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Effects of Urbanization on Sediment Microbial Communities in Lotic Ecosystems

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EFFECTS OF URBANIZATION ON SEDIMENT MICROBIAL COMMUNITIES IN LOTIC ECOSYSTEMS

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ABSTRACT

The world is becoming increasingly urbanized, with the majority of the world's population now living in urban areas. Urbanization has the potential to significantly alter lotic ecosystems and the services they provide. Benthic microbial communities are key components of lotic ecosystems due to their contributions to primary production and nutrient cycling. Two types of human inputs associated with urbanization that may impact benthic microbial communities in lotic ecosystems are the input of wastewater treatment effluent and the input of emerging contaminants, including pharmaceuticals and personal care products. This work examines the effects of treated WWTP effluent on benthic microbial communities obtained from a field study of two streams in the Chicago metropolitan area. In addition, the presence and effects of a widely used antimicrobial, triclosan, was studied both in the field and using the artificial stream facility located at Loyola University Chicago.

Our findings suggest that WWTP effluent significantly reduce both chemical and biological variation in the benthic ecosystems. These results raise questions about the impacts of anthropogenic ecosystem modifications and WWTPs on lotic ecosystems. Results also indicated that WWTPs were not significant point sources of triclosan, suggesting that non-point sources are more significant sources of triclosan into lotic ecosystems. However, sediment triclosan concentrations correlated closely with the degree of urbanization of the surrounding habitat. Using model streams we were able to

generate a triclosan resistant bacterial community that was similar in size to control streams from a single dosing of triclosan.

CHAPTER ONE

INTRODUCTION

In 2011 the world's population surpassed 7 billion (United Nations Department of Economic and Social Affairs, 2011). Of the over 7 billion people, the majority now live in cities and other urban areas following a shift to urban living, a trend that has been steadily increasing (Grimm et al., 2008). With this shift towards urbanization, many cities in the developed world now classify as megacities, areas that are home to more than 10 million people. Subsequently, such growth will place increased pressures on urban infrastructure, utilities such as electrical power and water treatment, and neighboring ecosystems (Grimm et al., 2008).

In highly urbanized areas, a common problem is the treatment of wastewater as to not contaminate drinking water sources. There are many processes by which wastewater can be treated, but highly urbanized areas in the developed world primarily use activated sludge wastewater treatment plants (WWTPs) for the treatment of domestic (residential) wastewater. In the United States, approximately 84% of wastewater is treated by activated sludge plants (US EPA, 1989). In heavily urbanized areas, these WWTPs can be large and numerous. For example, Cook County, IL (www.mwrd.org), which includes the city of Chicago and is the third most populous county in the United States (www.census.gov), is serviced by seven WWTPs, one of which, the Stickney Water Reclamation Plant, is the largest activated sludge WWTP in the world with a design

capacity of 1,200 million gallons per day (www.mwrd.org). Treated effluent from WWTPs is frequently discharged into lotic ecosystems, and in many cases WWTP effluent makes up a substantial portion of the flow of the receiving water body (Brooks et al., 2006). Therefore, in highly urbanized areas such as Cook County, IL, WWTP effluent represents a significant component of lotic ecosystems.

Wastewater Treatment Plants and Aquatic Ecosystems

Although WWTPs can be effective at the reduction of biochemical oxygen demand (BOD) and pathogen load, it is impossible for the composition of WWTP effluent to perfectly match the composition of water in the receiving system. Therefore, the potential exists for WWTP effluent to significantly impact the physical and chemical characteristics of the receiving ecosystem. A number of studies have documented various effects of WWTP effluent on receiving ecosystems, including increased nutrient loading (Waiser et al., 2011) and eutrophication (Gücker et al., 2006). Some studies have also examined the effects of WWTP effluent on bacterial populations in streams and rivers, although the majority of these studies have focused on bacteria within the water column (Garnier et al., 1992; Goñi-Urriza et al., 1999; Cébron et al., 2004). Relatively few studies have examined the potential effects of WWTP effluent on benthic microbial communities (Wakelin et al., 2008). This lack of focus on benthic microbial communities is surprising because bacterial density is typically much higher in freshwater sediments than in the overlying water (Sander & Kalff, 1993) and because benthic microbial communities are critical components of lotic ecosystems, as they contribute to nutrient cycling, decomposition of organic material, and bioremediation of a

variety of pollutants. Recent studies have presented some evidence that WWTP effluent may impact the function and structure of sediment microbial communities. For instance, (Lofton et al., 2007) reported a significant increase in denitrification rates in sediments collected downstream from a WWTP in North Carolina USA. In addition, Wakelin et al. used denaturing gradient-gel electrophoresis (DGGE) to demonstrate that effluent from a small WWTP altered the composition of sediment bacterial communities from a small rural stream in Australia. These studies have demonstrated some of the potential effects of WWTP effluent on benthic microbial communities, but no studies have examined the effects of WWTP effluent on sediment microbial community function and structure in heavily urbanized habitats.

WWTP Effluent Field Study

Chapter 2 of this thesis describes a study which examined the impacts of WTTP effluent on the size, activity and composition of sediment microbial communities in lotic ecosystems by examining two distinct field sites in the Chicago metropolitan region: a) a large river in a highly urbanized area receiving effluent from a large WWTP, and b) a smaller river in a less densely populated suburban area receiving effluent from a much smaller. My results demonstrate that both of these WTTPs had unexpectedly similar effects, including increases in inorganic nutrients, decreases in the population size of bacterial communities, and shifts in the composition of sediment bacterial communities. The decreases in bacterial abundance at the sites downstream of WWTP inputs were especially surprising, as we would have expected higher nutrient concentrations to increase bacterial population densities. We hypothesized that this negative effect of

effluent on bacterial populations might have been caused by organic enrichment, or some toxic compounds within the effluent. One class of compounds that might be present in WWTP effluent are pharmaceuticals and personal care products (PPCPs).

Personal Care Products in Aquatic Ecosystems

Pharmaceuticals and personal care products (PPCPs) are a class of compounds that are of growing concern to aquatic ecologists (Rosi-Marshall & Royer, 2012). PPCPs include prescriptions and over-the-counter therapeutic drugs such as antibiotics, antihistamines, and analgesics; antibacterial products such as soaps and detergents; and fragrances and cosmetics. Many PPCPs are used due to their specific biological effects. These compounds are often resistant to degradation in the environment (Singer et al., 2002) and are often not effectively removed by WWTPs (McAvoy et al., 2002; Singer et al., 2002; Bester, 2003; Kanda et al., 2003; Sabaliunas et al., 2003; Bendz et al., 2005; Bester, 2005; Thompson et al., 2005). PPCPs enter lotic ecosystems through WWTP effluent. The widespread use of PPCPs due to their incorporation in many consumer products has led to measureable quantities of these compounds in the ecosystem. There are numerous reports of PPCPs and their metabolites presence in lotic ecosystems (Daughton & Ternes, 1999; Kolpin et al., 2002; Kanda et al., 2003; Bendz et al., 2005; Bartelt-Hunt et al., 2009; Watkinson et al., 2009). However, studies are often designed only to detect and quantify these compounds in lotic environments. Very little is known about the effect these biologically active compounds have on non-target organisms and ecosystem function. For example, the antimicrobial compound triclosan was one of the most frequently detected compounds in a recent survey of streams and rivers in the

United States, being found in over half of the 139 streams tested in the study (Kolpin et al., 2002). Its effect on microbial community structure in streams is unknown.

Triclosan use in PPCPs

Triclosan (2,4,4'-trichloro-2'-hydroxydipheyl ether, CAS 3380-34-5) is a synthetic, lipophilic, phenolic, broad spectrum antimicrobial compound (Fig. 1). It was developed in the 1960s by Ciba-Geigy Company located in Basel, Switzerland and first used in surgical scrubs in 1972 (Jones et al., 2000). Today, triclosan is used in numerous consumer products including soaps, detergents, cleaners, toothpastes, and deodorants. A recent survey of liquid soaps available in the US found that 76% of soaps contained triclosan (Perencevich et al., 2001). Triclosan can also be incorporated into plastics and textiles, due to its thermal stability leading to its presence in products such as cutting boards, shopping carts, toys, shower curtains and socks (Adolfsson-Erici et al., 2002). Because triclosan has a low acute toxicity, it is relatively unregulated throughout the world, and global estimates of production exceed 1,500 tons annually (Singer et al., 2002).

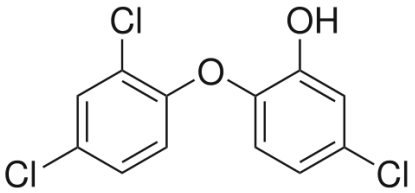

Figure 1. Molecular Structure of Triclosan

Triclosan is a broad spectrum antibacterial, and originally it was thought to integrate into and interfere with the bacterial cell membrane. Based on this lack of a specific intracellular target, it was considered to be unlikely that bacteria would develop resistance to triclosan. It was later discovered that triclosan does not disrupt the cell membrane, but in fact binds irreversibly to the enoyl-acyl carrier protein reductase (Heath

et al., 1998; Heath et al., 1999; Levy et al., 1999). This enzyme is an essential component of the fatty acid biosynthetic pathway for both Gram-positive and Gram-negative bacterial species but is not found in humans (Ledder et al., 2006).

Triclosan in the Environment

A number of recent studies have detected triclosan in lotic ecosystems (Adolfsson-Erici et al., 2002; Kolpin et al., 2002; Lindström et al., 2002; McAvoy et al., 2002; Singer et al., 2002; Sabaliunas et al., 2003; Morrall et al., 2004; Bester, 2005). However, most of these studies have measured triclosan concentrations in the water column, and few have measured triclosan concentrations in sediments (Singer et al., 2002; Morales et al., 2005). Due to the hydrophobic nature of triclosan, it is likely to bind to sediments and accumulate in higher quantities than in surface waters. Triclosan is also a stable compound that is highly resistant to degradation in sediments, and it has been detected in sediment cores >30 years old (Singer et al., 2002).

The route of entry of triclosan into aquatic ecosystems has not been determined. Because triclosan is found in a wide array of soaps, detergents, and other products that are destined for disposal via household drains, it will enter the domestic wastewater system directly after use. It has been measured in raw WWTP influent from ranges at 4.0-3100 µg L-1 (McAvoy et al., 2002; Bester, 2003; Kanda et al., 2003; Sabaliunas et al., 2003). Therefore, one possible route of entry of triclosan into lotic ecosystems would be through wastewater. Activated sludge WWTPs effectively remove >95% of triclosan from wastewater, with ~79% of the triclosan being degraded, and ~15% sorbing to the sludge (Federle et al., 2002; Singer et al., 2002; Bester, 2003; Kanda et al., 2003; Sabaliunas et

al., 2003; Bester, 2005; Thompson et al., 2005). Therefore, WWTP effluent may be a source of low concentrations of triclosan to the environment. Triclosan concentrations in treated effluent from activated sludge WWTPs ranging from 0.027-213 µg L-1 (McAvoy et al., 2002; Singer et al., 2002; Bester, 2003; Kanda et al., 2003; Sabaliunas et al., 2003; Thompson et al., 2005).

Another possible route of entry for triclosan into aquatic ecosystems is the direct release of untreated wastewater. For example, in many older cities in the United States, the underground sewage infrastructure is rapidly deteriorating and can constantly leak (Kaushal et al., 2011). In addition, combined sewer overflows (CSOs) discharge untreated water during storms, and are still widely used throughout the US.

Effects of Triclosan

The toxic effects of triclosan on bacteria have been well documented (Bradshaw et al., 1993; Suller & Russell, 2000; Chuanchuen et al., 2001; Hay et al., 2001; Fan et al., 2002; Orvos et al., 2002; Chuanchuen et al., 2003; Braoudaki & Hilton, 2004a; Braoudaki & Hilton, 2004b; Ledder et al., 2006; Yazdankhah et al., 2006). At low concentrations, triclosan is known to inhibit lipid biosynthesis by acting on the enoyl-acyl carrier protein reductase (McMurry et al., 1998). It is possible for Escherichia coli to develop resistance to triclosan either through mutations in the gene encoding this enzyme (fabI) (McMurry et al., 1998) or through overexpression of fabI (Fan et al., 2002). However, another study examining clinical significant strains such as Staphylococcus aureus found no change in triclosan resistance after one month of continuous sub-lethal exposure (Suller & Russell, 2000). Interestingly, the Gram-negative bacterium

Pseudomonas aeruginosa is intrinsically resistant to triclosan, and is isolated on agar plates containing 25 µg L-1 of triclosan. Triclosan resistance in P. aeruginosa is not conferred by a mutation in fabI, but instead is due to numerous efflux pumps in the cell membrane (Chuanchuen et al., 2003). Overexpression of efflux pumps due to triclosan exposure in P. aeruginosa also results in cross resistance to other clinically significant antibiotics (Chuanchuen et al., 2001).

Very few studies have examined the toxicity of triclosan to environmentally relevant organisms (Orvos et al., 2002; Marianne et al., 2004). Of those studied, it has been shown that algae are among the most susceptible organisms to triclosan (Orvos et al., 2002). No studies have explored the potential effects of triclosan on microbial communities in the environment.

Triclosan Field Study

Chapter 3 of this thesis describes a field study examining the potential ecological impacts of triclosan on aquatic ecosystems. I hypothesized that WWTPs would be a significant point source of triclosan to river sediments, so I collected sediment samples from two rivers in the Chicago region, both of which receive inputs of WWTP effluent. The North Shore Channel (NSC) was selected to represent a highly impacted urban river. NSC receives treated effluent from the North Side Water Reclamation Plant (NSWRP), an activated sludge plant that treats 245 million gallons of domestic wastewater per day (www.mwrd.org). The West Branch of the DuPage River (WBDR) located in DuPage County, IL was selected to represent a less highly impacted, suburban river. WBDR receives treated effluent from the West Chicago Wastewater Treatment Plant

(WCWWTP), an activated sludge plant that treats 5 million gallons of domestic wastewater per day (www.westchicago.org). I collected sediments from two sites on each river, one upstream and one downstream of the WWTPs. I also collected sediments from one site on Nippersink Creek (NC), in McHenry Country, IL. NC is a woodland river that receives no WWTP inputs upstream of the sampling site, which is located in a state park, and served as a control site due to its relatively low level of human impact. At each of the five sampling sites (two on NSC, two on WBDPR, and one on NC), I collected five replicate sediment samples (for details of sampling protocol see Chapter 3). Triclosan concentrations in the sediments were determined by my collaborator, John Scott, at the Illinois Sustainable Technology Center, Champaign, IL (for details of analysis see Chapter 3). Surprisingly, my results showed that triclosan concentrations in both NSC and WBDPR were higher upstream of the treatment plants as compared to downstream (Table 1). These results indicated that these two WWTPs were not significant point sources of triclosan and suggested that non-point sources (e.g. CSOs) may be more significant sources. Interestingly, the data revealed a very clear gradient of triclosan concentrations between the urban river (NSC Upstream

Table 1. Sediment Triclosan Concentrations

	ng g^{-1}*
North Shore Channel Upstream of WWTP	107 (18)
North Shore Channel Downstream of WWTP	33 (11)
West Branch DuPage River Upstream of WWTP	9 (4)
West Branch DuPage River Downstream of WWTP	4 (1)
Nippersink Creek	1 (0)

* Limit of Detection = 1 ng g^{-1}. Each data point represents mean value (n=3 to 5) with standard error values in parentheses.

Site), the suburban river (WBDPR Upstream Site) and the woodland river (NC). Therefore, Chapter 3 of this thesis focuses on these three sites as a field study on the impacts of triclosan on benthic microbial communities. My results indicated that there was a strong correlation between the concentrations of triclosan in the sediments and the percentages of bacteria in the sediments that were resistant to triclosan. The results also demonstrated significant differences in respiration rates and the composition and diversity of sediment bacterial communities. In order to determine if these differences in the bacterial communities were driven by triclosan as opposed to differences between the field sites, I conducted a model stream experiment designed to examine the effects of triclosan on benthic microbial communities. This model stream study is presented in Chapter 4 of my thesis. These results will provide valuable insights into the potential ecological effects of triclosan.

Model Stream Experiment

As described in Chapter 4 of my thesis, I conducted a model stream study to assess the effects of triclosan on sediment microbial communities. Results of this study indicated that five weeks after dosing with triclosan, the sediment bacterial communities within the treatment streams were similar in population density and levels of activity (respiration) to the communities within the untreated control streams. However, the communities in the triclosan amended streams showed much higher levels of resistance than the control stream communities. These results confirm observations from the field sites (as described in Chapter 3) and suggest that triclosan amendments shifted the composition of the sediment bacterial communities. Future work will include analysis of

the model stream bacterial communities by tag pyrosequencing of bacterial 16S rRNA genes, which will allow comparison of community shifts observed in the model stream experiment to shifts observed in the field study.

CHAPTER TWO

WASTEWATER TREATMENT EFFLUENT REDUCES CHEMICAL AND BIOLOGICAL VARIATION IN BENTHIC ECOSYSTEMS

In highly urbanized areas wastewater treatment plant (WWTP) effluent represents a significant component of lotic ecosystems. As it is impossible for the composition of WWTP effluent to perfectly match the composition of the water in the receiving system, the potential exists for WWTP effluent to significantly impact the chemical and biological characteristics of the receiving ecosystem. We assessed the impacts of WWTP effluent on the size, activity and composition of benthic microbial communities in lotic ecosystems by examining two distinct field sites in the Chicago metropolitan region: a river in a highly urbanized area receiving effluent from a large WWTP, and a river in a less densely populated suburban area receiving effluent from a much smaller WWTP. Our data demonstrate that these two WWTPs that differed dramatically in size had remarkably similar effects on the chemical and biological properties of the receiving rivers, including increases in inorganic nutrients, decreases in the population size of sediment bacterial communities, and shifts in the composition of sediment bacterial communities. The net effect of WWTP inputs was that two rivers that were distinct in chemical and biological properties upstream of the WWTPs were almost indistinguishable downstream. These results suggest that WWTP effluent may have the potential to reduce the natural chemical and biological variation of river ecosystems.

Introduction

Centralized wastewater treatment plants (WWTPs) are one of the most common systems for the treatment of domestic wastewater in the United States (US EPA, 2008). In highly urbanized areas with high population densities, WWTPs can be large and numerous. For example, Cook County, IL, which includes the city of Chicago and is the second most populous county in the United States (www.census.gov), is serviced by seven WWTPs, one of which, the Stickney Water Reclamation Plant, is the largest activated sludge WWTP in the world with a design capacity of 1,200 million gallons per day (www.mwrd.org). WWTPs frequently discharge effluent water into lotic ecosystems, and in many cases WWTP effluent makes up a significant proportion of the flow of the receiving water body (Brooks et al., 2006). Therefore, in highly urbanized areas like Cook County, WWTP effluent represents a significant component of lotic ecosystems.

Although WWTPs can be effective at reduction of biochemical oxygen demand (BOD) and pathogen load, it is impossible for the composition of the effluent to perfectly match the composition of the water in the receiving system. Therefore, the potential exists for WWTP effluent to significantly impact the physical and chemical characteristics of the receiving ecosystem, and numerous studies have documented the potential ecosystem effects of WWTP effluent, including increased nutrient loading (Waiser et al., 2011) and eutrophication (Gücker et al., 2006). Several previous studies have examined the effects of WWTP effluent on bacterial populations within the water column (Garnier et al., 1992; Goñi-Urriza et al., 1999; Cébron et al., 2004) and some have demonstrated the ability of microorganisms contained in the effluent to persist in the

water column of the receiving system (Cébron et al., 2004). However, few studies have examined the potential effects of WWTP effluent on benthic microbial communities (Wakelin et al., 2008) despite the fact that bacterial numbers are generally much higher in freshwater sediment that in the overlying water (Sander & Kalff, 1993) and despite the fact that benthic microbial communities are critical components of lotic ecosystems, as they contribute to organic matter decomposition, nutrient cycling, and bioremediation of a variety of pollutants. Several recent studies have presented some evidence that WWTP effluent may impact the function and structure of sediment microbial communities. For example, (Lofton et al., 2007) reported a significant increase in denitrification rates in sediment samples collected downstream from a WWTP in North Carolina, USA. In addition, (Wakelin et al., 2008) used denaturing gradient gel electrophoresis (DGGE) to demonstrate that effluent from a small WWTP altered the composition of sediment bacterial communities in a small rural stream in Australia. We are not aware of any study that has examined the effects of WWTP effluent on sediment microbial community function and structure in a highly urbanized habitat.

We assessed the impacts of WWTP effluent on the size, activity and composition of benthic microbial communities in lotic ecosystems by examining two distinct field sites in the Chicago metropolitan region: a) a river in a highly urbanized area receiving effluent from a large WWTP, and b) a river in a less densely populated suburban area receiving effluent from a much smaller WWTP. Our data demonstrate that these two WWTPs that differed dramatically in size had remarkably similar effects on the chemical and biological properties of the receiving rivers, including increases in inorganic

nutrients, decreases in the population size of sediment bacterial communities, and shifts in the composition of sediment bacterial communities. The net effect of WWTP inputs was that two rivers that were distinct in chemical and biological properties upstream of the WWTPs were almost indistinguishable downstream. These results suggest that WWTP effluent may have the potential to reduce the natural chemical and biological variation of river ecosystems.

Materials and Methods: Field Sites

The North Shore Channel (NSC) was selected to represent a highly impacted urban river. NSC is a 7.7 mile long canal that begins in the town of Wilmette, IL, USA and extends into the northeast section of the city of Chicago, IL. The canal was built in 1910 to bring water from Lake Michigan to the North Branch of the Chicago River. NSC has a drainage area of 25 square miles of which 63% is residential, 16.7% is commercial/industrial, 10% is forest/open land, 5.4% is institutional, and 3.5% is transportation/utility (HDR Engineering, 2011). (Aqua Terra Consultants, 2003) NSC receives treated effluent from the North Side Water Reclamation Plant (NSWRP), an activated sludge plant that receives domestic wastewater from over 1.3 million people residing in a 141 square mile area which includes the part of the city of Chicago (north of Fullerton Avenue) and the northern Cook County suburbs. NSWRP has an average flow of 245 million gallons per day (MGD) and a design capacity of 333 MGD. NSWRP treats wastewater with a series of physical and biological processes and effluent is not disinfected prior to release (www.mwrd.org). Two sampling sites on the NSC were chosen, one approximately 925 meters upstream of the input of effluent from the NSWRP

and one approximately 50 meters downstream of the effluent input.

The West Branch of the DuPage River (WBDR) located in DuPage County, IL, USA was selected to represent a less highly impacted suburban river. WBDR has a drainage area of 127 square miles of which 32.8% is residential, 17.4% is agricultural, 16.9% is vacant, 11.2% is forest/open land and less than 4% is industrial (Aqua Terra Consultants, 2003). WBDR receives treated effluent from the West Chicago WWTP (WCWWTP) which is located in West Chicago, IL. The WCWWTP receives domestic wastewater from the towns of West Chicago, IL and Winfield, IL. It treats 5 MGD and does not disinfect its effluent prior to release (www.westchicago.org). Two sampling sites on the WBDR were chosen, one approximately 275 meters upstream of the input of effluent from the WCWWTP and one approximately 50 meters downstream of the effluent input.

Materials and Methods: Sample Collection

Five replicate sediment samples and five replicate water samples were collected at each of the four sampling sites between August and September 2010. Each sediment sample consisted of a composite of ten individual sediment samples collected from randomly selected sections along the stream reach. Sediment samples were collected using a Petite Ponar Sampler (Wildlife Supply Company, Saginaw, MI) and large debris was removed by hand. Sediment samples were stored in sterile 400mL canning jars (Ball Corporation, Muncie, Indiana). Water samples were collected in sterile, pre-cleaned 1L amber glass jars (Thermo Scientific, Waltham, MA). All sediment and water samples were stored on ice for transport back to the laboratory.

Materials and Methods: Sample Characteristics

Dissolved organic carbon (DOC) in water samples was measured on a Shimadzu 5050 TC Analyzer as described by (Findlay et al., 2010). Ammonium, nitrate and phosphate concentrations in water samples were determined with a Lachat QuikChem 8000 by the phenate method (method #10-107-06-1-J, Lachat Instruments, Milwaukee, WI), the cadmium diazotization method (method #10-107-04-1-C, Lachat Instruments) and the phosphomolybdate method (method #10-115-01-1-M, Lachat Instruments) respectively. Sediment organic material was measured by loss on ignition at 500°C (Bear, 1955).

Materials and Methods: Microbial Respiration

Respiration was measured for each sediment sample using a standard method (Hill et al., 2002). Briefly, 10 mL of sediment was placed into a black HDPE 50mL centrifuge tube (Cole-Parmer, Vernon Hills, IL) filled to the top (no head space) with well water. Water temperature and initial dissolved oxygen (DO) were measured using a YSI ProODO meter (YSI Inc. Yellow Springs, OH). Centrifuge tubes were capped, eliminating all air bubbles and incubated at room temperature (25°C) in the dark for 2 hrs, after which final DO was measured and respiration rates were calculated as mg O2 consumed time-1. Respiration rates were then normalized by sediment surface area and by total heterotrophic plate counts.

Materials and Methods: Heterotrophic Plate Counts

Viable counts of heterotrophic bacteria were conducted for each sediment sample using a standard plate count method (Page, 1982). Briefly 10 g of sediment was placed in

a sterile 250mL centrifuge bottle containing 90 mL of sterile potassium phosphate buffer solution. Samples were agitated for 30 min at 300 rpm using a reciprocal shaker (New Brunswick Scientific, Edison, NJ). Samples were allowed to settle for 5 min, 1 mL of supernatant was serially diluted ten-fold to 10-5, and 100 µL of each dilution was plated on Soy Extract Agar (Becton Dickinson and Company, Sparks, MD) plates containing 100 mg L-1 cycloheximide (MP Biomedicals, Solon, OH) to inhibit fungal growth. Numbers of colony forming units were normalized based on grams of dry sediment.

Materials and Methods: Epifluorescence Counts

Direct counts of bacterial cells were performed using a modified standard method (Kepner Jr & Pratt, 1994). Cells were fixed by diluting sediment 1:50 in sterile DNA-free fixative solution (10mM NaPO4, 120mM NaCl, 10mM sodium pyrophosphate, 4% formaldehyde) (Gough & Stahl, 2003) in a sterile 50 mL centrifuge tube. Samples were placed in an ultrasonic ice water bath (Model 8845-30, Cole-Parmer, Vernon Hills, IL) and sonicated for 15 minutes at 60Hz. Following ultrasonic treatment, samples were diluted 1:1,000, 1:2,000 and 1:4,000 in 0.2 µm filtered deionized water. 2 mL of each diluted sample were filtered in duplicate onto 0.2 µm anodisc membrane filters (Whatman, Maidstone, UK) and stained with 100 µL of SYBR Gold (Invitrogen, Carlsbad, CA). Cells were counted at 400x magnification using an Olympus BH-2 Fluorescence Microscope (Olympus, Center Valley, PA). Cell numbers were normalized based on grams of dry sediment.

Materials and Methods: Tag Pyrosequencing

DNA was isolated from each of the sediment samples using the UltraClean Soil

DNA Kit (MoBio Laboratories, Carlsbad, CA). Successful DNA isolation was confirmed by agarose gel electrophoresis. For tag pyrosequencing of bacterial 16S rRNA genes extracted DNA was sent to the Research and Testing Laboratory (Lubbock, TX). 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, IN) with Titanium reagents. Sequences were processed using MOTHUR v.1.20.1 (Schloss et al., 2009). Briefly, any sequences containing ambiguities or homopolymers longer than 8 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 uniform length of 250 base pairs and chimeric sequences were removed using Chimeraslayer (Haas et al., 2011) run within MOTHUR. Sequences were grouped into phylotypes by comparison to the SILVA-compatible bacterial alignment database available within MOTHUR and chloroplast sequences were removed. After these pretreatment steps were completed the data set included a total of 173,842 sequences for an average of 8,692 sequences per sample. Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence identity using the average neighbor algorithm. The community compositions of the individual sampling sites were compared by calculating distances between sites based on the theta index (Yue & Clayton, 2005)

and visualizing the resulting distance matrix using non-metric multidimensional scaling (NMDS). MOTHUR was also used to calculate the Shannon diversity index (Shannon, 2001) and the Chao1 richness estimator (Chao, 1984).

Materials and Methods: Statistics

All data were analyzed by two-way ANOVA based on habitat type (urban vs. suburban) and location (upstream of effluent input vs. downstream). Analyses were run using Systat version 12 (Systat Software, Inc., San Jose, CA) and p values less than 0.05 were considered to be significant.

Results

There was a significant effect of habitat type (urban vs. suburban) on the water column nutrient concentrations in our two rivers, with the suburban river having higher concentrations of DOC, nitrate and phosphate, and a lower concentration of ammonium. There was also a significant effect of habitat type on the population size of the sediment bacterial communities as indicated by heterotrophic plate counts, with the suburban river having higher counts than the urban river (Fig. 2A). Direct epifluorescent counts of bacterial cells did not show this same trend, as there was no significant effect of habitat type on direct counts (Fig. 2B). There was also no significant effect of habitat type on microbial community respiration (Fig. 2A). Tag pyrosequencing of bacterial 16S rRNA genes revealed a significant difference in community composition between the suburban upstream and urban upstream sites (Fig. 3 and Table 2) but there was no significant effect of habitat type on bacterial diversity (Fig. 4A) or bacterial species richness (Fig. 4B).

WWTP effluent had significant effects on water column ammonium, nitrate, and

phosphate concentrations, as well as sediment organic content (Table 2). Specifically, WWTP effluent resulted in significant increases in water column nitrate and phosphate at both the urban and suburban sites and a significant increase in water column ammonium at the urban site. WWTP effluent also resulted in significant decreases in sediment organic material at both the urban and suburban sites. WWTP effluent had significant effects on the population size of the sediment bacterial communities as indicated by both heterotrophic plate counts (Fig. 2A) and direct counts (Fig. 2B). Specifically, WWTP effluent resulted in significant decreases in both plate counts and direct counts at both the urban and suburban sites. WWTP effluent had no effect on community respiration normalized by sediment surface area (Fig. 3A). However, the decreases in bacterial population size that was observed at both the downstream sites (Fig. 2 A and B) combined with this lack of change in overall respiration rates (Fig. 3A) suggested that these smaller populations were doing more respiration on a per cell basis. This hypothesis was supported by normalization of respiration rates by cell counts (Fig. 3B) which demonstrated a significant effect of WWTP effluent, with both downstream sites having higher per cell respiration rates than the corresponding upstream sites.

Tag pyrosequencing of bacterial 16S rRNA genes indicated that WWTP effluent resulted in a significant shift in the composition of sediment bacterial communities at both sites, with significant differences in community composition between both downstream sites and their corresponding upstream sites (Fig. 4 and Table 3). Tag pyrosequencing data also indicated that WWTP effluent had a significant effect on bacterial community diversity (Fig. 5A) and richness (Fig. 5B), with both of these

parameters decreasing at the downstream sites as compared to their corresponding upstream sites. Interestingly, there were no significant differences between the bacterial communities from the two downstream sites in terms of community composition (Fig. 4 and Table 3), diversity (Fig. 5A) or richness (Fig. 5B).

In terms of specific bacterial phyla, tag pyrosequencing revealed a significant effect of WWTP effluent on the relative abundance of proteobacterial sequences within the sediment bacterial communities, with both of the downstream sites having lower relative abundance of proteobacterial sequences than their corresponding upstream sites (Fig. 6). WWTP effluent also had significant effects on the relative abundance of Chloroflexi sequences, which decreased at downstream sites, Nitrospirae sequences, which increased at downstream sites, and Spirochaete sequences which decreased at downstream sites (Fig. 6). Looking more closely within the Proteobacterial phylum, WWTP effluent had significant effects on the relative abundance of alphaproteobacterial sequences, which increased at downstream sites, and deltaproteobacterial sequences, which decreased at downstream sites (Fig. 7). We also looked more closely within several of the proteobacterial classes. Within the alphaproteobacteria WWTP effluent resulted in a significant increase in Rhodobacterales sequences and a significant decrease in Rhodospirillales sequences. Within the betaproteobacteria WWTP effluent resulted in significant decreases in Rhodocyclales and Hydrogenophilales sequences. Finally, within the deltaproteobacteria WWTP effluent resulted in a significant increase in Desulfuromonadales sequences and a significant decrease in Syntrophobacteriales sequences.

Discussion

The two rivers analyzed in this study, one in an urban habitat and the other in a suburban habitat, differed significantly in chemical characteristics. Some of these differences may be related to differences in land use within the watersheds of these two rivers. For example, the watershed of the suburban river was 17.4% agricultural while the watershed of the urban river had no agriculture, so the use of fertilizers on the agricultural land may have contributed to the higher levels of inorganic and organic nutrients in the suburban river. Based on the higher nutrient concentrations, it is not surprising that higher numbers of heterotrophic bacteria were detected in the suburban river sediment as compared to the urban river sediment. The lower numbers of heterotrophic bacteria in the urban river sediment might also have been based on toxic contaminants in the urban river, as this river has a long history of industrial pollution. Based on the significant differences between the two rivers in chemical characteristics, it was also not surprising that the sediment bacterial communities from the two rivers differed significantly in species composition as revealed by tag pyrosequencing analysis.

The two rivers analyzed in this study both received WWTP effluent, but the WWTPs discharging to the two rivers were drastically different in size, with the urban WWTP being almost 50 times larger (in terms of volume of water treated) than the suburban WWTP. Both of these WWTPs significantly altered the downstream nutrient chemistry, specifically increasing concentrations of inorganic nitrogen and phosphorous. Increases in nitrogen and phosphorous due to WWTP effluent have been observed previously (Gücker et al., 2006). Based on these increases in inorganic nutrients, it was

very surprising that in both rivers the numbers of bacteria decreased downstream of the WWTPs. We would have expected increased nitrogen and phosphorous to have stimulated bacterial growth, and indeed other studies have demonstrated increased planktonic (Garnier et al., 1992; Goñi-Urriza et al., 1999) and benthic (Wakelin et al., 2008) bacterial numbers downstream of WWTP effluent inputs. The lower bacterial numbers at the downstream sites of the rivers examined in our study might have been related to the observed decreases in sediment organic matter, although this decrease in sediment organic matter was itself surprising, as we would have expected the large increases in inorganic nutrients at the downstream sites to have resulted in greater primary production. Other studies have demonstrated increased primary production downstream of WWTP inputs (Gücker et al., 2006). One hypothesis that could explain the decreases in bacterial numbers and sediment organic matter at the downstream sites of both rivers is that there might have been some toxic compounds in the WWTP effluent that inhibited microbial growth. There has been growing concern recently about the presence of a wide range of biologically active compounds, including antimicrobials, in rivers and streams in the United States (Kolpin et al., 2002). Many of these compounds can be detected in the domestic wastewater stream and many of them are not completely removed by wastewater treatment, so WWTPs can serve as point sources of these compounds (Bartelt-Hunt et al., 2009; Akiyama & Savin, 2010). If the effluent from the two WWTPs examined in this study contained compounds toxic to microorganisms, this could explain the fact that bacterial numbers and sediment organic matter decreased at our downstream sites despite the increases in inorganic nutrients. The presence of toxic

compounds could also explain the observed increases in per-cell respiration rates, as previous studies have indicated that respiration rates normalized by biomass increase for bacteria cells that are under stress (Anderson & Domsch, 1993). Toxic compounds in the effluent could also have contributed to the decreases in bacterial diversity and species richness that we observed at the downstream sites of both rivers. Decreases in sediment organic material could also contribute to the observed decreases in bacterial diversity and richness. Although quantification of all possible toxic compounds that could have been found within the WWTP effluents was beyond the scope of our study, these results provide support for future explorations of this topic.

WWTP effluent also resulted in shifts in bacterial community composition within both of the rivers examined in this project. For example, WWTP effluent resulted in a significant decrease in the relative abundance of proteobacteria, which are a ubiquitous and metabolically diverse group of gram negative bacteria. Within the proteobacteria we observed significant increases in the relative abundance of alphaproteobacteria at the downstream sites. The alphaproteobacteria includes many phototrophic bacteria (Brenner et al., 2005), and the increase in the relative abundance of these organisms may reflect the increased inorganic nutrients (N and P) at the downstream sites. Within the alphaproteobacteria we noted a significant relative decrease in Rhodospirillales, an order that contains many photoheterotrophic organisms (Brenner et al., 2005) whose growth might have been repressed by the lower organic matter concentrations in the downstream sediments. In contrast there was a relative increase in Rhodobacterales at the downstream sites, and this order contains many organisms capable of photoautotrophy (Brenner et al.,

2005) whose growth might have been stimulated by the increases in inorganic nutrients and not impacted by the decrease in sediment organic matter at the downstream sites.

We also observed that WWTP effluent had a negative effect on the relative abundance of deltaproteobacteria, a class that contains most of the known sulfate reducing organisms (Brenner et al., 2005). The decrease in deltaproteobacteria may reflect the increased concentration of nitrate at the downstream sites, as nitrate is a more energetically favorable electron acceptor than sulfate. Within the deltaproteobacteria we also observed shifts in relative abundance of some orders that include sulfate reducers (Brenner et al., 2005), specifically a relative increase in the order Desulfuromonadales and a significant decrease in the order Syntrophobacteriales. These shifts may reflect how well adapted these specific orders are to the conditions at the downstream sites.

Finally, our results indicated that Nitrospirae increased at downstream sites in both rivers. Nitrospirae are nitrite oxidizers that have been shown to be the dominant nitrite oxidizers within freshwater sediments (Altmann et al., 2003), so the increase in these organisms at our downstream sites may be due to the observed increases in inorganic nitrogen at the downstream sites caused by the WWTP effluent. However Nitrospirae have also been shown to be the dominant nitrite oxidizers within most WWTPs (Daims et al., 2001), so it is possible that the increase in Nitrospirae observed in our study at the downstream sites was the result of direct release of these organisms from the WWTPs, although most bacteria released in WWTP effluent do not survive for very long in lotic systems (Garnier et al., 1992).

Conclusions

What is most remarkable about the data presented in this study is the fact that two rivers that differed significantly in chemical and biological characteristics were driven towards almost identical chemical and biological states by inputs from two WWTPs which themselves differed drastically in size. These results indicate that for these two field sites, WWTP effluent reduced the chemical and biological variation in the benthic ecosystems. Given the ubiquity of WWTPs in urban regions of the United States, these results raise questions about the impacts of anthropogenic ecosystem modifications on stream ecosystems. Further investigations will be needed to determine if this pattern can be observed across a broader range of field sites.

Acknowledgements

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Table 2. Sampling Site Characteristics

	Mean (SE)[a]				ANOVA		
	Suburban Upstream	Suburban Downstream	Urban Upstream	Urban Downstream	Land Use	Effluent	Interaction
Water Column DOC (mg/L)	6.652 (0.052)	5.782 (0.306)	2.408 (0.085)	3.947 (0.072)	<0.001	0.060	<0.001
Water Column NH_4 (mg/L)[b]	0.060 (0.003)	N.D.	0.138 (0.007)	0.236 (0.005)	<0.001	<0.001	<0.001
Water Column NO_3^- (mg/L)[b]	2.742 (0.140)	4.662 (0.492)	0.232 (0.002)	4.696 (0.206)	<0.001	<0.001	<0.001
Water Column PO_4^{3-} (mg/L)[c]	0.268 (0.006)	0.466 (0.035)	0.003 (0.000)	0.410 (0.019)	<0.001	<0.001	<0.001
Sediment Organic Matter (%)	8.70 (1.20)	1.58 (0.12)	5.89 (0.43)	2.00 (0.21)	0.216	<0.001	0.025

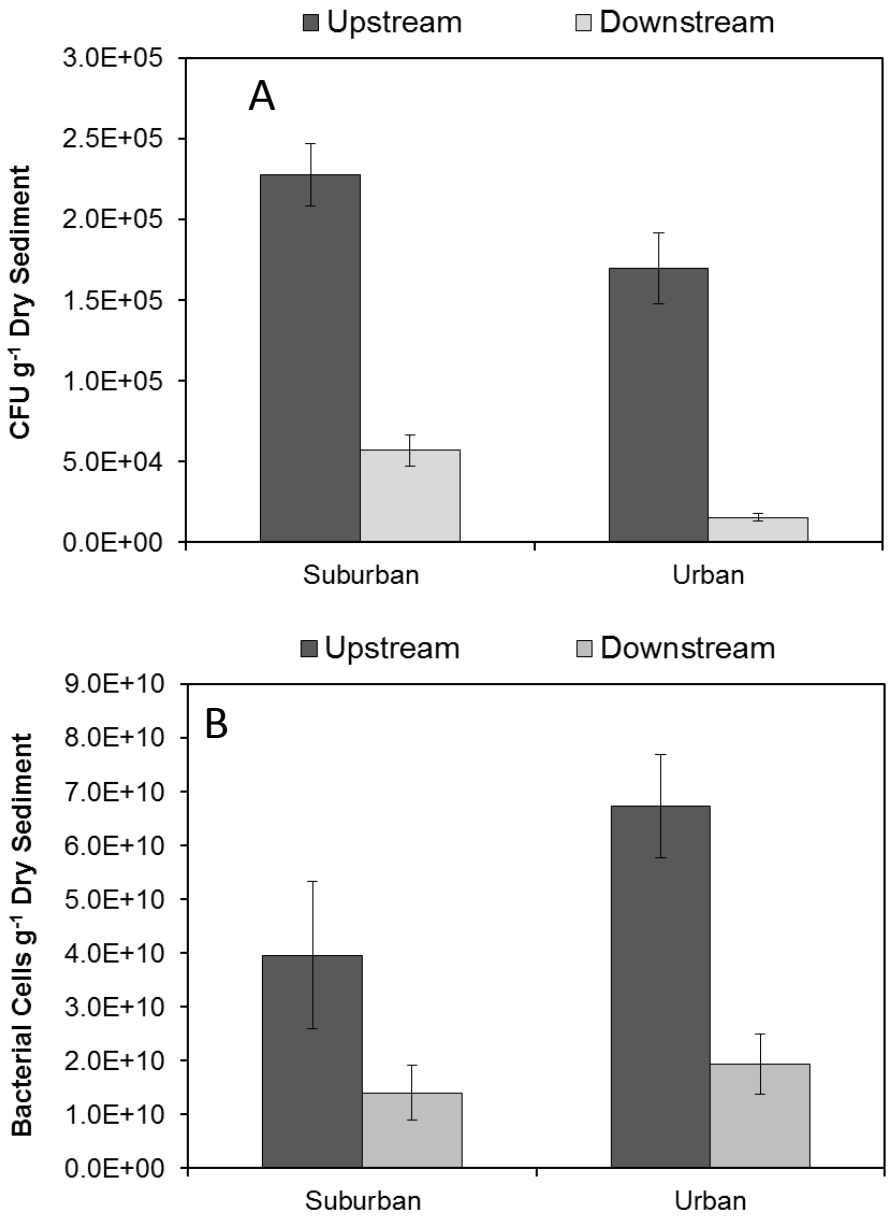
[a] Each data point is mean (n=5) standard error values in parentheses.

[b] NH_4 and NO_3^- Limit of Detection = 0.02mg L^{-1}

[c] PO_4^{3-} Limit of Detection = 0.002mg L^{-1}

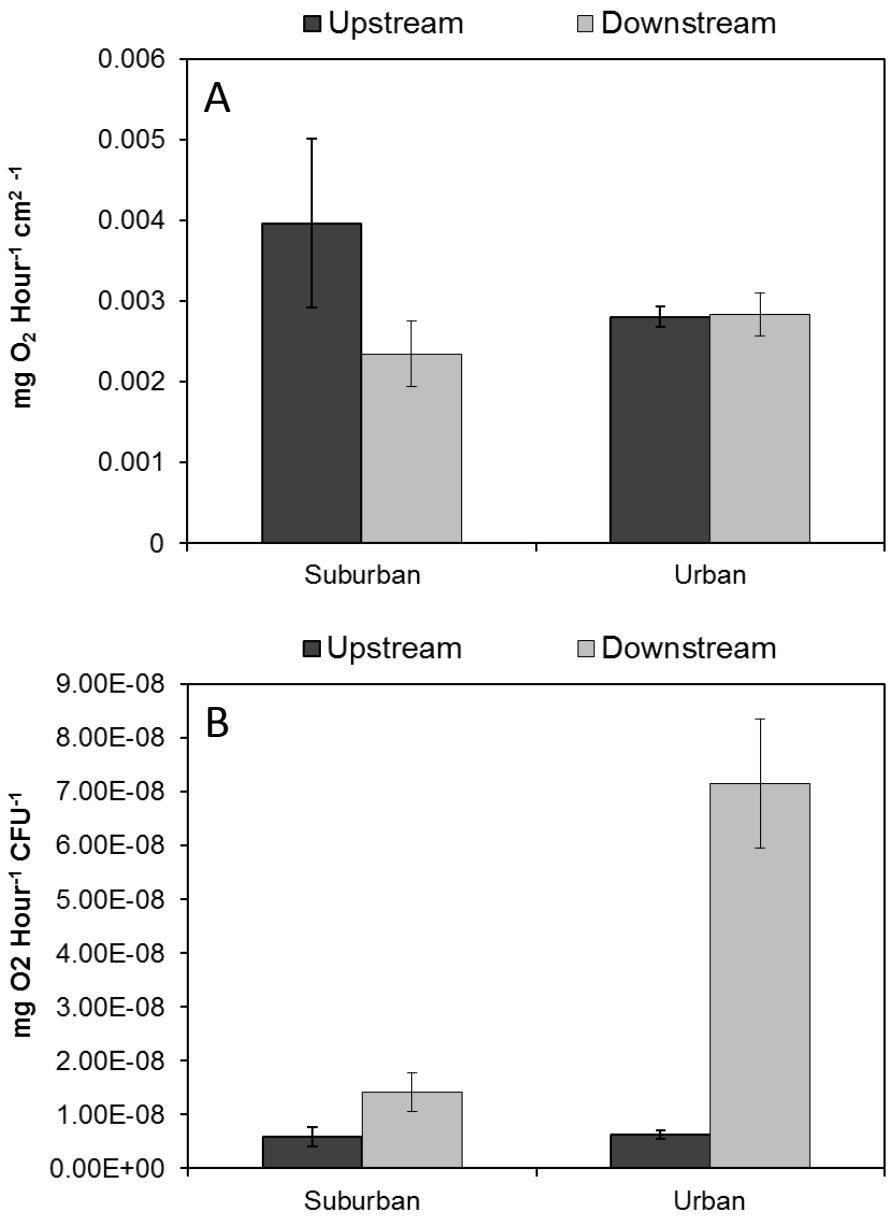
Nutrient concentrations of sediment samples collected from field sites.

Figure 2. Heterotrophic Plate Counts and Direct Bacterial Cell Counts



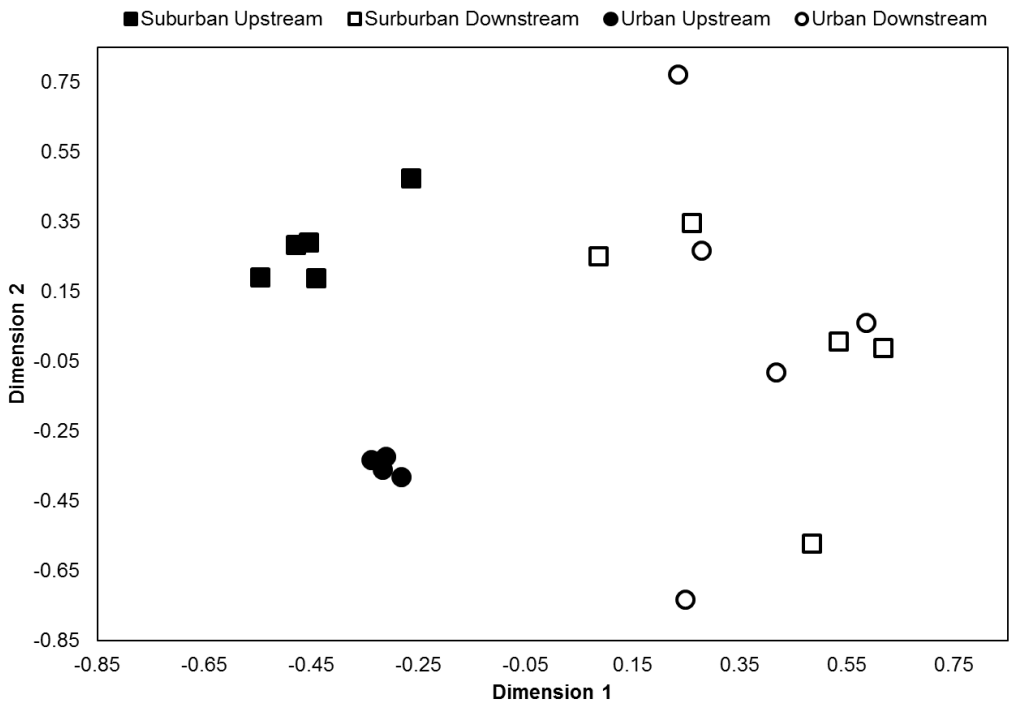
Heterotrophic plate counts (A) and direct bacterial cell counts for sediments collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Each data point is mean (n=5) ± standard error. Two-way ANOVA (A) demonstrated significant effect of land use (p=0.005) and effluent input (p=0.000) but no significant interaction effect (p=0.000). Two-way ANOVA (B) demonstrated no significant effect of land use (p=0.091), but a significant effect of effluent input (p=0.001) and no significant interaction effect (p=0.243).

Figure 3. Community Respiration



Community respiration normalized by surface area (A) and by bacterial cell numbers based on heterotrophic plate counts (B) for sediments collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Each data point is mean (n=5) ± standard error. Two-way ANOVA (A) demonstrated no effect of land use ($p=0.572$), effluent input ($p=0.189$) or interaction effect ($p=0.176$). Two-way ANOVA (B) demonstrated a significant effect of land use ($p=0.000$), effluent input ($p=0.00$) and a significant interaction effect ($p<0.001$).

Figure 4. NMDS Ordination of 16S Tag Pyrosequencing Data



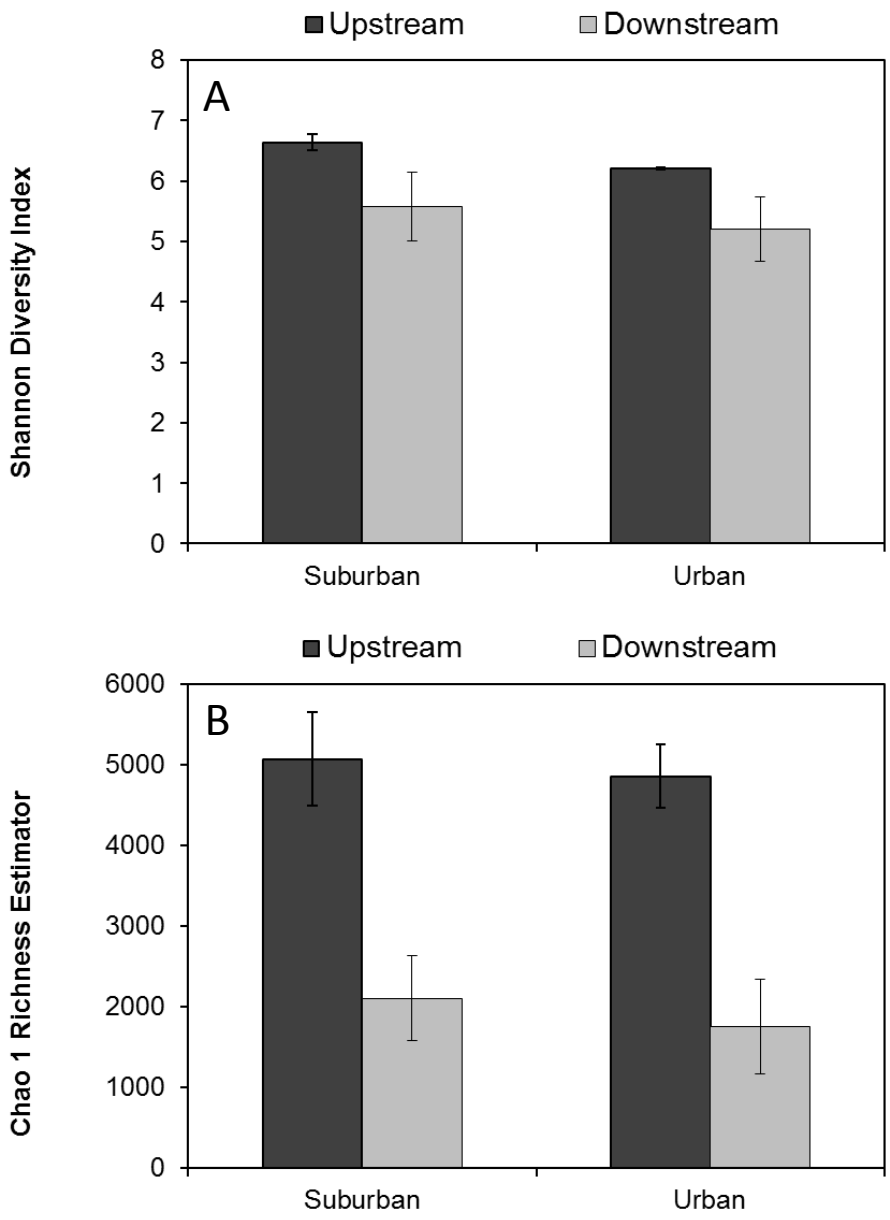
Ordination of 16S tag pyrosequencing data comparing community structure of sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs.

Table 3. Statistical Analysis of 16S Tag Pyrosequencing Data: WWTP Effluent

	P-value
Interaction Effect	<0.001
Suburban Downstream vs. Suburban Upstream	0.003
Suburban Downstream vs. Urban Downstream	0.92
Suburban Upstream vs. Urban Downstream	0.003
Suburban Upstream vs. Urban Upstream	<0.001
Urban Downstream vs. Urban Upstream	<0.001

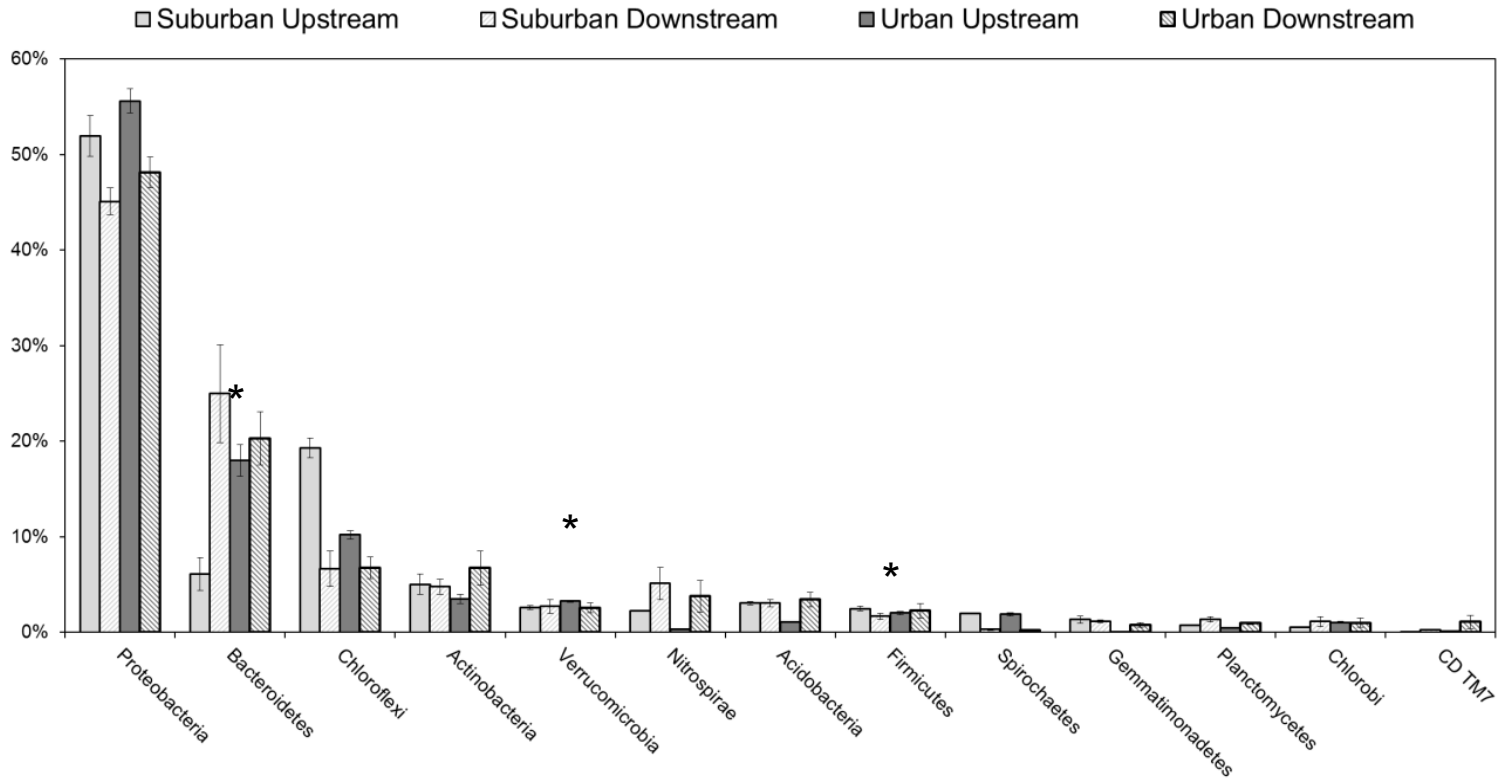
Statistical analysis of NMDS ordination of 16S tag pyrosequencing data presented above (Fig. 4).

Figure 5. Shannon Diversity Index



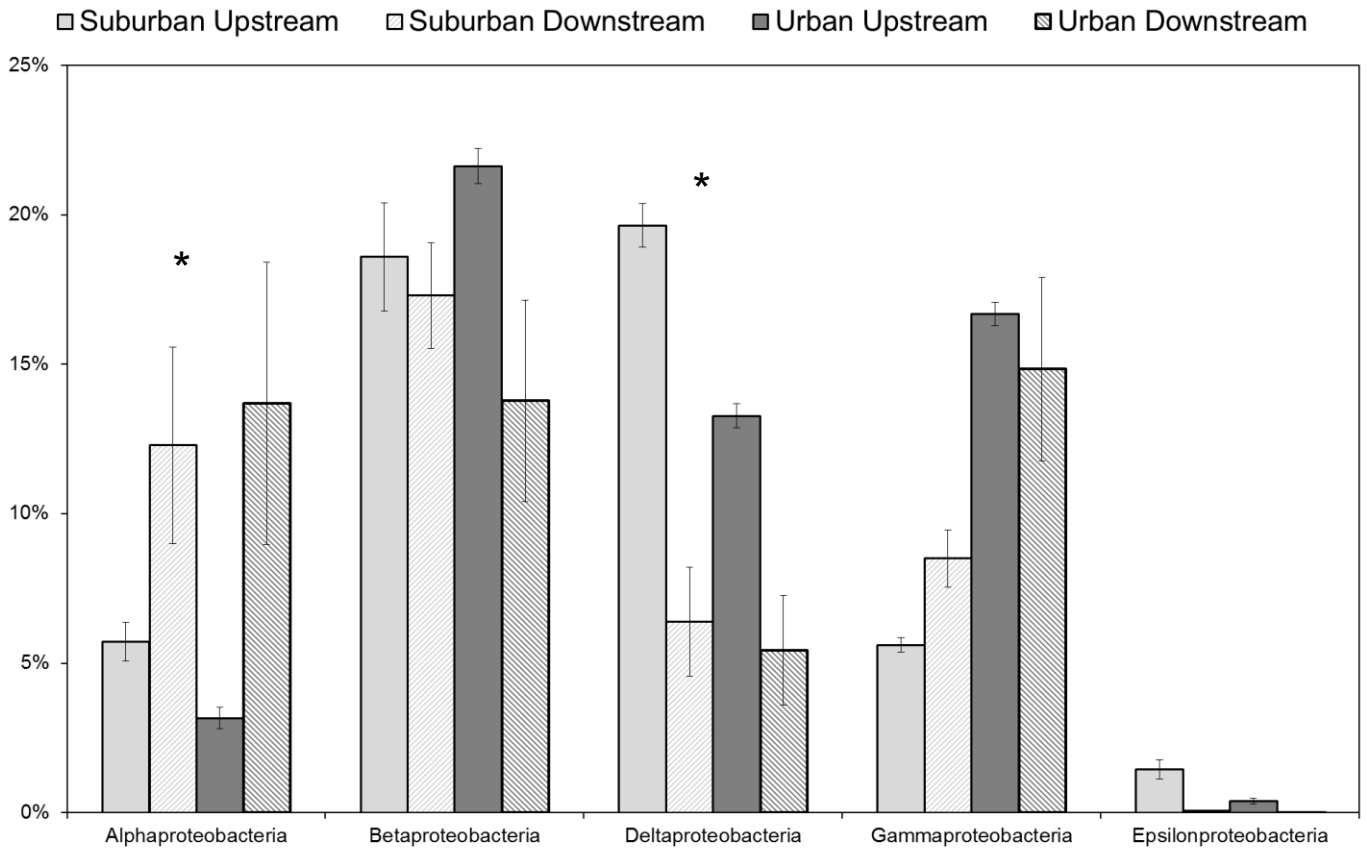
Shannon diversity index (A) and Chao 1 richness estimator (B) based on 16S tag pyrosequencing data for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Each data point is mean (n=5) ± standard error. Two-way ANOVA (A) demonstrated no significant effect of land use (p=0.328), but a significant effect of effluent input (p=0.019) and no significant interaction effect (p=0.936). Two-way ANOVA (B) demonstrated no significant effect of land use (p=0.604), but a significant effect of effluent input (p=0.000) and no significant interaction effect (p=0.895).

Figure 6. Phylotype Analysis of 16S Tag Pyrosequencing Data of Sediment Bacterial Communities



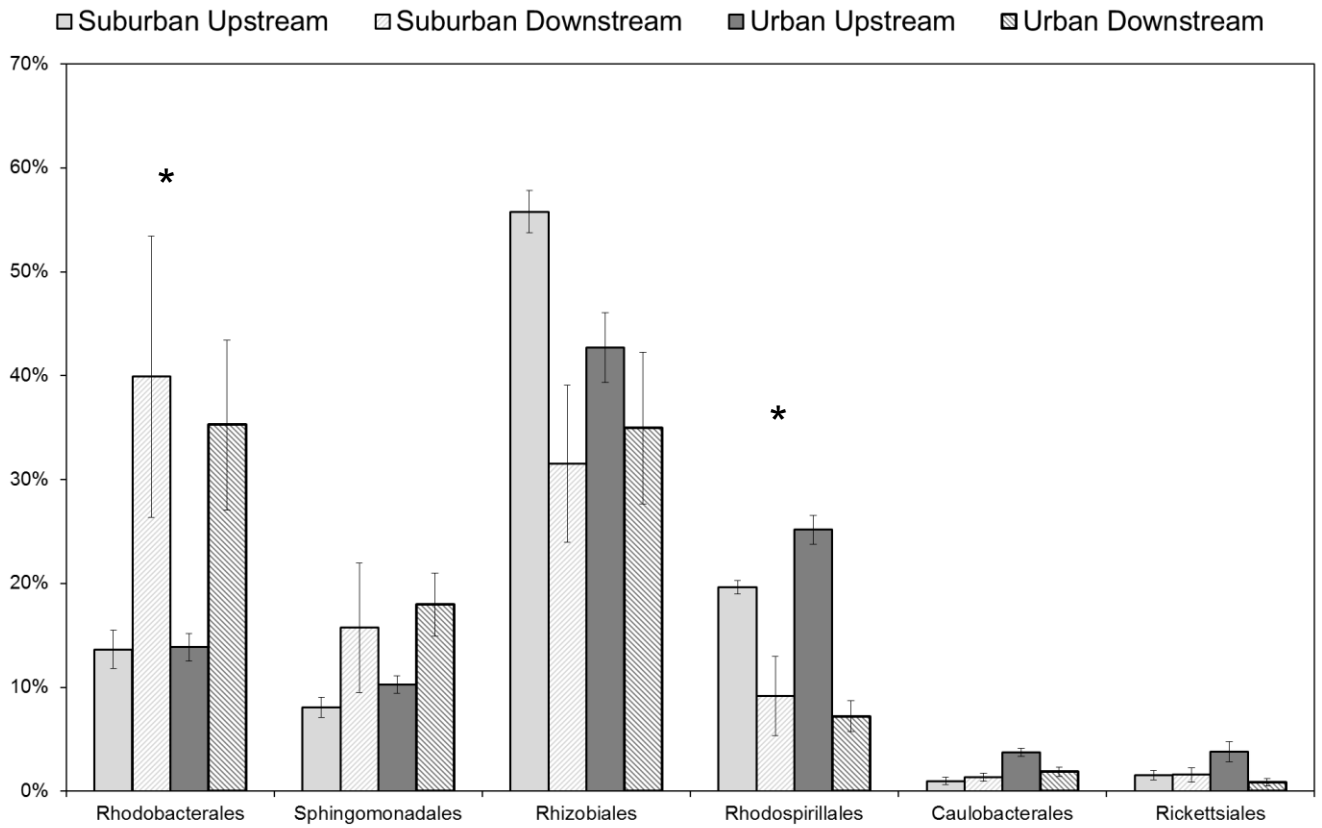
Phylotype analysis of 16S tag pyrosequencing data for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Y axis represents the percentage of all sequences within a sample that were within the phylotype listed on the x-axis. Asterisk (*) indicates a significant effect of effluent input ($p<0.05$).

Figure 7. Phylotype Analysis of Proteobacterial Sequences



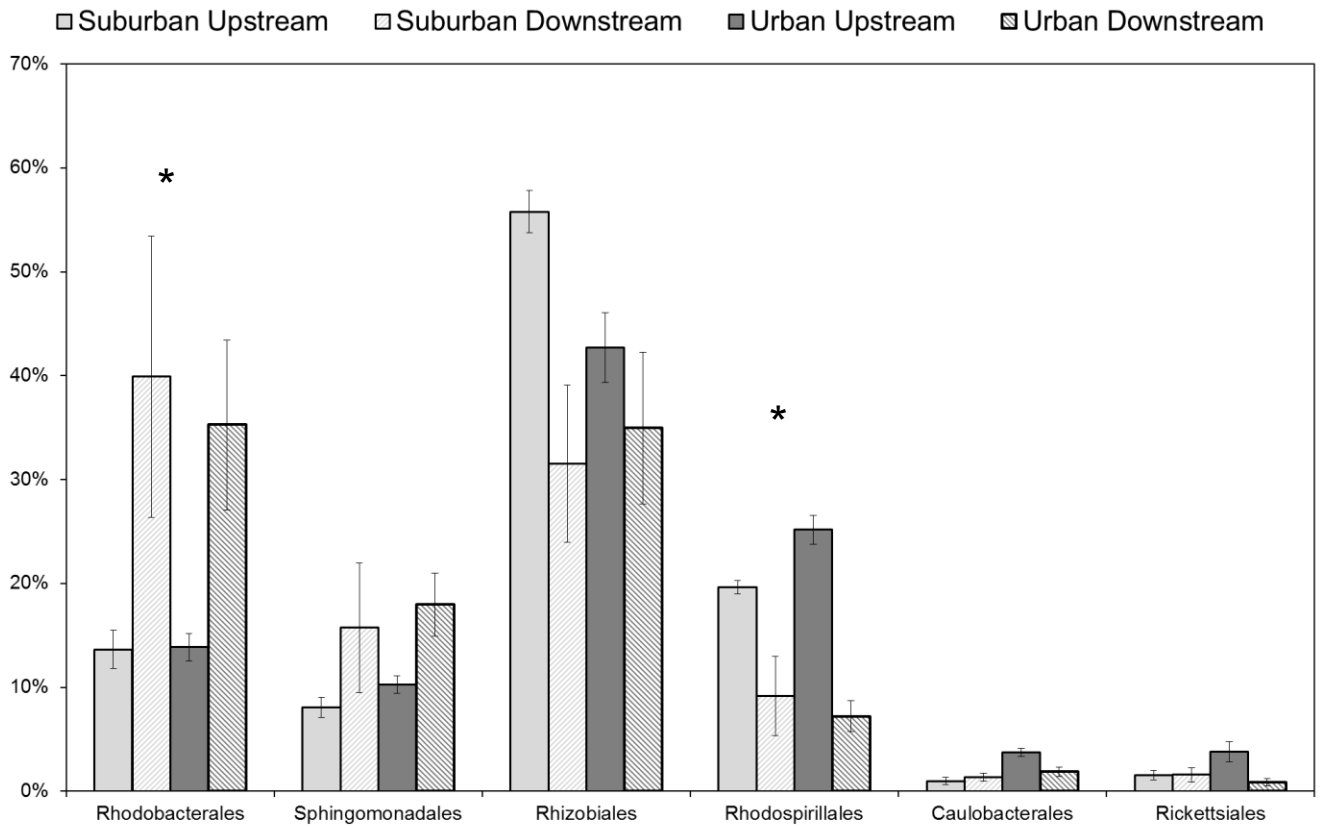
Phylotype analysis of proteobacterial sequences within 16S tag pyrosequencing data sets for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Y axis represents the percentage of all sequences within a sample that were within the proteobacterial phylotype listed on the x-axis. Asterisk (*) indicates a significant effect of effluent input ($p<0.05$).

Figure 8. Phylotype Analysis of Alphaproteobacterial Sequences



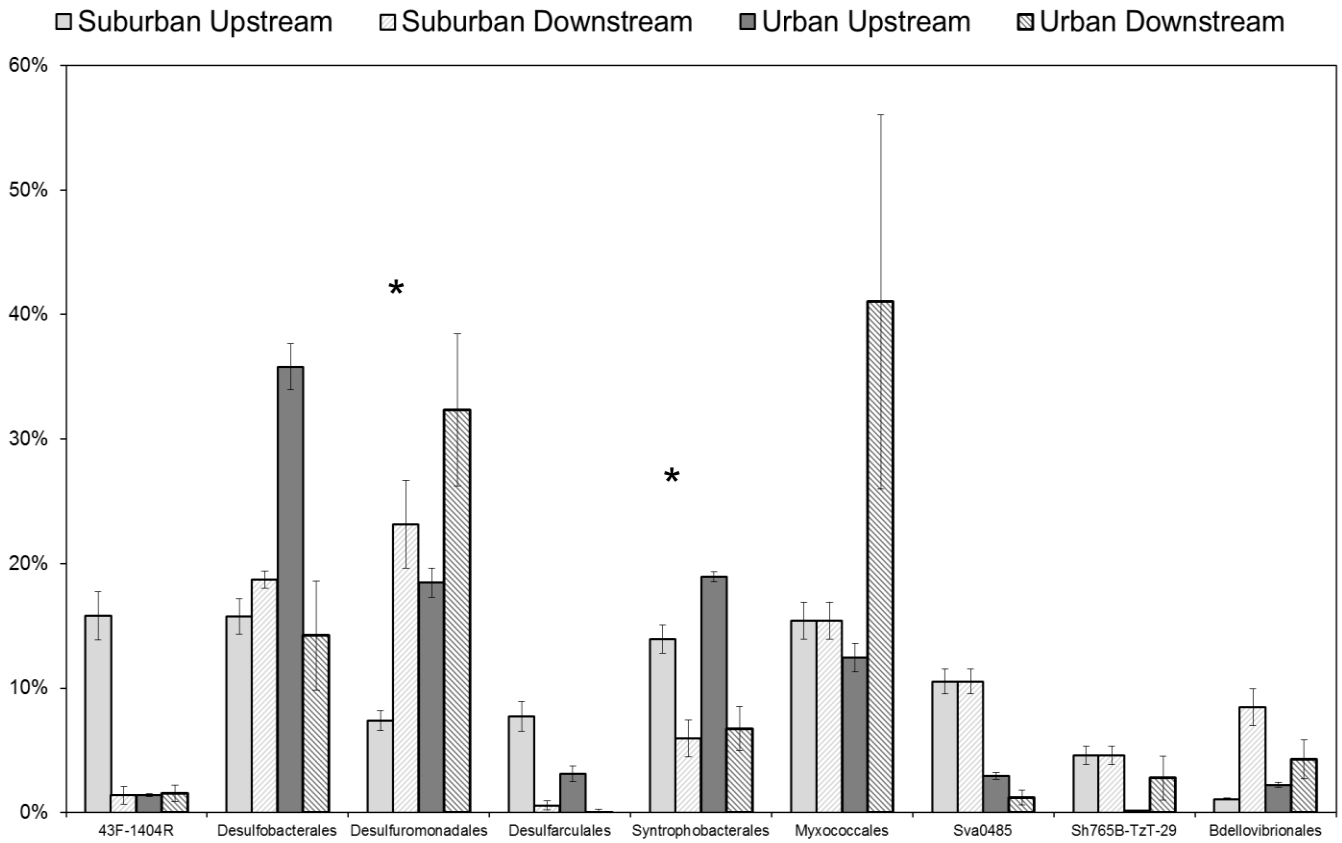
Phylotype analysis of alphaproteobacterial sequences within 16S tag pyrosequencing data sets for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Y axis represents the percentage of alphaproteobacterial sequences within a sample that were within the phylotype listed on the x-axis. Asterisk (*) indicates a significant effect of effluent input ($p<0.05$).

Figure 9. Phylotype Analysis of Betaproteobacterial Sequences



Phylotype analysis of betaproteobacterial sequences within the 16S tag pyrosequencing data sets for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Y axis represents the percentage of betaproteobacterial sequences within a sample that were within the phylotype listed on the x-axis. Asterisk (*) indicates a significant effect of effluent input ($p<0.05$).

Figure 10. Phylotype Analysis of Deltaproteobacterial Sequences



Phylotype analysis of deltaproteobacterial sequences within the 16S tag pyrosequencing data sets for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Y axis represents the percentage of deltaproteobacterial sequences within a sample that were within the deltaproteobacterial phylotype listed on the x-axis. Asterisk (*) indicates a significant effect of effluent input ($p<0.05$).

CHAPTER THREE

EFFECTS OF TRICLOSAN ON SEDIMENT MICROBIAL COMMUNITIES IN ILLINOIS RIVERS

Triclosan (2,4,4'-trichloro-2'-hydroxydipheyl ether, CAS 3380-34-5) is a broad-spectrum synthetic antimicrobial compound that is incorporated into numerous consumer products including soaps, detergents, cleansers, toothpastes, and deodorants (Schweizer 2001). There is growing concern about the potential ecological effects of triclosan and other pharmaceuticals and personal care products because many of these products affect biological systems and they have been detected in surface waters in the United States and Europe (Daughton & Ternes, 1999; Kolpin et al., 2002; Kanda et al., 2003; Bendz et al., 2005; Bartelt-Hunt et al., 2009; Watkinson et al., 2009). For example, between 1999 and 2000 the United States Geological Survey (USGS) analyzed water samples from 139 streams across 30 U.S. states and found contaminants including antibiotics and prescription and non-prescription drugs in approximately 80% of the streams (Kolpin et al., 2002). Triclosan was detected in approximately 58% of the streams, which ranked it in the top 10 of 95 water contaminants (Kolpin et al., 2002). The results of the USGS survey clearly demonstrate the potential environmental significance of triclosan. However this study was focused on sites with high human impacts, so it is difficult to generalize the results to streams in less impacted areas. In addition, the study only measured concentrations of contaminants in the water column. Triclosan is a lipophilic

compound with low aqueous solubility, so if triclosan is present in streams it is likely to be found at higher concentrations in the sediment. In addition, triclosan seems to be highly resistant to degradation in sediment, as it has been measured in sediment cores >30 years old (Singer et al., 2002), suggesting the possibility for long term accumulation.

If triclosan accumulates in sediments, it may influence the composition and function of sediment microbial communities because of its antimicrobial properties. Triclosan is toxic to bacteria and its mode of action is inhibition of a specific bacterial target, the enzyme enoyl-ACP reductase, which is an essential component of the fatty acid biosynthetic pathway in bacteria (Heath et al 1998). Therefore, the presence of triclosan in river sediments has the potential to negatively affect the numbers and activity of sediment bacteria. Bacteria can develop resistance to the effects of TCS through mutations in the gene encoding this enzyme, fabI (Heath et al 1999), overexpression of fabI (McMurray et al 1998) or via efflux pumps (Chuanchuen et al., 2003). Triclosan resistance is linked to resistance to other antibiotics (Chuanchuen et al., 2001; Braoudaki and Hilton, 2004), suggesting that triclosan exposure may select for resistance to therapeutically useful antibiotics. Therefore, accumulation of triclosan in sediments may drive shifts toward more triclosan resistant bacterial communities. Finally, triclosan negatively affect algal communities (Wilson et al. 2003, Proia et al 2011). The mechanism of triclosan toxicity to algae has not been identified (Proia et al 2011, Tatarazako et al. 2004). Therefore, triclosan in river sediment also has the potential to disrupt ecosystem function through negative effects on algae.

The goals of this project were to determine 1) whether triclosan could be detected in the sediments across a rural-urban gradient, and 2) if triclosan affects the sediment bacterial communities. To accomplish these goals we conducted a field study of three rivers in Illinois. The field sites were selected to represent a gradient of human impact because triclosan is a synthetic compound that is used extensively by humans but is not produced in nature. We predicted that the degree of human impact on an ecosystem to be a strong driver of triclosan contamination.

Materials and Methods: Field Sites

The North Shore Channel (NSC) is a highly impacted urbanized canal. The 12.4 km long canal that begins in Wilmette, IL, and flows south to the northeast section of the city of Chicago, IL. The canal was built in 1910 to bring water from Lake Michigan to the North Branch of the Chicago River. NSC has a drainage area of 64.8 square km of which 63% is residential, 16.7% is commercial/industrial, 10% is forest/open land, 5.4% is institutional, and 3.5% is transportation/utility (HDR Engineering, 2011). Sediment was collected from one site on the NSC that is approximately 7 km downstream of the origin of the channel at Lake Michigan (42.029496, -87.710032). This site is downstream of several combined sewer overflow (CSO) inputs and is approximately 925 m upstream of the input site of effluent from the North Side Water Reclamation Plant. This site was chosen based on preliminary data that indicated it had high concentrations of triclosan in the sediment.

The West Branch of the DuPage River (WBDR) located in DuPage County, IL is a suburban river. WBDR is 54.7 km and starts in Schaumburg, IL, flowing southward

through the county of DuPage, IL. WBDPR has a drainage area of 328.9 square km of which 32.8% is residential, 17.4% is agricultural, 16.9% is vacant, 11.2% is forest/open land and less than 4% is industrial (Aqua Terra Consultants, 2003). Sediment was collected from one site on the WBDR (41.866481, -88.189246) downstream of the inputs of five WWTPs and is approximately 275 meters upstream of the input site of effluent from the West Chicago WWTP. This site on WBDPR was chosen for analysis based on a preliminary study that indicated it had detectable, but low, concentrations of triclosan in the sediment.

Nippersink Creek (NC), located in McHenry County, IL is a woodland stream with low urban human impacts (www.il.nrcs.usda.gov). NC is a 37.1 km river that runs through Glacial Park, one of the many preserves/conservation areas managed by the McHenry County Conservation District. NC has a drainage area of 50.9 square km, of which 7.8% is residential, 63.1% is agricultural, 2.1% is vacant, 20.7% is open land and 0.1% is industrial (Watershed Resource Consultants Inc., Fluid Clarity Ltd., Nippersink Creek Watershed Planning Committee, 2008). Sediment was collected from one site on Nippersink Creek (42.417964, -88.344610) upstream of stream reaches where recreational activity is permitted. There are no WWTPs or CSOs on NSC upstream of our sampling location.

Materials and Methods: Sample Collection

Field work was conducted in July and August 2010. Five replicate sediment samples and five replicate water samples were collected at each of the three field sites. Each sediment sample was composed of 10 individual samples collected from randomly

selected sections along the stream reach and composited. Sediment was collected using a Petite Ponar Sampler (Wildlife Supply Company, Saginaw, MI) and large debris was removed by hand immediately after sampling. Sediment samples for triclosan analyses were stored in sterile 250mL brown amber bottles while samples for biological analyses were stored in sterile 400mL canning jars (TMs Ball Corporation, Muncie, Indiana). Water samples were collected in sterile, pre-cleaned 1L amber glass jars (Thermo Scientific, Waltham, MA). All samples were transported on ice to the laboratory and stored at 4°C. Samples for triclosan analysis were immediately shipped on ice to the Illinois Sustainable Technology Center, Champaign, IL.

Materials and Methods: Measurement of Triclosan Concentration

The triclosan concentration in the sediments was measured at the Illinois Sustainable Technology Center. Briefly, sediment samples were air dried overnight. Material larger than 1mm was removed with forceps and samples were sieved on a shaker to collect only material <0.165 cm. Sieved sediment was then homogenized with a mortar and pestle. One gram of the homogenate was extracted by an accelerated solvent extraction (ASE) procedure with a Dionex ASE 300 system. Carbon-13 enriched triclosan was spiked in to all samples to allow isotope dilution calculations and to monitor target compound loss. To minimize sample handling, the ASE method developed combined sample extraction and sample cleanup into a single step. After the sample was processed by the developed ASE method, the extract was concentrated to a final volume 2.0 mL prior to analysis. Extracts were analyzed by liquid chromatography tandem mass spectrometry (LC/MS-MS).

Materials and Methods: Sample Characteristics

Dissolved organic carbon (DOC) in water samples was measured on a Shimadzu 5050 TC Analyzer (Findlay et al., 2010). Ammonium, nitrate and phosphate concentrations in water samples were determined with a Lachat QuikChem 8000 by the phenate method (method #10-107-06-1-J, Lachat Instruments, Milwaukee, WI), the cadmium diazotization method (method #10-107-04-1-C, Lachat Instruments) and the phosphomolybdate method (method #10-115-01-1-M, Lachat Instruments) respectively. Sediment organic material was measured by loss on ignition at 500°C (Bear, 1955).

Materials and Methods: Triclosan Resistance of Bacterial Communities

The triclosan resistance of the bacterial communities was determined by performing heterotrophic plate counts on unamended soy extract agar and on soy extract agar amended with 16 mg L-1 triclosan. The percentage of the community resistant to triclosan was determined for each sample by dividing the counts obtained on the triclosan amended plates by the counts obtained on the unamended plates. Plate counts were performed by a standard method (Page, 1982). Soy extract agar plates were prepared based on the manufacturer's instructions (Becton Dickinson and Company, Sparks, MD) with the addition of 100 mg L-1 filter sterilized cycloheximide (MP Biomedicals, Solon, OH) to prevent fungal growth. Agar (1L) for triclosan plates was amended with 16 mg filter sterilized triclosan (Kansai Chemicals, Tokyo, Japan) suspended in 4mL of DMSO. For bacterial extraction, 10 g of sediment from each site was placed in a sterile 250mL HDPE container (Nalgene, Rochester, NY) containing 90mL of potassium phosphate buffer solution. Samples were agitated for 30 minutes at 300rpm using a reciprocal

shaker (New Brunswick Scientific, Edison, New Jersey). Samples were allowed to settle on the bench for 5 minutes and 1mL of supernatant was serial diluted ten-fold to 10-5. For each dilution, 100µL was plated and plates were incubated at room temperature for 48 hours. Numbers of colony forming units were normalized based on grams dry sediment.

Materials and Methods: Microbial Respiration

Respiration was measured for each sediment sample using a standard method (Hill et al., 2002). Briefly, 10 mL of sediment was placed into a black HDPE 50mL centrifuge tube (Cole-Parmer, Vernon Hills, IL) filled to the top (no head space) with well water. Water temperature and initial dissolved oxygen (DO) were measured using a YSI ProODO meter (YSI Inc. Yellow Springs, OH). Centrifuge tubes were capped, eliminating all air bubbles and incubated at room temperature (25°C) in the dark for 2 hrs, after which final DO was measured and respiration rates were calculated as mg O2 consumed time-1. Respiration rates were then normalized by sediment surface area.

Materials and Methods: Epifluorescence Counts

Direct counts of bacterial cells were performed using a method modified from Kepner Jr & Pratt (1994) (Kepner Jr & Pratt, 1994). Cells were fixed by diluting sediment 1:50 in sterile DNA-free fixative solution (10mM NaPO4, 120mM NaCl, 10mM sodium pyrophosphate, 4% formaldehyde) (Gough & Stahl, 2003) in a sterile 50 mL centrifuge tube. Samples were placed in an ultrasonic ice water bath (Model 8845-30, Cole-Parmer, Vernon Hills, IL) and sonicated for 15 minutes at 60Hz. Following ultrasonic treatment, samples were diluted 1:1,000, 1:2,000 and 1:4,000 in 0.2 µm filtered

deionized water. 2 mL of each diluted sample were filtered in duplicate onto 0.2 µm anodisc membrane filters (Whatman, Maidstone, UK) and stained with 100 µL of SYBR Gold (Invitrogen, Carlsbad, CA). Cells were counted at 400x magnification using an Olympus BH-2 Fluorescence Microscope (Olympus, Center Valley, PA). Cell numbers were normalized based on grams of dry sediment.

Materials and Methods: Tag Pyrosequencing

DNA was isolated from each of the sediment samples using the UltraClean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA). Successful DNA isolation was confirmed by agarose gel electrophoresis. For tag pyrosequencing of bacterial 16S rRNA genes extracted DNA was sent to the Research and Testing Laboratory (Lubbock, TX). 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, IN) with Titanium reagents. Sequences were processed using MOTHUR v.1.20.1 (Schloss et al., 2009). Briefly, any sequences containing ambiguities or homopolymers longer than 8 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 uniform length of 242 base pairs and chimeric sequences were removed using Chimeraslayer (Haas et al., 2011) run within MOTHUR. Sequences were grouped

into phylotypes by comparison to the SILVA-compatible bacterial alignment database available within MOTHUR and chloroplast sequences were removed. After these pretreatment steps were completed the data set included a total of 35,227 sequences for an average of 11,472 sequences per sample. Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence identity using the average neighbor algorithm. The community compositions of the individual sampling sites were compared by calculating distances between sites based on the theta index (Yue & Clayton, 2005) and visualizing the resulting distance matrix using non-metric multidimensional scaling (NMDS). MOTHUR was also used to calculate the Shannon diversity index (Shannon, 2001).

Materials and Methods: Statistics

All data were analyzed by one-way ANOVA based on land-use (urban vs. suburban vs. woodland). Pairwise comparisons were based on Tukey's honestly significant difference (HSD) test. Respiration and Shannon diversity index data were log transformed prior to ANOVA to account for unequal variances between treatments. Triclosan resistance data were arcsin-square root transformed prior to ANOVA. Correlations between selected variables were assessed by determining Pearson product-moment correlation coefficients and probabilities. All statistical analyses were run using Systat version 13 (Systat Software, Inc., San Jose, CA) and p values less than 0.05 were considered to be significant.

Results

There was a significant effect of habitat (urban vs. suburban vs. woodland) on water column nutrient concentrations and sediment organic matter in our three rivers, with the suburban river having the highest concentrations of nitrate, phosphate, and sediment organic material (Table 4). The urban site had the highest triclosan concentration, the suburban site had an intermediate concentration, and the woodland site had the lowest concentration (just above the limit of detection) ($p=0.00$; Table 5). A similar trend was observed for triclosan resistance, with the urban river having the highest resistance and the woodland the lowest (Fig. 11). The relationship between triclosan concentration and triclosan resistance of the sediment bacterial communities was confirmed by a logarithmic relationship and a significant correlation ($r=0.809$, $p=0.001$; Fig. 12).

There was no significant effect of site on the population size of sediment bacterial communities as indicated by heterotrophic plate counts (Fig. 13A) and direct counts (Fig. 13B). However, there was a significant effect of habitat on microbial community respiration, with the urban and suburban rivers having higher rates of respiration than the woodland stream (Fig. 14). The pattern observed for respiration resembled the pattern observed for sediment organic matter, and correlation analysis revealed a significant correlation ($r=0.660$, $p=0.007$) between respiration rates and sediment organic matter. NMDS analysis of 16S rRNA tag pyrosequencing data indicated that there were significant differences in the composition of sediment bacterial communities from each site (Fig. 15 and Table 6). Tag pyrosequencing also indicated the urban site had lower

diversity than the woodland and suburban sites (Fig. 16). In addition, the urban and suburban sites had significantly higher relative abundance of Proteobacteria than the woodland site ($p<0.001$; Fig. 17). The urban site had lower relative abundance of Nitrospirae than the woodland and suburban sites (Fig. 17). The woodland site had higher abundance of Firmicutes than the suburban and urban sites (Fig. 17). The woodland and suburban sites had more Alphaproteobacteria than the urban site (Fig. 18). Finally, the woodland and suburban sites had lower abundances of Gammaproteobacteria than the urban site (Fig. 18).

There were also some bacterial phyla that showed a significant habitat effect, but no trend across land-use gradient, including Bacteriodetes ($p<0.001$), Chloroflexi ($p<0.001$) (Fig. 17), and Deltaproteobacteria ($p<0.001$) (Fig. 18). For these groups, the suburban site was different than the woodland and urban sites. This trend follows some of the site physicochemical characteristics. For example, the suburban site was significantly different than the woodland and urban sites in water column $NO3^-$ and SRP concentrations and sediment organic matter (Table 4).

Discussion

Our project had two goals, first to determine whether triclosan could be detected in the sediments of Illinois rivers, and second, if triclosan was detected in sediments, to determine if triclosan was affecting the resident bacterial communities. The results of our study clearly confirmed that triclosan could be detected in rivers within Illinois, with the triclosan concentration in the sediments of our urban river exceeding sediment triclosan concentrations previously reported in the literature (Singer et al., 2002; Morales et al.,

2005). Our data also indicated that the concentration of triclosan in the river sediments increased with increasing urbanization. Specifically, the urban river, with 80% of it watershed being residential or commercial, had the highest concentration of triclosan. The suburban river, with 36% of its watershed being residential or commercial, had a much lower but still measureable concentration of triclosan. Finally, the woodland river with less than 8% of it watershed being residential and with no commercial land in the watershed, had triclosan concentrations just at or below the detection limit. This link between triclosan concentrations in river sediments and degree of urbanization has not been previously reported in the literature.

Triclosan in the sediments had a significant effect on the sediment bacterial communities. Triclosan resistance was highest at the site with the highest triclosan concentration (the urban site), and there was a significant correlation between triclosan concentration in the sediments and the triclosan resistance of the sediment bacterial communities. These data suggest that triclosan in the sediments is selecting for bacterial communities that are more resistant to triclosan. No previous studies we are aware of have demonstrated a biological effect of triclosan in the environment.

Several other aspects of the bacterial communities changed in ways that might suggest a connection to triclosan. For example, the bacterial communities from the three habitats were significantly different in respiration, with the communities from the urban and suburban rivers, which had higher concentrations of triclosan, showing higher respiration rates than the communities from the woodland river, which had almost undetectable levels of triclosan. Previous studies have indicated that respiration rates can

increase for bacterial cells that are under stress (Anderson & Domsch, 1993), so if triclosan is putting the sediment bacteria under stress it could explain the increase in respiration. However, we demonstrated that there was a significant correlation between sediment organic matter and respiration rates, a logical connection as sediment organic matter can provide a substrate for respiration of microbial and fungal cells.

In addition, the sediment bacterial communities from the three habitats differed in phylogenetic composition, with several bacterial groups, including Proteobacteria, Nitrospirae, Firmicutes, alphaproteobacteria, and gammaproteobacteria either increasing or decreasing in parallel with triclosan concentrations. These changes in bacterial community function and composition may be related to triclosan. However, it is also possible that these differences were due to other physical and chemical differences between the rivers, since there were significant differences between the three rivers in organic and inorganic nutrient content, and these factors could have significant effects on the function and composition of bacterial communities.

Conclusions

It is not possible with the data from this study alone to demonstrate that the observed changes in bacterial community composition and function were caused by triclosan and not by other differences in the field sites. In order to provide more insight into the potential biological effects of triclosan, we conducted a model stream study in which model streams were either amended with triclosan or retained as untreated controls in order to determine if we could reproduce any of the differences observed in these field sites in a controlled laboratory experiment. This model stream experiment is described in

Chapter 4 of this thesis.

Acknowledgements

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Table 4. Sampling Site Characteristics

	Value (SE)[a]		
	Woodland	Suburban	Urban
Water Column DOC (mg/L)[b]	6.313 (0.112) a	6.652 (0.052) b	2.408 (0.085) c
Water Column NH_4 (mg/L)[c]	N.D. a	0.060 (0.003) b	0.138 (0.007) c
Water Column NO_3^- (mg/L)[d]	0.014 (0.007) a	2.742 (0.140) b	0.232 (0.002) a
Water Column PO_4^{3-} (mg/L)[e]	0.003 (0.000) a	0.268 (0.006) b	0.003 (0.000) a
Sediment Organic Material (%)[f]	1.45 (0.10) a	8.70 (1.20) b	5.89 (0.43) c

[a] Each data point is mean (n=5) standard error values in parentheses.

[b] ANOVA for DOC demonstrated significant effect of habitat ($p<0.001$). Data points with different letters are significantly different based on Tukeys HSD test ($p<0.05$).

[c] NH_4 limit of detection = 0.02mg/L. ANOVA for NH_4 demonstrated significant effect of habitat ($p<0.001$). Data points with different letters are significantly different based on Tukeys HSD test ($p<0.05$).

[d] NO_3^- limit of detection = 0.02mg/L. ANOVA for NO_3^- demonstrated significant effect of habitat ($p<0.001$). Data points with different letters are significantly different based on Tukeys HSD test ($p<0.05$).

[e] PO_4^{3-} limit of detection = 0.002mg/L. ANOVA for PO_4^{3-} demonstrated significant effect of habitat ($p<0.001$). Data points with different letters are significantly different based on Tukeys HSD test ($p<0.05$).

[f] ANOVA for Organic Material demonstrated significant effect of habitat ($p<0.001$). Data points with different letters are significantly different based on Tukeys HSD test ($p<0.05$).

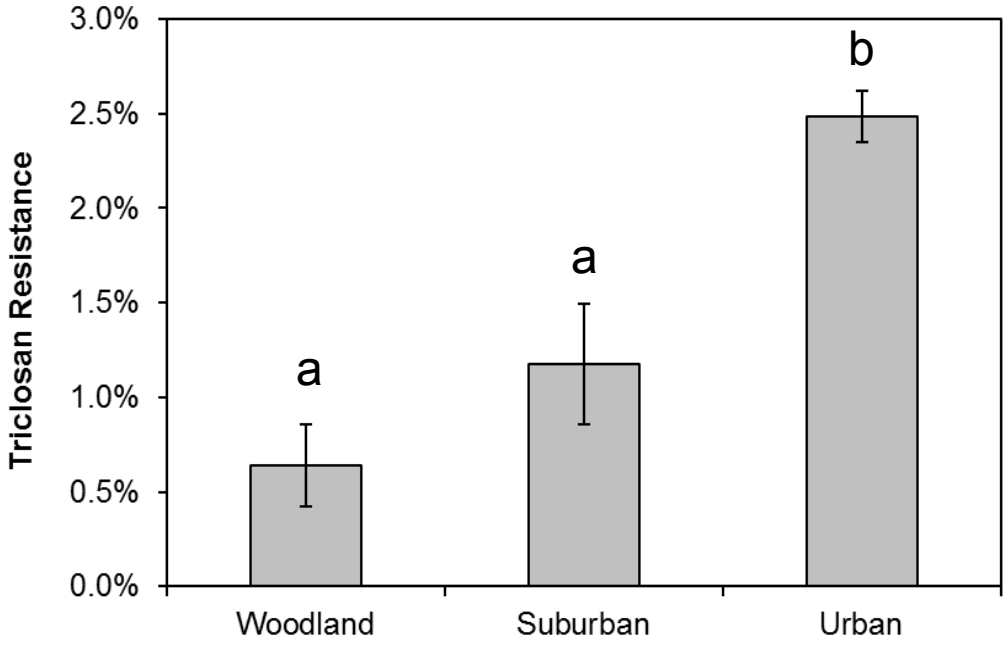
Table 5. Field Site Sediment Triclosan Concentrations

	Value (SE)[a]
	Sediment Triclosan Concentration (ng/g)[b]
Woodland	1.425 (0.014) a
Suburban	8.520 (3.288) a
Urban	106.632 (18.134) b

[a] Each data point is mean (n=3)with standard error values in parentheses

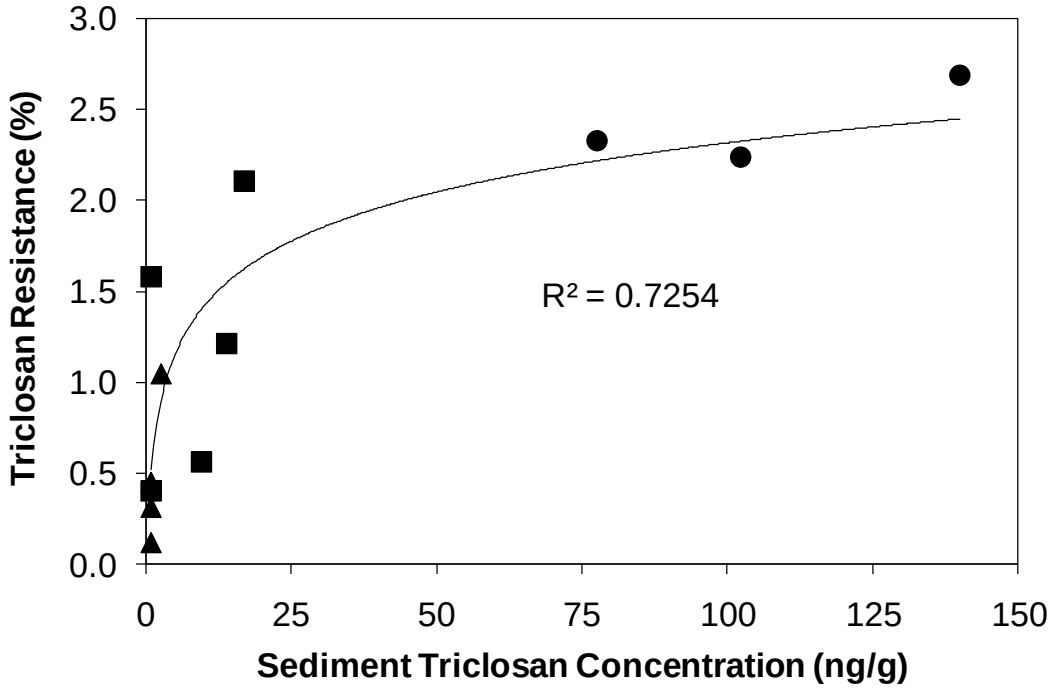
[b] Limit of detection = 1.0ng/g. ANOVA demonstrated significant effect of habitat ($p<0.001$). Letters after data points indicate significant differences based on Tukey's HSD test ($p<0.05$).

Figure 11. Percent Triclosan Resistance of Bacterial Communities from Field Sites



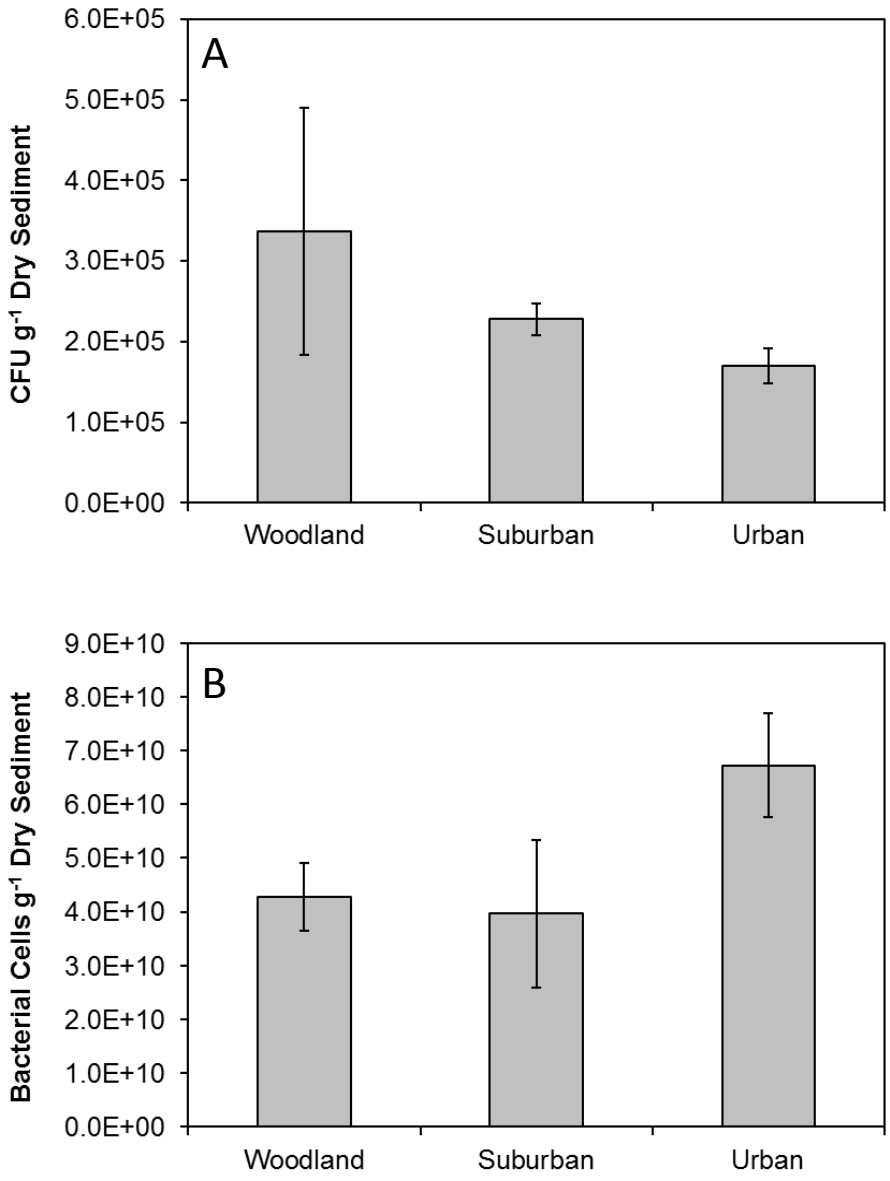
Percent triclosan resistance for sediments collected from rivers in three habitat types (woodland, suburban and urban). Each data point is mean (n=5) +/- standard error. ANOVA indicated significant effect of habitat ($p=0.002$). Data points labeled with different letters are significantly different based on Tukey's HSD test ($p<0.05$)

Figure 12. Correlation Analysis: Percent Triclosan Resistance



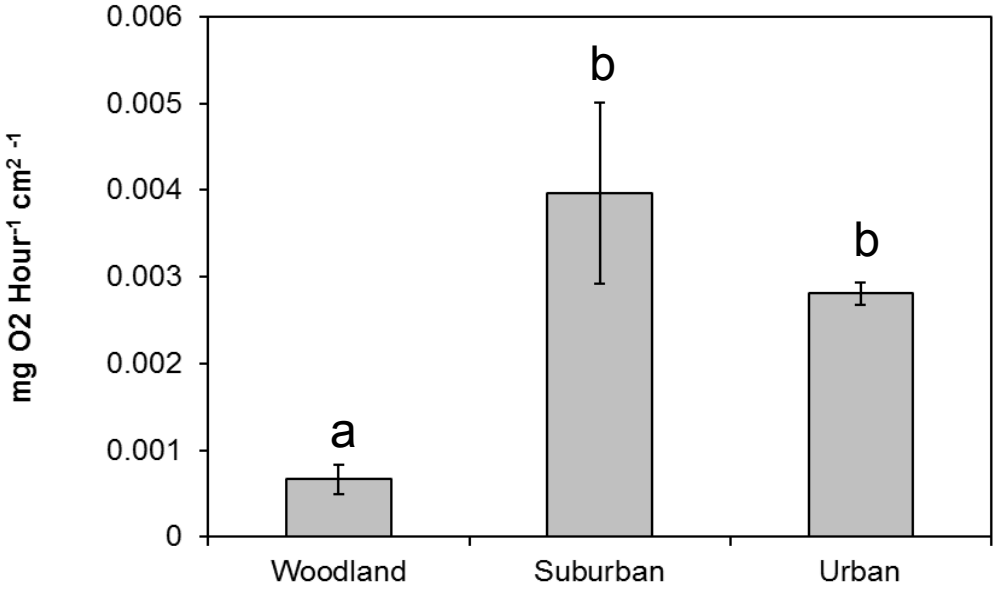
Relationship between sediment triclosan concentration and percent triclosan resistance of the sediment bacterial communities. Woodland (▲) suburban (■) urban (●). Line represents logarithmic regression. Correlation analysis resulted in r value of 0.809 and p=0.001.

Figure 13. Heterotrophic Plate Counts for Sediments Collected from Field Sites



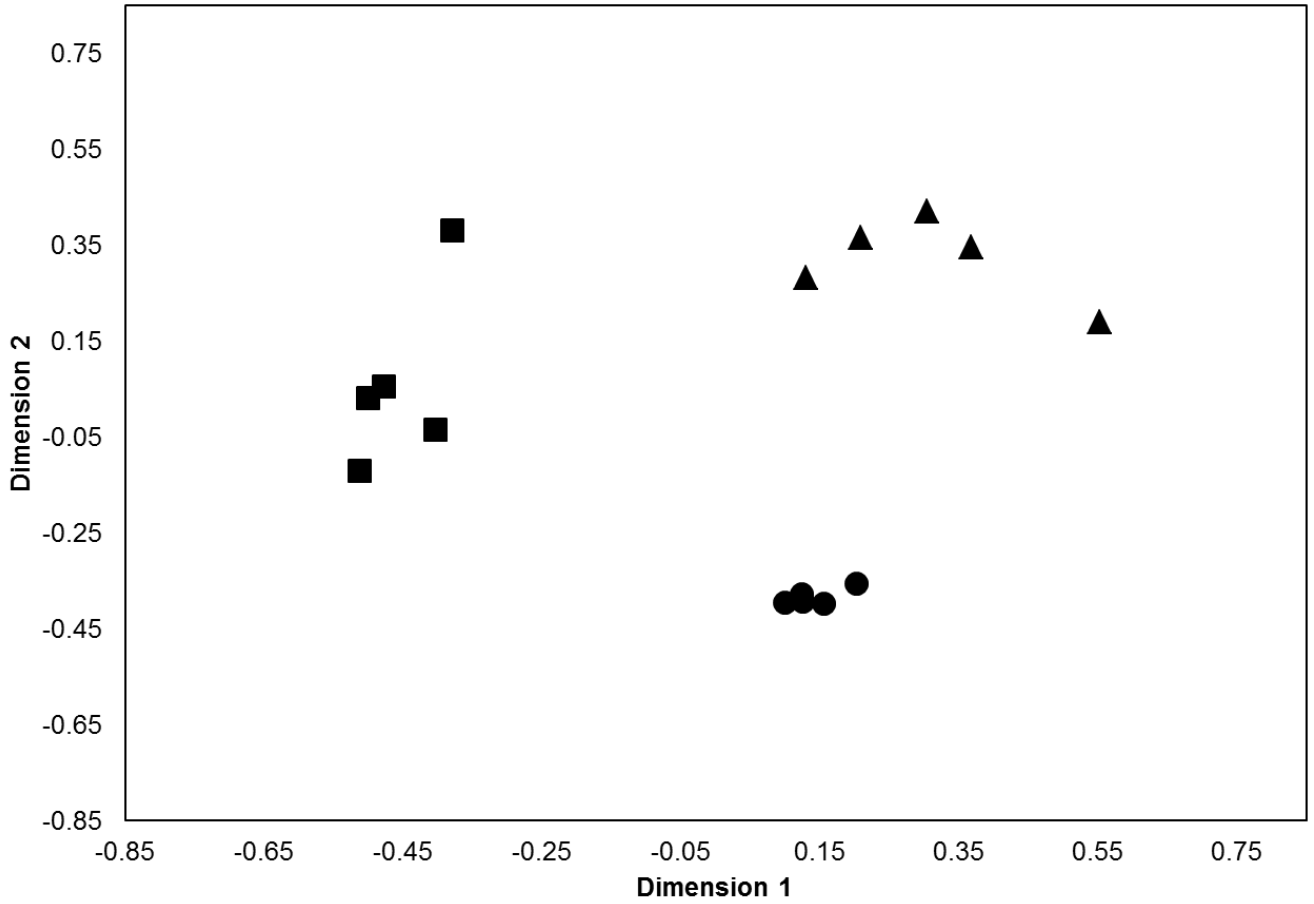
Heterotrophic plate counts (A) and direct bacterial cell counts (B) for sediments collected from rivers in three different habitats (woodland, urban and suburban). Each data point is mean (n=5) +/- standard error. ANOVAs demonstrated no significant effect of habitat on plate counts (p=0.440) or direct counts (p=0.160).

Figure 14. Community Respiration for Sediments Collected from Field Sites



Community respiration for sediments collected from rivers in three different habitats (woodland, urban and suburban). Each data point is mean (n=5) +/- standard error. ANOVA demonstrated a significant effect of habitat ($p=0.001$). Data points labeled with different letters are significantly different based on Tukey's LSD Test ($p<0.05$).

Figure 15. NMDS Ordination of 16S Tag Pyrosequencing Data from Field Sites



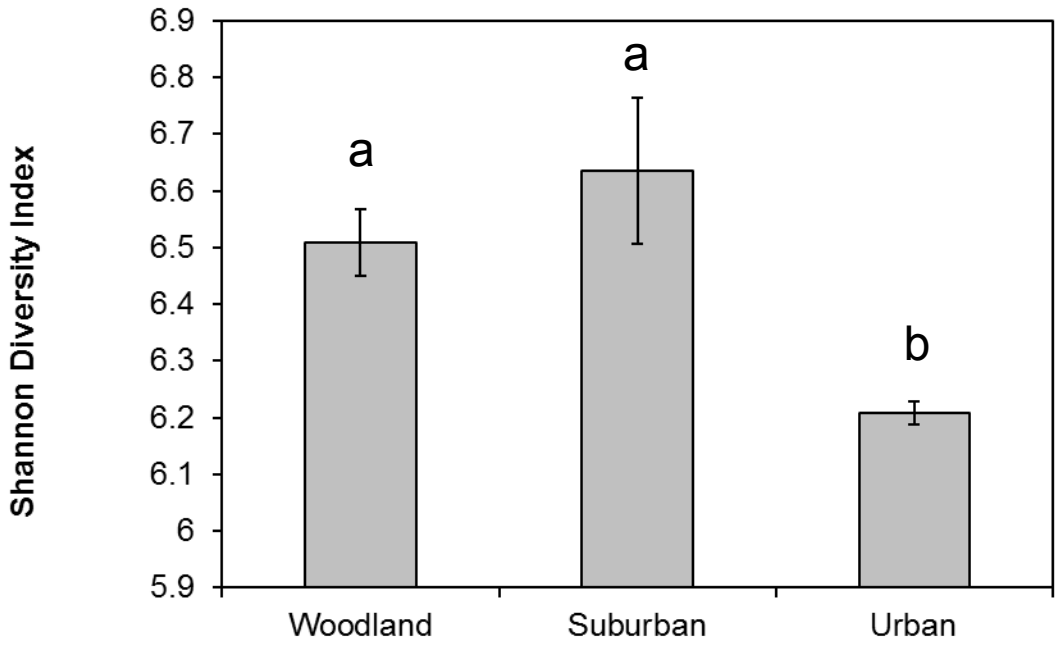
NMDS ordination based on 16S tag pyrosequencing data showing community structure of bacterial sediment communities collected from rivers in three habitat types (woodland, suburban and urban). Woodland (▲), suburban (■), urban, (●).

Table 6. Statistical Analysis of 16S Tag Pyrosequencing Data: Field Sites

	Value
	P-value
Interaction Effect	<0.001
Suburban vs. Urban	<0.001
Suburban vs. Woodland	0.001
Urban vs. Woodland	0.004

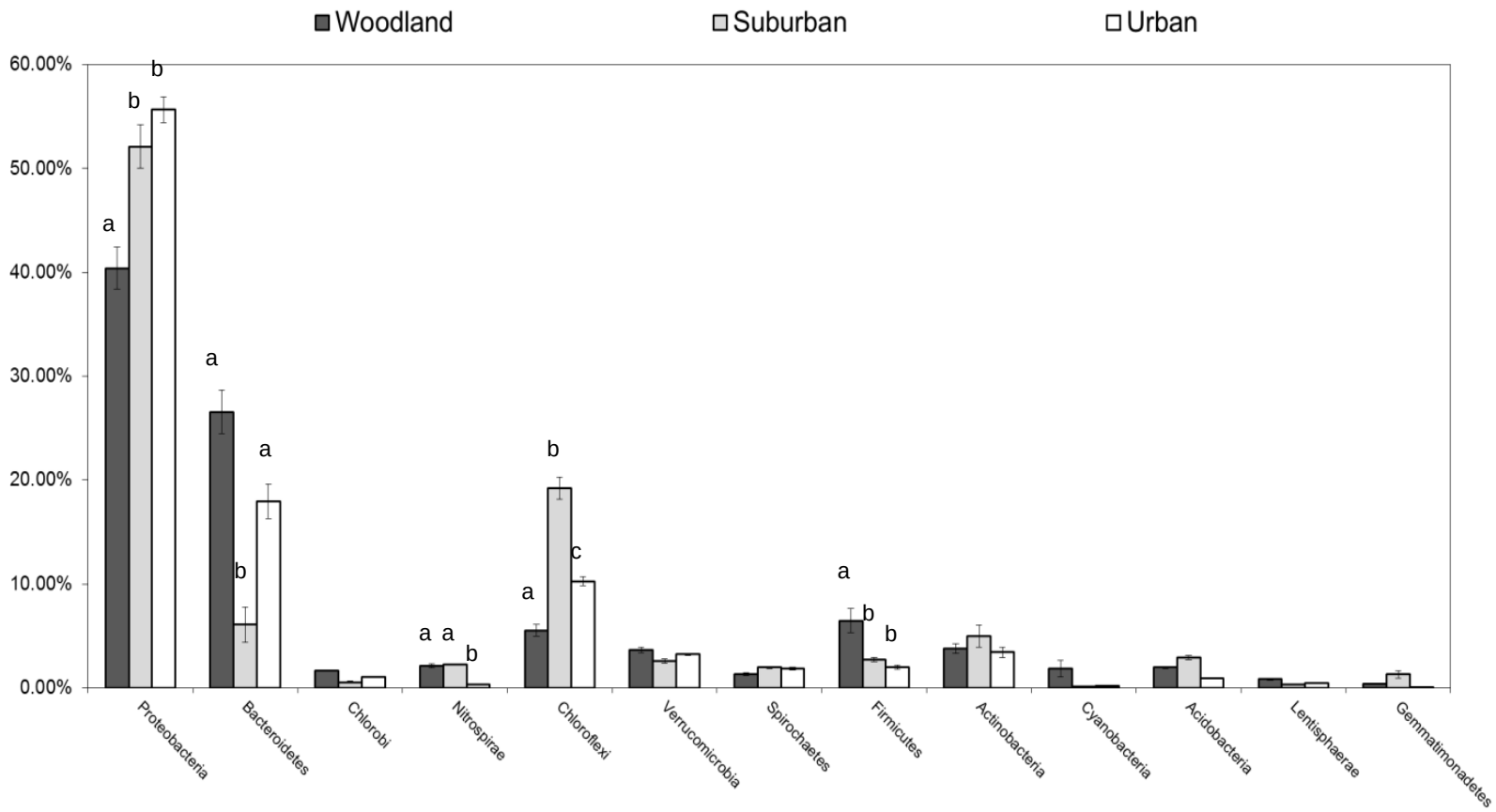
Statistical analysis of NMDS ordination of 16S tag pyrosequencing data presented in Figure 15.

Figure 16. Shannon Diversity Index of Field Site Sediments



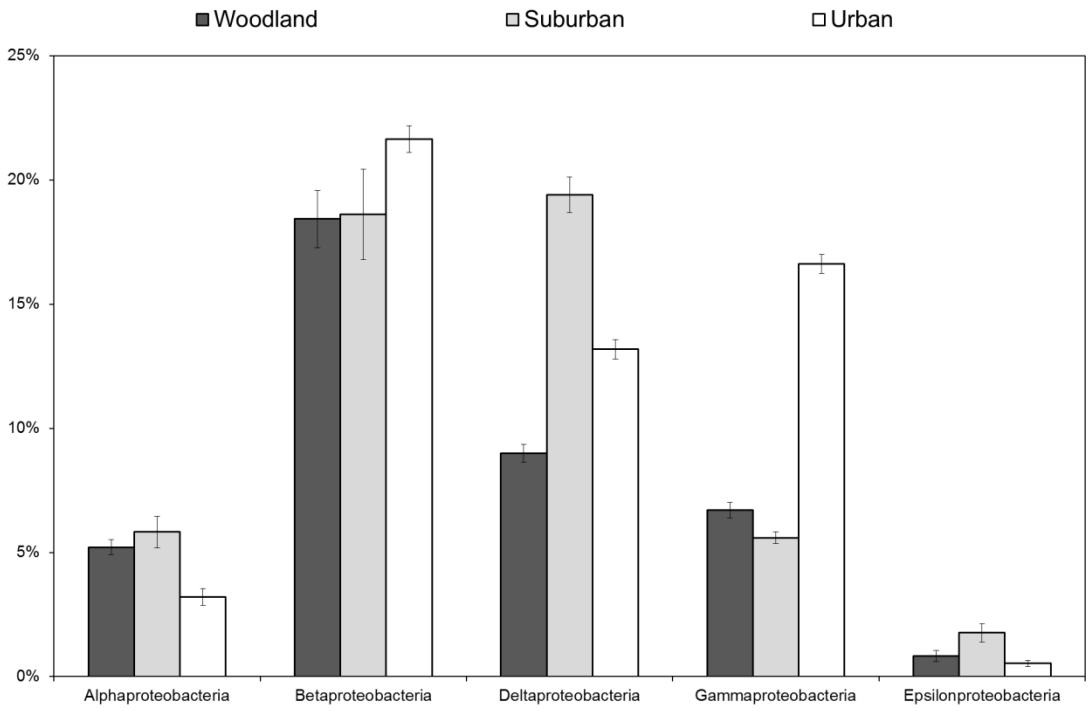
Shannon diversity index for sediments collected from rivers in three habitat types (woodland, suburban and urban). Each data point is mean (n=5) +/- standard error. ANOVA indicated a significant effect of habitat (p=0.008). Data points labeled with different letters are significantly different based on Tukey's HSD Test (p<0.05).

Figure 17. Phylotype Analysis of 16S Tag Pyrosequencing Data from Field Site Sediments



Phylotype analysis of 16S tag pyrosequencing data for sediment bacterial communities collected from rivers in three habitat types (urban, suburban and woodland). Y axis represents the percentage of all sequences within a sample that were within the phylotype listed on the x-axis. Phylotypes labeled with letters indicate significant habitat effect ($p<0.05$) and within a phylotype data points with different letters are significantly different based on Tukey's HSD Test ($p<0.05$).

Figure 18. Phylotype Analysis of Proteobacterial Sequences from Field Site Sediments



Phylotype analysis of proteobacterial sequences within 16S tag pyrosequencing data sets for sediment bacterial communities collected from rivers in three habitat types (urban, suburban and woodland). Y axis represents the percentage of all sequences within a sample that were within the phylotype listed on the x-axis. Phylotypes labeled with letters indicate significant habitat effect ($p<0.05$) and within a phylotype data points with different letters are significantly different based on Tukey's HSD Test ($p<0.05$).

CHAPTER FOUR

EFFECTS OF TRICLOSAN ON SEDIMENT MICROBIAL COMMUNITIES, A MODEL STREAM STUDY

Pharmaceuticals and personal care products (PPCPs) are a class of compounds that are of growing concern to aquatic ecologists (Rosi-Marshall & Royer, 2012). PPCPs include prescriptions and over-the-counter therapeutic drugs such as antibiotics, antihistamines, and analgesics; antibacterial products such as soaps and detergents; and fragrances and cosmetics. The widespread use of PPCPs due to their incorporation in many consumer products has led to detection of these compounds in the environment, including lotic ecosystems (Daughton & Ternes, 1999; Kolpin et al., 2002; Kanda et al., 2003; Bendz et al., 2005; Bartelt-Hunt et al., 2009). The antimicrobial compound triclosan was one of the most frequently detected compounds in a recent survey of streams and rivers in the United States, in approximately 58% of the 139 streams tested (Kolpin et al., 2002).

Triclosan (2,4,4'-trichloro-2'-hydroxydipheyl ether, CAS 3380-34-5) is a broad-spectrum synthetic antimicrobial compound that is incorporated into numerous consumer products including soaps, detergents, cleansers, toothpastes, and deodorants (Schweizer 2001). Triclosan has been detected in lotic ecosystems (Adolfsson-Erici et al., 2002; Kolpin et al., 2002; Lindström et al., 2002; McAvoy et al., 2002; Singer et al., 2002; Sabaliunas et al., 2003; Morrall et al., 2004; Bester, 2005). However, most of these

studies have measured triclosan concentrations in the water column, and few have measured triclosan concentrations in sediments (Singer et al., 2002; Morales et al., 2005). Due to the hydrophobic nature of triclosan, it is likely to bind to sediments and accumulate in quantities that exceed those that have been measured in surface waters. Triclosan is also a stable compound that is highly resistant to degradation once in sediments, and it has been detected in sediment cores >30 years old (Singer et al., 2002).

If triclosan accumulates in sediments, it may influence the composition and function of sediment microbial communities because of its antimicrobial properties. Triclosan is toxic to bacteria and its mode of action is inhibiting the enzyme enoyl-ACP reductase, which is an essential component of the fatty acid biosynthetic pathway in bacteria (Heath et al 1998). Therefore, the presence of triclosan in river sediments has the potential to negatively affect the number and activity of sediment bacteria. Bacteria can develop resistance to the effects of triclosan through mutations in the gene encoding this enzyme, fabI (Heath et al 1999), overexpression of fabI (McMurray et al 1998) or via efflux pumps (Chuanchuen et al., 2003). Several studies have linked triclosan resistance to resistance of other antibiotics (Chuanchuen et al., 2001; Braoudaki and Hilton, 2004), suggesting that triclosan exposure may select for resistance to therapeutically useful antibiotics. Therefore, accumulation of triclosan in sediments has the potential to drive shifts in bacterial communities toward more triclosan resistant groups. Triclosan also negatively affects algal communities (Wilson et al. 2003, Proia et al 2011). Although the mechanism of triclosan toxicity to algae has not been identified (Proia et al 2011) some studies have suggested that algae may be more sensitive to triclosan than bacteria

(Tatarazako et al. 2004). Therefore, triclosan in river sediments also has the potential to disrupt ecosystem function through negative effects on algae.

Despite the detection of triclosan in a wide variety of lotic ecosystems and the potential for triclosan to have negative effects on microbial communities, we are not aware of any study that has examined the effects triclosan on the composition and function of sediment microbial communities in these ecosystems. To address this knowledge gap, we conducted a field study (described in Chapter 3 of this thesis) in which we documented the presence of triclosan in rivers in northern Illinois, across an urban-rural gradient. There was a significant correlation between sediment triclosan concentration and the triclosan resistance of the sediment bacteria. In this field study we also observed some significant variation in the composition of the sediment bacterial communities among the three rivers. While some of these changes in bacterial taxa might have been driven by triclosan, it was not possible to separate the effects of triclosan from the effects of other physical and chemical variations in the three field sites.

The goal of this study was to assess the effects of triclosan on the composition and function of sediment bacterial communities through a laboratory experiment. Our prediction was that triclosan addition would select for a sediment bacterial community with elevated triclosan resistance, as we had observed in our field study in the highly contaminated urban river.

Materials and Methods: Artificial Streams

Model stream experiments were conducted from July through December 2011 using 6 artificial streams located in an indoor greenhouse facility at Loyola University

Chicago whose windows block 50% of incoming solar radiation. Oval, racetrack-style recirculating artificial streams (4 m x 15.5 cm x 15 cm) were constructed of composite fiberglass and have a streambed surface area of 0.62 m2. Model stream sediments were composed of 0.5kg pea gravel, 9.5kg sand, and 66.67g each of red maple, ginkgo and oak leaves that had been dried and leached to allow for complete removal of tannins. Addition of leaf material provided ~2% by weight organic material to stream sediments which approximates the sediment organic matter concentration of the woodland field site examined in our field study (see Chapter 3 of this thesis). Streams were filled with 60L of dechlorinated tap water and refilled each week to compensate for evaporative loss and sampling. Nutrient concentrations in the water were as follows: ammonium (NH_4^+): mean = 59 µg L-1, soluble reactive phosphorus (SRP): mean = 111 µg L-1, nitrate/nitrite (NO_3^-/NO_2^-): mean = 45 µg L-1. Current velocity was maintained at 0.18 m s-1 by a Dayton DC gear motor (model 42129b) and a Dayton DC speed control (model 5X412D) (Dayton DC Gear Motor, Niles, Illinois) connected to a stainless steel paddlewheel. To provide an inoculum of microbes each stream was amended with 100mL of sediment collected from the woodland field site examined in our field study (see Chapter 3 of this thesis) which had a very low sediment concentration of triclosan (1.4 ng g-1). Streams were covered with shade cloth (50% light reduction) to limit algal growth. Streams were run for two months prior to beginning triclosan treatments in order to allow for adequate colonization of the sediments by microbes.

Materials and Methods: Triclosan Treatment

After the two month pretreatment period three treatment streams were amended with triclosan and three control streams received no triclosan. The goal for the treatment streams was to bring the sediment triclosan concentration to 200ng g-1, a value approximately two times higher than the triclosan concentration we observed in a highly contaminated urban river in Chicago (see Chapter 3 of this thesis). To achieve this we added to each treatment stream an amount of triclosan that would exceed the aqueous solubility of triclosan (10mg L-1) (Morrall et al., 2004) by 200 ng g-1 sediment. 722mg triclosan (Kansai Chemicals, Tokyo, Japan) was dissolved in 25mL of dimethyl sulfoxide (DMSO; Fisher, Pittsburgh, PA) in a methanol rinsed container and this solution was added to the stream. Control streams received 25 mL DMSO with no triclosan.

Materials and Methods: Sample Collection

Sediment samples were collected through November and December (2011) from each stream immediately prior to dosing (day 0) and at approximately weekly intervals post-dosing. Sediment samples were collected prior to refilling stream water. Each sediment sample was a composite sample composed of 20 individual 5mL sediment samples collected from randomly selected locations along the length of each artificial stream. All biological assays were conducted on the same day samples were collected. Sediment samples for Triclosan analyses were stored in heat sterilized, methanol rinsed, 100mL brown amber jars (Fisher, Pittsburgh, PA) and stored at 4°C. Water samples for triclosan analysis were collected in 1L methanol rinsed, pre-cleaned I-Chem 200 Series glass amber bottles (I-Chem, Rockwood, TN) and stored at 4°C. Samples for triclosan

analysis were shipped on ice to Illinois Sustainable Technology Center, Champaign, IL.

Materials and Methods: Epifluorescence Counts

Direct counts of bacterial cells were performed using a modified standard method (Kepner Jr & Pratt, 1994). Cells were fixed by diluting sediment 1:50 in sterile DNA-free fixative solution (10mM NaPO4, 120mM NaCl, 10mM sodium pyrophosphate, 4% formaldehyde) (Gough & Stahl, 2003) in a sterile 50 mL centrifuge tube. Samples were placed in an ultrasonic ice water bath (Model 8845-30, Cole-Parmer, Vernon Hills, IL) and sonicated for 15 minutes at 60Hz. Following ultrasonic treatment, samples were diluted 1:1,000, 1:2,000 and 1:4,000 in 0.2 µm filtered deionized water. 2 mL of each diluted sample were filtered in duplicate onto 0.2 µm anodisc membrane filters (Whatman, Maidstone, UK) and stained with 100 µL of SYBR Gold (Invitrogen, Carlsbad, CA). Cells were counted at 400x magnification using an Olympus BH-2 Fluorescence Microscope (Olympus, Center Valley, PA). Cell numbers were normalized based on grams of dry sediment.

Materials and Methods: Triclosan Resistance of Bacterial Communities

The triclosan resistance of the bacterial communities was determined by performing heterotrophic plate counts on unamended soy extract agar and on soy extract agar amended with 16 mg L-1 triclosan. The percentage of the community resistant to triclosan was determined for each sample by dividing the counts obtained on the triclosan amended plates by the counts obtained on the unamended plates. Plate counts were performed by a standard method (Page, 1982). Briefly, soy extract agar plates were prepared based on the manufacturer's instructions (Becton Dickinson and Company,

Sparks, MD) with the addition of 100 mg L-1 filter sterilized cycloheximide (MP Biomedicals, Solon, OH) to inhibit fungal growth. Agar (1 L) for triclosan plates was amended with 16mg filter sterilized triclosan (Kansai Chemicals, Tokyo, Japan) suspended in 4mL of DMSO. For bacterial extraction, 10 grams of sediment from each site was placed in a sterile 250mL HDPE container (Nalgene, Rochester, NY) containing 90mL of potassium phosphate buffer solution. Samples were agitated for 30 minutes at 300rpm using a reciprocal shaker (New Brunswick Scientific, Edison, New Jersey). Samples were allowed to settle on the bench for 5 minutes and 1mL of supernatant was serial diluted ten-fold to 10-5. For each dilution, 100µL was plated and plates were incubated at room temperature for 48 hours. Number of colony forming units was normalized by grams dry sediment.

Materials and Methods: Microbial Respiration and Photosynthesis

Respiration was measured for each sediment sample using a standard method (Hill et al., 2002). Briefly, 10 mL of sediment was placed into a black HDPE 50mL centrifuge tube (Cole-Parmer, Vernon Hills, IL) filled to the top (no head space) with well water. Water temperature and initial dissolved oxygen (DO) were measured using a YSI ProODO meter (YSI Inc. Yellow Springs, OH). Centrifuge tubes were capped, eliminating all air bubbles and incubated at room temperature (25°C) in the dark for 2 hrs, after which final DO was measured and respiration rates were calculated as mg O2 consumed time-1. Respiration rates were then normalized by sediment surface area. Net primary production in stream water was measured based on net oxygen production by a modified method using clear HDPE 50mL centrifuge tubes that were incubated in the

light for 2hrs. Photosynthetic rates calculated as mg O2 produced time-1 were normalized by grams ash free dry mass.

Materials and Methods: Tag Pyrosequencing

DNA was isolated from each of the sediment samples using the UltraClean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA). Successful DNA isolation was confirmed by agarose gel electrophoresis. For tag pyrosequencing of bacterial 16S rRNA genes extracted DNA was sent to the Research and Testing Laboratory (Lubbock, TX).

Materials and Methods: Statistics

All data were analyzed by repeated measures ANOVA except data for photosynthesis (one sampling date) which was analyzed by one-way ANOVA. For heterotrophic plate counts (log transformed) and triclosan resistance the data for the triclosan treatments and control treatments was also analyzed individually by one-way ANOVA to assess changes within treatments over time. Analyses were run using Systat version 13 (Systat Software, Inc., San Jose, CA) and p values less than 0.05 were considered significant.

Results

There was no effect of triclosan on the size of the sediment bacterial communities as indicated by direct epifluorescence counts of bacterial cells (Fig. 19). However, triclosan amended streams showed higher heterotrophic plate counts on all days post-treatment ($p<0.005$; Fig. 20). The numbers of heterotrophic bacteria within the sediments of the triclosan amended streams increased one week after dosing, then steadily decreased, until by day 34 the numbers were equivalent to what they had been at day 0

(Fig. 20). In contrast, there was no difference in heterotrophic plate counts for the control streams over the sampling dates ($p=0.186$).

We noted a dramatic die-off of algae within the triclosan amended streams within the first week post-dosing. During the pretreatment period all of the streams had developed significant algal communities attached to the sides of the streams. In the triclosan amended streams these attached algal communities died and sloughed off the sides of the streams within the first week post-dosing and never reappeared in the triclosan streams during the course of the experiment. We measured photosynthesis in all of the streams on day 8 and found triclosan decreased net primary production ($p<0.05$; Fig. 21). Low primary production is reflected in lower dissolved oxygen ($p<0.005$) in triclosan streams at day 7 through day 34 (Fig. 22). This change in dissolved oxygen was likely driven by the relative decrease in photosynthesis as there was no significant effect of triclosan on rates of microbial community respiration ($p=0.785$; Fig. 23).

There was a significant increase in sediment bacteria that were resistant to triclosan ($p<0.001$) in the triclosan amended streams showing higher resistance levels on all days post-treatment (Fig. 24). In the triclosan amended streams, the percentage of sediment bacteria that were resistant to triclosan increased steadily throughout the course of the experiment ($p<0.001$; Fig. 24). In contrast the control streams showed no difference in the percentage of sediment bacteria that were resistant to triclosan over the sampling dates ($p=0.626$).

Discussion

This study was designed to introduce an environmentally relevant concentration of triclosan into model stream sediments and monitor the resulting effects on the size, composition and function of sediment communities. The results clearly demonstrated that triclosan amendments resulted in triclosan resistant bacterial communities in the model stream sediments. Planned analysis of the species composition of the bacterial communities of the triclosan amended and control streams via tag pyrosequencing of bacterial 16S rRNA genes will enable us to assess any bacterial community changes caused by triclosan. Unexpectedly, the percentage of resistant organisms in the triclosan amended streams continued to increase steadily during the 34 days of the study. This may reflect a gradual shift in the microbial communities, driven either by gradual changes in species composition toward more resistant species, or by horizontal transfer of triclosan resistance genes. This may also reflect changes in the triclosan concentration in the sediments during the course of the study, and forth coming analysis of triclosan concentrations in the water and sediments will provide insight into this issue.

Some of the results of the study were unexpected. For example, given the antibacterial properties of triclosan (Heath et al 1999) it was surprising that there was no effect of triclosan amendment on the size of the microbial communities as indicated by direct epifluorescence counts of bacterial cells. Triclosan increased bacterial cell mortality in laboratory incubated biofilms grown on glass slides (Proia et al 2011), but no studies we are aware of have determined the ability of triclosan to kill bacterial cells within a complex matrix like sediment. Therefore, the results of our study suggest that

triclosan's toxicity is not as high within sediment as it is in the water-column. Given triclosan's lipophilic nature, it is possible that the triclosan sorbed to organic material within the sediments, thus reducing its availability to sediment bacteria. However, the strong selection for triclosan resistant communities that was observed in this study does not support these conclusions, and instead indicates that triclosan impacts sediment bacterial communities. Therefore, it is more likely than changes in subpopulations of bacteria were occurring in response to triclosan amendments despite the lack of change in the size of the overall bacterial community. For example, we observed an increase in the numbers of heterotrophic bacteria in the triclosan amended streams within one week post-dosing. The plate count assay that we used to count the numbers of heterotrophic bacteria targets aerobic heterotrophic bacteria that can grow rapidly under high nutrient conditions (Zuberer 1994), which represents a small but significant component of the total sediment bacterial communities. Due to triclosan's antibacterial properties (Heath et al 1999) and previous work showing increased bacterial cell mortality in biofilms (Proia et al 2011) it was surprising that triclosan amendment resulted in an increase in heterotrophic bacteria in our study. However, the increase in these organisms may be connected to the die off of algae that was observed in the triclosan amended streams, as the dead algal cells would have provided an excellent carbon source for heterotrophic bacteria or removal of competitive pressure. This conclusion is supported by the temporary stimulation of heterotrophic bacteria in the triclosan amended streams, suggesting that it was to a one time increase in carbon triggered by the algal die off and not to a direct effect of triclosan. Algae may be more sensitive to triclosan than bacteria (Tatarazako et al. 2004; Proia et al

2011), but the link we observed between algal cell death and bacterial community dynamics has not been previously reported.

Conclusions

The impacts of triclosan of benthic microbial communities that were observed in this study have significant ecological implications, as benthic microbial communities are important drivers of primary production and nutrient cycling in benthic ecosystems. Alterations of these communities due to inputs of PPCPs such as triclosan have the potential to disrupt or modify ecosystem scale processes.

Future Work

Sediment and water samples from all of the streams and all of the time points were sent to our collaborators at the Illinois Sustainable Technology Center, Champaign, IL for quantification of triclosan. These data will provide insight into the actual concentrations of triclosan in our sediments at all sampling times.

DNA extracted from the sediments on days 0, 14 and 34 has been sent to the Research and Testing Laboratory (Lubbock, TX) for tag pyrosequencing of bacterial 16S rRNA genes. These data will be analyzed using MOTHUR following the approach described in Chapter 3 of this thesis. Comparison of the bacterial communities in the triclosan amended and control streams will enable us to determine if triclosan is capable of causing shifts in bacterial community composition. If shifts in community composition are observed, we will compare the species shifts observed in this lab study to those observed in the field study described in Chapter 3. If commonalities are observed between the lab and the field it may suggest that some of the differences in community

composition we observed at our field sites were driven by triclosan.

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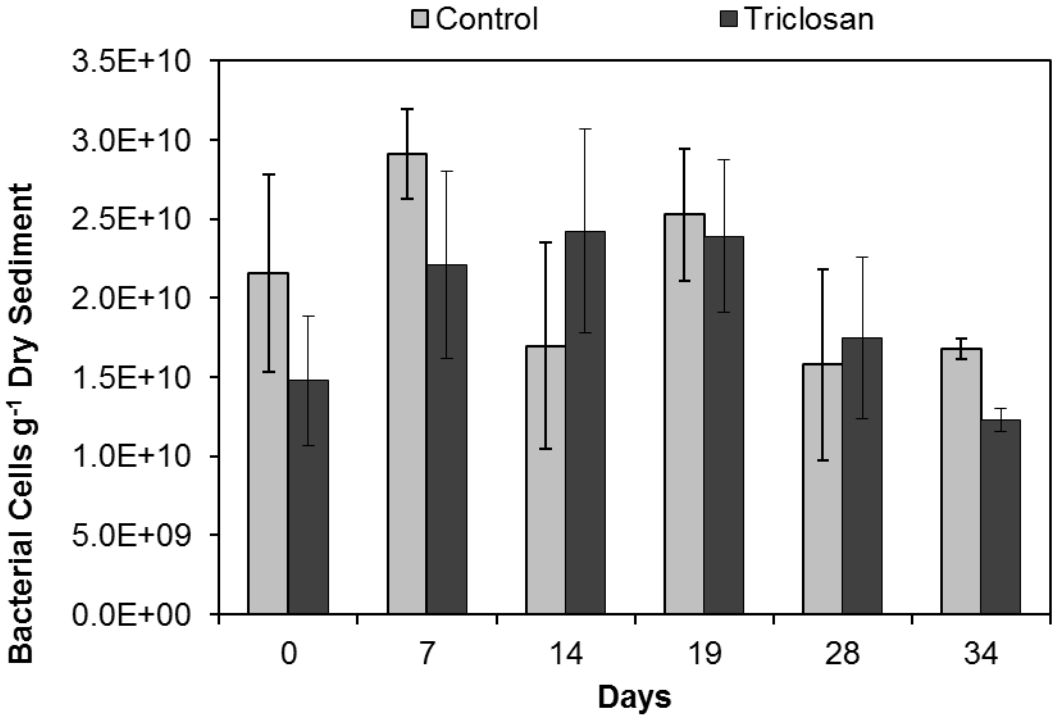
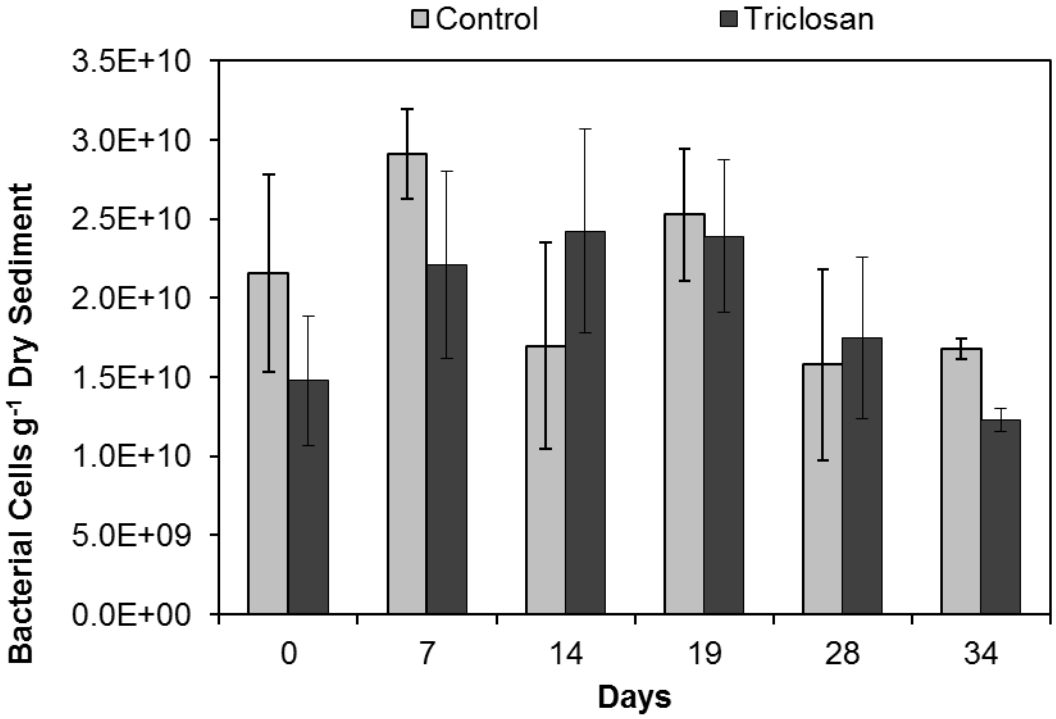
Figure 19. Direct Bacterial Cell Counts for Sediments Collected from Model Streams



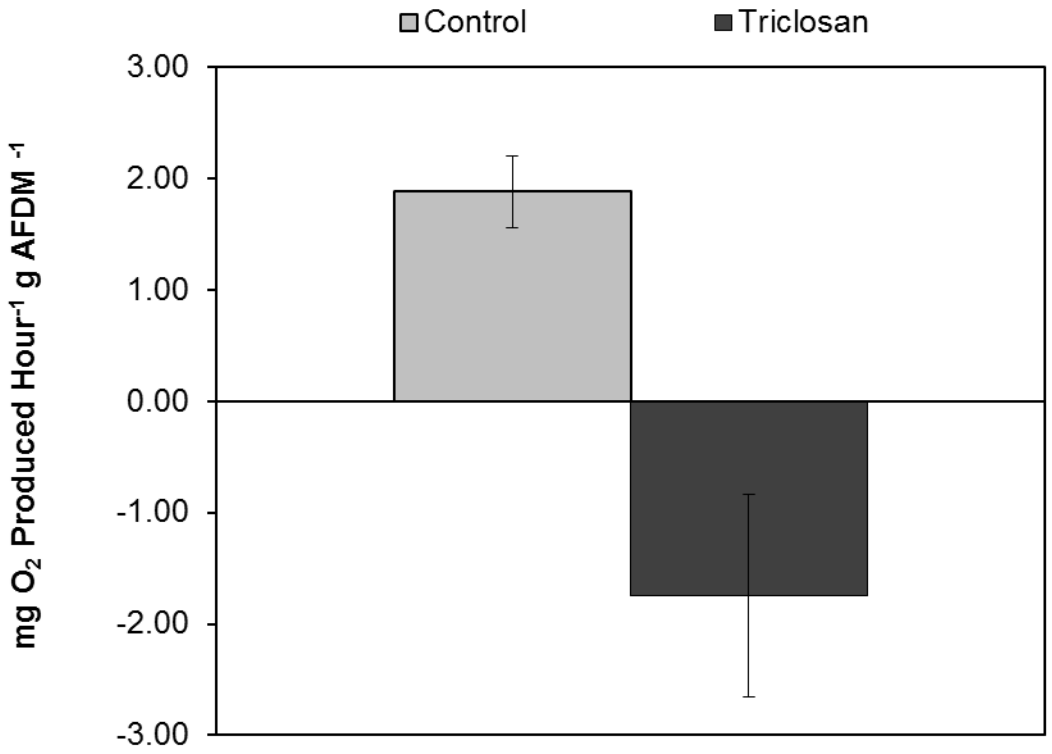
Direct bacterial cell counts for sediments collected from model streams. Treated streams received triclosan amendments on day 0 and control streams received no triclosan. Each data point is mean (n=3) +/- standard error. Repeated measures ANOVA indicated no significant effect of triclosan (p=0.540).

Figure 20. Heterotrophic Plate Counts for Sediments Collected from Model Streams



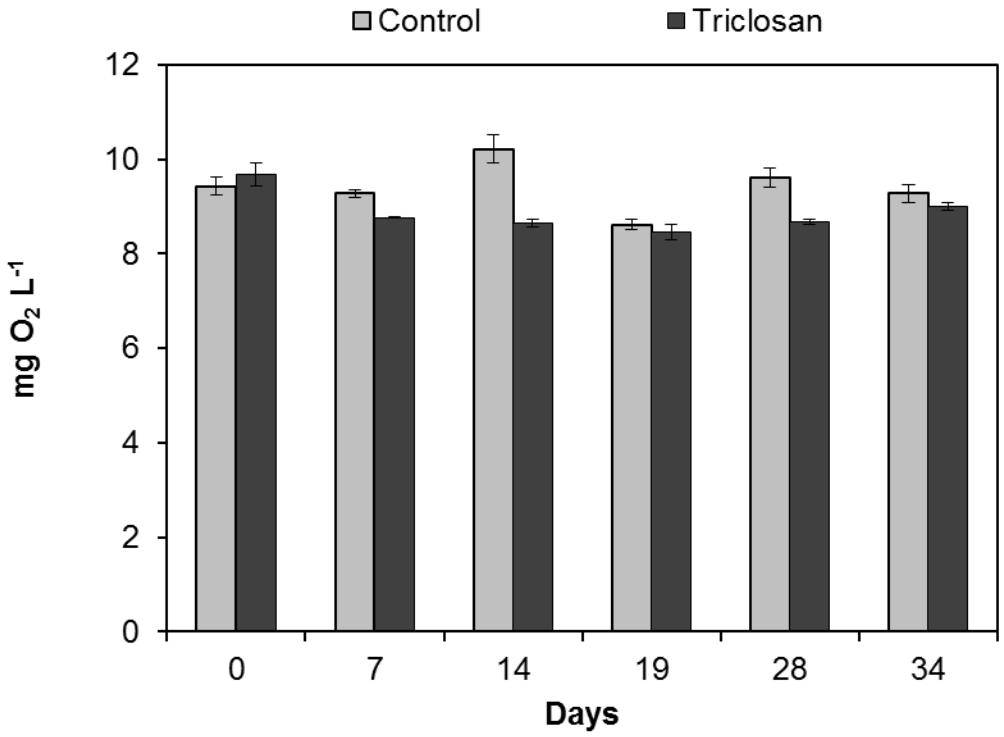
Heterotrophic plate counts for sediments collected from model streams. Treated streams received triclosan amendments on day 0 and control streams received no triclosan. Each data point is mean (n=3) +/- standard error. Repeated measures ANOVA indicated a significant effect of triclosan ($p<0.005$). ANOVA for individual treatments indicated no significant change in control treatments over time ($p=0.186$) but a significant change in triclosan treatments over time ($p<0.001$) with different letters indicating significant differences in the triclosan treatments between the sampling dates.

Figure 21. Photosynthesis Rates in Surface Water from Model Streams



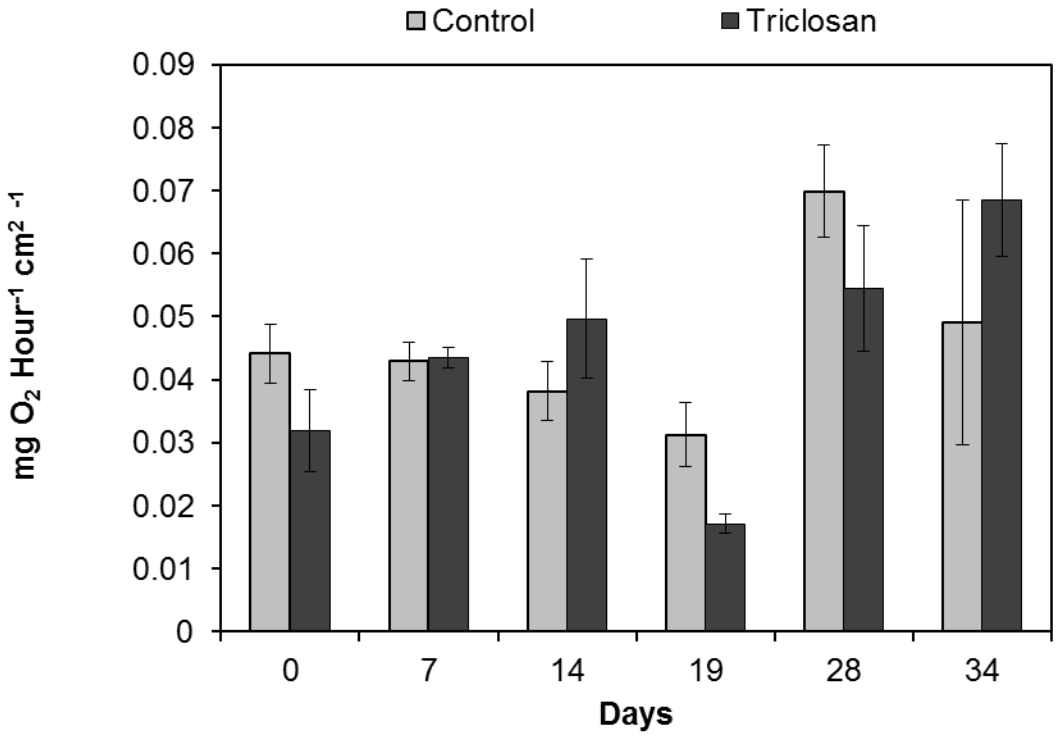
Photosynthesis rates in surface water collected from model streams 8 days after triclosan amendment. Treated streams received triclosan amendments while control streams received no triclosan. Each data point is mean (n=3) +/- standard error. ANOVA indicated a significant effect of triclosan ($p<0.05$).

Figure 22. Dissolved Oxygen Levels for Surface Waters of Model Streams



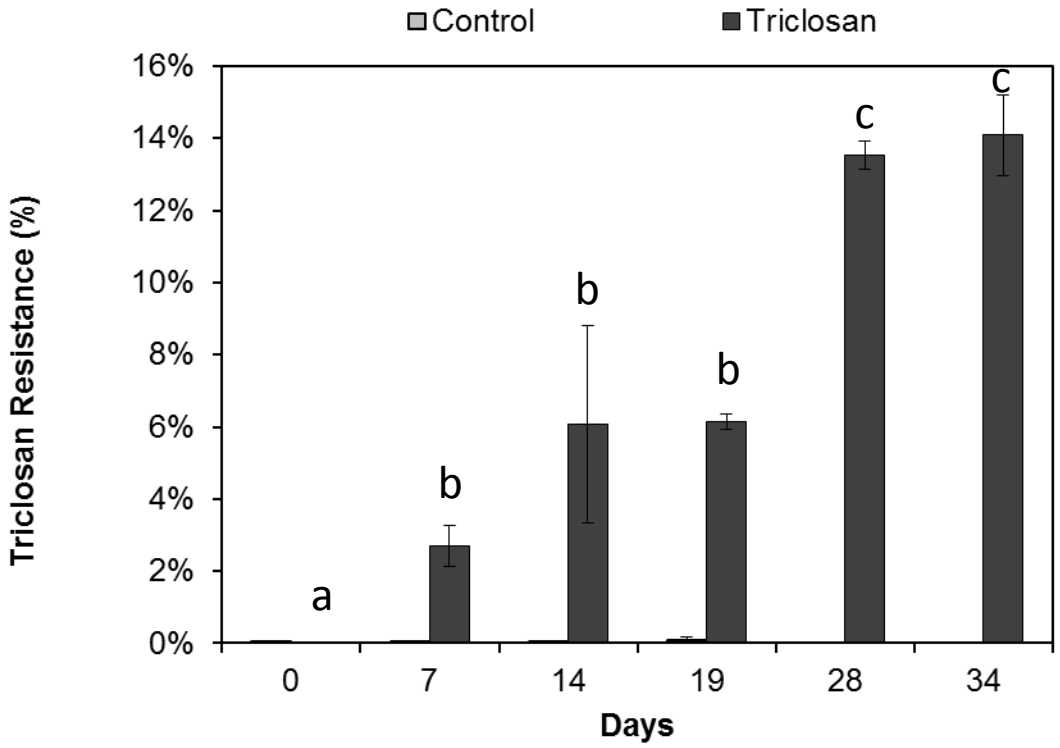
Dissolved oxygen levels for surface waters of model streams. Treated streams received triclosan amendments and control streams received no triclosan. Each data point is mean (n=3) +/- standard error. Repeated measures ANOVA indicated a significant effect of triclosan ($p<0.005$).

Figure 23. Community Respiration for Sediments Collected from Model Streams



Community respiration for sediments collected from model streams. Treated streams received triclosan amendments on day 0 and control streams received no triclosan. Each data point is mean (n=3) +/- standard error. Repeated measures ANOVA indicated no significant effect of triclosan (p=0.785).

Figure 24. Percent Triclosan Resistance for Sediments Collected from Model Streams



Percent triclosan resistance for sediments collected from model streams. Treated streams received triclosan amendments on day 0 and control streams received no triclosan. Each data point is mean (n=3) +/- standard error. Repeated measures ANOVA indicated a significant effect of triclosan ($p<0.001$). ANOVA for individual treatments indicated no significant change in control treatments over time ($p=0.626$) but a significant change in triclosan treatments over time ($p<0.001$) with different letters indicating significant differences in the triclosan treatments between the sampling dates.

CHAPTER FIVE

WASTEWATER TREATMENT EFFLUENT AND AN EMERGING POLLUTANT, TRICLOSAN

The world is becoming increasingly urbanized, with the majority of the world's population now living in urban areas. Urbanization has the potential to significantly alter lotic ecosystems and the services they provide. Benthic microbial communities are key components of lotic ecosystems due to their contributions to primary production and nutrient cycling. Two types of human inputs associated with urbanization that may impact benthic microbial communities in lotic ecosystems are the input of wastewater treatment effluent and the input of contaminants including pharmaceuticals and personal care products. In this thesis I have examined the potential ecological effects of wastewater treatment effluent and one pharmaceutical and personal care product that is of growing concern, triclosan. My thesis presents some unexpected findings that will advance our understanding of the effects of urbanization on sediment microbial communities in lotic ecosystems.

Effects of Wastewater Treatment Plant Effluent on Sediment Microbial Communities

In our field study, we examined two streams located in different habitats, one located in an urban area (North Shore Channel) and the other suburban (West Branch of the DuPage River). Both differed significantly in their chemical and biological

characteristics and received inputs from nearby wastewater treatment plants (WWTPs) that differed dramatically in size. Surprisingly, the sediment microbial communities downstream of these WWTPs were almost identical in their chemical and biological states. This is unexpected given the difference in sizes between the two WWTPs, and habitats of these streams. The results of our experiment show that for these two field sites, WWTP effluent significantly reduced both chemical and biological variation in the benthic ecosystems. These results raise questions about the impacts of anthropogenic ecosystem modifications and WWTPs on lotic ecosystems. However, further studies are needed to conclude whether this pattern can be observed across a larger range of sites.

Effects of Triclosan on Sediment Microbial Communities

To assess the effects of triclosan on sediment microbial communities, we examined three streams across a rural-urban gradient: woodland (Nippersink Creek), suburban (West Branch of the DuPage River), and urban (North Shore Channel). We had hypothesized that incomplete removal of triclosan in WWTP effluent would serve as a point source of triclosan into lotic ecosystems. From the data obtained from the field study, our results indicated that WWTPs were not significant point sources of triclosan, suggesting that non-point sources such as combined storm overflows (CSOs) are more significant sources of triclosan into lotic ecosystems. Also, sediment triclosan concentrations correlated closely with the degree of urbanization of the surrounding habitat. However, it was not possible to attribute changes in the sediment community structure and function to triclosan from the field study. To achieve this, we designed a

model stream experiment in which streams were amended with triclosan, or left as unamended controls to test a hypothesis generated from the field study in a controlled laboratory setting.

Amended artificial streams were dosed once with triclosan, and samples were taken weekly for five weeks following amendments. Following dosing, the triclosan resistance levels in amended streams was significantly higher than unamended streams, and continued to increase throughout the course of the experiment. By the end of the experiment, both amended and unamended streams contained similar size bacterial communities as indicated by heterotrophic plate counts and direct epifluorescent counts of bacterial cells. However, a significantly higher percentage of the bacterial community in the amended streams was resistant to triclosan when compared to unamended streams. This demonstrates that the addition of triclosan to sediments in a lotic ecosystem is sufficient to significantly change the composition of the residing microbial community. We are currently awaiting results of tag pyrosequencing to fully understand the resulting shifts in the microbial communities. Once complete, these results can be compared to those obtained in the field study to fully understand the effects of triclosan on sediment communities.

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