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ILLINOIS SUSTAINABLE TECHNOLOGY CENTER
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List of Abbreviations

ASE	accelerated solvent extraction
BOD	biochemical oxygen demand
CSO	combined sewer outfalls
DGGE	denaturing gradient gel electrophoresis
DO	dissolved oxygen
DOC	Dissolved organic carbon
ENR	enoyl-acyl carrier protein reductase
MGD	million gallons per day
NaCl	sodium chloride
$NaPO_4$	sodium phosphate
nMDS	non-metric multi-dimensional scaling
NSC	north shore channel
NSWRP	North Side Water Reclamation Plant
PAH	polynuclear aromatic hydrocarbons
PBDE	polybrominated diphenyl ethers
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
PFE	pressurized fluid extraction
PFE	pressurized fluid extraction
PPCP	pharmaceuticals and personal care products
rRNA	ribosomal ribonucleic acid
SPE	solid phase extraction
USEPA	United States Environmental Protection Agency
WBDR	West Branch of the DuPage River
WCWWTP	West Chicago Wastewater Treatment Plant
WWTP	wastewater treatment plant

Abstract

In urban areas wastewater treatment plant (WWTP) effluent can represent a significant component of freshwater ecosystems, and WWTP effluent can be a point source for a variety of pollutants, including pharmaceuticals and personal care products (PPCPs). We analyzed two field sites in the Chicago region: (1) an urban river receiving effluent from a large WWTP; and (2) a suburban river receiving effluent from a smaller WWTP. At both sites WWTP effluent had negative effects on the abundance and diversity of benthic bacterial communities. We then investigated the potential effects of one specific PPCP, triclosan, a broad-spectrum antimicrobial compound that is incorporated into numerous consumer products. We developed a method for the quantification of triclosan in sediment based on pressurized fluid extraction (PFE) by accelerated solvent extraction (ASE) and used this method to demonstrate that triclosan is present in streams in the Chicago region and that triclosan concentrations in stream sediments increased with degree of urbanization. Finally, we conducted a field survey and a lab-scale model stream experiment and demonstrated that triclosan exposure is linked to increases in triclosan resistance and decreases in biodiversity within benthic bacterial communities. These results indicate that widespread use of triclosan could have negative ecological consequences.

Chapter 1: Introduction

1.1 Urbanization and Freshwater Ecosystems

Over the last century the human population has undergone a shift from rural to urban living. In 1900 only 10% of the global population lived in urban areas. That percentage now exceeds 50% and will reach 60% in the next two decades, with the United States currently close to 80% of its population in urban areas (Grimm et al., 2008).

One challenge associated with increased urbanization is management of wastewater. Centralized wastewater treatment plants (WWTPs) are one of the most common systems for the treatment of domestic wastewater in the United States (USEPA, 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 these, the Stickney Water Reclamation Plant, is the largest activated sludge WWTP in the world with a design capacity of 1.2 billion 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). For example, treated municipal wastewater effluent is more than 70% of the annual flow in the Chicago Area Waterway System, which includes all segments of the Chicago River as well as the North Shore Channel (Illinois Department of Natural Resources, 2011). Therefore, in highly urbanized areas like Cook County, WWTP effluent represents a significant component of the water in lotic ecosystems.

Although WWTPs can be effective at reduction of biochemical oxygen demand (BOD) and pathogen load, it is impossible for the characteristics of the effluent to match the characteristics of the water in the receiving system. Therefore, the potential exists for WWTP effluent to significantly alter the physical and chemical properties of the receiving ecosystem. 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 also examined the effects of WWTP effluent on bacterial populations within the water column (Cébron et al., 2004; Garnier, et al., 1992; Goñi-Urriza et al., 1999) 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 and 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 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 Greensboro, NC. 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. However, 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 within a major city.

1.2 Pharmaceuticals and Personal Care Products

Over the last three decades ecologists have become increasingly concerned with the presence of emerging containments in streams and rivers associated with a variety of human activities, and more recently pharmaceuticals and personal care products (PPCPs) have received increased attention (Kolpin et al., 2002). PPCPs include prescription and over-the counter therapeutic drugs, antibacterial products such as soaps and detergents, and fragrances and cosmetics. PPCPs have the potential to enter the domestic wastewater stream directly after use (e.g., soaps and detergents), after passing through the human body (e.g., therapeutic drugs), or due to intentional disposal of expired or unwanted products.

Wastewater treatment plants are not designed to fully remove PPCPs (Ternes, 1998), resulting in their release to our nation's rivers and streams. The introduction of PPCPs into surface waters is likely to be especially significant in highly urbanized areas such as Chicago, IL, due to the high population density as well as the large amounts of wastewater discharged. Recently pharmaceutical chemicals have been detected in surface waters receiving wastewater effluent in highly urbanized watersheds (Kolpin et al., 2002; Gross et al., 2004), and a recent survey by the USGS detected numerous PPCPs in Illinois streams, including several antibiotics (Barnes et al., 2002).

Although it is known that PPCPs are extensively used and there is mounting evidence documenting their widespread distribution in aquatic ecosystems, relatively little is known about their ecological effects (Cunningham, 2006). This lack of knowledge is especially troubling as many of these compounds are used specifically because of their ability to interact in various ways with biological systems. Thus, there is a high likelihood that these biologically active compounds will interact in some way with biota in river and stream ecosystems. This interaction is especially true of antibacterial compounds, which by definition have negative effects on bacteria. Triclosan is an example of an antibacterial PPCP whose use has been dramatically increasing in recent years.

1.3 Triclosan

Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol] (Figure 1.1) is a broad spectrum antibacterial agent that is used in a number of PPCP products. Triclosan is a stable, lipophilic compound that is active against both gram-positive and gram-negative bacteria (Adolfsson-Erici et al., 2002). Triclosan was originally thought to act as a nonspecific biocide (Schweizer, 2001), but recent evidence has shown that triclosan acts on a defined bacterial target, the enzyme enoyl-acyl carrier protein reductase (ENR) (McMurray et al., 1998). This enzyme is an essential component of the fatty acid biosynthetic pathway of both gram-positive and gram-negative bacterial species (Ledder et al., 2006) but is not found in humans.

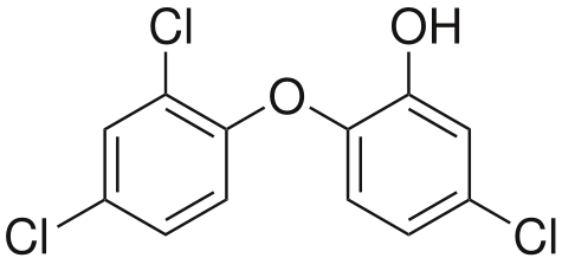

Figure 1.1: Molecular structure of triclosan.

Triclosan was first introduced in the 1960s (Ledder et al., 2006), but its use was limited to health care settings (Schweizer, 2001). However, between 1992 and 1999, over 700 consumer products with antibacterial properties, entered the market and the vast majority of them contained triclosan (Schweizer, 2001). Currently triclosan can be found in a diverse array of products, including hand soap, shampoo, toothpaste, mouthwash, lotions, deodorants, and detergents (Schweizer, 2001). The widespread use of these products is resulting in measureable concentrations of triclosan in the wastewater stream (Heidler and Halden, 2007).

Triclosan is known to be readily removed by wastewater treatment plants (WWTPs). A recent study reported a triclosan removal rate of 98% in an activated sludge WWTP in the United States (Heidler and Halden, 2007). However, this same study demonstrated that the majority of the triclosan removal was due to sequestering of the triclosan in the sludge and not to actual breakdown of the triclosan (Heidler and Halden, 2007). These data suggest that triclosan may be fairly resistant to decomposition. If this is the case, then long-term release of even small amounts of triclosan in WWTP effluent water has the potential to lead to triclosan accumulation in the environment (Singer et al., 2002). A recent survey of rivers in the United States detected water column concentrations of triclosan in 58% of the sampled rivers, which ranked triclosan in the top ten of 95 water contaminants in terms of prevalence (Kolpin et al., 2002). In addition, triclosan's low solubility in water suggests that triclosan may have a tendency to accumulate in sediments. In several recent studies triclosan was detected in marine sediment samples collected at the outflow of two urban WWTPs (Agüera et al., 2003) and in lake sediments (Singer et al., 2002).

The release of triclosan to rivers and streams could have significant impacts on bacterial communities in these habitats. For example, it could have broad, negative effects on the growth and activity of resident bacterial communities. This is a concern because bacteria are critical components of stream and river ecosystems.

1.4 Importance of Bacteria in Stream Ecosystems

Bacteria serve a number of essential functions in freshwater ecosystems. For example: (1) they contribute to the breakdown of organic material and the release of essential nutrients back to the nutrient pool; (2) they are responsible for the breakdown and detoxification of a variety of pollutants; and (3) they serve as an important food source for higher trophic level organisms

(e.g., aquatic invertebrates). Bacteria also serve as the key drivers of several essential biogeochemical cycles, including the sulfur cycle and the nitrogen cycle.

Bacteria catalyze several key steps in the nitrogen cycle, including nitrogen fixation, nitrification, and denitrification (Figure 1.2). Denitrification is an especially valuable process in streams because it converts nitrate, a soluble biologically-available form of nitrogen, to dinitrogen gas (N_2), which can diffuse from the stream to the atmosphere. Denitrification can thus effectively remove biologically-available nitrogen from aquatic ecosystems. The removal of nitrogen from streams is valuable because it can counteract the effects of nitrogen pollution.

Nitrogen pollution refers to the anthropogenic input to surface waters of excessive levels of nitrogen, mainly due to the use of commercially produced nitrogen fertilizers by the agricultural industry. Increased nitrogen inputs to freshwater systems can lead to decreased water quality, as high levels of nitrogen can cause excessive growth of algae, which can make lakes and rivers much less pleasant for recreational use. Excessive algal growth and subsequent decomposition of algal biomass can also lead to a decrease in the amount of dissolved oxygen (DO) in the water, a condition known as hypoxia (Nixon et al., 1996), which can cause the death of fish and other aquatic organisms (Carpenter et al., 1998; Goolsby et al., 2001). Nitrogen pollution is a significant problem in the United States. Compared to pre-industrial times, the amount of nitrogen entering rivers and lakes in the United States has increased 6- to 50-fold (Carpenter et al., 1998). Nitrogen pollution of rivers and streams is an especially significant issue for the state of Illinois due to the fact that almost 60% of the land in Illinois is used for agriculture (USDA, 2003).

Therefore, bacterial communities in rivers and streams provide many valuable ecosystem services, including denitrification, and PPCPs, including triclosan, have the potential to threaten these bacterial communities and the services they provide.

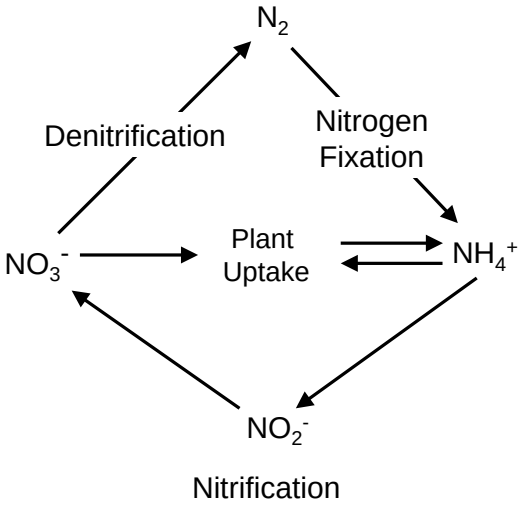

Figure 1.2: The nitrogen cycle.

1.5 Triclosan and Resistance

The release of triclosan to the environment also raises concerns about resistance. McMurry et al. (1998) demonstrated that *Escherichia coli* exposed to triclosan in the lab developed resistance though a mutation in the gene encoding the ENR enzyme. Therefore, it has been suggested that the release of triclosan to the environment could drive the development and spread of resistance among bacteria (Schweizer, 2001) and could make triclosan less useful as an antibacterial agent.

An even more worrisome aspect of triclosan release to the environment is the possibility that exposure to triclosan may select for bacteria with increased resistance not only to triclosan itself but also to other therapeutically useful antibiotics (Schweizer, 2001). Selection for cross-resistance is possible for two reasons. First, genes that confer resistance to triclosan are sometimes carried on plasmids that also bear antibiotic resistance genes. Thus, exposure to triclosan in the environment could increase the frequency and spread of these resistance plasmids (Levy, 1998). Second, bacterial efflux pumps can also confer resistance to triclosan (McMurry et al., 1998). Efflux pumps are very common in bacteria (Ledder et al., 2006), and in many cases these efflux pumps have relatively low specificity. For example, the AcrAB-TolC efflux pump found in *E. coli* can pump out a variety of compounds including certain antibiotics, cationic surfactants such as quaternary ammonium compounds, and pine oil (Ledder et al., 2006). Therefore, exposure to triclosan in the environment could increase the frequency and spread of efflux pumps which could confer resistance to triclosan and to other antibiotics. Experimental evidence supports the connection between triclosan and antibiotic resistance, as triclosan exposure has been shown to increase resistance to antibiotics in *Pseudomonas aeruginosa* (Chuanchuen et al., 2001) and *Escherichia coli* (Braoudaki and Hilton, 2004).

1.6 Questions addressed in this study

The following key questions were addressed by the project:

1. Does wastewater treatment plant (WWTP) effluent affect benthic bacterial communities in rivers in the Chicago metropolitan region?
    - This question was addressed by a field project, described in Chapter 2 of this report.
2. Are there detectable levels of triclosan in sediments of rivers in the Chicago metropolitan region?
    - One challenge to answering this question is the fact that extraction, detection, and quantification of PPCPs (including triclosan) can be difficult, especially from "dirty" samples such as soils and sediments. Therefore, we developed an analytical method (described in Chapter 3 of this report) for detection of triclosan in environmental samples, including soils and sediment.
3. Are the levels of triclosan in Illinois rivers and streams impacting the resident bacterial communities?
    - This question was addressed by a field project, described in Chapter 4 of this report.
4. How does triclosan impact the composition and function of sediment bacterial communities?

- This question was addressed by a laboratory-scale model stream experiment, described in Chapter 4 of this report.

1.7 Objectives

The main objectives of the project were the following:

1. Assess the potential effects of WWTP effluent on benthic bacterial communities
2. Develop an analytical method for detection of triclosan in environmental samples, including soil and sediment.
3. Assess the potential for environmental impacts of triclosan by quantifying triclosan concentrations and bacterial resistance levels in a variety of Illinois rivers and streams.
4. Determine the impacts of triclosan on the composition and function of bacterial communities via controlled laboratory experiments.

Chapter 2: Effects of Wastewater Treatment Plant Effluent on the Abundance and Diversity of Benthic Bacterial Communities in Urban and Suburban Rivers

2.1 Note on publication

The contents of this chapter have been published:

- Drury, B., Rosi-Marshall, E., and Kelly, J.J. 2013. Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers. Appl. Environ. Microbiol., 79:1897-1905.

2.2 Abstract

In highly urbanized areas, wastewater treatment plant (WWTP) effluent can represent a significant component of freshwater ecosystems. As it is impossible for the composition of WWTP effluent to match the composition of the receiving system, the potential exists for 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 by comparing two distinct field sites in the Chicago metropolitan region: a highly urbanized river receiving effluent from a large WWTP and a suburban river receiving effluent from a much smaller WWTP. At sites upstream of effluent input, the urban and suburban rivers differed significantly in chemical characteristics and in the composition of their sediment bacterial communities. Although effluent resulted in significant increases in inorganic nutrients in both rivers, surprisingly it also resulted in significant decreases in the population size and diversity of sediment bacterial communities. Tag pyrosequencing of bacterial 16S rRNA genes revealed significant effects of effluent on sediment bacterial community composition in both rivers, including decreases in Deltaproteobacteria, *Desulfococcus*, *Dechloromonas*, and Chloroflexi sequences, and increases in Nitrospirae and Sphingobacteriales sequences. The overall effect of the WWTP inputs was that the two rivers, which were distinct in chemical and biological properties upstream of the WWTPs, were almost indistinguishable downstream. These results suggest that WWTP effluent has the potential to reduce the natural variability that exists among river ecosystems and indicates that WWTP effluent may contribute to biotic homogenization.

2.3 Introduction

Centralized wastewater treatment plants (WWTPs) are one of the most common systems for the treatment of domestic wastewater in the United States (USEPA, 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 these, the Stickney Water Reclamation Plant, is the largest activated sludge WWTP in the world with a design capacity of 1.2 billion 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). For example, treated municipal wastewater effluent is more than 70% of the annual flow in the Chicago Area Waterway System, which includes all segments of the Chicago River as well as the North Shore Channel (Illinois

Department of Natural Resources, 2011). Therefore, in highly urbanized areas such as Cook County, IL, WWTP effluent represents a significant component of the water in lotic ecosystems.

Although WWTPs can be effective at reduction of biochemical oxygen demand (BOD) and pathogen load, it is impossible for the characteristics of the effluent to match the characteristics of the water in the receiving system. Therefore, the potential exists for WWTP effluent to significantly alter the physical and chemical properties of the receiving ecosystem. 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 also examined the effects of WWTP effluent on bacterial populations within the water column (Cébron et al., 2004; Garnier et al., 1992; Goñi-Urriza et al., 1999) 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 (1) bacterial numbers are generally much higher in freshwater sediment that in the overlying water (Sander and Kalff, 1993) and (2) 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 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 Greensboro, North Carolina. 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. However, 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 within a major city.

We assessed the impacts of WWTP effluent on the size, activity and composition of benthic microbial communities in lotic ecosystems in 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.

2.4 Materials and Methods

Field Sites

The North Shore Channel (NSC) was selected to represent a highly urbanized river. NSC is a 7.7 mile long canal that begins in the town of Wilmette, IL, 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 an average discharge of 0.93 m3 sec-1 (http://waterdata.usgs.gov) and a drainage area of 6,474 ha that is 63% residential, 16.7% commercial/industrial, 10% forest/open land, 5.4% institutional, and 3.5% transportation/utility (HDR Engineering Inc., 2011). 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 that includes part of the city of Chicago 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 into the channel.

The West Branch of the DuPage River (WBDR) located in DuPage County, IL, was selected to represent a suburban river. WBDR has an average discharge of 0.62 m3 sec-1 and a drainage area of 32,900 ha that is 32.8% residential, 17.4% agricultural, 16.9% vacant, 11.2% forest/open land and less than 4% industrial (Aqua Terra Consultants, 2003). WBDR receives treated effluent from the West Chicago WWTP (WCWWTP), which is located in West Chicago, IL. The WCWWTP is an activated sludge plant that receives domestic wastewater from the towns of West Chicago and Winfield, IL. It treats 5 MGD and does not disinfect the 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 into the river.

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 400 mL canning jars (Ball Corporation, Muncie, IN). 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.

Sample Characteristics

Dissolved organic carbon (DOC) in water samples was measured on a Shimadzu 5050 TC Analyzer as described previously (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, 1964).

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 50 mL 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 O_2 consumed time-1. Respiration rates were normalized by sediment surface area and by total heterotrophic plate counts.

Heterotrophic Plate Counts

Viable counts of heterotrophic bacteria were conducted for each sediment sample using a standard plate count method (Zuberer, 1994). Briefly 10 g of sediment was placed in a sterile 250 mL centrifuge bottle containing 90 mL of heat sterilized 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.

Epifluorescence Counts

Direct counts of bacterial cells were performed using a modified standard method (Kepner and Pratt, 1994). Cells were fixed by diluting sediment 1:50 in sterile DNA-free fixative solution (10 mM $NaPO_4$, 120 mM NaCl, 10 mM sodium pyrophosphate, 4% formaldehyde) (Gough and 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. Two milliliters 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.

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 using MOTHUR to calculate distances between sites based on the theta index (Yue

and Clayton, 2005) and visualize the resulting distance matrix using non-metric multidimensional scaling (NMDS). The significance of differences in theta index scores between sites was assessed by AMOVA run within MOTHUR. MOTHUR was also used to calculate the Shannon diversity index (Shannon, 2001) and the Chao1 richness estimator (Chao, 1984). SIMPER analysis run in Primer V.5 (Primer-E Ltd., Plymouth, United Kingdom) was used to identify the OTUs making the largest contributions to the variations between communities from each of the field sites.

Statistics
All data were analyzed by two-way ANOVA based on land use (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.

2.5 Results

Effects of land use
We examined the influence of land use on the two rivers by comparing data from the reaches above the wastewater effluent inputs. There was a significant effect of land use (urban vs. suburban) on water column nutrient concentrations, with higher concentrations of DOC, nitrate and phosphate, and a lower concentration of ammonium in the suburban river (Table 2.1).

There was also a significant effect of land use on the size of the sediment bacterial communities as indicated by heterotrophic plate counts, with the suburban river having higher counts than the urban river (Figure 2.1A). Direct epifluorescence counts of bacterial cells did not show this same trend, as there was no significant effect of land use (Figure 2.1B).

Table 2.1: Sampling site characteristics.

	Value (SE)[a]			
	Suburban Upstream	**Suburban Downstream**	**Urban Upstream**	**Urban Downstream**
Water Column DOC (mg L^{-1})	6.652 (0.052)	5.782 (0.306)*	2.408 (0.085)	3.947 (0.072)*
Water Column NH_4 (mg L^{-1})[b]	0.060 (0.003)	B.D.*	0.138 (0.007)	0.236 (0.005)*
Water Column NO_3^- (mg L^{-1})[b]	2.742 (0.140)	4.662 (0.492)*	0.232 (0.002)	4.696 (0.206)*
Water Column PO_4^{3-} (mg L^{-1})[c]	0.268 (0.006)	0.466 (0.035)*	0.003 (0.000)	0.410 (0.019)*
Sediment Organic Material (%)	8.70% (1.20%)	1.58% (0.12%)*	5.89% (0.43%)	2.00% (0.21%)*

a Each data point is mean (n=5) with standard error values in parentheses.
b NH4 and NO_3^- Limit of Detection = 0.02mg/L
c $PO4_3^-$ Limit of Detection = 0.002mg/L
* Indicates significant effect of effluent input (p<) based on two-way ANOVA
B.D. = below detection

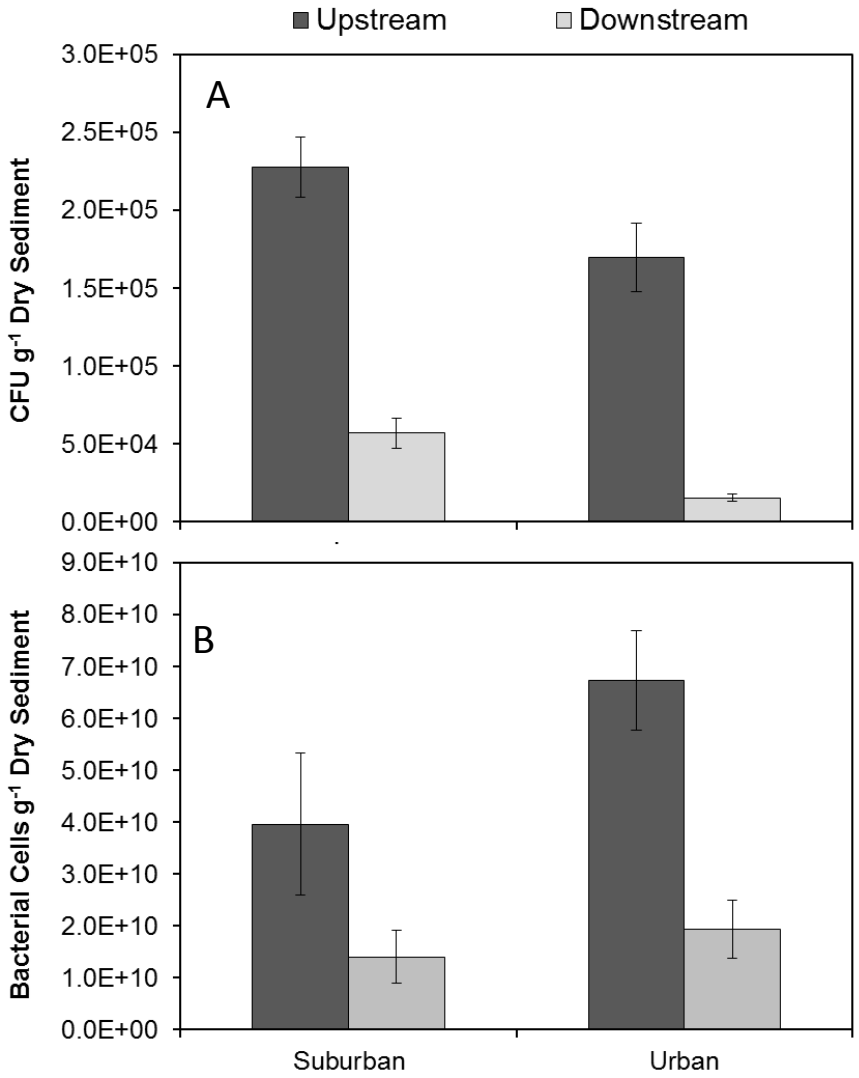

Figure 2.1: Heterotrophic plate counts (A) and direct bacterial cell counts (B) for sediments collected from rivers within two land use types (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Each data point is mean (n=5) ± standard error. Two-way ANOVA for plate counts demonstrated significant effect of land use (p=0.005) and effluent input (p=0.000) but no significant interaction effect (p=0.607). Two-way ANOVA for direct counts 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).

There was also no significant effect of land use on microbial community respiration (Figure 2.2A). NMDS analysis of tag pyrosequencing data revealed a significant difference in community composition between the suburban and urban rivers at the upstream sites ($p<0.001$) (Figure 2.3).

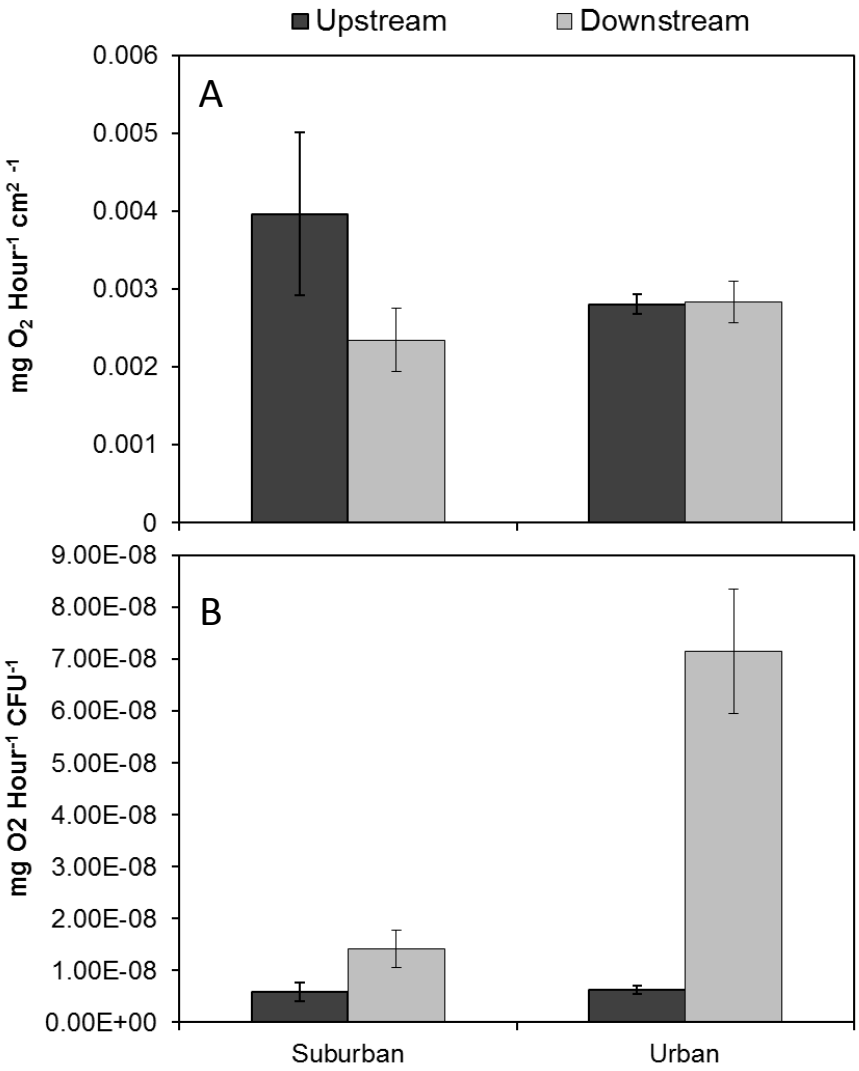

Figure 2.2: Community respiration normalized by surface area (A) and by bacterial cell numbers based on heterotrophic plate counts (B) for sediments collected from rivers within two land use types (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Each data point is mean (n=5) ± standard error. Two-way ANOVA for respiration normalized by surface area demonstrated no effect of land use (p=0.572), effluent input (p=0.189) or interaction effect (p=0.176). Two-way ANOVA for respiration normalized by cell numbers demonstrated a significant effect of land use (p=0.000), effluent input (p=0.000) and a significant interaction effect (p=0.000).

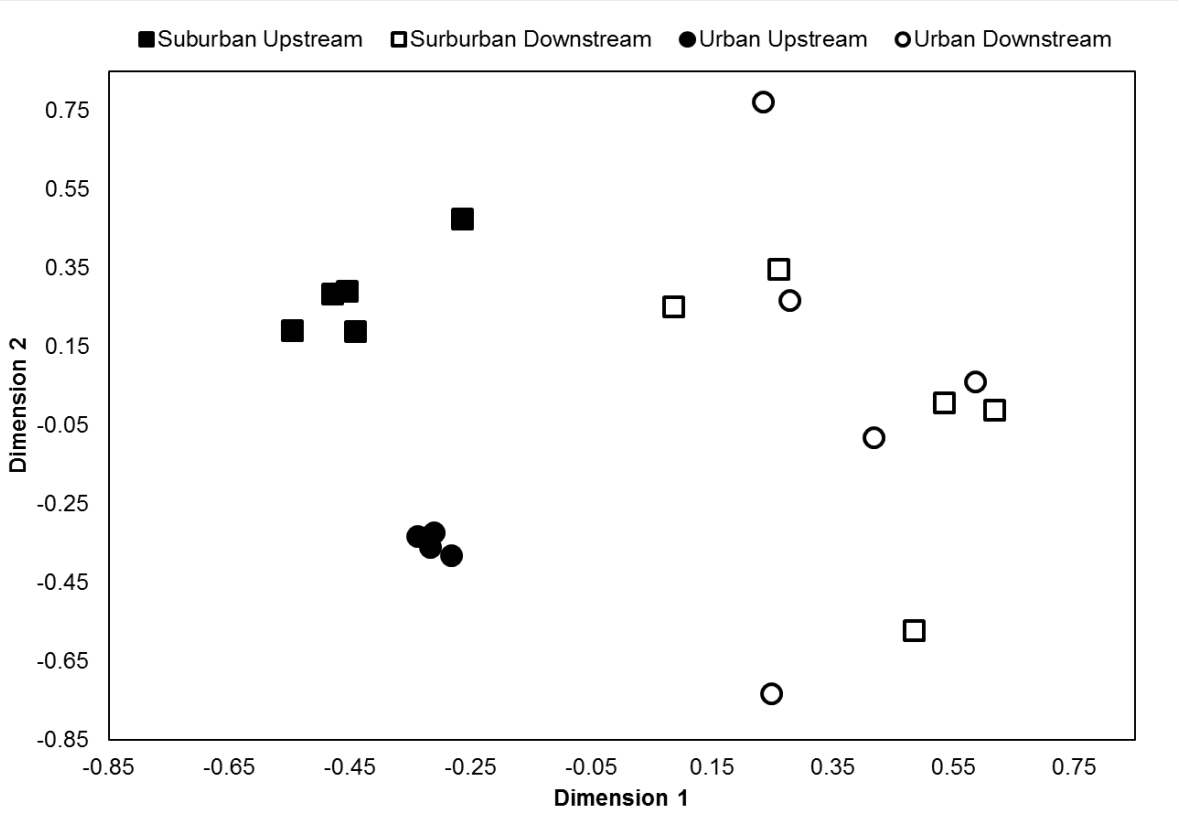

Figure 2.3: nMDS ordination of 16S tag pyrosequencing data comparing community structure of sediment bacterial communities collected from rivers within two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs.

Sediment communities from the upstream sites of both rivers were dominated by Proteobacteria, with Proteobacterial sequences accounting for more than 50% of the sequences (Figure 2.4). There was no significant difference in the overall abundance of Proteobacterial sequences between the two sites ($p=0.180$), but the urban upstream site showed significantly higher relative abundance of Bacteroidetes sequences ($p=0.001$) and significantly lower relative abundance of Chloroflexi sequences ($p<0.001$) (Figure 2.4). SIMPER analysis of the pyrosequencing data indicated 16 OTUs that accounted for greater than 20% of the variation in community composition between the suburban and urban upstream sites, and there were significant differences in the relative abundances of each of these OTUs between the two sites (Table 2.2).

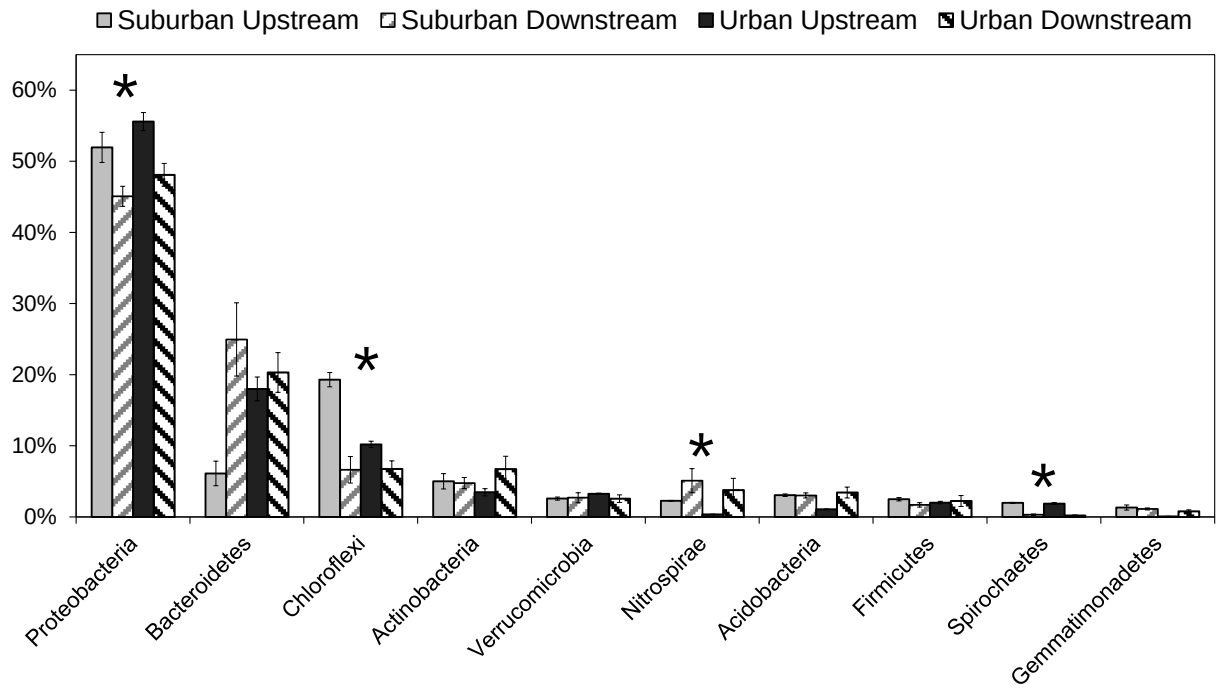

Figure 2.4: Phylotype analysis of 16S tag pyrosequencing data for sediment bacterial communities collected from rivers in two land uses (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Y axis represents the percentage of all sequences within a sample that were within the phylotype listed on the x-axis. Each data point is mean (n=5) ± standard error. Asterisk (*) indicates a significant effect of effluent input ($p<0.05$).

Effects of Effluent

WWTP effluent had significant effects on water column ammonium, nitrate, and phosphate concentrations, as well as sediment organic content in both rivers (Table 2.1). 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 (Figure 2.1A) and direct counts (Figure 2.1B). Specifically, WWTP effluent resulted in significant decreases in both plate counts and direct counts at both sites. In contrast, WWTP effluent had no effect on community respiration normalized by sediment surface area (Figure 2.2A). However, the decreased bacterial population size (Figure 2.1 A and B) combined with similar respiration rates (Figure 2.2A) suggests smaller populations respiring more on a per cell basis. When we normalized respiration rates by cell counts (Figure 2.2B), both sites downstream of WWTP effluent had higher per cell respiration rates than the upstream sites. This effect was much more pronounced at the urban site.

Table 2.2: Bacterial operational taxonomic units (OTUs) making the most significant contribution to variation between communities from suburban upstream and urban upstream sites.

	Relative Abundance (%)[a]					
Operational Taxonomic Units[c]	Suburban Upstream	Urban Upstream	p value[b]	Contribution to variation (%)	Cumulative Contribution to variation (%)	Taxonomic Identification[d]
Otu1	0.18	5.17	<0.001	3.55	3.55	*Dechloromonas*
Otu3	5.90	2.10	0.031	2.98	6.53	*Crenothrix*
Otu5	0.33	4.39	<0.001	2.89	9.41	*Thiobacillus*
Otu12	2.84	0.02	<0.001	2.01	11.42	*Comamonadaceae*
Otu16	0.93	2.41	0.001	1.05	12.47	*Proteobacteria* unclassified
Otu24	0.37	1.72	<0.001	0.96	13.43	*Alteromonadaceae*
Otu11	0.10	1.41	0.002	0.94	14.36	*Giesbergeria*
Otu22	0.07	1.35	<0.001	0.91	15.27	*Nitrospira*
Otu37	0.13	1.29	<0.001	0.83	16.10	*Caldilineaceae*
Otu29	0.21	1.11	<0.001	0.64	16.74	*Sinobacteraceae*
Otu17	0.00	0.90	0.001	0.64	17.38	*Sphingobacteria*
Otu30	1.01	0.11	<0.001	0.63	18.01	*Methylococcus*
Otu35	1.04	0.22	0.004	0.58	18.59	*Thiovirga*
Otu43	1.06	0.25	0.013	0.58	19.17	*Perlucidibaca*
Otu6	0.07	0.84	<0.001	0.55	19.72	*Deltaproteobacteria*
Otu176	0.78	0.02	<0.001	0.54	20.26	*Pseudomonas*

a Each data point is mean (n=5).
b p value based on ANOVA comparison of suburban upstream and urban upstream samples.
c OTUs were identified within 16S tag pyrosequencing data set based on 97% sequence identity.
d Taxonomic assignments were based on comparison to the SILVA-compatible bacterial alignment database.

NMDS analysis of tag pyrosequencing data indicated that WWTP effluent significantly changed the composition of the sediment bacterial communities at both the urban ($p<0.001$) and suburban ($p<0.005$) sites (Figure 2.3). These analyses also indicated that WWTP effluent significantly reduced bacterial community diversity (Figure 2.5A) and richness (Figure 2.5B). When we compared the bacterial communities of the two sites below the effluent inputs (i.e. urban downstream vs. suburban downstream), we found no significant differences in community composition ($p=0.982$) (Figure 2.3), diversity (Figure 2.5A) or richness (Figure 2.5B). In terms of broad bacterial phyla, tag pyrosequencing revealed that WWTP effluent significantly reduced the relative abundance of Proteobacterial sequences within the sediment bacterial communities (Figure 2.4). This overall decrease in Proteobacterial sequences was driven by a significant decrease in one Proteobacterial class, the Deltaproteobacteria ($p<0.05$) (data not shown). WWTP effluent also significantly reduced the relative abundance of Chloroflexi and Spirochaete sequences. In contrast, there was a significant increase in Nitrospirae sequences at downstream sites (Figure 2.4). Based on SIMPER analysis, there were 17 OTUs that accounted for 20% of the variation in community composition between the upstream and downstream sites, and there were significant differences in the relative abundances of some of these OTUs (Table 2.3). Notable differences included significantly higher relative abundances of Sphingobacteriales, Gallionellaceae, Verrucomicrobia, and Rhodobacter, and significantly lower relative abundances of Crenothrix, *Dechloromonas*, *Thiobacillus*, and *Desulfococcus* sequences at the downstream sites (Table 2.3).

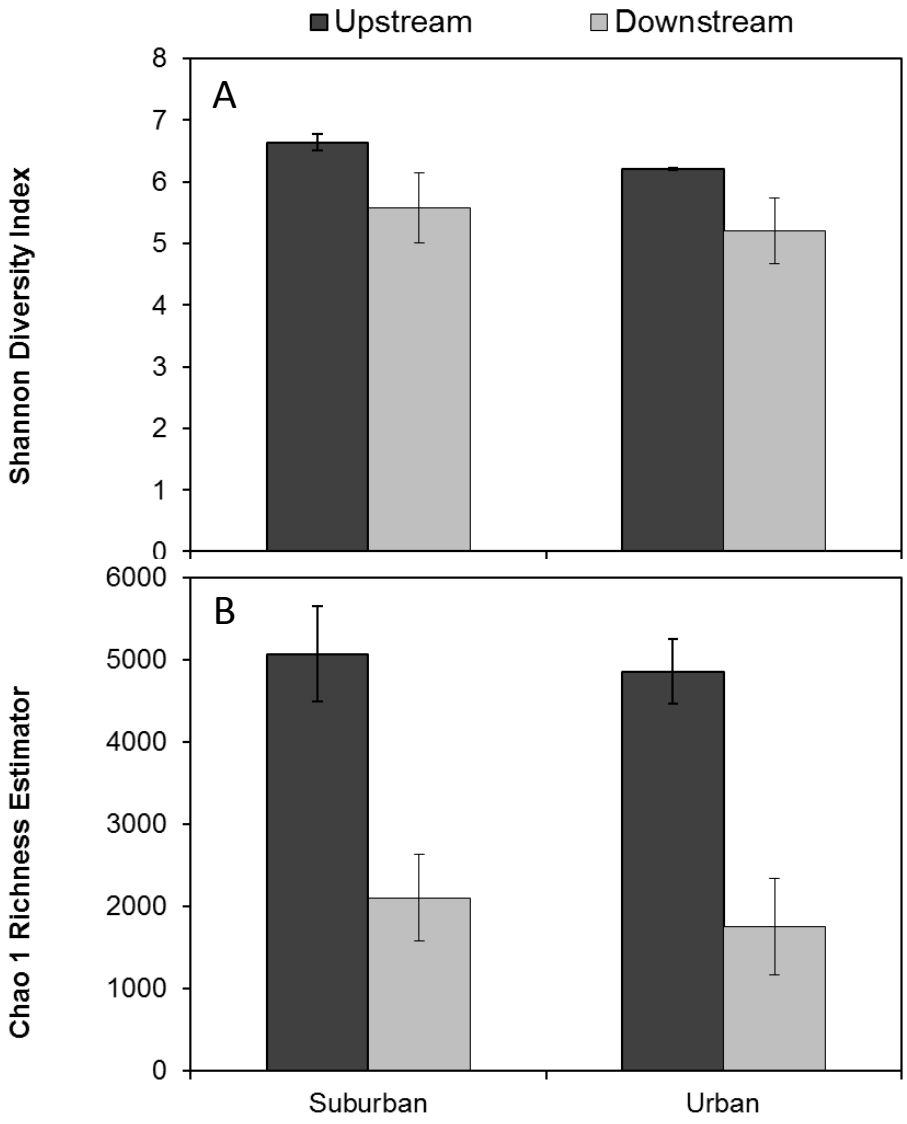

Figure 2.5: Shannon diversity index (A) and Chao 1 richness estimator (B) based on 16S tag pyrosequencing data for sediment bacterial communities collected from rivers within two land use types (urban and suburban) from locations upstream and downstream of WWTP effluent inputs. Each data point is mean (n=5) ± standard error. Two-way ANOVA of Shannon data 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 of Chao1 data 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).

Table 2.3: Bacterial operational taxonomic units making the most significant contribution to variation between communities from the upstream and downstream sites.

	Relative Abundance (%)[a]					
Operational Taxonomic Unit[c]	All Upstream Sites	All Downstream Sites	p value[b]	Contribution to variation (%)	Cumulative Contribution to variation (%)	Taxonomic Identification[d]
Otu4	0.16	4.35	0.043	2.51	2.51	*Sphingobacteriales*
Otu2	0.04	3.63	0.005	2.16	4.67	*Gallionellaceae*
Otu3	4.00	0.62	0.002	2.07	6.74	*Crenothrix*
Otu1	2.68	0.30	0.012	1.53	8.27	*Dechloromonas*
Otu42	0.38	2.73	0.002	1.41	9.69	*Verrucomicrobia*
Otu5	2.36	0.08	0.004	1.37	11.06	*Thiobacillus*
Otu8	2.97	0.71	<0.001	1.36	12.41	*Desulfococcus*
Otu39	0.10	2.10	0.272	1.22	13.64	*Alteromonadaceae*
Otu16	1.67	0.11	<0.001	0.94	14.58	*Proteobacteria, unclassified*
Otu33	0.87	2.09	0.003	0.86	15.44	*Rhodobacter*
Otu12	1.43	0.10	0.017	0.85	16.29	*Comamonadaceae*
Otu6	0.45	1.26	0.399	0.78	17.07	*Deltaproteobacteria, unclassified*
Otu10	0.00	1.26	0.106	0.76	17.83	*Oceanospirillales*
Otu7	0.03	1.23	0.239	0.73	18.55	*Methylophilaceae*
Otu9	0.02	1.17	0.321	0.70	19.25	*Flavobacteriaceae*
Otu15	0.00	1.06	0.331	0.63	19.89	*Sphingobacteriales*
Otu18	0.01	1.04	0.327	0.62	20.51	*Methylophilus*

a Each data point is the mean value (n = 5).
b P value based on ANOVA comparison of all upstream and all downstream samples.
c OTUs were identified within a 16S tag pyrosequencing data set based on 97% sequence identity.
d Taxonomic assignments were based on comparison to the SILVA-compatible bacterial alignment database.

2.6 Discussion

Effects of land use

Freshwater ecosystems are especially susceptible to changes in land use (Palmer et al., 2000), yet there is little information available on the effects of urbanization on benthic bacterial communities in lotic ecosystems (Perryman et al., 2008). Comparison of the reaches above the wastewater effluent inputs for the urban and suburban rivers used in this study provides insight into the effects of land use on these communities. For example, the suburban river has agriculture in its watershed (17% of total land use) compared to no agriculture in the urban watershed, so fertilizer use may have contributed to the higher concentrations of inorganic and organic nutrients in the suburban river. Based on these higher nutrient concentrations, it is not surprising that higher numbers of heterotrophic bacteria were detected in the suburban river sediment. In contrast, the watershed of the urban river has a higher proportion of land with impervious surfaces (residential, commercial and industrial land represented 80% of total land use). The urban river also receives inputs from several combined sewer overflows (CSOs) that release untreated wastewater and storm water during high rainfall (www.cityofchicago.org). Non-point source runoff from impervious surfaces and CSOs can be sources of anthropogenic

pollutants including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Lau et al., 2009; Polls et al., 1980; Walker et al., 1999) and elevated concentrations of PCBs have been reported in the NSC (Illinois EPA, 2004). Therefore, anthropogenic pollutants in the urban site may also have contributed to its lower numbers of heterotrophic bacteria.

Sediment bacterial communities from both rivers were dominated by Proteobacteria, a ubiquitous and metabolically diverse group of gram-negative bacteria frequently detected in freshwater sediments (Fazi, et al., 2005; Methe, et al., 1998; Tamaki, et al., 2005). Although there was no difference in the abundance of Proteobacterial sequences between the urban and suburban sites, Bacteroidetes sequences were significantly more abundant in the urban river sediment. Bacteroidetes are gram-negative heterotrophic bacteria that are common in freshwater ecosystems and are known to degrade high molecular weight organic compounds (Thomas et al., 2011) including petroleum hydrocarbons (Zhang et al., 2012). Therefore, the higher abundance of Bacteroidetes at the urban site might have been the result of higher concentrations of complex organic compounds including petroleum hydrocarbons (as discussed above). Another noteworthy difference in sediment bacterial communities was a 25-fold difference in the abundance of Dechloromonas sequences, which accounted for more than 5% of the total sequences in the urban sediment but less than 0.2% of the sequences in the suburban sediment. Dechloromonas are common in aquatic sediments and are known to oxidize aromatic compounds (Coates et al., 1980), so their higher abundance in the urban sediment may reflect higher concentrations of petroleum hydrocarbons. Finally, Nitrospira sequences were twenty-fold more abundant in the urban sediments. Nitrospira are gram-negative bacteria that catalyze the second step in the process of nitrification and are the dominant nitrite oxidizers within freshwater sediments (Altmann et al., 2003). The urban sediment had a significantly higher concentration of ammonium, which represents more substrate for nitrification and could explain the higher abundance of Nitrospira sequences. In summary, significant differences in the relative abundances of several bacterial taxa in the urban and suburban sediments, including Bacteroidetes, Dechloromonas and Nitrospira, may be linked to anthropogenic inputs resulting from differences in land use.

Effects of Effluent Addition

WWTP effluent significantly altered the downstream chemistry and bacterial communities of these rivers. In particular, the concentrations of inorganic nitrogen and phosphorus were higher downstream of the effluent, similar to what has been observed in a variety of ecosystems (Chambers and Prepas, 1994; Gücker et al., 2006; Marti et al., 2004; Spänhoff et al., 2007; Waiser et al., 2011). However, it was very surprising that despite the higher concentrations of inorganic nutrients, the numbers of sediment bacteria decreased downstream of the effluent inputs. Other studies have demonstrated that increased nitrogen and phosphorus associated with WWTPs stimulate planktonic bacterial growth (Garnier et al., 1992; Goñi-Urriza et al., 1999), and benthic (Wakelin et al., 2008) bacterial numbers. The downstream sites also had lower concentrations of sediment organic matter, which might explain the reduction in bacterial numbers. The decrease in sediment organic matter was in itself surprising, because increased inorganic nutrients often result in greater primary production (for review see Smith et al., 1999). However, in addition to elevated nutrients, toxic compounds may also be present in WWTP effluent and these may have inhibited bacterial populations. There is growing concern about the presence of a wide range of biologically active compounds, including antimicrobials, in rivers

and streams receiving WWTP effluent in the United States (Kolpin, et al., 2002). Many of these compounds are not completely removed by wastewater treatment, so WWTPs are point sources of these compounds (Akiyama and Savin, 2010; Bartelt-Hunt et al., 2009). If the effluent from the two WWTPs examined in this study contained compounds toxic to microorganisms (both algae and bacteria), this could explain both the decrease in bacterial numbers and the decrease in sediment organic matter. 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 bacterial cells that are under stress (Anderson and Domsch, 1993). Toxic compounds in the effluent could also contribute to the decreases in bacterial diversity and species richness at the downstream sites, which conflicted with previously published findings that demonstrated an increase in bacterial diversity downstream of a WWTP effluent input (Wakelin et al., 2008). Although quantification of toxic compounds in the effluents was beyond the scope of this study, future explorations of this topic are warranted.

WWTP effluent also resulted in shifts in bacterial community composition. The most striking effect was that the bacterial communities, which were clearly distinct at the upstream sites, were indistinguishable below WWTP effluents. This result provides an excellent illustration of the concept of biotic homogenization, which suggests that human modifications of the environment are reducing the biological differences that exist among natural ecosystems. As a result, there are now a series of human-altered ecosystems that consistently support a subset of naturally occurring species (McKinney, 2006). This process is predicted to result in a more homogenized biosphere with lower diversity at regional and global scales (McKinney and Lockwood, 1999). The phenomena of biotic homogenization has been demonstrated by numerous studies focused on plant and animal communities, but has been less well explored for microbial communities (McKinney, 2006). Our results suggest that WWTP effluent may be a driver of biotic homogenization of riverine bacterial communities.

Specific changes in bacterial community composition resulted from WWTP effluent inputs. For example, there was a significant decrease in the relative abundance of Proteobacterial sequences. This decrease was driven by a significant decrease in one Proteobacterial class, the Deltaproteobacteria, which includes most of the known sulfate reducing organisms (Madigan et al., 2009). For example, *Desulfococcus* is one sulfate- reducing genus within the Deltaproteobacteria, and *Desulfococcus* sequences were significantly lower downstream of the WWTP effluent. The decreases in Deltaproteobacterial and *Desulfococcus* sequences at the downstream sites may reflect the increased concentration of nitrate at those sites, as nitrate is a more energetically favorable electron acceptor than sulfate and an increase in nitrate would make sulfate reducers less competitive for available electron donors.

WWTP effluent also resulted in a significant decrease in Chloroflexi sequences. The phylum Chloroflexi includes a variety of anoxygenic phototrophic bacteria (Dworkin et al., 2006), so the decrease in the relative abundance of these organisms downstream of the WWTP input was somewhat surprising given the increases in nitrogen and phosphorus. This decrease in Chloroflexi suggests that these organisms may be sensitive to some component of the WWTP effluent. In contrast, Nitrospirae sequences increased downstream of the WWTP effluent. Nitrospirae catalyze the second step in nitrification, so their increased abundance may be a result of the increased ammonium concentrations at the downstream sites. In addition, Nitrospirae are

the dominant nitrite oxidizers within most WWTPs (Daims et al., 2001), so the observed increase in Nitrospirae may merely be the result of a direct release of these organisms from the WWTPs. This possibility is supported by the fact that the two WWTPs included in this study do not disinfect their effluent prior to its release, which is relatively uncommon for WWTPs in the United States (Krasner et al., 2009). However, previous studies have indicated that most bacteria released in WWTP effluent do not typically survive in lotic systems (Garnier et al., 1992), so it seems unlikely that direct inputs of Nitrospirae are the sole cause of higher populations downstream of WWTP effluent.

Two other notable effects of the WWTP effluent were a significant decrease in Dechloromonas sequences and a significant increase in Sphingobacteriales sequences. Dechloromonas sequences were most abundant at the urban upstream site, and as discussed above, this may have been related to the ability of Dechloromonas to oxidize aromatic compounds and the possible presence of anthropogenic aromatic compounds (e.g., PAHs and PCBs) originating from urban runoff and combined sewer discharges. The input of WWTP effluent lowered the abundance of Dechloromonas sequences almost 10-fold, suggesting that WWTP effluent may contain lower levels of these anthropogenic aromatic compounds than untreated urban runoff and combined sewer discharges. This hypothesis is supported by previous research that has demonstrated the ability of wastewater treatment processes to lower PCB and PAH concentrations in wastewater (Bergqvist et al., 2006; Pham and Proulx, 1997). WWTP effluent also resulted in higher abundance of Sphingobacteriales sequences. The Sphingobacteriales are gram-negative bacteria that are found in a wide array of habitats and are known for their ability to utilize unusual compounds including herbicides and antimicrobial compounds (Kämpfer, 2010). The increase in Sphingobacteriales sequences lends further support to the hypothesis that the WWTP effluent might have contained some anthropogenic compounds with antimicrobial properties.

2.7 Conclusions

Our data demonstrate that two rivers that differed significantly in chemical and biological characteristics showed similar responses to WWTP effluent inputs, including decreases in the abundance and diversity of sediment bacterial communities, with the result that bacterial communities that were clearly distinct at the upstream sites were indistinguishable below inputs of WWTP effluents. Given the ubiquity of WWTPs in the United States and worldwide, these results raise new questions about the effects of human modification of stream ecosystems. In addition, the effluent led to increased biotic homogenization and, to our knowledge, this is a new aspect of this phenomenon not previously explored. Further investigations are needed to study the universality of biotic homogenization due to WWTP effluent across a range of river ecosystems.

2.8 Acknowledgements

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Chapter 3: Development of an Analytical Method for Detection of Triclosan in Environmental Samples, Including Soil and Sediment

3.1 Introduction

Two sample preparation methods were explored for extraction of triclosan from sediment samples. The first method was a modified version of USEPA Method 1694 that employs a solid-liquid extraction followed by sample cleanup procedure by solid phase extraction (SPE) (USEPA, 2007). Figure 3.1 presents a flow diagram for the solid-liquid extraction method.

The second method is based on pressurized fluid extraction (PFE) by accelerated solvent extraction (ASE). PFE utilizes elevated temperatures and pressures to increase the efficiency of extraction. Furthermore, inline sample cleanup methods during PFE may be incorporated into the method to reduce sample handling, processing time, and cost. PFE by ASE has been successfully applied to extraction of polychlorinated biphenyls (PCBs), polynuclear aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), organochlorine pesticides, and countless other compounds in a wide range of matrices (Mensching et al., 2012; Ottonello et al., 2013; Xiaoming et al., 2013). In addition, these methods have been validated and are routine USEPA methodology (USEPA, 2007). Successful implementation of the PFE method has already been applied to triclosan extraction from sediments, soils, and biosolids (Cha and Cupples, 2012; Agüera et al., 2003). However, none of these methods included sample cleanup coupled to ASE extraction.

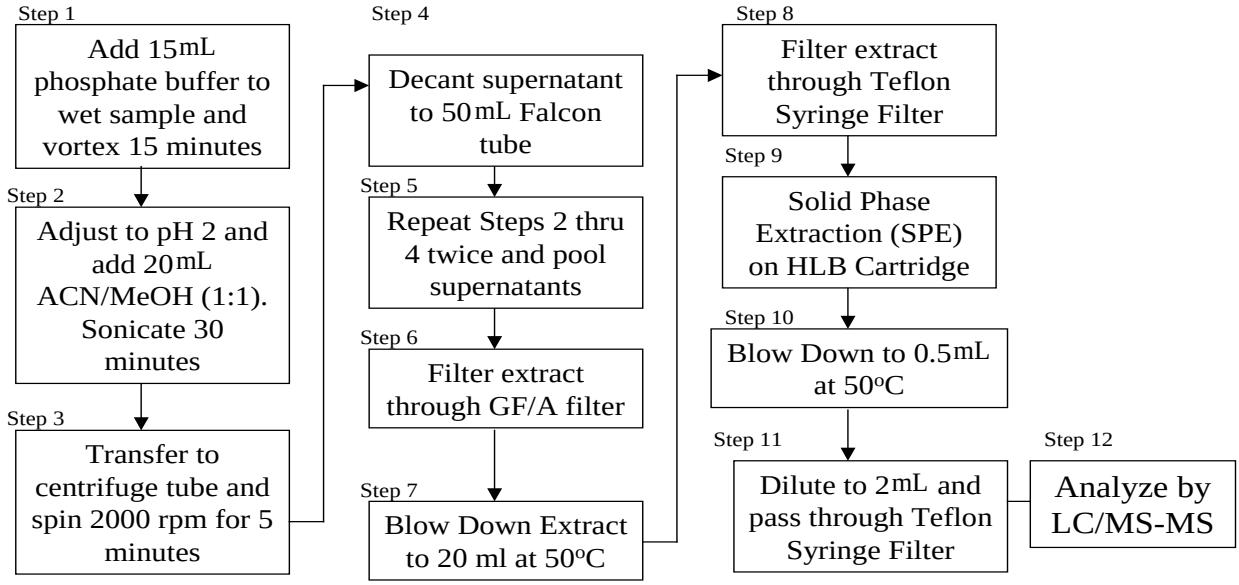

Figure 3.1: Solid-liquid extraction method for triclosan analysis in sediments.

Figure 3.2 presents the PFE method of extraction and analysis of triclosan from sediment samples. If sample cleanup with ASE inline material proves to be feasible, then sample extraction and analysis could be performed in three stages. If the inline sample cleanup is not possible, then a traditional clean method could be utilized with only adding two additional stages. Analyses of the extracts obtained from both methods were performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) per EPA method 1694. A Waters ® 2895 Separations Module coupled to Quattro Micro tandem mass spectrometer was utilized for the analysis. The instrument was calibrated daily with Irgasan reference material procured from Fluka (assay 97.0%). All standards prepared were spiked with carbon-13-enriched triclosan at the same concentration of sample extracts. The purpose of the addition of this internal standard is to monitor instrument drift and is required to calibrate the instrument for isotope dilution calculations. Verification of instrument calibration reference materials was achieved with an independent preparation of the Irgasan material. These check standards were analyzed post calibration and post sample injections. All other operating parameters can be located in Table 4.1 of this report.

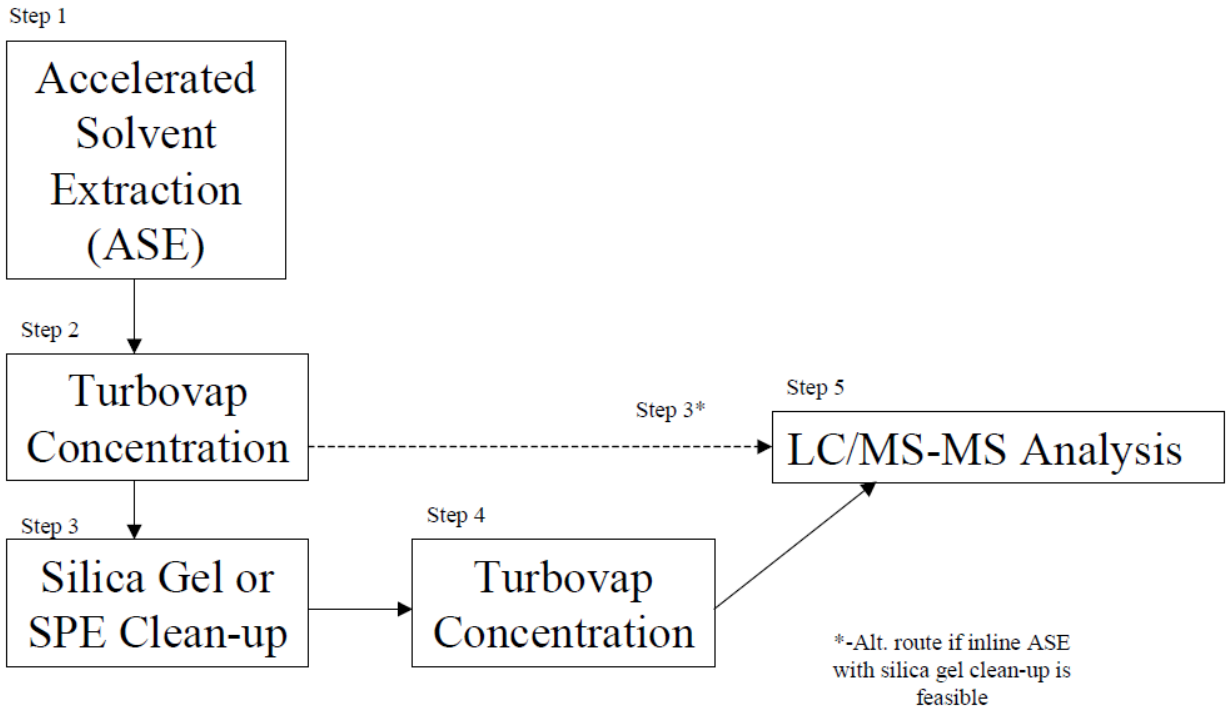

Figure 3.2: PFE extraction by ASE for triclosan in sediments.

3.2 PFE Method Optimization

Post Extraction Cleanup versus Inline Sample Cleanup
Sample cleanup is a necessary part of the sample preparation process and is required to remove components that interfere with triclosan measurements by LC-MS/MS. Silica gel and Oasis ® HLB resin were evaluated as possible cleanup materials. Silica gel is a standard cleanup material and is utilized in USEPA methodology for cleanup of sample extracts intended for analysis of chlorinated compounds (USEPA, 1996). Oasis ® HLB is the resin utilized for solid phase extraction cleanup of extracts intended for PPCP analysis by LC-MS/MS in USEPA methodology 1694. Traditionally sample cleanup is performed on the sample after the extraction process. The extract is normally concentrated and then cleaned up by a solid phase extraction (SPE) process. After the SPE process, the sample is once again concentrated to a known final volume. Inline sample cleanup has been demonstrated on several sample types (Do et al., 2013; Van Emon, 2013). This method involves packing of the PFE extraction cell with the cleanup materials. As the sample is extracted and the solvent flows through the cell, the extract is simultaneously cleaned-up by the resin. This process reduces preparation time and reduces sample handling. The inline sample cleanup and post extraction sample cleanup methods were performed in duplicate on a sediment sample. In addition, each method utilized silica gel, Oasis ® HLB resin, or a silica gel-HLB resin mixture (2:3) as possible cleanup materials.

Solvent to Sample Optimization
A PFE solvent to sample optimization experiment was performed with a sediment sample. A sediment sample was extracted in duplicate at solvent to sample ratios: 5 mL/g, 10 mL/g, 30 mL/g, 60 mL/g, and 80 mL/g.

Solid-Liquid and PFE Parallel Extractions
Extraction of a sediment sample was performed in triplicate by the optimized PFE procedure. The extraction was performed with an ASE 300 system (Dionex, Westmont, IL). Carbon-13-labeled triclosan was added to each sample to be used as a surrogate. The extraction parameters are presented in Table 3.1. After extraction the extracts were concentrated to a final volume of 2.0 mL at 50°C with a closed cell turbovap concentrator unit (Biotage).

Table 3.1: ASE extraction parameters for triclosan.

System	Dionex 300
Cell Sixe	33 ml
Pressure	1500 psi
Temperature	80°C
Static Time	5 min
Flush Volume	60%
Purge	100 sec
Cycles	5
Extraction Fluid	Methanol
Cleanup Material	Waters Oasis ® HLB Resin (3g) / Silica Gel (2g)

Extraction of the identical sediment sample from the PFE experiment was performed in triplicate by the solid-liquid extraction method, see Figure 3.1. Carbon-13-labeled triclosan was added to each sample at the same concentration utilized in the PFE extraction to be used as a surrogate. Extracts from both methods were analyzed by LC-MS/MS per USEPA Method 1694.

3.3 Results

The average absolute carbon-13-labeled triclosan recoveries for the PFE sample cleanup experiment are presented in Figure 3.3. No LC-MS/MS interferences were observed for any of the extracts produced in this experiment.

Figure 3.4 presents the results obtained from the solvent to sample optimization experiment. A solvent to sample ratio above 80 mL/g was above the processing capacity of the ASE extraction system. One possible way to increase this ratio is to reduce the sample mass processed, however, method detection limits would increase with a greater solvent to sample ratio.

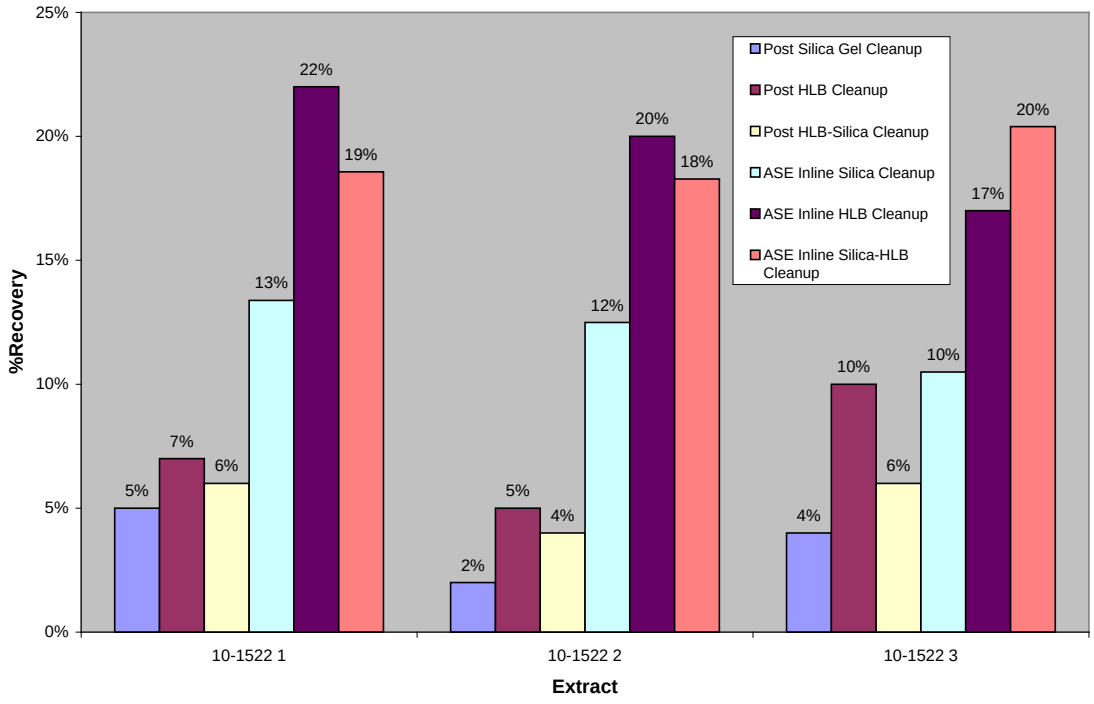

Figure 3.3: Absolute triclosan recoveries for ASE extracts with different cleanup methods.

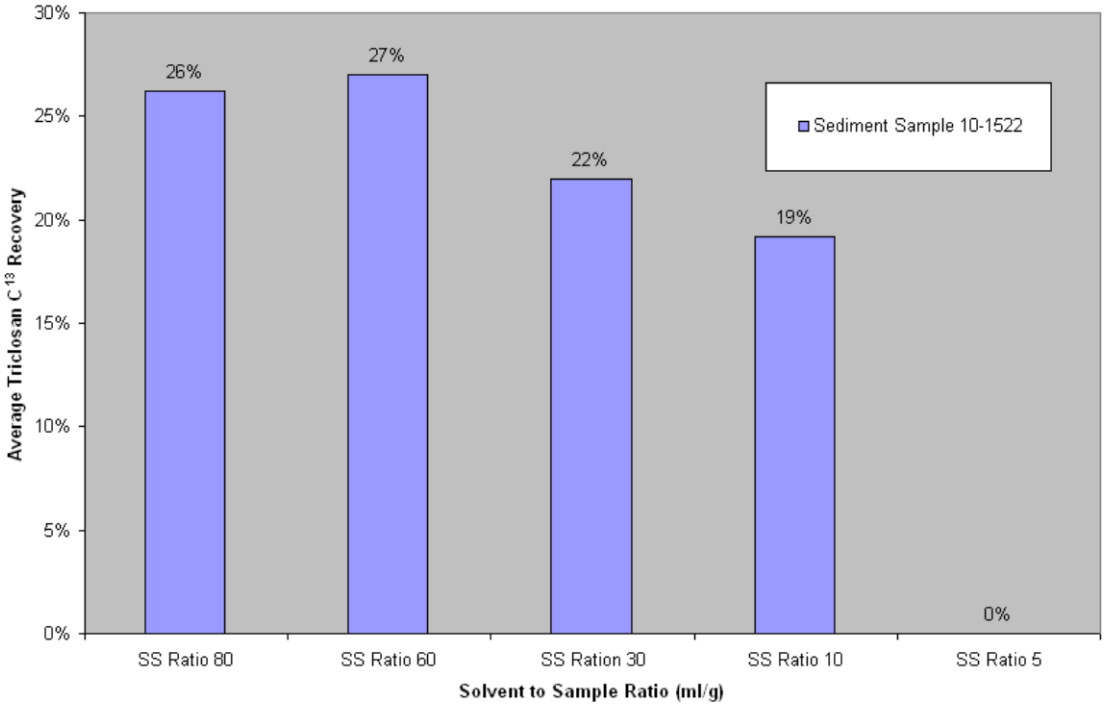

Figure 3.4: Average triclosan C^{13} recovery versus solvent to sample ratio for a sediment sample extracted by ASE.

The absolute triclosan recoveries for the solid-liquid extraction procedure had an average of 0.93% (SD= 1.1%). The absolute triclosan recoveries for the PFE extraction method had an average of 19% (SD= 1.0%). The absolute triclosan recoveries by the PFE extraction method were 20 times greater than those obtained by the solid-liquid extraction method. In addition, the standard deviation for triplicate PFE extraction was superior to the solid-liquid extraction method. Therefore, the developed PFE extraction method was used for extraction of triclosan for all sediment samples.

PFE extraction of the field sediment samples and the artificial stream sediment samples was performed in seven extraction batches. Carbon-13-labeled triclosan was spiked onto all samples before processing to be used for isotope dilution calculations. In addition, each exaction set contained a reagent blank, a sample duplicate preparation, a sample spike preparation, a soft soap sample spike sample, and a reagent blank spike as quality control parameters. Final triclosan results for the field sediment samples and artificial stream sediment samples are presented in Chapter 4 in Tables 4.2 and 4.3, respectively.

3.4 Discussion

Results obtained from the method development experiments indicate that significant triclosan loss occurs from the post extraction sample cleanup procedures. This supports the hypothesis that triclosan losses occur during sample processing. Because recoveries obtained from the inline sample cleanup methods were twice or more than that of the post extraction cleanup method, the

inline cleanup method was implemented for all samples processed. Silica gel material alone for both post extraction and inline extraction cleanup methods did not perform as well as the HLB resin and the silica gel-HLB resin mixture. The HLB resin alone and the silica gel-HLB resin performed about the same for inline sample cleanup. The silica gel-HLB resin mixture was used for all sample processing because it is less costly than HLB resin alone and it also has the potential to remove more of the sample matrix due to the polarity of silica gel.

The method development experiments also indicated that the ratio of methanol solvent to sample was optimal at about 60 mL/g. Recoveries for 80 mL/g were similar than those obtained for 60 mL/g and, because they used more solvent and took longer to concentrate, the 60 mL/g ratio was used for all ASE extractions. The solvent to sample ratio for the standard solid-liquid extraction method is only 12 mL/g. But that solvent-sample ratio may not be enough for complete extraction of triclosan and may be the cause for the lower recoveries observed by this procedure. Greater solvent to sample ratios may be achieved by lowering the sample mass extracted; however, by doing this, method detection limits would be increased. The instrument detection limit commonly reached by the LC-MS/MS ranged from 0.5 ng/mL to 1.0 ng/mL. When two grams of dried sediment sample was extracted by the ASE system with a solvent to sample ratio of 60 mL/g and concentration was performed to a final volume of 2.0 mL, a method detection limit of 0.002 mg/kg was achieved.

The optimized PFE extraction method outperformed the standard solid-liquid extraction method. Carbon-13-labeled triclosan recoveries by the PFE method were twice that of the solid-liquid extraction method. In addition, better precision was observed for the PFE extraction recoveries as well (%RSD for S-L Ext = 118% versus %RSD for PFE Ext = 5.3%). The most likely cause for the large %RSD associated with the solid-liquid extraction method is due to the massive triclosan loss during sample preparation. The solid-liquid extraction procedure developed by the EPA, method 1694, was designed to extract seventy or more target PPCPs from a solid sample. The sample preparation has a great deal of steps that increase sample handling and sample preparation time, while reducing absolute labeled triclosan recoveries. The optimized PFE extraction method with inline silica gel-HLB resin cleanup performed far more superior than the traditional solid-liquid extraction method. Absolute labeled triclosan recoveries were much greater, sample-processing time was reduced, and cost per sample was reduced as well. Increased recoveries are most likely due to a reduction in sample handling. Data generated in these experiments indicate that with each additional sample processing stage, a reduction of triclosan recovery occurs. Since triclosan is known for its hydrophobic property, along each sample processing step triclosan is lost to glassware and preparation materials. In addition, an increase of recovery by PFE may also be due to the elevated temperatures and pressures utilized by this extraction procedure.

Quality control parameters for the PFE extracted sediment samples were all within the acceptance criteria established in USEPA method 1694, Table 14. Processed sample duplicates had %RPD’s ranging from 3% to 26% for samples containing triclosan concentrations above the detection limit. Reagent blank spike recoveries ranged from 82% to 106% and sample spikes ranged from 92% to 116% for all spikes performed at appropriate concentration ranges.

3.5 Conclusions

A PFE extraction method was optimized for analysis of triclosan in sediment samples. Inline sample cleanup with ASE extraction was found to be superior to post extraction cleanup due to reduction in sample handling. Inline sample cleanup with silica gel-HLB resin (2:3) was found to perform as well as HLB resin alone and better than silica gel alone. The solvent to sample ratio for methanol by PFE was optimized at 60 mL/g sediment sample. The PFE extraction method was performed on seven batches of sediment samples and all quality control were within acceptable limits for EPA methodology 1694 for analysis of PPCPs. In addition, the developed method was quicker, less costly, and produced better absolute recoveries than the standard solid-liquid extraction method.

3.6 Acknowledgements

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Chapter 4: Effects of Triclosan Exposure on Benthic Bacterial Communities

4.1 Note on publication

The contents of this chapter have been published:

- Drury, B., Scott, J., Rosi-Marshall, E.J., and Kelly, J.J. 2013. Triclosan exposure increases triclosan resistance and influences taxonomic composition of benthic bacterial communities. Env. Sci. Techn., 47:8923-8930.

4.2 Abstract

Triclosan is a broad-spectrum antimicrobial compound that is incorporated into numerous consumer products. Triclosan has been detected in aquatic ecosystems across the United States, raising concern about its potential ecological effects. We conducted a field survey and an artificial stream experiment to assess effects of triclosan on benthic bacterial communities. Field sampling indicated that triclosan concentrations in stream sediments increased with degree of urbanization. There was significant correlation between sediment triclosan concentration and the proportion of cultivable benthic bacteria that were resistant to triclosan, demonstrating that the levels of triclosan present in these streams was affecting the native communities. An artificial stream experiment confirmed that triclosan exposure could trigger increases in triclosan resistance within cultivable benthic bacteria, and pyrosequencing analysis indicated that triclosan resulted in decreased benthic bacterial diversity and shifts in bacterial community composition. One notable change was a six-fold increase in the relative abundance of cyanobacterial sequences and a dramatic die-off of algae within the artificial streams. Selection of cyanobacteria over algae could have significant implications for higher trophic levels within streams. Finally, there were no observed effects of triclosan on bacterial abundance or respiration rates, suggesting that bacterial density and function were highly resilient to triclosan exposure.

4.3 Introduction

Triclosan (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, 2006). These products are widely used and there is growing concern about potential ecological effects of triclosan because it has been detected in surface waters across the United States (Bartelt-Hunt et al., 2009; Chalew and Halden, 2009; Halden and Paull, 2005; Kolpin et al., 2002; Watkinson et al., 2009) and in Europe (Adolfsson-Erici et al., 2002; Bendz et al., 2005; Lindström et al., 2002; Sabaliunas et al., 2003; Singer et al., 2002).

Triclosan can be introduced to natural aquatic environments via domestic wastewater. Because triclosan is incorporated into a wide variety of soaps and detergents, it will enter domestic wastewater through normal use, and several recent studies have confirmed the presence of triclosan in domestic wastewater (Bester, 2005; Halden and Paull, 2005; Kanda et al., 2003). Municipal wastewater treatment plants (WWTPs) remove the majority of triclosan from wastewater (Bester, 2005; Halden and Paull, 2005; Kanda et al., 2003; Sabaliunas et al., 2003), but removal is not complete, making it possible for WWTP effluent to provide a chronic low

dose of triclosan to aquatic ecosystems. Total annual loading of triclosan into U.S. surface waters has been estimated at 5,200-18,824 kg year-1 with 50-56% coming from WWTP effluent (Halden and Paull, 2005). Combined sewer overflows (CSOs), which release untreated wastewater during high rainfall events, could provide a more concentrated but sporadic dose of triclosan to aquatic environments (Halden and Paull, 2005). Finally, untreated wastewater released from leaking sewer infrastructure (Kaushal et al., 2011) represents another possible route for triclosan entry into aquatic ecosystems (Halden and Paull, 2005). Within aquatic habitats triclosan is likely to accumulate in sediments, as it is a lipophilic compound with low aqueous solubility (Halden and Paull, 2005). The aromatic nature of triclosan and its high chlorine content indicate that it should resist degradation and persist in the environment (Halden and Paull, 2005), and several studies have detected triclosan in sediment cores >30 years old (Cantwell et al., 2010; Miller et al., 2008; Singer et al., 2002).

Triclosan is toxic to bacteria via inhibition of the enzyme enoyl-ACP reductase, which is an essential component of the bacterial fatty acid biosynthetic pathway (Heath et al., 1998). There is extensive data on triclosan toxicity to pure bacterial cultures, but less is known about effects of triclosan on bacterial communities in the environment (Proia et al., 2011). Due to its antimicrobial properties, triclosan may negatively affect the abundance and activity of benthic bacteria, which could have broader ecosystem-level implications because benthic bacteria are key drivers of nutrient cycling (Hall and Tank, 2003). If bacterial taxa differ in triclosan sensitivity, triclosan may also act as a selective agent and drive changes in bacterial community composition, which can impact function (Ptacnik et al., 2008). In addition, triclosan can negatively affect algal communities (Proia et al., 2011; Wilson et al., 2003). 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).

Bacteria exposed to triclosan in the laboratory can develop resistance through mutations in the gene (fabI) that encodes the target enzyme (Heath et al., 1999), through overexpression of fabI (McMurry, et al., 1998) or through efflux pumps (Chuanchuen et al., 2003). The presence of triclosan might also contribute to the spread of resistance genes, as exposure to antimicrobial agents can lead to increased rates of genetic exchange via transformation (Prudhomme et al., 2006) or conjugation (Beaber et al., 2003). Therefore, accumulation of triclosan in sediments could increase prevalence of triclosan resistance among resident bacterial communities. This is a concern as it may lead to a reduction in efficacy of triclosan as an antimicrobial agent, but also because several studies have demonstrated a link between triclosan resistance and resistance to antibiotics (Birošová and Mikulášová, 2009; Braoudaki and Hilton, 2006; Chuanchuen et al., 2001), suggesting that triclosan exposure may select for resistance to therapeutically useful antibiotics. Several studies have demonstrated the ability of cultured bacteria to develop triclosan resistance based on triclosan exposure in the lab (26-28 hrs.), but no studies have linked triclosan exposure to resistance in the environment.

The goals of this project were (1) to determine if triclosan in urban stream sediments is affecting the resident benthic bacterial communities; and (2) to determine experimentally if triclosan exposure can influence the taxonomic structure, activity and triclosan resistance of benthic bacterial communities.

4.4 Materials and Methods

Field Sites and Sampling

North Shore Channel (NSC), which begins in Wilmette, IL, and extends into Chicago, IL, was selected to represent a highly urbanized river. NSC has a drainage area of 6,474 ha that is 63% residential, 16.7% commercial/industrial, 10% forest/open land, 5.4% institutional, and 3.5% transportation/utility (HDR Engineering Inc., 2011). NSC receives treated effluent from North Side Water Reclamation Plant (NSWRP), an activated sludge plant that has an average flow of 245 million gallons per day (MGD) and a design capacity of 333 MGD. Effluent from NSWRP is not disinfected prior to release (www.mwrd.org). There are also 29 combined sewer outfalls (CSOs) within the first 10 km of NSC. Two sampling sites on NSC were chosen. Urban site 1 (42.029496, -87.710032) is downstream of 25 CSO outfalls and is 950 meters upstream of the NSWRP. Urban site 2 (42.021514, -87.710198) is 50 meters downstream of NSWRP, and there are 4 additional CSOs between urban site 1 and site 2.

West Branch DuPage River (WBDR), located in DuPage County, IL, was selected to represent a suburban river. WBDR has a drainage area of 32,900 ha that is 32.8% residential, 17.4% agricultural, 16.9% vacant, 11.2% forest/open land and less than 4% industrial (Aqua Terra Consultants, 2003). Two sampling sites on WBDR were chosen, located upstream and downstream of West Chicago WWTP (WCWWTP), an activated sludge plant that treats 5 MGD and does not disinfect effluent prior to release (www.westchicago.org). Suburban site 1 (41.866481, -88.189246) is 275 meters upstream of the WCWWTP and approximately 9, 18 and 25 km downstream of three other suburban WWTPs that discharge to WBDR. Suburban site 2 (41.863812, -88.190416) is located 50 meters downstream of WCWWTP. There are no CSOs that discharge to WBDR (http://www.drscw.org/descriptions.html).

Nippersink Creek (NC), a woodland stream located in McHenry County, IL, was selected to represent a stream with minimal urbanization. NC has a drainage area of 5,095 ha that is 7.8% residential, 63.1% agricultural, 2.1% vacant, 20.7% open land and 0.1% industrial (www.nippersink.org). Sediment samples were collected from one site on NC (42.417964, -88.344610). There are no WWTPs or CSOs on NC upstream of the site.

Five replicate sediment samples were collected from each of the five field sites between July and August 2010. Each sediment sample was a composite of 10 individual samples collected using a Petite Ponar Sampler (Wildlife Supply Company, Saginaw, MI). Samples were transported to the laboratory on ice and stored at 4°C. Samples for triclosan analysis were shipped on ice to Illinois Sustainable Technology Center (ISTC), Champaign, IL.

Artificial Streams

Artificial stream experiments were conducted from July through December 2011 using six fiberglass artificial streams (4 m x 15.5 cm x 15 cm) located in a greenhouse. Stream sediments were composed of 0.5 kg pea gravel, 9.5 kg sand, and 66.67 g each of red maple, ginkgo and oak leaves that had been dried, shredded and leached to remove tannins. This amount of leaf material was chosen to approximate the sediment organic matter concentration of the woodland field site. Streams were filled with 60 L dechlorinated tap water and refilled each week to compensate for evaporation. Current velocity in the streams was maintained at 0.18 m s-1 by stainless steel

paddlewheels. To provide an inoculum of microbes, each stream was amended with 100 mL sediment from the woodland site. Streams were covered with shade cloth (50% light reduction) to limit algal growth. Streams were run for two months prior to triclosan treatments to allow for colonization of sediments by microbes.

After the two-month colonization period, three treatment streams were amended with triclosan and three control streams received no triclosan. The target was a triclosan sediment concentration of 12 ppm, which represents 25% of the highest value reported in the literature (Chalew and Halden, 2009). To achieve this in each stream, we added the amount of triclosan required to exceed the aqueous solubility of triclosan (10 mg L-1) (Morrall et al., 2004) by the amount needed to bring the sediment concentration to 12 ppm. Thus, after calculations, 720 mg triclosan (Kansai Chemicals, Tokyo, Japan) was dissolved in 25 mL of dimethyl sulfoxide (DMSO; Fisher, Pittsburgh, PA) and added to each of the treatment streams. While triclosan concentrations measured in stream ecosystems are several orders of magnitude lower than 10 mg L-1 (Kolpin et al., 2002a; Kolpin et al., 2002b; Kolpin et al., 2002c), this approach enabled us to achieve the targeted concentration in the artificial stream sediments. Control streams received 25 mL DMSO with no triclosan. Sediment samples were collected from each stream immediately prior to dosing (day 0) and subsequently at approximately weekly intervals. Biological assays were conducted the same day as the samples were collected. Sediment samples for triclosan analysis were shipped on ice to ISTC.

Measurement of triclosan Concentration in Sediments

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 smaller than 0.065 inches. The collected material was then mixed and extracted as is. Two grams of each sediment were extracted by an accelerated solvent extraction (ASE) procedure with a Dionex ® ASE 300 system. Methanol was utilized as the extraction fluid and each extraction cell was packed with a custom Waters resin. The resin allowed simultaneous sample cleanup and extraction. Specific extraction parameters are listed in Chapter 3, Table 3.1. This method reduced sample preparation time and target compound loss by decreasing sampling handling. Carbon-13-enriched triclosan was spiked into all samples before extraction to allow isotope dilution calculations and to monitor target compound loss. After the sample was processed by the ASE method, the extract was concentrated to a final volume 2.0 mL prior to analysis.

A Waters 2695 Separations Module coupled to Quattro Micro tandem mass spectrometer was utilized for analysis. The target compound was separated on a Waters Symmetry 2.1mm x 60mm, 3.5 μm C8 analytical column. Elution was performed isocratically with a water (pH 2)-methanol-acetonitrile (15:42:43) mobile phase. Native- and labeled-triclosan were detected by tandem mass spectrometry (MS/MS) using argon as a collision gas. Specific operating parameters are listed in Table 4.1. The instrument was calibrated with Irgasan reference material procured from Fluka (assay 97.0%). All standards prepared were spiked with carbon-13-enriched triclosan at the same concentration as sample extracts. The purpose of the addition of this internal standard was to monitor instrument drift and is required for calibration of the instrument for isotope dilution calculations. Verification of instrument calibration reference materials was achieved with an independent preparation of the Irgasan material. These check standards were analyzed post calibration and post sample injections.

Table 4.1: LC-MS/MS operating parameters.

Mobile Phaes	Water:Methanol:Acetonitrile (15:42:43)
Column	Waters Symmetry ® 2.1mm x 60mm, 3.5 mm C8
Flow Rate	0.2 ml/min
Injection Volume	10 µl
Number of injections per sample	3
MS Function Type	MRM
Ion Mode	ES-
Cone Voltage	18.00
Collision Energy (eV)	9.00
Triclosan Quant Ions (Da)	Parnt 287.00 - Dau 34.50
Labeled Triclosan Quant Ions (Da)	Parnt 298.80 - Dau 34.50

Triclosan Resistance of Bacterial Communities

Triclosan resistance of bacterial communities was measured by heterotrophic plate counts on soy extract agar amended with 16 mg/L triclosan. This concentration was chosen because it reduced growth of bacteria from the woodland field site by approximately 99% in a preliminary experiment. The percentage of bacteria resistant to triclosan was determined by dividing counts obtained on triclosan-amended plates by counts obtained on unamended plates. Plate counts were performed by a standard method (Page, 1982).

Epifluorescence Counts

Direct counts of bacterial cells were performed using a modified standard method (Kepner and Pratt, 1994). Cells were fixed by diluting sediment 1:50 in sterile fixative (Gough and Stahl, 2003). Samples were sonicated in an ultrasonic ice water bath for 15 min at 60 Hz and were then diluted 1:1,000, 1:2,000 and 1:4,000 in filter-sterilized deionized water. Diluted samples (2 mL) 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 dry sediment.

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 50 mL centrifuge tube filled to the top (no head space) with well water. Water temperature and initial dissolved oxygen (DO) concentrations were measured using an YSI ProODO meter (YSI Inc. Yellow Springs, OH). Tubes were capped and incubated at 25°C in the dark for 2 hours, after which final DO was measured and respiration rates were calculated. Respiration rates were normalized by sediment surface area.

Bacterial Community Composition Analysis

DNA was isolated from sediment samples using the UltraClean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA) and sent to Research and Testing Laboratory (Lubbock, TX) for tag

pyrosequencing of bacterial 16S rRNA genes. PCR amplification was performed using primers 530F and 1100R (Boon, et al., 2002). 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) as described previously (Drury, et al., 2013) and were grouped at the class level. The relative abundance of each bacterial class within each sample was imported into Primer software (Primer V.5, Primer-E Ltd., Plymouth, UK) (41). A similarity matrix was calculated using the Bray-Curtis coefficient (Bray and Curtis, 1957) and non-metric multi-dimensional scaling (nMDS) was used to ordinate the data. ANOSIM routine in Primer was used to assess differences between groups of samples. SIMPER routine in Primer was used to identify bacterial classes making the largest contributions to variations between communities. Diversity of communities was quantified based on inverse Simpson index (Simpson, 1949).

Statistics

Field data were analyzed by one-way ANOVA based on sampling location. When significant effects of location were observed pairwise comparisons were made by Tukey's post hoc HSD test. Correlations were assessed by determining Pearson product-moment correlation coefficients and Bonferroni-corrected probabilities. Artificial stream data were analyzed by repeated measures ANOVA. When significant effects of sampling day were observed one-way ANOVA followed by Tukey's HSD test was used to compare data within treatments. Artificial stream data were log transformed prior to analysis to correct for unequal variances between treatments. Statistical analyses were run using Systat 13 (Systat Software, Inc., San Jose, CA) and p values less than 0.05 were considered to be significant.

4.5 Results

Field Sites

There was a significant effect of site on the sediment triclosan concentrations ($p<0.001$) with the highest concentrations occurring at sites with the highest degree of urbanization (urban > suburban > woodland) (Table 4.2). The sediment triclosan concentration at urban site 1 was significantly higher than all of the other sites, most likely due to the high number of CSO outfalls upstream of this site. There were significantly lower sediment triclosan concentrations at urban site 2 than at site 1, suggesting that effluent from the intervening WWTP plant might be contributing to lower sediment triclosan concentrations at site 2. Although the mean triclosan concentrations from the two suburban sites were not significantly higher than the woodland site (Table 4.3), triclosan was detected more frequently at the suburban site (6 out of 8 samples) than at the woodland site (1 out of 4 samples), suggesting broader distribution of triclosan at the suburban sites.

Table 4.2: Triclosan concentrations in stream sediments from field sites.

Site	ng g^{-1} *	
Urban Site 1	107 ± 18.1	a
Urban Site 2	33 ± 11.0	b
Suburban Site 1	9 ± 3.3	bc
Suburban Site 2	4 ± 0.8	bc
Woodland Site	1 ± 0.3	c

* Limit of detection = 1 ng g-1. Each data point represents mean value (n=3 to 5) ± standard error. ANOVA indicated a significant effect of site ($p<0.001$). Data followed by different letters are significantly different ($p<0.05$) based on Tukey's post-hoc test.

There was a significant effect of site on triclosan resistance within sediment bacterial communities ($p<0.001$) with the sites with the highest degree of urbanization showing highest resistance (urban > suburban > woodland) (Fig 4.1A). There was also a significant correlation between sediment triclosan concentration and triclosan resistance ($p<0.001$) (Figure 4.1B), suggesting that triclosan exposure may increase the relative abundance of triclosan-resistant bacteria within aquatic sediments. There was a significant effect of site on total counts of bacteria within sediments (data not shown; $p=0.002$) but there was not a significant correlation between triclosan concentration and bacterial abundance ($p=0.485$; Fig 4.2A). Similarly, there was a significant effect of site on sediment respiration rates (data not shown; $p=0.005$) but there was not a significant correlation between triclosan concentration and respiration rates ($p=0.523$; Fig 4.2B). These data indicate that triclosan was not a significant driver of bacterial abundance or community respiration rates at these field sites.

Tag pyrosequencing of bacterial 16S rRNA genes was used to analyze taxonomic composition of sediment bacterial communities from field samples. After pretreatment the data set included a total of 214,711 high quality sequences for an average of 8,588 sequences per sample. The predominant sequences were those representing the phyla Proteobacteria (48% of total sequences), Bacteriodetes (19%) and Chloroflexi (10%). Pyrosequencing data were analyzed by nMDS and there was no relationship between overall community composition and triclosan concentration (data not shown). There was also no correlation between triclosan concentration and bacterial diversity (data not shown; $p=0.072$; $R2=0.177$).

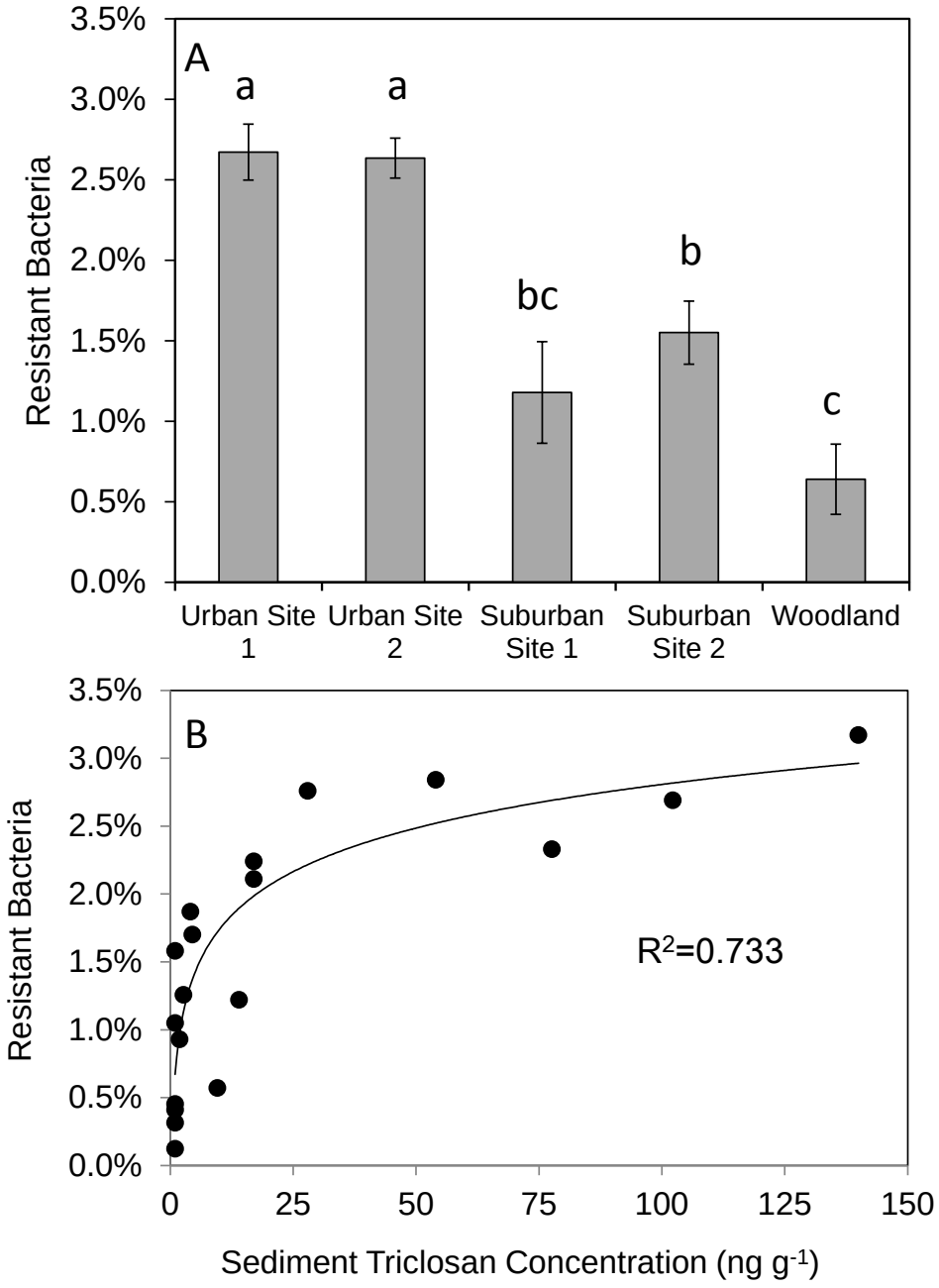

Figure 4.1: Bacterial resistance to triclosan. (A) Percentage of sediment bacteria resistant to triclosan for field samples. Each data point represents mean value (n=5) with standard error bars. ANOVA indicated a significant effect of location ($p<0.001$). Data points with different letters are significantly different based on Tukey's post-hoc test ($p<0.05$). (B) Relationship between sediment triclosan concentration and percentage of sediment bacteria resistant to triclosan for field samples. Pearson correlation analysis indicated a significant correlation between concentration and resistance ($p<0.001$).

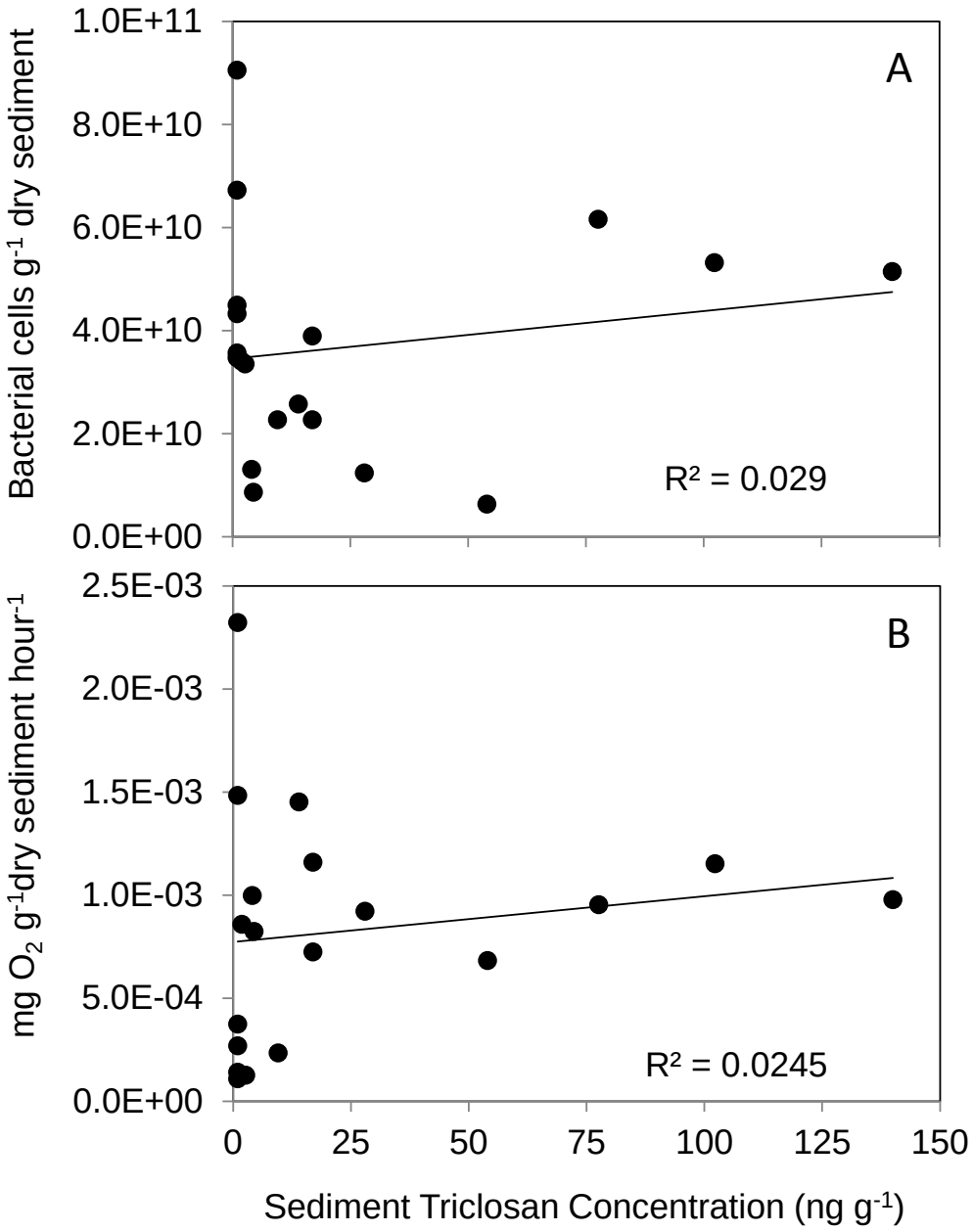

Figure 4.2: Relationship between sediment triclosan concentration and (A) bacterial abundance and (B) respiration in field samples. Pearson correlation analysis indicated no significant correlations between triclosan concentration and bacterial abundance (p=0.485) or respiration (p=0.523).

Artificial Stream Experiment

Triclosan amendment resulted in a significant increase in sediment triclosan concentrations within the artificial streams ($p<0.001$; Table 4.3). The average triclosan concentration in treated streams was 7.9 ppm, which was slightly below our target concentration of 12 ppm. The highest triclosan concentration was measured on day 7, but there was not a statistically significant difference in triclosan concentration between day 7 and later sampling dates, indicating that the sediment triclosan concentration remained stable over the course of the experiment.

There was no significant effect of triclosan amendment on the numbers of bacteria within artificial stream sediments (Figure 4.3A) or community respiration rates (Figure 4.3B). However, triclosan treatment did have a significant effect on the percentage of triclosan-resistant bacteria within sediments (Figure 4.4). Repeated measures ANOVA indicated a significant effect of triclosan on resistance ($p<0.001$), with triclosan treatments showing higher resistance than control streams for all days post-treatment. Repeated measures ANOVA also indicated a significant effect of sampling day on triclosan resistance ($p<0.001$) and a significant treatment by day interaction ($p<0.001$). One-way ANOVAs indicated a significant effect of sampling day on resistance levels in triclosan treated streams ($p<0.001$), with resistance levels increasing over time, whereas resistance levels in control streams showed no significant effect of sampling date ($p=0.626$).

Table 4.3: Triclosan concentrations in sediments from model streams (µg g-1). *

Day	Control Streams	Triclosan Streams
0	0.0022 a	0.0018 a
7	0.0040 a	17.3333 b
14	0.0175 a	8.0667 bc
19	0.0121 a	3.2667 c
28	0.0075 a	5.0333 bc
34	0.0076 a	5.7000 bc

* Limit of detection = 1 ng g-1. Each data point represents mean value (n=3) with standard error values in parentheses. Triclosan streams received triclosan amendment on day 0 and control streams received no triclosan. Repeated measures ANOVA indicated a significant effect of treatment ($p<0.001$), a significant effect of sampling day ($p<0.001$) and a significant interaction effect ($p<0.001$). Data points with different letters represent significant differences in triclosan concentration over time within a treatment (control or triclosan) based on one way ANOVA ($p<0.05$).

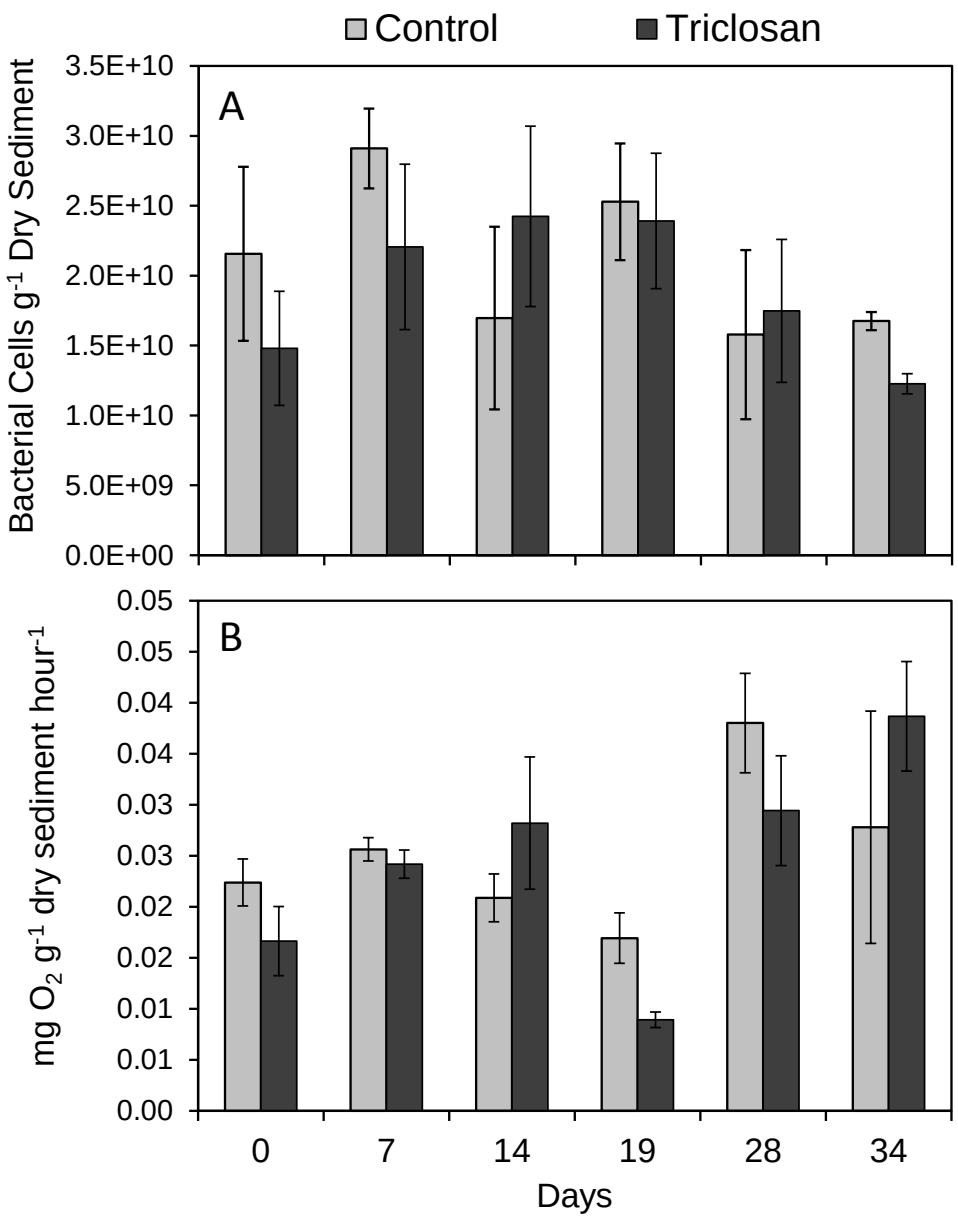

Figure 4.3: Bacterial abundance (A) and respiration rates (B) for sediments from artificial streams. Triclosan streams received a triclosan amendment on day 0 and control streams received no triclosan. Each data point is mean (n=3 streams) +/- standard error. Repeated measures ANOVA indicated no significant effect of triclosan on bacterial abundance (p=0.540) or respiration (p=0.785).

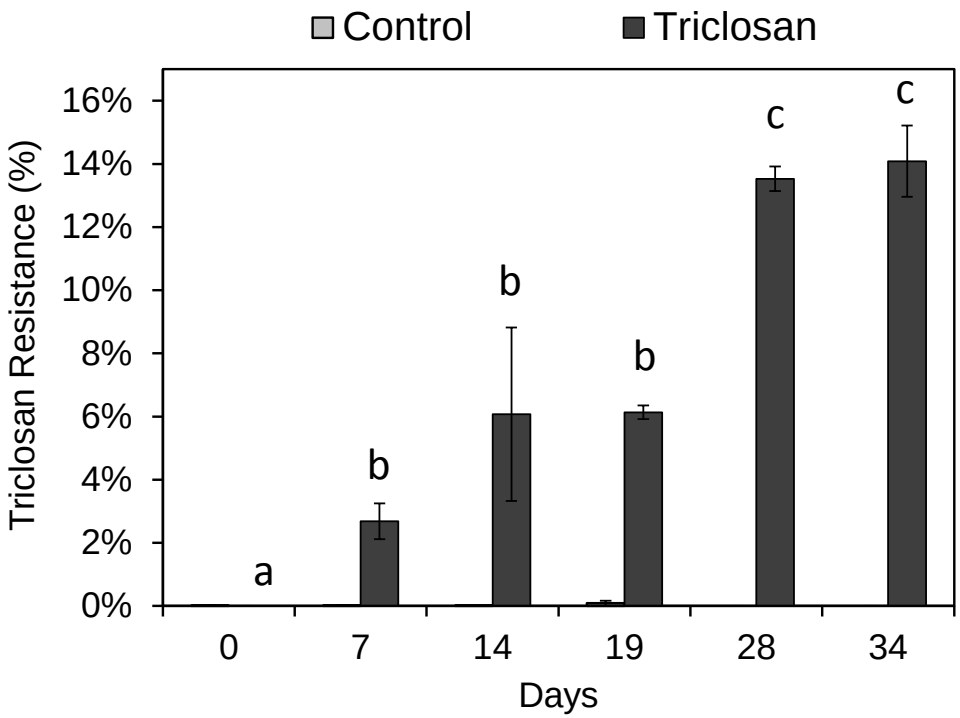

Figure 4.4: Percentage of sediment bacteria resistant to triclosan for artificial streams. Triclosan streams received a triclosan amendment on day 0 and control streams received no triclosan. Each data point is mean (n=3 streams) +/- standard error. Repeated measures ANOVA indicated a significant effect 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 between the sampling dates.

Tag pyrosequencing of bacterial 16S rRNA genes was used to analyze taxonomic composition of sediment bacterial communities within artificial streams on days 0, 14 and 34. After pretreatment the data set included a total of 197,208 high quality sequences for an average of 10,956 sequences per sample. The number of bacterial classes observed per sample ranged from 60 to 78 with a mean of 70.3. Depth of coverage at the class level was calculated for each sample by dividing the total number of bacterial classes observed by the estimated total number of bacterial classes within each sample (calculated using the Chao1 richness estimator). The depth of coverage for the samples ranged from 84 to 99% with a mean of 93.7%. The predominant sequences within all of the streams were those representing the phyla Chloroflexi (31% of total sequences), Proteobacteria (25%) and Bacteriodetes (25%), which were the same dominant groups as observed in the field sites. There was a significant effect of triclosan amendment on bacterial community diversity ($p<0.05$), with triclosan-treated streams showing significantly lower diversity ($p<0.05$) than control streams at 14 and 34 days post-treatment (Figure 4.5). The nMDS ordination (Figure 4.6) and ANOSIM analysis of pyrosequencing data indicated that there was no significant difference in bacterial community composition between triclosan and control streams on day 0 ($R= -0.222$, $p=0.9$), but there was a significant difference on day 14 ($R=0.667$, $p=0.10$) and a larger difference on day 34 ($R=0.815$, $p=0.10$). SIMPER analysis was used to identify bacterial taxa that made the most significant contributions to differences in bacterial community composition between triclosan and control streams on day 34. Nine bacterial taxa

accounted for 75% of the variation in bacterial community composition between triclosan and control streams (Table 4.4) and there were statistically significant differences in relative abundances of sequences from several of these taxa. For example, sediment bacterial communities from triclosan-amended streams showed significantly higher relative abundances of sequences representing Anaerolineae and Cyanobacteria, and significantly lower relative abundances of sequences representing Sphingobacteria, Betaproteobacteria, Deltaproteobacteria, and Bacteroidia as compared to control streams.

Comparison of Field and Artificial Stream Data

Based on results of the artificial stream experiment, we went back to the pyrosequencing data from the field sites to determine if there were significant correlations between triclosan concentrations and relative abundances of the bacterial groups that responded to triclosan addition in artificial streams (those listed in Table 3). Only one of these groups, the Gammaproteobacteria, showed a significant correlation between relative abundance of sequences and sediment triclosan concentration ($p<0.001$; $R2=0.7828$). Within Gammaproteobacteria, the predominant order in terms of sequence abundance was Methylococcales, and there was significant correlation between relative abundance of Methylococcales sequences and sediment triclosan concentration ($p<0.001$; $R2=0.930$; Figure 4.7). In artificial streams, there was no significant effect of triclosan addition on relative abundance of Methylococcales sequences at day 34 ($p=0.191$). However, the relative abundance of Methylococcales sequences in artificial streams was 0.8%, which was much lower than the 6% relative abundance of Methylococcales sequences at urban site 1 but was similar to the 0.5% relative abundance at the woodland field site, which was the source of the microbial inoculum for the artificial streams.

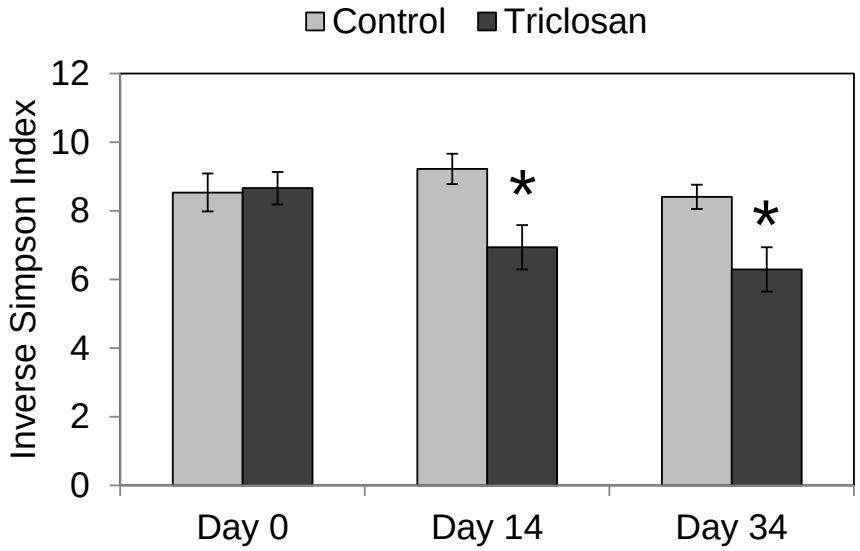

Figure 4.5: Diversity of sediment bacterial communities in artificial streams. Triclosan streams received a triclosan amendment on day 0 and control streams received no triclosan. Each data point is mean (n=3 streams) +/- standard error. Repeated measures ANOVA indicated a significant effect of triclosan ($p<0.05$) but no effect of day ($p=0.087$) and no interaction effect ($p=0.066$). Asterisks indicate significant differences between control and treatment streams on individual sampling days ($p<0.05$).

Table 4.4: SIMPER analysis of 16S tag pyrosequencing data.*

Bacterail Taxa	Relative Abundance (%) Control Streams	Relative Abundance (%) Triclosan Streams	p value	Contribution to variation (%)
Anaerolineae	25.92	34.73	0.038	21.27
Sphingobacteria	19.39	13.42	0.044	14.39
Cyanobacteria	0.94	5.55	0.034	11.61
Betaproteobacteria	5.11	2.46	0.002	6.39
Flavobacteria	6.15	7.61	0.354	5.13
Deltaproteobacteria	5.71	3.75	0.021	4.73
Bacteroidia	4.49	2.75	0.017	4.19
Alphaproteobacteria	4.99	4.07	0.478	4.00
Gammaproteobacteria	3.59	3.69	0.933	3.34

* indicating bacterial taxa making the most significant contributions to differences in community composition between day 34 samples from control and triclosan amended artificial stream sediments.

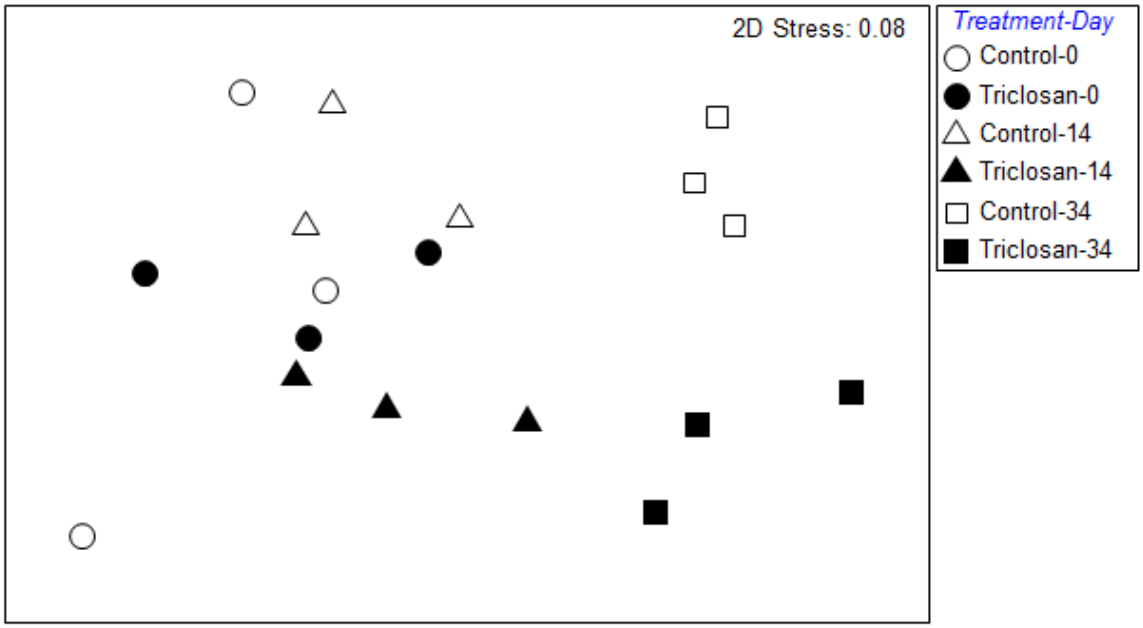

Figure 4.6: nMDS ordination of sediment bacterial communities from artificial streams. nMDS ordination is based on sample days 0, 14 and 34 and tag pyrosequencing of bacterial 16S rRNA genes. Triclosan streams received a triclosan amendment on day 0 and control streams received no triclosan. Each data point represents one model stream on one sampling date.

4.6 Discussion

Field Study

Triclosan concentrations in stream sediments in the Chicago metropolitan region increased with degree of urbanization. There was a strong correlation between triclosan concentrations in these sediments and relative abundance of triclosan-resistant bacteria, which provides strong support for the conclusion that the levels of triclosan detected in these stream sediments were high enough to have a direct biological effect, specifically by creating a selective pressure favoring triclosan-resistant bacteria. Several studies have demonstrated the ability of cultured bacteria to develop triclosan resistance based on triclosan exposure in the lab (Birošová and Mikulášová, 2009; Braoudaki and Hilton, 2006; Chuanchuen et al., 2001), but our work represents the first documented link between triclosan exposure and an increase in triclosan resistance in a complex bacterial community in the environment. It should be noted that the triclosan resistance assay used in this study was limited to the cultivable fraction of the bacterial communities. While it reasonable to hypothesize that the non-cultivable fraction of the bacterial communities in these sediments might also have shown an increase in triclosan resistance, further work would be needed to confirm this hypothesis.

There was no observed relationship between triclosan concentration in stream sediments and either bacterial abundance, respiration rates or diversity. However, these field sites varied in multiple parameters, ranging from physical factors including depth and discharge to chemical factors including concentrations of DOC and inorganic nutrients. Therefore, it is not surprising that triclosan was not the main driver of bacterial abundance, respiration or diversity at these sites. However, guided by the artificial stream results, we did detect a strong correlation between triclosan concentration and relative abundance of Methylococcales. A recent study demonstrated that *Methylobacillus* was the predominant organism utilizing triclosan as a carbon source in an enrichment culture from activated sludge (Lolas, et al., 2012). While Methylococcales (Gammaproteobacteria) and *Methylobacillus* (Betaproteobacteria) are not closely related phylogenetically, they are both methylotrophs. Methylotrophs, including species within the Gammaproteobacteria, have been shown to participate in cometabolism of a variety of environmental pollutants including chlorinated aliphatic and aromatic compounds through production of soluble methane monooxygenase (Jechorek, et al., 2003; Koh, et al., 1993). Therefore our data suggest that the observed correlation between triclosan concentration and Methylococcales abundance may be related to degradation of triclosan by these bacteria. However, since the field sites differ in a variety of parameters beyond triclosan concentration, further research will be needed to test this hypothesis.

The position of urban site 1 downstream from 25 CSOs strongly implicates these CSOs as major sources of triclosan, as has been suggested by others (Akiyamaand Savin, 2010). The sediment triclosan concentration at urban site 2 was significantly lower than urban site 1, suggesting that the intervening WWTP was contributing to lower sediment triclosan. Previously published data have demonstrated high efficiency of triclosan removal by various WWTPS (Akiyamaand Savin, 2010; Barnes et al., 2002; Beaber et al., 2003), and 99% removal of triclosan by NSWRP specifically (Fazi et al., 2005). Therefore these data indicate that at our urban site WWTP effluent is less of a contributor to sediment triclosan concentrations than CSOs.

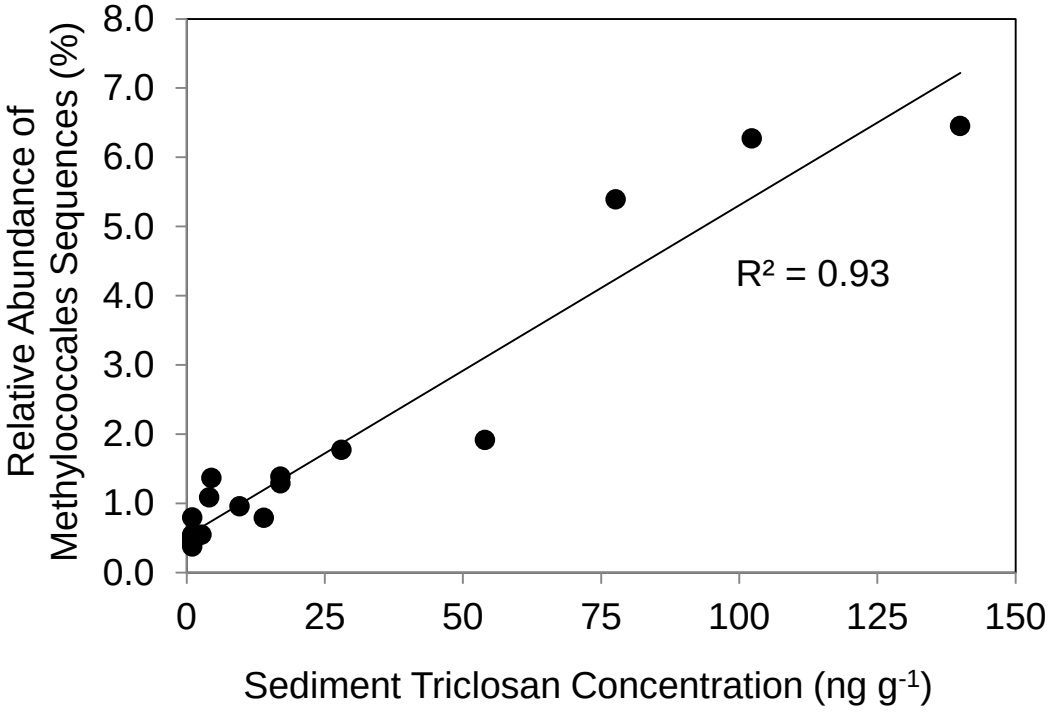

Figure 4.7. Relationship between sediment triclosan concentration and relative abundance of Methylococcales at field sites. Data is based on tag pyrosequencing of bacterial 16S rRNA genes; Pearson correlation analysis indicated a significant correlation ($p < 0.001$).

Artificial Stream Experiment

The goal of the artificial stream experiment was to determine whether experimental addition of triclosan could influence the structure and function of sediment bacterial communities. The sediment triclosan concentration in our artificial streams was approximately 15% of the highest sediment triclosan concentration that has been reported in the literature (Chalew and Halden, 2009), which was measured in a stream in Baltimore, MD, receiving direct sewage input due to a leaking sewer pipe (R. Halden, personal communication). Therefore, while the triclosan concentration used in our artificial stream experiment was extremely high and is not typical of streams even in urban environments, this concentration is a good model for a dose that can result from a sewer infrastructure failure such as the one that occurred in Baltimore. The U.S. EPA has predicted that the percentage of U.S. wastewater pipes that will be in "poor," "very poor," or "life elapsed" conditions will increase from 23% in 2000 to 45% in 2020 (USEPA, Office of Water, 2002), so sewer system failures such as the one observed in Baltimore are likely to become more common in the United States. In addition, this dose is a good model for the many areas of the world in which raw sewage is released to surface waters without treatment.

Within the artificial streams we did not observe any impact of this high triclosan concentration on bacterial cell abundance or community respiration rates. It is possible that 8 ppm triclosan in

the artificial stream sediments was not high enough to reduce bacterial abundance, but this seems unlikely as 16 ppm in agar resulted in dramatic reductions in bacterial growth. However, the triclosan was likely more available in the agar than in the sediment, due to the strong binding between triclosan and sediments. In addition, sediment is a much more complex matrix than agar and would likely lead to less uniform distribution of triclosan, with triclosan preferentially sorbing to organic matter, so it is likely that there were microsites within stream sediments that contained lower triclosan and might have served as refuges for sensitive bacteria. Another possible explanation for the lack of an observed effect on bacterial cell abundance or respiration is that the first sampling of artificial streams did not occur until seven days after triclosan addition, so there might have been a short-term bacterial response followed by a rapid recovery. This possibility is supported by a mesocosm study that showed that triclosan increased bacterial mortality within biofilms, but that bacterial numbers returned to baseline one week after exposure (Orvos et al., 2002; Orvos et al., 2002). Further work will be needed to elucidate more clearly the short-term responses of sediment bacterial communities to triclosan exposure, but our data suggest that sediment bacterial communities have a remarkable level of resilience.

Despite the lack of changes in overall bacterial community size or activity in the artificial streams in response to triclosan, there were other significant biological responses to triclosan. We measured a rapid and dramatic increase in relative abundance of triclosan-resistant bacteria in artificial stream sediments. Within the first week triclosan resistance within the artificial streams was equivalent to the resistance level observed at our most contaminated field site. Over the course of the five-week study the triclosan resistance level climbed to a maximum of 14%, suggesting that resistance levels in the field might also continue to rise if triclosan concentrations increase. The increase in resistance that was observed in bacterial communities within artificial stream sediments could have been caused by de-novo mutations conferring resistance, spread of existing triclosan-resistance genes through horizontal transfer, or a shift in community composition toward more triclosan-resistant taxa. While it is possible that all three of these mechanisms played a role in the increased resistance, we confirmed using pyrosequencing analysis of 16S rRNA genes that triclosan addition did result in a significant shift in taxonomic composition of sediment bacterial communities. Previous studies have demonstrated shifts in bacterial community composition in response to triclosan in biofilms (Lawrence et al., 2009; Yergeau et al., 2012) and WWTP effluent (Novo et al., 2013), but we are not aware of any studies that have reported changes in bacterial community composition in response to triclosan in freshwater sediments.

There was also a significant decrease in sediment bacterial diversity with triclosan addition. A previous study indicated that triclosan caused a decrease in diversity in a community of bacteria cultured from a sink drain and incubated in microcosms (McBain et al., 2003), but we are not aware of any study that has demonstrated a negative effect of triclosan on bacterial diversity under environmentally relevant conditions. The observed decrease in diversity raises concerns, as recent evidence demonstrates a link between diversity of phytoplankton communities in freshwater habitats and both stability and functioning of the communities (Ptacnik et al., 2008). Although a link between diversity and stability has not been established for benthic microbial communities, it suggests that further work is necessary to elucidate potential functional implications of these changes in diversity.

One of the specific population shifts we observed with triclosan addition to artificial streams was a dramatic increase in relative abundance of cyanobacterial sequences, from less than 1% of the total community to more than 5%. Although we did not quantify algae in this experiment, we did note that triclosan addition resulted in an immediate and clearly visible die-off of algae, and other studies have noted that triclosan negatively affects algal communities (Proia et al., 2011; Wilson et al., 2003). These results suggest that cyanobacteria are more resistant to triclosan than algae and that triclosan may act as a selective factor favoring growth of cyanobacteria over algae. Pure culture studies have demonstrated that algae are more sensitive to triclosan than cyanobacteria (Orvos et al., 2002), but we are not aware of any studies that have demonstrated this type of selection in a complex microbial community driven by triclosan. Selection of cyanobacteria over algae could have significant ecological implications as cyanobacteria are a less nutritious food for aquatic consumers (e.g., zooplankton) than algae (Lampert, 1987). In addition, many cyanobacteria produce toxins that negatively affect zooplankton (Christoffersen, 1996), so changes in relative abundance of cyanobacteria and algae in aquatic ecosystems could have significant effects on higher trophic levels (Karjalainen et al., 2007).

Triclosan addition also resulted in significant shifts in relative abundance of sequences representing several other bacterial taxa. For example, Betaproteobacteria, a bacterial class that is common in environmental samples, decreased in abundance in triclosan-amended streams. Another recent study demonstrated a decrease in abundance of Betaproteobacteria in WWTP effluent (Novo et al., 2013), suggesting that Betaproteobacteria may be especially sensitive to anthropogenic pollutants. Similarly, Bacteroidia, which are part of the Bacteriodetes phylum, decreased in abundance in triclosan-amended streams and a previous study demonstrated triclosan sensitivity of Bacteriodetes within biofilms in laboratory mesocosms (Lubarsky et al., 2012). Therefore, our data indicate that triclosan can act as a selective agent and modify the taxonomic composition of complex sediment bacterial communities under environmentally relevant conditions.

4.7 Acknowledgements

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