TROWBRIDGE, KIRSTEN ELISE, Ph.D. Impacts of Triclosan on Stream Microbial Communities: Investigating Mitigation and Multidrug Resistance. (2018)
Directed by Dr. Anne Evelyn Hershey 139 pp.

Countless chemical compounds and their derivatives are present in wastewater (WW) systems and other environments due to anthropogenic inputs from a vast array of product ingredients. In streams and WW treatment plants (WWTPs), microbial communities thrive and can interact with these compounds in a variety of ways. Triclosan is an example of a synthetic chemical with expansive use which has, in half a century, become nearly ubiquitous in the environment. Investigating triclosan mitigation by periphyton from a WW-associated and a forested stream, the WW-associated periphyton showed evidence of mitigation. Similar triclosan levels were observed in both streams, in most samples of periphyton and water. Among water samples, higher triclosan concentrations were measured in samples collected nearest the WWTP. In microcosms, periphyton were exposed to an environmentally-relevant level (10 µg/L) of triclosan. Bacterial isolates were purified from the unexposed and triclosan-exposed periphyton from each stream. Isolates were assayed for susceptibility to triclosan and five antibiotics using broth microdilution and identified to genus level via 16S rRNA sequence analysis. *Pseudomonas* was the dominant genus among identified isolates from all treatment groups and exposed groups had lower genus richness than unexposed. Multidrug-resistant (MDR^t) bacteria were detected in both streams, with more incidences of multidrug resistance (MDR) in the WW-associated stream. The environmentally-relevant triclosan exposure appeared to increase antibiotic resistance and MDR in the forested-stream periphyton community but not the WW-associated community. Due to the growing global challenge of MDR, the added contribution of triclosan is a noteworthy risk to human and environmental health.

IMPACTS OF TRICLOSAN ON STREAM MICROBIAL COMMUNITIES: INVESTIGATING MITIGATION AND MULTIDRUG RESISTANCE

by

Kirsten Elise Trowbridge

A Dissertation Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
2018

Approved by

_____
Committee Chair

DEDICATION

This work is dedicated to all who patiently supported me through this endeavor: for my parents and family who have encouraged me throughout life, for inspiring, helpful folks at UNCG, for enriching communities- brilliant, artistic and musical friends in song and dance who keep me rollin' with the flow, for found family who help each other …and help me and my amazing daughter, Kaleema Ilani. This is for her and all other bright, creative children of Earth. Wishes to all for whole wellness moving forward.

APPROVAL PAGE

This dissertation written by KIRSTEN ELISE TROWBRIDGE has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGMENTS

I want to sincerely thank Dr. Anne Evelyn Hershey, not only for her guidance, but also for the graceful, useful and entertaining ways through which she continually supported my learning and growth as an individual. I would also like to extend my heartfelt appreciation to the other phenomenal committee members who each brought their unique set of experience, knowledge and skills to assist in my endeavors as a graduate student: Nadja Cech, Zhenquan Jia and Parke Rublee. This is a brilliant and uplifting group of individuals. There are many other professors (such as Karen Katula, John Tomkiel Dean, David Remington), students (Emily Britton, Tatsiana Shymanovich, labmates and others) and support people (Laura Fondario, Megan Corum) at UNCG that have been extremely helpful throughout my UNCG graduate career and I hope they know how grateful I am that they invested their time to assist with this project. Conducting this research, I worked with many undergraduate research assistants. Training and working with students has been extremely rewarding, delightful and educational. Big thanks to Professor Joseph Falkinham at Virginia Tech who provided support on the design of susceptibility studies. I would also like to extend special appreciation to two wonderful humans who provided assistance in the completion of this dissertation without even being asked to. Brad Davis assisted with graphic design and collaborated with me on the creation of figures 2 and 3. Rebecca Rhodes has kindly reviewed this written work more than once.

PREFACE

infinite chemicals
exist in this world
in various places
they mix and are swirled
together with microbes
and water and genes
you'll find much resistance
if you run some screens
see these microbes won't be killed
by this drug or that
multidrug resistance
is growing, it's bad
I'd like to find better ways
to ensure they won't harm me
instead we fight them with tools
they use to beef up their army

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS

ARG – Antibiotic Resistance Gene

ARB – Antibiotic Resistant Bacteria

BLAST – Basic Local Alignment Search Tool

BMI – Body Mass Index

CEC – Contaminant of Emerging Concern

DDD – Defined Daily Doses

EDC – Endocrine Disrupting Compound

ECOFF – Epidemiologic Cutoff

MRSA – Methicillin-resistant *Staphylococcus aureus*

MDR – Multidrug Resistance

MDR^t – Multidrug-resistant

MEC – Measured exposure concentration

MIC – Minimum Inhibitory Concentration

MGE – Mobile Genetic Element

NCBI – National Center for Biotechnology Information

NOEC – No Observed Effect Concentration

OTC – Over-the-Counter

PPCPs – Pharmaceutical and Personal Care Products

PAHs – polyaromatic hydrocarbons

PCDDs – polychlorodibenzo-p-dioxins

PCR – Polymerase Chain Reaction

WW – Wastewater

WWTP – Wastewater Treatment Plant

CHAPTER I

INTRODUCTION AND BACKGROUND

Background on Triclosan and Related Antimicrobials

Triclosan is a synthetic diphenyl ether derivative (Fig.1) manufactured globally for its antimicrobial properties. Triclosan is added to sanitary products often at levels around 0.1-0.5% (Halden 2014). Most triclosan found in the environment enters through WW-associated processes, via discharges of effluent to surface waters, as well as the application of biosolids to agricultural lands (Dann and Hontela 2011; Huang *et al.* 2014; Pintado-Herrera *et al.* 2014).

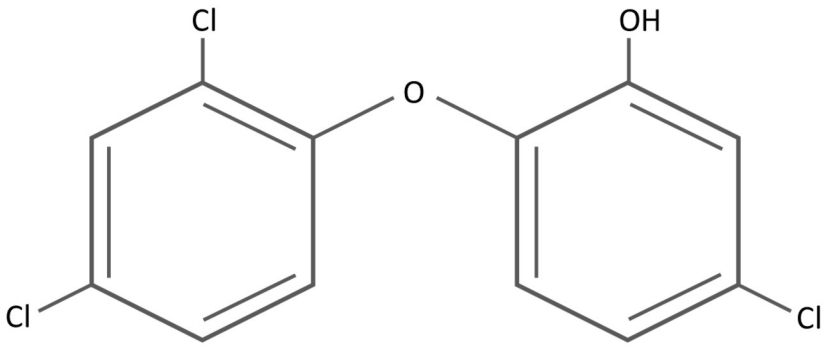

Figure 1. Structure of Triclosan, $C_{12}H_7Cl_3O_2$, a Chlorinated Biphenyl Ether.

Due to widespread use, triclosan is detectable in a multitude of samples including rivers, lakes and oceans (Dann and Hontela 2011; Singer *et al.* 2002; Xie *et al.* 2008). In surface waters, triclosan can be transformed through photodegradation and biodegradation, forming products such as chlorophenols, methyltriclosan (Chen *et al.* 2011; Tohidi and Cai 2017) and, occasionally, dioxins such as 2,8-Dichlorodibenzo-P-dioxin (Fang *et al.* 2010).

Triclosan and related organohalide biocides have interesting histories of production, regulation and numerous discoveries of adverse effects. Substituting chlorines for hydrogens on aromatic rings was found to result in biocidal compounds around the late 1930s and early 1940s. After large volume production of many of these compounds, detrimental impacts became apparent. Many of the compounds resulted in ecotoxic effects, human toxicity, bioaccumulation and environmental persistence. One example, hexachlorophene, was prominent during the first big push of antimicrobials in pharmaceutical and personal care products (PPCPs), being put into over 400 products in just a few years (Halden 2014). Hexachlorophene was soon banned (by the 1970s) from most uses because it was a suspected neurotoxin (N.W. 1972). In contrast, triclosan and the related compound, triclocarban, are less regulated, and have only very recently been banned in wash products (McNamara and Levy 2016). Triclosan was patented in 1964 and 10 years later, in a U.S. Food and Drug Administration (FDA) draft of an Over-the-Counter (OTC) Drug Monograph it was noted that there was not evidence that triclosan was safe or effective. In an update of the still tentative FDA Draft Monograph in 1994, antibacterial soaps were removed from the drug category (Halden 2014). This change led to the second big push of antimicrobials in PPCPs in the United States, resulting in an expansion from several dozen antimicrobial products in this country, to more than 2000 (Halden 2014). Triclosan has been added to antibacterial soaps and medical washes as well as a wide array of consumer products including carpets, clothes, cosmetics, deodorants, toothpastes, mouthwashes, detergents, paints, plastics for a plethora of uses: toothbrushes, tubs/showers, hot tubs, diaper changing stations, cutting boards, placemats and even items intended specifically for use by children like toys, school supplies and pacifiers (Dhillon *et al.* 2015; Halden 2014; Saleh *et al.* 2011; Young 2013).

The European Commission disapproved use of triclosan as a biocide in human hygiene products (EC 2016). Soon after, the U.S. FDA issued a final rule to stop the marketing of most antimicrobials, including triclosan and triclocarban, in OTC washes and stated these were not recognized as generally safe and effective (FDA 2016). However, they maintained that OTC consumer antiseptic rubs or antibacterial wipes that do not require rinsing and OTC antiseptics that would be used in health care are generally safe and effective and were not covered in the ruling. The final rule only covered antiseptic washes for use with water and does not cover the vast multitude of triclosan-containing products not addressed in the FDA's final rule (FDA 2016). Minnesota moved to ban triclosan in an effort to protect critical water resources when triclosan and polychlorodibenzo-p-dioxins (PCDDs) were detected in freshwater sediments (Anger *et al.* 2013; Venkatesan *et al.* 2012). PCDDs can be formed via a photochemical cyclization reaction with triclosan and are compounds known to be toxic and carcinogenic (van den Berg *et al.* 1998).

A large proportion of triclosan-containing products travel down drains shortly after their intended use. From there triclosan continues its journey through waterways to the WWTP. In a study in South Africa, average measured triclosan concentrations ranged from 2.01-17.6 µg/L in WW influents, 0.990-13.0 µg/L in WW effluents (Table 1), and 0.880-8.72 µg/L in the receiving river (Lehutso *et al.* 2017). It has been measured in WW influents in the range of 52-86200 ng/L (Bedoux *et al.* 2012). Varied WW treatment processes result in a range of removal efficiencies, generally 58-95% (Bester 2005; von der Ohe *et al.* 2012). Thus, triclosan is present in WW effluents and downstream waters (Table 1) (Barber *et al.* 2015; Gautam *et al.* 2014; Kumar *et al.* 2010; Morrall *et al.* 2004; Ying and Kookana 2007; Zhao *et al.* 2013) and sediments (Anger *et al.* 2013; Venkatesan *et al.* 2012).

Table 1. Review of Measured Triclosan Concentrations. Examples of triclosan levels reported in scientific literature. All liquid sample types give triclosan concentrations in µg/L. Any row that does not have a reference listed has data from the source listed in the row above.

Sample Details			Triclosan concentration (µg/L or µg/kg)				Reference
Sample Type		Source	min	median	mean(s)	max	
Wastewater	Influent	United States	13.703			86.161	Kumar *et al.* 2010
Wastewater	Effluent	United States	0.18			5.37	
Wastewater	Influent	Spain	1.3			37.8	Agüera *et al.* 2003
Wastewater	Effluent	Spain	0.4			22.1	
Wastewater	Influent	Greece				23.9	Stasinakis *et al.* 2008
Wastewater	Effluent	Greece				6.88	
Wastewater	Influent	South Africa			2-17.6		Lehutso *et al.* 2017
Wastewater	Effluent	South Africa			0.99-13		
Wastewater	Influent	United States	0.24			9.7	Barber *et al.* 2015
Wastewater	Effluent	United States	<0.01			1.4	
Wastewater	Influent	India			0.892	4.89	Balakrishna *et al.* 2017
Wastewater	Effluent	India			0.202	3.5	
Wastewater	Influent	India			2.5		Balakrishna *et al.* 2017
Wastewater	Effluent	India			2.5		
Wastewater	Influent	India			4.89		Balakrishna *et al.* 2017
Wastewater	Effluent	India			3.5		
Wastewater	Influent	Germany			7.3		Bester *et al.* 2005
Wastewater	Effluent	Germany			0.3		
Wastewater	Influent	Germany			4.8		Bester *et al.* 2005
Wastewater	Effluent	Germany			0.62		
Wastewater	Influent	Spain			0.488		Ricart *et al.* 2010
Wastewater	Effluent	Spain			0.071		
Wastewater		France		3.45		5.26	Gasperi *et al*. 2014
Wastewater	Effluent	Australia	0.023			0.435	Ying and Kookana 2007

Table 1. Review of Measured Triclosan Levels (continued). Examples of triclosan levels reported in scientific literature. Liquid sample types give triclosan concentrations in µg/L and solid samples give concentrations in µg/kg. Any row that does not have a reference listed has data from the source listed in the row above.

Sample Details			Triclosan concentration (µg/L or µg/kg)				Reference
Sample Type		Source	min	median	mean(s)	max	
Wastewater	Biosolids	Australia	90			16790	Ying and Kookana 2007
Wastewater	Biosolids	United States	1170	10200		32900	Kinney *et al.* 2006
Wastewater	Biosolids	South Africa			2.16-13.5		Lehutso *et al.* 2017
Surface water	WW-assoc. stream and AR River	United States	0.0039			0.0283	Gautam *et al.* 2014
Surface water	WW-associated river	Germany				0	Bester *et al.* 2005
Surface water		Germany	<0.003			0.01	
Surface water		United States	<0.01			0.28	Barber *et al.* 2015
Freshwater	Bed sediment	United States				85	Venkatesan *et al.* 2012
Urban Stream	Sediment	United States				107.0	Drury *et al.* 2013
Marine	Sediment	Spain	0.27			130.7	Agüera *et al.* 2003
Estuary	Water	Spain				0.3	Pintado-Herrera *et al.* 2014
Estuary	Sediment	Spain				9.6	
Indoor Dust	Classroom	United States			1003		Hartmann *et al.* 2016
Human mothers	Urine	Puerto Rico		26.2	29.9	2000	Meeker *et al.* 2013
Human females (18-40 y)	Urine	United States		14	18.7	2780	Meeker *et al.* 2013
Human females (18-40 y)	Urine	United States		13	16.9	2690	
Human mothers	Urine (triclosan households)	United States		916.1			Ribado *et al.* 2017
Human mothers	Urine (non-triclosan households)	United States		76			
Human infants	Urine (triclosan households)	United States		43.0			Ribado *et al.* 2017
Human infants	Urine (non-triclosan households)	United States		10.1			
Human mothers	Urine	United States			163.4		Pycke *et al.* 2014
Human children	Urine	India	0.2		9.55±314	2570	Xue *et al.* 2014

A study conducted over 8 km of river downstream of a Texas WWTP showed declining levels of triclosan with distance (104, 223, and 431 ng/L at 0.2, 2, and 8 km respectively) (Morrall *et al.* 2004). The group estimated approximately 19% loss due to sorption and settling. Different WW treatment processes can affect triclosan removal rates. Differences have been observed in triclosan removal efficiency through WW processing via Activated Sludge (96%) compared to Trickling Filter (71%) WW processes (McAvoy *et al.* 2002). There is a large variation in reported triclosan concentrations in WW effluents, which have been reported ranging from 23-22100 ng/L (Agüera *et al.* 2003; McAvoy *et al.* 2002; Halden and Paull 2005; Ying and Kookana 2007; Lehutso *et al.* 2017). Representative examples of measured triclosan levels are shown in Table 1. River discharges can lead to triclosan's presence in estuaries and oceans. Some reported values in estuarine waters range from 4.9-300 ng/L (Fair *et al.* 2009) and in seawater from 0.008-362 ng/L (Xie *et al.* 2008; Lydon *et al.* 2017). Higher levels are detected in biosolids (Armstrong *et al.* 2017; Kinney *et al.* 2006; Lehutso *et al.* 2017; Verlicchi and Zambello 2015; Ying and Kookana 2007). In another study comparing how triclosan responds through different WW treatment practices, 24-27% adsorbed to sludge and varying proportions of triclosan were biotransformed to toxic/persistent compounds (Tohidi and Cai 2017). When chlorination and UV disinfection were employed, 13% of triclosan was transformed to 2,8-DCDD and more to other degradation products (Tohidi and Cai 2017). In aerobic digestion, about 7.4% of triclosan was converted to methyl-triclosan (Tohidi and Cai 2017). Globally, there are countless systems with even more variables in terms of treatment practices, environmental factors and triclosan inputs. Observed variations in triclosan's fate from system to system and the broad ranges of detected concentrations in various environments are to be expected. Triclosan that remains in surface waters, or other environments such as agricultural fields, can interact with organisms found in

these environments causing a variety of impacts including endocrine disruption and contributing to increases in antimicrobial resistant microorganisms.

Environmental Health Connections

Triclosan is found in WW worldwide (Table 1) and is one of the most commonly detected organic WW contaminants in U.S. streams (Kolpin *et al.* 2002). Environmental presence of triclosan is widespread and exposures can impact organisms in a variety of ways. Lin *et al.* (2014) observed multiple toxic effects of triclosan in earthworms: reduced reproduction, upregulated expression of heat-shock protein gene, and increased DNA damage (Lin *et al.* 2014). Previous evidence of decreasing triclosan levels downstream of WWTPs (Morrall *et al.* 2004) and algal bioaccumulation (Coogan *et al.* 2007) motivated investigation of triclosan's mitigatory role in streams. Evidence presented in Aim 1 is in line with the possibility of triclosan mitigation by periphyton.

As both a broad-spectrum antimicrobial and an endocrine active compound, triclosan's effects on organisms are numerous and varied. In streams, aquatic organisms can also interact with anthropogenic inputs, such as triclosan, affecting the fate of these compounds. The current study provides additional evidence for a few of the ways in which triclosan and stream periphyton interact. In a mesocosm study investigating mitigation of biocides and fungicides, retention of compounds taken up by macrophytes depended on how lipophilic the compounds were (Stang *et al.* 2013). These macrophytes acted as a sink for triclosan, with average mass retention of 56 ± 7% (Stang *et al.* 2013). Triclosan exposure can impact microbial communities and lead to altered susceptibilities to antimicrobials (Nietch *et al.* 2013), topics addressed in Aims 2 and 3 respectively. Results in Chapter III provide evidence of potentially decreased microbial diversity due to triclosan exposure. If microbial diversity is decreasing, this could

impact the microbial community's contributions to ecosystem services, including mitigation. Some of the potential routes triclosan could travel in the environment are shown in Figure 2.

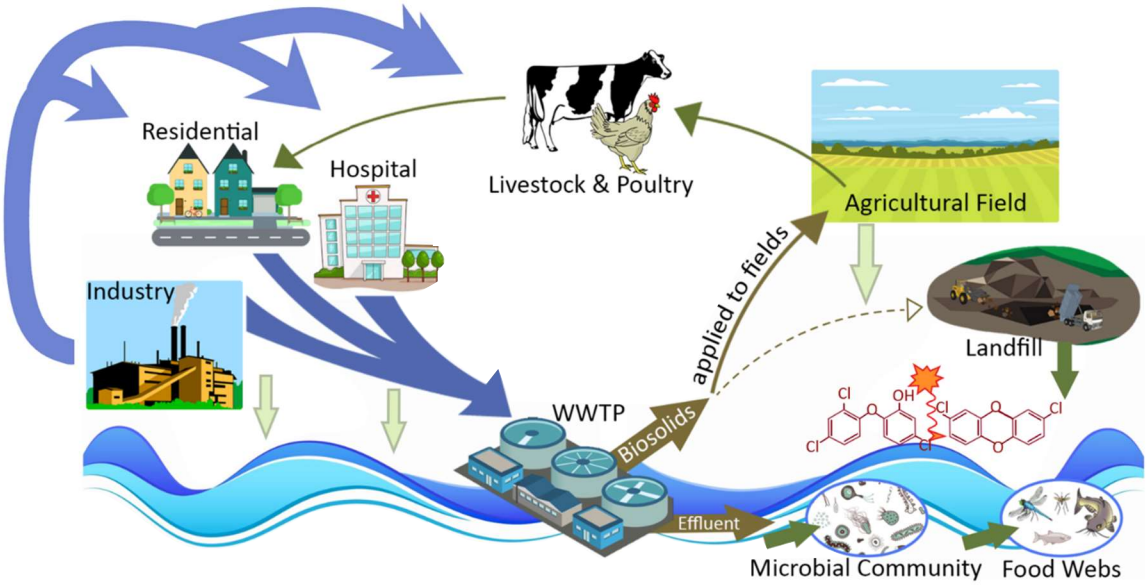

Figure 2. Pathways of Triclosan Exposure. All arrows indicate potential movements of triclosan (and other organic contaminants with similar chemistry). Arrows pointing down indicate movements toward surface and/or groundwater such as runoff and leaching. Red/orange icon indicates photodegradation as an example of transformation, though other transformations occur.

Microbial diversity and the assortment of factors within ecosystems that affect stream microbial communities vary widely from stream to stream (Jyrkänkallio-Mikkola *et al.* 2017; Vaz-Moreira *et al.* 2014). A decrease in microbial diversity can impact the community's ability to metabolize xenobiotic compounds (Hernandez-Raquet *et al.* 2013). So, there exists the possibility that periphyton mitigate triclosan, yet impacts of triclosan on periphyton affect the mitigation potential of the community.

Triclosan exposure has been shown to affect aquatic microbial communities in a variety of ways. In WW-associated stream microbial communities, exposure to 60 μg/L increased

bacterial mortality (45%) initially. Then after a week of exposure, bacterial mortality returned to normal values and diatom mortality was increased (41%) (Proia *et al.* 2011). Diatom and bacterial viability as well as photosynthetic efficiency were all decreased with triclosan exposure in another study on WW-associated river biofilms (Ricart *et al.* 2010). Investigations with chronically triclosan-exposed periphytic bacteria decreased cell densities were observed at 5 and 10 μg/L but increased cell densities we observed at 0.1, 0.5, and 1 μg/L (Nietch *et al.* 2013). Changes in community structure occurred in other triclosan exposure studies, in both suspended algal communities (Wilson *et al.* 2003) and in non-WWTP-associated river biofilm communities (Lawrence *et al.* 2009). Through algal community studies, authors demonstrated that with exposure to increasing triclosan concentrations, algal genus diversity became reduced (Wilson *et al.* 2003). They also noted significant changes in community structure of both suspended and attached algae collected from sites upstream and downstream of a WWTP. Some examples of noted community structure alterations upon 12-day triclosan exposure were significant reductions in *Chlamydomonas*, *Sphaerocystis*, cyanobacteria and an increase in *Synedra* (Wilson *et al.* 2003). Responses of WWTP-associated stream periphyton communities to chronic exposure at environmentally-relevant levels of triclosan have not been extensively studied. As systems, communities, and triclosan levels vary widely, additional evidence of triclosan's interactions with microbial communities will serve to provide a clearer picture of the possible outcomes of its presence in streams. Moreover, the environmentally-relevant exposure dose of 10 μg/L is useful for assessing potential effects of a level commonly occurring in the environment.

Background on Antimicrobial Resistance and Multidrug Resistance

Sources of bacteria and their genetic elements that lead to MDR in the environment are disturbingly vast. Human practices drive evolution of microbes, yielding antibiotic resistant bacteria (ARB) exhibiting acquired resistance encoded in antibiotic resistance genes (ARGs) often on mobile genetic elements (MGEs) that can be exchanged between bacteria of different genera or species. Global antibiotic consumption, expressed in defined daily doses (DDD), increased 65% (21.1–34.8 billion DDDs) between 2000 and 2015. Projections of future consumption depend on anticipated changes in use. The baseline prediction assumed no policy changes and had constant consumption rates set at current use levels and predicted a 15% increase from 2015-2030, (Klein *et al.* 2018), while other methods of estimation predicted much higher increases than this. Antimicrobial compounds have been broadly employed for use in healthcare, agriculture, industy, hygiene, apparel, building and households. In addition to antimicrobials, several other contaminants show evidence of co-selection for ARB (Gorovtsov *et al.* 2018; McArthur and Tuckfield 2000). MDR^t opportunistic pathogens from nonclinical environments (Quinn 1998; Gaynes and Edwards 2005) and increasing resistance to antibiotics pose healthcare challenges (Chang *et al.* 2015).

Worldwide, MDR has been increasing as a result of extensive use of antimicrobials. Many more examples than have been studied surely exist as human systems create environmental situations well-suited for resistance emergence. Some examples are WWTPs receiving inputs from hospitals, residents, businesses and industry. Still other examples include agriculture, livestock, aquaculture and waste in landfills. In these environmental systems, microbial communities can interact with antimicrobials and other compounds present, with varied results. As we have seen, acquired resistance is often the result of these interactions.

There are a seemingly endless number of reported cases of ARB and MDR in the literature and a few examples are listed below. MDR^{t} *Klebsiella pneumoniae* have spread globally and treatment options are limited. *K. pneumoniae* have shown resistance to colistin, a last resort antibiotic which had long been reserved mainly due to safety concerns of its nephrotoxicity and neurotoxicity (Granata and Petrosillo 2017).

Widespread use of fluoroquinolones in both human medicine and livestock production has resulted in global emergence of fluoroquinolone-resistant *Salmonella enterica* Typhimurium strains. MDR incidence in *Salmonella* Typhi ranged from 64.8-66.0% while incidence of fluoroquinolone resistance in clinical isolates ranged from 84.7-91.7%. *Salmonella* exhibiting MDR appears to be rapidly increasing. From 1999 to 2005 in India, MDR in *Salmonella* Typhi strains increased from 34% to 66% (Kumar *et al.* 2008). One human response to this rising resistance has been increased use of ciprofloxacin in typhoid fever treatment. As a result, ciprofloxacin-resistant strains began to spread in the early 1990s (Rowe *et al.* 1995). Before the 1990s came to a close, ciprofloxacin treatment failure reports followed (Maskey *et al.* 2008). In *Salmonella typhimurium* isolated from patients with infectious diarrhea, quinolone-resistant strains showed MDR and most of these harbored Class 1 integrons (Yuan *et al.* 2017). The spread of antimicrobial resistance is, in part, driven by horizontal gene transfer occurring between different bacterial species. ARGs can be transferred via mobile genetic elements (MGEs) such as plasmids and integrons (Gaze *et al.* 2011). Integrons are one type of MGE that can carry ARGs (Boucher *et al.* 2007; Wolters *et al.* 2015) and have been implicated in spreading ARGs in both environmental and clinical settings (Gillings 2014).

Antimicrobials have been used extensively for growth promotion in food animals since the 1950s. The emergence of antimicrobial resistance in animal production can lead to the

spread of ARB to humans via contact or through the food chain (Xiong *et al.* 2018). Aerially dispersed particulate matter from cattle feed yards has been shown to harbor antibiotics, ARB and ARGs (McEachran *et al.* 2015) (Fig. 3). Antimicrobial use in food animals correlates to increased ARB in humans (Schechner *et al.* 2013). The broad use of antimicrobials in animal production promotes antimicrobial resistance and MDR which is a threat to human health (Xiong *et al.* 2018). Resistance in animals was reported in 1951 in turkeys fed streptomycin. Streptomycin-resistant coliform bacteria were observed (Starr and Reynolds 1951).

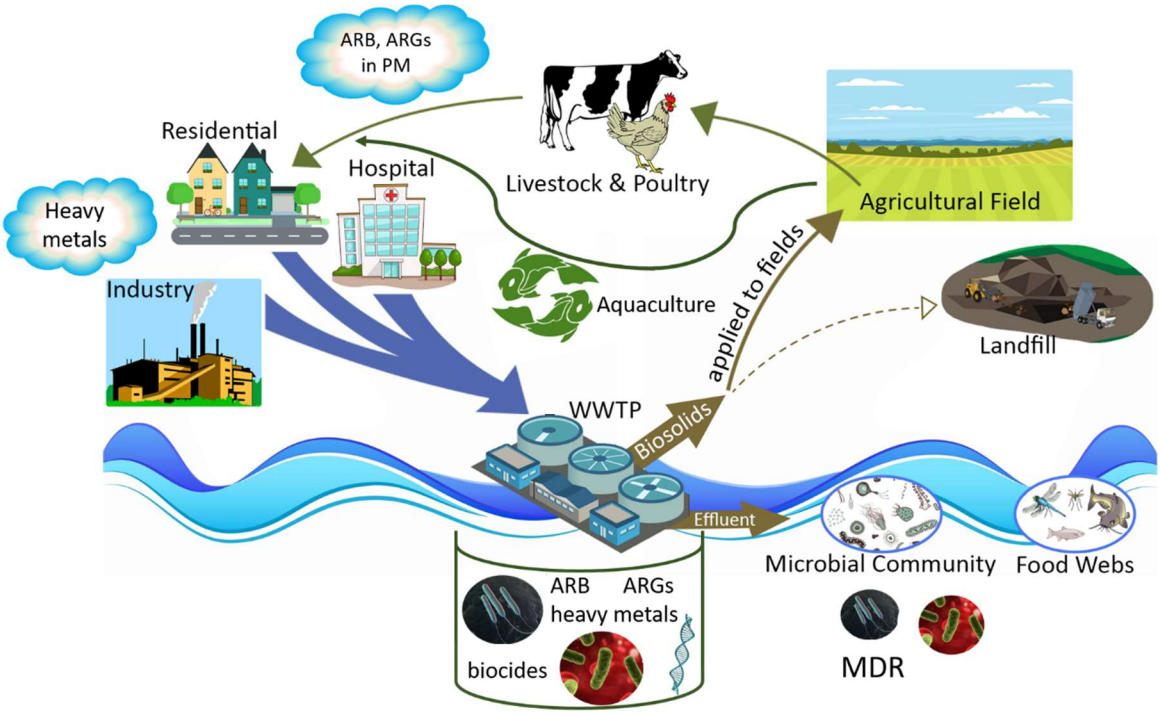

Figure 3. Cycles Amplifying Multidrug Resistance. Figure shows example pathways of enrichment of MDR^t bacteria due to triclosan exposure (and exposure to other anthropogenic inputs such as antibiotics and heavy metals). The collection of microbes, ARGs, and anthropogenic inputs shown beneath the WWTP is a relevant example. Similar scenarios occur in other environments as well.

Afterwards, similar results were seen when tetracycline was used as a growth promoter in chickens and resistance developed (Barnes 1958; Elliott and Barnes 1959). ARB as well as ARGs

conferring resistance constantly cycle through environments in water, soil, plants and animals. Resistant pathogens and ARGs pass through the meat industry as well as through contaminated crops, water and soil (Xiong *et al.* 2018). Examples of ARB reported in food production include *Escherichia coli* and *Salmonella* exhibiting MDR, vancomycin-resistant *Enterococcus*, methicillin resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase producing bacteria (Barton 2014). Antimicrobial agent levels strongly correlate with corresponding resistance in commensal *E. coli* isolates in swine, chickens and cattle (Chantziaras *et al.* 2014). Contributions of antimicrobial use in animals to development of resistance in human commensal bacteria has been shown using metagenomic data at the population level (Forslund *et al.* 2013). The use of cephalosporins in chickens may contribute to the development of resistant *E. coli* which have led to mortality in humans (Collignon *et al.* 2013) Annually, in the U.S. alone, ARB infect over 2 million people, resulting in over 23,000 deaths and $50 billion in management costs (CDC 2013).

In stream periphyton communities (urban or forested), and possibly to a greater extent at WWTPs, ARB harboring ARGs are diverse. Soluble chemical contaminants and ARB gather at WWTPs. Some relevant contaminants include: biocides, heavy metals, and ARGs. WWTPs are hotspots of emergence, development and spread of antibiotic resistance and MDR. This is because the microbial community along with inputs of additional bacteria from humans/animals in the WW as well as all the chemical contaminants are collected, allowing for complex interactions and effects (Martinez *et al.* 2009; Michael *et al.* 2013). ARGs can be exchanged between different types of bacteria (Amábile-Cuevas and Chicurel 1993; Chee-Sanford *et al.* 2001; Ciusa *et al.* 2012; Cooper *et al.* 2017; Salyers and Amábile-Cuevas 1997). In some cases, in what once was a strain of susceptible bacteria, these conditions lead to the production ARB. An extensive and growing list of ARGs have been detected in WW-associated systems (Berglund

2015; Chee-Sanford *et al.* 2009; Nnadozie *et al.* 2017). WW-associated microbial communities can show significant levels of ARB and MDR. In a WW-associated stream in New Jersey, ≈79% of fecal coliform isolates showed triclosan resistance and about 85% of these showed MDR to four classes of antibiotics (Middleton and Salierno 2013). WW-associated fecal coliform isolate samples from a nearby river showed a significant difference in multiple antibiotic resistance values between triclosan-sensitive and triclosan-resistant isolates with ≈90% of their samples containing triclosan-resistant isolates showing higher multiple antibiotic resistance values than those sensitive to triclosan (Middleton and Salierno 2013). This is indicative of cross-resistance development occurring in these WW-associated microbial communities, leading to triclosan resistance and MDR overlapping in environmental bacteria.

In addition to municipal WWTP, there are other sources of contamination intensifying the problem of MDR development. As mentioned above, heavy metal contamination has been shown to correlate with antibiotic resistance. Co-selection of ARGs by presence of heavy metals may occur when the resistance genes reside on the same MGEs (Gorovtsov *et al.* 2018). Soil microbial communities have shown evidence of selection by presence of polyaromatic hydrocarbons (PAHs) for ARB, as well as higher levels of expression of ARGs (Gorovtsov *et al.* 2018). Aquaculture also presents conditions favorable to the development and spread of ARB and MDR. In a study conducted in Brazil, bacteria from fish ponds had higher MDR when compared to those isolated from a water-fed canal. These bacteria showing MDR were more frequent and diverse in fish ponds than in the water-fed canal and there was also a positive correlation between antimicrobial resistance and metal tolerance (Alves Resende *et al.* 2012). Antibiotics used in aquaculture can persist and even at low concentrations, can select for ARB. This can lead to altering nearby microbial aquatic communities as well, impacting biodiversity in

sediment and water proximal to open aquaculture systems. Susceptible microbial communities can evolve to resistant ones (Watts *et al.* 2017).

Separating effects of antimicrobials from other environmental factors poses a major research challenge. In these habitats, there are many other factors at play, including but not limited to: WW treatment practices, type of organic substrates, dissolved oxygen content, salinity and temperature (Wang *et al.* 2012; Zhang *et al.* 2012). As a result of selective pressures, antimicrobial use in human practices (household, healthcare, agriculture, aquaculture, industry and others) and their dispersal to surrounding environments require careful consideration and pose environmental and public health threats in the form of rising resistance.

Public Health Connections

Environmental and toxicological concerns related to triclosan compelled the FDA to restrict its use in liquid soaps (FDA, 2016) though triclosan is an ingredient in thousands of other products, as previously mentioned. Triclosan is detectable in human samples (Adolfsson-Erici *et al.* 2002; Allmyr *et al.* 2007; Meeker *et al.* 2013; Ribado *et al.* 2017; Pycke *et al.* 2014; Toms *et al.* 2011; Xue *et al.* 2014) (Table 1). Epidemiological studies show correlations between increased urinary triclosan and a variety of detrimental health effects (Weatherly and Gosse 2017). Triclosan is associated with alterations of mammalian microbiomes (Bever *et al.* 2018; Hu *et al.* 2016), endocrine disruption (Crofton *et al.* 2007; Dann and Hontela 2011; Paul *et al.* 2010; Paul *et al.* 2012; Rodríguez and Sanchez 2010; Veldhoen *et al.* 2006; Zorrilla *et al.* 2009), increases in: abnormal sperm (Jurewicz *et al.* 2018; Lan *et al.* 2015; Sachan *et al.* 2015), total T3 levels (Koeppe *et al.* 2013), oxidative stress (Han *et al.* 2016; Wang *et al.* 2014), allergy/food sensitization/asthma (Bertelsen *et al.* 2013; Clayton *et al.* 2011), and spontaneous abortion rates (Wang *et al.* 2016), as well as decreases in: fecundity (Vélez *et al.* 2015), BMI (body-mass index)

(Li *et al.* 2015), and newborn weight, length and head circumference (Lassen *et al.* 2016; Philippat *et al.* 2014; Etzel *et al.* 2017). Unintended outcomes associated with triclosan use are generally outside of the scope of the current study, though, additional knowledge on environmental levels of triclosan is provided and could relate to some of these negative health effects. Reports of environmental triclosan levels provide additional pieces of evidence that reaffirm the ubiquitous nature of this Contaminant of Emerging Concern (CEC). Motivation for the current study was drawn from the collective knowledge that triclosan is one of many man-made compounds, disseminated into our environments via consumer/healthcare products, which poses risks to both environmental and human health. A major contributing factor to the presence of these compounds in the environment is the employment of products containing chemical ingredients, such as antimicrobials, in everyday household use. Production of triclosan by industry can also produce triclosan-containing waste, which can lead to contamination of water used for drinking or farming, representing another route of exposure to humans and other animals. Figure 2 shows some examples of ways anthropogenic contaminants may cycle through the environment. As the focal compound of this study, triclosan is highlighted in Figure 2 and this body of research. However, there are many other persistent contaminants that may follow similar patterns, affecting their own varied outcomes along the way.

In most cases there is no evidence of benefits of triclosan-containing products compared to products free of triclosan (Aiello *et al.* 2007). Many have weighed the effectiveness of triclosan in soaps and concluded that the risks of developing antimicrobial resistance outweigh potential benefits of use of these products (Giuliano and Rybak 2015; Halden *et. al* 2017). Regardless, humans are using triclosan-containing products, many of which end up going down drains and to WWTPs. At the WWTP, much of the triclosan remains with the biosolids

where it can play parts in other toxicological tales of chaotic chemical cocktails. Still, some makes it through the plant's processing and ends up in the downstream waterways. Here it can interact with the microbial community in several ways. Though the current study focused on stream bacteria, effects in related communities, such as in the biosolids (where a larger fraction of the triclosan ends up compared to effluents), may be of concern and show potential similarities. A key difference between the two, is where they go. Triclosan can travel from WWTPs to agricultural fields or sometimes forests or constructed wetlands via land application of biosolids (Figs 2 and 3). There, it can be taken up by plants, consumed by animals, and select for ARB and MDR. Multiple direct and indirect human health exposure pathways result from the practice of biosolid land application.

Community structure shifts have been noted and in one study with triclosan-dosed WW anaerobic digesters, these communities differed from the control community in such a way that more clades containing commensal and pathogenic bacteria were dominant in triclosan exposed groups (Carey *et al.* 2016). This suggests that triclosan exposure may enrich ARB resulting from previous exposure to high concentrations of triclosan on or in the human body (Carey *et al.* 2016). Community shifts increasing *Vibrio* were also observed in coastal microbial communities exposed to triclosan in seawater microcosms (Lydon *et al.* 2017). Results of environmentally-relevant triclosan exposure in stream periphyton presented here also showed some evidence of shifts toward genera containing opportunistic pathogens such as *Pseudomonas* and *Serratia*. The exposure level used in the study presented in this dissertation (10 μg/L) is lower than concentrations found in many tested samples from a variety of environments. The wide array of environmental exposures potentially experienced by a random microbe opens doors to a complex web of possible outcomes. It is clear that triclosan has been in use for decades, is

ubiquitously present and is connected to a plethora of health related impacts. The fate of triclosan inputs to the environment varies but a fraction of it persists, while other fractions transform into compounds that likely have their own set of related concerns. If WWTP inputs of triclosan continue, reports of evidence of associated health impacts will continue to flow in turn. When triclosan is assayed in samples from humans, more often than not it is detected (Calafat *et al.* 2008; Pycke *et al.* 2014; Xue *et al.* 2015). In a recent study in children in India, a group studying urinary levels of EDCs found triclosan in 100% of the samples which ranged from 0.220–2570 µg/L (Xue *et al.* 2015). In another example of microbial community shifts due to triclosan exposure (this time in humans), *Proteobacteria* species with broad antibiotic resistance were enriched in stool samples from mothers that used triclosan-containing toothpaste. Infants with higher triclosan levels showed an enrichment of *Proteobacteria* species as well (Ribado *et al.* 2017). Microbial community shifts are common with triclosan exposure as is development of MDR. Increases in MDR in the forested stream microbial community but not the WW-associated community may indicate that decades of chronic, low-level triclosan exposure in the urban community may have caused shifts prior to the study period. Though the forested stream contained comparable levels of triclosan, data on historical levels was not available. This work adds to the breadth of knowledge surrounding development of MDR related to low-level triclosan exposure. It appears that ARB and MDR are increasing and shifts to more commensal and pathogenic bacteria are occurring due to triclosan exposure. The collection of chemicals present in diverse and numerous environments is clearly cause for prudent pause.

CHAPTER II

AIM 1: TRICLOSAN DISTRIBUTION AND MITIGATION IN STREAMS

Abstract

Triclosan is nearly ubiquitous in the environment and is found in a broad range of concentrations in streams and other surface waters. Many organisms, including algae, have been shown to bioaccumulate triclosan. Community structure and function alterations have been observed in aquatic communities exposed to triclosan including stream periphyton communities. It is unclear to what extent these alterations affect potential ecosystem services provided by the periphyton community. The hypothesis that periphyton mitigate triclosan was tested in two stream periphyton communities: a non-urban, forested stream and an urban, WW-associated stream. A survey of triclosan occurrence in stream water and periphyton was also conducted along longitudinal gradients in the two study streams. Triclosan was detected in all samples from the two streams at levels in water ranging from 152-238 ng/L and in periphyton ranging from 71-1342 ng/L. In the WW-associated stream water, a higher concentration of triclosan after the outfall of the WW effluent was observed. WW-associated periphyton showed evidence of mitigation of triclosan while the forested-stream periphyton did not.

Introduction

Triclosan has been utilized for its antimicrobial properties in a vast array of products (Adolfson-Erici *et al.* 2002; Bedoux *et al.* 2012; Fang *et al.* 2010). It is most often included as an ingredient in products that ultimately end up going down our drains. Ultimately, much of this triclosan is gathered with wastewater and biosolids, along with many other anthropogenic

inputs, at WWTPs (Saleh *et al.* 2011; Singer *et al.* 2002; van Wijnen *et al.* 2018; Ying and Kookana 2007). Much of the triclosan adheres to the biosolids (Halden and Paull 2005), while some is converted to other compounds such as methyl-triclosan (Chen *et al.* 2011). Triclosan has been shown to bioaccumulate in algae (Coogan *et al.* 2007) and other organisms (Adolfson-Erici *et al.* 2002; Coogan *et al.* 2008; Higgins *et al.* 2011; Kinney *et al.* 2008; Macherius *et al.* 2014; Pannu *et al.* 2012). Research groups around the planet have measured levels of triclosan in a multitude of sample types, biotic and abiotic (Dann and Hontela, 2011; Fair *et al.* 2009 Fang *et al.* 2010; Heidler and Halden 2007; Kinney *et al.* 2008; Kolpin *et al.* 2002; Lawrence *et al.* 2009; Nietch *et al.* 2013; Ying and Kookana 2007). In surface waters, reported concentrations have a wide range from 1.4 ng/L to 40,000 ng/L triclosan (Montaseri and Forbes 2016). Triclosan is often found at low levels and the concentration tends to be higher when the water sample is WW-associated (Table 1). For the current study, it was hypothesized that triclosan levels are higher in WW-associated streams compared to non-WW streams. Additionally, it was predicted that triclosan levels would be highest near the WWTP discharge point. In previous studies, declining levels of triclosan have been observed along a gradient downstream of a WWTP (Morrall *et al.* 2004). This pattern, along with the knowledge that algae bioaccumulate triclosan (Coogan *et al.* 2007), led to a hypothesis that periphyton mitigate triclosan downstream of WWTPs. The hypothesis was tested using stream microcosms amended with triclosan at the environmentally-relevant level of 10 μg/L triclosan.

Triclosan is known to cause toxic effects and changes in periphyton communities (Drury *et al.* 2013; Eriksson *et al.* 2015; Johansson *et al.* 2014; Nietch *et al.* 2013; Proia *et al.* 2011; Ricart *et al.* 2010; Rosi-Marshal 2013; Wilson *et al.* 2003; Zhao *et al.* 2015). 48-hour exposures to increasing levels of triclosan decreased bacterial and diatom viability and photosynthetic

efficiency of a biofilm community (Ricart *et al.* 2010). If stream periphyton assist in mitigation of triclosan, but triclosan causes detrimental community shifts, then is chronic exposure to triclosan in the stream decreasing the community's ability to regulate the compound? Have adaptations occurred in periphyton in streams receiving WW effluent that have affected the community's ability to mitigate triclosan and/or other inputs? Further research is recommended to address these questions. The current study in periphyton communities from a forested and a WW-associated stream examines the distribution of triclosan in water and periphyton as well as the ability of periphyton to mitigate triclosan in stream water.

Methods

Study streams and sampling sites

Sampling sites were selected along longitudinal gradients for the triclosan distribution survey. The forested stream, North Double Creek, had three sampling sites. Sites 1, 2 and 3 were 19.61, 10.62 and 8.55 stream km upstream of the confluence with the Dan River in Stokes County, North Carolina. For all exposure studies (including Aim 2 and Aim 3 studies on stream bacterial isolates), forested-stream periphyton were grown, fully submerged, on stone tiles at site 3 at Simmons Rd in Pinnacle, NC, 36°26'27.7"N 80°19'51.3"W. This collection site is in a 2nd order section of North Double Creek. The upstream section of North Double Creek travels through an area northeast of Pilot Mountain, NC, with the stream passing through mostly forested and rural residential areas of Quaker Gap, NC.

The WW-associated stream had six sampling sites, two upstream and four downstream of North Buffalo Water Reclamation Facility (WWTP; now retired but active during study). The upstream sites, site 1 and site 2, are located 6.36 and 2.46 stream km upstream of the WWTP in the city of Greensboro in Guilford County, North Carolina. Site 3, just after the WWTP, is 0.20

stream km downstream of the effluent outfall. The other three downstream sites, sites 4, 5 and 6, are 2.60, 5.97 and 10.08 stream km below the outfall of the WWTP. WW-associated periphyton used in laboratory studies (including Aim 2 and Aim 3 studies on stream bacterial isolates) were colonized on unglazed stone tiles at site 5 in North Buffalo Creek just downstream of Rankin Mill Road, Greensboro, North Carolina 36°07'11.8"N 79°42'28.1"W. This stream drains the northern part of the city of Greensboro, passing through urban areas. Greensboro spans 296 km^2 where originating headwaters for the Cape Fear River Basin are located. This stream has been recognized as impaired based on impaired biological communities, instream habitat degradation and the presence of fecal coliform bacteria (NCDENR 2000). The collection site is located in a 4^{th} order section of North Buffalo Creek.

Periphyton colonization of tiles in streams

Stone tiles (1 cm thick with colonization surface 2.3 cm x 3.9 cm) were adhered to bricks with Amazing Goop adhesive and left submerged in each stream for periphyton to grow on tiles for approximately one month (tiles were left in streams for the same number of days for both streams). Bricks with tiles were transported to the laboratory fully submerged in stream water from their source stream.

Periphyton collection

Periphyton were scrubbed from a rectangular section of known surface area using a toothbrush and rinsed into pre-weighed vials, either with stream water or ultrapure water. For field samples used in the distribution survey, the area on submerged stream rocks was defined by an empty frame 24 mm by 36 mm and scrubbed to remove periphyton from the framed area. Three of these 864 mm^3 frames, collected from different areas of rock or different rocks, were pooled into a single vial for each periphyton sample. Three samples at each site were collected

for extraction and mass spectrometry analysis. Unfortunately one sample of each was compromised. Therefore results are given for two samples at each site. For mitigation studies, the top surface of 3 unglazed, stone tiles used as growth substrate defined the periphyton sample size. The top surface of each tile is 23 mm by 39 mm, for a marginally larger (as compared to frames used in distribution survey) 897 mm^3 colonization surface. Periphyton were rinsed with pure water from 3 tiles into a pre-weighed 50 ml screw cap tube with ultrapure water, periphyton were centrifuged at 4000 rpm for 5 minutes. Water was then removed prior to lyophilization (freeze-drying). Lyophilized samples were weighed and stored at -20° C until time of extraction.

Exposure to triclosan in microcosms

All laboratory experiments were conducted at the University of North Carolina at Greensboro in the Department of Biology. Tiles colonized with periphyton were carefully removed from bricks without touching the top surface and evenly distributed into microcosms containing 1100 ml water from the forested stream, North Double Creek. Periphyton and water were collected from microcosms prior to triclosan addition (0 hour samples) and again 11, 22, and 33 hours after triclosan addition to microcosms for analysis of triclosan levels. Each microcosm initially contained 21 tiles and was made of plastic with a doughnut-shaped tray with 30 cm diameter outer circle and a 12 cm inner circle. There were four air streams (2 on each side from 2 aquarium pumps) blowing across the surface of the water to keep the stream water circulating. Stream water was taken from North Double Creek on the day of periphyton collection. At the start of the exposure experiment, triclosan was added to raise the level of triclosan in the water to 10 μg/L above ambient stream level (ranging from undetectable to

0.175 µg/L, unpublished data). Microcosms were kept at 25°C with lights on in an environmentally-controlled room.

Triclosan extraction from water

Amber glass bottles used for water collection had previously contained only HPLC-grade solvents: water, methanol, or acetonitrile. The bottles were rinsed completely two times with acetone and three times with pure, deionized water and also rinsed with stream water prior to collection of stream water. 1 L samples were filtered through glass fiber filters and 100 µL of 10 ppm mass labeled internal standard, ^{13}C-Triclosan (Wellington Laboratories), was added. The entire 1 L was loaded through HLB Oasis 12cc cartridges (Waters Corporation) which had been preconditioned with 10 ml HPLC-grade methanol and 10 ml ultrapure water by letting each drip through slowly. A vacuum manifold was used to assist each 1 L water sample in passing through the extraction cartridge. Cartridges were air dried for at least 1 hour and if not immediately eluted, were stored at -20° C until time of elution. Samples were eluted 3 times with 5 ml (15 ml total) of a mixture of 1:1 acetone: methanol with 10 mM acetic acid. 15 ml eluates were transferred to 20 ml amber vials and test tubes rinsed with methanol into the vial also. These eluates were dried at room temperature under N_2 gas. Each extract was reconstituted in 1 ml HPLC-grade methanol through vortexing and sonication. These extracts were analyzed using mass spectrometry. As the extract of 1 L of water was concentrated into 1 ml of methanol, calculated amounts via mass spectrometry in the extracts in ppm range equate to the ppb range in the water sample (for example 1 ppm triclosan in extract shows there was 1 ppb triclosan in the water prior to extraction).

Triclosan extraction from periphyton

Lyophilized periphyton samples were combined with 1 ml ultrapure water and 100 µL of 10 ppm ^{13}C Triclosan as an internal standard for mass spectrometric analysis. Samples were homogenized with a tissue homogenizer. The following extraction process was repeated three times: samples were combined with 5 ml 1:1 acetone: methanol containing 10 mM acetic acid, vortexed for 1 minute, and sonicated for 15 minutes, then centrifuged at 2500 rpm for 5 minutes. The extract/supernatant was filtered through glass wool and collected in an amber glass scintillation vial. As stated, two additional rounds of this were conducted for a total of ≈15 ml extract per sample. Extracts were blown dry under nitrogen gas. Extracts were reconstituted in 1 ml HPLC-grade methanol each by vortexing and sonication, then analyzed using mass spectrometry. The extract of a known mass of periphyton was concentrated into 1 ml of methanol. The calculated triclosan concentrations measured through mass spectrometry (within the range of the standard curve) and the sample weights were used to calculate the triclosan concentration in each original sample.

Mass spectrometry analysis

Triclosan analysis was performed on a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray source (HESI-II) coupled to an Acquity Ultra-performance liquid chromatography (UPLC) system (Waters Corpation, Milford, MA). A 5 µL injection of each sample was eluted from a 2.1 x 50 mm Acquity UPLC BEH C18 column (Waters Corp.) using a binary solvent gradient consisting of Optima LC/MS grade water with 0.1% formic acid (solvent A) and Optima LC/MS grade methanol (solvent B) at a flowrate of 0.3 ml/min. The gradient initiated at an isocratic composition of 80:20 (A:B) for 2.0 min, increased linearly from 2.0–8.0 min. to 5:95 (A:B), followed by an isocratic hold at 5:95 (A:B)

from 8.0–10.0 min, gradient returned to starting conditions of 80:20 (A:B) from 10.0–10.1 min, and was held at this composition from 10.1–11 min. The mass spectrometer was operated in negative ionization mode over a scan range of 150–1000 with the following setting: spray voltage set at 3.0 kV, capillary temperature set at 320°C, s-lens RF level set at 50.00, sheath gas flow set at 50, and auxiliary gas flow set at 15.

Standard curves of triclosan and ^{13}C-triclosan were prepared using standards mixed in methanol at the following concentrations: 8096 µg/L, 4048 µg/L, 2024 µg/L, 1012 µg/L, 506 µg/L, 253 µg/L, and 126.5 µg/L. All samples were analyzed in triplicate and data processing was performed using the Xcalibur software (Thermo Fisher).

Results

Triclosan distribution survey

Both the forested North Double Creek and WW-associated North Buffalo Creek contained detectable levels of triclosan in both water (152-238 ng/L) (Fig. 4) and periphyton (71-1342 ng/L) (Fig. 5). Comparing forested and WW-associated samples using t-tests, there was not a significant difference between streams in the overall levels of triclosan measured in water ($p=0.577$) or periphyton samples ($p=0.195$) in the triclosan distribution survey.

Triclosan levels in stream water

Surface water sampled from the forested stream contained triclosan levels in the range of 152-208 ng/L (Fig. 4A). The slightly wider range of 143-238 ng/L observed in the WW-associated stream (Fig. 4B) reflects higher concentrations of triclosan found just downstream of the outfall of the WW effluent. These samples showed a characteristic peak of higher triclosan downstream of the WWTP, similar to previous studies (Morrall *et al.* 2004). Triclosan levels in

stream water were highest at the nearest sites downstream of the WWTP (218 ± 20 ng/L at site 3; 223 ± 12 ng/L at site 4) (Fig. 4B).

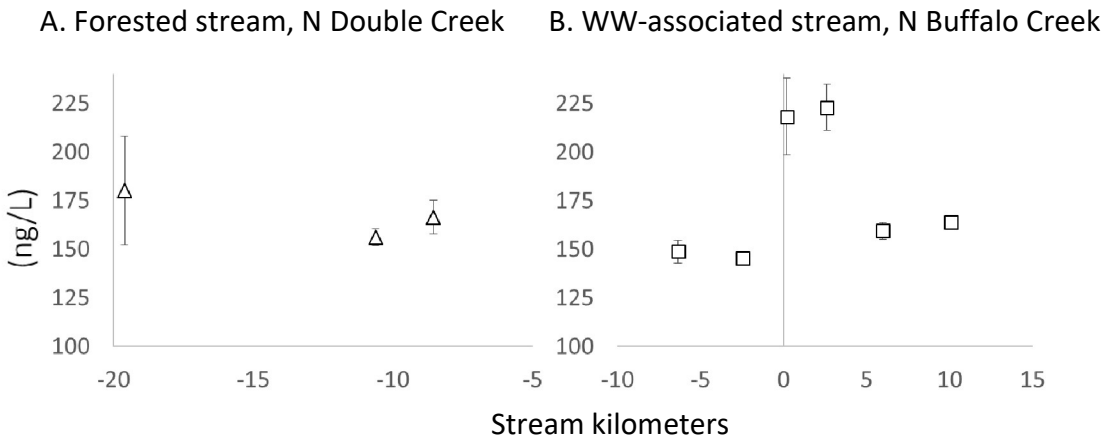

Figure 4. Triclosan Levels in Stream Water. (A) Triangles show average levels in water from North Double Creek. On the x-axis, negative distances indicate km upstream of the confluence with the Dan River (set at 0 km). (B) Squares show average levels in water from the North Buffalo Creek. On the x-axis, negative distances indicate kilometers upstream of the WWTP (set at 0 km), and positive values show kilometers downstream of the WWTP. Error bars show standard errors.

Triclosan levels in periphyton

Periphyton samples from the forested stream contained triclosan levels in the range of 178-1342 ng/g (Fig. 5A). WW-associated periphyton sampled from North Buffalo Creek showed a range of triclosan levels from 71-555 ng/g (Fig. 5B).

Triclosan mitigation study

Trials of the mitigation study showed decreasing triclosan levels in water over 33 hours (Fig. 6) in all microcosms. Increases in triclosan observed in periphyton occurred with WW-associated periphyton but not forested-stream periphyton (Fig. 7).

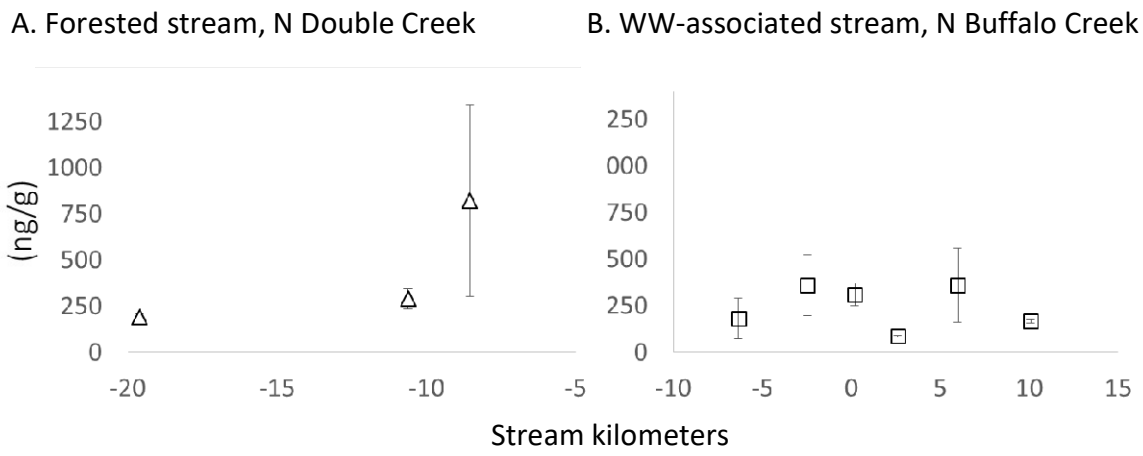

Figure 5. Triclosan Levels in Stream Periphyton. (A) Triangles show average levels in periphyton from rocks in the forested stream, North Double Creek. On the x-axis, negative distances indicate kilometers upstream of the confluence with the Dan River (set at 0 km). (B) Squares show average levels in periphyton from rocks in the WW-associated stream, North Buffalo Creek. On the x-axis, negative distances indicate kilometers upstream of the WWTP (set at 0 km), and positive values show kilometers downstream of the WWTP. Error bars show standard errors.

Triclosan levels in microcosm water

At time zero, all microcosms had water from the forested stream, amended with triclosan to reach approximately 10 μg/L + ambient concentration (10.2 ± 0.2 μg/L in Trial A; 9.2 ± 0.2 μg/L in Trial B). Microcosms with no periphyton showed a slight decrease in triclosan levels in water over the time of the experiment in trial A, and a more substantial decrease in triclosan levels in water in trial B (Fig. 6). Microcosms with WW-associated periphyton showed decreases in triclosan levels to a greater extent than microcosms containing no periphyton or forested-stream levels in water in trial B (Fig. 6). Microcosms with WW-associated periphyton showed decreases in triclosan levels to a greater extent than microcosms containing no periphyton or forested-stream periphyton, which also showed decreased triclosan in the water over 33 hours.

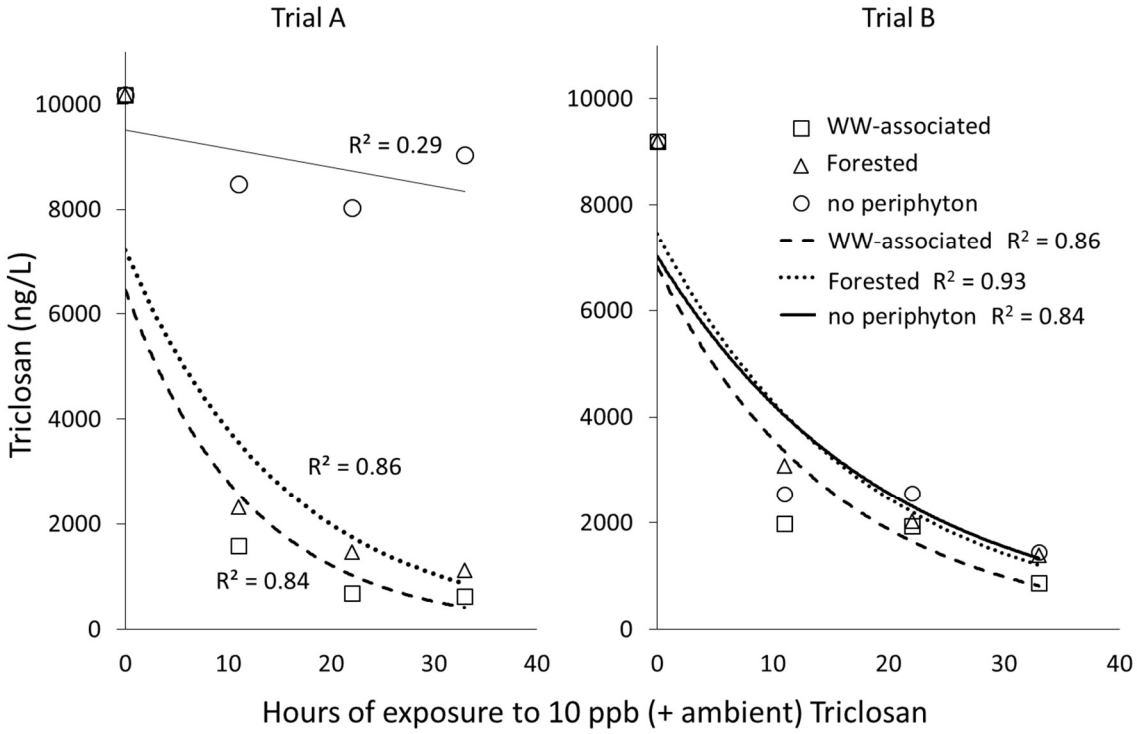

Figure 6. Triclosan Loss in Microcosm Water. Each graph shows measured triclosan levels in microcosm water from one trial of the triclosan mitigation study. Control microcosm levels are represented with circles with solid trendlines, showing triclosan levels in microcosms with stone tiles that had no periphyton. Levels in water from microcosms containing periphyton from the forested stream are represented by triangles with dotted trendlines, while the WW-associated levels are shown with squares and dashed trendlines.

Triclosan levels in microcosm periphyton

Triclosan levels increased over time in periphyton from the WW-associated stream (Fig.7) with no clear pattern shown in triclosan levels in periphyton from the forested stream. Forested-stream periphyton showed a higher triclosan concentration after 22 hours of exposure in trial A, though the measured concentration in periphyton after 33 hours of exposure was lower (Fig. 7) and this was not seen in trial B, where the triclosan levels in forested-stream periphyton did not appear to fluctuate greatly.

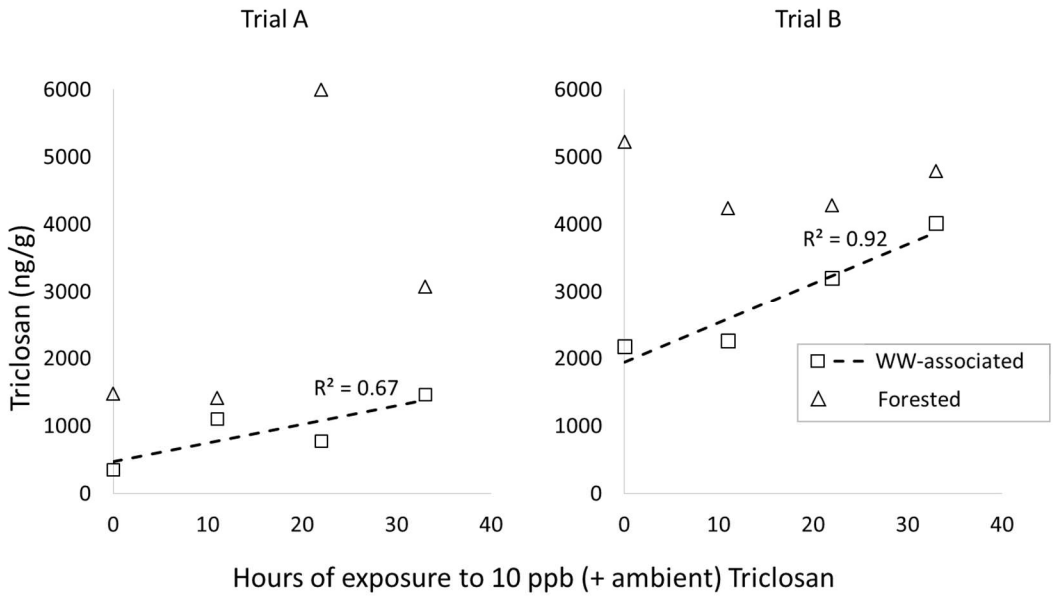

Figure 7. Triclosan Levels in Microcosm Periphyton. Each graph shows measured triclosan levels in microcosm periphyton from one trial of the triclosan mitigation study. Levels in periphyton from the forested stream are represented by triangles, while the WW-associated levels are shown with squares. Trendlines and R^2 values are shown for significant patterns (WW-associated stream only, with dashed trendlines).

Discussion

The highest measured triclosan levels observed in water samples in the current study were 238 ng/L and 235 ng/L, in water collected from 0.2 km and 2.6 km downstream of a WWTP on North Buffalo Creek. Peaks in levels of anthropogenic inputs at WWTPs, triclosan included, have been observed in previous studies (Barber *et al.* 2006; Coogan *et al.* 2007; Morrall *et al.* 2004; Ricart *et al.* 2010; Ying and Kookana 2007). As an urban area with a higher population density combining many inputs at the WWTP, it is understandable why, despite the majority of triclosan inputs from WW influents being presumably removed from water through WW processing, triclosan levels would be higher just downstream of the WWTP. However, this level was only 30 ng/L higher than the maximum measured level in the studied forested stream. Also,

the mean triclosan level measured in forested water samples (across all sites) tested was slightly higher than the mean of all tested WW-associated samples (176 ± 9 ng/L forested, 167 ± 10 ng/L WW-associated). Although higher overall triclosan levels were expected in the WW-associated stream, the two streams showed comparable levels of triclosan in both water and periphyton samples. Comparing triclosan levels in periphyton from the two streams, the average of all analyzed forested samples was higher than that in WW-associated periphyton samples tested (435 ± 183 ng/g forested, 243 ± 47 ng/g WW-associated). This indicates that triclosan can be present in streams in forested and non-urban areas, and at levels similar to those observed in urban streams. The results of this study support the hypothesis that triclosan levels are highest near WWTPs but did not support the hypothesis that overall triclosan levels are higher in WW-associated streams compared to non-WW streams.

In addition to population density and WW-input, some relevant factors that may influence triclosan concentration in stream water and periphyton include: photodegradation rates, adsorption to stream sediments and to biosolids in the WWTP, uptake by organisms, and conversion to methyl-triclosan, which is known to occur during WW processing (Chen *et al.* 2011). It is possible that despite higher inputs of triclosan at the WWTP, some of these processes are occurring to a greater extent in the WW-associated stream. Although outside of the scope of this study, photodegradation rates likely differ between the streams, as North Buffalo Creek is a 4th order stream with a more open canopy than the 2nd order, forested North Double Creek, which is mostly shaded at all sites along the study reach. There are various pathways through which triclosan can be transformed such as photodegradation and biotransformation. The amount of time it takes for triclosan breakdown to occur is highly dependent on the surrounding environmental conditions. The degradation time of triclosan

varies widely in different environments with estimated half-lives of: 1 day in air, 60 days in water, 120 days in soil and 540 days in sediment (Halden and Paull 2005). The half-life in other systems also vary. A study using water from the Tamar Estuary, UK showed a half-life of 4 days in seawater, as well as a half-life of 8 days in freshwater from St. John's Lake (Aranami and Readman 2007). Yet another study calculated triclosan's half-lives in two lakes to be 89 days and 148 days and triclosan's half-life in river water (Xiangxi River, China) to be 161 days (Huang *et al.* 2014). It seems likely that both sorption to tiles or microcosm trays and photodegradation led to decreases in triclosan across all microcosms. Photodegradation may have been higher in microcosms than reports in literature due to the shallow nature of the microcosms as well as the lights remaining on throughout the experiment. Neither photodegradation nor biotransformation were directly investigated in this study. It would be interesting to conduct a study analyzing products of photodegradation and biotransformation and investigating possible differences between different periphyton communities. Uptake by organisms is also likely to differ between the two streams, and there is evidence of this shown by the periphyton in the triclosan mitigation study, in which WW-associated periphyton showed increasing triclosan levels in microcosms but forested-stream periphyton did not (Fig. 7). To better understand the extent to which community composition plays a role in the mitigation potential of a periphyton community, additional studies with higher sample numbers as well as community structure analyses are warranted.

Chronic exposures to anthropogenic inputs such as triclosan may alter natural aquatic communities (Drury *et al.* 2013; Nietch *et al.* 2013; Proia *et al.* 2011; Ricart *et al.* 2010; Wilson *et al.* 2003; Zhao *et al.* 2015). Mitigation of triclosan is an ecosystem service that may be provided by some stream communities. However, there is also the potential that impacts of triclosan and

additional anthropogenic inputs alter effects on mitigation potential of these communities. The result that periphyton from the WW-associated stream took up triclosan more efficiently than the forested-stream periphyton invites further investigation. It is possible that different community compositions affect the mitigation potential of the communities, resulting in a difference between the WW-associated and forested stream. It is unclear why microcosms containing tiles only and no periphyton lost more triclosan over time in water in trial B relative to trial A. The setup was the same for both trials so photodegradation rates and adsorption rates (possibly to sides of microcosm trays as well as tiles) should have been similar.

In summary, field and lab results were consistent with mitigation, but more samples and trials would need to be run for a definitive conclusion. In the WW-associated stream water, there was a sharp drop in triclosan between the downstream stations near the WWTP and those further down. In microcosms showing evidence for mitigation, triclosan loss over time in water was greater than that of other microcosms (Fig. 6) and increasing triclosan over time was observed in the WW-associated periphyton (Fig. 7). Although more evidence should be collected for a conclusive result, this provides some evidence that WW-associated periphyton can provide a mitigation service for triclosan in WW-associated streams.

CHAPTER III

AIM 2: ANTIBIOTIC RESISTANCE GENES AND ISOLATE IDENTIFICATION

Abstract

Stream microbial communities and their ecosystems contain a complex mixture of components and inputs that can impact antibiotic resistance. Antimicrobial pollution, antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) are found in both urban and non-urban streams, though many are likely found at higher levels in WW- associated streams. The extent to which chronic, low-level exposures to triclosan affect microbial community composition and levels of ARGs in stream periphyton bacterial communities is not well understood. In stream periphyton communities (urban or forested), and possibly to a greater extent at waste water treatment plants (WWTPs), the combining ARB harboring ARGs, and antibiotics can lead to further development and dissemination of ARGs (Fig. 3). ARGs can be exchanged among different genera/species/strains of bacteria. In some cases, what once was a susceptible strain can develop into ARB. Chronic presence of triclosan may be exacerbating the issue of increasing antimicrobial resistance. To address the effects of triclosan on stream periphyton bacterial communities and ARG levels in these communities, stream bacterial isolates were cultured from periphyton communities sourced either from a WW-associated or a forested stream and with or without exposure to 10 µg/L triclosan in recirculating stream microcosms, and later identified to genus level. The endeavor to determine levels of triclosan resistance associated ARGs in these communities was met with limited procedural success. A single ARG target, *mexB*, was amplified in one of the exposed, forested-stream isolates. This

gene encodes a subunit of the MexAB–OprM efflux pump. 105 of 144 total stream bacterial isolates were identified to genus level via 16S rRNA sequencing and alignment using NCBI's BLAST. Based on identified isolates, all four treatment groups appear to exhibit dominance of the genus *Pseudomonas.* This genus and two other genera represented by multiple isolates in the current study (*Acinetobacter* and *Serratia*) are included in the World Health Organization's list of bacteria of critical concern (WHO 2017).

Introduction

Bacteria existing in various environmental compartments are often transported through water. In urban ecosystems, countless different types of bacteria reside in and can be exchanged between environments such as surface waters, drinking water and wastewater (Vaz-Moreira *et al.* 2014). In many cases, antibiotic resistant bacteria (ARB) are present and exhibit resistance to one or more antibiotics. The development and dissemination of ARB and ARGs in and between environmental compartments is considered a major threat to environmental health and the health and well-being of humans and other animals (Bush *et al.*, 2011; Forsberg *et al.*, 2012; Vaz-Moreira *et al.* 2014). Water systems are important microbial habitats and, in many cases, may act as sources of and reservoirs for ARGs. As an unwanted side-effect of human practices and product use, these habitats have developed into bioreactors where ARGs are exchanged between different types of bacteria (Baquero *et al.*, 2008; Poirel *et al.*, 2005; Rizzo *et al.*, 2013). WWTPs are known hotspots for selection of antibiotic resistance and transfer of ARGs (Rizzo *et al.* 2013). A vast and growing list of ARGs have been detected in WW-associated systems (Nnadozie *et al.* 2017; Tang *et al.* 2017; Xi *et al.* 2015). Among other genetic elements, integrons frequently carry ARGs (Boucher *et al.* 2007; Wolters *et al.* 2015) and have been implicated in spreading ARGs in both environmental and clinical settings (Gillings 2014). In

many cases, conventional treatment processes for wastewater are insufficient to reduce levels of ARB, often even increasing overall resistance in downstream effluents (Ferreira da Silva *et al.* 2006; Łuczkiewicz *et al.*, 2010; Novo *et al.*, 2013).

An array of routes resulting in triclosan resistance exist. Previous studies in pure cultures indicate that membrane resistance, target site modifications and efflux are the most common mechanisms of triclosan resistance (Carey and McNamara 2015). Properties of bacterial outer membranes can provide resistance to hydrophobic antimicrobials (Champlin *et al.* 2005; Tkachenko *et al.* 2007). In comparison between strains of *P. aeruginosa* possessing cell envelopes less permeable to hydrophobic chemicals and strains with highly permeable cell envelopes, those with the less permeable envelopes had higher intrinsic resistance (Champlin *et al.* 2005). Through investigations into a strain of *S. aureus* showing cross resistance with triclosan and ciprofloxacin, after eliminating several potential mechanisms of resistance, the authors attributed the cross resistance to altered gene expression affecting cell membrane structure and function (Tkachenko *et al.* 2007). The authors suggested that triclosan exposure elicits this gene expression response leading to possible increases in branched chain fatty acids in cell membranes that can help prevent agents from crossing the membrane. Any mechanism that indiscriminately inhibits hydrophobic chemicals from passing into cells could lead to resistance to triclosan and other antibiotics. There are numerous studies in bacterial isolates verifying the presence of triclosan-resistant isoenzymes for triclosan's target, the enoyl-acyl carrier protein reductase (reviewed in Carey and McNamara 2015; Zhu *et al.* 2010). Aside from isoniazid, which shares triclosan's target, resistant enoyl-acyl carrier protein reductases are not generally known to cause cross-resistance between triclosan and other antibiotics. This is still of concern as isoniazid is vital in tuberculosis management, and lower triclosan concentrations

specifically inhibit enoyl-acyl carrier protein reductase and resistance can develop (Giuliano and Rybak 2015). Export of antimicrobials via efflux pumps is a common adaptation. Triclosan resistance and resistance to antibiotics can be conferred through expression of efflux pumps such as AcrAB-TolC (Perez *et al.* 2007), MexAB–OprM (Carey *et al.* 2016; Yoneda *et al.* 2005), MexXY-OprM (Chuanchuen *et al.* 2008), and TriABC-OpmH (Mima *et al.* 2007). Associated genes targeted in the current study are: *AcrA, mexB, mexX,* and *triB.* Cross-resistance to other antibiotics, such as chloramphenicol and carbenicillin, has been observed in triclosan-resistant bacteria exhibiting constitutive upregulation of efflux pumps (Pycke *et al.* 2010). *P. aeruginosa* constitutively expresses two RND efflux pumps, MexAB–OprM and MexXY-OprM, and both of these systems can actively export fluoroquinolones, tetracycline and chloramphenicol (Sun *et al.* 2014). MexAB–OprM is the homolog of an efflux pump system in *E. coli* called AcrAB-TolC and it can also export novobiocin and b-lactams, such as carbenicillin and MexXY system can also export aminoglycosides such as erythromycin, gentamicin, neomycin and tetracylcine (Chuanchuen *et al.* 2008). The gene, *mexB*, encodes a proton antiporter subunit of the MexAB–OprM drug efflux pump that is known to confer MDR in *Pseudomonas aeruginosa* (Sun *et al.* 2014). There exists a highly complex web of interactions among microbial communities, our understanding of which is drastically limited by culturability (or lack thereof) of a large fraction of the microorganisms in these communities. Microbial community diversity is potentially important because microbial diversity can impact the community's ability to metabolize xenobiotic compounds (Hernandez-Raquet *et al.* 2013).

With MDR on the rise and threatening our health and well-being, the World Health Organization (WHO) has issued a list of priority pathogens (WHO 2017). At the top of this list are MDR^t bacteria posing threats in hospitals and among patients requiring care involving devices

such as ventilators and blood catheters. Genera on the most critical list include *Acinetobacter*, *Pseudomonas* and various Enterobacteriaceae including *Klebsiella*, *E. coli*, *Serratia*, and *Proteus* (WHO 2017). Broad-spectrum antimicrobials, such as triclosan, can act as selective agents for acquired resistance to triclosan as well as other antimicrobials. For example, in a study selecting for biocide-resistant strains of both *E. coli* and *S. enterica*, after only two sub-lethal exposures to triclosan, *E. coli* O157 strains acquired resistance to several antimicrobial agents such as biocides and antibiotics including chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim (Braoudaki and Hilton 2004). Acquired resistance to triclosan and other antibiotics can be transferred to pathogenic bacteria and is a threat to human and environmental health (Ciusa *et al.*2012). If chronic, sublethal exposures to triclosan are impacting stream microbial communities and/or ARG levels within these communities, this contributes to the major global health challenge of increasing antibiotic resistance.

Overall levels of ARGs may be higher in WWTP effluent compared to levels in sewage prior to treatment (Reinthaler *et al.*, 2010; Uyaguari *et al.*, 2011) due to selection of resistant bacteria during treatment processing. However, ARGs can also be found in stream communities not associated with WWTPs (Jacobs and Chenia 2007; Leff *et al.* 1993; Mohapatra *et al.* 2008; O'Flaherty and Cummins 2017). It was hypothesized that overall levels of ARGs would be higher in a WW-associated stream periphyton community compared to a forested stream periphyton community. It was also predicted that exposure to 10 µg/L triclosan would impact the distribution of ARGs in the stream periphyton community.

Methods

Sample sites, periphyton colonization and microcosm exposure

Isolates investigated were sampled from North Double Creek (forested, not WW-associated) or North Buffalo Creek (WW-associated) and the collection sites and methods related to periphyton collection on stone tiles are described in detail in the methods section of Chapter II. Exposed isolates described herein refer to isolates from colonies isolated after exposure to 10 µg/L triclosan (+ambient triclosan in North Double Creek). Microcosm set up is also described in the methods section of Chapter II.

Unexposed (0 hr) isolates were collected for pure culture isolation prior to addition of triclosan to microcosms. Exposed isolates were collected from microcosms at varying timepoints after addition of triclosan to microcosms (2, 11, 33 hours; 1, 2, 3, 4 weeks; with water replaced each week including a fresh addition of triclosan). Analyses described in study results group isolates as unexposed or exposed to 10 µg/L + ambient triclosan in microcosms and by periphyton source stream.

Bacterial culture conditions and DNA extraction

Isolate purification and microbiological analysis for isolate identification were conducted aseptically using sterile, low-nutrient agar or broth. Single colonies were isolated after periphyton were collected, diluted in sterile water and spread-plated on sterile, low-nutrient agar. Individual colonies were selected at random (using a grid and random number generator) and streaked individually on sterile, low-nutrient agar plates. From each of these a single colony was isolated to begin a pure liquid culture. Stocks were maintained in slants on sterile, low-nutrient agar containing 8% plate count agar. All liquid cultures were maintained in sterile, low-nutrient Mueller-Hinton broth (50% MH broth) made by dissolving the solid MH broth powder in

twice the recommended volume of distilled, deionized water and autoclaving. Therefore, the low-nutrient broth used for this study had 50% the normal concentration of nutrients in an attempt to simulate more relevant environmental conditions. Prior to ARG targeting and 16S rRNA sequencing, genomic DNA was extracted from pure liquid bacterial cultures using CTAB extraction method (Schaefer 1997).

Isolate identification

16S rRNA sequencing using universal primers (Table 2) was used to identify isolates to the genus level. Genomic DNA was extracted from pure liquid cultures using CTAB extraction method (Schaefer 1997). 16S rRNA sequencing was used to identify isolates to the genus level. Primers targeting the 16S gene are described in Table 2 (Barghouthi 2011; Klindworth *et al.* 2013). Specific primer pairs used for identification of each isolate are listed in results. Primers were ordered from IDT (Integrated DNA Technologies, Inc.) and 2X PCR Master Mix (reaction buffer, Taq Polymerase, dNTPs, $MgCl_2$) was obtained from Fisher Scientific (FERK0171). A given PCR well with a 31 μL total volume contained: 15.5 μL 2X master mix, 13.5 μL nuclease-free water, 0.5 μL forward primer, 0.5 μL reverse primer, and 1 μL genomic DNA. In some cases, larger volumes (up to 51 μL total volume) were set up when more product was needed. In these cases, proportions of ingredients were kept the same as the 31 μL reactions.

All touchdown PCR methods began with a 3 minute initial denaturation hold at 96°C, followed by 40 cycles of 12 seconds each at 93°C, 12 seconds at the annealing temperature, and 20 seconds at 72°C, with a final hold of 3 minutes at 72°C. Annealing temperatures vary with primer pairs, and were decreased by 1 degree per cycle from the first through the 19th cycle. Cycles 20-40 were repeated with the same low annealing temperature used at cycle 19. Initially, GM3/GM4 PCR was tested (Klindworth *et al.* 2013).

Table 2. Primers Used for 16S rRNA Amplification and Sequencing.
Table shows sequences and melting temperatures of universal 16S rRNA primers used for PCR and sequencing with the aim of identifying environmental isolates in this study.

Primer	Short name	PCR and sequencing primers	*Tm* (°C)	Reference
F-GM3-S-D-Bact-0008-a-S-16	GM3	5′-AGAGTTTGATCMTGGC-3′	47.0	Klindworth *et al.* 2013
R-GM4-S-D-Bact-1492-a-A-16	GM4	5′-TACCTTGTTACGACTT3-′	43.3	Klindworth *et al.* 2013
QUGP-F3	F3	5′-GATACCCTGGTAGTCCA-3′	52.80	Barghouthi 2011
QUGP-F5	F5	5′-CCTACGGGAGGCAGCAG-3′	54.54	Barghouthi 2011
QUGP-F6	F6	5′-GCAGCCGCGGTAATAC-3′	54.51	Barghouthi 2011
QUGP-R1	R1	5′-TACCTTGTTACGACTTCACCC-3′	57.90	Barghouthi 2011
QUGP-R2	R2	5′-GACGGGCGGTGTGTAC-3′	54.85	Barghouthi 2011
QUGP-R3	R3	5′-TGGACTACCAGGGTATC-3′	52.80	Barghouthi 2011
QUGP-R4	R4	5′-CGTACTCCCCAGGCGG-3′	59.55	Barghouthi 2011

Initial and final annealing temperatures were 54°C and 36°C for the GM3/GM4 PCR. 8 μL of each PCR product was run on a 1% agarose gel containing ethidium bromide via electrophoresis and viewed in UV light to determine success or failure of the PCR reaction. For those isolates that did not have successful 16S amplification with GM3/GM4, a mixture of primers was tested including equal amounts of each: F5, F6, R2 and R3 (Barghouthi 2011). Initial and final annealing temperatures were 69°C and 51°C for this mixture of 16S rRNA primer pairs. After analyzing the sizes of amplified products, PCRs with individual pairs of primers were run for the largest product amplified by the primer mixture for each isolate. PCRs with F5 /R3, F6/R2, F6/R3 and F6/R4 used the touchdown method described, with annealing temperatures declining from 69°C to 51°C. Any isolate that did not have successful amplification by methods described above was further tested with primer pairs: F3/R1 using initial and final annealing temperatures of 63°C and 45°C or F3/R2 using initial and final annealing temperatures of 66°C and 48°C. A PCR clean-up kit (DNA Clean & Concentrator, Zymo Research) was utilized to purify successful reactions to prepare products for sequencing. Cleaned PCR product samples were analyzed on a NanoDrop ND1000 spectrophotometer and diluted (with nuclease-free water) or further concentrated if necessary to provide samples with the proper DNA concentration range for sequencing. Sequencing was conducted by Eurofins Genomics. Trimmed sequences were analyzed via alignment on NCBI nucleotide BLAST and the top identified match was taken as the top match. This top match was the highest ranking sequence that was identified at least to genus in the BLAST results from alignment of a given environmental isolate query sequence. This along with subsequent matches on the BLAST results were used to determine the most likely genus for each isolate. Acceptable criteria were 350+ bases with discernable peaks on the chromatogram provided by Eurofins Genomics. Reported genera are based on trimmed sequences at least 350

bases long. For some isolates, the 16S region was successfully amplified, but without a chromatogram of 350+ bases of discernable peaks, so these were not included in the report of identified isolates.

Statistical analysis

To investigate exposure effects within each stream, a chi-square test was conducted using genus richness of the unexposed group as the expected ratio. To compare proportions of identified isolates that were in the genus *Pseudomonas* (or additionally, in the WW-associated stream, *Serratia*), a Z Score Calculator for 2 Population Proportions was conducted using the Social Science Statistics calculator (Stangroom 2018).

PCR targeting of ARGs

Antibiotic Resistance Genes (ARGs) related to triclosan resistance mechanisms were chosen for targeting (Table 3). Based on alignments of between 3 and 5 reference sequences from the NCBI database, primers were designed to target a portion of each of the chosen genes. Primers were ordered from IDT and 2X PCR Master Mix was obtained from Fisher Scientific. A given PCR well with a 21 µL total volume contained: 10.5 µL 2X master mix, 8.5 µL nuclease-free water, 0.5 µL forward primer, 0.5 µL reverse primer, and 1 µL genomic DNA. Attempting to make the primers more universal and work with more isolates from the environmental sample, bases showing mismatches in the alignments were designated as ambiguous bases and a mixture of primers varying at those positions was used. Touchdown methods were designed for primer pairs. All began with a 3 minute initial denaturation hold at 96°C, followed by 33 cycles of 12 seconds at 93°C, 12 seconds at the annealing temperature, and 20 seconds at 72°C, and finally a hold of 3 minutes at 72°C. Annealing temperatures vary with the different primer pairs, and were decreased by 1 degree per cycle from the first through the sixteenth cycle.

Table 3. Degenerate Primers Designed to Target ARGs.
Sequences for primers used to target ARGs are shown. Minimum, mean and maximum melting temperatures are shown as well as the probable length of product that would be amplified using the Forward primer and Reverse primer that is listed in the next row down.

Target gene	Primer name	Sequence	*min* Tm (°C)	*mean* Tm (°C)	*max* Tm (°C)	Target length
AcrA	AcrAF18	5′-CWSARGTYCGTCCWCARG-3′	50	53.8	58.1	447
AcrA	AcrAoR19	5′-CRACRTASATSGGRTCAAG-3′	47.6	51.5	55.5	
ErmX	ErmXpF22	5′-CGCTGTACTCCTCATGCAGTGG-3′		59.9		69
ErmX	ErmXoR20	5′-CTGAGCYGTCATCATCGTGC-3′	56	57.3	58.5	
FabG	FabGyF16	5′-TRGTYAAYAATGCYGG-3′	38.5	45.7	52.9	160
FabG	FabGpR17	5′-ATRNTRATNATWCGACC-3′	36.1	42.4	49.2	
FabI	FabIgF17	5′-GCNWTRCACCGNGAAGG-3′	48.9	55.1	60.8	299
FabI	FabIbR19	5′-AAGCTGTAKGMRCTGATGTC-3′	47.4	49.7	51.9	
FabI	FabIgF17	5′-GCNWTRCACCGNGAAGG-3′	48.9	55.1	60.8	215
FabI	FabIyR20	5′-GGWGCRAANCCRANNGAGTG-3′	50.8	57.5	63.4	
FabK	FabKgF20	5′-GCWGGWGGRATTGNNGATGG-3′	53.4	57.3	61.5	~350
FabK	FabKgR17	5′-CCYGCCATRAMDGADCC-3′	47.2	53.7	62.2	
FabL	FabLbF22	5′-TCGTMACMGGARGYASCCGCGG-3′	60.9	65.8	70.6	226
FabL	FabLyR19	5′-GACRATRTRCCCGCSRCCG-3′	55.6	61.4	67.2	
FabV	FabVrF20	5′-TCNCCWGTRCGTAAAMTGCC-3′	52.1	56.4	60.7	186
FabV	FabVpR16	5′-CCARTCTTSRCCRCCC-3′	49.7	54.7	59.8	

Table 3 continued. Degenerate Primers Designed to Target ARGs. Sequences for primers used to target ARGs are shown. Target length is the expected length of PCR product that would be amplified using the F and R primer that is listed in the next row down.

Target gene	Primer name	Sequence	*min* Tm (°C)	*mean* Tm (°C)	*max* Tm (°C)	Target length
FabV	FabVrF20	5′-TCNCCWGTRCGTAAAMTGCC-3′	52.1	56.4	60.7	189
FabV	FabVpR18	5′-TTSCCARTCYTSNCCRCC-3′	51.4	57.9	64.1	
FabV	FabVbF23	5′-CGTGAWGAYATTCARCARCACTG-3′	52.1	55.3	58.6	128
FabV	FabVoR17	5′-CCKTCRAYRCCAAAGCC-3′	47.7	54.1	60	
mexB	mexBgF17	5′-GAAGTRCARCGCCARGG-3′	50.6	55.3	59.9	203
mexB	mexBoR16	5′-AGCCAGATKCGCATMG-3′	48.4	52.2	56	
mexB	mexBaF21	5′-TGGTGATGTWCCTGTTCYTGC-3′	55	56.3	57.5	156
mexB	mexBbR20	5′-GCAARCCRATGGCSAGCACC-3′	60.2	62.4	64.7	
MexX	MexXrF19	5′-TGCTGTTCCNNATCGACCC-3′	54.4	57.3	60.6	276
MexX	MexXpaR17	5′-CGNCCSGMRATCGGCGC-3′	60	64.4	69	
MexX	MexXgF22	5′-TGCTGTTCCNNATCGACCCNGC-3′	59.3	62.9	66.8	276
MexX	MexXpaR17	5′-CGNCCSGMRATCGGCGC-3′	60	64.4	69	
triB	triBoF24	5′-AGGCRCARTNNATCRAYGCCCAGG-3′	57.3	63.6	69.8	145
triB	triByR20	5′-ACCGMSGCYTKGGCCTGGTC-3′	62.7	66.6	70.4	
triB	triByF19	5′-GACCAGGCCMARGCSKCGG-3′	61.4	65.5	69.5	126
triB	triBgR18	5′-CTGGCCGGCRSTSACCAC-3′	61.9	63.4	65	

Cycles 17-33 were repeated with the same low annealing temperature used at cycle 16. Initial and final annealing temperatures were 53°C and 38°C for FabGyF16 and FabGpR17. Initial and final annealing temperatures were 61°C and 46°C for: AcrAF18 and AcrAoR19, FabIgF17 and FabIbR19, FabVbF23 and FabVoR17, mexBgF17 and mexBoR16. Initial and final annealing temperatures were 65°C and 50°C for FabIgF17 and FabIyR20, FabKgF20 and FabKgR17, FabVrF20 and FabVpR16, FabVrF20 and FabVpR18, mexBaF21 and mexBbR20. Initial and final annealing temperatures were 67°C and 52°C for ErmXpF22 and ErmXoR20, MexXrF19 and MexXpaR17. Initial and final annealing temperatures were 67°C and 52°C for MexXgF22 and MexXpaR17, triBoF24 and triByR20, triByF19 and triBgR18. 24 isolates that showed some tolerance to triclosan (see Chapter IV) were used to test the primer pairs.

After touchdown PCR, products were separated through gel electrophoresis. 10 μL of each PCR was mixed with loading dye and separated on a 2% agarose gel containing ethidium bromide alongside a GeneRuler 1 kb Plus DNA Ladder, then visualized using UV light. Isolates used for testing all pairs of ARG primers were: WWun-4, WWun-7, WWun-8, WWun-10, WWun-11, WWun-14, WWun-16, WWun-17, WWun-18, WWun-19, WWun-20, WWx-2, WWx-3, WWx-4, WWx-5, WWx-35, FSx-1, FSx-5, FSx-6, FSx-7, FSx-23, FSx-39, FSx-41 and FSx-44. Additionally, WWun-3, WWun-24, WWx-1, WWx-6, WWx-16, WWx-19, WWx-21, WWx-23, WWx-27, WWx-36, WWx-38, WWx-39, WWx-40, WWx-46, FSun-3, FSun-11, FSun-15, FSun-21, FSx-4, FSx-5, FSx-19, FSx-24, FSx-34, FSx-36, FSx-42, FSx-46 and FSx-48 were used to test the primers mexBaF21 and mexBbR20.

Results

Isolate identification

Of 144 stream bacterial isolates cultured in the study, 105 were identified to genus level. Over a third of all identified isolates were found to be in the genus *Pseudomonas* (Table 4). *Acineotebacter* and *Plantibacter* were also dominant genera among identified isolates sampled. Many genera detected were found in both streams, while some were only found in one stream (Table 4). *Citrobacter, Rhizobium, Rhodococcus, Sphingobium* and *Sphingomonas* were only found in the forested stream community (Table 4). *Enterobacter, Plantibacter, Serratia*, and *Stenotrophomonas* were only found in the WW-associated community (Table 4). Of those genera unique (in this study) to the WW-associated stream, *Plantibacter* and *Serratia* were also among dominant genera. 18.8% (unexposed) to 20.6% (exposed) of identified WW-associated isolates were *Plantibacter* while 17.7% of identified exposed WW-associated isolates were *Serratia.* There were no identified members of *Serratia* in any of the other 3 treatment groups. Genera unique (in this study) to the forested stream did not show dominance, as each had a single representative in any given treatment group. Identification information on individual isolates, their genus, and the primer pair that gave successful 16S amplification are shown in Tables 5-8. There were no trends associated with exposure time that appeared to suggest increased effects on community diversity or dominant genera with exposure time over the timepoints used in this study, so any isolate exposed to triclosan in a microcosm, regardless of hours of exposure, was assigned to the exposed group. In the forested stream without triclosan microcosm exposure, genus richness was 52% while the exposed group of identified isolates from this stream showed only 29% genus richness. This suggests that triclosan exposure may have reduced diversity. A similar pattern was also seen in the WW-associated stream isolates

where genus richness in the unexposed group of isolates was 50% with that of the exposed group being lower at 35%.

Of the identified unexposed forested-stream isolates, less than a quarter were pseudomonads. Of the identified forested-stream isolates which underwent exposure to triclosan in microcosms, half were pseudomonads.

Table 4. Identified Isolate Genera.
Table shows number of isolates in the study of each identified genus. The most likely genus was determined by analysis and alignment of a partial 16S rRNA sequence.

Genus	Unexposed Forested	Exposed Forested	Unexposed WW-assoc.	Exposed WW-assoc.	Total
Acidovorax	1	1	1	1	4
Acinetobacter	2	2	3	3	10
Aeromonas	2	0	0	1	3
Bacillus	1	1	0	1	3
Chromobacterium	3	0	1	1	5
Citrobacter	1	0	0	0	1
Comamonadaceae	1	0	0	0	1
Enterobacter	0	0	1	0	1
Flavobacterium	0	4	1	2	7
Janthinobacterium	3	3	0	2	8
Pedobacter	0	0	1	1	2
Plantibacter	0	0	3	7	10
Pseudomonas	5	16	5	8	38
Rhizobium	0	1	0	0	1
Rhodococcus	0	1	0	0	1
Serratia	0	0	0	6	6
Sphingobium	1	1	0	0	2
Sphingomonas	1	0	0	0	1
Stenotrophomonas	0	0	0	1	1

Table 5. Unexposed Forested Stream Isolates. Table shows identified unexposed, forested isolates' genera and primer pair used for 16S amplification and sequencing.

Isolate	Genus	16S Primers used
FSun-17	*Acidovorax*	GM3 & GM4
FSun-1	*Acinetobacter*	F3 & R1
FSun-9	*Acinetobacter*	F6 & R3
FSun-7	*Aeromonas*	F3 & R1
FSun-10	*Aeromonas*	GM3 & GM4
FSun-24	*Bacillus*	F3 & R1
FSun-8	*Chromobacterium*	F5 & R3
FSun-12	*Chromobacterium*	F6 & R2
FSun-13	*Chromobacterium*	F5 & R3
FSun-14	*Citrobacter*	F3 & R2
FSun-16	*Comamonadaceae*	F5 & R3
FSun-18	*Janthinobacterium*	F5 & R3
FSun-23	*Janthinobacterium*	F5 & R3
FSun-25	*Janthinobacterium*	F3 & R1
FSun-3	*Pseudomonas*	GM3 & GM4
FSun-6	*Pseudomonas*	F6 & R3
FSun-11	*Pseudomonas*	F3 & R1
FSun-15	*Pseudomonas*	F3 & R1
FSun-21	*Pseudomonas*	F3 & R1
FSun-20	*Sphingobium*	F5 & R3
FSun-19	*Sphingomonas*	GM3 & GM4

Table 6. Exposed Forested Stream Isolates. Table shows identified exposed, forested isolates' genera and primer pair used for 16S amplification and sequencing.

Isolate	Genus	16S Primers used
FSx-45	*Acidovorax*	GM3 & GM4
FSx-9	*Acinetobacter*	GM3 & GM4
FSx-20	*Acinetobacter*	GM3 & GM4
FSx-33	*Acinetobacter*	F5 & R3
FSx-49	*Bacillus*	F5 & R3
FSx-17	*Flavobacterium*	F3 & R1
FSx-21	*Flavobacterium*	F3 & R2
FSx-22	*Flavobacterium*	GM3 & GM4
FSx-28	*Flavobacterium*	GM3 & GM4
FSx-12	*Janthinobacterium*	F6 & R2
FSx-15	*Janthinobacterium*	GM3 & GM4
FSx-27	*Janthinobacterium*	GM3 & GM4
FSx-38	*Pseudomonas*	F5 & R3
FSx-40	*Pseudomonas*	F5 & R3
FSx-1	*Pseudomonas*	GM3 & GM4
FSx-3	*Pseudomonas*	F6 & R2

This indicates an increased dominance of *Pseudomonas* species in the exposed isolates from the forested periphyton community (Z-Score = -2.1084; p=0.035). In contrast, for the WW-associated isolates sampled and identified, less than a third were pseudomonads in both exposed and unexposed isolate groups (slightly less in the exposed group at 23.5%). In this study, triclosan exposure did not significantly affect dominance of pseudomonads in the WW-

associated stream (Z-Score = 0.5806; p=0.562). Additionally, isolates in the genus, *Serratia,* were detected among exposed (17.6% of identified isolates) but not unexposed WW-associated isolates. This difference, however, was not significant (Z-Score = -1.7912; p= 0.073).

Table 6. Exposed Forested Stream Isolates, continued from previous page.
Table shows identified exposed, forested isolates' genera and primer pair used for 16S amplification and sequencing.

Isolate	Genus	16S Primers used
FSx-4	*Pseudomonas*	GM3 & GM4
FSx-5	*Pseudomonas*	GM3 & GM4
FSx-6	*Pseudomonas*	GM3 & GM4
FSx-7	*Pseudomonas*	GM3 & GM4
FSx-10	*Pseudomonas*	F5 & R3
FSx-23	*Pseudomonas*	F3 & R2
FSx-24	*Pseudomonas*	GM3 & GM4
FSx-34	*Pseudomonas*	F6 & R3
FSx-35	*Pseudomonas*	GM3 & GM4
FSx-36	*Pseudomonas*	F6 & R2
FSx-39	*Pseudomonas*	F5 & R3
FSx-42	*Pseudomonas*	GM3 & GM4
FSx-47	*Rhizobium*	F5 & R3
FSx-41	*Rhodococcus*	F5 & R3
FSx-43	*Sphingobium*	GM3 & GM4

Table 7. Unexposed WW-associated Isolates. Table shows the identified genus and 16S rRNA primer pair used for amplification and sequencing of each of the unexposed WW-associated (WW) isolates.

Isolate	Genus	16S Primers used
WWun-23	*Acidovorax*	F5 & R3
WWun-5	*Acinetobacter*	F5 & R3
WWun-6	*Chromobacterium*	F3 & R2
WWun-2	*Enterobacter*	F5 & R3
WWun-14	*Pedobacter*	GM3 & GM4
WWun-7	*Plantibacter*	GM3 & GM4
WWun-9	*Plantibacter*	F5 & R3
WWun-10	*Plantibacter*	F5 & R3
WWun-11	*Plantibacter*	F5 & R3
WWun-15	*Plantibacter*	F6 & R3
WWun-17	*Pseudomonas*	F5 & R3
WWun-18	*Pseudomonas*	F5 & R3
WWun-19	*Pseudomonas*	GM3 & GM4
WWun-20	*Pseudomonas*	GM3 & GM4
WWun-24	*Pseudomonas*	F5 & R3

There were 27 other isolates for which sequences were obtained and analyzed and, although deemed insufficient to confidently determine identity, alignment with NCBI's BLAST indicated a likely genus for each isolate. Of these 27, 14 appear to be Pseudomonads (FSun-5, FSx-13, FSx-19, FSx-26, FSx-32, FSx-46, WWun-1, WWun-3, WWun-13, WWun-16, WWx-19, WWx-23, WWx-36, WWx-40).

Table 8. Exposed WW-associated Isolates. (continued on next page) Table shows identified exposed, WW-associated isolates' genera and primer pair used for 16S amplification and sequencing. Table 8 is continued on the next page.

Isolate	Genus	16S Primers used
WWx-14	*Acinetobacter*	GM3 & GM4
WWx-22	*Acinetobacter*	F5 & R3
WWx-29	*Acinetobacter*	F5 & R3
WWx-6	*Aeromonas*	F3 & R1
WWx-37	*Bacillus*	F6 & R3
WWx-33	*Chromobacterium*	F5 & R3
WWx-18	*Flavobacterium*	F6 & R4
WWx-26	*Flavobacterium*	F5 & R3
WWx-45	*Janithinobacterium*	F5 & R3
WWx-15	*Janthinobacterium*	F6 & R3
WWx-3	*Plantibacter*	GM3 & GM4
WWx-4	*Plantibacter*	F5 & R3
WWx-5	*Plantibacter*	F6 & R4
WWx-7	*Plantibacter*	F6 & R3
WWx-12	*Plantibacter*	F6 & R3
WWx-2	*Plantibacter*	F5 & R3
WWx-9	*Plantibacter*	F6 & R3
WWx-38	*Pseudomonas*	F6 & R2
WWx-44	*Pseudomonas*	GM3 & GM4

Table 8 (continued). Exposed, WW-associated Isolates. This table shows identified exposed, WW-associated isolates' genera and primer pair used for 16S amplification and sequencing.

Isolate	Genus	16S Primers used
WWx-1	*Pseudomonas*	GM3 & GM4
WWx-16	*Pseudomonas*	F6 & R3
WWx-21	*Pseudomonas*	GM3 & GM4
WWx-27	*Pseudomonas*	GM3 & GM4
WWx-39	*Pseudomonas*	F3 & R1
WWx-46	*Pseudomonas*	GM3 & GM4
WWx-10	*Serratia*	GM3 & GM4
WWx-11	*Serratia*	F5 & R3
WWx-13	*Serratia*	GM3 & GM4
WWx-32	*Serratia*	F5 & R3
WWx-43	*Serratia*	F3 & R1
WWx-35	*Serratia*	GM3 & GM4
WWx-41	*Stenotrophomonas*	F5 & R3

Possible genera also represented in the group of isolates with sequences that did not quite fit the set of acceptable sequence quality criteria include *Acidovorax* (WWx-42, FSx-31), *Acinetobacter* (WWun-4), *Chromobacterium* (WWun-8), *Clavibacter* (WWun-21), *Comamonadaceae* (WWx-8), *Flavobacterium* (WWun-22), *Janthinobacterium* (WWx-34 and FSx-14), *Lysinibacillus* (FSun-22), *Microbacterium* (FSx-48) and *Pedobacter* (FSx-25 and FSx-37).

PCR targeting of ARGs

Due to PCR methods development complications, hypotheses regarding ARG levels were not successfully tested. Degenerate primers designed and tested through touchdown PCR on 24 or more isolates, for the most part, did not yield products. One exposed, forested isolate, FSx-34, revealed the presence of *mexB*. The portion of *mexB* flanked by mexBaF21 and mexBbR20 primers was amplified and sequenced (Eurofins). BLAST (NCBI) analysis showed a multitude of top matches with multidrug efflux pump genes, often encoding a permease subunit of an efflux pump, in varied species of *Pseudomonas*.

Discussion

There is evidence in the current study that triclosan exposure to stream periphyton has an effect on microbial diversity. Results among the isolates sampled indicate that triclosan exposure leads to loss of diversity regardless of the water source of the periphyton community. However, to obtain stronger evidence that triclosan exposure resulted in a reduction in diversity or clarify specific community shifts, a larger number of isolates should be isolated and identified. Environmental changes in microbial habitats are known to affect community structures and diversity. Salination of industrial wastewater evaporation ponds led to increased microbial diversity (Ben-Dov *et al.* 2008). In detrital food-web experiments, increased fungicide concentrations led to decreased species richness (Gardeström *et al.* 2016) which is similar to results presented here with triclosan exposure. WW-associated river sediments have been shown to have decreased microbial diversity in downstream waters as well (Drury, Rosi-Marshall, and Kelly 2013; Lu and Lu 2014). Though many factors are surely at play, it is likely that the presence of triclosan in the mix is one of the factors impacting microbial communities, and quite possibly through various mechanisms.

An increase in dominance of potentially pathogenic genera was observed in triclosan exposed periphyton bacteria. Measurements of *P. aeruginosa* have shown their presence in WW-associated river water, with higher levels in WW effluent and even higher levels in clinical WW (Schwartz *et al.* 2006). In dairy farm WW-associated river water, 5 common pathogens (*E. coli*, *Enterococcus*, *S. aureus*, *Shigella*, and *Salmonella*) were measured with decreasing levels downstream of the point pollution sources (Xi *et al.* 2015). In experimental anaerobic digesters containing triclosan, observed community shifts led toward clades containing commensal and pathogenic bacteria (Carey *et al.* 2016). It is possible that the presence of triclosan may enrich resistant organisms previously exposed to higher levels of triclosan or other antimicrobials used by humans. It would be interesting to see if *Serratia* enrichment in the exposed, WW-associated group would be observed in investigations using larger sample sizes. Although it is often present without causing problematic infections in individuals with competent immune systems, *P. aeruginosa* is known for its ability to resist a wide range of antimicrobials and can cause infection and conditions leading to mortality (Huhulescu *et al.* 2011; Micek *et al.* 2015). This species grows well under marginal conditions, in diverse environments including hot tubs (Crnich *et al.* 2003; Huhulescu *et al.* 2011; Yu *et al.* 2007), hand lotion (Becks and Lorenzoni 1995), cosmetics (Lundov and Zachariae 2008) and has even been observed colonizing apparatuses in hospitals such as respiratory equipment (Jadhav *et al.* 2013). *P. aeruginosa* is difficult to eradicate in places that have become contaminated. In a study on contamination of respiratory equipment in hospitals, 24.6% (15/61) of samples revealed the presence of pseudomonads (Jadhav *et al.* 2013). The authors, sampling inner surfaces of oxygen humidifiers and chambers of nebulizers, found other fungal and bacterial contaminants, including *Acinetobacter* species in 16.3% (10/61) of samples. *P. aeruginosa* is one of the organisms that

commonly causes nosocomial pneumonia which is associated with increased healthcare cost and prolonged hospitalization as well as mortality (Micek *et al.* 2015). *P. aeruginosa* nosocomial pneumonia patients with MDR^t strains showed higher hospital mortality rates compared to patients infected with non-MDR^t strains (Micek *et al.* 2015). In another hospital study, *Pseudomonas stutzeri* was detected in air samples in the bedside environment for patients using nebulizers in ICUs in South Africa (Van Heerden *et al.* 2017).

Much like *P. aeruginosa*, *Serratia marcescens* is a known nosocomial pathogen and is associated with mortality in immunocompromised patients (Hejazi *et al.*1997; Šiširak and Hukić 2013). *Serratia* was once thought to be nonpathogenic, but now exists in many MDR^t forms (Moradigaravand *et al.* 2016; Šiširak and Hukić 2013) and has contaminated triclosan-containing soap in a hospital setting (Barry *et al.* 1984). Some of the most at-risk patients of *Serratia* infections are those who have been treated with broad spectrum antimicrobials (Hejazi *et al.* 1997). Among other medical and military experimentation, it was dispersed by the US Navy in 1950 in San Francisco for monitoring as a biological warfare test agent (Mahlen 2011).

MDR efflux pumps are a common tool utilized by microbes to resist antimicrobials (Strateva and Yordanov 2009). *mexB* encodes the transporter of the MexAB-OprM efflux pump (Sun *et al.* 2014), known to export triclosan and certain antibiotics. Overexpression of *mexAB-oprM* can be prevalent in *P. aeruginosa* isolates resistant to carbapenems, ciprofloxacin, levofloxacin, and triclosan (Chuanchuen *et al.* 2001; Goli *et al.* 2016; Pan *et al.* 2016). In anaerobic digester reactors fed triclosan, relative abundance of *mexB* was increased compared to control digesters (Carey *et al.* 2016). In another study, 76% of MDR^t isolates showed overexpression of *mexB* (Goli *et al.* 2016). Attempts were made to amplify *mexB* and other ARGs, and *mexB* amplified in one isolate only. In the current study, it is unclear why the

designed primers and touchdown methods failed to amplify the targeted ARGs aside from *mexB*, or why *mexB* did not amplify in any of the other tested isolates. Previous studies have shown successful detection of *mexB* in clinical isolates with qPCR (Yoneda *et al.* 2005). The *mexB* sequences in sampled environmental isolates may vary from those aligned during the primer design, rendering the designed primers inadequate to assess presence/absence or quantity of *mexB* in these environmental samples in most cases. One *Pseudomonas spp.* isolate (FSx-34) from North Double Creek revealed the presence of *mexB*. Some examples of species showing matches to this *mexB* segment are *P. antarctica, P. brenneri, P. extremaustralis, P. fluorescens, P. mucidolens, P. orientalis, P. palleroniana, P. poae, P. trivialis, P. veronii* and *P. yamanorum (*NCBI). Detection of a multidrug efflux pump gene in a forested-stream bacterial isolate calls for additional study. Initially, more stream bacterial isolates should be tested. More extensive troubleshooting of PCR methods should be conducted, as well as development of quantitative PCR methods utilizing the FSx-34 *mexB* product as a DNA standard for the calibration curve.

It is possible that the small number of reference sequences (at the loci of the primers) used in each case did not happen to match well enough with sequences of environmental isolates chosen for initial testing; meaning, the designed primers were incompatible with sequences in isolates, even if genes were present. Of course, in some cases, the gene may not have been present and the lack of product is the correct result in those cases.

In diverse environments and with a complex assortment of antimicrobials, microbial species and resistance mechanisms, there exists the potential for enrichment of certain tolerant species and possibly bacteria harboring ARGs. Such occurrences in stream environments as well as many other environmental and clinical settings can increase difficulty of treating infectious disease. Additional discussion on these topics is found in Chapter I and Chapter IV.

CHAPTER IV

AIM 3: MULTIDRUG RESISTANCE AND SUSCEPTIBILITY OF STREAM BACTERIAL ISOLATES TO TRICLOSAN AND SELECTED ANTIBIOTICS

Abstract

Development and transmission of multidrug-resistant (MDR^t) pathogens is a major global health challenge. The potential for commonly used antimicrobials such as triclosan to increase levels of antimicrobial resistance and multidrug resistance (MDR) in environmental bacteria adds to this challenge. This study investigates antibiotic susceptibility profiles within two stream microbial communities and whether overall susceptibility is affected by exposure to 10 μg/L triclosan. It was predicted that triclosan exposure can impact susceptibility, not only to triclosan, but also to other antibiotics.

Wastewater-associated (WW-associated) and forested-stream microbial communities were hypothesized to have different overall susceptibility profiles. Susceptibility to triclosan and five antibiotics of different classes was assessed in isolates purified from these communities, with or without exposure to environmentally-relevant triclosan concentrations. Overall resistance and MDR was compared between triclosan-exposed and unexposed isolates and between WW-associated and forested-stream communities. Prior to experimental exposure to triclosan in microcosms, overall greater antimicrobial susceptibility was observed in the forested-stream community compared to the WW-associated community. Within the forested-stream microbial community, there was more overall resistance in the triclosan-exposed isolates compared to unexposed isolates. A higher proportion of isolates with 10% or less inhibition to 2

or more of the high doses of antimicrobial agents was observed in the exposed group of forested isolates compared to that in the unexposed group, indicating possible impacts of the triclosan exposure leading to increased resistance to multiple agents. No such significant difference between triclosan-exposed and unexposed isolates was noted for the WW-associated microbial community, where unexposed isolates showed high proportions of resistant isolates and MDR (3% or less inhibition to 3 or more agents). Triclosan is nearly ubiquitous in our surface waters and is an agent which has the potential to alter susceptibility patterns in stream environmental bacteria. Findings of the current study highlight the presence of MDR^t bacteria in both a forested and WW-associated stream, with more MDR in the WW-associated stream. Furthermore, some of the results draw links between environmentally-relevant triclosan exposure and possible increased antibiotic resistance and MDR in a forested-stream periphyton community.

Introduction

As a global community, we are confronted with numerous difficult to treat MDR^t pathogens, which can negatively impact our livelihood and survival. MDR^t bacteria have been increasing worldwide, and this trend is expected to continue. A contributing factor to this rising resistance is the over-utilization of broad spectrum antimicrobial agents such as triclosan, which bacteria often develop resistance to, sometimes in the process gaining co/cross resistance to additional antibiotics (Aiello *et al.* 2004; Braoudaki and Hilton 2004; Chuanchuen *et al.* 2001; Ciusa *et al.* 2012; Khan *et al.* 2016; McMurry *et al.* 1998; McMurry *et al.* 1999; Pi *et al.* 2017; Sanchez *et al.* 2005*;* Schmid and Kaplan 2004). Human practices can affect the development and transmission of bacteria and the resistance genes many of them carry. Each year, the global human community consumes roughly 70 billion standard units of antibiotics (Van Boeckel *et al.*

2014). Additionally, we use 63,151 ± 1560 tons/year for livest ock (Van Boeckel *et al.* 2015). It's been predicted that those numbers will increase by 30% in humans and by 67% in other animals within 15 years (Gelbrand *et al.* 2015). In addition to antibiotics used in human and veterinary medicine and animal husbandry, there is widespread use of broad spectrum antimicrobials. Triclosan is often found listed under other names such as Irgasan or Microban and is in thousands of products from antibacterial soaps and other personal care products to medical products and a wide array of plastic and textile goods. Triclosan is known to bioaccumulate in organisms, such as algae, fish, dolphins, and terrestrial animals (Chalew and Halden 2009; Coogan *et al.* 2007; Coogan and LaPoint 2008; Fair *et al.* 2009; Higgins *et al.* 2011; Miyazaki *et al.* 1984; Pannu *et al.* 2012; Tamura *et al.* 2013). Triclosan was the most commonly detected antimicrobial agent in a thorough investigation of surface waters and freshwater streams conducted by USGS (Kolpin *et al.* 2002). Samples tested from humans or from natural and engineered environments, typically contain detectable levels of triclosan (Bedoux *et al.* 2012; Benotti *et al.* 2009; Calafat *et al.* 2008; Gautam *et al.* 2014; Geer *et al.* 2017; Koeppe *et al.* 2013; Kolpin *et al.* 2002; Kumar *et al.* 2010; Mavri *et al.* 2012; Meeker *et al.* 2013; Miller *et al.* 2008; Pycke *et al.* 2014; Singer *et al.* 2002; Venkatesan *et al.* 2012; Weiss *et al.* 2015; Welsch and Gillock 2011; Xia *et al.* 2010; Ying and Kookana 2007).

There is a growing body of evidence indicating that the presence of commonly used antimicrobials in streams and at wastewater treatment plants (WWTPs) is a contributing factor to globally rising MDR. There are numerous avenues for transport of triclosan and other antimicrobials, MDR^t bacteria as well as antibiotic resistance genes (ARGs), into and around the environment where they can interact with various organisms including stream bacteria. MDR^t bacteria and ARGs have been detected in diverse environments (Graham *et al.* 2011; Jahne *et al.*

2015; Magalhães *et al.* 2016; Middleton and Salierno 2013; Osinska *et al.* 2016; Sapkota *et al.* 2006; Sayah *et al.* 2005; Schreiber and Kistermann 2013; Sjölund *et al.* 2008).

Genetic elements can be passed between different types of bacteria and viability of cells is not required for these elements to persist in the environment (Chee-Sanford *et al.* 2009). Studies have shown development of triclosan resistance and associated co/cross-resistance to other antibiotics in clinical isolates (Aiello *et al.* 2004; Braoudaki and Hilton 2004; Chuanchuen *et al.* 2001; Karatzas *et al.* 2007; Khan *et al.* 2016; McMurry *et al.* 1998; Pi *et al.* 2017; Sanchez *et al.* 2005*;* Schmid and Kaplan 2004). Regulatory mutations have occurred with triclosan exposure resulting in upregulation of multidrug efflux pumps (Chuanchuen *et al.* 2001). A study in New Jersey found high proportions (up to 89%) of MDR in highly triclosan-resistant isolates from WW-associated streams (Middleton and Salierno 2013). There is evidence that triclosan in the environment can alter antimicrobial resistance in stream periphyton bacteria (Drury *et al.* 2013; Lawrence *et al.* 2009; Nietch *et al.* 2013). In lab-scale anaerobic digester microbial communities, exposure to triclosan resulted in increases in *mexB*, a gene providing triclosan resistance (McNamara *et al.* 2014). Some bacteria have developed a multitude of mechanisms to combat antimicrobials, in some cases there are clusters of ARGs co-localized and ready to help the organism withstand a multitude of antimicrobial agents (Khan *et al.* 2016). For example, one observed ARG cluster included genes encoding: a triclosan-resistant enoyl-acyl carrier protein reductase, two multidrug efflux pump family proteins, and an aminoglycoside modifying enzyme (Khan *et al.* 2016). Triclosan-derived proliferation of MDR^t bacteria is a substantial threat to human and environmental health.

There are certain environments that can act as bioreactors for antibiotic resistance, such as WWTPs where a vast number of microbes and contaminants come together regularly. In

many cases, high levels of resistance and prevalence of diverse ARGs at/near WWTPs has been observed (Graham *et al.* 2011; Magalhães *et al.* 2016; Middleton and Salierno 2013) and higher triclosan signatures are generally found at WWTPs (Coogan *et al.* 2007; Venkatesan *et al.* 2012; Ying and Kookana 2007). Through an antibiotic risk assessment approach which reviewed multiple studies on antimicrobial resistance and environmental levels of common antibiotics and antimicrobials, triclosan was deemed the antimicrobial of greatest concern (Scott *et al.* 2016). Based on the excessive risk associated with ratios of measured exposure concentrations (MEC) to concentrations not known to affect organisms (the No observed effect concentration or NOEC) (MEC/NOEC > 1), monitoring this CEC in future studies was strongly recommended (Scott *et al.* 2016). Considering constantly evolving bacterial strains and genetically acquired resistance, our increasing knowledge on rising MDR, and the ability for different types of bacteria to exchange genetic material, there are major concerns surrounding impacts of anthropogenic antimicrobial pollutants such as triclosan on susceptibilities in stream microbial communities.

This study compares responses to triclosan exposure in microbial communities from two streams in the North Carolina Piedmont: one urban, WW-associated stream, North Buffalo Creek, and one non-urban, forested stream, North Double Creek. The forested-stream community was expected to be more sensitive to antibiotics than the WW-associated stream. This expectation led to the hypothesis that the microbial communities differ in their ambient level of antimicrobial resistance. Based on this hypothesis, it was predicted that there would be different responses to triclosan exposure from the WW-associated and forested-stream microbial communities. It was further predicted that more pronounced effects such as increased resistance would occur in the forested-stream community following triclosan exposure.

Triclosan resistance and MDR were hypothesized to be associated with triclosan exposure. Triclosan-resistant isolates were predicted to be more likely to show MDR. Susceptibility to triclosan and five antibiotics was assessed in isolates sampled from the two communities both before exposure and after microcosm exposure to 10 μg/L triclosan

Methods

Study streams, collection sites and periphyton colonization of tiles in streams

Stream and collection site locations and associated details, as well as methods for colonization of periphyton on stone tiles are described in detail in the methods section of Aim 1.

Exposure to triclosan in microcosms

Laboratory microcosm experiments were conducted at the University of North Carolina at Greensboro in the Department of Biology and microcosm set up is described in detail in the Methods section of Chapter II. Unexposed (0 hr) isolates were collected for pure culture isolation prior to addition of triclosan to microcosms. Exposed isolates were collected from microcosms at varying timepoints after addition to triclosan to microcosms (2, 11, 33 hours; 1, 2, 3, 4 weeks; with water replaced each week including a fresh addition of triclosan) and isolated in the manner described below. Analyses described in study results group isolates as unexposed or exposed to 10 μg/L ambient triclosan in microcosms and by periphyton source stream.

Bacterial strains and culture conditions

Isolate purification, antibiotic susceptibility testing and microbiological analysis for isolate identification were conducted aseptically after growth on or in sterile, low-nutrient agar or broth. Random selection of stream bacterial isolates and culturing methods are described in detail in the methods section of Chapter III. For susceptibility tests, 2 ml liquid cultures in 50%

MH broth in sterile, glass test tubes were cultured overnight in a shaker incubator at 200 rpm at 25°C.

Susceptibility tests

Pure cultures of stream bacteria were used for susceptibility testing with 6 agents: triclosan, carbenicillin, chloramphenicol, trimethoprim, erythromycin, and ciprofloxacin. Two doses of each agent were tested, in each case the high dose was 8-fold the concentration of the low dose. Low doses for each agent are as follows: 0.125 μg/ml Triclosan, 0.25 μg/ml Carbenicillin, 0.5 μg/ml Chloramphenicol, 1 μg/ml Trimethoprim, 0.625 μg/ml Erythromycin, and 0.125 μg/ml Ciprofloxacin. High doses for each agent are as follows: 1 μg/ml Triclosan, 2 μg/ml Carbenicillin, 4 μg/ml Chloramphenicol, 8 μg/ml Trimethoprim, 5 μg/ml Erythromycin, and 1 μg/ml Ciprofloxacin. Doses were chosen based on published Epidemiologic Cutoff (ECOFF) values (EUCAST 2016), with the aim of selecting doses that were near published minimum inhibitory concentration (MIC) values and at intermediate levels that would inhibit some isolates and be tolerated by others. Clinical Laboratory Standards Institute methods (Wikler *et al.* 2009) were used as guidance and modified as follows. Broth microdilution tests for susceptibility to antimicrobial agents were performed using 50% MH broth. Overnight cultures were diluted with sterile 50% MH broth to OD_{600} ≈0.257 to mimic a number 1 McFarland standard (approximately 3×10^8 CFU/ml). Isolate cultures were diluted to the range of $0.274 > OD_{600} > 0.240$ immediately before the start of the susceptibility assay.

Broth microdilution assays for susceptibility were conducted by preparing solutions in 96-well plates with a final well volume of 250 μL, 2% DMSO in 50% MH broth, and antimicrobial agent at the dose being tested. Antimicrobial solutions were added to 96-well plates in triplicate and at two concentrations for each antimicrobial agent tested. Vehicle only (2% DMSO in sterile

50% MH broth) was the negative control, and DMSO content was fixed at 2% in all wells. In a given culture well, 50 μL of a 24-hr 2 ml liquid culture of a single colony isolate (adjusted to OD_{600} ≈0.257 as described above) was added to the plate and grown at 25°C at 200 rpm for approximately 18 hours. Turbidity at 600nm (OD_{600}) was measured with a BioTek SynergyH1 microplate reader. To correct for background due to absorbance of the antimicrobial agent, the mean OD_{600} for each treatment without addition of bacteria (broth, antimicrobial agent at the given concentration, 2% DMSO) was subtracted from the mean OD_{600} of treated wells. This calculates the difference in absorbance due to the bacterial growth in presence of agent (Δ Agent). Turbidity of vehicle only wells (broth, 2% DMSO) was subtracted from that of wells containing bacterial culture and DMSO only to get absorbance of bacterial growth without agent (Δ Vehicle). The final calculation for % inhibition is [(Δ Vehicle - Δ Agent) / Δ Vehicle]*100.

Any isolate response showing more growth in wells containing an antimicrobial agent as compared to the vehicle-only liquid culture wells had their % inhibition values set to 0% inhibition. Any calculations that came out greater than 100 were set to 100% inhibition. % inhibition reported for each isolate in the study is the average of 3 wells and calculated as the percentage of growth prevented by the tested dose of antimicrobial agent.

Statistical analysis

There was not a significant trend associated with exposure time that would suggest increased effects on susceptibility over longer exposures in the course of this study. Therefore, analyses described in study results group isolates as unexposed or exposed to 10 μg/L ambient triclosan in microcosms and by periphyton source stream.

Mean percent inhibition in the four treatment groups was compared via 2-way ANOVA for each dose of each agent. The four treatment groups are: Unexposed Forested, Exposed

Forested, Unexposed WW-associated, and Exposed WW-associated. There were six agents each with two doses tested, so there are twelve 2-way ANOVAs run in total. 2-way ANOVAs and all t-tests were conducted utilizing statistical calculators (Lowry 2018). Results of t-tests comparing mean % inhibition were reported in cases where the 2-way ANOVA comparison showed no significant stream or exposure effects but the t-test for independent samples conducted comparing unexposed/exposed groups within one stream were significant. Paired t-tests were conducted comparing all isolate responses to the high dose of a particular agent to responses to the low dose of that agent. To compare proportions of isolates completely resistant to the given dose, isolates with low susceptibility to two or more agents, and MDR^t isolates, a Z Score Calculator for 2 Population Proportions was conducted using the Social Science Statistics calculator (Stangroom 2018).

Results

Susceptibility profiles of all 144 isolates revealed that the assortment of isolates sampled and tested for susceptibilities to six antimicrobial agents have varying responses to these agents. In many cases isolates were tolerant to more than one of the agents tested. Two thirds of the isolates resistant to triclosan (0-3% inhibition by 1 μg/ml) were also resistant to one or more of the other agents tested (Fig. 8). Identification of isolates revealed that a substantial number of pseudomonads were sampled (Table 4). Though these also showed variable susceptibility profiles, many were resistant to multiple antimicrobial agents.

Comparisons of mean % inhibition of treatment groups by each agent

Comparisons were made using 2-way ANOVAs comparing mean % inhibition in the four treatment groups (unexposed/exposed forested/WW-associated) by each dose of each agent.

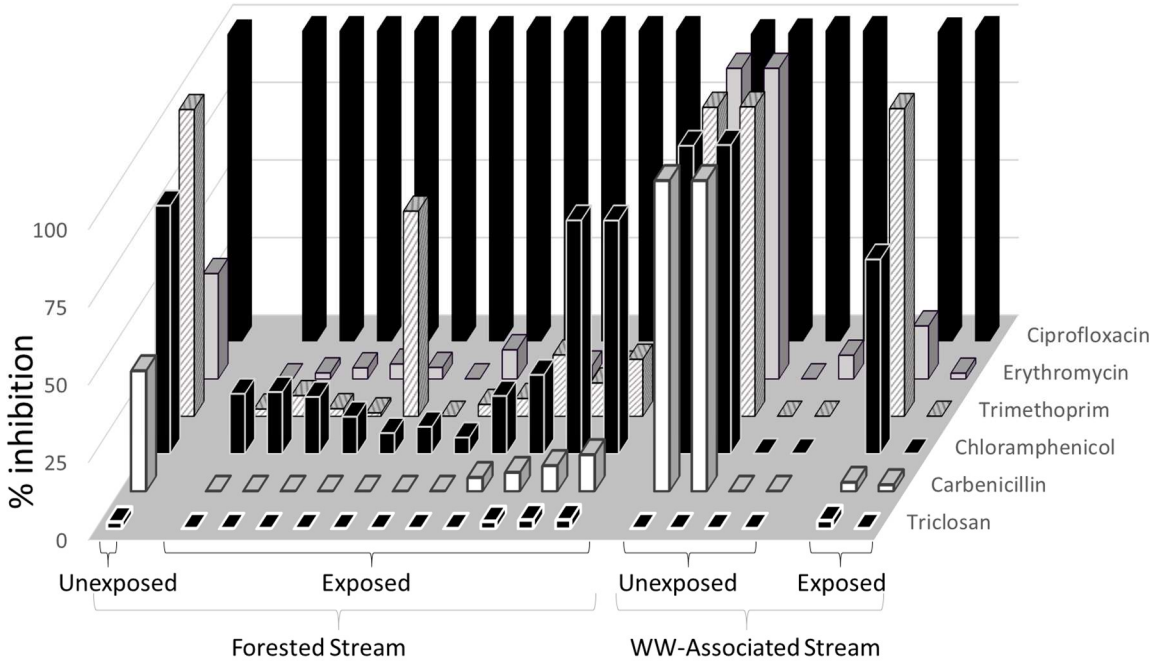

Figure 8. Percent Inhibition Profiles of Triclosan-resistant Isolates. Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the high doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics). Only isolates in the study which showed less than or equal to 3 percent inhibition by 1 µg/ml triclosan are shown.

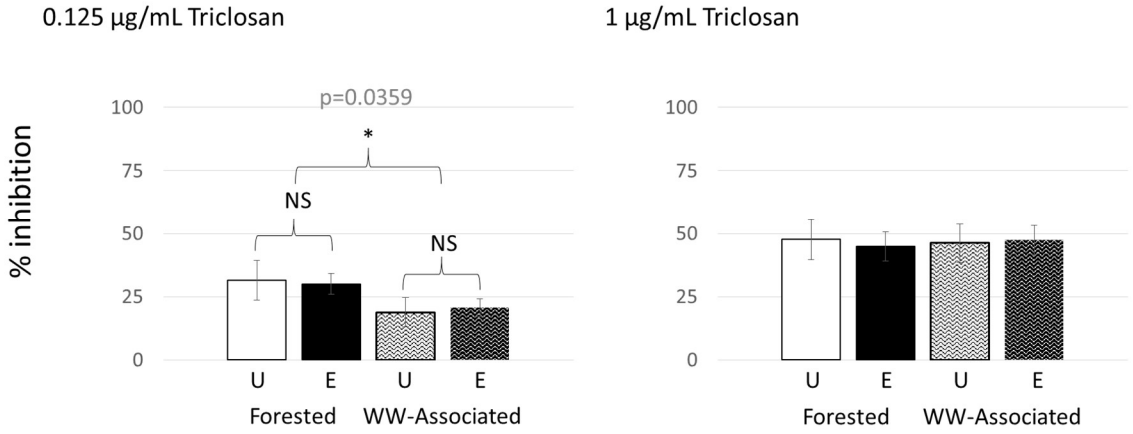

Figure 9. Mean Percent Inhibition by Triclosan. Figure shows mean % inhibition of each treatment group: unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested U: n=25; E: n=49) or North Buffalo Creek (WW-associated U: n=24; E: n=46). NS indicates no significant difference between means in these groups, while * indicates a significant difference and the p-value from 2-way ANOVA analysis is shown.

In susceptibility assays with 0.125 μg/ml triclosan, forested isolates were more susceptible overall compared to WW-associated isolates (p=0.0359), regardless of exposure (p=1; Fig. 9A). This was not the case with the higher dose of triclosan (1 μg/ml), in which there was no overall difference in susceptibility between streams (p=0.8627) or exposure status (p=0.8877; Fig. 9B).

There were no highly susceptible isolates (>50% inhibition) to 0.25 μg/ml carbenicillin in the WW-associated exposed group, and overall, there was no significant effect of stream (p=0.4399) or exposure (p=0.1495) in tests with this lower dose of carbenicillin (Fig. 10A). Susceptibility tests with 2 μg/ml carbenicillin showed significantly lower mean % inhibition in forested isolate groups compared to WW-associated groups (p=0.0232) but no effect of the microcosm exposure was noted (p=0.5666) (Fig. 10B).

Analyzing responses to chloramphenicol using 2-way ANOVA, there were not significant differences between streams (0.5 μg/ml chloramphenicol p=0.6398 Fig. 11A, 4 μg/ml p=0.6109 Fig. 11B) or exposure groups (0.5 μg/ml chloramphenicol p=0.1918, 4 μg/ml p=0.3553). However, t-tests on only the forested isolate groups show higher % inhibition in the unexposed group compared to the exposed group (0.5 μg/ml chloramphenicol p=0.036, 4 μg/ml p=0.019) indicative of higher resistance to chloramphenicol in the group that was exposed to triclosan in microcosms.

A similar trend was also noted in the forested isolate responses to trimethoprim, with the unexposed group showing significantly less resistance compared to the triclosan-exposed forested isolates when analyzed alone in a t-test (1 μg/ml Trimethoprim p=0.004 Fig. 12A, 8 μg/ml Trimethoprim p=0.025 Fig. 12B).

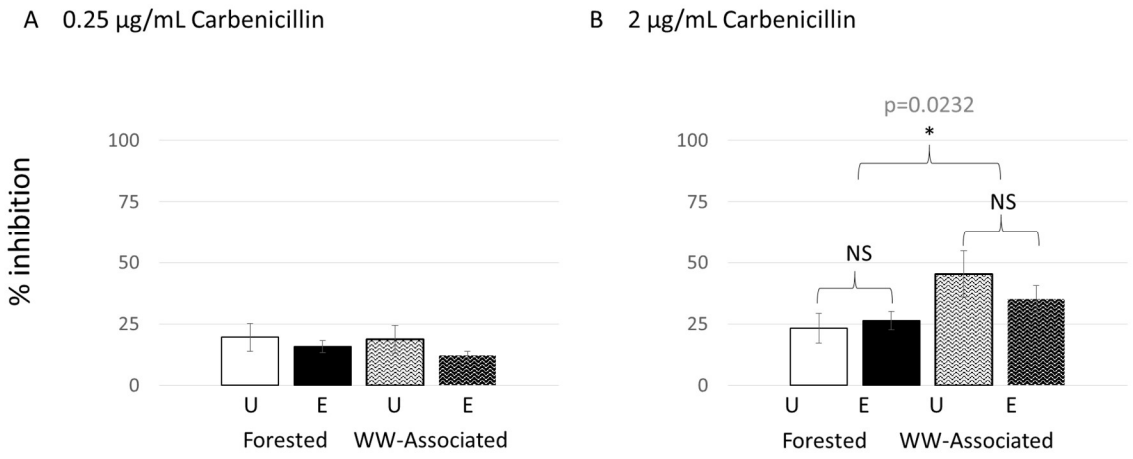

Figure 10. Mean Percent Inhibition by Carbenicillin. Figure shows mean % inhibition of each treatment group: unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested U: n=25; E: n=49) or North Buffalo Creek (WW-associated U: n=24; E: n=46). NS indicates no significant difference between means in these groups, while * indicates a significant difference and the p-value from 2-way ANOVA analysis is shown.

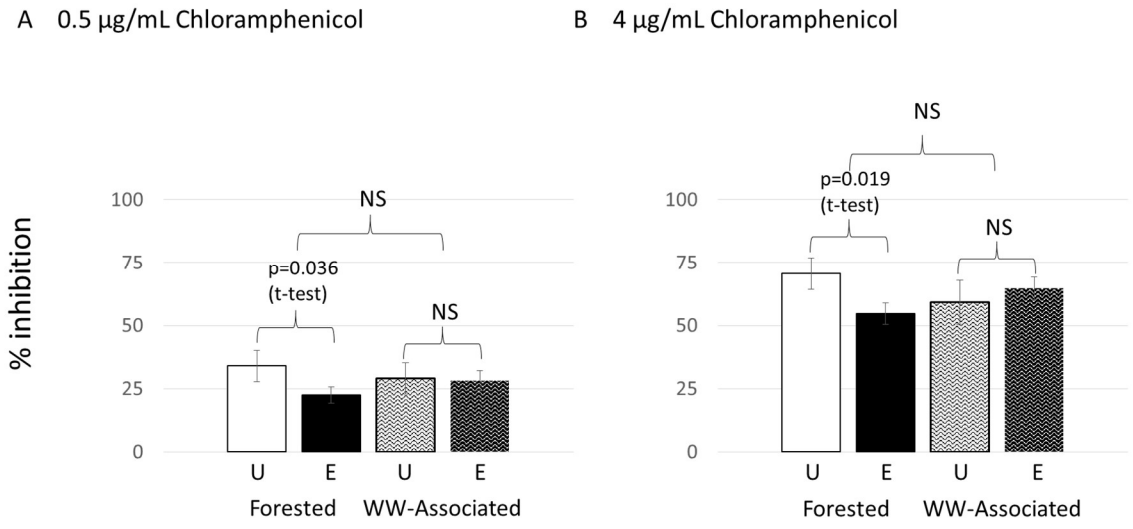

Figure 11. Mean Percent Inhibition by Chloramphenicol. Figure shows mean % inhibition of each treatment group: unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested U: n=25; E: n=49) or North Buffalo Creek (WW-associated U: n=24; E: n=46). NS indicates no significant difference between means in these groups. The given p-values refer to differences in unexposed forested isolates compared to exposed forested isolates via a t-test analysis.

When all four treatment groups are analyzed with 2-way ANOVA, no significant stream (1 µg/ml p=0.6898 Fig. 12A; 8 µg/ml p=0.1412 Fig. 12B) or exposure (1 µg/ml p=0.2094; 8 µg/ml p=0.1544 Fig. 12B) effects with these doses of trimethoprim were observed. However, there was a significant interaction effect with the lower dose (1 µg/ml Trimethoprim: Stream x Exposed p=0.015 Fig. 12A). The interaction effect occurred because the forested isolate mean % inhibition was lower in the exposed group but WW-associated isolate mean % inhibition was lower in the unexposed group.

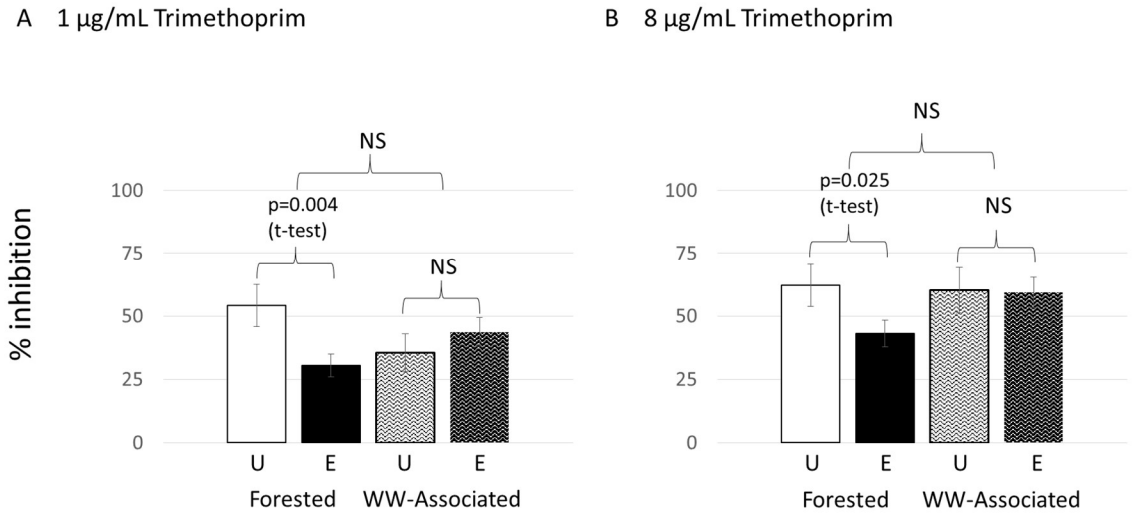

Figure 12. Mean Percent Inhibition by Trimethoprim. Figure shows mean % inhibition of each treatment group: unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested U: n=25; E: n=49) or North Buffalo Creek (WW-associated U: n=24; E: n=46). NS indicates no significant difference between means in these groups. The given p-values refer to differences in unexposed forested isolates compared to exposed forested isolates via a t-test analysis.

Isolate responses to 0.625 µg/ml Erythromycin showed higher mean % inhibition in the exposed forested isolates when comparing to unexposed forested isolates in a t-test (p=0.0134 Fig. 13A) but this trend was not repeated with the higher dose (p=0.225 Fig. 13B). There was no exposure effect in the WW-associated isolate groups. In comparisons between all treatment

groups via 2-way ANOVA, mean % inhibition differences were not statistically significant. Isolates from the WW-associated stream showed a trend toward higher mean % inhibition by 0.625 µg/ml Erythromycin compared to forested-stream isolates but it was not statistically significant (0.1 > p >0.05) (p=0.0505) and this trend was not repeated with the higher dose (5 µg/ml p=0.1737). 2-way ANOVA analysis did not uncover any exposure effects (0.625 µg/ml p=0.8877, 5 µg/ml p=0.093).

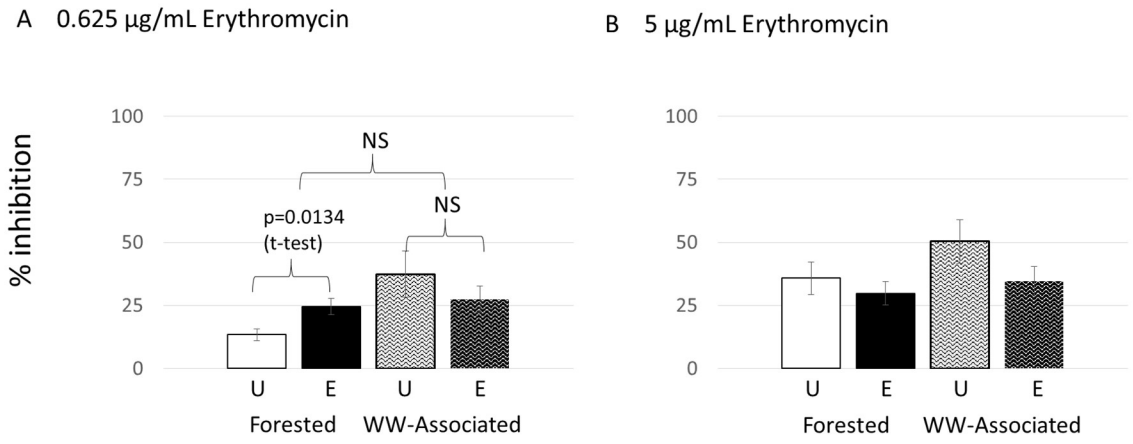

Figure 13. Mean Percent Inhibition by Erythromycin. Figure shows mean % inhibition of each treatment group: unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested U: n=25; E: n=49) or North Buffalo Creek (WW-associated U: n=24; E: n=46). NS indicates no significant difference between means in these groups. The given p-value refers to the difference in unexposed forested isolates compared to exposed forested isolates via a t-test analysis.

Ciprofloxacin doses used in this study more effectively inhibited isolates than doses of the other five agents. Tests with both doses of ciprofloxacin showed a significant exposure effect with higher mean % inhibition in the triclosan exposed groups triclosan (0.125 µg/ml Ciprofloxacin p=0.0395 Fig. 14A, 1 µg/ml Ciprofloxacin p=0.0056 Fig. 14B) suggesting that if the triclosan exposure affects susceptibility to ciprofloxacin, it does so in a counter-intuitive way, showing more ciprofloxacin susceptibility after exposure to triclosan. There were no significant

differences in ciprofloxacin susceptibility between the two streams (0.125 µg/ml Ciprofloxacin p=0.0702 Fig. 14A, 1 µg/ml Ciprofloxacin p=0.4851 Fig. 14B).

In paired t-tests, isolate responses to the high versus low dose of each antimicrobial agent were compared and generally, higher doses of agents were more effective at inhibiting growth than lower doses (triclosan p=0.008, carbenicillin p=0.057, chloramphenicol p<0.0001, trimethoprim p=0.022, erythromycin p=0.052, ciprofloxacin p=0.005).

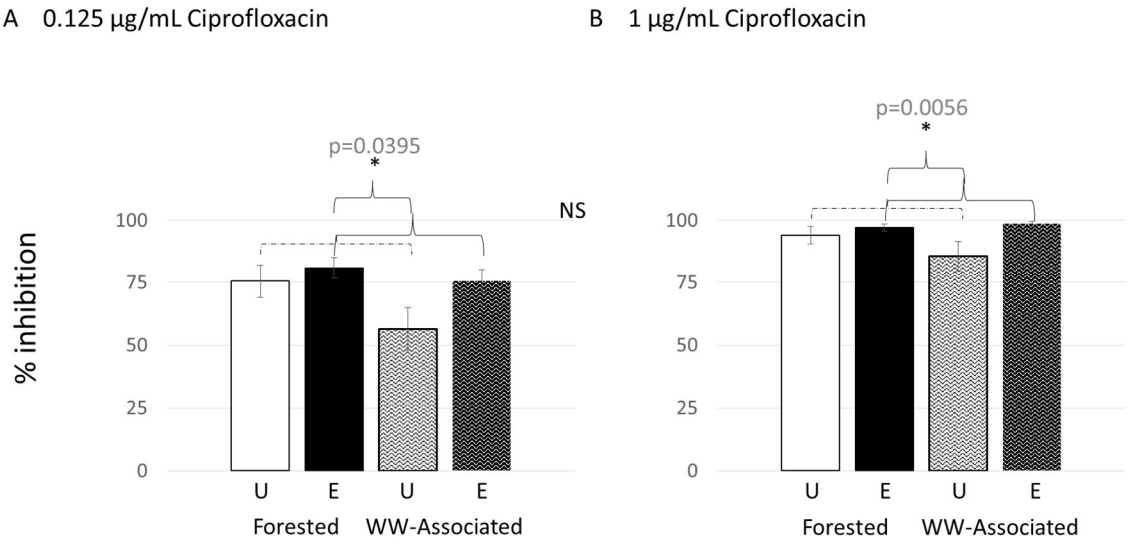

Figure 14. Mean Percent Inhibition by Ciprofloxacin. Figure shows mean % inhibition of each treatment group: unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested U: n=25; E: n=49) or North Buffalo Creek (WW-associated U: n=24; E: n=46). * indicates a significant difference and p-values from 2-way ANOVA are shown, here comparing unexposed vs exposed isolates (regardless of source stream).

Exposure effects on antibiotic resistance for each dose and stream

Proportions of isolates that showed more or equal growth in the presence of an antimicrobial agent (0% inhibition, thus isolates completely resistant to the given dose) within each treatment group were compared to one another. Comparisons between these proportions showed several noted differences between treatment groups based on exposure status or

stream source. The group of unexposed WW-associated isolates had a higher proportion (45.8% Fig. 15A) of isolates completely resistant to 0.125 µg/ml triclosan than exposed WW-associated group (17.4% p=0.011 Fig. 15A) and a similar pattern was also apparent in these isolates' responses to 1 µg/ml triclosan (unexposed: 16.7%; exposed 2.2%; Fig. 15A). The group of exposed WW-associated isolates had a lower proportion (2.2%) of completely resistant isolates to 1 µg/ml triclosan compared to exposed forested isolate group (16.3% p=0.019 Fig. 15A). The proportion of isolates able to grow as well or better with 1 µg/ml triclosan in susceptibility tests significantly differed between unexposed and exposed treatment groups in each stream (p=0.032 Forested, p=0.026 WW Fig. 15A). There were no unexposed forested isolates that showed complete resistance to the 1 µg/ml dose of triclosan, while in the exposed forested group, 16.3% of the isolates were completely resistant to this dose. Comparing proportions of completely resistant isolates between unexposed and exposed forested isolates with other agents did not reveal significant differences (Fig. 15B-F). WW-associated, unexposed isolates showed a trend toward high proportions of completely resistant isolates in all tests. These differences were significantly higher compared to the exposed WW-associated group in susceptibility tests with triclosan (Fig. 15A), 0.25 µg/ml carbenicillin (p=0.049 Fig. 15B) and 0.5 µg/ml chloramphenicol (p=0.003 Fig. 15C). Comparisons of proportions of 0% inhibited unexposed isolates sourced from the forested versus WW-associated stream show that among cultured, unexposed isolates, the WW-associated community showed higher proportions of completely resistant (0% inhibition) isolates than the forested community in susceptibility assays with triclosan (0.125 p=0.023, and 1 µg/ml p=0.033 Fig. 15A) and with 0.25 µg/ml carbenicillin (p=0.047 Fig. 15B) and 0.5 µg/ml chloramphenicol (p=0.008 Fig. 15C) and did not appear to be significantly different with other doses/agents (Fig. 15B-F).

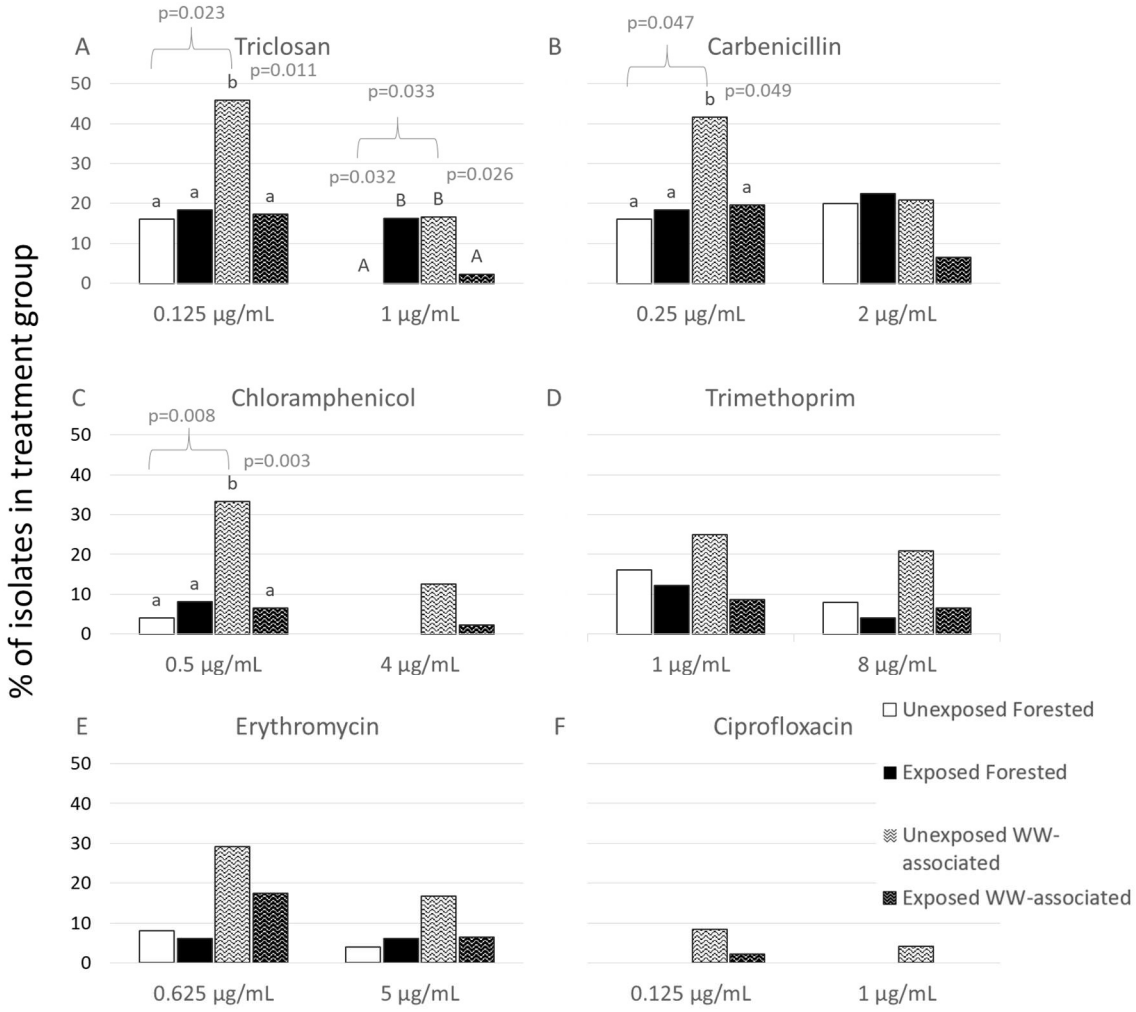

Figure 15. Percent of Isolates with 0% Inhibition. Figure shows % of isolates within each treatment group showing 0% inhibition to the given dose of agent. In each graph, white bars show unexposed forested isolates, black bars show exposed forested isolates, lighter patterned bars show unexposed WW-associated isolates, and darker patterned bars show exposed WW-associated isolates. Differing letters (a/A or b/B) above bars within the graph for a given dose of agent indicate significant differences between those groups' proportions (from z-test) with 2-tailed p-values shown. A brace pointing to a p-value is showing the comparison between the two groups below the two ends of the brace (not including the group in between).

Multidrug resistance (MDR)

Of all stream bacterial isolates in this study, 33.3% had low susceptibility (0-10% inhibition) to 2 or more of the higher doses and 58.3% exhibited 10% or less inhibition by 2 or more of the lower doses of antimicrobial agents. There were no significant differences between proportions of isolates in each treatment group showing low susceptibility to 2 or more of the low doses of antimicrobial agents (Fig. 16A). In response to the higher doses of agents, the exposed group of forested isolates exhibited a higher proportion (38.8%) of isolates with low susceptibility (0-10% inhibition) to 2 or more antimicrobial agents compared to that in the unexposed group (16%) (p=0.0455) (Fig. 16B) but there were no differences in susceptibility to 2 or more of the higher doses of agents due to triclosan exposure in the WW-associated isolates (p=0.7642).

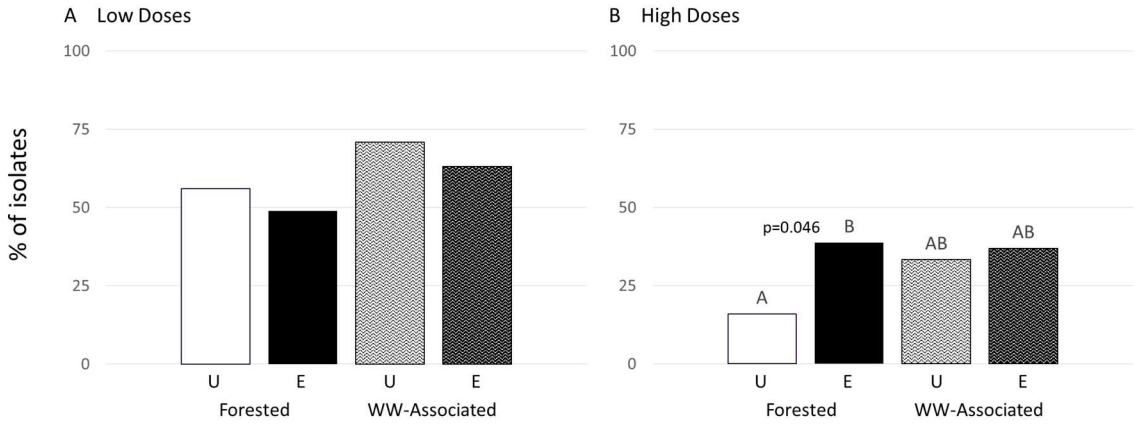

Figure 16. Percent of Isolates with Low Susceptibility to 2 or More Agents. Figure shows % of isolates within each treatment group exhibiting 0-10% inhibition by 2 or more of the given doses of antimicrobial agents. Bars represent treatment groups which were unexposed (U) or triclosan-exposed (E) isolates from North Double Creek (Forested) or North Buffalo Creek (WW-associated). Results from susceptibility assays with all low-dose agents are shown in the graph on the left (A), while results from high dose susceptibility assays are shown in the graph on the right (B). Comparisons of proportions were made through z-tests. Differing letters (A or B) above bars indicate significant differences between those groups' proportions, with the 2-tailed p-value shown.

Comparing proportions of MDR^t isolates showing 3% or less inhibition to 3 or more of the 6 agents, the unexposed WW-associated isolate group showed the highest proportions (29.2% with low doses Fig. 17A; 20.8% with high doses Fig. 17B) of MDR^t isolates. These proportions differed from the proportions in the exposed WW-associated group (8.7% MDR^t to low doses p=0.026 Fig. 17A; 4.4% MDR^t to high doses p=0.029 Fig. 17B). The difference in proportions of MDR^t isolates between unexposed isolates from the forested stream (0%) and WW-associated stream (20.8%) was more pronounced with high doses of antimicrobial agents (p=0.016) than with the low dose responses, where this difference was nearly significant (8.0% in forested versus 29.2% in WW-associated, p=0.05146).

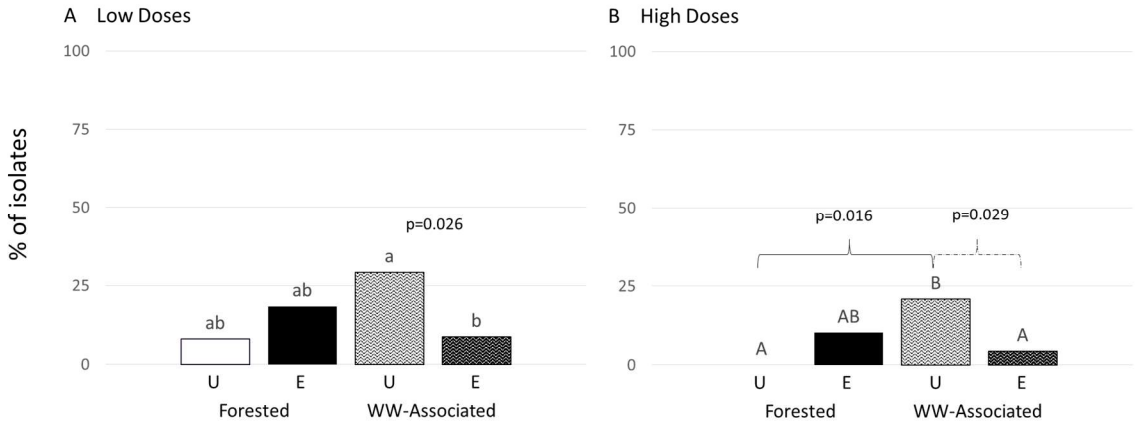

Figure 17. Percent of Isolates with Multidrug Resistance. Figure shows % of isolates in each treatment group showing 0-3% inhibition by 3 or more of the given doses of antimicrobial agents. Isolates were either unexposed (U) or triclosan-exposed (E) and from North Double Creek (Forested) or North Buffalo Creek (WW-associated). MDR results from susceptibility assays with all low-dose agents are shown in the graph on the left (A), while results from high-dose susceptibility assays are shown in the graph on the right (B). Comparisons of proportions were made through z-tests, each comparing only two groups. Differing letters (a/A or b/B) above bars indicate significant differences between those 2 groups' proportions, with the 2-tailed p-value shown.

Summary of results

In the sampled WW-associated microbial community, unexposed isolates showed as many or more instances of resistance and MDR compared to exposed isolates (Fig. 11, Fig. 12, Fig. 16B, Fig. 17B). While in the microbial community sampled from the forested-stream, there were more pronounced differences in antibiotic susceptibility with more instances of higher resistance and MDR^t isolates in the group of isolates collected from triclosan exposure microcosms revealing possible decreased susceptibility due to the triclosan exposure (Fig. 11, Fig. 12, Fig. 16B, Fig. 17B). Within the groups of isolates collected from the two streams that had no microcosm exposure to triclosan, high-dose MDR^t isolates (3% or less inhibition by high doses of 3 or more agents in this study) were collected from the WW-associated stream but not from the forested stream (Fig. 17B). There were low-dose MDR^t isolates collected from both streams (Fig. 17A). Susceptibility profiles (Figs. 18-27) and associated data tables for all isolates' % inhibition by the six agents tested are shown (Tables 9A-9D), as well as descriptive statistics and summaries of percent inhibition comparisons by ANOVA (Tables 10A-10L).

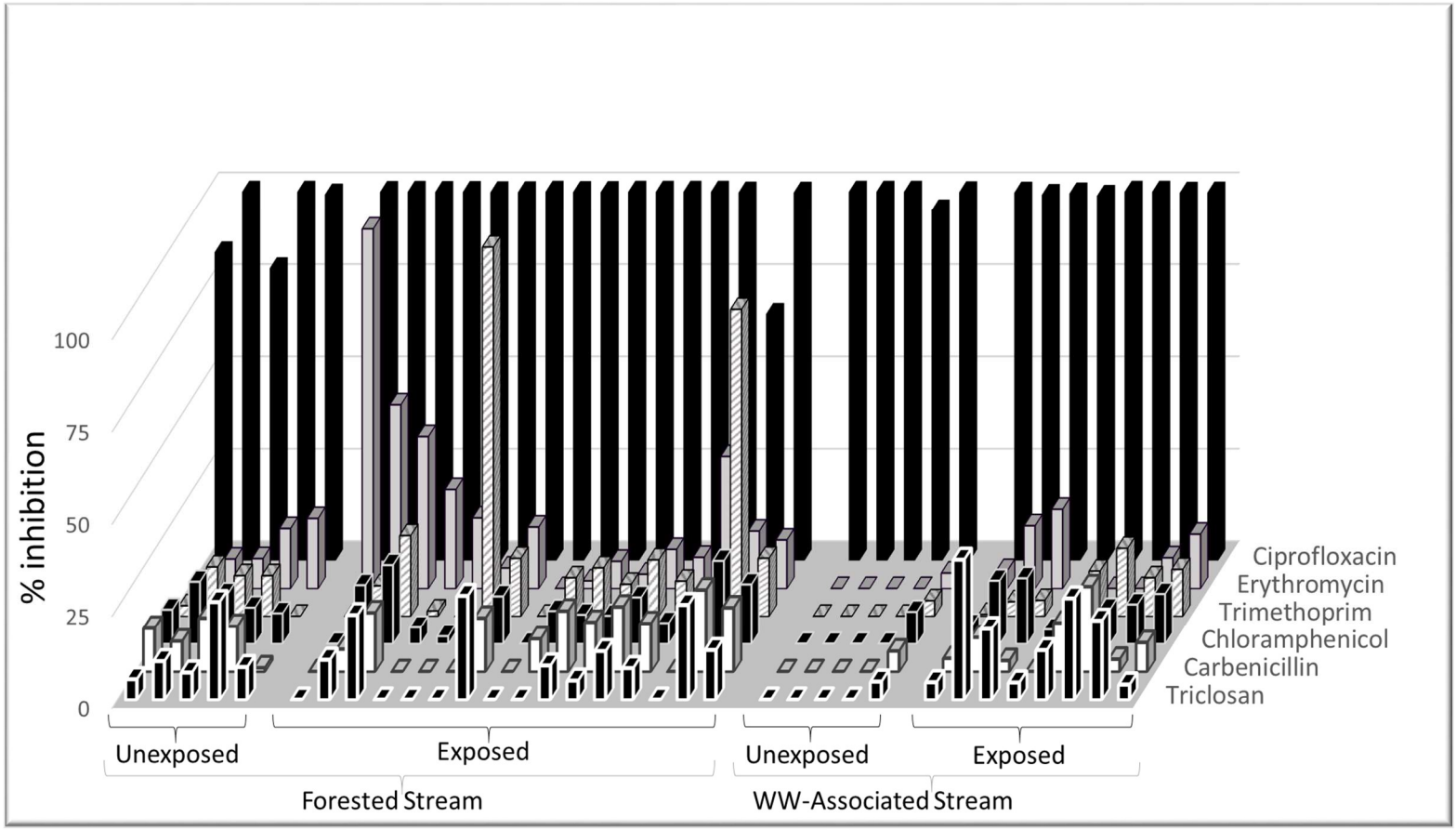

Figure 18. Susceptibility Profiles of Pseudomonads in Response to Low Doses of Agents.

Figure shows percent inhibition profiles of isolates identified to belong to the genus *Pseudomonas*. Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles shown here illustrate the isolates' % inhibition responses to the lower doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

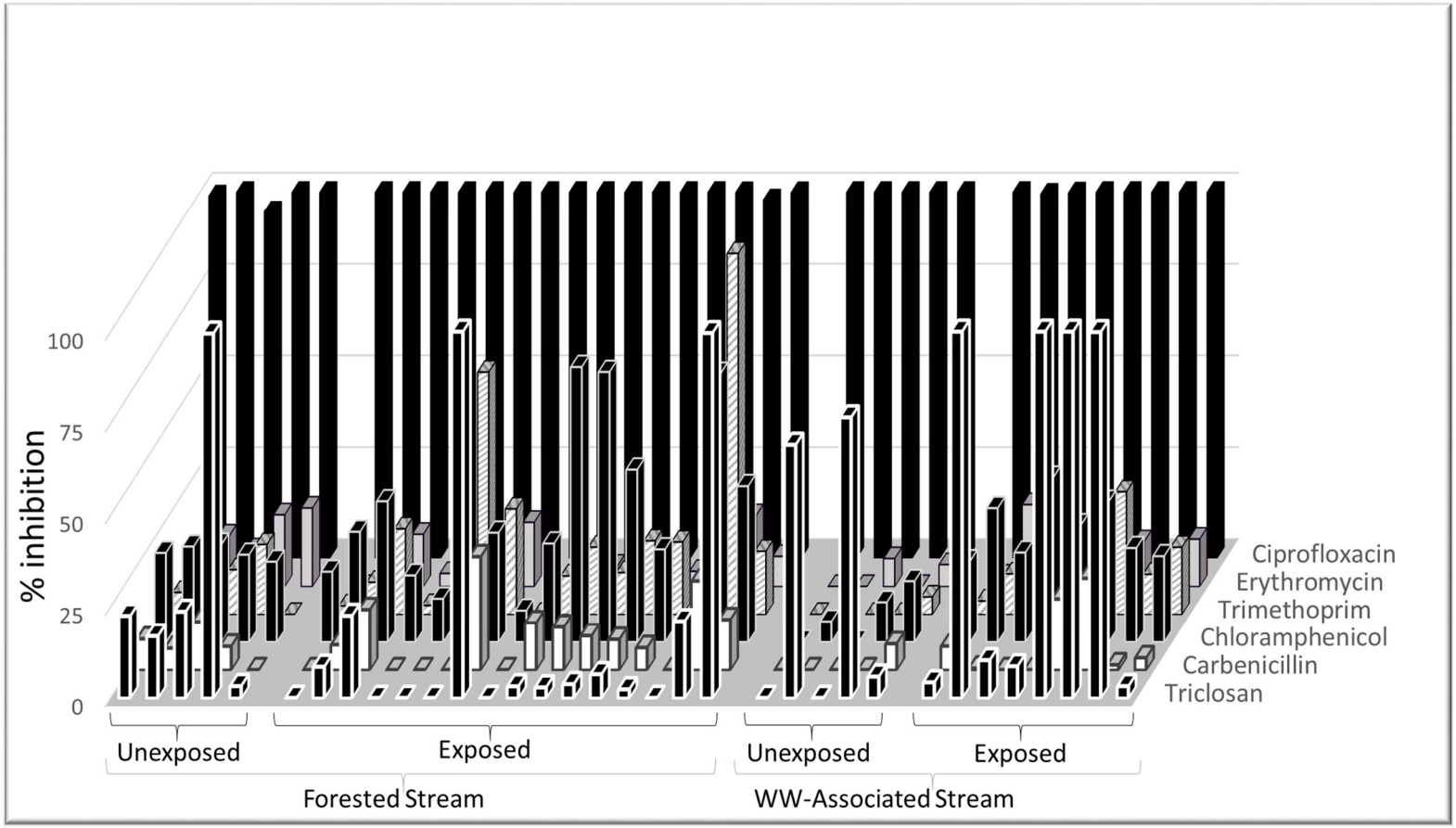

Figure 19. Susceptibility Profiles of Pseudomonads in Response to High Doses of Agents

Figure shows percent inhibition profiles of isolates identified to belong to the genus *Pseudomonas*. Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles shown here illustrate the isolates' % inhibition responses to the high doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

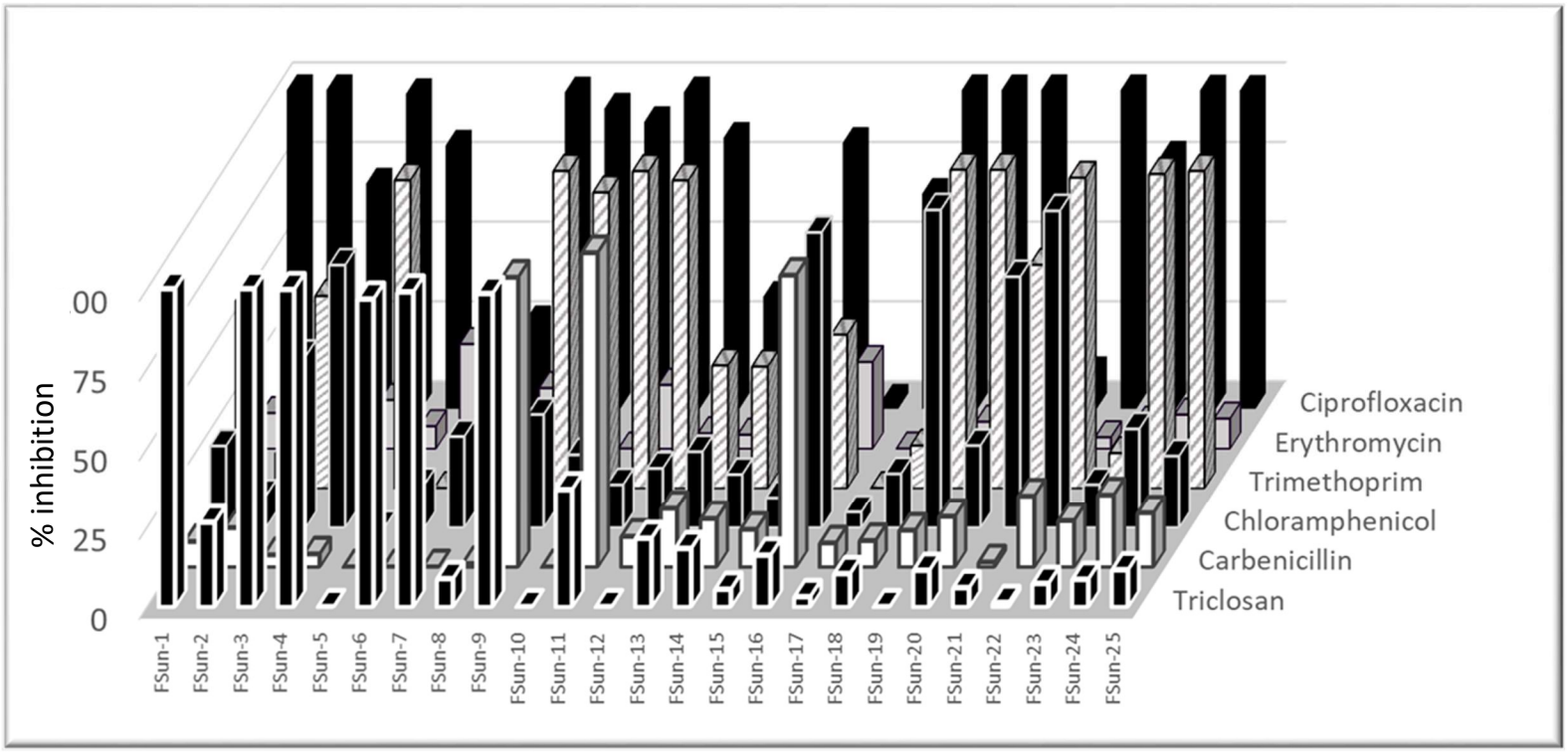

Figure 20. % Inhibition of Unexposed Forested Isolates by Low Doses of Agents

Figure shows percent inhibition profiles of all forested-stream, unexposed isolates in the study (n=25). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the low doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

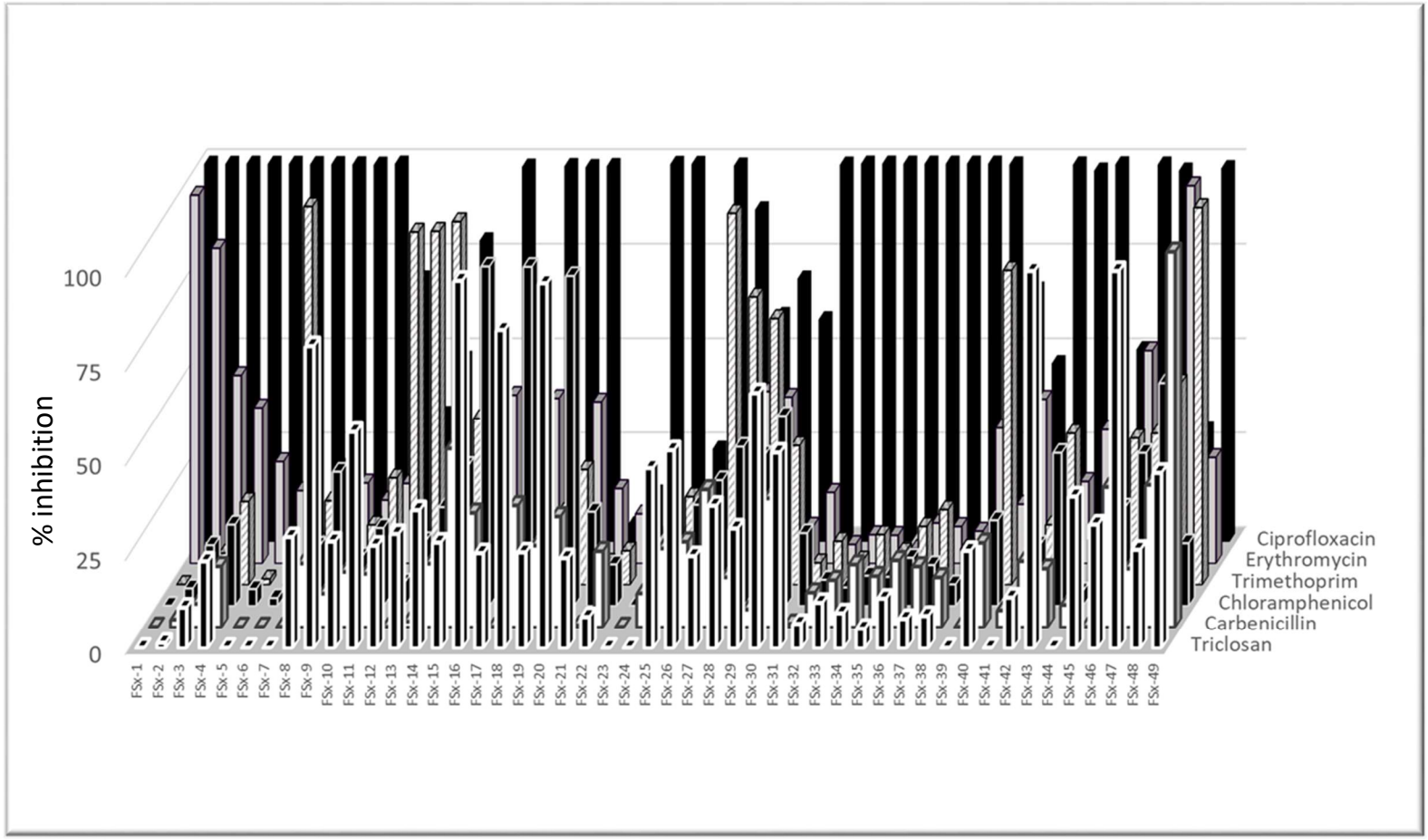

Figure 21. % Inhibition of Exposed Forested Isolates by Low Doses of Agents

Figure shows percent inhibition profiles of all forested-stream, triclosan-exposed isolates in the study (n=49). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the low doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

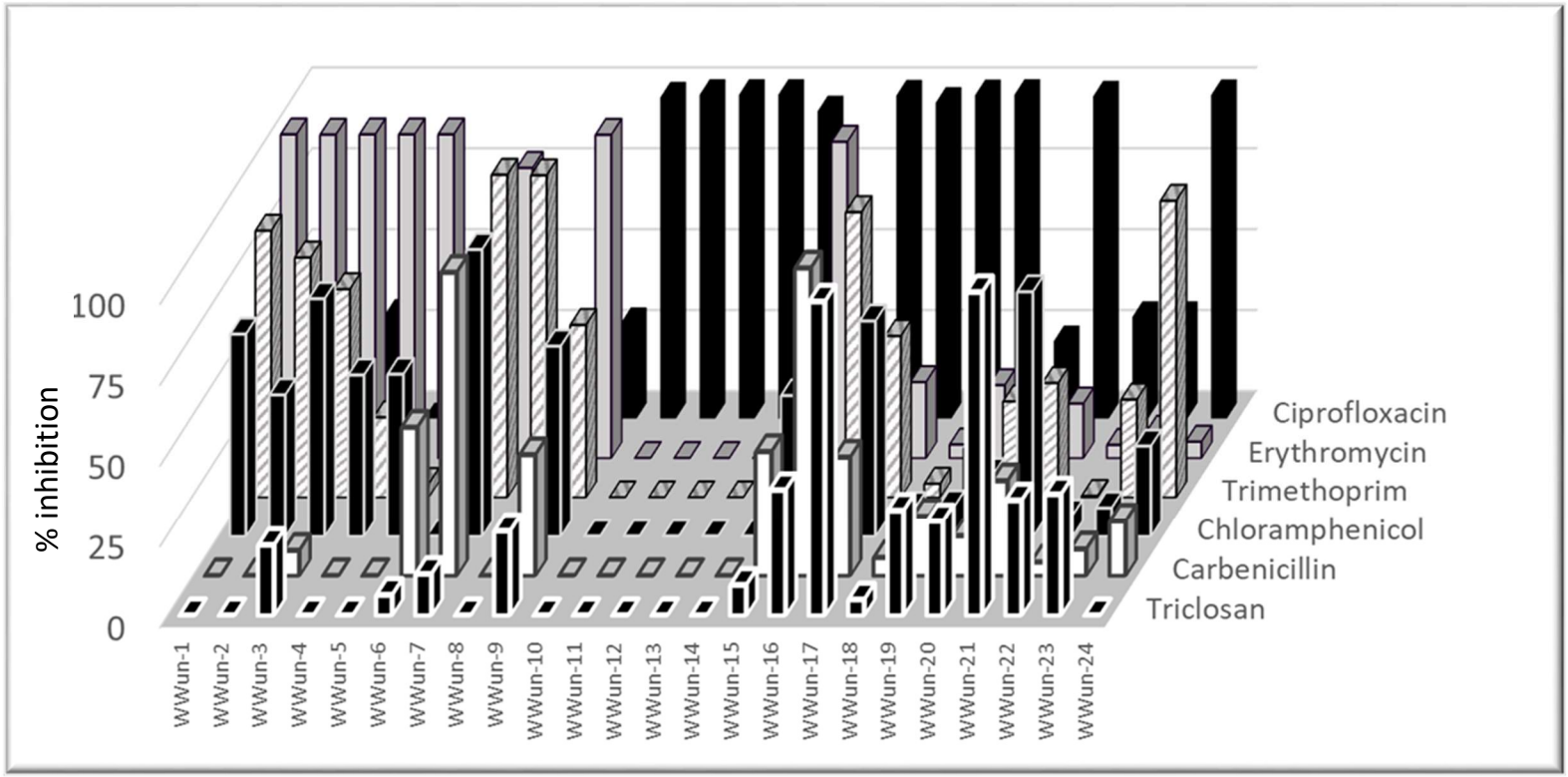

Figure 22. % Inhibition of Unexposed WW-associated Isolates by Low Doses of Agents

Figure shows percent inhibition profiles of all WW-associated, unexposed isolates in the study (n=24). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the low doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

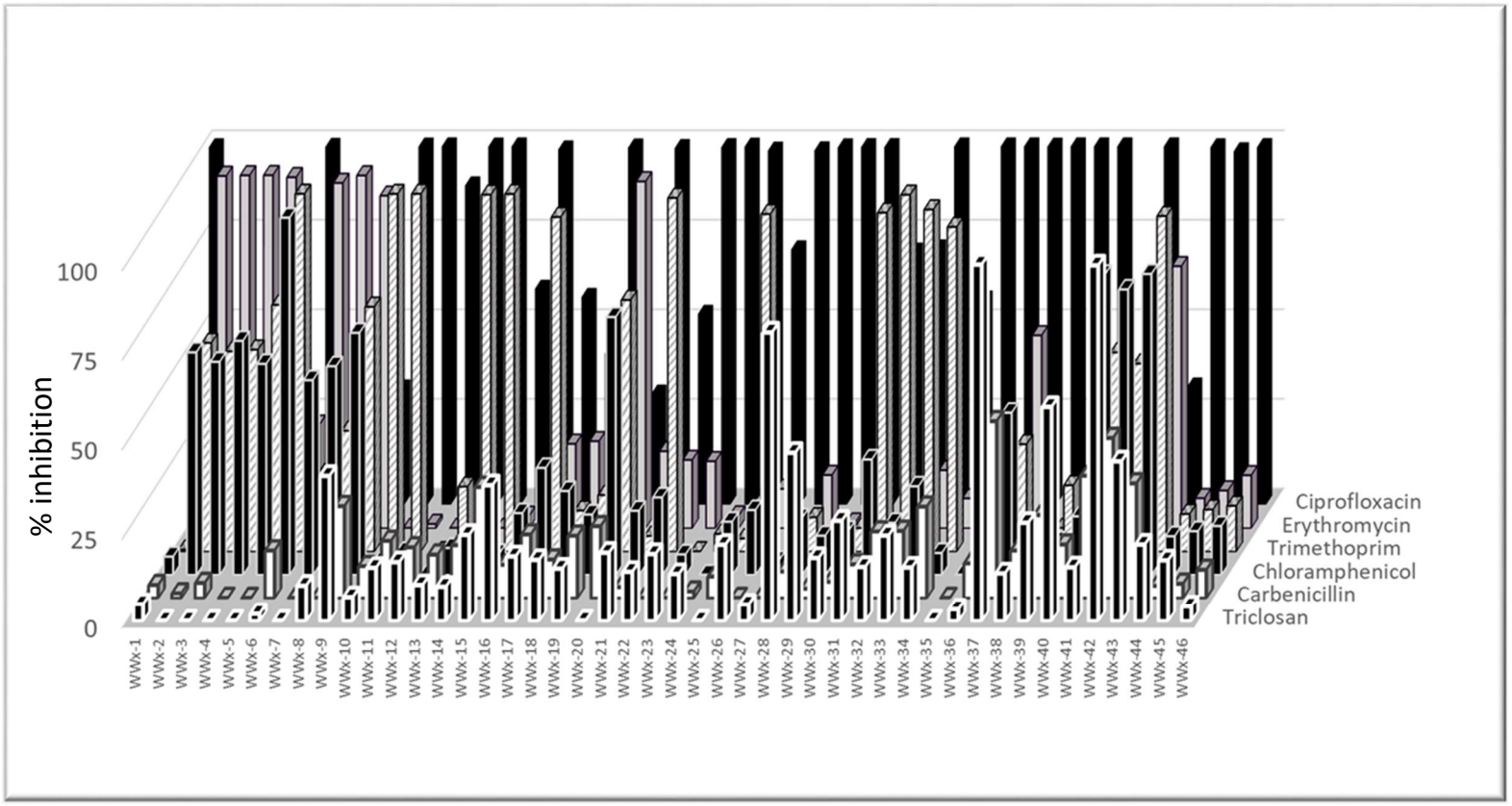

Figure 23. % Inhibition of Exposed WW-associated Isolates by Low Doses of Agents

Figure shows percent inhibition profiles of all WW-associated, triclosan-exposed isolates in the study (n=46). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the low doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

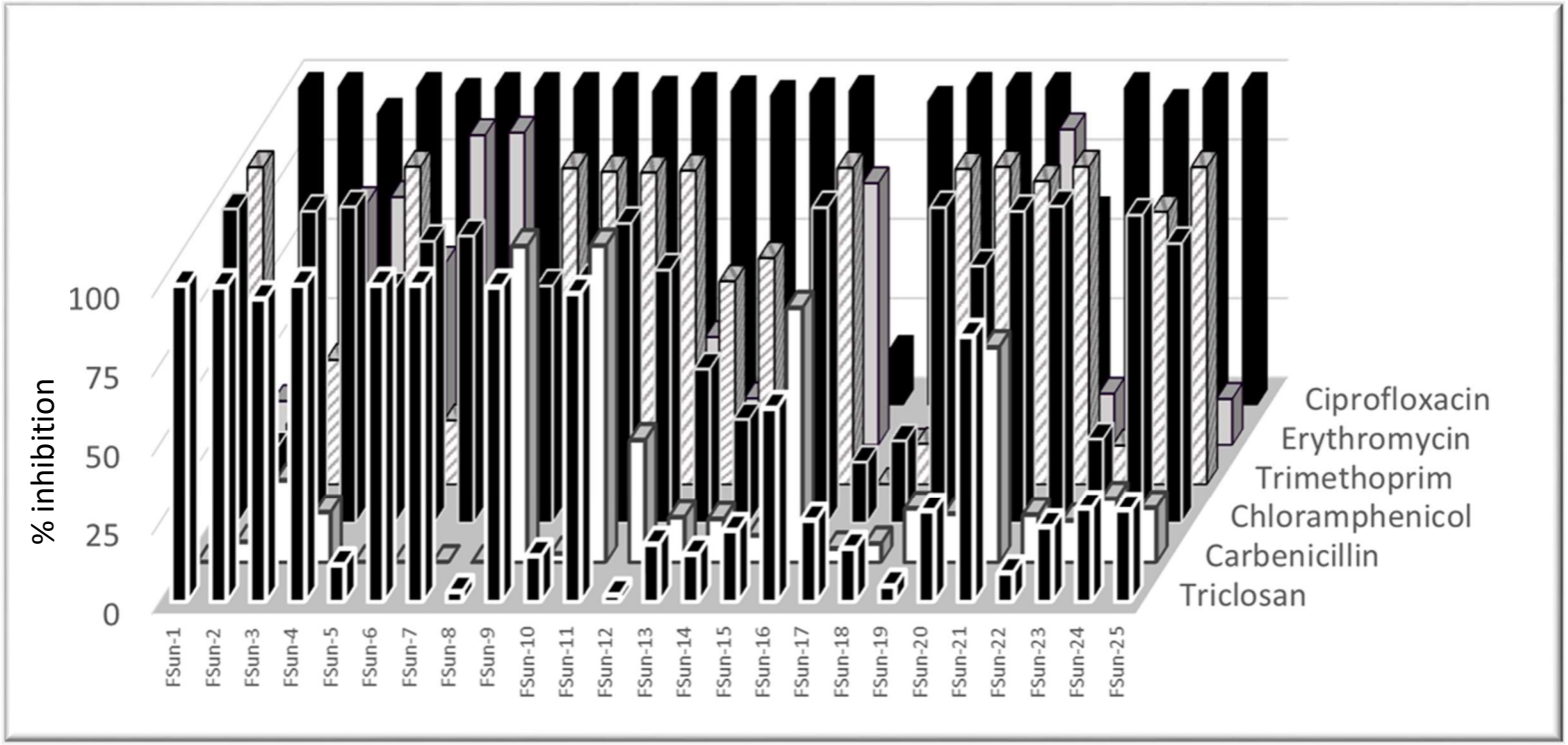

Figure 24. % Inhibition of Unexposed Forested Isolates by High Doses of Agents

Figure shows percent inhibition profiles of all forested-stream, unexposed isolates in the study (n=25). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the high doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

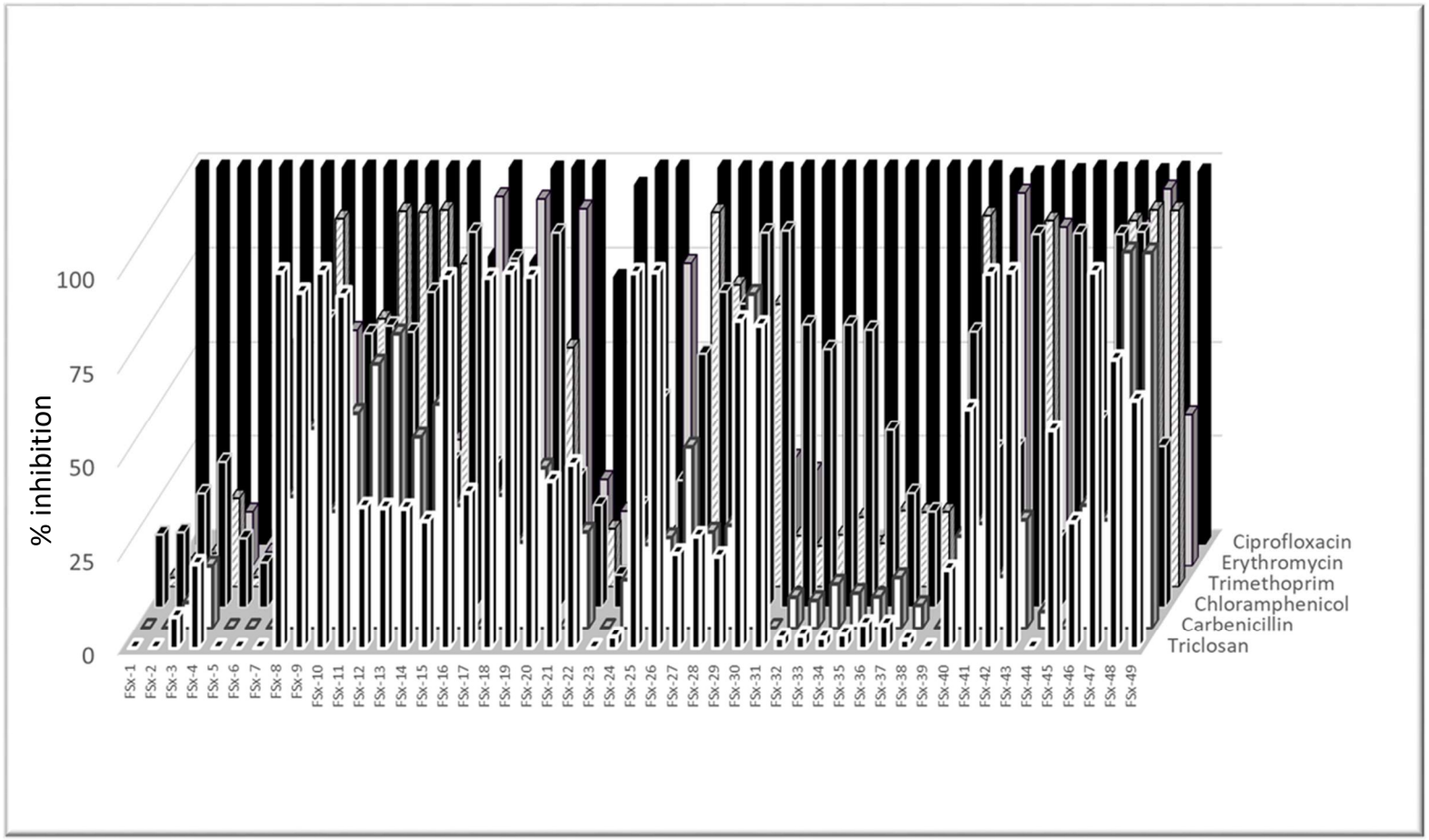

Figure 25. % Inhibition of Exposed Forested Isolates by High Doses of Agents

Figure shows percent inhibition profiles of all forested-stream, triclosan-exposed isolates in the study (n=49). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the high doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

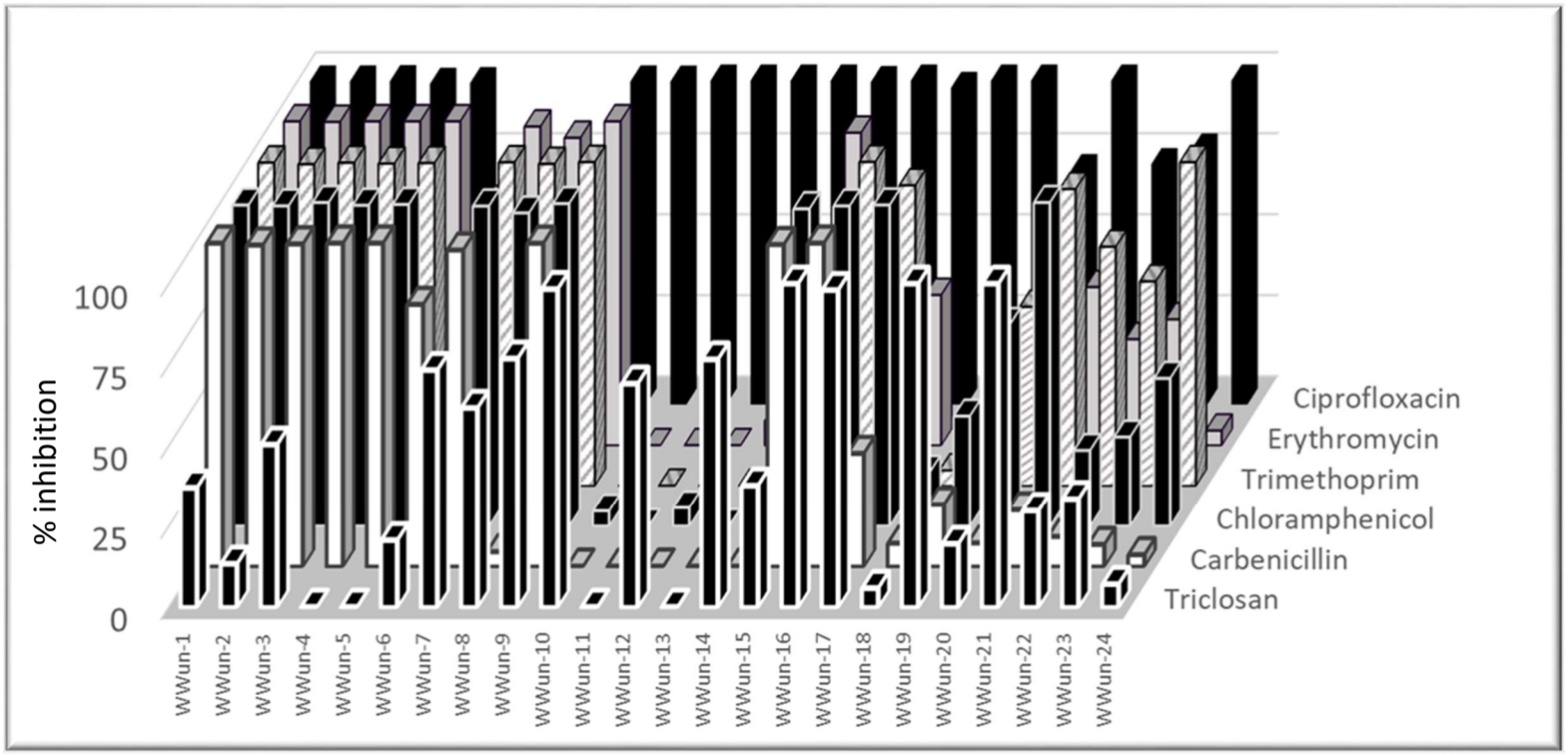

Figure 26. % Inhibition of Unexposed WW-associated Isolates by High Doses of Agents

Figure shows percent inhibition profiles of all WW-associated, unexposed isolates in the study (n=24). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the high doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

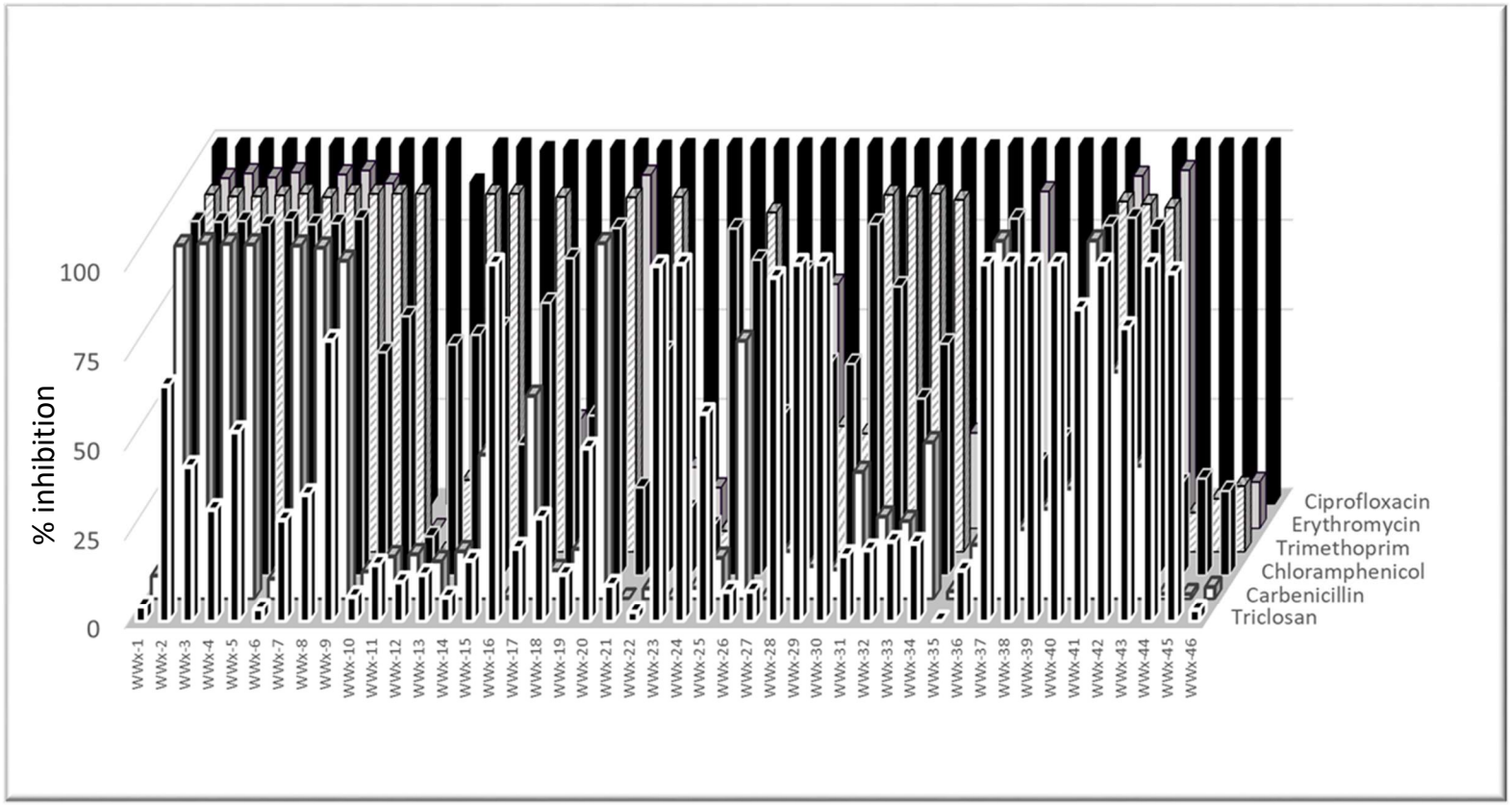

Figure 27. Susceptibility Profiles of Exposed WW-associated Isolates in Response to High Doses of Agents

Figure shows percent inhibition profiles of all WW-associated, triclosan-exposed isolates in the study (n=46). Each set of 6 bars extending into the z-axis represents a susceptibility profile of a single isolate. The susceptibility profiles in this graph show the isolates' % inhibition responses to the high doses of each of the 6 antimicrobial agents (triclosan and 5 antibiotics).

Tables 9A-9D. % inhibition data for all isolates

Table 9A. % Inhibition of Unexposed Forested Isolates by 6 Antimicrobial Agents
Columns headed with dose concentration show the mean % inhibition of that isolate (row) by that dose of agent (column) as calculated from 3 replicate wells. SE shows the standard error.

	Triclosan				Carbenicillin				Chloramphenicol				Trimethoprim				Erythromycin				Ciprofloxacin			
Isolate	0.125 µg/mL	SE	1 µg/mL	SE	0.25 µg/mL	SE	2 µg/mL	SE	0.5 µg/mL	SE	4 µg/mL	SE	1 µg/mL	SE	8 µg/mL	SE	0.625 µg/mL	SE	5 µg/mL	SE	0.125 µg/mL	SE	1 µg/mL	SE
FSun-1	100.0	0.3	100.0	0.1	8.0	0.4	0.0	2.6	25.7	3.2	99.1	0.2	58.9	0.6	99.8	0.0	11.2	2.1	13.7	3.8	100.0	0.1	100.0	0.0
FSun-2	26.5	3.0	99.5	0.1	12.3	1.9	6.4	1.0	9.9	5.3	23.9	2.5	11.0	5.1	19.1	4.0	16.3	3.2	19.5	2.3	100.0	0.0	100.0	0.0
FSun-3	100.0	2.9	95.7	1.4	3.8	4.3	26.1	15.2	53.8	14.7	98.4	1.9	60.3	8.2	39.1	5.2	7.6	5.6	76.1	13.2	70.7	16.0	91.8	0.5
FSun-4	99.8	0.1	100.0	0.1	4.1	3.8	15.8	1.7	82.5	1.3	99.8	0.1	19.3	3.4	4.2	2.3	15.2	3.3	78.0	1.4	98.8	0.4	100.0	0.0
FSun-5	0.0	3.6	11.6	6.8	0.0	3.4	0.0	19.9	0.0	8.8	74.0	3.8	96.7	0.7	100.0	0.1	7.0	5.0	57.3	2.9	82.6	4.9	98.1	0.5
FSun-6	96.8	1.6	100.0	0.2	0.0	1.3	0.0	4.4	13.6	4.3	88.9	0.8	0.0	6.8	20.1	10.4	32.8	1.4	97.5	0.2	16.9	6.5	100.0	0.0
FSun-7	99.0	0.7	100.0	0.2	0.0	3.0	0.0	0.0	28.7	5.2	90.6	0.2	0.0	7.6	29.0	10.4	42.6	1.8	98.3	0.0	27.5	4.7	100.0	0.2
FSun-8	8.8	2.8	3.2	3.9	1.0	1.7	0.0	2.4	8.3	4.9	22.0	2.2	0.0	5.5	0.0	2.8	19.0	3.4	21.4	4.3	99.2	0.1	100.0	0.0
FSun-9	98.5	0.2	99.4	0.0	91.2	1.3	99.8	0.1	35.6	1.6	74.9	2.3	99.6	0.0	99.5	0.1	22.3	3.0	21.4	0.9	94.2	0.4	100.0	0.0
FSun-10	0.0	0.4	14.4	3.1	0.0	8.6	2.7	4.2	22.9	0.8	76.5	1.4	92.9	0.4	98.4	0.2	0.0	1.0	9.6	9.3	89.9	0.6	98.8	0.1
FSun-11	36.9	2.0	97.4	0.5	98.9	0.9	100.0	0.1	13.5	1.2	94.5	1.8	99.5	0.2	98.2	0.1	20.0	2.8	15.7	5.7	99.3	0.3	100.0	0.2
FSun-12	0.0	7.4	2.1	9.5	9.6	12.9	38.7	6.2	18.7	0.3	79.9	2.9	96.6	1.3	98.8	0.8	4.8	4.5	33.9	10.4	85.0	4.4	98.9	0.3
FSun-13	21.5	3.9	18.1	1.0	18.4	3.5	14.2	1.6	23.9	5.3	48.7	3.6	38.6	2.8	63.9	4.2	4.3	0.6	14.6	1.0	35.1	2.7	97.5	0.9
FSun-14	18.3	2.4	14.9	2.3	15.2	4.0	13.6	2.0	16.7	0.7	32.9	2.1	38.2	0.5	71.2	1.0	3.8	0.3	22.1	0.4	43.6	2.0	98.3	0.4
FSun-15	5.5	0.5	22.4	4.0	11.8	3.1	8.4	0.6	9.4	2.6	24.4	2.3	2.9	1.3	6.0	0.3	2.0	0.7	7.4	0.3	83.4	0.7	99.0	0.2
FSun-16	16.2	1.8	61.2	2.6	91.8	3.8	80.4	10.8	92.8	0.9	99.5	0.4	48.2	2.2	99.6	0.1	27.2	3.7	82.3	4.2	2.8	4.6	17.2	2.3
FSun-17	3.2	0.8	25.7	0.4	7.5	4.7	4.3	3.1	4.8	0.9	19.4	2.6	0.0	2.2	0.0	2.3	0.0	0.8	0.0	0.4	67.4	1.3	95.6	0.4
FSun-18	10.4	2.4	16.9	0.6	8.3	0.2	5.9	0.7	16.9	1.8	26.1	0.4	13.4	0.5	12.7	0.3	8.0	2.5	13.8	0.7	100.0	0.0	100.0	0.0
FSun-19	0.0	6.2	5.0	3.0	11.5	3.7	17.0	3.6	99.8	0.1	99.5	0.0	100.0	0.0	99.3	0.6	8.4	1.4	33.0	1.4	99.9	0.0	100.0	0.0
FSun-20	11.4	2.8	28.7	1.0	15.9	2.3	15.3	0.5	25.9	1.1	81.1	0.1	99.9	0.1	99.9	0.1	11.2	0.3	18.0	0.8	100.0	0.0	100.0	0.0
FSun-21	6.0	1.4	83.9	2.2	1.6	1.6	68.0	2.8	78.9	1.9	98.3	0.1	70.1	6.4	95.5	3.6	37.8	3.2	99.3	0.2	11.5	5.2	60.8	2.7
FSun-22	1.3	1.8	9.1	1.6	22.4	1.9	15.0	6.1	99.5	0.1	99.9	0.0	97.4	2.1	100.0	0.1	3.5	3.4	16.1	0.6	100.0	0.0	99.9	0.1
FSun-23	7.3	2.9	23.6	1.7	14.6	0.5	13.2	1.2	13.5	1.0	26.6	0.8	11.0	0.3	12.2	2.6	8.1	0.4	10.0	0.5	79.1	0.5	94.6	0.5
FSun-24	8.6	2.1	29.5	1.2	22.7	3.0	20.2	1.7	31.1	1.8	97.2	0.4	98.6	0.3	85.8	10.7	10.6	1.1	20.3	0.3	99.9	0.1	99.9	0.0
FSun-25	11.6	0.2	28.9	2.2	17.0	1.5	17.2	0.5	22.5	0.5	88.2	0.4	99.6	0.1	99.9	0.1	9.4	0.8	14.4	2.1	99.8	0.1	100.0	0.0

Table 9B. % Inhibition of Triclosan-Exposed Forested Isolates by 6 Antimicrobial Agents

Columns headed with dose concentration show the mean % inhibition of that isolate (row) by that dose of agent (column) as calculated from 3 replicate wells. SE shows the standard error. The table is continued on the next page.

	Triclosan				Carbenicillin				Chloramphenicol				Trimethoprim				Erythromycin				Ciprofloxacin			
Isolate	0.125 µg/mL	SE	1 µg/mL	SE	0.25 µg/mL	SE	2 µg/mL	SE	0.5 µg/mL	SE	4 µg/mL	SE	1 µg/mL	SE	8 µg/mL	SE	0.625 µg/mL	SE	5 µg/mL	SE	0.125 µg/mL	SE	1 µg/mL	SE
FSx-1	0.0	3.6	0.0	2.6	0.0	1.4	0.0	2.1	0.0	1.4	19.3	1.3	0.0	1.3	2.3	2.1	97.4	1.2	0.0	2.4	100.0	0.0	100.0	0.1
FSx-2	1.3	1.9	0.0	0.9	1.9	2.3	0.0	3.3	4.6	1.4	19.9	2.5	1.2	1.8	6.6	3.2	83.4	9.6	1.9	1.8	99.9	0.0	100.0	0.1
FSx-3	10.8	1.9	8.5	1.2	6.1	2.0	6.8	0.8	16.0	3.7	30.2	1.2	8.3	0.7	8.8	0.3	49.7	9.6	4.6	2.9	100.0	0.0	100.0	0.1
FSx-4	23.0	4.4	22.5	2.9	16.0	2.4	16.5	0.1	21.4	1.1	38.5	3.1	21.9	2.0	23.4	0.8	41.1	6.0	14.2	2.5	99.9	0.0	100.0	0.0
FSx-5	0.0	1.1	0.0	0.3	0.0	0.8	0.0	1.1	4.4	0.9	18.3	1.1	1.4	1.1	2.4	0.3	26.8	3.1	3.5	0.9	99.9	0.0	100.0	0.1
FSx-6	0.0	1.4	0.0	2.8	0.0	0.6	0.0	0.9	2.0	1.0	11.9	1.1	0.0	1.2	1.1	1.6	19.1	1.3	4.8	1.1	99.9	0.0	100.0	0.0
FSx-7	0.0	1.7	0.0	1.5	0.0	1.3	0.0	0.5	0.0	0.5	6.6	0.5	100.0	0.1	66.1	33.9	5.6	0.8	3.7	1.3	99.9	0.1	99.9	0.2
FSx-8	29.4	13.1	99.9	0.1	16.8	2.1	34.9	2.3	15.9	3.2	36.9	1.9	22.1	0.6	35.4	3.7	20.0	4.0	26.6	1.5	99.8	0.1	100.0	0.0
FSx-9	80.0	0.7	94.6	0.2	8.8	0.3	53.0	0.8	35.4	2.5	76.6	0.8	33.5	1.2	97.6	0.2	21.2	2.4	62.5	1.1	99.7	0.0	100.0	0.0
FSx-10	28.2	14.3	100.0	0.0	14.6	3.9	31.0	0.3	13.0	2.4	29.9	3.8	15.7	0.8	28.8	2.1	16.7	4.6	17.5	0.1	100.0	0.0	100.0	0.0
FSx-11	57.2	20.1	93.9	0.2	14.1	3.9	57.2	0.5	20.5	4.0	72.8	1.4	28.1	5.5	71.1	1.2	21.0	7.7	45.3	1.1	69.7	2.5	99.9	0.0
FSx-12	27.1	26.2	37.6	2.7	0.0	2.1	70.2	2.8	6.1	3.9	74.8	3.3	93.3	0.1	99.6	0.1	0.0	1.9	35.7	7.1	33.8	3.4	99.9	0.1
FSx-13	30.2	25.2	37.3	4.7	1.4	1.3	78.2	2.9	17.1	0.6	73.2	2.3	93.5	0.4	99.4	0.1	2.1	2.4	33.8	5.8	48.4	4.3	99.8	0.0
FSx-14	36.5	26.4	37.2	4.2	16.7	0.5	50.7	1.0	26.0	2.3	83.7	2.4	96.0	0.1	99.9	0.0	10.9	3.6	33.4	1.3	79.7	1.2	99.9	0.0
FSx-15	27.9	0.9	33.9	3.0	47.2	1.5	59.6	0.5	37.0	1.2	39.0	1.7	43.9	1.9	85.8	1.1	15.9	0.4	45.0	5.0	32.7	16.7	76.5	3.6
FSx-16	97.2	0.3	98.7	0.1	30.1	2.1	32.6	4.1	89.8	0.7	99.7	0.1	26.4	2.7	67.8	0.6	44.5	0.6	98.0	0.5	99.2	0.3	100.0	0.1
FSx-17	25.1	5.7	41.3	4.7	0.0	0.8	0.0	0.4	21.8	2.4	38.1	1.0	4.9	3.6	8.0	5.2	22.8	0.4	23.4	4.6	0.5	1.6	74.9	0.8
FSx-18	84.2	13.3	98.5	0.2	32.1	3.7	35.3	3.6	89.7	0.6	92.6	7.1	30.3	2.2	75.5	1.6	43.6	0.4	97.3	0.2	99.3	0.1	99.7	0.4
FSx-19	25.3	0.5	100.0	0.0	21.5	3.2	22.8	2.4	19.4	0.3	32.5	0.5	19.2	2.3	23.7	2.4	9.9	0.6	10.1	0.5	99.1	0.1	100.0	0.0
FSx-20	96.6	0.1	98.8	0.0	29.2	5.7	42.5	1.1	87.3	0.6	99.4	0.0	30.3	0.6	63.5	3.2	42.7	0.8	94.7	1.4	99.4	0.1	100.0	0.0
FSx-21	23.8	5.5	44.5	2.4	0.4	0.8	0.0	1.9	24.8	2.0	34.8	4.3	5.9	1.7	9.5	2.9	19.7	3.0	22.8	4.9	4.5	2.2	71.0	1.0
FSx-22	8.2	3.7	48.8	3.3	20.0	2.3	25.5	2.0	10.9	1.9	27.1	2.3	8.9	1.7	15.3	0.9	13.0	1.0	14.4	2.1	12.9	5.5	95.4	0.5
FSx-23	0.0	0.3	0.0	2.6	0.0	0.8	0.0	2.0	0.0	0.2	8.6	0.5	0.0	3.3	0.0	1.1	0.0	0.3	0.0	9.6	99.9	0.1	100.0	0.0
FSx-24	0.0	5.9	3.2	2.5	8.9	2.6	12.9	1.6	9.0	1.1	27.0	1.6	10.5	1.0	10.5	1.5	2.1	0.4	5.6	1.3	99.9	0.0	100.0	0.0
FSx-25	47.6	5.0	99.8	0.1	20.7	1.9	22.2	2.0	14.5	1.4	55.7	1.1	23.2	0.4	13.8	5.6	8.8	3.2	80.3	2.0	24.5	1.3	44.5	0.8

Table 9B continued. % Inhibition of Triclosan-Exposed Forested Isolates by 6 Antimicrobial Agents
Columns headed with dose concentration show the mean % inhibition of that isolate (row) by that dose of agent (column) as calculated from 3 replicate wells. SE shows the standard error.

	Triclosan				Carbenicillin				Chloramphenicol				Trimethoprim				Erythromycin				Ciprofloxacin			
Isolate	0.125 µg/mL	SE	1 µg/mL	SE	0.25 µg/mL	SE	2 µg/mL	SE	0.5 µg/mL	SE	4 µg/mL	SE	1 µg/mL	SE	8 µg/mL	SE	0.625 µg/mL	SE	5 µg/mL	SE	0.125 µg/mL	SE	1 µg/mL	SE
FSx-26	52.3	0.4	100.0	0.1	22.5	6.2	24.0	5.9	26.7	3.5	33.7	0.8	16.6	5.8	27.9	3.0	4.3	2.4	3.6	2.1	99.5	0.4	100.0	0.3
FSx-27	24.4	2.1	25.2	4.8	36.3	2.9	48.0	2.5	33.2	1.5	67.4	1.1	98.2	0.1	99.3	0.2	18.1	0.5	21.9	2.8	87.9	1.3	99.8	0.1
FSx-28	37.6	6.3	29.8	2.8	13.2	3.6	25.5	6.4	42.0	1.4	83.8	0.7	76.1	0.2	80.1	0.6	45.5	2.7	22.0	14.5	60.0	2.8	99.6	0.1
FSx-29	31.7	3.2	24.5	7.0	4.6	2.2	27.3	2.7	39.9	2.5	80.7	0.9	70.4	0.9	75.4	1.1	44.0	2.2	19.7	14.5	69.6	1.3	99.4	0.2
FSx-30	67.5	2.3	87.2	1.1	34.0	2.9	88.7	0.7	50.2	4.3	99.4	0.0	36.7	3.5	75.0	13.0	9.8	2.1	28.5	3.6	58.9	2.9	100.0	0.1
FSx-31	51.7	1.1	85.9	0.2	0.8	0.7	0.0	1.2	19.2	1.6	100.0	0.1	5.7	2.0	13.4	3.6	18.7	0.1	25.0	3.8	99.7	0.1	100.0	0.1
FSx-32	6.4	1.7	2.8	0.3	9.1	0.2	8.2	2.8	6.9	1.8	75.1	1.2	11.4	4.5	10.7	1.8	5.0	1.5	4.1	1.4	100.0	0.0	100.0	0.1
FSx-33	12.0	0.2	3.4	1.1	12.4	1.4	7.3	2.3	4.9	1.6	68.6	1.8	6.4	1.5	13.7	3.1	7.5	0.3	3.5	1.8	100.0	0.0	100.0	0.0
FSx-34	9.2	0.9	2.9	3.2	16.5	0.8	11.7	0.6	8.0	2.2	75.1	1.0	13.2	1.5	18.3	3.1	7.4	2.0	5.5	1.0	100.0	0.0	100.0	0.0
FSx-35	5.1	0.1	3.8	1.1	13.4	2.0	9.3	1.5	7.7	1.1	73.7	1.4	8.7	1.3	11.4	3.0	3.9	1.2	2.8	1.0	100.0	0.0	100.0	0.0
FSx-36	13.1	1.4	6.4	2.7	17.6	0.6	8.3	1.7	12.7	1.7	47.1	1.8	15.3	1.5	20.1	2.3	10.6	0.4	9.8	1.7	100.0	0.0	100.0	0.0
FSx-37	7.7	1.3	6.3	1.3	16.0	2.0	13.4	1.5	10.8	0.6	30.3	1.3	19.8	0.6	19.6	1.9	9.7	1.9	7.3	2.9	100.0	0.0	100.0	0.0
FSx-38	8.5	1.3	2.3	1.5	13.1	0.9	6.0	2.6	5.7	0.7	25.4	1.3	9.6	1.7	19.8	0.5	8.5	0.2	4.1	1.6	100.0	0.0	100.0	0.0
FSx-39	0.0	8.9	0.0	8.7	0.0	7.2	0.0	6.3	2.1	8.2	5.2	2.3	0.0	10.8	3.8	1.5	35.7	32.0	9.4	4.5	99.8	0.0	99.9	0.0
FSx-40	25.7	1.4	21.0	3.9	22.2	2.7	24.4	0.2	22.6	1.4	73.2	1.7	83.1	3.0	98.4	0.2	15.6	4.4	19.3	1.2	66.7	2.7	97.9	0.6
FSx-41	0.0	3.6	63.7	0.8	4.4	5.7	27.8	9.6	0.0	4.2	42.0	3.8	0.0	2.0	0.0	4.9	43.4	3.8	98.9	0.7	47.3	5.8	98.4	0.1
FSx-42	13.4	1.1	99.6	0.1	17.4	1.9	13.8	2.8	16.2	2.1	42.6	1.1	15.8	1.0	17.2	2.4	13.1	2.7	8.2	0.9	99.8	0.1	99.9	0.0
FSx-43	99.9	0.2	100.0	0.1	15.4	4.5	28.7	4.4	40.4	2.3	99.0	0.1	40.0	0.9	97.1	0.1	21.6	6.0	89.9	1.2	98.2	0.3	99.0	0.2
FSx-44	0.0	4.4	0.0	5.2	5.8	1.7	4.4	2.1	3.8	2.6	18.6	6.0	11.8	1.3	5.7	2.8	35.3	32.2	0.0	5.5	99.8	0.0	99.9	0.0
FSx-45	40.1	1.9	58.0	2.3	0.0	1.2	0.0	3.1	21.3	1.3	99.3	0.3	0.0	3.9	52.5	21.4	0.0	2.8	27.7	1.2	51.0	1.2	99.4	0.0
FSx-46	32.7	1.2	33.6	0.7	37.0	3.8	32.6	3.7	25.9	2.4	49.9	0.6	38.7	0.7	37.3	4.8	56.3	21.9	37.6	3.1	99.8	0.1	99.9	0.0
FSx-47	99.9	0.2	100.0	0.1	15.4	4.5	28.7	4.4	40.4	2.3	99.0	0.1	40.0	0.9	97.1	0.1	21.6	6.0	89.9	1.2	98.2	0.3	99.0	0.2
FSx-48	26.0	4.7	76.9	2.6	37.6	2.2	100.0	0.0	58.9	0.4	99.7	0.1	53.3	4.4	100.0	0.1	99.9	0.0	100.0	0.0	29.5	13.0	99.7	0.1
FSx-49	46.3	0.8	66.0	1.4	99.3	0.1	99.7	0.0	16.5	1.1	42.7	0.4	99.7	0.1	99.8	0.2	27.9	0.1	40.2	1.9	98.8	0.1	99.0	0.2

Table 9C. % Inhibition of Unexposed WW-associated Isolates by 6 Antimicrobial Agents

Columns headed with dose concentration show the mean % inhibition of that isolate (row) by that dose of agent (column) as calculated from 3 replicate wells. SE shows the standard error.

	Triclosan				Carbenicillin				Chloramphenicol				Trimethoprim				Erythromycin				Ciprofloxacin			
Isolate	0.125 µg/mL	SE	1 µg/mL	SE	0.25 µg/mL	SE	2 µg/mL	SE	0.5 µg/mL	SE	4 µg/mL	SE	1 µg/mL	SE	8 µg/mL	SE	0.625 µg/mL	SE	5 µg/mL	SE	0.125 µg/mL	SE	1 µg/mL	SE
WWun-1	0	19.3	37	18.6	0	28.6	100	0.1	62.6	0.9	99.2	0.4	82.4	0.2	99.8	0.1	100	0.1	100	0.1	34.5	13	99.7	0
WWun-2	0	22.4	13.3	6.8	0	27.8	99.4	0.4	43.9	5.8	99	0.3	74.1	1.5	99.3	0.5	99.9	0.2	99.8	0	16.2	9.1	99.7	0.1
WWun-3	21.7	12.2	50.3	14.9	7.8	9.6	99.7	0.3	73.6	0.8	100	0	64.4	9	99.8	0.1	100	0.1	100	0.1	32.1	18.5	99.8	0
WWun-4	0	34.5	0	16.9	0	30.8	100	0	50	0.6	99.2	0.4	24.7	4.4	99.5	0.2	100	0.1	100	0	0	32.9	99	0.4
WWun-5	0	20.9	0	15.3	0	23.6	100	0.1	50.3	0.6	99.4	0.2	4.1	10.3	99.7	0.1	100	0.1	100	0.2	3.9	12.3	99.2	0.1
WWun-6	6.3	9.6	20.6	3.6	46	1.8	81.1	1.4	0	0	0	0	4.5	6.9	37.7	1.7	13.9	6.1	75.1	0.1	16.5	6.1	24.1	4.4
WWun-7	12.7	1.2	73.3	0.4	93.9	1.3	97.9	1	88.8	1.1	98.9	0.7	99.7	0	99.9	0	89.6	1	98.4	0.7	9.9	0.5	9.8	0.6
WWun-8	0	6.6	61.6	4.5	0	0.2	4.5	1.7	0	8.3	96.8	0.3	99.5	0.2	99.4	0	0	7.7	94.9	0.4	0	3.7	0	2.1
WWun-9	26	4.7	76.9	2.6	37.6	2.2	100	0	58.9	0.4	99.7	0.1	53.3	4.4	100	0.1	99.9	0	100	0	29.5	13	99.7	0.1
WWun-10	0	7.6	98.3	0.6	0	6.5	0	13.3	0	2.8	4.6	7.2	0	1.5	0	13.5	0	20.3	0	9.4	99.2	0.3	99.7	0.8
WWun-11	0	2.3	0	0.9	0	3.9	0	7.6	0	3.8	0	5.7	0	2.3	0	7.1	0	1.2	0	0.5	100	0.1	100	0.1
WWun-12	0	2.6	69.2	2.4	0	1.5	0	2.6	0	1	5.6	4.8	0	7.7	0	4	0	3.2	0	5	100	0.1	100	0.1
WWun-13	0	1.5	0	1.2	0	12.5	0	8	0	7.8	0	9	0	4.1	0	6.3	0	3.6	7.6	8.9	100	0.1	100	0.1
WWun-14	0	1.2	76.6	2.1	0	6.5	0	2.3	0	8.1	10.9	3.2	0	2.7	0	8.5	0	3.3	0	4	94.9	5.1	100	0.1
WWun-15	9.3	3.4	37.6	9.3	38.5	7.5	99.5	0.1	43.6	3.6	98.1	0.3	0	1	9	8.4	97.7	1.3	96.5	1.2	29.7	2.5	99.5	0
WWun-16	38.8	2.6	100	0.9	95.3	0.8	100	0.6	0	11.9	98.9	0.6	88.1	1.9	100	0.4	0	3.4	28.2	13.1	99.8	0.4	100	0.2
WWun-17	97.1	0.4	98	0.1	36.8	1.8	35	7.9	66.7	2.7	99.2	0.1	49.9	1.7	92.8	0.2	23.5	12.1	46.4	1.1	97.6	0.5	97.7	0
WWun-18	4.9	0.1	5.8	0.2	5.6	0.6	7.1	2.3	8.7	0.9	16.7	0.6	4.2	1.3	4.8	1.6	4.2	1.4	6	0.7	99.9	0.1	100	0.3
WWun-19	32.4	2.5	100	0.1	18.4	5.6	19.1	2.9	9.3	2.1	34	1.1	15.1	3.5	17.7	3.7	22.5	2.8	18.3	0.8	100	0	100	0
WWun-20	29.1	1.9	19.4	3.8	12.3	2.6	7.2	2.6	19.3	1.3	62.3	0.2	29.7	2.5	55.3	3.3	11.3	5.1	10.8	1.6	23.9	1.4	72.3	0.5
WWun-21	100	0	100	0.1	29.5	0.9	17.5	9.9	75.7	3.5	100	0.1	35.3	3	91.6	0.7	16.8	9.9	48.8	1.6	99.5	0.3	100	0.2
WWun-22	35.6	1.5	29.7	3.7	4.2	1.2	9.2	2.3	6.4	0.7	23.4	1.4	0.5	0.7	73.8	16	4.1	1.9	32.7	0.8	31.4	1.4	74.2	0.9
WWun-23	37.5	2.8	33.4	2.7	8.2	1.6	6.7	0.8	8.8	1.7	27.6	2	30.2	0.9	63.1	2.1	8.1	3	38.9	1.8	31.4	0.7	78.7	1.2
WWun-24	0	0.5	7	2	16.9	3.5	3.6	3.2	28	0.8	45.7	2.2	91.7	0.3	99.9	0	5.1	0.1	4.5	1.8	99.9	0.1	100	0

Table 9D. % Inhibition of Triclosan-Exposed WW-associated Isolates by 6 Antimicrobial Agents
Columns headed with dose concentration show the mean % inhibition of that isolate (row) by that dose of agent (column) as calculated from 3 replicate wells. SE shows the standard error. The table is continued on the next page.

	Triclosan				Carbenicillin				Chloramphenicol				Trimethoprim				Erythromycin				Ciprofloxacin			
Isolate	0.125 µg/mL	SE	1 µg/mL	SE	0.25 µg/mL	SE	2 µg/mL	SE	0.5 µg/mL	SE	4 µg/mL	SE	1 µg/mL	SE	8 µg/mL	SE	0.625 µg/mL	SE	5 µg/mL	SE	0.125 µg/mL	SE	1 µg/mL	SE
WWx-1	4.7	1.0	4.2	1.0	3.6	1.4	6.4	1.0	5.1	1.9	22.1	1.6	0.5	1.8	3.6	1.2	4.9	1.0	2.8	0.4	99.8	0.3	100.0	0.1
WWx-2	0.0	17.6	66.0	2.4	1.3	9.0	98.7	0.3	62.1	0.4	98.9	0.2	58.2	3.0	99.8	0.7	98.3	0.4	97.8	0.5	3.7	12.4	100.0	0.2
WWx-3	0.0	8.8	43.4	8.3	4.2	2.8	99.2	0.2	59.5	3.8	98.6	0.7	55.9	1.9	99.2	0.0	98.5	0.3	99.1	0.2	32.4	3.1	100.0	0.4
WWx-4	0.0	17.3	31.4	10.9	0.0	3.3	99.1	0.5	65.3	2.0	98.9	0.7	56.3	3.0	99.3	0.2	98.5	0.4	98.0	0.7	23.1	3.0	100.0	0.5
WWx-5	0.0	69.7	53.1	5.6	0.0	4.4	98.9	0.5	58.9	3.7	97.8	1.2	68.8	4.5	99.5	0.4	98.0	0.3	99.4	0.0	17.9	2.4	100.0	0.0
WWx-6	1.9	0.8	3.5	2.6	13.3	4.7	5.6	1.4	99.7	0.0	99.2	0.2	99.8	0.1	100.0	0.0	28.8	24.3	17.8	1.9	99.8	0.0	100.0	0.1
WWx-7	0.0	16.8	28.5	14.0	0.0	7.8	98.6	0.3	54.3	0.7	98.0	0.2	21.8	10.6	99.0	0.3	96.4	0.5	98.8	0.5	0.0	40.8	100.0	0.7
WWx-8	9.9	6.9	35.5	5.2	0.0	16.8	98.0	1.6	58.2	0.7	98.3	0.1	33.7	3.3	99.9	0.2	98.5	0.2	99.9	0.1	20.0	2.6	100.0	0.1
WWx-9	40.6	5.0	78.7	2.9	25.7	2.6	94.4	3.1	67.5	4.1	99.5	0.2	68.3	1.1	99.9	0.1	92.8	3.2	96.3	1.3	32.4	3.6	100.0	0.1
WWx-10	6.7	0.8	7.1	2.0	8.9	1.5	7.6	1.2	3.7	3.2	62.2	0.2	99.8	0.0	100.0	0.1	0.0	0.4	0.0	3.8	99.9	0.1	100.0	0.0
WWx-11	14.9	3.2	16.0	0.8	16.0	2.6	11.8	1.9	6.4	3.0	72.3	0.7	99.9	0.0	100.0	0.0	1.1	1.1	0.0	1.5	100.0	0.1	100.0	0.0
WWx-12	16.5	4.5	11.2	6.1	14.2	2.6	12.4	1.5	0.0	3.3	10.8	1.4	0.0	1.7	0.0	3.0	0.0	0.9	0.0	1.7	89.1	0.1	90.0	0.6
WWx-13	10.1	5.7	13.2	1.8	12.1	3.4	10.4	2.3	7.5	2.0	64.4	1.3	18.1	2.4	20.0	1.0	3.8	0.4	2.1	1.6	99.9	0.0	100.0	0.1
WWx-14	9.6	4.7	6.9	1.5	14.7	0.9	13.2	0.5	7.8	0.6	67.1	2.6	99.6	0.0	99.9	0.2	0.0	1.8	1.2	1.5	100.0	0.0	100.0	0.0
WWx-15	24.2	3.4	17.2	4.4	31.0	4.0	40.2	0.2	5.8	5.2	69.3	1.6	99.8	0.1	100.0	0.1	3.4	0.9	19.3	2.0	60.2	4.9	99.0	0.2
WWx-16	38.0	1.3	100.0	0.1	9.3	1.0	0.4	6.8	17.2	1.9	36.6	0.6	4.0	13.1	11.0	3.5	17.0	4.6	22.3	3.3	99.2	0.2	99.5	0.1
WWx-17	18.2	1.7	20.6	3.9	17.8	3.5	56.6	0.7	29.8	0.3	76.1	0.0	93.4	1.8	99.1	0.0	23.5	2.0	30.3	2.3	57.9	9.8	99.4	0.2
WWx-18	17.1	2.4	29.1	3.7	10.8	1.1	8.0	0.7	23.4	3.2	88.5	1.0	11.4	1.1	13.6	1.6	24.3	3.5	34.7	0.9	42.2	1.4	99.4	0.1
WWx-19	14.7	3.6	13.2	2.7	17.4	3.9	13.9	0.1	16.8	3.1	44.6	0.8	15.7	2.5	22.5	0.5	13.5	1.4	26.4	0.7	99.7	0.1	99.9	0.2
WWx-20	0.0	7.2	48.5	4.9	20.1	3.6	99.3	0.1	71.9	7.7	96.9	1.1	70.2	3.5	99.0	0.4	96.8	0.5	98.7	0.3	31.0	7.0	99.4	0.3
WWx-21	19.2	3.6	10.3	0.8	3.0	2.3	0.0	1.4	17.9	2.0	24.5	1.9	4.3	1.5	6.2	4.2	21.5	3.7	19.1	1.8	99.5	0.3	99.8	0.1
WWx-22	13.8	5.7	2.6	3.4	0.0	0.6	2.8	0.9	21.6	1.4	62.6	0.6	98.7	0.1	99.1	0.0	19.0	2.9	17.0	5.5	53.2	0.7	99.6	0.2
WWx-23	18.8	5.6	99.5	0.4	0.0	3.3	0.0	0.3	5.7	1.2	18.7	3.8	0.0	3.8	1.7	2.6	18.7	2.0	11.4	1.3	99.8	0.1	99.9	0.1

Table 9D continued. % Inhibition of Triclosan-Exposed WW-associated Isolates

Columns headed with dose concentration show the mean % inhibition of that isolate (row) by that dose of agent (column) as calculated from 3 replicate wells. SE shows the standard error.

	Triclosan				Carbenicillin				Chloramphenicol				Trimethoprim				Erythromycin				Ciprofloxacin			
Isolate	0.125 µg/mL	SE	1 µg/mL	SE	0.25 µg/mL	SE	2 µg/mL	SE	0.5 µg/mL	SE	4 µg/mL	SE	1 µg/mL	SE	8 µg/mL	SE	0.625 µg/mL	SE	5 µg/mL	SE	0.125 µg/mL	SE	1 µg/mL	SE
WWx-24	13.2	2.8	100.0	0.1	1.8	0.5	3.0	2.6	0.0	1.9	13.9	0.8	4.9	1.9	5.8	0.6	3.5	2.1	2.9	1.7	99.9	0.1	99.9	0.3
WWx-25	0.0	7.2	58.2	1.8	6.0	1.9	11.4	5.4	14.8	6.3	96.8	0.1	3.6	3.9	21.3	1.4	0.0	1.8	21.5	2.6	98.8	0.4	99.9	0.2
WWx-26	21.4	5.4	8.5	1.4	0.0	10.9	72.0	2.2	18.1	2.3	88.1	0.2	94.2	0.3	94.8	0.5	10.9	4.3	8.7	10.3	71.2	2.1	100.0	0.1
WWx-27	4.6	2.1	8.6	3.1	0.0	4.8	0.0	1.6	3.9	1.4	44.9	2.1	0.0	4.1	0.0	1.0	0.0	1.0	1.3	0.5	98.9	1.2	100.0	0.0
WWx-28	80.7	2.6	96.2	0.6	7.4	2.8	13.3	4.1	15.8	3.7	85.0	1.1	9.5	5.2	65.5	1.7	14.8	1.1	68.2	0.6	99.7	0.1	99.9	0.2
WWx-29	47.1	26.7	99.9	0.0	2.6	0.8	9.1	0.9	10.9	1.5	59.3	1.3	5.5	2.0	35.1	0.8	1.5	0.9	4.3	0.6	99.8	0.1	100.0	0.0
WWx-30	17.7	3.2	100.0	0.1	4.5	1.8	8.2	1.4	12.3	1.7	59.0	3.4	6.5	1.5	33.0	1.8	0.4	0.6	1.3	0.7	99.8	0.0	100.0	0.1
WWx-31	28.1	10.4	18.5	8.6	9.4	1.2	35.4	2.8	32.2	5.3	98.1	0.2	94.6	2.9	99.7	0.1	1.6	1.7	24.3	2.1	70.6	1.4	99.9	0.0
WWx-32	15.2	4.3	20.1	4.3	18.6	1.7	22.9	4.9	14.5	2.8	80.6	1.3	99.6	0.1	99.3	0.2	8.0	1.7	18.7	2.7	71.5	3.2	100.0	0.0
WWx-33	24.0	10.9	22.4	3.0	18.8	3.2	21.7	5.3	24.7	1.2	49.3	0.8	95.5	0.4	100.0	0.0	16.2	2.5	16.1	0.8	100.0	0.0	100.0	0.0
WWx-34	15.0	0.5	21.9	6.1	25.5	4.8	43.7	3.5	6.1	8.2	64.6	1.2	90.6	0.6	98.3	0.3	8.3	1.1	26.6	2.1	57.2	3.2	99.5	0.2
WWx-35	0.0	1.7	0.0	2.5	0.0	4.0	2.1	2.9	0.0	3.1	0.0	2.0	0.0	2.6	0.0	3.3	0.0	2.0	1.8	3.1	100.0	0.1	100.0	0.0
WWx-36	3.2	1.6	14.4	1.3	9.7	0.4	15.0	1.8	13.2	0.3	42.6	0.5	10.1	0.9	23.3	1.2	4.4	1.2	5.6	2.9	100.0	0.0	100.0	0.0
WWx-37	99.7	0.1	100.0	0.1	49.4	1.3	99.9	0.0	45.3	4.1	99.6	0.1	29.9	1.5	55.3	17.2	53.8	3.6	94.1	0.3	99.9	0.1	100.0	0.1
WWx-38	13.4	3.5	100.0	0.1	13.3	5.1	19.4	2.6	10.2	3.7	32.0	1.7	12.3	1.0	24.0	2.3	0.0	2.0	1.9	2.2	100.0	0.1	100.0	0.0
WWx-39	27.5	3.5	100.0	0.0	23.2	4.1	25.1	3.5	9.3	0.7	38.4	2.8	18.5	0.5	33.4	2.2	0.0	1.3	12.2	7.4	100.0	0.0	100.0	0.0
WWx-40	59.9	2.2	100.0	0.0	14.9	3.6	30.8	2.4	16.3	0.8	50.2	2.0	18.8	0.4	27.8	1.9	0.7	1.2	6.6	1.7	100.0	0.0	100.0	0.0
WWx-41	15.1	2.1	87.5	1.7	34.2	2.1	100.0	0.4	83.8	4.8	97.7	0.3	55.6	3.7	97.8	0.1	44.3	2.9	98.4	0.2	13.9	2.1	50.3	0.6
WWx-42	99.4	0.2	100.0	0.1	44.7	0.8	63.4	0.6	79.7	1.0	99.8	0.1	52.4	0.2	97.1	0.1	29.4	0.6	63.5	1.1	100.0	0.3	100.0	0.1
WWx-43	44.7	5.5	82.2	2.1	32.0	2.4	37.2	10.2	84.0	5.0	97.0	0.8	93.7	0.8	96.1	0.3	73.1	4.2	100.0	0.5	32.9	9.4	100.0	0.9
WWx-44	21.5	1.6	99.9	0.0	3.4	0.9	1.4	0.5	10.7	0.5	25.7	1.5	10.5	0.6	10.9	1.3	8.3	0.3	5.2	1.4	99.7	0.2	100.0	0.0
WWx-45	17.0	2.0	97.5	0.1	4.0	1.1	1.3	0.6	12.2	0.6	27.0	1.7	11.7	2.0	14.8	1.8	10.5	1.0	6.0	1.4	98.6	0.4	100.0	0.0
WWx-46	4.1	4.7	3.2	2.9	7.9	1.5	3.3	2.8	13.6	2.4	23.5	1.1	12.7	3.0	18.3	0.9	14.7	0.6	12.9	1.7	99.8	0.1	100.0	0.0

Tables 10A-10L ANOVA summaries of percent inhibition comparisons

Table 10A. Descriptive Statistics for 0.125 μg/ml Triclosan (TCS)

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	31.50	7.94	25
	Exposed (10 ng/ml TCS)	30.02	4.2	49
WW-associated	Unexposed	18.83	5.78	24
	Exposed (10 ng/ml TCS)	20.68	3.51	46

ANOVA Summary Table for 0.125 μg/ml Triclosan

Source	*df*	MS	*F*	*p*
Stream	1	3950.03	4.49	0.0359
Exposure Status	1	1.44	0	1
Str x Exp Interaction		1	90.6	0.1 0.7523
Error	140	880.36		
Total	143			

Note.—MS = Mean squares

Discussion

Low-level, chronic exposures to triclosan can drive evolution of bacteria. Triclosan exposure can lead to upregulation of multidrug efflux pumps (Chuanchuen *et al.* 2001; Chuanchuen *et al.* 2002) thus increasing MDR. When the minimum exposure levels in the environment are just above the NOEC, sensitive isolates of a given bacterial population will die, leaving behind the resistant isolates to persist within the environment. Additionally, exposure levels below the NOEC could lead to increased resistance as there is a higher potential for sublethal responses to the antimicrobial agent (Scott *et al.* 2016). The current study provides

additional evidence of triclosan's effects on stream microbial communities that can lead to increased resistance to antimicrobial agents. Several results herein indicate that an environmentally-relevant exposure to triclosan can affect a sensitive microbial community, while a WW-associated community showed resistance prior to microcosm exposure to triclosan.

Despite only one unexposed, forested isolate showing triclosan resistance (an isolate sensitive to the other five agents' high doses) (Fig. 8), over half (61%) of triclosan-resistant isolates in this study were cultured from the exposed, forested-stream periphyton. The higher proportion of exposed isolates showing triclosan resistance compared to unexposed isolates from the forested stream community suggests the microcosm exposure may have affected the composition of this community, leaving a higher proportion of triclosan-resistant isolates.

Table 10B. Descriptive Statistics for 1 μg/ml Triclosan

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	47.65	7.96	25
	Exposed (10 ng/ml TCS)	44.86	5.71	49
WW-associated	Unexposed	46.17	7.64	24
	Exposed (10 ng/ml TCS)	47.36	5.75	46

ANOVA Summary Table for 1 μg/ml Triclosan

Source	*df*	MS	*F*	*p*
Stream	1	47.47	0.03	0.8627
Exposure Status	1	23.29	0.02	0.8877
Str x Exp Interaction		1	127.66	0.08 0.7777
Error	140	1538.89		
Total	143			

Table 10C. Descriptive Statistics for 0.25 μg/ml Carbenicillin

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	19.50	5.79	25
	Exposed (10 ng/ml TCS)	15.72	2.46	49
WW-associated	Unexposed	18.79	5.65	24
	Exposed (10 ng/ml TCS)	12.06	1.78	46

ANOVA Summary Table for 0.25 μg/ml Carbenicillin

Source	*df*	MS	*F*	*p*
Stream	1	249.61	0.6	0.4359
Exposure Status	1	876	2.1	0.1495
Str x Exp Interaction		1	75.44	0.18 0.672
Error	140	417.77		
Total	143			

Note.—MS = Mean squares

Among cultured isolates from these two streams, two thirds of the triclosan-resistant isolates were also resistant to at least one other high dose agent tested (Fig. 8). This includes the majority (8/11) of the exposed, forested triclosan-resistant isolates, half (2/4) of the unexposed, WW-associated and both of the exposed, WW-associated triclosan-resistant isolates. This supports the hypothesis that triclosan resistance and resistance to additional antimicrobials are associated with triclosan exposure. Among forested isolates cultured in this study, more resistance and more MDR in exposed versus unexposed isolates (Fig. 11, Fig. 12, Fig. 13A) provides additional evidence in support of this hypothesis. Results among the isolates sampled show differences in responses between the two microbial communities (Fig. 12) which may be due to the forested-stream community being more sensitive to antimicrobials than the

pollution-tolerant community that resides in the urban, WW-associated stream. Overall, these more pronounced differences in measured susceptibilities between triclosan-exposed versus unexposed isolates observed in the forested-stream community suggest possible decreased susceptibility due to the microcosm exposure. Environmentally-relevant exposure leading to changes in susceptibilities of environmental bacteria highlights one of the key health risks associated with this chemical.

Table 10D. Descriptive Statistics for 2 μg/ml Carbenicillin

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	23.29	6.07	25
	Exposed (10 ng/ml TCS)	26.32	3.84	49
WW-associated	Unexposed	45.31	9.4	24
	Exposed (10 ng/ml TCS)	35.11	5.55	46

ANOVA Summary Table for 2 μg/ml Carbenicillin

Source	*df*	MS	*F*	*p*
Stream	1	47.47	0.03	0.8627
Exposure Status	1	23.29	0.02	0.8877
Str x Exp Interaction		1	127.66	0.08 0.7777
Error	140	1538.89		
Total	143			

There were also differences based on exposure status or stream source in proportions of completely resistant isolates (0% inhibition) (Fig. 15). No unexposed forested isolates showed complete resistance to 1 μg/ml triclosan, while 16.3% of the isolates in the exposed forested

group were completely resistant. The trend was the opposite for WW-associated groups, where unexposed WW-associated isolates showed higher proportions of completely resistant isolates (45.8% with 0.125 μg/ml triclosan and 16.7% with 1 μg/ml triclosan) than the exposed WW-associated group (17.4% with 0.125 μg/ml triclosan and 2.2% with 1 μg/ml triclosan Fig. 15). This result follows with the idea that the urban, WW-associated microbial community has become more tolerant of antimicrobial agents over time due to exposure to many anthropogenic inputs, while the forested stream community is more sensitive. Unexposed WW-associated isolates exhibited significantly higher proportions of completely resistant isolates than any other group in some susceptibility tests (Fig. 15).

Table 10E. Descriptive Statistics for 0.5 μg/ml Chloramphenicol

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	33.96	6.23	25
	Exposed (10 ng/ml TCS)	22.5	3.16	49
WW-associated	Unexposed	28.94	6.18	24
	Exposed (10 ng/ml TCS)	28.21	4.06	46

ANOVA Summary Table for 0.5 μg/ml Chloramphenicol

Source	*df*	MS	*F*	*p*
Stream	1	157.1	0.22	0.6398
Exposure Status	1	1256.17	1.72	0.1918
Str x Exp Interaction		1	924.04	1.27 0.2617
Error	140	728.61		
Total	143			

Note.—MS = Mean squares

Table 10F. Descriptive Statistics for 4 μg/ml Chloramphenicol

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	70.57	6.25	25
	Exposed (10 ng/ml TCS)	54.67	4.34	49
WW-associated	Unexposed	59.13	8.79	24
	Exposed (10 ng/ml TCS)	64.76	4.57	46

ANOVA Summary Table for 4 μg/ml Chloramphenicol

Source	*df*	MS	*F*	*p*
Stream	1	280.46	0.26	0.6109
Exposure Status	1	948.13	0.86	0.3553
Str x Exp Interaction		1	3739.9	3.41 0.0669
Error	140	1096.61		
Total	143			

In this pollution-tolerant, WW-associated microbial community, 10 μg/L triclosan did not lead to a shift to more resistance to agents tested in this study. Instead, lower proportions of completely resistant isolates and MDR were seen in the triclosan-exposed WW-associated group, suggesting that the microcosm exposure could somehow lower representation of resistant bacteria. Another possibility is that there is not a substantial effect on susceptibility levels in this tolerant community and the random selection of a relatively small sample of unexposed WW-associated isolates (n=24) led to a higher proportion of resistant isolates by chance. These possibilities could be clarified with an expanded study as mentioned above. Exposed WW-associated isolates had significantly lower proportions of resistant isolates to 1 μg/ml triclosan compared to exposed forested isolates, highlighting the different responses to

10 μg/L triclosan in the more sensitive, forested stream versus those of the pollution-tolerant, WW-associated community.

In addition to triclosan, the agents used for susceptibility tests were selected to represent different classes of antibiotics. Previous studies have shown development of cross-resistance between triclosan and these other agents. In one study, low-level triclosan resistance conferred through constitutive upregulation of various efflux pumps resulted in cross-resistance to chloramphenicol and carbenicillin, in some cases. They found that the degree of triclosan resistance was dependent upon the initial level of exposure (Pycke *et al.* 2010). A study that selected biocide-resistant strains of *E. coli*, found high levels of resistance after two sub-lethal exposures to triclosan.

Table 10G. Descriptive Statistics for 1 μg/ml Trimethoprim

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	54.11	8.28	25
	Exposed (10 ng/ml TCS)	30.39	4.53	49
WW-associated	Unexposed	35.48	7.55	24
	Exposed (10 ng/ml TCS)	43.67	5.77	46

ANOVA Summary Table for 1 μg/ml Trimethoprim

Source	*df*	MS	*F*	*p*
Stream	1	217.7	0.16	0.6898
Exposure Status	1	2153.33	1.59	0.2094
Str x Exp Interaction		1	8220.44	6.06 0.015
Error	140	1356.02		
Total	143			

Note.—MS = Mean squares

These strains then also showed decreased susceptibilities to several antimicrobial agents including chloramphenicol, erythromycin and trimethoprim (Braoudaki and Hilton 2004). Exposure to triclosan in grass shrimp led to increases in MDR^t *Vibrio* species, which showed resistance to six antibiotics, including erythromycin (DeLorenzo *et al.* 2014). Cross-resistance has also been detected between triclosan and quinolones such as ciprofloxacin (Hernandez *et al.* 2011; Sanchez *et al.* 2005).

Table 10H. Descriptive Statistics for 8 μg/ml Trimethoprim

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	62.09	8.31	25
	Exposed (10 ng/ml TCS)	43.05	5.34	49
WW-associated	Unexposed	60.13	8.93	24
	Exposed (10 ng/ml TCS)	59.22	6.19	46

ANOVA Summary Table for 8 μg/ml Trimethoprim

Source	*df*	MS	*F*	*p*
Stream	1	3630.49	2.19	0.1412
Exposure Status	1	3396.74	2.05	0.1544
Str x Exp Interaction		1	2615.73	1.58 0.2109
Error	140	1655.72		
Total	143			

Note.—MS = Mean squares

Several results supported the hypothesis that the forested and WW-associated community's ambient levels of antimicrobial resistance differed, leading to the two communities having

different responses to triclosan exposure. In the forested-stream community, but not the WW-associated community, there was increased overall resistance in the exposed group. Comparing mean % inhibition, regardless of exposure, there were a couple instances (0.125 µg/ml triclosan Fig. 9A and 2 µg/ml carbenicillin Fig. 10B) showing more resistance in the WW-associated community than the forested community. It is likely that WW-associated stream bacteria have had chronic exposure to low levels of triclosan and other antimicrobials in their stream environment.

Table 10I. Descriptive Statistics for 0.625 µg/ml Erythromycin

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	13.32	2.31	25
	Exposed (10 ng/ml TCS)	24.52	3.33	49
WW-associated	Unexposed	37.36	9.11	24
	Exposed (10 ng/ml TCS)	27.43	5.24	46

ANOVA Summary Table for 0.625 µg/ml Erythromycin

Source	*df*	MS	*F*	*p*
Stream	1	3666.89	3.89	0.0505
Exposure Status	1	22.35	0.02	0.8877
Str x Exp Interaction		1	3607.27	3.82 0.0526
Error	140	943.2		
Total	143			

Note.—MS = Mean squares

There are numerous documented cases of triclosan's presence in WW effluents (Gautam *et al.* 2014; Kolpin *et al.* 2002; Kumar *et al.* 2010; Middleton and Salierno 2013; Singer *et al.* 2002;

Ying and Kookana 2007) and based on its widespread use and incomplete removal in WW treatment processes, the likelihood of triclosan's presence in North Buffalo Creek over recent decades is high. Though this could also be the case with the forested stream, it is plausible that environmental exposures in the more rural, forested North Double Creek may have been lower than exposures in the urban, WW-associated stream. North Buffalo Creek has been shown to be impaired. From before triclosan use through completion of this study (1938 through October 2017) North Buffalo Water Reclamation Facility served the northern half of Greensboro, a city of about 281 thousand people at the time of this study. Pinnacle, NC had about 902 residents at the time of the study (U.S. Census Bureau 2016). This potentially higher and/or longer, chronic exposure to antimicrobials and other anthropogenic inputs may have led to higher antimicrobial resistance in this community, thus developing more tolerant bacteria.

Table 10J. Descriptive Statistics for 5 μg/ml Erythromycin

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	35.75	6.5	25
	Exposed (10 ng/ml TCS)	29.76	4.55	49
WW-associated	Unexposed	50.29	8.66	24
	Exposed (10 ng/ml TCS)	34.66	3.61	46

ANOVA Summary Table for 5 μg/ml Erythromycin

Source	*df*	MS	*F*	*p*
Stream	1	2442.59	1.87	0.1737
Exposure Status	1	3727.25	2.86	0.093
Str x Exp Interaction		1	717.46	0.55 0.4596
Error	140	1304.64		
Total	143			

This could explain why the unexposed isolates found in this WW-associated community show high levels of antimicrobial resistance and MDR.

The issue of rising antimicrobial resistance and increases in MDR^t bacteria is one of the greatest health challenges faced by today's world. There are many studies showing increasing MDR in bacterial pathogens (Andersen *et al.* 2015; Mahlen *et al.* 2011; reviewed in Chang *et al.* 2015) with less available data on MDR^t environmental bacteria, though there are many documented cases there as well.

Table 10K. Descriptive Statistics for 0.125 μg/ml Ciprofloxacin

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	75.46	6.47	25
	Exposed (10 ng/ml TCS)	80.96	4.28	49
WW-associated	Unexposed	56.24	8.45	24
	Exposed (10 ng/ml TCS)	75.50	4.91	46

ANOVA Summary Table for 0.125 μg/ml Ciprofloxacin

Source	*df*	MS	*F*	*p*
Stream	1	3747.68	3.33	0.0702
Exposure Status	1	4863.47	4.32	0.0395
Str x Exp Interaction		1	1484.46	1.32 0.2526
Error	140	1124.85		
Total	143			

Note.—MS = Mean squares

Bacteria are known to have the ability to share genetic information and ARGs can spread between different types of bacteria (Amábile-Cuevas and Chicurel 1993; Chee-Sanford *et al.*

2001; Ciusa *et al.* 2012; Cooper *et al.* 2017; Salyers and Amábile-Cuevas 1997). Often in nature, antimicrobial resistances initially increase in commensal bacteria to later be transferred to pathogens (Salyers *et al.* 2004; Sørum and L'Abée-Lund 2002). WW-treatment facilities are known hotspots of increasing antibiotic resistance and MDR (Graham *et al.* 2011; Magalhães *et al.* 2016; Middleton and Salierno 2013). Occurrences through wastewater treatment processing could be selecting for more resistant bacteria, as levels of ARGs can be higher in WWTP effluent than in pretreated sewage (Reinthaler *et al.* 2010; Uyaguari *et al.* 2011). In this study, only the WW-associated community revealed the presence of MDR^t isolates (3% or less inhibition by high doses of 3 or more agents) in the unexposed isolate group (Fig. 17B).

Table 10L. Descriptive Statistics for 1 μg/ml Ciprofloxacin

Stream	Exposure status	Mean	Std Error	N
Forested	Unexposed	94.02	3.57	25
	Exposed (10 ng/ml TCS)	96.98	1.41	49
WW-associated	Unexposed	85.56	6.14	24
	Exposed (10 ng/ml TCS)	98.59	1.09	46

ANOVA Summary Table for 1 μg/ml Ciprofloxacin

Source	*df*	MS	*F*	*p*
Stream	1	124.59	0.49	0.4851
Exposure Status	1	2015.35	7.93	0.0056
Str x Exp Interaction		1	815.39	3.21 0.0754
Error	140	254.13		
Total	143			

This plus evidence presented above suggest that a substantial portion of environmental bacteria inhabiting North Buffalo Creek can tolerate multiple antimicrobial agents. This is cause for concern for numerous reasons and anywhere downstream of a WWTP is likely to present similar issues. North Buffalo Creek is in the Cape Fear River basin and is upstream from many areas used for recreation and fishing. Its downstream waters also flow through farmlands, so even food sources aside from local fish could be at risk. The Haw River is also downstream and flows into Jordan Lake, an important drinking water resource. This study can serve as an example reporting on MDR in environmental stream bacteria with the knowledge that this is a global issue.

In this study, a forested stream microbial community exposed to an environmentally-relevant level of triclosan generally had increased MDR. The microcosm exposure level in this study, slightly higher than levels generally measured in streams, is lower than measured levels in some WW influents and effluents (reviewed in Chalew and Halden 2009; Kumar *et al.* 2010; Lehutso *et al.* 2017; Stasinakis *et al.* 2008) and substantially lower than many levels that have been measured in sediments (Agüera *et al.* 2003; Hale *et al.* 2000; Miller *et al.* 2008; Morales *et al.* 2005; Singer *et al.* 2002; Wilson *et al.* 2008), biota (Coogan *et al.* 2007; Coogan and LaPoint 2008; Mottaleb *et al.* 2009) and biosolids (Higgins *et al.* 2011; Pannu *et al.* 2012; Xia *et al.* 2010; Ying and Kookana 2007). Naturally, there is a complicated balance of microbial life, chemicals and other abiotic factors leading to different responses and various potential outcomes as these factors connect to direct development of resistance. Conducting a larger study on MDR^t environmental isolates would be ideal as it is quite clear this is not an issue unique to North Buffalo Creek or its watershed. Further studies with bigger sample sizes per sampling site and with a design that incorporates additional sampling sites such as more streams, additional

aquatic environments, pre-and post-treatment sewage samples and possibly expanding to terrestrial samples as well are recommended. Measuring responses from a wider range of environmentally-relevant exposure levels would further enhance our knowledge of what is occurring in natural microbial communities as a result of triclosan's presence.

In the current study, more pseudomonads were detected than other genera, and the majority (9/12) of MDR^t isolates were of this genus with another identified to the genus *Serratia*. Further study sampling larger numbers of isolates and expanding to add other streams (both WW-associated and forested) and additional sample types and testing susceptibilities to additional antimicrobial doses is warranted. It would also be interesting to expose the unexposed isolates in the study to triclosan then conduct susceptibility assays on them post-exposure to get a clearer picture of how specific environmental isolates respond to the 10 µg/L triclosan or to additional exposure levels. Additional testing of a larger selection of antimicrobial agents and antibiotics for MDR^t isolate screening would also provide further needed evidence of this global phenomenon.

Results of the current study show cases of tolerance to multiple antimicrobials in isolates from both streams. In response to the higher doses of antimicrobial agents tested, a third of all tested isolates showed either low to no susceptibility (0-10% inhibition) to at least two of the six agents, with over half of all isolates falling into this category when grown in the presence of the lower doses of antimicrobial agents. Among forested isolates, the fraction of exposed isolates showing low to no susceptibility (0-10% inhibition) to 2 or more of the high doses of antimicrobial agents is more than 2-fold that of the unexposed group (Fig. 16B). This adds to the evidence that there are more instances of resistance and MDR post-triclosan exposure in the forested microbial community. These results suggest that this environmentally-

relevant exposure has impacted the microbial community, driving up MDR. Comparing proportions of MDR^t isolates showing 3% or less inhibition to 3 or more agents, the unexposed WW-associated isolate group showed the highest proportions of MDR, with approximately one out of every five isolates exhibiting MDR at this level with the high doses (even higher proportion with low doses) (Figure 17). This group's higher proportions of MDR differed from the proportions in both exposed WW-associated isolates and unexposed forested isolates but did not significantly differ from the proportion of MDR^t triclosan-exposed forested isolates. Such a result could occur if exposure to triclosan is impacting the forested community, increasing their level of tolerance and inspiring more bacteria to acquire MDR, while the unexposed WW-associated group already had a higher subset of MDR^t isolates. Triclosan appears to be an agent of microbial evolution; instead of halting microbial growth, for many bacterial strains it has led to an increased ability to tolerate antimicrobial agents. From a human perspective, increases in MDR resulting from triclosan exposure are of concern as part of the global health issue of collectively rising MDR. MDR has become one of myriad threats to our health and well-being (Bertelsen *et al.* 2013; Cherednichenko *et al.* 2012; Crawford and Catanzaro 2012; Gee *et al.* 2008; Hu *et al.* 2016; Ishibashi *et al.* 2004; Jackson-Browne *et al.* 2018; James *et al.* 2010; Jung *et al.* 2012; Jurewicz *et al.* 2018; Kumar *et al.* 2009; Macedo *et al.* 2017; Matsumura *et al.* 2005; Raut and Angus 2010; Regnault at al. 2016; Rodríguez and Sanchez 2010; Savage *et al.* 2012; Stoker *et al.* 2010; Veldhoen *et al.* 2006; Wang *et al.* 2014; Zhang *et al.* 2017) associated with triclosan, which has been manufactured and distributed in goods in the interest of improved health and hygiene.

The WW-associated stream in the current study exhibits the presence of environmental stream bacteria with tolerance to multiple antimicrobial agents. Data herein also show higher

overall resistance and MDR after triclosan exposure in a more sensitive, forested stream microbial community, which was not observed in the WW-associated community. This highlights triclosan's potential for altering aquatic microbial communities as well as providing further evidence that MDR^t bacteria are present in our environment and typically found at greater levels downstream of where anthropogenic inputs combine at WWTPs. It is clear that the products we are using and the way our WW inputs are combining and being processed can contribute to the overall rise in MDR, but that MDR^t bacteria can also occur in streams not receiving WW due to triclosan exposure. This study is one of many demonstrating unintended impacts of a man-made chemical, designed and disseminated to improve our standard of living, which may be contributing to a major public health threat.

CHAPTER V

CONCLUSIONS

Results of the current study provide a clearer picture of some of the potential outcomes of environmental exposures to triclosan. Triclosan was detected at comparable levels at most sites in both a forested and an urban stream and results were in alignment with the hypothesis that triclosan levels peak at WWTPs (Fig. 4). WW-associated periphyton showed evidence of triclosan mitigation, with increasing levels in periphyton over time in microcosms (Fig. 7). Triclosan's interactions with stream microbial communities can lead to changes in diversity as well as development of antimicrobial resistance and MDR. A major aim of the current study was to gauge overall levels of antimicrobial resistance and MDR in a WW-associated periphyton community and a forested-stream periphyton community. Another goal was to draw comparisons between responses of isolates from these communities that were exposed to triclosan in microcosms and responses from isolates that were not exposed. The exposure dose of 10 μg/L was useful for investigating effects that could occur through exposures in natural or built environments. Results suggest that this environmentally-relevant exposure to triclosan could alter microbial communities by enriching genera containing opportunistic pathogens. Higher proportions of *Pseudomonas* (in the forested-stream) and *Serratia* (in the WW-associated stream) were observed in treatment groups from these microbial communities which had been exposed to triclosan in microcosms. In the WW-associated microbial community, *Pseudomonas* was equally dominant in both exposed and unexposed groups of isolates and it is possible that chronic exposure to anthropogenic inputs, likely including low-levels of triclosan,

could have impacted the microbial community structure prior to this exposure study. Additionally, the WW-associated microbial community showed higher levels of overall resistance and MDR in isolates that were not exposed to triclosan in microcosms. In contrast, the forested microbial community showed higher overall resistance and MDR in exposed isolates compared to unexposed isolates, suggesting that environmentally-relevant triclosan exposures may lead to altered susceptibility levels in more sensitive microbial communities. In both built and natural environments, microbial communities are experiencing chronic, sublethal exposures to triclosan which threaten human and environmental health as these exposures can drive evolution, leading to increased ARB and MDR in these communities. Assessing MDR in triclosan-resistant isolates, the majority were resistant to one or more additional agents tested (Fig. 8), highlighting the overlap of triclosan resistant and MDR^t bacteria in these stream microbial communities. Levels of exposure in certain environments are comparable to the level used in microcosm exposures here. It is clear that triclosan's presence and interactions with environmental bacteria are critical concerns. Environmental exposures to triclosan are potentially amplifying MDR in numerous bacteria residing in a wide variety of locations across the world. MDR has been observed in environmental bacteria from many sample types: streams (Magalhães *et al.* 2016), rivers (Graham *et al.* 2011; Mohanta and Goel 2014; Osinska *et al.* 2016), WW from drug manufacturing plants (Marathe *et al.* 2013), sediments (Graham *et al.* 2011; Morroni *et al.* 2016; Vignaroli *et al.* 2012), estuaries (Kim *et al.* 2011), marine shrimp (Kitiyodom *et al.* 2010), groundwater (Mohanta and Goel 2014), constructed wetlands for WW treatment processing from cattle feedlots (Jahne *et al.* 2015), pig manure (Zhu *et al.* 2013), and even in arctic birds (Sjolund *et al.* 2008). Globally, between the prevalence of MDR, continued everyday use of antimicrobials and the ability of bacteria to transfer ARGs, extensive monitoring

and reporting is needed. Additional strategies should be developed beyond use of products that provide selective pressures that aid microbial communities in expanding their arsenals of resistance mechanisms. The collective knowledge on development of MDR from this study and many others calls for improved monitoring, reporting, problem solving, and alternate solutions as we move forward and continue to explore ways to improve health worldwide.

REFERENCES

Adolfsson-Erici, Margaretha, Maria Pettersson, Jari Parkkonen, and Joachim Sturve. 2002. "Triclosan, a Commonly Used Bactericide Found in Human Milk and in the Aquatic Environment in Sweden." *Chemosphere* 46 (9): 1485–1489.

Aiello, A. E., Marshall, B., Levy, S. B., Della-Latta, P., Larson, E. 2004. "Relationship between triclosan and susceptibilities of bacteria isolated from hands in the community." *Antimicrobial Agents and Chemotherapy* 48 (8): 2973–2979.

Aiello, A. E., E. L. Larson, and S. B. Levy. 2007. "Consumer Antibacterial Soaps: Effective or Just Risky?" *Clinical Infectious Diseases* 45 (2): 147.

Agüera, Ana, Amadeo R Fernández-Alba, Luis Piedra, Milagros Mézcua, and M.José Gómez. 2003. "Evaluation of triclosan and biphenylol in marine sediments and urban wastewaters by pressurized liquid extraction and solid phase extraction followed by gas chromatography mass spectrometry and liquid chromatography mass spectrometry." *Analytica Chimica Acta* 480 (2): 193-205.

Allmyr, Mats, Margaretha Adolfsson-Erici, Michael S Mclachlan, and Gunilla Sandborgh-Englund. 2007. "Triclosan in Plasma and Milk from Swedish Nursing Mothers and Their Exposure via Personal Care Products." *The Science of the Total Environment* 372 (1): 87-93.

Alves Resende, J, V.L. Silva, C.O. Fontes, J.A. Souza-Filho, T.L. Rocha de Oliveira, C.M. Coelho, D.E. César, and C.G. Diniz. 2012. "Multidrug-Resistance and Toxic Metal Tolerance of Medically Important Bacteria Isolated from an Aquaculture System." *Microbes and Environments* 27 (4): 449–455.

Amábile-Cuevas, Carlos F., and Marina E. Chicurel. 1993. "Horizontal gene transfer." *American Scientist* 81 (4): 332-341.

Andersen, Jody, Gui-Xin He, Prathusha Kakarla, Ranjana KC, Sanath Kumar, Wazir Lakra, Mun Mukherjee, *et al.* 2015. "Multidrug Efflux Pumps from *Enterobacteriaceae*, *Vibrio cholerae* and *Staphylococcus aureus* Bacterial Food Pathogens." *International Journal of Environmental Research and Public Health* 12 (2): 1487-1547.

Anger, Cale T, Charles Sueper, Dylan J Blumentritt, Kristopher McNeill, Daniel R Engstrom, and William A Arnold. 2013. “Quantification of Triclosan, Chlorinated Triclosan Derivatives, and Their Dioxin Photoproducts in Lacustrine Sediment Cores.” *Environmental Science & Technology* 47 (4): 1833–1843.

Aranami, Kazushi, and James W Readman. 2007. “Photolytic Degradation of Triclosan in Freshwater and Seawater.” *Chemosphere* 66 (6): 1052–1056.

Armstrong, Dana L, Clifford P Rice, Mark Ramirez, and Alba Torrents. 2017. “Influence of Thermal Hydrolysis-Anaerobic Digestion Treatment of Wastewater Solids on Concentrations of Triclosan, Triclocarban, and Their Transformation Products in Biosolids.” *Chemosphere* 171 (3): 609–616.

Balakrishna, Keshava, Amlan Rath, Yerabham Praveenkumarreddy, Keerthi Siri Guruge, and Bikram Subedi. 2017. “A Review of the Occurrence of Pharmaceuticals and Personal Care Products in Indian Water Bodies.” *Ecotoxicology and Environmental Safety* 137 (3): 113–120.

Barber, Larry B., Jorge E. Loyo-Rosales, Clifford P. Rice, Thomas A. Minarik, and Ali K. Oskouie. 2015. "Endocrine disrupting alkylphenolic chemicals and other contaminants in wastewater treatment plant effluents, urban streams, and fish in the Great Lakes and Upper Mississippi River Regions.” *Science of the Total Environment* 517 (2): 195-206.

Barghouthi, Sameer A. 2011. "A Universal Method for the Identification of Bacteria Based on General PCR Primers.” *Indian Journal of Microbiology* 51 (4): 430-444.

Barnes, Ella M. 1958. “The Effect of Antibiotic Supplements on the Faecal Streptococci (Lancefield Group D) of Poultry.” *British Veterinary Journal* 114 (9): 333–344.

Barry, M. Anita, Donald E Craven, Theresa A Goularte, and Deborah A Lichtenberg. 1984. “*Serratia Marcescans* Contamination of Antiseptic Soap Containing Triclosan: Implications for Nosocomial Infection.” *Infection Control* 5 (9): 427–430.

Barton MD. 2014. “Impact of Antibiotic Use in the Swine Industry.” *Current Opinion in Microbiology* 19: 9–15.

Baquero F, Martínez JL, and Cantón R. 2008. “Antibiotics and Antibiotic Resistance in Water Environments.” *Current Opinion in Biotechnology* 19 (3): 260–265.

Bever, Candace S, Amy A Rand, Malin Nording, Diana Taft, Karen M Kalanetra, David A Mills, Melissa A Breck, Jennifer T Smilowitz, J. Bruce German, and Bruce D Hammock. 2018. “Effects of Triclosan in Breast Milk on the Infant Fecal Microbiome.” *Chemosphere* 203 (3): 467-473.

Becks VE, and Lorenzoni NM. 1995. “*Pseudomonas aeruginosa* Outbreak in a Neonatal Intensive Care Unit: A Possible Link to Contaminated Hand Lotion.” *American Journal of Infection Control* 23 (6): 396–398.

Bedoux, Gilles, Benoit Roig, Olivier Thomas, Virginie Dupont, and Barbara Le Bot. 2012. "Occurrence and toxicity of antimicrobial triclosan and by-products in the environment." *Environmental Science and Pollution Research International* 19 (4): 1044-1065.

Ben-Dov, Eitan, Orr H Shapiro, Ronen Gruber, Asher Brenner, and Ariel Kushmaro. 2008. "Changes in Microbial Diversity in Industrial Wastewater Evaporation Ponds Following Artificial Salination." *FEMS Microbiology Ecology* 66 (2): 437–446.

Benotti, Mark J, Rebecca A Trenholm, Brett J Vanderford, Janie C Holady, Benjamin D Stanford, and Shane A Snyder. 2009. "Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water." *Environmental Science & Technology* 43 (3): 597-603.

Berglund Björn. 2015. "Environmental Dissemination of Antibiotic Resistance Genes and Correlation to Anthropogenic Contamination with Antibiotics." *Infection Ecology & Epidemiology* 5 (9): 28564-1-10.

Bertelsen, R. J., M. P. Longnecker, M. Løvik, A. M. Calafat, K-H. Carlsen, S. J. London, and K. C. Lødrup Carlsen. 2013. "Triclosan exposure and allergic sensitization in Norwegian children." *Allergy* 68 (1): 84-91.

Bester, Kai. 2005. "Fate of Triclosan and Triclosan-Methyl in Sewage Treatment Plants and Surface Waters." *Archives of Environmental Contamination and Toxicology* 49 (1): 9-17.

Bioquell. "Serratia Marcescens." *Bioquell Incorporated*. 2018. http://healthcare.bioquell.com/en-us/resources-and-support/efficacy/bacteria/gram-negative-rods/serratia-marcescens. – to cite a photo used in creating Figure 3.

Boucher Y, Labbate M, Koenig JE, and Stokes HW. 2007. "Integrons: Mobilizable Platforms that Promote Genetic Diversity in Bacteria." *Trends in Microbiology* 15 (7): 301–309.

Braoudaki, M., and Hilton, A. C. 2004. "Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents." *Journal of Clinical Microbiology* 42 (1): 73-78.

Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, *et al.* 2011. "Tackling Antibiotic Resistance." *Nature Reviews. Microbiology* 9 (12): 894–896.

Calafat, A. M., Ye, X., Wong, L.-Y., Reidy, J. A., and Needham, L. L. 2008. "Urinary concentrations of TCS in the U.S. population: 2003-2004." *Environmental Health Perspectives* 116 (3): 303-307.

Carey, Daniel E, and Patrick J McNamara. 2015. "The Impact of Triclosan on the Spread of Antibiotic Resistance in the Environment." *Frontiers in Microbiology* 5 (1): 780-1-11

Carey D.E., Zitomer D.H., Kappell A.D., Choi M.J., Hristova K.R., and McNamara P.J. 2016. "Chronic Exposure to Triclosan Sustains Microbial Community Shifts and Alters Antibiotic Resistance Gene Levels in Anaerobic Digesters." *Environmental Science: Processes & Impacts* 18 (5): 1060–1067.

CDC (Centers for Disease Control and Prevention), National Center for Emerging and Zoonotic Infectious Diseases (NCEZID). "Antibiotic Resistance Threats in the United States, 2013." CDC. 2013. https://www.cdc.gov/drugresistance/biggest_threats.html.

Chalew, T. E. A., Halden, R. U. 2009. "Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban." *Journal of the American Water Resources Association* 45 (1): 4-13.

Champlin, Franklin R, Matthew L Ellison, James W Bullard, and Robert S Conrad. 2005. "Effect of Outer Membrane Permeabilisation on Intrinsic Resistance to Low Triclosan Levels in *Pseudomonas aeruginosa*." *International Journal of Antimicrobial Agents* 26 (2): 159–164.

Chang, Hsiao-Han, Ted Cohen, Yonatan H Grad, William P Hanage, Thomas F O'Brien, and Marc Lipsitch. 2015. "Origin and Proliferation of Multiple-Drug Resistance in Bacterial Pathogens." *Microbiology and Molecular Biology Reviews* 79 (1): 101-116.

Chantziaras, Ilias, Filip Boyen, Callens Bénédicte, and Jeroen Dewulf. 2014. "Correlation between Veterinary Antimicrobial Use and Antimicrobial Resistance in Food-Producing Animals: A Report on Seven Countries." *The Journal of Antimicrobial Chemotherapy* 69 (3): 827-834.

Chee-Sanford, Joanne C, R I Aminov, I J Krapac, N Garrigues-JeanJean, and R I Mackie. 2001. "Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities." *Applied and Environmental Microbiology* 67 (4): 1494-1502.

Chee-Sanford, Joanne C, Roderick I Mackie, Satoshi Koike, Ivan G Krapac, Yu-Feng Lin, Anthony C Yannarell, Scott Maxwell, and Rustam I Aminov. 2009. "Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste." *Journal of Environmental Quality* 38 (3): 1086-1108.

Chen, Xijuan, Jeppe Lund Nielsen, Karolina Furgal, Yaling Liu, Ihab Bishara Lolas, and Kai Bester. 2011. "Biodegradation of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions." *Chemosphere* 84 (4): 452-456.

Cherednichenko, Gennady, Rui Zhang, Roger A Bannister, Valeriy Timofeyev, Ning Li, Erika B Fritsch, Wei Feng, Genaro C Barrientos, Nils H Schebb, Bruce D Hammock, Kurt G Beam, Nipavan

Chiamvimonvat, Isaac N Pessah. 2012. "Triclosan impairs excitation-contraction coupling and Ca^{2+} dynamics in striated muscle." *Proceedings of the National Academy of Sciences of the United States of America* 109, 35 (8): 14158-14163.

Chuanchuen, Rungtip, Kerry Beinlich, Tung T Hoang, Anna Becher, RoxAnn R Karkhoff-Schweizer, and Herbert P Schweizer. 2001. "Cross-Resistance between Triclosan and Antibiotics in *Pseudomonas aeruginosa* is Mediated by Multidrug Efflux Pumps: Exposure of a Susceptible Mutant Strain to Triclosan Selects *Nfxb* Mutants Overexpressing MexCD-OprJ." *Antimicrobial Agents and Chemotherapy* 45 (2): 428–432.

Chuanchuen, R., Narasaki, C. T., and Schweizer, H. P. 2002. "The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of TCS." *Journal of Bacteriology* 184 (18): 5036-5044.

Chuanchuen, R., Wannaprasat, W., Ajariyakhajorn, K., and P. Schweizer, H. 2008. Role of the MexXY Multidrug Efflux Pump in Moderate Aminoglycoside Resistance in *Pseudomonas aeruginosa* isolates from *Pseudomonas* mastitis." *Microbiology and Immunology* 52 (4): 392-398.

Ciusa, M. L., Furi, L., Oggioni, M. R., Knight, D., Morrissey, I., Decorosi, F., Viti, C., Martinez, J. L. 2012. "A novel resistance mechanism to TCS that suggests horizontal gene transfer and demonstrates a potential selective pressure for reduced biocide susceptibility in clinical strains of *Staphylococcus aureus*." *International Journal of Antimicrobial Agents* 40 (3): 210-220.

Clayton EM, Todd M, Dowd JB, and Aiello AE. 2011. "The Impact of Bisphenol A and Triclosan on Immune Parameters in the U.S. Population, NHANES 2003-2006." *Environmental Health Perspectives* 119 (3): 390–396.

Collignon, Peter, Frank M Aarestrup, Rebecca Irwin, and Scott McEwen. 2013. "Human Deaths and Third-Generation Cephalosporin Use in Poultry, Europe." *Emerging Infectious Diseases* 19 (8): 1339–1340.

Coogan, M. A., Edziyie, R. E., La, P. T. W., and Venables, B. J. 2007. "Algal bioaccumulation of triclocarban, TCS, and methyl-TCS in a North Texas wastewater treatment plant receiving stream." *Chemosphere* 67 (10): 1911-1918.

Coogan, Melinda A, and Thomas W La Point. 2008. "Snail bioaccumulation of triclocarban, triclosan, and methyltriclosan in a North Texas, USA stream affected by wastewater treatment plant runoff ." *Environmental Toxicology and Chemistry* 27 (8): 1788-1793.

Cooper RM, L Tsimring, and J Hasty. 2017. "Inter-species population dynamics enhance microbial horizontal gene transfer and spread of antibiotic resistance." *ELife* 6 (11): 1-26.

Crawford, Brent R., and Denys de Catanzaro. 2012. "Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A." *Reproductive Toxicology* 34 (4): 607-613.

Crnich CJ, Gordon B, and Andes D. 2003. "Hot Tub-Associated Necrotizing Pneumonia Due to *Pseudomonas aeruginosa." Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 36 (3): 55–57.

Crofton, K. M., Paul, K. B., De Vito, M. J., Hedge, J. M. 2007. "Shortterm *in vivo* exposure to the water contaminant triclosan: Evidence for disruption of thyroxine." *Environmental Toxicology and Pharmacology* 24, 2 (9): 194−197.

Dann, Andrea B., and Alice Hontela. 2011. "Triclosan: environmental exposure, toxicity and mechanisms of action." *Journal of Applied Toxicology* 31 (4): 285-311.

DeLorenzo, M.E, J Brooker, K.W Chung, M Kelly, J Martinez, J.G Moore, and M Thomas. 2016. "Exposure of the Grass Shrimp, *Palaemonetes Pugio*, to Antimicrobial Compounds Affects Associated Vibrio Bacterial Density and Development of Antibiotic Resistance." *Environmental Toxicology* 31 (4): 469–77.

Dhillon GS, Kaur S, Pulicharla R, Brar SK, Cledón M, Verma M, and Surampalli RY. 2015. "Triclosan: Current Status, Occurrence, Environmental Risks and Bioaccumulation Potential." *International Journal of Environmental Research and Public Health* 12 (5): 5657–84.

Drury, Bradley, John Scott, Emma J Rosi-Marshall, and John J Kelly. 2013. "Triclosan Exposure Increases Triclosan Resistance and Influences Taxonomic Composition of Benthic Bacterial Communities." *Environmental Science & Technology* 47, 15 (7): 8923-8930.

Drury, Bradley, Emma Rosi-Marshall, and John J Kelly. 2013. "Wastewater Treatment Effluent Reduces the Abundance and Diversity of Benthic Bacterial Communities in Urban and Suburban Rivers." *Applied and Environmental Microbiology* 79 (6): 1897–97.

Elliott SD, and Barnes EM. 1959. "Changes in Serological Type and Antibiotic Resistance of Lancefield Group D Streptococci in Chickens Receiving Dietary Chlortetracycline." *Journal of General Microbiology* 20, 2 (4): 426–433.

Eriksson, K. Martin, C. Henrik Johansson, Viktor Fihlman, Alexander Grehn, Kemal Sanli, Mats X. Andersson, Hans Blanck, Åsa Arrhenius, Triranta Sircar, and Thomas Backhaus. 2015. "Long-term effects of the antibacterial agent triclosan on marine periphyton communities." *Environmental Toxicology and Chemistry* 34 (9): 2067-2077.

Etzel TM, Calafat AM, Ye X, Chen A, Lanphear BP, Savitz DA, Yolton K, and Braun JM. 2017. "Urinary Triclosan Concentrations during Pregnancy and Birth Outcomes." *Environmental Research* 156 (7): 505–511.

EUCAST 2016. "European Committee on Antimicrobial Susceptibility Testing." *European Society of Clinical Microbiology and Infectious Diseases*. http://www.eucast.org/mic_distributions_and_ecoffs/.

EC. European Commission. 2016 Jan 27. Commission Implementing Decision (EU) 2016/110 of 27 January 2016 not approving triclosan as an existing active substance for use in biocidal products for producttype 1. EUR-Lex; [accessed 2018 Oct 12]. https://eur-lex.europa.eu/eli/dec_impl/2016/110/oj

Fair, Patricia A., Hing-Biu Lee, Jeff Adams, Colin Darling, Grazina Pacepavicius, Mehran Alaee, Gregory D. Bossart, Natasha Henry, and Derek Muir. 2009. "Occurrence of triclosan in plasma of wild Atlantic bottlenose dolphins (*Tursiops truncatus*) and in their environment." *Environmental Pollution* 157 (8): 2248-2254.

Fang, J.L., R. Stingley, F. Beland, W. Harrouk, D. Lumpkins, and P. Howard. 2010. "Occurrence, Efficacy, Metabolism, and Toxicity of Triclosan." *Journal of Environmental Science and Health*, Part C 28 (3): 147–171.

Ferreira Da Silva, Miguel, Igor Tiago, Veríssimo António, Rui A. R Boaventura, Olga C Nunes, and Manaia Célia M. 2006. "Antibiotic Resistance of Enterococci and Related Bacteria in an Urban Wastewater Treatment Plant." *FEMS Microbiology Ecology* 55 (2): 322–329.

FDA. U.S. Food and Drug Administration. 2016. "FDA Issues Final Rule on Safety and Effectiveness of Antibacterial Soaps: Rule Removes Triclosan and Triclocarban from over-the-Counter Antibacterial Hand and Body Washes." *U.S. Department of Health and Human Services*. https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm517478.htm.

Forsberg, Kevin J, Alejandro Reyes, Bin Wang, Elizabeth M Selleck, Morten O A Sommer, and Gautam Dantas. 2012. "The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens." *Science* 337, 6098 (8): 1107–1111.

Forslund, Kristoffer, Shinichi Sunagawa, Jens Roat Kultima, Daniel R Mende, Manimozhiyan Arumugam, Athanasios Typas, and Peer Bork. 2013. "Country-Specific Antibiotic Use Practices Impact the Human Gut Resistome." *Genome Research* 23 (7): 1163-1169.

Gardeström Johanna, Matti Ermold, Willem Goedkoop, and Brendan G McKie. 2016. "Disturbance History Influences Stressor Impacts: Effects of a Fungicide and Nutrients on Microbial Diversity and Litter Decomposition." *Freshwater Biology* 61 (12): 2171–2184.

Gasperi J, Geara D, Lorgeoux C, Bressy A, Zedek S, Rocher V, El Samrani A, Chebbo G, and Moilleron R. 2014. “First Assessment of Triclosan, Triclocarban and Paraben Mass Loads at a Very Large Regional Scale: Case of Paris Conurbation (France).” *The Science of the Total Environment* 493 (6): 854–861.

Gautam P., Carsella J.S., and Kinney C.A. 2014. "Presence and transport of the antimicrobials triclocarban and triclosan in a wastewater-dominated stream and freshwater environment.” *Water Research* 48 (9): 247-256.

Gaynes, Robert and Jonathan R. Edwards and The National Nosocomial Infections Surveillance System. 2005. “Overview of Nosocomial Infections Caused by Gram-Negative Bacilli.” *Clinical Infectious Diseases* 41 (6): 848–854.

Gaze, William H, Lihong Zhang, Nouradin A Abdouslam, Peter M Hawkey, Leo Calvo-bado, Jeremy Royle, Helen Brown, *et al.* 2011. “Impacts of Anthropogenic Activity on the Ecology of Class 1 Integrons and Integron-Associated Genes in the Environment.” *The ISME Journal* 5 (8): 1253–1261.

Gee, R. H., A. Charles, N. Taylor, and P. D. Darbre. 2008. "Oestrogenic and androgenic activity of triclosan in breast cancer cells.” *Journal of Applied Toxicology* 28 (1): 78-91.

Geer LA, BFG Pycke, J Waxenbaum, DM Sherer, O Abulafia, and RU Halden. 2017. "Association of birth outcomes with fetal exposure to parabens, triclosan and triclocarban in an immigrant population in Brooklyn, New York.” *Journal of Hazardous Materials* 323 (2): 177-183.

Gelbrand, H., Miller-Petrie, M., Pant, S., Gandra, S., Levinson, J., Barter, D., *et al.* 2015. “The State of the World's Antibiotics 2015.*” Wound Healing Southern Africa*, 8: 30–34.

Geuenich HH, and Müller HE. 1984. “Das Vorkommen Von *Pseudomonas aeruginosa* Im Abwasser Und Ihr Verhalten Bei Der Biologischen Klärung.” Zentralblatt Fur Bakteriologie, Mikrobiologie Und Hygiene. 1. Abt. Originale B, *Hygiene* 179 (3): 259–65.

Gillings, Michael R. 2014. “Integrons: Past, Present, and Future.” *Microbiology and Molecular Biology Reviews* 78 (2): 257–57.

Giuliano, Christopher A, and Michael J Rybak. 2015. “Efficacy of Triclosan as an Antimicrobial Hand Soap and Its Potential Impact on Antimicrobial Resistance: A Focused Review.” *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 35 (3): 328–36

Goli, Hamid Reza, Mohammad Reza Nahaei, Mohammad Ahangarzadeh Rezaee, Alka Hasani, Hossein Samadi Kafil, Mohammad Aghazadeh, and Vajihe Sheikhalizadeh. 2016. “Contribution of mexAB-oprM and mexXY (-oprA) Efflux Operons in Antibiotic Resistance of Clinical *Pseudomonas aeruginosa* Isolates in Tabriz, Iran.” *Infection, Genetics and Evolution* 45 (8): 75–82.

Gorovtsov, Andrey Vladimirovich, Ivan Sergeevich Sazykin, and Marina Alexandrovna Sazykina. 2018. “The Influence of Heavy Metals, Polyaromatic Hydrocarbons, and Polychlorinated Biphenyls Pollution on the Development of Antibiotic Resistance in Soils.” *Environmental Science and Pollution Research* 25 (10): 9283–9292.

Graham, David W, Susana Olivares-Rieumont, Charles W. Knapp, Lazaro Lima, David Werner, and Emma Bowen. 2011. “Antibiotic Resistance Gene Abundances Associated with Waste Discharges to the Almendares River near Havana, Cuba.” *Environmental Science & Technology* 45 (2): 418–424.

Granata G, and Petrosillo N. 2017. “Resistance to Colistin in *Klebsiella pneumoniae*: A 4.0 Strain?” *Infectious Disease Reports* 9, 2 (4): 69-72.

Halden, Rolf U. 2014. “On the Need and Speed of Regulating Triclosan and Triclocarban in the United States.” *Environmental Science & Technology* 48 (7): 3603–3611.

Halden, Rolf U., and D.H. Paull, 2005. “Co-occurrence of triclocarban and triclosan in U.S. water resources” *Environmental Science & Technology* (39) 1420-1426.

Halden RU, Lindeman AE, Aiello AE, Andrews D, Arnold WA, Fair P, Fuoco RE, *et al.* 2017. “The Florence Statement on Triclosan and Triclocarban.” *Environmental Health Perspectives* 125 (6): 064501-1–13

Hale, Robert C., Craig L. Smith, Paul O. de Fur, Ellen Harvey, Elizabeth O. Bush, Mark J. La Guardia, and George G. Vadas. 2000. "Nonylphenols in sediments and effluents associated with diverse wastewater outfalls.” *Environmental Toxicology and Chemistry* 19 (4): 946-952.

Han, Jeonghoon, Eun-Ji Won, Un-Ki Hwang, Il-Chan Kim, Joung Han Yim, and Jae-Seong Lee. 2016. “Triclosan (TCS) and Triclocarban (TCC) Cause Lifespan Reduction and Reproductive Impairment through Oxidative Stress-Mediated Expression of the Defensome in the Monogonont Rotifer (*Brachionus Koreanus*).” *Comparative Biochemistry and Physiology, Part C* 185-186 (4): 131–137.

Hartmann, Erica M, Roxana Hickey, Tiffany Hsu, Betancourt Román Clarisse M, Jing Chen, Randall Schwager, Jeff Kline, *et al.* 2016. “Antimicrobial Chemicals Are Associated with Elevated Antibiotic Resistance Genes in the Indoor Dust Microbiome.” *Environmental Science & Technology* 50 (18): 9807-9815.

Heidler, Jochen, and Rolf U. Halden. 2007. "Mass balance assessment of triclosan removal during conventional sewage treatment.” *Chemosphere* 66 (2): 362-369.

Hejazi A, and Falkiner FR. 1997. “*Serratia Marcescens*.” *Journal of Medical Microbiology* 46 (11): 903–912.

Hernández, Alvaro, Federico M Ruiz, Antonio Romero, and Martínez José L. 2011. “The Binding of Triclosan to SmeT, the Repressor of the Multidrug Efflux Pump SmeDEF, Induces Antibiotic Resistance in *Stenotrophomonas maltophilia*.” *PLoS Pathogens* 7,1 (6): 1-12.

Hernandez-Raquet, Guillermina, Elodie Durand, Florence Braun, Cristiana Cravo-Laureau, and Jean-Jacques Godon. 2013. “Impact of Microbial Diversity Depletion on Xenobiotic Degradation by Sewage-Activated Sludge.” *Environmental Microbiology Reports* 5 (4): 588–94.

Higgins, C. P., Paesani, Z. J., Chalew, T. E. A., Halden, R. U., Hundal, L. S. 2011. “Persistence of triclocarban and triclosan in soils after land application of biosolids and bioaccumulation in *Eisenia Foetida*.” *Environmental Toxicology and Chemistry* 30 (3): 556−563.

Hu J, Raikhel V, Gopalakrishnan K, Fernandez-Hernandez H, Lambertini L, Manservisi F, Falcioni L, *et al.* 2016. “Effect of Postnatal Low-Dose Exposure to Environmental Chemicals on the Gut Microbiome in a Rodent Model.” *Microbiome* 4, 1 (6): 26–36.

Huang, Xiaolong, Chenxi Wu, Xiong Xiong, Kai Zhang, and Jiantong Liu. 2014. "Partitioning and Degradation of Triclosan and Formation of Methyl-Triclosan in Water-Sediment Systems.” *Water, Air, Soil Pollution: An International Journal of Environmental Pollution* 225, 9 (9): 1-10.

Huhulescu, S, M Simon, M Lubnow, M Kaase, G Wewalka, A T Pietzka, Stöger A, W Ruppitsch, and F Allerberger. 2011. “Fatal *Pseudomonas aeruginosa* Pneumonia in a Previously Healthy Woman Was Most Likely Associated with a Contaminated Hot Tub.” *Infection* 39 (3): 265–269.

Ishibashi, Hiroshi, Naomi Matsumura, Masashi Hirano, Munekazu Matsuoka, Hideki Shiratsuchi, Yasuhiro Ishibashi, Yuji Takao, and Koji Arizono. 2004. "Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin.” *Aquatic Toxicology* 67 (2): 167-179.

Jackson-Browne, Medina S., George D. Papandonatos, Aimin Chen, Antonia M. Calafat, Kimberly Yolton, Bruce P. Lanphear, and Joseph M.Braun. 2018. “Identifying Vulnerable Periods of Neurotoxicity to Triclosan Exposure in Children.” *Environmental Health Perspectives* 126, 5 (5): 1-9.

Jacobs, Liezl, and Hafizah Y Chenia. 2007. “Characterization of Integrons and Tetracycline Resistance Determinants in *Aeromonas* Spp. Isolated from South African Aquaculture Systems.” *International Journal of Food Microbiology* 114 (3): 295–306.

Jadhav, Savita, Tushar Sahasrabudhe, Vipul Kalley, Nageswari Gandham. 2013. “The Microbial Colonization Profile of Respiratory Devices and the Significance of the Role of Disinfection: A Blinded Study.” *Journal of Clinical and Diagnostic Research* 7 (6): 1021-1026.

Jahne, Michael A., Shane W. Rogers, Ivan P. Ramler, Edith Holder, and Gina Hayes. 2015. "Hierarchal clustering yields insight into multidrug-resistant bacteria isolated from a cattle feedlot wastewater treatment system." *Environmental Monitoring and Assessment* 187 (1): 1-15.

James MO, W Li, DP Summerlot, L Rowland-Faux, and CE Wood. 2010. "Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta." *Environment International* 36 (8): 942-949.

Johansson, C. Henrik, Lisa Janmar, and Thomas Backhaus. 2014. "Triclosan causes toxic effects to algae in marine biofilms, but does not inhibit the metabolic activity of marine biofilm bacteria." *Marine Pollution Bulletin* 84, 1-2 (7): 208-212.

Jung, Eui-Man, Beum-Soo An, Kyung-Chul Choi, and Eui-Bae Jeung. 2012. "Potential estrogenic activity of triclosan in the uterus of immature rats and rat pituitary GH3 cells." *Toxicology Letters.* 208 (2): 142-148.

Jurewicz, Joanna, Michał Radwan, Bartosz Wielgomas, Kałużny Paweł, Anna Klimowska, Paweł Radwan, and Wojciech Hanke. 2018. "Environmental Levels of Triclosan and Male Fertility." *Environmental Science and Pollution Research* 25 (6): 5484–5490.

Jyrkänkallio-Mikkola Jenny, Sandra Meier, Jani Heino, Tiina Laamanen, Virpi Pajunen, Kimmo T Tolonen, Mikko Tolkkinen, and Janne Soininen. 2017. "Disentangling Multi-Scale Environmental Effects on Stream Microbial Communities." *Journal of Biogeography* 44 (7): 1512–1523.

Karatzas, Kimon A G, Mark A Webber, Frieda Jorgensen, Martin J Woodward, Laura J V Piddock, and Tom J Humphrey. 2007. "Prolonged Treatment of *Salmonella enterica* Serovar *Typhimurium* with Commercial Disinfectants Selects for Multiple Antibiotic Resistance, Increased Efflux and Reduced Invasiveness." *The Journal of Antimicrobial Chemotherapy* 60 (5): 947–955.

Khan, Raees, Hyun Gi Kong, Yong-Hoon Jung, Jinhee Choi, Kwang-Yeol Baek, Eul Chul Hwang, and Seon-Woo Lee. 2016. "Triclosan Resistome from Metagenome Reveals Diverse Enoyl Acyl Carrier Protein Reductases and Selective Enrichment of Triclosan Resistance Genes." *Scientific Reports* 6 (8): 1-13.

Kim, Ji Hyung, Casiano H Choresca, Sang Phil Shin, Jee Eun Han, Jin Woo Jun, and Se Chang Park. 2011. "Occurrence and Antibiotic Resistance of *Vibrio vulnificus* in Seafood and Environmental Waters in Korea." *Journal of Food Safety* 31 (4): 518-524.

Kinney, Chad A, Edward T Furlong, Steven D Zaugg, and Mark R Burkhardt. 2006. "Survey of Organic Wastewater Contaminants in Biosolids Destined for Land Application." *Environmental Science & Technology* 40 (23): 7207–15.

Kinney, Chad A, Edward T Furlong, Dana W Kolpin, Mark R Burkhardt, Steven D Zaugg, Stephen L Werner, Joseph P Bossio, and Mark J Benotti. 2008. "Bioaccumulation of Pharmaceuticals and Other Anthropogenic Waste Indicators in Earthworms from Agricultural Soil Amended With Biosolid or Swine Manure." *Environmental Science & Technology* 42, 6 (2): 1863-1870.

Klindworth, Anna, Elmar Pruesse, Timmy Schweer, Jörg Peplies, Christian Quast, Matthias Horn, and Frank Oliver Glöckner. 2013. "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies." *Nucleic Acids Research* 41 (1): 1-11.

Kitiyodom, Sirikorn, Sirintip Khemtong, Janenuj Wongtavatchai, and Rungtip Chuanchuen. 2010. "Characterization of antibiotic resistance in *Vibrio spp*. isolated from farmed marine shrimps *Penaeus monodon*." *FEMS Microbiology Ecology.* 72 (2): 219-227.

Koeppe, Erika S., Kelly K. Ferguson, Justin A. Colacino, and John D. Meeker. 2013. "Relationship between urinary triclosan and paraben concentrations and serum thyroid measures in NHANES 2007-2008." *Science of the Total Environment* 445-446 (2): 299-305.

Kolpin, Dana W, Edward T Furlong, Michael T Meyer, and E Michael Thurman. 2002. "Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance." *Environmental Science & Technology* 36 (6): 1202-1211.

Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, Goossens H, and Laxminarayan R. 2018. "Global Increase and Geographic Convergence in Antibiotic Consumption between 2000 and 2015." *Proceedings of the National Academy of Sciences of the United States of America* 115, 15 (4): E3463-E3470.

Kumar, Kurunthachalam Senthil, S Mahalakshmi Priya, Aaron M Peck, and Kenneth S Sajwan. 2010. "Mass Loadings of Triclosan and Triclocarbon from Four Wastewater Treatment Plants to Three Rivers and Landfill in Savannah, Georgia, USA." *Archives of Environmental Contamination and Toxicology* 58 (2): 275-285.

Kumar S, Rizvi M, and Berry N. 2008. "Rising Prevalence of Enteric Fever Due to Multidrug-Resistant Salmonella: An Epidemiological Study." *Journal of Medical Microbiology* 57: 1247–1250.

Kumar, Vikas, Ajanta Chakraborty, Mool Raj Kural, and Partha Roy. 2009. "Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with triclosan." *Reproductive Toxicology* 27 (2): 177-185.

Lan, Zhou, Tae Hyung Kim, Kai Shun Bi, Xiao Hui Chen, and Hyung Sik Kim. 2015. "Triclosan Exhibits a Tendency to Accumulate in the Epididymis and Shows Sperm Toxicity in Male Sprague-Dawley Rats." *Environmental Toxicology* 30 (1): 83–91.

Lassen TH, Frederiksen H, Kyhl HB, Swan SH, Main KM, Andersson AM, Lind DV, *et al.* 2016. "Prenatal Triclosan Exposure and Anthropometric Measures Including Anogenital Distance in Danish Infants." *Environmental Health Perspectives* 124 (8): 1261–1268.

Lawrence, J. R., Zhu, B., Swerhone, G. D. W., Roy, J., Wassenaar, L. I., Topp, E., & Korber, D. R. 2009. "Comparative microscale analysis of the effects of TCS and triclocarban on the structure and function of river biofilm communities." *Science of the Total Environment* 407 (10): 3307-3316.

Leff, L. G., J. R. Dana, J. V. McArthur, and L. G. Shimkets. 1993. "Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA." *Applied Environmental Microbiology* 59, 2 (2): 417–421.

Lehutso, Raisibe F, Adegbenro P Daso, and Jonathan O Okonkwo. 2017. "Occurrence and Environmental Levels of Triclosan and Triclocarban in Selected Wastewater Treatment Plants in Gauteng Province, South Africa." *Emerging Contaminants* 3 (3): 107–114.

Li, Shengxu, Jinying Zhao, Guangdi Wang, Yun Zhu, Felicia Rabito, Marie Krousel-Wood, Wei Chen, and Paul K Whelton. 2015. "Urinary Triclosan Concentrations Are Inversely Associated with Body Mass Index and Waist Circumference in the US General Population: Experience in Nhanes 2003-2010." *International Journal of Hygiene and Environmental Health* 218 (4): 401–406.

Lin, Dasong, Ye Li, Qixing Zhou, Yingming Xu, and Di Wang. 2014. "Effect of Triclosan on Reproduction, DNA Damage and Heat Shock Protein Gene Expression of the Earthworm *Eisenia Fetida*." *Ecotoxicology* 23 (10): 1826–32.

Lowry, Richard. "T-Test for Independent or Correlated Samples." *Vassar Statistics*. 2018. http://vassarstats.net/index.html.

Lu XM, and Lu PZ. 2014. "Characterization of Bacterial Communities in Sediments Receiving Various Wastewater Effluents with High-Throughput Sequencing Analysis." *Microbial Ecology* 67 (3): 612–623.

Łuczkiewicz, A, K Jankowska, Fudala-Książek S, and Olańczuk-Neyman K. 2010. "Antimicrobial Resistance of Fecal Indicators in Municipal Wastewater Treatment Plant." *Water Research* 44 (17): 5089–97.

Lundov, M. D, and C Zachariae. 2008. "Recalls of Microbiologically Contaminated Cosmetics in Eu from 2005 to May 2008." *International Journal of Cosmetic Science* 30 (6): 471–74.

Lydon, KA, Glinski, DA, Westrich, JR, Henderson, WM and EK Lipp. 2017. Effects of triclosan on bacterial community composition and *Vibrio* populations in natural seawater microcosms. *Elementa: Science of the Anthropocene* 5 (5): 22-1-16.

Macedo, S, T Torres, and MM Santos. 2017. "Methyl-triclosan and triclosan impact embryonic development of *Danio rerio* and *Paracentrotus lividus*." *Ecotoxicology* 26 (4): 482-489.

Macherius, André, David R. Lapen, Thorsten Reemtsma, Jörg Römbke, Edward Topp, and Anja Coors. 2014. "Triclocarban, triclosan and its transformation product methyl triclosan in native earthworm species four years after a commercial-scale biosolids application." *Science of the Total Environment* 472: 235-238.

Magalhães, Mary, Gemilson Pontes, Paula Serra, Antonio Balieiro, Diogo Castro, Fabio Pieri, James Crainey, Paulo Nogueira, and Patricia Orlandi. 2016. "Multidrug resistant *Pseudomonas aeruginosa* survey in a stream receiving effluents from ineffective wastewater hospital plants." *BMC Microbiology* 16, 193 (10): 1-8.

Mahlen SD. 2011. "Serratia infections: from military experiments to current practice." *Clinical Microbiology Reviews.* 24 (4): 755-791.

Marathe, Nachiket P, Viduthalai R Regina, Sandeep A Walujkar, Edward R B Moore, D G Joakim Larsson, and Yogesh S Shouche. 2013. "A Treatment Plant Receiving Waste Water from Multiple Bulk Drug Manufacturers Is a Reservoir for Highly Multi-Drug Resistant Integron-Bearing Bacteria." *PLoS One* 8 (10): 1-11.

Martinez JL. 2009. "Environmental Pollution by Antibiotics and by Antibiotic Resistance Determinants." *Environmental Pollution* 157 (11): 2893–2902.

Maskey, Ashish P, Buddha Basnyat, Guy E Thwaites, James I Campbell, Jeremy J Farrar, and Mark D Zimmerman. 2008. "Emerging Trends in Enteric Fever in Nepal: 9124 Cases Confirmed by Blood Culture 1993-2003." *Transactions of the Royal Society of Tropical Medicine and Hygiene* 102 (1): 91–95.

Matsumura N, H Ishibashi, M Hirano, Y Nagao, N Watanabe, H Shiratsuchi, T Kai, T Nishimura, A Kashiwagi, and K Arizono. 2005. "Effects of nonylphenol and triclosan on production of plasma vitellogenin and testosterone in male South African clawed frogs (*Xenopus laevis*)." *Biological & Pharmaceutical Bulletin* 28 (9): 1748-1751.

Mavri, Ana, Marija Kurincic, and Sonja Smole Mozina. 2012. "The Prevalence of Antibiotic and Biocide Resistance among *Campylobacter coli* and *Campylobacter jejuni* from Different Sources." *Food Technology and Biotechnology* 50 (3): 371-376.

McArthur JV, and Tuckfield RC. 2000. “Spatial Patterns in Antibiotic Resistance among Stream Bacteria: Effects of Industrial Pollution.” *Applied and Environmental Microbiology* 66 (9): 3722–3726.

McAvoy, Drew C, Bert Schatowitz, Martin Jacob, Armin Hauk, and William S Eckhoff. 2002. “Measurement of Triclosan in Wastewater Treatment Systems.” *Environmental Toxicology and Chemistry* 21 (7): 1323–1329.

McEachran, Andrew D, Brett R Blackwell, J. Delton Hanson, Kimberly J Wooten, Gregory D Mayer, Stephen B Cox, and Philip N Smith. 2015. “Antibiotics, Bacteria, and Antibiotic Resistance Genes: Aerial Transport from Cattle Feed Yards via Particulate Matter.” *Environmental Health Perspectives* 123, 4 (4): 337-343.

McMurry, L. M., Oethinger, M., Levy, S. B. 1998. “Triclosan targets lipid synthesis.” *Nature* 394 (8): 531−532.

McMurry, LM, McDermott, PF, & Levy, SB. 1999. “Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for TCS.” *Antimicrobial Agents and Chemotherapy* 43 (3): 711-713.

McNamara, Patrick J, Timothy M LaPara, and Paige J Novak. 2014. "The Impacts of Triclosan on Anaerobic Community Structures, Function, and Antimicrobial Resistance.” *Environmental Science & Technology* 48, 13 (6): 7393-7400.

McNamara PJ, and Levy SB. 2016. “Triclosan: An Instructive Tale.” *Antimicrobial Agents and Chemotherapy* 60 (12): 7015–7016.

Meeker, John D, David E Cantonwine, Luis O Rivera-González, Kelly K Ferguson, Bhramar Mukherjee, Antonia M Calafat, Xiaoyun Ye, *et al.* 2013. "Distribution, Variability, and Predictors of Urinary Concentrations of Phenols and Parabens among Pregnant Women in Puerto Rico.” *Environmental Science & Technology* 47 (3): 3439-3447.

Micek S.T, Wunderink R.G, Kollef M.H, Chen C, Rello J, Chastre J, Antonelli M, *et al.* 2015. “An International Multicenter Retrospective Study of *Pseudomonas aeruginosa* Nosocomial Pneumonia: Impact of Multidrug Resistance.” *Critical Care* 19, 219 (5): 1-8.

Michael I, Rizzo L, McArdell CS, Manaia CM, Merlin C, Schwartz T, Dagot C, and Fatta-Kassinos D. 2013. “Urban Wastewater Treatment Plants As Hotspots for the Release of Antibiotics in the Environment: A Review.” *Water Research* 47 (3): 957–995.

Middleton, June H., and James D. Salierno. 2013. "Antibiotic resistance in triclosan tolerant fecal coliforms isolated from surface waters near wastewater treatment plant outflows (Morris County, NJ, USA).” *Ecotoxicology and Environmental Safety* 88 (11): 79-88.

Miller, Todd R, J Heidler, SN Chillrud, A DeLaquil, JC Ritchie, JN Mihalic, R Bopp, and Rolf U Halden. 2008. "Fate of Triclosan and Evidence for Reductive Dechlorination of Triclocarban in Estuarine Sediments." *Environmental Science & Technology* 42 (12): 4570-4576.

Mima, Takehiko, Swati Joshi, Margarita Gomez-Escalada, and Herbert P Schweizer. 2007. "Identification and Characterization of TriABC-OpmH, a Triclosan Efflux Pump of *Pseudomonas aeruginosa* Requiring Two Membrane Fusion Proteins." *Journal of Bacteriology* 189, 21 (11): 7600-7609.

Miyazaki, T., Yamagishi, T., Matsumoto, M. 1984. "Residues of 4-chloro-1-(2,4-dichlorophenoxy)-2-methoxybenzene(triclosan methyl) in aquatic biota." *Bulletin of environmental contamination and toxicology.* 32 (2): 227–232.

Mohanta, Tandra, and Sudha Goel. 2014. "Prevalence of antibiotic-resistant bacteria in three different aquatic environments over three seasons*." Environmental Monitoring and Assessment: An International Journal Devoted to Progress in the Use of Monitoring Data in Assessing Environmental Risks to Man and the Environment.* 186 (8): 5089-5100.

Mohapatra H, Mohapatra SS, Mantri CK, Colwell RR, and Singh DV. 2008. "*Vibrio cholerae* Non-O1, Non-O139 Strains Isolated Before 1992 from Varanasi, India Are Multiple Drug Resistant, Contain *Intsxt*, *Dfr18* and *Aada5* Genes." *Environmental Microbiology* 10 (4): 866–73.

Montaseri, H, and PBC Forbes. 2016. "A Review of Monitoring Methods for Triclosan and Its Occurrence in Aquatic Environments." *Trends in Analytical Chemistry* 85: 221–231.

Moradigaravand D, Boinett CJ, Martin V, Peacock SJ, and Parkhill J. 2016. "Recent Independent Emergence of Multiple Multidrug-Resistant *Serratia Marcescens* Clones Within the United Kingdom and Ireland." *Genome Research* 26 (8): 1101–1109.

Morales, S., P. Canosa, I. Rodríguez, E. Rubí, and R. Cela. 2005. "Microwave assisted extraction followed by gas chromatography with tandem mass spectrometry for the determination of triclosan and two related chlorophenols in sludge and sediments." *Journal of Chromatography A.* 1082 (2): 128-135.

Morrall, Donna, Drew McAvoy, Bert Schatowitz, Josef Inauen, Martin Jacob, Armin Hauk, and William Eckhoff. 2004. "A Field Study of Triclosan Loss Rates in River Water (Cibolo Creek, TX)." *Chemosphere* 54 (5): 653–660.

Morroni, Gianluca, A Di Cesare, L Di Sante, A Brenciani, C Vignaroli, S Pasquaroli, Eleonora Giovanetti, *et al.* 2016. "Enterococcus faecium ST17 from Coastal Marine Sediment Carrying Transferable Multidrug Resistance Plasmids." *Microbial Drug Resistance* 22 (7): 523-530.

Mottaleb MA, S Usenko, JG O'Donnell, AJ Ramirez, BW Brooks, and CK Chambliss. 2009. "Gas chromatography-mass spectrometry screening methods for select UV filters, synthetic musks, alkylphenols, an antimicrobial agent, and an insect repellent in fish." *Journal of Chromatography* 1216 (5): 815-823.

New England Historic Genealogical Society. "Webinar: Choosing a DNA Test for Family History Research." *New England Historic Genealogical Society*. 2018. https://www.americanancestors.org/dna#anchor-1. To cite DNA image in Fig. 3.

Nietch, Christopher T, Erin L Quinlan, James M Lazorchak, Christopher A Impellitteri, David Raikow, and David Walters. 2013. "Effects of a chronic lower range of triclosan exposure on a stream mesocosm community." *Environmental Toxicology and Chemistry.* 32, 12 (8): 2874-2887.

Nnadozie, C. F, S Kumari, and F Bux. 2017. "Status of Pathogens, Antibiotic Resistance Genes and Antibiotic Residues in Wastewater Treatment Systems." *Reviews in Environmental Science and Bio/Technology* 16 (3): 491–515.

Novo, Ana, Sandra André, Paula Viana, Olga C Nunes, and Célia M Manaia. 2013. "Antibiotic Resistance, Antimicrobial Residues and Bacterial Community Composition in Urban Wastewater." *Water Research* 47 (5): 1875–1887.

NCDENR North Carolina Department of Environment & Natural Resources. "Cape Fear Basinwide Water Quality Plan." 2000. *NC Environmental Management Commission*.

N.W. 1972. "Hexachlorophene Curbed." *Science* 177, 4055 (9): 1175.

O'Flaherty, Eithne, and Enda Cummins. 2017. "Antibiotic Resistance in Surface Water Ecosystems: Presence in the Aquatic Environment, Prevention Strategies, and Risk Assessment." *Human and Ecological Risk Assessment* 23 (2): 299–322.

Osińska, Adriana, M Harnisz, and Ewa Korzeniewska. 2016. "Prevalence of plasmid-mediated multidrug resistance determinants in fluoroquinolone-resistant bacteria isolated from sewage and surface water." *Environmental Science and Pollution Research* 23 (11): 10818-10831.

Pan, Ya-ping, Yuan-hong Xu, Zhong-xin Wang, Ya-ping Fang, and Ji-lu Shen. 2016. "Overexpression of Mexab-Oprm Efflux Pump in Carbapenem-Resistant *Pseudomonas aeruginosa*." *Archives of Microbiology* 198 (6): 565–571.

Pannu, Manmeet W, George A O'Connor, and Gurpal S Toor. 2012. "Toxicity and bioaccumulation of biosolids-borne triclosan in terrestrial organisms." *Environmental Toxicology and Chemistry.* 31 (3): 646-653.

Paul, K. B., Hedge, J. M., DeVito, M. J., Crofton, K. M. 2010. “Shortterm exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in young Long-Evans rats.” *Toxicological Sciences* 113 (2): 367−379.

Paul, K. B., Hedge, J. M., Bansal, R., Zoeller, R. T., Peter, R., DeVito, M. J., Crofton, K. M. 2012. “Developmental triclosan exposure decreases maternal, fetal, and early neonatal thyroxine: A dynamic and kinetic evaluation of a putative mode-of-action.” *Toxicology* 300 (1−2), 31−45

Pérez Astrid, Delia Canle, Cristina Latasa, Margarita Poza, Alejandro Beceiro, del Mar Tomás María, Fernández Ana, *et al.* 2007. “Cloning, Nucleotide Sequencing, and Analysis of the Acrab-Tolc Efflux Pump of *Enterobacter cloacae* and Determination of its Involvement in Antibiotic Resistance in a Clinical Isolate.” *Antimicrobial Agents and Chemotherapy* 51 (9): 3247–53.

Philippat C, Botton J, Calafat AM, Ye X, Charles MA, Slama R, and EDEN Study Group. 2014. “Prenatal Exposure to Phenols and Growth in Boys.” *Epidemiology* 25 (5): 625–635.

Pi, Borui, Dongliang Yu, Xiaoting Hua, Zhi Ruan, and Yunsong Yu. 2017. "Genomic and transcriptome analysis of triclosan response of a multidrug-resistant *Acinetobacter baumannii* strain, MDR-ZJ06.” *Archives of Microbiology* 199 (2): 223-230.

Pintado-Herrera, MG, Gonzalez-Mazo, E, Lara-Martin, PA, 2014. “Determining the distribution of triclosan and methyl triclosan in estuarine settings.” *Chemosphere* 95 (11): 478–485.

Poirel, Laurent, Jose-Manuel Rodriguez-Martinez, Hedi Mammeri, Alain Liard, and Patrice Nordmann. 2005. “Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA.” *Antimicrobial Agents and Chemotherapy* 49 (8): 3523–3525.

Proia L, Morin S, Peipoch M, Romaní AM, and Sabater S. 2011. “Resistance and Recovery of River Biofilms Receiving Short Pulses of Triclosan and Diuron.” *The Science of the Total Environment* 409 (5): 3129–3137.

Pycke, B. F., Crabbé, A., Verstraete, W., and Leys, N. 2010. “Characterization of TCS-resistant mutants reveals multiple antimicrobial resistance mechanisms in *Rhodospirillum rubrum* S1H.” *Applied and Environmental Microbiology* 76 (10): 3116-3123.

Pycke, Benny F G, Laura A Geer, Mudar Dalloul, Ovadia Abulafia, Alizee M Jenck, and Rolf U Halden. 2014. "Human Fetal Exposure to Triclosan and Triclocarban in an Urban Population from Brooklyn, New York.” *Environmental Science & Technology* 48 (6): 8831-8838.

Quinn JP. 1998. “Clinical Problems Posed by Multiresistant Nonfermenting Gram-Negative Pathogens.” *Clinical Infectious Diseases* 27 (8): 117–124.

Raut, S.A., and R.A. Angus. 2010. "Triclosan has endocrine-disrupting effects in male western mosquitofish, *Gambusia affinis*." *Environmental Toxicology and Chemistry* 29 (6): 1287-1291.

Regnault C, J Willison, S Veyrenc, A Airieau, P Méresse, M Fortier, M Fournier, P Brousseau, M Raveton, and S Reynaud. 2016. "Metabolic and immune impairments induced by the endocrine disruptors benzo[*a*]pyrene and triclosan in *Xenopus tropicalis*." *Chemosphere* 155 (7): 519-527.

Reinthaler, Franz Ferdinand, Gebhard Feierl, Herbert Galler, Doris Haas, Eva Leitner, Franz Mascher, Angelika Melkes, Josefa Posch, Ingrid Winter, Gernot Zarfel, Egon Marth. 2010. "ESBL-producing *E. coli* in Austrian sewage sludge." *Water Research* 44, 6 (3): 1981–1985.

Ribado JV, Ley C, Haggerty TD, Tkachenko E, Bhatt AS, and Parsonnet J. 2017. "Household Triclosan and Triclocarban Effects on the Infant and Maternal Microbiome." *EMBO Molecular Medicine* 9 (12): 1732–1741.

Ricart, Marta, Helena Guasch, Mireia Alberch, Damià Barceló, Chloé Bonnineau, Anita Geiszinger, Marinel·la Farré, Josep Ferrer, Francesco Ricciardi, Anna M. Romani, Soizic Morin, Lorenzo Proia, Lluis Sala, David Sureda, Sergi Sabater. 2010. "Triclosan Persistence through Wastewater Treatment Plants and Its Potential Toxic Effects on River Biofilms." *Aquatic Toxicology* 100 (9): 346–353.

Rizzo, L, C Manaia, C Merlin, T Schwartz, C Dagot, M.C Ploy, I Michael, and D Fatta-Kassinos. 2013. "Urban Wastewater Treatment Plants as Hotspots for Antibiotic Resistant Bacteria and Genes Spread into the Environment: A Review." *Science of the Total Environment* 447: 345–360.

Rodríguez, Pablo E A, and Mónica S Sanchez. 2010. "Maternal Exposure to Triclosan Impairs Thyroid Homeostasis and Female Pubertal Development in Wistar Rat Offspring." *Journal of Toxicology and Environmental Health Part A.* 73,24 (6): 1678-1688.

Rosi-Marshall, Emma J., Dustin W. Kincaid, Heather A. Bechtold, Todd V. Royer, Miguel Rojas, and John J. Kelly. 2013. "Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms." *Ecological Applications* 23 (3): 583-593.

Rowe B, Ward LR, and Threlfall EJ. 1995. "Ciprofloxacin-Resistant *Salmonella* Typhi in the UK." *Lancet (London, England)* 346, 8985 (11): 1302.

Sachan, R, JH Cheon, MS Kim, YJ Kang, KS Kim, JY Son, BM Lee, and HS Kim. 2015. "Triclosan Induces Alteration of Sperm Production and Morphology through its Accumulation in the Epididymis of Sprague-Dawley Male Rats." *Toxicology Letters: Supplement* 238 (2): 361-362.

Saleh, S, R.N.S Haddadin, S Baillie, and P.J Collier. 2011. "Triclosan - an Update." *Letters in Applied Microbiology* 52 (11): 87-95.

Salyers AA, and CF Amábile-Cuevas. 1997. "Why are antibiotic resistance genes so resistant to elimination?" *Antimicrobial Agents and Chemotherapy* 41 (11): 2321-2325.

Salyers AA, A Gupta, and Y Wang. 2004. "Human intestinal bacteria as reservoirs for antibiotic resistance genes." *Trends in Microbiology* 12 (9): 412-416.

Sanchez, Patricia, Eduardo Moreno, and Jose L Martinez. 2005. "The Biocide Triclosan Selects *Stenotrophomonas maltophilia* Mutants That Overproduce the Smedef Multidrug Efflux Pump." *Antimicrobial Agents and Chemotherapy* 49 (2): 781–782.

Sapkota, AR, KK Ojo, MC Roberts, and KJ Schwab. 2006. "Antibiotic resistance genes in multidrug-resistant *Enterococcus spp*. and *Streptococcus spp.* recovered from the indoor air of a large-scale swine-feeding operation." *Letters in Applied Microbiology* 43 (5): 534-540.

Savage, Jessica H, Elizabeth C Matsui, Robert A Wood, and Corinne A Keet. 2012. "Urinary levels of triclosan and parabens are associated with aeroallergen and food sensitization." *Journal of Allergy and Clinical Immunology* 130 (2): 453-460.

Sayah, Radia S, John B Kaneene, Yvette Johnson, and RoseAnn Miller. 2005. "Patterns of Antimicrobial Resistance Observed in *Escherichia coli* Isolates Obtained from Domestic- and Wild-Animal Fecal Samples, Human Septage, and Surface Water." *Applied and Environmental Microbiology* 71 (3): 1394-1404.

Schaefer, EF. 1997. "A DNA assay to detect the toxic dinoflagellate *Pfiesteria piscicida*, and the application of a PCR based probe." MS Thesis. University of North Carolina, Greensboro

Schechner V, Temkin E, Harbarth S, Carmeli Y, and Schwaber MJ. 2013. "Epidemiological Interpretation of Studies Examining the Effect of Antibiotic Usage on Resistance." *Clinical Microbiology Reviews* 26 (2): 289–307.

Schmid MB, and N Kaplan. 2004. "Reduced triclosan susceptibility in methicillin-resistant *Staphylococcus epidermidis*." *Antimicrobial Agents and Chemotherapy* 48 (4): 1397-1399.

Schreiber, Christiane and Thomas Kistermann. 2013. "Antibiotic resistance among autochthonous aquatic environmental bacteria." *Water Science & Technology* 67 (1): 117-122

Schwartz, Thomas, Holger Volkmann, Silke Kirchen, Wolfgang Kohnen, Schön-Hölz Katja, Bernd Jansen, and Ursula Obst. 2006. "Real-Time Pcr Detection of *Pseudomonas aeruginosa* in Clinical and Municipal Wastewater and Genotyping of the Ciprofloxacin-Resistant Isolates." *Fems Microbiology Ecology* 57 (1): 158–167.

Scott, Geoffrey I., Dwayne E. Porter, R. Sean Norman, C. Hart Scott, Miguel I. Uyaguari-Diaz, Keith A. Maruya, Steve B. Weisberg, *et al.* 2016. "Antibiotics as CECs: An Overview of the Hazards Posed by Antibiotics and Antibiotic Resistance." *Frontiers in Marine Science* 24 (3): 1-15.

Singer, Heinz, Stephan Muller, Celine Tixier, and Laurent Pillonel. 2002. "Triclosan: Occurrence and fate of a widely used biocide in the aquatic environment: Field measurements in wastewater treatment plants, surface waters, and lake sediments." *Environmental Science & Technology* 36 (23): 4998-5004.

Šiširak M, and Hukić M. 2013. "An Outbreak of Multidrug-Resistant *Serratia marcescens*: The Importance of Continuous Monitoring of Nosocomial Infections." *Acta Medica Academica* 42 (1): 25–31.

Sjölund M, J Bonnedahl, J Hernandez, S Bengtsson, G Cederbrant, J Pinhassi, G Kahlmeter, and B Olsen. 2008. "Dissemination of multidrug-resistant bacteria into the Arctic." *Emerging Infectious Diseases* 14 (1): 70-72.

Sørum, Henning, and Trine Marie L'Abée-Lund. 2002. "Antibiotic resistance in food-related bacteria—a result of interfering with the global web of bacterial genetics." *International Journal of Food Microbiology* 78 (1): 43-56.

Stang, Christoph, David Elsaesser, Mirco Bundschuh, Thomas A Ternes, and Ralf Schulz. 2013. "Mitigation of Biocide and Fungicide Concentrations in Flow-Through Vegetated Stream Mesocosms." *Journal of Environmental Quality* 42 (6): 1889-1895.

Stangroom, Jeremy. "Z Score Calculator for 2 Population Proportions." *Social Science Statistics*. 2018. https://www.socscistatistics.com/tests/ztest/Default2.aspx.

Stasinakis, Athanasios S, Georgia Gatidou, Daniel Mamais, Nikolaos S Thomaidis, and Themistokles D Lekkas. 2008. "Occurrence and Fate of Endocrine Disrupters in Greek Sewage Treatment Plants." *Water Research* 42 (6-7): 1796–1804.

Starr MP, and Reynolds DM. 1951. "Streptomycin Resistance of Coliform Bacteria from Turkeys Fed Streptomycin." *American Journal of Public Health and the Nation's Health* 41 (11): 1375–80.

Stoker TE, EK Gibson, and LM Zorrilla. 2010. "Triclosan exposure modulates estrogen-dependent responses in the female wistar rat." *Toxicological Sciences: an Official Journal of the Society of Toxicology* 117 (1): 45-53.

Strateva T and D Yordanov 2009. "*Pseudomonas aeruginosa* - a phenomenon of bacterial resistance." *Journal of Medical Microbiology* 58 (9): 1133-1148.

Sun, Jingjing, Deng, Ziqing, and Yan, Aixin. 2014. “Bacterial Multidrug Efflux Pumps: Mechanisms, Physiology and Pharmacological Exploitations.” *Biochemical and Biophysical Research Communications* 453 (2): 254-267.

Tamura, Ikumi, Kei-ichiro Kagota, Y. Yasuda, S. Yoneda, J. Morita, N. Nakada, Yutaka Kameda, Kumiko Kimura, Norihisa Tatarazako, and Hiroshi Yamamoto. 2013. "Ecotoxicity and screening level ecotoxicological risk assessment of five antimicrobial agents: triclosan, triclocarban, resorcinol, phenoxyethanol and p-thymol.” *Journal of Applied Toxicology* 33 (11): 1222-1229.

Tang M, Dou X, Wang C, Tian Z, Yang M, and Zhang Y. 2017. “Abundance and Distribution of Antibiotic Resistance Genes in a Full-Scale Anaerobic-Aerobic System Alternately Treating Ribostamycin, Spiramycin and Paromomycin Production Wastewater.” *Environmental Geochemistry and Health* 39 (6): 1595–1605.

Tattooos.biz. “Fish And Pisces Symbol Tattoos Stencil - 2016 / 2017 Ideas.” *Tattoo Ideas*. 2017. http://tattooos.biz/fish-and-pisces-symbol-tattoos-stencil.html. –Fish image on Figure 3.

Tkachenko, Olga, Jessica Shepard, Virginie M Aris, Amy Joy, Anangely Bello, Indira Londono, Jehona Marku, Patricia Soteropoulos, and Marcy A Peteroy-Kelly. 2007. “A Triclosan-Ciprofloxacin Cross-Resistant Mutant Strain of *Staphylococcus aureus* Displays an Alteration in the Expression of Several Cell Membrane Structural and Functional Genes.” *Research in Microbiology* 158 (9): 651–658.

Tohidi, F., Cai, Z., 2017. “Fate and mass balance of triclosan and its degradation products: comparison of three different types of waste water treatments and aerobic/anaerobic sludge digestion.” *Journal of Hazardous Materials* 323 (4): 329–340.

Toms, Leisa-Maree L, Mats Allmyr, Jochen F Mueller, Margaretha Adolfsson-Erici, Michael McLachlan, John Murby, and Fiona A Harden. 2011. “Triclosan in Individual Human Milk Samples from Australia.” *Chemosphere* 85 (11): 1682–1686.

US Census Bureau. “American Fact Finder: ACS DEMOGRAPHIC AND HOUSING ESTIMATES 2012-2016 American Community Survey 5-Year Estimates.” *United States Census Bureau*. 2016. https://factfinder.census.gov/faces/tableservices/jsf/pages/productview.xhtml?src=CF.

Uyaguari, M.I., E.B. Fichot, G.I. Scott, and R.S. Norman. 2011. “Characterization and Quantitation of a Novel β-Lactamase Gene Found in a Wastewater Treatment Facility and the Surrounding Coastal Ecosystem.” *Applied and Environmental Microbiology* 77, 23 (9): 8226–8233.

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A., Laxminarayan, R. 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases* 14 (8) 742–750.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., Laxminarayan, R. 2015." Global trends in antimicrobial use in food animals." *Proceedings of the National Academy of Sciences of the United States of America* 112, 18 (5): 5649–5654.

Van den Berg, M., Linda Birnbaum, Albertus T.C. Bosveld, Bjorn Brunstron, Philip Cook, Mark Feeley, Tim Zacharewski, *et al*. 1998. "Toxic Equivalency Factors (TEFs) for PCBs, PDCCs, PCDFs for Humans and Wildlife." *Environmental Health Perspectives* 106, 12 (12): 775–792.

Van Heerden, L., Van Aswegen, H., S. Van Vuuren, A. Duse, and R. Roos. 2017. "Contamination of Nebulisers and Surrounding Air at the Bedside of Mechanically Ventilated Patients." *Southern African Journal of Critical Care* 33 (1): 23–27.

van Wijnen, Jikke, Ad M.J Ragas, and Carolien Kroeze. 2018. "River Export of Triclosan from Land to Sea: A Global Modelling Approach." *Science of the Total Environment* 621 (10): 1280–1288.

Vaz-Moreira, Ivone, Olga C Nunes, and Manaia Célia M. 2014. "Bacterial Diversity and Antibiotic Resistance in Water Habitats: Searching the Links with the Human Microbiome." *FEMS Microbiology Reviews* 38 (4): 761–78.

Veldhoen, Nik, Rachel C. Skirrow, Heather Osachoff, Heidi Wigmore, David J. Clapson, Mark P. Gunderson, Graham Van Aggelen, and Caren C. Helbing. 2006. "The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development." *Aquatic Toxicology* 80 (3): 217-227.

Vélez, Maria P, Tye E Arbuckle, and William D Fraser. 2015. "Female Exposure to Phenols and Phthalates and Time to Pregnancy: The Maternal-Infant Research on Environmental Chemicals (MIREC) Study." *Fertility and Sterility* 103 (4): 1011–1020.

Venkatesan, Arjun K., Benny F.G. Pycke, Larry B. Barber, Kathy E. Lee, and Rolf U. Halden. 2012. "Occurrence of triclosan, triclocarban, and its lesser chlorinated congeners in Minnesota freshwater sediments collected near wastewater treatment plants." *Journal of Hazardous Materials* 229-230 (8): 29-35.

Verlicchi, P, and E Zambello. 2015. "Pharmaceuticals and Personal Care Products in Untreated and Treated Sewage Sludge: Occurrence and Environmental Risk in the Case of Application on Soil — a Critical Review." *Science of the Total Environment* 538 (12): 750–767.

Vignaroli, C, G M Luna, C Rinaldi, A Di Cesare, R Danovaro, and F Biavasco. 2012. "New Sequence Types and Multidrug Resistance among Pathogenic *Escherichia coli* Isolates from Coastal Marine Sediments." *Applied and Environmental Microbiology* 78 (6): 3916-3922.

Victoria, Joan. “Family Pseudomonadaceae.” *Emaze.* 2018. https://www.emaze.com/@ALTILZWL/Family-PSEUDOMONADACEAE. – to cite a photo used in creation of Fig. 3

von der Ohe, Peter Carsten, Mechthild Schmitt-jansen, Jaroslav Slobodnik, and Werner Brack. 2012. "Triclosan--the forgotten priority substance?" *Environmental Science and Pollution Research International* 19 (2): 585-591.

Waltman, Elise Lyn, Barney J Venables, and William T Waller. 2006. "Triclosan in a North Texas Wastewater Treatment Plant and the Influent and Effluent of an Experimental Constructed Wetland .” *Environmental Toxicology and Chemistry.* 25 (2): 367-372.

Wang, Xiaohui, Man Hu, Yu Xia, Xianghua Wen, and Kun Ding. 2012. “Pyrosequencing Analysis of Bacterial Diversity in 14 Wastewater Treatment Systems in China.” *Applied and Environmental Microbiology* 78 (10): 7042–7047.

Wang, Xiaonan, Zhengtao Liu, Wanhua Wang, Zhenguang Yan, Cong Zhang, Weili Wang, and Lihong Chen. 2014. "Assessment of toxic effects of triclosan on the terrestrial snail *Achatina fulica*." *Chemosphere* 108 (8): 225-230.

Wang, Xiaoli, Xiaojiao Chen, Xuejiao Feng, Fei Chang, Minjian Chen, Yankai Xia, and Ling Chen. 2016. “Triclosan Causes Spontaneous Abortion Accompanied by Decline of Estrogen Sulfotransferase Activity in Humans and Mice.” *Scientific Reports* 5 (12): 1-11.

Watts, J. E. M., Schreier, H. J., Lanska, L., and Hale, M. 2017. “The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions.” *Marine Drugs* 15, 6 (6): 158-1-16.

Weatherly, Lisa M, and Julie A Gosse. 2017. “Triclosan Exposure, Transformation, and Human Health Effects.” *Journal of Toxicology and Environmental Health. Part B, Critical Reviews* 20 (8): 447–469.

Weiss, Lorelle, Tye E. Arbuckle, Mandy Fisher, Tim Ramsay, Ranjeeta Mallick, Russ Hauser, Alain LeBlanc, Mark Walker, Pierre Dumas, and Carly Lang. 2015. "Temporal variability and sources of triclosan exposure in pregnancy.” *International Journal of Hygiene and Environmental Health* 218 (6): 507-513.

Welsch, Tanner, and Eric Gillock. 2011. "Triclosan-resistant bacteria isolated from feedlot and residential soils.” *Journal of Environmental Science and Health, Part A* 46 (4): 436-440.

Wikler, Matthew A. 2009. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard*. Wayne, Pa: Clinical and Laboratory Standards Institute.

Wilson, Brittan A, Val H Smith, Frank Denoyelles, and Cynthia K Larive. 2003. “Effects of Three Pharmaceutical and Personal Care Products on Natural Freshwater Algal Assemblages.” *Environmental Science & Technology* 37, 9 (3): 1713–1719.

Wilson B, J Zhu, M Cantwell, and CR Olsen. 2008. "Short-term dynamics and retention of triclosan in the lower Hudson River Estuary.” *Marine Pollution Bulletin* 56 (6): 1230-1233.

Wolters B, Kyselková M, Krögerrecklenfort E, Kreuzig R, and Smalla K. 2014. “Transferable Antibiotic Resistance Plasmids from Biogas Plant Digestates Often Belong to the IncP-1ε Subgroup.” *Frontiers in Microbiology* 5 (1): 1-11.

WHO. World Health Organization. “WHO Priority Pathogens List for R & D of New Antibiotics.” 2017. http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf.

Xi, Xiaoxia, Jiachao Zhang, Laiyu Kwok, Dongxue Huo, Shuzhen Feng, Heping Zhang, and Tiansong Sun. 2015. “Microbial Pollution Tracking of Dairy Farm with a Combined PCR-DGGE and qPCR Approach.” *Current Microbiology* 71 (6): 678–686.

Xia, Kang, Lakhwinder S Hundal, Kuldip Kumar, Kevin Armbrust, Albert E Cox, and Thomas C Granato. 2010. "Triclocarban, triclosan, polybrominated diphenyl ethers, and 4-nonylphenol in biosolids and in soil receiving 33-year biosolids application.” *Environmental Toxicology and Chemistry* 29 (3): 597-605.

Xie, Z., R. Ebinghaus, G. Flöser, A. Caba, and Wolfgang Ruck. 2008. "Occurrence and distribution of triclosan in the German Bight (North Sea).” *Environmental Pollution* 156 (3): 1190-1195.

Xiong, Wenguang, Yongxue Sun, and Zhenling Zeng. 2018. “Antimicrobial Use and Antimicrobial Resistance in Food Animals.” *Environmental Science and Pollution Research* 25 (19): 18377–18384.

Xue, Jingchuan, Qian Wu, Sivasubramanian Sakthivel, Praveen V Pavithran, Jayakumar R Vasukutty, and Kurunthachalam Kannan. 2015. “Urinary Levels of Endocrine-Disrupting Chemicals, Including Bisphenols, Bisphenol A Diglycidyl Ethers, Benzophenones, Parabens, and Triclosan in Obese and Non-Obese Indian Children.” *Environmental Research* 137(2): 120–128.

Ying, Guang-Guo, and Rai S. Kookana. 2007. "Triclosan in wastewaters and biosolids from Australian wastewater treatment plants.” *Environment International.* 33 (2): 199-205.

Yoneda, Kazuhiko, Hiroki Chikumi, Takeshi Murata, Naomasa Gotoh, Hiroyuki Yamamoto, Hiromitsu Fujiwara, Takeshi Nishino, and Eiji Shimizu. 2005. “Measurement of *Pseudomonas aeruginosa* Multidrug Efflux Pumps by Quantitative Real-Time Polymerase Chain Reaction.” *FEMS Microbiology Letters* 243 (1): 125–131.

Young, Saundra. “FDA Examining Antibacterial Soaps, Body Washes.” *CNN Health*. 2013. https://www.cnn.com/2013/12/16/health/fda-antibacterial/index.html.

Yu Y, Cheng AS, Wang L, Dunne WM, and Bayliss SJ. 2007. “Hot Tub Folliculitis or Hot Hand-Foot Syndrome Caused by *Pseudomonas aeruginosa.” Journal of the American Academy of Dermatology* 57 (4): 596–600.

Yuan, Jing, and Wei Guo. 2017. “Mechanisms of Resistance to Quinolones in *Salmonella typhimurium* from Patients with Infectious Diarrhea.” *Microbiology and Immunology* 61 (3): 138–143

Zhang, Tong, Ming-Fei Shao, and Lin Ye. 2012. “454 Pyrosequencing Reveals Bacterial Diversity of Activated Sludge from 14 Sewage Treatment Plants.” *The Isme Journal* 6 (6): 1137–1147.

Zhang X, X Zhang, Y Zhang, M Liu, J Jin, J Yan, X Shen, N Hu, and D Dong. 2017. "Mitochondrial uncoupler triclosan induces vasorelaxation of rat arteries.” *Acta Pharmaceutica Sinica* 7 (6): 623-629.

Zhao C, H Xie, J Xu, X Xu, J Zhang, Z Hu, C Liu, S Liang, Q Wang, and J Wang. 2015. "Bacterial community variation and microbial mechanism of triclosan (TCS) removal by constructed wetlands with different types of plants.” *The Science of the Total Environment* 505 (10): 633-639.

Zhao JL, Zhang QQ, Chen F, Wang L, Ying GG, Liu YS, Yang B, *et al.* 2013. “Evaluation of Triclosan and Triclocarban at River Basin Scale Using Monitoring and Modeling Tools: Implications for Controlling of Urban Domestic Sewage Discharge.” *Water Research* 47 (1): 395–405.

Zhu, Lei, Jinshui Lin, Jincheng Ma, John E Cronan, and Haihong Wang. 2010. “Triclosan Resistance of *Pseudomonas aeruginosa* PAO1 Is Due to FabV, a Triclosan-Resistant Enoyl-Acyl Carrier Protein Reductase.” *Antimicrobial Agents and Chemotherapy* 54 (2): 689–698.

Zhu, Yong-Guan, Timothy A Johnson, Jian-Qiang Su, Min Qiao, Guang-Xia Guo, Robert D Stedtfeld, Syed A Hashsham, and James M Tiedje. 2013. “Diverse and Abundant Antibiotic Resistance Genes in Chinese Swine Farms.” *Proceedings of the National Academy of Sciences of the United States of America* 110, 9 (2): 3435–3440.

Zorrilla, Leah M., Emily K. Gibson, usan C. Jeffay, Kevin M. Crofton, Woodrow R. Setzer, Ralph L. Cooper, Tammy E. Stoker. 2009. “The Effects of triclosan on puberty and thyroid hormones in male Wistar rats.” *Toxicological Sciences* 107 (1): 56−64.