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The Impact of Household Biocides and Antibiotics on Aquatic Microbial Community Composition

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THE IMPACT OF HOUSEHOLD BIOCIDES AND ANTIBIOTICS ON AQUATIC MICROBIAL COMMUNITY COMPOSITION

By

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List of abbreviations

Triclosan	TCS
Parts Per Million	PPM
Amplicon Sequence Variant	ASV
Polymerase Chain Reaction	PCR
Deoxyribonucleic acid	DNA
Ribonucleic acid	RNA
Real-time polymerase chain reaction	qPCR
Honest Significant Difference	HSD
Permutational multivariate analysis of variance	PERMANOVA
Analysis of variance	ANOVA
Principal coordinate analysis	PCoA
The Food and Drug Administration	FDA

Abstract

Triclosan (TCS) is antimicrobial agent that is used in a lot of consumer products, including toothpaste, liquid and bar soap, and cosmetics. TCS has also been found in many lakes and rivers in the United States. However, The Food and Drug Administration (FDA) banned TCS recently, and it will no longer be used in household products. Despite the recent ban, TCS is known to persist in the environment and may have long-term impacts. We conducted an experiment on using fresh water from three locations Houghton, Green Bay and the Huron Mountains. Our goals in the study is to assess the impact of TCS on environmental microbial communities, compare the response to an antibiotic and estimate the impact of human activity on the environment. We set up microcosms from each location. In these microcosms we attempted to mimic the natural environmental conditions while been in a controlled laboratory setting. One microcosm had 2 ppm TCS, another 6 ppm TCS and a third 2 ppm Tetracycline. An additional set of microcosms had no added chemicals and were used as a control. We filtered our water samples from 0 hour and 24 hours then 7 days and once a week until 50 days. From these samples, we measured the microbial community change using next-generation sequencing of the 16S rRNA. Our results indicate there is a significant difference between the two TCS treatments concentrations. Also, there was a significant difference between the biocide and antibiotic treatments. Finally, there was a large change in the microbial community in regions with different population sizes; the bigger the population the less change. For example, there is large change in the microbial community composition in response to TCS addition in Huron Mountains. In Houghton, there is also a change in the community composition, but not as big of a change as in the Huron Mountains. Finally, there was very little change in the microbial community in response to TCS in Green Bay. These findings combine to suggest that TCS can have a strong impact on the microbes in aquatic settings and that this response appears to vary based on population size.

1. Introduction

Antimicrobials are used in many industries such as food, health, and agriculture to sterilize equipment, solid surfaces and food packaging, beverages and drinking water, and to preserve health and productiveness. Microbial control is a fundamental component of preserving health, to prevent infection, and the spread of disease in hospitals. Also, antimicrobials are mixed in small percentage into household products (hand soap, toothpaste and shampoo) as a preservative to prevent microorganisms from growing, which could lead to spoiling of the product or cause infections [1, 2]. Antimicrobials are chemicals designed to control microbial growth. This includes compounds used for the purpose of disinfection, antiseptics, as well as antibiotics, which are used to cure infections resulting from bacterial or fungal activity in animals or humans [3]. Biocides are substances that can kill organisms and are often either chemicals or microorganisms. Biocides can be used to control the growth cycle of living organisms or reduce their harmful effects. So, the main purpose of a biocide is to stop the harmful living organism from influencing various organisms (such as humans, plants, animals) or consumer products (such as drinking water, food, and wood). Some biocides are used for disinfection and sterilization purposes such as quaternary ammonium compounds and chlorhexidine salts, while sensitive medical instruments such as ones used for endoscopy usually are disinfected by glutaraldehyde [3]. There are different classifications of biocides depending on the target organism, for example, insecticides, herbicides, fungicide, rodenticides, and bactericides. On the other hand, disinfectants, often used to sterilize surfaces, are specifically designed to kill microorganisms. The term pesticide is often misused to only refers to plant protection products or insecticides. Although the correct use is that pesticides are broader and stop the nuisance or diseases caused by other living organisms (such as microorganisms, nematodes) [4]. There are many personal care products made up of chemicals that are used by people including soaps, hair care products, teeth and skin care products and countless other examples [5]. Biocides are often added to these household products to preserve them from microbial activity. In America, in 2014, the value of biocides in the market is about 7.99 billion USD, with

some predictions showing that in 2020 there will be an annual growth of 5.2 per cent and the market size will be around USD 11.9 billion. Globally, the market is expected to reach 145.8 billion USD in 2022, an increase of 28.3 per cent from 2013. Since the market value in 2013 was USD 113.7 billion [6].

By washing and bathing, personal care products enter wastewater. Many water treatment facilities are not equipped to remove many of these chemicals. Thus, these active substances end up in receiving water bodies without any monitoring or testing of their impact on the environment. While most of these products, absorbs onto sediments and sludge during wastewater treatment due to high lipophilicity content, some of these chemicals make it into the water [5]. Prior 2017, the yearly market production of TCS was about 450,000-kilogram of which half of the bar soaps and liquid soaps in production contained TCS. TCS is usually used in small percentages which is about 0.1 to 0.3 percent, but in hospitals, the percentage is higher, up to 5 percent compared with other products [7]. There is widespread TCS pollution in the environment due to the large amount used for these antimicrobials. TCS was found in wastewater as high amounts as micromolar (μM) concentration. In particular, in the Great Lakes, TCS was present in Lake Michigan at concentrations ranging from 0.13 μg / L to 0.47 μg / L. This is due to its frequent presence in wastewater and their hydrophobic unique feature [8]. The goal of this study is to examine the impact of antimicrobial such as TCS and the antibiotic tetracycline on environmental microbial communities in order to better understand the environmental impacts of release of antimicrobials in to surface freshwater.

1.1 Definition of Biocide

There is a small variation between the legal and general definitions of biocides. According to Food and Drug Administration (FDA) a biocide is a substance that is intended to exert microbial toxicity [9]. According to the European legislation, biocides are known as "chemicals used to suppress organisms harmful to human or animal health or that cause damage to natural or processed materials" [4]. But when it comes to substances that protect plants from harmful effects, they are excluded from this definition because they indicate specifically to plant protection products. In the

United States, biocides are either chemical pesticides or antimicrobials [10]. “Biocide" is a common term illustrating a chemical factor, often a broad spectrum, which inhibits the activity of microorganisms and their ability to grow and react naturally. Since, biocides and antibiotics vary in their effects on microbial activity, there are more precise terms to describe the action. For example, "-static" indicates that the factor is able to prevent growth and give the immune system chance to kill the bacteria, but these biocides do not kill the bacteria (such as fungistatic, sporistatic, and bacteriostatic). The term "-cidal", is used in reference to the factors that kill the target object (such as bactericidal, sporicidal, and virucidal). For this study, biocides are defined as organic substances able to inhibit microbial growth. These compounds can be present in consumer products in a different concentrations.

Biocides can often be divided into three classes antiseptics, disinfectants, and preservatives. Nearly all antiseptics and disinfectants have the same actions, which is to kill or control the life cycle of microorganisms inside or outside living tissue. Antiseptics are often used on our skin (e.g., personal hand washes or shampoo) while disinfectants are used on inanimate objects or surfaces (e.g., cleaning the tables). Furthermore, disinfectants can be sporostatic but are not necessarily sporicidal. Sterilization is the use of chemical or physical activity that eliminates all microbes, as well as spores. Preservatives are used in pharmaceutical and food products to prevent the multiplication of microorganisms in these products [11].

1.2 Classification and Applications of Biocides

There is a great diversity of biocides that can utilized as disinfectants and antiseptics (Table1.1). These products can possess very different mechanisms of action against microorganisms. It is worth mentioning that factors such as formula effects, synergies, temperature, presence of organic load, dilution and test method may affect the activity of these antimicrobial agents [12]. Many biocides contain ammonium groups (such as quaternary, amines and aldehydes), halogen compounds (such as iodine and fluorine), oxidizing agents (such as biguanides, phenols and isothiazolones), as well as organic acids and alcohols (Table 1.1, Figure 1.1).

Usually, biocides have several biochemical interactions leading to their activity. As an example, several biocides target the membrane and will directly aim to destroy the cell envelope and inhibit microbial activity (Table 1.1). On the other hand, some biocides have the ability to inhibit cell growth and metabolism through affecting the proteins involved in the process of nucleic acid and protein production [12, 13].

Group	Examples[a]	Main target
Alcohols	• Ethanol • 2-Propanol • 2-Phenoxyethanol	• Membrane uncoupler • Protein denaturation
Aldehydes	• Glutaraldehyde • Formaldehyde • Glyoxal	• Cell wall • Protein denaturation
Amines	• Diethylamine • Glucoprotamin	• Cell wall • Cytoplasmic membrane
Biguanides	• Polyhexamethylen-biguanid (PHMB)	• Cytoplasmic membrane
Halogen compounds (oxidizing)	• Sodium hypochlorite • Chlorine dioxide • Calcium hypochlorite	• Nucleic acids
Isothia-zolinones	• Chlormethylisothia-zolinone / Methyl-isothiazolinone (CMIT/MIT)	• Inhibition of key enzymes
Organic acids and esters	• Parabens • Propionic acid • Formic acid • Benzoic acid • Salicylic acid	• Cytoplasmic membrane • Transport inhibition
Oxidizing agents	• Hydrogen peroxide • Sodium persulfate	• Nucleic acids
Phenolics	• Triclosan	• Cytoplasmic membrane • Inhibition of key enzymes
Quarternary ammonium compounds (QATs)	• Benzalkonium chloride (ADBAC) • Didecyldimethylammo-niumchlorid (DDAC)	• Cell wall • Cytoplasmic membrane
Silver compounds	• Silver and silver zeolite • Nanosilver	• Enzymes

Table 1.1. Table shows verity of biocides and the Main target adopted from [10]

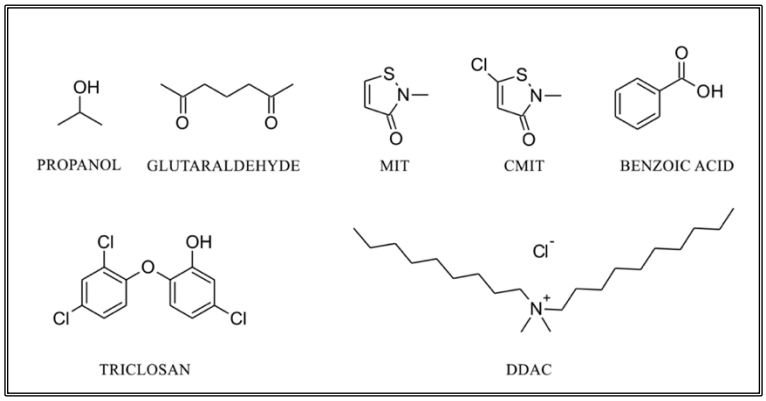

Fig. 1.1. Chemical structures of select biocides adopted from [10]

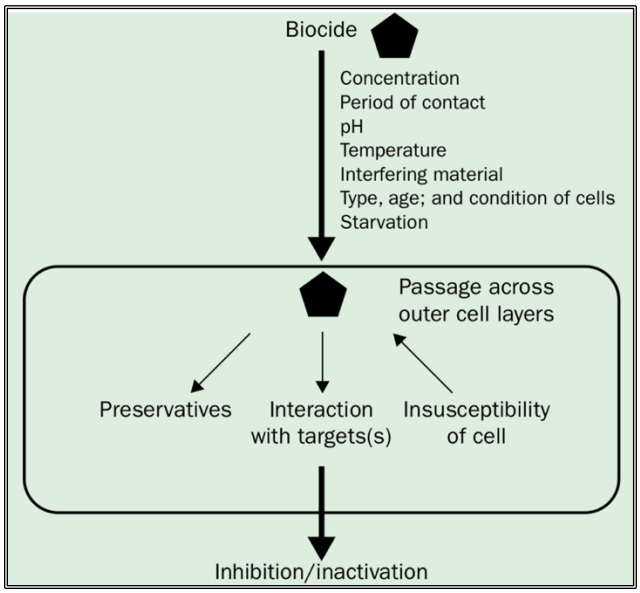

Fig 1.2 The final results that bacteria may experience when exposed to biocide adopted from [14]

Biocides are usually designed to have many targets based on concentration. Raising the concentrations will cause more significant damage, while low levels may create little effect (Fig 1.2) [15]. There are four general categories for the mechanisms of biocides (Fig 1.3). Oxidants agent are the fastest way of killing microorganisms by reactions to oxidize organic substances. The way an oxidizer strikes microorganisms is to interrupt nutrients crossing through the cell wall by oxidation (Transfer electrons from one atom to another). Some examples of very active oxidizes are chlorine, peroxides, and bromine [16-18]. The electrophilic biocides interact with the nucleophilic cell and have a synergistic interaction. This reaction aims to damage and deactivate the enzymes [16, 19]. Cationic biocides cause rapid cells lysis by damaging the membranes. These biocides are considered membrane-active biocides. One of the examples of cationic biocides are alcohols such as phenoxyethanol [20-22].

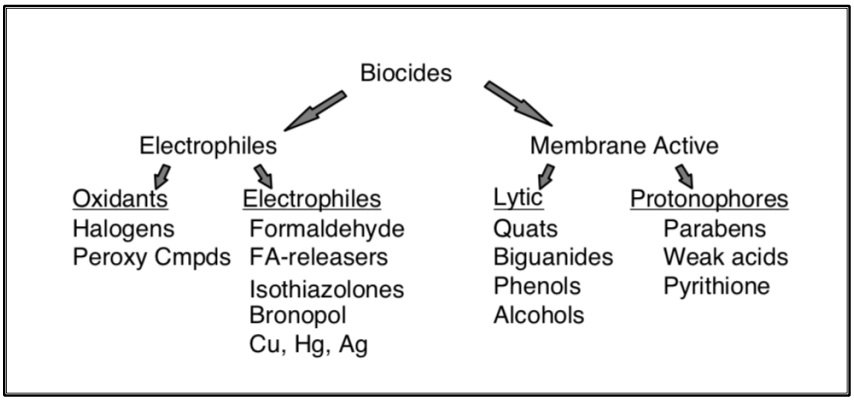

Figure 1.3 Mechanisms of action of biocides adopted from [19]

Biocides are designed to act upon key components of the bacterial cell. Consequently, there are three targets of interactions against bacteria: first biocides attack the cytoplasmic components; secondly biocides target external cellular components, and finally, biocides interact with cytoplasmic membrane. Nevertheless, there are some biocides that have two target in one product, to provide its antimicrobial actions. For example, amines target the cell wall and the cytoplasmic membrane [23].

1.3 Comparison Between Biocide and Antibiotics

Since both biocides and antibiotics are chemicals that are designed to control microbial growth, it is important to understand the similarities and differences between biocides and antibiotics. There is a difference in the mechanism of action of biocides and antibiotics. The antibiotic reaction is focused on a specific target involved in essential metabolism or structure of the bacterial cell. While biocide interact with targets in a more non-specific manner, attacking more than one target at the same time. For example, some antibiotics specifically target specific bacterial enzymes involved in essential processes such as translation (ribosomes) or synthesis of bacterial cell walls [24]. On the contrary, biocides interact with targets in a more non-specific manner, attacking more than one target at the same time. Biocides can

disrupt the regular activity of the cell membranes through interactions with amines and alcohol, interact with a genetic material by such biocides as aldehydes and halogens, or interact in an unclear method with a group of proteins such as compounds of oxygen [25]. Here are some examples that may explain the difference between the work of antibiotics and biocide. In antibiotics, tetracycline and mupirocin block protein synthesis by interacting with the 30S ribosome [26]. In regards to activity of biocides, glutaraldehyde has been known to attack two targets simultaneously, interacting with free amine groups of compound in both the cell membrane and cell walls, while cationic agents break the cell membrane integrity and ethylene oxide acts as an alkylation factor [27]. However, in some cases, biocides have very specific targets. For example, TCS works by specifically inhibiting the enoyl reductase enzyme, which is involved in fatty acid biosynthesis [28].

1.4 Mechanisms of Biological Resistance to Antimicrobials (biocides versus antibiotics)

Bacteria have the ability to rapidly adapt to their surrounding environment in order to survive when faced with severe pressure. The impact of an unfamiliar stressors on bacteria will cause a stress reaction that may also kill the cell or weaken its function (Fig 1.2). In some cases, this stress will produce a cell capable of tolerance and resistance to the stressor. Microorganisms in their normal environment are often growing at a slow pace due to environmental limitations [29]. The normal lifestyle of organisms in the environment requires microbes to respond to continuously changing pressures.

Recently, bacterial resistance to many antimicrobials has emerged as a common phenomenon. Bacterial resistance to antibiotics has developed rapidly and become threats to the effective use of antibiotics. Various mechanisms of resistance to antibiotics have spread easily to a diversity of bacterial strains. There are a number of mechanisms bacteria use to resist the activity of antibiotics including: (i) enzymes break down the antibacterial before it begins action, (ii) removal of the antimicrobial from the cell before any effect through the use of efflux pumps, (iii) restriction of the

antimicrobial compounds from entering the cell due to mutations that downregulation the porin genes, (iv) alterations of the target of the antimicrobial [30, 31]. This last mechanism is more prevalent in antibiotic resistance in which antibiotics target one specific target. Thus, mutation and selection or by obtaining coded genes resistant from other bacteria help natural bacteria gain antibiotic resistance. The spread of these mechanisms can be helped through the use of horizontal gene transfer, either by conjugation, transformation, or transduction [32].

1.5 Triclosan and Tetracycline

In this research two antimicrobial agent were used: TCS as a biocide and Tetracycline as an antibiotic. Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy) phenol) is a chemical compound that was used in hospitals in surgical wipes in 1972. Since then it has been used in a lot of personal care product such as toothpaste and in liquid hand soap. In the late 1990s, the annual production of TCS was approximately 1,500 tons in the world; the most significant share was for America and Europe, where the use amounted to about 400 tons [33, 34]. The Food and Drug Administration (FDA) declared that in September 2017, products containing TCS would no longer be used in health and personal care products [35]. TCS is a synthetic antimicrobial, broad-spectrum and can act as an antimycotic or as an antibiotic[36]. The mechanism of action of TCS varies depending on the concentration, so that if the concentration is low, it acts as bacteriostatic, which is used in commercial products, and is a targeted attack to inhibit the synthesis of fatty acids of the bacteria. When the concentration is high, it acts as a bactericidal and is attacked on multiple targets in the cytoplasmic membrane [12]. Despite the recent ban, TCS is known to persist in the environment and may have long-term impacts.

Tetracycline was discovered in 1948 and was extracted from *Streptomyces aureofaciens*. Since then, it has become the most widely used antibiotic since it has two advantages: it is considered one of the cheapest existing antibiotic classes, and its cost has become reduced due to advanced manufacturing technology. Also, it is considered as a broad-spectrum antimicrobial, which reacts against gram positive and

gram-negative bacteria. It is used in health clinics and is taken orally and also used in aquaculture and agriculture[37, 38]. Tetracyclines are thought to be found in the environment for a very long time as it is a polyketide synthase enzyme of actinomycete nonessential metabolism [39]. Malaria, cholera, and plague are examples of infections that can be treated by tetracycline. Tetracycline has been widely used to treat animal and human contagions because it has a distinctive character as it has no harmful side effects. In America and some other countries, tetracycline is combined in a less than the therapeutic dose to animal food as growth-promoting agents. Although tetracycline is a useful and necessary antibiotic that protects humans and animals from many diseases. However, excessive and unnecessary use may lead to bacterial resistance and possibly loss of efficacy [40].

1.6 Impact of Biocide on Microbial Resistance Community Composition

Biocides are found in a wide range of detergents and personal care products as it enters household wastewater through daily use, thus allowing access to natural aquatic environments through local wastewater effluent. Recent studies have confirmed that there are concentrations of TCS in domestic sewage ranging from 3,800 to 16,600 ppm. Therefore, municipal wastewater treatment plants are making numerous attempts to remove TCS [33, 34, 41]. However, the removal is most often incomplete, allowing for high concentrations of TCS in wastewater treatment waste such as activated sludge. These high concentrations will undoubtedly affect the aquatic ecosystems. In the United States alone, there are around 5,200-18,824 kilograms of TCS annually released in surface water, with waste from sewage treatment estimated to account for 50 to 57 percent of this mass [34]. Throughout high rainfall occurrence, it is possible to release sewage through combine sewer outflow flooding, so that aquatic environments may be exposed to an intense dose of TCS but intermittently [34]. In addition, untreated wastewater occasionally can leak out of the sewer system, creating an opportunity for TCS to enter the aquatic ecosystem in large and direct doses [42]. When TCS enters aquatic habitats, it has a

low solubility in water and is a lipophilic agent, such that it most probably ends up in the sediments.

Many studies have elucidated the ability of cultured bacteria to develop resistance against TCS in vitro [43, 44]. Because of this, it is possible for decreased efficacy of TCS as an antimicrobial agent. Also, many studies have shown that there is a link between resistance to TCS and antibiotic resistance [43, 45]. For example, a recently study demonstrated that treatment of sewage sludge with TCS resulted in an increase in genes involved in antibiotic resistance [46, 47]. Few studies have investigated the impact of TCS on microbial community composition using next-generation sequencing outside of wastewater treatment plants. One such study demonstrated the impacts of TCS on benthic microbial community composition [48]. TCS may negatively influence the activity and quantity of benthic bacteria because of its antimicrobial characteristic. Since benthic bacteria are the main drivers of nutrient flow, TCS exposure may have a wider ecosystem impact [49].

To address some of the gaps in our understanding we sought to use next-generation sequencing to characterize the impact of varying concentrations of TCS on aquatic microbial communities. Previous studies have shown that exposure history can impact the resilience of microbial communities to biocides [50]. We therefore examined this response in streams across a range of populations from pristine locations in the Huron Mountain Club to in the Fox river in Green Bay. To better understand the impact of TCS on microbial community composition, we sought to test the following hypotheses.

1.7 Study Goals

This research aims to look at the effects of biocides on the microbial communities in surface water by exposing the surface water to different concentrations of biocides and an antibiotic. Monitoring how the microbial environment will react to those different concentrations and different mechanism of action. Also, our goal was to observe whether bacteria will be able resist biocides or the antibiotic at these concentrations. Since the samples were taken from 3 places. (a town, city and an area

completely isolated), we will be able to compare the effect of population activity on rivers by testing microbial resistances. Therefore, we tested these following hypotheses by determining different factors:

1- By exposing the different samples to TCS at two different concentrations (2 ppm and 6 ppm) there will be a different reaction of the bacteria to these concentrations. Therefore, the TCS will attack the bacteria at different targets possibly producing a difference in the types and numbers of microbes.
2- The microbial response to biocides will be similar to the response to antibiotics. Antibiotic resistance is significantly affected by human influence. Efflux pumps are one of the resistance mechanisms that affect TCS and antibiotics. The samples were exposed to Tetracycline at 2 ppm concentration as an antibiotic agent. Later the bacterial resistance was measured by the bacterial growth curve and diversity on the microbial community.
3- Samples were taken from three places in different populations. The difference with the population size is linked to the number. The larger the population, the more likely the bacteria were to be exposed to antimicrobial agents and perhaps more resistant they would be. Also, the population number may have an effect on the microbial community.

2. Methods

2.1 Water Collection and Location

This study seeks to determine of the effect of different concentrations of biocides and antibiotic on the composition of freshwater microbial communities. Samples were taken from three different locations: a large city (Green Bay, WI), a small town (Houghton, MI) and pristine privately-owned land (Huron Mountain Club Lands, Big Bay, MI) to measure the effect of the population on the response of the microbial community to antimicrobial treatment. The water collection was done to study the impact population size on the microbial response to these treatments. The water collection was chosen to test the hypothesis that larger cities have higher volumes of treated wastewater effluent discharged into lakes and rivers from municipal sewage treatment plants and would hypothetically have higher exposure to antimicrobials.

The first set of samples were collected from Houghton, Michigan specifically from Keweenaw waterway near the Great Lakes Research Center (GLRC), and the second set of samples were from the Fox River in Green Bay, Wisconsin. These cities have large differences in the populations with the Houghton/Hancock area having a population of 7,888 and Green Bay having a population of 105,116 [51]. The third sample was taken from Huron Mountain and microcosms were set up by a previous undergrad student Andrew Baldwin. These samples will help to understand the impact of population size and potential exposure history on the microbial response to antimicrobials.

Water was collected in a 15 L water bottle and returned to the lab. Samples from Houghton were collected on February 2, 2018 and samples from Green Bay were collected on February 24, 2018. Upon return to the lab, water was immediately transferred to autoclaved 1 L media bottles that were divided into four groups. Each group had triplicate Pyrex Media Storage Bottles (1000 ml). The first group was the negative control and was just 1 L of river water in each bottle. The second group was treated with the biocide TCS at a concentration of 2 ppm. The third group was also treated with TCS but at a different concentration of 6 ppm. The fourth group was treated with 2 ppm of the antibiotic Tetracycline. The 6 ppm concentration of TCS

was previously used in a study by Patrick J. McNamara *et al.* 2014 [47] and represented levels found in a wastewater treatment plant. The 2 ppm tetracycline was chosen based on a study by Junsik et al, which observed that 2 ppm of tetracycline resulted in transfer of genes by conjugation with other bacteria and raise the possibility of trans conjugant in liquid media [52]. In total, we had 12 media bottles for Houghton and another 12 media bottles for Green Bay. All microcosms were kept in the dark and incubated at room temperature. Experimental set up and the workflow for the project are depicted in Fig 2.1.

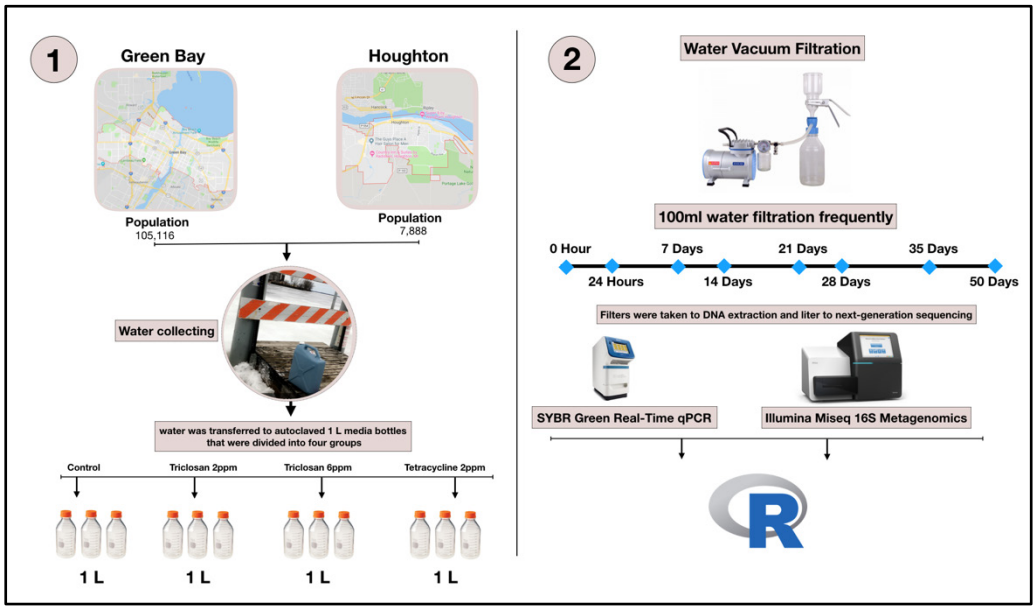

Fig 2.1 Sample Collection and Methodology

2.1 Water Filtration

Samples were collected at multiple time points for examination of microbial community composition. Initial samples were collected starting at experimental set up at 0 hour. The next sample was collected 24 hours after the start of the experiment and then once weekly till reaching the 50-day. Samples were filtered by using a Glass Vacuum Filtration Apparatus. A 100 ml sample of water from each replicate and condition was vacuumed through PES Membrane Filter, Pore Size: 0.22 µm, Diameter: 47 mm. After filtering, filters were cut in half using flame sterilized

scissors. Filters were then stored at -80 °C freezer inside a 2 ml microcentrifuge tubes until DNA extraction. One half of the filter was used in the DNA extraction and the other for storage.

2.2 DNA Extraction

Half of the filters were used to extract the DNA by using the ZymoBIOMICS DNA Miniprep Kit (D4301, Zymo Research Corporation, Irvine, CA USA) with the following modifications. The filters were beat in a Fast Prep 5G at 5.5 m/s for 100 s. DNA was eluted in 50 µl of water. By following the protocol that the company provides with the kit the DNA was isolated and purified.

2.3 Real Time qPCR

To quantify microbial abundance, (qPCR) was performed targeting the bacterial 16S rRNA gene. Environmental DNA was diluted by a factor of 10 to control for potential PCR inhibitors. Bacterial 16S rRNA genes were quantified using a protocol similar to the one described in Techtmann *et al.* 2017 [53]. Briefly the bacterial 16S rRNA gene was amplified using PCR primers that target region the V4 region of the 16S rRNA (bact314F and Uni519R described in Jorgenson et al 2012) [54]. SYBR® Green RT-PCR master mix was used. Triplicate reactions were set up with 2 µl of the diluted environmental DNA as a template. qPCR was performed using a StepOnePlus™ Real-Time PCR System (Thermos Fisher Scientific Waltham, MA USA). CT values were converted to copies of the 16S rRNA gene using a six-point standard curve. The CT values and concentration values were plotted as logCT and log(copies). The equation for our standard curve was y= -11.932x + 20.312 with an R^2 of 0.9594.

2.4 16S rRNA amplification and clean up

To profile the microbial community composition, 16S rRNA gene libraries were prepared from the environmental DNA. All reactions were done in 25 µL reactions. Per reaction volumes of reagents: 12.5 µL Phusion® High-Fidelity PCR Master Mix

(Thermo Fisher Scientific, Waltham, MA, USA). The master mix includes the nucleotides (dNTP's) – 200 mM each. Also, reaction buffer $MgCl_2$ and 1 Unit of high-fidelity polymerase enzyme (Phusion). All this were done in a 96 well PCR plates (VWR polypropylene plates). I used the 515Y forward primer and 926 reverse primer to amplify V4-V5 region [55]. Both primers used at a concentration (final) of 0.4 µM. The following temperature cycles were used to amplify the 16S rRNA gene. PCR settings: 95 °C 3 min, 25 cycles 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec, 72 °C for 5 min. PCR clean-up was done to each plate to remove the excess reagents and nucleotides other than the amplified DNA. I used Illumina MiSeq 16S rRNA metagenomic library prep guides (Illumina, San Diego, CA) and AxyPrep MAG PCR clean-up beads (Corning, Big Flags, NY, USA). The clean-up product was stored in 25 uL 10mM Tris buffer with a pH of 8.

2.5 Index PCR

Illumina adapters and sequencing indices were added to the amplicons from each sample using a low cycle PCR as described in the Illumina 16S rRNA metagenomic library prep guide. Briefly a forward and reverse primer were used where each contained the appropriate Illumina adaptors and a unique index sequence. 5 µl of the first round of PCR was added to each reaction. An eight cycle PCR was performed to add the appropriate sequencing adapters and index to each sample. PCR clean-up done here as well according to the protocol in the 16S rRNA metagenomic library prep guide and stored in 50 µL of 10mM Tris buffer with a pH of 8.

2.6 16S rRNA Gene Sequencing Analysis

Sequencing of the amplicons was done with the Illumina MiSeq (Illuming, San Diego, CA). All samples were pooled at roughly the same concentration into a single pool and diluted to 10 nM. Library pool containing all samples was denatured and prepared for sequencing using a MiSeq v2 500 cycle kit (2 x 250) following the standard methods for MiSeq sequencing (Illumina, San Diego, CA, USA).

2.7 Statistical Analysis

All data analysis and visualization were performed in R [56]. Diversity analysis was such as Alpha and Beta diversity to show the different between the sample sites was also performed in R. We used different packages like Phyloseq for the alpha and beta diversity and DESeq for differential abundance analysis.

2.7.1 16S rRNA Gene Data Analysis

The raw 16S rRNA sequencing reads processed in R through dada2. Merging, quality filtering, and finding sequencing errors were handled here in this package (dada2). Raw sequencing reads were demultiplexed by the Illumina MiSeq instrument prior to analysis. To process our overlapping reads, we used the dada2 package in R to merge, quality filter, to remove the phiX standard and to denoise reads to correct for sequencing errors. After generating read quality profiles, forward reads were trimmed to 240 nucleotides and reverse trimmed to 190 to ensure good quality across the reads. After trimming, the first 10 nucleotides of each read were removed before being merged into a single read and only samples that contained more than 50 merged reads were kept for downstream analysis. These sequencing reads were then dereplicated and processed to remove chimeric artifacts. Taxonomic assignment for each read was achieved through the RDP classifier implemented in dada2, trained against the silva database (version 132). This ultimately identified ~ 46026 Amplicon Sequence Variants (ASV) [57].

2.7.2 Alpha Diversity Analysis

We used phyloseq to calculate Shannon diversity from the rarified ASV tables. This was done to test if there was a significant difference in the variation between the Shannon diversity of the microbial communities. Various ANOVAs were conducted to test different hypothesis about the impact of treatment, time and location on Shannon diversity. Tukey Honest Significant Difference post hoc was used to find between which treatment, time, location there were substantial variation in Shannon diversity. ANOVA and Tukey HSD tests were conducted to see whether there was a

significant variation in the richness of treatments compared to controls, and if there were significant differences in the Shannon diversity between the locations. Statistical significance is achieved when the alpha balanced was $P \leq 0.05$.

2.7.3 Beta Diversity Analysis

The phyloseq package was used to compare the microbial community composition through plotting the microbial community composition between sites and treatments, Principal coordinates analysis (PCoA) was plotted. The PCoA plots were made using a Bray Curtis dissimilarity matrix built from an ASV table rarified to a depth of 1243. The vegan package was used in R, to conduct the statistics of permutational multivariate analysis of variance (PERMANOVA) to study the significance of response in microbial community composition. To test the hypothesis of a different community composition in each treatment PERMANOVA analysis was performed to compare the control between treatments. For p-value threshold level in PERMANOVA ≤ 0.05 is consider as significance change.

2.7.4 Differential Abundance Analysis

DESeq2 was used to analyze the differentially abundant ASVs using the complete (non-rarified) ASV tables. In order to identify the ASVs that were differentially abundant between treatment types for each sample across all sites, we used differential expression analysis through the DESeq2 package in R [58]. Volcano plots were used to show the results from DESeq. To identify which ASV were responsive to the control, differential abundance analysis was performed using DESeq. DESeq was performed using the ASV tables. DESeq estimates the mean on the variance in the number of the data from the high-throughput sequence and tests for differential abundance based on a model using negative-binomial distribution. ASVs were considered enriched if they had a $\log_2$ fold change of > 2 and an adjusted p-value of less than 0.05.

3. Results

3.1 Changes in 16S rRNA gene copies in response to treatments

Real time qPCR was done to quantify and to monitors the amplification and the change of 16S rRNA gene. we collected data during PCR amplification by measuring the fluorescence of SYBER- GREEN dye. Copies of the 16S rRNA gene were quantified using qPCR targeting the bacterial 16S rRNA gene. Copies of 16S rRNA were plotted to see how the microbial abundance changed in response to the different treatments. Also, the microbial abundance was compared between treatment and control. Changes in copies of the 16S rRNA gene were shown as a function of time for Houghton and Green Bay for each treatment. All replicate samples and technical replicates of the qPCR were used to come up with a value of box plot (Figure 3.1 and 3.2).

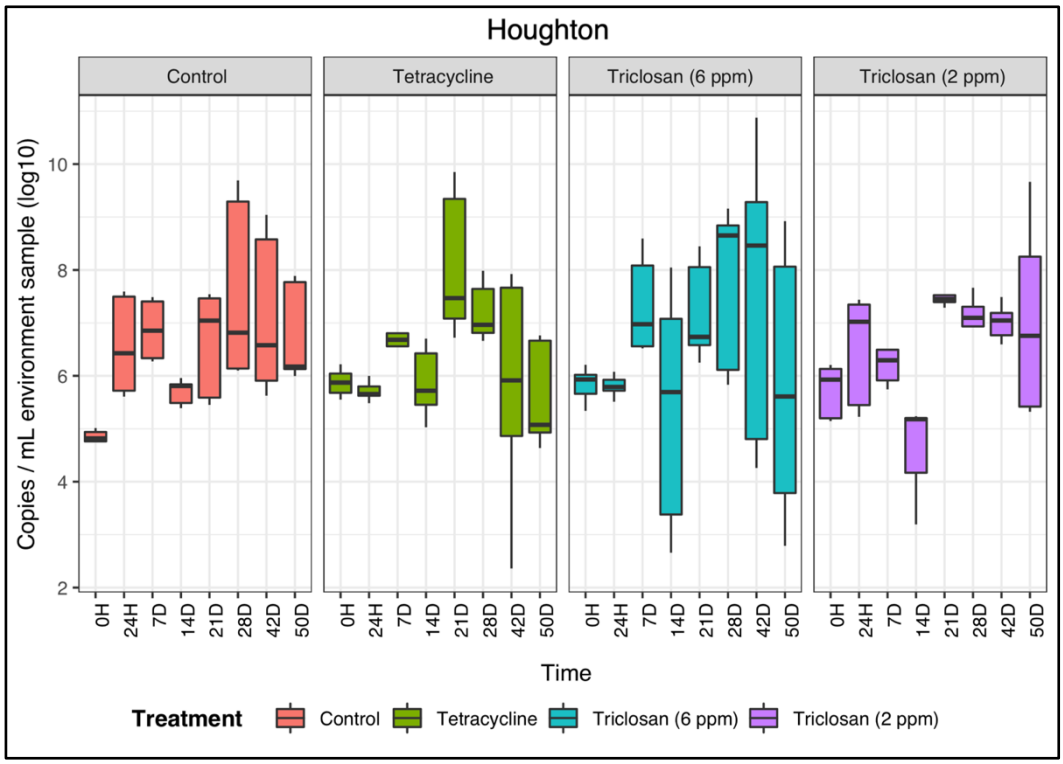

Figure 3.1.1 Microbial abundance in Houghton Microcosms

Microbial abundance in the treatments were compared to the controls for Houghton samples (Fig 3.1.1). Overall, the microbial abundance in the controls increased over time from 7.94 x 10^4 to 1.0 x 10^7 copies/ml of water. All of the treatments showed an increase in microbial abundance throughout the experiment. The highest number of copies observed in each treatment was 7.9 x 10^6 copies/ml for control, 2.5 x 10^7 copies/ml for Tetracycline, 6.3 x 10^8 copies/ml for TCS 6 ppm and 2.5 x 10^7 copies/ml for TCS 2 ppm. While we can see in the last three weeks, all samples show a continually decreasing. Also, we see that at Day 14 there was lower microbial abundance for TCS 6 ppm 1.2 x 10^5 copies/mL. While the first 0 hours of control recorded the lowest number of cells with a number of 7.9 x 10^4 copies/mL and the lowest number is 1.2 x 10^5 for Tetracycline which was the last week of the experiment. These numbers, however, are highly variable, which indicates that there may be some bottle effects that are impacting the overall microbial abundance determinations.

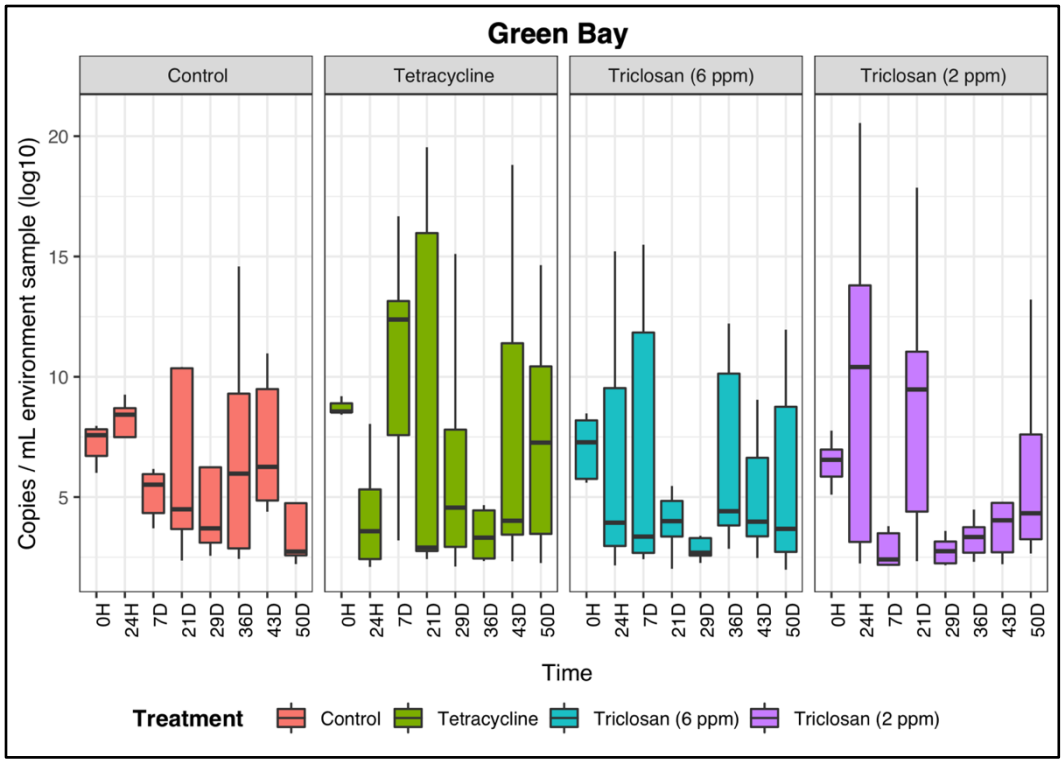

Figure 3.1.2 Microbial abundance in Green Bay Microcosms

Microbial abundance in the treatments were compared to the controls as copies/ml for the Green Bay samples (Fig 3.1.2). Overall, the microbial abundance in the controls decreased over time from 3.9×10^7 to 3.16×10^2 copies/mL and TCS 6 ppm likewise was decreased over time from 2.5×10^7 to 7.94×10^2 copies/mL. However, TCS 2 ppm and Tetracycline showed in increase throughout the experiment. The highest number for samples in the whole experiment where 1.0×10^8 for control, 2.5×10^{12} for Tetracycline, 1.9×10^7 for TCS 6 ppm and 2.5×10^{10} for TCs 2 ppm. Moreover, control and TCS 6 ppm show decrease in the last 3 weeks almost reaching the same microbial abundance from $3.98 \times 10^2 – 1.0 \times 10^3$ copies per ml. On the other hand, TCS 2 ppm and Tetracycline showed the opposite, which was increased in the last 3 weeks. We hypothesize that this increase in the TCS 2 ppm and the Tetracycline may be indicative of the presence of resistant bacterial strains in Green Bay.

Finally**,** there is a similarity when we compare the microbial abundance for the Tetracycline with TCS 6 ppm treatments, but compared to Tetracycline with TCS 2 ppm, there was a significant difference. The similar response to TCS 6 ppm and Tetracycline may be due to both antimicrobials being bacteriostatic at the tested concentrations or may suggest that bacterial communities repeatedly exposed TCS may be enriched for microbes that are able to resist tetracycline. However, the lower concentration of TCS may not result in as much cell death as the high concentration.

3.2 Taxonomic Diversity

To compare the diversity in bacterial communities between the three selected sites we used phyloseq to make Taxa Plots. To understand the taxonomic diversity and the changes in taxonomic diversity the relative abundance of the microbial classes in each treatment were plotted. **(Fig 3.2.1)**. To compare the data from Houghton and Green Bay with a pristine control location, we also analyzed data from an experiment performed by a previous undergrad student, Andrew Baldwin. His experiment only

examined the impact of TCS on the microbial community of the Huron Mountains **(Fig 3.2.1**).

Figure 3.2.1 shows an overall comparison of all locations. Similar trends were observed in the taxonomic composition of the controls from Houghton, Mountain Stream and Salmon Trout, while Green Bay was very different. In Green Bay, 71% of the microbial community in the controls were Bacteroidia and the second most abundant were the Gammaprotebacteria with 18%. In Houghton the three dominating classes were Alphaproteobacteria with 40%, then Bacteroidia (31%) and Gammaprotebacteria with 22 % of overall reads. In Mountain Stream the three dominating classes were Actinobacteria with 33%, Alphaproteobacteria with 3 % and Gammaprotebacteria with 24 % of overall reads. In Salmon Trout the three dominating classes were Gammaprotebacteria with 4 %, Alphaproteobacteria with 20% and Actinobacteria with 19% of overall reads. Hence, the starting microbial community composition was distinct in each of these locations.

Tetracycline was only applied to Green Bay and Houghton samples. However, in both locations, we first saw that the Gammaprotebacteria dropped out of the top 6 classes. In Green Bay Bacteroidia became 84 % of the overall reads, Alphaproteobacteria were the second most abundant with 6.8% and Actinobacteria with 6.5%. On the other hand, Houghton had two dominating classes which there are Bacteroidia with 48% and Alphaproteobacteria with 41%.

TCS 2 ppm was only applied to Green Bay and Houghton samples. In Green Bay, the most abundant three classes here were Bacteroidia with 62% of overall reads, Gammaprotebacteria with 21% and Alphaproteobacteria with 7.6%. In Houghton, we saw more abundance of Gammaprotebacteria with 33%, less of Bacteroidia 37% and Alphaproteobacteria with 22%.

TCS 6 ppm were applied to all locations. We can see here a switch ins the microbial community compared to controls. The Gammaprotebacteria in Salmon Trout Run increased to 80% abundance from 40% in the controls. This was double

the abundance, while the overall read assigned to Alphaproteobacteria and Actinobacteria decreased in the 6 ppm TCS. Additionally, the microcosms from Mountain Stream has a similar result as what happened to Salmon Trout, Gammaprotebacteria increased to 73% while it was 40% in the control. A similar reduction in the classes Alphaproteobacteria and Actinobacteria was observed. However, in Houghton, Alphaproteobacteria reduced from 40 to 16% and increased in the Bacteroidia from 31 to 42%. Also, an increase to the Gammaproteobacteria from 22 to 33% of overall reads. Finally, Green Bay has a similar increase and decrease to Houghton, which Gammaproteobacteria has increased from 18 to 33% while it has another decrease in Bacteroidia from 71 to 53%.

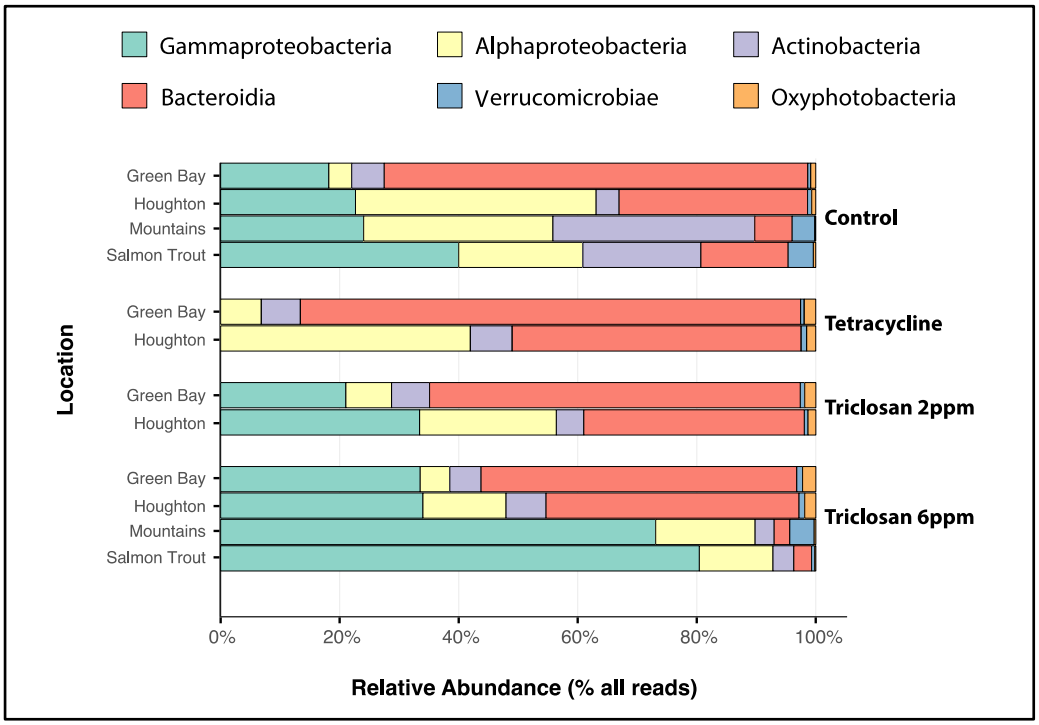

Figure (3.2.1) Taxa diversity presented as a bar graph of all reads of 6 classes that is most abundance. The colors characterize the different classes in the data set. Huron Mountain and Salmon Trout only have TCS reads. Green Bay and Houghton have TCS 2 ppm, 6 ppm and Tetracycline reads.

3.3 Alpha Diversity

Alpha diversity was used to measure the microbial richness which is the total number of microbes that are quantified in locations over time. Alpha diversity also can measure the microbial evenness, which is the number of individual microbes that are evenly divided between different each other over time. We used Shannon Diversity to plot the difference in richness and evenness between locations.

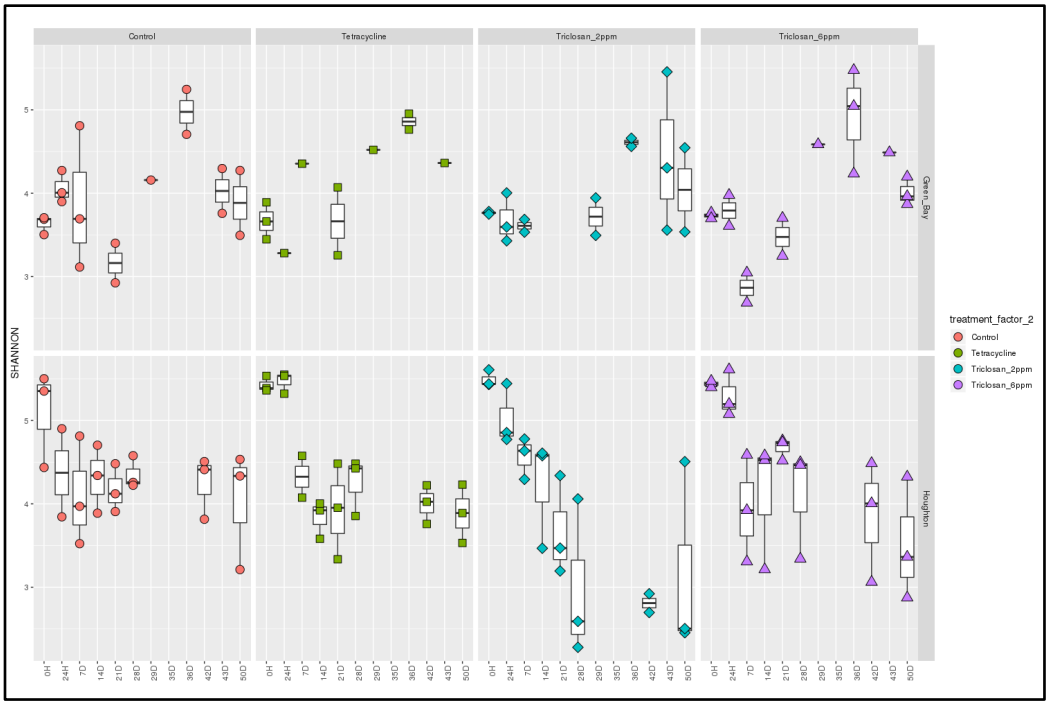

Figure (3.3.1) Shannon Index of microbial groups Alpha Diversity presented as box plot. The colors characterize the different classes in the data set. Two locations are plot as box bar to show the different between each other.

In Green bay samples **(Fig 3.3.1) t**he graph shows that there were large differences in trends in Shannon diversity between treatments over time. All samples started with about the same diversity, around 3.7. However, the highest diversity was 4.9 for control, 5.1 for TCS 6 ppm, 4.8 for Tetracycline and 4.6 for TCS 2 ppm. While the lowest diversity was TCS 6 ppm which was 2.8. overall, through the experiment there was obvious change in the diversity, but in the last week all

treatments range in a same richness which was around 3.8- 4.4. A two-way ANOVA comparing treatment and time indicated that there was only a slightly significant difference in the Shannon diversity between treatments over time in Green Bay (p-value 0.0528, F-stat = 2.005, degrees of freedom = 7) **(Table 3.3.1).**

In Houghton samples **(Fig 3.3.1)** Treatments started with a high richness around 5.3 to 5.5. However, there was a decrease in the richness through the whole experiments. Both TCS 2 ppm and 6 ppm showed a decrease over time and a large increase in the last week, TCS 2 ppm was 2.5 which was the lowest richness in the whole experiment and 3.4 for TCS 6 ppm. It is important to note that TCS 2 ppm continually decreased in richness from the first zero hour till the 28 day from 5.5 to 2.5 which almost more than the half of the diversity. A two-way ANOVA comparing treatment and time indicated that there was significant difference in the diversity between treatments across time in Houghton (p-value < $3.61e^{-12}$, F-stat = 11.025, degrees of freedom = 7) **(Table 3.3.1).** We used Tukey Post Hoc analysis to break down samples in the two locations by time **(TABLE 3.3.2 and table 3.3.3)**.

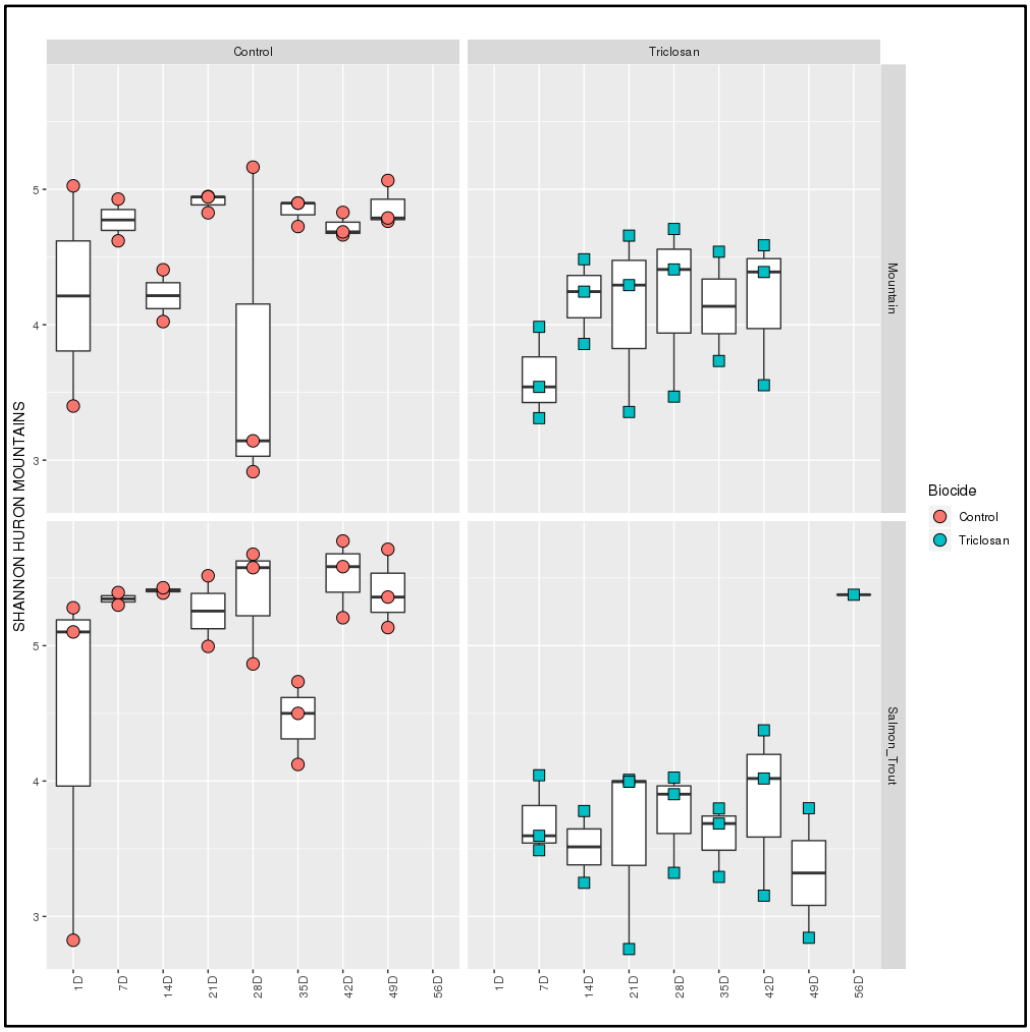

Figure (3.3.2) Shannon Index of microbial groups Alpha Diversity presented as box plot. The colors characterize the different classes in the data set. Two locations are plot as box bar to show the different between each other.

In Mountain Stream samples experiment there was no data for TCS in the first day. In the control we can see same in the richness in the whole experiment and it was ranging from 4.3 – 4.7. However, in the day 28 we see a large drop in the richness, to 3.2. But, in the last day the number of the community went back to almost the diversity observed at the start of the experiment. Assuming that the diversity on day zero was the same as the control, there was a substantial decrease in diversity from day zero to one week. After this initial drop, diversity in the TCS-treated samples

generally increased over time, from 3.6 at its lowest to 4.4 in the last day. Overall, the control had higher richness than the TCS-treated microcosms.

Similar to the Mountain Stream experiment, there was no data for TCS in the first day for Salmon Trout Run samples (Fig 3.3.2). Control samples on the first day had a richness of 5.1 and increased over time till the day 35 there was a big drop in the richness, but it went back to the normal number by day 42. If the richness observed on day zero was assumed to be similar to the richness at day zero for the controls (5.3), then there was a substantial decrease in richness observed between day zero and the first time point in the TCS-amended sampled (3.5). TCS-amended samples exhibited decreasing richness over time, starting from 3.6 and dropping all the way till the last week which was 3.2.

Region	***P.***	***F***
Green Bay	0.0528	2.025
Houghton	**< 0.00001**	11.005

Table 3.3.1 Two-way ANOVA test to see the significant change in the time between two different locations.

Time	***0H***	***24H***	***7D***	***21D***	***29D***	***36D***	***43D***	***50D***
0H		0.999	0.996	0.999	0.135	0.262	0.903	0.2839
24H			0.999	1.0	0.222	0.391	0.961	0.925
7D				0.9992	0.599	0.793	0.996	0.998
21D					0.201	0.362	0.951	0.909
29D						0.999	0.849	0.908
36D							0.959	0.981
43D								0.999
50D								

Table 3.3.3 Tukey's Honest Significant Difference (HSD) test is a post hoc test shows p-values. P-values that are bolded is showing a big difference in Green Bay samples starting from 0 hour till the 50 days.

Time	*0H*	*24H*	*7D*	*14D*	*21D*	*28D*	*42D*	*50D*
0H		0.489	**< 0.0219**	0.999	**< 0.00001**	**< 0.00001**	**< 0.0004645**	0.252
24H			0.792	0.187	**< 0.0042**	**< 0.0117**	0.247	0.999
7D				**< 0.0044**	0.534	0.712	0.998	0.956
14D					**< 0.00001**	**< 0.00001**	**< 0.0000555**	0.0753
21D						**< 0.00001**	0.861	**< 0.0247**
28D							0.956	0.057
42D								0.545
50D								

Table 3.3.2 Tukey's Honest Significant Difference (HSD) test is a post hoc test shows p-values. P-values that are bolded is showing a big difference in Houghton samples starting from 0 hour till the 50 days.

3.4 Beta Diversity

To understand the changes in community composition, we used PERMANOVA analysis for all locations (Green Bay, Houghton, Mountain Stream and Salmon Trout Run) to see if there is a significant difference in microbial community composition between the treatments and time **(Table 3.4.1)**. Statistical comparisons of treatment using the Pairwise PERMANOVA indicated that there was a significant difference in the microbial community composition between control and treatment samples in Houghton, Huron Mountain and Salmon Trout. Also, a significant difference was seen in Green Bay between treatments, but not as significant as the previous locations. **(Table 3.4.2, 3.4.3, and 3.4.4)**. Principal coordinate analysis (PCoA) was done to determine the variation between the taxonomic composition of the samples. PCoA showed that samples segregated primarily by time for Green Bay, with 30.5% of variance in the data explained by the two-dimensional plot **(Fig 3.4.1).** PCoA analysis was done also for Houghton samples with 29 % of the variance explained by the plot **(Fig 3.4.2).** PCoA analysis was done also for Huron Mountain and Salmon

Trout samples together in one plot with 45.7 % of the variance explained by the plot **(Fig 3.4.3).**

Region	*P.*	*F*
Green Bay	**0.009**	0.942
Houghton	**< 0.001**	1.703
Huron Mountain	**< 0.001**	0.272
Salmon Trout	**< 0.001**	0.166

Table 3.4.1 Overall PERMANOV by treatments in all sample locations

Treatment 1	*Treatment 2*	*R2*	*pvalFDR*
Control	***Tetracycline***	0.0315	0.834
Control	***TCS 2 ppm***	0.0375	0.900
Control	***TCS 6 ppm***	0.0370	0.483
Tetracycline	***TCS 2 ppm***	0.0371	0.744
Tetracycline	***TCS 6 ppm***	0.0315	0.875
TCS 2 ppm	***TCS 6 ppm***	0.0255	0.736

Table 3.4.2 PERMANOVA pairwise results comparisons between treatments in all Green Bay and Houghton samples

Treatment 1	*Treatment 2*	*R2*	*pvalFDR*
Control	***Tetracycline***	0.0331	0.447
Control	***TCS 2 ppm***	0.0341	0.561
Control	***TCS 6 ppm***	0.0403	0.726
Tetracycline	***TCS 2 ppm***	0.0426	0.382
Tetracycline	***TCS 6 ppm***	0.0371	0.508
TCS 2 ppm	***TCS 6 ppm***	0.0338	0.432

Table 3.4.3 PERMANOVA pairwise results comparisons between Green Bay treatments.

Treatment 1	*Treatment 2*	*R2*	*pvalFDR*
Control	***Tetracycline***	0.0728	0.002
Control	***TCS 2 ppm***	0.0884	0.002
Control	***TCS 6 ppm***	0.0760	0.002
Tetracycline	***TCS 2 ppm***	0.1020	0.002
Tetracycline	***TCS 6 ppm***	0.0517	0.027
TCS 2 ppm	***TCS 6 ppm***	0.0958	0.002

Table 3.4.4 PERMANOVA pairwise results comparisons between Houghton treatments.

		R2	*pvalFDR*
Control	***TCS***	0.234	0.001
Huron Mountain	***Salmon Trout***	0.111	0.001

Table 3.4.4 PERMANOVA pairwise results comparisons between treatments in Huron Mountain and Salmon Trout.

The results of the overall PERMANOVA by treatments in all sample locations showed that treatments in Houghton, and the Huron Mountains had a significant difference in microbial community composition. While, Green Bay shows an overall significant difference but not as the previous locations. The second table **(Table 3.4.2)** showed pairwise results comparisons between Houghton and Green Bay treatments that there was no significant difference in microbial community composition. The third table (**Table 3.4.3**) showed pairwise results comparisons between treatments in Green Bay samples, and those samples had no significant difference in microbial community composition. The fourth table (**Table 3.4.4**) showed pairwise results comparisons between treatments in Houghton samples, and those samples a significant difference in all treatments with a significant between Tetracycline and TCS 6 ppm in microbial community composition, but not big as the other treatments. The last table (**Table 3.4.4)** showed pairwise results comparisons between first the Control and TCS in both Mountain Stream and Salmon Trout and those samples had a significant difference. Also, a second comparisons between the both locations which showed a significant difference.

PCoA analysis indicated that in Green Bay all samples clustered together at the zero hour (Figure 3.4.1). At 24 hours, the samples started to cluster away from the zero hour between each other. At 7 days, the control clustered alone far away from the treatments. In particular the TCS 2 ppm and 6 ppm clustered near to each other at 7 days. In the 21 days, control is clustering near each other while we see TCS 6 ppm and Tetracycline is clustering far away from them. From 29 days, 36 days, 43 days and 50 days we see all sample including control were clustered near each other more.

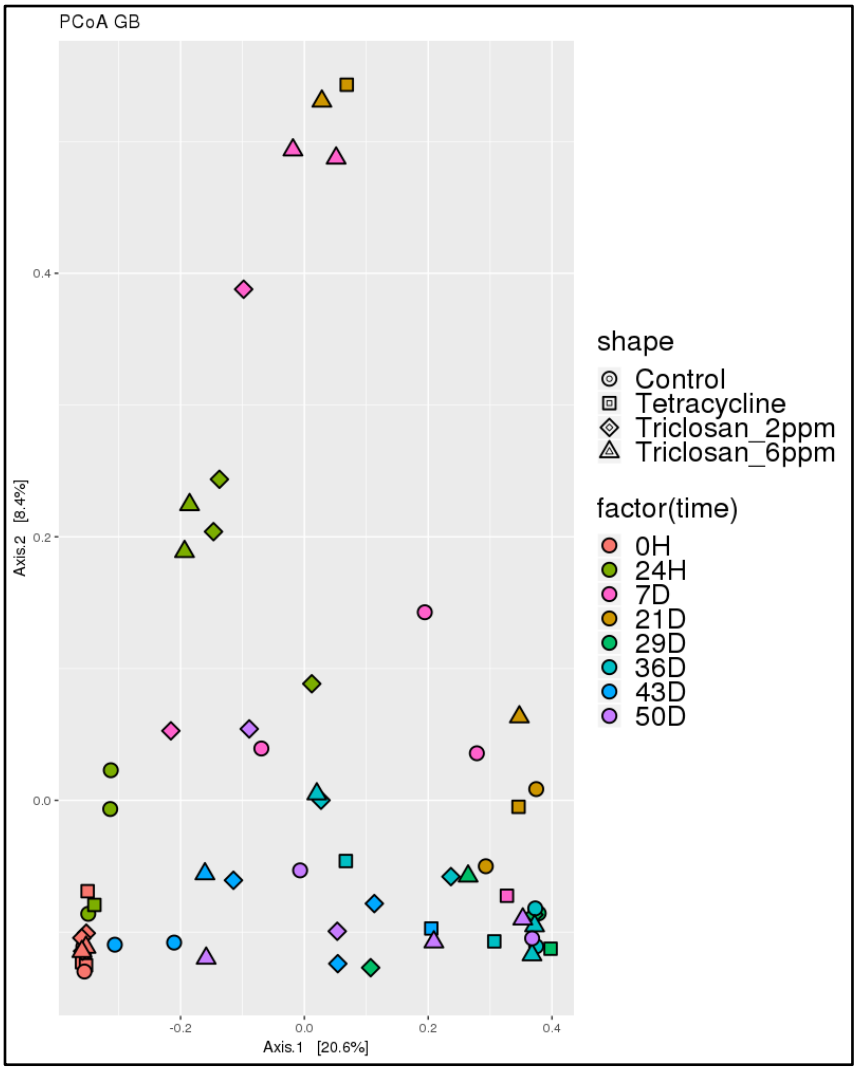

Figure (3.4.1) Principal coordinate analysis (PCoA) with Bray-Curtis showing the dissimilarity and similarity in Green Bay by time and treatment.

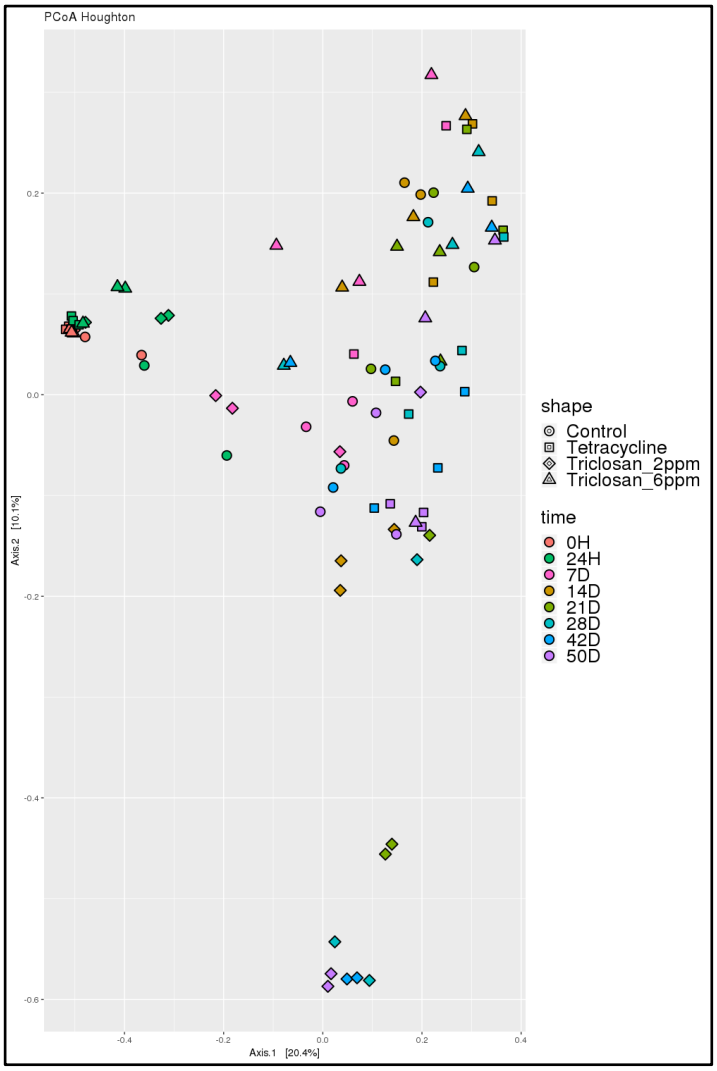

Figure (3.4.2) Principal coordinate analysis (PCoA) with Bray-Curtis showing the dissimilarity in Houghton separated by time and treatment.

PCoA analysis for samples from Houghton showed that at the Zero hour all conditions clustered near each other. **(Figure 3.4.2).** Likewise, the clustering continued with the 24 hours timepoints for all treatment. Suggesting that there was little change in the microbial community in the initial timepoints for these samples. At 7 days, TCS 6 ppm and Tetracycline samples started to cluster a little bit away from control and TCS 2 ppm. At 21 days, the TCS 2 ppm clustered far away from control, TCS 6 ppm and Tetracycline. In the 21 Days and 28 days TCS 2 ppm

continued to cluster far away from other samples, Tetracycline samples started to clusters nearer to the TCS 2 ppm samples and far away from the control and TCS 6 ppm. In the 42 days, TCS 2 ppm separated themselves from all other treatments. In addition, control, TCS 6 ppm and Tetracycline are clustering near each other. In 50 days, TCS 2 ppm samples clustered far away from other samples and control, TCS 6 ppm and Tetracycline were still clustered close to each other.

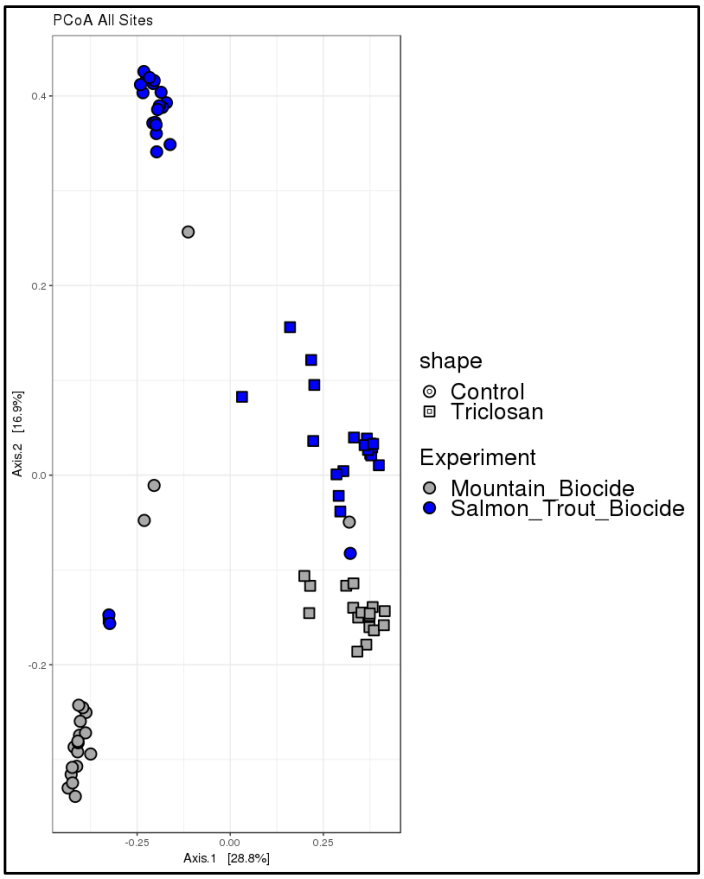

Figure (3.4.3) Principal coordinate analysis (PCoA) with Bray-Curtis showing the dissimilarity in Huron Mountain and Salmon Trout samples to TCS separated by treatment.

The strongest changes in community composition were observed in treatments from the Huron Mountains. The TCS-amended treatments from both Mountain Stream and Salmon Trout exhibited distinct community composition from their respective controls **Figure (3.4.3)**. In both sites the control samples clustered far

away from the TCS samples. Interestingly, while both sites started off with distinct community compositions, the TCS-impacted samples from both sites, clustered more closely together than their controls. This may suggest that TCS addition results in a selection for a similar set of microbes in the Huron Mountain Club lands.

Because of the wide differences between locations and in the structure of the bacterial community, a more specific analysis in the change in abundance of members of the microbial community was undertaken. We used DESeq to compare control samples to treatments and identify any ASVs that were differentially abundant. We rated that an ASV was enriched if the adjusted p value was less than 0.05 and the log2 fold change was greater than 2.

3.5 Differential Abundance Analysis and Taxa IDs

Differentially abundant ASVs were plotted as a volcano plot comparing significance of enrichment and fold change for each ASVs. To identify which ASVs responded to the treatments, we determined the differentially abundant ASVs between treatments and control conditions. ASVs that were enriched in the treatments would correspond to resistant taxa as they are higher in abundance in the antimicrobial treatments. Whereas, ASVs enriched in the controls would be considered sensitive as they are lower in abundance in the treatment compared to the control. There was a pairwise differences in the community composition for all sites, but we only found differentially abundant ASVs for Houghton and the Huron Mountain sites. For TCS 2 ppm from Houghton it was found that 21 ASVs were enriched in the presence of TCS 2 ppm, while there were 14 ASVs enriched in the controls these differences are plotted as a volcano plot **(FIGURE 3.5.1).** For TCS 6 ppm from Houghton it was found that 52 ASVs were enriched in the presence of TCS 6 ppm, while there were 45 ASVs enriched in the controls and these differences are plotted as a volcano plot **(FIGURE 3.5.2).** For Tetracycline from Houghton it was found that 36 ASVs were enriched in the presence of Tetracycline, while there were 12 ASVs enriched in the controls relative to Control. These differences are plotted as

a volcano plot **(FIGURE 3.5.3).** We also generated a plot for the Mountain Stream and Salmon Trout locations. For Mountain Stream it was found that 281 ASVs were enriched in the presence of TCS 6 ppm, while there were 284 ASVs enriched in the controls. **(FIGURE 3.5.4).** For Salmon Trout it was found that only Control were enriched 93 ASVs, while there were 268 ASVs enriched in the TCS 6ppm. **(FIGURE 3.5.5).**

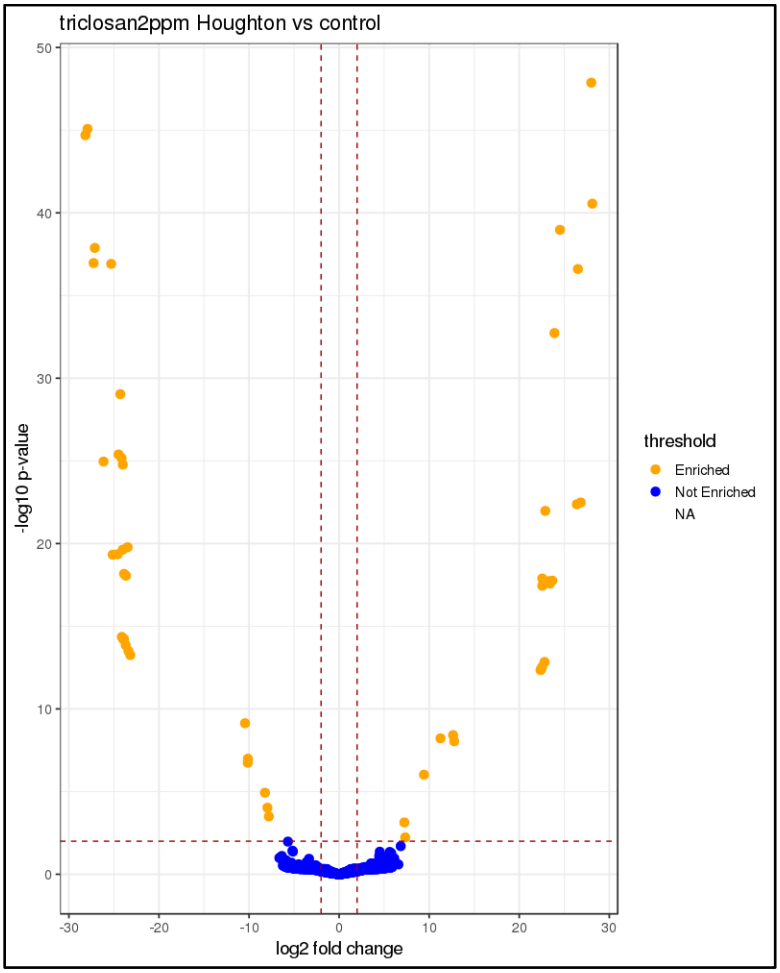

FIGURE 3.5.1 Volcano plot of enriched ASVs in TCS 2 ppm vs control in Houghton. The enriched ASVs in the presence of TCS 2 ppm are represented as the points on the positive while the control on the negative side of the plot. Enriched ASVs are shown in orange.

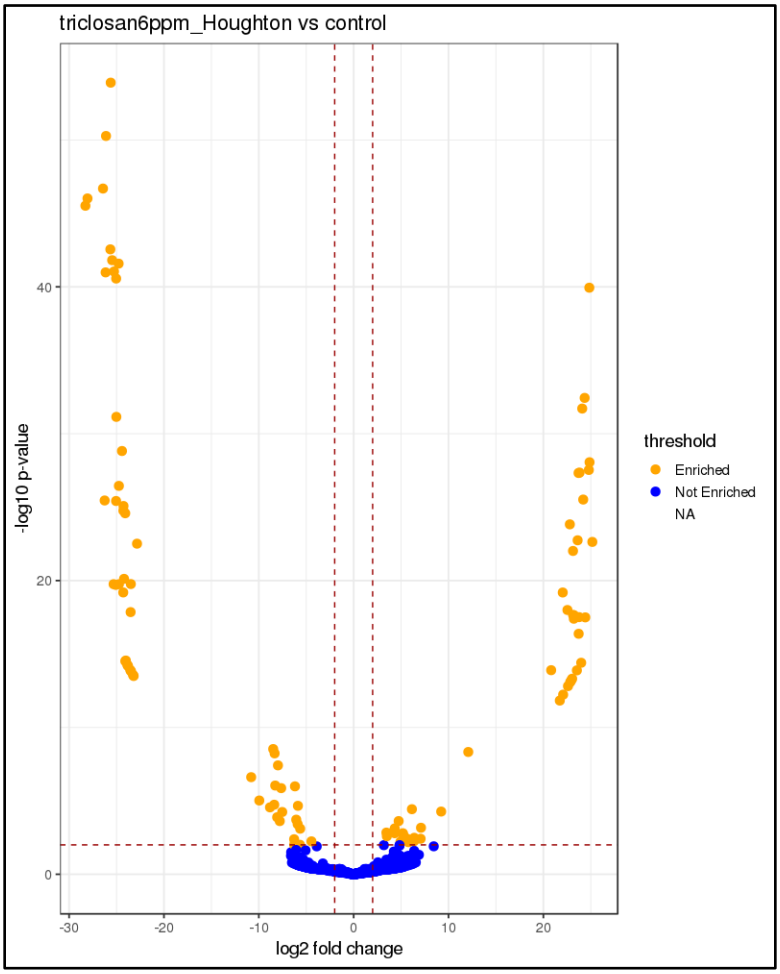

FIGURE 3.5.2 Volcano plot of enriched ASVs in TCS 6 ppm vs control in Houghton. The enriched ASVs in the presence of TCS 6 ppm are represented as the points on the positive while the control on the negative side of the plot. Enriched ASVs are shown in orange.

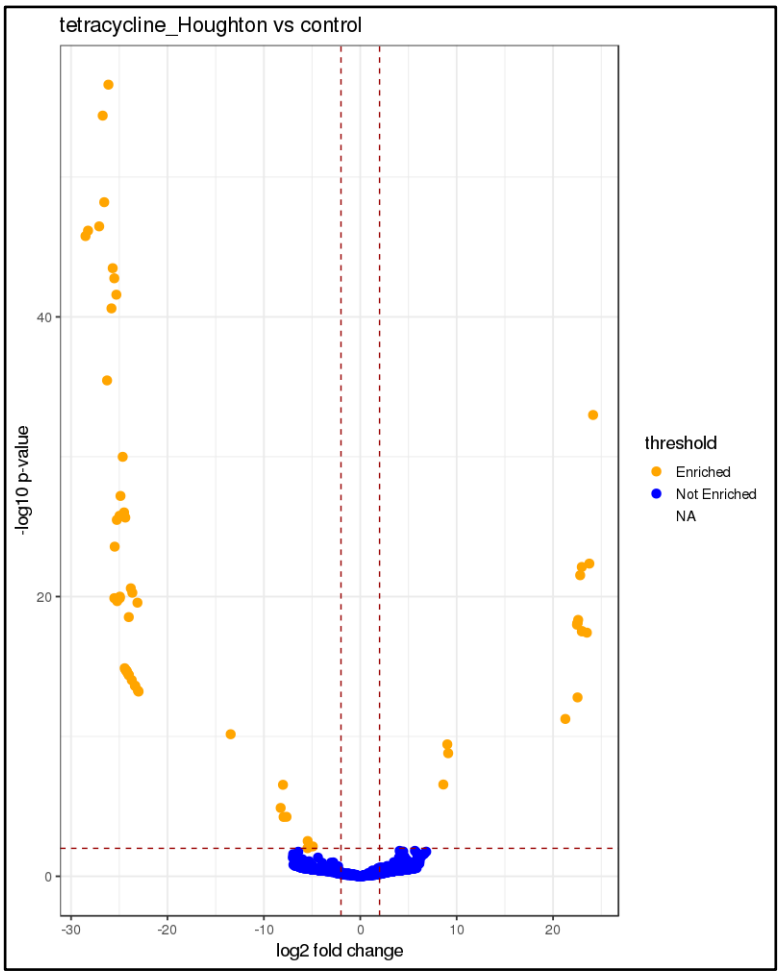

FIGURE 3.5.3 Volcano plot of enriched ASVs in Tetracycline vs control in Houghton. The enriched ASVs in the presence of Tetracycline are represented as the points on the positive while the control on the negative side of the plot. Enriched ASVs are shown in orange.

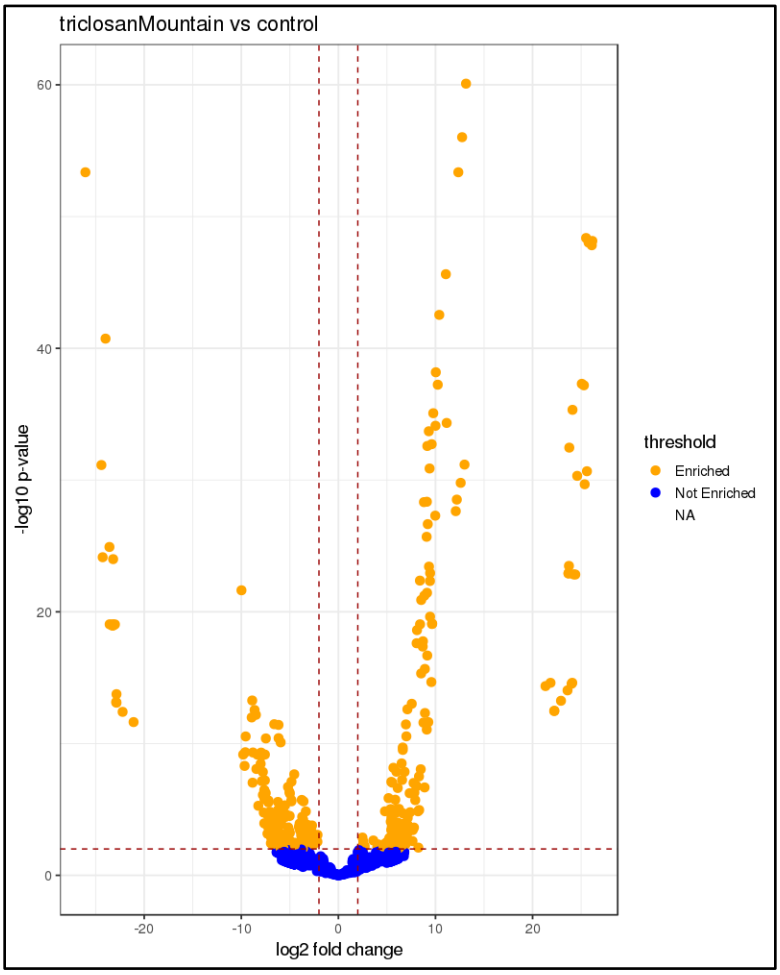

FIGURE 3.5.4 Volcano plot of enriched ASVs in TCS vs control in Huron Mountain. The enriched ASVs in the presence of TCS are represented as the points on the positive while the control on the negative side of the plot. Enriched ASVs are shown in orange.

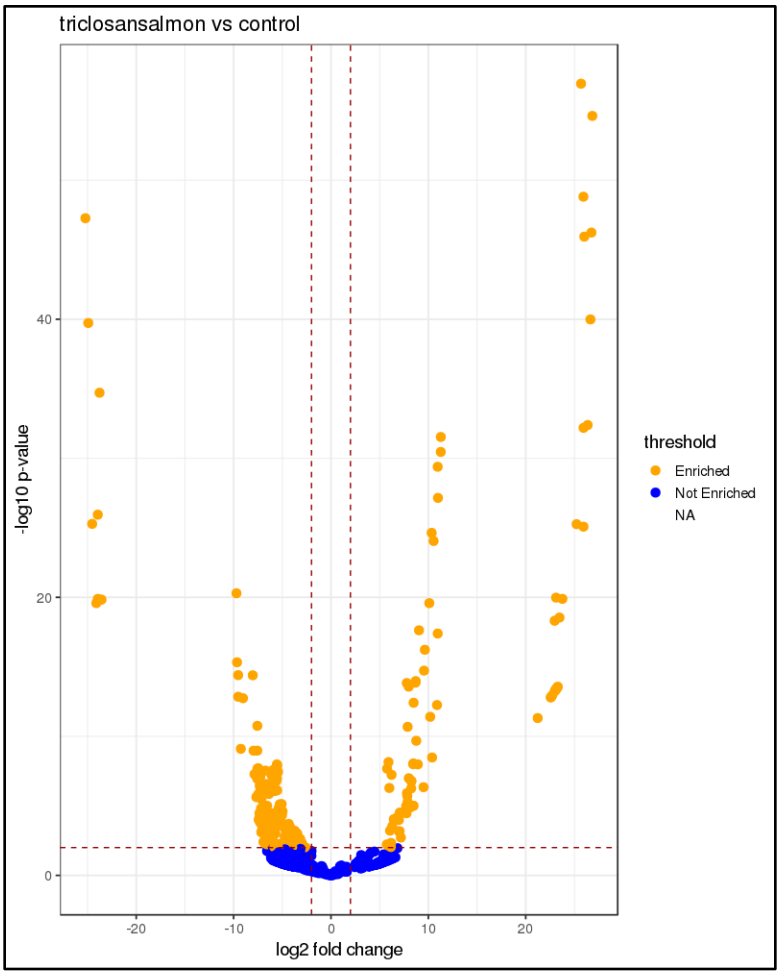

FIGURE 3.5.5 Volcano plot of enriched ASVs in TCS vs control in Salmon Trout. The enriched ASVs in the presence of TCS are represented as the points on the positive while the control on the negative side of the plot. Enriched ASVs are shown in orange.

Table 3.5.1 Table of top 5 enriched ASVs for Houghton with different treatments.

ASV IDs	log2 Fold Change	padj	Phylum	Class	Order	Family	Genus
Tetracycline							
sp348	19.58105978	1.11E-47	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	NA
sp677	18.44114264	3.31E-50	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
sp276	17.97001799	7.72E-76	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp466	17.6852991	3.46E-65	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp481	17.23760797	3.19E-76	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
Triclosan 2 ppm							
sp677	18.9913081	4.43E-54	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
sp229	18.0964664	5.75E-75	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp466	17.9421105	1.01E-67	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp343	17.7156297	7.21E-64	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp276	17.5617739	6.39E-73	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
Triclosan 6 ppm							
sp677	19.2508941	1.56E-55	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
sp229	18.5921847	1.35E-79	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp276	18.0859257	9.45E-78	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum
sp481	16.5486622	1.11E-71	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
sp348	13.509083	7.44E-23	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	NA

Table 3.5.2 Table of top 5 enriched ASVs for Salmon Trout and Huron Mountain location.

ASV IDs	log2 Fold Change	padj	Phylum	Class	Order	Family	Genus
Salmon Trout							
Sp110	26.84096371	2.38E-55	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium -Neorhizobium -Pararhizobium -Rhizobium
Sp120	26.74851986	5.84E-47	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium -Neorhizobium -Pararhizobium -Rhizobium
Sp143	26.64615673	1.03E-40	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas
Sp181	26.3634199	4.06E-33	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas
Sp138	26.01439841	1.15E-46	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas
Huron Mountain							
Sp333	26.1555402	7.12E-49	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
Sp351	26.1001774	1.52E-48	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
Sp415	25.76972877	9.40E-49	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
Sp379	25.60014356	2.15E-31	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium -Neorhizobium -Pararhizobium -Rhizobium
Sp497	25.51149962	4.26E-49	Actinobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas

The top 5 most enriched ASVs in treatment samples by each individual site **(TABLE 3.5.1 and 3.5.2).** Interestingly, we found that and ASV from the order Rickettsiales was the most enriched ASV in the Tetracycline treated samples from Houghton. Members of the Sphingomonadaceae were highly enriched in all treatments in Houghton. Additionally, members of the Azospirillum were also highly enriched in the treated samples from Houghton. ASVs tables in Houghton in these treatments TCS 2 ppm, 6 ppm and Tetracycline enrichment was the majority for the Alphaproteobacteria class, but we also have other classes like Bacteroidetes and Gammaproteobacteria **(TABLE 3.5.1)**.

In Huron Mountain we see in the top 5 enriched ASVs in TCS are from classes like Gammaproteobacteria and Alphaproteobacteria, members of the Xanthamonadales and the Rhizobiales. ASVs tables in overall Huron Mountain enrichment of TCS was showing so many different classes such as Alphaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacteroidia. However, the majority of the enrichment is Gammaproteobacteria. In Salmon Trout we see in the top 5 enriched ASVs in TCS are from classes like Gammaproteobacteria and Alphaproteobacteria, members of the Xanthamonadales and the Rhizobiales. ASVs tables in overall Salmon Trout enrichment was showing so many different classes such as Alphaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacteroidia **(Table 3.5.2)**.

4. Discussion

The objectives of this study were to examine the effect of household biocide and antibiotics on the aquatic microbial communities to better understand their impact on the environment. We had three hypotheses the first one was that the concentration of the biocide will have an impact on the microbial community. Second, we hypothesized that exposure to antibiotics in samples would cause a difference in the response of the microbial communities compared to biocide treatment. Third, we hypothesized that areas with a larger populations would have more human activity and thus result in increased resistance to the antimicrobials while microbial communities in areas with lower populations would be more impacted by antimicrobial addition.

4.1 Impact of TCS concentration

The first expectation of our hypothesis was that when TCS was added to the samples at different concentrations, there will be a change in the diversity of microbial communities, where the higher concentration would have the greatest influence compared to the lower concentration. This prediction was based on previous studies conducted in several concentrations of TCS which showed a different response of the microbial communities according to the concentration [47, 48]. However, we observed that the community response varied by location and time as well as the concentration of TCS. According to the PERMANOVA analysis, these different concentrations caused a different community composition as well as difference in the abundance of different classes at different sites. Our data show that there were more differentially abundant ASVs with higher concentration of TCS. We identified differentially abundant taxa present in Houghton, a greater number of taxa were found in TCS 6 ppm vs. TCS 2 ppm (21 in TCS 2 ppm, 51 in TCS 6 ppm). The most abundant microbial class in Houghton samples for the concentration 2 ppm and 6 ppm was Alphaproteobacteria, which dominated the top 5 classes. Moreover, the top five differentially abundant ASVs in TCS 2 ppm were from only two Orders

(Sphingomonadales and Azospirillales), while the top five differentially abundant ASVs in the TCS 6 ppm had the Orders (Sphingomonadales, Azospirillales and Rickettsiales). Previous studies have shown that Alphaproteobacteria often go through horizontal gene transfer more commonly than other Proteobacteria, and their extensive genomes are known to have a larger number of mobile elements [59]. Besides, Alphaproteobacteria are Gram-negative and, therefore, They are more resistant to antimicrobial due to their outer membrane, compared to gram-positive bacteria [50, 60]. These finding may explain the higher abundance or adaptation detected in the microbial communities in the TCS 2 ppm. This concentration might be able to kill other classes. However, these Alphaproteobacteria can seize the opportunity and resist the biocide, multiply and dominate. Also, members of the Rickettsiales order were the most enriched ASV in the Tetracycline treated samples from Houghton. whose members are linked to severe diseases in mammals, including humans [61]. This order, Rickettsiales, might have the ability to replicate in the lakes and rivers, and when they have a significant number, dominate the microbial community. They might find their way to be in the drinking water. Additionally, Members of the Sphingomonadaceae were highly enriched in all treatments in Houghton. There is study that show members of this order were used for environmental remediation, and they have the ability to degrade some aromatic compounds in some of their species [62]. Again, if these species stay a long time in the environment, they might change the quality of the water and might affect the normal life cycle for other species. In Green Bay, the TCS 2 ppm conditions showed an increase in the 16S rRNA gene copies in the last 4 weeks, while the TCS 6 ppm conditions decreased. In Houghton samples, both concentrations resulted in a decrease in the 16S rRNA gene copies in the last 4 weeks. However, there was stronger decrease in the TCS 6 ppm compared to TCS 2 ppm. That indicate that when microbial communities were exposed to a higher concentration of TCS, they start to lose their ability to survive and in the low concentration some of the bacteria start to fight back and can continue to grow. Previous studies have indicated that TCS is bacteriostatic at low concentrations, but higher levels are bactericidal. Showing that

TCS acts on a specific bacterial target at low concentrations and is nonspecific at higher concentrations [12].

4.2 Difference between biocide and antibiotic

Our second hypothesis in this study was that by exposing our samples to Tetracycline and TCS, there would be a different reaction in the bacterial communities between these two antimicrobials. This was expected because antibiotic and biocide agents attack bacterial communities differently and have a different mechanism of action. Based on our PERMNOVA results, there was a significant difference between the biocide and antibiotic treatments in Houghton. There was a similarity when we compared Tetracycline with TCS 6 ppm, but compared to Tetracycline with TCS 2 ppm, there was a significant difference. There appears to be a similar response in the microbial community to tetracycline and TCS 6 ppm. The similar response to TCS and Tetracycline may be due to both antimicrobials being bactericidal at the tested concentrations. However, the lower concentration of TCS may not result in as much cell death. This similar response may suggest that bacterial communities repeatedly exposed TCS may be enriched for microbes that are able to resist tetracycline. As the world has recently been faced with the problem of rapid development of antibacterial resistance, it may be that perhaps the use TCS has accelerated the resistance process. Previous studies have found a relationship between recurrent exposure to TCS and the spread of antibiotic resistance in the environment and how TCS could lead to cross-resistance to antibiotics [63 ,47 ,46]. Our data shows Gammaproteobacteria were some of the most enriched ASVs in the Huron Mountains, and also in other ASVs tables in other locations. A previous study showed that TCS in wastewater treatment plants (WWTPs) may select for some strategies for resistance, such as alterations to the outer membrane or expression of nonspecific efflux pumps, which may give bacteria cross-resistance to antibiotic drugs, as was observed in *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella enterica*. [46] However, our experiment was in fresh water, and our experiments

enriched other bacterial orders as well, but still they are the same class and might have the ability to cross-resistance to biocides and antibiotics.

4.3 Difference with population size

Our third hypothesis in this study was that the microbial response to these treatments would be related to the population size of the region. Three different locations were chosen that have a varied population size. Green Bay has a population of 105,116 people, Houghton 7,888 people, and the Huron Mountain club is an isolated land with only a seasonal population. We expected that the increase in the population would be related to an increase in human activity and thus effecting the environment. We think the big number of a population would lead to higher volumes of wastewater being released compared to other locations. For example, we would expect to have more waste water released in Green Bay and less in Houghton. Other studies have shown the presence of TCS in domestic wastewater, and TCS could escape wastewater treatment make it is way to the aquatic ecosystems [33, 34, 64]. Our data shows there is a minor community changes in taxa diversity in some sites while others have huge changes. For example, we saw the most a significant change in the microbial community composition in response to treatment in the Huron mountains. There were also significant changes in response to treatment in Houghton, but not big as seen in the Huron Mountains. There was little change in the Green Bay microbial communities in response to treatment. Due to its antimicrobial properties, TCS may negatively affect the abundance and activity of benthic bacteria, which could have broader ecosystem-level implications because benthic bacteria are key drivers of nutrient cycling. If bacterial taxa differ in TCS sensitivity, then TCS may also act as a selective agent and drive changes in bacterial community composition, which can impact function [49, 65]. These findings demonstrate that when there is a large population and the high human activity, these aquatic settings may have been subjected to previous exposure of antimicrobials. Therefore, the microbial communities may not be as affected by the concentrations used in this study. While the untouched environment, such as Huron Mountains, will be radically impacted by

these concentrations. Moreover, as we see in the Huron Mountains, there is a dramatic change in the microbial community of pristine locations in response to antimicrobials. This may support the idea that some human activities present a harsh environment for bacterial communities. Through this stress, human societies can begin to drive adaption to produce strains capable of continuity, resistance, and reproduction under these stressful conditions.

5. Conclusion

In the past 50 years we have seen the use of TCS intensively in our daily lives and this may cause it to accumulate in nature and to stay in lakes and rivers for a long time. The presence of TCS in nature is a concern because of the effects it can produce. TCS was found in measurable quantities, which proved its ability to overcome some water purification processes in wastewater treatments and ends in nature. These quantities may affect the ecosystem, human health and water quality. Sequencing and analysis of our bacterial communities illustrate the following result. Exposing bacterial communities to different TCS concentrations results in a significant difference between the samples and change the microbial communities. We had more abundance in samples that had a higher concentration of TCS, and less abundance in the samples that had a small concentration. This leads to a total change in the composition of the microbial community. Furthermore, after exposing the samples to two different combinations of antimicrobial substances, TCS and Tetracycline. We found that there is a difference between biocides and antibiotics because bacterial communities have a different reaction. Where there is less difference with the higher concentration of TCS compared to Tetracycline, and this difference increases with the lowest concentration of TCS. Finally, the difference with the population size is linked to the number. Where the population is reduced the change in microbial communities is more and while the population is higher, the change was small. Our results indicate there was a significant change in the microbial community in Huron Mountains. Houghton also showed a significant but not as much as in the Huron Mountains, and Green Bay only slightly changed. These findings suggest that biocide release has potential to dramatically affect aquatic microbial communities and repeated exposure to biocides may select for communities that are more resistant to biocides and other antimicrobials.

6. References list

1. Larson, E., *Antiseptics.* APIC Infection Control & Applied Epidemiology: Principles & Practices, 1996: p. 19.
2. Rutala, W.A., *Guidelines Committee: Draft Apic guideline for selection and use of disinfectants.* Ajic American Journal of Infection Control, 1995. **23**(3): p. 35A-67A.
3. McDonnell, G. and A.D. Russell, *Antiseptics and disinfectants: activity, action, and resistance.* Clinical microbiology reviews, 1999. **12**(1): p. 147-179.
4. Regulation, E., *No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products.* Official Journal of the European Union L, 2012. **167**.
5. Ternes, T.A., A. Joss, and H. Siegrist, *Peer reviewed: scrutinizing pharmaceuticals and personal care products in wastewater treatment*. 2004, ACS Publications.
6. Insights, R., *Biocides Market Analysis, Size, Share, Industry Report 2022*. 2015.
7. Hinther, A., et al., *Effects of triclocarban, triclosan, and methyl triclosan on thyroid hormone action and stress in frog and mammalian culture systems.* Environmental science & technology, 2011. **45**(12): p. 5395-5402.
8. Lozano, N., et al., *Fate of triclocarban, triclosan and methyltriclosan during wastewater and biosolids treatment processes.* Water research, 2013. **47**(13): p. 4519-4527.
9. Food, U. and D. Administration, *CFR-code of federal regulations title 21.* Current good manufacturing practice for finished pharmaceuticals Part, 2017. **211**.
10. Geueke, B., *Dossier – Biocides and food contact materials.* Food Packaging Forum, 2014.
11. Block, S.S., *Disinfection, sterilization, and preservation*. 2001: Lippincott Williams & Wilkins.
12. Russell, A., *Whither triclosan?* Journal of Antimicrobial Chemotherapy, 2004. **53**(5): p. 693-695.
13. Davin-Regli, A. and J. Pagès, *Cross-resistance between biocides and antimicrobials: an emerging question.* Revue Scientifique et Technique-OIE, 2012. **31**(1): p. 89.
14. Russell, A., *Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations.* The Lancet infectious diseases, 2003. **3**(12): p. 794-803.
15. Shannon, K., *Understanding Antibacterial Action and Resistance: 2nd edn. Russell, AD, Chopra, I. Ed. London: Ellis Hornwood, price£ 31.50. 292 pp. ISBN 0-13-124827-8.* Journal of Hospital Infection, 1996. **34**(3): p. 241-242.
16. Collier, P., et al., *Growth inhibitory and biocidal activity of some isothiazolone biocides.* Journal of Applied Bacteriology, 1990. **69**(4): p. 569-577.
17. Thurman, R.B., C.P. Gerba, and G. Bitton, *The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses.* Critical reviews in environmental science and technology, 1989. **18**(4): p. 295-315.
18. Dukan, S., S. Belkin, and D. Touati, *Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid.* Archives of biochemistry and biophysics, 1999. **367**(2): p. 311-316.
19. Chapman, J.S., *Biocide resistance mechanisms.* International Biodeterioration & Biodegradation, 2003. **51**(2): p. 133-138.
20. Broxton, P., P. Woodcock, and P. Gilbert, *A study of the antibacterial activity of some polyhexamethylene biguanides towards Escherichia coli ATCC 8739.* Journal of Applied Bacteriology, 1983. **54**(3): p. 345-353.
21. Gilbert, P., E. Beveridge, and P. Crone, *The lethal action of 2-phenoxyethanol and its analogues upon Escherichia coli NCTC 5933.* Microbios, 1977. **19**(76): p. 125-141.
22. CHAWNER, J.A. and P. Gilbert, *Interaction of the bisbiguanides chlorhexidine and alexidine with phospholipid vesicles: evidence for separate modes of action.* Journal of Applied Bacteriology, 1989. **66**(3): p. 253-258.
23. Morente, E.O., et al., *Biocide tolerance in bacteria.* International journal of food microbiology, 2013. **162**(1): p. 13-25.
24. Gillespie, S.H., *Antibiotic resistance: methods and protocols*. Vol. 48. 2001: Springer Science & Business Media.

25. Cloete, T.E., *Resistance mechanisms of bacteria to antimicrobial compounds.* International Biodeterioration & Biodegradation, 2003. **51**(4): p. 277-282.
26. Zhang, Y. and D.B. Young, *Molecular mechanisms of isoniazid: a drug at the front line of tuberculosis control.* Trends in microbiology, 1993. **1**(3): p. 109-113.
27. Denyer, S.P. and G. Stewart, *Mechanisms of action of disinfectants.* International biodeterioration & biodegradation, 1998. **41**(3-4): p. 261-268.
28. McMurry, L.M., M. Oethinger, and S.B. Levy, *Triclosan targets lipid synthesis.* Nature, 1998. **394**(6693): p. 531.
29. Suller, M. and A. Russell, *Antibiotic and biocide resistance in methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococcus.* Journal of Hospital Infection, 1999. **43**(4): p. 281-291.
30. Kojima, S. and H. Nikaido, *Permeation rates of penicillins indicate that Escherichia coli porins function principally as nonspecific channels.* Proceedings of the National Academy of Sciences, 2013. **110**(28): p. E2629-E2634.
31. Vargiu, A.V. and H. Nikaido, *Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations.* Proceedings of the National Academy of Sciences, 2012. **109**(50): p. 20637-20642.
32. Mcmanus, M.C., *Mechanisms of bacterial resistance to antimicrobial agents.* American Journal of Health-System Pharmacy, 1997. **54**(12): p. 1420-1433.
33. Bester, K., *Fate of triclosan and triclosan-methyl in sewage treatmentplants and surface waters.* Archives of Environmental Contamination and Toxicology, 2005. **49**(1): p. 9-17.
34. Halden, R.U. and D.H. Paull, *Co-occurrence of triclocarban and triclosan in US water resources.* Environmental science & technology, 2005. **39**(6): p. 1420-1426.
35. Food, U. and D. Administration, *FDA issues final rule on safety and effectiveness of antibacterial soaps.* FDA Web site, 2016.
36. Chemicals, C.S., *General Information on Chemical, Physical and Microbiological Properties of Irgasan DP300, Irgacare MP and Irgacide LP10*. 2001, Brochure.
37. Walsh, C., *Antibiotics: actions, origins, resistance*. 2003: American Society for Microbiology (ASM).
38. Thaker, M., P. Spanogiannopoulos, and G.D. Wright, *The tetracycline resistome.* Cellular and Molecular Life Sciences, 2010. **67**(3): p. 419-431.
39. Baltz, R.H., *Antimicrobials from actinomycetes: back to the future.* Microbe-American Society For Microbiology, 2007. **2**(3): p. 125.
40. Chopra, I. and M. Roberts, *Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance.* Microbiology and molecular biology reviews, 2001. **65**(2): p. 232-260.
41. McAvoy, D.C., et al., *Measurement of triclosan in wastewater treatment systems.* Environmental toxicology and chemistry, 2002. **21**(7): p. 1323-1329.
42. Kaushal, S.S., et al., *Tracking nonpoint source nitrogen pollution in human-impacted watersheds.* Environmental science & technology, 2011. **45**(19): p. 8225-8232.
43. Chuanchuen, R., et al., *Cross-Resistance between Triclosan and Antibiotics inPseudomonas 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, 2001. **45**(2): p. 428-432.
44. Prudhomme, M., et al., *Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae.* Science, 2006. **313**(5783): p. 89-92.
45. Birošova, L. and M. Mikulašova, *Development of triclosan and antibiotic resistance in Salmonella enterica serovar Typhimurium.* Journal of Medical Microbiology, 2009. **58**(4): p. 436-441.
46. Carey, D.E. and P.J. McNamara, *The impact of triclosan on the spread of antibiotic resistance in the environment.* Frontiers in microbiology, 2015. **5**: p. 780.
47. McNamara, P.J., T.M. LaPara, and P.J. Novak, *The impacts of triclosan on anaerobic community structures, function, and antimicrobial resistance.* Environmental science & technology, 2014. **48**(13): p. 7393-7400.

48. Drury, B., et al., *Triclosan exposure increases triclosan resistance and influences taxonomic composition of benthic bacterial communities.* Environmental science & technology, 2013. **47**(15): p. 8923-8930.
49. Robert Jr, O. and J.L. Tank, *Ecosystem metabolism controls nitrogen uptake in streams in Grand Teton National Park, Wyoming.* Limnology and Oceanography, 2003. **48**(3): p. 1120-1128.
50. Campa, M.F., et al., *Impacts of Glutaraldehyde on Microbial Community Structure and Degradation Potential in Streams Impacted by Hydraulic Fracturing.* Environmental science & technology, 2018. **52**(10): p. 5989-5999.
51. Bureau, U.S.C., *Green Bay city, Wisconsin; Houghton city, Michigan; UNITED STATES.* 2018, Feburary 10.
52. Oh, J., C.A. Medriano, and S. Kim, *The effect of tetracycline in the antibiotic resistance gene transfer before and after ozone disinfection.* Desalination and Water Treatment, 2016. **57**(2): p. 646-650.
53. Techtman, S.M., et al., *Comparison of Thaumarchaeotal populations from four deep sea basins.* FEMS microbiology ecology, 2017. **93**(11): p. fix128.
54. Jorgensen, S.L., et al., *Correlating microbial community profiles with geochemical data in highly stratified sediments from the Arctic Mid-Ocean Ridge.* Proceedings of the National Academy of Sciences, 2012: p. 201207574.
55. Parada, A.E., D.M. Needham, and J.A. Fuhrman, *Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples.* Environ Microbiol, 2016. **18**(5): p. 1403-14.
56. Team, R.C., *R: A language and environment for statistical computing.* 2013.
57. Callahan, B.J., P.J. McMurdie, and S.P. Holmes, *Exact sequence variants should replace operational taxonomic units in marker-gene data analysis.* The ISME journal, 2017. **11**(12): p. 2639.
58. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome biology, 2014. **15**(12): p. 550.
59. Le, P.T., P. Pontarotti, and D. Raoult, *Alphaproteobacteria species as a source and target of lateral sequence transfers.* Trends in microbiology, 2014. **22**(3): p. 147-156.
60. Epand, R.M., et al., *Molecular mechanisms of membrane targeting antibiotics.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2016. **1858**(5): p. 980-987.
61. Werren, J.H., L. Baldo, and M.E. Clark, *Wolbachia: master manipulators of invertebrate biology.* Nature Reviews Microbiology, 2008. **6**(10): p. 741.
62. Balkwill, D., J. Fredrickson, and M. Romine, *Sphingomonas and Related Genera. The Prokaryotes, A Handbook of the Biology of Bacteria. Volume 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria. Martin Dworkin*. 2006, SingaporE: Springer.
63. Karatzas, K.A., et al., *Prolonged treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness.* Journal of Antimicrobial Chemotherapy, 2007. **60**(5): p. 947-955.
64. Sabaliunas, D., et al., *Environmental fate of triclosan in the River Aire Basin, UK.* Water Research, 2003. **37**(13): p. 3145-3154.
65. Ptacnik, R., et al., *Diversity predicts stability and resource use efficiency in natural phytoplankton communities.* Proceedings of the National Academy of Sciences, 2008. **105**(13): p. 5134-5138.