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Evaluating Electronic Pulse Generators for Cooling Tower Sanitation

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Evaluating Electronic Pulse Generators for Cooling Tower Sanitation

By

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Science

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List of Abbreviations

ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BLD 14	Cooling tower within building 14
BLD 2	Cooling tower within building 2
BLD 76	Cooling tower within building 76
CFU	Colony-forming units
EET	Environmental Energy Technologies Inc.
EPG	Electronic Pulse Generator
EXP EPG	EXP Electrical Pulse Generator
HPC	Heterotrophic Plate Count
HVAC	Heating, ventilation, and air conditioning
mm	Millimeters
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PEF	Pulsed electrical fields
R2A	Reasoner's 2A agar
RIT	Rochester Institute of Technology
rpm	Revolutions per minute

SAB	Sabouraud Dextrose Agar
STD EPG	STD Electrical Pulse Generator
TSB	Tryptic Soy Broth
Tukey's HSD	Tukey's honesty significant difference test
UTI	Urinary tract infection

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Abstract

Cooling towers are possible sources of contamination to the environment, with implications to human health. Cooling towers are known sources for disease outbreaks. The cooling process releases aerosols, which if cooling towers are not adequately sanitized, pathogenic bacteria may be released and contaminate the environment. Compounding the risk of pathogenic bacteria release, cooling towers provide ideal conditions for biofilms to grow which encourage the exchange of antibiotic resistance genes. Current sanitation methods are unable to prevent or effectively remove biofilms in cooling towers. Therefore, a new sanitation method is necessary. This research explores the feasibility of using electrical pulse generators (EPG) manufactured by Environmental Energy Technologies Inc. (EET) to disinfect cooling tower water. This sanitation method constantly lyses bacterial cells by sending pulsed electrical fields (PEF) through the water. EET has developed a standard EPG (STD EPG) that is currently in use and an experimental EPG (EXP EPG) that is a purportedly improved version of the STD EPG. The purpose of this study was to determine if the EXP EPG was more effective than the STD EPG to sanitize cooling towers through evaluating the microbial CFUs/ ml and diversity. Water samples from each EPG treatment were collected from several building installation on the Rochester Institute of Technology (RIT) campus. These were examined for microbial richness, diversity, antibiotic resistance, and identity using the 16S rRNA gene. The results of the study suggest that there is antibiotic resistant bacteria present in cooling towers. In addition to this seasonality impacted species diversity where the fall had a lower diversity than the summer. Finally, it was determined the two EPG treatments both able to effectively sanitized cooling towers, but it was indistinguishable which treatment was more effective.

Chapter 1: Introduction and Literature Review

Introduction

Cooling towers are possible sources of contamination to the environment, with implications to human health. Their purpose is to absorb and release heat from air conditioning, industrial, and power generation processes (Engelhart et al., 2008). They function in counter current systems where cool water from the cooling tower basin absorbs heat from heat exchangers. Then, the water is cooled through evaporative cooling and collects in the basin to b recirculated. However, when the water returns to the basin, it contains a higher concentration of inorganic and organic material (Kurtz et al., 1982). As a result, cooling towers create an environment where bacteria can thrive. Thriving bacterial communities can result in the development of biofilms, which are difficult to remove through current sanitation treatments (Ozdemir and Ceyhan, 2010). Biofilms are also able to resist antibiotics and increase the chance of genetic exchange of antibiotic resistance genes (Slonczewski and Foster, 2017; Balcázar et al., 2015). Therefore, pathogens and antibiotic resistant bacteria may persist in cooling towers after sanitation treatments in biofilms, thus these bacteria can be released into the environment and cause contamination. Consequently, research addressing how to adequately disinfect cooling towers to prevent biofilm formation is an emerging field of study.

Pulsed electrical fields (PEF) may be a superior method to disinfect cooling water as a preventative treatment that does not result in harmful byproducts or electro-resistance in the bacteria (Gusbeth et al., 2009). Therefore, I proposed to study the effectiveness of electronic pulse generators (EPG) manufactured by Environmental Energy Technologies Inc. (EET). EPG devices use a PEF to lyse bacterial cells, which reduces bacterial loads thus preventing biofilm production and scale accumulation within cooling towers. In this study, I evaluated the standard electronic pulse generator (STD EPG) that is currently used in the installations of EET and the experimental electronic pulse generator (EXP EPG) which is a modification of the STD EPG to be more efficient. Using water samples from each EPG treatment and examining microbial colony-forming units/ ml (CFU/ml), diversity, and antibiotic resistance, this study evaluates how effective these two EPG treatments are to sanitize cooling towers and prevent harmful bacteria from contaminating the environment.

Cooling Towers Function

Cooling towers are part of the heating, ventilation, and air conditioning (HVAC) systems in buildings. Their purpose is to cool and hold water that is used to dissipate heat from heat exchangers (Center for Disease Control, 2017). In the HVAC system, water from the cooling tower, in the cooled state, is pumped out to absorb heat from heat exchangers. Once the water has absorbed the heat, the water enters the top of the cooling tower to be cooled through evaporative cooling. The warmed water is sprayed from the top of the tower onto a surface, such as splash bars. Concurrently, atmospheric air interacts with the water and a fan blows air up and out of the tower to increase cooling (Figure 1). Evaporative cooling releases heat from the system and produces aerosols which are released into the environment through openings in the tower. Finally, the cooled water collects at the base of the cooling tower to be recirculated (Kurtz et al., 1982; Milosavljevic and Heikkilä, 2001). Although this process is effective in removing heat, it also causes serious sanitation and contamination concerns because if pathogenic bacteria are able to thrive they could easily be released as an aerosol and cause contamination.

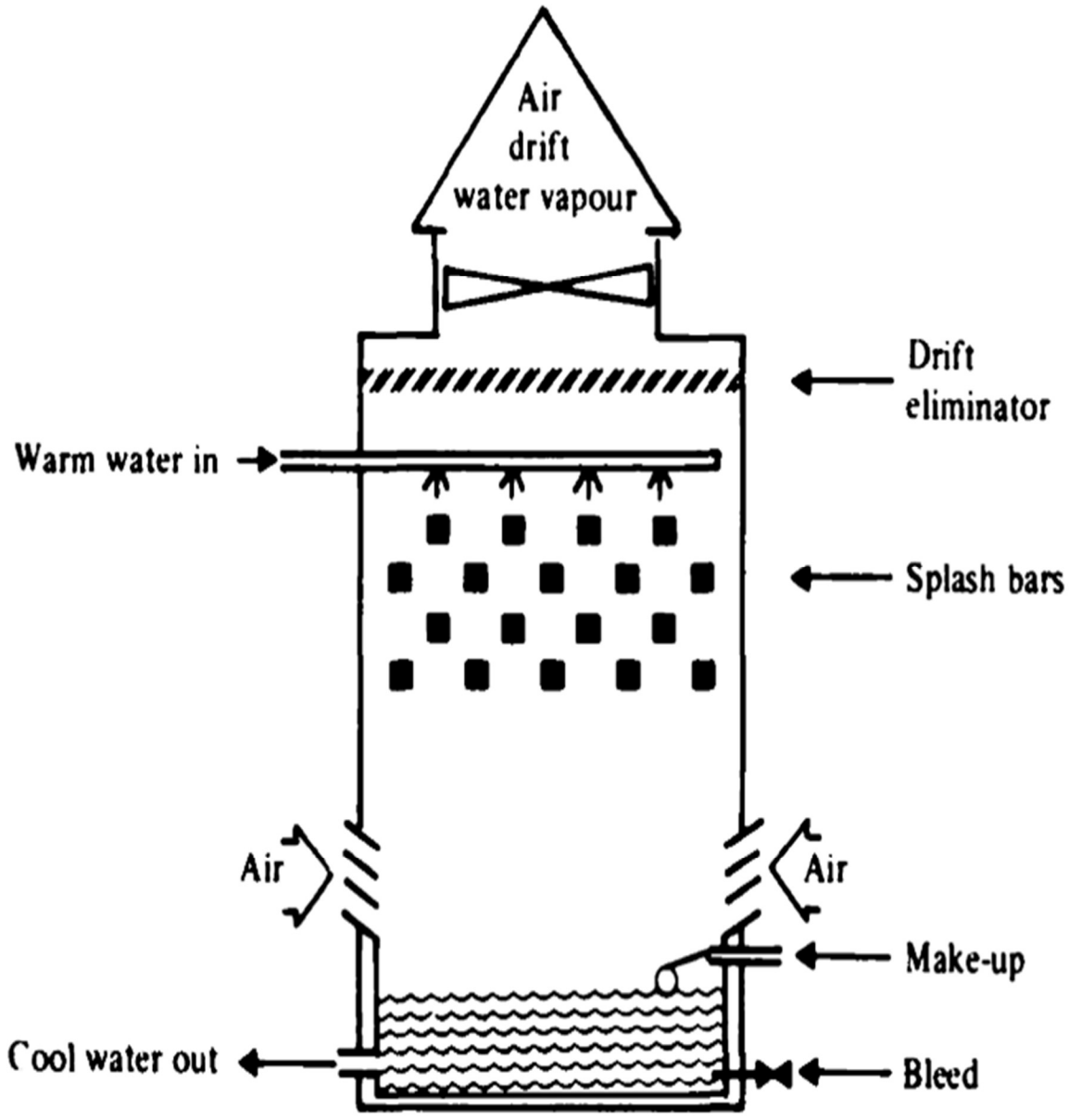


Figure 1: Schematic of cooling tower function (Kurtz et al., 1982).

Biofilms and Bacteria in Cooling Towers

Bacteria in cooling towers can persist in the water as planktonic or sessile organisms, however the majority are immobile bacteria in biofilm communities (Ozdemir and Ceyhan, 2010). Biofilms are accumulations of microorganisms, established on a surface, surrounded by an extracellular matrix made up of polysaccharide polymers, DNA, proteins, and inorganic materials (Slonczewski and Foster 2017). These form where nutrients and conditions are ideal for growth because it is more efficient to be sessile than hunt for food (Slonczewski and Foster, 2017). Cooling towers provide these conditions due to their large surface areas, low water flow speeds, and constant elevated temperature (Lin et al., 1998; Zacheus et al., 2000). The evaporative cooling process increases the concentration of organic and inorganic materials in water, which increases nutrients for bacteria, improving conditions for biofilm production (Kurtz et al., 1982). Additionally, construction materials of cooling towers may provide growth supporting factors. The leaching of biodegradable plasticizers can result in higher biomasses of biofilms (Colbourne, 1985). Consequently, these common cooling tower conditions provide habitat for bacteria to thrive and produce biofilms.

Biofilm formation poses many threats to cooling towers, the most pressing to this research is their ability to be reservoirs of pathogens and resist removal through disinfection (Ozdemir and Ceyhan, 2010; Wingender and Flemming, 2011). The exopolysaccharides of the biofilm protect the bacteria from environmental conditions, antibiotics, and sanitation treatments (Ozdemir and Ceyhan, 2010; Slonczewski and Foster 2017). Biofilms are able to resist sanitation treatments, while the same planktonic cells cannot, due to the biofilm matrix which blocks the inner channels of the biofilm structure and protects the dense aggregates of bacteria within the biofilm (Ozdemir and Ceyhan, 2010). As a result, pathogenic bacteria can persist and even multiply in biofilms. Then, when conditions are no longer ideal the bacteria may be released from the biofilms and cause contamination to the cooling tower and subsequently the environment through the evaporative cooling emissions (Wingender and Flemming, 2011).

Some known pathogens of concern in cooling towers are *Legionella pneumophila* and *Pseudomonas aeruginosa. L. pneumophila* is a pathogen of concern for cooling towers which causes Legionnaires Disease through inhaling the pathogen when aerosolized from the evaporative cooling process. It also utilizes biofilms and parasitizes amoebae to support its

growth. The parasitized amoebae and biofilm act as a protective environment around *L. pneumophila*. It prevents *L. pneumophila* from being removed during sanitation treatments and allows it to survive in conditions that are not ideal for the pathogen (Kuiper et al., 2004; Kurtz et al., 1982; Yamamoto et al., 1992). *P. aeruginosa* is also a bacteria of concern since it can synthesize biofilms in aquatic habitats, have amoebae associations, and produce biofilms in the lungs of cystic fibrosis patients which can lead to death (Branda et al., 2005; Brown and Barker, 1999). In addition, Karami et al., (2020) found that biofilm formation of *P. aeruginosa* reduces its antibiotic susceptibility. These bacterial interactions with biofilms and their ability to form biofilms are an issue because although a sanitation treatment may be effective at removing planktonic cells, the bacteria in biofilms are protected and can remain in the system (Ozdemir and Ceyhan, 2010). Therefore, for sanitation treatments to be effective they must treat biofilms or prevent biofilms from forming. The EPG treatments use mechanisms to prevent biofilms from forming to avoid the issues biofilms cause.

Antibiotic Resistance in Cooling Towers

Biofilm formation not only resists disinfection but can increase the presence of antibiotic resistant bacteria. The bacteria grown in a biofilm state may not be antibiotic resistant genetically, but in this state the bacteria are less susceptible to the antibiotics (Anderl et al., 2000). The biofilms may prevent antibiotics from affecting bacteria through several mechanisms. The polysaccharide matrix of the biofilm prevent antibiotics from penetrating into the biofilm to reach the bacteria, the bacterial cells may have a resistant phenotype, or the biofilm contains cells that are either slowly growing, not growing, or living in a stress response due to unfavorable conditions in the biofilm (Balcázar et al., 2015; Stewart, 2002; Stewart and Costerton, 2001; Slonczewski and Foster, 2017). These mechanisms are linked to the presence of antibiotic resistance genes (Anderl et al., 2000; Balcázar et al., 2015). Due to the close proximity of bacteria in biofilms there is an increased chance of genetic exchange of antibiotic resistance genes (Fux et al., 2005; Hausner and Wuertz, 1999; Li et al., 2002). Conjugation rates in biofilms are faster and larger biofilm surface/volume ratios are correlated with higher gene transfer efficiency (Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003). Consequently, since cooling towers are hot spots for biofilms they also tend to be hot spots for antibiotic resistance.

There is an enhanced risk to human health if there is a presence of antibiotic resistant pathogens in cooling towers due to their ability to share resistance genes and be dispersed into the environment as aerosols.

There are many different antibiotic classes that use different mechanisms to treat bacterial infections. There are serious concerns for bacteria with multiple drug resistances. This study set out to evaluate if there were antibiotic resistant bacteria in the planktonic bacterial community of cooling towers, which could cause harm if released into the environment. The antibiotics in this study span a broad spectrum of the antibacterial classes. In addition, they are common drugs used in hospitals for bacterial infections (Coates et al., 2011; Whitburn, 2019). Therefore, resistance to these antibiotics would be concerning because of their wide use.

Contamination to the Environment

The evaporative cooling process used by cooling towers to dissipate heat produces aerosols and releases them into the surrounding environment. This process can contaminate the environment when these aerosols contain pathogens or antibiotic resistant bacteria that were present in the cooling tower water. Legionnaires Disease caused by *L. pneumophila* are the most common illness borne from contaminated cooling towers. Outbreaks of this illness are well documented and were used as a case study to explain how far pathogens aerosolized from contaminated cooling towers can disperse disease. However this research did not focus on detecting *L. pneumophila*. In the largest *L. pneumophila* outbreak in 2001, a person passing through a 400 m radius of the contaminated cooling tower was very likely to contract Legionnaires Disease (García-fulgueiras et al., 2003). *L. pneumophila* outbreaks have been documented up to 3.4 km from contaminated cooling towers, indicating aerosolized pathogens were able to travel that far and still cause infection (Sala Ferré et al., 2009). These large zones of exposure put people who travel through them at severe risk of infection from pathogens cooling towers are contaminated with. Therefore, continuous sanitation of cooling towers is necessary to prevent this risk and protect public health.

Current Sanitation Methods

According to Kurtz et al. (1982), sanitation methods should be effective in a vast range of pH and temperatures, be fast, and inexpensive. The current sanitation methods attempt to follow these guidelines, but other factors in cooling towers may influence them and render the sanitation ineffective. Chlorination is the most widely used method of sanitation, however it has limitations and drawbacks. High temperatures or pH, common conditions for cooling towers, render chlorination ineffective (Tchobanoglous and Burton, 2002). Chlorination also has harmful consequences such as corrosion of cooling tower infrastructure and interacting with organics resulting in halogenated organics and carcinogens (Emmanuel et al., 2004; Lin et al., 1998). Other sanitation methods are UV irradiation and ozone. Ozone is more often a supplemental treatment with another disinfectant due to its rapid decomposition (Muraca et al., 1987). UV irradiation requires a large UV light for the water to pass by. However, if the water is turbid light cannot penetrate the water deep enough to effectively sanitize (Schwartz et al., 2003). Silver-copper ionization is a novel method of sanitation, which introduces electrically generated silver and copper ions into the water line to kill bacteria, specifically *L. pneumophila* (Lin et al., 1998). Unfortunately, an elevated pH can inactivate this method shifting the predominately positive copper ions to negative ions, preventing the ions from binding to the cells to lyse the cells (Lin et al., 2002). Consequently, all these sanitation methods are ineffective at consistently controlling bacterial growth within cooling towers and cannot prevent the accumulation and attachment of biofilms.

Electronic Pulse Generator (EPG)

The drawbacks of these current sanitation methods make the novel use of EPGs an appealing solution (Figure 2). These function to reduce bacterial CFUs/ml in cooling tower waters and prevent biofilm accumulation by continually sanitizing the cooling towers with short electrical pulses. This method is based on electroporation which uses a low electrical field to form pores in the lipid bilayer. Conventionally, these pores are reversible, where the membrane can rebound around the cell and close up after the electrical field is removed (Joshi and Schoenbach, 2000). Conventional electroporation is used for transfection to insert DNA, biologically active molecules into cells and is a nonthermal way of killing microorganisms (Sale

and Hamilton, 1967; Weaver and Chizmadzhev, 1996). The EPG uses this same concept, but attempts to make a pore that results in cell lysis. It sends high voltage PEF through the water to create pores in the cell membrane. The pulses in the electrical field increases the probability of producing large pores or multiple pores that coalesce into large irreversible pores (Joshi and Schoenbach, 2002). Irreversible pores prevent the cell membrane from rebounding around the cell and cause cell lysis (Neumann and Rosenheck, 1972; Weaver and Chizmadzhev, 1996).

This novel sanitation method does not have the limitations of the current sanitation methods. Short electrical pulses have negligible thermal heating, low power inputs with large electrical fields, and time scales that can be adjusted for the pulse width. Additionally, multiple high-intensity pulses are able to cause more irreversible damage than single-shot electrical pulses (Joshi and Schoenbach, 2002). EPGs function to continually sanitizing the bulk water in the basin of cooling towers. This method of continually sanitizing water prevents bacteria from accumulating in the towers and developing biofilms. In comparison, the sanitation frequency for chlorination is anywhere from every 18 hours to several times a week (Tsao et al., 2019). EPGs are advantageous since they are unlikely to cause chemical modification of the water to change the genotoxicity. Comparatively, the chlorination disinfection method changes the chemical compounds to produce halogenated organic compounds (Emmanuel et al., 2004). Also, the survival of bacteria after this treatment is not due to electro tolerance transferred from bacterial descendants but rather an extreme bacteria feature. These features could be cell size, physiologic state of the microorganism, and cytoplasmic chemical content during the treatment (Gusbeth et al., 2009). As a result this method is able reduce bacterial population sizes, thus preventing them from forming biofilms, with minimal limitations and side effects.

Figure 2: EPG developed by EET.

Other Applications of EPG

The use of EPGs on cooling tower sanitation is a novel method, but PEFs have been used in other areas to kill bacteria cells. Al-Sakere et al. (2007) used the PEF method as a minimally invasive way to treat tumors. In another study, PEFs were evaluated to sanitize hospital waste water to as a potential alternative to chlorination and prevent halogenation of organics within the waste water (Emmanuel et al., 2004). Finally, the most common use for PEFs is for electroporation to insert materials into the cell (Weaver and Chizmadzhev, 1996).

Study Focus

This study used two EPG units developed by EET to sanitize cooling towers. We evaluated the STD EPG unit EET uses for all their installations and an EXP EPG unit that has been modified to be more effective than the STD EPG (Figure 3). These units both treat the water with an electrical pulse, but with different pulse signals. The STD EPG has an exponential decay function signal and the EXP EPG has a square wave signal, so the electrical pulse could penetrate deeper into the water. The STD EPG with its exponential decay function signal allows it to be used as a coil wrapped or skid-based system, while the EXP EPG can only be used as a skid-based system interfaced with a cooling tower. EET will use this evaluation to determine if the changes to the EXP EPG have improved the ability of EPGs to sanitize cooling towers.

Figure 3: EPG installations STD EPG on left, EXP EPG on right.

Goals, Objectives, and Hypotheses

Goal: Prevent cooling towers from being a source of contamination and potential risk to public health.

Objectives:

1. Determine which device is more effective at removing bacteria by comparing the bacterial levels of the STD EPG to the EXP EPG.

Hypothesis: If the EXP EPG is effective, it will have lower bacterial CFUs compared to the STD EPG.

2. Determine if microbial populations remaining in the water systems are of similar diversity composition between the two treatments.

Hypothesis: If the EXP EPG is effective, it will have lower microbial diversity.

3. Determine abundance of antibiotic resistant bacteria in the water after treatment.

Hypothesis: The EXP EPG will have a lower presence of antibiotic resistant bacteria.

Chapter 2: Preliminary Methods and Results

This research was based off of findings from preliminary research. The first preliminary methods were conducted from November 2018 to July 2019 on the Rochester campus of the Rochester Institute of Technology (RIT). This period sampled three cooling towers within building 2 (BLD 2) which was the Frank Ritter Ice Arena, building 14 (BLD 14) the Hugh L. Carey Hall and building 76 (BLD 76) the Chester F. Carlson Center for Imaging Science. BLD 14 was treated with the EXP EPG, while BLD 2 and BLD 76 were treated with the STD EPG. This method tested for fungi using SAB plates, antibiotic resistance using the Kirby-Bauer disc diffusion method, and heterotrophic bacteria using Millipore HPC Red Test Samplers (HPC Samplers) and R2A plates (Figure 4). The results found no fungi present and no substantial difference between the EPG treatments. However, the heterotrophic bacteria below allowable levels, less than 5 log CFUs (Monash University, 2017) (Figure 5). Additionally, antibiotic

resistant bacteria was present in both EPG treatments (Figure 6). The antibiotics used in the preliminary methods were the same antibiotics used in experimental methods. These findings sparked a deeper study into antibiotic resistance of the cooling tower bacterial population and a change in the EXP EPG pulse signal. Therefore in the 2020 sampling period the EXP EPG had an increased pulse width as compared to the preliminary samplings.

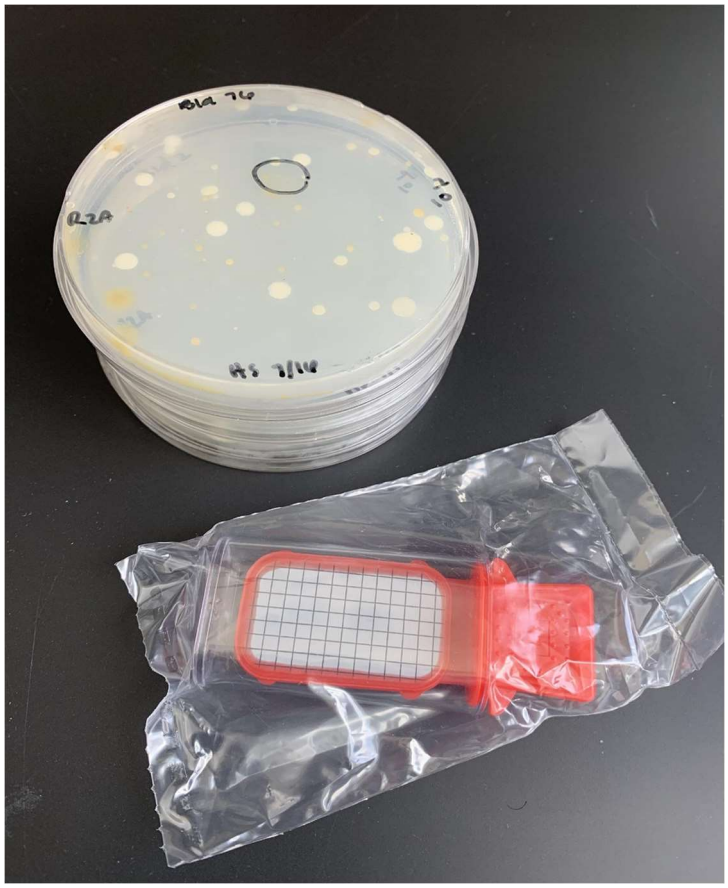


Figure 4: Media used to test microbial enumeration R2A plates (top) HPC Samplers (bottom).

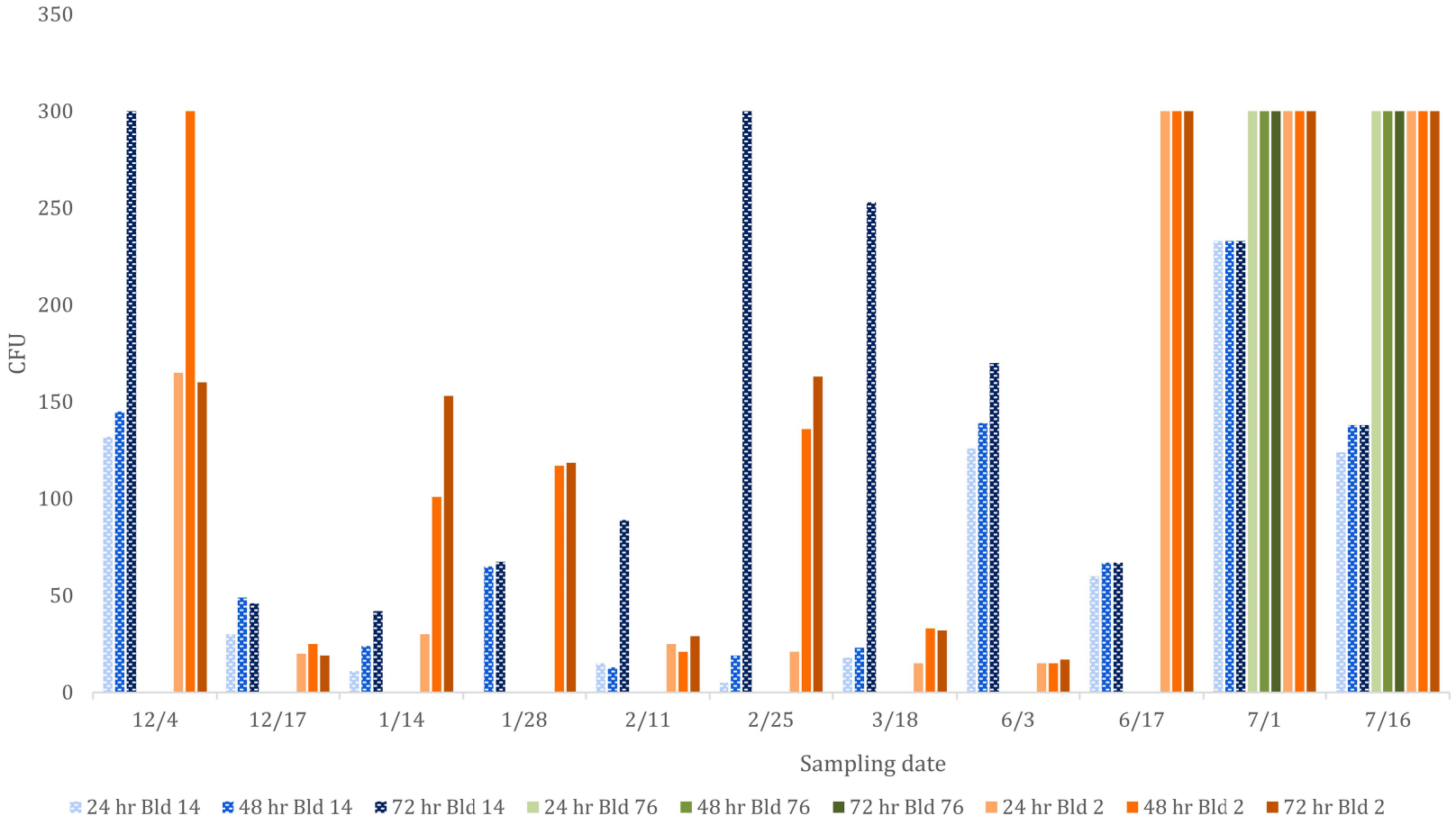


Figure 5: HPC Samplers CFU/ml at 24 hour increments. 300 CFUs was the maximum number of bacteria CFU/ml, bacteria values greater than this were those that were considered too numerous to count. BLD 2 and 76 had the STD EPG unit and BLD 14 had the EXP EPG unit.

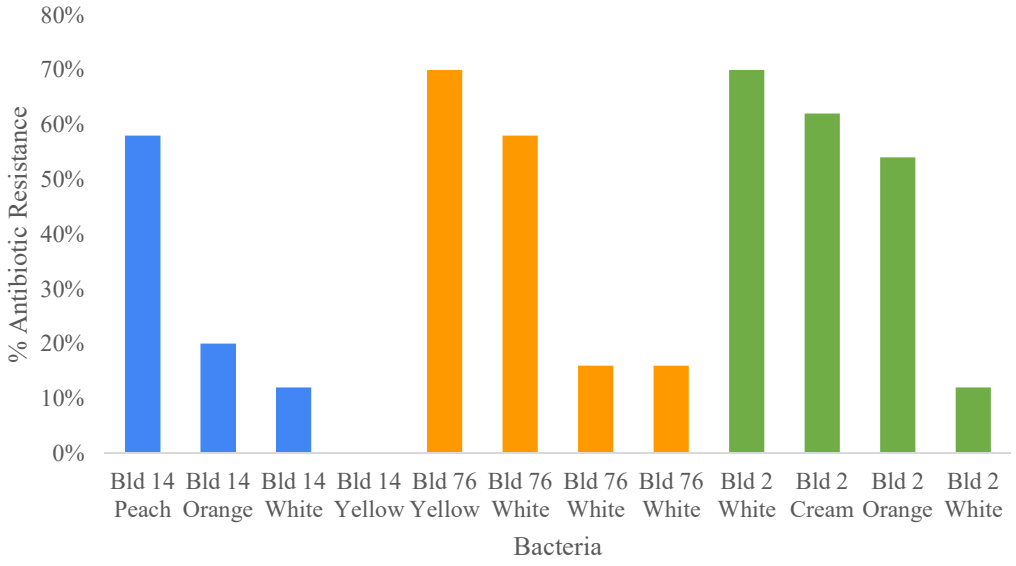


Figure 6: Antibiotic resistance levels of bacteria from BLD 14, BLD 76, and BLD 2. The bacteria were identified by their colony color. All cooling towers had antibiotic resistant bacteria and only one bacteria tested had no resistance to any of the 24 antibiotics tested.

The second preliminary method utilized the new EXP EPG and a split treatment system. This split system was equipped only on the cooling tower in BLD 14 because it was the only one running in the winter from December 2019 to February 2020, when this sampling occurred. The purpose of the split system was to test each EPG on the same cooling tower and remove variability possibly caused by unique environmental conditions of each particular tower. The split treatment system treated the water from the water basin in BLD 14 with both the STD and EXP EPG (Figure 7). Therefore, each EPG unit took a sample of the water, treated it, and returned it to bulk water in the tower. A sample was taken from each EPG unit immediately after treatment and tested for fungi, heterotrophic bacteria, and antibiotic resistance using the same methods as the first preliminary method.

The results of this second preliminary method continued to show the EPG treatments had very similar bacterial CFUs/ml (Figure 8, 9). This was attributed to the fact that after treatment by each unit, the water was replaced into the same basin. Additionally, even though samples were taken immediately after the EPG treatment there was likely not enough reaction time to isolate each particular treatment to compare their efficiency. Consequently, the mixing of the two treatments in the bulk water caused the samples EPG treatments to appear very similar. The Millipore Yeast and Mold Yellow Test Samplers consistently had little to no growth, indicating no presence of fungi in these cooling towers. Antibiotic resistance was also reported in these samples (Figure 10). Therefore this second method proved the split system was inadequate to isolate the each EPG treatment, and further confirmed the lack of fungi present in the cooling towers.

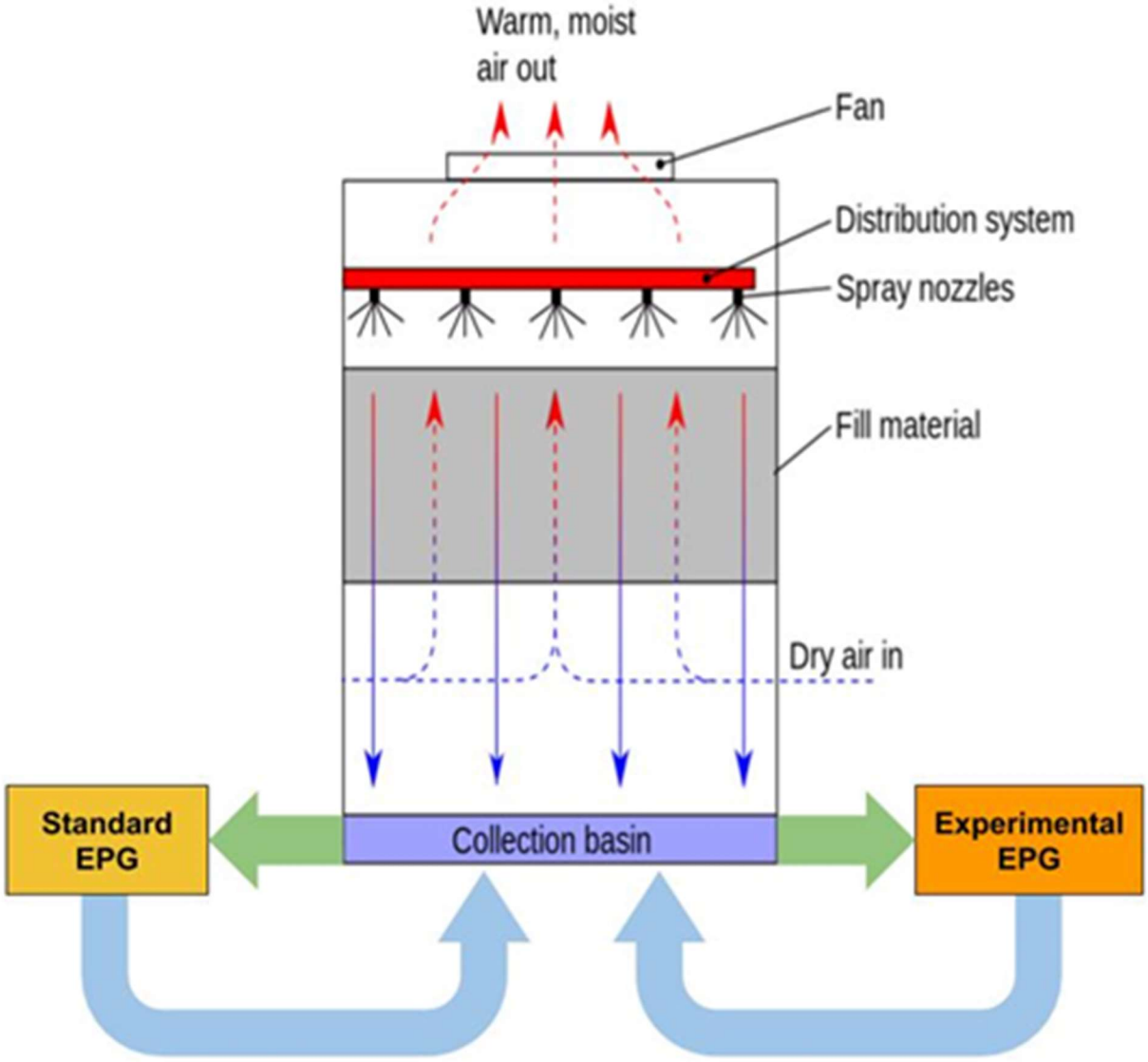


Figure 7: Split system treatment. Water in the collection basin is treated in one of the EPG units and returned to the collection basin (Edreher at English Wikipedia and Zerodamage, 2012). A modification was made to this image.

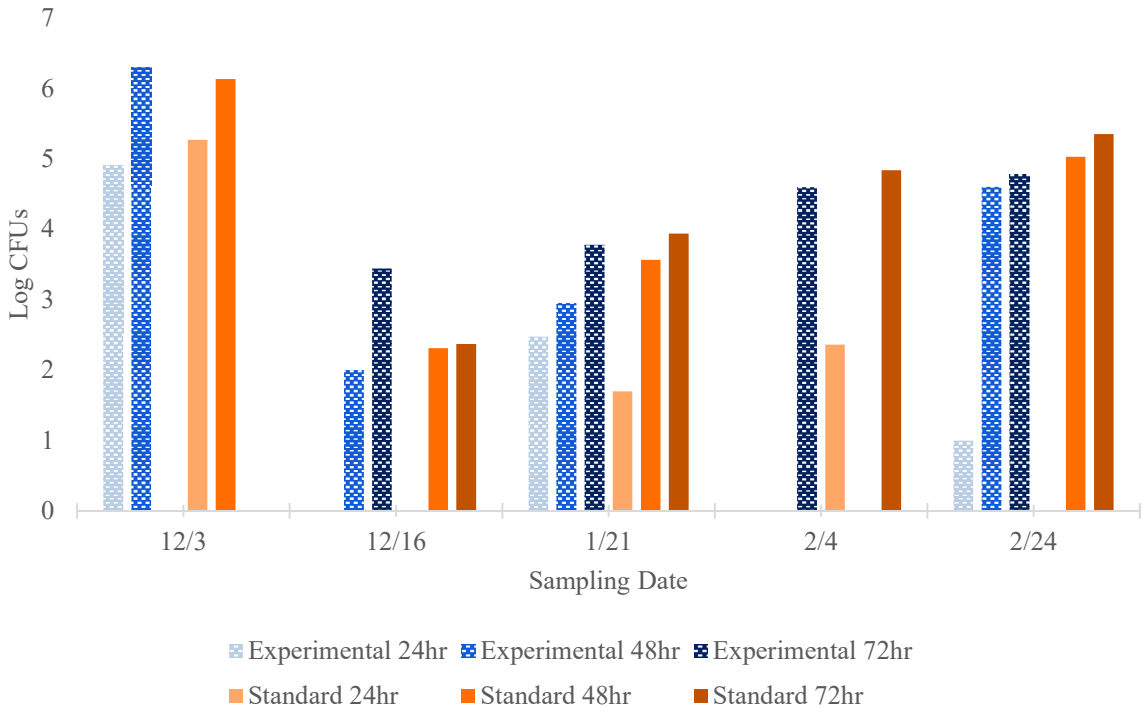


Figure 8: Split system 2019-2020 R2A Plates heterotrophic bacteria results for the STD and EXP EPG at 24hr increments. *lack of bars indicates CFU/ml of bacteria that were too numerous to CFU/ml.

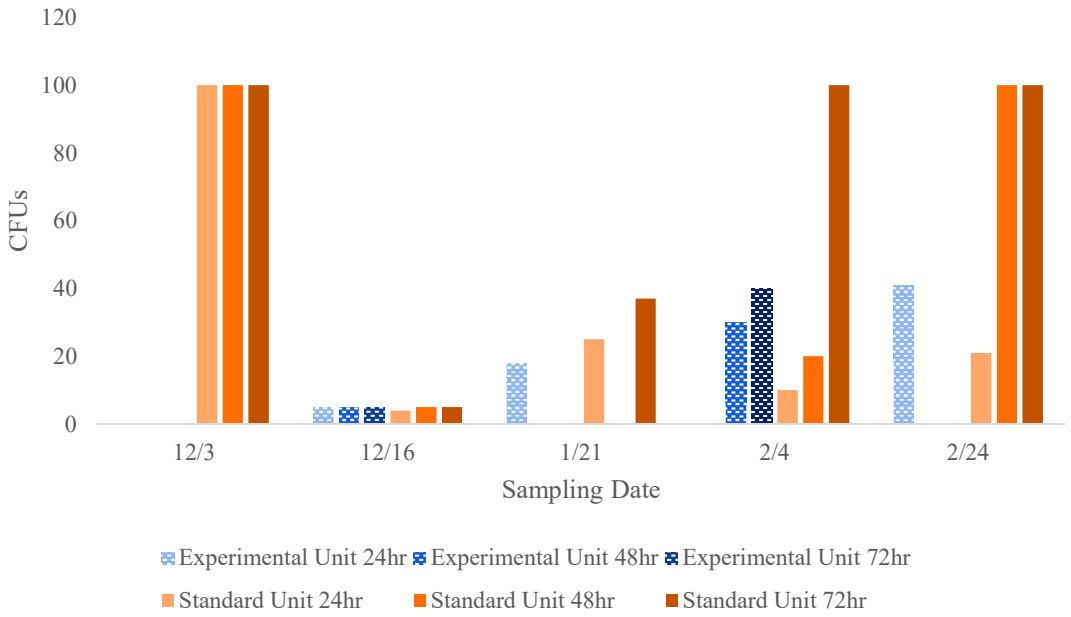


Figure 9: HPC Samplers results using the split system from 2019-2020. Graph represents the STD and EXP EPG CFUs at 24hr increments. *lack of bars indicates CFU/ml of bacteria that were too numerous to CFU/ml.

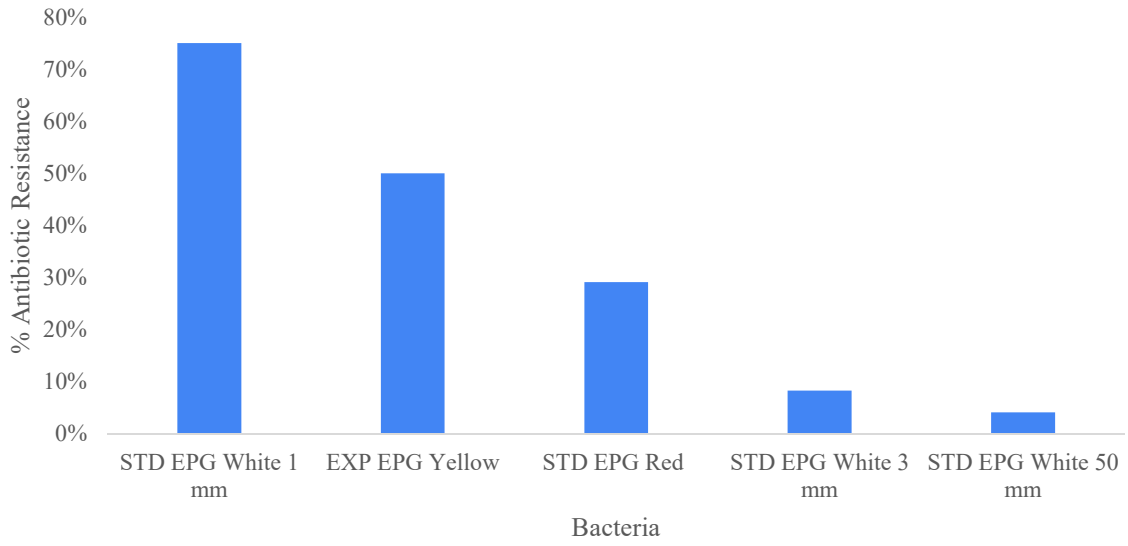


Figure 10: Antibiotic resistance in the split system 2019-2020. Bacteria had 4%-75% antibiotic resistance sparking that further study of antibiotic resistant bacteria in cooling towers.

Chapter 3: Experimental Methods

Sampling Sites

Sampling sites were chosen from RIT's Rochester campus due to their indoor and outdoor connecting cooling towers within a two mile square area to decrease variation in environmental conditions between cooling towers. BLD 2, the Frank Ritter Ice Arena was sampled from during the preliminary data collection period and was an indoor cooling tower equipped with the STD EPG. The cooling tower within BLD 14, Hugh L. Carey Hall, was selected because it was an indoor cooling tower thus only the cooling tower fills were exposed to the outside environment and this tower was functional all year round. BLD 14 was equipped with the EXP EPG since 2018, therefore was able to show how the EPG performs over a long period of time to reduce bacteria growth. From 2018-2019 the EXP EPG pulse signal was a square wave (step function) signal, preliminary data determined this signal did not improve the EXP EPG performance more than the STD EPG. As a result, the EXP EPG's pulse width was increased and this adjusted EXP EPG was used for the 2020 sampling period starting in July. The cooling tower in BLD 76, the Chester F. Carlson Center for Imaging Science, had the STD EPG unit installed and was used as a control. BLD 76 was a seasonally functional cooling tower from late spring to late fall. It had an outdoor connection such that the cooling tower fills and water basin of the cooling tower were exposed to the outside environment.

2020 Sample Collection

In the summer and fall of 2020 (the 2020 sampling period) water samples were collected from BLD 14 and BLD 76. The STD EPG unit in BLD 76 was sampled the entire time it was functional. The EXP EPG unit in BLD 14 was sampled from July to September, then the EXP EPG was turned off and replaced by the STD EPG unit which was sampled from October to December. At each site three, 300 ml samples of water were collected immediately after treatment every two weeks. Then, the samples were placed on ice and refrigerated at 4°C until analyzed (Figure 11). Samples were viable for 2 weeks, but analysis occurred as soon as possible, most frequently on the same day of collection.

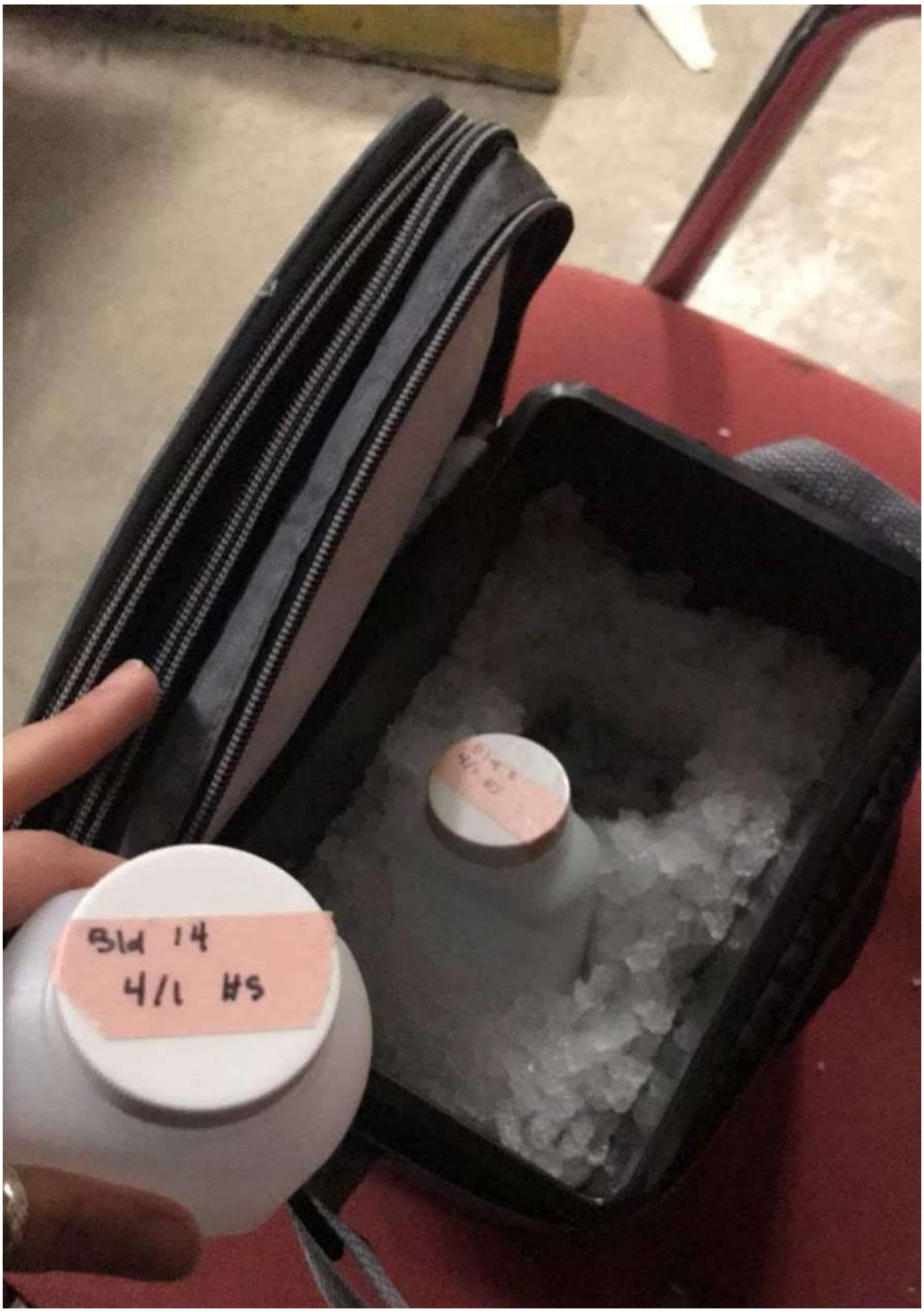


Figure 11: Field sampling materials included water collection containers and a cooler filled with ice for transport.

Microbial Enumeration

The microbial population of the water samples were tested for bacteria using nutrient agar and HPC Samplers. The HPC Samplers were used according to manufacturer's instructions. The indicated amount of sample water was poured into the container and the agar paddle was inserted into the water for 30 seconds. Then, the water was drained from the container and the HPC Samplers incubated for 3 days with bacteria counts taken every 24 hours (MilliporeSigma, 2019) (Figure 14). If the number of colonies on the HPC Samplers were too numerous to count after 24 hours, the samples were tested again with a 10^{-1} dilution using distilled water. These HPC Samplers are what the industry uses to enumerate bacterial CFU/ml of cooling towers.

The nutrient agar used for bacterial enumerations were R2A and PCA plates. R2A are the media recommended by the Center for Disease Control for water samples (Center for Disease Control, 2015). PCA plates are approved by the Public Health Association, Water Environmental Federation, and American Water Works Association for growing water sample bacteria (Baird et al., 2017). For this method, 0.1 ml of sample was serially diluted (10^{-2} to 10^{-4}) with NaCl (Kim et al., 2004) (Figure 12). Then, 0.1 ml of the sample and the dilutions were spread on the nutrient agar plates and incubated at 30 ℃ (Figure 13). Bacterial counts were taken every 24 hours for 3 days (Figure 14).

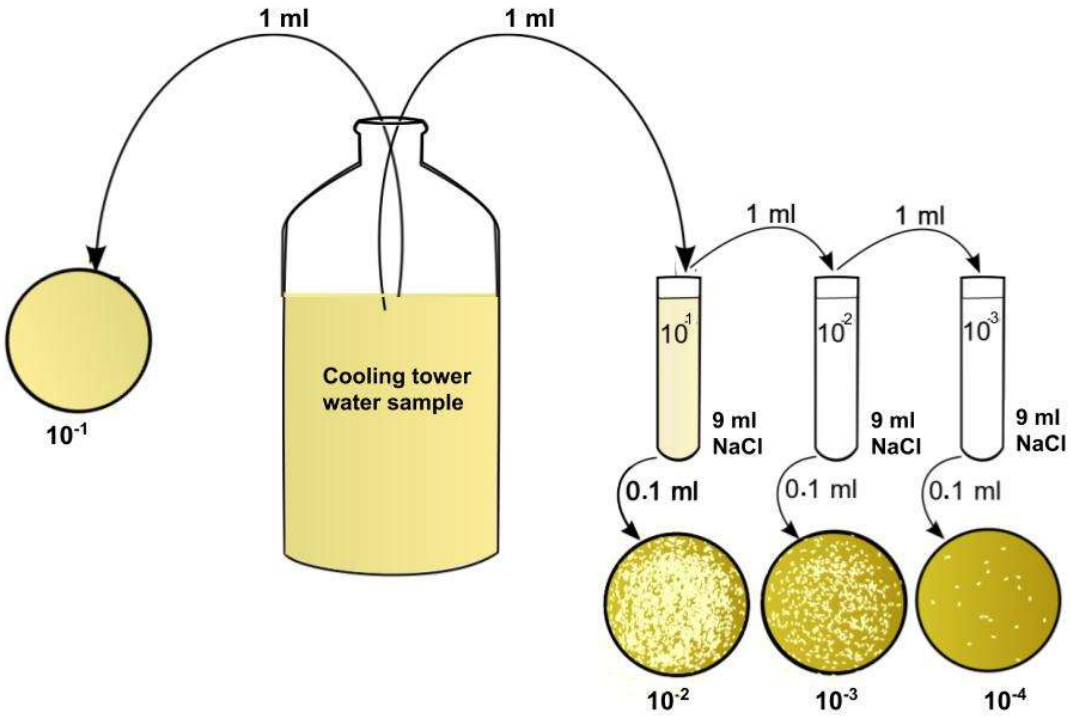


Figure 12: Serial dilutions performed. Tubes contain 9 ml of 0.9% NaCl and 1 ml of sample 0.1 ml of solution is plated on R2A plates (Leberechtc, 2010).

Modifications were made to this image.

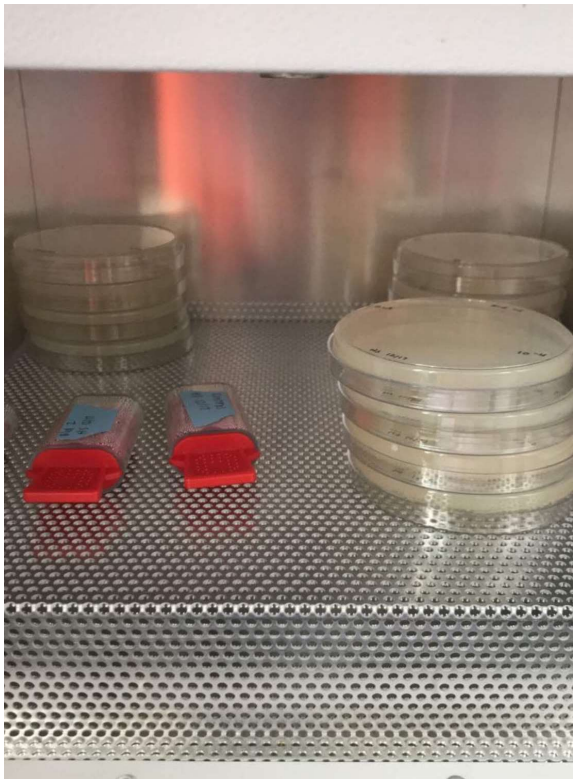


Figure 14: HPC Samplers (left) and nutrient agar plates (right) incubating at 30 ℃.

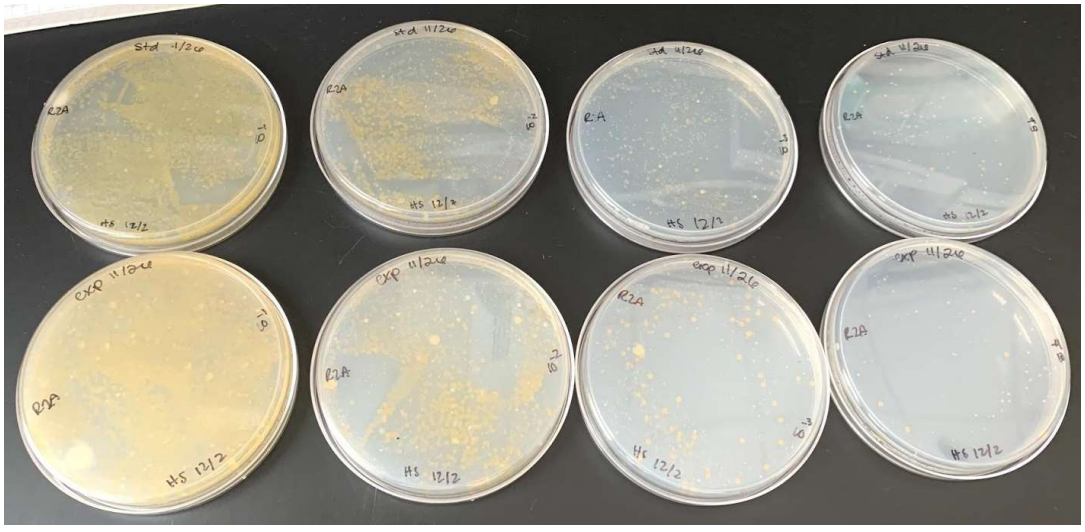


Figure 13: Serial dilutions 10^{-1} to 10^{-4} results of STD (top row) and EXP EPGs (bottom row) after 72 hours of incubations.

Identification of Isolates

Bacterial colonies were initially identified based on their macro physical characteristics such as colony color, colony morphology, and colony size (Ozdemir and Ceyhan, 2010). The bacteria identified were catalogued and given a letter designation. Then, the micro physical characteristics were used to further identify them based on their cell morphology and Gram-staining reaction (Ozdemir and Ceyhan, 2010). After the bacteria were identified they were catalogued for which samples they were present in. The top 20 bacteria that were most frequently present in the samples were then consistently isolated so further testing could be performed on them and further identify attributes of the bacteria.

Microbial Diversity

The diversity of bacteria present in the cooling towers was evaluated by species richness. Species richness was the number of different bacteria present in a particular sampling population. The abundance of the species was determined by how frequently an identified bacteria was present in samples. This value was not the number of colonies of a bacteria present in a sample, but the number of times is appeared in sampling populations (ex. EPG treatment, season).

Antibiotic Resistance

The bacteria with the top 20 frequency presence were tested for antibiotic resistance using the Kirby Bauer disc diffusion method (Hudzicki, 2016). Colonies were grown in 10 ml of TSB broth for 48 to 72 hours at 30 ℃, shaking at 140 rpm. Aseptic technique with a sterile swab was used to streak the broth culture on a Muller-Hinton agar plate to form a bacterial lawn. The plate was allowed to dry for about 5 minutes then, antibiotics were dispensed on the agar. The following antibiotics were used: ceftriaxone, amikacin, levofloxacin, ampicillin, amoxicillin/ clavulanic acid, ticarcillin, doxycycline, sulfamethoxazole/ trimethoprim, ciprofloxacin, mezlocillin, cefixime, lomefloxacin, carbenicillin, tobramycin, imipenem, cephalothin, oxacillin, piperacillin, cefepime, sulfadiazine, minocycline, and meropenem. These antibiotics were chosen since they are currently in clinical use in hospitals (Whitburn, 2019). To ensure attachment of each antibiotic disc flame-sterilized forceps pressed each antibiotic disc to the agar. The plates were then incubated for 3 to 4 days at 30 ℃. Next, the zones of inhibition were measured for

each bacterium's reaction to the antibiotic. A ruler recorded the diameter of the zones of inhibition in millimeters (Figure 15). Finally, the zones of inhibition were compared to published manufacture standards to determine the resistance to antibiotics of these bacteria (Hudzicki, 2016; Becton Dickinson and Company, 2011). Antibiotic zones of inhibition which determined antimicrobial susceptibility can vary based on specific bacteria (Becton Dickinson and Company, 2011). However, the bacteria identity was not determined prior to this test. Therefore, the reference zones of inhibition which determined antimicrobial susceptibility were based on the highest resistance zones of inhibition for each antibiotic, rather than specific bacteria. Antibiotic zones of inhibition are attached in Appendix A. The structures of these antibiotics are attached in Appendix B.

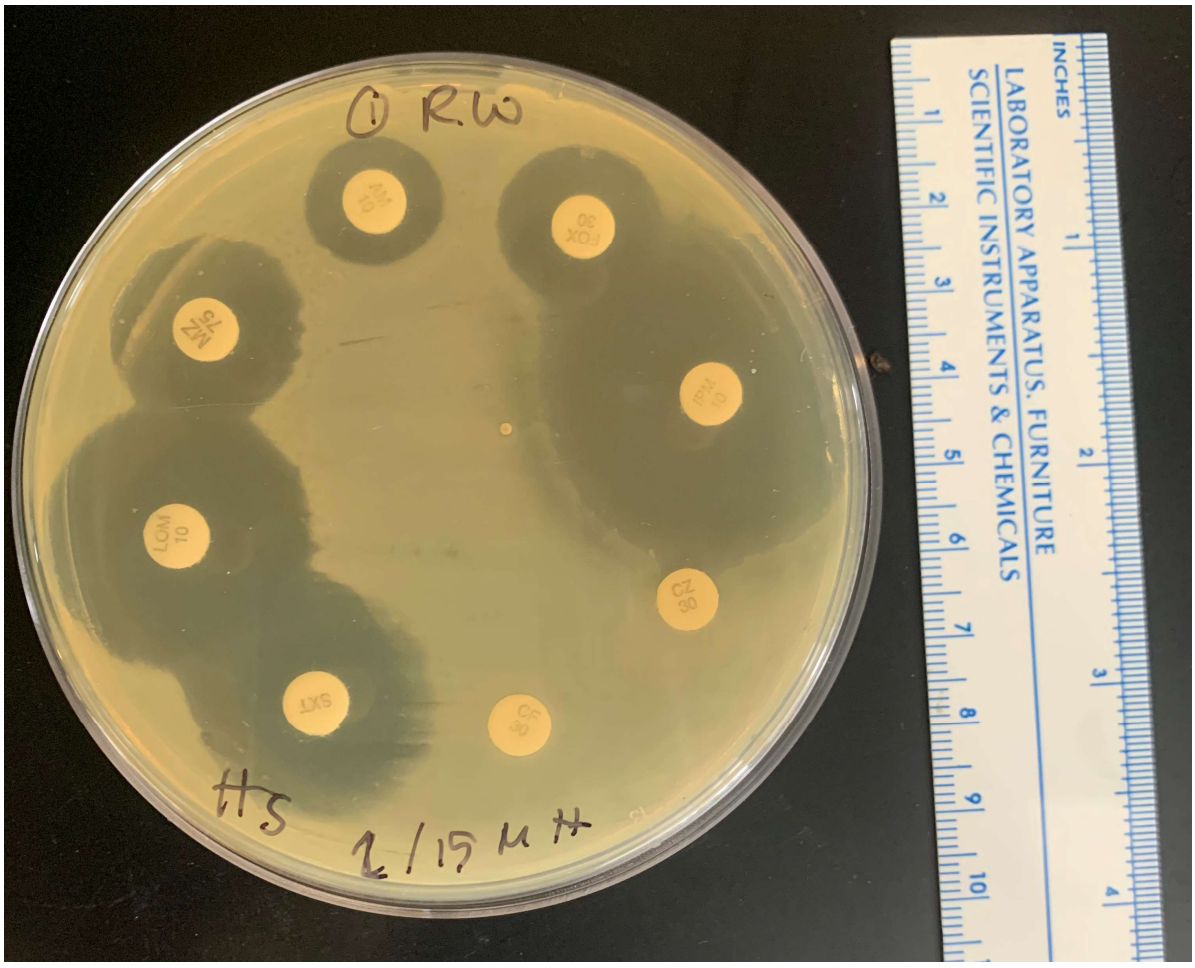


Figure 15: Kirby Bauer disc diffusion method. Bacteria was plated on Muller-Hinton plates with antibiotic discs. Bacterial resistance was measured by the diameter of the zone of inhibition around the antibiotic disc.

Bacterial Identification of the 16S rRNA Gene

A subset of the bacteria found in the water samples were identified using their 16S rRNA gene. The subset of bacteria was decided because they had a high frequency presence in the water samples and were more than 60% resistant to the 24 antibiotics. The 16S rRNA gene was chosen since it was a sufficient sequence length to reflect important sequence changes that identify bacterial genera. To isolate the bacteria for gene sequencing, pure cultures of the bacteria were grown on PCA plates for 72 hours and stored at 4℃. Then Polymerase Chain Reaction (PCR) was performed on the bacteria such that the 16S rRNA gene 3 and 4 variable regions (V3/V4) were amplified using the primers 314f (5'-CCTACGGGNGGCWGCAG-3') and 805r (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013; Parthasarathy et al., 2019). The PCR master mix solution used 10.5 µL H2O Rnase Free Sterile Water, 1 µL of the forward primer, 1 µL of the reverse primer, and 12.5 µL of the GoTaq™ Green (Promega) per each bacteria sample. Then a small amount of bacteria was added to the solution. Finally, the whole solution was placed in a thermocycler. The thermocycler PCR conditions were based on those used in Parthasarathy et al. (2019) edits to these methods were due to lab methods developed by Dr. Andre Hudson's lab at RIT. The first cycle was for two minutes at 95 ℃, then 30 cycles for 30 seconds at 95 ℃, 3 minutes at 72 ℃, five minutes at 72 ℃, and the temperature was held at 4 ℃. Next, gel electrophoresis and gel extraction was used to confirm the V3/V4 amplifications using the QIAquick Gel Extraction Kit (Qiagen) and Sanger nucleotide sequencing of the 314f and 805r primers (Parthasarathy et al., 2019). The gel for the gel electrophoresis was made using 0.7 g of Agarose and 100 mL of 1 x TAE. Then, 5 µL of the DNA samples and the ladder were inserted into the wells and the gel was run for 45 minutes. Then the gel was placed in a UV light box and the presence of fluorescent bands indicated successful DNA extraction (Figure 16). Next, the samples were purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Inc., 2015) (Figure 17). In an EZ-10 column 20 µL of the PCR product, previously used for the gel electrophoresis, was combined with 150 µL of binding buffer II and centrifuged at 10,000 rpm for 1 minute. Then, the flow through contents were discarded and 200 µL of wash solution was added to the column and centrifuged at 10,000 rpm for 2 minutes. The column was transferred into a 1.5 ml microfuge tube and the flow through was discarded. Twenty-five microliters of the elution buffer was added to the

column. The column and microfuge tube were spun in a centrifuge together at 10,000 rpm for 1 minute to elute the DNA. A NanoDrop fluorometer was used to find the DNA quantity of the PCR products. Preparation of the samples to send to Genewiz sequencing required to addition of 1 µL of the forward primer, H_2O RNASE free sterile water, and purified PCR product template DNA to a 1.5 ml microfuge tube. The equations 1 and 2 were used to determine the amount of purified PCR product template DNA and H_2O RNASE free sterile water. Finally, the samples were sent to Genewiz for sequencing and the Basic Local Alignment Search Tool (BLAST) identified the bacteria genera based on the sequence (Parthasarathy et al., 2019).

Equation 1: Purified PCR product template DNA

$$\text{Amount of template DNA} = 20 \div \text{Nucleic Acid (ng/µL)}$$

Equation 2: H_2O RNASE free sterile water

$$\text{Free sterile water} = 15 - \text{Template DNA} - \text{Forward Primer}$$

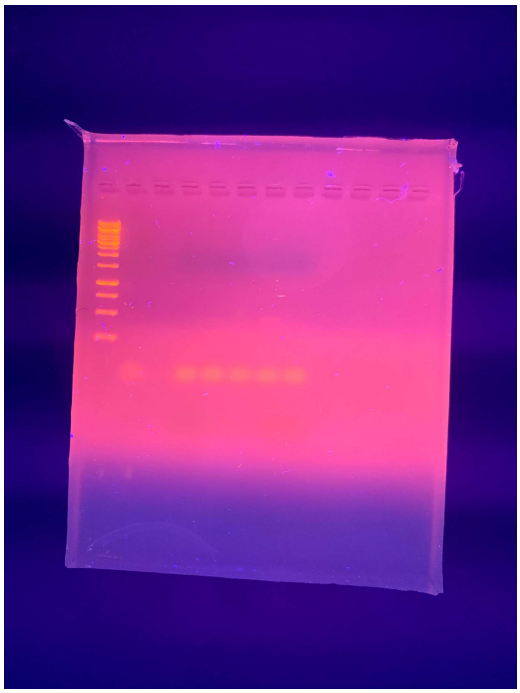


Figure 17: Electrophoresis gel results under UV light. The ladder was run on the farthest left well and the samples bands were run in the well to the right of it.

Figure 16: Solutions used for DNA preparation to send to Genewiz from the EZ-10 Spin Column PCR Products Purification Kit. From left to right: Binding Buffer II, Wash Solution, and Elution Buffer.

Statistical Analysis

A statistical analysis was performed using the statistical software R and Minitab. The cooling tower bacterial loads of CFUs/ml were evaluated statistically for sameness using one way blocked ANOVAs with incubation time as the blocking variable. The hypothesis for these tests were:

1) The bacterial CFUs from each building would be the same throughout the sampling period with the same EPG.
2) Seasonality would not affect bacterial CFUs therefore, CFUs/ml would be statistically the same throughout the fall and summer sampling periods in 2020.
3) The bacterial CFUs would be statistically different from each EPG treatment.

Tukey's honesty significant difference test (Tukey's HSD) followed these tests if there was a significant difference to determine which sample means were significantly different.

The Gram stain results were statistically evaluated to determine which bacteria Gram stain identification was most present in the cooling towers. One proportion Z tests and goodness of fit tests were respectively used to determine Gram stain presence and distribution. Finally, the EPG comparisons and comparisons between buildings were evaluated using two-sample t-tests.

Chapter 4: Bacteria Enumeration

Results

EPG Comparison of CFUs Results

The results from the EXP and STD EPG from December 2018 to December 2020 showed the bacteria levels fluctuate throughout the year and did not appear to have a seasonality trend (Figure 18, 19). Overall the STD EPG was able to keep bacterial levels below 6.6 log CFU/ml (Figure 18). The bacteria enumerations from the EXP EPG were all below 6 log CFU/ml indicating the EXP EPG was able to have a lower maximum bacteria CFU threshold as compared to the STD EPG (Figure 19). The EXP EPG also had a negative linear trend, thus as the EXP EPG use increased in time, the number of bacteria present in the tower decreased.

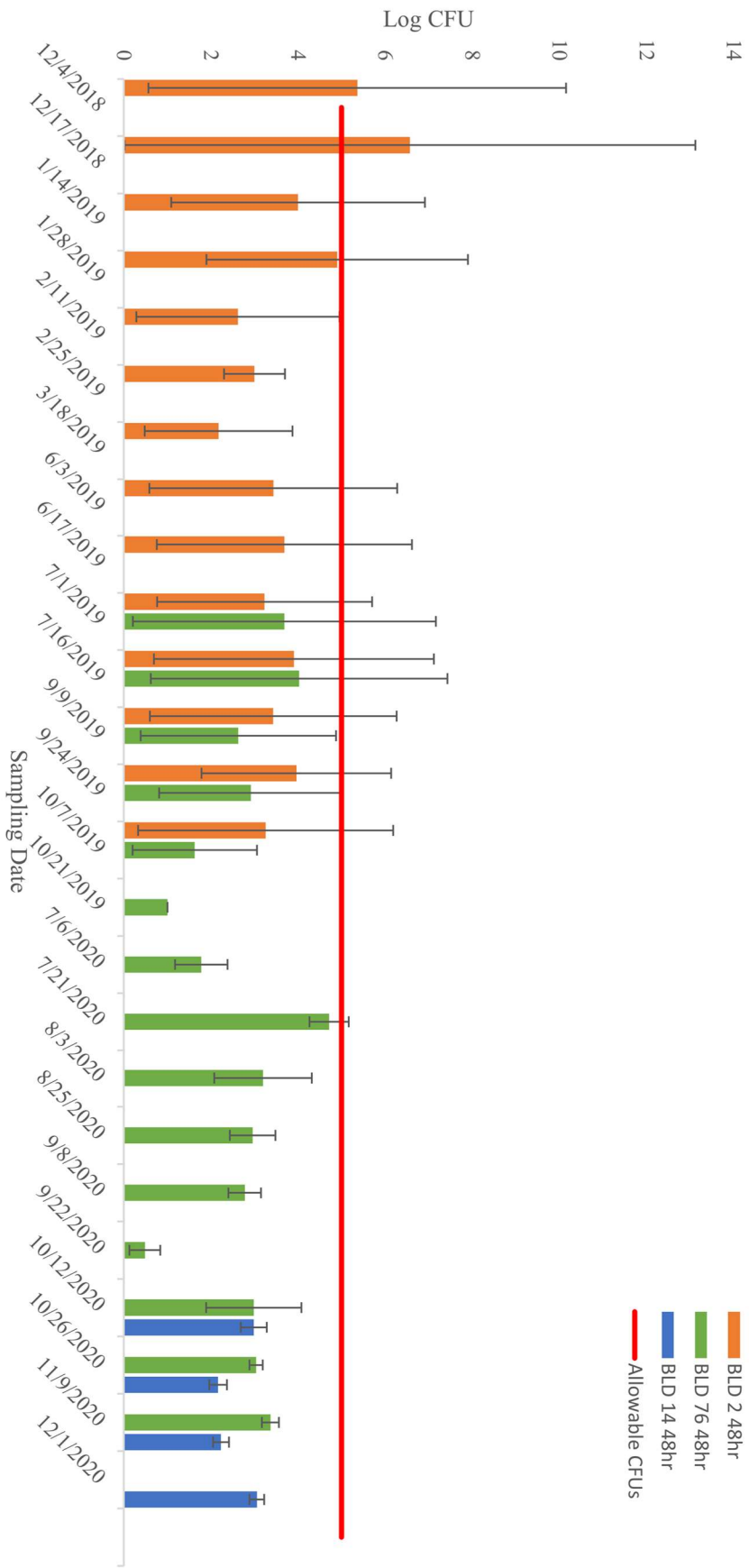


Figure 18: The STD EPG bacteria enumeration results from December 2018 to December 2020 of BLD 2, 14, and 76 after incubations of 48 hours.

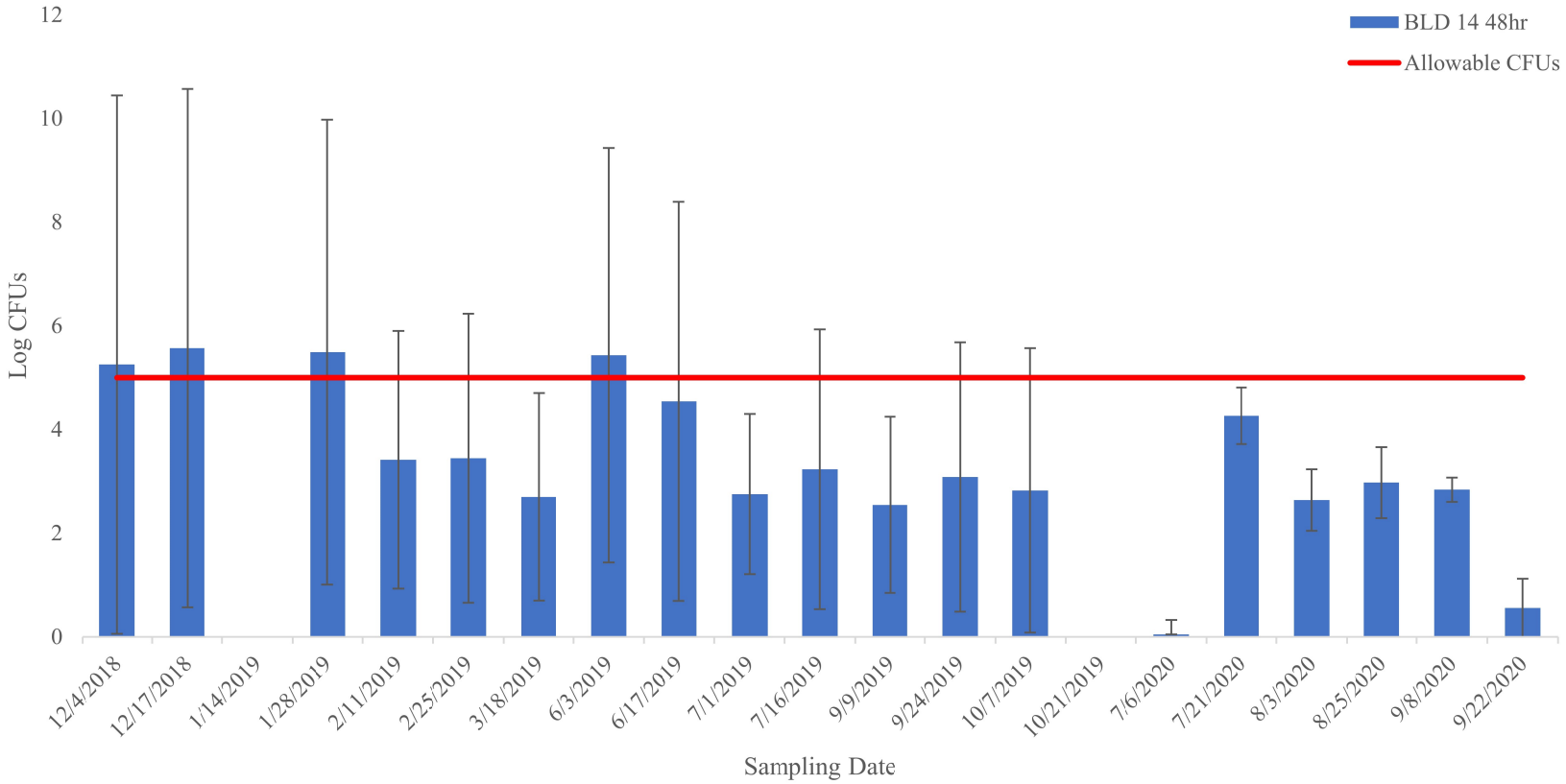


Figure 19: The EXP EPG bacteria enumeration results from December 2018 to December 2020 of BLD 14 after incubations of 48 hours.

Statistical Analysis of Bacteria Enumeration

The EPG devices were evaluated statistically using two sampling periods during 2020. The preliminary samplings before July of 2020 were excluded from this analysis because the EXP EPG had changes made to its PEF to improve its ability to lyse bacterial cells. The sampling period deemed summer sampled from July to September. During this period BLD 14 was treated with the EXP EPG and BLD 76 was treated with the STD EPG. The sampling period deemed fall was from October to December where both BLD 14 and 76 were treated with the STD EPG. The 2020 sampling period allowed for a paired comparison of the EPGs and buildings at the same dates.

Summer Sampling Period Statistical Results

The microbial enumerations of BLD 14 and 76 samples grown on nutrient agar during the summer sampling period indicated a large amount of variation with a sum of squares of 288.3 (Figure 20, Table 1). The samples taken within the same tower had a large variance. A one way blocked ANOVA that used incubation time as a blocking variable determined there were significant differences in the bacteria CFUs of each sample (Table 1). A Tukey's HSD found that many of the samples were significantly different from each other and there was not a trend which indicated if sampling date or building was the cause of these differences (Table 2). Due to the high frequency of variation between all the samples each tower was evaluated independently to determine if the variance was due to the EPG treatment or another factor. The independent analysis of BLD 14 and 76 had a significant p-value indicating the bacteria levels within the same building's cooling tower were different from each other (Table 1). Therefore, the bacterial CFUs throughout this sampling period were not uniform within each tower, which was unexpected.

The HPC samplers results were also statistically analyzed using the same tests as those conducted on the nutrient agar counts. These revealed that some of the sampling dates and buildings tested with the different EPG units were significantly different (Table 1). A Tukey's HSD showed the significantly different samples were samples from the same building and EPG and samples from different building and EPG, suggesting EPG was not influencing CFUs

significantly (Appendix C). A two-sample t-test also revealed the EPG treatments did not cause a significant difference in CFUs/ml of bacteria (Table 3).

Fall Sampling Period Statistical Results

During the fall sampling period both cooling towers were treated with the STD EPG. This was used to compare the bacterial levels within each building's cooling tower to see if the conditions of each cooling tower would impact CFUs/ml. The results of one way blocked ANOVA of all the samples in the fall sampling period from the nutrient agar counts and HPC test samplers both concluded there were a significant differences in CFU/ml between each sample (Table 6; Figure 21). The Tukey's HSD of the nutrient agar and HPC test sampler enumerations proved there was variance between the samples even though the EPG treatment was the same throughout the sampling period (Table 7, 9). The nutrient agar Tukey's HSD indicated there were significant differences were between BLD 14 and BLD 76 samples, where five of the 12 comparisons of the buildings were significantly different (Table 7). In comparison, the HPC test sampler differences were due to variances between the buildings and between the sampling dates 10/12 and 10/26 (Table 9). A two-sample t-test was run on the HPC test sampler results to compare the building CFUs which found the CFU levels between the buildings were distinct, where BLD 76 had higher CFUs than BLD 14 (Table 4, 5). Therefore, the individual cooling towers had an affinity to have unique bacterial loads even when treated with the same EPG device. However, a further analysis was performed independently on each buildings to confirm this.

A one way blocked ANOVA with a blocking variable of incubation time was run on the nutrient agar CFUs of BLD 76. This test was used to determine if there was a difference in the microbial CFUs between sampling dates. The tests results found there was not a difference between the samples, thus indicating the bacteria load within the BLD 76 using the STD EPG was uniform throughout the sampling period (Table 6). The same test was run on BLD 14, which resulted in a p-value that was less than the alpha indicating there was a significant difference in the samples (Table 6). Tukey's HSD indicated the samples from 12/1 were significantly different than the other samples taking during this sampling period (Table 8). Suggesting, BLD 14 had a

homogenous bacterial load expect in 12/1. Therefore, conditions in each cooling tower were distinct from each other yet homogenous within each tower.

Statistical Comparisons of EPGs Results

In comparing all the samples CFUs in the 2020 sampling period using a two-sample t-test it was determined the bacterial CFU levels did not differ by their EPG treatment (Table 10). In addition, a two-sample t-test was performed on the summer period and all the BLD 14 samples. The summer period was used to compare the different EPGs bacterial growth during the same time periods. The BLD 14 was used to compare the different EPGs bacterial growth within the same cooling tower to hold the building variable constant since each buildings environmental conditions are unique and may be influencing bacterial levels. In evaluating the CFUs from all the sampling in BLD 14 it was determined that EPGs had an equal effect on the cooling tower CFUs (Table 10). The same was found for the summer sampling period results which compared the EXP and STD EPGs (Table 3). Therefore, these devices were unable to show that one EPG was more effective at reducing the number of bacteria in the towers.

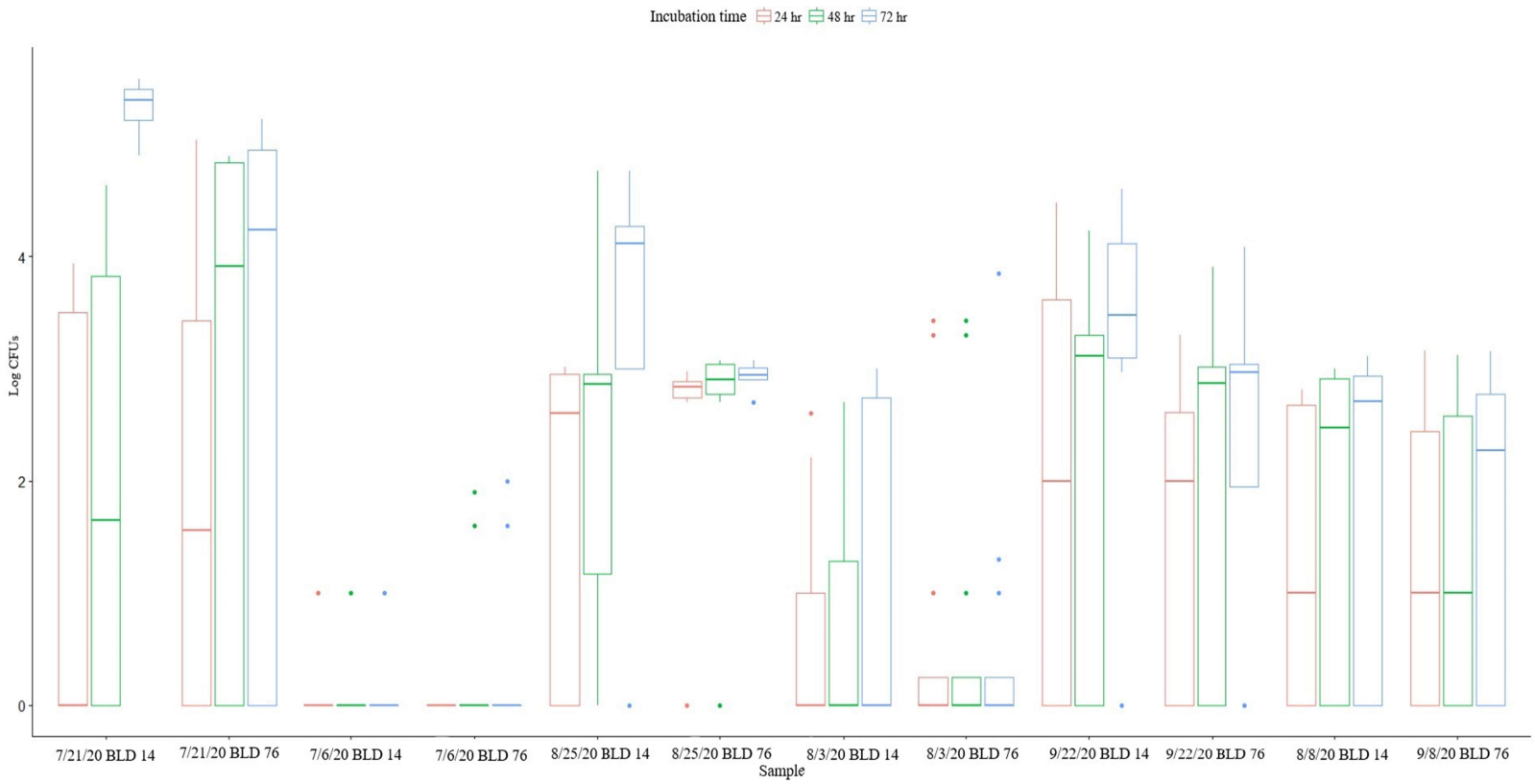


Figure 20: The bacterial populations of BLD 14 and 76 in the summer sampling period. During this period BLD 14 was treated by the EXP EPG and BLD 76 was treated by the STD EPG.

Table 1: One way blocked ANOVA with incubation time was used as the blocking variable for samples in the summer sampling period ($p \leq 0.05$).

One Way ANOVA Test Samples	Df	Sum Sq	Mean Sq	F value	P-value
All Samples Nutrient Agar CFUs	11	288.3	26.21	13.72	<2e-16 ***
Bld 14 Samples Nutrient Agar CFUs	5	157.4	31.482	15.22	2.08e-12 ***
Bld 76 Samples Nutrient Agar CFUs	5	129.53	25.905	14.846	4.2e-12 ***
All Samples HPC Samplers	11	105.703	9.6094	22.86	0.000

Table 2: Tukey's HSD in the summer sampling period for all samples of the nutrient agar CFUs. This table only represents the compared samples that were significantly different from each other.

Comparison of Samples	P-value
7/6/20 Bld 14-7/21/20 Bld 14	0
7/6/20 Bld 76-7/21/20 Bld 14	0
8/3/20 Bld 14-7/21/20 Bld 14	0
8/3/20 Bld 76-7/21/20 Bld 14	0
7/6/20 Bld 14-7/21/20 Bld 76	0
7/6/20 Bld 76-7/21/20 Bld 76	0
8/3/20 Bld 14-7/21/20 Bld 76	0
8/3/20 Bld 76-7/21/20 Bld 76	0
8/25/20 Bld 14-7/6/20 Bld 14	0
8/25/20 Bld 76-7/6/20 Bld 14	0
9/22/20 Bld 14-7/6/20 Bld 14	0
9/22/20 Bld 76-7/6/20 Bld 14	0
9/8/20 Bld 14-7/6/20 Bld 14	0
9/8/20 Bld 76-7/6/20 Bld 14	0
8/25/20 Bld 14-7/6/20 Bld 76	0
8/25/20 Bld 76-7/6/20 Bld 76	0
9/22/20 Bld 14-7/6/20 Bld 76	0
9/22/20 Bld 76-7/6/20 Bld 76	0
9/8/20 Bld 14-7/6/20 Bld 76	0
8/3/20 Bld 14-8/25/20 Bld 14	0
8/3/20 Bld 76-8/25/20 Bld 14	0
8/3/20 Bld 14-8/25/20 Bld 76	0
8/3/20 Bld 76-8/25/20 Bld 76	0
9/22/20 Bld 14-8/3/20 Bld 14	0
9/22/20 Bld 76-8/3/20 Bld 14	0.03

Table 2 Continued

Comparison of Samples	P-value
9/22/20 Bld 14-8/3/20 Bld 76	0
9/22/20 Bld 76-8/3/20 Bld 76	0

Table 3: Two-sample t-test on the HPC Samplers comparing CFU/ml from each EPG treatment during the summer sampling period ($p \leq 0.05$).

Two-sample t-test Results	Df	T- value	P-value
All Summer Samples HPC Samplers	105	0.09	0.930

Table 4: Two-sample t-test on the HPC Samplers comparing CFU/ml from each building in the fall sampling period ($p \leq 0.05$).

Two-sample t-test Results	Df	T- value	P-value
All Fall Samples HPC Samplers Compared by BLD	60	-3.93	0.00

Table 5: Descriptive statistics of two-sample t-test on the HPC Samplers comparing CFU/ml from each building in the fall sampling period ($p \leq 0.05$).

Building	N	Mean	StDev	SE Mean
Bld 14	36	475	786	131
Bld 76	27	1141	560	108

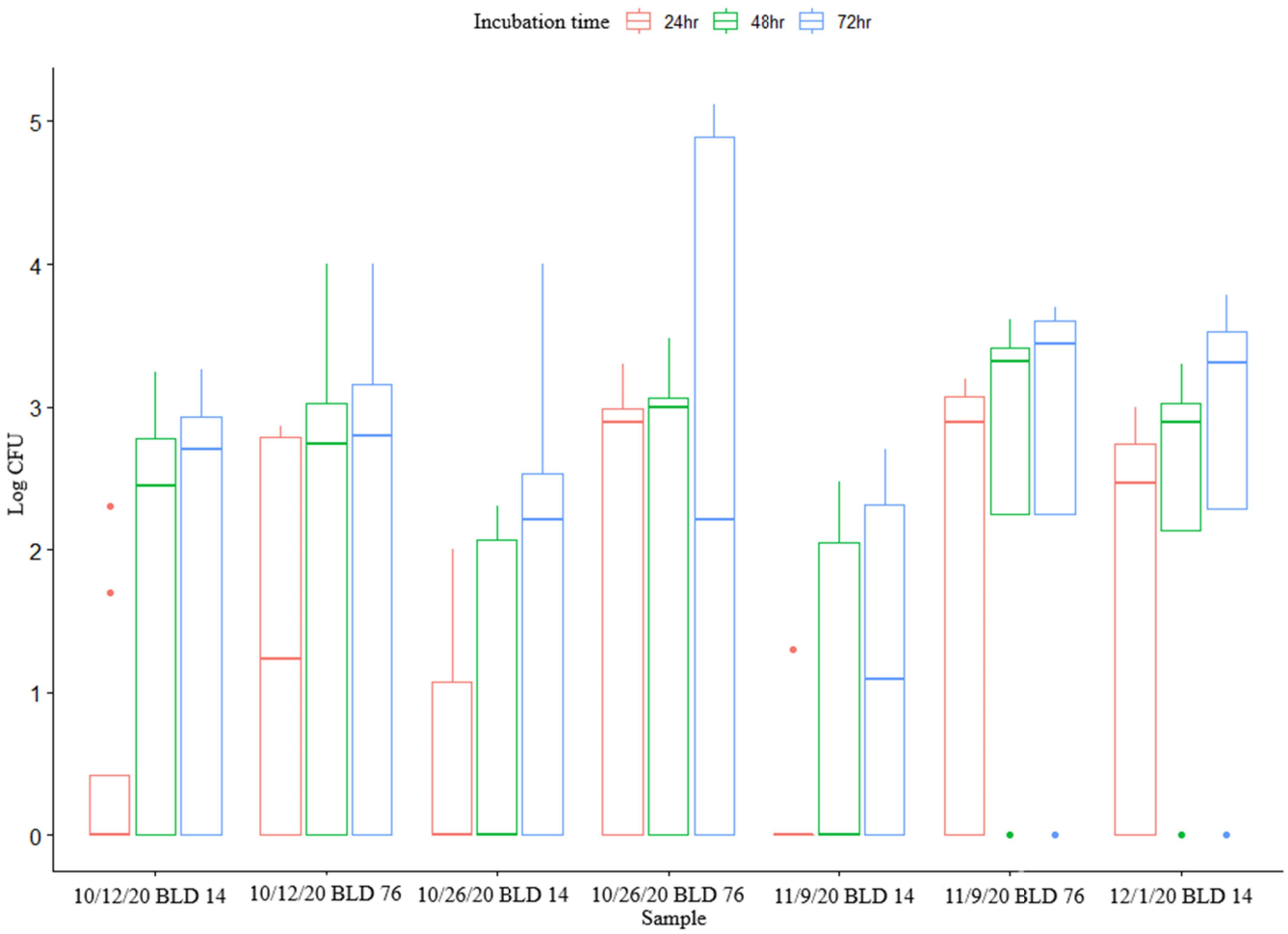


Figure 21: Bacterial log CFUs/ml in BLD 14 and BLD 76 cooling tower while treated by the STD EPG during the fall sampling period. The box plots represent the bacteria enumerations at each incubation time and sample.

Table 6: One way blocked ANOVA on nutrient agar CFUs in the fall sampling period with incubation time as blocking variable.

Sample Populations	Df	Sum Sq	Mean Sq	F value	P-value
All Fall Sampling Period Nutrient Agar CFUs	6	86.9	14.49	7.25	3.9e-07 ***
All Fall Sampling Period HPC Samplers CFUs	6	29.140	4.8566	8.51	0.000***
Fall BLD 76 Nutrient Agar CFUs	2	9.08	4.538	1.682	0.191
Fall BLD 14 Nutrient Agar CFUs	3	43.28	14.425	9.711	0.0000074***

Table 7: Tukey's HSD of all the fall 2020 samples of the nutrient agar CFUs. *bold values indicate a significant difference between samples

Comparison of Samples	P-value
10/12/20 Bld 76-10/12/20 Bld 14	0.8915605
10/26/20 Bld 14-10/12/20 Bld 14	0.9820173
10/26/20 Bld 76-10/12/20 Bld 14	0.116753
11/9/20 Bld 14-10/12/20 Bld 14	0.7582261
11/9/20 Bld 76-10/12/20 Bld 14	**0.0197647**
12/1/20 Bld 14-10/12/20 Bld 14	0.0729359
10/26/20 Bld 14-10/12/20 Bld 76	0.3972033
10/26/20 Bld 76-10/12/20 Bld 76	0.7786506
11/9/20 Bld 14-10/12/20 Bld 76	0.1071202
11/9/20 Bld 76-10/12/20 Bld 76	0.3678941
12/1/20 Bld 14-10/12/20 Bld 76	0.664001
10/26/20 Bld 76-10/26/20 Bld 14	**0.0107404**
11/9/20 Bld 14-10/26/20 Bld 14	0.9949631
11/9/20 Bld 76-10/26/20 Bld 14	**0.0010432**
12/1/20 Bld 14-10/26/20 Bld 14	**0.0056496**
11/9/20 Bld 14-10/26/20 Bld 76	**0.0009948**
11/9/20 Bld 76-10/26/20 Bld 76	0.995452
12/1/20 Bld 14-10/26/20 Bld 76	0.9999963
11/9/20 Bld 76-11/9/20 Bld 14	**0.0000669**
12/1/20 Bld 14-11/9/20 Bld 14	**0.0004673**
12/1/20 Bld 14-11/9/20 Bld 76	0.9993518

Table 8: Tukey's HSD of the BLD 14 fall sampling period nutrient agar CFUs. *bold values indicate a significant difference between samples.

Comparison of Samples	P-value
10/26/20 Bld 14-10/12/20 Bld 14	0.7730186
11/9/20 Bld 14-10/12/20 Bld 14	0.32072
12/1/20 Bld 14-10/12/20 Bld 14	**0.0069521**
11/9/20 Bld 14-10/26/20 Bld 14	0.8745764
12/1/20 Bld 14-10/26/20 Bld 14	**0.0002306**
12/1/20 Bld 14-11/9/20 Bld 14	**0.0000101**

Table 9: HPC test samplers fall sampling period Tukey's HSD. *bold values indicate a significant difference between samples

Difference of Sample Levels	P-Value
10/12/2020 Bld 76 - 10/12/2020 Bld 14	0.582
10/26/2020 Bld 14 - 10/12/2020 Bld 14	**0.012**
10/26/2020 Bld 76 - 10/12/2020 Bld 14	0.627
11/9/2020 Bld 14 - 10/12/2020 Bld 14	0.996
11/9/2020 Bld 76 - 10/12/2020 Bld 14	0.146
12/1/2020 Bld 14 - 10/12/2020 Bld 14	1.000
10/26/2020 Bld 14 - 10/12/2020 Bld 76	**0.000**
10/26/2020 Bld 76 - 10/12/2020 Bld 76	1.000
11/9/2020 Bld 14 - 10/12/2020 Bld 76	0.226
11/9/2020 Bld 76 - 10/12/2020 Bld 76	0.979
12/1/2020 Bld 14 - 10/12/2020 Bld 76	0.690
10/26/2020 Bld 76 - 10/26/2020 Bld 14	**0.000**
11/9/2020 Bld 14 - 10/26/2020 Bld 14	0.062
11/9/2020 Bld 76 - 10/26/2020 Bld 14	**0.000**
12/1/2020 Bld 14 - 10/26/2020 Bld 14	**0.007**
11/9/2020 Bld 14 - 10/26/2020 Bld 76	0.257
11/9/2020 Bld 76 - 10/26/2020 Bld 76	0.969
12/1/2020 Bld 14 - 10/26/2020 Bld 76	0.732
11/9/2020 Bld 76 - 11/9/2020 Bld 14	0.033
12/1/2020 Bld 14 - 11/9/2020 Bld 14	0.985
12/1/2020 Bld 14 - 11/9/2020 Bld 76	0.206

Table 10: Two-sample t-test of building comparisons from the all samples in the 2020 sampling period.

Comparison of EPGs	T-Value	DF	P-value
Nutrient Agar CFUs at 24 hr	-0.67	40	0.507
Nutrient Agar CFUs at 48 hr	0.41	30	0.686
Nutrient Agar CFUs at 72 hr	0.54	27	0.592
All 2020 Nutrient Agar CFUs Samples from BLD 14 at 48 hr	0.49	5	0.643
HPC Samplers CFUs at 48 hr	0.09	30	0.930

Discussion

EPG Comparison

The acceptable levels for heterotrophic bacteria in cooling towers are 5 log CFUs (Monash University, 2017). The EXP and STD EPGs both surpassed this level at one point or another but it was relatively infrequent and the bacteria load was quickly reduced below this level (Figure 18, 19). About 20% of the samples from the EXP EPG were above 5 log CFUs. However, the sampling period following the high bacterial load usually had CFUs that were reduced below this limit (Figure 19). In contrast the STD EPG had a higher CFU limit, 6.6 log CFUs but, this only occurred twice in the 30 STD EPG samples. This occurrence was during the preliminary data collection in BLD 2 where sampling was later discontinued due to the loss of sanitation contract. Excluding the two abnormally high CFUs in BLD 2, the STD EPG maximum CFUs was 4.7 log, therefore all of the STD EPG CFUs were below the allowable limit.

The cause of the excessive bacterial growth in either EPG was unclear, though it did not appear to be associated with seasonality. There were not trends that suggested seasonality affected bacterial CFUs because the same month could have very different CFUs (Figure 18, 19). Additionally, the same sampling date could have profoundly different CFUs in different cooling towers. The relative abundance of bacteria changed over time, however each cooling tower did not follow the same trend indicating that unique environmental conditions in each building were

responsible for these changes (Tsao et al., 2019). Therefore in comparison, the STD EPG was able to reduce the bacterial load below the allowable levels more frequently than the EXP.

Statistical Analysis of Microbial Enumeration

The bacterial community in the summer sampling period determined there was a difference in means among the different samples tested by the EXP EPG in BLD 14 and the STD EPG in BLD 76. However, this did not prove the CFUs difference was due to the EPG device. Tukey's HSD revealed a majority of the variation in this comparison was due to CFU variation within the same building. This indicated there was not uniformity within each building's cooling tower, thus a factor other than the EPGs was influencing bacterial growth. Factors such as wind, weather, and seasonal climates may be affecting the bacterial community in the cooling towers causing the CFUs to be higher or lower at the time of sampling (Tsao et al., 2019). Additionally, these towers were monitored by RIT facilities, if bacterial levels got too high the EPGs may be overridden by a chlorine treatment to kill the excess of bacteria. This may have occurred on 7/6/20 when bacteria CFUs were at 0. In conclusion, the statistical results were inconclusive as to why the summer period had so much variation, especially within their own towers.

The fall sampling period followed the same trend of variance as the summer sampling period. Even though all samples were treated by the STD EPG device during this period the bacterial loads were not statistically the same. This further confirmed that each cooling tower has a unique environment (Tsao et al., 2019). BLD 76 being an outdoor tower had different environmental conditions that may have influenced the bacterial community differently than BLD 14 which was an indoor cooling tower. When each building was evaluated independently, the samples within BLD 76 had similar bacterial loads, this differed from the summer sampling period. BLD 14 during the fall sampling period also had homogeneous CFUs except for its 12/1 sample, however this variance could have been caused by start of winter. This suggested the summer months caused bacteria loads to vary more significantly than fall months. Therefore, there may have been a seasonal effect where warmer months cause more variation in CFUs, than cooler months.

Overall the STD EPG and the EXP EPG showed they controlled bacterial growth the same. When compared to each other there was no significant difference between the treatments.

However, the enumerations alone showed that the STD EPG was able to more frequently keep bacterial CFUs below allowable levels. Therefore, the STD EPG may be able to reduce CFUs better than the EXP EPG, however this was not proved statistically.

Chapter 5: Bacterial Community

Results

Species Presence Results

The bacteria that appeared in the cooling tower samples varied vastly. Species presence was determined by counting the number of times a bacteria, as identified by Appendix D, appeared in a sample. The frequency presence within the bacteria community of all the samples determined that only 18 of the 77 bacteria appeared in a sample more than 5 times (Figure 22). The top five bacteria that appeared most frequently in all the samples were present in all seasons, EPG treatments, and cooling tower buildings. In comparison of the EPG treatments 34 of the same bacteria was present in both the STD EPG (54% of the population) and EXP EPG treatments (71% of the population) (Figure 23, 24). Only 24 bacteria were present throughout both the summer and fall sampling seasons. The summer sampling period had 60% unique bacteria in their population, while the fall sampling period had 41% unique bacteria in their population (Figure 25, 26). Consequently, the STD EPG and summer sampling period had a higher abundance of unique bacteria.

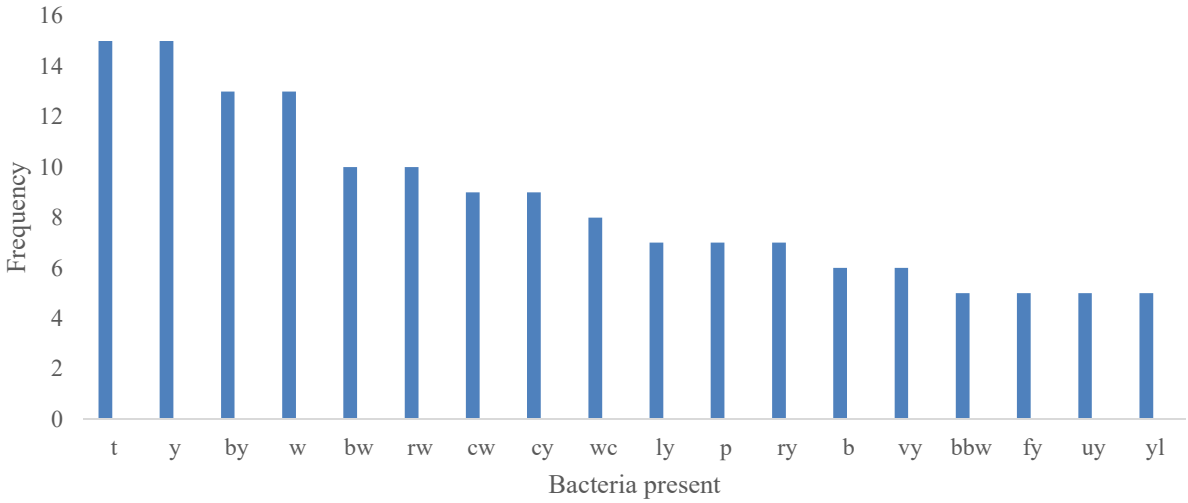


Figure 22: Frequency bacteria appeared in all samples. This graph only represents the bacteria with a frequency greater than five.

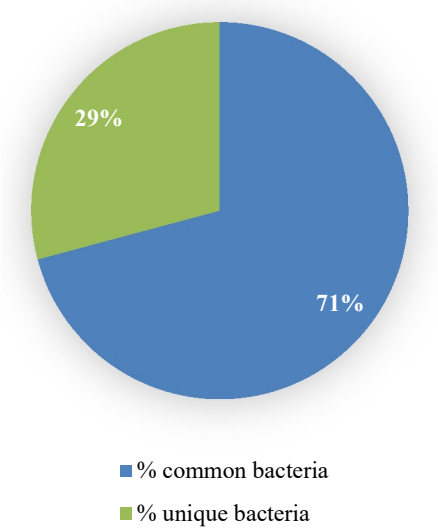


Figure 23: The common bacteria between the EPG treatments as a percent of the EXP EPG bacterial population in the summer sampling period.

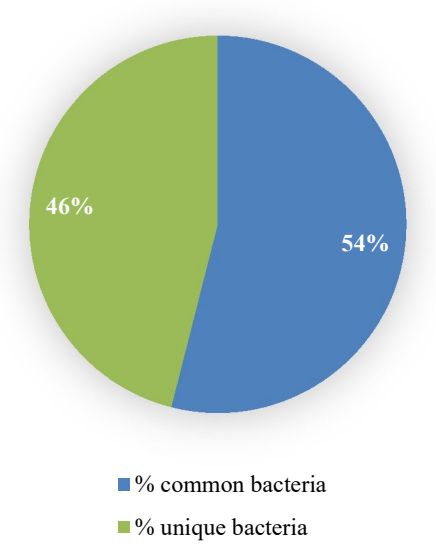


Figure 24: The common bacteria between the EPG treatments as a percent of the STD EPG bacterial population in the summer sampling period.

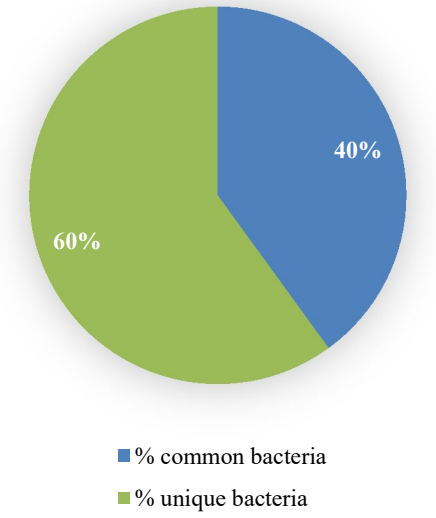


Figure 25: The common bacteria between seasons as a percent of the summer sampling period bacterial population.

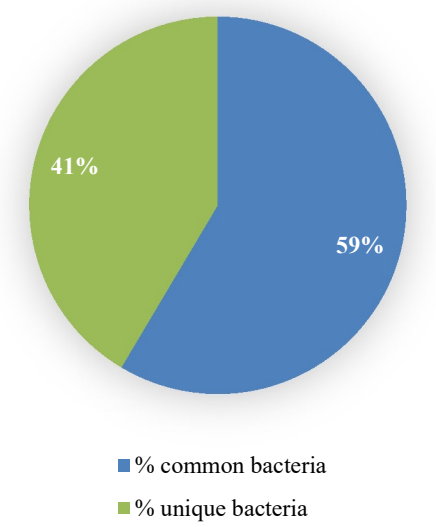


Figure 26: The common bacteria between seasons as a percent of the fall sampling period bacterial population.

Species Richness Results

The bacterial species richness of the samples was collected to determine the diversity of the bacterial communities within the cooling towers. The species richness was a count of the different bacteria that appeared in the samples. The overall richness of all the samples was 77 species. In comparison of the EPGs the STD EPG had a higher species richness than the EXP EPG (Table 11). The summer sampling period which compared the EPG devices on the same dates found the EXP EPG had a species richness of 48, while the STD EPG had a species richness of 42 in the summer sampling period. Therefore, the samples taken at the same time from the different EPGs showed a similar diversity. However in observing all the 2020 samples, the EXP EPG had a lower species richness (Table 11). This could be due to the fact that there was more data on the STD EPG inflating its diversity. The STD EPG overall in the fall sampling period had lower diversity than the summer. Therefore, the comparison in the summer period was more accurate to compare the EPG treatments.

In observing each building individually, BLD 76, which was only treated by the STD EPG, had a species richness of 55, while BLD 14 which was treated by both the EXP and STD EPG had a species richness of 59 (Table 11). Therefore, the bacteria diversity between these buildings did not vary vastly. Seasonality from summer to fall revealed the diversity reduced

significantly in the fall sampling period. The diversity in BLD 14 in the summer sampling period had a species richness was 48, however the fall sampling period showed a drop in diversity to 28. BLD 76 had a decline in species richness from 42 to 9 from the summer to fall sampling period. Seasonality proved the summer sampling period had a higher species diversity than the fall (Table 11).

Table 11: Bacterial species richness present in different sampling populations.

Sample Population	Species Richness
Bacteria Presence of All Samples (all BLDs, EXP & STD EPG)	77
STD EPG Richness	63
EXP EPG Richness	48
Summer Sampling Species Richness (Both EXP & STD EPG)	60
Fall Sampling Species Richness (STD EPG)	41
BLD 76 Richness All Samples (STD EPG)	55
Summer Sampling Richness of the STD EPG in BLD 76	42
Fall Sampling Richness of the STD EPG in BLD 76	9
BLD 14 Richness All Sampling (Both EXP & STD EPG)	59
Summer Sampling Richness of the EXP EPG in BLD 14	48
Fall Sampling Richness of the STD EPG in BLD 14	28

Gram Stain Results

From the cooling tower water samples, 77 distinct bacteria were identified. Of those 77 identified 72 were able to be successfully Gram stained, the inability to Gram stain was due to issues isolating particular bacteria. All Gram stains are described in Appendix E. The Gram stained bacteria had was a higher frequency (55%) of Gram negative bacteria (43), than Gram positive bacteria (29) (Table 12), although this was not a statistically significant majority (Table 13). Of the different treatments Gram negative bacteria held the statistical majority in the STD EPG treatment samples, while there was an even distribution of Gram negative and positive bacteria in the EXP EPG treatment samples (Table 13). Those that were Gram negative were mostly bacillus (30) rather than coccus (13). The Gram positive bacteria had an even distribution

of bacillus (15) and coccus (14) (Table 12). A goodness of fit test was run on the Gram stain and shape results to determine which Gram stain result was most present in the samples. This proved the distribution of the Gram stain results was not equal (p-value 0.013). The Gram negative bacilli had the highest presence and the most influence in disrupting the equal distribution of the bacteria Gram stain results since the observed frequency was much higher than the expected frequency of Gram negative bacilli (Table 14). These results showed that Gram negative bacilli were the dominant bacteria present in cooling towers and the EXP EPG was able to reduce Gram negative populations more effectively than the STD EPG.

Table 12: Summary of Gram stain results.

Gram Stain Results	**Frequency**
Gram Negative	**43**
Bacillus	30
Coccus	13
Gram Positive	**29**
Bacillus	15
Coccus	14
unable to isolate for gram stain	**5**

Table 13: 1 Proportion Z test on the Gram stains from the bacteria documented in the samples. These tests were performed such that Gram negative was $\neq 0.5$ to determine if Gram negative bacteria were the majority of the bacteria in the tower.

Samples	N	Event	95% Confidence Interval	Z-Value	P-Value
All Bacteria Gram Stains	72	43	0.4818067, 0.7027889	1.65	0.09896
STD EPG Bacteria Gram Stains	61	38	0.517716, 1.000000	3.6885	0.02739
EXP EPG Bacteria Gram Stains	43	23	0.3891564, 0.6748894	0.2093	0.6473

Table 14: Chi square goodness of fit Gram stain result contributions.

Category	Observed	Test Proportion	Expected	Contribution to Chi Square
negative bacillus	30	0.25	18	8.00000
negative coccus	13	0.25	18	1.38889
positive bacillus	15	0.25	18	0.50000
positive coccus	14	0.25	18	0.88889

Antibiotic Resistance Results

A subset of the bacteria that were frequently present in the cooling towers was tested for antibiotic resistance. These bacteria had the top 20 highest presence in the towers, meaning they appeared in the cooling tower samples more than four times with one appearing only three times. Half of the tested samples were resistant to 50% or more of the 24 antibiotics tested (Figure 27). Of the bacteria present in each EPG treatment which were tested for antibiotic resistance, 60% of the bacteria in the EXP EPG treatment and 50% of the bacteria in the STD EPG treatment were resistant to at least half of the antibiotics (Table 15, 16). The STD EPG contained more bacteria that were antibiotic resistant than the EXP EPG. Of the antibiotics tested, ampicillin and oxacillin were obsolete, all of the bacteria tested were 100% resistant to them. Imipenem, doxycycline, minocycline, and levofloxacin were most effective (85-95%) against the cooling tower bacteria, with 15% or less of resistance to these antibiotics (Figure 28).

The bacteria present in the cooling towers did not always have a uniform bacterial population. In some cases the bacterial community contained multiple populations that had different antibiotic sensitivities even though they were grown as a pure culture. Of the bacteria tested, 62% of them contained multiple populations that were resistant and sensitive to one or more antibiotic (Figure 29). In these cases the bacteria zones of inhibition were based on their most resistant antibiotic sensitivity.

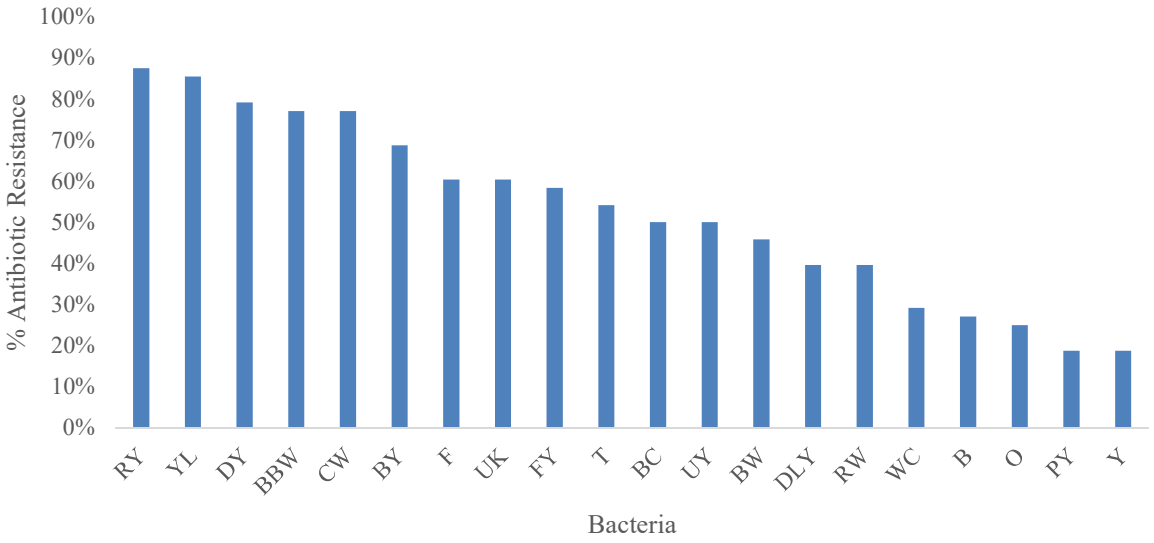


Figure 27: Cooling tower bacteria antibiotic resistance.

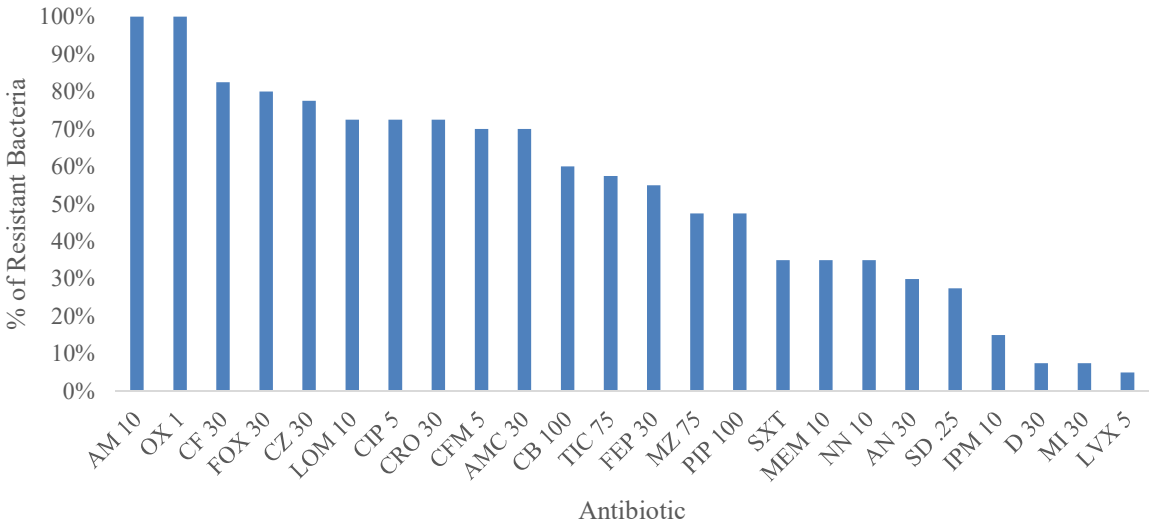


Figure 28: Percent of cooling tower bacteria which were resistant to an antibiotic.

Table 15: EXP EPG antibiotic resistance and bacteria frequency.

Bacteria Designation	Frequency in samples	% Antibiotic Resistance
RY	6	88%
YL	5	85%
DY	4	79%
BBW	4	77%
CW	6	77%
BY	10	69%
F	2	60%
UK	3	60%
FY	5	58%
T	10	54%
BC	4	50%
UY	3	50%
BW	7	46%
DLY	2	40%
RW	8	40%
WC	7	29%
B	3	27%
O	3	25%
PY	3	19%
Y	11	19%

Table 16: STD EPG antibiotic resistance and bacteria frequency.

Bacteria Designation	Frequency in samples	% Antibiotic Resistance
RY	1	88%
BBW	1	77%
CW	3	77%
BY	3	69%
F	2	60%
UK	1	60%
T	5	54%
UY	2	50%
BW	3	46%
DLY	1	40%
RW	2	40%
WC	1	29%
B	3	27%
O	1	25%
PY	1	19%
Y	4	19%

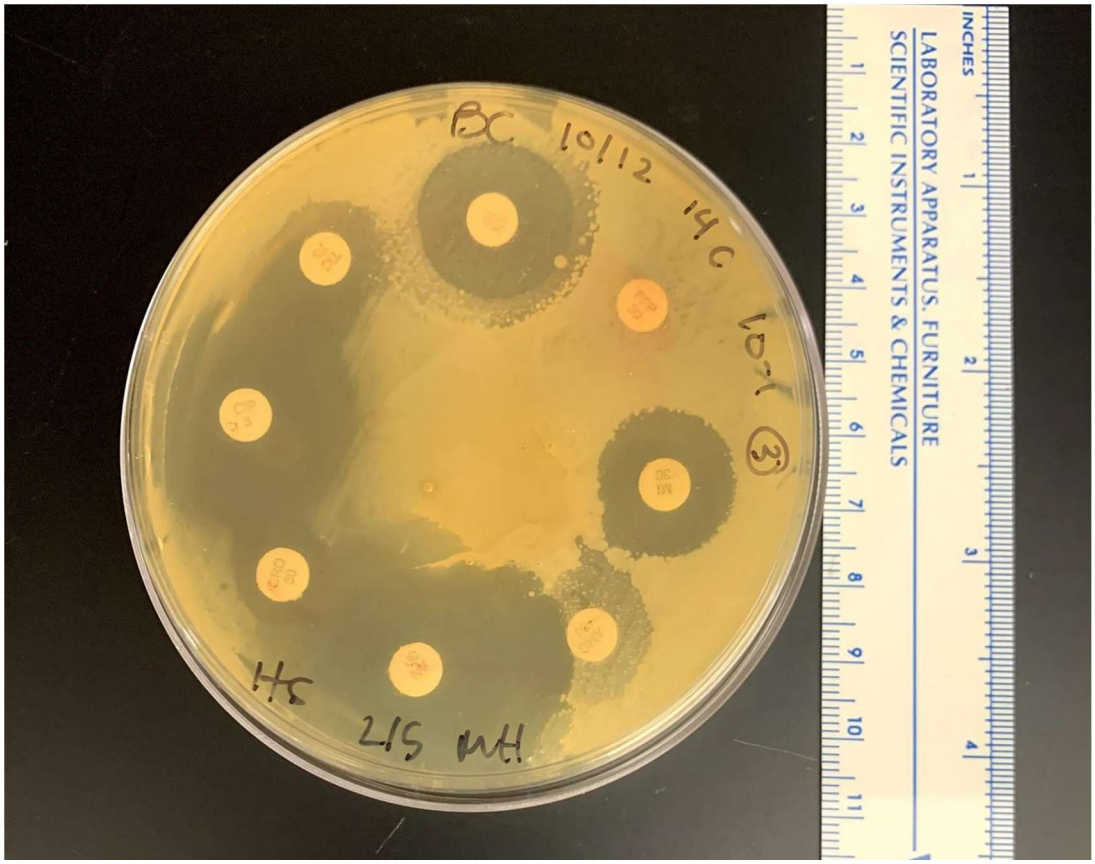


Figure 29: Image of antibiotic sensitivity test on BC bacteria showing multiple populations with different antibiotic sensitivities. The antibiotic under the BC label and going clockwise the 4th antibiotic also has 2 populations of bacteria with different resistances to the antibiotics.

16S rRNA Gene Bacterial Identification

The results of the 16S rRNA sequencing from Genewiz found the genera of each bacteria and in most cases the species or likely species. Therefore the species that were present in the cooling towers were *Morganella morganii, Chryseobacterium ureilyticum, Phenylobacterium* sp., *Chryseobacterium cucumeris, Stenotrophomonas maltophilia, Bacillus licheniformis,* and *Bacillus cereus* (Table 17). The Gram stains and images of these bacterial colonies are presented in Appendix F. The bacteria identified through the 16S rRNA gene had some discrepancies on the Gram stain results as compared to the reported Gram stain for the identified bacteria (Table 17). These bacteria were the most common bacteria and all were resistant to more than 46% of antibiotics tested on them (Table 17, 18).

Table 17: Bacteria that was identified by the 16S rRNA gene attributes.

Bacteria Designation	Bacteria Genera/ Species	EXP Gram stain	Documented Gram Stain	Antibiotic Resistance
BW	*Morganella morganii*	positive coccus	negative bacillus	46%
BY	*Chryseobacterium ureilyticum*	negative bacillus	negative bacillus	69%
CW	*Phenylobacterium* sp.	positive coccus	negative bacillus	77%
DY	*Chryseobacterium cucumeris*	negative bacillus	negative bacillus	79%
RY	*Stenotrophomonas maltophilia*	negative bacillus	negative bacillus	88%
T	*Bacillus licheniformis*	negative bacillus	positive bacillus	54%
UK	*Bacillus cereus*	positive bacillus	positive bacillus	60%

Table 18: Antibiotic resistance of the bacteria that had their 16S rRNA gene sequenced. R indicates resistant, S susceptible, and I intermediate antibiotic sensitivity.

	Bacteria Designation						
Antibiotic	BW	BY	CW	DY	RY	T	UK
Ampicillin	R	R	R	R	R	R	R
Amikacin	S	R	S	R	R	S	S
Amoxicillin/ Clavulanic acid	R	R	R	R	R	R	R
Carbenicillin	S	R	R	R	R	R	R
Cefazolin	R	R	R	R	R	I	R
Cefepime	S	S	R	R	R	R	R
Cefixime	S	R	R	R	R	R	R
Cefoxitin	R	R	R	R	R	S	R
Ceftriaxone	S	R	R	R	R	R	R
Cephalothin	R	R	R	R	R	R	R
Ciprofloxacin	I	I	R	R	R	I	I
Doxycycline	R	S	S	S	S	S	S
Imipenem	S	S	R	S	R	S	S
Levofloxacin	S	S	R	S	S	S	S
Lomefloxacin	R	I	I	R	R	R	I
Meropenem	S	R	R	R	R	S	S
Mezlocillin	I	R	R	R	R	R	I
Minocycline	S	S	S	S	S	S	S
Oxacillin	R	R	R	R	R	R	R
Piperacillin	R	R	R	R	R	R	R
Sulfadiazine	R	I	S	R	R	S	S
Sulfamethoxazole/ Trimethoprim	S	S	S	S	R	S	R
Ticracillin	S	R	R	R	R	R	R
Tobramycin	S	R	R	R	R	S	S

Discussion

EPG Diversity

In comparing all the samples taken in the 2020 sampling period the STD EPG had a higher species richness than the EXP EPG (Table 1). This could indicate the EXP EPG was able to reduce bacteria diversity more effectively than the STD EPG. However, there were more samples treated with the STD EPG than the EXP EPG which may have skewed the data. Therefore, the population of the summer sampling period was examined to compare diversity of each EPG treatment on the same sampling dates. This revealed that the EXP and STD EPG had very similar species richness. The increase of data for the STD EPG and the significant decline in diversity in the fall sampling period was likely why the comparison of all samples made the EXP EPG appear as though it reduced bacterial diversity more effectively than the STD EPG. The summer sampling period was a more robust analysis of diversity when comparing the EPGs, thus the EPGs have very little difference in their ability to reduce bacterial diversity and CFUs/ml.

Species Presence and Richness

Microbial diversity within cooling towers has been a neglected area of study. Few studies have evaluated bacteria within cooling towers beyond those in association with *L. pneumophila* presence (Tsao et al., 2019). This evaluation of diversity determined the species richness and frequency presence of bacteria within cooling towers in order to evaluate the EPG devices ability to reduce species diversity. Of the 77 species that were present in all the cooling towers only 23% of them appeared in the samples more than 5 times, thus species presence was variable. However, the top five bacteria that had the highest frequency presence were not affected by seasons, specific cooling tower building conditions, or the EPG treatment. Therefore, there were some bacteria that were constant no matter the conditions.

The majority of bacteria were consistent between the EPG treatments. The between the EXP and STD EPG there were 34 bacteria that appeared in both of the treatments. Based on the species richness of the EXP EPG, these 34 bacteria accounted for 71% of the population, while in the STD EPG they accounted for 54% of the population (Figure 23, 24). This suggests the

STD EPG fostered a more diverse bacterial community, while the EXP EPG had a simpler community. This was in agreement with the species richness results where the STD EPG had a higher species richness than the EXP EPG.

The reduction in diversity could be the result of seasonality. Twenty-four bacteria were present throughout both the summer and fall sampling seasons. Those 24 consistent bacteria accounted for 40% of the population in the summer sampling period, while in the fall sampling period it accounted for 59% of the bacterial population (Figure 25, 26). Therefore, there were more unique species in the summer than the fall, suggesting there was a trend in seasonality since there was more diversity in the summer than the fall.

Species richness also showed this seasonality trend. Species richness significantly decreased from the summer to fall sampling periods. The species richness in BLD 76 declined from 42 to 9 species, whereas the species richness in BLD 14 decreased from 48 to 28 species. In BLD 14 the EPG treatment also changed between the summer and fall sampling periods, yet this diversity decline was more likely from seasonality than EPG treatment since the same diversity decrease also occurred in BLD 76 where the EPG device was the same throughout the 2020 sampling period. Additionally, the EPG comparison in the summer sampling period found the EPG treatments resulted in similar diversities, thus the reduced diversity could not be due to EPG treatment but rather seasonality. The decrease in diversity could be due to the cooler weather conditions providing injurious conditions for some species (Tsao et al., 2019). The steeper decrease in the diversity in BLD 76 could be caused by its outdoor connection causing colder temperatures than within BLD 14 which is an indoor cooling tower. Therefore, seasonality had an effect on diversity. However, seasonality did not affect CFUs indicating that when some species were removed other species were able to thrive and grow to similar levels in their place. Lower diversities may be beneficial for sanitation since it results in fewer bacteria to be concerned about, yet they are in higher concentrations thus have a higher contamination potential if pathogenic.

Gram stain

Gram negative bacteria dominate cooling tower bacterial communities (Türetgen, 2004). Gram negative bacteria are the predominate bacteria in cooling tower biofilms and tend to be

opportunistic pathogens to immunocompromised humans (Center for Disease Control, 2015). The high frequency of Gram negative bacilli was concerning since cooling towers can contaminate large areas both indoors and outdoors and cause infections (Sala Ferré et al., 2009). The reduction of Gram negative bacteria in the EXP EPG as compared to the STD EPG indicated the EXP EPG can reduce the number of potentially pathogenic bacteria more effectively than the STD EPG. However, the bacteria tested in this study for antibiotic resistance had a relatively equal distribution of Gram negative and positive bacteria, which were both particularly resistant to the antibiotics tested (>60%) (Figure 15). As a result, although Gram negative bacteria is known to likely be pathogenic, in this study it did not indicate a higher affinity to antibiotic resistance.

Antibiotic Resistance

Although it is known that cooling towers are hotspots for biofilms where genetic exchange can occur, there are few studies that evaluate the antibiotic resistance within cooling towers (Fux et al., 2005; Hausner and Wuertz, 1999; Li et al., 2002; Molin and Tolker-Nielsen, 2003; Ozdemir and Ceyhan, 2010). This study found that cooling towers had a high presence of antibiotic resistant bacteria. The antibiotics recommended for cooling tower usage are imipenem, doxycycline, minocycline, and levofloxacin since they had the highest efficiency (85-95%) against the cooling tower bacteria (Figure 16). Comparatively, it is not recommended to use ampicillin or oxacillin since cooling tower bacteria are likely to be resistant to them. These results were of concern because these antibiotics are widely used in clinical settings (Whitburn, 2019).

The presence of multiple populations of the same bacteria with different susceptibilities to antibiotics suggests bacteria are actively gaining antibiotic resistance. Sixty-two percent of the bacteria tested had multiple populations that were resistant and sensitive to one or more antibiotic, thus there is a significant amount of genetic exchange occurring in cooling towers (Figure 17). Genetic exchange in cooling towers would result in even fewer antibiotics able to suppress bacteria. Outbreaks from cooling towers frequently occur in hospitals, thus these resistance levels and evidence of genetic exchange reduce the antibiotics healthcare providers are

able to use to treat bacterial infections resulting from cooling tower contamination (Dondero et al., 1980; Engelhart et al., 2008; García-fulgueiras et al., 2003).

The presence of antibiotic resistant bacteria did not suggest that one of the EPG treatments was more effective to remove antibiotic resistant bacteria. However, the frequency antibiotic resistant bacteria appeared in samples was lower in the STD EPG than the EXP EPG (Table 15, 16). Therefore, although the STD EPG may allow the presence of antibiotic resistant bacteria their abundance will be lower.

16S rRNA

Bacteria that had a high frequency presence in the cooling towers had their 16S rRNA gene sequenced to identify the bacteria and determine what implications it could have on the environment if released from the tower. There were discrepancies in the Gram stain results of the bacteria isolated from the cooling towers as compared to the reported Gram stains from the 16S rRNA identified bacteria (Table 17). The experimental Gram stains could have been erroneous due to over or under use of decolorizer resulting in a false Gram stain. In addition the variance in bacteria shape could simply have been a misidentification.

The species that were present in the cooling towers were *Morganella morganii, Chryseobacterium ureilyticum, Phenylobacterium* sp., *Chryseobacterium cucumeris, Stenotrophomonas maltophilia, Bacillus licheniformis,* and *Bacillus cereus* (Table 17). BW was determined to be *M. morganii,* however there was a discrepancy between the experimental Gram stain as Gram positive coccus, while the documented Gram stain is a Gram negative bacilli (Falagas et al., 2006). This error could be an excess of crystal violet or not enough decolorizer. In the cases of BY and CW, the bacilli may have been mistaken for coccus due to their small size.

Table 19: Summary of the contamination potential of the identified bacteria.

Bacteria	Contamination Potential
Morganella morganii	• Opportunistic pathogen • Broad resistance to antibiotics • Can easily transfer genetic material between bacteria. The isolate from this sample has additional antibiotic resistance than literature reports. • Not likely to cause infection through aerosol
Chryseobacterium ureilyticum	• Resistant to beta-lactams and carbapenems • Opportunistic pathogens
Chryseobacterium cucumeris	• Resistant to beta-lactams and carbapenems • Opportunistic pathogens
Phenylobacterium sp.	• Antibiotic resistance to ampicillin and carbenicillin • No known pathogenic properties
Stenotrophomonas maltophilia	• Resistant to a broad spectrum of antibiotics • This isolate had 88% antibiotic resistance • Can cause respiratory issues • Unlikely to survive in dry conditions

Table 19 Continued

Bacteria	Contamination Potential
Bacillus licheniformis	• Isolate with the highest frequency presence • Non-pathogenic • Forms spores • This isolate was sensitive to antibiotics it had known resistance to • Unlikely to transfer genes horizontally
Bacillus cereus	• Spore forming • Associated with food poisoning, respiratory tract infections, and nosocomial infections • Produce β-lactamases and carbapenemsases • NCCLS recommended the avoidance of cephalosporin for B. cereus treatment • Collected bacteria was susceptible to carbapenems

M. morganii is considered an opportunistic species that persists in the intestines of animals (Mbelle et al., 2020). It is known to cause urinary tract infections (UTI), meningitis, and septic arthritis (Falagas et al., 2006; Katz et al., 1987; Mbelle et al., 2020; Samonis et al., 2001). It also has resistance to a variety of antibiotics and multiple drug resistance (Rojas et al., 2006). In addition, *M. morganii* has the ability to easily transfer gene resistance between bacteria of the same or different species (Hsieh et al., 2015; Liu et al., 2016; Mbelle et al., 2020; Rojas et al., 2006). Literature has reported *M. morganii* to be resistant to oxacillin, ampicillin, amoxicillin, first and second cephalosporins, macrolides, lincosamides, glycopeptides, fosfomycin, fusidic acid, and colistin (Stock and Wiedemann, 1998).

This study agrees with these reports as *M. morganii* was resistant to ampicillin, amoxicillin/ clavulanic acid, and oxacillin, and cephalosporins: cephalothin, cefazolin, cefoxitin. In addition, *M. morganii* was resistant to doxycycline, piperacillin, lomefloxacin, and sulfadiazine (Table 18). Stock and Wiedemann (1998) also found *M. morganii* was susceptible to aztreonam, aminoglycosides, antipseudomonal penicillins, third-and fourth-generation cephalosporins, carbapenems, quinolones, trimethoprim/ sulfamethoxazole, and chloramphenicol. The results from this study are in line with these findings except this *M. morganii* was resistant to piperacillin and antipseudomonal penicillins, suggesting that while in the cooling tower *M. morganii* received genetic resistance genes.

The antibiotic resistance, susceptibility to obtain new antibiotic resistance genes, and high abundance of *M. morganii* raised concerns for *M. morganii's* presence in cooling towers. *M. morganii* is commonly known to cause UTIs in humans which is not communicated through breathing in an aerosol, however it has a high pathogenic potential in immunocompromised hosts (Samonis et al., 2001). Therefore, *M. morganii* in cooling towers are unlikely to pose a risk to human health directly, however their potential to gain and transfer antibiotic resistance to other pathogens raises a public health concern.

The bacteria designated BY and DY were identified as *Chryseobacterium ureilyticum* and *Chryseobacterium cucumeris*. *Chryseobacterium* is a quickly growing genera that is characterized by its yellow pigmented, Gram negative bacillus bacteria, and lack of motility. Generally, *Chryseobacterium* are resistant to beta-lactams and carbapenems (Kim et al., 2020). This study's results found DY, *Chryseobacterium cucumeris,* was resistant to 19 of the 24 antibiotics and BY, *Chryseobacterium ureilyticum,* was resistant to 15 of the 24 antibiotics. Most of the resistance of these bacteria was from antibiotics in the beta-lactams and carbapenems classes. The bacteria in this genera are found in diverse habitats such as freshwater, soil, or dairy products. These species have also been identified as pathogens in untreated drinking water and some are opportunistic pathogens to animals (Dworkin et al., 2006). Therefore the high frequency in cooling towers, documented resistance to antibiotics, and pathogenic potential of *Chryseobacterium* sp. identifies a pathogen of concern that could cause disease outbreaks originating from cooling towers.

The bacteria designated CW was identified as *Phenylobacterium* sp. which is a small novel genera that can inhabit soil or aquatic habitats (Tiago et al., 2005). They are a Gram negative rod bacteria known to degrade herbicide, specifically chloridazon (Lingens et al., 1985). This bacterium also had similar antibiotic resistance to literature reports with resistance to ampicillin and carbenicillin (Aslam et al., 2005). However, being such a novel genera *Phenylobacterium* currently does not have any known pathogenic properties. The presence of a bacteria with antibiotic resistance genes but is not pathogenic is still of concern because it has the potential to pass that information on to another bacteria that may be pathogenic. This is especially concerning for cooling towers since they are hot spots for genetic exchange due to their environmental conditions (Fux et al., 2005; Hausner and Wuertz, 1999; Li et al., 2002; Molin and Tolker-Nielsen, 2003).

Stenotrophomonas maltophilia was determined to be the identity of RY. This bacteria was a motile Gram negative bacillus with colonies that were smooth with a glistening yellow-white color and entire margins (Denton and Kerr, 1998). They have been documented to grow in a number of aquatic habitats. Infections of *S. maltophilia* can cause respiratory issues and can be transmitted through fecal carriage on hands (Denton and Kerr, 1998). *S. maltophilia* is known to be resistant to a broad spectrum of antibiotics. There is conflicting data of *S. maltophilia* sensitivity to the carbapenem class. Denton and Kerr (1998) state *S. maltophilia* had particular resistance to carbapenems while Cullmann and Dick (1990) stated that *S. maltophilia* only occasionally had resistance to carbapenems. The strain found in this study showed susceptibility to the antibiotics in the carbapenem class and disagreed with Denton and Kerr (1998). However, this study did prove that *S. maltophilia* is resistant to a broad spectrum of antibiotics with resistance to 88% of the antibiotics tested, the highest antibiotic resistance of all the bacteria tested. Therefore, the presence of this bacteria posed serious concern for public health because *S. maltophilia* is frequently present in cooling towers, highly resistant to antibiotics, and causes respiratory illnesses. However, *S. maltophilia* are unlikely to survive in dry conditions, thus if aerosolized from a contaminated cooling tower *S. maltophilia* may die before causing infection (Hirai, 1991; Moffet et al., 1967; Rosenthal, 1974).

Bacillus licheniformis was the identity of T, a Gram positive bacillus species that forms spores and occurs naturally in soil and had the highest presence frequently in all the cooling

tower samples (Duc et al., 2003). It is considered non-pathogenic to humans and is often used in commercial settings for animal feeds (Cutting, 2011; de Boer et al., 1994). Its ability to form spores could allow it to evade sanitation events and persist in cooling towers (Bottone, 2010). *B. licheniformis* has resistance genes against erythromycin, chloramphenicol, streptomycin, but these genes are unlikely to be transferred horizontally (Agersø et al., 2019). *B. licheniformis* also has carbapenemases which make it resistant to carbapenems, which have the widest activity spectrum of the β-lactam group (Carfi et al., 1995; Halat and Moubareck, 2020; Queenan and Bush, 2007).

B. licheniformis was resistant to 54% of the antibiotics in this study many of which were β-lactams, yet it was sensitive to the carbapenems of this group suggesting this strain did not contain genes for carbapenemases. Therefore, even though *B. licheniformis* had the highest bacteria presence and a 54% antibiotic resistance *B. licheniformis* does not pose a serious threat to cooling towers since this species is unlikely to transfer its resistance genes and is not considered pathogenic to humans (Agersø et al., 2019; de Boer et al., 1994).

Finally, UK was determined to be *Bacillus cereus*, a spore forming Gram negative bacillus. As a spore *B. cereus* can be inactive and essentially protected from an adverse environment (Bottone, 2010). In a cooling tower environment a spore of *B. cereus* could be protected during sanitation events and persist in the tower afterwards. It can inhabit different aquatic environments, decaying organic matter, and invertebrate intestinal tracts (Berkeley et al., 1984; Bottone, 2010). *B. cereus* is associated with food poisoning, respiratory tract infections, and nosocomial infections where HVAC systems could be bacterial reservoirs (Bottone, 2010; Bryce et al., 1993). *B. cereus* produce β-lactamases which renders them resistant to penicillins and cephalosporins (Bottone, 2010). They also produce carbapenemsases resulting in resistance to carbapenems (Carfi et al., 1995; Halat and Moubareck, 2020). It also has reported resistances to penicillin, ampicillin, cephalosporins, trimethoprim, erythromycin, oxacillin, and tetracycline (Bottone, 2010; Kiyomizu et al., 2008; National Committee for Clinical Laboratory Standards, 1984; National Committee for Clinical Laboratory Standards, 1985; Savini et al., 2009; Turnbull et al., 2004). *B. cereus* is so resistant to broad-spectrum cephalosporins that the National Committee for Clinical Laboratory Standards recommended that cephalosporin treatment use

should be avoided for suspected *B. cereus* infections (National Committee for Clinical Laboratory Standards, 1984; National Committee for Clinical Laboratory Standards, 1985).

These reports of antibiotic sensitivity were consistent with the antibiotic resistance of *B. cereus* in this study however, this strain was susceptible to carbapenems suggesting it did not contain resistance genes for carbapenems. This was interesting, since *B. cereus* has been described with carbapenemases (Carfi et al., 1995; Halat and Moubareck, 2020). The association with respiratory illnesses provides the potential for *B. cereus* to infect a wide range of people if released from cooling towers. The substantial amount of known antibiotic resistance of *B. cereus* makes infections of *B. cereus* difficult to treat. Finally, the ability to evade sanitation as a spore makes *B. cereus* a pathogen of concern in cooling towers since its release would pose a significant health risk.

Chapter 6: EPG Evaluation Conclusion and Recommendations for Future Research

In conclusion, the EPG devices were able to reduce bacterial CFUs to levels that are considered safe for cooling towers. Statistically one device did not perform better than the other in its ability to reduce the CFUs of bacteria present in the towers. However, the STD EPG was able to reduce CFUs slightly better than the EXP EPG. The bacterial diversity and antibiotic resistance between the two EPGs was also consistent. However, the STD EPG had a slightly lower species diversity and antibiotic resistance indicating it removed more diverse bacteria from the water. The STD EPG was able to reduce the presence of antibiotic resistant bacteria more effectively than the EXP EPG. The EXP EPG was able to more efficiently reduce Gram negative bacteria than the STD EPG. However, Gram stain did not indicate antibiotic resistance since there was an equal distribution of Gram positive and negative bacteria. The results of this study found that the STD EPG was able to reduce CFUs/ml and species diversity slightly more effectively than the EXP EPG. Therefore, the STD EPG was the superior EPG since it had an overall lower CFUs/ml, diversity, and abundance of antibiotic resistant bacteria. The bacteria this EPG allowed to persist had a lower potential to cause harm and were unlikely to produce biofilms as a result of the lower CFUs and cause fewer issues within the cooling tower.

Based on these results there is heightened concern about the bacteria present in these towers and their potential to contaminate the environments where cooling tower exhausts are

deposited. The bacteria in the cooling towers was very diverse and it was unclear what factors influenced their growth. Each cooling tower had unique environmental factors that were likely affecting the bacterial growth. Seasonality significantly affected species diversity, while CFUs were constant. However, seasonality did affect the variation within bacteria loads, with more variation in summer than fall. Further examination should be performed to understand other factors that affection bacterial diversity and growth. The presence of highly antibiotic resistant bacteria suggests that cooling towers are hot spots for genetic exchange. Additionally, the EPGs used the same method as electroporation for gene transfer, thus further study should be performed to evaluate if EPGs are facilitating the transfer of antibiotic resistance genes. Finally, further testing should be performed to determine if one EPG device is more effective to reduce the presence of antibiotic resistant bacteria.

Appendixes

Appendix A: Antibiotic Zones of Inhibition

Becton, Dickinson and Company Zones of Inhibition (Becton Dickinson and Company, 2011)

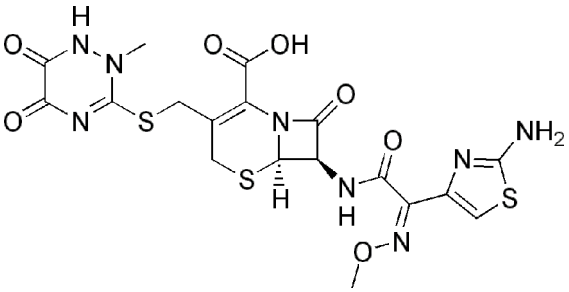
*most resistant zones of inhibition are represented

Antibiotic	Abbreviation	Resistant Zone of Inhibition (mm)	Intermediate Resistance Zone of Inhibition (mm)	Susceptible Resistance Zone of Inhibition (mm)
Amikacin	AN 30	≤14	15-16	≥17
Amoxicillin/ Clavulanic acid	AmC 30	≤13	14-19	≥20
Ampicillin	AM 10	≤13	14-28	≥29
Carbenicillin	CB 100	≤19	20-22	≥29
Cefazolin	CZ 30	≤14	15-17	≥18
Cefepime	FEP 30	≤ 21	22-23	≥24
Cefixime	CFM 5	≤15	16-17	≥19
Cefoxitin	FOX 30	≤24	25-26	≥27
Ceftriaxone	CRO 30	≤24	25-26	≥27
Cephalothin	CF 30	≤14	15-17	≥18
Ciprofloxacin	CIP 5	≤27	28-40	≥41
Doxycycline	D 30	≤12	13-15	≥16
Imipenem	IPM 10	≤13	14-15	≥16
Levofloxacin	LVX 5	≤13	14-16	≥17
Lomefloxacin	LOM 10	≤26	27-37	≥38
Meropenem	MEM 10	≤13	14-15	≥16
Mezlocillin	MZ 75	≤17	18-20	≥21
Minocycline	MI 30	≤14	15-18	≥19

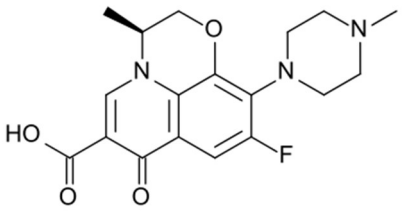
Appendix A Continued

Antibiotic	Abbreviation	Resistant Zone of Inhibition (mm)	Intermediate Resistance Zone of Inhibition (mm)	Susceptible Resistance Zone of Inhibition (mm)
Oxacillin	OX 1	≤17	-	≥18
Piperacillin	PIP 100	≤17	18-20	≥21
Sulfadiazine	SD 0.25	≤10	11-14	≥15
Sulfamethoxazole/ Trimethoprim	SXT	≤15	16-18	≥19
Ticracillin	TIC 75	≤14	15-19	≥20
Tobramycin	NN 10	≤12	13-14	≥15

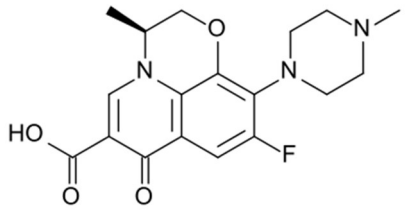
Appendix B: Antibiotic chemical structures



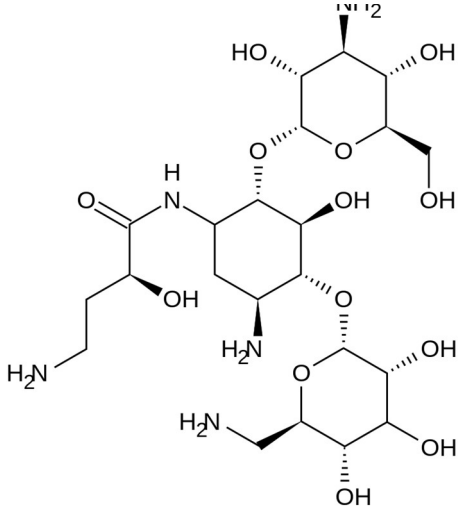
Ceftriaxone (Edgar181, 2007)



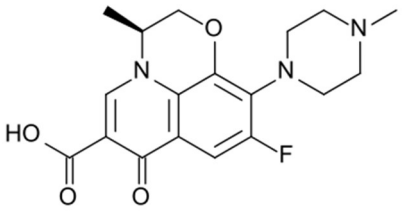
Levofloxacin (Derksen, 2007)



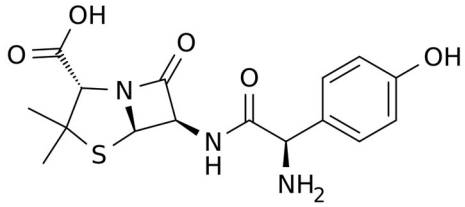
Levofloxacin (Derksen, 2007)

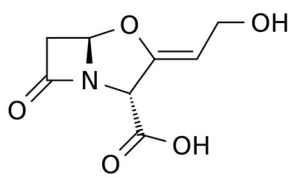


Amikacin (Fvasconcellos, 2006)

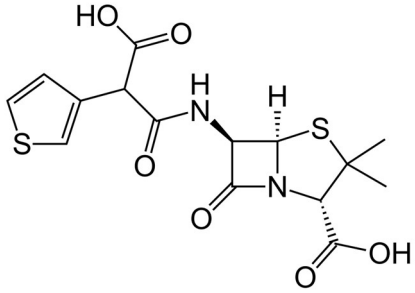


Ampicillin (Mysid, 2007)

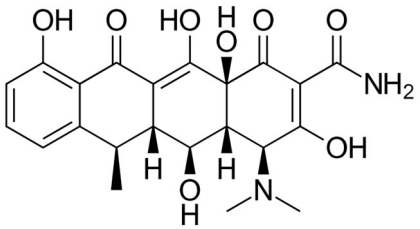




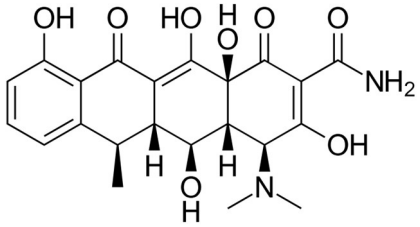
Amoxicillin/ Clavulanic acid (Fuse809, 2014)



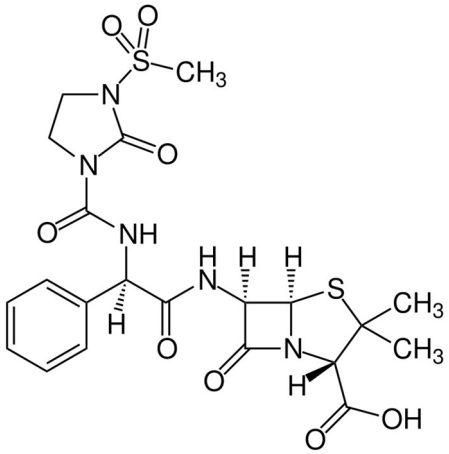
Ticarcillin (Fvasconcellos, 2006)



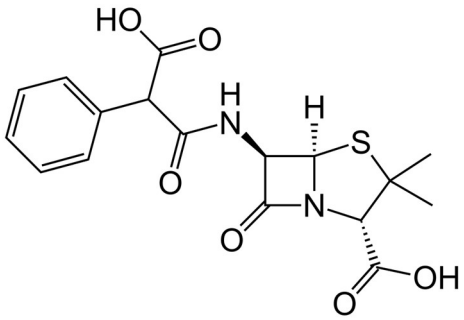
Doxycycline (Vaccinationist, 2017)



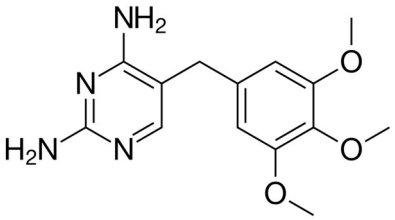
Ciprofloxacin (Fvasconcellos, 2008)



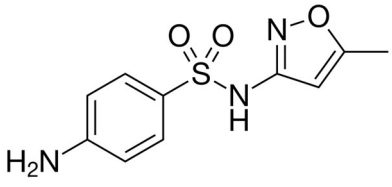
Mezlocillin (Jü, 2017)

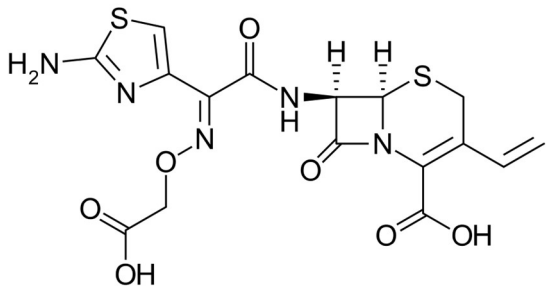


Carbenicillin (Fvasconcellos, 2006)

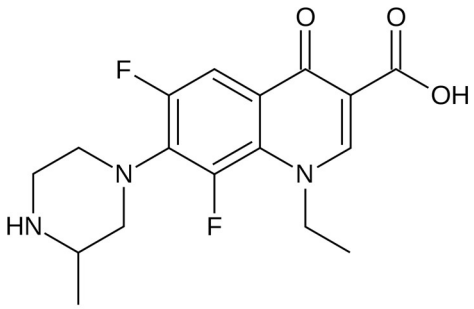


Su 15)

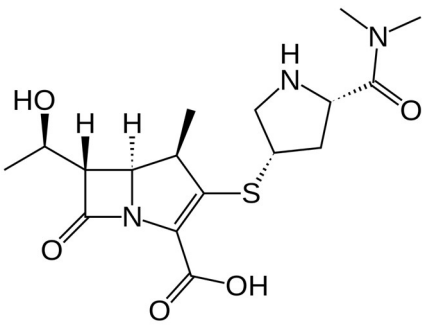




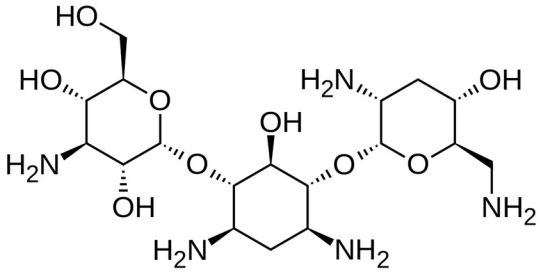
Cefixime (JaGa, 2008)



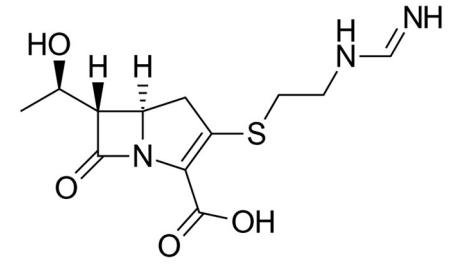
Lomefloxacin (Fvasconcellos, 2006)



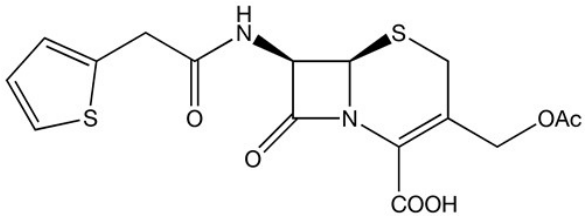
Meropenem (Fvasconcellos, 2006)



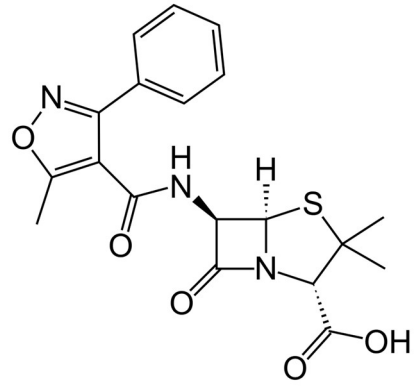
Tobramycin (Fvasconcellos, 2008)



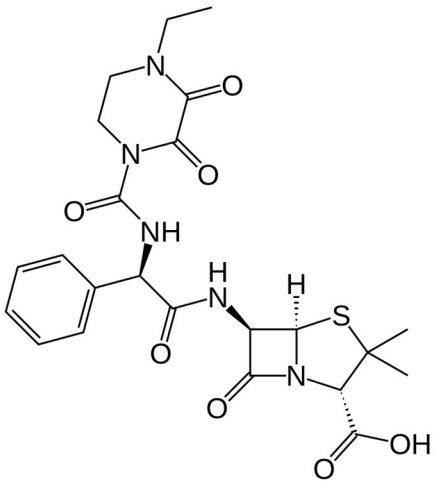
Imipenem (Fvasconcellos, 2007)



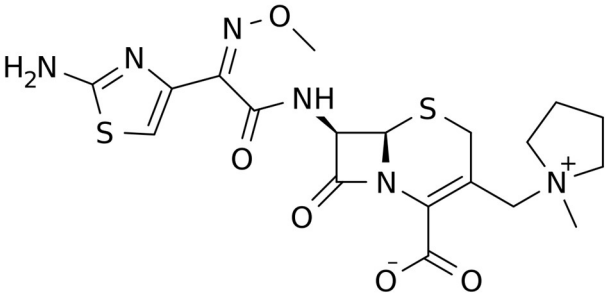
Cefalotin (GNU, 2008)



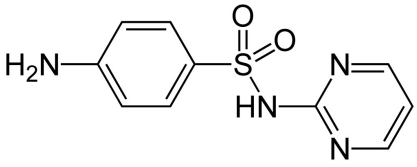
Oxacillin (Fvasconcellos, 2007)



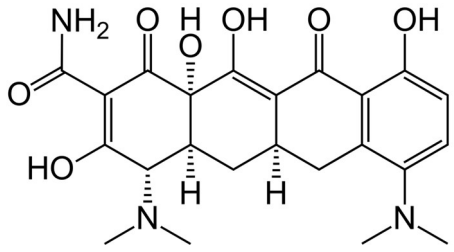
Piperacillin (Fvasconcellos, 2008)



Cefepime (Fuse809, 2014)



Sulfadiazine (public domain)



Minocycline (Fvasconcellos, 2011)

*Appendix C: Tukey's HSD on HPC Samplers Summer Sampling. *Bold indicates significant difference*

Difference of Sample Levels	P-Value
7/6/2020 Bld 14 - 7/21/2020 Bld 14	**0**
7/6/2020 Bld 76 - 7/21/2020 Bld 14	**0**
7/6/2020 Bld 14 - 7/21/2020 Bld 76	**0**
7/6/2020 Bld 76 - 7/21/2020 Bld 76	**0**
8/25/2020 Bld 14 - 7/6/2020 Bld 14	**0**
8/25/2020 Bld 76 - 7/6/2020 Bld 14	**0**
8/3/2020 Bld 14 - 7/6/2020 Bld 14	**0**
8/3/2020 Bld 76 - 7/6/2020 Bld 14	**0**
9/22/2020 Bld 14 - 7/6/2020 Bld 14	**0**
9/22/2020 Bld 76 - 7/6/2020 Bld 14	**0**
9/8/2020 Bld 14 - 7/6/2020 Bld 14	**0**
9/8/2020 Bld 76 - 7/6/2020 Bld 14	**0**
8/25/2020 Bld 14 - 7/6/2020 Bld 76	**0**
8/25/2020 Bld 76 - 7/6/2020 Bld 76	**0**
8/3/2020 Bld 14 - 7/6/2020 Bld 76	**0**
8/3/2020 Bld 76 - 7/6/2020 Bld 76	**0**
9/22/2020 Bld 14 - 7/6/2020 Bld 76	**0**
9/22/2020 Bld 76 - 7/6/2020 Bld 76	**0**
9/8/2020 Bld 14 - 7/6/2020 Bld 76	**0**
9/8/2020 Bld 76 - 7/6/2020 Bld 76	**0**
8/3/2020 Bld 76 - 7/21/2020 Bld 76	**0.002**
8/25/2020 Bld 76 - 7/21/2020 Bld 76	**0.011**
9/22/2020 Bld 76 - 7/21/2020 Bld 76	**0.018**
9/8/2020 Bld 14 - 7/21/2020 Bld 76	**0.018**
8/25/2020 Bld 14 - 7/21/2020 Bld 76	**0.049**
7/21/2020 Bld 76 - 7/21/2020 Bld 14	0.091

Appendix C Continued

Difference of Sample Levels	P-Value
9/22/2020 Bld 14 - 8/3/2020 Bld 76	0.096
9/8/2020 Bld 76 - 7/21/2020 Bld 76	0.109
8/3/2020 Bld 14 - 7/21/2020 Bld 76	0.22
9/22/2020 Bld 14 - 8/25/2020 Bld 76	0.316
9/22/2020 Bld 76 - 9/22/2020 Bld 14	0.402
9/8/2020 Bld 14 - 9/22/2020 Bld 14	0.404
9/22/2020 Bld 14 - 8/25/2020 Bld 14	0.639
9/22/2020 Bld 14 - 7/21/2020 Bld 14	0.789
9/8/2020 Bld 76 - 9/22/2020 Bld 14	0.829
8/3/2020 Bld 76 - 8/3/2020 Bld 14	0.889
9/22/2020 Bld 14 - 8/3/2020 Bld 14	0.947
9/8/2020 Bld 76 - 8/3/2020 Bld 76	0.972
9/22/2020 Bld 14 - 7/21/2020 Bld 76	0.979
8/3/2020 Bld 76 - 7/21/2020 Bld 14	0.982
8/3/2020 Bld 14 - 8/25/2020 Bld 76	0.994
8/3/2020 Bld 76 - 8/25/2020 Bld 14	0.997
9/22/2020 Bld 76 - 8/3/2020 Bld 14	0.998
9/8/2020 Bld 14 - 8/3/2020 Bld 14	0.998
8/25/2020 Bld 14 - 7/21/2020 Bld 14	1
8/25/2020 Bld 76 - 7/21/2020 Bld 14	1
8/3/2020 Bld 14 - 7/21/2020 Bld 14	1
9/22/2020 Bld 76 - 7/21/2020 Bld 14	1
9/8/2020 Bld 14 - 7/21/2020 Bld 14	1
9/8/2020 Bld 76 - 7/21/2020 Bld 14	1
7/6/2020 Bld 76 - 7/6/2020 Bld 14	1
8/25/2020 Bld 76 - 8/25/2020 Bld 14	1
8/3/2020 Bld 14 - 8/25/2020 Bld 14	1
9/22/2020 Bld 76 - 8/25/2020 Bld 14	1

Appendix C Continued

Difference of Sample Levels	P-Value
9/8/2020 Bld 14 - 8/25/2020 Bld 14	1
9/8/2020 Bld 76 - 8/25/2020 Bld 14	1
8/3/2020 Bld 76 - 8/25/2020 Bld 76	1
9/22/2020 Bld 76 - 8/25/2020 Bld 76	1
9/8/2020 Bld 14 - 8/25/2020 Bld 76	1
9/8/2020 Bld 76 - 8/25/2020 Bld 76	1
9/8/2020 Bld 76 - 8/3/2020 Bld 14	1
9/22/2020 Bld 76 - 8/3/2020 Bld 76	1
9/8/2020 Bld 14 - 8/3/2020 Bld 76	1
9/8/2020 Bld 14 - 9/22/2020 Bld 76	1
9/8/2020 Bld 76 - 9/22/2020 Bld 76	1
9/8/2020 Bld 76 - 9/8/2020 Bld 14	1

Appendix D: Bacteria Physical Descriptions

Designation	Description
3W	Beige/white translucent colony color, entire margin, 3 mm
A	White colony color, undulating edge with rings, flat with a raised center, 10 mm
AP	Aggregates of yellow translucent pinhead-like colonies that resulted in an appearance of a dark yellow center and lighter edges in a gradient, 5 mm
B	White colony color, raised edge with sunken middle, 2 mm
BB	Blue/white colony color, irregular margin, 3mm
BBW	Beige colony color, flat, entire, 3-5mm
BC	White colony color, circular edge, ringed, outer ring has a gradient to the edge, center solid with irregular bumps and dotted texture
BW	flat, white, 3-5 mm
BY	Big yellow colonies, entire, convex, 5 mm
C	White undulating bullseye, white gradient, dips into agar, 10 mm
CB	Beige, convex, 1 mm, opaque
CW	White, convex, entire, 1-4 mm
CY	Light yellow, highly convex with a height of 1 mm, entire margin, 1-10 mm
DLY	Light yellow, entire, convex, divot in center, like dy but lighter and without lines, 4 mm
DW	Opaque white irregular edge, wrinkly texture that grows upward, 2-3 mm up to 11 mm

Appendix D Continued

DY	Yellow, slight indent at center, entire, lines in the center that resemble an iris, 4mm
EGG	Circular white center with translucent outer edge that undulates, looks like a fried egg
F	White frosty colored, flat, 4-10 mm
FB	Murky white, flat, undulating, bullseye, flat, edge dry looking, 15 mm
FW	White, flat, 2 mm, entire, so flat looks level with the agar ring around edge
FY	Yellow, irregular edge, flat, uneven texture (like fruit leather), radial gradient
G	Greenish (yellow/blue), lowly convex, entire 1-4 mm
GB	Translucent green with a brown like inside, undulating edge
GK	White translucent, irregular edge, textured with a bumpy appearance like after pulling off a sticker with a gradient edge, middle is sunken edge is raised, irregular edge that is mostly entire with some undulations, 10-20 mm
H	Yellow, transparent/clear edge, yellow center and predominant color, can grow in/on other colonies, 1-2 mm
IB	Beige, flat top, raised, entire edge, iridescent lines on top, 8 mm
IG	iridescent green
IRB	Beige, irregular edge, flat uneven texture, 7 mm
IW	White, irregular edge, wrinkly, 4 mm
IWA	White color, translucent light white irregular edge boundary, then a solid white irregular ring around the edge, and a wormy/ brain like texture center, 20 mm

Appendix D Continued

IY	Yellow beige, convex mostly entire, with some undulating and some iridescence in the center
J	Transparent/clear, elevated, bumps like warts irregularly placed on top, 5 mm
K	Beige, translucent, flat, textured with a bumpy appearance like after pulling off a sticker, irregular edge that is mostly entire with some undulations, 10-20 mm
L	White/beige, opaque, circular, fuzzy edge, ring pattern in center, 30 mm
LO	Opaque light orange, flat, 5 mm
LW	White, entire, raised 1 mm above agar, slight indent in center, slightly wrinkled texture on surface, 4 mm
LY	Light yellow, raised, 1-5 mm
LYB	Yellow, raised ~1 mm, divot in center, 2 mm
M	White, with a white opaque circular center, undulating textured edges in an oblong shape
N	Orange, flat, dry appearance, raise lip around edge, 12 mm
O	Orange, convex, <1 mm
P	Pinhead, translucent, <1 mm
PO	Pinhead orange, entire, <1 mm
PR	Pinhead red, entire, <1 mm
PY	Yellow pinhead, entire, <1 mm
R	Red, undulating, 5 mm

Appendix D Continued

RC	Red, entire, flat, 1.5 mm
RK	Beige, translucent, flat, textured with a bumpy appearance like after pulling off a sticker, smooth textured undulating ring around the edge, 10-20 mm
RT	Red, translucent, entire, 1 mm
RU	White colony, undulating center with an undulating ring around it, flat, 13 mm
RW	White opaque, raised but with a flat top, entire, 3 mm
RWA	White colony with a wormy/brain texture and elevated ring around inner and undulates, 8 mm
RY	Ringed yellow/white, raised, convex, entire, 2-5 mm
T	Transparent, flat, 1 mm
TB	Translucent yellow/brown, irregular edge, 3 mm, flat
TP	Translucent pink, entire, 1 mm
TU	Transparent beige color, undulating edge, flat, textured like the “Sally Hansen Fuzzy Coat Textured Nail Color” with short rods in the colony, 40mm
TW	Transparent, wrinkly, irregular edge, 3 mm
TY	Translucent yellow, uneven circular edge
U	Beige, undulating, 32 mm
UB	White, undulating with a bullseye, raised, 10 mm
UF	White frosted, undulating, 6 mm

Appendix D Continued

UK	Translucent white/beige, flat undulating edge, textured with a bumpy appearance like after pulling off a sticker, smooth textured undulating ring around the edge, 24 mm
UT	Translucent, white middle, undulating edge
UW	White, undulating, flat, 8 mm
UY	Yellow, raised, irregular edge almost circular
VY	Yellow, highly convex, entire, 2mm
W	White, opaque, 1 mm
WA	White, circular (not entire but almost) wiggly wormy/ brain like texture, flat, 11mm
WB	Beige, wrinkly, entire, bullseye rings colony formation
WC	White, wrinkly/ undulating edge
WP	Light pink color, ring texture, 2 mm
WW	White/opaque beige outer, solid white fuzzy inner, solid grey fuzzy center, convex, 1 mm
WY	White-yellow, raised with a flat top, 1-4 mm
Y	Yellow, opaque, raised, 1mm
YB	Yellow, entire with elevated rings (raised edge with sunken middle)
YL	Light yellow, translucent, lowly convex, entire, 4 mm

Appendix E: Gram Stain Results of Isolated Bacteria from Cooling Tower Samples

Designation	Gram Stain	Bacteria Shape	Notes
3W	Negative	bacillus	
A	Negative	bacillus	in spindly rows
AP	Negative	coccus	
B	Positive	bacillus	small and clustered
BB	Negative	bacillus	
BBW	Negative	bacillus	
BC	Positive	bacillus	
BW	Positive	coccus	
BY	Negative	bacillus	
C	Negative	bacillus	
CB	Negative	bacillus	
CW	Positive	coccus	
CY	Negative	bacillus	in clumps
DLY	Negative	coccus	
DW	Negative	coccus	
DY	Negative	bacillus	
EGG	Positive	coccus	in strings
F	Positive	bacillus	
FB	Positive	bacillus	in lines
FW	Positive	bacillus	
FY	Negative	bacillus	
G	Negative	coccus	

Appendix E Continued

GB	Unable to isolate to gram stain		
GK	Positive	bacillus	in lines/ fibrous
H	Negative	bacillus	
IB	Positive	bacillus	
IG	Negative	bacillus	
IRB	Negative	bacillus	
IW	Positive	bacillus	
IWA	Positive	bacillus	
IY	Positive	bacillus	
J	Positive	coccus	
K	Positive	coccus	
L	Positive	bacillus	
LO	Positive	coccus	
LW	Positive	coccus	
LY	Negative	bacillus	small bacillus
LYB	Negative	bacillus	
M	Negative	coccus	
N	Unable to isolate to gram stain		
O	Negative	bacillus	
P	Negative	coccus	
PO	Unable to isolate to gram stain		
PR	Unable to isolate to gram stain		
PY	Negative	bacillus	
R	Negative	coccus	

Appendix E Continued

RC	Negative	coccus	
RK	Positive	bacillus	
RT	Negative	coccus	in clusters
RU	Negative	coccus	
RW	Positive	coccus	
RWA	Negative	bacillus	
RY	Negative	bacillus	
T	Negative	bacillus	
TB	Positive	coccus	
TP	Unable to isolate to gram stain		
TU	Negative	coccus	
TW	Negative	bacillus	
TY	Negative	bacillus	
U	Positive	bacillus	
UB	Positive	coccus	diplococci
UF	Negative	bacillus	
UK	Positive	bacillus	
UT	Negative	bacillus	
UW	Positive	coccus	
UY	Negative	bacillus	
VY	Negative	bacillus	
W	Negative	coccus	
WA	Positive	coccus	
WB	Negative	bacillus	

Appendix E Continued

WC	Positive	coccus	
WP	Positive	bacillus	
WW	Positive	coccus	
WY	Negative	bacillus	
Y	Negative	bacillus	
YB	Negative	bacillus	
YL	Negative	coccus	

Appendix F: Gram Stains and Bacteria Morphology of Bacteria Sequenced for their 16S rRNA Gene

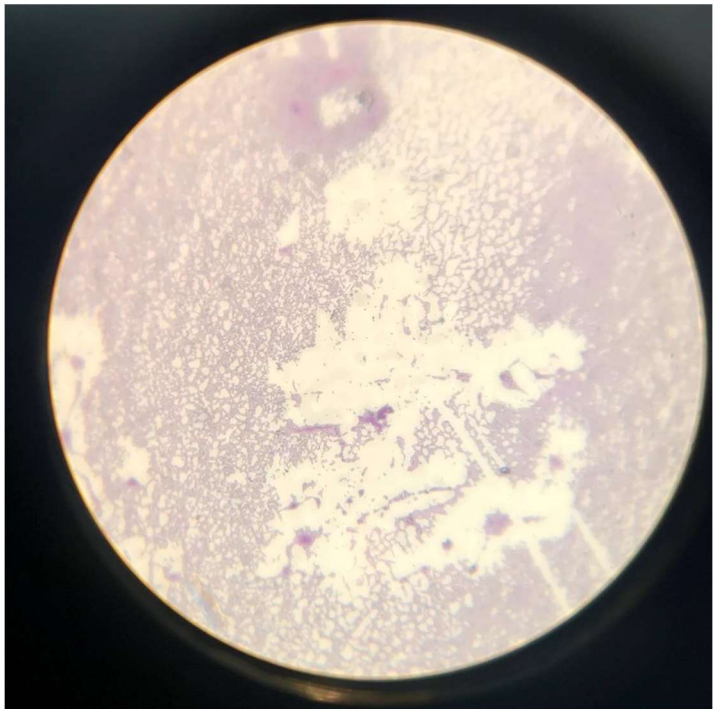


Figure S- 1: Gram stain of BW, *Morganella morganii*

Figure S- 2: Morphology of BW, *Morganella morganii*

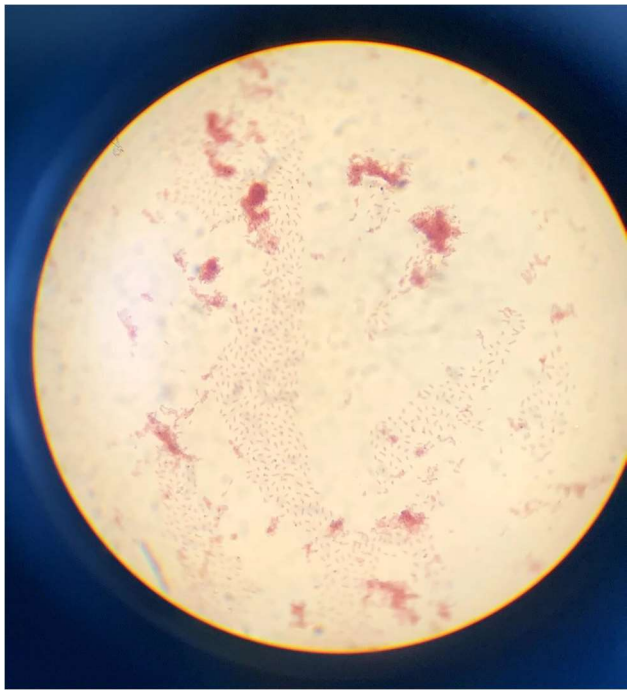


Figure S- 3: Gram stain of BY, *Chryseobacterium ureilyticum*

Figure S- 4: Morphology of BY, *Chryseobacterium ureilyticum*

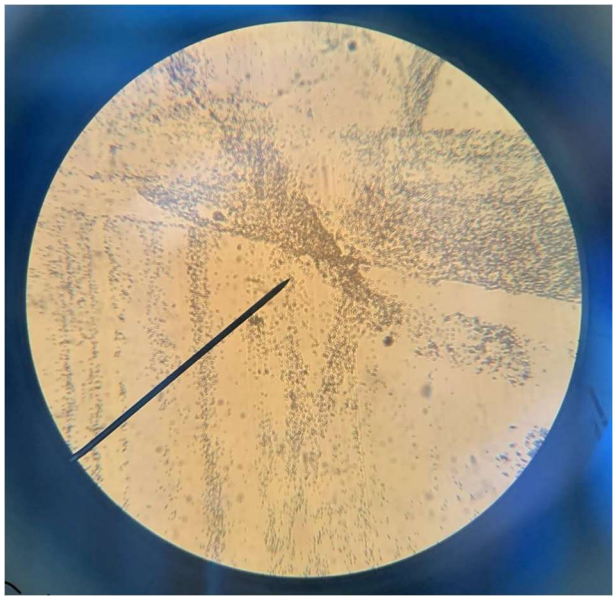


Figure S- 5: Gram stain of CW, *Phenylobacterium* sp.

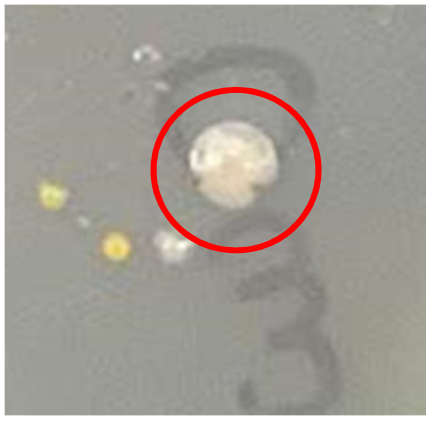


Figure S- 6: Morphology of CW, *Phenylobacterium* sp.

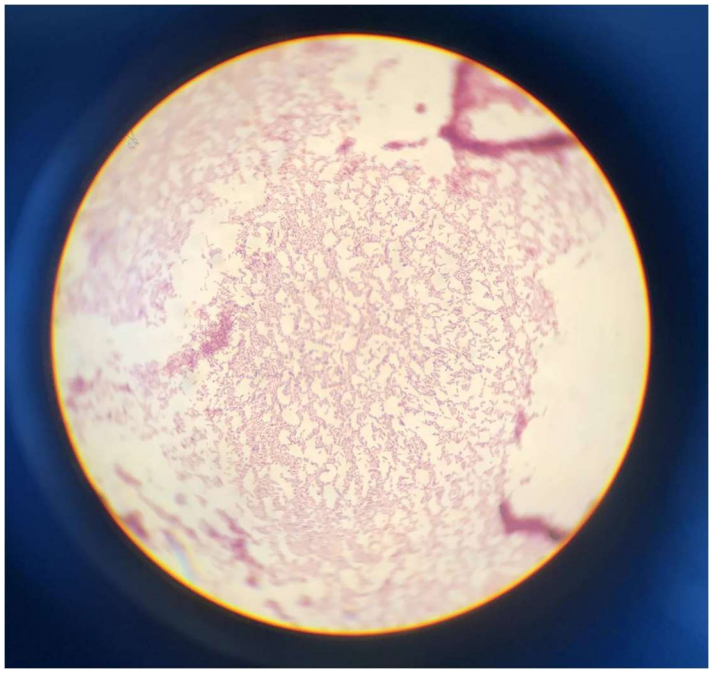


Figure S- 7: Gram stain of DY, *Chryseobacterium cucumeris*

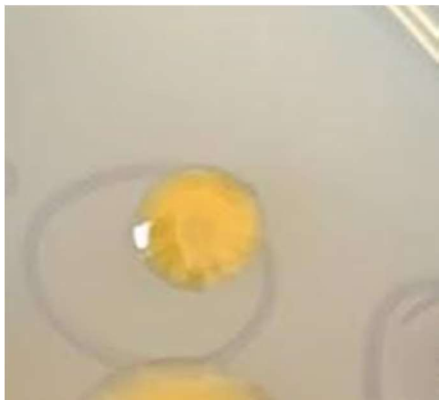


Figure S- 8: Morphology of DY, *Chryseobacterium cucumeris*

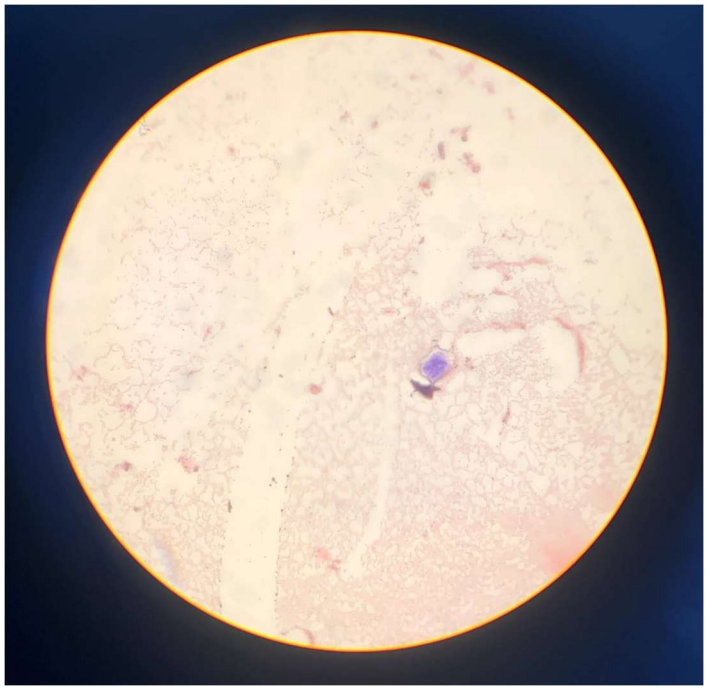


Figure S- 9: Gram stain of RY *Stenotrophomonas maltophilia*

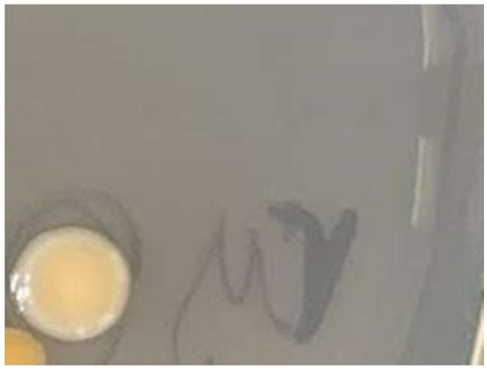


Figure S- 10: Morphology of RY *Stenotrophomonas maltophilia*

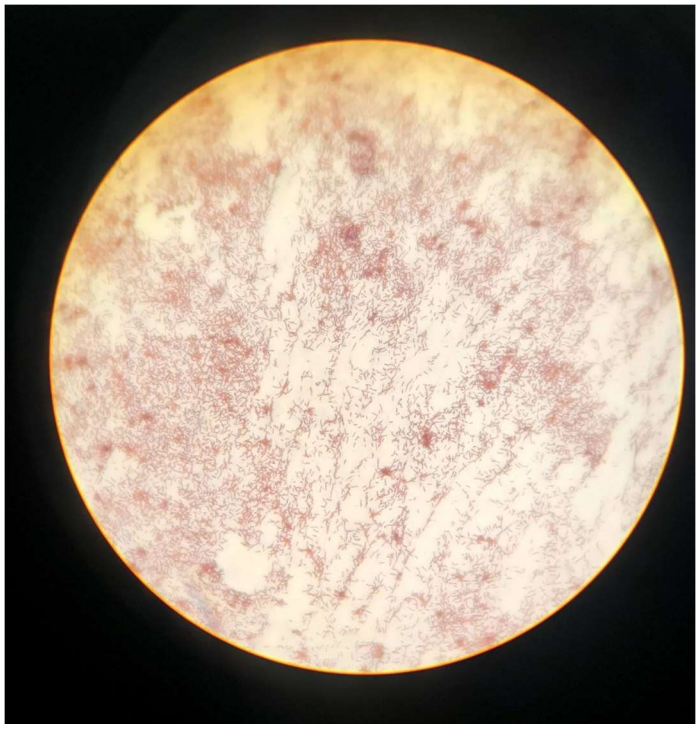


Figure S- 11: Gram stain of T, *Bacillus licheniformis*

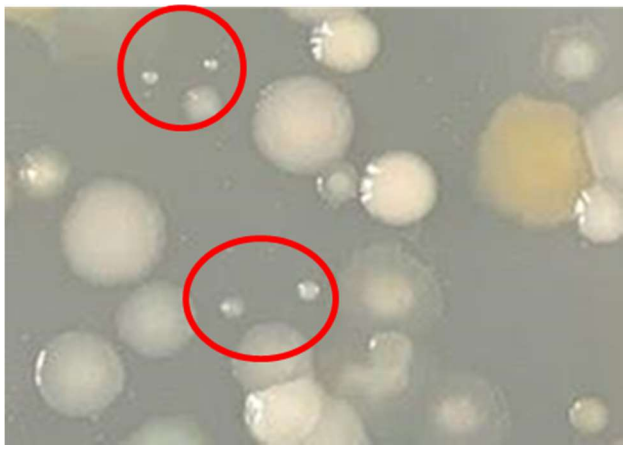


Figure S- 12: Morphology of T, *Bacillus licheniformis*

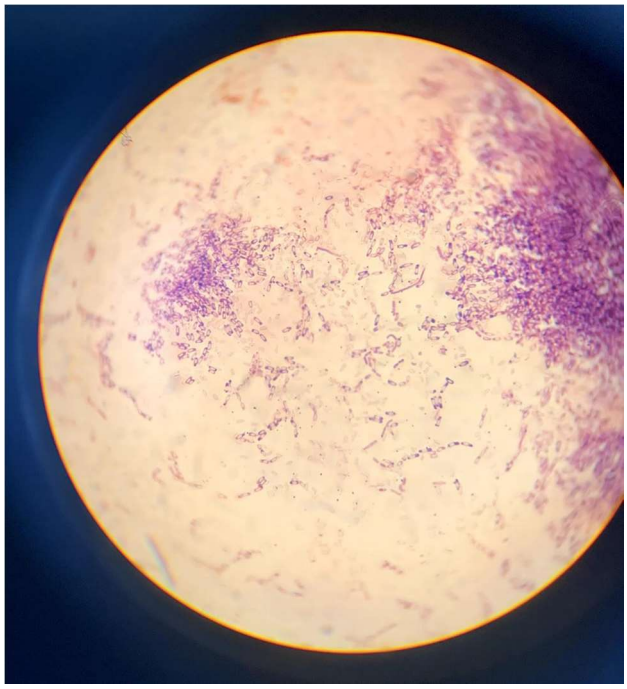


Figure S- 13: Gram stain of UK, *Bacillus cereus*

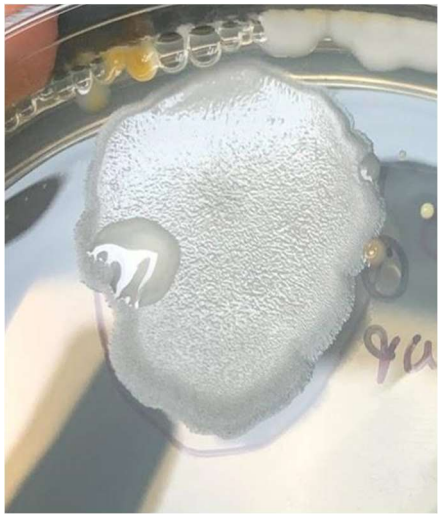


Figure S- 14: Morphology of UK, *Bacillus cereus*

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