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MICROBIAL CONTAMINATION OF ICE AT FOOD ESTABLISHMENTS IN LAS VEGAS, NEVADA

By

Jerry Allen Wills

Bachelor of Science - Neuroscience
University of Nevada, Reno
2012

A thesis submitted in partial fulfillment
of the requirements for the

Master of Public Health

Department of Environmental and Occupational Health
School of Community Health Sciences
Division of Health Sciences
The Graduate College

University of Nevada, Las Vegas
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Thesis Approval

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The University of Nevada, Las Vegas

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Microbial Contamination of Ice at Food Establishments in Las Vegas, Nevada

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ABSTRACT

Microbial contamination of ice at food establishments in Las Vegas, Nevada

By

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When food establishments make ice for consumption, it is important to use water free of pathogens and to employ hygienic practices. Freezing can reduce the number of culturable microorganisms present in water but does not eliminate them completely. Coliform bacteria are used as an indicator of water quality and are used to test for fecal contamination. The U.S. Environmental Protection Agency (EPA) establishes standards for drinking water, and has set a goal for zero total coliforms. Another commonly used tool to measure water quality is the heterotrophic plate count (HPC). The EPA limit for HPC is 500 colony forming units (CFU) per milliliter. The objective of this study was to determine the microbial contamination of ice at food establishments in Las Vegas, NV. Ice and water samples were collected from local food establishments and analyzed using EPA standard methods. A convenience sample was collected from 14 food establishments near the main campus of the University of Nevada, Las Vegas. Samples were processed and analyzed for the presence of *E. coli*, total coliforms, and HPC using culture analysis. A molecular method, Polymerase Chain Reaction (PCR) was also used to test

for *E. coli*. A total of 19 of the 28 (67.9%) samples collected exceeded the EPA limit for HPC. Coliform and *E. coli* analysis showed 10 of the 14 ice samples (71.4%) contained coliforms, and two out of 14 (14.3%) ice samples also contained *E. coli*. The current methods for inspecting ice at food establishments are insufficient for determining microbial contamination. Contaminated ice can contain pathogens and is a public health concern. Further research should be conducted to reduce the public health risk of ice and drinking water contamination in food establishments.

ACKNOWLEDGEMENTS

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DEDICATION

I would like to dedicate this thesis to the memory of my dear friend Harvi Singh. His courage was an inspiration as he never shied away from a challenge. His laughter often filled the room and his altruistic behavior inspired me. You are a large reason I am on my current path. I am so thankful to have had you in my life. You are missed.

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CHAPTER 1

BACKGROUND

Water is an essential element for biological life. The human body is made up of 60% water (United States Geological Survey (USGS), 2015) making it the most prevalent compound in the body. Commonly, adult humans require between 2.2 to 3 liters of water every day to sustain healthy physiological function. Water serves many functions for living organisms including: acting as a building material for cells, regulating our internal body temperature, transporting macronutrients throughout the body by way of the bloodstream, metabolic and nutrient waste excretion, helping the brain and spinal cord absorb shock, forming saliva, and lubricating joints (USGS, 2015). These biological functions are only made possible due to the unique chemistry of water molecules and its interactions with other compounds.

Water molecules create hydrogen bonds with each other, and this contributes to its various characteristics unique to this molecule. Hydrogen bonds form an organized lattice network which is responsible for ice having less density than water and allowing the solid form to float and act as an insulator. Without the ability of ice to float and act as an insulator, life would not be possible. If oceans and lakes froze from the bottom up, the wide range of life present in oceans would not exist, as lakes and oceans could freeze solid shortening the water supply for consumption and environmental stability. Ice can also be used to slow the growth and reproduction of microorganisms which can reduce food spoilage by reducing the kinetic energy of the organism slowing metabolic and growth processes.

When the advantage of storing food on ice to prevent food spoilage was realized, people began to harvest ice from frozen lakes and rivers to sell it around the world. Before the 1850s

natural ice was the only option to prevent food spoilage by refrigeration. The ice stock was dependent on the season and location, and there were many events that could lead to ice shortages. Ice was traditionally more available above the 40th parallel in North America and scarce below the 30th dictating primary diets of certain cultures. The first commercial ice making machine was invented in 1854 (Reif-Acherman, 2012) allowing the preservation of food to be more readily available and aiding in ease of transportation of food stocks that were susceptible to spoilage. Ice is more commonly used to cool drinks, preserve foods, and regulate temperature, but it can also be used medically to reduced swelling and pain from injuries.

It is difficult to track how much ice is consumed in the United States. Ice machines are abundant in homes, businesses, and food establishments. According to the Food and Drug Administration (2010) the average American consumes four bags of ice each year. Ice can come in different shapes such as block, cubed, crushed, and shaved. Due to the large amount of ice that Americans consume each year, it is of the upmost importance that ice is clean and safe for ingestion. Equipment that comes into contact with ice should be clean to avoid ice contamination and the spreading of communicable diseases.

Disease-causing microorganisms can be transmitted through drinking water. There are an estimated 502,000 deaths worldwide each year due to contaminated drinking water (World Health Organization, 2014). Ice made from contaminated water will also contain disease-causing microorganisms. It is important for public health that ice is made from uncontaminated water to prevent the spread of disease.

CHAPTER 2

INTRODUCTION

The ability of ice to be a vehicle for the transmission of pathogenic microorganisms has been recognized for some time (Dickens, DuPont & Johnson, 1985; Stout, Yu, & Muraca, 1985). When food establishments make ice for consumption, it is important to use water free of pathogens and to employ hygienic practices. Ice may become contaminated through the use of contaminated water, by food service staff, by customers, and due to environmental factors within ice machines.

Freezing can reduce the number of culturable microorganisms present in water but does not eliminate them completely. A study that placed contaminated ice in beverages found that, even with high alcohol content, not all pathogens were eliminated (Dickens et al., 1985). In the late 1990s, after an outbreak on three cruise ships affected more than 1,300 individuals, an epidemiological investigation was launched. Isolates of enterotoxigenic *Escherichia coli* (ETEC) were recovered from stool samples. The investigation identified contaminated water used to make ice as the vehicle for ETEC transmission (Daniels et al., 2000).

There have been several well-known case reports that demonstrate contaminated ice machines spread disease. Ice from a poorly maintained machine at a Louisiana correctional facility is suspected for causing a *Francisella novicida* outbreak among inmates (Brett et al., 2014). In this outbreak, three immunocompromised inmates were infected with the pathogen and one died after being admitted to the hospital. Several epidemiological investigations in hospitals have implicated ice machines as the most likely cause for the spread of *Legionella* (Bencini et al., 2005; Graman, Quinlan, & Rank, 1997; Bangsborg, Uldum, Jensen, & Brunn,

1995). An outbreak of cryptosporidiosis in a hospital in Denmark that affected immunocompromised patients and caused several deaths was attributed to prolonged diarrhea traced to an ice machine (Ravn et al., 1991).

The total impact of waterborne disease outbreaks due to contaminated ice is difficult to estimate. The Centers for Disease Control and Prevention (CDC) reported 33 outbreaks attributed to contaminated water in the United States from 2009-2010, resulting in 1,040 illnesses, 85 hospitalizations, and 9 deaths (CDC, 2013). Two conditions are needed to define a waterborne disease outbreak; two or more individuals needed to be exposed to the same water source, at the same location, and at approximately the same time, and epidemiological evidence must implicate water as the probable source (CDC, 2013). Due to limitations of the reporting system and challenges associated with identifying waterborne illness, the total number of waterborne illness episodes each year is unknown.

Colford et al. (2006) estimated that acute gastrointestinal illness (AGI) caused by drinking water affects 4.26 – 11.69 million individuals annually. As part of the Safe Drinking Water Act (SDWA) amendment of 1996, the Environmental Protection Agency (EPA) attempted to determine the number of AGI cases caused by drinking water and created a model which estimated 16.4 million cases per year (Messner et al., 2006). Due to the challenges associated with indicating water as vehicle for AGI, it is even more difficult to determine the burden of AGI due to ice.

The United States has high quality standards for drinking water. Since the passage of the SDWA by the U.S. Congress in 1974, cases of waterborne illness have decreased dramatically (EPA, 2014). The SDWA gives the EPA the authority to set limits on naturally occurring

contamination and manmade chemicals. The EPA works with local water authorities to ensure these levels are met for public safety. The EPA standards for drinking water include a list of Maximum Contaminant Levels (MCL) for specific impurities and instructions on how to remove contaminants. Coliform bacteria are often used as an indicator of water contamination and are used to test for fecal contamination (Falcão, J., Dias, Correa, & Falcão D., 2002). Total coliforms are groups of closely related bacteria and include fecal coliform bacteria, which are normally found in the intestines of warm-blooded animals. The presence of fecal coliforms indicates that human or animal fecal material, and pathogens associated with fecal material, are present. Due to the variety of bacteria, parasites, and viruses that can cause disease when ingested, coliforms are used as an indicator of sanitary quality. Testing for each pathogen individually is too expensive and impractical.

Coliforms present in ambient water may be stressed by environmental factors, such as lack of nutrients or chlorination of water. The susceptibility of coliforms to water treatment makes them a useful indicator of water contamination (Cabral, 2010). In 1990, the Total Coliform Rule (TCR) became effective and established goals and legal limits for the number of samples of drinking water that could test positive for coliforms (EPA, 2013). The TCR applies to all public water systems (PWS) and is designed to protect the public's drinking water supply. The MCL goal for total coliforms is zero, because even with low levels of coliforms waterborne disease outbreaks have occurred (EPA, 2013). PWS that serve less than 1,000 people are required to test once a month and smaller systems test even less frequently. Systems that serve 50,000 customers must test at least 60 times per month and those with 2.5 million customers must test at least 420 times per month. An MCL violation is triggered if a PWS that tests less than 40 times per month has two or more samples test positive for coliforms. PWS that test more

than 40 samples per month will receive a violation if more than 5% of samples test positive for coliforms. Violations require additional water treatment to remove contaminants. All samples that test positive for total coliforms are also analyzed for fecal coliforms or *E. coli*.

Another commonly used tool to measure water quality is the heterotrophic plate count (HPC). Heterotrophs are a broad group of microorganisms that include bacteria, yeasts, and molds. The defining trait of the group is that they require organic carbon for growth. There are numerous tests that can be utilized to culture these microorganisms from water samples. The microorganisms cultured from HPC can vary greatly between locations, seasons, and consecutive samples at the same location (Chowdhury, 2012). Although concentrations of heterotrophic bacteria may not have a direct health effect, they can be used as an analytical tool to determine the amount of bacterial contamination in the water, and lower concentrations of bacteria may indicate a well maintained system (EPA, 2014). The EPA limit for HPC is 500 colony forming units (CFU) per milliliter. High concentrations of heterotrophs can also interfere with the detection of pathogens (Allen, Edberg, & Reasoner, 2004).

Conventional methods of culturing microorganisms to determine water contamination have limitations. Current culture methods are time consuming and may underestimate bacterial contamination. Damaged microorganisms may be viable but nonculturable, causing samples to falsely appear negative (Agudelo et al., 2010). Molecular biological methods, such as polymerase chain reaction (PCR), which can detect the DNA of target microorganisms, can be used to supplement detection of microorganisms by culture. PCR is a molecular biological technique that amplifies DNA sequences. The target nucleic acid sequences are specific to the organism of interest. Short DNA molecules known as primers attach to the matching target DNA segment. The primers and a heat stable polymerase are used to make copies of the nucleic

acid sequence. In real-time PCR, DNA amplification of the target sequence is detected using a fluorescent probe. The PCR assay is very sensitive for detecting microorganisms in environmental samples, but cannot distinguish between living and dead cells.

The Southern Nevada Water Authority (SNWA) is responsible for water quality in Clark County, which includes the cities of Las Vegas, North Las Vegas, and Henderson. In 2014, approximately 36,000 samples were collected throughout the region and 327,000 analyses were performed on drinking water (SNWA, 2015). Continuous monitoring and treatment of regional water ensures the quality of drinking water in Las Vegas meets the highest standards to avoid AGI. Therefore, tap water used to make ice should be of high quality.

Contamination may occur at any point of use within a water or ice dispensing system and can taint water products, causing water to become a vehicle for the transmission of pathogens. The formation of biofilms can cause contamination in ice machines. Biofilms, also known as slime, are a complex community of microorganisms growing together in a matrix of polysaccharides (Vert et al., 2014). Biofilm formation can occur in water lines. Bacterial cells from biofilms may be released as they come into contact with the water, causing the water to become contaminated. Biofilms protect bacteria within the matrix, which allows them to survive in hostile environments. This protective function helps bacteria have a reduced susceptibility to antibiotics, dehydration, phagocytosis, acid exposure, metal toxicity, and biocides (Otter et al., 2015). Biofilms can show reduced sensitivity to antibiotics and other disinfectants, which makes their physical removal challenging. This resilience may be due to the inability of molecules to penetrate the matrix (Mah, & O'Toole, 2001). Once these microorganisms come into contact with food equipment and biofilm formation begins, there is an increased potential for contamination of the food and heightened risk of foodborne illness.

Poor hygiene practices can lead to ice contamination. Contamination occurs through human and environmental factors. Human causes for contamination can arise through cross contamination, inadequate handwashing, storage and cooking temperatures, and contamination of food by animal waste. Dirt and dust from the environment can be responsible for environmental contamination. Proper sanitary behaviors include: hand washing with soap and water, keeping clothing clean and ensuring it does not come into contact with ice, using clean ice scoops, touching only scoop handles, ensuring ice scoops are not left in the ice container, and disposing of unused ice instead of returning it to the machine. In order to prevent contamination of food, equipment should be cleaned and sanitized often.

The Southern Nevada Health District (SNHD) is responsible for regulating the sanitation of food establishments. SNHD defines ice as a food; therefore, all regulations pertaining to food pertain to ice (SNHD, 2010). Regulations pertaining to utensils in Chapter 3 - 304.12 of the SNHD Regulations Governing the Sanitation of Food Establishments outline the proper handling of ice scoops to avoid contamination. Ice machines must be cleaned using a three step process, which includes washing with soapy water, rinsing with clean water, and using a sanitizer. The cleaning frequency of ice machines is not directly defined by SNHD and manufacturer's directions should be followed. SNHD restaurant inspectors currently perform a visual inspection of ice machines during restaurant inspections. Currently, there are no established guidelines for determining the quality of ice in food establishments. Determining microbial contamination of ice could lead to improved sanitation practices which could improve public health.

Objective

The objective of this study was to determine the microbial contamination of ice at food establishments in Las Vegas, NV. Ice and water samples were collected from local food establishments and samples were analyzed for *E. coli*, total coliforms, and heterotrophs using EPA standard methods. PCR was also used for the detection of *E. coli*.

Research Questions

1) Will the concentration of microorganisms cultured from ice samples exceed EPA drinking water standards for total heterotrophic bacteria?
2) Will the concentration of microorganisms cultured from ice samples exceed EPA drinking water standards for total coliform bacteria?
3) Will PCR produce comparable results to culture methods for detection of *E. coli*?
4) Will the contamination of ice be comparable to the contamination of drinking water from the same establishment?

Hypotheses

H^1_0: Heterotrophic bacterial concentrations present in water and ice samples are less than or equal to EPA standards for drinking water.

H^1_a: Heterotrophic bacterial concentrations present in water and ice samples are greater than EPA standards for drinking water.

H^2_0: Drinking water and ice from the same food establishment are of the same microbial quality as determined by coliform concentrations.

H^2_a: Drinking water and ice from the same food establishment are not of the same microbial quality as determined by coliform concentrations.

H^3_0: Drinking water and ice from the same food establishment are of the same microbial quality as determined by heterotroph concentrations.

H^3_a: Drinking water and ice from the same food establishment are not of the same microbial quality as determined by heterotroph concentrations.

CHAPTER 3

MATERIALS AND METHODS

Study Design

A convenience sample was collected from 14 food establishments near the main UNLV campus. Samples were taken from zip codes 89119 and 89169, and were within a 3 mile radius of UNLV. A single ice and water sample was taken from each location. A total of 28 samples, which included 14 ice and 14 water samples, were collected. Samples were processed and analyzed for the presence of *E. coli*, total coliforms, and HPC using culture analysis. Samples were also analyzed using PCR to assess the presence of *E. coli* that may be viable but nonculturable.

Sample Collection

EPA approved procedures were used to collect water and ice samples (Standard Methods for the Examination of Water and Wastewater [SMEWW] 9060, 1998) (Figure 1). Nalgene® 500 ml wide mouth bottles were used for sample collection. Bottles were sterilized directly under a UV light for 20 minutes. A minimum of 500 ml of ice and water were collected from each location. When samples were received from employees in a disposable drinking cup provided by the establishment, samples were transferred to sterile containers before transport. Samples were transported to the Emerging Diseases Laboratory at UNLV in a cooler with ice packs. Sample volume was measured using a sterile graduated cylinder and 1 µl of a 10% sodium thiosulfate solution per milliliter of sample was added to neutralize the chlorine found in tap water (EPA, 2002). Ice samples were thawed before sodium thiosulfate was added. All samples were stored at 4°C and processed within 24 hours of collection.

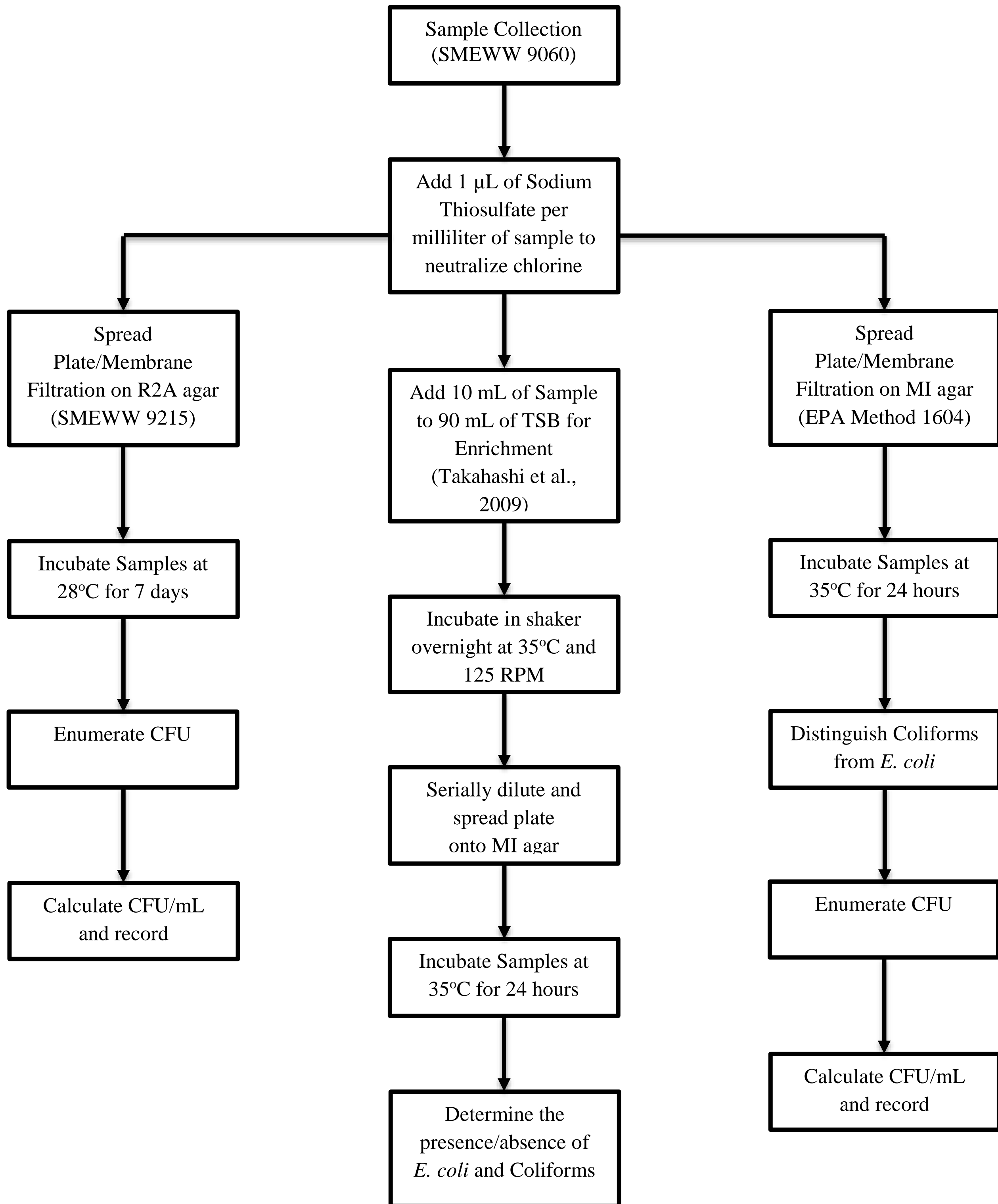

Figure 1. Flow Chart for ice and water sample collection and analysis on R2A and MI agar

Culture Analysis

EPA (2002) "Method 1604: Total Coliform and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)" was used for the detection and enumeration of total coliforms and *E. coli* in water and ice samples (Figure 1). MI agar (BD Laboratories, Sparks, MD) is selective and differential for total coliforms and *E. coli*. MI agar has a 95.7% specificity for *E. coli* and 93.1% specificity for total coliforms (EPA, 2002). This method has been shown to be precise and accurate by reducing the number of false positives and negatives when compared to previously approved EPA methods (Brenner, Rankin, Sivaganesan, & Scarping, 1996). Samples were inoculated in duplicate onto MI agar by spread plating or membrane filtration and incubated at 35°C for 24 hours. Colony forming units (CFU) on MI agar were confirmed as coliform growth by fluorescence under UV light. The presence of *E. coli* was determined by the appearance of blueish green colonies (Figure 2). MI agar plates were incubated at 35°C ± 2°C for 20-24 hours. The detection limit for culture of coliforms and *E. coli* was 1 CFU/100 ml of sample through membrane filtration.

Heterotrophic bacteria were cultured and analyzed using EPA approved methods (SMEWW 9215, 1998) (Figure 1). R2A agar (BD Laboratories, Sparks, MD) was inoculated to enumerate heterotrophic concentrations. R2A agar is recommended for culturing heterotrophs (Reasoner & Geldeich, 1985). The agar has low nutrient concentrations and, when incubated at lower temperatures for a longer time, bacteria stressed from chlorine treatment can be cultured. Samples were inoculated onto R2A agar by spread plating or membrane filtration and incubated at 28°C for 7 days. Replicate plates of each sample were analyzed and the number of heterotrophic bacterial colonies per milliliter of water was determined. The detection limit for culture of heterotrophs was 1 CFU/10 ml of sample.

Membrane filtration was used to concentrate samples for enumeration. Samples were concentrated using volumes of 100 ml, 10 ml, and 1 ml for *E. coli* and total coliforms. Samples were concentrated in 10 ml and 1 ml amounts for HPC. Serial dilutions and spread plating were also performed to facilitate enumeration. Dilutions of 1:10, 1:100, and 1:1000 ml were used for *E. coli,* total coliforms, and HPC. Spread plate methods were utilized to inoculate dilutions onto designated media. All plates were cultured in duplicate. After plates were incubated for the determined time, CFU were counted and averaged for each sample. A mean value from each sample was obtained and an average CFU/ml was calculated.

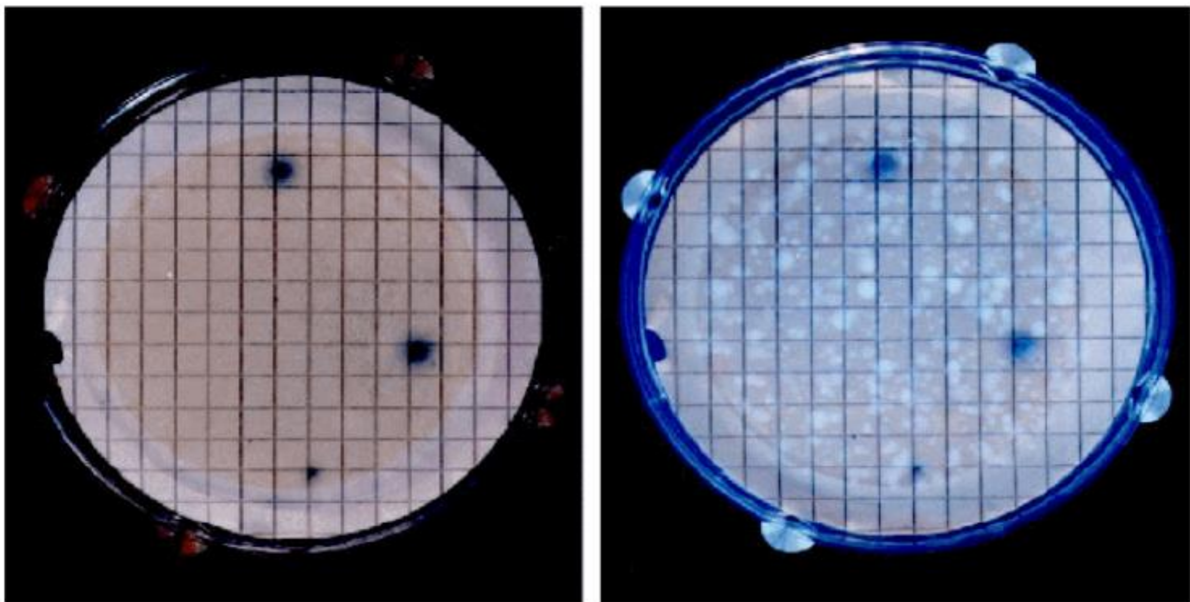

Figure 2. Photographs of *E. coli* and total coliforms on MI agar. Ambient light (left) and UV light (right). The blue colonies are *E. coli*. The coliforms are transparent and difficult to see under ambient light, but they fluoresce under UV light.

Sample Enrichment

An enrichment procedure was used to increase the ability to culture injured or stressed *E. coli* cells from samples (Figure 1). Ten milliliters of each sample was placed into 90 ml of trypticase soy broth (Takahashi et al., 2009) (BD Laboratories, Sparks, MD). The enrichment was placed into a sterile 250 ml Erlenmeyer flask and incubated at 35°C and 125 RPM overnight. Dilutions of 1:100 were made from the enrichment sample. MI agar was inoculated at dilutions of 1:10, 1:1000, and 1:100,000 ml and incubated at 35°C ± 2°C for 20-24 hours. The presence or absence of total coliforms and *E. coli* CFU was recorded.

DNA Extraction and Purification

A volume of 100 ml of each sample was concentrated by filtration onto a 0.45 µm HAWG filter membrane (EMD Millipore, Billerica, MA) using a sterile swinnex cartridge and 60 ml syringe. The filter was washed with 8 ml of 0.01M phosphate buffer with tween (pH 7.0) and aseptically removed from the swinnex filter holder and placed into a bead beater tube containing 50 mg of 425-600 µm and ≤ 106 µm diameter glass beads. DNA was extracted and concentrated with the Amicon DNA extraction and purification kit (EMD Millipore), following the manufacturer's protocol. A final volume of approximately 100 µl of DNA was obtained and stored at -70°C for future use.

Real-Time Polymerase Chain Reaction

All samples were analyzed by real-time PCR to determine the presence of *E. coli* DNA (Figure 3). DNA samples were analyzed in duplicate using the 7900 HT Fast PCR system (Applied Biosystems, Foster City, CA) and positive and negative controls were used for each amplification. The primers and probe target the *uidA* gene found in *E. coli*. The *uidA* gene

encodes for β-D-glucuronidase, which has only been detected in *E. coli* and *Shigella* (Takahashi et al., 2009). The published sequence for the forward primer was 5'GCAAGGTGCACGGGAATATT3' and the sequence for the reverse primer was 5'CAGGTGATCGGACGCGT3' (Takahashi et al., 2009). A TaqMan® probe was used with the sequence 6-FAM-5'CGCCACTGGCGGAAGCAACG3'-TAMRA. An internal positive control (IPC) (Applied Biosystems) was used to test each sample for inhibition. Samples showing inhibition were diluted to remove inhibitors and reanalyzed. Each PCR reaction had a total volume of 25 µl, which contained nuclease free water (Promega, Madison, WI), 1X of TaqMan® Universal Master Mix (Applied Biosystems), 0.4 µM of *E. coli* forward primer, 0.4 µM of *E. coli* reverse primer (Eurofins Operon, Huntsville, AL), 0.25 µM of the *E. coli* probe (Applied Biosystems), and 5 µl of template DNA. The 7900 HT Fast PCR system (Applied Biosystems) was operated in standard mode with the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Positive samples were reanalyzed under alternative cycling conditions to increase specificity of the *E. coli* primers and probe and verify results. Alternative cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds followed by 63°C for 1 minute. Amplification of the DNA was analyzed using SDS software version 2.3 (Applied Biosystems). The software analyzes fluorescence of the reporter dye attached to the 5' end of the TaqMan® probe to indicate the presence of the specific nucleic acid sequence. The detection limit for PCR was based on a sensitivity of 1 to 10 templates of *E. coli* DNA, and was between 0.2 and 2 *E. coli* cells per milliliter of sample.

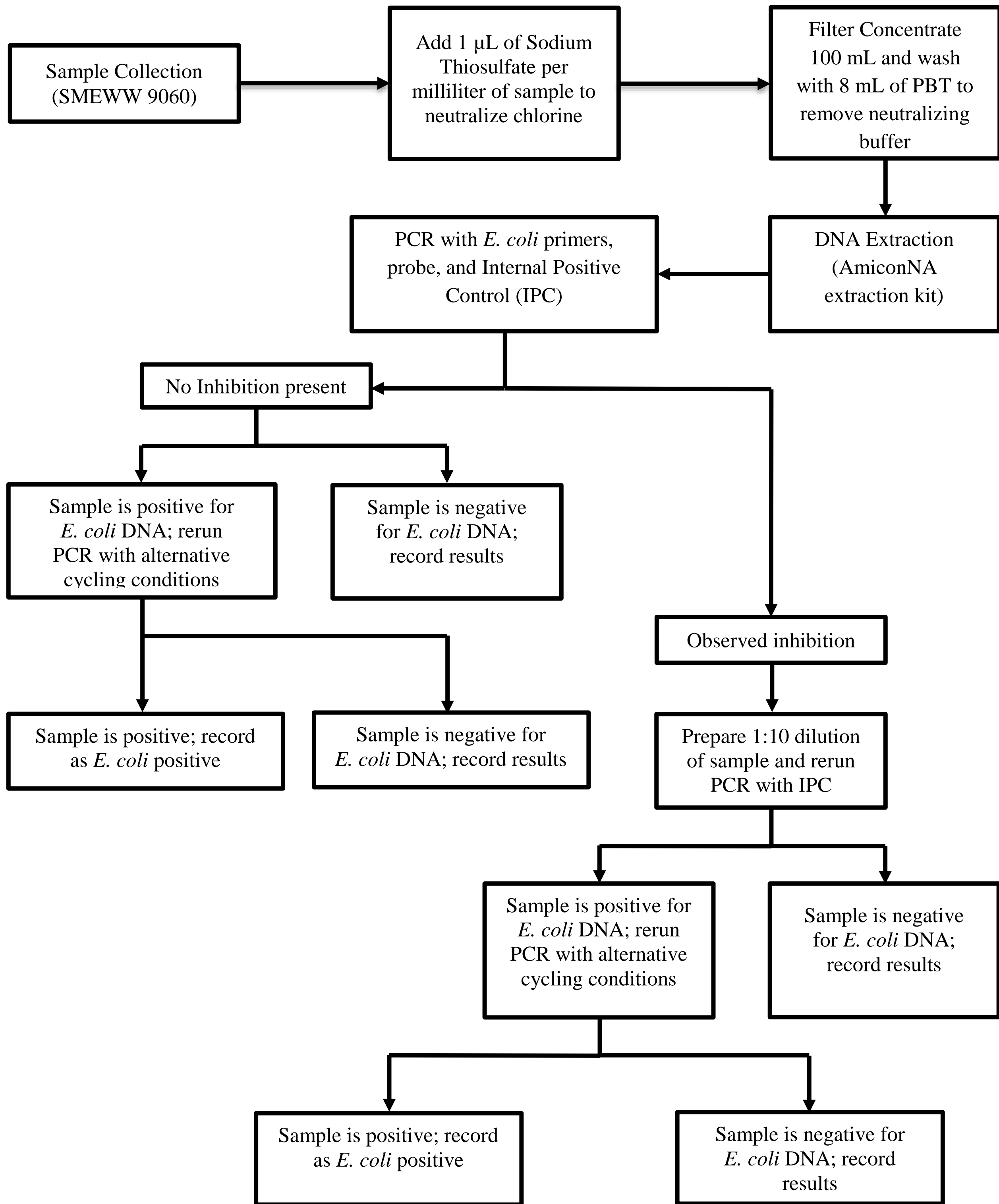

Figure 3. Flow chart for sample processing and PCR analysis

Data Analysis

A total of 28 samples were collected from 14 food establishments. Concentrations and dilutions of each sample were made to enumerate CFU/ml. The Shapiro-Wilk's test ($p > 0.05$) test was used to determine normality of the data. Then the data were analyzed using a means comparison method chosen based on the data distribution. The Wilcoxon signed-rank test was used to compare HPC to EPA standards of 500 CFU/ml. The Mann-Whitney U test was used to compare total coliforms and HPC means from ice and water. The number of samples that tested positive for coliforms was compared to the EPA limit of 5% of samples allowed to test positive for coliforms. IBM SPSS Statistics version 22 was used to calculate descriptive statistics.

CHAPTER 4

RESULTS

Microbiological Analysis

Heterotrophic Bacteria

All 28 samples were cultured on R2A agar. The mean CFU of replicate plates was determined. Plate counts between 30 and 300 CFU were used to determine the CFU/ml. CFU/ml ranged between 65 and 104,000. Rates of samples exceeding the EPA standard for heterotrophic bacteria of > 500 CFU/ml were observed (Table 1). A total of 19 of the 28 (67.9%) samples collected exceeded the EPA limit. Nine (64.3%) of the samples from water and 10 (71.4%) of the samples from ice exceeded 500 CFU/ml. Eight of the water and ice samples that exceeded the EPA limit were collected from the same location. The data from heterotrophic plate counts were analyzed using a Shapiro-Wilk W test for normality. The outcome showed the data were not normally distributed using $\alpha = 0.05$ level ($p < 0.001$). A Wilcoxon signed rank test was the non-parametric statistical method used to compare EPA limits for heterotrophic bacteria and heterotrophic plate counts from the samples of water and ice using SPSS version 22. The results showed the median value of the ice sample (4,150 CFU/ml) and the median of the water sample (5,300 CFU/ml) were both significantly greater than 500 CFU/ml ($p = 0.0065$ for ice, and $p = 0.0165$ for water). Therefore, hypothesis 1 was rejected. These results indicate that ice and water dispensing machines may not be undergoing regular maintenance and cleaning.

Table 1. Sample CFU determined by Heterotrophic Plate Count of samples cultured in duplicate on R2A agar.
(TNTC = Too Numerous to Count and applies to plates with more than 300 colonies;
CFU = Colony Forming Unit; i = ice; W = water; **Bold** = samples above EPA limits).

	Mean CFU per Dilution Factor					
Sample	**10**	**10^0**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**CFU/mL**
1i	TNTC	TNTC	256.5	33	1.5	**3,300**
1W	TNTC	TNTC	TNTC	TNTC	40	**40,000**
2i	TNTC	TNTC	40.5	6	1.5	405
2W	TNTC	TNTC	42	10.5	0	420
3i	TNTC	TNTC	TNTC	TNTC	26	**26,000**
3W	TNTC	TNTC	TNTC	82.5	2.5	**8,250**
4i	TNTC	TNTC	TNTC	41.5	5	**4,150**
4W	TNTC	TNTC	TNTC	191.5	27.5	**19,150**
5i	TNTC	TNTC	TNTC	68	13	**6,800**
5W	TNTC	TNTC	TNTC	53	4.5	**5,300**
6i	TNTC	TNTC	TNTC	160.5	20.5	**16,050**
6W*	TNTC	TNTC	TNTC	TNTC	104	**104,000**
7i	TNTC	TNTC	TNTC	104.5	8	**10,450**
7W	TNTC	TNTC	TNTC	30	4.5	**3,000**
8i	TNTC	74	6	1	0	74
8W	TNTC	68.5	23.5	0	0	68.5
9i	TNTC	TNTC	TNTC	72.5	9	**7,250**
9W	TNTC	134	46.5	6	1	134
10i*	TNTC	TNTC	TNTC	149.5	38.5	**38,500**
10W	TNTC	TNTC	TNTC	TNTC	58.5	**58,500**
11i	TNTC	TNTC	TNTC	41.5	3.5	**4,150**
11W	TNTC	91	7.5	0	0	91
12i	TNTC	TNTC	TNTC	144.5	11.5	**14,450**
12W	TNTC	TNTC	TNTC	TNTC	30	**30,000**
13i	TNTC	65	14.5	0	0	65
13W	TNTC	96	6	0	0	96
14i	TNTC	TNTC	31.5	1	1	315
14W	TNTC	TNTC	TNTC	207.5	13.5	**20,750**

*Treated as an outlier and not included in statistical analysis
Outlier = 1.5 times the interquartile range

Total coliforms and E. coli

All 28 samples, 14 ice and 14 water, were cultured on MI agar and examined. Analysis of MI agar showed 10 of the 14 ice samples (71.4%) contained coliforms (Figure 4). Two out of 14 (14.3%) ice samples also contained *E. coli* (Table 2). The water samples tested did not contain *E. coli.* Analysis of water samples showed that three out of 14 (21.4%) contained coliforms (Table 3). There is no acceptable limit for coliforms, and 10 of the 14 locations contained coliforms in ice. For comparison, EPA standards for drinking water contamination specify that a violation will be triggered if a PWS that tests more than 40 times per month has > 5.0% positive for total coliforms. For PWS that test less than 40 times per month, a violation will be triggered if two or more samples test positive for total coliforms. Therefore, based on a single test of each site, the presence of coliforms indicates the contamination of ice and/or water.

Table 2. *E. coli* CFU determined by Culture Analysis of samples cultured in duplicate on MI agar.
(CFU = Colony Forming Unit; i = ice; W = water; **Bold** = samples above EPA limits).

	CFU per Dilution Factor						
Sample	**100**	**10**	**100**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**CFU/mL**
10i	10.5	0.5	0	0	0	0	**0.105**
10W	0	0	0	0	0	0	0
12i	1	0	0	0	0	0	**0.01**
12W	0	0	0	0	0	0	0

Table 3. Total coliform sample CFU determined by membrane filtration and spread plating cultured in duplicate on MI agar.
(TNTC = Too Numerous to Count and applies to plates with more than 300 colonies
CFU = Colony Forming Unit; i = ice; W = water; **Bold** = samples above EPA limits).

	Mean CFU per Dilution Factor						
Sample	**100**	**10**	**1**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**CFU/mL**
1i	0	0	0	0	0	0	0
1W*	TNTC	47.5	6	0	0	0	**4.75**
2i	170.5	19	2.5	0.5	0	0	**1.705**
2W	0	0	0	0	0	0	0
3i	169	20.5	1.5	0	0	0	**1.69**
3W	0	0	0	0	0	0	0
4i	45	5	0	0	0	0	**0.45**
4W	0	0	0	0	0	0	0
5i	10	1	0	0	0	0	**0.1**
5W	0	0	0	0	0	0	0
6i	1.5	0.5	0	0	0	0	**0.015**
6W	155	19	3	0.5	0	0	**1.55**
7i	40	2.5	0	0	0	0	**0.4**
7W	0	0	0	0	0	0	0
8i	1.5	0	0	0	0	0	**0.015**
8W	0	0	0	0	0	0	0
9i	0	0	0	0	0	0	0
9W	0	0	0	0	0	0	0
10i*	TNTC	TNTC	TNTC	289	45	5	**2,890**
10W*	TNTC	TNTC	234.5	29	5	0	**234.5**
11i	0	0	0	0	0	0	0
11W	0	0	0	0	0	0	0
12i	203.5	20.5	4	0	0	0	**2.05**
12W	0	0	0	0	0	0	0
13i*	TNTC	135	16	0	0	0	**13.5**
13W	0	0	0	0	0	0	0
14i	0	0	0	0	0	0	0
14W	0	0	0	0	0	0	0

*Treated as an outlier and samples were not included in statistical analysis
Outlier = 1.5 times the interquartile range

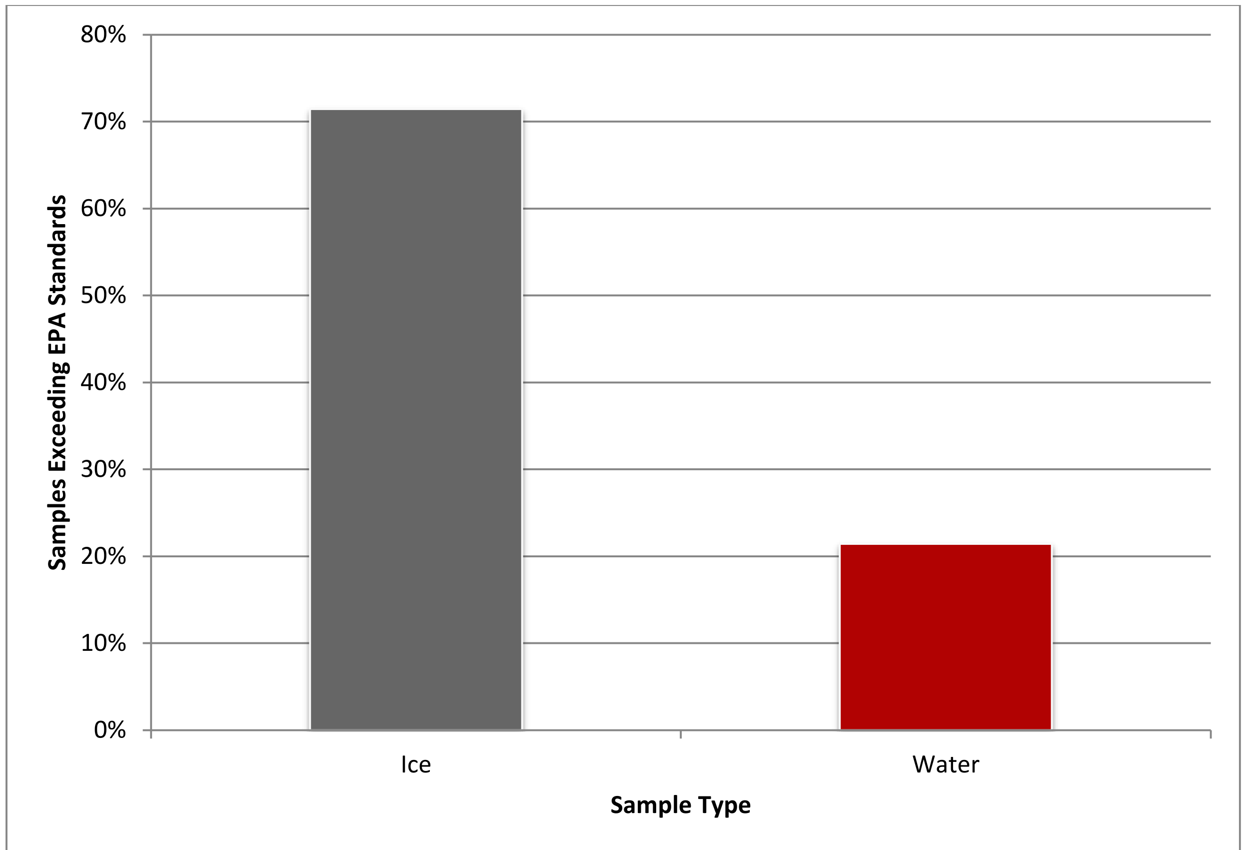

Figure 4. Percentage of samples containing coliforms with error bars representing ± 1 standard error.

PCR Analysis

All 28 samples were tested for the presence of *E. coli* using PCR analysis. Initially, an undiluted DNA sample extract was analyzed with an internal positive control to test for inhibition. A total of 27 out of 28 samples showed complete or partial inhibition (data not shown). A 1:10 dilution of the DNA sample extract removed inhibition for all samples, but all of the samples were negative for the presence of *E. coli* compared with two samples that were positive for *E. coli* with culture analysis. DNA was then extracted from isolates of samples that were *E. coli* positive on MI agar. These samples underwent PCR analysis that confirmed the identification of *E. coli*.

Comparing Ice and Water

Coliform Bacteria

The CFU/ml of coliforms in ice and water samples were averaged. The average coliform colony count for ice samples was 0.54 CFU/ml (Figure 5). The water samples had an average of 0.13 CFU/ml. The ice sample from location 10 contained 2,890 CFU/ml and the ice sample from location 13 contained 13.5 CFU/ml of total coliforms. The water sample from location 10 contained 290 CFU/ml and the water sample from location 1 contained 4.75 CFU/ml of total coliforms. The values of these four samples are above 1.5 times the interquartile range (IQR). Therefore, these samples were treated as outliers and were not included in the statistical analysis of total coliforms.

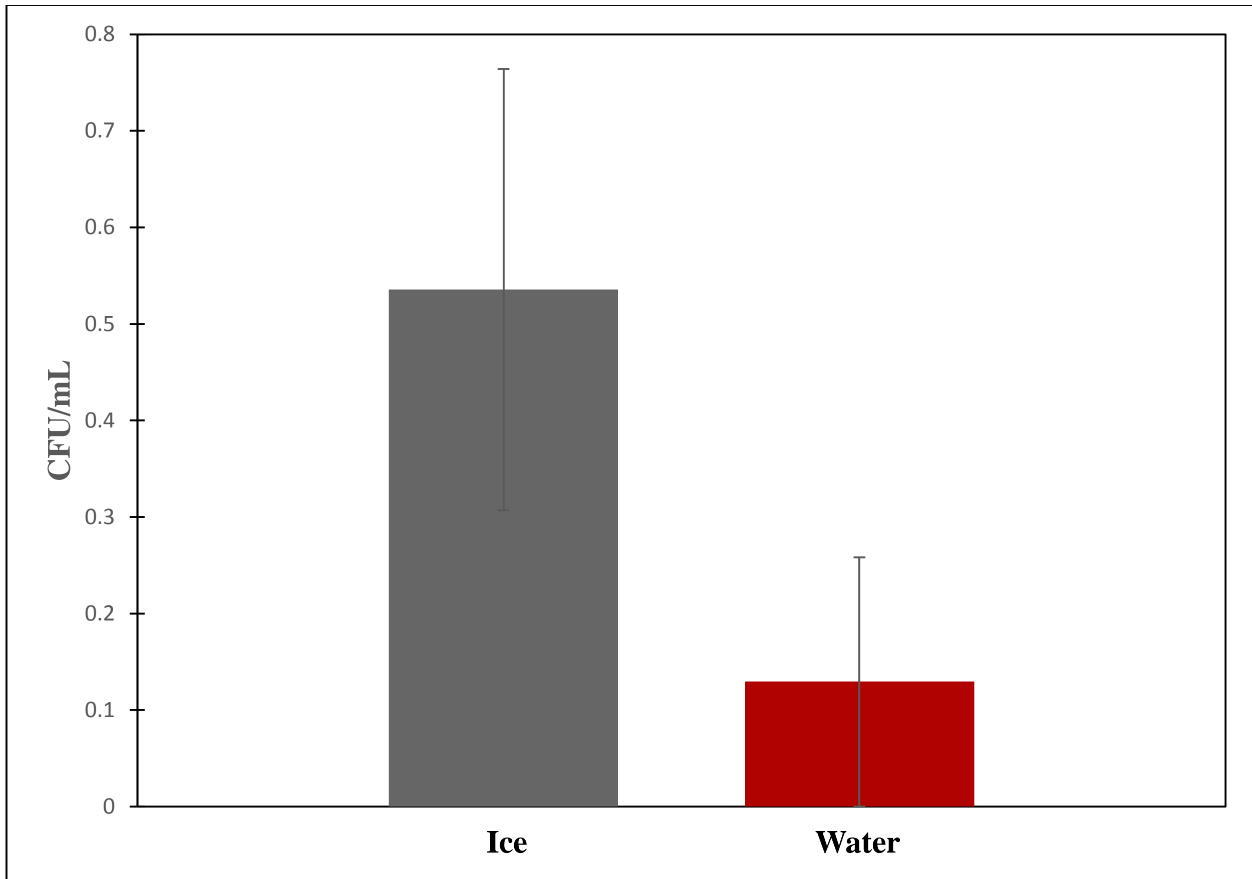

Figure 5. Comparison of total coliforms (CFU/ml) between ice and water with error bars representing ± 1 standard error. *Locations # 10i, 13i 10W, and 1W were treated as outliers and data were not included.

A Shapiro-Wilk W test was used to test for normality of the coliform data. The test determined the data were not normally distributed using $\alpha = 0.05$ level ($p = 0.001$ for ice and $p < 0.001$ for water). Consequently, the non-parametric Mann-Whitney U test was used to compare the average CFU/ml between ice and water using SPSS version 22. The results showed there was a significant difference between ice and water from the same establishment ($p = 0.017$). Therefore, hypothesis 2 was rejected.

Heterotrophic Bacteria

The heterotrophic plate counts from ice and water were averaged and compared. The ice samples had an average 7,189 CFU/ml and water samples had an average of 14,289 CFU/ml (Figure 6). The ice sample from location 10 contained 38,500 CFU/ml and the water sample from location 6 contained 104,000 CFU/ml of heterotrophs. These values are 1.5 times above the IQR and were treated as outliers and not included in the statistical analysis. The data were analyzed to determine the normality of the distribution using a Shapiro-Wilk W test. The data were not normally distributed using $\alpha = 0.05$ level ($p = 0.035$ for ice and $p = 0.006$ water). Therefore, a non-parametric Mann-Whitney U test was performed to compare the averages of heterotrophic plate counts of ice and water. The results showed that there was no significant difference in the number of heterotrophic CFU/ml between ice and water at $p = 0.650$. Therefore, hypothesis 3 was not rejected.

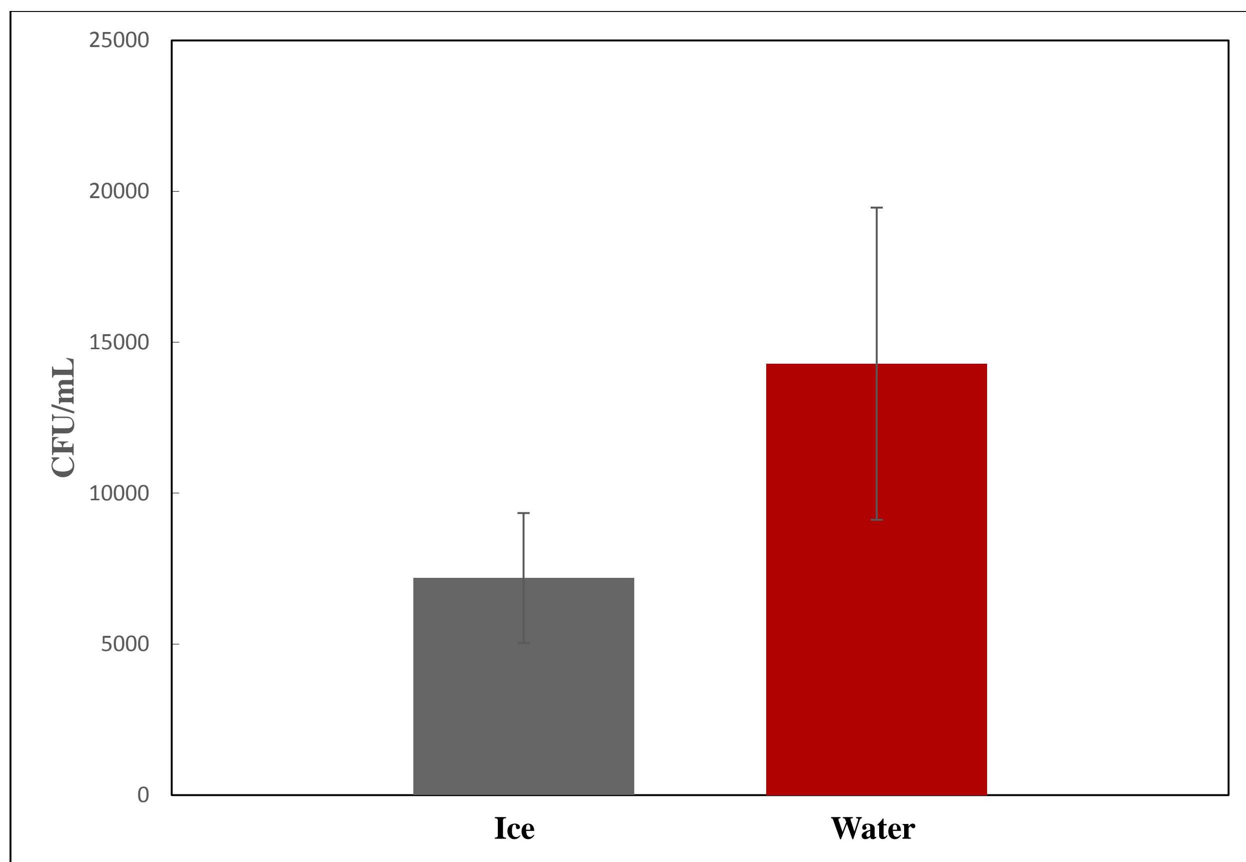

Figure 6. Comparison of heterotrophic CFU/ml between ice and water with error bars representing ± 1 standard error. *Locations # 10i and 6W were treated as outliers and data were not included.

Enrichment

All 28 samples underwent the enrichment procedure and were placed in an incubator shaker overnight. Samples that were clear and showed no signs of turbidity were not cultured. For samples showing turbidity, two 1:100 dilutions were prepared and used to inoculate MI agar plates. Turbidity was observed in 21 of the enrichment samples. A total of 13 of the 21 enrichment samples showed the presence of coliforms. A total of 11 of the 13 enriched samples were also positive for coliforms before enrichment. Therefore, in two samples coliforms were

detected only after enrichment. The enrichment procedure did not increase the detection of *E. coli* in any of the samples.

CHAPTER 5

DISCUSSION

Microbial Contamination of Ice

The objective of this study was to determine the microbial contamination of ice at food establishments in Las Vegas, Nevada. The contamination of ice and water at food establishments was compared to the EPA standards for water quality. EPA approved culture methods were used to analyze ice and water. Heterotrophs and indicator organisms, such as coliforms, were used to determine the sanitary quality of ice and water. The results showed high concentrations of heterotrophic bacteria and high frequencies of coliform bacteria were present in ice at food establishments in Las Vegas.

In 2007, The Food Safety Authority of Ireland (FSAI) tested 1,044 ice samples from food establishments. The samples were tested for *E. coli,* coliforms, and enterococci. The results of the study showed that 27.1% of the samples contained at least one of the three aforementioned groups of organisms and did not meet drinking water regulations (FSAI, 2007). In this study, of food establishments in Las Vegas, NV, 71.4% of ice samples were contaminated and failed to meet drinking water regulations. In the previous study, a correlational relationship was observed that was statistically significant between the way the ice was handled and stored and the presence of coliforms. It was undetermined if there was any type of causality, but storage conditions, storage of utensils, presence of melt water, and whether the ice was covered may have contributed to the sanitary conditions of the ice. A study in Northern Ireland conducted by Wilson, Hogg, & Barr (1997) examined the microbiological contamination of ice in hospitals and the community. Researchers tested 27 samples from hospital ice machines and 194 samples

from food establishments, including bars and hotels. Organisms from hospital samples were identified and the researchers discovered that most of the organisms in the ice were from environmental contamination and some could be opportunistic pathogens. A total of 31% of the samples taken from the community contained coliforms or *E. coli* (Wilson et al., 1997). The higher rate of ice contamination found in this study compared with previous research may be due to a smaller sample size.

A U.S. study conducted by Mako, Harrison, Sharma & Kong (2014) examined bagged ice from various locations throughout the state of Georgia. Two hundred and fifty bags of ice were purchased from retail locations and self-serve vending machines and 25 bags of ice were purchased from a packaged manufacturer. International Packaged Ice Association (IPIA) guidelines were used to determine microbiological standards for ice. The IPIA heterotrophic limit is less than 500 Most Probable Number (MPN)/100 ml of water. The IPIA tolerable level for coliforms and *E. coli* is less than 1 MPN/100 ml of water. Analysis of all 25 samples from the packaged manufacturers showed microbiological levels lower than IPIA standards. A total of 6.4% of the samples taken from self-serve and retail locations exceeded the IPIA standards for HPC (Mako et al., 2014). Analysis for coliforms and *E. coli* found that 37.2% of samples were contaminated and three of the samples from self-serve locations contained *E. coli* (Mako et al., 2014). This study of food establishments in Las Vegas, NV found 67.9% of the samples contained HPC higher than 500 CFU/ml and 71.4% of the ice samples contained coliforms or *E. coli*, a much higher percentage than what was observed in the Mako et al. (2014) study.

The SNWA distributed information about contaminants found in the drinking water from the previous year in the 2015 Water Quality Report (SNWA, 2015). This report includes samples from: Big Bend Water District, City of Boulder City, City of Henderson, City of North

Las Vegas, and Las Vegas Valley Water District. In 2014, approximately 36,000 samples were collected and more than 327,000 analyses were performed. The samples were collected from 367 sampling stations throughout the distribution system. Some sampling stations are above ground and others are installed in customers' meter boxes to ensure the quality of the water is maintained. The average of the percent of samples that were positive for coliforms per month was 0.3%. The maximum number of samples that were positive for coliforms was 1.4% per month and the minimum was 0 (SNWA, 2015). These results indicate that contamination of water and ice at food establishments in Las Vegas are not due to the quality of the water that is coming into the facility.

The EPA limit for HPC is 500 CFU/ml of water. Analysis of ice and water from Las Vegas food establishments showed that 19 (67.9%) of the 28 samples of ice and water contained CFU counts above 500 CFU/ml. This may indicate that water and ice systems at food establishments are not consistently maintained or cleaned. Although HPC is regularly used as a measure of the cleanliness of a system, this test does have some limitations. For example, consistent monitoring of HPC patterns can be more indicative of an issue with a water system, but a numerical count itself may have little indication of how it will affect health (Payment, Sartory & Reasoner, 2003). The food establishments in this study were not monitored over time; therefore, the microorganism count from HPC represents only a single measurement.

Coliform and *E. coli* counts from Las Vegas food establishments were high. Ten (71.4%) of the 14 ice samples contained coliforms. Analysis of the samples determined that two (14.3%) of these samples also contained *E. coli*. Examination of the water samples showed that 3 contained coliforms, but the presence of *E. coli* was not detected in any water sample. The

number of positive coliform samples indicated ice machines can become contaminated and may be a public health issue.

PCR analysis of samples from Las Vegas food establishments did not detect *E. coli*. PCR was used to detect the DNA of *E. coli* that were potentially stressed from disinfection techniques and may not be culturable. Two samples determined to be positive for *E. coli* with culture analysis, were negative with PCR. Because inhibition was observed in DNA extracts, dilutions of the extracts may have resulted in *E. coli* DNA concentrations below the detection limit of the PCR assay; the detection limit was between 0.2 and 2 cells per milliliter without inhibition, which is below the detection limit of the PCR assay as used in this study. The average CFU/ml of samples containing *E. coli* ranged from 0.01 CFU/ml to 0.10 CFU/ml. Concentrating 100 ml of the sample for DNA extraction would have resulted in 1 to 10 cells of *E. coli* per extract. Presumptive *E. coli* isolates determined by culture analysis were confirmed by PCR.

There was a significant difference in the number of coliforms in ice compared to water, indicating that drinking water used to make ice is not the source of contamination. In two of the samples, the number of coliforms were considerably higher in the water sample than the ice sample. This may be attributed to a local contamination near where the water is dispensed. The large difference in the number of coliforms found in ice compared to water indicates that ice machines may not be cleaned as often as recommended and nozzles where water is dispensed may be contaminated. In this study, five ice samples were taken from ice bins using ice scoops, and placed in a cup provided by the food establishment. All five of these ice samples were contaminated with coliforms. Accessories, such as ice scoops and buckets, may have contamination which would explain the difference between water and ice samples. Some

samples were placed in a cup, which could have been contaminated as well. Decreased access to the nozzle or having a recessed nozzle may reduce contamination.

The enrichment procedure did not effectively increase the detection of *E. coli*. In the samples where *E. coli* was detected through membrane filtration, *E. coli* was not detected post enrichment. This may be due to the low levels of *E. coli* cells present in samples or injury to the cells. The sample with the highest concentration of *E. coli* contained 0.105 CFU/ml. The enrichment procedure called for 10 ml of the sample to be placed in the enrichment broth. Therefore, there may have only been one or two cells of *E. coli* placed into the enrichment broth. *E. coli* may have been outcompeted by coliforms or there might not have been any *E. coli* cells placed into the enrichment initially. There were two samples where coliforms were not detected through membrane filtration, but did show the presence of the coliforms after the enrichment procedure. The detection level for membrane filtration for coliforms and *E. coli* was 1 cell per 100 ml of sample. This indicates that there was less than 1 coliform per 100 ml in the negative samples.

This convenience sample shows that ice and water from food establishments may not meet requirements for drinking water. Food establishments in Las Vegas, NV may need to more regularly clean and inspect their ice storage and dispensing machines. The contamination found in this study was higher than previous studies. This may have been due to the limitations of a small sample size. These results may not be representative of Las Vegas food establishments. Further research should be conducted to determine the source of contamination and how to reduce the microbial risk of ice and drinking water contamination in food establishments.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The ability of ice to be a vehicle for transmission of pathogenic microorganisms has been documented (Dickens et al., 1985; Daniels et al., 2000; Brett et al., 2014). It is estimated that contaminated water causes 4.26 to 16.4 million cases of acute gastrointestinal illness (AGI) annually (Colford et al., 2006; Messner et al., 2006). There are many limitations associated with diagnosing waterborne disease, such as individuals not seeking medical attention, as the disease is usually self-limiting. This makes it even more challenging to determine if disease is linked to ice. Coliforms and *E. coli* are used as indicator organisms to test water for pathogens. The presence of these organisms is an indication that the water is contaminated and may contain pathogens that are harmful to human health. The EPA has set a limit of zero coliforms or *E. coli* cells for drinking water; even at low levels these microorganisms have been known to cause disease outbreaks (EPA, 2013). Testing for heterotrophs is also useful as an analytical tool to determine the amount of microorganisms in the water, and a low HPC can indicate a system that is well maintained.

The objective of this study was to determine the microbial contamination of ice at food establishments in Las Vegas, Nevada. There are various ways that microorganisms can be introduced into water and ice. Therefore, both ice and water were collected from each food establishment in the study. PCR analysis was unable to detect *E. coli* in any of the water or ice samples. Culture analysis showed that 10 (71.4%) of the ice samples contained coliforms with two (14.3%) ice samples also containing *E. coli*. HPC testing indicated that 19 of the 28 (67.9%) samples exceeded EPA limits for heterotrophs. The current methods for inspecting ice at food establishments are insufficient for determining microbial contamination.

SNHD is responsible for inspecting ice machines at food establishments. Restaurant inspectors perform a visual assessment on ice machines to evaluate cleanliness, but this type of inspection is inadequate for determining microbial contamination. Bacterial detection methods or stricter maintenance procedures should be implemented to ensure ice is of the highest quality and free of pathogens. The source of bacterial contamination should be further investigated and samples should be taken from selected locations to determine the amount of ice contamination at food establishments in Las Vegas, Nevada.

This was a pilot study using a convenience sample. Ice contamination levels found in this study does not necessarily represent all the food establishments in Las Vegas, Nevada. However, the results indicate that contamination of ice at food establishments is a problem. The bacterial source for coliforms and *E. coli* found in the ice and water samples is unknown. Due to the contamination found in ice compared with water during sample analysis, it is unlikely that the water used to make ice is the source of these microorganisms. The nozzles, pitchers, scoops, and buckets that come into contact with ice maybe a source of bacterial contamination. Contaminated ice can contain pathogens and may become a public health concern. Therefore, further investigation of the contamination of ice at food establishments in Las Vegas, Nevada is needed.

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