MICROBIOLOGICAL QUALITY AND MICROBIAL COMMUNITY OF PACKAGED ICE

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ABSTRACT

Ice is defined as a food and is frequently used in direct contact with food and beverages. Packaged ice is commercially produced and can be easily found in grocery and convenience stores. However, the quality and safety of packaged ice products is not consistent. The Packaged Ice Quality Control Standards manual (PIQCS) published by the International Packaged Ice Association provides the quality and processing standards for packaged ice produced by its members. Packaged ice produced on the premise of stores (on-site packaged ice) is not required to be in compliance with these standards. In this study, packaged ice produced by manufacturing plants or by in-store bagger (ISB) machines and on-site packaged ice were compared for their microbiological quality, microbial diversity and their viability. Our results revealed that 19% of the 120 on-site packaged ice samples did not meet the PIQCS microbial limit of 500 CFU/mL (or g) and also the absence of coliforms and *Escherichia coli*. Staphylococci were found in 34% of the on-site packaged ice samples had unacceptable microbial levels, and all were devoid of staphylococci. *Salmonella* was absent in all samples analyzed in this study. Microbial community analysis of based on 16S/18S rRNA targeted sequencing revealed a much higher microbial diversity and abundance in the on-site packaged ice than in the ISB ice. *Proteobacteria*, especially *Alphaproteobacteria* and *Betaproteobacteria*, were the dominant bacterial groups in all samples tested. Furthermore, *Proteobacteria* dominated the viable profile, especially *Betaproteobacteria* showed the highest survivability in both samples. Most of these bacteria were oligotrophic; however, a few viable opportunistic or potential pathogens were found at low levels in the on-site packaged ice but not in the ISB packaged ice. The types of microbes identified may provide information needed to
investigate potential sources of contamination. Our data also suggest a need for enforcement of processing standards during the on-site packaging of ice.
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CHAPTER 1

Introduction

1. What is packaged ice?

The history of commercial ice trading began in the early 19th century by a New England entrepreneur named Fredric Tudor who envisioned exporting ice on a commercial basis. Tudor’s plan was to sell ice to the wealthiest people for their drinks and food. Two centuries later, ice has become a staple in American households.

Ice is frozen water that has many applications including on its own or mixed with beverages, food processing and storage, and chemical manufacturing. For consumption, ice is produced in three main types, such as flake, tubular and regular cubes, typically packaged in 5 to 10 lb bags, and sold in stores. Furthermore, packaged ice can be produced in a wide variety of methods and settings, including commercial-scale manufacturing plants (manufactured ice), self-sufficient in-store bagger machines (ISB), and production on convenience store premises (on-site packaged ice). According to the International Packaged Ice Association (IPIA), approximately 2 billion packaged ice bags are sold from retail, wholesale, and vending producers each year in the U.S. alone. From the total sales, 800 million bags are packaged on store premises and 200 bags are from vending machine sales (packagedice.org).

2. Packaged Ice Quality Control Standards

The IPIA published the Packaged Ice Quality Control Standards manual (PIQCS) as mandatory practices for its members to provide high quality and food quality safe ice for consumers. PIQCS is based on the Food and Drug Administration’s Federal Good Manufacturing Practices (GMP’s) for food products and is adjusted specifically to packaged ice. The implementation of a hazard analysis and critical control is not
required by the FDA; however, the implementation of HACCP and HACCP prerequisites are required by IPIA.

The IPIA requires all manufacturers of packaged ice to be held to the highest food quality standards with adequate regulatory oversight. For example, the PIQCS microbial limit for the total plate count (TPC) or total hydrophilic count (THC) is 500 colony forming unit (CFU) per milliliters (or 500 CFU/g), and the total coliform level (including *Escherichia coli*) should be zero in 100 mL. Coliforms are aerobic or facultative anaerobic, non-spore forming, rod shaped, and Gram-negative bacteria that ferment lactose to acids with gas production in 24 to 48 hours at 35-37°C. They are found in soil and intestines of warm blooded animals. The presence of coliforms in water might indicate that the water is polluted and possibly contains pathogenic microorganisms.

According to the IPIA, approximately 800 million bags of packaged ice are produced with PIQCS packaged ice processing standards in the United States each year; however, 1.2 billion bags of packaged ice are not produced under such standards, especially bags of ice that are sold directly from vending machines or packaged on site in retail stores with an ice machine.

3. **Safety concerns of packaged ice and drinking water**

   Ice is a food. According to the World Health Organization (WHO), ice is expected to be at the same quality and safety level as drinking water if ice is to be consumed or makes direct contact with the food that is to be consumed. Ice is frozen water; therefore, microbiological quality and sanitation of drinking water must satisfy WHO’s Guidelines for Drinking Water Quality (Fourth edition, 2011). Despite improving trends, many people still have no access to improved sanitation, and 2.2 million children still die from
water related diarrheal diseases each year (WHO). The majority of deaths involved children under 5 years old in developing countries (Medema et al., 2001).

Microorganisms, especially those found in human or animal feces, are responsible for most waterborne infections and are among the oldest health threats to drinking water. Unfortunately, contaminated drinking water is not limited to developing countries. In the U.S.A., about seven million people suffer from microbial waterborne infections each year, which leads to 12,000 deaths per year (Medema et al., 2001). The same public health concern should apply to packaged ice, especially for those that do not comply with the PIQCS standards.

3.1. **Studies on microbiological quality of ice**

Unfortunately, there are only a few studies on the quality of ice that have been reported. According to a study that was conducted from Iowa in 1991, out of 22 packaged ice samples, 8 samples (36%) exceeded the TPC limit established by the IPIA, 16 (68%) were positive for coliforms, and 13 (59%) were positive for mold species (Moyer et al., 1991). All of the unsatisfactory samples did not comply with the IPIA’s PIQCS. The bacteria isolated in the study are common opportunistic pathogens, such as *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Alcaligenes*, and *Corynebacterium* (Moyer et al., 1991). Furthermore, chemical standards were satisfactory, except for five samples that were outside of the standard pH range of 6.5 to 8.5. The analysis of ice quality in Florida reported in 1999 revealed coliforms in 13.5% of on-site packaged ice and 3.6% manufactured ice, but the chemical and physical qualities were comparable between the two types of packaged ice products (Schmidt & Rodrick, 1999).
A recent study in Georgia revealed that manufactured ice was much higher in microbiological and chemical quality than was on-site packaged ice from retail stores and vending machines (Mako, Harrison, Sharma, & Kong, 2014). Of the 250 on-site packaged ice samples, 6.4% of the on-site samples had unsatisfactory TPCs and 37% contained coliforms. The foodborne pathogen *Salmonella* was found in one ice sample from a food service establishment and *Enterobacter agglomerans*, a potential pathogen, was present in ice from a self-service vending machine (Mako et al., 2014). The presence of potential pathogens may raise safety concerns.

A study from the United Kingdom examined the microbiological quality of ice from retail and catering premises. A total number of 4,346 ice samples were tested, 3,528 samples used to cool drinks, 144 samples from food displays, and 674 samples of unknown origin. Among the samples that are used to cool drinks, 9% showed presence of coliforms, 1% *E. coli*, and 1% enterococci in excess of $10^2$ CFU/100 ml, and 11% had an aerobic plate count over $10^3$ CFU/100 ml (Nichols & Gillespie, 2000). Interestingly, ice used in food displays showed poorer microbiological quality than ice used to cool drinks. 23% contained coliforms, 5% *E. coli*, 8% enterococci at $10^2$ CFU/100 ml or more, and 29% samples showed an aerobic plate count greater than $10^3$ CFU/ml (Nichols and Gillespie, 2000). The presence of *E. coli* and enterococci indicate potential fecal contamination, which is a health concern for consumers.

Another similar study, microbiological quality of ice used to cool drinks and foods was examined in Greece. Out of 100 samples, 31 samples contained coliforms and *E. coli* was detected in 22 samples. Furthermore, foodborne pathogens were found in some of ice samples (Gerokomou et al., 2011). 35 percent of samples had presence of spore forms of
Clostridium perfringens and P. aeruginosa and Yersinia spp. were found in three samples each. The presence of foodborne pathogens as well as coliforms suggested that ice may cause a serious illness to the consumer.

The microbiological quality of edible ice study was conducted in Hong Kong. Among 89 samples from retail businesses, 36 samples contained at least one colony of coliforms and 3 samples exceeded the criteria for aerobic colony counts (<\(10^3\) CFU/ml) whereas 12 samples from ice manufacturing plants met the microbiological criteria. Of 36 samples that contained coliforms, 8 samples contained more than 100 CFU/100 ml. The authors suggested that the contamination of packaged ice in retail businesses might come from unhygienic handling, improper storage or cross-contamination.

The microbial quality of ice may correlate directly to its water source. It may be further impacted by low temperature and the contaminations during the ice making process. Falcão et al. (2002), examined 60 samples of commercial ice and ice used to refrigerate seafood for their microbiological quality from the city of Araraquara, Brazil. The heterotrophic counts were performed at 37°C and 4°C, and the results showed that the average CFU/ml of mesophiles was higher than psychrophiles, implying a potential contamination from warm blooded animals or humans (J. Falcão, 2002). Furthermore, 50 strains of E. coli were identified from 23 ice samples. Among the 50 isolates, 12 were classified as enteroaggregative E. coli (EAEC), which can cause acute and persistent diarrheal diseases (J. P. Falcão et al., 2004).

3.2. Studies on microbiological quality of drinking water

To address the problem of drinking water, many studies were conducted to evaluate the microbiological quality of water. A study on microbiological quality of drinking
water sold by roadside vendors in Delhi, India revealed that the coliform bacteria were present in all 36 samples (Chauhan, Goyal, Varma, & Jindal, 2015) ranged 14 to >1600 per 100 ml. Furthermore, opportunistic human pathogenic bacteria such as Escherichia coli (61%), Salmonella (25%), Staphylococcus aureus (14%) and Pseudomonas aeruginosa (53%) were found. Another interesting study was done to identify fecal contamination of drinking water in Zagazig city, Egypt. In this study, a total of 300 water samples were collected, 36 (12%) samples were found to be contaminated coliforms, and among 36 samples, 16 (5.3%) samples were positive for presence of E. coli. In addition, E. coli isolates showed high level of resistance to multiple antibiotics, and eight isolates harbored at least one virulence gene (Fakhr et al., 2016). As expected, developing countries still seem to struggle with supplying clean drinking water to consumers. There should be more strict regulations and enforcements on drinking water.

A study from Oregon in 1980 examined the presence of Staphylococcus aureus in rural drinking water. S. aureus is one of the most common indictors of poor food handling and common agents of food poisoning. Out of 320 samples, a total of 144 samples of rural drinking water had the appearance of typical staphylococcal colonies. Furthermore, coagulase was performed to differentiate between S. aureus and other Staphylococcus species. As a result, 20 samples (6.25%) had a coagulase positive reaction, and of the 20 samples, 8 samples were positive for enterotoxin A (Lechevallier & Seidler, 1980).

In 2008, the microbial evaluation of 35 different brands (16 spring water, 11 purified/fortified tap water, 5 carbonated water and 3 distilled water) of commercial bottled water were examined in Houston, Texas (Saleh et al., 2008). As a result, six
brands of spring waters showed microbial growth in the range of 5-50 CFU/mL, 3 bottled tap waters with a range of 30-50 CFU/mL, and none for carbonated and distilled water. Furthermore, the identified bacteria in the spring waters include 6 Gram negative bacterium, *Klebsiella terrigena*, *Ralstonia pickettii*, *Cidovorax temperans*, *Acidovorax delafeldii*, *Agrobacterium rhizogenes*, and *Burkholderia glumae*, and one Gram-positive *Bacillus thermoglucosidsiu*. These microorganisms are commonly found in environments such as soil, rivers, and lakes. Based on this study, bottled waters are generally safe to drink. However, some of the identified bacteria in spring waters have been reported to cause infections to people who are immunocompromised.

4. **Limitation of culturing methods**

Culture-dependent approaches, including isolation and characterization of microorganisms using growth media, have been used in microbiology laboratories for many years to study microbial communities. However, the inevitable major limitation of culture-based technique is that greater than 99 percent of the microorganisms in any of environment cannot be cultured ex situ (Amann *et al.*, 1995; Hugenholtz, 2002). Numerous Improved culture media have been implemented to mimic natural environments in laboratory settings; however, many of them still remain unculturable (Oliver, 2005). These viable but nonculturable state (VBNC) organisms are viable in their natural environments but do not grow under traditional laboratory conditions (Oliver, 2005). VBNC is used as a survival strategy for some non-sporulating bacteria facing adverse environment stresses. During the VBNC state, the bacteria modify their morphology and metabolic state such as reduction in size, changes in cell membrane composition, and decreased metabolic activity (Signoretto *et al*. 2000; (Nowakowska &
Oliver, 2013). The VBNC concept was first introduced in 1982 (Xu et al., 1982) and, since then, the importance of the VBNC organisms has caught people’s attention when studying microbial community in the environments.

5. **New methods to study microbial communities**

Microbial communities represent the largest proportion of the Earth’s biodiversity. Microbes are involved in our lives, shaping environments to our own body. With current molecular and biotechnology techniques, we can study the ubiquitous and complex microbial communities in unprecedented details. In recent years, culture independent techniques have been rapidly developed, providing true diversity of microbial communities by eliminating the cultivation step. Such techniques include analysis of whole genomes or selected genes such as 16S and 18S rRNA (ribosomal RNA) for prokaryotes and eukaryotes, respectively. Moreover, these molecular techniques to explore microbial diversity structure and function can be grouped into two major categories, partial community and whole community. Below are a few examples of partial community analyses. Section 6 will discuss the whole community analysis using metagenomics.

5.1. **Clone library assays**

Partial community analysis approaches generally include amplification of 16S or 18S rRNA gene using polymerase chain reaction (PCR). One of the ways to study partial community analysis is using clone library assays. Once sample is cloned after PCR amplification, the individual gene fragments are compared to known sequences in a database such as Ribosomal Database Projects (RDP), Greengenes, and GenBank (DeSantis et al., 2006). Clone library assays have been widely used to examine microbial
communities; however, it is a time-consuming method and unsuitable for high-throughput analyses dealing with a large number of samples.

The United States Environmental Protection Agency (USEPA) conducted a study on microbial communities of drinking water systems using 16S rDNA cloning method in 2004. Microbial diversity of water samples was dominated by the groups of $\alpha$-, $\beta$-, $\gamma$-proteobacteria and Hyphomicrobi um sp. A notable opportunistic bacterial pathogen, Legionella sp, was found (Williams et al., 2004). Another similar study was conducted by the USEPA in 2006 showing members of $\alpha$-Proteobacteria (43%) and Mycobacterium sp. (48%) dominated the samples. Furthermore, members of potential pathogens in the genera of Legionella, Pseudomonas, and Agrobacterium were identified with low percentages in the samples (Humrighouse et al., 2006).

5.2. Molecular Fingerprinting

Another popular method for investigating partial microbial communities are molecular fingerprint techniques, such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism analysis (T-RFLP). Molecular fingerprinting generates a rapid profile of microbial communities based on PCR fragments’ different sequences and/or sizes and are separated by their differing mobility on a gel or in a capillary. Once the patterns (molecular fingerprints) are generated, the community diversity and relationships can be compared using computer assisted cluster analysis such as GelCompar (Gilbride et al., 2006). Furthermore, phylogenetic information can be obtained by sequencing the DNA fragments; however, the resolution is low due to short oligonucleotide probes. In addition, molecular fingerprinting technique is limited on
quantifying microbial community because the gene expression patterns heavily vary on growth and environmental conditions.

There are many studies that performed molecular fingerprinting methods to test microbiological quality of drinking water. Dewettinck’s group performed a DGGE analysis on the bottled mineral water and groundwater samples (Dewettinck, Van Houtte, Geenens, Van Hege, & Verstraete, 2001). In comparison to the bottled mineral water samples, groundwater showed more bands on DGGE, showing more diverse microbial community. Interestingly, different samples of water showed different patterns of fingerprints, indicating each water sample contained its own specific bacterial community.

5.3. **Fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) is another popular way to study partial microbial communities using a probing method that does not require nucleic acid extraction. When whole cell is hybridized with oligonucleotide probes that are as small as 20-40 or up to 1,000 base pairs long, the FISH allows us to study individual microbial cells’ in situ phylogenetic identification and enumeration (Amann *et al*., 1995). Once fluorescently labeled FISH probes are bound to cellular rRNA, epifluorescence microscopy can visualize the abundance and distribution of microorganisms in environmental community (R. Amann & Fuchs, 2008). The FISH techniques; however, have a limitation of being expensive, low signal intensity, background fluorescence noise, and target inaccessibility. Brighter fluorochromes were used to improve these problems which is amplifying the signal using reporter enzymes and hybridizing with probes carrying multiple fluorochromes (Rogers *et al*., 2007).
A study was done in Europe to use FISH to detect *Mycobacterium avium*, an environmental opportunistic human pathogen, in potable water. They used peptide nucleic acid oligonucleotide sequence for detecting *M. avium*, as well as other several mycobacteria species, and mycolic acid containing bacteria. As a result, the enumeration of bacteria using the FISH method was $1.8 \times 10^7$ bacteria/liter, and the standard plate count of heterotrophs was $2.7 \times 10^7$ CFU/liter. They were also able to detect viability of the PNA-hybridized cells by observing their fluorescence signal (Lehtola, Torvinen, Miettinen, & Keevil, 2006).

6. **Whole community analysis using metagenomics and marker gene sequencing**

Metagenomics studies have revolutionized novel aspects of modern microbiology. For example, studies of the human microbiome have shown possible links between gut microbiome and diseases such as diabetes (Devaraj *et al.*, 2013) and depression (Marin *et al.*, 2017). Furthermore, metagenomics allowed us to investigate very complex communities in different places such as soil (Hartmann *et al.*, 2014), glaciers (Anesio, Lutz, Chrismas, & Benning, 2017), air (Adams *et al.*, 2015), and low-biomass cleanroom (Vaishampayan *et al.*, 2012).

Metagenomics begins with collecting samples from an environment and simply reading all DNA sequences from the microorganisms present in a sample. This approach is reshaping various research areas that are investigating microbiomes. Similar to partial community analysis, metagenomics approaches do not require cultivation of the organisms. Furthermore, it allows us to identify the microbes at a taxonomic level or reveal the potential functions such as metabolic pathways, enzymatic and gene functions of the microbial community. Metagenomics can be performed by two general approaches
to achieve the characterization of taxonomic content of environmental samples. Marker
gene analysis sequences PCR amplicons corresponding to phylogenetic marker genes
such as 16S or 18S rRNA. Another method is called shotgun metagenomics, and it
sequences all gDNA in the community.

6.3. Shotgun Metagenomics

Shotgun metagenomics can reveal more comprehensive functional knowledge about
microbial communities by analyzing the complete sequences of protein coding genes and
full operons in the sequenced genomes. In order to view the complex sequenced
microbial community, shorter reads need to be assembled into genomic contigs (a set of
overlapping segments of DNA), and positioning contigs into scaffolds (chains of
contigs). The contigs can be assembled by either reference based assembly or de novo
assembly depending on their complexity.

In reference based assembly (or reference assisted assembly), a previously assembled
genome is used as a reference in order to create contigs using tools such as MetaAMOS
MIRA 4, or Newbler (Roche). These contigs represent genomes or parts of genomes
belonging to specific genus or species. Reference based assembly does not require
intensive computational analyses and works well when metagenomics samples are
obtained from greatly studied areas. During the assembly process, sequences from online
databases will be used as references using genome browser tools such as Artemis
(Rutherford *et al*., 2000). However, when the large gaps are seeing as a result of the
assembly, it can be inferred that the assembly is incomplete or the reference genome is
not as closely related to the community. *De novo* assembly involves the generation of
assembled contigs without the aid of a reference genome. A *de novo* assembly aligns
reads that overlap into contigs and some number of mismatches will be made due to sequencing error or biological variation. Unlike reference based assembly, de novo method is computationally expensive, and it requires to use highly sophisticated graph theory algorithms, such as de-Brujin. In addition, de novo assembly takes up a lot of memory in computers and generally takes a long time to execute because it is more of an ideal approach when the size of genome is medium to large scale.

Another important step is to group the contigs into operational taxonomic units (OTUs) and assign the groups to specific genus, species, or subspecies (Chatterji et al., 2008). This is called binning, and this process helps us to understand what functional roles each microorganism plays and how the taxonomic profiling work in the environment. The binning methods can be broadly classified into two categories. First category is taxonomy dependent, which reference databases are required for the assignment from contigs. The national Center for Biotechnology Information (NCBI), Uniprot (Apweiler, 2004) or Pfam (Punta et al., 2012) is usually used as the reference database. In comparison, taxonomy independent requires no additional reference databases or taxonomic information. This method requires similarity measurements from GC content, the frequency of tetranucleotides or hidden Markov models. However, the binning process still remains one of the significant challenges in metagenomics analysis for many reasons, including pre-processing stage (DNA extraction, sequencing protocols or contaminated sequences), limitation of algorithm and knowledge on microbial genomes.
6.4. **16S/18S rRNA targeted community analysis**

16S/18S rRNA targeted community analysis (or marker gene metagenomics) is a fast way to study the microbial diversity in natural environments using evolutionarily conserved marker genes, such as the 16S (Tringe & Hugenholtz, 2008) or 18S (Andersen et al., 1998) rRNA gene for prokaryotes and eukaryotes, respectively. One of the technologies that revolutionized studying the microbial community was 454 pyrosequencing (detects pyrophosphate release on nucleotide) of the 16S rRNA gene and became the gold standard in the field of microbial ecology. However; Roche has shut down the 454 pyrosequencing division in mid-2016 due to its bias being acritical insertions and deletions. Nonetheless, all the software that was developed to analyze pyrosequencing has been adjusted to analyze data obtained by other platforms such as Illumina MiSeq or Ion Torrent.

Illumina MiSeq or any other marker gene metagenomics technology has three important processes during metagenomics data analysis. The first part is called denoising. The low quality reads will be truncating based on the appearance of low signal intensities, and chimeras (artificial recombinants between two or more parental sequences) will be identified and removed by using an open-source software such as QIIME, Mothur, or DADA2. The next step is Operational Taxonomic Units (OTUs) clustering and taxonomic assignment. For prokaryotes (bacteria and archaea), greater than 97% sequencing similarity is expected for OTU picking. There are generally two ways to generate OTUs. First method is called alignment based clustering, and it uses query sequences against pre-aligned reference sequences (DeSantis et al., 2006). Second method is called alignment-free clustering, which compares motifs directly based on
shared features, and it is more broadly used in picking OTUs by using tools such as UCLUST (Edgar, 2010). Lastly, identifying each individual sequence is a crucial step in microbial community analysis. Basic local alignment search tool (BLAST) is one of the well-known algorithms to search for the best match to an OTU. This step requires reference databases of marker genes, such as Greengenes (DeSantis et al., 2006), Ribosomal Database Project (Cole et al., 2009), and Silva (Quast et al., 2013).

7. Detection of viable microbial communities

Partial and whole community techniques can detect VBNC and provide genetic diversities; however, these methods cannot differentiate between viable and dead bacteria (Elizaquível et al., 2014). Previously, accurate and reliable molecular methods such as reverse transcription PCR (RT-PCR) and Live/Dead BacLight viability assay have been developed to differentiate viable from dead cells. However, these methods are not suitable for studying complex microbial communities. Ethidium monoazide (EMA) and propidium monoazide (PMA) are then introduced as DNA intercalating dyes that can be used to distinguish viable from dead bacteria (Elizaquível et al., 2012). These DNA intercalating dyes enter bacterial cells with damaged membrane (dead cells) and bind covalently to dsDNA after exposure to bright light, thus inhibiting the DNA from PCR amplification. Studies showed that PMA is more sensitive in the selective removal of dead cells than EMA (Nocker et al., 2006).

PMA dye has been used in various applications such as pathogenic bacterial detection to next generation sequencing. According to Cattani et al (2016), the PMA induced qPCR was able to detect B. cereus group species in 44 samples (32.6%) whereas no PMA treatment qPCR detected 78 positive samples out of 135 ultra-heat treatment milk
samples (57.8%) (Cattani et al., 2016). Furthermore, Chiao et al (2014) from Ann Arbor, Michigan monitored the changes of viable bacterial diversity in drinking water system using pyrosequencing after the monochloramine disinfection (Chiao et al., 2014). As a result, *Mycobacterium*, *Sphingomonas*, and *Coxiella* were present in higher abundance in the live bacterial communities following monochloramine disinfection. Lastly, viable and total biofilm communities after 4 step nitrification treatment of chloriminated drinking water were revealed using 454 pyrosequencing. First step was dominated by *Bradyhizobium* while *Legionella* increased during second step of nitrification. Interestingly, nitrite-oxidizing bacteria, *Nitrospira moscoviensis*, was degreased to <0.02% from 2% after 3 step of nitrification. Furthermore, *Methylobacterium* and *Nitrosomonadaceae* were most prevalent after third step. Lastly, the relative abundance of *Afipia*, haloacetic acid degrading bacteria, increased from <2% to 42% during fourth step.

8. **Focus and important findings of present studies**

   Ice is defined as a food and is regulated by the FDA. The PIQCS published by the IPIA provides the quality and processing standards for packaged ice produced by its members. For example, ice manufacturers must monitor cleanliness of employees and equipment and use sanitary water. Unfortunately, FDA does not inspect retail stores (gas stations or convenience stores) that make ice directly for the consumers. In addition, FDA does not inspect establishments that make ice for drinks or cooling food. Small packaged ice producers are subject to regulation by the state and local authorities; however, it is nearly impossible to keep the consistent quality and safety of packaged ice products. The focus of my study was to compare and measure the microbiological quality of
manufactured, ISB, and on-site packaged ice samples collected from various areas in southern California. Furthermore, microbiological diversity of selected samples was revealed by performing next generation sequencing.

Our first objective was to test microbiological quality of packaged ice (manufactured, ISB, and on-site) samples from different counties in southern California using culturing methods such as TPCs, enumeration of *Staphylococcus*, coliforms and *E. coli*, and presence of *Salmonella* using qPCR. Overall, the on-site samples exhibited lower microbiological quality than manufactured and ISB packaged ice samples, which led us to investigate their microbial diversity in selected samples.

Our second objective was to compare the difference in microbial communities between two on-site and one ISB packaged ice samples using non-culturing method, the 16S and 18S rRNA targeted community analysis using next generation sequencing. According to our results, on-site samples showed microorganisms that are commonly found in environments, guts of human and animals whereas ISB contained microorganisms that are found in an oligotrophic environment.

Since NGS cannot discriminate dead and viable cells, this led us to our third objective, which was to investigate viable, non-culturable, and dead cells in two on-site packaged ice samples. PMA dye was induced to inhibit dead cells from amplification. 16S/18S rRNA gene targeted MiSeq sequence revealed that the viability of microbe ranged from 50% to 90% in samples.

In this study, we found that the overall microbiological quality of on-site packaged ice was poorer than manufacture or ISB packaged ice. Poor food handling indicator organisms, *Staphylococcus* species and possible fecal contamination indicator coliforms
were only found in on-site packaged ice. Furthermore, on-site packaged ice showed more diverse microbial communities, and a few viable opportunistic or potential pathogens were found at low levels in the on-site packaged ice but not in the ISB packaged ice. The types of microbes identified may provide information needed to investigate potential sources of contamination. Our data also suggest a need for enforcement of processing standards during the on-site packaging of ice.
CHAPTER 2

Materials and Methods

1. Sample collection

On-site samples were collected from various stores from six different counties in southern California: Imperial, Los Angeles, Orange, Riverside, San Bernardino, and San Diego. These counties were further grouped together based on their geographical location and population density. A total of thirty on-site packaged ice samples were collected from each geographical area, 120 samples in total. The on-site packaged ice was verified by absence of the IPIA logo on bags and was also confirmed with the store employees. On the other hand, six bags of manufactured packaged ice with IPIA complied from two different brands were collected from various retail stores. Furthermore, six bags of in-store bagger (ISB) packaged ice from each four different brands, two being IPIA complied, were collected from different locations in southern California.
Table 1. Sample collection information and location

<table>
<thead>
<tr>
<th>Source of ice</th>
<th>Label</th>
<th>County/Description</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>On-site packaged ice</td>
<td>1</td>
<td>Los Angeles</td>
<td>30</td>
</tr>
<tr>
<td>(convenient stores/gas station)</td>
<td>2</td>
<td>Orange</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>San Bernardino &amp; Riverside</td>
<td>30</td>
</tr>
<tr>
<td>Manufactured packaged ice</td>
<td>4</td>
<td>San Diego &amp; Imperial</td>
<td>30</td>
</tr>
<tr>
<td>In-Store Bagger (ISB)</td>
<td>5</td>
<td><strong>IPIA complied</strong></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><strong>IPIA complied ISB</strong></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td><strong>Non-IPIA complied ISB</strong></td>
<td>12</td>
</tr>
<tr>
<td><strong>Total number of samples</strong></td>
<td></td>
<td></td>
<td><strong>156</strong></td>
</tr>
</tbody>
</table>

2. **Sample preparation**

After purchase, each sample was bagged in a sterile 5-kg WhirlPak bag to avoid cross contamination with other samples and placed in a cooler to minimize melting. For each collection, sample’s condition was noted, such as damage, shape of ice, weight, type of sealer, and location of the freezer. Upon arrival to the laboratory, ice samples were aseptically transferred to another sterile 5-kg WhirlPak bag and thawed overnight at room temperature. After completion of thawing each sample, samples were thoroughly mixed.
before microbial analysis. Sample preparation was completed within 24 hours of collecting the sample.

3. Microbiological analyses

Total coliform and *E. coli* count (CEC), total plate count (TPC), staphylococci counts, and *Salmonella* detection were performed for each sample following the procedures described below. The data were graphed and analyzed by one way nonparametric ANOVA using Graphpad Prism software (La Jolla, CA).

3.1. Total plate count

Total plate counts were performed using the SimPlate TPC (BioMedix, Pomona, CA). The SimPlate TPC measures the number of viable microorganisms based on the most probable number (MPN) using statistic estimation. The sample (up to 10 mL) was mixed with power media that contained nutrients for microorganisms to grow. It was then poured onto a plate with 84 wells. After 20 – 24 hour incubation at 37°C, the number of pink/purple, which indicates a positive growth, wells were counted then converted to MPN/mL based on the statistic estimation table provided by the manufacturer.

3.2. Enumeration and detection of total coliforms and *E. coli*

CEC counts were measured using membrane filtration and MI medium (Becton Dickinson Co., Franklin Lakes, NJ) as described in EPA method 1602. MI agar detects and enumerates total coliforms and *E. coli* in drinking water by using a chromogenic/fluorogenic medium. After 100 mL of sample was filtered, the membrane filter was aseptically removed and placed on a MI agar. After incubation at 35°C for 20-24 hours, the color of colonies were recorded. The visualization of total coliforms and *E. coli* was possible because MI agar detects the presence of the bacterial enzymes β-
galactosidase and β-glucuronidase produced by total coliforms and *E. coli*, respectively. Under ambient light, total coliforms will appear as a tan colony color whereas *E. coli* will appear as blue. Under the long-wave ultraviolet light (366 nm), all coliforms including *E. coli* will appear blue/white fluorescent colonies whereas non-coliform bacteria will not appear as fluorescent colonies.

3.3. **Baird-Parker agar and Coagulase test**

Staphylococci counts were performed by filtering 100 ml of melted ice samples through Microfil 0.45 μm membranes (EMD Milipore, Billerica, MA), which were then placed on Baird-Parker plates (Hardy Diagnostics, G96). After incubation at 37°C for up to 48 hours, positive growth, indicated by black colonies, was recorded. Baird-Parker agar contains potassium tellurite and egg yolk, which allow staphylococci to have black colonies and clear zones around colonies, respectively. When black colonies appeared, they were tested for coagulase reaction using CoaguStaph™ (Hardy Diagnostics, Z020). The coagulase test detects the presence of exoenzyme coagulase. *S. aureus* produces free coagulase whereas *S. epidermidis* does not. On the coagulase slide test, the cells will clump together, which indicates a positive coagulase test.

3.4. **Detection of Salmonella**

Samples were analyzed for *Salmonella* using the FDA PRL-SW validated qPCR procedure with a detection limit of 0.08 to 0.2 CFU/g. Composited samples were used in the initial screening by filtering 5-10 samples of 100 ml each through a membrane. The membrane was placed in modified buffered peptone water for enrichment at 35 ± 2°C for 18-24 hours.
4. Microbial community analysis by 16S/18S rRNA sequencing

Selected samples were analyzed for their microbial community composition using Illumina MiSeq next generation sequencing. Due to limitations of resources and the amount of DNA available in the ice, our analysis was limited to three packaged ice samples, including two on-site packaged ice and one IPIA-compiled ISB packaged ice products, designated as on-site A, on-site B, and ISB, respectively. On-site A and the ISB samples were chosen among the samples with satisfactory microbiological quality that could produce sufficient DNA for the sequencing analysis. On-site B was chosen to represent the samples that did not pass the PIQCS microbiological standards. None of the manufactured ice produced sufficient DNA for positive sequencing reads. From 300 ml to several liters of melted ice were filtered to obtain enough cells for DNA extraction using the PowerWater DNA isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer’s procedure. Purified DNA was subjected to amplification using the Bacteria/Archaea universal primers, 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGTWTCTAAT), which covers the V4 region of the 16S rRNA gene and have been recommended for environmental samples, followed by sequencing on the Illumina MiSeq sequencer at the Research and Testing Laboratory (Lubbock, Texas). The Eukarya specific primers, TArEuKf (CCAGCASCYGCCTTCC) and TReuKr (ACTTTCGTTCTTGATYRA), were used to amplify the V4 region of 18S rRNA DNA.

16S and 18S rRNA raw amplicons were processed according to the Schloss’s MiSeq standard operating procedure using MOTHUR v1.36.1. More specifically, 250 bp paired MiSeq reads were combined, following the removal of reads shorter than 200 bp or containing ambiguous base pairs. Remaining reads were aligned to SILVA v119 followed
by reducing PCR errors using precluster. Additionally, chimeric sequences identified by UCHIME, sequences not classified at kingdom level, mitochondrial and chloroplast sequences were excluded from the dataset. For 16S rRNA analysis remaining, high quality reads were de-gapped and used for closed references operational taxonomic unit (OTU) picking using Greengenes v13.5 in QIIME v1.91, where sequences were clustered at 97% sequence similarity using UCLUST and classified by SortMeRNA. The OTU table was passed to the core diversity workflow using nonphylogenetic diversity parameter and an e-value of 3,065 sequences per sample. Environmental clustering using principal coordinates analysis (PCoA) plots based on a Bray-Curtis distance matrix was performed for beta-diversity analysis while observed-OTUs and Chao1 was used for alpha-diversity evaluation.

5. **PMA study: sample collection, preparation, and DNA purification**

A total of eight on-site packaged ice samples were randomly collected from each of the San Diego or Los Angeles County. For each county, the eight samples were combined to provide sufficient DNA for the viable microbial community analysis by PMA treatment using Illumina MiSeq next generation sequencing. About 10 liters of melted ice (from the eight bags) were filtered using filter membranes. Each filtered membrane sample was washed with 1000 µl of sterilized PBS in a 50 mL Falcon tube by vortexing for 5 minutes before separating into two aliquots of 500 µl each. One of the two identical aliquots was subjected to PMA pre-treatment (viable DNA), and the other one was untreated (total DNA). A total of four samples, on-site C Total, on-site C Viable, on-site D Total, and on-site D Viable, were subjected to DNA extraction using the PowerSoil
DNA isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer’s procedure.

6. **PMA treatment**

   A 500 µl of filter-concentrate sample suspension was treated with 1.25 µl of PMA (20 mM; Biotium, Inc., Hayward, CA, USA) to a final concentration of 50 µM (Nocker *et al.*, 2010). Tubes, including non-PMA treated samples, were incubated in the dark for 5 minutes at room temperature (Vaishampayan *et al.*, 2013). During this step, PMA penetrates the dead cell membranes then bind to the DNA of cell membrane compromised bacteria. The tubes were inverted 3-4 times every minute. After incubation, PMA and non-PMA treated sample tubes were laid flat on a piece of foil on top of an ice bath. The tubes were treated with 500-W halogen lamp set 20 cm above for 15 minutes. The placement of foil was to maximize the exposure of the light. This step allowed PMA to inactivate DNAs from dead cells (Vaishampayan *et al.*, 2013). The tubes were inverted 5-6 times every minute to ensure homogenous light exposure. Samples were centrifuged at 5000 x g for 10 minutes. Supernatant was removed and cells were resuspended in 200 µl of sterile PBS and stored at -20°C until DNA extraction.
CHAPTER 3

Results

1. Microbiological quality of packaged ice samples

   All samples were analyzed for their microbiological quality using TPCs, coliform and 
   *E. coli* counts, staphylococci counts, and presence or absence of *Salmonella*. In addition, 
   many yeasts/molds also grew on the Baird Parker plates for staphylococci. Therefore, 
   presence of yeasts/molds was noted. The results are summarized in Table 2, and the 
   distributions of the TPCs and coliform counts are plotted in Figure 1. For the on-site 
   packaged ice products from stores in all four geographic areas in southern California, 
   11% of the samples had unacceptable TPCs, but none of the ISB and manufactured ice 
   samples had TPCs above the PIQCS compliant limit of 500 CFU/mL. Among the four 
   areas, samples from Orange County had the lowest prevalence of unacceptable TPCs (2 
   of 30 samples, 7%) based on the PIQCS limit. Orange County samples also had a lower 
   average count of 77 most probable number (MPN)/mL (Fig. 1). In contrast, Los Angeles 
   (LA) County had the highest prevalence of unacceptable TPCs (13%) and a significantly 
   higher average count at 919 MPN/mL ($P < 0.05$), which is above the PIQCS limit (Table 
   2 and Fig. 1). The highest TPC was $>10^4$ MPN/mL in a sample collected from LA 
   County, and its melted liquid was distinctively turbid. Repeated samples collected from 
   the same store 2 months apart had similar TPCs, indicating a persistent problem (data not 
   shown). Compared with the on-site packaged ice, ISB and manufactured ice had much 
   lower mean TPCs of 3 to 14 MPN/mL compared with 77 to 919 MPN/mL for the on-site 
   packaged ice (Fig. 1). Overall, the on-site packaged ice samples were more likely to be of 
   unsatisfactory quality, and the average TPCs were higher than those for the ISB and
manufactured ice samples tested in this study. Samples from L.A., San Bernardino and Riverside, and San Diego and Imperial counties had higher TPCs than did at least one group of the non-on-site packaged ice samples ($P < 0.05$, Fig. 1).

Figure 1. Scatterplots of total heterotroph counts and coliforms. Scatterplots of total heterotroph counts (A) and coliform counts (B) sorted by sources and types of packaged ice products. On-site packaged ice samples were analyzed by geographical area: Los Angeles (LA), Orange County (OC), San Bernardino and Riverside counties (SB/R), and San Diego and Imperial counties (SD/I). These samples were compared with ice samples packaged by the manufacturer and those obtained from the in-store bagger (ISB), which were both IPIA compliant (IPIA) and noncompliant (non-IPIA). Samples with a zero value were plotted as 0.1 on the log scale for convenience of comparison. Horizontal bars indicate this means of each sample group. Dotted line indicates the PIQCS acceptable limit of $\leq$500 MPN/mL for heterotrophs. Brackets with asterisks indicate statistical significance: *$P < 0.05$; ***$P < 0.001$.

Of the 30 samples tested from each area, there are 3, 3, and 6 samples from L.A., Orange, and San Diego and Imperial counties, respectively, were positive for coliforms, as indicated by the fluorescent colonies on the MI plates (Table 2). On-site packaged ice samples from San Bernardino and Riverside counties and manufactured and ISB ice samples had no detectable coliforms. None of the samples tested were positive for E. coli, indicating a low likelihood of fecal contamination in the samples analyzed. The one-way
nonparametric ANOVA revealed no significant difference in coliform counting among the seven groups \((P > 0.05)\), probably because of smaller number of samples in the ISB and manufactured ice groups. However, when analyzing the on-site packaged ice samples versus the combined non-on-site packaged ice samples (ISB and manufactured combined), samples from San Diego and Imperial counties had significantly higher coliform counts than did samples from San Bernardino and Riverside counties \((P = 0.035)\) or the non-on-site packaged samples \((P = 0.023)\).

When applying the PIQCS microbiological criteria for TPCs of \(\leq 500\) MPN/mL and zero coliforms and \textit{E. coli} per 100 mL, the on-site packaged ice had an overall acceptable rate of 81%; the samples with the lowest counts originated in convenience stores in San Bernardino and Riverside counties (90%), followed by Orange County (83%), L.A. Country (80%), and San Diego and Imperial counties (70%) (Table 2). All manufactured and ISB ice samples (100%) were acceptable.

Additional tests were performed to determine the presence of \textit{Salmonella} and staphylococci (Table 2). \textit{Salmonella} was detected using the FDA PRL-SW validated qPCR procedure with a detection limit at 0.08 to 0.2 CFU/g (Ruby et al., 2009). None of the 156 samples were positive for \textit{Salmonella}. 
Table 2. Microbiological quality of 156 packaged ice samples collected in southern California

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of samples</th>
<th>unacceptable TPCs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Coliforms&lt;sup&gt;b&lt;/sup&gt;</th>
<th>unacceptable PIQCS quality&lt;sup&gt;a&lt;/sup&gt;</th>
<th>staphylococci</th>
<th>Yeasts/molds</th>
</tr>
</thead>
<tbody>
<tr>
<td>On-site packaged ice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Los Angeles County</td>
<td>30</td>
<td>4 (13)</td>
<td>3 (10)</td>
<td>6 (20)</td>
<td>11 (37)</td>
<td>22 (73)</td>
</tr>
<tr>
<td>Orange County</td>
<td>30</td>
<td>2 (7)</td>
<td>3 (10)</td>
<td>5 (17)</td>
<td>12 (40)</td>
<td>15 (50)</td>
</tr>
<tr>
<td>San Bernardino/ Riverside Counties</td>
<td>30</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>3 (10)</td>
<td>10 (33)</td>
<td>17 (57)</td>
</tr>
<tr>
<td>San Diego / Imperial Counties</td>
<td>30</td>
<td>4 (13)</td>
<td>6 (20)</td>
<td>9 (30)</td>
<td>8 (27)</td>
<td>13 (43)</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td>13 (11)</td>
<td>12 (10)</td>
<td>23 (19)</td>
<td>41 (34)</td>
<td>67 (56)</td>
</tr>
<tr>
<td>In-Store Bagger ice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-IPIA Compliant</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (25)</td>
</tr>
<tr>
<td>IPIA compliant</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Manufactured ice, IPIA compliant</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> PIQCS limits to be acceptable: TPC (total plate count) ≤ 500 MPN/mL and zero coliforms and E. coli

<sup>b</sup> None of the coliforms tested were identified as E. coli
Staphylococci were detected (based on the growth on Baird Parker agar) in 34% of the on-site packaged ice samples but not in the ISB or manufactured ice samples. The highest staphylococcal count was 528 CFU/100 mL, which was found in one of the L.A. County samples. Among the samples from San Diego and Imperial counties, which had the poorest quality based on TPCs and counts of coliforms and E. coli (Table 2), the number of samples positive for staphylococci was relatively low (8 of 30) compared with 10 or more positive samples from the other counties. The staphylococcal isolates were further tested for coagulase activity as an indication of the enterotoxigenic foodborne pathogen *Staphylococcus aureus*. Four of 41 staphylococcal isolates from these samples had weak coagulase activity, suggesting a positive result for *S. aureus*.

Interestingly, some common yeasts and molds were able to grow on Baird Parker plates for staphylococci detection. Since it is not designed to enumerate and detect yeasts and molds, the observation could only be used as a reference. The presence and absence of molds and yeasts colonies were noted and the results showed 56% of the on-site packaged ice samples to be positive for yeasts and molds growth on the Baird Parker plates, while all but three non-IPIA complied ISB ice samples showed negative in yeasts and molds on our plates.

2. **Microbial community analysis overview**

To examine the microbial diversity and abundance in packaged ice products, next generation sequencing analysis was performed using 16S and 18S rRNA gene targeted sequencing to determine the prokaryotic (bacteria and archaea) and eukaryotic microbial communities, respectively. Two on-site packaged and one IPIA-compliant ISB packaged ice products were sampled: on-site A, on-site B, and ISB, respectively. The on-site B
sample was chosen to represent the samples that did not meet the PIQCS standards. None of the manufactured ice produced sufficient DNA for positive sequencing reads. The diversity of microbial species is presented as the total OTUs. Each OTU represents a unique 16S or 18S rRNA taxonomic identity. All three samples generated at least 17,000 sequences from the 16S rRNA analysis. The two on-site packaged ice products had 194 and 136 OTUs, respectively. In contrast, the ISB packaged ice had much lower microbial diversity, with only 29 OTUs, most likely because its production occurred in a closed environment without human handling. Lastly, 18S rRNA analysis produced 10 times fewer sequencing reads than 16S rRNA data for all three samples and revealed lower eukaryote diversity, at 2, 15, and 6 OTUs for on-site A, on-site B, and ISB samples, respectively.

Principal coordinates analysis plots (PCoA) were used to visualize the correlations among the sample sets based on Bray-Curtis dissimilarity, observed OTUs, and Chao 1 (Fig. 2). The 16S rRNA profiles of the two on-site packaged ice samples are similar to each other, as indicated by the close locations on the plots with either the α- or β-analysis. The 16S rRNA profile of ISB packaged ice is distinctive from those of the on-site packaged ice products, as indicated by the distal location of the coordinates on both plots. The 18S rRNA eukaryote profiles of all three samples are distinct from the bacterial and archaeal profiles as expected. A Venn diagram was created to compare the unique and shared prokaryote OTUs among the three samples. The two on-site packaged ice samples shared 41 OTUs, whereas the ISB sample shared fewer than 5 OTUs with either of the two on-site samples (Fig. 3).
Figure 2. Principal coordinate analysis of microbial communities: On-site A, On-site B and ISB. Principal coordinates analysis plots of microbial communities based on Bray-Curtis dissimilarity analysis (A and B). Open and Closed symbols represent 16S and 18S rRNA sequencing results, respectively. (A) Principal component 1 (PC1) which explains 20.72% variance, is plotted against (PC2) (20.12%) variance explained). (B) PC1 is plotted against PC3, which explains 20.06% variance.

Figure 3. A Venn diagram of bacterial OTUs shared among the three samples
3. **16S rRNA sequence analysis of bacterial and archaeal communities in ice**

The identities of bacteria and archaea were determined by mapping the sequences to the Greengenes v. 13.5 16S rRNA database. The abundance data were calculated based on the sequence counts of each OTU divided by the copy number of each single species. *Proteobacteria* was the dominant phylum for all three samples, at approximately 90% for the two on-site packaged ice samples and almost 100% for the ISB packaged ice sample analyzed. Other than *Proteobacteria*, the on-site A and B samples also included OTUs in the phyla *Actinobacteria, Bacteroidetes, Firmicutes*, and *Verrucomicrobia*.

Within the *Proteobacteria*, *Betaproteobacteria* dominated the on-site A sample (72%) and *Alphaproteobacteria* dominated the on-site B and ISB samples at 63.55 and 99.97%, respectively (Fig. 4). The *Gammaproteobacteria*, including coliforms and many others, had low prevalence in all three samples analyzed, at 5.0, 0.6, and 0.006% of the on-site A, on-site B, and ISB samples, respectively (Fig. 4).

The relative abundance of the major genera or families (when the genera were not identified) is summarized in Table 3. Members of *Caulobacteraceae* (51%) and *Sphingobium* (43%) were the majority of bacteria in the ISB packaged ice analyzed in this study. These two taxa were also present in the two on-site packaged ice samples, at <2% relative abundance, implicating their common presence in the packaged ice samples.
Figure 4. Pie charts of relative abundance of bacterial communities: On-site A, On-site B and ISB FIGURE 4. Relative abundance of bacterial communities at the class level for the two on-site packaged ice sample (site A and site B) and the ISB packaged ice sample analyzed using 16S rRNA targeted sequencing. Individual OTUs with >1% abundance were plotted; OTUs with <1% were included in the “other” category.
Both of the on-site packaged ice samples analyzed in this study contain a diverse collection of bacterial OTUs (Figs. 3 and 4 and Table 3). The on-site A sample, which was compliant with the PIQCS, was dominated by methylotrophs such as *Methylophilaceae, Methylibium,* and *Comamonadaceae* at 31, 30 and 5% relative abundance, respectively (Table 3).

The on-site B ice sample, which was not PIQCS compliant, was dominated by photosynthetic purple non-sulfur *Alphaproteobacteria* in the family of *Rhodospirillaceae* at 53% relative abundance (including the genus *Phaeospirillum*) (Table 3). Twelve unique OTUs were identified for this family, indicating its abundance and diversity in the on-site B ice sample.

Some potential or opportunistic pathogens were found at low abundance in the two on-site packaged ice samples, suggesting a potential public health threat. Among the significant taxa, the *Firmicutes, Staphylococcus,* and *Burkholderia* were present in the on-site B sample at low abundance (≤ 0.1%) but were not found in the on-site A or ISB packaged ice. The results are in agreement with our staphylococci selective plating data (data not shown). The on-site A sample contained *Mycobacterium* at 4.6% (Table 2). *Legionella, Rickettsiales,* and *Pseudomonas* were found in both on-site A and B packaged ice samples at <0.1 % abundance (data not shown).
Table 3. Bacterial identification and relative abundance in the packaged ice samples analyzed by 16S rRNA sequencing

<table>
<thead>
<tr>
<th>Family or genus*</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>On site A</strong></td>
<td></td>
</tr>
<tr>
<td><em>Methylophilaceae</em> (β)</td>
<td>31.3</td>
</tr>
<tr>
<td><em>Methylibium</em> (β)</td>
<td>30.1</td>
</tr>
<tr>
<td><em>Comamonadaceae</em> (β)</td>
<td>5.23</td>
</tr>
<tr>
<td><em>Mycobacterium</em></td>
<td>4.64</td>
</tr>
<tr>
<td><em>Polaromonas</em> (β)</td>
<td>3.58</td>
</tr>
<tr>
<td><em>Rhodobacter</em> (α)</td>
<td>3.26</td>
</tr>
<tr>
<td><em>Bradyrhizobiaceae</em> (α)</td>
<td>3.09</td>
</tr>
<tr>
<td><em>Nevskia</em> (γ)</td>
<td>2.70</td>
</tr>
<tr>
<td><em>Xanthomonadaceae</em> (γ)</td>
<td>1.71</td>
</tr>
<tr>
<td><em>Sphingomonadales</em> (α)</td>
<td>1.46</td>
</tr>
<tr>
<td><em>Flectobacillus</em></td>
<td>1.32</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (α)</td>
<td>1.24</td>
</tr>
<tr>
<td><em>Oxalobacteraceae</em> (β)</td>
<td>1.18</td>
</tr>
<tr>
<td><em>Mycoplasma</em> (α)</td>
<td>1.08</td>
</tr>
<tr>
<td><strong>On-site B</strong></td>
<td></td>
</tr>
<tr>
<td><em>Rhodospirillaceae</em> (α)</td>
<td>46.1</td>
</tr>
<tr>
<td><em>Betaproteobacteria</em> (β)</td>
<td>12.8</td>
</tr>
<tr>
<td><em>Comamonadaceae</em> (β)</td>
<td>8.41</td>
</tr>
<tr>
<td><em>Phaeospirillum</em> (α)</td>
<td>6.98</td>
</tr>
<tr>
<td><em>Hyphomicrobiaceae</em> (α)</td>
<td>5.01</td>
</tr>
<tr>
<td><em>Prosthecochirus</em></td>
<td>3.00</td>
</tr>
<tr>
<td><em>Rhodocyclaceae</em> (β)</td>
<td>2.97</td>
</tr>
<tr>
<td><em>Methylibium</em> (β)</td>
<td>1.82</td>
</tr>
<tr>
<td><em>Lachnospiraceae</em></td>
<td>1.38</td>
</tr>
<tr>
<td><strong>ISB</strong></td>
<td></td>
</tr>
<tr>
<td><em>Caulobacteraceae</em> (α)</td>
<td>51.4</td>
</tr>
<tr>
<td><em>Sphingobium</em> (α)</td>
<td>43.0</td>
</tr>
<tr>
<td><em>Caulobacter</em> (α)</td>
<td>3.80</td>
</tr>
<tr>
<td><em>Sphingomonadales</em> (α)</td>
<td>1.60</td>
</tr>
</tbody>
</table>

*a Relative abundance was sorted at the genus level. Family names are listed only when genus names were unclassified. *Proteobacteria* classes are designated as α, β, or γ in parentheses. The list is sorted by the abundance >1% at the genus level.
4. **18S rRNA eukaryote community analysis**

The compositions of eukaryotic microorganisms were analyzed using 18S rRNA gene sequencing for the two on-site packaged ice samples and one ISB ice sample. The relative abundance of each OTU is listed in Table 4. The majority of the eukaryotic microorganisms identified were fungi, although some unicellular algae and protists were also identified. ISB packaged ice had one dominant yeast species, *Bensingtonia yamatoana* (98%), and five other minor species of fungi, algae, and protists.

The on-site A sample contained only two OTUs of the same yeast species, *Saccharomyces cerevisiae* (Table 4). The on-site B ice, which was not PIQCS compliant, contained diverse eukaryotic microbes, as expected. The dominant groups were the uncultured *Tremllales* yeasts (48%) and the two algal OTUs, a chrysophytic alga (46%) and the *Spumella* like flagellate 49D3 (3%) (Table 4). A separate OTU (*Cryptococcus carnescens*) was found in the same sample at 0.1% prevalence (Table 4). Furthermore, additional evidence of water and/or soil contamination in the on-site B sample was the presence of three protozoan species, *Vermamoeba vermiformis*, *Protostelium nocturnum*, and *Cercozoa* (Table 4). Lastly, the on-site B sample contained genes from sesame plants and/or seeds and from tree shrews, which may further indicate that the water was contaminated with material from animals, plants, and/or foods.
Table 4. Eukaryotic OTUs and relative abundance in the packaged ice samples analyzed by 18S rRNA sequencing

<table>
<thead>
<tr>
<th>OTU</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>On site A</strong></td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> M22</td>
<td>94.2</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> (baker’s yeast)</td>
<td>5.74</td>
</tr>
<tr>
<td><strong>On-site B</strong></td>
<td></td>
</tr>
<tr>
<td><em>Tremellales</em></td>
<td>47.9</td>
</tr>
<tr>
<td>Chrysophytes</td>
<td>46.4</td>
</tr>
<tr>
<td><em>Spumella</em>-like flagellate</td>
<td>3.39</td>
</tr>
<tr>
<td><em>Vermamoeba vermiformis</em></td>
<td>0.49</td>
</tr>
<tr>
<td><em>Protostelium nocturnum</em></td>
<td>0.39</td>
</tr>
<tr>
<td><em>Cercozoa incertae sedis</em></td>
<td>0.36</td>
</tr>
<tr>
<td><em>Sesamum indicum</em> (sesame)</td>
<td>0.20</td>
</tr>
<tr>
<td><em>Basidiomycota</em></td>
<td>0.16</td>
</tr>
<tr>
<td><em>Tupaia belangeri</em> (tree shrew)</td>
<td>0.16</td>
</tr>
<tr>
<td><em>Pheosphaericeae</em></td>
<td>0.10</td>
</tr>
<tr>
<td><em>Cryptococcus carnescens</em></td>
<td>0.10</td>
</tr>
<tr>
<td>Phialophora sp. GHP 1105</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Cryptococcus carnescens</em></td>
<td>0.07</td>
</tr>
<tr>
<td>Stramenopiles</td>
<td>0.07</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> M22</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>ISB</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bensingtonia yamatoana</em></td>
<td>98.1</td>
</tr>
<tr>
<td><em>Paracercomonas</em> sp.</td>
<td>1.53</td>
</tr>
<tr>
<td>Chrysophyte</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Sporobolomyces</em></td>
<td>0.10</td>
</tr>
<tr>
<td><em>Microbotryum violaceum</em></td>
<td>0.07</td>
</tr>
<tr>
<td><em>Rhodotorula nymphaea</em></td>
<td>0.03</td>
</tr>
</tbody>
</table>
5. **Viability study of packaged ice: overview**

A total of two compositied on-site packaged ice samples were subjected to PMA viability analysis, on-site C (from Los Angeles County) and on-site D (from San Diego County). Each sample was further divided to non-PMA treated (total DNA) and PMA treated (viable DNA) samples. The microbial community in the samples was assessed using 16S rRNA gene-targeted MiSeq sequencing. A total of 67,576 bacterial reads (mean ± standard deviation, 16,894 ± 8,121) were generated from the 4 samples (on-site C Total, on-site C Viable, on-site D Total and on-site D Viable). A total of 234 genera and 678 distinct OTUs (using a threshold of 97% identity) were identified.

Between the two PMA-untreated samples, on-site C yielded higher number of sequences than on-site D sample (Table 5). As expected, the un-treated PMA samples accounted for about 64 percent of the total sequences, whereas 36 percent of the sequences were retrieved from PMA-treated samples. Among the PMA treated samples, on-site C and on-site D samples accounted for 56% and 44%, respectively.

The compositions of dead and viable eukaryotic microorganisms were analyzed using 18S rRNA gene-targeted MiSeq sequencing. In total, 36,261 eukaryote reads (9065 ± 1933) comprising a total of 151 distinct eukaryotic OTUs were generated from the four samples. Similar to our earlier results for on-site A and B samples, the eukaryote OTUs were less diverse. Among the samples sequenced, eukaryote reads and the number of OTUs were more abundant in on-site C samples than on-site D samples (Table 5).

Overall, PMA treatment did reduce the numbers of reads and OTUs for both 16S and 18S rRNA microbial analyses for both samples studied, allowing us to differentiate the viable cells from the dead cells (Table 5).
Overall, on-site C showed more microbial diversity and generated more reads than on-site D. Furthermore, on-site C showed higher percentage of viability than on-site D. As expected, higher number of OTUs were generated than on-site A and on-site B in our second objective of this study because both on-site C and D were composited from 8 packaged ice samples each.

Table 5. Total reads and OTUs of 16S and 18S rRNA MiSeq sequencing for on-site C and on-site D

<table>
<thead>
<tr>
<th></th>
<th>On-site C</th>
<th>On-site D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Viable</td>
</tr>
<tr>
<td>Prokaryotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total reads</td>
<td>28,833</td>
<td>13,776</td>
</tr>
<tr>
<td>OTUs</td>
<td>612</td>
<td>435</td>
</tr>
<tr>
<td>Eukaryotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total reads</td>
<td>11,732</td>
<td>9,133</td>
</tr>
<tr>
<td>OTUs</td>
<td>112</td>
<td>84</td>
</tr>
</tbody>
</table>

6. Viability study of packaged ice: 16S rRNA analysis of bacteria communities

The 16S rRNA targeted gene Illumina sequencing data analysis revealed the altered viable microbial community profiles in the packaged ice. In the on-site C Total sample, profiles at the phylum level constituted 88% or more by Proteobacteria and Bacteroidetes and similarly, on-site C Viable sample was dominated by Proteobacteria and Bacteroidetes (Fig. 5). At the class level, the changes in total and viable microbial community between on-site C Total and on-site C Viable were more noticeable. In on-site C Total sample, Betaproteobacteria, Alphaproteobacteria, Gammaproteobacteria, and Cytophagia were most prevalent. In contrast, total reads of Betaproteobacteria
relatively stayed the same whereas *Alphaproteobacteria* had a dramatic drop. *Actinobaceteria* was dropped from 3.1% to 0.7% and as well as *Cytophagia* in the on-site C Viable sample. Interestingly, *Gammaproteobacteria* remained almost the same. At the Genus level, the profiles were dominated by *Proteobacteria* (*Pseudomonas, Mitsuaria, Polaromonas, Methylophilus, Janthinobacterium, Sphingomonas, etc.*) and *Bacteroidetes* (*Cytophagaceae* and two unclassified/unknown *Bacteroidetes* OTUs). The unknown indicates that the taxonomic classification at a certain classification level was not determined due to low confidence level. In the on-site C Viable sample, *Proteobacteria* was dominant once again; however, the abundance of individual genera was shifted within the *Proteobacteria* phylum (*Mitsuaria, Pseudomonas, Janthinobacterium, Polaromonas, Massilia, Methylophilus, Sphingomonas, Aquabacterium, and Brevundimonas*). The Cytophagaceae (*Bacteroidetes*) was decreased from 7.4% to 3.3% whereas *Flavobacterium* increased from 0.8% to 1.7% (Table 5).

![Figure 5. The total reads of on-site C at the phylum level. Total reads were combined and listed as others if too low.](image)
Figure 6. Total reads of on-site C sample at class level. Classes that were in low abundance were composited as ‘Others’.
Table 6. Bacterial identification, total reads and relative abundance of total and viable percentage in the packaged ice on-site C sample analyzed by 16S rRNA sequencing

<table>
<thead>
<tr>
<th>Family or Genus(^a)</th>
<th>Total reads (relative abundance %)</th>
<th>Total</th>
<th>Viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Pseudomonas} (γ)</td>
<td>3,369 (11.9)</td>
<td>1,594 (11.9)</td>
<td></td>
</tr>
<tr>
<td>\textit{Mitsuaria} (β)</td>
<td>2,488 (8.79)</td>
<td>1,877 (14.0)</td>
<td></td>
</tr>
<tr>
<td>\textit{Cytophagaceae}</td>
<td>2,103 (7.44)</td>
<td>438 (3.30)</td>
<td></td>
</tr>
<tr>
<td>\textit{Polaromonas} (β)</td>
<td>1,924 (6.80)</td>
<td>1,212 (9.07)</td>
<td></td>
</tr>
<tr>
<td>\textit{Methylophilus} (β)</td>
<td>1,680 (5.94)</td>
<td>976 (7.30)</td>
<td></td>
</tr>
<tr>
<td>\textit{Janthinobacterium} (β)</td>
<td>1,612 (5.70)</td>
<td>1,348 (10.1)</td>
<td></td>
</tr>
<tr>
<td>\textit{Sphingomonas} (α)</td>
<td>1,583 (5.69)</td>
<td>429 (3.21)</td>
<td></td>
</tr>
<tr>
<td>\textit{Aquabacterium} (β)</td>
<td>684 (2.63)</td>
<td>310 (2.32)</td>
<td></td>
</tr>
<tr>
<td>\textit{Brevundimonas} (α)</td>
<td>470 (1.66)</td>
<td>137 (1.02)</td>
<td></td>
</tr>
<tr>
<td>\textit{Massilia} (β)</td>
<td>435 (1.54)</td>
<td>766 (5.73)</td>
<td></td>
</tr>
<tr>
<td>\textit{Rhodobacteraceae} (α)</td>
<td>337 (1.19)</td>
<td>34 (0.25)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Relative abundance was sorted at the genus level. Family names are listed only when genus names were unclassified. \textit{Proteobacteria} classes are designated as α, β, or γ in parentheses. The list is sorted by the abundance >1% at the genus level.

Similar to on-site C Total samples, \textit{Proteobacteria} was dominant at the phylum level in on-site D Total sample, and on-site D Viable sample was dominated by \textit{Proteobacteria} and \textit{Firmicutes}. Furthermore, at the class level, \textit{Betaproteobacteria}, \textit{Alphaproteobacteria} and \textit{Gammaproteobacteria} were present in the on-site D Total sample. When examining the on-site D Viable, based on reads, \textit{Betaproteobacteria} had the highest survivability, \textit{Alphaproteobacteria} reads dropped drastically which indicates many cells are dead, and most \textit{Gammaproteobacteria} cells were alive (Fig. 8). Interestingly, the abundance of \textit{Bacilli} was significantly increased from 0.04 to 20.1%. Within the \textit{Bacilli} Class in On-site D Viable sample, Genus \textit{Bacillus} dominated the profile with 20.4%; however, \textit{Bacillus} was not present in the on-site D Total sample. This may be due to a sequencing error or a DNA isolation technical error. At the genus level, \textit{Janthinobacterium}, \textit{Sphingomonas}, \textit{Mitsuaria}, \textit{Polaromonas}, unknown, \textit{Methylophilus}, and \textit{Aquabacterium} were most dominantly present in on-site D Total sample. In comparison,
*Janthinobacterium, Bacillus, Mitsuaria, Polaromonas, Sphingomonas,* unknown, and *Methylophilus* were present in on-site D Viable sample (Table 6).

Furthermore, the viability of opportunistic or potential pathogens were evaluated in each sample. In on-site C sample, most notable opportunistic or potential pathogens were present but dead include *Bacillus* and *Staphylococcus*. Some of them were viable such as *Brucella, Clostridium botulinum, Legionella, Mycobacterium,* and *Pseudomonas.* Furthermore, the viable potential/opportunistic pathogens in on-site D sample include, *Neisseria, Pseudomonas* and *Staphylococcus.* whereas dead ones include *Mycobacterium* sp. and *Legionella* sp.. The relative abundance of these potential/opportunistic pathogens were very low (less than 0.2, except *Pseudomonas*); however, their presence still raises concerns for consumer’s safety. Lastly, a group of *Enterobacteriaceae,* a possible indicator of enteric bacteria, was found at low relative abundance. *Erwinia rhapontici* was viable in on-site C sample and *Buchnera aphidicola* was present in on-site D Total only which indicates its death. *Cronobacter dublinensis* was viable in both on-site C and D (data not shown). They are not known as pathogens/opportunistic pathogens.

![Figure 7](image.png)

Figure 7. Total reads of on-site D sample at phylum level. The total reads were combined and listed as others if too low.
Figure 8. Total reads of on-site D sample at class level. If total reads were too low, they were composited as others.
**Table 7. Bacterial identification and relative abundance of total and viable percentage in the packaged ice on-site D sample analyzed by 16S rRNA sequencing**

<table>
<thead>
<tr>
<th>Family or Genus</th>
<th>Total reads (relative abundance %)</th>
<th>Viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Janthinobacterium (β)</td>
<td>4,249 (30.0)</td>
<td>4,821 (45.2)</td>
</tr>
<tr>
<td>Sphingomonas (α)</td>
<td>2,528 (18.2)</td>
<td>414 (3.88)</td>
</tr>
<tr>
<td>Mitsuaria (β)</td>
<td>1,440 (10.2)</td>
<td>599 (5.62)</td>
</tr>
<tr>
<td>Polaromonas (β)</td>
<td>941 (6.64)</td>
<td>522 (4.90)</td>
</tr>
<tr>
<td>Pseudomonadales (γ)</td>
<td>638 (4.50)</td>
<td>331 (1.40)</td>
</tr>
<tr>
<td>Methylophilus (β)</td>
<td>393 (2.77)</td>
<td>234 (2.19)</td>
</tr>
<tr>
<td>Cytophagaceae</td>
<td>273 (1.93)</td>
<td>24 (0.22)</td>
</tr>
<tr>
<td>Aquabacterium (β)</td>
<td>258 (1.82)</td>
<td>2 (0.01)</td>
</tr>
<tr>
<td>Pseudomonas (γ)</td>
<td>243 (1.71)</td>
<td>149 (1.40)</td>
</tr>
<tr>
<td>Methylobacterium (α)</td>
<td>237 (1.67)</td>
<td>90 (0.38)</td>
</tr>
<tr>
<td>Bradyrhizobium (α)</td>
<td>224 (1.58)</td>
<td>21 (0.20)</td>
</tr>
<tr>
<td>Bacillus</td>
<td>0 (0.0)</td>
<td>2,234 (21.0)</td>
</tr>
</tbody>
</table>

*Relative abundance was sorted at the genus level. Family names are listed only when genus names were unclassified. Proteobacteria classes are designated as α, β, or γ in parentheses. The list is sorted by the total reads at least.

7. **Viability study of packaged ice: top viable bacteria classes**

In order to investigate the viability of the major bacteria groups in on-site C and on-site D, bacterial classes that consisted of more than 7 OTUs were retrieved and calculated their viability percent. For example, 30 (on-site C Viable) out of 32 OTUs (on-site C Total) under Sphingobacteria classes remained viable in On-site C sample. It indicates that 93.8% (30/32 OTUs) of the OTUs under Sphingobacteria class is viable and shows the robust nature of this class of microorganism.

Among the other top viable classes are, Acidobacteria, Cytophagia, Flavobacteria, Betaproteobacteria, and Gammaproteobacteria and the classes with the lowest viability are Planctomycetia, and Actinobacteria. In comparison, the highest percentage of viability belonged to Betaproteobacteria in on-site D sample. In addition, Gammaproteobacteria, Alphaproteobacteria, Cytophagia, and Actinobacteria were
viable in sample on-site D. Overall, Gram-negative bacteria showed the highest percentage of viability in both samples.

Table 8. Number of OTUs at class level in on-site C and on-site D samples

<table>
<thead>
<tr>
<th>Class</th>
<th>On-site C</th>
<th></th>
<th>On-site D</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Viable %</td>
<td>Total</td>
<td>Viable %</td>
</tr>
<tr>
<td>Acidobacteriia</td>
<td>7</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>32</td>
<td>37.5</td>
<td>3</td>
<td>20.0</td>
</tr>
<tr>
<td>Cytophagia</td>
<td>32</td>
<td>90.6</td>
<td>10</td>
<td>30.0</td>
</tr>
<tr>
<td>Flavobacteria</td>
<td>20</td>
<td>90.0</td>
<td>4</td>
<td>25.0</td>
</tr>
<tr>
<td>Sphingobacteria</td>
<td>32</td>
<td>93.8</td>
<td>9</td>
<td>11.1</td>
</tr>
<tr>
<td>Planctomycetia</td>
<td>33</td>
<td>39.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>133</td>
<td>69.9</td>
<td>23</td>
<td>37.5</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>32</td>
<td>93.8</td>
<td>15</td>
<td>62.5</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>43</td>
<td>79.1</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>GammaProteobacteria</td>
<td>36</td>
<td>91.7</td>
<td>10</td>
<td>45.0</td>
</tr>
</tbody>
</table>

8. Viability study of packaged ice: 18S rRNA analysis of eukaryotes

In on-site C sample, there were diverse groups of eukaryotic microorganisms with various ranges of viable percentages. For sake of the simplicity, Protist kingdom will include all unclassified Eukaryota, no hit, and unknown due to sequencing nature. A diverse number of OTUs have been identified in all eukaryotic kingdoms in on-site C Total, including 13, 20, 18, and 60 OTUs in Animalia, Fungi, Plantae, and Protists, respectively (Table 8). Among the members at kingdom level, Animalia showed the highest viability percentage with 92.3%. Plantae, Fungi and Protists showed 72.2%, 65.0% and 61.5%, respectively.

The on-site D sample, similar to 16S rRNA targeted gene sequencing, showed less diverse groups of eukaryotic microorganisms present in the sample. As a result, a total number of 13, 18, 18, and 28 OTUs were present in Animalia, Fungi, Plantae, and
Protists, respectively (Table 8). Furthermore, On-site D sample showed less viability than the On-site C sample. However, similarly, Animalia had the highest viability percentage at 69.2%, followed by Fungi at 50%, Protists at 46%, and Plantae at 22.2%.

We also analyzed the relative abundance of each eukaryotic group in the “Total” and “Viable” samples in order to further to reveal the viability state of each sample. When incorporating the relative abundance data, Fungi and Animalia were dominating, totaling 59.0% and 22.6%, respectively, in on-site C Total sample. Interestingly, the relative abundance of Fungi decreased to 36.9, and Animalia increased to 52.6% in on-site C Viable sample, implying a likelihood of fungi being dead in this sample (Table 9). Similarly, Fungi and Animalia dominated the On-site D Total sample. Unlike the on-site C sample, in on-site D Viable, the relative abundance of Animalia decreased whereas Fungi increased, indicating the viability state is unique to individual samples and may vary due to the type of species in the sample.

At species level, many OTUs were identified as unknown or unclassified; therefore, they were recorded with the closest identifiable level. In on-site C Total sample, top four eukaryotic genera with the highest abundance were Microbotryomycetes, Bdelloidea, Plectidae, and Streptophyta (Table 10). Microbotryomycetes showed over 50% relative abundance in the sample containing both viable and dead cells (C Total), but has decreased to ~35% in the viable population (C Viable). Interestingly, Bdelloidea showing ~15% in the Total sample has dramatically increased to over 44% relative abundance in the viable population, indicating its high viability state in the sample.

In on-site D samples, the top five eukaryotic species (or OTUs) with the highest relative abundance were Microbotryomycetes, Bradysia, Bdelloidea, Filobasidiaceae,
and Chrysophyceae. In comparison, the relative abundance of Microbotryomycetes and Bdelloidiea were increased in on-site D Viable sample, indicating their superior survivability in ice (Table 11).

In summary, on-site C and on-site D samples showed diverse eukaryotic communities. Animalia showed the highest viability in on-site C sample whereas Fungi had the highest viability in on-site D sample. Interestingly, Plantae had the lowest viability percentage in both samples. Microbotryomycetes, a Basidiomycota fungal group, make up over one third of the viable and total population in both of our on-site packaged ice samples (Table 10 and 11). Bdelloidiea, a microscopic zooplankton in fresh water, showed a drastic increase of its relative abundance in the viable population for both samples, probably due to its capability in surviving the ice processing environment.

Table 9. The number of OTUs by identification of On-site C and On-site D

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Number of OTUs</th>
<th>On-site C</th>
<th>%</th>
<th>On-site D</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Viable</td>
<td></td>
<td>Total</td>
<td>Viable</td>
</tr>
<tr>
<td>Animalia</td>
<td>13</td>
<td>12</td>
<td>92.3</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Fungi</td>
<td>20</td>
<td>13</td>
<td>65.0</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Plantae</td>
<td>18</td>
<td>13</td>
<td>72.2</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Protists*</td>
<td>60</td>
<td>46</td>
<td>76.7</td>
<td>28</td>
<td>13</td>
</tr>
</tbody>
</table>

* Protists include OTUs that were classified under “Eukaryota Kingdom” in Green Genes Database.
### Table 10. Total reads and relative abundance of eukaryotes in On-site C and On-site D

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>On-site C Total</th>
<th>Viable (relative abundance %)</th>
<th>On-site D Total</th>
<th>Viable (relative abundance %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animalia</td>
<td>2,655 (22.6)</td>
<td>4,801 (52.6)</td>
<td>3,383 (41.7)</td>
<td>2,824 (38.8)</td>
</tr>
<tr>
<td>Fungi</td>
<td>6,920 (59.0)</td>
<td>3,374 (36.9)</td>
<td>3,531 (43.5)</td>
<td>3,857 (53.0)</td>
</tr>
<tr>
<td>Plantae</td>
<td>1,048 (8.93)</td>
<td>230 (2.52)</td>
<td>481 (5.93)</td>
<td>289 (3.97)</td>
</tr>
<tr>
<td>Protists</td>
<td>1,109 (9.47)</td>
<td>718 (7.98)</td>
<td>721 (8.87)</td>
<td>310 (4.23)</td>
</tr>
<tr>
<td>Total reads</td>
<td>8,116</td>
<td>7,280</td>
<td>11,732</td>
<td>9,133</td>
</tr>
</tbody>
</table>

### Table 11. Total reads and relative abundance of top four OTUs in On-site C sample

<table>
<thead>
<tr>
<th>OTUs*</th>
<th>Total reads (relative abundance %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbotryomycetes</td>
<td>6,470 (55.1)</td>
</tr>
<tr>
<td>Bdelloidea</td>
<td>1,750 (14.9)</td>
</tr>
<tr>
<td>Plectidae</td>
<td>755 (6.43)</td>
</tr>
<tr>
<td>Streptophyta</td>
<td>713 (6.07)</td>
</tr>
</tbody>
</table>

*Name of the lowest identifiable level was used.

### Table 12. Total reads and relative abundance of top five On-site D sample

<table>
<thead>
<tr>
<th>OTUs*</th>
<th>Total reads (relative abundance %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbotryomycetes</td>
<td>2,748 (33.9)</td>
</tr>
<tr>
<td>Bradysia</td>
<td>2,542 (31.2)</td>
</tr>
<tr>
<td>Bdelloidea</td>
<td>665 (8.20)</td>
</tr>
<tr>
<td>Filobasidiaceae</td>
<td>526 (6.48)</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td>296 (3.65)</td>
</tr>
</tbody>
</table>

*Name of the lowest identifiable level was used
CHAPTER 4

Discussion

According to FDA, average American buys about four bags of packaged ice each year. Ice is classified as a food by the FDA, and if safety practices are neglected, it can make us ill. A recent study by Leeds Beckett University was reported in June, 2017 on the microbiological quality of ice from the United Kingdom’s biggest coffee chains such as Starbucks, Costa Coffee, and Caffe Nero. According to the study, 13 out of 30 ice samples contained opportunistic pathogens or fecal coliforms, and these findings caused major concerns for costumers. Similar to the UK’s study, but in a much larger scale, our first objective was to test microbiological quality of packaged ice (ISB, manufactured, and on-site) from southern California.

Result of the culture based microbiological quality analysis of the packaged ice samples indicated that there is a significant difference between ISB, manufactured, and on-site samples. On-site sample showed poor microbiological quality. The contamination can occur at various points. During the sample collection, it was noted some on-site packaged ice were placed with other types of foods, the packaging was done poorly, and some were melting because the freezer was not working properly. Furthermore, using contaminated water or not cleaning the ice machine filter might have caused poor microbiological quality of on-site packaged ice. In comparison, ISB and manufactured packaged ice satisfied the PIQCS’s expectations. Our results confirm the high microbiological quality of manufactured ice, as previously reported from other regions (Mako et al., 2014) and Moyer et al., 1993). We also determined that the ISBs (whether IPIA compliant or not) produced ice products of satisfactory microbiological quality,
possibly because of lack of human handling. When comparing results of TPC analyses of on-site packaged ice from different studies, 11% of our on-site samples had unacceptable TPCs (Table 2), whereas the unacceptable TPC rate was 6% in Georgia (Mako et al., 2014), 36% in Iowa (Moyer et al., 1993), and 11% in the United Kingdom (Nichols and Gillespie, 2000).

The presence of coliforms in food may indicate poor environment sanitation or unhygienic practices during or after food production since coliforms are normally found in the intestines of warm blooded animals or in the environment such as soil, vegetation and water (Nkere et al., 2011). Among coliforms, E. coli is one of the well-known fecal coliforms because they are commonly found in the intestinal tracts of all warm-blooded animals, including humans. In our study, E. coli was not detected in all packaged ice samples despite of the sources (Table 2). In comparison, 5% of ice used in food displayed contained E. coli in the United Kingdom (Nichols and Gillespie, 2000) and 22% of ice used to cool drinks and foods in Greece (Gerokomou et al., 2011). In another study, 50 strains of E. coli were identified from 23 ice samples, 12 were enteroaggregative E. coli which can cause severe diarrhea (J. P. Falcão et al., 2004). For coliforms, the percentage of samples positive for coliforms was 10% for our on-site packaged ice samples (Table 2), 37% in both Georgia and Greece (Gerokomou et al., 2011; Mako et al., 2014), 13.5% in Florida (Schmidt and Rodrick, 1999), 4.5% in Iowa (Moyer et al., 1993), and 9% in the United Kingdom (Nichols and Gillespie, 2000) and Hong Kong (Hong Kong, 2005). The variations among these studies may be attributed to the sources of water, the nature of the samples, and the testing methods. Nevertheless, in all studies the microbiological quality of the on-site packaged ice was lower than the manufactured ice.
Salmonella can cause food poisoning called salmonellosis. Food can be infected with Salmonella during unhygienic food processing or poor food handling (South et al., 2002). According to our results, Salmonella was not found in any of the samples including the on-site packaged ice. This result was similar to those found previously; Salmonella was found in only 1 of 275 samples in Georgia (Mako et al., 2014) and was not found in other studies (Hong Kong 2005; Moyer et al., 1993; Nichols and Gillespie, 2000; Schmidt and Rodrick, 1999). In Greece, Salmonella was found in 4% of the ice used to cool drinks, which may be a severe public health concern (Gerokomou et al., 2011).

Staphylococci are part of the human skin microbiota and are routinely used as indicators of contamination attributed to food handlers (Gabutti et al., 2000; Lechevallier and Seidler, 1980) Staph food poisoning symptoms such as vomiting, nausea, stomach cramps, and diarrhea, usually develop within 30 minutes to six hours due to their fast acting Staphylococcal toxins (Do Carmo et al., 2004). The presence of Staphylococcal colonies in 34% of our on-site packaged ice raises a concern of Staph food poisoning. Furthermore, the highest staphylococcal count was from one of the LA County convenient store with 528 CFU/ml. The presence of staphylococci was detected in a second sample collected 2 months later (data not shown), which indicated the persistence of the problem. In addition, more than 300 CFU/ml of staphylococcal colonies were detected in one of the on-site packaged ice samples in SB/RS County. These findings suggest that the on-site packaging process probably introduced these bacteria into the ice through contacting with food handlers who may not wear gloves or using contaminated water for making packaged ice. Staphylococci were also isolated from ice samples in Iowa (Moyer et al., 1993) and from 144 of 320 rural drinking water samples evaluated at
Oregon State University (Lechevallier and Seidler, 1980); among these 144 positive samples, 20 samples contained \textit{S. aureus}.

Next generation sequencing methods have fundamentally enhanced our understanding of microbial communities in the environment. As a second objective, the difference in microbiological communities between on-site and ISB packaged ice were examined, for the first time to our knowledge, using 16S/18S targeted gene sequencing by Illumina MiSeq sequencing. Overall, we found that there is more prokaryote diversity in the packaged ice samples when compared to the eukaryotic microorganisms. Other studies also have shown that eukaryotic organisms are less prevalent than bacterial organisms in water (Uyaguari-Diaz \textit{et al.}, 2016; Gomez-Alvarez \textit{et al.}, 2012).

We further analyzed the microbial community composition in packaged ice samples. We observed that the on-site packaged ice differed considerably from ISB packaged ice based on the PCoA plots (Figure 4) and Venn diagram (Figure 3) and analyses. PCoA is a method to visualize and summarize and represent between objects of their (dis)similarity on a dimensional chart. Objects that are close to one another are more similar than those are placed farther away (Callahan \textit{et al.}, 2016). Our results revealed that on-site A and on-site B samples were placed close to each other whereas ISB sample was far away from them. This is probably because on-site A and on-site B have dynamic ice making variables (water, ice making, storage and handling) whereas ISB ice is produced in a closed system with filtered water and machine without operators.

The Venn diagram revealed that on-site A and on-site B shared 41 OTUs together whereas ISB sample shared only 2 and 4 OTUs with on-site A and on-site B, respectively. When comparing the bacterial diversity, on-site packaged ice has a higher
number of OTUs than ISB packaged ice (Figure 3). The ISB packaged ice sample had a less diverse microbial community and mainly constituted with oligotrophic microbes because ice is made and packaged by the machine, and water is not contaminated. Although manufactured packaged ice was not included in our sequencing analysis due to insufficient DNA, they would probably place near ISB sample in PCoA plot and have low OTUs due to similar ice making processes.

Interestingly, the number of OTUs is not always correlated with the TPCs. For example, the TPC for the on-site A sample was 128 MPN/mL and 2.4 X 10^4 MPN/mL for on-site B. Despite its lower TPC, the on-site A sample was more diverse (194 OTUs) than on-site B sample (136 OTUs) (Figure 3). This is might have occurred because a small number of species are dominating the high TPC sample.

We further analyzed the microbial community in the packaged ice samples. The phylum Proteobacteria dominates all three samples, making up of more than 90% of the microbial populations in the on-site A, on-site B and ISB samples (Figure 4). Several studies reported that Proteobacteria is dominating the microbial communities in the freezing environments such as Arctic ice (Brinkmeyer et al., 2003), Arctic tundra soil (Männistö et al., 2009), glaciers (Foght et al., 2004) and Greenland ice core (Miteva et al., 2015). Proteobacteria are the primary inhabitants of the ground water system (Karwautz and Lueders, 2014). It was not surprising that our results echo many previous microbial community analyses of drinking water. Within the Proteobacteria, Alphaproteobacteria and Betaproteobacteria are most prevalent in most of our samples and the distributions between these two classes varied by samples (Figure 4, 6, and 8). Similar results were reported in studies on drinking water. Alphaproteobacteria was
represented 43% of the total clones examined from drinking water (Humrighouse et al., 2006) and 51% in chlorinated distribution system water in Ohio (Revetta et al., 2007). In addition, water with monochloramine treatment was dominated by *Alphaproteobacteria* (23%) and *Betaproteobacteria* (25%), and free-chlorine treated water was dominated largely by *Alphaproteobacteria* (35%) (Gomez-Alvarez et al., 2012). Furthermore, *Betaproteobacteria* were reported as 80 to 98% of the bacteria in bottled mineral water produced in Europe as determined by fluorescence in situ hybridization and 16S rRNA sequence analysis (Loy et al., 2005).

Besides the dominance of *Proteobacteria*, the phylum *Actinobacteria, Bacteroidetes, Firmicutes*, and *Verrucomicrobia* are present in one or more of the four on-site samples studied (Figure 4, 5, and 7). All of these phyla are known to abundantly inhabit soil, environments, and guts of humans and animals (Jovel et al., 2016 and Penton et al., 2016). *Actinobacteria* was present in monochloramine water (28%) (Gomez-Alvarez et al., 2012). The bulk water for drinking contained *Bacteroidetes* (25%) and *Actinobacteria* (16%) in Germany (Henne et al., 2012). *Firmicutes* were largely represented in all sewage treatment plants (Mclellan et al., 2013). *Verrucomicrobia* was found in biofilm and bulk water samples from drinking water distribution system (Martiny et al., 2005). The presence of these phyla further suggests that on-site packaged ice samples have more microbial diversity. None of the ice products had any Archaea, in corroboration with other reports that Archaea are rare (at low levels) in drinking water system (Gomez-Alvarez et al., 2012).

Members of the *Caulobacteraceae* and *Sphingobium* dominated the microbial composition of ISB sample. Both *Caulobacteraceae* and *Sphingobium* are
Alphaproteobacteria and can be abundant in chlorinated drinking water, bottled mineral water, ground water, and glacial ice (Balkwill et al., 2006; Gomez-Alvarez et al., 2012; Karwautz and Lueders, 2014; Loy et al., 2005; Simon et al., 2009). These microorganisms can also be abundant in aquatic environments as part of biofilms (Vaz-Moreira et al., 2011). The presence of Caulobacteraceae and Sphingobium in our ISB packaged ice samples suggests the presence of biofilms in the ice machine and/or the system from which the water originated. No health concerns have been associated with these microorganisms.

When analyzing at the family/genus level, methylotrophs (Methylophilaceae, Methylibium, and Comamonadaceae) were dominating the on-site A packaged ice (Table 3). The genus Methylibium is a relatively new genus described in 2006 for methylotrophs that are able to use methanol as a sole carbon source (Nakatsu et al., 2006). Methylibium strains isolated from gasoline-contaminated aquifers or compost-filled filters have been shown to degrade gasoline additive ethers, such as methyl tert-butyl ether (MTBE) (Nakatsu et al., 2006; Szabó et al., 2015). The family Comamonadaceae has been reported as the primary member of the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)-degrading denitrifying bacteria in activated waste water sludge (Khan et al., 2007). Therefore, it is possible that Comamonadaceae, including the genus Methylibium, originated from waste water treatment processes that use methanol or PHBV to enrich denitrifying bacteria (Khan et al., 2007). Methylophilaceae, the second largest family in the On-site A ice, is also a methylotroph prevalent in surface water, terrestrial and marine environment and is known to play a role in carbon cycling (Beck et al., 2014). The presence of primarily simple methylotrophs in the ice product was expected due to the
extremely oligotrophic condition of drinking water and ice (Karwautz and Lueders, 2014). Therefore, these methylotrophs could have been enriched and thus survived the water treatment process.

Members of the *Rhodospirillaceae*, the purple non-sulfur bacteria, can be found in anaerobic aquatic environments such as mud and stagnant water and has been found to dominate our on-site B packaged ice sample (Table 3). These bacteria have been found in ground and well water at up to 10% abundance (Karwautz and Lueders, 2014). The source water for the on-site B ice sample probably was either untreated or polluted with ground and/or surface water, resulting in contamination with *Rhodospirillaceae*. The water also may have been subjected to abusive conditions that encouraged the growth of *Rhodospirillaceae* and *Comamonadaceae* to high levels.

*Pseudomonas, Legionella, Rickettsiales*, and *Mycobacterium* were found in all on-site samples analyzed. Even though these opportunistic or potential pathogens were found at a low abundance, the presence of pathogens has raised concern for consumers. *Mycobacterium* and *Legionella* cause some respiratory infections and were found in monochloramine-treated drinking water and chlorinated water samples, respectively (Gomez-Alvarez et al., 2012). *Pseudomonas* is a large group of free-living bacteria that can be found naturally in the ground and drinking water (Hardalo and Edberg, 1997; Mena and Gerba, 2009) that might cause a wide range of infections (Mena and Gerba, 2009). Unfortunately, the species was not revealed for our *Pseudomonas* OTUs; therefore, the pathogenicity was unclear. Furthermore, the Order *Rickettsiales* are among the groups of *Proteobacteria* and has some notable pathogens (*Wolbachia, Neorickettsia* and *Rickettsia*) (Yu and Walker, 2006). Lastly, *Enterobacteriaceae*, including coliforms
and pathogens such as *Salmonella* and *E. coli*, were absent from ISB and on-site packaged ice A and B samples, indicating that fecal contamination was unlikely in the samples analyzed. The lack of *Enterobacteriaceae* was also reported in studies of drinking water (Karwautz and Lueders, 2014).

The eukaryotic profiles was not as abundant as prokaryotes. The dominance of *Bensingtonia yamatoana* accounted for 98% of the ISB packaged ice. *B. yamatoana* is a basidiomycetous budding yeast that has been found on the surfaces of plants (Nakase, 2000) and is phylogenetically related to *Rhodotorula arctica* found in arctic soil (Vishniac and Takashima, 2010). However, its prevalence in water and other sources has not been reported. The ISB ice also contained other yeasts such as *Rhodotorula*, *Microbotryum violaceum*, and *Sporobolomyces*, which are known to be prevalent in oligotrophic water and deep ground water and can originate from the microbial effluents of plants (Nagahama, 2006).

In on-site A sample, which only had two eukaryotic OTUs belonging to the species, *Saccharomyces cerevisiae*. *S. cerevisiae* can be found in human environments because it’s common use in brewing and baking and in its natural habitat on the surface of fruits and trees (Liti, 2015). The eukaryote profile of on-site B was more complex than other two samples, as expected. There were two dominant groups in the sample: uncultured *Tremellales* yeasts (48%) and Chrysophytic alga (46%). *Tremellales* is a group of basidiomycetous yeasts that include opportunistic pathogens such as *Cryptococcus* (Liu *et al.*, 2015). It has occasionally been associated with opportunistic infections (Ferreira-Paim *et al.*, 2014; Takashima *et al.*, 2003). *Cryptococcus* has been found in indoor dust (Hanson *et al.*, 2016). Chrysophytes and *Spumella* are major groups of nanoflagellates in
fresh waters and soil of various geographical regions (Boenigk et al., 2005). Additional evidence of water and/or soil contamination in the on-site B sample was the presence of three protozoan species, *Vermamoeba vermiformis*, *Protostelium nocturnum*, and *Cercozoa* (Table 4). *V. vermiformis* is a free-living amoeba prevalent in the environment and in treated drinking water, especially in hot water systems, probably because of its resistant cysts (Buse et al., 2013; Fouque et al., 2014). These amoebae could be fed by biofilms and waterborne pathogens such as *Legionella* (Lau and Ashbolt, 2009). In our study, several *Legionella* OTUs were found in both the on-site A and on-site B ice samples, indicating a potential interaction between the amoebae and the potentially pathogenic bacteria. The on-site B sample also contained genes from sesame plants and/or seeds and from tree shrews, which may further indicate that the water was contaminated with material from animals, plants, and/or foods. The analysis of the eukaryotic microbial community again supports the hypothesis that the on-site B ice may have been made from surface or well water, may have been cross-contaminated with environmental material, and/or may have been abused for an extended time.

Overall, the two clean ice samples, on-site A and ISB, show the presence of primarily yeasts and protists that are commonly found in source water or plant effluents. Many species are known to survive chemical treatments and are oligotrophic, allowing them to live in minimal nutritional environments. The dirty sample, on-site B, showed diverse eukaryotic microorganisms in all three groups of unicellular eukaryotes: algae, fungi, and protists. Eukarya had much lower species diversity as compared to bacteria: 2 in on-site A, 15 in on-site B, and 6 in ISB ice (Table 4). Because 18S rRNA sequencing was performed independently of the 16S rRNA sequencing, the relative abundance data
cannot be compared between the two sets of data. However, in can be inferred that the eukaryotic microorganisms were much less abundant than the bacteria based on the lower number of reads obtained.

Our third objective of this study was to determine the viability of microbiomes in the on-site packaged ice samples. To our knowledge, comprehensive characterization (16S/18S rRNA targeted gene sequencing) of viable microbial communities in the packaged ice has not yet been studied before. The microbes in the packaged ice face adverse environment factors: freezing temperature and chemicals in water. Back in 1955, a study was conducted to examine the survivability of bacteria upon repeated freezing and thawing (Harrison, 1955). *L. fermenti*, *E. coli*, and *S. marcescens* were employed as testing organisms and as a result, the rate of survivability varied; however, none of the test organisms completely died. Furthermore, *Pseudomonas* sp., *Bacillus* sp., and *Arthrobacter* sp., bacterial isolates from an artic soil were tested. Upon freezing and thawing, *Pseudomonas* was most susceptible and *Arthrobacter* was the most resistant out of three isolates (Nelson and Parkinson, 1978). In sum, the survivability of freezing varies depending on the type of microorganisms. Another environmental factor influencing microorganisms in the packaged ice is the added chemicals in water. Tap water is constantly treated with chemicals such as chlorine, aluminum sulfate, calcium hydroxide, and many other chemicals to kill microorganisms. These factors will have an impact on the viability of microorganisms in packaged ice.

In order to study the viable microbiome, propidium monoazide (PMA) dye was used to discriminate the DNAs from dead cells. In this part of study, we combined the on-site packaged ice samples into two composited samples and each subjected to no PMA and
PMA treatment. Our results show that *Proteobacteria* was the most dominant taxa in both of on-site packaged ice samples (on-site C and on-site D), which is consistent with our earlier results on on-site A and on-site B. Similar results have been reported in studies of the psychrophilic environment where the Gram-negative of *Alpha-, Beta-, and Gamma-proteobacteria* and the *Cytophaga-Flavobacterium-Bacteriodes* phylum are most abundant (D’Amico *et al.*, 2006). Furthermore, within the *Proteobacteria* phylum, it was noted that the relative abundance of *Alphaproteobacteria* was decreased in the viable profiles by 70% and 50% in on-site D and on-site C samples, respectively. In comparison, *Betaproteobacteria* was increased by 14% and 52% in on-site D and on-site C samples, respectively, indicating its capability in surviving ice making process. Unfortunately, there is not any study that performed the viability of microbial community in packaged ice using next generation sequencing. In Greenland ice core study (Miteva *et al.*, 2015), their live/dead culture staining revealed that the viability of microorganism present in ice core samples ranged from 39% to 84.5%. They also reported that *Betaproteobacteria* and *Firmicutes* constituted more than one million reads each out of 3.1 million reads generated from 16S rRNA next generation sequencing (Miteva *et al.*, 2015). Another study measured the effect of freeze-thaw cycles on Arctic tundra soil (Männistö *et al.*, 2009). Their clone analysis of reverse transcribed 16S rRNA gene sequencing revealed that the bacterial community was dominated by *Bacteroidetes*, *Gamma-, Beta-, and Alphaproteobacteria; Verrucomicrobia;* and *Acidobacteria*. After 5 freeze-thaw cycles, the relative abundance of *Betaproteobacteria* decreased whereas *Alphaproteobacteria* and *Bacteroidetes* increased (Männistö *et al.*, 2009). In combining with our findings, it is likely that *Betaproteobacteria* may survive well in ice as shown in
our viability study, however, they may not survive after repetitive freeze-thaw process. Further investigation is needed to validate how Betaproteobacteria is affected by the freeze-thaw cycle in a packaged ice condition.

As shown above, microbes are versatile, and they are able to inhabit extreme environments such as ice. As defense mechanisms, microorganisms respond to sudden temperature drops by overexpressing proteins, cold-adopted enzymes and cryoprotectant such as cold-shock proteins (Phadtare and Inouye, 2004), antifreeze proteins (Jia and Davies, 2002), and exopolysaccharides (EPSs) which have been found in Antarctic marine bacteria (Nichols et al., 2005) and in Arctic winter sea ice (Krembs et al., 2002). Therefore, all of above stress defense mechanisms may contribute to the survivability of individual microorganisms in packaged ice.

Ice is the frozen state of water. Ice should be the same quality as drinking water if it is consumed. The United States has one of the safest drinking water supplies in the world. During water treatments such as filtration and disinfection, microorganisms and chemicals will be filtered out, and a disinfectant such as chlorine or chloramine may be added to kill any remaining microorganisms. However; microorganisms can still survive and might cause sickness and disease. In regulatory perspective, investigation of viable microbial community is a lot more meaningful for food safety concerns. Several studies have incorporated PMA in their microbial community analysis in drinking water. Chiao et al (2014) used 16S rRNA targeting gene sequencing with PMA treatment to evaluate the microbial community alteration after monochloramine disinfection in drinking water. After monochloramine disinfection, the relative abundance of Legionella, Escherichia, Geobacter, Mycobacterium, Sphingomonas, and Coxiella were increased whereas
*Methylotenera, Chlorobium, and Dechloromonas* were susceptible (Chiao et al., 2014). Among the genera that exhibited the resistance to disinfection, they all belonged to the phylum *Proteobacteria* except *Mycobacterium* (*Actinobacteria*). *Proteobacteria* show their resistance to extreme conditions in another study. In Gomez-Alvarez *et al* (2012), they evaluated the viable and total biofilm communities during 4 chloraminated drinking water nitrification steps (Gomez-Alvarez *et al*., 2012). As a result, *Proteobacteria* showed greater resistance against different chemical treatments.

Among the top three most abundant viable microbes in on-site C and D samples, *Mitsuaria* and *Janthinobacterium* were present in both, and *Pseudomonas* and *Bacillus* were present in on-site C and on-site D, respectively (Table 6 and 7). *Mitsuaria* and *Janthinobacterium* both belong to *Betaproteobacteria* and commonly found in soil (Amakata *et al*., 2005; Gong *et al*., 2017). The presence of these organisms indicates that on-site packaged ice samples were exposed to soil environment due to poor packaging or storage. *Pseudomonas* are commonly found in soil and evolved to tolerate a variety of physical conditions such as treated drinking water (Mena and Gerba, 2009), hot tub (Ratnam *et al*., 1986) and glacier cryoconite in the Alps mountain (Shin *et al*., 2012). Lastly, *Bacillus* is a versatile microbe. Studies have reported that *Bacillus* have been found in surface waters (Østensvik *et al*., 2004), ice core from Greenland and Antarctica (Knowlton *et al*., 2013; Shivaji *et al*., 2013), and disinfected drinking water (Raber and Burklund, 2010; Morrow *et al*., 2008). Due to the presence of endospores, *Bacillus* can survive at such extreme environments. Overall, these prevalent microbes showed the highest relative abundance in on-site C and D Viable samples indicating they can adopt to the cold and oligotrophic environments in order to survive.
When comparing between viable and total relative abundance, we noticed that *Bacillus* sp. was not present in on-site D Total sample but the relative abundance rose to 21% in the Viable sample. We suspect that the DNA extraction was not successful in on-site D Total sample because bacterial endospores may easily escape the extraction of nucleic acid (Rose *et al*., 2011). Another possibility was a technical error during sample preparation and PMA treatments. Even though PMA has the highest potential to discriminate dead and viable cells (Rawsthorne *et al*., 2009), previous studies on bacterial spores have shown that inhibition of dead cells using PMA dye is not perfect yet. For example, PMA dye was not able to differentiate between dead and intact spores, and it bounded to intact spores instead (Vaishampayan *et al*., 2012). The high abundance of *Bacillus* in the on-site D viable sample may need to be further verified.

PMA dye treatment was also used effectively in discriminating viable and dead eukaryotic species. In Nocker *et al*. study in 2006, they successfully evaluated the communities of live and dead infectious fungal from the environmental samples using PMA treatment (Nocker *et al*., 2006). Ever since then, the evaluation of viable and dead eukaryotic communities using PMA treatment has been extensively studied at different environments such as soil (Muzafar *et al*., 2016), air and water (Vesper *et al*., 2008), international space station (Venkateswaran *et al*., 2014), and foods (Vendrame *et al*., 2014).

In our study, 18S rRNA targeted gene NGS was performed to evaluate the viable and dead communities of eukaryotic species in packaged ice. According to our results, eukaryotic community was less diverse than bacterial community. Since we composited more samples, there was a significant increase in number of OTUs compared to the
second objective of our study. Only 2 and 15 OTUs were detected in on-site A and on-site B samples, while on-site C and D samples have much higher numbers of OTUs at 112 and 78, respectively (Table 4 and 5). This indicates that the number of OTUs varies depending on the quality of packaged ice.

The process of screening viable eukaryotic species is very important because the presence of viable infectious species may lead to illness to the immunocompromised people. The viability percentage, in terms of OTUs, for On-site C sample was 75.0% whereas On-site D sample was at 44.9%. Top two most abundant viable eukaryotes of both samples belonged to Microbotryomycetes and Bdelloidea groups. The class Microbotryomycetes is not well known besides being a pathogenic in plants and mycoparasitic meaning a fungus that is parasitic to another fungus. A few studies reported that Microbotryomycetes was found in water supply reservoirs (Zhang et al., 2015), dust particles from spacecraft facilities (Checinska et al., 2015), South African acid mine water (Kamika et al., 2016). Bdelloidea belongs to the class of rotifers and is a microscopic zooplankton that commonly found in freshwater habitats and in moist soil. The presence of these viable eukaryotic organisms indicates that on-site samples were exposed to the soil environment and ice was made using unsanitary water.
CHAPTER 5

Conclusion

In this study, microbiological quality and microbial diversity of on-site, ISB and manufactured packaged ice were evaluated from southern California. The viability of microorganisms was examined by using PMA dye to differentiate dead and live cells in on-site packaged ice. As a result, all of manufactured and ISB packaged ice samples satisfied the PIQCS’s requirement. In comparison, some of on-site packaged ice samples showed poor microbiological quality by violating the PICQS’s requirements. Analysis of the 16S/18S gene targeted MiSeq sequencing further confirmed that on-site packaged ice showed poor microbiological quality than ISB packaged ice. In on-site packaged ice, there were many microorganisms that are commonly found in soil, stagnant water and even potential or opportunistic pathogens. In contrast, microbial diversity of ISB packaged ice mainly constituted oligotrophic microorganisms. Lastly, PMA analysis of 16S rRNA gene targeted MiSeq sequencing revealed that the bacterial viability was at about 49% and 74% for the on-site C and D samples, respectively. On-site C sample has more microbial diversity than on-site D sample. Betaproteobacteria showed the highest relative abundance in the viable samples of both on-site C and on-site D samples. The presence of potential and opportunistic pathogens in the viable populations is a concern for consumer’s health. Further studies will be required to examine how these microorganisms can survive at harsh conditions and how to prevent or remove them effectively.
CHAPTER 6

Here are some of the potential future studies to examine the presence of microorganisms in on-site packaged ice.

1. Examining the source of contamination and biofilms in the ice making process, such as ice machine, storage, and water filters by using culturing techniques.

2. Further evaluation of how Proteobacteria especially Betaproteobacteria can survive at harsh conditions such as freezing temperature and chemicals.
REFERENCES


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Vendrame, M., Manzano, M., Comi, G., Bertrand, J., & Iacumin, L. (2014). Use of propidium monoazide for the enumeration of viable Brettanomyces bruxellensis in wine and beer by quantitative PCR. *Food Microbiology, 42*, 196–204. https://doi.org/10.1016/j.fm.2014.03.010


