SHELF LIFE EXTENSION OF SEAFOOD USING SANITIZED ICE

A Thesis

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By

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ABSTRACT

The cross-contamination of foods, especially raw fish and seafood products, is an important food safety problem. Seafood products can be cross-contaminated at the working environment, when food contact surfaces (e.g. processing equipment, utensils, knives, etc) bear a high number of microorganisms. In addition, raw seafood products can become contaminated when food handlers do not follow good manufacturing practices. Another important source of cross-contamination can be the ice in contact with raw seafood products and the water from this melted ice. Since ice is extensively used as a preservation method, ice prepared with safe sanitizers could be a promising alternative to reduce the potential for cross-contamination from raw fish and seafood products.

The first part of this thesis (Chapter 2) reports an investigation on the stability (melting rate) of ice prepared with neutral electrolyzed water (NEW) and PRO-SAN® sanitizers and compared them with the stability of ice prepared with tap water. This first part also evaluated the efficacy of the sanitized ice in reducing the natural microbial burden on whole fish samples and in the ice used to store the fish. In addition, the efficacy of sanitized ice in reducing *Escherichia coli* K-12, *Listeria innocua* and *Pseudomonas putida* populations on Tilapia fish fillet samples and in storage ice was alsoevaluated. This was done by enumerating each bacterial species on the fish fillet and in the water from the melted ice at 12 and 24 hour intervals.

This study found that sanitizing the ice did not affect its stability when compare with the control. Also, this study revealed that ice prepared with PRO-SAN® was effective in reducing the natural microflora washed off from the whole fish in both the water from the melted ice and in the ice that was in contact with the fish. In addition, this study found that ice prepared with PRO-SAN® and the NEW sanitizers had the ability to produce at least a 4 log reduction in *E. coli*, *L. innocua* and *P. putida* populations in the water collected when the ice melted. However, the overall reductions in the bacterial species on the fish fillet samples stored on sanitized ice were not different than the reductions obtained for the ice prepared with tap water.

The second part of this thesis (Chapter 3) reports an investigation of the mechanisms used by PRO-SAN® and the NEW sanitizers to cause injury to the cells of *E. coli* K-12 and *L. innocua*. This was done by examining the bacterial cells using the transmission electron microscopic technique. The transmission electron microscope (TEM) images showed that the sanitizers altered the cell wall, internal membrane of *E. coli* and *L. innocua*. The results obtained for *L. innocua* revealed that this bacterium was more resistant to the bactericidal action of the NEW and PRO-SAN® sanitizers. This second part of this thesis confirmed that the mechanism used by various sanitizers against target microorganisms can be studied and understood using TEM.

DEDICATION

To my family and to my friend Rebecca. Thanks for your support and for always been there when I needed the most.

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CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Food safety is a major issue in the United States and concerns about it has increased within the food industry, regulatory agencies and consumer groups. Microbial pathogens are the cause of millions of cases of foodborne illnesses each year and this results in many hospitalizations and deaths (Lin et al., 2005). In 2008, the CDC's Foodborne Diseases Active Surveillance Network (FoodNet) confirmed a total of 18,499 laboratory cases of infection in 10 US states. *Salmonella*, *Campylobacter*, *Shigella*, *Cryptosporidium*, *Listeria*, *E. coli* O157:H7, *Yersinia*, *Vibrio* and *Cyclospora* were the major pathogens responsible for the infections (CDC, 2009). A substantial proportion of foodborne illnesses have been attributed to improper food handling, preparation and consumption practices by consumers. Some of these include the consumption of raw and undercooked, or unsafe foods (Redmond and Griffith, 2003; Lin et al., 2005). For example, a considerable amount of the seafood products that are consumed in the US are imported. For many products (e.g. raw fish fillets) no cooking, pasteurizing, or retorting step is performed by the processor, thus making the consumer the one responsible for the cooking (FDA, 2001b). During processing operations, pathogenic as well as spoilage

bacteria (e.g. *Pseudomonas* spp.) can attach themselves to processing equipments, food preparation utensils, and other food contact surfaces. Some of these microorganisms can produce a biofilm which can serve as a barrier against the effect of sanitizers and subsequently results in the contamination of the product (Wirtanen et al., 2000; Skandamis et al., 2009).

Even though the amount of microorganisms (including pathogens) on raw fish and other seafoods may be low, the potential for growth and toxin production warrants consideration (FDA, 2001d). To avoid the growth of undesirable microorganisms on these products, ice is commonly used as a preservation method. However, the ice is not meant to eliminate those microorganisms, and as a result certain types of bacteria could potentially lead to foodborne illnesses (FDA, 2001e). Therefore, the incorporation of sanitizers into the ice could be used as an additional safety step to inhibit the growth of spoilage and pathogenic bacteria. The literature provides sufficient information regarding the efficacy of various sanitizers in reducing the amount of bacterial cells from food processing equipments, food contact surfaces, etc. But limited information about the mechanism of action of those sanitizers against bacterial cells is available. Hence, there is a need for understanding how bacterial cells respond to chemical sanitizers in order to be inactivated. This can be achieved by using a transmission electron microscopy (TEM) technique, which will be discussed in more detail in section 1.12 and in Chapter 3.

In addition to the health risk associated with the microbial contamination of seafoods, chemical agents employed during sanitization practices are also a concern. This is so because wastes from food processing industries can carry a substantial amount of

chemical sanitizers, such as chlorine compounds (Islam et al., 2004). For instance, fish that are harvested from waters that have been exposed to processing wastes have a higher probability to accumulate toxic compounds in their tissues (FDA, 2001c; Lopes, 2004). For this reason, alternatives to traditional sanitizers are needed. These alternative sanitizers should be as effective as the traditional ones in reducing microbial numbers, but they should not have a negative impact on the environment or on the health of the public.

Thus, the objectives of this study were to (1) Perform a literature review of the subject of pathogens of food safety concerns (especially on seafood products), advantages and disadvantages of various sanitizers and potential alternatives; (2) Investigate the efficacy of neutral electrolyzed water and PRO-SAN® (an acidic sanitizer) to reduce bacterial numbers on fish and related products; and (3) Gain an understanding of the mechanism of action of the test sanitizers to kill or to cause injury to bacterial cells.

1.2. Foodborne Illness and its prevalence

Consumer demands for safe and more nutritious food products are current major challenges facing the food industry and regulatory agencies. Food and beverages can serve as vehicles for bacteria and other pathogens that can cause foodborne illness. According to the World and Health Organization (2007), foodborne illnesses are caused by agents (either infectious or toxic in nature) that enter the body as a result of the ingestion of contaminated food. These infectious diseases can be serious and potentially life-threatening for high risk groups such as young children, pregnant women, elderly,

and people with compromised immune systems (USDA-FSIS, 2006). Public health officials and the scientific community are concerned because the food supply is changing in ways that can promote foodborne illness. Thus, there exists a need for comprehensive data to explain at what point pathogens are introduced into the food supply.

Foodborne illness of microbial origin is the most serious food safety problem in the United States (Collins, 1997). Approximately 76 million cases of foodborne diseases occur each year, resulting in 325,000 hospitalizations and at least 5,000 deaths (Mead, et al., 1999). Figure 1.1 shows the most common causes of foodborne disease outbreaks in the United States between the years 1993 to 1997. Factors influencing these were temperature abuse, poor personal hygiene, cross-contamination, unsafe food source and inadequate cooking.

The prevalence of foodborne illnesses in society could potentially result in severe economic disruptions. Diseases caused by the major pathogens alone are estimated to cost up to $35 billion annually in medical costs and lost productivity in the United States (WHO, 2007). Despite numerous activities aimed at preventing foodborne human infections, progress toward the national health objectives has plateaued, suggesting that fundamental problems with bacterial and parasitic contamination are not being resolved (CDC, 2009). Robert Tauxe, deputy director of the CDC's Division of Foodborne, Bacterial and Mycotic Diseases, said that several factors are influencing the prevalence of foodborne illnesses, including the intricacy of the U.S. food chain, the changing nature of the contaminating bacteria and the rise in imported food (Layton, 2009). This represents a

threat to public health that needs to be addressed and new innovations aimed at ensuring the safety of food products should be incorporated as control and prevention measures.

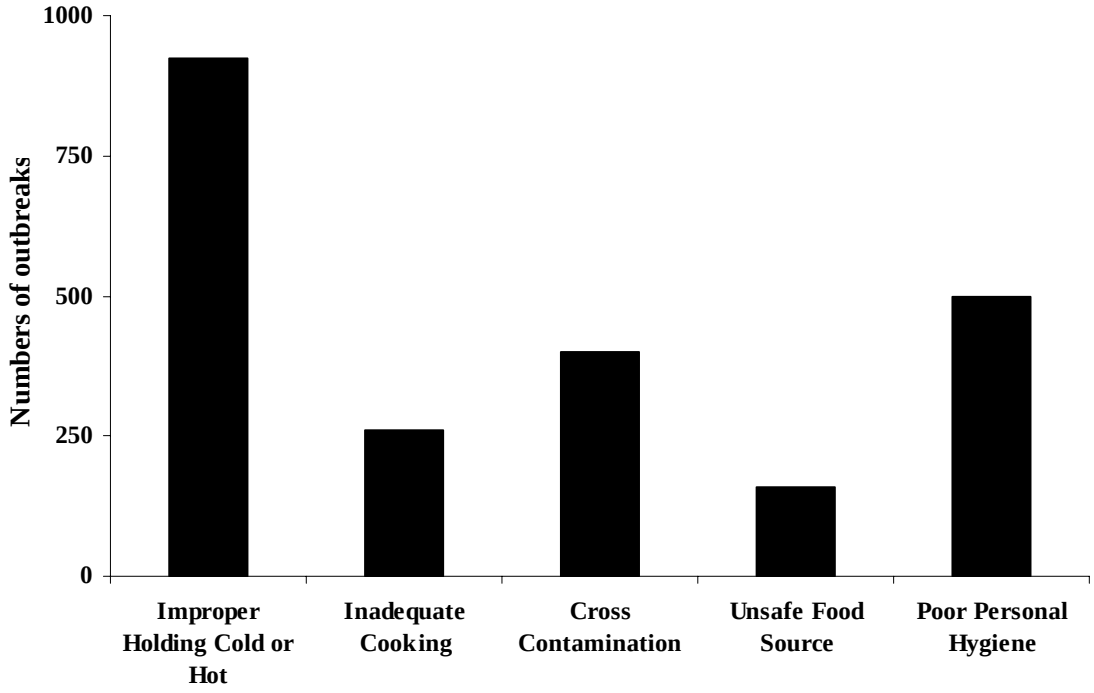

Figure 1.1: Contributing factors to foodborne outbreaks from 1993-1997 (Olsen et al., 2000; McCabe-Sellers, et al., 2004).

In addition to contaminated food products, there is also a concern about the nation's drinking water and its association with foodborne illness. Potable or drinking water is a commodity that meets the U.S. National Primary Drinking Water Regulations (40 CFR 141), the World Health Organization's International Standards for Drinking Water, or other recognized equivalent standards (Wang, et al., 2002). This water source is made available to the public and the industry by State and municipal authorities. Potable

water is often used for drinking, the washing, heating, and cooling of food stuffs as well as the cleaning and sanitation of equipment and facilities that come in contact with food commodities such as meat, poultry, fish, fruits, vegetables and cheese (National Research Council et al., 1985; Palumbo et al. 1997, Wang et al., 2002). However, the quality of the water used for food processing is critical because it could be a potential source of contamination. This is so because contaminated drinking water has been implicated in some foodborne illness cases. In 1999-2000, 39 outbreaks associated with U.S. drinking water, were reported. During this occurrence, 2,068 persons and 2 deaths were reported. From these 39 outbreaks, 20 were of known infectious etiology including parasites, bacteria and viruses (Lee et al., 2002).

1.3. Relevance of seafood and fish products to foodborne illnesses

The worldwide growth in the human population has resulted in an increase demand for more food. Data have shown that the consumption of fishery products has increased and many people include them in their daily diet because of the health and nutrition benefits that they offer. However, seafood products could be a potential source of foodborne diseases. In the United States, more than 80% of the seafood consumed is imported from different countries around the world. These include Canada, China, Thailand, Chile, Ecuador and Vietnam as the top 6 seafood importing countries (GAO, 2004). As a result of this, seafood products consumed in the United States have a high probability to become contaminated at several points in the food chain supply. This is so because seafood may contain pathogens obtained from the marine environment, from

which they were harvested (Jay, 1992a; Price, 2009). Bacteria in polluted marine environments can arise from the discharge of untreated sewage that passes into rivers, lakes and coastal waters. Because many enteric pathogens are capable of surviving sewage processing, they are also able to contaminate seafood products (Scoging, 1991; Kirby, et al, 2003).

Contamination of seafood products may also arise from handlers, equipment or from the work environment. Data collected by the Center for Science in the Public Interest (CSPI) between 1990 and 2003 revealed that food most commonly linked to outbreaks (when a number of events happen simultaneously), were seafood, produce, poultry, beef, and eggs (Dewaal et al., 2006). Figure 1.2 shows the findings obtained by the CSPI, in which the outbreak data were organized by food source instead of by pathogen. Likewise, Wallace and collaborators (1999) reviewed reports submitted to the New York State Department of Health (NYSDH) relating seafood-associated outbreaks that occurred during 1980-1994. Their results showed that seafood-associated outbreaks accounted for 19% of all reported foodborne cases and 10% of foodborne illness. The studies thus concluded that seafood products were responsible for large numbers of foodborne disease cases.

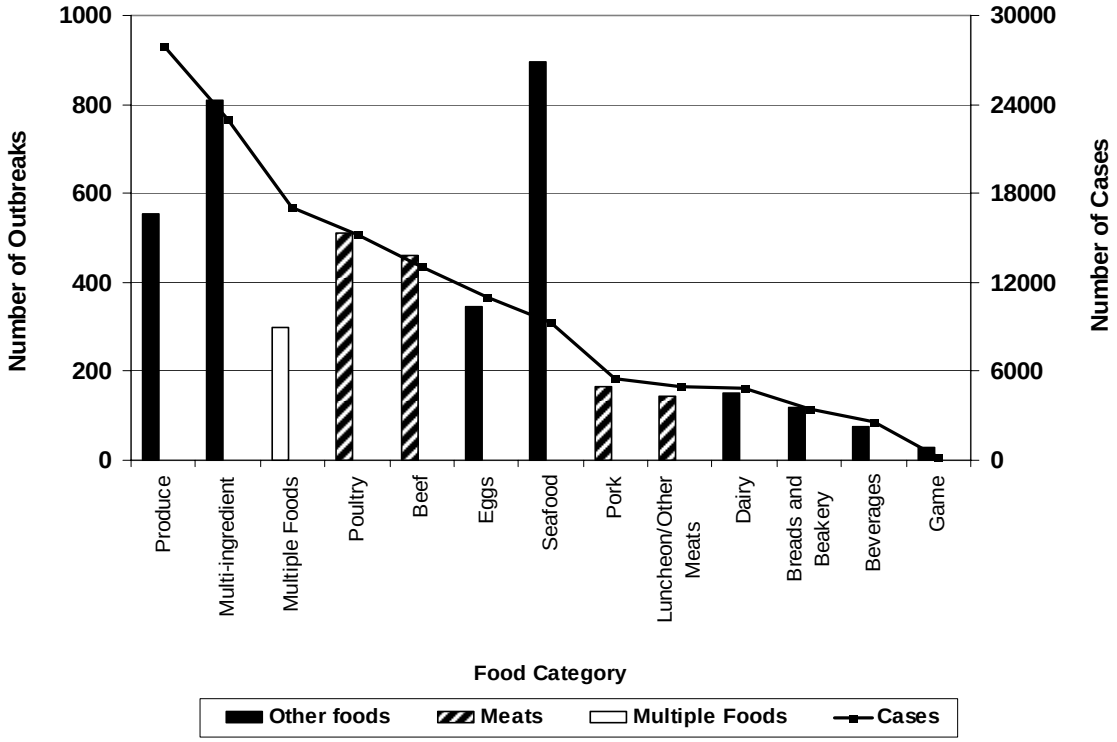

Figure 1.2: Cases linked to foodborne illness outbreaks, 1990-2003 (Dewaal, et al., 2006)

1.4. Fresh fish and market

An estimated 25% of primary agriculture and fishery products are lost every year, mostly because of chemical deterioration and microbial spoilage of the harvested commodity (Baird-Parker, 2000). Once fish has been caught from sea or fresh water, they are handled and, in most cases, processed without the use of additives or chemical preservatives and finally distributed with chilling or freezing as the only means of preservation (Feldhusen, 2000). Fresh fish are very perishable and they require an appropriate storage temperature to control the growth of bacteria that could multiply under inappropriate storage conditions. If the storage temperature is not properly

controlled, exposure of fresh fish to temperature abuse can cause serious deterioration in quality (Jeremiah, 1996). Although fish products should be kept under refrigeration temperatures to maintain their freshness and the overall quality of the products, the inability of refrigeration to completely eliminate microorganisms from the fish products represents a potential health risk (Ashie, et al., 1996). Also, if fish undergoes further processing, such as cutting and filleting, it will have a higher probability to be contaminated. As a result, it is recommended that fish and seafood products be thoroughly cooked in order to reduce the risk of foodborne illness.

1.5. Bacteria likely to be found in fresh fish

Bacteria known to be found on fresh fish include *Acinetobacter*, *Aerobacter*, *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Proteous*, *Pseudomonas* and *Vibrio* (Chen, 1995). The amount of these bacteria on the fish is highly influenced by the environment (e.g. the quality of the water where the fish was harvested), the nature of its skin or digestive tract. It has been shown that different species of fish caught during the same season and in the same environment have similar microflora, whereas fish caught in different environments have different microflora, reflecting the influence of the microflora of the water in which they are caught (Jay, 1992a; Chen, 1995; Feldhusen, 2000; Al Balushi, 2008). Likewise, the temperature of the water, the fishing method used, and the storage conditions can also affect the microbial flora of the fish (Cahill 1990; Chen 1995). For example, fish caught in cold, clean waters carry lower numbers of

bacteria whereas fish caught in warm waters have slightly higher counts (Huss, 1995). Because of this temperature effect, the types of bacteria tend to be slightly different for fish caught in cold waters versus fish caught in warmer waters. Usually fish from cold waters carry mostly psychrotrophic bacterial species on their surface or skin whereas fish from warmer waters carry mainly mesophilic species on their skin and gills (Cahill, 1990). Thus, the amount of the bacteria on fresh fish can vary from country to country and from place to place.

The manner in which fish is handle after being caught can contribute to the contamination of the product and could alter the normal flora adversely, resulting in more rapid deterioration and therefore a reduction in the quality of the fish (Cahill, 1990; Reilly and Käferstein, 1997; Feldhusen, 2000; Reynisson et al., 2008). The flesh of the newly-caught fish is basically sterile, but the outer surfaces such as skin and gills are not (Huss, 1995). For this reason, the handling practices after the fish has been caught play an important role in avoiding the proliferation of the bacteria that are indigenous to the aquatic environment or to reducing the chances of introducing bacteria that are not naturally present on the fish. It is imperative to remember the importance and implications of the handling of fresh fish because mishandled product could cause foodborne illness.

1.5.1. Spoilage bacteria

Spoilage bacteria are those that can affect different types of food products causing them to deteriorate and to develop unpleasant odors, tastes and textures (USDA-FSIS,

2006). Food spoilage may be caused by a wide range of reactions including some that are mainly physical or chemical, others due to action of enzymes or microorganisms (Huis in't Veld, 1996). Spoilage bacteria may be harmless and hence may not affect the health of the public. In contrast, the economic impact due to the deterioration of food products can be significant to different sectors of the economy and individuals in the food industry.

Foods that are a good source of protein such as meat, poultry, fish, shellfish, milk and some dairy products can spoil rapidly. The reason for this is that such foods are highly nutritious, possess a neutral or slightly acid pH and have higher moisture content, supporting the growth of a wide range of microorganisms (Huis in't Veld, 1996). Fish contains little carbohydrate but a high content of free amino acids, which enable spoilage organisms to grow and to produce spoilage metabolites such as trimethylamine (TMA), which contribute to the characteristic ammonia-like and fishy off-flavors (Gram and Dalgaard, 2002). Origin, catching techniques, initial handling and temperature during storage will certainly influence the spoilage rate and shelf-life of seafood and fish products (Olafsdottir, et al., 2006). The spoilage of fish begins soon after they are caught, so in order to preserve the quality of these, they should be refrigerated immediately (Campos et al., 2005). While alive, fish has bacteria on the skin, gills and in the guts but because of its immune system bacteria are prevented from contaminating the muscle. After rigor mortis (when muscles are stiff and rigid), a gradual tenderization of fish meat occurs and high-molecular weight compounds such as proteins, lipids and glycogen are gradually degraded into low-molecular-weight compounds, which can be utilized more readily by microorganisms, through autolysis by indigenous enzymes (Hamada-Sato et al.,

2005). During this stage, bacteria on the skin and gill surfaces increase gradually and begin the process of invading the flesh, which is an ideal medium for their growth and their multiplication (Hamada-Sato et al., 2005; Peralta, 2007).

It is well known that high temperatures enhance spoilage, promoting microbial activity and enzymatic breakdown, resulting in quality changes in muscle foods (Pedrosa-Menabrito and Regenstein, 1990; Zugarramurdi, et al., 2004). On the contrary, low temperatures reduce the proliferation of spoilage bacteria and deterioration of foods (Johnston et.al., 1994). Since temperature control can help in limiting the rate of deterioration in fish and seafood products, the utilization of ice as a cooling system is common. Nevertheless, spoilage bacteria can represent a problem to these type of products, especially those organisms that are able to spoil food at low temperatures, ranging between -1ºC and + 7ºC (Ray, 2004).

1.5.1.1. *Pseudomonas*

The fact that psychrotrophic spoilage bacteria can multiply under cold storage conditions represents a problem to the seafood and fish industries. *Pseudomonas* species are Gram-negative rod-shaped bacteria that are motile by the use of polar flagella. These bacteria can be found in various environments such as soil and water sources. Some species within this genus are pathogenic (i.e. *Pseudomonas aeroginosa*) while others are non pathogenic. *Pseudomonas* is one of the most common spoilage bacteria that can be found in cold environments and is a specific bacterium of iced fresh fish, regardless of the origin of the fish (Gram and Huss, 1996; Gram and Dalgaard, 2002; Pacquit et al.,

2006). Since the composition of the microflora of the fish and seafood products changes dramatically during storage, it is important to understand that under iced storage conditions, the population of *Pseudomonas* spp. is more likely to predominate (Huss, 1995; Oral et al, 2008). Therefore, this organism could be used as a marker to estimate the shelf life and quality of iced fish in storage.

Another problem associated with *Pseudomonas* spp. is their ability to form biofilms. This contributes to the persistence of *Pseudomonas* spp. on food equipment surfaces (Wirtanen et al., 2000), making them more resistant and harder to be eradicated by cleaning and disinfection procedures (Russell and Chopra, 1996; Bagge-Ravn et al., 2003; Langsrud et al., 2003; Mazzola et al., 2006; Simões et al., 2009). For this reason, the sanitary practices and handling procedures during the processing of fish and seafood products are important in order to prevent the raw products from becoming cross-contaminated by the processing equipment or other food contact surfaces.

1.5.2. Pathogenic bacteria

Human food sources are of plant and animal origin and since foodborne illnesses remain an important public health problem in the US (CDC, 2009), efforts to reduce the incidence of illnesses are needed. The Foodborne Diseases Active Surveillance Network (FoodNet) of CDC's Emerging Infections Program collects data from 10 US states (California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee) in order to monitor diseases caused by enteric pathogens transmitted commonly through food. This program works in partnership with the U.S.

Department of Agriculture and the Food and Drug Administration (CDC, 2006). FoodNet surveillance includes foodborne pathogenic bacteria such as *Vibrio*, *Yersinia enterocolitica*, *Shigella, Campylobacter, E. coli* O157:H7*, Listeria monocytogenes,* and *Salmonella.* In addition, parasites such as *Cryptosporidium* and *Cyclospora* are also included in this surveillance program.

During the years 1998 - 2002, a total of 2,167 of the 6,647 outbreaks were reported to CDC, where 128,379 persons were reported to become ill (Lynch et al., 2006). Among the 2,167 (33%) outbreaks for which the etiology was determined, bacterial pathogens were responsible for the largest percentage (55%). Among the bacterial pathogens, *Salmonella* serotype Enteritidis accounted for the largest number of outbreaks and outbreak-related cases, whereas *L. monocytogenes* accounted for the majority of deaths of any pathogen (Lynch et al., 2006). In 2006, compared with the 1996-1998 baseline period, significant declines occurred in the estimated incidence of *Campylobacter*, *Listeria*, *Shigella,* and *Yersinia* infections (CDC, 2007). However, the incidence of *Listeria* infections remained higher in 2006 when compared with the number of incidences in 2002 (CDC, 2007). The number of incidence infections caused by *E. coli* O157:H7 and *Salmonella* did not decrease significantly whereas an increase in *Vibrio* infections was noted (CDC, 2007). According to a recent report from the CDC (2009), the incidence of *Campylobacter*, *Cryptosporidium*, *Listeria*, *Salmonella*, *Shigella*, *E. coli* O157:H7, and *Yersinia* infections have not changed when compared with previous years (2005-2007). Besides, the number of incidence remains highest among children aged < 4 years. For most of the pathogens mentioned above, persons aged ≥ 50 years appear to be

at a greater risk than are other age groups for hospitalization and death (CDC, 2009). Table 1.1 shows a brief overview of some of these pathogens associated with foodborne illnesses.

Bacterial organism	US cases annually	Associated foods	Symptoms of illness
Salmonella	2–4 million	Raw meats, poultry, eggs, milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces and salad dressing, cake mixes, cream-filled desserts and toppings, dried gelatin, peanut butter, cocoa, and chocolate	Nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. Chronic consequences—arthritic symptoms may follow 3–4 weeks after onset of acute symptoms
Campylobacter jejuni	At least 2–4 million	Raw chicken, raw milk, non-chlorinated water	Diarrhea, fever, abdominal pain, nausea, headache and muscle pain. The illness usually occurs 2–5 days after ingestion. Illness generally lasts 7–10 days, but relapses are not uncommon (about 25% of cases)
Escherichia coli O157:H7	20,000 and 40,000	Undercooked or raw hamburger (ground beef), alfalfa sprouts, unpasteurized fruit juices, dry-cured salami, lettuce, game meat, cheese curds, raw milk	Severe cramping (abdominal pain) and diarrhea that becomes grossly bloody. Occasionally vomiting occurs. Fever is either low-grade or absent. Lasts for an average of 8 days
Listeria monocytogenes	1600 cases	(Foods with high or moderate risk) Unpasteurized and pasteurized fluid milk, deli meats, frankfurters (not reheated), cheeses (particularly soft-ripened varieties), high-fat and other dairy products, pâté and meat spreads, smoked seafood, cooked and ready-to-eat crustaceans	Septicemia, meningitis (or meningo-encephalitis), encephalitis, spontaneous abortion or stillbirth, fever, nausea, vomiting, and diarrhea

Table 1.1: Four major foodborne pathogens: incidence, associated foods, and symptoms (adapted by Lin et al., 2005)

In order to prevent the contamination of food products with pathogens, particularly bacteria, preservation methods, including thermal processing, dehydration, irradiation, refrigerated storage, chemical ingredients and the utilization of packaging have been employed with the aim of prolonging the shelf life, storage and wholesomeness of foods (Jay, 1992b; Aberle et al, 2001). For example, acidification of many foods is one of the factors that help to prevent the growth of food pathogens. Yet, some microorganisms such as *Shigella* spp., *Escherichia coli* O157:H7 and *L. monocytogenes* can tolerate acidic conditions and survive (Schoolnik, 2002; Gandhi and Chikindas, 2007; Skandamis et al., 2009). Similarly, the use of salt to lower the water activity of foods is a common method of preservation. However, pathogens like *Listeria monocytogenes* are able to adapt and survive in high salt concentrations (Aase et al., 2000; Gandhi and Chikindas, 2007; Posfay-Barbe and Wald, 2009).

Currently, the fact that pathogenic microorganisms can survive or that they are resistant to certain preventive treatments, has important implications to the food industry. Microorganisms can exist in the environment as communities in biofilms. Since biofilms are capable of forming on food contact surfaces, areas where food is stored or on food processing surfaces such as conveyor belts, counter tops and stainless steel equipments (Farrell et al, 1998; Kumar and Anand, 1998; Skandamis et al., 2009). The chance to contaminate foods with pathogenic microorganisms is high, but a person needs to ingest a large quantity of the infectious agent in order to develop a foodborne illness (FMI, 2004). Nevertheless, *Listeria* and *Escherichia coli* O157:H7 are exceptions. The infectious dose associated with listeriosis is estimated to be 10^4–10^6 organisms per gram of ingested

product, but may be lower in susceptible individuals (Swaminathan and Gerner-Smidt, 2007; Posfay-Barbe and Wald, 2009). On the other hand, the infectious dose for *Escherichia coli* 0157:H7 is possibly less than 10 cells (Aberle et al., 2001). For this reason, special handling and preventive measures should be taken with foods, especially raw foods of animal origin, which are most likely to be contaminated with pathogens (CDC, 2005).

The fact that *Listeria monocytogenes* and *E. coli* O157:H7 have shown resistance to certain food processing treatments, makes it necessary to examine them in more details. *L. monocytogenes* has been a major cause of death and outbreaks caused by *E. coli* O157:H7 remains prominent (Lynch et al., 2006). These microorganisms are of current concern and represent a threat to society, especially children and the elderly, which appear to be at higher risk (CDC, 2009).

1.5.2.1. *Escherichia coli* spp.

Escherichia coli represent one of the most commonly studied microorganisms. It is a Gram-negative bacillus that is usually motile by use of peritrichous flagella, which project in all directions. *E. coli* strains are common inhabitants of the gastrointestinal tracts of birds and mammals (Aberle et al., 2001; Johnson et al., 2004; Ishii et al., 2007; Vogel et al., 2007), but they can also be found in soil and sediments (Byappanahalli et al., 2006; Ishii et al., 2006; Ishii et al., 2007), and treated wastewater effluent (Ishii et al., 2007; Boutilier et al, 2009). This group of enterobacteria comprises both non-pathogenic and pathogenic species. Most *E. coli* strains in the gut are non-pathogenic, but certain

strains may carry virulence genes which enable them to cause intestinal infections such as diarrhea or hemorrhagic colitis, or to cause extra-intestinal infections such as neonatal meningitis, septicemia, hemolytic uremic syndrome (HUS), urinary tract and surgical site infections (Falagas and Gorbach, 1995; Ahmed et al., 2007; Badri et al., 2009). The pathogenic or enterovirulent strains of *E. coli* can be categorized a s enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998; Ahmed et al., 2007). Figure 1.3 illustrates how some of these virulent strains interact and cause illness in epithelial cells in the gut of a host.

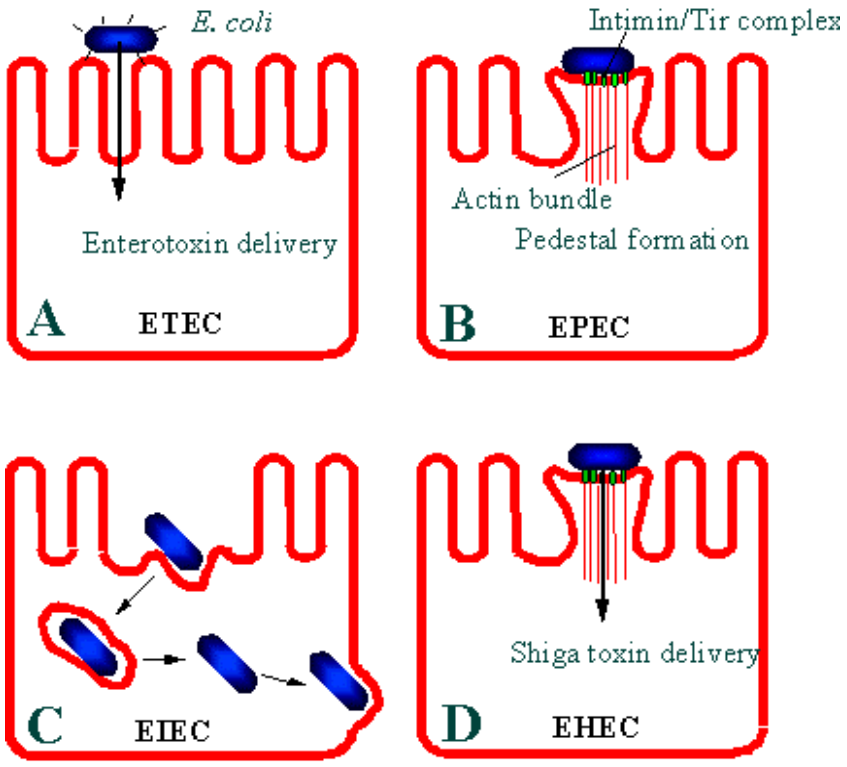

Figure 1.3: Interaction of various pathogenic *E. coli* with the epithelial cells in the gut of a host. (http://www.bms.ed.ac.uk/research/others/smaciver/Bacteria%20Inv.htm)

A. ETEC binds loosely via fimbriae, secretes toxins (like Cholera toxins) into the gut. This subsequently gains entry into the cell without disruption of cytoskeleton. **B.** EPEC destroys the brush outer limits microvilli, and becomes firmly attached through a pedestal consisting of actin and actin binding proteins. **C**. EIEC, gains entry into the cell, escaping from the immune system by digesting the phagolyosome. EIEC then grows and divides in the cell cytoplasm and gains entry to neighbouring cells by bursting through and digesting membranes. **D**. EHEC, operates like EPEC, but in addition, Shiga toxins are liberated, taken up by the epithelial cells in coated pits and then transferred to the Golgi bodies within the cell. The toxins then travel from the Golgi to the endoplasmic reticulum (E.R.) where they destroy the ribosomes by the removal of a single adenine residue from the 28SrRNA. This results in the death of the cell.
(http://www.bms.ed.ac.uk/research/others/smaciver/Bacteria%20Inv.htm).

E. coli has been used as a fecal contamination indicator in natural environments such as fresh water lakes and streams (Byappanahalli et al., 2003; Power et al., 2005). Pollution of water bodies by fecal discharges from humans and animals may transport a variety of pathogens that can affect the human population at large. For this reason, fecal indicator bacteria (FIB) such as *Escherichia coli* are used, since their abundance correlate with the density of other pathogenic microorganisms of fecal origin. This is used as an indicator of the sanitary risk associated with water utilizations, particularly for drinking and food processing (Field and Samadpour, 2007; Servais et al., 2007).

The processing of seafood and fish produces a large bulk of by-products and wastes. These are mixtures of trimmings from the muscles, fins, scales and shells, soluble proteins, fats and oils, partially decomposed organic matters, different chemical substances, and pathogenic bacteria among others (Islam et al., 2004). Although recent trends show that much of these waste products are made into various value added substances, considerable quantities are discharged as processing effluent together with large volumes of water. Most of these are discharged into the nearby coastal waters through discharge channels and, therefore, represent a potential hazard to the environment (Islam et al., 2004). The impact of this processing waste can become a problem to live fish and seafood products. This is so, because the microbial load in a given seafood/fish depends greatly on the environmental conditions and microbiological quality of the water from where these animals are harvested (Jay, 1992a; Chen, 1995; Feldhusen, 2000; Al Balushi, 2008).

1.5.2.1.1. *E. coli* 0157:H7 (enterohemorrhagic *E. coli* or EHEC**)**

Escherichia coli O157:H7 is a member of the enterohemorrhagic group of pathogenic *E. coli* and is recognized as a major food and waterborne pathogen of public health concern (Karpman, 2002). The serotype O157:H7 is a rare variety of *E. coli* that produces large quantities of one or more related, potent toxins (verotoxin and shiga-like toxin) which can cause severe damage to the lining of the intestine (FDA, 2009). This bacterium was first recognized as a human pathogen in 1982, when two outbreaks of hemorrhagic colitis occurred due to the consumption of fast food hamburgers in Oregon and Michigan (CDC, 1982; Riley et al., 1983; Wells et al., 1991; Karch et al., 1999; Park et al., 2001). Although undercooked/contaminated ground beef appears to be the primary source of human *E. coli* O157:H7 infections, other sources such as salami, yoghurt, raw milk, unpasteurized cheese, apple cider, radish sprouts, swimming and drinking tap water as well as person-to-person transmission have been reported (Mead and Griffin, 1998; Karpman, 2002). Other outbreaks caused by *E. coli* O157:H7 have been reported in the US and in other parts of world, including United Kingdom, Canada and Japan (Watanabe et al., 1996; Caprioli et al., 1997; Simmons, 1997; Tarr et al., 1997; Rowe et al., 1998; Michino et al. 1998; Karch et al., 1999; Park et al., 2001; Karpman, 2002). The concern about this microorganism is that complications such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) or hemorrhagic colitis could develop in individuals during an outbreak (Nataro and Kaper, 1998; Park et al., 1999; Park et al., 2001; Snider et al., 2009). Young children are a highly susceptible population to *E. coli* O157:H7 infections and can develop HUS, a leading cause of acute renal failure

in childhood (Kaplan et al., 1990; Griffin and Tauxe, 1991; Constantinescu et al., 2004).

The most common manifestation of the infection is diarrhea, which accounts for at least 95% of the HUS cases in children (Remuzzi, 1995; Ruggenenti et al., 2001), and may cause sporadic or epidemic disease. On the contrary, TTP occurs mainly in adults. Besides, it appears to be more common in females, with a peak incidence occurring in the fourth decade of age (Tsai and Lian, 1998). The mortality rate with this infection exceeds 90% when no therapy or treatment is received (Allford et al., 2003). This uncommon multisystem disorder is characterized by hemolysis, thrombocytopenia (few platelets in blood), renal failure, neurological problems, and a fluctuating fever (Proesmans, 1996; Eldor et al., 1998; Michael et al., 2009). Even though TTP and HUS are related conditions with similar clinical features (Michael et al., 2009), TTP is associated with the central nervous system (CNS), which is an important factor in the differentiation of the two infections (Park et al., 2001).

Healthy cattle are the most important reservoir of *E. coli* O157:H7, so contamination of beef carcasses with this bacterium through fecal shedding is important for the dissemination of the pathogen (Snider, et al., 2009). Organic acid spraying, hot water spray washing, and steam vacuuming are some of the treatments that have been applied in order to reduce the microbial load on carcasses (Dormedy et al., 2000; Delmore et al., 2000; Castillo et al., 2001; Kang et al., 2001; De Martinez et al., 2002; Koutsoumanis et al., 2004; Logue et al., 2005). However, *E. coli* O157:H7 has shown the ability to develop acid resistance in response to acidic conditions (Jordan et al., 1999; Marques et al., 2001; Ruiz-Cruz et al., 2007). The survival of this bacterium in these

types of conditions represents a problem. This is so because colonization of slaughtering plant equipment surfaces by *E. coli* O157:H7 during and after processing may result in persistent strains with increased acid resistance, especially in sites where meat runoff fluids may accumulate and temperatures allow them to grow freely (Skandamis et al., 2009). Besides, acid adapted biofilm cells of *E. coli* O157:H7 may be distributed in the processing environment via aerosols or liquids, or may cross-contaminate food products via direct contact (Skandamis et al., 2009).

E. coli O157:H7 is capable of surviving in water for long periods of time. Thus, the longer this pathogen survives in the environment, the more likely it is to contaminate water sources (Kirby et al., 2003). *E. coli* 0157:H7 can be introduced into the environment via: 1) run-off from cattle farms; 2) during or after flooding of industry, residential or farm lands; 3) from irrigation water and the application of manure to farms; and 4) from wildlife and birds (Wallace et al., 1997; Suhalim et al., 2008). Since animal manures, particularly bovine, is used as pond fertilizers in some places, there is a risk that pathogenic strains of *E. coli* can be distributed to pond water by this method (Feldhusen, 2000).

1.5.2.2. *Listeria monocytogenes*

Today, the presence of *Listeria species* in food products is a major concern to regulatory authorities and food manufactures. These Gram-positive bacterial species are ubiquitous in all types of environments and can be isolate from soil, sewage, vegetation, and other sources, including water. *Listeria* is also found in sea water and as a result, it is

not surprising that fish and seafood products have been found to contain the bacteria (Jemmi and Stephan, 2006). The fact that *Listeria* can be isolated from these products makes it a threat to the public health. Although not all species of *Listeria* are harmful, the isolation of non-pathogenic species is of great concern. That is so because if non-pathogenic species grow in an environment, there is a possibility that *L. monocytogenes* could also survive and grow under those same conditions. The genus *Listeria* includes six species, and these comprise *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii* and *L. grayi.* However, *L. monocytogenes* is the major pathogenic species in both animals and humans (Zaytseva et al., 2007). *Listeria* spp. is an important cause of zoonoses, infecting many types of animals (domestic pets, livestock, other mammals, rodents, amphibians, fish, and arthropods) and more than 17 avian species (Posfay-Barbe and Wald, 2009), thus, representing a threat to society, especially the food chain supply.

L. monocytogenes is an invasive opportunistic foodborne pathogen that remains one of the leading causes of mortality from foodborne infections (Buchrieser et al., 2003). The concern with this bacterium is that it can cause listeriosis, a foodborne disease that most frequently affects pregnant women, new-born infants, children and immunocompromised populations (Rocourt et al., 2000). The immunocompromised category includes people with AIDS or those who use immunosuppressive drugs such as corticosteroids for cancer treatment. These drugs are known to reduce T-cell mediated immunity (Rocourt and Cossart, 1997; Rocourt el al., 2000). In the US, *L. monocytogenes* infections are responsible for the highest hospitalization rates (91%) among known foodborne pathogens (Mead et al., 1999). Once contaminated food is ingested, it passes

to the stomach where the acid environment may kill many of the *L. monocytogenes* cells (McLauchlin et al., 2004). However, the buffering capacity of some food types may be important in facilitating the survival of the bacterium, which may then invade other sites further along the gastrointestinal tract (McLauchlin et al., 2004). *Listeria* gains entry into mammalian cells by phagocytosis and subsequently is released from the membrane-bound vacuole and begins to multiply. The pathogen uses actin polymerization (actin filament tail) for intracellular movement and cell-to-cell spread, infecting a vast range of host tissues, with the liver being the main site of infection (Rocourt and Cossart, 1997). The incubation time for listeriosis is long, so it is difficult to identify the pathogen and trace the contaminated food (Gandhi et al., 2007; Swaminathan and Gerner-Smidt, 2007). The intracellular nature of *L. monocytogenes* may allow incomplete eradication and survival of the bacterium, which could result in successive invasion of other organs. This may explain the relatively long incubation periods (up to 3 months) shown in some patients after consumption of contaminated foods (Linnan et al., 1988; McLauchlin et al., 2004).

L. monocytogenes possesses properties that favor it as a foodborne pathogen. This bacterium has the ability to adapt and survive extreme environments such as high salt concentration (10% NaCl), a broad pH (from 4.5 to 9.0) and a wide temperature range. This bacterium has the ability to grow between 1ºC and 45ºC, which increases the contamination risk for refrigerated foods, especially fish and seafood products (Rocourt et al., 2000; Buchrieser et al., 2003; Pal et al., 2009). Ready-to eat (RTE) foods are commonly stored at refrigeration temperatures as a control mechanism against the growth

of *L. monocytogenes* and the maintenance of product quality. Since low temperatures can only reduce the growth rate of this psychrotrophic microorganism (Pal et al., 2008), these types of foods are at highest risk if they become contaminated with *Listeria.* This is so because relatively long periods in refrigeration storage could still enabling *Listeria* to grow and reach infective doses (Rocourt et al., 2000; Rosset et al., 2004).

Milk and dairy products, meat and meat products, vegetables and seafood can be contaminated with *Listeria* (CDC, 1999; Rocourt et al., 2000; Schlech, 2000; Posfay-Barbe and Wald, 2009). In the case of seafood products, their safety is influenced by a number of factors such as the origin of the fish, microbial ecology of the product, handling and processing practices and traditional preparation before consumption (Rocourt et al., 2000). An aspect to consider is the fact that *Listeria* is ubiquitous in nature and can easily contaminate raw products. It has been shown that *L. monocytogenes* can contaminate food products in the processing environment, where this bacterium can persist for several years (Aase et al., 2000; Senczek et al., 2000; Lundén et al., 2003 Bagge-Ravn et al., 2003). This could be attributed to its ability to form biofilms (Farrell et al., 1998; Borucki et al., 2003). The formation of biofilms in the processing plants is a problem, because surviving microflora can contaminate the surfaces of equipment and subsequently cross-contaminate food products (Gandhi et al., 2007). For example, conveyor belts and stainless steel surfaces of equipment are commonly found to be contaminated even after sanitizing treatments (Midelet and Carpentier, 2002). Hence, if raw food products, such as fish or other seafood products are not cooked properly prior to consumption, the chances for listeriosis infection are much higher.

1.6. Tilapia fish

Tilapia has become an important fish in the US market. Its availability and relatively low costs make Tilapia accessible to consumers, restaurants and grocery stores throughout the year. The global production of Tilapia is basically dominated by aquaculture (Figure 1.4), and this appears to be replacing the wild catch harvesting (Fitzsimmons, 2005). In recent years aquaculture has been one of the fastest growing sections for primary production of Tilapia (Rana, 1997). It has the advantage of producing higher amounts of fish under controlled conditions, and this allows it to meet the higher demands for Tilapia products worldwide. Although aquaculture products are as safe and wholesome as wild-caught species, there are some public health hazards associated with ignorance, abuse, and neglect in aquaculture technology (Garrett et al., 1997). For example, if the aquaculture production system is intensive and depends on the addition of agricultural by-products (such as animal wastes from pigs, poultry, or cow manure) to provide feed for the fish being raised, there is a possibility that potential human pathogens might remain on or in the product harvested from the ponds (Cahill, 1990; Kirby et al., 2003).

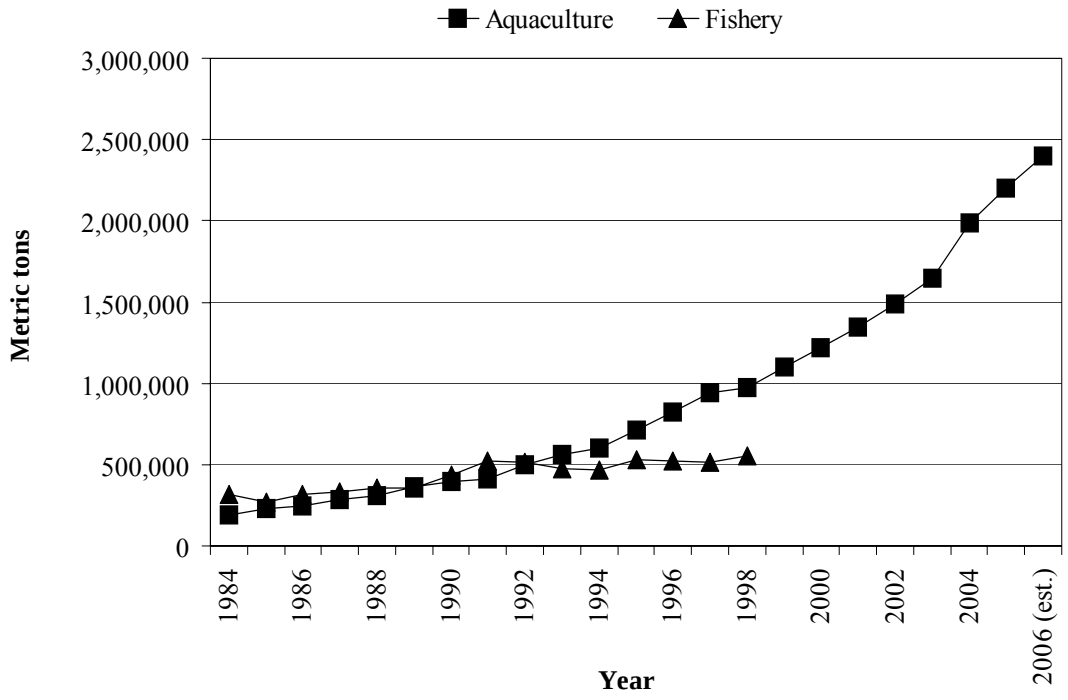

Figure 1.4: Global aquaculture production of Tilapia (Fitzsimmons, 2005).

Most of the Tilapia consumed in the US is imported (Figure 1.5). China is the world's major producer of tilapia and together with Taiwan, they have incorporated agricultural wastes as part of their aquaculture system (Cahill, 1990). The concern with this is that some pathogens can survive and contaminate the final product, which eventually could reach the retail market and could become a hazard to the consuming public. An example of this was seen in a study conducted by Chow et al., (1996), in which they investigated the microorganisms in Tilapia and milkfish obtained from fish ponds, supermarkets and traditional retail markets in Taiwan. They found that a high number of *E. coli* infection cases were due to water pollution in the fish ponds. This study agreed with Fujioka et al., (1988), who said that high numbers of *Enterobacteriaceae*

may be found in polluted waters, that *E. coli* could survive for long periods in tropical waters, and once introduced, they could become indigenous to that environment.

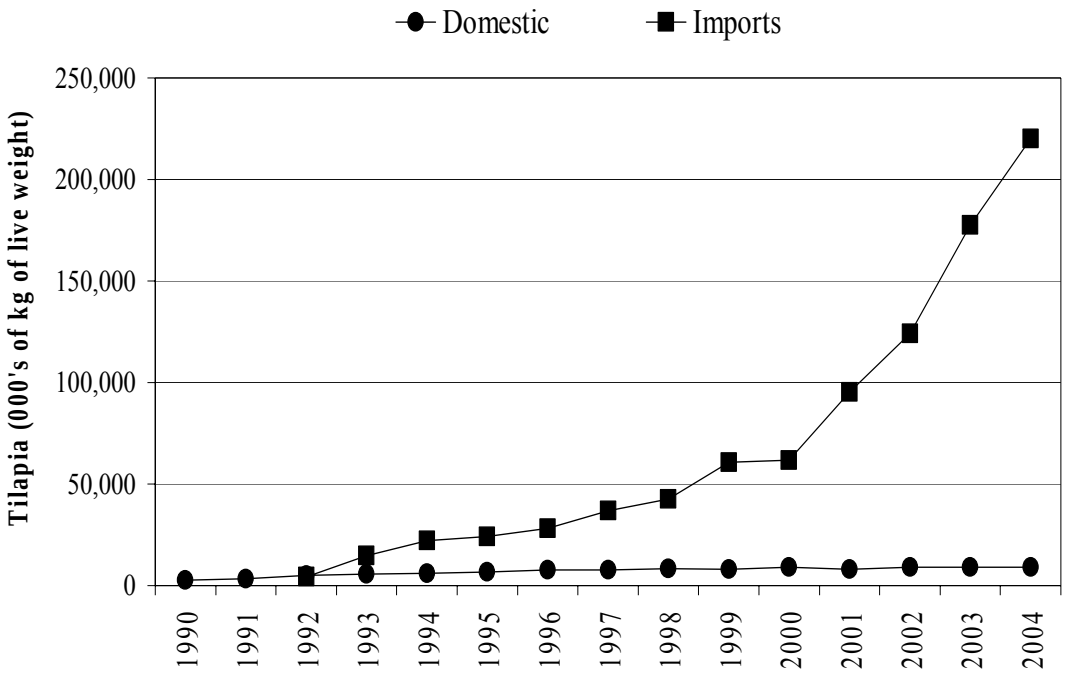

Figure 1.5: US consumption of Tilapia from domestic and imported sources (Fitzsimmons, 2005).

Tilapia can be imported to the US as frozen or fresh fillets, and as frozen whole Tilapia. Southeast Asian exporters dominate these frozen markets, with Taiwan, China, Indonesia, and Thailand being the main suppliers. Costa Rica, Ecuador, Colombia, Jamaica and Honduras are the major suppliers of fresh fillets (Teichert-Coddington and Green, 1997; Young and Muir, 2002). Fillets are available in different sizes and packages and they are available with or without skin, and can be ozone-dipped, carbon monoxide-

treated, individually quick frozen or smoked (Fitzsimmons, 2004). This demonstrates that the market for Tilapia products is diverse and large, and this demands a need for new innovations to ensure a high quality product and to avoid contamination of the final commodity.

1.7. On-board fishing vessels

It is well known that fish and seafood products are extremely perishable and if not handled or stored properly, they will spoil sooner than when is expected. Because spoilage bacteria can proliferate quickly, the sooner the fish is stored under refrigeration temperatures, the longer would be its shelf life. The use of ice for preserving newly-caught fish is critical to maintaining its quality and its shelf life. But, if ice is not efficiently used and on-board fishing vessels do not use proper storage (e.g. insulated ice boxes, containers and fish holds where ice is stored), the catch will not be preserved adequately, and this could result in lower economic returns (Shawyer and Medina Pizzali, 2003). For this reason, prevention measures should be taken on board, since poor post-harvest practices have been identified as one of the causes of higher solid waste loads produced during trimming and filleting (Islam et al., 2004).

The use of ice on board fishing vessels has proven to be an effective handling method for the following reasons: 1) It is available in many fishing ports; 2) it has a very high cooling capacity; 3) it can maintain a very definite temperature; 4) it is harmless and relatively cheap; 5) it can keep fish moist and can wash surface bacteria from the fish as it melts; 6) it can be moved from place to place and its refrigeration effect can be taken to

wherever it is needed; 7) it can be made on shore and used at sea (Shawyer and Medina Pizzali, 2003).

1.8. Sanitizers

Food processing and handing equipment should be properly cleaned in order to minimize the growth of microorganisms on food contact surfaces. This will minimize contamination of the product, enhance shelf life, and reduce the risk of foodborne illnesses (Wirtanen and Salo, 2003). In cleaning operations, food processing plants use a combination of detergents, acid/alkali rinses, and sanitizers/disinfectant treatments followed by final rinsing to control bacterial presence and cross-contamination from utensils, working food contact surfaces and equipment (Venter et al., 2006). The selection of a sanitizer and disinfectant in the food industry depends on the efficacy, safety and rinsibility of the agent as well as its corrosive nature and its effect on the sensory properties of the products manufactured (Wirtanen and Salo, 2003). A sanitizer is an agent that reduces microbial contaminants in the inanimate environment to levels considered safe by the Public Health Ordinance (Scientific Advisory Panel, 1997). On the contrary, disinfectants are meant to completely eliminate microorganisms. To obtain a rapid rate of bacterial kill, these are generally used at very high concentrations relative to their minimal inhibitory concentration (Chapman, 2003). However, the effectiveness of disinfectants can be affected by the presence of organic material such as fats, sugars, and protein-based materials (Wirtanen and Salo, 2003).

Sanitizing can be described as: 1) adequately treating food contact surfaces by a process that is effective in destroying vegetative cells of microorganisms of public health significance, and significantly reducing the numbers of other microorganisms; and 2) a process that does not negatively affect food products or their safety for consumers once applied (Gavin and Weddig, 1995). Thermal treatments and/or irradiation are some of the sanitization methods used in the food industry. Pasteurization could be an example of thermal sanitization. Thermal sanitation is effective in destroying certain microorganisms, but steam and hot water are relatively expensive to generate and excessive heat can be damaging to some food processing equipment (Guzel-Seydim et al., 2004). Another sanitizer that has been used is ozone. This sanitizer has good antimicrobial properties and can be used to sanitize food surfaces, food plant equipment, and to treat waste water intended to be used (Guzel-Seydim et al., 2004). However, ozone toxicity is a limitation, especially if it is intended for use in the food industry. This is so because it can affect the respiratory tract and produce symptoms such as headache, coughing, dizziness, a burning sensation in the eyes and throat, and induces a sharp taste and smell to food products (Guzel-Seydim et al., 2004). Besides, the initial cost of this technology is high because of the need to purchase, an in situ ozone generator (Martínez-Sánchez et al., 2006). Chemical sanitizers such as peracetic acid, hydrogen peroxide, iodophors, quaternary ammonium compounds (QAC) and chlorine have been used to treat industrial process waters and as disinfectants in the health, food, and consumer industries (Gavin and Weddig, 1995; Chapman, 2003; Aarnisalo et al., 2007). Peracetic acid is a strong disinfectant with a wide spectrum of antimicrobial activity, but its high cost and the

increase of organic matter in effluent due to acetic acid, are the major limitations for its use (Kitis, 2004). One of the most common uses of hydrogen peroxide is as a sterilant in packaging materials (Hsu et al., 2008). Although the hydrogen peroxide has shown to be effective in reducing native microbial and pathogen populations on some fruits and produce (Sapers 2003; Artés, 2007b), it is not yet approved by the FDA as a sanitizing agent for fresh produce (Artés et al., 2007a; Artés et al., 2009). Table 1.2 illustrates a summary of the advantages and disadvantages of iodophors, QAC and chlorine-based compound sanitizers.

Sanitizer Type	Advantages	Disadvantages
Iodophors	Non-corrosive, easy to use, non-irritating, broad activity spectrum	Expensive; food flavors and odors can be affected if used at high concentrations; forms purple compounds with starch. Are not stable above 120ºF-140ºF, because the iodine vaporizes.
Quaternary ammonium agents	Effective, non-toxic, prevents regrowth, supports microbial detachment, non-irritating, non-corrosive, odorless, flavorless. Stable to heat. Less affected by organic matter than is chlorine.	Inactivated in low pH and by salts (Ca^{2+} and Mg^{2+}), resistance development, ineffective against Gram-negative bacteria
Chlorine	Effective in low concentration, broad microbial spectrum, easy to use, supports microbial detachment, cheap	Toxic by-products, resistance development, residues, corrosive, reacts with EPS (exopolysaccharide), discoloration, explosive gas
Hypochlorite	Cheap, effective in a broad microbial spectrum, easy to use, supports detachment	Unstable, toxic, oxidative, corrosive, rapid regrowth, no prevention of adhesion, discoloration of products
Chlorine dioxide	Effective in low concentration, can be produced on-site, low dependency in pH	Toxic by-products, explosive gas

Table 1.2: Advantages and disadvantages of some disinfectants used in the food processes (Gavin and Weddig, 1995; Wirtanen and Salo, 2003)

1.8.1. Advantages and disadvantages

Several benefits are obtained when food sanitizers are used on food commodities or on food contact surfaces. Since they can reduce the microbial burden in the working environment, sanitizers also help to reduce the incidence of foodborne illness. Several researches have reported on the use of sanitizers to reduce microbial load on fresh produce such as carrots, cilantro, spinach, lettuce and tomatoes (Zhuang and Beuchat, 1996; Park et al., 2001; Koseki et al., 2003; Gonzalez et al., 2004; Ruiz-Cruz et al., 2006, 2007; Park et al., 2008; Stopforth et al., 2008; Allende et al., 2009; Keskinen et al., 2009). Others have reported their use on animal carcasses or derived products such as beef, poultry, and seafood/fish (Kim et al., 1994, 1996; Xiong et al., 1998; Blaszyk, and Holley, 1998; Park et al., 2002; Su et al., 2003; Russell and Axtell, 2005; Huang et al., 2006). For example, fruits and vegetables may harbor a variety of microbes from the environment where they grow, and washing is a critical step for the maintenance of quality and the safety of these products. Besides, sanitized washing is often the only measure taken to reduce microbial populations and to remove contaminants during the preparation of fresh-cut produce (Simons and Sanguansri, 1997; Ruiz-Cruz et al., 2007). However, tissue injuries make fresh-cut produce more vulnerable to microbial growth and quality deterioration than the uncut produce, and if the quality of the water used for cleaning is not the same as drinking water, it could contribute to the contamination of the fresh produce (Francis et al., 1999; Beltrán et al., 2005; Doyle, 2005).

Another aspect to consider is the effect of sanitizers on the quality attributes of the food products. For example, decontamination processes may cause undesirable

physical changes to the color or texture of meats or other types of food (Hegerding et al., 2005; Lin and Chuang, 2001). Also, if the sanitizer remains on the surface at high residual levels after rinsing, it could be toxic and/or affect the taste of the product. These changes can affect the perception of consumers when they are about to decide whether or not they are making the right choice to purchase the product. In some cases, the product could be perfectly fine but if its appearance is unacceptable to consumers, then the effort and the investment are worthless.

1.8.2. Chlorine-based compounds

Despite the antimicrobial effects that chlorine-based compounds have demonstrated, drawbacks associated to their use have been reported (Bower and Daeschel, 1999; Klaiber et al., 2005, Inatsu et al., 2005; Allende et al., 2008; Lee and Baek, 2008; Allende et al., 2009). Potable drinking water within the United States comes from surface (lakes, rivers, and reservoirs) and underground sources (springs, wells, aquifers) (Rose et al., 2001). To be safe for consumption, water from these sources need to be disinfected in order to inactivate microbial pathogens, such as bacteria, viruses and protozoa. This is so because some pathogens are often found in water as a result of: 1) fecal matter from sewage discharges; 2) leaking septic tanks; and 3) runoff from animal feedlots into bodies of water (EPA, 2008). Depending on its initial microbial load, treatment of contaminated water with disinfectant chemicals such as chlorine reduces the population of pathogenic bacteria but may not eliminate them altogether (Kirby et al., 2003; EPA, 2008). Public water systems are regulated under the Safe Drinking Water Act

(SDWA), and the U.S. Environmental Protection Agency (EPA) is authorized to set national standards to protect dinking water and its sources against contaminants (Lee et al., 2002). According to the EPA, (2001), disinfectants are effective in controlling many microorganisms, but they can react with natural organic and inorganic matter in the water to form potentially harmful disinfection byproducts (DBPs) such as trihalomethanes (THMs), which are potential carcinogens (Aieta et al., 1984; Fawell, 2000; Allende et al., 2009). Many of these DPBs can cause cancer, reproductive and developmental problems in laboratory animals (EPA, 2001). Because more than 200 million people consume water that is disinfected, the EPA has been mandated by the SDWA to establish maximum contaminant levels for DBPs and maximum residual disinfectant levels for chlorine, chloramines, and chlorine dioxide under the Disinfectants and Disinfection Byproducts Rule (EPA, 1998). Chlorine is a severe nose, throat and upper respiratory tract irritant and if people are exposed to high concentrations, they can develop severe respiratory tract damage. These can also include bronchitis and pulmonary edema which can be fatal (Chaiyakosa et al., 2007).

The resistance some microorganisms have shown against the effect of chlorine-based and other sanitizers employed in the food industry is another important factor to take into consideration (Frank and Koffi, 1990; Lee and Frank, 1991; Russell, 1997; Bower and Daeschel, 1999; Aase et al., 2000; To et al., 2002; Lundén et al., 2003; Aarnisalo et al., 2007; Vandekinderen et al., 2009). When a microorganism develops resistance to a sanitizer solution or disinfectant, it means that the microbe has the ability to remain viable and/or multiply under conditions that would destroy or inhibit other

members of the strains (Cloete, 2003). This resistance can be achieved by mutation, acquisition of new genetic information, expression of previously silent genes, and growth in biofilms, for example (Chapman, 2003). The possibility that biofilms may not be inhibited by high levels of residual chlorine has significant implications. This causes an increase in energy consumption, mechanical blockage of pipes and the acceleration of corrosion to metal surfaces (Kumar and Anand, 1998; Shi and Zhu, 2009). For these reasons, safer and more efficient sanitizers are needed to ensure an improved quality of wholesome foods.

1.9. Cross-contamination of seafood products

Cross-contamination is one factor that contributes to the spread of harmful microorganisms from one surface to another. It can occur at any point in the food chain such as on the farm, in the processing plant, at the retail level or in the kitchen (Dewaal, 2006). Different factors are known to contribute to the contamination of raw seafood products. These include: 1) the food handlers; 2) practices at work; 3) insanitary conditions of the work environment, including utensils used; and 4) the ice that is used during refrigeration or low temperature storage. The findings from Rashid et al. (2000) are in agreement with the statement previously exposed. According to them, the bacterial load on shrimps for example, increased along the different steps in processing, mainly due to contamination from ice, water, contact surface of utensils and the worker's hands.

At the retail level, the most common way to introduce microorganisms into seafood is via workers. If workers do not follow good sanitary practices during the

handling of a raw product, it can become contaminated with both pathogenic and or spoilage microorganisms and this could cause foodborne illnesses and spoilage of product (Todd et al., 2007). However, if measures are taken to handle seafood products properly then cross-contamination can be prevented (USDA-FSIS, 2006). The concern with the mishandling of the seafood products is that pathogenic bacteria can contaminate not only other raw products that are handled in the same area but also food contact surfaces and processing equipment. Bagge-Ravn et al. (2003) reported that the processing equipment of food industries can harbor a microbial ecosystem both during production and even after cleaning and disinfection, especially if biofilms develop.

Seafoods are frequently displayed on ice once they reach the retail market. Melting ice has a tendency to wash bacteria off the fish, but if the water is not drained properly, and the fish remains soaking in it, a build up of slime could cause the fish to spoil quickly (Shawyer and Medina Pizzali, 2003; Moody, 2009). Besides, this melted ice could also be a source of pollution to the environment if it is filled with a high population of pathogenic bacterial cells. Thus, melting ice could be a potential safety hazard. If bacteria remain viable during storage conditions, the contact of fish and seafood with the melting ice could allow the water to develop a high bacterial load. If this water contacts RTE foods, a potential health hazard could develop. For example, since *E. coli* can remain viable during ice storage, there could be a chance for pathogenic strains within this group (e.g. *E. coli* 0157:H7) to contaminate food and to cause illness. Since ice commercially made with potable water alone will not eliminate pathogenic bacteria,

additional barriers or hurdles are therefore needed (in combination with refrigeration) to reduce the bacterial load in melted ice that come into contact with raw seafood products.

1.10. Studies with ice

The incorporation of a sanitizer in the ice used to preserve seafoods and fish is a promising alternative to extend the shelf life of these products and to reduce the risks associated with them. The use of sanitized ice to preserve seafoods and to extend their shelf life has been studied and reported in the literature. For example, Oral et al., (2008), studied the preserving effect of ice containing wild-thyme hydrosol (plant extract) for extending the shelf life of fish. Their results were favorable, and consumers appeared to favor fish treated with this extract because of the pleasant odor and taste. In another study conducted by Campos et at., (2005), the microbial quality and sensory attributes of sardines treated with ozonised slurry ice was evaluated. Results showed the sanitized ice improved the sensory, microbiological and biochemical quality of sardines when compared with storage in conventional flaked ice.

In another study, antimicrobial ice containing chlorine dioxide (ClO_2) was utilized to control foodborne pathogens on fish skin. This study, conducted by Shin et al., (2004), evaluated the effect of the antimicrobial ice on the reduction of *E. coli* O157:H7, *Salmonella* Typhimurium, *L. monocytogenes*. Results obtained showed that when antimicrobial ice (100 ppm ClO_2) was applied to fish skin for 120 min, the total reduction of *E. coli* O157:H7, *Salmonella* Typhimurium and *L. monocytogenes* was 4.8, 2.6 and 3.3 log_{10}, respectively. Likewise, Phuvasate and Su (2009) evaluated the effect of ice

prepared with electrolyzed oxidizing (EO) water (100 ppm chlorine) against histamine-producing bacteria on yellowfin tuna. These microorganisms are known to growth and produce a toxin (histamine) if the fish is stored improperly, usually at temperatures higher than 7.2ºC [45ºF] (López et al., 1996; FDA, 2001a). The results for that study showed that EO ice was capable of reducing the histamine-producing bacteria (*Enterobacter aerogenes* and *Morganella morganii*) by 2.4 and 3.5 log CFU/m2, respectively, on the fish skin after 24 hours.

Although these studies show the advantages of ice containing sanitizers or antimicrobial agents, limited information is found regarding the microbial burden in the waters collected as the ice melts. Since this can be a potential source of cross-contamination for seafood and other products and for the environment, the effect of sanitizers on these microbial loads requires further studies.

1.11. Alternatives

1.11.1. PRO-SAN®

PRO-SAN® is an organic acid sanitizer manufactured by Microcide Inc. It is approved by both the USDA and FDA for the cleaning of fruits and vegetables. When used on food contact surfaces, PRO-SAN® is not required to be rinsed as long as its concentration is less than 1%. This sanitizer is shelf-stable, clear, colorless, odorless, biodegradable, and free of animal products (Microcide Inc., 2004). The main active ingredients are citric acid and sodium dodecylbenzene sulfonate. As an organic acid, citric acid has antimicrobial activity, due in part to its pH lowering ability (Chien, 1992;

DiPersio et al., 2004) and its ability to inhibit essential metabolic reactions in microorganisms (DiPersio et al., 2004). The antibacterial effectiveness of organic acids is thought to stem from the fact protonated acids are membrane soluble, and can enter the cytoplasm of the cell by simple diffusion (Lambert and Stratford 1999; Ricke, 2003). This may be possible because the near-neutral intracellular pH favored by most bacteria results in immediate acid dissociation and release of protons and anions inside the cell (Carpenter and Broadbent, 2009). As a result, the intracellular pH of the cell is lowered and the metabolism of the microorganism is inhibited (Diez-Gonzalez and Russell, 1997).

On the other hand, sodium dodecylbenzene sulfonate (SDBS) acts as a surfactant agent. It can be defined as being heterogeneous and with long-chain molecules containing both hydrophilic (head) and hydrophobic (tail) parts (Paria, 2008). These can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds (Prince, 1997; Mulligan, 2005). Therefore, SDBS helps to release the water-insoluble contaminants that are strongly bound to surfaces (Neupane and Park, 1999; Lee et al., 2007). Surfactants are also known to interact with microbial proteins and can be manipulated to modify enzyme conformation in a manner that alters the enzyme's activity, stability and/or specificity (Kamiya et al., 2000). These agents may promote exocytosis (intracellular organelles are released out of the cell) by interaction with cell and organelle lipid membrane components (Ishikawa et al., 2002; Chatterjee et al., 2002; Simões et al., 2005; Simões et al., 2008).

1.11.2. Electrolyzed water

Electrolyzed water is a sanitizer that has been used mainly in Japan, and it has been shown to have antimicrobial activity against different microorganisms (Fabrizio and Cutter, 2003; Kim et al., 2000a, 2000b; Kim, et al., 2001; Kiura et al., 2002; Park and Beuchat, 1999; Park, et al 2002; Venkitanarayanan et al, 1999; Vorobjeva et al., 2004). Recently, electrolyzed water is now widely used in sectors such as agriculture, dentistry, medicine and the food industry in many countries (Huang et al., 2008). Many studies have also been conducted to evaluate the efficacy of electrolyzed water as a disinfectant for poultry (Fabrizio et al., 2002; Park et al., 2002), cutting boards (Venkitanarayanan et al., 1999), vegetables (Izumi, 1999; Koseki et al., 2001, 2004), fruits (Al-Haq et al., 2002; Koseki et al., 2004) and food contact surfaces (Park et al., 2002; Handojo et al., 2009).

1.11.2.1. Production of electrolyzed water

The electrolyzed water is produced by the electrolysis of a diluted salt solution (sodium chloride and tap water) in a chamber, where a semi-permeable membrane separates an anode from a cathode electrode (Figure 1.6). Acidic electrolyzed water (AEW) can be obtained from the anode side. This resulting water has a high oxidation reduction potential (ORP) with values higher than 1000 mV and a pH lower than 3.0. Basic electrolyzed water (BEW) is obtained from the cathode side and has a higher pH (10.0 – 11.5) and an ORP ranging from -800 mV to -900 mV (Huang et al., 2008).

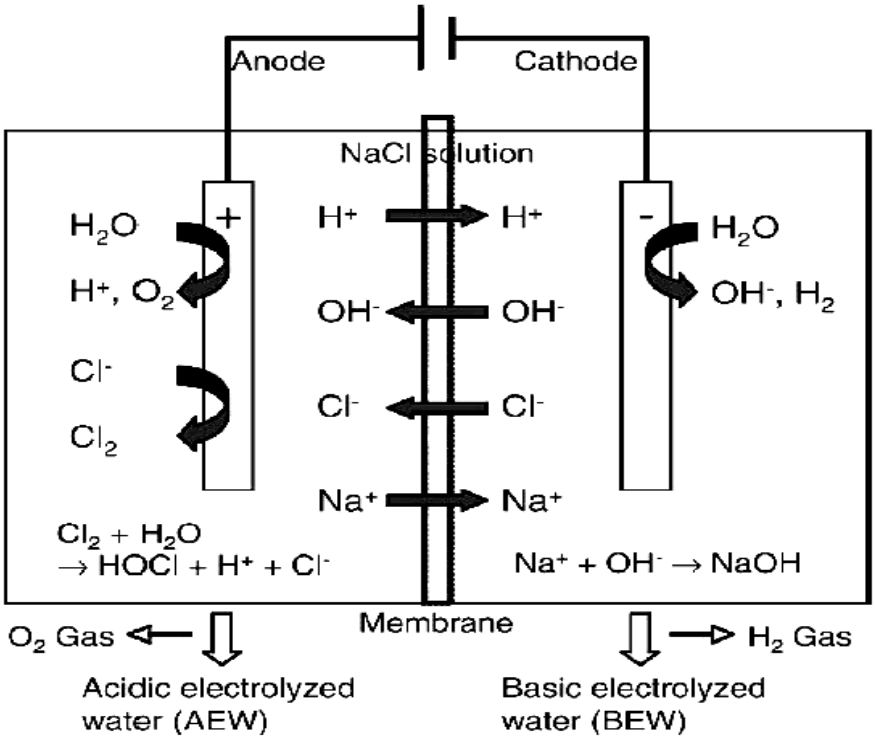

Figure 1.6: Diagram of the generation of electrolyzed water (Hricova et al., 2008)

1.11.2.2. Advantages and disadvantages of AEW

Although the literature mentions that the antimicrobial effect of AEW comes from the combined action of the hydrogen ion concentration, ORP and the amount of free chlorine (mainly hypochlorous acid) present in the solution (Huang et al., 2008), it is believed that the main contributors to the antimicrobial activity of AEW come from chlorine and the high ORP (Al-Haq, et al., 2005). Since the antimicrobial activity of electrolyzed water and ORP increase when the pH decreases, AEW is commonly used as a sanitizer while the BEW is used as a degreaser to remove dirt from various kitchen utensils (Hsu, 2005; Huang et al., 2008). The ORP indicates the ability of a solution to acts as a reducing or oxidizing agent. For the purpose of this study, the ORP of the

electrolyzed water indicated its effectiveness as a sanitizer against microorganisms. Thus, solutions with higher ORP values will have a greater oxidizing strength and will be more effective in reducing microbial numbers. Usually, bacteria grow in a pH range of 4-9, and aerobic bacteria typically grow at an ORP range of +200 to 800 mV. The ORP range for anaerobic bacteria is -700 to +200 mV (Huang et al., 2008). Because of its low pH, it is believed that AEW reduce the growth of bacteria by making the bacterial cell more sensitive to the active chlorine. This is caused by modifications to the outer membrane of the cell and it facilitates the entry of HOCl, which eventually cause the death of the organism (Park et al., 2004).

When compared with other chlorine-based sanitizers, AEW is safer to use because it is not corrosive to skin, mucous membrane or organic matter, therefore, it is harmless to humans and does not have a negative impact on the environment (Mori et al., 1997; Nakagawara et al., 1998). Likewise, Ayebah and Hung (2005) also indicated that AEW did not have any adverse effect on stainless steel and that it can still be safely used as a sanitizer to inactivate bacteria on food contact surfaces made from stainless steel in food processing. Since AEW reverts to normal water after its used, there is no need for special handling, storage, or transportation of concentrated chemicals that are a potential health hazard (Al-Haq et al., 2005). Its production only involves the use of sodium chloride and it does not produce harmful byproducts when in contact with organic matter (Kim et al., 2000a; Huang et al. 2008). Another aspect to take into consideration is that AEW does not promote the development of bacterial resistance, because of its nonselective antimicrobial properties (Al-Haq et al., 2005; Vorobjeva, et al., 2004). However,

Guentzel et al., (2008) and Huang et al., (2008) reported that problems, such as chlorine gas emission, metal corrosion, and synthetic resin degradation, due to its strong acidity and free chlorine content have been a matter of concern. In addition to this, Tanaka et al., (1999) reported that metal corrosion and synthetic resin degradation have occurred on hemodialysis equipment, although this reaction was not significant.

1.11.2.3. Neutral electrolyzed water (NEW)

The incorporation of NEW as a sanitizer in foods and food contact surfaces is a promising alternative to chlorine-based sanitizers. The principles for the production of NEW are the same as those that are used to produce AEW. However, in the case of NEW, part of the product formed at the anode side of the EO water generator is redirected towards the cathode chamber during the electrolytic process (Pernezny et al., 2005; Guentzel et al., 2008). This mixing causes the NEW generator to produce a neutral solution (pH 5.0 – 6.5) in which the most effective form of chlorine compound is hypochlorous acid (~95%) having strong antimicrobial activity (Cui et al. 2009). Besides, the resulting solution has a high ORP (Huang et al., 2008) and does not contribute to the corrosion of processing equipment or to the irritation of hands (Abadias et al., 2008).

1.12. Transmission Electron Microscope

Electron microscopes are instruments used to examine objects on a very fine scale in order to obtain information about the topography, morphology and composition of given specimen. Transmission electron microscope (TEM) is a type of electron

microscope that was originally designed to give structural information about a sample, by forming a magnified image or diffraction pattern (Egerton and Malac, 2005). For this reason, TEM has become a useful tool for the scientific community, especially for researchers that are interest in studying the morphology of microorganisms and how the structure of these organisms can be affected.

The increase incidences of foodborne illnesses demand innovations and preventive measures in order to reduce the risk on infections that can become life-threatening. The utilization of food-graded sanitizers as a tool for reducing bacterial populations at the work environment has been an approach to this matter. Even though enumeration of bacterial populations before and after treatment provides information on the treatment effect on viability, limited information is available on the type or extent of morphological or physical damage that occur to the bacterial cells (Hajmeer, et al., 2006). Therefore, visual information is useful to provide insight on the microstructure of the cell and also to characterize the type and magnitude of changes that occur to the cell's composition in response to treatments (Hajmeer, et al., 2006). This can help researchers and investigators to understand how effective a treatment is and what is the mechanism involved to kill the microorganisms. Jeong et al. (2006) and Liao et al. (2007) observed the morphological changes of *E. coli* cells after exposure to electrolyzed water treatment. In another study, Feng et al. (2000) observed the antibacterial effect of silver ions on *E. coli* and TEM images allowed them to directly observe the morphological changes of the internal structure of the bacterial cells after the silver ion treatment.

Prior to the development of the TEM, cells and bacteria could be observed by the use of light microscopy. However, the resolution of light microscopy (approximately 200 nm) was a limitation (Curry, et al., 2006). This is so because the small size of bacteria (close to the resolution limit of conventional light microscopy), impeded morphological investigations (Eltsov and Zuber, 2006). TEM can be used to investigate tissues and the organization of organelles within large cells at nanometer scale resolution (Müller et al., 2008). It utilizes electrons as light sources and their much lower wavelength makes it possible to get a resolution a thousand times better than with a light microscope (Nobel Foundation, 2009). Besides, since TEM allows the examination of specimen cross-sections, gradients in structure from the surface to interior regions can be observed (Simmons and Thomas, 1998). However, TEM observation requires the preparation of thin samples (<100 nm). This is so because the penetrating power of the electron beam is poor (Brown, et al., 1998), but thin specimen sections limit scattering of the electrons and allow proper resolution (Egerton and Malac, 2005). Conventional TEM is based on chemical fixation, which transforms the biological material into a chemically cross-linked gel and prevents the degradation of the cellular components (Eltsov and Zuber, 2006). The fixed sample is dehydrated by an organic solvent and embedded in a resin, which allows thin sectioning and provides resistance against the TEM environment. Besides, the sections are usually impregnated with heavy metals to enhance the electron scattering and to obtain a better image contrast (Eltsov and Zuber, 2006).

Since researchers and scientists have been able to integrate TEM as a tool to understand the effect of different stressors in bacterial cells, TEM can be a promising tool

to study pathogenic bacteria, both Gram-positive and Gram-negative in nature. Because these two groups have different cell structures, the high resolution of TEM will provide valuable information for the understanding and behavior of these bacteria when exposed to treatments that are meant to destroy them.

CHAPTER 2

EFFICACY OF SANITIZED ICE IN REDUCING BACTERIAL LOAD ON FISH FILLET AND IN THE WATER COLLECTED FROM THE MELTED ICE

2.1. Abstract

The first part of this study compared the stability of ice prepared with various sanitizers with the stability of ice prepared with tap water (control). An organic acid formulation (PRO-SAN® at 0.1%, 0.5% and 1% concentrations) and neutral electrolyzed water (NEW) were used as the chemical sanitizers. This was done by comparing the melting rates of the ice samples at room temperature (25ºC). The second part of this study investigated the efficacy of the sanitizers to reduce the natural microflora washed off from the whole fish samples. This was conducted by testing the water from the melted ice and the ice that was in contact with the whole fish for their bacterial load. The last part of this study investigated the efficacy of sanitized ice to reduce *Escherichia coli*, *Listeria innocua* and *Pseudomonas putida* populations on fish fillet samples as well as in the waters from the melted ice. This was done by enumerating the amount of each bacterial species on the fish and in the water samples at 12 and 24 hours intervals, respectively. The objective of the last part of this study was to evaluate the incorporation of ice prepared with various sanitizers to reduce the potential for cross-contamination of fish fillets and other seafood products under refrigeration storage. It also aimed to reduce the

microbial burden in the working environment. Results showed that the sanitizers did not affect the stability of ice when compared with the control. Therefore, the melting rate of the sanitized ice was not significantly different ($p > 0.05$) from the control. For the whole fish experiment, the results showed that sanitized ice significantly ($p < 0.05$) reduced the natural microbial burden washed off from the whole fish and was lodge in both the water from the melted ice and in the ice that was in contact with the fish. On the other hand, the results collected from the fish fillets on sanitized ice showed that the bacterial load reductions for *E. coli* and *L. innocua* were not relatively different than the reductions obtained with the control. However, significant differences ($p < 0.05$) were observed for the mean overall reductions in the *P. putida* population obtained with PRO-SAN® at 0.1% (0.23 log CFU/g) when compared with the control (0.49 log CFU/g). For the water samples, the results showed that the sanitizer treatments significantly ($p < 0.05$) reduced all the bacterial species used under this study when compared with the control. It could be concluded that the potential for cross-contamination of fish or other types of raw seafood products could be significantly reduced by the use of sanitizers such as PRO-SAN® and the NEW in the ice.

2.2. Introduction

The market for seafood and fish products is diverse, large and important. Fish is an important source of protein and provides many health benefits. One such benefit is its high level of omega-3(n-3) fatty acids which is known to reduce cholesterol levels and the incidence of stroke, heart disease, and pre-term delivery (Daviglus et al., 2002; Konig

et al., 2005; Willet, 2005). In the US, more than 2 billion kg of seafood were consumed in 2002, and consumption has risen steadily over the past several decades from an average of 4.5 kg per person in 1960 to about 7 kg in 2002 (Butt et al., 2004). Globally, more than 63.5 million tons of seafood are caught and consumed each year (Butt et al., 2004).

Despite all the health benefits these products provide, there are some risks and concerns associated to their consumption. Most of the seafood consumed in the US is imported from different countries around the world, and at least half of these are produced by aquaculture production systems (GAO, 2004; NOAA, 2008). An example of this is the Tilapia fish. In some aquaculture facilities, production is intensive and depends on the addition of agricultural by-products (such as animal wastes from pigs, poultry, or cow manure) to provide feed for the fish being raised. This practice raises the possibility that potential human pathogens could contaminate the fish harvested from these ponds (Kirby et al., 2003). For instance, healthy cattle are the main reservoir for *E. coli* O157:H7, and if the manure from these animals is used to feed the fish being raised or as a pond fertilizer, the risk that this pathogen may be present increases substantially. Besides, since *E. coli* O157:H7 is capable of surviving in water for long periods of time, it is more likely to contaminate not only the water sources but also the fish (Kirby et al., 2003).

Foodborne illness is a major cause of concern in the US, and approximately 76 million illnesses occur annually, leading to 325,000 hospitalizations and 5000 deaths (Mead et al., 1999). A large number of foodborne illness cases are associated with the consumption of raw or lightly cooked seafood and fish (Wallace et al., 1999; Rocourt et

al., 2000; Feldhusen, 2000; But et al., 2004; Dewaal et al., 2006). Seafood and fish products are highly susceptible to deterioration and to microbial contamination. To prevent the proliferation of pathogens on these products, ice is extensively used as a preservation method. If potable water is used to make this ice, at best, it will allow the bacterial load on the fish to survive. However, if ice made with sanitized water is used to store the fish, it has the potential to be bactericidal to the microorganisms. Sanitized ice prepared with chlorinated water could help in achieving this goal, but there are concerns about chlorine-based chemicals interacting with organic matter to form potentially harmful disinfection byproducts (DBPs), such as trihalomethanes (Aieta et al., 1984; Fawell, 2000; EPA, 2001; Allende et al., 2009). Many of these DPBs have been demonstrated to cause cancer, reproductive and developmental symptoms in laboratory animals (EPA, 2001). Besides, the impact these sanitizers could have on the environment is an issue demanding attention, actions and innovation from the food industry and pertinent regulatory agencies.

This study investigated the use of ice made with two different sanitizers for the storage of fish and fish products. This ice was made with PRO-SAN® (an organic acidic formulation) and NEW sanitizers. These were selected because they did not show potential to bioaccumulate in the environment (Mori et al., 1997; Nakagawara et al., 1998; Kim et al., 2000; Lopes, 2004; Microcide Inc., 2004; Lee et al., 2007; Huang et al. 2008; Handojo et al., 2009). In addition, these sanitizers are not toxic to human when correctly used. They are safe on the human skin and do not corrode metal. However, studies by Lee et al., (2007) and Handojo et al., (2009), show that these sanitizers are as potent as

the traditional ones. By reducing the bacterial load on fish, the risk of cross-contamination and associated foodborne illnesses would be minimized. Additionally, the bacterial burden in the environment is also reduced when the sanitized ice melts.

Since these two sanitizers have not been extensively studied for their efficacy in reducing bacteria load on seafood products or to reduce cross-contamination in seafood processing environments, the objectives of this study were: 1) to investigate the ability of chemically sanitized ice to reduce the natural bacterial load in melted ice used during the storage of whole fish; 2) to monitor the survival of *Listeria innocua*, *Escherichia coli* and *Pseudomonas putida* populations on fish fillet samples stored on sanitized ice compared with ice made from tap water; and 3) to monitor the survival of these bacteria in the waters collected from the sanitized and unsanitized melted ice used to store the fish fillets.

2.3. Materials and Methods

2.3.1. Bacterial cultures

Three different species of bacteria were used for this study: *Listeria innocua* (ATTC 33090), *Escherichia coli* K-12 (ATTC 29181), and *Pseudomonas putida* (ATTC 49451). *E. coli* and *Pseudomonas putida* were nalidixic acid resistant strains. Since Gram-negative bacteria in general are sensitive to nalidixic acid (Regnault et al., 2000), it was necessary to obtain strains resistant to this compound. To develop the resistance to nalidixic acid, *E. coli* K-12 and *P. putida* were incubated on Tryptic Soy Agar (Difco Laboratories, Sparks, MD) containing 50µg/ml nalidixic acid and 10 µg/ml of acriflavine. Bacterial colonies that were able to grow on this media were then harvested and incubated under the same conditions already mentioned (TSA 50µg/ml nalidixic acid and

10 µg/ml of acriflavine), and this procedure was repeated for four days until a pure culture for each bacterial species (*E. coli* and *P. putida*) was obtained. Once obtained, they were kept in the freezer (-80ºF) until ready for use. *P. putida* was used to evaluate the efficacy of sanitized ice against common spoilage bacteria found in fish and other seafood products. In evaluating the ice treatment for efficacy against *Listeria*, PALCAM media containing an antimicrobial supplement was used to prevent the growth of other Gram-positive bacteria. This thus created a medium selective for *Listeria innocua*.

The pure bacteria cultures were stored frozen (-80ºC) in 30% (v/v) sterile glycerol (Fisher Scientific, Fair Lawn, NJ). Each bacterial species was cultured individually by transferring a loopful of *E. coli*, *L. innocua,* and *P. putida* into 20 ml of sterile Tryptic Soy Broth (Difco Laboratories, Sparks, MD) containing 0.3% (w/w) yeast extract (Fisher Scientific, Fair Lawn, NJ) (TSBYE) using a 10 µl inoculation loop. The bacteria were incubated for 24 hours at 37ºC (*L. innocua* and *E. coli*) and 30ºC (*Pseudomonas putida*), respectively. Afterwards, the bacteria were transferred from the TSBYE to a Tryptic Soy Agar (Difco Laboratories, Sparks, MD) slant supplemented with 0.3% (w/w) yeast extract (TSAYE) by loop inoculate and incubated for 24 hours at 37ºC (*L. innocua* and *E. coli*) and 30ºC (*P. putida*), respectively. Following the 24 hour incubation period, the slants were kept under refrigeration temperatures (4ºC) and used as a stock culture.

Prior to each experiment, a loopful of the respective bacteria was taken from the slant and transferred into a 20 ml sterile TSBYE and incubated for 24 hours at 37ºC and 30ºC, depending on the bacterial species. Following incubation, each bacterial cell's broth was transferred into a sterile 50 ml plastic centrifuge tube and sedimented at 7,000

rpm for 10 minutes (Sorvall® RC 5C Plus, Kendro Laboratory Products, Newtown, CT). The supernatant was decanted after centrifugation, and the pellets were suspended in 20 ml of sterile potassium phosphate buffer (pH = 7.2). The buffer stock solution was prepared by mixing 0.1M potassium phosphate monobasic (Acros, New Jersey, NJ) and 0.1 M potassium phosphate dibasic (Fisher Scientific, Fair Lawn, NJ) until they reached a pH of 7.2. Once the buffer solution was added, the solution was mixed by a vortex mixer (Vortex-Genie®2, Scientific Industries, Inc., Bohemia, New York). This was done prior to inoculation of the fish fillet samples.

2.3.2. Whole fish and fillet samples preparation

Whole gutted Tilapia fish (average weight = 466g), in a frozen state, was obtained from a local supermarket in Columbus, Ohio. The fish samples were transported to the Ohio State University – Food Science and Technology Department and stored in the freezer (-40 ºC) until use. Tilapia fish fillets were similarly obtained, transported to the lab and were cut in squares (wt = 5 ± 0.3 g) using a fillet knife sterilized with ~ 90% alcohol and flame. After being weighed, the samples were vacuum-packed into polyethylene bags and stored in the freezer at -40º C.

2.3.3. Sanitizer solutions and ice preparation

The NEW solution containing 150 ppm chlorine, with a pH of 6.5-7.0, was prepared by the electrolysis of a saturated sodium chloride (Morton International Inc., Chicago, IL) solution, using an electrolyzed water generator (STEL-80) provided by

Hobart Corporation (Troy, OH). This was done at a setting of 29 ± 1 Amperes (A), and 20.0 ± 0.6 Voltage (V). The free available chlorine content, pH and oxidation–reduction potential (ORP) of NEW were determined using a HI 95771 Clorine Ultra High Range Meter (Hanna Instruments, Ann Arbor, MI), a pH Indicator Paper (Whatman International Ltd, Maidstone, Kent, UK) and an ORP meter (titrator model DL70ES, Mettler Toledo, Columbus, OH), respectively. The NEW ice was prepared by freezing the NEW water into sealed freezer plastic bags immediately after production and held inside a -40ºC freezer until use.

Another treatment tested was PRO-SAN®, an organic acid sanitizer (containing citric acid and sodium dodecylbenzene sulfonate) obtained from Microcide Inc. (Troy, MI). The sanitizer was supplied as a powdered concentrate, which needed reconstitution before use. Three different concentrations of this sanitizer were prepared (0.1%, 0.5% and 1%). To prepare the concentrations, 1g, 5g and 10g of the powdered concentrate were weighed and mixed with 1000 ml of tap water to obtain the 0.1%, 0.5% and 1%, respectively. Once prepared, each of the solutions was poured into ice plastic trays, and held inside the freezer (-40ºC) overnight. Tap water ice cubes were also made as controls and stored under the same conditions.

2.3.4. Stability of ice treatments

For each of the ice treatments, the speed of melting was determined and this was used to estimate their stabilities. This was done by placing the ice cubes into plastic trays that were left uncovered at room temperature (25ºC). At 30 minutes intervals, the volume

of water from the melting ice was measured until the ice was completely melted. From the data collected and the rate of melting for each ice treatment was determined.

2.3.5. Whole fish storage on sanitized ice

This test was done to determine the ability of various ice treatments to reduce natural flora from whole fish. Four different ice treatments were used for this experiment- tap water (control) and three different concentrations of PRO-SAN® sanitizer (0.1%, 0.5% and 1%). Approximately 610 g of crushed ice were placed into a perforated plastic tray. The whole gutted Tilapia fish (~ 466g) samples were thawed in a refrigerator (4ºC) for around 20 hours, washed with tap water, and stored in the respective ice treatment for 8 hours. The total viable cells of the natural microflora from the whole fish were enumerated immediately after the 8 hours of storage.

2.3.6. Fish fillets storage on ice

The fish fillet samples were thawed in a refrigerator (4ºC) for 24 hours. The samples (n= 48) were then placed in an sterilized glass cutting board using tweezers (sterilized with ~ 90% alcohol and flame) and inoculated with 100 µl of the respective bacteria (*L. innocua*, *P. putida* or *E. coli*) to produce a contamination level of 10^{6-7} CFU/g. Four extra fish fillet samples were set apart. Two of the samples were inoculated with each bacterial species and were used to determine the amount of microbial load on the surface of the sample before treatment. The other two were not inoculated and were used to check the microbial load of the samples before inoculation. All samples were allowed

to dry inside a biological safety cabinet at room temperature for 15 minutes. Once dried, the samples were divided into the respective ice treatments [control, PRO-SAN® (0.1 and 0.5%), and NEW], covered with ice and stored in a refrigerator at 4ºC.

2.3.7. Microbial analysis

2.3.7.1. Whole fish stored on crushed ice

After eight hours storage, water from the melted ice of each treatment was sampled for microbial loads. The ice left in the plastic trays was melted and its microbial load was also tested. The water samples collected for each treatment were serial diluted (1:10 dilutions, water sample: 0.1% peptone water) and plated into Plate Count Agar (PCA) containing neutralizer (1:10, neutralizer: agar). The 10x neutralizer solution was prepared by mixing lecithin (0.07%), Tween 80 (0.5%) and sodium thiosulfate (0.1%) in distilled water (w/v). All these chemicals were obtained from Fisher Scientific (Fair Lawn, NJ). The plates were incubated at 30ºC for 24 hours and the microbial numbers determined by counting the colonies using a Darkfield colony counter (American Optical, Buffalo, NY). The bacterial numbers were expressed as CFU/ml.

2.3.7.2. Fish fillets stored on crushed ice

The efficacy of sanitized ice in reducing *L. innocua, E. coli* and *P. putida* was conducted with Tilapia fish. Each of these bacterial species was separately inoculated onto twelve sets of fish fillet samples inoculated with the bacterium to be tested (10^{6-7} CFU/g). Each set of samples were placed in perforated plastic trays containing ~ 610g of

crushed ice prepared with the sanitizers (PRO-SAN® [0.1% and 0.5%], NEW and tap water [control]). The plastic trays and the receptacle used to collect the water from the melting ice were sanitized with 85% proof alcohol prior to use. The sample sets were then covered with a thin layer of each of the ice treatments and stored in a refrigerator at 4ºC. At 12 hour intervals, two fish samples were taken from each ice treatment and aseptically placed in sterile stomacher bags (individually) using sterile tweezers (~ 90% alcohol and flame). The stomacher bags contained 45 ml of 0.1% peptone water with a 10x neutralizer (1:10, neutralizer: peptone water). The mixture was homogenized for two minutes in a stomacher (Tekmar Stomacher Lab-Blender 80, Cincinnati, Ohio) and serial dilutions prepared with 0.1% peptone water in order to determine the amount of bacteria viable cells after each treatment. On the other hand, at 24 hour intervals, 4 water samples were taken from each of the treatments in order to determine the bacterial load of the water collected from the melted ice. *L. innocua* plate counts were determined by pour plating onto PALCAM medium base agar with antimicrobic supplement (Difco Laboratories, Sparks, MD) and incubating for 36 hours at 37ºC. *E. coli* plate counts were determined by pour plating onto Triptic Soy Agar containing 50µg/ml nalidixic acid and 10 µg/ml of acriflavine (Difco Laboratories, Sparks, MD) and incubated for 36 hours at 37ºC. *P. putida* numbers were determined in a similar manner to *E. coli*, except that the plates were incubated at 30ºC for 48 hours. The bacterial cells were enumerated using a Darkfield colony counter (American Optical, Buffalo, NY). All treatments were repeated at least three times for each bacterial species under study.

2.3.8. Statistical analysis

Data obtained for the stability of the ice treatments were analyzed using two-way ANOVA and Dunnett's test. These analyses were conducted to determine if the melting rate of sanitized ice was different from than of unsanitized ice. For the whole fish stored on ice, results of the microbial test were transformed into log values (to normalize the data) and the data were analyzed using two-way ANOVA and Dunnett's test. The statistical analyses were conducted to analyze the significance of the bacterial load in the melted ice with and without sanitizers. In the case of the fish fillet stored on ice, the results of the microbial tests were transformed into log values [$\log_{10}$ (CFU/g and CFU/ml - for the fish and water samples, respectively)]. The data were analyzed by two-way ANOVA and Tukey's multiple comparison tests in order to determine how effective the sanitized ice was in reducing each bacterial species on the surface of the fish when compared with the control. In addition, the statistical analysis was conducted to evaluate the efficacy of the treatments in reducing the microbial burden in the waters drained after the ice melted. Significant differences were established at $p < 0.05$ using an SPSS, version 16, statistical software program (SPSS Inc., Chicago, IL).

2.4. Results and Discussion

2.4.1. Effect of the sanitizers on the stability of ice

The stability of ice prepared with the various sanitizers was compared with the stability of ice prepared with tap water as a control. Figure 2.1 shows the amount of water colleted from the sanitized ice at 30 minutes intervals. According to this graph, tap water appeared to melt faster than the sanitizers. For example, after 60 minutes at room

temperature, the volume of water collected from the control ice was 115.63 ml whereas 59.67, 84.50, 67.33, and 52.33 ml were collected from NEW and PRO-SAN® at 0.1, 0.5 and 1% concentrations, respectively. After 120 minutes, the volume collected for all the treatments were similar. After that point, the volume of water collected from the control seemed to be smaller than that of the sanitizers, giving the impression that the sanitized ice had melted faster than the control. Figure 2.2 illustrates the melting rate of the sanitized ice compared with the control. The results from this graph showed that the control appeared to melt faster during the first 90 minutes but after that time it melted at a constant rate. However, the melting rate for all the sanitized ice treatments appeared to increase slightly near the end of the test.

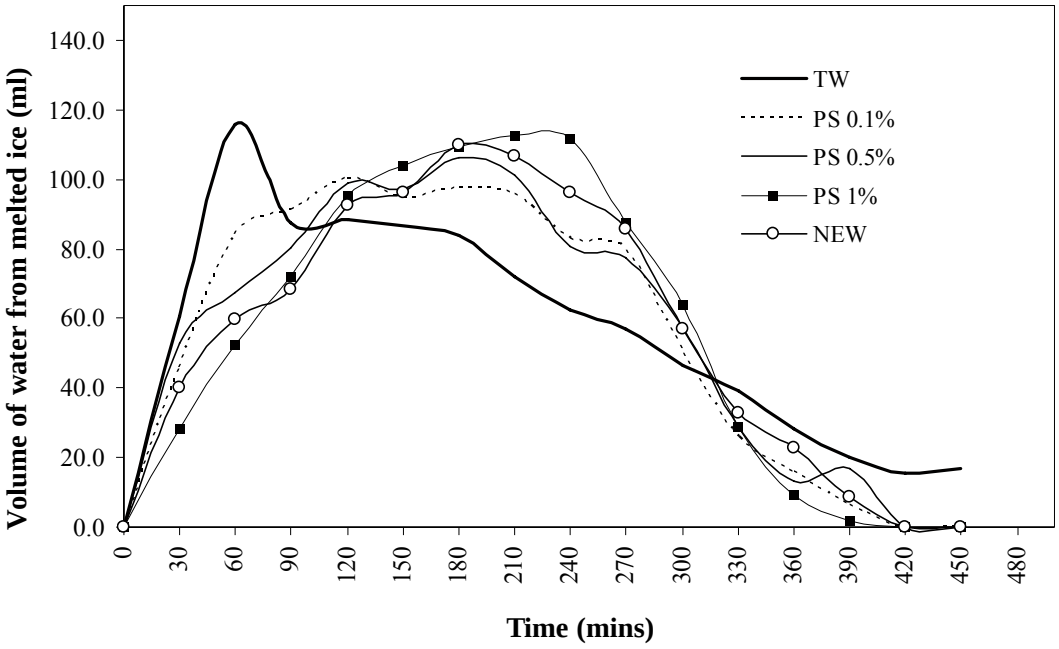

Figure 2.1: Volume of water collected from the ice treatments during the melting process

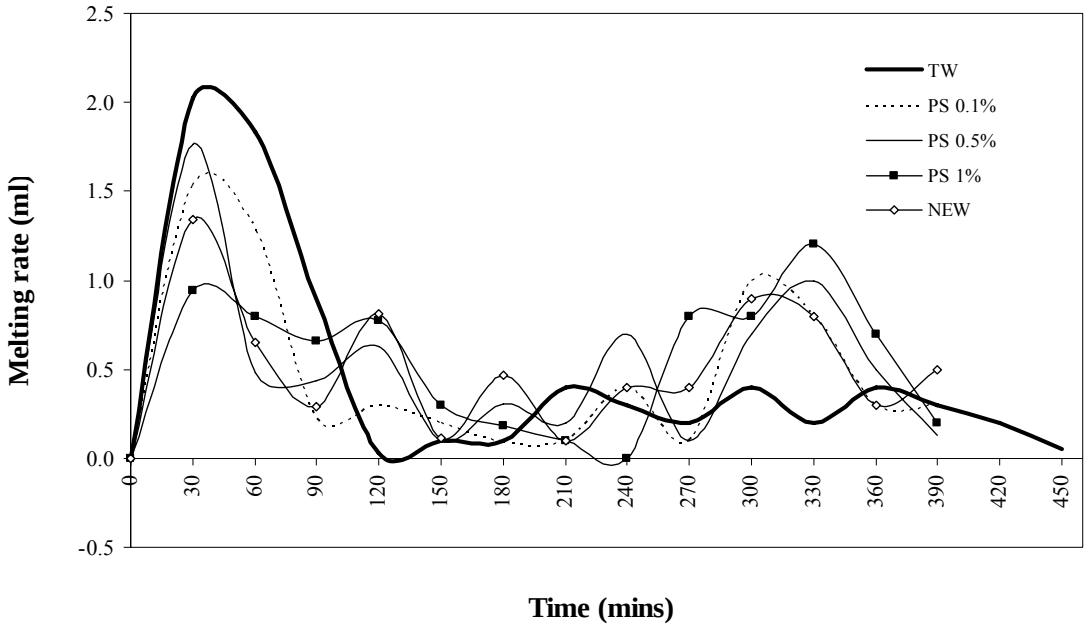

Figure 2.2: Melting rate for ice prepared with NEW and PRO-SAN® sanitizers when compared with the control.

In order to compare the melting behavior of the sanitized ice when compared to the control, the amount of liquid collected over time was subtracted from the initial volume of the ice (Figure 2.3). The results in this graph showed a similar behavior for all the ice treatments during the melting process. The ANOVA and Dunnet's methods were performed to determine the significance in the difference between the control and the sanitized ice. These statistical analyses revealed that the sanitizers did not have a significant ($p>0.05$) effect in the stability of ice when compared with the control.

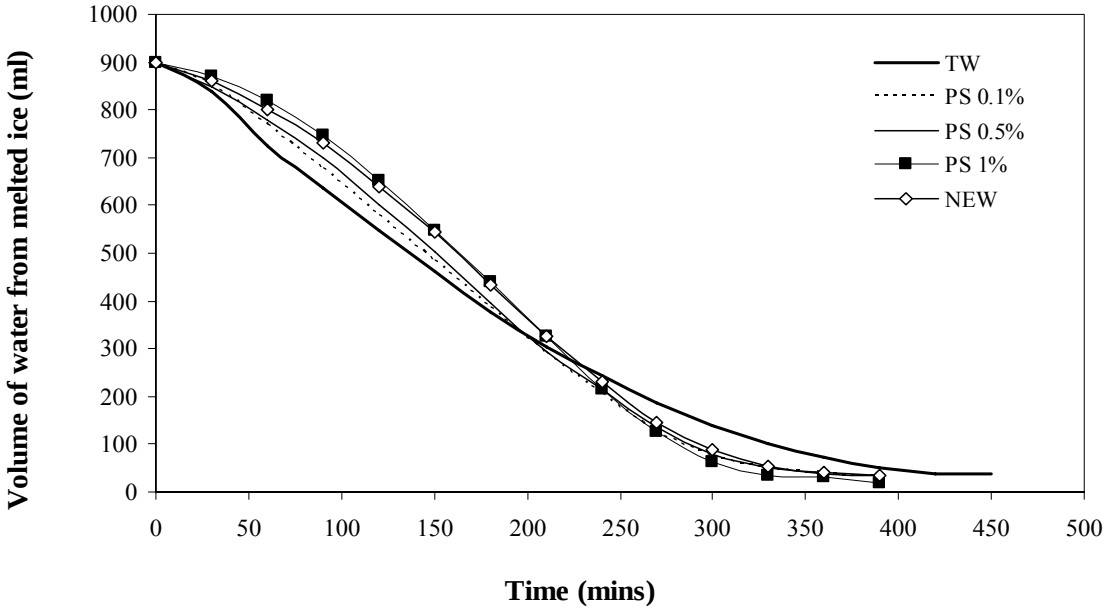

Figure 2.3: Reduction of the initial volume of ice as it was transformed from solid to liquid state.

2.4.2. Effect of PRO-SAN® sanitizer on the microflora of whole fish samples

The amount of bacteria in the ice left in the trays after storage of the whole fish samples and in the water from the melted ice were enumerated after 8 hours of exposure (Figure 2.4). For all treatments, the results showed significant differences ($p<0.05$) in the bacterial load in the ice used to store the fish when compared with the load in the water from the melted ice. Similarly, the results showed that the bacterial count in the tap water treatment were significantly higher ($p<0.05$) than the counts from the PRO-SAN® treatments. In the case of the PRO-SAN® sanitizer, no bacteria were detected in the water from the melted ice. However, the ice in contact with the fish had a bacterial load of 0.85, 1.02 and 1.15 log CFU/ml for the 0.1%, 0.5% and 1% concentrations, respectively. On the other hand, the tap water treatment had a bacterial load of 1.47 log CFU/ml in the

water from the melted ice whereas the bacterial load in the ice was 2.18 log CFU/ml. These results agreed with a previous study conducted by Gonzalez et al., (2004), in which different sanitizers, including PRO-SAN®, were exposed to total aerobic bacteria in shredded carrots. When the carrots were washed with water alone, up to 5.3 log CFU/ml of total aerobic bacteria were recovered. However, no recovery of bacteria was obtained from the sanitizing solutions used to treat the shredded carrots. This result confirms that sanitized ice can indeed reduce the natural microflora of fresh fish. In this present study, the reduction was seen in the water from the melted ice used to store the fish. This thus reduces the potential for cross-contamination to other seafoods, food contact surfaces and even to the environment.

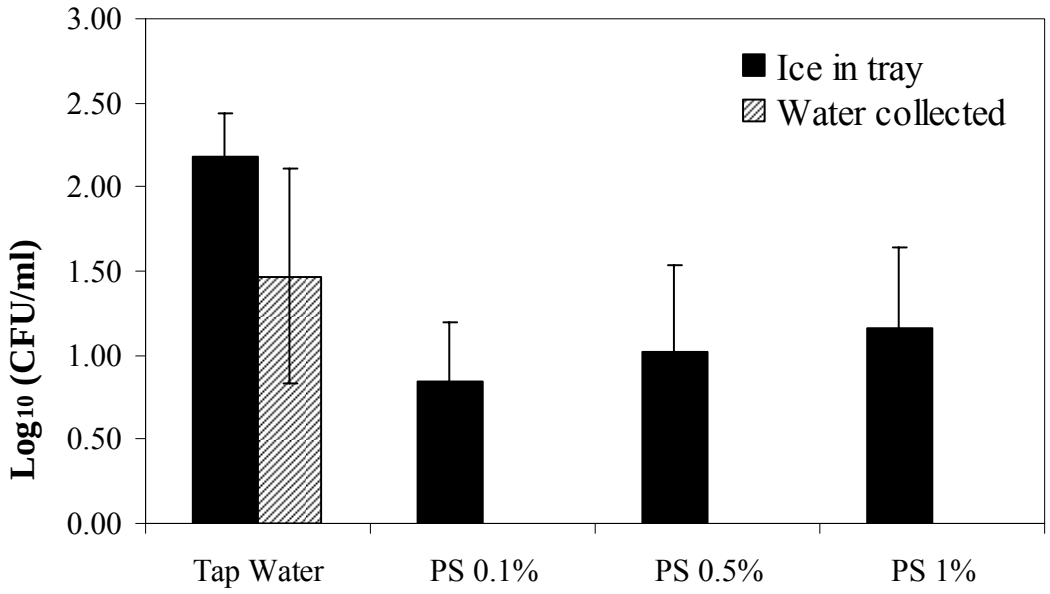

Figure 2.4: Natural microflora numbers in the waters from the melted ice and in the ice left after 8 hours storage of whole fish samples on PRO-SAN® and tap water ice.

2.4.3. Efficacy of sanitized ice on bacterial load reduction on fish fillet samples stored at 4ºC

To evaluate the efficacy of sanitized ice against foodborne pathogens of current public health concern, non-pathogenic *E. coli* K-12 (Gram-negative) and *L. innocua* (Gram-positive) were used as surrogates for *E. coli* O157:H7 and *L. monocytogenes*, respectively. The *E. coli* K-12 and *P. putida* strains used under this study were resistant to nalidixic acid.

Figure 2.5 illustrates how *E. coli* K-12 responded when exposed to the various ice treatments. The total bacterial reductions obtained for *E. coli* cells on the fish fillet samples were 0.36, 0.34, 0.45 and 0.62 log CFU/g after 72 hours exposure to tap water (control), PRO-SAN® (0.1% and 0.5%), and NEW ice treatments, respectively. Statistically, there were no significant differences ($p>0.05$) in the bacterial reductions between the ice treatments prepared with tap water and PRO-SAN® (0.1% and 0.5%), but the reductions were significantly different ($p<0.05$) for NEW ice compared with the other ice treatments. The data collected showed that the mean reductions for the treatments were 0.26, 0.22, 0.28 and 0.45 log CFU/g for tap water, PRO-SAN® (0.1% and 0.5%), and NEW, respectively.

Figure 2.6 shows the response of *L. innocua* after exposure to the different ice treatments. The total bacterial reductions obtained for *L. innocua* cells on the fillets were 0.65, 0.77 and 0.83 log CFU/g after 72 hours exposure to tap water and PRO-SAN® 0.1% and 0.5% treatments, respectively. The total bacterial reduction for *Listeria* was 0.72 log CFU/g) after 72 hours exposure to the NEW ice treatment. Statistically, no significant differences ($p>0.05$) were found in the bacterial cells reduction for *L. innocua* between

the treatments. The data collected showed that the mean reductions were 0.52, 0.57, 0.59 and 0.57 log CFU/g for tap water, PRO-SAN® (0.1% and 0.5%) and NEW, respectively.

The reductions obtained for *P. putida* on the surface of the fillet samples are shown in Figure 2.7. The statistical analysis revealed that the treatments had a significant effect ($p<0.05$) on the bacterial reduction on the fish samples. The initial count of *P. putida* before the treatments was 7.11 log CFU/g and the total viable cells after 36 hours of exposure were 6.57, 6.70, 6.66 and 6.71 log CFU/g for the control, PRO-SAN (0.1% and 0.5%,) and NEW, respectively. However, the results in Figure 2.7 show that *Pseudomonas* had a tendency to increase in numbers over time. For instance, the total viable cells of *P. putida* were higher on the sanitized ice treatments than in the control after 72 hours. The numbers obtained for PRO-SAN® (0.1% and 0.5%) were 7.04 and 6.91 log CFU/g, respectively whereas the total viable cells for the NEW-ice were 6.81 log CFU/g. According to these results, *Pseudomonas* appeared to be more resistant to PRO-SAN® at a 0.1% concentration. Statistically, significant differences ($p<0.05$) were observed for the mean overall reductions in the *P. putida* population obtained with PRO-SAN® at 0.1% (0.23 log CFU/g) when compared with the control (0.49 log CFU/g). For the 0.5% PRO-SAN® (0.35 log CFU/g) and the NEW (0.40 log CFU/g) ice treatments, no significant differences ($p>0.05$) were observed when compared with the PRO-SAN® 0.1% or with the control.

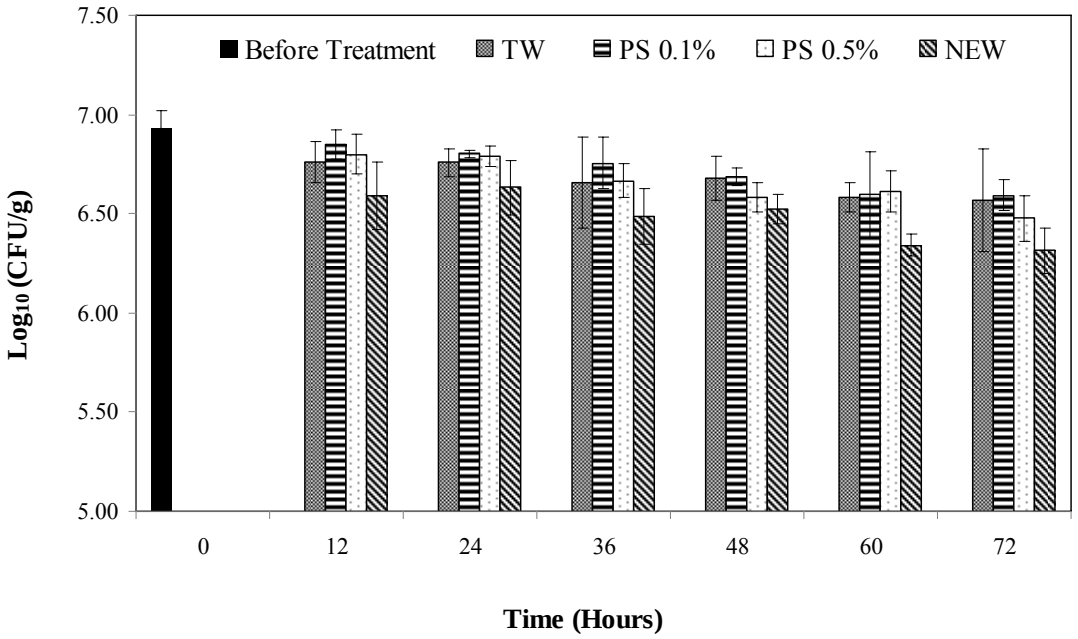

Figure 2.5: Effect of sanitized ice on the reduction of *E. coli* K-12 on the surface of fish fillets

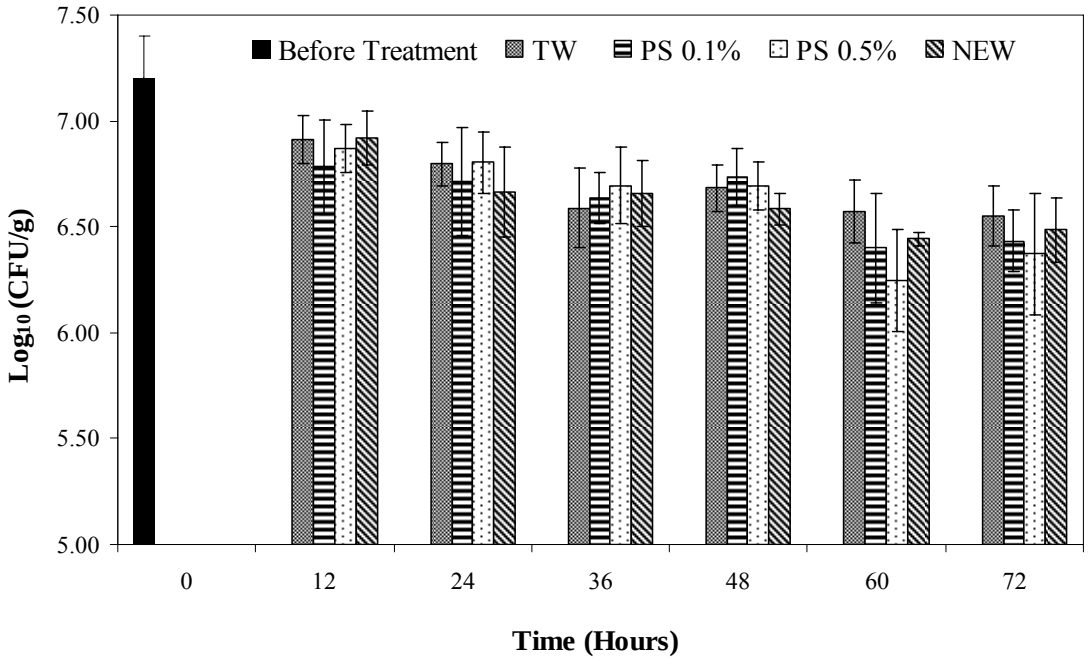

Figure 2.6: Effect of sanitized ice on the reduction of *L. innocua* on the surface of fish fillets

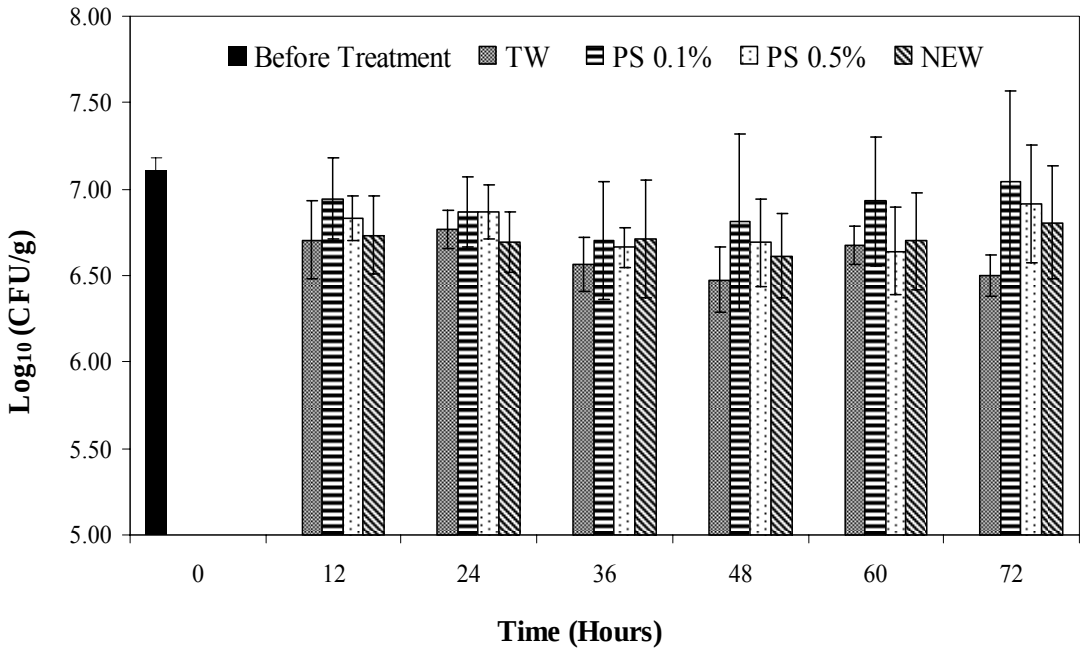

Figure 2.7: Effect of sanitized ice on the reduction of *Pseudomonas putida* on the surface of fish fillets during storage conditions (4ºC)

The results from Figures 2.5 and 2.6 show that *E. coli* and *L. innocua* reductions on the bacterial load on the fish fillets using sanitized ice were not relatively different than the reductions achieved with the control, except when *E. coli* was exposed to the NEW-ice. In the case of *Pseudomonas*, this bacterium appeared to be more resistant to the sanitized ice treatments, although the reductions were not significantly different ($p>0.05$) among the ice treatments, except for PRO-SAN® 0.1% when compared with the control.

In a study conducted by Koseki et al., (2004), ice prepared with acidic electrolyzed water (AEW) was evaluated for the reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce after 24 hours of exposure to the treatment at 20ºC. Ice containing 70-240 ppm available chlorine reduced *L. monocytogenes* populations by 1.5

log CFU/g after 24 hours. In contrast, *E. coli* O157:H7 cells were reduced by 2.0 log CFU/g when ice containing 70 – 150 ppm available chlorine was used. When the results from Koseki et al. (2004) study are compared with those from this present study, some inconsistencies are observed. The *E. coli* K-12 in this studty was reduced by 0.30 log CFU/g whereas *L. innocua* was reduced by 0.40 log CFU/g. Although the reductions are much lower than those obtained by Koseki et al., (2004), some factors should be taken into consideration before making any assumptions. For example, that the type of food (lettuce versus fish) were different and could have possibly affected the bactericidal action of the sanitizer. Vegetable and fruits are foods rich in carbohydrates whereas animal products (e.g. fish, meat) are food rich in proteins and/or lipids (Vandekinderen et al., 2009). It has been reported that even low amounts of proteins or fats are capable for reducing the antimicrobial efficacy of sanitizers (Verhaeg et al., 1996; Lee et al., 2007b; Vandekinderen et al., 2009). This is so because high levels of fat and protein can protect bacterial vegetative cells from the effect of sanitizers (Guzel-Sedim et al., 2004; Artés et al., 2009). Therefore, sanitization of animal products may be more complicated than the sanitization of fruit or vegetable products.

Huang et al., (2006), evaluated the efficiency of electrolyzed oxidizing (EO) water in reducing *E. coli* cells on Tilapia fish skin. In that study, the Tilapias were immersed in a solution containing 120 ppm free available chlorine. The pH and ORP of the EO water solution were 2.47, and 1159 mV, respectively. After 1 min in the solution treatment, *E. coli* achieved an additional 0.7 log CFU/cm^2 reduction when compared with tap water (6.54 versus 7.19 log CFU/cm^2, respectively). No changes in the reductions

were achieved when the treatment was extended to 10 minutes. Although greater in reduction, the results obtained by Huang et. al, (2006) are similar to what was obtained for *E. coli* during this present study after 12 hours. Huang et al., (2006) obtained a result of 0.45 log CFU/cm^2 compared with a microbial reduction of 0.34 log CFU/g obtained in this study. In Huang et al., (2006) study, an EO water solution was used to immerse the fish skin instead of sanitized ice. Previous studies have shown that freezing can reduce the chlorine content of the EO water and this has been attributed to the evaporation of Cl_2 (Koseki et al., 2004; Kim et al., 2006). During our study, the NEW-ice was kept in the freezer and taken out prior to the experiment. Therefore, this could have affected the concentration of the initial chlorine content since the NEW-ice was not used immediately after its preparation.

Although the pH (2.47) of the EO water used to treat the Tilapia fish skins (in Huang et al., 2006) was lower than the one used in this present study (~7.0), the differences in the bacterial reductions are not believed to be attributed to this factor. This may have occurred because studies have shown that EO water is effective against *E. coli* O157:H7 and *L. monocytogenes* in a wide pH ranging from 2.6 to 7.0, indicating that the effect of chlorine on bacterial inhibition is more significant than the effect of pH (Park et al., 2004). However, the oxidation reduction potential (ORP) of the EO water solution can be an important factor for its bactericidal effect. The ORP of a solution is an indicator of its ability to oxidize or reduce, with positive and higher ORP values correlated to greater oxidizing strength (Robbs et al., 1995; Venkitanarayanan et al., 1999). It has been reported that an ORP of +200 to +800 mV is optimal for growth of aerobic

microorganisms, whereas an optimum range of -200 to -400 mV is favored for growth of anaerobic microorganisms (Venkitanarayanan et al., 1999). In this present study, the ORP of the NEW solution was around +700 mV, whereas the ORP in the study conducted by Huang et al., (2006), was around +1100 mV. Therefore, the higher the ORP, the higher the oxidative effect of the solution against bacteria. Kim et al., (2006b) also reported that the ORP of EO water could be the primary factor responsible for its bactericidal effect. This is so because the high ORP of the EO water could cause modifications to the metabolic fluxes and ATP production in bacterial cells, and this may be due to a change in the electron flow (Huang et al., 2008). However, Len et al., (2000), reported that the relative concentrations of chlorine, HOCl, hypochlorite ion (OCl^-) and chlorine gas (Cl_2) could also be responsible for the effectiveness of EO water as a bactericidal agent.

As is the case with other sanitizers, EO water deteriorates in the presence of organic materials, which include amino acids and protein (Oomori et al., 2000). When this happens, the free chlorine of EO water reacts with the organic materials and becomes combined available chlorine, which has a lower bactericidal activity than the free form of chlorine (White, 1992; Ayebah et al., 2006; Huang et al., 2008). In addition to this, temperature also influenced the efficacy of the sanitized ice against bacteria tested in this present study. This is supported by studies done by Venkitanarayanan and collaborators (1999) when they evaluated the efficacy of EO water for the inactivation of *E. coli* O157:H7 and *L. monocytogenes.* They found that *E. coli* O157:H7 and *L. monocytogenes* were more rapidly inactivated by EO water at 35 or 45ºC than at 4 or 23ºC. This temperature effect occurs because the cell membranes of Gram-negative bacteria (*E. coli*

and *Pseudomonas*), are composed of phospholipids and are more fluid at higher temperatures. As a result, the sanitizer enters the cell at a faster rate than at lower temperatures. For example, at 4ºC the membrane is more rigid (Fabrizio and Cutter, 2003).

When the outer membrane of a Gram-positive bacterium (e.g. *Listeria*) is considered, it is mainly composed of peptidoglycan, which imparts resistance to environmental stresses (Fabrizio and Cutter, 2003). During this present study, the bacterial reduction counts obtained for *L. innocua* on tap water ice were not significantly different ($p>0.05$) to the reductions obtained from the sanitized ice. These results demonstrate the resistance this bacterium has against stressors, which makes it harder to remove it from food contact surfaces and from the surface of foods such as those of animal origin.

The efficacy of organic acids can also be affected by temperature. Virto et al., (2005) studied the efficacy of organic acids (lactic and citric acid) in inactivating *Yersinia enterocolitica* (Gram-negative) at different temperatures. They found that the organic acid concentrations that did not have bactericidal activity against *Y. enterocolitica* at 4ºC were effective at higher temperatures (20 and 40ºC). Lee et al., (2007a), studied the efficacy of PRO-SAN® (an organic acid formulation) in reducing *L. innocua* and *E. coli* on metal cans and packaging materials. Their results also demonstrated that higher temperatures increase the bacterial reductions when compared with lower temperatures. On animal products, such as carcasses, organic acids have been used as sanitizers and

they are more effective at higher temperatures, when the carcass of the animal is still warm (Huffman, 2002).

Bacteria from the *Pseudomonas* group are mainly known for their deteriorative effects on foods, especially fish and fish products (Gram and Huss, 1996; Pacquit et al., 2006). The proliferation of this particular group under refrigeration storage is not surprising as was seen in this present study. Other studies have shown that bacteria from the *Pseudomonas* group tend to be more resistant to the effect of some antimicrobial agents (Holley and Patel, 2005). For example, Rong et al., (2009), studied the bactericidal effect of ozonated water against the microbial load on raw oysters. The results from that study revealed that Gram-negative bacteria (mainly *Pseudomonas*) increased significantly in numbers (42%) after ozonated water treatment. In another study, Vandekinderen et al., (2009) determined the susceptibility of various microorganisms (including *Pseudomonas fluorescens*) to chlorine dioxide. According to their results, *P. fluorescens* showed an anomalous resistance for which no explanation could be offered.

Kim et al., (2006) compared the effect of ice prepared with electrolyzed water with ice prepared from tap water. The results of that study revealed that storing fish on ice prepared with EO water (100 ppm) for 24 hours reduced *Enterobacter aerogenes* and *Morganella morganii* (histamine-producing bacteria) on Tuna fish skin by 2.4 and 3.5 log/cm^2, respectively. This is an important fact because ice prepared with EO water was able to reduce histamine-producing bacteria and this shows that the probability exists that it could produce higher reductions on other fish spoilage bacteria as well. Histamine-

producing bacteria are known to cause severe cases of foodborne illness and as a result, they are of food safety concern (Phuvasate and Su, 2009).

Factors that could have affected the results obtained for *P. putida* in this present study include: 1) the chlorine concentration of the NEW-ice. This could have been affected by its long period of frozen storage; 2) the bactericidal activity of the organic acid formulations could have been reduced as a consequence of the low temperature; 3) the fish samples stored on 0.1% PRO-SAN® ice for 36 hours showed the formation of foam; and 4) foam formation was more prominent at the 0.5% PRO-SAN® concentration.

2.4.4. Efficacy of sanitized ice on the bacterial load reduction in the water from the melted ice collected during the fish storage at 4ºC

Figure 2.8 shows the amount of *E. coli* cells recovered in the waters collected from the ice treatments. After 24 hours storage, the total *E. coli* viable cells recovered from the control and the 0.1% PRO-SAN® treatments were 5.03 and 3.91 log CFU/ml, respectively. For the 0.5% PRO-SAN®, the total *E. coli* cells enumerated in the water samples was 2.81 log CFU/ml. For the NEW treatment a 3.11 log CFU/ml were recovered. Little changes in the bacterial counts were observed during the test after 48 and 72 hours. These were relatively minor when compared with those obtained at 24 hours. The statistical analysis confirmed that the sanitizers had a significant effect ($p<0.05$) in reducing *E. coli* populations in the waters from the melted ice when compared with the control. The mean *E. coli* cells recovered from the control treatment (4.80 log CFU/ml) was statistically ($p<0.05$) higher than the other sanitizers. For the sanitizer treatments the mean bacterial cells enumerated were 3.29, 2.82 and 2.61 for

PRO-SAN® 0.1%, NEW and PRO-SAN® 0.5%, respectively. No significant differences ($p>0.05$) were found between NEW and PRO-SAN® 0.5%. Trends observed after an examination of the data suggest that as long as *E. coli* remained in contact with the sanitizer, a lower load of this bacterium were recovered under the storage conditions. However, the bacterial load in the water samples remained fairly constant after 48 hours of exposure to the sanitizers. According to the statistical analysis, no significant differences ($p>0.05$) were found in the mean *E. coli* cells recovered between 48 and 72 hours (3.21 and 3.07 log CFU/ml), respectively.

Figure 2.9 shows the efficacy of the ice treatments on the residual *L. innocua* cells in the water samples. The *Listeria* populations remained constant during the 72 hours storage when exposed to the control treatment. However, *L. innocua* was significantly ($p<0.05$) reduced when exposed to sanitized ice when compared with the control. According to the results obtained in this present study, both treatments and time had a significant effect ($p<0.05$) on the amount of *Listeria* counted in the water samples. For the treatments, 0.5% PRO-SAN® appeared to be more effective against *L. innocua* than the other sanitizers, but no significant differences ($p>0.05$) were found between 0.5% and 0.1% PRO-SAN®. The overall mean counts for *L. innocua* were 2.87 and 2.64 log CFU/ml for the 0.1% and 0.5% PRO-SAN concentrations, respectively. For the control, the overall mean count was 5.28 log CFU/ml, while the NEW treatment had an overall mean count of 3.27 log CFU/ml. These results suggested that the sanitizer treatments achieved additional reductions in *L. innocua* populations in the water from the melted ice

when compared with the control treatment. The additional reductions that were achieved were at least 2 log CFU/ml.

The effect of time was also important on the amount of *Listeria* cells recovered in the water samples. After 24 hours storage, the overall bacterial load was 3.95 log CFU/ml. The mean *L. innocua* cells enumerated after 48 hours was 3.63 log CFU/ml and 3.10 log CFU/ml at the 72 hours time point. At the 24 hours time point, the bacterial load in the waters from the 0.5% PRO-SAN® treatment was 2.71 log CFU/ml, thus achieving an additional 2.60 log CFU/ml reduction when compared with the control. The trends observed from this data suggested that the longer the exposure to the sanitizers' solutions, the lower the bacterial population of *L. innocua* in the water samples.

Figure 2.10 shows the response of *P. putida* when exposed to the various sanitizers at low temperature. The sanitizers had a significant effect ($p<0.05$) on *Pseudomonas*. The data showed that PRO-SAN® treatments achieved reductions for the *P. putida* cells (> 2 log) at 24 hours of exposure (0.1% = 2.83 log CFU/ml and 0.5% = 2.08 log CFU/ml, respectively) when compared with the counts obtained with the control (4.68 log CFU/ml). The bacterial load in the waters from the control treatment was 4.68 log CFU/ml at 24 hours of exposure. On the other hand, the total viable cells of *P. putida* detected at 24 hours of exposure to NEW was 3.71 log CFU/ml. According to the results, PRO-SAN® sanitizers were more effective against *P. putida* than were NEW and tap water. The mean overall *P. putida* viable cells recovered during this test were as followed: tap water = 4.47; 0.1% PRO-SAN® = 2.02; 0.5% PRO-SAN® = 2.36; and NEW = 3.39 log CFU/ml. The statistical analysis revealed that the mean for the *P. putida* cells

enumerated during this test were significantly different ($p < 0.05$) for the PRO-SAN® concentrations when compared with the NEW and tap water treatments. Even though the amount of *Pseudomonas* populations was lower for the PRO-SAN® treatments when compare with the tap water and the NEW treatment at 24 hours, the results showed a tendency for the bacterial load to increase in numbers as the time progressed. Although this trend was observed, the statistical analysis shows no significant differences ($p > 0.05$) in the counts after exposure to all sanitizers after the 24 hour time point.

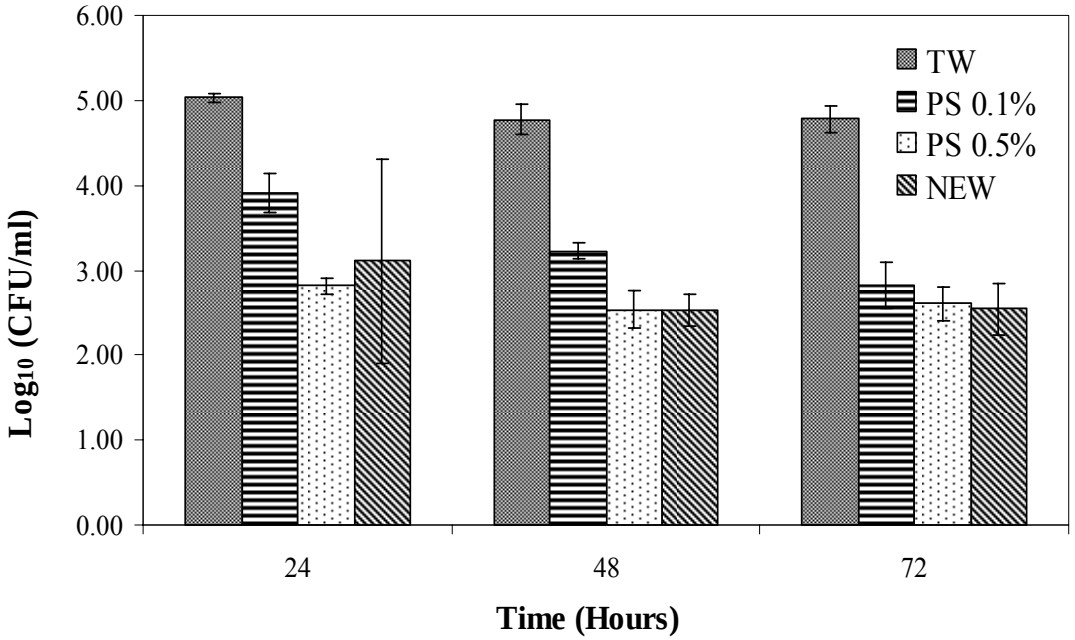

Figure 2.8: Effect of sanitized ice on the reduction of *E. coli* K-12 in the waters from the melted ice during the fish samples storage (4ºC)

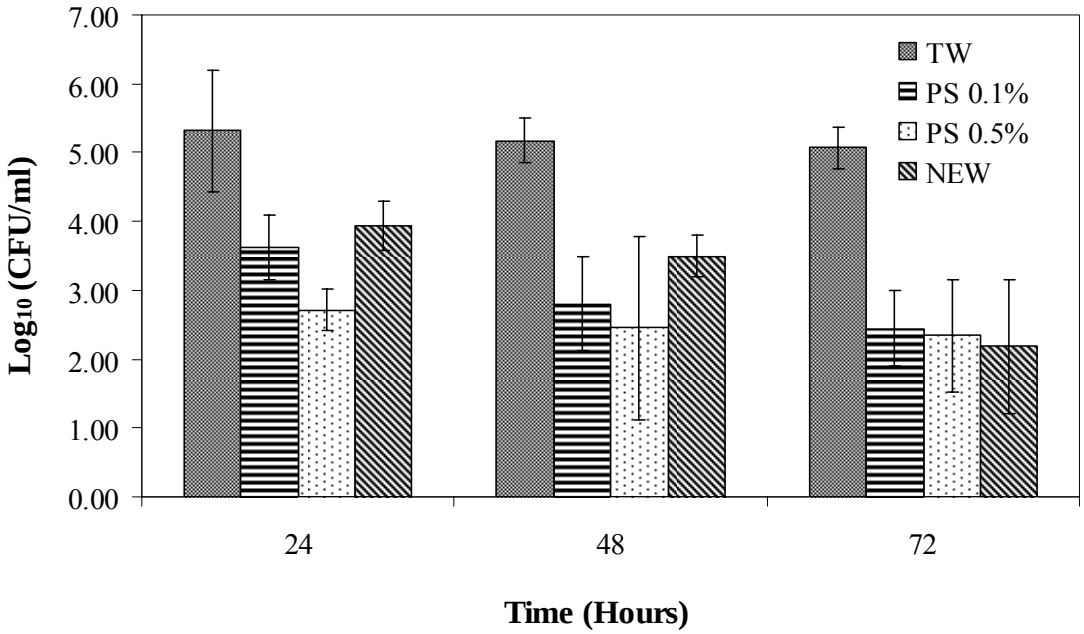

Figure 2.9: Effect of sanitized ice on the reduction of *L. innocua* in the waters from the melted ice during the fish samples storage (4ºC)

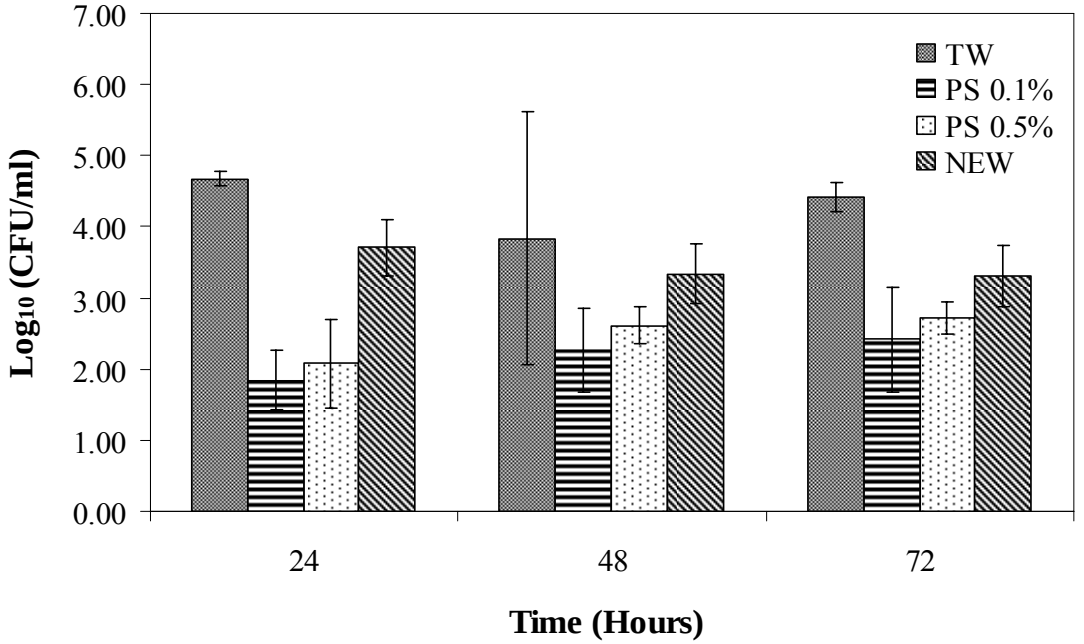

Figure 2.10: Effect of sanitized ice on the reduction of *Pseudomonas putida* in the waters from the melted ice during the fish samples storage (4ºC)

The efficacy of the sanitizers in reducing bacterial cells was greater in the water from the melted ice than on fish fillet samples (Figures 2.5-2.7). This can be attributed to the fact that sanitizers can perform better against microorganism when they are in suspension instead of on a food surface (Fabrizio and Cutter, 2003; Gonzalez et al., 2004; Ayebah et al., 2006). *E. coli* K-12 and *L. innocua* were used as surrogates for the pathogenic bacteria *E. coli* 0157:H7 and *L. monocytogenes*, respectively. Since these surrogates bacteria were significantly reduced under refrigeration storage conditions, it can be assumed that the potential of these sanitizers to minimize the presence of pathogenic bacteria (e.g. *E. coli* O157:H7 and *L. monocytogenes*) is promising and valuable. *L. monocytogenes* is most likely to contaminate raw fish during its handling and processing steps (Herrera et al., 2006), but the potential to contaminate other raw products or ready-to-eat foods that may come into contact with melted ice can be minimized by the use of sanitized ice. Likewise, *E. coli* O157:H7 bacterial cells can potentially be reduced by the utilization of PRO-SAN® and NEW sanitizers. Thus, the likelihood of releasing considerable amounts of foodborne pathogens into the environment through waste discharge is also diminished.

2.5. Conclusions

Fish and seafood products are usually chilled with ice to retain quality and to limit the growth of bacteria during storage. The findings during this study confirmed that melting ice in contact with raw fish or other types of raw seafood products can potentially become a source of cross-contamination if not discarded properly. This study showed that

this potential could be significantly reduced by the use of a sanitizer in the ice. Although the sanitized ice appeared not to significantly reduce the bacterial load on fish fillets, the number of organisms did not increase more than what was observed for the unsanitized ice. It could be concluded that the NEW and PRO-SAN® are sanitizers that could be added to ice to reduce the bacterial load in the water as it melts.

CHAPTER 3

TRANSMISSION ELECTRON MICROSCOPIC ANALYSIS OF BACTERIAL CELLS TREATED WITH ELECTROLYZED WATER AND AN ACIDIC SANITIZER

3.1. Abstract

The effects of neutral electrolyzed water (NEW) and PRO-SAN® sanitizers on *Escherichia coli* K-12 and *Listeria innocua* were investigated in this study. This was done by inoculating two tryptic soy broth solutions, supplemented with yeast extract (TSBYE), one with *E. coli* and the other with *L. innocua*. After 24 hours incubation, the bacterial cells were centrifuged and their pellets treated with the sanitizers and the control (tap water) for 10 minutes. *E. coli* and *L. innocua* cells were examined using a transmission electron microscopic (TEM) technique in order to investigate a cross-section of the bacterial cells before and after the treatments. The results showed that NEW and PRO-SAN® sanitizers both caused changes to the cell wall and cytoplasm of *E. coli* cells. These changes were more pronounce in the cytoplasm. In contrast, *L. innocua* appeared to be more resistant to the bactericidal activity of the sanitizers because smaller changes were observed in its cell wall as well as to its cytoplasm. These results demonstrated that the TEM technique can be used to better understand the mechanism of action of various sanitizers and other bactericidal agents. The results also showed that the efficacy of

various sanitizers against bacteria can be evaluated by the morphological changes occurring with their cells.

3.2. Introduction

Many studies have been conducted to evaluate the efficacy of different sanitizing solutions for the reduction or elimination of major pathogens of current FDA and USDA concerns. Organic acids, electrolyzed water and chlorinated compounds are some of the various sanitizing solutions that have been evaluated to produce reductions or complete inactivation of pathogens in food commodities (Han et al., 2000; Lundén et al., 2002; Stopforth et al., 2003; Escudero et al., 2003; Fukuzaki et al., 2004; Ayebah et al., 2005). However, the literature provides limited information about how certain sanitizers inactivate bacteria.

Transmission electron microscopy allows the examination of bacteria by providing a high resolution image of the tissues and internal structures of the cells (Müller et al., 2008). For instance, TEM can provide images of the bacteria cell walls, which can characterize the response of the organisms after exposure to the sanitizers. The outer membrane of bacteria plays an important role in their protection from the environment. This is so because the membrane serves as a permeable barrier to prevent the entry of noxious compounds and, at the same time, allow the influx of nutrient molecules (Nikaido, 2003). The probable mechanism of the sanitizers in killing bacteria could be by damaging, disintegrating or by producing holes (perforations) in the cell wall. This could eventually result in the death of the bacteria if the sanitizer is allowed to get into the cell (Nikaido, 2003; Hajmeer et al., 2006).

In order to understand the response of different bacteria when expose to chemical agents, it is necessary to understand the structures or main components of these bacterial cells. For instance, Gram-negative bacteria (e.g. *E. coli*) contains two distinct membranes, an outer and an inner (cytoplasmic) membrane separated by the periplasm, a hydrophilic compartment that includes a thin layer of peptidoglycan (Hancock and Rozek, 2002; Sperandeo et al., 2009). The outer membrane is an asymmetric lipid bilayer with phospholipids forming the inner leaflet and lipopolysaccharides (LPS) forming the outer leaflet (Sperandeo et al., 2009). The internal membrane, however, is a symmetric phospholipid bilayer in which proteins are embedded (Sperandeo et al., 2009). When compared with Gram-negatives, Gram-positive bacteria (e.g. *Listeria* spp.) do not have the phospholipid bilayer membrane, but they possess a thicker layer of peptydoglygan. This layer offers protection by imparting resistance to environmental stresses (Fabrizio and Cutter, 2003). For this reason, the objective of this chapter is to study the effects that neutral electrolyzed water (NEW) and PRO-SAN® sanitizers have on the cell envelope of Gram-negative (*E. coli* K-12) and Gram-positive bacteria (*L. innocua*). To achieve this goal, transmission electron microscopy (TEM) will be used as a tool to better understand the effect of these sanitizers on the bacteria cell structures.

3.3. Materials and Methods

3.3.1. Bacterial cultures

Escherichia coli K-12 (ATTC 29181) and *Listeria innocua* (ATTC 33090) were used for this study as surrogates for *Escherichia coli* O157:H7 and *Listeria monocytogenes*. These microbial species were selected because of the ability of the

pathogenic bacteria to survive in processing plants and the challenges they represent when they survive sanitization treatments. Once received in the laboratory, the surrogate cultures were stored frozen (-80ºC) in 30% (v/v) sterile glycerol (Fisher Scientific, Fair Lawn, NJ). A loopful of each bacterial species was individually transferred into 20 ml sterile Tryptic Soy Broth (Difco Laboratories, Sparks, MD) containing 0.3% (w/w) yeast extract (Fisher Scientific, Fair Lawn, NJ). The bacteria were incubated for 24 hours at 37ºC. Following the 24 hours incubation period, a loopful of each microorganism was transferred into a Tryptic Soy Agar (Difco Laboratories, Sparks, MD) slant supplemented with 0.3% (w/w) yeast extract (TSAYE). The inoculated slants were kept under refrigeration temperatures (4ºC) and used as a stock culture.

3.3.2. Sanitizer solutions preparation

PRO-SAN®, an acidic compound manufactured by Microcide Inc., Troy, MI, was used as one of the sanitizing solutions. One concentration of this sanitizer was prepared by adding 0.1% (w/v) of the powdered compound into tap water. The solution was prepared prior to the experiment and covered with aluminum foil until used at room temperature (25ºC).

Another treatment tested was NEW. The solution was prepared using an electrolyzed water generator (STEL-80) provided by Hobart Corporation (Troy, OH). Solutions containing 150 ppm chlorine were prepared using a setting of 29 ± Amps (A), and 20.6 ± Volts (V). The free available chlorine was determined using a HI 95771 Chlorine Ultra High Range Meter (Hanna Instruments, Ann Arbor, MI). The pH and ORP of the solutions were determined using a pH indicator Paper (Whatman International Ltd,

Maidstone, Kent, UK) and an ORP titrator model DL70ES (Mettler Toledo, Columbus, OH), respectively. The NEW solutions were prepared within two hours prior to the experiment. The control used during this study was tap water and together with the NEW solutions were covered with aluminum foil after preparation.

3.3.3. Culture preparation and treatment

Prior to the experiment, a loopful of each bacterial species was transferred from the slant stored in the refrigerator into 100 ml of TSBYE and incubated for 24 hours at 37ºC. After incubation, TSBYE containing *E. coli* cells was aseptically transferred into a total of four sterile 50 ml plastic centrifuge tubes to yield a total of 20 ml of the mixture. The tubes were centrifuged at 7,000 rpm for 10 minutes (Sorvall® RC 5C Plus, Kendro Laboratory Products, Newton, CT). Following centrifugation, the supernatants were decanted and the treatments [control, 0.1% PRO-SAN and NEW] were added into the pellets to reach a total volume of 20 ml. These solutions were mixed by a vortex mixer (Vortex-Genie®2, Scientific Industries, Inc., Bohemia, New York) and the bacterial cells were allowed to sit in the tubes for 10 minutes. After these treatments, 10% (w/v) of a 10x neutralizer solution was added to the tubes and they were vortexed in order to stop the reaction of the sanitizers against the bacterial cells. The 10x neutralizer solution was prepared by mixing lecithin (0.07%), Tween 80 (0.5%) and sodium thiosulfate (0.1%) in distilled water (w/v). An identical procedure was followed to treat the *L. innocua* cells.

3.3.4. Sample preparation for TEM

After adding the neutralizers, the samples were centrifuged at 7,000 rpm for 10 minutes and the supernatant decanted. The pellets from each treatment were transferred into sterile 1.5 ml centrifuge tubes and re-suspended into 1 ml of a fixative solution provided by The Ohio State University - Campus Microscopy and Imaging Facility (Columbus, OH). The fixative solution contained a mixture of 2.5% glutaraldehyde and 0.1M sucrose in a 0.1M phosphate buffer, at a pH of 7.4. The glutaraldehyde was added to preserve the structure of the cells at the time of fixation and minimize alterations during embedding, sectioning, or exposure to the electron beam of the TEM (Hajmeer et al., 2006). The samples were fixed overnight at 4ºC and centrifuged the next morning at 7,000 rpm for 5 minutes. The supernatants were decanted and the pellets were rinsed in suspension 3x (5 minutes each) by adding 1 ml of phosphate buffer (0.1M phosphate with 0.1M sucrose, pH 7.4) at ambient temperature. After the last centrifugation, the bacteria were post-fixed in the suspension (1% sodium tetroxide) for 1 hour in phosphate buffer for 90 minutes. The bacteria were then rinsed 2x in the buffer with centrifugation and re-suspension. Following centrifugation and removal of most of the buffer, the cells were suspended in a microfuge tube in 2% warm low temperature gelling agarose and centrifuged at high speed. The tubes were put into an ice bath to gel the agar, and then cut out of the tubes. The fixed samples were cut into 1 mm cubed blocks in buffer. The samples were stored in the buffer overnight at 4ºC.

The samples tissues were rinsed 2x in distilled water and put into 1% uranyl acetate for 90 minutes. The samples were rinsed 2x in distilled water before dehydration in ethanol. The samples were dehydrated by successive soakings in ethanol according to

the following schedule: 50% for 10 minutes, 70% for 10 minutes, 80% for 15 minutes, 95% for 15 minutes, 100% for 20 minutes with 3 changes (dilutions should be changed at least once during the specific time). After dehydration, the samples were soaked in propylene oxide for 20 minutes. The dried cell blocks were infiltrated by a mixture of 1:1 (v/v) propylene oxide and eponate 12 resin for 1 hour at 37ºC, then by a mixture of 1:2 (v/v) polypropylene/resin overnight at room temperature on a rotator. Finally, the cells were infiltrated in 2 changes of 100% eponate 12 resin over 2-6 hours at 37ºC. Following infiltration, plastic capsules were used to embed the tissue blocks, which were then polymerized at 60ºC overnight.

3.3.5. TEM testing method

Ultra thin sections (70 nm) were prepared using an ultramicrotome (Leica EM UC6, Leica Microsysteme, Vienna, Austria). The sections were stained in 2% aqueous uranyl acetate for 20 minutes, washed with distilled water, stained in Reynold's lead citrate for 15 minutes and washed again with distilled water. After air-drying, TEM images of the bacteria were obtained using a FEI^{TM} Tecnai G2 Spirit transmission electron microscope at 80 kV.

3.4. Results and Discussion

3.4.1. Effect of the sanitizers on *E. coli* and *L. innocua* cells

Results from the test on *E. coli* cells are shown in Figures 3.1A - 3.1D. For the control (tap water), the TEM image (Figure 3.1B) shows that the outer membrane of the *E. coli* cell was slightly affected by the chlorine in the tap water. This is so because the

TEM image of the untreated cell (Figure 3.1A) appears to have a smooth and well defined cell wall. When compared with the TEM image for the control treatment (Figure 3.1B), a slight wrinkling of the cell wall appeared to have occurred. The effect of the PRO-SAN® and the NEW sanitizers on the *E. coli* cells are shown in Figures 3.1C-3.1D. In comparison with the control, the sanitizers appeared to alter the internal content of the bacterial cells, causing changes to the cytoplasm and the appearance of inner vacuoles. This agrees with the findings of Tong et al., (2005), when they studied the antibacterial effects of Cu (II)-exchanged montmorillonite on *E. coli* cells. In that study they found that the structural integrity of *E. coli* cells were affected by the antibacterial agent and it resulted in an increase in the density of the cytoplasm. That research also showed that the permeability of the cell membrane was also affected, causing leaching of the nutrients to occur. This is also supported by Liu et al., (2004) where they reported that damages to the cell membrane of *E. coli* (caused by bactericidal agents) can result in their destruction.

A test of the tap water used in this study showed that it contained ~ 2 ppm of free available chlorine, had a neutral pH (~7.0) and an ORP of 630 mV. Even though the amount of chlorine was low, Park et al., (2004) reported that a concentration as low as this could be enough to alter the bacterial cell membrane. Besides, since the ORP in the tap water was relatively high, this explains why the tap water contributed to the activity against the cell membrane of *E. coli* (Figure 3.1B).

When the TEM images of *E. coli* cells were compared with those of *L. innocua*, it showed that the *L. innocua* cells responded differently when exposed to the tap water (Figure 3.2B). *L. innocua* appeared to maintain the integrity of their cell wall after the tap water treatment. Figures 3.2A- 3.2D illustrate the TEM images for *L. innocua* cells. The

images show that the bacterial cells of *L. innocua* had less structural changes after exposure to the sanitizers (Figures 3.2C-3.2D), when compared with the control (Figure 3.2B). However, a close examination of the images show a lower level of sharpness in the cell wall definition, and the wall appeared to lose some smoothness after exposure to the sanitizers. Similar results were obtained by Calderón-Miranda et al., (1999), when they exposed *L. innocua* to pulse electric field (PEF). In that study they found little obvious wrinkling in the outer cell wall, although some blurring and roughing appeared. The Calderón-Miranda et al., (1999) study also showed that the inactivation of *L. innocua* was a consequence of damages that the cell membrane suffered after the PEF treatment.

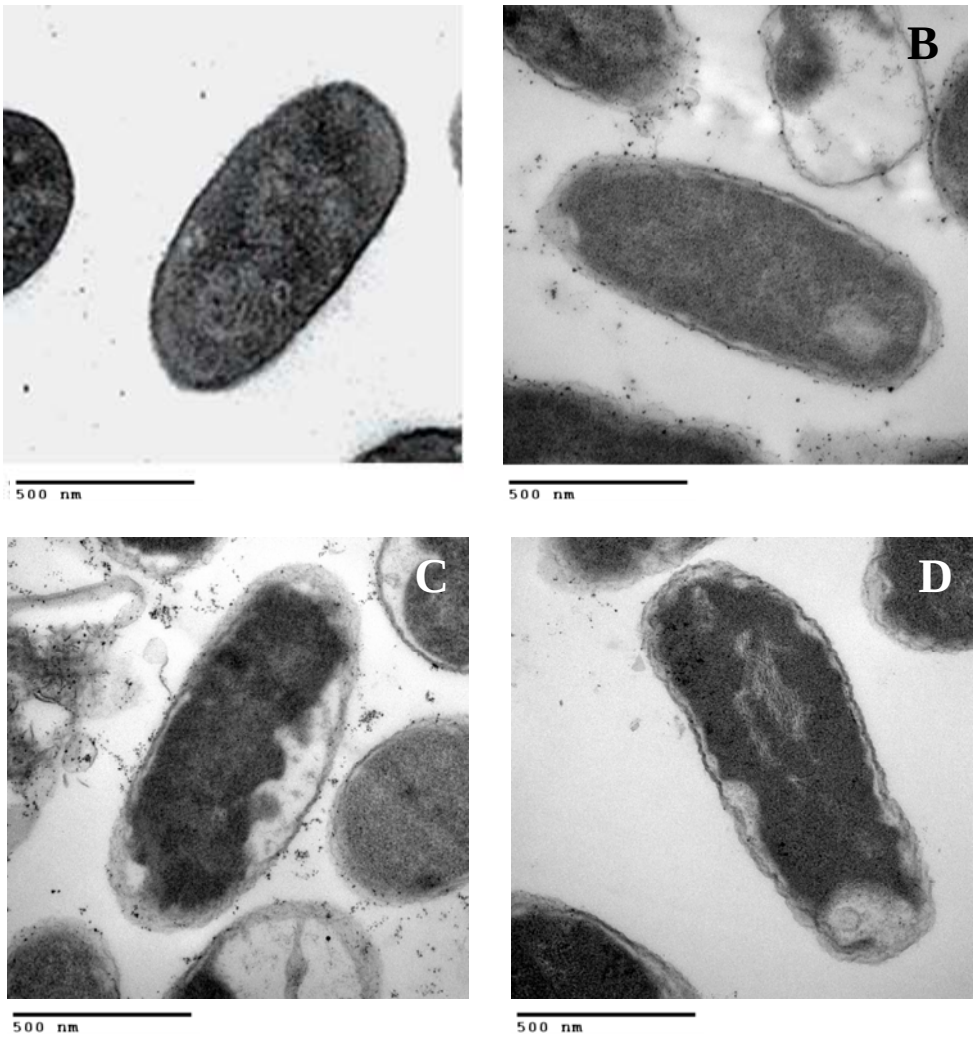

Figure 3.1. TEM images of *E. coli* cells (A) untreated, after 10 minutes treatment with (B) tap water, (C) 0.1% PRO-SAN®, and (D) NEW.

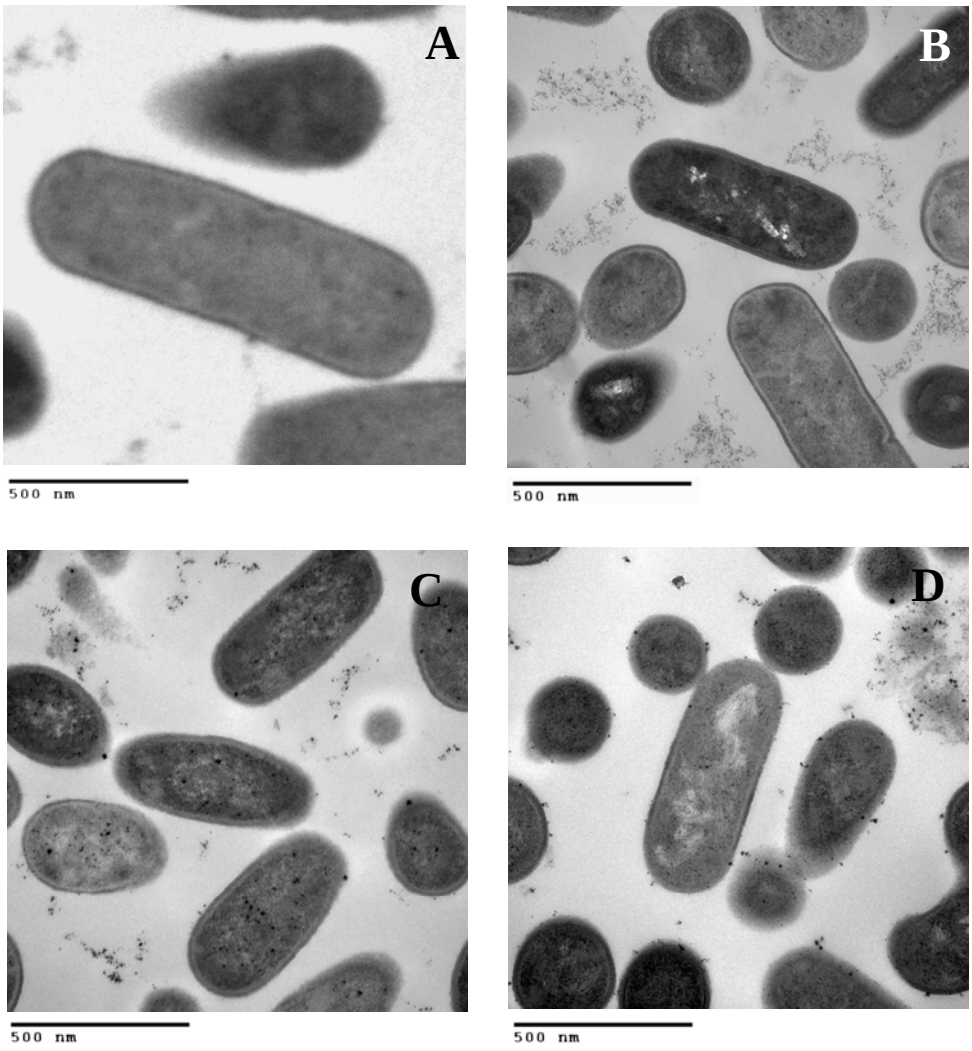

Figure 3.2. TEM images of *L. innocua* (A) untreated, after 10 minutes treatment with (B) tap water, (C) 0.1% PRO-SAN®, and (D) NEW.

In this present study, PRO-SAN® (organic acid based sanitizer) appeared to be more effective in altering the structure of the *E. coli* cells, whereas it appeared to be less effective on *L. innocua*. These findings are in disagreement with previous studies which revealed that organic acids tend to be more effective against Gram-positive when compare with Gram-negative bacteria (Skrivanova et al., 2006). This has been attributed

to the fact that Gram-positive bacteria are more susceptible to the action of compounds interfering with the transport of ions across the cell membrane due to a lack of an extra outer membrane (Nagajara, 1995). The inability of organic acids to attack Gram-negative bacteria have been hypothesized to be related to the protective outer membrane, which covers the cytoplasmic membrane and the peptidoglycan layer of the cells (Belfiore et al., 2007).

In an attempt to understand the results obtained, some additional factors should be taken into consideration - such as the concentration of the sanitizer. According to Damodaran (1996), the bactericidal effect of organic acids at low concentrations is reduced, but when the concentration of the acid increases, it can affect the cytoplasm of the cell by causing protein denaturation. This is so because undissociated forms of organic acids can penetrate the lipid membrane of bacterial cells and dissociate within the cells. Bacteria are known to maintain a neutral pH of the cytoplasm, but the export of excess protons from the dissociated organic acid consumes cellular ATP and causes depletion of the organism's energy (Ricke, 2003; Skrivanova et al., 2006). Another factor to take into consideration is the time of exposure to the treatment. PRO-SAN® has shown a pattern in its mode of action, where a minimum amount of time is required before its bactericidal action is evident (Gonzalez et al., 2004). However, it is important to mention that PRO-SAN® contained sodium dodelcylbenzene sulfonate (SDBS) as an active ingredient. SDBS acts as a surfactant (chelating agent) and since chelators are compounds able to sequester metal ions by forming stable metal complexes, they chelate Ca^{2+} and Mg^{2+} ions from the outer membrane of Gram-negative cells, destabilizing its structure and altering its permeability (Hancock and Rozek, 2002).

The literature reports that when the outer membrane of a bacterium is damaged, it inhibits the cell's ability to act as a controlled permeable barrier. This allows harmful substances to come into contact with the cytoplasmic membrane, leading to leakage of the cell contents (Blankenship, 1981; Thompson and Hinton, 1996). This mechanism of action is the one that has been proposed for the electrolyzed water (EO water) sanitizer. Since EO water has a high ORP, it could cause modifications to the metabolic fluxes and ATP production within the cell, probably due to changes in the electron flow (Huang et al., 2008). Thompson and Hinton (1982) proposed that bacteria are generally known to form filaments within the cytoplasm as a response to DNA damage during acid treatment. Thus, the condensed fibril formation in the central regions of both *E. coli* (Figure 3.1D) and *L. innocua* (Figure 3.2D) cells possibly indicated that the DNA structures were affected by the NEW treatments. This filament formation can be seen as lightly colored areas in mid region of the bacterial cells shown in Figures 3.1D and 3.2D.

As reported earlier the NEW sanitizer appeared to have a greater impact on structural changes to the *E. coli* cells when compared with *L. innocua*. Since the cell membrane of a Gram-positive bacterium is mainly composed of peptydoglycan (Fabrizio and Cutter, 2003), this could have protected the *L. innocua* cells from the effect of the NEW sanitizer. Another factor that could have limited the bactericidal activity of the NEW sanitizer could be the pH. This is so because a low pH is known to sensitize the outer membrane of bacterial cells, allowing the entry of other toxic compounds (McPherson, 1993; Huang et al., 1998). Thongbai et al., (2006), studied the mechanism of nisin against Gram-negative bacteria using scanning electron microscopy (SEM). They found that *Salmonella* cells treated with the nisin-based solution at a low pH (4.5) caused

extensive morphological changes in the cell envelope. These changes resulted in a lost to the original shape and the development of indentations on the cell surface. However, a study conducted by Yang et al., (2003) confirmed that Gram-positive bacteria (*L. monocytogenes*) appeared to be more resistant to acidic conditions (EO water at pH 4) when compared with Gram-negative bacteria (*E. coli* O157:H7). This was determined using a SEM technique when they evaluated the effect of the EO water on lettuce inoculated with *E. coli* O157:H7.

3.5. Conclusions

The transmission electron microscopy technique can be used to better understand the mechanism of action of various sanitizers or other chemical agents against bacterial cells. In addition, it can be a valuable tool for developing new products intended to sanitize and disinfect different types of food contact surfaces and processing equipment. This technique can also help to evaluate the efficacy of antimicrobial treatments that are currently employed in the food industry to sanitize fruits, vegetables and raw animal products. Thus, it can provide significant information that can be used to enhance the safety of the food supply and as a result, reduce the occurrence of foodborne illness.

CHAPTER 4

CONCLUSION

This study demonstrated that ice prepared with the PRO-SAN® and the NEW sanitizers could significantly reduce the potential for cross-contamination of fish and other seafood products. This study also demonstrated that the stability of ice prepared with sanitizers was similar to the stability of the unsanitized ice (tap water). The ice prepared with the PRO-SAN® sanitizer was shown to be effective in reducing the natural microbial burden on the whole fish samples. As a result, it reduced the bacterial load in both the water from the melted ice and in the ice that was in contact with the fish. The PRO-SAN® and the NEW sanitizers had the ability to produce at least a 4 log reductions on *E. coli*, *L. innocua* and *P. putida* populations in the water from the melted ice.

The transmission electron microscopic technique can be used to evaluate the efficacy of sanitizers against various microorganisms, including pathogens that can cause foodborne illnesses. This study demonstrated that *L. innocua* (Gram-positive) was more resistant to the bactericidal activity of the PRO-SAN® and the NEW sanitizers, whereas *E. coli* (Gram-negative) was more sensitive. Therefore, when evaluating the efficacy of different sanitizers, the type of microorganism to be target should be taken into consideration.

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APPENDIX A
RAW DATA AND STATISTICAL ANALYSIS

Time		Before Treatment		Tap Water		PS 0.1%		PS 0.5%		NEW	
Hours	Replication	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g
	1	27050000	7.43								
0	2	15925000	7.20								
	3	16175000	7.21								
	4	8800000	6.94								
	1			11100000	7.05	11450000	7.06	10350000	7.01	12300000	7.09
12	2			7250000	6.86	7250000	6.86	7650000	6.88	8250000	6.92
	3			8950000	6.95	4400000	6.64	6750000	6.83	7600000	6.88
	4			6100000	6.79	3700000	6.57	5550000	6.74	6050000	6.78
	1			8200000	6.91	10550000	7.02	9450000	6.98	7350000	6.87
24	2			4650000	6.67	6700000	6.83	6450000	6.81	3800000	6.58
	3			6450000	6.81	3000000	6.48	6400000	6.81	6500000	6.81
	4			6200000	6.79	3450000	6.54	4200000	6.62	2566667	6.41
	1			7100000	6.85	5900000	6.77	9250000	6.97	7550000	6.88
36	2			2993333	6.48	5050000	6.70	4050000	6.61	4500000	6.65
	3			3850000	6.59	3350000	6.53	4150000	6.62	3250000	6.51
	4			2750000	6.44	3450000	6.54	3850000	6.59	3900000	6.59

(continued)

Table A.1. Raw data for *L. innocua* counts on fish fillet samples after treatment with sanitized ice.

	1			6600000	6.82	8050000	6.91	5650000	6.75	3000000	6.48
48	2			4700000	6.67	6050000	6.78	4900000	6.69	4050000	6.61
	3			3550000	6.55	4150000	6.62	3450000	6.54	4050000	6.61
	4			4900000	6.69	4350000	6.64	6200000	6.79	4400000	6.64
	1			5100000	6.71	4400000	6.64	3233333	6.51	2610000	6.42
60	2			3600000	6.56	3850000	6.59	2396667	6.38	2950000	6.47
	3			4550000	6.66	1307500	6.12	975000	5.99	2603333	6.42
	4			2345000	6.37	1782500	6.25	1272500	6.10	2907500	6.46
	1			3143333	6.50	2743333	6.44	4800000	6.68	4500000	6.65
72	2			2972500	6.47	3900000	6.59	2656667	6.42	2365000	6.37
	3			4800000	6.68	1867500	6.27	1017500	6.01	2666667	6.43
	4			2170000	6.34	3600000	6.56	1612500	6.21	1980000	6.30

Time		Before Treatment		Tap Water		PS 0.1%		PS 0.5%		NEW	
Hours	Replication	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g
	1	9750000	6.99								
0	2	9575000	6.98								
	3	6700000	6.83								
	1			4450000	6.65	5850000	6.77	5800000	6.76	3800000	6.58
12	2			6000000	6.78	7850000	6.89	8150000	6.91	5850000	6.77
	3			7100000	6.85	7800000	6.89	5250000	6.72	2650000	6.42
	1			5700000	6.76	6150000	6.79	5550000	6.74	3083333	6.49
24	2			6700000	6.83	6700000	6.83	6050000	6.78	5750000	6.76
	3			4900000	6.69	6200000	6.79	6950000	6.84	4500000	6.65
	1			4100000	6.61	4600000	6.66	4250000	6.63	3850000	6.59
36	2			2853333	6.46	5050000	6.70	4050000	6.61	2107500	6.32
	3			8100000	6.91	8000000	6.90	5850000	6.77	3483333	6.54
	1			4500000	6.65	4350000	6.64	4400000	6.64	3400000	6.53
48	2			3800000	6.58	5050000	6.70	4000000	6.60	3923333	6.59
	3			6300000	6.80	5300000	6.72	3150000	6.50	2800000	6.45
	1			3190000	6.50	2566667	6.41	3150000	6.50	2047500	6.31
60	2			3950000	6.60	3500000	6.54	4900000	6.69	2032500	6.31
	3			4500000	6.65	6800000	6.83	4450000	6.65	2526667	6.40
	1			1935000	6.29	3260000	6.51	2986667	6.48	2337500	6.37
72	2			4100000	6.61	4700000	6.67	3900000	6.59	1517500	6.18
	3			6350000	6.80	3900000	6.59	2300000	6.36	2450000	6.39

Table A.2. Raw data for *E. coli* K-12 counts on fish fillet samples after treatment with sanitized ice

Time		Before Treatment		Tap Water		PS 0.1%		PS 0.5%		NEW	
Hours	Replication	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g	CFU/g	Log CFU/g
	1	11100000	7.05								
0	2	17050000	7.23								
	3	12150000	7.08								
	4	11950000	7.08								
	1			3130000	6.50	6500000	6.81	5950000	6.77	3450000	6.54
12	2			9066667	6.96	19650000	7.29	10350000	7.01	8950000	6.95
	3			3450000	6.54	7850000	6.89	5500000	6.74	3470000	6.54
	4			6850000	6.84	6050000	6.78	6250000	6.80	8100000	6.91
	1			6000000	6.78	5100000	6.71	7133333	6.85	2706667	6.43
24	2			8100000	6.91	14200000	7.15	11200000	7.05	5900000	6.77
	3			4350000	6.64	7636667	6.88	7750000	6.89	5750000	6.76
	4			5550000	6.74	5450000	6.74	4750000	6.68	6350000	6.80
	1			2180000	6.34	2780000	6.44	3916667	6.59	2412500	6.38
36	2			3850000	6.59	15650000	7.19	6900000	6.84	15000000	7.18
	3			4800000	6.68	3203333	6.51	4036667	6.61	3800000	6.58
	4			4550000	6.66	4650000	6.67	4100000	6.61	5150000	6.71

(continued)

Table A.3. Raw data for *P. putida* counts on fish fillet samples after treatment with sanitized ice.

	1			2092500	6.32	3016667	6.48	7003333	6.85	1780000	6.25
48	2			3450000	6.54	37000000	7.57	8950000	6.95	5900000	6.77
	3			2142500	6.33	3560000	6.55	2543333	6.41	5143333	6.71
	4			5200000	6.72	4350000	6.64	3650000	6.56	5200000	6.72
	1			3360000	6.53	18950000	7.28	3545000	6.55	2930000	6.47
60	2			5900000	6.77	16000000	7.20	10450000	7.02	9550000	6.98
	3			5550000	6.74	5000000	6.70	3210000	6.51	8023333	6.90
	4			4500000	6.65	3400000	6.53	3100000	6.49	2800000	6.45
	1			2267500	6.36	2433333	6.39	7823333	6.89	2956667	6.47
72	2			2833333	6.45	41000000	7.61	21700000	7.34	17850000	7.25
	3			3650000	6.56	18800000	7.27	8150000	6.91	5600000	6.75
	4			4200000	6.62	7800000	6.89	3250000	6.51	5650000	6.75

Time		Before Treatment		Tap Water		PS 0.1%		PS 0.5%		NEW	
Hours	Replication	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
	1	27050000	7.43								
	2	15925000	7.20								
0	3	16175000	7.21								
	4	8800000	6.94								
	1			10000	4.00	4725	3.67	266	2.43	5750	3.76
	2			537500	5.73	876	2.94			3225	3.51
24	3			585000	5.77	7400	3.87	500	2.70	14188	4.15
	4			592500	5.77	10075	4.00	1035	3.01	19700	4.29
	1			76625	4.88	414	2.62	10	1.00	1910	3.28
	2			85750	4.93	86	1.93			2611	3.42
48	3			377500	5.58	1493	3.17	536	2.73	2195	3.34
	4			198250	5.30	3088	3.49	4228	3.63	8775	3.94
	1			73750	4.87	130	2.11			92	1.96
	2			56500	4.75	85	1.93	25	1.40	10	1.00
72	3			257429	5.41	400	2.60	510	2.71	289	2.46
	4			174125	5.24	1384	3.14	825	2.92	2110	3.32

Table A.4. Raw data for *L. innocua* counts in waters from the melted ice collected during fish samples storage on ice (4ºC)

Time		Before Treatment		Tap Water		PS 0.1%		PS 0.5%		NEW	
Hours	Replication	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
	1	9750000	6.99								
	2	9575000	6.98								
0	3	6700000	6.83								
	1			94875	4.98	4475	3.65	735	2.87	55	1.74
	2			110125	5.04	10275	4.01	743	2.87	10063	4.00
24	3			119250	5.08	11738	4.07	508	2.71	3838	3.58
	1			38000	4.58	1476	3.17	578	2.76	554	2.74
	2			61500	4.79	2201	3.34	206	2.31	308	2.49
48	3			86125	4.94	1480	3.17	349	2.54	231	2.36
	1			39375	4.60	320	2.51	295	2.47	741	2.87
	2			69875	4.84	1071	3.03	324	2.51	190	2.28
72	3			79375	4.90	866	2.94	678	2.83	303	2.48

Table A.5. Raw data for *E. coli* K-12 counts in waters from the melted ice collected during fish samples storage on ice (4ºC)

Time		Before Treatment		Tap Water		PS 0.1%		PS 0.5%		NEW	
Hours	Replication	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
	1	11100000	7.05								
	2	17050000	7.23								
0	3	12150000	7.08								
	4	11950000	7.08								
	1			64625	4.81	56	1.75	20	1.30	9550	3.98
	2			40000	4.60	20	1.30	78	1.89	2425	3.38
24	3			42500	4.63	131	2.12	248	2.40	2200	3.34
	4			46375	4.67	163	2.21	534	2.73	13225	4.12
	1			37250	4.57	35	1.54	383	2.58	1539	3.19
	2			57750	4.76	136	2.13	223	2.35	2428	3.39
48	3			68625	4.84	273	2.44	345	2.54	793	2.90
	4			15	1.18	906	2.96	943	2.97	7725	3.89
	1			17500	4.24	70	1.85	249	2.40	464	2.67
	2			34333	4.54	1826	3.26	715	2.85	4175	3.62
72	3			17833	4.25	58	1.76	580	2.76	3088	3.49
	4			42500	4.63	624	2.80	749	2.87	2600	3.41

Table A.6. Raw data for *P. putida* counts in waters from the melted ice collected during fish samples storage on ice (4ºC)

Water collected from melted ice								
	Tap Water		**PS 0.1%**		**PS 0.5%**		**PS 1%**	
Replication	CFU/ml	Log	CFU/ml	Log	CFU/ml	Log	CFU/ml	Log
		CFU/ml		CFU/ml		CFU/ml		CFU/ml
1	11	1.02	ND		ND		ND	
2	83	1.92	ND		ND		ND	
Ice left in trays								
	Tap Water		**PS 0.1%**		**PS 0.5%**		**PS 1%**	
Replication	CFU/ml	Log	CFU/ml	Log	CFU/ml	Log	CFU/ml	Log
		CFU/ml		CFU/ml		CFU/ml		CFU/ml
1	99	2.00	4	0.60	5	0.65	7	0.81
2	230	2.36	13	1.10	24	1.38	31	1.50

*ND = Non-detected

Table A.7. Raw data for the natural microflora counts in waters from the melted ice and in the ice left in the trays after 8 hours storage of whole fish.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.225E7	19	1170857.964	629.106	.000
Intercept	6.217E7	1	6.217E7	33406.036	.000
Time	2.223E7	15	1481883.498	796.221	.000
Treatment	18048.848	4	4512.212	2.424	.049
Error	381534.969	205	1861.146		
Total	8.448E7	225			
Corrected Total	2.263E7	224			

Table A.8. Test of between-subjects effects: Two-way ANOVA for the stability of ice.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	18.806	4	4.701	30.767	.000
Intercept	22.207	1	22.207	145.330	.000
Treatment	12.456	3	4.152	27.172	.000
Source	6.350	1	6.350	41.553	.000
Error	4.126	27	.153		
Total	45.139	32			
Corrected Total	22.932	31			

Table A.9. Test of between-subjects effects: Two-way ANOVA for the natural microflora of the whole fish enumerated in the water from the melted ice

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7.682	9	.854	24.439	.000
Intercept	6205.154	1	6205.154	177661.406	.000
Treatment	.136	3	.045	1.297	.277
Time	5.051	5	1.010	28.924	.000
Error	6.636	190	.035		
Total	8850.923	200			
Corrected Total	14.318	199			

Table A.10. Test of between-subjects effects: Two-way ANOVA for *L. innocua* populations on fish fillet samples

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.874	9	.319	17.495	.000
Intercept	4577.870	1	4577.870	250798.349	.000
Treatment	1.078	3	.359	19.686	.000
Time	1.275	5	.255	13.973	.000
Error	2.537	139	.018		
Total	6576.183	149			
Corrected Total	5.411	148			

Table A.11. Test of between-subjects effects: Two-way ANOVA for *E. coli* K-12 populations on fish fillets

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.595	9	.399	5.097	.000
Intercept	6329.678	1	6329.678	80754.514	.000
Treatment	1.706	3	.569	7.254	.000
Time	.872	5	.174	2.224	.054
Error	14.814	189	.078		
Total	9086.592	199			
Corrected Total	18.410	198			

Table A.12. Test of between-subjects effects: Two-way ANOVA for *P. putida* populations on fish fillets

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	211.692	5	42.338	137.055	.000
Intercept	2006.941	1	2006.941	6496.740	.000
Treatment	191.621	3	63.874	206.768	.000
Time	28.751	2	14.375	46.535	.000
Error	50.044	162	.309		
Total	2394.729	168			
Corrected Total	261.736	167			

Table A.13. Test of between-subjects effects: Two-way ANOVA for *L. innocua* in water from the melted ice

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	123.040	5	24.608	194.518	.000
Intercept	1649.073	1	1649.073	13035.326	.000
Treatment	105.561	3	35.187	278.140	.000
Time	17.479	2	8.740	69.084	.000
Error	17.458	138	.127		
Total	1789.571	144			
Corrected Total	140.498	143			

Table A.14. Test of between-subjects: effects Two-way ANOVA for *E. coli* K-12 in water from the melted ice

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	171.650	5	34.330	190.925	.000
Intercept	1739.265	1	1739.265	9672.831	.000
Treatment	171.649	3	57.216	318.205	.000
Time	.155	2	.077	.430	.651
Error	32.366	180	.180		
Total	1980.717	186			
Corrected Total	204.016	185			

Table A.15. Test of between-subjects effects: Two-way ANOVA for *P. putida* in water from the melted ice

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NEW	Tap Water	-12.1067	9.09492	.478	-34.4890	10.2757
PS 1%	Tap Water	-9.8222	9.09492	.655	-32.2046	12.5601
PS 0.5%	Tap Water	4.3978	9.09492	.969	-17.9846	26.7801
PS 0.1%	Tap Water	12.0311	9.09492	.483	-10.3512	34.4134

Table A.16. Multiple comparisons of Dunnett test for the stability of the ice treatments

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PS 0.1%	Tap Water	-1.5043*	.19545	.000	-1.9906	-1.0179
PS 0.5%	Tap Water	-1.4558*	.19545	.000	-1.9422	-.9695
PS 1%	Tap Water	-1.3435*	.19545	.000	-1.8298	-.8572

Table A.17. Multiple comparisons of Dunnett test for the natural microflora of the whole fish enumerated in the water from the melted ice

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Treatment	Tap Water	.5260018*	.07136876	.000	.3294414	.7225621
	PS 0.1%	.5780622*	.07136876	.000	.3815019	.7746226
	PS 0.5%	.5975811*	.07136876	.000	.4010207	.7941415
	NEW	.5783802*	.07136876	.000	.3818198	.7749406
Tap Water	Before Treatment	-.5260018*	.07136876	.000	-.7225621	-.3294414
	PS 0.1%	.0520605	.03814821	.651	-.0530055	.1571264
	PS 0.5%	.0715793	.03814821	.334	-.0334866	.1766453
	NEW	.0523784	.03814821	.646	-.0526875	.1574444
PS 0.1%	Before Treatment	-.5780622*	.07136876	.000	-.7746226	-.3815019
	Tap Water	-.0520605	.03814821	.651	-.1571264	.0530055
	PS 0.5%	.0195188	.03814821	.986	-.0855471	.1245848
	NEW	.0003179	.03814821	1.000	-.1047480	.1053839

(continued)

Table A.18 .Multiple comparisons of Tukey test for *L. innocua* on fish fillet samples

PS 0.5%	Before Treatment	-.5975811*	.07136876	.000	-.7941415	-.4010207
	Tap Water	-.0715793	.03814821	.334	-.1766453	.0334866
	PS 0.1%	-.0195188	.03814821	.986	-.1245848	.0855471
	NEW	-.0192009	.03814821	.987	-.1242669	.0858650
NEW	Before Treatment	-.5783802*	.07136876	.000	-.7749406	-.3818198
	Tap Water	-.0523784	.03814821	.646	-.1574444	.0526875
	PS 0.1%	-.0003179	.03814821	1.000	-.1053839	.1047480
	PS 0.5%	.0192009	.03814821	.987	-.0858650	.1242669

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Treatment	Tap Water	.2545374*	.05969688	.000	.0895404	.4195345
	PS 0.1%	.2157049*	.05957542	.004	.0510436	.3803662
	PS 0.5%	.2751651*	.05957542	.000	.1105038	.4398264
	NEW	.4433564*	.05957542	.000	.2786951	.6080177
Tap Water	Before Treatment	-.2545374*	.05969688	.000	-.4195345	-.0895404
	PS 0.1%	-.0388325	.03207106	.745	-.1274742	.0498091
	PS 0.5%	.0206277	.03207106	.968	-.0680140	.1092693
	NEW	.1888190*	.03207106	.000	.1001773	.2774606
PS 0.1%	Before Treatment	-.2157049*	.05957542	.004	-.3803662	-.0510436
	Tap Water	.0388325	.03207106	.745	-.0498091	.1274742
	PS 0.5%	.0594602	.03184440	.340	-.0285550	.1474754
	NEW	.2276515*	.03184440	.000	.1396363	.3156667

(continued)

Table A.19 .Multiple comparisons of Tukey test for *E. coli* K-12 on fish fillet samples

PS 0.5%	Before Treatment	-.2751651*	.05957542	.000	-.4398264	-.1105038
	Tap Water	-.0206277	.03207106	.968	-.1092693	.0680140
	PS 0.1%	-.0594602	.03184440	.340	-.1474754	.0285550
	NEW	.1681913*	.03184440	.000	.0801761	.2562065
NEW	Before Treatment	-.4433564*	.05957542	.000	-.6080177	-.2786951
	Tap Water	-.1888190*	.03207106	.000	-.2774606	-.1001773
	PS 0.1%	-.2276515*	.03184440	.000	-.3156667	-.1396363
	PS 0.5%	-.1681913*	.03184440	.000	-.2562065	-.0801761

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Treatment	Tap Water	.4877393*	.10691430	.000	.1932661	.7822125
	PS 0.1%	.2267893	.10691430	.215	-.0676839	.5212625
	PS 0.5%	.3452529*	.10707666	.013	.0503325	.6401733
	NEW	.3964217*	.10691430	.003	.1019485	.6908949
Tap Water	Before Treatment	-.4877393*	.10691430	.000	-.7822125	-.1932661
	PS 0.1%	-.2609500*	.05714810	.000	-.4183526	-.1035475
	PS 0.5%	-.1424864	.05745127	.100	-.3007240	.0157512
	NEW	-.0913176	.05714810	.501	-.2487202	.0660849
PS 0.1%	Before Treatment	-.2267893	.10691430	.215	-.5212625	.0676839
	Tap Water	.2609500*	.05714810	.000	.1035475	.4183526
	PS 0.5%	.1184636	.05745127	.241	-.0397740	.2767012
	NEW	.1696324*	.05714810	.028	.0122298	.3270349

(continued)

Table A.20. Multiple comparisons of Tukey test for *P. putida* on fish fillet samples

PS 0.5%	Before Treatment	-.3452529*	.10707666	.013	-.6401733	-.0503325
	Tap Water	.1424864	.05745127	.100	-.0157512	.3007240
	PS 0.1%	-.1184636	.05745127	.241	-.2767012	.0397740
	NEW	.0511688	.05745127	.900	-.1070688	.2094064
NEW	Before Treatment	-.3964217*	.10691430	.003	-.6908949	-.1019485
	Tap Water	.0913176	.05714810	.501	-.0660849	.2487202
	PS 0.1%	-.1696324*	.05714810	.028	-.3270349	-.0122298
	PS 0.5%	-.0511688	.05745127	.900	-.2094064	.1070688

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Tap Water	PS 0.1%	2.4107*	.11600	.000	2.1096	2.7119
	PS 0.5%	2.6395*	.13033	.000	2.3012	2.9778
	NEW	2.0055*	.11784	.000	1.6997	2.3114
PS 0.1%	Tap Water	-2.4107*	.11600	.000	-2.7119	-2.1096
	PS 0.5%	.2288	.12807	.284	-.1037	.5612
	NEW	-.4052*	.11533	.003	-.7046	-.1058
PS 0.5%	Tap Water	-2.6395*	.13033	.000	-2.9778	-2.3012
	PS 0.1%	-.2288	.12807	.284	-.5612	.1037
	NEW	-.6339*	.12973	.000	-.9707	-.2972
NEW	Tap Water	-2.0055*	.11784	.000	-2.3114	-1.6997
	PS 0.1%	.4052*	.11533	.003	.1058	.7046
	PS 0.5%	.6339*	.12973	.000	.2972	.9707

Table A.21. Multiple comparisons of Tukey test for *L. innocua* in the water from the melted ice

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Tap Water	PS 0.1%	1.5108*	.08383	.000	1.2928	1.7289
	PS 0.5%	2.1910*	.08383	.000	1.9730	2.4091
	NEW	1.9796*	.08383	.000	1.7615	2.1976
PS 0.1%	Tap Water	-1.5108*	.08383	.000	-1.7289	-1.2928
	PS 0.5%	.6802*	.08383	.000	.4622	.8982
	NEW	.4687*	.08383	.000	.2507	.6867
PS 0.5%	Tap Water	-2.1910*	.08383	.000	-2.4091	-1.9730
	PS 0.1%	-.6802*	.08383	.000	-.8982	-.4622
	NEW	-.2115	.08383	.061	-.4295	.0065
NEW	Tap Water	-1.9796*	.08383	.000	-2.1976	-1.7615
	PS 0.1%	-.4687*	.08383	.000	-.6867	-.2507
	PS 0.5%	.2115	.08383	.061	-.0065	.4295

Table A.22. Multiple comparisons of Tukey test for *E. coli* K-12 in the water from the melted ice

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Tap Water	PS 0.1%	2.4530*	.08799	.000	2.2248	2.6812
	PS 0.5%	2.1110*	.08799	.000	1.8828	2.3391
	NEW	1.0831*	.08656	.000	.8586	1.3075
PS 0.1%	Tap Water	-2.4530*	.08799	.000	-2.6812	-2.2248
	PS 0.5%	-.3420*	.08940	.001	-.5738	-.1102
	NEW	-1.3699*	.08799	.000	-1.5981	-1.1418
PS 0.5%	Tap Water	-2.1110*	.08799	.000	-2.3391	-1.8828
	PS 0.1%	.3420*	.08940	.001	.1102	.5738
	NEW	-1.0279*	.08799	.000	-1.2561	-.7998
NEW	Tap Water	-1.0831*	.08656	.000	-1.3075	-.8586
	PS 0.1%	1.3699*	.08799	.000	1.1418	1.5981
	PS 0.5%	1.0279*	.08799	.000	.7998	1.2561

Table A.23. Multiple comparisons of Tukey test for *P. putida* in the water from the melted ice