THE PRESENCE OF BACTERIAL PATHOGENS IN RECIRCULATING AQUACULTURE SYSTEM BIOFILMS AND THEIR RESPONSE TO VARIOUS SANITIZERS

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
In partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Food Science and Technology

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April 13, 2001
Blacksburg, Virginia

Key words: Biofilms, recirculating aquaculture, green fluorescent protein, sodium hypochlorite, quaternary ammonium compounds, ozone, peracetic acid

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ABSTRACT

Recirculating aquaculture offers a prospect for successful fish farming, but this form of aquaculture presents a great potential for pathogenic microorganisms to become established in the system through the formation of biofilms. Biofilms are capable of forming on all aquaculture system components, incorporating the various microflora present in the water. Pathogenic microorganisms released from the biofilms are capable of causing recurring exposure to disease in both fish and humans. With the increased consumption of raw and rare fish, the presence of these bacteria in or on the fish could lead to ingestion of pathogens. There is also the possibility of cross-contamination during processing. The objectives of this study were to increase the understanding of pathogen incorporation into biofilms in recirculating aquaculture systems and to determine the effectiveness of various sanitizers in eliminating biofilms.

Seven freshwater and two saltwater facilities were sampled, with eight different types of materials tested. Pathogenic bacteria were identified using Bacteriological Analytical Manual methods and rapid commercial test kits. Most of the pathogenic bacteria identified were opportunistic organisms ubiquitous in an aquatic environment. The most significant human pathogens were *Bacillus cereus*, the *Shigella* species and the *Vibrio* species. The major piscine pathogens of concern were *Photobacterium damsela*, the *Vibrio* species, and *Aeromonas hydrophila.* The most significant variation in biofilm pathogens was observed between facilities and not construction material.

Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, glass, fiberglass and stainless steel disks were suspended in 79.2 liter (20 gallon) aquariums stocked with Nile tilapia (*Oreochromus niloticus)*. The tanks were inoculated with a known amount of green fluorescent protein (GFP) modified *Escherichia coli* and samples were removed on days 1,3, 7 and 15. The modified *E. coli* were isolated on Luria Broth Agar and plate counts were performed under ultraviolet light. There was no significant difference in the growth of the surrogate pathogen on the different materials. The GFP *E. coli* was isolated in the largest numbers 24 hours after inoculation of the tanks, with an approximate 1-log decrease after day 1. Days 3, 7, and 15 showed equivalent growth of the target organism.

Two sets of disks were suspended in another six 79.2 liter (20 gallon) aquariums. The tanks were inoculated with a known amount of the surrogate pathogen, GFP *E. coli,* and after 24 hours one set of disks was removed from each tank. The second set of disks was removed and treated by spraying with water, alkaline cleanser, sodium hypochlorite, quaternary ammonium compound, or peracetic acid. Ozone was bubbled directly into one tank to treat another set of disks. The modified *E. coli* were isolated and counted. Total aerobic plate counts and Enterobacteriaceae counts were performed. Statistical analysis indicated that the type of material had no significant affect on the effectiveness of the sanitizers. It was determined that sodium hypochlorite (99.4591 overall reduction) and peracetic acid (98.8461 % overall reduction) were the most effective sanitizers overall, and ozone (32.9332 % overall reduction) was the least effective.

ACKNOWLEDGEMENT

The author would like to thank the Virginia Sea Grant College Program, the Commercial Fish and Shellfish Technologies Program, Virginia Polytechnic Institute and State University (VPI & SU), and the National Fisheries Institute, Inc., Arlington, VA, for their financial support as well as the commercial aquaculture facilities that allowed me to obtain samples from their systems. I would also like to thank all the members of my committee for their support and assistance. My sincere appreciation goes to all the staff of the Food Science and Technology Department for their assistance, especially to John Chandler for his help in making all the disks used in this study and Tatiana Lorca for the use of her GFP *E. coli* isolate. I am also grateful to Lee Weigt of the DNA Laboratory, VPI & SU, for his time in training and performing the polymerase chain reaction section of this study.

The author is also grateful for the educational leave of the U.S. Department of Defense, U.S. Army Veterinary Corps, to pursue a doctoral degree program.

Finally, I would like to thank my husband, David L. Smith, for his patience, assistance, and encouragement while I pursued this doctoral degree.

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INTRODUCTION

With the decreasing numbers of wild fisheries (Pauly et al. 2000), the demand for seafood will need to be filled, and commercial aquaculture will help to meet that demand. Aquaculture is the fastest growing agricultural industry today. Different species of finfish and shellfish are raised using diverse methods including ponds, raceways, net pens and recirculating systems. The advantages of acquiring seafood from aquaculture are that the products are of uniform size and quality, with less physical damage present. There is a high potential for aquaculture products to be of better physical and biochemical quality than wild-caught seafood (Reilly and Käferstein 1999).

Aquaculture systems generally employ intensive culture methods involving dense populations of fish. These can produce large quantities of waste often released into natural bodies of water. The refuse consists largely of unused feed and excretory waste. This dense waste leads to algae blooms causing a decrease in the growth of natural flora and fauna. Some algae blooms produce toxins that can further decrease the natural fish populations and lead to disease in humans (Austin 1999). Bacteria are an additional pollutant released from aquaculture systems (Austin and Austin 1989). The bacteria in the waste can lead to infections in wildlife as well as exposure of humans to possible pathogens.

Another issue is the presence of antibiotic and chemical residues used illegally as prophylactics or as treatment for piscine diseases in the effluent. The only government-approved route of antibiotic administration is orally by incorporation into the feed. These medicated feeds may be unpalatable to the fish or the fish may be too sick to consume the normal amounts resulting in more feed in the waste. The excess treated feed may be

consumed by wild fish, leading to potential exposure of the public. Antibiotic use in aquaculture systems may also contribute to antibiotic resistance in bacteria. The American Society of Microbiology (1999) includes the use of antibiotics in aquaculture as an important factor leading to the evolution of antibiotic-resistant bacteria.

The presence of pesticides in aquaculture is another potential source of pollution. Chemical usage in aquaculture in the United States is regulated. Release of these chemicals into the environment would affect the growth of normal, necessary microorganisms, causing a detrimental effect on the area. Under the Federal Pollution Act of 1990, the order of preference to manage pollution is prevention, recycle and reuse the waste, treatment of waste, and disposal of waste into the environment. Reducing the amount of pollution at the source requires improved management techniques. Using recirculating aquaculture systems is one method of source reduction. Recirculating aquaculture reuses water more than once before discharging it. It requires limited resources and provides greater environmental control. For these reasons it offers increased control of potential pollutants.

Food safety issues associated with aquaculture products vary according to the method of production, management practices and environmental conditions (Reilly and Käferstein 1999). Being unaware of microorganisms present in aquaculture products can affect human health as evidenced by the transmission of streptococcal infections from tilapia to humans resulting in several cases of meningitis in Canada (Prevention 1996).

Despite the advantages, recirculating systems pose a latent disease and public health risk. Part of the biological filtration necessary for removal of harmful toxins involves the biofilm that forms on all components of a recirculating system. Because the

water is reused, pathogens introduced into the system could remain indefinitely through incorporation into the biofilm, leading to recurring exposure of fish to pathogens and the presence of asymptomatic carriers. In studies by Karunasagar et al. (1994, 1996), antibiotic resistant *Vibrio harveyi* persisted in the larval tanks of a shrimp hatchery, most probably as biofilm bacteria and therefore not easily eliminated by sanitizer treatment. Because people are eating more raw, rare and cold-smoked seafood, this could lead to consumption of infected fish or cross contamination during processing. Understanding how these pathogens persist in the biofilm and how they respond to sanitizers will help the aquaculturist decrease the threat to public health.

LITERATURE REVIEW

1. Recirculating Aquaculture Systems

Recirculating aquaculture is the most intensive level of fish production. Recirculating systems recondition and recycle almost all of the water delivered into the fish-rearing units, and can be semi-closed or closed, with almost 90 to 100% of the water being reused, respectively (Stickney 1993). Closed aquaculture systems have been proposed for production of fish in areas of low water availability or unfavorable water conditions for specific fish species, as well as to produce fish closer to their markets (Stickney 1993).

Maintaining healthy fish in a recirculating system involves establishing adequate dissolved oxygen levels, removal of solid wastes, and sufficient ammonia nitrification. The overall rate of oxygen consumption in a system is the sum of the respiration rate of the fish, the oxygen demand of bacteria breaking down organic wastes and uneaten food, and the oxygen demand of nitrifying bacteria in the filter (Losordo et al. 1992). Proper dissolved oxygen levels are generally maintained using aerators to introduce air into the water. This delivers oxygen into the water where it becomes accessible to the fish by contact with the gill surface. Another method becoming more popular for use in high-density systems is the injection of pure oxygen into the water (Losordo et al. 1992). This increases the rate of oxygen addition and allows for a higher oxygen utilization rate.

High amounts of solid material in recirculating aquaculture systems cloud the water, clog filters and cause irritation of gill epithelium (Stickney 1993). Organic solids are generated by fish as fecal solids, uneaten feed and may include microorganisms. When these solids accumulate to concentrations sufficient to clog mechanical filters they

begin to decay, leading to a build up of anaerobic conditions (Stickney 1993). The bacterial colonies established on that filter then emit hydrogen sulfide, ammonia, and other toxic substances, as well as release water that is very low in oxygen (Stickney 1993). Removal of these solids should be a primary consideration in aquaculture design.

The methods of solid waste removal are dependent on the type of solid being removed. Sedimentation removes large particles that settle to the bottom of the tank. In round tanks these solids accumulate in the center, where a sump is placed for easy removal (Stickney 1993). Mechanical filtration is often used to remove smaller particles that stay suspended in the water column. Screens and granular media are the two types of filters used to remove suspended solids (Stickney 1993). Dissolved solids and fine suspended solids are difficult to remove via sedimentation or mechanical filtration. These particles are often removed with foam fractionation or protein skimming (Stickney 1993). In this process, air is bubbled through a closed column of water. As the bubbles rise to the surface, the dissolved solid particles attach to the surface, forming foam. The foam is then channeled off as waste.

Generation of nitrogen in aquaculture systems occurs with the breakdown of proteins from excess feed, excretion from the gills of fish as they utilize feed, and decomposition of organic waste by bacteria. Total ammonia-nitrogen (TAN) consists of ammonia and ammonium ions (Losordo et al. 1992). Ammonia is more toxic than ammonium ions, and the amount present depends on the pH and temperature of the water (Stickney 1993). Ammonia-nitrogen must be removed at a rate equal to production (Losordo et al. 1992). There are a number of technologies available to remove ammonia-nitrogen, but the most commonly used is biological filtration (Losordo et al. 1992). The

concept of biological filtration is to provide a substrate with a large surface area to encourage the growth of autotrophic bacteria capable of oxidizing ammonia to nitrite and nitrite to nitrate (Kaiser and Wheaton 1983). The nitrifying bacteria as well as heterotrophic bacteria present in the system form biofilms on all surfaces in the system (Stickney 1993). Increasing the surface area in the filter increases the number of bacteria leading to more efficient nitrification of ammonia and allowing denser fish populations. Gravel, sand, plastic beads and rings, and plastic plates are the substrates most commonly used. *Nitrosomas* species oxidize ammonia to nitrites. Nitrite-nitrogen is still harmful to aquatic animals and must be removed. *Nitrobacter* bacteria utilize nitrite-nitrogen as an energy source and produce nitrates as a by-product. Nitrates are relatively non-toxic unless the levels are extremely high and they are usually flushed from the system during maintenance.

When a recirculating system is started or restarted after harvesting or cleaning, time must be allowed for colonization of the biofilter (Stickney 1993). This is a critical time in recirculating systems, because ammonia levels increase faster than their removal (Stickney 1993). Biofilters are often started by placing inorganic ammonia into the system thereby allowing establishment of the filtering microorganisms prior to stocking with fish (Stickney 1993). This would also be expected to encourage growth of autotrophic bacteria because without an organic load, competition for attachment surfaces from faster growing heterotrophs would be minimal (Wickens 1983).

2. Biofilms

Biofilms are common in nature and grow at the water/solid interface in most all biological systems. They are found on medical implants, on surfaces in streams, and lead to plaque on teeth (Costerton et al. 1987; Wilderer and Characklis 1989; Geesey et al. 1992). Biofilms are responsible for the deterioration of ship hulls and underwater building supports (Blenkinsopp and Costerton 1991; Geesey et al. 1992). Research has also investigated the presence of biofilms formed by *Listeria monocytogenes* on food preparation surfaces (Smoot and Pierson 1998a). Biofilm formation is a response by microorganisms to alterations in growth rate, exposure to subinhibitory concentrations of certain antibiotics, and growth on solid surfaces (Brown and Gilbert 1993; Sasahara and Zottola 1993; Yu and McFeters 1994; Smoot and Pierson 1998a, 1998b; Kerr et al. 1999).

The sessile cells of biofilms are very different from planktonic cells. There are differences noted in cellular enzymatic activity, cell wall composition, and surface structures between bacterial cells adherent to surfaces and planktonic cells of the same organism (Costerton et al. 1987). It was found that exposing *L. monocytogenes* in a biofilm to sublethal treatments of antimicrobials or stresses would result in a unique adaptive response and the stress of starvation was often accompanied by an increase in cell surface hydrophobicity and adhesiveness, with a decrease in cell size (Costerton et al. 1987; Smoot and Pierson 1998a, 1998b; Wong 1998). The molecular composition of bacterial cell walls is essentially plastic and is very responsive to the cell's growth environment (Costerton et al. 1987). Environmental signals and cellular structures

required for adhesion to intestinal epithelium, nonnutritive abiotic surfaces and nutritive, abiotic surfaces are distinct (de Franca and Lutterbach 1996; Watnick et al. 1999).

Biofilm bacteria live in glycocalyx-enclosed microcolonies whose location, size and shape are determined by nonrandom species-specific factors (Costerton 1995). The glycocalyx functions as an ion-exchange column and excludes large, highly charged molecules (Brown and Gilbert 1993). Primary colonizers may not all produce an exopolymer, but those that do may attract other bacteria that have no propensity for attachment from the planktonic phase. Sasahara and Zottola (1993) found that *L. monocytogenes* did not form an exopolymer, and attached better in the presence of *Pseudomonas fragi* cells that were already attached to the surface. Other research found that *L. monocytogenes* attachment was inhibited in the presence of a proteolytic enzyme (trypsin) indicating that proteins are involved in the initial attachment of this organism (Smoot and Pierson 1998a).

Multispecies biofilms form highly complex structures with cells arranged in clusters or layers with anastomosing water channels that bring nutrients to the lower layers and remove waste products (Costerton 1995). In thick biofilms the upper layers are aerobic and the lower layers are anaerobic (Blenkinsopp and Costerton 1991; Geesey et al. 1992). Fastidious organisms can be maintained within the microcolonies, and anaerobic organisms can contribute to the overall activity of the consortium. Microbial metabolic processes in biofilms are stratified, with aerobic oxidation only occurring in the shallow layer near the surface (Yu and Bishop 1998). Sulfate reduction occurs in the anaerobic zone (Yu and Bishop 1998). Yu and Bishop (1998) demonstrated that the redox potential in a biofilm decreased sharply from a positive potential to a negative

potential with a very narrow band near the interface between the aerobic zone and the sulfate reduction zone. Aerobically pre-grown biofilm cultures of *Shewanella putrefaciens* initially use oxygen as electron acceptors, then switch to using both sulfite and oxygen as terminal electron acceptors when dissolved oxygen is less than 1.5 mg/L (Dawood et al. 1998). This mechanism is energetically wasteful, because oxygen as the electron acceptor requires less energy and growth is promoted (Dawood et al. 1998). Another study shows that after 21 days the biofilm includes the greatest number of microorganisms, including microalgae contributing to the generation of conditions favorable to colonization by sulfite-reducing bacteria, aerobic bacteria producing most of the exopolysaccharide, and microaerophilic and anaerobic bacteria (de Franca and Lutterbach 1996).

The major advantage of biofilm formation is that the biofilms provide protection from the effects of an adverse environment and host immune defenses. The multispecies culture can provide and maintain the appropriate physical and chemical environments for growth and survival. In many cases the organisms in the biofilm develop a resistance to antimicrobials including surfactants, heavy metals, antibiotics, phagocytic predators and drying (Brown and Gilbert 1993; Ronner and Wong 1993; Yu and McFeters 1994; Costerton 1995; Liltved and Landfald 1995; Watnick et al. 1999).

One mechanism of resistance is the presence of the glycocalyx. Whether the glycocalyx is a physical barrier depends upon the nature of the antimicrobial, the binding capacity of the glycocalyx towards it, the levels of agent used and the rate of growth of the microcolony relative to the antibiotic diffusion rate (Nichols 1989; Brown and Gilbert 1993). More complex biofilm consortia may respond differently to disinfection because

of variations in cellular physiology and greater production of extracellular polymers (Yu and McFeters 1994). For chemically reactive antimicrobials the glycocalyx and outlying cells react with and quench the biocide (Brown and Gilbert 1993). Losses of the biocide through chemical or enzymatic inactivation will decrease the diffusion coefficient and may facilitate resistance (Brown and Gilbert 1993). If the adsorptive capacity of the glycocalyx is high with respect to the biocide, more protection may exist.

Another adaptive response in biofilm bacteria is the ability to decrease growth rate, with development of atypical phenotypes (Nichols 1989; Brown and Gilbert 1993). When cells grow at reduced rates they grow with different properties than in laboratory media. Nutrient deprivation causes cells to respond with decreased use of the deficient nutrient and causes alterations in the cell surface including increased competitive transport mechanisms (Brown and Gilbert 1993). These changes influence an organism's susceptibility to antimicrobials and antibiotics. The action of some antimicrobial agents can be altered by up to 1000-fold with changes in growth rate (Brown and Gilbert 1993). Older biofilms have a decreased growth rate, and show a decreased susceptibility to antimicrobials (Anwar et al. 1990; Geesey et al. 1992).

Attachment to surfaces causes the cell to derepress/induce genes associated with sessile existence with coincidental alteration of antimicrobial susceptibility (Brown and Gilbert 1993). Some of the phenotypic changes that allow cells to change their cell surface characteristics involve target molecules for biocides, antibiotics, antibodies and phagocytes, and external structures that control the access of these agents to the targets (Costerton et al. 1987). This results in resistance to a wide variety of antimicrobials.

It is believed that attachment of bacteria to form a biofilm is dependent on the type of substrate. Kerr et al. (1999) found that heterotrophic bacteria grew faster on cast iron pipe material than on two plastic pipe materials. The effectiveness of detergents and sanitizers is also dependent on the surface, especially a rough surface. In a study testing the ability of *Vibrio harveyi* to form biofilms, the biofilm cells were more resistant to treatment by sanitizers on concrete and high-density polyethylene than on stainless steel (Karunasagar and Otta 1996). In other studies, it was determined that *L. monocytogenes* grew slower on Buna-N rubber surfaces than stainless steel with an increased resistance noted in the biofilm cells on the rubber (Smoot and Pierson 1998b; Wong 1998).

3. Specific Antimicrobials

To reduce or eliminate microorganisms from surfaces, physical or chemical measures are used. Physical methods include heat treatment, high-pressure sprays and mechanical cleaning, such as with brushes (Bal'a et al. 1999). The predominating microorganisms present, as well as water hardness and the protein load determining clean and dirty conditions, determine efficacy of disinfectants (Bessems 1998). The mechanism of action of the disinfectant and type of microorganism present determines concentration and contact time necessary. For example, the contact time and concentration of the membrane-active sanitizers are regulated by the presence of Gram-negative bacteria and the same factors when using oxidizing disinfectants are regulated by the presence of Gram-positive bacteria (Bessems 1998). In aquaculture systems, sanitizers are employed to eliminate the transfer of pathogenic microorganisms between tanks on equipment (Stickney 1993) and to disinfect wastes (Barnabe' 1990).

Many of the chemicals used in aquaculture are applied directly to the water. This use is regulated under the jurisdiction of the Environmental Protection Agency (EPA) or the Food and Drug Administration (FDA) depending on the intended use of the product (Aquaculture 1994). Some compounds can accumulate in the fish causing illegal chemical residues (Aquaculture 1994). The proper use of regulated products in aquaculture production, handling, and processing promotes human, target organism, and environmental safety; ensures the effectiveness of the products used; and prevents illegal residues in edible products available for human consumption. Food safety and quality, and the public perception of the safety of food, influence the long-term development of all food production. Proper use of regulated products in the aquaculture industry can ensure food safety and public trust (Aquaculture 1994).

Because the presence of organic material can decrease the efficacy of many sanitizers, sanitation procedures in the food industry are usually accompanied first by cleaning. "When treated with only a sanitizer, the chemical is inactivated by the deposited soil and becomes ineffective in reaching and destroying all of the microorganisms" (Zottola 1994). Richards (2000) found that when sanitizers alone were used on *L. monocytogenes* biofilms they were not as effective as when cleaning was performed prior to sanitizing. Biofilm bacteria, which attach to many surfaces in aquaculture systems including water storage tanks and water pipes, are not easily eliminated by sanitizers alone, emphasizing the need for physical removal of biofilms on tank surfaces with periodic drying to reduce the presence of pathogenic bacteria in the system (Karunasagar and Otta 1996).

A. Ozone

Ozone (O_3) is a three-atom allotrope of oxygen held together by unstable bonds. A single oxygen atom breaks away easily and reacts with most of the organic and inorganic molecules that it contacts (Lawson 1995). This makes ozone a powerful oxidizing agent, reacting directly and indirectly with other compounds. Indirect reactions occur when ozone decomposes into radicals and these react with other compounds (Sugita et al. 1992; Lawson 1995). When free ozone and ozone decomposition products react with organic molecules, the reaction often takes place at bonds that are not easily oxidized by biological degradation. These compounds then degrade at a faster rate due to smaller molecular size and decreased numbers of higher-order covalent bonds (Summerfelt and Hochheimer 1997). These reactions make elimination of solids from the system easier.

The pathway of the ozone reaction depends on the properties and concentrations of the other compounds, as well as the quality of the water, including pH, bicarbonate level, the level of total organic carbon and temperature. These factors affect the decomposition of ozone and therefore the effective oxidation power and oxidation rate of ozone (Lawson 1995; Summerfelt and Hochheimer 1997). For example, free ozone will react directly with ammonia to form nitrate, but at a slow rate. Ozone radicals also react with ammonia to form nitrate, but at a much faster rate (Summerfelt and Hochheimer 1997). Therefore if the goal of ozone use in an aquaculture system is to aid in denitrification, treatment design should be such that formation of ozone radicals is favored.

Ozone oxidation increases at high pH levels when there is low alkalinity because ozone forms the highly reactive hydroxyl radical (Stachelin and Hoigne 1985). If there are high levels of alkalinity at a high pH, the bicarbonate and carbonate ions present will scavenge the hydroxyl ions, decreasing the effectiveness of ozone (Legube et al. 1986).

Ozone is used in aquaculture systems to improve water quality and overall system performance in terms of increased fish growth and decreased effluent discharge. The goals of ozone use in aquaculture are removal of dissolved organic and inorganic waste removal, enhanced nitrification, control of suspended solids, and disinfection (Paller and Lewis 1988; Lawson 1995; Liltved and Landfald 1995; Summerfelt and Hochheimer 1997). Ozone is very effective in water clarification, denitrification, and dissolving nonbiodegradable organic material (Summerfelt and Hochheimer 1997). Ozone is also toxic to humans (Lawson 1995; Summerfelt and Hochheimer 1997) and because of this toxicity, residual ozone concentration should be measured in the water and in the ambient air (Lawson 1995; Summerfelt and Hochheimer 1997).

Ozone effectively destroys bacteria, viruses, fungi, algae and protozoa by disrupting cell membrane function, entering the cell and destroying the nuclear chemistry of the cell (Lawson 1995). The effectiveness of ozone as a disinfectant is a function of dosage and contact time (Lawson 1995). The target organism and water quality determine the required concentration of ozone and the necessary contact time (Lawson 1995; Summerfelt and Hochheimer 1997). Microbial

reductions are limited by the ability to maintain a specific ozone concentration for the time needed (Summerfelt and Hochheimer 1997).

Microorganisms are considered part of the total particulate matter load and when dissolved and particulate organic compounds are together, hydroxyl radicals react preferentially toward dissolved organic compounds (Lawson 1995). Thus, in recirculating aquaculture systems, where the levels of dissolved organic matter is often high due to large amounts of fish feed, nitrite production by the biofilters and the sloughing of suspended solids, maintaining adequate residual ozone levels for disinfection can be difficult (Lawson 1995; Summerfelt and Hochheimer 1997). In some systems the ozone demand can be so high that the ozone and its decomposition products may be depleted before microorganisms can be killed (Liltved and Landfald 1995; Summerfelt and Hochheimer 1997).

Use of ozone in freshwater systems can be beneficial, but other factors must be considered before use in saltwater systems. Ozone reacts with bromide and chloride ions in the seawater to form toxic hypochlorite and hypobromite ions, so removal of residuals must be allowed before replacing water into the system (Sugita et al. 1992; Lawson 1995). Saltwater also has a higher pH, so ozone decomposition is faster (Lawson 1995). Ozone decomposition can also deplete trace elements in saltwater, especially manganese and calcium (Lawson 1995).

B. Sodium Hypochlorite

Of the chemical disinfectants currently available, chlorine is most likely the cheapest and easiest to obtain. It is used in the form of chlorine gas, calcium hypochlorite or sodium hypochlorite. Of all these forms, sodium hypochlorite is the most common (Lawson 1995; Boothe 1998). Chlorine sanitizers are truly broad-spectrum with germicidal activity against viruses, acid-fast and non-acid-fast bacteria, spores, fungi, algae and protozoa, while presenting a minimal environmental hazard (Cords and Dychdala 1993; Boothe 1998). However, chlorine toxicity to microorganisms varies widely and is dependent on water conditions, temperature and species of target organism (Lawson 1995).

Sodium hypochlorite disassociates in water, forming the hypochlorite ion (OCl^-) or hypochlorous acid (HOCl). These compounds are strong oxidizing agents and react with a variety of organic and inorganic substances, including microorganisms (Lawson 1995). The mechanism of action against microorganisms is to oxidize peptide links, denaturing proteins, and allowing intracellular accumulation, which further inhibits essential enzyme systems (Boothe 1998).

Two significant environmental factors affecting the biocide activity of chlorine are pH and the concentration of organic material (Boothe 1998). The pK_a of HOCl is 7.54 (Lawson 1995), so when the pH of the solution is less than that there is more of the undissociated hypochlorous acid present, which has a greater bactericidal action than the dissociated form (Cords and Dychdala 1993; Boothe 1998). As with ozone, an increased organic load can deplete available

chlorine before microorganisms are killed, decreasing its bactericidal activity (Price; Boothe 1998). In a study by Liltved (1995), using sodium hypochlorite to disinfect aquaculture wastewater, bactericidal effects of chlorine were reduced as water quality decreased.

In aquaculture systems, an additional factor must be considered in determining the effectiveness of sodium hypochlorite. Hypochlorous acid in water reacts with ammonia nitrogen and organic amines to form chloramines (Lawson 1995; Price 1995). Chloramines also have germicidal activity, but because they form hypochlorous acid and organic salts slowly, a long contact time is required to make them effective sanitizers (Cords and Dychdala 1993; Price 1995). Chloramines are more stable and are less irritating to personnel and less corrosive to equipment (Cords and Dychdala 1993; Lawson 1995). Hypochlorous acid and OCl^- are considered free residual chlorine, and chloramines are regarded as combined residual chlorine. The total residual chlorine refers to the sum of the free and combined forms (Lawson 1995). If a rapid kill rate is desirable, the dose of sodium hypochlorite necessary is generally determined by the concentration of free chlorine present. In an aquaculture system, the concentration of free chlorine is dependent on the ammonia concentration in the water (Lawson 1995).

Fish and invertebrates are highly sensitive to even low concentrations of free and combined forms of chlorine (Lawson 1995). It has also been found that chlorine reacts with organic materials in water forming carcinogenic trihalomethane compounds (Cords and Dychdala 1993; Liltved and Landfald 1995). Because of this, chlorine must be removed from water before it can be

used in aquaculture systems. There are several dechlorination options, including the use of reducing agents, activated carbon, ultraviolet (UV) irradiation, aeration and ozonation (Lawson 1995). Reducing agents reduce free chlorine to inactive compounds but have little effect on chloramines. Examples of reducing agents are sodium thiosulfate, sodium sulfite and ferrous salts. These compounds have toxic properties as well and should be used cautiously (Lawson 1995). Activated carbon removes free and combined chlorine, is safe for fish and biofilter bacteria, but must frequently be reactivated (Lawson 1995). Aeration for 24 to 48 hours is often recommended but this method is ineffective for dechlorination if the water has a high organic load. It is doubtful if aeration removes chloramine residuals (Lawson 1995). Ultraviolet irradiation and ozonation can remove limited amounts of residual chlorine (Lawson 1995).

C. Quaternary Ammonium Compounds

Quaternary ammonium compounds (QAC) are a group of chemical substances considered surface-active cations (Cords and Dychdala 1993; Boothe 1998). The basic chemical structure of the cation consists of a nitrogen molecule with four covalently bound alkyl groups of various sizes and structures. The anion is generally chloride or bromide (Cords and Dychdala 1993). These compounds are divided into eight classifications based on the structure of the alkyl groups (Cords and Dychdala 1993).

Quaternary ammonium compounds bind irreversibly to the negatively charged phospholipids and proteins of the cell membranes of microorganisms,

impairing membrane permeability (Frank and Koffi 1990; Boothe 1998). The presence of lipopolysaccharides and lipids in cell membranes are fundamental to penetration of hydrophobic molecules such as QAC (Guerin-Mechin et al. 1999). Ionic aggregates form on the cell surface after binding causing changes in conductivity, surface tension and solubility (Cords and Dychdala 1993). The most efficacious QAC are those compounds containing alkyl groups in the range of C_{12} to C_{16}, with C_{14} having maximum activity (Cords and Dychdala 1993).

Due to the diversity of the QAC family, generalizing about bactericidal efficacy is difficult. These compounds have been shown to be effective against bacteria, yeast, mold, protozoa and viruses, however there is greater activity against Gram-positive organisms with greater resistance exhibited among Gram-negative organisms (Cords and Dychdala 1993; Price 1995; Boothe 1998). Gram-negative bacteria exhibit two main pathways by which antimicrobials enter the cells: one is hydrophilic (porin-mediated) and one is hydrophobic (diffusion across the membrane by lipids and lipopolysaccharides) (Russell and Gould 1988). In a study by Guerin-Mechin et al. (1999), it was found that *Pseudomonas aeruginosa*, an indicator bacteria for the evaluation of bactericidal activity in Europe, developed a specific variation in cell membrane fatty acid composition in the presence of long-chain QAC, allowing resistance to develop.

Some environmental factors affect the bactericidal activity of quaternary ammonium compounds. One significant factor is the effect of temperature. Higher temperatures enhance the activity of QAC, and this effect seems to be more pronounced for QAC than other traditional sanitizers, such as hypochlorites

and iodophors (Cords and Dychdala 1993). One study found that a quaternary ammonium may be ineffective against some *Listeria* species at cold temperatures, but was germicidal at 25^{o}C (Tuncan 1993). It is unclear why some disinfectants exhibit temperature effects, though it may be because microorganisms lower their metabolic activity at lower temperatures or the onset of cold shock may enhance resistance (Taylor et al. 1999).

The literature contains conflicting reports about the effect of organic matter on the germicidal activity of QAC. It is felt that at concentrations approved for use in the food industry, QAC are considered superior to hypochlorite with respect to organic matter tolerance (Cords and Dychdala 1993). Because QAC react with lipids and lipopolysaccharides, the presence of high amounts of lipids in inorganic material can inactivate QAC (Boothe 1998). A study by Taylor et al. (1999) comparing disinfectants used in the food industry, showed that a QAC failed to achieve a five-log reduction in viable bacterial counts under dirty conditions. Another study testing the effects of a QAC on normal heterotrophic bacteria in the Rhine River showed that the presence of suspended matter reduced the germicidal activity of the QAC (Tubbing and Admiraal 1991).

Another environmental factor affecting the bactericidal activity of QAC is water hardness. Hard water refers to the presence of magnesium and calcium ions that can inactivate QAC (Price 1995; Boothe 1998), though tolerance varies significantly with the type of quaternary used (Cords and Dychdala 1993). Due to

this effect, many QAC produced today have chelating agents present to decrease the adverse affect of these cations (Cords and Dychdala 1993).

Quaternary ammonium compounds are often used in sanitation procedures in the food industry. They are not heavily used in aquaculture so reports of toxicity to cultured organisms are not prevalent. A study by Tubbing and Admiraal (1991) showed that when the nitrifying bacteria and normal phytoplankton in the Rhine River were exposed to low concentrations of a QAC there was a significant effect. Concentrated solutions of 10% or higher of QAC are toxic to humans, but it is felt that even the cumulative effect of food contaminated through residues on food contact surfaces was not a problem (Cords and Dychdala 1993). Quaternary ammonium sanitizers penetrate porous substances but they may leave a film on equipment in cold water, allowing the presence of residues and the possibility of organisms persisting under that layer (Price 1995; Boothe 1998). A thorough rinsing is often necessary to eliminate a persistent layer of germicide.

D. Peracetic Acid

Peracetic acid (PAA) is considered an organic peroxide containing at least one pair of oxygen atoms bonded by a single covalent bond (Cords and Dychdala 1993). The decomposition products of peracetic acid in water are acetic acid, hydrogen peroxide and oxygen (Cords and Dychdala 1993; Blanchard et al. 1998). Peracetic acid is a very strong oxidizing agent with a broad spectrum of antimicrobial activity, effective against both Gram-positive and Gram-negative

bacteria, as well as fungi, viruses and bacterial spores (Cords and Dychdala 1993; Price 1995).

The single bond between oxygen atoms, the peroxygen bond, decomposes to form free radicals (Blanchard et al. 1998). These free radicals have an oxidative capability to disrupt sulphydryl and sulfur bonds in proteins and enzymes in the cells (Cords and Dychdala 1993; Blanchard et al. 1998). Peracetic acid also disrupts the bacterial cell wall, which leads to alteration of the chemiosmotic function of the cell membrane and oxidation of the enzymes leading to impairment of cellular biochemical pathways (Cords and Dychdala 1993; Blanchard et al. 1998). Destruction of the microbial cell by peracetic acid is grouped into three different mechanisms: (1) denaturation of cell proteins and interruption of cell transport, (2) inactivation of enzymes needed for cell metabolism, and (3) disruption of cell membranes and their permeability (Cords and Dychdala 1993).

Though peracetic acid is very effective in most environmental conditions, the efficacy is still affected by some factors. Temperature and pH are of significant importance to the bactericidal effectiveness of PAA. The concentration of organic material present is also important (Cords and Dychdala 1993).

The pH determines the degree of dissociation of the peracetic acid and, consequently, the concentration of the undissociated active species (Sanchez-Ruiz et al. 1995). Peracetic acid is said to be effective over a broad pH range (Price 1995), however optimum antimicrobial activity occurs in an acid environment,

with activity decreasing when the pH is greater than 7 to 8 (Cords and Dychdala 1993; Sanchez-Ruiz et al. 1995). Higher pH levels result in the slower kill of organisms, and to obtain equivalent biocidal activity within 30 seconds at a pH of 9 to 11, a much higher concentration of PAA is required (Cords and Dychdala 1993).

Peracetic acid solutions exhibit significant bactericidal activity even when tested at 5^{o}C, making this compound particularly effective against psychrotrophs (Cords and Dychdala 1993; Price 1995). An increased temperature improves the efficacy of PAA and sporicidal activity of peracetic acid is much greater at higher temperatures (Assoc. 1982; Cords and Dychdala 1993). However peracetic acid explodes at temperatures above 110^{o}C, so care should be taken (Cords and Dychdala 1993; Price 1995).

The presence of organic material adversely affects the germicidal activity of peracetic acid. When testing the effectiveness of PAA on *Mycobacterium bovis* in 1) an aqueous solution, 2) the presence of sterile bovine feces, and 3) a protein-rich medium, the concentration of PAA needed to get a 100% reduction of *M. bovis* in the contaminated solutions was double to quadruple that needed in the aqueous solution (Pavlas 1967). When peracetic acid is used to disinfect wastewater, a great variability in the efficiency of PAA is noted (Sanchez-Ruiz et al. 1995). When comparing PAA to other oxidative sanitizers however, the bactericidal activity of PAA is least affected by organic contamination (Cords and Dychdala 1993).

In 1986, the FDA amended the food additive regulation to include the safe use of PAA in a sanitizing solution on food contact surfaces in food processing plants (CFR 1990; Cords and Dychdala 1993). The decomposition products are considered non-toxic when introduced to food or the environment and do not adversely affect wastewater treatment systems (Cords and Dychdala 1993; Sanchez-Ruiz et al. 1995). PAA has been used in urban wastewater treatment with the goal of guaranteeing the microbiological quality of the estuarine environment into which the treated water would be sent (Sanchez-Ruiz et al. 1995). Peracetic acid is not used extensively in aquaculture systems, however the decomposition products of PAA, acetic acid and hydrogen peroxide, are used as dip treatments for external parasites on fish (Stoskopf 1988).

4. Use of Green Fluorescent Protein Modified *Escherichia coli*

Because *Escherichia coli* is ubiquitous in the stools of humans, in 1892 it was suggested that this organism be used as indicator organisms of fecal contamination (Hitchins et al. 1992). In 1914, the standard was changed to include the whole coliform group (Hitchins et al. 1992). The coliform group includes aerobic and facultative anaerobic, Gram-negative, nonsporeforming rods that ferment lactose (Hitchins et al. 1992; Hitchins et al. 1995). The group may contain organisms not included in the Enterobacteriaceae group, such as *Aeromonas* species (Hitchins et al. 1992).

This indicator group has since been limited to the fecal coliform group, which is restricted to organisms which grow at elevated temperatures such as those found in the gastrointestinal tract of humans and other warm-blooded animals (Hitchins et al. 1992).

The fecal coliforms include *Escherichia* species, *Klebsiella* species, and *Enterobacter* species (Hitchins et al. 1992). Water and food products being tested for fecal coliforms are incubated at temperatures of 44.5 to 45^{o}C (Hitchins et al. 1992).

In Europe the indicator group commonly used when testing food for contamination is the Enterobacteriaceae (Hitchins et al. 1992). These organisms are fermentative, Gram-negative bacteria and include *Salmonella* species, *Escherichia* species, *Enterobacter* species, and *Klebsiella* species. It has been proposed that Enterobacteriaceae are important when incorporated in biofilms in drinking water distribution systems (Jones and Bradshaw 1996).

Because some strains of *E. coli* are pathogenic and the *Klebsiella* species are more ubiquitous than previously believed, some people feel *E. coli* should again be made the indicator organism for fecal contamination (Hitchins et al. 1992). *Klebsiella pneumoniae*, when sloughed off biofilms into drinking water, can cause false-positive coliform results (Jones and Bradshaw 1996). The Environmental Protection Agency in its National Primary Drinking Water Regulations (1999) requires public water systems to test for total coliforms. If there is a positive test, further testing must be done for either fecal coliforms or *E. coli*. The United States Department of Agriculture in its Final Rule on Pathogen Reduction and Hazard Analysis Critical Control Points (1996) require meat and poultry plants to test for generic *E. coli* to verify the HACCP systems in place because generic *E. coli* is found in all animal feces and is more effective as an indicator of fecal contamination than *Salmonella*. When testing seafood from cold water for contamination, *E. coli* is not as reliable an indicator of fecal pollution because of a rapid decline of the bacteria's presence in low temperatures (Nickelson and Finne 1992).

Green fluorescent protein (GFP) is a light emitting cytoplasmic protein originally isolated from the jellyfish, *Aequorea aequorea* (Shimomura et al. 1962). Green fluorescent protein is composed of 238 amino acids and emits green light at a wavelength of 508 nm when excited by blue light with a wavelength of 395 nm. It can be detected by ultraviolet radiation and requires no cofactors or substrates for activity. The cDNA of GFP was cloned to develop a plasmid used to produce GFP as a marker for detecting bacterial growth and viability (Prasher et al. 1992). The plasmid is associated with an ampicillin resistance gene for easier isolation. The protein is expressed when the transformed bacteria is incubated in media containing isopropyl-β-D-thiogalactoside (IPTG). Fluorescence produced by GFP is stable; independent of bacterial species used, can be monitored non-invasively in living cells, and persists in fixed cells (Chalfie et al. 1994; Anonymous 1998). However, Skillman et al. (1998) found that the bacterial cells would lose plasmid when ampicillin was not used for selection. The modification of cells with GFP appears to be non-toxic to the cells and does not seem to alter the cells virulence and other cellular functions including growth (Chalfie et al. 1994). When using GFP modified *Edwardsiella tarda* to study invasion pathways in fish, Ling et al. (2000) found that transformed cells had similar LD_{50} values and affected host cells the same as untransformed strains. These factors all make microorganisms transformed to express green fluorescent protein effective for monitoring bacterial presence and growth.

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IDENTIFICATION OF BACTERIAL PATHOGENS IN BIOFILMS OF RECIRCULATING AQUACULTURE SYSTEMS.[1]

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Abstract

Recirculating aquaculture offers good potential for successful fish farming since it requires limited resources and is often independent of environmental conditions. However, this form of aquaculture presents a potential for pathogenic microorganisms to become established in the system through the formation of biofilms. Biofilms are capable of forming on all aquaculture system components, incorporating the various microflora present in the water. Pathogenic microorganisms released from the biofilms are capable of causing recurring diseases. The project objective was to increase the understanding of pathogen incorporation into biofilms in recirculating aquaculture systems by determining the relationship of the various materials used in construction of those systems and the presence of piscine and human pathogenic bacteria.

Seven freshwater and two saltwater facilities were sampled, with eight different types of materials tested. Pathogenic bacteria were identified using Bacteriological Analytical Manual methods and rapid commercial test kits.

Most of the pathogenic bacteria identified were opportunistic organisms ubiquitous in an aquatic environment. The most significant

human pathogens were Bacillus cereus, Shigella spp. and the Vibrio spp. The major piscine pathogens of concern were Photobacterium damsela, Vibrio spp., and Aeromonas hydrophila. The most significant variation in biofilm pathogens was observed between facilities and not construction material.

This study implicates biofilms as another reservoir for pathogenic bacteria in recirculating aquaculture systems. More research is necessary to determine the potential for disease and how to decrease the number of pathogens in the biofilm below the infective dose.

Keywords: Aquaculture, bacteria, biofilms, pathogens, piscine, reservoir.

Introduction

As one of the most rapidly growing segments of agriculture, recirculating aquaculture offers a great potential for providing aquatic products to consumers. It requires limited resources, because large populations of fish are raised in a small area. Recirculating aquaculture is independent of environmental conditions because most facilities are indoors and the fishes' environment is constant despite the weather.

Biofilms form at the water/solid interface of all components of aquaculture systems. They are also found in streams, on medical implants, and cause plaque on teeth (Costerton et al. 1987; Wilderer and Characklis 1989; Geesey et al. 1992). Bacterial biofilms are used in wastewater treatment plants in some countries to aid in cleaning the water. Some bacteria found in aquaculture system biofilms are essential in removal of metabolic toxins harmful to fish, such as ammonia and nitrites.

Studies have shown that bacteria in biofilms can alter cellular functions as an adaptive response to adverse environmental conditions, such as sublethal antimicrobial treatments or poor nutritional levels (Brown and Gilbert 1993; Sasahara and Zottola 1993; Yu and McFeters 1994; Smoot and Pierson 1998a, 1998b; Kerr et al. 1999; Stoodley et al. 1999). The differences between sessile biofilm cells and their planktonic

counterparts include different cellular enzymatic activity, cell wall composition, and surface structures (Smoot and Pierson 1998a, 1998b; Wong 1998). Biofilm bacterial cells have been found to be resistant to antimicrobials including antibiotics, surfactants (or detergents), heavy metals, phagocytic predators and drying (Brown and Gilbert 1993; Ronner and Wong 1993; Yu and McFeters 1994; Liltved and Landfald 1995).

The presence of pathogenic bacteria in a recirculating aquaculture system can make the system a potential unacceptable public health risk. The periodic sloughing of pathogens may also cause recurring disease in stressed fish or can lead to the presence of infected asymptomatic fish being consumed. Another, perhaps more hazardous occurrence, would be the possibility of more pathogens with increasing antibiotic resistance if chemotherapeutics were employed to treat symptomatic fish.

The primary objective of this study was to determine the presence of pathogenic bacteria, both piscine and human, in biofilms of recirculating aquaculture systems. It was also determined if a correlation existed between bacterial presence and the type of material used in system construction.

Materials and Methods

Nine different recirculating facilities were tested. Two were mariculture or saltwater facilities raising summer flounder (Paralichthys dentatus). Six were freshwater facilities, all raising tilapia (Oreochromus spp.), and one was a hydroponics facility with tilapia and various leafy vegetables. Samples were obtained from nine different materials that included fiberglass, plastic, PVC, glass, stainless steel, rubber, aluminum, foam and cement.

The biofilm was sampled by swabbing surfaces just above water level. Sampling inside pipes was accomplished when the water flow was temporarily stopped. Tank, pipe and biofilters surfaces were the primary structures sampled. Samples were streaked on tryptic soy agar (TSA) (Becton Dickinson Microbiology Systems, Becton Dickinson Company, Sparks, MD 21152), blood agar (Difco Laboratories, Detroit, MI 48232) with 5% defibrinated sheep blood (Becton Dickinson Microbiology Systems, Becton Dickinson Company, Sparks, MD 21152), and, if the sample was taken at a saltwater facility, marine agar (Difco Laboratories, Detroit, MI 48232). Incubation was at 35^{o}C for 24 hours. Individual colonies differing by morphology were again streaked on TSA and incubated another 24 hours at 35^{o}C. After the second incubation, the

organisms were Gram stained to determine which Becton-Dickinson ID kit (Becton Dickinson Microbiology Systems, Becton Dickinson Company, Cockeysville, MD 21030) to use - the Gram Negative Non-fermenting test kit or the Gram Positive test kit. These were incubated another 18 - 24 hours at 35^{o}C.

Data was evaluated using an unbalanced random block statistical model using the computerized SAS System (SAS Institute, Inc., Cary, NC) to test whether the type of material, the type of facility, and the history of piscine disease influenced pathogen growth.

Results and Discussion

Table 1 lists the species of piscine and human bacterial pathogens identified in this study. They were all ubiquitous to an aquatic environment. Vibrio species include V. parahaemolyticus and V. vulnificus. When the test indicated either one or two Vibrio species and one or two Aeromonas species, the organism was labeled Vibrio-like species.

The most significant human pathogens found were Bacillus cereus, Shigella species and Vibrio species. All of these can cause severe gastrointestinal disease. Bacillus cereus is becoming more of a concern in

the food industry because it is a spore-forming organism and is very hardy, capable of surviving harsh conditions including high temperatures. Shigella spp. cause dysentery and are generally associated with poor sanitation. Vibrio cholera is found in water and can persist in shellfish and plankton beds that have been contaminated with polluted effluent. Vibrio parahaemolyticus can also cause GI distress when undercooked or raw seafood has been consumed. Vibrio vulnificus is associated with wound infections and septicemia in humans, though usually only in immunocompromised patients.

Photobacterium damsela, previously known as Pasteurella piscicida, causes a pseudotuberculosis type of disease in marine fish. It is interesting to note that in a study by Toranzo et al. (1982), this particular bacteria did not survive more than 4-5 days in estuarine water, so it was concluded that the bacterial reservoir was carrier fish. However, in this study, the organism was isolated from biofilms in the marine systems, indicating persistence in the environment, thereby creating another reservoir.

Vibriosis also occurs in fish and is most common in marine fish. It can also be present in freshwater fish (Roberts 1989), and Vibrio species were isolated from one freshwater facility. Disease outbreaks vary with

temperature, strain virulence and the amount of environmental stress present. Aeromonas hydrophila can be a significant fish pathogen, but it is generally considered opportunistic. The other bacteria isolated were opportunistic human and piscine pathogens that are not commonly found as primary etiological agents.

Many of the organisms isolated from the biofilms could not be definitively identified. One of the shortcomings of the rapid test kits is that they are designed to identify specific human pathogens. If the bacteria are strictly fish pathogens or if they are normal, non-pathogenic flora, the kits may not identify them, or could improperly identify them. This raises the possibility of the presence of other pathogens that were unidentifiable with these methods.

When comparing the types of material, the p-value was well above the 5% significance level, indicating the type of material was not a significant factor in whether pathogenic bacteria were present. A similar response was obtained when comparing the history of disease. However, when comparing facilities, the p-value was 0.0027, showing a significant difference in pathogen growth between facilities.

Conclusion

Many piscine and human pathogenic bacteria are present in biofilms of recirculating aquaculture systems. Most of these are opportunistic and ubiquitous in aquatic environments. If bacteria alter their chemical functions to survive as sessile organisms, they may not respond to the chemical tests in a predictable manner. This, as well as test shortcomings, may cause under-representation of the numbers and types of bacteria.

The potential for food-borne illness resulting from consumption of fish raised in recirculating systems is unclear, but with the increased consumption of undercooked fish and sushimi and the increased population of immunocompromised persons, there is a potential risk. More importantly, perhaps, is the possibility of cross-contamination between incoming and processed product in processing plants.

Raising fish in recirculating aquaculture systems exposes them to high density populations and other stressors. The presence of pathogenic bacteria in biofilms in these systems increases the possibility of recurring disease, with resultant economic losses to the aquaculturist.

In this study, bacteria grew on all surfaces tested. When using multiple comparisons to determine which facilities showed a significant

amount of pathogen growth, it did not matter whether the facility was a mariculture or freshwater facility. It could be hypothesized that the differences between facilities was due to different husbandry and biosecurity techniques, but more research would need to be done to verify this.

The next step for research in this area would be to determine the most effective methods to decrease pathogen presence in biofilms. The aquaculture industry is heavily regulated with regards to the use of antibiotics and chemicals in the water. The proliferation of antibiotic resistant organisms is of great concern because the antibiotics currently legally available are becoming increasingly ineffective. The environmental effect of antimicrobial treatment of wastewater must also be considered. Developing methods of pathogen elimination will require a better understanding of the viability of pathogens in biofilms, as well as a better knowledge of the sensitivity of sessile versus planktonic organisms to antimicrobials, including sanitizers and disinfectants.

Developing biosecurity measures in the aquaculture industry similar to those in the poultry industry will be essential to the future success of those facilities. Keeping facilities pathogen-free is an impossible task, but reducing levels of pathogens to below infective levels,

should decrease the chance of fish becoming clinically infected. Thus, for economic and public health reasons, the aquaculturist should strive for this goal.

Acknowledgement

The authors would like to thank the Virginia Sea Grant College Program and the Commercial Fish and Shellfish Technologies Program of the Virginia Polytechnic Institute and State University for their financial support. We would also like to thank the commercial aquaculture facilities that participated in this study.

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Table 1. List of pathogenic bacteria identified in commercial aquaculture facilities with the number of facilities from which each pathogen was isolated and materials on which they were growing. (H) represents human pathogens, (F) represents piscine pathogens.

Bacteria		**No. of Facilities**	**Different Materials**
Acinetobacter spp.	H	4	F, A, P
Aeromonas hydrophila	F/H	7	F, A, P, Pl, R, C, S
Aeromonas spp.	F/H	5	F, A, P, Pl, R, C, S
Bacillus cereus	H	3	F, P, Pl, C
Bacillus spp.	F/H	1	F, A
Citrobacter freundii	F/H	4	F, P, Pl, S
Enterobacter cloacae	H	2	F, A, Pl, Fo
Enterococcus spp.	H	2	F, P, Pl
Escherichia coli	H	3	F, P, R
Photobacterium damsela	F/H	2	F, Pl
Plesiomonas shigelloides	F/H	2	P, Pl
Serratia marcescens	H	2	F, P, R, C
Shewanella putrefaciens	F/H	3	F, A, P, Pl
Shigella spp.	H	1	P
Sphingobacterium multivorum	H	2	F, C
Sphingomonas spp.	H	3	F, A, P, Pl
Vibrio cholerae	H	2	F, R
Vibrio spp.	F/H	3	A, P, C
Vibrio-like spp.	F/H	4	F, A, R, C

F-Fiberglass A-Aluminum P-PVC
Pl-Plastic R-Rubber C-Cement
S-Stainless steel Fo-Foam

Table 2. Number of pathogenic bacteria found in each facility by substrate, and the history of disease of the facility. N=No history of disease.

Materials	**Facilities**								
	1	**2[a]**	**3**	**4**	**5**	**6**	**7[a]**	**8**	**9**
Fiberglass	-	10	7	-	8	4	7	16	-
Plastic	2	7	2	1	4	-	-	2	3
PVC	3	6	2	6	11	2	1	-	3
Aluminum	-	-	-	-	-	-	-	16	-
Rubber	-	-	-	4	-	2	-	-	3
Cement	-	-	-	6	-	-	2	-	-
Stainless Steel	-	-	-	-	-	3	-	-	-
Foam	1	-	-	-	-	-	-	-	-
History of Disease	N	N	N	Strepti-coccosis	N	N	Vibriosis	S. iniae A. hydrophila M. marinum	Bacterial growth on scales

[a]Mariculture facilities

THE USE OF GREEN FLUORESCENT PROTEIN E. COLI TO MONITOR PATHOGEN INCORPORATION INTO BIOFILMS OF RECIRCULATING AQUACULTURE SYSTEMS.[2]

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Abstract

With the decreasing numbers of wild fisheries, aquaculture provides increasing aquatic products to the consumer. Recirculating aquaculture offers a good potential for successful fish farming because it requires limited resources, and is independent of environmental conditions. However, this form of aquaculture may present a potential unacceptable public health risk. With growing concerns of increased antibiotic resistant organisms, controlling pathogenic microorganisms is paramount. Biofilms form on all aquaculture components, incorporating microflora present in the water. Pathogenic microorganisms can be found in this biofilm, causing recurring exposure to disease agents. The project objective was to increase the understanding of pathogen incorporation into biofilms in recirculating aquaculture systems by determining the relationship of the various materials used in construction of those systems and the presence of a surrogate pathogen, E. coli, modified to express a green fluorescent protein (GFP E. coli).

Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, glass, fiberglass, and stainless steel disks, were placed in six 79.2 liter (20 gallon) aquariums stocked with one Nile tilapia (Oreochromus niloticus) each. The disks remained suspended for one year prior to testing. The

tanks were inoculated with a known amount of GFP E. coli and samples were taken on days one, three, seven and fifteen post-inoculation. Within 24 hours post-inoculation, a mean log count of approximately 2.0 was observed growing on all materials. The growth decreased by day three and remained consistent during the remaining period. There was no significant difference between the different materials studied. The modified bacteria grew in equivalent amounts on all substrate surfaces.

This study demonstrated that a surrogate pathogen can become incorporated into recirculating aquaculture system biofilms and thus there is a potential for pathogen colonization of biofilms in commercial aquaculture systems. As a result, methods need to be identified that decrease the pathogen load in biofilms, leading to the establishment of effective biosecurity practices.

Keywords: Aquaculture, bacteria, biofilms, E. coli, green fluorescent protein, pathogens.

Introduction

With the decreasing numbers of wild fisheries (Pauly et al. 2000), the demand for seafood will need to be filled, and aquaculture enterprises will help meet that demand. Recirculating aquaculture systems reuse water, making them environmentally friendly because there is less waste produced, less water used, and the systems require less space. A disadvantage of recirculating systems is if a pathogenic organism is introduced into the system, it may survive in the system indefinitely. This can lead to economic losses for the facility, the possibility of asymptomatic fish being ingested by humans, or cross-contamination of foods during processing.

Biofilms are common in nature and grow at the water/solid interface in most all biological systems. They are found on medical implants, on surfaces in streams, and lead to plaque on teeth (Costerton et al. 1987; Wilderer and Characklis 1989; Geesey et al. 1992). Biofilms are responsible for the deterioration of ship hulls and underwater building supports (Blenkinsopp and Costerton 1991; Geesey et al. 1992). Research has also investigated the presence of biofilms formed by Listeria monocytogenes on food preparation surfaces (Smoot and Pierson 1998a).

Biofilm formation is a response by microorganisms to alterations in growth rate, exposure to subinhibitory concentrations of certain antibiotics, and growth on solid surfaces (Brown and Gilbert 1993; Sasahara and Zottola 1993; Yu and McFeters 1994; Smoot and Pierson 1998a, 1998b; Kerr et al. 1999). The main advantage of biofilm formation is that the biofilms provide protection from the effects of an adverse environment and host immune defenses. The multispecies microbial culture can provide and maintain the appropriate physical and chemical environments for growth and survival. In many cases the organisms in the biofilm develop a resistance to antimicrobials including surfactants, heavy metals, antibiotics, phagocytic predators and drying (Brown and Gilbert 1993; Ronner and Wong 1993; Yu and McFeters 1994; Costerton 1995; Liltved and Landfald 1995; Watnick et al. 1999).

There are many organisms in the aquatic environment affecting the health of aquaculture-raised fish that can lead to disease in humans. Most are opportunistic fish and human pathogens, living freely in the environment and only causing disease if the individual is immunocompromised or if environmental conditions are sufficient. A few of these organisms are obligate pathogens. Obligate pathogens usually do

not remain viable in the environment for long periods, though it is unknown if pathogens survive longer in biofilms.

The indicator group of bacteria for determination of fecal contamination is limited to the fecal coliform group, which includes Escherichia spp., Klebsiella spp., and Enterobacter spp. (Hitchins et al. 1992). Because some strains of E. coli are pathogenic and Klebsiella spp. are more ubiquitous than previously believed, some people feel E. coli should again be made the indicator organism for fecal contamination (Hitchins et al. 1992). The Environmental Protection Agency in its National Primary Drinking Water Regulations (1999) requires public water systems to test for total coliforms, with further testing for fecal coliforms or E. coli if the total coliform test is positive.

Green fluorescent protein (GFP) is a light emitting cytoplasmic protein originally isolated from the jellyfish, Aequorea aequorea (Shimomura et al. 1962), with the cDNA of GFP cloned to develop a plasmid used to produce GFP as a marker for detecting bacterial growth and viability (Prasher et al. 1992). GFP fluorescence is stable, independent of bacterial species used, can be monitored non-invasively in living cells with ultraviolet irradiation, requires no cofactors or substrates for activity, and persists in fixed cells (Chalfie et al. 1994; Anonymous

1998). The modification of cells to express GFP appears to be non-toxic toward cells and does not seem to alter the cells virulence and other cellular functions (Chalfie et al. 1994). When using GFP modified Edwardsiella tarda to study invasion pathways in fish, Ling et al. (2000) found that transformed cells had similar LD_{50} values and affected host cells the same as untransformed strains.

When conditions in an aquaculture system are right, planktonic cells are released from biofilms. If the fish are in a stressed condition, a disease outbreak may occur. The purpose of this research was to determine the potential survival and growth of pathogenic bacteria in biofilms on different substances in laboratory conditions, using antibiotic-resistant bacteria expressing the green-fluorescent protein.

Materials and Methods

Four millimeter thick disks were obtained from 39 mm wide rods of Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, and fiberglass. Glass disks with the same measurements were machine made and stainless steel was represented by the use of 39 mm washers. A 6 mm hole was drilled in each material. Twenty-four sets of disks were then autoclaved at 121^{o}C for 30 minutes. Four sets of disks were suspended in

six 79.2 liter (20 gallon) aquariums with an average water temperature of 24.8±0.6^{o}C. Each tank was stocked with one Nile tilapia (Oreochromus niloticus). The disks remained suspended for 1 year before testing.

The challenge strain of E. coli, ATCC 25922, represented a surrogate pathogen and was used because E. coli and coliform bacteria are most often used as indicators of contaminated water (Hitchins et al. 1992). The Clontech plasmid pGFP (Clontech Laboratories, Inc., Palo Alto, CA) was used to transform the model pathogen and a known amount (10.0 log CFU/ml) was placed in the aquariums. One set of disks was removed from each tank and sampled on days one, three, seven and fifteen post-inoculation.

Each disk was placed in 25 ml of sterile water in a sterile sampling bag and stomached (Stomacher 400 Lab Blender, Seward Medical, London, England) for two minutes at medium speed (Gagnon and Slawson 1999). The samples were spread on plates of Luria Broth Agar (20 mg Luria broth [Difco, Becton Dickinson Microbiology Systems, Becton Dickinson Company, Sparks, MD], 15 mg agar, 1 ml 1N NaOH/L) with 50 mg/L ampicillin and 560 µl/L 420 mM isopropyl-β-D-thiogalactoside (IPTG) to enhance production of fluorescent green protein. After

incubation at 35^{o}C for 24 hours, the plates were examined under long-wave ultraviolet light to count green fluorescent colonies.

Statistical analysis was performed using the computerized SAS System (SAS Institute, Inc., Cary, NC). A randomized complete block design was used, with a two-way factorial treatment structure. There were six blocks in this study consisting of each tank used. The treatments were the day of sampling and the material composing each disk. The significance level was p=0.05, or 5%.

Results and Discussion

The mean log count of green fluorescent protein E. coli for each substrate over the 15 day period tested is shown in Figure 1. The mean values for each substrate from all six tanks were plotted together. Examination of the chart shows that there was only approximately a 0.5 log difference between the substance with the lowest and the highest numbers of GFP E. coli. There was about a 2 log count/cm^2 on each test disk within 24 hours post-inoculation, which was significant when compared to the number of the surrogate pathogen on the other days. On day 3 all substances showed decreased numbers to about 1 log count/cm^2, and over the remainder of the period these numbers remained steady on all

materials but the cPVC. Rubber consistently had the highest numbers, except on day 7 when the count on both types of PVC increased. The microbial count on glass was consistently the lowest. The presence of GFP E. coli on each disk was found to be independent of the type of material ($p>0.05$) used as substrate, with equivalent growth on all substrates.

There are conflicting reports concerning the effect of substrate on biofilm growth. In a study performed by Kerr et al. (1999) comparing the growth of heterotrophic bacteria on different pipe material, more bacterial biofilms formed on pipes composed of cast iron than on plastic pipe materials such as unplasticised polyvinyl chloride or medium density polyethylene. There was no significant difference in biofilm growth on the plastics. Krysinski et al. (1992) found that the type of surface (stainless steel, polyester, or polyester backed with polyurethane) had little effect on the rate of cell attachment of Listeria monocytogenes. Smoot and Pierson (1998) found L. monocytogenes attached to Buna-N rubber and stainless steel within short contact times at all temperatures and pH levels tested. However, Ronner and Wong (1993) found there was a lag in the growth rate of a number of microorganisms including E. coli O157:H7 on Buna-N rubber compared to stainless steel, leading to the conclusion

that the rubber exhibited a bacteriostatic effect. After 48 hours however, the recovery of biofilm bacteria from the rubber was equivalent to the number of organisms recovered from the stainless steel.

There was a substantial amount of variation in the amount of pathogen recovered between tanks (Figure 2). This fluctuation was most probably due to the variable environment in each tank. Though each system was treated identically, there were slight differences. There was a variable temperature between tanks with the average water temperature 24.8^{o}C and a range of 1.2^{o}C. Temperature is considered the most important rate controlling parameter for biofilm accumulation (LeChevallier et al. 1990). In this study, the average temperature difference between tanks was not more than 1.2^{o}C, so it seems unlikely this difference influenced the growth of the E. coli in the biofilm.

The amount of organic material present in each aquarium was inconsistent. In some tanks the water became very cloudy due to excess food and there was more fungal growth on all surfaces. In the cleaner tanks, the fish ate all of the food and kept algae and fungal growth to a minimum, though the growth of fungus and algae was not measured definitively. De Franca and Lutterbach (1996) observed that fungal cells in multiorganism biofilms reached a constant number even though

bacterial numbers fluctuated. Another study determined that dominant heterotrophs in the biofilms changed over time reflecting continuously changing bacterial populations because of changes in environmental parameters and competition with new biofilm members (Kerr et al. 1999). The presence of the fish added to the environmental parameters of the system. The amount of surrogate pathogen present in the biofilms within each tank was almost certainly dictated by how much competition between microorganisms was present.

Conclusions

The use of green fluorescent protein modified E. coli as a surrogate pathogen for monitoring bacterial incorporation into biofilms of recirculating aquaculture systems is effective. Skillman et al. (1998) found that the bacterial cells would lose plasmid when there was no ampicillin present. In this study there appeared to be no loss of the plasmid. Even though the presence of the GFP E. coli decreased after 24 hours, the numbers of bacteria on the test disks remained constant over the remaining time. It is difficult to know how long the bacteria might remain in the biofilm. The presence of dominating heterotrophic bacterial

colonies changes and the amount of the GFP E. coli could also be affected by these trends.

The results of this study demonstrate that E. coli can become incorporated into recirculating aquaculture system biofilms with the possibility of forming another reservoir for bacterial infections. It is felt that E. coli is not as reliable an indicator of fecal pollution when testing seafood from cold water because of a rapid decline of the bacteria's presence in low temperatures (Nickelson and Finne 1992). In this study, the E. coli could be isolated from the biofilms possibly because the water temperature encouraged growth in biofilms. More research should be done investigating the avenues available to pathogenic bacteria incorporated in recirculating aquaculture system biofilms in causing recurring infections and possible contamination of food obtained from these systems.

Acknowledgement

The authors would like to thank the Virginia Sea Grant College Program, the Commercial Fish and Shellfish Technologies Program of the Virginia Polytechnic Institute and State University, and the National Fisheries Institute, Inc., Arlington, VA, for their financial support. We

would also like to thank John Chandler of the Department of Food Science and Technology for his assistance in machining the disks for this project. We also give our sincere appreciation to Tatiana Lorca from the same department for the use of her GFP E. coli isolate.

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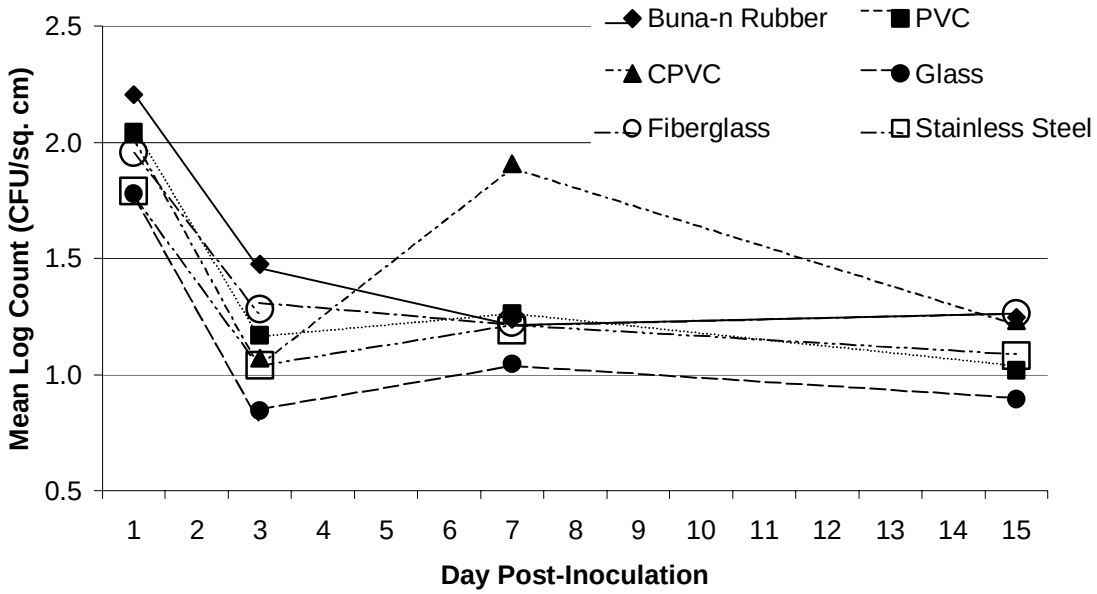
Figure 1 – Percent reduction of various sanitizers on biofilms of recirculating aquaculture systems. TPC-Total Plate Count, EB-Enterobacteriaceae count, GFP-GFP E. coli. Error bars were omitted due to extreme overlapping making the graph less understandable.



THE RESPONSE OF BACTERIAL BIOFILMS IN RECIRCULATING AQUACULTURE SYSTEMS TO VARIOUS SANITIZERS[3]

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Abstract

With the decreasing numbers of wild fisheries, aquaculture provides increasing aquatic products to the consumer. Recirculating aquaculture offers a good potential for successful fish farming because it requires limited resources, and is independent of environmental conditions. However, this form of aquaculture may present a potential for pathogenic microorganisms to become established in the system through the formation of biofilms. Biofilms are capable of forming on all aquaculture components, incorporating the various microflora present in the water. Pathogenic microorganisms released from the biofilms are capable of causing recurring diseases. The project objective was to determine the effectiveness of various sanitizers in eliminating biofilms.

Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, glass, fiberglass, and stainless steel disks were placed in six 79.2 liter (20 gallon) aquariums stocked with one Nile tilapia (Oreochromus niloticus) each. The disks remained suspended for one year prior to testing. The tanks were inoculated with a known amount of the surrogate pathogen, GFP E. coli, and after 24 hours one set of disks was removed from each tank. A second set of disks was treated by spraying with water, alkaline cleanser,

sodium hypochlorite, quaternary ammonium compound, or peracetic acid. Ozone was bubbled directly into one tank to treat another set of disks.

The type of material had no significant affect on the effectiveness of the sanitizers. Sodium hypochlorite and peracetic acid were the most effective sanitizers, with an overall percent reduction of approximately 99%. Ozone was the least effective. The quaternary ammonium compound demonstrated a moderate effectiveness. Water demonstrated a 99% reduction of the total plate count, suggesting that some form of mechanical cleaning was achieved. Keywords: Aquaculture, bacteria, biofilms, E. coli, green fluorescent protein, pathogens, sanitizers.

Introduction

With the decreasing numbers of wild fisheries (Pauly et al. 2000), the demand for seafood will need to be filled, and aquaculture will help meet that demand. Recirculating aquaculture systems reuse water, making them environmentally friendly because there is less waste produced, less water used, and the systems use less space. A disadvantage of recirculating systems is that if a pathogenic organism is introduced into the system, it may survive in the system indefinitely. This can lead to tremendous economic losses for the facility, the possibility of asymptomatic fish being ingested by humans, or cross-contamination of foods during processing.

Biofilms are common in nature and grow at the water/solid interface in most all biological systems. They are found on medical implants, on surfaces in streams, and lead to plaque on teeth (Costerton et al. 1987; Wilderer and Characklis 1989; Geesey et al. 1992). Biofilms are responsible for the deterioration of ship hulls and underwater building supports (Blenkinsopp and Costerton 1991; Geesey et al. 1992). Research has also investigated the presence of biofilms formed by Listeria monocytogenes on food preparation surfaces (Smoot and Pierson 1998a).

Biofilm formation is a response by microorganisms to alterations in growth rate, exposure to subinhibitory concentrations of certain antibiotics, and growth on solid surfaces (Brown and Gilbert 1993; Sasahara and Zottola 1993; Yu and McFeters 1994; Smoot and Pierson 1998a, 1998b; Kerr et al. 1999). The main advantage of biofilm formation is that biofilms provide protection from the effects of an adverse environment and host immune defenses. The multispecies microorganism culture can provide and maintain the appropriate physical and chemical environments for growth and survival. In many cases, the organisms in the biofilm develop a resistance to antimicrobials including surfactants, heavy metals, antibiotics, phagocytic predators and drying (Brown and Gilbert 1993; Ronner and Wong 1993; Yu and McFeters 1994; Costerton 1995; Liltved and Landfald 1995; Watnick et al. 1999).

There are many organisms in the aquatic environment affecting the health of aquaculture-raised fish that can lead to disease in humans. Most are opportunistic pathogens, living freely in the environment and only causing disease if the individual is immunocompromised or if environmental conditions are sufficient. A few of these organisms are obligate pathogens. Obligate pathogens usually do not remain viable in

the environment for long periods, though it is unknown if pathogens survive longer in biofilms.

When conditions in an aquaculture system are optimum, planktonic cells are released from biofilms. If the fish are in a stressed condition, a disease outbreak may occur. The overall purpose of this research was to determine the potential survival of pathogenic bacteria in biofilms on different substances in laboratory conditions after treatment with various sanitizers. This research project meets the demands for data pertaining to the farm-fish industry with regards to good agricultural practices leading to prevention of recurring disease in aquaculture systems by identifying sanitizers that might decrease or eliminate pathogenic bacteria from biofilms.

Materials and Methods

Pre-treatment Samples

Four-millimeter thick disks were sawn off 39 mm wide cylindrical rods of Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, and fiberglass. Glass disks with the same measurements were machine made and stainless steel was represented by the use of 39 mm washers. A 6 mm hole was drilled in each material. The disks were then autoclaved at

121°C for 30 minutes before being suspended in 79.2 liter (20 gallon) aquariums by using plastic strips tied to aluminum rods. Each tank had an average water temperature of 24.8±0.6°C and was stocked with one Nile tilapia (Oreochromus niloticus). The disks remained suspended for 1 year before testing was initiated.

Challenge strains of E. coli were used in laboratory testing. This microorganism was used because E. coli and coliform bacteria are most often used as indicators of contaminated water (Hitchins et al. 1992). The bacteria were transformed to develop a plasmid used to produce GFP as a marker for detecting bacterial growth and viability (Prasher et al. 1992). GFP fluorescence is stable; is independent of bacterial species used; can be monitored non-invasively in living cells, and persists in fixed cells (Chalfie et al. 1994; Anonymous 1998). The modification of cells with GFP appears to be non-toxic to the cells and does not seem to alter the cells virulence and other cellular functions including growth (Chalfie et al. 1994). All these factors contribute to make microorganisms transformed to express green fluorescent protein effective for monitoring bacterial presence and growth.

The Clontech plasmid pGFP (Clontech Laboratories, Inc., Palo Alto, CA) was used to transform the surrogate pathogen, E. coli ATCC

25922, and a known amount (9.95 log CFU/ml) was placed in three tanks. One set of disks was removed and sampled 24 hours post-inoculation. The disks were stomached (Stomacher 400 Lab Blender, Seward Medical, London, England) for two minutes at medium speed in 25 ml of sterile water to remove the biofilm (Gagnon and Slawson 1999). The samples were spread on plates of Luria Broth Agar (20 mg Luria Broth [Difco, Becton Dickinson Microbiology Systems, Becton Dickinson Company, Sparks, MD], 15 mg agar, 1 ml 1N NaOH/L) with 50 mg/L ampicillin to improve selection and 560 µl/L 420 mM isopropyl-β-D-thiogalactoside (IPTG) to enhance production of fluorescent green protein. After incubation, the plates were examined under long-wave ultra-violet light to count fluorescent colonies. Aerobic Count Plate and Enterobacteriaceae Count Plate Petrifilm™ (3M Microbiology Products, St. Paul, MN) were also used for each sample to determine the total plate count and the presence of Enterobacteriaceae family bacteria.

Sanitizer Treatment

To determine if there was any mechanical removal of bacteria during sanitizer treatment, water was used as a control. Twenty-four

hours post-inoculation, another set of disks was removed from the same three tanks as above. The disks were sprayed with water at 5.3L/minute for 5 minutes using a sprayer (No-mix Liquid Hose End Sprayer Model 2101, HD Hudson Manufacturing Co., Hastings, MN) and garden hose. Water was obtained from the Town of Blacksburg municipal water supplies. Samples were then taken as described.

Because ozone is commonly used in aquaculture to clean and disinfect water, another set of disks was treated with ozone. A portable ozone generator (Activated Oxygen Generator, Golden buffalo, Orange, CA) was used to bubble ozone (0.9g/L) into tanks at a flow rate of 2.4L/minute for 5 minutes. Samples were then taken in the same manner as the pre-inoculation samples.

Various sanitizing treatments were used in this study. The chemical sanitizers were used in accordance to manufacturer's instructions, resulting in various concentrations and contact times. In the food and dairy industries, clean-in-place systems are often used. Generally, an alkaline cleanser is used first, followed by an acidic sanitizer. To test the effect of an alkaline cleanser on biofilm bacteria, a sodium carbonate alkaline cleanser (FastPac 425, Klenzade Division, Ecolab, Inc., St. Paul, MN) was mixed according to manufacturer

instructions of one packet weighing 13.88 grams per 15.1 liters (4 gallons) of water. Contact time was 2 minutes. Samples were placed in 25 ml of Universal Quenching Agent (containing [g/L distilled water] peptone 1, sodium thiosulphate 1, lecithin 0.7, Tween 80 5, and adjusted to pH 7) (Jones et al. 1992) before being stomached and sampled as the pre-inoculation samples.

Sodium hypochlorite is also used for sanitation treatments in the aquaculture industry. Another set of disks was treated with cleanser, and then the disks were sprayed with a 8.4% sodium hypochlorite solution (XY-12, Ecolab Food & Beverage Division, St. Paul, MN) at a concentration of 10 ml solution/gallon water (2.6 ml/L) for a contact time of 2 minutes, rinsed with water, then placed in the Universal Quenching Agent and treated as previously described.

Quaternary ammonium compounds (QAC) are not frequently used in the aquaculture industry, but these compounds are common in the food industry. After treatment with cleanser as explained, another set of disks was sprayed with a 10% solution of n-alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride (Ster-bac, Ecolab Food & Beverage Division, St. Paul, MN) at a final concentration of 10 ml solution/gallon of

water (2.6 ml/L) and a 1 minute contact time. The disks were rinsed with water, then placed in Universal Quenching Agent and treated as described.

Another sanitizing compound used in the food industry is peracetic acid. This compound has not been approved for use in the aquaculture industry, but it is very effective as a sanitizer, so its effectiveness against biofilm bacteria was tested in this study. A 4.4% peroxyacetic acid solution, with 6.9% hydrogen peroxide and 3.3% octanoic acid (Vortexx, Ecolab Food & Beverage Division, St. Paul, MN) was sprayed on the disks for 5 minutes at a final concentration of 3 ml of solution/gallon of water (0.8 ml/L). These disks were not rinsed with water before being placed immediately in the Universal Quenching Agent. They were then treated as the other sanitizer samples.

Statistical Analysis

Statistical analysis was performed using the computerized SAS System (SAS Institute, Inc., Cary, NC). A randomized unbalanced block design was used, with a two-way factorial treatment structure. There were three blocks in this study consisting of each tank used. The treatments were the sanitizers and the material composing each disk. The significance level was p=0.05, or 5%.

Results and Discussion

The overall reductions of the total plate count on the Buna-N rubber was significantly different from the glass, with rubber showing the least amount of reduction and glass the most. However, there appeared to be no difference overall in how effective the sanitizers were in decreasing the total plate counts, Enterobacteriaceae counts and GFP E. coli counts based on the substrate.

When comparing the percent reduction of the various treatments (Figure 1) on the total plate count, all treatments except ozone demonstrated over 90% reduction. The peracetic acid (PAA) solution, at 99.9971% reduction showed the largest decrease. PAA was most effective on glass and stainless steel and least effective on Buna-N rubber. The sodium hypochlorite solution and water demonstrated greater than a 99% reduction, or a 2 – 3 log decrease. The QAC and cleanser performed equivalently with over a 90% reduction, equivalent to a 1-log decrease in microorganisms. Ozone showed a significant difference as the least effective of all the sanitizers on all substrates. The percent reduction never rose above 20 % and on PVC, cPVC, fiberglass and stainless steel there was no reduction at all.

In the reduction of Enterobacteriaceae, the peracetic acid and sodium hypochlorite solutions were significantly more effective from the other sanitizers. Members of the family Enterobacter were reduced by a 3 – 4 log reduction (99.9-99.99%) on all substances by both sanitizer treatments. All other treatments demonstrated less than 90% reduction. Water did not reduce the numbers of Enterobacteriaceae on glass, and ozone had a 0% reduction on PVC and fiberglass.

There was a lot of variation in the reduction of GFP E. coli between the sanitizers. Peracetic acid and sodium hypochlorite were the most effective in reducing GFP E. coli from all substrates. AS with the Enterobacteriaceae, all other treatments performed in an equivalent manner, at well below 90% reduction.

To reduce or eliminate microorganisms from surfaces, physical or chemical measures are used. Physical methods include heat treatment, high-pressure sprays and mechanical cleaning, such as with brushes (Bal'a et al. 1999). In an attempt to simulate sanitation practices in an aquaculture facility, a garden hose was used with a concentration sprayer. A high-pressure hose increases the possibility of aerosolizing bacteria as well as the chemical sanitizers being used. Mechanical cleaning with brushes will help to decrease the bacteria present, especially on rough

surfaces, but it can be time consuming, requiring substantial man-hours. Spraying with water alone decreased the total plate count, Enterobacteriaceae, and the surrogate pathogen on most of the substrates, indicating that using a sprayer has some mechanical effect in bacterial elimination. There could be some antimicrobial affects in the water as well, because municipal water supplies are often chlorinated.

Because the presence of organic material can decrease the efficacy of many sanitizers, sanitation procedures in the food industry are usually accompanied first by cleaning. "When treated with only a sanitizer, the chemical is inactivated by the deposited soil and becomes ineffective in reaching and destroying all of the microorganisms" (Zottola 1994). Richards (2000) found that when sanitizers alone were used on L. monocytogenes biofilms they were not as effective as when cleaning was performed prior to sanitizing. Biofilm bacteria, which attach to many surfaces in aquaculture systems including water storage tanks and water pipes, are not easily eliminated by sanitizers alone, emphasizing the need for physical removal of biofilms on tank surfaces with periodic drying to reduce the presence of pathogenic bacteria in the system (Karunasagar and Otta 1996).

Of the three oxidizing sanitizers used in this study, ozone was the least effective in eliminating all types of bacteria. Ozone (O_3) is a powerful oxidizing agent, reacting directly and indirectly with other compounds. The pathway of the ozone reaction depends on the properties and concentrations of the other compounds, as well as the quality of the water, including pH, bicarbonate level, the level of total organic carbon and temperature. Microorganisms are considered part of the total particulate matter load and when dissolved and particulate organic compounds are together, hydroxyl radicals react preferentially toward dissolved organic compounds (Lawson 1995). Thus, in recirculating aquaculture systems, where the levels of dissolved organic matter is often high due to large amounts of fish feed, nitrite production by the biofilters and the sloughing of suspended solids, maintaining adequate residual ozone levels for disinfection can be difficult (Lawson 1995; Summerfelt and Hochheimer 1997). In some systems the ozone demand can be so high that the ozone and its decomposition products may be depleted before microorganisms can be eliminated (Liltved and Landfald 1995; Summerfelt and Hochheimer 1997). In the aquarium systems used in this study, ozone was bubbled into the system where there were varying amounts of organic matter. The disks were also not cleaned prior to

sanitizing. These factors probably contributed to the relative ineffectiveness of ozone in decreasing the bacteria in the biofilm.

The other oxidizing sanitizers were sodium hypochlorite and peroxyacetic acid. The PAA was more effective in decreasing the aerobic bacteria of the total plate count, but both were equally effective in decreasing the Enterobacteriaceae and the GFP E. coli. Peracetic acid is a very strong oxidizing agent with a broad spectrum of antimicrobial activity (Cords and Dychdala 1993; Price 1995). Chlorine sanitizers are also broad-spectrum germicides (Cords and Dychdala 1993; Boothe 1998). Both PAA and sodium hypochlorite act on microorganisms by denaturing proteins in the cell walls, leading to alteration of the chemiosmotic function of the cell membrane and oxidation of the enzymes leading to impairment of cellular biochemical pathways (Cords and Dychdala 1993; Blanchard et al. 1998; Boothe 1998).

Quaternary ammonium compounds performed moderately. These compounds are considered surface-active cations that bind irreversibly to the negatively charged phospholipids and proteins of the cell membranes of microorganisms, impairing membrane permeability (Frank and Koffi 1990; Boothe 1998), therefore the presence of lipopolysaccharides and lipids in cell membranes are fundamental to penetration of hydrophobic

molecules such as QAC (Guerin-Mechin et al. 1999). It has been noted that there is a greater resistance to QAC exhibited among Gram-negative organisms (Cords and Dychdala 1993; Price 1995; Boothe 1998). In a study done by Guerin-Mechin et al. (1999), it was found that Pseudomonas aeruginosa, an indicator bacteria for the evaluation of bactericidal activity in Europe, developed a specific variation in cell membrane fatty acid composition in the presence of long-chain QAC, allowing resistance to develop. In an aquatic environment there would most likely be Gram-negative bacteria present, causing a possible decrease in the effectiveness of the sanitizing capability of QAC.

Another reason for the decreased effectiveness of QAC could be the presence of excess organic material. The literature cites conflicting reports about the effect of the presence of organic matter on the germicidal activity of QAC. Because QAC react with lipids and lipopolysaccharides, the presence of high amounts of lipids in inorganic material can inactivate quaternary ammonium compounds (Boothe 1998). A study done by Taylor et al. (1999) comparing disinfectants used in the food industry, showed that a QAC failed to achieve a five-log reduction in viable bacterial counts under dirty conditions. Another study testing the effects of a QAC on normal heterotrophic bacteria in the Rhine River showed the

presence of suspended matter reduced the germicidal activity of the QAC (Tubbing and Admiraal 1991).

Conclusion

This study shows that sodium hypochlorite and peracetic acid can reduce the numbers of pathogenic bacteria in biofilms of recirculating aquaculture systems. Quaternary ammonium compounds are not as effective. It may not be necessary to include cleaning with an alkaline cleanser prior to sanitizing, but the importance of some kind of mechanical cleaning has been demonstrated. These sanitizers may not be as effective when used independently.

Ozone was not effective in this study, but if its use is instituted in the initial stages of developing an aquaculture facility when there is less organic material present, the results may be significantly different. Occasional cleaning with brushes may still be necessary to assist in the effectiveness of the sanitizing process with ozone.

The complete decrease in total aerobic bacterial count in aquaculture systems is perhaps not such a desirable endpoint. Nitrifying bacteria are essential for elimination of toxins produced when waste decomposes in the systems. However, when considering the initiation of

any sanitation program, there is going to be the loss of some biofilter bacteria expected. Therefore it is essential to develop effective biosecurity measures to prevent the introduction of pathogens into the systems, so use of sanitizers and other antimicrobials can be kept to a minimum.

Another consideration is that the sanitizers tested in this study can be abrasive to various materials. When considering all factors necessary to institute a successful biosecurity plan, the types of materials used in construction of the commercial facilities are important.

Acknowledgement

The authors would like to thank the Virginia Sea Grant College Program, the Commercial Fish and Shellfish Technologies Program of the Virginia Polytechnic Institute and State University, and the National Fisheries Institute, Inc., Arlington, VA, for their financial support. We would also like to thank John Chandler of the Department of Food Science and Technology for his assistance in machining the disks for this project. We also give our sincere appreciation to Tatiana Lorca from the same department for the use of her GFP E. coli isolate.

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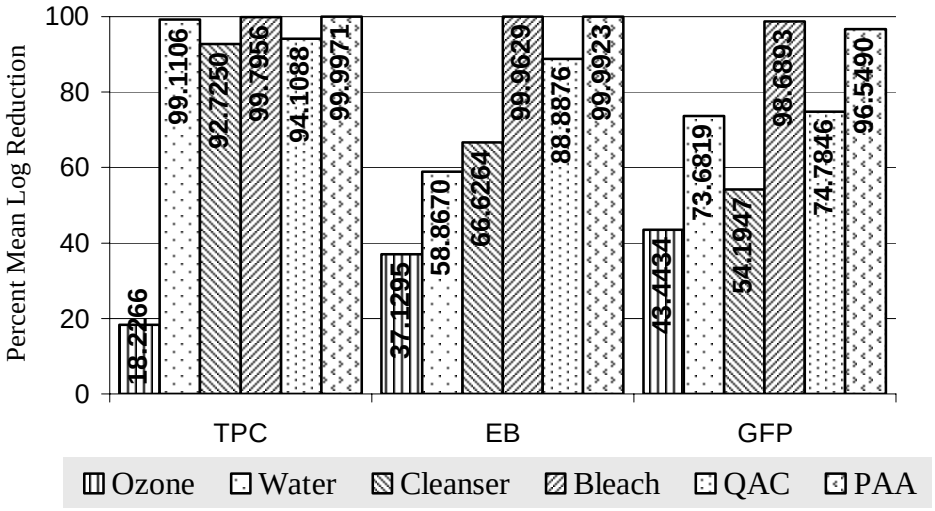
Figure 2 – Percent reduction of various sanitizers on biofilms of recirculating aquaculture systems. TPC-Total Plate Count, EB-Enterobacteriaceae count, GFP-GFP E. coli



THE USE OF POLYMERASE CHAIN REACTION TO DETECT *STREPTOCOCCUS AGALACTIAE* IN BIOFILMS OF RECIRCULATING AQUACULTURE SYSTEMS

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Abstract

Recirculating aquaculture offers a good potential for successful fish farming, but this form of aquaculture poses a latent disease and public health risk. Biofilms form on all components of a recirculating system. Pathogens introduced into the system could remain indefinitely by incorporation into the biofilm, leading to recurring exposure of fish to disease.

Biofilms are common in nature and grow at the water/solid interface in most biological systems forming highly complex structures. Much research has been carried out with single organism biofilms dealing with growth and response to disinfection. Multi-organism biofilms are a complex consortium of different types of microorganisms and more precise methods for determining the presence of pathogens within those biofilms has yet to be ascertained. A potential method for bacterial detection is the use of polymerase chain reaction (PCR), a technique for the *in vitro* amplification of specific segments of DNA. The primary objective of this study was to evaluate the use of PCR to detect the presence of *Streptococcus agalactiae* in biofilms of recirculating aquaculture systems in a laboratory setting.

One pair of synthetic 16S rRNA specific oligonucleotide primers targeting a 282-bp fragment of the 16S rRNA gene of *S. agalactiae* was designed. Two other specific primers targeting a 409-bp fragment of the capsular polysaccharide biosynthesis gene of *S. agalactiae* type Ia were also developed. Disks of Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, fiberglass, glass and stainless steel were obtained, autoclaved and suspended in seven 20 gallon (79 liter) aquariums. Each tank was stocked with one Nile tilapia (*Oreochromus niloticus*).

The positive control for the 16S rRNA primers was a series of dilutions of DNA extracted from pure cultures of *S. agalactiae.* One PVC disk was removed from a non-

inoculated control tank and the whole biofilm sample was used to determine whether the target microorganism was present. The six other tanks were inoculated with *S. agalactiae*. One disk of each material was taken from all seven tanks, including the non-inoculated control tank, and treated as previously described. To prepare wash and biofilm samples used with the capsular polysaccharide biosynthesis gene primers, samples were plated on Columbia Nutrient Agar (CNA) prior to amplification. DNA extraction was performed using Qiaprep Spin Miniprep Kit. Amplification with the 16S rRNA primers was accomplished with 45 total cycles. The amplification for the capsular primers used 35 cycles.

All samples analyzed using the 16S rRNA primers indicated positive results for the desired length of DNA including the assumed negative control. The capsular polysaccharide biosynthesis gene primers with biofilm and wash samples demonstrated the majority of positive results for amplicon, including samples from the control tank. The positive control, a pure culture of *S. agalactiae*, showed a strong positive response, and the negative control, distilled water, showed no response. There was the possibility that *S. agalactiae* was present in the tanks. Some of the positive samples were identified as *Staphylococcus epidermidis*. The few very bright bands in the gel, indicating strong positive responses, were identified as *S. agalactiae.*

These results reveal that more research needs to be done examining the use of species-specific primers when performing PCR on multiorganism biofilms. Other PCR techniques, such as multiplex PCR, reverse transcriptase PCR, and restriction fragment length polymorphisms (RFLP) should be studied.

Introduction

Biofilms are common in nature and grow at the water/solid interface in most biological systems. They are found on medical implants, on surfaces in streams, and lead to plaque on teeth (Costerton et al. 1987; Wilderer and Characklis 1989; Geesey et al. 1992). Biofilms are responsible for the deterioration of ship hulls and underwater building supports (Blenkinsopp and Costerton 1991; Geesey et al. 1992). Biofilm formation is a response by microorganisms to alterations in growth rate, exposure to subinhibitory concentrations of certain antibiotics, and growth on solid surfaces (Brown and Gilbert 1993; Sasahara and Zottola 1993; Yu and McFeters 1994; Smoot and Pierson 1998a, 1998b; Kerr et al. 1999). Multispecies biofilms form highly complex structures with cells arranged in clusters or layers with anastomosing water channels that bring nutrients to the lower layers and remove waste products (Costerton 1995). Biofilm bacteria live in glycocalyx-enclosed microcolonies whose location, size and shape are determined by nonrandom species-specific factors (Costerton 1995).

The major advantage of biofilm formation is that the biofilms provide protection from the effects of an adverse environment and host immune defenses. The multispecies culture can provide and maintain the appropriate physical and chemical environments for growth and survival. In many cases the organisms in the biofilm develop a resistance to antimicrobials including surfactants, heavy metals, antibiotics, phagocytic predators and drying (Brown and Gilbert 1993; Ronner and Wong 1993; Yu and McFeters 1994; Costerton 1995; Liltved and Landfald 1995; Watnick et al. 1999).

It is believed that attachment of bacteria to form a biofilm is dependent on the type of substrate. Kerr et al. (1999) found that heterotrophic bacteria grew faster on cast iron pipe material than they did on two plastic pipe materials. In other studies it was determined that

Listeria monocytogenes grew slower on Buna-N rubber surfaces than stainless steel with an increased resistance noted in the biofilm cells on the rubber (Smoot and Pierson 1998b; Wong 1998). The effectiveness of detergents and sanitizers is also dependent on the surface, especially when the surface is rough. In a study testing the ability of *Vibrio harveyi* to form biofilms, the biofilm cells were more resistant to treatment by sanitizers on concrete and high-density polyethylene than on stainless steel (Karunasagar and Otta 1996).

Recirculating aquaculture is one of the most intensive levels of fish production. Recirculating systems recondition and recycle almost all of the water delivered into the fish-rearing units, and can be semi-closed or closed, with almost 90 to 100% of the water being reused, respectively (Stickney 1993). Closed aquaculture systems have been proposed for production of fish in areas of low water availability or unfavorable water conditions for specific fish species, as well as to produce fish closer to their markets (Stickney 1993).

Despite the advantages, recirculating systems pose a latent disease and public health risk. Part of the biological filtration necessary for removal of harmful toxins involves the biofilm that forms on all components of a recirculating system. Because the water is reused, pathogens introduced into the system could remain indefinitely by incorporation into the biofilm, leading to recurring exposure of fish to disease. Studies by Karunasagar et al. (1994, 1996) showed antibiotic resistant *Vibrio harveyi* persisted in the larval tanks of a shrimp hatchery, most probably as biofilm bacteria and therefore not easily eliminated by sanitizer treatment. This could lead to consumption of infected fish or cross contamination during processing. Understanding how these pathogens persist in the biofilm and how they respond to antimicrobials would help the aquaculturist decrease the threat to public health.

There has been much research on biofilms dealing with growth and response to disinfection. Most of this research has been carried out with single organism biofilms. Multi-organism biofilms are a complex consortium of different types of microorganisms and more precise methods for determining the presence of pathogens within those biofilms has yet to be ascertained. A potential method for bacterial detection is the use of polymerase chain reaction (PCR). PCR is a technique for the *in vitro* amplification of specific segments of DNA (Hill 1996). A million-fold increase of a particular region of DNA can often be realized, allowing the sensitive detection of genes specific to taxonomic groups of bacteria, virulence genes of bacteria, as well as DNA from viruses and parasites (Hill 1996). A three-step cycling process requiring two synthetic oligonucleotide primers complementary to regions on opposite strands that flank the target DNA sequence, a thermostable DNA polymerase and the four deoxyribonucleotides is used (Glick and Pasternak 1994). The first step in amplification is denaturation, where the temperature is raised to about 95^{o}C enabling separation of the double stranded DNA, and production of the single stranded template (Glick and Pasternak 1994; Promega 1996). Annealing decreases the temperature in the reaction tube to between 40 – 60^{o}C allowing annealing of the primers to their complementary sequences of the template DNA (Glick and Pasternak 1994; Promega 1996). The final step of the cycle is the polymerization of DNA. The temperature is increased to about 75^{o}C, the optimum for the *Taq* DNA polymerase to attach the four deoxyribonucleotides to the complementary template strand of DNA (Glick and Pasternak 1994; Promega 1996). The reaction is placed in a thermal cycler, an automated instrument taking the reaction through the temperatures of the cycle for varying amounts of time (Promega 1996). Each PCR cycle theoretically doubles the amount of target DNA present, so ten cycles would theoretically multiply the amplified strand by a factor of one thousand (Promega 1996).

The primary objective of this study was to evaluate the feasibility of using PCR to detect the presence of *Streptococcus agalactiae* in biofilms of recirculating aquaculture systems in a laboratory setting.

Methods and Materials

Primer Design and Preparation

One pair of synthetic 16S rRNA specific oligonucleotide primers (RKF, S623R) targeting a 282-bp fragment of the 16S rRNA gene of *S. agalactiae* ATCC13813 was used in the PCR. The primers were designed by computer analysis using published sequences of the 16S rRNA gene of *Streptococcus* species (Genbank Accession #U02908)(Greisen et al. 1994). The specificities of the primers were confirmed by the Genbank database 'Blast' program (Altschul et al. 1997). The primers used in this study are given in Table 1. Two other specific primers (ScpsF, ScpsR) targeting a 409-bp fragment of the capsular polysaccharide biosynthesis gene of *S. agalactiae* type Ia (Genbank Accession #AB028896)(Yamamoto et al. 1999) were also designed and confirmed in the same manner.

Table 4-1. Sequences of oligonucleotide primers

Region[1]	Size[2]	Primer[3]	Sequence
341-366	282	RKF	5'-CCAGACTCCTACGGGAGGCAGCAGT-3'
623-598	282	S623R	5'-CAGCCTTTAACTTCAGACTTATCA-3'
165-188	409	ScpsF	5'-GCGCCGTCAACAAAAGAAACACTC-3'
568-546	409	ScpsR	5'-CTGGTGCGGCTAATTTGCTGACA-3'

[1] Positions of the first and last nucleic acids of the primer in the targeted fragment.
[2] Number of nucleotides in the amplified fragment.
[3] RKF-S623R and ScpsF-ScpsR are primer pairs.

Sample Preparation and DNA Extraction

Four-millimeter thick disks were obtained from 39 mm wide rods of Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, and fiberglass. Glass disks with the same measurements were machine made and stainless steel was represented by the use of 39 mm washers. A 6 mm hole was drilled in each material. The disks were then autoclaved at 121^{o}C for 30 minutes before being suspended in seven 79.2 liter (20 gallon) aquariums by using plastic strips tied to aluminum rods. Each tank had an average water temperature of 24.8±0.6^{o}C and was stocked with one Nile tilapia (*Oreochromus niloticus*). The disks remained suspended for 3 months before sampling.

The positive control for the 16S rRNA primers was a series of dilutions of DNA extracted from pure cultures of *S. agalactiae.* One PVC disk was removed from a non-inoculated control tank to use as a negative control. The disk was placed in a sterile sampling bag with 5 ml of 0.9% sterile peptone. The sample was agitated by hand so any loosely attached cells would detach from the biofilm. This wash was discarded. The disk was then removed from the bag aseptically, and the biofilm sample was formed by scraping the disk with a sterile scalpel blade and placing the material in 5 ml of 0.9% sterile peptone.

The six other tanks were inoculated with 10 ml of 1.5x10^{8} CFU/ml of *S. agalactiae*. At 24 hours post-inoculation, one disk of each material was taken from all seven tanks, including the non-inoculated control tank, and placed in individual sterile sampling bags with 5 ml of 0.9% sterile peptone. The samples were agitated by hand so any loosely attached cells would detach from the biofilm, forming the wash sample. The disks were then removed from the bags aseptically and treated as described forming the biofilm sample.

While using the 16S rRNA primers, the whole biofilm sample from the control tank was used to determine whether the target microorganism was present. The wash and biofilm samples used with the capsular polysaccharide biosynthesis gene primers were spread on tryptic soy agar (TSA) (Difco, Becton Dickinson Microbiology Systems, Sparks, MD) with 5% defibrinated sheep's blood, 10 mg colistin sulfate/L and 10 mg nalidixic acid/L as a selective media for *S. agalactiae* (Plumb 1999). The plates were then incubated for 24 hours at 30^{o}C. Typical *Streptococcus* spp. colonies were placed in Todd Hewitt broth (Difco, Becton Dickinson Microbiology Systems, Becton Dickinson and Company, Sparks, MD) and incubated for 24 hours at 30^{o}C. For extraction of DNA all samples were treated with the Qiaprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA).

DNA Amplification

The amplification reactions were performed using a DNA thermal cycler (M.J. Research, Inc., Watertown, MA) Model PTC-100, and Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NY) in 0.5 ml microcentrifuge tubes. The reaction mixture for sampling (25 µl total volume) consisted of 10 µl of sample DNA, 11 µl of sterile water, 2 µl of each primer RKF and S623R, and one PCR bead. The samples were subjected to 45 total cycles of amplification. Preincubation was at 94^{o}C for 2 minutes. Five cycles were run with denaturation at 94^{o}C for 1.5 minutes, primer annealing at 56^{o}C for 2 minutes, and DNA extension at 72^{o}C for 2 minutes. Another 40 cycles were run under the same conditions except the primer annealing temperature was 58^{o}C. After the last cycle, the PCR tubes were incubated for 5 minutes at 72^{o}C. Five microliters of the reaction mixture was analyzed in 1.5% agarose gel with 0.04 µl/ml of ethidium bromide for staining, enabling visualization. Gel electrophoresis

was run at 100 V/cm. Different concentrations of pure *S. agalactiae* culture were used, as well as biofilm sample from a non-inoculated fish tank for positive and negative controls respectively.

The amplification for the capsular primers was run in a similar manner as the 16S rRNA primers, except that 35 cycles were run. The reaction mixture for sampling (25 µl total volume) consisted of 5 µl of sample DNA, 16 µl of sterile water, 2 µl of each primer ScpsF and ScpsR, and one PCR bead. Preincubation was at 94^{o}C for 2 minutes. Denaturation took place for 30 seconds at 94^{o}C, the annealing step was at 65^{o}C for 15 seconds, and extension occurred at 72^{o}C for 30 seconds. After the last cycle, the PCR tubes were incubated for 5 minutes at 72^{o}C. Five microliters of the reaction mixture was analyzed in 1.5% agarose gel with 0.04 µl/ml of ethidium bromide for staining, enabling visualization. Gel electrophoresis was run at 100 V/cm.

Results and Discussion

Figure 4-1. Representation of the gel electrophoresis of the amplification of the 16S rRNA amplicon. Lane 1 is the 100 bp ladder with the brightest line represents 500 – 525 bp. Lanes 2 – 5 are decreasing dilutions of S. agalactiae DNA from pure cultures. Lane 6 is DNA from a biofilm sample.

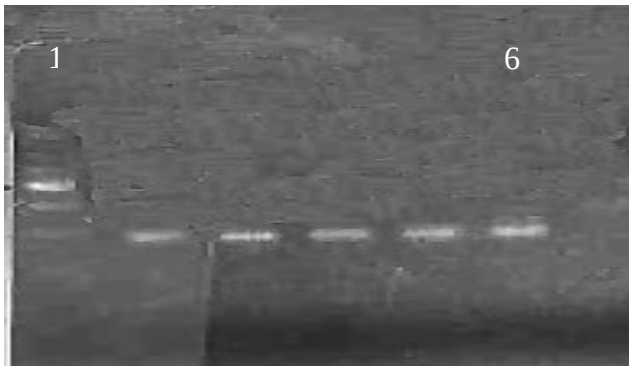

Figure 4-1 shows the agarose gel from the study using the 16S rRNA primers. Lane 1 is a 100-bp ladder used to determine the size of the amplified fragment. Lanes 2 through 6 are different concentrations of pure *S. agalactiae* culture and lane 6 is biofilm from the negative control tank. All five samples are positive for a DNA fragment approximately 300-bp long. It was expected the biofilm sample would be negative. It is possible *Streptococcus* spp. could have been brought into the tanks with the fish. There is also the

possibility of contamination of the equipment, though subsequent samples completed with the same equipment gave expected results.

Another possibility for the unexpected positive result is that unknown organisms in the multispecies biofilm included a DNA sequence that was similar or related to the target organism's DNA. Bäckman et al. (1999) found some Group C and G streptococci produced bands of an identical size to *S. agalactiae* using semi-nested PCR and amplifying a fragment of the 16S rRNA gene, leading to the conclusion that the streptococcal group of bacteria is conservative in critical sequences for the PCR assay of the 16S rRNA gene. Most applications of PCR in microbiology to date have concentrated on the identification of a single species from a pure culture, or differentiation of multiple organisms, all with known DNA sequences (Hall et al. 1995; Parrish and Greenberg 1995; Hill 1996; Khan and Cerniglia 1997; Bäckman et al. 1999). Primers have accordingly been chosen only with those species in mind. However, it has already been demonstrated that primers recognizing sequences in the ribosomal RNA genes can amplify fragments from many species (Greisen et al. 1994; Hall et al. 1995). The advantages of using the 16S rRNA gene as a distinct signature for bacteria are the universal nature of the gene and the fact that it contains extremely conserved regions of nucleotides alternating with regions that are variable between species but constant within a species, making these sequences convenient targets for primers (Gray et al. 1984; Lane et al. 1985; Wilson 1992; Radstrom et al. 1994). In this study there are probably many microorganisms in the biofilms that have not had their DNA sequenced, making primer design difficult. This makes primer design for a specific organism in biofilms more complex, and sometimes impossible.

Table 4-2. Number of samples amplified with capsular polysaccharide biosynthesis primers by material, with the number of samples with positive amplicon detection.

Material	Biofilm		Wash	
	Total # of Samples	Number Positive	Total # of Samples	Number Positive
Rubber	6	4	6	5
PVC	5	4	5	4
cPVC	6	5	6	6
Glass	6	4	6	5
Fiberglass	4	4	5	4
Concrete	4	4	5	4
Stainless Steel	4	3	4	3

The capsular polysaccharide biosynthesis gene primers with biofilm and wash samples demonstrated a majority of positive results for amplicon, including the control tank. The positive control, a pure culture of *Streptococcus agalactiae,* showed a strong positive response, and the negative control, distilled water, showed no response. This eliminated the possibility of contaminated equipment and reagents.

Again, there was the possibility that *Streptococcus agalactiae* was present in the tanks. To verify this possibility, positive samples were Gram stained and tested using the Becton-Dickinson Gram-positive ID kit (Becton Dickinson Microbiology Systems, Becton Dickinson Company, Cockeysville, MD 21030). The most common identification of bacteria was *Staphylococcus epidermidis*. The few very bright bands in the gel, indicating strong positive responses, were identified as *S. agalactiae* and included the positive control.

These results reveal that more research needs to be done examining the use of species-specific primers when performing PCR on multiorganism biofilms. Other PCR techniques, such as multiplex PCR, reverse transcriptase PCR, and restriction fragment length polymorphisms (RFLP) should be studied. Another way to increase sensitivity and eliminate false positive

reactions is to decrease the number of cycles (Radstrom et al. 1994). This increases the sensitivity of the reaction by decreasing the possibility of the primers attaching to a strand of template DNA that is not an exact complement of it. This method should also be investigated to optimize primers.

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APPENDIX A

DISEASE HISTORY QUESTIONNAIRE

Please complete by checking the appropriate box and returning the completed form either by mail in the attached postage paid envelope or fax. Thank you in advance for your assistance in this matter.

What species of fish are being farmed at this facility?

Tilapia Striped bass

Catfish Rainbow trout

Yellow perch Flounder

Other (describe)_____

How many tanks does your facility have?

_____

What is the construction material(s) of the tanks?

Concrete Stainless steel
Rubber liner Plastic
Fiberglass
Other (describe)_____

What type of filtration system(s) is being used?

RBC Biological tower
Fluidized bed
Other (describe)_____

Is ozone being used for disinfection?

Yes_____ No_____

Are you using any other type of disinfectants in the system?

No_____ Yes (describe)_____

_____

How many fish per gallon of water does your facility hold?

_____

Are new fish put into tanks with old ones?

Yes_____ No_____

Has there been any disease present in the last 10 years?

No_____ Yes_____

If so, was it diagnosed?

No_____ Yes_____

If diagnosed, was it treated and with what?

No_____ Yes_____

_____

Was the treatment effective?

No_____ Yes_____

How often is the system water tested?

Daily Weekly
Monthly Yearly
Not at all

What is the water tested for and what is considered normal for your facility?

Normal Range

pH_____

Nitrites_____

Ammonia_____

Temperature_____

Specific gravity (salinity)_____

Nitrates_____

Copper_____

Oxygen_____

Other (describe)_____

APPENDIX B

Table B1. Green fluorescent protein E. coli growth on different substrates by aquarium tank**.**

Tank	Material	Mean Log Count			
		Day 1	Day 3	Day 7	Day 15
A2	Rubber	2.47±0.32	0.00	0.00	0.00
	PVC	2.02±0.18	0.00	0.00	0.00
	cPVC	2.00±0.21	0.00	1.95±0.49	0.00
	Glass	1.57±0.81	0.00	0.00	0.00
	Fiberglass	2.77±0.13	0.00	0.00	0.00
	Stainless	1.74±0.37	0.00	0.00	0.00
A3	Rubber	2.32±0.13	2.53±0.28	1.00±0.00	2.24±0.09
	PVC	1.96±0.08	1.50±0.28	1.16±0.28	1.00±0.00
	cPVC	2.38±0.69	1.54±0.34	2.13±1.40	2.48±0.35
	Glass	2.24±0.46	1.63±0.46	2.71±0.00	0.00
	Fiberglass	1.48±0.67	2.53±0.43	0.00	1.60±0.43
	Stainless	2.30±0.12	2.00±0.00	2.33±1.20	1.77±0.10
A7	Rubber	1.50±0.71	1.30±0.00	0.00	1.47±0.58
	PVC	1.39±0.12	0.00	0.00	2.09±0.08
	cPVC	1.49±0.50	0.00	0.00	2.16±0.22
	Glass	2.00±0.00	0.00	0.00	2.45±0.13
	Fiberglass	1.00±0.00	0.00	0.00	3.00±0.49
	Stainless	1.00±0.00	0.00	0.00	1.00±0.00
B3	Rubber	2.97±0.23	2.14±0.31	2.56±0.00	1.00±0.00
	PVC	2.65±0.24	2.40±0.10	1.90±0.00	0.00
	cPVC	2.67±0.16	1.94±0.14	1.80±1.13	0.00
	Glass	2.21±0.21	1.15±0.21	0.00	0.00
	Fiberglass	2.80±0.34	2.09±0.34	1.30±0.00	0.00
	Stainless	1.93±0.21	1.65±0.07	1.85±0.00	1.30±0.00
B4	Rubber	1.00±0.00	0.00	0.00	0.00
	PVC	1.70±1.20	0.00	1.00±0.00	0.00
	cPVC	1.00±0.00	0.00	1.98±0.71	0.00
	Glass	0.60±0.00	0.00	0.00	0.00
	Fiberglass	1.18±0.31	0.00	2.30±1.83	0.00
	Stainless	1.00±0.00	0.00	0.00	0.00
B5	Rubber	2.97±0.18	2.88±0.24	3.88±0.00	2.77±0.34
	PVC	2.54±0.56	3.11±0.26	3.51±0.24	3.01±0.48
	cPVC	2.68±0.17	2.95±0.07	3.59±0.14	2.76±0.15
	Glass	2.64±0.68	2.29±0.44	3.57±0.30	2.92±0.23
	Fiberglass	2.68±0.01	3.07±0.44	3.74±0.76	2.97±0.71
	Stainless	2.77±0.16	2.59±0.35	2.97±0.03	2.41±0.48

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APPENDIX C

Table C-1. Percent Reduction of the total plate count (TPC), Enterobacteriaceae (EB), and green fluorescent protein E. coli (GFP) by different sanitizers on various substrates.

		Sodium Hypochlorite				Ozone				Cleanser			
		Mean Log Reduction	SD	% Reduction	SD	Mean Log Reduction	SD	% Reduction	SD	Mean Log Reduction	SD	% Reduction	SD
Rubber	TPC	3.98	0.89	99.9641	0.06	0.37	0.47	42.8676	43.62	3.22	1.19	99.5563	0.74
	EB	4.86	1.62	99.9750	0.04	0.84	0.98	64.0146	55.44	2.65	4.18	66.6660	57.73
	GFP	1.38	0.33	95.2467	3.27	1.78	0.41	97.9040	1.37	0.65	2.21	49.6903	70.27
PVC	TPC	4.54	1.31	99.9883	0.01	0.00	2.27	0.0000	15.79	1.29	3.02	66.5533	57.64
	EB	5.51	2.48	99.9255	0.13	0.00	2.47	0.0000	36.24	2.48	3.16	66.6479	57.72
	GFP	1.31	0.24	94.6772	2.81	0.00	0.75	0.0000	35.15	1.44	2.04	49.9347	70.62
cPVC	TPC	3.67	1.37	99.7987	0.33	0.00	0.09	0.0000	8.56	2.80	1.95	90.9977	15.57
	EB	6.1	1.39	99.9989	0.00	0.59	0.62	59.0135	51.11	2.64	3.92	66.6632	57.73
	GFP	1.25	0.35	93.4189	4.83	0.95	1.43	49.4421	69.92	2.07	0.84	98.2232	2.20
Glass	TPC	3.24	0.86	99.8070	0.30	1.61	1.86	66.4918	57.58	3.70	0.46	99.9728	0.02
	EB	4.75	1.39	99.9884	0.02	2.77	4.52	66.6512	57.72	1.61	2.75	66.4634	57.56
	GFP	0.79	0	83.8935	0.00	0.00	1.55	0.0000	0.00	0.26	3.95	49.9553	70.65
Fiberglass	TPC	2.32	0.52	99.2726	0.72	0.00	0.22	0.0000	9.19	3.14	1.45	99.6425	0.37
	EB	5.03	1.04	99.9963	0.01	0.00	0.55	0.0000	0.00	2.87	4.13	66.6663	57.73
	GFP	1.85	0	98.5940	0.00	0.83	1.48	65.1019	56.39	0.00	0.00	0.0000	0.00
Stainless Steel	TPC	4.05	1.64	99.9426	0.06	0.00	0.96	0.0000	0.00	2.79	0.69	99.6274	0.52
	EB	4.65	2.22	99.8935	0.18	0.00	4.22	33.0976	57.33	2.53	3.43	66.6515	57.72
	GFP	1	0	90.0000	0.00	0.30	2.69	49.6850	70.27	1.48	0.00	96.6657	0.00

		Water				QAC				PAA			
		Mean Log Reduction	SD	% Reduction	SD	Mean Log Reduction	SD	% Reduction	SD	Mean Log Reduction	SD	% Reduction	SD
Rubber	TPC	2.44	0.24	99.6121	0.20	3.63	1.51	99.5595	0.76	4.75	1.48	99.9870	0.02
	EB	2.50	3.54	49.9995	70.71	3.00	3.69	66.6652	57.73	4.76	1.90	99.9656	0.06
	GFP	0.00	0.00	0.0000	0.00	0.86	1.50	44.9090	50.60	2.94	0.66	99.8138	0.21
PVC	TPC	2.80	0.25	99.8271	0.09	4.90	1.62	99.9797	0.03	4.85	0.66	99.9974	0.00
	EB	0.96	1.35	49.3877	69.84	6.38	0.93	99.9999	0.00	5.47	1.83	99.9888	0.02
	GFP	0.94	1.33	49.3317	69.77	2.71	1.06	99.4626	0.46	1.31	1.48	63.2932	55.04
cPVC	TPC	5.48	0.13	99.9997	0.00	4.03	2.16	99.6975	0.51	5.51	0.47	99.9995	0.00
	EB	2.72	2.43	94.9982	7.07	5.08	1.48	99.9954	0.01	5.90	1.00	99.9996	0.00
	GFP	0.89	0.92	69.7362	38.77	2.57	0.47	99.5998	0.43	1.37	1.59	63.3074	55.05
Glass	TPC	3.89	0.23	99.9863	0.01	4.42	1.92	99.9436	0.09	5.99	0.19	99.9999	0.00
	EB	0.00	0.00	0.0000	0.00	6.02	1.44	99.9995	0.00	6.88	0.26	100.0000	0.00
	GFP	1.08	0.11	91.4684	2.08	2.70	1.56	96.6323	5.74	3.37	0.00	99.9576	0.00
Fiberglass	TPC	2.64	0.20	99.7578	0.11	4.44	1.23	99.9780	0.03	5.48	0.82	99.9991	0.00
	EB	2.50	3.54	49.9995	70.71	5.59	0.59	99.9996	0.00	6.76	0.37	100.0000	0.00
	GFP	1.29	0.35	93.9979	4.41	1.67	1.21	82.3475	29.75	2.11	1.17	96.3175	5.49
Stainless Steel	TPC	1.35	0.12	95.4804	1.19	1.74	3.60	65.4947	56.75	6.07	0.07	99.9999	0.00
	EB	1.50	2.12	49.9500	70.64	3.13	3.58	66.6662	57.73	6.45	0.23	100.0000	0.00
	GFP	0.81	0.06	84.3710	2.08	1.39	0.42	94.6100	4.16	1.72	1.60	66.3104	57.43

APPENDIX D

Table D-1. The mean log count of total plate count (TPC), Enterobacteriaceae (EB), GFP E. coli (GFP) before and after treatment with sodium hypochlorite.

Sodium Hypochlorite

	Substrate	Mean Log Count		% Mean Log Reduction	Overall % Mean Log Reduction	Standard Deviation
		Before	After			
TPC	Rubber	5.719	2.718	52.47	61.29	8.18
		6.719	2.503	62.75		
		6.892	2.161	68.64		
	PVC	5.898	2.290	61.17	73.04	10.73
		5.245	1.265	75.88		
		7.365	1.321	82.06		
	cPVC	5.412	1.598	70.47	61.05	14.28
		7.301	2.332	68.06		
		5.000	2.769	44.62		
	Glass	5.790	2.243	61.26	64.49	20.91
		4.500	0.593	86.82		
		5.000	2.731	45.38		
	Fiberglass	5.096	2.807	44.92	49.07	15.27
		5.000	3.185	36.30		
		4.339	1.476	65.98		
	Stainless Steel	4.791	1.887	60.61	66.83	12.49
		5.667	2.343	58.66		
		7.295	1.371	81.21		
EB	Rubber	5.000	1.870	62.60	78.54	19.30
		7.000	1.889	73.01		
		6.349	0.000	100.00		
	PVC	4.000	1.349	66.28	88.76	19.47
		7.000	0.000	100.00		
		6.891	0.000	100.00		
	cPVC	7.000	0.000	100.00	100.00	0.00
		6.790	0.000	100.00		
		4.500	0.000	100.00		
	Glass	3.500	0.000	100.00	100.00	0.00
		4.500	0.000	100.00		
		6.239	0.000	100.00		
	Fiberglass	5.000	0.000	100.00	100.00	0.00
		6.075	0.000	100.00		
		4.000	0.000	100.00		

	Stainless Steel	2.500	0.000	100.00	100.00	0.00
		4.500	0.000	100.00		
		6.941	0.000	100.00		
GFP	Rubber	1.612	0.000	100.00	66.67	57.74
		1.151	0.000	100.00		
		0.000	0.000	0.00		
	PVC	1.136	0.000	100.00	66.67	57.74
		1.477	0.000	100.00		
		0.000	0.000	0.00		
	cPVC	1.000	0.000	100.00	66.67	57.74
		1.500	0.000	100.00		
		0.000	0.000	0.00		
	Glass	0.793	0.000	100.00	33.33	57.74
		0.000	0.000	0.00		
		0.000	0.000	0.00		
	Fiberglass	1.852	0.000	100.00	33.33	57.74
		0.000	0.000	0.00		
		0.000	0.000	0.00		
	Stainless Steel	1.000	0.000	100.00	33.33	57.74
		0.000	0.000	0.00		
		0.000	0.000	0.00		

Table D-2. The mean log count of total plate count (TPC), Enterobacteriaceae (EB), GFP E. coli (GFP) before and after treatment with a quaternary ammonium compound.

Quaternary Ammonium Compound

		Mean Log Count		Mean Log Count	Overall % Mean Log Reduction	Standard Deviation
		Before	Before			
TPC	Rubber	4.325	2.444	43.49	52.74	8.35
		8.216	3.697	55.00		
		7.502	3.022	59.72		
	PVC	5.381	2.159	59.88	70.23	10.28
		8.012	1.567	80.44		
		7.164	2.123	70.37		
	cPVC	5.241	1.546	70.50	64.09	18.94
		8.029	1.687	78.99		
		4.798	2.746	42.77		
	Glass	5.201	2.397	53.91	73.58	17.62
		7.448	0.901	87.90		
		4.956	1.044	78.93		
	Fiberglass	5.107	1.900	62.80	65.42	4.78
		7.995	2.323	70.94		
		7.115	2.666	62.53		
	Stainless Steel	0.000	1.710	0.00	35.61	35.34
		7.730	2.267	70.67		
		4.020	2.566	36.17		
EB	Rubber	0.000	1.182	0.00	48.52	43.85
		7.239	2.880	60.22		
		6.817	1.000	85.33		
	PVC	0.000	0.000	0.00	61.09	53.56
		7.041	0.000	100.00		
		6.876	1.151	83.26		
	cPVC	0.000	0.000	0.00	51.09	45.22
		7.129	1.000	85.97		
		6.000	1.962	67.30		
	Glass	5.000	0.000	100.00	66.67	57.74
		7.031	0.000	100.00		
		0.000	0.000	0.00		
	Fiberglass	0.000	0.000	0.00	53.66	46.60
		7.156	1.151	83.92		
		6.717	1.540	77.07		
	Stainless Steel	0.000	1.000	0.00	60.43	53.17
		6.626	1.239	81.30		
		5.000	0.000	100.00		

GFP	Rubber	2.252	2.431	0.00	20.22	35.97
		4.256	1.671	60.74		
		2.379	2.192	7.86		
	PVC	2.051	0.000	100.00	100.00	0.00
		3.932	0.000	100.00		
		2.148	0.000	100.00		
	cPVC	2.697	0.000	100.00	100.00	0.00
		2.965	0.000	100.00		
		2.050	0.000	100.00		
	Glass	3.025	0.000	100.00	100.00	0.00
		4.059	0.000	100.00		
		1.000	0.000	100.00		
	Fiberglass	2.550	0.000	100.00	69.59	52.68
		3.243	2.959	8.76		
		2.170	0.000	100.00		
	Stainless Steel	1.301	0.000	100.00	75.57	42.32
		3.790	2.778	26.70		
		1.841	0.000	100.00		

Table D-3. The mean log count of total plate count (TPC), Enterobacteriaceae (EB), GFP E. coli (GFP) before and after treatment with peracetic acid.

Peracetic Acid

	Substrate	Mean Log Count		Mean Log Count	Overall % Mean Log Reduction	Standard Deviation
		Before	After			
TPC	Rubber	7.679	3.243	57.77	67.01	14.21
		5.761	2.310	59.90		
		7.638	1.270	83.37		
	PVC	7.657	2.097	72.61	71.66	12.52
		5.663	0.925	83.67		
		7.250	2.995	58.69		
	cPVC	7.551	2.571	65.95	79.04	11.67
		6.887	1.185	82.79		
		6.625	0.770	88.38		
	Glass	7.079	1.301	81.62	86.51	4.47
		6.940	0.867	87.51		
		6.773	0.651	90.39		
	Fiberglass	7.555	2.009	73.41	76.87	9.72
		6.666	2.043	69.35		
		7.131	0.867	87.84		
	Stainless Steel	7.462	1.476	80.22	83.70	4.57
		6.898	0.767	88.88		
		7.421	1.336	82.00		
EB	Rubber	7.094	2.604	63.29	83.48	18.63
		3.000	0.000	100.00		
		7.774	1.000	87.14		
	PVC	7.134	1.301	81.76	93.92	10.53
		3.477	0.000	100.00		
		7.086	0.000	100.00		
	cPVC	6.977	0.000	100.00	95.03	8.60
		5.000	0.000	100.00		
		6.711	1.000	85.10		
	Glass	6.584	0.000	100.00	100.00	0.00
		7.000	0.000	100.00		
		7.047	0.000	100.00		
	Fiberglass	7.059	0.000	100.00	95.46	7.86
		6.867	0.000	100.00		
		7.344	1.000	86.38		
	Stainless Steel	6.705	0.000	100.00	95.49	7.81
		6.253	0.000	100.00		
		7.392	1.000	86.47		

GFP	Rubber	2.477	0.000	100.00	66.67	57.74
		3.410	0.000	100.00		
		0.000	0.000	0.00		
	PVC	0.000	0.000	0.00	66.67	57.74
		2.919	0.000	100.00		
		1.000	0.000	100.00		
	cPVC	0.000	0.000	0.00	66.67	57.74
		3.109	0.000	100.00		
		1.000	0.000	100.00		
	Glass	0.000	0.000	0.00	33.33	57.74
		3.373	0.000	100.00		
		0.000	0.000	0.00		
	Fiberglass	2.000	0.000	100.00	100.00	0.00
		3.324	0.000	100.00		
		1.000	0.000	100.00		
	Stainless Steel	2.000	0.000	100.00	66.67	57.74
		3.162	0.000	100.00		
		0.000	2.301	0.00		

Table D-4. The mean log count of total plate count (TPC), Enterobacteriaceae (EB), GFP E. coli (GFP) before and after treatment with water.

Water

	Substrate	Mean Log Count		Mean Log Count	Overall % Mean Log Reduction	Standard Deviation
		Before	After			
TPC	Rubber	7.95	5.34	32.81	30.20	3.69
		8.25	5.97	27.59		
	PVC	7.85	5.22	33.41	34.54	1.60
		8.33	5.36	35.67		
	cPVC	8.57	3.00	64.99	64.61	0.55
		8.39	3.00	64.22		
	Glass	7.72	3.67	52.52	51.12	1.98
		7.50	3.77	49.72		
	Fiberglass	7.96	5.18	34.92	32.56	3.34
		8.27	5.77	30.20		
	Stainless Steel	7.02	5.75	18.11	18.61	0.72
		7.50	6.07	19.12		
EB	Rubber	8.00	3.00	62.50	31.25	44.19
		0.00	3.00	0.00		
	PVC	7.50	5.59	25.49	12.75	18.03
		0.00	5.62	0.00		
	cPVC	7.43	3.00	59.64	42.32	24.50
		4.00	3.00	25.00		
	Glass	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00		
	Fiberglass	0.00	3.98	0.00	31.25	44.19
		8.00	3.00	62.50		
	Stainless Steel	0.00	0.00	0.00	18.75	26.52
		8.00	5.00	37.50		

GFP	Rubber	1.56	2.15	0.00	0.00	0.00
		1.56	1.99	0.00		
	PVC	1.87	0.00	100.00	0.00	70.71
		0.48	1.00	0.00		
	cPVC	1.55	0.00	100.00	100.00	0.00
		0.24	0.00	100.00		
	Glass	1.00	0.00	100.00	100.00	0.00
		1.15	0.00	100.00		
	Fiberglass	1.54	0.00	100.00	100.00	0.00
		1.04	0.00	100.00		
	Stainless Steel	0.77	0.00	100.00	100.00	0.00
		0.85	0.00	100.00		

Table D-5. The mean log count of total plate count (TPC), Enterobacteriaceae (EB), GFP E. coli (GFP) before and after treatment with ozone.

Ozone

	Substrate	Mean Log Count		Mean Log Count	Overall % Mean Log Reduction	Standard Deviation
		Before	After			
TPC	Rubber	7.056	6.824	3.29	4.87	5.82
		6.793	6.818	0.00		
		7.893	7.000	11.31		
	PVC	6.993	6.869	1.77	0.00	1.17
		6.790	6.639	2.22		
		6.477	10.274	0.00		
	cPVC	6.787	6.889	0.00	0.00	0.62
		6.534	6.464	1.07		
		7.000	7.000	0.00		
	Glass	5.902	6.420	0.00	20.96	18.23
		7.183	4.801	33.16		
		9.961	7.000	29.73		
	Fiberglass	7.540	7.700	0.00	0.00	0.64
		6.751	6.676	1.12		
		8.389	8.758	0.00		
	Stainless Steel	6.376	8.025	0.00	0.00	0.00
		4.867	6.534	0.00		
		7.000	7.000	0.00		
EB	Rubber	6.301	6.593	0.00	14.29	12.38
		6.333	5.000	21.05		
		6.781	5.301	21.82		
	PVC	7.000	6.571	6.13	0.00	3.54
		6.075	6.270	0.00		
		3.000	7.120	0.00		
	cPVC	6.026	6.151	0.00	8.68	7.52
		7.151	6.195	13.37		
		7.301	6.376	12.67		
	Glass	3.000	5.000	0.00	66.67	57.74
		6.978	0.000	100.00		
		3.333	0.000	100.00		
	Fiberglass	6.636	7.609	0.00	0.00	0.00
		6.151	6.151	0.00		
		7.173	7.212	0.00		
	Stainless Steel	5.826	5.867	0.00	11.65	20.19
		0.000	6.000	0.00		
		6.151	4.000	34.96		

GFP	Rubber	1.492	0.000	100.00	100.00	0.00
		2.244	0.000	100.00		
		1.602	0.000	100.00		
	PVC	1.940	1.534	20.92	0.00	10.89
		2.602	2.193	15.73		
		1.000	1.894	0.00		
	cPVC	1.952	0.000	100.00	33.33	57.74
		1.239	1.301	0.00		
		0.000	0.000	0.00		
	Glass	1.534	1.753	0.00	0.00	0.00
		0.000	2.415	0.00		
		0.000	0.000	0.00		
	Fiberglass	1.866	0.000	100.00	66.67	57.74
		1.000	1.867	0.00		
		1.477	0.000	100.00		
	Stainless Steel	2.201	0.000	100.00	33.33	57.74
		0.000	1.602	0.00		
		0.000	0.000	0.00		

Table D-6. The mean log count of total plate count (TPC), Enterobacteriaceae (EB), GFP E. coli (GFP) before and after treatment with an alkaline cleanser

Cleanser

	Substrate	Mean Log Count		Mean Log Count	Overall % Mean Log Reduction	Standard Deviation
		Before	After			
TPC	Rubber	6.552	2.991	54.35	47.02	14.17
		7.497	3.297	56.02		
		6.151	4.264	30.68		
	PVC	6.845	3.339	51.22	29.69	26.57
		6.632	4.122	37.85		
		0.000	2.145	0.00		
	cPVC	6.888	3.248	52.85	41.59	23.82
		7.237	3.062	57.69		
		4.000	3.431	14.23		
	Glass	6.793	3.188	53.07	59.32	5.42
		6.695	2.498	62.69		
		5.301	2.004	62.20		
	Fiberglass	8.164	3.359	58.86	49.74	11.36
		6.680	4.207	37.02		
		4.000	1.866	53.35		
	Stainless Steel	6.775	3.775	44.28	46.36	8.54
		6.000	2.655	55.75		
		5.151	3.139	39.06		
EB	Rubber	7.000	2.360	66.29	47.11	41.04
		7.265	1.812	75.06		
		0.000	2.151	0.00		
	PVC	5.900	2.646	55.15	43.75	39.31
		6.811	1.628	76.10		
		0.000	1.000	0.00		
	cPVC	6.239	2.241	64.08	47.37	41.61
		7.292	1.602	78.03		
		0.000	1.778	0.00		
	Glass	5.000	2.778	44.44	39.97	37.93
		5.301	1.301	75.46		
		0.000	1.389	0.00		
	Fiberglass	7.957	2.880	63.81	48.15	42.54
		6.725	1.301	80.65		
		0.000	1.900	0.00		
	Stainless Steel	5.826	2.480	57.43	52.48	50.18
		5.477	0.000	100.00		
		0.000	1.239	0.00		

GFP	Rubber	1.778	2.688	0.00	33.33	57.74
		2.208	0.000	100.00		
		0.000	0.000	0.00		
	PVC	3.623	0.739	79.60	26.53	45.96
		0.000	0.000	0.00		
		0.602	0.602	0.00		
	cPVC	3.761	1.102	70.70	56.90	51.41
		1.477	0.000	100.00		
		0.000	0.000	0.00		
	Glass	3.200	0.151	95.28	31.76	55.01
		0.000	0.000	0.00		
		0.000	2.534	0.00		
	Fiberglass	0.000	1.000	0.00	0.00	0.00
		0.000	0.000	0.00		
		0.000	0.000	0.00		
	Stainless Steel	0.000	0.000	0.00	33.33	57.74
		0.000	0.000	0.00		
		1.477	0.000	100.00		

Curriculum Vitae
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Professional Experience

Squad Officer-in-Charge, 72nd Medical Detachment (VS), US Army Veterinary Corps, Augsburg, Germany. October 1995 – June 1998.

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Branch Chief, San Diego Branch, US Army Veterinary Corps, San Diego, CA. June 1993 – September 1995.

Associate Veterinarian, Anne Arundel Veterinary Clinic, Baltimore, MD. June 1992 – June 1993.

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Professional Affiliations

American Veterinary Medical Association

Institute of Food Technologist, IFT Student Representative Seafood Division Executive Committee, 1998-2000.

International Association for Food Protection

Professional Honors and Awards

Elected to Gamma Sigma Delta and Phi Zeta honoraries

AVMA Outstanding Student Award; September 1993; Veterinary Corps Officers, Medical Officers Basic Course, U.S. Army Medical Department Center and School

Waste Policy Institute Summer Fellowship; April 1999; Virginia Polytechnic Institute and State University

Award winner; Seafood Technology Graduate Research Paper Competition; June 2000; Institute of Food Technologists