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Frequency of *Aeromonas* spp. detection in rainbow trout and recirculation aquaculture systems and the storage stability of fillets

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**Frequency of *Aeromonas* spp. Detection in Rainbow Trout and
Recirculation Aquaculture Systems and the Storage Stability of Fillets**

Tabetha Marie Littler

**Thesis submitted to the
Davis College of Agriculture, Forestry, and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Animal and Nutritional Sciences**

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ABSTRACT

Frequency of *Aeromonas* spp. Detection in Rainbow Trout and Recirculation Aquaculture Systems and the Storage Stability of Fillets

Tabetha Marie Littler

Recirculating aquaculture systems enable increased fish production per unit volume of water. Unfortunately, recirculation systems also provide conditions for bacterial growth. *Aeromonas*, a genus of bacteria ubiquitous in the environment, contains fish pathogens, human pathogens, and/or spoilage organisms. Of the salmonid species, rainbow trout are the most susceptible to Motile Aeromonad Septicemia (MAS), the fish disease caused by *Aeromonas* spp. In humans, illnesses such as gastroenteritis, septicemia, and wound infections are caused by ingestion or exposure to *Aeromonas* spp. The objective of this study was to survey *Aeromonas* spp. present in the production system, on the fish, and on the butterfly fillet. Psychotropic plate counts of fillets were also determined as part of a 6-d shelf life study.

Rainbow trout and water samples were collected from The Conservation Fund's Freshwater Institute (TCFFI) in Shepherdstown, WV over a period of eight month. Source water, a natural spring, and three production systems (flow-through, 80% reuse, and 95% reuse) were sampled. Three fish from each system were rinsed in 100 mL Butterfield's Phosphate Buffer (BPB) and analyzed for *Aeromonas* spp. and psychrotrophic plate count. Three additional fish per system were filleted, divided into equal halves, and randomly assigned to 0 or 6-d storage. Core samples were taken following 0 and 6 d storage at 4 ± 2 °C, blended with 25 mL BPB, and processed. Psychrotrophic plate counts were performed for whole-fish rinsates and fillet cores on d 0 and 6 of refrigerated storage.

Presumptive positive *Aeromonas* isolates were obtained from the fillets, rinsates, and water samples via enrichment in Tryptic Soy Broth with Ampicillin (TSBA) and isolation on Starch Ampicillin Agar (SAA) incubated at 28 ± 2 °C for 24 h. The oxidase test, resistance to the vibriostatic agent O/129, and starch hydrolysis with Lugol's solution were determined for isolates before species identification with API 20NE strips. Water samples were enumerated for *Aeromonas* spp. by filtering 100 mL through 0.45 µm filters and placing the filter on SAA plates. Water SAA plates were flooded with Lugol's solution after incubation at 28 ± 2 °C for 24 h, and presumptive positive *Aeromonas* spp. were counted.

Fifty-seven *Aeromonas* isolates were collected over an eight month period. The highest frequency of positive samples ($P < 0.05$) occurred in January (16/21), whereas the lowest occurred in November and February (4/21). In January, two *Aeromonas* spp. were isolated from the source water, two isolates from the 80% reuse water, and one isolate from the 95% reuse water. Four rinsates were positive, two from each the 80% reuse and 95% reuse systems. Two core samples were positive at d 0 from the 95% reuse, and five were positive after 6 d, three from the 80% and two from the 95% reuse systems.

The November sampling generated one positive each from the source, 80% reuse water, 80% reuse fish rinsate, and 95% reuse rinsate. In February, three positive samples were isolated from the source and one positive from the 80% reuse rinsate. Psychrotrophic plate counts of fillets increased ($P < 0.05$) with refrigerated storage, while counts for fish rinses were highest ($P < 0.05$) for the 95% reuse system. The flow-through system produced fish that gave the highest ($P < 0.05$) percent fillet yield. Frequency of *Aeromonas* spp. detection was affected by sampling period and water reuse. Of particular note, prevalence of *Aeromonas* spp. increased in January, coinciding with discontinuation of UV light and ozone treatments for the 95% reuse system. Implementing pre-harvest controls for *Aeromonas* can decrease fillet contamination, resulting in a longer shelf-life and a safer product for consumers.

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INTRODUCTION

The demand for fish and fish products in the United States continues to rise. Due to the increase in aquatic food production, water reuse has become an important option in aquaculture, particularly when water resources are limited. In a flow-through system, fresh water passes continuously through the system, requiring an enormous amount of water for fish production. Recirculating some water back through the system increases production per unit volume of water.

In order to ensure quality products, producers should recognize that bacterial growth occurs in the system, on the fish, and in the fish gut. Many genera of microorganisms are natural inhabitants of the aquatic environment. Some of these may be pathogenic to fish, pathogenic to humans consuming food derived from fish, and/or cause spoilage. Bacteria that are pathogenic to fish are controlled through fish health programs. Potential human pathogens are monitored where fish are grown, in processing facilities, and during distribution.

Aeromonas spp. are ubiquitous in the environment. They are present in water, food, and soil throughout the world. Some members of this genus are spoilage organisms, while other species are fish or human pathogens. Because of human health risks associated with *Aeromonas* spp., it is important to monitor their levels at fish farms. Controlling the prevalence of these organisms during production is imperative in maintaining fish health and producing high quality aquatic foods safe for human consumption. This study surveyed levels of *Aeromonas* spp. present in rainbow trout, recirculation aquaculture systems to establish a foundation for preharvest control of this pathogen/spoilage organism.

LITERATURE REVIEW

Importance of Aquaculture

More than 220 species of finfish and shellfish are farmed worldwide. In 2000, fish produced from aquaculture accounted for over 1/4 of fish directly consumed by humans. As the United States and world populations keep growing, the importance of fish as a source of protein will continue to increase (Naylor and others 2000). With an expanding market for fish and fish products, the demand for seafood is projected to exceed 3 million tons in the US by 2020 (Pitney and Bopp 2006). Alternative methods of large-scale fish production, such as recirculated aquaculture systems, are being implemented to further this development where water resources are limited.

Partial Reuse System

Water reuse is becoming progressively more important as aquaculture operations increase aquatic food production. In areas with limited high-quality water resources, partial-reuse systems are efficient in culturing coldwater species, such as rainbow trout and arctic char. Circular, partial-reuse, fish-culture tanks have an advantage over traditional culture methods because water is able to completely mix in a circular tank, making the water quality uniform throughout the tank (Summerfelt and others 2004).

In a partial-reuse system, particles are settled or filtered from the water exiting the culture tank. Eighty percent or more of the filtered water is pumped to the head of the system, stripped of carbon dioxide (CO₂), supplemented with pure oxygen (O₂), and then reused. Fish production in any intensive, tank-based

system is limited by the availability of dissolved O₂ and by the density of fish per unit volume of water (Summerfelt and others 2004).

In order to decrease the amount of bacteria reentering the system, recirculated water is exposed to ultraviolet (UV) light. Bacterial proteins and nucleic acids absorb the UV light, causing photochemical changes, and resulting in lethal nucleic acid mutations (Jay 2003). Adsorption of UV light relies on the absence of dissolved compounds for effectiveness; consequently, removal of particles larger than 70 µm by filtration is recommended. At a wavelength of 253.7 nm, UV light can effectively disinfect water. The dose requirement (intensity x time) is related to the size and transparency of the target organism. For example, 3600 mW/cm²/s is required to kill *Aeromonas salmonicida* (Colt and Tomasso 2001). Because of its poor penetrating ability, UV light is limited to surface applications for food use (Jay 2003). Lamps can either be inside a pressurized chamber or in an open channel, but they must be replaced yearly (Colt and Tomasso 2001).

Another treatment used to decrease bacteria is ozone, a colorless molecule with a pungent odor containing 3 atoms of oxygen (O₃) (Colt and Tomasso 2001). Ozone is an oxidizing agent capable of reducing levels of microorganisms on food and food contact surfaces (Crapo and others 2004) by stripping away electrons. In 1997, the use of ozone for food was granted the status of GRAS (generally regarded as safe) (Jay 2003). Because ozone is a strong oxidizing agent, it is not recommended for use on foods with high lipid content (Jay 2003; Crapo and others 2004). Crapo and others (2004) reported

that ozone was not effective in controlling bacteria on salmon fillets and roe, but rather accelerated the development of rancidity of these products.

Ozone is relatively unstable, so it must be generated on site. It can react with ammonia (NH_4^+), nitrite (NO_2^-), reduced Fe and Mn, Br, dissolved organic compounds, and microorganisms. Under ambient pH, ozone can oxidize 5-15% NH_4^+ to NO_2^- and can then rapidly oxidize NO_2^- to nitrate (NO_3^-). Ozone effectively inactivates bacteria, viruses, and protozoa at 0.5-1.5 mg/L. The disinfecting reactions of O_3 may involve proteins within the cell membrane, although these reactions are not well understood. No side effects have been found in salmonid eggs and fry at 10-20 $\mu\text{g/L}$ O_3 under production conditions (Colt and Tomasso 2001).

Ammonia (NH_4^+) is the primary nitrogenous by-product of protein metabolism in aquatic animals. Removal of NH_4^+ can increase production from a given water source, because in its unoxidized form (NH_3), ammonia can build up and become toxic. In a water reuse system, NH_4^+ is oxidized to NO_3^- by certain bacteria in a two-step process. *Nitrosomonas* oxidizes NH_4^+ to NO_2^- and water, and then *Nitrobacter* oxidizes NO_2^- to NO_3^- . The overall NH_4^+ oxidation-reduction reaction is $\text{NH}_4^+ + 2\text{O}_2 \rightarrow \text{NO}_3^- + 2\text{H}^+ + \text{H}_2\text{O}$ which requires 4.57 mg O_2/mg total ammonia nitrogen (TAN) oxidized (Colt and Tomasso 2001).

A biofilter in a water reuse system uses nitrifying bacteria to remove NH_4^+ and its by-products. Because these bacteria attach to surfaces, increasing surface area in the biofilter by adding media such as sand, crushed oyster shells, and rocks allows for improved NH_4^+ removal. Typically, water is trickled through

the biofilter, solids settle to the bottom, and the nitrifying bacteria oxidize NH_4^+ as the water flows through the filter. Performance of the nitrifying filter depends on temperature, filter depth, NH_4^+ concentration, and surface area (Colt and Tomasso 2001).

Rotational water velocity in a circular tank can be adjusted to an optimum level for fish health and removal of solid waste. Solids are removed through a center bottom drain by the 'tea-cup' solids transport mechanism produced by rotating water flow in the tank. With this mechanism, solids are concentrated in the center of the tank by the circulating water and flushed through the bottom drain. The culture tanks at The Conservation Fund's Freshwater Institute (TCFFI) use a dual-drain technology to accomplish solids removal.

Approximately 82% of the total suspended solids (TSS) produced are collected in the partial-reuse system and accumulated in an off-line settling tank (Summerfelt and others 2004). Davidson and Summerfelt (2005) reported that using a swirl separator or a radial-flow settler to treat the effluent in the settling tank removed $37.1 \pm 3.3\%$ and $77.9 \pm 1.6\%$ mean TSS, respectively ($P < 0.001$). After treatment and addition of makeup water, the recirculated water contained an average of 2.4 ± 0.5 mg/L and 2.7 ± 0.3 mg/L TSS from the swirl separator and radial-flow settler, respectively (Davidson and Summerfelt 2005).

Traditional aquaculture utilizes large water supplies to raise fish in culture ponds and raceways. In locations where water supply is abundant, but space is limited, such as the Snake River Valley in Idaho, coldwater species are typically cultured in serial-use raceways. Water quality progressively deteriorates from

raceway to raceway with this system. Particulate matter accumulates along the bottoms and quiescent zones at the end of the raceway, making it difficult to capture more than 25-51% TSS in the water. In comparison to the partial-reuse system, raceways are less efficient in the capture of particulate waste (Summerfelt and others 2004).

Rapid and effective solids removal can improve culture conditions for salmonid species in recirculating systems. The accumulation of solids in culture tanks can promote the proliferation of fish pathogens and decrease water quality. Solids that are not promptly removed can break down into smaller particles that leach nutrients, degrade water quality, and exert a biological O₂ demand which increases CO₂ levels. These smaller particles can cause gill irritation, leading to a reduced immune response, and possibly a disease outbreak. In addition, solids that are not effectively removed may adversely affect essential system components such as aeration columns, screens, and spray nozzles, resulting in system failure (Davidson and Summerfelt 2005).

At TCFFI, the partial-reuse system was able to sustain a production level of 35-45 kg/year of rainbow trout for every 1 L/min of make-up water, which equals 11,000-17,000 kg/year. This level of production is approximately 6-7 times greater than the serial-reuse raceway systems in the Snake River Valley that produces 6 kg of trout per year for every 1 L/min of water (Summerfelt and others 2004). Water recirculation in a partial-reuse system increases production per unit volume of water, thus allowing large scale production when water supply is not abundant.

Water pollution laws and limited availability of high-quality water resources are two factors that have promoted an increased interest in alternative fish culturing methods. These alternative methods may economically sustain and/or increase fish production, while using less water, in addition to capturing waste more efficiently. The partial-reuse system at TCFFI is able to support high fish densities and efficiently capture waste using only 20% of the water required for a flow-through raceway system (Summerfelt and others 2004).

Rainbow Trout Management

Hatchery management is the most effective way to prevent bacterial infections in fish populations. Ozonation and filtration, in combination with ultraviolet irradiation, are effective in reducing the occurrence of *Aeromonas hydrophila* in hatcheries. Recently infected fish should be avoided when stocking hatcheries, while eggs should be surface disinfected to decrease the chance of contaminating the facility and other fish. The most effective antibiotics include chloramphenicol, oxytetracycline, chlortetracycline, and a mixture of penicillin and streptomycin added to water at 10-15 mg/L (Cipriano 2001).

Productivity is largely dependent on water quality (Klontz 1991). For rainbow trout, the optimal temperature for growth is 16.5-17.2 °C and 10.0-13.0 °C for spawning. The minimal dissolved O₂ concentration of the production raceway is 6.0 mg/L, and the medial-lethal concentration of NH₃ for 96 h old fingerlings at 12-14 °C is 0.60 mg/L (Colt and Tomasso 2001). Levels for finfish should be maintained at 6.7-8.5 pH, 30-200 mg/L alkalinity as CaCO₃, <2.0 mg/L CO₂, >50 mg/L Ca, <0.04 mg/L Zn at pH 7.5, <0.006 mg/L Cu in soft water, and <0.3 mg/L Cu in hard water. Other important factors should be kept at <1.0 mg/L

Fe, <0.03 mg/L constant NH_3 , <0.05 mg/L intermittent NH_3 , <0.55 mg/L NO_2^- , <100% N saturation, <80 mg/L suspended solids, and 50-200 mg/L dissolved solids (Klontz 1991).

Currently, there are no regulations for the minimum allowable amount of bacteria in fish productions systems. Shellfish growing areas in costal waters are routinely monitored for coliforms as an indicator of sewage contamination; however, freshwater fish are not regulated. The fecal coliform:fecal streptococci ratio is used to infer the source of fecal contamination in water. These bacteria originate from warm-blooded animals such as humans, livestock, domestic pets, wild animals, and birds. Because fish are cold-blooded, any fecal coliform contamination is brought into the aquaculture system through the water (Tomasso 2002). At TCFFI, effluent water is treated so that it contains no more than 400 CFU/100 mL when discarded.

Rainbow Trout Intestinal Microflora

In order to produce safe and wholesome products, producers need to recognize the extent and dynamics of bacterial growth in their system and its effect on fish gut microflora. There are naturally occurring bacteria in the digestive tract of fish. The aquatic environment is a determinant of the number and type of microorganisms on and in the fish (González and others 1999). These environmental factors such as the time of year, including seasonal changes, affect fish rearing conditions (Huber and others 2004).

Only a fraction of microorganisms observed on fish or in their gut are culturable. Skin contained less than 0.01% culturable bacteria (Spanggaard and others 2000; Huber and others 2004). This observation may be a result of

bacteria cultured from aquatic environments being less than 1% since most isolates found on fish skin are also found in the surrounding water. The microflora of fish intestines consists of bacteria that are present in water, but are able to live and multiply in the gut. These organisms are predominately aerobic or facultatively anaerobic (Huber and others 2004).

It is estimated that up to 10^8 aerobic, heterotrophic bacteria/g and approximately 10^5 anaerobic bacteria/g can colonize the fish gut. These numbers fluctuate depending on season and location in the gastrointestinal tract. Austin and Al-Zahrani (1988) found that populations of aerobic heterotrophic bacteria progressively declined along the digestive tract from the esophagus to the lower intestine; however, the highest counts were recovered from intestinal contents and feces. Anaerobes were mostly restricted to the upper intestine and its contents. Scanning electron micrographs showed that bacteria were not attached to the gut wall, but instead were associated with intestinal contents. Bacterial populations in the tank water ranged from 6.0×10^4 to 2.9×10^5 aerobes mL^{-1} (Austin and Al-Zahrani 1988).

Austin and Al-Zahrani (1988) identified 12 genus groupings when examining aerobic bacteria in the digestive tract of rainbow trout. The majority of the isolates (65%) were gram negative consisting of *Acetobacter* spp., *Alcaligenes denitrificans*, *Providencia stuartii*, *Aeromonas hydrophila*, *Acinetobacter calcoaceticus*, *Flavobacterium* spp., and *Pseudomonas* spp. The gram positive bacteria present were *Kurthia* spp., *Micrococcus varians*, *Microbacterium* spp., gram positive irregular shaped rods, coryneforms, *Bacillus*

circulans, *Bacillus megaterium*, and *Staphylococcus* spp. Of these organisms, four isolates of *Aeromonas hydrophila* were found in the stomach and feces of the trout (Austin and Al-Zahrani 1988).

In a study by Spanggaard and others (2000), the culturability of intestinal microflora of rainbow trout on classical agar plates was compared to direct microscopic counts using DAPI (4',6-diamidino-2-phenylindole) staining. On average, 50% of the intestinal microflora was cultured using conventional techniques. DAPI stain counts were approximately 0.5 logs higher than conventional plate counts, indicating that culturable microorganisms dominated the microflora. The bacteria accounting for the 0.5 log higher DAPI stain counts may be viable but not culturable. The dominant groups of bacteria cultured belonged to the γ -subclass of *Proteobacteria* (genera *Citrobacter*, *Aeromonas*, and *Pseudomonas*), the gram-positive bacteria with low G + C content (genus *Carnobacterium*), and the β -subclass of *Proteobacteria* (Spanggaard and others 2000).

Rainbow trout studies using electron microscopy and direct fluorescence *in situ* hybridization (FISH) analysis of gut microflora concluded that most microorganisms live in the lumen instead of colonizing the gut wall. Huber and others (2004) used FISH to identify and quantify the microbial community in the gastrointestinal tract of rainbow trout. They found that the γ - subclass of *Proteobacteria*, consisting primarily of *Aeromonas* and *Enterobacteriaceae*, occupied most of the intestine. They also concluded that approximately 20% of organisms had not yet been cultured (Huber and others 2004).

Huber and others (2004) also detected the entire *Aeromonas* genus in 56 isolates when using the 16S rRNA probe AER642 to examine samples from rainbow trout microflora. Of these isolates, 10 were identified as *A. salmonicida* by the ASA446 probe and 24 isolates were identified as *A. sobria* by the ASO642 probe. Isolates of *Acinetobacter*, *Pseudomonas*, *Shewanella*, *Plesiomonas*, and *Proteus* were also identified. Other isolates included those from the β -subclass of *Proteobacteria*, gram-positive bacteria with high G + C content, and gram-positive bacteria with low G + C content. In contrast, intestinal microflora of some rainbow trout was dominated by an uncultivated, anaerobic organism. Overall, DAPI staining revealed 10-50% more microorganisms than aerobic counts (Huber and others 2004).

***Aeromonas* Characteristics**

The genus *Aeromonas* is officially classified as a member of the family *Aeromonadaceae* (Isonhood and Drake 2002). *Aeromonas* spp. are ubiquitous microorganisms that have been isolated from environmental, clinical, and food samples. There are 14 named species in the genus, all of which are highly similar (~98-100%) in their 16S rDNA sequence (Cipriano 2001; Isonhood and Drake 2002; Lee and others 2002). Motile aeromonads have adapted to a number of environments with varying conductivity, turbidity, pH, salinity and temperature (Cipriano 2001).

Most *Aeromonas* spp. are capable of growing at a pH of 5.5-9.0 and temperatures <5 °C (Isonhood and Drake 2002). Rouf and Rigney (1971) classified 33 strains of *Aeromonas* spp. as either psychrophiles or mesophiles.

Six *A. hydrophila* and one *A. shigelloides* strains were classified as psychrophiles, with optimal temperatures of 15-20 °C, minimum of 0-5 °C, and maximum of 40-45 °C. All other strains were classified as mesophiles with an average optimal temperature of 35 °C. Rouf and Rigney (1971) concluded that most *Aeromonas* spp. can grow well at 20 °C. In particular, the optimum growth temperature for *A. hydrophila* is 28 °C, but it is capable of growing from 1-42 °C (Isonhood and Drake 2002).

Some aeromonads have been reported to be viable but not culturable, similar to some *Vibrio* spp. (Isonhood and Drake 2002). Cells are typically 1.0 µm in diameter and 2.0-4.5 µm in length, but there are a few coccoid forms and short rods. They usually occur as singles or pairs, and sometimes form short chains up to 8.0 µm in length (Post 1987; Isonhood and Drake 2002). The majority of motile aeromonads are non-pigmented, although some produce a brown to brown-red water soluble pigment (Post 1987). *Aeromonas* spp. do not have a salt requirement (Palumbo and others 2001), but can be found in marine environments.

These gram-negative rods can be motile via a monotrichous polar flagellum, are non-acid fast, do not form spores, and usually do not have a capsule (Post 1987). *Aeromonas* spp. are oxidase positive, catalase positive, facultatively anaerobic, and resistant to the water soluble vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine phosphate). The oxidase test allows *Aeromonas* spp. to be distinguished from the *Enterobacteriaceae*; whereas, the O/129 test distinguishes them from the *Vibrio* genus. *Aeromonas* spp. are inositol

negative, mannitol positive, ornithine decarboxylase negative, and have 57-63% mol DNA G + C content. The bacteria produce amylase, protease, gelatinase, lipase, phospholipase, and DNase, but lack the enterobacterial antigen (Palumbo and others 2001).

Aeromonas spp. produce 2,3-butanediol, reduce nitrate to nitrite, and metabolize glucose aerobically and anaerobically, with or without the production of gas (Cipriano 2001). Acid or acid with gas is produced from glucose, fructose, galactose, maltose, mannitol, trehalose, dextrin, glycogen, starch, mannose, and glycerol (Cipriano 2001; Isonhood and Drake 2002). Glucose fermentation is a critical biochemical reaction that differentiates motile aeromonads from species of *Pseudomonas*. Production of acid from arabinose, salicin, cellobiose, sucrose, and lactose is varied. *A. hydrophila*, specifically, is able to hydrolyze albumin, casein, fibrinogen, gelatin, hemoglobin, and elastin. No *Aeromonas* species are capable of hydrolyzing collagen (Cipriano 2001).

Motile aeromonads adhere to eukaryotic cells by producing fimbriae that allow them to attach. The three most virulent species, *A. hydrophila*, *A. sobria*, and *A. caviae*, adhere to mucus receptors on host cells. *A. hydrophila* and *A. sobria* produce enterotoxins, dermonecrotic factors, and hemolysins. Both species are able to lyse red blood cells when grown on blood agar plates at 30 °C, but *A. hydrophila* is also capable at 10 °C. Environmental temperature may partially account for the difference in virulence between the two species, especially in salmonid fish that live in cold water (Cipriano 2001).

Aeromonas spp. are the most antigenically and taxonomically diverse bacterial pathogens in fish. Studies have revealed the diversity of somatic (O) and flagellar (H) antigens and the serologic specificity of extracellular antigens. There are 12 O-antigens and 9 H-antigens, each of which have been further divided into several more serotypes. Although several strains of *Aeromonas* spp. have common somatic antigens, there is no single antiserum that can agglutinate more than just a small percentage of species (Cipriano 2001). Variation in the immunological and biochemical characteristics of *Aeromonas* spp. makes it difficult to identify the majority of species with a single test.

These bacteria have a broad host range of warm- and cold-blooded animals, including humans and fish, respectively. *Aeromonas* spp. can be spread on the plumage of birds, by terrestrial animals, and by amphibians and reptiles (Post 1987). Strains isolated from the environment seem to differ from strains isolated from humans with infection only if virulence factors are present. Certain species are more frequently isolated from patients with gastroenteritis and from diseased fish than from environmental samples (Vivekanandhan and others 2005).

Human Disease

The genus *Aeromonas* consists of 2 major sub-divisions, the mesophilic, or motile aeromonads, and the non-motile group. Motile aeromonads, which include *A. hydrophila*, *A. sobria*, and *A. caviae*, are of primary concern to public health (Palumbo and others 2001). *A. hydrophila*, *A. caviae*, *A. jandaei*, *A. veronii*, and *A. schubertii* are considered pathogenic to humans. These organisms cause intestinal infections and are a source of diarrheal disease

(Paniagua and others 1990; Janda 1991; Lee and others 2002). In particular, infection with *Aeromonas hydrophila* can result in serious complications, especially in immunocompromised hosts, the elderly, and children (Palumbo and others 2001; Isonhood and Drake 2002).

The importance of *Aeromonas* spp. as human enteric pathogens continues to rise (Daily and others 1981; Palumbo and others 1985; Deodhar and others 1991). Motile aeromonads, *A. hydrophila*, *A. sobria*, and *A. caviae*, are capable of causing infections through open wounds or illness by ingestion of a number of organisms in food or water. These bacteria can act as an infectious or enterotoxigenic pathogen causing gastroenteritis, wound infections, septicemia, endocarditis (Isonhood and Drake 2002), urinary tract infections, cellulitis, hepatobiliary problems, and ear infections (Vila and others 2003). In extraintestinal disease, *A. hydrophila* and *A. sobria* appear to be more invasive and pathogenic than *A. caviae*, although variations in strain-to-strain virulence exists (Janda 1991).

Two types of gastroenteritis are associated with *A. hydrophila*. A cholera-like illness yields watery diarrhea often described as rice and water. Secondly, a dysenteric illness is characterized by diarrhea containing blood and mucus, which in severe cases, may last for several weeks. In individuals with an impaired immune system, bacteria may spread throughout the body and cause septicemia (US Food and Drug Administration 1991). Other complications in humans due to *A. hydrophila* are arthritis, corneal ulcers, and meningitis. In most cases, *Aeromonas* infections are sporadic rather than associated with large

outbreaks of illness (Cipriano 2001); however, substantial clinical and microbiological research supports the epidemiologic evidence that some *Aeromonas* spp. can cause gastroenteritis (Villari and others 1999).

Approximately 1% of humans are carriers of *Aeromonas*. Infections frequently result from exposure to water containing these bacteria (Daily and others 1981). Daily and others (1981) examined 15 *Aeromonas* isolates from various human infections and 9 from polluted water. Of the 15 clinical strains, 13 were *A. sobria* and two were *A. hydrophila*. Two environmental isolates were *A. sobria* and seven were *A. hydrophila*. *Aeromonas* isolates from environmental and clinical samples were tested for adherence mechanisms and several virulence factors. Isolates without pili were bound to buccal cells, suggesting that *Aeromonas* spp. may have alternative mechanisms for adhering to human epithelial cells. The majority of clinical specimens in this study (13/15) were cytotoxigenic, enterotoxigenic *A. sobria* (Daily and others 1981).

The infectious dose of *A. hydrophila* is still unknown. Scuba divers that ingested a small amount of water became ill and had the organism isolated from their stool. In contrast, studies where volunteers ingested large amounts, up to 10^{11} cells, have not produced illness. Nevertheless, *A. hydrophila*'s presence in feces of individuals with diarrhea, without other enteric pathogens present, is reason to believe that it may play some role in gastroenteritis. *A. caviae* and *A. sobria* are also associated with diarrheal disease, but still remain as unproven causative agents (US Food and Drug Administration 1991).

Diarrheal diseases caused by *Aeromonas* spp. have been reported worldwide. A study in Thailand reported an isolation rate of 31% from individuals with traveler's diarrhea and 27% from children. *Aeromonas* strains were isolated from 3.7% (21/561) of children with gastroenteritis and 2.1% (12/576) of healthy children in a study performed in Italy (Kraft 1992). In Bangladesh, a high rate of *Aeromonas* was found in the feces of a control population (Deodhar and others 1991). The frequency of disease due to *A. hydrophila* in the United States is unknown; although, efforts are being made to quantify its occurrence (US Food and Drug Administration 1991).

In India, *Aeromonas* spp. have been identified as an enteric pathogen in 1.8% of patients with diarrhea (Vila and others 2003). Deodhar and others (1991) isolated 45 strains of *Aeromonas* from 2,480 patients in Bombay, India with acute gastroenteritis. Of these 45 isolates, 35 (77.8%) were *A. hydrophila*, 7 (15.5%) were *A. sobria*, and 3 (6.7%) were *A. caviae*. All strains were enterotoxin and hemolysin positive. Patients' symptoms improved when traces of *Aeromonas* spp. disappeared from their stools. This study suggests that *Aeromonas* spp. are potential enteric pathogens that possess virulence mechanisms (Deodhar and others 1991).

Motile aeromonads possess virulence factors associated with enteric disease and gastroenteritis in children and adults (Deodhar and others 1991; Palumbo and others 2001). These virulence factors may include enterotoxins, hemolysins, cytotoxins (Deodhar and others 1991; Schuman and others 1997), adhesins (Deodhar and others 1991), hemagglutinin (Schuman and others

1997), proteases, lecithinases, nucleases, amylases, elastases (Daily and others 1981), invasions, endotoxin lipopolysaccharide, fimbriae, pili, capsules, S-layers, and siderophores (Janda 1991; Isonhood and Drake 2002). Virulence is particularly prevalent in warmer months when motile aeromonads are highest in aquatic habitats (Palumbo and others 2001).

The virulence of *Aeromonas hydrophila* is associated with extracellular toxins and enzymes including hemolysins, cytotoxins, enterotoxins, and proteases. This pathogenic organism is able to colonize tissues of the host by adherence mechanisms. Some of these mechanisms are additional protein layers, O antigens, fimbriae, and other unidentified means. Nonetheless, the particular virulence mechanism and the role of *A. hydrophila*'s pathogenicity factors have not been clearly established (Paniagua and others 1990).

A study by Cumberbatch and others (1979) examined toxin production by *Aeromonas hydrophila* and its relationship to diarrhea. Sixty-six (69%) of 96 isolates were positive for cytotoxin and hemolysin production. Of diarrheal isolates, 32/40 (80%) were toxigenic compared to 9/22 (41%) of non-diarrheal isolates ($P = 0.004$). There was no correlation between toxigenicity and the presence or absence of plasmids. Cumberbatch and others (1979) concluded that the enteropathogenic potential of *A. hydrophila* is mediated by a cytotoxic enterotoxin and may be a cause of diarrheal disease.

Antibiotic resistance is a major concern with human pathogens. Most species of *Aeromonas* are susceptible to cephalosporins, aminoglycosides, carbapenems, tetracyclines, trimethoprim-sulfamethoxazole, and quinolones

(Gunsalam and others 2006). In a study by Fass and Barnishan (1981), 32 antimicrobial agents were tested against 20 strains of *A. hydrophila*. Most isolates were susceptible to chloramphenicol, tetracycline, aminoglycosides, and trimethoprim-sulfamethoxazole, and resistant to penicillins, cephalosporins, erythromycin, and polymyxins. Moxalactam, a member of the β -lactam antibiotic family, was the most active antimicrobial against *A. hydrophila* (Fass and Barnishan 1981).

In a study by Vila and others (2003), 863 patients with traveler's diarrhea were evaluated in Barcelona, Spain. *Aeromonas* spp. were isolated in 18 (2%) of these patients with symptoms of persistent watery diarrhea, fever, and abdominal cramps. *A. sobria* was isolated from nine patients, *A. caviae* from seven, *A. jandaei* and *A. hydrophila* from one patient each. When antibiotic susceptibility was evaluated, all isolates were susceptible to cefotaxime, ciprofloxacin, and nalidixic acid. They were resistant to ampicillin and variably resistant to chloramphenicol, tetracycline, and cotrimoxazole (Vila and others 2003).

Eight antibiotic resistant *Aeromonas hydrophila* isolates from infected fish were evaluated in a study by Rahim and others (1984). Isolates were also tested for enterotoxin production, hemolysin production, and the correlation between enterotoxin and hemolysin production. All *A. hydrophila* strains were susceptible to streptomycin, neomycin, gentamicin, and polymyxin B. Two were resistant to chloramphenicol, trimethoprim-sulfamethoxazole, and cefamandole. Three were resistant to tetracycline and erythromycin, and 5 were resistant to vancomycin. If fish infected with antibiotic resistant *A. hydrophila* are consumed without proper

cooking, then enterotoxigenic diarrhea may result (Rahim and others 1984). It is important to monitor antibiotic sensitivity/susceptibility of food-borne pathogens, like *Aeromonas*, because of their significance to human health.

Fish Disease

Aeromonas infections cause hemorrhagic septicemia in freshwater fish and some marine fish species. Motile *Aeromonas* Septicemia (MAS), the disease caused by infection with *Aeromonas* spp., is one of the most common and problematic diseases of cultured fish (Post 1987; Paniagua and others 1990). *A. hydrophila*, *A. caviae*, and *A. sobria* are three species that cause MAS in fish (Winton 2001). Fish culture facilities may develop MAS regardless of season because water temperature is maintained at a relatively constant temperature (Post 1987).

Stress such as overcrowding, rough or excessive handling, and poor water quality may cause fish to be more susceptible to bacteria (Post 1987, Winton 2001). Nutrient deprivation, injured or damaged skin, and water with high amounts of feces increase susceptibility to infection (Post 1987). Elevated water temperature, low dissolved O₂ concentration, or high ammonia and CO₂ concentrations are a few water quality stressors for fish, thus increasing their susceptibility to infection (Cipriano 2001).

Motile aeromonads are transmitted orally, through skin or gill abrasions, or in conjunction with external fish parasites that cause skin abrasions. Organisms multiply in the intestine and/or at the site of invasion, and then spread throughout the body via the blood stream. Incubation period is dependent on environmental temperature or body temperature of the host. Acute cases may appear within 4-

10 d, while sub-acute or chronic cases may take longer to develop. Latent cases and carrier fishes are particularly problematic in *A. hydrophila* infections.

Infection can spread rapidly, especially if the fish are stressed or very young.

Morbidity among fish populations with MAS may approach 100%. Mortalities can be as high as 80% among young, stressed, or injured fish, and 20-35% in older fish (Post 1987).

Of the salmonids, rainbow trout are the most susceptible to MAS (Cipriano 2001; Winton 2001). Normally, bacteria enter through abrasions on the skin or intestinal tract. It is also possible for some aeromonads that are natural inhabitants of the gut microflora to become pathogenic when the fish are stressed and their immune response is lowered. The severity of the disease is dependent on bacterial virulence, type and degree of stress, physiological condition (gender, age, size) of the host, and genetic resistance or predisposition within the fish population. MAS is most severe among fish grown under intensive culture conditions (Cipriano 2001). Because *Aeromonas* spp. are members of the normal intestinal microflora of trout, they are present on many trout farms (Lee and others 2002).

There are several signs of MAS infection that include small hemorrhages at the base of the fins or on the skin, protruding eyes, swollen liver and spleen, and distended and fluid-filled intestines (Post 1987). Fluid in the abdominal cavity, causing the abdomens to become distended, may be opaque or bloody (Post 1987, Winton 2001). The liver may become pale or greenish, the kidneys may be swollen and soft, and the intestines may be filled with yellow mucus.

Usually, the lower intestine and vent are swollen and inflamed with a bloody discharge (Winton 2001).

Pathological conditions attributed to MAS infection include: tail or fin rot, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease, and scale protrusion disease. Clinical signs of infection are reddening of the skin and fluid filled scale pockets. This causes the scales to protrude, giving a 'washboard' effect (Cipriano 2001). Fish may have grayish-red ulcerations on the skin and eyes with inflammation and erosion in and around the mouth (Cipriano 2001; Winton 2001). Orbits of the eye may rupture due to thrombosis and inflammation, leading to blindness. If left untreated, leptomeningeal congestion may occur in the brain, possibly leading to death (Cipriano 2001).

Aeromonas hydrophila, *A. sobria*, and *A. caviae* are the most common clinical isolates associated with fish. They adhere to eukaryotic cells that have mucus receptors via fimbriae regardless of their virulence. In rainbow trout, strains of *A. hydrophila* that are highly virulent share a common O-antigen. In comparison, there is no common O-antigen for low-virulent strains of *A. sobria*. *A. hydrophila* hydrolyzes esculin and ferments salicin and arabinose; whereas, *A. sobria* does not utilize these compounds. Both species are able to produce hemolysis on blood agar plates at 30 °C, but *A. hydrophila* is also able to do so at 10 °C as well (Cipriano 2001). Strains of *A. hydrophila*, isolated from healthy and diseased fish, are more hemolytic, dermonecrotic, and virulent than strains of *A. sobria* (Leblanc and others 1981; Cipriano 2001).

There are many virulent, avirulent, and attenuated aeromonads that possess hemorrhagic factors and lethal toxins with different levels of toxicity (Cipriano 2001). Paniagua and others (1990) reported that 77.02% of *Aeromonas hydrophila* strains were virulent for trout when an intramuscular injection was given. The 50% lethal dose of the most virulent strain, having a 100% mortality rate, was 10^7 cells. Using the same method of pathogenicity testing, 63.6% of *Aeromonas sobria* isolates were also virulent. Fish death occurred within 48-72 h after injection with lesions usually progressing to hemorrhagic during the course of infection. When examined postmortem, hemorrhages of the internal abdominal wall, a pale colored liver, pronounced and sometimes sanguinous ascites, and necrotic lesions of the skeletal muscle at the site of injection were observed. Strains of aeromonads tested were always recovered from the kidneys and external necrotic lesions of the fish (Paniagua and others 1990).

The *Aeromonas hydrophila aroA* live vaccine is effective against strains of *A. hydrophila* and *A. salmonicida* in rainbow trout. The vaccine is an auxotrophic mutant derived from a virulent strain of *A. hydrophila* that is administered through an intraperitoneal injection. Ideally, the vaccine elicits a high and long-lasting immune response that enables the vaccine to persist in the tissues and express protective antigens (Vivas and others 2005). Antibodies are then produced to recognize these specific antigens. Unfortunately, this product is not yet licensed for use in the USA; however, several antibiotics are available.

A few antibiotics are commonly used for treating MAS in fish; oxytetracycline (terramycin) is the most effective. It has been approved to treat salmonid species when orally administered at a daily rate of 50-75 mg/kg of fish over a 10 d period. Chloramphenicol (chloromycetin) is effective in treating fish, but is prohibited for use in food fish. Antibiotic resistant strains could develop and compromise the effectiveness of this antibiotic for treatment of human disease (Cipriano 2001). Sulfamerazine at 264 mg/kg of fish administered in food for 3 d followed by 154 mg/kg of fish for an additional 11 d is also an effective treatment (Post 1987).

The use of antimicrobials in aquaculture is of concern because of the risk of multiple antibiotic resistance commonly associated with fish pathogens and aquatic bacteria (Saavedra and others 2004). Austin and Al-Zahrani (1988) examined several bacterial isolates from the gastrointestinal tract of rainbow trout for antibiotic resistance. Among the organisms tested was *Aeromonas hydrophila*. When tested for erythromycin resistance, there was an increase in *A. hydrophila* at d 1, then a decrease by d 5. Penicillin G and sulphafurazole resistance yielded the same trends as erythromycin (Austin and Al-Zahrani 1988).

Infections caused by antibiotic resistant *Aeromonas* spp. are a common problem in fish raised in recirculation systems. In a study by Saavedra and others (2004), 51 strains of *Aeromonas* were isolated from 20 rainbow trout skin, kidney, and raceway water samples. Fish symptoms included lesions or alterations in skin and kidney tissues. Antibiotics of the β -lactam family

(penicillins, cephalosporins, monobactams, and carbapenems) that are used in clinical treatment of humans were evaluated for resistance. The least effective antibiotics were amoxicillin, carbenicillin, and ticarcillin (Saavedra and others 2004). The transfer of resistance to organisms of human and/or veterinary significance could be detrimental to human and fish health.

***Aeromonas* in Food and Water**

Since 1960, *Aeromonas* spp. have been considered opportunistic human pathogens. In 1984, the US Food and Drug Administration (FDA) introduced *A. hydrophila* as a 'new' food-borne pathogen (Isonhood and Drake 2002). In December 1995, FDA implemented a mandatory quality assurance program for fish and fishery products based on the Hazard Analysis and Critical Control Points (HACCP) concept. Today, *Aeromonas* spp. are considered an emerging human pathogen of importance because they are ubiquitous in aquatic environments, have multiple virulence factors, and are psychrotrophic in nature (Schuman and others 1997; Isonhood and Drake 2002; Vivekanandhan and others 2005).

Psychrotrophs are cold tolerant bacteria that prefer to grow in temperatures $>0^{\circ}\text{C}$, a maximum of $30\text{--}35^{\circ}\text{C}$, and $12\text{--}15^{\circ}\text{C}$ optimal. Psychrotrophic food-borne pathogens, such as *Aeromonas hydrophila*, can grow and/or produce toxins at 5°C (Kraft 1992). In addition to being a safety concern for consumers, *Aeromonas* spp. are spoilage organisms and can affect the quality of refrigerated food (Kraft 1992; Fernandes and others 1998). Like other spoilage bacteria, aeromonads can produce compounds such as H_2S , proteases,

and lipases that can cause off odor and flavor development (González and others 2001).

Fish muscle is highly susceptible to spoilage organisms because its pH is closer to neutrality (~6.2) and rich in available nutrients (Kraft 1992; Fernandes and others 1998). Fernandes and others (1998) inoculated cultured rainbow trout fillets with $10^{4.7}$ CFU *A. hydrophila*/100 g fish, stored them at 2-4 °C, and monitored aerobic plate counts and *A. hydrophila* counts for 15 d. Aerobic plate counts and *A. hydrophila* counts increased ($P < 0.05$) from 6.0 to 9.0 log CFU/100 g and 4.5 to 7.2 log CFU/100 g, respectively. This growth not only spoiled the fillets after 13 d, but is also a food safety risk (Fernandes and others 1998).

Many *Aeromonas* spp. are able to grow at temperatures as low as 5 °C in most foods (Palumbo and others 2001; Isonhood and Drake 2002). Since refrigeration temperature is 4-7 °C, *A. hydrophila*, with its growth range of 0-41 °C, is a concern to public health (Isonhood and Drake 2002; Vivekanandhan and others 2005). Vivekanandhan and others (2005) found that *A. hydrophila* was able to grow at a sufficiently rapid rate to be competitive with other psychrotrophic bacteria in foods. Because of its frequent isolation from various foods and its psychrotrophic nature, *A. hydrophila* is considered to be a significant food-borne pathogen (Palumbo and others 1985; Schuman and others 1997).

A primary concern is the growth of psychrotrophic pathogens at refrigeration temperatures and the possibility of cross-contamination with ready-

to-eat foods in grocery store display cases or at home (Pin and others 1994; Fernandes and others 1998). The sale of fish, shellfish, and fish products contaminated with psychrotrophic pathogens, such as *Aeromonas hydrophila*, is hazardous. They can multiply to dangerous levels on refrigerated fresh fish during normal storage time and cause food-borne disease (Fernandes and others 1998). Since motile aeromonads can grow at 5 °C, they may also grow on animal carcasses in abattoirs (Pin and others 1994) or become a serious risk to muscle food safety as a post processing contaminant.

Aeromonas spp. occur widely in both fresh and processed foods. Most likely, it is a result of food coming in contact with a water source containing the bacteria. Many *Aeromonas* isolates from water and several foods possess virulence factors that are typical of most enteric pathogens. This pathogenicity is especially a concern in immunocompromised individuals such as patients with underlying malignancies, elderly, young children, people traveling in developing countries (Palumbo and others 2001) and patients with hepatic illnesses (Janda 1991). In some cases when immunocompromised patients are involved, the infection may be fatal (Daily and others 1981).

Pathogenic strains of motile aeromonads have been isolated from a high percentage of aquatic habitats including, but are not limited to, freshwater, brackish water (Palumbo and others 2001; US Food and Drug Administration 1991), and marine environments (Palumbo and others 2001). They are frequently found in chlorinated and unchlorinated drinking water (Pin and others 1994; Palumbo and others 2001), frogs (Rouf and Rigney 1971; Cipriano 2001),

marine and freshwater fish, shellfish, fish products, beef (Pin and others 1994; Schuman and others 1997; Cipriano 2001; Palumbo and others 2001; US Food and Drug Administration 1991), raw milk (Pin and others 1994; Schuman and others 1997; Cipriano 2001), poultry (Schuman and others 1997; Cipriano 2001; Palumbo and others 2001; US Food and Drug Administration 1991) pork, lamb (Schuman and others 1997; Palumbo and others 2001; US Food and Drug Administration 1991), eggs, veal, butter, and whipped cream (Schuman and others 1997). Motile aeromonads have also been found in sausage (Rouf and Rigney 1971; Vivekanandhan and others 2005), meat offal, a variety of uncooked and ready-to-eat foods, sewage (Vivekanandhan and others 2005), fresh vegetables (Pin and others 1994; Palumbo and others 2001; Isonhood and Drake 2002), unripened cheese (Pin and others 1994), spices (Palumbo and others 2001), sinks, drains, and household effluents (Gunsalam and others 2006).

There are several effective means of inhibiting the growth of aeromonads other than refrigeration. Most *Aeromonas* spp. can easily be destroyed by irradiation, heat, and other food processing treatments (Palumbo and others 2001). They are heat and acid labile, and they do not grow below pH 5 or above 3.5% NaCl. Polyphosphates with NaCl, sorbates, and smoking are also methods of inactivating *Aeromonas* spp. (Isonhood and Drake 2002). Sodium nitrite in combination with 2.5-3.5% NaCl at pH $\leq$ 6.3 and 5-12 °C inhibits growth of *A. hydrophila* (Kraft 1992).

The specific infectious mechanisms that cause gastroenteritis are unknown; however, *A. hydrophila* and *A. caviae* have been recognized as the

most common species of *Aeromonas* associated with diarrhea. In the US, it has been estimated that up to 13% of gastroenteritis cases are caused by *Aeromonas* spp. Because of their ability to adapt to a wide range of environmental conditions, aeromonads are able to grow on a variety of foods. This contaminated food, if ingested, may possibly lead to illness (Isonhood and Drake 2002).

Although the infectious dose of *Aeromonas* spp. in food has not been identified, adequate cooking should inactivate these bacteria (González and others 2001). Schuman and others (1997) determined the level of thermal inactivation needed to eliminate *A. hydrophila* in raw liquid whole egg. The D values (time needed to reduce bacterial population by 90%, 1 log, at a specified temperature), z_D values (temperature change in °C needed to give a 10-fold change in the D value), and E_a (activation energy) were determined for initial populations of 7.0-8.3 log CFU *A. hydrophila* heated over a range of 48-60 °C. D values at 48 °C were 3.62-9.43 min and 0.026-0.040 min at 60 °C. Decimal reduction time curves yielded z_D values of 5.02-5.59 °C and E_a values of 380-343 kJ/mol. The z_D values were within the 4-6 °C, similar to other non-spore-forming bacteria (Schuman and others 1997).

The majority of *Aeromonas*-implicated, food-borne disease outbreaks between 1977 and 1991 involved seafood such as oysters, sashimi, prawns, and shrimp (Isonhood and Drake 2002). Several FDA surveys noted *Aeromonas hydrophila* in shellfish growing areas in the state of Washington and on the West Coast. *A. hydrophila* counts ranged from 3 to 4,600/100 g in oysters and 3 to

2,400/100 mL in water. In West Coast estuaries, 200/400 samples of shellfish and sediment contained *Aeromonas* spp. In Louisiana in 1982, 472 cases of gastroenteritis were associated with *A. hydrophila* contaminated oysters that had been frozen at -72 °C for 1.5 years. Oysters from the same growing area in Louisiana were later implicated in another outbreak in Florida (Kraft 1992).

Gunsalam and others (2006) isolated 19 *Aeromonas hydrophila* strains from fresh milk (2/34), water (4/35), and prawn (13/32) samples from a wet market in Selangor, Malaysia. Of these isolates, 17 (89.5%) contained plasmids that could be grouped into 8 profile patterns based on multiple antibiotic resistance. The high number of *A. hydrophila* in prawn samples indicate that either the prawns were cultured in polluted water, or they were contaminated. Bacterial contamination could have occurred as a result of unhygienic practices during harvest, transportation, or storage at the wet market (Gunsalam and others 2006).

Pin and others (1994) purchased 80 food samples and tested them for the presence of motile *Aeromonas* spp. Poultry had the highest frequency of detection with 100% positive. Lamb samples were 60% positive, while 20% of raw milk and unripened cheese samples were positive. Forty percent of the beef and pork samples were positive. Shellfish and finfish samples were 45% and 36% positive, respectively. No motile aeromonads were present in pre-prepared salads. Most isolates were *Aeromonas hydrophila*, although *A. sobria*, *A. caviae*, and unclassified *Aeromonas* spp. were also observed (Pin and others 1994).

In a study by Lee and others (2002), water samples were collected from a trout farm, a sedimentation pond at the farm, and upstream and downstream of the farm in January, May, August, and November. Heterotrophic plate counts of samples collected in the warmer months (May and August) were higher than those from the colder months (January and November); although, *Aeromonas* spp. were isolated from all water samples. *A. hydrophila* and *A. caviae* were the most frequently isolated species, especially during the warmer months. *A. salmonicida* was also widely distributed and dominant at lower temperatures during the colder months. Because of human health risks associated with *Aeromonas* contaminated trout, it is important to monitor their levels at fish farms (Lee and others 2002).

González and others (2001) examined 171 isolates of *Aeromonas* spp. from the skin, gills, and intestines of farmed rainbow trout, wild brown trout, wild pike, and water samples. Of the isolates, 88% were identified as known species of *Aeromonas* with 29% *A. hydrophila*, 19% *A. sobria*, 18% *A. bestiarum*, 16% *A. jandaei*, 2% *A. caviae*, 2% *A. schubertii*, and 0.6% unnamed. The remaining 12% were similar to *A. schubertii*, but were not recognized as a specific species. Whether the fish were wild or farm-raised influenced the initial levels of *Aeromonas* present on the skin, gills, and in the intestines. Farmed rainbow trout had significantly lower ($P < 0.05$) Starch Ampicillin Agar (SAA) counts than wild brown trout and pike. The clinically important isolates to human health, *A. hydrophila*, *A. sobria*, and *A. caviae*, were isolated from farmed rainbow trout. Not only are these bacteria a concern for public health, particularly with the

potential for cross-contamination, but they also contribute to the deterioration of fillet quality (González and others 2001).

Because *Aeromonas* spp. can grow at psychrotrophic and mesophilic temperatures, it is important to monitor their levels and define specific temperature requirements for this organism. McAdams and others (2005) determined psychrotrophic and mesophilic, aerobic plate counts of rainbow trout. There was no significant difference ($P > 0.05$) between whole rainbow trout or rainbow trout fillets between five different aquaculture facilities. Sampling season also showed no significant difference ($P > 0.05$). Psychrotrophic counts ranged from 1.6×10^4 to 1.5×10^5 CFU/g for whole trout and 1.7×10^4 to 3.7×10^5 CFU/g for fillets. Mesophilic counts ranged from 1.7×10^4 to 2.0×10^5 CFU/g for whole trout and 3.0×10^4 to 4.9×10^5 CFU/g for fillets. These levels reflect the bacterial microflora present on the surface of the fish which may be transferred to the fillet during processing (McAdams and others 2005).

Variations in *A. hydrophila* levels for food from different regions of the world may be a result of handling (Vivekanandhan and others 2005). Sources of fillet contamination may be the outer surface of the fish, the intestinal tract, or the environment during processing (Kraft 1992). Secondary contamination may occur during transportation, storage, or distribution. Contaminated water may also be a reason for the presence of *Aeromonas* spp. (Vivekanandhan and others 2005; Gunsalam and others 2006). Rainbow trout, like all fish, can harbor microorganisms potentially pathogenic to humans (McAdams and others 2005). Decreasing bacterial load in the water and using stringent sanitation during

processing may reduce fillet contamination, thus resulting in a longer shelf-life and safer products.

Sampling Procedures

Detection of food-borne pathogens in food is limited by the sampling method. Several methods are typically used for sampling the bacterial microflora associated with the surfaces of finfish. Samples can be prepared by excising and blending skin and/or muscle tissue, rinsing the whole carcass, or swabbing the skin surface. The method used is often a personal preference of the researcher (Nedoluha and others 2001).

Nedoluha and others (2001) compared three sampling methods used for detection of microorganisms in finfish fillets. Aerobic plate counts of rainbow trout fillets rinsed in Butterfield's Phosphate Buffer (BPB) were slightly lower than counts from fillets blended in a stomacher with BPB. Sampling method did not affect plate counts on media selective for *Aeromonas* spp.; however, fillet rinses yielded a lower percentage ($P < 0.05$) of *Aeromonas* isolates than stomached samples and samples rinsed before being stomached. This study supports previous findings that stomaching/blending is superior to rinsing for enumerating bacteria from fish fillets, although both methods are suitable for determining the predominant microorganisms present on finfish surfaces (Nedoluha and others 2001).

Palumbo and others (1985) developed a selective and differential medium to quantitatively recover bacteria in the *Aeromonas hydrophila* group (*A. hydrophila*, *A. sobria*, and *A. caviae*) present in food. Starch Ampicillin Agar (SAA) consists of phenol red agar base, 10 g/L soluble starch, and 10 mg/L

ampicillin. Starch and ampicillin were incorporated as differential and selective agents, respectively, because *Aeromonas* spp. are resistant to ampicillin and can hydrolyze starch. After incubation for at 28 °C for 24 h, the plates are flooded with Lugol's iodine solution. Presumptive *Aeromonas* colonies are identified as being amylase positive (having a clear zone around them), 3-5 mm in diameter, and yellow to honey colored when Lugol's is added. The SAA enables isolation of *A. hydrophila* from foods with large numbers of competing bacteria present (Palumbo and others 1985).

In a study by Vivekanandhan and others (2005), 536 fish and 278 prawn samples were collected from a major fish market in Coimbatore, South India. The samples were analyzed for the presence of *Aeromonas hydrophila* using Starch Ampicillin Agar (SAA) incubated at 37 °C for 18-24 h. The plates were flooded with Lugol's Iodine solution to reveal amylase positive yellow to honey colored colonies. These colonies were considered presumptive positive and were isolated on Nutrient Agar (NA). The isolates were then confirmed as *A. hydrophila* by their biochemical characteristics. The prevalence of *A. hydrophila* was 17.62% in prawns and 33.58% in fish, with a higher frequency during the monsoon season (Vivekanandhan and others 2005).

Aeromonas spp. can be isolated in a number of different media such as MacConkey Agar, Hektoen Enteric Agar, and Xylose Lysine Desoxycholate Agar since they are not extremely fastidious. Amylase activity and ampicillin resistance are used as selective characteristics to isolate *Aeromonas* spp. from foods. Media such as Glutamate Starch Penicillin Agar, Ampicillin Dextrin Agar,

and Starch Ampicillin Agar utilize these selective methods (Isonhood and Drake 2002). Glutamate Starch Phenol Red Agar, an alternate isolation medium, is selective and differential. Suspected *Aeromonas* colonies are round and yellow surrounded by a yellow zone (Gunsalam and others 2006). This medium may be more user friendly than SAA when isolating and/or enumerating *Aeromonas* spp.

Villari and others (1999) compared several culture media for the membrane filter enumeration of *Aeromonas* spp. in water. Water samples were seeded with suspensions of *Aeromonas* spp., fecal material, and raw sewage, and filtered through 0.45 µm ester filters. Filters were placed on a variety of different media and incubated aerobically or anaerobically for 37 or 30 °C for 24 or 48 h to determine the most suitable methodology for the quantitative detection of *Aeromonas* spp. The best results were obtained when plates were incubated aerobically at 30 °C for 24 h. Villari and others (1999) concluded that if bacterial contamination is likely to be low, then sensitive culture media such as SAA, Rippey Cabelli Agar (mA), Ampicillin Dextrin Agar (ADA), or Pril Ampicillin Dextrin Ethanol Agar (PADE Agar) is suggested. In contrast, if background microflora is expected to be high, then a more selective medium such as Ampicillin Bile Salts Inositol Xylose Agar (MIX Agar) is recommended (Villari and others 1999).

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CHAPTER

Frequency of *Aeromonas* spp. Detection in Rainbow Trout and Recirculation Aquaculture Systems and the Storage Stability of Fillets

ABSTRACT

Recirculation aquaculture increases trout production per unit volume of water. *Aeromonas* spp. are pathogenic to fish and humans plus contribute to fillet spoilage. The objective was to survey the presence of *Aeromonas* spp. in water reuse systems (source water, flow-through, 80% reuse, and 95% reuse), on the fish, and on the fillet. Psychrotrophic plate counts of fish rinsates and butterfly fillets were also determined during the eight month study. Three fish per system were rinsed in Butterfield's Phosphate Buffer (BPB) and analyzed for *Aeromonas* spp. and psychrotrophic plate count. Three additional fish per system were filleted, halved, and stored at 4 ± 2 °C for 0 or 6-d before being analyzed. *Aeromonas* spp. were isolated on Starch Ampicillin Agar (SAA) and confirmed with API 20NE strips. Psychrotrophic counts were determined initially and after 6-d refrigeration. The occurrence of *Aeromonas* spp. was greatest in January (16/21), whereas the least were in November and February (4/21). Fillet psychrotrophic counts increased ($P < 0.05$) with refrigerated storage and water reuse, while rinsate psychrotrophic counts were highest for 95% reuse. The flow-through system produced fish that gave the highest ($P < 0.05$) percent fillet yield. *Aeromonas* spp. detection was affected by sampling period and water reuse. Prevalence of *Aeromonas* spp. increased in January, coinciding with the discontinuation of UV light and ozone treatments for the 95% reuse system. Implementing pre-harvest controls for *Aeromonas* can decrease fillet contamination, resulting in a longer shelf-life and a safer product for consumers.

Key Words: water reuse, *Aeromonas*, rainbow trout

INTRODUCTION

With an expanding market for fish and fish products, the demand for seafood is projected to exceed 3 million tons in the US by 2020 (Pitney and Bopp 2006). In areas with limited high-quality water resources, alternative methods of large-scale fish production, including recirculated aquaculture systems, are being implemented to fill this demand. Coldwater species, such as rainbow trout, have been efficiently cultured in partial-reuse systems (Summerfelt and others 2004), thus increasing trout production per unit volume of water. The partial-reuse system at The Conservation Fund's Freshwater Institute (TCFFI) was able to sustain a production level of 35-45 kg/year of rainbow trout for every 1 L/min of make-up water, which equals 11,000-17,000 kg/year. This level of production is approximately 6-7 times greater than the serial-reuse raceway systems in the Snake River Valley of Idaho that produces 6 kg/year of trout for every 1 L/min of water (Summerfelt and others 2004).

In order to produce safe and wholesome aquacultured products, producers need to recognize the extent and dynamics of bacterial growth in their system and its effect on fish gut microflora. The microflora of fish intestines consists of predominately aerobic or facultatively anaerobic bacteria that are also present in water (Huber and others 2004). The genus *Aeromonas*, consisting of 14 species, is officially classified as a member of the family *Aeromonadaceae* (Isonhood and Drake 2002). They are ubiquitous microorganisms that have been isolated from environmental, clinical, and food samples (Cipriano 2001; Isonhood and Drake 2002; Lee and others 2002) and are found in the

gastrointestinal tract of fish (Austin and Al-Zahrani 1988, Spanggaard and others 2000, Huber and others 2004).

Aeromonas spp. are considered emerging human pathogens of importance because they are ubiquitous in aquatic environments, have multiple virulence factors, and are psychrotrophic in nature (Isonhood and Drake 2002; Vivekanandhan and others 2005). Motile aeromonads can act as infectious or enterotoxigenic pathogens causing gastroenteritis, wound infections, and septicemia (Isonhood and Drake 2002). The three most virulent species of *Aeromonas*, all of which are motile, (*A. hydrophila*, *A. sobria*, and *A. caviae*) (Cipriano 2001) are of primary concern to public health (Palumbo and others 2001) because they are a source of intestinal infections and diarrheal disease when contaminated food or water is ingested (Paniagua and others 1990; Janda 1991; Isonhood and Drake 2002; Lee and others 2002). They are also the most common clinical isolates associated with fish (Cipriano 2001).

Since *Aeromonas* spp. are members of the normal intestinal microflora of trout and several health risks are associated with *Aeromonas* contaminated trout, it is important to monitor their levels on fish farms (Lee and others 2002). Not only are these bacteria a concern for public health, particularly with the potential for cross-contamination, but they also contribute to the deterioration of fillet quality (González and others 2001). Sources of fillet contamination may be the outer surface of the fish, the intestinal tract, or the environment during processing (Kraft 1992). Decreasing bacterial load in the water and using stringent

sanitation during processing may reduce fillet contamination, thus resulting in a longer shelf-life and safer products.

In addition to being a safety concern for consumers, *Aeromonas* spp. are spoilage organisms and can affect the quality of refrigerated food (Kraft 1992; Fernandes and others 1998). Vivekanandhan and others (2005) found that *A. hydrophila* was able to grow at a sufficiently rapid rate to be competitive with other psychrotrophic bacteria in foods. Although the optimum growth temperature for *A. hydrophila* is 28 °C, this psychrotrophic food-borne pathogen is capable of growing from 1-42 °C (Isonhood and Drake 2002) and produce toxins at ≥ 5 °C (Kraft 1992).

Pathogenic motile aeromonads have been isolated from freshwater, brackish water (Palumbo and others 2001; US Food and Drug Administration 1991), and marine environments (Palumbo and others 2001). They have also been found in chlorinated and unchlorinated drinking water (Pin and others 1994; Palumbo and others 2001), fish, shellfish, fish products, beef (Pin and others 1994; Schuman and others 1997; Cipriano 2001; Palumbo and others 2001; US Food and Drug Administration 1991), raw milk (Pin and others 1994; Schuman and others 1997; Cipriano 2001), poultry (Schuman and others 1997; Cipriano 2001; Palumbo and others 2001; US Food and Drug Administration 1991) pork, lamb (Schuman and others 1997; Palumbo and others 2001; US Food and Drug Administration 1991), eggs, veal, butter, whipped cream (Schuman and others 1997), sausage (Rouf and Rigney 1971; Vivekanandhan and others 2005), fresh

vegetables (Pin and others 1994; Palumbo and others 2001; Isonhood and Drake 2002), and unripened cheese (Pin and others 1994).

In the US, it has been estimated that up to 13% of gastroenteritis cases are caused by *Aeromonas* spp. (Isonhood and Drake 2002). Because of its frequent isolation from various foods and its psychrotrophic nature, *A. hydrophila* is considered to be a significant food-borne pathogen (Palumbo and others 1985; Schuman and others 1997). Infection with *Aeromonas hydrophila* can result in serious complications, especially in immunocompromised hosts, the elderly, and children (Palumbo and others 2001; Isonhood and Drake 2002). The frequency of disease due to *A. hydrophila* in the US is unknown; although, efforts are being made to quantify its occurrence (US Food and Drug Administration 1991).

Because *Aeromonas* spp. are pathogenic to humans and fish as well as cause spoilage, it is important to monitor their occurrence in fish productions systems. The objectives of this study were to survey the *Aeromonas* spp. present in the production system, on the fish, and on the butterfly fillet. Psychrotrophic plate counts of rainbow trout fillets were also determined as part of a 6-d shelf life study.

MATERIALS AND METHODS

Partial Reuse System

Rainbow trout and water samples were collected from aquaculture production facilities at The Conservation Fund's Freshwater Institute (TCFFI) in Shepherdstown, WV, USA. Fish were reared in Cornell-type, dual-drain partial water reuse culture tank systems. This design provides an alternative to serial reuse raceway systems in salmonid production (Summerfelt and others 2004). Three levels of water reuse were monitored from June 29, 2006 to February 20, 2007: 1) 100% fresh water in eight, 0.5 m³ flow-through culture tanks, 2) 80% reuse and 20% fresh water in three, 10 m³ tanks (Fig. 1), and 3) 95% reuse and 4-6% fresh water in a large, 150 m³ tank (Fig. 2).

All water is eventually replaced in a water reuse system. The water exchange rate is maintained at 1100-1900 L/min to ensure that waste metabolites are flushed away and that a sufficient amount of dissolved O₂ is delivered to the fish (Summerfelt and others 2004). As an example, if 5% fresh water is being circulated in the 95% reuse system, 230 L of water are being replaced each min. In 1 h, 13,800 L of fresh water are replacing reused water (60 min x 230 L = 13,800 L/h). In one day, 331,200 L of water total are used (24 h x 13,800 L/h = 331,200 L). Since the 95% reuse system is 151,400 L, the total water is exchanged over twice per day.

Water intended for the 95% reuse system is discharged through a sidewall drain and is processed through a series of steps before it is recirculated back into the system (Fig. 2). Initially, the discharged water is filtered across a microscreen drum filter, biofiltered for nitrogen products, and then pumped back

to the head of the system. Secondly, carbon dioxide (CO₂) is stripped and oxygen (O₂) is supplemented. Water flowing to the 95% reuse tank is treated with ultraviolet light and ozone (O₃). The remaining uncirculated water exits through a center bottom drain, flows through an external standpipe, held in the radial clarifier, refiltered, and discarded as waste.

Water quality was monitored throughout the study. These measurements included: temperature, pH, dissolved CO₂, dissolved O₂, TAN (total ammonia nitrogen), and TSS (total suspended solids). Dissolved CO₂ for the flow-through system ranged from 15-25 mg/L, dissolved O₂ was 9-11 mg/L, pH was 7.29-7.66, and temperature was 12.6-13.2 °C. Total ammonia nitrogen and TSS were not measured for this system. For the 80% partial reuse system, dissolved CO₂ was 14-24 mg/L, dissolved O₂ was 10-14 mg/L, pH was 7.49-7.91, temperature was 12.6-13.2 °C, TAN was 0.7-1.7 mg/L, and TSS was 0.8-3.07 mg/L. In the 95% reuse system, dissolved CO₂ was 10.8-28 mg/L, dissolved O₂ was 9.3-11.6 mg/L, pH was 7.21-7.83, temperature was 13.8-16.3 °C, TAN was 0.9-1.8 mg/L, and TSS was 1.02-5.03 mg/L.

Treatment of the 95% reuse water with ozone and UV light occurred irregularly throughout the study (Fig. 3). During the three initial sampling periods (June 29, August 8, and September 5, 2006), water had not been exposed to ozone. Ultraviolet light use began on June 30, 2006; consequently, only the June 29 sample was unaffected. Ozone treatment began on September 6, 2006 and continued through September 27, but was discontinued for the October 3 sampling. Ozonation resumed on October 13, 2006 and was functioning for the

November 8 and December 5, 2006 sampling periods. Ozonation and UV light were off from December 21, 2006 to January 12, 2007, but they were running for the January 18 sampling. Again, these water treatments were discontinued on February 8 and remained off until after the last sampling on February 20, 2007.

Fish Management

Rainbow trout stocked in the various systems were part of the all female 1105RBT cohort that were received and stocked in November 2005. The fish were reared in the fry system consisting of 12, circular, flow-through tanks with a maximum volume of 0.5 m³. Normal husbandry protocols were followed for this cohort of rainbow trout to ensure maintenance of an optimal environment for growth. Fish were handled minimally for routine weight measurements to assess growth response and for culling to maintain densities below 80 kg/m³. The Freshwater Institute's staff was responsible for fish husbandry and care as specified in TCFFI's Standard Operating Procedures for the Care and Use of Research Animals (Salmonid Fish) (Freshwater Institute 2003).

Fish density increased over time, but was kept below 80 kg/m³. Fish were stocked at relatively low densities (10-20 kg/m³), and continued to grow without harvesting until reaching about 80 kg/m³. At this point, the tanks' biomass, or fish quantity, was reduced to approximately 60-70 kg/m³. Fish growth continued until densities reached 80 kg/m³ once again. At this time, tanks were harvested to achieve a density of 60-70 kg/m³.

All fish were fed a slow-sinking extruded high energy trout feed (Zeigler Brothers, Gardners, Pennsylvania, USA) to satiation using automatic feeders (Sternier Fish Tech AS, N-1405 Langhus, Norway). Feed protein:fat ratios range

from 50% protein:15% fat for smaller fish to 42% protein:16% fat for larger fish. In this study, fish were fed 42% protein:16% fat from June 29 to November 29, 2006 and 45% protein:20% fat from November 30, 2006 to February 20, 2007. Fish were fed based on bodyweight, feed tables, and appetite-induced feeding behavior. Smaller fish (~100 g) were fed more than 2% bodyweight per d, while larger fish (≥ 600 g) were fed about 1% bodyweight per d. Fish in the flow-through system were fed every other h, fish in the partial reuse system were fed 8-10 times per d, and fish in the reuse system were fed about 8 times per d.

Sampling Procedure

Rainbow trout and water samples were collected from TCFFI at one month intervals from the end of June 2006 to the middle of February 2007 for a total of 8 samplings. Water samples were collected in triplicate from three systems differing in level of make-up water until October 2006. Thereafter, samples were only collected from the 80% and 95% reuse systems. The spring water source was sampled at all time periods. Three fish were sampled from each system for the first two samplings. Three additional fish per system were collected for the remaining sampling periods to determine psychotropic plate counts of the butterfly fillets. Trout feed was also enriched for *Aeromonas* spp.

Trout were caught using a net, immobilized by percussive stunning, and placed in a sterile, 3-L Whirl pak® bag (Fisher Scientific). A total of 102 fish were collected for the duration of the study. Measures were taken to minimize cross contamination when sampling. Water samples were collected in pre-sterilized bottles and 70% ethanol was sprayed on the net and club between fish. Gloves were changed between tanks and sprayed with ethanol between fish. Water and

fish samples were placed on ice for the 3-h transport to WVU lab facilities.

Samples were held on ice in a walk-in cooler (4 ± 2 °C) until processed. All samples were processed within 3-4 h of collection.

Enrichment and Isolation of *Aeromonas* spp.

Aeromonas spp. were isolated according to procedures described in the Compendium of Methods for the Microbiological Examination of Foods (Palumbo and others 2001) with a slight modification. This modification consisted of picking characteristic colonies from starch ampicillin agar and restreaking them on new agar before adding Lugol's solution to confirm presumptive positive colonies. Since Lugol's is an iodine solution consisting of iodine and potassium iodide, bacteria die when the solution is added to the plate. Media and incubation temperatures were followed as described in the Compendium.

Each Whirl pak® bag containing a fish was aseptically opened, and 100 mL of sterile, Butterfield's Phosphate Buffer (BPB) was added to the bag. Each fish was manually manipulated in the diluent for 10 s. In duplicate, 1 mL of the rinsate was transferred to 9 mL of Tryptic Soy Broth with Ampicillin (TSBA) to enrich for *Aeromonas*. Ampicillin was used to suppress the background microflora because *Aeromonas* spp. are resistant to it. All TSBA tubes were incubated at 28 ± 2 °C for 22-24 h.

After vortexing, growth from these tubes was streaked to isolation on Starch Ampicillin Agar (SAA) and incubated for 22-24 h at 28 ± 2 °C. Characteristic white, rough colonies were picked from each plate and restreaked to isolation on fresh SAA. These plates were subsequently flooded with Lugol's solution to check for a halo of starch hydrolysis around yellow, stained colonies

contrasted against dark brown, stained agar. If a halo formed around the previously selected colony on the original plate, then the restreaked plate was kept for further evaluation. If the characteristic colony was not surrounded by a halo of starch hydrolysis, then the restreaked plate was discarded.

Restreaked plates kept for further evaluation were incubated at 28 ± 2 °C for 22-24 h. A characteristic colony was picked from the restreaked plate and placed on a Nutrient Agar (NA) slant. Once again, the plate was flooded with Lugol's solution to re-confirm halos of starch hydrolysis. If the colonies picked formed a halo, the slant was kept because these starch hydrolyzing colonies are presumptive positive *Aeromonas* spp. If no halo formed around the colony, the slant was discarded. All slants that were kept were incubated at 28 ± 2 °C for 22-24 h.

Isolates on nutrient agar slants were used as a source of pure culture for several tests that followed. An oxidase test was performed to distinguish the oxidase positive *Vibrio* family, which includes *Aeromonas*, from the oxidase negative *Enterobacteriaceae* family. The 0/129 test, a water soluble vibriostatic agent (2,4-diamino-6,7-diisopropylpteridine phosphate), was used to distinguish 0/129 resistant *Aeromonas* isolates from the susceptible *Vibrio* genus. In order to perform the 0/129 test, full lawns of growth for all oxidase positive isolates were spread onto NA plates, and a 150 µg, 0/129 disk was placed on the surface. Plates were incubated at 28 ± 2 °C for 22-24 h before assessing whether or not a zone of inhibition was formed.

Isolates resistant to the 0/129 vibriostatic agent were considered presumptive positive *Aeromonas*. A Gram stain was performed on isolates obtained from the first collection to confirm that they were gram-negative rods. All presumptive positive isolates were confirmed by their biochemical characteristics using API 20NE strips. *Aeromonas* isolates were regrown on NA slants and were used to inoculate microprotect beads for long-term storage at -80 °C.

Trout feed was also analyzed for the presence of *Aeromonas* spp. Ten g of feed was ground using a mortar and pestle, placed in a stomacher bag, and blended in a stomacher for 30 sec with 90 mL BPB. One mL of the blended material was enriched in 9 mL TSBA in duplicate and incubated at 28 ± 2 °C for 22-24 h. After vortexing, liquid from these tubes were streaked to isolation on SAA and incubated at 28 ± 2 °C for 22-24 h. No growth resulted on these plates and analysis was complete.

Detection of *Aeromonas* spp. from Water Samples

Water samples were collected, in triplicate, in sterile bottles. The water source, a natural spring, was sampled along with water from each of the systems (0%, 80%, and 95% reuse). Spring water was collected prior to entering the pump station. Water samples for each system were collected from the bottom drain of each tank. This water is pumped from the bottom of the tank prior to treatment and recirculation; therefore, it may contain the highest bacterial load. Water and fish samples were placed on ice for the 3-h transport to WVU.

Each water sample was enumerated, enriched for *Aeromonas* spp., and tested for pH. Enumeration was carried out in duplicate by aseptically passing

100 mL of water through a pre-sterilized 47 mm 0.45 μm Whatman nylon membrane filter suctioned through a Gelman Magnetic Filter Funnel and placing it on a SAA plate. Presumptive positive *Aeromonas* spp. were counted on the water-filter SAA plates by flooding the plate with Lugol's solution and counting the yellow colonies with characteristic halos of starch hydrolysis. To determine the presence/absence of *Aeromonas*, 1 mL of water was enriched in 9 mL of TSBA in duplicate. TSBA tubes and SAA plates were incubated at 28 ± 2 °C for 22-24 h. The TSBA enrichment tubes were treated in the same manner as enrichments from fish rinses and fillet cores. The API 20NE identified, *Aeromonas* isolates were stored on microprotect beads at -80 °C. Water pH was tested, in duplicate, using a Corning pH/ion analyzer 350 meter.

Psychrotrophic Plate Counts

Three fish from each water reuse level were processed to produce butterfly fillets. Special care was taken to be aseptic in the filleting process since fillets were not rinsed before testing. Using clean knives, fish were filleted on a plastic cover that had been sanitized with ethanol. Gloves were either changed or sprayed with ethanol, and the work surface was wiped and sprayed with ethanol between each fish. Each fillet was equally divided into right and left halves and randomly assigned to either 0 or 6-d storage at 4 ± 2 °C. The remaining half, assigned to 6-d storage, was placed on a tray, covered with an oxygen permeable, polyvinyl chloride overwrap (PVC), and refrigerated for 6 d at 4 ± 2 °C.

Using a sterilized metal handheld corer, two, 11.4 cm² cores were taken from half of the trout butterfly fillet and blended in a stomacher for 30 s with 25 mL of BPB. Each core contained a flesh and a skin side; therefore, 22.8 cm² of flesh surface and 22.8 cm² of skin surface were sampled from each fillet half, initially and after 6-d storage. One mL of the blended material was enriched in 9mL of TSBA and incubated for 22-24 h at 28 ± 2 °C. Growth from the TSBA tubes were streaked onto SAA and these samples were treated in the same manner as the fish rinses and water samples.

Psychrotrophic plate counts were determined for fish rinses and core samples, before and after refrigeration for 6 d, in addition to being enriched for the presence/absence of *Aeromonas* spp. Psychrotrophic plate counts were performed using Plate Count Agar (PCA) and serial BPB dilutions, ranging from 10⁻¹ to 10⁻². The dilutions were spread plated onto PCA and incubated for 48 h at 26 ± 2 °C. After incubation, colonies were counted and recorded.

Following storage for 6 d at 4 ± 2 °C, the remaining half of the butterfly fillets were removed from the refrigerator and processed as previously described for *Aeromonas* enrichment. Psychrotrophic plate counts were also performed using serial BPB dilutions from 10⁻¹ to 10⁻⁴ on PCA. All *Aeromonas* isolates confirmed by API 20NE were transferred to microprotect beads and stored at -80 °C.

Statistical Methods

The independent variables in this experiment were level of water reuse, sampling period, and storage period. The dependent variables were psychrotrophic plate counts for fish rinses and fillet cores; water pH; fillet and

whole fish weights; presence/absence testing for *Aeromonas* in water, whole fish rinses, and cores; and presumptive positive *Aeromonas* enumeration for water samples. Data were analyzed using the General Linear Models platform of JMP® Statistical Analysis System produced by SAS® Institute, Cary, NC. Analysis of variance (ANOVA), variable by variable pairwise correlations, and regression analyses to determine linear fit were performed using log₁₀ counts. Significance was accepted as $P < 0.05$.

RESULTS AND DISCUSSION

Water Filters

Presumptive positive *Aeromonas* spp. on water filters (Fig. 4) differed ($P < 0.05$) with percent of water reuse. Water from the 80% reuse system had the lowest occurrence ($0.37 \log_{10}$ CFU/100 mL) compared to 1.00, 1.14, and $0.43 \log_{10}$ CFU/100 mL, respectively for the source, 95%, and flow-through systems. Counts ranged from 0.79-1.06, <0 -0.94, <0 -1.49, and 0.55-1.33 $\log_{10}$ CFU/100 mL for the flow-through, 80% and 95% reuse systems, and the source, respectively.

The amount of presumptive positive colonies also differed ($P < 0.05$) with sampling period (Fig. 4). On the average, presumptive positive counts decreased by $0.26 \log_{10}$ CFU/100 mL per sampling period. The highest frequency of presumptive positive *Aeromonas* spp. occurred in October for the source, flow-through, and 80% reuse systems (1.33, 1.06, and $0.94 \log_{10}$ CFU/100 mL, respectively) and November for the 95% reuse system ($1.49 \log_{10}$ CFU/100 mL). In contrast, the lowest frequency of presumptive positive *Aeromonas* spp. occurred in January for the source and the 80% reuse system (0.18 and $<0 \log_{10}$ CFU/100 mL, respectively), November for the flow-through ($0.79 \log_{10}$ CFU/100 mL), and February for the 95% reuse system ($<0 \log_{10}$ CFU/100 mL).

There was a significant correlation ($P < 0.05$) between sampling period and presumptive positive *Aeromonas* spp. on water filters because the number of presumptive positives decreases with time. Water at TCFFI was maintained at a relatively neutral pH, ranging from 6.9-8.2 throughout the duration of the study

(Table 1). The pH increased ($P < 0.05$) by 0.031 units per sampling period and varied with percent of water reuse. Source water pH (7.19 ± 0.045) was lower ($P < 0.05$) than flow-through (7.62 ± 0.060), 80% (7.60 ± 0.044), and 95% (7.35 ± 0.045) reuse systems.

Alonso and Garay (1989) enumerated motile *Aeromonas* spp. in sewage contaminated seawater using membrane filtration. Filters were incubated at 30 °C for 48 hours on two media, Ampicillin Dextrin Agar (ADA) and Starch Agar (SA), modified by adding 50 mg/L vibriostatic agent 0/129 and 16 mg/L ampicillin. Motile aeromonad counts ranged from 40×10^4 to 65×10^5 CFU/100 mL and 33×10^4 to 63×10^5 CFU/100 mL for mADA(0/129) and mSA(0/129), respectively. *A. caviae* was the most frequently isolated species, followed by *A. hydrophila*, and *A. sobria* (Alonso and Garay 1989). In comparison, the highest water filter counts for the current study (Fig. 4) are 2.1-3.4 $\log_{10}$ CFU/100 mL lower than *Aeromonas* spp. counts reported by Alonso and Garay (1989).

In the current study, *A. hydrophila* was the most frequently (98.7%) isolated organism from enrichment, whereas Alonso and Garay (1989) isolated more *A. caviae*. Seidler and others (1980), who sampled river water, isolated strains of *A. hydrophila* and *A. sobria* over a period of 10 mo. *A. hydrophila* was the most frequently isolated *Aeromonas* spp. (Seidler and others 1980), which is in agreement with the current study. Certain environmental characteristics such as salt requirement and available nutrients in polluted seawater for the Alonso and Garay (1989) study may have been factors that influenced *A. caviae* to proliferate.

***Aeromonas* Species Detection**

Enriched water, fish rinses, and rainbow trout fillet core samples were evaluated for the presence/absence of *Aeromonas* spp. Sampling period was significant ($P < 0.05$) for presence/absence, with average probabilities of detection decreasing by 0.23 for fish rinses and increasing by 0.23 for water samples per month. The highest frequency of *Aeromonas* spp. detection (Fig. 5) occurred on January 18, 2007 (16/21), whereas the lowest occurred on November 8, 2006 and February 20, 2007 (4/21). Frequency of detection decreased ($P < 0.05$) with increasing water reuse. Trout feed was also analyzed for the presence of *Aeromonas* spp., but no growth resulted. In conclusion, the trout feed did not harbor any *Aeromonas* spp. and was not a source of these bacteria.

Presence/absence probabilities were lowest for the 95% reuse system (1.05 ± 0.25), followed by the 80% reuse (2.12 ± 0.25), and flow-through (2.47 ± 0.47) systems. The source water had the highest frequency of detection (62.5%, 15/24) with 19 isolates identified from these 15 positive samples (Fig. 5). The probability of *Aeromonas* spp. being present on fillets after 6 d storage at 4 ± 2 °C (0.23 ± 0.054), was approximately 3 times higher ($P < 0.05$) than on d 0 (0.07 ± 0.054). Fillet core samples of the 80% reuse system before refrigeration had the lowest prevalence (0%, 0/18). Cores for the flow-through and 95% reuse systems before refrigeration also had low detection rates (16.7%, 1/6) and (11.1%, 2/18), respectively. There was a significant ($P < 0.05$) presence/absence fillet core – fish rinse variable by variable correlation. This correlation coefficient of 0.4769 suggests that *Aeromonas* spp. present on the

exterior of the fish, i.e. the scales, gills, fins, etc., may contaminate the fillet during processing.

A. hydrophila was isolated from the source water and all three systems. Of the 78 *Aeromonas* isolates identified by API 20NE strips, *A. hydrophila* accounted for 98.7% and *A. sobria* for 1.3%. Source water may have contributed to the presence of *Aeromonas* spp. in the flow-through, 80%, and 95% reuse systems; however, specific strains were not identified. Without strain identification, *Aeromonas* spp. present in the source water can not be definitively linked to *Aeromonas* spp. in the systems.

Lee and others (2002), collected water samples from a trout farm, a sedimentation pond at the farm, and upstream and downstream of the farm in January, May, August, and November. Heterotrophs, bacteria that utilize organic molecules as a carbon source, were monitored. Heterotrophic plate counts of samples collected in the warmer months (May and August) were higher than those from the colder months (January and November); although, *Aeromonas* spp. were isolated from all water samples. *A. hydrophila* and *A. caviae* were the most frequently isolated species, especially during the warmer months. *A. salmonicida* was also widely distributed and dominant at lower temperatures during the colder months (Lee and others 2002).

Because of potential human health risks associated with *Aeromonas* contaminated trout, it is particularly important to monitor the levels of these bacteria in fish production systems (Lee and others 2002). Even if the fish are not showing symptoms of disease, *Aeromonas* spp. can be present in the

system. If those fish are consumed, then illness may result. Pin and others (1994) purchased food samples and tested them for the presence of motile *Aeromonas* spp. Forty-five percent of shellfish and 36% of fish samples were positive. Most isolates were *Aeromonas hydrophila*, although *A. sobria*, *A. caviae*, and unclassified *Aeromonas* spp. were also observed (Pin and others 1994).

Motile aeromonads have adapted to a number of environments with varying conductivity, turbidity, pH, salinity and temperature (Cipriano 2001). Many *Aeromonas* isolates from water and several foods possess virulence factors that are typical of enteric pathogens (Palumbo and others 2001). The three most virulent species (*A. hydrophila*, *A. caviae*, and *A. sobria*) are all motile (Cipriano 2001). Some motile *Aeromonas* spp. can metabolize several of the same compounds and have a few of the same virulence factors as non-motile species, although it seems to be more species dependent.

González and others (2001) examined 171 isolates of *Aeromonas* spp. from the skin, gills, and intestines of farmed rainbow trout, wild brown trout, wild pike, and water samples. Of the isolates, 88% were identified as known species of *Aeromonas* with 29% *A. hydrophila*, 19% *A. sobria*, 18% *A. bestiarum*, 16% *A. jandaei*, 2% *A. caviae*, 2% *A. schubertii*, and 0.6% unnamed. The remaining 12% were similar to *A. schubertii*, but were not recognized as a specific species (González and others 2001).

In this study, ozone and UV light were effective in reducing the frequency of *Aeromonas* spp. detection. When ozone and UV light were not being used to

treat the recirculated water (12/21/06 to 1/12/07), the next sampling period (1/18/07) had the highest occurrence of *Aeromonas* spp. compared to all other samplings within the 8-mo period (Figs. 3 and 5). Colt and Tomasso (2001) report that ozone can effectively inactivate bacteria, viruses, and protozoa at 0.5-1.5 mg/L by reacting with cell membrane proteins. Ultraviolet light, at a wavelength of 253.7 nm, is also an effective disinfectant when particles larger than 70 μm are removed by filtration. The dose requirement (intensity x time) for UV light is related to the size and transparency of the target organism (Colt and Tomasso 2001). Efficacy of UV light treatment varies as a function of bacterial characteristics such as cell wall, capsule, and cell membrane composition.

Psychrotrophic Plate Counts

Psychrotrophic plate counts of fish rinses (Fig. 6) were higher ($P < 0.05$) for the 95% reuse system. The flow-through system had the lowest occurrence ($3.77 \pm 0.42 \log_{10} \text{CFU/mL}$) followed by the 80% ($4.03 \pm 0.25 \log_{10} \text{CFU/mL}$) and 95% ($5.35 \pm 0.25 \log_{10} \text{CFU/mL}$) reuse systems. Counts (Appendix 1) ranged from 4.08-6.0 $\log_{10} \text{CFU/mL}$ for the 95% reuse and 3.86-5.03 $\log_{10} \text{CFU/mL}$ for 80% reuse system during the study. The amount of recirculated water may have caused a larger quantity of psychrotrophic bacteria to be present in the 95% reuse system. Since more water is maintained in the system for recirculation, the bacteria have longer to acclimate to their environment. A longer retention time in the recirculation system might be all that is needed for more bacteria to transition from the log to the lag phase of growth. This increase in logarithmic growth may

account for the higher numbers of psychrotrophic bacteria present in the 95% reuse fish rinses.

Psychrotrophic plate counts of rainbow trout fillets (Table 2) were higher ($P < 0.05$) after 6-d storage at 4 ± 2 °C ($4.80 \pm 0.13 \log_{10}$ CFU/cm²) than at d 0 ($2.48 \pm 0.13 \log_{10}$ CFU/cm²). Counts (Appendix 1) for the flow-through system ranged from 1.54-3.56 $\log_{10}$ CFU/cm² at d 0 and 4.53-6.12 $\log_{10}$ CFU/cm² at d 6 during the study. The 80% and 95% reuse systems also had higher psychrotrophic plate counts on d 6 than d 0. They ranged from 1.95-3.74 $\log_{10}$ CFU/cm² on d 0 and 4.29-5.78 $\log_{10}$ CFU/cm² on d 6 for the 80% reuse system and 1.81-4.18 $\log_{10}$ CFU/cm² on d 0 and 3.09-6.13 $\log_{10}$ CFU/cm² on d 6 for the 95% reuse system.

González-Rodríguez and others (2001) reported higher counts of psychrotrophic bacteria on prepackaged rainbow trout fillets after 4 and 7-d storage at 3 °C than initial counts. Plates were incubated at 25 and 30 °C to account for the discrepancy between two incubation temperatures recommended for determining microbiological limits for fresh fish. Initial aerobic counts for trout fillets (5.27 ± 0.57 and $4.87 \pm 0.80 \log$ CFU/g at 30 °C and 25 °C, respectively) were significantly ($P < 0.05$) lower than counts after 4 d storage (7.45 ± 0.90 and $7.51 \pm 1.14 \log$ CFU/g at 30 °C and 25 °C, respectively) and 7 d storage (8.76 ± 0.32 and $8.97 \pm 0.39 \log$ CFU/g at 30 °C and 25 °C, respectively). Trout fillets were scored “poor freshness quality” after 7 d storage and were showing signs of spoilage including sour smell, off coloring, slightly soft tissue, and moderate drip (González-Rodríguez and others 2001).

McAdams and others (2005) determined psychrotrophic plate counts of rainbow trout. There was no significant difference in counts ($P > 0.05$) between whole rainbow trout or rainbow trout fillets from 5 different aquaculture facilities. Samples were taken once in September and once in June, but time of year did not affect ($P > 0.05$) these counts. Psychrotrophic counts ranged from 1.6×10^4 to 1.5×10^5 CFU/g for whole trout and 1.7×10^4 to 3.7×10^5 CFU/g for fillets. These levels reflect the bacterial microflora present on the surface of the fish which may be transferred to the fillet during processing (McAdams and others 2005).

According to Giménez and others (2002), it is accepted that fish fillets are considered to be spoiled at approximately 10^7 CFU/cm². Regardless of reuse level, psychrotrophic plate counts of fillets in the current study did not reach spoilage levels after 6-d storage at 4 ± 2 °C. González-Rodríguez and others (2001) obtained higher psychrotrophic counts in rainbow trout fillets where spoilage resulted after 7-d storage at 3 °C. In that study, the fish were purchased at a supermarket versus freshly caught. Processing, handling, and transportation are a few factors that may explain the higher counts for purchased fish.

Water reuse increased ($P < 0.05$) the psychrotrophic plate counts of fillets (Fig. 7). The 95% reuse system generated a higher mean ($4.03 \pm 0.14 \log_{10}$ CFU/cm²) than the 80% reuse ($3.40 \pm 0.14 \log_{10}$ CFU/cm²) or flow-through ($3.50 \pm 0.21 \log_{10}$ CFU/cm²) systems. On December 5, 2006 (Appendix 1), the 80% reuse system gave higher counts at both d 0 and d 6 than the 95% reuse system. On September 5, 2006, the 80% reuse counts on d 6 were higher than those for

the 95% reuse system. In all other instances, rainbow trout fillets from the 95% reuse system yielded higher counts than the 80% reuse system. Fluctuations in psychrotrophic plate counts and *Aeromonas* spp. detection for the 95% reuse system may be a result of discontinuous use of UV light and ozone.

Confounding Effects

This study surveyed *Aeromonas* spp. present in the recirculated aquaculture systems at TCFFI during existing, ongoing research at the facility. Balanced data were not collected because TCFFI's research agenda dictated the discontinuation of the flow-through system. Consequently, that treatment was terminated after 11/8/06. Since the tanks were not a uniform size across recirculation treatments, fish size, tank volume, water flow, and the amount of feces generated in each tank may vary; nevertheless, fish density was kept below 80 kg/m³ during the course of the study. Water for the 95% reuse system goes through a process where it is biofiltered to remove excess nitrogen, treated with UV light, ozonated, and oxygenated before being recirculating back into the system. Effects of water reuse were confounded with the use of UV light and ozone because the flow-through and 80% reuse systems did not receive these treatments. Water quality of the source may have also changed with the seasons and change in temperature.

The ratio of fish mass to buffer volume (Table 3) varied with the size of the fish. In hindsight, this variable should have been controlled, but it does not affect the number of positive samples per sampling period (Fig. 5). For example, the average mass of the fish increased approximately 530 g from 9/5/06 to 2/20/07

(Table 3); however, the frequency of *Aeromonas* spp. detection during that time remained relatively consistent (Fig. 5) except for a peak at 1/18/07. During the course of this study, fish mass ranged from 253.78 g to 1,321.78 g with a mean of 666.07 ± 246.40 g yielding fillets with a mean of 446.84 ± 170.77 g (Fig. 9). This variation may have influenced the amount of bacteria recovered from the rinsates, thus influencing psychrotrophic plate counts and the amount of *Aeromonas* bacteria enriched in TSBA. In contrast, fish mass may not be an accurate measure of surface area.

Water reuse was the only significant variable ($P < 0.05$) when fillet yield was analyzed (Fig. 8). The flow-through system produced larger trout fillets with the highest fillet yields (68.08 ± 0.74 % yield) in comparison to the 95% (65.65 ± 0.40 % yield) and 80% (65.58 ± 0.43 % yield) reuse systems. This may have been because the fish in the flow-through system were bigger, had more muscle, and thus yielded a larger dressing percentage when they were filleted.

Not all treatments were maintained in the same manner though the duration of this study. The flow-through treatment was discontinued after the October 3 sampling, so only the 80% and 95% reuse systems were sampled for the November 8, 2006 though February 20, 2007 samplings. There were a few harvest and stocking events that occurred (Fig. 3); however, not all systems were stocked or harvested at the same time. Fish were harvested from the 80% reuse and stocked in the 95% reuse systems on June 29, 2006. There was a small stocking of the 95% reuse system on September 5, 2006 and a harvest on January 18, 2007. These differences in harvest and stocking events may impact

the fecal load, and thus affect the level of *Aeromonas* spp. present in the water and on the fish.

CONCLUSIONS

Understanding how human and fish pathogens grow and persist in recirculated aquaculture systems and on the fish needs to be further investigated. Water reuse significantly affected ($P < 0.05$) the water pH, presumptive positive *Aeromonas* spp. on the water filters, psychrotrophic plate count of the fish rinses and fillets, as well as fillet yield. There was a fillet core by fish rinse correlation ($P < 0.05$) in the presence/absence of *Aeromonas* spp. This correlation sanctions further expansion of the connections between fish production in recirculated systems and fillet quality as it relates to food safety and spoilage. *Aeromonas hydrophila* was the predominant (98.7%) species detected in this study. This finding is important because *A. hydrophila* is a significant water and food-borne pathogen of concern to public health. Isolate strains need to be determined before *Aeromonas* spp. in the source water can be linked to strains isolated from the systems. This data suggests that pre-harvest controls are warranted to control this emerging human pathogen.

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FIGURE 1 – Diagram of the 80% Reuse System.

(Summerfelt and others 2004)

FIGURE 2 – Diagram of the 95% Reuse System.

(Davidson and Summerfelt 2005)

FIGURE 3 – Timeline of Sample Dates, Ozone and UV Light Usage, and Stocking and Harvesting Events for the 95% Reuse System.

TABLE 1 – Water pH for the Source and 3 Systems.

†Sampling Period	Source	Flow-through	Medium 80%	High 95%
6/29/06	6.96 ± 0.046	7.41 ± 0.019	7.51 ± 0.003	7.54 ± 0.006
8/8/06*	-	-	-	-
9/5/06	7.10 ± 0.016	7.31 ± 0.031	7.37 ± 0.100	7.03 ± 0.096
10/3/06	7.15 ± 0.035	7.59 ± 0.006	7.48 ± 0.074	7.49 ± 0.031
11/8/06	7.14 ± 0.146	8.00 ± 0.048	7.76 ± 0.019	7.50 ± 0.029
12/5/06*	7.59 ± 0.054	-	7.97 ± 0.181	7.49 ± 0.022
1/18/07*	7.23 ± 0.013	-	7.55 ± 0.024	7.17 ± 0.041
2/20/07*	7.21 ± 0.068	-	7.59 ± 0.004	7.32 ± 0.023
<i>Overall Mean</i>	7.19 ± 0.045 ^a	7.62 ± 0.060 ^c	7.60 ± 0.044 ^c	7.35 ± 0.045 ^b

n = 3

*pH was not sampled for 8/8/06 and flow-through after 11/8/06

†Sampling period caused an increase ($P < 0.05$) in pH by 0.031 over time.

^{a,b,c} indicates that percent of water reuse caused differences ($P < 0.05$) in pH.

FIGURE 4 – Presumptive *Aeromonas* spp. on filters from the Source Water and 3 Systems.

* <0 log₁₀ CFU/100 mL is <1.0 CFU/100 mL

†Presumptive positive counts decreased ($P < 0.05$) on the average by 0.26 log₁₀ CFU/100 mL per sampling period.

^{a,b} indicates that presumptive positive *Aeromonas* spp. on water filters were different ($P < 0.05$) with water reuse.

FIGURE 5 – Samples Positive for *Aeromonas* spp. in Water, Fish Rinses, and Fillets at d 0 and d 6.

†Frequency of *Aeromonas* spp. detection probabilities decreased ($P < 0.05$) by 0.23 and increased by 0.23 per sampling period for fish rinse and water, respectively.

^{a,b} indicates that the frequency of *Aeromonas* spp. detection was higher ($P < 0.05$) after 6-d storage at 4 ± 2 °C.

FIGURE 6 – Effect of Water Reuse on Fish Rinse Psychrotrophic Plate Counts.

^{a,b} indicates that psychrotrophic counts of fish rinses increased ($P < 0.05$) with water reuse.

FIGURE 7 – Effect of Water Reuse on Fish Fillet Psychrotrophic Plate Counts.

^{a,b} indicates that psychrotrophic counts of fillets increased ($P < 0.05$) with water reuse.

TABLE 2 – Psychrotrophic Plate Counts of Fillets Before and After Refrigerated Storage.

Effect of Storage on Fillet Psychrotrophic Counts (log ₁₀ CFU/cm ²)	
Day 0 ^a	2.48 ± 0.130
Day 6 ^b	4.80 ± 0.131

n = 18

^{a,b} indicates that psychrotrophic counts of fillets increased ($P < 0.05$) with refrigerated storage.

FIGURE 8 – Effect of Water Reuse on Fillet Yield.

^{a,b} indicates that fillet yields decreased ($P < 0.05$) with water reuse.

FIGURE 9 – Changes in Mass of Whole Fish and Butterfly Fillets.

TABLE 3 – Mass:Volume Ratio of Whole Fish/100 mL BPB.

Sampling Period	Whole Fish Mass (g)	Mass:Volume (g/mL)
9/5/06	420.89 ± 135.20	4.21 ± 1.35
10/3/06	599.71 ± 234.94	6.00 ± 2.35
11/8/06	701.60 ± 170.44	7.02 ± 1.70
12/5/06	652.76 ± 233.32	6.53 ± 2.33
1/18/07	807.67 ± 153.87	8.08 ± 1.54
2/20/07	951.81 ± 226.88	9.52 ± 2.27

n = 3

APPENDIX 1

Psychrotrophic Plate Counts

Sampling Period	Water Reuse	Fish Rinses (log ₁₀ CFU/mL)	Fillets d 0 (log ₁₀ CFU/cm ²)	Fillets d 6 (log ₁₀ CFU/cm ²)
9/5/06	FT	-	2.45 ± 0.31	4.53 ± 0.72
	80%	-	1.95 ± 0.50	4.29 ± 0.53
	95%	-	2.56 ± 0.28	3.09 ± 0.73
10/3/06	FT	4.08 ± 0.37	3.56 ± 1.37	6.12 ± 0.98
	80%	4.20 ± 0.35	2.99 ± 0.62	5.63 ± 0.62
	95%	TNTC	3.64 ± 0.36	6.13 ± 0.45
11/8/06	FT	4.08 ± 0.45	1.54 ± 0.13	4.59 ± 0.49
	80%	4.20 ± 0.48	3.15 ± 0.95	4.51 ± 0.36
	95%	5.26 ± 0.09	3.56 ± 0.60	5.85 ± 0.86
12/5/06	FT	-	-	-
	80%	5.03 ± 2.31	2.54 ± 0.68	5.78 ± 1.13
	95%	4.08 ± 0.12	1.81 ± 0.31	4.46 ± 0.26
1/18/07	FT	-	-	-
	80%	4.81 ± 0.32	3.74 ± 1.05	5.30 ± 1.00
	95%	5.32 ± 0.26	3.93 ± 0.99	6.05 ± 0.40
2/20/07	FT	-	-	-
	80%	3.86 ± 0.04	2.04 ± 0.18	5.35 ± 0.13
	95%	5.51 ± 0.03	4.18 ± 0.29	6.06 ± 0.11

FT = flow-through

TNTC = too numerous to count

APPENDIX 2

Procedure for Enrichment and Isolation of *Aeromonas* spp.

1. If sampling a whole fish, rinse it in a known amount of Butterfield's Phosphate Buffer (BPB). If sampling a fillet, add a known amount of fillet sample to a known amount of BPB and blend in a stomacher for 30 s. If sampling water, skip this step.
2. In duplicate, transfer 1 mL of the rinsate, blended material, or water sample to 9 mL of Tryptic Soy Broth with Ampicillin (TSBA) and incubate them at 28 ± 2 °C for 22-24 h.
3. Vortex the tubes for 10 s.
4. Streak growth from these tubes to isolation on Starch Ampicillin Agar (SAA) and incubate them for 22-24 h at 28 ± 2 °C.
5. Pick the characteristic white, rough colonies from each plate and restreak them to isolation on fresh SAA.
6. Flood the plates with Lugol's solution to check for a halo of starch hydrolysis. They will surround yellow, stained colonies contrasted against dark brown, stained agar. If a halo forms around the previously selected colony on the original plate, then keep the restreaked plate for further evaluation. If a halo of starch hydrolysis does not appear around a characteristic colony, then discard the plate.
7. Incubate the restreaked plates at 28 ± 2 °C for 22-24 h.
8. Pick a characteristic colony from the restreaked plate and place it on a Nutrient Agar (NA) slant.

9. Flood the restreaked plate with Lugol's solution to re-confirm halos of starch hydrolysis. If the colonies picked forms a halo, then keep the slant. If no halo forms around the colony, discard the plate.
10. Incubate NA slants at 28 ± 2 °C for 22-24 h.
11. Perform an oxidase test to distinguish the oxidase positive *Vibrio* family, which includes *Aeromonas*, from the oxidase negative *Enterobacteriaceae* family.
12. Perform the 0/129 test to distinguish 0/129 resistant *Aeromonas* isolates from the susceptible *Vibrio* genus by spreading a full lawn of growth for oxidase positive isolates onto NA plates, place the 150 µg 0/129 disk on the surface, and incubate the plates at 28 ± 2 °C for 22-24 h. Assess whether or not a zone of inhibition forms around the disk.
13. Complete a Gram stain on the isolates to confirm that they are gram-negative rods.
14. Confirm presumptive positive isolates by their biochemical characteristics using API 20NE strips.
15. Regrow *Aeromonas* isolates on NA slants and store them on microprotect beads at -80 °C.

APPENDIX 3

Procedure to Determine Psychrotrophic Plate Counts

1. If sampling a whole fish, rinse it in a known amount of Butterfield's Phosphate Buffer (BPB). If sampling a fillet, add a known amount of fillet sample to a known amount of BPB and blend in a stomacher for 30 s. If sampling water, skip this step.
2. Serially dilute rinsate, blended material, or water samples in BPB.
3. Spread plate the dilutions onto Plate Count Agar (PCA).
4. Incubate them for 48 h at 26 ± 2 °C.
5. Count and record the number of colonies.
6. Repeat steps 1-5 after storage for the desired number of days at 4 ± 2 °C.

APPENDIX 4

Procedure to Enumerate *Aeromonas* spp. in Water

1. In duplicate, aseptically pass 100 mL of water through a pre-sterilized 47 mm 0.45 μm Whatman nylon membrane filter suctioned through a Gelman Magnetic Filter Funnel and place it on a SAA plate.
2. Incubate the SAA plates at 28 ± 2 °C for 22-24 h.
3. Flood the plate with Lugol's solution.
4. Count and record the yellow colonies with characteristic halos of starch hydrolysis as presumptive positive *Aeromonas* spp.

CURRICULUM VITAE

Professional Preparation

West Virginia University Biochemistry B.S. 2005

West Virginia University Animal and Nutritional Science M.S. 2007

Appointments

West Virginia University Graduate Research Assistant Fall 2005 –
Summer 2007

Published Abstracts

Presence of antibiotic resistant *Salmonella* in commercially raised organic
chicken. – Poultry Science meeting 2005

Prevalence and antibiotic resistant profiles of *Salmonella* from fresh retail
chicken. – Poultry Science meeting 2005

Presentations

Antibiotic resistant Salmonellae in retail processed chicken. – Poster at
Undergraduate Research Day at the Capitol March 2005

Meat Quality of Steers Finished On Pasture or In Confinement. – Talk at Davis
College Research Day April 2006

Meat Quality of Steers Finished On Pasture or In Confinement. – Talk at IFT
meeting July 2007

Frequency of *Aeromonas* spp. Detection in a Rainbow Trout, Water Reuse
System. – Poster at IFT meeting July 2007

Selected Honors/Awards

Undergraduate Research Day at the Capitol, Charleston WV – March 2005

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Beef and Poultry Hazard Analysis and Critical Control Points

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