

**THE DETERMINATION OF MICROBIAL QUALITY AND PRESENCE OF
PATHOGENS AND CHEMICAL CONTAMINANTS IN AQUACULTURED
RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FILLETS AND WHOLE FISH
FROM DIFFERENT AQUACULTURE PRODUCTION SYSTEMS**

by

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Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

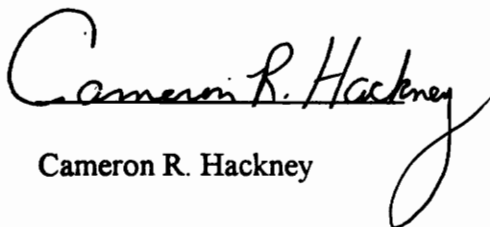
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Food Science and Technology

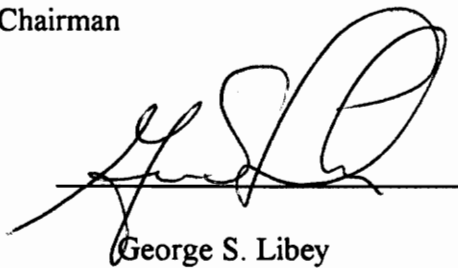
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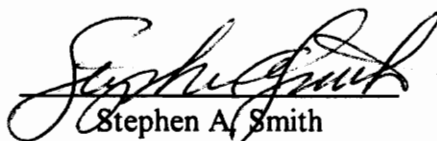
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April 1996

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(ABSTRACT)

Qualitative and quantitative levels of human pathogens (*Listeria monocytogenes*, *Clostridium botulinum*, *Salmonella* species, *Vibrio cholerae*, *Yersinia enterocolitica* and *Y. pseudotuberculosis*) and general microbial quality of whole aquacultured rainbow trout (*Oncorhynchus mykiss*) were compared in differing aquacultural systems and growing seasons. *Listeria* levels and general microbial quality were determined on fillets from three facilities and compared to whole fish. Secondly, in each facility and season, levels of select minerals and heavy metals in edible tissue were determined. Aerobic plate counts for whole fish and fillets ranged from 3-6 logs. Coliform counts were high in many cases, but fecal coliform, and *E. coli* were comparatively lower. Both whole fish and fillets showed similar *Listeria* incidence (25-90%) and respective levels of 1.12-21 and 0.36-4.83 MPN/g. Presumptive *C. botulinum* incidence was 45-95% with levels of 0.46-2.33 MPN/g. Coliform, fecal coliforms, and *E. coli* counts and *Listeria* and *C. botulinum* incidence and counts were significant in plant/year interaction ($p < 0.05$). A significant

increase from whole fish to fillets in mesophilic APC, coliforms, and *Listeria* counts ($p < 0.05$) was observed. No *Salmonella*, *Vibrio cholerae*, or *Yersinia* were isolated from any sample. Levels of Na, Zn, S, Ca, Mg, Mn, and Fe were generally within normal ranges. The heavy metals- As, Hg, Ni, Co, Cr, Mo, Pb, and Cd when detected, were below 0.5 ppm, with the exception of Cd in one sample (60.49 ppm). The presence of both *C. botulinum* and *Listeria* would be of concern to processors, although no heavy metals were at levels to cause concern.

DEDICATION

This work is dedicated to the memory of my grandfather, Mr. James Henergar Wright. In the twenty-four short years I knew him, he taught me a lifetime of lessons and gave a world of love. I will miss him.

ACKNOWLEDGEMENTS

I would first like to convey my gratitude to my Committee members who provided guidance and encouragement throughout this study. I would like to express my sincere appreciation to my major professor and advisor, Dr. George J. Flick, Jr. His unwavering support and trust in my abilities were a great source of motivation in my time in the department, and will continue to influence my work in other endeavors.

I would also like to thank Robert Reinhard who spent long hours in the lab with me during the running of the samples, and Sharma Puella who was invaluable during the *C. botulinum* toxin study. I would like to express my gratitude to Dr. William Eigel and Dr. Cameron Hackney, who, when space became limited, volunteered the use of their labs for the continuation of my research, and Dr. Susan Duncan who provided input during the preparation of the taste panel.

My appreciation is extended to both Kathy Lawler and Brian Smith for all the time, patience, and help they gave during the *C. botulinum* study, Dr. Stephen Smith whose time and help made possible the *Mycobacterium* portion of my work, and Dr. Custodio Fernandes, for an occasional “push in the right direction” in dealing with methodology.

I would also like to extend my heartfelt thanks to my best friend, Angela Reeves, for her support and constant words of encouragement throughout my research and writing, and to my parents who have always been there and have provided the foundation of love and support for which I am eternally grateful.

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INTRODUCTION

In 1989, the total world seafood supply was estimated to be around 90 million tons and increasing. Although it has been referred to for some time as the wave of the future, it has only been in the past ten years that the aquaculture industry has had a major impact on the world supply of seafood (Redmayne, 1989). The rising demand of seafood products coupled with overfishing of the world's oceans has forced the world to turn to aquaculture to help satisfy this demand.

Many species of fish, such as catfish, salmon, and trout, have been successfully adapted to aquaculturing practices. While catfish and salmon comprise a larger share of the U. S. market than does trout, this by no means lowers the importance of trout in the seafood supply. Trout still has a substantial niche in the market for aquacultured products.

Rainbow trout (*Oncorhynchus mykiss*) has been successfully raised via aquaculture for many years. However, the rearing of this fish can not be accomplished in many areas in a manner that is economically feasible. The optimum temperature for rainbow trout is 16°C, and 20°C is the maximum temperature in which the fish are likely to survive for any length of time (Stevenson, 1989). Temperature also effects the oxygen solubility of the water, at a fully saturated state containing 9.00 ppm at 20°C and 12.88 ppm at 4°C. Trout require minimum dissolved oxygen content of 6.00 ppm, although this level is undesirable and should be held at full saturation (Stevenson, 1987).

Two main aquacultural systems are employed in the rearing of rainbow trout. These systems are either flow-through, which primarily use dirt bottomed ponds or

concrete raceways, or recirculating, which reuses a portion of the water. The flow-through system relies on large quantities of water to supply adequate amounts of oxygen to the fish as well as ridding the system of waste products. It has been stated that typically in temperate regions, a flow rate of at least 5 l/s is needed for the production of one ton of trout (Sedgwick, 1990). This figure depends largely on the temperature of the water which affects the ability of the water to hold oxygen. The recirculating system relies on artificial aeration to supply the necessary oxygen and filtration to remove waste products and impurities (Stevenson, 1987).

There are a variety of human pathogens that have been associated with seafood products. *Listeria monocytogenes*, which usually only poses a threat to certain "at risk" populations such as the immunocompromised or pregnant women and their fetuses, has generated much concern in the food industry over the past ten years. *Listeria* is ubiquitous in its distribution and has been isolated from a variety of seafood products (Farber, 1991; Hartemink and Georgsson, 1991; Weagent et al., 1988). The organism represents a large threat during food processing and post-processing handling due to its distribution and ability to grow at refrigeration temperatures.

The spore-forming anaerobic organism, *Clostridium botulinum* is widely found throughout the environment, and has been shown to have caused problems in the past in rainbow trout, especially in the smoked fish industry (Bach et al., 1971; Cann et al., 1975; Burns and Williams, 1975; Huss et al., 1974). Although studies have shown the isolation of other types, type E is regarded as the most frequently type isolated from aquatic

environments and most often implicated in botulism traced to seafood products (Hackney and Dicharry, 1988).

Salmonella as well as most pathogenic strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are commonly transmitted to fish when the fish are raised in polluted environments. Brown and Dorn (1977) stated that raw whole fish in general, except when harvested from polluted waters, rarely contained bacteria pathogenic to humans. *Yersinia enterocolitica* is commonly found in the environment, and studies have shown swine to be the main reservoir of the bacteria, although the organism has been isolated from other animals (Doyle, 1988).

The disease, cholera, caused by the organism *Vibrio cholerae* antigen type O1 infections, is rare, although illnesses from non-O1 cholerae are relatively common (Wilson et al., 1981). *V. cholerae* has been shown to exist in both fresh and salt waters and aquatic sediment (Kaysner et al., 1987; Venkateswaren et al., 1989; Eyles and Davey, 1988), although the organism is found predominately in marine areas such as oceans and estuary regions. Both types of *V. cholerae* have been found in the feces of aquatic birds which suggests that these birds could serve to spread the organism to other waters (Ogg et al., 1989).

The health concerns from both chronic levels and bioaccumulation of heavy metals are well documented (Friberg et al., 1979; Goyer et al., 1995; Stoeppler, 1992). Heavy metals are distributed throughout the environment by sources such as leaching from the soil as well as non-point pollution. By these routes, the water table and subsequently the

aquifers that provide the water for aquaculture facilities may become contaminated.

In January of 1994, the United States Food and Drug Administration (FDA) purposed a rule for the safe processing of the fish and fishery products. In this proposal, which has been published in the Federal Register, the FDA called for the implementation of mandatory quality assurance program based on the HACCP (Hazard Analysis Critical Control Point) concept. This plan encompasses microbial, physical, and chemical hazards. With the 1997 implementation of the FDA's HACCP system in the seafood industry on the horizon, sound scientific data on the hazards (microbial and chemical) associated with all seafood products, more specifically, aquacultured products must be assessed.

The objective of this study was to determine the presence of *Listeria monocytogenes*, *Clostridium botulinum*, *Salmonella* species, *Vibrio cholerae*, *Yersinia enterocolitica* and *Y. pseudotuberculosis* in aquacultured rainbow trout (*Oncorhynchus mykiss*). A second purpose was to compare differing aquaculturing systems and growing seasons (late spring-early summer vs. late summer-early fall) on the basis of qualitative and quantitative levels of pathogens and general microbial quality. Additionally, the general microbial quality data and qualitative and quantitative *Listeria* levels on whole fish were compared to those of fillets. The final objective was to determine levels of select heavy metals present in the different aquaculture facilities, noting any trends in the heavy metal or pesticide/herbicide levels when examined during the growing seasons of late spring-early summer and late summer-early fall.

Section I: REVIEW OF LITERATURE

A. *Listeria spp.*

1. Historical Background

First described in 1924, Murray et al. (1924) isolated the organism, *Bacterium monocytogenes*, from infected rabbits. In 1926, Pirie (1940), isolated the organism from an infected gerbille and named it *Listerella hepatolytica*. Later, when it was found that *Bacterium monocytogenes* and *Listerella hepatolytica* were in fact the same organism, the name was changed to *Listerella monocytogenes*, and subsequently the name *Listeria monocytogenes* was adopted (Pirie, 1940). Since its adoption as a genus, *Listeria* has been divided from the original single species genus to eight separate species.

In 1981, the first confirmed case of food transmitted listeriosis were reported involving contaminated coleslaw (Schlech et al., 1983). In California, in 1985, Mexican style soft cheese was implicated in more than 142 cases of listeriosis, at least 40 of which resulted in death (Linnan et al., 1988). Following the reported outbreaks, the rising concerns of the food industry and the public as well, government regulatory agencies established a zero tolerance criteria for *Listeria* in ready-to-eat foods (Paranjpye et al., 1992).

2. Taxonomy, nomenclature and morphology

Listeria monocytogenes is a Gram-positive, non-spore forming, facultative anaerobic, rod-shaped bacterium with blunt ends, typically measuring 0.5-2.0 μm X 0.5

μm in fresh smooth isolates to 6-20 μm X 0.5 μm observed in rough strains that usually develop in older cultures. The organism is catalase positive and oxidase negative. Colonies of the organism exhibit β -hemolysis on blood agar, and on nutrient agar appear bluish-gray under normal illumination on nutrient agar, and take on a blue-green sheen when observed under oblique light (Seeliger and Jones, 1986). The *Listeria* cells, at a temperature of 37°C, are motile by means of one polar flagellum and exhibit a characteristic slow tumbling motility, while at a decreased temperature of 20 to 25°C, four peritrichous flagellum are formed (Willet, 1992). Young and old isolates of the organism reveal several differing characteristics such as morphology, Gram staining, and cell appearance. Young isolates (incubation up to 48 hrs.), are Gram-positive, smooth, short rods, while older isolates (incubation over 24 hrs.) may exhibit Gram-negative staining and a rougher more filamentous appearance (Seeliger and Jones, 1986).

In the 9th edition of Bergey's Manual of Determinative Bacteriology, the following organisms were recognized as *Listeria* species: *L. denitrificans*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. murrayi*, *L. seeligeri*, and *L. welshimeyer*. Recently, however, evidence has supported the removal of the species *L. denitrificans* from the genus and placed it in the new genus, *Jonseia* (Jones and Seeliger, 1986).

Listeria monocytogenes is separated into 13 separate serovars (serotypes) on the basis of the O and H antigens. Most serovars also contain several different strains (Willet, 1992).

3. Growth characteristics

It is generally accepted that *Listeria monocytogenes* grows at temperatures between 1 and 45°C with optimum growth between 30 and 37°C as noted in Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986). In trials where smoked fish were inoculated with *L. monocytogenes*, neither an increase nor a decrease was observed in fish held at 4°C for 20 days where an increase, as much as 6 logs, was observed in fish stored at 8-10°C (Jemmi and Keusch, 1992). Lovett and Tweldt (1988) noted that *L. monocytogenes* is capable of doubling every 1.5 days at 4°C.

One of the main concerns in the food industry regarding *Listeria* spp., is its ability to grow at refrigeration temperatures, as well as the survival of cells exposed to sub-freezing temperatures (Weagent et al., 1988; Harrison et al., 1991). Because of this, special care must be taken to assure that no *Listeria* is introduced into the product. Refrigeration effectively controls the spoilage of products such as fish, but if *Listeria* is present, it could continue multiplying unchecked as refrigeration does not control the proliferation of the organism, and freezing is ineffective to destroy it.

Listeria monocytogenes has also been shown to be very resistant to high salt concentrations. Bergey's Manual of Systematic Bacteriology reports *Listeria* growth in nutrient broth containing 10% NaCl (Seeliger and Jones, 1986). This ability to withstand higher salt concentrations provides the smoked fish industry with a need for concern, given the zero tolerance in the food industry for *Listeria* in ready-to-eat products.

The minimum pH required for growth of *Listeria* has been shown to be highly

variable. George et al. (1988) showed delayed growth of the organism at pH values as low as 4.39. The minimum pH for the organism is influenced by conditions such as incubation temperature and the type of acid used to lower the pH (Ryser and Marth, 1991).

McCarthy (1992) noted a resistance to biocides in *Listeria* cells that were attached to chitin. Survival of attached *Listeria* exposed to differing amounts of three disinfectants for up to twenty minutes was enhanced. Chitin is the structural component of the exoskeleton of crustaceans such as shrimp, a regular ingredient in fish feed. Because *Listeria* is able to attach itself to chitin and in doing so exhibits an increased resistance to the biocides, care must be taken to thoroughly sanitize surfaces that come in contact with the viscera of the fish due to the presence of undigested fish feed that may harbor *Listeria*.

4. Pathology

Of the *Listeria* spp. only the hemolytic species (*L. monocytogenes*, *L. seeligeri*, and *L. ivanovii*) are known to be pathogenic, and of these, only *L. monocytogenes* is consistently so (Lovett and Tweldt, 1988). Following ingestion of virulent bacteria, the organism invades macrophages. The *Listeria* cells then escape from the host vacuoles and enter the cytoplasm where rapid multiplication occurs. Bacteria then move to the inner cell membrane where they are secreted by pseudopod structures. Following this, they can again be phagocytized, starting the process over, resulting in septicemia (Portnoy et al., 1992). It is at this stage that *Listeria* has access to all other areas of the body, including the fetus. Depending on the stage of pregnancy, the infection may result in abortion,

stillbirth, or neonatal sepsis. In adults, septicemia is the most common manifestation of listeriosis. Symptoms include fever, malaise, or enteric symptoms. Death in healthy adults is rare, although in newborns, the very young, or immunocompromised individuals, the death rate can be as high as 30%. Other manifestations of the disease include meningitis and encephalitis, which may have a mortality rate as high as 70%. All manifestations of the disease are more common for the groups at risk (Lovett and Tweldt, 1988).

One may question, that if *Listeria* is such a widespread pathogen, found in a variety of foods, why does it not result in more reported outbreaks? Listeriosis can take several different forms in humans, from no effect to discomfort due to diarrhea to death. In healthy humans, *Listeria* usually has no effect, or causes very mild cases of diarrhea. It is when the organism attacks an immunocompromised individual or pregnant woman that the symptoms become more severe. The mechanism by which *Listeria monocytogenes* causes its infections is still not well understood, but there are several factors that affect the organisms pathogenicity. The first of these is cell morphology. Smooth strains of the serovar 4b are the most virulent, while the rough phenotype of the serovar appears to have lost its ability to cause disease (Schlech, 1988). The production of hemolysins are crucial in the pathogenicity of the organism. Hof (1984) noted a loss of pathogenicity with the loss of the organisms ability to produce hemolysin. The same study also noted that the nonhemolytic strains exhibited the rough morphology.

It must also be noted that the serotype of the organism is a good indication of the organism's virulence. The most invasive of the *Listeria* serotypes tend to be clustered

around the serotypes 1-4 and 7. The serotype that has been attributed to the recent foodborne outbreaks is the serotype 4b (Schlech, 1988). Willet (1992a) noted that 90% of the clinical manifestations of listeriosis worldwide are presently caused by three strains; 1/2a, 1/2b, and 4b, with 4b being the predominant strain.

5. Epidemiology

The primary mode of distribution for *Listeria monocytogenes* is dirt, and because of its ubiquitous distribution in nature, the organism represents a large threat during food processing and post-processing handling. Also, Lovett and Tweldt (1988) noted that as many as 5% of people who are not actively showing symptoms of the disease may be actively shedding *Listeria* cells.

The incidence of *Listeria* in various food products has been examined rather closely. Nitcheva et al. (1990) found that the incidence in 170 frozen beef and pork samples was 17.2 %, demonstrating not only the freeze tolerance of the organism, but also supporting the fact that the occurrence of *Listeria* in a given food product is highly variable. *Listeria* has also been isolated from a variety of seafood and fishery products. Such products include, but are not limited to, raw and cooked shrimp, raw and cooked crabmeat, lobster tails, langostinos, scallops, squid, raw fish, smoked fish, fish salads, and shellfish, just to name a few (Farber, 1991; Hartemink and Georgsson, 1991; Weagent et al., 1988). *Listeria* has also been isolated from various places throughout processing facilities, particularly from wetter areas of the facilities (Slade, 1995).

Fish and seafood products have been the subject of much concern because of the

ability of *Listeria* to grow at low temperatures, its freeze and heat tolerance, as well as its ability to survive higher salt concentrations. These abilities have not only made *Listeria* a concern in fresh fish products, but also in frozen products as well as smoked fish products.

Listeria has been isolated in both hot and cold smoked fish. These products often are eaten "as is" with little or no additional heat treatment, and are thereby considered "ready to eat" products. Dillon et al. (1994) found *Listeria* spp. in 25.4% of hot-smoked products and 6% of the cold smoked products. The hot smoking process should prove adequate to destroy *Listeria* spp. found on the fish, however, the author noted that processors examined, often deviate from the outlined hot smoking protocol. Fuchs and Nicolaides (1994) also isolated *Listeria* spp. from hot and cold smoked fish at levels of 8.7% and 8.6% respectively, citing post-smoking contamination as the cause in the hot-smoked product.

In experimentally contaminated hot-smoked trout, Jemmi and Keusch (1992) found that when exposed to hot-smoke temperatures (core temp. 65°C for 20 min.) the initial surface counts dropped from 106 MPN/g before smoking to undetectable levels after smoking continuing through the storage time of 20 days. In trials where the fish were inoculated after the smoking, neither an increase nor a decrease was observed in fish held at 4°C for 20 days where an increase, as much as 6 logs, was observed in fish stored at 8-10°C. Farber (1991) examined artificially contaminated smoked salmon held at 4°C and observed as much as a 2 log increase in 15 days. The difference in these two experiments could mean that salmon offers a better substrate for the growth of *Listeria*

monocytogenes than does trout.

In experiments on fish fillets, under different packaging conditions, no difference was noted in the packaging type and the organisms survival or growth when held on ice for 21 days or 3 months at -20°C. In fact, a decrease of less than 1 log was noted after 3 months at -20°C (Harrison et al., 1991). Leung et al. (1992) also noted no difference at 4°C after 21 days, although an initial growth was noted over the first 12 days, the counts had once again decreased to the initial levels by day 16. This decrease was attributed possibly to growth of other spoilage microorganisms.

6. Methods of isolation

The methods of isolation for *Listeria* put forth by the FDA and the USDA are the methods used most frequently by those striving to detect the organism. The two methods have undergone many changes in the past years and both have made an effort to encompass the quickest, most reliable methods available for isolation. The FDA procedure was first developed by Lovett et al. (1987), and originally consisted of a seven day cold enrichment in FDA Enrichment broth, diluted 1:10 in 0.5% KOH, and streaked onto FDA-Modified McBride *Listeria* Agar. Later the procedure was changed in favor of a shorter two day enrichment, the KOH dilution was eliminated, and Lithium Chloride-Phenyletanol-Moxalactam Agar (LPM) was added as a second isolation agar. (Lovett and Hitchens, 1988). The current procedure (Hitchens, 1992) consists of enrichment in *Listeria* Enrichment Broth (LEB). LEB is incubated for 24 and 48 hours at 30°C and subsequently streaked onto LPM and Oxford Agar (OXA) incubated at 30°C and 35°C

respectively for 24-48 hours. Suspect colonies from each agar, black with a black halo on OXA and sparking blue on LPM when viewed under oblique light, are then transferred to a nonselective agar for biochemical testing.

The USDA method, in comparison, uses the approach put forth by McClain and Lee (1987, 1988, 1989). The method uses a 24 hour primary enrichment in LEB at 30°C, followed by a secondary enrichment in which the LEB is transferred to Fraser Medium (FM) and incubated for an additional 24 to 48 hours. FM, originally developed by Fraser and Sperber (1988) uses LEB as its base, but contains increased levels of acriflavin-HCl, ferric ammonium citrate, and lithium chloride. The lithium chloride is added to deter the growth of enterococci, which are also known to hydrolyze esculin. This esculin hydrolysis is detected by the ferric ammonium citrate, which along with the hydrolyzed esculin, forms the characteristic black color thus indicating *Listeria* spp. Following a positive indication using FM, the sample is streaked onto Modified Oxford Agar (Curtis et al., 1989) and examined after 24 to 48 hours of incubation at 35°C for the characteristic *Listeria* colony described previously. Characteristic colonies are checked for hemolysis and biochemical tests (McClain and Lee, 1989).

The FDA and USDA methods have been compared on several occasions for selectivity and ability to isolate *Listeria* from a variety of food products under different conditions. In isolation of the organism from brie cheese, Swaminathan et al. (1988) found the FDA method to be superior to that of the USDA, but the supplementation of the USDA's selective enrichment broth with D-glucose greatly enhanced the growth of the

organism. When comparing the two methods for the isolation of the organism from fresh and heat treated seafood, such as shrimp, surimi, and crab, the USDA method was found to be preferable for raw seafood. The 48 hr. or 7 day FDA enrichment methods were found to be preferable when the cells have been stressed (Lovett et al., 1991).

Traditionally, to differentiate between the species of *Listeria*, the Christie-Atkins-Munch-Peterson (CAMP) test is used. The test is performed by streaking hemolytic *Staphylococcus aureus* and a *Rodococcus equi*, parallel to one another, on a plate of Sheep Blood Agar. The test strains are streaked at a right angle to and between these initial streaks. Based on the reaction of the test strains to the growth of the *S. aureus* and *R. equi* as well as the hemolysis, the species of *Listeria* can be differentiated (Hitchens, 1992). A direct link between virulence in *L. monocytogenes* and the CAMP reaction has been investigated, but as stated in a review by McKellar (1994) the evidence for such a link remains "unconvincing."

Several commercially available methods have been developed for the detection of *Listeria* spp. in foods including enzyme-linked immunosorbent assay (ELISA), and DNA hybridization assays. Listeria-Tek (Organon Teknika Corp., Durham, NC), ELISA can be used to detect *Listeria* spp. within 3 days. This test uses monoclonal antibodies, developed by Butman et al. (1988) to target and bind to specific antigens common to *Listeria* spp. Samples, enriched using either the FDA or USDA method, are added to the microtiter plate wells coated with antibodies. After binding has occurred, a different enzyme labeled monoclonal antibody is added and incubated, allowing the enzyme to bind

to the *Listeria* specific antigen. The plate is then washed and incubated with tetramethylbenzidine (TMB). A stop solution (H_2SO_4) is added and if the sample is positive for *Listeria* spp. a yellow color is formed. This color is checked photospectromically (Dever et al., 1993).

The Gene-Trak *Listeria* Assay (Gene-Trak Systems, Framingham, MA) is used to detect *Listeria* from both food and environmental samples (King et al., 1989). The newest version uses *Listeria* specific DNA probes to detect *Listeria* by a colormetric instead of a radioactive response. Following the enrichment, using either the FDA or USDA method, the cells are lysed to release the ribosomal RNA (rRNA). The test uses two oligodeoxynucleotide probes which hybridize to adjacent target regions of the rRNA, the advantage being that 1,000 to 10,000 copies of rRNA are present in each cell, thus intensifying the response. The capture probe binds to a specific sequence of the target and using its polydeoxyadenylate binds the complex to the polydeoxythymidine coated solid support. The detector probe, while the other fluorescein labeled probe binds to another specific sequence of the target, provides the colormetric response after subsequent enzyme treatment. The response is brought about by treatment with horseradish peroxidase labeled anti-fluorescein, followed by visualization by means of a substrate (Farber and Peterkin, 1991). The detection limit of this test is approximately 10^6 CFU/ml (Dever et al., 1993). Klinger et al. (1988) demonstrated the inclusivity and exclusivity of the probe by testing samples representing all known species of *Listeria* and 73 non-*Listeria* bacteria. Of the 139 *Listeria* strains tested, 138 gave positive results, and the false negatives from

the assay were lower than those of the conventional method. Also noted were the low numbers of false positives as well as the high sensitivity.

The need for rapid isolation of *Listeria* spp. from a food product led to the development of the Listertest by VICAM (Watertown, MA). The test makes use of immunomagnetic beads. These antibody coated beads bind with a *Listeria* in the sample after only two hours and are subsequently removed from the sample with the use of a magnet. These beads can then be run through the Listertest Lift which identifies all *Listeria* spp., or Listertest Mac which identifies pathogenic *Listeria*. Both of these tests can be completed within 24 hours and, depending on the sample, are sensitive to < 1 CFU/g (Dever et al., 1993).

Several tests are available for the biochemical testing of *Listeria*. Two of the three test kits to be mentioned rely on the additional use of the CAMP test to differentiate between *Listeria innocua* and *Listeria monocytogenes*. The MICRO-ID *Listeria* (Organon Teknika Corp., Durham, NC) system identifies *Listeria* to species within 24 hours. Bannerman et al. (1992) found that the kit, when combined with the CAMP test correctly identified 98.9% (409 out of 414) of the *Listeria* spp. Though rapid, some of the tests in the kit were considered unneeded, and the kit itself still relied on the use of the CAMP test (Dever et al., 1993).

The API *Listeria* system (BioMerieux, La Balmeles-Grottes, France) is a ten-well testing strip used to identify *Listeria* isolates to the genus, species, and subspecies within 24 hours. The system relies on the arylamidase test (DIM) instead of the CAMP test to

differentiate between *L. monocytogenes* and *L. innocua*. The test correctly identified 541 of 646 (85%) *Listeria* strains at the species and subspecies level. It also identified 252 of 258 (97.7%) strains of *L. monocytogenes* and 99.4% of *L. innocua* strains (Dever et al., 1993).

B. *Clostridium botulinum*

1. Historical Background

Recognized as a foodborne disease for more than 1,000 years, it was not until 1896 that Professor E.P.M. van Ermengem recognized *Clostridium botulinum*, known as *Bacillus botulinum* by Ermengem, as the cause of the disease affecting consumers of inadequately cured ham. This disease was recognized as a problem long associated with sausages, so it was aptly named *Bacillus botulinus*, from the Latin for sausage, *botulus* (Pierson and Reddy, 1988)

2. Taxonomy, nomenclature and morphology

Clostridium botulinum is described by Bergey's Manual of Systematic Bacteriology as Gram-positive, obligately anaerobic, rod-shaped bacteria with the capability of forming oval endospores. The species includes seven toxin types, A, B, C, D, E, F, and G, which are differentiated by the specific antigenic nature of the toxins (Cato et al., 1986). Although they produce similar effects on the host, each toxin is distinctly different; they can, however, be divided into 4 categories based on similarities. These categories are as follows (Hatheway, 1993):

Group I- types A, B, and F. Organisms in this group are proteolytic, have spores with a high heat resistance, and grow optimally at 37°C.

Group II- types B, E, and F. Organisms are non-proteolytic, have spores of low heat resistance, grow optimally at 30°C or less including refrigerator temperatures, and produce toxins that are enhanced by trypsinization.

Group III- types C and D. Organisms are generally non-proteolytic, grow optimally at 40°C, and have spores that are intermediately resistant to heat.

Group IV- type G. Organisms are vastly different from the afore mentioned groups in that it is lipase negative and asaccharolytic.

3. Growth characteristics

There are many different factors, of interest to processors, that affect *C. botulinum*'s ability to grow and form toxins. These factors differ between the different types of the organism, and most dramatically between the proteolytic and non-proteolytic. Several of these characteristics such as minimum temperature, pH and a_w , appear in Table I-1. It does, however, bear mentioning that Group II is non-proteolytic, produces heat labile spores, and does not produce putrid odors (Hackney and Dicharry, 1988). It is this characteristic that could allow non-proteolytic strains to grow undetected in a food product.

The acid used to decrease the pH has been shown to be a contributing factor in the organism's ability to proliferate and survive in lower pHs. Smelt et al. (1982) demonstrated that hydrochloric and citric acids were less able to inhibit growth than lactic or acetic acid. It has also been shown that the organism can grow at pHs lower than 4.6. This is of little concern to most processors of low pH foods because demonstration of this characteristic has occurred only in the presence of high protein concentrations (Gibson and Eyles, 1989).

Smoking, which is an adequate process to remove most microorganisms, may, if

other deterrents are not adequate, serve to aid in the development of *C. botulinum* toxins. The smoke that is deposited on the surface of the smoked fish serves to lower the oxidation-reduction potential of the product, accounting for the growth of the organism and subsequent development of toxins (Brown and Dorn, 1977).

Temperature plays an important role in proliferation control and toxin production by the organism. Hobbs (1976) stated that usually toxin production in unprocessed fish at temperatures below 10°C is so slow that the fish is spoiled before detectable amounts of the toxin are produced. After a review of numerous studies, Eyles and Marth (1981) noted that there was a margin of safety between spoilage and toxin production in fish, but that this safety margin decreased as the product's temperature increased from 0 to 10°C and above.

Although an important achievement in food preparation, modified atmosphere packaging (MAP) may serve to enhance the growth of *C. botulinum* in products. Even if a MAP does not directly affect the growth of the organism, it could promote the growth by changing the microbial flora of the product, resulting in less competition for *C. botulinum* from other organisms. The extended shelf-life of these products may also cause a problem. If fish are stored in a MAP, toxin growth could occur before the product was rejected due to spoilage (Gibson and Eyles, 1989). Post et al. (1985) found that in cod fillets stored at 8°C, rejection due to spoilage occurred at 6 days, while the toxin production did not occur until day 10 or greater. In a vacuum package, spoilage and toxin production occurred at 16 and 20 days respectively. In 100% CO₂, spoilage occurred 4

days after toxin production (23 and 19 days), and with 65% CO₂, toxin was produced on day 9, while spoilage occurred at day 16.

TABLE I - 1. Growth requirements and characteristics for types A,B, E and F of *Clostridium botulinum*

	Group	
	I Proteolytic	II Non proteolytic
Toxin types	A, B, F	B, E, F
Inhibitory pH	4.6	5.0
Minimum a _w	0.94	0.97
Inhibitory NaCl conc.	10%	5%
Minimum temp.	10°C	3.3°C
Maximum temp.	45-50°C	40-50°C
Spore heat resistance	High (D _{100°C} appr. 25 min.)	Low (D _{100°C} <0.1 min.)

(Gibson and Eyles, 1989)

4. Pathology

The food poisoning associated with *Clostridium botulinum* is traditionally an intoxication. Botulinum toxins are produced as high molecular weight complexes with molecular weights of 150-167 kDa. The toxins consist of a light and heavy chain joined by a disulfide bridge, approximately 100 and 50 kDa respectively. To become biologically active, the complex must be split by a proteolytic enzyme (Hatheway, 1993).

The toxin works by irreversibly binding to the peripheral nerve endings, blocking the release of acetylcholine at the neuromuscular junctions. Symptoms of the intoxication generally occur within 12-72 hours of toxin ingestion. Symptoms include nausea, fatigue, dizziness, headaches, dryness of skin, mouth and throat, constipation, paralysis, and difficulties in breathing. Duration of the illness ranges from 1 to 10 days depending on such factors as type and amount of toxin ingested and host resistance. Treatment includes administration of antitoxin and supportive care. If untreated, the toxin results in causing the muscles that control breathing to become nonresponsive and suffocation ensues. The death rate from this disease is now less than 10% (Gibson and Eyles, 1989; Pierson and Reddy, 1988).

There is also another illness caused by *C. botulinum* that can be transmitted via food, toxico infection (also known as infant botulism). This illness is caused by the organism actively infecting the intestinal tract of the victim and secreting the toxin. This illness primarily occurs in infants under the age of 6 months, but has been shown to occur to a lesser extent in the elderly, as well as the general population (Gibson and Eyles, 1989).

5. Epidemiology

Clostridium botulinum is widely found throughout the environment, from soils to water as well as the intestinal tract of animals. Different types of the organism are found to various degrees in different places. Type A occurs frequently in the western United States, while type B is found more frequently in Europe and the eastern U.S. (Pierson and

Reddy, 1988). The incidence of *C. botulinum* in freshly-caught fish varies greatly between geographic locations. A study of fish from the Great Lakes revealed the incidence of *Clostridium botulinum* to be anywhere from 1 to 56% (Pace and Krumbiegel, 1973), while a study, conducted in Nigeria on both fresh and smoked fish, revealed no *C. botulinum* (Okafor and Nzeako, 1985).

In 1971, an outbreak of fatal human botulism, reported in Germany (Bach et al., 1971), was traced to the consumption of smoked trout. This discovery spurred the efforts to determine the incidence of *C. botulinum* in trout farms and farmed trout (Cann et al., 1975), as well as the incidence, growth and toxin production in smoked fish. In a study conducted to determine the incidence of *C. botulinum* in farmed trout in Great Britain, Cann et al. (1975) found that of 17 farms visited in Scotland, Wales, and England, the organism was found in 13 of the farms, ranging from 2.9 to 100%. The incidence in 1,400 whole trout and viscera was 9.4 and 11.0% respectively. Types C, B, E, and F were isolated, with type C being the most prevalent. In a similar study by Huss et al. (1974), it was found that the incidence of the organism varied from 5-100% in the winter to 85-100% in the late summer. Here it was found that type E was the predominant type isolated, but other types (A and B) were found in 4 of the 550 fish sampled. *C. botulinum* type E was also isolated from the mud in the ponds, the water, and invertebrates found.

Burns and Williams (1975) found slightly different results when examining fish farms in Scotland. This study found 1.4% of the 69 fish sampled to be positive (1 fish, 1 farm), and 20% of the mud positive. Interestingly enough, the organism was only isolated

from the mud bottom and not the concrete raceways. The only type that was isolated was type B.

Although the studies showed the isolation of other types, type E is regarded as the most frequently isolated type from aquatic environments and most often implicated in botulism traced to seafood products (Hackney and Dicharry, 1988).

6. Methods of isolation

The current method of isolation as put forth by the United States Food and Drug Administration (Kautter et al., 1992), calls for an enrichment in two different broths if a mixture of proteolytic and non-proteolytic are suspected, followed by a mouse bioassay for the presence of the toxin. The sample is enriched in both Cooked Meat medium (CM) and Trypticase-Peptide-Glucose-Yeast Extract Broth (TPGY). CM is incubated at 35°C and used to promote growth of proteolytic strains, while TPGY, incubated at 27°C, is used to promote the growth of non-proteolytic strains. Both broths are incubated for anywhere from 7 to 17 days. The cultures are examined for typical clostridial cells and sporulation. For isolation of a pure culture, 1 or 2 ml of the broth is added to an equal amount of sterile absolute ethanol and incubated for 1 hour at room temperature to kill vegetative cells, leaving only spores. The liquid is then streaked onto either Liver-Veal-Egg Yolk Agar or Anaerobic Egg Yolk Agar and incubated at 35°C for 48 hours in an anaerobic environment and subsequently examined for typical colonies. For the detection of the toxin, the sample is diluted with an equal amount of gel-phosphate buffer and centrifuged. The supernatant is then used to test for the presence of toxin, and thus the

presence of viable clostridial cells. One portion of the supernatant is heat treated to destroy the toxin, one is treated with trypsin to activate the toxin of non-proteolytic samples, and one portion of the supernatant is used "as is". Each of the treatments (0.5 ml) is then injected intraperitoneally into separate mice and examined for death and the associated symptoms of the disease; the mouse injected with the heat treated supernatant should not die because the toxin was inactivated by heating. The supernatant of the positive samples can then be typed using mice and specific antitoxins.

Other methods have been examined for use in the detection of *Clostridium botulinum* in food. One such recently developed method is the polymerase chain reaction (PCR). This method relies on the amplification of specific sequences of DNA that code for toxin production. The test can be conducted after enrichment in either of the two broths described previously. In the study by Szabo et al. (1993), *C. botulinum* types A to E were examined using this method. The study revealed that the PCR assay reliably identified the *C. botulinum* types, as well as multiple strains having the same neurotoxin type. It was also found that as little as 10 fg (femtogram) of DNA, which equates to approximately three clostridial cells, was detected. It also bears noting that neurotoxin types A, B, and E (most common cause of human botulism) could be amplified from DNA extracts, vegetative cells, as well as spore preparations.

Another method of detecting *C. botulinum* positive food samples was reported by Snygg et al. (1979). This technique used gas chromatography to examine the headspace of pond-raised trout, incubated in evacuated plastic pouches. No one compound was

unique to either the positive or negative samples. However, by means of a specific pattern recognition, positive samples were able to be placed in one group and negative samples in another. The results from this study suggested that headspace analysis, coupled with the pattern recognition, may prove to be a valuable tool in screening food containing vegetative *C. botulinum* cells.

C. *Vibrio cholerae*

1. Historical Background

Vibrio cholerae was first described by Pacini, in Florence in 1854, and first isolated in pure culture by Robert Koch in 1883 (Finkelstein, 1986). This organism, the causative agent of the enteric disease, cholera, and endemic to many areas of the world, has long been a problem in areas where there is inadequate water and sewage treatment. Since the year 1817, this disease has swept the world in seven great pandemics (Zwadyk, 1992b).

2. Taxonomy, nomenclature and morphology

Bergey's Manual of Systematic Bacteriology defines *Vibrio cholerae* as Gram-negative, facultative anaerobic, straight or curved rod shaped bacteria in which growth is stimulated by sodium ions. Movement of most species is due to a single polar flagellum (Baumann et al., 1986).

Vibrio cholerae is usually divided into three groups, O1 and non-O1 serotypes, based on the organism's virulence as well as its ability to agglutinate in antisera directed against the O1 somatic antigen, part of the liposaccharide component of the cell wall of the organism (Zwadyk, 1992b). The virulent agglutinating, or O1 group, can further be divided into two distinct subgroups, classical or cholerae and El Tor biotypes. This division is made based on the such characteristics as susceptibility of the organism to polymyxin B and the Voges-Proskauer reaction (Gorzynski, 1982) (Table I-2). These biotypes are the cause of classical epidemic cholerae.

Table I - 2. Differentiating characteristics of *Vibrio cholerae* biotypes: Classical and El Tor

Characteristics	<i>V. cholerae</i> biotypes	
	Classical	El Tor
Growth in NaCl-free broth	+	+
Sucrose fermentation	+	+
Susceptibility to polymyxin B (50 units)	+	-
Susceptibility to bacteriophage IV	+	-
Voges-Proskauer reaction	-	+
Hemolysis of sheep red blood cells	-	+

(Gorzynski, 1982)

The second group, atypical or nontoxigenic O1, is biochemically the same as the virulent O1 strains of cholerae, but do not produce the cholera toxin. Finally, the third group of the organism does not agglutinate when exposed to the O1 antisera, but is otherwise indistinguishable from the O1 group (Zwadyk, 1992b).

3. Growth characteristics

Unlike other members of the family Vibrionaceae, *Vibrio cholerae* does not require salt to survive, and has been found to thrive in areas of both high and low salt concentrations. According to Madden (1988), the organism can grow in salt concentrations from 0 to 10%, but the organism has been recovered from environments of higher salt concentrations. Kaysner et al. (1987), recovered the organism from salinities ranging from 0-35.6% and reported that the incidence of the organism was not related to

the salinity of the aquatic environment.

Temperature plays an important role in the survival of *Vibrio cholerae*. When held at very low temperatures, between 0° and 5°C, the organisms die, in addition to being fairly sensitive to rapid temperature changes (Liston, 1990). This characteristic was also noted by Amako et al. (1987), that when suspensions of various strains of *V. cholerae* O1 were kept at 0°C for extended lengths of time, numbers decreased rapidly, becoming undetectable by day three, and survival time increased with an increased initial inoculum. The organism is rarely isolated from the environment below 10°C (Kaysner et al., 1987; Ogg et al., 1989).

In artificially contaminated foods held at temperatures ranging from 45 to 60°C for up to an hour, viable cells of *V. cholerae* were recovered, in some cases after 24 hours of incubation on Thiosulphate Citrate Bile Sucrose Agar (TCBS), while in other cases, 48 hours were required for growth of colonies. This observation indicates that temperature most likely injures the cells, and that in some cases, when trying to isolate *V. cholerae*, depending on the condition, the normal 24 hours of incubation may not prove adequate to recover injured cells (Makukutu and Guthrie, 1986).

V. cholerae is a facultative anaerobe, and is able to survive in anaerobic conditions. Youngren-Grimes et al. (1988), however, demonstrated *V. cholerae*'s ability not only to survive strict anaerobic conditions, but also capable of active metabolism and growth even after prolonged anaerobic incubation.

It has also been shown that the presence of both soluble and insoluble forms of

chitin in quantities as little as 0.04% (wt/vol) greatly extended low temperature survival times of the inoculum; as much as seven or more days in some instances. Furthermore, various amino acids as well as peptides were found to increase the low temperature survival time of the organism (Amako et al., 1987).

4. Pathology

The requirement for the ingestion of extremely large numbers of cells is the most probable reason for the low occurrence of acute enteritis, or cholera, in the United States, as well as other areas of the world with good sanitation and clean water supplies. According to Gorzynski (1982), in most volunteers, 10^{11} viable cells, are usually needed to overcome stomach acid and cause overt infection, although 10^6 organisms proved effective when ingested with 2g of NaHCO_3 . Doyle and Cliver (1990c), however estimated the infective dose as somewhere between 10^8 - 10^9 cells, although certain strains are shown to be more virulent. They also made note of the susceptibility of individuals who consumed antacid, and reported an infective dose as little as 10^3 in some strains of El Tor.

Cholera is characterized by abdominal pain and profuse watery diarrhea, "rice water stool," as much as several liters in a day, which results in severe dehydration, characterized by intense thirst and other symptoms of dehydration (Doyle and Cliver, 1990c). Treatment of the disease consists of replacing the large amounts of water and electrolytes lost from the body. Antibiotics are useful in shortening the duration of the disease, but by themselves are an insufficient treatment for the disease (Finkelstein, 1986).

Secretion of the cholera toxin does not in itself ensure the onset of the disease. The organism must first become established in the small intestine by adhering to the lining. The mechanism by which the organism adheres is not well understood, but it appears that motility may play a part, since only motile toxigenic strains are known to cause the disease (Zwadyk, 1992b). Pili, possessed by vibrios, as well as mucinases, secreted by *V. cholerae* may also play a part in the organisms adherence (Gorzynski, 1982).

The cholerae toxin itself is a heat labile enterotoxin comprised of two subunits, with an overall molecular weight of approximately 84,000 Da. The toxin acts by binding one of the subunits to the cells lining the small intestine, at which time the other subunit dissociates and crosses the cell membrane. Once inside the cell, the subunit stimulates adenylcyclase activity by inhibiting its turnoff mechanism. This stimulated activity causes increased levels of intracellular cyclic AMP; the result being the rapid secretion of electrolytes and water from the cells to the small intestine (Zwadyk, 1992b).

V. cholerae non-O1 are known to also produce illness in humans, although not to the degree of the O1 group. Symptoms of foodborne outbreaks of disease caused by this group of *V. cholerae*, diarrhea and vomiting, generally last less than three days (Doyle and Cliver, 1990c), and are caused by an enterotoxin related to the cholera toxin (Finkelstein, 1986). In immunocompromised individuals, however, septicemia via gastrointestinal infections can occur. This septicemia may be accompanied by nausea, but not usually by diarrhea (Madden, 1988).

5. Epidemiology

Vibrio cholerae is known to be an endemic part of the flora of many aquatic systems as well as the intestines of various animals. The organism has been shown to exist in both fresh water and waters of varying degrees of salinity, as well as in the sediment from these waters. In these cases, the differences in salinity did not correlate to differences in the vibrios detected (Kaysner et al., 1987; Venkateswaren et al., 1989; Eyles and Davey, 1988). Eyles and Davey (1988) did, however, find a seasonal variation in the incidence of *Vibrio cholerae*, with the highest incidence recorded in the fall, and the organism not being detected during winter and spring for 3-4 months. *Vibrio cholerae*, both O1 and non-O1, has also been found in the feces of aquatic birds, suggesting that these birds could serve to spread the organism over a wide area and contaminate other waters (Ogg et al., 1989).

Since the year 1817, there have been seven pandemic outbreaks of the disease, cholera. Both the fifth and six outbreaks were attributed to the classical biotype, however, in the latest and still ongoing outbreak, the biotype has been found to be El Tor (Zwadyk, 1992b).

The United States reported no cases of cholera from 1911 until 1973, when there was a lone case reported in Texas (Weissman et al., 1974). In 1978, eleven cases of cholera broke out in Louisiana, identifying cooked crabs caught in Louisiana as the source of the infection (Blake et al., 1980).

Cholera caused by *V. cholerae* O1 infections is rare, illness from non-O1 cholerae

are relatively common. In 1979, the first reported outbreak of a *V. cholerae* non-O1 gastroenteritis infection, associated with consuming raw oysters, was reported in Florida (Wilson et al., 1981). The organism has been traced to a variety of foods such as crabs, oysters, shrimp and crawfish (Bradford, 1984).

6. Methods of isolation

The FDA's Bacteriological Analytical Manual (BAM) isolation of *Vibrio cholerae* calls for an enrichment in Alkaline Peptone Water (APW) at 35-37°C for between 6 and 8 hrs, followed by streaking for isolation of sucrose positive colonies on Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS). From this agar, suspect colonies are streaked onto an agar with at least 1%, but not more than 2% NaCl. Subsequently, biochemical tests confirming *V. cholerae* can be performed. The BAM states that in lieu of conventional media, API strips may be used for identification (Elliot et al., 1992).

Because of the seriousness of the disease, rapid identification of O1 group *V. cholerae* can be accomplished by directly evaluating suspect colonies from TCBS with the *V. cholerae* O1 antisera. If no agglutination takes place, however, additional biochemical tests still need to be performed because *V. cholerae* non-O1 may be the cause of the illness (Hoover, 1985).

There has, however, been evidence presented in favor of an elevated temperature method of detection. This method employs the accepted FDA methodology, however, in the primary enrichment, the APW is incubated at 42°C instead of the normal 35°C. This method is designed to increase the specificity in isolation by the conventional media by

inhibiting microflora that may interfere with the growth of the organism. The results of this investigation showed significantly better isolation of *V. cholerae* at 42°C than that incubated at 35°C (DePaola et al., 1987).

TCBS has been criticized for inadequate selectivity for *Vibrio cholerae*, and was compared to both Sodium Dodecyl Sulfate-Polymoxin-Sucrose (SPS) Agar and Cellobiose-Polymoxin B-Colistin (CPC) Agar. The latter of these agars takes advantage of both the cellobiose as well as the polymoxin B resistance of *V. cholerae* and *V. vulnificus*. The CPC agar proved superior to both TCBS and SPC in the differentiation of *V. cholerae*, both O1 and non-O1, and *V. vulnificus* from other vibrios (Massad and Oliver, 1987). *V. cholerae*, both O1 and non-O1, did, however, grow on TCBS, and therefore TCBS could prove adequate in the isolation of *V. cholerae* from freshwater sources since other vibrio are dependent on salt for growth.

The outstanding characteristic of virulent *V. cholerae* O1 is its ability to form the cholerae toxin (CT), and therefore detection of this toxin is of the utmost importance. The Y-1 adrenal gland assay uses adrenal gland cells to test for CT or for the cytotoxin formed by *V. cholerae*. The cells are exposed to the culture supernatant and incubated overnight in a CO₂ incubator, after which, the cells are examined for characteristic appearance due to CT or the cytotoxin (Elliot et al., 1992).

Another traditional test for this toxin, an ELISA, has been used since the 1970's to look for cholera toxin. This method uses burro anti-CT as the capture molecule, rabbit anti-CT as the second antibody, and alkaline phosphatase-labeled goat anti-rabbit

immunoglobulin as the indicator molecule. The ELISA assay was compared to a latex agglutination assay for CT, which uses latex sensitized to CT with rabbit immunoglobulin G. The results of this comparison showed that the latex agglutination was 98% effective when compared to the ELISA test (Almeida, 1990).

Other tests available to test for CT, include the Y-1 adrenal gland assay, various ELISA tests, as well as gene probes available for CT-like gene sequences (Elliot et al., 1992).

D. *Yersinia*

1. Historical Background

Yersinia pestis, the cause of bubonic plague, has long been known to cause human disease and suffering. It was not until 1939 that Schleiferstein and Coleman, calling it *Bacterium enterocoliticum*, recognized another member of the genus *Yersinia*, *Yersinia enterocolitica*, as a human pathogen (Schofield, 1992). Even then, it wasn't until the 1970's that the organism's significance became apparent. The reported cases of yersiniosis worldwide increased from less than 25 in 1966, to over 4,000 only eight years later. In some places, infections caused by *Yersinia* became a more prevalent type of enteric infection, surpassing even *Salmonella* (Doyle, 1985), although the cases of human infection in the U.S. is infrequent (Doyle, 1988). This rise in reported cases as well as reported isolation from foods is probably due to two major factors: increased awareness of the disease as well as improved methods of isolation (Schofield, 1992).

2. Taxonomy, nomenclature and morphology

According to Bergey's Manual of Systematic Bacteriology, *Yersinia* are Gram-negative, facultative anaerobic, 0.5-0.8 μm , rod shaped to coccobacilli organisms of the family *Enterobacteriaceae*. *Yersinia* spp., with the exception of the non-motile *Y. pestis*, exhibit motility when grown below 30°C but are non-motile when grown at 37°C (Bercovier and Mollaret, 1986). Motility is due to either paripolar or peritrichious flagella (Gutman, 1992).

There are currently 27 known serotypes of *Y. enterocolitica*; the distinction made

on the basis of their O and H antigens. Currently there are six known serotypes of *Y. pseudotuberculosis*, each characterized by type specific O and H antigens. Some of the O antigens are also present in the B and D groups of *Salmonella*, and are thus responsible for cross reactions of these organisms (Gutman, 1992).

3. Growth characteristics

Bergey's Manual of Systematic Bacteriology states that all species of *Yersinia* can grow at a temperature range of 4-42°C with an optimum temperature of 28-29°C. The pH range for the various organisms differs with the species. *Y. pseudotuberculosis* can grow at a pH range of 5.0-9.6, while *Y. enterocolitica* requires a pH range of 4.0-10. It should also be noted that *Y. pseudotuberculosis* can only tolerate salt concentrations of up to 3.5% (Bercovier and Mollaret, 1986).

Doyle (1988) reports that *Y. enterocolitica* is sensitive to heat, 50°C and above, and sensitive to salt concentrations above 5%, as well as a pH of 4.6 and below. The organism is generally considered to be inactivated by conditions adequate to kill another enterobacter, *Salmonella*.

The resistance to relatively high pH, as well as the ability to grow at refrigerator temperatures are valuable in the isolation of the organism from samples. Most enrichments are performed at low temperatures to inhibit the growth of competing microflora, while allowing *Yersinia* to grow unchecked. Most isolation procedures also employ a transfer of the enrichment broth to a solution of KOH before streaking onto a selective agar. Typically the KOH concentration is 0.5% which is sufficient to kill many of

the competing microorganisms (Doyle and Cliver, 1990b).

4. Pathology

Although a variety of serotypes can readily be isolated in the environment, only those belonging to the serotypes O:3, O:5, O:8, and O:9 are considered to be pathogenic in humans (Schofield, 1992).

The organism is known to produce a heat stable toxin similar to *Escherichia coli*, but its role in food borne intoxications has not been thoroughly explored. The pathogenicity factor of the organism, although not well understood, is known to be plasmid-mediated (Schofield, 1992).

The onset of the illness is usually about 1 day. The illness is usually characterized by gastrointestinal symptoms such as diarrhea, and mesenteric lymphatic involvement, as well as other systematic symptoms such as headache, general feeling of malaise, and fever which could be associated with convulsions. Both *Y. enterocolitica* and *Y. pseudotuberculosis* are also known to produce severe abdominal pain, usually in the lower right region, which when coupled by fever, is often mistakenly diagnosed as appendicitis. Milder cases of simple gastroenteritis are often indistinguishable from those caused by *Salmonella* or *Shigella*. One complication associated with the disease is a type of arthritis (erythema nosodum), which is rare in the United States and Canada. Other complications include septicemia and liver abscesses, but these complications are commonly associated with diabetics, those with preexisting liver problems, those receiving corticosteroids, or those under the age of 1 year. Diagnosis can be made from cultures

taken from feces and blood, as well as other infected organs. Treatment consists of using commonly used antibiotics, based on that particular strain's susceptibility, as well as maintaining the electrolytic and fluid balance of the patient (Gutman, 1992; Kanazawa et al., 1987).

Age also plays a major role in the manifestations of the disease. In children and adolescents, gastroenteritis, mesenteric lymphitis, and pseudoappendicitis are the dominant manifestations of the disease. In adults, cases of acute abdominal disorders, diarrhea and arthritis primarily occur. In the elderly, erythema nosodum or arthritis is the primary symptom (Palumbo, 1986; Sato, 1987).

5. Epidemiology

Yersinia enterocolitica is commonly found in the environment, and studies have shown swine to be the main reservoir of the bacteria, although the organism has been isolated from other animals. Studies regarding the incidence of *Yersinia pseudotuberculosis* in foods number much fewer than those of *Y. enterocolitica*, most probably because the former is not as ubiquitous in the environment as the latter. Regardless of where it was isolated, the majority of the strains of *Yersinia enterocolitica* found are non-pathogenic, with the exception of those isolated from pigs and may explain why there are relatively few food related outbreaks of yersiniosis reported (Doyle, 1985, 1988). Although pork is the main reservoir of virulent *Y. enterocolitica*, pork has yet to be implicated in a foodborne outbreak (Doyle, 1988). In some European countries, there has been evidence that pigs play a major role in the transmission of serotypes O:3 and O:9,

although these serotypes are uncommon in the U.S. where the predominant pathogenic serotype is O:8 (Schofield, 1992).

In 1981, there was an outbreak in Washington state among 87 people. The patients involved had all consumed tofu that was packed in unchlorinated spring water. From the patients *Y. enterocolitica* was isolated and subsequently serotyped and found to be serotype O:8. Water was tested from the processing plant and found to contain the same serotype as that of the patients, thus implicating the water as the disease source (Aulisio et al., 1983).

Yersinia enterocolitica, has been isolated from fish and seafood, although few studies have been done to examine the incidence. Most likely the reason for this is that as of yet, no outbreaks have occurred associated with fresh fish, and the isolation of most of the strains in seafood have proved to be non-virulent. Bruce and Drysdale (1989) found that the incidence of *Yersinia* spp. in fresh salmon was 50%, in smoked salmon 47.5%, 55.6% in rainbow trout, and 47.8% in mussel samples. The majority of the isolates were either Biotype 1 or closely related. All of the samples from the salmon consisted of serotypes not previously associated with disease.

Although water and environmental samples may harbor *Yersinia*, the significance of isolation from these sources still needs to be established. By doing so, the pathogenic potential of these sources as well as that of food may be made more clear (Schofield, 1992).

6. Methods of isolation

According to the FDA Bacteriological Analytical Manual (BAM), 7th ed., the isolation of the organism begins with an enrichment in Peptone Sorbitol Bile Broth (PSBB) for 10 days at 10°C. The enrichment, 0.1 ml, is added to 1 ml of 0.5% KOH in 0.5% saline and mixed for 5 to 10 seconds before streaking onto both MacConkey Agar and Cefsulodin-Irgasan-Novobiocin Agar (CIN). Both agars are incubated for 48 hours at room temperature. Typical colonies are transferred to Lysine Arginine Iron Agar (LAIA), Christensen's Urea Agar, and Bile Esculin Agar slants. It should be noted at this time that all of the currently known pathogenic strains of *Y. enterocolitica* do not rapidly hydrolyze esculin within 24 hours. Isolates that meet the criteria described in the BAM are then streaked onto Anaerobic Egg Yolk Agar (AEY) and incubated at room temperature. From this agar, the biochemical tests may be performed. According to the BAM, in lieu of many of the biochemical tests, the Minitek Minikit biochemical test kit may be used, or the API 20 E identification may be used for *Yersinia* identification (Weagent et al., 1992). Other biochemical test kits not mentioned in the BAM may also be used, providing that they distinguish between different members of the family *Enterobacteraceae*.

The plasmid that is identified with many of the traits associated with pathogenicity of the organism can be tested for in several ways. The autoagglutination test is carried out in duplicate tubes of MR-VP, incubated at room temperature and 35°C. The tube incubated at room temperature shows turbidity, while the tube at 35°C shows clumping.

Any other autoagglutination pattern is considered negative for the virulence plasmid.

Another test for the virulence plasmid is the binding of crystal violet to the cell wall when incubated at 37°C but not at 25°C. Other virulence assays include DNA hybridization specific for a plasmid in both *Y. enterocolitica* and *Y. pseudotuberculosis*, low calcium response to Congo Red Agarose virulence test, as well as a suckling mouse bioassay (Weagent et al., 1992).

E. *Salmonella*

1. Historical Background

The first member of the species, *Salmonella*, to be described was *Salmonella choleraesuis*; described by bacteriologist Salmon, for whom the genus is named (Neter, 1988). *Salmonella* is the most commonly reported cause of foodborne illness accounting for 45% of outbreak associated cases from 1973-1987 (Bean and Griffin, 1990). Until the mid 20th century, concern with *Salmonella* hinged on the organism's most severe manifestation, typhoid fever. Since then, control of carriers of the disease, water treatment and improved sewage disposal have reduced the numbers of those affected by the disease in the U.S. from over 5,000 in 1942 to 460 in 1989. Seventy percent of these cases were acquired when people traveled abroad. Today, the number of people estimated to become ill with the milder gastroenteritis is thought to be somewhere around 2 million per year, with only around 3% reported (Zwadyk, 1992a).

2. Taxonomy, nomenclature and morphology

Members of the genus, *Salmonella*, are Gram-negative, facultative anaerobic, 0.7-1.5 μm , straight rod shaped of the family *Enterobacteriaceae*. *Salmonella* is usually motile by means of peritrichious flagella (Le Minor, 1986).

There are currently over 2,000 known serovars of *Salmonella*, each with distinctive O and H antigens. The distinction between the serovars is made by the examination of the somatic antigen, and the two flagellar or H antigens, known as phase 1 and phase 2 H antigens (Le Minor, 1986). Evidence from genetic studies revealed that all

Salmonella and those organisms previously classified in the genus *Arizona* belonged to the same species, *S. enterica*. This species has been divided into six subspecies: *enterica*, *salamae*, subgroup 3a and 3b (*arizonae* and *diarizonae*), *bongori*, and *indica*. Within these subspecies, occur the more than 2,000 serovars (Zwadyk, 1992a).

3. Growth characteristics

Temperature is a major factor in the control and destruction of *Salmonella*. The organism can grow at a temperature range between 5-45°C and possibly as high as 47°C, with an optimal range of 35-37°C. Palumbo (1986) stated that the minimum temperature for growth was a function of cultural conditions as well as the food itself, citing growth in foods with temperatures as low as 4.4 and 5.2°C although the rate of growth was greatly decreased. The organism does not survive freezing as well as other organisms, and generally, a 1-2 log decrease can be achieved by freezing and thawing the sample one time (Doyle and Cliver, 1990a). *Salmonella* are also heat sensitive organisms which are generally killed at temperatures attained by such heat processes as ordinary pasteurization or cooking in most high moisture products, and occurrences on such products are usually a result of post-processing contamination (Flowers, 1988). For *S. typhimurium* in whole eggs, the D value at 60°C is about 0.27 minutes (Doyle and Cliver, 1990a).

Other factors used to control the growth and aid in the destruction of *Salmonella* are a reduction in the pH of the food or increase in the salt level, thereby effectively lowering the A_w . *Salmonella* grows at a pH of around 4-9, depending on the strain and the type of acid present in the substrate. Also the closer the pH is to the limits listed, the

slower the organism will grow. *Salmonella* has been shown to grow in a water activity in the range of 0.945-0.999 (Doyle and Cliver, 1990a). For the most part, salmonellosis can be attributed to several key factors: improper cooling, inadequate thermal processing, consumption of raw products, or cross contamination (Flowers, 1988).

4. Pathology

Salmonella produce a large number of virulence factors that contribute to the overall invasiveness of the organism, the role of each, differing between the various serovars of the organism. *Salmonellae* produce virulence factors such as surface antigens, endotoxins and enterotoxins. The O antigenic side chains as well as the Vi antigen aids the organism in being able to attach to the host cells and survive phagocytosis. The O antigen in some strains appears to be important for the bacteria's penetration into the subepithelial tissue of the small intestine. Recent findings have also shown that the organism synthesizes new proteins in the presence of mammalian cells and these new proteins aid in the organism's attachment and subsequent penetration of the small intestine. The role of the endotoxin and enterotoxins formed by some strains is unclear, but *Salmonella* also produce a cytotoxin associated with the outer membrane. The fact that this cytotoxin is associated with the outer membrane leads to the reasoning that it is important in invasiveness and cellular destruction (Zwadyk, 1992a).

Typhoid fever is the most severe of the diseases caused by *Salmonella*. This disease is most often caused by the serotype *typhi*, but may also be caused by other strains such as *paratyphi* A and *paratyphi* B, although these forms are generally milder. The

disease is first characterized by malaise, fever, aches, and constipation, during which time the organism is moving from the intestine to the lymphatic, bloodstream, and other systems. The next symptoms occur as a tender abdomen, diarrhea, and a fever of around 104°F. The death rate from this disease ranges from 2-10% (Zwadyk, 1992a).

Gastroenteritis caused by a *Salmonella* infection of the colon usually shows manifestation between 6 and 48 hours after ingestion of the organism, but generally occurs between 12-36 hours. The infection is characterized by diarrhea, fever, vomiting, and abdominal pain, and usually lasts from 1 to 7 days, but could last as long as several weeks. Most cases of gastrointestinal salmonellosis are usually not reported, but instead are attributed to "stomach flu." Once the symptoms subside, the organism can still be isolated from the feces of those infected, sometimes as long as several weeks, but chronic carriers of the disease are rare. Treatment of the gastrointestinal disease should be confined to maintaining fluid/electrolyte balance. Antibiotic treatment only serves to prolong the carrier state and increase the chances of a secondary case (Flowers, 1988; Zwadyk, 1992a).

5. Epidemiology

Brown and Dorn (1977) stated that although most fish related foodborne illness in the U.S. are due to a select few microorganisms, raw whole fish in general, except when harvested from polluted waters, rarely contained bacteria pathogenic to man. In cases of *Salmonella* poisoning, the fish is contaminated during processing or in polluted waters, harboring the organism in its intestinal tract or on its skin. The Center for Disease Control

(CDC) reported that from 1977-1981, fishery products accounted for only 0.9% of the outbreaks of salmonellosis (CDC, 1982).

Research has been conducted to assess the bacteriological quality of wastewater aquacultural systems. Cloete et al. (1984) found that although large numbers of potential pathogens in the water that the waste treatment system reduced their presence by more than 99.6%. Although the intestines, skin, and gills the fish raised in this system contained pathogens, fish of natural populations contained similar counts. The organism was not found in the blood or flesh of the fish. Baker et al. (1983) noted a 95% decrease during the first 6 hours of viable cells of *S. typhimurium* in the water column of culture pools fertilized with fresh swine waste. The organism was detected in the viscera of the fish as well as the water at 16 but not 32 days. No *Salmonellae* were isolated from the flesh or blood of the fish. Similar results were found by Phelps and Stiebel (1991), who found *Salmonella* at levels of 23.3% in water samples but on examination of carp from the water found an incidence of only 3.2%. The study also noted that no *Salmonella* was found in either the mucus (skin), or the kidneys, but was instead confined to the intestine.

Products grown using more accepted methods than those of wastewater aquaculture systems have been found to harbor *Salmonella*. The United States Food and Drug Administration found *Salmonella* in retail catfish fillets in the southern U.S., but at an incidence of less than 5% (Andrews et al., 1977). In an examination of a variety of fresh and frozen fish in Kochi, an incidence of 5.76% of the fresh fish and 8.66 of the frozen fish were found to be contaminated with *Salmonella* (Nambiar and Mahadevaiyer,

1991). *Salmonella* was also isolated from the intestine and to a lesser degree from the gills and skin of naturally grown common carp (Cloete et al., 1984). Upon examining *Tilapia nilotica*, Youssef et al. (1992) found that only 3.9% harbored *Salmonella* in the intestine.

Caution needs to be exercised to not group fish together in the risks associated with potential pathogens. The most concrete thing that can be said concerning fish and *Salmonella* is that isolation from fresh fish is variable. Fish have demonstrated differing levels of *Salmonella* when grown under differing conditions, as well as those fish obtained from natural habitats. Research needs to be done to carefully assess microbial quality of fresh rainbow trout related to the unique conditions under which it is reared.

Smoked fish have also been the subject of studies concerning *Salmonella*. Dodds et al. (1992) in a survey of both cold and hot-smoked fish products, found no incidence of *Salmonella* in any sample. Similar results were found by Okafor and Nzeako (1985) who also examined fresh fish and found no incidence of *Salmonella*.

6. Methods of isolation

The current method of isolation put forth by the United States Food and Drug Administration (Andrews, 1992) begins with a pre-enrichment in a nonselective broth to aid in the recovery of stressed microorganisms for 24 hours at 35°C, after which the organism is transferred to selective enrichment broths. These broths, Selenite Cystine Broth (SC) and Tetrathionate Broth (TT) which are incubated at the same temperature and duration as was for the pre-enrichment. Selective agars {(Hektoen Enteric Agar

(HE), Bismuth Sulfite Agar (BS), and Xylose Desoxycholate Agar (XLD)} are then streaked from each of the selective broths, incubated, and examined for typical *Salmonella* colonies. Typical colonies are then subjected to preliminary biochemical tests on Triple Sugar Iron Agar (TSI) and Lysine Iron Agar. Isolates giving typical results are then biochemically tested by conventional testing media or by the use of 5 accepted biochemical systems, including the API 20E system. Rapid confirmation may also be accomplished by such procedures as agglutination which test for the presence of somatic antigens.

This method, from initial pre-enrichment to the completion of initial biochemical testing, takes 5 or more days, and is thereby considered very time consuming, especially when testing the quality of foods before shipping. Other methodology, as well as media, is being examined for its ability to aid in a more rapid screening of samples or a more sensitive detection of the organism.

G. Further Processing

When accessing the microbial hazards associated with rainbow trout, it needs to be taken into account that the product will receive additional heat treatment before consumption. These processes are divided into three different types: conventional cooking, microwave cooking, and smoking.

1. Conventional cooking

Conventional cooking is probably the most common end treatment of rainbow trout, and when compared to microwaving, heats the product more uniformly. Of the nonspore pathogens examined, *L. monocytogenes* is considered to be the most heat resistant. Although the D-values of all appear in Table I-3.

As shown in the table, a fillet, if cooked to well done would be adequate to destroy *L. monocytogenes* that may be present. It should be noted that although *Mycobacterium* was not included in the table, the organisms are no more resistant to moist heat than other bacteria and are easily destroyed by temperatures present during the pasteurization process (Willet, 1992). Although *Clostridium botulinum* may survive the process, adequate cooking should serve to destroy levels of the toxin that may be present, thus minimizing the risk.

TABLE I - 3. Heat resistance (expressed as D-value) and corresponding food medium for select microbial pathogens as reported in various studies

Organism	D-value	Food	Source
<i>Clostridium botulinum</i> Proteolytic	$D_{100^{\circ}\text{C}} = 25 \text{ min.}$	not given	Gibson and Eyles, 1989
<i>Clostridium botulinum</i> Non-proteolytic	$D_{100^{\circ}\text{C}} = <0.1 \text{ min}$	not given	Gibson and Eyles, 1989
<i>Listeria monocytogenes</i>	$D_{71.7^{\circ}\text{C}} = 1 \text{ sec}$	raw milk	Bahk and Marth, 1990
	$D_{55^{\circ}\text{C}} = 12.00 \text{ min}$	blue crab	Harrison and Huang, 1990
	$D_{60^{\circ}\text{C}} = 2.61 \text{ min}$		
<i>Salmonella</i> spp.	$D_{60^{\circ}\text{C}} = 0.27 \text{ min}$	whole eggs	Doyle, 1990
<i>Vibrio cholerae</i>	$D_{60^{\circ}\text{C}} = 2.65 \text{ min}$	crabmeat homogenate	Shultz et al., 1984
	$D_{71^{\circ}\text{C}} = 0.30 \text{ min}$		
<i>Yersinia enterocolitica</i>	$D_{55^{\circ}\text{C}} = 4.6 \text{ min}$	skim milk	Pavlov, 1989

2. Microwaving

Several studies have examined the effectiveness of microwave cooking on the destruction of pathogens in food products. These studies found that pathogen destruction was based on several intrinsic properties of the foods. These properties include the moisture and ionic contents of the food, product density and shape and load volume of the product. An increased ionic content of the food serves to decrease the amount of penetration of microwaves into the food product, thus resulting in an unevenly heated

food. It was also stated that in general the higher the moisture content of the food, the more efficiently the food will heat. Although larger items absorb more microwave energy than do smaller ones, the larger items heat more slowly because time is needed for the temperature to equilibrate due to conduction. Shape plays an important role also, as objects with corners show more localized heating (Heddleson and Dorres, 1994).

Luend et al. (1989) investigated the survival of *Listeria monocytogenes* in stuffed chicken and found that although the product was cooked to a high temperature, and these temperatures accounted for a 10^6 reduction in *Listeria* counts, differences in the temperature within the stuffing (72 to 85°C) allowed for the isolation of viable organisms from the finished product.

Huang et al. (1993) examined the fate of *Listeria monocytogenes* on microwaved catfish fillets. The fillets were cooked to well-done (70°C), just done (60°C), and slightly underdone (55°C), by measuring the temperatures in different parts of the fillet. Because of different heating rates in the product, the destruction of microorganisms differed also. As expected, the thinnest edges of the food heated first, causing increased lethality in that area. Covering the fillets with polyvinylchloride films during cooking also helped to reduce the numbers of viable *Listeria* found in the product. Initial counts of *Listeria* in the product were 10^6 cells/cm². Cooking uncovered at each temperature reduced the numbers of *Listeria* found in the product except that with an end temperature of 55°C. No uncovered cooking process completely destroyed all viable *Listeria* cells. The counts for the covered fillets were all reduced and cooking at 70°C reduced the counts below the

limit of detection. The authors concluded that fillets should be cooked to well done and the product should be cooked covered to aid in even heating.

3. Smoking

Smoking is a process in which fish are brine smoked, partially cooked and dried, resulting in products that have extended shelf-life or a relatively shelf stable product. Although various authors have examined the incidence of *Listeria* in smoked products, there have been few studies to examine the survival of *Listeria* during the smoking process (Fuchs and Nicolaides, 1994). *Listeria* has been isolated in both hot and cold smoked fish. These products often are eaten "as is" with little or no additional heat treatment, and are thereby considered "ready-to-eat" products. Dillon et al. (1994) found *Listeria* spp. in 25.4% of hot-smoked products and 6% of the cold smoked products. The hot smoking process should prove adequate to destroy *Listeria* spp. found on the fish, however, the author noted that processors in the area, often deviate from the outlined hot smoking protocol. Fuchs and Nicolaides (1994) also isolated *Listeria* spp. from hot and cold smoked fish at levels of 8.7% and 8.6% respectively, citing post processing contamination as the cause in the hot-smoked product.

In experimentally contaminated hot-smoked trout, Jemmi and Keusch (1992) found that when exposed to hot-smoke temperatures (core temp. 65°C during 20 min.) the initial surface counts dropped from 106 MPN/g before smoking to undetectable levels after smoking and throughout the storage time of 20 days. In trials where the fish were inoculated after smoking, neither an increase nor a decrease was observed in fish held at

4°C for 20 days where an increase, as much as 6 logs, was observed in fish stored at 8-10°C. Farber (1991) when examining artificially contaminated smoked salmon held at 4°C, did observe as much as a 2 log increase in 15 days. The difference in these two experiments could mean that salmon offers a better substrate for the growth of *Listeria monocytogenes* than does trout.

Smoking of the product, however, if not adequate to destroy the spores of the organism present, may provide a product that could prove to be hazardous due to toxin formation and no additional heat treatment before consumption. A smoking process that provides a temperature of 82.2°C for a minimum of 30 minutes is considered to be sufficient in the destruction of 10^7 spores of *C. botulinum* type E (Hackney and Dicharry, 1988). Proteolytic types of *Clostridium botulinum* are much more resilient than the non-proteolytic strains, and smoking may not be adequate to destroy the spores of these types. Other precautions therefore need to be taken by processors to ensure that in the event the spores survive the treatment, the organism will not have adequate conditions to produce toxin. These conditions may be avoided by adequate storage of the product at least below 10°C (minimum temperature for proteolytic types), but more preferably at temperatures below 3.3°C which would prevent the outgrowth of non-proteolytic type toxins, the spores of which could have survived the smoking treatment. These conditions, coupled with a minimal salt concentration of 3% ensure wholesomeness in excess of 7 days and allow a safety factor of 1 day at room temperature for possible mishandling of the product (Cann and Taylor, 1979).

Brown and Dorn (1977) noted that smoking, which is an adequate process to remove most microorganisms, if other deterrents are not adequate, may serve to aid in the development of *C. botulinum* toxins. The smoke that is deposited on the surface of the smoked fish serves to lower the oxidation-reduction potential of the product, accounting for the growth of the organism and subsequent development of toxins.

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Section II: The Incidence of Pathogenic Microorganisms in Aquacultured Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Qualitative and quantitative levels of human pathogens (*Listeria monocytogenes*, *Clostridium botulinum*, *Salmonella* species, *Vibrio cholerae*, *Yersinia enterocolitica* and *Y. pseudotuberculosis*) and general microbial quality of whole aquacultured rainbow trout (*Oncorhynchus mykiss*) were used to compare differing aquacultural systems and growing seasons. *Listeria* levels and general microbial quality were also determined on fillets from three facilities and compared to levels on whole fish. Aerobic plate counts for whole fish and fillets ranged from 3-6 logs. For fillets, coliform levels were high in some cases (3.09-543 MPN/g), fecal coliforms (Non detectable-4.98 MPN/g) and *E. coli* counts (Non detectable-3.58 MPN/g) were lower with *E. coli* only isolated from one plant in one season. *Listeria* incidence for whole fish ranged from 25-90% with average levels from 0.36-4.83 MPN/g, and 60-90% with levels of 1.12-21 MPN/g for fillets. Presumptive *C. botulinum* incidence ranged from 45-95% with levels averaging 0.46-2.33 MPN/g. Coliform, fecal coliforms, and *E. coli* counts and *Listeria* and *C. botulinum* incidence and

counts were found to be significant in plant/year interaction ($p < 0.05$). This interaction indicates trends and differences seen in one plant may not be observed in others. There was a significant increase in mesophilic APC, coliforms, and *Listeria* counts for fillets ($p < 0.05$). No *Salmonella*, *Vibrio cholerae*, or *Yersinia* were isolated from any samples tested. The presence of both *C. botulinum* and *Listeria* would be of concern to processors.

INTRODUCTION

In December of 1995, the United States Food and Drug Administration (FDA) published a rule for the safe processing of fish and fishery products. In this regulation, the FDA called for the implementation of a mandatory quality assurance program based on the Hazard Analysis Critical Control Point (HACCP) concept. With the approaching deadline (December 1997) for the implementation of HACCP, there is a need for sound data identifying the biological hazards contained in fishery products including, the incidence of microbial pathogens. While the microbial hazards of some fishery products have been extensively reported, there are some fish species or fishery products where only limited, if any, information exists.

Listeria monocytogenes, which usually only poses a threat to certain "at risk" populations such as the immunocompromised or pregnant women and their fetuses, has generated much concern in the food industry over the past ten years. *Listeria* is ubiquitous in its distribution and has been isolated from a variety of seafood products

(Farber, 1991; Hartemink and Georgsson, 1991; Weagent et al., 1988). The organism represents a large threat during processing and post processing handling due to its ability to grow at refrigeration temperatures.

Clostridium botulinum, the causative agent of the intoxication, botulism, is widely found throughout the environment, from soils to water, as well as the intestinal tract of animals (Pierson and Reddy, 1988). The organism has caused illness due to the consumption of seafood products, although the type usually implicated in seafood is of the nonproteolytic type E (Hackney and Dicharry 1988).

Salmonella as well as the most pathogenic strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* can be transmitted to fish when the fish are raised in polluted environments. Swine has been shown to be the main reservoir of *Yersinia enterocolitica*, although the organism has been isolated from other animals.

The disease, cholera, caused by the organism *Vibrio cholerae* antigen type O1 infections is rare, although illnesses from non-O1 cholerae are relatively common (Wilson et al., 1981). *V. cholerae* has been shown to exist in both fresh and salt waters and aquatic sediment (Kaysner et al., 1987; Venkateswaren et al., 1989; Eyles and Davey, 1988); although, the organism is found predominately in marine areas such as oceans and estuary regions. Both types of *V. cholerae* have been found in the feces of aquatic birds which suggests that these birds could serve to spread the organism to other waters (Ogg et al., 1989).

The objective of this study was to determine the presence of *Listeria*

monocytogenes, *Clostridium botulinum*, *Salmonella* species, *Vibrio cholerae*, and *Yersinia enterocolitica* in aquacultured rainbow trout (*Oncorhynchus mykiss*). Secondly, the purpose was to compare differing rearing systems and growing seasons on the basis of qualitative and quantitative levels of pathogens and general microbial quality. Finally, the general microbial data and qualitative and quantitative *Listeria* levels on whole fish were compared to those of fillets.

MATERIAL AND METHODS

Selection of facilities and sampling periods

The facilities for the study were chosen based on two factors: type of aquaculture system employed and presence of processing operations. Four of the facilities chosen employed a flow-through aquaculture system, while the fifth used a system recirculating approximately 80% of its water. Facilities were chosen, where possible, that processed their own fish, although no facilities which used the recirculating system processed fish. A total of five facilities were sampled for whole fish. Fillets from three of the five facilities were also sampled. Sampling took place from late-August through early October 1994 which is typically a low water flow period and from mid-May through early July 1995, typically a high flow period. Water temperature remained relatively constant, approximately 13°C, throughout both sampling periods.

Collection of Samples

Twenty fish from each of the aquaculture facilities were obtained, transferred to

individual sterile stomacher bags, and placed on ice for transport. A sample of the rearing water was also obtained in a sterile container which was opened and sealed under water and transported on ice to the Department of Food Science and Technology at Virginia Tech in Blacksburg, Virginia. Samples were held on ice in a walk-in refrigerator until analysis. The samples were examined within 36 hours of collection. From each of three facilities that processed fish, twenty trout fillets were taken and treated in the same manner as the whole fish.

Microbiological analysis

The whole fish were removed from the stomacher bag and aseptically eviscerated. The fish were then weighed and an equal amount of Butterfield's phosphate buffered dilution water was added to the bag, after which the fish was massaged for one minute. The rinse solution was then used for the microbial analysis. The fillets were treated in the same method as the whole fish.

General microbial analysis

Five arbitrarily selected samples from each plant were tested for aerobic plate count (psychrotrophic and mesophilic) while twenty samples were tested for the presence of pathogens. The water was tested for coliforms, fecal coliforms, and *Escherichia coli*. Twenty fillets were tested for *Listeria monocytogenes*, five of which were also tested for aerobic plate counts, coliforms, fecal coliforms, and *Escherichia coli*. All serial dilutions were performed in Butterfield's phosphate buffered dilution water.

Coliform, fecal coliform and *E. coli* tests were performed according to the liquid

medium method of isolation described in the Food and Drug Administration's Bacteriological Analytical Manual (BAM) using the three tube Most Probable Number (MPN) method (Hitchens et al., 1992). Biochemical testing was carried out using API Rapid 20E test kits (bioMérieux Vitek, Hazelwood, MO).

Presence of the selected pathogens, with the exception of *C. botulinum*, was determined by enrichment of 25 ml of the sample in 225 ml of the enrichment medium. Enumeration of all pathogens was carried out using the three-tube MPN method. Dilutions were carried out in Butterfield's phosphate buffered dilution water.

Listeria

Listeria was isolated according to the methodology described in the BAM (Hitchens, 1992). This method includes a 48 hr. incubation at 30°C in *Listeria* Enrichment Broth, followed by isolation on Modified Oxford Agar. Biochemical testing for identification was carried out using API *Listeria* (bioMérieux Vitek, Hazelwood, MO).

Clostridium botulinum

Enumeration of probable *C. botulinum* cells was performed using the three-tube MPN method. Enrichment was accomplished in both Cooked Meat Medium and Trypticase Peptone Glucose Yeast Extract Broth. After enrichment, the MPN tubes were tested for anaerobic spores by treating a portion of the sample with an equal amount of absolute ethanol and streaking onto Anaerobic Egg Yolk Agar. The plates were then incubated under anaerobic conditions using anaerobe jars and GasPak Plus™ anaerobic system envelopes with a paladium catalyst (BBL®). The plates were then checked for

typical *C. botulinum* colonies, and counts of probable *C. botulinum* were made using the three tube MPN method. A representative group of probable *C. botulinum* positive MPN tubes were selected at random and examined for toxin production using the mouse bioassay. For each sample tested, three mice were used: one receiving a heat treated sample, one receiving a trypsinized sample, and one receiving an untreated sample (Kautter et al., 1992).

Salmonella

Salmonella enrichment was carried out in Lactose broth, followed by isolation on Xylose Lysine Desoxycholate Agar and Bismuth Sulfite Agar. Typical colonies were selected and transferred to both Lysine Iron Agar and Triple Sugar Iron Agar (Andrews et al., 1992). Subsequent biochemical identification testing was accomplished using API Rapid 20E test strip (bioMérieux Vitek, Hazelwood, MO).

Vibrio cholerae

An enrichment procedure was carried out in Alkaline Peptone Water with subsequent isolation on Thiosulphate Citrate Bile Sucrose Agar. Colonies were then tested for salt tolerance on T₁N₁ Agar and tested for the presence of oxidase (Elliot et al., 1992). Subsequent biochemical testing was not necessary because colonies either failed to grow with the increased salt concentration or those that did grow were oxidase negative.

Yersinia enterocolitica and *Yersinia pseudotuberculosis*

An enrichment procedure was carried out in Peptone Sorbitol Bile Broth incubated at 4°C for seven days. A dilution of 1 to 10 was then made in 0.5% KOH in 0.5% saline,

and streaked for isolation onto Yersinia Isolation Agar with CIN antimicrobial supplement. Colonies were then transferred to Lysine Arginine Iron Agar, Esculin Bile Agar, Christensen's Urea Agar slants (Weagent et. al, 1992). Subsequent biochemical identification was performed on API Rapid 20E test strip (bioMérieux Vitek, Hazelwood, MO).

Analysis of Data

Data generated was analyzed using the Statistical Analysis System (SAS) software. The quantitative data was subjected to an analysis of variance testing for differences in the factors of plant, season, plant coupled with season, as well as fish form (whole or fillet) where applicable. Levels of incidence (*Listeria* and *C. botulinum*) were subjected to a logistic regression was tested using logistic regression testing for differences in the factors of plant, season, plant coupled with season, as well as fish form (whole or fillet) where applicable. Data from each of the flow-through systems was compiled and treated as one facility when compared to the recirculating facility.

RESULTS AND DISCUSSION

Aerobic Plate Count

The anaerobic plant counts (APC), both psychrotrophic and mesophilic, were found to be consistent throughout the different facilities as well as during the different times of the year (Table II-1). The average counts for the different facilities typically ranged between 3 and 6 logs.

When comparing the APC (mesophilic and psychrotrophic) of the two aquaculture

systems, there was no significant difference in the observed counts for the two systems ($p>0.05$). There was however slight significance with regard to plant ($p<0.05$) indicating that from which plant the samples were taken had an effect on what the levels were, some plants having consistently higher or lower counts than others. In the contrast of cut (fillets vs. whole fish), APC were significantly higher in the fillets than in the whole fish ($p<0.05$); the season or plant in which sampling occurred had no significant effect on the counts observed ($p>0.05$).

The aerobic plate counts observed were consistent with those reported by Cloete et al. (1984) and Okafor and Nzeako (1985) who found levels of 0.3×10^5 and $4 \times 10^5 \text{ g}^{-1}$, respectively. These microbial levels reflect the natural bacterial flora in the slime coat on the trout. Large numbers of microorganisms in the coat are transferred to the fillets during processing operations.

Coliforms, Fecal coliforms, and E. coli

Coliform counts for Plants 1, 2, and 3 as well as those of the recirculating system (plant 5) were all very low (Table II-2; Rearing Water). The counts were all below 2 logs and ranged from 0.91 to 46 MPN/ml of water. The counts in plant 4 were much higher than those of the other facilities and ranged from 110 to 240 MPN/ml. As was the case for coliforms, fecal coliforms and *E. coli* counts (Table II-2) in Plant 4 were higher than those of the other facilities. The source of this contamination could not be ascertained, however, the system could have a polluted aquifer. Fecal counts in the recirculating system were below the limit of detection.

When compared to the low flow period counts of 1994, there was no change in the coliform counts, except for plant 4 which showed counts of 1 log lower than the previous year. In the case of fecal counts, all facilities showed a decrease in fecal coliform and *E. coli* counts, with the exception of Plants 1 and 5 which remained constantly low and non detectable, respectively.

When examining the coliform, fecal coliform, and *E. coli* counts for the differing aquaculturing systems, a significant difference was noted in the coliform and fecal coliform counts ($p < 0.05$), but not in the *E. coli* counts ($p > 0.05$). There was also a significance noted in the plant/year interaction for coliforms, fecal coliforms, and *E. coli*. The significance of the data indicates that each plant may have dissimilar counts from other plants seasonally, some plants showing an increase in counts from one season to the other, while others show a decrease. Variability is such that changes seen in one plant may not be seen in another.

Except for Plant 2B in 1995, the fillets from both the low flow period in 1994, and the high flow period in 1995 showed mean coliform counts that were fairly high. Fecal coliforms, however, from these plants were very low in most cases, ranging from below the limit of detection to 9.3 MPN/g. *E. coli* was not isolated during 1994, and only from Plant 3B in 1995. In this plant, the organism was only isolated from 3 of the 5 samples tested, and the mean level was 5.9 MPN/g.

In comparing the rearing water and fillets, the coliform counts for the fillets were significantly higher than those in the rearing water ($p < 0.05$). In fecal coliforms and *E. coli*

counts, however, no significant difference ($p>0.05$) was noted when the counts for whole fish were compared to those of the fillets. The only factor that was significant in the processing facilities was which facility was sampled ($p<0.05$). As has been noted, some plants generally showed higher counts than others, even when the plants were in the same region.

Although the evidence in this study shows that no coliform, fecal coliform, and *E. coli* counts reached levels that would be cause for concern, care needs to be taken by facilities to protect the sources of the water used for the rearing of the fish to make sure they do not become polluted. It should be noted that the high coliform counts could be attributed to the presence of decaying vegetation at the water source. Also, variability seen in the counts could have been affected by the differences in stocking densities in the different facilities. Wildlife, i.e. muskrats, living in the streams are at the springhead of the sources, as well as runoff from animal production in close proximity to the aquaculture facilities could provide contamination to aquifers. In general, fecal counts as well as *Escherichia coli* counts, were markedly lower for the fillets when compared to the counts of the rearing water, indicating that care was taken to ensure that no additional organisms were introduced into the product. Measures do need to be taken or continued by the processing facilities to ensure minimization of organisms introduced into the product either by cross contamination with in-coming product or workers using unacceptable sanitation practices.

Pathogens

Listeria monocytogenes

Listeria monocytogenes was isolated from samples from each facility to varying degrees during the two seasons (Tables II-3 and 4). The percentage of samples positive for *Listeria* species ranged from 40-90% in 1994 and 25-80% in 1995, with most probable number counts of positive samples ranging from 0.36-4.83 MPN/g in 1994 and 0.73-3.98 in 1995. Plant 5 (recirculating facility) showed an incidence level of 40% and an average of 0.39 MPN/g in 1994, and an incidence of 45% and an average 3.98 MPN/g in 1995. These incidence levels and average counts were in the range of those observed in the flow-through aquaculture systems for both seasons. The non-pathogenic species, *Listeria innocua*, was also isolated from whole fish in all plants and seasons with the exception of Plant 1 in 1994. Incidence of this species ranged from 10 to 65%. The presence shows both pathogenic and nonpathogenic species of *Listeria* occurring together. This indicates that although *Listeria* can be isolated from a product, the distinction between species needs to be made to know the true threat that *Listeria* presents.

The statistical analysis of the quantitative and quantitative data for the recirculating and flow-through systems showed no significant difference in the mean counts for the two systems ($p>0.05$). A significant difference was noted in the mean *Listeria* counts with regard to the factor of the plant/seasonal interaction ($p<0.01$). Again, this means that a seasonal change in mean counts for a particular plant is different than the change in mean counts for another. Although the incidence of the organism is high in some cases during

both seasons, the corresponding average counts for the positive samples in all cases were low.

The fillets showed similar percentages of *Listeria* positive samples as well as MPN counts with the exception of the Plant 3B in 1995, which showed a higher count than any of the other plants (21 MPN/g). The non-pathogenic strain, *L. seeligeri* was isolated in 10% of the samples in Plant 3B in 1994. No other non-pathogenic strains of *Listeria* were isolated in 1994, however, the non-pathogenic strain *L. innocua* was isolated in each of the 3 processing facilities in 1995. The incidence of *L. innocua* in 1995 ranged from 25 to 85%. A significant difference was noted in both the incidence and quantitative levels of *Listeria* on the basis of whole fish vs. fillet ($p < 0.05$). In general, the incidence as well as the quantitative levels were higher in the fillets than in the whole fish. Levels of incidence observed in this study are not unlike those found in other raw fish (Hartemink and Georgsson, 1991). In this study, the incidence of the organism may well have been over 50%, but the overall levels of the pathogens were low. Quantitative levels found in this study, even the higher levels observed in Plant 3B, do not present a concern for consumers who will cook their product at home. Care, however, must be taken to ensure that temperature abuse which could result in the proliferation of the bacteria to very high levels does not occur. In the case of rainbow trout, the product will be receiving a terminal heat treatment that will serve to destroy levels of *Listeria* present; heating raw meat at 70°C for two minutes would be sufficient to inactivate any *Listeria monocytogenes* present (Gaze et al., 1989). Processors need to make sure that their processing temperature is

adequate to any *Listeria* that may be present, and be take the necessary precautions to ensure that cross-contamination of processed product and raw product does not occur. Concern for *Listeria monocytogenes* emanates from the potential of contaminating facilities that further process the fish, and smoking operations, where a zero tolerance for the product has been established, as well as the fact that *Listeria* has been isolated from smoked fish (Fuch and Nicolaides, 1994).

Clostridium botulinum

The percentage of samples positive for presumptive *Clostridium botulinum* spores ranged (enrichment in Cooked Meat Medium as well as TPGY) from 45 to 95% in 1994 and 60 to 85% in 1995 (Table II-5). For the quantitative counts made from Cooked Meat Medium, there was no significant difference between plant types ($p>0.05$). There was, however, a significant difference when examined on the basis of the plant/year interaction ($p<0.05$). For the quantitative counts using the TPGY enrichment, there was a significant difference observed between the two systems ($p<0.05$), however, there was also a significance observed in the plant/year interaction. Average counts for presumptive positive data were all very low; 4.31 MPN/g being the highest of the counts and 0.46 MPN/g the lowest. With regard to counts of positive samples, differing plant types or differing sampling seasons were not significantly different ($p>0.05$).

Of the 85 samples that were tested using the mouse bioassay, 8 were positive for presence of the toxin. Of these eight samples, 7 demonstrated toxicity only after trypsinization, indicating the presence of nonproteolytic type toxin. These results indicate

that although the counts of presumptive positive *C. botulinum* were very low, the actual counts, as well as the percentage of samples positive for *C. botulinum* were probably, in fact, much lower. Also, of the samples positive for the *Clostridium botulinum* toxin, very few of these positive samples are likely of a proteolytic type of *C. botulinum*.

The incidence of *C. botulinum* found in this study is supported by studies of trout farms in other areas that found similar levels of incidence (Cann and Taylor, 1975; Huss et al., 1974). These studies also found that the predominant types of *C. botulinum* isolated was a non-proteolytic type, which was supportive of the data found in this study.

The risk represented by *C. botulinum*'s presence in foods should be evaluated based on several criteriae. First of all, it needs to be established whether or not the storage of the product is such that it promotes the outgrowth of toxin. Storage of the product below 3.3°C would prevent the outgrowth of non-proteolytic type toxins (Cann and Taylor, 1979), although toxin production at temperatures below 10°C is so slow that the fish would spoil before detectable amounts of the toxin are produced (Hobbs, 1976). Secondly, the end treatment of the product needs to be established. Generally fish will receive a heat treatment during cooking which serves to destroy any toxin that may have formed, however, temperatures used in the hot smoking of fish, are inadequate to inactivate the spores of the more heat-sensitive nonproteolytic type E spores (Pelroy et al., 1982), and proteolytic forms of *C. botulinum* are more resistant to heat. Care must be taken to maintain the product at temperatures (<38°F) to prevent toxin outgrowth of spores that may have survived. Also, it is important to have the minimum water phase salt

concentration as recommended by the Association of Food and Drug Officials (AFDO) as an additional safety factor.

Other pathogens

No *Salmonella* spp., *Vibrio cholerae*, or *Yersinia enterocolitica* and *Y. pseudotuberculosis*, were isolated from any of the samples of whole fish tested. The absence of the pathogens indicates that these organisms do not pose a risk in aquacultured rainbow trout.

CONCLUSIONS

Of the pathogens isolated in this study, none were present at levels sufficient to pose a risk to consumers who will be cooking their own products. This, however is not the case with processors who will be creating ready to eat products. *Clostridium botulinum* has been shown in the past to be a problem in the smoked fish industry. Care needs to be taken to ensure that the conditions are present to adequately inhibit the outgrowth of toxin in both aerobically and modified atmospheric packaging (proper water phase-salt and storage temperature). A proper processing temperature for the ready-to-eat product also needs to be reached that would destroy any *Listeria monocytogenes* present, and secondly care needs to be taken to ensure that cross contamination of processed and raw product does not occur.

Pathogen levels and the general microbial quality remained unchanged with respect to season. There was also no evidence that would support the safety of one aquaculture

system over another. This fact may prove useful in regions of the United States where culture of rainbow trout is severely limited by the lack of water sources that supply the high flow rate needed to raise trout.

Acknowledgements

This investigation was sponsored by CFAST (through funding from the Commonwealth of Virginia) and the National Oceanic and Atmospheric Administration (NOAA), Virginia Sea Grant College Program (This work is a result of research carried sponsored under Federal Grant No. NA90AA-D-SG045). The authors also wish to express their gratitude to the Virginia Trout Growers Association and Freshwater Institute for their assistance in this study. The authors would also like to thank the Statistical Consulting Center at Virginia Polytechnic Institute and State University for their assistance.

Table II-1: Plate Counts (Mesophilic and Psychotrophic) for Whole Rainbow Trout and Fillets (CFU/g: Log transformation)

Plant	Whole Fish				Fillets			
	Late Summer (1994)		Late Spring (1995)		Late Summer (1994)		Late Spring (1995)	
	Mes.	Psych.	Mes.	Psych.	Mes.	Psych.	Mes.	Psych.
1	1.74x10 ⁴	2.95x10 ⁴	2.29x10 ⁴	3.09x10 ⁴	4.68x10 ⁴	5.62x10 ⁴	7.24x10 ⁴	1.05x10 ⁵
2	3.55x10 ⁴	2.51x10 ⁴	1.95x10 ⁴	1.55x10 ⁴	2.63x10 ⁵	3.72x10 ⁵	4.9x10 ⁵	1.66x10 ⁴
3	2.04x10 ⁵	7.76x10 ⁴	5.37x10 ⁴	1.1x10 ⁵	2.95x10 ⁴	3.8x10 ⁴	5.75x10 ⁴	1.07x10 ⁵
4	1.15x10 ⁵	1.48x10 ⁵	1.95x10 ⁴	3.02x10 ⁴	-	-	-	-
5**	2.00x10 ⁴	7.41x10 ⁴	5.01x10 ⁴	7.24x10 ⁴	-	-	-	-

** indicates recirculating system

- firm did not have processing operations

Table II-2: Coliform, Fecal Coliform, and *Escherichia coli* counts for rainbow trout rearing water and fillets (MPN/g o ml: Log Transformation)

Plant	Rearing Water (MPN/ml)						Fillets (MPN/g)					
	Late Summer (1994)			Late Spring (1995)			Late Summer (1994)			Late Spring (1995)		
	Col.	Fecal Col.	<i>E. coli</i>	Col.	Fecal Col.	<i>E. coli</i>	Col.	Fecal Col.	<i>E. coli</i>	Col.	Fecal Col.	<i>E. coli</i>
1	1.90	0.47	0.47	15.30	2.45	0.40	204.00	4.98	0.00	54.46	0.86	0.00
2	22.66	0.69	0.58	4.02	0.26	0.00	583.20	0.07	0.00	3.09	0.00	0.00
3	21.86	2.62	1.82	3.50	0.00	0.00	99.00	0.00	0.00	292.00	3.58	3.58
4	188.00	41.60	25.92	39.50	0.00	0.00	-	-	-	-	-	-
5**	1.54	0.00	0.00	15.28	0.00	0.00	-	-	-	-	-	-

** indicates recirculating system
 - firm did not have processing operations
 ND- Not Detected

Table II-3: *Listeria* Incidence and Quantitative Levels on Whole and Filleted Rainbow Trout in Late Summer (1994)

	Whole fish					Fillet		
	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5**	Plant 1B	Plant 2B	Plant 3B
Number of Samples Positive for <i>Listeria</i> spp. (20 samples)	18 (90%)	10 (50%)	8 (40%)	15 (75%)	8 (40%)	18 (90%)	12 (60%)	14 (70%)
Average Counts for Positive Samples (MPN/g) Log Trans.	4.83	1.02	0.359	0.396	0.391	4.23	1.12	1.08
Samples positive for <i>Listeria monocytogenes</i>	18 (90%)	7 (35%)	7 (35%)	8 (40%)	7 (35%)	18 (90%)	12 (60%)	13 (65%)
Other <i>Listeria</i> spp. Isolated	none	<i>L. innocua</i> 5 (25%)	<i>L. innocua</i> 3 (15%)	<i>L. innocua</i> 7 (35%)	<i>L. innocua</i> 2 (10%)	none	none	<i>L. seeligeri</i> 2 (10%)

** indicates recirculating system

Table II-4: Data for *Listeria* Incidence and Quantitative Levels on Whole and Filleted Rainbow Trout in Late Spring (1995)

	Whole Fish						Fillet		
	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5**		Plant 1B	Plant 2B	Plant 3B
Number of Samples Positive for <i>Listeria</i> spp. (20 samples)	14 (70%)	5 (25%)	16 (80%)	9 (45%)	9 (45%)		16 (80%)	16 (80%)	18 (90%)
Average Counts for Positive Samples (MPN/g) Log trans.	1.83	1.27	2.30	0.73	3.98		1.91	1.50	21
Samples Positive for <i>Listeria monocytogenes</i>	9 (45%)	4 (20%)	11 (55%)	8 (40%)	8 (40%)		11 (55%)	15 (75%)	14 (70%)
Other <i>Listeria</i> spp. Isolated	<i>L. innocua</i> 10 (50%)	<i>L. innocua</i> 2 (10%)	<i>L. innocua</i> 13 (65%)	<i>L. innocua</i> 4 (20%)	<i>L. innocua</i> 7 (35%)		<i>L. innocua</i> 11 (55%)	<i>L. innocua</i> 5 (25%)	<i>L. innocua</i> 17 (85%)

** indicates recirculating system

Table II-5: *Clostridium botulinum* Incidence and Quantitative Levels on Whole Rainbow Trout in Late Summer (1994) and Late Spring (1995)

	Plant 1	Plant 3	Plant 4	Plant 2	Plant 5**
Number of Samples Positive for presumptive <i>C. botulinum</i> spores	13 (65%)	14 (70%)	16 (80%)	9 (45%)	19 (95%)
Late Summer (1994)					
Late Spring (1995)	13 (65%)	12 (60%)	17 (85%)	16 (80%)	12 (60%)
Average Counts for Positive Samples (MPN/g)	1.60	0.46	0.54	1.76	1.69
Log trans.	0.68	0.89	2.33	4.31	1.40
Late Summer (1994)					
Late Spring (1995)					

** indicates recirculating system

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Section III: An Assessment of Inorganic Constituents in Aquacultured Rainbow Trout (*Oncorhynchus mykiss*) Muscle Tissue

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ABSTRACT

Muscle tissue of aquacultured rainbow trout, *Oncorhynchus mykiss*, from different aquaculture facilities in the Appalachian mountains of Virginia and West Virginia was examined to determine levels of select minerals and heavy metals. Levels were then examined to note any trends on a seasonal basis. Moisture and ash levels ranged from 71.29 - 81.49% and 0.88- 2.21%, respectively. Levels of Na were above the limit of detection in all cases but two (210.7 and 223.5 ppm). Quantification of Zn, S, Ca, Mg, and Mn showed levels of 2.829-7.335, 54.98-128.0, 27.14-100.8, 111.0-174.7, 0.0463-0.1303 ppm. With the exception of two cases (15.03 and 75.81 ppm), Fe levels were typically around 2 ppm. The heavy metals, As, Hg, Ni, Co, Cr, Mo, and Pb, when at detectable levels, were all consistently low (below 0.5 ppm). With the exception of one sample (60.49 ppm), the Cd levels were below 0.06 ppm. Although high, this level should pose no risk to the consumer due to the infrequent consumption of rainbow trout and low uptake of ingested Cd by the body. None of the data examined suggests any trends in the levels on a seasonal basis.

INTRODUCTION

The health concerns from both chronic levels and bioaccumulation of heavy metals are well documented. The distribution of heavy metals throughout the environment is widespread and may be present due to pollution as well as naturally occurring reservoirs. By routes such as leaching from the soil, as well as non-point pollution, the water table and subsequently the aquifers that provide the water for aquaculture facilities may become contaminated.

In January of 1994, the United States Food and Drug Administration purposed a rule for the safe processing of the fish and fishery products. In this proposal, the FDA called for the implementation of mandatory Hazard Analysis Critical Control Point (HACCP). This plan, not only encompasses microbial and physical hazards, but also those of a chemical nature, such as pesticides and heavy metals. Although work has been conducted with regard to trace metal analysis, the work has been limited, and levels of various metals, as well as those of pesticides tend to vary with location. With the HACCP plan already mandated, data is needed so that the chemical hazards associated with the seafood industry, more specifically, aquacultured trout may be assessed.

The objective of this study was to determine the levels of select heavy metals, pesticides, and herbicides present in the muscle tissue of rainbow trout, *Oncorhynchus mykiss*, from different aquaculture facilities in the Appalachian mountains of Virginia and West Virginia. Secondly, the purpose was to note any trends in the heavy metal levels when examined during the growing seasons of late spring-early summer and late summer-

early fall.

MATERIAL AND METHODS

Selection of facilities and sampling periods

The facilities for the study were chosen based on two factors: type of aquaculture system employed and presense processing operations. Four of the facilities that were chosen employed a flow-through aquaculture system, while the fifth used a system recirculating approximately 80% of its water. A total of five facilities were sampled, three of which were also sampled for fillets. Sampling took place mid-August through early October 1994 which is typically low flow period and mid-May through early July 1995, typically higher flow period.

Collection of Samples

Twenty fish from each of the aquaculture facilities were obtained, killed by thermal shock in ice water, transferred to individual sterile stomacher bags, and placed on ice for transport. Five randomly sampled fish were filleted, skinned, and the muscle tissue ground three times to form a composite sample.

Heavy Metal Analysis

Approximately 100g (wet weight) of each composite sample was dried overnight at 85°C. The resulting dried sample was then placed in a platinum crucible. A small amount of nitric acid was added to each of the crucible as an ashing aid (Rowe, 1973).

The samples were then placed in a muffle furnace at 500°C for not less than 14 hours, until a white ash was achieved. The resulting ash was then dissolved by heating to near boiling in concentrated nitric acid. Next, the solution was filtered through a Whatman No. 40 filter paper after which the crucible and the filter paper were repeatedly rinsed. The solution was then brought up to a volume of 11 ml and added to 89 ml of deionized water yielding a solution of 10% nitric acid which was used for elemental analysis. The samples were analyzed using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) system. Testing for Ca, Fe, Mg, Mn, Na, S, and Zn was carried out using the Jarrell Ash ICAP 61 simultaneous spectrometer, while testing for As, Cd, Co, Cr, Hg, Mo, Ni, and Pb was performed using the Jarrell Ash Atomscan 2400 sequential spectrometer.

RESULTS AND DISCUSSION

Moisture, ash, and select mineral levels

Moisture contents and ash percentages appear in Table III-1. Moisture levels showed some diversity, varying over a range of 10% (71.29 - 81.49%). The percentage of ash also showed some variation. The levels showed a range of 1.3% (0.88- 2.21%). The moisture as well as the ash percentages were consistent with those of Lazos et al. (1989) who reported findings from eleven species of freshwater fish, and Anthony et al. (1983) who analyzed 6 varieties of marine fish. Although there was variation in the percentages, from the data observed, the variation could not be attributed to the growing season or the rearing method, but more likely to the variations in the fish themselves.

Select mineral levels for muscle tissue appear in Table III-2, and are reported in ppm (wet weight). Calcium levels ranged from 27.14 to 100.8 ppm. The levels of calcium found in this study are very similar to those reported in other studies (Anthony et al., 1983; Teeny et al., 1984). Magnesium levels range from 111.0 to 174.7 ppm, slightly lower than the levels found in other studies (Anthony et al., 1983; Teeny et al., 1984).

Levels of iron were found to be low in most cases, typically around 2 ppm; very similar to those reported by Teeny et al. (1984). In two of the cases, however, the levels were found to be 15.03 and 75.81 ppm. Although higher than those reported in the aforementioned study, the levels were lower than those reported by Anthony et al. (1983).

Detectable amounts of manganese were found, however, levels were found to be very low (0.0463 to 0.1303 ppm). Manganese levels were found to be consistent or slightly lower in some cases than those found by Lazos et al. (1989). Sodium levels were found to be above the limit of detection in all cases but two. In these cases, however, the levels were 210.7 and 223.5 ppm.

Zinc and sulfur were detected at levels ranging from 2.829 to 7.335 ppm and 54.98 to 128.0 ppm respectively. Zinc levels were very close to those found by Teeny et al. (1984).

The mineral levels reported in this study exhibit no seasonal trends. Rather the varying level found in the fish are most likely due to variations in the fish themselves or other outside variables such as water..

Heavy Metals

Only in two cases were levels of arsenic present at detectable levels (0.1757 and 0.1031 ppm). At these levels, the ingestion of fish would pose no significant risk. Mercury and nickel levels for the differing plants during each rearing season were generally below the limit of detection. Only in one sample for each of the two aforementioned metals was the metal present at detectable levels, and even then the levels, as was the case with arsenic, were quite low. The samples contained 0.4276 and 0.2475 ppm for mercury and nickel respectively.

Nearly all of the samples tested showed detectable levels of the metals cobalt, chromium, molybdenum, and lead. Although present in most cases, the levels of each of the metals were consistently low for each facility during the two different growing seasons, and represented no danger due to consumption of the product. Levels of cobalt ranged from 0.0150 to 0.0369 ppm, while molybdenum ranged from below the limit of detection (<0.0061 ppm) to 0.0369 ppm. Chromium and lead ranged from 0.1644 to 0.3061 ppm and below the limit of detection (<0.1028 ppm) to 0.3079 ppm, respectively.

Cadmium levels were all consistently low (0.0031 - 0.0552 ppm) with the exception of one facility during one growing season. Plant 5 during the late summer months contained a level of 60.49 ppm. Although this level is high, the product would not be considered adulterated by the FDA, which does not have an action level for cadmium in seafood and seafood products. The provisional tolerance daily intake limit (PTDI) for cadmium established by the Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO) has been set at 57-72 $\mu\text{g/day}$. This level was

calculated, however, using the provisional tolerance weekly intake limit (PTWI) (Committee on the Evaluation of the Safety of Fishery Products, 1991). Even though the PTDI would be exceeded upon ingestion of the sample, the fish should not pose any threat to the consumer from bioaccumulation of the metal, since rainbow trout is not generally consumed on a daily basis, as well as the low uptake of ingested cadmium by the body (5-10%).

It should also be noted that although the level of cadmium for Plant 5 was high in one instance, the second observation showed levels that were low and more consistent with those of the other facilities. There is no way of pinpointing the exact cause of the cadmium contamination in this case, although cadmium is frequently found in phosphate fertilizers (Ryan et al. 1982) and could have entered the aquifer by this route. Also the fact that Plant 5 was a recirculating aquacultural facility meant that once in the system, the cadmium would have remained there for some time, thereby increasing the exposure of the fish.

Of the metals examined, the FDA established an action level (1.0 ppm) for mercury. Although other metals are known to cause illness upon extended exposure, the inconsistent consumption of seafood products by the United States as a whole makes risk due to exposure to fishery products minimal. None of the data for heavy metals suggest any trends in the levels on a seasonal basis.

Acknowledgments

This investigation was sponsored by CFAST (through funding from the Commonwealth of Virginia) and the National Oceanic and Atmospheric Administration (NOAA), Virginia Sea Grant College Program (This work is a result of research carried sponsored under Federal Grant No. NA90AA-D-SG045). The authors also wish to express their gratitude to the Virginia Trout Growers Association and Freshwater Institute for their assistance in this study.

Table III-1: Moisture and Ash Content of Aquacultured Rainbow Trout (*Oncorhynchus mykiss*) Muscle Tissue

Plant	Year	Dry Sample	Wet Sample	Ash	%Moisture	%Ash
1	94	24.1891	96.2183	2.1282	74.86	2.21
1	95	25.0536	98.602	1.2095	74.59	1.23
2	94	17.1619	92.7358	0.8157	81.49	0.88
2	95	23.5793	82.1419	1.4073	71.29	1.71
3	94	24.4712	96.4705	1.0355	74.63	1.07
3	95	27.4512	98.4603	0.8697	72.12	0.88
4	94	26.93	97.3075	1.1861	72.32	1.22
4	95	25.3398	96.4206	1.1837	73.72	1.23
5	94	22.8716	97.4586	1.9108	76.53	1.96
5	95	25.9041	97.4721	1.2293	73.42	1.26

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1	95	25.0536	98.602	1.2095	74.59	1.23
2	94	17.1619	92.7358	0.8157	81.49	0.88
2	95	23.5793	82.1419	1.4073	71.29	1.71
3	94	24.4712	96.4705	1.0355	74.63	1.07
3	95	27.4512	98.4603	0.8697	72.12	0.88
4	94	26.93	97.3075	1.1861	72.32	1.22
4	95	25.3398	96.4206	1.1837	73.72	1.23
5	94	22.8716	97.4586	1.9108	76.53	1.96
5	95	25.9041	97.4721	1.2293	73.42	1.26

Table III-2: Select Mineral Content of Rainbow Trout Muscle Tissue (ppm wet weight)

Plant	Year	Ca	Fe	Mg	Mn	Na	S	Zn
1	Late Summer (1994)	100.8	1.933	111.0	0.0462	>207.9	54.979	4.0543
1	Late Spring (1995)	81.70	1.545	152.7	0.0590	>202.8	77.007	5.9918
2	Late Summer (1994)	86.77	1.792	111.4	0.0480	210.7	73.920	2.7519
2	Late Spring (1995)	75.16	1.858	168.1	0.0542	223.5	54.564	3.8433
3	Late Summer (1994)	39.02	2.098	149.7	0.0745	>207.3	55.945	4.1992
3	Late Spring (1995)	27.14	15.03	150.4	0.1113	>203.1	78.204	5.8937
4	Late Summer (1994)	62.40	2.166	114.9	0.1303	>205.5	70.704	4.3666
4	Late Spring (1995)	47.67	2.569	145.0	0.0604	>207.4	77.152	7.3345
5	Late Summer (1994)	60.84	75.81	121.0	0.5483	>205.2	68.829	2.8289
5	Late Spring (1995)	77.44	1.807	174.7	0.0527	>205.2	128.04	5.0691

Table III-3: Select Heavy Metal Concentrations of Rainbow Trout Muscle Tissue (ppm wet weight)

Plant	Year	As	Cd	Co	Cr	Hg	Mo	Ni	Pb
1	Late Summer (1994)	<0.1039	0.0039	0.0196	0.1644	0.4276	0.0339	<0.1039	0.1569
1	Late Spring (1995)	<0.1014	0.0114	0.0369	0.1885	<0.1014	0.0308	<0.1014	0.1367
2	Late Summer (1994)	<0.1078	0.0031	0.0150	0.2466	<0.1078	0.0369	<0.1078	0.1161
2	Late Spring (1995)	0.1757	0.0074	0.0254	0.3061	<0.1217	<0.0061	<0.1217	0.2060
3	Late Summer (1994)	<0.1037	0.0073	0.0311	0.1986	<0.1037	0.0093	0.2475	0.1918
3	Late Spring (1995)	<0.1016	0.0551	0.0394	0.1682	<0.1016	0.0122	<0.1016	0.2158
4	Late Summer (1994)	<0.1028	0.0086	0.0313	0.2637	<0.1028	0.0321	<0.1028	<0.1028
4	Late Spring (1995)	<0.1037	0.0054	0.0240	0.2414	<0.1037	0.0101	<0.1037	0.1913
5	Late Summer (1994)	<0.1026	60.49	0.2186	0.2698	<0.1026	0.0309	<0.1026	0.3079
5	Late Spring (1995)	0.1031	0.0080	0.0340	0.2040	<0.1026	0.0243	<0.1026	0.1700

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Appendix 1: Sensory Evaluation of Aquacultured Rainbow Trout After Prolonged Freezer Storage

The objective of this study was to examine the effect of prolonged freezer storage on human sensory perception of aquacultured rainbow trout.

Materials and methods

Several trout fillets, collected from a processing facility that produced their own fish and packaged in zip-lock bags, were frozen at -20°C for eight months. The fillets were then thawed and cut into squares of 1 inch, and wrapped in aluminum foil. Fresh fillets from the same facility were cut and treated in the same manner as were the frozen fillets. All samples were then labeled and baked until the internal temperature of the fish reached a temperature of $\geq 160^{\circ}\text{F}$ (70°C) (AOAC, 1990). A triangle taste panel was then conducted on the samples to assess any difference in taste that may have resulted from the prolonged storage.

Results and Discussion

Twenty-five people were given a triangle taste test in which they were asked to taste two sets of trout, from which they were asked to pick the sample from each group that tasted different. Of the 50 judgements, 22 were correct. This number fell short of the

minimum number required for significance at the 5% level (American Society for Testing and Materials, 1968). These results indicate that although there were instances where people appeared to have been able to tell the difference in the samples, there was in fact no significant difference in the taste of the frozen and fresh fillets.

Conclusions that can be drawn from the study are that the freezing of the fillets have no deleterious effect on the flavor of the fillets, even when held at freezer temperatures for eight months. Although oxidation occurs at lower temperatures, freezing is adequate to slow the rate enough so that the effects of oxidation are not noticed.

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Appendix 2: Analysis of Pesticides and Herbicides

Preparation of Samples

Samples were collected and prepared as was described in “Collection of Samples” in Section III. Samples from only the low flow period (1994) were examined.

General Pesticide Analysis

A 40 g sample of the fish was subjected to an 8 hr. soxhlet extraction, dried, and redissolved in 5 ml of petroleum ether (Saha and Sumner, 1978). A C-18 solid phase extraction column was used for sample clean up. Methanol was used to precondition the column, and 1 ml of filtered dionized water was added to the sample, vortexed and delivered to the column. Three ml of methylene chloride/acetonitrile/hexane (50:3:47) was used to elute the sample from the column. One ml of iso-octane was then added and evaporated to 0.5 ml on an N₂ evaporator. The sample was then diluted with hexane and evaporated to 1 ml for GC analysis (Marble and Delfino, 1988).

The sample was analyzed using a Tracor Gas Chromatograph Model 540 with an electron capture detector. A 6'6" 1/4" ID column of 1.5/1.95% SP2250/2401 80/100 Supelcoport and a carrier gas of nitrogen was used. The oven temperature was 195°C with an inlet temperature of 235°C and a detector temperature of 350°C

Acid Herbicide

Analysis was carried out according to the protocol of Glas (1978). In this procedure, 10 g of fish were ground with an 170 ml 0.1 N NaOH and transferred to a

centrifuge bottle, after which the mixer was rinsed with 20 and 10 ml of NaOH. The sample was separated by centrifugation. Twenty ml of the supernatant was removed to which 7g NaCl, 1 ml 6N H₂SO₄ and 15 ml of 3:7 ethyl ether:hexane was added. The mixture was again separated by centrifugation and the upper organic layer was removed and extraction of the aqueous phase was repeated twice with 15 ml of 3:7 ethyl ether:hexane. The sample was evaporated with nitrogen gas to a volume of 0.5 ml. Methylation was carried out using 2 ml diazomethane (CH₃N₂) for 30 min., and the sample was evaporated to 1 ml, and diluted to 2 ml with hexane prior to analysis.

The sample was analyzed using a Tracor Gas Chromatograph Model 540 with an electron capture detector. A 6'6" 1/4" ID column of 1.5/1.95% SP2250/2401 100/120 Supelcoport and a carrier gas of nitrogen was used. The oven temperature was 195°C with an inlet temperature of 235°C and a detector temperature of 350°C

Carbofuran and Sevin

Extraction, derivatization and detection carried out according to the methodology of Shuttleworth (1973). In this procedure, 10 g of sample was ground for 2 minutes with 100 ml of acetone and filtered, after which the filter cake was ground with 100 ml of acetone and added to the original filtrate. The flask was then placed in a -10°C freezer until the fat had separated from the sample and then filtered through glass wool and rinsed with 100 ml of cold 0.25 N HCl. To the filtrate, 100 ml of methylene chloride was added, shaken, allowed to separate, and the lower layer was drained through a sodium sulfate

column. This extraction was repeated twice with 100 ml of methylene chloride. Three drops of keeper solution (1% paraffin in hexane) was added and the sample was evaporated to near dryness on rotary evaporation. For derivatization, the sample was evaporated to 0.1 ml on N₂ evaporation and 0.5 ml FSNBC (1-Flouro-2, 4-dinitrobenzene in acetone) and 5 ml of sodium borate buffer was added and heated for 1 hour at 70°C. The sample was then cooled to room temperature and 5 ml of hexane was added, after which the sample was vortexed.

The sample was analyzed using a Tracor Gas Chromatograph Model 540 with an electron capture detector. A 6'6" 1/4" ID column of 1.5/1.95% OV-17/OV-210 Chromosorb WHP 100-120 mesh and a carrier gas of nitrogen was used. The oven temperature was 240°C with an inlet temperature of 235°C and a detector temperature of 375°C

Lannates

Analysis was carried out according to the protocol of Pease and Kirkland (1968). In this procedure, 10 g of sample was mixed with 100 ml of ethyl acetate . The sample was separated by centrifugation, decanted through glass wool and saved. Another 100 ml aliquot of ethyl acetate was added to the centrifuge bottle, shaken, centrifuged, and added to the other fraction after decanting through glass wool. This step was again repeated, after which 50 ml of distilled water was added to the ethyl acetate fractions and evaporated at room temperature for 2-3 hours. Five ml of 1N H₂SO₄ was then added to

the sample after transferring to a separatory funnel and the beaker was rinsed with hexane. The sample was then separated and the lower layer was drained, the upper layer discarded, and the lower layer returned to the funnel. This process was repeated twice with 50 ml of hexane. Fifty ml of chloroform was added, shaken for two minutes, and the lower layer (chloroform) was drained, and the process was repeated twice. The sample (chloroform) was then evaporated to 20 ml and 50 ml 0.1N NaOH was added and the chloroform was evaporated on a flask evaporator at 45°C. The sample was then heated for 15 min. at 45°C, allowed to cool, and acidified to ~pH 2 with 1N H₂SO₄. The solution was then transferred to a separatory funnel with 2 rinses of 10 ml of distilled water, after which, 30 ml ethyl acetate was added. The solution was shaken for two minutes and the bottom layer (ethyl acetate) was collected through sodium sulfate. This step was repeated twice. Triethylamine (0.1 ml) was added to the ethyl acetate fractions and evaporated to 5 ml. Triethylamine was again added and evaporated at room temperature using a N₂ evaporator. The final volume was adjusted to 2 ml using hexane.

Samples were analyzed using a Tracor 540 GLC with a 10% OV101 100/120 Supelcoport column and a FPD sulfur filter detector. The inlet temperature was held at 225°C, the column temperature was at 175°C and the detector temperature was 250°C. Nitrogen was used as the carrier gas while hydrogen and air were the combustible gases.

Aldicarbs

Analysis was carried out according to the protocol of *Anonymous* (1984). In this

procedure, 10 g of sample was obtained, to which 275 ml 3:1 acetone:water and 5 ml of 35% peracetic acid was added and blended. The sample was then filtered and rinsed with 50 ml of the solvent mixture. Fifty ml of 10% sodium bicarbonate was then added to the sample, stirred for 30 min, and evaporated to 100 ml at 45°C. To the sample, 50 ml of methylene chloride was added, shaken, and allowed to separate. The bottom layer was then drained through a sodium sulfate column. This extraction was repeated twice with 50 ml of methylene chloride. The sample was then evaporated to near dryness at 45°C. Cold acetone was added, evaporated to 0.5 ml, and readjusted to 2 ml with acetone.

Samples were analyzed using a Tracor 540 GLC with a 10% OV 101 100/120 Supelcoport column and a FPD sulfur filter detector. The inlet temperature was held at 220°C, the column temperature was at 160°C and the detector temperature was 225°C. Nitrogen was used as the carrier gas while hydrogen and air were the combustible gases.

Results and Discussion

The results from each of the analyses appear in tables app.2-1 through app.2-3. Only ppDDE and Endrin were isolated from the samples tested (Table app.2-1b). These pesticide residues did not exceed the FDA action limits (Committee on the Evaluation of the Safety of Fishery Products, 1991).

Table App.2-1a: Select organochlorine and organophosphate pesticide residues in rainbow trout muscle tissue (ppm)

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
1221 PCB	ND	ND	ND	ND	ND
1254 PCB	ND	ND	ND	ND	ND
Technical Chlordane	ND	ND	ND	ND	ND
HCB	ND	ND	ND	ND	ND
alpha-BHC	ND	ND	ND	ND	ND
Diazinon	ND	ND	ND	ND	ND
Lindane	ND	ND	ND	ND	ND
Atrazine	ND	ND	ND	ND	ND
Simazine	ND	ND	ND	ND	ND
Heptachlor	ND	ND	ND	ND	ND
Aldrid	ND	ND	ND	ND	ND
Lasso	ND	ND	ND	ND	ND
Lexone	ND	ND	ND	ND	ND

ND- Not Detected

Table App.2-1b: Select organochlorine and organophosphate pesticide residues in rainbow trout muscle tissue (ppm) - continued

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
Dursban	ND	ND	ND	ND	ND
Oxychlordane	ND	ND	ND	ND	ND
Dual	ND	ND	ND	ND	ND
Malathion	ND	ND	ND	ND	ND
Heptachlor Epoxide	ND	ND	ND	ND	ND
Ethyl parathion	ND	ND	ND	ND	ND
gamma-Chlordane	ND	ND	ND	ND	ND
alpha-Chlordane	ND	ND	ND	ND	ND
ppDDE	0.004	ND	0.004	ND	ND
Dieldrin	ND	ND	ND	ND	ND
Endrin	ND	ND	0.008	ND	ND
ppDDD	ND	ND	ND	ND	ND
ppDDT	ND	ND	ND	ND	ND

ND- Not Detected

Table App.2-2: Select acid herbicide residues in rainbow trout muscle tissue (ppm)

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
2,4-D	ND	ND	ND	ND	ND
Silvex	ND	ND	ND	ND	ND
2, 5, 5-T	ND	ND	ND	ND	ND
Triclopyr	ND	ND	ND	ND	ND
Bentazon	ND	ND	ND	ND	ND
Dicamba	ND	ND	ND	ND	ND

ND- Not Detected

Table App.2-3: Levels of carbofuran, sevin, lannate, and aldicarb residues in rainbow trout muscle tissue (ppm)

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
Carbofuran	ND	ND	ND	ND	ND
Sevin	ND	ND	ND	ND	ND
Lannate	ND	ND	ND	ND	ND
Aldicarb	ND	ND	ND	ND	ND

ND- Not Detected

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Appendix 3: *Mycobacterium tuberculosis*

1. Historical Background

In 1886, Robert Koch first established the tubercle bacillus as the cause of the disease tuberculosis (Nolte and Metchock 1995). Tuberculosis, once referred to as the white plague, was greatly reduced in the United States by the advent of pasteurization in the milk industry. Pasteurization served to eliminate the organism, *Mycobacterium bovis*, the cause of bovine tuberculosis and when ingested by humans, a form of human tuberculosis indistinguishable from the kind caused by *M. tuberculosis* (Johnson, 1990; Collins and Grange, 1983). The disease tuberculosis was so important to medicine, that other acid-fast bacilli found were largely ignored and believed to be of no consequence to humans (Collins et al., 1984). Mycobacteriosis in fish was first described in 1897 when an acid fast bacilli was isolated from tumor in a carp. Since then, it has been shown that some of the same mycobacteria that cause mycobacteriosis in fish can also cause an infection in humans (Dulin, 1979; Willet, 1992)

2. Taxonomy, nomenclature and morphology

Members of the family *Mycobacteriaceae*, are acid-fast, nonsporogenous, nonencapsulated, straight or slightly curved rod shaped bacteria usually ranging in size from 0.3-0.6 x 1-4 μm . Growth of the organism occurs in a serpentine, parallel pattern, which form cordlike masses (Wayne and Kubica, 1986; Willet, 1992).

The bacteria are difficult to stain with Gram stain, but are generally considered to be Gram-positive (Willet, 1992). Staining of the bacteria is accomplished by using a strong dye such as carbol fuchsin coupled with heat. The organism is then exposed to acid alcohol (3% HCl in 95% ethanol) which removes the dye from all bacteria except those that are acid-fast (Beutner and Miner, 1982).

Although *M. tuberculosis* and *M. bovis* are the most common cause of the disease, tuberculosis, there are other species of mycobacteria able to infect humans and cause similar infections. These organisms include, but are not limited to the following: slow growing mycobacteria-*M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. xenopi*, and *M. avium*, and fast growing mycobacteria-*M. fortuitum* and *M. chelonae* (Willet, 1992).

3. Growth characteristics

Mycobacteria can generally be divided into two groups based on their growth times. Mycobacteria taking more than 7 days to show visible colonies are designated slow growers,. Similarly, those acid-fast bacilli requiring less than 7 days to show visible colonies are known as fast growing mycobacteria. The genus, *Mycobacterium* contain a wide range of differing nutritional types among the different species, such as saprophytes and parasites (Willet, 1992).

4. Pathology

Mycobacteriosis manifests itself in several ways. The most common manifestation of the organism is one of a pulmonary nature, however, the organism has

been known to effect nearly every organ in the body, including both the lymphatic system and the skin. Both *Mycobacterium marinum* and *M. fortuitum* have been shown to cause abscesses at the site of a small abrasion or cut (Willet, 1992).

5. Epidemiology

Pathogenic *Mycobacterium* other than *M. tuberculosis* and *M. bovis* are considered opportunistic pathogens. These organisms can enter the body in the same way as most *M. tuberculosis* infections, although it is possible that the organisms may enter the body during the ingestion of food and drink or through contact with the organism through an abrasion. Some mycobacteria, which cause the condition lymphadenitis, common to children, are more likely to enter from sources such as food, water, and enter through areas such as the tonsils (Grange et al., 1982; Collins et al., 1984).

In general, mycobacteria are widely distributed throughout the environment. Although mycobacteria may indeed reproduce in aquatic environments, the principle place of replication is most likely in slow moving or stagnant water which contains rotting vegetation that is associated with the organism. Collins et al. (1984) feel that the source of most free-living mycobacteria is not an aquatic environment, but most likely wet soil or stagnant water in contact with wet soil. These organisms most likely enter the aquatic environment due to runoff or floods. Several species of mycobacteria, such as *M. marinum* and *M. fortuitum* cause disease in both fish and humans (Dulin, 1979; Landell et al., 1993; Henderick et al., 1987). In fish, the manifestation of the disease often includes

the formation of granulomas. These nodules can be found in any organ or tissue, but are usually found on the liver, kidney and spleen (Dulin, 1979). Handling infected fish exposes workers to a source of infection, insomuch as the organisms are not confined to causing disease in fish.

6. Methods of isolation

There are several different solid media available for the isolation of mycobacteria. These media, all of which contain malachite green to suppress the growth of competing bacteria, are of two types: egg-potato-base media and agar-base media; Löwenstein-Jensen media and Middlebrook 7H-10 media, respectively (Willet, 1992). Of the two types of media, the clearer agar based media may provide earlier results when viewed microscopically. These agars also have a shorter shelf life and are susceptible to deterioration when exposed to light.

The egg based media have longer shelf lives and resist drying during prolonged incubation. Of these media, Löwenstein-Jensen (LJ) is the most commonly used. Another type, Pentraghani media, which contains almost two times the amount of malachite green as does LJ, is most commonly used for recovery from specimens that are contaminated heavily, although it can inhibit some strains of mycobacteria. Still another media is the American Trudeau Society media which contains less malachite green than LJ, but is very susceptible to overgrowth by contaminants (Nolte and Metchock, 1995).

Vitae

Thomas James McAdams was born on August 16, 1971 in South Boston, Virginia. He is the son of Annette Wright McAdams and Harold Thomas McAdams. In 1989, he graduated from Halifax County Senior High School, and in the fall of that year entered East Carolina University as a biology major. In August of 1991, James transferred to Virginia Polytechnic Institute and State University (VPI&SU) where he continued his studies receiving a Bachelor of Science degree in Biology in May of 1993.

Deciding to further his education, James entered the Master's program in the Department of Food Science and Technology at VPI&SU in the fall of 1993 under the direction of Dr. George J. Flick. As a student, he was a member of the Institute of Food Technologists .

PRESENTATIONS AT PROFESSIONAL MEETINGS:

Institute of Food Technologists

Annaheim, California, June 1995

- Presentation of paper “A Comparison of Microbiological Quality of Fresh Aquacultured Rainbow Trout (*Oncorhynchus mykiss*) Fillets and Whole Fish, and a Comparison of Pathogenic Microorganisms in Whole Fish to Rearing Methods”

International Association of Milk, Food, and Environmental Sanitarians

Pittsburgh, Pennsylvania, July 1995

- Presentation of paper "The Incidence of Pathogenic Microorganisms in Aquacultured Rainbow Trout (*Oncorhynchus mykiss*)"

PUBLICATIONS:

Reinhard, R.G., T.J. McAdams, G.L. Flick, R.F. Wittman, R.E. Croonenberghs, and Diallo. 1995. "Qualitative and quantitative analysis of *Campylobacter jejuni* and *Campylobacter coli* in fresh Blue Crab (*Callinectes sapidus*) meat."

J. Aquatic Food Prod. Tech. 4(4): 31-36.