

**The Effects of Modified Atmosphere Packaging on Toxin Production by  
*Clostridium botulinum* in Raw Aquacultured Flounder Fillets and Fully  
Cooked Breaded and Battered Pollock Portions**

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Abstract

Fish products under vacuum (VAC) and/or modified atmosphere packaging (MAP) conditions can have a significantly extended shelf life. Prevention of toxin production by *Clostridium botulinum* is essential for processors of VAC and MAP refrigerated fishery products. The objective of this study was to determine if *C. botulinum* toxin development precedes microbiological spoilage and sensory rejection in fully cooked breaded and battered Alaskan Pollock or raw aquacultured flounder fillets.

Aquacultured summer flounder (*Paralichthys dentatus*) fillets and fully cooked breaded and battered Alaskan pollock (*Theragra chalcogramma*) were either aerobically packed (Oxygen Transmission Rate (OTR) of 3,000 cc/m<sup>2</sup>/24h@70°F for flounder and 6,000 cc/m<sup>2</sup>/24h@70°F for Pollock), vacuum packed or MAP packaged in a 100% CO<sub>2</sub> atmosphere (OTR of 7.3 cc/m<sup>2</sup>/24h@70°F). Flounder fillets were stored at either 4 or 10°C while pollock portions were stored at 8 and 12°C. Based on the time to spoilage (counts >10<sup>7</sup> CFU/g), additional samples

were inoculated with five strains of nonproteolytic *C. botulinum* and analyzed qualitatively for botulinum toxin using a mouse bioassay.

For flounder at 4°C, toxin formation did not occur after 35 days in aerobically packed fillets. Vacuum packed and 100% CO<sub>2</sub> fillets produced toxin before spoilage at days 20 and 25, respectively. In the aerobic packages at 10°C, toxin production occurred after spoilage at day 8, but before spoilage in the vacuum and 100% CO<sub>2</sub> packages at day 9. Sensory evaluation of toxic vacuum and 100% CO<sub>2</sub> packages at 4°C revealed toxin production proceeded spoilage and absolute sensory rejection. However, at 10°C toxin production was evident only after absolute sensory rejection and microbiological spoilage for aerobically packed fillets. Vacuum packages and 100% CO<sub>2</sub> packages were toxic during spoilage but before absolute sensory rejection.

Toxin was not present in the aerobically and 100% CO<sub>2</sub> packed pollock samples at 8°C and the 100% CO<sub>2</sub> packed samples at 12°C after 35 days. Aerobically packed portions stored at 12°C first produced toxin at day 25; toxicity occurred after absolute sensory rejection and before spoilage. The vacuum packed portions first formed toxin at day 25 for 8 and 12°C storage before spoilage and absolute sensory rejection.

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## I. Introduction

Currently the world's consumption of seafood is approximately 80 million metric tons. However, it has been projected that by 2010 the demand will be 110 to 120 million metric tons. Technology must continue to be developed to meet with the demand as the world's natural supply dwindles (Garrett et al., 2000). Decreasing fishery supplies of the natural flounder population as well as an increased market demand particularly with the cultivation of Japanese flounder, *Paralichthys olivaceus* (Matsuoka, 1995) have led to an increase in flatfish research and production. In the U.S. one common type of flounder is the summer flounder (*Paralichthys dentatus*). It is a highly valued fish naturally located in the waters of the Atlantic coast from Nova Scotia to southern Florida particularly from Cape Cod, Massachusetts to Cape Hatteras, North Carolina (Rogers and Van den Avyle, 1983).

Consumer demands for cooked ready-to-eat (RTE) refrigerated food products are an emerging growth area for all food products. In 2002, the per capita consumption of value added fish sticks and portions were approximately 0.8 lbs./person. The U.S. production of fish portions and sticks was 235.4 million pounds with an estimated value of \$288.6 million. Fish portions alone accounted for 187.3 million pounds with a value of \$236.6 million. Alaskan Pollock fillets and blocks led all species being 59% of the total amount of fillets, steaks, and blocks (NMFS, 2002). Lambert (1990) suggested that, besides convenience,

additional reasons for the continued growth may be increased numbers of freezers and microwave ovens in homes, less meal preparation time with two or more individuals working and changing tastes. New refrigerated foods with extended shelf life present a highly profitable opportunity for food processors.

The traditional frozen batter and breaded seafood portion is a low risk food safety concern. The final product is usually fully cooked prior to consumption by the consumer. In addition, bacterial pathogen growth is unlikely to occur during frozen storage. For these reasons the National Seafood Alliance for Training and Education HACCP Curriculum (1997) has identified only two Critical Control Points (CCPs) for this product. (i.e., *Staphylococcus aureus* growth in the batter operation if the batter is held too long at elevated temperatures, and possible metal fragments from the saw and conveyor belts). Nevertheless, an increasing number of supermarkets and convenience stores are interested in selling refrigerated fully cooked batter and breaded portions. There is also commercial interest in vacuum and modified atmosphere packaged refrigerated, (never frozen) raw and fully cooked batter and breaded products.

Packaging products under vacuum (VAC) and modified atmosphere packaging (MAP) conditions can significantly extend shelf life of refrigerated fish products. However, there are food safety and quality issues that companies must consider in marketing refrigerated foods especially with extended shelf life (Doyle, 1998). Doyle went on to state that processors should be careful to fully evaluate and

prevent hazards that may exist in refrigerated foods with extended shelf lives before marketing such products. Microbiological concerns with refrigerated foods focus on psychrotrophic and mesophilic organisms that have an opportunity to grow during extended storage at refrigeration temperatures. Several pathogens can grow at refrigerated temperatures and for some pathogens, only a few cells can cause illness when ingested (Marth, 1998). Shelf life is increased by the inhibition of aerobic spoilage bacteria, but VAC and MAP will not inhibit the growth of *Clostridium botulinum* (ICMSF, 1996). Fish inoculated with high numbers of spores and stored under VAC or MAP conditions have become toxic within 6-8 days during refrigerated storage when temperatures approached 50° F (10°C) (NACMCF, 1992). This is a concern, since in distribution and retail storage, product temperatures have been found to fluctuate between 40-50° F (4.4-10°C), (NACMCF, 1992).

Kalish (1991) reported in retail operations only 37% of food products were stored at the recommended temperature range of 32-38°F, with many cases averaging 44°F and some as high as 55°F. Consumers were also at risk, since refrigerator temperatures in consumer homes ranged from 32°F to 55°F (Beard, 1991). Studies have shown that at the retail level, product rotation procedures were inadequate, as sales of product dictated product rotation frequency (Kalish, 1991). Approximately, 2000 retail stores, including back-room storage facilities and chill cases were checked. Kalish reported that only 37% of products were stored within the recommended 32-38°F (0-3.3°C) range. Products were also

found stacked on the floor without any refrigeration and the temperature of many refrigerated cases were 44°F with some as high as 56°F.

## II. Review of Literature

### A. Seafood of increasing marketability

#### 1. Aquacultured Flounder (*Paralichthys dentatus*)

Currently the world's consumption of seafood is approximately 80 million metric tons. However, it has been projected that by 2010 the demand will be 110 to 120 million metric tons. Technology must continue to be developed to meet with the demand as the world's natural supply dwindles (Garrett et al., 2000). Decreasing fishery supplies of the natural flounder population as well as an increased market demand particularly with the cultivation of Japanese flounder, *Paralichthys olivaceus* (Matsuoka, 1995) have led to an increase in flatfish research and production. In the U.S. one common type of flounder is the summer flounder (*Paralichthys dentatus*). It is a highly valued fish naturally located in the waters of the Atlantic coast from Nova Scotia to southern Florida particularly from Cape Cod, Massachusetts to Cape Hatteras, North Carolina (Rogers and Van den Avyle, 1983).

#### 2. Value added products

Consumer demands for cooked ready-to-eat (RTE) refrigerated food products is an emerging growth area for all food products. In 2002, the per capita consumption of value added fish sticks and portions were approximately 0.8 lbs./person. The combined production of fish sticks and portions was 235.4 million lbs. valued at \$288.6 million (NMFS, 2002). Lambert (1990) suggested

that besides convenience additional reasons for the continued growth may be increased numbers of freezers and microwave ovens in homes, less meal preparation time with two or more individuals working and changing tastes. New refrigerated foods with extended shelf life present a highly profitable opportunity for food processors. Figueiredo (2000) stated that today's consumer is demanding speed, ease and convenience along with nutrition, health, and food safety.

Traditional battered and breaded seafood products are one type of value added product and are typically sold and prepared from the frozen, raw state. An increasing number of retail outlets and convenience stores are interested in selling refrigerated fully cooked batter and breaded fish portions packaged under vacuum (VAC) or modified atmosphere (MAP) due to consumer perceptions that non-frozen fish is of better quality and faster to prepare. Microbiological concerns with these refrigerated foods focus on psychrotrophic organisms that can grow during extended storage at refrigeration temperatures. Cooked foods, in which competitive spoilage bacteria have been destroyed, may also allow pathogens such as *Clostridium botulinum* to grow during refrigerated storage resulting in a higher risk of foodborne illness for the consumer.

## B. *Clostridium botulinum*

### 1. Taxonomy

*C. botulinum* is a Gram positive, anaerobic, spore-forming foodborne pathogen included in the family *Bacillaceae* that typically produces one of six antigenically different types of toxin named A, B, C, D, E or F. Cells ranging in size from 0.3-0.7 X 3.4-7.5 µm are motile with peritrichous flagella (ICMSF, 1996). Spores are oval shaped and are formed subterminally. The species *C. botulinum* can be broken down into four metabolic subgroups (Groups I, II, III and IV). Group II is the non-proteolytic, psychrotrophic group comprised of *C. botulinum* B, F, and E that ferment both mannose and glucose.

### 2. Ecology

*C. botulinum* is found throughout the environment in soil, water, vegetables, meats, dairy products, fish, shellfish, crustaceans, etc. *C. botulinum* is indigenous to the aquatic environment and can be isolated from water, aquatic sediments and organisms. Off the coasts of Alaska, Washington and Oregon, it is the most frequent environmentally isolated type (Eklund, et al., 1993). Fish is an excellent substrate for growth of *C. botulinum* Type E (Gram and Huss, 2000; Lund and Peck, 2000). Estimated concentrations of *C. botulinum* Type E range from 1 - 200 per kg of seafood, although some studies have indicated 2,000-3,000 spores/kg (Lund and Peck, 2000). Huss et al. (1974) reported levels as high as 5.3 spores per gram in farmed trout.

### 3. Pathogenesis

*C. botulinum* growth and toxin production can occur under the following conditions 1) if a food is contaminated with spores or vegetative cells, 2) the processing treatment is inadequate to inactivate spores or the product is recontaminated after processing, 3) the food supports growth and toxin formation when temperatures exceed 3.3°C and 4) the food is consumed without cooking or after inadequate heating to inactivate preformed toxin (Eklund, 1993). Type E toxin in foods is released from the bacteria as an inactive protein and must be cleaved by proteases to expose the active site. This can be achieved by contact with body fluids of the exposed host. It has also been shown that some bacterial enzymes can increase the toxicity of Type E toxin (Sakaguchi and Tohyama, 1955).

Delay time for onset of symptoms can be from several hours up to ten days however it most commonly occurs between 18-36 hours. A small amount of toxin absorbed into the intestinal mucosa is all that is needed to be effective. The lethal dose is approximately 1ng/kg in adults. The toxin enters into the blood stream and the lymphatic system to become fixed in cranial and peripheral nerves where it inhibits the release of acetylcholine at synapses resulting in flaccid paralysis. The cranial nerves are affected first followed by a symmetric descending paralysis of motor nerves. Initial symptoms include difficulty with sight, hearing and speech. Muscle weakness increases and eventually the nerves involved with respiration are affected resulting in death by suffocation

although cardiac failure may be a primary cause (Wells and Wilkins, 1996). In the United States approximately 110 botulinal illnesses occur each year with about a quarter of those being attributed to the consumption of intoxicated food particularly home canned foods (CDC, 2004).

#### 4. Factors affecting growth

##### a. Temperature

*C. botulinum* Type E is of concern because of its ability to grow at temperatures as low as 3.3°C (Eklund et al., 1967; 1982; 1993) or even down to 3.0°C (Graham et al., 1997). Optimum growth temperature for Group II *C. botulinum* is between 28 and 30°C. Growth and toxin production is not associated with typical objectionable odors as with many botulinal types due to the absence of deterioration caused by proteolysis (McClure et al., 1994). Spores of *C. botulinum* are resistant to freezing and can survive in frozen storage indefinitely. Botulinal toxins present in foods are also tolerant to freezing and retain their activity.

##### b. Water activity and pH

*C. botulinum* metabolic activity reduces with lower of water activity ( $A_w$ ). If the  $A_w$  is less than 0.97 or the equivalent of 5% sodium chloride in water, Group II strains cease to grow at temperatures of 16, 21, and 30°C after 365 days (Segner et al. 1966). *C. botulinum*, regardless of the strain, can grow and produce toxin down to a pH of 5.2. Group II strains stop growing at a pH of about 5.1

(Graham et al., 1996). Eklund et al. (1993) conducted studies on growth of *C. botulinum* Type E and toxin formation in hot smoked fish packaged in an oxygen permeable film (i.e., 1.5 mil polyethylene, oxygen transmission rate (OTR) 7195 cc/m<sup>2</sup>/24h at 760 mm Hg 23° C and 0%RH; CO<sub>2</sub> transmission 22,858 cc/m<sup>2</sup>/24h), and under vacuum in an oxygen impermeable film (i.e., OTR 108 cc/m<sup>2</sup>/24h; CO<sub>2</sub> transmission 526 cc/m<sup>2</sup>/24h). Current FDA regulations identify a film OTR of 10,000 cc/m<sup>2</sup>/24h at 760mm Hg and 23°C at 0% RH or higher as an oxygen permeable film. Eklund et al. (1993) reported that higher levels of NaCl were needed to inhibit *C. botulinum* Type E in oxygen permeable films compared with oxygen impermeable films. Several studies have used a combination of Aw and pH to inhibit the growth of *C. botulinum* Type E strains (Graham et al., 1996; McClure et al., 1994; Hauschild and Hilsheimer, 1979).

### c. Atmosphere

Many studies have been conducted on the manipulation of atmosphere to prevent the growth and subsequent toxin production of *C. botulinum* Type E in fresh fish. In addition to several other studies a five-year project including 927 experiments and 18,700 samples (Baker and Genigeorgis, 1990) indicated atmosphere alone is not effective to prevent toxin production. Ito and Seeger (1980) observed a 99.9% reduction of spores using ozone for 2 min. and chlorine dioxide for 13 and 14 min. on Type A 62A and proteolytic Type B 231B.

#### d. Antimicrobial treatments

In seafood, 0.0, 2.4 and 4.8% sodium lactate was used as a preservative with sous-vide salmon inoculated with  $10^4$  per sample of non-proteolytic *C. botulinum* Types B and E incubated at 12°C resulting in toxicity at 12, >40 and >40 days respectively (Meng and Genigeorgis, 1994).

#### 5. Destruction of spores and toxin

Botulinal toxin is easily destroyed by heat. Spores of *C. botulinum* however can survive high cooking temperatures particularly proteolytic strains. However, heat resistance varies between Types and Groups (e.g. proteolytic or non-proteolytic). Scott and Bernard (1982) reported destruction of non-proteolytic 17B spores at a temperature of 82.2°C, a D-value of 16.7 min. and z-value of 6.5°C. In 1985, he reported a temperature of 82.2°C, a D-value of 28.21 min. and no reported z-value in M/15 phosphate buffer. Peck et al. (1993) used the same strain and substrate heated to temperatures of 85, 90 and 95°C D-values of 100, 18.7 and 4.4 min. and z-values of 7.6, 7.6 and 7.6 respectively were reported for spore destruction. It would be expected for these values to increase in actual product. Lynt et al. (1977) reported Type E destruction in blue crab meat at temperatures of 74, 76.7, 79.4, 82.2 and 85°C with D-values of 6.8-13.0, 2.4-4.1, 1.1-1.6, 0.49-0.74 and 0.29, min., respectively. Again Lynt et al. (1983) used crab meat and Type E with temperatures ranging from 73.8 to 85°C, D-values of 0.3 to 11.2 min. and z-values of 10.5-12.8°C respectively for spore destruction. Rhodehamel et al. (1991) used and Type E menhaden surimi with temperatures of 73.9, 76.7,

79.4 and 82.2°C, D-values of 8.66, 3.49, 2.15 and 1.22 min. and z-values of 9.78, 9.78, 9.78 and 9.78°C respectively for spore destruction. Lynt et al. (1979) used Type F strain 202 and temperatures of 76.6, 79.4, 82.2 and 85°C. D-values of 9.5, 3.55, 1.16 and 0.53 min. and z-values of 10.27, 10.27, 10.27 and 10.27°C respectively were reported for spore destruction in crab meat.

Ito and Seeger (1980) introduced one strain of non-proteolytic Type B, four strains of Type E and two strains of non-proteolytic Type F in the amount of  $10^4$ /ml into phosphate buffer and used 4.5 mg/L of free chlorine achieving a 99.9% destruction in 5.5, 6.0 and 7 min. respectively at a pH of 6.5 and incubation temperature of 25°C. Chlorine in the form of  $\text{Ca}(\text{OCl})_2$  in the amount of 4.0 µg/ml in acetate buffer at a pH of 3.5 and 5.0 achieved a 99.9% reduction of  $10^4$ /ml Type E spores in 1.1 and 2.8 min. respectively. The same experiment was repeated in phosphate buffer at a pH of 6.5, 8.0 and 10.0 producing the same destruction in 4.0, 17.0 and >30 min. respectively. The spores had been heated to 60°C for 35 min (Ito et al., 1968). Irradiation has also been used to reduce four strains of Type E spores at 1.7°C in haddock producing a D-value (kGy) of 0.8-2.2 (Segner and Schmidt, 1966).

### C. Control of *Clostridium botulinum* in seafood

#### 1. Vacuum (VAC) and Modified Atmosphere Packages (MAP)

Due to the relatively low body temperature of fish the optimum environment for the growth of naturally occurring spoilage bacteria exists (Brody, 1989). Bacteria

such as *Acinetobacter*, *Flavobacterium*, *Moraxella* and *Pseudomonas* are dominant (Eddy and Jones, 2002) and once processed begin to proliferate on nutrient rich proteins producing off-flavors and odors associated with a short shelf-life (Reddy et al., 1992).

The advantages of extended shelf-life and quality as well as the disadvantages including providing conditions conducive to botulinal toxin production of packaging fresh fish with VAC and MAP have been outlined (Wolfe 1980, Yambrach 1987 and Cann 1988). A CO<sub>2</sub> atmosphere extends the lag phase and generation time of aerobic bacteria that in turn decreases the growth rate and extends shelf-life (Finne, 1982; Parkin and Brown, 1982; Genigeorgis, 1985).

The inhibition of bacterial growth in food packaged with CO<sub>2</sub> increases as the storage temperature decreases (Reddy et al., 1992). A 100% CO<sub>2</sub> atmosphere has been shown to inhibit *C. botulinum* at chill temperatures (Gibson et al., 2000). Detrimental quality effects may also occur including the lowering of pH, absorption of CO<sub>2</sub> into the flesh causing a deflation of the package, pigmentation changes (Parkin and Brown, 1982) and increased drip from the alteration in the water holding capacity brought on by the CO<sub>2</sub> solubility (Davis, 1998).

The ratio of CO<sub>2</sub> atmosphere to volume of food in the package is also a concern (G/P ratio). Randell et al. (1995) showed a G/P ratio of one increased shelf-life versus a ratio of 0.4, which had no differences when compared to vacuum. Compared to air packaging a ratio of 2, and a 50% CO<sub>2</sub> atmosphere doubled

shelf-life in hake (Pastoriza et al., 1998). To inhibit the growth of organisms a certain amount of CO<sub>2</sub> must be dissolved into the product (Gill and Penney, 1988). The antimicrobial activity of CO<sub>2</sub> may be attributed to a surface pH change caused by the absorption of CO<sub>2</sub> into the fish flesh and the subsequent ionization of carbonic acid, the toxic affects of the acid itself and the inhibition of decarboxylating enzymes, which disrupt the metabolic processes of resident microorganisms (Genigeorgis, 1985). Therefore, the proper ratio of G/P should be 2:1 to 3:1 (Sivertsvik et al., 2002).

For sections a., b., c., d. and e. Appendix 1 contains a list of important characteristics for each journal article referenced.

a. Headspace gas

Reddy et al. (1996) reported air package oxygen concentrations decreased from 20.9% to 3.7% on day three, 3.5% on day six and 5.0% on day 10 for 16, 8 and 4°C, respectively with tilapia fillets. Headspace CO<sub>2</sub> concentrations increased from <1.0% to >15.0% on the day of spoilage for all three temperatures.

Modified atmosphere (MA) 75:25 CO<sub>2</sub>:N<sub>2</sub> revealed that within the first few days of storage CO<sub>2</sub> concentrations decreased to 55% at 8°C and 4°C at 3 and 6 days, respectively. On day one at 16°C levels decreased to 56%. However by the time spoilage was achieved the concentration had increased to >60% for all temperatures.

With pond raised catfish, Reddy et al. (1997a) noted in air packages the O<sub>2</sub> concentration decreased from 20.9% to 0.7%, 1.8% and 0% on days 3, 10 and 13 for 16, 8 and 4°C, respectively for inoculated samples. Uninoculated samples decreased to 0% in similar numbers of days at 3, 9 and 16 for 16, 8 and 4°C, respectively.

In the MA packages, O<sub>2</sub> concentrations increased from 0% to 3.8% on day one then decreased to 0% the day of spoilage. The increase was due to O<sub>2</sub> leaching from the soakpad and foam tray. The higher the incubation temperature the more rapid the O<sub>2</sub> decrease occurred. The initial CO<sub>2</sub> concentration was 75% but decreased to 17.5, 18.9 and 18.7% at day one at 16, 8 and 4°C, respectively. However, at all storage temperatures, CO<sub>2</sub> concentration increased to >58% by the day of spoilage.

Reddy et al. (1997b) reported similar results with aquacultured salmon. In air packages, O<sub>2</sub> concentration decreased from 20.9% to 0% on days 4, 10 and 17 at 16°C, 8°C and 4°C, respectively for inoculated samples. Uninoculated samples decreased to 0% at days 4, 8 and 13 for 16°C, 8°C and 4°C, respectively.

In the MA packages O<sub>2</sub> concentrations initially were 0% but increased to 4.2% by day one then decreased to 0% on the day of spoilage. Initial CO<sub>2</sub> concentration was 75%, which decreased to 9.7%, 11.9% and 11.3% at day one at 16°C, 8°C

and 4°C, respectively. However, for all storage temperatures, CO<sub>2</sub> concentration was greater than 64% on the day of spoilage.

Reddy et al. (1999) indicated that the initial concentration of O<sub>2</sub> in air packaged cod was 20.9%, which decreased to 0% by day 4, 10 and 17 at 16°C, 8°C and 4°C, respectively. Carbon dioxide concentrations increased from <1.0% to >18.0% on the day of spoilage for all storage temperature and packaging combinations. In the MA packages, initial O<sub>2</sub> concentrations were at 0% but increased to 4.2% on day one, decreased down to 0% by the day of spoilage.

In the MA packages, initial CO<sub>2</sub> concentration was 75% but decreased to 22.5%, 23.2% and 20.5% at day at 16°C, 8°C and 4°C, respectively. However, at all storage temperatures the CO<sub>2</sub> concentration was >58% on the day of spoilage.

Garcia et al. (1987) also reported an initial decrease in headspace concentration of CO<sub>2</sub> in both 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub> (MA) packages. Product at all temperature combinations denoted by package shrinkage. Shrinkage was most pronounced at 4°C due to an increased saturation rate of CO<sub>2</sub> into the tissue. Carbon dioxide concentration increased over time and O<sub>2</sub> was not detected. In the MA packages, O<sub>2</sub> concentrations decreased from 2.9% to non-detectable levels on the 21<sup>st</sup> day of storage at 8°C, on the 18<sup>th</sup> day of storage at 12°C, on the third day of storage at 30°C, and 0.6% at 4°C after 60 days of storage.

Although no initial decline in CO<sub>2</sub> concentrations were observed a similar trend was observed by Lyver et al. (1998) and Lyver and Smith, (1998). In uninoculated and inoculated raw and cooked surimi nugget packages, O<sub>2</sub> concentrations decreased from 20% to <1% at 4°C, 12°C and 25° C. Initial headspace CO<sub>2</sub> concentrations were <1% increasing to 30%, 44% and 60% for uninoculated samples and to 30%, 45% and 80% for inoculated samples at 4°C, 12°C and 25°C raw nuggets, respectively. For cooked nuggets, initial CO<sub>2</sub> concentrations were <1% increasing to 25% and 40% for uninoculated samples and 23% and 80% for inoculated samples at 12°C and 25°C, respectively

Air and oxygen absorbent packages used with inoculated and uninoculated raw and cooked nuggets kept O<sub>2</sub> concentrations <1% for 28 days at all temperatures. The same packages resulted in initial CO<sub>2</sub> concentrations of <1% to increase to 4%, 10% and 80% for uninoculated samples at 5%, 32% and 40% for inoculated samples at 4°C, 12°C and 25°C raw nuggets, respectively. Cooked nuggets CO<sub>2</sub> concentrations remained at <1% at 12°C and increased to 40% for 25°C (Lyver et al. 1998 and Lyver and Smith, 1998).

Stier et al. (1981) reported during storage at 4.4°C concentration of CO<sub>2</sub> decreased from approximately 60% to 20% by day 22 in the 60:25:15 CO<sub>2</sub>:O<sub>2</sub>:N<sub>2</sub> packages of salmon fillets. Oxygen concentrations increased to approximately 30% at day 14 and then decreased to approximately 10% by day 22.

Conversely, rainbow trout packages with OTR of 4,371, 4923, and 10,043 cc/m<sup>2</sup>/24h at 24°C, 0% RH showed a similar pattern for aerobically packed fillets. In packages backflushed with 85:15 CO<sub>2</sub>:N<sub>2</sub> there was a headspace decrease in CO<sub>2</sub> and an increase in O<sub>2</sub>. Headspace analysis of packages (OTR of 11.6cc/m<sup>2</sup>/24h at 24°C, 0% RH) revealed a decrease in O<sub>2</sub> and increase in CO<sub>2</sub> (Dufresne et al. 2000). Concentrations of CO<sub>2</sub> and O<sub>2</sub> remained relatively constant between 77.5-80.1% and 0.2-2.0%, respectively at 4°C and 10°C in MA with 80:20 CO<sub>2</sub>:O<sub>2</sub>. (Cai et al.1997).

#### b. pH

Reddy et al. (1997a) reported that initial average surface pH was 6.59 for catfish fillets but on the day of spoilage for the air packages the pH increased by 0.19-0.26 at 4°C and 16°C and decreased 0.04 at 8°C. In MA packages pH initially decreased then increased above initial levels by the day of spoilage.

For aquacultured tilapia, Reddy et al. (1996) found for all combinations of package and temperatures a decrease in surface pH during early storage and an increase in later storage. For all combinations, the pH values ranged from 6.12 to 7.04.

Salmon fillets had an initial average surface pH of 6.42 and 6.61 for uninoculated and inoculated samples, respectively. On the day of spoilage, for the uninoculated air packages, salmon pH increased by 0.05-0.12 at 4°C, 8°C and

16°C. In inoculated packages salmon pH no consistent pattern. Final pH values were lower than initial values at 4°C and 8°C. In MA packages salmon pH initially decreased then increased above initial levels by the day of spoilage. The initial pH decrease may have been caused by the saturation of CO<sub>2</sub> into the tissue of the fillet during early storage (Reddy et al. 1997b).

Conversely, no initial decrease of pH occurred in 100% CO<sub>2</sub> packages with salmon in a study by Garcia et al. (1987). Initial pH concentration was 6.2 and varied between 5.95-6.8 for most samples at all storage temperatures for MA packages. There was no change in the pH of cod fillets from the initial value of 7.1 for any temperature or atmosphere combination (Reddy et al. 1999).

Additionally, Dufresne et al. (2000) used to reported the initial pH of rainbow trout fillets was approximately 6.5, and no significant changes occurred due to the buffering capacity of fish proteins. Cai et al. (1997) also found little difference in pH at any storage temperature or packaging type.

Lyver et al. (1998) and Lyver and Smith (1998) reported a decrease in pH with similar trends seen in raw inoculated surimi package study. Values of pH began at approximately 6.5 and declined to 5, 4.5 and 4 for 4°C, 12°C and 25°C, respectively. In cooked samples little change was apparent after 28 days for 12°C and 25°C.

### c. Toxigenesis

Lalitha and Gopakumar (2001) used mullet and shrimp tissue homogenates to determine time to toxicity. In the mullet tissue the earliest days of toxin production was day 1, 3, 8 and 28 for 30°C, 15°C, 10°C and 4°C, respectively. Shrimp tissue produced toxin at days 2, 5, 10, and 35 for temperatures of 30°C, 15°C, 10°C and 4°C, respectively.

### d. Toxin production with relation to sensory rejection

Post et al. (1985) packaged cod, whiting and flounder in atmospheres of air, vacuum, 100% N<sub>2</sub>, 100% CO<sub>2</sub>, 90:8:2, CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub>, 65:31:4, CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> were applied to packages of cod fillets stored at 26°C and 8°C. Air, vacuum, 100% N<sub>2</sub> and 100% CO<sub>2</sub> atmospheres were used in conjunction with 12°C. Only 100% CO<sub>2</sub> was used with 4°C and packages being cycled from 26°C to 8°C or 4°C. Cod fillets inoculated with 5.0 X 10<sup>1</sup> spores/g developed toxin before absolute rejection in all temperature and atmosphere combinations except 26°C 90% CO<sub>2</sub>: 8% N<sub>2</sub>: 2% O<sub>2</sub> packages, 12°C air packages, 8°C air and 100% N<sub>2</sub> packages. Toxin production coincided with absolute sensory rejection in the 26°C air packages.

Similar time and temperature combinations were used for whiting fillets. Only 8°C air packages were spoiled before toxin production. Spoilage coincided with toxin production for 26 and 8°C vacuum and 100% N<sub>2</sub> packages. All other package and temperature combinations were toxic before absolute spoilage.

Air, vacuum, 100% N<sub>2</sub> and 100% CO<sub>2</sub> atmospheres were used in conjunction with 12°C, 8°C and 4°C in flounder fillets. For all temperature and atmosphere combinations fillets became absolutely spoiled before toxin production.

Toxin was produced in cod fillets at days >7, 7 and 7 for 16°C, >41, >60 and 17 at 8°C and >60, >90 and >55 for 4°C in air, MA and vacuum packages, respectively (Reddy et al. 1999). Sensory rejection occurred at days 3-4, 6 and 3-4 at 16°C, 13-17, 24-27 and 13 at 8°C, 20-24, 55-60 and 24-27 at 4°C for air, MA and vacuum packages, respectively. In every combination sensory rejection proceeded toxin production.

In air and vacuum packaging crawfish tails sensory rejection occurred before toxin production at all storage temperatures and packaging combinations. They used the same sensory scale as Reddy et al. (1999)(Lyon and Reddmann, 2000).

Concentrations of CO<sub>2</sub> ranged from 0-100% and packaging film range from OTR of 11.6cc/m<sup>2</sup>/24h at 24°C, 0% RH for trout fillets inoculated with 10<sup>2</sup> spores stored at 12°C (Dufresne et al. 2000). At all levels of CO<sub>2</sub> concentration, fillets were toxic in 5 days following sensory rejection. Packages with OTR of 4,371, 4923, and 10,043 cc/m<sup>2</sup>/24h at 24°C, 0% RH were also used and became toxic at 4-5 days, after sensory rejection.

Dufresne (2000) conducted product shelf life studies for both hot and cold smoked trout vacuum packaged (VAC) and MA products. Microbiological shelf life was approximately 21-28 days at 4°C, with no toxin formation in any sample. At 8°C, spoilage occurred between 7-14 days, and toxigenesis occurred after 28 days in cold smoked trout packaged in films with OTRs of 4923 and 10,043 cc/ m<sup>2</sup>/24h. At 8°C, hot smoked products spoiled between 21-28 days, and toxigenesis occurred at 14, 21, and 28 days when packaged in films with OTRs of 2952, 4923, and 11.6 cc/ m<sup>2</sup>/24h.

At 12° C, toxin formation and spoilage coincided at 14 days of storage for cold smoked trout packaged in films with OTRs of 11.6, 2951, and 4923 cc/ m<sup>2</sup>/24h. Cold smoked trout packaged in films with an OTR of 10,043 cc/ m<sup>2</sup>/24h, spoiled after 7 days of storage, but toxin formation in these products did not occur until 14 days. Toxin formation in hot smoked trout coincided with spoilage in products packaged with a film OTR of 4923 cc/ m<sup>2</sup>/24h. Toxin formation occurred prior to spoilage in hot smoked trout packaged with a film OTR of 11.6 and 2951 cc/ m<sup>2</sup>/24h.

e. Toxin production with relation to microbiological count and sensory rejection

In inoculated shrimp in psychrotrophic counts by increased 3.5 log and anaerobic counts increased by 4.3 log during 21 days of storage at 4°C (Garren et al. 1994). Spoilage, based on off-odors, occurred between 6-9 days. The microbial

counts at this time were approximately 6 log CFU/g. Toxin was not detected in either package at this temperature. At 10°C there was a 3.6 and a 4.9 log increase in psychrotrophic and anaerobic plate counts, respectively during 14 days of storage. Sensory rejection occurred between days 3 and 6, however microbial counts did not exceed 6.0 log CFU/g, and toxin was present at day 6.

In inoculated aquacultured rainbow trout, anaerobic populations increased 3.3 log cycles and psychrotrophic counts increased 5.4 log cycles in oxygen permeable vacuum skin packages at 4°C by day 21 (Garren et al. 1995). Fillets were spoiled between days 12 and 15 (~3-5 log CFU/g) but toxin never developed. In oxygen barrier vacuum skin packages anaerobic populations increased 3.4 log cycles and psychrotrophic counts increased 5.5 log cycles at 4°C by day 21, but again toxin never developed.

Anaerobic populations increased 4.0 log cycles and the psychrotrophic counts increased 5.1 log cycles at 10°C in the oxygen barrier vacuum skin packages by day 21. Toxin developed by day 6 but spoilage occurred at day 3 with psychrotrophic counts of ~6 log CFU/g and off-odors being apparent.

In packaged salmon fillets and fillet sandwiches (two fillets) stored at 4.4°C total counts at sensory rejection on day 6 and 12 were 9 log CFU/g for air and MA packages, respectively (Stier et al. 1981). An aerobic count of 7 log CFU/g occurred at day 4. Sensory rejection for two sandwiched fillets occurred at day

14 with counts of approximately 7.86 log and 8 log CFU/g for air and MA packages, respectively. Toxin was not detected for either product at any temperature combination. At a storage temperature of 22.2°C, sensory rejection occurred at day 1 with microbial counts of approximately 9 log CFU/g for fillets of both atmospheres and day 2 for both atmospheres at a level of approximately 8.3 log CFU/g. Toxin was produced at day 2 which coincided with sensory rejection in the MA package and proceeded it in the air package.

Aquacultured tilapia aerobic counts at sensory rejection for the 30-day study were 8.54, 8.04 and 8.15 log CFU/g for air packages stored at 4, 8 and 16°C, respectively. Aerobic counts at sensory rejection for MA packages were >8.36, 8.54 and 6.69 log CFU/g at 4, 8 and 16°C, respectively. Toxin production coincided with sensory rejection for the vacuum and MA packages, and occurred one day later for the air package at 16°C. For all other storage temperatures spoilage proceeded toxin production. At the lower storage temperatures, intervals between toxicity and sensory spoilage increased (Reddy et al. 1996). Uninoculated pond raised catfish initial counts were 4.9, 3.6 and 3.2 log CFU/g and increased >8.5, >7.0 and >6.5 log CFU/g for aerobes, facultative anaerobes and coliforms respectively by spoilage (Reddy et al. 1997a).

Aquacultured salmon initial microbial counts were 4.9, 3.6 and 3.2 log CFU/g but to increased >8.8, >8.5 and >8.5 log CFU/g for aerobes, anaerobes and psychrotrophs respectively by spoilage in the uninoculated study. Inoculated

samples aerobic, anaerobic and psychrotrophic counts increased to >8.3 log CFU/g on the day of spoilage (Reddy et al. 1997b).

Aquacultured tilapia, catfish and salmon packaged in MA developed toxin at days >90, >75 and >80 at 4°C, days 40, 18 and 24 at 8°C and day 4 at 16°C, respectively. Sensory rejection occurred at day 80, days 38-40 and 55-62 at 4°C, days 17, 13 and 20-24 at 8°C and days 4, 4 and 5-6 at 16°C, respectively.

Sensory rejection preceded toxin production in all scenarios except for the 8°C stored salmon, the 16°C stored tilapia and the 16°C stored catfish (toxin production coincided with sensory rejection). For the 16°C stored salmon toxin production preceded sensory rejection (Reddy et al. 1996, 1997a, 1997b, 1999).

Salmon fillets packaged in VAC and MA had initial aerobic counts of 2.48 log CFU/g fillet which increased to 7.7-8.7 log CFU/g (Garcia et al. 1987). The earliest toxin formation occurred at 30°C on day 1 for vacuum, 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub> at spore levels of 10<sup>0</sup>. For samples stored at 12°C, toxin formed at days 3, 6 and 3 at lowest spore levels of 10<sup>1</sup>, 10<sup>2</sup> and 10<sup>3</sup> for vacuum, 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub>, respectively. At 8°C, earliest toxicity occurred at days 6, 9 and 6 at lowest spore levels of 10<sup>1</sup>, 10<sup>2</sup>, and 10<sup>4</sup> for vacuum, 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub>, respectively. Only vacuum packages became toxic at 4°C on day 15 at a level of 10<sup>4</sup>. Using the same sensory scale as Post et al (1985) toxin production coincided with sensory rejection at 30°C but at 8 and 12°C sensory rejection came after toxin production. At 4°C sensory scores were at rejection

before toxin production and at 1°C they remained acceptable through day 60 where no toxin was detected.

For uninoculated channel catfish fillets stored at 4°C, psychrotrophic plate counts exceeded 7 log CFU/g before day 6 in the overwrapped packages while in the master bag (MB) and modified atmosphere (MA) this level was reached by day 9 (Cai et al. 1997). At the same temperature anaerobic counts, for inoculated and uninoculated fillets increased 5.5 and 5.3 log cycles more than initial levels in overwrapped packages and 4.3 and 5.0 log cycles more in MA and MB packages. Inoculated samples psychrotrophic counts reached spoilage by day 6 in all three packages at 4°C. In uninoculated and inoculated fillets, microbiological spoilage occurred on day 2 for overwrapped packages, on day 6 for MB, and after day 6 for MA at 10°C. Anaerobic populations increased 4.4 log cycles over the initial levels in the overwrapped, MA and MB samples by day 12 (Cai et al. 1997).

Inoculated and uninoculated samples showed no difference in relation to the number of days to spoilage. At 4°C in overwrapped packages, toxin production coincided with sensory rejection but proceeded microbiological spoilage (Cai et al. 1997). In MA packages toxin production coincided with microbiological spoilage and sensory rejection. Toxin production did not occur in the MB samples up to day 30. Sensory rejection and spoilage occurred between days 9-12.

In overwrapped fillets stored at 10°C, toxin production occurred during or before sensory rejection but after microbial spoilage. In MA packaged fillets, toxin production coincided with microbial spoilage but before and after sensory rejection. Toxin production coincided with both sensory rejection and microbial spoilage for MB samples. MB samples that were relocated from 4°C to 10°C did not produce toxin (Cai et al. 1997).

Lyver et al. (1998) and Lyver and Smith (1998) reported in cooked inoculated battered and breaded surimi nuggets packaged in air *Bacillus* spp. counts increased from 1 log CFU/g to approximately 4 log CFU/g and 6 log CFU/g at 12 and 25°C, respectively. After 28 days, the cooked surimi samples were not rejected by sensory at 12°C or 25°C in either the air or the air with oxygen absorbent packaging. Toxin did not develop at 4°C, 12°C or 25°C before day 28 in raw or cooked inoculated surimi nuggets. It was hypothesized the prevention of toxin production in the cooked surimi was caused by the presence of *Bacillus* spp. which was the only bacteria in high numbers during microbiological analysis. However, in the sterilized surimi nuggets toxin developed in air packages and air packages with oxygen absorbent materials at days 14 and 28 at 25°C and 12°C, respectively.

## 2. Food safety and regulatory concerns

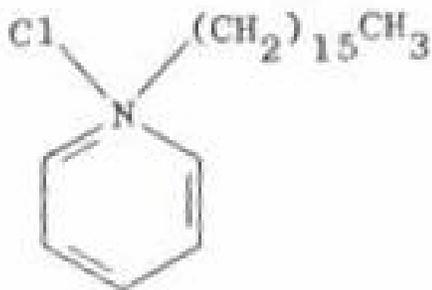
Fish inoculated with high numbers of *C. botulinum* spores have become toxic within 6-8 days during refrigerated storage when storage temperatures approach

50° F (10°C) (NACMCF, 1992). This is a concern, since during distribution and retail storage, product temperatures have been found to fluctuate between 40-50° F (4.4-10°C), (NACMCF, 1992). Kalish (1991) reported that in retail operations, only 37% of food products were stored at the recommended temperature range of 32-38°F (0°-3.3°C), with many cases averaging 44°F (6.7°C) and some as high as 55°F (13.3°C). Approximately, 2000 retail stores, including back-room storage facilities, and chill cases were checked. Also, products were also found stacked on the floor without any refrigeration. Consumers may have increased risk of pathogen growth in these products. For example, in one study, refrigerator temperatures in consumer homes ranged from 32°F to 55°F (0°C - 13.3°C) (Beard, 1991). Studies have shown that at the retail level, product rotation procedures were inadequate, as sales of product dictated product rotation frequency (Kalish, 1991). If continuous refrigerated storage of products below 3.3°C could be assured, there would not be any potential botulinum hazards with these products. However, food products have a high likelihood of being temperature abused throughout distribution and by the consumer (Eklund et al., 1982). Products and storage conditions must be carefully assessed to ensure that growth and toxin formation does not occur during the shelf life of the product.

Information is needed concerning fish species, size of spore inoculum, packaging, gas compositions, potential abuse temperatures during distribution and storage, shelf life, etc. in order to properly evaluate the safety of any

modified package (Eklund et al., 1982). Currently the FDA recommended minimum oxygen transmission rate for reduced oxygen packaged (ROP) refrigerated raw fishery products is 10,000 cc/m<sup>2</sup>/24h at 70°F. Exceptions are when time and temperature indicators are placed on each package or unless scientific data can be presented to support alternative OTRs (FDA, 2001a). The data obtained should follow the recommendations of the NACMCF for the examination of *C. botulinum* in foods (NACMCF, 1992). Additional antimicrobial treatments could be used in combination with MA and reduced OTR to achieve an extended shelf-life product without the formation of toxin.

### 3. Cetylpyridinium chloride (CPC)



#### a. Chemical description

Cetylpyridinium chloride, or 1-hexadecyl pyridinium chloride, is a quaternary ammonium compound with antimicrobial properties against many microorganisms. “It is classified

as a cationic surface-active agent and contains a cetyl radical substituted for hydrogen atom on position 1. In hydrochloric acid it forms a chloride salt. The cetyl radical renders the molecule lipophilic, contributing to the lipophilic/hydrophilic balance which is necessary for the antimicrobial activity of such quaternary nitrogenous compounds.” (FDA, 1998b). Currently it is used in some commercial mouthwashes to prevent the formation of dental plaque

(Ashley, et al, 1984; Barnes, et al. 1976; Frost and Harris, 1994). The use of CPC in spraying or dipping poultry products commercially is disclosed in a patent application (Lattin, et al. 1994). Cecure™ (Safe Foods Corp., Little Rock, AR) is a commercial formulation of CPC and has been approved for use in poultry up to 0.3g per pound (Federal Register Docket No.2002F-0181).

#### b. Mechanism of action

Antimicrobial activity is dependent on the charged cetyl radical being positioned with a bacterial cell possessing a net negative charge. This allows the hydrophilic portion of the molecule to react with the cell membrane resulting in the leakage of the cellular components, disruption of cell metabolism, prevention of growth and replication, and cell death (FDA, 1998b). At a pH of approximately 7.2, CPC is not corrosive to metal, does not add to phosphate waste, and is not a severe health hazard. The melting point for CPC (depending upon purity) is approximately 79°C (Sigma-Aldrich, 2002). This does not affect the chemical structurally therefore it will survive cooking processes including frying.

#### c. Previous studies

Most previous reports have focused on the use of CPC as an antimicrobial spray against bacterial pathogens on poultry. Yang, et al. (1998) applied a 0.5% CPC solution to pre-chill chicken carcasses and reduced *Salmonella* by 3.62 log<sub>10</sub> CFU/ml. Breen, et al. (1997) compared four levels of CPC (0.1, 0.2, 0.4, and 0.8%) and three reaction (contact) times of 1, 3, and 10 min. *Salmonella*

reductions of 0.59 to 4.91 log<sub>10</sub> CFU occurred when the bacteria were applied before the treatment. More specifically when the bacteria were applied before the treatment the average log<sub>10</sub> reductions across the three contact times were 1.05 for 0.1 CPC and 3.63 for 0.4% CPC. They also applied the bacteria after the treatment where the average log<sub>10</sub> reductions across the three contact times were 0.91 for 0.1% CPC and 2.74 for 0.4% CPC. Under the high concentration, long contact time conditions (0.8%, 10 min) CPC inhibited attachment by 4.9 log<sub>10</sub> CFU. Also, Arritt et al. (2001) reported that a 0.5% CPC spray was superior to 10% trisodium phosphate (TSP), 0.1% acidified sodium chlorite (ASC), and 0.1% cetylpyridinium chloride (CPC) for reducing the number of viable *Campylobacter jejuni* on chicken breast skin using a skin attachment model.

### III. Chapter One:

TITLE: The Effects of Modified Atmosphere Packaging on Toxin Production by  
*Clostridium botulinum* In Raw Aquacultured Summer Flounder Fillets  
(*Paralichthys dentatus*)

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cetylpyridinium chloride

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## A. Abstract

The Effects of Modified Atmosphere Packaging on Toxin Production by  
*Clostridium botulinum* in Raw Aquacultured Summer Flounder Fillets  
(*Paralichthys dentatus*)

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Packaging fishery products under vacuum (VAC) and/or modified atmosphere packaged (MAP) conditions can significantly extend the shelf life of raw refrigerated fish products. There is considerable commercial interest in marketing VAC and MAP refrigerated, (never frozen) raw fish fillets. The objective of this study was to determine if *C. botulinum* toxin development precedes microbiological spoilage in raw, refrigerated flounder fillets. Such information is important in evaluating the safety of these products.

Aquacultured flounder (*Paralichthys dentatus*) fillets were air packaged (Oxygen Transmission Rate (OTR) of 3,000 cc/m<sup>2</sup>/24h@70°F), vacuum packaged or MAP packaged in a 100% CO<sub>2</sub> atmosphere (OTR of 7.3 cc/m<sup>2</sup>/24h@70°F). Fillets were stored at either 4°C, 10°C or 21°C. Spoilage was determined on separate fillets using >10<sup>7</sup> CFU/g (initial counts were 10<sup>2</sup>-10<sup>3</sup> CFU/g) as the criteria for

spoilage. At 10°C, spoilage occurred at an average of 6, 8, and 15 days for air, vacuum and for 100% CO<sub>2</sub> packaged fish, respectively. At 4°C, spoilage occurred at an average of 13, 30, and >37 days for aerobic, vacuum and 100% CO<sub>2</sub> packages, respectively. At 21°C, spoilage occurred at an average of 2, 3, and 4 days for air, vacuum and for 100% CO<sub>2</sub> packaged fish, respectively.

Based on time to spoilage, additional samples were analyzed qualitatively for botulinum toxin using a mouse bioassay. An external inoculation of approximately 10<sup>2</sup>-10<sup>3</sup> /g non-heat shocked spores consisting of five strains of nonproteolytic *C. botulinum* was used. At 4°C, toxin formation did not occur even after 35 days for the air packaged (OTR of 3,000cc/m<sup>2</sup>/24h@70°C) fillets. The vacuum packaged and 100% CO<sub>2</sub> packaged fillets were toxic before spoilage at days 20 and 25, respectively. In the air packages stored at 10°C, toxin production occurred after spoilage at day 8, but before spoilage in the vacuum and 100% CO<sub>2</sub> packages at day 9.

Sensory evaluation of the vacuum and 100% CO<sub>2</sub> packages stored at 4°C showed toxin production occurred before microbiological spoilage and total sensory rejection. However, for air packed fillets at 10°C, toxin was present after total sensory rejection and microbiological spoilage for air packaged fillets. Toxin formation coincided with microbiological spoilage but before total sensory rejection in vacuum packaged and 100% CO<sub>2</sub> packaged fillets.

## B. Introduction

The world's current consumption of seafood is approximately 80 million metric tons with a prediction of 110 to 120 million metric tons by 2010. Technology must continue to be developed to meet increasing demand (Garrett et al., 2000).

Decreasing fishery supplies of wild flounder populations, an increased market demand particularly with the cultivation of Japanese flounder, *Paralichthys olivaceus* (Matsuoka, 1995) has resulted in increased flatfish research and production studies. In the U.S., the summer flounder (*Paralichthys dentatus*) is a highly valued fish found off the waters of the Atlantic coast from Nova Scotia to southern Florida (Rogers and Van den Avyle, 1983).

The use of MAP and VAC can increase the shelf life of refrigerated fishery products (Reddy et al., 1992). Nevertheless, there are food safety and quality issues that must be considered when marketing refrigerated foods especially with extended shelf life (Doyle, 1998). Processors should carefully evaluate and prevent hazards that may exist in refrigerated foods with an extended shelf life before marketing such products (Doyle, 1998). Microbiological concerns with refrigerated foods focus on psychrotrophic and mesophilic organisms that have an opportunity to grow during extended refrigerated storage. Several pathogens can grow at refrigerated temperatures and for some pathogens, only a few cells can cause illness when ingested (Marth, 1998). Shelf life is increased by the inhibition of aerobic spoilage bacteria, but VAC and MAP will not inhibit the growth of *Clostridium botulinum* (ICMSF, 1996). Fish inoculated with high

numbers of spores and stored under VAC or MAP conditions have become toxic within 6-8 days during refrigerated storage at temperatures of 50° F (10°C) (NACMCF, 1992). This is a concern, since in distribution and retail storage, product temperatures have been found to fluctuate between 40-50° F (4.4-10°C), (NACMCF, 1992).

Eklund (1993) identified the following conditions needed for foodborne illness from *C. botulinum* to occur: (1) the food must be contaminated with spores or vegetative cells; (2) the processing treatment must be inadequate to inactivate spores or the product is recontaminated after processing; (3) the food must support growth and toxin formation when temperatures exceed 3.3°C; and the food is consumed without cooking or after inadequate heating to inactivate preformed toxin. *Clostridium botulinum* is indigenous to the aquatic environment and fish is an excellent substrate for growth. Spores can survive cooking and the organism also can grow at temperatures as low as 3.3°C (Eklund et al., 1982; 1993) or even 3.0°C (Graham et al., 1997).

The objective of this study is to determine if aquacultured flounder can be packaged in a reduced oxygen package (ROP) to extend shelf-life without compromising safety.

## C. Materials and Methods

### 1. Flounder

Flounder (*Paralichthys dentatus*) fillets were from adult fully grown finfish at the aquaculture facility of the Virginia Seafood Agricultural Research and Extension Center, Hampton, VA. All flounder were fed the same diets under the same culture conditions, and were the same age. Fish were netted from tanks and chill killed in a cooler of ice water. They remained in the cooler until they were filleted (~10 min). Four boneless and skinless fillets were obtained from each fish (~350g total). Each fillet was rinsed quickly in tap water to remove excess blood, placed into a gallon size resealable plastic bag inside a cooler of ice and immediately returned to the lab for packaging and analysis. Once a resealable bag was full, it was removed from the cooler, drained to remove excess water and packed in ice prior to transport.

### 2. Modified Atmosphere Storage

Individual fish fillets were either packed with air (-5 in. Hg) in a package with an oxygen transmission rate (OTR) of 3,000 cc/m<sup>2</sup>/24h at 73°F (Cryovac Super L Bag, Duncan, SC.), vacuum packed (-27 in. Hg) or MAP in 100% CO<sub>2</sub> (OTR of 7.3 cc/m<sup>2</sup>/24h at 70°F). The vacuum package and the 100% CO<sub>2</sub> package film were High Barrier Deli 3-mil. composed of 0.75-gauge nylon with EVOH and 2.25-gauge polyethylene (Koch Supplies Inc., Kansas City, MO). The air and vacuum packages were packed with an Ultravac<sup>®</sup> vacuum packager (KOCH Industries, Kansas City, MO). The modified atmosphere packages were

prepared by flushing packages to their capacity with a 100% CO<sub>2</sub> gas tank (Holox Limited, Roanoke, VA.) for 4 consecutive replications. A 3-foot Nalgene hose terminated with a 5 inch, 24-gauge canula delivered the modified atmosphere. The canula was placed to one side of the package during filling and sterilized with ethanol between each package. Gas was expelled by hand from each bag three times prior to sealing. Gas was prevented from escaping by folding over the open end of the package while it was double sealed using a portable tabletop contact heat sealer (Kapack cooperation Minneapolis, MN). The final gas to product ratio was at least 6:1. Packaged fillets were stored at 4°C, 10°C or 21°C with a minimum of one-inch separation between packages to allow for respiration.

Several different temperatures were chosen in order to represent different storage scenarios. A 4°C storage temperature represents “recommended” refrigeration temperature. A 10°C storage temperature is the protocol required of the NACMSF for performing *C. botulinum* toxin testing in MAP raw fishery products. Finally, a 21°C storage temperature duplicates strict abuse conditions (e.g. a product is left unrefrigerated). A 4°C and 10°C storage temperature were used for *C. botulinum* toxin assay and CPC antimicrobial treatment in the raw flounder fillets. Garren et al. (1995) used these temperatures for *C. botulinum* toxin testing in a vacuum packaged rainbow trout fillets as well as Cai et al., (1997) in a channel catfish aerobic packages and an 80% CO<sub>2</sub>:20% N<sub>2</sub> packages. Reddy et al. (1996, 1997a, 1997b, and 1999) used 4°C, 8°C and

16°C in air, vacuum and 75% CO<sub>2</sub>: 25% N<sub>2</sub> in tilapia, catfish, salmon, and cod, respectively.

### 3. Headspace gas analysis

At 4°C, two fillets were sampled for the air and 100% CO<sub>2</sub> packages after 0, 20, 25, 30 and 35 days storage. Sampling for the 10°C aerobic occurred on days 0, 4, 6, 7, 8 and 9. At 10°C, sampling for the 100% CO<sub>2</sub> packages were on days 0, 9, 12, 15, 17 and 18. A ½ inch section of ¼ inch thick adhesive weather stripping was placed on the surface of each bag. A 30cc syringe (Becton Dickinson, Franklin Lakes, NJ) with a air valve mechanism and a 1 inch, 21-gauge needle (Becton Dickinson) was inserted into each package. The air valve was opened and the plunger was withdrawn filling the syringe to capacity with the atmosphere mixture from the package. The air valve was closed and the needle was removed. After removing the needle below the air valve, the syringe-air valve apparatus was placed into a Illinois Instruments 6600 Headspace Oxygen/Carbon Dioxide Analyzer (Ingleside, IL). The plunger was depressed to achieve the proper gas intake for the machine while the headspace composition was analyzed.

### 4. pH determination

Storage temperatures, replications and sampling days were identical to those previously mentioned in section 3. Packages were removed, cut open and a 1:1 ratio of HPLC grade water (VWR International, West Chester, PA) was added.

The samples were hand massaged and then placed in a laboratory stomacher (Seward Stomacher 400 Circulator, Thetford, Norfolk, England) for 2 min at 230 rpm. The pH was taken with an Accumet pH meter 915 (Fisher Scientific Company, St. Louis, MO) using a Symphony pH electrode (VWR).

## 5. Shelf-life study

Aerobic, anaerobic and psychrotrophic plate counts (in triplicate) were conducted for air, vacuum and 100% CO<sub>2</sub> packages after storage at 4°C, 10°C and 21°C. Sampling days were designed for 0, 75, 100, 125% of estimated spoilage. In this study, microbiological spoilage was defined as at least 7.0 log CFU/g for any plate count analysis (ICMFS, 1978). Analytical procedures followed the Food and Drug Administration Bacteriological Analytical Manual. Samples were pulverized in a laboratory stomacher (Seward Stomacher 400 Circulator) for 2 minutes at 230 rpm with a 1:4 ratio (wt/vol) of sample to Butterfield's buffer (International Bio Products, Bothell, WA). One ml of sample was serially diluted with Butterfield's buffer then plated onto Petrifilm<sup>®</sup> (3M Microbiology, St. Paul, MN) in duplicate. Films were incubated for 48h at 36°C except psychrotrophic films were incubated at 21°C for 3-4 days.

## 6. Botulinum toxin assay

### a. Spore Crop Generation

The strain of Type F 83F was obtained from the culture collection of the Department of Food Science and Technology at Virginia Polytechnic Institute and

State University (VPI&SU) and spore crops were created using biphasic procedures as described in Anellis, et. al.(1972). Five ml. of initial spore suspension was added to 20 ml of sporulation medium composed of (w/vol) 5% tryptone peptone (Difco Laboratories, Detroit, MI), 0.5% peptone (Difco) and 0.625%  $K_2HPO_4$  (Sigma). A 0.075% (w/vol) solution of  $NaHCO_3$  (Sigma) was filter sterilized into the media before inoculation. The inoculated medium was incubated for 24h at 30°C. After incubation 5 ml was removed and placed into another 20 ml of sporulation medium and incubated for 4h. This procedure was repeated for another 4h incubation. After final incubation, the entire 25ml was added to the liquid phase of a biphasic medium in a Fernbach flask and incubated at 30°C for up to 11 days. The flask was gassed with  $N_2$  for five minutes before and after inoculation. The liquid phase consisted of 0.1% w/vol. solution of yeast extract (Difco) in 250 ml. The solid phase was composed of 5% (w/vol.) tryptone peptone (Difco), 0.5% peptone (Difco), 0.125%  $K_2HPO_4$  (Sigma), 0.1% yeast extract (Difco), 3% agar (Difco) and 1 L of sterile distilled water. Phases were autoclaved then cooled in a 30°C water bath then gassed with  $N_2$  for five minutes before and after inoculation with one ml of initial spore crop. Spores were harvested by removing 10 ml of liquid phase and combining it with 10 ml of sterile Butterfield's buffer (International Bio Products). The subsequent suspension was centrifuged using a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments, Wilmington, DE). Spore suspensions were spun for 20 min. at 4°C with a Sorvall SS-34 rotor head at 2420 x g. Supernatant was removed and the spores were re-suspended with 10

ml of sterile Butterfield's buffer (International Bio Products) and centrifuged again. This process was repeated three consecutive times before 10 ml allocations of washed spores were placed into sterile glass vials and stored in an -80°C freezer until needed.

#### b. Spore crop enumeration

Five strains of nonproteolytic *C. botulinum*; 17 Type B, Beluga, Minnesota, Alaska Type E and 83 Type F were obtained from the culture collection of the Department of Food Science and Technology at VPI&SU. Spore crops were enumerated using serial dilutions in sterile Butterfield's buffer (International Bio Products) and anaerobically incubated in trypticase peptone glucose yeast extract (TPGY) roll tubes. Tubes were incubated for up to ten days at 30°C. Final equivalent dilutions were made with sterile Butterfield's buffer (International Bio Products) to produce a 5 strain cocktail in a concentration of  $10^2$ - $10^3$ /ml then stored in vials at -80°C until needed. Identifying a rainbow iridescent or pearly reaction on anaerobically incubated McClung-Toabe Egg Yolk Agar at 30°C confirmed purity of cultures (Eklund, 2002).

#### c. Spore inoculation

Shaking the cultures at room temperature with sterilized glass beads was used to break apart any coagulated spores during thawing of the frozen 5 strain cocktail (Eklund, 2002). An external inoculation of approximately  $10^2$ - $10^3$  non-heat shocked spores per gram was applied by dropwise addition with a 1cc tuberculin

syringe (Becton Dickinson) on the outside (bone side) of each fillet. The distribution was applied as uniformly as possible. The droplets were spread with a sterile L-shaped glass rod into a thin layer (NACMCF, 1992). In between inoculations, the thawed cocktail was stored in icepacks to prevent the mixture from warming to room temperature. Control inoculations were performed using sterile distilled water. Five to seven replications of all temperature and packaging combinations were performed.

#### d. Toxin determination

Detection of *C. botulinum* toxin followed the general protocol as outlined in the Food and Drug Administration Bacteriological Analytical Manual (FDA, 1998c). Samples were pulverized in a laboratory stomacher for 2 minutes at 230 rpm with a 1:1 ratio of gel phosphate buffer. One ml of sample was serially diluted into Butterfield's buffer (International Bio Products) then plated onto Petrifilm<sup>®</sup> (3M Microbiology) in duplicate. Twenty ml of slurry were removed and centrifuged (DuPont Instruments) for 10 min. at 11,951x g. Supernatant was filtered using a sterile 25mm diameter .2 µm pore size syringe tip filter (Acrodisk<sup>®</sup>, Gelman Laboratory, East Hills, NY). Samples were typically frozen (-20°C) at this point. Within a week the following steps were taken after the samples were thawed out at 4°C. The freezing process as well as dilution and filtering is also used to reduce the event of non-specific death in the mouse biotoxin assay in severely spoiled foods due to the presence of additional toxic compounds (ICMSF, 1996). Trypsin solution (0.2 ml) was added to two 1.8 ml aliquot of supernatant for each

sample. (Trypsin was prepared with 0.5g Difco 1:250 trypsin added to 10 ml sterile distilled water). The subsequent treated solutions were incubated at 35-37°C with gentle agitation for 1 hour. One of these aliquots was heated to 100°C for thirty minutes as a control. One ml of trypsinized, non-trypsinized, and heated trypsinized supernatant was loaded into three separate tuberculin syringes (Becton Dickenson. Five 15-18g male mice (*Mus musculus*) (Harlan Sprague Dawley) (2 trypsinized, 2 non-trypsinized, 1 control) were given an intraperitoneal injection with 0.5 ml of supernatant. Mice were observed periodically for symptoms of botulism for 48 h. Botulism signs typically began within the first 24 h with ruffling of fur, followed in sequence by labored breathing, weakness of limbs, total paralysis with gasping for breath, and finally death due to respiratory failure. Any mice not succumbing to the effects of the toxin were sacrificed with CO<sub>2</sub> gas. Carcasses were autoclaved to denature any toxin then frozen to await disposal by university agents. This project was approved by the Virginia Tech Animal Care Committee, which complies with the Animal Welfare Act and has been approved by the American Association for the Accreditation of Laboratory Animal Care (ACC proposal # 02-046-FST).

e. Toxin study aerobic plate count

Storage temperatures, replications and sampling days were identical to those previously mentioned in section 3. Samples were pulverized in a laboratory stomacher (Seward) for 2 minutes at 230 rpm with a 1:1 ratio of gel phosphate buffer composed of 2g gelatin (Oxoid Ltd., Basingstoke, Hampshire, England), 4g

Na<sub>2</sub>HPO<sub>4</sub> (Sigma Chemical Corporation, St. Louis, MO) and 1 L of sterile distilled water pH adjusted to 6.2 with 1N NaOH (Sigma) or HCl (Sigma). One milliliter of sample was serially diluted into Butterfield's buffer (International Bio Products) then plated onto Petrifilm<sup>®</sup> (3M Microbiology) in duplicate. Films were incubated for 48h at 36°C.

## 7. Sensory Evaluation

Sensory analysis was performed in conjunction with the microbiological analysis and toxin detection. The fillets were held frozen (-80°C) until the day of the sensory analysis. Samples were thawed at room temperature, allowed to equilibrate at 4°C and presented as whole fillets to three untrained panelists, who were vaccinated against *C. botulinum* toxin. The panelists held packaged fish samples a minimum distance of 8 in. from the nose. If an odor could not be identified, the package was brought closer to the nose. A 5-point hedonic scale was used to determine characteristics of odor and appearance (1-little or no odor; 5-putrid, trash like odor, musty off odor). Normal rejection was given a numerical value of 4 while a score of a 5 was reserved for a product that had deteriorated to the point of absolute rejection. (i.e., if the consumer had purchased it and were starving to death they would not consider consuming it) (Post, et. al.,1985). For sensory rating definitions see Table 1.

## 8. CPC

### a. Preparation

Cetylpyridinium chloride (CPC)(Sigma) solutions were prepared out in sterile 25 ml volumetric flasks sterile distilled water to create a wt./vol. solution. Solutions were prepared in an aqueous 10 times concentration. A second set of CPC solutions was derived using propylene glycol (Sigma) in an effort to prevent the crystallization of the chemical at colder temperatures (Waldrop, 2003). Final dilutions of these concentrations were made using sterile distilled water. For flounder application a sterile 100 ml volumetric flask was used to make a 15% concentration.

### b. Determination of Experimental CPC level

Preliminary studies determining the minimum concentration of CPC required to inhibit *C. botulinum* were necessary due to the absence of literature on CPC and *C. botulinum* interaction. A series of tubes was used with 9 ml Brain Heart Infusion agar (BHI) and 1 ml concentrated CPC in a vol/vol solution to create different concentrations of aqueous CPC first beginning at 1.0% and increasing by 0.5% increments until a 4.0% solution was reached. Four replications were performed using both non-heat shocked and heat shocked spores. Heat shocking was performed by placing the cocktail in a 60°C water bath for ten minutes. The time period began once the temperature of the cocktail reached water bath temperature. Approximately  $10^5$  CFU/ml of a four strain *C. botulinum* spore cocktail (Alaska, Beluga, Minnesota, and 17 B) was added to each solution

and incubated at 30°C for 13 days. Ten ml of incubated solution was added to 10 ml of sterile gel-phosphate buffer and placed into sterilized 50ml centrifuge tubes. Centrifuge tubes were immediately placed in a 4°C Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments, Wilmington, DE) and spun for 10 min. at 11,951xg.

c. Spore inoculation CPC

An external inoculum of approximately  $10^3$ - $10^4$  spores per gram was used to achieve a final inoculation of  $10^2$ - $10^3$  spores per gram after being dipped. Confirmation of this procedure was determined by inoculating control fillets with increasing concentrations of spores and providing a 15 second immersion in sterile distilled water until the appropriate target amount of inoculated spores was achieved. Spore counts on fillets were enumerated using serial dilutions in sterile Butterfield's buffer (International Bio Products) and anaerobically incubated in TPGY roll tubes for up to 10 days at 30°C. Control inoculations were performed using sterile distilled water.

d. CPC application

One hundred ml of a 15% CPC solution in propylene glycol (wt/vol) was added to 900 ml of sterile distilled water to achieve a final concentration of 1.5%. The solution was placed into a sterilized ceramic lined tray. Each individual fillet was immersed in this solution for 15 seconds on a sterilized thin metal grate, allowed to drain for 15 seconds, then placed into a 4°C incubator on an ethanol sterilized

foam tray for two minutes. This allowed any fluid from the inoculation to be absorbed and the spores to settle on the tissue in an attempt to prevent them from being rinsed off during the dipping procedure. After the two-minute period the fillets were packaged in the same manner as previously described. CPC treated samples were placed in an ice water bath after centrifugation and supernatant isolation for 30 min. to facilitate the crystallization of the chemical. Chilled syringes and filters that had been stored in the freezer were also used keep the chemical out of solution to ensure total removal of CPC, which proved to cause non-specific death of the mice used in the biotoxin assay.

## **D. Results and Discussion**

### 1. Aquacultured Flounder

#### a. Shelf-life

Initial APCs for flounder fillets were between 100 and 1000 CFU/g. At 21°C spoilage occurred at an average of 2, 3, and 4 days for air, vacuum and 100% CO<sub>2</sub> packages, respectively. At 10°C spoilage occurred at an average of 5, 9, and 15 days for air, vacuum and 100% CO<sub>2</sub> packaged fillets, respectively. At 4°C, spoilage occurred on average a 15, 22, >37 days for air, vacuum and 100% CO<sub>2</sub> packaged fillets, respectively (Table 2).

Vacuum packaging increases shelf-life by approximately 4 and 7 days 10°C and 4°C. The 100% CO<sub>2</sub> flushed fillets had shelf-life extensions of 22, 10 and 2 days

compared with the air packaged fillets and 15, 6 and 1 days compared with the vacuum packaged fillets at 4°C, 10°C and 21°C, respectively (Table 2). It required 15 days for spoilage in the air packaged fillets at 4°C and the same number of days at 10°C for the 100% CO<sub>2</sub> packaged fillets.

There was no significant microbiological difference (>1 log CFU/g) between aerobic, anaerobic and psychrotrophic plate counts in any of the three package types. (Table 2) Anaerobic counts were more comparable to the aerobic and psychrotrophic plate counts in the vacuum package and 100% CO<sub>2</sub> (Table2). Results suggest a dramatic extension of shelf life using modified atmosphere and refrigeration. However the initial microbial counts were low making the shelf-life extension longer than other studies with higher initial microbial counts. Average soluble protein and crude fat on a dry matter basis for triplicate samples was 30.5% and 11.2%.

#### b. Headspace gas

The average gas to product ratio (G/P) for air and 100% CO<sub>2</sub> packages to aquacultured flounder were 6:1. In uninoculated air packaged fillets at 10°C, O<sub>2</sub> concentrations initially were 21.3% but reduced to <1.0% by day 6 and to <0.25% by day 7, where the concentrations remained until termination of the study at day 9. Initial concentrations of CO<sub>2</sub> were 0.8% but increased to 16.6% by day 6 and peaked on day 8 at 21.1% only to decline to 18.5% on the final 9<sup>th</sup> day (Figure 1).

For 100% CO<sub>2</sub> packaged fillets, the concentration of CO<sub>2</sub> at 10°C and 4°C remained at 100% until termination of the study on day 18 and 35, respectively. Conversely, O<sub>2</sub> concentrations remained negligible at <1.0% throughout the duration of the study. Initially there was a small decrease in the total volume of the 100% CO<sub>2</sub> packages caused by gas being absorbed into the tissue particularly at 4°C. This partial deflation was also described by Garcia et al. (1987) CO<sub>2</sub> in both 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub> (MA) packages at different temperature combinations.

On day 20 at 4°C, O<sub>2</sub> concentrations in uninoculated air packaged fillets were <1.0% from an initial concentration of 21.3%. The concentration fluctuated very little for the remainder of the study, which ended on day 35. The CO<sub>2</sub> concentration for the same packages increased from 0.8% to 21.3% by day 20 and achieved a concentration of 22.3% by day 35. Changes in the composition of headspace gases were probably a direct result of post-mortem metabolic and microbial processes.

Similarly, Reddy et al. (1996) reported O<sub>2</sub> concentrations dramatically decreased for tilapia fillets, aquacultured salmon (Reddy et al. 1997a), pond raised catfish (Reddy et al. 1997b) and cod (Reddy et al. 1999). Headspace CO<sub>2</sub> concentrations increased from <1.0% to >15.0% at spoilage in all four studies.

Concentrations of CO<sub>2</sub> and O<sub>2</sub> for channel catfish packaged in MA with 80:20 CO<sub>2</sub>:O<sub>2</sub> remained relatively constant (Cai et al. 1997). In modified atmosphere (MA) (75:25 CO<sub>2</sub>:N<sub>2</sub>) for tilapia fillets (Reddy et al. 1996), aquacultured salmon (Reddy et al. 1997b), pond raised catfish (Reddy et al. 1997a) and cod (Reddy et al. 1999) CO<sub>2</sub> day zero concentration was 75%. It initially decreased and then increased to >58% by the day of spoilage. Garcia et al. (1987) also reported an initial decrease in headspace concentration for CO<sub>2</sub> in both 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub> (MA) packages at every temperature combination.

Conversely, Stier et al. (1981) reported that for salmon fillets stored under 60:25:15 CO<sub>2</sub>:O<sub>2</sub>:N<sub>2</sub>, CO<sub>2</sub> concentrations declined from approximately 60% to approximately 20% however the O<sub>2</sub> concentrations reacted differently than previously mentioned articles increasing to approximately 30% then decreasing to approximately 10%.

Dufresne et al. 2000 in rainbow trout packages with OTRs of 4,371, 4923, and 10,043 cc/m<sup>2</sup>/24h at 24°C, 0% RH backflushed with 85:15 CO<sub>2</sub>:N<sub>2</sub> there was a headspace decrease in CO<sub>2</sub> and an increase in O<sub>2</sub>. Headspace analysis on packaging having an OTR of 11.6cc/m<sup>2</sup>/24h at 24°C, 0% RH revealed a headspace decrease in O<sub>2</sub> and increase in CO<sub>2</sub>.

### c. pH

Initial pH values in uninoculated flounder fillets at 4 and 10°C for both air and 100% CO<sub>2</sub> packages averaged 6.76. At 10°C, pH slowly declined in air packaged fillets to 6.16 by the final day 9, coinciding with absolute rejection. Normal rejection occurred on day 7 with an average pH value of 6.27. For 100% CO<sub>2</sub> packaged fillets, pH decreased to 6.03 by day 9 where the values generally remained until the termination of the study on day 18. Neither absolute nor normal sensory rejection occurred for these packages by the final day.

Initial sampling for air packaged fillets at 4°C was at day 20 with an average pH value of 6.35. Subsequent analyses were all below the initial pH of 6.76. Fillets packaged in 100% CO<sub>2</sub> were initially sampled at day 20 with an average pH value of 6.00. The pH increased approximately 0.1 every 5 days. Values reached 6.3 by termination of the study at day 35 where normal sensory rejection occurred. Absolute sensory rejection never occurred.

Parkin et al. (1982) found a decrease in pH for MA rockfish fillets packaged in 80:20 CO<sub>2</sub>:O<sub>2</sub>. In 1999, Reddy et al. (1999) and Cai et al (1997) found no change in the pH of the cod and channel catfish fillets stored at any temperature or atmosphere combination, respectively. Additionally, Dufresne et al. (2000) reported no significant changes in pH in rainbow trout fillets due to the buffering capacity of the fish proteins. Similarly, there was no initial decrease in pH in salmon fillets stored under 100% CO<sub>2</sub> (Garcia et al. 1987).

Conversely, Reddy et al. (1997a) reported that the initial average surface pH for catfish fillets on the day of spoilage in air packages at 4°C and 16°C increased, but decreased at 8°C. In MA packages, pH decreased then increased above initial levels by the day of spoilage. For all combinations of package and temperature for aquacultured tilapia (Reddy et al. 1996) and salmon fillets (Reddy et al. 1997b) surface pH decreased during early storage and increased later. The initial pH decrease was assumed to be caused by the saturation of CO<sub>2</sub> into the tissue of the fillet during early storage. However, for all atmosphere combinations and types of species, pH values ranged from 6.12 to 7.04. In uninoculated air packaged salmon fillets, pH increased by 0.05-0.12 at 4°C, 8°C and 16°C.

The range of pH values is consistent with those reported in the literature. Differences in initial pH values and final pH values may be attributed to different species, surface pH versus internal pH, packaging atmospheres, relative fish freshness, fish stress (e.g., amount of lactic acid in the musculature at time of death), etc. The results indicate that pH is a poor indicator of microbial spoilage, time to sensory rejection and/or toxin production.

#### d. Toxin production with relation to sensory rejection

Toxin production and absolute sensory rejection in inoculated flounder fillets stored at 10°C in air packages occurred on day 8, but only one out of 7 samples

were toxic. In the vacuum packaged fillets, two out of 5 samples were toxic at day 9, but normal rejection was identified by one of three panelists. On day 9, 4 out of 5 samples in 100% CO<sub>2</sub> were toxic. Only one panelist found normal rejection on one of these samples. Complete sensory scores for appearance and odor and corresponding APCs are in Table 3.

Post et al. (1985) found at all temperature and atmosphere combinations flounder fillets became absolutely spoiled before toxin production. Cod fillets developed toxin before absolute rejection in all temperature and atmosphere combinations except 90:8:2 CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> packages at 26°C, air packages at 12°C, air and 100% N<sub>2</sub> packages at 8°C. Toxin production coincided with absolute sensory rejection in the 26°C air packages. For whiting fillets, only air packages at 8°C were spoiled before toxin production. Spoilage coincided with toxin production at 26°C and 8°C for vacuum and 100% N<sub>2</sub> packages. All other package and temperature combinations became toxic before absolute spoilage.

Conversely, in cod fillets (Reddy et al. 1999), crawfish tails (Lyon and Reddmann, 2000), and trout fillets (Dufresne et al. 2000), sensory rejection proceeded toxin production for every temperature and package combination. However, Dufresne (2000) reported that toxigenesis in smoked trout fillets coincided with sensory rejection at some temperatures, OTRs, and atmosphere combinations.

e. Toxin production with relation to microbiological count and sensory rejection

Inoculated flounder fillets stored at 10°C in air packages did not form toxin until day 8 and day 9. On day 8 one sample out of 7 was toxic and on day 9 one out of 5 was toxic. Although mean appearance scores remained acceptable odor scores were 5.0 with an APC of 8.3 CFU/g on day 8. Both microbial spoilage and absolute sensory rejection occurred before toxin formation (Table 3).

Vacuum packaged samples were toxic as early as day 9 with two of 5 samples being toxic with a corresponding mean odor score of 2.7, an appearance score of 1.0, and APCs of 7.8 log CFU/g. Samples were also toxic on days 10 and 11 with 4 of 5 and 5 of 7 positive samples, respectively. Mean sensory scores, appearance scores and APC for days 10 were 3.3, 1.3 and 8.2 log CFU/g. On day 11, they were 4.2, 1.5 and 8.1 log CFU/g. Although APCs were equivalent to microbiological spoilage levels, sensory scores were not considered rejection level. Day 11 sensory scores were at normal rejection, but toxin was present at days 9 and 10 (Table 3).

Samples packaged under 100% CO<sub>2</sub> developed toxin as early as day 9 (4 out of 5 packages were toxic). Toxin was also detected on day 12 with two out of 5 samples being positive. For days 15, 17 and 18 toxin was present in 4, 4 and 5 samples out of 7, respectively with mean odor scores being ≤2.0, appearance scores of less than 1.8 and APC of ~7 log CFU/g. Again APC indicated spoilage

but toxin development occurred before sensory rejection. Table 3 summarizes the data for these three packaging conditions with inoculated flounder fillets stored at 10°C.

After 35 days of storage at 4°C, aerobic packaged fillets did not form toxin. Vacuum packaged fillets developed toxin on day 20 with two of 5 packages being positive and again on days 25, 30 and 35 with 1, 2 and 2 of 7 samples positive, respectively. Inoculated flounder fillets packaged in 100% CO<sub>2</sub> were toxic at days 25, 30 and 35 with one sample out of 7 at each sample day containing toxin. Table 4 summarizes the data for these three packaging conditions with inoculated flounder fillets at 4°C.

Aerobic plate counts are a poor indicator of toxin production in inoculated aquaculture flounder fillets packaged in air, vacuum or 100% CO<sub>2</sub>. There was no correlation found between APCs on days fillets were toxic. For every package and temperature combination, only in the 10°C air packages did sensory rejection occur before toxin formation. In all other temperature package combinations toxin production proceeded sensory rejection (Table 5).

Garcia et al. 1987 packaged salmon fillets in VAC and MA packages with initial aerobic counts of 2.48 log CFU/g fillet increasing to 7.7-8.7 log CFU/g. Using the same sensory scale as Post et al (1985), toxin production coincided with sensory rejection at 30°C but at 8°C and 12°C sensory rejection came after toxin

production. At 4°C sensory scores indicated rejection before toxin production. At 1°C sensory scores remained acceptable through day 60 where no toxin was detected.

Inoculated shrimp at 4°C spoiled when microbial counts were approximately 6 log CFU/g but no toxin was detected in either package (Garren et al., 1994). At 10°C sensory rejection occurred before microbial counts exceeded 6 log CFU/g but coincided with toxin production. Additionally, Garren et al. (1995) inoculated aquacultured rainbow trout and at 10°C toxin developed after spoilage. Psychrotrophic counts were ~6 log CFU/g and off-odors were present.

Total plate counts for packaged salmon fillets and fillet sandwiches comprised of two fillets at 4.4°C were 9 log CFU/g at time absolute sensory rejection (Stier et al. 1981). Toxin was not detected in either type of packaged fillet for any temperature combination. Toxin production coincided with sensory rejection in the MA packaged fillets and toxin production preceded spoilage in the air packaged fillets at 22.2°C. Microbial counts were approximately 9 log CFU/g for fillets in air and MA.

For aquacultured tilapia, catfish and salmon packaged in MA, sensory rejection preceded toxin production in all scenarios except salmon stored at 8°C, tilapia at 16°C and catfish at 16°C when toxin production coincided with sensory rejection. Toxin production, in the salmon stored at 16°C, preceded sensory rejection. In

all cases microbial counts were  $>8.0$  log CFU/g (Reddy et al. 1996, 1997a, 1997b, 1999).

Cai et al. (1997), reported inoculated and uninoculated samples spoiled at the same rate. At 4°C in overwrapped packaged samples, toxin production coincided with sensory rejection but proceeded microbiological spoilage. In MA packaged samples, toxin production coincided with microbiological spoilage and sensory rejection. Toxin production did not occur in the MB (MB- individual overwrap then MA of 5-7 fillets) samples up to day 30. Sensory rejection and spoilage occurred between days 9-12.

At 10°C toxin production occurred in overwrapped packaged samples during or before sensory rejection but after microbial spoilage. In MA packaged fillets, toxin production coincided with microbial spoilage but before and after sensory rejection. Toxin production coincided with both sensory rejection and microbial spoilage for MB samples. MB samples relocated from 4°C to 10°C did not produce toxin.

f. Toxin production with relation to microbiological count, sensory rejection and added CPC.

Inoculated flounder fillets dipped in 1.5% wt/vol CPC stored at 10°C in vacuum packages did not have toxin until days 9, 11 and 19. On day 9 only one out of two samples was toxic. On days 10 and 11, both samples were toxic. In 100%

CO<sub>2</sub> packaged fillets toxin developed on days 15, 17 and 18 whereby one out of two samples were toxic each day (Table 6).

With samples dipped in 1.5% CPC/propylene glycol wt/vol vacuum packaged fillets were toxic on the first day of sampling (day 12) and again on day 15. Both samples were toxic with a corresponding mean odor sensory score of 4.8, appearance scores of 1.7 and 2.0, and an APCs of 7.6 and 7.9 log CFU/g, respectively. Packages flushed with 100% CO<sub>2</sub> developed toxin at day 15 with 2 out of 2 packages being toxic. Mean odor scores were 2.7, appearance score was 1.5 and APC was 6.8 log CFU/g. Microbiological counts did not indicate spoilage before toxin development or absolute sensory rejection for either chemical combination or packaging type (Table 6).

There were no previous studies on the effect of CPC on inhibition of toxin production from *C. botulinum*. In a study with the same cocktail mixture of organisms at a level of 5.0 log CFU/g at 30°C in BHI broth CPC inhibited toxin production at all concentrations from 1.0% to 4.0%. Unfortunately, this chemical begins to crystallize at 12°C (Waldroup, 2002) preventing it from inhibiting botulinal growth. Considering seafood is stored at colder temperatures this chemical will not be useful unless it can be modified to remain in solution without losing its antimicrobial properties.

## 2. Safety of aquacultured flounder

This research and previously published studies had variations in the OTR, atmospheres, fish species, G/P ratio, storage temperatures, sampling times, length of study, initial sampling days, spore strain, etc. Additionally, the low initial microbiological counts of aquacultured flounder fillets resulted in an extended shelf-life compared with other research that used fillets with higher initial microbial counts. Due to these factors direct correlation between this research and others is difficult. However, some general observations can be made. *C. botulinum* responds differently on various fish species. Some species of fish are more inclined to become toxic than other species. It has been suggested that fat and protein content may play a role (Reddy et al. 1999) in addition to natural microbial flora and atmosphere. Also, *C. botulinum* is sensitive to microenvironment. Due to the potency of the toxin only a small number of spores are sufficient to cause illness. If these spores are subjected to conditions conducive to growth on the microscopic level germination and subsequent toxin formation will occur no matter what the package environment, microbial count or pH is on the macroscopic level.

In aquacultured flounder fillets stored at both 4°C and 10°C in air, vacuum and 100% CO<sub>2</sub> packages, only the air packaged fillets failed to produce toxin at 4°C. The probability of toxin production in vacuum and 100% CO<sub>2</sub> packaged fillets with an OTR 7.3 cc/m<sup>2</sup>/24h at 70°F on this species is high. The probability of toxicity for air packaged with OTR of 3000 cc/m<sup>2</sup>/24h at 70°F for this species is

low. At 10°C toxin developed in the air packaged fillets and spoilage coincided with toxicity on the 7<sup>th</sup> replication. If the protocol by NACMCF (i.e., only five replications) were followed toxicity would not have been reported. Temperature remains the most important factor for controlling *C. botulinum*. This study indicates that under certain conditions *C. botulinum* can grow at 4°C. Also, the recommended OTR of 10,000 cc/m<sup>2</sup>/24h at 70°F for ROP refrigerated raw fish should be considered high based on this study for this species, as toxin was not formed before spoilage in packages with an OTR of 3000 cc/m<sup>2</sup>/24h at 70°C.

## E. Tables and Figures

**Table 1: Sensory rating definitions for aquacultured flounder fillets**

Rating	Appearance	Odor
1	Flesh firm, totally translucent to totally opaque, color light to dark varying shades of pink	Little or no odor
2	Flesh firm, slight darkening in color	Slight fishy odor
3	Flesh firm, off colors evident (yellow, green, gray)	Fishy or slight off-odor, not objectionable
4	Tissue deterioration evident, definite darkening of color	Noticeable odor, strong fish of off odor
5	Definite tissue breakdown, flesh dark	Putrid, trash-like odor, strong musty odor

(variation of table in Post et al., 1985)

**Table 2: Average number of days to microbiological spoilage<sup>a</sup> determined by microbial plate count for aquacultured flounder**

Atmosphere	Storage Temperature (°C)	Aerobic plate count	Anaerobic plate count	Psychrotrophic plate count
Air <sup>b</sup>	4	15	16	15
	10	5	5	4
	21	2	2	2
Vacuum <sup>c</sup>	4	>22	>22	>22
	10	9	9	9
	21	3	2	3
100% CO <sub>2</sub> <sup>c</sup>	4	>37	>37	>37
	10	15	15	15
	21	4	4	4

<sup>a</sup> >10<sup>7</sup> log CFU/g, n=3

<sup>b</sup> OTR 3000 cc/m<sup>2</sup>/24h at 73°F

<sup>c</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

**Table 3: Positive replications out of total replications for each time and atmosphere combination with corresponding sensorial scores and aerobic plate count<sup>a</sup> in flounder stored at 10°C**

Atmosphere	Days Storage	Toxin Detected <sup>b</sup>	Mean Odor Score	Mean Appearance Score	Mean APC Log CFU/g
Air <sup>c</sup>	4	0/5			
	6	0/5			
	7	0/5			
	8	1/7	5.0	3.0	8.3
	9	1/5			
Vacuum <sup>d</sup>	7	0/5			
	9 <sup>f</sup>	2/5	2.7	1.0	7.8
	10 <sup>e</sup>	4/5	3.3	1.3	8.2
	11 <sup>e</sup>	5/7	4.2	1.5	8.1
100% CO <sub>2</sub> <sup>d</sup>	9	4/5	2.0	1.7	7.8
	12	2/5			
	15 <sup>e</sup>	4/7	1.7	1.0	7.3
	17 <sup>f</sup>	4/7	2.0	1.0	7.0
	18 <sup>e</sup>	5/7	1.5	1.8	6.8

<sup>a</sup> only toxic samples were used to determine sensorial and APC means

<sup>b</sup> Samples positive/Total number of samples

<sup>c</sup> OTR 3000 cc/m<sup>2</sup>/24h at 73°F

<sup>d</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

<sup>e</sup> only 2 toxic samples were used to determine sensorial and APC means

<sup>f</sup> only 1 toxic sample was used to determine sensorial and APC mean

**Table 4: Positive replications out of total replications<sup>a</sup> for each time and atmosphere combination for flounder samples stored at 4°C**

Atmosphere	Sample Days			
	20	25	30	35
Air <sup>b</sup>	0/7	0/5	0/5	0/5
Vacuum <sup>c</sup>	2/5	1/7	2/7	2/7
100% CO <sub>2</sub> <sup>c</sup>	0/5	1/7	1/7	1/7

<sup>a</sup> Samples positive/Total number of samples

<sup>b</sup> OTR 3000 cc/m<sup>2</sup>/24h at 73°F

<sup>c</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

**Table 5: Sensory panel rejection and microbial spoilage determination at the time that toxin was first detected for raw aquacultured flounder stored under different atmospheres and incubation temperatures.**

	Microbial spoilage (APC > 10 <sup>7</sup> /CFU/gm)	No microbial spoilage (APC < 10 <sup>7</sup> /CFU/gm)
Rejection by sensory panel	10°C, aerobic	
Not rejected by sensory panel	10°C, vacuum 10°C, CO <sub>2</sub>	4°C, vacuum <sup>a</sup> 4°C, CO <sub>2</sub> <sup>a</sup>

<sup>a</sup> Sensory panel evaluation did not occur at the time that toxin was first detected. Spoilage determination based on initial shelf life study with uninoculated samples.

**Table 6: Effect of Cetylpyridinium Chloride on time to toxin detection with relation to sensorial scores and aerobic plate count for vacuum and 100% CO<sub>2</sub> packages<sup>d</sup> for flounder stored at 10°C**

Treatment	Atmosphere	Days Storage	Toxin Detected <sup>c</sup>	Mean Odor Score	Mean Appearance Score	Mean APC Log CFU/g
CPC <sup>a</sup>	Vacuum	7	0/2			
		9	1/2			
		10	2/2			
		11	2/2			
	100% CO <sub>2</sub>	12	0/2			
		15	1/2			
		17	1/2			
		18	1/2			
CPC <sup>a</sup> + PG <sup>b</sup>	Vacuum	12	2/2	4.8	1.7	7.6
		15	2/2	4.8	2.0	7.9
	100% CO <sub>2</sub>	12	0/2	2.0	1.7	7.7
		15	2/2	2.7	1.5	6.8
		17	0/2	2.7	1.8	7.4
		18	0/2	2.8	1.5	7.8

<sup>a</sup> Cetylpyridinium chloride (1.5%)

<sup>b</sup> Propylene glycol

<sup>c</sup> Samples positive/Total number of samples

<sup>d</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

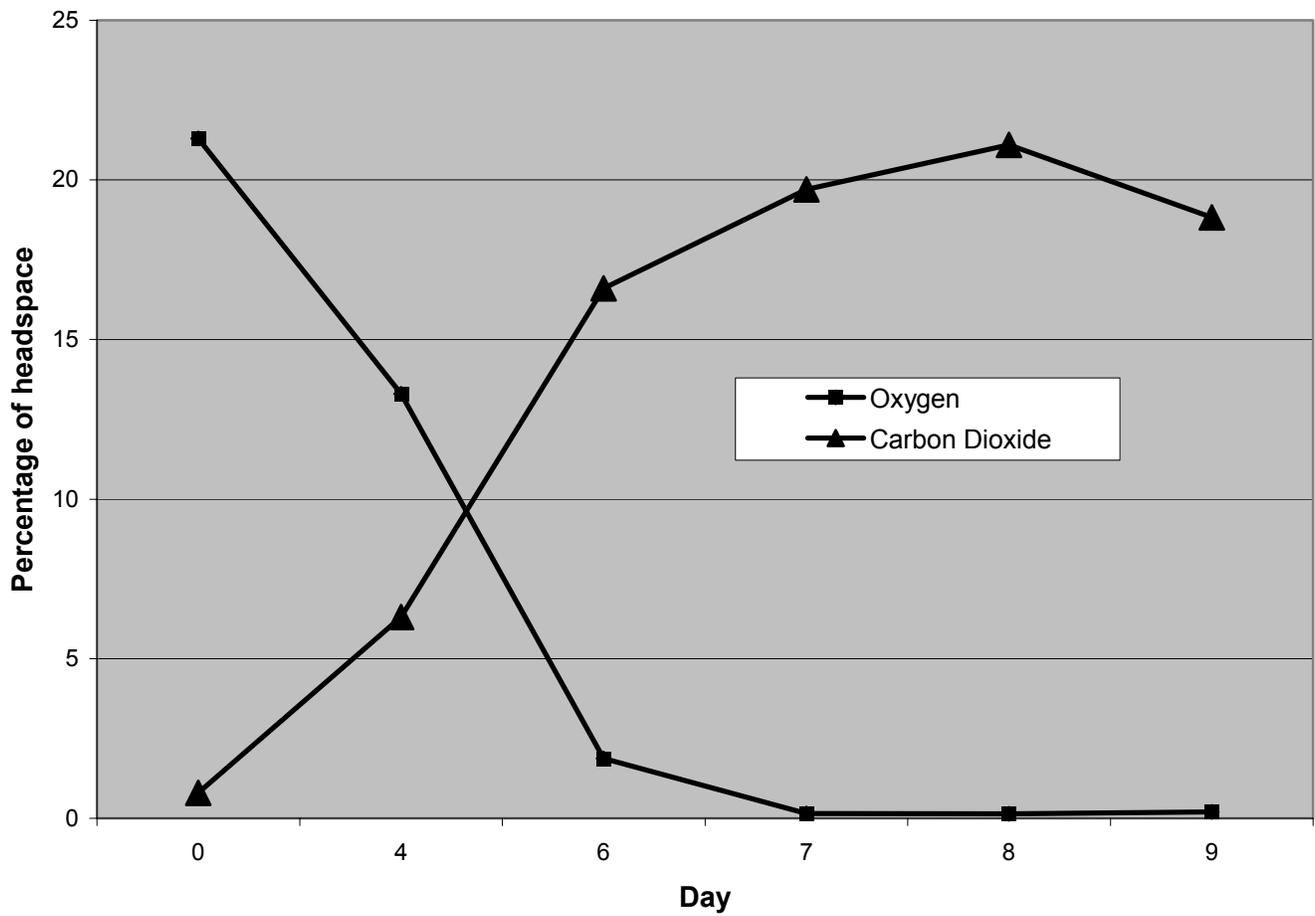


Figure 1: Aerobic package<sup>a</sup> headspace gas analysis at 10°C for aquacultured flounder

<sup>a</sup> OTR 3000 cc/m<sup>2</sup>/24h at 70°F

#### **IV. Chapter Two:**

TITLE: The Effects of Modified Atmosphere Packaging on Toxin Production by *Clostridium botulinum* in Fully Cooked Breaded and Battered Alaskan Pollock (*Theragra chalcogramma*)

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## A. Abstract

The Effects of Modified Atmosphere Packaging on Toxin Production by  
Clostridium botulinum in Fully Cooked Breaded and Battered Alaskan Pollock  
(Theragra chalcogramma)

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Packaging products under vacuum (VAC) and/or modified atmosphere packaged (MAP) conditions can significantly extend the shelf life of fish products.

Prevention of toxin production by *Clostridium botulinum* is essential for processors of VAC and MAP refrigerated fishery products. Since, there is commercial interest in marketing VAC and MAP refrigerated, value added products the objective of this study was to determine if *C. botulinum* toxin development precedes microbiological spoilage and sensory rejection in fully cooked breaded and battered Alaskan pollock.

Fully cooked breaded and battered Alaskan pollock (*Theragra chalcogramma*) were air packed (Oxygen Transmission Rate (OTR) of 6,000 cc/m<sup>2</sup>/24h@70°F for pollock), vacuum packed or MAP packaged in a 100% CO<sub>2</sub> atmosphere (OTR of 7.3 cc/m<sup>2</sup>/24h@70°F). Pollock portions were stored at either 8 or 12°C.

Spoilage was determined on separate samples using microbiological criteria of bacterial counts  $>10^7$  CFU/g (initial counts were  $10^2$ - $10^3$  CFU/g). Based on the time to spoilage, additional samples were analyzed qualitatively for botulinal toxin using a mouse bioassay. An external inoculation of approximately  $10^2$ - $10^3$  non-heat shocked spores per gram of a five strains mixture of nonproteolytic *C. botulinum* was used.

Microbiological spoilage of Alaskan pollock portions did not occur in any temperature or package combination in 35 days with the exception of days 30 and 35 in air packed portions at both 8 and 12°C. Toxin was not present in the air and 100% CO<sub>2</sub> packed samples at 8°C and the 100% CO<sub>2</sub> packed samples at 12°C after 35 days. However, air packed portions at 12°C first produced toxin at day 25, but toxicity occurred after absolute sensory rejection but before microbial spoilage. The vacuum packed portions first formed toxin at day 25 for 8 and 12°C storage before spoilage and absolute sensory rejection.

## B. Introduction

Consumer demands for cooked ready-to-eat (RTE) refrigerated food products are an emerging growth area for all food products. In 2002, the per capita consumption of value added fish sticks and portions were approximately 0.8 lbs./person. The U.S. production of fish portions and sticks was 235.4 million pounds with an estimated value of \$288.6 million. Fish portions alone accounted for 187.3 million pounds with a value of \$236.6 million. Alaskan pollock fillets and blocks led all species being 59% of the total amount of fillets, steaks, and blocks (NMFS, 2002). Lambert (1990) suggested that, besides convenience, additional reasons for the continued growth may be increased numbers of freezers and microwave ovens in homes, less meal preparation time with two or more individuals working and changing tastes. New refrigerated foods with extended shelf life present a highly profitable opportunity for food processors.

The traditional frozen batter and breaded seafood portion is a low risk food safety concern. The final product is usually fully cooked prior to consumption by the consumer. In addition, bacterial pathogen growth is unlikely to occur during frozen storage. For these reasons the National Seafood Alliance for Training and Education HACCP Curriculum (1997) has identified only two Critical Control Points (CCPs) for this product. (i.e., *Staphylococcus aureus* growth in the batter operation if the batter is held too long at elevated temperatures, and possible metal fragments from the saw and conveyor belts). Nevertheless, an increasing

number of supermarkets and convenience stores are interested in selling refrigerated fully cooked batter and breaded portions. There is also commercial interest in vacuum and modified atmosphere packaged refrigerated, (never frozen) raw and fully cooked batter and breaded products. Packaging products under vacuum (VAC) and modified atmosphere packaging (MAP) conditions can significantly extend shelf life of refrigerated raw and fully cooked batter and breaded fish products.

Consumer demand for cooked ready-to-eat (RTE) refrigerated products is an emerging growth area for all food products. Figueiredo (2000) stated that today's consumer is demanding speed, ease and convenience along with nutrition, health, and food safety. The refrigerated and frozen food segment is gaining an increasing share of the consumer meal consumption business. Demand exists from the retail food industry and from consumers for more convenient refrigerated raw and fully cooked batter and breaded seafood products. Also, consumers are demanding fresh, easy to prepare products that are safe and wholesome.

Extended shelf life refrigerated foods are those that have received minimal processing or pre-cooking and have an enhanced but limited shelf life, with refrigeration being a key preservation technique (Marth, 1998). Nevertheless, there are food safety and quality issues that companies must consider in marketing refrigerated foods especially with extended shelf life (Doyle, 1998).

Doyle went on to state that processors should be careful to fully evaluate and prevent hazards that may exist in refrigerated foods with extended shelf lives before marketing such products. Microbiological concerns with refrigerated foods focus on psychrotrophic and mesophilic organisms that have an opportunity to grow during extended storage at refrigeration temperatures. Common bacterial pathogens that can grow at refrigeration temperatures associated with finfish products include *Clostridium botulinum* Type E, *Yersinia enterocolitica*, and *L. monocytogenes*. Application of Good Manufacturing Practices (GMPs) can help to control and prevent the introduction of many of these pathogens to seafood products. Several pathogens can grow at refrigerated temperatures and for some pathogens, only a few cells can cause illness when ingested (Marth, 1998). *C. botulinum* is found throughout the environment and occurs naturally on many raw seafood products. Contamination of cooked products with these pathogens is also difficult to control through application of GMPs and SSOPs. In addition, under certain conditions, these pathogens may grow on refrigerated batter and breaded products with extended shelf lives.

Refrigerated raw or fully cooked batter and breaded seafood products face some of the same potential food safety concerns as do other processed products. Techniques used to extend the shelf life of fully cooked batter and breaded fish portions can also increase the possible outgrowth of pathogens on the product. For example, the use of VAC or MAP for shelf life extension increases the risk for outgrowth of *Clostridium botulinum*, in the product. As these new value added

products are developed, it is important that the possible food safety hazards associated with these products are identified and fully understood.

Packaging products under VAC and MAP conditions can significantly extend shelf life of refrigerated raw and fully cooked batter and breaded fish products. Such products require temperature control to reduce the likelihood of pathogen growth. However, Kalish (1991) reported in retail operations only 37% of food products were stored at the recommended temperature range of 32-38°F (0.0-3.3°C), with many cases averaging 44°F (6.7°C) and some as high as 55°F (12.8°F). Consumers were also at risk, since refrigerator temperatures in consumer homes ranged from 32°F to 55°F (0.0 to 12.8°C) (Beard, 1991). Studies have shown that at the retail level, product rotation procedures were inadequate, as sales of product dictated product rotation frequency (Kalish, 1991). Approximately, 2000 retail stores, including back-room storage facilities and chill cases were checked. Products were found stacked on the floor without any refrigeration and the temperature of many refrigerated cases were 44°F (6.7°C) with some as high as 56°F (13.3°C).

### **C. Materials and Methods**

#### **1. Fully cooked breaded and battered Alaskan pollock**

Breaded battered fish portions were made from Alaskan pollock (*Theragra chalcogramma*). The fish were filleted and frozen using a plate freezer into 16½ lb. 10" x 19" X 2½" blocks and stored. Within a six-month period, the blocks were

shipped to the plant for further processing. The blocks were cut into rectangles approximately 2 ounces in size fitting the dimensions of a 2 ¾" x 4" rectangle consisting of 60 +/- 2% fish flesh. A mildly seasoned "Italian style" batter was applied followed by breading made of Japanese breadcrumbs to achieve a precooked weight of 3.25 oz. The product was boxed and shipped to another facility where the portions were fried in a continuous cooking process at 350°F (176.7°C) in partially hydrogenated soybean oil for approximately 2½ minutes. After reaching an internal temperature of 140°F (60°C), the product was removed from the oil during which the internal temperature rose an additional 20°F (6.7°C) over the next 5-6 minutes to a final temperature of 160°F (71.1°C). The portion was held for a minute and then blast frozen for 1½ minutes. The final prepackaged cooked weight was 3 oz (84g). Each portion serving size consisted of 170 calories, 80 calories from fat 9g total fat, 2g saturated fat, 40 mg cholesterol, 240 mg sodium, 10g total carbohydrate, 2g dietary fiber, 3g sugars and 12g protein.

## 2. Modified Atmosphere Storage

Portions were stored frozen at -20°C, removed prior to packaging and thawed at room temperature for 30 min. Individual fish fillets were either packed with air (-5 in. Hg) in a package with an oxygen transmission rate (OTR) of 6,000 cc/m<sup>2</sup>/24h at 73°F (Cryovac, Duncan, SC.), vacuum packed (-27 in. Hg) or MAP in 100% CO<sub>2</sub> (OTR of 7.3 cc/m<sup>2</sup>/24h at 70°F). The vacuum package and the 100% CO<sub>2</sub> package film were High Barrier Deli 3-mil. composed of 0.75-gauge nylon with

EVOH and 2.25-gauge polyethylene (Koch Supplies Inc., Kansas City, MO.). The air and vacuum packages were packed with an Ultravac<sup>®</sup> vacuum packager (Koch Industries, Kansas City, MO). The modified atmosphere packages were prepared by flushing packages to their capacity with a 100% CO<sub>2</sub> anaerobe grade gas tank (Holox Limited, Roanoke, VA.) for 4 consecutive replications. A 3-foot Nalgene hose terminated with a 5 inch, 24-gauge canula delivered the modified atmosphere. The canula was placed to one side of the package during filling and sterilized with ethanol between each package. Gas was expelled by hand from each bag three times prior to sealing. Gas was prevented from escaping by folding over the open end of the package while it was double sealed using a portable tabletop contact heat sealer (Kapack cooperation Minneapolis, MN). The final gas to product ratio was at least 6:1. Packaged portions were stored at 8°C or 12°C with a minimum of one-inch separation between packages to allow for respiration.

Several different temperatures were chosen in order to represent different storage scenarios. Storage at 8°C represents mild temperature abuse. The 12°C storage represents moderate temperature abuse. (e.g., fillet is set on the counter overnight and the placed in the refrigerator in the morning). Finally, 21°C is a strict abuse situation (e.g., unrefrigerated product). Dufresne et al. (2000) stored rainbow trout at 12°C, Garren et al. (1987) stored salmon fillets at 8°C and 12°C, Lyver et al. (1998) and Lyver and Smith (1999) stored raw and cooked breaded and battered surimi at 12°C, Post et al. (1985) stored whiting and flounder at 8°C

and 12°C , and Reddy et al. (1996, 1997a, 1997b, 1999) stored tilapia, catfish, salmon, and cod at 4°C, 8°C and 16°C in air, vacuum and 75% CO<sub>2</sub>: 25% N<sub>2</sub>, respectively.

### 3. Headspace gas analysis

At 8°C and 12°C, two portions were sampled for the air and 100% CO<sub>2</sub> packages at days 0, 20, 25, 30 and 35. A ½ inch section of ¼ inch thick adhesive weather stripping was placed on the surface of each bag. A 30cc syringe (Becton Dickinson, Franklin Lakes, NJ) with a air valve mechanism and a 1-inch, 21-gauge needle (Becton Dickinson) was inserted into each package. The air valve was opened and the plunger was withdrawn filling the syringe to capacity with the atmosphere mixture from the package. The air valve was closed and the needle was removed. After removing the needle below the air valve, the syringe-air valve apparatus was placed into an Illinois Instruments 6600 Headspace Oxygen/Carbon Dioxide Analyzer (Ingleside, IL). The plunger was depressed to achieve the proper gas intake for the machine while the headspace composition was analyzed.

### 4. pH determination

Storage temperatures, replications and sampling days were identical to those previously mentioned in section 3. Packages were removed, cut open and a 1:1 ratio of HPLC grade water (VWR International, West Chester, PA) was added. The samples were hand massaged and then placed in a laboratory stomacher

(Seward Stomacher 400 Circulator, Thetford, Norfolk, England) for 2 min at 230 rpm. The pH was taken with an Accumet pH meter 915 (Fisher Scientific Company, St. Louis, MO) using a Symphony pH electrode (VWR).

## 5. Shelf-life study

Aerobic and psychrotrophic plate counts (in triplicate) were conducted for aerobic, vacuum and 100% CO<sub>2</sub> packages after storage at 4°C, 8°C and 12°C. Sampling days were designed for 0, 75, 100, 125 % of estimated spoilage. In this study, microbiological spoilage was defined as at least 7.0 log CFU/g for any plate count analysis (ICMFS, 1978). Analytical procedures followed the Food and Drug Administration Bacteriological Analytical Manual. Samples were pulverized in a laboratory stomacher (Seward Stomacher 400 Circulator) for 2 minutes at 230 rpm with a 1:4 ratio (wt/vol) of Butterfield's buffer (International Bio Products, Bothell, WA). One ml of sample was serially diluted with Butterfield's buffer then plated onto Petrifilm<sup>®</sup> (3M Microbiology, St. Paul, MN) in duplicate. Films were incubated for 48h at 36°C except psychrotrophic films were incubated at 10°C for 7 days.

## 6. Botulinum toxin assay

### a. Spore Crop Generation

The strain of Type F 83F was obtained from the culture collection of the Department of Food Science and Technology at Virginia Polytechnic Institute and State University (VPI&SU) and spore crops were created using biphasic

procedures as described in Anellis, et. al.(1972). Five ml. of initial spore suspension was added to 20 ml of sporulation medium composed of (w/vol) 5% tryptone peptone (Difco Laboratories, Detroit, MI), 0.5% peptone (Difco) and 0.625%  $K_2HPO_4$  (Sigma). A 0.075% (w/vol) solution of  $NaHCO_3$  (Sigma) was filter sterilized into the media before inoculation. The inoculated medium was incubated for 24h at 30°C. After incubation, 5 ml was removed and placed into another 20 ml of sporulation medium and incubated for 4h. This procedure was repeated for another 4h incubation. After final incubation, the entire 25ml was added to the liquid phase of a biphasic medium in a Fernbach flask and incubated at 30°C for up to 11 days. The flask was gassed with  $N_2$  for five minutes before and after inoculation. The liquid phase consisted of 0.1% w/vol. solution of yeast extract (Difco) in 250 ml. The solid phase was composed of 5% (w/vol.) tryptone peptone (Difco), 0.5% peptone (Difco), 0.125%  $K_2HPO_4$  (Sigma), 0.1% yeast extract (Difco), 3% agar (Difco) and 1 L of sterile distilled water. Phases were autoclaved, cooled in a 30°C water bath and then gassed with  $N_2$  for five minutes before and after inoculation with one ml of initial spore crop. Spores were harvested by removing 10 ml of liquid phase and combining it with 10 ml of sterile Butterfield's buffer (International Bio Products). The subsequent solution was centrifuged using a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments, Wilmington, DE). Spore suspensions were spun for 20 min. at 4°C with a Sorvall SS-34 rotor head at 2420 x g. Supernatant was removed and the spores were re-suspended with 10 ml of sterile Butterfield's buffer (International Bio Products) and centrifuged again.

This process was repeated three consecutive times after which 10 ml aliquots of washed spores were placed into sterile glass vials and stored in an -80°C freezer until needed.

#### b. Spore crop enumeration

Five strains of nonproteolytic *C. botulinum*; 17 Type B, Beluga, Minnesota, Alaska Type E and 83 Type F were obtained from the culture collection of the Department of Food Science and Technology at VPI&SU. Spore crops were enumerated using serial dilutions in sterile Butterfield's buffer (International Bio Products) and anaerobically incubated in trypticase peptone glucose yeast extract (TPGY) roll tubes. Tubes were incubated for up to ten days at 30°C. Final equivalent dilutions were made with sterile Butterfield's buffer (International Bio Products) to produce a 5 strain cocktail in a concentration of  $10^2$ - $10^3$ /ml then stored in vials at -80°C until needed. Identifying a rainbow iridescent or pearly reaction on anaerobically incubated McClung-Toabe Egg Yolk Agar at 30°C confirmed the purity of cultures (Eklund, 2002).

#### c. Spore inoculation

Shaking the cultures at room temperature using sterilized glass beads broke apart any coagulated spores during thawing of the frozen 5 strain cocktail (Eklund, 2002). A 1cc tuberculin syringe (Becton Dickinson) was used to administer 0.1 ml of inoculum into the product. The inoculum was injected along the length and width on one large surface of the portion equidistantly in

approximately nine locations by slowly retracting the needle out of the product as the inoculum was being dispensed. Special care was taken to ensure that the geometric center of the product had been inoculated.

#### d. Toxin determination

Detection of *C. botulinum* toxin followed the general protocol as outlined in the Food and Drug Administration Bacteriological Analytical Manual (FDA, 1998b). Samples were pulverized in a laboratory stomacher for 2 minutes at 230 rpm with a 1:1 ratio of gel phosphate buffer. One ml of sample was serially diluted into Butterfield's buffer (International Bio Products) then plated onto Petrifilm<sup>®</sup> (3M Microbiology) in duplicate. Twenty ml of slurry were removed and centrifuged (DuPont Instruments) for 10 min. at 11,951x g. Supernatant was filtered using a sterile 25mm diameter .2 µm pore size syringe tip filter (Acrodisk<sup>®</sup>, Gelman Laboratory, East Hills, NY). Samples were then frozen at -20°C. The freezing process as well as dilution and filtering is used to reduce the event of non-specific death in the mouse biotoxin assay in severely spoiled foods due to the presence of additional toxic compounds (ICMSF, 1996). Within a week, after the samples were thawed at 4°C, the subsequent steps were followed. Trypsin solution (0.2 ml) was added to two 1.8 ml aliquot of supernatant for each sample. (Trypsin was prepared with 0.5g Difco 1:250 trypsin added to 10 ml sterile distilled water). The subsequent treated solutions were incubated at 35-37°C with gentle agitation for 1 hour. One of these aliquots was heated to 100°C for thirty minutes as a control. One ml of trypsinized, non-trypsinized, and heated

trypsinized supernatant was loaded into three separate tuberculin syringes (Becton Dickenson. Five 15-18g male mice (*Mus musculus*) (Harlan Sprague Dawley) (2 trypsinized, 2 non-trypsinized, 1 control) were given an intraperitoneal injection with 0.5 ml of supernatant. Mice were observed periodically for symptoms of botulism for 48 h. Botulism signs typically began within the first 24 h with ruffling of fur, followed in sequence by labored breathing, weakness of limbs, total paralysis with gasping for breath, and finally death due to respiratory failure. Any mice not succumbing to the effects of the toxin were sacrificed with CO<sub>2</sub> gas. Carcasses were autoclaved to denature any toxin then frozen to await disposal by university agents. This project was approved by the Virginia Tech Animal Care Committee, which complies with the Animal Welfare Act and has been approved by the American Association for the Accreditation of Laboratory Animal Care (ACC proposal #02-046-FST).

e. Toxin study aerobic plate count

Storage temperatures, and sampling days were identical to those previously mentioned in section 3 except five replications were performed. Microbiological analyses only included aerobic plate counts. Sampling days at 8 and 12°C occurred on days 0, 20, 25, 30 and 35. Samples were pulverized in a laboratory stomacher (Seward) for 2 minutes at 230 rpm with a 1:1 ratio of gel phosphate buffer composed of 2g gelatin (Oxoid Ltd., Basingstoke, Hampshire, England), 4g Na<sub>2</sub>HPO<sub>4</sub> (Sigma Chemical Corporation, St. Louis, MO) and 1 L of sterile distilled water pH adjusted to 6.2 with 1N NaOH (Sigma) or HCl (Sigma). One milliliter of

sample was serially diluted into Butterfield's buffer (International Bio Products) and plated onto Petrifilm<sup>®</sup> (3M Microbiology) in duplicate. Films were incubated for 48h at 36°C.

## 7. Sensory Evaluation

Sensory analysis was performed in conjunction with the microbiological analysis and toxin detection. The portions were held frozen (-80°C) until the day of the sensory analysis. Samples were thawed at room temperature, allowed to equilibrate at 4°C and presented as whole fillets to three untrained panelists, who were vaccinated against *C. botulinum* toxin. The panelists held packaged fish samples a minimum distance of 8 in. from the nose. If an odor could not be identified, the package was brought closer to the nose. A 5-point hedonic scale was used to determine characteristics of odor and appearance (1-little or no odor; 5-putrid, trash like odor, musty off odor). Normal rejection was given a numerical value of 4 while a score of a 5 was reserved for a product that had deteriorated to the point of absolute rejection (i.e., if the consumer had purchased it, and were starving to death, they would not consider consuming it) (Post, et. al., 1985). For sensory rating definitions see Table 7.

## D. Results and Discussion

### 1. Shelf life

Initial APCs were between 100 and 1000 CFU/g. At 12°C spoilage occurred at days 17-20, 20-25, and >35 for air, vacuum and 100% CO<sub>2</sub> packaged portions,

respectively. At 8°C spoilage occurred at days 20-25, >35, and >35 days for air, vacuum and 100% CO<sub>2</sub> packaged portions, respectively. Finally, at 4°C, spoilage occurred at >35 days for all three atmospheres (Table 8).

Vacuum packaging added about 3-8 days of shelf-life at 12°C and 5-10 days of shelf-life at 8°C. The 100% CO<sub>2</sub> flushed package extended shelf-life 10-13 days and 5-10 days compared with the air package, and 5-10 days at 12°C compared with the vacuum packaged sample (Table 8). It required >35 days for spoilage to occur in the air package at 4°C, and at 12°C for the 100% CO<sub>2</sub> packaged samples.

There was no significant difference (>1 log CFU/g) between aerobic and psychrotrophic plate counts in any of the three atmospheric conditions (Table 8). Results suggest a dramatic extension of shelf life using modified atmosphere and refrigeration.

## 2. Headspace gas

The average gas to product (G/P) ratio for air and 100% CO<sub>2</sub> to breaded and battered pollock in packages incubated at 8°C and 12°C was 6:1. In uninoculated air packaged portions at 12°C O<sub>2</sub> concentrations initially were 21.3% but decreased to 16.7% by day 35. Initial concentrations of CO<sub>2</sub> were 0.8% but increased to 1.55% by the final 35<sup>th</sup> day.

In the 100% CO<sub>2</sub> packaged portions, the concentration of CO<sub>2</sub> at 12°C and 8°C was initially 100% where it remained until termination of the study on day 35. Conversely, O<sub>2</sub> concentrations remained negligible at <1.0% throughout the study. Initially there was a small decrease in the total volume of the 100% CO<sub>2</sub> packages caused by gas being absorbed into the tissue particularly at 8°C. This partial deflation was also described by Garcia et al. (1987) CO<sub>2</sub> in both 100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub> (MA) packages at every temperature combination. Results were similar to a study by Cai et al. (1997) on channel catfish where concentrations of CO<sub>2</sub> and O<sub>2</sub> remained relatively constant between 77.5-80.1% and 0.2-2.0%, respectively for both 4 and 10°C in MA with 80:20 CO<sub>2</sub>:O<sub>2</sub>.

Uninoculated air packages stored at 8°C were first sampled on day 20, when O<sub>2</sub> concentration diminished to 18.3% from an initial concentration of 21.3%. The O<sub>2</sub> concentration decreased to 13.9% by day 35. The CO<sub>2</sub> concentration for the same packages increased from 0.8% to 1.6% by day 20 and was 2.5% by day 35. Changes in the composition of headspace gases were probably a direct result of post-mortem metabolic and microbial processes.

These results are similar to those found for flounder. Portions flushed with 100% CO<sub>2</sub> packaged under a film with an OTR of 7.3 cc/m<sup>2</sup>/24h at 70°F behaved very similarly. Although the concentrations of CO<sub>2</sub> were higher and O<sub>2</sub> concentrations were lower in air packages of flounder the trends were the same. The OTR for flounder was 3000 cc/m<sup>2</sup>/24h at 70°F versus the OTR for Pollock at 6000

cc/m<sup>2</sup>/24h at 70°F. The increased gaseous exchange rate could account for the reduced build up of CO<sub>2</sub> and the slowed reduction of O<sub>2</sub>. Disparity of results in headspace gas concentrations to the literature can be attributed to speciation, atmosphere, relative freshness, storage temperature, OTR, etc.

Lyver et al. (1998) reported an increase and decrease of O<sub>2</sub> and CO<sub>2</sub> concentrations depending upon atmosphere and packaging for value added products. However, the change in concentration was much more dramatic for air packages and air packages with oxygen absorbent.

Similarly, Reddy et al. (1996) reported air package oxygen concentrations dramatically decreased with tilapia fillets, aquacultured salmon (Reddy et al. 1997), pond raised catfish (Reddy et al. 1997) and cod (Reddy et al. 1999). Headspace CO<sub>2</sub> concentrations increased from <1.0% to >15.0% on the day of spoilage for all four studies.

Cai et al. (1997) reported packaged channel catfish packaged in MA with 80:20 CO<sub>2</sub>:O<sub>2</sub> concentrations of CO<sub>2</sub> and O<sub>2</sub> remained relatively constant. In modified atmosphere (MA) 75:25 CO<sub>2</sub>:N<sub>2</sub> for tilapia fillets (Reddy et al. 1996), aquacultured salmon (Reddy et al. 1997b), pond raised catfish (Reddy et al. 1997a) and cod (Reddy et al. 1999) the day zero concentration for CO<sub>2</sub> was 75% but initially decreased only to recover to >58% by the day of spoilage. Garcia et al. (1987) also reported an initial decrease in headspace concentration for CO<sub>2</sub> in both

100% CO<sub>2</sub> and 70:30 CO<sub>2</sub>:N<sub>2</sub> (MA) packages at every temperature combination denoted by package shrinkage.

Conversely, Stier et al. (1981) reported that salmon fillets packaged under 60:25:15 CO<sub>2</sub>:O<sub>2</sub>:N<sub>2</sub> packages the concentration of CO<sub>2</sub> declined from approximately 60% to approximately 20%, but O<sub>2</sub> concentration increased to approximately 30% then declined to approximately 10%.

Dufresne et al. (2000) reported in rainbow trout packaged with OTRs of 4,371, 4923, and 10,043 cc/m<sup>2</sup>/24h at 24°C, 0% RH backflushed with 85:15 CO<sub>2</sub>:N<sub>2</sub> there was a headspace decrease in CO<sub>2</sub> and an increase in O<sub>2</sub>. Headspace analysis for packaging with an OTR of 11.6cc/m<sup>2</sup>/24h at 24°C, 0% RH had a headspace decrease in O<sub>2</sub> and increase in CO<sub>2</sub>.

### 3. pH

Initial pH values for air and 100% CO<sub>2</sub> packaged uninoculated fully cooked breaded and battered pollock portions at 8°C and 12°C averaged 6.86. For air packaged portions stored at 12°C, pH remained constant varying only by 0.1% until the final day 35. In 100% CO<sub>2</sub> packaged portions stored at 12°C, the pH decreased to 6.23 at day 20, and then varied only by 0.08% until the termination of the study on day 35.

The initial sampling time for air-packaged portions stored at 8°C was day 20 with an average pH value of 6.74. The pH decreased to 6.38 by day 35. Portions packaged in 100% CO<sub>2</sub> had an average pH value of 6.2 at day 20. The pH varied only by 0.13% throughout the study.

Lyver et al. (1998) reported a decrease in pH in raw inoculated surimi packaged in air and in packages with air and oxygen absorbent packages. In cooked samples, little change in pH occurred after 28 days of storage at 12°C and 25°C.

Parkin et al. (1982) found a decrease in pH of MA rockfish fillets packaged in 80:20 CO<sub>2</sub>:O<sub>2</sub>. Reddy et al. (1999) and Cai et al (1997) found no change in the pH of the cod and channel catfish fillets stored at any temperature or atmosphere combination, respectively. Additionally, Dufresne et al. (2000) reported no significant changes in pH in rainbow trout fillets due to the buffering capacity of the fish proteins. Similarly, there was no initial decrease in pH in salmon fillets stored under 100% CO<sub>2</sub> (Garcia et al. 1987).

Conversely, Reddy et al. (1997a) reported that the initial average surface pH for catfish fillets on the day of spoilage in air packages at 4°C and 16°C increased, but decreased at 8°C. In MA packages, pH decreased then increased above initial levels by the day of spoilage. For all combinations of package and temperature for aquacultured tilapia (Reddy et al. 1996) and salmon fillets (Reddy et al. 1997b) surface pH decreased during early storage and increased

later. The initial pH decrease was assumed to be caused by the saturation of CO<sub>2</sub> into the tissue of the fillet during early storage. However, for all atmosphere combinations and types of species, pH values ranged from 6.12 to 7.04. In uninoculated air packaged salmon fillets, pH increased by 0.05-0.12 at 4°C, 8°C and 16°C.

The range of pH values is consistent with those reported in the literature. Differences in initial pH values and final pH values may be attributed to different species, surface pH versus internal pH, packaging atmospheres, relative fish freshness, fish stress (e.g., amount of lactic acid in the musculature at time of death), etc. The results indicate that pH is a poor indicator of microbial spoilage, time to sensory rejection and/or toxin production.

#### 4. Toxin production with relation to sensory rejection

Toxin production in air packed inoculated pollock portions stored at 12°C occurred on day 30, in which one out of 5 samples were toxic. Toxin detection coincided with absolute sensory rejection. In vacuum packaged portions, three out of 5 samples were toxic on day 25. Normal sensory rejection was observed by one of three panelists. Packages with 100% CO<sub>2</sub>, never became toxic up to 35 days. Sensory scores for appearance and odor and corresponding APC values are found in Table 9.

In vacuum packaged portions stored at 8°C three of 5 samples were toxic on day 25 and day 30. Mean sensory odor scores were 3.8 and 3.3 respectively (Table 10). On day 25, one toxic sample was rated by one panelist as normally rejected. For the second positive sample, two panelists indicated normal rejection. The final positive sample, two panelists indicated absolute rejection while one panelist indicated normal rejection. The samples packaged under 100% CO<sub>2</sub> did not become toxic throughout 35 days of storage at 8°C.

Post et al. (1985) found at all temperature and atmosphere combinations flounder fillets became absolutely spoiled before toxin production. Cod fillets developed toxin before absolute rejection in all temperature and atmosphere combinations except 90:8:2 CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> packages at 26°C, air packages at 12°C, air and 100% N<sub>2</sub> packages at 8°C. Toxin production coincided with absolute sensory rejection in the 26°C air packages. For whiting fillets, only air packages at 8°C were spoiled before toxin production. Spoilage coincided with toxin production at 26°C and 8°C for vacuum and 100% N<sub>2</sub> packages. All other package and temperature combinations became toxic before absolute spoilage.

Conversely, in cod fillets (Reddy et al. 1999), crawfish tails (Lyon and Reddmann, 2000), and trout fillets (Dufresne et al. 2000), sensory rejection proceeded toxin production for every temperature and package combination. However, Dufresne (2000) reported that toxigenesis in smoked trout fillets

coincided with sensory rejection at some temperatures, OTRs, and atmosphere combinations.

#### 5. Toxin production with relation to microbiological count and sensory rejection

Inoculated air packaged pollock portions stored at 12°C, one out of 5 samples were toxic at day 30. Mean appearance score remained acceptable, odor score was 5.0, and APC was 9.1 log CFU/g. Vacuum packaged product stored at 12°C was toxic as early as day 25, with three of 5 samples being toxic. The mean odor score was 2.0, appearance score was 2.3, and APC was 5.8 log CFU/g. Samples were also toxic on days 30 and 35 with three of 5 positive portions. Mean sensory scores, appearance scores and APC for days 30 were 2.3, 2.6 and 6.7 log CFU/g and day 35 were 3.0, 2.3 and 7.0 log CFU/g. Both microbial count spoilage levels and sensory scores were not at the rejection level. Packages flushed with 100% CO<sub>2</sub> never developed toxin even after 35 days at 12 °C. Table 9 summarizes the data for these three packaging conditions with inoculated fully cooked breaded and battered pollock portions stored at 12°C.

After 35 days of storage at 8°C, air packaged portions did not form toxin although APCs were 7.6 log CFU/g and mean appearance scores were 3.9 which is just shy of normal sensory rejection. Vacuum packaged portions were toxic on day 25 and day 30, with three of 5 packages being positive for toxin. Mean odor scores were 2.8 and 2.5 with a corresponding APCs of 5.3 and 6.6 log CFU/g on

days 25 and 30, respectively. For vacuum packaged portions stored at 8°C, toxin developed before sensory rejection and microbial spoilage. Inoculated fully cooked breaded and battered pollock portion packages flushed with 100% CO<sub>2</sub> never developed toxin even after 35 days at 8°C. Table 10 summarizes the data for these three packaging conditions.

Aerobic plate count is a poor indicator of toxin production for fully cooked breaded and battered pollock portions packaged in air, vacuum or 100% CO<sub>2</sub>. There was no correlation between APC and toxic fillets. Only in air-packaged pollock portions stored at 12°C, did sensory rejection coincide with toxin production. For all other temperature package combinations, toxin production proceeded sensory rejection (Table 9).

Lyver et al. (1998) reported similar results for raw or cooked inoculated surimi nuggets in which toxin did not develop after 28 days at 4°C, 12°C or 25°C. However, in sterilized surimi nuggets, toxin developed in air-packaged nuggets and air packaged nuggets with oxygen absorbent materials at day 14 and day 28 at 25°C and 12°C, respectively. For, cooked inoculated surimi nuggets packaged in air, *Bacillus* spp. increased from 10<sup>1</sup> to approximately 10<sup>4</sup> and 10<sup>6</sup> at 12°C and 25°C, respectively. After 28 days, the cooked surimi samples in air or the air with oxygen absorbent materials were not rejected by sensory evaluation at 12°C or 25°C.

In this study for the 100% CO<sub>2</sub> packaged pollock portions, and in the Lyver et al. (1998) study for air and air with oxygen absorbent materials headspace gas O<sub>2</sub> concentrations were >1.0%. There was no toxin production at all storage temperatures. These studies used internal inoculations and high barrier films. Lyver hypothesized the prevention of toxin production in the cooked surimi was from the presence of high numbers of *Bacillus* spp. Lyver et al. (1998) also, inoculated autoclaved cooked samples and packaged them in air packages with oxygen absorbent material. It was reported that these samples became toxic. Lyver et al. (1998) isolated *Bacillus* species and plated them directly with strains of *C. botulinum* and determined that some species of *Bacillus* inhibited botulinal growth.

This study suggests that vacuum packaging removes O<sub>2</sub> from inside the product leaving only a small amount of O<sub>2</sub> resulting in an anaerobic environment. The 100% CO<sub>2</sub> packages may not have produced toxin due to the abundance of O<sub>2</sub> still inside the product. Even though the outside of the product was surrounded by an abundance of 100% CO<sub>2</sub>, the inside of the product where the inoculated spores were located was still aerobic, as indicated by the small decrease in pH for the sample after 35 days suggesting that CO<sub>2</sub> had not permeated inside the sample. Additionally, when Lyver et al. (1998) autoclaved the samples to remove background *Bacillus* species the O<sub>2</sub> was also removed therefore spores were inoculated into an anaerobic environment.

Garcia et al. (1987) packaged salmon fillets in VAC and MA packages with initial aerobic counts of 2.48 log CFU/g fillet increasing to 7.7-8.7 log CFU/g. Using the same sensory scale as Post et al (1985), toxin production coincided with sensory rejection at 30°C but at 8°C and 12°C sensory rejection came after toxin production. At 4°C sensory scores indicated rejection before toxin production. At 1°C sensory scores remained acceptable through day 60 where no toxin was detected.

Inoculated shrimp at 4°C spoiled when microbial counts were approximately 6 log CFU/g but no toxin was detected in either package (Garren et al., 1994). At 10°C sensory rejection occurred before microbial counts exceeded 6 log CFU/g but coincided with toxin production. Additionally, Garren et al. (1995) inoculated aquacultured rainbow trout and at 10°C toxin developed after spoilage. Psychrotrophic counts were ~6 log CFU/g and off-odors were present.

Total plate counts for packaged salmon fillets and fillet sandwiches comprised of two fillets at 4.4°C were 9 log CFU/g at time absolute sensory rejection (Stier et al. 1981). Toxin was not detected in either type of packaged fillet for any temperature combination. Toxin production coincided with sensory rejection in the MA packaged fillets and toxin production preceded spoilage in the air packaged fillets at 22.2°C. Microbial counts were approximately 9 log CFU/g for fillets in air and MA.

For aquacultured tilapia, catfish and salmon packaged in MA, sensory rejection preceded toxin production in all scenarios except salmon stored at 8°C, tilapia at 16°C and catfish at 16°C when toxin production coincided with sensory rejection. Toxin production, in the salmon stored at 16°C, preceded sensory rejection. In all cases microbial counts were >8.0 log CFU/g (Reddy et al. 1996, 1997a, 1997b, 1999).

Cai et al. (1997), reported inoculated and uninoculated samples spoiled at the same rate. At 4°C in overwrapped packaged samples, toxin production coincided with sensory rejection but proceeded microbiological spoilage. In MA packaged samples, toxin production coincided with microbiological spoilage and sensory rejection. Toxin production did not occur in the MB (MB- individual overwrap then MA of 5-7 fillets) samples up to day 30. Sensory rejection and spoilage occurred between days 9-12.

At 10°C toxin production occurred in overwrapped packaged samples during or before sensory rejection but after microbial spoilage. In MA packaged fillets, toxin production coincided with microbial spoilage but before and after sensory rejection. Toxin production coincided with both sensory rejection and microbial spoilage for MB samples. MB samples relocated from 4°C to 10°C did not produce toxin.

## 6. Safety of fully cooked breaded and battered Alaskan pollock

*C. botulinum* grows differently on various fish species. Some species of fish are more inclined to become toxic than other species. It has been suggested that fat and protein content may play a role (Reddy et al. 1999) in addition to the effect of natural microbial flora and atmosphere mixture. Also, *C. botulinum* is sensitive to the surrounding microenvironment. Due to the potency of the toxin only a small number of spores are sufficient to cause illness. If these spores are subjected to conditions conducive to growth on the microscopic level, germination and subsequent toxin formation occur independent of package environment, microbial count or pH on the macroscopic level.

Fully cooked breaded and battered Alaskan pollock products should be packaged only after they have been given time to equilibrate to ambient atmosphere temperature. This will help replenish the O<sub>2</sub> that may have been partially reduced during cooking, or the inside of the fillet may remain partially anaerobic allowing *C. botulinum* to grow. At both 8°C and 12°C, high barrier vacuum packaged products are likely to become toxic. Vacuum packaging removes O<sub>2</sub> from inside the fillet producing an anaerobic environment. At 8°C both the air and 100% CO<sub>2</sub> packaged portions failed to become toxic. Air packaged products are unlikely to become toxic. Additional research on 100% CO<sub>2</sub> packaged portions should be conducted addressing external inoculations of *C. botulinum* spores before conclusions on safety can be stated (Table 11).

At 12°C, toxin formed in air-packaged samples, but the samples were moldy long before toxicity developed (Table 11). Under, 100% CO<sub>2</sub>, the fully cooked batter and breaded pollock portions were not toxic at 8°C or 12°C. However, additional research is needed on external inoculations of *C. botulinum* spores before the safety of these products can be verified. The results indicate that temperature control is the effective method to control growth of *C. botulinum*.

## E. Tables

**Table 7: Sensory rating definitions for fully cooked breaded and battered Alaskan pollock portions.**

Rating	Appearance	Odor
1	Portion firm, color light to dark varying shades of beige	Little or no odor
2	Portion firm, slight darkening in color	Slight fishy odor
3	Portion firm, off colors beginning (yellow, green, gray)	Fishy or slight off-odor, not objectionable
4	Portion deterioration evident, definite darkening of color, small patches of mold	Noticeable odor, strong fish or off odor
5	Definite mold growth, discoloration, portion breaking apart	Putrid, trash-like odor, strong musty odor

(variation of table in Post, et. al., 1985)

**Table 8: Average number of days to spoilage<sup>a</sup> in air<sup>b</sup>, vacuum and 100% CO<sub>2</sub> packaged<sup>c</sup> fully cooked battered and breaded Alaskan pollock determined by microbial plate count**

Atmosphere	Storage Temperature (°C)	Approximate Days to Spoilage	APC Log CFU/g	PPC Log CFU/g
Vacuum <sup>c</sup>	4	> 35	< 3.0	< 3.0
	8	> 35	6.7	6.7
	12	20-25	7.3	6.8
Air <sup>b</sup>	4	>35	4	5.1
	8	20 -25	7.6	7.6
	12	17-20	8.3	7.3
100% CO <sub>2</sub> <sup>c</sup>	4	> 35	< 3.0	< 3.0
	8	> 35	4.0	4.0
	12	> 35	5.5	5.6

<sup>a</sup> >10<sup>7</sup> log CFU/g, n=3

<sup>b</sup> OTR 6000 cc/m<sup>2</sup>/24h at 73°F

<sup>c</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

**Table 9: Positive replications out of total replications for each time and atmosphere combination with corresponding sensorial scores and aerobic plate count<sup>a</sup> in breaded and battered Alaskan pollock stored at 12°C**

Atmosphere	Days Storage	Toxin Detected <sup>b</sup>	Mean Odor Score	Mean Appearance Score	Mean APC Log CFU/g
Air <sup>c</sup>	20	0/5	2.9	5.0	4.5
	25 <sup>e</sup>	1/5	3.0	5.0	4.0
	30 <sup>e</sup>	1/5	2.0	5.0	9.1
	35	0/5	2.6	5.0	8.1
Vacuum <sup>d</sup>	20	0/5	1.8	1.9	5.5
	25 <sup>e</sup>	3/5	2.0	2.3	5.8
	30 <sup>e</sup>	3/5	2.3	2.6	6.7
	35 <sup>e</sup>	3/5	3.0	2.3	7.0
100% CO <sub>2</sub> <sup>d</sup>	20	0/5	3.2	1.0	4.3
	25	0/5	1.2	1.5	3.1
	30	0/5	1.8	1.3	5.3
	35	0/5	1.5	1.2	6.1

<sup>a</sup> 5 samples were used to determine sensorial and APC means

<sup>b</sup> Samples positive/Total number of samples

<sup>c</sup> OTR 6000 cc/m<sup>2</sup>/24h at 73°F

<sup>d</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

<sup>e</sup> only toxic samples were used to determine sensorial and APC means

**Table 10: Positive replications out of total replications for each time and atmosphere combination with corresponding sensorial scores and aerobic plate count<sup>a</sup> in breaded and battered Alaskan pollock stored at 8°C**

Atmosphere	Days Storage	Toxin Detected <sup>b</sup>	Mean Odor Score	Mean Appearance Score	Mean APC Log CFU/g
Air <sup>c</sup>	20	0/5	2.0	2.7	5.0
	25	0/5	1.9	3.1	4.5
	30	0/5	2.0	3.9	7.3
	35	0/5	2.7	3.9	7.6
Vacuum <sup>d</sup>	20	0/5	1.8	1.7	5.1
	25 <sup>e</sup>	3/5	3.8	1.8	6.0
	30 <sup>e</sup>	3/5	3.3	1.8	7.3
	35	0/5	1.5	2.0	6.2
100% CO <sub>2</sub> <sup>d</sup>	20	0/5	1.3	1.3	4.6
	25	0/5	1.1	1.2	4.9
	30	0/5	1.6	1.4	2.7
	35	0/5	1.7	1.1	2.0

<sup>a</sup> 5 samples were used to determine sensorial and APC means

<sup>b</sup> Samples positive/Total number of samples

<sup>c</sup> OTR 6000 cc/m<sup>2</sup>/24h at 73°F

<sup>d</sup> OTR 7.3 cc/m<sup>2</sup>/24h at 70°F

<sup>e</sup> only toxic samples were used to determine sensorial and APC means

**Table 11: Sensory panel rejection and microbial spoilage determination at the time that toxin was first detected for battered and breaded pollock stored under different atmospheres and incubation temperatures.**

	Microbial spoilage (APC > 10 <sup>7</sup> /CFU/gm)	No microbial spoilage (APC < 10 <sup>7</sup> /CFU/gm)
Rejection by sensory panel		8°C, vacuum 12°C, aerobic
Not rejected by sensory panel		12°C, vacuum

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## VI. Appendix

For each journal article referenced for section II. C. 1. of the literature review the following is a list of important common characteristics for each study.

Cai et al. (1997)

Fish: Channel Catfish

Temperatures: 4, 10 and 4 then 10°C

OTR: overwrap 125 cm<sup>3</sup>/m<sup>2</sup>/24h/atm at 20°C, 0%RH: B-bag 3-6

cm<sup>3</sup>/m<sup>2</sup>/24h/atm at 4.4°C, 0%RH

Atmosphere: overwrap, B-bag 80:20 CO<sub>2</sub>:N<sub>2</sub> (MA), individual overwrap then B-bag (5-7 fillets) (Master Bag).

Inoculated counts: psychrotrophic, anaerobic and aerobic

Initial microbial counts: 3.0-4.0 log CFU/g

G/P ratio: not given

*C. botulinum* spore inoculum: 10<sup>3</sup>-10<sup>4</sup>/g

Dufresne et al. (2000)

Fish: Rainbow Trout

Temperatures: 12°C

OTR: 11.6, 4,371, 4,923 and 10,043 cc/m<sup>2</sup>/24h at 1 atm

Atmosphere: 11.6: air, 0, 25, 50,75,100% CO<sub>2</sub>; air, vacuum, 85:15 CO<sub>2</sub>:N<sub>2</sub>

Uninoculated and inoculated counts: not done

Initial microbial counts: N/A

G/P ratio: not given

*C. botulinum* spore inoculum:  $10^2$ /g

Garcia et al. (1987)

Fish: Salmon fillets

Temperatures: 1, 4, 8, 12 and 30°C

OTR: 30-50 cc/m<sup>3</sup>/24h at 1atm at 22.8°C

Atmosphere: vacuum, 100% CO<sub>2</sub>, 70:30 CO<sub>2</sub>:N<sub>2</sub>

Uninoculated counts: aerobic

Initial microbial counts: 2.48 log CFU/g fillet

G/P ratio: 1:5

*C. botulinum* spore inoculum:  $10^{-1}$ ,  $10^0$ ,  $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ /50g fillet

Garren et al. (1994)

Fish: Shrimp

Temperatures: 4 and 10°C

OTR: oxygen barrier

Atmosphere: vacuum skin

Inoculated and uninoculated counts: psychrotrophic and anaerobic

Initial microbial counts: 2.0-3.0 log CFU/g

G/P ratio: not given

*C. botulinum* spore inoculum:  $10^3$ - $10^4$ /g

Garren et al. (1995)

Fish: Aquacultured Rainbow Trout

Temperatures: 4 and 10°C

OTR: oxygen barrier and oxygen permeable

Atmosphere: vacuum

Inoculated and uninoculated counts: psychrotrophic and anaerobic

Initial microbial counts: 2.0-3.0 log CFU/g

G/P ratio: not given

*C. botulinum* spore inoculum:  $10^3$ - $10^4$ /g

Lalitha and Gopakumar (2001)

Fish: Mullet and Shrimp tissue homogenates

Temperatures: 4, 10, 15 and 30°C

OTR: not given

Atmosphere: vacuum

Uninoculated counts: none

Initial microbial counts: none

G/P ratio: N/A

*C. botulinum* spore inoculum:  $10^6$ /g type A, B, C, D and  $10^3$ /g type E

Lyon and Reddmann, (2000)

Fish: Crawfish tails

Temperatures: 4 and 10°C

OTR: 3-6 cc/m<sup>2</sup>/24h@1atm and 4.4°C (vacuum), air permeable bag (air)

Atmosphere: air and vacuum

Inoculated counts: aerobic and anaerobic

Initial microbial counts: 2.0 log CFU/g

G/P ratio: not given

*C. botulinum* spore inoculum: 10<sup>3</sup>/g

Lyver et al. (1998)

Fish: Raw and cooked breaded and battered surimi

Temperatures: 4, 12 and 25°C for raw and 12 and 25°C for cooked

OTR: 3-6 cc/m<sup>2</sup>/24h at 4.4°C and 0% RH

Atmosphere: air and air with Ageless SS oxygen absorbents

Inoculated microbial counts: lactic acid and *Bacillus spp.*

initial counts: 1.0 log CFU/g *Bacillus spp.*

G/P ratio: not given

*C. botulinum* spore inoculum: 10<sup>4</sup>/g

Lyver et al. (1999)

Fish: Raw and cooked breaded and battered surimi

Temperatures: 4, 12 and 25°C for raw and 12 and 25°C for cooked

OTR: 3-6 cc/m<sup>2</sup>/24h at 4.4°C and 0% RH

Atmosphere: air and air with Ageless SS oxygen absorbents

Inoculated microbial counts: lactic acid and *Bacillus spp.*

initial counts: 1.0 log CFU/g *Bacillus* spp.

G/P ratio: not given

*C. botulinum* spore inoculum:  $10^4$ /g

Post et al. (1985)

Fish: Cod, Whiting and Flounder

Temperatures: 8, 12 and 26: 4, cycled 4-26, cycled 8-26: 8 and 26°C

OTR: air: 980 ml/100in<sup>2</sup>/1atm/24h, vacuum and MA 2.6+/- 0.1

ml/100in<sup>2</sup>/1atm/24h

Atmosphere: air, vacuum, 100% N<sub>2</sub>, 100% CO<sub>2</sub>, 90:8:2 CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub>, 65:31:4

CO<sub>2</sub>: N<sub>2</sub>:O<sub>2</sub>

Uninoculated and inoculated counts: none

Initial microbial counts: N/A

G/P ratio: none given

*C. botulinum* spore inoculum:  $5.0 \times 10^1$ /g

Reddy et al. (1996)

Fish: Aquacultured Tilapia

Temperatures: 4, 8 and 16°C

OTR: 3-6 cc/m<sup>2</sup>/24h at 1 atm

Atmosphere: 75:25, CO<sub>2</sub>: N<sub>2</sub>, air and vacuum

Uninoculated microbial counts: aerobic and anaerobic

Initial microbial counts: 3.0-4.0 log CFU/g

G/P ratio: 7.71

*C. botulinum* spore inoculum:  $10^2/g$

Reddy et al. (1997a)

Fish: Pond raised Catfish

Temperatures: 4, 8 and 16°C

OTR: 3-6 cc/m<sup>2</sup>/24h at 1 atm

Atmosphere: 75:25 CO<sub>2</sub>:N<sub>2</sub>, air and vacuum

Uninoculated and inoculated counts: aerobic, facultative anaerobes, coliforms

Initial microbial counts: uninoculated: 4.9, 3.6 and 3.2, inoculated: 4.7, 3.8

(no coliforms) log CFU/g, respectively.

G/P ratio: 6

*C. botulinum* spore inoculum:  $10^2/g$

Reddy et al. (1997b)

Fish: Aquacultured Salmon

Temperatures: 4, 8 and 16°C

OTR: 3-6 cc/m<sup>2</sup>/24h at 1 atm at 4.4°C

Atmosphere: 75:25 CO<sub>2</sub>:N<sub>2</sub>, air and vacuum

Uninoculated and inoculated counts: aerobic, anaerobes and psychrotrophs

Initial microbial counts: uninoculated: 4.6, 3.1 and 4.6, inoculated: 4.9, 4.7

and 3.2 log CFU/g, respectively.

G/P ratio: 6

*C. botulinum* spore inoculum:  $10^2/g$

Reddy et al. (1999)

Fish: Cod and aquacultured Tilapia, Catfish, and Salmon

Temperatures: 4, 8 and 16°C

OTR: 3-6 cc/m<sup>2</sup>/24h at 1 atm

Atmosphere: 75:25 CO<sub>2</sub>: N<sub>2</sub>, air and vacuum

Uninoculated counts: aerobic, anaerobic and psychrotrophic

Initial microbial counts: 5.0, 4.3 and 4.3 log CFU/g, respectively

G/P ratio: 6

*C. botulinum* spore inoculum:  $10^2/g$

Stier et al. (1981)

Fish: Salmon fillets and 2 fillet sandwiches

Temperatures: 4.4 and 22.2°C

OTR: 4.8 cc/100in<sup>2</sup>/24h at 22.8°C

Atmosphere: air and 60:25:15 CO<sub>2</sub>:O<sub>2</sub>:N<sub>2</sub>

Uninoculated counts: aerobic, Gram positive, and Gram negative

Initial microbial counts: ~ 5.84 log CFU/g

G/P ratio: not given

*C. botulinum* spore inoculum:  $3 \times 10^3/g$

## **VII. VITA**

Fletcher Marion Arritt, III was born on January 5, 1975 in Fork Union, Virginia, the son of Fletcher M., Jr. and Betty J. Arritt. He graduated from Fork Union Military Academy in May, 1993.

He studied Biology at Virginia Polytechnic Institute and State University where he received a Bachelor of Science with a minor in Chemistry and an option of microbiology and immunology in May, 1998. He continued graduate studies there in August, 1998 in Food Science and Technology, and completed his masters degree in May, 2001.

He is a member of the Institute of Food Technologists, the International Association for Food Protection, Gamma Sigma Delta Honor Society and the Phi Sigma Society. Upon completion of his doctorate he plans to continue to perform research on pathogenic bacteria to enhance microbiological food safety.