

*Clostridium botulinum* toxin development in refrigerated reduced oxygen packaged  
Atlantic croaker (*Micropogonias undulatus*)

By

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**Abstract**

The purpose of this study was to determine the effects of storage temperature and film oxygen transmission rate (OTR) on toxin development by *Clostridium botulinum* in refrigerated raw vacuum packaged croaker fillets, and to determine if toxin development precedes microbiological and/or organoleptic spoilage. Raw croaker fillets were vacuum packaged in oxygen-permeable films (OTR of 10,000 cc/m<sup>2</sup>/24hr or 3,000 cc/m<sup>2</sup>/24hr) and stored at either 4°C or 10°C. Type 83F, 17 Type B, Beluga, Minnesota, and Alaska nonproteolytic strains of *C. botulinum* were used to inoculate fish prior to vacuum packaging. At both temperatures, microbial spoilage preceded toxin production in fillets vacuum packaged in both film types. At 4°C microbial spoilage occurred after approximately 7 days for fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film and after 8 days for fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film. However, toxin was not detected until day 8. At 10°C microbial spoilage occurred after approximately 3 days for fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film, while toxin production occurred on day 5. For fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film microbial spoilage occurred after 4 days. However toxin production did not occur until day 6. In contrast, at both temperatures toxin production preceded or coincided with organoleptic spoilage in fillets vacuum packaged in both film

types. At 4°C organoleptic spoilage occurred after 10 days for fillets packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film and after 9 days in the 3,000 cc/m<sup>2</sup>/24hr OTR film, while toxin production occurred on day 8. At 10°C organoleptic spoilage occurred after 6 days for fillets packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film, and toxin was detected on day 5. For fillets packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film and stored at 10°C, organoleptic spoilage occurred after 6 days, while toxin production occurred on day 6. Although toxin production preceded or coincided with organoleptic spoilage in both film types, this may have been because samples were presented on ice, which could have masked potential odors. This study shows that there are not significant differences between these film types when it comes to microbial and organoleptic spoilage. Therefore lower OTR films, such as 3,000 cc/m<sup>2</sup>/24hr film, may be used to vacuum package Atlantic croaker.

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## **Introduction and Justification**

Fresh fish provides is a natural environment for growth of psychrotrophic spoilage microorganisms. When lipids and other seafood proteins are degraded by these microorganisms, the result is a highly pronounced off-flavor and off-odor resulting in a short shelf life and monetary losses for seafood companies. In the past, ice and refrigeration were the most common methods used to hinder microbial and biochemical spoilage (29). However, there have been reports that ice harbors bacteria, and may contaminate the fresh fish when it melts, thus accelerating spoilage and decreasing shelf life (20, 27).

Alternative methods to increase the shelf life of fish include the use of vacuum (VAC) packaging. Vacuum packaging refers to packaging in containers where all air has been removed prior to the final sealing of the container. There is considerable interest in VAC packaging technology for raw fish to extend shelf life by preventing the growth of naturally occurring, aerobic spoilage microorganisms. Increased shelf life allows the fresh fish market to expand to new markets beyond the coastal regions (26).

Vacuum packaging of fresh fish can increase food safety problems by providing ideal anaerobic conditions for the possible growth and toxin formation by *Clostridium botulinum*, found on both saltwater and freshwater fish. There are reports that for some species of fish, botulinum toxin production precedes sensory rejection by consumers (8, 25, 28). Furthermore, since psychrotrophic spoilage microorganisms are inhibited by the anaerobic environment provided by VAC packaging, *C. botulinum* may successfully compete and grow on the fish.

To minimize the possibility of toxin production in VAC packaged fresh fish, the U.S. Food and Drug Administration mandated, as one of three approaches, that fishery products are packaged in a film with an oxygen transmission rate (OTR) of 10,000 cc/m<sup>2</sup>/24hr. Use of this film may prevent toxin formation, but shelf life is shorter compared with products packaged under a film a lower OTR.

Inoculated studies on vacuum packaged fresh fish have not been conducted on Atlantic croaker. The National Advisory Committee on the Microbiological Criteria of Food recommends that inoculated pack studies be performed for each species of fish (25).

The objectives of this study were to determine if botulinum neurotoxin develops before organoleptic and/or microbial spoilage in VAC packaged croaker fillets, and also to determine the effects of film oxygen transmission rate (OTR) of VAC packaged croaker fillets on toxin production by *C. botulinum*.

## Literature Review

### *Clostridium botulinum* and its toxins

*Clostridium botulinum* is an anaerobic, Gram positive, rod-shaped, spore-forming bacterium that produces a neurotoxic protein. There are at least three genetically distinct groups of *C. botulinum* which are alike in their ability to produce neurotoxins, but have diverse serologic properties (toxin types A, B, C, D, E, F, and G) (32, 34). These groups can be differentiated on the basis of biochemical and physiological characteristics, and can be identified by completely neutralizing the toxins and using the homologous antitoxin (34). Types A, B, E, and F are pathogenic to humans and can be divided into two groups – the proteolytic types (A, B, and F) and the nonproteolytic types (B, E, and F). The proteolytic types are heat resistant, mesophilic, and salt tolerant. The nonproteolytic types are heat sensitive, psychrotolerant, and salt sensitive (15).

The toxin produced by *C. botulinum* consists of a 100-kd heavy chain joined to a 50-kd light chain by a single disulfide bond. The heavy chain contains portions for binding to peripheral motor neurons and for toxin internalization. The light chain is a “zinc metalloprotease, specific for proteins involved in acetylcholine” (33). First, the toxin is ingested and absorbed into the intestinal wall. After absorption in the intestine and stomach occurs, the toxin binds irreversibly to the presynaptic nerve endings of the peripheral nervous system and cranial nerves, preventing the release of acetylcholine, which is involved in the transmission of nerve impulses in the body. The toxin mechanism of action involves binding to a receptor on the nerve cell membrane at the neuromuscular junction, followed by an internalization of a section of the toxin, and finally “cleavage of protein components of the neuroexocytosis apparatus within the cell”

(32). The neurotoxin produced by *C. botulinum* is considered to be the most potent lethal substance known, with a lethal dose of 1 ng/kg; 15,000 to 100,000 times more toxic than sarin, a potent organophosphate used in a terrorist attack in Tokyo in 1995.

Although the toxins affect a wide variety of vertebrates, the evolutionary utilization of the toxin to the host organism is unknown (32).

Botulinum neurotoxins bind with high affinity to neuronal membranes, and it has been hypothesized that the heavy chain binds to complex gangliosides located in the presynaptic cleft. Proteins such as synaptotagmin may be receptors for the toxin, however because synaptotagmin is found at every nerve ending, they are probably not the sole binding sites. Many believe both high affinity and low affinity receptors are necessary to transport the toxin into neurons (31).

### Botulism

There are four clinical types of botulism that occur in humans – foodborne botulism, wound botulism, infant botulism, and in rare instances, adult infectious botulism. Wound botulism occurs when the anaerobic conditions present in an abscessed wound allow the germination of *C. botulinum* spores, followed by the growth of the bacteria, and production and absorption of toxin. Infant botulism occurs in infants when *C. botulinum* spores are ingested, colonize the gastrointestinal tract, and produce the toxin. Adult infectious botulism also occurs as a result of the ingestion of spores, in a similar manner to that of infant botulism (32).

Foodborne botulism is a serious form of food poisoning caused by ingesting foods that contain the preformed toxin produced by *C. botulinum* (32, 34). Although there are few recorded botulism outbreaks in the United States, its mortality rate is high.

Of the 2,320 reported cases of botulism in the United States from the years 1899-1990, 1,036 of those cases resulted in death (34). Symptoms usually develop within 12 to 36 hours (15) and include blurred vision, difficulty in swallowing, and dysarthria. Botulism is characterized by “symmetric, descending, flaccid paralysis of motor and autonomic nerves, usually beginning with the cranial nerves”(32). However, botulism is often underdiagnosed because many physicians are unfamiliar with the disease and tend to mistake it for more common syndromes such as Guillain-Barré syndrome or stroke (32). Botulism type E usually has the most rapid onset of symptoms while type A botulism tends to have the most severe symptoms (15).

Suspected cases of botulism are confirmed at the Centers for Disease Control and Prevention (CDC), and other state laboratories, and treatment includes hospital care, as well as trivalent equine antitoxin, which may prevent death if dispensed early. Trivalent equine antitoxin works by neutralizing toxins that are not yet bound to nerve endings. Severe cases of botulism may require mechanical ventilation, which has drastically decreased mortality rates in the past 40 years. If wound botulism is suspected, the dead tissue should be removed from the wound and antibiotics such as penicillin should be administered. The management of large outbreaks, such as a deliberate exposure, would be to treat victims with mechanical ventilators and administer the antitoxin rapidly. The botulism toxoid vaccine may also be used to protect against exposure. However, administration is impractical since the vaccination process must be started months before exposure to the toxin, and because the vaccine is unlicensed. Therefore, it is only administered to those who are at high risk to exposure, such as laboratory personnel

working with the microorganism or those in the military who may be exposed during battle (32).

### Outbreaks

There have been a number of botulism outbreaks due to the consumption of contaminated fish and fish products, and in general type E was the responsible type (15). More recent episodes are primarily affiliated with processing and non-commercial traditional foods.

In November 1987, a 39-year-old Russian immigrant and his 9-year-old son were diagnosed with botulism after consuming ungutted, salted, air-dried whitefish known as either ribyetz or kapchunka. The whole fish was purchased from a delicatessen in Queens, New York City. The mechanism of contamination could not be established, however the fact that the fish was not eviscerated may have contributed to the intoxication. That same month, the CDC received a report from the Ministry of Health in Jerusalem, Israel of five other patients with suspected cases of botulism; one case was fatal. All of the patients had eaten ribyetz purchased from a grocery store in Brooklyn, New York City (2).

In July 1990, the Hawaii Department of Health was informed that three adults from the same family had been hospitalized with symptoms indicative of botulism after ingesting palani (surgeon fish). The first patient, a Hawaiian woman, complained of double vision, difficulty swallowing, and muscle weakness. Samples of the leftover fish were tested at the CDC and found to contain type B botulinum toxin. The palani had been purchased fresh and previously cleaned from a retail fish market. The woman's husband grilled the palani on both sides. He then opened the fish with his fingers and

noticed remnants of the intestines inside the fish. The fish had been sold to the retailer by a local fisherman, but the length of time the palani had been kept at the market could not be determined. A thorough inspection of the market revealed that fish were kept on ice in a display freezer case which did not have functional cooling equipment. The internal temperature of the fish found on top of the ice was determined to be 11°C. Since there was inadequate refrigeration at the market, the internal temperature of the fish may have been elevated for an extensive period of time. Furthermore, the conditions around the retained gut may have provided an ideal anaerobic environment. These factors may have led to the growth and toxin production by *C. botulinum* (3).

In New Jersey in May 1992, the Department of Health investigated a case of botulism associated with an uneviscerated, salt-cured fish product. On May 4<sup>th</sup> through 5<sup>th</sup>, a 32-year-old man visited the hospital emergency room three times complaining of dizziness, facial drooping, dry mouth, weakness, and respiratory problems. On May 6<sup>th</sup>, three other family members developed similar symptoms and were hospitalized. The source of botulism was traced to an ethnic preparation of an uneviscerated, salt-cured fish product known as moloha. The family purchased the fish from a local retail fish market and consumed the moloha without cooking or heating. Botulinum toxin type E was isolated from the leftover fish and in the stool specimen of the 32-year-old man. A family friend who also consumed the fish product but did not develop botulism symptoms was treated with the antitoxin as a precautionary measure. When the market was visited, no moloha were found and the owner denied selling this type of fish (4).

In January 1997, two Germans were diagnosed with botulism after consuming VAC packaged hot-smoked Canadian whitefish. The patients experienced symptoms of



dizziness, blurred vision, dyspnea, headache, dysarthria, nausea, abdominal cramps, and vomiting. After using Polymerase Chain Reaction to analyze the remains of the hot-smoked whitefish eaten by the patients, the type of toxin was identified as E. The fish was processed from frozen whitefish imported to Finland from Canada, and the pulsed-field gel electrophoretic pattern of the isolated strain indicated that the fish was contaminated by *C. botulinum* in Canada. The fish was probably held at sufficiently high temperature for a sufficiently long period of time after processing for the strain to produce the toxin (18). Another case involving VAC packaged smoked whitefish occurred in Finland in June 2006 after a 65-year-old woman fell ill with symptoms indicative of botulism. Her initial symptoms were vomiting and diarrhea, followed by muscle weakness in her upper and lower limbs. She was admitted to the hospital and serum samples analyzed from the patient using the mouse bioassay confirmed the presence of botulinum toxin. Neutralization tests suggested toxin type E was present. The incriminated fish product was imported from Canada, but smoked and packed in Finland. There was no leftover fish to test for toxin presence, and samples from the fish's plastic packaging were negative for *C. botulinum*. Although inspections of the storage temperatures throughout the entire processing chain revealed no factors that could have increased the risk of toxin production, investigators hypothesized that storage temperature abuse may have occurred at the home or at the retail market (22).

#### Source and distribution of *Clostridium botulinum* in seafood

*Clostridium botulinum* is widely distributed in soils throughout the world (34). *Clostridium botulinum* toxin type E is found mainly in aquatic environments and is primarily associated with occurrences of botulism outbreaks in seafood products (11).

However, types A-F have all been isolated from aquatic environments as well. Type E is prevalent in numerous parts of the world, including the Northern Japanese Islands, the North Sea adjacent to Scandinavia, the U.S. Great Lakes, and Alaska south to the 38<sup>th</sup> N parallel (8).

The incidence of *C. botulinum* spores in fish is extremely variable, and is highly dependent on geographical location, as well as whether the fish are farm-raised or wild-caught (8, 12, 24). For example, fish farming may increase the prevalence and number of spores present on fish. Product contamination with spores may occur when feed is heavily contaminated, if farming is conducted in ponds with earthen bottoms, and if ponds are overloaded with excess organic matter due to overfeeding (10). Surveys have also shown the incidence of *C. botulinum* in the intestines of fish caught in various parts of the Great Lakes differed from 0.7% to over 50% (8). However because botulism is an intoxication, food products must be assessed closely to determine the combination of parameters that will prevent spore germination, growth, and toxin production (11).

Fish supply both the ideal nutritional and anaerobic requirements for the growth of *C. botulinum* (34). The anatomy of fish muscle differs from that of terrestrial animals in that fish muscle cells run parallel and are connected to sheaths of connective tissue referred to as myocommata, which are attached to the skeleton and the skin (14). This provides an optimal anaerobic environment for *C. botulinum* growth and toxin production.

#### Control of *C. botulinum* toxin production

The heat-resistant spores of this bacterium survive traditional food preservation methods used to kill nonsporulating organisms. They will produce the deadly neurotoxin

under anaerobic and low acid (a pH greater than 4.6) conditions, and low solute concentrations (34). For these reasons, a 12D thermal process (i.e. a process equivalent to twelve decimal reductions in the population of *C. botulinum* spores) is employed for low acid canned foods to protect consumers from significant health risks associated with botulism (38).

The optimum temperature for growth and toxin production by nonproteolytic strains is 26-28°C. However, refrigeration will not prevent growth and toxin formation unless the temperature is strictly controlled and kept below 3°C. Therefore, foods (with the exception of canned foods) that have been processed and packaged to prevent spoilage but have not been adequately refrigerated are the most common culprits of botulism (34). Although freezing has no effect on spores, it is a proficient way to completely prevent germination and growth of *C. botulinum*. Freezing may also delay adverse effects on sensory quality in VAC packaged products (11).

*Clostridium botulinum* growth may be controlled in foods by manipulating cooking and storage temperatures, pH, water activity, salt, redox potential, and added preservatives. Proteolytic types A, B, and F can grow at a minimum temperature of 10 °C, pH of 4.6, and water activity of 0.94. Nonproteolytic types B, E, and F can grow at a minimum temperature of 3.3 °C, pH 5.0, and water activity of 0.97 (16). To safely inactivate any toxin type in concentrations of  $10^5$  LD/g present in foods, time and temperature combinations of 20 minutes at 79°C or 5 minutes at 85°C is recommended (15).

Although some aerobic bacteria may enhance the risk of toxin formation by depleting oxygen levels, the growth of other aerobic microorganisms may actually inhibit

the growth and toxin formation of *C. botulinum*. For example, certain *Bacillus* species as well as lactic acid bacteria may hinder toxin formation. However, relying solely on competition from naturally occurring microflora is not an effective way to prevent growth and toxin formation by *C. botulinum* (11).

The addition of nitrite, lactate, and sorbate may also help prevent *C. botulinum* toxin development in some foods. Although the exact mechanism is not known, nitrite inhibits or delays toxin formation. However because there have been concerns that these types of compounds may be carcinogenic, they are not widely used outside the meat industry. Lactate affects toxin production by greatly increasing the lag phase of *C. botulinum* growth, especially when used in conjunction with NaCl. Sorbate has been shown to inhibit spore germination and vegetative cell growth and because it has generally recognized as safe status in the food industry, its use is being further explored (11).

### Seafood Spoilage

#### *Microbial spoilage of seafood*

The bacterial flora present on recently caught fish depends on the environment from which the fish were harvested, rather than the fish species. For example, fish caught in cold waters contain lower numbers of bacteria, whereas fish caught in warmer waters have slightly higher bacterial counts. The microflora present on temperate water fish is dominated by psychrotrophic, Gram negative, rod-shaped bacteria such as *Pseudomonas*, *Moraxella*, *Acintebacter*, *Shewanella*, and *Flavobacterium* species. However, Gram positive bacteria such as *Micrococcus*, *Clostridium*, and *Lactobacillus* may also be found (14).

The skin of live, healthy fish or recently caught fish contains extremely low numbers of bacteria because the immune system of the fish prevents bacterial growth. However, when the fish dies, the immune system fails, and bacteria grow. During storage, bacteria invade the flesh of fish by moving between the muscle fibers (14). The large concentrations of polyunsaturated fatty acid compounds in fish make them highly susceptible to oxidation by bacteria (13, 14).

Although fish spoil at different rates, microbial activity is greatly influenced by temperature since many bacteria are unable to grow at temperatures below 10°C (14). Therefore, the quality of fresh fish has focused on the importance of temperature effect on bacteria and their activities on fish (16). The shelf life of fish is extended when fish are stored at low temperatures (14). The microflora responsible for spoilage of fresh fish changes with increased storage temperatures. Only a few hours exposure to high temperatures may accelerate spoilage (15). In fact one study showed that at ambient temperature, most fish species spoiled within 12-24 hours (16). Therefore, keeping the temperature strictly controlled throughout all steps of fish processing, from catch to distribution to the consumer, is critical (15).

#### *Organoleptic spoilage of seafood*

Sensory characteristics of fish such as appearance, odor, taste, and texture are the most important factors that determine a consumer's choice of fish (37). Spoilage of any finfish may be measured organoleptically by a sensory panel using sensory characteristics related to seafood products. The sensory process can be divided into three steps: 1) detection of a certain stimulus by human sense organs; 2) a mental evaluation and interpretation; and 3) a response by the assessor to the stimuli. Variations in the response

of the same stimuli can differ among individuals. These variations may lead to a non-conclusive answer of the test (14). The main cause of product spoilage is the generation of off-flavors and off-odors produced when bacteria metabolize proteins present in the fish (19).

When developing a sensory profile to describe the sensory characteristics of fish products, flavor and odor intensity are usually used and measured on a attribute scale. Lindstrom et al. (21) conducted sensory evaluations on VAC packaged hot-smoked rainbow trout in which the intensities of sensory attributes were rated on 11-point category scales. Intensities of flavor and odor (0 – weak; 10- strong), juiciness (0 – dry; 10 – juicy), firmness (0- soft; 10 – firm), and degree of cooking (0- undercooked; 5 – cooked to the correct degree; and 10 – overcooked) were used to describe the sensory attributes of the fish product.

The difference-from-control sensory test may also be useful in measuring organoleptic spoilage in fish and other seafood products since many people do not like the smell of fresh fish (7). This test is used to determine whether a difference exists between samples and a control and to estimate the size of any such differences. During the difference-from-control test, one sample is designated as the “control” sample, and all other samples are evaluated with regard to how different each is from the control sample. To determine the shelf life of fresh fish, information on the relative size of a difference from a control is useful (23).

#### Packaging of fresh fish

Reduced oxygen packaging is “the reduction of the amount of oxygen in a package by removing oxygen; displacing oxygen and replacing it with another gas or

combination of gases; or otherwise controlling the oxygen content to a level below that normally found in the surrounding, 21% oxygen atmosphere” (36). Reduced oxygen packaging includes VAC packaging, MAP, and controlled atmosphere packaging. Modified atmosphere is packaging “in which the atmosphere of a package of food is modified so that its composition is different from air but the atmosphere may change over time due to the permeability of the packaging material or the respiration of the food” (36).

Modified atmosphere packaging (MAP) was developed in the 1930s to lengthen the shelf life of fresh fish. In the last 25 years it has been implemented in the United Kingdom with considerable success. In the past 20 years MAP has seen considerable use for various food products in the United States (29).

Controlled atmosphere packaging is achieved when “the atmosphere of a package of food is modified so that until the package is opened, its composition is different from air, and continuous control of that atmosphere is maintained, such as by using oxygen scavengers or a combination of total replacement of oxygen, nonrespiring food, and impermeable packaging material” (36). Vacuum packaged products are products in “which air is removed from a package of food and the package is hermetically sealed so that a vacuum remains inside the package” (36). High barrier films with reduced OTRs are generally used for VAC packaging of fresh seafood products.

Studies have shown that aerobic conditions result in faster spoilage compared with VAC packaging (5). Vacuum packaging extends the shelf life of fresh fish by inhibiting or slowing the growth of aerobic spoilage bacteria by extending the lag phase. In some cases of VAC packaged fresh fish, the lag phase of the microorganisms is

extended by 12 days (30). By extending the shelf life, the product can be transported longer distances and new markets can be penetrated, thus increasing revenues. Economic losses are also reduced due to decreased spoilage (10, 29). Vacuum packaging technology may also allow processors to harvest from remote places which are rich in fish (10).

Psychrotrophic bacteria such as *Pseudomonas*, *Flavobacterium*, *Micrococcus*, and *Moraxella* produce trimethylamine, total volatile nitrogen, hypoxanthine, and ammonia – common chemical spoilage indicators. However, the growth of these common aerobic spoilage bacteria is inhibited by the anaerobic environment produced by VAC packaging (29). In VAC packaged products, residual oxygen is utilized by resident aerobic bacteria to produce carbon dioxide. This tends to cause the surface oxidation reduction potential ( $E_h$ ) to become negative. The atmospheric change and change in surface  $E_h$  will repress the growth of aerobic psychrotrophic spoilage bacteria (10). The conditions that result will favor the slow growth of facultative anaerobic organisms such as lactic acid bacteria, therefore delaying spoilage (5, 10)

#### *Safety concerns of Modified Atmosphere Packaging (MAP)*

Modified atmosphere packaging presents some unique food safety concerns, since this packaging technique is designed to maintain sensory and microbial quality for long periods of time. Some studies show the shelf life may be extended by 100% or more for fishery products that are packaged under modified atmospheres high in carbon dioxide and refrigerated at 8°C or below (29). Nevertheless, the destruction or inhibition of aerobic spoilage microorganisms can permit slow-growing Gram positive bacteria such as *Clostridium* to reach high populations in the absence of competition.



Fundamental safety concerns are related to temperature abuse of products, allowing the growth and toxin production by *C. botulinum* nonproteolytic types B, E, and F, which are naturally associated with fish (25). Psychrotrophic strains of *C. botulinum* can grow and produce toxin at temperatures as low as 3.3°C (26). Scientific literature indicates reveal that botulinum toxin production can precede or coincide with consumer rejection of the product due to obvious spoilage (6, 17, 30). Although fish contaminated with type E spores may become toxic even under aerobic conditions, it has been shown that reduced oxygen environments, such as those provided by VAC packaging, greatly increases the risk of toxin production by *C. botulinum* (10). A study by Eyles and Warth (8) showed that as storage temperatures of VAC packaged fish increased from 0°C to 10°C, the time period between products becoming unacceptable due to spoilage and toxin production is shortened (8).

One approach to address the critical safety concerns associated with VAC packaging is for fishery products to be packaged in oxygen-permeable film. Oxygen-permeable film is defined as film with an OTR of 10,000 cc/m<sup>2</sup>/24hr or greater. (Note: A reduced oxygen package is one in which the OTR is lower than 10,000 cc/m<sup>2</sup>/24 hr.) Oxygen-permeable packaging allows aerobic spoilage bacteria to grow, which may spoil the product before toxin is produced under abused temperatures. However the use of an oxygen-permeable package will not compensate for such practices such as packing product in oil or in deep containers (35).

## Analytical methods for the detection of botulinal neurotoxin

### *Mouse bioassay*

The current FDA process for determining the presence of botulinum neurotoxin is explained in detail in the Bacteriological Analytical Manual (34). This method utilizes the mouse bioassay procedure. During this procedure the biological activity of prepared toxin from food samples is measured by comparing its potency to that of a control. The current procedure outlined by FDA requires three stage testing in which mice are injected intraperitoneally and then observed for 48 hours. In the first stage, a number of sample dilutions are generated and injected into mice. The mice are then observed for 48 hours. In stage two, if the mice die of botulism symptoms, the sample is further diluted, and again injected into the mice until a dilution that does not kill the mice is found (9). Botulism symptoms in mice include ruffled fur, followed by labored breathing, weakness of limbs, and total paralysis (31). In stage 3, after the end point has been reached, the type of toxin present is determined using a toxin neutralization assay.

The mouse bioassay has several advantages over other analytical tests used to detect botulinum neurotoxin. The mouse bioassay is extremely sensitive and has been shown to detect botulinum type A neurotoxin at levels as little as 0.02 to 0.03 ng. This procedure may also be applied to a large range of toxin concentrations. The mouse bioassay will only detect toxin that is active and contamination by food, era, and fecal matter will not affect the test (31).

Because of concerns that the mouse bioassay might cause pain and suffering to animals, individuals claim that the assay should not be used without substantial justification for its use. There are also personal hazards involved when injecting animals.

The mouse bioassay is extremely time-consuming. It can take several days just to perform the assay since it requires establishing the minimum lethal dose to estimate toxicity, separate tests for the presence of botulinum neurotoxins, and neutralization assays to determine the type of botulinum toxin present (31). Since some clostridia produce toxins (unlike those produced by *C. botulinum*) that also kill mice, false positives may arise (1). This assay is also expensive and requires animal housing facilities (9). Due to the numerous disadvantages of the mouse bioassay, it cannot meet the present needs of rapid detection of the toxin, and it is impractical to use it to detect deliberate food or water contamination (31).

#### *Lateral flow device*

Lateral flow devices, also referred to as immunochromatographic assays, are rapid detection tests. The principle of the lateral flow device is that any ligand can be bound to a solid support that is detected visually, for example dyed microspheres. In these types of assays, a distinct line is created when a liquid containing the biological agent of interest is applied to a test well. The sample migrates due to capillary action along the nitrocellulose membrane. If the sample is positive, it reacts with the agent-specific labeled antibody that is bound to the membrane, and thus the visible line is shown. However, if the sample is negative (i.e. in the absence of the specific agents), no line will develop when the agent-specific antibody conjugate is bound. There are numerous advantages to the lateral flow device. It is very easy to use and results can be obtained in a short period of time, usually 15 minutes or less. Immunochromatographic assays can be used in the field where more sophisticated equipment is unavailable. The test remains

stable over time and over a wide range of climatic conditions, and is relatively inexpensive (33).

The lateral flow device does have its limits, for detecting botulinum neurotoxin, as there is limited literature available on the validity compared to other traditional assays. Immunochromatographic assays can only detect toxin levels at 10-20 ng/ml, and are less sensitive than the mouse bioassay and enzyme-linked immunosorbent assay (ELISA). The limit of detection is many times higher than the toxic dose. Lateral flow devices also may give false positives due to contaminants present in the food. Confirmatory tests and follow-up assays must be implemented when a positive result is found (33).

#### *Immunofluorescence assay and biosensors*

Biosensors are analytical devices which combine the molecular recognition capabilities of biomolecules with electronics for signal measurement. A fiber optic-based biosensor uses the formation of a fluorescent complex of the botulinum toxin, a capture antibody which has been immobilized, and a fluorescent antibody. This biosensor technique is unique because it only takes one step to rapidly detect the botulinum toxin and allows continuous monitoring. The sensor is extremely sensitive and can detect toxin type A at levels of 1 ng/ml. The fiber optics biosensor can be modified further by using fluorescence-based assays. These sensors were developed for the detection of large molecules, and have been shown to detect botulinum toxins at approximately 0.02 nM. A sensor that can simultaneously detect all serotypes of the botulinum toxin has also been invented. This particular biosensor has been used to detect botulinum toxin at 50 ng/ml.

### *Polymerase Chain Reaction*

Polymerase chain reaction (PCR) assays have also been used to detect the toxins produced by *C. boulinum* in foods. During PCR assays, DNA polymerase, an enzyme that is active only within a certain temperature range, is used in conjunction with synthetic primers to quickly amplify a target DNA sequence. First, double-stranded DNA is denatured, then each strand is annealed with primers, and finally DNA polymerase elongates the strands. Therefore, two stands of DNA can be produced from every strand. Polymerase chain reaction is extremely rapid, sensitive, and reliable. Since it does not require the organism to be cultured, it significantly reduces incubation times to determine the presence of *C. botulinum*. However, because the PCR assay only targets the botulinum neurotoxin gene, it is limited to detecting the organism carrying the gene, rather than the toxin itself. Furthermore, the primers have a broad range of melting temperatures; therefore separate tests must be completed for each toxin serotype; increasing costs and total required time of the assay.

### *Mass Spectrometry*

Botulinum neurotoxins are differentiated using mass spectrometry since each botulinum toxin serotype has a unique cleavage site on a unique peptide. Therefore the product peptides detected by mass spectrometry distinguish active serotypes. First, the botulinum toxin is incubated with substrate peptides that are specific for that serotype. Then these serotype specific product peptides are detected using mass spectrometry (1).

### *ELISA*

ELISA methods detect a variety of protein antigens from pathogenic organisms by quantifying the interaction between antigens and antibodies. The ELISA procedure

involves five steps. In the first step, the wells of a plastic microtiter plate are covered with an appropriate concentration of antibody against each protein antigen of the pathogen of interest. The antibody is absorbed by the plastic and stays attached to the well surface. During the second step, any sites on the plastic that are still able to bind protein are inhibited by the addition of an unrelated protein such as bovine serum albumin. This step reduces nonspecific binding of primary or secondary antibodies to sites in the plate wells. Food sample extracts suspected of containing the toxin antigen are added to the well during the third step so that any toxin antigen present in the food will bind to the antibody. Remaining antigens and other proteins present in the sample are removed by washing the plate with a buffer solution. The fourth step involves the detection of the bound antigen by the addition of an enzyme-conjugated antibody. The antibody binds to the antigen but does not react with the blocking protein. In the last step of the ELISA, the substrate for the enzyme bound to the antibody is added to the wells. The amount of enzyme-conjugated antibody bound in each well can be measured by how well the enzyme can hydrolyze a colorless substrate to give a colored product. This color intensity can be measured quantitatively using a spectrophotometer (33).

ELISA is a method that is rapid, sensitive, and specific. The total time required to run a set of samples by ELISA, including preparation of the sample, is approximately 5 or 6 hours. It requires less time than the traditional mouse bioassay and does not require trypsin activation (33). An experiment conducted by Joseph Ferreira, FDA, determined that “all botulinal toxigenic strains found positive by the mouse bioassay were also positive with the amp-ELISA” (9). Moreover, the FDA has developed an ELISA that can detect *C. botulinum* neurotoxins A, B, E, and F (33, 34).

However, ELISA may give false positive and false negative results. For example, false positives may occur if the step to prevent nonspecific binding is not completed correctly. False positives may also arise if the substrate is contaminated or exposed to light. False negatives may occur if the plate is not initially coated with the correct concentration of antibodies. False negatives can also be due to microtiter plate wells being scratched by pipette tips or washing tips. When using ELISA, results may need to be verified using the mouse bioassay (1).

#### *Electrochemiluminescence (ECL)*

Electrochemiluminescence involves the attachment of antibodies on magnetic particles in a large volume of sample suspension. The antibodies are then trapped and bound in a small area. In this sense it is unlike ELISA where antibodies are directly bound to a polystyrene or polyvinyl microplate (33).

Electrochemiluminescence is more sensitive than other methods due to the high luminescent signal-to-noise ratios. When ECL is compared to ELISA, the magnetic beads are found to have a larger surface area, which provides for a large amount of antibody immobilization. In contrast to the ELISA, the beads are not fixed, and this enhances the kinetics of the antibody-antigen reaction. Electrochemiluminescence allows a large number of food samples to be tested in a short period of time. Although ECL may seem like an ideal method to test for botulinum neurotoxin, its validity has not been authenticated in a wide variety of food products, particularly solid and semi-solid foods. It has also received limited development for its use in detecting the toxin produced by *C. botulinum* (33).

## REFERENCES

1. Barr, J. R., H. Moura, A. E. Boyer, A. R. Woolfitt, S. R. Kalb, A. Pavlopoulos, L. G. McWilliams, J. G. Schmidt, R. A. Martinez, and D. L. Ashley. 2005. Botulinum neurotoxin detection and differentiation by mass spectrometry. *Emerg. Infect. Dis.* 11:1578-1583.
2. Centers for Disease Control and Prevention. 1987. International outbreak of type E botulism associated with ungutted, salted whitefish. *Morb. Mortal. Wkly. Rep.* 36:812-813.
3. Centers for Disease Control and Prevention. 1991. Epidemiologic notes and reports fish botulism - Hawaii, 1990. *Morb. Mortal. Wkly. Rep.* 40:412-414.
4. Centers for Disease Control and Prevention. 1992. Outbreak of type E botulism associated with an uneviscerated, salt-cured fish product--New Jersey, 1992. *Morb. Mortal. Wkly. Rep.* 41:521-522.
5. Dondero, M., F. Cisternas, L. Carvajal, and R. Simpson. 2004. Changes in quality of vacuum-packed cold-smoked salmon (*Salmo salar*) as a function of storage temperature. *Food Chem.* 87:543-550.
6. Dufresne, I., J. P. Smith, J. N. Liu, and I. Tarte. 2000. Effect of films of different oxygen transmission rate on toxin production by *Clostridium botulinum* type E in vacuum packaged cold and hot smoked trout fillets. *J. Food Saf.* 20:251-268.
7. Duncan, S. 2006. Personal communication.
8. Eyles, M. J. and A. D. Warth. 1981. Assessment of the risk of botulism from vacuum-packaged raw fish: a review. *Food Technol. Aust.* 33:574-580.
9. Ferreira, J. L. 2001. Comparison of amplified ELISA and mouse bioassay procedures for determination of botulinum toxins A, B, E, and F. *J. AOAC Int.* 84:85-88.
10. Genigeorgis, C. A. 1985. Microbial and safety implications of the use of modified atmospheres to extend the storage life of fresh meat and fish. *Int. J. Food Microbiol.* 1:237-251.
11. Gram, L. 2001. Potential hazards in cold-smoked fish: *Clostridium botulinum* type E. *J. Food Sci.* 66:S1082-S1087.
12. Hielm, S., J. Bjorkroth, E. Hyttia, and H. Korkeala. 1998. Prevalence of *Clostridium botulinum* in Finnish trout farms: pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. *Appl. Environ. Microbiol.* 64:4161-4167.
13. Huss, H. H. 1994. Assurance of seafood quality, p. 46-48. Food and Agriculture Organization, Rome.
14. Huss, H. H. 1995. Quality and quality changes in fresh fish, p. 11, 51-72, 130-131. Food and Agriculture Organization, Rome.
15. Huss, H. H., L. Ababouch, and L. Gram. 2004. Assessment and management of seafood safety and quality, p. 31-34, 178. Food and Agriculture Organization, Rome.
16. Huss, H. H., M. Jakobsen, and J. Liston. 1992. Quality assurance in the fish industry, p. 93-95, 199-200. Elsevier, Amsterdam.



17. Ikawa, J. Y. and C. Genigeorgis. 1987. Probability of growth and toxin production by nonproteolytic *Clostridium botulinum* in rockfish fillets stored under modified atmospheres. *Int. J. Food Microbiol.* 4:167-181.
18. Korkeala, H., G. Stengel, E. Hyytia, B. Vogelsang, A. Bohl, H. Wihlman, P. Pakkala, and S. Hielm. 1998. Type E botulism associated with vacuum-packaged hot-smoked whitefish. *Int. J. Food Microbiol.* 43:1-5.
19. Koutsoumanis, K. and G. J. Nychas. 2000. Application of a systematic experimental procedure to develop a microbial model for rapid fish shelf life predictions. *Int. J. Food Microbiol.* 60:171-184.
20. LaBombardi, V. J., A. M. O'Brien, and J. W. Kislak. 2002. Pseudo-outbreak of *Mycobacterium fortuitum* due to contaminated ice machines. *Am. J. Infect. Control.* 30:184-186.
21. Lindstrom, M., M. Nevas, S. Hielm, L. Lahteenmaki, M. W. Peck, and H. Korkeala. 2003. Thermal inactivation of nonproteolytic *Clostridium botulinum* type E spores in model fish media and in vacuum-packaged hot-smoked fish products. *Appl. Environ. Microbiol.* 69:4029-4036.
22. Lindstrom, M., M. Vuorela, K. Hinderink, H. Korkeala, E. Dahlsten, M. Raahenmaa, and M. Kuusi. 2006. Botulism associated with vacuum-packed smoked whitefish in Finland, June-July 2006. *Euro. Surveill.* 11:E060720-060723.
23. Meilgaard, M., Vance Civille, G., Carr, B.T. 1999. Sensory evaluation techniques, p. 87-94. CRC Press, Boca Raton.
24. Merivirta, L. O., M. Lindstrom, K. J. Bjorkroth, and H. J. Korkeala. 2006. The prevalence of *Clostridium botulinum* in European river lamprey (*Lampetra fluviatilis*) in Finland. *Int. J. Food Microbiol.* 109:234-237.
25. National Advisory Committee on the Microbiological Criteria for Foods (NACMCF). 1992. Vacuum or modified atmosphere packaging for refrigerated raw fishery products. Report of the NACMCF. Adopted March 20, 1992. In NACMCF, U.S. Food and Drug Administration, Washington, D.C.
26. National Advisory Committee on the Microbiological Criteria for Foods (NACMCF). 1993. Report on vacuum packaging and associated processes. In NACMCF, U.S. Food and Drug Administration, London.
27. Panwalker, A. P. and E. Fuhse. 1986. Nosocomial *Mycobacterium gordonae* pseudoinfection from contaminated ice machines. *Infect. Control.* 7:67-70.
28. Post, L. S., D. A. Lee, M. Solberg, D. Furgang, J. Specchio, and C. Graham. 1985. Development of botulinal toxin and sensory deterioration during storage of vacuum and modified atmosphere packaged fish fillets. *J. Food Sci.* 50:990-998.
29. Reddy, N. R. and D. J. Armstrong. 1992. Shelf-life extension and safety concerns about fresh fishery products packaged under modified atmospheres: a review. *J. Food Saf.* 12:87-118.
30. Reddy, N. R., H. M. Solomon, H. Yep, M. G. Roman, and E. J. Rhodehamel. 1997. Shelf life and toxin development by *Clostridium botulinum* during storage of modified-atmosphere-packaged fresh aquacultured salmon fillets. *J. Food Prot.* 60:1055-1063.

31. Scarlatos, A., B. A. Welt, B. Y. Cooper, D. Archer, T. DeMarse, and K. V. Chau. 2005. Methods for detecting botulinum toxin with applicability to screening foods against biological terrorist attacks. *J. Food Sci.* 70:121-130.
32. Shapiro, R. L., C. Hatheway, and D. L. Swerdlow. 1998. Botulism in the United States: a clinical and epidemiologic review. *Ann. Intern. Med.* 129:221-228.
33. Sharma, S. K. and R. C. Whiting. 2005. Methods for detection of *Clostridium botulinum* toxin in foods. *J. Food Prot.* 68:1256-1263.
34. Soloman, H. M. and J. T. Lilly. 2001. *Clostridium botulinum*. In, Bacteriological analytical manual, 8th ed. AOAC International, Arlington, Va.
35. U.S. Food and Drug Administration. 2001. *Clostridium botulinum* toxin formation, chap. 13. In, Fish and fisheries products hazards and controls guidance, 3rd ed. U.S. Food and Drug Administration. Available at: <http://www.cfsan.fda.gov/~comm/haccp4.html>.
36. U.S. Food and Drug Administration. 2001. Reduced oxygen packaging, chap. 1. In, Food Code. U.S. Food and Drug Administration. Available at: <http://www.cfsan.fda.gov/~dms/fc01-1.html#1-1>.
37. Warm, K., J. Nelsen, and G. Hyldig. 2000. Sensory quality criteria for five fish species. *J. Food Quality.* 23:583-601.
38. Warne, D. 1988. Manual on fish canning. Food and Agriculture Organization, Rome.

## Chapter II

Spoilage and shelf life of refrigerated reduced oxygen packaged Atlantic croaker

*(Micropogonias undulatus)*

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## ABSTRACT

The purpose of this study was to determine the effects of storage temperature and film oxygen transmission rate (OTR) on microbial shelf life and organoleptic shelf life in refrigerated raw vacuum packaged croaker fillets. Raw croaker fillets were vacuum packaged in oxygen-permeable films (OTR of 10,000 cc/m<sup>2</sup>/24hr or 3,000 cc/m<sup>2</sup>/24hr) and stored at either 4°C or 10°C. For the microbial shelf life study, psychrotrophic plate counts were determined for up to 12 days storage; and microbial spoilage was defined as at least 7.0 log CFU/g. For the organoleptic shelf life study, the odor difference was compared with a control sample, and organoleptic spoilage was defined as a rating of at least 3.0 on the difference-from-control scale. For both microbial and organoleptic spoilage, the effect of temperature on spoilage was statistically significant ( $P < 0.05$ ). For example, the microbial shelf life of the vacuum packaged fish was extended by 4 days when stored at 4°C versus 10°C, while the organoleptic shelf life of the vacuum packaged fish was extended by 3 days when stored at 4°C versus 10°C. When both temperatures were examined, the effect of film type on microbial spoilage was not statistically significant ( $P > 0.05$ ). At 4°C and 10°C, the 3,000 cc/m<sup>2</sup>/24hr OTR film extended the microbial shelf life by 1 day. For the organoleptic shelf life study at both 4°C and 10°C, the effect of film type on organoleptic spoilage was also not statistically significant ( $P > 0.05$ ). At 4°C and 10°C, in this case, the 10,000 cc/m<sup>2</sup>/24hr OTR extended the organoleptic shelf life of the vacuum packaged fillets by 1 day. These data suggest that the most important factor affecting the microbial and organoleptic shelf life of vacuum packaged croaker fillets is temperature.

## INTRODUCTION

Fresh fish is a natural environment for growth of psychrotrophic spoilage microorganisms. When lipids and other seafood proteins are degraded by these microorganisms, the result is a highly pronounced off-flavor and off-odor, resulting in a short shelf life and monetary losses for seafood companies. In the past, ice and refrigeration were the most common methods used to hinder microbial and biochemical spoilage (13). However, there have been reports that ice harbors bacteria, and may contaminate the fresh fish when it melts, thus accelerating spoilage and decreasing shelf life (6, 12).

Alternative methods to increase the shelf life of fish include the use of vacuum (VAC) packaging. Vacuum packaging refers to packaging in containers where all air has been removed prior to the final sealing of the container. There is considerable interest in VAC packaging technology for raw fish to extend shelf life by preventing the growth of naturally occurring, aerobic spoilage microorganisms. Increased shelf life allows the fresh fish market to expand to new markets beyond the coastal regions (11).

The FDA has mandated that refrigerated fishery products be packaged in film providing an oxygen transmission rate (OTR) of 10,000 cc/m<sup>2</sup>/24hr to allow microbial spoilage before possible *Clostridium botulinum* toxin development (15). Additional research is needed, however, to determine if films with lower OTRs significantly extend the shelf life of fresh fish, and increase the safety risks associated with possible toxin formation from *C. botulinum* preceding spoilage.

## **MATERIALS AND METHODS**

**A. Fish** Atlantic croaker (*Micropogonias undulatus*) were pound netted from the Chesapeake Bay near Whitestone, VA by commercial fishermen. Whole fish were placed on ice and offloaded in Hampton, VA. The fish were transported on ice in coolers to the Department of Food Science and Technology at Virginia Tech in Blacksburg, VA, and held frozen at -20°C until ready to be filleted. After thawing at 4°C for 24 hours, the fish were aseptically hand filleted and skinned. Each fillet was rinsed with sterile distilled water to remove excess blood. Two fillets were obtained from each fish.

**B. Vacuum packaging** Individual fish fillets (~ 30-100 g) were vacuum packaged in oxygen-permeable packaging material with a guaranteed oxygen transmission rate (OTR) of 10,000 cc/m<sup>2</sup>/24hr or 3,000 cc/m<sup>2</sup>/24hr. The 10,000 cc/m<sup>2</sup>/24hr packaging material was a highly permeable multilayered, coextruded polyolefin formulation (Cryovac<sup>®</sup> HP2700 bag, Duncan, SC). The 3,000 cc/m<sup>2</sup>/24hr packaging material was a multilayered, coextruded polyolefin with high shrink energy (Cryovac<sup>®</sup> FC-805 bag, Duncan, SC). Individual fish fillets were vacuum packed at 99% vacuum for 2.4 second duration of sealing using an Ultravac<sup>®</sup> vacuum packager (KOCH Industries, Kansas City, MO). All packages maintained their reduced oxygen atmosphere throughout sampling.

### **C. Shelf life studies**

**1. Microbial spoilage** Psychrotrophic plate counts were conducted for the vacuum packaged fillets (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr film type) after storage at one of two temperatures (4°C or 10°C). For fillets stored at 4°C, samples were

analyzed at days 0, 2, 4, 6, 8, 10, and 12. For fillets stored at 10°C, samples were analyzed at days 0, 1, 2, 3, and 4. Sampling days were selected to represent 0, 75, 100, and 125% estimated shelf life (1). Three different individual fish fillets per film type were used per sampling day.

For this study, microbial spoilage (i.e. end of shelf life) was defined as a psychrotrophic plate count of at least 7.0 log CFU/g (5). Following the analytical procedures outlined in the Food and Drug Administration Bacteriological Analytical Manual, samples were pulverized in a laboratory stomacher (Seward Stomacher® 400 Circulator, Worthing, UK) for 2 min at 230 rpm with 99 mL of Butterfield's buffer (Biotrace International, Munice, IN). Three consecutive 10 fold dilutions per sample were plated on Aerobic Plate Count Petrifilm® (3M Microbiology, St. Paul, MN). Petrifilms® were incubated at 10°C for 7 days prior to enumeration (8).

**2. Organoleptic spoilage** The difference-from-control test was used to determine the organoleptic shelf life of the vacuum packaged fillets (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr film types) after storage at 4°C or 10°C. For fillets stored at 4°C, sensory sessions were conducted on days 2, 4, 6, 8, 10, and 12. For fillets stored at 10°C, sensory sessions were conducted at days 1, 2, 3, and 4. Three different individual fish fillets per film type were used per sampling day.

For this study, organoleptic spoilage was defined as a minimum score of 3.0 on a six-point difference from control scale (Table 1). Following difference-from-control test methods, subjects were presented a control sample (Day 0 sample) and two test samples (fillets vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr and 3,000

cc/m<sup>2</sup>/24hr) (9). Control fillets were held frozen (-40°C) until the day of the sensory analysis and were allowed to thaw and equilibrate at 4°C. Each of the three fish fillets were cut into 10 equal portions and presented to panelists in 2 oz. Soufflé Cups (Mongram Company, Columbia, MD) which were stored on crushed ice.

Thirty untrained panelists were used per sensory session. Subjects were first asked to pick up the control sample (labeled C) with their right hand, hold it 6 inches from their nose, use their left hand to fan odor towards their nose, and smell the control sample. If an odor could not be identified, the sample was brought closer to the nose. Subjects were then asked to repeat the steps mentioned above for the samples vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr (labeled with a 3-digit code). A six attribute descriptive scale was used to determine the difference in odor from the control. Finally, subjects were asked to mark the scale to indicate the overall size of the difference in odor relative to the control sample (9).

## **RESULTS**

### *Microbial spoilage shelf life study*

Inverse prediction was used to predict the time at which microbial spoilage occurred (i.e. when the microbial count reached at least 7.0 log CFU/g). At 4°C, Atlantic croaker fillets vacuum packaged in film with an oxygen transmission rate (OTR) of 10,000 cc/m<sup>2</sup>/24hr reached a microbial log count of 7.0 CFU/g at 7 days, while fillets vacuum packaged film providing a 3,000 cc/m<sup>2</sup>/24hr OTR reached this microbial log count at 8 days. At 10°C, Atlantic croaker fillets vacuum packaged in film providing an OTR of 10,000 cc/m<sup>2</sup>/24hr reached a microbial log count of 7.0 CFU/g at 3 days, while



fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film became microbially spoiled at 4 days (Table 2). When storage temperature was examined independently of film type, fish fillets stored at 4°C reached a microbial log count of 7.0 CFU/g at 7 days while fillets stored at 10°C reached a microbial log count of 7.0 CFU/g at 3 days.

For storage at 4°C, the effect of film type on microbial spoilage was not statistically significant ( $P = .09$ ), which indicates that microbial spoilage is not significantly affected by film type (Figure 1). For storage at 10°C, the effect of film type on microbial spoilage was also not statistically significant ( $P = .41$ ), again indicating that microbial spoilage is not significantly affected by film type (Figure 2). The effect of temperature on microbial spoilage was highly statistically significant ( $P < 0.01$ ). This indicates that microbial spoilage is significantly and substantially affected by storage temperature (Figure 3).

#### *Organoleptic spoilage shelf life study*

Inverse prediction was used to predict the time at which organoleptic spoilage occurred (i.e. when the difference-from-control rating reached at least 3.0). At 4°C, Atlantic croaker fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film reached a difference-from-control rating of at least 3.0 at 10 days, while fillets vacuum packaged in the film providing an OTR of 3,000 cc/m<sup>2</sup>/24hr reached organoleptic spoilage at 9 days. Although sensory sessions were only conducted up to 4 days, inverse prediction was used to determine that at 10°C, Atlantic croaker fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr OTR film reached a difference-from-control rating of at least 3.0 at 6 days (Table 3). When storage temperature was examined independently of film type, croaker fillets stored at 4°C reached a difference-from-control rating of at least

3.0 at 9 days, while fillets stored at 10°C reached a difference-from-control rating of at least 3.0 at 6 days (Note: As previously mentioned sensory sessions were only conducted up to 4 days; inverse prediction was used to predict organoleptic spoilage).

For storage at 4°C, the effect of film type on the difference-from-control rating (i.e. organoleptic spoilage) was not statistically significant ( $P = .74$ ), which indicates that organoleptic spoilage is not significantly affected by film type (Figure 4). For storage at 10°C, the interactions between film type and organoleptic spoilage were not statistically significant ( $P = .81$ ), indicating that organoleptic spoilage is not significantly affected by film type (Figure 5). The effect of temperature on organoleptic spoilage was statistically significant ( $P = 0.04$ ), which indicates that organoleptic spoilage is somewhat affected by storage temperature (Figure 6).

## **DISCUSSION**

### *Microbial spoilage shelf life study*

The purpose of microbial shelf life studies in fish is to determine the possible presence of bacteria in order to give an overall sense of quality of the product. Microbiological examinations of fresh fish may provide insight into the overall cleanliness during harvesting and processing, as well as whether or not temperature abuse has occurred (4). As anticipated, the results of the current study show that the microbial shelf life of Atlantic croaker fillets vacuum packaged in both film types (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr) decreased as the storage temperature and storage time increased. As the temperature and the storage time increased, microbial log counts increased, leading to microbial spoilage (defined as a count of at least 7.0 log CFU/g).

These results are consistent with a similar study done by Reddy et al. which found that microbial populations of fish packaged in modified atmospheres stored at 8°C and 16°C increased more rapidly than fillets stored at 4°C (14). A study done by Lalitha and Gopakumar also found that as the storage temperature is lowered, the lag phase of microbial growth is extended (7). When the two storage temperatures (4°C and 10°C) were compared, the microbial shelf life of fish stored at 4°C was extended by 4 days. It is interesting to note that contrary to what was anticipated, this study showed that at both 4°C and 10°C, film type had little effect on the microbial shelf life of the vacuum packaged croaker fillets. In fact, at 4°C, when compared to fish fillets packaged in film with an OTR 10,000 cc/m<sup>2</sup>/24hr, fish fillets vacuum packaged in film with an OTR of 3,000 cc/m<sup>2</sup>/24hr had an extended microbial shelf life of 1 day. Similar results were also found for fish fillets stored at 10°C. Other studies have shown that the most important factor affecting the microbial shelf life of fresh fish is temperature (3, 10).

#### *Organoleptic spoilage shelf life studies*

Because the consumer is the final judge of fish, sensory evaluations must be used to assess the quality of fresh fish. However, people can vary widely in their responses to odor stimuli (4). Although sensory sessions were only conducted for up to 4 days, and the product did not reach organoleptic spoilage within this time frame, inverse prediction could be used to estimate the organoleptic shelf life.

The results of the organoleptic shelf life study were consistent with those of the microbial shelf life study in that the organoleptic shelf life of Atlantic croaker fillets vacuum packaged in both film types (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr) increased as the storage temperature and storage time decreased. Lower sensory scores were seen

at the lower temperature and shorter storage times. In fact, the organoleptic shelf life was extended by 3 days when vacuum packaged croaker fillets were stored at 4°C versus 10°C. These results are consistent with other studies that also showed that as temperature and storage time increased, organoleptic shelf life decreased (2, 14). At 4°C film type had a slight effect on the organoleptic shelf life of the vacuum packaged fillets. The croaker fillets vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr had an increased organoleptic shelf life of 1 day versus the fillets packaged in a film with an OTR of 3,000 cc/m<sup>2</sup>/24hr. These results differ from a similar study conducted by Dufresne et al. which found that vacuum packaging hot and cold smoked trout in films with a lower OTR (5,000 cc/m<sup>2</sup>/24hr) significantly increased the organoleptic shelf life of the fish when compared to films with an OTR of 10,000 cc/m<sup>2</sup>/24hr. At 10°C film type also had little effect on the organoleptic shelf life of the vacuum packaged fillets.

Because often sensory panelists do not like the smell of fish, the difference-from-control method can be a useful tool for predicting the shelf life of fishery products. However because the samples were presented to panelists in such small portions, this may have affected the results of the organoleptic shelf life study. In addition, because the samples were presented on ice, this may have also masked potential odors. If the samples were presented to panelists at a warmer temperature, they could possibly reject the samples sooner. Perhaps if different sized portions, a different rating scale, or different sensory test were used, the results may have been different.

### *Conclusion*

These data suggest that microbial and organoleptic spoilage of croaker fillets is significantly affected by storage temperature. Both the microbial and organoleptic shelf

life of vacuum packaged croaker fillets were not significantly affected by film type at both temperatures (4°C and 10°C). In this study, microbial spoilage generally occurred 2-3 days earlier than organoleptic spoilage, regardless of storage temperature or film OTR. The results of this study show that because film type does not substantially affect the microbial or organoleptic shelf life of vacuum packaged croaker fillets, lower OTR films may be used. Safety risks associated with possible toxin formation of *C. botulinum* preceding spoilage may not be a concern since spoilage occurs at approximately the same time for both film types (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr).

## REFERENCES

1. Arritt, F. M., J. D. Eifert, M. L. Jahncke, M. D. Pierson, and R. C. Williams. 2007. Effects of modified atmosphere packaging on toxin product by *Clostridium botulinum* in raw aquacultured Summer Flounder fillets (*Paralichthys dentatus*). *J. Food Prot.* 70:in press.
2. Dufresne, I., J. P. Smith, J. N. Liu, and I. Tarte. 2000. Effect of films of different oxygen transmission rate on toxin production by *Clostridium botulinum* type E in vacuum packaged cold and hot smoked trout fillets. *J. Food Saf.* 20:251-268.
3. Gelman, A., L. Glatman, V. Drabkin, and S. Harpaz. 2001. Effects of storage temperature and preservative treatment on shelf life of the pond-raised freshwater fish, silver perch (*Bidyanus bidyanus*). *J. Food Prot.* 64:1584-1591.
4. Huss, H. H. 1995. Quality and quality changes in fresh fish, p. 11, 51-72, 130-131. Food and Agriculture Organization, Rome.
5. International Commission on Microbiological Specifications for Foods (ICMSF). 1978. p. 92-104. *In* Microorganisms in foods. 2. Sampling for microbial analysis: principle and specific applications. University of Toronto Press, Toronto, Ontario, Canada.
6. LaBombardi, V. J., A. M. O'Brien, and J. W. Kislak. 2002. Pseudo-outbreak of *Mycobacterium fortuitum* due to contaminated ice machines. *Am. J. Infect. Control.* 30:184-186.
7. Lalitha, K. V. and K. Gopakumar. 2001. Growth and toxin production by *Clostridium botulinum* in fish (*Mugil cephalus*) and shrimp (*Penaeus indicus*) tissue homogenates stored under vacuum. *Food Microbiology.* 18:651-657.
8. Maturin, L. J. and J. T. Peeler. 2001. Aerobic plate count, chap. 3. *In*, Bacteriological analytical manual, 8th ed. AOAC International, Arlington, VA.
9. Meilgaard, M., Vance Civille, G., Carr, B.T. 1999. Sensory evaluation techniques, p. 87-94. CRC Press, Boca Raton.
10. Molin, G. and I. M. Stenstrom. 1984. Effect of temperature on the microbial flora of herring fillets stored in air or carbon dioxide. *J Appl Bacteriol.* 56:275-282.
11. National Advisory Committee on the Microbiological Criteria for Foods (NACMCF). 1993. Report on vacuum packaging and associated processes. *In* NACMCF, U.S. Food and Drug Administration, London.
12. Panwalker, A. P. and E. Fuhse. 1986. Nosocomial *Mycobacterium gordonae* pseudoinfection from contaminated ice machines. *Infect. Control.* 7:67-70.
13. Reddy, N. R. and D. J. Armstrong. 1992. Shelf-life extension and safety concerns about fresh fishery products packaged under modified atmospheres: a review. *J. Food Saf.* 12:87-118.
14. Reddy, N. R., H. M. Solomon, H. Yep, M. G. Roman, and E. J. Rhodehamel. 1997. Shelf life and toxin development by *Clostridium botulinum* during storage of modified-atmosphere-packaged fresh aquacultured salmon fillets. *J. Food Prot.* 60:1055-1063.
15. U.S. Food and Drug Administration. 2001. *Clostridium botulinum* toxin formation, chap. 13. *In*, Fish and fisheries products hazards and controls guidance, 3rd ed. U.S. Food and Drug Administration. Available at: <http://www.cfsan.fda.gov/~comm/haccp4.html>.

Table 1. *Organoleptic difference-from-control rating definitions for raw vacuum packaged croaker fillets (Micropogonias undulatus)<sup>a</sup>*

| Rating | Odor difference from control |
|--------|------------------------------|
| 0      | No difference                |
| 1      | Very small difference        |
| 2      | Slight/moderate difference   |
| 3      | Moderate                     |
| 4      | Moderate/large difference    |
| 5      | Large difference             |
| 6      | Very large difference        |

<sup>a</sup> Variation of table in Meilgaard et al (9).

Table 2. *Microbial spoilage in raw vacuum packaged croaker fillets (Micropogonias undulatus)*

| Storage Temperature | Packaging Film OTR             | Approximate day to microbial spoilage <sup>a</sup> |
|---------------------|--------------------------------|--|
| 4°C                 | 10,000 cc/m <sup>2</sup> /24hr | 7  |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 8  |
| 10°C                | 10,000 cc/m <sup>2</sup> /24hr | 3  |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 4  |

<sup>a</sup> APC,  $\geq 10^7$  CFU/g



Table 3. *Organoleptic spoilage in raw vacuum packaged croaker fillets (Micropogonias undulatus)*

| Storage Temperature | Packaging Film OTR             | Approximate day to organoleptic spoilage <sup>a</sup> |
|---------------------|--------------------------------|---|
| 4°C                 | 10,000 cc/m <sup>2</sup> /24hr | 10  |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 9   |
| 10°C                | 10,000 cc/m <sup>2</sup> /24hr | > 4 <sup>b</sup>                                      |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | > 4 <sup>b</sup>                                      |

<sup>a</sup> Difference-from-control rating  $\geq 3.0$

<sup>b</sup> Sensory sessions were conducted only up to day 4 – inverse prediction was used to approximate day to organoleptic spoilage as 6 for both film types

Figure 1. 4°C storage - the interactions between film type and microbial growth (i.e. spoilage) (n=3)

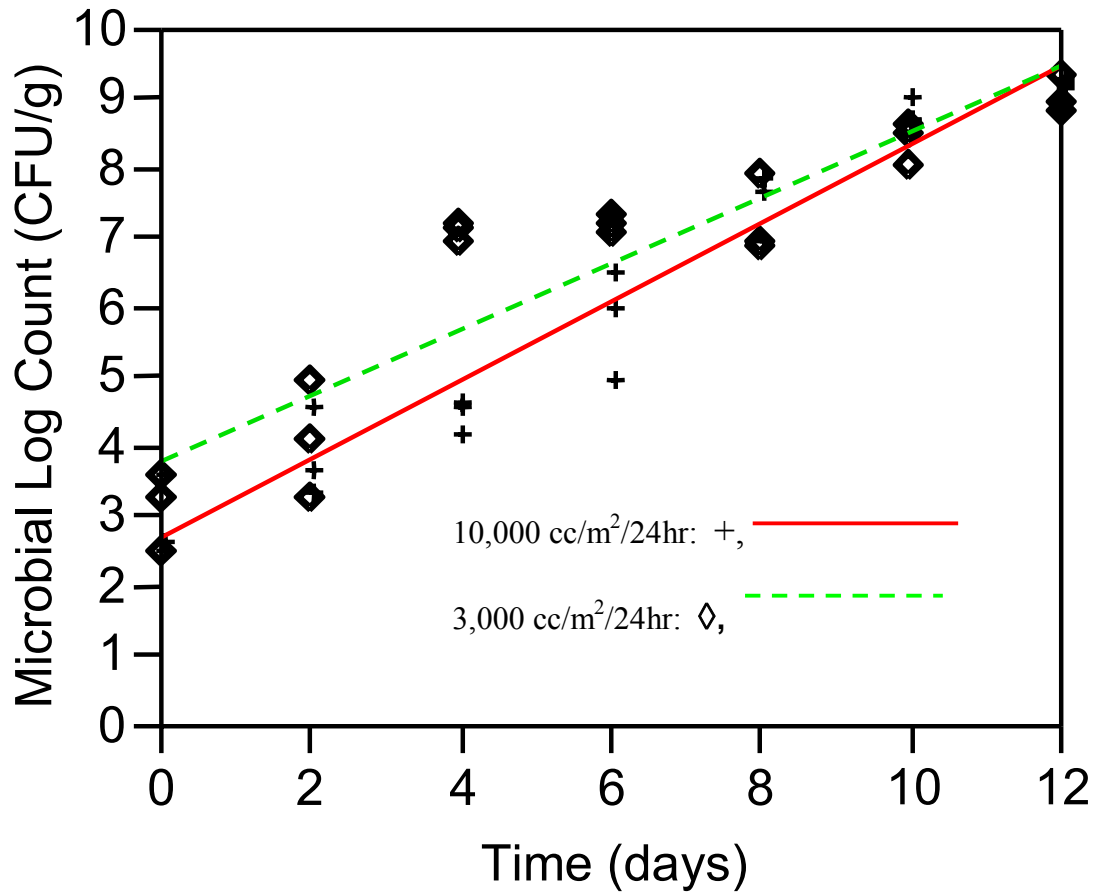


Figure 2. 10°C storage - the interactions between film type and microbial growth (i.e. spoilage) (n=3)

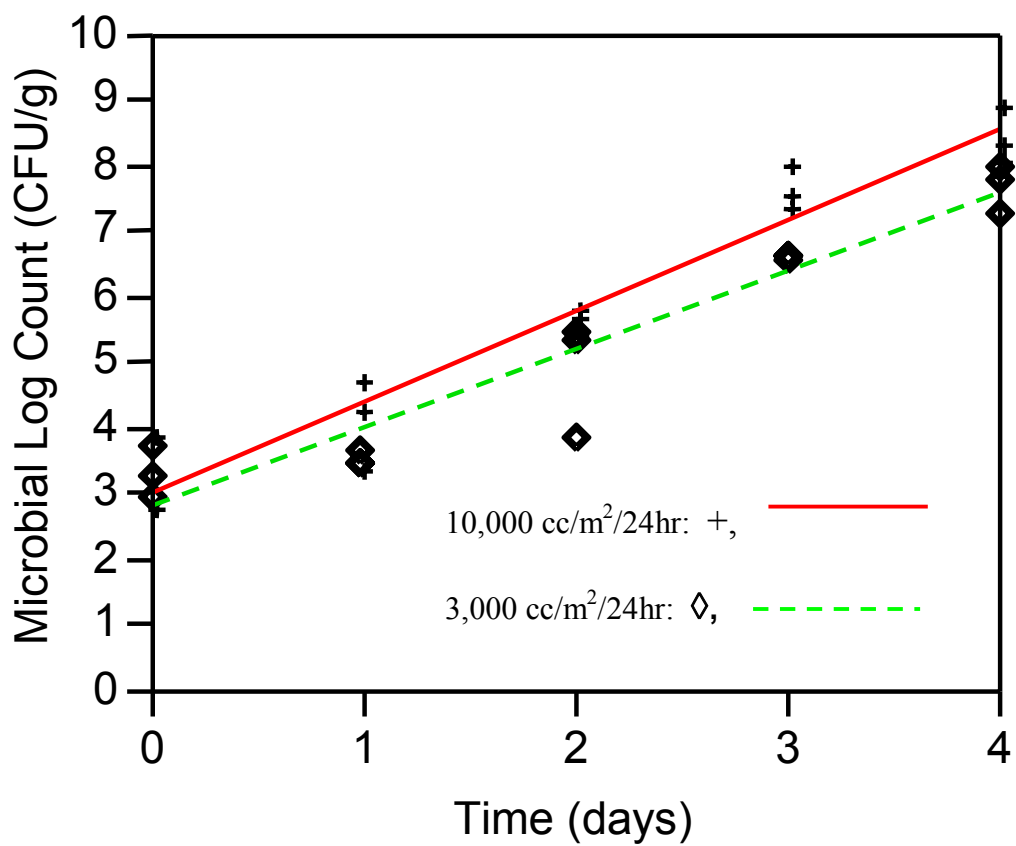


Figure 3. *The interactions between temperature and microbial growth (i.e. spoilage)*  
( $n=3$ )

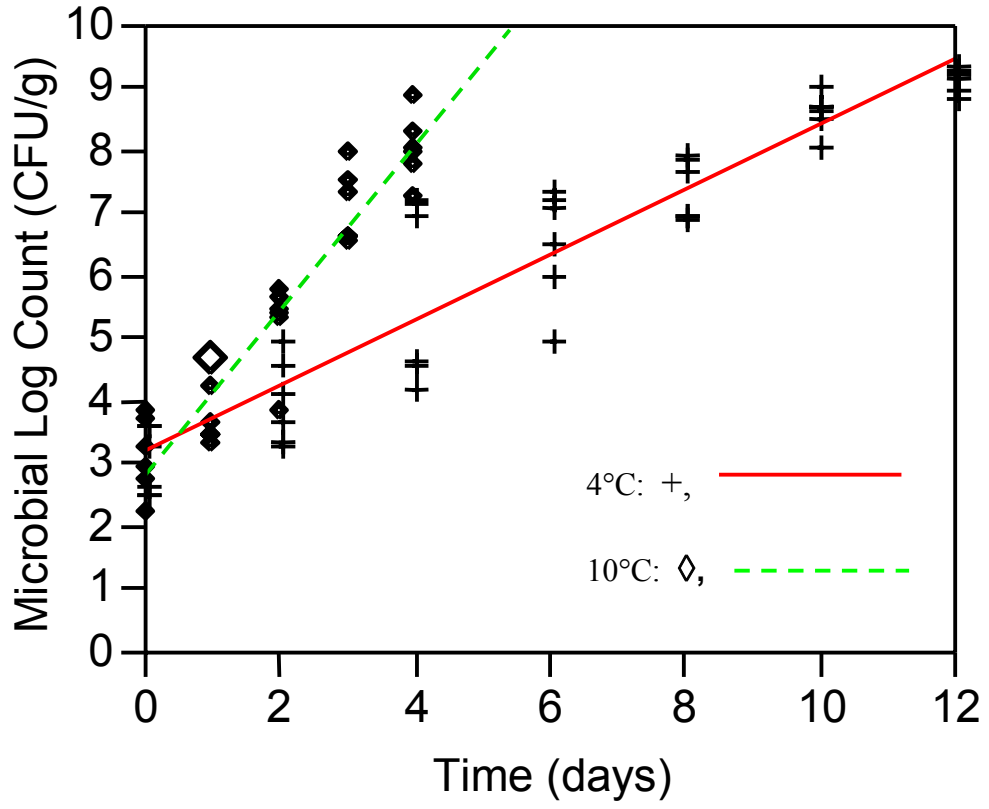


Figure 4. 4°C storage - the interactions between film type and organoleptic spoilage (n=10 panelists per fillet)

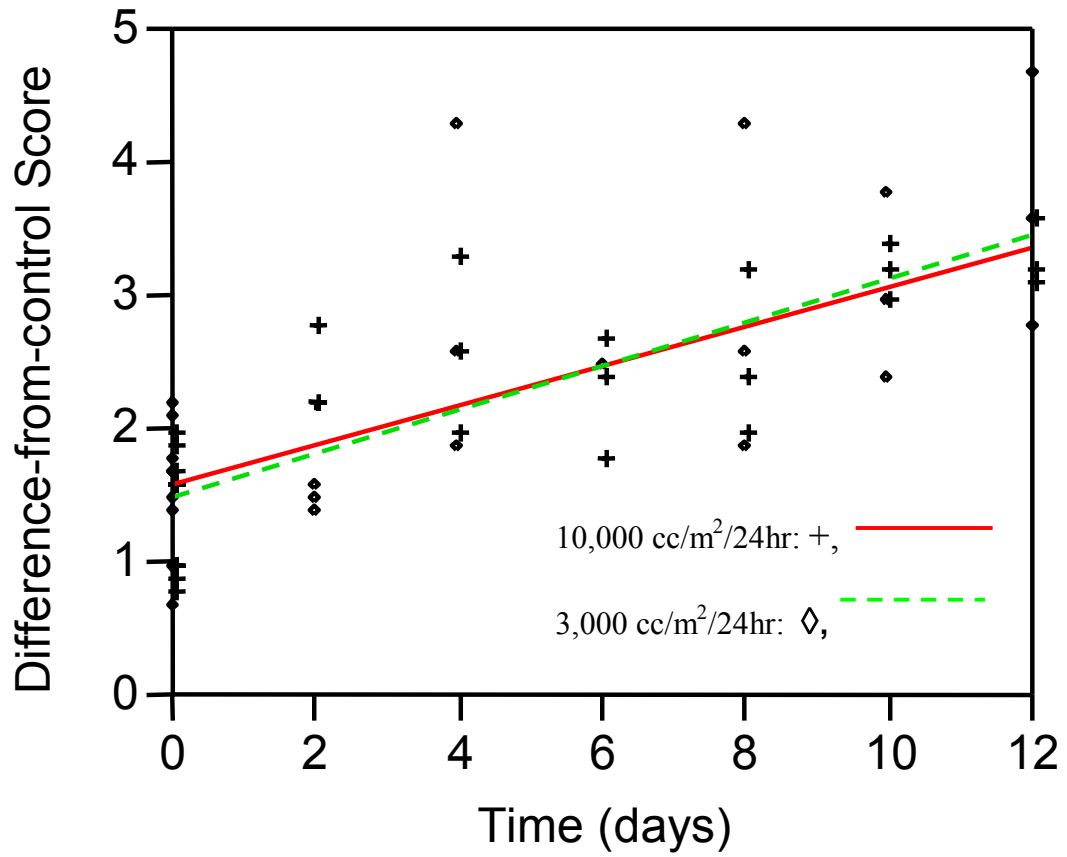


Figure 5. 10°C storage - the interactions between film type and organoleptic spoilage (n=10 panelists per fillet)

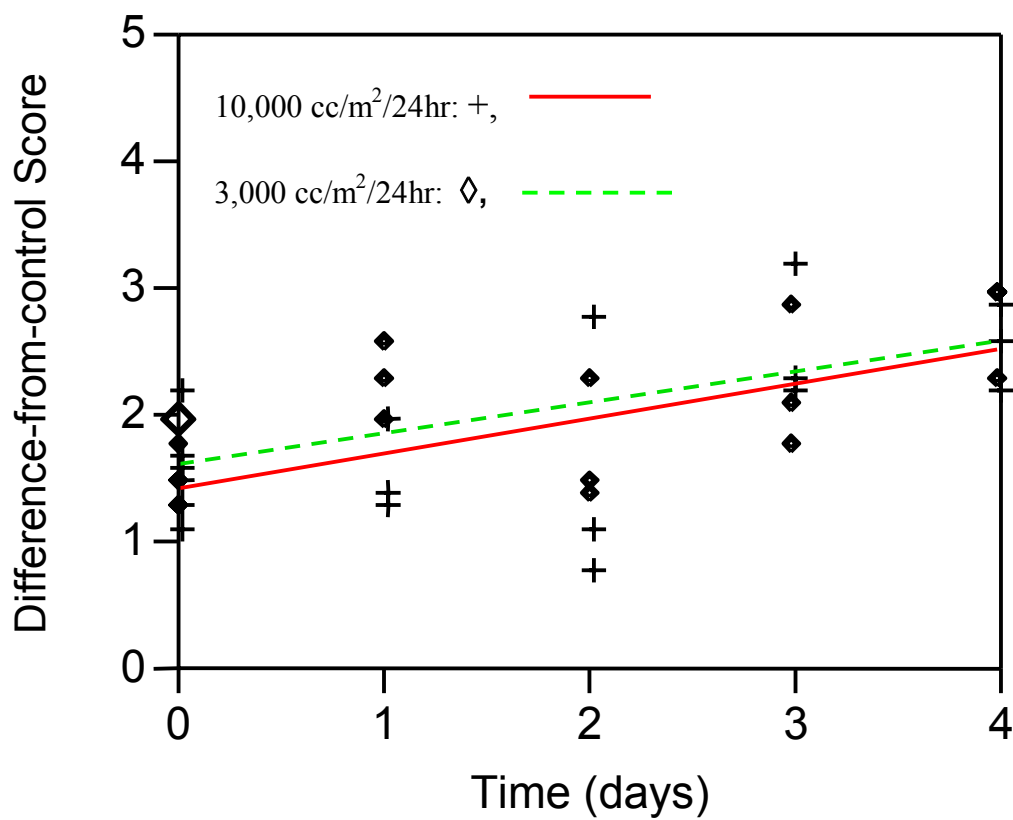
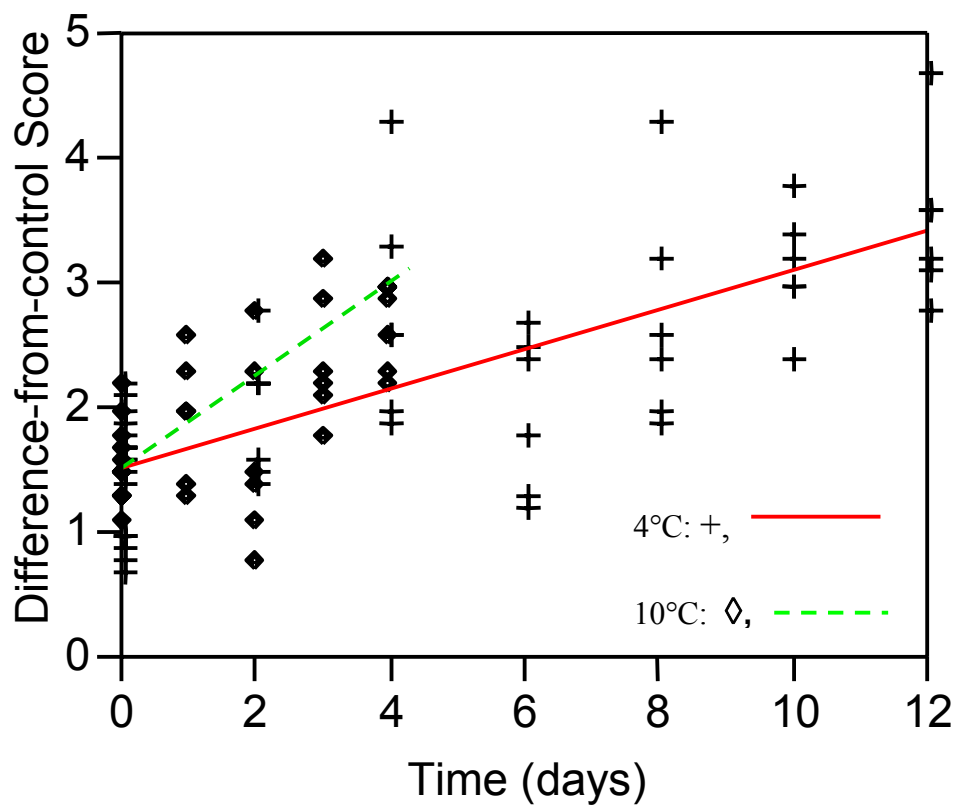


Figure 6. *The interactions between temperature and organoleptic spoilage (n=10 panelists per fillet)*



### Chapter III

*Clostridium botulinum* toxin development in refrigerated reduced oxygen packaged

Atlantic croaker (*Micropogonias undulatus*)

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## ABSTRACT

The purpose of this study was to determine the effects of storage temperature and film oxygen transmission rate (OTR) on toxin development by *Clostridium botulinum* in refrigerated raw vacuum packaged croaker fillets, and to determine if toxin development precedes microbiological and/or organoleptic spoilage. Raw croaker fillets were vacuum packaged in oxygen-permeable films (OTR of 10,000 cc/m<sup>2</sup>/24hr or 3,000 cc/m<sup>2</sup>/24hr) and stored at either 4°C or 10°C. Type 83F, 17 Type B, Beluga, Minnesota, and Alaska nonproteolytic strains of *C. botulinum* were used to inoculate fish prior to vacuum packaging. At both temperatures, microbial spoilage preceded toxin production in fillets vacuum packaged in both film types. At 4°C microbial spoilage occurred after approximately 7 days for fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film and after 8 days for fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film. However, toxin was not detected until day 8. At 10°C microbial spoilage occurred after approximately 3 days for fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film, while toxin production occurred on day 5. For fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film microbial spoilage occurred after 4 days. However toxin production did not occur until day 6. In contrast, at both temperatures toxin production preceded organoleptic spoilage in fillets vacuum packaged in both film types. At 4°C organoleptic spoilage occurred after 10 days for fillets packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film and after 9 days for fillets packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film, while toxin production occurred on day 8. At 10°C organoleptic spoilage occurred after 6 days for fillets packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film, and toxin was detected on day 5. For fillets packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film and stored at 10°C organoleptic

spoilage occurred after 6 days, while toxin production occurred on day 6. Although toxin production preceded or coincided with organoleptic spoilage in both film types, this may have been because samples were presented on ice, which could have masked potential odors. This study shows that there are not significant differences between film types when it comes to microbial and organoleptic spoilage. Therefore lower OTR films, such as 3,000 cc/m<sup>2</sup>/24hr film, may be used to vacuum package Atlantic croaker.

## INTRODUCTION

Fresh fish provides a natural environment for growth of psychrotrophic spoilage microorganisms. When lipids and other seafood proteins are degraded by these microorganisms, the result is a highly pronounced off-flavor and off-odor resulting in a short shelf life and monetary losses for seafood companies. In the past, ice and refrigeration were the most common methods used to hinder microbial and biochemical spoilage (15). However, there have been reports that ice harbors bacteria, and may contaminate the fresh fish when it melts, thus accelerating spoilage and decreasing shelf life (7, 13).

Alternative methods to increase the shelf life of fish include the use of vacuum (VAC) packaging. Vacuum packaging refers to packaging in containers where all air has been removed prior to the final sealing of the container. There is considerable interest in VAC packaging technology for raw fish to extend shelf life by preventing the growth of naturally occurring, aerobic spoilage microorganisms. Increased shelf life allows the fresh fish market to expand to new markets beyond the coastal regions (12).

Vacuum packaging of fresh fish can increase food safety problems by providing ideal anaerobic conditions for the possible growth and toxin formation by *C. botulinum*, found on both saltwater and freshwater fish. There are reports that for some species of fish, botulinum toxin production precedes sensory rejection by consumers (3, 11, 14). Furthermore, since psychrotrophic spoilage microorganisms are inhibited by the anaerobic environment provided by VAC packaging, *C. botulinum* may successfully compete and grow on the fish.

To minimize the possibility of toxin production in VAC packaged fresh fish, the U.S. Food and Drug Administration mandated, as one of three approaches, that fishery products are packaged in a film with an oxygen transmission rate (OTR) of 10,000 cc/m<sup>2</sup>/24hr to allow microbial spoilage before possible *C. botulinum* toxin development (19). Use of this film may prevent toxin formation, but shelf life is shorter compared with products packaged under a film with a lower OTR.

Inoculated studies on vacuum packaged fresh fish have not been conducted on Atlantic croaker. The National Advisory Committee on the Microbiological Criteria of Food recommends that *C. botulinum* inoculated pack studies must be performed for each species of fish (11).

## **MATERIALS AND METHODS**

**A. Fish** Atlantic croaker (*Micropogonias undulatus*) were pound netted from the Chesapeake Bay near Whitestone, VA by commercial fishermen. Whole fish were placed on ice and offloaded in Hampton, VA. The fish were transported on ice in coolers to the Department of Food Science and Technology at Virginia Tech in Blacksburg, VA, and held frozen at -20°C until ready to be filleted. After thawing at 4°C for 24 hours, the fish were aseptically hand filleted and skinned. Each fillet was rinsed with sterile distilled water to remove excess blood. Two fillets were obtained from each fish.

**B. Vacuum packaging** Individual fish fillets (~ 30-100 g) were vacuum packaged in oxygen-permeable packaging material with a guaranteed oxygen transmission rate (OTR) of 10,000 cc/m<sup>2</sup>/24hr or 3,000 cc/m<sup>2</sup>/24hr. The 10,000 cc/m<sup>2</sup>/24hr packaging material was a highly permeable multilayered, coextruded polyolefin formulation (Cryovac<sup>®</sup> HP2700 bag, Duncan, SC). The 3,000 cc/m<sup>2</sup>/24hr packaging material is a multilayered, coextruded polyolefin with high shrink energy (Cryovac<sup>®</sup> FC-805 bag, Duncan, SC). Individual fish fillets were vacuum packed at 99% vacuum for 2.4 second duration of sealing using an Ultravac<sup>®</sup> vacuum packager (KOCH Industries, Kansas City, MO). All packages maintained their reduced oxygen atmosphere throughout sampling.

### **C. Shelf life studies**

**1. Microbial spoilage** Psychrotrophic plate counts were conducted for the vacuum packaged fillets (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr film type) after storage at one of two temperatures (4°C or 10°C). For fillets stored at 4°C, samples were

analyzed at days 0, 2, 4, 6, 8, 10, and 12. For fillets stored at 10°C, samples were analyzed at days 0, 1, 2, 3, and 4. Sampling days were selected to represent 0, 75, 100, and 125% estimated shelf life (1). Three different individual fish fillets per film type were used per sampling day.

For this study, microbial spoilage (i.e. end of shelf life) was defined as a psychrotrophic plate count of at least 7.0 log CFU/g (6). Following the analytical procedures outlined in the Food and Drug Administration Bacteriological Analytical Manual, samples were pulverized in a laboratory stomacher (Seward Stomacher® 400 Circulator, Worthing, UK) for 2 min at 230 rpm with 99 mL of Butterfield's buffer (Biotrace International, Munice, IN). Three consecutive 10 fold dilutions per sample were plated on Aerobic Plate Count Petrifilm® (3M Microbiology, St. Paul, MN). Petrifilms® were incubated at 10°C for 7 days prior to enumeration (9). One purpose of the microbial shelf life study was to provide spoilage estimations to reduce the frequency of sampling for the inoculated studies and toxin determination studies (1).

**2. Organoleptic spoilage** The difference-from-control test (10) was used to determine the organoleptic shelf life of the vacuum packaged fillets (10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr film types) after storage at 4°C or 10°C. For fillets stored at 4°C, sensory sessions were conducted on days 2, 4, 6, 8, 10, and 12. For fillets stored at 10°C, sensory sessions were conducted at days 1, 2, 3, and 4. Three different individual fish fillets per film type were used per sampling day.

For this study, organoleptic spoilage was defined as a minimum score of 3.0 on a six-point difference from control scale (Table 1). Following difference-from-control test

methods, subjects were presented a control sample (Day 0 sample) and two test samples (fillets vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr) (10). Control fillets were held frozen (-40°C) until the day of the sensory analysis and were allowed to thaw and equilibrate at 4°C. Each of the three fish fillets were cut into 10 equal portions and presented to panelists in 2 oz. Soufflé Cups (Mongram Company, Columbia, MD) which were stored on crushed ice.

Thirty untrained panelists were used per sensory session. Subjects were first asked to pick up the control sample (labeled C) with their right hand, hold it 6 inches from their nose, use their left hand to fan odor towards their nose, and smell the control sample. If an odor could not be identified, the sample was brought closer to the nose. Subjects were then asked to repeat the steps mentioned above for the samples vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr and 3,000 cc/m<sup>2</sup>/24hr (labeled with a 3-digit code). A six attribute descriptive scale was used to determine the difference in odor from the control. Finally, subjects were asked to mark the scale to indicate the overall size of the difference in odor relative to the control sample (10).

#### **D. *Clostridium botulinum* analyses**

**1. Generation of spore crops** As recommended by the National Advisory Committee for the Microbiological Criteria of Foods (NACMCF), type 83F, 17 Type B, Beluga, Minnesota, and Alaska nonproteolytic strains of *C. botulinum* were chosen to conduct the inoculated study (11). Strains were obtained from the culture collection present in the Department of Food Science and Technology at Virginia Tech in Blacksburg, VA.

**2. Enumeration of spore crops** Spore crops were enumerated using serial dilutions in sterile 9.9 mL anaerobic peptone blanks. Three consecutive 10 fold dilutions per strain were inoculated into Brain Heart Infusion Roll Tubes, in duplicate. Roll tubes were incubated anaerobically for 3-4 days at 30°C prior to enumeration. Following enumeration, dilutions were made with sterile distilled water to produce a 5 strain cocktail of equal spore concentrations. The 5 strain cocktail was stored in a glass vial at 20°C until ready for use (1).

**3. Spore inoculation of fish fillets** Prior to inoculation, final equivalent dilutions were made using distilled water to produce a 5 strain cocktail in a concentration of  $10^2$ - $10^3$  spores/mL. Spore inoculation of fish fillets followed procedures outlined in the recommendations by the NACMCF (11). Prior to vacuum packaging, fish were surface inoculated uniformly using non-heat-shocked spores by dropwise (up to 0.1 ml per drop) addition using a 1 cc tuberculin syringe (American Pharmaseal Laboratories, Glendale, CA) (11). Fillets were also internally inoculated using the same methods as mentioned above. Non-heat-shocked spores were used because temperatures during the processing of raw fish are far below 80°C, the temperature required to heat shock spores. Approximately  $1.6 * 10^3$  total spores were applied per fillet.

Based on the results of the organoleptic and microbial shelf life studies, fillets stored at 4°C were sampled on days 0, 4, 6, 8, 10, and 12 and fillets stored at 10°C were sampled on days 0, 3, 4, 5, 6, and 7. As recommended by the NACMCF, five different fish fillets were used per sampling day (11). Sterile distilled water inoculations were used as controls.



**4. Positive controls** To determine if *C. botulinum* spores were capable of germination, growth, and toxin production in Atlantic croaker, two positive control fillets were inoculated with  $10^2$ - $10^3$  spores using the methods mentioned above. The fillets were then vacuum packaged twice in films with an OTR of 3,000 cc/m<sup>2</sup>/24hr and incubated at room temperature for approximately 2 weeks. To verify that *C. botulinum* spores were capable of germination, growth, and toxin production in Atlantic croaker, both the mouse bioassay and the DIG-ELISA method were used to detect toxin presence. Detection of *C. botulinum* toxin followed the mouse bioassay procedure as outlined in the FDA Bacteriological Analytical Manual (17). Toxin extraction followed the same procedures as mentioned in section 5. Because toxins of nonproteolytic types, if present, may need trypsin activation to be detected, 0.2 ml of aqueous trypsin solution was added to 1.8 ml of each supernatant fluid to be tested for toxicity. Samples heated for 10 min at 100°C served as controls. Tests were conducted simultaneously with trypsin-treated samples, untreated samples, and heat treated controls in duplicate. Portions of untreated sample fluids and trypsinized sample fluids were diluted in gel-phosphate buffer. Each separate pair of mice was injected intraperitoneally with 0.5 mL of trypsin-treated samples, untreated samples, and heat treated negative control samples. All mice were observed periodically for 48 hours. Symptoms and deaths were recorded since death of mice without clinical symptoms of botulism is not sufficient evidence that injected material contained botulinal toxin. Characteristic botulism signs in mice include ruffling of fur, followed by labored breathing, weakness of limbs and total paralysis with gasping for breath, and finally death due to respiratory failure (17).

## 5. Toxin determination by DIG-ELISA Detection of *C. botulinum* toxin

followed the DIG-ELISA procedure as outlined in the FDA Bacteriological Analytical Manual (17). To extract the toxin, an equal amount of sample (50 g fish fillet) and casein buffer (Pierce, Rockford, IL) (50 ml) were stomached for 2 min at 230 rpm. The fish fillet and buffer slurry (1:2 dilution) was transferred to centrifuge bottles (Nalgene® Labware, Rochester, NY) and centrifuged at 7,000 x g for 20 min at 4°C using a Sorvall® RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Newtown, CT). The aqueous supernatant was removed and placed in glass vials. The pH was adjusted to 7.4-7.6, if necessary, using 1 N NaOH or 1 N HCl. The glass vials were stored at -20°C until the assay was performed.

For each temperature and packaging film, one 96 well microtiter plate was used per sampling day. Each well of the microtiter plate was coated with 100 µL of goat type E and F and rabbit type B antitoxin diluted in bicarbonate buffer (FDA-SRL, Atlanta, GA). The plate was stored overnight at 4°C with a plastic seal to prevent evaporation. After the plate was removed from 4°C storage, it was washed 5 times in phosphate buffered saline (Fisher Scientific International, Inc., Fair Lawn, NJ) with 0.005% Tween 20 (Acros Organics, Fair Lawn, NJ) wash buffer (PBST) with a 45 second hold between each aspiration using a WW004 Wellwash 4 microplate washer (Denley Instruments, Ltd., West Sussex, United Kingdom). The plate was then blocked in casein buffer by filling all the wells to the top of the plate (~300 µL/well) and incubated for 60-90 minutes at 35°C. After incubation, the blocked plate was washed as above and 100 µL of each of the five samples, positive controls, negative controls, and ELISA food inhibition controls were added to the wells. Standard toxins types B, E, and F (Metabionics, Inc.,

Madison, WI) diluted in casein buffer at a concentration of 2 ng/mL were used as positive controls. Casein buffer was used as a negative system control. To demonstrate if the food product inhibits the ELISA, types B, E, and F neurotoxins were used to spike the supernatant obtained from the fish fillet-casein buffer slurry at 2 ng/mL. Non spiked supernatant obtained from the fish fillet-casein buffer slurry served as a negative ELISA food inhibition control. The samples and controls were incubated for 2 hours at 35°C. After two hours, the plates were washed as above and 100 µL of diluted digoxigenin-labeled goat antibodies (FDA-SRL, Atlanta, GA) were added to each well. The plate was incubated for 60 minutes at 35°C. The plate was washed as above and 100 µL of anti-digoxigenin poly HRP conjugate (Roche Diagnostics, Indianapolis, IN) diluted 1:5,000 in casein buffer was added to each well. The plate was incubated for 60 minutes at 35°C. After removing the plate from the incubator, the plate was washed 5 times in PBST and tamped on a paper towel to remove any residual wash buffer. Finally, 100 µL of tetra methyl benzidine (Ultra-TMB) (Roche Diagnostics, Indianapolis, IN) solution was added to the plate and the plate was incubated for 20-30 minutes at 35°C. After positive controls were shown to give an absorbance of  $\geq 1.0$  and negative controls were shown to give an absorbance not greater than  $\sim 0.39$ , 100 µL of the stop reagent (0.5 M H<sub>2</sub>SO<sub>4</sub>) was added to the wells. Immediately afterwards, the absorbance was measured at 450 nm on a Reader 520 (Organon Teknika, Durham, NC). A positive result was an absorbance value that was  $> 0.20$  above the absorbance observed in the negative controls.

**E. Statistical analysis** An analysis of covariance was used to model the data. The time at which microbial and organoleptic spoilage occurred, as well as the time of toxin

formation, was determined using an inverse prediction model with JMP™ IN statistical software (SAS Institute, Cary, NC). An analysis of covariance using JMP™ IN statistical software (SAS Institute, Cary, NC) was also used to assess the effects of storage temperature, film type, and their interactions on the relationship between spoilage and storage times. A P-value of 0.05 was used.

## **RESULTS**

### *Positive Control Results*

Positive control fillets were tested for toxin presence using the mouse bioassay as well as ELISA. For both fillets, the mice injected with the trypsinized fillet samples and untreated fillet samples experienced botulism symptoms within 24 hours which included labored breathing, weakness of limbs, total paralysis, followed by death. The mice injected with the heat treated negative control samples did not experience botulism symptoms, and remained alive after 48 hours.

The DIG-ELISA method was also used to determine toxin presence in positive control fillets. Both fillet samples gave absorbance values >0.20 above the absorbance value observed in the negative controls, indicating a positive result.

### *Toxin production in relation to microbial spoilage*

At 4°C, Atlantic croaker fillets vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr reached a microbial log count of 7.0 CFU/g (i.e. became microbially spoiled) at 7 days, which preceded toxin formation. One of five of the inoculated fillets vacuum packaged in film with an OTR of 10,000 cc/m<sup>2</sup>/24hr stored at 4°C was toxic at day 8. At 4°C, croaker fillets vacuum packaged in film providing a 3,000 cc/m<sup>2</sup>/24hr OTR became

microbially spoiled at 8 days, which also preceded toxin formation. In this case, one of the five inoculated croaker fillets vacuum packaged in film providing a 3,000 cc/m<sup>2</sup>/24hr OTR was toxic (Table 2, Table 3). None of the uninoculated control fillets tested positive for toxin.

At 10°C, croaker fillets vacuum packaged in film providing a 10,000 cc/m<sup>2</sup>/24hr OTR reached a microbial log count of 7.0 CFU/g at 3 days. However, toxin formation did not occur until day 5, where it was found that two of the five inoculated fillets were toxic. Croaker fillets stored at 10°C and vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film became microbially spoiled at 4 days, but toxin production did not occur until day 6. At 10°C on day 6, one of the five inoculated fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film was toxic (Table 2, Table 3). None of the uninoculated control fillets tested positive for toxin.

#### *Toxin production in relation to organoleptic spoilage*

At 4°C, Atlantic croaker fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film reached a difference-from-control rating of at least 3.0 (i.e. became organoleptically spoiled) at 10 days, which occurred after toxin formation. At 4°C on day 8, one of the five inoculated Atlantic croaker fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film was toxic. On day 10, three of the five fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film were toxic. Fillets stored at 4°C that were vacuum packaged in film providing an OTR of 3,000 cc/m<sup>2</sup>/24hr reached organoleptic spoilage at 9 days, which also occurred after toxin formation. At 4°C, one of the five inoculated fillets that were vacuum packaged in the film providing an OTR of 3,000 cc/m<sup>2</sup>/24hr became toxic

on day 8. At 4°C on day 10, four of the five inoculated fillets were toxic (Table 3, Table 4). None of the uninoculated control fillets tested positive for toxin.

At 10°C, Atlantic croaker fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film reached a difference-from-control rating of at least 3.0 at 6 days, which occurred after toxin formation. At 10°C on day 5, two of the five inoculated Atlantic croaker fillets vacuum packaged in the 10,000 cc/m<sup>2</sup>/24hr OTR film were toxic, while on day 6, three of the five inoculated fillets were toxic. Atlantic croaker fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film and stored at 10°C became organoleptically spoiled at 6 days, which coincided with toxin formation. At 10°C on day 6, one of the five inoculated Atlantic croaker fillets vacuum packaged in the 3,000 cc/m<sup>2</sup>/24hr OTR film was toxic, while on day 7, four of the five inoculated fillets were toxic (Table 3, Table 4). None of the uninoculated control fillets tested positive for toxin.

## **DISCUSSION**

### *Toxin production in relation to microbial spoilage*

In the current study, at both temperatures (4°C and 10°C) and both film types (10,000 cc/m<sup>2</sup>/24hr OTR and 3,000 cc/m<sup>2</sup>/24hr OTR) microbial spoilage preceded toxin formation in vacuum packaged Atlantic croaker fillets. These results differ from the results of a similar study conducted by Arritt et al. which found that at 4°C vacuum packaged flounder fillets became toxic before microbial spoilage occurred and that at 10°C toxin formation coincided with microbial spoilage (1). However, studies have shown that *C. botulinum* toxin production varies among species of fish and that some species of fish are more prone to toxin formation than others (1, 8). Variations may also

be due to the natural microflora present on the fish species. While some naturally occurring microorganisms present on fish may cause the product to spoil before toxin formation occurs, others may use considerable amounts of the oxygen present in the environment, thus aiding in the growth and toxin formation of *C. botulinum* (4). The method of spore inoculation onto the fish may also effect when toxin forms. Some studies have shown that fish that are deep inoculated with spores become toxic faster than fish that are only surface inoculated (5).

#### *Toxin production in relation to organoleptic spoilage*

The current study shows that at both temperatures (4°C and 10°C) and both film types (10,000 cc/m<sup>2</sup>/24hr OTR and 3,000 cc/m<sup>2</sup>/24hr OTR) organoleptic spoilage occurred either after toxin had formed or coincided with toxin formation in vacuum packaged Atlantic croaker fillets. These results differ substantially from the results of the microbial spoilage in relation to toxin production study, which showed that toxin production always occurred after microbial spoilage. These results differ from studies conducted by Dufresne et al. and Reddy et al. which showed that at 4°C, organoleptic spoilage preceded toxin formation in both inoculated cold and hot smoked vacuum packaged trout fillets and aquacultured salmon fillets (2, 16). However at higher temperatures, these studies have found that toxin production either preceded or coincided with organoleptic spoilage in vacuum packaged fishery products (2, 16). These results are consistent with the findings of the current study. As stated earlier, variations in results between studies may be due to the fish species studied, the inoculum size, the method of inoculation, and the analyses used to detect product decomposition and toxin production (11).

Because often sensory panelists do not like the smell of fish, the difference-from-control method can be a useful tool for predicting the shelf life of fishery products. However because the samples were presented to panelists in such small portions, this may have affected the results of the organoleptic shelf life study. In addition, because the samples were presented on ice, this may have also masked potential odors. If the samples were presented to panelists at a warmer temperature, they could possibly reject the samples sooner. Perhaps if different sized portions, a different rating scale, or different sensory test were used, the results may have been different.

### *Conclusion*

The U.S. Food and Drug Administration has mandated that fishery products be packaged in a film with a relatively high oxygen transmission rate ( $10,000 \text{ cc/m}^2/24\text{hr}$ ), in order to allow spoilage to occur before possible growth and toxin formation by *C. botulinum* (19). The current study shows that while both films (i.e. OTR of  $10,000 \text{ cc/m}^2/24\text{hr}$  and  $3,000 \text{ cc/m}^2/24\text{hr}$ ) allow microbial spoilage to occur before growth and toxin formation by *C. botulinum*, toxin formation occurred before organoleptic spoilage in vacuum packaged Atlantic croaker. However because the  $3,000 \text{ cc/m}^2/24\text{hr}$  OTR film type did not significantly extend the microbial or organoleptic shelf life of the vacuum packaged fillets, it may be used to vacuum package Atlantic croaker. Studies have shown that time-temperature indicators can be used to pinpoint when and if temperature abuse has occurred (18). The use of time-temperature indicators in conjunction with lower OTR films may also increase the safety of these food products by assuring that these products do not become temperature abused.



## REFERENCES

1. Arritt, F. M., J. D. Eifert, M. L. Jahncke, M. D. Pierson, and R. C. Williams. 2007. Effects of modified atmosphere packaging on toxin product by *Clostridium botulinum* in raw aquacultured Summer Flounder fillets (*Paralichthys dentatus*). *J. Food Prot.* 70:in press.
2. Dufresne, I., J. P. Smith, J. N. Liu, and I. Tarte. 2000. Effect of films of different oxygen transmission rate on toxin production by *Clostridium botulinum* type E in vacuum packaged cold and hot smoked trout fillets. *J. Food Saf.* 20:251-268.
3. Eyles, M. J. and A. D. Warth. 1981. Assessment of the risk of botulism from vacuum-packaged raw fish: a review. *Food Technol. Aust.* 33:574-580.
4. Huss, H. H., L. Ababouch, and L. Gram. 2004. Assessment and management of seafood safety and quality, p. 31-34, 178. Food and Agriculture Organization, Rome.
5. Ikawa, J. Y. and C. Genigeorgis. 1987. Probability of growth and toxin production by nonproteolytic *Clostridium botulinum* in rockfish fillets stored under modified atmospheres. *Int. J. Food Microbiol.* 4:167-181.
6. International Commission on Microbiological Specifications for Foods (ICMSF). 1978. p. 92-104. *In* Microorganisms in foods. 2. Sampling for microbial analysis: principle and specific applications. University of Toronto Press, Toronto, Ontario, Canada.
7. LaBombardi, V. J., A. M. O'Brien, and J. W. Kislak. 2002. Pseudo-outbreak of *Mycobacterium fortuitum* due to contaminated ice machines. *Am. J. Infect. Control.* 30:184-186.
8. Lalitha, K. V. and K. Gopakumar. 2001. Growth and toxin production by *Clostridium botulinum* in fish (*Mugil cephalus*) and shrimp (*Penaeus indicus*) tissue homogenates stored under vacuum. *Food Microbiology.* 18:651-657.
9. Maturin, L. J. and J. T. Peeler. 2001. Aerobic plate count, chap. 3. *In*, Bacteriological analytical manual, 8th ed. AOAC International, Arlington, VA.
10. Meilgaard, M., Vance Civille, G., Carr, B.T. 1999. Sensory evaluation techniques, p. 87-94. CRC Press, Boca Raton.
11. National Advisory Committee on the Microbiological Criteria for Foods (NACMCF). 1992. Vacuum or modified atmosphere packaging for refrigerated raw fishery products. Report of the NACMCF. Adopted March 20, 1992. *In* NACMCF, U.S. Food and Drug Administration, Washington, D.C.
12. National Advisory Committee on the Microbiological Criteria for Foods (NACMCF). 1993. Report on vacuum packaging and associated processes. *In* NACMCF, U.S. Food and Drug Administration, London.
13. Panwalker, A. P. and E. Fuhse. 1986. Nosocomial *Mycobacterium gordonae* pseudoinfection from contaminated ice machines. *Infect. Control.* 7:67-70.
14. Post, L. S., D. A. Lee, M. Solberg, D. Furgang, J. Specchio, and C. Graham. 1985. Development of botulinal toxin and sensory deterioration during storage of vacuum and modified atmosphere packaged fish fillets. *J. Food Sci.* 50:990-998.

15. Reddy, N. R. and D. J. Armstrong. 1992. Shelf-life extension and safety concerns about fresh fishery products packaged under modified atmospheres: a review. *J. Food Saf.* 12:87-118.
16. Reddy, N. R., H. M. Solomon, H. Yep, M. G. Roman, and E. J. Rhodehamel. 1997. Shelf life and toxin development by *Clostridium botulinum* during storage of modified-atmosphere-packaged fresh aquacultured salmon fillets. *J. Food Prot.* 60:1055-1063.
17. Soloman, H. M. and J. T. Lilly. 2001. *Clostridium botulinum*. In, Bacteriological analytical manual, 8th ed. AOAC International, Arlington, VA.
18. Skinner, G.E. and J.W. Larkin. Conservative prediction of time to *Clostridium botulinum* toxin formation for use with time-temperature indicators to ensure the safety of foods. *J. Food Prot.* 61: 1154-1160.
19. U.S. Food and Drug Administration. 2001. *Clostridium botulinum* toxin formation, chap. 13. In, Fish and fisheries products hazards and controls guidance, 3rd ed. U.S. Food and Drug Administration. Available at: <http://www.cfsan.fda.gov/~comm/haccp4.html>.

Table 1. *Organoleptic difference-from-control rating definitions for raw vacuum packaged croaker fillets (Micropogonias undulatus)*<sup>a</sup>

| Rating | Odor difference from control |
|--------|------------------------------|
| 0      | No difference                |
| 1      | Very small difference        |
| 2      | Slight/moderate difference   |
| 3      | Moderate                     |
| 4      | Moderate/large difference    |
| 5      | Large difference             |
| 6      | Very large difference        |

<sup>a</sup> Variation of table in Meilgaard et al. (10)

Table 2. *Microbial spoilage in relation to C. botulinum toxin development in raw vacuum packaged croaker fillets (Micropogonias undulatus)*

| Storage Temperature | Packaging Film OTR             | Approximate day to microbial spoilage <sup>a</sup> | Day of toxin formation |
|---------------------|--------------------------------|--|------------------------|
| 4°C                 | 10,000 cc/m <sup>2</sup> /24hr | 7  | 8                      |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 8  | 8                      |
| 10°C                | 10,000 cc/m <sup>2</sup> /24hr | 3  | 5                      |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 4  | 6                      |

<sup>a</sup> APC,  $\geq 10^7$  CFU/g

Table 3. Fraction of Atlantic croaker fillets (*Micropogonias undulatus*) in which *C. botulinum* toxin was detected after storage at 4°C or 10°C<sup>a</sup>

| Storage Temperature | Packaging Film OTR             | Days of storage | No. of toxic fillets detected/no. of inoculated fillets |
|---------------------|--------------------------------|-----------------|---|
| 4°C                 | 10,000 cc/m <sup>2</sup> /24hr | 4               | 0/5   |
|                     |                                | 6               | 0/5   |
|                     |                                | 8               | 1/5   |
|                     |                                | 10              | 3/5   |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 4               | 0/5   |
|                     |                                | 6               | 0/5   |
|                     |                                | 8               | 1/5   |
|                     |                                | 10              | 4/5   |
| 10°C                | 10,000 cc/m <sup>2</sup> /24hr | 3               | 0/5   |
|                     |                                | 4               | 0/5   |
|                     |                                | 5               | 2/5   |
|                     |                                | 6               | 3/5   |
|                     |                                | 7               | 2/5   |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 3               | 0/5   |
|                     |                                | 4               | 0/5   |
|                     |                                | 5               | 0/5   |
|                     |                                | 6               | 1/5   |
|                     |                                | 7               | 4/5   |

<sup>a</sup> Variation of table in Arritt et al. (1)

Table 4. *Organoleptic spoilage in relation to C. botulinum toxin development in raw vacuum packaged croaker fillets (Micropogonias undulatus)*

| Storage Temperature | Packaging Film OTR             | Approximate day to organoleptic spoilage <sup>a</sup> | Day of toxin formation |
|---------------------|--------------------------------|---|------------------------|
| 4°C                 | 10,000 cc/m <sup>2</sup> /24hr | 10  | 8                      |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | 9   | 8                      |
| 10°C                | 10,000 cc/m <sup>2</sup> /24hr | > 4 <sup>b</sup>                                      | 5                      |
|                     | 3,000 cc/m <sup>2</sup> /24hr  | > 4 <sup>b</sup>                                      | 6                      |

<sup>a</sup> Difference-from-control rating  $\geq 3.0$

<sup>b</sup> Sensory sessions were conducted only up to day 4 – inverse prediction was used to approximate day to organoleptic spoilage as 6 for both film types

## **VITA**

Courtney Rheinhardt is from Virginia Beach, VA and earned a Bachelor of Science degree in Biology from Virginia Tech. As an undergraduate she was a research laboratory assistant in the department of Biological Sciences. In this position she assisted in graduate research to determine the effects of various rig materials on microbial growth in water treatment systems. She also prepared various types of media such as Brain Heart Infusion Broth, R2A, and Triple Sugar Iron Agar. After graduation, Courtney took an internship at QuanTech, Inc. where she worked as a Staff Biologist. At this internship, she assisted in the management of a large scale fisheries-related data collection program in nine states from Virginia to New Hampshire. Courtney also oversaw the recruitment, training, hiring, and supervision of data collection staff. In 2005, Courtney began working towards her Master of Science with a concentration in Food Microbiology at Virginia Tech. As part of her graduate research assistantship, she is a lead teaching assistant for Undergraduate Food Microbiology during the spring semester. As a lead teaching assistant, Courtney provides individual instruction on microbiological methods and prepares laboratory media and materials as well as written materials. She is expected to graduate in spring 2007.