SEASONAL OCCURRENCE AND THE USE OF NON THERMAL TECHNOLOGIES TO CONTROL GROWTH AND TOXIN FORMATION OF *Bacillus cereus* IN TUNA

By

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To my parents and family in Ecuador

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LIST OF ABBREVIATIONS

BAM	Bacteriological analytical manual
BE	Big eye
CFU	Colony forming units
FDA	Food and Drug Administration
MYP	Mannitol egg yolk polimixin agar
SJ	Skip jack
STD	Standard deviation
TFTC	Too few to count
UV	Ultraviolet
YF:	Yellow fin

Abstract of Thesis Presented to the Graduate School
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There is an increasing concern about the hazardous presence of *B. cereus* in seafood. Some outbreaks caused by a diarrheal toxin produced by *B. cereus* in fish have been reported. For this reason all seafood processors must follow FDA guidelines which specify that to avoid outgrowth of *B. cereus*, the period of time for holding seafood after cooking must not exceed 3 hours.

In this investigation tuna samples of the species Skip Jack, Yellow Fin, and Big Eye were taken from the coasts of Ecuador and analyzed for presence of *B. cereus* vegetative cells and spores every other month during 1 year. A significant ($P < 0.05$) seasonal variation of the amount of *B. cereus* vegetative cells was found in the months June to August as opposed to the rest of the months in each of the species. No significant ($P > 0.05$) difference was found in the amount of *B. cereus* vegetative cells among tuna species. No significant occurrence ($P > 0.05$) was found on the levels of *B. cereus* spores in any of the sampled months.

To find an efficient non-thermal technology capable to reduce *B. cereus* vegetative cells in tuna, Yellow Fin tuna samples were inoculated with known amounts of *B. cereus* vegetative cells and treated with ultraviolet (UV) light, Ozone, and lactoperoxidase system.

The first set of samples was treated with 8.5 mJ/cm^2 and 17 mJ/cm2 doses of UV light. A significant ($P < 0.05$) reduction in the number of *B. cereus* vegetative cells was achieved with

these doses. No significant ($P > 0.05$) difference was found between the two doses. The second set of samples was treated with 0.65 mg/L of Ozone. A significant ($P < 0.05$) reduction was achieved with this treatment. The third set of samples was treated in a one liter solution containing 100 µL of a 1 mg/mL solution of the enzyme lactoperoxidase, 20 µL of a 30% hydrogen peroxide solution, and 1.8 grams of KSCN. No significant ($P > 0.05$) reduction in the number of *B. cereus* vegetative cells was achieved by this treatment. Effects of these treatments on the color of the tuna were also evaluated. All treatments showed a significant ($P < 0.05$) variation in the a* value when compared with untreated tuna.

CHAPTER 1
REVIEW OF LITERATURE

Bacillus Cereus

General Characteristics

Bacillus cereus is a gram-positive, spore-forming, aerobic bacterium that grows well anaerobically. *B. cereus* produces two kinds of foodborne illnesses: diarrheal and emetic. The diarrheal toxin is heat labile and has to be produced in the small intestine to cause the disease whereas the emetic toxin is heat stable and can be produced in some foods to cause intoxication (Granum 2001). Besides food borne illnesses *B. cereus* may also cause diseases like endocarditis and endophthalmitis (Drobniewski 1993).

Reservoirs

Bacillus cereus is ubiquitous, but is most commonly found in soils and growing plants from which it can be spread to foods. It can also be found in fish and other marine species (Granum 2001).

Diseases

Bacillus cereus is the etiologic agent of two types of food-borne disease, diarrhea and vomiting illnesses. Both types are caused by toxins: the diarrheal type by protein toxins which must be formed in the intestinal tract by growing organisms, and the emetic type by a peptide toxin that is preformed in the food (Agata and others 1995).

The diarrheal type of disease is characterized by diarrhea within 6 to 15 hours after consumption of the suspected food (FDA 2003). Other symptoms are abdominal cramp and pain, and nausea. The emetic type of disease is characterized by vomiting and nausea within half to 6 hours after the consumption of the suspected food. For both types of disease the total duration of clinical signs is about 24 hours (FDA 2002; Kramer and Gilbert 1989).

Diarrheal toxin

The diarrheal type of disease is caused by *B. cereus* species that are able to produce enterotoxins. There are four toxins that are produced by *B. cereus* and cause diarrhea: Haemolytic BL toxin (HBL), Non Haemolytic Enterotoxin (NHE), enterotoxin T, and cytotoxin K. Three of these toxins are related to food borne outbreaks; the fourth, enterotoxin T, is not (Granum and Lund 1997;Lund and others 2000;Agata and others 1995).

Haemolytic BL toxin is a hemolytic toxin consisting of three protein subunits: B, L1 and L2. The protein subunit B is the one that binds the toxin to the cells whereas the proteins L1 and L2 cause the cellular lysis (LM Wijnands and others 2002). The toxin shows dermonecrotic activity as well as activity towards vascular permeability, and causes fluid accumulation in ligated rabbit ileal loops (Granum and Lund 1997). All three components are necessary for maximal enterotoxic activity (Beecher and others 1995).

Non Haemolytic Enterotoxin consists also of three protein subunits: nheA, nheB and nheC. In this case the nhC protein is the binding factor whereas the other two are cause lysis (LM Wijnands and others 2002). Although binary combinations of the subunits show some biological effect, maximal activity is achieved when all three components are present (Lund and Granum, 1997).

Enterotoxin-T has been named such based on cloning and immunoblot experiments. This toxin has not been related to outbreaks of food borne disease so far. It is a single component protein enterotoxin with activity towards vascular permeability. Enterotoxin T causes fluid accumulation in the ligated rabbit ileal loop test, and is lethal to mice after intravenous injection (Agata and others 1995a).

Cytotoxin-K is the most recently described enterotoxin from *B. cereus*. It was detected after a food poisoning outbreak in an elderly home in France. In total 44 people were ill, 6 of

these patients had bloody diarrhea, and three of these six died (Lund and others 2000 reviewed by LM Wijnands and others 2002). Cytotoxin-K is a single component protein enterotoxin showing necrotic and hemolytic activity, and is highly toxic to epithelial cells (Hardy and others 2001 reviewed by LM Wijnands and others 2002).

Emetic Toxin

Emetic poisoning is characterized by vomiting and occurs within 1 to 5 hours after ingestion of contaminated foods (Kramer and Gilbert 1989). Emetic syndrome is usually mild but rare fatal cases have been reported (Malher and others 1997). The emetic toxin cereulide is produced in the food and poisoning occurs after ingestion of the toxin.

The emetic toxin cereulide is a small cyclic peptide (Agata and others 1994). Because cereulide is very stable, it may persist in heat treated foods after death of the *B. cereus* cells.

All *B. cereus* strains involved in emetic foodborne infections produce cereulide. Emetic *B. cereus* are unable to hydrolyze starch, so incidence of starch negative *B. cereus* could provide an estimate of emetic *B. cereus*.

Starch negative *B. cereus* represented at most 2% to 11% of strains isolated from dairy products and from dairy farms (Te Giffel and others 1995). Since emetic intoxication occurs through ingestion of emetic toxin (cereulide) preformed in the food, determining conditions in the foods that would lead to production of cereulide by emetic *B. cereus* is important for risk assessment of emetic intoxication. Cereulide is not easily destroyed by heat treatments. For instance, it can resist 90 min at 126°C (Turnbull and others 1979; ICMSF 1996). It is also resistant to acid conditions. Cereulide will therefore not be eliminated from foods in which it had been produced.

Conditions permitting emetic toxin production in foods by emetic strains of *B. cereus* are still not elucidated.

The few conclusions that can be drawn from published work are the following:

- Cereulide became detectable at the end of the growth of *B. cereus* (Häggblom and others 2002).
- The range of conditions permitting cereulide production is narrower than conditions permitting growth of *B. cereus*. Anaerobic conditions and temperatures above 37°C did not permit cereulide production (Finlay and others 2000, Finlay and others 2002, Jääskeläinen and others 2004).
- Not all foods permit cereulide production even if growth of *B. cereus* is possible (Agata and others 2002). Milk, cooked rice and pasta supported important cereulide production at 30°C (Finlay and others 2002).
- Production of cereulide below 10°C does not seem possible. This clearly shows that presence and even growth of emetic *B. cereus* does not always mean cereulide accumulation in foods.

Spore

B. cereus is a sporeformer microorganism. Sporulation is stimulated under low nutrient conditions (Granum 2001).

"Phase-contrast microscopic studies have indicated that the germination response of a single *B. cereus* spore consists of a lag-phase (microlag) and a biphasic event in which the actual germination reactions take place" (Hashimoto and others 1969 reviewed by Vries 2006)

"Heat activation stimulates the germination of spores primarily by reducing the microlag times, and the kinetics of germination of spore suspensions are most critically influenced by the microlag time of each member of that population. More recent analysis confirmed that the microlag was affected by heat activation treatment and indicated that the germination phase varied considerably with germination temperature" (Vries 2006).

Foodborne Outbreaks

Most outbreaks produced by this microorganism have been related to rice and other starchy foods, but recently it has been taken into consideration *B. cereus* presence in other kind of food like fish (National Restaurant Association 2004).

It is well known that *B. cereus* diarrheal infection can be associated with the consumption of high amounts of *B. cereus* in fish (Granum 2001); however the required levels of *B. cereus* to cause infection have not been established yet.

Some outbreaks have been related to *B. cereus* in fish. For example in 1996 some cases of fish borne intoxication with *B. cereus* were reported in Japan (FAO 1998). Other outbreaks have been reported in small numbers in USA, The Netherlands, Canada and England (Notermans and others 1998).

Potential Problems in the Seafood Industry

The ubiquitous nature of *B. cereus* suggests that it is present in a vegetative form the fish before catching, then during freezing it sporulates, so it can resist the low temperatures. Further research is needed to understand how *B. cereus* reaches the fish, in which part of the fish it is mainly located; and to confirm the hypothesis that *B. cereus* is present in a vegetative form in the fish after catching and before freezing.

The main problem in fish processing is that many fish processes just require a mild cooking— i.e. 130 F during 50 minutes—which only can kill other competitive bacteria. On the other hand this kind of heat treatments causes *B. cereus* spore germination (Granum 2001).

As a preventing measure, producers must not exceed a 3 hours holding time at room temperature after mild cooking (FDA 2001). This short amount of time is known to cause some loss in texture quality and efficiency, and hence an increase in production costs.

No data exist on the quantification of the presence of *B cereus* in tuna. It is also necessary to know if B cereus is present in tuna under vegetative or spore conditions.

For this reason it is necessary to find out other ways to control *B. cereus* growth. Additional heating, pH and Aw reduction will alter in some ways the sensory quality of the fish,

so it would be necessary to try out other kind of treatments such as ozone, UV light, freezing, and lactoperoxidase treatments.

Methods for *B. cereus* Inhibition in Food

Sorbates

Smoot and Pierson (1981) were the first to report that 3900 micrograms of sorbate per milliliter of sodium-potassium buffer at a pH of 5.7 will inhibit *B. cereus* growth in culture media. This amount of sorbate is too high compared to the levels sorbates are commonly used in the food industry.

Temperature

Vries (2006) determined the D values of several *B. cereus* spores. Those results are shown in Figure 1-1.

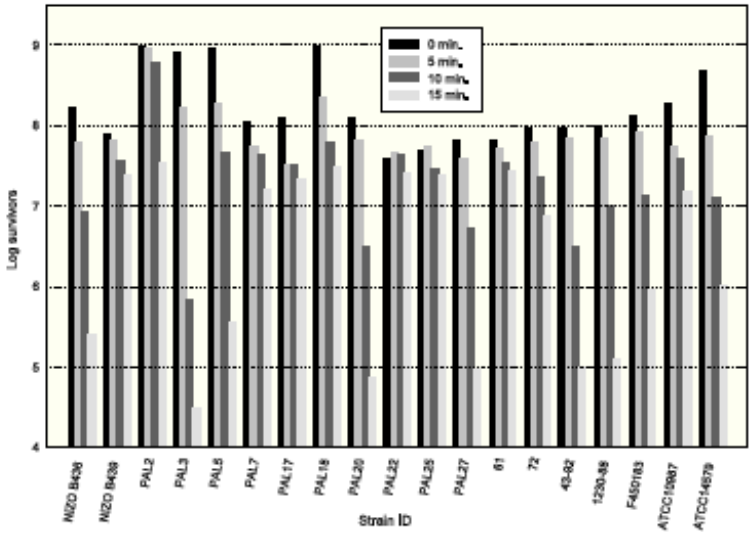

Figure 1-1. Survival of spores exposed to 95 ºC for 0, 5, 10 and 15 minutes (Vries 2006)

Based on the results obtained in Figure 1-1, Vries (2006) determined the D values for each analyzed strain. Those results are shown in Figure 1-2. Data in Figure 1-2 show a high variability of the resistance of *B. cereus* to heat treatment, being the highest D value of 80 minutes a very

large amount of time to treat a food. For this reason it is important to find alternatives to reduce *B. cereus* in food.

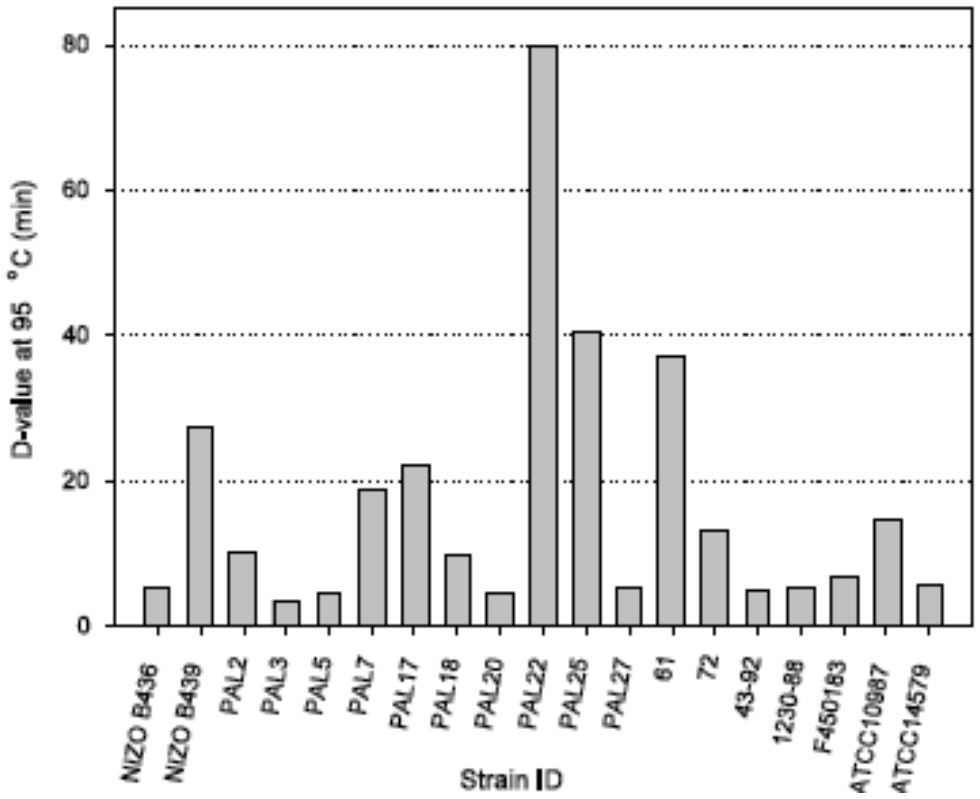

Figure 1-2.D values for different strains of spores of *B. cereus* (Vries 2006).

Ultraviolet Light

Ultraviolet processing involves the use of radiation from the ultraviolet region of the electromagnetic spectrum in order to reduce the number of microorganisms in the surface of a food. Typically, the wavelength for UV processing ranges from 100 to 400 nm. This range may be further subdivided (Bolton 1999 cited by FDA 2000) into UVA (from 315 to 400 nm), UVB (280 to 315 nm); UVC (from 200 to 280 nm) called the germicidal range since it effectively inactivates bacteria and viruses, and the vacuum UV range—from 100 to 200 nm—that can be absorbed by almost all substances and thus can be transmitted only in a vacuum (FDA 2000).

The germicidal properties of UV irradiation are mainly due to DNA mutations induced through absorption of UV light by DNA molecules.

Benoit and others (1990) found that *B. cereus* spores can be reduced in a 90% by applying UV doses of the order of 738 J/m2 in distilled water and several culture broths; however no experiments in tuna have been done to inactivate *B. cereus*.

Ozone

Another important alternative is the use of ozone. The strong antibacterial characteristics of ozone are due to a combination of its high oxidizing potential and its ability to diffuse through biological membranes (Hunt and Marinas 1996). Oxidation reactions of ozone in water follow two major pathways: direct oxidation by molecular ozone and indirect oxidation by free radical species formed from the auto decomposition of ozone. Also reactions between ozone and some inorganic and organic compounds can produce important antibacterial compounds (Hoigne and Bader 1976 cited by Hunt and Marinas 1997).

Broadwater and others (1973) found that ozone concentrations of 0.19 mg/L or higher can inactivate vegetative cells of *B. cereus* in water; however no complete data exist regarding inactivation of this microorganism in tuna.

Lactoperoxidase System

Finally, an important tool against gram positive pathogens is the use of the enzyme lactoperoxidase. Lactoperoxidase (LPO) is an enzyme that catalyses some oxidation reactions at the expense of hydrogen peroxide. This enzyme is widely distributed in the nature and can be found in plants and animals including man. LPO is a part of some human secretions such as saliva and tears (Kussendager and van Hooijdonk 2000). This enzyme has been evaluated as an antimicrobial agent, and its use has been suggested as a preservative in food (Bosch and others 2000).

LPO catalyses the following reaction (Kussendager and van Hooijdonk 2000):

$$2SCN^- + H_2O_2 + 2H^+ \xrightarrow{LPO} (SCN)_2 + 2H_2O$$
$$(SCN)_2 + H_2O \longrightarrow HOSCN + H^+ + SCN^-$$
$$HOSCN \longrightarrow OSCN^- + H^+$$

Hypothiocyanite ($OSCN^-$) and hypothiocyanus acid (HOSCN) are oxidizing products of this reactions and are responsible of the inhibition of some microorganisms by the oxidation of sulphydryl groups of important bacterial proteins (Kussendager and van Hooijdonk 2000). Tenovuo and others (1985) proved the efficacy of the lactoperoxidase system against *B. cereus* in phosphate buffer, but no research in tuna has been done.

Tuna Processing

Overview

The term tuna correspond to several species of fish from the family Scombridae and the genus *Thunnus*. One of tuna's most known characteristic is that its flesh is red—as oppose many other fish that have a white flesh—because of its higher amount of myoglobin in tuna's tissues (Wikipedia 2007). Tuna can be processed in several ways, the most common tuna products are canned tuna, precooked tuna loins, and raw tuna.

According to Foodmarket Exchange (2003) countries with the highest level of caught tuna are Japan and Taiwan. Ecuador and Mexico have the highest level of caught tuna in the eastern Pacific.

Data from Foodmarket Exchange (2003) shows that US is the world's largest market for canned tuna, with consumption estimated at about 46 million cases in 2001, or 28 percent of the world's consumption.

Canned Tuna

Canned tuna is one of the most popular ways of processing tuna since it has a longer shelf life and can be shipped to longer distances. Canned tuna is produced from fresh or frozen tuna in

accordance with requirements in the Canned Tuna Standard of Identity, 21 CFR 161.190 (FDA 2001).

Several tuna species are packed in water or oil and may be seasoned with salt, vegetable broth, hydrolyzed protein, or other optional ingredients. Bones, scales, skin, and other undesirable fish portions are removed from tuna. The filled cans are hermetically sealed and processed in retorts in accordance with processes scientifically designed to render them commercially sterile. The finished products are shelf stable low acid canned foods with pH above 6.0.

Tuna species used in tuna canning operations in Ecuador include Albacore, Yellow fin, Skipjack, and Big Eye. Other species provided for in the Canned Tuna Standard of Identity may be used (FDA 2001). Tuna are caught via a variety of methods, including long line, jig boat (hook and line), pole and line, and purse seine nets. Fishing boats are equipped with blast or brine freezing systems to quickly lower backbone temperatures of fish to the freezing point and below. Some fish are delivered to canneries directly from the boats utilized to catch them. Some are transferred from catch vessels to refrigerated carriers or vans and then delivered to canneries.

Tuna are unloaded from fishing boats, refrigerated carriers, or vans into steel fish boxes. During unloading, the fish are segregated into lots that identify the supplier, well, or van from which the fish were unloaded, and species. To be considered acceptable in the FDA standard, tuna lots must have histamine levels below FDA's established guidelines for canned tuna which is 50 ppm (2001). Tuna boxes are transferred to cannery cold storage and maintained at temperatures near 0°F (-18°C) until needed for processing (FDA 2001).

When tuna lots are scheduled for processing, tuna boxes are brought out of cold storage and thawed to backbone temperatures sufficient to facilitate evisceration and organoleptic

evaluation. After thawing, viscera are removed and trained staff for physical characteristics associated with decomposition or contamination evaluates fish. Any fish exhibiting unacceptable characteristics is rejected.

After evisceration and organoleptic evaluation, fish are placed on racks and transferred to large ovens called precookers where they are processed until backbone temperatures are sufficient to facilitate cleaning of the fish.

Precooked fish are cooled under controlled conditions and then transferred to the packing room for cleaning. The cleaning operation consists of manual removal of the head, tail, skin, bones, and dark flesh known as red meat.

Cleaned loins are fed into filling machines where prescribed amounts of fish are placed into cans. Using a separate system, empty cans are conveyed to filling machines after being inverted and flushed with air jets and/or water sprays.

When cans leave the filling machine, they are conveyed to locations where packing media and other ingredients are added. Several types of packing media are used in canned tuna processing. These include spring water, water or vegetable oil used alone or in combination with hydrolyzed protein and/or vegetable broth. Sodium acid pyrophosphate and/or salt may also be added.

Filled cans are conveyed to seaming machines where ends are put in place and the cans hermetically sealed. Each can or end is affixed with a production code that identifies manufacturing plant, product, date packed, batch, and other information necessary for product tracing purposes. Seamed cans are then retorted under controlled conditions designed by process authorities to render the can contents commercially sterile. Both the seaming and retorting

operations are carried out in strict compliance with Low Acid Canned Food regulations in 21 CFR 113 (FDA 2001).

After retorting, cans are partially cooled, in accordance with good manufacturing practice GMP establish in 21 CFR 113.5 (FDA 2001), and removed from retorts. Cans are then further cooled and delivered to labeling lines and are labeled, cased, and palletized. Cases and pallets are appropriately marked to facilitate product tracing and pallets are either shipped or staged in warehouses for later shipment.

Precooked Tuna Loins

Tuna Loins are produced from fresh or frozen tuna in accordance with requirements in the Canned Tuna Standard of Identity, 21 CFR 161.190 (FDA 2001). Tuna species, color designations (e.g., white or light), and forms of pack (e.g., solid or chunk) are as provided for in the standard. Bones, scales, skin, and other undesirable fish portions are removed from tuna, in keeping with good manufacturing practices, and the cleaned fish is packed into plastic bags from which most of the air is removed.

Tuna are unloaded from fishing boats, refrigerated carriers, or vans into steel fish boxes. During unloading, the fish are segregated into lots that identify the supplier, well, or van from which the fish were unloaded, and species. Lots considered potentially acceptable must have histamine levels below FDA's established guidelines for canned tuna. Tuna boxes are transferred to cannery cold storage and maintained at temperatures near 0°F (-18°C) until needed for processing.

When fish lots are scheduled for processing, fish boxes are brought out of cold storage and thawed to backbone temperatures sufficient to facilitate evisceration and organoleptic evaluation. After thawing, viscera are removed and fish are evaluated by trained staff for physical

characteristics associated with decomposition or contamination. Any fish exhibiting unacceptable characteristics is rejected.

After evisceration and organoleptic evaluation, fish are placed on racks and transferred to large ovens called precookers where they are processed until backbone temperatures are sufficient to facilitate cleaning of the fish.

Precooked fish are cooled under controlled conditions and then transferred to the packing room for cleaning. The cleaning operation consists of manual removal of the head, tail, skin, bones, and dark flesh known as red meat.

The precooked cleaned loins may be packed into plastic bags from which most of the air is removed, frozen and transferred by frozen containers to another plant for thawing and packing as canned tuna.

Objectives

The overall objective of the research was to determine the seasonal occurrence of *B. cereus* spores and vegetative cells in Skip Jack, Yellow Fin, and Big Eye tuna; as well as achieve *B. cereus* inactivation using non thermal technologies, and develop a HACCP plan.

- **Objective 1:** to develop the *B. cereus* growth curve in tuna at 28C
- **Objective 2:** to determine the doubling time of *B. cereus* in tuna at 21, 28, and 37 C
- **Hypothesis 1:** tuna can be held for more than 3 hours at ambient temperatures under adequate conditions without any risk of emetic toxin formation.
- **Hypothesis 2:** *B. cereus* is present only as vegetative cells—no as spores—in tuna. Several samples of tuna are to be analyzed for presence of spores in a monthly basis.
- **Hypothesis 3:** *B. cereus* presence in tuna is affected by the season in which the tuna is caught. Tuna samples are to be taken in different seasons and analyzed for presence of *B. cereus.*
- **Hypothesis 4:** *B. cereus* can be inactivated in tuna by non thermal treatments. Ozone, UV light, freezing and lactoperoxidase system are going to be tested to determine which of these alternatives is the best for reducing the number of *B. cereus* cells

CHAPTER 2
MATERIALS AND METHODS

Microbiological Materials and Methods

Media

Mannitol Egg Yolk Polimixin agar (MYP agar) was obtained from Oxoid Ltd., Lenexa, Kansas; polimixim B sulfate from Fisher Scientific Inc., Miami, Oklahoma; phosphate buffer from Fisher Scientific Inc., Miami, OK; and the egg yolk emulsion was prepared by soaking raw eggs in 70% ethanol, cracking the eggs, separating the yolk, and mixing it with equal volume of a 0.85% sodium chloride solution as described in the US Food and Drug Administration Bacteriological Analytical Manual (2001).

Microorganisms

Bacillus cereus strains were isolated from tuna samples from Seafman C.A.—a tuna processing company located in Manta, Ecuador—as follows: Tuna samples of the species *Katsuwonus pelamis* (skipjack tuna), *Tunnus albacares* (yellow fin tuna), and *Tunnus obesus* (big eye tuna) were obtained in the Pacific coasts of Ecuador in South America. *Bacillus cereus* was isolated from these samples by inoculation in MYP agar enriched with egg yolk emulsion and polimixim B sulfate. Confirmation test were executed by following FDA guidance on *B. cereus* given in the Bacteriological Analytical Manual (2001).

Enumeration

One hundred grams of each sample was dissolved in 900 ml of phosphate buffer to achieve 10^{-1} dilution. Following dilutions were made by adding 1 ml of the previous dilution to 9 ml of phosphate buffer. Each sample was diluted and run in triplicate by adding 0.1 ml of the dilution to the solidified MYP agar and spreading it with a glass spreader. Growth was observed after 24, 48, and 72 hours and reported as colony forming units per gram of tuna (cfu/g). Plates yielding

colony numbers from 25 to 200 were counted. Dilution was noted and used to calculate colony forming units per gram of tuna.

Seasonality Analysis

For the seasonal analysis, 10 samples of each of the species *Katsuwonus pelamis* (skipjack tuna), *Tunnus albacares* (yellow fin tuna), and *Tunnus obesus* (big eye tuna) were taken per month in two month intervals during one year. The samples were taken in the Pacific coasts of Ecuador. . Levels of *B. cereus* were determined by surface plating on MYP agar as described above.

Spore Assessment

Ten samples of each of the three tuna species mentioned above were taken in two month intervals during one year. The samples were taken in the pacific coasts of Ecuador. One hundred grams of each sample was dissolved in 900 ml of phosphate buffer to achieve 10^{-1} dilution. This dilution was divided in two halves: the first half was further diluted by adding 1 ml of the previous dilution to 9 ml of phosphate buffer and inoculating by triplicate in MYP agar as described above. The second half was heat treated at 80°C for 60 seconds and then diluted and inoculated as done with the first half. The difference in growth between the first and second half is the number of spores present in the sample)

Growth Assessment

Tuna samples of the specie *Tunnus Albacares* (Yellow fin tuna) were obtained from Norwest Seafood in Gainesville, Fl. The samples were inoculated with a known concentration of *B. cereus* cells and incubated at 28°C. Fifty grams of the sample were taken every 30 minutes during 6 hours and analyzed for *B. cereus* quantification by using Mannitol Egg Yolk Polimixin Agar as described in the FDA Bacteriological Analytical Manual (2001).

The growth curve and the doubling time of *B. cereus* in tuna were determined by using the traditional equations:

$$\mu = \frac{\ln N - \ln No}{t} \qquad \text{(equation 1)}$$

$$td = \frac{0.693}{\mu} \qquad \text{(equation 2)}$$

Where,

N = Final amount of *B. cereus* colonies per gram of tuna after the period of time t

No = Initial amount of *B. cereus* colonies per gram of tuna after the time t

t= Time in hours in which the exponential growth part of the curve is clear.

Td = Doubling time. Amount of time in hours that *B. cereus* needs in order to duplicate its number.

μ = slope of the exponential logarithmical exponential growth curve.

Bacillus Cereus Inactivation by Processing Methods

Ultraviolet Light Processing

Samples of Yellow Fin tuna were inoculated with known amounts of *B. cereus*. The inoculated samples received several doses of ultraviolet light irradiation (250 nm) and the amount of surviving *B. cereus* was measured by spread plating in MYP Agar as described above.

The 250 nm of wavelength ultraviolet light source was a 20 watts lamp from a Type 3 safety cabinet from Fischer Scientific Inc., Miami, OK. The amount of ultraviolet radiation was measured using an Ultraviolet Light Meter (Professional Equipment Inc., Janesville, WI).

Ozone Processing

Yellow Fin tuna samples were inoculated with known levels of *B. cereus* vegetative cells and treated with different levels of ozone. The ozone generator was from Tersano Inc., Buffalo, New York. There were two groups of Yellow Fin tuna samples. The first group was inoculated

with a known number of *B. cereus* vegetative cells and treated with several concentrations of ozone. The second group was inoculated with known amounts of *B. cereus* vegetative cells, frozen, and then treated with the same ozone concentrations used for the sample that was not frozen. In both cases, the number of surviving *B. cereus* was measured by spread plating on MYP agar as described above and reported in colony forming units per gram of tuna.

Lactoperoxidase System Processing

Yellow Fin tuna samples were inoculated with known amounts of *B. cereus* vegetative cells and treated in a one liter solution containing 100 µl of a 1mg/ml solution of the enzyme lactoperoxidase, 20 µl of a 30% hydrogen peroxide solution, and 1.8 grams of KSCN. The samples were treated and analyzed after zero, fifteen, and thirty minutes of contact time. The number of *B. cereus* surviving were measured by spread plating on MYP agar and were reported in colony forming units per gram of tuna.

Color Analyses

Yellow Fin tuna samples were analyzed before and after each of the three treatments by using a Color Machine Vision system from the Department of Food Science and Human Nutrition at University of Florida, Gainesville, Florida.

The L*(light-dark scale), a* (red-green scale), and b* (yellow-blue scale) values were recorded and analyzed looking for statistical differences.

Statistical Analysis

All experiments were run in triplicates. The means of microbial counts of all repetitions were calculated for the seasonality, spore assessment, and for all the treatments. All statistical analysis were run by using the Statistical Analysis System software SAS 9.0 from SAS Institute Inc., Cary, North Carolina, and significance was reported at levels of P value lower than 0.05.

CHAPTER 3
RESULTS

Seasonality and Spore Assessment

In order to find the effect of the sea temperature in the occurrence of *B. cereus* in the species skip jack, yellow fin, and big eye ten samples of each species were taken every two months and analyzed for presence of *B. cereus* vegetative cells and spores. The sampling and analysis procedure are detailed in the materials and methods section.

All the samples that tested positive, all the *B. cereus* vegetative cells were found in the surface of the tuna only.

Figure 3-1 shows the seasonal occurrence of *B. cereus* in the most commonly used tuna species: skip jack (SJ), yellow fin (YF), and big eye (BE)

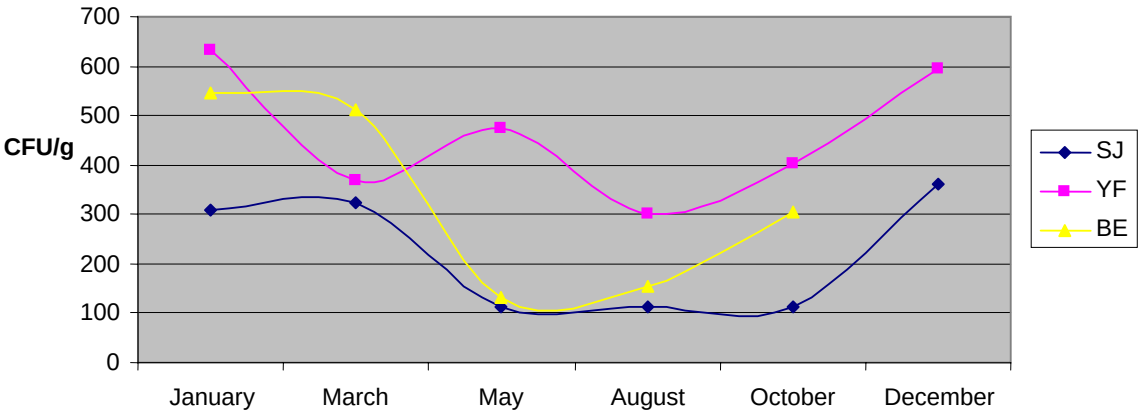

Figure 3-1. The seasonal occurrence of B. Cereus in the species skip jack (SJ), yellow fin (YF), and Big eye (BE) tuna

Data in Figure 3-1 reveals that the highest levels of *B. cereus* are generally present in the yellow fin specie, except during the month of March, in which the big eye specie had the highest level of *B. cereus*.

The data show a clear seasonality trend in *B. cereus* levels in the Skip Jack specie.

The highest *B. cereus* levels for the Skip Jack specie occurred in the months of January and December, in which the water temperature is the highest of the year (about 32°C).

The coldest month of the year—from May to August—show the lowest amount of *B. cereus* in the Skip Jack specie.

The Big Eye specie also shows a seasonality pattern similar to that of the Skip Jack specie. The highest values of *B. cereus* in the Big Eye specie were found in the month of January—the Big Eye specie was not sampled and analyzed in the month of December—and the lowest levels were also found between the months of May and August.

The Yellow Fin specie did not show the same seasonality trend as the Skip Jack and Big Eye species did, however the highest levels of *B. cereus* were found in the months of January and December, and the lowest in the month of August.

The individual bimonthly data are detailed in Tables 3-1 to 3-6

Table 3-1. *Bacillus cereus* occurrence in the Skip Jack, Yellow Fin, and Big Eye tuna species in the month of January 2006. All values are the means of the positive samples out of 10 samples expressed as colony forming units per gram of tuna. Standard deviations are also shown

Specie	Positive samples	Average vegetative cells (cfu/g)	Average spores
Skip Jack	9	$310 \pm 136_a$	0
Yellow Fin	6	$633 \pm 175_a$	0
Big Eye	9	$544 \pm 305_a$	0

Values with the same subscript a, b, or c are not significant different ($P > 0.05$)

Table 3-1 shows the results for the month of January.

All the samples were collected in the same location in the Ecuadorian sea. The average weight of the Skip Jack, Yellow fin, and Big Eye samples was 10 lb, 50 lb, and

20 lb respectively. No significant difference (P > 0.05) was found among the levels of *B. cereus* in any of the species.

Table 3-2. *Bacillus cereus* occurrence in the Skip Jack, Yellow Fin, and Big Eye tuna species in the month of March 2006. All values are the means of the positive samples out of 10 samples expressed as colony forming units per gram of tuna. Standard deviations are also shown.

Specie	Positive samples	Average vegetative cells (cfu/g)	Average spores
Skip Jack	10	$323 \pm 188_a$	0
Yellow Fin	9	$369 \pm 319_a$	0
Big Eye	9	$512 \pm 297_a$	0

Values with the same subscript a, b, or c are not significant different (P > 0.05)

Table 3-2 shows the results for the month of March. The average weight of the Skip Jack, Yellow fin, and Big Eye samples was 10 lb, 50 lb, and 20 lb respectively. No significant difference (P > 0.05) was found among the levels of *B. cereus* in any of the species

Table 3-3. *Bacillus cereus* occurrence in the Skip Jack, Yellow Fin, and Big Eye tuna species in the month of May 2006. All values are the means of the positive samples out of 10 samples expressed as colony forming units per gram of tuna. Standard deviations are also shown

Specie	Positive samples	Average vegetative cells (cfu/g)	Average spores
Skip Jack	6	$112 \pm 112_a$	0
Yellow Fin	3	$475 \pm 150_a$	0
Big Eye	5	$133 \pm 189_a$	0

Values with the same subscript a, b, or c are not significant different (P > 0.05)

Table 3-3 shows the results for the month of May. All the samples were collected in the same location in the Ecuadorian sea.

The average weight of the Skip Jack, Yellow fin, and Big Eye samples was 10 lb, 50 lb, and 20 lb respectively. No significant difference (P > 0.05) was found among the levels of *B. cereus* in any of the species.

Table 3-4. *Bacillus cereus* occurrence in the Skip Jack, Yellow Fin, and Big Eye tuna species in the month of August 2006. All values are the means of the positive samples out of 10 samples expressed as colony forming units per gram of tuna. Standard deviations are also shown.

Specie	Positive samples	Average vegetative cells (cfu/g)	Average spores
Skip Jack	5	$112 \pm 98_a$	0
Yellow Fin	4	$301 \pm 145_a$	0
Big Eye	5	$154 \pm 202_a$	0

Values with the same subscript a, b, or c are not significant different ($P > 0.05$)

Table 3-4 shows the results for the month of August. All the samples were collected in the same location in the Ecuadorian sea.

The average weight of the Skip Jack, Yellow fin, and Big Eye samples was 10 lb, 50 lb, and 20 lb respectively. No significant difference ($P > 0.05$) was found among the levels of *B. cereus* in any of the species.

Table 3-5. *Bacillus cereus* occurrence in the Skip Jack, Yellow Fin, and Big Eye tuna species in the month of October 2006. All values are the means of the positive samples out of 10 samples expressed as colony forming units per gram of tuna. Standard deviations are also shown

Specie	Positive samples	Average vegetative cells (cfu/g)	Average spores
Skip Jack	5	$112 \pm 12_a$	0
Yellow Fin	9	$401 \pm 202_a$	0
Big Eye	5	$305 \pm 98_a$	0

Values with the same subscript a, b, or c are not significant different ($P > 0.05$)

Table 3-5 shows the results for the month of October. All the samples were collected in the same location in the Ecuadorian sea.

The average weight of the Skip Jack, Yellow fin, and Big Eye samples was 10 lb, 50 lb, and 20 lb respectively.

No significant difference ($P > 0.05$) was found among the levels of *B. cereus* in any of the species

Table 3-6. *Bacillus cereus* occurrence in the Skip Jack, Yellow Fin, and Big Eye tuna species in the month of December 2006. All values are the means of the positive samples out of 10 samples expressed as colony forming units per gram of tuna. Standard deviations are also shown

Specie	Positive samples	Average vegetative cells (cfu/g)	Average spores
Skip Jack	10	$445 \pm 198_a$	0
Yellow Fin	10	$605 \pm 312_a$	0
Big Eye	NA*	NA*	NA*

*NA = Not analyzed. Values with the same subscript a, b, or c are not significant Different ($P > 0.05$)

Table 3-6 shows the results for the month of December. All the samples were collected in the same location in the Ecuadorian sea. The Skip Jack samples weighed 10 pounds on average, the Yellow Fin ones 50 pounds and the Big Eye ones 20 pounds. No significant difference ($P > 0.05$) was found among the levels of *B. cereus* in any of the species.

Bacillus Cereus Growth Assessment in the Yellow Fin Tuna Specie.

In order to determine the doubling time of *B. cereus* in Yellow Fin tuna under processing temperature (28°C), samples of Yellow Fin tuna were inoculated with *B. cereus* and its growth at 28°C was monitored every 30 minutes. Figure 3-2 shows the results at 28°C. From the data point in Figure 3-2 the slope value—or μ—can be obtained from the regression equation: $\mu = 0.34\ hours^{-1}$; then the doubling time was calculated using the μ value: $td = \frac{0.693}{0.34} = 2hours$

This information can be used to calculate the time that *B. cereus* needs to increase from 10^2 to 10^4 CFU/gram by working equation 1 (see materials and methods section) for t:

$$t = \frac{\ln 10^4 - \ln 10^2}{0.34} = 13.54h$$

This result shows that if we have an initial population of *B. cereus* of 10^2 CFU/g it would take it 13.54 hours to reach the critical value of 10^4 CFU/g

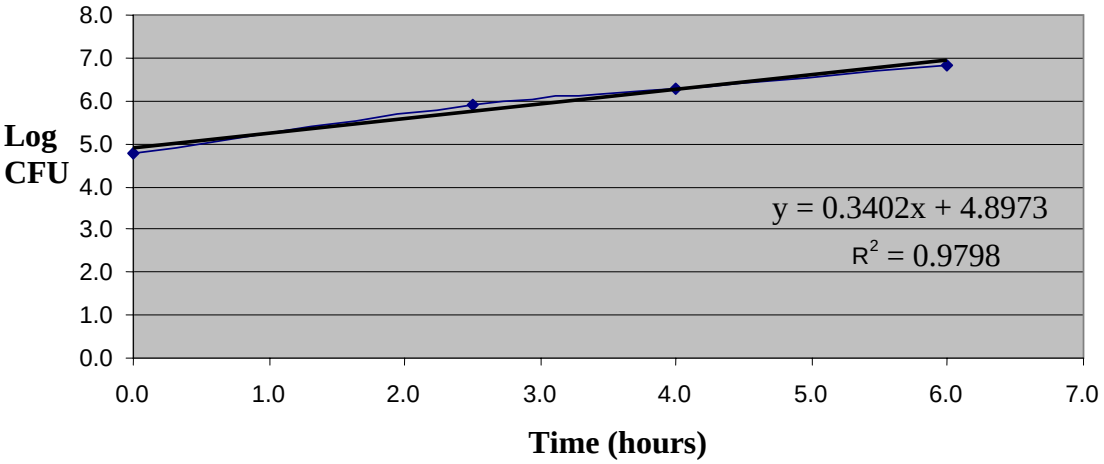

Figure 3-2. Exponential growth of B. cereus in tuna at 28°C. The regression equation and coefficient are also shown.

Bacillus Cereus Inactivation

Inactivation by Ultraviolet Light

To evaluate the effects of ultraviolet light against *B. cereus* in Yellow Fin tuna, several Yellow Fin tuna samples were inoculated with known levels of *B. cereus*. These samples were then treated with several ultraviolet radiation doses and the surviving amount of *B. cereus* was measured in colony forming units per gram of Yellow Fin tuna.

Table 3-7 shows the different ultraviolet radiation doses that were used in this study.

Table 3-7. *Bacillus cereus* inactivation by Ultraviolet light in Yellow Fin tuna at 28°C.

Before Treatment	After 8.5 mJ/cm^2	After 17 mJ/cm^2
7.04_a	5.85_b	5.48_b
6.36_a	4.92_b	4.60_b
7.98_a	4.70_b	3.0_b

Values with the same subscript a, b, or c are not significant different (P > 0.05). All the values in the Table are in Log_{10} of colony forming units of *B. cereus* per gram of Yellow Fin tuna.

Data in Table 3-7 show that a significant ($P < 0.05$) reduction can be achieved by ultraviolet light. Table 3-7 also shows that more than 2 log reduction can be obtained by the ultraviolet light doses shown in the Table. There is no significant difference ($P > 0.05$) between treatments at 8.5 mJ/cm^2 and 17 mJ/cm^2. Figure 3-3 shows the progressive reduction of *B. cereus* vegetative cells in Yellow Fin tuna at 28°C.

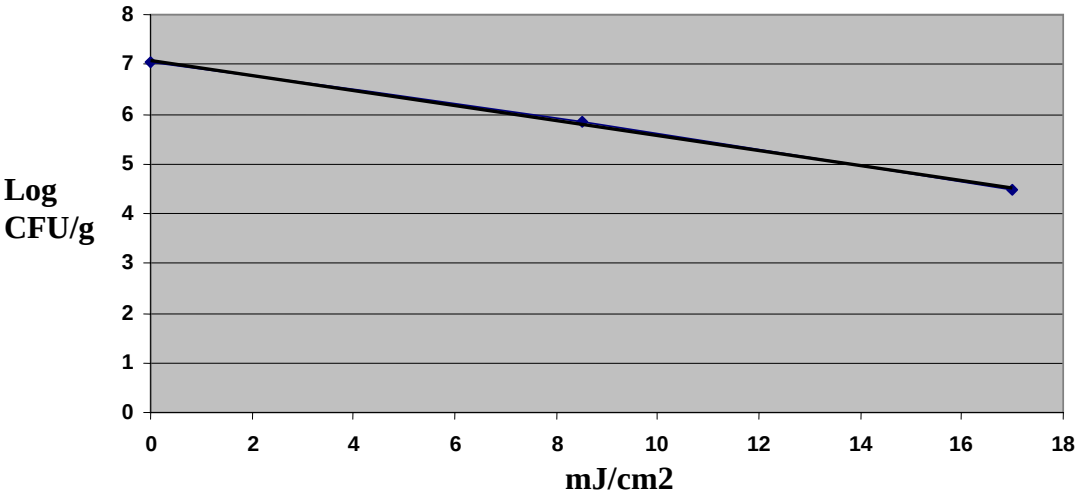

Figure 3-3. Inactivation of *B. cereus* in Yellow Fin tuna by several doses of ultraviolet light.

As we can see in Figure 3-3 as we plot the logarithm of the colony forming units per gram of tuna versus the UV doses, the plot follows a straight line pattern.

Bacillus Cereus **Inactivation by Ozone**

Yellow Fin tuna samples were inoculated with known amounts of *B. cereus* vegetative cells and treated with different concentrations of ozone. Table 3.8 shows the results of several concentrations treatments.

Table 3-8. Reduction of *B. cereus* in Yellow Fin tuna at several ozone concentrations

Ozone concentration (mg/L)	Treatment time (min)	Log reduction
0.3	30	0.138_a
0.4	30	0.45_b
0.5	30	0.98_c

Values with the same subscript a, b, or c are not significant different ($P > 0.05$)

Data in Table 3-8 shows that the log reduction increased as the ozone concentration increased.

There is a significant difference ($P < 0.05$) between each of the treatments at the ozone concentrations specified in the Table

Bacillus Cereus Inactivation by Lactoperoxidase System

Yellow Fin tuna samples were inoculated with known amount of *B. cereus* vegetative cells and treated in a one liter solution containing 100 µl of a one mg/ml solution of the enzyme lactoperoxidase, 20 µl of a 30% hydrogen peroxide solution, and 1.8 grams of KSCN. The samples were treated and analyzed after zero, fifteen, and thirty minutes of contact time. Table 3-9 shows the results of the treatment.

Table 3-9. Reduction of *B. cereus* at several contact times by a solution containing 100 µl of a one mg/ml solution of the enzyme lactoperoxidase, 20 µl of a 30% hydrogen peroxide solution, and 1.8 grams of KSCN

Contact time	Initial count of *B. cereus* (Log_{10} CFU/g)	Final count of *B. cereus* (Log_{10} CFU/g)	Log reduction
15 min	5.08_a	4.7_a	0.4_b
30 min	5.08_a	4.2_a	0.87_b

Values with the same subscript a, b, or c are not significant different ($P > 0.05$)

Data in Table 3-9 shows the amount of log reduction that can be achieved by different contact times by using the lactoperoxidase system described above, however this reduction is not significant ($P > 0.05$).

Also no significant difference ($P > 0.05$) was found among the two contact times.

Color Analysis of Tuna after Each Treatment

In order to evaluate the effects of each of the analyzed treatment on the color and visual appearance, each treated sample was analyzed by using a Machine Vision system, and the results were compared to non treated tuna.

Color Analysis after UV Treatment

Figure 3-4 shows photographs of typical tuna loins before and after being treated with the UV light doses described in Table 3-7

A

B

Figure 3-4. Photographs of typical tuna samples before (A) and after UV treatment (B)

Color variations were analyzed by sampling the tuna before and after treatment with UV light.

Table 3-10 shows the average results of the L*, a*, and b* values of the treated and non treated samples.

Data in Table 3-10 shows that the a* values after the treatment are significantly higher ($P < 0.05$) than those of the untreated sample, suggesting a color closer to the brownish region.

Table 3-10. Average results of the L*, a*, and b* values of the UV treated and non treated Yellow Fin tuna samples

	Before treatment	UV treated
L*	50.77_a	46.88_a
A*	23.25_b	28.36_c
B*	12.62_d	14.42_e

Values with the same subscript a, b, c, d or e are not significant different ($P > 0.05$)

The b* value also presented a significant difference ($P < 0.05$) among the treated and non treated samples whereas the L* value does not show significant difference ($P > 0.05$) among the treated and non treated samples.

Color analysis after Ozone treatment

Color variations were analyzed by sampling the tuna fillets before and after treatment with ozone. Tuna samples were treated with the ozone concentrations shown in Table 3-8 and the a*, L*, and b* values were measured. Table 3-11 shows the average results of the L*, a*, and b* values of the treated and non treated samples. Data in Table 3-11 shows that the a* values after the treatment are significantly higher ($P < 0.05$) than those of the untreated sample, suggesting a color closer to the brownish region. The L* and b* values do not show significant difference ($P > 0.05$) among the treated and non treated samples.

Table 3-11. Average results of the L*, a*, and b* values of the ozone treated and non treated Yellow Fin tuna samples

	Before treatment	Ozone treated
L*	50.77_a	47.25_a
a*	23.25_b	26.42_c
b*	12.62_d	12.53_d

Values with the same subscript a, b, c or d are not significant different

Figure 3-5 shows photographs of typical tuna fillets before and after being treated with the ozone doses described in Table 3-8

A

B

Figure 3-5. Photographs of typical tuna samples before (A) and after ozone treatment (B)

Color Analysis after Treatment with Lactoperoxidase System

Figure 3-6 shows photographs of typical tuna fillets before and after being treated with the ozone doses described in Table 3-9

A

B

Figure 3-6. Photographs of typical tuna fillet samples before (A) and after lactoperoxidase system treatment (B)

Color variations were analyzed by sampling the tuna before and after treatment with lactoperoxidase system and then tested by using a Machine Vision device to contrast those colors.

Table 3-12 shows the average results of the L*, a*, and b* values of the treated and non treated samples.

Table 3-12. Average results of the L*, a*, and b* values of the lactoperoxidase system treated and non treated Yellow Fin tuna samples

	No treatment	Lactoperoxidase system treatment
L*	50.77_a	47.32_a
a*	23.25_b	30.07_c
b*	12.62_d	15.53_e

Values with the same subscript a, b, c, d or e are not significant different

Data in Table 3-12 shows that the a* values after the treatment are significantly higher ($P < 0.05$) than those of the untreated sample, suggesting a color closer to the brownish region.

The b* values of the tuna treated with lactoperoxidase system are also significantly different ($P < 0.05$) than those of the non treated tuna samples.

Only the L* value does not show significant difference ($P > 0.05$) among the treated and non treated tuna samples.

Plan HACCP Flow Chart and Critical Control Points for Processing of Tuna

Figure 3-7 shows the flow chart for processing precooked tuna loins. As we can see only one critical control point can be detected.

Table 3-13 shows the critical limits, monitoring actions, and corrective actions for the processing of tuna loins. Notice that tuna can also be processed on several other ways, being canned tuna the most processed tuna product in the world; however because of the high temperatures involved, *B. cereus* may not be a threat in canned tuna.

Since only one critical limit was detected (histamine at reception), all the corrective action determined in the HACCP plan are related to the prevention of the occurrence of this hazard.

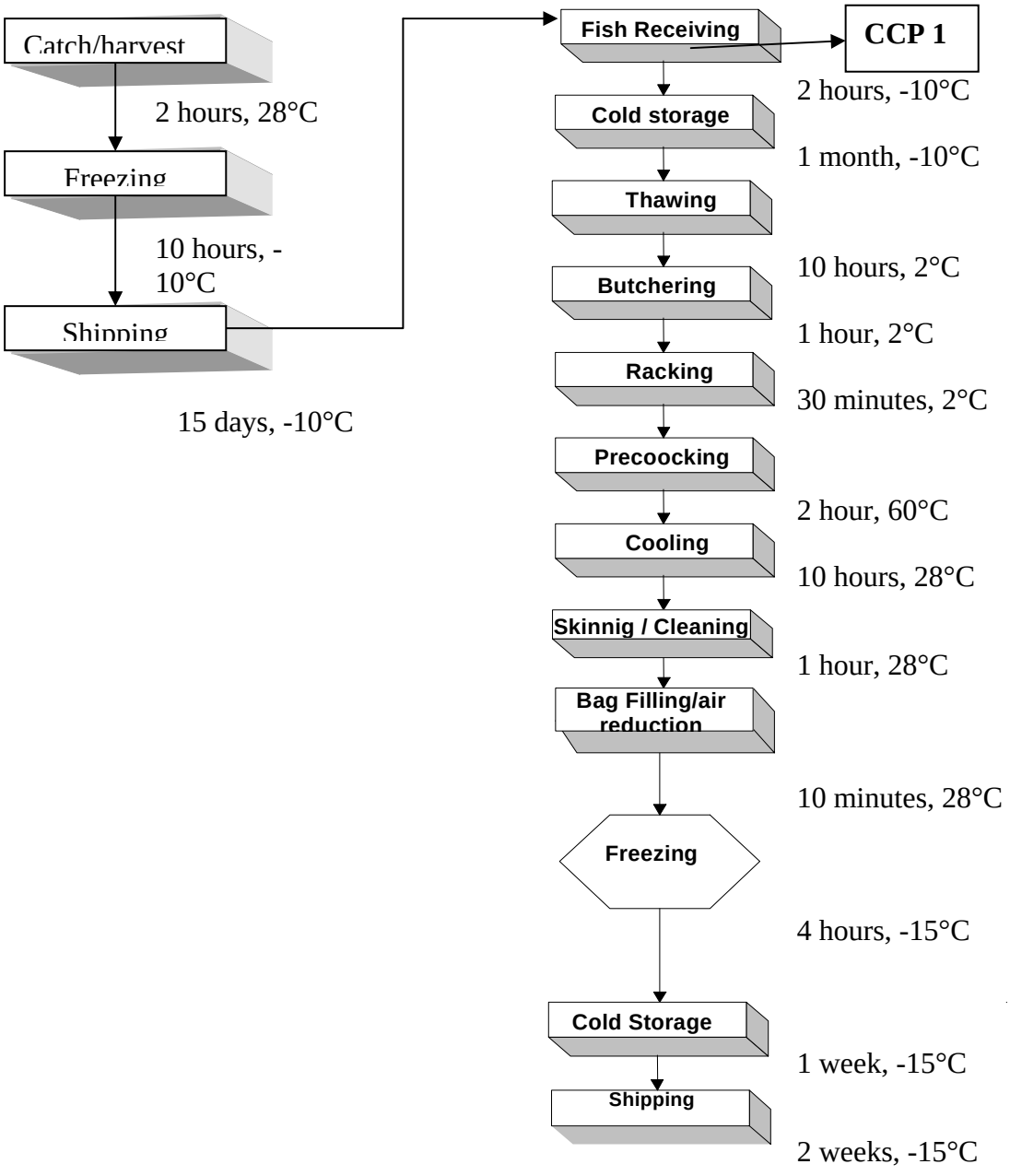

Figure 3-7.Hazard Analysis and Critical Control Points flow chart

Table 3-13. Precooked tuna loins HACCP summary

Critical control point	Hazard	Critical limit(s)	Monitoring			Corrective actions	Records	Verification
Fish receiving	Chemical	Histamine levels shall be less than FDA action level of 5 mg% (50ppm) (FDA 2001)	Histamine levels of incoming loin lots.	Collect samples and analyze for histamine content.	Every loin lot.	Quality control	Reject loins with histamine levels in excess of 4.9 mg% (49 ppm) or divide into sub-lots of 25 TM max. ant test 60 fish for histamine. Reject sub-lot if any histamine greater than 4.9 mg% (49 ppm) (FDA 2001)	Histamine analytical results Histamine sub-lots results	Verify accuracy of histamine analytical results; Review records within one week

CHAPTER 4
DISCUSSION

Seasonality and Spore Assessment

In Figure 3-1 there is a significant ($P < 0.05$) variation in the number of *B. cereus* vegetative cells in the months of May to August compared with the rest of the months in all the three species Skip Jack, Yellow Fin, and Big Eye tuna. The months from May, June, July and some days of August are colder in the region of Ecuador compared with the rest of the year. During this time of the year the temperature in the coast of Ecuador is around 20°C which is more than 10°C lower than the rest of the year. This lower temperature might have caused the number of vegetative cells of *B. cereus* to be lower in tuna, since they cannot multiply at the same rate they do at above 30°C.

Another hypothesis is that since during those months, the catching of tuna is not as abundant as the rest of the months—probably because many of them migrate to warmer waters, and some times the International Commission of Tuna bans the catching of tuna during those months to assure future sustainability—therefore the fishing vessels have lower amount of tuna being cough, so they can handle the tuna in a more quicker and efficient manner thus preventing the exposure of the catch to high temperatures during high periods of time before the tuna is placed in frozen storage.

We can also notice from Table 3-1 that the highest amount of *B. cereus* vegetative cells are present in Yellow Fin tuna, followed by Big Eye tuna, and the last one is Skip Jack tuna. These three species of tuna do not differ much in their composition, and feeding and migrating habits; however one major difference between the three species is the average weight of the caught. The weigh of the Yellow Fin and Big Eye tuna species tend to be larger than the Skip Jack specie. Specifically, the average size of the sampled Yellow Fin tuna was 50 pounds, whereas the Big

Eye tuna specie was 20 pounds, and for the Skip Jack one was 10 pounds. This size differences may have had an influence in the number of *B. cereus* vegetative cells present in the sample, giving a positive correlation between the amount of *B. cereus* vegetative cells present in a whole tuna and the original tuna size, since the bigger the tuna the higher the number of *B. cereus* vegetative cells.

No spores were found in any of the samples analyzed. The absence of *B. cereus* spores may be because vegetative cells of *B. cereus* have sufficient nutrients and therefore do not need to sporulate in tuna.

Another important fact to notice is that the highest level of *B. cereus* found was in the order of 10^2 colony forming units per gram of tuna, which is not enough to produce the toxin. The lowest level of *B. cereus* vegetative cells needed to produce the toxin is 10^4 (10,000) colony forming units per gram of food (Granun 2001). Even though those levels are not likely to be found in fresh tuna according to this research, time and temperature abuse may occur during processing, and levels of 10,000 or greater in might be reached in tuna.

Growth Assessment

From Figure 3-2 we can see that at 28^{o}C *B. cereus* needs approximately 2 hours to increase its population in 1 log in Yellow Fin tuna. In other words, since the seasonality data shows that the regular levels of *B. cereus* in tuna are in the 10^2 (100) levels of colony forming units per gram of Yellow Fin tuna, a time and temperature abuse of two hours at 28°C may cause the *B. cereus* population increases to 10^4 in tuna and cause the illness. This is an important finding because some processors do not control their processing time and therefore the amount of *B. cereus* may increase in the production lines if they do not hold the tuna at the proper time-temperature while processing it.

Figure 3-2 shows the growth of *B. cereus* in Yellow fin tuna at 28C. We can see that it is much slower in growth with a doubling time of 2 hours. This might be due to the complexity of the protein composition in Yellow Fin tuna, which forces *B. cereus* to use its proteolytic enzymes in order to obtain free amino acids that can be used by the bacteria as food. Yellow Fin tuna samples have a moisture contend of about 75% and a slightly acid pH of 6.5 as an average. These conditions are conducive for *B. cereus* enzymes to break down nutrients; however those conditions are not optimal

Bacillus Cereus Inactivation and Color Analysis

Ultraviolet Light

As mentioned earlier, all the samples that were found to be contaminated with *B. cereus* were only contaminated in the surface, therefore any antibacterial port harvest treatment that affects the surface of the tuna may be successful in lowering the number of bacteria. In addition, the seasonality study revealed that no spores are found under regular catching and processing circumstances, so the post harvest antibacterial treatments were focused to reduce *B. cereus* vegetative cells only.

Figure 3-3 shows the kinetics of inactivation of *B. cereus* vegetative cells in the surface of Yellow fin tuna. As we can see in Figure 3-3, the higher the dose of UV radiation, the higher the inactivation of *B. cereus* vegetative cells. There is a significant ($P < 0.05$) reduction in the number of *B. cereus* vegetative cells after applying UV treatment.

The reduction may be because of the following reasons:

- *Bacillus cereus* is mostly present in the surface of the tuna in the early stages of the process
- *Bacillus cereus* is found in tuna on its vegetative stage only
- Tuna surface does not provide a significant protection against UV light, which is usually provided by fatty compounds.

For these reasons the UV radiation can easily penetrate trough *B. cereus* membrane and cause DNA mutations induced through absorption of UV light by DNA molecule.

Another important point is that UV light has a low penetration power, so it would not cause any important change in the tuna composition. However there may be an effect on the myoglobin composition of the tuna since a significant difference ($P < 0.05$) was found in the a* value of the UV treated tuna when compared to the non treated ones.

Some authors have suggested that this phenomenon is due to the fact that UV radiation stimulates the formation of metmyoglobine which has a brownish color (Djenane and others 2001).

Ozone

Bacillus cereus vegetative cells were targeted in the surface of some Yellow Fin tuna samples. Data in table 3-8 shows that the higher the ozone concentration, the higher the level of inactivation of *B. cereus* in tuna.

The reduction in the levels of this bacterium may be due to direct oxidation of *B. cereus*' membrane and citoplasmatic compounds by molecular ozone and indirect oxidation by free radical species formed from the auto decomposition of ozone and its reaction with some tuna compounds as described in the review of the literature (Hunt and Marinas 1997).

One of the main problems found when treating tuna with ozone was the oxidation of some pigmented compounds in the tuna. This caused a significant ($P < 0.05$) variation in the a* value probably due to oxidation reaction on the myoglobine molecules in the tuna leading to the production of metmyoglobin.

Lactoperoxidase System

The effect of lactoperoxidase system was found to be not significant ($P > 0.05$) when compared with untreated controls. The lack of effect of this treatment may be due to the fact that

some bacteria use compounds in the food surface to reduce the contact area and provide protection against lactoperoxidase antimicrobial products as suggested by Min and others (2005). Color changes in the tuna samples were also noticed, due to the oxidation reactions of the hydrogen peroxide which is one of the components of the lactoperoxidase system. A significant difference ($P < 0.05$) was found in the a* value of the sample treated with lactoperoxidase system compared to tuna samples that were not treated.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Concerns about the presence of *B. cereus* in tuna can be supported by this research since the seasonal results show that the average levels of *B. cereus* in tuna can be as high as 63×10^1 CFU/g of tuna especially in the months of January and December. The Yellow Fin and Big Eye species were the ones that presented the highest levels of *B. cereus* probably because they are usually bigger in size and weight than the Skip Jack specie.

Even though the likelihood of finding *B. cereus* in tuna is very high, the levels found do not represent any threat to human health, since they were below 10^4 CFU/g which is the lowest amount of *B. cereus* needed to produce intoxication. For this reason FDA suggests a holding time at room temperature of 3 hours or less, so the *B. cereus* levels are kept below this value.

No spores of *B. cereus* in tuna were found in a one year period, suggesting that *B. cereus* has all the nutrients they need in order to stay in a vegetative stage. For this reason all efforts to reduce *B. cereus* in tuna should be directed to the vegetative stage only.

In the second part of this research the doubling time was determined for *B. cereus* in tuna at room temperature (28°C). The doubling time obtained was 2 hours and the μ value 0.34 h^{-1}. This allows us to calculate the actual amount of time that processors can hold the tuna before it reaches the critical *B. cereus* level of 10^4 CFU/gram of tuna. The calculated time (assuming a starting level of 10^2 CFU/g) is 13.5 hours. This shows that the 3 hours limit may actually be underestimated.

The third part of the research was aimed to find a method to reduce the levels of *B. cereus* in tuna with the least change in the color of the tuna. The processing methods used in this study were non thermal, since the final tuna product is intended for raw or precook usages.

The first processing method we tested was ultraviolet light. Ultraviolet light is known to be effective when the microorganism is mainly present in the surface of the product (which is the case of *B. cereus* in tuna). Several ultraviolet doses (doses between 8.5 mJ/cm^2 and 17 mJ/cm^2 were used) were tested producing significant ($P < 0.05$) reduction in the levels of *B. cereus* in tuna. The doses needed to produce this effect were achieved by a regular ultraviolet light lamp from a type 2 laminar flow cabinet. This suggests that the dosages needed are too low, so it would be something of low cost to apply in a bigger industrial scale. Even though UV light achieved a significant log reduction in the levels of *B. cereus* in tuna, there is an effect on the myoglobin composition of the tuna since a significant difference ($P < 0.05$) was found in the a* value of the UV treated tuna when compared to the non treated ones.

The second processing method we tested was ozone. Relatively low ozone concentrations (between 0.1 and 0.5 ppm of ozone) were tested yielding to significant ($P < 0.05$) log reduction of *B. cereus* in tuna. Ozone is known to cause oxidation in several cytoplasmatic compounds of *B. cereus*. One of the main problems found when treating tuna with ozone was the oxidation of some pigmented compounds in the tuna. This caused a significant ($P < 0.05$) variation in the a* value probably due to oxidation reaction on the myoglobine molecules in the tuna leading to the production of metmyoglobin.

The third processing method used was the lactoperoxidase system. Lactoperoxidase is an enzyme that takes hydrogen peroxide and some tyocianates to produce tyocianic acid, which is further broken into bactericide compounds. Yellow Fin tuna samples were inoculated with known amount of *B. cereus* vegetative cells and treated in a one liter solution containing 100 µl of a one mg/ml solution of the enzyme lactoperoxidase, 20 µl of a 30% hydrogen peroxide solution, and 1.8 grams of KSCN. No significant ($P > 0.05$) log reduction was observed in the

levels of *B. cereus* in tuna. Color changes in the tuna samples were also noticed, due to the oxidation reactions of the hydrogen peroxide which is one of the components of the lactoperoxidase system. A significant difference ($P < 0.05$) was found in the a* value of the sample treated with lactoperoxidase system compared to tuna samples that were not treated.

In conclusion, treating tuna with moderate doses of UV light (doses between 8.5 mJ/cm^2 and 17 mJ/cm^2 are recommended) is effective in reducing the levels of *B. cereus* in tuna, however it causes a small change in the a* value.

Treating tuna with concentrations above 0.5 ppm of ozone is also effective in reducing the levels of *B. cereus* in tuna but a small change in the a* value is also observed. Further research need to be done to find if this significant ($P < 0.05$) changes in the color of the tuna are actually perceivable by the human eye.

APPENDIX
SEASONALITY AND SPORE ASSESSMENT RAW DATA

Table A-1. Raw data for the seasonal analysis for the month of January 2006.

Sample	Skip Jack	Yellow Fin	Big Eye
1	269	429	406
2	342	649	599
3	427	905	824
4	66	455	133
5	360	704	647
6	344	656	605
7	445	TFTC	872
8	119	TFTC	8
9	418	TFTC	801
10	TFTC	TFTC	TFTC
Average	310	633	544
STD	135	175	305

TFTC: Too few to count. All values are in CFU/g

Table A-2. Raw data for the seasonal analysis for the month of March 2006.

Sample	Skip Jack	Yellow Fin	Big Eye
1	297	930	289
2	392	108	663
3	504	207	167
4	82	809	537
5	416	566	728
6	395	266	671
7	528	49	177
8	100	221	319
9	493	164	1057
10	23	TFTC	TFTC
Average	323	369	512
STD	189	320	298

TFTC: Too few to count. All values are in CFU/g

Table A-3. Raw data for the seasonal analysis for the month of May 2006.

Sample	Skip Jack	Yellow Fin	Big Eye
1	75	338	25
2	199	451	129
3	298	636	461
4	25	TFTC	25
5	49	TFTC	25
6	25	TFTC	TFTC
7	TFTC	TFTC	TFTC
8	TFTC	TFTC	TFTC
9	TFTC	TFTC	TFTC
10	TFTC	TFTC	TFTC
Average	112	475	133
STD	112	150	189

TFTC: Too few to count. All values are in CFU/g

Table A-4. Raw data for the seasonal analysis for the month of August 2006.

Sample	Skip Jack	Yellow Fin	Big Eye
1	25	287	25
2	109	359	54
3	262	450	500
4	25	108	25
5	139	TFTC	166
6	TFTC	TFTC	TFTC
7	TFTC	TFTC	TFTC
8	TFTC	TFTC	TFTC
9	TFTC	TFTC	TFTC
10	TFTC	TFTC	TFTC
Average	112	301	154
STD	98	145	202

TFTC: Too few to count. All values are in CFU/g

Table A-5. Raw data for the seasonal analysis for the month of October 2006.

Sample	Skip Jack	Yellow Fin	Big Eye
1	129	323	301
2	114	443	333
3	95	582	401
4	112	84	142
5	110	473	348
6	TFTC	447	TFTC
7	TFTC	612	TFTC
8	TFTC	77	TFTC
9	TFTC	568	TFTC
10	TFTC	TFTC	TFTC
Average	112	401	305
STD	12	202	98

TFTC: Too few to count. All values are in CFU/g

Table A-6. Raw data for the seasonal analysis for the month of December 2006. All values are in CFU/g

Sample	Skip Jack	Yellow Fin
1	350	463
2	482	668
3	636	908
4	427	593
5	515	720
6	486	674
7	669	960
8	240	156
9	620	884
10	25	25
Average	445	605
STD	198	312

All values are in CFU/g

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BIOGRAPHICAL SKETCH

Juan Manuel Cevallos was born in Manta, Ecuador on August 24th 1981. He is the sixth of seven children born to Eddie Cevallos and Esperanza Cevallos. He attended Julio Pierregrosse elementary and high school and graduated with honors being the best student graduating in 1999. He was admitted to do an undergrad program in food engineering and received a full tuition waiver at the Escuela Superior Politecnica del Litoral in Guayaquil, Ecuador. He graduated with honors being the best student graduating with a food engineering degree in 2004.

In early 2004 he started working in the quality assurance department for one of the biggest tuna processing companies in Ecuador: Sociedad Ecuatoriana de Alimentos y Frigorificos Manta C.A. (SEAFMAN C.A.)

In 2005 he was awarded a Fulbright fellowship to attend University of Florida to pursue a Master's degree in food science. At the same time he pursued a master's degree in agribusiness. He was awarded both degrees in 2007 and plans to begin a Ph.D. program in food science at the University of Florida.