ABSTRACT

Allen, Dennis Grey, Jr. Regulatory control of histamine production in North Carolina harvested mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacares*): a HACCP-based industry survey. (Under the direction of Drs. David Green and Lee-Ann Jaykus)

Histamine poisoning is one of the most common chemically induced seafood borne illnesses reported in the United States today. Generally it is believed that the causative agents are biogenic amines (histamine, putrescine and cadaverine) produced by Gram negative bacteria. Under the U.S. Food and Drug Administration's HACCP program, growth of histamine-producing bacteria in potentially hazardous fish is controlled primarily by limiting time and temperature conditions. The purpose of this study was to determine if current regulatory guidelines are being meet for the control of histamine production in North Carolina harvested mahi-mahi and yellowfin tuna, and if not, what potential food safety risks may likely occur.

Twenty-nine composite fish muscle samples were obtained from 18 mahi-mahi and 11 yellowfin tuna troll-caught and analyzed for their histamine content. No sample analyzed exceeded 2 ppm histamine, the detection threshold for Neogen's ELISA-based Veratox® rapid test. Fish internal temperatures were continuously monitored from point of harvest through primary processing to determine individual fish cooling rates. Mahi-mahi were chilled on ice within 12 hrs of harvest as required under the federal HACCP guidelines. Generally, yellowfin tuna (60%) did not meet the HACCP requirement [uneviscerated tunas exceeding 20 lbs (9.1 Kg) in weight] of achieving an internal temperature of $\leq 50^{o}F$ ($10^{o}C$) in 6 hrs.

Three hundred and eighty-six composite fish muscle and environmental samples were screened for the presence of histamine-producing bacteria. Twenty-six percent of 549 isolates selected based on their morphological characteristics tested positive on Niven's media. Sixty-three Niven's positive isolates were Gram negative rods and 58 were Gram positive. The Beckon Dickinson BBL Crystal method was used primarily for identification of Gram positive isolates since the API 20E Enterobacteriaceae identification test is specific for the identification of Gram negative bacteria. Neither API 20E test nor BBL Crystal method was able to identify every Niven's positive isolate.

Only five of forty-three isolates tested were confirmed and classified as low histamine producers (<250 ppm in 48 hrs at >15^{o}C). Three Gram negative isolates were identified as *Enterobacter cloacae*. Two Gram-positive isolates were identified as *Staphylococcus kloosii*. This study contradicts the general belief that Gram-negative bacteria are solely responsible for histamine production in potentially hazardous fish.

The confirmation of histamine-producing bacteria found in this study demonstrates the potential risk for histamine production. However, no detectable levels were found in the fish muscle samples analyzed, even though yellowfin tuna did not meet the regulatory HACCP guidelines. Therefore no food safety risks were found under commercial conditions studied.

REGULATORY CONTROL OF HISTAMINE PRODUCTION IN NORTH CAROLINA HAVESTED MAHI-MAHI (*CORYPHAENA HIPPURUS*) AND YELLOWFIN TUNA (*THUNNUS ALBACARES*): A HACCP-BASED INDUSTRY SURVEY

By

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BIOGRAPHY

Dennis Grey Allen, Jr. was born on April 7, 1979 in Raleigh, NC. He is the eldest son of Dennis G. Allen, Sr., a retired computer consultant for the Department of Agricultural Economics at NC State University, and Jane F. Allen, a first grade teacher at the Raleigh School for Children.

He first started his career in food science as a high school senior, where he worked on a project at NC State University to concentrate calcium-binding caseinate under supervision of Dr. Jonathan Allen. Through this experience his love of research flourished and he followed through to enroll as an undergraduate in the Food Science department. During this time he continued to work for Dr. Jonathan Allen and Dr. Daniel Carroll, as well as under Dr. Lee-Ann Jaykus on a project designed to concentrate *Listeria monocytogenes* and *Samanella* to better detect these organisms in Cheddar Cheese. In spring of 2001 Grey earned a BS degree in Food Science as well as Biochemistry

During the summer following graduation Grey continued his studies by working on a project developing a receptor based quantification test for PCB's and Dioxins at Hybrizyme Corporation, located in Raleigh, NC.

In August of 2001, Grey began his masters degree at North Carolina State University in Food Science, focusing on seafood safety and toxicology. In conjunction with Dr. David Green, the majority of his research was conducted at the Center for Marine Sciences and Technology at Morehead City, NC.

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INTRODUCTION

Biogenic amines are low molecular weight organic bases which normally serve several human physiological functions, such as regulation of body temperature, stomach volume, stomach pH, brain activity, and in high concentrations can cause allergic reactions (16, 48). Biogenic amines include the compounds ethanolamine, putrescine, cadaverine, spermidine, phenylethlamine, tyramine, and histamine (23). Most biogenic amines that are of a concern in foods are produced by endogenous microorganisms that decarboxylate amino acids (16). The most important biogenic amines from a food safety perspective are putrescine, formed from ornithine; cadaverine, formed from lysine; and histamine, formed from histidine. Illness resulting from the consumption of foods high in biogenic amines is generally called histamine poisoning. Alternatively, it may be referred to as scrombroid poisoning or scombrotoxicosis because of its common association with fish species in the suborder Scombriodei (i.e., tuna and mackerel).

BACKGROUND

Epidemiology and Outbreaks

From 1973 through 1987, 697 outbreaks reported to the Centers for Disease Control and Prevention (CDC) in Atlanta were caused by chemical agents, 29 percent of which were scombroid fish poisoning (37). From 1993 to 1997, 69 outbreaks caused 297 cases of scombroid poisoning. The total number of outbreaks and cases caused by chemical agents were 148 and 576, respectively, meaning that over half of the cases of

food borne disease caused by chemical agents was histamine poisoning (59). One incident of scombrotoxicosis occurred in February 1973, affecting 232 people in four states. All victims became ill about 45 minutes after consumption of one of two lots of commercially canned tuna, and their symptoms lasted about 8 hours (54).

Scombroid poisoning occurs not only in the United States, but the entire world. From 1976 to 1979, Britain had 50 reported scombroid poisoning outbreaks, from which almost 200 people fell ill (34). In one case in Taiwan, shortly after lunch on July 4, 1986, 41 employees of a department store were hospitalized with symptoms of scrombrotoxicosis (47).

Symptoms

The most common symptoms of histamine poisoning include rash, flushing and sweating, and burning of the mouth, sometimes described as oral tingling. This manifestation occurs seconds after consumption, declining rapidly in the first 10 minutes, and then slowly afterwards (18, 57). Nausea, vomiting, diarrhea, and stomach pain have also been associated strongly with histamine poisoning. Other possible symptoms include dizziness, and swelling of the tongue and face (57). Lethal doses of histamine in guinea pigs produce gasping respiratory movements, followed by convulsions, with autopsies revealing perforation of the gastric wall (14). Symptoms usually begin 10 minutes to 2 hours after consumption of toxic substances (57). The severity of symptoms can differ from person to person or with other variables. For example, in one study fish

implicated in an outbreak that led to hospitalization of several people was later fed to volunteers who reported only mild responses (18).

Histamine poisoning can be differentiated from an allergic reaction using three main criteria. The first criterion is that the inflicted individual had no previous allergy to the food in question. A second parameter is a high attack rate in outbreaks. Another guideline used for discriminating allergic reactions from histamine poisoning is the presence of a high concentration of histamine in the implicated food. Levels of immunoglobulin E (IgE) in afflicted individuals can also be used to discriminate allergic reactions from scombroid poisoning (16).

At Risk Food Products

Many foods can support significant histamine formation. Fermented products, such as salami, cheese, and canned sauerkraut have been shown to have histamine concentrations high enough to cause illness, because many of the lactic acid bacteria species needed to produce these products can also produce histamine (70). Fish of the suborder Scombroidei are good candidates for histamine formation because of the suborder's high concentration of free histidine in muscle tissue (16, 54). The suborder includes tunas, dolphin, mackerels, and many other fish. Histidine content might differ within a single species of fish, and may even differ with stage of reproduction at harvest, leading to a greater or lesser risk of conditions favorable to the production of histamine (35).

Associated Microorganisms

There are numerous species of bacteria that produce histamines, and many belong to the *Enterobacteriaceae* and *Bacillaceae* families (23). In general, species in the *Bacillus, Citrobacter, Clostridium, Escherichia, Klebsiella, Lactobacillus, Pediococcus, Photobacterium, Proteus, Pseudomonas, Salmonella, Shigella, and Streptococcus* genera have all been shown to produce amino acid decarboxylase activity (16). *Proteus spp.* and *Klebsiella spp.* were isolated and classified as strong histamine formers in samples of skipjack tuna and jack mackerel, with the most abundant bacteria being *Proteus morganii* (60). *Morganella morganii* and *Proteus mirabilis*, both mesophilic Gram negative rods, have been isolated from mahi-mahi and both species formed greater than 1 mg histamine/ml when grown in tryptic soy broth supplemented with 2% histidine monohydrochloride for 24 hours at 37 ^{o}C (32). *Morganella morgani* and *Proteus mirabilis* were also isolated from sardine, along with *Proteus vulgaris*, *Providencia stuartii*, and unidentified species of *Proteus* (2). From samples of mackerel collected from Barcelona, Spain markets, *Citobacter freundii*, *Enterobacter agglomerans*, *Morganella morganii, Proteus mirabilis*, *Serratia fonticola*, and *Serratia marcescens* have been isolated (51). In addition to these, *Enterobacter cloacae*, *Enterobacter intermedium*, *Hafnia alvei*, *Klebsiella oxytoca*, *Plesiomonas shigelloides*, *Proteus vulgaris*, *Pseudomona fluorescens*, *Serratia liquefaciens*, and *Serratia plymuthica* have all been isolated from samples of tuna collected from markets in the same area (51). Likewise, bonito samples contained *Enterobacter agglomerans*, *Enterobacter cloacae*, *Klebsiella pheumoniae*, and *Pseudomona fluorescens* (51). *Hafnia alvei* and *Proteus spp.*

were isolated and classified as weak histamine formers of skipjack tuna and jack mackerel (60). *Vibrio alginolyticus*, a mesophilic, Gram negative rod, was isolated from fresh mahi-mahi as a weak histamine former, producing less than 1mg histamine/10 ml of tryptic soy broth supplemented with 2% histidine monohydrochloride in 24 hrs at 37 ^{o}C (32). *Stenotrophomonas maltophia* was recently isolated from albacore (*Thunnus alalconga*) (13). *Photobacterium spp.*, a psychrophilic organism, has also been suggested as a significant histamine producer (16). *Alteromonas putrefaciens*, also a psychrotrophic, Gram negative rod, was isolated from mahi-mahi, and determined to be a weak histamine former, producing less than 1mg histamine/10ml histidine supplemented growth medium (32). *Lactobacillus buchneri*, isolated from Swiss cheese samples implicated in a food poisoning outbreak, was found to produce a significant amount of histamine (67).

The presence of high numbers of histamine-producing bacteria does not necessarily correlate with high histamine levels in samples. In one study, a very low percentage (7%) of isolates from one tuna specimen were histamine producers, but the level of histamine from that tuna sample was higher than that in a second tuna specimen with a much higher percentage (59%) of histamine-producing isolates (60). The most likely explanation for this observation is that the bacteria responsible for the histamine production were out competed or where inactivated over the course of product storage. Other possible explanations include the presence histamine reducing substances or bacteria, or that the bacterial isolates vary in their speed and ability to produce the compound (60).

The optimum conditions for growth of histamine producing bacteria are variable due to the wide range of microorganisms that produce the compound. For smoked fish from New Zealand, isolates that grew at 35, 20, and 5°C were found to contain 12.6, 7.5, and 22.8% histamine producers, respectively, showing a large range of microorganisms with the capability of producing histamine under different temperature conditions (30). There have been varying reports on how temperature affects biogenic amine production (65). *Klebsiella pneumoniae* was found to grow and produce histamine at 37, 25, and 10°C, with the shortest generation time being 0.64 hours at 37°C. At 37°C *Klebsiella pneumoniae* produced about 5% more histamine than the organism did at 25°C (8). Histamine production by three species of *Proteus* were all optimal at pH 5, sodium chloride concentration of 4%, and at 25°C, when tested at combinations of pH (5-7), sodium chloride concentration (0-8%), and temperature (4-35°C) (2). One strain of *Stenotrophomonas maltophilia* was found to produce significant levels of histamine at 4°C within six days (13).

Some of the other conditions that affect the rate of production of histamine and other biogenic amines include the availability of free amino acids, and the presence of organisms capable of producing these biogenic amines (16). Aksnes et al. (3) found that capelin, stored at 6°C, and treated with antibiotics to eliminate bacterial growth, contained 30% of amino acids in a free form. By having such a high concentration of available amino acids, this study confirms that capelin, and other fish with similar meat chemistry, are excellent environments for the production of biogenic amines.

Optimum Conditions for Histidine Decarboxylase Enzyme Activity

Optimum conditions for histidine decarboxylase enzyme activity are not completely clear, largely because of the many factors that need to be interpreted, including bacterial cell propagation, initial cell concentrations, and initial composition of the microflora. Substrate-specific decarboxylase enzymes from microorganisms in the food carry out the production of amines in foods, but the rate of production is not necessarily linked to bacterial growth (16). For instance, *Morganella morganii* produced substantial amounts of histamine at temperatures between 10 and 25^{o}C. After incubation at 25^{o}C for 24 hours, this same organism was found to produce even more histamine during subsequent low temperature storage (0-5^{o}C), exceeding the level produced by storage at 25^{o}C. At these low temperatures, *Morganella morganii* did not actively grow (44). Similarly, histamine was formed by resting cells, or cells that are alive but not reproducing, of *Klebsiella pneumoniae* at 2^{o}C (8). Haaland et al (35) found that histamine was produced at 2^{o}C, but at a lower rate than at 20^{o}C, by bacteria present in mackerel. Fernandez-Salguero et al. (28) also reported the production of toxic levels of histamine at 0^{o}C in fillets, minced meat, and liver samples of mackerel by storage day 18.

Not only is there variation in the optimal temperature for histamine production, but there is also variation in the oxygen and pH requirement for production of histamine. Ferencik (27) found that histamine producing *Hafnia* strains produce the compound under anaerobic conditions, but the process has a longer lag time than when the cells were cultured in an aerobic environment. On the other hand, Dapkevicius et al. (21) states that the best condition for decarboxylation of histidine is in a low oxygen

environment. The optimum pH for the production of histamine by *Streptococcus cremoris* has been found to be about 5.5 and the optimum temperature for both growth and production of histamine was found to be 30^{o}C. (7). Histidine decarboxylase from *Klebsiella pneumoniae* was found to have an optimum activity at a pH of 4.0 with a reduction to 70% activity at a pH of 6.0 (8). In general, a low pH is preferred for optimal decarboxylase activity (21). As perhaps expected, there was a shorter lag period when histamine was produced by bacteria inoculated into fish homogenate compared to actual flesh samples (27).

Normal Function of Biogenic Amines

In multicellular animals, histamine is produced in cell types called mast cells, enterochromaffin-like cells and in blastophils. In humans, histamine is also produced in platelets (22, 62). Most release of histamine, also called degranulation, is mediated through the immune system, through the action of interleukins and IgE immunoproteins. Besides those directly related to the immune system, there are other substances that cause histamine release, such as opioids, and certain short chain peptides (62). The peroxidation of rat pleural and peritoneal mast cell lipids has also been found to cause degranulation and release of endogenous histamine (53).

Histamine is an important messenger compound in the body. It is used by enterochromaffin-like cells in the stomach to stimulate the release of acid from parietal cells (22). There is also evidence that histamine release in the frontal cortex is linked to sleeping (62). Histamine and other biogenic amines have been shown to be an essential

chemical for tissue growth. For example, dietary histamines have been shown to cause an increase in growth of blue shrimp, but only to a point. At very high concentrations, dietary histamine appears to suppress growth (69). Also, polyamines have been found to be necessary for cellular metabolism and to be directed to tissues in a state of rapid growth (9). One study showed that even though histamine is essential for cell growth, it also might have a suppressive effect on wound healing by suppressing T-cell mediated functions, which could lead to the formation of cancer (42).

Decomposition of Histamine

Histamine is primarily broken down by two enzymes, histamine methyl transferase (HMT) and diamine oxidase (DAO), forming N-methyl histamine and imidazole acetic acid, respectively. Monoamine oxidase further degrades N-methyl histamine to form N-methyl imidazole acetic acid as its primary metabolite. Just under 50% of histamine recovered from human males is in this form. Less than twenty five percent of recovered histamine in human males consists of the compound produced when imidazole acetic acid is processed by conjugating with the sugar, ribose (62). HMT is found throughout the body including in the islets of Langerhans cells, alveolar cells, and in tubules of the kidney (68). HMT is also the major enzyme that acts on histamine in the stomach (61). Histamine break down by DAO not only produces imidazole acetic acid, but also produces hydrogen peroxide, which can form a free radical and cause lipid peroxidation (36, 50, 52). Diamine oxidase activity has been found in order of highest activity to lowest, in the ileum, jejunum, caecum, and colon of rats (39).

Metabolism and Toxicity

Early theories about scrombotoxicosis suggested that toxicity is caused by histamine alone. For a time a compound called "saurine" was thought to be a secondary toxin that acted like histamine, but it was found that this substance was simply a histamine salt (31). More recently, it has been demonstrated that histamine alone is unlikely to be the sole cause of scombrotoxicosis (18). Putrescine and cadaverine interact with amine oxidases, decreasing detoxification efficiency, and leading to a higher sensitivity to histamine (16). Klausen (46) postulated that because there were higher amounts of cadaverine produced in mackerel than in herring, with equal production of histamine, that the potentiation theory could explain why mackerel is more often implicated in scombroid poisoning. Consumption of cadaverine with histamine has also been found to produce symptoms like that of scombroid poisoning, but the ratio of cadaverine to histamine found to potentiate toxicity was far higher than that in most spoiled fish, indicating that there may be other potentiators (14). One suggested mechanism for potentiation is that other biogenic amines, specifically spermine, spermidine, putrescine and cadaverine, inhibit the binding of the histamine to mucin, a histamine binding barrier in the gut, allowing more histamine to pass the intestinal wall and come in contact with cell membrane receptors (20). MAO inhibitors might be able to decrease effective doses. Alcoholic beverages might also cause this effect, because of their ability to make the intestinal wall more permeable (16).

One report concluded that histamine dose does not correlate with severity of illness in subjects that consumed toxic levels in fish. H1 receptor blockers, such as

Benadryl™, were used to confirm histamine as the cause of illness, and in these cases histamine blockers reduced or eliminated symptoms. These investigators theorized that the mechanism for illness was mast cell degranulation, creating high levels of endogenous histamine in the subjects (40). Clifford et al (18,19) found that there was no real correlation between concentrations of six biogenic amines (cadaverine, histamine, putrescine, spermidine, spermine, and tyramine) both alone and combined in pairs, and the production of scombrotoxic symptoms. From this data it was concluded that dietary histamine might interact with endogenous histamine, but the primary toxin produced causes mast cells to degranulate.

METHODS OF DETECTION

Detection of Histamine-producing Bacteria

Isolation of histamine forming bacteria is usually done using Niven's agar, a media containing tryptone, yeast extract, L-histidine dihydrochloride, sodium chloride, calcium carbonate, agar and a pH indicator bromocresol purple, such that purple colonies indicate possible histamine production (13, 30, 58). There have been modifications in the concentration of these various components over the years, but mechanisms of diagnosis are the same, i.e., the decarboxylation of histidine to histamine makes acidic conditions more basic, resulting in a color change. After isolation and incubation, further methods for detection of histamine, discussed later, can be used to confirm histamine production (30). Although Niven's agar is a generally accepted method for screening, it can give not only false positive but also false negative results. For instance, Kim et al

(43) cultured an isolate that was negative on Niven's agar but turned out to be the most active histamine producer isolated from the study. These same investigators also found that many positive isolates on Niven's agar produced no detectable histamine. Fletcher et al (30) also had many false positive isolates, slightly more than 15 percent, where as Lopez-Sabater et al. (51) recorded over 60% false positives on Niven's agar.

Histamine forming bacteria can be detected and the activity of their decarboxylase enzymes quantified by measuring changes in the electric potential of a histidine decarboxylating media in which they are grown. The confirmation of a significant histamine producer can be determined within 24 hours using this method. This method was found to be rather specific and comparatively fast, but it has not been extensively tested, due to the high price of the equipment needed to perform the procedure (45).

Detection of Histamine and other Biogenic Amines

The original official method for detection of histamine involved inoculation of a section of guinea pig intestine. The histamine was extracted from unknown samples by incubating the homogenized sample in a weak hydrochloric acid solution, followed by filtering and neutralizing steps. A standard curve was produced using different concentrations of histamine, and this was compared to the unknown sample, the measurement being the muscle's reaction to the solution (4).

Chronologically, the second official method for detecting and quantifying histamine in seafood started with a complex chemical extraction using methanol, benzaldehyde, a sodium hydroxide solution, and a benzene-n-butanol mixture, after

which the extracted sample was passed through a crude cotton acid succinate column. The eluted fraction was collected and quantified by reading at 475 nm in a spectrophotometer (5). Lerke and Bell (49) developed the basic design of the most recent official method of histamine determination in seafood by using an ion exchange column step for sample extraction, after which was applied a flourescence based detection method (6). Extraction of biogenic amines using 75% methanol, as in the second official method, was later added to the most current official method (63).

Other methods for detection of biogenic amines are available, though they are not the official standard. Chromatographic methods are the most commonly used for analyzing biogenic amine concentrations. For instance, similar to the AOAC method, liquid chromatography, followed by fluorometric detection is a reliable method with good detection limits (10 ppm) (11). A common method of biogenic amine analysis has been the use of high pressure liquid chromatography (HPLC), which can accurately quantify histamine at concentrations as low as 0.5-2.5 ppm, although for some products, such as canned anchovies in oil, the detection limit was higher (10-55 ppm) (38, 55,73). For HPLC methods, Ben-Gigirey et al (12) found that extraction of the biogenic amines using 0.4 M perchloric acid provided better peak separation than the traditional use of 10% trichloroacetic acid, making interpretation of results more efficient. Thin layer chromatography (TLC) has also been used to separate histamine from fish tissue using ninhydrin or Pauly's reagent to visualize spots. The lower detection limit of this method (20 ppm) restricts its use to screening purposes only, but due to its speed and low cost it

remains a valuable detection approach (65). Putrescine and cadaverine can be quantified using gas chromatography (GC) technology (63).

Histamine can also be quantified based on electrical potential, which is conceptually the same as that used to identify decarboxylase producing bacteria. The major differences between these methods is that for direct detection of biogenic amines an enzyme must be added, either MAO or DAO, and a prior perchloric acid extraction is also needed. This method appears to be rather sensitive, being able to quantify an increase of only 2μmol/L of biogenic amines in a sample. A problem with this method is that it is not very specific and will respond to all biogenic amines (24). Histamine concentration has also been evaluated using DAO applied to an aqueous extraction from tuna. This results in the formation of hydrogen peroxide, followed by quantification of the hydrogen peroxide using a colormetric method (50). Until recently this method has not been very accurate because other biogenic amines can also be oxidized. However, Lopez-Sabater et al (52) has applied it with relatively good results using a perchloric acid extraction method, and a more specific DAO enzyme, reporting a curve that can be correlated with a standard curve in the range of 3-30 ppm. Capillary zone electrophoresis has been used after a methanol extaction to separate histamine from other sample components. This was followed by spectrophotometric detection at 210 nm yielding a quantification range between 0.5 ppm and 100 ppm (56). After a methanol extraction similar to the AOAC method, the strong copper chelation activity of histamine can also be used to determine histamine concentration. By adding copper and a dye to a purified sample extract, a color change results. This method has a detection limit in the low ppm

range, making it useful as an industry confirmation method because it provides sensitivity without the need for additional equipment (10). Odor detection has even been applied to the detection of certain biogenic amines. Putrescine was found to have an odor threshold of about 10^{-5} M, while spermine and spermidine were found to both have thresholds of less than 10^{-4} M in soybean flour (71). However since sensory thresholds vary from person to person, this is not a very accurate or reliable method of screening for biogenic amines.

Histamine test kits are now available that are based on enzyme-linked immunosorbent assay technology. Most of these tests are good for little more than screening, but some, such as Neogen Corporation's (Lansing, MI) Veratox histamine kit have shown to accurately measure a wide range (2-50 ppm) of histamine concentrations (64).

CONTROL MEASURES

Control of Microbial Growth

Temperature is the most effective method for controlling the growth and production of histamine by relevant bacterial strains (26). For instance, investigators have reported that storage of sardines on ice for 8 days was found to be approximately equal to storage at ambient temperature for 24 hours with respect to APC and the production of histamine and cadaverine (1). The role of anaerobic storage was illustrated when Haaland et al. (35) reported that the amino acid composition of whole mackerel did not change significantly under anaerobic conditions at 2^{o}C. Cooking fresh fish that have

had little or no temperature abuse is another way to control microbial growth, and hence prevent further increases in histamine levels. For example, hot smoking has been shown to provide a high enough temperature for a long enough time to eliminate *Hafinia alvei* from seafood (15).

Storage time at frozen temperatures is another key factor in control of histamine and other biogenic amines. It has been found that after storage of albacore tuna for 3 months at either –18°C or –25°C, histamine levels decreased dramatically, but after 9 months of storage in –18°C the levels rose to their original concentration. Putrescine showed the same drop in concentration for a 6 month period in both temperatures, but after 9 months of storage at both temperatures, the concentrations had increased to far more than the initial levels (12). It is possible that components, chemical or enzymatic, break down the biogenic amines faster than they can be produced at these temperatures, but then the components responsible for breaking down biogenic amines are either used up, or inactivated, whereas the enzymes producing biogenic amines remain active for a longer period of time.

In addition to temperature and time, there are other methods of control. One variable that has been extensively investigated is when and how to further process fish. Whole, ungutted haddock have been found to deteriorate more slowly than fillets held on ice or at 5°C, indicating that keeping the fish in a more intact state may help control histamine levels in the edible product. Conflicting data was found in the same study with herring where iced whole, ungutted fish consistently had higher histamine levels than did gutted fish held at 5°C. Putrescine and cadaverine development shows similar variability

with respect to temperature and fish species, although there has been no correlation with histamine production (29). Likewise, tuna fish obtained from Barcelona, Spain, which were cut into pieces before market, had higher histamine concentrations than did whole bonito samples, which in turn, had higher histamine levels than did whole mackerel, all purchased from the same area (51).

The development of high bacterial and histamine levels can be controlled by using 8% salt in iced sardines (1). In fact, there is evidence that greater than 0.5% sodium chloride can notably decrease the rate of histamine production by *Streptococcus cremoris* in M-17 broth (7). Modified atmosphere at a constant concentration of 80% CO_2 has been shown to slow the increase of histamine levels in the short term, but by the third day (the longest trial period executed in the study), histamine levels of experimental and control groups were essentially equal. The levels of the biogenic amines examined in the study (tyramine, cadaverine, and putrescine) were also significantly higher for modified-atmosphere stored products (72). Antibiotics have been used in research to eliminate histamine forming bacteria in capelin, and as a result prevent histamine production in the product (3).

Control Methods Targeting the Chemical

Historically, the control of histamine has focused on controlling the growth of microbial populations that produce the compound. The reason for this is that most biogenic amines are heat stable, even under retort conditions (16, 26). Also, some of the

enzymes that form amines can retain their activity even after pasteurization processing (16). Recently, control methods that target the histamine itself have been advocated.

One such method is the use of diamine oxidase (DAO). DAO producing lactic acid bacteria have been shown to deteriorate biogenic amines when used in the early stages of fish silage processing. Determination of the optimal conditions for DAO treatment is difficult, since each organism has its own optimum condition for producing DAO and the DAO's of different organisms have their own conditions for optimum activity. In one study, purified DAO from an unidentified bacterial isolate performed optimally at 37^{o}C with a range of activity from 20^{o}C to 63^{o}C (21). A relatively thermostable DAO produced by *Vigna radiara* seedlings had reactivity between 25^{o}C and 85^{o}C and could withstand heat treatment of 85^{o}C for 30 minutes with no significant loss of activity (17). Draisci et al (24) found that optimal deterioration of different biogenic amines is pH-dependent when using a single DAO.

In some cases, a whole organism that has significant DAO activity can be used in place of the purified enzyme. When planning to use the entire organism, even more factors, such as factors affecting growth and production of the enzyme, play a role in optimizing conditions for the oxidation of biogenic amines. Gale (33) found differences in *Ps. pyocyanea* and *E. coli* DAO activity under different conditions. Under optimal conditions, *Ps. pyocyanea* was found to completely oxidize putrescine and cadaverine but histamine was only partially oxidized. Even though the histamine was only partially oxidized, the benzene and iminazole rings were eliminated, inactivating the compound's toxicity (33).

Pretreating spoiled fish samples with antihistamines has been shown to dramatically reduce or eliminate symptoms of scombroid poisoning in consuming individuals. In one study, a female subject consumed half of a sample pretreated with antihistamine, presenting only mild symptoms of histamine poisoning. When she later consumed the remaining, untreated portion, she was hospitalized for several hours with classic symptoms (40).

Conclusion and Future Research Needs

Although our understanding of scrombroid poisoning has improved over the years, much remains unknown. Three main areas which could use further study include: (1) the significance of histamine and other biogenic amines relative to scrombroid poisoning; (2) the specific microorganisms responsible for scombrotoxosis, their origin, and the conditions necessary for toxin production; and (3) control of toxin production and/or toxin elimination.

Federal Regulation of Histamine in Seafood

In September 1982, the US Food and Drug Administration (FDA) established a defect action level (DAL) for histamine in fish at 200 ppm, meaning that when a product was found to have 200 ppm or more histamine corrective action (discard) had to be taken. In 1995, the FDA reduced the the histamine DAL to 50 ppm (25). The reasoning for this reduction was due to the variability in histamine concentrations from section to section in a fish. If there is a 50 ppm concentration of histamine in a sample, the FDA reasoned that

there could be a sufficient concentration to cause illness (500 ppm) in another section of the fish. Also in 1995, the FDA promulgated new food safety regulations based on the Hazard Analysis Critical Control Points (HACCP) approach that required industry to implement written HACCP plans by December 1997 (26).

HACCP is based on seven principles, which are designed to set limits at points in a process to control the risk of a health hazard, and then makes sure the limits are met by monitoring and record keeping to verify that the HACCP plan is being followed (41). For fish susceptible to histamine formation, the FDA has suggested three critical points where controls should be exercised: (1) receiving by the primary processor (includes first receiver); (2) receiving by the secondary processor; and (3) during processing (including storage). The limits suggested for the primary processor depends mainly on the water temperature at harvest, the size, and the species of the fish. For harvesters that do not collect records at capture, the FDA suggests that a sample of the fish be tested for histamine levels to ensure compliance with DAL levels. On receipt by the secondary processor, either transportation records showing that the fish were held at or below 4.4^{o}C, or else the presence of sufficient cooling media surrounding the product at time of delivery, are suggested. Critical limits for processing steps depend on whether the fish have been previously frozen. If the fish were not previously frozen, they should not be exposed to temperatures above 4.4^{o}C for more than 8 cumulative hours. If at any time, fish are exposed to temperatures above 21^{o}C the cumulative time above 4.4^{o}C can not exceed 4 hours. If the fish were previously frozen, they should not be exposed to temperatures above 4.4^{o}C for more than 24 cumulative hours. If at any time the

temperature is above 21°C, the cumulative time above 4.4°C can not exceed 12 hours (16).

Commercial Handling Practices

Both mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacares*) fisheries support a significant commercial industry in North Carolina. The primary method of capture is trolling using hook and line techniques. Several long-line operations currently target and off-load both species of fish in North Carolina as well. Most commercial capture of mahi-mahi occurs as a by-catch from snapper-grouper boats. This means that most of the sizable fish will be bled and gutted on board, but this is not always the case. Handling procedures vary from region to region, boat to boat, and even day to day due to captains' preferences, traditions, and time restraints. The FDA encourages evisceration of large tuna (≥20 lbs) as another control mechanism for histamine production by using temperature guidelines. Evisceration of these fish while onboard may reduce the microbial load in fish but could also result in accidental cutting of the digestive tract, further contaminating the fish with histamine-producing bacteria. Due to the variation in handling, harvest methods need to be monitored and, if possible, improved to control histamine formation in these species.

Goals and Objectives

Therefore, it is prudent to observe commercial practices in the mid-Atlantic region to better our understanding of product safety risks associated with the harvesting

and processing of these important fish species. The purpose of this study was to determine if current regulatory guidelines are being meet for the control of histamine production in North Carolina-harvested mahi-mahi and yellowfin tuna, and if not, what potential food safety risks may likely occur.

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Control of Histamine Production in Current Commercial Fishing Operations for Mahi-mahi (*Coryphaena hippurus*) and Yellowfin Tuna (*Thunnus albacares*) in North Carolina

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ABSTRACT

Histamine fish poisoning represents one of the most common risks to seafood consumers in the United States today. In 1997, the U.S. Food and Drug Administration (FDA) implemented industry guidelines for the control of histamine production based on the Hazard Analysis Critical Control Point (HACCP) program. The primary concern for FDA is the proper handling and cooling of fish in order to reduce the growth of bacteria capable of supporting histamine production. The purpose of this study was to determine if current regulatory guidelines are being meet for the control of histamine production in North Carolina harvested mahi-mahi and yellowfin tuna, and if not, what potential food safety risks may likely occur.

Twenty-nine composite fish muscle samples were obtained from 18 mahi-mahi and 11 yellowfin tuna troll-caught in North Carolina and analyzed for their histamine content. No sample analyzed exceeded 2 ppm histamine. Generally mahi-mahi cooled faster to $\leq 10^{o}C$ (avg. 2.5 hrs, n=18) than yellowfin tuna (avg. 7.5 hrs, n=10). This may be due to the relative smaller size of mahi-mahi (avg. 3.4 Kg, n=12) compared to yellowfin tuna (avg. 14.2 Kg, n=8). According to the FDA's HACCP guidelines, fish should be placed on ice within 12 hrs of capture (death) or brought to an internal temperature of $\leq 10^{o}C$ in 12-24 hrs. Uneviscerated tuna $\geq$ 20 lbs (9.1 Kg) should be chilled to $\leq 10^{o}C$ within 6 hours. In this study, all mahi-mahi were placed on ice within the recommended HACCP guideline of < 12 hrs. The majority of the yellowfin tuna (60%) however, did not reach the FDA's recommended temperature of $\leq 10^{o}C$ within 6 hours of harvest.

Three hundred and eighty-six composite fish muscle and environmental samples were screened for the presence of histamine-producing bacteria. Twenty-six percent of 549 isolates selected based on their morphological characteristics tested positive on Niven's media. Sixty-three Niven's positive isolates were Gram negative rods, and 58 Gram positive. The Beckon Dickinson BBL Crystal method was used primarily for identification of Gram positive isolates because the API 20E Enterobacteriaceae identification test is specific for the identification of Gram negative bacteria. Neither API 20E test nor BBL Crystal method was able to identify every Niven's positive isolate. A total of 60 Niven's positive isolates were identified at > 90 percent confidence level.

Only five of forty-three isolates tested were confirmed and classified as low histamine producers (<250 ppm in 48 hrs at >15^{o}C). Three Gram negative isolates were identified as *Enterobacter cloacae*. Two Gram-positive isolates were identified as *Staphylococcus kloosii*. This study contradicts the general belief that Gram-negative bacteria are solely responsible for histamine production in potentially hazardous fish.

The confirmation of histamine-producing bacteria found in this study demonstrates the potential risk for histamine production. However, no detectable levels were found in the fish muscle samples analyzed, even though yellowfin tuna did not meet the regulatory HACCP guidelines. Therefore no food safety risks were found under commercial conditions studied.

INTRODUCTION

Histamine fish poisoning represents one of the most common risks to seafood consumers in the United States today (24). In 1997, the U.S. Food and Drug Administration (FDA) implemented industry guidelines for the control of histamine production based on the Hazard Analysis Critical Control Point (HACCP) program (8). The most recent guidelines recommend specific time and temperature limits for potentially hazardous fish based on the species, size, and water temperature at harvest (8). FDA recommends that fish be placed in a cooling medium, or cooled to a specific temperature, within a prescribed time (Figure 1). Primary processors bear the burden of proof and are expected to complete any cooling necessary to achieve a core temperature of 4.4^{o}C or less and maintain this temperature throughout handling, processing and distribution (Figure 2).

Histamine is commonly found in humans, serving as a cell messenger for regulating vascular and bronchial diameter as well as other normal bodily functions (6). It is chemically produced by decarboxylation of histidine, a naturally occurring amino acid (6). Histamine production is performed by one of two types of decarboxylase enzymes, a pyridoxal phosphate dependant enzyme found in animals as well as Gram negative bacteria and a pyruvoyl dependant enzyme found in Gram positive bacteria (14). Human illness may occur when a high concentration of histamine is ingested with potentially hazardous food such as meat products, dairy products, wine, beer, vegetables, fruits, nuts, chocolate, and most notably certain fish, both scombroid (i.e., tunas and mackerels) and some non-scombroid fish (i.e., bluefish) (6, 18, 26). The production of

histamine in potentially hazardous fish is generally believed to result from the growth of Gram negative, rod shaped bacteria capable of producing pyridoxal phosphate dependent decarboxylase enzymes (7).

The primary concern for FDA is the proper handling and cooling of fish in order to reduce the growth of bacteria capable of supporting histamine production. While the FDA has jurisdiction over primary processors, the agency does not have regulatory authority over commercial harvesters. This presents a challenge for both regulatory and industry members alike who are concerned with product safety and regulatory compliance. Commercial industry practices can vary significantly by geographic region, fisheries, vessel and gear type and time of year. In the mid-Atlantic region, mahi-mahi and yellowfin tuna represent economically important commercial fisheries.

Therefore, it is prudent to observe commercial practices in the mid-Atlantic region to better our understanding of product safety risks associated with the harvesting and processing of these important fish species. The purpose of this study was to determine if current regulatory guidelines are being meet for the control of histamine production in North Carolina harvested mahi-mahi and yellowfin tuna, and if not, what potential food safety risks may likely occur.

MATERIALS AND METHODS

FISH SAMPLES

Mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacares*) were harvested by trolling aboard North Carolina commercially licensed fishing vessels using hook and line techniques. A total of five day trips were made for collecting samples of mahi-mahi and three day trips for collecting yellowfin tuna samples. A minimum of four fish were landed on each occasion in order to observe variations in size, harvest conditions, and commercial handling techniques. Mahi-mahi was sampled during the summer, 2002 and spring, 2003. Yellowfin tuna was sampled in the spring and summer of 2003 and in the spring, 2004.

HARVEST CONDITIONS

Air and water temperatures were recorded at time of harvest during commercial operations. An internal temperature profile for each fish was determined using a Datatrace (Mesa Laboratories, Lakewood, CO) temperature probe inserted into the fish at the dorsal area. Probes were inserted at a depth of four to six inches in mahi-mahi and yellowfin tuna at the backbone just behind the head (Figure 3), and slightly anterior to the middle of the fish (Figure 4), respectively. Probes were secured using plastic cable-ties anchored in the fish at the dorsal skin area. The Datatrace probes were pre-programmed in the laboratory to record temperature at five minutes intervals from point of harvest to

time of processing at the commercial cutting house. Prior to processing, weight, total length (from head to tip of tail) and girth were measured for each fish.

Environmental Samples

Environmental swabs were taken of fish and fish contact surfaces onboard the vessel, at receiving on the dock and at the primary processing facility to screen for the presence of potential histamine-producing bacteria. Samples were obtained by swabbing an area of ca. ten square centimeters with a sterile calcium alginate tipped applicator and placing it into a sterile tube with 10 ml of sterile neutralizing buffer. Approximately 24 samples were obtained on the vessel, 10 at receiving and 24 at the processing facility. All environmental samples were placed immediately on ice after collection for transport back to the NC State University Seafood Laboratory at Morehead City, NC. Analyses were performed within 48 hrs of sample collection.

Muscle Sampling and Preparation

Sampling of muscle tissue was performed for each fish at the primary processing facility. Approximately 50 g were obtained from each of three locations (head, belly, and tail) (Figure 5) and placed in separate sterile Whirl pack bags (Seward Medical, London). A total of 12 tissue (three from each fish) samples were collected from each day, placed immediately on ice and processed within 12 hours of collection in the North Carolina State Seafood Laboratory at Morehead City, NC.

A composite tissue sample from each fish was prepared. Approximately 10 g of tissue (mahi-mahi, skin-on; tuna, skin-off) was aseptically sub-sampled from each of

three locations and combined in a sterile stomacher bag. Fish tissue samples were diluted 1 to 10 (w/v) with sterilized water in a Laboratory Blender Stomacher 400 (Seward Medical, London) and blended for one minute at normal speed (230 rpm) with thirty second intervals between each of three cycles. Immediately after blending, three 100 µl samples were obtained using a micropipette (Oxford Benchmate™, St. Louis, MO) fitted with a wide bore tip for use in microbiological analyses.

HISTAMINE DETERMINATION

The blended samples were placed under refrigeration for ca. 15 minutes to allow the suspensions to settle. Approximately 5 ml of supernatant was collected and filtered using a glass wool syringe into polyethylene tubes according to the Neogen Corp. (Lansing, MI) Veritox histamine test kit. The filtered samples were processed as described in the ELISA based Veritox histamine assay using a pre-programmed histamine determination test on the Stat Fax (Awareness Technologies Inc, Palm City, FL) microtiter well reader.

MICROBIOLOGICAL ANALYSES

Composite samples of fish tissue were analyzed for total aerobic plate count by serial dilution with normal saline solution to obtain 10^{-2} to 10^{-4} concentrations. The samples were plated in duplicate on tryptic soy agar (Beckon Dickinson, Sparks, MD) supplemented with 2% sodium chloride (Fisher Scientific, Fair Lawn, NJ) ($TSAN_2$).

Environmental samples were analyzed for total aerobic plate count by serial dilution with normal saline solution to obtain 10^{-1} to 10^{-3} concentrations. The samples were plated in duplicate on $TSAN_2$. Fish tissue and environmental samples were incubated at 37°C for 48 hours.

Screening Bacterial Isolates for Histamine Production

Bacterial isolates were selected from $TSAN_2$ plates based on their morphological differences. Between one and fifteen isolates were selected from $TSAN_2$ plates based on both sample location and time of collection. Isolates were assigned four digit identification numbers according to the type of fish, trip, location and time. Isolates were streaked on $TSAN_2$ plates and incubated at 37°C for 48 hrs. Single colonies were picked, re-streaked and incubated on $TSAN_2$ plates to obtain pure cultures. The pure cultures were transferred to $TSAN_2$ slants and incubated at 37°C for 24 hours. A sample of each isolate was plated on Niven's agar and incubated at 37°C for 48 hrs to screen for histamine production (22).

Niven's positive cultures were maintained on $TSAN_2$ slants at 8-10°C as well as frozen at –10°C in suspension (v/v) with Tryptic Soy Broth (2x) supplemented with 2% sodium chloride and 40-50% glycerol.

Bacterial Identification

Niven's positive isolates were Gram stained and examined under oil-immersion on a compound microscope (1000X) to determine bacterial shapes. Gram negative rods were identified with the API 20E Enterobacteriaceae (bioMerieux Vitek, Inc.,

Hazelwood, MO) and the enteric/nonfermenter Beckon Dickinson BBL Crystal identification (Sparks, MD) tests. Gram positive isolates were identified using the Gram positive Beckon Dickinson BBL Crystal identification method. All confirmed histamine-producing isolates were analyzed by Dianne Bourne at Virginia Polytechnic Institute and State University by a cellular fatty acid identification method as described in Moore et al. (21) and Ghanem et al. (13).

HISTAMINE CONFIRMATION

Positively identified Gram negative and Gram positive isolates were incubated in $TSBN_2$ at 37°C for 18-24 hrs. The isolates were streaked in triplicate on $TSAN_2$ plates supplemented with 0.1% histidine (histidine monohydrochloride monohydrate, Research Chemicals Ltd.) ($TSAN_2$+histidine) and incubated at 37°C for 18-24 hrs. A representative colony from each $TSAN_2$+histidine plate was inoculated into 9 ml of Tryptic Soy Broth supplemented with 2% NaCl, 2% histidine (histidine monohydrochloride monohydrate, Research Chemicals Ltd.) and 0.0005% pyridoxal-HCl (pH 5.8) (TSB+) and incubated at 37°C for 24 hrs. A 1 ml sample of each TSB+ suspension was transferred into a tube of fresh TSB+ media and incubated at 37°C for 48 hrs. A 3 ml sample of the final culture was transferred into polypropylene centrifuge tubes and centrifuged at 6,500 RPM for 20 minutes. Supernatants were diluted 1 to 10 in water and histamine concentrations determined using the Neogen Veritox histamine test kit (Neogen Corp., Lansing, MI).

HISTAMINE PRODUCTION RATES

Positively identified histamine-producing isolates were further characterized by their rate of histamine production at 25 and 37°C. A known histamine producer, *Raoultella planticola* ATCC 43176, was used as a positive control. Histamine-producing isolates and the control were incubated in $TSBN_2$ at 37°C for 24 hrs. Samples of each culture were streaked in triplicate on $TSAN_2$+histidine plates and incubated at 37°C for 18-24 hrs. A single colony from each $TSAN_2$+histidine plate was selected and inoculated into 9 ml of TSB+ and incubated at 37°C for 24 hrs. A negative control (blank 9 ml tube of TSB+) was incubated in triplicate at 37°C for 24 hrs.

Representative 1 ml samples from each TSB+ culture tube was transferred into 9 ml of fresh TSB+ media and incubated separately at 25 and 37°C for 48 hrs. A 3 ml sample of each culture at 25 and 37°C was transferred into polypropylene centrifuge tubes and centrifuged at 6,500 RPM for 20 minutes. Supernatants were diluted 1 to 10 in water and histamine concentrations determined using the Neogen Veritox histamine test kit (Neogen Corp., Lansing, MI).

RESULTS AND DISCUSSION

FISH SAMPLES

Histamine Content

Twenty-nine composite fish muscle samples were obtained from 18 mahi-mahi and 11 yellowfin tuna troll-caught in North Carolina and analyzed for their histamine content. No sample analyzed exceeded 2 ppm histamine, the detection threshold for Neogen's ELISA-based Veratox® rapid test. This low level of histamine in the fish samples was not unexpected due to their storage conditions. Fernandez-Salguero et al. (9) found similar results in haddock (*Melanogrammus aeglefinus*) and herring (*Clupea harengus*) held for short periods of time on ice. However, they did find moderate histamine content in fish held on ice for a long period of time ($\geq$ 8 days). Also, Ababouch et al. (1) found high histamine content in sardines (*Sardina pilchardus*) within a few hours where gross temperature abuse of fish occurred ($\geq 25^{o}C$).

The primary cause of histamine production in scombroid fish is the growth of histamine-producing bacteria. Factors affecting growth of histamine-producing bacteria are time and temperature dependent and may include the type and size of associated fish, handling techniques used and cooling method employed. This study focused on mahi-mahi and yellowfin tuna in North Carolina, a predominately hook and line fishery with few commercial vessels employing long lining techniques. All fish used in the study had a relatively short period of time ($\leq$ 36 hrs) between harvest (death) and primary processing. The fish were placed on ice within 30 min of capture on board the vessels and

adequate temperature controls (re-icing $\leq$ 30 min) were observed at first receipt (packing house) and in handling at the processing facility. The total length of time between harvest and quantification of histamine in tissue samples was $\leq$ 48 hrs for the 29 samples analyzed.

Cooling Rates

Generally mahi-mahi cooled faster to $\leq 10^{o}C$ (avg. 2.5 hrs, n=18) than yellowfin tuna (avg. 7.5 hrs, n=10). This may be due to the relative smaller size of mahi-mahi (avg. 3.4 Kg, n=12) compared to yellowfin tuna (avg. 14.2 Kg, n=8). Craven et al. (5) found that large albacore tuna (9.1 Kg) required more time (ca. 2 hrs) to cool to $\leq 10^{o}C$ than smaller fish (6.8 Kg) when placed in seawater/ice slurry conditions. A representative sample of cooling rates observed for mahi-mahi and yellowfin tuna are shown in Figures 6 and 7 respectively.

Figure 8 shows variations in temperature of mahi-mahi harvested on the same day and adjusted for the weight of fish. The data reveals that weight alone can not account for the variation of temperatures. Other factors may contribute to variation in temperatures and include composition and shape of the fish, cooling medium, and method of chilling.

All vessels used ice to chill their catch with most vessels using less ice than the amount of fish harvested (w/w). One vessel used a thermally insulated cover, in addition to ice, to help maintain the cooling effects of ice on fish. Craven et al. (5) demonstrated improved cooling rates for albacore tuna chilled in seawater/ice slurries, a practice

commonly used in the harvest of other species of fish in the mid-Atlantic region. General commercial practice observed in this study was chilling of fish on ice alone.

Handling Considerations

The FDA encourages bleeding and gutting large tuna $\geq$ 20 lbs (9.1 Kg) or requires that strict time and temperature controls (internal temperature of $\leq 10^{o}$C within 6 hrs of death) be met if gutting is not performed on board the vessel. In this study, no vessel targeting tuna was observed bleeding and gutting their catch. Therefore, large tuna are expected to be chilled rapidly in order to achieve the recommended internal temperature of $\leq 10^{o}$C within 6 hrs. This recommendation may be difficult to achieve for very large fish due to their mass and shape characteristics.

After receipt, handling practices were adequate to maintain or further decrease the fish internal temperatures to $\leq 4.4^{o}$C. All fish were held overnight in ice and processed the next day. In general, mahi-mahi fillets and tuna loins were cut by hand at the fish house and placed back on ice within 30 min. of processing.

HACCP Guidelines

According to the FDA's HACCP guidelines, fish should be placed on ice within 12 hrs of capture (death) or brought to an internal temperature of $\leq 10^{o}$C in 12-24 hrs. Uneviscerated tuna $\geq$ 20 lbs (9.1 Kg) should be chilled to $\leq 10^{o}$C within 6 hours. In this study, we observed that all mahi-mahi were placed on ice within the recommended

HACCP guideline of < 12 hrs. The majority of the yellowfin tuna (60%) however, did not reach the FDA's recommended temperature of $\leq 10^{o}C$ within 6 hours of harvest (Figure 9). This may be due to an insufficient amount of ice used to chill the fish or the inability to chill large fish under the method of cooling employed (ice alone).

Five of the mahi-mahi were recorded with elevated temperatures ($> 4.4\,^{o}C$) for brief periods of time after chilling. The amount of time "out of temperature" was generally less than one hour, but this violates the HACCP guidelines for maintaining fish at $\leq 4.4\,^{o}C$. The elevated temperature observed for mahi-mahi is not likely a substantial safety risk but slow cooling observed for yellowfin tuna is more serious, especially for first receivers who must comply with the HACCP guidelines.

HISTAMINE-PRODUCING BACTERIA

Isolation and Identification

Three hundred and eighty-six composite fish muscle and environmental samples (252 for mahi-mahi and 134 for yellowfin tuna) were screened for the presence of histamine-producing bacteria. Of the 549 isolates selected based on their morphological characteristics, 26 percent were positive on Niven's media. Eighty Niven's positive isolates were associated with mahi-mahi sampling and 65 were associated with yellowfin tuna sampling. Sixty-three Niven's positive isolates were identified as Gram negative rods, 41 from mahi-mahi and 22 from yellowfin tuna. In addition, 58 Niven's positive isolates were identified as Gram positive, 23 from mahi-mahi and 35 from yellowfin tuna.

The API 20E rapid biochemical test identified 21 of the Gram negative isolates, 18 from mahi-mahi and three from yellowfin tuna. The Becton Dickinson BBL Crystal method identified 19 Gram negative isolates, 11 from mahi-mahi and eight from yellowfin tuna. In addition, the BBL Crystal method was used to screen 58 Gram positive isolates, resulting in 18 identified from mahi-mahi and 26 identified from yellowfin tuna.

The BBL Crystal method was used primarily for identification of Gram positive isolates since the API 20E test is specific for the identification of Gram negative. Neither the API 20E test nor the BBL Crystal methods were able to identify every Niven's positive isolate. A total of 73 Niven's positive isolates were identified at >50 percent confidence level. Of those 73 isolates, 60 were identified at > 90 percent confidence level. Three Gram negative isolates were identified but as different organisms by the API and BBL Crystal tests. Of the 57 identified isolates, only 43 remained viable after freezing in Tryptic Soy Broth (2x) supplemented with 2% sodium chloride and 40-50% glycerol.

Five cultures of the 43 viable isolates were confirmed as histamine producers and identified as *Citrobacter frundii* by the API and BBL Crystal tests for Gram negative rods or *Staphylocccus epidermidis* and *Aerococcus urinae* by the BBL Crystal test for Gram positives (Table 1). The five histamine-producing isolates were subjected to further analyses using cellular fatty acid profile (CFAP) identification to confirm their identities (4). The CFAP technique identified the three Gram negative isolates as *Enterobacter cloacae* which are closely related to *Citrobacter frundii*. The Gram negative isolates gave

negative H_2S production, weakly positive Voges-Proskauer reaction and lacked the *Citrobacter* odor characteristic. The CFAP technique identified both Gram positive isolates as *Staphylocccus kloosii*. The CFAP identification test is considered more appropriate for use in environmental sampling compared to the API 20E and BBL Crystal tests, which were developed for clinical use.

Many researchers have isolated and identified a number of Gram negative histamine-producing bacteria. Gram negative bacteria are believed to be the primary cause of histamine development in scombroid fish (6). Tsai et al. (27) isolated and identified 14 different histamine-producing Gram negative (rods) bacteria in the genera *Proteus, Enterobacter, Aninetobacter, Klebsiella* and *Rahnella*. Frank et al. (10) identified *Morganella morganii*, *Proteus mirabilis*, and *Vibrio alginolyticus* from the 27 different histamine-producing bacteria they were able to isolate. Omura et al. (23) also isolated histamine producing *Morganella morganii*, *Proteus spp*, and *Klebsiella spp*, as well as *Hafnia alvei,* from skipjack tuna and jack mackerel. Other researchers have identified *Citrobacter spp.* as histamine producers (6). However, the CFAP findings in this study may bring into question results from previous studies identifying *Citrobacter spp*. as histamine producers. Kanki et al. (16) recently discovered that several histamine producing cultures believed to be *Klebsiella pneumoniae* were incorrectly identified strains of *Raoultella planticola*.

Histamine Confirmation

The Niven's positive isolates (43 viable) identified with > 90 percent confidence level were subjected to further examination to verify their ability to produce histamine. Only five of 43 isolates or 12% were confirmed as histamine producers. This low percentage is consistent with other studies of Niven's positive bacterial isolates where histamine confirmation by quantification methods confirmed 15, 18 and 37 percent of isolates (17, 19, 27).

Kim et al. (17) identified a histamine producing strain of *Morganella morganii* that gave a false negative when cultured on Niven's media. The low histamine confirmation level (false positives) and potential for false negatives reveals weakness in the use of Niven's media for screening environmental samples for the presence of histamine-producing bacteria. The Niven's method is based on a pH shift in the media that causes a color change from a brown-green to purple (22). It is known than many metabolic processes of microorganisms can result in pH changes in culture media that may lead to reports of false positives for the production of histamine.

Even with only 12% of Niven's positive isolates confirmed as histamine producers, the number of potential histamine-producing bacteria at harvest, receiving and processing of scombroid fish is large. For example, taking the total number of Niven's positive isolates (145 presumptive isolates) and extrapolating for histamine producing bacteria, about 17 isolates would be expected to produce histamine. The fact that histamine-producing bacteria were isolated from fish contact surfaces as well as the fish

themselves, reinforces the FDA's guidelines for strict temperature controls to reduce the potential for growth of histamine-producing bacteria on scombroid fish.

Histamine Production Rates

The five histamine-producing isolates identified in this study were further characterized by their rate of histamine production at 25 and 37°C (Figure 10). Because all five bacterial isolates were slow to grow, incubation time was increased from 24 to 48 hrs. This observation is consistent with results reported by Kim et al (17) for a strain of *Morganella morganii* that produced higher histamine concentration in 48 hrs at 37°C than it did in 24 hrs at either 25 °C or 37°C. However, Babu et al. (3) found that histamine production by *Streptococcus cremoris* decreased from 24 to 48 hrs when incubated at 37°C, suggesting that each bacterial isolate may have different histamine production profiles.

None of the five bacterial isolates identified were high histamine producers [>1000 ppm in 24 hrs at >15°C (2, 27)]. *Raoultella planticola* (ATCC 43176) was found to be a medium producer of histamine (250-1000 ppm in 48 hrs at $\geq$ 15°C) under the two incubation temperatures studied. Contrary to our results, Kanki et al. (16) reported that *Raoultella planticola* (ATCC 43176) produced histamine levels of 4,550 ppm using 2 ml media volume and an incubation of 18 hrs at 30°C. Bacteria isolated by Tsai et al. (27) and Ababouch et al. (2) reported histamine production rates between 1000 to 4000 ppm in 24 hrs at 37°C. Frank et al (10) reported that mesophilic histamine producing bacteria could achieve concentrations of $\geq$10,000 ppm in 24 hrs at 32 °C.

The five bacterial isolates were low histamine producers, defined as <100 ppm in 24 hrs at $\geq$ 15°C or <250 ppm in 48 hrs at $\geq$ 15°C (2,27). However, isolates 4077 and 4086 did produce > 100 ppm histamine at 37°C but in 48 hrs rather than the 24 hrs as reported in previously published studies. Tsai et al. (26) reported low histamine-producing Gram negative rods around 100 ppm in 24 hrs at 37°C and Ababoch et al. (2) found similar levels produced at 35°C. *Streptococcus cremoris* was reported by Babu et al. (3) to produce a concentration of 43 ppm in 24 hrs at 30°C, a concentration lower than any of the Gram negative isolates identified in this study. The majority of bacterial isolates reported by Frank et al. (10) produced histamine concentrations between10-50 ppm, with about a third acquiring concentrations of 10,000 ppm or more in 24 hrs at 32°C.

Few Gram positive bacteria have been studied in association with histamine production in fish. A strain of *Lactobacillus buchneri* was found in Swiss cheese to have a histamine-producing rate of 420 ppm in 24 hrs at 37°C (26). This concentration is more than the histamine levels produced by *S. epidermidis* and *A. urinae* identified in this study. However, the concentration is close to the average concentration found with *S. epidermidis* if replicates where no histamine production was found are excluded. Our strain of *S. epidermidis* consistently produced negative and positive histamine production from the same culture. To ensure our culture was not mixed, samples were streaked and replated on $TSAN_2$ several times and the BBL Crystal identification test was performed on both positive and negative histamine-producing cultures. All of the isolates tested were pure cultures and identified by BBL Crystal as *S. epidermidis*. All bacterial cultures

exhibiting histamine-production were retested for histamine-production with similar mixed positive and negative results. One explanation for this observation may a genetic variation within the strain, where the gene coding for the enzyme responsible for histamine production is lost or its expression somehow suppressed. These phenomena may also be why Gram positive histamine producing bacteria have not been studied as much due to their inconsistency in producing histamine.

There are several possible reasons why bacterial isolates identified in this study do not appear to be as prolific histamine producers as bacterial isolates in other studies. One reason may be that other studies used strains previously confirmed as high histamine producers and used them as a model to study growth and histamine production characteristics (1,3,17,26). Others may have isolated histamine-producing bacterial strains from thermally or temporally-abused fish rather than those strains obtained under normal commercial harvest conditions that were observed in this study.

Babu et al (3) used a strain of *Staphylocccus cremoris* (National Center of National Dairy Research Institute, Karnal, India) specifically for its histamine producing capability. Sumner et al. (26) isolated the *Lactobacillu buchneri* strain from Swiss cheese that was implicated in an outbreak of histamine poisoning. Kim et al. (17) and Ababouch et al. (1) studied bacterial isolates from temperature-abused fish samples that may possibly allow time for a greater number and more prolific histamine-producing bacteria to grow.

Another possible reason for low histamine producers identified in this study could be the specific growth media used. We incorporated 2% sodium chloride in the culture

media to enable the growth and isolation of *Vibrio sp*. that are known to produce histamine. However, Babu et al. (3) found that addition of salt reduced the production of histamine in a strain of *Staphylocccus cremoris*.

HISTAMINE DEFECT ACTION LEVELS

The U.S. FDA's defect action level (DAL) for histamine in fish is 50 ppm. Our study found no fish muscle samples exceeding the DAL, therefore no food safety risks were found under commercial conditions studied. However, the potential for growth of histamine-producing bacteria and histamine development occurred. The FDA is concerned with mild temperature abuse of fish that may pre-dispose fish to greater risk of histamine development by selecting for growth of a greater number of histamine-producing bacteria (25). Previous studies by a number of authors have shown rapid production of histamine under mild to high temperatures and moderate to long exposure times (1, 9).

HACCP IMPLICATIONS

It has been generally accepted that Gram negative bacteria are the sole cause of histamine production in scombroid fish and that reducing growth of histamine-producing bacteria is the primary control measure for preventing histamine poisoning. This study identified two Gram positive histamine-producing isolates that were found on fish contact surfaces where the potential to contaminate the fish may occur. In addition, three Gram

negative isolates were identified at the primary processing facility. Two isolates were obtained from fish surfaces and the third was obtained from a knife used to cut fish. While histamine was not detected in the 29 composite fish samples analyzed, temperature profiles of fish obtained and isolation of bacteria capable of producing histamine demonstrates potential risks associated with these fish.

In order for North Carolina's commercial industry to meet current federal HACCP guidelines, better records are necessary at the point of harvest. For mahi-mahi, time and temperature records of fish on board the vessel are not necessary because all vessels were at the docks within the 12 hour HACCP guideline. For yellowfin tuna, harvesters did not gut the fish. Therefore, time and temperature records are necessary on board the vessel to document the internal temperature of fish within the FDA's recommended 6 hour HACCP guideline.

One improvement in handling of fish that was evident in this study was to increase the amount of ice used to chill fish or employ a more rapid cooling technique such as the saltwater/ice slurry method employed with albacore tuna. Consistent cooling of fish may be achieved through better utilization of slush ice instead of ice alone (5,15). The FDA is also strongly encouraging the practice of gutting large fish ($\geq$ 20 lbs, 9.1 Kg) in order to reduce the number of spoilage bacteria.

An additional control that may reduce risks is the use of hand sanitizers and gloves to reduce cross-contamination of fish. Periodic sanitation on the harvest vessel could reduce the risks, as would processors employing the use of a sanitizing rinse step for knives in-between cutting of individual fish. Gingerich et al. (11) found histamine

producing *Klebsiella ozaenae* in a sanitizing soluiton used to store knives, demonstrating that for a knife dip to be effective it must be changed regularly.

Another improvement may be incorporation of a second hurdle to reduce growth of histamine-producing bacteria present on fish. Currently, time and temperature are the only hurdles available for use by industry to reduce the risk of histamine formation by growth of bacteria. Other options for reducing in growth of histamine-producing bacteria may be incorporation of anti-microbial agents in the ice or saltwater/ice slurry used to chill fish. Acidified sodium chlorite in refrigerated seawater systems has been used for extending the shelf life of fresh fish in Alaska (22). Preliminary studies have been reported on the use of buffered sodium chlorite solutions in ice for the control of histamine producing bacteria in fish (12).

Any further reduction in risks associated with histamine development in potentially hazardous fish will depend upon development of new control strategies or changing commercial practices, which depend upon cost, ease of implementation, modifying human behaviors or regulations.

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Table 1: A summary of five histamine-producing isolates by type, location, and organism.

ID#	4077	4083	4086	2015B	5059
Fish Species	Mahi-mahi	Mahi-mahi	Mahi-mahi	Mahi-mahi	Yellowfin Tuna
Step	Post-Processing	Post-Processing	Post-Processing	Preharvest	Receiving
Sample Type	Fish-Surface	Fish-Surface	Environ	Environ	Environ
Location	Fish 4.1	Fish 4.2	Knife	Fish basket	Packing Crate
Organism (API)	*Citrobacter freundii*	*Citrobacter freundii*	*Citrobacter freundii*	NA	NA
Organism (Crystal)	*Citrobacter freundii*	*Citrobacter freundii*	*Citrobacter freundii*	*Staph. epidermidis*	*Aerococcus urinae*
Organism (CFAP)	*Enterobacter cloacae*	*Enterobacter cloacae*	*Enterobacter cloacae*	*Staph. kloosii*	*Staph. kloosii*
Comments	No H_2S Production	No H_2S Production	No H_2S Production	None	None

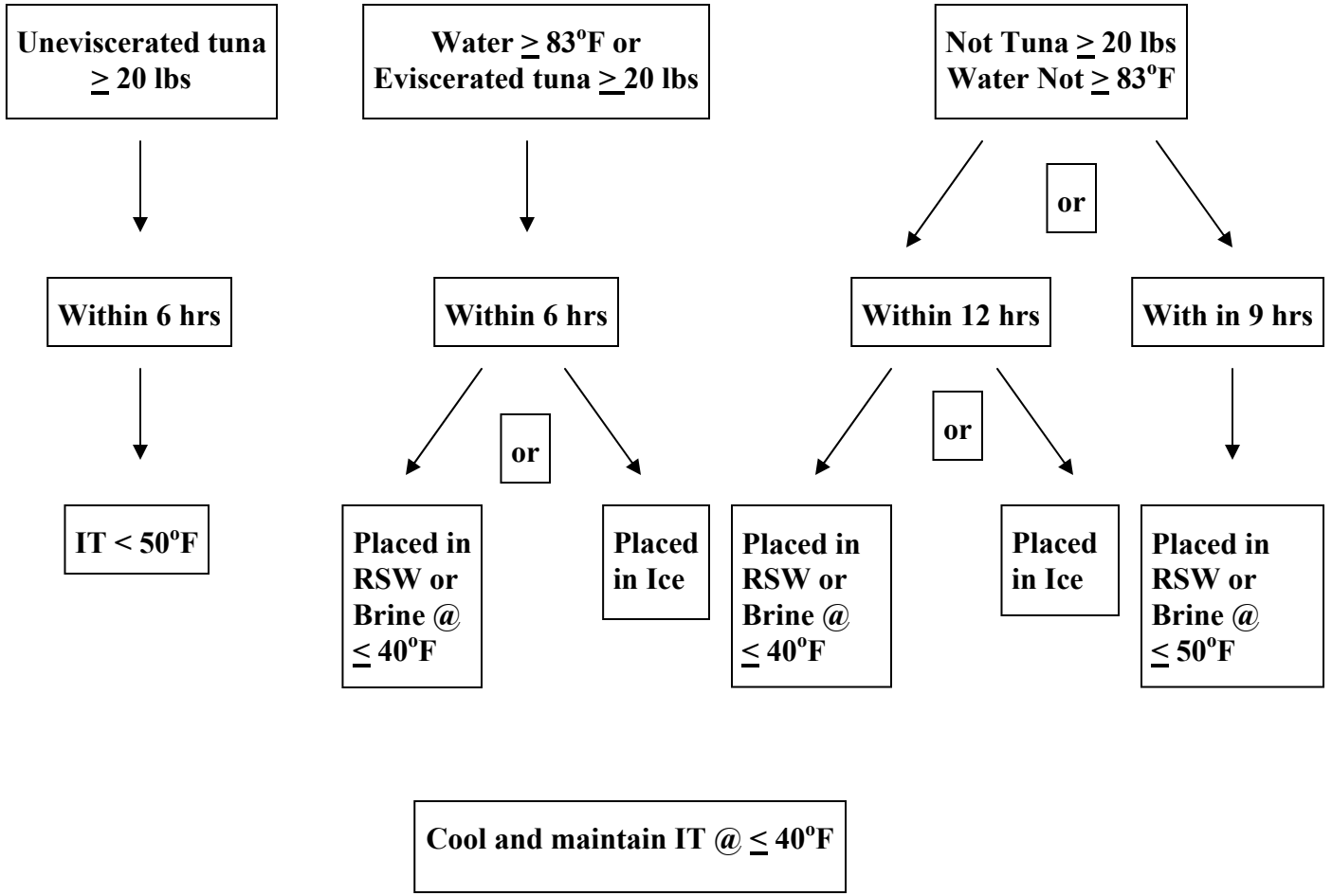


Figure 1: The US FDA's current HACCP guidelines for chilling fish on a vessel.

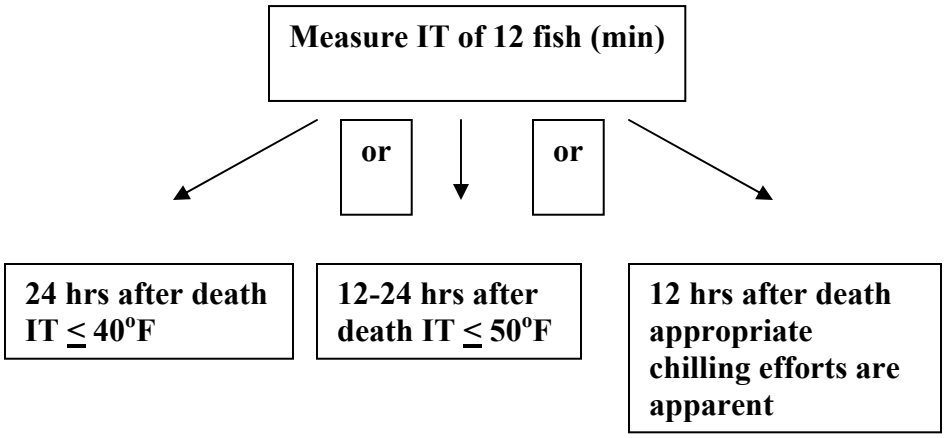


Figure 2: The US FDA's current HACCP guidelines for chilling fish at receiving.

Figure 3: Mahi-mahi with secured temperature probe.

Figure 4: Yellowfin tuna with secured temperature probe.

Figure 5: Mahi-mahi showing sampling location of muscle tissue.

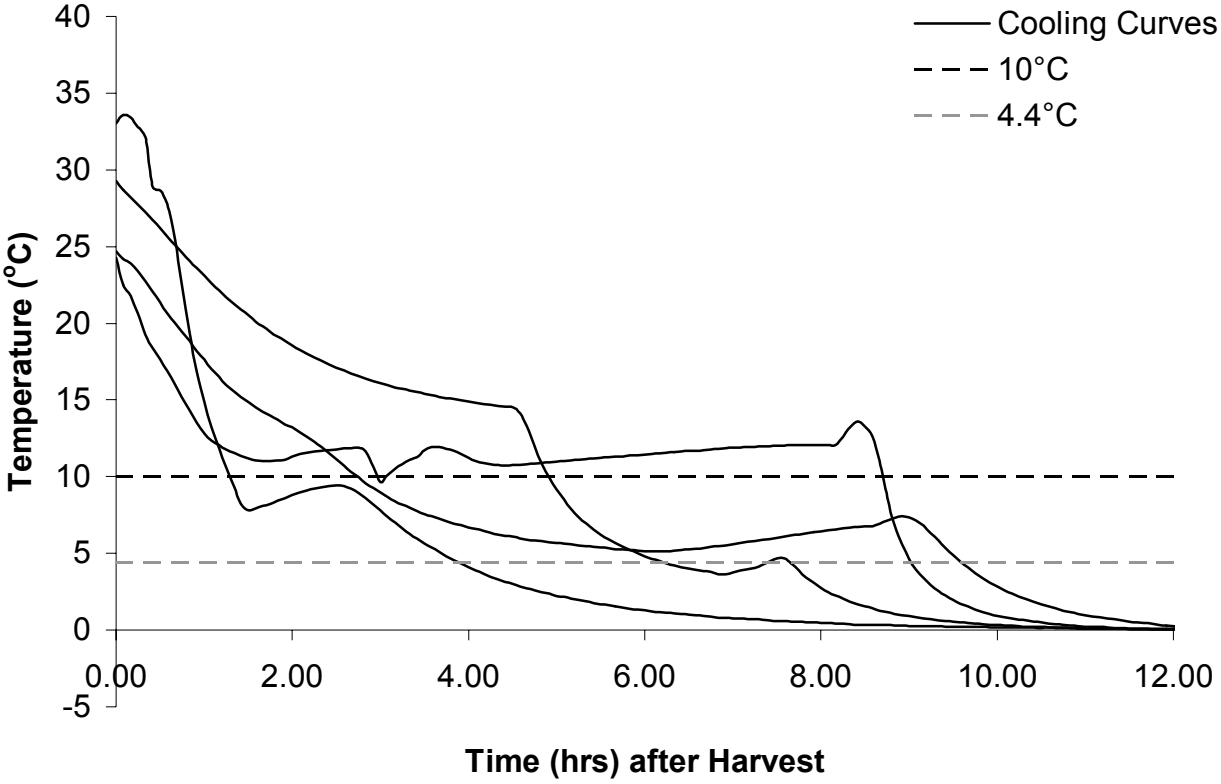


Figure 6: Representative cooling rate curves of 4 mahi-mahi harvested by North Carolina vessels.

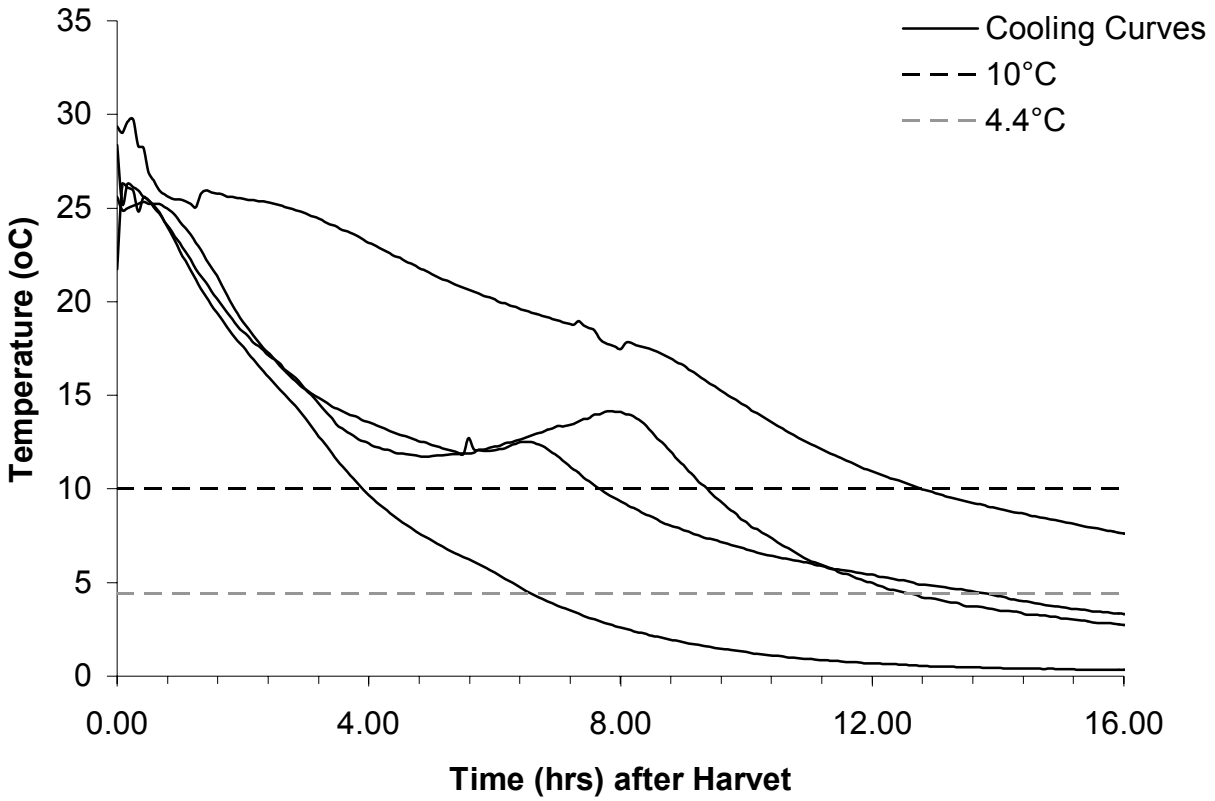


Figure 7: Representative cooling rate curves of 4 yellowfin tuna harvested by North Carolina vessels.

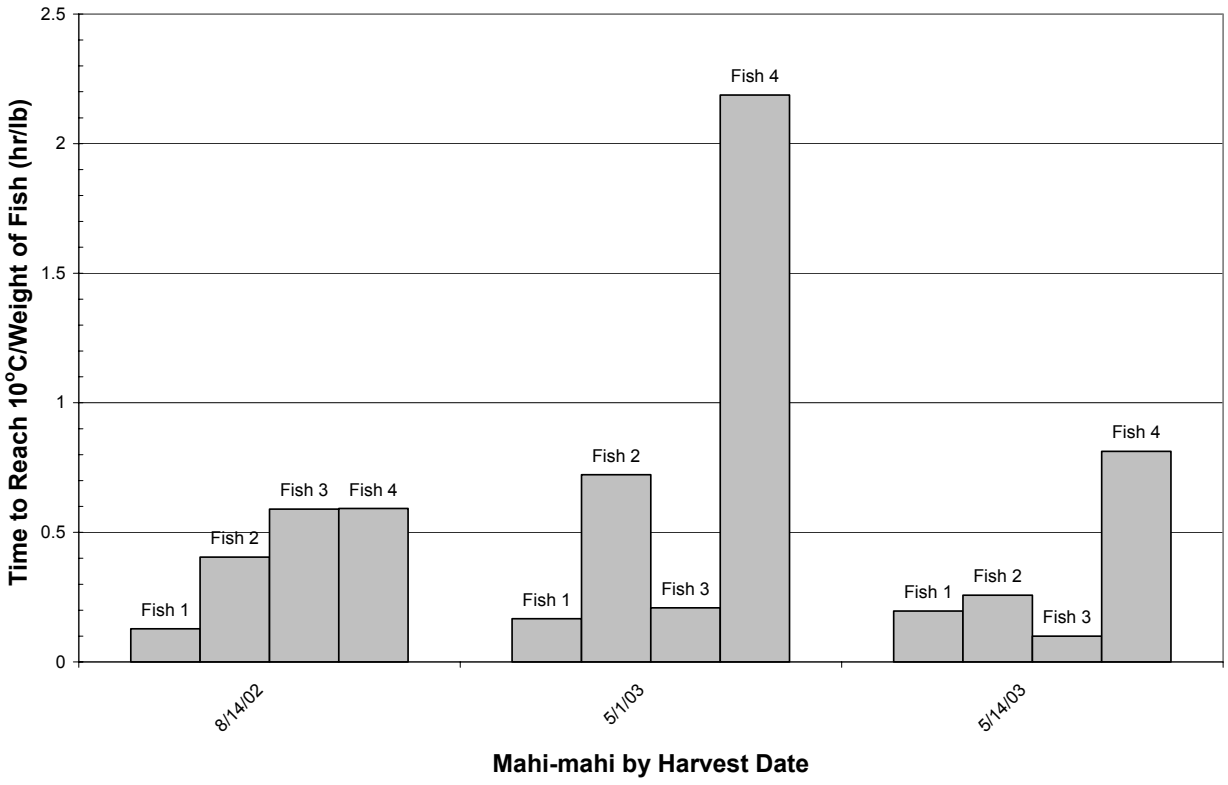


Figure 8: Time required for mahi-mahi to reach 10°C from point of harvest.

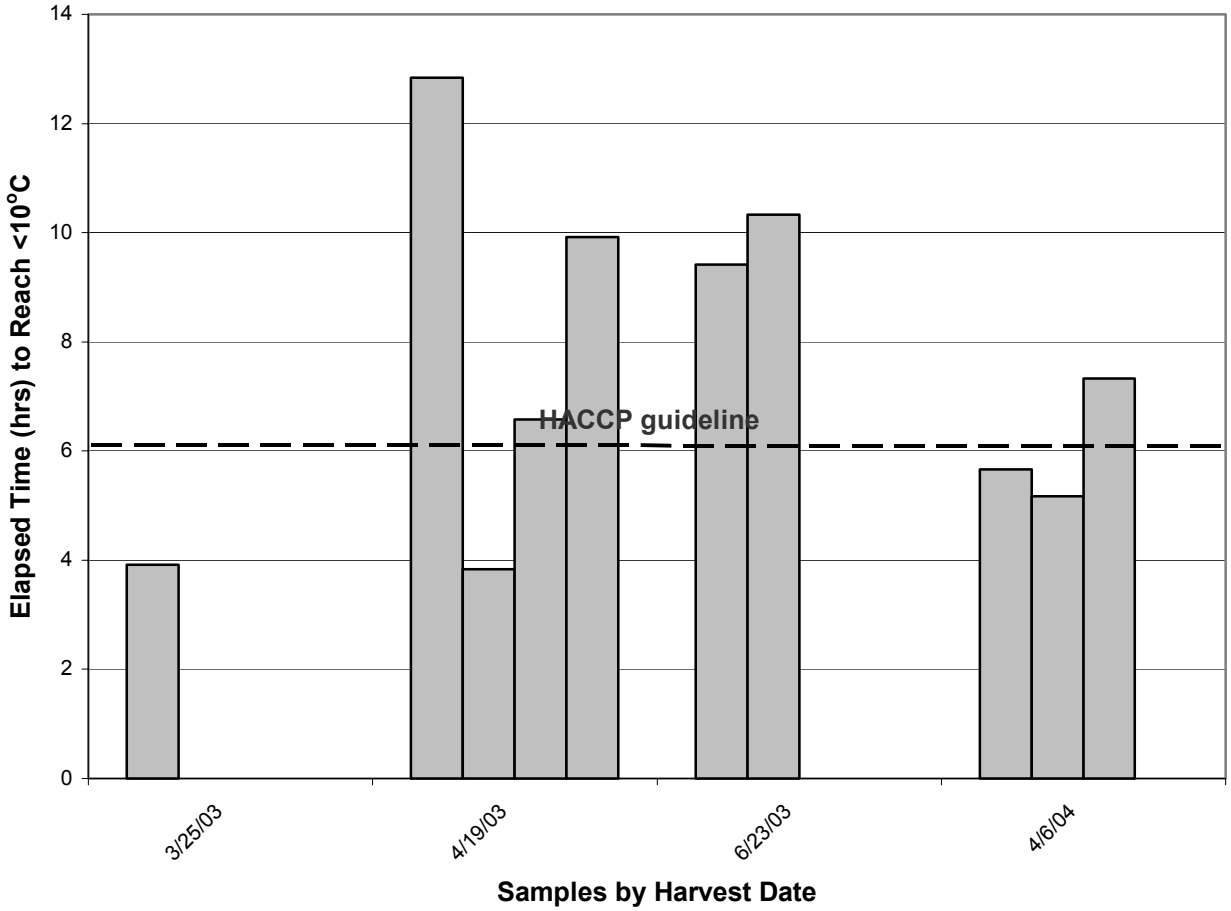
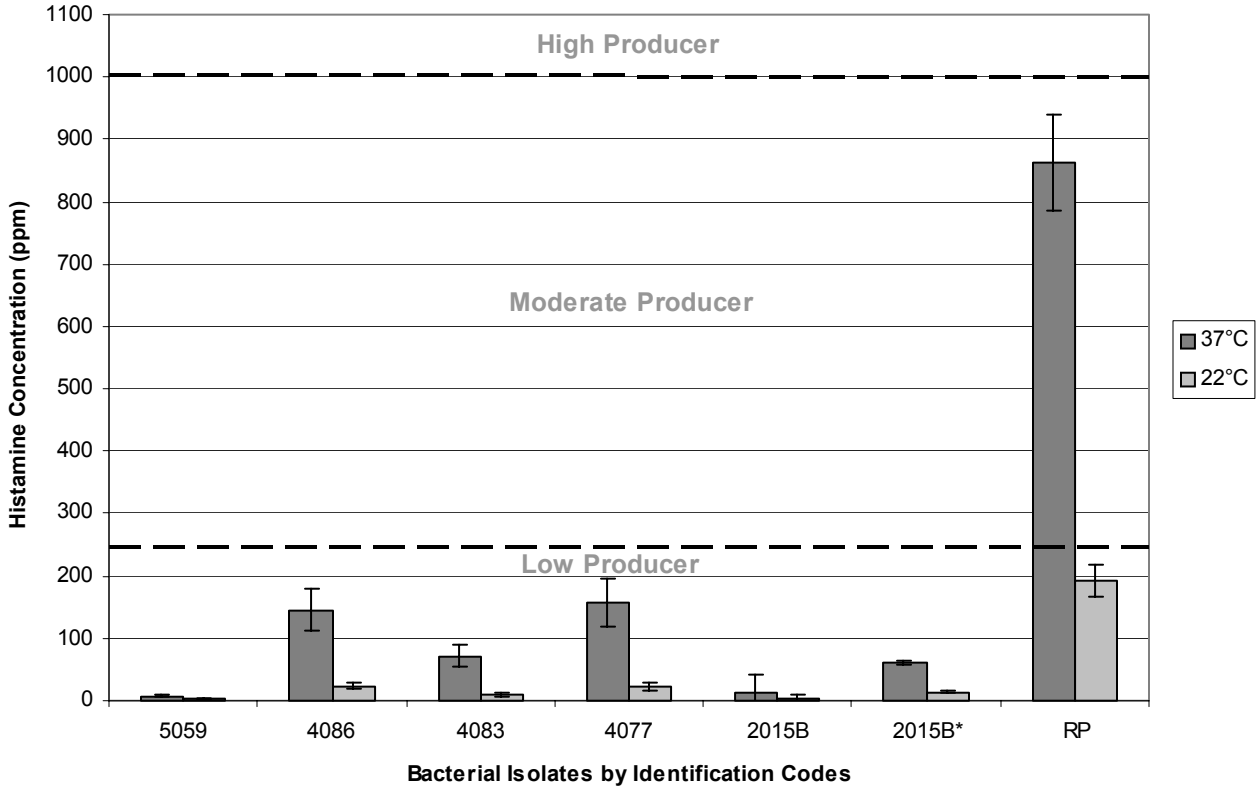


Figure 9: Time required for yellowfin tuna to reach 10°C from point of harvest.



*Adjusted average

Figure 10: Histamine production rate of identified bacterial isolates and *Raoultella planticola* incubated at 37°C and 22°C for 48 hrs

CONCLUSION AND FUTURE RESEARCH NEEDS

Although our understanding of histamine fish poisoning has improved over the years, much remains unknown. Three main areas of further study may include: (1) the significance of histamine and other biogenic amines relative to scrombroid poisoning; (2) the specific microorganisms responsible for scombrotoxosis, their origin, and the conditions necessary for toxin production; and (3) control of toxin production and/or toxin elimination.

One aspect of research that would increase our knowledge base about histamine poisoning are studies that identify the mechanisms of toxicity and affected organs. This information may allow for better treatment of afflicted patients. Another area of research would be to study the genetic make-up of biogenic amine-producing microorganisms. This research may help identify specific mechanisms in production of histamine and other related biogenic amines that could be targeted for intervention. Although focusing on basic issues would lead to a better understanding of histamine production, the factors surrounding histamine poisoning may take longer to resolve.

Therefore, a more practical approach is needed to control histamine-related foodborne outbreaks. One research area that needs to be examined is cooling rates for large pelagic fish such as yellowfin, bigeye and bluefin tunas. Work in this area needs to be performed under a number of different harvesting conditions and cooling media. The information obtained would be useful to FDA in assigning reasonable cooling rates for

potentially hazardous fish that can be achieved by the commercial fishing industry. Investigating an alternative or second hurdle for biogenic amine production would be of use in preventing consumer illness. Possible methods include targeting the responsible organisms through the use of anti-microbial agents or inactivating the decarboxylase enzymes as well as the biogenic amines themselves. Current research is being done to determine the efficacy of inactivating histidine decarboxylase enzymes in fish using high hydrostatic pressure techniques. Until efficacy of these methods is determined in a commercially relevant environment, they remain only possibilities.

APPENDIX

Table 1: Summary of physical data.

Date	Species	Fish ID	weight (lbs)	length (cm)	Histamine (ppm)	Time >50C	Times >40C
6/5/02	Mahi	1	NA	NA	Below limit	4hrs. 35 min	1
6/5/02	Mahi	2	NA	NA	Below limit	55min	1
6/5/02	Mahi	3	NA	NA	Below limit	1hr 25min	1
6/5/02	Mahi	4	NA	NA	Below limit	45min	1
7/16/02	Mahi	1	NA	NA	Below limit	1hr 55min	2
7/18/02	Mahi	1	NA	53.5	Below limit	1hr 15min	1
8/1/02	Mahi	1	NA	51	Below limit	55 min	1
8/1/02	Mahi	2	NA	52	Below limit	30 min	1
8/1/02	Mahi	3	NA	54.5	Below limit	1hr. 35 min	1
8/1/02	Mahi	4	NA	65	Below limit	4hrs 55min	2
8/14/02	Mahi	1	9.1	91	Below limit	1hr 10min	1
8/14/02	Mahi	2	3.3	57	Below limit	1hr 20min	1
8/14/02	Mahi	3	4.1	60	Below limit	2hrs 25min	1
8/14/02	Mahi	4	3.8	63	Below limit	2hrs 15min	1
3/25/03	Tuna	1	25	NA	Below limit	3hrs 55min	1
4/19/03	Tuna	1	50	NA	Below limit	12hrs 50min	1
4/19/03	Tuna	2	30	NA	Below limit	3hrs 50min	1
4/19/03	Tuna	3	40	NA	Below limit	6hrs 35min	1
4/19/03	Tuna	4	30	NA	Below limit	9hrs 55min	1
4/19/03	Mahi	1	NA	NA	NA	NA	NA
5/1/03	Mahi	1	9	73.5	Below limit	1hrs 30min	1
5/1/03	Mahi	2	6	72	Below limit	4hrs 20min	2
5/1/03	Mahi	3	4	56.5	Below limit	50 min	2
5/1/03	Mahi	4	4	62	Below limit	8hrs 45min	1
5/14/03	Mahi	1	14	85.5	Below limit	2hrs 45min	1
5/14/03	Mahi	2	11	83.5	Below limit	2hrs 50min	1
5/14/03	Mahi	3	15	94	Below limit	1hr 30min	2
5/14/03	Mahi	4	8	71	Below limit	6hrs 30min	1
6/23/03	Tuna	1	NA	NA	Below limit	9hrs 25min	1
6/23/03	Tuna	2	NA	NA	Below limit	NA	NA
6/23/03	Tuna	3	NA	NA	Below limit	10hrs 20min	1
6/23/03	Tuna	4	NA	NA	Below limit	NA	NA
4/6/04	Tuna	1	26.1	97.5	Below limit	5hrs 40min	1
4/6/04	Tuna	3	22.9	93	Below limit	5hrs 10min	1
4/6/04	Tuna	4	27.1	97.5	Below limit	7hrs 20min	1

Table 2: Summary of Niven's positive isolate data collected from mahi-mahi.

ID#	Grm	Shape	Step	Type Sample	Location	Organism (API)	Assurance	Organism (Crystal)	Assurance
1000	-	Rod	Preharvest	Environ	Prefish Cooler	NA	NA	NA	NA
1005	-	Rod	Preharvest	Environ	Hold	NA	NA	NA	NA
1009	-	Rod	Preharvest	Environ	Hold	*Steno. maltophilia*	75%	NA	NA
1018	+	Cocci	Harvest	Fish-Surface	Fish 1.3	NA	NA	NA	NA
1033	-	Rod	Receiving	Fish-Surface	Fish 1.1	*Pantoea spp.*	98.60%	NA	NA
1037	+	Cocci	Receiving	Fish-Surface	Fish 1.3	NA	NA	NA	NA
1040	-	Rod	Receiving	Environ	Ice Bucket	*Pantoea spp.*	98.60%	NA	NA
1049	-	Rod	Pre-Processing	Environ	Table-precutting	NA	NA	NA	NA
1053	+	Cocci	Pre-Processing	Fish-Surface	Fish 1.2	NA	NA	NA	NA
1065	-	Rod	Post-Processing	Fish-Surface	Fish 1.2	*Flavi. oryzihabitans*	81.20%	NA	NA
2000	-	Rod	Preharvest	Environ	Deck	NA	NA	NA	NA
2003	?	Cocci	Preharvest	Environ	Gaff	NA	NA	NA	NA
2004	?	Cocci	Preharvest	Environ	Boat ice shovel	NA	NA	NA	NA
2006	-	Rod	Post-Harvest	Environ	Deck	*Flavi. oryzihabitans*	40.10%	NA	NA
2008	-	Rod	Preharvest	Environ	Boat ice shovel	Bord./Alc/Mor spp. *	65.90%	NA	NA
2016	+	Cocci	Harvest	Fish-Surface	Fish 2.4	NA	NA	*Staph. aureus*	99.62%
2017	-	Rod	Harvest	Fish-Surface	Fish 2.4	Non-fermenter spp.	67.00%	NA	NA
2021	?	Cocci	Harvest	Fish-Surface	Fish 2.3	NA	NA	NA	NA
2024	-	Rod	Harvest	Fish-Surface	Fish 2.2	NA	NA	NA	NA
2025	-	Rod	Harvest	Fish-Surface	Fish 2.2	NA	NA	NA	NA
2026	-	Rod	Harvest	Fish-Surface	Fish 2.2	NA	NA	NA	NA
2049	+	Cocci	Receiving	Environ	Fish Bucket	NA	NA	*Micrococcus spp.*	99.21%
2054	+	Rod	Pre-Processing	Environ	Gutting table	NA	NA	*Corynebacterium spp.*	98.53%
2067	-	Rod	Pre-Processing	Environ	Knife-fillet	*Flavi. oryzihabitans* *	40.10%	NA	NA
2074	+	Rod	Pre-Processing	Fish-Surface	Fish 2.1	NA	NA	*Corynebacterium spp.*	95.72%
2095	-	Rod	Post-Processing	Fish-Tissue	Fish 2.2	NA	NA	NA	NA
2095	-	Rod	Post-Processing	Fish-Tissue	Fish 2.2	NA	NA	NA	NA
3001	-	Cocci	Preharvest	Environ	Hold	NA	NA	NA	NA

Table 2: (continue)

ID#	Grm	Shape	Step	Type Sample	Location	Organism (API)	Assurance	Organism (Crystal)	Assurance
3003	-	Rod	Preharvest	Environ	Deck	*Pantoea spp.*	99.90%	*Enterobacter cancerogenus* *	85.77%
3006	+	Cocci	Preharvest	Environ	Greg's Hands	NA	NA	*Staph warneri*	99.24%
3013	-	Rod	Harvest	Fish-Surface	Fish 3.2	*Ps. fluo./putida**	75.40%	*Psedomonas putida*	91.66%
3019	-	Cocci	Harvest	Fish-Surface	Fish 3.3	NA	NA	NA	NA
3024	-	Cocci	Harvest	Fish-Surface	Fish 3.4	NA	NA	NA	NA
3030	-	Cocci	Post-Harvest	Environ	Hold	NA	NA	NA	NA
3031	+	Cocci	Post-Harvest	Environ	Hold	NA	NA	*Micrococcus luteus* *	87.72%
3034	-	Cocci	Post-Processing	Fish-Tissue	Fish 3.1	NA	NA	NA	NA
3039	-	Cocci	Post-Processing	Fish-Tissue	Fish 3.4	NA	NA	NA	NA
3047	+	Cocci	Pre-Processing	Fish-Surface	Fish 3.1	NA	NA	*Staph. saprophyticus*	99.99%
3061	-	Cocci	Pre-Processing	Fish-Surface	Fish 3.3	NA	NA	NA	NA
3070	+	Cocci	Post-Processing	Fish-Surface	Fish 3.4	NA	NA	*Staph. vitulinus*	99.99%
3079	-	Rod	Post-Processing	Environ	Table	*Pantoea spp.*	99.30%	*Enterobacter gergoviae*	98.34%
3082	-	Rod	Post-Processing	Fish-Surface	Fish 3.1	*Pantoea spp.*	99.90%	*Enterobacter gergoviae*	93.25%
4001	-	Rod	Preharvest	Environ	Deck	NA	NA	NA	NA
4008	-	Rod	Harvest	Fish-Surface	Fish 4.1	*Ps. fluo./putida**	75.40%	*Psedomonas aeruginosa**	67.97%
4011	-	Cocci	Harvest	Fish-Surface	Fish 4.2	NA	NA	NA	NA
4013	-	Rod	Harvest	Fish-Surface	Fish 4.2	*Ps. fluo./putida**	75.40%	NA	NA
4017	+	Cocci	Harvest	Fish-Surface	Fish 4.3	NA	NA	*Staph. vitulinus*	78.99%
4018	-	Cocci	Harvest	Fish-Surface	Fish 4.3	NA	NA	NA	NA
4020	-	Rod	Harvest	Fish-Surface	Fish 4.4	*Ps. fluo./putida**	75.40%	NA	NA
4021	-	Cocci	Harvest	Fish-Surface	Fish 4.4	NA	NA	NA	NA
4025	-	Rod	Post-Harvest	Environ	Hold	*Flavi. oryzihabitans*	81.2	NA	NA
4026	-	Rod	Post-Harvest	Environ	Hold	NA	NA	*Flavimonas oryzihabitans*	90.10%
4028	-	Cocci	Post-Harvest	Environ	Hold	NA	NA	NA	NA
4031	+	Cocci	Post-Processing	Fish-Tissue	Fish 4.1	NA	NA	*Staph. saprophyticus*	86.82%
4032	+	Cocci	Post-Processing	Fish-Tissue	Fish 4.1	NA	NA	*Staph. warneri*	99.73%
4036	+	Cocci	Post-Processing	Fish-Tissue	Fish 4.4	NA	NA	*Staph. epidermidis*	99.99%

Table 2: (continue)

ID#	Grm	Shape	Step	Type Sample	Location	Organism (API)	Assurance	Organism (Crystal)	Assurance
4038	-	Rod	Receiving	Fish-Surface	Fish 4.1	NA	NA	NA	NA
4039	+	Rod	Receiving	Fish-Surface	Fish 4.1	NA	NA	*Corynebacterium psedodiphtheriticum*	94.98%
4041	+	Cocci	Receiving	Fish-Surface	Fish 4.1	NA	NA	NA	NA
4043	-	Rod	Receiving	Fish-Surface	Fish 4.2	NA	NA	NA	NA
4046	+	Rod	Receiving	Fish-Surface	Fish 4.2	NA	NA	*Corynebacterium spp.*	98.54%
4048	-	Rod	Receiving	Fish-Surface	Fish 4.3	NA	NA	NA	NA
4051	-	Rod	Receiving	Fish-Surface	Fish 4.3	NA	NA	NA	NA
4052	+	Rod	Receiving	Fish-Surface	Fish 4.3	NA	NA	NA	NA
4053	-	Rod	Receiving	Fish-Surface	Fish 4.4	*Ps. fluo./putida**	44.50%	NA	NA
4055	-	Rod	Receiving	Fish-Surface	Fish 4.4	*Steno. maltophilia*	99.30%	NA	NA
4061	-	Rod	Pre-Processing	Fish-Surface	Fish 4.1	*Ps. fluo./putida**	75.40%	NA	NA
4062	-	Rod	Pre-Processing	Fish-Surface	Fish 4.2	NA	NA	*Pseudomonas aeruginosa*	67.97%
4063	+	Cocci	Pre-Processing	Fish-Surface	Fish 4.2	NA	NA	*Staph. capitis*	98.06%
4065	-	Cocci	Pre-Processing	Fish-Surface	Fish 4.3	NA	NA	NA	NA
4067	-	Rod	Pre-Processing	Fish-Surface	Fish 4.3	NA	NA	*Pantoea agglomerans*	98.99%
4069	+	Rod	Pre-Processing	Fish-Surface	Fish 4.4	NA	NA	*Corynebacterium spp.*	95.72%
4071	-	Cocci	Pre-Processing	Fish-Surface	Fish 4.4	NA	NA	NA	NA
4077	-	Rod	Post-Processing	Fish-Surface	Fish 4.1	NA	NA	*Citrobacter freundii*	99.88%
4083	-	Rod	Post-Processing	Fish-Surface	Fish 4.2	*Citrobacter freundii*	98.90%	*Citrobacter freundii*	99.70%
4085	+	Cocci	Post-Processing	Fish-Surface	Fish 4.4	NA	NA	*Staph. warneri*	99.99%
4086	-	Rod	Post-Processing	Environ	Knife	NA	NA	*Citrobacter freundii*	99.32%
4088	-	Rod	Post-Processing	Environ	Knife	Bord./Alc/Mor spp. *	59.90%	Misc Gm- Bacilli	99.44%
4089	-	Rod	Post-Processing	Environ	Table	*Citrobacter braakii*	96.40%	3 possibilities	NA
2015B	+	Cocci	Preharvest	Environ	Fish basket	NA	NA	*Staph. epidermidis*	99.99%

* More than one possible identity due to clarity of test reactions

Table 3: Summary of Niven's positive isolate data collected from yellowfin tuna.

ID#	Grm	Shape	Step	Type Sample	Location	Organism (API)	Assurance	Organism (Crystal)	Assurance
5008	+	Cocci	Pre-Harvest	Environ	Deck	NA	NA	*Kytococcus sedentarius*	82.55%
5010	+	Cocci	Pre-Harvest	Environ	Deck	NA	NA	*Staph. Aureus*	99.58%
5014	-	Cocci	Harvest	Fish-Surface	Mahi	NA	NA	NA	NA
5016	-	Cocci	Harvest	Fish-Surface	Mahi	NA	NA	NA	NA
5017	+	Rod	Harvest	Fish-Surface	Fish 5.1	NA	NA	*Gardnerella vaginalis*	81.25%
5018	-	Rod	Harvest	Fish-Surface	Fish 5.1	Bord./Alc./Mor. spp.*	38.70%	*Pseudomonas aeruginosa*	98.16%
5027	-	Rod	Harvest	Fish-Surface	Fish 5.4	Non-fermenter *	32.10%	NA	NA
5028	+	Rod	Harvest	Fish-Surface	Fish 5.4	NA	NA	!!!Many possibilities!!!	NA
5029	+	Cocci	Harvest	Fish-Surface	Fish 5.4	NA	NA	*Staph. warneri*	99.89%
5035	-	Cocci	Post-Harvest	Environ	Hold	NA	NA	NA	NA
5038	+	Cocci	Post-Processing	Fish-Tissue	Fish 5.1	NA	NA	*Staph. vitulinus*	99.99%
5044	+	Cocci	Post-Processing	Fish-Tissue	Fish 5.3	NA	NA	*Micrococcus luteus*	90.48%
5046	-	Rod	Post-Processing	Fish-Tissue	Fish 5.4	*Ent. amnigenus*	90.20%	*Enterobacter cloacae*	93.27%
5047	-	Rod	Post-Processing	Fish-Tissue	Fish 5.4	NA	NA	*Pantoea agglomerans*	99.32%
5051	-	Cocci	Post-Harvest	Environ	Hold	NA	NA	NA	NA
5052	+	Rod	Post-Harvest	Environ	Deck	NA	NA	*Corynebacterium bovis*	78.85%
5053	-	Rod	Post-Harvest	Environ	Deck	Non-fermenter *	32.10%	*Pseudomonas spp.*	96.75%
5058	-	Rod	Post-Harvest	Environ	Hold	Non-fermenter *	32.10%	*Pseudomonas aeruginosa*	99.60%
5059	+	Cocci	Receiving	Environ	Packing Crate	NA	NA	*Aerococcus urinae*	99.99%
5060	+	Cocci	Receiving	Environ	Cooler	NA	NA	*Micrococcus luteus*	87.72%
5061	+	Rod	Pre-Processing	Environ	Table	NA	NA	*Bacillus cereus*	99.99%
5063	-	Cocci	Pre-Processing	Environ	Table	NA	NA	NA	NA
5068	-	Rod	Pre-Processing	Environ	Knife	*Ent. amnigenus* *	96.50%	*Enterobacter cloacae*	95.44%
5069	+	Cocci	Pre-Processing	Environ	Knife	NA	NA	*Micrococcus luteus*	96.15%
5071	-	Rod	Pre-Processing	Environ	Knife	NA	NA	*Pantoea agglomerans*	90.41%
5073	+	Cocci	Pre-Processing	Fish-Surface	Fish 5.4	NA	NA	*Staph vitulinus*	99.99%
5079	+	Cocci	Pre-Processing	Fish-Surface	Fish 5.1	NA	NA	NA	NA
5080	+	Cocci	Pre-Processing	Fish-Surface	Fish 5.2	NA	NA	*Staph vitulinus*	99.99%

Table 3: (continue)

ID#	Grm	Shape	Step	Type Sample	Location	Organism (API)	Assurance	Organism (Crystal)	Assurance
5081	+	Cocci	Pre-Processing	Fish-Surface	Fish 5.3	NA	NA	*Bacillus magaterium*	99.86%
5084	-	Rod	Post-Processing	Fish-Surface	Fish 5.4	*Pantoea spp.*	99.90%	*Pantoea agglomerans* *	44.48%
5085	+	Cocci	Post-Processing	Fish-Surface	Fish 5.4	NA	NA	*Staph. warneri*	99.99%
5088	+	Cocci	Post-Processing	Fish-Surface	Fish 5.1	NA	NA	!!!Many possibilities!!!	NA
5089	+	Cocci	Post-Processing	Fish-Surface	Fish 5.2	NA	NA	*Staph. aureus*	99.99%
5091	+	Rod	Post-Processing	Fish-Surface	Fish 5.3	NA	NA	*Corynebacterium spp.**	*
5092	-	Cocci	Post-Processing	Fish-Surface	Fish 5.3	NA	NA	NA	NA
5093	-	Cocci	Post-Processing	Fish-Surface	Fish 5.3	NA	NA	NA	NA
5095	+	Cocci	Post-Processing	Fish-Surface	Fish 5.2	NA	NA	*Staph. warneri*	91.21%
6004	-	Rod	Post-Processing	Fish-Tissue	Fish 6.C1	NA	NA	NA	NA
6005	-	Rod	Post-Processing	Fish-Tissue	Fish 6.C1	NA	NA	NA	NA
6008	+	Cocci	Post-Processing	Fish-Tissue	Fish 6.C2	NA	NA	NA	NA
6009	-	Rod	Post-Processing	Fish-Tissue	Fish 6.C2	NA	NA	NA	NA
6010	-	Rod	Post-Processing	Fish-Tissue	Fish 6.C2	NA	NA	NA	NA
6013	-	Rod	Post-Processing	Fish-Tissue	Fish 6.C3	NA	NA	NA	NA
6021	+	Cocci	Harvest	Fish-Surface	Fish 6.4	NA	NA	NA	NA
6023	-	Rod	Harvest	Fish-Surface	Fish 6.4	NA	NA	NA	NA
6024	-	Rod	Harvest	Fish-Surface	Fish 6.4	NA	NA	NA	NA
6027	-	Rod	Harvest	Fish-Surface	Fish 6.4	NA	NA	NA	NA
6035	+	Cocci	Post-Harvest	Fish-Surface	Fish 6.1	NA	NA	NA	NA
6043	-	Rod	Post-Harvest	Fish-Surface	Fish 6.4	NA	NA	NA	NA
6044	+	Cocci	Post-Harvest	Fish-Surface	Fish 6.4	NA	NA	NA	NA
6046	-	Cocci	Receiving	Environ	Ice Shovel	NA	NA	NA	NA
6047	-	Rod	Post-Processing	Environ	Table	NA	NA	NA	NA
6051	-	Rod	Post-Processing	Environ	Knife	NA	NA	NA	NA
6052	+	Rod	Post-Processing	Environ	Knife	NA	NA	NA	NA
5098A	+	Rod	Post-Processing	Environ	Table	NA	NA	*Bacillus megaterium*	99.99%
5098B	+	Rod	Post-Processing	Environ	Table	NA	NA	*Bacillus megaterium*	99.52%

Table 3: (continue)

ID#	Grm	Shape	Step	Type Sample	Location	Organism (API)	Assurance	Organism (Crystal)	Assurance
7004	-	Rod	Harvest	Fish-Surface	Fish 7.1	NA	NA	Many	NA
7006	+	Cocci	Harvest	Fish-Surface	Fish 7.3	NA	NA	*Staph. warneri*	99.75%
7009	-	Rod	Post-Harvest	Environ	Hold	NA	NA	Many	NA
7011	+	Cocci	Pre-Processing	Fish-Surface	Fish 7.1	NA	NA	*Staph. simulans*	61.76%
7012	+	Cocci	Pre-Processing	Fish-Surface	Fish 7.1	NA	NA	*Staph vitulinus*	92.29%
7016	+	Cocci	Pre-Processing	Fish-Surface	Fish 7.3	NA	NA	*Staph. warneri*	multiple%
7030	+	Cocci	Post-Processing	Fish-Tissue	Fish 7.1	NA	NA	*Staph. pasteuri*	91.44%
7034	+	Cocci	Post-Processing	Fish-Tissue	Fish 7.3	NA	NA	*Staph. Sp.*	>96.71%
7036	+	Cocci	Post-Processing	Fish-Tissue	Fish 7.4	NA	NA	*Staph. warneri*	91.50%

* More than one possible identity due to clarity of test reactions

Date	Fish	Number of Samples taken	Fish	Environmental	Number of Isolates from Fish						Number of Isolates from the Environment					
					Isolates	Niven's+	Gm-	Gm+	Rod	Cocci	Isolates	Niven's+	Gm-	Gm+	Rod	Cocci
8/1/02	Mahi	68	36	32	37	5	2	3	2	3	37	5	5	0	5	0
8/14/02	Mahi	52	28	24	42	9	NA	NA	7	2	58	9	NA	NA	4	5
4/17/03	Tuna	60	30	30	55	24	10	14	7	17	46	16	13	3	10	6
5/1/03	Mahi	60	28	32	62	9	7	2	2	7	27	5	3	2	2	3
5/14/03	Mahi	68	36	32	67	30	19	11	18	12	24	7	7	0	6	1
6/23/03	Tuna	41	20	21	42	13	9	4	9	4	11	4	3	1	3	1
4/6/04	Tuna	37	15	22	19	8	1	7	1	7	17	1	1	0	1	0

Table 4: Summary of sampling data.