AN EVALUATION OF THE ROLE OF STORAGE TEMPERATURE ON THE SAFETY AND QUALITY OF RAW SHELLSTOCK OYSTERS AND BLUEFISH

by

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Part 1. Role of Temperature on the Safety and Quality of Raw Shellstock Oysters (*Crassostrea virginica*)

(ABSTRACT)

Raw oyster shellstock was subjected to abuse conditions (7, 13, and 21°C) and sampled over a ten day storage period to gather scientific data to aid in determining whether spoilage occurred in the raw product over time before proliferation of pathogenic flora (*Vibrio vulnificus*) made the product unsafe. Spoilage was evaluated through pH measurements of a homogenate of the shucked meat and liquor. The olfactory acceptability of the raw oysters was evaluated in concert with the microbial and chemical evaluations. At all storage conditions, halophilic bacteria outgrew *V. vulnificus* by a minimum of 1 log CFU/g oyster (Colony Forming Units per gram) ($p < 0.05$). Olfactory acceptability was below 40% when *V. vulnificus* growth was at its highest ($p < 0.05$). Refrigerated storage should be considered a CCP for raw shellstock since even moderate temperature control kept *V. vulnificus* below 10^4, approximately 1-2 Logs below the estimated infective dose for the majority of the population.

To my best friend and companion on the road of life, Scott Drake,
without whose emotional
support this work would not have been possible

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Table of Contents

Part 1. Role of Temperature on the Safety and Quality of Raw Shellstock Oysters

CHAPTER I. INTRODUCTION

Raw shellstock oysters have been vehicles for many foodborne diseases, including *Vibrio vulnificus* induced infections. Infections from *V. vulnificus* range from mild gastroenteritis in healthy individuals, to life-threatening septicemia in immunocompromised patients. *V. vulnificus* is part of the normal microflora of oysters. Its presence is not due to fecal contamination, therefore fecal safety indicators cannot be used to estimate levels. Oyster samples with low levels of organisms used as such sanitation indicators may have elevated levels of natural *V. vulnificus,* thus posing a potential threat to human health if consumed. Control of the microflora and pH of oysters is linked to control of temperature during storage and transport. When these controls fail, for example when temperatures exceed the guidelines established by the National Shellfish Sanitation Program (NSSP), natural flora proliferate, inducing a rise in the pH of the product as spoilage bacteria begin to decompose oyster tissues. Categories describing oyster quality have been correlated with pH values and may be used to classify an oyster sample as wholesome or spoiled. Since the natural flora of oysters also includes *V. vulnificus*, it is important to determine the effect of temperature abuse on both the pH and level of the pathogen. If the pH reaches unacceptable levels before elevated *V. vulnificus* numbers are found, the oyster would be considered spoiled and therefore not pose a threat to the general population. On the other hand, if temperature abuse induces proliferation of the pathogen while the oysters are within acceptable pH limits, the general population may be at risk of *V. vulnificus* induced infections. Researchers estimate the minimum infective dose for healthy individuals to be approximately 10^4. The minimum infective dose for immunocompromised individuals has not been determined to date (24). The following research was undertaken to determine whether temperature abuse during storage of raw shellstock produces a spoiled product before it becomes unsafe.

CHAPTER II. REVIEW OF THE LITERATURE

A. Oyster industry in the United States

The eastern oyster, *Crassostrea virginica***,** is a molluscan bivalve growing in estuarine waters. It is a sessile filter feeder, pumping surrounding waters in through the gills to obtain oxygen and food; concentrating matter (including toxins and microorganisms) encountered within its tissues. Acceptably-sized particles travel into the mouth to the esophagus, where they are trapped in a mucus layer and moved to the stomach by ciliary action of the epithelial cells lining the esophagus (62). In the caecum (within the stomach), particles are sorted by size, surface, or chemical characteristics. Those particles rejected here (the pseudofeces) are shuttled to the opening of the mid-gut from the caecum by an intestinal groove or pushed out between mantle ridges or out of the shell by rapid opening and closing action. Those particles not rejected continue their trek through the digestive system, being mixed with digestive enzymes in the anterior part of the stomach and then with digestive acids in the posterior portion by the crystalline style.

The life cycle of the oyster is similar to that of most mollusks. The temperature of the growth waters directs the development and maturation of the reproductive organs. Once complete, spawning and external fertilization occur. Larvae develop into spat and attach to oyster shells (culch) lining the bottom of beds and grow (15). Growth to market size (ca. 90 mm in length) is typically about 14 months, depending on the temperature and salinity of the growth waters. Growth is more rapid in warm waters; for example estuaries in the Gulf of Mexico can produce a marketable oyster in approximately 2 years (87). Researchers have long suggested oysters required a minimum of 10 ppt (parts per trillion) salinity level in order to grow at normal rates, with stunting or non-existent growth below 7.5 ppt (87).

1. Production

In the Atlantic area of the US, the oyster industry is concentrated in the Gulf of Mexico. The National Marine Fisheries Services estimates commercial landings at 81,871.6 metric tons between 1990 and 1996, generating over $550 million (48). Oysters are typically farmed and harvested by raking or dredging the bottom of estuary beds. Once harvested, they are given a quick wash and placed into dry sacks and will remain alive as shellstock for approximately two weeks under refrigeration (21). NSSP requires interstate shipments of shellstock be made under mechanical refrigeration, at or below 7.2 °C (45°F) (33). Forcible removal of the oyster body from the shell (shucking) by hand, washing of the meat, and packaging occurs at the processing facility. Refrigerated, on ice, or frozen transport are the methods employed in relaying oysters to secondary processors.

2. Consumption as food

The whole body of the oyster is consumed either cooked (baked, boiled, steamed, fried) or raw. A 3.5 oz. (100 g) uncooked serving contains 7.9 g of protein, 3.5 g of carbohydrates, 2 g of saturated fat, 0.057 g of cholesterol, and 75 calories. In addition, this serving size supplies 65%, 35%, 100%, and 100% of the USRDA (Unites Sates Recommended Daily Allowance) for niacin, iron, vitamin B_{12}, and zinc, respectively (80).

3. Spoilage

Spoilage in oysters is primarily fermentative and involves the breakdown of glycogen, an energy storage molecule found in high concentrations in the tissues. The glycogen content of oysters has been shown to be seasonal, with a larger concentration during the late summer and fall and a lower concentration in early summer and again in the winter (69). Mixed microflora initiate glycogen breakdown, producing lactate. Initial spoilage seems to be started by members of the *Serratia, Pseudomonas, Proteus, Clostridium, Bacillus, Aerobacter,* and *Escherichia* (35). Accumulation of acid in the tissues results in a drop in pH, making the environment favorable for the proliferation of *Lactobacilli, Streptococci,* and yeasts (23,13,53). At late stages of decomposition, when the pH ranges between 5.0 and 4.6, *Lactobacilli, Streptococci,* and yeasts compose the microflora almost exclusively (35). Hunter and Linden (53) concluded that spoilage of oysters depended more on the presence and outgrowth of certain types of microorganisms rather than on the total number of microorganisms present.

The relationship between the pH of a homogenate of shucked meat and oyster liquor and spoilage has been used as a basis for evaluating wholesomeness. Samples with a pH range between 5.6 and 6.1 are considered to be passing from “good” to “stale”, those between 4.9 and 5.3 considered “stale” to “sour” or “putrid”, and those with a pH below 5.0 are considered to be in a state of “advanced decomposition” (53). Liuzzo et al. (64) found the correlation between pH of both the oyster liquor and meat to organoleptic scores to be statistically significant, supporting the use of pH as an indicator of quality.

4. Classification of shellfish growing areas

Shellfish growing areas are classified by ordinances set forth by the National Shellfish Sanitation Program (NSSP) (34) and classified by the Division of Shellfish Safety (DSS). Areas are classified as approved, conditional (approved or restricted), restricted, or prohibited based on shoreline survey results providing an overview of potential pollution sources and strict seawater sampling programs which provide bacteriological data in terms of indicator bacteria. Shellfish may or may not be harvested for interstate commerce depending on this initial classification of the growing waters. The most commonly used indicator organisms have been the coliforms and fecal coliforms and are expressed in terms of the approved procedure used and the nature of the specimen (51). The total coliform group is comprised of aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rod-shaped bacteria that ferment lactose to yield acid and gas within 48 hours at 35°C (68). Fecal coliforms is considered to be a more specific indicator of fecal contamination (51). Fecal coliforms are enteric pathogens. They are those coliforms which

produce acid and gas from lactose at a higher temperature of 44.5 ± 0.2 °C when incubated for 24 ± 2 hours (68).

Shellfish harvested from approved waters are considered safe for the direct marketing of shellfish. Shellfish harvested from these areas are unlikely to cause illness attributed to fecal contamination. These waters are not subject to unsafe levels of fecal contamination and are not contaminated with pathogens, poisonous or deleterious substances, or biotoxins. In addition, proper and repeated testing of the waters show they meet the bacteriological requirements for the classification; that is, the water cannot exceed < 70 total coliforms / 100 ml (MPN (Most Probable Number)/100 ml water) with no more than 10% of the samples exceeding an MPN of 230 MPN/100ml (5 tube decimal dilution series) or 330 MPN/100ml (3 tube decimal dilution series) and must contain less than 14 fecal coliforms/100 ml (34).

Growing areas classified as conditionally approved or conditionally restricted are those that are subject to predictable pollution incidents. Incidents include effluent from wastewater treatment facilities or direct contamination by tourists or fishing fleets. These areas are considered approved during the absence of pollution events and restricted during the presence of such events under the guidance of the DSS. Shellfish may be harvested for direct sale during both periods, but must be relayed or depurated if such a practice is approved. Shellfish may be depurated if total coliforms do not exceed 700 MPN/100 ml or fecal coliforms 88 MPN/100 ml (34).

Restricted areas are those that are subject to limited pollution by pathogens, deleterious substances, or fecal pollution. Shellstock is made safe for human consumption through relaying or depuration, under the supervision of the state or local shellfish authority. Shellfish may be depurated if total coliforms do not exceed 700 MPN/100 ml or fecal coliforms 88 MPN/100 ml (34).

Shellfish may not be harvested for human consumption from prohibited areas. These areas include those adjacent to sewage treatment plants (or other pollution sources detrimental to public health), those subject to unpredictable pollution sources, contaminated with fecal waste, dangerous levels of biotoxins, or contaminated with deleterious or poisonous substances (34). Hunt (51) described the disposal of domestic wastes into shellfish growth and harvest waters as the major health hazard potential of consuming shellfish.

B. Natural microflora

Because oysters are filter feeders, their natural flora is dependent on the nature and quality of the growing waters which is affected in turn by salinity, temperature, and pollution (106). In an attempt to characterize the natural flora of raw oysters, researchers have isolated numerous organisms from oysters since the turn of the century, including spirochetes, yeasts, viruses, and Gram-positive and Gram-negative rods and cocci. Human illnesses associated with mollusks include, but are not limited to, typhoid fever, Hepatitis A, gastroenteritis, neurotoxic and paralytic

shellfish poisoning, and cholera (50).

Dimitroff (29) found spirochete commensals (*Saprospira* spp., *Cristispira* spp., and *Spirillium* spp.) in approximately 91% of oysters sampled in his survey of Baltimore seafood markets. Tall and Nauman (100) found *Cristispira* sp. to be associated with the crystalline style of oysters harvested from the Chesapeake Bay. Pathogenic yeasts have been found, though no human mycoses have yet been linked to raw oyster consumption (23).

Buck et al. (12) isolated both human-associated (HAY) and non-human associated (NHAY) yeasts from oysters harvested from Long Island Sound. *Candida parapsilosis*, *C. tropicalis*, and *Torulopsis grablata* were those HAY most frequently isolated. *C. albicans* was found in the most heavily polluted waters during the coldest months. Hood (43) suggested *Rhodotorula hubra* to be part of the normal flora of *C. virginica* upon finding the non-toxic pink yeast in 86% of all freshly harvested oyster samples during the course of a study of the Apalachiacola and Tampa Bays between April 1980 and August 1981. At a mean level of 3.0 x 10^1 CFU/g, the researcher found time and temperature of storage and the salinity of harvest waters as the major factors influencing the growth of the yeast in oyster samples.

Human enteric viruses (Norwalk, Hepatitis A) have been found at harvest, but they do not multiply in the bivalve tissues; though they may remain viable during the storage of the shell stock (23). In 1978, 2000 cases of mollusk-associated gastroenteritis throughout Australia were traced to oysters tainted with the Norwalk virus. Murphy et al. (74) noted Norwalk-induced illnesses occurred irrespective of consumption of the oysters as raw or cooked. The Norwalk virus was implicated in a 1993 outbreak of oyster-associated gastroenteritis in Louisiana, Maryland, Mississippi, and North Carolina (17). At least 180 cases were documented, with 90 persons from Maryland exhibiting acute diarrhea (92%), vomiting (71%), nausea (67%), abdominal cramps (61%), and fever (40%). Again, illness occurred regardless of whether the oysters were consumed raw or cooked (steamed). The human enteroviruses Coxakie and echovirus were isolated from *C. virginica* samples collected from both approved and closed harvesting areas of Long Island in a 1976-77 study of harvest waters by Vaughn et al. (108). The researchers noted no correlation between coliform levels and virus levels (as measured in Plaque Forming Units/g oyster homogenate [PFU/g]). Tierney et al. (103) found no reduction in virus particles (PFU/g oyster homogenate) occurred for approximately 28 days (the estimated time between harvest and consumption of shellstock or shucked oysters stored at 5°C), during a 30-77 day storage study of oysters stored as shellstock and shucked. Live oysters were either artificially inoculated in the laboratory or allowed to self-contaminate during feeding in water tanks containing feces associated poliovirus particles.

Gram-negative rods (including *Vibrionaceae*, *Pseudomonadaceae*, *Flavobacterium/Cytophaga*, *Aeromonaceae*, *Neisseriaceae*, *Alcaligenes*, *Achromobacter*, *Enterobacteriaceae*) and Gram-negative cocci (*Moraxella*) have been isolated by numerous researchers. Hunter and Linden (53) found members of the genera *Achromobacter*, *Flavobacterium*, and *Eberthella* in oysters collected from the Potomac River. Colwell and Liston

(22) found that *Pseudomonas* and *Vibrio* (including *V. alginolyticus V. parahaemolyticus* and Kanagawa positive *V. parahaemolyticus*) accounted for approximately 50% of the natural flora of *Crassostrea gigas* harvested from the coasts of Washington state. Lovelace et al. (65) found *Vibrio* spp., *Pseudomonas* spp., *Achromobacter* spp., *Corynebacterium* spp., *Cytophaga/Flavobacterium* spp., *Proteus* spp., and *Enterobacter* spp. from Chesapeake Bay oysters in a 1968 study. Vasconcelos and Lee (107) found *Flavobacterium/Cytophaga, Vibrio/Pseudomonas* type II, *Pseudomonas* type III or IV, *Acinetobacter/Moraxella,* Gram-positive cocci, and *Bacillus* to comprise over 90% of the microflora of *C. gigas.* Vanderzant et al. (104) found *Vibrio, Aeromonas,* and the Gram-negative coccus *Moraxella* predominated in Galveston Bay oysters. In a study of the bacterial flora of *C. virginica* collected from estuaries off the coast of Long Island in 1968, Murchelano and Brown (72) noted that all isolates consisted of Gram-negative, short or pleomorphic rods and no Gram-positive bacteria were found at all. Of these, 35.4% were pigmented yellow or orange, and 57.3% were motile with all but one having a monotrichous polar flagellum. Upon classifying the isolates, the researchers noted that 31.2% were *Pseudomonads*, 26% were *Flavobacterium/Cytophaga*, 25% *Vibrios*, and 17.7% *Achromobacter/Alcaligenes.* Abeyta et al. (1) recovered *Aeromonas hydrophila* from oyster samples that came from the same growing area as those that had been associated with 472 cases of gastroenteritis in Louisiana in 1982. Though the samples had been stored at -72°C for one and a half years, the researchers detected the pathogen at levels of 9.3 MPN/100g oysters.

In a case report review of 333 patients with raw oyster-associated illnesses in Florida between 1981 and 1994, Hlady (41) reported an annual incidence of 10.1 *Vibrio* infections per 1,000,000 adults. Further analysis revealed that 28% of the cases were attributed to *V. vulnificus* with the rest (72%) to non-vulnificus *Vibrios*.

Other bacteria isolated form oysters include *Clostridia, Salmonellae,* and *Yersinia.* Sobsey et al. (93) found a log mean average of 1 *Salmonella* CFU/100 ± 40g oyster homogenate in both open and closed beds during a one year study of oysters harvested from North Carolina coastal harvest areas. They found that oysters from closed beds had higher *S. aureus*, total and fecal coliform, fecal streptococci, and aerobic plate counts (APC, 35°C , 48 hrs.) than did the open beds. Lee (63) isolated *Y. enterocolitica* from four of seventeen oyster samples collected during a 1973 study investigating the incidence of the pathogen in Ontario Canada. In a 1990 investigation of Japanese foods, environmental samples, and human fecal samples, Saito (84) isolated 223 strains of *C. perfringens* from 23 raw oysters (of 41 sampled). Of the 223 strains, nine were found to be enterotoxigenic, leading the researcher to suggest raw oysters as a potential vehicle for *C. perfringens* induced foodborne illness. Colburn et al. (21) isolated many potential pathogens from *C. gigas* harvested from Puget Sound during a 1984 study. Seventy-eight percent of oysters sampled contained *Aeromonas hydrophila. Salmonella typhimurium* was detected in 2.7% of samples. *Vibrio cholerae* non-O1 and *V. fluvialis* were isolated from 7.4% of samples. *Yersinia* spp., including a non-pathogenic strain of *Y. enterocolitica,* were isolated from approximately 21% of oysters sampled.

Gram-positive cocci and rods (Micrococcus, Coryneforms, *Bacillaceae, Staphylococcus*)

have been isolated in numerous studies. Colwell and Liston (22) found that approximately 20% of the microflora of *C. gigas* harvested off the coast of Washington State was composed of assorted Gram-positive rods and cocci. Approximately 7% of the total flora consisted of Coryneforms, less than 1% consisted of *Micrococcus*, *Staphylococcus*, and *Bacillaceae*. Lovelace et al. (65) isolated both *Micrococcus* and *Bacillus* spp. from Chesapeake Bay oysters in a 1968 study. Vanderzant et al. (104) detected low levels of *Micrococcus* in Galveston Bay oysters. Son and Fleet (94) isolated *Bacillus cereus* form *C. commercialis* harvested from five approved locations in New South Wales, Australia (mean level 389 CFU/g oyster homogenate), noting the low likelihood of such low levels of the pathogen (10^5 CFU/g less than the typical infective dose) producing a direct public health risk. During a 1980 study to determine the occurrence of enteric bacteria and viruses in *C. virginica* collected from both closed and approved harvest areas of North Carolina, Sobsey et al. (93) found a log mean level of 90 CFU/100 g oyster homogenate in open beds and a log mean level of 170 CFU/100 g oyster homogenate in closed beds.

Researchers have reported a predominance of halophilic bacteria in shellstock and shucked samples. Colwell and Liston (22) noted many of the bacteria isolated from *C. gigas* required salt for growth.

Biotoxins produced by algae (Phytoplankton spp.) commonly accumulate in oyster tissues once phytoplankton are ingested as food by the mollusks. Toxic phytoplankton are causative agents of shellfish induced Paralytic Shellfish Poisoning (PSP) (50).

Wholesome oysters (those not considered to be in a state of decomposition) typically have elevated aerobic plate counts. Hunter and Linden (53) found ca. 10^7 CFU/g in shellstock collected from the Potomac River. In a 1977 study of the microbiological quality of fresh and frozen seafood products, Foster et al. (36) noted geometric means of 3.8×10^7 APC/g (Aerobic Plate Count at 35°C) and coliform levels of 4.8×10^3 MPN/g (Most Probable Number at 35 °C) in fifty-nine fresh shellstock samples. Vanderzant et al. (104) found halophilic aerobic plate counts (3% NaCl added to TSA incubated at 25°C for 48 hrs) of oysters freshly harvested from Galveston Bay ranged from 2.3×10^4 - 3.0×10^7 CFU/g, with an average of 4.0×10^3 CFU/g. Similar results were found by Wentz et al. (109) in 1983. In a survey of sixty-five randomly selected retail outlets representing standard metropolitan statistical areas, geometric means of 3.9×10^5 APC CFU/g and 8.9×10^5 APC (at 30°C) CFU/g were found from fresh shucked oyster meat collected from sampled retail outlets. Improper handling and abuse during storage were cited as possible reasons for finding 52.7% of all oysters sampled met or exceeded the NSSP wholesale guidelines for APC (<500,000 CFU/g oyster) and 93% for fecal coliforms (<230 MPN/100g). During a 1989 study of *C. gigas* harvested from Puget Sound, Colburn et al. (21) noted the average APC was 2000 CFU/100 g oyster. Coliform levels averaged 525 MPN/100g and fecal coliform 155 MPN/100g oyster. Camire et al. (13) suggested season, season-route interaction, and oyster sample influenced the APC of oysters during a study of oyster distribution. The researchers also suggested the time lapse and holding conditions contributed to a rise in SPC and fecal coliform levels.

NSSP guidelines for total coliform and SPC's (or APC's) are useful tools for the wholesaler and researcher in ascertaining the wholesomeness, storage, and handling of market oysters and other shellfish (52). Table 1 lists the bacteriological levels and interpretations for wholesale market shellfish, providing samples are examined in accordance to the methods described in the *Compendium of Methods for the Microbiological Examination of Foods*. Upon following proper laboratory procedure, shellfish found to contain low fecal coliform and low SPC levels are considered a high quality product (in terms of bacteriology). High sanitary standards and practices were followed during harvesting, processing, storage, and shipping.

1. Storage effects

Hood et al. (45) observed the effects of storage on loads of natural flora of *C. virginica* in a 1983 study. In oysters stored as shellstock at 2, 8, 20, and 35°C for seven days, mean levels of total bacteria increased (SPC [Standard Plate Count] at 35°C for 48 hrs.). After seven days of storage at 8 and 20°C, *Vibrio* levels increased exponentially and then decreased after 14 and 21 days. The researchers noted that traditional storage methods, such as refrigeration, resulted in an overall increase in bacterial load while *Vibrio* levels increased only in oysters stored as shellstock. Cook and Ruple (26) documented microbial load changes during shellstock handling in 1989. SPC (CFU/g oyster meat) increased four-fold from harvest to the processing plant and fecal coliform levels increased upon subsequent incubation of shellstock above 50°F (10°C).

2. Depuration and relaying effects

Oyster depuration (self cleansing of oysters through submersion in pH and salt controlled tanks) and re-laying (moving live shellstock from polluted to non-polluted waters for a period of time) are frequently used to reduce the overall bacterial load (83). Bacteria removed from surrounding waters are trapped in the mucus of the oyster's gill, mantle, and palp surfaces upon which bacteria are transported to the labial palps. It is at the labial palps where bacteria and particulates are sorted into pseudofeces (materials rejected and expelled readily from the shell) and those materials that will be injected by the oyster and travel through the digestive tract (81). Perkins et al. (81) observed naturally contaminated *C. virginica* shellstock depurated from levels as high as 39,000 MPN/100 g to < 50 fecal coliforms/100g oysters in 48 hr. Son and Fleet (94) investigated the effect of relaying *C. commercialis* shellstock harvested from polluted areas (the Georges River New South Wales Australia; *E. coli* levels of 30 MPN/100 ml water) to non-polluted waters (Quibray Bay, New South Wales Australia; *E. coli* levels of < 3 MPN/100 ml water). The researchers found average *E. coli* levels were reduced from 5.9 to 1.3 MPN/100g oyster homogenate after 2 days and then to non-detectable levels after 6 days. *Salmonella*, *B. cereus, V. parahaemolyticus,* and *C. perfringens* levels (measured as CFU/100 g oyster homogenate) were reduced from 0.4, 335, 18, and 18 to non-detectable levels, 67, 4, and 4 after two days and non-detectable levels, 67, 2.5 (5 in sample 1 and non-detectable in sample 2), and to non-detectable levels after six days, respectively. In their investigation of UV light assisted depuration of oysters artificially inoculated with pathogens, Son and Fleet (94) noted that both *S. typhimurium* and *S. senftenberg* were quickly depurated from heavily contaminated oysters and

Table 1. National Shellfish Sanitation Program guidelines for the interpretation of bacteriological results of market shellfish. [a]

Bacterial levels		Interpretation of results			
FC [b]	SPC [c]	Harvest waters	Quality	Practices followed / possible problems	Notes / Action
Low	Low	approved	high	High sanitary standards during harvest, processing, storage, shipping	-
low	high	approved	inferior	Excessive storage in processing or transit to market	probably pathogen free; Notify producer state for investigative/corrective action
high	low	improperly classified area	potentially hazardous	direct fecal contamination	Notify producer state for investigative/corrective action
FC	SPC	Harvest waters	Quality	Practices followed / possible problems	Notes / Action
high	high	+/- approved	potentially hazardous; poor	Direct contamination (handlers/equipment); inadequate refrigeration; mixing of old and fresh shellfish; poor re-packing; excessive storage times	Notify producer state for investigative/corrective action

[a] From Hunt et al. (52).
[b] Fecal coliforms
[c] Standard Plate Count

by two days initial counts had been reduced by 300 to 1,000 fold. Samples with initial levels of greater than 10^3 CFU/g homogenate were still found to contain low levels of *Salmonella* after three days of depuration. *B. cereus* and *V. parahaemolyticus* levels were reduced from approximately 10^3 CFU/g to < 20 CFU/g after two days of depuration. In two days of depuration, *C. perfringens* levels were reduced to < 10 CFU/g from an initial 10^3 -10^5 CFU/g. Tamplin and Capers (101) found that even UV light assisted depuration did not reduce natural loads of *V. vulnificus* in live shellstock oysters. Groubert & Oliver (39) noted the same lack of depuration of natural *V. vulnificus* populations from shellstock, adding the observation that artificially inoculated *V. vulnificus* readily depurated. Vasconcelos and Lee (107) noted that the microbial flora of *C. gigas* generally resembled that of untreated seawater in shellstock sampled during a three day depuration study (UV light assisted). The researchers found coliforms and most *Pseudomonads* depurated out from shellstock fairly readily after 72 hours, but that Gram-positive cocci and *Vibrio* spp. remained at elevated levels for a longer period of time.

3. Relationship between indicator bacteria and potentially pathogenic species

The relationship between levels of indicator bacteria (total coliforms and fecal coliforms) and potentially pathogenic bacterial species and pathogenic viruses has been investigated by numerous researchers. Because levels of indicator bacteria are used to classify growing waters and determine whether oyster samples are fit for wholesale levels (51, 34, 33), the importance of correlating the levels of indicator bacteria with certain pathogens is of great importance. Overall, the literature points to mixed correlations between indicator bacteria and pathogens. Hood et al. (44) found low fecal coliform levels in both shellstock and stored oysters correlated to the absence of *Salmonella* spp. in oysters harvested from approved, conditionally approved, prohibited, and unapproved harvest waters of the Apalachiacola and Tampa Bays. The researchers noted, however, that high fecal coliform levels could not be used to predict the absence of *Salmonella* spp. from such samples since some samples with high fecal coliform levels were found to be positive for *Salmonella* spp. while some found to be negative for the pathogen. Gerba et al. (37) failed to find a strong relationship between the presence of enteroviruses in harvest water and oyster shellstock with total coliform levels, fecal coliform levels, pH, or salinity (of either harvest waters or shellfish). The researchers noted the prevalence of viruses in Galveston Bay waters considered to meet bacteriological guidelines of 70 coliforms/100 ml water. Ellender et al. (31) did not find statistically significant correlations between NSSP guidelines and bacterial and viral levels determined from *C. virginica* harvested from the Gulf Coast in a 1980 study. The researchers isolated twelve and 146 viruses from approved and prohibited sites, respectively. Eight of the twelve viral isolates from the approved site where classified as poliovirus type 1. Poliovirus types 1 and 2 and echovirus type 24 where isolated from the prohibited waters.

4. Processing effects

The search for increased distribution range and profits have led many to experiment with processing aids as methods to increase the shelf life of oysters without compromising the organoleptic quality consumers expect from the raw product. To date, cold storage, freezing,

heating, vacuum packaging, irradiation, and the use of preservatives have been examined. Cook and Ruple (28) noted natural *V. vulnificus* populations dropped to non-detectable levels upon heating oysters for 10 min in 50°C water without producing a noticeable cooked appearance or flavor. Parker et al. (79) found a 2-3 log APC (MPN/g oyster meat in Alkaline Peptone Water supplemented with 1% NaCl) drop in shucked oysters during a 70 day frozen storage study (-20°C), noting 3.5 log and 4 log decreases in natural *V. vulnificus* populations and those oysters artificially inoculated with a type strain of *V. vulnificus* culture, respectively. The researchers also found the combination of vacuum packaging and frozen storage (-20°C) produced a 3-4 log APC (CFU/g oyster meat) decrease over the 70 day study, though they did not evaluate the sensory acceptability of the treated oysters. In 1994 Sun and Oliver (97) observed the effects of several GRAS (Generally Recognized As Safe) compounds on *V. vulnificus,* noting diacetyl, lactic acid, and BHA (butyl-hydroxy acid) to be highly antimicrobial *in-vitro* while diacetyl (min. 0.05%) highly effective *in-vivo*. In a later study, the researchers found that Tabasco™ brand hot sauce successfully reduced surface *V. vulnificus* populations upon 10 minute subsequent incubation at room temperature, but had no effect on *V. vulnificus* populations within the meat (96).

5. *Vibrio vulnificus*

Vibrio vulnificus is an organism common in estuarine and marine environments and found on the surface and intestinal contents of marine animals. Two biotypes have been identified, one a human and the other an eel pathogen (6). *V. vulnificus* is unrelated to pollution, commonly isolated from marine and estuarine waters as well as from shellfish and sediment (76). In 1992, Tamplin and Capers (101) found the largest concentrations of the organism in the digestive tract, gills, and adductor muscle, followed by the mantle and hemolymph of the eastern oyster.

V. vulnificus is a facultatively anaerobic Gram-negative curved rod belonging to the family *Vibrionaceae*. It is motile in liquid media with a single sheathed polar flagellum (5). Its biochemical characteristics are summarized by Elliot et al. (32) in Table 2. An obligate halophile, *V. vulnificus* requires 1-3% NaCl for growth in laboratory media (75). Generation time in BHI or HI broth is 22-30 min with cell density resulting in $6x10^8$ to $2x10^9$ *Vibrios*/ml (75). Tamplin and Capers (101) found the optimum temperature for growth to be 37°C, with the organism possibly experiencing metabolic damage at lower temperatures. Its sensitivity to cold was first noted by Boutin et al. (9) in 1985. Their studies showed that five *V. vulnificus* strains held in shrimp homogenate would undergo a 3 log reduction if subjected to a 4°C chill during a two week storage period. When subjected to low temperatures (4°C), it has been documented that the organism enters a viable but non-culturable state (75). In 1997 Whitesides and Oliver (110) suggested this state (VBNC) was reversible upon subsequent heat treatment. In their study, VBNC cells were subjected to a seventeen degree temperature upshift (from 5 - 22 °C) for a twenty-four hour period. Populations previously subjected to cold shock to induce the VBNC state produced visible colonies after a twenty-four hour heat treatment. Tamplin and Capers (101) found that during the coldest months *V. vulnificus* was not isolated from freshly harvested oysters but was detectable in large numbers when the same shellfish are incubated at 25°C for 24 hours. Cook and Ruple (27) noted the death of the organism when exposed to temperatures

above 45°C.

Two morphotypes linked to virulence have been observed, opaque and translucent colony development (90). *V. vulnificus* capable of developing an acidic polysaccharide capsule form virulent opaque colonies, whereas the rest form avirulent translucent colonies (90). Yoshida et al. (115) observed in a 1985 study, using isogenic strains with translucent and opaque morphotypes, differences in capsular composition among morphotypes. Electron microscopy in concert with ruthenium red staining showed that all opaque strains had capsular materials, one of four translucent strains had incomplete capsular materials, and the remaining three translucent strains contained no capsular materials. Evaluation of invasiveness in the subcutaneous tissue of guinea pigs (via timed controlled tissue blots onto LB agar) showed opaque colonies as more invasive than translucent ones. Phagocytotic resistance and resistance to serum (human and guinea pig) was common to all opaque morphotypes. Less than 10% of translucent strains compared to all opaque morphotypes were recovered from the blood of mice (10^6-10^7 CFU/mouse, intravenously) ten minutes after administration. The average LD_{50} (log_{10}) (lethal dose) among opaque strains was calculated at 6.0, approximately 2.4 logs lower than for translucent, confirming the enhanced virulence of opaques over translucents. Studies of eight strains (both clinical and environmental) by Kim et al. (57) in 1997 revealed that morphotype is related to the organism's susceptibility to heat and suggested capsular materials may function as cellular insulators. The D-values (time required to reduce viable populations of a strain by 90%) and Z-values (the absolute value of temperature required to reduce the D-value by 1 log) for opaque strains were greater than those for transluscents. Z-values ranged from 1.7 - 2.5 °C, prompting the researchers to suggest heat treatment as a relatively effective method for eradicating *V. vulnificus*. The researchers also observed morphotype shifts on heart infusion agar. Shifting of opaque to translucent morphotypes occurred at a rate of 10^{-5} - 10^{-4} while the reverse shift from translucent to opaque did not occur. The rate of shifting was defined as the number of colonies showing the opposite morphotype divided by the total number of colonies. Table 3 compares the D-values between the two morphotypes.

In a susceptible host, the organism is highly invasive once ingested or introduced into tissue through puncture. Boudre et al. (8) suggested in 1981 that the invasiveness of the organism, as evaluated by vascular permeability of *V. vulnificus* in the host tissue, required direct contact between host cells and viable *Vibrio* cells rather than being caused by the action of an extracellular toxin. They found that only viable bacteria produced edema or mortality in mice while sterilized edema fluid (from mice inoculated with ca. 10^7 CFU), culture supernatant, killed cells, nor disrupted cells produced edema or mortality.

Table 4 lists agents reputed to be involved in the pathogenicity of the organism. Though these are listed as separate entities for clarity, Miyoshi et al. (71) in a 1993 review of the literature grouped the separate toxic agents into actions produced by three exocellular toxic factors, the

Table 2. Phenotypic traits of *V. vulnificus.* [a]

Characteristic / test	Reaction	Characteristic	Reaction
Gram	neg.	Fermentation of:	
Straight / curved rods	pos.	raffinose	neg.
Motile (single polar flagellum)	pos.	L-rhamnose	neg.
Fermentative metabolism	pos.	maltose	pos.
Kovac's oxidase	pos.	D-xylose	neg.
Bioluminescence	neg .[b]	trehalose	pos.
Pigment	neg.	cellobiose	pos. [e]
Catalase	pos.	erythritol	neg.
Fermentation of:		L-arabinose	neg.
glucose (gas production)	pos. (-)	melibiose	neg.
lactose	pos .[c]	D-arabitol	neg.
sucrose	neg. [d]	D-mannose	pos.
D-mannitol	pos.	D-galactose	pos.
dulcitol	pos.	D-galacturonate	neg
salicin	pos. [e]	levulose	pos.
adonitol	neg.	amygdalin	pos.
myoinositol	neg.	glycerol	neg.
D-sorbitol	neg.	α-methyl-D-glucoside	neg.
arabitol	neg.	Methyl red	pos.
Nitrate reduction	pos.	H_2S production	neg.
Nitrite reduction	neg.	Spreading growth	pos.
Indole	pos.	Hydrolysis of:	
Vogues-Proskauer	neg.	ONPG	pos.
Lysine decarboxylase	pos.	Simmon's Citrate	pos. [e]
Ornithine decarboxylase	pos.	urea	neg.

Characteristic / test	Reaction	Characteristic	Reaction
Arginine dihydrolase	neg.	gelatin	pos.
Phenylalanine dicaminase	variable	starch	pos.
Growth in broth with:		agar	neg.
0 % NaCl	neg.	esculin	variable
0.5 % NaCl	pos.	Sensitivity to:	
2 % NaCl	pos.	penicillin	pos.
6 % NaCl	pos.	colistin	neg. [e]
8% NaCl	neg.	o/129	pos.
DNase	pos.	polymyxin B	neg.
Lipase (corn oil)	pos.	Growth at:	
Coagulase	neg.	40°C	pos.
String test	pos.	25°C	pos.
		5°C	neg.

[a] From Oliver, 1989 (52).
[b] Luminescent strain
[c] May be delayed several days and is negative on some media. Ability to ferment lactose may be indicated by ability to hydrolyze ONPG
[d] 15% may ferment sucrose
[e] Strains with the opposite reaction have been reported

protease *vvp*, the cytotoxin *vvc*, and a phospholipase.

Vvp is a 45 kDa N-type (refers to ability to hydrolyze the peptide bond at the P_1' amino acid residue) zinc-requiring metalloprotease which is secreted during the late log and early stationary phases of growth. The protease has an isoelectric point of 5.8. Researchers believe the protease contributes to the virulence and invasiveness of *V. vulnificus* through degradation of biologically important proteins and through interference with host defense mechanisms. The extensive tissue damage observed in patients with *V. vulnificus* infections may be accounted by the collagenase and elastase activities attributed to *vvp* (60,92). Type I collagen, elastin, fibrinogen, plasma proteins, and heme proteins have been shown to be susceptible to *Vvp* activity (92).activation in immunity than *V. parahaemolyticus*, a closely related and less invasive Vibrio. This suggested an immunological reason for the enhanced susceptibility of cirrhotic patients to *V. vulnificus* infections since it is the alternative pathway that cirrhotic patients are most dependent on to fight bacterial attack. Nineteen ninety three studies by Maeda et al. (66) suggested that proteases produced by *V. vulnificus* enhance the bradykinin generating cascade in mice injected inter-peritonealy with ca. 10^7 CFU/mouse, facilitating the systemic dispersal of the microorganism and accelerating the death rate in mice. Activation of the bradykinin generating cascade produces pain, shock, and facilitates septicemia (30). Kinin is involved in inflammatory reactions following tissue injury, vasodialation, smooth muscle contraction, as well as increasing vascular permeability (30). Maeda et al. (66) noted only an 8% survival in mice given an i.p. injection of 10^7 CFU/mouse + 100 μg mouse bradykinin versus a 75% survival in mice given 10^7CFU/mouse alone. In a 1986 study, Miyoshi et al. (71) showed *vvp* directly activated isolated rat mast cells, leading to degranulation and histamine release. Studies with guineapigs led Miyoshi et al. (71) to conclude *vvp* complexed with α-macroglobulin in the bloodstream upon detection of the protease. This complex is then unable to degrade elastin and other large structural proteins (as would free vvp), blocking the progression of vascular permeability and the development of hemorrhagic skin lesions. The formation of α-macroglobulin-*vvp* complexes occurs in the bloodstream of healthy animals. It is important to note that patients with primary septicemia have as a consequence of the disease reduced α-macroglobulin levels and thus have higher levels of free *vvp* in the bloodstream and are more susceptible to free *vvp*.

Temperature and chemical inhibition of virulence characteristics attributed to *vvp* have been studied by many. Kothary and Kreger (60) reported the loss of caseinolytic and elastase activities upon 30 minute exposure to 100°C. Protease inhibition by EDTA and other chelating agents was reported by Smith and Merkel (92) as well as by Kothary and Kreger (60).

Vvc is a 56 kDa heat labile (56 °C, 30 min.) hydrophobic single-chain polypeptide which is linked to hemolytic activity and increased vascular permeability (38). The cytolysin is secreted during logarithmic growth and has a pI of 7.1 (71). Yamamoto et al. (114) in a 1990 work noted genetic homology between the *vvha* gene regions with the structural gene for the *Vibrio cholerae*

Table 3. Comparison of D-values between opaque and translucent morphotypes of *Vibrio vulnificus*. [a]

Temp	D-val (ave. ± SE) for morphotype:		
°C	opaque (n = 4)	translucent (n = 4)	P-value [b]
45	48.25 ± 2.60	40.58 ± 0.60	0.028
47	3.55 ± 0.11	3.28 ± 0.10	0.122
49	0.55 ± 0.03	0.44 ± 0.03	0.024
51	0.19 ± 0.00	0.18 ± 0.01	0.194

[a] From Kim et al. 1997 (57)

[b] Statistically significance of the difference between the two morphotypes was evaluated with Student's unpaired t-test. Significance level $p < 0.05$.

Table 4. Possible causative agents in diseases caused by *Vibrio vulnificus*. [a]

Agent	Action
Cytotoxin	Binds to human intestinal cells
Bacterial translocation	Allows movement from the duodenum to circulatory system within 4 hrs.
Polysaccharide capsule	Increased resistance of MO to serum bactericidal effects, decreased rate of phagocytic activity by reticuloendothelial cells; subcutaneous invasiveness
Exocellular metalloprotease	Edematous; increases permeability & skin lesions in interstitial tissue space
Tumor necrosis factor ∝	Aids in cellular death

[a] from Miyoshi et al., 1993 (71)

El Tor hemolysin though the two polypeptides produce much different pathological effects. Purified and characterized by Gray and Kreger (38) in 1985, the cytolytic protein lyses mature erythrocytes in a two-step process. The first step involves temperature independent binding of the cytolysin to cholesterol molecules embedded in the cell membrane of the host erythrocyte. A temperature dependent disruption of the host erythrocyte follows as *vvc* forms pores on the membrane surface and allows a potassium efflux. The intracellular osmotic pressure increases, facilitating bursting of the cell and release of the host cell hemoglobin. Gray and Kreger (38) noted an LD_{50} rate of 3 µg/kg purified cytolysin when injected into mice intravenously. They also found *vvc* to be active against erythrocytes of seventeen different animal species (with the cytolysin most effective against pig, monkey, burro, cat, sheep, pigeon, and mouse) and Chinese Hamster Ovary cells (CHO) in tissue culture. Inactivation studies with EDTA and other chelating agents have proved fruitless (no inactivation observed), with only high levels of cholesterol showing any inactivation of the cytolysin. The pathogenicity of *vvc* was put into question in 1991 by Wright and Morris (113). The researchers were not able to confirm the pathogenic role of the cytolysin. After inactivating the structural gene for the cytolysin (*vvh*A) in fully virulent strains, they found no significant differences in production of lethality and tissue damage in mice between cytolysin-positive and cytolysin-negative strains, leading them to conclude that *vvc* may be a less important player in lethality than other etiolic agents. Though the researchers were not convinced of the pathogenic role of *vvc*, their studies revealed the whole polypeptide to be unique to *V. vulnificus* and in 1993 developed an alkaline-phosphatase labeled oligonucleotide probe with a 3.2 kb DNA fragment of the cytolysin gene for rapid analysis of samples on non-selective media (113). Chang et al. (20) reported sequencing and cloning a new hemolysin/cytolysin gene (*vll*Y) from *V. vulnificus* in 1997, suggesting the polypeptide encoded by the newfound gene produced the cytolytic effects observed in *vvh*A-negative strains of *V. vulnificus* by Wright and Morris in 1991. The new protein was found to be distributed in the cytoplasmic and periplasmic portions of the cell. BLAST GenBank searches revealed a homology between *vll*Y and a legiolysin produced by *Legionella pneumophila* which is responsible for hemolysis, pigment production, and fluorescence. The *vll*Y gene encoded a 40 kDa (with an estimated pI of 5.02) which induced hemolysis and color production in recombinant *Escherichia coli* cells (transformed with the *vll*Y gene through several controlled plasmid transfers from *V. vulnificus* cells deficient in the *vvh*A gene sequence). The existence of a phospholipase and additional collagenases have been suggested in the literature, though none have been isolated nor purified to date.

In 1983, Simpson and Oliver (89) studied host iron acquisition in *V. vulnificus*, discovering the ability of the microorganism to produce both hydroxamate and phenolate siderophores. The organism produced these siderophores to obtain iron for growth and pathogenicity in environments in which the element is not readily available, such as the mammalian system where it is bound to transferrin and therefore not available for use by the organism. These low molecular weight chelators (siderophores) compete with transferrin for iron, bind the element, then transfer it to *V. vulnificus*. Stelma et al..(95) noted in 1992 that avirulent strains did not produce phenolate siderophores nor utilized transferrin-bound iron. Because the organism crosses the intestinal mucosa rapidly, septicemia can quickly produce visible cutaneous

bullae (19).

i. Disease: characteristics and epidemiology

Manifestation of the diseases caused by *V. vulnificus* depends on the vehicle of transmission, that is whether the organism enters the body through ingestion or puncture of the epidermis. The severity of the illness is dependent on host factors of the victim. Researchers have found that patients with preexisting hepatic diseases (such as cirrhosis, chronic hepatitis, and fatty liver, a history of alcoholism) and underlying chronic illnesses (such as malignant lymphoma, pulmonary tuberculosis, rheumatoid arthritis, duodenal ulcer, and diabetes melitus) had a higher incidence of primary septicemia (7,77). Infection by *V. vulnificus* most often manifests itself as primary septicemia (via ingestion) in vulnerable subjects or as wound infections (through direct contact such as exposure to an existing wound or physical injury with contaminated objects), though it has been found as the causative agent in many other diseases (7). Table 5 lists the pathologies and clinical references gathered by Kumamoto and Vukich (61) in their 1998 review of the literature.

V. vulnificus infection by ingestion leads to primary septicemia, gastroenteritis , and in some cases death (7,19,32,75,78). Symptoms of primary septicemia are summarized in Table 5. Tacket et al. (99) described secondary skin lesions found in patients with septicemia during a study of cases reported to the CDC Atlanta between 1980 and 1981. He described them as distinctive large hemorrhagic bullae on the trunk and extremities which later developed into necrotic ulcers. The infective dose for healthy subjects has not been determined, though the relationship between incidence of infection and time of year during which warm water temperatures prevailed led Cook (24) to suspect it to be high. Cases of gastroenteritis typically go unreported due to the self-limiting nature of the disease in healthy subjects (86). Incubation time for primary septicemia varies, from 7 hours to several days, though the median for cases reviewed by Oliver (75) was 16-38 hours. The strong link between the severity of *V. vulnificus* infection and pre-existing medical conditions among victims led physicians in the early 1980's and ‘90's to recommend that individuals with compromised immune systems not consume partially cooked or raw shellfish and take barrier precautions when exposed to seawater and when opening shellfish (7,18,19,27,41,42,45,50,54,75,99). Though physician warnings about the dangers of raw oyster consumption by at risk populations became widespread throughout coastal states in 1980, the results of the 1988 Florida Behavioral Risk Factor Survey estimated that 3 million persons in Florida consumed oysters raw, with 2.4 % of those polled believing they suffered from cirrhosis (the leading risk factor in oyster-associated septicemia) (16). Blake et al. (7) linked the development of septicemia after raw oyster consumption to diseases characterized by elevated host iron levels in a 1979 review of 39 cases reported to the CDC between 1964 and 1977. Johnston et al. (54) also noted a statistically significant association of certain underlying diseases and the development of primary septicemia after raw oyster consumption during a case controlled study of *V. vulnificus* infections in Southern Louisiana between 1980 and 1981. Of the nine patients developing *V. vulnificus* induced primary septicemia, more than 6 suffered from previous liver disease, hematopoietic disorder, chronic renal disorder, and or gastric disease. In a review of

Table 5. Other pathologies attributed to infection by *Vibrio vulnificus*. [a,b].

Disease
Spontaneous bacterial pentonitis
Meningitis
Corneal ulcers
Epiglottitis
Myositis
Pneumonia
Osteomyelolitis
Rhabdomyolysis

[a] Excluding primary septicemia and wound infections typically manifested by the organism.
[b] From Kumamoto and Vukich , 1998 (61)

cases reported to the Florida Department of Health and Rehabilitative Services between 1981 and 1992, Hlady et al. (42) noted patients with liver disease had a 63% death rate when compared to victims without pre-existing liver disease. Stelma et al. (95) in 1992 set out to study the virulence characteristics of both environmental and clinical isolates on serum overloaded mice (injected with 250 mg of iron dextran per kg of body weight) as a model for patients with high serum overloads. After finding no significant differences in virulence between clinical and environmental strains, they concluded that host factors (such as iron overload and immunosuppression) to be more important risk factors in lethality than the characterization of strains as virulent or avirulent. They found a much lower LD_{50} ($\geq$3.5 $\log_{10}$ CFU) via intra-peritoneal injection was needed by these iron-overloaded mice versus control mice. Isolates classified as virulent were found to be more resistant to serum complement inactivation than avirulent strains. In addition, the ability to produce phenolate siderophores to utilize transferrin-bound iron was limited to virulent strains. In a 1999 study, Hor et al. (46) further investigated the link between the severity of *V. vulnificus* infection and liver disease as measured by the *in-vitro* post-injection survival/recovery of the organism in whole blood samples of patients with varying degrees of liver disease. Patients were injected with c.a. 10^4 CFU/ml. After a 5 hour incubation period, the number of CFU/ml recovered from blood samples increased with the severity of the donor's liver disease. This number differed significantly between heptoma patients and healthy volunteers. Overall, they found the a positive correlation between recovery of the organism and serum ferritin levels, a negative correlation with serum C_4 concentrations (part of the classical pathway of immunity), and negatively with levels of neutrophilic phagocyte levels. The researchers concluded that once the microorganism enters the bloodstream, it survives better and multiplies faster in persons with low phagocytotic activity (associated with poor hepatic functional reserve) and serum ferritin levels. The researchers concluded that both phagocytotic activity and serum ferritin levels were significant independent predictors of the survival of *V. vulnificus* in the blood and increased host susceptibility to the microorganism.

From epidemiological data, Koenig et al. (59) suggested the normally higher iron levels in over pre-menopausal females may play a role in increased host susceptibility to *V. vulnificus* infection by males. Septicemia cases for males outnumbered females by 4:1 by 1991. Shapiro et al. (86) and the Vibrio Working Group also noted a prominence in male cases of *V. vulnificus* induced septicemia, with 86 % patients of 422 cases reviewed being male. Tacket et al. (99) and Blake et al. (7) also commented on the higher distribution of *V. vulnificus* infection among males. The effects of immunosuppression on lethal dose were studied by Stelma et al. (95) in a 1992 work. The researchers noted a lower LD_{50} dose for mice treated with 150 mg/kg body weight cyclophosphamide (an immunosuppressor) versus control mice. The immunosuppressive characteristics of primary disease on V. vulnificus infection was observed in the case of a child with Diamond Blackfan disease (2). The child fell ill to *V. vulnificus* infection after consuming raw oysters, his immune system weakened by Diamond Blackfan Disease.

ii. Treatment of *V. vulnificus* infections

Treatment for primary septicemia must be early in the diagnosis and aggressive. Studies

have shown that delay in antibiotic treatment is linked to severity of the progression of the disease and increased lethality. In a study of mice given ca. 10^8 *V. vulnificus* (subcutaneously per mouse) the severity of edema increased as the time lapse between injection and tetracycline treatment increased (8). The researchers concluded that 50 μg tetracycline injected subcutaneously at the site of *V. vulnificus* injection prevented death only if given within 2 hours of inoculation. After 18 hours, no mice survived (n = 0/7). Once the organism is detected in a clinical sample (in most cases, fluid from a lesion or a blood sample), antibiotic therapy can be effective. Studies and clinical trials have shown *V. vulnificus* to be sensitive to tetracycline, aminoglycosides, third generation cephalosporins, and ciprofraxin (60). Tetracycline inhibits protein synthesis by selectively binding to tRNA receptor sites on several ribosomal subunits of the microorganism (55). It may also interfere with the action of extracellular enzymes (lipase and protease) (55). This broad-spectrum antibiotic causes dental staining in developing teeth and is therefore not used to treat young patients with *V. vulnificus* infection. In one case involving a 9 year old boy with Diamond-Blackfan syndrome, Albayan et al. (2) recommended treatment for *V. vulnificus* infection with a combination therapy consisting of an aminoglycoside (also a protein synthesis inhibitor) and a third generation cephalosporin or chloramphenicol rather than tetracycline. Current treatment involves administration of intra-venous doxycycline (a synthetic derivative of tetracycline, 100 mg q 12 hr.) and ceftazidimide (2.0 g q 8 hr., a third-generation cephalosporin, a β-lactam antibiotic which disrupts cell wall synthesis) (61,85). Primary septicemia requires supportive care in addition to antibiotic therapy. Septic shock is treated with crystalloid and pressor agents. In addition, vigilance for development of rhabdomyolysis and DIC is recommended (18,19,61,77). Wound infections usually require surgical removal or amputation of the affected area, depending on the severity of the infection (75,19,59).

iii. Laboratory analysis

Examination of shellfish suspected to be responsible for *V. vulnificus* outbreaks is performed by following selective and differential plating procedures described by Elliot et al. (32) in the *Bacteriological Analytical Manual*. Procedures established for the examination of whole shellfish are used to examine the shellfish and interpret results (52).

iv. Isolation of *V. vulnificus*

Vibrio vulnificus grows readily on a wide variety of laboratory media, though their halophilic nature normally requires supplementing growth media with 1-3% NaCl. Selective enrichment involves the use of alkaline broths, such as Alkaline Peptone Water (APW 57, 5,89,32). Glucose-Salt-Teepol was the standard for use and included in the sixth edition of the BAM until teepol was no longer commercially available. HAE (arabinose-ethyl violet based broth) was developed by Horie et al. in 1964. MNS was developed by Monsur in 1963. Salt Polymyxin Broth was used by Hagen et al. (40) to successfully recover *V. vulnificus* from artificially inoculated shrimp, oysters, lobster, crab, and shark. In a comparison of five enrichment broths for the recovery of *V. vulnificus* from oyster samples (APW, MRN, Horie's, Monsur's, and GST), Sloan et al. (91) noted APW with subsequent plating onto CPC (Colystin Polymyxin

Cellobiose Agar) to produces the best results.

Selective and differential plating typically follows inoculation of an MPN three tube series (32). Arguing the 3 tube MPN procedure was imprecise, time consuming, and its reliance on antibiotics at plating reduced recovery, Miceli et al. (69) developed a 2-4 day procedure for isolation and identification of the organism from oyster samples consisting of direct plating on Vibrio Vulnificus Enumeration agar(VVE) with subsequent biochemical tests for identification. This medium is both selective and differential for *V. vulnificus*, with the organism producing blue to greenish blue colonies upon hydrolyzing the chromogenic analog of lactose, X-Gal. Oliver (75) described the appearance of the organism on Blood agar plates as opaque colonies immediately surrounded by a narrow zone of beta hemolysis, in turn surrounded by a larger zone of alpha hemolysis. *V. vulnificus* colonies on Sodium Dodecyl Sulfate-polymyxin B-Sucrose agar (SDS) are approximately 2-4 mm in diameter, are of purple color, and have opaque halos (11,91). This selective and differential medium is touted for its ease of use in the direct isolation of the microorganism from shellfish samples within 24 hours. Sucrose acts as a non-fermentable sugar source for the microorganism (producing the purple color), the antibiotic polymyxin acts as a selective agent, and sulfate allows for detection of alkyl-sulfatase activity, an exocellular enzyme produced by *V. vulnificus* (11,91). Of twenty *V. vulnificus* strains tested, Bryant et al. (11) found that nineteen produced visible opaque halos, indicating alkyl-sulfatase activity. A differential and selective medium, SPS (Sodium dodecyl polymyxin sulfate), was created when researchers combined the knowledge of polymyxin resistance and sodium dodecyl sulfate hydrolysis by *V. vulnificus* (75).

Azanza et al. (4) noted that 0.1% peptone with 3% NaCl (PS) was a better diluent for use in enumerating *V. vulnificus* than PBS. One environmental and one type strain were used to show that PBS dilutions repeatedly gave lower counts than PS dilutions upon subsequent plating onto both TCBS (Thiosulfate Citrate Bile Salts Agar) and PCAN (Plate count agar + 3% NaCl). The counts of diluted cultures held in PBS for 10, 30, 60, and 120 minutes at room temperature (21-25 °C) prior to plating were all lower than the equivalent PS dilutions, with PBS dilutions held for 120 minutes showing a 2 log decline in viable populations. Because *V. vulnificus* does not ferment glucose, the organism produces green colonies on Thiosulfate Citrate Bile Salts plates (TCBS), a medium selective for *Vibrionaceae* (32,75,11,26,102). *V. vulnificus* does not ferment sucrose, the sole sugar source, thereby differentiating itself from sucrose-fermenting *Vibrios* which would produce yellow colonies on TCBS (32,75). The medium Colystin Polymyxin Cellobiose agar (CPC) was developed by Massad et al. (67) in 1987 and is typically used for isolation (32,67,97). This medium takes advantage of *V. vulnificus* resistance to polymyxin and colystin and its ability to ferment cellobiose, allowing the use of CPC as both a selective and differential medium. The organism produces dark yellow colonies from the acidic fermentation of cellobiose. In 1995, Sun and Oliver (98) compared VVE with CPC, confirming the differential usefulness of CPC over VVE. Sloan et al. (91) compared five enrichment broths with plating on two differential agars, finding the combination of enrichment in APW with subsequent plating on CPC agar produced the best results.

v. Rapid methods

Rapid methods for the detection, identification, and enumeration of *V. vulnificus* have been applied and developed based on biochemical (e.g. API 20E system), immunological (anti-H) and molecular (or DNA analysis) techniques. Identification of the organism with API (BioMeriux), France) and micro-ID systems reduced the labor-intensive nature of conventional phenotypic analysis. Researchers can confirm the identity of a presumptively positive *Vibrio vulnificus* with similar systems in approximately 24 hours with mixed success rates.

In 1986, Simonson and Siebeling (88) applied the knowledge that the core of the organism's flagellum contained species-specific H-antigenic determinants to the development of a rapid-serological slide co-agglutination confirmation test for clinical and environmental isolates. They attached and anti-H antibody (produced in rabbits inoculated with *V. vulnificus* core protein) to the Fc receptors on the cell wall of a heat-killed carrier particle (*Staphylococcus aureus* Cowan 1), thus forming a co-agglutination reagent. By placing one drop of the Co-Ag reagent on a glass slide and mixing with a bacterial suspension of *V. vulnificus* in PBS they were able to detect visible agglutination among positive samples within three minutes. Using this technique, Simonson and Siebeling were able to correctly identify 432 out of 435 *V. vulnificus* isolates. ELISA techniques have been developed to enumerate the organism in oyster and environmental samples. In 1994 Parker and Lewis (78) created a sandwich ELISA using rabbit and goat anti-*vvh* gene (sequence coding for cytolysin/hemolysin *vvc*) antibodies for detection of *V. vulnificus* in environmental samples. Of 340 presumptive *V. vulnificus* colonies, their assay correctly detected 95 % of the confirmed colonies while also not reacting with 99% of the non-*V. vulnificus* colonies which would otherwise produce a high percentage of false positives. The specificity of the assay allows the researcher to directly enumerate the organism from food and environmental samples, though the use of radio labeling places constraints on wide usage in laboratories. The most widely accepted for confirming *V. vulnificus* isolates is the method developed by Tamplin (102) which uses monoclonal antibody FRBT37 in an ELISA assay. Gene probes and GC analysis of fatty acid composition are also confirmation methods accepted by)the FDA (32).

Upon characterizing *vvc* and its encoding gene sequence, Wright et al. (112) developed an alkaline-phosphatase-labeled oligonucleotide probe specific for the organism. With the probe and plating onto non-selective L agar the researchers were able to detect and enumerate viable *V. vulnificus* cells in seawater and oyster homogenates in less than twenty-four hours. By using an enzyme label rather than a ^{32}P-label for the oligonucleotide, the researchers expended the usability of the probe to facilities not able to use radioactivity. One important limitation of using the AP-*vvh*A probe is its inability to detect VBNC cells which would pose a threat to susceptible consumers if the oysters were temperature abused after cooling (112). Keysner et al. (56) in 1993 modified existing hydrophobic grid membrane filtration techniques (HGMF) by using a ^{32}P radiolabeled whole plasmid probe specific to the same *V. vulnificus* cytotoxin-hemolysin gene for overnight enumeration of the microorganism from spiked samples through DNA-DNA colony hybridization. This four day procedure gives a theoretical detection level of 100 CFU/g in a

complex and particulate sample. The use of a species-specific probe allowed the researchers to distinguish the organism from the closely-related and much less pathogenic *V. parahaemolyticus*.

Several Polymerase Chain Reaction (PCR) techniques have been developed since 1993. To date, PCR amplification techniques have been limited to *in-vitro* whole cell lysate applications because of possible interference encountered between oyster particulates in a homogenated sample and the *Taq* polymerase enzyme used in PCR techniques. Inhibition of *Taq* would interfere with amplification of the target gene sequence (112). Wright et al. (112) suggested the development of a 24 hour direct enumeration technique based on nonselective plating in concert with PCR amplification of the *V. vulnificus* cytolysin gene. Brauns and Oliver (10) in 1994 amplified the same *vvh* gene utilized in Parker and Lewis' assay by using whole cell lysates of a type strain as the source of the DNA primer templates. Using this technique, they were able to detect one *V. vulnificus* cell *in vitro*. Anono et al. (3) compared the usefulness of PCR analysis (*vvh*A oligonucleotide probe) with phenotypic evaluation (API 20E, BioMeriux) for identifying *V. vulnificus* isolates from biological samples (seawater, sediment, oysters, and goby) citing PCR analysis was able to correctly identify phenotypically different *V. vulnificus* isolates based on their homology to the *vvh*A gene oligonucleotide.

CHAPTER III. LITERATURE CITED

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CHAPTER IV. The Effect of Refrigerated Storage on the Safety and Quality of Raw Oysters (*Crassostrea virginica*)

Manuscript formatted for publication in the Journal of Food Protection

A. TITLE PAGE
The effect of storage on oysters...

The Effect of Refrigerated Storage on the Safety and Quality of Raw Oysters (*Crassostrea virginica*)

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Effect of storage on oysters...

Key Words: oyster, *Vibrio vulnificus*, bacteria, sensory, spoilage

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B. ABSTRACT

Temperature abuse during raw oyster harvesting and storage may allow for the multiplication of natural spoilage flora as well as microbial pathogens; thus posing a potential health threat to susceptible consumers and/or compromising product quality. The objective of the study was to provide a scientific basis for determining whether different refrigeration temperatures for raw oysters will result in a spoiled product before it becomes unsafe. Raw shellstock oysters (*Crassostrea virginica*) purchased from a commercial Virginia processor were subjected to different temperature abuse conditions (7°C, 13°C, and 21°C) over a ten day storage period. Salinity, pH, HPC (halophilic plate count), total *Vibrio* counts, and *V. vulnificus* counts were determined at each abuse condition. *V. vulnificus* isolates were confirmed by a specific ELISA. Olfactory analysis was performed in order to determine consumer acceptability of the oysters at each abuse stage. The pH of the oysters decreased over time in each storage condition. HPC increased while olfactory acceptance decreased over time. The length of storage had a greater effect on the bacterial counts and olfactory acceptance of the oysters ($p < 0.05$) over time than did the storage temperature ($p < 0.05$).

C. INTRODUCTION

Commercial landings of the eastern molluscan bivalve, *Crassostrea virginica*, were estimated at 80,000 metric tons from 1990 through 1996 harvests, generating over $550 million (1). Once harvested, the oysters remain alive as shellstock for approximately two weeks under refrigeration (5). The National Shellfish Sanitation Program (NSSP) requires interstate shipments of shellstock to be made under mechanical refrigeration, at or below 7.2°C (45°F) (13).

Exceeding the required temperature during transport and storage can lead to chemical decomposition of tissue glycogen as well as growth of microorganisms. The filter feeding nature of oysters and the quality of surrounding growth waters determine their natural flora (19). Organisms typically found include spirochete commensals, yeasts, viruses, Gram-positive rods and cocci, and Gram-negative rods (including members of the family *Vibrionaceae*) (5,4,10,15,19). *Vibrio vulnificus* is of concern to susceptible individuals. This facultatively anaerobic halophilic curved rod is highly invasive once ingested, producing a hemolysin cytotoxin and albuminases which help the organism quickly penetrate the intestinal mucosa (17). Researchers estimate the minimum infective dose for healthy individuals to be approximately 10^4 CFU/g (6,17). The minimum infective dose for immunocompromised individuals has not been determined (6). Data from the Centers for Disease Control (CDC) links ingestion of *V. vulnificus* laden raw oysters to fatal primary septicemia and gastroenteritis in individuals with pre-existing conditions such as elevated serum iron levels (3). No clinical reports have linked *V. vulnificus* infections to consumption of cooked seafood, suggesting the organism's upper temperature tolerance is similar to other non-sporeforming Gram-negative rods. Cook and Ruple (9) estimated the D_{50} value of *V. vulnificus* in oysters at 39.8 s, emphasizing the role of adequate cooking in preventing foodborne *V. vulnificus* infection.

Spoilage in oysters is primarily fermentative and involves the breakdown of glycogen, an energy storage molecule found in high concentrations in the tissues. Accumulation of lactic acid in the tissues results in a drop in pH, making the environment favorable for the proliferation of *Lactobacilli*, *Streptococci*, and yeasts (2,5,15). The relationship between the pH of a homogenate of shucked meat and oyster liquor and spoilage has been used as a basis for evaluating wholesomeness. Samples with a pH range between 5.6 and 6.1 are considered to be passing from "good" to "stale", those between 4.9 and 5.3 considered "stale" to "sour" or "putrid", and those with a pH below 5.0 are considered to be in a state of "advanced decomposition" (15).

It has been suggested that refrigerated storage of raw oyster shellstock be a CCP in industry HACCP plans. A CCP is defined as a step at which control can be applied and is essential to prevent, eliminate, or reduce a food safety hazard to an acceptable level. Flourishing of *V. vulnificus* prior to outgrowth of natural spoilage flora would produce an unsafe product, whereas predominant growth of natural spoilage flora would yield spoiled oysters. The former is clearly a safety issue while the latter a quality issue. Due to the health threats *V. vulnificus* poses and its natural occurrence in raw oysters, it is of importance that researchers provide a scientific basis for determining whether or not different refrigeration temperatures for raw oysters will

result in a spoiled product before it becomes unsafe.

D. MATERIALS & METHODS

1. Oyster samples

Raw shellstock (*Crassostrea virginica* freshly harvested from the Yeocomico River) purchased from a commercial processor in the Northern Neck region of Virginia were transported in a chilled Styrofoam ice chest to the laboratory at Virginia Tech (VPI&SU) in Blacksburg, VA within 24 hours of harvest. Live oysters were washed under cold running tap water, sorted into storage lots (approx. 200 whole shellstock), placed into open shallow plastic tubs, and stored at 7°C, 13°C, and 21°C within incubators (Precision Scientific Low Temperature Incubator model 815, Fisher Scientific Low temperature Incubator model 307). Only live oysters were used for the study. Microbiological and sensory analyses were performed on days zero (control lot, immediately sampled upon arrival at the laboratory), one, three, six, and ten. The parameters observed were pH of an oyster homogenate, the salinity of river from where the oysters were harvested, storage temperature, HPC, total *Vibrio* count, *V. vulnificus* count, and olfactory acceptance of the raw samples.

2. Microbiological evaluation

Microbiological evaluation of the raw oysters followed the work of Elliot *et al.* (11). Ten to fifteen oysters randomly selected from one storage lot were aseptically shucked, with intact bodies and liquor placed into a sterile stomacher bag (Seward model 400, 7" x 12", London) and massaged through the bag by hand for 1 min. Fifty g meat and liquor was measured (Metler-Toledo model BB 3000, NJ), poured into the sterile glass jar of a Warring Blender (Warring, model 91-203 306, CN), and homogenized for 2 min with 450 ml sterile phosphate buffered saline (PBS pH 7.4 (11)). Serial dilutions were made in sterile 9 ml PBS blanks which were used to inoculate Trypticase Soy Agar + 2.5% NaCl plates (for HPC) (TSA, Difco # 0369-17-6, MI) and a 3 tube MPN series (Most Probable Number, 10ml aliquots of alkaline peptone water APW, pH 8.5 (11). TSA plates were read for halophilic standard plate count (HPC) after 48 hr incubation at 35°C. After 24 hr incubation at 35°C, 1 loopful from the top inch of fluid of each APW tube was streaked onto Thiosulfate Citrate Bile Salts agar (TCBS [11] Difco # 0650-17-4, MI) and onto modified Cellobiose Polymyxin Colistin agar (mCPC [11]). After 24 hr incubation (35°C and 39°C, respectively), TCBS plates were checked for any growth to indicate growth of total *Vibrios* and mCPC plates were checked for growth of yellow colonies indicating the presumptive positive growth of *V. vulnificus*. Yellow (cellobiose positive) isolated colonies from mCPC were subcultured onto long term storage medium and confirmed as *V. vulnificus* isolates using the ELISA procedure described by Tamplin et al. (19). Growth and confirmation data were combined with MPN results to enumerate total *Vibrios* and *V. vulnificus* present in the raw shellstock.

The above procedure was followed to examine shellstock samples (40 - 60 oysters per time and temperature treatment for a total of 520 - 780 samples per study) from all storage lots during the ten day study. The study was replicated a total of five times (for a total of 2600 - 3900

oysters sampled). For each replication, sample analysis was performed in duplicate.

4. Sensory evaluation

Olfactory evaluation was performed by an untrained panel of consumers of raw shellstock oysters (n = 51 (males = 20, females = 31), ages = 22 - 60) recruited from the Blacksburg campus of Virginia Tech. Each panelist was provided with one live shucked oyster and its liquor which was placed into a sterile plastic Petri dish (Fisher Scientific #08-757-12, PA) for each treatment. Samples were coded with randomly generated 3-digit numbers and presented to panelists in random balanced order under fluorescent light at room temperature. Panelists were asked to evaluate the samples by smell only, then indicate on scorecard provided whether or not they would consume each sample raw.

5. Statistical analysis

Data analysis was performed by J. Huffman and D. Eno (Virginia Tech Dept. of Statistics). GLM by SAS (SAS Institute Inc., Cary, NC) was used to evaluate trend analysis and variable effect ($\propto$ = 0.05 %) in the microbiological observations and Pearson Correlation Analysis was used to evaluate the sensory data ($\propto$ = 0.05 %). Values for microbiological counts are represented as geometric means.

E. RESULTS & DISCUSSION

Quality & pH. Oysters at and below pH 5.0 are considered spoiled and therefore unfit for consumption. As seen in Table 1, the pH decreased steadily over time; consistent with the work of Hunter and Linden (15), who found the pH of shucked oysters stored in their liquor dropped with time and storage temperature. A 0.14 pH unit drop was observed in oysters stored at 7 °C between day zero and day 10. A more dramatic drop was observed in those stored at 21°C, with an approx. 0.30 pH unit decrease. The pH of the shellstock samples never fell below an average pH of 5.9, even for samples stored at 21 °C for 10 days. The pH of the most temperature abused oysters would prompt researchers to classify them as “good”, even though HPC and most importantly *V. vulnificus* reached elevated levels (Figs. 1 & 3).

Halophilic microflora. Halophilic microflora (HPC) of the shellstock increased approximately 2 - 4 logs for all temperature conditions during the ten day storage period (Fig. 1). HPC values increased as time and temperature increased ($p<0.05$). Hood et al. (14) noted a similar effect on total bacteria (SPC) in both shellstock and shucked oysters stored at selected temperatures (2, 8, 20, and 35°C). Cook (7) showed that APC (grown in PCA supplemented with 1% NaCl to support halophilic growth, incubated at 25°C) increased over time in shellstock oysters exposed to ambient temperatures after harvest. The effect of the variable time on halophilic flora and pH was found to be statistically significant ($p<0.05$). The combined effects of variables time and storage temperature on HPC were not statistically significant ($p>0.05$). Trend analysis showed a quadratic effect between log HPC and time ($p = 0.05$).

Total Vibrios. Total *Vibrio* levels rose over time as the storage temperature increased (Fig. 2). Cook and Ruple (8) noted a similar rise in *Vibrio* levels in postharvest shellstock oysters stored above 10°C (22 and 30°C), but reported no increases at 10°C during a 5 day sampling period. The present study showed no apparent decrease in *Vibrionaceae* levels, though a 1 - 2 log decrease was observed on day six in oysters stored at 21°C, followed by a spike to ca. 7 - 8 logs. The highest level in *Vibrionaceae* counts was reached on the tenth day of 21°C storage. *Vibrio* levels were held at approx. 5 logs or below in shellstock held at both 7 and 13°C, indicating that even moderate temperature control has the ability to keep *Vibrionaceae* levels low. The effect of time on *Vibrio* growth was statistically significant ($p<0.05$) and the combined effect of time and temperature were not ($p>0.05$), although the effect of temperature in this case was found to be statistically significant ($p<0.05$). Trend analysis indicated a quadratic relationship between log *Vibrio* and time.

***Vibrio vulnificus*.** Levels of *V. vulnificus* increased as both storage time and storage temperature increased (Fig. 3. The ability of the microorganism to flourish within oysters after harvest and during controlled storage has been documented (6,7). Cook (6,7) found levels of *V. vulnificus* to be significantly greater in oysters held at both 18°C and ambient temperatures than in oysters sampled at harvest, leading the researcher to suggest prompt refrigeration after harvest be used as a means to prevent the postharvest growth of the pathogen. Storage at 21°C produced the greatest rise in *V. vulnificus* levels, with the pathogen reaching 10^4 CFU/g by day six and ~$10^{7.5}$CFU/g by day ten. Researchers have reported the pathogen declines in number during prolonged storage on ice and at 18°C (7,8). This was not observed in the present study, rather *V. vulnificus* levels appeared to rise over time and with storage temperature, though a few 0.5 to 1 log decreases were observed on day three in samples stored at 13 and 21°C. The declines were followed by a quick 1 log rise by the sixth day. Storage of shellstock at 13°C kept *V. vulnificus* levels around 4 logs while storage at 7°C kept levels at 3.5 logs or below. This suggests cool storage (~7°C) is adequate at keeping levels of the pathogen to low levels. The effect of temperature on the growth of *V. vulnificus* was statistically significant ($p<0.05$), whereas the effect of time was not ($p=0.07$).

Sensory analysis. Figure 4 illustrates the results of olfactory evaluation during the storage period. All microbiological variables displayed negative Pearson correlations to storage time (~ -0.3), indicating that as storage time increased the olfactory acceptability of the shellstock decreased. This confirms the results obtained by Liuzzo and Novak (16). The researchers showed that oyster samples with elevated bacterial counts (in their case both psychrotrophic and mesophilic counts) were negatively correlated to organoleptic quality. Since all bacterial counts obtained in the present study increased over time and with storage temperature, the observation that olfactory acceptance decreased accordingly was of no surprise. Elevated *V. vulnificus* levels were observed in samples held at both 13 and 21°C before 100% sensory rejection was obtained (Table 1). Elevated storage could pose a significant health hazard to all consumers since even samples held at 13°C reached the inoculum level for the pathogen. These findings suggest that simple olfactory analysis of raw shellstock may not be an adequate means to prevent oyster-associated *V. vulnificus* infections.

Halophilic Plate Count (HPC) outgrew *V. vulnificus* by a minimum of 1 log at all storage conditions. All microbiological counts increased logarithmically over time and storage temperature. Many researchers have noted the same effect of temperature and time in oysters stored as both shellstock and shucked (6,9,12). The pH of shellstock oysters never fell below 5.9, indicating the oysters were still considered to be of acceptable quality even though levels above 10^4 *V. vulnificus*/g were reached in samples stored at 21°C for the duration of the study. Olfactory acceptability and storage time were negatively correlated. Olfactory acceptability was below 40% when *V. vulnificus* growth was at its highest. It is the opinion of the author that refrigerated storage should be considered a CCP for raw shellstock. Storage of samples at 7°C held *V. vulnificus* levels below 10^4 CFU/g, the inoculum level for healthy consumers established by researchers (4,17). Storage of shellstock at elevated temperatures allowed for the proliferation of the pathogen without altering the chemical quality (as evaluated by pH) of the oysters. Because both pH levels and olfactory acceptability of oysters stored at elevated temperatures fell within the acceptable range, they could have the effect of masking elevated *V. vulnificus* levels, thus posing a heath threat to healthy consumers. Researchers indicate the minimum inoculum level of a pathogen for an immunocompromised individual can be 2-3 logs lower than that for a healthy individual. If this is so, one may conclude that the level found in all oyster samples could pose a potential health threat to susceptible individuals. Statements alerting susceptible individuals to the possible health risks of consuming raw oysters should keep oyster-associated *V. vulnificus* infections to low levels.

F. ACKNOWLEDGEMENTS

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H. TABLES AND FIGURES

Table 1. pH change of oyster shellstock during ten day storage study [a]

Storage time (days)	Storage temp (°C)	average pH
0	-	6.27 ±0.02
1	7	6.27 ±0.02
	13	6.22 ±0.02
	21	6.14 ±0.04
3	7	6.22 ±0.08
	13	6.21 ±0.13
	21	6.11 ±0.12
6	7	6.17 ±0.01
	13	6.09 ±0.04
	21	5.97 ±0.04
10	7	6.13 ±0.08
	13	6.05 ±0.08
	21	5.90 ±0.06

[a] average salinity of harvest waters = 9.1 ± 2.7 ppt, provided by Virginia Department of Health Division of Shellfish Sanitation

Fig. 1 - Halophilic Plate Count (HPC) over ten day storage study. Oysters stored as shellstock at 7 (**F**), 13 (**G**), and 21°C (Δ).

Fig. 1. top

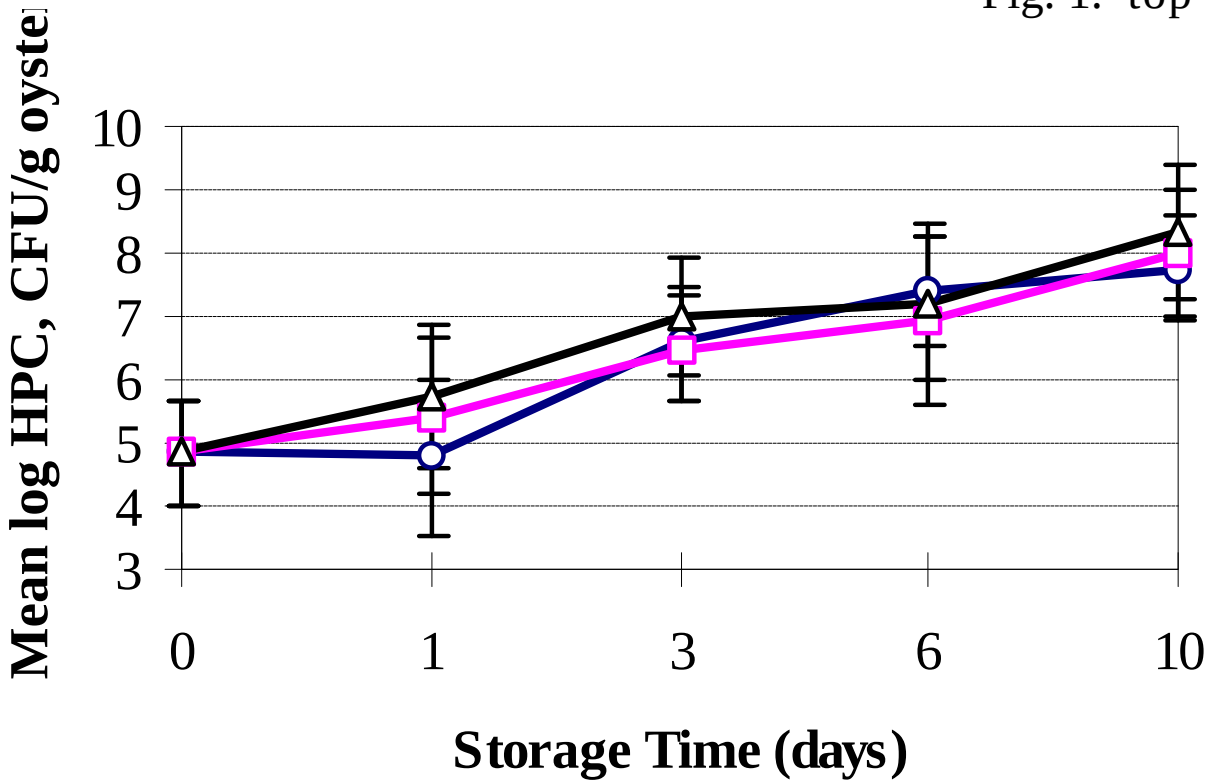


Fig. 2 - Growth of total *Vibrios* during 10 day storage study. Oysters stored as shellstock at 7 (**F**), 13 (**G**), and 21°C (Δ).

Fig. 2. top

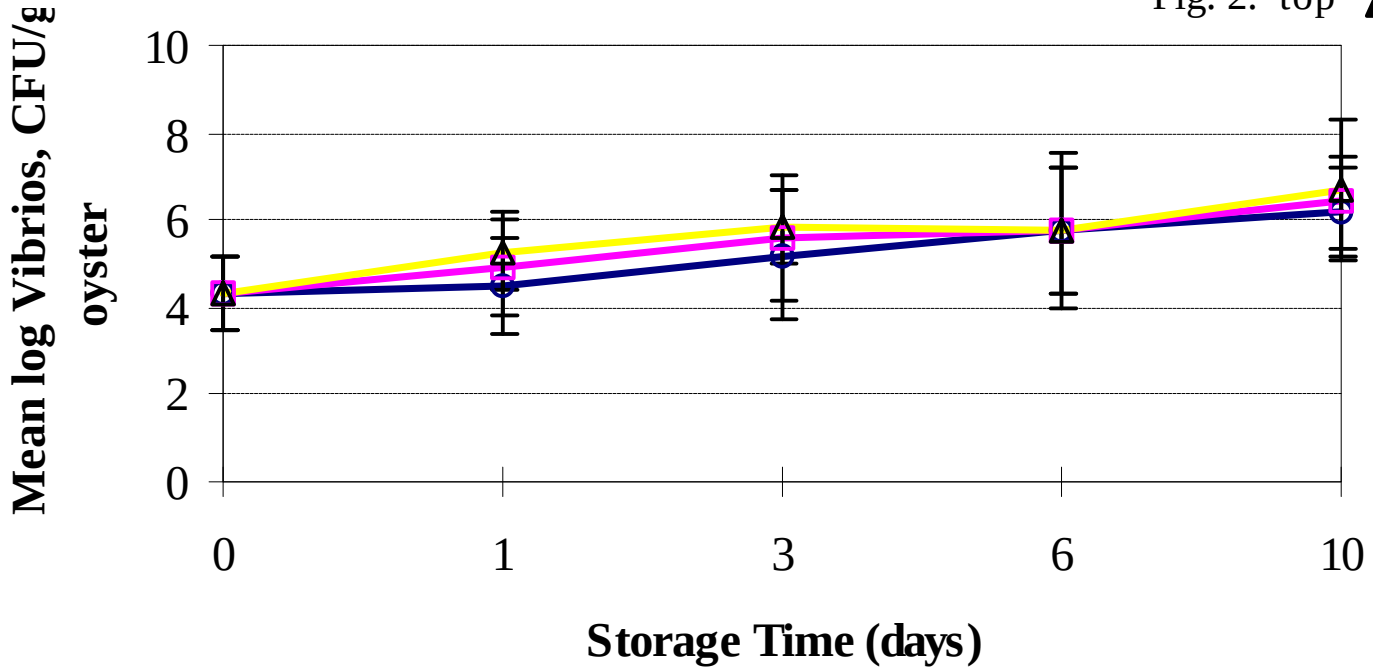

Mean log Vibrios, CFU/g oyster
10
8
6
4
2
0
0
1
3
6
10
Storage Time (days)

Fig. 3 - Growth of *V. vulnificus* during 10 day storage study. Oysters stored as shellstock at 7 (**F**), 13 (**G**), and 21°C (Δ).

Fig. 3. top

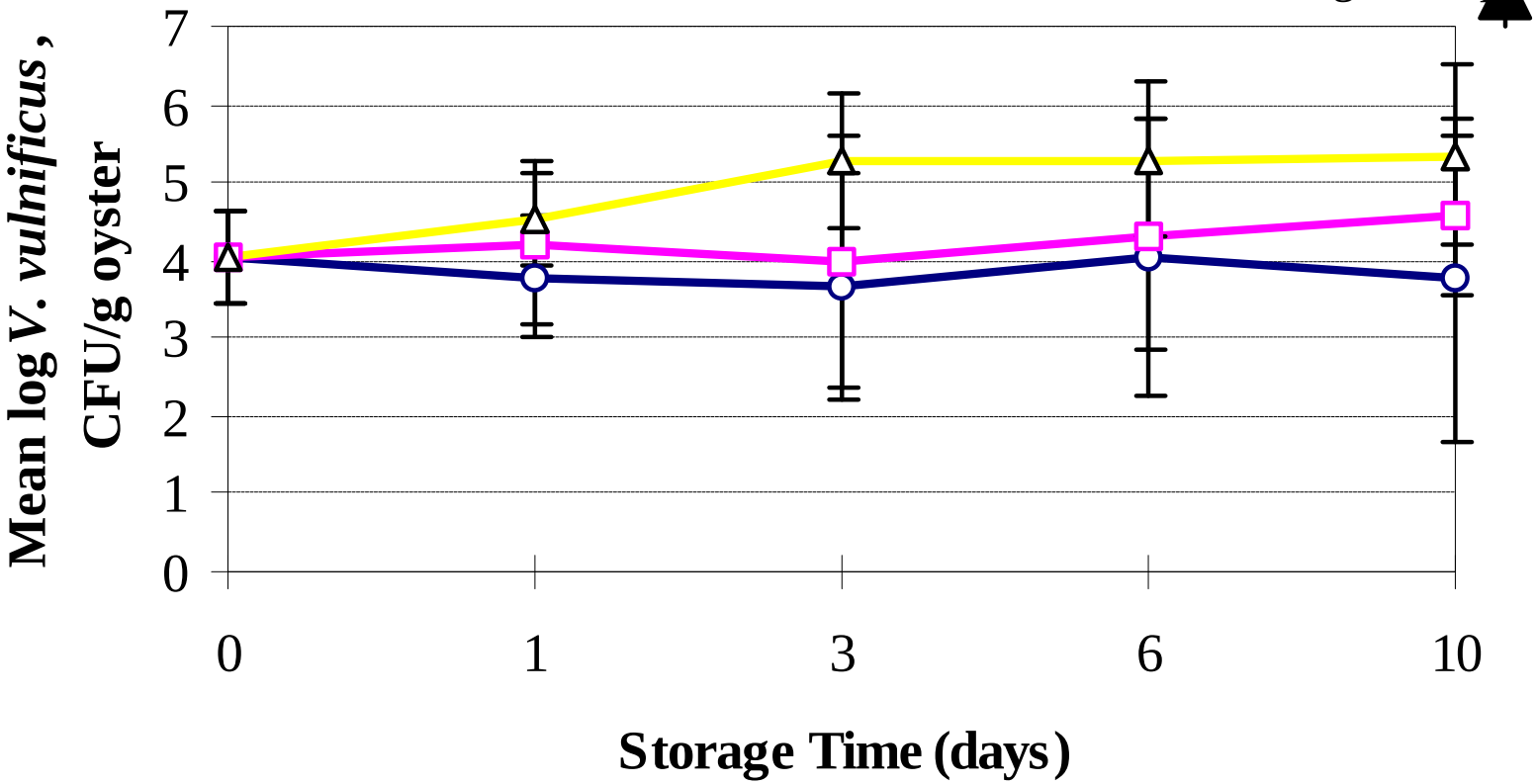
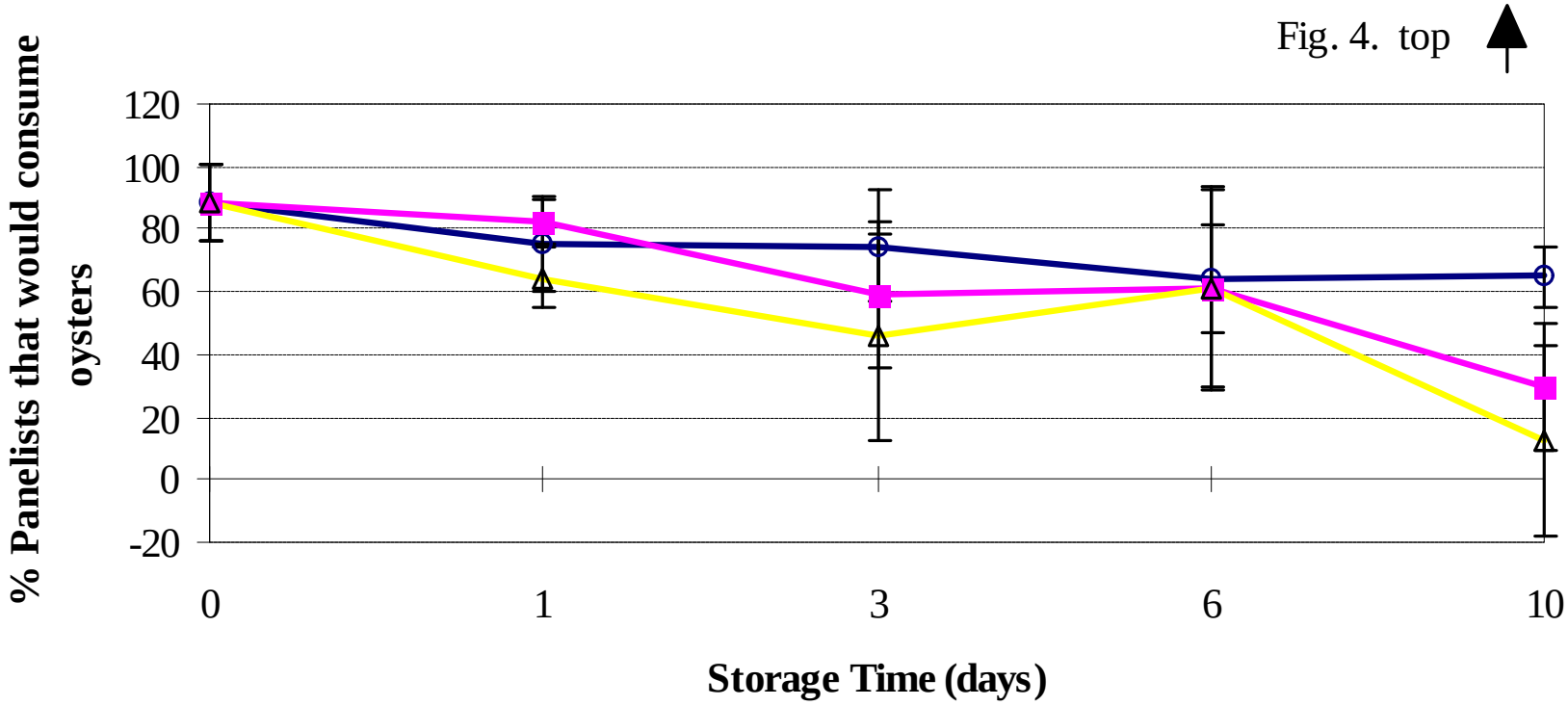

Mean log V. vulnificus, CFU/g oyster
7
6
5
4
3
2
1
0
0
1
3
6
10
Storage Time (days)

Fig. 4. Organoleptic acceptability of oysters under controlled conditions during ten day storage study. Oysters stored as shellstock at 7 (**F**), 13 (**G**), and 21°C (Δ).

Fig. 4. top


% Panelists that would consume oysters
120
100
80
60
40
20
0
-20
0
1
3
6
10
Storage Time (days)

Part 2. Role of Temperature on the Safety and Quality of Raw Bluefish (*Pomatomus saltatrix*)

CHAPTER V. ABSTRACT

The production of histamine and the competitive growth of *Morganella morganii* (a known histamine former) as a function of time and temperature was observed in fresh bluefish fillets. Bluefish fillets inoculated with *Morganella morganii*, were examined daily during an eight day storage period at 5,10, and 15°C. Microbial isolates from *M. morganii* inoculated and uninoculated fillets were screened for histidine decarboxylase activity and identified. Olfactory acceptance was performed by an informal sensory panel. Histamine levels were quantified via HPC and fluorescence detection. Histamine concentration and bacterial counts increased while olfactory acceptance decreased over time. The storage temperature had a statistically significant effect on the histamine levels, bacterial counts, and olfactory acceptance of the bluefish over time. Fillets inoculated with *M. morganii* developed significantly more histamine over time than did uninoculated fillets *(p. <0.0001)*.

CHAPTER VI. INTRODUCTION

Scombroid (or histamine) poisoning is a benign self-limiting foodborne illness responsible for substantial economic losses to the seafood industry. Bluefish (*Pomatomus saltatrix*) have been implicated in histamine poisoning outbreaks. The free histidine found in large concentrations in the dark muscle is susceptible to bacterial decarboxylase activity once sufficient levels of these bacteria colonize the exposed surface of the muscle. These enzymes decarboxylate free histidine into histamine, leading to a large accumulation of the toxic byproduct in the fish. The Food and Drug Administration (FDA) has established an advisory level of 50-ppm. to be hazardous to human health (4). It is at and above this level that humans begin to experience the allergy-like symptoms of the disease (6). Histamine is heat stable and is not detectable through organoleptic analysis by even trained panelists (5). Control of histamine formation must therefore be achieved through suppression of the outgrowth of microorganisms capable of decarboxylating histidine. The following research was undertaken to examine the growth of *Morganella morganii* (a known histamine former) and its ability to produce histamine in fresh bluefish fillets when stored under temperature abuse conditions. In addition, histidine decarboxylating microorganisms were isolated and identified from uninoculated samples.

CHAPTER VII. REVIEW OF THE LITERATURE

A. Scombroid poisoning

Scombroid (or histamine) poisoning is a short-lived and usually benign food-borne chemical intoxication typically associated with the consumption of food products containing large levels of histamine (38,54,60). The predominance of spoiled members of the family *Scombridae* and *Scombersociadae* among fish implicated in reported cases supports the name scombroid poisoning for this allergy-like chemical intoxication, though the frequency of incidents involving non-scombroid fish or other non-fish foods ultimately rules it a misnomer. Hughes and Merson (22) classified the illness as an ichthyosarcotoxism (a fish poisoning) caused by bacterial action on the fish flesh.

Though no deaths linked to histamine poisoning have been reported, the illness does produce a serious economic impact to fish processors and distributors (38). These parties must trace and then destroy entire lots implicated in outbreaks. Not only is the immediate loss of product a great expense, but suppliers and processors lose the confidence and business of customers with each outbreak.

1. Histamine

Histidine decarboxylase

Histidine ➜ Histamine

Dietary histamine (not associated with normal immune reactions) is formed through enzymatic degradation of the amino acid histidine by either endogenous or exogenous enzymes in a food system. Histidine is converted to histamine through the enzymatic release of a carbonyl group. As exogenous enzymes, bacterial decarboxylases play a much larger role than endogenous enzymes in the formation of histamine.

The role of histamine in foodborne intoxications like scombrotoxicosis was not immediately apparent to researchers until the late 70's. Prior to that, they searched for the presence of unique toxins in fish implicated in outbreaks as possible culprits of the disease even though the symptomology and treatment of the disease were consistent with physiological reactions to histamine. Early doubts regarding the biological action of orally administrated histamine were planted by Weiss et al. (64) in 1932 as they claimed to observe no direct effect (change in pulse rate, blood pressure, or facial flushing) in five healthy subjects given large levels of histamine phosphate (200 - 500 mg). Studies by Kawabata et al. (26) revealed larger concentrations of a previously unknown vagustimulant (termed saurine) than histamine in foods implicated in scombroid poisoning outbreaks. The researchers then concluded that saurine was the etiologic agent, rather than histamine. The work of Foo et al. (16) brought the role of histamine back to the forefront in when the researchers characterized saurine as a phosphate salt of histamine through paper chromatography and vagastimulation of guinea pig ilium. The

researchers then suggested for the first time that histamine poisoning was brought about by the synergistic action of histamine and another unidentified compound. Motil and Scrimshaw (37) further solidified the role of exogenous histamine in scombrotoxicosis with their 1979 tests on healthy human volunteers. Upon feeding subjects grapefruit juice or tuna fish sandwiches spiked with measured levels of histamine (0, 100, 150, and 180 mg/ 100g or ml sample), the researchers found the symptoms (severe headaches and facial flushing the most commonly reported) exhibited to be consistent with scombroid poisoning in subjects who consumed spiked samples.

a. Detoxification of dietary histamine

Low levels of dietary histamine are easily detoxified by the body through enzymatic breakdown of the amine. The majority of these enzymes are found in the intestinal tract of warm blooded animals. The oxidative deamination pathway is regulated primarily by the enzymes diamine oxide (DAO), monoamine oxidase (MAO), and histamine-N-methyl-transferase (HMT) (54). Histamine is converted into N^+-methylhistamine by HTM. MAO then converts N^+-methylhistamine into N^+-imidazoleacetic acid. DAO directly converts histamine into imidazoleacetic acid, which is in turn conjugated to ribose prior to excretion. The end products of histamine metabolism are excreted into the urine (54).

b. Potentiation of histamine by other chemicals

Augmentation of the toxicity of histamine by other chemicals has been suggested by many researchers in an effort to explain the number of studies showing the low toxicity of plain histamine in man. The presence of other biogenic amines in addition to histamine led researchers to test the additive effects of these biogenic amines and histamine. In studies published in 1978, Bjeldanes et al. (8) suggested the amino acid cadaverine may act synergistically with histamine to produce the symptoms observed in victims of scombroid poisoning. They noted the simultaneous administration of histamine (150 mg/kg body weight) and relatively low levels of cadaverine increased the LD_{50} of guinea pigs by a minimum of 30%, when compared to the LD_{50} resulting from the administration of histamine (150 mg/kg, $LD_{50} = 0$) or cadaverine (500 mg/kg, $LD_{50} = 0$) alone. Controlled decomposition studies by Frank et al. (17) in 1985 indicated the formation of cadaverine and putrescine in addition to histamine in mahimahi (*Coryphaena hippurus*) loin tissue. Studies by Clifford et al. (11) led the researchers to conclude that histamine alone was the causative agent in scombrotoxicosis as they observed no significant biological effects (both instrumental and subjective) on healthy human volunteers fed fresh mackerel spiked with varying levels of exogenous histamine or spoiled mackerel from a batch implicated in a scombrotoxicosis outbreak (containing 300 mg histamine/100 g fish)

Chemical agents with histaminase activity interfere with the body's natural histamine detoxification mechanisms. Histaminases like isonazid have the effect of compromising these natural reactions, leaving those undergoing treatment with such agents vulnerable to histamine poisoning from sources with otherwise benign histamine levels (24).

2. Foods implicated

Scombroid fishes are typically implicated as the agent causing production of histamine in cases of scombroid poisoning (38,54). The high level of free histidine in their dark muscle is susceptible to bacterial decomposition and thus to an accumulation of histamine. The formation and accumulation of histamine in whole fish is uneven. Lerke et al. (28) showed levels varied along the length of spoiling tuna with a greater concentration near the abdominal cavity in a 1978 examination of sashimi implicated in a 1977 outbreak.

Implication of non-fish foods such as cheeses in histamine poisoning outbreaks led researchers to apply analyses designed for the examination of fish to survey retail foods for histamine content and their potential threat to human health. For example, Chambers and Staruszkiewicz (9) evaluated histamine levels in retail cheeses in the Washington D.C. area in 1978. Using AOAC fluorometric methods, the histamine content for eight types of commercially available cheeses was determined. Table 1 provides a selected list of fish and Table 2 non-fish foods implicated in cases of histamine poisoning**.**

3. Regulatory guidelines

The Food and Drug Administration (FDA) (4) has established 50 mg histamine / 100g fish (500 ppm) to be hazardous to human health, though levels of 20 mg/100g fish (200 ppm) have been reported to produce symptoms consistent with histamine poisoning (6). In 1982, the Defect Action Level of 20 mg/100 g scombroid fish product was established to indicate mishandling of the product. The Defect Action Levels in fish depend on the presence or absence of accompanying off odors, with 10 mg histamine/ 100 g(100 ppm) fish and 20 mg/100 g (200 ppm) fish, respectively (25). Due to the typically uneven distribution of histamine in fish, the FDA set a guidance level of 5 mg/100 g fish in 1996 (4,28). If the guidance level is found within part of a sample, it is assumed other parts of the fish will likely contain levels of 50 mg/100 g (500 ppm) or above.

4. Disease: symptoms

Taylor places the symptoms into four categories: cutaneous, gastrointestinal, hemodynamic, and neurological (54,60). These symptoms are brought about by the body's immunological response to the metabolic byproduct of the amino acid histidine. Rashes, swelling (edema), and hives (urticaria) constitute cutaneous infections. Histamine acts to dilate small blood vessels and capillaries as well as stimulating the contraction of larger vessels, thus producing cutaneous symptoms. A 1932 study revealed an intravenous injection of 0.01 - 0.03 mg histamine phosphate per kg of body weight was the minimal single dose required for an adult male with a normal circulatory system to display facial flushing (64). Nausea, vomiting, diarrhea, and abdominal cramps are included in the gastrointestinal symptoms brought about by the induction of smooth muscle contraction. Hemodynamic (hypotension) and neurological symptoms (including headache, palpitations, tingling, flushing, itching, and a burning sensation in

Table 1. Fish implicated in histamine poisoning cases.

Fish	Histamine levels / 100 g fish	Reference
tuna (including canned)	583-728 mg	31 2, 13, 16, 21, 65
skipjack tuna		2, 5, 7,8,34,65
mackerel (including smoked)	>200 mg	56 6
anchovies		15
bluefish	250 mg	43,57
processed Moroccan		44
bonito		21, 34
sailfish		30, 36
mahimahi	~ 50 mg (50-160 range)	31 17
salmon (including canned)	<1 mg & 17mg	56
sardines		25, 28
herring, kipper, sprat, sild		56
gefilte fish		56
herring & cod roe		56
huss		56
cod		56
halibut		56

Table 2. Non-fish foods implicated in histamine outbreaks.

Food	Reference
Extra sharp cheddar	24
salchichon / ripened sausage	46, 47
sauerkraut	35
crab	56
Swiss cheese	58

the mouth) are associated with the stimulant action of histamine on nerve endings and the dilation action on capillaries and blood vessels. The histamine-induced dilation of small blood vessels and capillaries produces headaches and flushing, while the action of the chemical on the heart produces palpitations. In a 1987 review of 258 suspected cases of histamine poisoning reported to disease surveillance agencies in England (Food Hygiene Laboratory and the Communicable Disease Surveillance Centre) between October 1976 and December 1986, Bartholomew et al. (6) summarized symptoms reported in 101 confirmed cases where >5 mg% histamine was detected in the fish samples. Among the cutaneous, 45% reported a bright red rash, 3% swelling of the lips (edema), tongue, or face. Forty-five percent of patients complained of diarrhea, 30% of vomiting, 27% nausea, and 17% reported stomach pains among the gastrointestinal symptoms. Among neurological symptoms, 38% of the patients felt flushed and experienced excessive perspiration, 37% complained of headache, 24% experienced burning sensations in the mouth, 7% felt dizzy, 3% reported palpitations, 2% shaking and shivering, 1% tingling, and 1% chest pain. A ten person outbreak was traced to baked Pacific amberjack fish (a.k.a. yellowtail and kahala) prepared in three restaurants in Alabama and Tennessee between December 1985 and January 1986 (50). DA analysis of 20 pounds of amberjack collected from area restaurants by the distributor revealed 257-430 mg% histamine in 19 or 20 subsamples. Four of the ten victims gave detailed descriptions regarding the nature of their symptoms, all characteristic of histamine poisoning. Twenty-three minutes was the median onset of illness following consumption or handling the amberjack, with a median duration of 14 hours. Three victims sought medical attention. All suffered from a facial or body rash lasting a median of 3 hours. Among these included one restaurant cook who developed a rash on the hands after handling the fish during preparation; half of the victims complained of severe headache; one victim experienced oral tingling (paresthesias); half of the victims shortness of breath; one had vomiting; and three had diarrhea (50).

The specific pharmacological effects of histamine dictate the symptoms experienced by victims of histamine poisoning. Shalaby (49) summarized the effects in a 1996 publication. Histamine has been shown to stimulate sensory and motor neurons, excite smooth muscle (in the uterus, intestine, and respiratory tract), release adrenaline and noradrenaline, and controls the secretion of gastric acid.

The severity of illness is dependent on many factors, including overall immune health. Patients undergoing therapeutic treatment for existing illnesses with known histaminases require less than 50 mg/100g foodstuffs to become ill, as the natural ability to detoxify histamine in the gut is compromised. It has been suggested that patients undergoing isonazid treatment should be warned to avoid foods containing naturally high levels of histamine. One such patient in Ottowa Canada fell ill after consuming extra-sharp cheddar cheese containing only 40 mg histamine /100 g cheese (24), 10 mg / 100 g food less than the FDA hazard level.

The incubation period for scombroid poisoning is typically short, occurring 10 minutes to two hours after consumption of the food, and is consistent with a typical chemical intoxication. Hughes and Merson (22) cited the median incubation among 27 outbreaks reported to the CDC between 1970 and 1976 to be 30 minutes with a median duration of 4 hours.

a. Treatment

Treatment of histamine poisoning is dependent on the symptoms experienced. Since the illness is short lived and usually mild, many patients do not pursue medical treatment (38). Hughes and Merson (22) recommended the administration of oral antihistamines in most mild cases for relief. In severe cases, vomiting and diarrhea is induced to expel unabsorbed toxin from the system. The use of bronchodilators is recommended for use when bronchospasms occur.

b. Control

Because histamine is heat stable, thorough cooking of fish containing the chemical is not sufficient to prevent an outbreak (60). Control of histamine in fish must begin at catch and continue to the consumer's table. The FDA (4) recognizes catch as one of the initial critical steps in the formation of histamine, stating that the immunological defenses of fish on a line fall as they struggle (with a rise in activity producing a rise in internal body temperature) and thus they become less able to fend off bacterial attack. Proper and prompt refrigeration of freshly caught fish prior to canning, cooking, or freezing is the key to controlling outbreaks (7,22,38). In a 1980 study of histamine forming bacteria isolated from spoiled skipjack tuna, Arnold et al. (5) reported the lack of histamine formation by all isolates at 1°C. This led the researchers to suggest that rapid cooling of the fish flesh to 1°C would suppress histamine formation. In a 1986 survey of commercially processed fish in Morocco, Ababouch et al. (2) found canned fish products from canneries in regions furthest from catch sites (which received their fish in open trucks without ice or refrigeration) to have a 7.1 % of product containing greater than 50 mg histamine /100g fish, as compared to 1.3 and 4.4 for regions closer to catch sites. In 1982, Behling and Taylor (7) noted histamine production was below the level of detection for all histamine producing bacteria in tuna fish infusion broth (inoculum level of ca. 10^7 CFU/ml) with the exception of *Klebsiella pneumoniae*, which produced 715 nmol/ml after 158 hours of incubation at 0°C.

Work performed by Klausen and Huss (27) support the recommendation that fresh fish containing high levels of histidine be kept at constant refrigeration temperatures prior to processing or consumption. Samples of fresh mackerel and histidine decarboxylase broth (HDB) inoculated with *Morganella morganii* at levels of approximately $10^{5.6}$ CFU/g of mackerel or ml HDB, respectively were subjected to storage at elevated temperatures (10 and 25°C) followed by incubation at 0°C for predetermined time periods. In HDB samples, no histamine was detected throughout the 100 + hour sampling period. In tubes incubated at 25°C followed by 5°C incubation after 19 and 23 hours, histamine was detected at 1200 ppm and 2700 ppm, respectively. Mackerel samples stored at 10°C prior to 0°C incubation showed similar results. Those stored for 24 hours at 10°C produced no detectable histamine throughout six additional days of storage at 0°C. In samples stored for 48 hours at 10°C followed by 5 days at 0°C, 100-270 ppm histamine was detected. For samples stored for 72 hours at 10°C followed by 4 days at 0°C 600-1400 ppm was detected. This suggested that prolonged temperature abuse (as 10 and 25°C would imply) prior to refrigeration could spur the production of histamine, rendering the fish unsafe for consumption.

Ferencik (15) suggested that hazardous levels of histamine developed in fish when the following four conditions were met: the presence of a microorganism with the potential to decarboxylate histidine, the presence of histidine decarboxylase, free histidine, and an environment which encouraged the growth and proliferation of said microflora. Conditions reducing the exposure of fish to histidine decarboxylating microorganisms has been seen as a method to control scombrotoxicosis since the early 70's, as implied by Ferencik's work.

HACCP plans mandated by the FDA require temperature control of raw fish from the time of docking to receipt by the first processor, but do not regulate the method of catch nor storage conditions practiced before the fish is brought to dock (4,36). Loss of control at this stage was implicated to be the causative agent in a 1998 Pennsylvania outbreak of Scombroid poisoning. Officials form the Pennsylvania Department of Health and the Pennsylvania Department of Agriculture found no deviations from HACCP procedures occurred during the distribution of the implicated fish from wholesale to retail, concluding loss of control occurred between time of catch and arrival to the dock (34).

5. Epidemiology

One of the earliest recorded and characterized cases of histamine poisoning was reported in 1830 in London. Five members of an English ship crew fell ill after consuming bonito (*Scomber pelamis*) which had been stored on the ship's deck without means of cooling or preservation. The sailors had been consuming the fish for several days before becoming ill. Reports indicated that those who ate the most fish became the most severely ill (20).

Lerke et al. (28) described a 1977 outbreak from sashimi prepared from raw tuna and consumed in three San Francisco Japanese restaurants. Fifteen people reported symptoms consistent with scombroid poisoning. Murray et al. (39) reported 71 cases of scombrotoxicosis resulting from consumption of canned tuna-fish in England between 1979 an 1980. A spinach-tuna salad prepared from fresh tuna was reported to be the culprit in a 1998 Pennsylvania scombrotoxicosis outbreak involving four victims (34). Samples of the fish were found to contain levels greater than 50 ppm (34).

In 1987, Bartholomew et al. (6) published a review of 258 suspected incidents of scombrotoxic fish poisoning in Britain between 1976 and 1986. Of the reported cases, 80% or more involved fewer than five victims; only 13 cases involved 5-10 victims, and five involved more than 10 victims. Seventy percent of all incidents were traced to smoked mackerel and canned tuna, while the rest to non-scombroid fish, namely sardine, pilchard, salmon, and herring.

Mahi mahi has been implicated in many cases of scombroid poisoning. One 1988 outbreak reported in Chicago was traced to mahimahi in dill sauce prepared and consumed in a private club. Six samples from the same lot as the fish consumed had histamine levels greater than or equal to 50 mg/100 g fish (50-160 range) (38). Grilled mahi was implicated in an Albuquerque outbreak in which samples were found to contain only 20 mg/100 g fish (45). In another 1988

outbreak, nine cases of scombroid poisoning in South Carolina were traced to yellowfin tuna (38). Two samples tested by the FDA contained 728 mg histamine/100g and 583 mg/100 g. In 1981, five persons fell ill with scombroid poisoning after consuming bluefish (36 - 138 mg histamine/100 g fish), a fish not previously implicated in such an outbreak (25). Bluefish were again implicated in a 1987 outbreak involving five participants of a New Hampshire medical conference (14). Analysis of the fish indicated the presence of 250 mg histamine, 30 mg putrescine, and 74 mg cadaverine, per 100 g fish.

Thirty states and the District of Columbia participate in surveillance and reporting. Between 1973 and 1986, 178 outbreaks affecting 1096 people were reported to the CDC (38). Hawaii reported 51, California 29, New York 24, Washington 19, and Connecticut 9. Mahimahi was the fish most commonly implicated (in 66 cases), followed by tuna (42 cases), and bluefish (19 cases). One hundred and forty five outbreaks involving 811 victims were reported to the CDC between 1988 and 1997 (38). No fatal cases have been reported to date.

6. Sensory clues

Although no evidence has been gathered on characteristic organoleptic clues of fish containing high levels of histamine, a few victims in isolated outbreaks reported detecting similar flavors in the offending fish. Victims in a 1988 outbreak involving mahi mahi reported the fish to have a hot, spicy, or Cajun taste, though it was not prepared with spices which would produce such flavors (38). A slight peppery taste and a metallic taste was reported by victims in a 1988 outbreak involving yellow-fin tuna prepared in a South Carolina restaurant (38). Four victims in a 1998 outbreak reported the presence of a metallic taste in tuna-spinach salad that was implicated as the source of scombroid fish poisoning (34). Lerke et al. (28) found that raw tuna (implicated in a 1977 outbreak of scombroid poisoning) with very high levels of histamine had been judged as acceptable by persons with experience handling, preparing, and consuming tuna sashimi. They also noted samples of the raw tuna had only a very mild off odor even though they contained high levels of histamine.

B. Histidine decarboxylating bacteria

The natural flora of most fish, reflects the quality of its aquatic habitat. Taylor (53) listed *Pseudomonas, Flavobacterium, Vibrio, Bacillus*, coryneforms, and *Acinetobacter-Moraxella* as organisms comprising the natural flora of freshly caught fish. Among these, few posses the ability to breakdown histidine.

Many bacterial species have the ability to transform histidine to histamine through the action of histidine decarboxylases. Most prominently represented are the members of the family *Enterobacteriaceae.* Rawles et al. (44) provided a comprehensive table (Table 3) of isolates showing the ability to decarboxylate histidine. *Morganella morganii* is one of the most frequently isolated microorganisms from foods implicated in scombroid poisoning (1,5,17,30,31,32,33,41,59). Other microorganisms have been found, including *Klebsiella*

pneumoniae and psychrotrophic *Pseudomonas* (*fluorescens* and *putida*) which produced less than 3 mg/100 ml broth (30,46,48,55,66).

Histidine decarboxylating organisms do not normally make up the natural flora of the slime layer of fish, a portion of the fish which most often traps bacteria during contact with water and storage media (54). Typically, these organisms are filtered out of the fishing waters through the gills and mouth or adhere to the slime layer through contact with contaminated surfaces during harvest, transport, and storage. They find their way from the gills and intestinal tract to the muscles of healthy fish as a consequence of post-catch and or post-processing contamination (54). Fujii et al. (18) found halophilic psychrotrophs capable of decarboxylating histidine in frozen tuna samples.

In addition to histidine decarboxylating microorganisms, mesophilic and psychrotrophic spoilage bacteria capable of breaking down lysine and ornithine have been isolated in decomposing mahimahi loin tissue (17). Decarboxylation of the two amino acids leads to the formation of putrescine and cadaverine, respectively.

1. *Morganella morganii*

Morganella morganii is a motile Gram-negative facultative anaerobic rod with a length of 1.0-1.7µm and diameter of 0.6-0.7µm (42). Table 4 lists the phenotypic traits of the organism. This organism naturally occurs in mammalian and reptilian feces and takes the role of an opportunistic pathogen in bacteremias, respiratory, wound, and nosocomial urinary tract infections (43). Previously classified as *Proteus morganii* (1939-1978), DNA base pair composition analyses in 1978 showed the organism to be more closely related to *Escherichia* and *Salmonella* rather than *Proteus* (42). *M. morganii* shows *in-vitro* resistance to many antibiotics, including colistin, erythromycin, penicillin, ampicillin, cephalotrin, nalidixic acid, carbenicillin, aminoglycosides, chloramphenicol, tetracycline, and sulfonamides (42). The organism is typically isolated using standard media and techniques used to isolate *Enterobacteriaceae* and stored onto Trypticase Soy Agar (TSA) slants (42).

C. Laboratory analysis

1. Histamine

Many laboratory techniques for isolation and quantification of histamine in food samples have been developed, with chromatographic techniques being most widely used. These techniques involve extraction (typically with an alcohol), selective conjugation to fluorometric chemicals, and subsequent identification or quantification of the conjugates by way of a visual color change or through spectrophotometry.

Four principal chromatographic methods have been developed and further modified for use: thin-layer chromatography (TLC), Liquid Chromatography (LC), High Pressure Liquid

Table 3. Microbial isolates showing histidine decarboxylase activity in carboxylase broth supplemented with histamine. [a]

Identification of isolate	Decomposition temperature(s) at which found (°C)	Source
Acinetobacter lwoffi	0,15	Beef
Aeromonas hydrophila	0,15	Skipjack tuna
Citrobacter freundii	15	Skipjack tuna
Clostridium perfringens	15,30	Mahimahi
Edwardsiella sp.	15	Skipjack tuna, tuna, mahimahi, pork, beef
Enterobacter aerogenes	15,30	Food, tuna
Enterobacter sp.	30	Tuna
Escherichia coli	15,30	Tuna
Hafnia alvei	15,30	Tuna, Skipjack tuna, mackerel, food
Klebsiella pneumoniae	15	Tuna, mahimahi, mackerel
Klebsiella sp.	15	Skipjack tuna, mackerel
Morganella morganii	0,15,30	Scombroid fish, pork, turkey, mahimahi
Proteus mirabilis	15	Pork, turkey, tuna, skipjack tuna
Proteus vulgaris	30	Beef, pork, turkey, tuna
Proteus sp.	15	Fish, feces, tuna, mackerel, food, milk
Pseudomonas fluorescens/putida	15	not given
Pseudomonas putrefaciens	0,15,30	not given
Pseudomonas sp.	0,15	Food
Vibrio sp.	15	Mackerel
Vibrio alginolyticus	0,15,30	Skipjack tuna

[a] From Rawles 1996 (44)

Chromatography (HPLC), and Gas Chromatography (GC).

In the 1970's, Abdel-Monen and Ohno (3) adapted existing TLC techniques to the quantification of diamines and polyamines (putrescine and spermine, respectively) in urine samples. By changing the derivatization compound and the fluorescent molecule, scientists later used the technique to measure histamine levels. Lieber and Taylor (29) tested twelve solvent and thin-layer systems for rapid screening of tuna fish samples, finding a methanol-ammonia (20:1) and a chloroform-methanol-ammonia (2:2:1) solvent system to work best with silica gel-layered plates followed by development with ninhydrin and glacial acetic acid. The methods currently in use for TLC analysis have not been appreciably altered since Lieber & Taylor's original work. An aliquot of fish extract or broth containing suspected histamine producing bacteria (after 24- 48 h incubation) is dispensed onto a TLC plate coated with a 0.25 mm layer of silica gel G60. The plate is then exposed to a 2:2:1 mixture of chloroform, methanol, and ammonia. The presence of histamine is confirmed by the appearance of dark spots upon the addition of the Pauly reagent (1, 41,44).

For LC the extract or inoculated broth is first passed through a cation exchange column to separate the histamine-containing phase with pH 4.62 acetate buffer (1). Once the phase is eluted from the column with H_2SO_4, histamine is joined to diazonium through a series of washes. The histamine-diazonium derivative is quantified by measuring the optical density at 475 nm spectrophotometrically.

Officially recognized, standardized, and widely used HPLC methods are described in the AOAC manual. Behling and Taylor (7), among others, used the AOAC method when quantifying bacterial histamine production levels. This method begins with extraction of amines and like chemicals with methanol for 15 minutes at 60°C followed by rapid cooling. The eluant is then fractionated with an ion exchange column and the histamine portion selectively bound to the fluorometric molecule o-phthalaldehyde. The level of complexed histamine is determined upon 360 nm excitation followed by spectrophotometric measurement of emission at 450 nm. The relationship between the optical density and histamine concentration is linear (13,28,47,63). Fluorescamine is another fluorescent molecule which may be used for detection of primary amines, used successfully by Gingerich et al.(19) in 1999 (62).

Enzymic tests exploit the formation of byproducts from enzymes and histamine. One of the most widely used methods requires the breakdown of histamine by diamine oxidase to produce imidazole acetaldehyde, ammonia, and hydrogen peroxide (53). The addition of crystal violet and horseradish peroxidase produce a purple color in the presence of H_2O_2. The presence of histamine is indicated by a purple color and confirmed by spectrophotometric analysis at 596 nm (30,31,46,47).

2. Histidine decarboxylating microorganisms

Identification of histidine decarboxylating microorganisms involved in scombrotoxin outbreaks involves isolation and screening, quantification of histamine production, and

Table 4. Phenotypic traits of *Morganella morganii*. [a]

Characteristic / test	Reaction	Characteristic	Reaction
Gram	neg.	Acid production from:	
Straight rod	pos.	Glucose	pos.
Motile (single peritrichous flagellum)	pos.	Trehalose	pos.
Fermentative metabolism	variable	mannose	pos.
Phenylalanine deaminase	pos.	Gas production from Glucose	variable
urease	pos.	lactose, sucrose, L-arabinose	neg.
Growth in KCN	pos.	raffinose, L-rhamnose., D-xylosem, cellobiose, α methyl-glucoside, melibiose, salicin, esculin, mucate	neg.
amino acid decarboxylases:		Tartrate utilization	pos.
Ornithine decarboxylase		H_2S produced in triple sugar iron	neg.
Lysine decarboxylase	pos. (-)	gelatin liquefaction	pos.
Arginine dihydrolase	pos .[c]	Simmon's citrate utilization	neg.
Methyl red test	neg. [d]	ONPG-Hydrolysis	neg.
Voges-Proskauer test	pos.	Deoxyribonuclease	neg.
reduction of NO_3- to NO_2-	pos.	Lipase	neg.
tyrosine clearing	pos. [e]		
Oxidase test	neg.		

[a] from Penner, J. 1984(42)

biochemical or genetic characterization. Food samples are first plated onto non-selective media like Trypticase Soy Agar (TSA) or broth (17,32,66). The use of fish infusion broths in the isolation was widespread in early investigations and is still widespread. Researchers made their own fish broths from wholesome tuna (Tuna Fish Infusion Broth) (TFBI) and sardines (Sardine Infusion Broth) (1,28,41,59). The dependence on wholesome fish and the variability in histidine levels among different fishes led to most researchers amending traditional microbiological broths such as Trypticase Soy Broth with histidine (61).

Buchanan and Gibbons were among the first researchers to use TFIB to grow histidine decarboxylating species. Taylor et al. (56) used TFIB in conjunction with TSBH to quantify histamine production by 38 bacterial species. Arnold et al. (5) created Skipjack Infusion Broth (SIB) in their characterization of *Proteus* and *Hafnia* species isolated from frozen skipjack tuna. Taylor & Woychik (61) developed TSBH (trypticase soy broth amended with 2% histidine, pH 6.3) to quantify histamine production by *Enterobacteriaceae*. The ease of formulation, low cost, and selectivity make it a popular medium for use. In their study of time and temperature effects on bacterial histamine production by histidine decarboxylating bacteria, Behling & Taylor (7) reaffirmed the usefulness of the medium.

After initial growth, bacteria are then subcultured on TSA for isolation (in some cases amended with histidine), and then screened by growing the isolates on media containing histidine and indicator dyes. Niven et al. (40) developed one of the first screening media for histidine decarboxylating bacteria in 1981. By amending a solid medium containing traditional microbiological nutrients with histidine and bromcresol purple (pH 5.0), the researchers were able to differentiate histidine decarboxylating bacteria from non-decarboxylators by the appearance of a deep purple color adjacent to colonies in positive isolates. The accumulation of the alkaline histamine in the medium induced a color change in the indicator dye corresponding to an approximate rise in 1.5 pH units. Smith et al. (51) modified the pH in Niven's medium to allow for growth of microorganisms at pH 5.25. By increasing the histidine concentration (from 2.7 - 3%) and the agar concentration (from 2 - 2%) the researchers noted the development of a more dramatic purple color and a stabilization of gel matrix in the modified medium respectively. Yoshinaga and Frank (66) further raised the pH of Niven's medium to 6.5 to allow for growth of less acid-tolerant species. The researchers further investigated the decarboxylating and sugar-reducing ability of histidine decarboxylating bacteria by stabbing isolates into modified Niven's medium tubes and inoculating liquid Niven's tubes (containing inverted Durham tubes) and looking for anaerobic histidine metabolism and CO_2 production, respectively. Isolates were deemed histidine decarboxylase positive if CO_2 evolved or the characteristic purple color change occurred. Yamani and Unterman (65) developed a liquid medium which allowed the researchers to screen out histidine decarboxylating organisms between 24-48 hrs. A color change from light green to violet, based on a rise in pH upon decarboxylation of histidine, indicates a positive result.

Isolates are identified through biochemical reactions listed in reference books such as *Bergey's Manual for Systematic Bacteriology* (42) or through the use of widely available biochemical test kits such as the API series (20E, 20NE, bio-Merioux) or PASCO Identification Systems(Gram Positive and Negative, Difco) (1,31,33,46,59).

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CHAPTER IX. Growth and histamine formation of *Morganella morganii* in determining the safety and quality of bluefish (*Pomatomus saltatrix)*

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Growth and histamine formation of *Morganella morganii* in determining the safety and quality of bluefish (*Pomatomus saltatrix)*

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Key words: bluefish, histamine, *Morganella morganii*, safety, *Pomatomus saltatrix*

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ABSTRACT

The objective of this study was to determine the effect of normal microflora and *Morganella morganii* on histamine formation and olfactory acceptability in raw bluefish under controlled storage conditions. Fillets inoculated with and without *M. morganii* were stored at 5°, 10°, and 15° C for seven days. Microbial isolates from surface swabs were identified and screened for histidine decarboxylase activity. Olfactory acceptance was performed by an informal sensory panel. Histamine levels were quantified using HPLC and fluorescence detection. While olfactory acceptance decreased , histamine concentration and bacterial counts increased. Storage temperature had a significant effect on histamine levels, bacterial counts, and olfactory acceptance of the bluefish. Inoculation with *M. morganii* had a positive significant effect on histamine formation ($p<0.0001$). The results of the study will serve in supporting FDA regulations regarding guidance and hazard levels of histamine in fresh bluefish.

INTRODUCTION

Scombroid poisoning is associated with the consumption of dark fleshed fish that contain elevated levels of histamine in their tissues. Scombroid fish are typically implicated in histamine poisoning cases, hence the name scombroid poisoning. Clinical symptoms are typically short lived and involve cutaneous (rash, inflamation, redness), gastrointestinal (discomfort, nausea, vomiting, diarrhea), hemodynamic (hypotension), and neurological reactions (headache, flushing, tingling) (19). Bluefish (*Pomatomous saltatrix*) are oily dark fleshed saltwater fish that were implicated in more than 19 cases of histamine poisoning between 1973 and 1986 (5,15). The high levels of histidine in their dark muscles are susceptible to bacterial decomposition, leading to a high concentration of histamine. Histamine at a level equal to or greater than 50 mg histamine /100g fish (500 ppm) is considered hazardous by the FDA (1), though levels as low as 20 mg/100g fish (200 ppm) have been shown to produce symptoms of histamine poisoning (1). Although the FDA currently regulates only the histamine level in dark fleshed fish, researchers and the agency agree that other biogenic amines such as putrescine and cadaverine may also play a role in scrombotoxicosis (1, 8).

Histidine decarboxylating organisms do not normally make up the natural flora of the slime layer of a healthy fish which typically include *Pseudomonas, Achromobacter, Flavobacterium, Vibrio, Micrococcus,* and coryneforms (19). Histidine decarboxylating organisms are filtered out of the fishing waters through the gills and mouth. *Morganella morganii*, a Gram negative facultative anaerobic rod, is one of the leading histidine decarboxylating organisms isolated from fish implicated in histamine poisoning cases (2,13,14,16,20). Histidine decarboxylating organisms can adhere to the slime layer through contact with contaminated surfaces during harvest and transport (16). Cross contamination also occurs during processing as the organisms can be spread from the GI tract and gills to sliced surfaces (19). The objective of this study was to determine the effect of normal microflora and *M. morganii* on histamine formation and olfactory acceptability of raw bluefish fillets under controlled storage conditions.

MATERIALS & METHODS

Fish samples

Whole fresh bluefish (*P. saltatrix)* were purchased from commercial processors in Hampton, VA. The fish were packaged into commercial wax lined corrugated cardboard boxes provided by processors and transported on ice (within six hours) to the laboratory at Virginia Tech in Blacksburg, VA for immediate evisceration, filleting, and analyses (n=100 fillets, approx. 50 g each).

Sample treatment

Whole fillets were placed into 530 ml sterile polyethylene Whirl-pak™ bags.

Inoculated samples

For half of the samples (50), the non-skin surface of each fillet was inoculated with 0.1 ml of *Morganella morganii* (ATCC 43176, American Type Culture Collection, Rockville, MD) in Log phase (ca.10^6 CFU/ml, in Tryptic Soy Broth TSB: Difco Laboratories, MI) with a sterile pipette. The inoculum was evenly distributed over the surface and the inoculated fillet placed back into a sterile polyethylene Whirl-pak™ bag. Uninoculated and inoculated samples were stored at 5, 10, and 15°C. Chemical, microbiological, and sensory analyses were performed daily for seven days or until 100% sensory rejection was reached, whichever occurred first. Samples were discarded upon reaching 100% sensory rejection and after testing. The experiment was replicated three times, with a total of 300 fillets tested.

Chemical analysis

Histamine analysis followed procedures previously described (8). Briefly, 50 g samples were removed from the fillets and homogenized using a Waring Blender. A 10 g portion of the homogenated sample was extracted with a total volume of 60 ml 5% trichloroacetic acid (T9151 Fisher Scientific, Fairlawn, NJ). Biogenic amines were separated using HPLC and pre-column derivatization with 4.0 mM fluorescamine (F9015 Sigma, St. Louis, MO). Histamine was quantified using fluorescence detection. The minimum level of detection was 1 ppm.

Microbiological evaluation

A nine cm^2 area of the sample's filleted surface (the non-skin side) was swabbed with a sterile calcium alginate cotton-tipped swab (14-959-80 Fisher Scientific, Fairlawn NJ). The swab was then dipped into sterile 0.1 % trypticase peptone solution (Becton Dickinson, Cockeysville MD), the tip broken off into 9 ml 0.1 % peptone, vortexed, and serially diluted. Dilutions were pour plated onto Trypticase Soy Agar (TSA) (Difco # 0369-17-6, MI) and spread plated onto Eosin Methylene Blue Agar (EMB) (Becton Dickinson # 11221, Cockeysville MD), and incubated 48 hrs. at 35°C. Surface levels of Standard Plate Count bacteria (SPC/ cm^2) and *Enterobacteriaceae* (EPC/ cm^2) were determined form TSA and EMB, respectively. Isolated colonies were transferred onto TSA slants, and incubated 48 hrs. at 35°C. Isolates were screened for histidine decarboxylase activity using Niven's agar (16). Isolates found negative for histidine decarboxylase activity were excluded from the study. Organisms were identified using API 20E , API 20NE (bioMerieux Vitek, Hazelwood MO),Gram reaction, and morphology.

Sensory evaluation

One cm^3 piece of uninoculated or inoculated sample was placed into individual 1 oz. capped plastic souffle cups (Solo Cup Co., # P100, Urbana IL) and labeled with randomly generated 3 digit codes. Samples were presented to an untrained sensory panel (n=20, with at least 12 volunteers experienced in seafood products) in balanced random order under fluorescent light at room temperature. Panelists were asked to evaluate the olfactory acceptability of each sample in comparison with a reference sample (1 cm^3 portion of freshly thawed bluefish, which was frozen immediately after filleting). In total, eighty to one hundred 1 cm^3 samples were evaluated per treatment (combination of inoculation, time, temperature).

Statistical analysis

Data analysis was performed using SAS's PROC Mixed procedure for both chemical and microbiological data (split-plot design with mixed models) and SAS's Pearson Correlation analysis to evaluate sensory data (SAS Institute Inc., Cary, NC).

RESULTS & DISCUSSION

Histamine formation. Uninoculated bluefish fillets contained a histamine level of 74 ± 15 ppm at day zero (Table 1), indicating the fish may have been exposed to elevated temperatures post harvest. The FDA (1) recognizes catch as one of the initial critical steps in the formation of histamine, stating that the immunological defenses of fish on a line weaken as they struggle and the fish become less able to fend off bacterial attack. Bluefish have been shown to contain elevated histamine at the wholesale and retail level (8,10).

Histamine levels rose in the majority of uninoculated samples during storage time, with the greatest increase in histamine found in those fillets stored at 15°C (35 ppm ± 26ppm on day 1 to 938 ppm ± 200 ppm on day 3). Both the separate and combined effects of time and temperature on the formation of histamine in uninoculated fillets were statistically significant ($p<0.05$). The average amount of histamine detected (ppm) in the uninoculated and the *M. morganii* inoculated samples are shown in Tables 1 and 2, respectively. The effects of time and temperature on average histamine formation were statistically significant for both inoculated and uninoculated fillets ($p < 0.05$), concurring with the findings of Behling and Taylor (3) . Crapo & Himelbloom (4) noted a rise in histamine level in Pacific herring stored at 10°C from undetectable to 30 ppm after four days. During initial studies, both inoculated and uninoculated bluefish stored directly on ice did not produce detectable levels of histamine (data not shown), a phenomenon previously observed in spoiled skipjack tuna by Arnold et al. (2). Behling and Taylor (3) reported non-detectable histamine production by bacterial species in Tuna Fish Infusion Broth when incubated at 1°C and below. In this study, the natural flora of the bluefish fillets assisted in the formation of histamine in the uninoculated fillets stored at elevated temperatures. Studies performed by Ferencik (6) showed that sterile uninoculated tuna fish flesh produced no detectable histamine even after eight days of storage, therefore the presence of histidine decarboxylating microorganisms would most likely account for the formation and rise of histamine in the uninoculated samples.

In general, histamine levels in *M. morganii* inoculated fillets were significantly higher ($p <0.05$) than in the uninoculated fillets. The organism has been frequently isolated from fish implicated in scombrotoxicoses and its ability to decarboxylate histidine to form histamine has been documented (3,7,11,19,20). The highest level of histamine was found in the inoculated fillets stored at 15°C (2200 ppm on day 3). At 15°C the generation time of the microorganism in trypticase broth is 2.6 hrs (11). It is likely the microorganism not only reached elevated numbers in the fillets incubated at 15°C but that the amount of and the activity of histidine decarboxylase produced by *M. morganii* accounted for the observed spike in histamine. Neither *M. morganii* inoculated nor uninoculated samples stored on ice for 24 days produced any detectable histamine (data not shown).

The microorganism *Klebsiella pneumoniae* was initially included with *M. morganii* as challenge microorganisms in the study. The strain (*K. pneumoniae* ATCC:13883) used did not produce significant levels of histamine and yielded a pleasant odor to the inoculated fillets over time, therefore it was removed from the final study. Researchers have included *K. pneumoniae* as

a prodigious histamine former and have isolated it from fish implicated in scombrotoxicoses with frequency (12,18,19).

Microbiological evaluation. Generally, the SPC and EPC levels rose in an exponential manner as time and storage temperatures increased (Tables 1 and 3). This concurs with typical exponential growth models of most bacteria and with the work of Crapo and Himelbloom (4). The effects of time and temperature on SPC and EPC were statistically significant ($p<0.01$) and the combined effect of time an temperature on both SPC and EPC was also found to be statistically significant ($p<0.001$). The effect of *M. morganii* on the growth of the normal microflora was not statistically significant ($p <0.41$). The highest level of SPC were found in uninoculated samples held at 10°C for 5 days (1.1×10^8 CFU/cm^2). Both inoculated and uninoculated samples held at 5°C maintained SPC values ca. 10^7 CFU/cm^2.

Bacterial isolates. Histidine decarboxylating bacteria isolated from bluefish were predominantly Gram negative rods and cocci (Table 3). The findings concur with previously published studies. *Enterobacter* spp. have been isolated from bonito and tuna (14), skipjack tuna (2,21), and herring (4). Researchers have isolated *M. morganii* from many fish sources with frequency, including skipjack tuna (2,16,20,21), tuna (14), mackerel (14), mahimahi (7). Tuna destined for canning contained *Acinetobacter lwoffi* at approx. 5.7 Logs, though did not produce histamine when cultured in the laboratory (13). *Citrobacter freundii* has been isolated from both tuna and mackerel purchased at the retail level (14), as well as from frozen skipjack tuna (20). Researchers have not previously reported the presence of the nosocomial pathogen *Chryseomonas luteola* in fish samples implicated in scombroid poisoning outbreaks, though the ubiqutous nature of the organism may account for its presence in the bluefish samples (9).

Sensory evaluation and consumer safety. As expected, the acceptability of samples decreased as the storage temperature increased (Tables 1 and 2). Samples containing 50 - 100 ppm histamine were scored acceptable by greater than 50% of the panelists, indicating that simple olfactory evaluation for rejection of a sample containing hazardous levels of histamine is not an adequate method. These results are similar to those found by Lopez-Sabater et al. (12) in a study evaluating the organoleptic acceptability and histamine level of raw tuna stored at 0, 8, and 20°C. These researchers found that tuna stored at 8°C was still deemed as organoleptically acceptable (score 1 = fresh through 6 = spoiled) after four days of storage eve though histamine levels of 120 - 150 mg/100 g (1200 - 1500 ppm) were found. Samples removed from the posterior portion of the tuna were still deemed acceptable at day three (~ 1750 ppm), having exceeded the FDA hazard level of 50 ppm. Samples stored at 20°C were deemed spoiled by 36 hrs, reaching histamine levels between 50 mg and 100 mg/100 g fish (500 - 1000 ppm). Results of this study indicate that raw bluefish and other dark-fleshed fish subjected to mild temperature abuse conditions (10°C as in this study and 8°C in the work of Lopez-Sabater) are capable of producing hazardous levels of histamine before organoleptic rejection of the fish is reached. Because histamine is heat stable, post-processing treatments such as cooking or smoking do not eliminate the toxin once it has been formed; therefore raw fish with elevated histamine produces a hazardous finished product with elevated histamine.

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Table 1. Olfactory acceptability, histamine, standard plate count, and *Enterobacteriaceae* counts during controlled storage of uninoculated bluefish.

Temperature (°C)	Storage Time (days)	Acceptability (%)	Histamine (ppm)	Standard Plate Count log (CFU/cm^2)	*Enterobacteriaceae* Plate Count log (CFU/cm^2)
-	0	N.D.	74 ± 15	6.1 ± 0.4	3.0 ± 1.6
5	1	74 ± 17	58 ± 27	4.6 ± 0.2	3.5 ± 0.4
	2	76 ± 43	50 ± 43	4.3 ± 0.0	4.1 ± 0.2
	3	63 ± 36	44 ± 63	4.3 ± 0.1	3.6 ± 0.8
	4	76 ± 8	42 ± 43	4.7 ± 0.3	5.1 ± 0.2
	5	56 ± 24	99 ± 18	7.2 ± 3.1	5.6 ± 1.1
	6	36 ± 11	18 ± 10	7.5 ± 0.5	6.6 ± 0.4
	7	25 ± 12	25 ± 44	8.2 ± 1.9	7.2 ± 0.3
10	1	56 ± 21	52 ± 33	3.7 ± 1.3	4.5 ± 1.1
	2	44 ± 14	41 ± 6	5.2 ± 1.1	5.4 ± 0.4
	3	17 ± 11	44 ± 21	7.3 ± 1.2	6.1 ± 1.3
	4	5 ± 7	12 ± 14	7.3 ± 0.3	7.1 ± 0.3
	5	0 ± 0	33 ± 37	7.5 ± 0.5	7.7 ± 0.3
15	1	40 ± 17	35 ± 26	5.9 ± 1.2	5.3 ± 0.5
	2	10 ± 12	55 ± 42	6.7 ± 0.4	6.8 ± 0.3
	3	0 ± 0	938 ± 200	7.6 ± 0.2	7.6 ± 0.1

[1] Percent panelists rating product as acceptable

[2] N.D. - Not done

Table 2. Olfactory acceptability, histamine, standard plate count, and *Enterobacteriaceae* counts during controlled storag

bluefish inoculated with *Morganella morganii*.

Temperature (°C)	Storage Time (days)	Acceptability [1] (%)	Histamine (ppm)	Standard Plate Count log (CFU/cm^2)	*Enterobacteriaceae* Plate Count log (CFU/cm^2)
5	1	61 ± 35	102 ± 10	4.1 ± 0.8	4.4 ± 1.4
	2	76 ± 43	68 ± 74	5.3 ± 1.6	3.6 ± 0.6
	3	63 ± 38	68 ± 44	4.2 ± 0.3	3.8 ± 1.1
	4	69 ± 5	47 ± 57	5.5 ± 0.4	5.3 ± 1.1
	5	51 ± 18	116 ± 0	7.1 ± 2.9	5.6 ± 1.1
	6	19 ± 11	80 ± 37	7.3 ± 1.4	8.0 ± 0.2
	7	19 ± 5	16 ± 15	8.9 ± 1.8	7.5 ± 0.2
10	1	41 ± 28	42 ± 17	5.3 ± 0.1	4.6 ± 0.6
	2	31 ± 34	54 ± 13	5.5 ± 0.9	6.0 ± 0.8
	3	24 ± 12	31 ± 12	7.1 ± 0.3	7.1 ± 0.4
	4	3 ± 3	338 ± 13	7.3 ± 0.0	7.3 ± 0.0
	5	0 ± 0	886 ± 41	8.0 ± 0.0	8.0 ± 0.0
15	1	20 ± 26	286 ± 261	6.5 ± 0.6	6.0 ± 0.7
	2	0 ± 0	2154 ± 606	8.0 ± 0.4	7.1 ± 0.8
	3	0 ± 0	2200 ± 0	6.7 ± 0.0	6.4 ± 0.0

[1] Percent panelists rating product as acceptable

Table 3. Microorganisms isolated from bluefish samples.

Organism	Occurrence in Samples
Acinetobacter iwolffi	1
Chryseomonas luteola	3
Citrobacter freundii	2
Enterobacter cloacae	1
Morganella morganii	1
Providencia alcalifaciens	2

CHAPTER X. VITA

Tatiana A. Lorca

EDUCATION

Doctorate of Philosophy, Food Science and Technology, Expected May 2002
Virginia Polytechnic Institute and Sate University (Virginia Tech), Blacksburg, VA 24060
***Dissertation*:** The effects of high pressure shock waves on spoilage and pathogenic bacteria in meats
Advisor: Merle D. Pierson

Master of Science, Food Science and Technology, November 7, 2000
Virginia Polytechnic Institute and Sate University (Virginia Tech), Blacksburg, VA 24060
***Thesis*:** An evaluation of the role of storage temperature on the safety and quality of raw shellstock oysters and bluefish
Advisor: Merle D. Pierson

Bachelor of Science, Biology; Minor: Chemistry; Minor: Spanish, May 1995
Virginia Polytechnic Institute and Sate University (Virginia Tech), Blacksburg, VA 24060

AFFILIATIONS

American Society for Microbiology, member, 1997 - present
Institute of Food Technologists, student member 1995 - present
International Association for Food Protection (formerly IAMFES), member, 1998 - present
Virginia Tech Graduate Student Assembly, Food Science and Technology department delegate, 1996 -1998
Virginia Tech Food Science Club, coordinator for elementary school outreach program, 1997 - present
Virginia Tech Agriculture Club Council, Food Science Club delegate. 1996-1997
Gamma Sigma Delta Agriculture & Life Sciences Honor Society, 1997
Phi Sigma Biological Honor Society 1994
Sigma Delta Pi Spanish Honor Society, 1994-95
Phi Sigma Iota Language Honor Society, 1994

RESEARCH INTERESTS

HACCP in the food industry
Rapid methods in the microbiological analysis of foods
Environmental sampling of food production areas

TEACHING INTERESTS

Introductory microbiology
Food microbiology

EXPERIENCE

Research

Ph.D. Research, Department of Food Science and Technology, Virginia Tech
Blacksburg, VA, January 1998 - present

- Designed and conducted experiments for dissertation project
- Traditional microbiological evaluation of natural flora in model systems
- Package integrity studies
- Engineered tracer microorganisms for use in surface penetration studies in whole intact steaks - *Escherichia coli* with GFP expression and *E. coli* with rifampicin resistance
- Laser Scanning Confocal Microscopic (LSCM) examination of core samples from whole steaks in concert with Cryostat sectioning
- Traditional microbiological examination of core samples form whole steaks in concert with Cryostat sectioning

M.S. Research, Department of Food Science and Technology, Virginia Tech
Blacksburg, VA, May 1996 - December 1997

- Designed and conducted experiments for thesis project
- Traditional microbiological examination of raw oysters
  Maintenance of stock cultures - *Vibrio vulnificus*, *Klebsiella pneumoniae*, *Morganella morganii*
- ELISA technique for confirmation of *V. vulnificus*
- Organoleptic evaluation of raw oysters
- Growth curves of stock cultures
- Traditional microbiological examination of raw bluefish
- Organoleptic evaluation of raw bluefish

Teaching

Instructor, General Microbiology , Department of Biology, Virginia Tech, Blacksburg, VA
Summer semester I 2000

Laboratory Instructor, Food Microbiology 4604 , Department of Food Science and Technology, Virginia Tech, Blacksburg, VA
Spring semester 1996, 1997, 1998, 1999, 2000

PUBLICATIONS

Gingerich, T., Lorca, T., Flick, G., Pierson, M., McNair, H. 1999. Biogenic amine survey and organoleptic changes in fresh, stored, and temperature-abused bluefish (*Pomatomus saltatrix*). J. Food Prot. 62: 1033-1037.

Lorca, T., Pierson, M., Flick, G., and Hackney, C. The effect of refrigerated storage on the safety and quality of raw oysters (*Crassostrea virginica*). (submitted for publication in the Journal of Food Protection)

Lorca, T., Gingerich, T., Pierson, M. and Flick, G. Growth of *Morganella morganii* and changing histamine levels in determining the safety and quality of bluefish (*Pomatomus salatrix*). (submitted for publication in the Journal of Food Protection)

ABSTRACTS

Lorca, T., Gingerich, T., Pierson, M. and Flick, G. 1998. Growth of *Morganella morganii* and changing histamine levels in determining the safety and quality of bluefish (*Pomatomus salatrix*). Institute of Food Technologists Conf., Atlanta, GA.

Lorca, T., Pierson, M., Flick, G., and Hackney, C. 1998. The effect of refrigerated storage on the safety and quality of raw oysters (*Crassostrea virginica*). International Association for Food Protection (formerly IAMFES) Conf., Nashville, TN.