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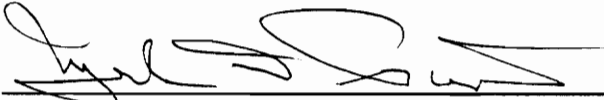
Growth and Survival of Clostridium botulinum type E  
in Pasteurized Oysters

by

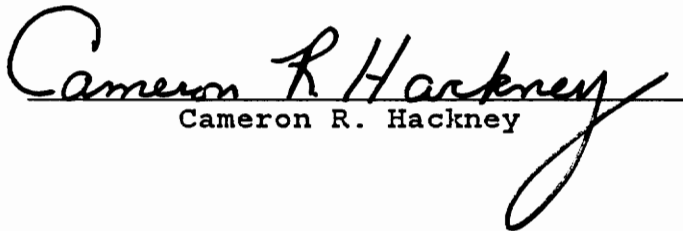
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Growth and Survival of Clostridium botulinum type E  
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by

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Food Science and Technology

(ABSTRACT)

The risk of toxin production by Clostridium botulinum type E in pasteurized oysters was evaluated. Thermal death time studies for type E spores in a oyster homogenate showed that the spores survived pasteurization at 55°C; D-values ranged from 65 to 100 min at 70°C and 880 to 1300 min at 55°C. When tubes of oyster homogenate were inoculated with  $1 \times 10^3$  type E spores/ml and stored at 3, 6, 12, and 30°C, no growth was observed; however, when the inoculum was increased to  $1 \times 10^4$  spores/ml, toxin was produced during storage at 30°C, but not at lower temperatures. For containers of whole oysters inoculated with a low spore level, toxin production occurred during incubation at 6, 12, and 30°C.

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## I. Introduction

Fresh oysters have a short shelf-life and thus their sale is often limited to coastal areas. A pasteurization process was developed at V.P.I & S.U. [Butler, 1984] which extended the shelf-life of oysters (45 to 60 days versus 10 to 14 for fresh) while maintaining comparable sensory characteristics to that of fresh raw oysters. This process involved both heating oysters to 55°C and the addition of 1.0% salt and 0.13% sorbate.

One of the primary concerns in pasteurized food products is growth of the bacterium Clostridium botulinum type E, which is the causative agent of the foodborne intoxication botulism. This psychrotrophic sporeformer may be capable of surviving mild heat treatments given in pasteurization processes. Surviving spores may grow and produce toxin in products they are stored at temperatures above 3.3°C.

The purpose of this research was to evaluate the effect of the V.P.I. & S.U. oyster pasteurization process on C. botulinum type E. This objective was achieved in two parts. First, the heat resistance of type E spores was determined in oyster homogenate. The purpose of this was to determine whether spores will survive the heating process of pasteurization. Since spores may survive this process, it was essential to evaluate the potential for C. botulinum

spore outgrowth and toxin production in pasteurized oysters at different storage temperatures.

## II. Literature Review

### A. Oysters

#### 1. General

For centuries, the oyster has been an important food source. There are three species that are commercially harvested in the United States. Crassostrea virginica is the species found on the East and Gulf coasts. The other two oyster species are found on the West coast; Ostrea lurida, once an important species until about a century ago, and Crassostrea gigas, the Pacific oyster, which was imported to the west coast from Japan.

Of the three, the most important species is the eastern oyster. The harvest of this oyster has decreased steadily within this century, from a record high in 1908 of 152,046,000 pounds to 35,013,000 pounds in 1986. The 1986 harvest however, was valued close to 78,068,000 dollars. Much of the decrease in harvest has been due to overfishing, increased pollution of the harvesting waters, storms, shoreline development, and disease (dermo and MSX).

Oysters are bivalved mollusks that are found in shallow waters between tidal levels and estuaries where they attach themselves to rocks and feed by filtering microscopic plants and animals from the water. They are commercially harvested from these waters primarily by two methods, tonging and dredging. Tongs are two long tooth rakes hinged together

midway on the length of the handles. Oysters are scooped from the bottom by the teeth of these rakes. The dredge is a metal frame with a toothed bar across the front with a chain mesh connected behind. This bar is drug across the bottom, dislodging the attached oysters which get caught in the chain mesh.

Freshly harvested oysters are sold as shellstock or as processed. After harvesting, the shellstock are broken into singles and may be washed before chilling. More oysters are sold shucked; those in which the meats have been removed from the shell. After shucking, the freed meats are first blown, a process of washing in an air-agitated bath to remove shell and debris. After this they are sized, packed in glass, metal or plastic containers, and shipped on ice. Shucked oysters are also quick frozen to make them available throughout the whole year.

Shucked Crassostrea virginica are sold on the basis of size. The Standard of Identity is based on the amount of oyster meats per gallon. Extra large is not more than 160 per gallon; large (extra selects), 160-210; medium (selects) 210-300; small (standards), 300-500; and very small, greater than 500.

The U.S. FDA has established market guidelines for the microbiological quality of oysters to be sold on the fresh market. Criteria include having less than  $5 \times 10^5$

microorganisms per gram based on aerobic plate count at 35°C. Fecal coliforms should be  $\leq$  230/100gram. The pH should also be above 6.0.

The decomposition of oysters which results in the reduction in quality is due primarily to spoilage bacteria. These spoilage bacteria are part of the natural flora initially present in the oysters when harvested. Oysters contain a large amount of carbohydrates, mainly glycogen, in comparison to other seafoods. As spoilage occurs, fermentation of these carbohydrates causes a decrease of pH [Jay, 1988].

A number of studies have examined the flora of the oyster [Colwell et al., 1960; Goldmintz et al., 1983; Keuh et al., 1985; Lovelace et al., 1968; Murchelan et al., 1968; and Pace et al., 1988]. Genera that have been isolated include Vibrio, Pseudomonas, Achromobacter, Cytophagia, Corynebacterium, Acinetobacter-Moraxella, Bacillus, Lactobacillus, Micrococcus, and Clostridium. Of the species isolated, the gram positive organisms comprise only about 10% of the species found [Goldmintz et al., 1983]. Also, there are a few species of yeast that have been isolated [Hood et al., 1983b].

Based on the high incidence of the same gram negative organisms, it is believed that there is a commensal flora in oysters. Commensal flora are found in the gut of higher

species, however, there it is comprised of mainly gram positive organisms [Colwell et al., 1960; Vasconcelos et al., 1972]. Also, unlike higher animals, the flora of oysters does not appear to be permanently attached. Garland et al. [1982], using electron microscopy, did not find microorganisms attached to the epithelium and rarely associated with the adhesive mucous.

The water from which the oysters are harvested has a great affect on the types of bacteria isolated. Factors such as salinity, water temperature, proximity of freshwater as well as sewage outfall sources influence the types of bacteria found in water. This, in turn, is seen in the organisms isolated from the oysters.

For fresh shucked oysters, the number of bacteria range from  $10^4$  to  $10^6$  organisms per gram [Kueh et al., 1985; and Wentz et al., 1983]. Higher numbers are often seen in oysters harvested from warmer waters [Vanderzant et al., 1973]. During refrigerated storage, the concentrations of most species continue to increase, but there is a decline in species of Vibrio [Goldmintz et al., 1983; Hood et al., 1983a]. Pseudomonas and Moraxella species predominate and are primarily responsible for early stages of spoilage [Jay, 1988]. During the estimated two week shelf-life, the number of bacteria will increase an average of about two log units and the pH will drop below 6.0 [Hoff et al., 1967].

As decomposition continues, the pH will continue to decrease as oyster glycogen is fermented. As the pH approaches 4.0, lactic acid bacteria and yeast become the predominate organisms [Hunter et al., 1925].

## 2. Risks associated with oyster consumption

Oysters and other shellfish have been indicated as vehicles of a number of foodborne diseases. These mollusks are filter feeders and have the ability to concentrate microorganisms. Oysters from fecally contaminated water have been shown to concentrate coliforms up to 25 times the concentration found in overlying waters [Perkins et al., 1980]. This can be a problem if there are pathogenic microorganisms in the water such as Salmonella or enteric viruses. This problem is further compounded by the fact that oysters are often eaten raw, therefore, these pathogens are not destroyed in heat preparation. To assess the risk of foodborne illness associated with the consumption of oysters, it is important to look at the likelihood of contamination (bacterial or viral), survival and growth of such contamination, and the effect any processing may have on its survival [Byran, 1988].

There are a great number of pathogenic organisms which have the potential of contaminating oysters and being passed onto the consumer. Shellfish contamination is classified

into three categories based on the primary source of this agent; a) agents naturally present in the aquatic environment; b) sewage pollution of the aquatic environment; and c) contamination from workers, equipment, or the environment of food processing or foodservice establishments [Bryan, 1980].

Pathogens which are normally present in aquatic environment include members of the family Vibrionacea and Clostridium botulinum type E [Hackney and Dicharry, 1988]. Pathogens in the family Vibrionacea include members of the genus Vibrio as well as Aeromonas hydrophila and Plesiomonas shigelloides.

There are eleven members in the genus Vibrio which are pathogenic to humans. Of these, six have been associated with foodborne disease: V. parahaemolyticus, V. cholerae, V. vulnificus, V. mimicus, V. hollisea, and V. furisii. The first three mentioned are the most important.

Vibrio parahaemolyticus, an aquatic bacterium, is often found in waters from which oysters are harvested as well as oysters themselves [Bartley and Slanetz, 1971; Beuchat, 1982; Kaneko and Colwell, 1973; and Thompson and Vanderzant, 1976a]. The incidence of this organism is dependent on the water temperature, only being isolated from warmer waters. During the cooler months, it is found in the sediment [Kaneko and Colwell, 1973]. Although frequently isolated,

the majority of V. parahaemolyticus isolates are found to be nonpathogenic. Virulence is determined by a hemolysin test, the Kanagawa reaction, which differentiates pathogenic strains by their ability to produce heat-stable hemolysin. The majority of the strains isolated from marine sources are Kanagawa negative [Hackney et al., 1980, Thompson and Vanderzant, 1976c].

V. parahaemolyticus has been shown to be quite sensitive to hot and cold temperatures [Beuchat, 1982]. The concentration of this organism decreases rapidly in refrigerated oysters [Hood et al., 1983b; and Thompson and Vanderzant, 1976b]. Delmore and Crisley [1979] found a low thermal resistance of V. parahaemolyticus in clam homogenate. At 55°C, the D-value ranges between 0.03 and 0.24 minutes. Sodium chloride has been shown to have a protective effect for this organism in regard to the organisms sensitivity to temperature extremes [Covert and Woodburn, 1972].

Vibrio cholerea, an aquatic pathogen associated with oysters, is divided into two groups, serotypes 01 and non-01. Toxigenic strains of serotype 01 are responsible for the disease cholera while strains of the group non-01 are associated with gastroenteritis. Of the two, the non-01 species are much more prevalent in U.S. coastal waters [Madden, 1982]. Kaper et al. [1979] surveyed the Chesapeake

Bay for V. cholerea and isolated this organism from 13 of the 21 stations sampled, generally within water of a salinity between 4‰ and 17‰. Concentrations in waters are low (1 to 10 cells/liter). They also isolated this species from oysters, but in low numbers. Kaysner et al. [1987a] had similar results in a survey of waters on the West coast.

Unlike V. parahaemolyticus, V. cholerea will survive for up to one week or more at refrigeration temperatures [DePaola, 1981]. Heat resistance of this organism in crab homogenate was measured. At 54°C, the D-value is 2.65 minutes [Shultz et al., 1984]. Boutin et al. [1982] demonstrated that common cooking recipes reduced the number of V. cholerea by 5 logs/gram.

V. vulnificus has recently become a great concern because of its high invasiveness. Infection by this organism is rare in normal healthy people but is particularly virulent in individuals with high iron levels such as those with liver disorders. The mortality rate for those infected is between 40 and 60 %. Infection occurs during digestion of contaminated food; most commonly, raw oysters.

V. vulnificus has been isolated from seawater, usually in the summer months [Kelly, 1982; and Oliver et al., 1982]. It has also been isolated from oysters [Kaysner et al.,

1987; and Kelly et al., 1985]. While this organism may accumulate in oysters, it has not been shown to multiply there.

Plesiomonas shigelloides and Aeromonas hydrophila are two other members of the family Vibrionaceae that have been implicated in oyster-associated gastroenteritis [Abeyta et al., 1986; and Miller and Koburger, 1985]. There are no known studies examining the growth of Plesiomonas in oysters, while Aeromonas has been shown to be capable of growth in oysters at refrigeration temperatures [Miller and Koburger, 1986].

One pathogen commonly found in the aquatic environment is Clostridium botulinum Type E. While other types of C. botulinum (A, B, and F) can be isolated from water, it is type E which causes most seafood related botulism outbreaks. While spores of type E are much less heat resistant than those of type A and nonproteolytic type B, its ability to grow at refrigeration temperatures is its greatest threat, especially in seafood processed such that type E would survive. The incidence of this bacterium in oysters is low [Presnell et al., 1967; and Sayler et al., 1976], and there has been no known botulism outbreak due to oysters. C. botulinum type E will be examined more closely in the second half of this review.

Pathogens not naturally found in seawater can enter into harvesting waters from pollution such as sewage contamination. Bacterial species and enteric viruses from contamination then may be concentrated by oysters.

In the past, Salmonella typhi, the causative agent of typhoid fever, was often associated with oysters harvested from heavily polluted waters. Today, however, there are fewer carriers of the disease and sewage treatment facilities are much more effective, therefore there is less chance of oyster contamination unless the oysters are harvested from grossly polluted waters [Bryan, 1980]. There is still the possibility of other, more common Salmonella species, those responsible for foodborne gastroenteritis, to contaminate harvesting waters and thus oysters. The incidence of these Salmonella species is dependent on fecal contamination and is therefore correlated with fecal indicators [Sayler et al., 1976]. However, Fraiser and Koburger [1984] suggest that based on the serotypes isolated and consistent recoveries throughout the year, Salmonella may represent a natural portion of the natural background flora. Survival studies in refrigerated oysters indicate that Salmonella may survive over 14 days [Hood et al., 1983b].

There are other pathogens which are associated with sewage that have the potential to cause oyster linked

illness, although no known related illness has been identified. Arumugwany et al. [1987] examined the incidence of Campylobacter jejuni and Campylobacter coli in the Sydney Rock Oysters. The two species were detected in 14% of the samples. Other pathogens which may be found in sewage include Clostridium perfringens, Yersinia enterocolitica, and Listeria monocytogenes. All of these pathogens are associated with warm blooded animals and sewage contamination and can be isolated from oysters.

Gerba [1988] recently reviewed viral disease transmission by seafood. Shellfish have been known to be a vehicle of viral diseases since the early 1950's. Of the 100 known enteric viruses, those shown epidemiologically to cause shellfish related disease include hepatitis A, non A, non B hepatitis, Norwalk, Snow Mountain Agent, Astroviruse, calicuvires, and small round viruses. Of these, the most important are hepatitis A and Norwalk virus. It is believed that enteric viruses find their way into estuaries by means of direct or indirect flow of sewage. Some can survive for over 100 days in marine waters. This survival may be increased if the viruses becomes associated with sediment.

Shellfish can rapidly accumulate viruses to much higher concentrations than that found in the surrounding waters. Maximum accumulation in oysters can occur within a few hours. These viruses may survive in refrigerated oysters

for several months [Gerba and Goyal, 1978].

After harvesting, contamination of oysters by pathogenic organisms may occur during handling, processing, and packing. Types of organisms include many of those which have been previously mentioned, such as Salmonella species and enteric viruses [Bryan, 1980]. Others pathogens such as Staphylococcus aureus and Shigella may also contaminate oysters during shucking. Contamination can be prevented by following proper sanitary procedures.

While it is impossible to prevent contamination by pathogens naturally present, it is possible to control pathogens from pollution. This would reduce the incidence of pathogenic illnesses due to oysters.

Oysters from beds which have been contaminated with sewage should not be harvested. To identify contaminated beds, indicator organisms for fecal contamination are used; these are the fecal coliforms. There are standards for harvesting waters and guidelines for oyster meats [Hunt, 1980]. These standards and guidelines set limits of less than 14 fecal coliforms/100ml for harvesting waters by the MPN procedure and a limit of 230 fecal coliforms/100g of oyster meat also by the MPN procedure.

While these standards and guidelines correlate well with Salmonella and thus are good indicators of such contamination [Andrews et al., 1975; and Hood et al.,

1983a], there are some drawbacks in using them. One drawback is the inability to accurately predict viral contamination of oysters. Investigators found little or no correlation between fecal coliforms and the presence of enteric viruses in oyster meats or overlying waters [Cole et al., 1986 and Ellender et al., 1980]. Viruses have been found in oysters harvested from water which met bacteriological standards [Gerba et al., 1980; and Vaughn et al., 1980]. A possible explanation is that virus survival in waters is much longer than that of fecal coliforms. Viruses can become associated with sediment particles which enhance survival and may also serve as a reservoir for viruses [Gerba and Goyal, 1978]. Fecal coliforms also show little correlation with bacterial pathogens naturally found in water such as the Vibrionaceae [Eyles and Davey, 1988; Hackney et al., 1980; Sharis et al., 1987; and Thompson and Vanderzant, 1976a]. Another problem is that non-E. coli fecal coliforms that increase in concentration in harvesting waters during the summer are not indicative of fecal contamination [Paille et al., 1987].

A way to rid harvested oysters of potential pathogens is through relaying and depuration. This topic has been extensively reviewed by Richards [1988]. Relaying is a process where shellfish from contaminated water are transferred to clean shellfish growing areas. Here the

oysters are allowed to purge themselves of contamination. Depuration is similar to relaying but instead of the natural environment, oysters are purged in man made facilities. These methods are effective in reducing levels of bacteria and viruses that are concentrated in shellfish. However, there has been outbreaks of Vibrio related illness due to contamination of shellfish. This may be due to the fact that Vibrio is not effectively removed by depuration. The same has been seen for viruses [Richards, 1988].

### 3. Further Processing

There is a possibility for many of the pathogens discussed above to infect the consumer via oysters. Further processing by a heat treatment is probably the best way to rid oysters of pathogenic microorganisms. This can be done by the consumer through cooking the oysters during food preparation or by a food processor in some type of value added product. The safety of the cooked or processed oyster will not only depend on the thoroughness of the heating process, but also the heat resistances of the types of pathogenic organisms present (Table 1). Although an oyster may be cooked, it does not necessarily destroy all potential pathogens. Some of these organisms are heat resistant enough to survive some types of processing which may not be stringent enough. The problem of surviving organisms may be

Table 1. D-values<sup>a</sup> ( min ) for selected pathogenic microorganisms.

| Organism                                      | D-value<br>150°F | D-value<br>131°F   | z-value<br>°F | Heating<br>medium | References               |
|-----------------------------------------------|------------------|--------------------|---------------|-------------------|--------------------------|
| <u>Aeromonas hydrophila</u>                   | 0.02             | 0.62               | 13.8          | saline            | Palumbo et al. [1987]    |
| <u>Campylobacter jejuni</u>                   | 0.02             | 1.00               | 11.4          | skim milk         | Doyle and Roman [1981]   |
| <u>Clostridium botulinum</u><br>type E spores | 192 <sup>b</sup> | 8050 <sup>b</sup>  | 11.7          | crabmeat          | Lynt et al. [1982]       |
| <u>Listeria monocytogenes</u>                 | 0.19             | 28.8               | 13            | milk              | Bradshaw et al. [1986]   |
| <u>Salmonella senftenburg</u> 775W            | 1.70             | 49.2               | 13            | oyster            | Goldmintz et al. [1978]  |
| <u>Salmonella typhimurium</u>                 | 0.06             | 5.9                | 9.5           | milk              | Bradshaw et al. [1987]   |
| <u>Staphylococcus aureus</u>                  | 2.00             | 158.               | 10            | various           | Stumbo [1973]            |
| <u>Vibrio cholerae</u>                        | 1.08             | 4.32               | 28            | crabmeat          | Shultz et al. [1983]     |
| <u>Vibrio parahaemolyticus</u>                | 0.03             | 0.24               | 22.3          | clam              | Delmore et al. [1979]    |
| Viruses (rotaviruses)                         | 1.78             | 470.2 <sup>b</sup> | 7.8           | media             | Van Donsel et al. [1986] |
| <u>Yersinia enterocolitica</u>                | 0.31             | 36.2               | 9.2           | milk              | Lovett et al. [1982]     |

<sup>a</sup> D-values are calculated from reported D and z-values.

<sup>b</sup> Values calculated are extrapolations outside the range of actual experimental temperatures.

compounded by any abuse that may occur after being processed. Another point is that oysters are often eaten raw and with any heating, they lose that "raw" flavor desired by the consumer. Hence, there is great risk of foodborne illness due to the consumption of raw or not fully cooked oysters. Butler [1984] developed a pasteurization process for oysters in order to maintain the raw taste qualities while adding some additional shelf-life and perhaps some safety against shellfish related foodborne illness.

There is great interest in a process that would extend the relatively short shelf-life of fresh oysters. Increasing the two week shelf-life to perhaps several months would not only allow for greater product distribution, but would also help to stabilize the industry by allowing better utilization of oversupply during the off season.

There have been several processes which have been developed for shelf-life extension. They utilize chemical preservatives, vacuum packaging, a mild heat treatment, or a combination of these. Although processed, these products are not commercially sterile and therefore must be kept under refrigeration conditions. None of these processes are used commercially today.

Abbey et al. [1957] and Lartigue et al. [1960] found that by washing in low levels of chlorotetracycline (1 to 30

ppm.), oysters remained organoleptically acceptable longer than untreated controls. The treated samples also have lower bacterial counts.

One of the more practical methods for the shelf-life extension of oysters is pasteurization. This may be just a heat treatment by itself or in combination with another treatment such as chemical additives. However, there are some constraints which must be considered in development of such a product. Unlike seafood products such as crabmeat, raw oysters are much more sensitive to processes such as high heat. There is also the possibility pathogenic bacteria may survive heat treatment and then grow. Growth may possibly occur more readily in these treated foods due to the absence or reduction in numbers of competing spoilage microorganisms. There is always the likelihood of temperature abuse to a product which "appears" to be more shelf stable.

Goldmintz et al. [1978], established the times and temperatures for the destruction of Salmonella senftenburg 775W in oyster homogenate and oyster shellstock. They found that at 140°F (60°C), this bacterium had a D-value of 10 minutes and a z-value of 13°F (7.2°C) in oyster homogenate. Actual tests in shellstock showed that thermal destruction was similar to that in the homogenate. Using this bacterium as an indicator, they showed that its destruction would

offer a wide safety margin for less heat resistant gram negative organisms: Esherichia coli, Pseudomonas fluorescens, and Salmonella typhimurium.

Goldmintz et al. [1984] using the thermal destruction data of Salmonella senftenburg 775W, developed a pasteurization process that would destroy any gram negative pathogens and hopefully any enteric viruses. In this process, shellstock was steamed to a temperature of 62°C and then rapidly cooled. This process increased the shelf-life for up to 8 weeks. The bacteriological data revealed that at 62°C, total counts were reduced from  $10^5$  to  $10^2$  organisms/gram. These surviving organisms were mainly gram positive, mostly Bacillus species. There was concern about the growth of the gram positive sporeformer C. botulinum. When C. botulinum spores were inoculated into oysters at levels up to  $10^5$  spores/gr, and stored at 3°C, there was no toxin production. No toxin production was noted when spore levels of  $10^2$  spores/gr were used in inoculated pack studies and the product was stored at 10°C or 30°C. However, toxin was found at elevated spore levels stored at 10 and 30°C.

Ward [1980] observed that with the pasteurization of shellstock, there was the problem of cross contamination during shucking. This could reduce some of the benefit gained in the heating process.

Chai et al. [1984] developed a process utilizing both a sanitizing chemical treatment, vacuum packaging, and a heat treatment. Shucked oysters were first washed in 150 ppm chlorine water for 20 minutes followed by vacuum packaging in flexible pouches. These pouches were then pasteurized at 72°C for 8 minutes and rapidly cooled in ice water. Sensory evaluation after pasteurization revealed no significant difference between treated and untreated oysters in odor, appearance, flavor, and texture. Although the pH decreased to unacceptable levels after two months of storage, the pasteurized oysters still had an acceptable appearance. Both aerobic and facultative anaerobic counts drastically decreased but enzymes such as lipases were still present and may have been the main cause of deterioration.

The effect of the process developed by Chai et al. [1984] on the bacteriological flora was examined by Pace et al. [1988]. They showed a 99.9% reduction of the bacterial flora during heating. The remaining flora was composed of gram positive organisms including Bacillus, Clostridium, Listeria, Peptostreptococcus, and Staphylococcus. The predominant organism was Bacillus. Because there were no gram negative organisms, the cause of spoilage would be different than that of raw oysters. Swelling of the products occurred after 5 months of storage.

Shaw [1981] investigated the shelf-life extension of oysters by the use of potassium sorbate. Oysters were blown for 2 minutes in both a 1% and a 3% potassium sorbate solution. The 3% solution yielded the lowest mesophilic and psychrotrophic counts. While the use of potassium sorbate gave an off aroma only initially, the variable with added potassium sorbate was generally preferred, although there was no difference in overall acceptability.

Shaw also examined the addition of a heat treatment in conjunction with the addition of potassium sorbate. Looking at the thermal death rate of Pseudomonas fluorescens in oyster homogenate with the addition of potassium sorbate, it was postulated that a mild pasteurization would be feasible.

In a follow up to Shaw's work, Butler [1984] investigated the shelf-life extension by the use of a mild heat treatment, 55°C for 10 minutes. The effect of 1% added salt and a combination of 1% salt and 0.13% potassium sorbate was also investigated. Pasteurization by itself increased the shelf-life up to 45 days. The addition of salt improved acceptance but did not improve shelf-life. The addition of the combination of salt and sorbate realized a shelf-life approaching 60 days. Although these heated and chemically treated oysters had acceptable aerobic and psychrotrophic plate counts and a pH above 6.0, the "raw" sensory scores dropped after 45 days of refrigerated storage

at 2°C.

Recently the FDA reviewed the oyster pasteurization process put forth by Butler utilizing a heat treatment (55°C for 10 minutes) and a combination of 1% salt and 0.13% potassium sorbate [Anonymous, 1987]. They opposed the addition of sorbate in the process and expressed some concern about the safety of the packages as well as the possible use of "old" shellfish by unscrupulous processors.

#### B. Incidence and Growth of Clostridium botulinum Type E

##### 1. Introduction

Clostridium botulinum is the etiological agent of the foodborne disease botulism. The disease is caused by the ingestion of a exotoxin (protoplasmic) toxin which is produced by this bacterium.

C. botulinum is a gram positive, anaerobic, sporeforming rod. The genus is divided into seven types (A,B,C,D,E,F, and G) based upon the serological specificity of the exotoxins. Of the seven types, only A, B, E, and F have been shown to cause disease in humans. There is also some question whether type G is a human pathogen.

Although there is some major physiological differences between the strains, the toxin they produce, termed a neurotoxin, has a similar mode of action. The toxin is a

protein comprised of two subunits which is formed inside the cell as a progenitor toxin and is released upon cell lysis. To achieve toxicity, this progenitor must be cleaved, or activated, by proteolytic enzymes. Some strains produce proteolytic enzymes which are capable of activating the toxin. Nonproteolytic strains need exogenous proteolytic enzymes added for the activation of toxin. Once activated, these proteins act as a neurotoxin, interrupting neurotransmission in the victim.

The strains are divided into groups based on proteolytic activity. Types A, some B, and some F produce proteolytic enzymes. Nonproteolytic types include type E as well as some B and F strains. Lynt et al. [1982] reviewed some of the major differences between the proteolytic and nonproteolytic strains. Proteolytic strains have been shown to be more resistant to heat and salt, while nonproteolytic strains are capable of growing at a lower temperature (3.3°C versus 10°C for proteolytic).

The primary concern with the nonproteolytic strains occurs in nonsterile foods that can support the growth of C. botulinum. Processing short of sterilization such as a mild pasteurization destroys much of the competing spoilage organisms, while spores of heat resistant types of C. botulinum may survive. The nonproteolytic strains of this organism then might be capable of growth during storage at

refrigerated temperatures greater than 3.3°C. Also, off odors may not be present and foods may appear acceptable. To prevent food poisoning by the nonproteolytic types, it is important to examine the distribution of these organisms, their heat resistance, their growth in relation to intrinsic and extrinsic food characteristics (pH, salt, competing organisms, storage temperature, etc.) and the effect any processing may have. Taking these factors into consideration, the risk of C. botulinum type E growth and toxin production can be estimated and preventative action taken.

## 2. Distribution

Huss [1981] reviewed the ecology of C. botulinum type E in the environment. Unlike types A and B, this type is almost exclusively associated with marine habitats, mainly in the subarctic and temperate climatic zones.

The numbers for all types of C. botulinum found in waters sampled are usually low. The highest counts are found in the sediment ( less than 100 per gram ). The incidence in marine fish usually follows patterns as seen in the bottom sediments. Presnell et al [1967] examined the incidence in Mobile Bay, Alabama. C. botulinum was found in only 4.1% of the sediment samples and 2.7% of the oyster

samples. Ward et al. [1967a] surveyed the U.S. Gulf Coast. They found 3% to 5% of fish and 5% to 8% of the sediment sampled to contain C. botulinum. In further work, Ward et al. [1967b] found a slightly lower incidence on the Atlantic Coast. Cockey et al. [1974] found C. botulinum in 21 of 24 samples from the Chesapeake Bay. In these surveys, type E was usually the predominate type.

### 3. Heat resistance

Heat destruction of microorganisms is considered to follow first order kinetics and therefore assumes a logarithmic order of death. Relative heat resistance is compared by plotting a survivor curve, which relates the log of the number of organisms surviving at a given temperature and the time in minutes. The inverse slope of the line gives the D-value or decimal reduction time, which is the time required to kill 90% of the population at a particular temperature. To compare the heat resistance of an organism at different temperatures requires the use of the z-value. More specifically, the z-value is the temperature required to change the D-value by 90% or one log cycle. Stumbo [1973] has a monograph which deals with this area of thermobacteriology in much greater detail.

The heat resistances of bacteria, vegetative cells or spores varies with the species of bacteria, conditions under

which the cells are grown, and the inoculum level. The heating menstruum, or medium in which the spores are tested, also influences the heat resistance. Factors affecting bacterial heat resistance include water content, pH, and the chemical composition ( fats, salts, proteins, and carbohydrates ) of the heating menstruum.

In measuring heat resistance at a particular temperature, cells are heated and the survivors are enumerated at given time intervals. The enumeration can be based on either direct plating of the heating menstruum or by the end point method which examines replicate dilutions of the menstruum at different heating times for growth versus no growth. Growth in the end point method may be determined by either subculturing into a nutrient medium or by directly incubating the heating menstruum and examining for signs of growth. After enumeration, a survivor curve is drawn plotting the log of cells/spores surviving versus the time of heating at a given temperature.

Stumbo [1973] described several methods for conducting the thermal resistance determinations. Listed below are a few of the more common methods used for C. botulinum type E.

1) Thermal Death Time (TDT) Tube Method - Inoculated menstruum is distributed in test tubes and then sealed. These tubes are placed in a heated bath for a given amount of time, after which, they are removed, cooled, and

enumerated directly or indirectly by end point growth. While this method is inexpensive, problems exist with the inability to recover all survivors from the tube and with heating and cooling lags.

2) TDT Cans - Similar to above, but small cans (208 x 006) are used. While this method is more similar to actual processing, subculturing is more difficult and when incubated directly, growth is not as easy to detect.

3) Capillary Tube Method - Again similar to the tube method but small capillary tubes are used. This reduces thermal lags but subculturing may be more difficult.

4) Flask Method - The heating menstruum is contained within round bottom flasks which are heated to process temperature. While being constantly stirred, spores are added. Samples are then withdrawn at predetermined intervals and inoculated into media for direct enumeration. This method is rapid and convenient. Heating and cooling lags can be assumed to be negligible. However, this method can only be used for bacteria whose resistances are below the boiling point of water. Also, care must be taken not to splash the contents on the walls of the flask, which would cause unequal heating.

There are problems that exist in measuring heat resistances besides those mechanical ones already mentioned, one of which is the possibility of injured bacteria.

Vegetative cells or spores may survive a heat treatment, but may not be recoverable on the media being used [Foegeding and Busta, 1981]. Another problem is how to handle survivor curves which are not linear. Many researchers have found these survivor curves to have a tailing of the line. This tailing may be an artifact due to technique or may be due to variation in heat resistances of the spore population [Cerf 1977].

The thermal resistances for the nonproteolytic strains, in particular type E, have been found to be much less than that of the proteolytic types. Roberts and Ingram [1965] examined the heat resistance of type E spores in an aqueous solution. The  $D_{176^{\circ}\text{F}(80^{\circ}\text{C})}$  ranged from less than 0.33 minutes to 1.25 minutes. The z-value of the eight strains tested ranged between  $9.0^{\circ}\text{F}$  ( $5.0^{\circ}\text{C}$ ) and  $10.7^{\circ}\text{F}$  ( $5.9^{\circ}\text{C}$ ). Ito et al. [1967], using phosphate buffer as the heating menstruum in TDT tubes, had  $D_{77^{\circ}\text{C}}$  values between 0.77 minutes and 1.95 minutes. Crisley et al. [1968] examined the heat resistance of type E in crabmeat using TDT tubes. They found  $D_{176^{\circ}\text{F}(80^{\circ}\text{C})}$  values to range from 1.6 to 4.3 minutes and Z values to be between  $10.3^{\circ}\text{F}$  ( $5.7^{\circ}\text{C}$ ) and  $13.6^{\circ}\text{F}$  ( $7.6^{\circ}\text{C}$ ). Lynt et al. [1977] examined the heat resistance for five strains of type E in the meat of the blue crab using TDT tubes. The  $D_{180^{\circ}\text{F}(82.2^{\circ}\text{C})}$  was between 0.51 and 0.74 minutes and the z-value was between  $13^{\circ}\text{F}$  ( $7.2^{\circ}\text{C}$ ) and

15°F (8.3°C). Licciardello [1983], found similar z-values for different heating media using capillary tubes and direct enumeration. For strain 8E, z-values were 9.2°F (5.1°C) in phosphate buffer, 9.3°F (5.2°C) in clam liquor, and 9.0°F (5.0°C) in haddock. Comparison of methods for the determination of z-values by direct and endpoint enumeration were also investigated by Licciardello. The Detroit strain had a z-value of 8.2°F (4.6°C) by direct enumeration and 17.0 by end point enumeration.

Scott and Bernard [1982] compared the heat resistances of nonproteolytic type B spores to those of type E. For four strains of nonproteolytic type B spores, D<sub>180°F</sub>(82.2°C) values ranged from 1.49 to 32.3 minutes in phosphate buffer, while for the Saratoga strain of type E, it was 0.33 minutes.

Lynt et al. [1983] addressed the effect of delayed germination by heat damaged spores on estimates of heat resistance of C. botulinum type E. By comparing a 10 week incubation to a 1 week incubation in TDT tubes, there was a 5 to 12% increase in tubes showing positive growth. This gave a general rise in the D values but had little effect on the z-value.

Alderton et al. [1974] looked at the effect of lysozyme on the recovery heated spores. By adding lysozyme to the

recovery medium, there was an 1800 fold increase in the measured heat resistance of type E spores. Without lysozyme,  $D_{175^{\circ}\text{F}}(80^{\circ}\text{C})$  was 1.3 minutes. With lysozyme in the medium,  $D_{195^{\circ}\text{F}}(90.6^{\circ}\text{C})$  was 13.5 minutes. Scott et al. [1985] found similar results with nonproteolytic type B and type E, although the increase was not as great. For nonproteolytic type B, the measured heat resistance increased 16 to 160 times, while that for type E increased 73 times. It is postulated that lysozyme might replace some thermally inactivated germination enzymes.

#### 4. Factors affecting growth

If spores are present in a food and there is no process such as an adequate heat treatment, to destroy them, then there exists the possibility for these spores to grow and produce toxin. Growth is dependant, though, on the intrinsic and extrinsic properties of the food.

It has long been assumed that storage of foods at refrigeration temperatures would prevent growth of pathogenic microorganisms, such as C. botulinum. In the early 1960's however, type E was found to be the causative agent in fish-related outbreaks of botulism. Investigators became concerned since this organism seemed to be capable of growth at refrigeration temperatures [Palumbo, 1986].

One of the first studies to address the psychrotrophic growth of type E was that of Schmidt et al. [1961]. In this study they established a growth minimum of 3.3°C. Roberts and Hobbs [1968] reviewed some of the other early studies showing that nonproteolytic strains as well as type E are capable of growth between 3.5°C and 5°C. Solomon et al. [1977] demonstrated that while  $1 \times 10^3$  spores/gram could grow as low as 4°C in broth, the lowest growth temperature in crabmeat was 12°C. The other nonproteolytic strains showed similar results [Solomon et al., 1982]. Unlike crabmeat, however, Patel et al [1978] found toxin production in oysters stored at 4°C after 90 days.

Blocher and Busta [1983] reviewed bacterial spore resistance to acid. Generally, a pH of 5.0 was shown to be the minimum pH for type E. One of the lowest pH levels allowing growth in a food product was 4.95 in pH adjusted crabmeat [Lerke, 1973]. Recently, Tsang et al. [1985] was able to detect toxin at pH 4.2 in a citric acid modified broth system. However under the same conditions, no toxin production was seen in pH adjusted seafood products at either pH 4.20 or 4.60 [Post et al., 1985].

The established  $A_w$  for inhibition of type E is at 0.97 [Sperber, 1982]. The type of humectant used will influence this  $A_w$  level. For example, a 5.0% NaCl broth ( $A_w = 0.975$ ) was inhibitory for growth compared to a 22.5% glucose broth

(Aw = 0.970) [Emodi and Lechowich, 1969b].

Chemicals are often added to food products to either extend their shelf-life or inhibit growth of bacteria. One food additive studied extensively in relationship to C. botulinum is sorbate. This chemical has been found to affect sporeformers at various stages of their life including germination, outgrowth, and cell division [Sofos et al., 1986]. Seward et al. [1982] investigated the effect of sorbate on type E spores. At pH 5.8 to 6.0, 1.0%, 1.5%, and 2.0% sorbate inhibited either the emergence of vegetative cells or elongation and cell division of those cells that did divide. At pH 7.0 to 7.2, these same levels resulted in aberrant cells with defective cell division.

In food products, there is concern when sorbate is used in lower levels than those found to inhibit C. botulinum. While these levels inhibit spoilage organisms, they may have little effect on C. botulinum especially at higher pH levels. In fact at higher pH levels, sorbate was once considered as a selective factor in media for isolation of C. botulinum [York and Vaughn, 1954].

To better evaluate the risk of growth and toxin production, investigators have looked at the interactive effect of these intrinsic and extrinsic parameters. Baird-Parker and Freame [1967] examined the combined effect of Aw, pH, and temperature on the growth of C. botulinum. It was

noted that by lowering the pH or  $A_w$  of the medium, the minimum temperature at which type E spores could germinate increased. Segner et al. [1966] showed that as the temperature decreased to 8°C, the minimum inhibitory salt concentration decreased from 5.0% to 4.5%. They also showed that the minimum pH allowing growth at 8.0°C is 5.9, but not 5.7. Emodi and Lechowich [1969a] showed similar relationships between NaCl, pH, and growth temperature. At 5°C, the lowest pH allowing growth was between 6.2 and 6.4. Lund and Wyatt [1984] reported the relationship between redox potential and sodium chloride. Lund et al. [1985], and Graham and Lund [1987] studied the effect of low temperature and pH on growth. In general, these investigations demonstrate that these factors have an interactive effect and that to prevent growth, each one does not have to be at the optimal inhibitory level. Rather, a combination of parameters can be used with each at some suboptimal level. This is the basis of the hurdle effect [Farber, 1986].

## 5. Processes that extend shelf-life

C. botulinum type E is of major concern in those food products that have been treated in some manner to extend the shelf-life, but where the product is not commercially sterile. These products are processed to reduce the number

of spoilage organisms or inhibit their growth, and then are stored at refrigeration temperatures to keep growth of remaining spoilage organisms at minimal levels. These processes, however, may effect C. botulinum to a lesser extent. Type E spores, if present, are capable of growth at refrigeration temperatures. In addition, growth of type E may be no longer impeded by the once competitive spoilage organisms due to drastically reduced number or function.

Seafood products are often pasteurized to extend the shelf-life. Pasteurization is the application of a mild heat treatment to either eliminate pathogenic organisms or to reduce the number of spoilage organisms present in the product. The effect on type E spores will depend on the amount of heat the product receives. Blue crab heated at 185°F (85°C) for 1 minute reduced Type E spores by at least 8 log cycles. Upon subsequent storage at 40°F (4.4°C) for 6 months, no toxin was formed [Cockey et al., 1974]. In another study, crabmeat was inoculated with  $1 \times 10^3$  type E spores/ml, heated to 180°F (82.2°C) for 5 minutes and then stored at 40°F (4.4°C). Toxin was produced in 30 to 40 days [Lerke and Farber, 1971]. Goldmintz et al. [1984] heated oyster shell stock inoculated with different levels of C. botulinum type E spores to 62°C. No unheated oysters, or pasteurized oysters stored at 5°C became toxic. However, toxin was produced in pasteurized oysters stored at the

abuse temperature of 10°C and 30°C.

To extend shelf-life of seafood, other methods besides pasteurization are used. These include hot process smoking, ionizing radiation, and modified atmospheric storage. Like pasteurization, these processes reduce or inhibit the spoilage organisms but may have little effect on C. botulinum. The risk associated with these processes and C. botulinum type E are covered in a review by Eklund [1982].

Chemical additives are often used in conjunction with other process such as pasteurization and vacuum packaging for shelf-life extension. Fletcher et al. [1988] investigated toxin production by types A, B, E, and F spores in vacuum packaged scallops with 0.1% sorbate added. Type A toxin was found in vacuum packaged scallops (pH 6.2 to 6.4), with and without added sorbate, stored at 27°C. Toxin was found to form more rapidly in those with added sorbate. Vacuum packaging created an anaerobic environment and sorbate acted as a selective factor, inhibiting spoilage organisms while having little effect on C. botulinum.

## PART ONE

### Heat Resistance of Clostridium botulinum type E in Oysters

#### III. Materials and Methods

##### A. Bacteria

Clostridium botulinum type E, the Beluga strain, was obtained from the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University. This strain was chosen because it's spores have been shown to be more heat resistant than spores of most other type E strains [Lynt et al., 1983].

##### B. Spore Production

Spores were produced by growing cells in a biphasic system as described by Bruch et al. [1976]. Spores were harvested by centrifugation (2000g for 20 minutes) and then washed three times in distilled water. Further cleaning to remove debris from spores was accomplished by using a modification of the method used by Rowley and Feeherry [1970]. The spore suspension was enzymatically treated with lysozyme (Sigma Chemical Co., St. Louis, MO) and trypsin (Sigma) (100 µg/ml and 50 µg/ml respectively) in 0.5M phosphate buffer (pH 8.1) for 2 hours at 45°C. The spores were then washed 6 more times in distilled water by repeated suspension and 5 minute centrifugation at 4068g. The

cleaned spores were suspended in distilled water and stored at 2°C.

Before use, spores were filtered through a 3µm filter (Nucleopore Corp. Pleasanton, CA) to remove spore clumps. This was done to help prevent any tailing effect in the survivor curve that clumped spores may cause [Cerf, 1974]. Viable spore counts were then done by the roll tube method of Pierson et al. [1974] and the spore suspension was adjusted to approximately  $1.5 \times 10^5$  spores/ml.

#### C. Oyster Homogenate Preparation

For homogenate preparation, frozen oysters were obtained from various sources on the Atlantic Coast. On the day of use, oysters were defrosted and drained. Drained oyster meat (200 ml) was put into beakers (1000 ml) and liquor was added back to the meat to obtain a 85:15 meat to liquor ratio. This was then autoclaved at 121°C for 15 minutes. The hot sterile oysters were then blended for 30 seconds. The pH and the salt were measured for each batch. The pH was adjusted with sterile 0.2N NaOH to 6.20, the pH of fresh oysters [Jay, 1986].

#### D. Salt and pH Analysis of the Homogenate

NaCl was assayed by the method described by Hackney et al. [1988]. One gram of the homogenate was added to 20 ml

of distilled water. This was titrated with silver nitrate (0.171N) using dichlorofluorescein as the indicator.

The pH of each sample was determined by placing the probe of a pH meter in a tube containing a small amount of the oyster homogenate.

#### E. Thermal Resistance Determinations

Homogenate (150ml) was added to round bottom flasks (500 ml). These were submerged in a water bath heated by a circulating heater. Stirring of the flask contents was accomplished by a stir plate located beneath the water bath. Temperature was monitored in the bath by the use of a thermometer with 0.1°C gradations. Also, a sterile thermometer was placed in the flask to be sure that the desired temperature was reached in the homogenate. It was removed at the time of addition of the spores to the homogenate.

After flasks had reached the desired temperature, approximately 2 ml of spores were added to attain a concentration of  $2 \times 10^4$  spores/ml in the homogenate. At selected time intervals, a sample (1 ml) was withdrawn using a wide mouth pipet and enumerated.

Spores were tested in 4 homogenate variables: control, 1% salt, 0.13% potassium sorbate (KS), and a combination of 1% salt and 0.13% KS. For the 1% salt variable, NaCl was

added in granular form to the homogenate before autoclaving to a level of 1% w/v of the homogenate. For 0.13% sorbate variable, a filter sterile potassium sorbate solution (0.975 g/ml) was added to the homogenate to achieve the desired concentration. The addition of sorbate was after autoclaving and immediately before the addition of the spores.

Heating temperatures used were 50°C, 55°C, 60°C, 70°C, and 80°C. For the lower temperatures (50°C through 60°C), flasks were stirred simultaneously in a large water bath over a multistir plate. At the two upper temperatures, flask variables were tested one at a time.

#### F. Enumeration of Surviving Spores

Samples were diluted in prereduced 0.1% peptone buffer. Roll tubes containing prereduced peptone yeast glucose (PYG) agar were used to determine anaerobic counts.

The inoculated tubes were incubated at 32°C for 48 hours. Colonies were counted using a dissecting microscope.

#### G. Calculation of D-values and z-values

The log number of survivors was plotted against time and the D-value was calculated. Using this procedure, no correction was made for the thermal lag [Stumbo, 1973]. Using these calculated D-values, a phantom thermal death

time curve was calculated and z-values were estimated from this. Statistical analysis was done using SAS [1986].

#### IV. Results and Discussion

##### A. Chemical Analysis of Oysters

The salt content of the oysters ranged from 0.2% to 0.7% and with an average of 0.4%. As expected the addition of 1% salt increased the measured salt concentration of each sample by 1%.

The pH of oysters dropped 0.1 to 0.3 units during autoclaving to an average pH of 5.93. Oysters with salt added were on average 0.1 units lower. NaOH (0.2N) was added to the homogenate to adjust the pH to 6.20 that of fresh oysters. After addition, the mean pH for both salt and no salt variables was 6.19. However, more NaOH was required for the adjustment of those with added salt than those without (3.8 ml versus 2.3 ml of 0.2N NaOH).

##### B. D-value determination

Survival curves for both trials are given in the Appendix. To evaluate the heat resistances of C. botulinum type E spores in oyster homogenate variables, D-values were calculated from the slopes of these survivor curves for each variable in two trials at five temperatures. Regression analysis was performed on the points visually falling in the straight line portion of each survivor curve. These values are given in Table 2.

Table 2. D-values\* for C. botulinum type E spores in oyster homogenate.

| Treatment | Heating Temperature |         |         |         |        |
|-----------|---------------------|---------|---------|---------|--------|
|           | 50°C                | 55°C    | 60°C    | 70°C    | 80°C   |
| Trial 1   |                     |         |         |         |        |
| Control   | 21000A,a            | 1300A,a | 840A,a  | 65A,a   | 1.2B,a |
| Sorbate   | 19000A,a            | 1200A,a | 1100A,a | 67A,a   | 0.8A,a |
| Salt      | 280000B,a           | 1200A,a | 860A,a  | 100B,a  | 2.0C,a |
| Sal+Sor   | 23000A,a            | 1200A,a | 1100A,a | 67A,a   | 1.0B,a |
| Trial 2   |                     |         |         |         |        |
| Control   | 3000A,b             | 1200A,a | 720A,b  | 84A,B,b | 0.5A,b |
| Sorbate   | 2900A,b             | 1200A,a | 750A,b  | 77A,b   | 0.5A,b |
| Salt      | 2100B,b             | 920B,a  | 560B,b  | 100C,a  | 0.5A,b |
| Sal+Sor   | 1900B,b             | 880B,a  | 560B,b  | 93B,C,b | 0.3B,b |

\* D-value in minutes

A-C letters that are different represent significant difference ( $p \leq 0.05$ ) among variables at a given temperature for each trial.

a,b letters that are different represent significant difference ( $p \leq 0.05$ ) among corresponding variables between trials at a given temperature.

These points fit well into the linear model of thermal death as evidenced by the R-square value which was generally greater than 0.90. The first trial at 50°C showed the greatest deviation with R-square values as low as .01. This may be due to the visually high consistency of the homogenate which made enumeration difficult.

No obvious tailing of the survivor curve was evident in any of the survivor curves. Tailing is a major problem in determining thermal resistance because of the difficulty in determining the straight line portion of the curve. One potential reasons for tailing is clumped spores [Cerf, 1977], which was of great concern in this study because of their common occurrence in our spore preparations. By first filtering the spore preparation through a 3µm filter, clumps were removed.

The flask method proved to be a very convenient and economical tool for the elucidation of thermal resistance of type E spores. Heating and cooling lags are considered insignificant because spores are added directly into the heating menstruum so come up time is negligible. Samples are removed in small amounts by pipets so cooling is rapid.

One major problem with this method is temperature differences which may occur. This may be seen as cool temperature pockets in the menstruum or in the water bath. Cooling may also occur when flask contents are splashed onto

the side of the flask.

Besides possible causes of inaccurate determinations of heat resistance inherent in the system, there were some encountered in our design. When conducting trials at 80°C, the thermal death rate was so rapid that it was difficult to withdraw samples quickly in the time before the destruction of all spores. Thus the number of data points is small. This can be seen in the second trial at 80°C. Also, the possibility of thermal lag becomes significant during cooling because the high temperature may have some effect even though the homogenate cools rapidly in the pipet.

Homogenate variation, both physical and chemical, may also change the thermal resistances of bacteria. This problem was obvious in Trial 1 at 50°C where the consistency was different from the other samples. Here, the thicker homogenate yielded D-values which were larger than anticipated. The thicker homogenate may have added additional protection to spores from thermal destruction. Although not evident in other samples, variation in homogenate may have subtle effects on the heat resistances of the spores. The cause of homogenate variation is mainly due to differences naturally seen in oysters.

Direct enumeration of spores in a solid media had some drawbacks. Lynt et al. [1983] demonstrated that many spores exhibit delayed germination. Therefore, broth is more ideal

in that you can incubate for longer periods when looking for growth versus no growth. In direct enumeration using solid media, counts are made after 48 hours of incubation because of possible overgrowth. This, therefore, does not take in account spores exhibiting delayed germination. In addition, solid media is more stressful on bacteria, so recovery may be decreased especially in the case of injured bacteria.

Counting of colonies was a problem even though a dissecting microscope was used. Colonies were often mistaken for oyster particles and visa versa. This posed a great problem because of the low spore load used in this study. Much of the counting was done at low dilutions where oyster pieces were prevalent.

Despite possible inaccuracies in values that may have been caused by methodology, the thermal destruction rates calculated from the survivor curves are consistent with those given in the literature. In general, expected D-values at 80°C should range from 0.3 minutes to 3.3 minutes [Angelotti, 1968]. In this investigation, D-values at 80°C ranged from 0.3 minutes to 2.0 minutes and at 70°C, values ranged between 65.3 and 100.0 minutes. In comparison to a specific heat resistance study in food, Lynt et al. [1983] reported D-values for the Beluga strain in clam homogenate at 165°F (73.9°C) to be 11.22 minutes and at 175°F (79.4°C) to be 1.19 minutes. Using the z-value of 11.7°F which they

calculated, the computation of a D-value at 80°C is 0.98 minutes while at 70°C, it would be 33.5 minutes. However, the latter temperature is below the range studied by these investigators.

There appears to be no known reports on the thermal destruction rates for type E spores below 60°C. Thermal death did occur at these lower temperatures, however rates were so slow that it is unlikely that any food products would be processed for such a time. At 60°C for example, D-values ranged from 500 to 1000 minutes.

The addition of salt increased the D-values significantly at 70 and 80°C. This would indicate that the salt is protecting the spore against thermal inactivation, and hence increasing its survival. This effect has been reported previously [Pflug and Holcomb, 1983]. At the lowest three temperatures in Trial 2, the addition of 1% salt had the opposite effect in that it significantly decreased the D-value. This may indicate that spores undergo different types of inactivation in the two temperature ranges.

The addition of sorbate alone had no significant effect on the D-values of type E spores except at 80°C in Trial 1. The effect when used in combination with salt is most likely due to the salt. While inclusion of sorbate in the heating

menstruum can decrease the thermal resistance of vegetative cells [Shaw, 1981], it has not been shown to have an effect on the heat resistance of spores [Shibasaki and Tsuchido, 1973].

There was a significant difference between the heat resistance of spores used in Trials 1 and 2; heat resistance values were generally higher in Trial 1. The difference may be related to the age of the spores. In Trial 1, spores were used before 2 months of storage. In the Trial 2, the same spores were used after 3 months of storage. There was also a different source for the oysters, therefore possible variation in homogenate.

### C. Z-value Calculations

The z-value can be obtained from the slope of a plot of the logarithm of the D-value versus temperature. This plot is called the phantom thermal death time curve. Figures 1 and 2 are plots of the D-values of Trials 1 and 2. As can be seen, these phantom death time curves are concave up. Pflug and Holcomb [1983] describe similar incidences of this trend.

While one overall slope can be calculated, the curves are biphasic and two slopes can be calculated; one using the upper two temperatures (70 to 80°C) and another slope using the values from the lower temperatures. Z-values calculated

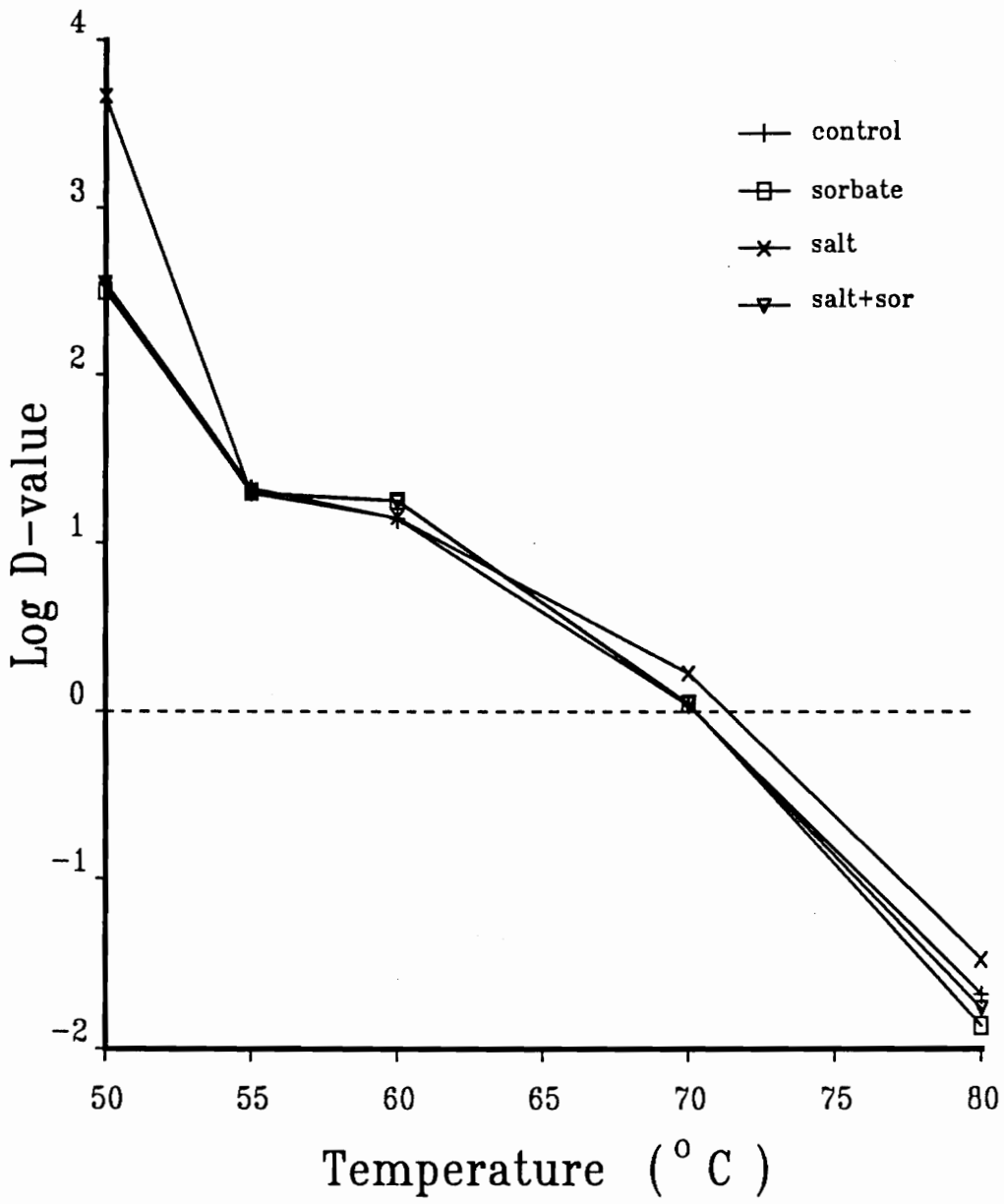


Figure 1. Phantom thermal death time curve for Trial 1.

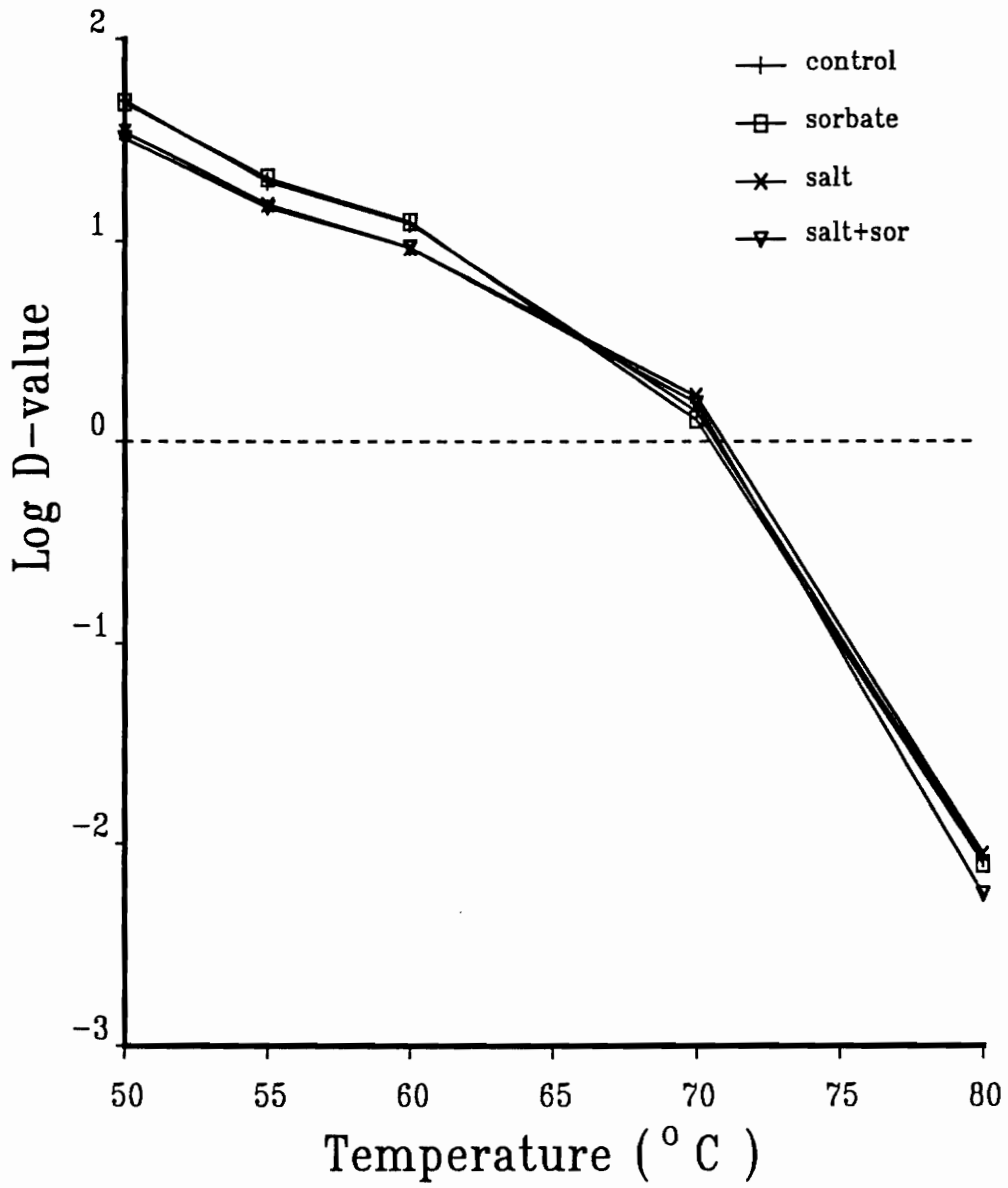


Figure 2. Phantom thermal death time curve for Trial 2.

without inclusion of D-values from 50°C are preferred based on better fit of the line. Z-values are presented in Table 3.

Z-values for type E spores generally range between 7.4 and 17 [Licciardello, 1983]. Z-values can be calculated for the entire temperature range tested (Table 3, 50 to 80°C) and these values fall in the range of z-values previously reported. However, these overall values would not represent the data correctly. Z-values calculated from the phantom TDT curves using data points from the upper two temperatures (70 and 80°C) range from 7.4 to 10.6°F (4.1 to 5.9°C). These values are consistent with those previously found for type E. Another set of z-values can be calculated from those points of the 55 to 70°C range. The calculated values are considerably much larger than those determined for the higher temperature range, generally above 20.0°F (11.1°C). It is believed that thermal death at temperatures less than 70°C follows a different mechanism of inactivation than is seen at temperatures greater than 70°C. A possible explanation for such large values in this lower range is that inactivation at these temperatures is not due thermal destruction of the heat resistant spores as seen at the upper temperatures. Rather, spores become activated, germinate with subsequent loss in heat resistance, and then

Table 3. z-values for Trials 1 and 2.

| Treatment             | Temperature Range (°C)               |             |             |            |
|-----------------------|--------------------------------------|-------------|-------------|------------|
|                       | 50-80                                | 50-70       | 55-70       | 70-80      |
| Trial 1               |                                      |             |             |            |
| Control               | 7.7 <sup>a</sup> (13.8) <sup>b</sup> | 8.6 (15.5)  | 11.2 (20.2) | 5.8 (10.4) |
| Sorbate               | 7.4 (13.3)                           | 8.8 (15.8)  | 11.2 (20.2) | 5.2 (9.4)  |
| Salt                  | 6.8 (12.3)                           | 6.6 (11.9)  | 13.4 (24.1) | 5.9 (10.6) |
| Sal+Sor               | 7.5 (13.5)                           | 8.6 (15.5)  | 11.3 (20.3) | 5.5 (9.9)  |
| Trial 2               |                                      |             |             |            |
| Control               | 8.3 (14.9)                           | 13.1 (23.6) | 13.9 (25.0) | 4.5 (8.1)  |
| Sorbate               | 8.2 (14.7)                           | 12.8 (23.0) | 12.0 (21.6) | 4.5 (8.1)  |
| Salt                  | 8.8 (15.8)                           | 15.4 (27.7) | 15.3 (27.5) | 4.4 (7.9)  |
| Sal+Sor               | 8.4 (15.1)                           | 15.4 (27.7) | 14.9 (26.8) | 4.1 (7.4)  |
| Combination of Trials |                                      |             |             |            |
| Control               | 8.0 (14.4)                           | 10.4 (18.7) | 11.8 (21.2) | 5.1 (9.2)  |
| Sorbate               | 7.8 (14.0)                           | 10.4 (18.7) | 11.6 (20.9) | 4.8 (8.6)  |
| Salt                  | 7.7 (13.9)                           | 9.3 (16.7)  | 14.2 (25.6) | 5.0 (9.0)  |
| Sal+Sor               | 7.9 (14.2)                           | 11.1 (20.0) | 12.9 (23.2) | 4.7 (8.5)  |

<sup>a</sup> z-value in °C

<sup>b</sup> z-value in °F

are inactivated. Other possible explanations may relate to differences in enzyme inactivation kinetics.

There was no significant difference in z-values between the different variables. More experimental data would be needed to make such determinations.

#### D. The Effect of Oyster Pasteurization

Comparing thermal inactivation rates for type E spores and the heat process given to oysters during pasteurization, it can be concluded that there will be little if any inactivation to spores present. It would require approximately 60 hours as compared with the heating time of 27.7 to 42.6 minutes actually received during pasteurization to inactivate 1000 C. botulinum type E spores at 55°C. To design a process which would eliminate the threat of botulism from type E spores, process temperatures would need to be much higher.

One possible effect that could occur to dormant spores during heating is activation. Once activated, these spores may germinate, grow and produce toxin if stored under the proper conditions. Proper refrigeration of the pasteurized oysters should prevent this.

## Part Two

### Toxin Production by Clostridium botulinum type E in Pasteurized Oysters

#### IV. Materials and Methods

##### A. Spores

Five strains of Clostridium botulinum type E (Beluga, Terry 4249 Tenno, VPI 1623, VPI 4206, and VPI 2796) were obtained from the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University. Spores of each strain were prepared as described previously. A stock suspension was made by mixing spores of each strain to get a suspension with all five strains in equal concentration. Similar suspensions were used in both inoculated tube and inoculated container designs.

##### B. Oysters

For both studies, fresh oysters were shipped overnight from Back River Seafood Co., Poquoson, VA. The salt concentration and pH were determined before use.

##### C. Overall Design

In order to determine whether C. botulinum type E can grow and produce toxin at different incubation temperatures, a combination of five strains of C. botulinum type E spores

were used in two types of inoculated pack designs. As a preliminary experiment, spores were inoculated into tubes containing oyster homogenate, pasteurized, and then incubated at various temperatures. At predetermined times, homogenate in tubes was assayed for botulinum toxin. Based on results from the inoculated tube experiments, an inoculated pack study was designed. For the latter study, the combination of spores were inoculated into whole oysters, packaged in 12 fl oz (354.9 ml) containers, pasteurized, and then incubated at different temperatures. At predetermined times, containers of pasteurized oysters was assayed for botulinum toxin.

#### D. Inoculated Tube Study

##### 1. Oyster homogenate

Fresh oysters were drained and liquor was added back to achieve an 85:15 ratio of drained meat to liquor.

For additive variables (1% salt, 0.13% sorbate, and 1% salt plus 0.13% sorbate) salt and potassium sorbate in dry form were added to the liquor so that when added back to the meat, the proper concentrations of salt and sorbate were achieved.

Oysters were then blended for 2 minutes in a Waring blender. Using a pipet, homogenate (10ml) was added to test tubes (16 mm x 100 mm). As the tubes were being filled,

they were simultaneously flushed with anaerobic grade CO<sub>2</sub> by the use of a canula inserted into the mouth of the tube. After each tube was filled, the canula was removed and a screw cap was tightened on. These tubes were then pasteurized.

For samples that were preinoculated with spores, a small volume (less than 2ml) of spore suspension was added to oysters during blending to obtain desired concentration. For tubes that received post-pasteurization inoculations, heat shocked spores (60°C for 15min) were added (0.2ml) into the pasteurized tubes below the level of the homogenate. Anaerobic grade CO<sub>2</sub> was simultaneously flushed into tubes during inoculation. After the cap was secured these tubes were vigorously vortexed.

## 2. Pasteurization of tubes

Pasteurization of oyster homogenate in tubes was accomplished by putting capped tubes in racks capable of holding 72 tubes. Two racks at a time were lowered into a waterbath preheated to 55°C. These tubes were kept in the waterbath for 40 minutes to achieve an  $F_{140} = 5.8$ , the same process as calculated by Butler [1984]. After this time, tubes were removed and cooled in an ice bath.

Monitoring of temperature in tubes and bath was accomplished by the use of copper-constantan thermocouples. Temperatures were measured on a Omega Model OM 271 Data Logger (Omega Engineering, Stamford, CN). Thermocouples were placed at the cold point (2.9 cm from the bottom of the tube) of six tubes and three thermocouples were placed at different locations in the waterbath.

### 3. Incubation and sampling

After tubes were cooled, they were stored at appropriate temperatures. At given times, three tubes were removed for each time/temperature/variable. The pH was checked and toxin was assayed. For toxin analysis, an equal amount of gel phosphate buffer was added to the homogenate and mixed.

A few tubes that were negative for botulinum toxin were tested for surviving type E spores. Tubes were heated to 60°C for 15 minutes and an MPN in cooked meat media was done.

### 4. Experimental design

#### a. Experiment I and II

Experiments I and II were similar in design. Tubes of homogenate were inoculated to a level of  $1 \times 10^3$  spores/ml. There were six variables:

a) not inoculated, pasteurized

- b) inoculated, not pasteurized
- c) inoculated, pasteurized
- d) inoculated, pasteurized, 1% salt
- e) inoculated, pasteurized, 0.13% sorbate
- f) inoculated, pasteurized, 1% salt and 0.13% sorbate

Test storage temperatures were carried out at 3, 6, 9, and 12°C. Sampling times 0, 5, 10, 15, 30, 45, and 60 days after storage for all temperatures. Three tubes per variable were assayed for toxin at each time and temperature.

#### b. Experiment III

This study was done as a follow up to Experiments I and II to determine the effects of incubation at 30°C, compare the effect of different spore inoculum levels ( $1 \times 10^3$ ,  $1 \times 10^4$ , and  $1 \times 10^5$  spores/ml), and post-pasteurization inoculation versus inoculation before pasteurization. Variables and their incubation temperatures were as follows:

- a) uninoculated
  - 1. pasteurized, and stored at 30°C
- b) inoculated to  $10^3$  spores/ml
  - 1. 1.0% salt, pre- and post-pasteurization, and stored at 30°C
  - 2. 0.13% sorbate, pre- and post-pasteurization, and stored at 30°C

3. 1.0% salt + 0.13% sorbate, pre- and post-pasteurization, and stored at 30°C
  4. pre- and post-pasteurization, and stored at 6, 12, 30°C
  5. unpasteurized, and stored at 6, 12, 30°C
- c) inoculated to  $10^4$  spores/ml
1. pre- and post-pasteurization, and stored at 6, 12, 30°C
- d) inoculated to  $10^5$  spores/ml
1. pre-pasteurization, and stored at 6, 12, 30°C

Three tubes were sampled for each variable incubation temperature combination at predetermined times for pH and botulinum toxin.

#### E. Inoculated Pack Study

##### 1. Oyster

Fresh extra select oysters were shipped overnight from Back River Seafood Co. Poquoson, VA. Before use, salt and pH were determined.

##### 2. Inoculation and packaging

Oysters were drained and the both meat and liquor were inoculated with spores. The meat, 10.2 fl oz (301.7 ml), was added to 12 fl oz (354.9 ml) Duratek polypropylene cups (Steeltin Can Corporation, Baltimore, MD). Liquor was added

back to the point of overfilling. This gave a ratio of 85:15 meat to liquor.

For the salt added variable, granulated salt (1% of the volume of oysters) was added directly to the oysters before draining. The oysters were stirred to completely dissolve the salt.

For inoculation of oysters, spores were injected into the body of the drained oyster meat and spores were added to the separated liquor. Spores were injected into the body by the use of a syringe-type automatic pipetor (4 ml). Spores were diluted to obtain a level of  $9.0 \times 10^4$  spores/ml. This spore mixture was injected (0.2 ml/oyster) into 10 oysters (average weight of 40 grams). The ten inoculated oysters were added to a container. Noninjected oysters were used to fill the container to the 10.2 fl oz level. Liquor, inoculated to  $5 \times 10^2$  spores/ml, was then added to overfilling.

Containers were sealed with a 307 diameter aluminum pull tab ends on a Dixie Automatic Can Sealer, model 25-2000, using a single roller operation. Containers were randomly numbered according to variable.

### 3. Pasteurization

The eighty-eight containers were loaded into crates in a fashion to allow maximum flow between containers. These

crates were then lowered into open end retorts containing water preheated to 60°C and agitated by compressed air.

Container temperature was monitored by the use of thermocouples placed at the cold point in the same manner as was done by Butler [1984]. After all thermocouples reached 55°C, the containers were held at that temperature for 10 minutes. After this time expired, the crates were removed and the containers were placed in an air agitated ice bath. After all thermocouples reached 12°C, the containers were removed and placed at the appropriate incubation temperatures (6, 12, and 30°C).

#### 4. Sampling

On each sampling day, two containers for each variable were examined for pH, aerobic plate count, anaerobic plate count, and toxin (microbiological analysis was done until a point of gross deterioration). C. botulinum counts were done at day 0.

For microbiological analysis, 75 grams of oysters was added to an equal amount of 0.1% phosphate buffer and then homogenized for 2 minutes in a 400 Stomacher (Teckmar Co., Cincinnati OH). To prepare samples to be analyzed for botulinum toxin, 75 grams of oysters was mixed with an equal amount of gel phosphate buffer (pH 6.1) and homogenized for 2 minutes.

## 5. Experimental design

Two similar trials were performed based on Experiments I and II from the inoculated tube study. In each trial, three variables were examined; uninoculated control, inoculated, inoculated with the addition of salt. Incubation temperatures were 6, 12, and 30°C. Sampling times were dependant on the incubation temperature.

## F. Salt and pH Analysis

Salt content was determined as described in Part A.

The pH of the sample was determined by inserting a combination probe of a pH electrode into a tube containing a small amount of undiluted oyster liquor and the pH determined (Corning Digital 109, Corning, Midfield, MA).

## G. Microbiological Analysis

### 1. Total aerobic count

Appropriate dilutions were made in 0.1% peptone and samples were poured in duplicate using Standard Methods Agar (BBL). Pour plates were incubated at 32°C for 48 hours.

### 2. Total anaerobic count

Appropriate dilutions were made in prereduced 0.1% peptone water. Strict anaerobic conditions using the VPI Anaerobic Culture System (Belco Glass Co. Vineland, NJ) were

used to inoculate all prereduced medium [Holdeman et al., 1977]. Counts were done in roll tubes using PYG medium [Pierson et al., 1974]. Tubes were incubated at 32°C for 48 hours.

### 3. C. botulinum count

To determine the presence of C. botulinum in samples, a Most Probable Number (MPN) procedure was done with prereduced chopped meat glucose (CMG) broth. Appropriate dilutions were made by anaerobic techniques as previously described. Samples were heat shocked for 15 minutes at 60°C. All MPN tubes that were positive for growth were then tested for botulinum toxin by the mouse assay. A three MPN table was used to calculate the total C. botulinum count for each sample.

### H. Analysis for Presence of C. botulinum Toxin

All oyster samples to be checked for botulinum toxin were initially diluted 1:1 in gel phosphate buffer (pH 6.2). After mixing, this suspension was centrifuged at 10,000 g for 20 minutes. The supernatant fluid was checked for toxin by the mouse bioassay.

Mice used in this assay were Dub[ICR] strain, white males weighing 16 to 22 grams. They were obtained from Dominion Labs, Dublin, VA. For each sample, two mice (one

mouse for MPN) were injected with trypsin treated supernatant. As a control, one other mouse was injected with sample extracts which had been boiled for 10 minutes. Trypsin treatment was accomplished by adding trypsin (1:250) to the sample to obtain a final concentration of 1/1000.

After injection, mice were observed for 72 hours. A positive presumptive test for botulinum toxin is death with typical symptoms for botulism occurring in the mice injected with the trypsin treated sample and survival of the mouse receiving the heated sample.

For selected inoculated container samples, identification of the sample was accomplished by mixing sample extract with type A, B, and E antitoxin. Botulinum toxin analysis is covered in more detail in the Bacteriological Analytical Manual [FDA, 1984].

Statistical analyses were done by the methods of Snedecor and Cochran [1967]

## V. Results and Discussion

### A. Inoculated Tube Study

#### 1. Salt and pH analysis

The pH of oysters is one measure of quality. Fresh oysters should have a pH greater than 6.2. A pH of 5.9 is viewed as acceptable [Jay, 1986]. The pH of fresh oysters used in Experiments I and II had a pH of about 6.2. When stored at 3°C, the pasteurized homogenate maintained a pH of 5.9 or greater. The pH of unpasteurized homogenate dropped below this after 10 days. The addition of 0.13% sorbate to homogenate caused the pH to remain above 6.0 for 60 days. On the other hand, the addition of 1% salt maintained a pH slightly lower than the control; between 5.8 and 5.9. The combination of both 1.0% salt and 0.13% sorbate maintained a pH slightly greater than that with just added salt but not as high as the pH with only sorbate added.

As the storage temperature increased, the drop in pH became greater. In pasteurized controls at 6°C, there was only a slight decrease below those stored at 3°C. At 9°C, the pH was below 5.9 after 20 days, and at 12°C, the pH was below this mark before 10 days of storage. The effect of the variables was consistent with increased storage temperature.

In experiment III, the pH of the homogenate stored at 30°C dropped below 5.0 by day 5, the first day of sampling. At 6°C and 12°C, similar patterns were seen to experiment I and II except in tubes that were positive for toxin production.

The pH of homogenate stored at 3°C was similar to that of whole oysters stored at 2°C as reported by Butler [1984]. The addition of 1% salt to homogenate resulted in a lower pH than was seen in whole oysters.

## 2. Microbiological analysis

Anaerobic counts of the oyster homogenate (pasteurized and unpasteurized, inoculated and uninoculated) for Experiments I, II, and III are given in Table 4. Counts for the unpasteurized homogenate ranged from  $8.6 \times 10^2$  cfu/gr for Experiment I to  $5.7 \times 10^4$  cfu/gr for Experiment III.

Pasteurization reduced the number of anaerobic bacteria by 1 and 2 log cycles. In inoculated tubes, anaerobic counts increased as expected by at least 2 logs, which would be due to C. botulinum spores added. For the inoculated product, it would be expected that, C. botulinum type E spores comprised the major portion of the anaerobic microflora in the pasteurized tubes.

The flora surviving pasteurization were not identified. However, the types of organisms from the natural flora

Table 4. Initial total anaerobic counts<sup>a</sup> for inoculated tube study.

| Treatment                      | Experiment        |                   |                    |
|--------------------------------|-------------------|-------------------|--------------------|
|                                | I                 | II                | III                |
| Unpasteurized                  | $8.6 \times 10^2$ | $4.4 \times 10^3$ | $5.7 \times 10^4$  |
| Inoculated                     | $3.0 \times 10^3$ | $3.8 \times 10^3$ | $>5.0 \times 10^4$ |
| Pasteurized                    | $1.0 \times 10^1$ | $2.5 \times 10^1$ | $4.2 \times 10^3$  |
| Inoculated ( $1 \times 10^3$ ) | $5.1 \times 10^2$ | $9.7 \times 10^2$ | $1.4 \times 10^2$  |
| 1.0% salt                      | $7.6 \times 10^2$ | $3.7 \times 10^3$ | --                 |
| 0.13% sorbate                  | -- <sup>b</sup>   | $2.5 \times 10^3$ | --                 |
| salt + sorbate                 | $3.8 \times 10^3$ | $1.7 \times 10^3$ | --                 |

<sup>a</sup> Colony forming units per gram.

<sup>b</sup> Analysis not done.

expected to survive the heating process given during pasteurization would be mainly gram positive, although some gram negatives would be expected to survive. Heating oyster shellstock to 55°C, Goldmintz et al. [1983] found that the gram negative organisms were decreased from 50 to 70%. When heated to 62°C, there was greater than a 95% reduction in the number of gram negatives. Bacillus and gram positive anaerobes had the greatest percentage of survivors. Pace et al. [1988] found no gram negatives when shucked oysters were heated to 72°C in pouches. The flora was comprised of gram positives, mainly Bacillus.

### 3. Toxin analysis

In experiments I and II, the inoculation of  $1 \times 10^3$  spores/ml into oyster homogenate variables with subsequent incubation at 3, 6, 9, and 12°C, there was no toxin was detected.

The third experiment was designed to investigate the effect of increased inoculum level, inoculation after pasteurization, and incubation at 30°C. The results are given in Table 5.

As in Experiment I and II, homogenate without added salt or sorbate and inoculated to  $1 \times 10^3$  spores/ml prior to pasteurization yielded no toxin production. In tubes inoculated with  $1 \times 10^4$  spores/ml before pasteurization and

Table 5. Toxin detection in Experiment III.

| Sample Description                         | Storage Temperature |        |        |
|--------------------------------------------|---------------------|--------|--------|
|                                            | 6°C                 | 12°C   | 30°C   |
| Unpasteurized, uninoculated                | 0 (60) <sup>a</sup> | 0 (60) | 0 (15) |
| Inoculation to 1x10 <sup>3</sup> spores/ml |                     |        |        |
| 1. Pre-pasteurization                      | 0 (60)              | 0 (60) | 0 (15) |
| a. 1.0% salt                               |                     |        | 0 (15) |
| b. 0.13% sorbate                           |                     |        | 3 (5)  |
| c. salt + sorbate                          |                     |        | 3 (5)  |
| 2. Post-pasteurization                     | 3 (30)              | 3 (20) | 3 (5)  |
| a. 1.0% salt                               |                     |        | 3 (5)  |
| b. 0.13% sorbate                           |                     |        | 3 (5)  |
| c. salt + sorbate                          |                     |        | 1 (5)  |
| 3. Unpasteurized                           | 3 (30)              | 3 (20) | 3 (5)  |
| Inoculated to 1x10 <sup>4</sup> spores/ml  |                     |        |        |
| 1. Pre-pasteurization                      | 0 (60)              | 0 (60) | 1 (5)  |
| 2. Post-pasteurization                     | 3 (30)              | 2 (20) | 2 (5)  |
| 3. Unpasteurized                           | 3 (30)              | 3 (20) | 3 (5)  |
| Inoculated to 1x10 <sup>5</sup> spores/ml  |                     |        |        |
| 1. Pre-pasteurization                      | -- <sup>b</sup>     | 3 (20) | 3 (3)  |

<sup>a</sup> Number represents the number of containers toxic out of 3 samples tested. (First day toxin detected or last day sampled if negative)

<sup>b</sup> Analysis not done.

incubated at 30°C, toxin was detected. When  $1 \times 10^5$  spores/ml was the inoculum, toxin was found in tubes incubated at both 30 and 12°C. (No sample was tested at 6°C for this inoculum level.) Toxin production was seen in homogenate inoculated to  $1 \times 10^3$  when there was the addition of 0.13% sorbate (with and without added salt). Toxin was also detected in tubes that were inoculated with heat shocked spores after pasteurization. This was the case for all three spore inoculum levels in homogenate incubated at 6, 12, and 30°C. Unlike the first two experiments, however, tubes of unpasteurized homogenate were positive for toxin production.

These three experiments showed that as the inoculum size was increased, the temperature minimum for growth was less. Inoculum level has been previously shown by Roberts et al. [1982] to have a significant effect on toxin production. Inoculating spores in a pork slurry system with adverse growth conditions such as high salt and low incubation temperatures, it was found that as the inoculum size increased, the probability of toxin production increased.

Other factors may contribute to the probability of toxin production by C. botulinum in oyster homogenate along with low incubation temperature and inoculum size. These include pH, initial flora, and oxidation reduction potential.

The initial pH of the homogenate ranged from 6.0 to 6.1. With the growth of spoilage organisms, the pH decreases. It has been shown in laboratory medium that at 5°C, the minimum pH allowing growth of type E was 6.2. At 7.2°C, the minimum pH lowered to 5.8 [Emodi and Lechowich, 1969a]. Segner et al. [1966] found spore outgrowth to occur at pH 5.9 but not 5.7 when stored at 8°C. Ohye and Christian [1966] examined the effect of pH on growth in broth inoculated at  $1 \times 10^3$  type E spores/ml and incubated at 10°C. Growth occurred at pH 6.0 but not at pH 5.0.

The type of growth medium also has been found to differ from food systems. Solomon et al. [1977] found growth in broth at 12, 8, and 4°C, but in crabmeat, growth occurred no lower than 12°C. Therefore, caution is needed when applying growth studies in broth to food systems.

Oxidation reduction potential is another factor that may influence the growth of an anaerobic bacterium such as C. botulinum type E. Upon mixing there may be some incorporation of air into the broth. Another likely occurrence is the utilization of oxygen by spoilage organisms making the conditions more anaerobic and hence more favorable to C. botulinum growth.

The competing microflora can have a great impact on the growth of C. botulinum type E. Not only the number of spoilage organisms is important, but the type of metabolism

they possess. Organisms can make conditions more anaerobic by utilizing available oxygen. But on the other hand, they may be producing acid byproducts which lowers the pH, a condition not favorable to outgrowth of type E spores. The growth rate of these organisms is also important. This will determine whether they can outcompete C. botulinum for available nutrients and the amount of inhibitory byproducts.

The reason for growth of C. botulinum type E, as detected by toxin production in nonpasteurized homogenate, in Experiment III while not in Experiment I and II is unclear. This may relate to some of the points just mentioned. It may also be that the inoculum could have been slightly higher than what was used in the first two experiments. A similar study showing growth in raw oysters was that of Patel et al. [1978]. Raw oysters inoculated with  $1 \times 10^4$  spores/ml were positive for toxin after 90 days at 13, 10, 7, and 4°C. Goldmintz et al [1983], unlike Patel et al [1978], found no toxin production in raw oysters when inoculated to  $1 \times 10^5$  spores/gram.

The effect of microflora on toxin production can be seen by the addition of 0.13% sorbate to homogenate. In tubes with added sorbate, there was toxin production in tubes of homogenate inoculated with  $1 \times 10^3$  spores/ml incubated at 30°C. No toxin was detected in similar tubes without the added sorbate. These results are similar to those published

by Fletcher et al. [1988]. It was shown that toxin production occurred more rapidly in C. botulinum inoculated vacuum packaged scallops with 0.1% added sorbate than those without added sorbate. It is believed that sorbate inhibits gram negative spoilage organisms more than C. botulinum. In fact at one time, sorbate was considered as a selective factor for C. botulinum when used in such concentrations.

The use of sorbate in oysters for shelf-life extension has been questioned by the FDA [Anonymous, 1987]. They opposed the addition of sorbate in the pasteurization process of oysters because it is felt that the incidence of use of sorbate is too widespread.

Although it is more likely that spores would enter into product before pasteurization, one variable in Experiment III was designed to investigate the effect of post-pasteurization inoculation. Heat shocked spores were injected into tubes of pasteurized homogenate, resealed, and then vortexed. The result was that while tubes inoculated before pasteurization yielded no toxin, those inoculated after pasteurization were positive. While heat resistance studies show that type E spores would easily survive such a heating process, perhaps the heating during pasteurization with subsequent storage had a more negative effect on outgrowth of the spores than was seen in thermal resistance studies. Possibly some chemical constituent in the oysters

is acts as an antimicrobial agent. On the other hand, there may have been a higher degree of spore activation during the heating in buffer then was received during the pasteurization of oyster homogenate. If this is the case, then a lower number of spores in the containers become activated during the pasteurization process and thus less are available for outgrowth. This may explain why a higher inoculum level gives a higher probability of toxin production. If only a certain proportion of spores are becoming activated, the larger the number to start, the more that become activated. This raises the question of why there was growth as evidenced by toxin production in unpasteurized samples in Experiment III.

To check the survival of spores in those tubes in Experiment II exhibiting no toxin production, an MPN for C. botulinum was carried out on a sampling of tubes stored at both 3 and 12°C. It was found that an average of  $1.7 \times 10^4$  C. botulinum/ml surviving in the tubes after 60 days. The number for both temperatures was similar. The average total anaerobic count at the outset of the experiment for all of the batches tested by MPN was  $2.6 \times 10^3$  cfu/ml. While growth during storage is a possible explanation for the difference between initial and final counts, it is believed that it may be the difference between the counting methods. The MPN is done with a broth which is less stressful and the incubation

time allotted for growth is five days versus two days incubation for the roll tube method.

The inoculated tube study demonstrated that as the inoculum level was lowered, the probability for toxin production decreased. Pasteurization did not destroy spores, but rather may activate them. Most importantly, however, these spores of C. botulinum can grow and produce toxin in oyster at 6, 12, and 30°C.

#### B. Inoculated Container Study

##### 1. Salt and pH analysis

NaCl measured in samples ranged from 0.2 to 0.3%. The addition of 1% w/v NaCl raised the measured level to an average concentration of 1.05%.

Average pH for both initial samples are given in Table 6. The initial pH of oysters in Trial I and II was 6.24 and 6.12, respectively. Upon canning and pasteurization, the pH remained relatively the same, decreasing about 0.1 pH units.

The decline of pH was greater in containers than was seen in tubes. At a storage temperature of 6°C, the pH in uninoculated containers was less than 5.5 at 10 days while in tubes, it took between 20 and 30 days. At 6°C, the pH in tubes remained above 5.8 for 60 days while in uninoculated containers the pH was an average of 5.62.

Table 6. Initial analysis of oysters in Trial I and II of inoculated container study (N = nonpasteurized, C = uninoculated control, I = inoculated, and S = inoculated + 1.0% salt).

| Variable | pH   | %NaCl | APC <sup>a</sup>  | Anaerobica <sup>a</sup> |
|----------|------|-------|-------------------|-------------------------|
| Trial 1  |      |       |                   |                         |
| N        | 6.24 | 0.25  | $4.3 \times 10^4$ | $2.8 \times 10^4$       |
| C        | 6.14 | 0.25  | $4.4 \times 10^2$ | $1.5 \times 10^2$       |
| I        | 6.17 | --b   | $3.6 \times 10^2$ | $5.8 \times 10^2$       |
| S        | 6.10 | 1.0   | $4.5 \times 10^2$ | $1.0 \times 10^3$       |
| Trial 2  |      |       |                   |                         |
| N        | 6.12 | 0.3   | $7.8 \times 10^5$ | $9.7 \times 10^5$       |
| C        | 6.10 | 0.25  | $5.7 \times 10^2$ | $1.0 \times 10^2$       |
| I        | 6.12 | 0.25  | $9.8 \times 10^2$ | $7.3 \times 10^2$       |
| S        | 6.00 | 1.1   | $6.3 \times 10^2$ | $7.4 \times 10^2$       |

<sup>a</sup> Colony forming units per gram.

<sup>b</sup> Analysis not done.

## 2. Microbiological analysis

Total aerobic and anaerobic counts for initial sampling times are given in Table 6. As can be seen, aerobic plate counts dropped two logs in Trial 1 and almost 3 logs in Trial 2. This same trend was seen for anaerobic counts. This is similar to the reduction seen by Butler [1984]. Containers inoculated with spores had an increased count of  $6 \times 10^2$  cfu/ml. This would indicate the increase added by added spore inoculum. The MPN for C. botulinum indicates similar results although there is variation between the two. Inoculation of supernatant from the MPN into mice indicates that the majority of anaerobes counted were C. botulinum.

Counts done after incubation show great variation. Most were greater than the  $5 \times 10^5$  limit by the first day of sampling.

## 3. Toxin analysis

The days for sampling for each temperature were based mainly on the results seen in the inoculated tube study, Experiments I and II. These sample time are shown in Table 7.

Toxin assays demonstrate that toxin production does occur in oysters stored at 6, 12, and 30°C (Table 8). In Trial 1, toxin was detected on the first day of sampling for each temperature. At 30°C, toxin was seen at 2 days; at

Table 7. Initial sampling times and temperatures for inoculated container study.

| Storage |                | Sampling Day |    |    |    |    |
|---------|----------------|--------------|----|----|----|----|
| Temp    | 0              | 5            | 15 | 30 | 45 | 60 |
| Initial | x <sup>a</sup> |              |    |    |    |    |
| 30°C    |                | X            | X  |    |    |    |
| 12°C    |                |              | X  | X  | X  |    |
| 6°C     |                |              |    | X  | X  | X  |

<sup>a</sup> Day on which toxin is assayed.

Table 8. Toxin analysis of containers at different storage temperatures for each variable ( C = uninoculated control, I = inoculated, and S = inoculated + 1.0% salt ).

| Storage Temp   | Vari-<br>able | Sample Day       |     |     |     |                  | Total <sup>c</sup> |
|----------------|---------------|------------------|-----|-----|-----|------------------|--------------------|
|                |               | 2                | 5   | 10  | 15  | 30               |                    |
| <u>Trial 1</u> |               |                  |     |     |     |                  |                    |
| 30°C           | C             | 1/2 <sup>a</sup> |     |     |     |                  | 1/2                |
|                | I             | 2/2              |     |     |     |                  | 2/2                |
|                | S             | 2/2              |     |     |     |                  | 2/2                |
| 12°C           | C             |                  |     | 1/2 |     |                  | 1/2                |
|                | I             |                  |     | 2/2 |     |                  | 2/2                |
|                | S             |                  |     | 2/2 |     |                  | 2/2                |
| 6°C            | C             |                  |     |     |     | 0/2 <sup>b</sup> | 0/6                |
|                | I             |                  |     |     |     | 2/2              | 5/6                |
|                | S             |                  |     |     |     | 1/2              | 3/6                |
| <u>Trial 2</u> |               |                  |     |     |     |                  |                    |
| 30°C           | C             | 1/2              |     |     |     |                  | 1/2                |
|                | I             | 2/2              |     |     |     |                  | 2/2                |
|                | S             | 2/2              |     |     |     |                  | 2/2                |
| 12°C           | C             |                  | 0/2 | 1/2 |     |                  | 1/4                |
|                | I             |                  | 2/2 | 2/2 |     |                  | 4/4                |
|                | S             |                  | 2/2 | 2/2 |     |                  | 4/4                |
| 6°C            | C             |                  |     |     | 0/2 | 0/2 <sup>b</sup> | 0/8                |
|                | I             |                  |     |     | 1/2 | 2/2              | 7/8                |
|                | S             |                  |     |     | 0/2 | 2/2              | 5/8                |

<sup>a</sup> Number of containers toxic/number of containers sampled

<sup>b</sup> Samples were also taken on days 45 and 60 at 6°C

<sup>c</sup> Total at 6°C also includes sample days 45 and 60

12°C, day 10; and at 6°C, toxin was detected at day 30.

In the second trial, some adjustments were made midway to check earlier times. Toxin was found to be produced in oysters on day 2 when stored at 30°C, day 5 when stored at 12°C, and day 15 when stored at 6°C. (In this case only 1 of 2 mice died in 1 of 4 inoculated samples.)

Toxin was detected in four uninoculated containers. In trial I, one container stored at 30°C and one stored at 12°C tested positive for toxin. Toxin was also detected in Trial II; one stored at 30°C and one at 12°C. Although the incidence of C. botulinum in oysters has been shown to be low [Ward et al., 1967 and Presnell et al., 1967], this indicates that it is possible for C. botulinum to be present in oysters at a level which can cause problems.

All controls positive for toxin production as well as one inoculated sample (with and without salt) from each temperature were typed for toxin. In all cases (except two where the toxin in these samples was evidently diluted too much during preparation), the toxin proved to be type E.

C. botulinum type E has been shown to grow at temperatures as low as 3.3°C in broth [Schmidt et al. 1961]. Results of this investigation show that C. botulinum type E does have the capability to grow in pasteurized oysters at a temperature as low as 6°C. Patel et al. [1978] found type E

to grow and produce toxin in fresh oysters as low as 4°C. Toxin production by this organism has been reported in other seafood products such as fresh herring and smoked vacuum-packaged fish [Palumbo, 1986].

In a similar pasteurized product, Goldmintz et al. [1983] examined toxin production in shellstock rather than shucked oyster. Inoculated oysters pasteurized to 42, 45, 55, and 62°C and incubated at 30°C were positive for toxin production. However, no toxin was detected in pasteurized shellstock stored at either 5 or 10°C. Also, type E spores inoculated at a similar level in crabmeat would not grow below 12°C [Solomon et al., 1978].

Containers containing 1.0% added salt appeared to have a lower percentage of those containing toxin. While the difference is insignificant, the reason may be due to the lower starting pH.

In Trial I, all containers were swollen by the time of the first sample date for that temperature. One exception was a container incubated at 6°C. In this inoculated sample, no toxin was detected. In Trial II, sample times for containers stored at 6 and 12°C were moved ahead so that sampling of nonswollen containers would be possible. Uninoculated containers stored at 12°C on day 5 were not swollen, while inoculated containers did exhibit slight gas

production. At 6°C on day 15, none of the containers sampled were swollen, while on day 30, two of the 6 containers sampled were swollen. In both Trial I and Trial II, only two of the nonswollen containers contained toxin. Both were inoculated samples stored at 6°C; one sampled at day 15 and the other at day 30. The pH of all swollen containers was below 5.9, while the pH of all nonswollen containers was above 5.9. This demonstrates that container swelling may not be used to detect possible C. botulinum contamination in pasteurized oysters.

Basing sample times for each temperature on the inoculated tube study proved to be an error. Another problem was that a temperature which inhibited C. botulinum growth in whole containers was not identified. The time required for toxin production at 12°C should be better characterized.

Discrepancies between inoculated tube and container methods also indicate that there is a difference between growth conditions in homogenate and whole oysters in containers. Growth in whole pasteurized oysters proved to be superior to that seen in oyster homogenate. Factors which may have influenced this growth include pH, initial flora, and redox potential. While the pH of both are similar, there may be some differences in other parameters.

One difference may be the flora which survives in each. The redox potential on the interior of the oyster may be lower than that found in blended oyster. These as well as other factors may positively influence C. botulinum growth in whole oysters. Investigators should use caution when using results from a blended form of a product as a basis for prediction of microbiological safety.

## VII. Summary and Conclusions

The risk of toxin production by Clostridium botulinum type E in pasteurized oysters is dependent upon two factors: 1) survival of spores during pasteurization, and 2) growth of this bacterium in pasteurized oysters during storage. Survival of spores during the heating process is dependant upon the heat resistance inherent to that strain of bacterium as well as the medium in which the spores are heated. If spores should survive, toxin production may occur if the proper growth conditions are met. These include suitability of the medium as well as incubation temperature.

The thermal resistance of C. botulinum type E spores was determined by the flask method. Two sets of survivor curves were plotted for five temperatures. At 80°C, D-values ranged from 0.3 minutes to 2.0 minutes; at 70°C, 65 to 100 minutes; at 60°C, 560 to 1100 minutes; at 55°C, the temperature at which oysters are pasteurized, the D-value was 880 to 1300 minutes. Addition of 0.13% potassium sorbate had no significant effect on the measured heat resistance of type E spores while salt had a protective effect at 70°C and 80°C.

Phantom thermal death time curves were plotted from these D-values and z-values calculated. The two resulting curves were biphasic and therefore two z-values calculated

for each curve. Z-values calculated for the curve between 70°C and 80°C ranged from 7.4 to 10.6°F. Z-values calculated for the lower range, 55°C to 70°C, were greater than 20°F. While the z-values calculated at the upper two temperatures fall within the range previously published, those calculated for the lower temperatures are much higher.

The heat process given to oysters during pasteurization has an  $F_{140^\circ\text{F}}(60^\circ\text{C})$  of between 5.6 and 8.6 minutes. Based on these results, the effect of pasteurization will have little if any lethal effect on type E spores. To have an effect, the time and/or the temperature would have to be greatly increased.

Since survival of spores is highly probable, it was essential to examine the conditions needed for growth and toxin production in pasteurized oysters. An inoculated tube study using oyster homogenate was first done to set the parameters for an inoculated container study utilizing whole oysters.

In the inoculated tube study, toxin was not produced in homogenate inoculated to  $1 \times 10^3$  spores/ml and incubated at temperatures at 3, 6, 9, and 12°C. Also, no toxin was found at 30°C except for the variable with 0.13% added sorbate. It is believed that the sorbate inhibited the competitive spoilage organisms while having little effect on C. botulinum.

As the inoculum size increased, the temperature minimum for toxin production decreased. At  $1 \times 10^4$  spores/ml, toxin production was seen at 30°C, and at  $1 \times 10^5$  spores/ml, toxin production occurred at 12°C.

Unlike spores added before pasteurization, heat activated spores inoculated to  $1 \times 10^3$  spores/ml to tubes of homogenate after pasteurization produced toxin at the three temperatures tested: 6, 12, and 30°C. This may have been due to a larger amount of activated spores present in the growth medium.

An inoculated container study was designed based upon results from the tube study. Oysters inoculated with type E spores (500 to 1000 spores/g) were packaged in 12 oz containers, pasteurized, and incubated at 6, 12, and 30°C. Toxin was produced by C. botulinum type E in oysters stored at all three temperatures. At 30°C, toxin was produced within 2 days. At 12°C toxin was present at day 5, while at 6°C, toxin was present between 15 and 30 days.

This research demonstrated that C. botulinum type E can survive pasteurization and then grow and produce toxin in pasteurized oysters that are not held at low refrigeration temperatures. The shelf-life of oysters can be extended using the proposed pasteurization process; however, the product has an associated botulism risk if it is not properly refrigerated.

Other psychrothrophic pathogens such as Listeria monocytogenes that are capable of surviving pasteurization and then growing in the product need to be examined. The survival of enteroviruses should also be determined.

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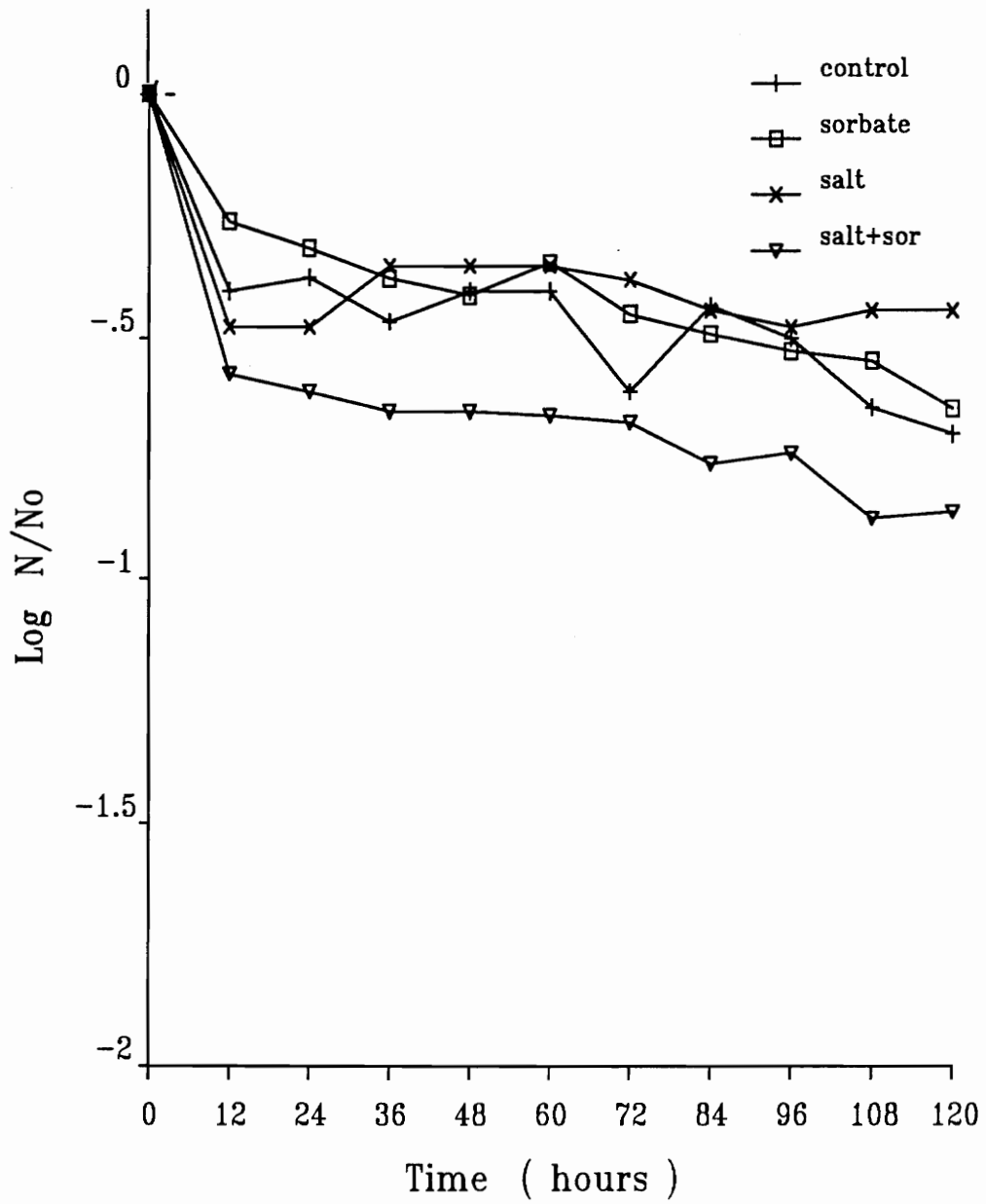
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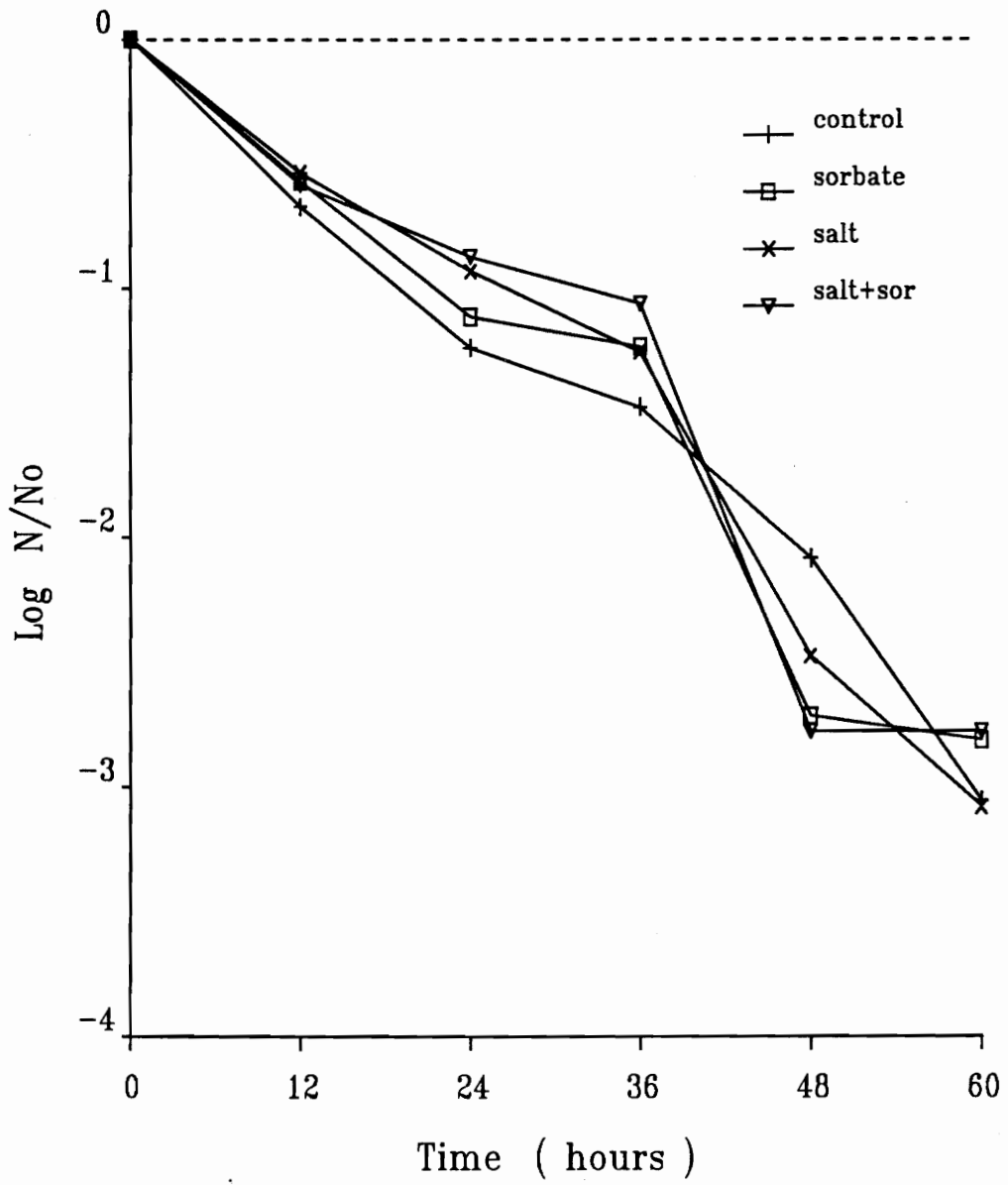
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## Appendix

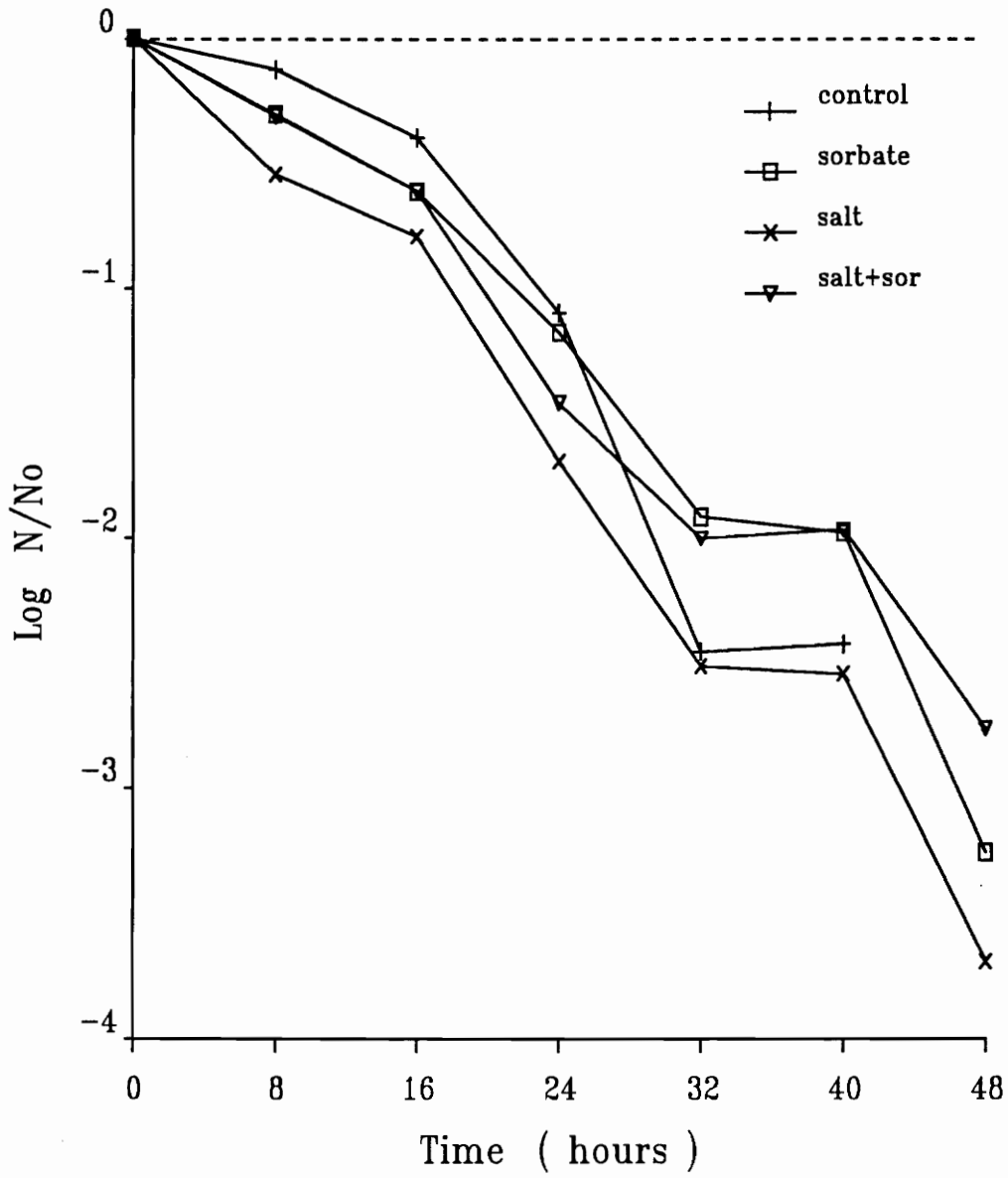
Plots of survivor curves for Trial 1 and 2 in Part One.  
N denotes the anaerobic count for the given time while  $N_0$   
denotes the initial count.



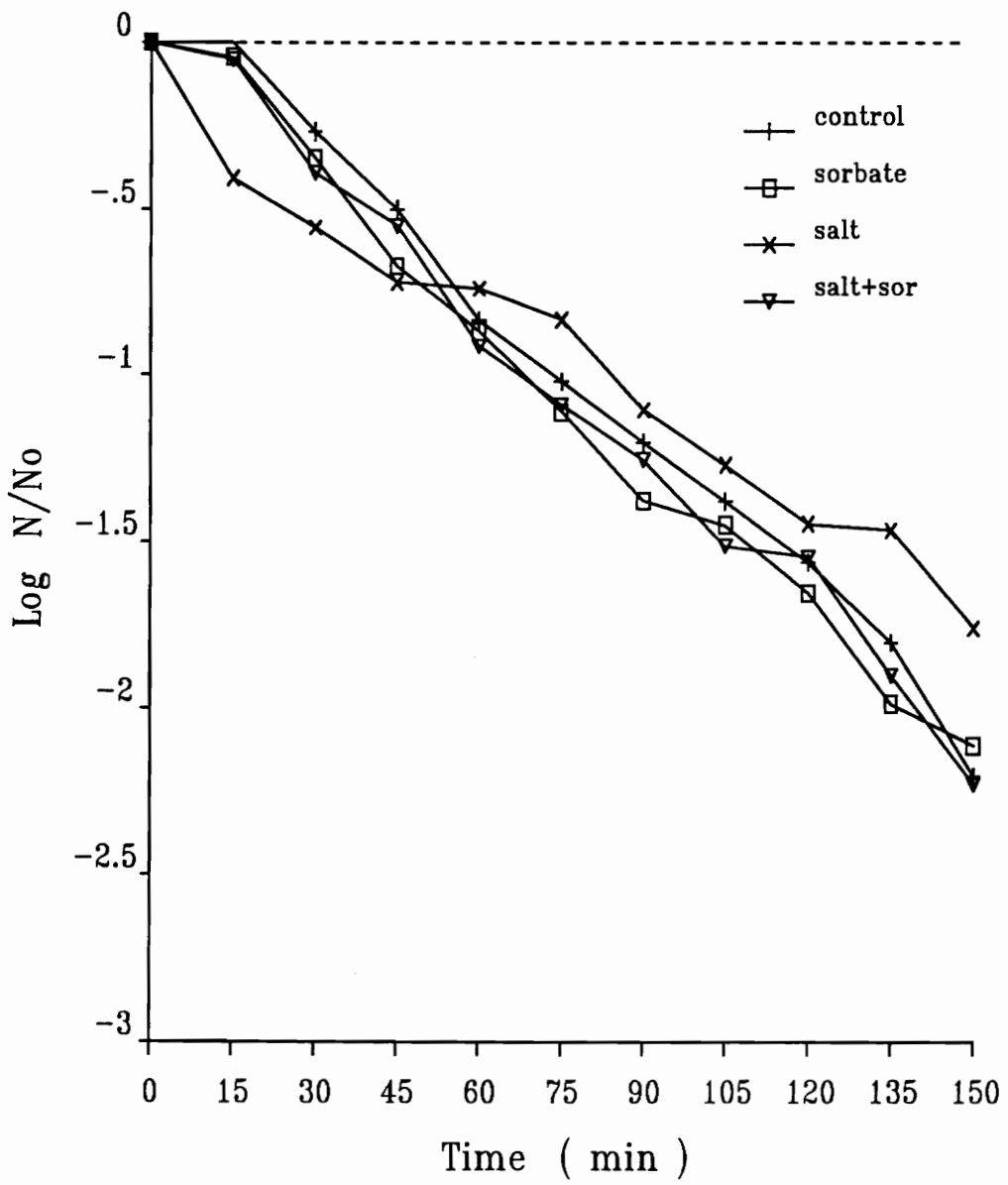
Survivor curve for Trial 1, 50 °C



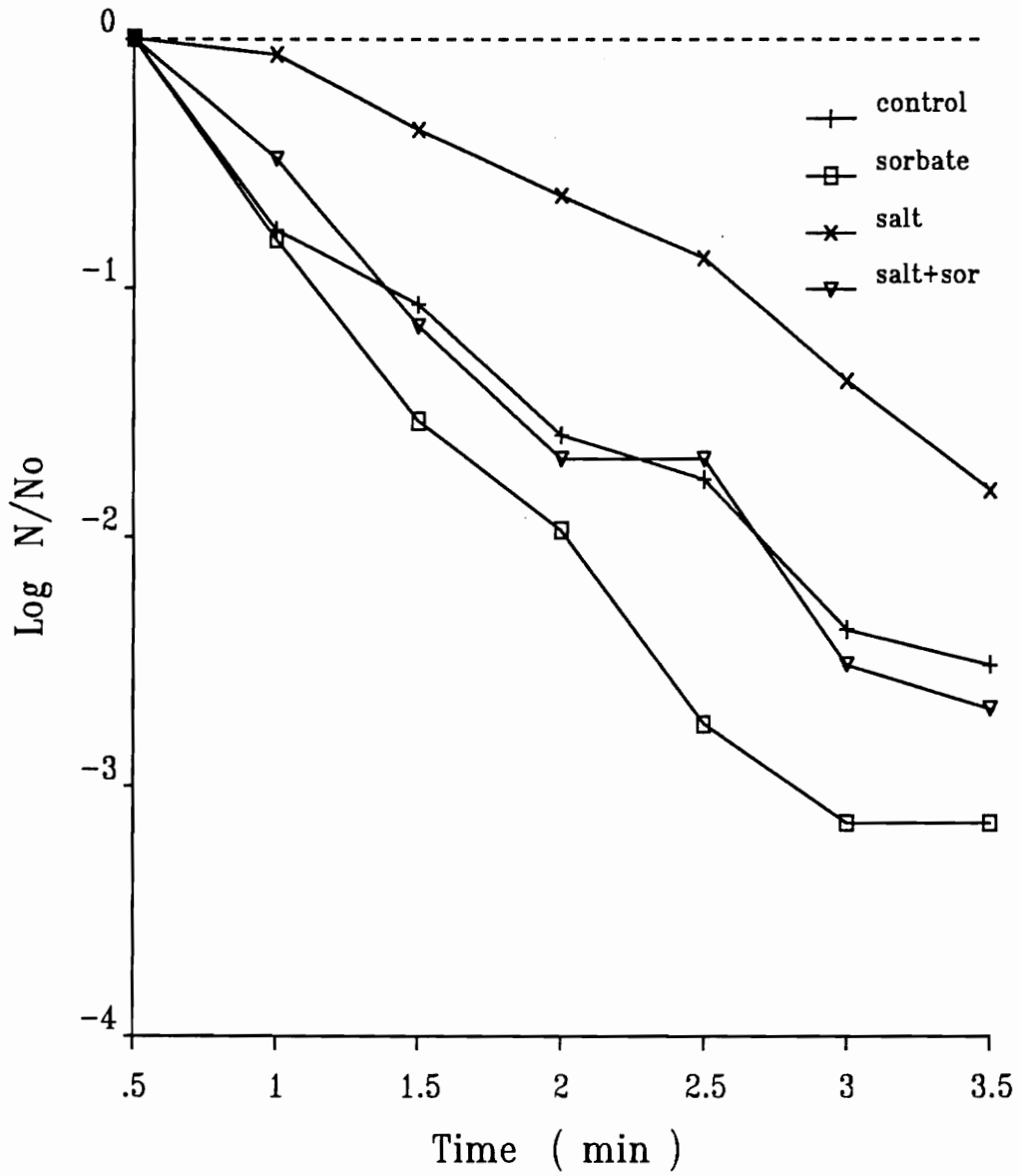
Survivor curve for Trial 1, 55 °C



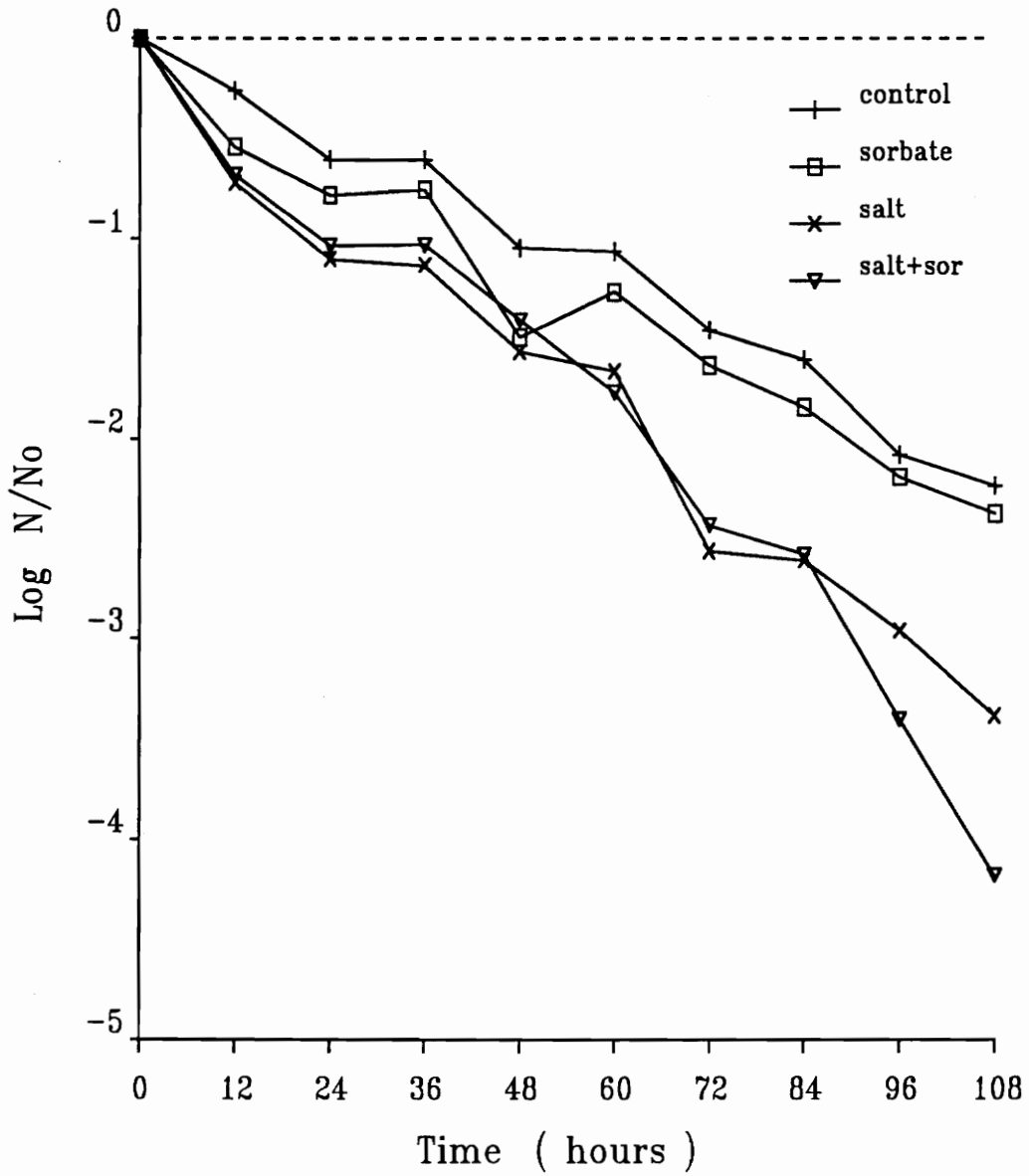
Survivor curve for Trial 1, 60 °C



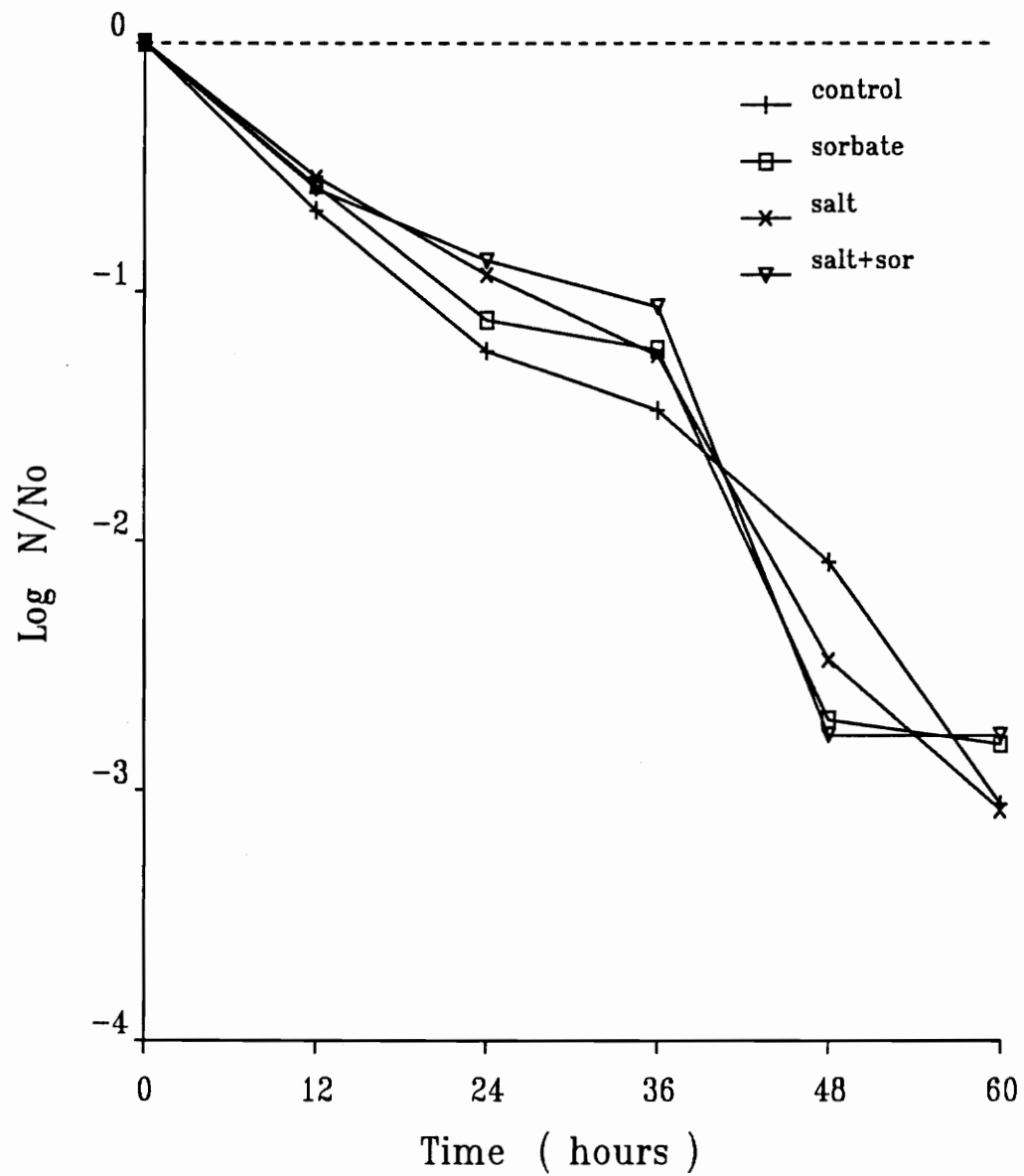
Survivor curve for Trial 1, 70 °C



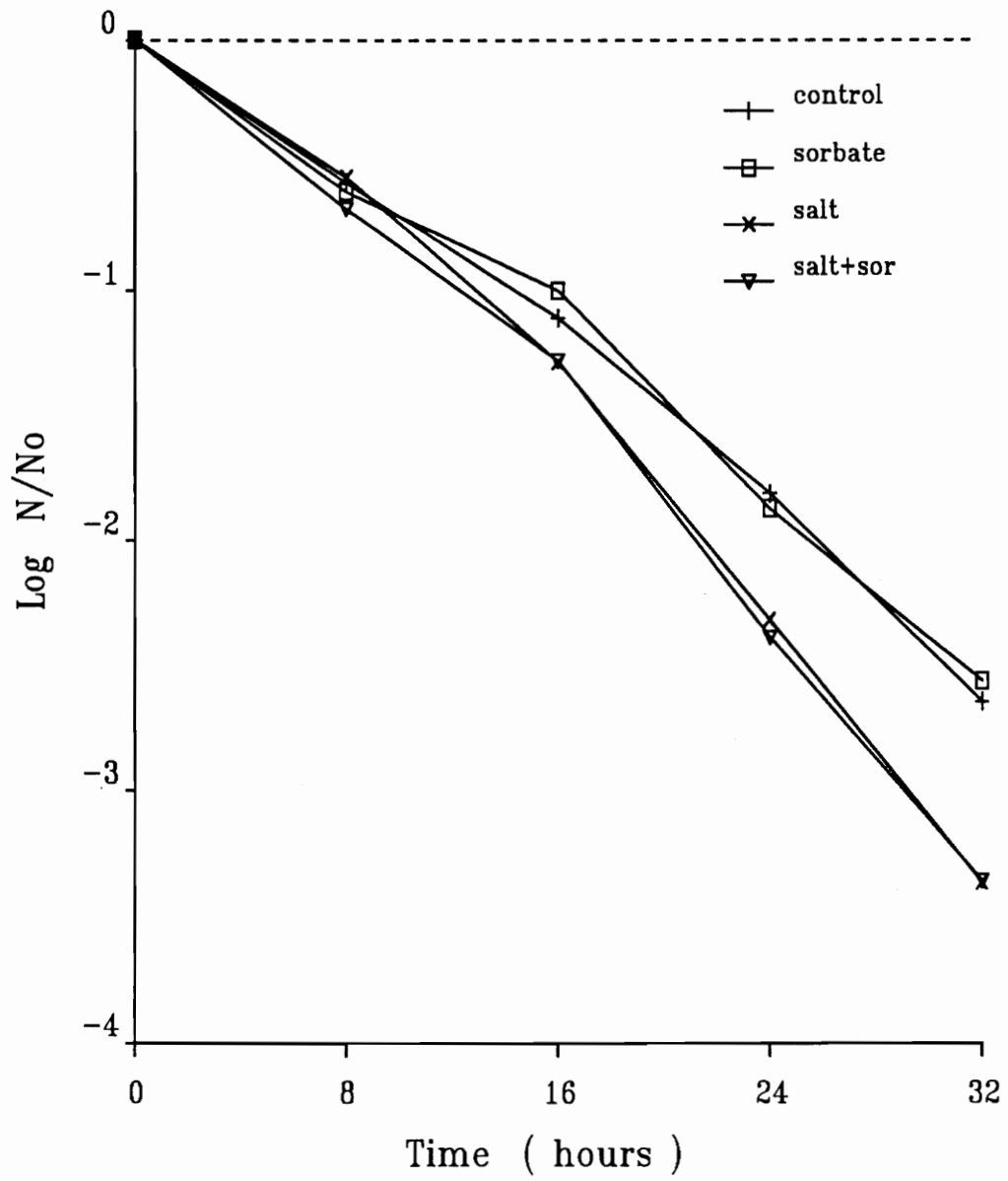
Survivor curve for Trial 1, 80 °C



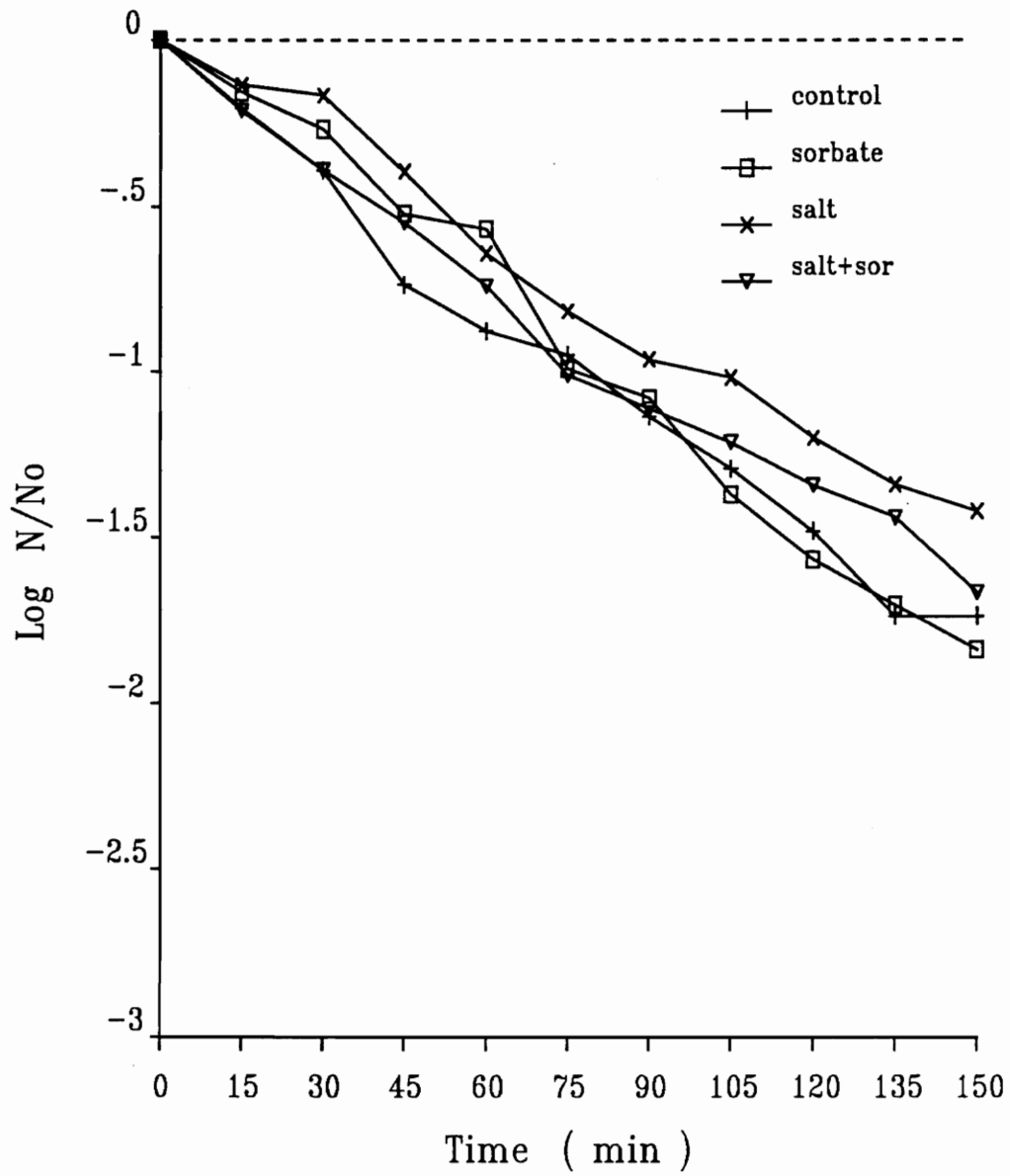
Survivor curve for Trial 2, 50 °C



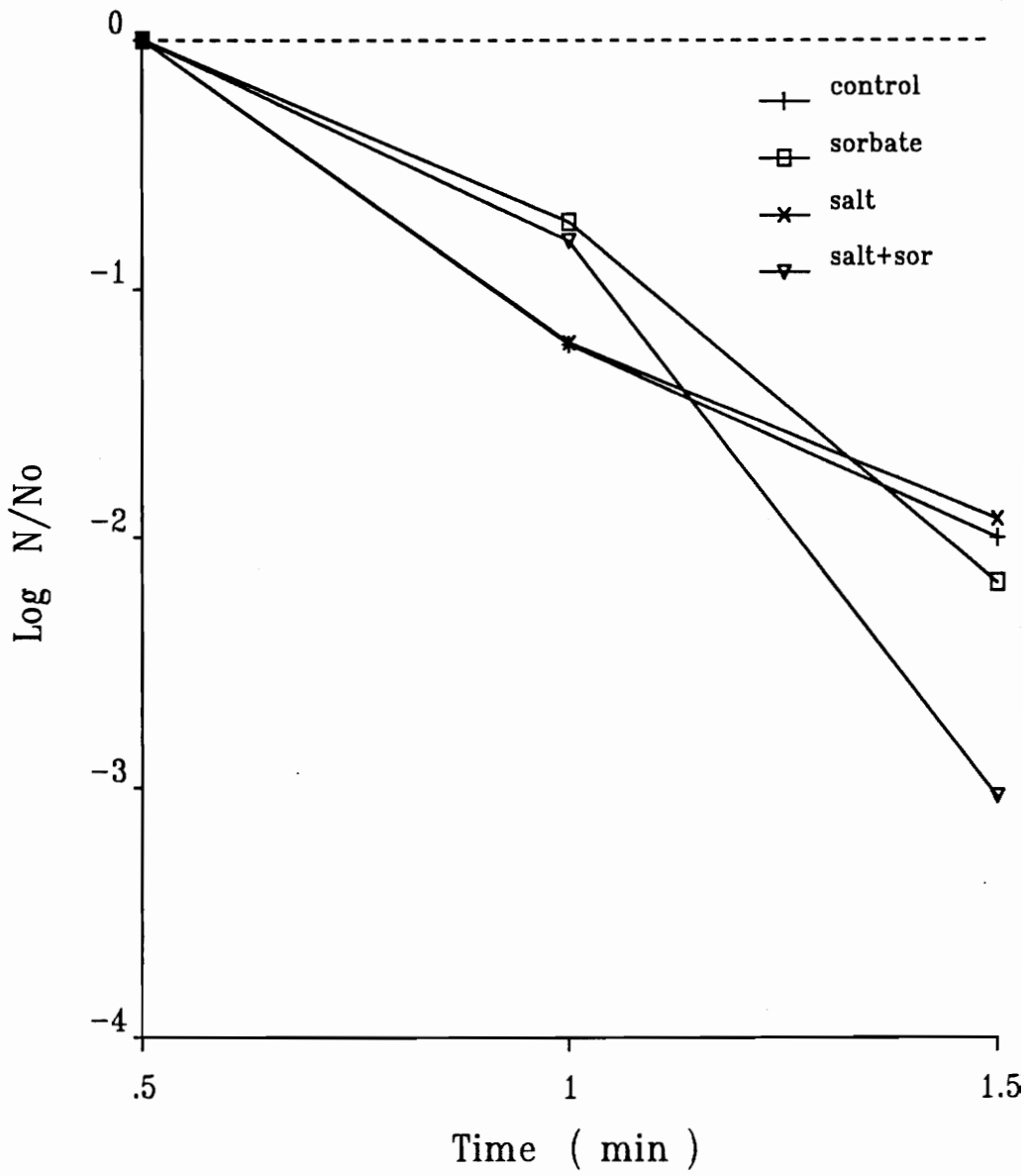
Survivor curve for Trial 2, 55 °C



Survivor curve for Trial 2, 60 °C



Survivor curve for Trial 2, 70 °C



Survivor curve for Trial 2, 80 °C

## VITA

The author of this thesis, Martin W. Bucknavage, Jr., was born June 18, 1963 in Plains, Pa. He is the son of Martin and Joan Bucknavage. He attended Coughlin High School in Wilkes-Barre, Pa. and the Pennsylvania State University, University Park, Pa., where he received his Bachelor of Science degree in Microbiology in May, 1985. He entered the Graduate School of Virginia Polytechnic Institute and State University in September, 1985 and is currently pursuing the Master of Science degree in Food Science and Technology.

Martin is a member of Institute of Food Technologists (IFT), Phi Sigma Delta, and the American Society of Microbiology (ASM).

A handwritten signature in cursive script, reading "Martin W. Bucknavage, Jr.", located in the lower right portion of the page.