AN EXAMINATION OF THE BEHAVIOUR OF LISTERIA MONOCYTOGENES DURING THE STORAGE AND HEAT PROCESSING OF SHRIMP (PANDALUS BOREALIS)

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AN EXAMINATION OF THE BEHAVIOUR OF *LISTERIA MONOCYTOGENES* DURING THE STORAGE AND HEAT PROCESSING OF SHRIMP (*PANDALUS BOREALIS*)

by

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Abstract

L. monocytogenes is ubiquitous in nature, has been isolated from many different types of food, has human health implications and can survive at very low temperatures. Additionally, the infectious dose of the pathogen is unknown, thus this bacteria is a concern in ready-to-eat seafood products. Currently, there is a lack of available information on the thermal resistance of *Listeria monocytogenes* in seafood, particularly in shrimp (*Pandalus borealis*), and on the pathogen's behaviour under different conditions of storage in seafood products. Furthermore, due to the lack of available data, it has been quite difficult for guidelines, regarding the processing times and temperatures required to control the presence of *L. monocytogenes* in foods, to be established. Hence, this study investigated the behaviour of *L. monocytogenes* on inoculated raw shrimp stored at -20°C, on ice (0-1°C), 4°C and 10°C and examined its thermal resistance in shrimp. Additionally, the total aerobic colony count and incidence of *L. monocytogenes* on raw shrimp was examined.

Low levels of total aerobic bacteria, averaging 429.8 CFU/g, were found on the raw shrimp and no *L. monocytogenes* cells were isolated from the samples. For the storage experiments, shrimp samples were inoculated with low (10^2 CFU/g) and high (10^6 CFU/g) levels of the bacteria and stored at the various temperatures in sealed plastic bags. Overall, *L. monocytogenes* was able to survive, but not grow, when stored on shrimp at -20°C and on ice (0-1°C). However, the pathogen was able to grow, quite significantly, when stored at temperatures of 4°C and 10°C, with generation times of 3.60 and 3.08 min at 4°C and 1.1

and 1.96 min at 10°C, for low and high inoculation levels, respectively. The highest increase in *L.monocytogenes* numbers was on shrimp inoculated with 10^2 CFU/g of the bacterium and stored at 10°C. Hence, refrigeration and freezing are no safeguards against this pathogen.

For the thermal resistance portion of this study, the capillary tube method was used. Triplicate thermal inactivation trials were carried out and D and *z* values determined for temperatures of 65, 68, 70 and 72°C. *L. monocytogenes* were enumerated using both *Listeria*-selective agars, Oxford and PALCAM, and a non-selective agar, TSA. D values were calculated using $D = -slope^{-1}$, obtained from the linear regression analysis of the survivor curve (log_{10} survivors vs. time). Average D values obtained in this study for *L.monocytogenes* in shrimp were 0.591, 0.266, 0.144 and 0.0202 min, for temperatures of 65, 68, 70 and 72°C, respectively. A *z* value of 5.07°C was also determined. Also, D values of 5.73, 2.31, 0.0063 and 0.00065 min were predicted for 60, 62, 75 and 80°C, using the D values and *z* value obtained from the experiments. The results of this study are compared to those of other studies.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

A_w	Water activity
CFU	Colony forming units
GMP	Good manufacturing practice
HTST	High-temperature-short-time
LEB	*Listeria* enrichment broth
LPM	Lithium chloride-phenylethanol-moxalactum medium
MPN	Most probable number
MFB	Modified Fraser secondary enrichment broth base
OXA	Oxford medium base
PAL	Polymyxin acriflavin lithium-chloride ceftazidime aesculin mannitol agar
PCA	Plate count agar
TSA	Trypticase soy agar
TSA-YE	Trypticase soy agar with yeast extract
TSB	Trypticase soy broth
TSB-YE	Trypticase soy broth with yeast extract

List of Appendices

Chapter One

Introduction

1.1 Microorganisms in Seafood: A Background

The consumption of seafood has dramatically increased in recent years, which, in turn, has raised public health concern over its safety (Liston, 1990; Mu *et al.*, 1997). Seafood, like any other food, is associated with a variety of nonpathogenic and pathogenic microorganisms (Ward and Hackney, 1991). Due to their ability to cause illness in consumers, only the pathogenic microorganisms are of concern and will be discussed here. The pathogens most commonly associated with seafood include *Clostridium botulinum*, *Vibrio* species, *Aeromonas* species, *Plesiomonas shigelloides*, *Listeria monocytogenes*, *Salmonella* species, *Campylobacter jejuni*, *Shigella* species, *Yersinia enterocolitica*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and viruses (Oblinger, 1988; Liston, 1990; Ahmed, 1992; Sinell, 1995; Garrett *et al.*, 1997).

L. monocytogenes is one of the more important of these pathogens since it is ubiquitous in nature, able to withstand environmental stresses such as salt and acid, and can grow at refrigeration temperatures of less than 4°C, which were once thought to prevent the growth of any food borne pathogens (Ahmed, 1992; McCarthy, 1997; Wang and Johnson, 1997). Accordingly, this pathogen is of major concern in ready-to-eat seafoods that are minimally processed or not cooked before consumption, e.g., cooked and peeled shrimp. If *L. monocytogenes* is present on the finished product, it can survive storage at refrigeration

temperatures and, since the product will not be cooked prior to consumption, the pathogen will not be subsequently destroyed before eating. Thus, if present in high enough numbers, it has the potential to cause illness. Furthermore, *L.monocytogenes* has been isolated from many ready-to-eat foods including cooked shrimp and crabmeat, smoked fish and surimi-based seafood (McCarthy, 1997).

1.2 Rationale and Objectives

Many countries, including Canada, have imposed a zero tolerance for *L.monocytogenes* in cooked, ready-to-eat seafood products, such as cooked and peeled shrimp (Curtis and Lee, 1995; Budu-Amoako *et al.*, 1999). It has been suggested that cooking foods to an internal temperature of 70°C for two minutes would be sufficient to ensure complete destruction of *L. monocytogenes* (Mackey and Bratchell, 1989; Farber and Peterkin, 1991; Huss *et al.*, 2000). There are, however, no official guidelines regarding the cooking times and temperatures required to effectively eliminate *L. monocytogenes* in seafood products. The cooking of seafood to an internal temperature of 80°C has been suggested as a good manufacturing practice (GMP) in the shrimp processing industry. This temperature should be sufficient to eliminate the pathogen from ready-to-eat seafood products and this guideline has, to varying degrees, been used in the shrimp processing industry. This temperature, however, is thought to be unnecessary and too high, by shrimp processors, as it can result in overcooking, leading to low yields and poor product texture. Furthermore, this practice has not been established as a legislative requirement or guideline,

and, as indicated, there is currently a lack of scientific information on which to evaluate its effectiveness. Therefore, the main objective of this research is to investigate the thermal resistance of *L. monocytogenes* in shrimp (*Pandalus borealis*) and identify effective times and temperatures required to destroy this pathogen in this seafood.

L. monocytogenes is able to survive under adverse conditions and grow at refrigeration temperatures. This has raised concerns for the potential hazard faced if an inadequate thermal processing step is employed since seafood is usually stored under refrigeration conditions after this step. Furthermore, raw seafood is also refrigerated, frozen or stored on ice prior to processing. Additionally, there is very little scientific information available regarding the behaviour of *L. monocytogenes*, on seafood, during storage at these different conditions. Due to this lack of information concerning the behaviour of this pathogen in seafoods, and consumer and regulatory concerns regarding food safety, as well as *L. monocytogenes*'s ability to grow over a broad temperature range, the second objective of this research will examine the behaviour of *L. monocytogenes* on raw shrimp, inoculated with both low and high levels of the pathogen, under different conditions of storage (-20°C, ice, 4°C and 10°C).

Furthermore, there is concern about the presence and numbers of *L. monocytogenes* in cooked, ready-to-eat foods, such as shrimp, crab meat and smoked fish, because of the potential for growth during storage through the cold chain (Elliot and Kvenberg, 2000). Finally, determination of the total aerobic colony counts and *L. monocytogenes* incidence on raw shrimp samples is the third objective of this study.

1.3 Thesis Structure

Following this introduction, Chapter Two is a literature review, which gives an overview of *L. monocytogenes*, including its characteristics and sources, as well as describing listeriosis, the disease caused by the pathogen. It also discusses the control of the pathogen, in terms of its storage and thermal resistance. Finally, the chapter reviews the regulations and guidelines in place in the shrimp processing industry to deal with this pathogen. Chapter Three outlines the methodological requirements of this study, giving a step-by-step account of the data collection process for three major objectives; the bacteria, including *L. monocytogenes*, present on raw shrimp, storage experiments and thermal resistance experiments. Chapter Four presents the results. Chapter Five discusses the results, compares them with other similar studies and evaluates the methods used. The chapter also discusses the applicability of the data to the control of *L. monocytogenes* in the shrimp processing industry. Finally, Chapter Six summarizes the findings in this study and a discussion of the perceived need for future work in this area.

Chapter Two

Listeria and Listeriosis

2.1 Introduction

One of the most important and widely discussed foodborne pathogens is *Listeria monocytogenes*. This interest is due to the ability of this microorganism to grow at refrigeration temperatures, which were once thought to prevent pathogen growth, and to the recent outbreaks of the *Listeria*-related illness, listeriosis, linked to seafood. This chapter presents a review of the literature related to this pathogen, specifically its characteristics, sources, storage and thermal resistance, as well as human health implications and regulatory issues.

2.2 Characteristics of *Listeria monocytogenes*

Listeria monocytogenes was first described by Murray *et al.* in 1926 (Huhtanen *et al.*, 1989). It is a gram-positive, non-sporeforming, facultatively anaerobic, rod-shaped bacterium (Brackett, 1988; Cole *et al.*, 1990; Ryser and Marth, 1991). It is able to grow at temperatures between -0.4 and 50°C (Farber and Peterkin, 1991; Zemser and Martin, 1998), with an optimal growth temperature of 30 to 37°C (Lovett, 1990; Ryser and Marth, 1991). The pH range for growth of this organism is 4.2 to 9.5 and it is able to grow in salt concentrations up to 10% (Bonnell, 1994; Farber, 2000; Ross *et al.*, 2000), but it can survive at salt concentrations of 25% (Ahmed, 1991).

Although seven species of the genus *Listeria* are currently recognized: *L.monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. murrayi*, and *L.grayi* (Jay, 1992; Oh and Marshall, 1993), *L. monocytogenes* is the only species considered to be an important human pathogen, and is currently responsible for nearly all cases of *Listeria*-related illness, i.e., listeriosis (Jones, 1990; Ryser and Marth, 1991).

2.3 Sources of *Listeria monocytogenes*

2.3.1 Natural Sources

L. monocytogenes is ubiquitous in nature and has been isolated from numerous sources, such as water, soil, vegetation and raw and treated sewage and silage (McCarthy *et al.*, 1990; Bremer and Osborne, 1995; Zemser and Martin, 1998). Furthermore, various animal species also serve as hosts for *L. monocytogenes*, including domestic animals such as cattle, sheep, and goats. Additionally, various avian species such as domestic fowl, songbirds, birds of prey and seagulls also act as hosts for the pathogen (Brackett, 1988; Bonnell, 1994; Gecan *et al.*, 1994).

2.3.2 Presence in Food

L. monocytogenes has also been isolated from many different foods. In fact, due to the numerous outbreaks of foodborne listeriosis, there has been a significant amount of research undertaken to determine the presence of *Listeria* species, especially *L.monocytogenes*, in various foods. Dairy products have received a vast amount of attention

as they have been identified as the vehicle of infection in several cases of listeriosis. *L.monocytogenes* has been recovered from raw milk (Fleming *et al.*, 1985; Farber *et al.*, 1988; Ryser and Marth, 1991), various types of cheese (Farber and Peterkin, 1991; Rorvik and Yndestad, 1991; Delgado da Silva *et al.*, 1998) and ice cream (Greenwood *et al.*, 1991; Ryser and Marth, 1991). *L. monocytogenes* has also been isolated from meat, poultry (Brackett, 1988; Rorvik and Yndestad, 1991; Ryu *et al.*, 1992; Arumugaswamy *et al.*, 1994) and egg products (Leasor and Foegeding, 1989; Farber and Peterkin, 1991; Schuman and Sheldon, 1997). Additionally, fruits and vegetables have been identified as sources of *L.monocytogenes*. Cabbage was the vehicle of transmission in the 1981 outbreak of listeriosis in Nova Scotia, Canada (Schlech *et al.*, 1983). Heisick *et al.* (1989) detected *L.monocytogenes* in cabbage, cucumbers, potatoes and radishes. Fresh lettuce, celery and tomatoes have also been suggested to play a role in transmission of the pathogen (Brackett, 1988). Hence, *L. monocytogenes* is as ubiquitous in foods as it is in nature.

2.3.2.1 Presence in Seafood

The presence of *Listeria* in seafood, however, has only recently been investigated. The first broad scale survey on the prevalence of *Listeria* spp. in frozen fish products was published by Weagant *et al.* (1988). Fifty-seven samples were analyzed, of which 35 (61%) contained *Listeria* spp. and 15 (26%) were positive for *L. monocytogenes*. The positive samples included raw shrimp, cooked and peeled shrimp, cooked crabmeat, lobster tail, fin fish, and surimi-based seafood. Fuchs and Surendran (1989) tested 35 tropical fish and

fishery products for the presence of *Listeria* and detected *L. innocua* in 8 (23%) of the samples. Jeyasekaran *et al.* (1996) also investigated the incidence of *Listeria* in tropical fish. *Listeria* spp. were detected in 72.4% and 44.4% of the fresh finfish and shellfish, respectively, while the percentages of *L. monocytogenes* were 17.2% and 12.1%, respectively.

In an examination of 113 wholesale level seafood products, Farber (1991) found 15 to be positive for *L. monocytogenes*, including ready-to-eat shrimp, crab and smoked salmon products. Similarly, in a study using 128 samples of seafood on the Icelandic market, Hartemink and Georgsson (1991) detected the presence of *Listeria* spp. in 56% of the raw fish samples, 29% of the smoked fish samples, 9% of the frozen shrimp samples and 32% of the fish salad samples. Salads positive for *Listeria* included gravad salmon, smoked salmon, shrimp and herring. *L. monocytogenes* was detected in 46% of the positive samples listed above. However, no *Listeria* spp. were found in the shellfish or dried fish.

Ryu *et al.* (1992) purchased seafood, including raw tuna, raw prawn, salted raw seafood and cooked seafood, from supermarkets or department stores in or around Tokyo and tested these samples for the presence of *Listeria*. They found that 6.1% of the 114 samples of fish and fish products contained *L. monocytogenes*. In a study of 3331 samples of cooked shrimp from 26 Icelandic factories, over a six year period, Valdimarsson *et al.* (1998) found *Listeria* spp. in 270 (8.1%) of the samples, of which 26.5% was *L. monocytogenes*.

Motes (1991) found that 11% of the live shrimp samples he tested contained *L.monocytogenes*, while none of the oysters sampled were positive for *Listeria*. Similarly,

Weagant *et al.* (1988) were unable to isolate *Listeria* from oysters. Monfort *et al.* (1998) also investigated the incidence of *Listeria* spp. in live shellfish collected from 9 growing sites in western France. Of the 120 samples analyzed, they found 55% of the samples to contain *Listeria* spp., of which 9.2% was positively identified as *L. monocytogenes*. The shellfish tested included oysters, mussels and cockels.

Adesiyun (1993) studied the incidence of *Listeria* spp. in meat and raw seafood (fish and shrimp) in Trinidad. Of the 102 fish samples tested, 11 (10.8%) were positive for *Listeria* spp. and *L. monocytogenes* was detected in 2 (2.0%) of the samples tested. Additionally, Arumugaswamy *et al.* (1994) found that a high proportion (44%) of the fresh raw prawns they tested contained *L. monocytogenes*. This organism was also present in 22% of the ready-to-eat squid, prawns, chicken and clams. However, they did not state the exact number of each of these foods tested, or the number positive. In a comparison of the microbiological quality of raw shrimp from China, Ecuador and Mexico, Berry *et al.* (1994) found *L.monocytogenes* in 6.7% of the samples.

Dillon *et al.* (1994) investigated the prevalence of *Listeria* spp. in hot and cold smoked seafood products commercially available in Newfoundland. Of the 258 samples tested, 43 (16.7%) contained *Listeria* spp. *L. monocytogenes* made up 27.9% of these 43 isolates. The samples that contained *L. monocytogenes* were cod and herring. Other species of *Listeria* were found in mackerel, caplin and salmon, while trout, eel and charr were not found to contain the bacteria. Heinitz and Johnson (1998) also studied the incidence of *Listeria* spp. in smoked seafood, including finfish and shellfish. *L. monocytogenes* was

isolated from 14% of the 1080 samples examined. Similarly, Jorgensen and Huss (1998) examined ready-to-eat seafood from Danish production sites for the presence of *L.monocytogenes*. They found the highest prevalence to be in cold-smoked fish (34-60%), while the lowest was found in heat-treated and cured seafood (4-12%). For all of the above studies, a higher incidence of *L. monocytogenes* was found in cold-smoked fish products.

In a study carried out by Rorvik and Yndestad (1991) in Norway , 7 (12%) of the 57 seafood samples tested contained *L. monocytogenes*. These products included smoked salmon (9%), shrimp (18%) and minced fish (12%). Fuchs and Sirvas (1991) analyzed 32 samples of ceviche, a South American acidified fish product, for the presence of *Listeria*. *L.innocua* was isolated from 24 (75%) of the samples, while 3 (9%) of the samples were positive for *L.monocytogenes*. Finally, Gecan *et al.* (1994) examined domestic (United States) and imported fresh and frozen shrimp for the presence of *Listeria*. Of the 205 samples analyzed, 14 (6.8%) were positive for *Listeria spp.* of which nine contained *L.monocytogenes*.

2.4 Human Health Implications

2.4.1 Listeriosis

The disease caused by the pathogen *Listeria monocytogenes* is known as listeriosis. This disease is severe and often fatal in susceptible hosts (Busch and Donnelly, 1992). While the first case of listeriosis in humans was confirmed by Nyfeldt in 1929 (Ryser and Marth, 1991), *L. monocytogenes* has only been recognized as an important agent of foodborne

illness in the last 20 years (Ben Embarek, 1994; Carlier *et al.*, 1996).

There is no unique set of symptoms characterizing human listeriosis as the course of the disease depends upon the individual's susceptibility. In less serious cases of the disease, symptoms may be limited to influenza-like symptoms, such as fever, headache and chills or gastrointestinal symptoms, such as nausea, vomiting and diarrhea. However, these can precede a more serious illness. The most commonly occurring symptoms in susceptible adults are meningitis or meningoencephalitis (brain infections), septicaemia (bacteria in the bloodstream), encephalitis (Kerr *et al.*, 1993; Bonnell, 1994), and, in pregnant women, intrauterine or cervical infections, which may result in spontaneous abortion, premature birth, stillbirth or mental retardation in the baby (Bahk and Marth, 1990; Food Institute of Canada, 1992).

Listeriosis is rare in healthy, non-pregnant adults. Individuals most susceptible to the disease include pregnant women and their fetuses, newborns, people over 65 years and immunocompromised individuals, such as cancer patients, transplant recipients, AIDS patients, diabetics, asthmatics, ulcerative colitis patients and alcoholics or drug addicts (Food Institute of Canada, 1992; Jay, 1992). Most healthy people are either unaffected by *Listeria*, or experience only mild flu-like symptoms (Cole *et al.*, 1990; Jay, 1992). Foodborne listeriosis has a relatively high mortality rate of 20 to 30% in symptomatically infected people (Patchett *et al.*, 1996; Rocourt *et al.*, 2000). The principal route of transmission of listeriosis is via the consumption of food contaminated with the causative organism, *L.monocytogenes* (Broome *et al.*, 1990; Snelling *et al.*, 1991; Kerr *et al.*, 1993) and human

consumption of such food has lead to numerous outbreaks of listeriosis.

2.4.2 Outbreaks of Listeriosis

The first reported outbreak in North America in which food was positively identified as the vehicle of infection occurred in Nova Scotia in 1981. Coleslaw made from cabbage grown on a farm fertilized with manure from sheep infected with *Listeria*, was the vehicle of transmission, and of the 41 individuals infected, there were 18 deaths (Schlech *et al.*, 1983). Since that time *L. monocytogenes* has received a great deal of attention, and numerous cases of foodborne outbreaks have been documented.

In 1983 there were 49 cases of listeriosis in Boston, of which 14 resulted in death. The vehicle of transmission was pasteurized milk obtained from *Listeria*-infected dairy cows (Fleming *et al.*, 1985). Mexican-style cheese, manufactured with contaminated milk, was implicated as the vehicle of transmission in a listerosis outbreak in California in 1985 which involved 142 cases and resulted in 48 deaths (Jay, 1992). Other documented outbreaks include a perinatal epidemic in Auckland, New Zealand in 1980, which resulted in 29 cases, including five deaths. Raw fish and shellfish were suggested to have been the source of infection, but this was not proven conclusively (Fuchs and Sirvas, 1991). In Canton de Vaud, Switzerland, 122 cases, with 31 deaths, were reported from 1983-1987. Swiss soft cheese was suggested to be the vehicle of transmission (Jay, 1992; Fenlon *et al.*, 1996). Ice-cream and salami were linked to a listerosis outbreak, including 16 deaths in 36 cases, in Philadelphia in 1986-1987 (Jay, 1992). Another outbreak, in the United Kingdom, which

included 300 cases, was linked to imported pate (Farber, 2000).

In 1989 shrimp was linked to an outbreak that included nine cases and one death in Connecticut (Reido *et al.*, 1990). Seafood, was also linked to one case of listeriosis in Italy in 1989 and to 2 cases in Australia in 1991. Smoked mussels were thought to be the caustaive agent for the latter (Rocourt *et al.*, 2000). Another occurrence of listeriosis involving 4 cases in New Zealand, in 1992, was also linked to smoked mussels. Pork tongue in jelly was implicated as the major vehicle of transmission in an outbreak in France in 1992 that involved 279 cases (Carlier *et al.*, 1996).

Six to nine cases, with 2 deaths, occurred in Sweden between 1994 to 1995. Gravad rainbow trout was implicated as the vehicle of infection (Ericsson *et al.*, 1997). In 1996 in Ontario, Canada, imitation crab meat was linked to 2 cases of listeriosis (Farber *et al.*, 1997). Finally, cold-smoked rainbow trout was thought to be the causative agent in 5 cases of listeriosi in Finland (Miettinen *et al.*, 1999).

2.5 Controlling *Listeria monocytogenes* in Foods

Due to the fact that humans most commonly contract listeriosis by consuming food that has been contaminated with *L. monocytogenes* (Rorvik and Yndestad, 1991; Bremer and Osborne, 1995) and the human health implications of *Listeria* consumption, there has been a significant amount of research into the presence and abundance of *L. monocytogenes* in various types of food products. The necessity of effective measures to control *Listeria* in food is also widely recognized, particularly in relation to processing and storage techniques

required to ensure that food products do not contain this pathogen.

L. monocytogenes may be present on the raw material, may be introduced to the food product during the various stages of processing (e.g., handling), or it may become contaminated after processing (e.g., during packaging or shipping). Regardless of the source of contamination, the occurrence of *L. monocytogenes* in ready-to-eat products can only be due to either post-processing or an inadequate thermal processing step. Since the implementation of effective cleaning and sanitizing regimes and the enforcement of good personal hygiene can virtually eliminate post-processing contamination, the critical control point in the production of ready-to-eat foods and one of the most important ways of controlling *Listeria* in food products, is to ensure that an adequate thermal processing step is employed prior to the food being passed on to the consumer. As a result, much of the research conducted regarding *Listeria* has focussed on the thermal resistance of *L.monocytogenes*, and specifically, the processing times and temperatures required to eliminate the pathogen from food. The development and implementation of effective measures to control *L. monocytogenes* in food is, therefore, dependent upon an understanding of the thermal resistence of the pathogen The following section provides an overview of various studies carried out on this topic, focussing primarily on seafood.

2.5.1 Thermal Resistance of *Listeria monocytogenes*

Most of the information currently available on the thermal resistance, i.e., the ability of the microorganism to resist or survive various types of heat treatment, of

L.monocytogenes in food deals with dairy products and red meat. Studies that have examined the thermal resistance of *L. monocytogenes* in milk have produced somewhat conflicting results. Doyle *et al.* (1987) suggested that the pathogen was able to survive high-temperature-short-time (HTST) pasteurization, in which heat is applied for 15 seconds at 71.7°C. While others have concluded that it cannot survive such treatment (Farber *et al.*, 1988; Lovett *et al.*, 1991; Farber *et al.*, 1992). Little is presently known, however, about the thermal resistance of *L. monocytogenes* in seafood.

One of the first studies that examined the thermal resistance of *L. monocytogenes* in seafood was done by Harrison and Huang (1990) on blue crab meat. The strain *L.monocytogenes* Scott A, inoculated onto blue crab at levels of approximately 10^7 cells/g, was found to have a D value (decimal reduction time or time required to destroy 90% of the organisms) of 2.61 min at 60°C, 12.00 min at 55°C and 40.43 min at 50°C and a z value of 8.4°C. The D value is numerically equal to the number of minutes required for the survivor curve to traverse one log cycle, while the z value refers to the degrees Celsius required for the thermal destruction curve to traverse one log cycle. This value provides information on the relative resistance of an organism to different destructive temperatures, while the D value reflects the resistance of an organism to a specific temperature (Jay, 1992). McCarthy *et al.* (1990) examined the recovery of heat-stressed *L.monocytogenes* from experimentally and naturally contaminated shrimp. Shrimp tail meat was internally inoculated to contain 10^5 *L.monocytogenes* cells/g and boiled for time intervals of one, three and five minutes. The pathogen was detected in inoculated shrimp tails, but not in naturally contaminated shrimp

(approximately 10^3-10^5 cells/g). No D values were given. They suggested that naturally occurring *Listeria* are more heat-sensitive than experimental *Listeria*, possibly due to their growth environment or other unknown stresses. Dorsa *et al.* (1993) determined the thermal resistance of *L. monocytogenes* in crawfish tail meat. The meat was inoculated with 10^8-10^9 cells/g levels and heat processed at three different temperatures. The D values obtained for 55, 60 and 65°C, were 10.23, 1.98 and 0.19 min, respectively. Also, a z value of 5.5°C was determined.

In a study of the thermal resistance of *L. monocytogenes* in experimentally contaminated trout, Jemmi and Keusch (1992) found that *L. monocytogenes*, inoculated at 10^6 cells/g levels, did not survive a hot-smoking process in which the trout was smoked at 65°C for 20 minutes. Bremer and Osborne (1995) also studied the thermal resistance of *L.monocytogenes* in a smoked seafood product. Green shell mussels were inoculated with 10^6 cells/g of the pathogen and heat treated at several temperatures. D values at 56, 58, 59, 60 and 62°C were determined to be 48.09, 16.25, 9.45, 5.49 and 1.85 min, respectively, with a z value of 4.25°C.

In a study on the heat resistance of *L. monocytogenes* in vacuum packaged pasteurized fish fillets, Ben Embarek and Huss (1993) determined D values for cod and salmon fillets. D values for 60°C of 1.95-1.98 min were obtained for cod and values of 4.23-1.48 were obtained for the salmon fillets. Budu-Amoako *et al.* (1999) found that heating cans of cold-pack lobster meat, inoculated with 10^3 and 10^5 cells/g of *L. monocytogenes*, at 60°C for five minutes and at 65°C for two and 10 minutes, resulted in decimal reductions of one

to three logs.

The regular isolation of *L. monocytogenes* from ready-to-eat seafood products and the few and varying results pertaining to the thermal resistance of the organism has prompted concern about the adequacy of thermal processing treatments employed by the seafood industry (Ben Embarek, 1994; Ryser and Marth, 1991). Additionally, *L. monocytogenes* is as heat resistant as or more heat resistant than most other vegetative foodborne pathogens (Mackey and Bratchell, 1989; Fairchild and Foegeding, 1993; Juneja and Eblen, 1999), which makes this issue all the more important.

2.5.2 Regulatory Issues

Many countries, including Canada, have imposed a zero tolerance for *L.monocytogenes* in cooked ready-to-eat seafood products, such as cooked and peeled shrimp (Curtis and Lee, 1995; Heinitz and Johnson, 1998; Budu-Amoako *et al.*, 1999). It has been suggested that cooking any type of food to an internal temperature of 70°C for two minutes would be sufficient to ensure complete destruction of *L. monocytogenes* (Mackey and Bratchell, 1989; Farber and Peterkin, 1991; Huss *et al.*, 2000). Upon reviewing published heat resistance data for *L. monocytogenes*, a decimal reduction time or D value of 2 minutes at 60°C and an average *z* value of 6°C appears to be typical (Farber, 1989; Mackey and Bratchell, 1989; Juneja and Eblen, 1999). There are, however, no official guidelines regarding the cooking times and temperatures required to effectively eliminate *L.monocytogenes* in seafood products. There has been some speculation that cooking food

to an internal temperature of 80°C is sufficient to eliminate the pathogen from ready-to-eat seafood products, and this has, to varying degrees, been used as a good manufacturing practice (GMP) in the shrimp processing industry. This temperature, however, is thought to be unnecessary and too high by many in the seafood industry, as it can result in overcooking, leading to low yields and poor product texture. Furthermore, this practice has not been established as a legislative requirement or guideline, and, as indicated, there is currently a lack of scientific information on which to evaluate its effectiveness.

2.6 Growth of *Listeria monocytogenes* in Seafood

Raw and processed seafoods are excellent substrates for the growth of most common agents of foodborne disease, especially if the food is held at improper temperatures (Ryser and Marth, 1991). *L. monocytogenes* has the ability to withstand harsh environmental conditions, such as pH extremes and heat, and is able to survive freezing and grow at refrigeration temperatures. The pathogen is able to grow in product under conditions that would normally inactivate other microorganisms (Oblinger, 1988). Hence, it is important to control its growth during processes such as cooling, freezing or storage in cool rooms (Membre *et al.*, 1999). The behaviour of this pathogen in seafoods, however, has not been extensively studied and, thus far, relatively few studies have been published in this area. Additionally, little is known about the survival and growth characteristics of *L.monocytogenes* in ready-to-eat seafoods (McCarthy, 1997).

In a study done by Lovett *et al.* (1990), *L. monocytogenes* grew quickly, with a generation time of approximately 12 hours, in raw shrimp, crab, surimi and whitefish samples inoculated with about 10^3 CFU/g (colony forming units per gram) of the pathogen. In all four products, the levels of *L.monocytogenes* reached a maximum of more than 10^8 CFU/g (an increase of 5 logs) after 14 days of storage at 7°C. In a study done by Harrison *et al.* (1991) on the fate of *L.monocytogenes* on packaged, refrigerated and frozen seafood, the pathogen was able to survive, but not grow on two raw Atlantic whitefish species stored on ice for 3 weeks. Also, *L. monocytogenes* populations on frozen products (-20°C) decreased by less than 1 log after 3 months.

Leung *et al.* (1992) detected marginal growth of *L. monocytogenes* on inoculated catfish fillets stored at 4°C for 16 days. Similarly, Ben Embarek and Huss (1992) found that *L. monocytogenes* grew only marginally on raw salmon stored at 5°C for 6 days, while the pathogen grew rapidly at 10°C storage, increasing in numbers by approximately 2.2 logs in 4 days. In a similar study, Wang and Shelef (1992) indicated that *L. monocytogenes* Scott A was able to grow on inoculated raw cod fillets stored at 5°C and grew quite fast at 20°C with the population increasing by 5 logs in 2 days. Also, the *L. monocytogenes* population remained constant for 10 days and then increased by more than one log by day 17, the end of storage.

L. monocytogenes grew fairly well on inoculated cooked lobster, shrimp, crab and smoked salmon, increasing by about 2-3 logs within 7 days of storage at 4°C, in a study by Farber (1991). In the same study, the seafood products were stored at room temperature for

6 hours, to simulate consumer abuse, during which time the *L. monocytogenes* increased in numbers by about 1.0, 1.0, 0.2 and 0 to 1.0 logs, for shrimp, crab, salmon, and lobster, respectively.

Brackett and Beuchat (1990) found that *L. monocytogenes* did not increase in population size on crab meat inoculated with the pathogen after 7 days of storage at 5°C. Numbers of the pathogen did increase by approximately 2 to 2.8 logs, however, after 12 to 14 days of storage. Furthermore, they also found that the pathogen grew quite well on inoculated crabmeat stored at 10°C, increasing by more than 4 logs by day 8.

In an investigation into the growth of inoculated psychrotrophic pathogens on refrigerated fillets of aquacultured rainbow trout and channel catfish, Fernandes *et al.* (1998) found that *L. monocytogenes* counts increased significantly by 2 log CFU/100g during 15 days of storage at 4°C. Also, they found that *L. monocytogenes* counts increased from $10^{5.1}$ CFU/100g on day 1 to $10^{6.9}$ CFU/100g on day 15.

Dalgaard and Jorgensen (1998) studied the growth of *L. monocytogenes* in naturally contaminated cold-smoked salmon stored at 5°C. They only observed modest growth, from 0 to 2.1 log (MPN/g), which corresponded to 0 to 0.7 log (MPN/g) per week. McCarthy (1990) examined the effects of storage on the survival of *L. monocytogenes* on crawfish and crabmeat. She found that, at 22°C, externally localized *L. monocytogenes* cell numbers increased by 1.4 log units after 6 hours and, at 30°C, numbers increased by 1.9 log units after 6 hours. Additionally, she found that *L. monocytogenes* could survive, but not grow, when the inoculated crawfish was stored at 6°C and -20°C. The bacteria did grow at 6°C, however,

when inoculated onto cooked crabmeat. A large increase of 3.8 log MPN/g was seen in this instance. Finally, no change in *L. monocytogenes* numbers was seen when inoculated smoked salmon was stored at -20, 6 and 22°C.

Rawles *et al.* (1995) also reported an increase in growth of *L. monocytogenes* inoculated onto fresh blue crabmeat when stored at refrigeration temperatures. They found that *L. monocytogenes* growth rate increased with increasing refrigeration temperatures. Finally, Hudson and Mott (1992) reported significant growth of this pathogen at 5 and 10°C within the shelf life of vacuum-packaged salmon.

2.7 Summary of the Literature Review

L. monocytogenes is as ubiquitous in seafoods as it is in nature. It has been regularly isolated from numerous types of seafood, including ready-to-eat seafood, and has been linked to several outbreaks of listeriosis thought to have been caused by different seafood products. Furthermore, this pathogen is as heat resistant as or more heat resistant than most other vegetative foodborne pathogens. It can be controlled by ensuring that an adequate thermal processing step is employed and by inhibiting growth of the bacterium during storage prior to and after processing. There is, however, very little scientific data available on the thermal resistance of this pathogen in seafood, particularly shrimp, thus the difficulty in establishing processing times and temperatures that will completely destroy any *L. monocytogenes* present on the raw seafood. With regard to storage, again, there is a lack of consensus in the published data and this is an area of great concern since raw and processed seafoods are

excellent substrates for growth of pathogenic bacteria, such as *L. monocytogenes*, especially if the seafood is stored or held at improper temperatures. Due to all of the reasons discussed above, the thermal resistance of *L. monocytogenes* in seafood and its behaviour during storage are areas where more investigation is essential.

Chapter Three

Materials and Methods

3.1 Test Organism

The organism used in this study was *Listeria monocytogenes* ½b (HPB #395), obtained from the Memorial University of Newfoundland stock collection. The bacterium was lyophilized for long term storage and periodically subcultured onto Trypticase Soy Agar (TSA) plates and stored at 4°C.

3.2 Media

Unless otherwise specified, all the media used in this work were of reagent or laboratory grade. The following media were obtained from Sigma Chemicals, Oakville, Ontario: plate count agar (PCA), peptone, *Listeria* enrichment broth (LEB), modified Fraser secondary enrichment broth base (MFB) and Fraser *Listeria* supplements, *Listeria* identification agar base - PALCAM agar (PAL) and supplements, acriflavin, moxalactum, trypticase soy broth (TSB), blood agar, sodium chloride, dextrose, esculin, maltose, rhamnose, methyl-α-D-mannopyranoside, ferric ammonium citrate and hypochlorite.

Trypticase soy agar (TSA), Oxford medium base and Oxford antimicrobic supplements (OXA), lithium chloride-phenylethanol-moxalactum agar base (LPM), Fraser broth base, modified and Fraser broth additive were obtained from Becton Dickinson and Company, Cockeysville, Maryland.

The following media were obtained from Fisher Scientific Ltd., Dartmouth, Nova Scotia: motility test medium, purple broth base, yeast extract, acetone, ethanol, nalidixic acid and glass capillary tubes (Kimax, 0.8 - 1.10 x 90 mm). Mannitol and xylose were obtained from BDH Chemicals, Dartmouth, Nova Scotia.

3.3 Shrimp Samples

Fresh and frozen raw shrimp samples were obtained from Fishery Products International Ltd, Port Union, Newfoundland. All fresh samples were stored at 4°C and analyzed within one day of receipt. Frozen samples were stored at -20°C until use.

3.4 Presence of Bacteria on Raw Shrimp

3.4.1 Aerobic Colony Count Protocol

The Health Protection Branch of Health Canada protocol, as outlined by Szabo (1998), was used to determine the aerobic colony count or the numbers of viable microorganisms per gram of fresh shrimp sample. Using aseptic techniques, eleven grams of fresh shrimp, weighed using a top-loading balance (Precisa 3500D), was transferred to a sterile stomacher bag, diluted with 99 mL of 0.1% peptone water diluent, and homogenized for 2 minutes at high speed in a Seward Stomacher 400 laboratory blender (Seward Medical, London, UK). The pH of this homogenate was then measured with a pre-standardized pH meter (Accumet pH Meter, Model No. 915, Fisher Scientific) and adjusted to 7.0 with sterile NaOH or HCl, if outside the range of 5.5 to 7.6. This homogenate was further serially diluted

as required. 1.0 mL and 0.1 mL aliquots of each dilution were transferred to labeled duplicate Petri plates and 12-15 mL of tempered plate count agar was added to each plate. At this point, one change was made to the Health Protection Branch Protocol, 3% salt was added to the plate count agar to allow the growth of marine bacteria, since the shrimp being analyzed came from salt water. Plates were allowed to solidify and incubated in the inverted position at 35°C for 48 hours. Colonies were numerated using a colony counter (Model 225, Technilab Instruments, Pequannock, New Jersey).

3.4.2 *Listeria monocytogenes* Incidence Protocol

The Health Protection Branch of Health Canada protocol, as outlined by Farber and Warburton (1998), was used to detect viable *L. monocytogenes* in fresh shrimp samples (Figure 3.1). The primary enrichment step consisted of the aseptic addition of twenty-five grams of fresh shrimp, weighed using a top-loading balance (Precisa 3500D), and 225 mL of *Listeria* enrichment broth (LEB) to a sterile stomacher bag and homogenizing for 2 minutes at high speed in a Seward Stomacher 400 laboratory blender (Seward Medical, London, UK). The homogenate was then transferred to a sterile Erlenmeyer flask and incubated at 30°C for 48 hours. The isolation procedure consisted of streaking LEB onto *Listeria*-selective agars, Oxford, PALCAM and LPM agar plates at 24 and 48 hours. Oxford and PALCAM plates were incubated at 35°C for 24-48 h, and LPM plates were incubated at 30°C for 24-48 h. The second enrichment step consisted of inoculating 0.1 mL of the LEB culture into 9.9 mL of modified Fraser Broth (MFB) at 24 and 48 hour intervals. The MFB

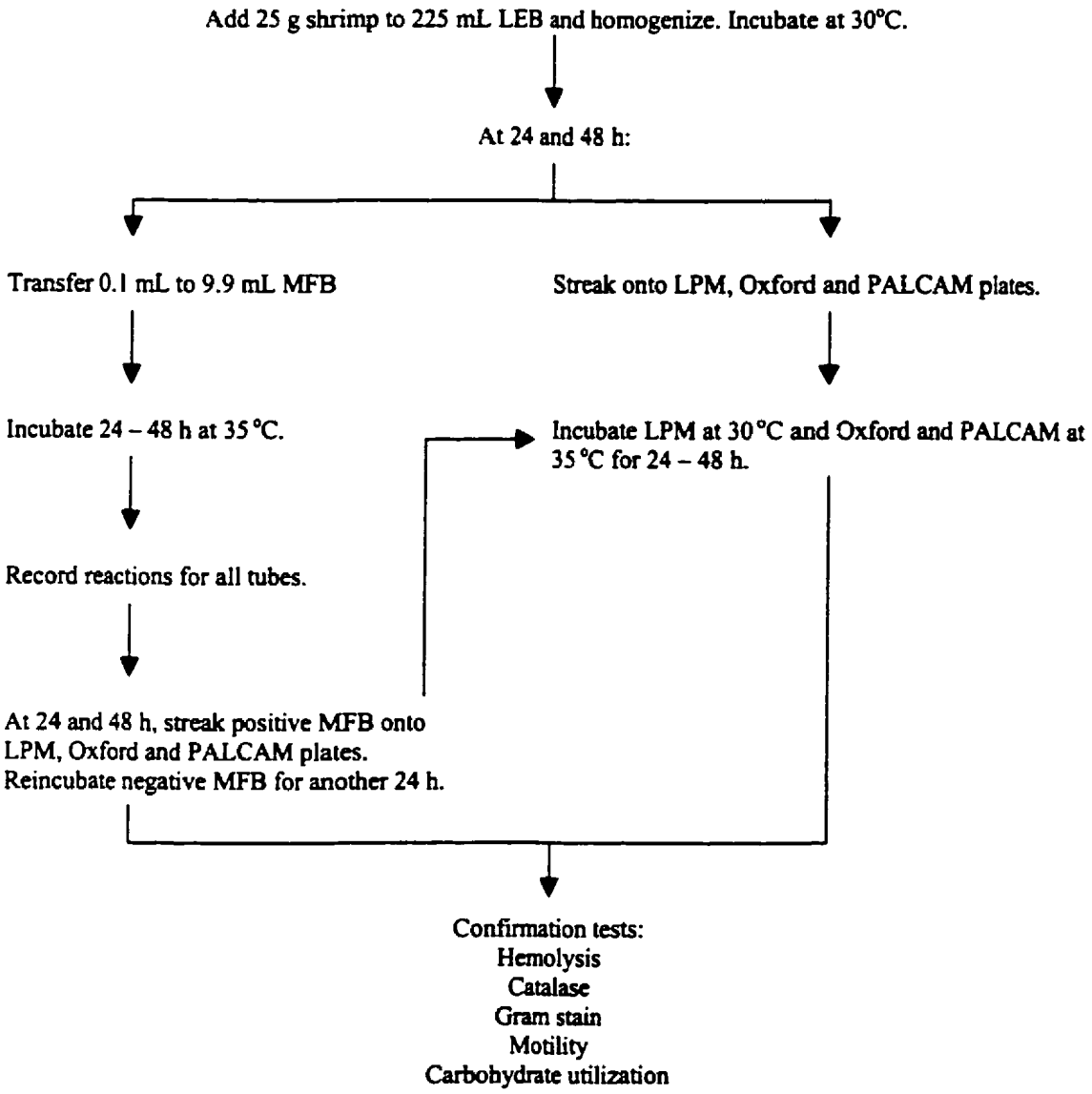

Figure 3.1: A Flow Diagram for the Protocol Used to Isolate *L. monocytogenes* From Shrimp Samples (*Adapted from Farber and Warburton, 1998*).

enrichment was then incubated for 24 to 26 hours at 35°C. At 24 hours, the MFB was vortexed, reincubated for 2 hours and the broth was then observed to determine if the reaction was positive or negative. If positive (broth had darkened and was black, dark brown or dark green), the MFB was streaked onto Oxford, PALCAM and LPM plates. These plates were incubated as above. If negative (straw colour of newly made broth), the MFB was reincubated for another 24 hours and then streaked as described above. All plates were examined for typical *L. monocytogenes* colonies. On PALCAM agar *L. monocytogenes* forms 2 mm grey-green colonies with a black sunken centre and a black halo on a cherry-red background. On Oxford agar, *L. monocytogenes* forms 1 mm diameter black colonies surrounded by black haloes after 24 h. At 48 h, colonies are 2-3 mm in diameter, black with a black halo and sunken centre. On LPM agar, *L. monocytogenes* colonies appear as whitish piles of crushed glass often showing mosaic-like internal structures occasionally having blue-grey iridescence that tends to sparkle, when viewed using beamed white light powerful enough to illuminate the plate well.

A minimum of 5 typical colonies were streaked onto trypticase soy agar with 0.6% yeast extract (TSA-YE) and incubated at 30°C for 24 h or until satisfactory growth was achieved. These cultures were used to carry out further confirmatory tests including hemolysis, Gram staining, motility, catalase and carbohydrate utilizations tests. The carbohydrate utilization tests were carried out in purple broth base with 0.5% solutions of dextrose, esculin, maltose, mannitol, rhamnose, α-methyl-D-mannoside and xylose.

3.4.2.1 Media Preparation for *Listeria monocytogenes* Incidence Protocol

LEB: 1.0 mL of a 1.2% acriflavin stock solution was added to 1000 mL of LEB immediately before use.

MFB: 0.1 mL of acriflavin (0.25%) and 0.1 mL of ferric ammonium citrate (5%) stock solutions were added to 10 mL of MFB immediately before use.

Oxford agar: 1 vial of manufacturer's supplements (Oxford antimicrobic supplement) was added to 1 L of Oxford agar before pouring plates. The vial required rehydration with 5 mL ethanol and 5 mL of sterile distilled water.

PALCAM agar: 2 vials of manufacturer's supplement (*Listeria* selective supplement - PALCAM), reconstituted with 5 mL of sterile distilled water, were added to 1 L of PALCAM agar immediately before pouring plates.

LPM: 2 mL of moxalactum stock solution was added to LPM agar before pouring plates. This solution consisted of 1 g of moxalactum sodium salt in 100 mL of 0.1 M potassium phosphate buffer, pH 6.0. 1 mL aliquots of the filter sterilized stock solution was stored at -20°C in Eppendorf tubes.

Blood agar (for hemolysis): Horse blood plates consisted of 5% horse blood in TSA.

Carbohydrate fermentation broth: 9 ml of purple broth base was dispensed into 16 x 125 mm tubes each containing a Durham tube. The tubes were then autoclaved at 121°C for 15 minutes. All carbohydrates, except esculin, were prepared as 5% solutions and filter sterilized. One mL of carbohydrate solution was added to 9 mL broth base to yield a final concentration of 0.5% carbohydrate in broth. Esculin was added directly to the broth base to

make a 0.5% solution and autoclaved at 121°C for 15 minutes.

Motility test medium: This medium was rehydrated and sterilized according to the manufacturer's directions. Six mL was dispensed into 16 x 125 mm screw-cap test tubes.

TSA-YE: 40 g of trypticase soy agar and 6 g of yeast extract were added to 1 L of distilled water, autoclaved at 121°C for 15 minutes and dispensed into Petri plates.

TSB-YE: 30 g of trypticase soy agar and 6 g of yeast extract were added to 1 L of distilled water, autoclaved at 121°C for 15 minutes and dispensed into 16 x 100 mm screw-cap test tubes.

3.5 Salt Content

Eleven gram shrimp samples, to which 99 mL distilled water was added, were homogenized in a stomacher for one minute. A salt analysis was carried out on the blended samples using the prestandardized Salt Meter 10 (Presto-Tek Corporation); the probe of the meter was simply placed in the stomacher bags containing the samples. This was done for five samples.

3.6 pH

Eleven gram shrimp samples, to which 99 mL distilled water was added, were homogenized in the stomacher for 1 minute. The pH was measured with a prestandardized pH meter (Accumet pH Meter, Model No. 915, Fisher Scientific); the probe was simply placed in the stomacher bags containing the samples. This was done for five samples.

3.7 Water Activity

Shavings of the shrimp samples (five samples) were placed in the containers used for water activity readings in a Rotronic Water Activity Unit (Rotronic Instruments, Huntington, New York, Model AWCM3L). Efficiency of the water activity unit was verified with a saturated salt solution.

3.8 Storage Experiment

3.8.1 Inoculation of Shrimp Samples

Fresh shrimp received at the lab was frozen, stored at -20°C, and thawed at 4°C overnight when required. For the two inoculation levels for each storage condition, approximately 500 g of thawed shrimp was dipped in an inoculation bath (1 L) containing 0.85% sterile saline and *L. monocytogenes* at levels of approximately 2 x 10^3 CFU/g and 2 x 10^7 CFU/g, to achieve inoculation levels of 10^2 CFU/g and 10^6 CFU/g, respectively. After complete submersion for 5 minutes, the shrimp was aseptically drained and ready for use in the storage experiments described in the next section (3.8.2) . Inoculation baths were prepared just immediately prior to use. Inoculation levels were determined using the procedure outlined in section 3.8.3.

3.8.2 Storage of Shrimp Samples

The growth and survival of *L. monocytogenes* was examined on raw shrimp stored at 4 storage temperatures: -20°C, on ice (0 to1°C), 4°C and 10°C. Two levels of inoculation

were used, a low level of 10^2 CFU/g and a high level of 10^6 CFU/g of raw shrimp. For each of the -20, 4 and 10°C storage temperatures, approximately 250 g of inoculated shrimp were aseptically transferred to sterile plastic bags, sealed and stored at the different temperatures. For storage on ice, the inoculated shrimp was aseptically placed on ice in a sterile plastic container and subsequently added to a Styrofoam carton filled with ice and stored in a refrigerator. Water from the melting ice was removed at two day intervals with ice replenished as necessary. Initially, and after 1, 2, 3, 4, 6, 8, 10 and 12 days of storage, duplicate 10 g samples were aseptically removed and analyzed for viable *L. monocytogenes*. This enumeration procedure is described in detail in the next section (3.8.3). Each storage was done in triplicate.

3.8.3 Enumeration of Shrimp Samples for *Listeria monocytogenes*

The Health Protection Branch of Health Canada protocol, as outlined by Farber and Daley (1995), was used to determine the number of viable *L. monocytogenes* in shrimp samples. In the first step, 11 g of shrimp, weighed using a top-loading balance (Precisa 3500D), and 99 mL of 0.1% peptone water were added to a sterile stomacher bag, homogenized for 2 minutes at high speed in a Seward Stomacher 400 laboratory blender (Seward Medical, London, UK) and serially diluted as required. Appropriate aliquots of the dilutions were then spread onto two *Listeria*-selective agars, Oxford and PALCAM agar plates. The plated were then incubated at 35°C for 24-48 h.

All plates were examined for typical *L. monocytogenes* colonies. On PALCAM agar *L. monocytogenes* forms 2 mm grey-green colonies with a black sunken centre and a black halo on a cherry-red background. On Oxford agar, *L. monocytogenes* forms 1 mm diameter black colonies surrounded by black haloes after 24 h. At 48 h, colonies are 2-3 mm in diameter, black with a black halo and sunken centre. Colonies were counted and the number of organisms present in each sample were expressed as colony forming units per gram of sample (CFU/g).

A minimum of 5 typical colonies were streaked onto trypticase soy agar with 0.6% yeast extract (TSA-YE) and incubated at 30°C for 24 h or until satisfactory growth was achieved. These cultures were used to carry out further confirmatory tests including hemolysis, Gram staining, motility, catalase and carbohydrate utilizations tests. The carbohydrate utilization tests were carried out in purple broth base with 0.5% solutions of dextrose, esculin, maltose, mannitol, rhamnose, α-methyl-D-mannoside and xylose.

3.8.3.1 Media Preparation for the Enumeration of *Listeria monocytogenes* Protocol

Peptone water: 1 g of peptone was added to 1 L of distilled water. 90 mL was dispensed into dilution bottles after sterilization at 121°C for 15 minutes.

Oxford agar, PALCAM agar, Blood agar (for hemolysis), carbohydrate fermentation broth, motility test medium, TSA-YE and TSB-YE were prepared as described in 3.4.2.1.

3.9 Thermal Resistance Experiments

3.9.1 Steam Cooker Apparatus

The purpose of this research was to obtain thermal resistance data for *L.monocytogenes* in shrimp using a method that was designed to simulate actual processing conditions used in commercial shrimp processing facilities. These facilities cook the shrimp using live steam injection and shrimp are cooked whole. Additionally, a method in which the "come-up" times, i.e., those at which the shrimp reached the desired internal temperatures, were relatively low, was also desirable. However, after much experimentation, several problems, which will be discussed, were encountered and such a design has proved impossible.

The first attempt used a steam cooker (Pressure Cooker, Model MKVIT-2, Gaunt Steel Products Limited) at the Centre for Aquaculture and Seafood Development processing plant of the Marine Institute of Memorial University of Newfoundland. This required the construction of an aluminum plate, containing an opening, that could be placed over the front of the cooker and clamped into place (Figure 3.2). A thermocouple, which was attached to a data logger (Data Logger System, Doric 345, Beckman Industrial) and recorded the temperature every eight seconds, was placed in the centre of the shrimp sample. The sample was then attached to a paddle and inserted into the cooker through the opening in the aluminum plate. Using the plate and steam valve, the steam inside the cooker could be adjusted. Using this method several different time-temperature profiles were obtained for many shrimp samples. These are presented in Chapter 4.

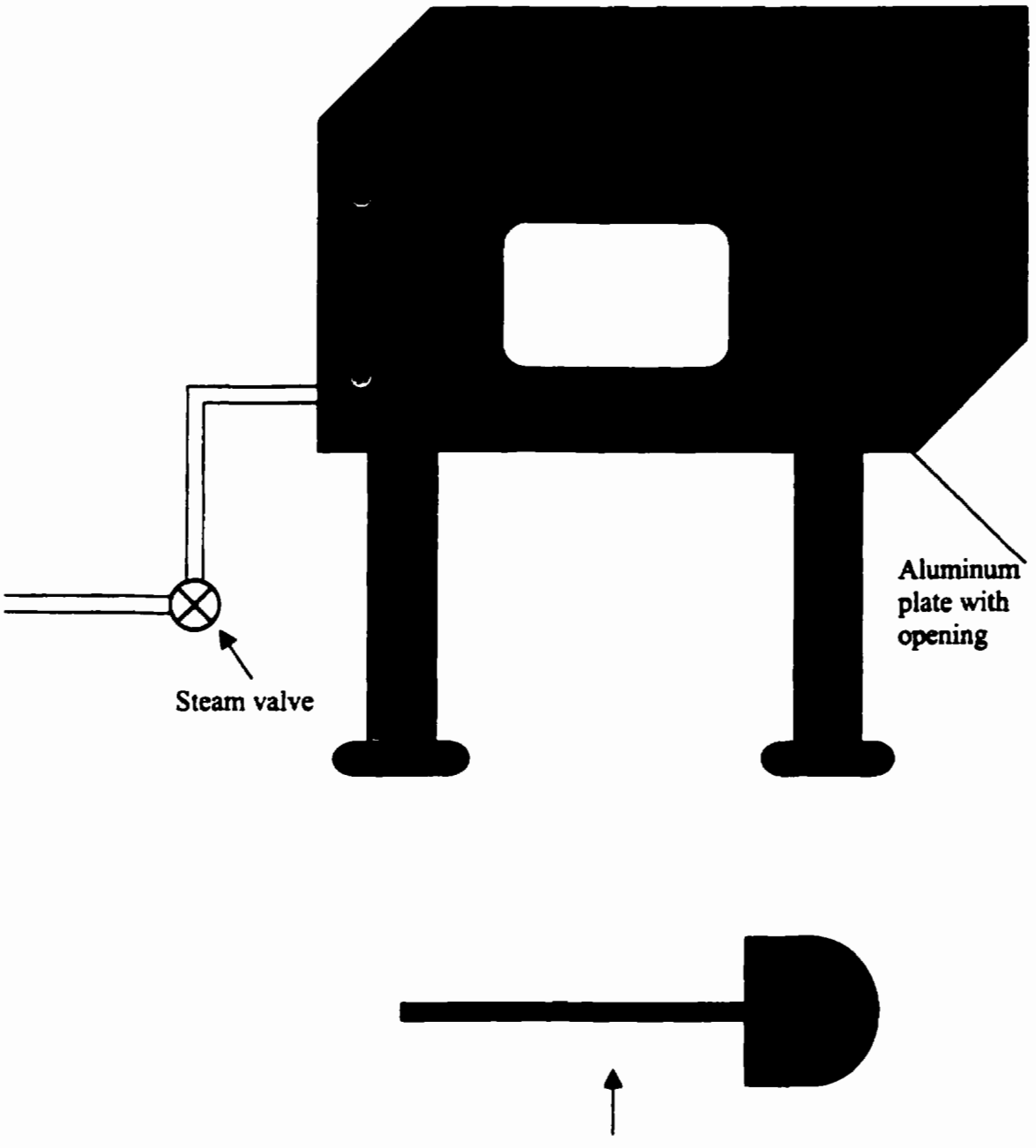

Figure 3.2: Schematic of the Steam Cooker Apparatus at the Seafood Processing Plant of the Marine Institute of Memorial University of Newfoundland.

However, in order to obtain thermal resistance data, i.e. D and z values, the shrimp needed to be inoculated with *L. monocytogenes*, added to the cooker, removed at different time intervals and enumerated for surviving *L. monocytogenes*. Two problems were encountered. First, the temperature of the cooker could not be adjusted and kept constant at specific temperatures using the above procedure. Second, the shrimp would have to be cooked in some sort of sealed bag or container to ensure that the plant equipment and/or environment would not be contaminated with the pathogen. Vacuum-sealed bags were used to contain the shrimp samples and the resulting time/temperature profiles are presented in Chapter 4.

Due to equipment problems encountered in trying to design a method of obtaining thermal resistance data (in which shrimp were processed as similarly as possible to commercial facilities), a capillary tube method (Foegeding and Leasor, 1990) was used (See Section 3.9.2).

3.9.2 Capillary Tube Experiment

The method outlined by Foegeding and Leasor (1990) was used to evaluate the thermal resistance of *L. monocytogenes* in shrimp. Immediately prior to carrying out these experiments, a shrimp broth was made by homogenizing 10 g of shrimp and 90 mL of 0.85% sterile physiological saline. This broth was then inoculated with an appropriate solution of *L. monocytogenes* ½b and 0.85% saline to obtain an inoculation level of 10^5 CFU/g. To evaluate the thermal resistance of the pathogen in shrimp, 0.05 mL of the shrimp broth was

inoculated as described above and added, by syringe, to glass capillary tubes (0.8 - 1.1 x 90 mm, Kimble Products No. 34507). For each temperature and inoculation level tested, tubes were heat-sealed using a Bunsen burner flame and submerged into a water bath (Fisher Versa-Bath, Model 138. Fisher Scientific Company, USA) set at the appropriate temperature. At each sampling time interval, which was every 10 seconds for up to 2 minutes, one tube was removed from the water bath and immediately placed into ice water for 5 minutes. The tube was then transferred to 200 ppm hypochlorite for 15 minutes, rinsed with distilled water and aseptically crushed with a sterile glass rod in a glass test tube containing 5 mL sterile 0.1% peptone water and enumerated for *L. monocytogenes* as described below in Section 3.9.2.1. Triplicate thermal inactivation trials were carried out and D and *z* values were determined for temperatures of 65, 68, 70, 72, 75 and, if required, 80°C. Time/temperature combinations that rendered the shrimp free of *L. monocytogenes* were identified from this experiment.

3.9.2.1 Enumeration of Shrimp Samples for *Listeria monocytogenes*

For each tube removed from the water bath, the number of viable *L. monocytogenes* was enumerated as previously described in section 3.8.3. In addition to Listeria-selective agar plates, TSA plates were also used to ensure isolation of any heat-injured cells.

3.9.2.2 D and z Value Determination

The results of the thermal inactivation trials were plotted and analyzed using Microsoft® Excel software (Version 97 SR-1). The slopes of the linear regression lines of the survivor curves ($\log_{10}$ number of survivors vs. time) were used, where possible, to calculate D values, where D is equal to the negative reciprocal of the slope. Average D values for the three inactivation trials are reported in the results. The *z* values were determined from the slopes of linear regression lines of the decimal reduction time curves (log average D vs. temperature) as z is equal to the negative reciprocal of the slope.

The D value is the decimal reduction time, or the time required to destroy 90% of the organisms. It is numerically equal to the number of minutes required for the survivor curve to traverse one log cycle. The z value refers to the degrees Celsius required for the thermal destruction curve to traverse one log cycle. This value provides information on the relative resistance of an organism to different destructive temperatures, while the D value reflects the resistance of an organism to a specific temperature (Jay, 1992).

Mean and standard deviation values were obtained using a scientific calculator with statistical functions (Texas Instruments, TI-34 II).

Chapter Four

Results

4.1 Aerobic Colony Count

Each time raw shrimp was received in the laboratory, the total aerobic colony count of the shrimp samples was determined in triplicate. Table 4.1 presents the results obtained from the six different shrimp samples analyzed, as described previously, for total aerobic bacteria. The results indicate that the total aerobic bacteria present on the raw shrimp are quite low, ranging from 360 ± 14.2 to 515 ± 16.1 CFU/g. The average of all three trials for all six samples was 429.8 ± 65.4 CFU/g for all samples. Thus, high levels of background bacteria were not present to interfere with the *L.monocytogenes* inoculated onto the shrimp for experimental purposes as the shrimp has very low aerobic counts and appears to be relatively sterile.

4.2 *Listeria monocytogenes* Incidence

Shrimp samples were also analyzed for the presence of *L. monocytogenes* each time shrimp was received in the lab. Six samples (in triplicate) were analyzed in total, of which none were positive for *L. monocytogenes*. Thus, the shrimp caught in the cold waters of the Atlantic ocean appear to be free of *L. monocytogenes*.

Table 4.1: Results Obtained from the Total Aerobic Colony Count Analysis of Six Different Batches of Shrimp Samples Provided by a Newfoundland Shrimp Processor.

Sample Number	Colonies observed (CFU/g*) Trial 1	Colonies observed (CFU/g*) Trial 2	Colonies observed (CFU/g*) Trial 3	Mean and standard deviation†
1	500	532	513	515 ± 16.1
2	376	349	355	360 ± 14.2
3	405	388	392	395 ± 8.9
4	327	372	402	367 ± 37.8
5	429	470	457	452 ± 21.0
6	500	486	484	490 ± 8.7

* CFU/g = colony forming units of bacteria per gram of sample.
† Significance at $p<0.05$.

4.3 Storage Experiment

The growth and survival of *L. monocytogenes* was examined on raw shrimp stored at four storage temperatures, -20°C, on ice (0 to 1°C), 4°C and 10°C. Two levels of inoculation were used, a low level of 10^2 CFU/g and a high level of 10^6 CFU/g of raw shrimp.

4.3.1 Storage at -20°C

As can be seen in Figures 4.1 and 4.2, during the 12 days of storage at -20°C, the population of *L. monocytogenes*, for both low and high inoculation levels, did not increase or decrease significantly. It remained relatively stable, there was a 0.35 log decrease in the low inoculum sample and a 0.05 log increase in population in the high inoculum sample (Table 4.2). Although, the *L. monocytogenes* population was able to survive frozen storage, it did not grow at this low temperature condition.

4.3.2 Storage on Ice (0-1°C)

For both inoculation levels, 10^2 CFU/g and 10^6 CFU/g, the population of *L.monocytogenes* on shrimp stored on ice (0-1°C) decreased by approximately 0.70 log and 0.64 log (Table 4.2), respectively, until around day four of storage, after which point the population levelled off and stayed relatively stable over the remainder of the experiment (See Figures 4.3 and 4.4). There was no increase in *L. monocytogenes* during storage of shrimp on ice. As with frozen storage (-20°C), the *L. monocytogenes* cells survive the low

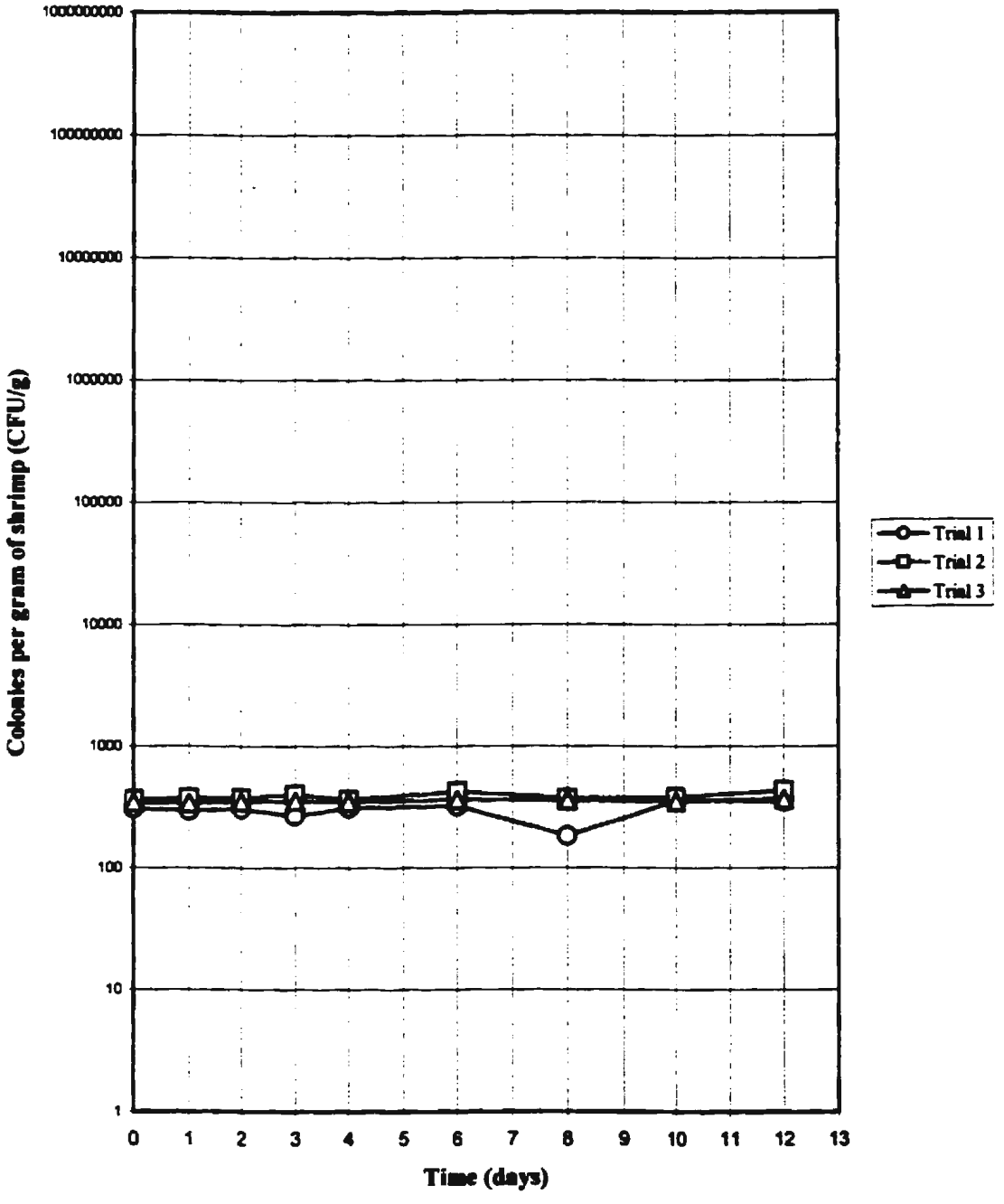

Figure 4.1: Survival of *Listeria monocytogenes* (Initial Innoculum of 10^2 CFU/g) on Shrimp Stored at -20°C for 12 Days

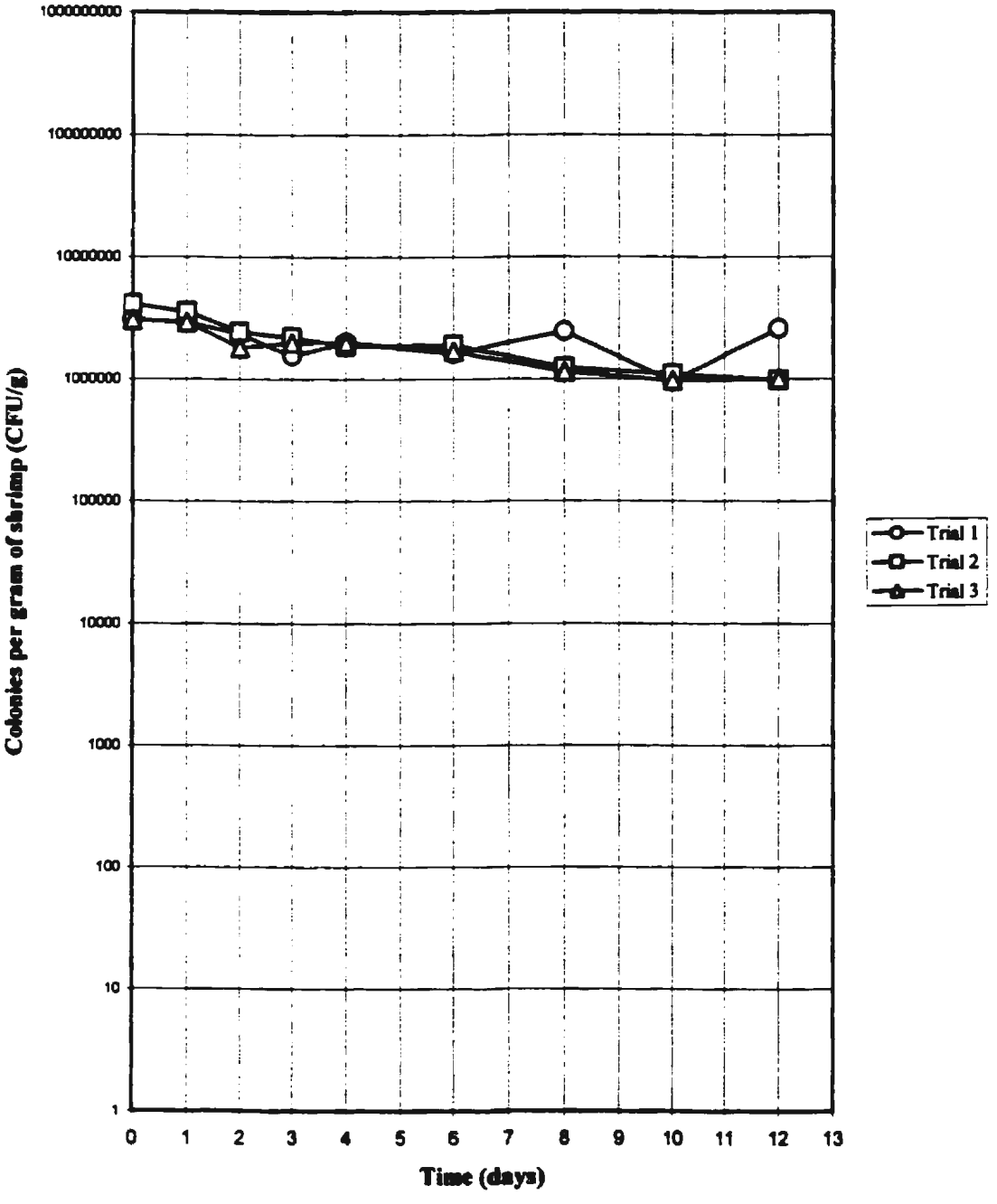

Figure 4.2: Survival Of *Listeria monocytogenes* (Initial Innoculum of 10^6 CFU/g) on Shrimp Stored at -20°C for 12 Days

Table 4.2: Growth Data Obtained from the Storage Experiment Growth Curves.

Inoculation Level (CFU/g*) /Storage Temperature (°C)	Number of Generations (generations)	Generation Time (days)	Initial Population (log_{10}CFU/g*)	Final Population (log_{10}CFU/g*)	Log Increase or Decrease (log)
10^2/-20°C	na	na	6.53	6.18	0.35 decrease
10^6/-20°C	na	na	2.53	2.58	0.05 increase
10^2/Ice (0-1°C)	na	na	2.52	1.82	0.70 decrease
10^6/Ice (0-1°C)	na	na	6.37	5.73	0.64 decrease
10^2/4°C	3.33	3.60	2.56	5.48	2.92 increase
10^6/4°C	3.89	3.08	6.46	8.67	2.21 increase
10^2/10°C	10.86	1.1	2.46	7.26	4.80 increase
10^6/10°C	6.11	1.96	6.43	8.79	2.33 increase

*CFU/g = colony forming units of bacteria per gram of sample.
The values in the above table are averages of three trials.

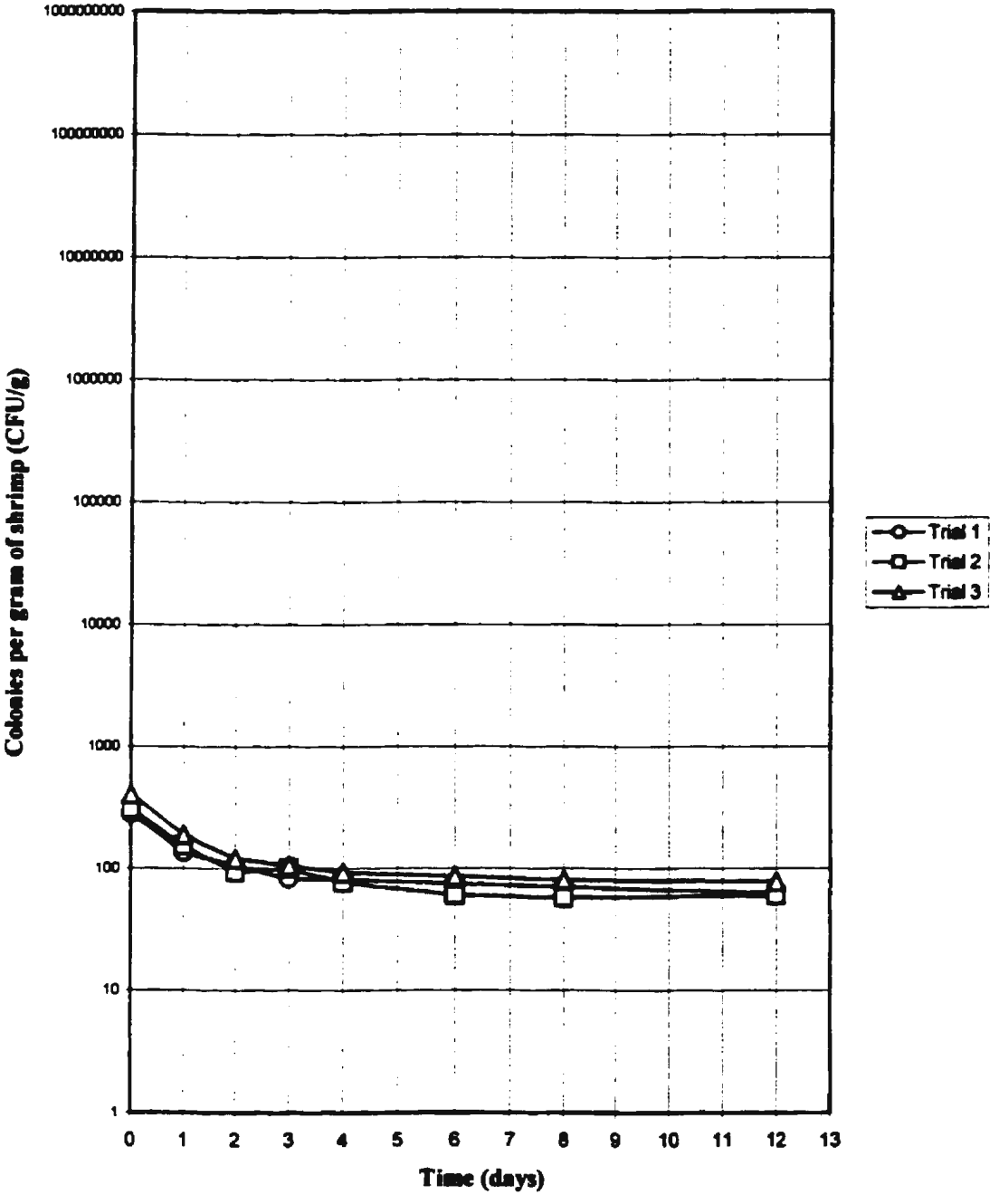

Figure 4.3: Survival of *Listeria monocytogenes* (Initial Innoculum of 10^2 CFU/g) on Shrimp Stored on Ice (0-1°C) for 12 Days

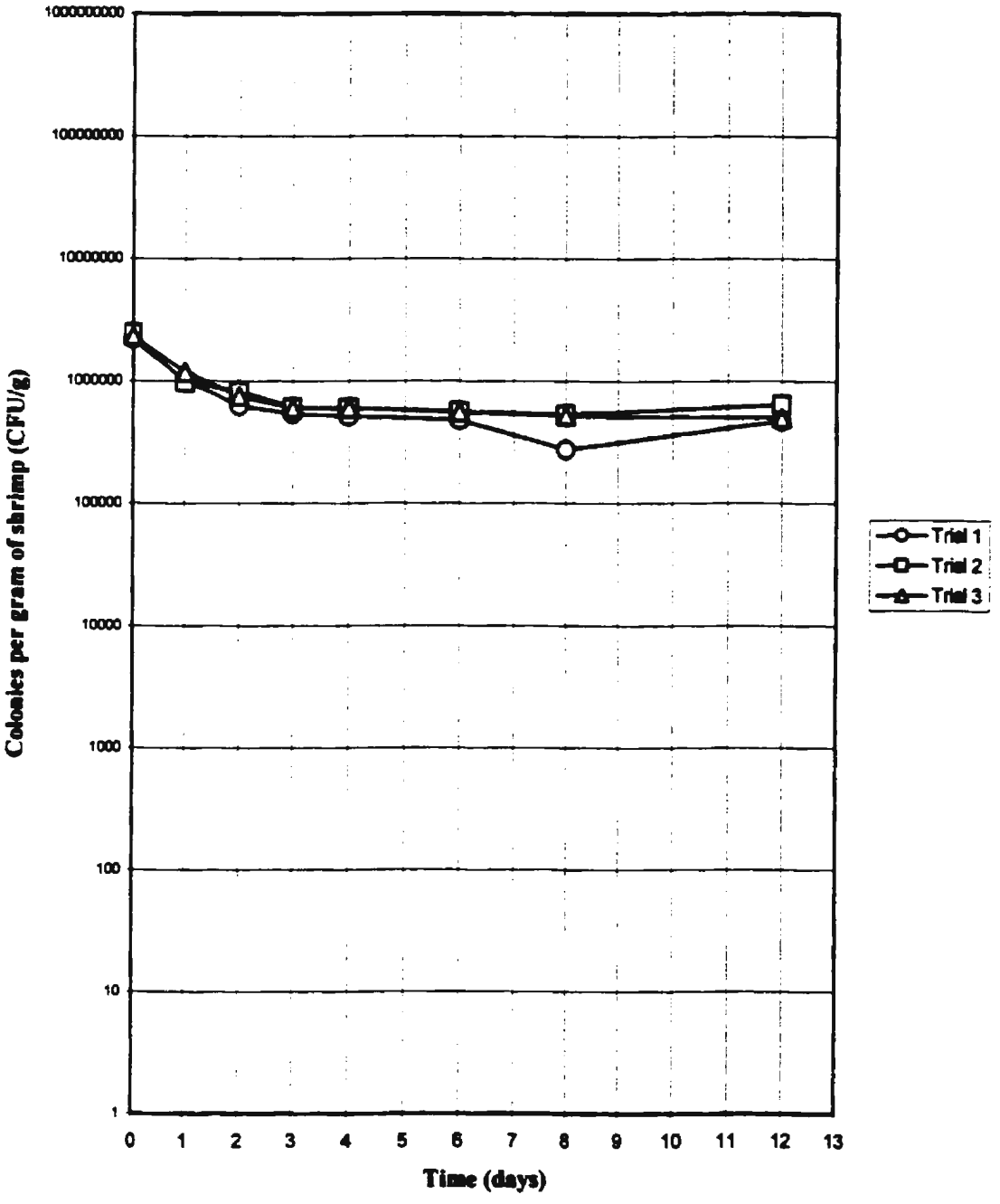

Figure 4.4: Survival of *Listeria monocytogenes* (Initial Innoculum of 10^6 CFU/g) on Shrimp Stored on Ice (0-1°C) for 12 Days

temperatures of the ice. Similar patterns of cell counts were observed in each of the three trials.

4.3.3 Storage at 4°C

Figure 4.5 shows the growth curve for shrimp stored at 4°C with an initial inoculum of 10^2 *L. monocytogenes* cells per gram. The population counts rise from 2.56 $\log_{10}$CFU/g to 5.48 $\log_{10}$CFU/g in the 12 days of storage. The number of generations that have developed over a particular time period and the generation time was calculated using mathematical equations as described by Pelczar *et al.* (1993) and the log phase of growth obtained from the graphs. Also, the log increase or decrease was calculated for each storage condition and level of inoculation. These values are presented in Table 4.2. A generation time (the time taken for a population of microorganisms to double) of 3.60 days was obtained, as well as a 2.92 log increase. These values indicate that the *L. monocytogenes* population increased significantly when stored at refrigeration temperatures.

The initial population of 10^6 CFU *L. monocytogenes* per gram of shrimp stored at 4°C slowly increases and levels off to a relatively stable cell count around day six of storage (Figure 4.6). The population counts increase from 6.46 $\log_{10}$CFU/g to 8.67 $\log_{10}$CFU/g in 12 days. Similar to the results obtained for the lower initial inoculum levels (10^2 CFU/g), a generation time of 3.08 days and a 2.21 log increase was determined (Table 4.2). While there is a significant increase in *L. monocytogenes*, the increase is not as large as the increase for the initial inoculum of 10^2 cells/g under the same conditions of storage.

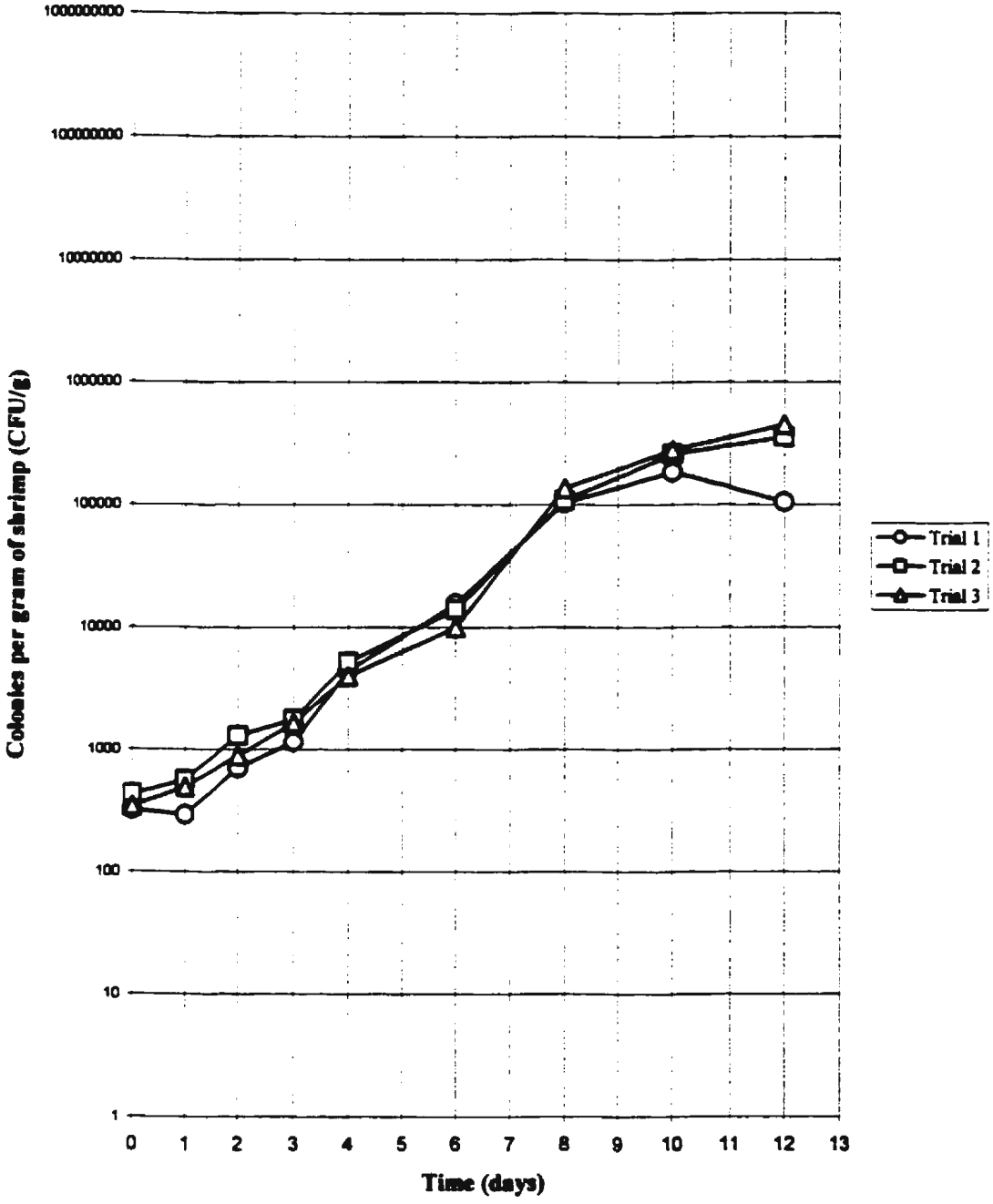

Figure 4.5: Survival of *Listeria monocytogenes* (Initial Innoculum of 10^2 CFU/g) on Shrimp Stored at 4°C for 12 Days

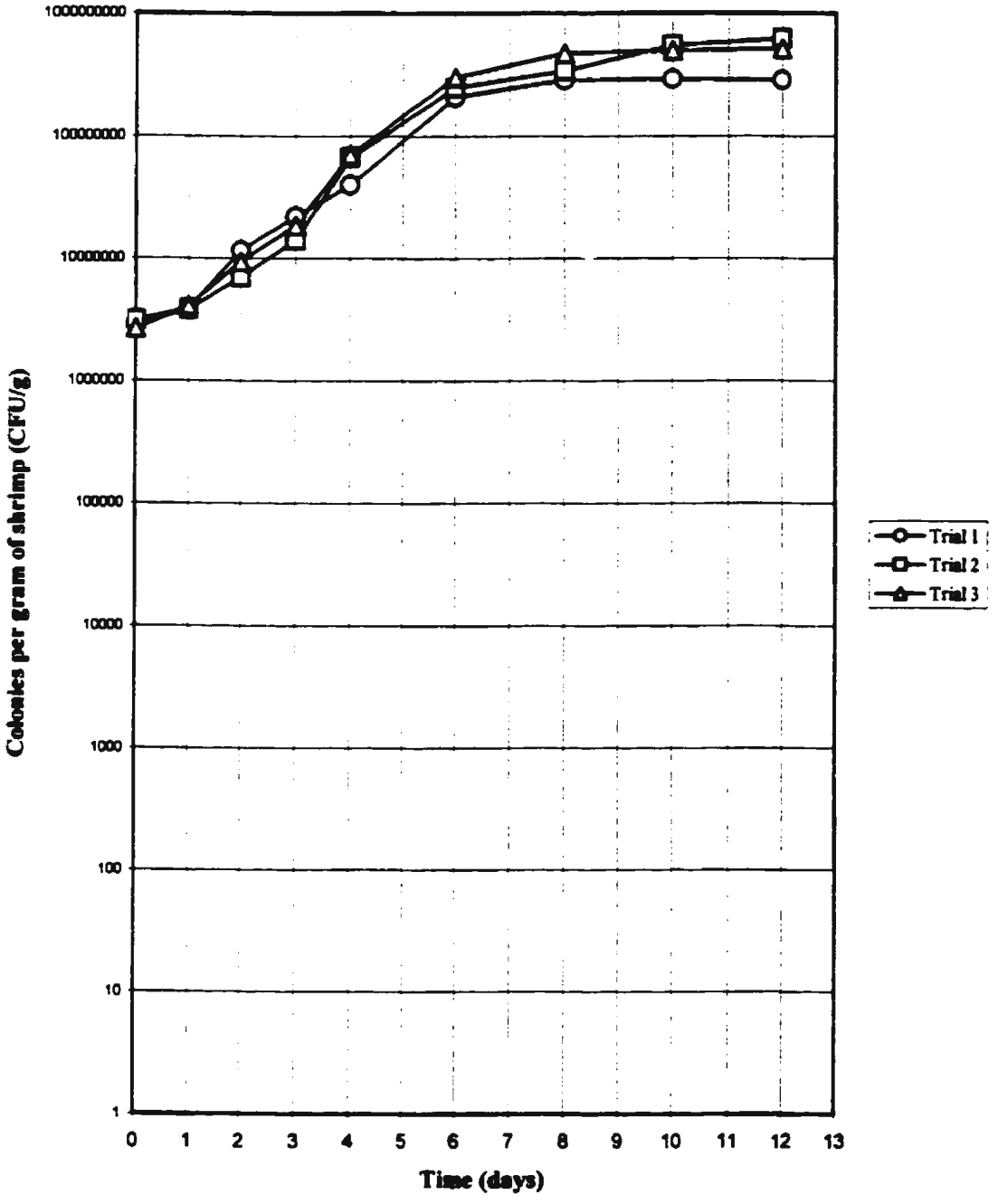

Figure 4.6: Survival of *Listeria monocytogenes* (Initial Innoculum of 10^6 CFU/g) on Shrimp Stored at 4°C for 12 Days

4.3.4 Storage at 10°C

As can be seen in Figures 4.7 and 4.8, the *L. monocytogenes* population greatly increased from 2.46 $\log_{10}$CFU/g to 7.26 $\log_{10}$CFU/g and from 6.43 $\log_{10}$CFU/g to 8.76 $\log_{10}$CFU/g for inoculation levels of 10^2 and 10^6, respectively, when stored at 10°C. The shortest generation time of 1.1 days and the largest log increase of 4.80 was observed for the low initial inoculum levels of 10^2 CFU/g (Table 4.2). A log increase in population similar to the one obtained for storage at 4°C, was obtained for high inoculum levels of 10^6 CFU/g, i.e., a 2.33 log increase. However, a much shorter generation time of 1.96 days was observed for storage at 10°C and a high inoculum level.

4.4 Thermal Resistance Experiments

4.4.1 Steam Cooker Apparatus

As described previously, a steam cooker, whose front was covered with an aluminum plate containing an opening, was used to obtain time-temperature profiles. A thermocouple, which was attached to a data logger system, was placed in the geometric centre of a shrimp sample, which was then attached to an aluminum paddle and inserted into the cooker through the opening. The steam inside the cooker was adjusted using the plate and the steam valve, which controlled the amount of steam entering the cooker. Using this method, many different time-temperature profiles were obtained for the shrimp samples. These profiles are presented graphically in Figures 4.9 to 4.15.

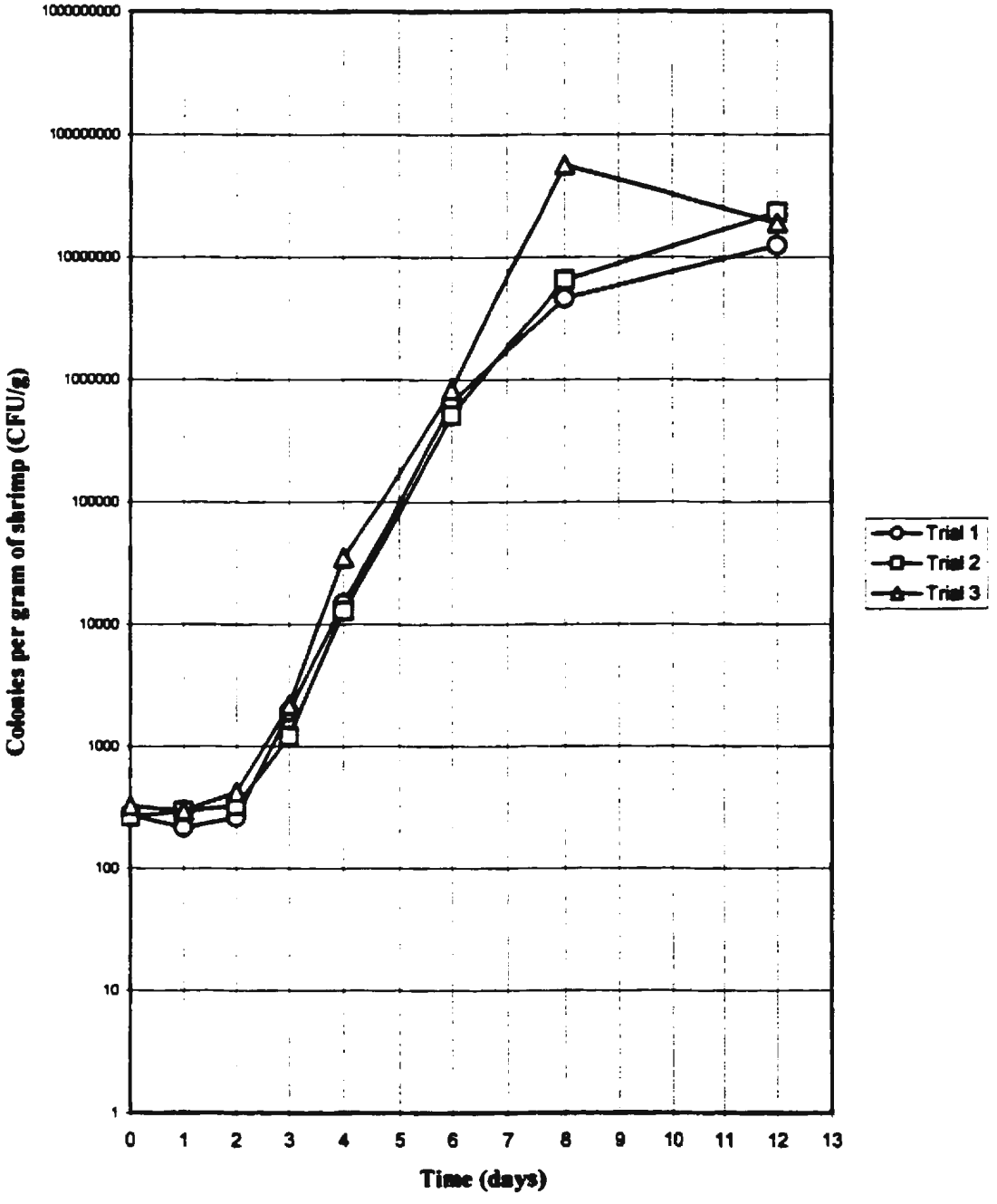

Figure 4.7: Survival of *Listeria monocytogenes* (Initial Innoculum Of 10^2 CFU/g) on Shrimp Stored at 10°C for 12 Days

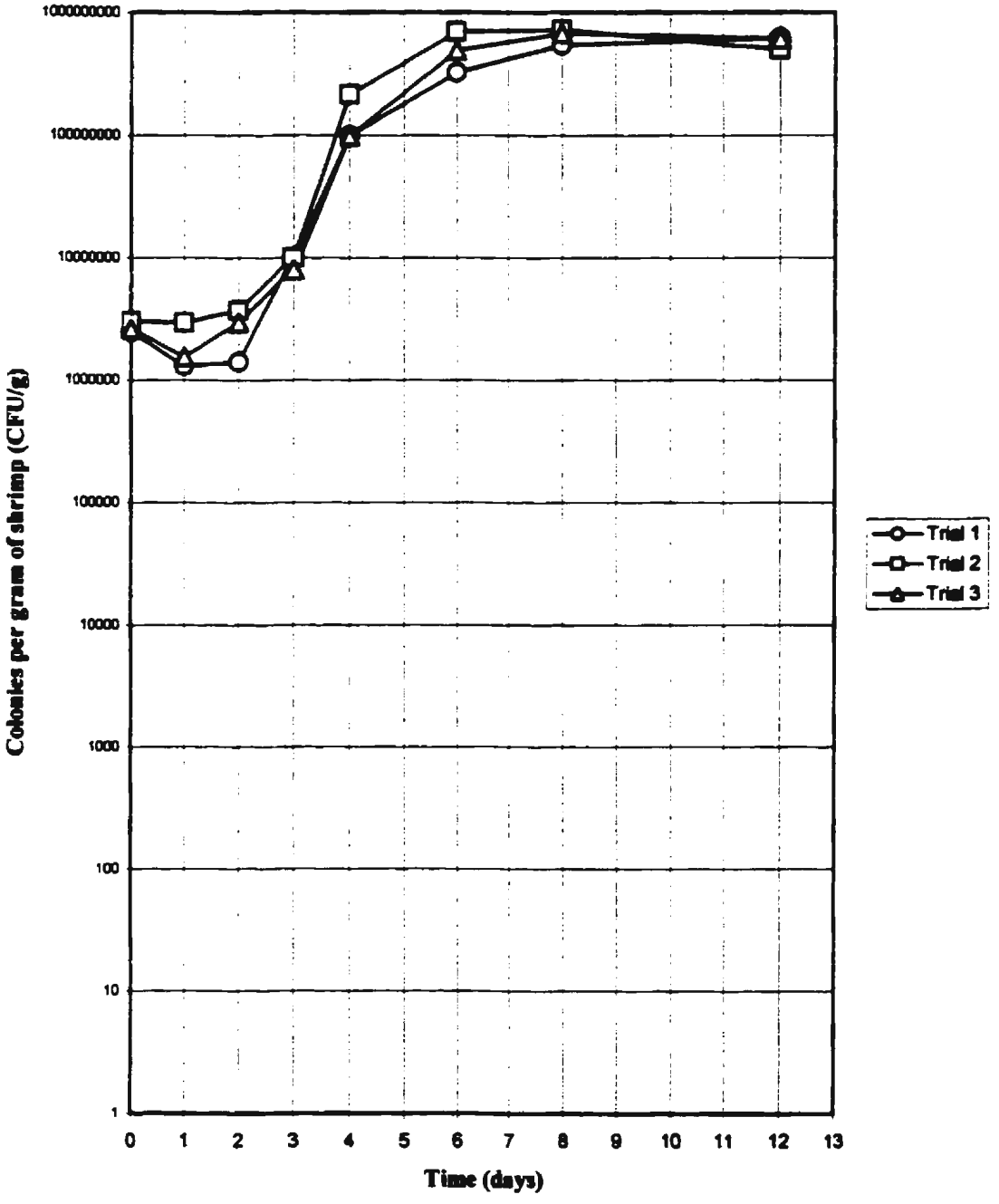

Figure 4.8: Survival of *Listeria monocytogenes* (Initial Innoculum of 10^6 CFU/g) on Shrimp Stored at 10°C for 12 Days

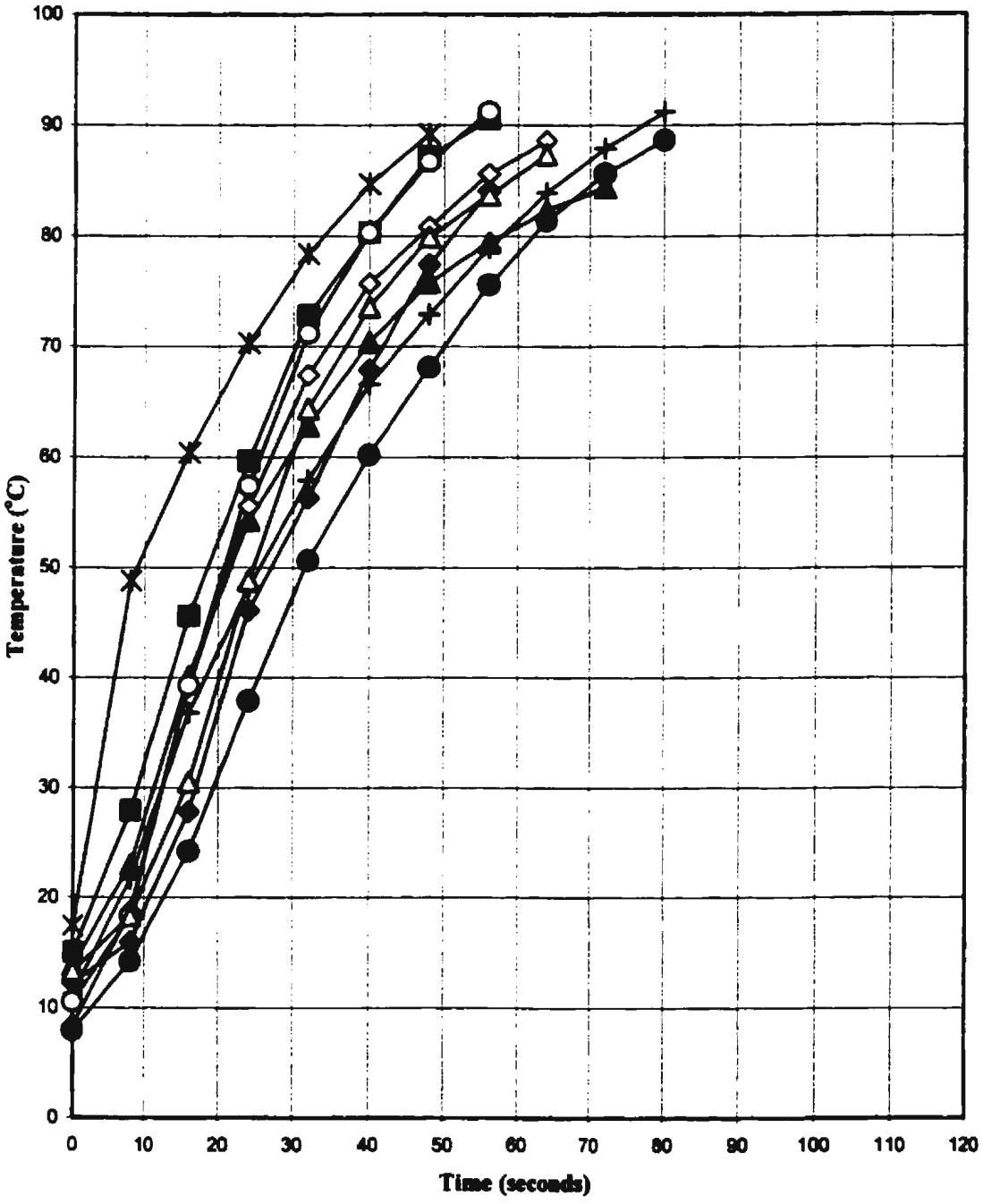

Figure 4.9: Time-temperature Profiles of Shrimp Obtained from a Steam Cooker with a Plate Fully Covering Cooker Opening and Steam Turned on Full (Each Colour Represents One Shrimp Sample)

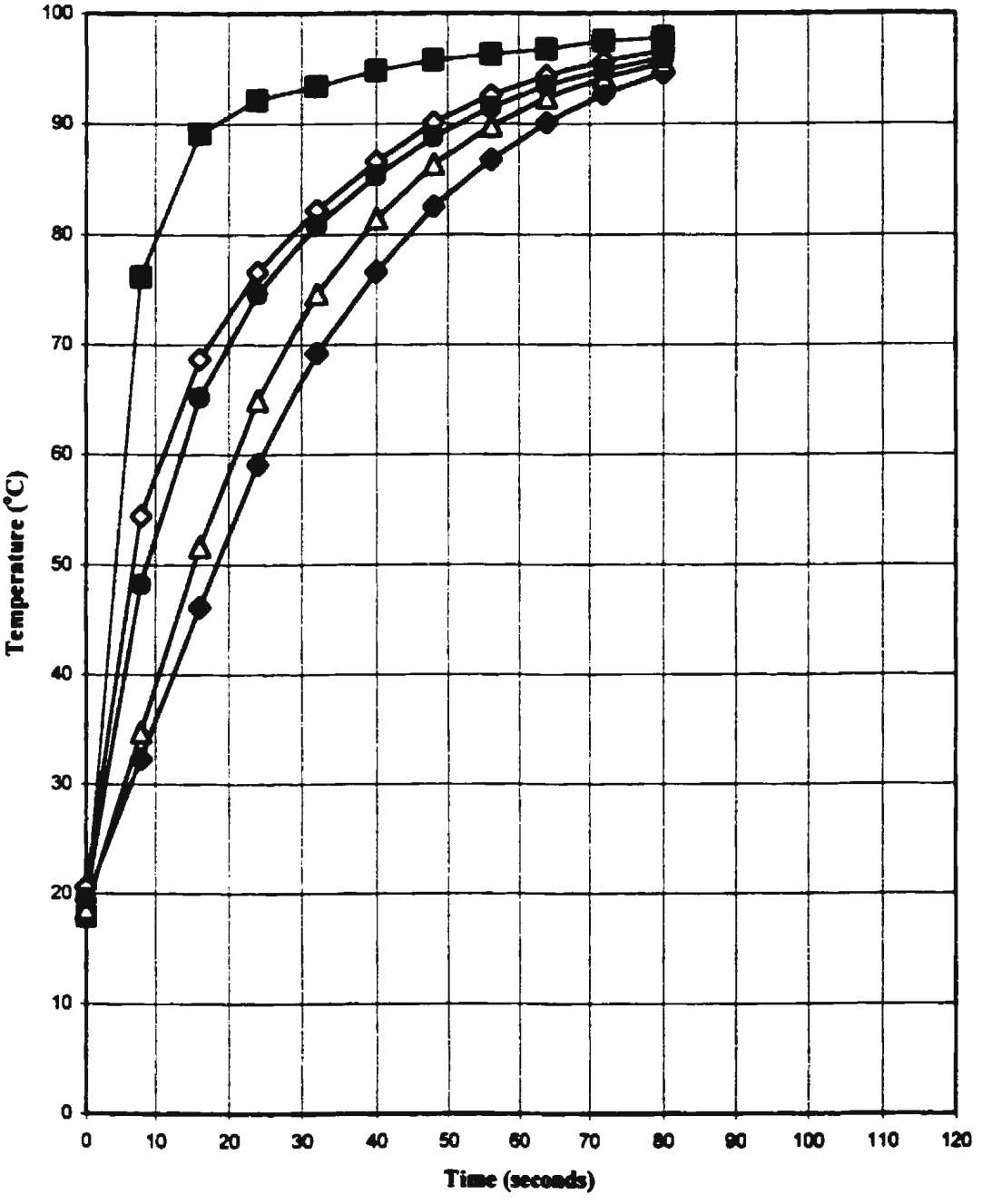

Figure 4.10: Time-temperature Profiles of Shrimp Obtained from a Steam Cooker with a Plate Fully Covering Cooker Opening and Steam Turned Down Three Turns (Each Colour Represents One Shrimp Sample)

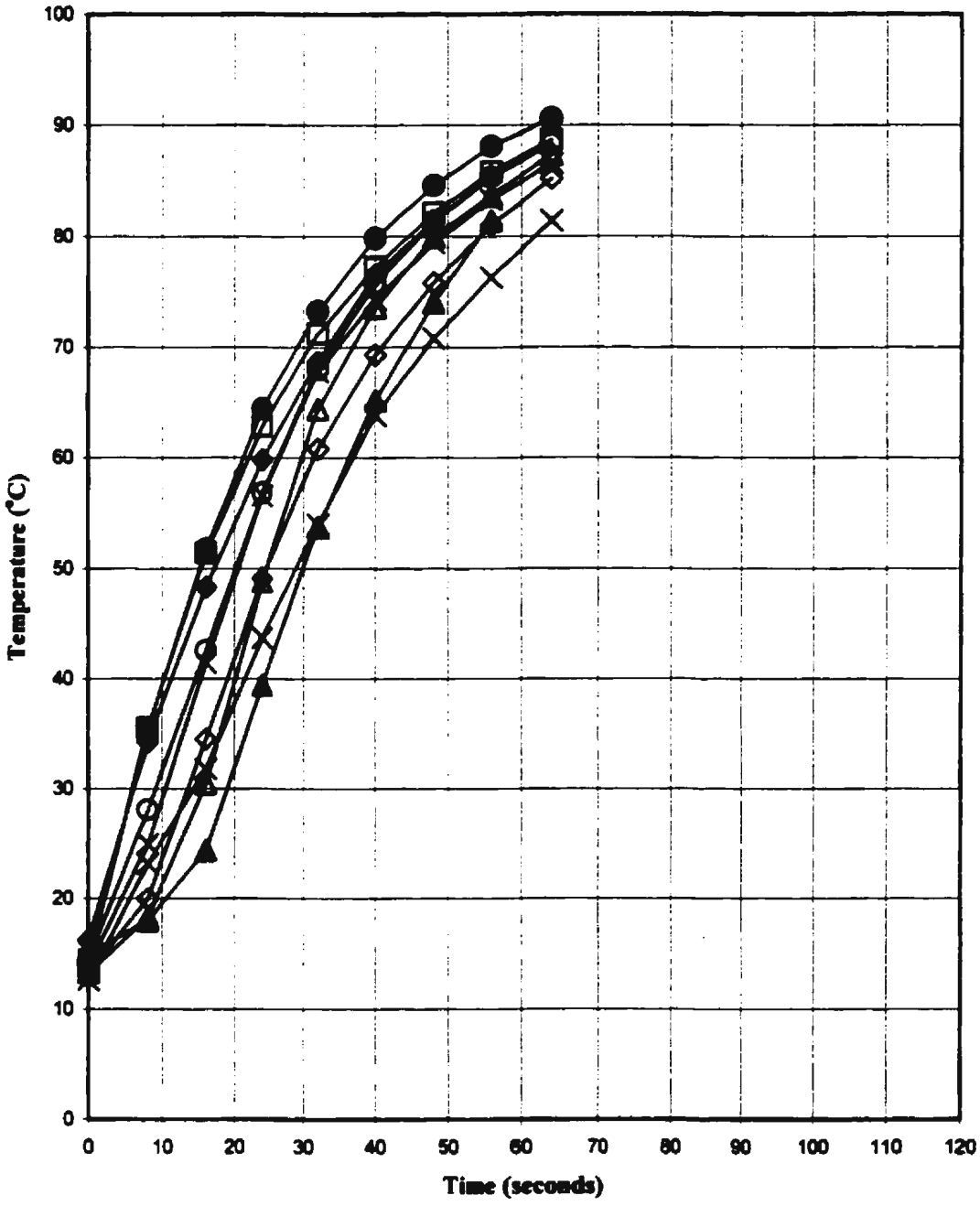

Figure 4.11: Time-temperature Profiles of Shrimp Obtained from a Steam Cooker with a Plate Fully Covering Cooker Opening and Steam Turned Down Four Turns (Each Colour Represents One Shrimp Sample)

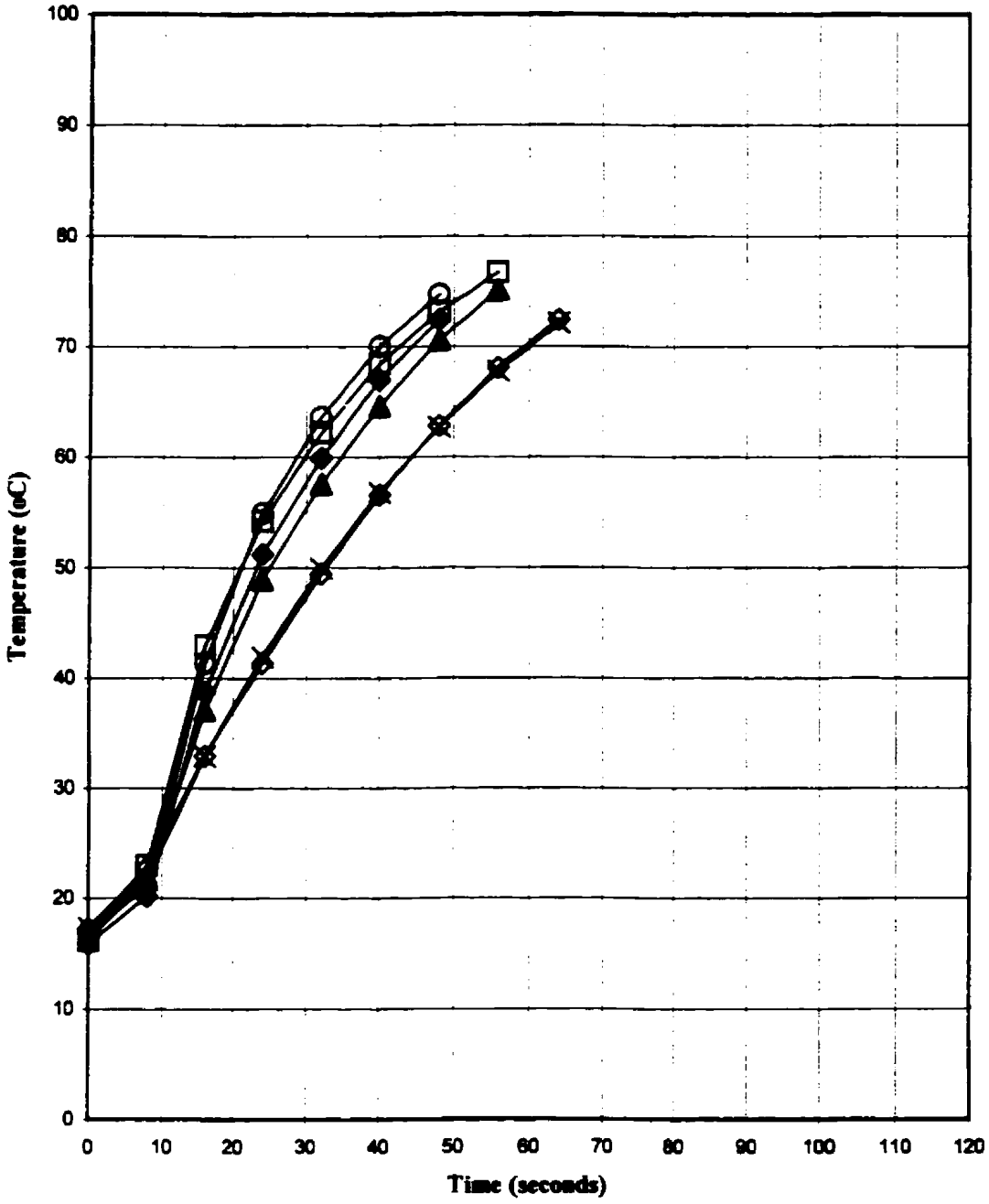

Figure 4.12: Time-temperature Profiles of Shrimp Obtained from a Steam Cooker with a Plate Partially Covering Cooker (2.5 cm Opening) and Steam Turned Down Two and a Half Turns (Each Colour Represents One Shrimp Sample)

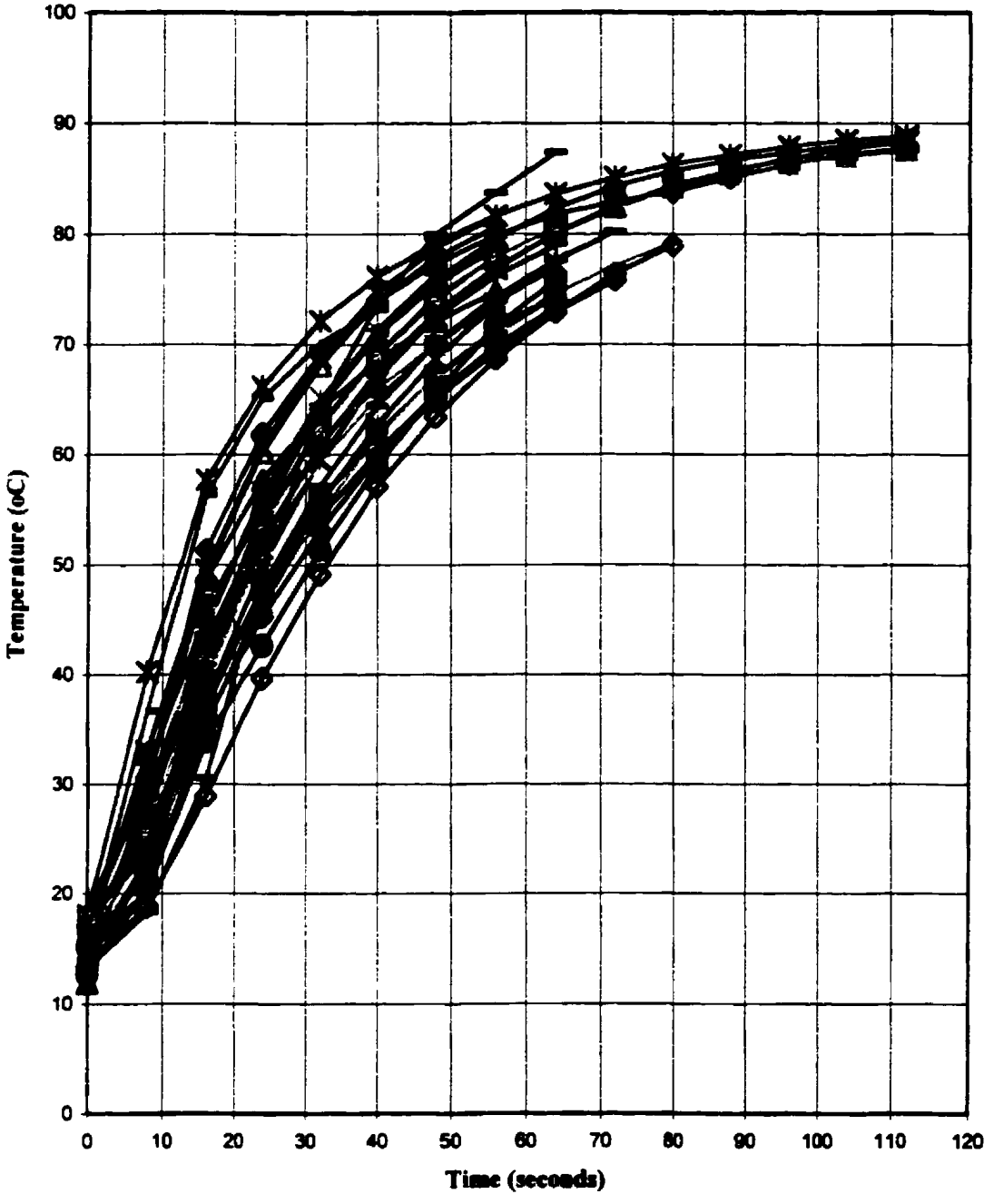

Figure 4.13: Time-temperature Profiles of Shrimp Obtained from a Steam Cooker with a Plate Partially Covering Cooker (2.5 cm Opening) and Steam Turned Down Three Turns (Each Colour Represents One Shrimp Sample)

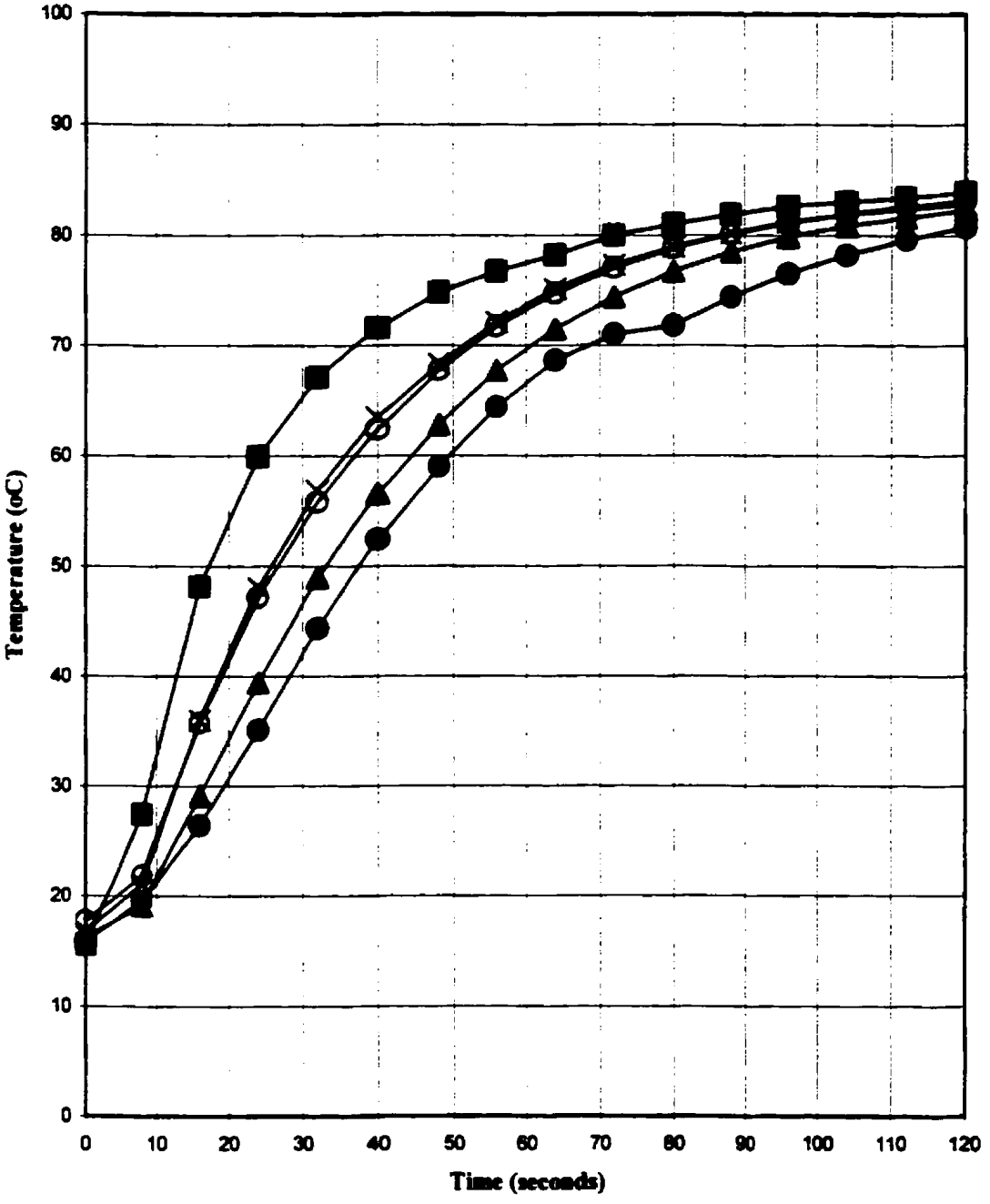

Figure 4.14: Time-temperature Profiles of Shrimp Obtained from a Steam Cooker with a Plate Partially Covering Cooker (2.5 cm Opening) and Steam Turned Down Four Turns (Each Colour Represents One Shrimp Sample)

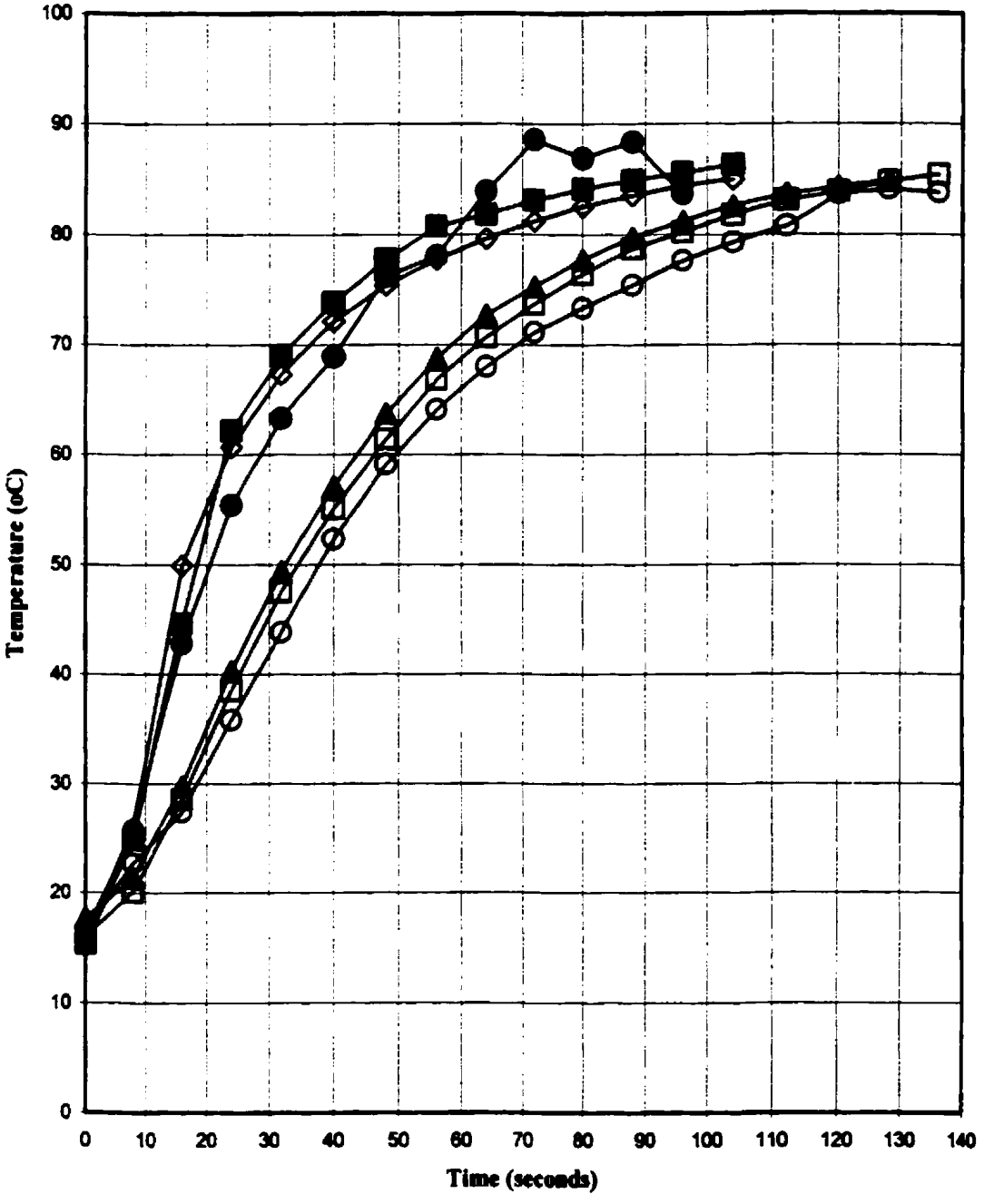

Figure 4.15: Time-temperature Profiles of Shrimp Obtained From a Steam Cooker with a Plate Partially Covering Cooker (3.4 cm Opening) and Steam Turned Down Three Turns (Each Colour Represents One Shrimp Sample)

As can be seen in Figures 4.9 to 4.11, the profiles obtained using conditions in which the aluminum plate completely covered the front of the cooker and only the steam valve was adjusted do not differ dramatically. The time taken for the shrimp to reach temperatures between 65 and 75°C (temperatures that will be investigated in this study) typically ranged from 20 - 50 seconds, which is relatively quick.

Another series of profiles were obtained using a condition in which the aluminum plate was adjusted to give a 2.5 cm opening (through which steam could escape the cooker) and adjusting the steam valve by 2.5, 3 and 4 turns down (Figures 4.12 to 4.14). For these conditions, again, the time-temperature profiles did not differ significantly. The time taken to reach target temperatures of 65 to 75°C, ranged from approximately 25 to 60 seconds. This is similar to the results obtained for the conditions described previously, in which the time to reach these temperatures was between 20 and 50 seconds, slightly less then the 30 to 60 seconds observed here.

Figure 4.15 depicts the last condition examined, in which there was an opening between the plate and cooker of 3.4 cm and the steam valve was turned down three turns. In this case, it took between 30 and 80 seconds for the shrimp sample to reach 65 to 75°C, which was longer than the previous times. Thus, as the opening between the plate and the cooker increased, the come-up times increased.

These profiles were obtained to identify the condition that most resembled the conditions of thermal processing use in a commercial shrimp processing facility. However,

other factors of this experiment had to be taken into consideration to determine if this set-up would work.

In order to obtain thermal resistance data, i.e., D and *z* values, the shrimp needed to be inoculated with *L. monocytogenes*, added to the cooker, removed at different time intervals and enumerated for surviving *L. monocytogenes*. Using this data, survivor curves could then be plotted from which D values could be calculated. Two problems were encountered. First, the temperature of the cooker could not be adjusted and kept constant at specific temperatures using the above procedure. The temperature continued to rise until the steam temperature was reached. The shrimp would have to be cooked in some sort of sealed bag or container to ensure that the plant equipment and/or environment would not be contaminated with the pathogen. Vacuum-sealed bags were used to contain the shrimp samples but the heating "come-up" times were too high (50 to 110 seconds to reach targeted temperatures of 65 to 75°C) and the temperature could not be held once the desired internal temperature was reached in the shrimp (See Figure 4.16). An ideal method of obtaining decimal reduction times and other thermal resistance data would be one in which the heating "come-up times" were very low. Finally, this method was not used because the facility decided that *L. monocytogenes* could not be brought into the plant after discussing the issue with regulatory agencies.

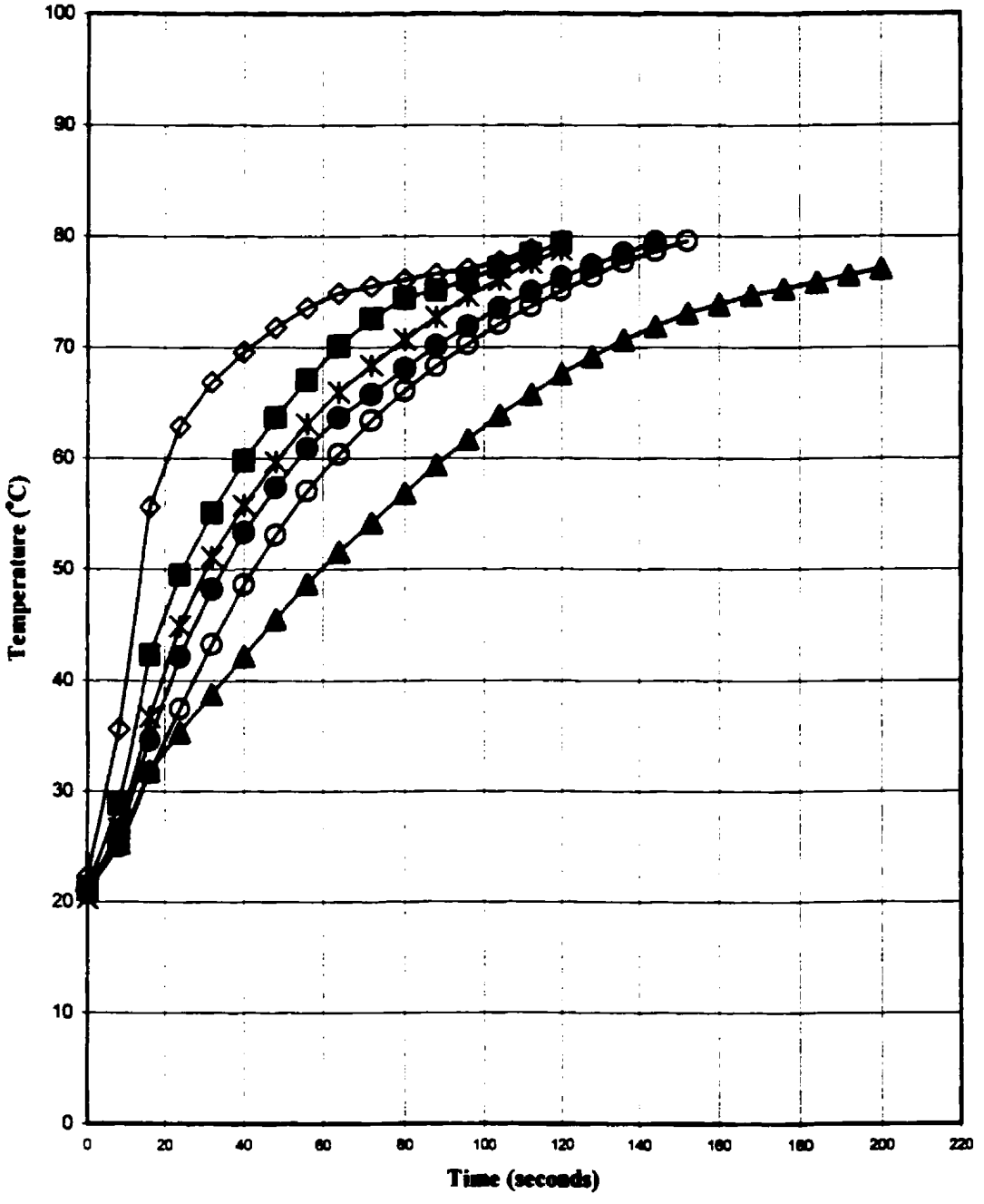

Figure 4.16: Time-temperature Profiles of Shrimp in Vacuum-sealed Bags Obtained From Steam Cooker with a Plate Partially Covering Cooker (2.5 cm Opening) and Steam Turned Down 3 Turns (Each Colour Represents One Shrimp Sample)

4.4.2 Capillary Tube Experiment

Due to the problems outlined above with the use of a modified steam cooker apparatus for obtaining thermal resistance data, an alternative method had to be used. The method selected was the capillary tube method, described in Section 3.9.2. This method has been consistently used by many researchers to investigate the thermal resistance of different bacteria. A more detailed discussion of this method will be undertaken in Section 5.4.1.

Triplicate thermal inactivation trials were carried out and D and *z* values were determined for temperatures of 65, 68, 70, 72, 75 and, if required, 80°C. Surviving bacteria were enumerated for each 10 second interval up to 2 minutes. However, for 72°C, 5 second intervals were used due to the very fast rate of inactivation of *L. monocytogenes* at this temperature.

Figures 4.17 to 4.19 show the results of three trials in which *L. monocytogenes* was inoculated onto shrimp and subjected to a heat treatment of 65°C. D values for the three inactivation trials, conducted for each temperature, are presented in Table 4.3. As can be seen in this table, D values of 0.606, 0.589 and 0.579 min were obtained for Trials 1 to 3, respectively, with a mean D value of 0.591 ± 0.0137 min.

For the 68°C heat treatment, lower D values of 0.296, 0.227 and 0.276 minutes, were obtained for Trials 1 to 3, respectively. These results are displayed in Figures 4.20 to 4.22. A mean D value of 0.266 ± 0.0355 min was calculated (Table 4.3). Thus, it would take 0.266 minutes or 15.96 seconds for the *L. monocytogenes* population to be reduced by 90%.

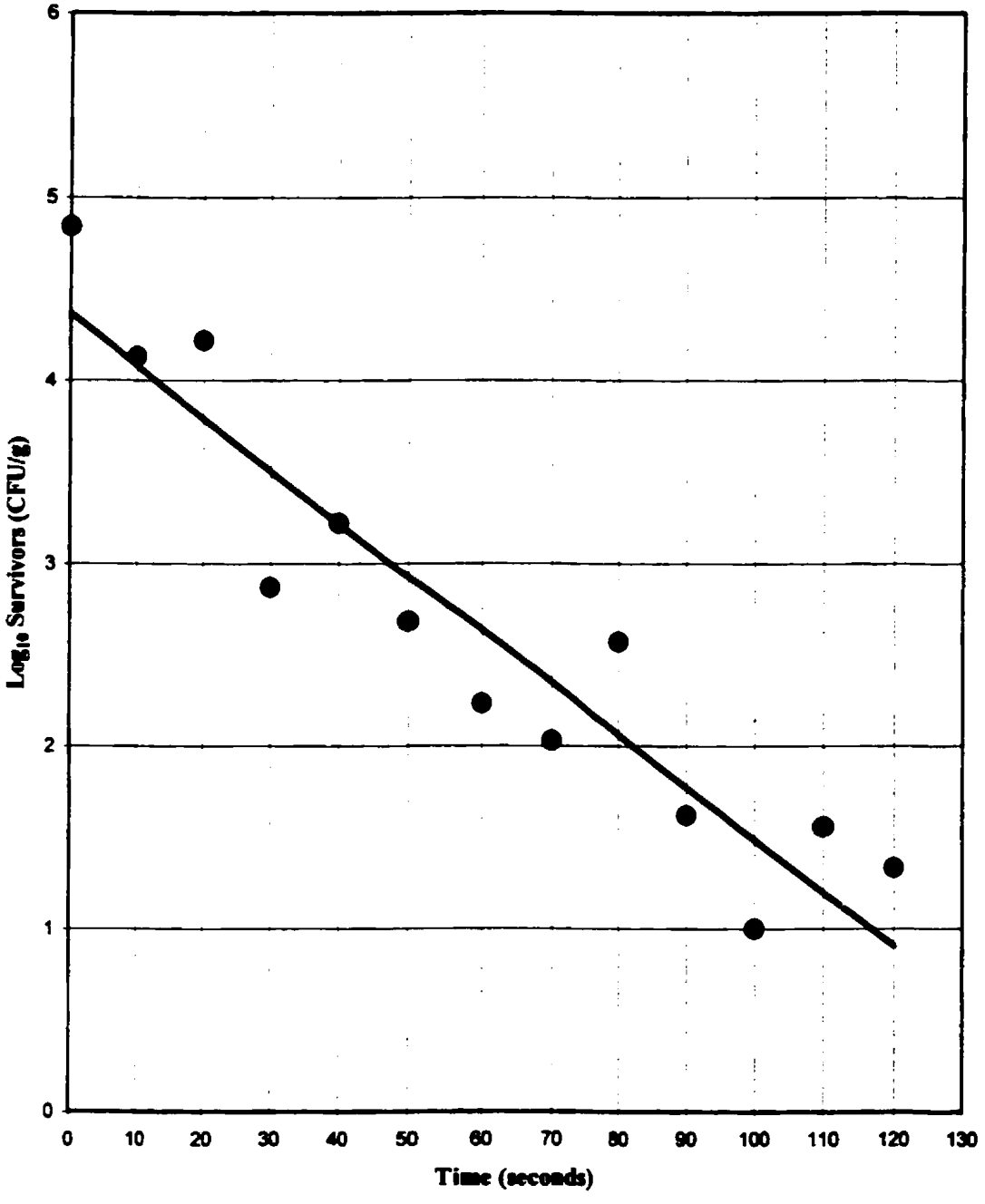

Figure 4.17: *Listeria monocytogenes* Surviving a 65°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 1)

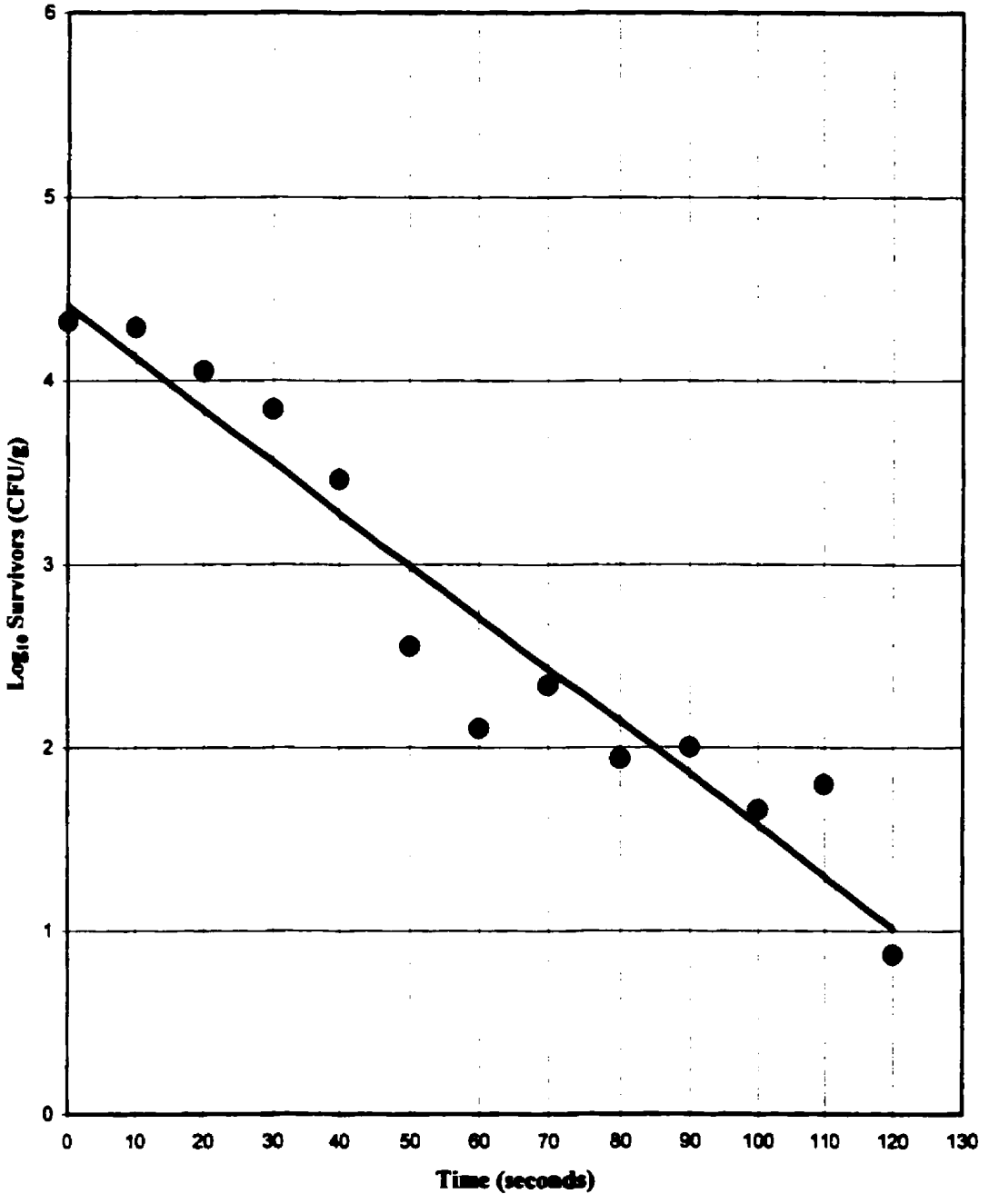

Figure 4.18: ***Listeria monocytogenes*** **Surviving a 65°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 2)**

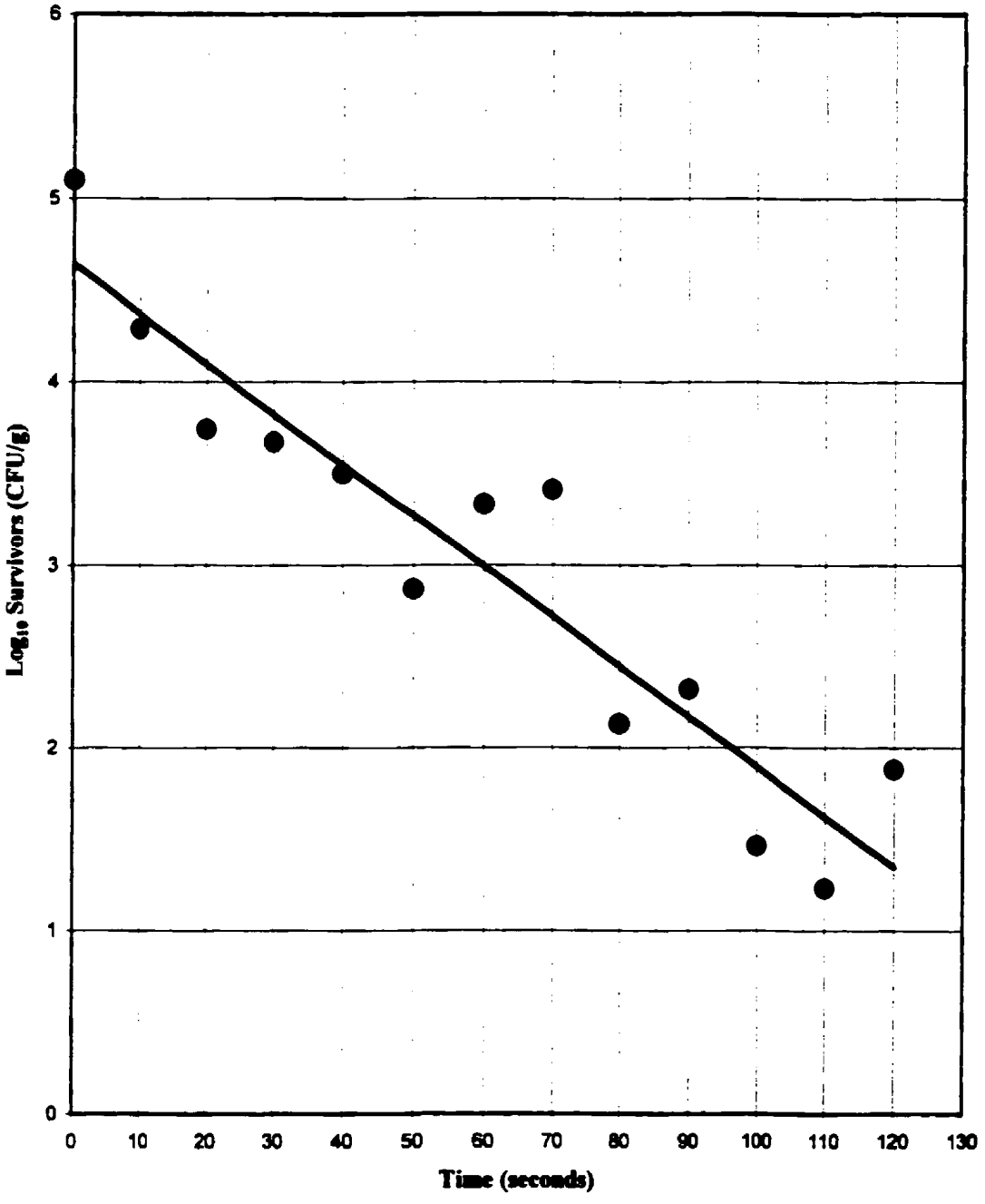

Figure 4.19: *Listeria monocytogenes* Surviving a 65°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 3)

Table 4.3: Decimal Reduction Times (D Values) of *Listeria monocytogenes* in Cooked Shrimp*

Temperature (°C)	Trial 1 D Value (min)	Trial 2 D Value (min)	Trial 3 D Value (min)	Mean and Standard Deviation†
65	0.606	0.589	0.579	0.591 ± 0.0137
68	0.296	0.227	0.276	0.266 ± 0.0355
70	0.153	0.147	0.132	0.144 ± 0.0108
72	0.0207	0.0209	0.0189	0.0202 ± 0.0011

* A z value = 5.07°C was obtained. Also, D values could not be calculated for 75°C and 80°C since these temperatures inactivated *L. monocytogenes* at rates too quick to accurately measure thermal death times.
†Significance at $p<0.05$.

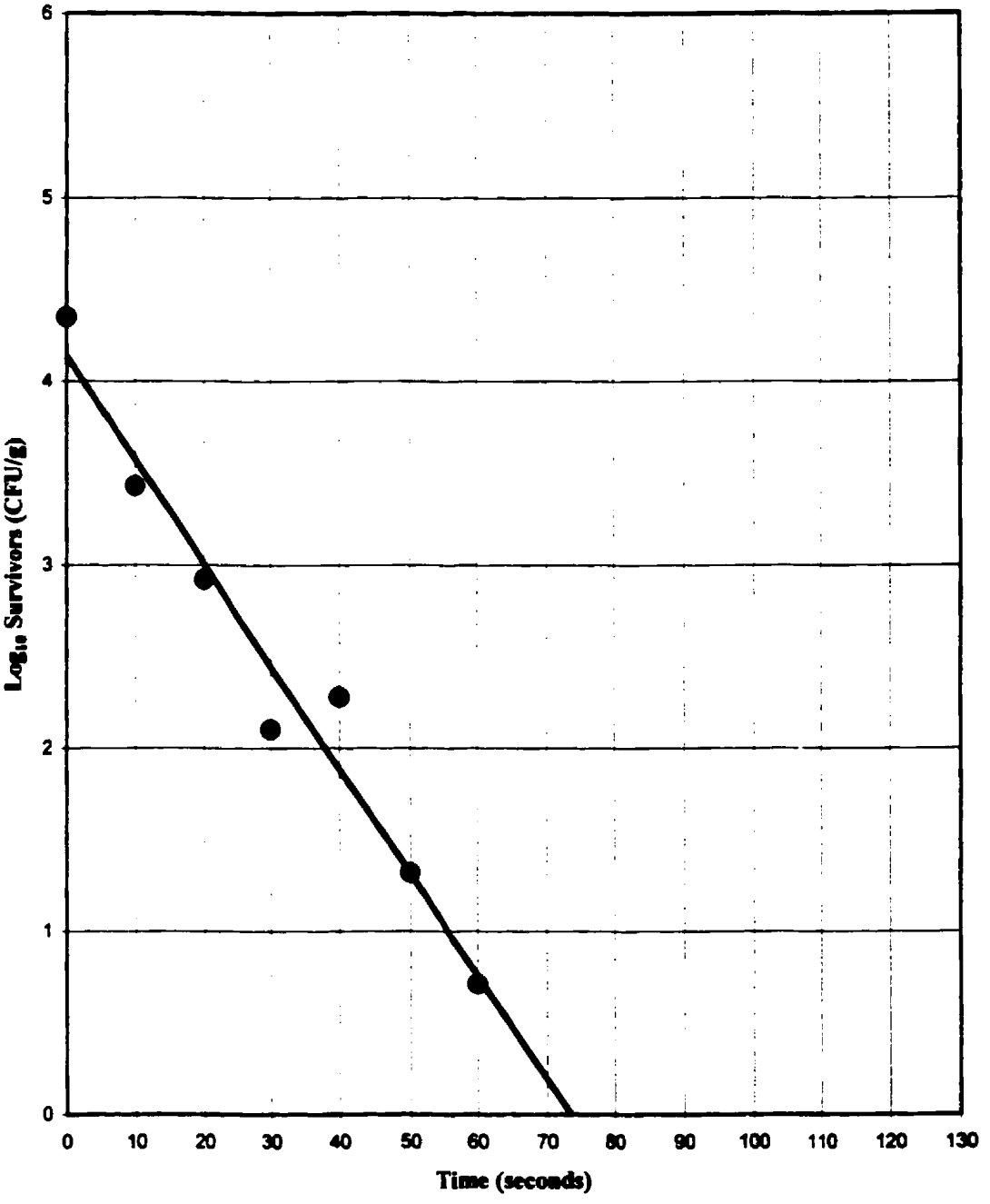

Figure 4.20: *Listeria monocytogenes* Surviving a 68°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 1)

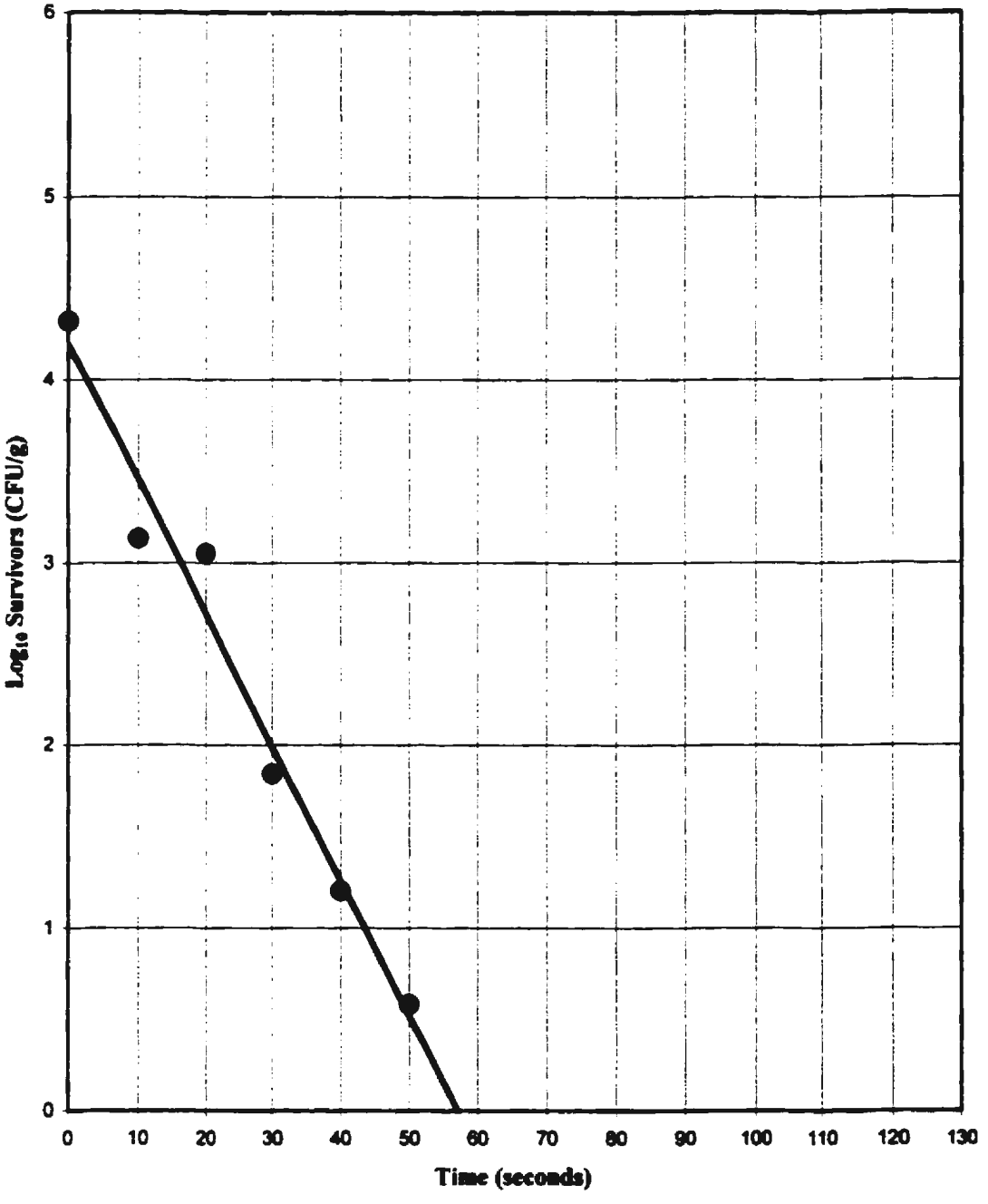

Figure 4.21: *Listeria monocytogenes* Surviving a 68°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 2)

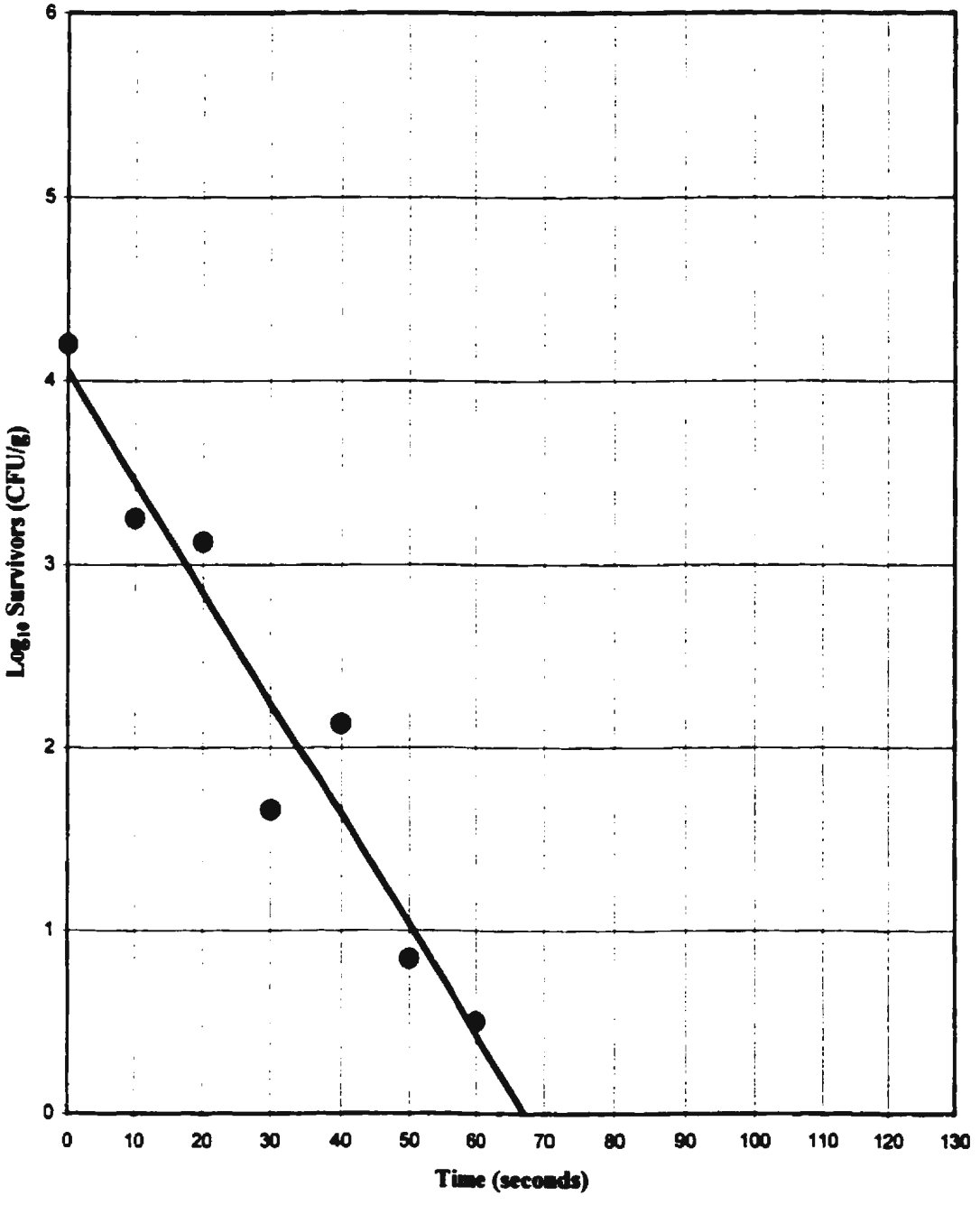

Figure 4.22: *Listeria monocytogenes* Surviving a 68°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 3)

The thermal inactivation curves for the three trials conducted for the heat treatment of *L. monocytogenes* on shrimp at 70°C are shown in Figures 4.23 through 4.25. From the negative reciprocal of the slope of these curves, D values for Trial 1, 2 and 3 were calculated to be 0.153, 0.147 and 0.132 min, respectively, with a mean of 0.144 ± 0.0108 min (Table 4.3).

Very low D values of 0.0207, 0.0209 and 0.0189 min were determined from Figures 4.26 to 4.28, respectively, for heat treatments at 72°C. A mean D value of 0.0202 ± 0.0011 min was calculated (Table 4.3). Thus, a very short time period, i.e. only 1.21 seconds, would be required to destroy 90% of a population of *L. monocytogenes* if heat processed at 72°C. At this temperature, there were no survivors after 5 seconds.

For all of the above temperatures, Table 4.4 shows the y-intercept, slope and R^2 values obtained from the line of linear regression, from which D values were determined. Figure 4.29 shows the thermal death curve for *L. monocytogenes* in shrimp (log D value vs. temperature). A *z* value of 5.07°C was calculated using the negative reciprocal of the slope of the linear regression line of this curve. Thus, an increase in heating temperature of 5.07°C will change the D value by a factor of 10. As can be seen from these results, *L. monocytogenes* becomes less resistant to heat (lower D value) as the temperature is increased.

For the next two temperatures examined, i.e., 75 and 80°C, there were no *L. monocytogenes* survivors at only 5 seconds after beginning the heat treatment. Thus, thermal inactivation curves could not be completed, since a time period shorter than 5 seconds could

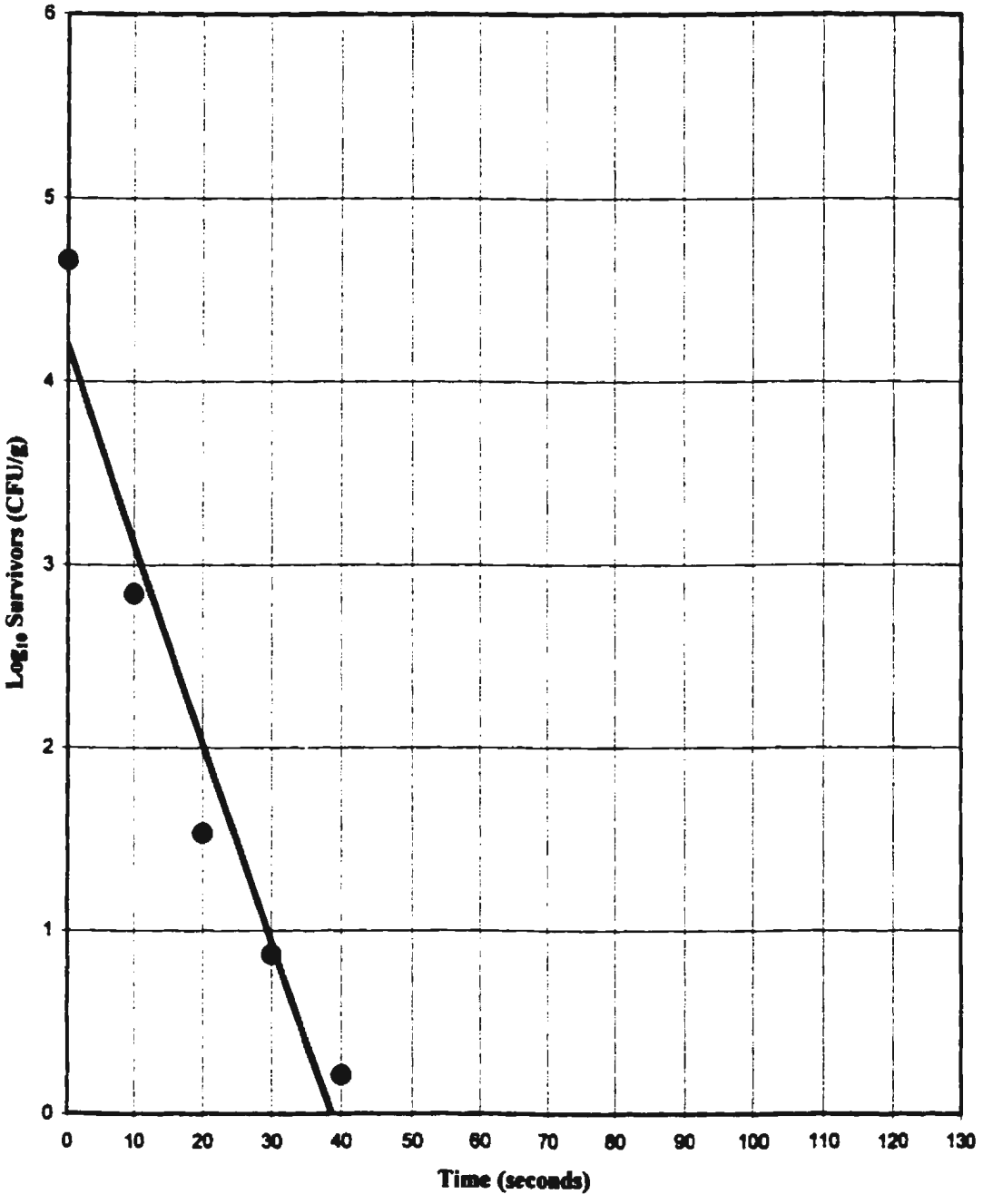

Figure 4.23: *Listeria monocytogenes* Surviving a 70°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 1)

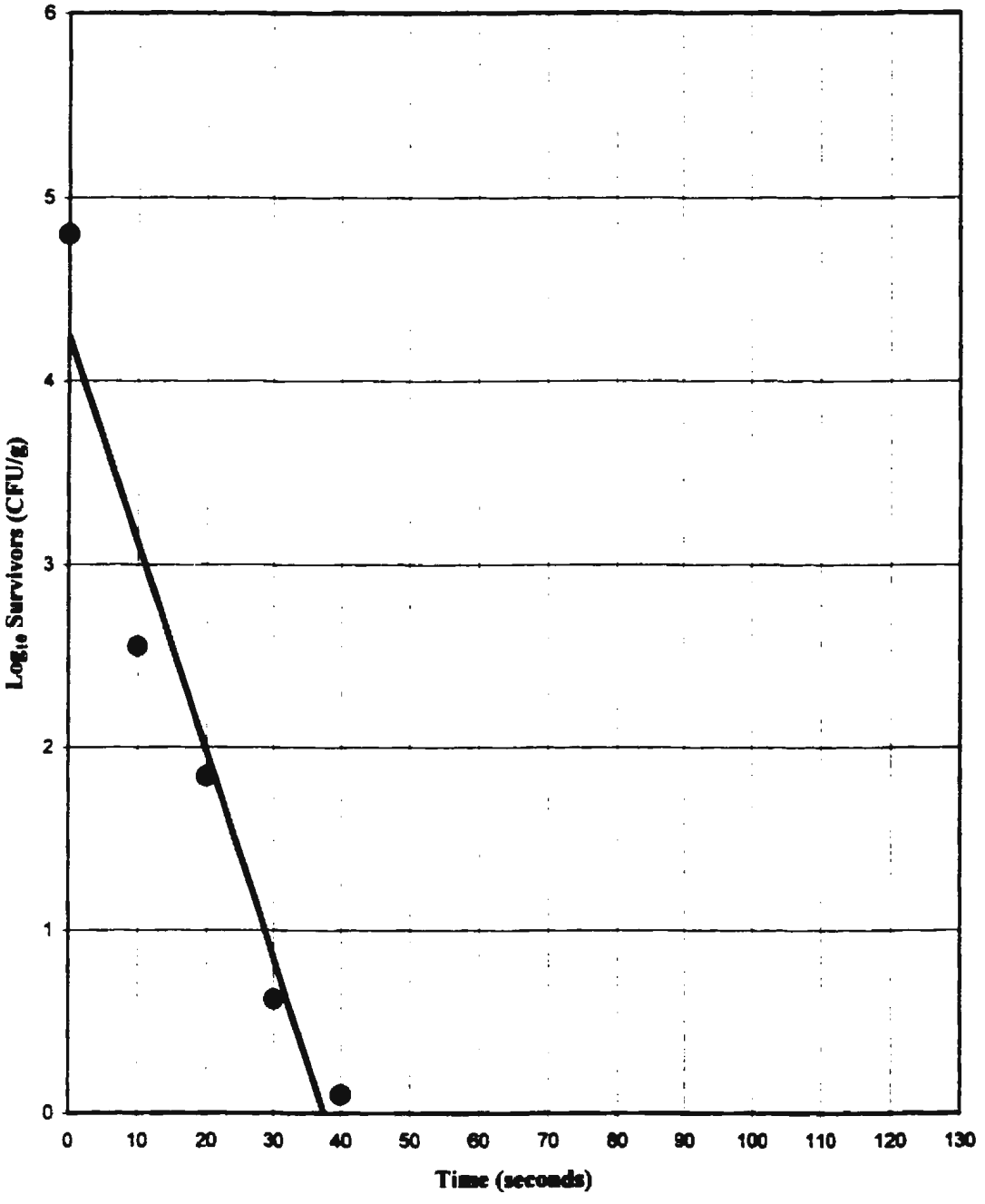

Figure 4.24: *Listeria monocytogenes* Surviving a 70°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 2)

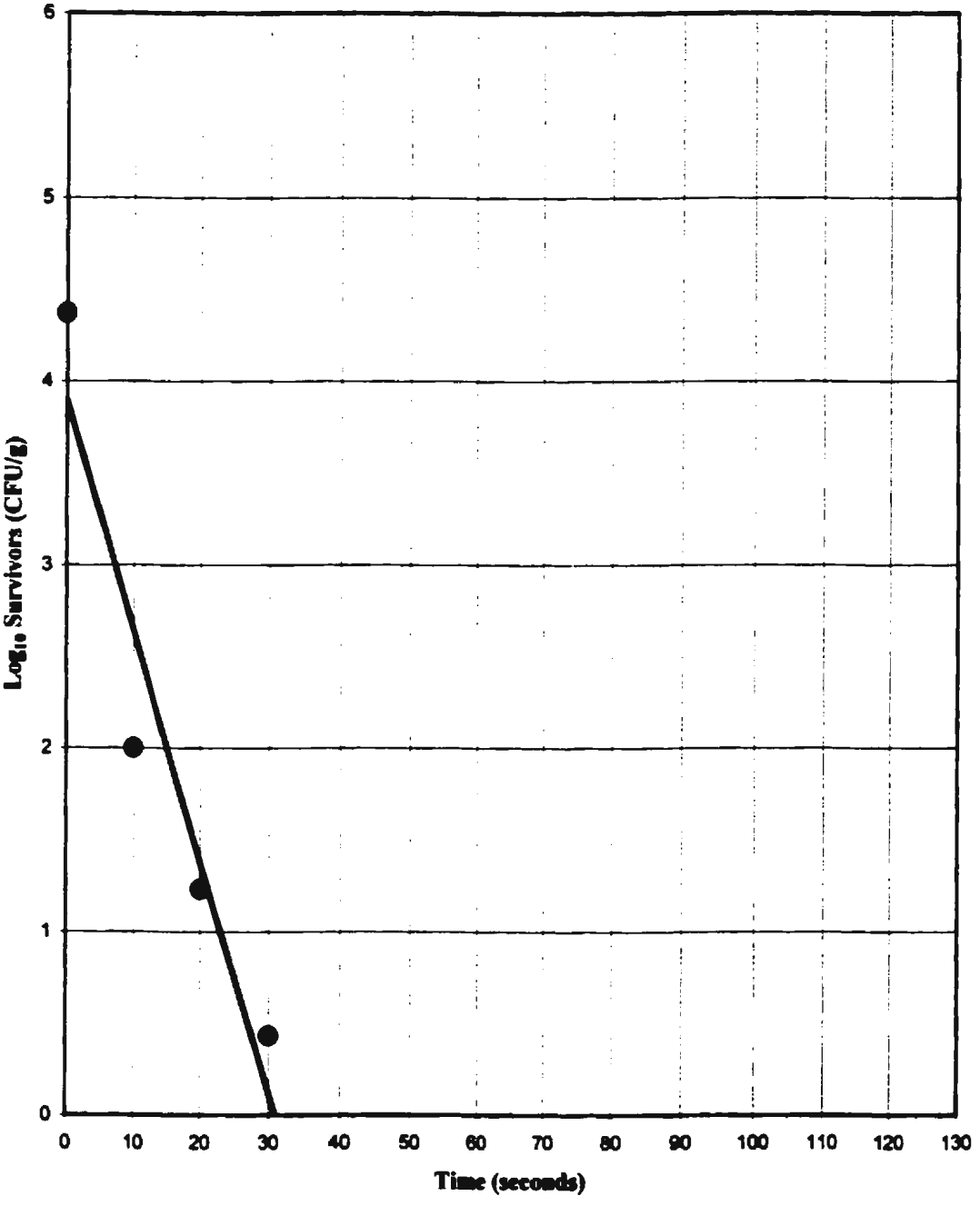

Figure 4.25: *Listeria monocytogenes* Surviving a 70°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 3)

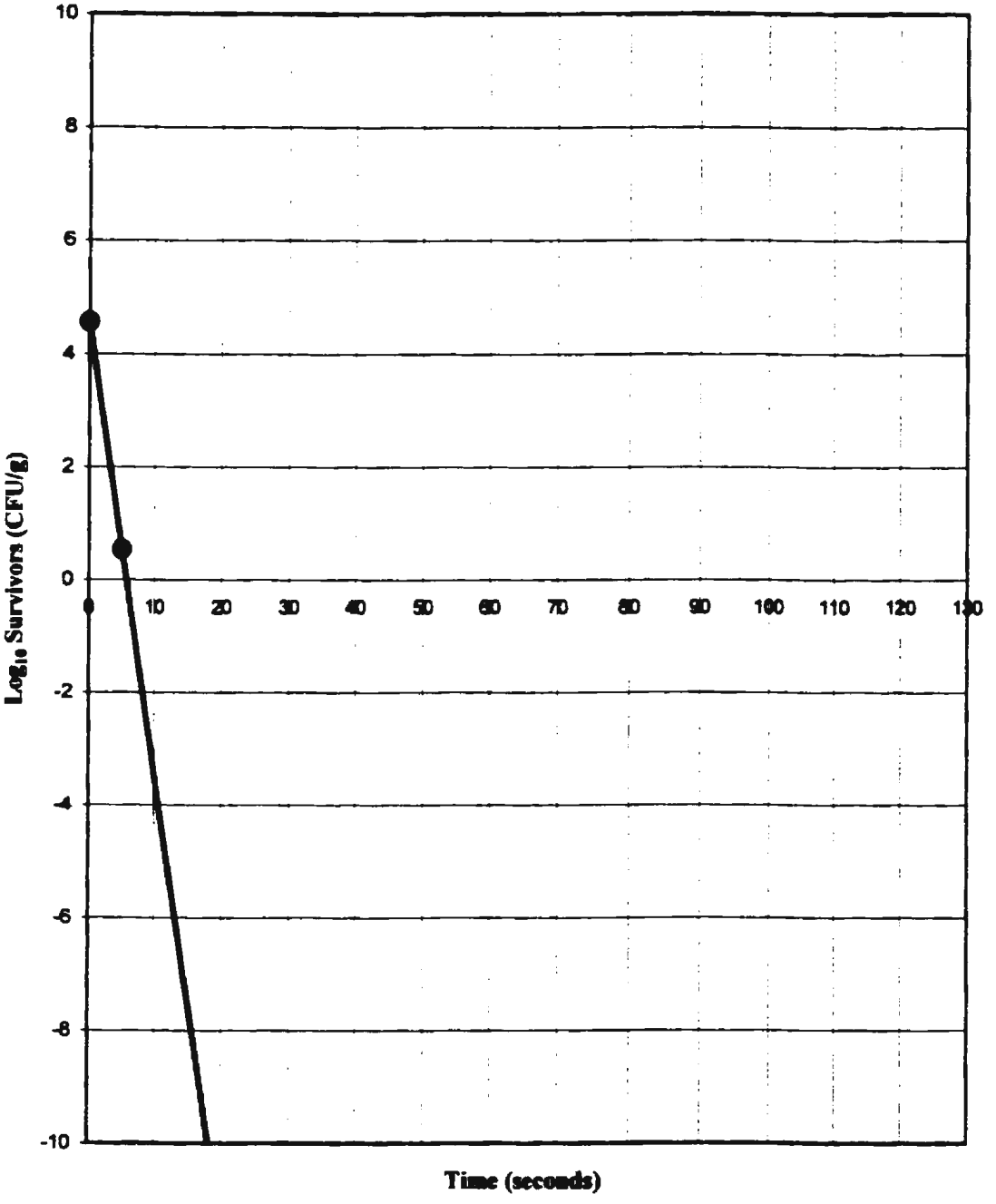

Figure 4.26: *Listeria monocytogenes* Surviving a 72°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 1)

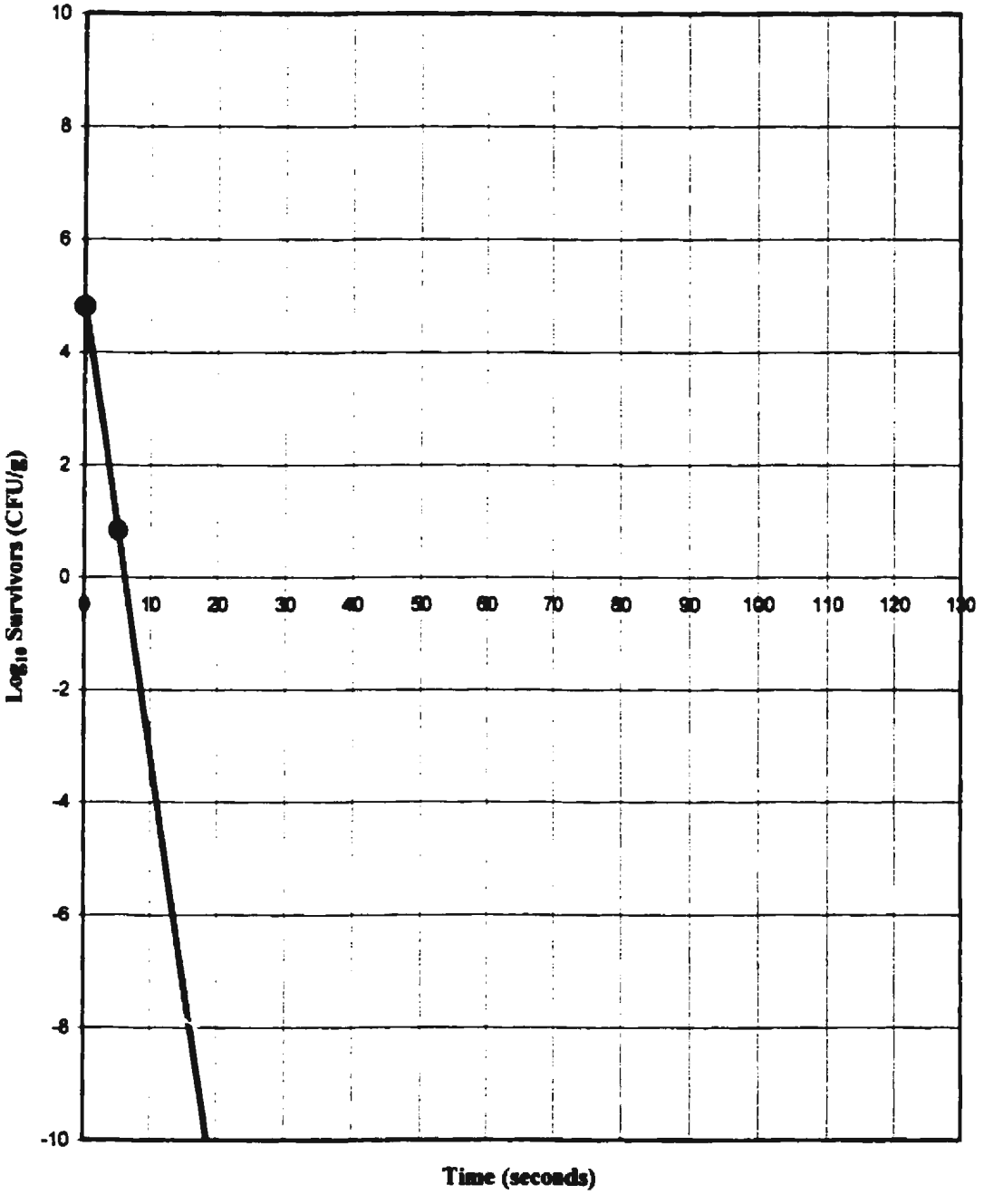

Figure 4.27: *Listeria monocytogenes* Surviving a 72°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 2)

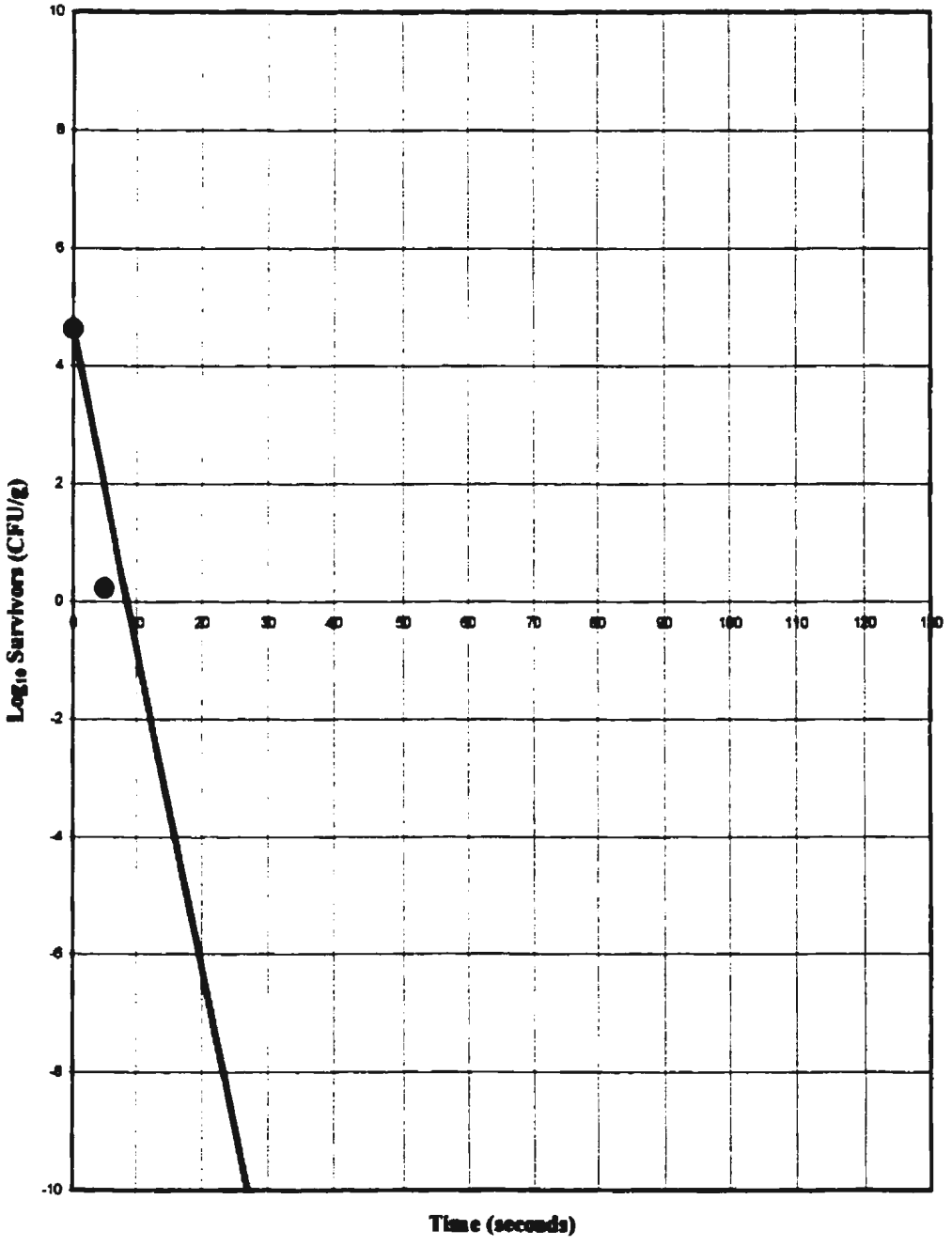

Figure 4.28: ***Listeria monocytogenes*** **Surviving a 72°C Heat Treatment in Shrimp Inoculated with 10^5 CFU/g (Trial 3)**

Table 4.4: Least Squares Regression Factors and Goodness of Fit for Decimal Reduction Times (D Values) of *Listeria monocytogenes* in Cooked Shrimp

Temperature (°C)	Trial	Y-intercept (log CFU/g)	Slope (seconds)	R^2
60	1	4.3689	-0.0288	0.8850
60	2	4.4064	-0.0283	0.9292
60	3	4.6426	-0.0275	0.8806
68	1	4.135	-0.0564	0.9617
68	2	4.189	-0.0734	0.9733
68	3	4.0546	-0.0604	0.9308
70	1	4.196	-0.1087	0.9467
70	2	4.248	-0.1133	0.9383
70	3	3.896	-0.1259	0.9136
72	1	4.56	-0.804	1
72	2	4.81	-0.796	1
72	3	4.63	-0.882	1

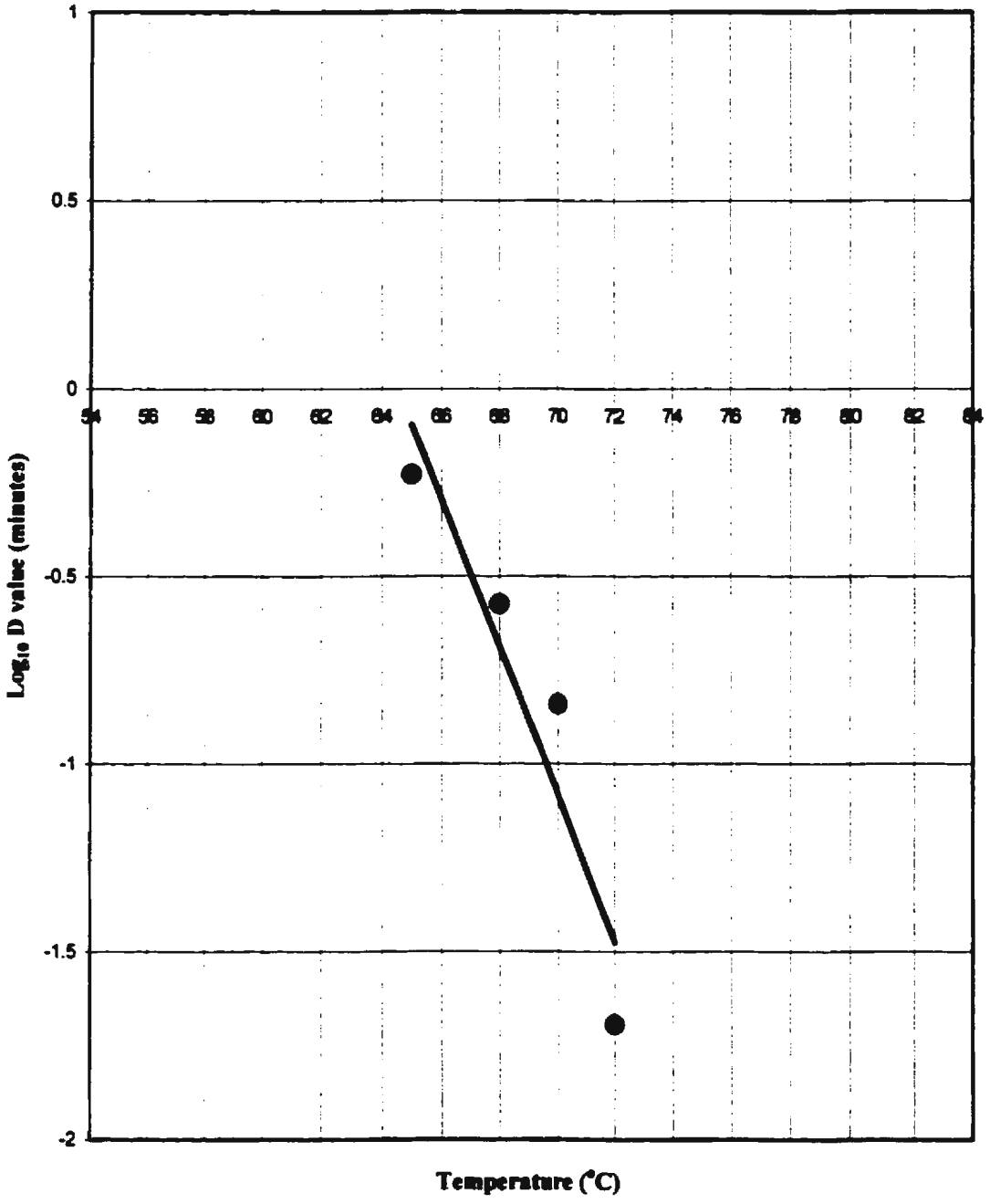

Figure 4.29: Thermal Death Time Curve for *Listeria monocytogenes* in Shrimp

not be accurately enumerated. Hence, the heat treatment temperatures of 75 and 80°C were not able to be carried out. However, using the D values obtained for the other temperatures examined and the *z* value of 5.07°C obtained, D values for these and other temperatures could be predicted using the following equation: $D_2 = 10^{\log D1-((T2-T1)/z)}$. Table 4.5 shows predicted D values for the temperatures of 60, 62, 75 and 80°C. With regards to come up times, the average time taken for the suspension in the capillary tube to reach the desired internal temperature was very fast, between 1.5 and 2 seconds.

4.5 Water Activity, Salt Content and pH

A total of five shrimp samples were tested to determine water activity, salt content and pH. The results obtained from these analyses are shown in Table 4.6. The water activity ranged narrowly from 0.981 to 0.989, with a mean value of 0.987 ± 0.003. The salt content readings ranged from 2.52 % to 2.67 %, with a mean value of 2.60 % ± 0.056. The pH readings also had a narrow range of 6.75 to 6.83, averaging 6.80 ± 0.031.

4.6 Summary

This study had three main objectives: determining total aerobic colony counts and *L. monocytogenes* incidence in raw shrimp, examining the behavior of *L. monocytogenes* on raw shrimp inoculated with low and high levels of the pathogen and stored at -20°C, on ice (0-1°C), 4°C and 10°C (an abuse temperature), and to investigate the thermal resistance of *L. monocytogenes* in shrimp.

Table 4.5: Predicted D Values for Various Temperatures Using a z Value of 5.07°C and a D_{65}* of 0.591 minutes.

Temperature (°C)	D Value (min)
60	5.73
62	2.31
75	0.0063
80	0.00065

* D_{65} = The D value obtained for the temperature of 65°C.

Table 4.6: Water Activity (A_w), pH and Salt Content of the Raw Shrimp Samples Supplied by a Newfoundland Shrimp Processor.

Trial	A_w*	pH	Salt (%)
1	0.988	6.75	2.63
2	0.986	6.82	2.52
3	0.981	6.83	2.58
4	0.989	6.79	2.67
5	0.989	6.80	2.61
Mean ± Standard Deviation†	0.987 ± 0.003	6.80 ± 0.031	2.60 ± 0.056

*A_w = Water activity.
†Significance at $p<0.05$.

In examining the bacteria naturally present on raw shrimp, no *L. monocytogenes* cells were detected on the raw shrimp and a very low number of 429.8 ± 65.4 CFU/g was obtained for the total aerobic colony count of the raw shrimp.

For the storage experiments, overall, *L. monocytogenes* was able to survive, but not grow, when stored on shrimp at -20°C and on ice (0-1°C). However, the pathogen was able to grow quite significantly, when stored at temperatures of 4°C and 10°C. The highest increase in *L. monocytogenes* numbers was on shrimp inoculated with 10^2 cells/g of the bacterium and stored at 10°C. This temperature would be considered an abuse temperature at which shrimp should definitely not be stored. Even at refrigeration temperatures, at which shrimp is normally held prior to processing (for up to six days), *L. monocytogenes* is able to survive and grow. Thus, the concern regarding *L. monocytogenes* growth in seafood stored at these temperatures is justified.

With regard to the thermal resistance portion of this study, as the temperature of the heat treatment increased the thermal resistance of *L. monocytogenes* decreased, as indicated by the decreasing D values with increasing temperatures. Average D values of 0.591, 0.266, 0.144 and 0.0202 min were obtained for temperatures of 65, 68, 70 and 72°C, respectively.

Chapter Five

Discussion

5.1 Aerobic Colony Count

The total aerobic colony counts ranged from 360 to 515 CFU/g for the six samples analyzed in triplicate, with an overall mean of 429.8 CFU/g. Thus, the shrimp is relatively sterile and high levels of background bacteria were not present to interfere with the *L.monocytogenes* inoculated onto the shrimp for experimental purposes. Shrimp naturally have low levels of *L. monocytogenes* (Farber, 1991; Motes, 1991; Ryser and Marth, 1991; Jemmi and Keusch, 1992). The marine bacteria that are most likely to be present on crustaceans that grow in cold seawater are *Vibrio* spp., *Plesiomonas shigelloides* (Hackney and Dicharry, 1988; Huss, 1994), *Flavobacterium*, *Moroxella* and *Aeromonas* (Ray, 1996). However, the bacteria that grew on the PCA plates used in this portion of the research were not identified.

5.2 *Listeria monocytogenes* Incidence

Six samples were analyzed in total, of which none were positive for *L.monocytogenes*. Thus, the shrimp caught in the cold waters of the Atlantic ocean appear to be free of *L. monocytogenes*.

Similar results were obtained by a fish inspection lab of the Canadian Food Inspection Agency. No *L. monocytogenes* was found on any of the 347 seafood samples

analyzed from 1997-1999 (Farber, 2000). They also reported that most of the seafood that tests positive for the presence of *L. monocytogenes* is imported seafood. Similarly, Hartemink and Georgsson (1991) did not isolate any *Listeria* spp. from the shrimp samples tested. In contrast, however, many other investigators have isolated *L. monocytogenes* from fresh, raw shrimp (Weagant *et al.*, 1988; Ryu *et al.*, 1992; Adesiyun, 1993; Berry *et al.*, 1994; Gecan *et al.*, 1994; Destro *et al.*, 1996). None of the shrimp samples analysed in these studies were obtained from Canada.

The protocol used in this study was the one outlined by the Health Protection Branch of Health Canada. As described in Section 3.4.2, it involves a two-stage enrichment, a 48 h primary enrichment in *Listeria* enrichment broth (LEB) and a 48 h secondary enrichment in modified Fraser broth (MFB). After this enrichment, aliquots of the broth are then plated onto *Listeria*-selective agar plates. After reviewing the culture media and methods for the isolation of *L. monocytogenes*, Curtis and Lee (1995) recommended a three-stage procedure consisting of pre-enrichment, selective enrichment and selective plating steps. Other researchers have also found a two-enrichment step procedure to be more effective at isolating *L.monocytogenes* than a single enrichment step (Lovett *et al.*, 1991; Warburton *et al.*, 1991a; Warburton *et al.*, 1991b; Ben Embarek, 1994). In the present study, three selective agars were used, LPM, Oxford and PALCAM. Oxford, modified Oxford and LPM media are the ones used most widely in North America, while PALCAM and Oxford media are most popular in Europe (Farber and Peterkin, 1991; Farber, 2000). Thus, the methods used in this study are effective in isolating *L. monocytogenes* present on the shrimp samples and the

results obtained should be accurate.

5.3 Storage Experiment

5.3.1 Storage at -20°C

During the 12 days of storage at a temperature of -20°C, the *L. monocytogenes* population did not significantly increase or decrease, but remained stable at initial inoculum levels. Similarly, McCarthy *et al.* (1990) recovered *L. monocytogenes* from laboratory contaminated shrimp, at levels of 10^5 CFU/g, after 90 days of storage at -20°C. In another study by McCarthy (1990), the exoskeletons of crawfish were inoculated with 3.0 log *L.monocytogenes* cells/g and stored at -20°C. *L. monocytogenes* survived, but did not grow, under these conditions. The total storage time was 15 days. Finally, Harrison *et al.* (1991) studied the fate of *L. monocytogenes* on packaged, refrigerated and frozen seafood. For the frozen storage experiment, fish and shrimp were inoculated by dipping in a suspension of the bacteria, packaged and stored at -20°C for a 3 month period. Samples were taken and *L.monocytogenes* numbers determined at zero and three months. *L. monocytogenes* populations decreased slightly after three months of storage, regardless of the type of packaging type. *L. monocytogenes* numbers for the shrimp samples only decreased from initial populations of 4.68 $\log_{10}$ CFU/ml to 3.74, 4.20 and 4.08 $\log_{10}$ CFU/ml, for the three types of packaging examined. This decrease was not significant and thus, similar to the results obtained in this study.

Although, the *L. monocytogenes* population was able to survive frozen storage in these studies, it did not grow at this low temperature condition.This survival is likely due to the protective effect of the chitin in the shell of the shrimp. Chitin is the major polysaccharide component of crustacean exoskeletons and can protect bacteria from long periods of freezing. Additionally, *Listeria* can grow at these harsh temperatures by attaching and multiplying on the chitin found in the crustacean carapace which may act as the sole carbon source (Platt *et al.*, 1995; Nascumento *et al.*, 1998). Many strains of *L.monocytogenes* possess chitinase activity (Ryser and Marth, 1991). In a study by Fuad *et al.* (1989) that evaluated the ability of *L. monocytogenes* to survive in an estuarine environment, *Listeria* populations decreased in chitin-free filtered and unfiltered seawater, but the addition of chitin to both types of water stimulated the growth of *Listeria*. These findings, as well as those of Van Wagner (1989), who found *L. monocytogenes* on the exoskeleton, but not in the digestive tract, of shrimp that were exposed to high levels of the pathogen in aquaculture tanks, indicate that *L.monocytogenes* may be ecologically adapted to chitin (Ryser and Marth, 1991).

Golden *et al.* (1988) also found this bacterium to be relatively resistant to damage due to freezing. They observed very little decrease in *L. monocytogenes* when stored for 14 days at -18°C in tryptose phosphate broth. With respect to the methodology used, *Listeria*-selective media were used to enumerate the pathogen, as was used in this study, and they reported that freezing did not affect the ability of the *L. monocytogenes* to grow on this media. For the same temperature, Lammerding and Doyle (1990) conducted experiments

using ice-cream as the storage substrate and a storage time of five months at -18°C. They also reported that the bacteria could be readily recovered on both selective and non-selective media. Thus, freezing of shrimp is no safeguard against the transmission of foodborne listeriosis as this pathogen is evidently quite resistant to subfreezing temperatures.

5.3.2 Storage on Ice (0-1°C)

During storage of the shrimp on ice, the initial population of *L. monocytogenes* decreased slightly by approximately 0.70 log and 0.64 log, for both low and high inoculation levels, respectively, until around the fourth day of storage, after which point the population levelled off and stayed relatively stable. As with frozen storage, the *L. monocytogenes* cells survive the low temperatures of the ice, but do not grow. Similar findings were also reported by Harrison *et al.* (1991) in their study of the fate of *L. monocytogenes* on packaged, refrigerated and frozen seafood, i.e., raw shrimp and fin fish. The samples were dipped in a *L. monocytogenes* suspension, packaged and stored in a 2:1 mix of ice: fish or shrimp and placed in ice chests. Water from the melting ice was removed every second day and ice was replenished as necessary. The *L. monocytogenes* populations were determined at 0, 7, 14 and 21 days. The *L. monocytogenes* population did not increase during storage on the shrimp or fish on ice. They also noted that, in most cases, there was a decrease in *L. monocytogenes* population of less than one log, i.e., for shrimp, initial populations of 4.68 $\log_{10}$ CFU/ml decreased to populations of 3.56, 3.86 and 3.63 $\log_{10}$ CFU/ml, respectively, for the three packaging types. The initial decrease of less than one log in the number of surviving cells,

which was also observed in this study, may have been due to a dilution effect, i.e., some of the cells that were not attached to the shell of the shrimp may have gone into the water from the ice. However, as the storage time increased a greater number of cells may have attached themselves to the shrimp shells. This attachment may be possible via the production of chitinase by the *Listeria* cells (Ryser and Marth, 1991; Platt *et al.*, 1995). Also, Mu *et al.* (1997) discussed the ability of *L. monocytogenes* to attach to various types of surfaces, including shrimp shell. They reported that *L. monocytogenes* cells form a strong attachment onto the shells of shrimp and these cells are not readily removed by rinsing with water and that this attachment is enhanced at 4°C.

5.3.3 Storage at 4°C

For initial inoculum levels of 10^2 *L. monocytogenes* cells/g of shrimp, population counts increased from 2.56 $\log_{10}$CFU/g to 5.48 $\log_{10}$CFU/g in the 12 days of storage. A generation time, or time taken for the population to double, of 3.60 days was obtained, as well as a 2.92 log increase. As can be seen from these values, the *L. monocytogenes* population increased significantly when stored at refrigeration temperatures. For the higher inoculation level, the initial population of *L. monocytogenes* (10^6 CFU/g) slowly increased and levelled off to a relatively stable cell count around day six of storage. The population counts increased from 6.46 $\log_{10}$CFU/g to 8.67 $\log_{10}$CFU/g in 12 days. Similar to the results obtained for the 10^2 CFU/g inoculation level, a generation time of 3.08 days and a 2.21 log increase was determined. While this is a significant increase in *L. monocytogenes*, it is not

as significant an increase as was obtained for the 10^2 CFU/g inoculation level, under the same conditions of storage. Similarly, Dalgaard and Jorgensen (1998) found that the initial numbers of the organism had a profound effect in growth in their study of the growth of *L.monocytogenes* on cold-smoked salmon.

Farber (1991) examined the potential for growth of *L. monocytogenes* in fish products including lobster, crabmeat, shrimp and smoked salmon. The various seafood samples were inoculated by dipping for one minute in an inoculation bath containing levels of 2×10^3 CFU/ml of *L. monocytogenes*. It was found that the organism grew fairly well on all fish products tested, in most cases increasing about 2 to 3 logs after seven days of storage at 4°C. After 15 days of storage at 4°C, initial *L. monocytogenes* populations of about three log CFU/g increased to between 4.5 log CFU/g and eight log CFU/g for all seafood tested. The initial bacteria levels of about 2 to 2.5 log CFU/g increased by about 2 logs to 4 to 4.5 log CFU/g for the seven days of storage of the shrimp samples. These results are similar to the ones obtained in the present study. In contrast, Brackett and Beuchat (1990) found that *L.monocytogenes* numbers did not increase on inoculated crabmeat after seven days of storage at a temperature of 5°C. However, after a longer period of storage, i.e., 12 to 14 days, numbers increased by about 2 to 2.8 logs.

Lastly, Fernandes *et al.* (1998) also studied the growth of *L. monocytogenes* on refrigerated fillets of trout and catfish. Fillets were inoculated with the pathogen, stored at 4°C, and enumerated for *L. monocytogenes* at days 1, 3, 6, 8, 10, 13 and 15. They found *L.monocytogenes* counts to rise during storage at 4°C. They increased from $10^{5.2}$ CFU/g to

$10^{6.5}$ CFU/g on trout fillets and from $10^{5.1}$ CFU/g to $10^{6.9}$ CFU/g on catfish fillets in 15 days of storage.

5.3.4 Storage at 10°C

During storage on shrimp at 10°C, the *L. monocytogenes* population greatly increased from 2.46 $\log_{10}$CFU/g to 7.26 $\log_{10}$CFU/g and from 6.43 $\log_{10}$CFU/g to 8.76 $\log_{10}$CFU/g for inoculation levels of 10^2 and 10^6, respectively. The shortest generation time of 1.1 days and the largest log increase of 4.80 was observed for the low initial inoculum levels of 10^2 CFU/g. A log increase in population similar to the one obtained for storage at 4°C, was obtained for high inoculum levels of 10^6 CFU/g, i.e., a 2.33 log increase. However, a much shorter generation time of 1.96 days was observed for storage at 10°C and a high inoculum level.

In a similar study, Brackett and Beuchat (1990) inoculated crabmeat with 10^4 *L.monocytogenes* cells/g of crabmeat, placed the crabmeat in zip-lock storage bags and incubated at 10°C. Samples were analyzed for *L. monocytogenes* at days 0, 2, 5, 8, 12 and 14. After the two-week storage period, *L. monocytogenes* populations increased from about 4.2 $\log_{10}$CFU/g to about 9.3 $\log_{10}$CFU/g, a 5.1 log increase. These results are similar to the 4.80 increase of *L. monocytogenes* population observed in the present study for low inoculation levels. An increase in *L. monocytogenes* numbers was also reported by Ben Embarek and Huss (1992) in a study in which they stored inoculated raw salmon at 10°C. Growth occurred rapidly with an increase in numbers from approximately 2.2 logs in four days. Finally, Jemmi

and Keusch (1992) also examined the behaviour of *L. monocytogenes* during the storage of experimentally contaminated hot-smoked trout. They found that initial inoculation levels of 10^1 CFU/g significantly increased to levels of 10^7 CFU/g after 20 days of storage.

Thus, the findings of the present and other studies, indicate that moderate to severe temperature abuse of seafood products contaminated with *L. monocytogenes* may significantly enhance the growth potential of the pathogen on these products.

5.3.5 Summary

Overall, *L. monocytogenes* was able to survive, but not grow, when stored on shrimp at -20°C and on ice (0-1°C). However, the pathogen was able to grow, quite significantly, when stored at temperatures of 4°C and 10°C. The highest increase in *L. monocytogenes* numbers was on shrimp inoculated with 10^2 cells/g of the bacterium and stored at 10°C. This temperature would be considered an abuse temperature at which shrimp should definitely not be stored. Even at refrigeration temperatures, at which shrimp is normally held prior to processing (for up to six days), *L. monocytogenes* is able to survive and grow. Thus, concerns relating to the growth of *L. monocytogenes* in seafood stored at these temperatures is justified. The psychrotrophic characteristics of this organism are of great concern to seafood processors. Moreover, since the infectious dose is unknown, any outgrowth of surviving *Listeria* should be prevented (McCarthy, 1990).

5.4 Thermal Resistance

5.4.1 Steam Cooker Apparatus

As previously discussed, this study initially attempted to obtain thermal resistance data for *L. monocytogenes* in shrimp using a method that simulated actual conditions used in commercial shrimp processing facilities, which cook the shrimp using live steam injection and shrimp are cooked whole. Other than cooking via steam, the chosen heating method should be able to achieve desired internal temperatures (of the shrimp) in very short times, as is the case with steam.

We attempted to design such a method through the use of a steam cooker apparatus (Figure 3.2). However, due to various factors, previously discussed in Section 4.4.1, no method for determining the thermal resistance of *L. monocytogenes* using a steam cooker could be carried out. Thus, an alternative method, the capillary tube method, was used, which will be discussed in the next section.

5.4.2 Capillary Tube Experiment

The capillary tube method of determining the thermal resistance of a particular bacterium has been used extensively for many different bacteria and media. With respect to *L. monocytogenes*, it has been used primarily to investigate the pathogen's behaviour in milk and milk products (Fairchild and Foegeding, 1993). It has, however, been used for other food products, such as liquid egg products (Foegeding and Leasor, 1990; Schuman and Sheldon, 1997; Michalski *et al.*, 2000) and ham (Carlier *et al.*, 1996).

Several authors have used a method that uses sealed bags or flexible pouches for the heating medium. The food and bacteria being examined are added to the pouches, which are heated in a water bath. This method has been used to investigate the thermal resistance of *L.monocytogenes* in foods such as pork and beef sausage (Farber and Brown, 1990), pork chops (Mussa *et al.*, 1999) and beef and potatoes (Doherty *et al.*, 1998). However, using this method, as compared to the capillary tube method, come-up times were much higher. For example, Mussa *et al.* (1999) reported heating come-up times of 2 minutes for their study on the heat resistance of *L. monocytogenes* in pork. They used sterilization pouches. Similarly, Doherty *et al.* (1998), using vacuum sealed bags, also reported come-up times of approximately 2 minutes in their examination of the thermal resistance of *L. monocytogenes* in meat and potato substrates. In contrast, come-up times between 1.5 and 2 seconds in the present study were significantly lower. It would be more difficult to use short time intervals for removing samples because of the time required and awkwardness (larger size as compared to capillary tubes) involved in removing the bags from the water bath and placing them in ice. Thus, the capillary tube method was chosen as a more appropriate method for investigating the thermal resistance of *L. monocytogenes* in shrimp.

Fujikawa *et al.* (2000) compared the use of capillary and test tube procedures in thermal inactivation studies. They reported that the capillary tube method is superior for obtaining thermal resistance curves of microorganisms since the come-up times of the microbial suspension are short and since the temperature becomes uniform rapidly. Screw-capped test tubes have been used in some studies rather than capillary tubes. These tubes are

not fully submerged in the water bath. In order to obtain accurate and reliable heat resistance data, however, tubes must be submerged. If not, the bacteria that are on the tube walls or the cap will not be exposed to the inactivation temperatures; thus, they will survive and be counted, causing inaccurate results and overestimated D values (Sorqvist, 1989; Donnelly, 1990; Fujikawa *et al.*, 2000; Michalski *et al.*, 2000). This problem is avoided using sealed capillary tubes, which are completely submerged below the surface of the water bath and allow almost instantaneous heating to the desired temperature.

With respect to the enumeration of *L. monocytogenes* using this method, it has been suggested that the use of selective media for the enumeration of surviving bacteria in thermal inactivation studies may be inaccurate, as heat-injured cells will not grow on selective media (Mackey and Bratchell, 1989; Harrison and Huang, 1990; Ben Embarek and Huss, 1993; Dorsa *et al.*, 1993). Therefore, in the present study, both *Listeria*-selective media and non-selective media (TSA) were used to enumerate surviving bacteria to ensure that all survivors, including heat-injured ones, would be accounted for.

In terms of shrimp inoculation, the levels of *L. monocytogenes* used to inoculate the shrimp in this study, approximately 10^5 CFU/g, represent a worst-case scenario, since *L.monocytogenes* levels naturally present on shrimp are quite low, if present at all.

The average D values obtained in this study for *L. monocytogenes* in shrimp were 0.591 ± 0.0137, 0.266 ± 0.0355, 0.144 ± 0.0108 and 0.0202 ± 0.0011 min, for temperatures of 65, 68, 70 and 72°C, respectively. Correlation coefficients (R^2) ranged from 0.8806 to 1, with an average of 0.9466 ± 0.0419, indicating that the inactivation curves (log survivors vs.

time) were linear and D values could be calculated directly from them.

The comparison of the results of this study to other similar studies is difficult, since the number of studies that have been conducted on the thermal resistance of *L.monocytogenes* in seafood, more specifically shrimp, to date is limited. In the literature reviewed, only one study was found that examined the thermal resistance of *L.monocytogenes* in artificially and naturally contaminated shrimp. In this study, McCarthy *et al.* (1990) boiled shrimp tail meat, artificially and naturally contaminated with *L.monocytogenes*, for 1-5 minutes at 100°C. The organism was recovered from the artificially inoculated (10^5cells/g) shrimp, but not from the naturally contaminated shrimp. D values were not reported. These results are quite different from the ones obtained in this study, since at 65°C (D value = 0.591 min), levels of 10^5 cells/g of *L. monocytogenes* would have been completely destroyed in about 3 minutes. Thus, it is obvious that *L. monocytogenes* would not have been recovered if 100°C was tested in the current study.

Harrison and Huang (1990) examined the thermal resistance of *L. monocytogenes* in blue crab meat. The strain, Scott A, inoculated onto the crab at levels of approximately 10^7 CFU/g, was found to have D values of 40.43, 12.00 and 2.61 min for 50, 55 and 60°C, respectively, as well as a *z* value of 8.40°C. While the current study did not test these temperatures, a D value of 5.73 min was predicted for 60°C based on the observed D value for 65°C and the *z* value of 5.07°C calculated. This value is much higher than the value of 2.61 min obtained by Harrison and Huang (1990), but the *z* value of 5.07°C is quite lower than the *z* value of 8.40°C they obtained.

In another study on the thermal resistance of *L. monocytogenes* in seafood, Dorsa *et al.* (1993) obtained D values of 10.23, 1.98 and 0.19 min for 55, 60 and 65°C, respectively, when they inoculated crawfish tail meat with 10^8-10^9 CFU/g levels of *L. monocytogenes*. A *z* value of 5.5°C was calculated. These D values are very similar to the ones obtained by Harrison and Huang (1990), which were presented above. In comparing these results to the current study, these values are, again, much lower. The D value of 0.591 min for 65°C obtained in the current study is quite lower than the D value of 0.19 min obtained by these authors.

Budu-Amoako *et al.* (1999) reported a D value of 2.39 min for lobster cooked at 60°C. Cans of cold-pack lobster meat were inoculated with 10^3 and 10^5 cells/g of *L.monocytogenes* and cooked at 60°C for five minutes. In a study of thermal death times of *L. monocytogenes* in green shell mussels, D values of 48.09, 16.25, 9.45, 5.49 and 1.85 min were found for temperatures of 56, 58, 59, 60 and 62°C, respectively (Bremer and Osborne, 1995). In comparing D values for 60°C, the value of 5.49 min is close to the predicted value of 5.73 min for 60°C in the current study. With respect to *z* values, the *z* value of 4.25°C reported by Bremer and Osborne (1995) is similar to the *z* value of 5.07°C obtained in this study.

Finally, Ben Embarek and Huss (1993) investigated the heat resistance of *L.monocytogenes* in vacuum packaged cod and salmon fillets. D values for 60°C of 1.95-1.98 min were obtained for cod and values of 4.23-1.48 min were reported for salmon fillets. Also, *z* values of 5.65 and 6.4°C were calculated for salmon and cod, respectively. These *z*

values are also similar to the *z* value of 5.07°C obtained in the present study.

Thus, results are varied between studies. However, it is difficult to accurately compare these studies, or, in fact, any other studies on the thermal resistance of *L.monocytogenes* in seafood for various reasons. First, differences in the type and composition of the seafood samples may influence the results. The pH (Juneja *et al.*, 1998), fat content (Dorsa *et al.*, 1993; Ben Embarek, 1994) and salt content (Bremer and Osborne, 1995) of the product being examined, influence the thermal resistance of *L. monocytogenes*. Additionally, the physical form of the product, such as fresh or frozen, may also influence the thermal properties of *Listeria* in food (Bremer and Osborne, 1995).

Furthermore, findings regarding the thermal resistance of bacteria can also be influenced by the particular methodologies and apparatus used by individual researchers (e.g., the media used to recover heat-stressed *Listeria*; the conditions in which the bacteria are grown; the inoculation method, etc.). For example, Ben Embarek and Huss (1993) and Dorsa *et al.* (1993) used *Listeria*-selective media (*Listeria* enrichment broth and lithium chloride-phenylethanol-moxalactam). In the current study, however, non-selective media (TSA), as well as *Listeria*-selective media, was used, as suggested by several researchers, since injured cells are not recoverable on selective media (e.g., Farber and Peterkin, 1991; Busch and Donnelly, 1992; Ben Embarek, 1994). Additionally, different inoculation procedures, i.e., surface inoculation vs. injection, and the growth of *L. monocytogenes* in different environmental conditions may cause variation between the results of different studies. Even subtle variations in the specific techniques used may account for differences

in results between studies.

Based on the results obtained in this study, the suggestion that foods should be cooked to an internal temperature of 70°C for 2 minutes (Mackey and Bratchell, 1989) is more than adequate to render ready-to-eat shrimp products safe from *L. monocytogenes*, since a D value of 0.144 min was obtained for this temperature. Thus, a heat treatment of 2 minutes at 70°C would result in a 14 log reduction of *L. monocytogenes*. It is extremely unlikely, however, that *L. monocytogenes* populations would reach such high levels, hence, this suggested heating time may be overestimated and unnecessary. Similarly, the GMP of cooking shrimp to an internal temperature of 80°C is also more than adequate to eliminate *L.monocytogenes* in shrimp. Since the texture and quality of the shrimp may be adversely affected at this temperature, this GMP should be reviewed.

The findings of this study are useful in that they contribute to an understanding of the processing times and temperatures necessary to eliminate *L. monocytogenes* in shrimp, however, they cannot necessarily be applied directly to other food products, for the reasons previously discussed.

In addition, it is not possible to propose regulatory times and temperatures at which the seafood industry should heat process shrimp based on the findings of this study alone for several reasons. First, it was not possible to accurately design a thermal inactivation experiment that simulated plant processing conditions, for reasons already described. Also, in processing plants whole shrimp are cooked by direct contact with live steam. In this study, however, the shrimp was homogenized and placed in small capillary tubes that were heated

in a water bath. This may have influenced the results.

Also, the inoculum levels of *L. monocytogenes* used to contaminate the shrimp in this study likely differ from levels which would be naturally present on the raw materials used by shrimp processing plants. Farber (1991), for example, found levels of *L. monocytogenes* on shrimp to be very low. Accordingly, the inoculum levels used in this study (10^5) likely exceeded natural levels. In addition, based on the results of their study, McCarthy *et al.* (1990) suggest that naturally occurring *Listeria* are more heat-sensitive than experimental *Listeria* due to their growth environment or other unknown factors, which would mean that the *L. monocytogenes* used in this study would be more heat resistant and thus, have a higher survival rate than the naturally occurring bacteria found on shrimp. The use of high inoculum levels of experimental *L. monocytogenes* in this investigation therefore created a somewhat extreme case, which is beneficial since the optimal cooking times and temperatures found in this study to eliminate the pathogen from shrimp would be more than adequate to eliminate the bacteria naturally occurring on shrimp, and would therefore provide a margin of safety. Hence, since shrimp are more likely to be contaminated externally with relatively low levels of *Listeria*, these findings suggest that present cooking methods are adequate to eliminate these organisms from raw shrimp. Post processing contamination is the most likely cause of finding *Listeria* in cooked shrimp (Ryser and Marth, 1991; Farber *et al.*, 1992; Jemmi and Keusch, 1992).

5.5 Water Activity, Salt Content and pH

Listeria's ability to survive in various foods depends on several combined parameters such as temperature, pH, salt content and water activity. *L. monocytogenes* has been reported to survive in up to 25% salt (Ahmed, 1991), thus, is very salt resistant. The average salt concentration of 2.56 % in the shrimp used in this study had no effect on the organism's ability to proliferate. *Listeria* is also capable of growing in a wide pH range from 4.2 to 9.5 (Bonnell, 1994; Farber, 2000; Ross *et al.*, 2000). Hence, the average pH of 6.79 of the shrimp used in this study should have had no influence on the organism. The minimum water activity for *Listeria* growth was recorded at 0.92 (Health and Welfare Canada, 1990; Jemmi and Keusch, 1992). Additionally, Farber (1991) reported a minimum water activity of 0.90 for growth at 30°C. For the shrimp used here, the average water activity was 0.977, hence it should have had no effect on the growth of the organism.

Chapter Six

Conclusions

L. monocytogenes has been isolated from many seafood products, including both raw and ready-to-eat shrimp. The greatest concern is the threat of listeriosis from these ready-to-eat products that do not require further cooking by the consumer. However, this risk can be prevented through an effective heating step and by preventing cross-contamination after heating. Since a critical point in the processing of seafood is the heat processing step, the thermal resistance of *L. monocytogenes* is an area of great interest. With regards to seafood products, however, there are currently very few studies published on the thermal resistance of *L. monocytogenes* in seafood. The availability of accurate and reliable data on the thermal resistance of *L. monocytogenes* is essential in determining and defining the parameters of a safe heat treatment, especially since this and other studies have shown that refrigeration and freezing of the food products will not prevent growth of the pathogen if the heat step was not effective.

Additionally, there is a lack of understanding of the infectious dose of *L.monocytogenes*, to both healthy and susceptible individuals. This information is essential to the setting of tolerance levels, which will also influence the heat treatments needed to reduce *L. monocytogenes* in seafood to acceptable levels. Therefore, research is required in both of these areas, i.e. examining the thermal resistance of *L. monocytogenes* in different seafood products and determining the level of *L. monocytogenes* necessary to cause

listeriosis.

Another issue is the different methodologies used by the various researchers. Research needs to be undertaken to determine a standard method of conducting thermal inactivation trials, as well as recovering and enumerating healthy and heat-stressed *L.monocytogenes,* which will ensure comparability of results. The fundamentals of the protocols should be consistent and well established.

Furthermore, it is difficult to apply data obtained on the thermal resistance of *L.monocytogenes* to processing situations unless the study accurately simulates the processing conditions. If the results are to be used in the design of effective regulations and guidelines to be applied to the food processing industry, the methods used should simulate, as closely as possible, the processing techniques for the specific product under consideration. It would, for example, be more accurate to monitor *L. monocytogenes* in shrimp under actual process conditions, but it may be undesirable to risk working with pathogens under such conditions, as the *L. monocytogenes* could possibly survive and multiply in various areas of the plant, contaminating it.

With respect to the storage component of this study, the findings of this research are quite useful since they provide valuable information on the behaviour of *L. monocytogenes* on shrimp during different storage conditions. For instance, if *L. monocytogenes* is present on shrimp when it is received in a facility, it can grow rapidly when stored at 4°C, which is the usual procedure, or at an abuse temperature of 10°C. Also, the pathogen is able to survive freezing and storage on ice. These results emphasize the concern relating to the presence of

this pathogen in seafood, due to its ability to survive and grow at low temperatures, which were once thought to prevent the growth of pathogens in foods. These results also demonstrate the importance of preventing post-processing contamination of cooked ready-to-eat shrimp, since the bacteria can flourish under refrigeration conditions and survive at freezing temperatures. To do this, a strict sanitation and cleaning program must be developed

Although this concern may seem somewhat unjustified, since no *L. monocytogenes* were detected on raw shrimp in the *L. monocytogenes* incidence experiment, the shrimp may become contaminated during handling or storage and thus, *L. monocytogenes* would be present on the shrimp prior to heat processing. Hence, there is cause for concern and a need to determine effective heat processing protocols for the complete elimination of this pathogen from the shrimp.

The results of this investigation has provided valuable insight into the thermal resistance of *L. monocytogenes* in shrimp. However, there is clearly a need for additional research on this subject, particularly if effective industry regulations and guidelines are to be developed. These guidelines cannot be based on one study alone. Based on the results of the present study, however, it is suggested that an internal temperature of 70°C during 0.72 minutes (43.2 seconds) in processing shrimp will lead to a 5 $\log_{10}$ reduction of *L.monocytogenes*. This is based on the worst case scenario of a 10^5 CFU/g contamination level. Since contamination levels will likely be much less than this, this heat treatment should be more than adequate to ensure a "*Listeria*-free" shrimp product after the heating step.

Moreover, a heat treatment such as this would not adversely affect shrimp texture and quality.

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Appendix 1

Formulations of Media used in the Protocol for the Isolation of *Listeria monocytogenes* from all Food and Environmental Samples and the Protocol for the Enumeration of *Listeria monocytogenes* in Food

Carbohydrate fermentation broth:

Purple broth base	16.00 g
Distilled water	900 mL

***Listeria* enrichment broth (LEB):**

Tryptose	10.00 g
Yeast extract	5.00 g
Beef extract	5.00 g
Sodium chloride	20.00 g
Disodium hydrogen phosphate	9.60 g
Monopotassium phosphate	1.35 g
Esculin	1.00 g
Nalidixic acid	0.02 g
Acriflavin hydrochloride	0.012 g
Distilled water	1000 ml

Lithium chloride-phenylethanol-moxalactum medium (LPM):

Pancreatic digest of casein	5.00 g
Proteose peptone No. 3	5.00 g
Beef extract	3.00 g
Sodium chloride	5.00 g
Lithium chloride	5.00 g
Glycine anhydride	10.00 g
Phenylethanol	2.50 g
Agar	15.00 g
Distilled water	1000 mL

Moxalactum solution:	2.00 mL
Moxalactum	1.00 g
Potassium phosphate buffer, 0.1 M, pH 6.0	100 mL

Fraser secondary enrichment broth base:

Proteose peptone	5.00 g
Casein enzymic hydrolysate	5.00 g
Yeast extract	5.00 g
Beef extract	5.00 g
Sodium chloride	20.00 g
Lithium chloride	3.00 g
Disodium phosphate	12.00 g
Monopotassium phosphate	1.35 g
Esculin	1.00 g
Ferric ammonium citrate	0.50 g
Distilled water	990 mL

Fraser Listeria supplement:
Nalidixic acid (2% solution in NaOH) and
Acriflavin (0.25% solution in distilled water) 10 mL

Motility test medium:

Tryptose	10.00 g
Sodium chloride	5.00 g
Agar	5.00 g
Distilled water	1000 mL

Oxford medium base (OXA):

Columbia blood agar base	39.00 g
Esculin	1.00 g
Ferric ammonium citrate	0.50 g
Lithium chloride	15.00 g
Agar	2.00 g
Distilled water	1000 mL

Oxford Antimicrobic supplements:

Colistin sulfate	20.00 mg
Acriflavin	5.00 mg
Cefotetan	2.00 mg
Cycloheximide	400.00 mg
Fosfomycin	10.00 mg
Ethanol	5.00 mL
Distilled water	5.00 mL

PALCAM (Polymyxin acriflavin lithium-chloride ceftazidime aesculin mannitol) agar (PAL):

Peptic digest of animal tissue	23.00 g
Starch	1.00 g
Sodium chloride	5.00 g
Mannitol	10.00 g
Ammonium ferric citrate	0.50 g
Esculin	0.80 g
Dextrose	0.50 g
Lithium chloride	15.00 g
Phenol red	0.08 g
Agar	13.00 g
Distilled water	1000 mL

Listeria selective supplement (PALCAM):

Polymyxin B	100,000 IU
Ceftazidime	20.00 mg
Acriflavine hydrochloride	5.0 mg
Distilled water	10 mL

Trypticase soy agar with 0.6% yeast extract (TSA-YE):

Trypticase soy agar	40.0 g
Yeast extract	6.0 g
Distilled water	1000 mL

Trypticase soy broth with 0.6% yeast extract (TSB-YE):

Trypticase soy broth	30.0 g
Yeast extract	6.0 g
Distilled water	1000 mL