Control Method for Invasive Aquatic Species introduced via Ballast Water: Effects of Carbon Dioxide Supersaturation on Survivorship of *Digesia tigrina* (Planaria: Maculata) and *Lirceus brachyurus* (Isopoda: Crustacea)

and

Effect of High Hydrostatic Pressure Processing on Freely Suspended and Shellfish Associated T7 Bacteriophage

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(ABSTRACTS)

Control Method for Invasive Aquatic Species introduced via Ballast Water: Effects of Carbon Dioxide Supersaturation on Survivorship of *Digesia tigrina* (Planaria: Maculata) and *Lirceus brachyurus* (Isopoda: Crustacea)

Survivorship of an aquatic species of planaria (*Digesia tigrina*) and isopods (*Lirceus barchyurus*) to elevated levels of carbon dioxide (CO_2) was determined. Both planaria and isopods were exposed to levels of freshwater supersaturated with carbon dioxide, and percent mortality was calculated for various exposure durations, and at various pressure levels. The data collected were graphically analyzed to determine the time necessary to produce mortality in 50% (LT_{50}) of any given sample of specimens tested at a certain pressure level. At 38.6 kPa, 103.4 kPa and 172.4 kPa, the LT_{50} for planaria was calculated to be 150.3 ± 10.1, 58.6 ± 11.1, and 27.8 ± 6.2 minutes, respectively. At 38.6 kPa, 103.4 kPa and 172.4 kPa , the LT_{50} for isopods was calculated to be 181.1 ± 52.5, 79.7 ± 21.9, and 40.5 ± 17.0 minutes, respectively. These results suggest that CO_2 supersaturation may be an easily applied, efficient method that would end the unwanted introduction of nonnative aquatic species to habitats via ballast water released from shipping vessels.

Effect of High Hydrostatic Pressure Processing on Freely Suspended and Shellfish Associated T7 Bacteriophage

The effectiveness of hydrostatic pressure processing (HPP) for inactivating viruses has only been evaluated in a limited number of studies and most of the work has been performed with freely suspended viruses. In this work, the inactivation of freely suspended, as well as shellfish associated bacteriophage T7, by HPP was studied. T7 was selected in hopes that it could potentially serve as a model for animal virus behavior. Both clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) were homogeneously blended separately and inoculated with bacteriophage T7. The inoculated shellfish meat, as well as freely suspended virus samples, were subjected to HPP under the following conditions: 2, 4 and 6 min durations; 241.3, 275.8 and 344.7 Megapascals (MPa) pressure levels; and temperature ranges of 29.4 – 35, 37.8 – 43.3 and 46.1 – 51.7˚C. Plaque forming unit (PFU) reductions of 7.8 $\log_{10}$ (100% inactivation) were achieved for freely suspended T7 at 344.7 MPa, 2 min and 37.8 – 43.3˚C. At 46.1 – 51.7˚C, T7 associated with either clams or oysters was inactivated at nearly 100% (> 4 $\log_{10}$) at all pressure levels and durations tested. The results indicated that T7 is readily inactivated by HPP under the proper conditions, may be protected or made more susceptible by shellfish meat, and may serve as a viable model for the response of several animal viruses to HPP.

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Chapter 1
Literature Reviews

1.1 Literature Review: Control Method for Invasive Aquatic Species introduced via Ballast Water: Effects of Carbon Dioxide Supersaturation on Survivorship of *Digesia tigrina* (Planaria: Maculata) and *Lirceus brachyurus* (Isopoda: Crustacea)

One of the most pervasive and ecologically damaging effects of human activities is the widespread movement of species beyond their natural range. In many countries, 100 -10,000 nonindigenous species have been documented and their numbers are increasing (Lodge 1993). In North America, hundreds of exotic plants and animals have become established in aquatic habitats during this century (Mills et al. 1993*a*, 1996; Cohen and Carlton 1998). These biological invasions will continue to occur, particularly as expanding global trade increases the volume of flora and fauna that is shuttled from one geographic realm to another (Carlton and Geller 1993). Although many of the introduced exotic species do not have any measurable effect on the new environment, it only takes a few species to have detrimental effects. Aquatic species have had success invading ecosystems, and have been recently introduced to various locales throughout the world. The marine shipping industry offers a uniquely efficient method for the transplantation of various marine species: ballast water.

Ships have used water as ballast regularly since the 1880s, drawing ambient water into ballast tanks and floodable holds for balance and stability. The ballast is necessary for a ship's safe operation, and may be taken on and discharged at the port of departure, during the voyage, and at the arrival port. Ballast is defined as any solid or liquid placed in a ship that increases the depth of submergence of the vessel, to change the trim, to maintain stability, or to maintain adequate

stress loads throughout the ship. Ballast water taken aboard may contain planktonic organisms in the water column. Thus, plankton assemblages may be entrained by vessels and then released within days or weeks on a continent or island thousands of kilometers away. Although modern ships may have living organisms on the hull, or in the pumping systems within the ship, ballast water has had a clear and identified role in the spread of nonindigenous species throughout the water. Ships draw ballast water in bays, estuaries, and inland waters and then release this water into ports of call around the world that have similar environments.

Recently, it was estimated that the world's major cargo vessels transfer 8–10 billion tons of ballast water per year (Carlton and Geller 1993), and that on average 3,000 to 4,000 species are transported by ships each day (Carlton and Geller 1993). The invasion of the Asian clam *Potamocorbula amurensis* in San Francisco Bay (Carlton et al 1990), the zebra mussels *Dreissena polymorpha* and *Dreissena sp.* in the Laurentian Great Lakes (Griffiths et al. 1991, 1992), and the comb jelly *Mnemiopsis leidyi* in the Black Sea (Vinogradov et al. 1989) are dramatic examples of the catastrophic impact of ballast water introductions. Knowledge of a potential invasive specie's biology and ecological niche in its natural habitat, does not allow its potential for invasive devastation to be completely predicted (Castri et al. 1990; Drake et al. 1989; Groves 1986; Hengeveld 1989; Kornberg et al. 1986; Mooney 1986). Due to the high rate of introduction of nonnative marine species and the unpredictability of an invasive organisms impacts, bays, estuaries, and inland waters with deep water ports may be considered among the most threatened ecosystems in the world.

Efforts to reduce the risk of future introductions have focused on ballast water management. The U.S. Congress passed P. L. 101-646, The Nonidigenous Aquatic Nuisance Prevention and Control Act of 1990. Many of the provisions directly address ballast water as the primary mechanism for the introduction of exotic invasive species.

Voluntary ballast water exchange (BWE) guidelines were initiated by the Canadian government in 1989 and made mandatory by U.S. legislation in 1993 (United States Coast Guard 1993). The BWE procedure is intended to purge brackish water and freshwater organisms in ballast tanks and to kill those remaining by osmotic stress (United States Coast Guard 1993). Vessels that carry ballast water originating from freshwater or coastal brackish-water ports outside of the Great Lakes and that declare "ballast on board" (BOB) status are subject to these regulations and are requested either to exchange ballast during their ocean voyage or to seal ballast tanks while operating on the Great Lakes (United States Coast Guard 1993).

Legislation covering the Great Lakes was implemented in 1993 by the United States, and effectively mandates that all ships carrying ballast water originating in foreign, freshwater, or brackish water sources conduct ballast water exchange while at least 320 km offshore in water not less than 2000 m depth (United States Coast Guard 1993). This policy was predicated on the concept that virtually all freshwater organisms carried in ballast tanks would be expelled during ballast discharge, while those remaining in residual water would be killed by highly saline water following exchange (Locke et al. 1991, 1993).

It is unlikely that ballast water exchange or risk-based management strategies can fully protect the world's waterways from additional invasions for a number of reasons. First, compliance with the ballast water legislation may not be absolute, and, in addition, some species may survive in ballast tanks either as live organisms or viable resting stages (e.g., Locke et al. 1993; Aquatic Sciences, Inc. 1996). At least one species, *C. pengoi*, has been successfully introduced to Lake Ontario following implementation of the mandatory ballast water exchange program (MacIsaac et al. 1999).

It seems that, even with the current technologies and enforced standards, invading aquatic species will continue to cause extensive ecological damage and economic loss (Hall and Mills 2000; Mack et al. 2000; Ricciardi and MacIsaac 2000). It is even expected that the invaders will be the major drivers of changes in freshwater biodiversity over the next 100 years (Sala et al. 2000).

Ballast tanks are viable habitats for any number of freshwater, brackish or saltwater species. For example, Carlton and Gellar (1993) sampled ballast water from 159 cargo ships in Coos Bay, Oregon. The ships and their ballast water originated from 25 Japanese ports. Plankton from these vessels included 16 animal and 3 protist phyla, and 3 plant divisions. All major and most minor phyla were represented, including 47 ordinal or higher taxa and a minimum of 367 distinctly identifiable taxa. The supraspecific diversity demonstrates the wide taxonomic spectrum represented and emphasizes the broad implications of this phenomenon.

The potential for diversity within the ballast tanks is vast. Nearly all organisms less than 1 cm in size, and adjacent to the vessel can be drawn into the ballast tanks. This includes all naturally swimming organisms, any organisms stirred up from the sediment or rubbed off from the harbors pilings. Of course, the maximum size of the organism depends on the method of ballasting and the size of the intake screens. The pumped water typically passes through intake screens, and the impeller may kill some organisms. However, with gravity ballast systems, no pumps are necessary, which increases the chances of ballasting more organisms.

Although high frequency of release does not necessarily lead to successful invasions, it is thought that there have been far more introductions of polychaetes, flatworms, and diatoms than have been reported. Invasions of intensely studied larger-size animals (such as fish, mollusks, and decapods) are more apparent and thus more noticeable. More invasions of both large and small organisms will be recognized when susceptible regions are investigated and, in addition, new invasions will be discovered in well-studied regions (Carlton and Gellar 1993).

Throughout their sampling, Carlton and Gellar (1993) noted that all major marine trophic groups were represented within ballast tanks. This included carnivores, herbivores, omnivores, deposit feeders, scavengers, suspension feeders, primary producers, and parasites. Taxa characteristic of most temperate shallow-water marine communities were represented, including those from infaunal, soft and hard bottom epifaunal, epibiotic, and planktonic habitats. The ballast biota included meroplankton organisms spending part of their life cycle in the water column), holoplankton (spending all of their lives in the water column), demersal plankton (benthic

species that vertically migrate into the water), and tychoplankton (suspended benthic organisms). Ballast water therefore acts as a phyletically and ecologically nonselective transport vector.

Despite the lack of selectivity, certain taxa predominate. Five phyla accounted for more than 80% of taxa recorded: crustaceans (31% of all taxa present), polychaete annelids (18%), turbellarian flatworms (14%), cnidarians (11%), and mollusks (8%). Taxa found in many or most vessels included copepods (present in 99% of ships), polychaetes (89%), barnacles (83%), bivalve mollusks (71%), flatworms (65%), diatoms (93%), gastropod mollusks (62%), decapod crustaceans (48%), and chaetognaths (47%) (Carlton and Gellar 1993). It is important to note that flatworms were present in 65% of all ballast tanks sampled, and accounted for 14% of all the biota throughout the ballast tanks. *Dugesia tigrina* (planaria), is a common, free living aquatic flatworm that has regenerative capacities. *Dugesia tigrina* (Girard 1850) planaria are flatworms of the phylum Platyhelminthes, order Tricladida, family Planariidae.

The impending threat of continued biological invasion, and the potential devastation of freshwater biodiversity requires a process to control exotic invasive species. Shipboard treatment of ballast water is considered to be the ultimate solution to reducing introduction of nonindigenous aquatic species (NRC 1996). The National Research Council developed criteria to evaluate treatment methods. The two primary criteria for any treatment method are safety and effectiveness. The method must be effective in the inactivation of a broad spectrum of organisms potentially found in ballast water, and the method must be applied in a manner deemed safe for the ship and crew. By first exploring the effectiveness of an alternative

shipboard treatment method, it may be possible to implement a feasible, safe and efficient method to control the introduction of nonindigenous marine species.

1.2 Literature Review: Effect of High Hydrostatic Pressure Processing on Shellfish Associated T7 Bacteriophage

Although numerous foods have been linked to viral disease outbreaks, molluscan shellfish are one of the most prominent sources of viral contamination (Richards 1987). Infectious diseases attributable to the consumption of raw and lightly cooked molluscan shellfish are caused by bacterial agents that are native to the marine environment and by viral and bacterial agents from sewage effluents and other sources that contaminate environmental waters. As filter-feeding organisms, shellfish magnify public health problems associated with environmental contamination because they accumulate microbial pathogens, including viruses, at densities significantly higher than those found in overlying waters. The current public health problems of greatest concern to consumers of molluscan shellfish are associated with viral, and suspected viral, pathogens. The numbers of cases and outbreaks caused by these pathogens far exceed those of all other infectious diseases (Rippey 53). As a result of these viral pathogens, raw shellfish have been implicated in outbreaks of foodborne viral gastroenteritis in the United States, Europe, and Australia (Appleton 1990, Gill et al. 1983, Grohmann et al. 1980, Gunn et al. 1982, Morse et al. 1986, Murphy et al. 1979). Human enteric pathogenic viruses are known to be present in treated wastewater and, as a result, contaminate various waterways, including seawater and shellfish habitats (Melnick and Metcalf 1985).

The association of shellfish consumption and infectious disease has been known or suspected for many years. In 1816, more than 40 years before Pasteur advanced his germ theory of disease, the French physician Pasquier described typhoid fever in a group of people who had consumed oysters harvested from a coastal area contaminated by raw sewage (Fisher 1927, Pasquier 1818). In the United States, infectious bacterial disease associated with molluscan shellfish consumption was first reported in 1894 with two cases of typhoid fever described in Connecticut from shellfish harvested from its coastal waters. No documented cases of infectious disease were reported in the United States before that time, although other types of shellfish-associated illnesses (caused by marine biotoxins) were reported in the late 1700s (McFarren 1960).

An increase in the number of shellfish-associated illnesses after 1900 was the result of urbanization. The construction of storm water or sewerage systems, which began during the mid- to late 1800s in urban centers, resulted in the consolidation of human-derived wastes in collection systems and their eventual release into near coastal environments (Fair et al. 1966). This practice resulted in the progressive contamination of commercial and recreational shellfish-growing areas and outbreaks of enteric disease associated with shellfish harvested from them.

The majority of the world's population is found along the coasts. Often, wastewater is disposed directly or indirectly into coastal waters. Approximately 9.3×10^7 people (37%) of the total U.S. population reside in coastal areas and discharge about 1.0×10^{10} gallons of treated wastewater day^{-1} (National Research Council 1993). In 2000, there were over 11,000 beach closings or advisories (freshwater and marine beaches) in the United States, a number that had almost doubled from the previous year, and a majority of these closings were due to wastewater

pollution (National Research Defense Council 2001). On a global scale, coastal development is twice that of inland sites, with ~90% of the generated wastewater being released untreated into marine waters (Crossette 1996, Henrickson et al. 2001).

Outbreaks resulting from foodborne viral pathogens occur following consumption of shellfish harvested from waters contaminated with human sewage (Gunn et al. 1982, Guzewich and Morse 1986, Richards 1985, Truman et al. 1987). The discharge of viral pathogens in treated sewage is not regulated, and monitoring relies on bacterial indicator detection to predict virus contamination (Griffin et al. 1999). Virus levels in wastewater, measured by cell culture assay, range from 1.82×10^2 to 9.2×10^4 liter^{-1} in untreated sewage and from 1.0×10^{-3} to 1.0×10^2 liter^{-1} in treated wastewater depending on the level of treatment (National Research Council 1993, Rose 1986, Rose et al. 1996). Some of the wastewater that is discharged into the marine environment is only partially treated and is not disinfected (examples of partially treated discharges where only the undissolved solids are removed before release of the sewage effluent from the plant can be found in Los Angeles, San Diego, and Hawaii). Combined sewer overflows, which are systems that receive rainwater and untreated wastewater and overflow during high precipitation events, have been sources of coastal pollution in areas like Puget Sound, Wash. (Rose et al. 1998). Finally, communities with high-density septic tanks (on-site, individual disposal systems) also contribute to poor water quality and increased viral pollution of coastal waters (Griffin et al. 1999, Lipp et al. 2001).

Oysters, clams, and other shellfish filter virus particles from contaminated water and accumulate them in their tissues. Shellfish are harvested from these contaminated waters and are frequently

eaten raw. Human enteric viruses, such as Norwalk-like viruses (Noroviruses), can persist within oyster tissue for extended periods and are considered environmentally stable (Grohmann et al. 1981), creating a foodborne disease. Even at low concentrations, Noroviruses potentially cause a wide range of human illnesses including, paralysis, meningitis, respiratory disease, epidemic vomiting, diarrhea and hepatitis (Anderson and Strenstrom 1987, Fleisher 1998). Noroviruses are the major known etiologic agents of acute nonbacterial gastroenteritis, resulting in an estimated 23,000,000 infections, 50,000 hospitalizations and 300 deaths per year (Mead 1999). In addition, these viruses are a major cause of shellfish-associated disease and may be the most significant cause of adult viral gastroenteritis (Ando et al. 1995, Ando et al. 1997, Dowell et al. 1995, Fankhauser et al. 1998, Hafliger et al. 1997, Honma et al. 2001, Lawson et al. 1991, Linton and Patterson 2000, Lipp and Rose 1997, Lodder et al. 1999, Simmons et al. 2001).

The term “foodborne disease” encompasses a variety of clinical conditions whose etiology may be actively or passively transmitted by food. Food may serve as a medium for many pathogenic or nonpathogenic organisms and in some circumstances may support the growth of the etiologic agent. In many situations, food probably has a passive role and may be defined as a fomite; that is, the etiologic agent(s) does not grow in the food, but is merely transmitted to humans through it. Food, then, may act as a vehicle by transmitting viruses, protozoa, and even some bacteria, which are present but unable to replicate on food, to humans who ingest it. Food unquestionably constitutes the bulk of "foreign" material encountered daily by humans and in many circumstances is an unavoidable, ingestible fomite.

Once the shellfish is contaminated, there are a limited number of treatment options to inactivate infectious viruses within shellfish, while still maintaining the raw, marketable characteristics of the food. Depuration, a process in which live shellfish stock are placed in clean seawater for a period of days, is proven to be incapable of removing a number of enteric viruses (Chironna et al. 2002, Franco et al. 1990, Grohmann et al. 1981, Kingsley and Richards 2003, Sobsey 1987). Irradiation can negatively affect the shellfish taste, appearance and shelf-life (DiGirolamo 1972, Harewood et al. 1994). Cooking is recognized as the only reliable method to inactivate viruses within shellfish meat, however, many consumers prefer the characteristics of raw shellfish.

Recently, hydrostatic pressure processing (HPP) has been used as a rapid, uniform method of inactivating microorganisms in foods (Linton and Patterson 2000). High hydrostatic pressure is viewed as one of the more promising nonthermal methods for inactivating microbes in food (Hayashi 1992, Hover 1997). As an isothermal process, HPP as high as 400 Megapascals (MPa) does not alter the food's taste, odor or texture from its original state (Lopez-Caballero et al. 2000). In addition, the foods require little processing time and final products are additive-free. Presently, HPP is being applied commercially on the U.S. Gulf and West Coasts (Gold Seal Oysters Inc., Homa, LA; Nisbet Oyster Inc., Bay Center, WA) at pressures of 275 MPa.

Several studies suggest that viruses may be susceptible to HPP. Pontes et al. (1997) reported a 10^4 plaque forming unit (PFU) reduction of simian rotavirus in isotonic cell culture media after a treatment of 250 MPa for 30 minutes. Herpes simplex virus type 1 and human immunodeficiency virus type 1 were inactivated at 400 MPa for 10 minutes with 8 $\log_{10}$ PFU and

5.5 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) per/ml being eliminated, respectively (Shigehisa et al. 1996). In addition, feline calicivirus, a norovirus surrogate, can be inactivated by HPP at pressures of 275 MPa (Kingsley et al. 2002), and the San Miguel sealion virus-17, another norovirus surrogate, can also inactivated by HPP and reduced by 0.04, 1.57, 3.35, and > 3.97-$\log_{10}$ PFU/ml, at 200, 250, 275 and 300 MPa, respectively, when pressurized for 1 minute.

However, the full extent of HPP capabilities has yet to be investigated, and requires that many more individual viruses be tested for their susceptibility under varying pressures, times and temperatures. In addition, the effectiveness of HPP on the inactivation of shellfish-associated viruses has not been fully investigated.

Chapter 2
Control Method for Invasive Aquatic Species introduced via Ballast Water: Effects of Carbon Dioxide Supersaturation on Survivorship of *Digesia tigrina* (Planaria: Maculata) and *Lirceus brachyurus* (Isopoda: Crustacea)

2.1 Introduction

One of the most pervasive and ecologically damaging effects of human activities is the widespread movement of species beyond their natural range. Ballast water discharge from vessels in the marine shipping industry is a major pathway for the introduction of invasive marine species (Carlton 1985). It was estimated that the world's major cargo vessels transfer 8–10 billion tons of ballast water per year, and that on average 3,000 to 4,000 species are transported by ships each day (Carlton and Geller 1993). Although many of the introduced exotic species do not have any measurable effect on the new environment, it only takes a few species to have detrimental effects. The invasion of the Asian clam *Potamocorbula amurensis* in San Francisco Bay (Carlton et al. 1990), the zebra mussels *Dreissena polymorpha* and *Dreissena sp*. in the Laurentian Great Lakes (Griffiths et al. 1991, Hebert et al. 1991, May and Marsden 1992), and the comb jelly *Mnemiopsis leidyi* in the Black Sea (Vinogradov et al. 1989) are examples of the specie introductions via ballast water that have had significant negative effects.

Generally, two ballast water management methods are suggested to limit the number of introduced nonindigenous aquatic species. The first method, known as the "voyage approach"

concerns drawing up and discharging ballast water (NRC 1996) at various points throughout the vessel's voyage. It is intended to purge most organisms and kill those remaining with osmotic stress (United States Coast Guard 1993). However, compliance with the ballast water legislation may not be absolute, and, in addition, some species may survive in ballast tanks either as live organisms or viable resting stages (Locke et al. 1993; Aquatic Sciences, Inc. 1996). The second method relies on shipboard treatment options to inactivate stowaway organisms within the ballast water including filtration, biocides, thermal treatments, ultraviolet treatments and even acoustic systems (NRC 1996). However, all of these alternatives have at least one disadvantage according to an extensive paper concerning invasive species introduced via ballast water, written by the National Research Council (1996).

It is apparent that, even with the current technologies and enforced standards, invading aquatic species will continue to cause extensive ecological damage and economic loss (Hall and Mills 2000; Mack et al. 2000; Ricciardi and MacIsaac 2000). It is predicted that the invaders will be the major drivers of changes in freshwater biodiversity over the next 100 years (Sala et al. 2000). However, by exploring alternative shipboard treatment methods, it may be possible to implement a safe and efficient method to control the introduction of nonindigenous marine species.

Shipboard treatment of ballast water is considered to be the ultimate solution to reducing the introduction of nonindigenous aquatic species (NRC 1996). The purpose of this study was to evaluate the effectiveness of carbon dioxide supersaturation as a shipboard treatment method for inactivation of a surrogate model of an invasive species. Previous studies prove the effectiveness of CO_2 as an inactivation method for various species of mollusks (McMahon et al 1995). We

intend to determine the effectiveness of CO_2 to inactivate both a flatworm specie and an isopod specie.

The invasion of an aquatic flatworm specie is conceivable. Carlton and Gellar (1993) noted the prominence of flatworms throughout their sampling of ballast tanks from 159 cargo ships in Coos Bay, Oregon; flatworms were present in 65% of all ballast tanks sampled, and accounted for 14% of all the biota in the ballast tanks. In this study, *Digesia tigrina*, a common flatworm, as well as *Lirceus barchyurus*, a common isopod, were placed within bench-scale ballast tanks and pressurized with carbon dioxide at 38.6, 103.4 and 172.4 kPa for various durations. The effectiveness of inactivation was evaluated by determining the LT_{50} at each pressure.

2.2 Materials and Methods

This study was conducted at the United States Geological Survey Science Center (LSC), in Leetown, West Virginia, U.S.A.

2.2.1 Experimental Setup

The experimental bench apparatus (Figure 1) consisted of five 3 L cylindrical hyperbaric chambers. Freshwater from the spring source at LSC circulated at approximately 19 L • min^{-1}, maintained by a Dywer rotameter, through a closed loop that included each of these five

cylinders, as well as a packed tower that was used to introduce CO_2 into the water. Pressure settings were controlled by a regulator on the gas supply lines that service the trickling column. Each of the five holding cylinders could be isolated from the circulating loop and depressurized, which allowed the remaining cylinders to maintain pressure for continued testing. A McMaster-Carr cooling coil (part no. 35113K21), with a 0.2 m^2 surface area, maintained the temperature within the closed loop at approximately 15˚C. A Cole-Palmer inline electrode with probe guard (part no. P-27301-21) was used to measure pH and temperature. A Cole-Palmer polargraphic oxygen electrode (part no. 53202-00) was used to measure oxygen saturation and oxygen concentration. Figure 1 shows a schematic of the testing apparatus and indicates the relative location of the cylinders, regulators and gas column. Two separate, identical benches were used throughout the experiment to facilitate efficiency in testing.

Twenty planaria specimens and at least 10 isopods specimens were tested per cylinder at various replicated parameters. The planaria specimens were placed in a fine mesh bag that was sealed with a stainless steel clamp. The isopods were placed in a stainless steel mesh ball, identical to those used in brewing single cups of tea. One mesh bag filled with 20 planaria and one mesh ball filled with at least 10 isopods were placed in each of the five cylinders at each of the two benches. Each of the two devices allowed complete circulation of the supersaturated water to the specimens. The first cylinder at each bench was isolated from the circulating water loop and gas concentrations, and was maintained as a control. Each of the remaining four cylinders was subjected to pressure levels at 38.6 kPa, 103.4 kPa, and 172.4 kPa. One of the remaining four cylinders during the 38.6 kPa was depressurized at 110 min, the second cylinder was depressurized at 140 min, the third at 170 min, and the last cylinder at 200 min. Cylinders

throughout the 103.4 kPa trials were depressurized at 30 min, 60 min, 90 min, and 120 min. At 172.4 kPa the vessels were depressurized at 20 min, 40 min, 60 min, and 80 min. The varied durations of exposure provide enough data to determine the LT_{50} using probit analysis procedures for each pressure setting.

1,000 planaria were tested at 38.6 kPa, 800 at 103.4 kPa and 800 at 172.4 kPa. Since a minimum of 10 isopods were tested in each vessel during each trial, at least 500 isopods were tested at 38.6 kPa, 300 at 103.4 kPa and 400 at 172.4 kPa. Upon depressurization, the specimens were removed from the holding cylinders and allowed to recover in fresh spring water for 60 minutes. Generally, specimens were observed for signs of movement after the recovery period using a 5x sterio microscope. Duration increments at each pressure level were determined by trial and error, and were selected due to trial results prior to testing that produced mortality levels above and below 50% for planaria only. Since a probable LT_{50} was established for only planaria prior to testing, the isopod specimens were added to the testing vessels without any expectation for inactivation.

2.2.2 Planaria Specimens

One of the two species tested in this experiment was *Dugesia tigrina* (Girard 1850), a free living aquatic flatworm (planaria) that has regenerative capacities. *Dugesia tigrina* are flatworms of the Phylum Platyhelminthes, Order Tricladida, Family Planariidae. Species were collected at the Leetown Science Center (LSC) from an abandoned fishway that had a limited inflow of degassed

springwater. The planaria were maintained in a wet laboratory at the LSC that had access to the same natural freshwater source. Test specimens had a mean wet weight of approximately 6.1 mg (N = 39) and a length of 13.5 mm ± 2.7 mm.

2.2.3 Isopoda Specimens

The second specie tested in this experiment was *Lirceus Brachyurus* (Harger 1876), a free living aquatic organism that is usually found in the substrate of a slow moving or stagnant water body. *Lirceus Brachyurus* (isopoda) are isopods of the Phylum Arthropoda, Order Isopoda, Family Asellidae. Species were collected and maintained by the same methods as the planaria specimens. Test specimens had a mean wet weight of approximately 12.5 mg (N = 30) and a length of 9.8 mm ± 2.2 mm.

2.2.4 Statistical Methods

Testing consisted of at least eight trials for planaria and at least six trials for isopods at all pressure levels and durations. Percent mortality, as a result of each trial, was calculated for each cylinder. The values for percent mortality were converted to a probit scale and plotted versus a logarithmic scale of time (minutes). Table 1 shows the values for percent mortality and the probit value equivalent. The LT_{50} for each data set was determined by using the mean and standard deviation of the data set. A linear regression provided an equation to determine the

LT_{50} for each trial, an example is shown as Figure 2. The equation was then used with the probit value that coincided with each LT_{50}. Since the distribution of data was not Gaussian, the LT_{50} was not always equivalent to a probit value of 5.0. Once a LT_{50} value was obtained for each trial, the overall LT_{50} value for each pressure setting was determined by averaging the LT_{50} values that had a correlation of at least 0.77 (Tables 2-7). Correlation values above 0.77 were chosen to reduce variance of data, and maintain at least 4 points of data for each specimen at any given pressure.

2.3 Results and Discussion

Carbon dioxide has many advantages as an inactivating agent. It is a highly soluble gas (Figure 3), and its solubility insures that most of the toxicant reaches the target organism to induce gas bubble disease. In addition, the Occupational Safety and Health Administration has considerable higher limitations for air quality regarding the concentration of carbon dioxide, when compared to other gases such as chlorine or ozone that may also be considered as an inactivating agent. Carbon dioxide is typically viewed as a waste product in many processes, and because of this, it may be assumed that carbon dioxide will be available in large quantities at a relatively lower cost, compared to chlorine or ozone.

Other studies have determined CO_2 to be an inactivating agent for aquatic organisms, but most concentrate on the inactivation mollusk species. In one study, Elzinga and Butzlaff (1994) reported inactivation concentrations for Asian clam and zebra mussels. McMahon et al. (1994)

also subjected zebra mussels and Asian clams to hypercapnic, anoxic and normoxic water at 25°C and demonstrated that by elevating Pco_2 to 10.0 kPa complete mortality of zebra mussels results (LT_{50} = 78h). These data suggest carbon dioxide alone or in combination with other gases can be used as an environmentally neutral molluscicide. However, few other studies have considered the invasive threat of other aquatic organisms, and no studies have shown CO_2 inactivation data for planaria and isopods.

Besides mollusks, any number of species have the potential to invade. Carlton and Gellar (1993) noted throughout their study, that flatworms were present in 65% of all ballast tanks sampled, and accounted for 14% of all the biota throughout the ballast tanks. The results in this study show that, if implemented, the use of CO_2 as an onboard treatment of ballast water has the potential to inactivate multiple organisms, besides various mollusk species. Table 8 shows that 50% of the population of planaria can be inactivated in less than 30 minutes (172.4 kPa). During the recovery period after testing, it was noted that the majority of inactivated planaria were not completely intact. After testing, the once recognizable black flatworm had been distorted into a mass of mucous and body parts. In contrast, inactivated isopod specimens were visually observed under a microscope to be intact (although many inactivated isopod specimens had an altered color: isopods had a pale brown color prior to testing, and an orange-brown coloration after testing). This may suggest that the body structure and composition of flatworms may be more susceptible to gas bubble disease.

In this study, LT_{50} results were calculated from performing between 6 – 10 trials, depending on the specie. For planaria at 38.6 kPa, 10 trials were conducted, however only 8 trials were used

(based on a correlation rate above 0.77) to calculate the LT_{50}. The final LT_{50} value of 150.2 min, is the result of data used from the testing of 800 planaria specimens. At 103.4 kPa, 8 trials were conducted (800 planaria specimens), and the data from all of the trials was used to calculate the final LT_{50} value of 59.1 min. At 172.4 kPa, 8 trials were conducted and 7 were used (corresponding to 700 planaria specimens) to calculate the final LT_{50} value of 27.8 kPa. For isopods at 38.6 kPa, 10 trials were conducted, however only 4 trials were used (200 isopods) to calculate the final LT_{50} value of 181.1 min. At 103.4 kPa, 6 trials were conducted, and 4 trials (200 isopod specimens) were used to calculate the final LT_{50} value of 79.7 min. At 172.4 kPa, 8 trials were conducted and 5 were used (corresponding to 250 isopod specimens) to calculate the final LT_{50} value of 40.5 kPa.

The results indicate that CO_2 is an effective agent for the inactivation of the selected planaria and isopods, and may prove to be an effective method for the reduction of other exotic species. Although its effectiveness should be directly tested on numerous exotic organisms, the data collected in this study reveal that CO_2 at 172.4 kPa achieved nearly complete inactivation of planaria and isopods in less than 100 minutes. Table 9 shows slight changes in the characteristics of the water contained within the control cylinders. Although the specimens were exposed to these changes, no mortalities were recorded throughout the controls. This is an indication of the experimental apparatus's ability to reduce external stresses on the specimens, and indicates that inactivation of the specimens was the result of CO_2 supersaturation only. Overall, the data for isopods may be limited by the availability of specimens, but the results displayed in Table 8 support the hypothesis that an increase in CO_2 saturation will decrease the time for inactivation of both species.

It should be noted once again, that the experimental design was developed by methods that would determine the LT_{50} of planaria, not isopods. Despite this, inactivation of isopods was still observed within the same time frame as planaria (based on standard deviations) and this is revealed in Figure 4. Isopod LT_{50} is consistently higher than that of planaria, but within approximately 30 minutes at 38.6 kPa, app. 20 min. at 103.4 kPa, and 15 minutes at 172.4 kPa. By testing multiple species at the same time, and within the same experimental parameters, the capabilities of CO_2 as an inactivating agent are further revealed.

The relatively large standard deviations (Table 8), as well as the data that lacked significant correlation (Tables 5 – 7), associated with isopod species may be the result of a combination of two possibilities. First, it may be the result of less inhibiting parameters within the experimental design. If the experiment intended to inactivate isopods as its primary focus, then increased pressure levels or increased time of exposure may have been more appropriate. (It should be noted that the isopod specimens, although not inactivated, displayed narcotized behavior during the recovery period. As a result of any movement within the specimens, the results were recorded as "not inactivated.") The large standard deviations may also be the result of the ability of the isopod specie to resist an overall inactivation by CO_2. This possibility suggests that other organisms be selected based on their potential to invade via ballast water and be tested by the same methods with different experimental parameters.

The impending threat of continued biological invasion, and the potential devastation of freshwater biodiversity demands a mechanism to control exotic invasive species. It seems that,

even with the current technologies and enforced standards, invading aquatic species will continue to cause extensive ecological damage and economic loss (Hall and Mills 2000; Mack et al. 2000; Ricciardi and MacIsaac 2000). With specie invasions predicted to be the major drivers of changes in freshwater biodiversity over the next 100 years (Sala et al. 2000), and considering that the shipboard treatment of ballast water is considered to be the ultimate solution to reducing introduction of nonindigenous aquatic species (NRC 1996), it is imperative to develop an effective shipboard control method. First, the effectiveness of an alternative shipboard treatment method for inactivation of various species must be proven. Once potential invaders are identified, and tested for their susceptibility of inactivation, it may be possible to implement a feasible, safe and efficient method to control the introduction of nonindigenous marine species.

2.4 Conclusions

1. LT_{50} for both planaria and isopods occurs approximately within 3 hours when supersaturated with CO_2 at 38.6 kPa.

2. LT_{50} were reduced for planaria and isopods when CO_2 pressure levels were increased.

3. Isopod specimens (*Lirceus brachyurus*) generally have a longer LT_{50} than planaria (*Digesia tigrina*).

4. Planaria (*Digesia tigrina*) may be more susceptible to gas bubble disease.

5. CO_2 supersaturation can inactivate multiple organisms that are tested collectively within the same vessel.

2.5 Figure and Table Legends

Fig. 2.1. Experimental apparatus

Fig. 2.2. Equation obtained from the first trial of the planaria at 38.6 kPa. Each data point corresponds to a hyperbaric chamber and the percent mortality observed for the planaria specimens within the vessels.

Fig. 2.3. The concentration of CO_2 based on pH, according to the Henderson-Hasselbach equation.

Fig. 2.4. A comparison between planaria and isopods. The figure represents the relation between CO_2 pressure levels and LT_{50} for each specie.

Table 2.1. Data obtained from the ten trials for planaria at 38.6 kPa. The probit values were then plotted versus log time to obtain the corresponding LT_{50}.

Table 2.2. LT_{50} values for planaria trials when tested at 38.6 kPa. The equation is derived from the data points of the trial and is used by substituting in the probit value (5.07) that coincides with the LT_{50} value.

Table 2.3. LT_{50} values for planaria trials when tested at 103.4 kPa. The equation is derived from the data points of the trial and is used by substituting in the probit value (4.79) that coincides with the LT_{50} value.

Table 2.4. LT_{50} values for planaria trials when tested at 172.4 kPa. The equation is derived from the data points of the trial and is used by substituting in the probit value (4.24) that coincides with the LT_{50} value.

Table 2.5. LT_{50} values for isopod trials when tested at 38.6 kPa. The equation is derived from the data points of the trial and is used by substituting in the probit value (5.64) that coincides with the LT_{50} value.

Table 2.6. LT_{50} values for isopod trials when tested at 103.4 kPa. The equation is derived from the data points of the trial and is used by substituting in the probit value (4.79) that coincides with the LT_{50} value.

Table 2.7. LT_{50} values for isopod trials when tested at 172.4 kPa. The equation is derived from the data points of the trial and is used by substituting in the probit value (4.62) that coincides with the LT_{50} value.

Table 2.8. LT_{50} values at each pressure level for each specie.

Table 2.9. Characteristics of the control cylinders measured before testing and after the final cylinder was depressurized. The control cylinder were measured for 38.6 kPa, 103.4 kPa, and 172.4 kPa trials at 80 minutes, 120 minutes and 200 minutes, respectively.

2.6 Figures and Tables

Figure 2.1

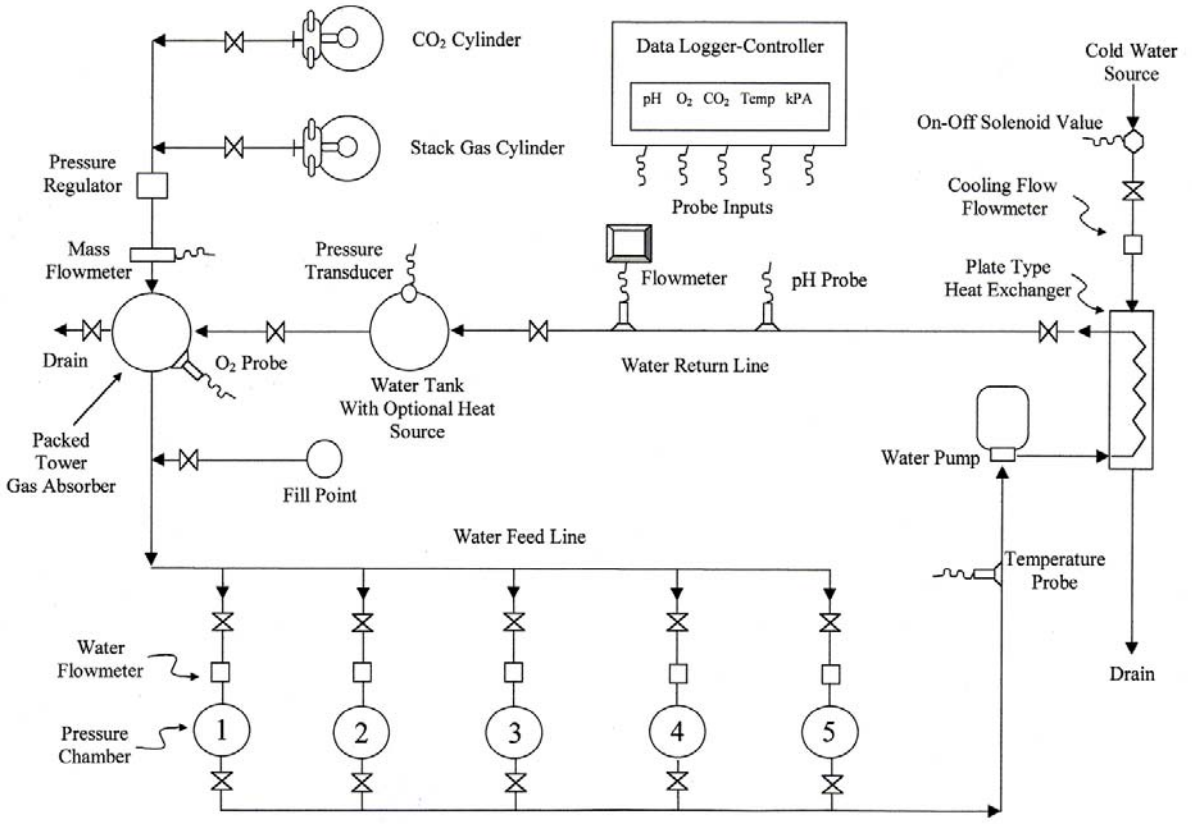


Figure 2.2

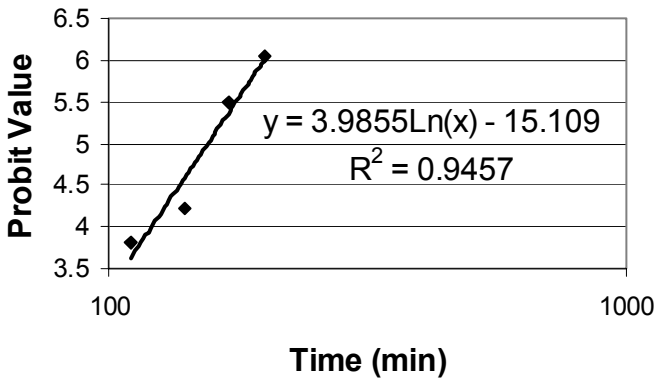

6.5
6
5.5
5
4.5
4
3.5
Probit Value
y = 3.9855Ln(x) - 15.109
R2 = 0.9457
100
1000
Time (min)

Figure 2.3

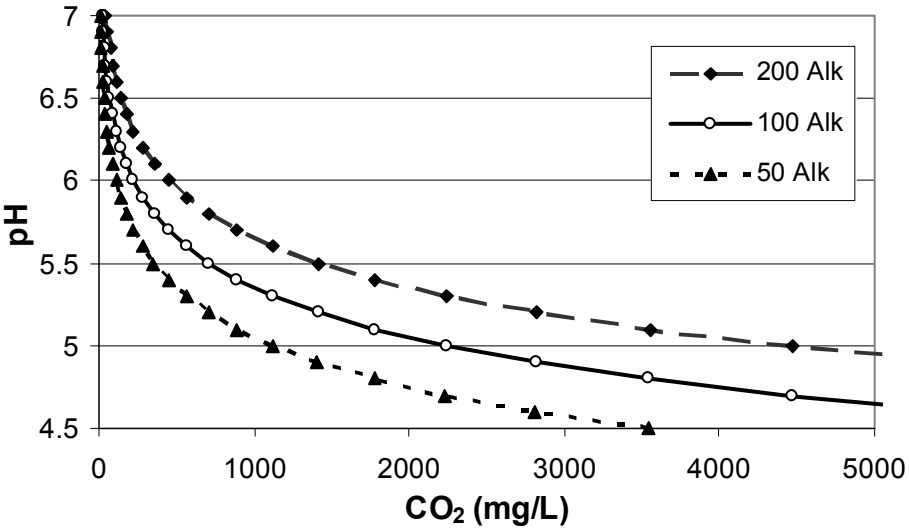

7
6.5
6
5.5
5
4.5
pH
0
1000
2000
3000
4000
5000
CO2 (mg/L)
200 Alk
100 Alk
50 Alk

Figure 2.4

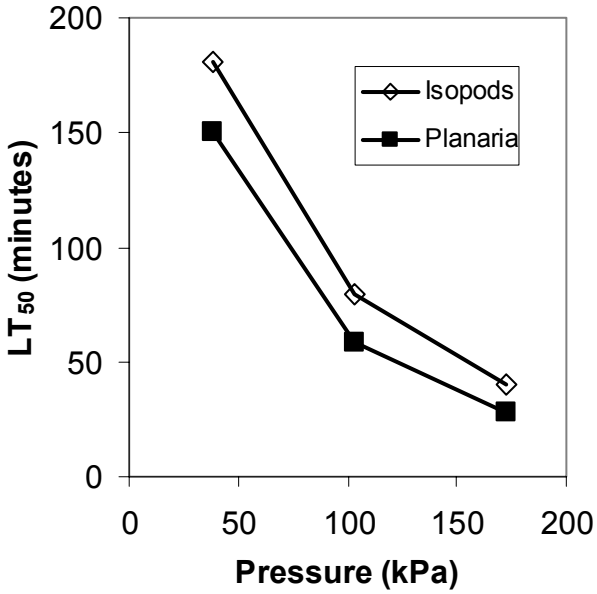

200
150
100
50
0
LT50 (minutes)
0
50
100
150
200
Pressure (kPa)
Isopods
Planaria

Table 2.1

Trial	Time (min)	Mortality (%)	Probit Value	Trial	Time (min)	Mortality (%)	Probit Value
1	110	5.0	3.80	**6**	110	5.0	3.80
	140	20.0	4.23		140	55.0	5.21
	170	65.0	5.49		170	85.0	6.06
	200	85.0	6.06		200	100.0	6.48
2	110	0.0	3.66	**7**	110	5.0	3.80
	140	35.0	4.65		140	5.0	3.80
	170	60.0	5.35		170	65.0	5.49
	200	95.0	6.34		200	95.0	6.34
3	110	5.0	3.80	**8**	110	5.0	3.80
	140	25.0	4.37		140	15.0	4.08
	170	60.0	5.35		170	20.0	4.23
	200	80.0	5.92		200	75.0	5.77
4	110	10.0	3.94	**9**	110	20.0	4.23
	140	5.0	3.80		140	30.0	4.51
	170	40.0	4.79		170	75.0	5.77
	200	90.0	6.20		200	90.0	6.20
5	110	10.0	3.94	**10**	110	15.0	4.08
	140	20.0	4.23		140	75.0	5.77
	170	70.0	5.63		170	90.0	6.20
	200	95.0	6.34		200	100.0	6.48
	Mean[a]	47.5					
	St. Dev[b]	35.52					
	LT_{50}[c]	5.07					

[a]Mean value for all percent mortality

[b]Standard deviation value for all percent mortality

[c]The probit value that corresponds to the LT_{50}, based on the data

Table 2.2

Trial No.	LT_{50}[a] at 38.6 kPa (min)	Equation	(R^2)[c]
1	158.01	3.9855Ln(x) - 15.109	0.95
2	153.71	4.3589Ln(x) - 16.877	0.99
3	160.28	3.6507Ln(x) - 13.464	0.98
4	152.55	4.2376Ln(x) - 16.234	0.92
5	141.04	4.5248Ln(x) - 17.323	0.98
6	158.45	4.5446Ln(x) - 17.95	0.85
7	146.85	3.5628Ln(x) - 12.706	0.92
8	131.11	3.9379Ln(x) - 14.131	0.90
Mean	150.25		0.93
(S.D.)[b]	10.06		0.05

[a] LT_{50} coincides with a probit value of 5.07

[b] Standard deviation

[c] R^2 values less than 0.77 were not included in the calculation of LT_{50}

Table 2.3

Trial No.	LT_{50}[a] at 103.4 kPa (min)	Equation	(R^2)[c]
1	57.26	1.714Ln(x) - 2.1461	0.90
2	54.17	1.7818Ln(x) - 2.3218	0.91
3	59.26	1.5784Ln(x) - 1.6497	0.90
4	56.00	1.7004Ln(x) - 2.0529	0.93
5	60.07	2.1684Ln(x) - 4.089	0.90
6	43.08	1.4245Ln(x) - 0.5688	0.79
7	80.25	1.721Ln(x) - 2.7555	0.78
Mean	58.58		0.87
(S.D.)[b]	11.11		0.06

[a] LT_{50} coincides with a probit value of 4.79

[b] Standard deviation

[c] R^2 values less than 0.77 were not included in the calculation of LT_{50}

Table 2.4

Trial No.	LT_{50}[a] at 172.4 kPa (min)	Equation	(R^2)[c]
1	30.08	1.8826Ln(x) - 2.1707	0.96
2	22.89	1.3483Ln(x) + 0.0159	0.98
3	33.77	2.2379Ln(x) - 3.6389	0.96
4	31.40	2.001Ln(x) - 2.6601	0.96
5	29.28	1.9557Ln(x) - 2.367	0.92
6	31.16	2.0717Ln(x) - 2.8875	0.84
7	15.89	1.1114Ln(x) + 1.1563	0.95
Mean	27.78		0.94
(S.D.)[b]	6.23		0.05

[a] LT_{50} coincides with a probit value of 4.24

[b] Standard deviation

[c] R^2 values less than 0.77 were not included in the calculation of LT_{50}

Table 2.5

Trial No.	LT_{50}[a] at 38.6 kPa (min)	Equation	(R^2)[c]
1	162.58	5.2416Ln(x) - 21.042	0.86
2	158.24	5.7172Ln(x) - 23.309	0.77
3	144.56	5.3712Ln(x) - 21.071	0.78
4	259.02	2.0302Ln(x) - 5.638	0.96
Mean	181.10		0.84
(S.D.)[b]	52.51		0.09

[a] LT_{50} coincides with a probit value of 5.07

[b] Standard deviation

[c] R^2 values less than 0.77 were not included in the calculation of LT_{50}

Table 2.6

Trial No.	LT_{50}[a] at 103.4 kPa (min)	Equation	(R^2)[c]
1	62.99	2.4072Ln(x) - 4.5721	0.99
2	109.59	1.0476Ln(x) + 0.4809	0.85
3	63.78	1.4644Ln(x) - 0.684	0.94
4	82.24	1.998Ln(x) - 3.4093	0.80
Mean	79.65		0.89
(S.D.)[b]	21.85		0.09

[a] LT_{50} coincides with a probit value of 4.79

[b] Standard deviation

[c] R^2 values less than 0.77 were not included in the calculation of LT_{50}

Table 2.7

Trial No.	LT_{50}[a] at 172.4 kPa (min)	Equation	(R^2)[c]
1	15.91	1.0572Ln(x) + 1.6925	0.78
2	53.63	1.264Ln(x) - 0.4159	0.89
3	58.94	1.4756Ln(x) - 1.3979	0.94
4	39.99	2.0749Ln(x) - 3.0365	0.77
5	33.90	2.0281Ln(x) - 2.5285	0.96
Mean	40.47		0.87
(S.D.)[a]	17.04		0.09

[a] LT_{50} coincides with a probit value of 4.24

[b] Standard deviation

[c] R^2 values less than 0.77 were not included in the calculation of LT_{50}

Table 2.8

			CO_2 Pressure		
Specimen			(kPa)		
	38.6		103.4		172.4
Planaria	150.3		58.6		27.8
S.D.[a]	10.1		11.1		6.2
Isopods	181.1		79.7		40.5
S.D.[a]	52.5		21.9		17.0
[a] Standard deviation					

Table 2.9

		Before			After		
	0_2 ppm	8.7	±	0.7	7.7	±	0.5
38.6 kPa	0_2 %sat.	84.7	±	7.1	83.9	±	5.2
	Temp ˚C	15.2	±	0.8	20.8	±	0.8
	02 ppm	9.4	±	1.2	8.47	±	0.6
103.4 kPa	02 %sat.	91.8	±	12.2	91.9	±	5.5
	Temp ˚C	15.5	±	0.8	20.06	±	1.0
	02 ppm	7.8	±	0.4	7.6	±	0.3
172.4 kPa	02 %sat.	77.1	±	3.8	82.1	±	3.7
	Temp ˚C	15.5	±	0.9	19.5	±	1.0

Chapter 3
Effect of High Hydrostatic Pressure Processing on Freely Suspended and Shellfish Associated T7 Bacteriophage

3.1 Introduction

Although numerous foods have been linked to viral disease outbreaks, molluscan shellfish are one of the most prominent sources of viral contamination (Pontes et al. 1997). Human enteric pathogenic viruses are known to be present in treated wastewater and, as a result, contaminate various waterways, including seawater and shellfish habitats (Melnick 1985). Shellfish are harvested from these contaminated waters and are frequently eaten raw. Human enteric viruses, such as Norwalk-like viruses (Noroviruses), are not completely depurated after 48 hours and are considered environmentally stable (Grohmann 1981). Even at low concentrations, these viruses potentially cause a wide range of human illnesses including, paralysis, meningitis, respiratory disease, epidemic vomiting, diarrhea and hepatitis (Anderson and Strenstrom 1987, Fleisher et al. 1998). Noroviruses are the major known etiologic agents of acute nonbacterial gastroenteritis, resulting in an estimated 23,000,000 infections, 50,000 hospitalizations and 300 deaths per year (Mead et al. 1999).

Hydrostatic pressure processing (HPP) has been used as a rapid, uniform method of inactivating microorganisms in foods (Linton and Patterson 2000). High hydrostatic pressure is viewed as one of the more promising nonthermal methods for inactivating microbes in food (Hayashi 1992, Hoover 1997). As an isothermal process, HPP as high as 400 MegaPascals (MPa) (58,015 psi) generally does not alter the food's taste, odor or texture from its original state (Lopez-Caballero

et al. 2000). In addition, the foods require little processing time and final products are additive-free. The process is currently being commercially used in the United States at pressures up to 275 MPa (Gold Seal Oysters Inc., Homa, LA; Nisbet Oyster Inc., Bay Center, WA) to facilitate the shucking process and to reduce populations of spoilage bacteria (He et al. 2002). However, based on reviews of current published studies, only a limited number of viruses have been tested for inactivation under high pressure. In addition, a study of HPP effectiveness has not been conducted to determine the comparative efficiency of inactivating a virus associated with two different shellfish species.

Several studies suggest that viruses may be susceptible to HPP. Pontes et al. (1997) reported a 10^4 plaque forming unit (PFU) per ml reduction of simian rotavirus in isotonic cell culture media after a treatment of 250 MPa for 30 minutes. Also, herpes simplex virus type 1 and human immunodeficiency virus type 1 were inactivated at 400 MPa for 10 minutes with 8 $\log_{10}$ PFU and 5.5 $\log_{10}$ 50% tissue culture infectious doses per ml being eliminated, respectively (Shigehisa et al. 1996). In addition, feline calicivirus, a norovirus surrogate, can be inactivated by HPP at pressures of 275 MPa (Kingsley et al. 2002). It has also been demonstrated that San Miguel sealion virus-17 (SMSV-17), another norovirus surrogate, is susceptible to inactivation by HPP. In oyster homogenate, SMSV-17 titer was reduced by 0.04, 1.57, 3.35, and > 3.97 $\log_{10}$ PFU/ml, at 200, 250, 275 and 300 MPa, respectively, when pressurized for 1 minute (Calci et al. 2002). In addition to viruses, it has also been demonstrated that HPP can effectively inactivate 6 strains of the pathogenic bacterium, *Vibrio*, tested at 200 – 300 MPa for 5 to 15 minutes at 25˚C (Berlin et al. 1999).

To determine the effectiveness of HPP for viral inactivation, T7 bacteriophage (or phage) was evaluated in this study. It is hoped that the T7 bacteriophage will serve as a reasonable surrogate for animal viruses. Experiments were conducted with freely suspended phage in aqueous solutions and phage associated with shellfish (oysters and clams). The independent variables considered were pressure, duration of applied pressure, and temperature. In each temperature range, a sample was tested at each of the three durations and three pressure levels.

3.2 Material and Methods

3.2.1 Experimental Design

The experimental matrix for pressure, temperature and duration, provided in Table 1, was repeated three times, once for freely suspended virus samples, and once each for virus samples with oyster meat and clam meat. In addition, four trials were replicated to confirm results. Samples were tested in the high pressure processing unit (model 35L-600, Quintus Food Press, having a capacity of 35 L and a maximum pressure setting of 600 MPa). Additional elution experiments were also conducted to show the extent of viral association with the two types of shellfish. A comparison of results revealed the effects of each of the following parameters: temperature, pressure, duration and extent of viral association with the shellfish. Duration of exposure does not include the typical 1 minute required by the high pressure processing unit to reach the desired pressure level.

3.2.2 Microbe Propagation

Escherichia coli ATCC 11303 was cultivated and used for the propagation the bacteriophage T7 ATCC 11303-B7. *E. coli* was propagated using a nutrient broth (Difco 234000) plus 0.5% NaCl. After an incubation period of 12-18 hours at 35˚C, approximately 1.5 L of the broth inoculated with *E. coli* was then inoculated with T7. After 4-6 hours, the supernatant was withdrawn and filtered through a 0.22 μm Millipore filter. Both *E. coli* and T7 were separately maintained in plastic Isc Bioexpress, Snap Seal Microcentrifuge tubes (1.7 ml) at -80˚C in 1.5 ml aliquots.

3.2.3 Shellfish Preparation

Oysters (*Crassostrea virginica* from York River, Virginia) and hard shell clams (*Mercenaria mercenaria*: Cherrystone Aquafarms, Plantation Creek, Virginia) were shucked. The meat of approximately 35 of each shellfish was blended separately in a laboratory blender (model 31BL91; Waring, New Hartford, Conn.) at the high setting for 3 min. Twenty gram aliquots of each shellfish were then frozen in glass vials at -80˚C for future use. The blended solution of oysters had a measured total solids of 16%, a salinity of 0.8 ppt, and a pH of 6.6. The blended solution of clams had a total solids of 13%, a salinity of 1.4 ppt, and a pH of 6.8.

3.2.4 Sample Preparation

After thawing, 20 g of the shellfish was mixed for 1 hour with 180 ml of distilled water and 1 ml of the virus stock (about 4 x 10^8 PFU/ml) . This suspension represented the control stock for test comparisons. Approximately 10 ml of the inoculated shellfish solution was placed in a 3 mm, standard barrier, nylon/PE vacuum pouch and heat sealed (model A 300/16, Multivac Sealer). The freely suspended virus samples were prepared by diluting 1 ml of virus stock in 100 ml of distilled water, and were packaged for HPP testing using the same methods.

3.2.5 Sample Analysis

Processed samples were serially diluted and analyzed using the soft agar overlay technique (Adams 1959) in 100 x 15 mm petri dishes. The nutrient agar base was composed of Difco nutrient agar 213000 (2.3%) and 0.5% NaCl, and the soft agar overlay consisted of Difco nutrient agar (1.0%) and nutrient broth (Difco 234000) (0.7%). The completed petri dishes were incubated at 37˚C, and plaques were counted after 4 hours. Plaque counts of samples were compared to the plague counts of the controls, and percent inactivation and log reduction calculations were used to quantify inactivation of virus populations.

3.2.6 Percent Association

The distribution of viruses between meat solids and the aqueous solution (percent association) was evaluated for samples containing shellfish meat. The sample was prepared by combining 20 g of the shellfish, 180 ml of distilled water and 1 ml of virus stock. The shellfish/viral suspension was centrifuged at 10,000 x *g* at 4˚C for 5 min (DuPont Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant was plated, and the pellet was resuspended with 10 ml of distilled water and plated. In addition, the supernatant of another centrifuged sample was plated, and the pellet was resuspended with 10 ml of a glycine buffer, pH 9.5 (0.1 M glycine, 0.3 M NaCl) to further facilitate elution of the virus. The percent association and percent recovery was calculated using a control for each centrifuged sample. The percent association was calculated by dividing the number of viruses in the pellet by the number of viruses in the mixture prior to centrifugation. It was also calculated by dividing the number of viruses in the pellet by the sum of the viruses assayed in both the supernatant and the pellet. The procedure was duplicated to confirm the results.

3.3 Results and Discussion

Table 1 shows experimental parameters that produced 27 data points based on the various combinations of exposure time, pressure and temperature. Freely suspended, oyster associated and clam associated viruses were studied in this test matrix. Percent adsorption was also calculated for each shellfish meat using viral extraction methods described by Kingsley and Richards (2001). Table 2 shows elution results for clams and oysters. Typically, clams exhibited 7 – 8% viral adsorption, whereas oysters adsorbed 16 – 18% of the viruses. Nearly

100%, of the viruses were recovered in these experiments for both clams and oysters. Results from using glycine failed to prove more effective than elution with distilled water.

Inactivation of T7 were reported in terms of log PFU reduction and percent inactivation (Tables 3 – 5 and Fig. 1-3). Although there were a few exceptions (e.g. freely suspended T7 response at 275.8 MPa, 4 min, 29.4 – 35.0˚C), the general trends were that increases in virus inactivation were seen with increases in temperature, time of exposure and pressure. For example, the data in Table 3 reveal that freely suspended T7 was not inactivated at the lowest pressure, for any of the times of exposure, and the lowest temperature. Whereas, all of the viruses were inactivated at the highest pressure, middle and high temperature range, and all durations considered. In between these extremes, there is a gradient of T7 response to the various test variables.

Studies have been conducted to evaluate the effectiveness of HPP on various shellfish to extend shelf life and inactivate infectious microbes (He et al. 2002, Lopez-Caballero et al. 2000), however, a direct comparison of the association propensity of viruses to two different shellfish meat, and the effects of HPP based on the different shellfish has not been thoroughly documented. When oyster tissue was introduced, keeping in mind that the viral sorption level was in the area of 16 – 18%, overall inactivation trends were similar to those seen in the freely suspended T7 trials. However, it seemed that the oyster meat promoted inactivation of the viruses at the lower temperature range and pressures. As the temperature and pressure were increased, a threshold was reached where 100% of the viruses were inactivated in both the freely suspended and oyster associated systems. This is interesting because it was originally thought

that the oyster meat might afford the viruses some protection throughout all experimental conditions.

In the clam associated experiment, as in the freely suspended T7 experiments, little to no viral inactivation was seen at the lowest pressure and temperature. However, as the temperature was increased at the lowest pressure, the clam solids seemed to enhance inactivation of T7 somewhat. The effect was not as great as seen when contrasting freely suspended with oyster associated viruses, but then again, less virus associated with clam tissues (7 – 8%) than oyster tissue. At the middle temperature range and middle pressure, clam associated T7 seemed to be somewhat more protected than freely suspended T7. Some protection was also noted at the highest pressure considered.

Thus, the role that shellfish tissue might play in protecting viruses is more complex than originally thought. Under some conditions, inactivation will be enhanced and in other cases, the virus might be protected. The mechanism of inactivation therefore becomes very important and requires further study. In any event, it was demonstrated that 100% of T7, in all the systems studied, can be inactivated in 4 min, at 344.7 MPa and 46.1 – 51.7˚C. The results of this study also revealed that HPP can effect 7.8 $\log_{10}$ PFU/ml reductions (100.00% inactivation) for freely suspended T7 phage at 344.7 MPa for 2 min between the temperatures of 37.8 – 43.3˚C (Table 3).

As noted earlier, only a few studies have shown the potential of HPP to inactivate viruses. For example, Kingsley et al. (2002) demonstrated that hepatitis A suspended in cultured media was

susceptible to HPP. In other studies, investigators have demonstrated the potential of HPP to inactivate simian rotavirus (Pontes et al. 1997), herpes simplex virus type 1 and human immunodeficiency virus type 1 (Shigehisa et al. 1996) feline calicivirus (Kingsley et al. 2002), San Miguel sealion virus-17 (Calci et al. 2002), and *Vibrio* bacteria (Berlin et al. 1999, Cook 2003). Thus, much more research is needed to fully appreciate the response of viruses to HPP.

As previously mentioned, the Noroviruses, feline calcivirus and SMSV-17, were inactivated using HPP. It is difficult to compare the results of our study with what is reported in the literature. The conditions were not the same and/or temperatures were not reported by the other authors. However, some parallels in the data can be drawn. For instance, feline calcivirus was inactivated at 275 MPa (Kingsley et al. 2002) and, in this study, freely suspended T7 was quite susceptible to inactivation at 275.8 MPa (>98%) when the temperatures were above 37.8˚C (Table 3). In addition, SMSV-17 was reduced by >3.97 $\log_{10}$ PFU/ml at 300 MPa when pressurized for 1 min (Calci et al. 2002). At the higher two ranges of temperatures, it seems reasonable that T7 levels would be reduced by 4 logs or more by 300 MPa (Table 3). More recently, a study has shown that hepatitis A virus (HAV), when associated with oysters, can be inactivated by HPP. PFU reductions of >1 $\log_{10}$ were observed for 1 min treatments at 8.7 – 10.3˚C at 350 MPa (Calci et al. 2005). In this study, at 2 min exposure to 344.7 MPa and 29.4 – 35.0˚C produced a 1.6 $\log_{10}$ reduction of T7 associated with oyster. Although few studies have been published and the experimental conditions cannot be matched exactly, T7 now appears to be a reasonable surrogate model for the HPP processing of animal viruses.

Unlike the experiments in this study, performed with shucked and blended product, typical commercial high pressure units process whole clams and oysters. Typically, viruses would become associated to the product via live uptake and, as a result, might exist at higher or more concentrated levels within various parts of the bivalve. Despite the documentation that states that fat content, salt concentration and other characteristics of solid foods can affect HPP inactivation rates (San Martin et al. 2002), one would expect inactivation rates to be similar between blended and whole product, given the uniform application of hydrostatic pressure. However, the fact that lower inactivation rates for the shellfish associated T7 (when compared to freely suspended T7) were noted under certain conditions, indicates that further investigation is needed to verify the hypothesis that shucked and blended products will behave similarly under HPP.

3.4 Conclusions

The conclusions derived from this study are as follows:

1. T7 bacteriophage exhibited 7 – 8% association with the blended meat of clams, and 16 – 18% association with the blended meat of oysters.

2. Despite having a lower association percentage, T7 associated with clam was generally more protected from HPP than T7 associated with oyster. And, it seemed that the oyster meat promoted inactivation of the viruses at the lower temperature range and

pressures. Whereas, enhancement of T7 inactivation in the clam experiments was only noted as the temperature was increased at the lower pressure considered (241.3 MPa).

3. Except for a few instances, inactivation of T7 increased when either temperature, time of exposure or pressure was increased.

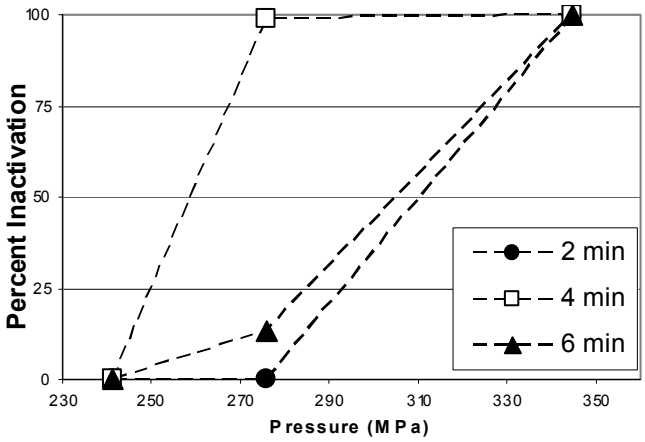
4. At 46.1 – 51.7˚C, T7 associated with either clams or oysters can be inactivated at nearly 100% at all pressure levels (241.3, 275.8 and 344.7 MPa) and durations (2, 4 and 6 min) tested.

5. Although few studies have been published and the experimental conditions cannot be matched exactly, T7 appears to be a reasonable surrogate model for the HPP processing of animal viruses.

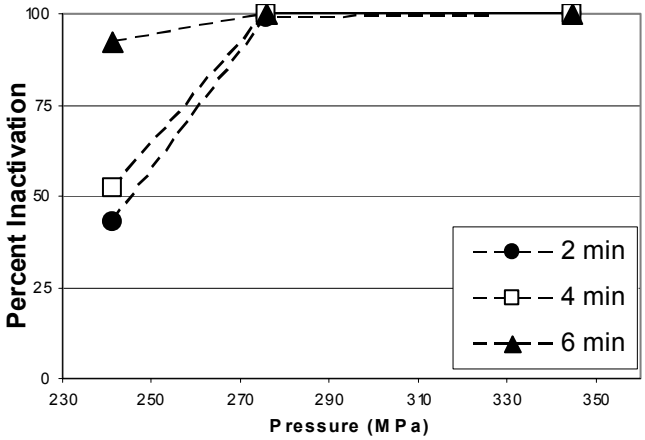
Figures 3.5

Figure 3.1.

a)



b)



c)

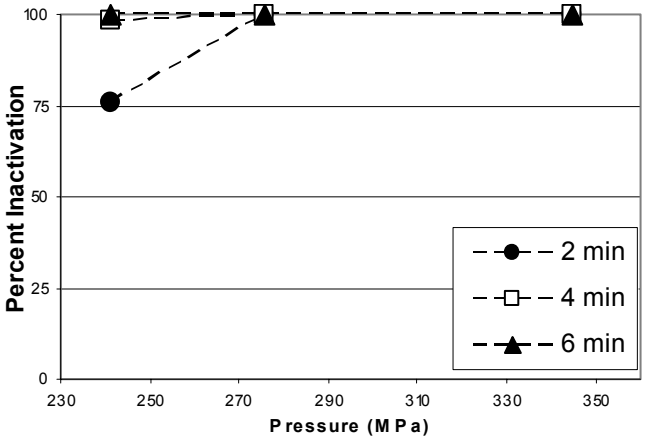
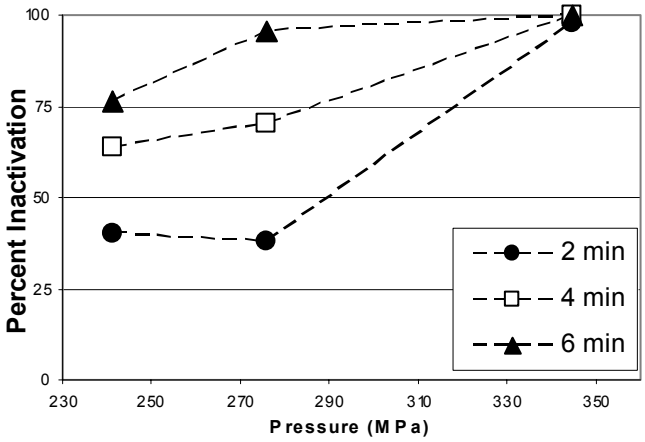
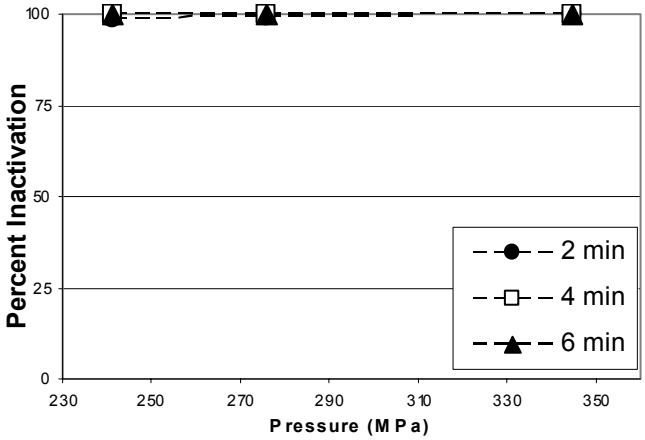


Figure 3.2.

a)



b)



c)

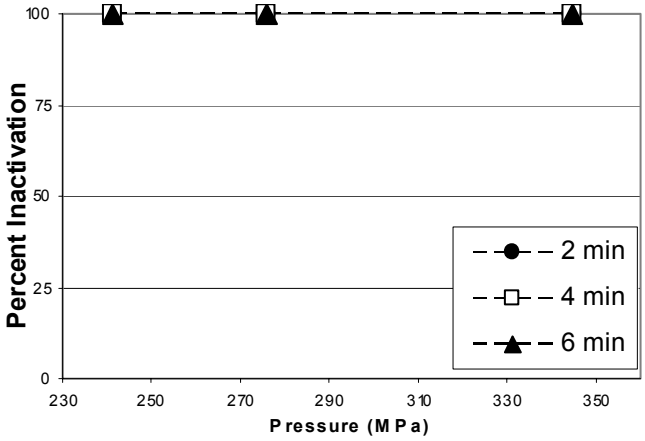
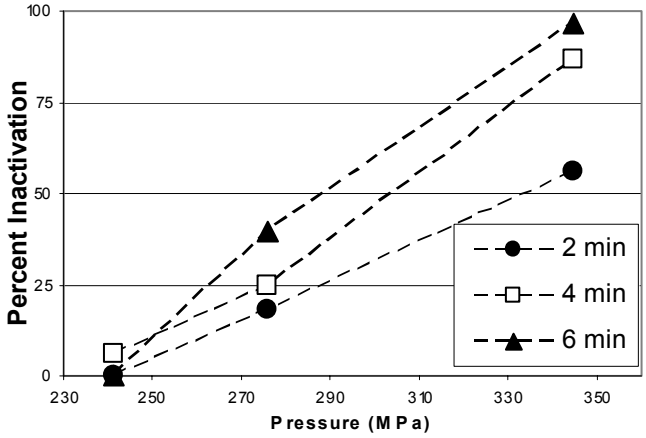
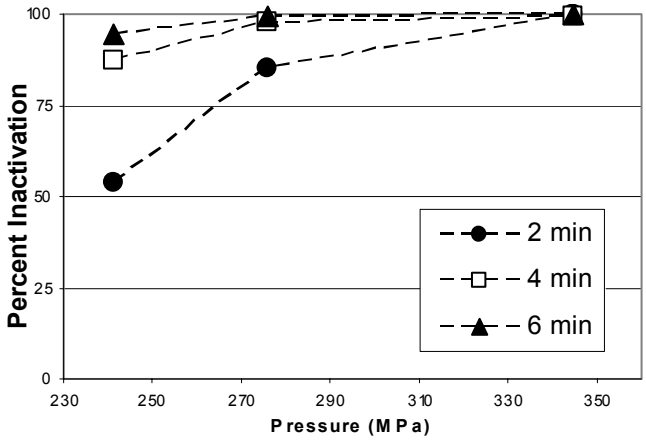


Figure 3.3

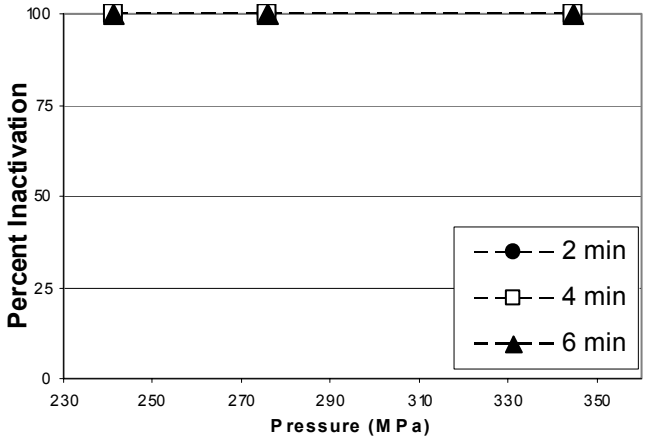
a)



b)



c)



3.6 Figure Legends

Figure 3.1.

Inactivation of freely suspended T7 at a) 29.4 – 35.0 ˚C, b) 37.8 – 43.3 ˚C and c) 46.1 – 51.7 ˚C.

Figure 3.2.

Inactivation of T7 associated with the meat of oysters at a) 29.4 – 35.0 ˚C, b) 37.8 – 43.3 ˚C and c) 46.1 – 51.7 ˚C.

Figure 3.3.

Inactivation of T7 associated with the meat of clams at a) 29.4 – 35.0 ˚C, b) 37.8 – 43.3 ˚C and c) 46.1 – 51.7 ˚C.

3.7 Tables

Table 3.1

TABLE 1. Duration of applied pressure in minutes at the three pressure and three temperatures ranges considered

Pressure (MPa)	Temperature Range (˚C)		
	29.4 - 35.0	37.8 - 43.3	46.1 - 51.7
	2 [1]	2	2
241.3	4	4	4
	6	6	6
	2	2	2
275.8	4	4	4
	6	6	6
	2	2	2
344.7	4	4	4
	6	6	6

[1]Duration of exposure in minutes

Table 3.2

TABLE 2. T7 adsorption to shellfish based on Aqueous and Glycine Elutions							
Shellfish	Association (%), Aqueous Eluted		Association (%), Glycine Eluted		Recovery(%)	
	Method 1[a]	Method 2[b]	Method 1[a]	Method 2[b]	Aqueous	Glycine
Clam	7.6	7.4	7.2	7.5	103.1	94.8
(S.D.)[c]	0.2	0.5	2.1	1.9	10.6	3.4
Oyster	17.6	16.2	16.5	16.9	112.7	101.0
(S.D.)[c]	2.6	3.5	3.7	6.3	40.2	15.5

[a] Calculated by dividing the concentration of the centrifuged pellet by the concentration of the control

[b] Calculated by dividing the concentration of the pellet by the sum concentration of the pellet and supernatant

[c] Standard deviation based on duplicates

Table 3.3

TABLE 3. Inactivation of freely suspended T7 reported by log reduction and percent inactivation							
Pressure	Duration	Temperature Range (˚C)					
(MPa)	(min)	29.4 - 35.0		37.8 - 43.3		46.1 - 51.7	
	2	0.0[a]	0.00[b]	0.2	42.92	0.6	75.85
241.3	4	0.0	0.00	0.3	51.97	1.7	98.10
	6	0.0	0.00	1.1	92.48	3.6	99.98
	2	0.0	0.00	1.9	98.76	2.9	99.87
275.8	4	1.9	98.66	3.2	99.93	3.8	99.98
	6	0.1	13.33	4.6	100.00	6.9	100.00
	2	2.1	99.28	7.8	100.00	6.9	100.00
344.7	4	3.9	99.99	7.1	100.00	6.7	100.00
	6	4.9	100.00	7.1	100.00	6.9	100.00
[a] Log reduction							
[b] Percent inactivation							

Table 3.4

TABLE 4. Inactivation of oyster associated T7 reported by log reduction and percent inactivation

Pressure	Duration	Temperature Range (˚C)					
(MPa)	(min)	29.4 - 35.0		37.8 - 43.3		46.1 - 51.7	
	2	0.2[a]	40.00[b]	1.9	98.87	5.9	100.00
241.3	4	0.4	63.78	3.0	99.89	5.8	100.00
	6	0.6	76.38	3.5	99.97	5.9	100.00
	2	0.2 / 0.3[c]	30.00 / 46.71[c]	1.9 / 2.5[c]	98.69 / 99.79[c]	5.9	100.00
275.8	4	0.5	70.45	3.5	99.97	5.8	100.00
	6	1.3	95.38	3.8	99.98	5.9	100.00
	2	1.6	97.68	4.9	100.00	5.9	100.00
344.7	4	2.8	99.85	5.5	100.00	5.8	100.00
	6	4.4	100.00	5.9	100.00	5.9	100.00

[a] Log reduction

[b] Percent inactivation

[c] Multiple values indicate that the data were experimentally replicated

Table 3.5

TABLE 5. Inactivation of clam associated T7 reported by log reduction and percent inactivation

Pressure	Duration	Temperature Range (˚C)					
(MPa)	(min)	29.4 - 35.0		37.8 - 43.3		46.1 - 51.7	
	2	0.0[a]	0.00[b]	0.3	53.95	5.9	100.00
241.3	4	0.0 / 0.1[c]	0.00 / 12.11[c]	0.9	87.54	5.8	100.00
	6	0.0	0.00	1.2	94.36	5.8	100.00
	2	0.1	18.38	0.8	84.98	5.0	100.00
275.8	4	0.0 / 0.3[c]	0.00 / 49.03[c]	1.6	97.74	5.8	100.00
	6	0.2	39.37	2.3	99.51	5.4	100.00
	2	0.4	56.29	2.9	99.89	4.0	99.99
344.7	4	0.9	86.94	2.5	99.67	5.0	100.00
	6	1.5	96.72	3.3	99.95	5.8	100.00

[a] Log reduction

[b] Percent inactivation

[c] Multiple values indicate that the data were experimentally replicated

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