

THE EFFECTS OF DIFFERENT TEMPERATURES ON THE GROWTH OF *VIBRIO CHOLERAE*, TOTAL VIABLE MICROORGANISMS AND HYDROGEN SULPHIDE (H₂S) PRODUCING BACTERIA

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ABSTRACT

The aim of this project is to study the effects of different temperatures on growth of *Vibrio cholerae*, total viable microorganisms and hydrogen sulphide (H₂S) producing bacteria. The study was conducted in two phases, in the first phase (experiment I) a sample of minced redfish was contaminated with *V. cholerae* and stored at different temperatures of -1.5 °C, 0 °C, 2 °C, 4 °C, 8 °C and 17 °C respectively. In this experiment *V. cholerae* was not detected. In the second phase (experiment II), 1 kg of redfish mince was contaminated with 1 ml of pure strains of *V. cholerae* and stored at 0 °C, 8 °C, 17 °C, 22 °C, 25 °C and 30 °C. Only *V. cholerae* was measured in this study. For *V. cholerae* two procedures were used; alkaline peptone water (APW) enrichment and direct inoculation of thiosulphate citrate biles salts sucrose agar (TCBS). Superchilled storage improved the shelf-life of minced redfish by 6 – 10 days compared with traditional chilled mince at 0 °C. *V. cholerae* entered a viable but nonculturable state during storage time at low temperature but did grow well at high temperatures. However, higher numbers of *V. cholerae* counts were detected in APW enrichment than direct inoculation of TCBS agar. At high storage temperatures, bacterial growth was much faster than at low storage temperatures.

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1 INTRODUCTION

Mozambique is situated on the eastern coast of southern Africa and borders Tanzania in the north, Malawi and Zambia in the northwest, Zimbabwe and South Africa in the west and Swaziland and South Africa in the south. The coastline is about 2470 km, bathed by the Indian Ocean. Mozambique has a land area of 786,380 km², of which 13,000 km² are inland waters. The climate in Mozambique is inter-tropical. The population is estimated at 20.5 million according to the 2007 census, the capital is Maputo with the official language Portuguese (INE). Mozambique is endowed with many natural resources among them are the hydroelectric power, gas, coal, minerals, timber and land for agriculture. The main products for export are shrimp, cotton, cashew nuts, sugar, tea and dried coconut.

The coastline can be divided into three regions. The northern region characterised by corals and rocks, the central sandy region and the southern region composed mainly of dunes. Partly, this division determines the distribution and potential of the marine ecosystems, their access and influence on the choice of both fishing methods and equipment (Lopes 2003).

Fishing is a very important activity as it provides local employment. In addition, it is an important source of animal protein, and brings valuable foreign income. The main marine resources are crustaceans (prawns, lobsters and crabs), pelagic and demersal fish and marine algae (Lopes 2003). Mozambique exports its products to the EU, Japan, China and some African countries like Malawi, Zimbabwe and South Africa. Every day consumers are becoming more demanding and good services and product quality is needed to compete, especially in the international market.

1.1 Laboratory of fish inspection

The National Institute of Fish Inspection, which is subordinate to the Ministry of Fisheries, has done a great job at the industrial level and artisanal fishery in ensuring the quality of fishery products. For this purpose it has introduced international standards, such as the codex alimentarius and ISO. The Laboratory of Fish Inspection forms part of the National Institute of Fish Inspection. Its role is to support the system of fish inspection and provide services to the industry. This helps the companies monitor their own quality system.

1.1.1 Mission

The Laboratory of Fish Inspection is designated to support the Competent Authority in inspection of fish and fishing and fish processing industries. In addition, it is responsible for studying and developing new analytical techniques in the field of quality control of fishery products. The laboratory is involved in projects for training of university students and maintains a technical-scientific exchange on a national level and research projects in the area of fishery products. The laboratory is equipped for chemical, microbiological and sensorial analyses. The approach of quality in the Laboratory of Fish Inspection is based on the adoption and implementation of a quality system in accordance with ISO 17025. Analyses are based on internationally recognised methods. Internal audits are carried out and the laboratory participates in proficiency tests in order to evaluate its performance.

1.2 Product quality

Shelf-life can be defined as the period of time during which a perishable product under a given appropriate set of conditions can be stored or displayed until it is considered as

unsuitable for sale or consumption. Shelf-life is highly variable among different types of food products and several quality and safety parameters determine its values. These parameters can be divided into the categories of microbiological, chemical and sensory properties (Duun *et al.* 2008).

Freshness is one of the most important aspects of fish quality because consumers have a strong tendency to select very fresh fish (Cyprian *et al.* 2008). The extension of shelf-life of fresh fish and fishery products is therefore of importance to allow transport of products to distant markets. Shelf-life of fishery products is usually limited by microbial activities that are influenced most importantly by storage temperature (Cyprian *et al.* 2008). Some microbes are a part of the natural flora of fish, but may negatively affect the quality of products if they find good growth conditions. It is therefore important to control and prevent the microbial growth during handling, processing and storage. To verify the presence or absence of microorganisms it is necessary to do a proper laboratory analysis.

Among the several pathogenic microorganisms that can affect the quality of the fish is *Vibrio spp.* The causative agent of cholera, *Vibrio cholerae*, is a water-borne bacterium that is a natural inhabitant of brackish aquatic environments. Cholera is an acute infection caused by the colonisation and multiplication of *V. cholerae* O1 or O139 within the human small intestine. People contract cholera when they ingest an infective dose of *V. cholerae* from contaminated water or food. Many developing countries still endure frequent outbreaks due to the lack of basic sanitation services and clean water (Emch *et al.* 2008). A total of 131,943 cases, including 2272 deaths, were reported from 52 countries during 2005 (WHO 2006) and the low level of personal hygiene was the most implicated cause in cholera epidemics. In Mozambique cholera is a public health problem. Different strains of *Vibrio* occur in city sewage which is drained into the sea, and also in industrial parks.

To fight spoilage and pathogenic microorganisms in fish products, it is not enough to have a good laboratory; one also needs to have the technical expertise that can carry out analysis in accordance with international standards. To meet the series of requirements, provision of adequate facilities, validated methods and calibration of equipment is needed in addition to skilled technicians capable of performing the analysis with precision. The aim of the present study was to analyse the effects of different temperature on growth of *V. cholerae*, total number of viable microorganisms (TVC) and H₂S-producing bacteria in fish mince.

2 OBJECTIVES

- To study growth of *V. cholerae* in minced redfish at different temperatures and different storage times.

To observe the growth of total viable microorganisms and H₂S-producing bacteria in minced redfish at different temperatures during storage.

3 LITERATURE REVIEW

It is well known that both enzymatic and microbiological activities are greatly influenced by temperature. However, in the temperature range from 0 to 25 °C, microbiological activity is relatively more important and temperature changes have greater impact on microbiological growth than on enzymatic activity (Huss 1995).

Cholera infections vary greatly in frequency, severity and duration and the endemic of cholera in different parts of the world is dynamic. Cholera is firmly endemic in some areas such as the South Asian countries of Bangladesh and India where cholera infections occur every year. In contrast, other regions such as parts of South America and Africa have historically had only sporadic epidemics (Emch *et al.* 2008).

3.1 The bacterium

3.1.1 Description of genus

Members of the genus *Vibrio* are defined as Gram-negative, asporogenous rods that are straight or have a single, rigid curve. They are motile; most have a single polar flagellum when grown in a liquid medium. Most produce oxidase and catalase and ferment glucose without producing gas. Three species, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are well-documented human pathogens. *V. mimicus* is a recognised pathogen with similar characteristics to *V. cholerae*, except an ability to ferment sucrose (Table 1). Other species within the genus, such as *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. metschnikovii* and *V. hollisae* are occasional human pathogens. Most vibrios are sensitive to the vibriostatic agent 0129 which is used as a diagnostic test (Igbinosa *et al.* 2008).

Table 1: Different characteristics of the species most often associated with human illness related to seafood consumption (Kaysner *et al.* 2004)

TABLE 1. Biochemical characteristics of human pathogenic Vibrionaceae commonly encountered in seafood*									
	<i>V. alginolyticus</i>	<i>V. cholerae</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. hollisae</i>	<i>V. metschnikovii</i>	<i>V. mimicus</i>	<i>V. parahae-molyticus</i>	<i>V. vulnificus</i>
TCBS agar	Y	Y	Y	Y	NG	Y	G	G	G
mCPC agar	NG	P	NG	NG	NG	NG	NG	NG	Y
CC agar	NG	P	NG	NG	NG	NG	NG	NG	Y
AGS	KA	Ka	KK	KK	Ka	KK	KA	KA	KA
Oxidase	+	+	+	+	+	-	+	+	+
Arginine dihydrolase	-	-	+	+	-	+	-	-	-
Ornithine decarboxylase	+	+	-	-	-	-	+	+	+
Lysine decarboxylase	+	+	-	-	-	+	+	+	+
Growth in (w/v):	0% NaCl	-	+	-	-	-	+	-	-
	3% NaCl	+	+	+	+	+	+	+	+
	6% NaCl	+	-	+	+	+	+	-	+
	8% NaCl	+	-	V	+	-	V	-	+
	10% NaCl	+	-	-	-	-	-	-	-
Growth at 42°C	+	+	V	-	nd	V	+	+	+
Acid from:	Sucrose	+	+	+	+	-	+	-	-
	D-Cellobiose	-	-	+	-	-	-	V	+
	Lactose	-	-	-	-	-	-	-	+
	Arabinose	-	-	+	+	+	-	-	+
	D-Mannose	+	+	+	+	+	+	+	+
	D-Mannitol	+	+	+	+	-	+	+	V
	ONPG	-	+	+	+	-	+	+	+
Voges-Proskauer	+	V	-	-	-	+	-	-	
Sensitivity to:	10 µg O/129	R	S	R	R	nd	S	S	R
	150 µg O/129	S	S	S	S	nd	S	S	S
	Gelatinase	+	+	+	+	-	+	+	+
	Urease	-	-	-	-	-	-	-	V

* Adapted from Elliot *et al.* (31)** *Aeromonas hydrophila*, *Plesiomonas shigelloides*

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; mCPC, modified cellobiose-polymyxin B-colistin; AGS, arginine-glucose slant;

Y = yellow NG = no or poor growth S = susceptible nd = not done

G = green V = variable among strains R = resistant P = purple, V = variable

KK = Slant alkaline / Butt alkaline KA = Slant alkaline /Butt acidic, Ka = Slant alkaline/ Butt slightly acidic

3.1.2 *V. cholerae*

V. cholerae was first described as the cause of cholera by Pacini in 1854. *V. cholerae*, the type species of the genus *Vibrio*, is the causative agent of cholera outbreaks and epidemics. Various biochemical properties (Table 1) and antigenic types characterise it. It can be differentiated from other *Vibrio* species, except *V. mimicus*, by its obligate requirement for sodium ion (Na⁺) which can be satisfied by the trace amounts present in most media constituents. *V. cholerae* can survive in an environment with a pH ranging from 6 to 11 (Le

Roux 2006). It grows in the presence of relatively high levels of bile salts. It is a facultative anaerobe and grows best under alkaline conditions. *Vibrio* grows in temperatures between 10°C and 43°C, but grows fast at the higher temperature. Pathogenic *V. cholerae* produces a heat-sensitive enterotoxin that causes the characteristic cholera symptoms, including “rice water stool”. The species comprises several somatic (O) antigen groups, including O-group-1, which is associated with classical and El Tor biotypes. *V. cholerae* O1 may have several serotypes, including Inaba, Ogawa, and Hikojima. *V. cholerae* non-O1 also can cause gastrointestinal disease, though typically less severe than that caused by *V. cholerae* O1. Serotype O139 is an exception, and produces classic cholera symptoms. This serotype was first identified in 1992 as the cause of a new epidemic of cholera in India and Bangladesh. Non-O1 *V. cholerae* is found more readily in estuarine waters and seafood in the United States than is the O1 serogroup, however, the O139 serogroup has not yet been found. Because O139 can grow in media lacking sodium chloride, it is not considered a halophilic *Vibrio*, although traces of sodium are required for growth (FDA 1995). Cholera enterotoxin (CT) is the primary virulence factor of the disease cholera (Kaysner *et al.* 2004) and as little as 5µg cholera toxin (CT) administered orally caused diarrhoea in humans volunteers (Huss 1994). Not all *V. cholerae* O1 strains produce cholera toxin (Le Roux 2006).

3.1.3 *Vibrio cholerae* in the environment

Environmental control of growth and persistence of vibrios in the aquatic environment is poorly understood even though members of the genus *Vibrio* are globally important pathogens (Eiler *et al.* 2007). For example, warm water temperatures appear to have a positive effects on the abundance of human-invasive pathogens, which tend to have mesophilic growth optima. *V. cholerae* inhabit seas, estuaries, brackish waters, rivers and ponds of coastal areas of the tropical world. They flourish in dense organic matter, algae and zooplankton of the Ganges delta and similar ecosystems. Environmental parameters such as salinity, temperature, nutrients and solar radiation influence the survival and proliferation of *Vibrio spp.* directly by affecting their growth and death rates and indirectly through ecosystem interactions. The survival of the contaminant *Vibrio spp.* in water environments has been shown to decrease with elevated sunlight, high salinity and increased temperature. However, elevated nutrients and particle associations have been shown to promote the survival of water body contaminants (Igbinsosa *et al.* 2008). There is increasing evidence that many pathogens found as pollutants in water body environments can survive harsh environmental conditions for prolonged periods of time in a spore-like, “viable but non-culturable” (VBNC) state (Igbinsosa *et al.* 2008). Bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity (Oliver 2005). When the bacteria are exposed to adverse conditions of salinity, temperature or nutrient deprivation, they can be reversibly injured and they cannot be detected by standard bacteriological methods. However, when given optimal conditions they can return to normal “culturable” state (Huss 1994).

Major cholera outbreaks are recorded in eastern African nations including Djibouti, Kenya, Mozambique, Somalia, Uganda and Tanzania where the majority of outbreaks occur following rainfall and/or floods (Emch *et al.* 2008).

3.1.4 Cholera

V. cholerae O1 is excreted in great numbers in the faeces of cholera patients and convalescents. The disease is transmitted primarily by the faecal-oral route, indirectly through

contaminated water supplies. Direct person-to-person spread is not common (FDA 2004). Food supplies may be contaminated by the use of human faeces as fertilizer or by freshening vegetables for market with contaminated water and the consumption of vegetables badly washed which is the more common route of contamination in developing countries. The regions of eastern and southern Africa face one of the lowest coverage of water and sanitation in the world, according to the latest report by UNICEF Progress for Children: Water, Sanitation and Objectives of Development of the Millennium. It is also stated in the document that Madagascar, Malawi and Mozambique are the three countries of the region showing the greatest progress in the provision of access to facilities for improved sanitation between 1990 and 2004 and the full coverage of water in Mozambique is 43% (UNICEF 2006). Cholera outbreaks in several countries, including the USA, are thought to have resulted from the consumption of raw, undercooked, contaminated or recontaminated seafood (Kaysner *et al.* 2004).

The incubation period of cholera can range from several hours to five days, and is dependent on inoculum size and the immune state of the individual. Vibrios are easily destroyed by heat, proper cooking is sufficient to eliminate most vibrios (Huss 1995).

3.1.4.1 Symptoms of cholera

V. cholerae causes mild to severe gastrointestinal illness, and may cause patient dehydration, leading to death. Common symptoms include profuse watery diarrhoea, anorexia and abdominal discomfort, and vomiting is often present. In cholera gravis, the rate of diarrhoea may quickly reach 500 to 1000 ml/hour, leading rapidly to tachycardia, hypotension and vascular collapse due to dehydration (WHO 2005). One exception is infections with *V. vulnificus*, which are primarily characterised by septicaemias (Huss 1994).

3.1.4.2 Treatment of cholera

Cholera is an easily treatable disease. The prompt administration of oral rehydration salts to replace lost fluids nearly always results in cure. In especially severe cases, intravenous administration of fluids may be required to save patients' lives.

3.1.4.3 Cholera vaccine use

Better organisation of the health system in Mozambique in recent years has led to reduced mortality rates. In 2003, the Ministry of Health in Mozambique decided to engage in the first demonstration project using oral cholera vaccines in Beira City in which they were involved with different partners, such as WHO, international vaccine institute (IVI) and *médecins sans frontieres* (MSF) /Epicentre (WHO 2006).

3.2 The situation of cholera in Mozambique

The first epidemic of cholera in Mozambique was in 1859, on the island of Mozambique (Matos *et al.* 2006). Since 1973, cholera has always been present in Mozambique. During the years 1992, 1993, 1998, 1999 and 2004 notified cholera cases from Mozambique represented between one third and one fifth of all African cases. In 1998, cholera outbreaks in Mozambique, which started in Beira City, reported 42,672 cases and 1353 deaths with a case fatality rate (CFR) of 3.2%. The already poor sanitary conditions in Beira had deteriorated following a storm that affected the central region of the country.

In 1999, an outbreak occurred in Tete Province, with 4725 registered cases and 148 deaths (CFR 3.1%). In late September resurgence occurred in Macanga, a district of Tete.

In 2004, the Ministry of Health reported a total of 9391 cases and 61 deaths from 20 December 2003 to 16 of February 2004 in six provinces. Maputo City was the most affected area, reporting 65% of all cases. The outbreak started to decrease only with diminishing rainfalls.

WHO recorded a total number of 2226 cases with 24 deaths in Mozambique in 2005 (WHO 2006). Matos *et al.* (2006) found that Cholerae 01, El Tor, serotype Inaba (predominant) and Ogawa (sporadic) was responsible for this epidemic.

Cholera epidemics mainly occur during the period from December to May/June, therefore coinciding with the rainy season. Emch *et al.* (2008) reported cholera in high numbers in sea water off the northern parts of southern Africa from the last week of January to mid-March.

3.3 Total viable microorganisms and H₂S-producing bacteria

3.3.1 Total viable microorganisms

Fish caught in very cold, clean waters carry lower numbers of microorganisms whereas fish caught in warm waters have slightly higher counts (Huss 1995). Many different bacterial species can be found on the fish surfaces. In warmer waters, higher numbers of mesophiles can be isolated. The water temperature is naturally having a selective effect on types of bacteria which can grow in different areas. The more mesophilic types (*V. cholerae*, *V. parahaemolyticus*) are representing part of the natural flora on fish from coastal and estuarine environments of temperate or tropical zones (Huss 1994).

Newly caught fish and shellfish typically have a species-specific flavour that disappears after a few days of chilled storage. Further storage results in development of off-flavours which are often ammonia-like, sulphurous, malt-like or rancid. The off-flavours are typically caused by microbial metabolites and they increase in intensity during storage resulting in spoilage as determined by sensory methods.

Extended shelf-life of fresh food products can be obtained by superchilling (partial freezing). The terms “superchilling” or “partial freezing” are used to describe a process where a minor part of the products water content is frozen. During superchilling, the temperature of the food stuff is lowered, often 1-2°C, below the initial freezing point of the product. After initial surface freezing, the ice distribution levels out to a predefined value and the product obtains a

uniform temperature which is maintained during storage and distribution (Magnussen *et al.* 2008).

At temperatures below superchilling, most microbial activity terminates or is strongly inhibited. Chemical and physical changes may progress however and in some cases can even be accelerated (Magnussen *et al.* 2008).

3.3.2 Hydrogen sulphide (H_2S) producing bacteria

During storage a characteristic flora develops, but only a part of this flora contributes to spoilage (Huss 1994). Spoilage of fish and shellfish results from changes caused by oxidation of lipids, reactions caused by activities of the fishes' own enzymes, and the metabolic activities of microorganisms. Fish and shellfish are highly perishable, because of their high water activity, neutral pH, and presence of autolytic enzymes (Sivertsvik *et al.* 2002). The spoilage of fresh fish is usually microbial. The specific spoilage organisms (SSO) are producers of the metabolites responsible for the off odours and off flavours associated with spoilage (Huss 1994).

Shewanella putrefaciens (*S. putrefaciens*) is characterised as sodium-requiring. *S. putrefaciens* has been identified as the specific spoilage bacteria of marine temperate-water fish stored aerobically in ice. Strains of *S. putrefaciens* can also be isolated from freshwater environments (Huss 1995).

Some *Pseudomonas spp.* are the specific spoilage bacteria of iced stored tropical freshwater fish (Huss 1995) and are also, together with *S. putrefaciens*, spoilage bacteria of marine tropical fish stored in iced (Huss 1995). The spoilage flora of iced tropical fish from marine waters is composed almost exclusively of *Pseudomonas spp.* and *S. putrefaciens*.

The principle of modified atmosphere packaging (MAP) is the replacement of air in the package with a different fixed gas mixture. CO_2 is the most important gas used in MAP of fish, because of its bacteriostatic and fungistatic properties. MAP extends shelf-life of most fishery products by inhibiting bacterial growth and oxidative reactions. The combination of superchilling storage with MAP increases the sensorial and microbial shelf-life of the fillets when compared to air stored fillets at chilled temperature (Bahaud *et al.* 2008).

The achievable extension of shelf-life depends on species, fat content, initial microbial population, gas mixture, the ratio of gas volume to product volume and most importantly, storage temperature. The spoilage of MAP cod is caused by the growth and metabolism of the CO_2 -resistant bacterium *Photobacterium phosphoreum* and this explains why shelf-life is only slightly extended by MAP. This SSO grows to high levels in different MAP fish but is inactivated by freezing at $-20\text{ }^\circ\text{C}$ and the shelf-life of thawed MAP cod can be substantially extended (Emborg *et al.* 2002). The SSO of MAP cod at $0\text{ }^\circ\text{C}$ has been found to be *P. phosphoreum* (Sivertsvik *et al.* 2002).

A large number of bacteria (10^7 - 10^8 /g) are normally found on spoiling fish, but only part of this flora may be classified as active spoilers (Gram *et al.* 1987). Volatile sulphur-compounds are typical components of spoiling fish and most bacteria identified as specific spoilage bacteria produce one or several volatile sulphides. *S. putrefaciens* and some Vibrionaceae produce H_2S from the sulphur containing amino-acid l-cysteine (Huss 1995). Specific counts of fish spoilage organisms at both low and high temperatures could therefore be obtained

directly on an iron agar containing thiosulphate and cysteine where bacteria capable of forming H₂S from either source of sulphur would appear as black colonies (Gram *et al.* 1987).

The microflora responsible for spoilage of fresh fish changes with changes in storage temperature. At low temperatures (0-5 °C), *S. putrefaciens*, *P. phosphoreum*, *Aeromonas* spp. and *Pseudomonas* spp. cause spoilage. However, at high storage temperatures (15-30 °C) different species of Vibrionaceae, Enterobacteriaceae and Gram-positive organisms are responsible for spoilage (Huss 1995).

4 MATERIAL AND METHODS

4.1 Experimental design

Since the experiments were done in Iceland, redfish was picked for the experiments as it is readily available and a relatively fatty pelagic fish which resembles the redfish caught in the Indian Ocean near Mozambique, sold in the local market and also exported to the EU.

4.1.1 Experiment –I

Iced redfish (*Sebastes mentella/marinus*) was filleted and minced. The sample was contaminated with *V. cholerae*, the mixture was distributed in six sterile containers and stored at different temperatures: -1.5, 0, 2, 4, 8 and 17°C, respectively; for analysis of total viable counts, H₂S-producing bacteria and *V. cholerae*. Table 2 shows the sampling plan after the initial samples (at the time of contamination) were taken.

Table 2: Days of analysis of samples stored in different temperatures of experiment-I

Temperature/day	Sampling days							
	1 st	2 nd	3 rd	6 th	7 th	10 th	14 th	20 th
-1.5 °C								
0 °C								
2 °C								
4 °C								
8 °C								
17 °C								

4.1.2 Experiment –II

In the case of *V. cholerae*, there was no growth in experiment-I. For three weeks, while experiment-I was ongoing, the culture stock of *V. cholerae* was kept in the laboratory at room temperature. A further experiment was later conducted using the same culture of *V. cholerae* to contaminate samples, and the mixture stored at different temperatures of 0, 8, 17, 22, 25 and 30 °C respectively, for analysis of *V. cholerae*. Table 3 shows the sampling plan after the initial samples (at the time of contamination) were taken.

Table 3: Days of analysis of samples stored in different temperatures of experiment-II

Temperatures / day	Sampling days		
	2 nd	4 th	6 th
0 °C			
8 °C			
17 °C			
22 °C			
25 °C			
30 °C			

4.2 Methods

4.2.1 Total viable counts (TVC) and H₂S-producing bacteria

Procedure:

For TVC and H₂S- producing bacteria, 20 g of each sample were placed in a stomacher bag and 180 ml of peptone water solution added to obtain a 10-fold dilution. Blending (homogenization) was done in stomacher for 1 minute. After homogenization serial dilutions were prepared (1/10). Total viable psychrotrophic counts (TVC) and counts of H₂S-producing bacteria were evaluated on iron agar (IA) as described by Gram *et al.* (1987) with the exception that 1% NaCl was used instead of 0.5% with no overlay. Plates were incubated at 17 °C for four to five days. Bacteria forming black colonies on IA produce H₂S from sodium thiosulphate and/or cysteine.

4.2.2 *V. cholerae*

A comparison was made to determine the sensitivity of direct inoculation onto TCBS agar and alkaline peptone water (APW) enrichment prior to plating on TCBC agar. For this purpose, 1 ml of pure strains of *V. cholerae* broth was mixed with 1 kg of the minced redfish.

4.2.2.1 Procedure- I

APW enrichment

1 g of minced redfish was placed into a tube containing 9 ml of APW and incubated at 42 °C for 2 hours. After that serial dilutions were prepared (1/10). Sample (0, 1 ml) solution was transferred to the surface of a dried thiosulphate citrate salt sucrose agar (TCBS) plates, spreading on surface using sterile triangle rod. Plates were incubated at 37 °C for two days. Typically colonies of *V. cholerae* on TCBS agar are large (2 to 3 mm) and yellow.

4.2.2.2 Procedure –II

Direct inoculation on TCBS agar

20 g of minced redfish was placed in stomacher bag and 180 ml of peptone water solution added to obtain a 10-fold dilution. Blending (homogenization) was done in stomacher for 1 minute. After homogenization serial dilutions were prepared (1/10). 0.1 ml of solution was transferred to the surface of a dried TCBS plates, spreading on surface using sterile triangle rod. Plates were incubated at 37 °C for two days (NMKL 1997).

Colonies on plates that had 25 to 250 colonies were counted and counts were multiplied with the relevant dilution factor.

5 RESULTS

The samples were stored at different temperatures and analysed for TVC, H₂S-producing bacteria and *V. cholerae*. The figures below show the counts of each bacterial group at different storage temperatures and a comparison of growth of *V. cholerae* using two different procedures.

Figure 1 shows low initial bacterial loads (TVC) on day 1 of storage and an increasing trend during storage. TVC was significantly lower in low temperature compared to storage at higher temperatures. At 0 °C and 2 °C, the number of bacteria started to decrease after 14 and 10 days.

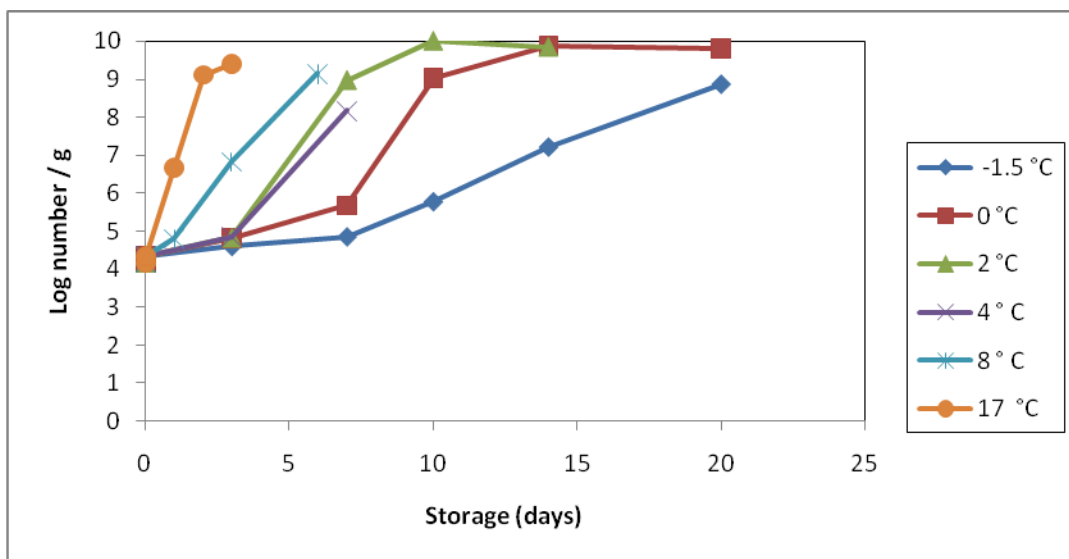


Figure 1: Growth of total viable microorganisms in minced redfish at different storage temperatures, experiment-I.

The results show low proportions of H₂S-producing bacteria on the first day. After three days, the number of H₂S-producing bacteria was much higher in storage at high (8 °C and 17 °C) temperatures compared to low storage temperatures (-1.5 °C and 0 °C) (Figure 2).

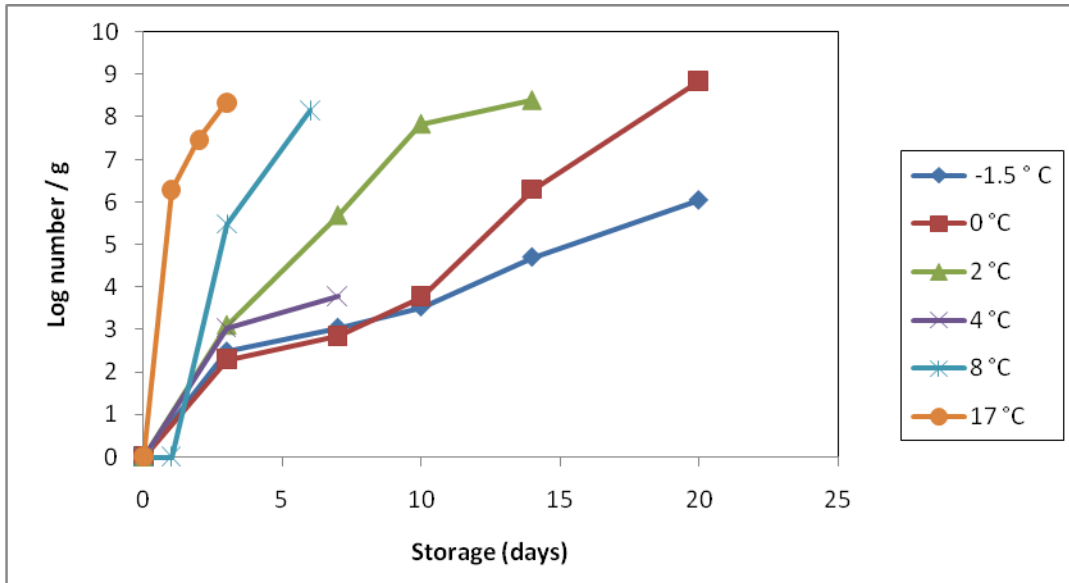


Figure 2: Growth of H₂S-producing bacteria in minced redfish at different storage temperatures, experiment-I.

Figure 3 and Figure 4 show that at 0 °C and 8 °C *V. cholerae* did not grow at all. With both methods, the number of colonies increased until day 4, after which it started to decrease except at 30 °C after enrichment (Figure 4), which started to decrease after day 2. Without the enrichment, the growth was slower at all temperatures and at 17 °C *V. cholerae* was first detected on day 2. At high storage temperatures *V. cholerae* grew faster than at low storage temperatures.

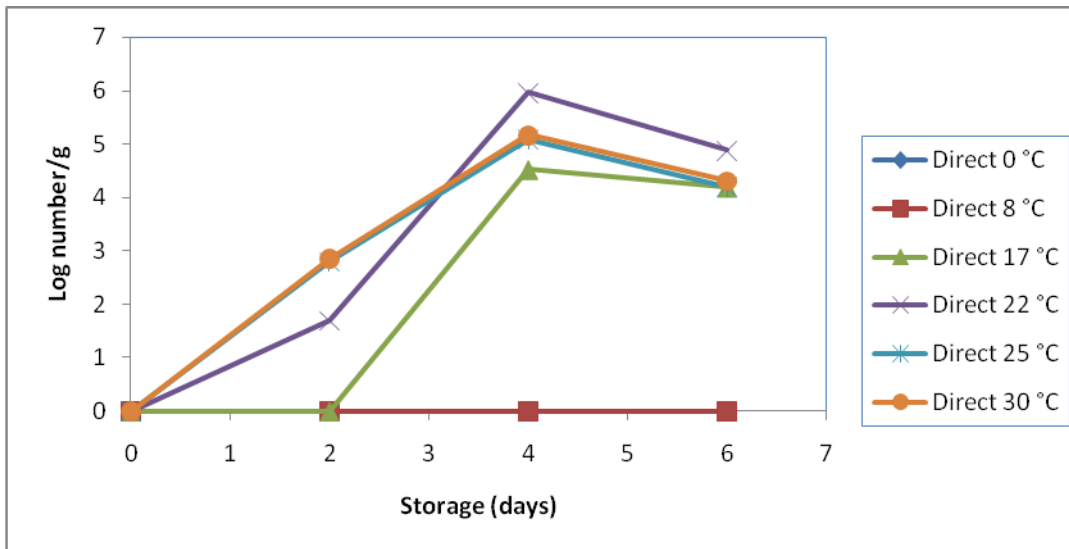


Figure 3: Growth of *V. cholerae* in minced redfish at different storage temperatures, experiment-II.

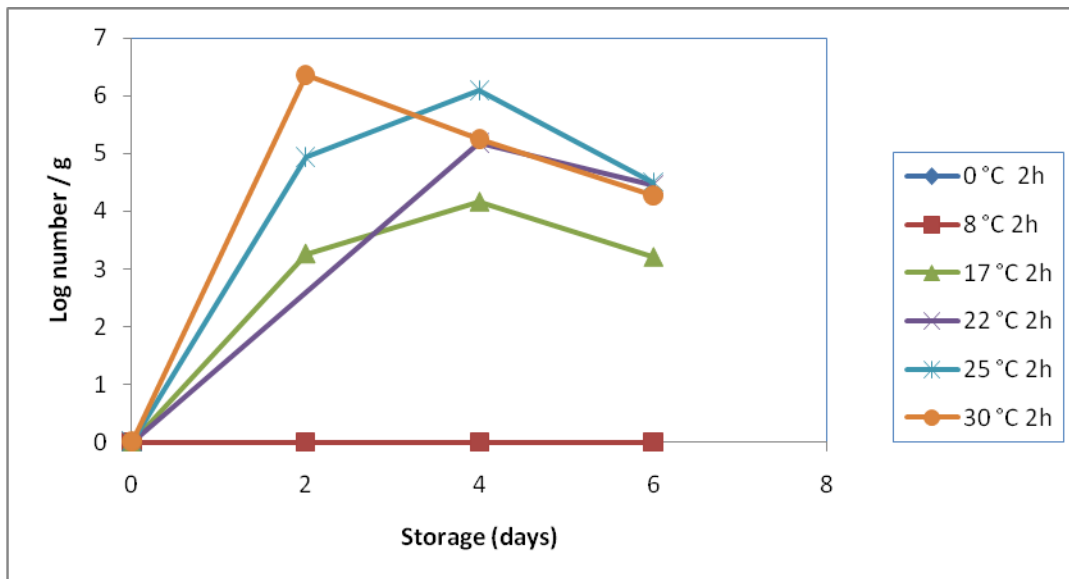


Figure 4: Growth of *V. cholerae* in minced redfish at different storage temperatures after 2 hours of enrichment, experiment –II.

Comparing the results of APW enrichment and direct inoculation of TCBS agar, it was observed that the number of bacteria in APW enrichment was higher than the number of bacteria in direct inoculation of TCBS at 25 °C and 30 °C (Figure 5).

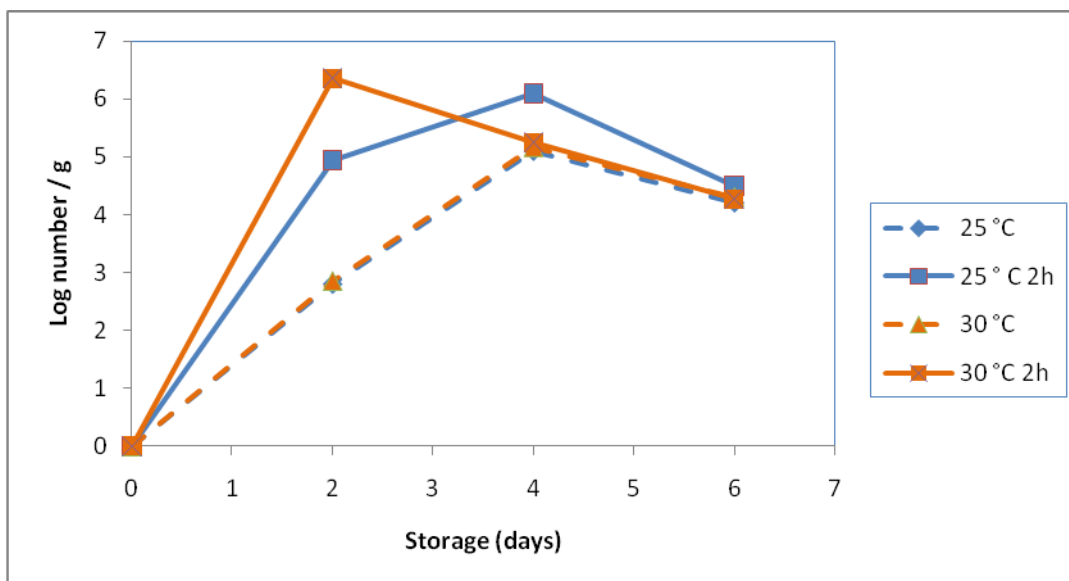


Figure 5: Comparison between growths of *V. cholerae* in minced redfish at different storage temperatures using two different procedures. Broken lines show growth with direct inoculation, solid lines show growth after 2 hours of enrichment.

6 DISCUSSION

The bacteria grew much faster at high temperatures of storage than at low temperatures (Figure 1), and the superchilled sample always had the lowest TVC. Bacteria in the sample kept at 17 °C reached log 9.4/g on day 3 of storage (Appendix 1). On day 10, the TVC in the samples stored at 2 °C exceeded log 9/g and sample kept at 0 °C on day 14. At that time (day 14), TVC in the superchilled sample was about log 7/g. At 0 °C the growth rate is less than one-tenth of the rate at the optimum growth temperature. Many bacteria are unable to grow at temperatures below 10 °C and even some psychrotrophic organisms grow slowly, and sometimes with extended lag phases, when temperatures approach 0 °C (Huss 1995).

Another important aspect that we can see from Figure 1 is that the TVC in samples stored at 0 °C and 2 °C after 20 and 14 days respectively started to show a slight decrease in number. When the population increases in size, there is an increase in the consumption of nutrients with a consequent increase in production of secondary products of metabolism. Whereas these metabolites at low levels have little effect on rates of cell division, in high concentrations they can inhibit cell division and kill the cells, resulting in decreased numbers of bacteria.

In general, much lower growth curves were obtained for H₂S-producing bacteria than for TVC during the storage period. The initial number of H₂S-producing bacteria was much lower than the initial total viable count. This is probably due to the fact that on newly processed fresh fish, specific spoilage organisms (SSO), are usually present in very low concentrations and constitute only a minor part of the total microflora (Figure 2). The number of H₂S-producing bacteria increased fastest at 17 °C to log 8.36/g colonies forming units (CFU) on day 3, as compared to a maximum value of log 6.04/g CFU, which was reached first after 20 days at -1.5 °C (Appendix 2). The low total counts in early storage days are because the flesh of newly caught fish is sterile since the immune system of the fish prevents the bacteria from growing in the flesh, but when the fish dies, the immune system collapses and consequently during storage bacteria invade the flesh (Sveinsdottir *et al.* 2002). Olafsdottir *et al.* (2006) found that in cod fillets, the amount of H₂S-producing bacteria was log 7.6 units CFU/g after 15 days of -1.5 °C storage, which is comparable to the log 4.7 units CFU/g at day 14 in the present study.

Super-chilling has a potential of prolonging the shelf-life of minced redfish during storage. According to microbial activity the super-chilled storage improved shelf-life of minced redfish by about 6-10 days compared to chilled mince kept at 0 °C.

A large number of human pathogenic bacteria can enter a viable but non-culturable (VBNC) state. Bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity (Oliver 2005). Cells enter the VBNC state as a response to some form of natural stress, such as starvation, incubation outside the temperature range of growth, elevated osmotic concentrations, oxygen concentration, or exposure to white light (Oliver 2005). In experiment-I *V. cholerae* did not grow, probably because it was in the VBNC state. After these results appeared, further experiments were conducted, including storage at high temperatures. For these later experiments (experiment II), the pure strain of *V. cholerae* broth used, had been stored at room temperature for three extra weeks, which may have helped to revitalise the bacteria.

V. cholerae both in direct inoculation on TCBS agar and after APW enrichment grew at 17, 22, 25 and 30 °C but not at 0 and 8 °C. Growth was faster at high temperatures than low temperatures of storage (Figure 3 and Figure 4).

At 17 °C, on the second day of sampling *V. cholerae* was not detected in sample for direct inoculation of TCBS (Figure 3 and Appendix 3) but in APW enrichment the number was log 3.27 CFU/g (Figure 4) and

Appendix 4). This probably happened because 17 °C is not an optimal temperature for *V. cholerae* and after storage the bacteria were stressed and may have started to enter the VBNC state but when APW enrichment was used, it was possible to recover the same bacteria. Smith *et al.* (2006) found that during three in situ experiments in which *V. vulnificus* cells were loaded into membrane diffusion chambers and incubated in cold (< 15 °C) estuarine waters, all three *V. vulnificus* strains entered the VBNC state, decreasing from 10^6 to 10^7 CFU/ml to <0.1 CFU/ml over 14 days.

The APW enrichment had a significant effect on the number CFU of detectable *V. cholerae*. The experiment revealed significant differences between temperatures, with higher numbers of CFU at higher temperatures (Figure 5).

However, faster initial growth rates can be seen at all temperatures, 17-30°C (Figure 3-Figure 5) and high counts (>log 5), are reached two days earlier at the highest temperatures (Figure 5) because all growth curves of bacteria have four phases of population growth that are lag phase, exponential phase, stationary phase and death phase. In this case on day 4 the death phase started where the cell loss was higher than the rate of division and the number of viable cells decreased exponentially.

All bacteria have their optimal temperature for growth and as they approach the optimum, the growth is faster (Figure 1, Figure 2, Figure 3 and Figure 4).

Enrichment in APW allows *V. cholerae* growing to detectable levels prior to plating on a suitable culture medium, such as TCBS agar. The sodium chloride in APW promotes growth of *V. cholerae*, whilst its alkalinity inhibits most unwanted background flora. Possibly, this method needs to be universally applied, whenever testing for *V. cholerae* in samples that have been exposed to temperatures below 15 °C anytime before the test is performed. Experiment I shows that without the enrichment, *V. cholerae* in the VBNC state can be overlooked with conventional methods.

7 CONCLUSIONS

A higher number of CFU of *V. cholerae* was detected when using the APW enrichment procedure.

Super-chilling has a potential of prolonging the shelf-life of minced redfish during storage. According to microbial activity the super-chilled storage improved shelf-life of minced redfish by about 6-10 days compared to chilled mince kept at 0 °C.

These results suggested that *V. cholerae* entered a viable but nonculturable state during storage time at low temperature of 0 °C and 8 °C. The temperature has been identified as the most important variable regulating *V. cholerae*, total viable microorganisms and hydrogen sulphide-producing bacterial growth.

8 RECOMMENDATIONS

Based on the results of this study the following is recommended:

- To maintain the freshness of fish products, they should be stored at low temperatures to delay or prevent the growth of bacteria.
- To obtain more reliable results of *V. cholerae* counts it is recommended to use alkaline peptone water enrichment at 42 °C for 2 hours prior to plating on TCBC agar.
- For fish and fish products that have been stored at low temperatures great care should be taken not to expose these products to high temperatures (close to 40°C), not even for a short time (2 hours) as this may start a fast growth of *V. cholerae* that would otherwise stay dormant.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the UNU-FTP staff for the opportunity to attend this programme, the microbiological laboratory that assisted me with my project work, in particular my supervisors Hannes Magnusson and Pall Steinthorsson, for their endless support and professional opinions at each step of this project towards its success in the end.

I also thank Cyprian Odoli for assisting me in the use of the English language. Last but not least thanks to all who contributed directly and indirectly to the success of this project.

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APPENDIX**Appendix 1: Growth of total viable microorganisms in redfish mince at different storage temperatures, experiment-I. Log 10 of counts.**

Days	0	1	2	3	6	7	10	14	20
-1.5 °C	4.35			4.61		4.86	5.78	7.22	8.87
0 °C	4.35			4.81		5.67	9.03	9.86	9.79
2 °C	4.35			4.83		8.97	10.00	9.84	
4 °C	4.35			4.86		8.17			
8 °C	4.35	4.8		6.83	9.15				
17 °C	4.35	6.67	9.11	9.41					

Appendix 2: Growth of H₂S-producing bacteria in redfish mince at different storage temperatures, experiment-I. Log 10 of counts.

Days	0	1	2	3	6	7	10	14	20
-1.5 °C	0.00			2.48		3.04	3.53	4.70	6.04
0 °C	0.00			2.30		2.85	3.77	6.30	8.85
2 °C	0.00			3.11		5.70	7.85	8.41	
4 °C	0.00			3.04		3.78			
8 °C	0.00	0.00		5.48	8.15				
17 °C	0.00	6.30	7,48	8.36					

Appendix 3: Growth of *V. cholerae* in redfish mince at different storage temperatures, experiment-II. Log 10 of counts.

Days	0	2	4	6
0 °C	0.00	0.00	0.00	0.00
8 °C	0.00	0.00	0.00	0.00
17 °C	0.00	0.00	4.52	4.19
22 °C	0.00	1.70	5.96	4.88
25 °C	0.00	2.80	5.09	4.20
30 °C	0.00	2.86	5.17	4.31

Appendix 4: Growth of *V. cholerae* in redfish minces at different storage temperatures after 2 hours of enrichment, experiment –II. Log 10 of counts.

Days	0	2	4	6
0 °C 2 hours	0.00	0.00	0.00	0.00
8 °C 2 hours	0.00	0.00	0.00	0.00
17 °C 2 hours	0.00	3.27	4.17	3.21
22 °C 2 hours	0.00		5.19	4.46
25 °C 2 hours	0.00	4.93	6.09	4.50
30 °C 2 hours	0.00	6.36	5.25	4.27

Appendix 5: Comparison between growths of *V. cholerae* in minced redfish at different storage temperatures using two different procedures, experiment-II. Log 10 of count.

Days	0	2	4	6
25 °C	0.00	2.80	5.09	4.20
25 °C 2 hours	0.00	4.93	6.09	4.50
30 °C	0.00	2.86	5.17	4.31
30 °C 2 hours	0.00	6.36	5.25	4.27