CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Namibia is one of the leading fishing countries in the world. Hake, pilchards and horse mackerel are the leading economically important species (MFMR, 2002). All products are frozen in factories and kept at -18°C before distribution to either home markets or exported. In Namibia certification of the exported fish follows a rigorous quality control system to meet strict specifications in accordance with consumer demands. Fish quality is measured by using both microbiological and chemical parameters to estimate the shelf life (Chang et al., 1998; Gram, 1992). The current quality control protocol follows a number of routine tests. All microbiological tests are done according to the EU directive 19–493 EEC (Iyambo, 1995). Quality control is enforced by the Ministry of Trade and Industry as the Competent Authority, and the testing and certification is done by the Namibia Standards Institute (NSI).

The safety of seafood is increasingly becoming an important public health issue. This is due to the global rise in the number of people vulnerable to disease during the last two decades, coupled with the customary tendency of the world population to consume raw or improperly cooked seafood. Minimizing the amount of pathogens associated with seafood is therefore becoming increasingly important. To ensure seafood safety the presence of food–borne pathogens such as the *Clostridium botulinum*, *Aeromonas hydrophyla*, *Bacillus cereus*, *Salmonella* spp., *Yersinia enterocolitica*, *Listeria monocytogenes*, and human pathogenic *Vibrio* species such as *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus,* is monitored and controlled (Church and Parson, 1995; Drake et al., 2007; Fabber, 1991; Giménez et al., 2002; Masniyom et al., 2002).

Seafood is prone to contamination by spoilage and pathogenic bacteria due to the ubiquity of many of these microorganisms in marine environments. Another major source in the contamination and spread of bacteria is the cross contamination from utensils and surfaces during food processing as well as the water used during processing (Wekell et al., 1994). In fish processing plants the spread of spoilage bacteria and food-borne pathogens are typically controlled by the use of Good Manufacturing Processes (GMP) (Vasconcellos,

2004a) and Hazard Analysis and Critical Control Points (HACCP) principles (Vasconcellos, 2004b; Ward, 2002). Proper cleaning and sanitation, on a continuous basis, is a basic requirement. The use of process water free of any pathogens is also crucial to ensure the microbial quality and safety in a seafood processing environment (Ray, 2003) especially when seawater is used during processing.

1.2 PROBLEM STATEMENT

In 1999 there was a serious controversy surrounding the safety of hake exported from Namibia. One of the major issues was the suspicion that some of the exported hake were contaminated by pathogenic *Vibrio* species. This concern was primarily raised by the European countries after these bacteria were detected in a few consignments which were returned to Namibia. This incident called for a thorough investigation to verify the extent of this problem and trials to come up with possible solutions. At the same time seafood spoilage is commonly encountered in the Namibian fishing industry, but the exact cause of spoilage has received very little attention. The industry is especially worried about quality deterioration experienced with hake whereby the fish fillet becomes soft and prematurely looses its firmness. The scientific literature has not been a good source of information as it primarily contains information on the microbial quality of fish from temperate regions and information on tropical marine fish spoilage and pathogenic micro-organisms seems to be limited.

1.3 JUSTIFICATION OF THE STUDY

Quality control plays a significant role in the fishing industry especially when dealing with seafood intended for export. Enumeration and identification of the pathogenic and spoilage organisms associated with the fish will give some indication of the possible source of processed fish contamination. This information will enable the design of appropriate control methods to extend the product shelf life, minimise economic losses due to the rejection of exports and improve the safety of the product.

Apart from some incidents where *Vibrio* spp. have been detected on hake, the occurrence of these bacteria on fish processed in Namibia has not yet been determined. These species are widespread in aquatic environments and the general oceanographic conditions off the coast of Namibia are favourable for their survival and growth. The possibility of these bacteria contaminating processed fish therefore does exist. Although no food-borne cases linked to pathogenic *Vibrio* species transmitted via seafood have so far been reported in Namibia between 1990 and 2005 it might have gone unnoticed, as they are not included amongst the notifiable diseases in Namibia. In addition, lack of expertise, facilities, and the difficulties encountered in the use of classical methods of isolation and identification of these bacteria might have hampered the availability of data on these organisms in Namibian seafood. As there have recently been outbreaks of cholera in Namibia linked to contaminated fresh water after floods hit the country between 2006 and 2009 (Smith et al., 2008) it is important to carry out a survey on the microbiological quality of processed fish to ensure that Namibia maintains a marine fishing industry free of pathogenic *Vibrio* species.

1.4 AIMS AND OBJECTIVES

The aim of this study was to investigate the microbiological quality (using safety and shelf life indicators) of the three economically most important processed marine fish species (hake, pilchard and horse mackerel) in Namibia in order to formulate measures aimed at counteracting contamination and improving the shelf life and safety of the processed marine fish.

The objectives of this study therefore were:

1.4.1   To determine the general microbiological quality of processed hake, using total viable aerobic plate counts and methods for the isolation of selected common fish spoilage organisms (*Shewanella putrefaciens*, *Pseudomonas species*, *Aeromonas* and *Enterobacteriaceae*).

1.4.2   To determine the microbial quality of hake during the various steps of processing.

1.4.3 To determine the occurrence of potential human pathogenic *Vibrio* species along a hake processing line.

1.4.4 To demonstrate the ability of selected *Vibrio* isolates to form biofilms in sea water and to investigate measures to control these biofilms.

1.5 HYPOTHESES

The hypotheses of the study were as follows based on the objectives:

1.5.1 Hake, pilchard and horse mackerel processed in Namibia have higher bacterial counts than those normally found on freshly caught fish.

1.5.2 The microbiological quality of hake deteriorates during processing.

1.5.3 Potential pathogenic *Vibrio* spp. increase on hake during processing and originate from the treated sea water used during processing.

1.5.5 Selected *Vibrio* isolates form biofilms in sea water and cannot be controlled by chlorination.

1.6 ORGANISATION OF THE THESIS

The thesis is organised into six chapters. The current chapter is followed by a literature review dealing with the general information on marine fishing and related commercial activities in Namibia and quality and safety issues of seafood. The chapter also highlights the most prevalent bacterial pathogens and spoilage organisms associated with marine fish. It lays emphasis on the four most common pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*) focusing on available literature about their prevalence in marine environments, their route of transmission from aquatic habitats and the available method of isolation and identification. The final part of the chapter deliberates on related issues such efforts to control microbial contamination within fish processing facilities and the methods used in combating biofilms in food

processing establishments and water distribution networks.

Chapter three reports on a pilot study carried out during 2005 with the aim to unravel and establish the extent of microbial contamination of processed hake. In this chapter the contamination of hake with *Vibrio* spp. was also identified. Chapter four deals with the identification of randomly selected *Vibrio* isolates obtained from processed hake, pilchards and horse mackerel. Chapter five constitutes experiments that demonstrated the ability of selected pathogenic *Vibrio* species to form biofilms in artificial sea water in a continuous flow laboratory system. The ability of three disinfectants (chlorine, ozone and hydrogen peroxide) to prevent or eliminate these biofilms was also investigated. The last chapter concludes the research and provides some overall recommendations based on the study.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The abundance of fish along the coast of Namibia is a natural resource that forms part of the backbone of the country's economy. In order to maintain and grow its market share compliance with various international food safety guidelines is essential (Scharm, 1998). It is therefore important to monitor the microbiological parameters of harvested marine fish to ensure that the product does not pose health hazards to end users and that the levels of potential spoilage organisms is within the limits that permit acceptable product shelf life.

Sea food spoilage occurs mainly as a result of bacterial metabolism of the fish muscle producing metabolites that decrease the quality of fish. The most common spoilage reaction is the production of biogenic amines of which trimethylamine is the most important (Gram and Huss, 1996; Kyrana and Lougovois, 2002). In terms of safety, human pathogenic *Vibrio* species are of great concern (Nilsson and Gram, 2002; Wekell et al., 1994). These pathogens are common inhabitants of the natural marine environment and may enter the final product via contamination from processing equipments and surfaces, food handlers or via water used in the processing plant (DePaola et al., 2000; Joseph et al., 1982).The situation might be aggravated when quality assurance and quality control tests fail to correctly identify the pathogens due to atypical genetic or phenotypic traits hence conferring a false sense of security (Farmer et al., 2004; Oliver and Kaper, 1997). Adequate plant sanitation and product temperature control as well as maintaining personal hygiene are important in minimising the risk of product contamination.

Some pathogens and spoilage organisms may form attached microbial communities that flourish on the surfaces of water distribution systems (Momba, 1997; September et al., 2006) causing deterioration of the quality of processing water and contamination of the final end product. Formation of these structures (biofilms) is normally a response to unfavourable conditions such as low temperatures and nutrient scarcity. Bacteria in biofilms are embedded in a semipermiable complex formed as a result of increased secretions and deposition of extracellular polymers that promote resistance of the

microorganisms to disinfectants such as chlorine and UV irradiation. In water distribution systems biofilms are normally formed as a result of surface attachment after repair and multiplication of sub-lethally injured microorganisms (Momba, 1997). Although chlorine is the conventional chemical sanitizer used in fish processing establishments other disinfectants such as ozone, and hydrogen peroxide may prove more efficient. In addition these two sanitizers are legislated and do not leave toxic by-products (Kim et al., 1999b; Lück and Jager, 1997).

2.2 MARINE FISHING INDUSTRY IN NAMIBIA

2.2.1 Commercial activities

Marine fishing is the second most important sector in the country after mining in terms of export values and its contribution to GDP (MFMR, 2004). Marine fishing is carried out from the Exclusive Economic Zone (EEZ), a 200 nautical miles wide zone extending along the whole coastal length (Hamunyela and Tjihuiko, 1996). Fishing vessels are dispatched from Walvis Bay and Lüderitz Bay to carry out commercial fishing. Both domestic and international commercial fishing companies harvest all fish types. There were 163 fishing rights holders by 2002 using 335 licensed fishing vessels with the fishing rights duration of seven, ten, fifteen and twenty years (MFMR, 2004).

Over 70% of the fish export goes to the EU, Japan, China, and Malaysia and to a lesser extent West, East and other Southern African countries. Some fish species are sold unprocessed, while others are processed and transported frozen to home markets or exported to other countries. Most of the Namibian fish and fish products are sold through two trade agreements; the Lomè convention and the South African Custom Union (SACU) to European and Southern African countries respectively. International trade in fisheries earn the country about 25% of its foreign exchange (European Research Office, 2003; Namibia Brief: Focus on Fisheries and Research, 1998).

2.2.2 Important Fish Species.

The three major fish species, namely hake (*Merluccius capensis* and *Merluccius paradoxus*), pilchard (*Sardinops sargax*) and Cape horse mackerel (*Trachurus trachurus*) occupy varying geographical positions and depths depending on the fish species and the age of the fish. Cape hake is found throughout the Namibian and South African waters, while deep water hake is more common in the south. Both species co-exist at a depth of between 150–800m. The third hake species, Benguela hake (*Merluccius polli*) is mostly found in Angolan waters. Both shallow and deep water hake are caught by bottom trawls with by catches of monk, kingklip, jacopivo, snoek and sole. Hake make up the bulk of the demersal fish species. Other demersal species are monk fish (found at depths of 150-500m), orange roughy (at depths of 600-1000m) and alfonsino (at depths of 400-700m) (Boyer and Hampton, 2001).

Cape horse mackerel is found in two distinct localities, one off the northern Namibia/Angolan border and another off the western coast of South Africa near the Cape. It is believed that these two stocks are recruited from separate spawning grounds. Cape horse mackerel is mainly caught by trawling occupying a mid water position. The juvenile horse mackerel are mainly found near the coast at about 200m below surface. Together with the sardines commonly referred to as pilchard, anchovy and round herring they make up the pelagic fish stocks harvested by purse–seine type of fishing gear.

Pilchard and anchovy have a wide geographic distribution in Southern Africa, from the northern Namibia/Angolan borders up to Kwa Zulu–Natal on the east coast of South Africa (Boyer and Hampton, 2001). There has been a marked decline in total landings for pilchards since the late sixties which can be ascribed to uncontrolled fishing activities before independence in 1990 as well as adverse environmental conditions such as the Benguela Ninõ events (Boyer and Hampton, 2001). The trend was reversed after independence through introduction of a strict resource management policy that enforced rational fishing practice.

Data for the past fourteen years show that three marine fish species dominated the industry in terms of landings and export, i.e. horse mackerel, hake and pilchard. Though the landings of horse mackerel for this period were higher than those of hake, the latter had higher values in terms of export to Europe and the Far East (MFMR. 2002). Horse mackerel is popular both in the local markets and for exports to a number of African countries (The Namibian, 2004). Pilchard has relatively fewer landings than the other two species, (MFMR, 2004; Namibia Brief: Focus on Fisheries and Research, 1998).

The type of processing and preservation methods used varies depending on the fish species. For hake, the fish are gutted and headed offshore and thereafter kept on ice and brought to the factories where further processing into fillets and packaging takes place. Hake is mainly preserved by the simple method of over wrap and subsequent freezing. Exported fish is usually kept at -18°C. Demersal horse mackerel is harvested by demersal fish licence holders and after washing the fish is packaged and frozen onshore ready for both the local markets and for export. Horse mackerel is preferred over white fish and is an important part of the diet within the low income groups.

2.2.3 Quality assurance in the Namibian Fishing Industry

Internationally, the methods used in the examination of foods to determine principal food pathogens, or limits of indicator organisms have been standardised by a number of organisations such as the International Standard Organisation (ISO) (in the Codex Code of Hygiene Practise), the International Dairy Federation (IFD), the International Commission on Microbiological Specification for Food (ICMSF), the Association of Official Analytical Chemists, (AOAC)and the American Public Health Association (APHA). A joint ISO/AOAC/IDF working group was established in 1975 to harmonise these methods (ICMSF, 1986). The legislation on food export was defined by the Codex Alimentarius Commission in 1981 in the 'General Principles for the Establishment of Microbiological Criteria for Foods'. Microbiological criteria are therefore a worldwide

prerequisite for international trade in foods and have five components which are included in all the sampling plans used for routine inspections for food trade (ICMSF, 1986):

a) "Statement of the food to which the criteria applies."

b) "Statement of the food contaminants of concern."

c) "The analytical methods to be used".

d) "The samples to be taken from the lot or from the critical control point."

e) "Microbiological limits in which the values of *n, c*, *m* and *M* are defined" (ICMSF, 1986).

In order to maintain its position as one of the major fish exporting countries the Namibian government represented by the Ministry of Trade and Industry together with the fishing industry are obliged to observe the legislation on food export as laid down in the above mentioned document (ICMSF, 1986; Scharm, 1998). In Namibia certification of the exported fish follows a rigorous quality control system to meet strict specifications in accordance with the consumer demands (Iyambo, 1995).The current quality control protocol follows a number of routine tests. All microbiological tests are done according to the EU Directive 91–493 EEC, chapter (II), article II, No. 3b, 3c, and 3d (Iyambo, 1995). Quality control is enforced by the Ministry of Trade and Industry as the Competent Authority while the Namibia Standards Institute (NSI) is responsible for testing and certification. The tests commonly performed include total viable count (TVC), total coliforms, faecal coliforms, *Vibrio* species, *Staphylococcus aureus* and *E. coli*. General hygienic conditions both on board of fishing vessels and in the factories were upgraded following the EU inspection mission to the fishing industry in 1994 (Iyambo, 1995). At present the Namibian fishing industry strives to carry out Good Manufacturing Practices and implements HACCP concept in all its fishing establishments (Iyambo, 1995; Scharm, 1998).

2.3 QUALITY AND SAFETY OF SEAFOOD

2. 3.1 Spoilage of Seafood

Quality deterioration is responsible for huge economic losses (up to 25%) in the food industry all over the world. Seafood spoilage is of biochemical and/or microbial origin and results in limited shelf life and the eventual sensory rejection of the food (Gennari et al., 1999; Gram, 1992; Gram and Dalgaard, 2002; Huis in't Veld, 1996). Fish spoilage occurs as a result of autolysis and lipolysis due to the activity of endogenous enzymes or contamination by metabolically active microorganisms (Chang et al., 1998; Chytiri et al., 2004; Ordóñez et al., 2000; Pastoriza et al., 1996). Bacteria are pivotal in the process of seafood spoilage by either initiating or accelerating the spoilage process (Gennari et al., 1999; Gram, 1992; Tryfinopoulon et al., 2002).

The number of total viable bacteria is a measure of the general microbiological quality of the food. While the mesophilic count is regarded as an indication of the sanitary condition under which the food is produced, the pshychrotrophic count may indicate the number of bacteria present per unit food that is capable of causing spoilage during refrigeration storage (ICMSF, 1978; Mol et al., 2007). High mesophilic counts indicate poor sanitary practise during processing in food intended to be distributed at ambient temperatures. It also signals that the food might have been exposed to conditions that permit multiplication of disease causing microorganisms. Most pathogenic microorganisms are mesophiles; high counts of mesophiles therefore may indicate a health hazard. On the other hand mesophilic counts usually depend on the type of food and number present during harvesting; it may also indicate that a slow spoilage process is taking place (ICMSF, 1978).

For the food that is preserved by low temperature storage (chilling and freezing) a count obtained at lower incubation temperature is required to predict the shelf life of the product. Psychrotrophic bacteria belonging to the class γ- Proteobacteria group are well documented as spoilage organisms in fresh and preserved fish (Chytiri et al., 2004;

Gennari et al., 1999; González et al., 1999; Gram and Huss, 1996; Himelbloom et al., 1991; Koutsoumanis and Nychas, 1999; Ordóñez et al., 2000; Pastoriza et al., 1996; Tryfinopoulou et al., 2002). The total viable aerobic plate count (TVC) ranges between 10^3-10^6 cfu/gram/cm^2 in fresh fish (Chang et al., 1998; Giménez et al., 2002; Mahmoud et al., 2004). Fish spoilage is usually associated with total counts of 10^7–10^8 of the specific spoilage organisms (Gram and Huss, 1996; Ordonéz et al., 2000).

Seafoods in their natural environments are associated with a variety of microorganisms. Many researchers have shown that fresh fish contain large numbers of microorganisms on the skin (10^4-10^6), in the gills, (10^4-10^7 cfu/gram) (Gennari et al., 1999), and in the intestines (10^3-10^5) (Nickelson and Finne, 1992). Studies carried out on fresh fish and ice-stored fish from temperate regions show that the predominant micro flora include *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Brochotrix thermosphacta*, *Pseudomonas* species, *Aeromonas* species and lactic acid bacteria (LAB) (Chytiri et al. 2004; Tryfinopoulou et al., 2002). Similar studies on initial micro flora on sardines from the Adriatic Sea have given similar, but not exactly the same results. *Pseudomonas fluorescens*, *Pseudomonas putida*, *Shewanella putrefaciens, Achromobacter, Acinetobacter, Psychrobacter* and *Flavobacterium* isolates were found (Gennari et al., 1999). Fish from warm waters mainly carry Gram-positive bacteria such as coryneforms, micrococci and *Enterobacteriaceae* as the predominant Gram negative micro flora (Gennari et al., 1999). *Shewanella* and *Pseudomonas* are the predominant microorganisms in seafood stored on ice under aerobic conditions regardless of its origin (Gram and Huss, 1996, Ordóñez et al., 2000). Vennemann et al. (1994) isolated *Moraxella, Pseudomonas, Corynebacterium* and *Micrococcus,* as the initially bacteria from Cape hake and deep water hake; while *Pseudomonas* species became the predominant spoilage bacteria after the fish was stored on ice. Kyrana and Lougovois (2002) found *Vibrio* species and *Enterobacteriaceae* instead of *S. putrefaciens* as the major spoilage organism on sea bass stored at ambient temperatures especially when the fish were harvested from polluted water.

Factors that influence these microbial identities including contamination from the environment are the intrinsic factors (water activity, pH, nutritional composition of the fish, and redox potential), the extrinsic factors (temperature, surrounding environment in packages), the processing factors (lightly versus heavily preserved fish, slicing and grinding, the methods of preservation) and implicit factors (conditions of storage, transport and distribution), the biochemical reactions within the residual groups, and their interactions; (synergism, antagonism) (Gram, 1993; Gram and Dalgaard, 2002; Gram and Huss, 1996; Huis in't Veld, 1996; Tryfinopoulou et al., 2002).

Enterobacteriaceae counts are used as an index to measure the degree of sanitation in food. High numbers of this group of bacteria in food is believed to be as result of unsanitary handling or temperature abuse. The number detected in a particular food does not always correlate with the extent of contamination from the original source, due to the ability of the bacteria to growth at varying rates in different food commodities (ICMSF, 1978). The coliform group is not well defined and results obtained can vary depending on the specimen, medium used, incubation temperature and methods used to read results. The use of *Enterobacteriaceae* test was therefore introduced due to the discrepancies associated with the use of coliforms as indicators of safety in foods (ICMSF, 1978).

2.3.2 Microbial safety of fish

Apart from spoilage the safety of seafood also has to be controlled in terms of the presence of possible food–borne pathogens such as the human pathogenic *Vibrio* species, *Clostridium botulinum*, *Aeromonas hydrophyla*, *Bacillus cereus*, *Salmonella* spp. *Yersinia enterocolitica*, *Listeria monocytogenes*. In 1978, 10.5% of all disease outbreaks and 3.6% of all cases of seafood diseases in the United States of America were linked to the consumption of both shellfish and fin fish (Wekell et al., 1994). *Vibrio* species are of major concern in seafood as they occur naturally in marine environments and will be discussed in more detail in the following section. Human pathogens of exogenous origin in seafood include *E .coli*, *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Campylobacter* spp. and *Bacillus cereus* (Wekell et al., 1994) These pathogens may come into contact

with fish through faecal contaminated water, as a result of poor personal hygiene or through cross contamination with other contaminated foods such as rice. *Staphylococcus aureus* may contaminate fish during handling and may grow in cooked or processed fish due to lack of competition from spoilage organisms.

2.3.3 Biogenic Amines

The proteinaceous nature of fish favours formation of biogenic amines some of which may cause intoxication in humans (Karovičová and Kohanjdová, 2005; Reviewed by Santos, 1996). Some biogenic amines (agmatine, spermine and spermidine) can react with nitrites to form pro-carcinogenic nitrosamines (Santos, 1996). *Proteus morganii*, *Klebsiella pneumoniae, Klebsiella oxytoxa, Hafnia alvei, Staphylococcus hominis* and *Enterococcus hirae* produce histamine at a fast rate and are important in the microbiological quality of fish., while *Photobacterium phosphoreum* and *Photobacterium damsela* are psychrotolerant and mesophiles respectively that produce biogenic amines in scombroid fish species at low temperatures and at ambient temperature (Economou et al., 2007; Kanki et al., 2007; Santos, 1996). Another adverse effect of biogenic amines to humans is the triggering of allergic reactions and scombrotoxicosis of which histamine is the primary mediator. Scombroid poisoning is, however, a rare disease in properly handled fish. The amine is resistant to freezing and cooking (CDC, 2007; Economou et al.*,* 2007).

The most common cause of histamine accumulation in fish muscle is temperature abuse during fish harvesting, processing, transport and storage e.g. when the fish is held at temperatures above $7^{o}C$ for several hours (Auerswald et al., 2006; CDC, 2007; Economou et al., 2007). The levels of allowable histamine concentrations in food range from 50–200ppm in Australia and USA respectively (Auerswald et al., 2006).

2.4 *VIBRIO* SPECIES AS FOOD-BORNE PATHOGENS

2.4.1 Introduction

Species of the genus *Vibrio* belong to the Phylum *Proteobacteria* and class *Gamma Proteobacteria* (Farmer et al., 2004). They occur naturally in large numbers in aquatic environments including fresh and coastal marine habitats such as oceans, rivers and estuaries. They are also commonly found as commensals on the surfaces and in the digestive tracts of fish and zooplankton (Drake et al., 2007; Montanari et al., 1999).

Vibrios are responsible for a number of clinical conditions such as cholera, gastroenteritis, septicaemia and wound infections (Jay et al., 2005; Oliver and Kaper, 1997; Thompson et al., 2004).Twelve *Vibrio* species have been documented as potential food-borne disease agents in humans: *V. cholerae*, *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. funissii, V. fluvialis, V. damselae, V. mimicus, V. hollisae, V. cincinatiencis, V. harveyi* and *V. metchnikovii* (Adams and Moss; 2008; ICMSF, 1996; Jay et al., 2005; Thompson and Swings, 2006). A few species are pathogens of fish, while some other species are involved in coral bleaching (Thompson et al., 2004).

Vibrio species are transmitted to humans mostly via sewage contaminated water or seafood (fish, molluscs and crustaceans) when consumed raw or partially cooked (DePaola et al., 2000; Feacham 1981; ICMSF, 1986; ICMSF, 1996; Joseph et al., 1982;; Oliver and Kaper, 1997). Though *Vibrio* species have been isolated from marine environments, poor processing practises are regarded as the major cause of the food contamination (Kaysner et al., 1992). The organisms may also be introduced during and after processing by food handlers in factories. The bacteria may persist in the food depending on storage temperatures, pH and the product water activity (ICMSF, 1996) until the food is consumed, thereby causing disease. Pathogenic *Vibrio* species are a health concern especially in fish harvested from poor quality waters (ICMSF, 1986). The levels of these pathogens in shellfish and in water does not correlate with the level of

indicator organisms, necessitating direct detection of each species in order to ensure public health and food safety (Harwood et al., 2004).

2.4.2 Pathogenic *Vibrio* species associated with food

2.4.2.1 *Vibrio cholerae*

V. cholerae is the most commonly occurring pathogenic *Vibrio* species, followed by *V. parahaemolyticus*. Cholera is characterised by profuse watery diarrhoea with flakes and mucus, dehydration and sometimes death when adequate medical intervention is not instituted (Adams and Moss, 1995; Jay et al., 2005; Kaper et al., 1995). Huq et al. (1990) have suggested that *V. cholerae* is wide spread in estuarine and marine waters around the world, although the numbers may be low in sea water throughout the year (1-10cfu/litre). The bacteria are found in areas where salinity is between 4-17% and their presence does not correlate with either *E. coli* or *Salmonella*. They establish symbioses with planktons as a means of overcoming low temperatures that prevail during winter in temperate regions (Adams and Moss, 1995; Huq et al., 1990; Montanari et al., 1999).

The bacterium is sensitive to high temperatures, higher than 45°C, and to many disinfectants used in the food industry. Studies elsewhere on the survival patterns of *V. cholerae* in different food types have shown that the bacteria remained viable for 14 days in refrigerated raw vegetables and at room temperature (28-30°C) for 28 days. On dry cereals (maize, rise and biscuits) the survival time was 1-5 days at 4°C. On the same cooked food item survival times were found to increase up to 14 and 24 days for room temperature and refrigeration storage respectively. The survival times where shown to be longer under refrigeration conditions, with 4-9 days in raw, and 2-21 days in cooked fish (ICMSF, 1996).

2.4.2.2 *Vibrio parahaemolyticus*

V. parahaemolyticus associated gastroenteritis manifests by profuse, watery diarrhoea free from blood and mucus, abdominal cramps, nausea, and vomiting (Joseph et al.*,* 1982; Adams and Moss, 1995; Jay et al., 2005). Outbreaks of *V. parahaemolyticus* food poisoning are associated with consumption of raw molluscs (oysters, clams), cooked crustaceans (shrimp, crab and lobsters) in America and Europe, but in Japan, South East Asia, India and Africa raw fish is always implicated as a vehicle.

Vibrio parahaemolyticus is usually resident in coastal waters (Adams and Moss, 2008). It is halophylic requiring 3%-10% NaCl for growth. It is distinguished from other *Vibrio* species by its inability to ferment sucrose. It is mesophilic hence isolated only in summer in temperate regions, but in warm water (19-20°C), it is detected throughout the year (ICMSF, 1996). It is mostly associated with crustaceans such as shrimp and crab, but it is also found in molluscan shell fish and free swimming fish at a concentration of 10^2-10^3 cells per gram, while in very warm water there may be 10^6 cfu per gram. About 98% of the bacteria isolated from marine animals and seawater are Kanagawa negative and non pathogenic (ICMSF, 1996). It is rarely isolated in water where temperatures are below 15°C (Matches et al., 1971). The bacteria are moderately sensitive to freezing and can persist in frozen food for long periods (Vasudevan et al., 2002). *Vibrio parahaemolyticus* is very sensitive to heat (killed at 47°-60°C) and to ionizing radiation, as well as to halogens (Adams and Moss, 2008). Food poisoning associated with this organism is said to arise from gross mishandling during preparation leading to cross contamination between raw and cooked food and from temperature abuse (Nickelson and Finne, 1992).

Vibrio parahaemolyticus has been isolated from shellfish (Bauer et al. 2006; DePaola et al., 1990; DePaola et al., 2000; Drake et al., 2007; Hervio-Heath et al., 2002; Vieira and Iaria, 1993; Yang 2008). The levels in oysters correlated with the levels in corresponding waters (DePaola et al., 1990) pointing at the filter feeding habit as the way by which the bacteria enter the oysters. *V. parahaemolyticus* was also detected in lobster samples

collected from a supermarket and from a fish processing factory at 3-21 cfu/g (Vieira and Iaria, 1993). The survival of these organisms in lobster samples from super markets was mostly as result of inadequate processing methods used, post process contamination and temperature abuse as most samples showed signs of decomposition before samples were taken. Some of environmentally isolated *V. parahaemolyticus* were Kanagawa test positive (Vieira and Iaria, 1993). The minimum levels of detection for *V. parahaemolyticus* in chilled or raw crustaceans are 10^3/g (Vieira and Iaria, 1993). *Vibrio parahaemolyticus* and *V. vulnificus* have been isolated from bivalve molluscs, but their numbers did not correlate with the number of coliforms (Normanno et al., 2005).

2.4.2.3 *Vibrio vulnificus*

V. vulnificus is regarded as an emerging pathogen, infection in humans was first reported in 1964 in the USA and in 1987 in Taiwan (Harwood et al., 2004; Hsueh et al., 2004). It is an opportunistic pathogen in the elderly, immunocompromised or in individuals with impaired liver function, or underlying disease such as, diabetes mellitus or those on steroid therapy (Drake et al., 2007; Harwood et al., 2004). Infections are usually acquired through consumption of raw or improperly cooked shellfish or through contact with seawater (Hsueh et al., 2004). *V. vulnificus* causes three important disease syndromes; septicaemia, necrotising wound infections and gastroenteritis with a mortality rate of 40-50% occurring one to two days after onset of the symptoms (Harwood et al., 2004; Hsueh et al., 2004).

V. vulnificus is a common inhabitant of seawater, but the levels are not correlated with those of indicator organisms (Harwood et al., 2004). Hsueh et al. (2004) have shown that the bacteria are most prevalent in seawater during summer when the temperatures are between 26^oC–29^oC. It occurs in environments with salinity of 0.5–2.5% (Harwood et al., 2004). *V. vulnificus* is sensitive to low pH's and acid treatment is suggested for effective control of the survival of this pathogen in seafood (Lee et al., 1997). *V. vulnificus* is found in high numbers in shellfish. Tamplin and Capers (1992) have demonstrated the presence of *V. vulnificus* (10^3–10^5 cfu /mℓ) in sea water and (10^2 and 10^3 cfu/g) oyster

samples collected from the Gulf of Mexico. They are also found associated with other shellfish, (clams, and mussels) fish, sediments and planktons which are believed to act as reservoirs (Harwood et al., 2004). The USA Interstate Shellfish Sanitation Conference has set a limit of 30 *V. vulnificus* per gram oyster (Harwood et al., 2004). Tamplin and Capers (1992) showed that the organisms could not be cleared from the oyster tissues by normal depuration procedures when UV treated and filtered water was used.

2.4.2.4 *Vibrio alginolyticus.*

Vibrio alginolyticus is largely an opportunistic pathogen causing systemic infections in persons with underlying diseases such as the immunocompromised individuals, those with severe burns, cancers or with a history of alcohol abuse (Oliver and Kaper, 1997), though it has occasionally been associated with cases of gastroenteritis and diarrhoea. In healthy individuals *V. alginolyticus* is associated with extra intestinal infections such as wound or ear infections. The bacterium was also isolated from the blood of a leukaemia patient alongside *Pseudomonas aeruginosa* (Oliver and Kaper, 1997). *V. alginolyticus* is also an important food spoilage organism producing histamine by the decarboxylation of histidine and is responsible for scombroid poisoning characterised by nausea, vomiting, abdominal cramps, neurological disorders and skin irritations (Ray and Bhunia, 2008).

Vibrio alginolyticus is the most commonly isolated *Vibrio* species in marine environments from all over the world. Its numbers correlate with increases in temperatures (Oliver and Kaper, 1997). *Vibrio alginolyticus* has been isolated from both fin fish and shell fish. Hervio–Heath et al. (2002) had isolated *V. alginolyticus* as the most predominant *Vibrio* species from mussels and water samples from the coastal areas in France. Di Pinto et al. (2006) analysed 38 shellfish samples and detected *V. alginolyticus* from 76% of those samples while only 42% of their samples were positive for *V. parahaemolyticus.* Gonzales–Escalona et al. (2006) and Xie et al. (2005) detected *V. parahaemolyticus* virulence associated genes in some *V. alginolyticus* strains.

2.4.3 Isolation of pathogenic *Vibrio* species

Detection of *Vibrio* species in food and water typically relied on isolation of the bacteria followed by identification by means of classical biochemical tests (Croci et al., 2007; Harwood et al., 2004) *Vibrio* species are non fastidious and grow readily on basic laboratory media, but some need supplementation of vitamins, amino acids and minerals (Thompson et al.*,* 2004, Farmer and Hickmann-Brenner, 1991). They grow better at alkaline pH (7.5-8.5) and require added NaCl. The optimum growth temperature ranges from 15°-30^oC (Thompson et al.*,* 2004).

Most *Vibrio* species grow on Mac Conkey agar, but do not ferment lactose (Farmer et al., 2004). Isolation of *Vibrio* species from environmental sources usually is done by a pre-enrichment step in alkaline Peptone Water (APW), pH 8.6 supplemented with 1-2% NaCl (Harwood et al., 2004; ICMSF, 1978; Kaysner et al., 1992), followed by plating on a solid growth medium such as Thiosulphate Citrate Bile salts Sucrose (TCBS) agar. Enrichment media are normally incubated at room temperatures, (18–22^oC), while solid media are incubated at 25^oC (Farmer and Hickmann-Brenner, 1991).

TCBS is a selective differential media that incorporates bile salts, alkaline pH (8.6) and 1% NaCl as selective agents, sucrose as a fermentable sugar and bromothymol blue as the pH indicator (Farmer and Hickmann-Brenner, 1991; Harwood et al., 2004). On TCBS sucrose fermenters form yellow colonies, while non-sucrose fermenters are green (Farmer and Hickmann-Brenner, 1991; Kaysner et al., 1992). *Enterobacteriaceae*, *Pseudomonas* and Gram positive bacteria are inhibited on TCBS (Harwood et al., 2004). The problems encountered with TCBS are that some species do not grow well on it, the selectivity and performance of the medium may vary from batch to batch or between manufacturers (Sakazaki and Ballows, 1991), bacteria other than *Vibrios* may grow on it (Farmer and Hickmann- Brenner, 1991; Harwood et al., 2004) and sometimes TCBS may be too inhibitory for some species (especially *V. vulnificus*) and may reduce the amount of *Vibrio* species isolated compared to non selective media. Other media used in the

isolation of *Vibrios* include Tryptone Soy Agar to which 1-2% NaCl is added and Marine Agar. Luria-Bertani (LB) broth is used for the enrichment of psychrotrophic species (Thompson et al., 2004). Lee et al. (1997) showed that Brain Heart Infusion broth (BHI) was a better enrichment medium for *V. vulnificus* than L-B broth, Cellobiose Polymyxin– B Colistin (CPC) broth or Alkaline Peptone Water (APW).

The media that best suit isolation of *V. vulnificus* from shellfish and other environmental sources include amongst others *Vibrio vulnificus* (VV) agar , CPC agar and its modification, as well as Sodium Dodecyl Sulphate-Polymyxin-B - Sucrose (SPS) agar (Harwood et al., 2004).

2.4.4 Identification of *Vibrio* species

2.4.4.1 General taxonomy

The taxonomy of *Vibrios* was initially based on the classical methods of classification, identification and nomenclature where morphological features (cell shape and presence of extracellular appendages such as flagellae) and biochemical reaction played an important role (Thompson and Swings, 2006). The current taxonomy of *Vibrios* is based on the polyphasic approach that includes phenotypic and molecular methods (Arias et al., 1997; Thompson and Swings, 2006).

The DNA–DNA hybridisation and phylogenetic relationship studies based on the 16S rRNA comparison has been extensively used in *Vibrio* classification (Thompson and Swings, 2006). However the latter method has not been very successful in delineating *Vibrios* to species level as most *Vibrio* species have more than 90% 16S rDNA similarities (Aznar et al., 1994).

Multilocus Sequence Analysis (MLSA) is currently used to provide better differentiation of *Vibrio* isolates into respective species (Thompson and Swings, 2005). Although the current family *Vibrionaceae* comprises eight genera (Thompson et al., 2004) the

phylogenetic analysis of this group based on the concatenated genes viz., 16S rRNA, *recA* and *rpoA* has proposed four different families e.g. *Vibrionaceae*, *Photobacteriaceae*, *Enterovibrionaceae* and *Salinivibrionaceae* (Thompson and Swings, 2006). The species pathogenic to humans will, however, remain within the genus *Vibrio*.

2.4.4.2 Phenotypic identification

The initial phenotypic traits used for the identification of *Vibrio* species are the Gram reaction, oxidase test where *Vibrios* are always positive and the oxidation/fermentation (OF) test in which *Vibrio* species are facultatively fermentative. *Vibrio* species can be differentiated from one another and from *Aeromonas* species by the sensitivity test to the *Vibrio* static agent O/129 (Famer et al., 2004). Miniaturised biochemical test systems such as API 20E, and Biolog can also be used for final identification. These methods are, however, often slow and unreliable, since some stains exhibit atypical phenotypic characteristics. More rapid and precise means of detection have been sought. One of the main obstacles in species identification is to correctly differentiate *V. alginolyticus* from *V. parahaemolyticus*. *V. alginolyticus* has 60-70 % DNA homology with *V. parahaemolyticus*, and was initially classified as a biotype of *V. parahaemolyticus*. The two species can only be differentiated on the basis of a few phenotypic characters (Farmer et al., 2004; Oliver and Kaper, 1997) as shown in Table 2.1.

2.4.4.3 Immunological based methods

Immunological methods are based on the reaction of antibodies with specific antigens to form immune complexes. Serotyping has been used as a tool for the terminal confirmation step during the identification of human pathogenic *Vibrio* species, especially *V. cholerae*. *V. cholerae* is divided into a number of serovars with O1 being the most important. The O1 strains are highly pathogenic and have so far caused seven cholera pandemics. Strain O139 Bengal is a non O1 strain first isolated in 1992 from the coastal waters of the Bay of Bengal during a cholera epidemic in India, Bangladesh, and in Thailand and is also of concern. The same technique was used in typing the pathogenic

strains of *V. parahaemolyticus* O3:K6 and O1: K UT (Iida et al., 2001; Myers et al., 2003). DePaola et al. (2003) used species specific antisera to differentiate pathogenic strains of *V. parahaemolyticus* into 27 serotypes which were also confirmed by multiplex PCR as being pathogenic.

Table 2.1 Phenotypic traits used to differentiate between *V. parahaemolyticus* and *V. alginolyticus*. (Farmer et al., 2004; Oliver and Kaper, 1997)

Phenotypic test	*Vibrio parahaemolyticus*	*Vibrio alginolyticus*.
Voges-Proskauer (VP) test in1% NaCl	80-95%	0%
Urea hydrolysis	15%	0%
Cellobiose	5%	3%
Dulcitol	3%	0%
Sucrose	1%	99%
ONPG	5%	0%
L- Arabinose	80-89%	0-1%
Growth in Nutrient broth with 10% 12%	0-2% 0-1%	69-100% 17-100%

2.4.4.4 DNA based methods

2.4.4.4.1 Hybridisation

Oligonucleotide probes are used to detect complementary genes or gene fragments in cultures as a means of identifying these cultures. Probes directed to the variable region of the 16S rRNA gene have been developed, but these were not very useful for *Vibrio* species identification partly due to cross reactions of some probes with strains other than their specific targets and the specificity of some probes had not yet been tested across the whole *Vibrio* genus. This may be problematic as some *Vibrio* species may share 100%

16S rDNA homology (Thompson et al., 2005). With the advent of PCR technology the use of hybridisation has largely been replaced.

2.4.4.4.2 PCR detection of unique gene fragments

Several methods employing *in vitro* amplification of specific gene fragments by the Polymerase Chain Reaction (PCR) and derivatives of this method have been used for the identification of *Vibrio* species. PCR technique exploits the specificity of short synthetic DNA fragments to bind to complementary sequences and the ability of the DNA polymerase enzymes to directly synthesize the opposite strand under a defined set of conditions using the available DNA as a template (Cha and Thilly, 1995). The process is robust, specific and fast hence enabling detection of target genes, gene sequences or specific DNA sequences in test samples.

Hoshino et al. (1998) developed a multiplex PCR consisting of three primer pairs targeting the *rfb*) (gene region specific for O1 and O139) and the cholera toxin (*ctxA*) gene. The *rfb* gene based PCR could detect up to 65 and 200 O1 and O139 cfu per assay in clinical samples respectively. Keasler and Hall (1993) designed a multiplex PCR simultaneously detecting the cholera toxin (*ctxA*) gene in pathogenic and environmental *V. cholerae* O1 Classical and El Tor biotypes and differentiating the two biotypes through their differences in the toxic co-regulated pilus (*tcpA)* genes. Theron et al. (2000) developed and evaluated the performance of a seminested ctxAB gene specific PCR for the detection of pathogenic *V. cholerae* in environmental water and drinking water sources. This protocol was shown to be highly sensitive, specific and rapid producing results within 10 hours.

In a quest for a broader spectrum detection protocol due to the fact that non-epidemic strains could also cause disease, the outer membrane protein (*ompW*) gene was targeted. This gene forms part of the *tox*R regulon and was shown to be present in all *V. cholerae* strains and conserved across different biotypes and serogroups, but absent from all other *Vibrio* species studied (Nandi et al.*,* 2000). Oligonucleotide primers specific to the *ompW*

gene were therefore designed and tested for their ability to amplify the specific gene in both clinical and environmental strains. The specificity of these primers was confirmed using DNA probes (Nandi et al., 2000). Le Roux et al. (2004) have evaluated and identified a combination of three primers for the detection of *V. cholerae* ompW gene in environmental isolates. Their work has shown that the PCR approach is more specific than the API 20E and VITEK 32 systems in identifying environmental *V. cholerae* strains.

Other approaches that have been followed include a multiplex Real Time (RT- PCR) targeting four *V. cholerae* potential virulence genes (Gubala, 2006) and a similar fourplex Real Time PCR targeting *V. cholerae* specific genes e.g. repeat in toxin (*rtxA*), extracellular secretory protein (*epsM*), the toxic co-regulated pilus A (*tcpA*) and *ompW* gene with a view to enable detection of both toxigenic and non toxigenic strains (Gubala and Proll, 2006). More recently Fykse et al. (2007) designed real time nucleic acid sequence based amplification (NASBA) PCR that amplifies specific RNA for a number of virulence factors and housekeeping genes. The NASBA is an improved method that detects only actively metabolising cells, as opposed to DNA amplification that might have originated from dead cells.

A number of *V. parahaemolyticus* genes have been used as targets to develop species specific PCR based detection. These genes include the thermolabile direct haemolysin, *tl* (Baffone et al.*,* 2006; Croci et al., 2007), the thermostable direct haemolysin, *tdh*, the thermostable direct haemolysin related haemolysin, *trh* (Baffone et al.*,* 2006*)*, the phosphatidyl serine synthetase gene (pR72H fragment) (Lee et al., 1995), the gyrase B gene (Venkateswaran et al., 1998), the metalloprotease gene (Luan et al., 2007) and the collagenase gene (Di Pinto et al., 2006). Venkateswaran et al, (1998) cloned and sequenced the gyrase B (*gyrB*) gene of *V. parahaemolyticus* and of its close genetic relative, *V. alginolyticus*. They (Venkateswaran et al., 1998) subsequently developed oligonucleotide primers (Vp-1 and Vp-2r) that amplify a 285bp fragment from the *V. parahaemolyticus gyrB* gene by PCR. All *V. parahaemolyticus* strains were recognised by this primer set, and false positives were not detected. Lee et al. (1995) developed a

pair of oligonucleotide sequences (Vp32 and VP33) that bind to opposite ends of a 320 - 387bp DNA fragment termed *pR72H*, from the chromosome of *V. parahaemolyticus* that was shown to be found only in this species. This is a fragment of unknown function located after an rRNA operon and composed of a non coding region and a phosphatidyl serine synthetase gene that was found conserved in *V. parahaemolyticus* (Lee et al., 1995; Robert-Pillot et al., 2002). Hervio–Heath et al. (2002) used the *V. parahaemolyticus* (Vp32/Vp33), the *tdh* and the *trh* specific primers to identify suspect isolates and to determine their pathogenicity respectively.

Kim et al. (1999a) developed *V. parahaemolyticus* specific primers that detected the species specific Tox–R gene. Low amplification signals were, however, also obtained with closely related species, *V. alginolyticus* and *V. vulnificus*. They (Kim et al., 1999a) recommended that detection of this gene as an identification tool should be supplemented with screening the suspect isolates for *V. parahaemolyticus* virulence specific genes (*tdh* and *trh*) so as to confirm the results. Luan et al. (2007) developed PCR primers specific for the *V. parahaemolyticus* metalloprotease gene and used 101 bacterial strains, 85 of which were identified by phenotypic methods as *V. parahaemolyticus* to assess the specificity of their new primers. When the specificity and sensitivity of the new primer *VPM1* and *VPM2* were compared to three other primer sets that were already in use (including primer pairs directed against three other known virulence genes; (*tl, tdh* and *trh*) the designed metalloprotease specific primer pair gave the best results with a sensitivity of up to 4pg DNA. Unlike the L-*tdh*/R-*tdh* and L-*trh/*R-*trh* primer sets that gave false negatives, *VPM1*/*VPM2* detected all the *V. parahaemolyticus* strains tested, and did not react with other bacteria. Reverse transcriptase (R-T) PCR for *tdh, trh1* and *trh2* have also been designed (Mothershed and Witney, 2006).

Di Pinto et al. (2006) used three oligonucleotide primer pairs specific for either *V. parahaemolyticus*, *V. cholerae* or *V. alginolyticus* collagenase gene and have demonstrated a simultaneous detection of the two species (*V. parahaemolyticus* and *V. alginolyticus*) that were present in alkaline peptone water (APW) enriched shellfish tissue homogenates. In addition the researchers recommended the use of these primer pairs in

discriminating between *V. alginolyticus* and *V. parahaemolyticus*. This multiplex-PCR was able to detect the presence of these two bacterial species in some culture negative samples circumventing the low sensitivity inherent in culturing and the inability of these phenotypic tests to identify isolates with atypical biochemical profiles. Qian et al. (2008) cloned two proteins from *V. alginolyticus* (*OmpK* and *OmpW*) expressed them in *E. coli* and designed specific primers to the genes. More recently Dalmaso et al. (2009) designed a multiplex primer extension reaction (PER) PCR protocol directed against *rpoA* gene to simultaneously detect and identify six human pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *V. fluvialis*) in fishery products.

The gyrase B gene was also targeted (Kumar et al., 2006) for *V. vulnificus* identification using the primer set *gyr-vv1* and *gry-vv2*. Arias et al., (1995) developed a highly sensitive nested PCR specific for the 23S rDNA of *V. vulnificus* and Lee et al. (1998) developed another nested PCR directed against the *V. vulnificus* haemolysin gene (*vvh*). Real Time PCR (RT-PCR) was later developed to detect *V. vulnificus* in sea water and oyster tissue homogenates targeting the species specific *vvh* gene (Panicker et al., 2004). Chakraborty et al. (2006) developed a species specific PCR targeting the *ToxR* gene of the less characterised human pathogen, *Vibrio fluvialis* facilitating a successful differentiation of this pathogen from the closely related *Aeromonas* species.

2.4.4.4.3 Molecular typing techniques

Various molecular techniques have been used to type the strains belonging to the various *Vibrio* species. These methods include Amplified Fragment Length Polymorphism (AFLP), Rapidly Amplified Polymorphic DNA (RAPD), Restriction Fragment length Polymorphism (RFLP), Ribotyping, Repetitive Extragenic Palindromic (Rep) sequences and Multilocus Sequence Typing (MLST). Most of these techniques could also be used for the identification of specific *Vibrio* species. This is, however, only possible once a database containing the various type strains and other important reference isolates has been created (Thompson and Swings, 2006).

2.4.4.4.4 Sequencing of 16 S rDNA

The traditional methods for the identification of bacteria uses phenotypic traits; structure and morphology, biochemical and physiological behaviour, ecological characters and antigenic make up. The use of artificial growth media for isolation was examined (Lim, 1998). These methods are slow, labour intensive, expensive and in some cases inaccurate. Modern methods of identification that compare nucleotide sequences of group specific genes have gained popularity within the last three decades. The 16S rRNA gene is used for both phylogenetic studies and as a taxonomic marker (Thompson et al., 2005). Typically the whole gene or variable regions of the 16S rRNA gene are sequenced through the use of various primer pair combinations. Ideally comparison of the obtained nucleotide sequences with the sequences available in the GenBank, BCCM or other gene banks facilitates identification of unknown isolates. The 16S rDNA PCR method increases the efficiency of bacterial identification due to its rapid, reproducible nature (Coenye et al., 1999). This notion was supported by Petti et al. (2005) who showed that the 16S rDNA sequencing was able to correctly identify bacteria including pathogens that had been misidentified by traditional methods. Harris and Hartley (2003) developed a broad range 16S rDNA PCR for use in identifying bacteria isolated from various clinical specimens and compared the results with cultural and serological methods. PCR amplification of the 16S rDNA detected many potentially pathogenic organisms from culture negative samples implying that the 16S rDNA PCR improves the identification process as compared to cultural methods. Complete 16S RNA gene sequences of many V*ibrio* species have been determined and *Vibrio* species 16S rRNA gene specific primers and probes have been developed, as cited by Maeda et al. (2003) who developed a clustering scheme based on *Vibrio* species 16S rRNA gene specific PCR that clustered 46 *Vibrio* species into 16 groups.

There are also several problems associated with the use of the 16S rDNA for identifying bacteria. No universally accepted criteria exist for the required level of homology to delineate isolates of the same species or genus. Some bacteria with different phenotypic characters may share up to 100% 16S rDNA sequences whereas less than 99% 16S rDNA

sequence homology has been observed in bacteria belonging to the same species (Harris and Hartley, 2003). In some species multiple heterogenous copies of the 16S rRNA gene operons exist (Case et al., 2007, Pontes et al.*,* 2007). *Bacillus subtilis* has ten, *E. coli* and *Salmonella* Typhimurium have seven, *Clostridium perfringens* and *V. cholerae* have nine, *Mycobacterium* species have two and *Nocardia* species have 3-5 copies (Conville and Witebsky, 2007), *V. parahaemolyticus* has eleven (Harth et al.*,* 2007). Another serious problem is that the databases used in sequence comparison for identification are public facilities hence sequences that are incorrectly identified may also be erroneously published and could lead to misidentification of accurately read sequences.

2.4.4.4.6 Multilocus sequence analysis

With the problems experienced with the prokaryotic identification schemes (phenotypic traits) and 16S rRNA gene sequences, as well as with the DNA- DNA hybridisation (DDH), multilocus sequence analysis (MLSA) is gaining popularity as a promising taxonomic tool to differentiate between closely related bacterial species. MLSA uses gene sequences from more than one locus, generally of protein coding genes that are widely distributed among bacteria to be studied and have single copy in the genome (Pontes et al., 2007, Thompson et al., 2005). The genes should also be long enough to give sufficient information, but should be of the length that permits easy sequencing (Thompson et al., 2005).

The genetic loci that were initially found suitable in MLSA for taxonomic studies of *Vibrio* species included the 16S rRNA gene, *rpoA* (RNA polymerase alpha subunit) , *recA* (recombinant repair protein) and *pyrH* (uridylate kinase gene) (Thompson et al.*,* 2005). Recently Thompson et al. (2007) have developed an MLSA scheme to distinguish *Vibrio harveyi* from the closely related *Vibrio campbellii* species using seven housekeeping genes; *recA*, *topA* (topoisomerase I), *pyrH, ftsZ* (a cell division protein), *mreB* (actin like cytoskeleton protein), *gapA* (glyceraldehydes-3 phosphate dehydrogenase) and the *gyrB* DNA (gyrase B gene beta subunit). The resulting 3596 nucleotide long DNA gave a better resolution than when only three genes; *gyrB, reA* and

gapA were used. MLSA therefore acts as a buffer against mutations and horizontal gene transfer (HGT) problems associated with the 16S rDNA sequences (Thompson et al., 2005).

2.5 CONTROL OF BACTERIAL CONTAMINATION DURING FISH PROCESSING.

2.5.1 Introduction

Seafood contamination occurs naturally from the environment where the fish is harvested, during harvesting, processing or during food preparation. Cross contamination may occur during food processing or preparation where bacteria are transferred from raw fish and/or contaminated surfaces and/or from utensils to hygienically safe seafood (Wekell et al., 1994). During processing contaminated water may also introduce microorganisms including pathogens into the food. In some cases the levels present in the food may not be critical as to pose a health hazard to consumers. Improper methods of handling (poor general and/or personal hygiene) and distribution (time-temperature abuse) (Wekell et al., 1994) may provide ideal conditions for the pathogens to proliferate and reach infective levels. A number of pathogens belonging to different microbial taxa are predominant in marine environments and find their way into seafood. These include *Aeromonas, Plesiomonas*, *Clostridium botulinum*, *Listeria monocytogenes* and *Vibrio species* (Wekell et al., 1994).

The first line of control of the presence of bacterial pathogens in seafood is the use of Good Manufacturing Processes (GMP) (Vasconcellos, 2004a). Secondly most seafood processors have an effective safety and quality assurance system in place in most cases based on the Hazard Analysis and Critical Control Points (HACCP) principles (Vasconcellos, 2004b; Ward, 2002). Several methods to remove, reduce, kill or inhibit the growth of pathogens in seafood and water are employed and one of the main functions of the HACCP system is to ensure the effectiveness and robustness of these methods. Proper sanitation of the food processing environment (manufacturing plant, surfaces where the food is handled, all equipments and utensils, materials used as

additives and preservatives), using the right sanitizers in correct concentrations, upholding the personal hygiene of the personnel and training in sanitation methods, as well as the use of potable clean water in all cleaning and sanitation procedures are paramount to keeping the initial microbial load to the minimum. Once this goal has been achieved other methods to be used in successive steps will be successful in reducing or eliminate spoilage organisms and pathogens to acceptable limits hence attaining the desired shelf life and safety of the final product (Ray and Bhunia, 2008).

2.5.2 Role of water in the processing of frozen fish

In fish that is only filleted, deboned, packaged and frozen before distribution exposure of the fish to water constitutes one of the critical steps that must be optimally controlled in order to prevent the introduction of pathogens. The water used in the processing of the fish needs to be clean with low levels of bacteria and free of pathogens. For this purpose it is essential that biofilm formation is minimized to ensure optimal microbial quality of the water during distribution and use.

2.5.3 Biofilm formation in water distribution systems

Biofilms are hydrated, (85–95% water) (Chmielewski and Frank, 2003) multicellular structures occurring in oligotrophic environments as a result of the adhesion, growth and metabolism of microorganisms on the surfaces and covered in complex extracellular polymeric substance (EPS) matrix that protect the cells from adverse effects of a changing environment and from antimicrobial agents such as sanitizers, antibiotics and host defence mechanisms (Costerton, 2005; Hall-Stoodley and Stoodley, 2005; O' Toole et al., 2000). Biofilm formation is influenced by a number of environmental factors including nutrient availability (O' Toole et al., 2000), which in water distribution systems may be in a form of biodegradable dissolved organic carbon (BDOC), the water temperature, the concentration of disinfectant (Momba et al., 2002). A nutrient rich environment promotes biofilm formation, but cells tend to detach from such structures

and favour the free swimming form that enables them to scavenge for nutrients during nutrient scarcity (O' Toole et al., 2000).

Once attached cells undergo a series of phenotypic changes that emanate from the gene expression level that enable them to adapt to the new environment and to carry out unusual metabolic activities including secretion of exopolysaccharides that renders them resistant to disinfectants (reviewed by Langsrud et al., 2003; O' Toole et al., 2000; Prigent-Combaret and Lejeune, 1999). Retardation of molecular movement through the biofilm matrix and reduced bacterial growth rates are some of the mechanisms by which resident bacteria mediate resistance to antimicrobial agents (Costerston et al., 1987; Donlan and Costerton, 2002). The EPS may also concentrate nutrients, sequester metals and toxins and protect resident bacteria from desiccation (Chmielewski and Frank, 2003). Chu et al. (2003) using a laboratory scale continuous flow unit demonstrated that though addition of chlorine to the experimental system reduced the number of colony forming units recovered from biofilms, this disinfection process did not completely inhibit biofilm formation.

EPS consist of polysaccharides, proteins, phospholipids, teichoic and nucleic acids (Chmielewski and Frank, 2003). Bacterial biofilms may consist of a single layer of attached cells (Chmielewski and Frank, 2003) or may have defined three dimensional structures with species specific architectural organisation that may (Lawrence et al., 1987) or may not (Lawrence et al., 1991) consist of microcolonies (flocks or aggregates) (Chmielewski and Frank, 2003) interspersed by water channels. The architecture of a mature biofilm depends on the hydrodynamics of the surrounding fluid (Chmielewski and Frank, 2003).

2.5.4 Bacterial pathogens in biofilms

Some bacteria are able to form biofilms in isolation while others establish synergistic associations termed co-aggregates, in which one organism acts as a primary attachment candidate and exopolysaccharide producer providing a favourable environment and protection for the other species (Palmer et al., 2007). Bacterial pathogens can form part of biofilms and pose a challenge to public health and food safety. Biofilms provide a favourable microenvironment for pathogens and act as a stable source of a large number of pathogens while the flow of water facilitates pathogens dispersal in a form of clumps or clusters (Hall-Stoodley, and Stoodley, 2005). Biofilms therefore confer a selective advantage to pathogens by enabling them to persist, multiply and disseminate (Hall-Stoodley and Stoodley, 2005). It is also assumed that the first prokaryotic cells had the propensity to exist in biofilms as the natural stable structures and that free swimming cells evolved for the sake of dispersal in environments (McDougal and Kjellberg, 2006) The ability of the pathogens to grow and form biofilms in vitro depends on the nature of the surface; rough surfaces are more difficult to clean properly than smooth surfaces, on the composition of the growth medium, on genetic factors and the environmental conditions (Reisner et al., 2006). Variations within individual strains of the same species are also common (Reisner et al., 2006).

The bacterial pathogens well known to form biofilms include *Pseudomonas aeruginosa*, *E. coli* (Banning et al., 2003; Dewanti et al., 1995; Reisner et al., 2006), *Vibrio cholerae* (Mueller et al., 2007), *Vibrio parahaemolyticus* (Enos-Berlage et al., 2005), *Listeria monocytogenes* (Kim and Frank, 1995) *Salmonella* (Chmielewski and Frank, 2003), *Legionella pneumophila* (Armon et al., 1997; Carvalho et al., 2007, Marao et al., 1993), many strains of pathogenic and environmental *E. coli* (Reisner et al., 2006) *Campylobacter jejuni* (Buswell et al., 1999) and *Helicobacter pylori* (Mackay et al., 1999).

A number of *Vibrios* including pathogenic species; *V. cholerae* (Faruque et al., 2006), *V. parahaemolyticus* (Enos–Berlage et al., 2005), *V. vulnificus* (Joseph and Wright, 2004) and *V. alginolyticus* (Kogure et al., 1998) are able to form biofilms. *V. cholerae*, one of the most prevalent human pathogenic *Vibrio* species has been shown to form biofilms on both biotic and abiotic surfaces (Mueller et al., 2007). Bacterial attachment to surfaces improves their survival potential, and transmission in a hostile environment. Mueller et al. (2007) demonstrated 30 gene loci in *V. cholerae* that are involved in biofilm formation in addition to the mannose sensitive haemagglutinin factor Type IV pilus. However there is high genetic and phenotypic variability among different strains in their requirements for surface attachment. Enos–Berlage et al. (2005) demonstrated that both opaque and translucent colonies of *V. parahaemolyticus* form biofilms in micro titre plates. *V. alginolyticus* formation of biofilms is mediated by the Na^+ dependent polar flagellum (Kogure et al.*,* 1998). Joseph and Wright (2004) showed that capsular polysaccharides (CPS) inhibit biofilm formation by *V. vulnificus*. The polysaccharides differ from those of *V. cholerae* in chemical composition that affect the net surface charge being highly negative in *V. vulnificus* CPS.

2.5.5 Control of biofilm formation of water used for fish processing

Biofilm control entails prevention of biofilm formation by suspended microorganisms and removal of matured biofilms from food processing equipment, from inside the water pipes and from medical equipment**.** Various disinfectants have been used in food processing plants including ultraviolet light, potassium permanganate, bromine, hydrogen peroxide and permono sulphuric acid, ozone and chlorine (Degrėmont, 1991).

2.5.5.1 Chlorination of water for fish processing

Chlorine compounds are broad spectrum antimicrobials active against vegetative forms of bacteria, fungi, spores and viruses (Ray, 2003). Chlorine acts by oxidising sulfhydryl (-SH) groups of enzymes and structural proteins, by causing damage to cell membranes, by disrupting protein synthesis, reacting with nucleic acids, and interference with total cell

metabolism (Ray, 2003). Liquid chlorine and hypochlorites mediate their germicidal activity through formation of hypochlorous acid (HOCl) that is most potent at acidic pH due its oxidising power, the smaller molecular weight and electrical neutrality (Walker, 1978). In solution at pH above neutrality and ambient temperatures HOCl becomes less effective as it dissociates into H^+ and hypochlorite anions (OCl^-). The OCl^- is less effective as its negative charge seems to interfere with cell penetration (Walker, 1978).

Different forms of chlorine are used as disinfectants, (Sodium hypochlorite) liquid chlorine, chlorine gas (Cl_2), hypochlorites (salt) (CaOHCl), organic and inorganic chloramines and chlorine dioxide (Momba et al., 2002; Ray, 2003). In fish processing only chlorine gas and hypochlorite solutions are typically used (Bonnell, 1994). Three components are important in chlorination regimes; chlorine dosage (amount of chlorine added), chlorine demand (amount of chlorine that reacts with impurities in water) and total residual chlorine (total amounts of chlorine that remains loosely combined with nitrogenous compound and the free amounts of chlorine that remains and are available to kill microorganisms). The recommended level of free residual chlorine is 5–7 ppm for fish processing water and 15–25ppm for surface cleaning (Bonnell, 1994). Residual chlorine should have a retention time of at least 20-30 minutes. When sea water, brine or brackish water is readily available a self contained hypochlorinator that generates NaHOCl through the decomposition of sea water or sodium chloride solution is often more economical for disinfection since the addition of liquid chlorine is not necessary (Walker, 1978). Chlorine concentration decreases in disinfected potable water systems due to a natural decay related to the chlorine demand of the pipe wall itself and the bulk decay which is the water chlorine demand (Momba et al., 2002). The use of chloramines for disinfection in potable water is gaining popularity over chlorine as the use of chloramines generates less toxic compounds (Momba et al., 2002). Chloramines also have a longer half life in water than chlorine especially in waters with high content of organic matter. Chloramines could be safely used in high dosage to prevent development of bad tastes (Momba et al., 2002).

Chlorine compounds suffer several disadvantages, they are less effective in the presence of organic matter and in hard water, less stable at higher temperatures, they are corrosive to metals and can cause discolouration of food through lipid oxidation (Ray, 2003). The use of chlorine and its derivatives is also associated with the formation of toxic and possibly carcinogenic compounds commonly referred to as disinfection by products e.g. trihalomethanes (THM), bromate, haloacetonitriles and haloacetic acid (HAA) formed when chlorine reacts with organic compounds naturally found in water (LeChevallier and Au, 2004; Gopal et al., 2007; Momba et al., 2002; Wang et al., 2007). Chloramines are weak disinfectants and less effective against protozoa, bacteria and viruses hence mostly used as secondary disinfectants. Chloramines produce volatile compounds that impart taste and odour (Gopal et al., 2007; Momba et al., 2002).

2.5.5.2 UV irradiation of water for fish processing

The UV light occupies the 40-400nm region of the electromagnetic spectrum (LeChevallier and Au, 2004). It is produced using low pressure or high pressure mercury vapour lamps. The disinfection system normally consists of a mercury vapour lamp surrounded by a protective coat such as quartz. The device is inserted into a tube or cylinder or can be used in a tank connected to the water supply. In the cylindrical form the water to be disinfected is passed over the UV lamp where the water rapidly absorbs the radiation as it flows past the lamp (Mahapatra et al., 2005).

UV light causes damage to the DNA by cross linking the thymidine bases through a double bond forming dimers that inhibits DNA replication and transcription (LeChevallier and Au, 2004). The cytoplasmic membrane is also affected (Mahapatra et al., 2005). Microorganisms are able to repair damaged DNA by photo reactivation in the presence of light and 'dark repair' in the absence of light. The repair mechanisms compromise the action of the UV light, high doses are therefore necessary to ensure that the process of microbial inactivation has been done beyond repair (LeChevallier and Au, 2004). Also its effect is not long lasting due to the lack of a residual (Degrémont, 1991). Bacteria are more sensitive to UV light than viruses and are killed by exposure to 0.65-31

mW-sec/cm^2. *V. cholerae* is highly sensitive, a 4-log reduction is attained by exposure to 0.65 mW-sec/cm^2. Spores of Gram positive bacteria are most resistant; a 4-log reduction can be effected by exposure to 31 mW-sec/cm^2 (LeChevallier and Au, 2004). The use of UV irradiation in fish processing facilities has not been documented.

2.5.5.3 Ozonation of water for fish processing

Ozone is an allotrope of oxygen that contains three oxygen molecules (O_3) (Degrèmont, 1991). Ozone acts by oxidising microbial cells due to its high positive oxidising potential (+0.27V) (Mahapatra et al., 2005). Ozone has antibacterial, antifungal and antiviral properties (Khadre et al., 2001). The primary target of ozone is the bacterial cell surface. Once in contact with the cell surface its oxidising activity causes damage of essential components in the cell membrane through damage to the double bond in unsaturated fatty acids, the lipoproteins and lipopolysaccharides in the cell wall of Gram negative bacteria by reactive oxygen species. It also damages glycolipids, glycoproteins and nucleic acids present in the cell membrane causing flocculation of cellular proteins, interference with the respiratory system, oxidation of the sulfhydryl groups to form disulfide bonds and damage to DNA primarily of the pyrimidine bases and eventually leakage of cytoplasmic contents (Guzel-Seydim et al.*,* 2004; Kim et al., 1999b). Ozone causes oxidation of amino acids of proteins, enzymes and peptides to shorter peptides; polyunsaturated fatty acids are oxidised to acid peroxides (Guzel-Seydim et al., 2004). Ozone is effective against a wide range of both Gram positive and Gram negative bacteria in both vegetative and spore forms including *E. coli*, *Sh. putrefaciens, Salmonella*, *St. aureus*, *L. monocytogenes* at concentrations of 0.05 - 2 mg/ℓ; against fungi and fungal spores at concentrations of 6–9 mg/ℓ, and against viruses at concentrations of 600 mg/ℓ. It is also effective against bacterial endospores when the relative humidity of the treatment medium is increased to 50% (Guzel-Seydim et al., 2004; Kim et al., 1999b; Mahapatra et al., 2005). In Gram negative bacteria the lipoprotein and lipopolysaccharides are particularly susceptible (Guzel-Seydim et al., 2004).

However the effectiveness of ozone depends of the presence of suspended organic particles and on the pH of the medium. Low pH values enhance the lethality of ozone (Kim et al., 1999b). Ozone is used as a sanitizer of food surfaces for many foods; cheese, eggs, poultry, fruits and vegetables, fish (Kim et al., 1999b) mostly with positive results in extending the shelf life of products and improving the sensory quality such the colour of fish products.

There are three methods for producing ozone. The most widely used method is the electrical discharge method. Dried clean air or oxygen at dew point (-60°C to -80°C) (Degrėmont, 1991) is passed through two high voltage electrodes, also called a corona, ozonator or plasma. The electrodes are in a form of either concentric rings or parallel plates (Chawla, 2006, Mahapatra, et al., 2005, Degrėmont, 1991; Degrėmont, 2007). The high voltage (15000-20000 Volts) (Guzel-Seydim et al., 2004; Walker, 1978) electrical discharge in the form of an alternating current is passed through a small gap (Chawla, 2006; Degrėmont, 1991; Fielding and Bailey, 2005) between the electrodes where the air or gas containing oxygen is present. This high electrical field causes electron excitation and the formation of unstable oxygen radicals (O^{-}), which spontaneously combine with intact oxygen molecules to form Ozone (O$_3$) (Degrėmont, 1991; Mahapatra et al., 2005). Ozone could also be produced by the electrochemical method or when using ultraviolet light with a wavelength of 185 nm (Fielding and Bailey, 2005).

In the USA ozone was given the status of generally regarded as safe (GRAS) status by the FDA in 2001 and could now be used in both gaseous and aqueous form for food processing and preservation (Guzel-Seydim et al., 2004; Mahapatra et al., 2005). Ozone is therefore used as a direct decontaminant on both food surfaces and equipments. Several studies have demonstrated the effectiveness of ozone as a decontaminant in the food processing environments. It was successfully used in the decontamination of equipment and food contact surfaces in a cheese processing factory. Both the aerobic plate count and the level of *Enterobacteriaceae* were reduced after exposure of the whole plant to 2 mg/ℓ ozone overnight over a period of two months. When the treatment with ozone was discontinued the levels of the *Enterobacteriaceae* and total counts returned to

unacceptable levels (Fielding and Bailey, 2005). It was also used in disinfection of poultry chill water and carcasses, in treating fruits and vegetables to reduce microbial load including moulds and to increase the shelf-life (reviewed, by Guzel-Seydim et al., 2004; Kim et al., 1999b).

Ozone is more effective in low ozone demand liquid media than on food surfaces. The composition of the food surface, the type of microbial contaminant and the degree of association of the microorganisms dictate the effectiveness of ozone. Higher moisture content in food promotes ozone action (Kim et al., 1999b). It has been proved to significantly reduce counts of *Clostridia*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and fungi at > 2mg/ℓ. In gas mixtures it has been shown to have a synergistic inhibitory effect with UV light and H_2O_2, but it had little effect on *Microbacterium*, *Lactobacillus* and *P. fluorescence* and *Leuconostoc* on beef surfaces (Kim et al., 1999b).

In all experiments conducted ozone caused significant improvement in food quality and hygiene by either lowering the microbial load or by improving the shelf life or taste of the food products. Dew (2005) demonstrated that off flavours caused by methylisoboneol on catfish could be reduced when fillets from fresh catfish spiked and stored for 12 hours were treated with ozone for 30-60 minutes. Overall ozone has no effect on chemical and sensory properties of foods, except on dry foods where a decrease in essential oils and a negative sensory quality of spices was experienced (Kim et al., 1999b). The use of ozone in preservation has now been patented for spices, herbs, fruits, vegetables, fish, beef and for food process water (Kim et al., 1999b).

Lower concentrations and shorter contact time of ozone are required in food, food plant and water sanitation than chlorine (Kim et al., 1999b). Unlike chlorine that leaves potentially carcinogens in the environment such as trihalomethanes (THM) and haloacetic acids (HAA) (Chawla, 2006; Guzel-Seydim et al., 2004) ozone does not leave any residues hence it is ideal for use as a terminal sanitizer of food surfaces and process water in industries. Also unlike chlorine where continuous use of high amounts may trigger development of resistance in some bacteria and viruses, ozone reacts quickly

killing microorganisms, hence giving them no chance to develop resistance (Chawla, 2006). Ozone has been shown to effectively remove biofilms where other disinfectants have failed (Fielding and Bailey, 2005). Ozone also removes bad tastes, odours and colour in water. It is more effective than trisodium phosphate and acetic acid, but comparable to H_2O_2 at lower concentrations (Fielding and Bailey, 2005).

Ozone has low solubility in water, thus proper mixing and high doses are required to attain optimum efficiency (Walker, 1978). Ozone is highly reactive thus it reacts with any organic materials including textiles, organic dyes, metals, plastic, paint and natural rubber, but this side effect can be reduced by the addition of antiozonants (Fielding and Bailey, 2005). It is, however, not more corrosive than chlorine. The short half life of ozone necessitates that it is used together with other disinfectants if a residual disinfectant is required to inhibit biofilm formation within distribution networks (Chawla, 2006). In water treatment ozone demand increases as the amount of suspended particles and pH increase (Kim et al., 1999b).

If not handled correctly ozone could impact on human health. Doses of 0.1mg/ℓ can cause sharp irritation of the nose and throat, 0.1–0.5 mg/ℓ exposure for 6 hours can result into loss of vision. At 1-2 mg/ℓ ozone could cause headaches, dizziness, weakness, decreased memory and other symptoms associated with irritation of the upper respiratory tract, while high doses (5-10mg/ℓ) and 50mg/ℓ or more could cause oedema of the lungs and death respectively (Mahapatra et al., 2005, Guzel-Seydim et al., 2004). The Occupational Safety and Health Administration (OSHA), USA has set limits for ozone exposure as 0.01-0.05 mg/ℓ for detectable odour and 0.1 mg/ℓ as exposure limit over an 8 hour period (Mahapatra et al., 2005).

2.5.5.4 Use of Hydrogen peroxide in food sanitation

Hydrogen peroxide is a chemical compound that contains two atoms of hydrogen and two atoms of oxygen (H_2O_2). The compound is produced naturally by certain bacteria including *Pediococcus, Lactobacillus* and *S. pneumonia* or in media exposed to light and

oxygen causing inhibition on other bacterial species (Juven and Pierson, 1996; Pericone et al., 2000). It is also produced photosynthetically in cyanobacteria and in plants (Roncel et al., 1989). Gaseous H_2O_2 is produced naturally by photochemical reactions in the atmosphere, but is also produced artificially by dissolving barium peroxide in water (Schumb et al., 1955).

H_2O_2 has a wide range of house hold and industrial applications; as a food preservative (Brul and Coote, 1999; Juven and Pierson, 1996) and in industrial and drinking water dechlorination (Worley, 2000). H_2O_2 is used to control biofouling and other pollutants in drinking water, soil, air and waste water distribution systems; to remove, sulphides, metals and other easily oxidisable materials (solids, gasoline and pesticides) and industrial solvents; for detoxification and deodorisation and for cosmetics and laundry applications (Asano et al., 2007; Degrèmont, 2007). The combinations of H_2O_2 with ozone (peroxones) and with UV light are used for ground water, drinking water and waste water disinfection (Lenntech, 2008). In industries concentrations of 30–50% are generally used as sanitising agents (Clyde Co-op, 2008). It is also used in combination with other additives such as potassium sorbate. It is used to sterilise containers for aseptic packaging (Juven and Pierson, 1996; Lück and Jager, 1997). In low concentrations (0.02 and 0.05 %) H_2O_2 was used to kill pathogens and spoilage bacteria during the pre-Pasteurisation era and it is still used in the USA to treat cheese and milk (Lück and Jager, 1997). H_2O_2 is used for sanitising hatching eggs and to sterilise liquid whole egg (Juven and Pierson, 1996). The residual H_2O_2 is normally inactivated by addition of catalase (Lück and Jager, 1997), but this practice presents a drawback due to the peroxide damage to vitamins. However H_2O_2 is not recommended in swimming pool disinfection unless when used in combination with other disinfectants (UV, ozone, silver salts or quaternary ammonium compounds). In this respect it is therefore assumed to be less effective than chlorine, bromine and ozone. It was recommended for Anthrax spores decontamination after the 2001 bioterrorism attack on the USA (EPA, 2007). It can be used as surface decontaminant in either aqueous or vapour forms (Juven and Pierson, 1996).

Lillard and Thomson (1983) showed that 5300–12000 mg/ℓ H_2O_2 reduced aerobic counts and *E coli* in water by 95-99%. Kim and Day (2007) reported that a combination of H_2O_2 with sodium bisulphide and thymol was effective in removing attached *E. coli* and S.Typhimurium from chicken carcasses. Drogui et al. (2001) have demonstrated that electroperoxidation removed dissolved organic carbon from solutions of phenol, salicylic acid, benzoic and humic acids.

H_2O_2 is a strong oxidising agent due to its ability to generate extremely biocidal reactive oxygen and hydroxyl radicals ($O^{\bullet-}$, OH^-) (Brul and Coote, 1999; Sias, 2003). In the presence of superoxide radical ($O2^{2-}$) and transition metal ions such as Fe^{2+}) H_2O_2 forms hydroxyl radical (OH^-) by the Fenton like and Haber-Weiss reactions respectively (Juven and Pierson, 1996). Secondly the lactoperoxidase system naturally occurring in milk and other biological fluids produces hypothiocyanate ($OSCN^-$) through the oxidation of the thiocyanate ion (SCN^-) by H_2O_2 (Juven and Pierson, 1996). The free hydroxyl radicals and singlet oxygen attack microbial cells causing irreversible damage to essential cell components including membrane lipids, DNA, and enzymes, (Brul and Coote, 1999; Lück and Jager, 1997). H_2O_2 is a more powerful oxidising agent than chlorine, chlorine dioxide and potassium permanganate, it has a lower oxidation potential than ozone, hydroxyl radical and fluorine (Lück and Jager, 1997).

The antimicrobial action of H_2O_2 is temperature and pH dependent (Brul and Coote, 1999) and it is potentiated by reducing agents and by peroxidases (Juven and Pierson, 1996). The free oxygen radicals decompose pollutants, while the H_2O_2 molecule is reduced to oxygen and water (Lenntech, 2008). At low concentrations H_2O_2 is more effective against bacteria, while yeasts and moulds require higher concentrations. In solution it enhances the antimicrobial effect of heat against vegetative cells and spores (Lück and Jager, 1997).

In the presence of organic material hydrogen peroxide decomposes completely forming water and oxygen, hence does not leave any undesirable or toxic chemical residues; decomposition is enhanced by the presence of pollutants and high temperatures (Lück

and Jager, 1997). H_2O_2 is, however, highly corrosive reacting with a number of substances (Lenntech, 2008). Human exposure to damp or mist can cause irritation of the eyes, skin, and mucous membranes. Concentrations of 5% or more can cause permanent eye damage; contact with skin causes painful blisters, burns and skin whitening. "Organs most susceptible to H_2O_2 exposure are the lungs, intestines and thymus" (Lenntech, 2008). Tests done on laboratory animals show that H_2O_2 is a potent carcinogen; it has also been shown to cause mutations in bacteria through damage to DNA (Lenntech, 2008). H_2O_2 reacts with food constituents such as vitamins and has a bleaching effect. According to Lück and Jager (1997) H_2O_2 is used to bleach starch, gelatine and fish marinades to suppress bacterial spoilage and odours in some countries. Although the use of hydrogen peroxide is not regulated under the EU legislation 98/83/EC, in the USA H_2O_2 is registered as a pesticide by Environmental Protection Agency (EPA) in 1977 hence used directly on dairy/cheese processing plants, on eggs, as a decontaminant of packaging materials, in agricultural premises, medical facilities, home bathrooms, on food processing equipment, and as Pasteurisers in breweries, wineries, and beverage plants (Brul and Coote, 1999; EPA, 2007; Lenntech, 2008).

2.6 CONCLUSION

Bacterial contamination is of paramount importance to the safety and shelf life of processed marine fish. The initial microbiological quality of marine fish is depended upon the microbial load of the marine water where the fish is harvested as governed by the prevailing physicochemical parameters (pH, salinity, nutrient content and temperature). The predominant microorganisms that persist in the finished products to cause spoilage and or food borne diseases depend on the intrinsic factors of the fish in question; the extrinsic factors of the food processing, storage and distribution environment, the explicit factors of the microbial population, and the processing factors.

To optimise the microbiological quality of marine fish it is important to implement acceptable Good manufacturing practices through adequate temperature control, training of personnel on personal hygiene and a sound HACCP system and the use of clean and

hygienically controlled equipment, utensils and materials including water used during processing.

Bacterial metabolism of the final product can cause significant quality deterioration while the presence of pathogens can be hazardous to human health. The current laboratory methods used in quality assurance and quality control are sometimes ineffective due to low sensitivity or atypical phenotypic or genotypic characters of target organisms.

Microorganisms are also constantly changing to adapt to hostile environments created by processing, preservation and sanitation methods. One of such adaptation strategies is attachment of bacteria to water distribution pipes and food processing surfaces, forming multispecies layers or three dimensional structures covered in matrices with limited permeability to chemical substances used in sanitation. Within biofilms bacteria undergo a series of genetic and phenotypic changes as a means of adaptations that often leads to selection for virulent strains mostly exhibiting adaptive traits such as increased ability to attach to surfaces and exopolysaccharide secretion. These biofilms act as nodes of water and product recontamination and a mode of pathogen persistence and dispersal in circulating aqueous environments and are important in disease transmission and outbreak of epidemics.

Sea water is abundant and readily available to the marine fish industry in Namibia. Most factories make use of sea water at one or more steps during processing. Although the water is subjected to conventional water treatment regimes, using mostly chorine and UV irradiation as the method of sanitation, these disinfectants may prove ineffective in controlling spoilage and pathogenic bacteria especially those that are able to attach to surfaces forming biofilms resulting into water recontamination and final product quality deterioration.

The most commonly used disinfectants, in the fishing industry are associated with a number of disadvantages. Chlorine is ineffective in providing proper control and can form substances that are believed to be hazardous to human health and the environment.

The UV radiation has low penetrability and no long term effect. Novel disinfection methods such as the use of ozone and H_2O_2 for biofilm control therefore need to be explored as to improve the microbial quality and safety of the fish.

Both ozone and hydrogen peroxide are strong oxidising agents without toxic residues and act instantaneously causing irreversible damage to microbial cell surface and cytoplasmic constituents. They are more potent than chlorine, economical and easily accessible. They are used in many parts of the world in decontamination of food processing environments and in improving the shelf life of a wide range of food items through controlling both suspended and sessile microorganisms. Trial studies therefore need to be conducted in assessing the benefits that the fishing industry might reap by using these disinfectants.

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