UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

Occurrence and control of *Vibrio* spp. as contaminants of processed marine fish

by

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Supervisor: Prof SN Venter
Co-supervisor: Dr PM Chimwamurombe

November 2010

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Declaration

I, the undersigned, hereby certify that the work contained in this thesis is my own original work and has not previously been submitted by me in its entirety or in part to any other university for a degree.

Signature:......................

Date:……………………..

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SUMMARY

Marine water contains large numbers of fish spoilage bacteria and pathogens including *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus*. Consumption of contaminated seafood could lead to the transmission of these pathogens to humans. Accurate identification of suspected pathogens and spoilage organisms is important to ensure consumer safety and a long shelf life. This project assessed the bacterial quality of hake during processing. Some attention was also given to pilchards and horse mackerel. The results showed a sharp increase in the mesophilic and sucrose fermenting *Vibrio* species counts in hake after filleting. It has been suggested that this contamination occurred during processing from biofilms present in the sea-water distribution system.

During the study 257 strains isolated from Thiosulphate citrate bile salts sucrose (TCBS) agar were screened to determine the presence of the pathogenic *Vibrios* amongst these isolates. It was difficult to distinguish between *V. alginolyticus* and *V.*

parahaemolyticus species due to high sequence similarity in their 16S rRNA genes. Final identification of the isolates required a polyphasic approach and it was found that none of the pathogenic *Vibrios* were present but that the *Vibrio* isolates mainly belonged to *V. alginolyticus*.

As it was suspected that the main source of contamination was the treated sea water used during processing the ability of chlorine, ozone and hydrogen peroxide to prevent biolfilm formation was examined. The behaviour of two *V. alginolyticus* strains (V590 and V595) isolated from the processed hake was evaluated. These strains formed biofilms faster than the *V. alginolyticus* LMG4409 type strain, but were similar to *V. parahaemolyticus* LMG2850. Biofilms formed by these bacteria were resistant to 4 mg/l chlorine and to 2 mg/l ozone, but were inhibited by 0.05 % and 0.2 % hydrogen peroxide for biofilms initiation and mature biofilms respectively. The close resemblance of atypical *V. alginolyticus* isolates to *V. parahaemolyticus* may indicate the ability of pathogens to survive under similar conditions. Effective decontamination and quality assurance strategies are therefore required when processing fish to prevent disease outbreaks.

DEDICATION

This work is dedicated my late father Jason Ndinoshiho Shikongo, to my late husband, John Simaneka Nambabi, to my late brothers, Isack Ndeshitiwa Pondo Shikongo and Andreas Kashinghola Shikongo, to my late sister, Elisabeth Shikongo, and to my late academic supervisor, Alexander Von Holy who have not lived to witness this important milestone in my life.

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LIST OF ABBREVIATIONS

AA	-	Aeromonas agar
ADH	-	Alanine dehydrogenase
APW	-	Alkaline Peptone water
ANOVA	-	Analysis of Variance
ABM	-	After (baader) filleting machine
APHA	-	American Public health Association
API 20E	-	Analytical Profile Index 20 for *Enterobacteriaceae*
APW	-	Alkaline Peptone Water
AOAC	-	Association of Official Analytical Chemists
ASW	-	Artificial Sea Water
BCCM	-	Belgium Culture Collection, Laboratorium voor Microbiologie
BDOC	-	Biodegradable dissolved organic carbon
BM	-	Basal Medium
BLASTn	-	Basic Local Alignment Search Tool for nucleotides
BPW	-	Buffered Peptone Water
CaOHCl	-	Calcium hypochloride
CPS	-	Capsular polysaccharides
CDC	-	The Centre for Disease Control and Prevention
Cl_2	-	chlorine gas
CFC	-	Cephaloridin Fusidin Cyclosporin Agar
cfu	-	Colony forming units
cm	-	centimetre
^oC	-	Degrees Celsius
DNA	-	deoxy Ribonucleic Acid
EEC	-	European Economic Community
EU	-	European Union
EEZ	-	Exclusive Economic Zone
EPA	-	Environment protection Agency, USA
EPS	-	Extracellular polymeric substances

FP - Hake fillets finished packaged products
GDP - Gross domestic Product
GSTB - Glucose Salt Teepol Broth
GMP - Good Manufacturing Processes
H^+ - Proton
HACCP- Hazard analysis and critical Control Point
HAA - haloacetic acid
OCl^- - hypochlorite anions
HOCl - hypochlorous acid
H&G/HG- Headed and gutted hake fish
ICMSF- International Commission on Microbiological Specification for Foods
IFD - International Dairy Federation
ISO - International Standard Organisation
JAMA - The Journal of the American Medical Association
K antigens -capsular antigens
LAB - Lactic Acid Bacteria
LDC - Lysine decarboxylase
L - litre
MFMR- Ministry of Fisheries and Marine Resources, Windhoek, Namibia
g - gram
GRAS - generally regarded as safe
H_2O_2 - Hydrogen peroxide
mg - milligram
mg/l - milligrams per litre
m - metres
mW-sec/cm^2- MilliWatts per second per square centimetre
NaCl - sodium chloride
nm - nanometres
NA - Nutrient Agar
NCBI - National Centre for Biotechnology Institute
NSI - Namibia Standards Institute
NSW - Natural Sea water
ODC - Ornithine decarboxylase
OSHA - Occupational Safety and Health Administration

O_3 -	ozone
O antigen-	somatic antigen
RT-PCR-	Real time PCR
R-T PCR-	Reverse Transcriptase PCR
SWA -	sea water agar

PUBLISHED ARTICLES

SHIKONGO-NAMBABI MNNN, KACHIGUNDA B and VENTER SN (2010) Evaluation of oxidising disinfectants to control *Vibrio* biofilms in treated sea-water used for fish processing. *Water SA* **36**: 215-220.

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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

2.1INTRODUCTION

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°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°C–29°C. 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°C (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°C), while solid media are incubated at 25°C (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 Dodesyl 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%	0-2%	69-100%
12%	0-1%	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 (Degrėmont, 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) (Degrėmont, 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; Degrėmont, 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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CHAPTER 3

SHIKONGO-NAMBABI MNNN, CHIMWAMUROMBE PM and VENTER SN (2010) **FACTORS IMPACTING ON THE MICROBIOLOGICAL QUALITY AND SAFETY OF PROCESSED HAKE**. *African Journal of Biotechnology*. **9** 8405-8411

3.1 ABSTRACT

Problems with the safety and shelf life of hake intended for export have been raised in the Namibian fishing industry. This prompted an investigation into the factors that may have an impact on the microbiological quality and safety of processed hake. Samples were collected at three stages along the processing line; the general microbiological quality (mesophilic and psychrotrophic aerobic plate counts), total *Vibrio* species and common fish spoilage bacterial counts were performed. The results constantly showed relatively high counts for the psychrotrophic and spoilage bacteria, indicating that most of these bacteria already formed part of the incoming fish. Hake is headed and gutted on board of fishing vessels and delivered to the factory only after 7–8 days for final processing. It is likely that this practise of heading and gutting the hake may have a negative effect on microbiological quality of the final product. A sharp increase in the mesophilic and sucrose fermenting *Vibrio* species counts were observed after filleting. It has been suggested that this contamination could be due to biofilms present in the distribution system for the treated sea-water used during processing. Although sea-water could be an alternative source of water for marine fish processing plants, the treatment and the quality of the water needs to be carefully managed.

Key Words: hake fish, aerobic plate counts, *Vibrio* species, mesophiles, psychrophiles, spoilage bacteria.

3.2 LIST OF ABBREVIATIONS

AA: aeromonas agar
ABM-F: after filleting machine hake fillets washed with fresh water
ABM-S: after filleting machine hake fillets washed with sea-water
APW: alkaline peptone water pH 8.4
APHA: American Public Health Association
BM: basal medium
CFC: cetrimide fusidin cephaloridin agar
EU: European Union
FP: hake fish finished products
H and G: headed and gutted hake fish
ICMSF: international commission on microbiological specifications for foods of the international association of microbiological societies
LFHF: laminated and folded hake fillets
PCA: plate count agar
PW: peptone water
SWA: sea water agar
TVC: total viable counts
TCBS: thiosulphate citrate bile salts sucrose agar
VRBG: violet red bile glucose agar

3.3 INTRODUCTION

Fishing is the second largest export industry after mining and earned about 25% of the total export value for Namibia in 2002 (Meyn, 2005). Of these exports hake constituted about 45% of the total export value of the Namibian fishing industry by 1998 (Ministry of Fisheries and Marine Resources, 2004). Hake is initially processed off shore where the head and intestines are removed on board of vessels and the fish is kept frozen for 7-8 days before being delivered for processing into fillets at the land based facilities. At the processing plant the fish is first defrosted before being sliced into fillets by the filleting machine (Baader, Germany) followed by trimming of the fillets and rinsing with water before final packaging and freezing for export. All exported fish including hake is subjected to microbiological tests to ensure compliance with the EU Directive EU 91/493/EEC (Iyambo, 1995) in order to ensure both the quality and safety of the product. As part of the evaluation total viable counts (TVC), total coliforms, faecal coliforms, *Vibrio* species, *Staphylococcus aureus* and *Escherichia coli* levels have to be determined.

Concerns have been raised by the fishing industry with regard to the microbial quality of hake since premature spoilage and fillets loosing their firmness have been noted. Deterioration of the processed product is part of a global problem that causes huge economic losses (Gram and Dalgaard, 2002; Huis in't Veld, 1996). Although spoilage could be due to endogenous enzymes (Chang et al., 1998, Chytiri et al., 2004; Ordóňez et al., 2000), it is widely found that bacteria play an important role (Gennari et al., 1999; Gram, 1992; Tryfinopoulon et al., 2002). It was reported that the number and identity of the initial fish microflora and those present after processing play an important role in determining the shelf life of the fish (Gram and Huss, 1996; Koutsoumanis and Nychas, 1999). In temperate regions fish spoilage is caused by a range of Gram negative bacteria including *Shewanella putrefaciens*, *Photobacterium phosphoreum, Pseudomonas*, *Aeromonas*, *Acinetobacters*, *Psychrobacter*, *Flavobacterium*, and *Brochothrix thermosphacta* (Chytiri et al., 2004; Gennari et al., 1999; Tryfinopoulon et al., 2002). *Moraxella*, *Corynebacterium*, *Pseudomonas, Micrococcus* and *Shewanella* predominate

under cold storage in seafood harvested from both temperate and tropical regions (Gram and Huss, 1996; Ordóňez et al., 2000).

Another concern for the producers and export countries alike is the safety of the hake harvested from Namibia. Questions with regard to the presence of pathogens such as pathogenic *Vibrio* species have been raised. Should *V. cholerae* or any of the other pathogenic *Vibrio* spp. (*V. parahaemolyticus*, and *V. vulnificus*) be detected on the hake by importing countries they will reject the whole consignment that will result in huge economic losses to the fishing industry. Human vibriosis is typically acquired through consumption of contaminated seafood. These pathogens may be present due to either the ubiquity of the causative agents in aquatic environments (DePaola et al., 2003; Harriague et al., 2008) or through contamination during processing.

The aim of this study was to determine and highlight potential factors that may lead to the deterioration of the microbial quality of hake during processing and impact on the safety of the final product. The microbiological quality of the hake was assessed at three sampling points during processing. Apart from the incoming fish, fillets after processing by the filleting machine (ABM-S) and the finished product after packaging (FP) were also sampled. The filleting machine was targeted as a possible source of contamination due to its compact mechanical nature that could prove difficult during cleaning and sanitation. The initial samples were taken at the time when sea-water was used for washing the fish during filleting. A second limited sampling programme was performed later when fresh water was used for the same purpose. The results from these two sampling periods were also compared.

3.4 MATERIALS AND METHODS

3.4.1 Sampling

During the first sampling period, hake samples were taken from three points along the processing line. These samples included the headed and gutted fish kept on ice (H and G) after delivery from the fishing boat, fillets after processing by the filleting machine (ABM) and the finished product after packaging and ready for freezing (FP). All samples were collected in food sampling bags, transported to the laboratory and either analysed immediately or kept frozen at -20°C until analysed within 24 hours. Frozen samples were thawed into the refrigerator at 2-5°C for not more than 18 hours (ICMSF, 1978a).

During the first sampling period, treated sea-water was used for defrosting incoming fish and the washing of filleted fish at filleting. A total of 120 hake samples were analysed; 20 headed and gutted fish, 20 samples after the filleting machine (ABM-S), and 60 finished products (FP). The FP sampled were either the laminated and folded hake fillets (LFHF), fillets with the skin on or the Mascato packs. The samples consisted either of a whole fish or fillets with a weight of about 300 g. The sample descriptions and number of samples are summarized in Table 3.1. During a smaller follow-up study twenty (20) fillets were re-sampled at the ABM point. At this time only fresh water was used for washing of the fillets after they were filleted (ABM-F). Each sample was analysed in five replicates.

3.4.2 Mesophilic and psychrotrophic plate counts

The total aerobic plate count was performed according to the method described by ICMSF (1978a) and Kaysner et al. (1992) for psychrotrophic and mesophilic counts respectively. A resuscitation step was included to aid recovery of potentially stressed or damaged cells including those present in the frozen samples. For resuscitation 25 g of fish tissues were transferred to a stomacher bag containing 225 mℓ of 0.1% peptone water

(PW) (Oxoid) and hand minced for one minute at room temperature (22°C) to obtain a homogenous suspension. Serial decimal dilutions of homogenates were prepared up to 10^{-6} and plated on plate count agar (PCA) (Oxoid) and Sea Water Agar (SWA) (Farmer and Hickman-Brenner, 1991) prepared using natural purified sea-water in place of artificial sea-water. Plates were incubated at 35°C for 24 h (mesophilic count) and 22°C for 72 h (psychrotrophic count) respectively. All colonies were counted.

3.4.3 Sucrose fermenting *Vibrio* species

Tissues (25 g) were aseptically excised, minced in 225 mℓ alkaline peptone water (APW) pH 8.4 (ICMSF, 1978a ; Kaysner et al., 1992) and incubated at 22°C for 6 hours according to Farmer and Hickman-Brenner, (1991) to aid in the recovery of any damaged cells. Serial decimal dilutions of the homogenates were prepared in APW up to 10^{-3}. One hundred µℓ aliquots of each dilution were spread in duplicate on Thiosulphate Citrate Bile salts Sucrose (TCBS) agar plates (Oxoid), and incubated at 35°C for 24 hour (ICMSF, 1978a; Kaysner et al., 1992). Colonies that appeared on TCBS agar as large, smooth and yellow, with flattened centres and translucent peripheries were counted.

3.4.4 Sucrose non-fermenting *Vibrio* species

Fifty grams (50 g) of fish tissue, were aseptically excised, hand minced in 450 mℓ 3% NaCl (Kaysner et al., 1992). Serial decimal dilutions were prepared in 3% NaCl up to 10^{-4}. Aliquots of the serial dilutions (10 mℓ) were inoculated into 10 mℓ double strength glucose salt teepol broth (GSTB) (Kaysner et al., 1992) and incubated at 35°C for 6 hours. Aliquots of the serial dilutions in GSTB (0.1 mℓ) were surface plated onto TCBS (Oxoid), and incubated at 35°C for 24 h. Round, bluish or green colonies 2-3 mm in diameter (Arias et al., 1998; Kaysner et al., 1992) were recorded.

Table3.1 Fish samples analysed

Sample	Description	Number of samples analysed
H and G	Headed and gutted fish stored on ice	20
ABM-S	Filets taken after filleting using sea water	20
ABM-F	Filets taken after filleting using Fresh water	20
FP (finished product)	Laminated, folded hake Fillets	20
	Fillets with skin on	20
	Mascato packs	20
Total Samples analysed		120

3.4.5 *Enterobacteriaceae*

Fish tissue (10 g) was aseptically excised, mixed with 90 mℓ of buffered peptone water (BPW) in sterile polythene bags and hand minced. The samples were decimally diluted in series up to 10^{-4} in BPW. After thoroughly mixing, dilutions were incubated at 35°C for 6 hours (ICMSF, 1978b). Aliquots (1mℓ) of the dilutions were transferred in duplicates to sterile 90 mℓ Petri dishes. Fifteen mℓ of cooled violet red bile glucose (VRBG) agar (Oxoid) was added and immediately mixed with the sample. After the agar had set a second layer (10 mℓ) of VRBG agar was added, allowed to set and the plates were incubated at 35°C for 24 hours (Chouliara et al., 2004; Paleologos et al., 2004) after which the number of pink colonies were recorded.

3.4.6 *Pseudomonas, Shewanella putrefaciens* and *Aeromonas*

For these analyses 25 g of fish tissue were placed in sterile stomacher bags containing 225 mℓ basal medium (BM) (Baumann and Baumann, 1991) and hand minced.

Homogenates were incubated at 22°C for 6 hours to aid in the recovery of any damaged cells. Thereafter serial dilutions were made in BM up to 10^{-4}. Aliquots (0.1 mℓ) of each dilution were transferred to cetrimide fusidin cephaloridin agar (CFC) (Oxoid) supplemented with supplement SR 103, (Oxoid) for culturing potential *Pseudomonads* (Chouliara et al., 2004; Chytiri et al., 2004; Paleologos et al., 2004). The CFC plates were incubated at 20°C for 2 days. Small grey round colonies on CFC were scored as *Pseudomonas* spp..

For *Shewanella putrefaciens*, 1 mℓ of the same dilutions was added to 10 mℓ of molten (45°C) iron agar (IA) (Oxoid), poured into 90 mℓ Petri plates and allowed to set. After setting a further 10 mℓ of IA was added as a seal, allowed to set and the plates were then incubated at 20°C for 4 days. Black colonies forming on IA were scored as presumptive *Shewanella putrefaciens* colonies (Chytiri et al., 2004).

For *Aeromonas* 0.1 mℓ aliquots of the dilutions enriched in APW as described for *Vibrio* species enrichment were spread in duplicate onto *Aeromonas* agar (Oxoid) to which supplement SR 151 (Oxoid) was added. Plates were incubated at 25°C for 48 hours according to Farmer et al. (1991). Pale green colonies were scored as presumptive *Aeromonas* spp. Average logs values from counts of presumptive *Pseudomonas*, *Shewanella* and *Aeromonas* were calculated after 5 replicate samples were processed.

3.4.7 Statistical analysis

A number of statistical methods were used. In order to be able to compare the data from the different stages during the processing, average log values of counts were first determined for all the microbial analyses and the standard deviations were calculated using Microsoft excel. ANOVA for Randomised complete Block Design and the least significant difference technique to separate means was also used. In some instances the nested hierarchal approach was used in cases where the factors were nested.

3.5 RESULTS

The microbial data for the sampling period when sea-water was used in the processing plant are shown in Figure 3.1. At the beginning of the processing line (H and G) the mean mesophilic aerobic plate count was log 3.73 cfu/g, increasing to log 6.50 cfu/g and 6.24 cfu/g after the filleting machine (ABM-S) and in the final products (FP) respectively. The increase in mesophilic count by nearly 3 logs along the processing line suggests either loss of temperature control or exogenous contamination along the processing line. The mean psychrotrophic aerobic plate count remained fairly constant during processing (Figure 3.1). The measured count for the incoming fish (H and G) was log 7.03 cfu/g. At ABM-S and FP the recorded psychrotrophic counts were log 6.85 cfu/g and 6 18 cfu/g respectively.

No bacteria grew on TCBS after APW enrichment of the incoming fish (H and G). At the filleting machine (ABM-S) and in the final product the level of sucrose fermenting bacteria rose sharply with an average log of 5.63 cfu/g and 6.61 cfu/g respectively when treated sea water was used for processing. This increase in potential *Vibrio* levels indicated exposure of hake to some form of contamination during processing. Sucrose fermenting *Vibrio* species were not detected when fresh water was used (Figure 3.1). No non-sucrose fermenting bacteria with characteristic appearance of *Vibrio parahaemolyticus* were detected in any of the samples.

Enterobacteriaceae counts on the fish were the same at the beginning (H and G) and at the end of the processing line (FP) with levels of log 5.00 cfu/g and 5.03 cfu/g respectively. A lower value of log 3.20 cfu/g was noted at the intermediate stage (ABM-S). High counts of *Pseudomonas,* similar to those observed for the psychrotrophic counts, were observed throughout processing. The mean levels were log 7.48 cfu/g (H and G), log 6.34 (ABM-S) and log 6.62 cfu/g (FP). The *S. putrefaciens* counts decreased during processing and were log 6.17 cfu/g (H and G), log 5.68 cfu/g (ABM-S) and log 4.5cfu/g

(FP) respectively. The *Aeromonas* levels remained fairly constant at log 5.69 cfu/g (H and G), log 6.11 cfu/g (ABM-S) and log 5.8 cfu/g (FP).

As the use of treated sea-water to wash the fish after filleting was discontinued another set of samples were taken. In most cases the bacterial levels differed significantly from those measured during the first sampling period (Figure 3.1). The mean mesophilic count recorded was log 3.41 cfu/g, 3 logs lower than the previous count obtained when sea-water was used at this point. The count of *Enterobacteriaceae* was log 2.26 cfu/g, that of *Pseudomonads* log 0.63 cfu/g, *S. putrefaciens* log 3.14 cfu/g and *Aeromonas* log 3.35cfu/g. All of these results were significantly different from the original levels (p=0.001).

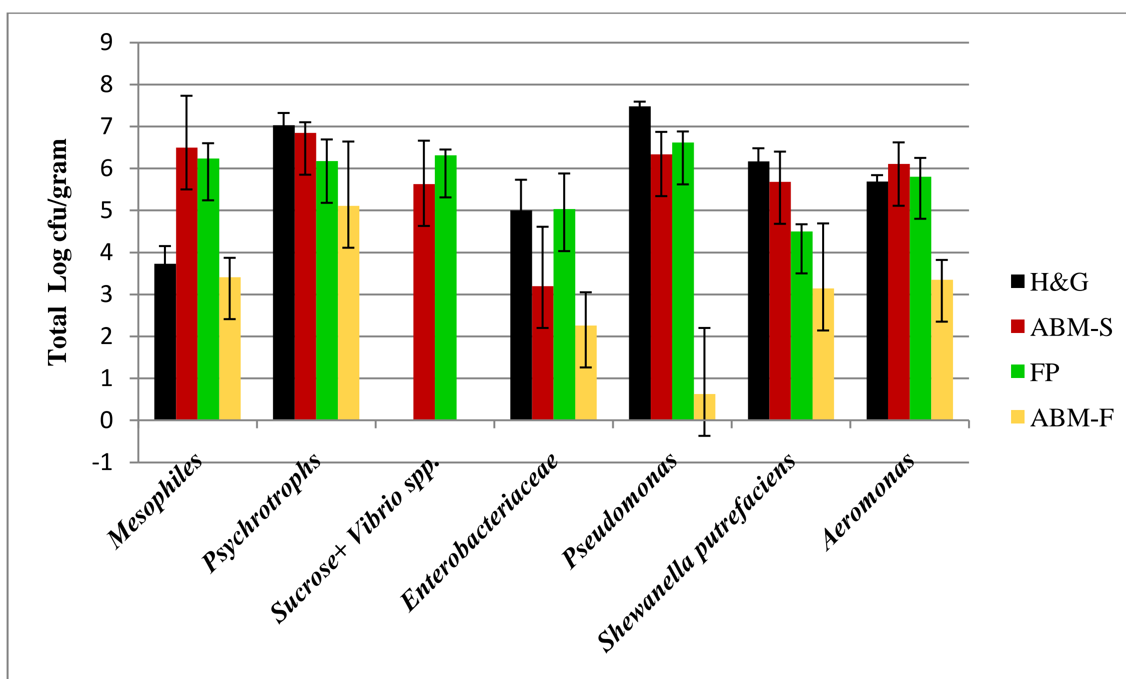

Figure 3.1 Total viable bacterial counts on hake. H and G= Headed and gutted, ABM-S= after filleting machine with sea water, FP= Finished packed hake fish.

3.6 DISCUSSION

The microbial quality of processed fish is usually determined by a number of factors, including the levels of microbes on the raw product, the microbial contamination during processing and the exposure of the product to conditions that will allow for the multiplication of the existing microbes on the product. High mesophilic counts in marine fish are usually indicative of the existence of such conditions and may signal a potential spoilage or health hazard as many spoilage and pathogenic bacteria are mesophilic (ICMSF, 1978a). Total viable aerobic counts on seafood are normally in the ranges of (ca. 10^4 -10^6cfu/g) on the skin, (10^4-10^7 cfu/g) in the gills (Gennari et al., 1999), and (10^4-10^6cfu/g) in the intestines (Austin and Austin, 1987).

In this study the mean viable mesophilic count was log 3.73 cfu/g on the raw product (H and G) but increased dramatically to a value of higher than log 6 cfu/g at the intermediary stage (ABM-S) and in the finished product (FP). The levels determined for the headed and gutted fish, kept on ice on board of the fishing vessels for several days before the fish is delivered to the factory, compared well with other studies (Cakli et al., 2006; Pastoriza et al., 1996; Tzikas et al., 2007).

The mean psychrotrophic counts obtained in this study remained high at nearly the same level during all the stages of processing. High psychrotrophic counts observed could have originated from the natural flora of hake that multiplied from the time of the fish catch to the time of delivery to the factory indicating potential problems on board of the fishing vessels. The psychrotrophic counts are usually representative of normal spoilage organisms such as *Pseudomonas* and *Shewanella* spp. (Gram and Dalgaard, 2002) that can grow at refrigeration and ambient temperatures. This was confirmed by Ordóňez, et al. (2000) who also showed that *Pseudomonas* and *Shewanella* were the predominant spoilage bacteria on gutted hake stored on ice. In this study the *Pseudomonas* counts of log 7.48cfu/g in the incoming fish and log 6.62 cfu/g in the final hake products, were

similar to those measured for the total psychrotrophic counts. These values may indicate a short product shelf life.

Aeromonas spp. could also form part of the psychrotrophic bacteria and have been isolated from a number of marine and fresh water species fish (Papadopoulou et al., 2007). They are also fish spoilage organisms and may produce H_2S. In this study counts ranged from log 5 to log 6 cfu/g and hake spoilage due to this group of bacteria can therefore not be excluded.

S. putrefaciens is typically one of the predominant microflora of ice stored fish from temperate regions (Chytiri et al., 2004; Gennari et al., 1999; Paarup et al., 2002). In this study the levels of sulphate reducing bacteria (SRB) including *S. putrefaciens* in H and G and FP hake were log 6.17 and log 4.50cfu/g respectively. Some reduction in the levels of *S. putrefaciens* was noted as the fish moved along the processing line. Despite this reduction the levels are still of concern and it should be noted that the method of keeping fish on board for several days before delivery to the factory for final processing may have a negative effect on microbiological quality and could lead to spoilage.

Some of the counts were higher than what was reported in literature (Vennemann, 1991; Tsikas et al., 2007). This could be linked to a recovery step that was included in the analysis of some of our samples. This step was included as Tsikas et al. (2007) have observed a lag phase in the growth of total viable bacteria performed on Mediterranean horse mackerel and blue jack mackerel muscle done after 4 to 6 days of fish storage on ice. Within fish processing environments bacteria are also continually exposed to stressing situations such as chill temperatures, and the presence of sanitizers that cause sublethal injury to bacteria. An enrichment step often assists with the recovery of these bacteria (Foegeding and Ray, 1992; ICMSF, 1978b)

Human pathogens are typically mesophilic bacteria with an optimum growth range between 30-45°C (Forsythe and Hayes, 1998). An increase in the mesophilic count is therefore of potential health concern. *Enterobacteriaceae* are widely distributed in

aquatic environments including marine waters (Papadopoulou et al., 2007) and could be one of the reasons for the observed increase. High counts of *Enterobacteriaceae* typically indicate potential faecal contamination (ICMSF, 1978a). During this study the *Enterobacteriaceae* initial counts for hake were log 5.00 cfu/g (H and G), and similar count was observed in the finished products. These counts were similar to those obtained by Ordóňez et al. (2000) on hake steaks before storage, and by Economou et al. (2007) in tuna fish which was kept at 20°C. Himelbloom et al. (1991) found lower (10^2 cfu/g) *E. coli* counts on Alaskan finfish. In this study it was therefore demonstrated that the levels were within the expectable norms, and faecal contamination of the processed fish was not suspected to be the reason for the deterioration in the mesophilic counts.

The sharp increase in mesophilic counts observed during processing was still a cause for concern as this trend corresponded with a similar increase in the levels of sucrose fermenting *Vibrio* spp. No sucrose fermenting *Vibrio* species were detected in the incoming hake (H and G), but this group of bacteria suddenly appeared at high levels in the ABM-S and FP samples. Further investigations indicated that this sharp increase in potential *Vibrio* spp. could be a result of the introduction of the bacteria by means of the treated sea-water used during processing. There were indications that the major source of these bacteria was not inefficient treatment of the raw water but the subsequent formation of biofilms in the distribution network in spite of the presence of residual chlorine (Shikongo-Nambabi et al., 2010a). Identification of the isolated sucrose fermenting bacteria confirmed that these strains were not *V. cholerae* but *V. alginolyticus* and that they did not pose any immediate health risk to any of the consumers (Shikongo-Nambabi et al., 2010b)

The impact of sea-water was further investigated when the factory was refurbished and started to use fresh water as the major source of water during processing. No sucrose fermenting *Vibrio* species were detected in any of the products tested. The differences observed for all the microbial parameters were of statistical significance indicating a positive impact on the overall quality of fish. This improvement should significantly increase the shelf life as well as the safety of the hake processed in the plant.

3.7 CONCLUSION

The microbial quality as observed during the initial study period raised a number of concerns and warranted a closer investigation to ascertain that good manufacturing practises are strictly adhered to from the time the fish is caught up to the point of processing of the final product. The results indicated that some deterioration in quality during processing could be due to contamination during processing while others may have originated with the fish supplied to the plant since all fish samples analysed during this study were not freshly caught.

This study has shown that *Pseudomonas*, *S. putrefaciens* and *Aeromonas*, and typical spoilage organisms, form part of the bacterial population on the hake. *Pseudomonas* and *Aeromonas* were present at the same level while *S. putrefaciens* levels were slightly lower. The results indicated that these organisms already formed part of the incoming fish and that process did not increase their levels dramatically. It is highly likely that the method of keeping fish on board the fishing vessels for several days before delivery to the factory for final processing may have a negative effect on microbiological quality and could lead to spoilage.

Comparison of viable bacterial counts obtained from the three stages along the processing line has revealed higher mesophilic counts in hake after filleting. Of particular interest was the sucrose fermenting *Vibrio* species that were not detected in the incoming (H and G) fish, but were detected in high numbers ca. log 6.31 cfu/g when sea-water was used to wash the hake fillets before trimming and packaging. A link was made to the treated sea - water used during processing as the most likely source of contamination. This was confirmed when a significant improvement was observed when fresh water was used to wash fish at the same point during processing. Although sea-water could be an alternative source of water for marine fish processing plants, the treatment and the quality of the water needs to be carefully managed.

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CHAPTER 4

SHIKONGO-NAMBABI MNNN, CHIMWAMUROMBE PM and VENTER SN (2010) **IDENTIFICATION OF *VIBRIO SPP.* ISOLATES OBTAINED FROM PROCESSED MARINE FISH.** *Proceedings of the National Symposium 15-17 September Safari Hotel Windhoek, Namibia* (In print)

4.1 ABSTRACT

Vibrio species are ubiquitous in marine environments and contaminate marine fish during processing. *V. cholerae*, *V. parahaemolyticus, V. vulnificus* are human pathogens causing cholera, gastroenteritis, septicaemia and wound infections that can be fatal in humans. *V. alginolyticus* is an opportunistic pathogen and maybe of less importance. The presence of these bacteria in fish could pose serious health risks to consumers and creates trade barriers for Namibia. The aim of this study was to identify 243 Gram negative bacteria isolated from hake, pilchards and horse mackerel during processing using Thiosulphate Citrate Bile salts Sucrose (TCBS) agar. Initially basic phenotypic tests such as oxidase and O-F tests were done. This indicated that 92 (38%)of the isolates were typical *Vibrionaceae.* Secondly the isolates were screened for genes specific to the human pathogenic *Vibrio* species by Polymerase Chain Reaction (PCR) targeting the *ompW* of *V. cholerae*, *pR72H* fragment of *V. parahaemolyticus* and the cytolysin-haemolysin gene of *V. vulnificus*. This was followed by sequencing the 16S rRNA gene and drawing Neighbour Joining phylogenetic trees based on the data. All suspect isolates were further characterised by a combination of selected phenotypic tests and the *V. alginolyticus* specific collagenase gene PCR. None of the bacteria isolated from hake, pilchard or horse mackerel were identified as *V. cholerae, V. parahaemolyticus* or *V. vulnificus*. Out of the 80 isolates that were identified as *Vibrionaceae* by their 16S rRNA gene sequences sixty-three could be identified as *V. alginolyticus*. The remaining 17 isolates belonged to the genera *Listonella, Shewanella* and *Aeromonas.* The study therefore indicated that the three fish species examined do not pose an immediate health hazard to consumers. It also showed that when identifying *Vibrio* spp., particularly when it is necessary to differentiate between *V. alginolyticus* and *V. parahaemolyticus,* a polyphasic approach is still most desirable.

KEYWORDS: *V. parahaemolyticus, V. alginolyticus,* V. *vulnificus*, identification, PCR, 16S rRNA, hake, pilchards and horse mackerel.

4.2 INTRODUCTION

The family *Vibrionaceae* represents a number of genera including *Vibrio*, *Grimontia*, *Photobacterium* and *Salinivibrio* (Thompson et al., 2005). Amongst the *Vibrio* species there are at least 13 species pathogenic to humans (*V. cholerae*, *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. funissii, V. fluvialis, V. damsela, V. mimicus, V. hollisae, V. cincinnatiensis, V. harveyi, V. metchnikovii* and *V. carchariae*) causing cholera, gastroenteritis, septicaemia and wound infections (Drake et al., 2007). Many *Vibrios* are natural inhabitants of the marine environment, largely in association with fish and zooplankton (Montanari et al., 1999) and may contaminate marine fish during processing (Wekell et al., 1994). Temperature abuse often results in the uncontrolled proliferation of these bacteria in sea food and represents one of the most common routes of transmission of these pathogens to humans (Joseph et al., 1982).

The identification of *Vibrio* species is commonly done using classical phenotypic methods and 16S rRNA sequence comparisons are often included (Thompson and Swings, 2006; Drake et al., 2007). Classical techniques, however, take a long time to conduct and may sometimes be inaccurate as some strains exhibit atypical phenotypic characteristics (Oliver and Kaper, 1997). Comparison of the 16S rRNA sequences can in most cases only correctly identify *Vibrio* isolates up to genus level due to high interspecies similarities. It has, for example been difficult to correctly differentiate *V. alginolyticus* from the closely related *V. parahaemolyticus* which share between 60 -70% DNA homology (Oliver and Kaper, 1997; Farmer et al., 2004). Molecular methods targeting and amplifying unique genes with PCR have also been used, sometimes with limited success (Drake et al., 2007). We have recently reported that the microbiological quality of hake deteriorated during processing at a marine fish processing facility in Walvis Bay, Namibia. Sucrose fermenting *Vibrios* were isolated at the intermediate stage of the processing line while none were detected on the incoming fish (Shikongo-Nambabi et al., 2010a). *Vibrio cholerae* is predominantly a sucrose fermenter and accurate identification of these isolates was therefore very important. Should this or any related pathogens be present in the processed fish exported to the European

countries, the whole consignment would be returned back to Namibia as a sign of non-compliance with the trade agreement reached between Namibia and these countries. It was also noted that the isolated *Vibrio* species were able to form multicellular complexes that defy the action of oxidizing agents commonly used in the fishing industry as sanitizers (Shikongo-Nambabi et al., 2010b). Accurate identification of these isolates would help eliminating any doubt surrounding the presence of pathogenic *Vibrio* species and the safety of the Namibian hake.

During this study 247 bacteria isolated from processed fish were identified to establish whether any pathogenic *Vibrio* spp. are associated with these products. The isolates obtained during TCBS isolations (both sucrose and non sucrose fermenters) were identified by a polyphasic approach. This included phenotypic methods (Gram stain reaction, oxidase, and O-F tests), amplification of unique genes and sequencing of the 16S rDNA. One hundred and eighty-four of the isolates were obtained from processed hake, 27 isolates were from pilchards and 15 from horse mackerel (HM). Many of the isolates gave conflicting reactions to different identification methods and could only be identified once the collective data were analysed.

4.3 MATERIALS AND METHODS.

4.3.1 Bacterial isolates

All 247 isolates, as previously described in Chapter 3, were obtained from hake, pilchard, or horse mackerel. Samples were plated on Thiosulphate Citrate Bile salts Sucrose (TCBS) agar after pre-enrichment in either (Alkaline peptone water) APW pH 8.6 or in 3% NaCl-Glucose Salt Teepol Broth (GSTB). Apart from the 247 isolates obtained from the fish, four reference strains were also included in the study to serve of positive controls. *Vibrio cholerae* isolate (C453) was kindly supplied by Prof. SN Venter, Department of Microbiology and Plant Pathology, University of Pretoria. Type strains of *Vibrio parahaemolyticus* (LMG 2850), *V. vulnificus* (LMG13545) and *V. alginolyticus* (LMG 4409) were obtained from the Belgium Culture Collection, Laboratorium voor Microbiologie, Ghent (BCCM/LMG) University of Ghent, Belgium.

4.3.2 The Gram stain differentiation

A single colony from an overnight pure culture was suspended in 50 µl of 10% KOH to determine the Gram reaction of the isolates. Cultures that showed an increase in viscosity in the suspension and the formation of a characteristic slimy string were recorded as being Gram negative. If no string was seen the culture was recorded as Gram positive (Buck, 1982). A *Micrococcus* spp. isolate was used as a negative control.

4.3.3 The Oxidase test

The oxidase test was performed according to Quin et al. (1994). The colony to be tested was picked with a sterile toothpick and streaked across filter paper placed in a Petri dish. The filter paper was flooded with a freshly prepared 1% (w/v) Oxidase (N-N-N-N-Tetra methyl phenylene diamine dihydrochloride) solution. The test was recorded as positive

when the cells turned dark purple within 5-10 seconds. *Vibrio cholerae* isolate (C453) was used as a positive control.

4.3.4 The Oxidation/Fermentation (O/F) Test

The ability of isolates to utilize glucose under both aerobic and anaerobic conditions was tested with O-F medium (Biolab). The semisolid medium (1%) was prepared according to the manufacturer's instructions. The medium was sterilised by autoclaving at 121°C for 20 minutes. As carbon source, 10 mℓ of a filter sterilized 10% D-Glucose solution was added to the autoclaved medium. The medium (5 mℓ) was dispensed into sterile test tubes. For each test culture two tubes were prepared, the anaerobic tube was overlaid with 1 mℓ sterile mineral oil. The test was performed by stab inoculating the medium and then incubating it at 37°C for 24 hours. When both tubes turned yellow the isolate was taken as being facultative anaerobic or fermentative. When only the tube without oil turned yellow, the isolate was regarded as aerobic or oxidative. If none of the test tubes turned yellow the isolate was regarded as glucose inactive (Quin et al., 1994). *Vibrio cholerae* isolate (C453) was used as a positive control.

4.3.5 Polymerase Chain Reaction (PCR)

A total of 247 cultures suspected to be *Vibrio* species by their growth on TCBS agar, were screened for specific genes associated with the three major human pathogenic *Vibrio* species (*V. cholerae, V. parahaemolyticus* and *V. vulnificus*). *Vibrio parahaemolyticus* (LMG 2850), *V. vulnificus* (LMG13545) and *V. cholerae* (C453) were included as positive controls. Isolates that reacted in *V. parahaemolyticus* specific PCR were screened for the species specific virulence genes, thermostable direct haemolysin (*tdh*) and thermostable direct haemolysin related haemolysin (*trh*). All isolates that reacted with any of the pathogen specific PCR tests were subjected to 16S rDNA sequencing (Coenye et al., 1999).

4.3.5.1 Chromosomal DNA extraction

DNA required for PCR or sequencing was extracted as described by Le Roux et al. (2004). Bacterial cultures (either test or reference strains) were grown overnight on Nutrient Agar supplemented with 3% sodium chloride (NaCl) or on sea water agar (SWA) (Atlas 2006; Farmer and Hickmann-Brenner, 1991). DNA was extracted by suspending a loopful of the culture in 500 µℓ of sterile distilled water (SDW). After vortex mixing to obtain a homogenous suspension, the cells were boiled for 10 minutes. Cell debris was removed by centrifugation at 1000 rpm for 60 seconds. The supernatant (200 µℓ) was transferred to a sterile 1.5 mℓ Eppendorf tube and frozen at -20°C till used. In some cases DNA was extracted with the Qiagen DNA extraction kit according to the manufacturer's instructions.

4.3.5.2 Oligonucleotide Primers

Oligonucleotide primers used to amplify species specific genes and pathogenicity factors or the sequencing of the16S rRNA are listed in Table 4.1. All primers were synthesised by Inqaba Biotechnology, Pretoria, Republic of South Africa (RSA).

4.3.5.3 PCR conditions

The *V. cholerae* targeting primers (VIB1 and VIB3) were used to directly amplify a 305 bp fragment of the *ompW* gene (Nandi et al., 2000). The PRC was carried out in a 20 µℓ reaction mixture containing 2.0 µℓ 10 × PCR Buffer, 25 µM dNTP's (Thermo Scientific), 2.5 mM $MgCl_2$, 0.125 µM of each primer (VIB1 and VIB3), 0.0025 IU/µℓ Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology) and 5.0 µℓ of template DNA. The rest of the volume was filled with nuclease free water. The PCR cycle consisted of an initial denaturation step at 94°C for 3 minutes, final strand extension at 72°C for 7 minutes and 30 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minute and DNA extension at 72°C for 1 minute.

For the *Vibrio parahaemolyticusp R72H* fragment, PCR reactions were carried out in 30 µℓ reaction mixtures consisting of 3.0 µℓ of 10 × PCR buffer, 20 µM of dNTP's (Thermo Scientific), 1.7 mM $MgCl_2$, 3.3 µM of each primer solutions, (Vp32 and Vp33), 0.001 IU/µℓ Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), and 5 µℓ template DNA. The rest of the volume was filled with nuclease free water. The PCR cycle consisted of an initial denaturation step at 94°C for 5 minutes, and a final extension at 72°C for 7 minutes, with 35 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and primer extension at 72°C for 1 minute (Lee et al., 1995; Robert-Pillot et al., 2002).

Detection of *V. parahaemolyticus* virulence genes, *tdh* and *trh* was done in a reaction mixture consisting of 5.0 µℓ 10 × PCR Buffer, 20.0 µM of dNTP's (Thermo Scientific), 0.15 mM $MgCl_2$ 0.4 µM of each of the primers, (L-*trh* and R-*trh*) for the *trh* gene and (L-*tdh* and R-*tdh*) for the *tdh* gene, 0.0006 IU/µℓ Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), 2 µℓ of template DNA and nuclease free water to a final volume of 50 µl. The PCR cycle consisted of an initial denaturation step of 94°C for 5 minutes, final extension at 72°C for 5 minutes, thirty cycles, each consisting of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute, and primer extension at 72°C for 1 minute (Bej et al., 1999).

For the amplification of the *V. vulnificus* cytolysin-haemolysin gene, the PCR was performed in a 50 µℓ reaction mixture consisting of 10 µℓ of 10 × PCR Buffer, 40.0 µM dNTP's (Thermo Scientific), 2.0 µM of $MgCl_2$, 1.0 µM of each of the primers, (*Choi-1* and *Choi-2*), 0.15 IU/µℓ DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), 2.0 µℓ of test DNA and nuclease free water to the final volume. The PCR cycle consisted of an initial denaturation at 94°C for 3 minutes, and final primer extension at 72°C for 10 minute with 45 cycles each consisting of denaturation, at 94°C for 1minute, primer annealing at 60°C for 1 minutes and primer extension at 72°C for 2 minutes (Lee et al., 1997).

Table 4.1 Primer sequences and their associated gene targets

Primers	Target gene and organism	Amplicon Size (bp)	Primers Sequence (5' to 3')	Reference
VIB1 (Forward)	*ompW* *V. cholerae*	305	CACCAAGAAGGTGACTTTAATTGTG	Nandi et al., (2000)
VIB3 (Reverse)			GGTTTGTCGAATTAGCTTCACC	Nandi et al., (2000)
Vp32 (Forward)	pR72H *V. parahaemolyticus*	387 or 320	TGCG AATTCGATAGGGTGTTAACC	Lee et al., (1995);
Vp33 (Reverse)			CGAATCCTTGAACATACGCAGC	Robert-Pillot et al., (2002)
Choi1 (Forward)	Cytolysin-haemolysin *V. vulnificus*	704	GACTATCGCATCAACAACCG	Lee and Choi (1995)
Choi2 (Reverse)			AGGCGAGTATTACTGCC-3'GTA	Lee and Choi (1995)
pA (16F27)	16SrRNA of prokaryotes	1458	AGA GTT TGA TCC TGG CTC AG	Coenye et al., (1999)
MH2 (16R1485)			TAC CTT GTT ACG ACT TCA CCC CA	Coenye et al., (1999)
*pD (16F536)	16S rRNA sequencing	849	CAGCAGCCGCGGTAATAC	Coenye et al., (1999)
VA-F (Forward)	Collagenase *V. alginolyticus*	737	CGAGTACAGTCACTTGAAAGC	Di Pinto et al., (2006)
VA-R (Reverse)			CACAACAGAACTCGCGTTACC	Di Pinto et al., (2006)
L-trh (Forward)	*TDH-related haemolysin of V. parahaemolyticus*	500	TTGGCTTCGATATTTTCAGTATCT	Bej et al., (1999)
R-trh (Reverse)			CATAACAAACATATGCCCATTTCC	Bej et al., (1999)
L-tdh (Forward)	*Thermostable direct haemolysin of V. parahaemolyticus*		GTAAAGGTCTCTGACTTTTGGAC	Bej et al., (1999)
R-tdh (Reverse)		269	TGGAATAGAACCTTCATCTTCACC	Bej et al., (1999)

For the *V. alginolyticus* collagenase gene, PCR amplification of the gene was carried out in a reaction mixture consisting of 5.0 μℓ 10 × PCR Buffer, 20.0 μM dNTP's (Thermo Scientific), 1.5 μM $MgCl_2$, 0.2 μM of each of the Primers, (*VA-F* and *VA-R*), 0.001 IU/μℓ of Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology, 3.0 μℓ of DNA. The volume was made up to 50 μℓ with nuclease free water. The PCR cycle consisted of initial denaturation at 95°C for 15 minutes, and the final primer extension at 72°C for 5 minutes, 35 cycles, each consisting of denaturation at 94°C for 30 seconds, primer annealing at 57°C for 30 seconds and primer extension at 72°C for 1 minute (Di Pinto et al., 2006).

The 16S rDNA was amplified in a total volume of 50 μℓ, consisting of 5 μℓ of 10 × PCR buffer, 2.0 μM of dNTP's (Thermo Scientific), 2.0 mM of $MgCl_2$, 5.0 μM of each primer, 0.0005 IU/μℓ of Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology) 2.0 μℓ of template DNA and nuclease free water to make up the volume. The PCR cycle included the following steps. Initial denaturation at 94°C, a final primer extension at 72°C for 5 minutes and 30 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1minute and primer extension at 72°C for 5 minutes (Coenye et al., 1999). Bands of the desired size (1458 bp) and intensity were cut out of the gel and purified using the Qiagen Gel purification kit.

In all PCR reactions the appropriate type strain was included as the positive control. The DNA was substituted for nuclease free water in the negative control. All PCR mixtures were held at 4°C after the final primer extension step. The DNA amplicons were observed by running the PCR reactions on 1.5% (w/v) agarose gel (Amersham Pharmacia Biotech) in 1×TE (Tris phosphate EDTA) buffer at 80 V, 200 mA and 100 W for 40–45 minutes. Bands were observed using an Upland CA 9178 USA transilluminator Model M5. Gel photos were taken using the Vilber Lourmat camera with Vida max screen.

The 16S rDNA sequencing PCR was carried out in a 10 μℓ reaction mixture consisting of the following reagents. Nuclease free water (5 μℓ), 1 μℓ of 5 × sequencing buffer, 1 μℓ of the sequencing primer *pD (Forward), 2 μℓ of Big dye Chain Terminator mixture and 1 μℓ

template DNA to approximately a final concentration of 150 ng. The PCR cycle consisted of initial denaturation at 96°C for 5 seconds, a final extension at 60°C for 4 minutes, and 25 cycles, each consisting of denaturation at 96°C for 10 seconds, primer annealing at 55°C for 5 seconds and the primer extension at 60°C for 4 minutes.

4.3.6 Sequence alignment and phylogenetic analysis.

Sequence alignments were carried out using Clustal X software (Thompson et al., 1997) and the sequences were edited and trimmed in Bioedit Sequence alignment Editor v. 5.0.9 (Hall, 1999). Phylogenetic trees of the 16S rDNA sequences were draw with PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 2000) using the Neighbour –Joining (NJ) method (Saitou and Nei, 1987). Confidence limits were determined using Bootstraps (Efron, 1979). Thirty-one sequences of known species, mainly type strains, obtained from GenBank (http://www.cnbi.nlm.nih.gov/Genbank/) were included in the phylogenetic trees to assist with the final grouping of the unknown sequences (Sneath, 1989).

4.3.7 API 20E and other biochemical tests

Due to unresolved identifications for many of the isolates based on the 16S rRNA sequencing, extra phenotypic data were collected for these isolates. The phenotypic data included the Analytical Profile Index (API) 20E in conjunction with individual biochemical tests such as cellobiose and sucrose fermentation and growth in Nutrient broth containing 10 and 12% sodium chloride (Farmer et al., 2004; Oliver and Kaper, 1997).

The API 20E (BioMérieux) was selected to assist with the identification especially to help differentiate between *V. parahaemolyticus* and *V. alginolyticus* strains (Oliver and Kaper, 1997; Farmer et al., 2004) For this purpose special attention was given to the Voges-Proskauer (VP) test, ornithine decarboxylase activity and arabinose fermentation. The API 20E test was done according to the supplier's instructions and proposed species identification was obtained using the API lab software.

4.3.8 Phenotypic comparison

The 24 phenotypic characters for 52 isolates that clustered with *Vibrios* were also imported into Bionumerics and a similarity coefficient dendrogram was drawn (Austin and Colwell, 1977) to demarcate isolates as either *V. parahaemolyticus* or *V. alginolyticus*. These characters include 20 miniaturized biochemical tests on API 20E, the cellobiose fermentation test determined according to Jones and Wise, (1925) in a sterile 1% cellobiose solution supplemented with 3% sodium chloride and 0.15% Bromocresol purple as pH indicator, sucrose fermentation test by growing the isolates on TCBS, the oxidase test as described by Quin et al. (1994) and the ability to growth in nutrient broth containing 10% sodium chloride (Farmer et al., 2004; Oliver and Kaper, 1997;). *V. parahaemolyticus* LMG 2850 and *V. alginolyticus* LMG4409 were used as controls.

4.4 RESULTS.

A total of 247 bacteria were selected from TCBS agar and subjected to a number of identification protocols; Gram reaction, oxidase and Oxidative/Fermentative (O/F) tests, PCR detection of species specific genes (*V. cholerae ompW*, *V. parahaemolyticus pR72H* fragment, and the *V. vulnificus vvh* and the *V. alginolyticus* collagenase gene), 16S rRNA gene sequence alignment, phylogenetic analysis using the 591 bp fragment of the 16S rDNA, API20E, and the numerical taxonomy using 24 phenotypic attributes.

4.4.1 Gram reaction, sucrose fermentation, oxidase and O-F tests

Two hundred and forty-three (98. 3%) isolates formed the characteristic string in the KOH test and were confirmed as Gram negative bacteria. Only four (1.6%) of the 247 isolates were negative for the KOH test and were considered to be Gram positive (Table 4.4). The four Gram positive isolates were excluded from other tests that followed since our concern lay with the Gram negative bacteria of the γ subclass of the *Proteobacteria*. Only 243 isolates will be discussed further. One hundred and twenty-eight (52.6%) of the Gram negative bacterial isolates were sucrose fermenters, while one hundred and fifteen (47.3%) were sucrose non fermenters.

Based on the oxidase and O-F tests performed on 243 isolates only 81 (33.3%) of the isolates were oxidase positive, fermentative or facultative anaerobic (F/O-F) hence belonging to the family *Vibrionaceae* (including genera such as *Vibrio*, *Aeromonas* and *Shewanella*). Eight (3.31%) of the isolates were oxidase positive, oxidative and possibly belonging to the family *Pseudomonadaceae*. Fifty-three (21.8%) isolates were oxidase positive but glucose inactive hence regarded as either *Vibrionaceae* or *Pseudomonadaceae*. Four (1.6%) isolates were oxidase negative and glucose inactive and were suspected to be *Enterobacteriaceae*. Eighty-four (34.6%) of the isolates were oxidase negative facultative anaerobic and were grouped into the family *Enterobacteriaceae*, of the genera such as *Morganella, Pectobacterium, Citrobacter, Providencia,* and *Proteus*. Thirteen (5.3%) of the isolates were oxidase positive, but gave doubtful results in the O-F test (Figure 4.1).

Twenty-eight (11.5%) of the 243 bacteria were isolated from headed and gutted (H and G) hake fish, fifty-eight (23.9%) were from hake fish after the filleting stage (ABM-S), ninety-six (39.5%) were from hake fish packaged products (FP), twenty-one (8.6%) were isolated from the headed and gutted pilchards Jitney (tall line) (TL), twenty-five (10.3%) were isolated from washed and packaged pilchards bait line (BL) and fifteen (6.2%) were isolated from headed and gutted demersal horse mackerel (HM).

4.4.2 Identification of isolates to species level

Due to the unusual phenotypic variability of the bacteria isolated from TCBS e.g. the inability to ferment glucose and because of problems experienced in the past by other researchers to accurately identify *Vibrio* species to species level (Colwell, 1970; Molitoris et al., 1985) a polyphasic taxonomy approach of identifying the isolates (Oosthuizen, 1998) was taken employing both genotypic and phenotypic traits. Screening of all the isolates for the presence of genes typically associated with the human pathogenic *Vibrio spp*. was used as the starting point.

4.4.2.1 Detection of species specific genes using PCR.

None of the 243 cultures screened were positive for the amplification of the 305 bp fragment of the *V. cholerae* ompW gene. Fifty-seven (23.5 %) isolates reacted with the Vp32 / Vp33 primer pair specific for the *pR72H* fragment of *V. parahaemolyticus*. Thirty three of these isolates produced two bands, 387 and 400 bp in size while 24 produced the expected single band of 387 bp (Table 4.2).

Eighty-eight (36.2%) of all the isolates reacted with the *V. vulnificus* specific Choi1 / Choi2 primer pair targeting the cytolysin/haemolysin gene, 19 of these isolates also reacted with the *V. parahaemolyticus* specific primers (Figure 4.2). Of the 88 Choi1 / Choi2 primer pair positive isolates, thirty-seven formed a band of 704 bp which was similar in size to the band formed by the positive control strain *V. vulnificus* LMG 13545. Twenty-four isolates

formed bands smaller than that of the positive control and eleven formed a band larger than the typical 704 bp product. Another sixteen isolates formed bands either equal and greater or equal and smaller to the band produced by the positive control (Table 4.2).

4.4.2.2 PCR for detection of *V. parahaemolyticus* virulence genes

Only 7 (12.1%) of the 57 isolates that were positive for the *pR72H* fragment produced a product for the L-*trh* / R-*trh* primer pair. Strong bands were detected although these bands were about 550bp and 600bp in size (results not shown).The amplified products were all of higher molecular weight than the typical 500 bp product obtained from the *V. parahaemolyticus* strain LMG2850. None of the isolates reacted with the R-*tdh*/L-*tdh* primer pair.

4.4.2.3 16S rRNA gene sequence analysis

The partial 16S rRNA genes of 119 of the 127 isolates that reacted with either the Vp32/Vp33 and/or the Choi1/Choi2 primers pairs were sequenced. Eight isolates were not sequenced because they were lost during the sub culturing process.

Table 4.2 Grouping of isolates that reacted with *V. parahaemolyticus* and *V. vulnificus* specific (*VP32/Vp33* and *Choi1/Choi2*) primer pairs according to genera

Amplification of species specific genes	*pR72H* (bp)	*vvh* (bp	Isolate/s	Number	Genera
	387	704	381, 577, 622	3	*Vibrio, Shewanella*
	387 + 400	704	445, 447, 482, 523, 524	5	*Vibrio*
	387	<704	412, 422, 430	3	*Vibrio*
	387 +400	<704	414, 415, 462, 464, 483, 522	6	*Vibrio*
	387	>704	406, 624	2	*Vibrio*
	387	-ve	382, 383, 425, 432, 435, 499, 526, 588, 590, 601, 602 606, 608, 614, 617, 619, 634,	17	*Vibrio, Shewanella*
	387 + 400	-ve	386, 388, 389, 407, 409, 410, 411, 421, 423, 426, 427, 434, 436, 439, 440, 510, 511, 517, 595, 611, 638, 645	22	*Vibrio, Citrobacter, Shewanella*

Table 4.2 continued

Amplification of species specific genes	*pR72H* (bp)	*vvh* (bp	Isolate/s	Number	Genera
	-ve	704	343, 372, 374, 377, 391, 394, 398, 408, 416, 420, 424, 428, 429, 449, 463, 465, 472, 479, 480, 481, 486, 487, 490, 525, 578, 589, 598, 599, 633, 640	29	*Aeromonas, Citrobacter, Enterobacter, Morganella, Providencia, Shewanella, Vibrio,*
	-ve	<704	385, 441, 469, 477, 569, 575, 576, 593, 600, 615, 618, 623, 626, 632, 636	15	*Enterobacter, Morganella, Shewanella, Providencia, Vibrio,*
	-ve	>704	475, 478, 494, 501, 574, 583, 596, 613, 616	9	*Morganella, Providencia, Shewanella, Vibrio,*
	-ve	≤ / ≥ 704	341, 390, 397, 431, 437, 445, 446, 448, 459, 460, 467, 473, 476, 500, 639, 582	16	*Aeromonas Citrobacter, Morganella, Providencia, Pseudomonas, Vibrio,*
Total	58	88		127	

4.4.2.3.1 Sequence similarities

A BLASTn search of the 591 bp sequence of the 16S rRNA of 119 isolates was compared to similar sequences in Genbank database. The results indicated that these strains were closely related to a number of known bacterial species. Thirty one isolates were found to have between 97% and 100% 16S rDNA sequence homology to *V. alginolyticus* strain X56576. Twenty-two (18.5%) of the isolates'partial 16S rRNA sequence was closely aligned to *V. parahaemolyticus*, 3 (15.0%) of which had 100%, 14 (70.0%) had 99%, 2 (10.0%) had 98%, and 3 (15.0%) had 97% 16S rDNA sequence similarity with *V. parahaemolyticus* strain AF388386. Eighty-three (69.7%) of the isolates were identified by the BLASTn as belonging to the family *Vibrionaceae* and of these isolates sixty-six

(55.5%) were suggested to belong to the genus *Vibrio*, thirteen (10.9%) to the genus *Shewanella*, and four (3.4%) to the genus *Aeromonas*. Thirty-one (26.1%) of the isolates were proposed to belong to the family of *Enterobacteriaceae*. Five of the isolates were closely aligned with *Pseudomonas* spp. (Table 4.3).

4.4.2.3.2 Phylogenetic analysis

After all the partial 16S rRNA sequences were trimmed to yield a 591bp sequence for each isolate, two trees were constructed using the Neighbour joining method to group the unknown isolates. One tree consisted of 80 *Vibrionaceae* related isolates and the second tree consisted of the 30 isolates grouped as belonging to the *Enterobacteriaceae*. Four sequences were not included in the trees because they did not produce good sequences of the desired fragment size. Also five sequences belonging to *Pseudomonas* were excluded from the trees because they were very different from the sequences of the *Vibrionaceae* and *Enterobacteriaceae*.

The NJ method assigned the 80 *Vibrionaceae* to 14 clusters. Fifty-eight isolates grouped in cluster 1 with *V. alginolyticus, V. campbellii, V. natriegens, V. mytili, V. rotiferianus, V. diabolicus, V. parahaemolyticus* and *V. nereis* type strains. Four isolates grouped together in cluster 2, while one formed a separate group in cluster 3. Cluster 4 consisted of *V. fluvialis*, cluster 5 consisted of *V. harveyi* and cluster 6 consisted of *V. proteolyticus* type strains. None of the isolates clustered within these groups. Cluster 7 consisted of *V. albensis*, *V. cholera,* and *V. mimicus*. Again none of the isolates were found in these clusters. One isolate clustered within the *Listonella anquillarum* group (cluster 8).

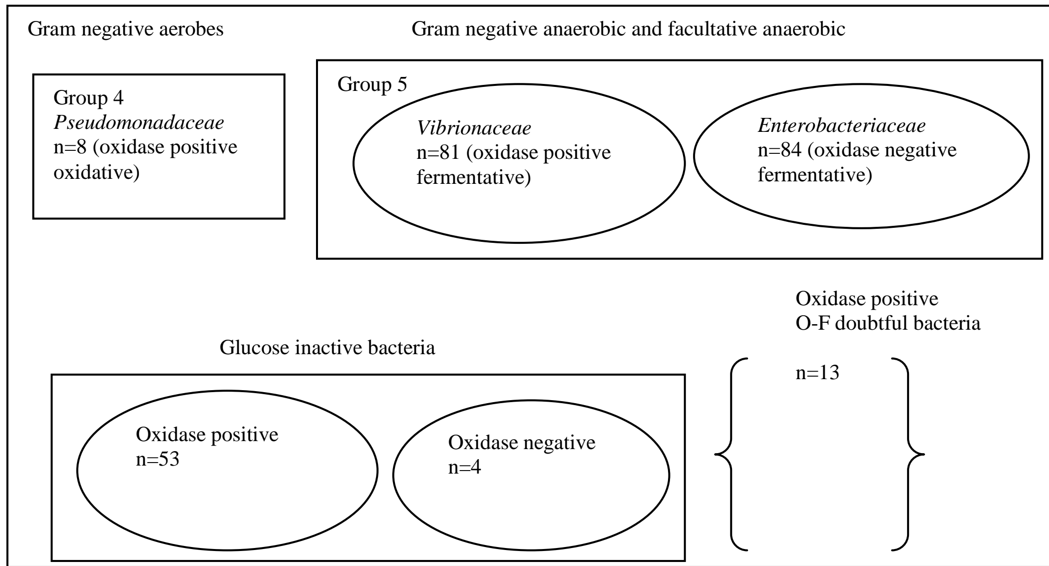

Figure 4.1 Potential groups where Gram negative bacteria isolated from TCBS could belong to. Four isolates were Gram positive

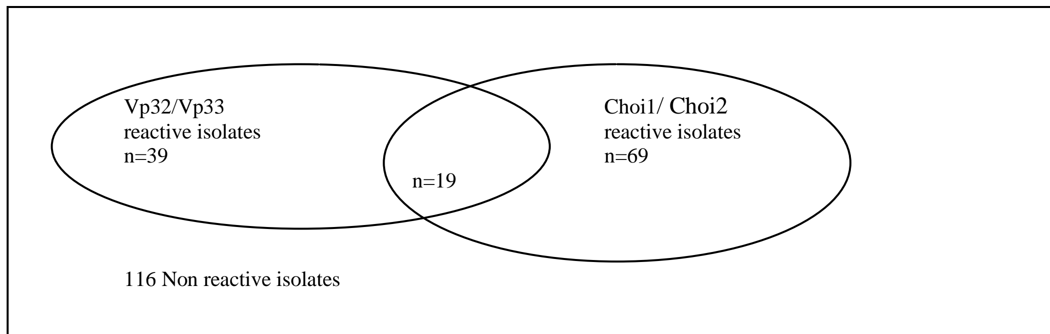

Figure 4.2 Grouping of isolates from TCBS that reacted with the *VP32/Vp33* and *Choi1/Choi2* primer pairs

Cluster 9 consisted of eight isolates belonging to the genus *Shewanella.* Three isolates clustered together with *Shewanella haliotis* in group 10. One of the isolates was found within cluster 11 together with *Shewanella algae*. *Shewanella putrefaciens* formed a separate cluster 12, but none of the isolates were found in this group. Cluster 13 was a distinct group consisting of the *Aeromonas veronii* and three isolates, cluster14 consisted of *Aeromonas hydrophyla* type strains with one isolate. Most of these groups were supported with high bootstrap values of between 55-100%. *Aeromonas punctata* was used as an outgroup (Figure 4.3). In the second tree the 30 *Enterobacteriaceae* isolates were divided into four groups; fifteen isolates clustered with *Providencia alcalifaciens*, *Providencia rustigianii*, *Providencia heimbachae* and *Providencia rettgeri* in group 1.Ten (10) isolates clustered together with *Morganella morganii* subspecies *morganii* and *Morganella morganii* subspecies *sibonii* in group 2. Four (4) isolates clustered together with *Citrobacter braakii*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Citrobacter werkmanii* in group 3. One isolate clustered with *Enterobacter hormaechei in* group 4. The four groups were supported by high bootsrap values of between 52 and 98. *Serratia marcescens* was used as an outgroup (Figure 4.4).

Table 4.3 Isolates that reacted with *Vp32/Vp33* and *Choi1/Choi2* primer pairs grouped according to their similarities to families and genera based on a BLASTn search.

Taxon identification based on BLASTn results	Number of isolates	Family	Genus
V. alginolyticus	31	*Vibrionaceae*	*Vibrio*
V. parahaemolyticus	22	*Vibrionaceae*	*Vibrio*
Vibrio spp	11	*Vibrionaceae*	*Vibrio*
V. harveyi	1	*Vibrionaceae*	*Vibrio*
Vibrio natriegens	1	*Vibrionaceae*	*Vibrio*
Shewanella alga	13	*Vibrionaceae*	*Shewanella*
Aeromonas hydrophyla	2	*Vibrionaceae*	*Aeromonas*
Aeromonas media.	1	*Vibrionaceae*	*Aeromonas*
Aeromonas molluscorum	1	*Vibrionaceae*	*Aeromonas*
Citrobacter freundii	3	*Enterobacteriaceae*	*Citrobacter*
Enterobacter aerogenes	2	*Enterobacteriaceae*	*Enterobacter*
Enterobacter spp	1	*Enterobacteriaceae*	*Enterobacter*
Enterobacteriaceae bacterium	1	*Enterobacteriaceae*	*Enterobacteriaceae*
Morganella morganii	10	*Enterobacteriaceae*	*Morganella*
Pectobacterium (Erwinia) carotovorum)	1	*Enterobacteriaceae*	*Erwinia*
Providencia alcalifaciens	3	*Enterobacteriaceae*	*Providencia*
Providencia heimbachae	2	*Enterobacteriaceae*	*Providencia*
Providencia rustigianii	8	*Enterobacteriaceae*	*Providencia*
Pseudomonas spp.	5	*Pseudomonadaceae*	*Pseudomonas*

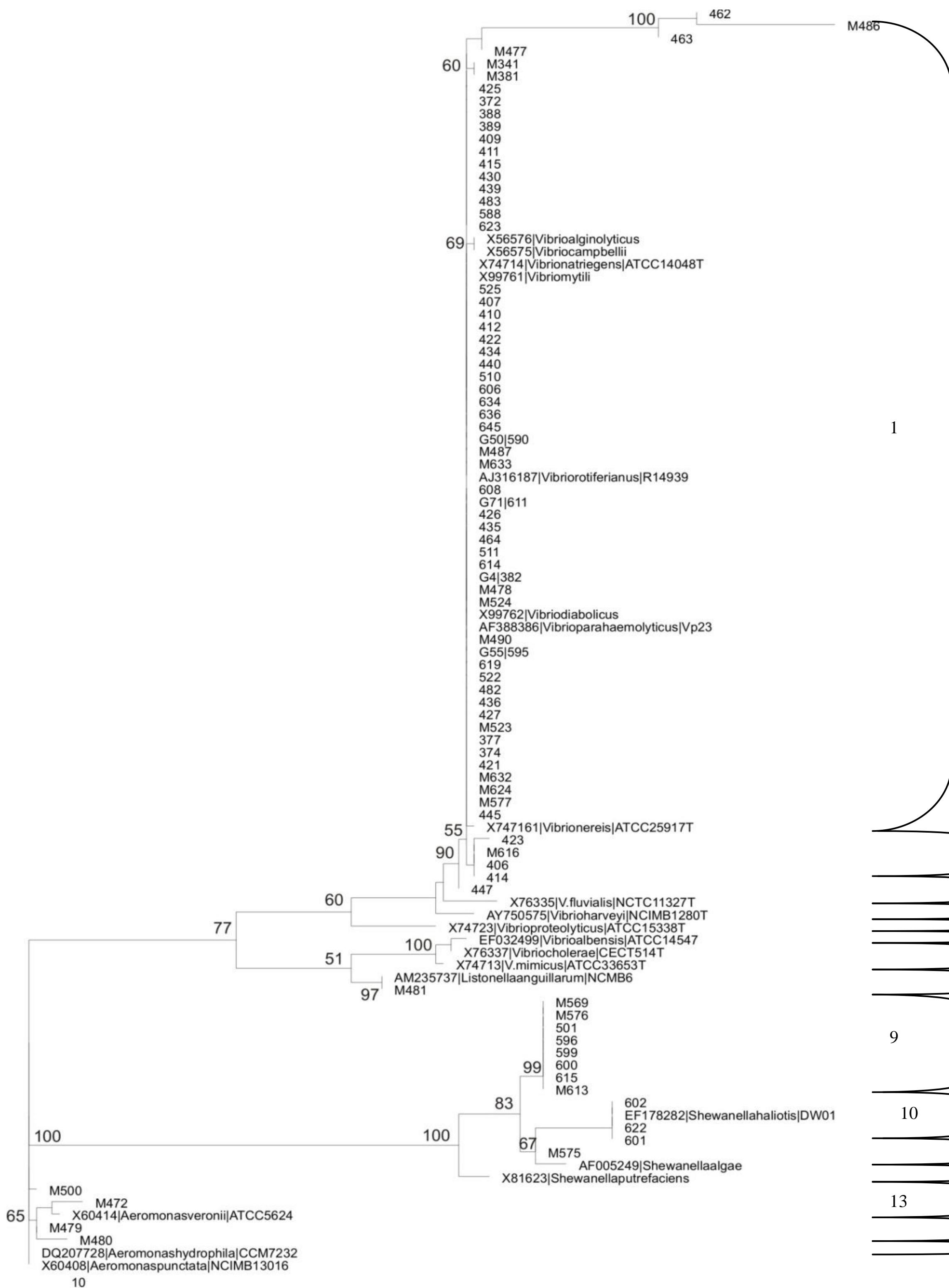

Figure 4.3 Neighbour-joining tree of 80 isolates and 21 type strains belonging to the *Vibrionaceae* based on a591 bp sequence of the 16S rRNA. Bootstrap values based on a 1000 repeats. *Aeromonas punctata* was used as an outgroup. Not all the clusters are indicated with brackets.

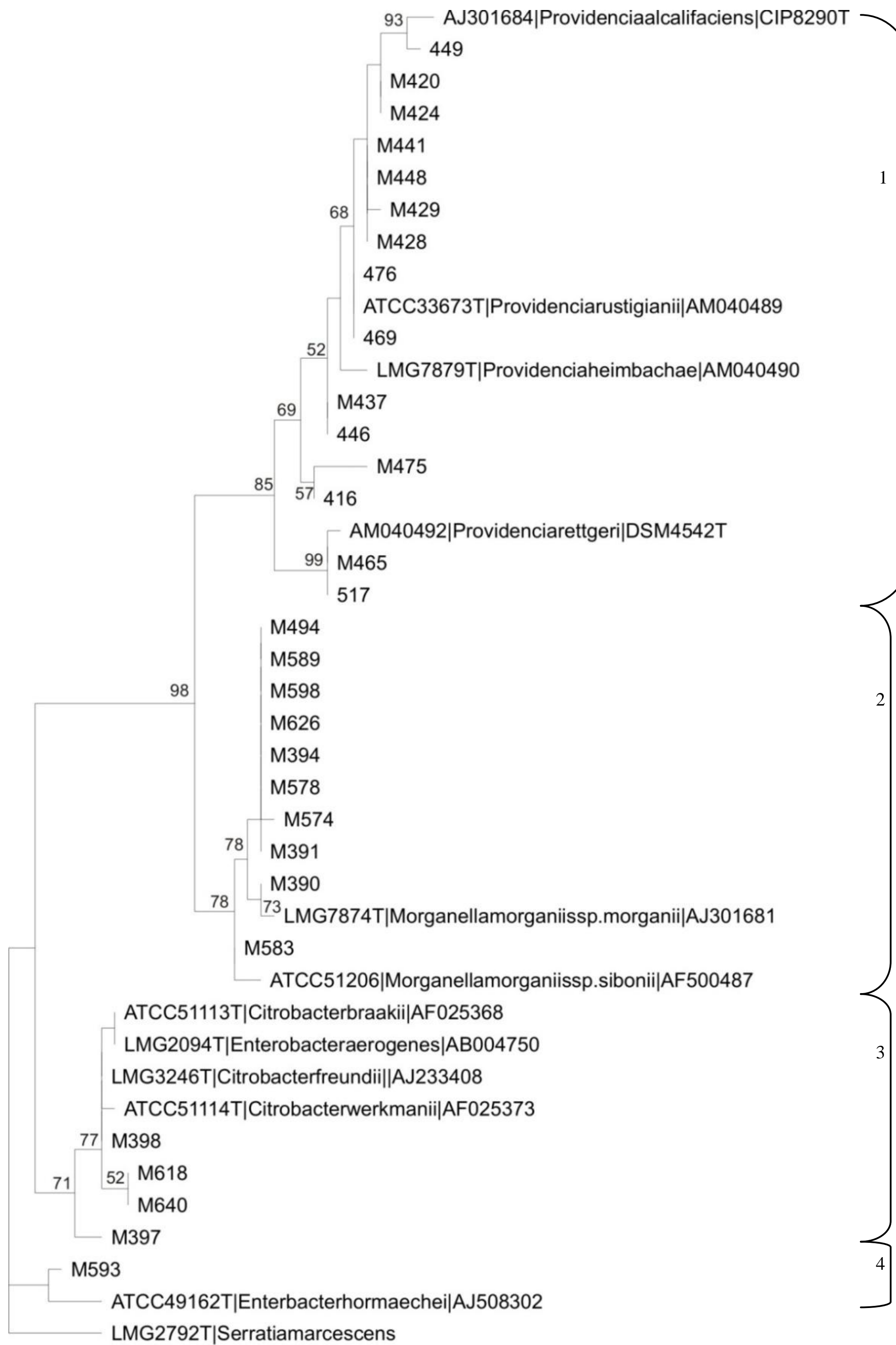

Figure 4.4 Neighbour-joining tree of 30 isolates and 21 type strains belonging to the family *Enterobacteriaceae* based on a591 bp sequence of the 16S rRNA. Bootstrap values based on a 1000 repeats. *Serratia marcescens* was used as an outgroup.

4.4.3 Confirmation of isolates identities

To confirm their identities the 63 isolates that clustered within the *V. parahaemolyticus*/ *V. alginolyticus group* were subjected to API 20E and a few other phenotypic tests (VP, ODC tests, L-Arabinose, cellobiose and sucrose fermentations as well as growth in 10% NaCl) to enable demarcation of the two species (Farmer et al., 2004; Oliver and Kaper, 1997).

4.4.3.1 API 20E and other biochemical tests

Based on the API lab software database 58 (92.1%) of the 63 isolates, for which it was impossible to distinguish between *V. Alginolyticus* or *V. parahaemolyticus* in the NJ method eighteen (18) of these isolates also aligned with *V. parahaemolyticus* in the BLASTn of their 16S rRNA sequences, were identified as *V. alginolyticus,* four (6.3%) (406, 407, 415, and 511) were identified as *A. hydrophyla* and one (634) was identified as *Pasteurella pneumotropica*. Twenty-six of these isolates were, however, only 49.5% similar to the standard *V. alginolyticus*. These isolates differed from the typical *V. alginolyticus* biochemical profile in tests such as cellobiose and amygladin fermentation, the VP and lysine decarboxylase (LDC) tests some of which are important in the differentiation of *V. parahaemolyticus* from *V. alginolyticus*. *V. alginolyticus* LMG4409 did not ferment cellobiose or amygladin and was VP and LDC tests negative.

4.4.3.2 Phenotypic comparison

The biochemical profiles of 52 selected isolates that clustered within the *V. Parahaemolyticus/ V. alginolyticus* group were imported into Bionumerics and a dendrogram was constructed using the Jaccard coefficient and unweighted pair group with arithmetic average (UPGMA) (Figure 4.5). *V. alginolyticus* LMG4409 and *V. parahaemolyticus* LMG2850 were used as controls. The examined isolates were analysed for 24 traits. All isolates clustered closer to *V. alginolyticus* LMG4409 and were clearly distinct from *V. parahaemolyticus* LMG2850. Isolate 406 differed substantially from the other *V. alginolyticus* strains as it fermented arabinose, produced urease and H_2S. It was also TDA (tryptophan deaminase) positive and the isolate did not produce indole from tryptophan (Figure 4.5)

4.4.3.3 *V. alginolyticus* specific collagenase gene PCR.

According to the BLASTn results 22 of the isolates that amplified the *pR72H* fragment, were potential *V. parahaemolyticus* isolates. These isolates were screened for the *V. alginolyticus* specific collagenase gene. Only three (13.6 %) of the isolates did not react with *V. alginolyticus* specific collagenase gene primers. Nineteen of these isolates produced the 737 bp fragment of *V. alginolyticus* collagenase gene. *V. alginolyticus* LMG4409 gave the same reaction. The type strains of *V. parahaemolyticus* LMG2850*, V. vulnificus* LMG 13545 and an environmental strain of *V. cholerae* C453 did not react (results not shown).

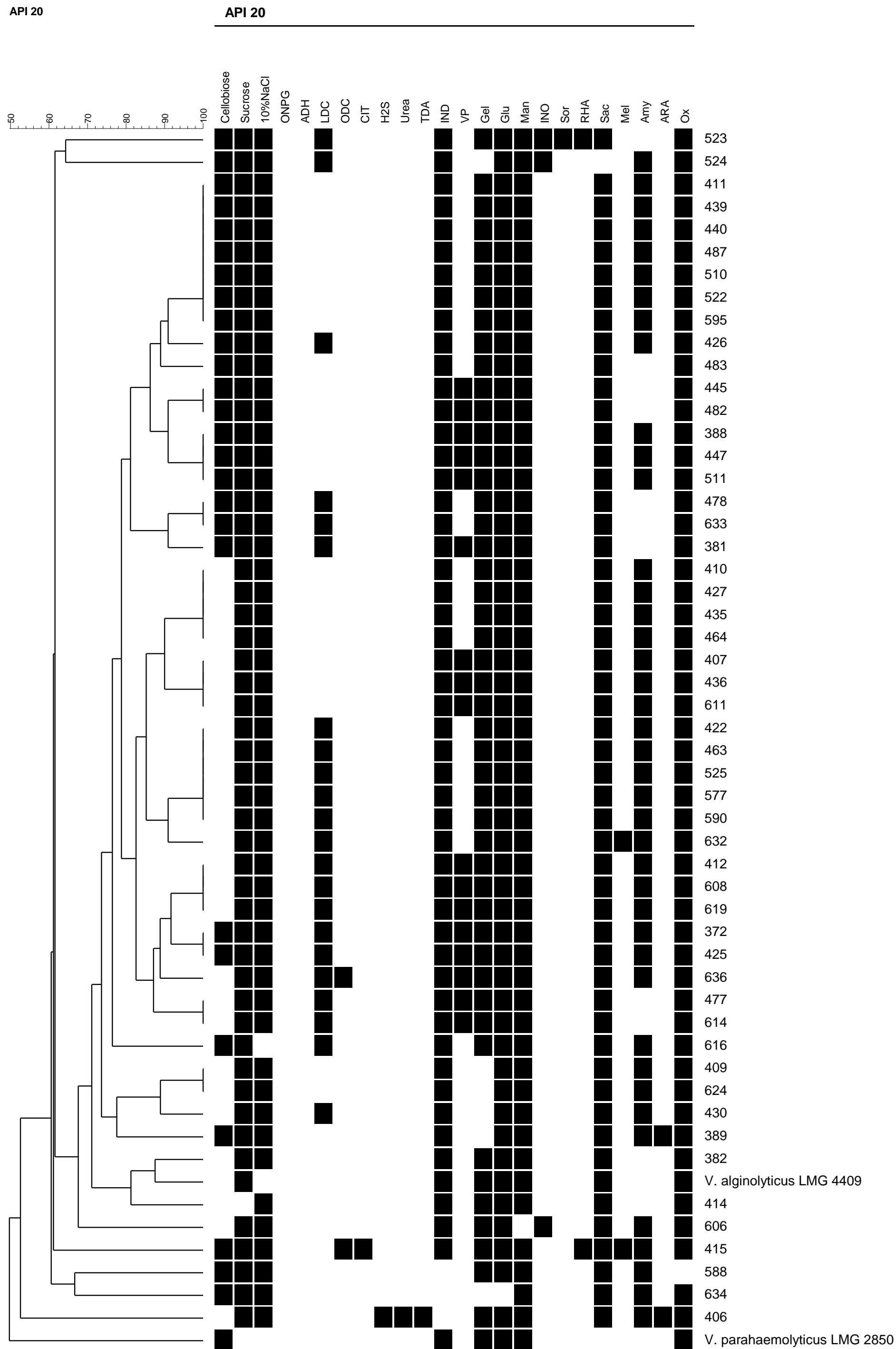

Figure 4.5 Dendrogram showing the levels of phenotypic similarity amongst the 52 selected *Vibrio* species isolates where a clear differentiation between *V. parahaemolyticus* and *V. alginolyticus* could not be made based on the 16S rRNA sequence data.

4.4.4 Final identities of isolates that were selected from TCBS.

Of all the isolates investigated 4 were Gram positive and 243 were Gram negative. The initial grouping of the sucrose negative and sucrose fermenting isolates according to the bacteria families they belonged to are shown in Table 4.4. Out of the 243 isolates fifty-two were confirmed to be *V. alginolyticus* according to the combined results. The other *Vibrionaceae* could be *V. campbellii, V. natriegens*, *V. mytili*, *V. rotiferianus*, *V. diabolicus*, *V. nereis*, or *Listonella anquillarum*. The *Enterobacteriaceae* belonged mainly to *Providencia*, *Morganella* and *Citrobacter* genera.

The majority of the isolates belonged to the family *Vibrionaceae* (*Vibrios, Shewanella)* and *Aeromonadaceae* followed by the family *Enterobacteriaceae*. Sucrose negative isolates that showed nonspecific amplification of the *pR72H* fragment of *V. parahaemolyticus* and the cytolysin–haemolysin gene of *V. Vulnificus* were *Shewanella*, *Morganella*, *Aeromonas*, *Providencia* spp. and *Pseudomonadaceae*.

Table 4.4 Grouping of 247 bacteria isolated from TCBS grouped based on the sucrose fermentation

Gram	Sucrose	Heading	Isolates	Number	Family
negative	positive	Oxidase+ve and O	459, 460, 484	3	*Pseudomonadaceae*
		Oxidase +ve and OF/F	341, 372, 374, 377, 381, 382,383, 386, 388, 399, 406, 407, 409, 411, 415, 419, 421, 423, 434, 438, 440, 461, 462, 464, 481, 488, 490, 499, 502, 509, 510, 521, 522, 523, 524, 525, 526, 528, 530, 531, 532, 533, 534, 535, 536, 541, 542, 544, 545, 546, 547, 548, 590, 594, 595, 606, 608, 611, 614, 619, 632, 633, 634, 635, 638, 645, 648, 649	68	*Vibrionaceae*
		Oxidase+ve and Glu$^-$ve	343, 379, 401, 402, 404, 412, 432, 435, 436, 454, 455, 463, 482, 483, 486, 495, 504, 511, 512, 516, 518, 543, 553, 570, 572, 586, 588, 597, 612, 616, 623, 624	32	*Pseudomonadaceae* *Vibrionaceae*

Table 4.4 continued

Gram	Sucrose	Heading	Isolates	Number	Family
negative	positive	Oxidase-ve and OF/F	389, 400, 403, 405, 408, 424, 441, 445, 449, 529, 538, 593	12	*Enterobacteriaceae*
		Oxidase-ve and Glu-ve	452, 644,	2	*Enterobacteriaceae*
		Oxidase+ve and doubtful	410, 422, 425, 426, 427, 430, 439, 447, 478, 487, 577, 636,	12	*Vibrionaceae*
	Sucrose negative	Oxidase+ve and O	568, 582, 617, 628, 629	5	*Pseudomonadaceae*
		Oxidase +ve and OF/F	385, 431, 443, 451, 468, 472, 479, 480, 496, 500, 520,	11	*Vibrionaceae*
		Oxidase+ve and Glu-ve	467, 473, 501, 514, 537, 550, 551, 569, 571, 575, 576, 596, 599, 600, 601, 602, 613, 615, 622, 641, 642	21	*Pseudomonadaceae* *Vibrionaceae*

Table 4.4 continued

Gram	Sucrose	Heading	Isolates	Number	Family
negative	negative	Oxidase-ve and OF/F	384, 387, 390, 391, 392, 394, 396, 397, 398, 417, 418, 433, 437, 446, 448, 465, 466, 469, 475, 476, 477, 492, 493, 494, 497, 498, 503, 505, 506, 507, 515, 517, 519, 527, 539, 540, 549, 574, 578, 579, 580, 581, 583, 584, 585, 587, 589, 591, 592, 598, 603, 604, 605, 607, 609, 610, 618, 620, 621, 637, 639, 643, ,640, 626, 630, 631, 646, 647	68	*Enterobacteriaceae*
		Oxidase-ve and Glu-ve	416, 420, 428, 429, 453, 456, 457, 513	8	*Enterobacteriaceae*
		Oxidase+ve and doubtful	414	1	*Vibrionaceae*

Table 4.4 continued

Gram positive	Sucrose positive	Heading	Isolates	Number	Family
		Oxidase+ve and O	444	1	*Micrococci*?
		Oxidase-ve and NR	450	1	*Micrococci*?
	Sucrose negative	Oxidase+ve and O	458	1	*Micrococci*?
		Oxidase –ve and OF	380	1	*Streptococcus*?

Abbreviations: Oxidase+ve and O= Oxidase positive and oxidative, Oxidase +ve and OF/F = Oxidase positive and fermentative, Oxidase+ve and Glu^{-}ve = oxidase positive and glucose inactive, Oxidase-ve and OF/F = Oxidase negative and fermentative, Oxidase-ve and Glu-ve= oxidase negative and glucose inactive, Oxidase+ve and doubtful= oxidase positive and glucose fermentation doubtful.

4.5 DISCUSSION

From a consumer health perspective it was important to establish the identities of the bacterial species associated with processed hake, pilchard and horse mackerel. *Vibrio* species are one of the most important groups of bacteria that cause food borne diseases as a result of the consumption of contaminated fish. The diseases associated with human pathogenic *Vibrio* species; (cholera, gastroenteritis, septicaemia and wound infection) cause huge economic losses and sometimes are fatal (Thompson et al., 2004b). The fatality rate in *V. vulnificus* infections is 40-50% (Hsueh et al., 2004). In addition the presence of human pathogenic *Vibrio* species in these fish species creates an impediment in international trade. According to the current trade agreement hake would be rejected in case *V. cholerae* was detected in the fish by the recipient countries especially those of the EU.

TCBS is one of the recommended media used for the selective isolation of *Vibrio* species (Ottaviani et al., 2003). The selectivity of this medium, may, however, vary (Sakazaki and Ballows, 1991). *Vibrios* form characteristic colonies (yellow or green) based on the ability of the isolate to ferment sucrose. In certain instances TCBS medium may be too inhibitory and suppresses the growth of some species of *Vibrio*. Bacteria other than *Vibrios* such as *Enterobacteriaceae*, *Proteus*, *Aeromonas* and *Staphylococci* may also grow (Joseph et al., 1982). During this study both sucrose and sucrose non fermenting bacteria were identified because both groups are found as human pathogens often transmitted to humans via consumption of fish or through sea water (Drake et al., 2007). In addition the three human pathogenic *Vibrio* species do not have specific sucrose fermenting characteristics (Arias et al., 1998; Joseph et al., 1982; Nagao et al., 2006; Oliver et al., 1992;).

Of the bacteria isolated from TCBS 243 isolates belonged to the class γ-Proteobacteria, (Holt et al., 1994), and only four isolates were Gram positive confirming that TCBS is not 100% selective for Gram negative bacteria as was also found by others (Gomez-Gil and Roque, 2006). Gram positive bacteria form an important part of the bacterial

population on fish. It has been reported that isolates from the genus *Micrococcus* were part of the predominant bacteria on fish from warm waters (Genarri et al., 1999). *Moraxella, Pseudomonas, Corynebacterium* and *Micrococcus* were isolated from hake fish of the Atlantic waters (Vennemann et al., 1994). Out of 243 isolates 133 were sucrose fermenters while 110 were sucrose non fermenters. However pathogenic *Vibrio* species belong to both groups and variability within a species with regard to sucrose fermentation is common; hence the sucrose fermentation test is not an important trait in the taxonomy of *Vibrio* species. In addition this test was unable to differentiate *Vibrio* species from *Enterobacteriaceae* and *Pseudomonadaceae*. Although some sucrose negative colonies were isolated no typical *V. parahaemolyticus* colonies were detected on TCBS.

After the Gram stain differentiation, the first step to group and identify the strains isolated from TCBS was to carry out the oxidase and O-F tests. A substantial number of the bacteria isolated during this study, 53 (21.8%) were oxidase positive and glucose inactive, while 4.1% were oxidase positive, oxidative and possibly belonging to the family *Pseudomonadaceae.* The 16S rRNA gene sequence alignment indicated that some of these isolates did belong to the genera *Vibrio* and *Shewanella* while others belonged to the *Enterobacteriaceae*. The inability of these isolates to ferment glucose confirms the unusual nature of the bacteria isolated from marine waters. This study has isolated four bacterial families using TCBS; *Vibrionaceae*; (*Vibrios* and *Shewanella)*, *Aeromonadaceae, Enterobacteriaceae* (*Morganella*, *Providencia*, *Enterobacter* and *Citrobacter*) and *Pseudomonadaceae*.

Confirmation of the *Vibrio spp*. identity is normally carried out by biochemical tests and by serological methods. Cultural methods, however, have disadvantages such as delays in obtaining final results, and that atypical isolates are difficult to identify due to variations in important phenotypic characteristics. This approach has in many laboratories been superseded by molecular methods that identify bacteria at the gene level or by protein profiling.

In this study it was decided to screen all isolates directly using the species specific PCRs for the three selected human pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) regardless of the sucrose fermentation properties of the isolates. The main reason for this decision was that strains with atypical sucrose fermentation patterns have been reported for all three of these species. A few strains of *V. parahaemolyticus* (Joseph et al., 1982) and *V. vulnificus* (Arias et al., 1998; Nagao et al., 2006; Oliver et al., 1992) have been reported to ferment sucrose. On the other hand sucrose negative *V. cholerae* O139 strains have also been documented (Ansaruzzaman et al., 1995).

When screened with the selected species specific primers many isolates reacted with the *V. parahaemolyticus* and *V. vulnificus* specific primer pairs and some isolates even reacted with both. Amongst these isolates some showed either non-specific reactions or the formation of atypical sized bands. This study has shown that the *pR72H* fragment and the *V. vulnificus* specific cytolysin-haemolysin genes are widely distributed amongst *Vibrionaceae* and *Enterobacteriaceae*. While the Phosphatidyl serine synthetase gene (Lee et al., 1995) is present in *V. alginolyticus* and few *Shewanella* species, parts of the cytolysin-haemolysin sequence of *V. vulnificus* (Lee and Choi, 1995) are widely distributed among other species of *Vibrionaceae* including *Shewanella* and *Aeromonas* as well as amongst the *Pseudomonadaceae*. A number of *Enterobacteriaceae* species including *Providencia*, *Morganella* and *Citrobacter* also carry this gene. *V. alginolyticus* was the most predominant species that carries sequences belonging to both of these genes. The reaction of *V. alginolyticus* strains to *V. parahaemolyticus* specific genes has confirmed previous findings where this was observed (Gonzales–Escalona et al., 2006; Robert-Pillot et al., 2002; Xie et al., 2005). It was, however, the first time that the *V. vulnificus* specific cytolysin-haemolysin based sequences were being detected in other bacterial species.

The problem encountered in detecting genes in different species using species specific primers could possibly be attributed to a high frequency of gene mobility in marine environments. Environmental strains might also be more prone to genetic changes due to natural selection processes in unfavourable environments. Tests used to accurately

identify clinical strains might therefore not be appropriate for bacteria isolated from the environment. It was therefore necessary to use supplementary molecular methods (detection *V. alginolyticus* specific collagenase gene), and phenotypic data so as to validate the results obtained with the initial phenotypic tests (API 20E), and from the contradictory genotypic results (detection of the *pR72H* fragment and the cytolysin-haemolysin gene for *V. parahaemolyticus* and *V. vulnificus* respectively).

As a follow-up the partial 16S rRNA sequences were determined for these strains. The 16S rRNA sequences, however, did not offer final identifications due to a high level of interspecies 16S rRNA nucleotide sequence homology displayed within the genus *Vibrio* (Thompson et al., 2005). More than 19% of the *V. alginolyticus* isolates tested had between 97 and 100% 16S rDNA sequence homology with *V. parahaemolyticus.* The 16S rRNA sequences did help to differentiate *Vibrio* species from other *Vibrionaceae* such as *Aeromonas* and *Shewanella*. The sequences were also used to differentiate the *V. parahaemolyticus*/ *V. alginolyticus* group from the *V. cholerae, V. mimicus* group, but it was unable to differentiate *V. parahaemolyticus* from *V. alginolyticus*.

Identification of *Vibrio* species especially differentiation between *V. alginolyticus* and *Vibrio parahaemolyticus* by phenotypic tests is difficult because the two species have similar characteristics. The most common test used is their ability to ferment sucrose, but final identification is based on the VP, urease, cellobiose, dulcitol, sucrose, ONPG, Arabinose test and growth in 10-12% NaCl (Farmer et al., 2004; Oliver and Kaper, 1997;). Even these tests do not give clear cut differences between the two species since some strains still behave differently.

To resolve the contradiction of the sixty-three isolates that clustered within the *V. alginolyticus V. parahaemolyticus* group a polyphasic approach was applied to obtain the final identities of isolates to species level. The results from the phylogenetic trees were compared with those from the API lab software database and by determining their phenotypic similarities. The *V. alginolyticus* collagenase specific PCR was also included. API 20E identified 55 isolates from the *V. alginolyticus*- *V. parahaemolyticus* group in

the phylogenetic tree as *V. alginolyticus*. Only one isolate was misidentified as *Pasteurella pneumotropica* while the second was misidentified as *A. hydrophyla*. The collagenase specific gene gave the same results, but three isolates were negative for this gene. This is in line with the work of Cai et al. (2009) who demonstrated that this gene is only present in fish pathogenic *V. alginolyticus* strains. During this study specific phenotypic tests assisted the most in differentiating between *V. parahaemolyticus* and *V. alginolyticus*. This finding is in line with the study of West et al. (1986) who used biochemical characters and were able to clearly differentiate the two species using the same methods as we used. Some *V. alginolyticus* strains isolated during this study did not ferment glucose. This finding is in contradiction with the findings of West et al. (1986) where all species of *Vibrios* were found to ferment glucose. This situation stresses the unusual character of the isolates from marine fish processing plants.

V. alginolyticus strains isolated from processed marine fish ready for distribution were found to carry sequences belonging to *V. parahaemolyticus* (*pR72H* fragment and the *trh*) an important pathogen transmitted through shellfish and responsible for gastroenteritis in humans. Robert-Pillot et al. (2002) stressed that the pR72H gene fragment is confined to *V. parahaemolyticus,* and its detection is strongly associated with this species. It is likely that these isolates acquired foreign genetic material from the marine environment due to its dynamic nature. The evolution of pathogens in the environment is believed to take place as a result of horizontal gene transfer between pathogenic and non pathogenic strains (Vezzulli et al., 2008). The fish isolated *V. alginolyticus* strains could therefore have the potential to cause gastroenteritis normally associated with *V. parahaemolyticus*. The high phenotypic and genetic similarity of *V. alginolyticus* isolates to *V. parahaemolyticus* could also indicate the ability of the virulent strains of *V. parahaemolyticus* to thrive in processed marine fish under similar conditions. This situation is a cause of concern and could pose a health threat to consumers.

4.6 CONCLUSION

The taxonomy of *Vibrios* and related genera is a complex and controversial subject that is subjected to constant changes depending on the current findings and improvements in taxonomic methods (Ruimy et al., 1994). None of the 247 bacteria isolated from hake, pilchard or horse mackerel was identified as *V. cholerae, V. parahaemolyticus or V. vulnificus*. The study implies that the three examined fish species are safe and do not pose a health hazard to consumers. This study confirms that enrichment in APW and GSTB and plating on TCBS can still be used in isolating *Vibrio* species from marine samples, but the selectivity of TCBS needs to be improved to minimise growth of other bacteria that are not *Vibrios* such as *Pseudomonas, Aeromonas*, *Shewanella* and *Enterobacteriaceae*. The *V. parahaemolyticus* and *V. vulnificus* primers are not specific and therefore were not useful in differentiating between *Vibrio* species. Both the *pR72H* fragment and the cytolysin haemolysin gene primers also showed non specific binding with some of the *Enterobacteriaceae* and *Pseudomonadaceae* isolates. Neither the classical method nor the detection of species specific genes alone is 100% reliable in identifying *V. alginolyticus* and *V. parahaemolyticus* to species level. However the PCR targeting the *ompW* of *V. cholerae* continues to be a valuable tool to differentiate between sucrose fermenting strains of *V. cholerae* and *V. alginolyticus*. The use of the NJ method of the 16S rDNA in combination with biochemical profiles from API20E facilitates accurate identification of *Vibrio* species.

4.7 RECOMMENDATIONS

For identification of *Vibrio,* particularly when differentiating *V. alginolyticus* and *V. parahaemolyticus*, a polyphasic approach is always recommended as a baseline using phenotypic tests such as the API 20E combined with PCR for species specific genes such as the collagenase gene of *V. alginolyticus*, so as to avoid ambiguity in the final results. *Vibrio alginolyticus* is an important fish pathogen and some of its strains may carry *V. parahaemolyticus* virulence associated sequences (Gonzales–Escalona et al., 2006; Xie et al., 2005). Some strains of *V. alginolyticus* are important fish pathogens in aquaculture (Balebona et al., 1998; Liu et al., 2004) while others are used as probiotics in the same systems (Austin et al., 1995; Balcázar et al., 2006; Verschuere et al., 2000). Accurate identification of this species is therefore very important. Further studies need to be carried out to establish the role of *V. alginolyticus* in marine fish processing factories. Pathogenicity studies could also be carried out in animal models to determine its safety implications on consumers.

4.8 ACKNOWLEDGEMENTS

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CHAPTER 5

SHIKONGO-NAMBABI MNNN, KACHIGUNDA B and VENTER SN (2010) **EVALUATION OF OXIDISING DISINFECTANTS TO CONTROL *VIBRIO* BIOFILMS IN TREATED SEAWATER USED FOR FISH PROCESSING**. *Water SA* **36** 215-220 3

5.1 ABSTRACT

Marine fish-processing plants often use sea-water during their operations. Chlorination and UV are commonly used for disinfection of this water but may not be effective in preventing biofilm formation within the water distribution network. These biofilms negatively impact on water quality and could lead to contamination of fish products. During a recent study *Vibrio alginolyticus* strains were detected on processed hake. The presence of most *Vibrio* spp. on fish products is of consumer safety concern and needs to be minimised. Water treatment strategies effective for sea-water disinfection but with minimal negative effect on fish quality are required. In this study the effectiveness of chlorine, ozone and hydrogen peroxide (H_2O_2) to inhibit mature biofilms or biofilm formation in natural sea-water was investigated. Two *V. alginolyticus* strains (V590 and V595) isolated from hake fish as well as the type strains of *V. alginolyticus* LMG4409 and *V. parahaemolyticus* LMG2850 were used. Chlorine was ineffective as experiments showed that strains V590, V595 and *V. parahaemolyticus* LMG2850 could form biofilms even in the presence of 4 mg/ℓ chlorine. When ozone was used, biofilm initiation and formation of only 2 strains of *V. alginolyticus*, i.e. LMG 4409 and V590, were completely inhibited at 1.6 mg/ℓ or 0.8 mg/ℓ ozone, respectively. Hydrogen peroxide performed the best of all the disinfectants evaluated in this study. Inhibition of biofilm formation was observed for all strains at 0.05% H_2O_2. The mature biofilms were more resistant to H_2O_2 but they were all eliminated at 0.2% concentrations. This study indicated that H_2O_2 is the most effective biocide to prevent biofilm formation in sea-water distribution networks and could potentially be used as an alternative or supplementary disinfectant of sea-water in marine fish-processing plants.

KEYWORDS: *V. alginolyticus*, *V. parahaemolyticus,* biofilms, H_2O_2, disinfection, sea-water

5.2 INTRODUCTION

The use of sea-water instead of freshwater during marine fish processing is an economical alternative especially in arid countries such as Namibia which are often faced with severe fresh water shortages. The water is typically used for activities such as cooling of the product or washing of surfaces and apparatus in the plant. Sea-water may, however, contain human pathogenic bacteria including *Vibrio* species such as *V. parahaemolyticus*, *V. cholera*, *V. vulnificus* and *V. alginolyticus* (Thompson et al., 2004; Wekell et al., 1994). The water, therefore, requires treatment and disinfection before being used for the processing of fresh fish. Currently chlorination and UV treatment are most commonly used to disinfect the water.

A recent study at a fish-processing plant showed that the quality of hake deteriorated along the processing line (Shikongo-Nambabi et al., 2010a). *Vibrio* species were isolated at the intermediate stages of processing. None of these species were isolated from the fish which had just been delivered to the factory for processing. From the study it was clear that *Vibrio alginolyticus* strains were introduced by the treated sea-water used during processing. Indications were that the major source of these bacteria was not inefficient treatment of the raw water but the subsequent formation of biofilms in the distribution network in spite of the presence of residual chlorine. *V. cholerae,* (Faruque et al., 2006; Mueller et al., 2007), *V. vulnificus* (Joseph and Wright, 2004). *V. alginolyticus* (Kogure et al., 1998) and *V. parahaemolyticus* (Enos-Berlage et al., 2005) are well known to form biofilms.

The presence of *Vibrio* spp. in the sea-water used during processing of the fish poses a potential health hazard to consumers and should be minimized and controlled. Chlorine, or chloramines are typically used to control bacterial biofilms in freshwater distribution systems (DeQueiroz and Day, 2007; Momba et al., 2002), but many studies have described the ineffectiveness of chlorine in controlling biofilm formation (Chu et al., 2003; Momba, 1998). There is, however, little information in the literature on alternative

disinfectant strategies to control biofilm formation in sea-water systems. The type and level of disinfectant to be used are, however, restricted as high concentrations of disinfectants have been shown to cause discolouration of the fish due to oxidation of the myoglobin (Kim et al., 2000).

The aim of this study was to evaluate 3 different oxidising disinfectants to control biofilm formation by selected *Vibrio* isolates in sea-water distribution systems. For this purpose chlorine, ozone and hydrogen peroxide were tested at a range of concentrations. Improvement in the treatment, disinfection and microbiological quality of the sea-water used for fish processing would help to ensure the safety of the final product and protect the consumers.

5.3 MATERIALS AND METHODS

5.3.1 Sampling and analysis of treated sea-water

Samples of sea-water used in a fish-processing facility, in Walvis Bay, Namibia, were collected to evaluate the current treatment system. Samples were taken from the raw water, after flocculation and chlorination, after UV treatment at 300 J/cm^2 at 254 nm (UV Pure, Cape Town) and at 2 points within the factory after distribution through the network. Thiosulphate (0.04% w/v) was added to all samples to neutralize the residual chlorine. Samples were refrigerated during transport and storage. After serial decimal dilutions samples were plated in duplicate onto nutrient agar (NA) (Oxoid), sea-water agar (SWA) (Atlas, 2006) and thiosulphate citrate bile salts sucrose agar (TCBS) (Farmer and Hickmann-Brenner 1991). NA and TCBS plates were incubated at 37°C while SWA plates were incubated at 22°C.

The studies to evaluate the ability of *Vibrio* strains to form biofilms and to determine the effect of selected disinfectants on the inhibition and control of biofilm formation were conducted using either natural or artificial sea-water. The artificial sea-water (ASW) consisted of water in which 0.4 M NaCl, 0.1 M $MgSO_4.7H_2O$, 0.02 M KCl and 0.02 M $CaCl_2.2H_2O$ was dissolved (Farmer and Hickman-Brenner, 1991).

5.3.2 Bacterial cultures and inoculums

Four bacterial isolates were included in this study. Two of the isolates were *Vibrio alginolyticus* strains previously isolated from the processing plant. Isolate V590 was isolated from hake after filleting by the Baader machine and V595 from the finished product. These strains were identified using the API 20E (BioMérieux) test and amplification of the collagenase gene according to the method of Di Pinto et al. (2006). The type strains of *V. alginolyticus* (LMG 4409) and *V. parahaemolyticus* (LMG 2850)

were also included for comparative purposes. Both these strains were initially isolated from food- poisoning incidents related to seafood.

Strains were cultured individually on sea-water agar (SWA) by incubation overnight at 37°C. This culture was then used to inoculate 250 mℓ sterile nutrient broth (NB) containing 3% NaCl and incubated unshaken at 37°C for 24 h. The cell suspension (75 mℓ) was harvested by centrifugation at 4 000 r/min for 30 min and washed twice with 0.85% (w/v) NaCl. The pellet was re-suspended in 15 mℓ 0.85% NaCl to obtain an inoculum containing $\pm 10^7$ cfu/mℓ.

5.3.3 Biofilm formation in artificial sea-water

The ability of the selected bacteria to form biofilms in sea-water was evaluated using a Pedersen's device (Pedersen, 1982). The device contained 20 clean microscope glass slides (7.6 × 2.6 cm) and was connected by means of silicone tubing via a peristaltic pump to a reservoir containing artificial sea-water. Sterile artificial sea-water (ASW) instead of natural sea-water was used for these experiments due to logistical constraints of transporting and storing large volumes of water. The ASW was supplemented with bacteriological peptone (Biolab) and nutrient broth (NB) (Biolab) at 1 g/ℓ each. The effluent was collected, disinfected and discarded. The system was disinfected by perfusing the systems for 24 h with tap water containing 2.5 g/ℓ residual chlorine at a rate of 500 mℓ/h. Afterwards the chlorine was neutralised by allowing 12 ℓ of sterile tap water containing 1.0 mg/ℓ sodium thiosulphate to run through the systems for another 24 h. The system was then rinsed with 10 ℓ of sterile tap water. Sterility was assessed by plating 0.1 mℓ of the effluent onto TCBS agar and incubating the plates at 37°C for 24 h.

At the start of each experiment ASW was allowed to flow through the Pedersen's device for 1 h. After inoculation with 5 mℓ of the cell suspension the flow of ASW was maintained at 500mℓ/h. Slides were withdrawn at 24 h intervals up to 96 h. Each slide was washed in sterile water, transferred to a 100 mℓ screw-cap bottle containing 10 mℓ sterile 0.85% (w/v) NaCl solution supplemented with 0.01 mg/l sodium thiosulphate, and

sonicated (Integral systems) for 5 min. The suspension was diluted, plated in duplicate on TCBS agar plates and incubated at 37°C for 24h after which the culturable counts were determined. The degree of biofilm formation was calculated as the density of bacteria per cm^2 (Momba et al., 2002).

5.3.4 Disinfectants

The disinfectant concentrations used during this study corresponded to levels previously indicated as suitable for food processing. Previous studies showed that H_2O_2 could be used at a higher concentration than the other two disinfectants without negatively affecting the product quality (Kim et al., 2000). Chlorine concentrations were prepared by adding varying amounts of commercial sodium hypochlorite solution (3.5% m/v) to 250 mℓ NSW to give final concentrations of 0.2, 0.4, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 or 4.0 mg/ℓ free residual chlorine. The chlorine concentrations were measured using N, N-diethyl-p-phenelenediamine (DPD) Tablets No1 (The Tintometer (Ltd), England) and a Lovibond colour comparator. Ozone was generated using the Ozone Air and Water System, (Bulkmatech, Cape Town) to give ozone concentrations of 0.4, 0.8, 1.0, 1.6 and 2.0 mg/ℓ. Ozone concentrations were measured by the indigo colorimetric method (*Standard Methods*, 1998). H_2O_2 concentrations were prepared by adding varying amounts of 35% H_2O_2 (Merck) to 250 mℓ NSW for final concentrations of 0.05, 0.08, 0.1, 0.2, 0.3, 0.4 and 0.5%.

5.3.5 Inhibition of biofilm formation

Chlorine, ozone or H_2O_2 solutions were prepared at the concentrations described above in screw-top bottles containing 250 mℓ sterile NSW. Sea-water without any disinfectant added served as the control. Microscope slides were inserted in all the bottles. After inoculation with 1 mℓ of the test culture suspension, the bottles were kept at 22°C. Slides were withdrawn after 72 h. All slides were washed in sterile ASW and examined for the presence of biofilms through culturable-count determination as described above.

5.3.6 Inhibition of mature biofilms

Chlorine, ozone or H_2O_2 were prepared at the concentrations described above in 250 mℓ sterile NSW. Sea-water without any disinfectant added was used as a control. Four slides containing mature (72h) monoculture biofilms of the selected strains were obtained from the Pedersen's device systems described above. The slides were rinsed in sterile ASW and then immersed into the bottles, swirled and allowed to stand at room temperature. A slide was withdrawn from each bottle after 1 h. Slides were examined for the presence of biofilms by determining the culturable counts using TSBC agar incubated at 37°C for 24 h.

5.3.7 Statistical analysis

The experimental set-up was a randomised complete block design. The statistical analysis was conducted by ANOVA using Genstat Release 7.2. The mean, the least significant difference, and coefficient of variation were calculated to determine the significance in responses among treatments.

5.4 RESULTS

5.4.1 Microbial quality of treated sea-water

The aerobic heterotrophic, psychrotrophic and *Vibrio* culturable counts were determined for the treated sea-water used in the Namibian fish-processing plant and are presented in Table 5.1. The heterotrophic and psychrotrophic counts for the untreated sea-water (S1) were 2.0×10^2 (cfu/mℓ) and 5.6 10^3 (cfu/mℓ) respectively. Only 10 cfu/mℓ presumptive *Vibrio* colonies were detected. Similar heterotrophic and psychrotrophic results were observed after dissolved air flotation (DAF) and chlorination. At this point of the treatment no *Vibrio* ssp. were detected. The water leaving the plant after UV disinfection had very low numbers of both heterotrophic (60 cfu/mℓ) and psychrotrophic bacteria (35 cfu/mℓ). No *Vibrio* spp. were detected in this sample. Inside the processing plant the value of both the heterotrophic bacteria and the psychrotrophic bacteria had risen sharply to the similar levels detected in the raw water. Growth of *Vibrio* spp. above the detection limit of 250 cfu/mℓ was also observed on the TSBC plates after 72 h of incubation (Table 5.1).

Table 5.1 Average bacterial counts (cfu/mℓ) of sea-water used in the processing plant after various treatments and distribution to the plant

Sample	Sampling point	Heterotrophic count (Nutrient agar)	Psychrotrophic count (Sea-water agar)	*Vibrio* spp. count
S1	Raw sea-water	2.0×10^2	5.6×10^3	10
S2	After DAF and chlorination	2.5×10^2	1.2×10^3	ND
S3	After UV disinfection	6.0×10^1	3.5×10^1	ND
S4	Distribution Line 1	9.8×10^3	6.4×10^3	@72 hrs +++[a]
S5	Distribution Line 2	1.7×10^2	9.7×10^3	@72 hrs +++

Abbreviations: a =Abundant growth with counts higher than 250cfu/mℓ which was the detection limit for this analysis. ND = Not detected, DAF = Dissolved air flotation

5.4.2 Ability of *Vibrio* isolates to form biofilms in sea-water

All *Vibrio* strains included in this study were able to form biofilms on glass slides in artificial sea-water (ASW) supplemented with bacteriological peptone and nutrient broth (Figure 5.1). The average bacterial densities in the biofilms peaked between $10^7$and 10^9 cfu/cm^2 after 72 h, changing only slightly over the next 24h.

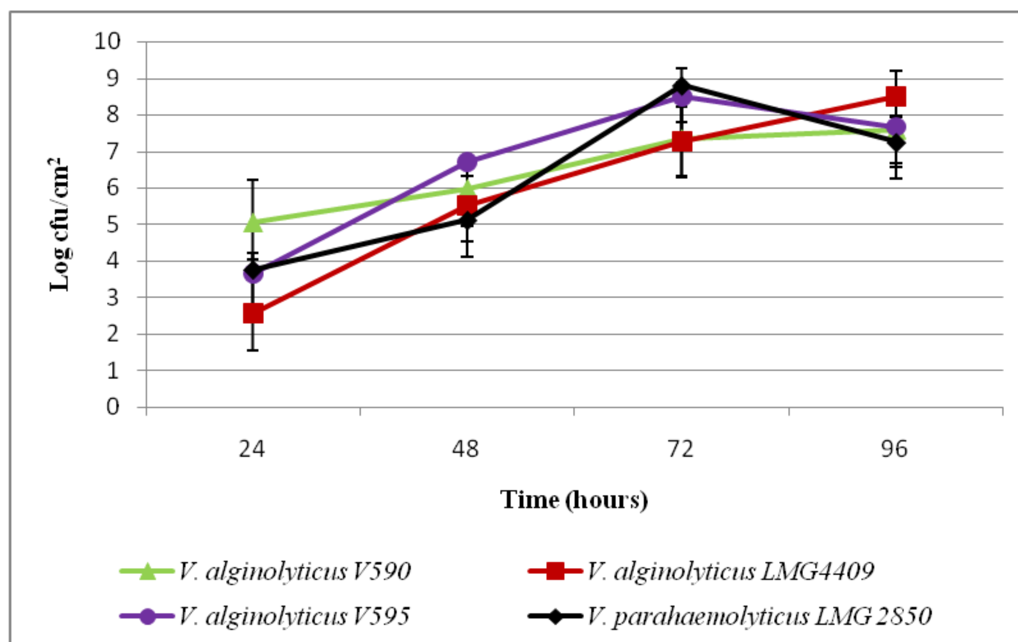

Figure 5.1 Biofilm formation of selected *Vibrio* isolates in artificial sea water. Counts based on 3 replicates.

5.4.3 Effect of disinfectant on biofilm formation

The development of biofilms in the presence of different pre-determined concentrations of disinfectant was measured. After 72 h of exposure to the chlorine concentrations biofilm formation was observed for almost all the strains (Fig. 5. 2a). At the lower chlorine concentrations (1.5 mg/ℓ and below) there was no significant reduction in bacterial densities, however, there was a significant difference in the mean bacterial

counts between the control without chlorine and the highest chlorine concentration (4 mg/ℓ) used (LSD=1.24) (Fig. 5.2a). The bacterial densities of strains V590, V595 and LMG 2850 dropped to log 3.73, 2.33 and 2.88, respectively. while *V. alginolyticus* type strain (LMG 4409) did not form biofilm at a chlorine concentration of 4mg/ℓ.

Biofilm formation varied substantially between the different *Vibrio* strains (Fig. 5.2b) at the different concentrations of ozone used. There was a significant interraction between *Vibrio* strains and the disinfectant ($p<0.001$). For the *V. alginolyticus* isolates, Strains V590 and LMG 4409, bioflim formation was completely inhibited at 0.8 mg/ℓ and 1.6 mg/ℓ, ozone respectively. Biofilm formation was, however, still observed for *V. parahaemolyticus* (LMG 2850) and one of the *V. alginolyticus* fish isolates (V595) after 72 h of exposure to 2.0 mg/ℓ ozone (Fig. 5.2b). Statistically the overall response of V595 was similar to that of *V. parahaemolyticus* LMG 2850. Their responses were, however, significantly different from that of *V. alginolyticus* LMG 4409 and V590. No formation of biofilms could be detected after 24 h at any of the H_2O_2 concentrations tested.

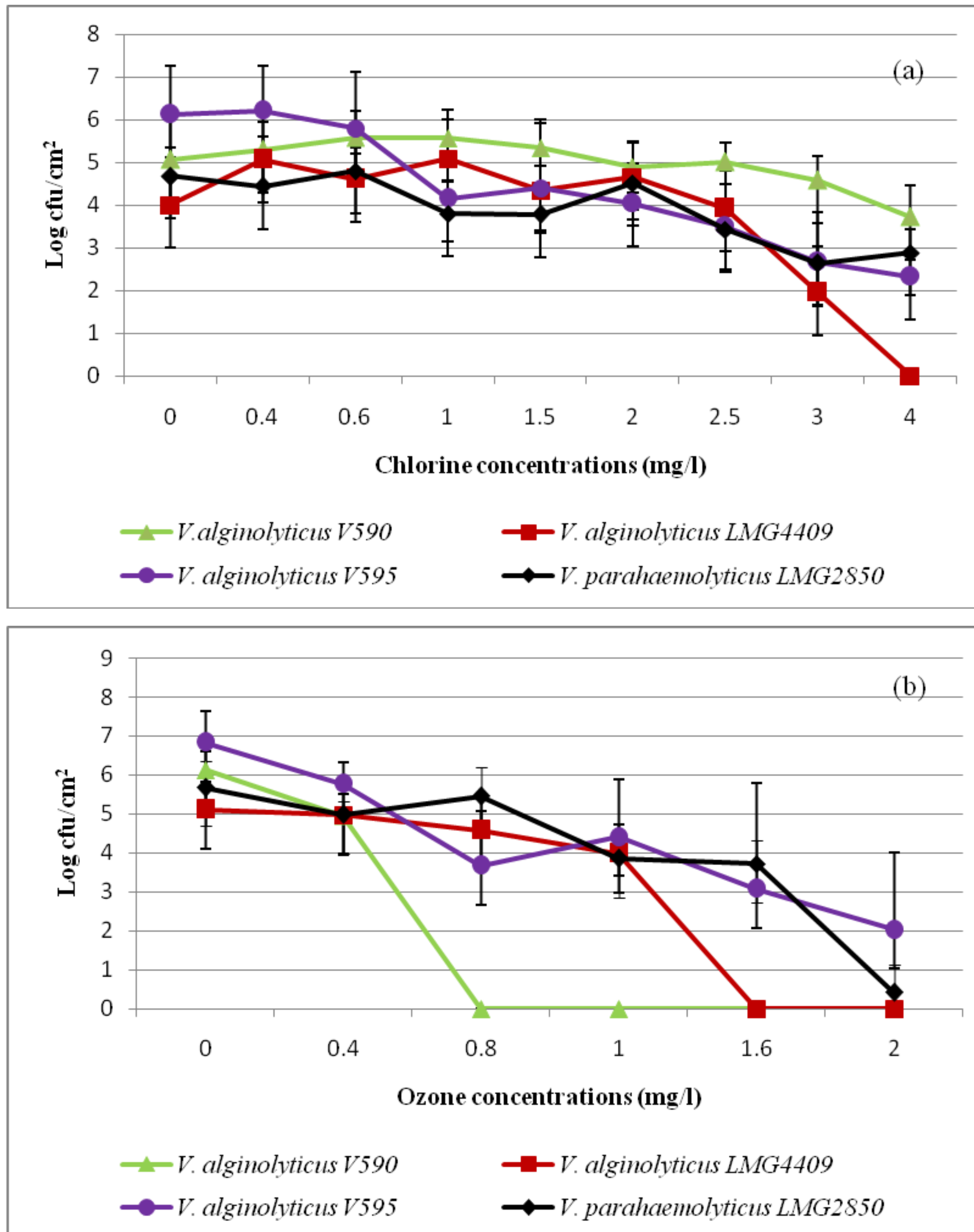

Figure 5.2 Biofilm densities of selected *Vibrios* after 72 h incubation in the presence of chlorine (a) and ozone (b). Values reflect the average of three independent experiments.

5.4.4 Effect of disinfectants on mature biofilms

Chlorine had a limited impact on the bacterial levels of all 3 *V. alginolyticus* biofilms after one hour of exposure (Fig. 5.3a) when compared to the control value. *V. parahaemolyticus* LMG 2850 was more sensitive to chlorine with a significant reduction in bacterial numbers. After 1h of exposure to 4 mg/ℓ the bacterial density dropped from 8.03×10^7 to 9.43×10^4 cfu/cm^2 (Fig. 5.3a). At 4 mg/ℓ the reactions of the type strains, *V. parahemolyticus* LMG2850 and *V. alginolyticus* LMG 4409 to chlorine were not significantly different, but it differed from those of the 2 fish isolates who were more resistant to chlorine. The ozone treatments resulted in a typical 1 log reduction in the bacterial levels, independent of the ozone concentration used (Fig. 5.3b) and these reductions were not statistically significant ($p=0.166$).

There was a significant reduction in bacterial counts of the mature biofilms of all the *Vibrio* isolates (Fig. 5.3c) as the H_2O_2 concentration increased ($p<0.001$). Of all the strains tested *V. parahaemolyticus* strain LMG 2850 was the most sensitive and the mature biofilm could be inhibited after 1 h of exposure to 0.08% H_2O_2. The *V. alginolyticus* type strain (LMG 4409) biofilms were inhibited at 0.1% H_2O_2. The two *V. alginolyticus* stains isolated from the facility (V590 and V595) were more difficult to remove and were only inhibited after one hour exposure to 0.2% H_2O_2 (Fig. 5.4c).

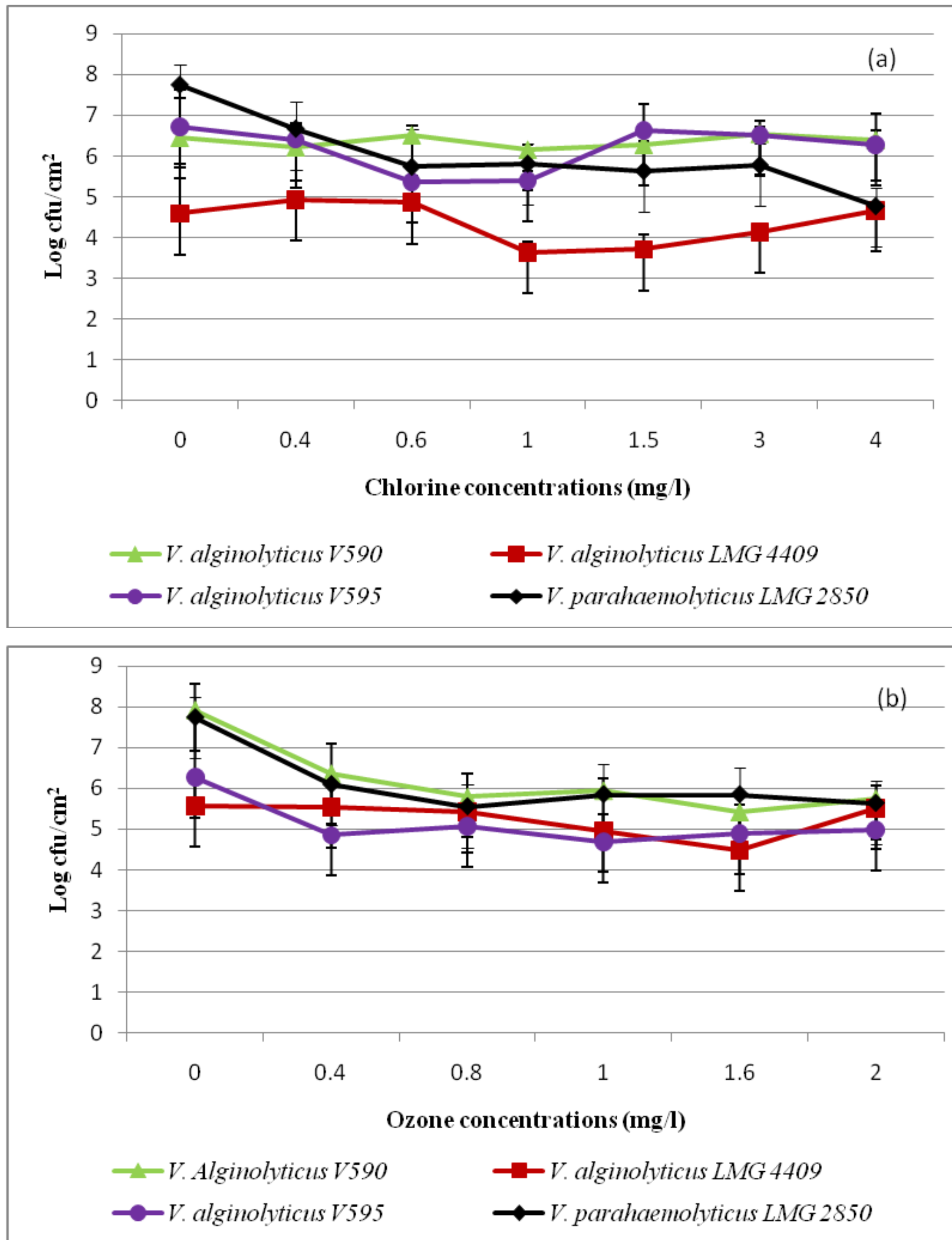

Figure 5.3 **Continued on page 158**

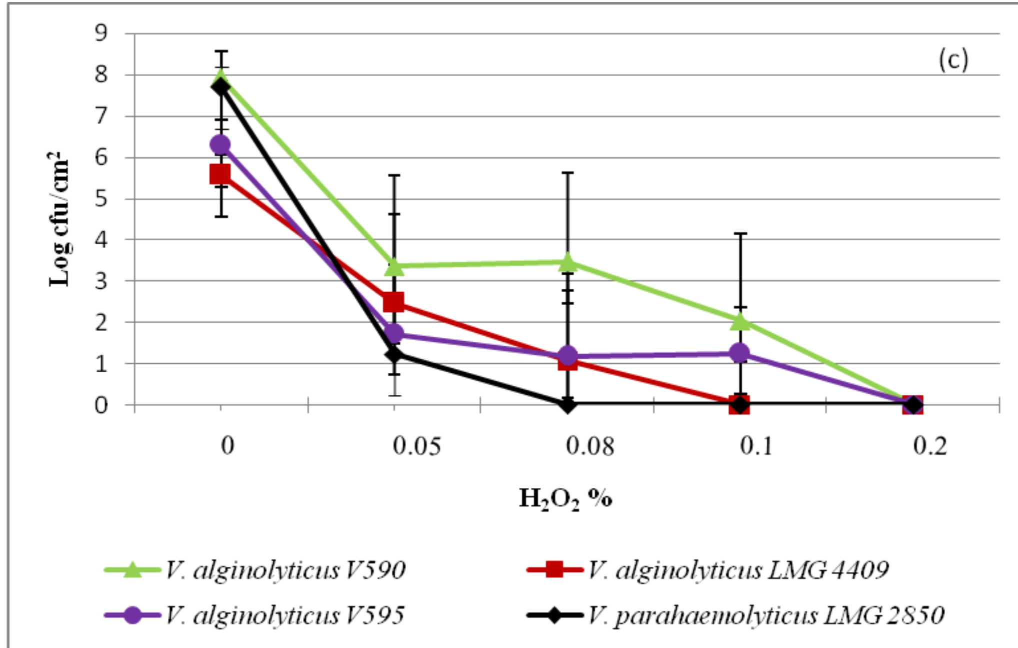

Figure 5.3 Survival of mature biofilms of selected *Vibrio* isolates when exposed to different levels of (a) chlorine, (b) ozone or (c) H_2O_2 as measured after one hour of exposure. Values reflect the average of three independent experiments.

5.5 DISCUSSION

For many marine fish-processing facilities the use of treated sea-water during operations is a viable economic alternative to the use of freshwater. The microbial quality of the water should be well managed as it may have a negative impact on the quality and safety of the final product. During an investigation into the deterioration of the microbial quality of hake during processing, *V. alginolyticus* strains most likely introduced by the treated sea-water used during processing were isolated (Shikongo-Nambabi et al., 2010b). Although certain strains of *V. alginolyticus* have been shown to be pathogenic, it is their close relationship to *V. parahaemolyticus,* a pathogen widely associated with food-borne infections and outbreaks linked to seafood (Thompson et al., 2004) that is of even greater concern. The ability of these *V. alginolyticus* strains to survive and grow in treated sea-water and the subsequent contamination of the hake may, therefore, be indicative of a similar behaviour of *V. parahaemolyticus*, should this pathogen be present. Control of this route of contamination is, therefore, of great importance for the efforts to minimize potential health hazards to consumers.

The 1st focus of the study was to survey the quality of the sea-water used at the facility and to investigate whether it could serve as a source of contamination. Results showed that chlorination combined with UV irradiation drastically reduced both the mesophilic and psychrotrophic bacteria in sea-water. Although the quality of the water directly after these treatments was very good it deteriorated during distribution and some *Vibrio* spp. could be detected. It was clear from these data that the residual chlorine was ineffective in inhibiting biofilm formation in the distribution network. This was not unexpected as biofilm formation is common in water distribution networks (September et al., 2007) and detachment of the biomass can lead to deterioration of the microbial quality of the water.

Under a defined set of conditions of temperature, pH and a limited supply of nutrients the 4 *Vibrio* isolates selected for this study were able to form monoculture biofilms on glass slides in the Pedersen's device. This was not unexpected as biofilm formation by *V. alginolyticus* has previously been demonstrated by Kogure et al. (1998). The biofilm

formation of the fish isolates (V595 and V590) was similar to that of the *V. parahaemolyticus* (LMG2850) and *V. alginolyticus* (LMG 4409) type strains. These results support the hypothesis that the *Vibrio* species detected on hake fish could have originated from bacteria released from biofilms that formed in the water distribution network after the initial treatment. It also showed that there was little difference in the overall behaviour of the *V. alginolyticus* strains isolated from the facility and the *V. parahaemolyticus* type strain. All isolates were quite similar in terms of their ability to form biofilms and their resistance to specific disinfectants.

Chlorine is not very effective against biofilms formed by either atypical *V. alginolyticus* isolates or the *V. parahaemolyticus* type strain LMG 2850 in sea-water. The effectiveness of chlorine against microorganisms in freshwater depends on a number of factors including the residual concentration, contact time, temperature, pH, and aggregation (Obi et al., 2008). Not much attention has, however, been given to the possible additional inhibitors of chlorine that might exist in water with high salt concentrations such as sea-water. From the historical data kept at the factory it is clear that a residual chlorine concentration of 0.2 mg/ℓ was constantly maintained in the system. These conditions might have selected for strains with an ability to tolerate high chlorine concentrations as was previously demonstrated by Ridgeway and Olson (1982). From the responses of the *V. parahaemolyticus* type strain it can be deducted that this bacterium will behave similarly in the sea-water distribution system and may, therefore, contaminate the final product whenever present.

Failure of chlorine to inhibit biofilm formation and mature biofilms was not due to the effect of pH. The pH of the NSW was 7.4, a level at which both HOCl and OCl^- exist in various proportions (LeChevallier and Au, 2004). In this study the CT (concentration × exposure time) value for the highest concentration used (4mg/ℓ) after 72 h exposure was 288, a value that was much higher than the 15 to 150 CT values recommended for drinking water (Debore and Von Gunten, 2008). The use of chlorine at concentrations higher than 4mg/l was not considered due to the potential effect that higher chlorine

levels might have on costs, acceptability of the final product, corrosion in the plant and the potential to generate possible carcinogens (Gopal et al., 2007; Wang et al., 2007).

The ability of ozone to inhibit biofilm formation varied between the strains tested. Again *V. alginolyticus* V595 was the most resistant to ozone and biofilm formation was not inhibited at 2 mg/ℓ ozone. Although biofilm formation could be inhibited for *V alginolyticus* V590 at an ozone concentration of 0.8 mg/ℓ, none of the mature biofilms were inhibited at the highest concentrations of ozone used (2mg/ℓ) in this study. Higher ozone concentrations to remove mature biofilms are, however not recommended. Reports have shown that at high concentrations ozone reacts with organic matter in water, generating nutrients that could stimulate bacterial attachment to surfaces and formation of biofilms (Clark et al., 1994). Ozone is also not stable for long periods and may not provide the level of residual disinfectant required to inhibit existing biofilms (Khadre et al., 2001; Guzel-Seydim et al., 2004)

Hydrogen peroxide (H_2O_2) was very effective in inhibiting biofilm formation at a concentration of 0.05 % (500mg/ℓ) H_2O_2. Mature biofilms of all four strains tested could be killed at concentrations between 0.08% and 0.2% H_2O_2. This suggested that H_2O_2 at higher concentrations could be used to remove existing mature biofilms from sea-water distribution systems during shock dose treatments. During this study $H_2O_2$was more effective in killing bacteria than during a study done by Kim et al. (2000) on channel catfish carried out in freshwater. The discrepancy found in H_2O_2 action between sea-water and freshwater could be ascribed to different disinfection environments implying that mineral ions present in sea-water are essential in maximising the action of H_2O_2 against bacteria. Pedahzur et al. (1995) found such a synergistic effect between silver ions and H_2O_2 when inactivating *E. coli* in phosphate buffer.

Part of the success of H_2O_2 was that it could be used at a higher concentration than the other two disinfectants without negatively affecting the product quality. Kim et al. (2000) investigated the effectiveness of 0.7% (7000 mg/ℓ) H_2O_2 on reducing bacterial counts on catfish fillets and found no significant differences between controls and H_2O_2 treated

fillets with regard to appearance, colour, and odour scores. The levels of H_2O_2 used by Kim et al. (2000) were 10 times higher than the concentrations used in this study.

5.6 CONCLUSION

This study has shown that although sea-water could be an alternative source of water for marine fish-processing plants, the treatment and the quality of the water needs to be carefully managed. The conditions in this seawater distribution network have selected for *V. alginolyticus* strains that can form biofilms in the presence of a residual chlorine concentration of 0.2 mg/ℓ. Once present in the system these bacteria may be released from the biofilm and would contaminate the fish during processing. The presence of *V. alginolyticus* on its own is not of a great health concern as it is rarely associated with cases of diarrhoea or gastroenteritis. *V. alginolyticus* is, however, closely related to the common food borne pathogen, *V. parahaemolyticus,* and may be predicative of the growth, behaviour and survival of this seafood pathogen in the water system. The current study has confirmed that this is the case as there was little difference between the behaviour of strains representing these 2 species in the experiments conducted. This finding also emphasised the need for control of biofilm growth in distribution systems even though it may not currently pose a significant health threat.

Evaluation of 3 oxidising disinfectant showed that chlorine and ozone are ineffective in preventing biofilm formation and in removing mature biofilms formed by *Vibrio* species in sea-water at their permissible concentrations. The only disinfectant that showed some promise was H_2O_2. Bench-scale experiments indicated that it would be possible to control biofilm formation at a concentration of 0.05 % (500 mg/ℓ) H_2O_2 and that existing biofilms could be removed by shock doses or 0.2%. This still needs to be investigated with larger-scale experiments run over a longer period of time. Results published by Kim et al., (2000) strongly support the notion that the proposed levels of H_2O_2 would not have any negative effect on the quality of the processed hake, but further studies would be required to confirm these conclusions.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Microbiological quality of marine fish plays an important role in determining product shelf life and safety to end users. Controlling the quality and safety of the final processed fish is no easy feat as a number of bacteria present in marine waters are either pathogenic to humans or cause spoilage of stored fish. Many bacterial species are able to metabolise the fish proteins resulting in a reduction in shelf life through the generation of byproducts that lead to fish spoilage often associated with bad odour and taste, change in texture and discolouration (Gram et al., 1987; Huis in't Veld, 1996). Amongst the pathogenic bacteria, the *Vibrio* spp. are the most important pathogens associated with seafood and are often transmitted through the consumption of raw or improperly cooked produce. These pathogens cause diseases such as cholera, gastroenteritis and septicaemia. (Harwood et al*.,* 2004; Hsueh et al, 2004; Oliver and Kaper, 1997).

Monitoring the levels of spoilage and pathogenic bacteria during fish processing is essential to ensure that bacterial contamination is kept to a minimum. For this purpose accurate diagnostic and identification tools are required as misidentification of pathogens may pose health risks to consumers or lead to loss of income through rejections of products. Monitoring also forms an integral part of quality assurance procedures such as HACCP and GMP implemented by seafood processing facilities to ensure consumer safety and compliance with microbiological criteria controlled by trade agreements. Little information with regard to the quality of fish harvested off the Namibian coast is, however, available.

This project has investigated microbiological contamination of fish during processing. A total of 120 fish samples were analysed for spoilage bacteria and potential human pathogens, focusing mainly on the human pathogenic *Vibrio* species. The quality of marine fish harvested off the Namibian coast was assessed by carrying out total counts of indicator organisms (total mesophilic counts and *Enterobacteriaceae* counts), total *Vibrio* species and total counts of mesophilic and psychrotrophic bacteria that would indicate potential spoilage of marine fish during storage.

This study has shown that typical psychrotrophic spoilage bacteria such as *Pseudomonas, Shewanella putrefaciens* and *Aeromonas*, already formed part of the microbial population of the incoming fish and that processing did not increase their levels dramatically. It was postulated that keeping fish on board the fishing vessels for several days before delivery to the factory for final processing may have increased the level of spoilage bacteria on the unprocessed fish. The study, however, revealed higher mesophilic counts in hake after filleting. Of particular interest was the sucrose fermenting *Vibrio* species that were not detected in the incoming (H&G) fish, but were detected in high numbers (ca. log 6.31 cfu/gram) at the various stages during processing.

Neither the biochemical tests nor the molecular biology methods used were 100% accurate in identifying *Vibrio* isolates to the species level. The 16S rRNA sequence comparison was accurate in differentiating the *Vibrio* species from other genera, but could not identify *Vibrio* isolates up to species level. Amongst the presumptive *Vibrio* isolates no human pathogens commonly associated with marine fish were present. *Vibrio alginolyticus* were the predominant *Vibrionaceae* bacteria isolated from hake as well as pilchards and horse mackerel. These isolates showed unusual pheno and genotypes and were difficult to distinguish from *V. parahaemolyticus.* Specific phenotypic traits combined with the *V. alginolyticus* collagenase gene specific PCR were used to make a final identification. In future improved methods such as DNA-DNA hybridisation (DDH) (Gomes-Gil et al., 2004) and MLSA employing other genes such as *recA* (Thompson et al., 2004) or multiple genes (Thompson et al., 2007) could be used to give better resolution in the identification of *Vibrio spp.* isolated from marine environments. The MSLA approach (Thompson et al., 2007) could supersede the polyphasic approach.

At the time of sampling, sea-water was used to wash the hake fillets before trimming and packaging and indications were that the sea water was the most likely source of contamination. To support this notion the source of *Vibrio alginolyticus* present on the processed hake was investigated. Water samples were analysed at various points within the treatment and distribution network. It was established that the levels of bacteria in water increased to values higher than the initial levels found before disinfection. The fact

that sucrose fermenting *Vibrio* species were not detected on the incoming hake fish also suggested that the water distribution system served as the source of *V. alginolyticus* to hake fish during processing. The factory used 0.2 mg/ℓ free chlorine as the residual sanitizer to prevent bacteria regrowth. Increases in bacterial counts after disinfection was a clear indication that the levels of chlorine used were ineffective in controlling bacterial attachment to surfaces in water pipes and subsequent multiplication thereafter. In addition the 300 J/cm^2 at 254 nm UV treatment given during water disinfection had no residual effect beyond the point of treatment. The problem of biofilms formation in the presence of residual chlorine is well known (Momba and Binda, 2002). In biofilms, bacteria are embedded in complex structures and protected from the adverse effect of sanitizers by the EPS (Hall-Stoodley and Stoodley, 2005).

This project has shown that chlorination and UV irradiation were unable to inhibit biofilms formed by *V. alginolyticus* prevalent in sea water and on processed marine fish. Although the fish processing plant used methods recommended for efficient treatment and biofilm control in fresh water distribution systems (chlorination and UV irradiation), these methods were ineffective for the same purpose in sea water. Methods specific for sea water therefore need to be extensively researched and defined. When studying the interactions between *Vibrio* biofilms and various disinfectants, it was shown that the *V. alginolyticus* isolates, obtained from the sea water system, formed biofilms in artificial sea water. In the laboratory scale experimental system it was demonstrated that chlorine could not prevent these strains from forming biofilms in natural sea water even at concentrations up to 4 mg/ℓ. The same level of chlorine was also not able to remove mature biofilms formed by the same bacteria.

Ozone and H_2O_2 were more effective in biofilm control formed by *Vibrio* species in sea water. Ozone has already been successfully used in the food industry to control bacterial contamination with minimal side effects to humans (Guzel-Seydim et al., 2004) and is recommended for use in slurry ice to extend the shelf life of marine fish (Campos et al., 2006). $H_2O_2$was the most effective and is therefore recommended for use in marine fish processing factories to inhibit biofilm formation in the distribution network. H_2O_2 has a

number of benefits including the lack of a residue in water as opposed to chlorine (Simpson, 2008). Further trials on the use of the two disinfectants at a larger scale over a longer time period is still required to substantiate these findings.

The presence of *V. alginolyticus* on its own is not of a great health concern as it is rarely associated with cases of diarrhoea or gastroenteritis. The current study has, however, shown that the *V. alginolyticus* isolates, obtained from the plant, were able to form biofilms in artificial sea water at rates similar to the type strain of *V. parahaemolyticus* (LMG 2850) The similarity in behaviour between the *V. alginolyticus* isolates and the pathogenic *V. parahaemolyticus* type strain may therefore indicate that *V. parahaemolyticus* stands a good chance of forming biofilms once present in sea water used in the factories. This finding emphasised the need for control of biofilm growth in distribution systems even though it may not currently pose a significant health threat. Sea-water could only be an alternative source of water for marine fish-processing plants if the treatment and the quality of the water are carefully managed.

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