# INVESTIGATION OF THE PATHOGENICITY OF *VIBRIO ALGINOLYTICUS* STRAINS ISOLATED FROM PROCESSED HAKE AND SEAWATER IN NAMIBIA AND VALIDATION OF HYDROGEN PEROXIDE ($H_2O_2$) FOR THE DISINFECTION OF SEAWATER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

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BY

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# Abstract

In Namibia seawater is employed by marine fish factories during processing. Previous studies indicated that chlorine and Ultra Violet (UV) light are ineffective in keeping seawater free from bacteria throughout the distribution system. However, laboratory studies showed that hydrogen peroxide eliminates both sessile and attached bacteria from seawater. Secondly it is not known whether *Vibrio alginolyticus* bacteria isolated from the Namibian marine waters and from hake fish whose detection results into rejection of export products are pathogenic or not. This study was aimed at validating the ability of hydrogen peroxide ($H_2O_2$) to eliminate *V. alginolyticus* in seawater and to detect *Vibrio* species associated virulence factors in *V. alginolyticus* strains isolated from seawater and from hake fish products. Seawater used by factory B was dosed with 0.05% $H_2O_2$ and allowed to pass through pipes to collection points after which *Vibrio* counts in water and in fish products were determined. Secondly, *V. alginolyticus* strains isolated from seawater and hake fish and identified using 16S rRNA and *rpo*B genes were screened for *tox*R, *tlh* and *tdh* genes using PCR. *V. parahaemolyticus* LMG 2850 and *V. alginolyticus* Bcc892 were used as positive and negative controls respectively. High bacterial counts were detected from seawater treated with chlorine alone. *Vibrio alginolyticus* was detected in the fish processing factories were seawater was used and on fish products. *Vibrio* species were eliminated from seawater distribution system by 0.05% $H_2O_2$. Only one (1.9 %) out of 54 strains, tested *tlh* positive, but all the strains tested were *tox*R and *tdh* negative. This study, confirmed that these strains do not

contain specific virulence genes known in other *Vibrio* species. Furthermore, this study confirmed that $H_2O_2$ is an effective disinfectant of seawater.

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# ABBREVIATIONS

| | |
|---|---|
| UV: | Ultra Violet light |
| TCBS: | Thiosulpahate Citrate Bile salts Sucrose |
| $H_2O_2$: | Hydrogen Peroxide |
| GDP: | Gross Domestic Product |
| WTO: | World Trade Organization |
| NSI: | Namibia Standards Institution |
| FAO: | Food Agricultural Organization |
| WHO: | World Health Organization |
| HACCPs: | Hazard Critical Control Points |
| GHP | General Hygiene Practice |
| TVC: | Total Viable Colony |
| USFDA: | United States Food and Drug Administration |
| TTHMs: | Total Trihalomethanes |
| CBP: | Carcinogenic By-Products |
| TRC: | Total Residual Chlorine |
| CI: | Chlorine Institute |
| ACGIH: | American Conference of Governmental Industrial Hygienists |
| IMO: | International Maritime Organization |
| PCR: | Polymerase Chain Reaction |
| T3SS: | Type III secretion System |
| VBNC: | Viable But Non-Culturable |

| | |
|---|---|
| KP: | Kanagawa Phenomenon |
| $LD_{50}$: | Lethal Dose |
| toxR: | Toxins Regulator Gene |
| CT: | Cholera Toxin |
| TCP: | Toxins Coregulated Pilus |
| VIPPAD: | Veterinary Import Permit for Pet Animals with special Dispensation |
| SNA: | Saline Nutrients Agar |
| ASPW : | Alkaline Saline Peptone Water |
| TSAT: | Soya Peptone Triphenyl tetrazolium chloride agar |
| NaCl: | Sodium Chloride Solution |
| FABI: | Forestry and Agricultural Biotechnology Institute |
| EtoH: | Ethanol |
| Tlh: | Thermolabile haemolysin |
| Tdh: | Thermostable Direct Haemolysin |
| CFU: | Colony Forming Unit |
| bp: | base pair |

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# DEDICATION

I dedicate this thesis to all professionals (Teachers) at Amutanga Combined School (Oshana Region) who have dedicated their lives to instil values and knowledge into learners at the early stage of their career development.

To students who have not yet realized the potential and importance of the career they have chosen; and,

To our leaders all over Namibia  who work every day at making their career a success and our world a much better place to live in;

Finally, I dedicate this thesis to my family for every support and guidance they have given to me. It's a privilege to have them in my life, I always think fondly about them, thank you.

# Declarations

I, Dionisius Shetunyenga, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education. No part of this thesis may be reproduced, stored in any retrieval system, or transmitted in any form without prior permission of the author, or The University of Namibia.

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Dionisius Shetunyenga

# CHAPTER 1: INTRODUCTION

## 1.1. General Introduction

The Namibian standards institution (NSI) Fisheries Inspectorate Division, is a regulatory conformity body, accredited against ISO/IEC 17020:1998, which provides inspection and surveillance as well as sampling and product inspection services to the fish processing factories, fishing vessels and evaluation of food processing systems for compliance to the legislative requirements and to qualify Namibian products to international trade markets. The Namibian Standards Institution food testing center is accredited against ISO/IEC 17025:2005 and is responsible for the chemical analyses together with biotoxins in shell fish and microbiology testing services, for conformity purposes to enable and facilitate the exportation of products and safety of consumers.

FAO, (2005) reported that veterinary checks on all consignment at the border control points comprises documentary, identity and physical checks. The first point at the veterinary check point for all consignments embrace documentary check, where necessary documentation including the health certificates that are checked for existence and completeness. The second point verifies whether consignment complement the information in the documentation and check the health mark, which are traceable to the country and company of origin. Physical check is carried out on all consignments, however, for products fully controlled by import rules; a physical check is carried out according to the percentage of consignments. The percentage would differ based on the products and country of origin. The physical inspection include scrutiny of the contents

of the consignment to ensure that there are no risks to animal, public health and quality defects and samples are taken to the laboratories when essential. Physical checks carried out on products of animal origin are grouped according to the product type and the level of sampling required, however, particular countries have special arrangements with the European Union.

During fish quality control at the Namibian Standards Institution food testing center and at Swift Microbiology Laboratories in South Africa, *Vibrio alginolyticus* species were frequently detected in fish samples from Namibian marine fishery in Walvis Bay, between 2010 and 2013. The data from the Namibian Standards Institution's fisheries inspectorate division indicated that a number of fish consignments have been rejected for export due to various reasons, 34 cases of fish products rejections occurred between 2006 and August 2011 due to bad odors and flavours. Six rejections cases were due to high Total Viable Colony (TVC) counts, one case was due to high *Escherichia coli* counts and one due to detection of the abundant *V. alginolyticus* bacteria.

The scenario of products rejection due to detection of the abundant *V. alginolyticus* bacteria in hake fish triggered tension between fishing companies and the Namibian Standards Institution's Fishery Inspectorate Division. The disagreement was due to unavailability of scientific evidence as to whether *V. alginolyticus* bacteria found abundant in Namibian marine environment (fish and seawater) are pathogenic to humans or not. Rejections of fish and fishery products, due to detection of *V. alginolyticus* species, have therefore been suspended until substantive evidence on the pathogenicity

of this group of bacteria has been found. The presence of *V. alginolyticus* in processed hake therefore was of consumers' safety concern and posed great threats to the market. Shikongo-Nambabi *et al*. (2010b) found that the microbial quality of hake fish sampled from fish factories in Walvis Bay deteriorated along the processing line and that *V. alginolyticus* was frequently detected and most likely to have been introduced by treated seawater. According to Shikongo-Nambabi *et al*. (2010), the ability of *V. alginolyticus* to survive and grow in treated and disinfected seawater, preceded to successive contamination of hake fish, henceforth, be symbolic of related *V. parahaemolyticus*, should this pathogen be present. It is therefore, significant that this center of contamination be controlled to diminish the potential health hazard to consumers (Shikongo-Nambabi *et al*., 2010).

According to some government regulations on *Vibrio* species in seafood, specification of acceptance varies from one species to another. The United States Food and Drug Administration (USFDA) would remove seafood from the market if any pathogenic strain of *Vibrio cholera* and *Vibrio vulnificus* is detected (USFDA, 2001 cited by Ababouch *et al*., 2005). The maximum and critical limit for *V. parahaemolyticus* ingestion is 10 000 cfu/g, whether pathogenic or not pathogenic (USFDA, 2011). According to the Health Protection Agency, (2009) cited by Oberbeckmann *et al*., (2011), the limit for *V. parahaemolyticus* in ready to eat food is 1 000 cfu/g in the United Kingdom. Based on the thresholds, the researchers (Oberbeckmann *et al*., 2011)

found that the level of *Vibrio* species at Helgo Lands Roads (German Bight) was of no health threat to humans.

## 1.2. Fish Processing and use of Seawater

Jespersen *et al*. (2000), described the general fish processing industry as a business consisting of a diversity of items, in terms of operation, scales of production and outputs. Approximately, 75% of the world fish catch is predestined for human consumption purposes, whereas 25% is employed in the production of oil and fish meal production. Fish processing is abundant at onshore processing facilities, followed by those carried out on board fishing vessels. Most factories process fish as whole, while others process fish into fillets, blocks, mince, canned fish, fish meal, fish oil and other products as a way of value addition.

George *et al*. (2001), states that the existence and growth of bacteria in food processing environments could introduce contamination to finished products that lead to reduced microbiological safety and quality. The microbiological contamination can be hosted by raw material, processing equipment, manufacturing activities, cleaning and maintenance practices, operators, waste, animal and insect pests and microbial growth niches implanted in equipment and in structural components of the building as well as processing and cleaning water.

The water quality plays a direct role on food quality (Kirby *et al*., 2002; Wujie *et al*., 2011). Wujie *et al*. (2011), further explained that contaminated water may cause

modifications in food colour, abnormal odor, turbidity, precipitation, crystallization as well as infectious diseases. It is therefore a requirement for the processed water quality, to meets the national drinking water standards to assure that food quality is not compromised. The national drinking water standard require that the water should not contain pathogenic microorganisms and the chemicals in the water must not be harmful to human health. The water quality appearance is obligatory to be colourless, odourless and there should be no suspended solids and/or swimming organisms.

In Namibia, currently there are 20 hake fish processing companies and 2 fish canneries processors, located along the west coastal line in Walvis Bay and Lüderitz. The majority (14) of these processing factories regularly make use of fresh water to conduct their fish processing activities. Eight others including fish cannery employ both fresh water and seawater treated with either chlorine (4) or chlorine dioxide (4) and Ultra Violet (UV) light.

Shikongo-Nambabi *et al*., (2010), pointed out that the use of seawater as a substitute for fresh water in marine fish processing is an economical alternative in dry countries such as Namibia. Fish filleting and canning utilizes large volumes of water during processing. Jespersen *et al*. (2000) and Wujie *et al*. (2011), pointed out that water is applied during several activities along the processing line such as hand sanitation, cleaning of floors, automated equipment, conveyor belts, raw materials, de-icing, thawing, cleaning of products and cooling towers.

According to Thompson *et al*. (2004), seawater is the first choice of water source to marine fish processing companies. The abundance of seawater along the coastal line of Namibia, where 100% of the fish processing factories are located, contributes to an economic advantage over fresh water. Seawater is also believed to maintain the taste (sensory quality) of marine fish and fishery products. However, seawater could be a source of human pathogenic bacteria including *Vibrio* species such as *V. parahaemolyticus, V. cholera, V. vulnificus* and *V. alginolyticus,* when not properly disinfected. Utilization of seawater by fish processing factories therefore necessitates effective treatment and effective disinfection before use on the processing line (Figure 1.1) (Shikongo-Nambabi *et al.*, 2010).

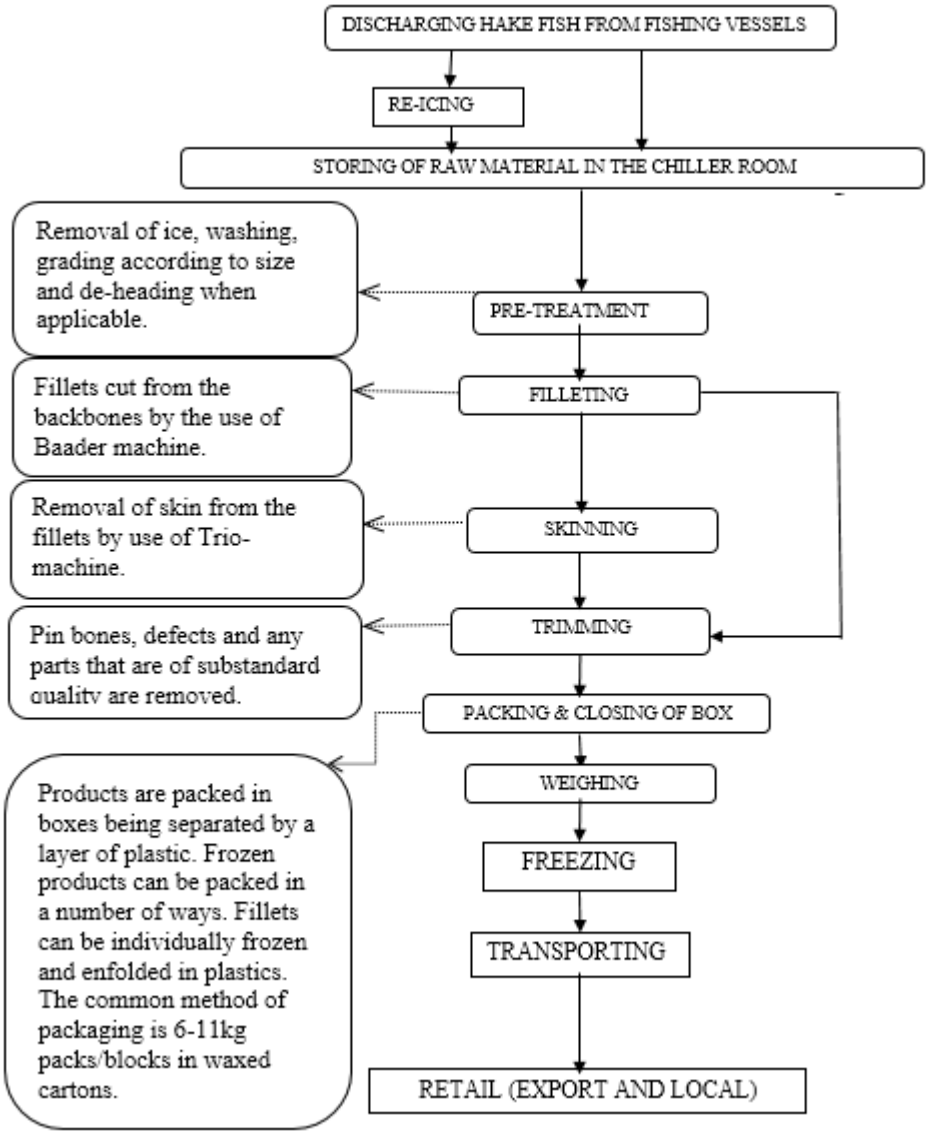



**Figure 1.1.** A typical process flow diagram for the Hake fish filleting factory B in Namibia.

### 1.2.1. Seawater Treatment and Disinfection

Seawater treatment methods may include the following; physical processes, thermal treatment and chemical treatment. During chemical treatment methods, chlorine and ozonation have been commonly utilized as effective, economical and steadily available biocide for various purposes (Huang *et al*., 1997 cited by Da Pozzo, 2008; White, 1999; Cesey *et al*., 2012). However, previous studies have indicated the inability of chlorine to eradicate bacteria from seawater systems (Goldman *et al*., 1978; Shikongo-Nambabi *et al.*, 2010).

Only a few disinfectants have shown to be effective in eliminating bacteria biofilms in seawater (Shikongo-Nambabi *et al*., 2010). Due to its ability to dissociate into harmless products, oxygen ($O_2$) and water ($H_2O$), hydrogen peroxide ($H_2O_2$) is regarded as one of the most environmentally friendly chemicals/disinfectants that gives maximum disinfection including oxidation of organic matter (Da Pazzo *et al*., 2008). Studies carried out by Marine Biological Laboratory in collaboration with Eltron Research (2011) showed that at 5-7 mg/L hydrogen peroxide has broad effectiveness on algae, fish, and invertebrates, planktonic and bacterial organisms within the first 200-500 mins of contact time. Shikongo-Nambabi *et al*. (2010), found that total initiation of new biofilms and inhibition of matured biofilms generated by strains of *V. alginolyticus* were achieved at 0.05%/L and 0.2%/L of hydrogen peroxide respectively.

The aim of this study was to investigate the pathogenicity of *V. alginolyticus* strains isolated from hake fish and seawater using mice models, detection of virulence genes by

PCR. The identity of the isolates was established using Gram reaction, oxidase test, API 20E, PCR detection of species specific *rpoB* and 16S rRNA gene sequences. In addition, the study also aimed at validating the use $H_2O_2$ as an effective biocide that eliminate, biofilms from seawater used by marine fish factories using *V. alginolyticus* species as indicator bacteria.

### 1.3. Statement of the problem

The problems being investigated were:

1. Unavailability of evidence whether strains of *V. alginolyticus* from the Namibian marine waters (seawater) and from raw fish and processed products are pathogenic to humans or not. The concern about the presence of *V. alginolyticus* in fish and fishery products needed to be addressed to determine the safety of these products for human consumption. Export rejections of fish and fishery products by Namibian Standards Institution fishery inspectorate division due to detection of *V. alginolyticus* species have therefore been suspended until substantial evidence of the pathogenicity of this group of bacteria has been found.

2. It was not clear whether $H_2O_2$ could perform better than chlorine for the disinfection of seawater. Shikongo-Nambabi *et al*. (2010), pointed out that fish processing plants make use of chlorinated and UV irradiated seawater. However, this method is not effective enough to eliminate bacteria biofilm from water pipes causing product contamination when the water is distributed in factories. This problem has raised

concerns with regard to the quality and safety of fish products to consumers and poses great threats to the market with regards to consumer demand. A number of hake fish consignments have been rejected for export by the Namibian Standards Institution fishery inspectorate division due to various reasons regarding low quality of fish and fish products. The Namibian economy through GDP is often affected when exportation of fish and fish products decreases. Fish processing companies retrench employees, leading to higher rates of unemployment and poverty. In Walvis Bay, one company was shut down due to loss of revenue since 2013 to date.

Previous studies have reported that seawater can be a potential source of contamination to fish and fish products (Thompson, 2004). Thus, to ensure the acceptable quality of fish and fish products processed and exported, the benchmark experimentation of the disinfection capacity of hydrogen peroxide demonstrated by Shikongo-Nambabi *et al*. (2010) needed to be validated in real life situations, within marine fish factories using *in situ* experiments. This study will make a definite contribution to the fishing industry in Namibia by increasing revenue through increased export. The development of a disinfection method that is specific for seawater would optimize seafood safety and shelf life as the present methods of water disinfection (Chlorination and UV irradiation) are ineffective. It would create a platform that promotes the use of seawater in the marine fishing industry at large. Availability of safe seawater would save costs and would facilitate sustainable and economical marine fish processing curbing water shortages sporadically experienced

by the fishing industry. Furthermore, improvement in fish quality and safety will guarantee returns from the international market by maintaining regular export hence augmenting national and global efforts to improve food security.

## 1.4. Aim and Objectives

The aim of this study was to assess the pathogenicity of *V. alginolyticus* strains isolated from hake fish processing factories and to demonstrate the effectiveness of $H_2O_2$ in Seawater disinfection.

Specific Objectives

1. To determine the pathogenicity of different *V. alginolyticus* strains isolated from Namibian marine waters as well as from raw and processed hake fish by experimental infection of mice and detection of virulent genes (*toxR, tlh and tdh)* by polymerase chain reaction (PCR).

2. To validate the use of $H_2O_2$ as an alternative disinfectant, to chlorine that effectively reduces bacteria loads and eliminates biofilms from seawater distribution systems using in Total Viable Colony (TVC) counts of *V. alginolyticus* as indicator organisms.

Hypotheses

($H_{o1}$): Strains of *V. alginolyticus* isolated from Namibian marine water (seawater) and processed hake fish are non-pathogenic.

($H_{o2}$): There are no significant differences in Total Viable Colony (TVC) counts of *V. alginolyticus* in seawater, fish and fishery products between samples processed using seawater treated with chlorine and those processed using seawater treated with hydrogen peroxide ($H_2O_2$) and Ultra Violet light.

# CHAPTER 2: LITERATURE REVIEW

## 2.1. Background

The Namibian economy depends on sustainable utilisation of natural resources. The country's economy is thriving on available natural resources, mainly on agriculture, fisheries and mining. The fishing sector contributed 5.3% and 4.7% to the Gross Domestic Product (GDP) in 2007 and 2008 respectively, indicating a decrease in the fisheries output (MFMR, 2009). In 2009 the ministry of fisheries contribution to GDP increased to 5% and further decreased to 3.7% in 2010 (NPC, 2012, cited by MFMR, 2010/11). The factors which contributed to the poor performance of the fishing industry include unfavorable oceanic conditions, high oil prices, as well as reduced fish stocks (NPC, 2012). The fishing industry is the second biggest export earner of foreign currency after mining (NPC, 2012).

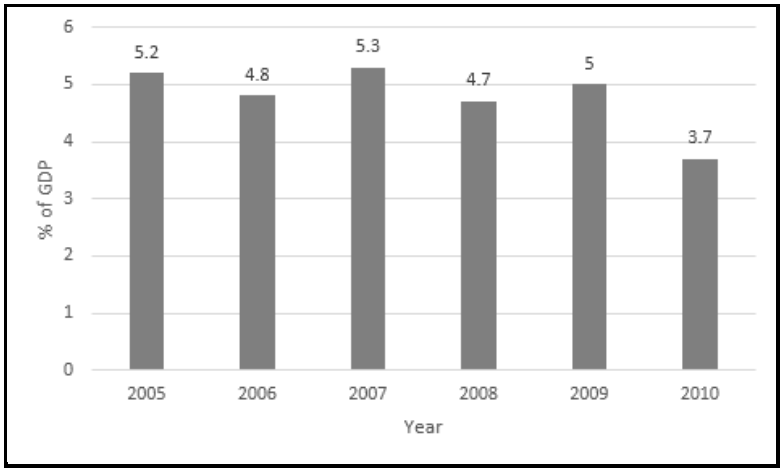



**Figure 2.1.** The contribution of the fisheries subsector to the GDP in Namibia. (Source: NPC, 2011, cited by MFRF, 2009; MFMR, 2010/2011).

The Marine fishing sector is located along the west coast of Namibia, with greatest concentration in Walvis Bay and Lüderitz which lie off the Namib Desert (Figure 2.2). Namibia has productive fishing grounds due to the Benguela upwelling current system that sustains rich stocks of demersal and small pelagic species (O'Toole, 1977; Agenbag, 1980; Boyd and Cruickshank, 1983; Agenbag and Shannon, 1988, cited by Boyer and Hampton, 2001; Boyer *et al*., 2000).

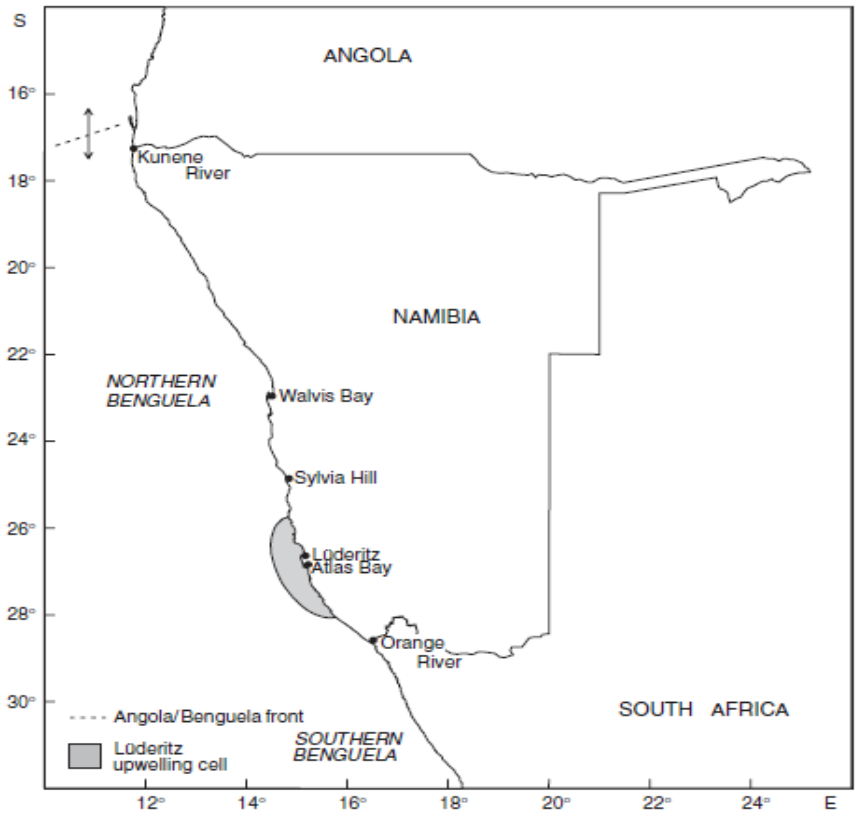



**Figure 2.2.** Map of Namibia, showing towns of Walvis Bay and Lüderitz along the west coast where marine fishing sector is concentrated (Source: Boyer *et al.*, 2000).

The following fish species are harvested and processed in Namibia; hake (*Merluccius capensis* and *M. paradoxus*), horse mackerel (*Trauchurus capensis*), monkfish (*Lophius volmerinus*), pilchards (*Sardinops sagax*), Cape anchovy (*Engraulis capensis*), tuna (*Katsuwonus pelamis*), swordfish (*Xiphias gladius*), sharks (*Isurus oxyrinchus, Prionace glauca* and *Alopias vulpinus*), snoek (*Thyrsites atun*), kingklip (*Genypterus capensis*), orange roughy (*Hoplostethus atlanticus*), silver kob (*Argyrosomus inodorus*), west coast steenbras (*Lithognathus Aureti*), rock lobster (*Jasus lalandii* ) and deepsea red-crab

(*Chaceon maritae*) (Smolenski *et al*., 1993; Punt and Japp, 1994; Boyer and Hampton, 2001; MFMR, 2010/11). See figure 2.3.

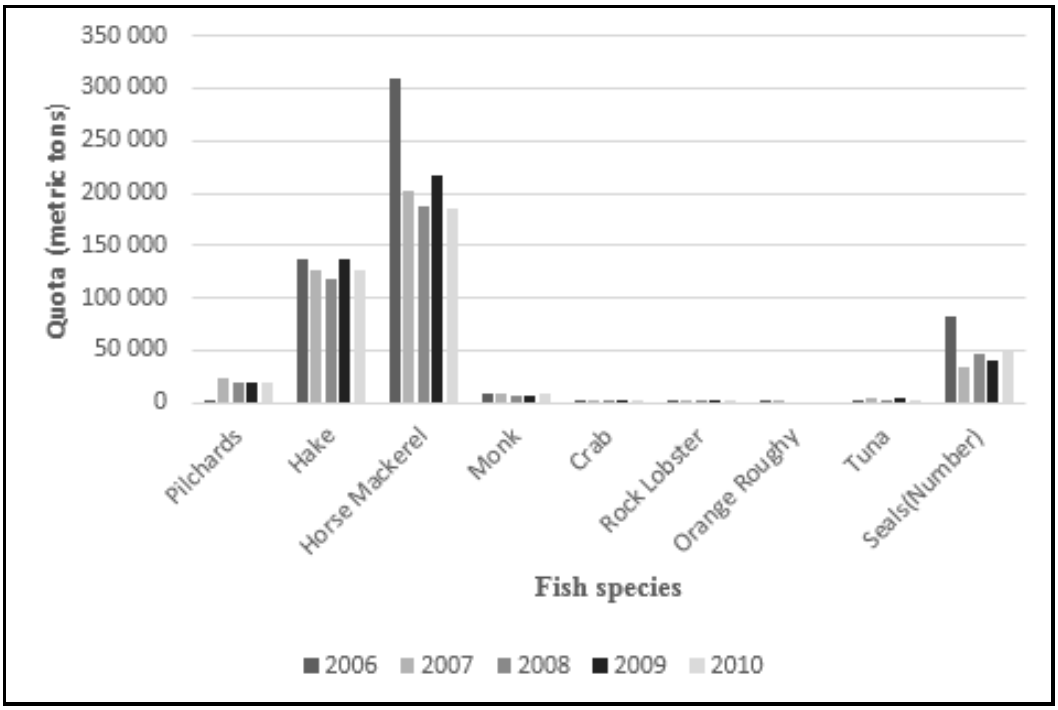



**Figure 2.3.** Namibia landings of quota Species (metric tons) in 2006 to 2010 (statistics obtained from the MFMR, 2010/11)

2.1.1. Export of fish and fish products

The natural freshwater bodies in Namibia suitable for commercial utilization are not notable. Thus, the marine commercial fishing industry has the most fish and fishery products dominated by hake fishery; hence, products are mainly exported to the European markets such as Spain and distributed to other markets such as Italy, Portugal,

France, Germany and Holland, where they fetch high prices. The demand for fish and fishery products continues to increase in the European Market with Namibia being one of the leading suppliers for frozen hake in terms of both volume and value (MFMR, 2010/11).

The World Trade Organization's (WTO) agreement on technical barriers to trade, facilitate the conduct of international trade by improving efficiency of production through international standards, technical regulations and conformity assessment. Technical regulations and standards involve procedures for conformity assessment, packaging, marking, and labeling requirements (WTO, 1994).

2.1.2. Microbiological quality control and fish testing

Quality refers to all physical, chemical, microbiological and sensory characteristics that affect the value of products to the consumer, either positively or negatively. Examples of negative attributes are such as spoilage manifested by off-odors, off flavours, off texture, discoloration and contamination with pathogens or chemicals. Positive attribute include colour, flavours, texture and fish processing method. A number of different factors that contribute to the possible hazards include poor hygiene, lack of precautionary controls, misapplication of chemicals, use of contaminated raw materials, ingredients and water used during processing as well as shipping and storage shipping and storage under inadequate conditions (FAO/WHO, 2001).

European Commission (2005), described quality control as an act by which all quality attributes are measured and identified to ensure maintenance of food safety. Thus, most fish processing factories use hazard critical control points as tools to evaluate, identify and create critical control systems that prevent impairment instead of depending on finished product testing. Furthermore, the implementation of hazard critical control points system normally depends on scientific evidence of risk to human health and brings forth good benefits such as regular microbiological testing, inspection by regulatory bodies, hence, promoting global trade by increasing confidence in food safety.

Frohberg *et al*. (2006), emphasized that in the food sector, there is a wide range of different standards and regulations such as hygienic standards and sanitary standards. Certification is a universally acceptable approach to standards that considers a large number of standards and regulations related to food quality, environmental and social issues. Certification delivers superior information to consumers about characteristics and quality of food products, therefore increasing market transparency. It is therefore crucial that standards of practice are established to ensure that general hygiene practice (GHP) has been applied (European Commission, 2001, cited by FAO, 2005).

### 2.2. *Vibrio alginolyticus*

*Vibrio alginolyticus* belongs to the genus *Vibrio*. It is a Gram negative, asporogenous rod that is either straight or has a single rigid curve. Cells are motile, mostly having singular polar flagellum when grown in liquid medium. Most members of the genus

*Vibrio* produce oxidase and catalase enzymes and ferment glucose without producing gas; besides being pathogenic (Kaysner and DePaola, 2001).

*Vibrio* species are indigenous to marine and estuarine environments, and are commonly associated with fish and crustacean (Thompson *et al*., 2004; Ababouch *et al*., 2005; Worden *et al*., 2006; Hsieh *et al*., 2008). A large number of these species are mesophilic and tend to bloom during warm seasons. The distribution of *Vibrio* species is heavily affected by pH, salinity, temperature as well as availability of nutrients in water (Huq *et al*., 1983; Thompson *et al*., 2004; Blackwell and Oliver, 2008). In some cases, the distribution and life span of *Vibrio* species depends on the available host organisms such as copepods, crustaceans, plants, and algae (Colwell *et al*., 1977; Huq *et al* 1983; Thompson *et al*., 2004; Deter *et al*. 2010; Oberbeckmann *et al*., 2011a).

According to the latest (2014) list of Bacteria Names with Standing Nomenclature (http://www.bacterio.net/uw/vibrio.html), members of the genus *Vibrio* have been increasing, with more than 100 recognized species. Nevertheless, only about 13 of these species (*V. alginolyticus*, *V. carchariae*, *V. cholera*, *V.costicola*, *V. cincinnatiensis*, *V. hollisae*, *V. fluvialis*, *V. furnissii*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus*, *V. vulnificus,* and *V. damselae* that has now been renamed to"*Photobacteriumdamselae* subspecies. *Damselae*") are considered human pathogens. Seafood-borne diseases are predominantly caused by *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholera* (Ababouch *et al*., 2005). According to the American Centers for Disease Control and

Prevention (CDC, 2013), there has been an increase (43%) in *Vibrio* related infections since 1996 till 2012.

*Vibrio alginolyticus* is an opportunistic pathogen to human and marine animals (Balebona *et al*., 1998; Schets *et al*., 2006; Campanelli *et al*., 2008; Austin, 2010; Zhao *et al*., 2010 Reilly *et al*., 2011). The outbreaks of *V. alginolyticus* infections increases rapidly during summer seasons (Morris, 1985; Sganga *et al*., 2009). Most of these infections stemmed from contact of cuts to contaminated seawater (Reilly *et al*., 2011). Infection by *V. alginolyticus* causes otitis, endophthalmitis and wound infections (Pezzlo *et al*., 1979; Sganga *et al*., 2009; Austin, 2010; Reilly *et al*., 2011). Sganga *et al*. (2009), thus suggested a need for the characterization of virulence mechanisms of *V. alginolyticus*.

Zhao *et al*. (2010), characterized multiple mechanisms (induction of rapid apoptosis, cell rounding and osmotic lysis) as being responsible for inducing cell death in fish cells, when infected with a *V. alginolyticus* strain (ZJO). The researchers further explained that the bacterial cells required their type III secretion system (T3SS) to induce rapid death of infected fish cells. They found that croaking fish cells depicted attributes of apoptotic cells, such as nuclear condensation, DNA fragmentation as well as membrane blabbing which further led to membrane pore formation and discharge of cellular contents, evidenced by lactate dehydrogenase release and the absorption of a membrane-impermeable dye. Hueck (1998), described the type III secretion system as a conserved tool among gram negative bacteria that delivers proteins, known as effectors, openly into

the host cells. Coburn *et al*. (2007), confirmed that many of the above mentioned effectors are virulence factors that prompt host-cell death and maneuver with the innate immune system. Other pathogens such as *Salmonella*, *Shigella*, *Pseudomonas aeruginosa* and *Yersinia* species have been described to make use of the T3SS mechanism in causing host cell death (Monak *et al*., 1997; Dacheux *et al*., 2001; Nonaka *et al*., 2003; Fink and Cookson, 2006).

Furthermore, a large number of *Vibrio* species are pathogenic to humans, marine vertebrates and invertebrates, by producing and secreting various virulence factors such as enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive onto surfaces and haemagglutinins (Lida and Honda, 1997; Austin and Austin, 1999; Shinoda, 1999). The most common type of virulence factors among *Vibrio* species is a haemolysin, an enterotoxin (Lida and Honda, 1997; Shinoda, 1999) which strikes the erythrocyte membranes causing them to lyse and leading to emancipation of iron-binding proteins (haemoglobin) as well as lysing of the blood cells (Lida and Honda, 1997; Shinoda, 1999).

*Vibrio* species can survive under unfavorable environmental conditions such as low temperatures, exposure to disinfectants and nutrient depletion, by the formation of biofilms, attachment to algae, crustaceans and by utilization of nutrients released by these organisms (Huq *et al*., 1983; Ababouch *et al*., 2005). Another survival strategy of *Vibrio* species is by undergoing a viable but non-culturable (VBNC) state during which metabolically active bacterial cells do not grow on any culture media, but may remain

pathogenic and resistant to environmental stress (Colwell *et al*., 1985; Baffone *et al*., 2003; Du *et al*., 2007).

Public Health Laboratories experience challenges during identification of *Vibrio* species. Conventional phenotypic methods employed during the identification of *Vibrio* species, are found to be time consuming and not so accurate. For instance, in 1997, two isolates cultured from shrimps and submitted by the Canadian Food Inspection Agency in St John's, Newfoundland to the National Microbiology Laboratory in Canada for confirmation were both presumptively identified as *V. parahaemolyticus* using conventional biochemical tests. In addition, the 16S rRNA gene molecular analysis was unable to confirm the presumptive identification because of high sequence identity between *V. parahaemolyticus* and *V. alginolyticus.* It is recommended that additional methods, for the identification of *Vibrio* species, including molecular techniques are necessary (Kwok *et al*., 2002). According to Ki *et al*. (2009), the gene *rpo*B (encoding the RNA polymerase beta subunit) was found to give better identity than the 16S rRNA gene sequencing.

Based on a mini review by Janda and Abbott (2007), the 16S ribosomal RNA (rRNA) is a highly conserved gene in all prokaryotic organisms. It has been used in phylogenetic and taxonomic studies for several reasons; it exist in every bacterial genome, its function does not change over time that made it a good indicator for evolution and it is long enough (1500 bp) for informatics or genetic determinations.

The *rpoB* is employed as an alternative tool for universal bacteria genotypic identification to the 16S rRNA (Mollet *et al*., 1997).

### 2.3. Acquisition of pathogenicity by *Vibrio* species

Bacteria may change their genotypes through DNA recombination on chromosomes with foreign DNA that they have picked up from the environment. Genetic transformation was discovered in *Streptococcus pneumonia* when non-pathogenic mutants were rendered pathogenic by a concomitant injection with virulent strains which caused cell death in mice (Lengeler *et al*., 1999). Genetic recombination can be artificially induced *in vitro* sometimes with very high transformation frequencies, following cells that are rendered competent by treatment with physical or chemical agents to enhance uptake of DNA carrying foreign genes (Lengeler *et al*., 1999).

The determinants of virulence distribution between species are of major importance and affect the evolution of known and emerging pathogens. Bacterial pathogens are known to obtain virulence by a number of different mechanisms that ensure their survival. (Fitzgerald and Musser, 2001).

Mordacq and Ellington (2004), explained that there are several ways by which bacteria acquire foreign genes including those that encode for virulence factors; by genetic recombination in bacteria, transformation, transduction and conjugation. During transformation bacterial cells pick up genetic materials from environments while

transduction occurs when a virus conveys a genetic material from one bacterial cell to another, where it gets incorporated into the host chromosome. Through conjugation, the genetic material of one bacterial cell (donor) is transferred to another bacterial cell (recipient).

A large number of non-pathogenic *Vibrio* species, are considered to be reservoirs of virulence genes; toxin regulator (*tox*R), thermostable direct hemolysin (*tdh)* which through successful gene mobility may transform nonpathogenic to pathogenic strains (Nishibuchi *et al*., 1996). The researchers concluded that only certain *V. parahaemolyticus* strains carry pathogenic genes and are pathogenic to humans. *V. alginolyticus* species examined were found to be *tlh* positive, while *tox*R was commonly detected among *V. parahaemolyticus* strains (Oberbeckmann *et al*., 2011). According to Rodrieguez-Castro *et al*. (2010) and Whitaker *et al*. (2010), cited by Oberbeckmann *et al*. (2011), the pathogenicity (*tdh or trh*) of *V. parahaemolyticus* is negatively or inversely affected by environmental factors, mainly; water temperature and salinity. Oberbeckmann *et al*., (2011) specified that *tdh* or *trd* are virulent-associated genes and summer was associated with highest risks for infections caused by *V. parahaemolyticus*. However, there was no detection of virulence-associated genes in the strains from Helgoland Roads (German Bight). Martinez-Ultaza *et al*. (2008) and Rodrieguez-Castro *et al*. (2010), highlighted that the virulence associated genes are very limited in environmental strains. Izutsu (2008), affirmed that single gene is not sufficient to differentiate between pathogenic and non-pathogenic strains. In addition, other genes are involved in the pathogenicity process of *V. parahaemolyticus* (Caburlotto *et al*., 2009,

2010b). Oberbeckmann *et al*. (2011), indicated that other environmental factors such as sediment, water bodies and changing of the environmental parameters are regarded as reservoirs for pathogenicity transmission, environmental monitoring of *Vibrio* species, is consequently required. Based on sample results produced by the Namibian Standards Institution' food laboratory in Walvis Bay and Swift microbiology laboratories in Cape town with regard to outsourced samples from Namibia, there has never been a single incident of detection of *Vibrio cholera* and *V. parahaemolyticus* since 2009 to July 2014, which is a good indication that the cold Benguela current does not support the growth of most pathogens as well as expression of pathogenic genes.

Some strains of *V. alginolyticus* isolated from Alaskan oysters where found to contain *trh* previously reported to be associated with *V. parahaemolyticus* only (Gonzalez-Escalona *et al*., 2006).

### 2.4. Determination of pathogenicity and detection of virulence associated genes in *Vibrio* species

The virulence genes commonly occurring in *V. parahaemolyticus* and *Vibrio cholera* have been described to be widely distributed among *V. alginolyticus* in the coastal mariculture systems in Guangdong, China. The pathogenicity of *V. alginolyticus* strains to aquatic animals is assumed to have been derived from *V. parahaemolyticus* and *V. cholera* signifying a possible reservoir of the virulent genes (Xie *et al*., 2005).

Furthermore, the presence of virulence associated genes in *Vibrio* species has been demonstrated using detection of specific virulence associated genes (Xie *et al*., 2005; Oberbeckmann *et al*., 2011).

2.4.1. Detection of Thermostable Direct Hemolysin (*tdh*) and Thermostable Direct related Hemolysin (*trh*) genes.

Detection of *tdh* and its homolog *trh* is regarded to be the simplest method and commonly used as indicative of pathogenicity in *Vibrio* species (Parveen *et al*., 2008; Broberg *et al*., 2011; Paranjype *et al*., 2012; West, 2012). About 90% clinical strains and 1% environmental isolates of *V. parahaemolyticus* were found to carry *tdh* virulence factor (Miyamoto *et al*., 1969; Nishibuchi and Kaper 1985; Nishibuchi *et al*., 1992; Okuda *et al*., 1997, cited by Mclaughlin *et al*., 2005). The genetics of *tdh* and *trh* genes are reflected in a wide range of studies, their amino acid sequences are approximately 67% indistinguishable, and the two genes are expected to work in a similar way (Broberg *et al*., 2011; Ohnishi *et al*., 2011). The molecular structures of the *tdh* and *trh* genes is related to their pathogenic functions; they facilitate the outward conveyance (efflux) of divalent cations and other solutes from the bacteria cells, as well as inward carriage (influx) of water molecules from and into intestinal cells. These movements of substances into and out of the bacterial cells is expedited by their possession of tetrameric proteins that serve as porins (Yanagihara *et al*., 2010; Broberg *et al*., 2011; Ohnishi *et al*., 2011). Numerous studies have pointed out that the *tdh* and *trh* are detected almost exclusively in clinical strains cultured and isolated from patients

suffering from *V. parahaemolyticus* gastroenteritis (Miyamoto *et al*., 1969; Shirai *et al*., 1990). The capability of the *tdh* gene to break down the erythrocytes on a high salt content Wagatsuma agar is termed the Kanagawa phenomenon (Nishibuchi and Kasper, 1995). *V. parahaemolyticus* strains carrying the *trh* gene were found to be Kanagawa negative (Honda *et al*., 1990). This phenotypic method has been used to screen for pathogenic *V. parahaemolyticus* in sea food as well as patient samples (Xie *et al*., 2005). However, its accuracy is highly depend on pH, salinity and erythrocyte type, it has therefore been suggested that this method is not always dependable (Nishibuchi and Kasper, 1995; Xie *et al*., 2005).

2.4.2. Detection of the Thermolabile Hemolysin gene (*tlh*)

The *tlh* gene was first cloned and characterized in 1986; it encodes for thermolabile hemolysin, a phospholipase (Taniguchi *et al*., 1986; Zhang and Austin, 2005). This gene is considered to be species specific in *V. parahaemolyticus* (Bej *et al*., 1999; DePaola *et al*., 2003). Several studies have recommended this gene as a target gene for the identification of *V. parahaemolyticus* (Bej *et al*., 1999; Ward and Bej, 2006; Nordstrom *et al*., 2007). Despite the fact that this gene has been regarded as a virulence factor, its contribution to *V. parahaemolyticus* pathogenicity is not known. However the manifestation of this gene depends on conditions prevailing in the human intestines (Gotoh *et al*., 2010; Broberg *et al*., 2011). Thermolabile hemolysin (*tlh*) was found to be toxic to zebra fish. Thus, it was suggested that "*tlh* could potentially be developed as a vaccine and used as diagnostic tool for vibriosis" (Jia *et al*., 2010). Furthermore, when

erythrocytes of sea bream were exposed to *tlh* they showed signs of apoptosis and necrosis such as increase in caspase activities and DNA fragmentations, During the same experiments the toxin induced membrane vesiculation, detachment of the membrane vesicles and cell membrane disintegration which triggered leakage of cell contents (hemoglobin and other cytoplasmic contents) (Wong *et al*., 2012). Apoptosis was described and exemplified by morphological cell contraction and hyperchromatic nuclear fragment as well as biochemically by chromatin cleavage into nucleosomal oligomers (Willie *et al*., 1980, cited by Hong *et al*., 1998).

2.4.3. The role of Toxin Regulator gene (*toxR*) in *Vibrio* species pathogenicity

ToxR is a conserved virulence gene regulator in Vibrios, and it was detected in a pathogenic strain of *V. alginolyticus* (ZJ51-O), that was isolated from diseased fish (Chang *et al*., 2011). *ToxR* gene was first identified in *Vibrio cholera* species (Miller and Mekalanos, 1984). Toxigenic *cholera* is one of the bacteria that is mostly responsible for causing severe diarrhea disease that kills large numbers of people (Faruque *et al*., 1998; Bina *et al*., 2002). The two genes believed to be accountable for the virulence are cholera enterotoxin (CT) which causes cholera typical diarrhoea and toxin coregulated pilus (TCP) which is responsible for attachment and colonization of intestinal epithelia (Miller *et al*., 1987; Herrington *et al*., 1988; Faruque *et al*., 1998; Bina *et al*., 2002).

These two genes belong to a group (ToxR regulon) of pathogenic genes whose communication is controlled by transcriptional regulators encoded by the *toxRS, tcpPH,* and *toxT* genes (Bina *et al*., 2002). *ToxRS* takes part in the activation of *toxT,* ctxAB, and

OmpU. The expression of CT is inhibited by crude bile, possibly by inhibition of *ToxT*. The *ToxT,* an independent branch of *ToxRS* is activated by bile acids (Hung and Mekalanos, 2005). *ToxR* is a molecule that regulates the complex mechanisms for virulence factor expression pathway in pathogenic bacteria. *ToxR* aids pathogenic bacteria to differentiate between time and place (Ottemann and Mekalanos, 1995; Whitaker *et al*., 2012).The *tox*R regulates other genes through another activator called *toxT* promoter, and is modulated by *in vitro* growth conditions that regulates expression of the *toxR* operon (Higgins *et al*., 1992; Bina *et al*., 2002; Dirita, 2006). It has been established that *ToxT* is a transcriptional regulator belonging to the AraC familly of proteins. The two outer membrane proteins, *OmpU* and *OmpT* are also being directly regulated by *toxR* (Higgins *et al*., 1992). The *toxR* gene was originally known for its functional transcriptional activator of *ctx*AB in a heterogous *Escherichia coli system*. However, the activation of the *ctx*AB promoter has not been established in *V. cholera* (Champion *et al*., 1997).

During gene expression, a group of operons regulated by one common and specific regulator called regulon, optimize production quantity of each gene when necessary, thus genes are switched on and off purposefully by DNA recombination (Lengeler *et al*., 1999).

The *toxR* virulence gene has been detected in; *Vibrio cholera* (Miller *et al*., 1987), *V. parahaemolyticus* (Lin *et al*., 1993), *V. alginolyticus*, *V. hollisae*, *V. mimicus* and *V, fluvialis* (Osorio and Klose, 2000), *Vibrio anguirallum* (Okuda *et al*., 2001), *Vibrio*

*ficheri* (Reich and Schoolnik, 1994) as well as *V. vulnificus* (Lee *et al*., 2000). Previously, *tox*R has been successfully detected by PCR (Kim *et al*., 1999; Takashi *et al*., 2005). The illustration of the network of virulence gene regulators is shown in Figure 2.1 below.

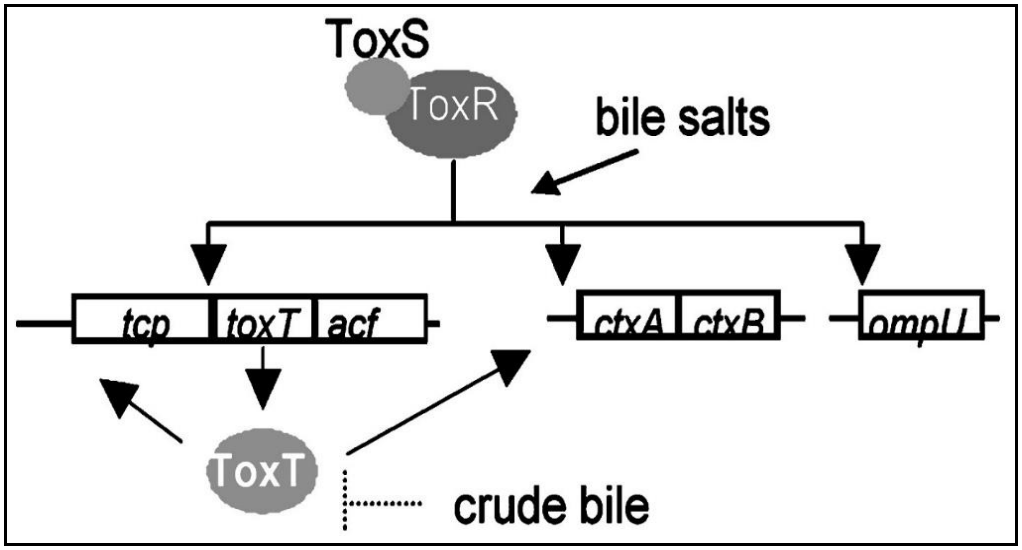



**Figure 2.4.** Exemplary function of *ToxR* in *Vibrio cholera* (Source: Hung and Mekalanos, 2005).

### 2.5. Outbreaks of *V. alginolyticus* related disease outbreaks

The documented *V. alginolyticus* associated disease outbreaks affect marine fin fishes and crustaceans with only a few cases were connected to human disease. Austin *et al*. (1995), emphasized that *V. alginolyticus* species form part of the normal marine flora.

However, other findings indicated its virulence to aquatic animals (Lighter, 1993). A large number of pathogenic organisms are found in most aquaculture practices (Defoirdt *et al*., 2004). *Vibrio* species are the most common agents of infection in fish, abalone, sea urchins and oysters (Vanderberghe *et al*., 2002). Vibrios may be transmitted to humans through contact with marine ecosystems or consumption of contaminated fish and other sea food (Aoki, 1997).

2.5.1. *V. alginolyticus* infections in fish

Schroeder *et al.* (1985), established that *V. alginolyticus* caused severe lesions and ulcerations in the muscles of cranial insertion area of pectoral fins in Atlantic Bottle Dolphin housed in an open ocean pen. The ulcers were oval located at the interior insertion of the pectoral fin with sizes ranging between 2.5 and 5.0 cm in diameter but sometimes more severe. Despite the treatment carried out, recurrence happened within 4 to 6 months. Furthermore, *V. alginolyticus* was implicated as the cause of gill disease in 1992 and was found prevalent in juvenile turbot (*Scophthalmus maximus*) conserved in an aquarium during spring and summer seasons. The bacteria induced gill disease and fin rots that led to progressive low-levels of mortalities. The gills of some infected turbot were pale in colour and were fused while others diseased fish had pink skin, loose muscle and swollen abdomen. Internal organs of infected fish were not affected. The disease outbreak was accompanied by biofilm around the side of the tank (Austin *et al*., 1993). In 1993, an outbreak of Vibriosis in Taiwan was associated with the common *V.*

*alginolyticus* isolated from diseased kuruma prawn (*Penaeus japonicas*) with clinical signs of bacterial septicemia (Lee *et al*., 1996).

In 2001 to 2002, two incidents of mortality of cultured carpet juvenile and larvae shell clams (*Ruditapes decussatus*) were noted at a commercial hatchery located in Spain. During the two occasions, the bacterial isolates from the diseased organisms were identified as *V. alginolyticus* (Gomez-Leon *et al*., 2004). Sharma *et al*. (2011), reported an outbreak of vibriosis in Asian seabass (*Lates calcarifer*), which were cultured in open sea floating cages in India. The diseased fish were found to contain hemorrhage and ulcers. Further histological examination indicated congestion, hemorrhage and necrosis in vibrant organs. *V. alginolyticus* species was isolated from the liver, gill, kidney, brain and blood of the diseased fish. In 2008 and 2009, *V. alginolyticus* outbreak along the coast of Tunisia affected cultured Gilthead sea bream (*Sparus aurata L*.) and Sea bass (*Dicentrarchus labrax*). The bacteria strains were identified using phenotypic and molecular characterizations methods (Sadok *et al*., 2012).

2.5.2. *V. alginolyticus* infections in humans

According to Schmidt *et al.* (1979) findings, *V. alginolyticus* has been occasionally isolated from human infections and from coastal waters. The organism is abundantly associated with self-limited wound and ear infections. It causes septicemia and fatal infections in patients with severe burns and those with harshly compromised immune systems. In October 1978, *V. alginolyticus* was isolated from a purulent eye discharge of a 43 year old man who was hospitalized due to pneumococcal pneumonia and who

worked as a fish cutter and was a heavy alcohol consumer. *V. alginolyticus* was together with non-group A beta hemolytic *Streptococci*, *Proteus mirabilis*, and *Proteus morganii* from a stamp ulcer below knee amputation of a 58 years old man who experienced the pain for 17 years and eventually developed purulent discharge in the stump (Schmidt *et al*., 1979).

In 2006, three people suffered *V. alginolyticus* infections after swimming in the Oosterschelde, a large inlet on the North Sea of the Netherlands. One patient was a 73 years old lady who cut on her knee and *V. alginolyticus* was isolated from the synolvial fluid of the knee joint. The second patient was an 18 years old lady, with injuries on her hands and the bacteria was cultured from the injured site. The third patient was an 11 years old baby boy, who acquired otitis media in one ear, after swimming in the sea. The bacteria were isolated and identified as *V. alginolyticus.* Seawater samples were also collected from the Oosterschelde for microbiological analyses and *V. alginolyticus* as well as *V. parahaemolyticus* were regularly detected. During the three cases, the patients were treated with amoxicillin-clavulate and speedily healed after antibiotic treatment (Schets *et al*., 2006).

In 2011, a woman over 70 years old, from Guernsey was diagnosed with *V. alginolyticus* infection, after visiting a clinic with a wound on a lower leg that was refusing to heal. The lady initially injured herself on the leg after working in the garden, and she continued her habit of swimming at sea in British waters. The patient was treated using

doxycycline (100g, twice a day) and the wound was healed, after three weeks. The Figure 2.5 below indicate the infected wound (Reilly *et al*., 2011).

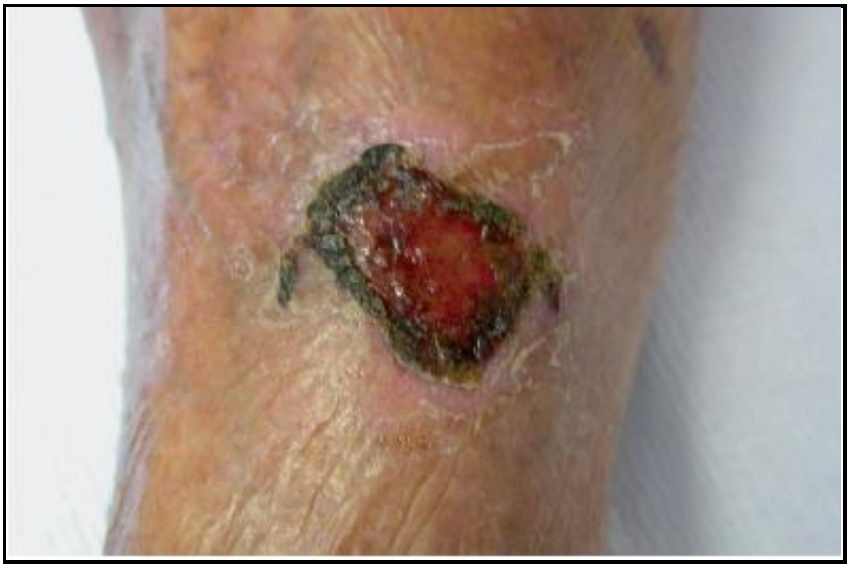


**Figure 2.5.** Wound on the Guernseyan woman's leg, with crusting and surrounding erythema indicating signs of infection (source: Reilly *et al*., 2011).

## 2.6. Seawater treatment and disinfection In Namibia

The use of treated seawater is a typical approach used to meet requirements of water for fish processing. The overall process entails several stages and procedures, before clean seawater is pumped and used in the fish processing factories. The first stage include pumping of raw seawater from a depth (2m) below the sea surface. Raw seawater is then pumped into the coagulant/flocculent tank containing the coagulant chemical. The common coagulant chemical being used is called potassium aluminum sulphate (KAl

$(SO_4)_2.12H_2O$) which hydrolysed in water to form a colloid (Griffiths, 2003; Bratby, 2007; Wujie *et al*., 2011). The colloid has neutralization and absorption properties which absorbs the natural colloid and suspended solids in water (Wujie *et al*., 2011).

In the devolved air flotation (DAF) tanks where the coagulation process takes place, the colloid particles in the water become compressed into thick flocs which eventually sink to the bottom (Wujie *et al*., 2011). Clean water is found in the middle of the DAF tanks and it flows down to the intermediate tank, driven by the inclination and gravitational force. In the intermediate tank, seawater is stored temporally then pumped through the pressure sand filters. The pressure sand filters consist of sand layer and carbon filters, which separate and get rid of suspended solids and colloid impurities from the mother liquor (Wujie *et al*., 2011). After the sand filters, seawater is disinfected using chlorine gas that is dosed automatically into the seawater system. The amount of chlorine dosed into the seawater is measured automatically using a residual chlorine meter. Disinfected seawater is stored in the final tank where it is kept until such time the water get pumped to the fish processing factory for use. When seawater is pumped to the fish processing fish processing factory, it goes through UV light, a very effective water disinfection step. The best UV light wavelength for water disinfection is 2600A. During the irradiation of microorganisms, their proteins and nucleic acid absorbs spectrum energy that cause cells denaturation (Wujie *et al*., 2011). Figure 2.6 below, indicate a standard seawater treatment and chlorine disinfection system in Namibia.

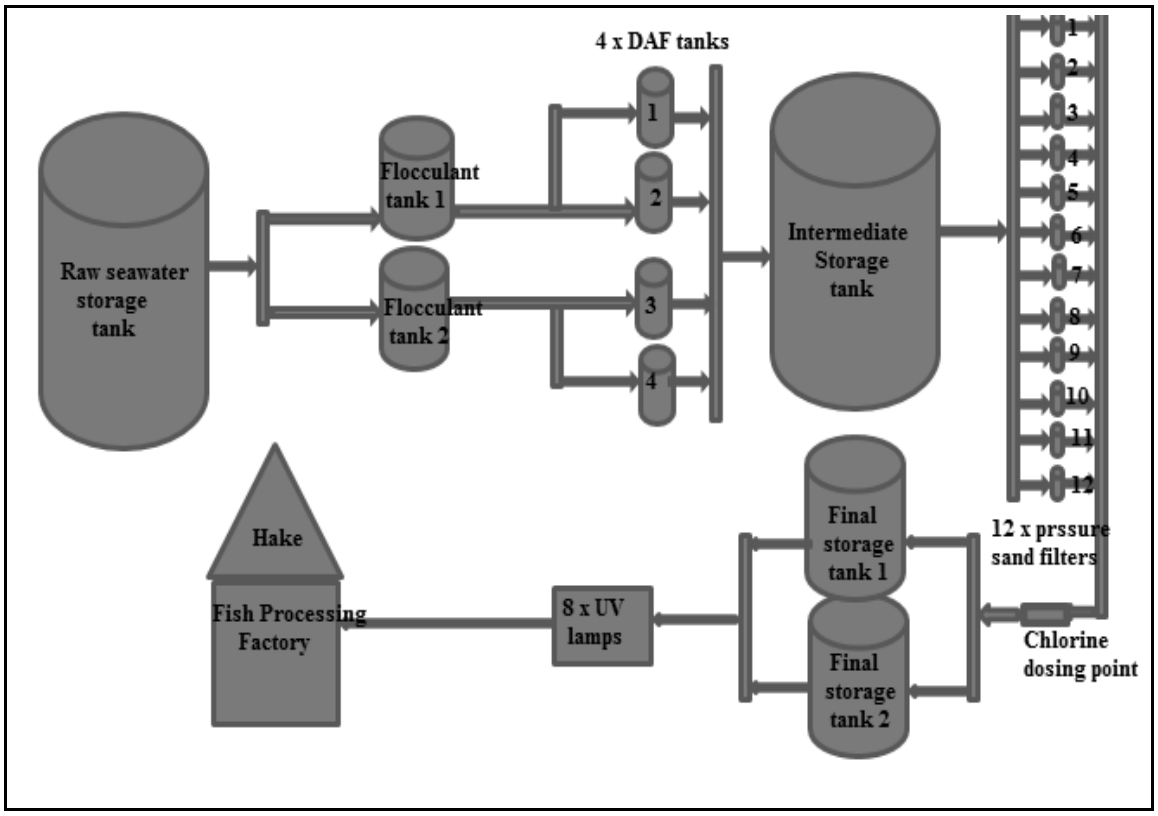



**Figure 2.6.** Typical seawater treatment and chlorine disinfection plant at factory B in Namibia

2.6.1. The use of chlorine gas in seawater disinfection

Shikongo-Nambabi *et al*. (2010), demonstrated that bacteria persisted in seawater distribution systems in spite of the use of chlorine and ultra violet light as disinfectants. Chlorine is defined as an element that belonged to halogen family. It is characterized by its high solubility in water, bactericidal effect and its capability to react and form compounds in nature (Wallis-Lage, 2010). The reduced chlorine biocide activity in

seawater is due to its ability to rapidly react with bromide ions to form hypobromite detectable as the major species of residual chlorine as well as total trihalomethanes (TTHMs) and other different constituents in the water to which it is dosed, hence, continuous reduction in the residual free chlorine prevails (Wong and Davidson, 1977; Goldman *et al*., 1978; Kutty *et al*., 1995; Hofstetter and von Gunten, 2007; Cesey *et al*., 2012). In the case whereby reduced bromide chloroform ions ($Br^-$) are of minimal concentrations in the systems, extensive concentrations of TTHMs increases (Kutty *et al*., 1995).

Furthermore, higher chlorine concentration dosage in water systems is associated with generation of other hazardous and possible carcinogenic by-products (CBP) and toxicity of total residual chlorine (TRC) (*Assessment of the water quality implications of phased implementation of HATS stage 2*, accessed 17.03.2014; Gibson and Laha, 1999; Cedegren *et al.*, 2002; Sadiq and Rodrigues, 2004 cited by Da Pazzo *et al*., 2008).Thus, it is suggested that special attention should be taken into consideration when handling chlorine during disinfection of any type of water.

Liquid chlorine causes irritation and severe injury to eye and skin when brought into contact with the aforementioned surfaces; however, the impact could be very diverse if ingested in any possible way. The Chlorine gas is accountable for triggering irritation of the following organs; mucous membranes, respiratory system as well as to the eyes. The concentration of chlorine regulates the severity of impartment. The Chlorine Institute (CI) recommends medical surveillance of all personnel who becomes exposed to

chlorine concentration higher than the American Conference of Governmental Industrial Hygienist (ACGIH) standard concentration of 0.5 ppm during production, use or handling. Recommendations apply to 1ppm for short term exposure limit (Wallis-Lage Cindy, 2010).

According to Penru *et al*. (2012), for the past thirty years, researchers around the world are working around the clock to develop effective disinfection technologies for marine waters (seawater and brackish water). Ever since 2004, international maritime organization (IMO) convention, implemented a law to manage and regulate the disinfection of ballast water and sediments, that is mandatory for the prevention of spread of harmful aquatic organisms from region to region (IMO, 2004, cited by Penru *et al*., 2012).

2.6.2. The effect of hydrogen peroxide ($H_2O_2$) on biofilms during seawater disinfection

Biofilms are a protected state of microbial growth during which micro-organisms are able to survive in hostile environments due to their physiological and behavioral differences from their planktonic counterparts (Simoes *et al*., 2009). Bacteria biofilms are formed in both sea – and fresh water distribution systems. Emtiazi *et al*. (2003), found that in fresh water distribution systems biofilms developed within 1-2 m distance after UV light disinfection. This method (UV irradiation) did not have an effect on planktonic bacteria in municipal drinking water resulting in occasional detection of opportunistic pathogens such as *Mycobacteria*, *Pseudomonas aeroginosa* and *Legionella* species The sensitivity of a wide range of bacteria to disinfectants decreases after

nutrient limitation, thus biofilm cells are less susceptible to disinfectants than planktonic cells (Pyle and Mcfeters, 1989; Stewart and Olson, 1992a). A representative example of biofilm formation is shown in Figure 2.7, below.

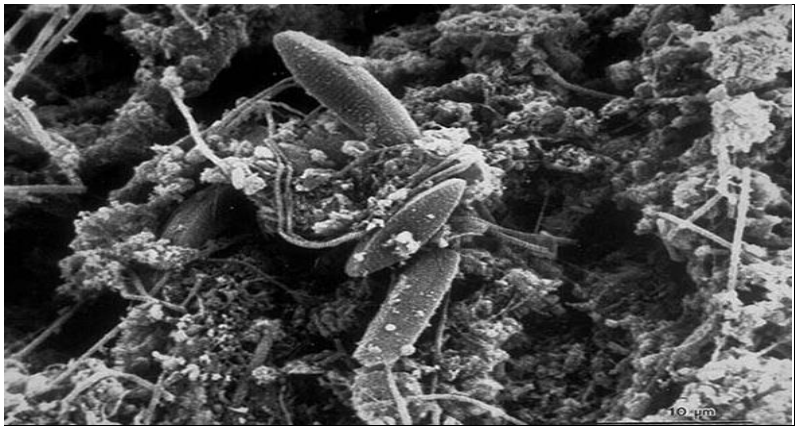



**Figure 2.7** Scanning electron micrograph of a natural biofilm that developed on a mild steel coupon (Source: Donlan, 2000, Licenced for use, American Society for microbiology microbes library. Available from URL:http://www.microbelibrary.org/)

In 1818, hydrogen peroxide ($H_2O_2$) was discovered by Louis Thenard (Thenard, 1818, cited by Penru *et al*., 2012). Its practice as a disinfectant was first projected by B.W. Richardson in 1891 (Richardson, 1891). For the past thirty years, researchers have been putting exertions to develop technologies for marine water disinfections (Penru *et al*., 2012).

Hydrogen peroxide ($H_2O_2$) is considered as one of the most environmentally friendly disinfectant. During its chemical reaction in water, it dissociates into oxygen ($O_2$) and water ($H_2O$), free of harmful compounds and producing maximum oxidation of organic matter on its path (Da Pozzo *et al*., 2008; Linley *et al*., 2012). Another advantage of hydrogen peroxide as compared to other disinfectants is the fact that it could be used at high concentrations without undesirably affecting the product quality. Kim *et al*. (2000), cited by Shikongo-Nambabi *et al*. (2010), investigated 0.7% (7000 mg/L) concentration of $H_2O_2$ to effectively reduce bacteria counts on catfish fillets, and found no significant difference in characteristics (appearance, colour, and odour scores) between controls and $H_2O_2$ treated fillets.

Yanong (2008), established that $H_2O_2$ is regarded as highly reactive, strong oxidizing and decolorizing (whitening) agent that is classified as corrosive at concentrations greater than 20%. The degradation of hydrogen peroxide is speeded up by light, heat as well as higher pH. Tort *et al*. (2003), cited by Yanong (2008), Carried out an experiment at different temperatures (15℃ and 20℃), to study the rate of degradation of hydrogen peroxide in fish rearing tanks, by dosing 10 and 100 mg/L in separate aerated water tanks containing organic matter and found that the concentrations were not measurable after 2-3 days, when water conditions were changed to stagnant with no aeration or organic matter. The concentrations were halved on the sixth day and undetectable by day 10.

Hydrogen peroxide is broadly applied as a biocide, destroying wholly living cells in its path (Da Pozzo *et al*., 2008; Linley *et al*., 2012). Linley *et al*. (2012), explained that predominantly, the application of $H_2O_2$ is required where its decomposition into non-toxic by-products is significant. It was further expounded that regardless of increasing information about its biocidal efficacy, there is limited understanding with respect to its prominent biocidal mechanism of action. Linley *et al*. (2012), reported that hydrogen peroxide extinguishes living cells by producing increased oxidation of their genetic materials (DNA), proteins and membrane lipids. Furthermore, due to the broad spectrum activity of $H_2O_2$, its application is increasingly used in medical, food and water treatment industries. Hydrogen peroxide is also used during water disinfection in aquaculture practices (OIE, 2009). Generally, Seawater contains a large quantity of pathogens including *V. alginolyticus*. Shikongo-Nambabi *et al*. (2010), reported that seawater used during processing is implicated as one of the origins of contamination to fish and fishery products. Furthermore, it was stated that the number of mesophilic and sucrose fermenting *Vibrio* species increased after filleting along the processing line. It is presumed that, this contamination comes from biofilms (adhesion of individual cells to solid surface) existing within the seawater distribution systems (Shikongo-Nambabi *et al*., 2010).

The physico-chemical properties of biofilms protect bacterial cells against dehydration, cleaning and disinfecting agents, subsequently surfaces with continuous exposure to seawater such as water tanks, pipes, and cooling towers may contain extreme forms of

biofilms (Tompkin, 2002). Total initiation of new biofilms and inhibition of matured biofilms generated by strains of *V. alginolyticus* were achieved at 0.05% (500 mg/L) and 0.2% (2 000 mg/L) of hydrogen peroxide respectively (Shikongo-Nambabi *et al*., 2010). In 2007, the American Food and Drug Administration approved 35% PEROX-AID® (Eka Chemicals, Marrieta, Georgia) product, which is available at 35% strength to be used in aquaculture, to control the mortality of reared fish (Yanong, 2008).

Da Pozzo *et al*. (2008), compared seawater disinfection efficacy between commercial hydrogen peroxide ($H_2O_2$) and electro generated hydrogen peroxide with combined use of ferrous ions, using *Escherichia coli* and total coliforms as indicator bacteria for biological contamination and testing to measure Hydrogen peroxide concentrations reflectrometrically by means of Merk analytical tests. The researchers used 300 mg/L doses of commercial and electro generated hydrogen peroxide. The electro generated $H_2O_2$ removed 50% of total bacteria load after 30 mins, when only 90 mg/L of hydrogen peroxide was detected in the solution. Elimination of the entire bacterial load was attained after 200 mins, during which 500 mg/L was detected in the solution. In the case of commercial hydrogen peroxide, 50% of the bacterial load was eradicated after 2 hours of contact time. After 4 hours, 70% of bacteria disinfection was accomplished and 250 mg/L residual concentration of commercial hydrogen peroxide was detected.

However, there was no further improvement with increased contact time. It was further explained that the improved efficacy of the electro generated hydrogen peroxide even at the low concentrations as compared to commercial hydrogen peroxide is due to

simultaneous action of different radical species used during production mechanism. The results indicated powerful oxidation of organic matter in both cases that can be successfully utilized to treat seawater and waste water (Da Pozzo *et al*., 2008).

Originalities of exotic species in water bodies all over the world are being introduced through cargo shipments by ballast water (Eno, 1996; Gollasch and Leppakowski, 1999). The use of $H_2O_2$ for the ballast water disinfection was due to its environmental friendliness and due to the fact that it can be produced by electrochemically on board the vessel (Kuzirian *et al*., 2001). In 2004, the International Maritime Organization (IMO) convention for the management of ship's ballast waters and sediments, implemented a law for all ship's ballast water treatment, to regulate any possibility of spread of pathogenic aquatic organisms from one side of the world to another (IMO, 2004). Studies carried out by Marine Biological Laboratory in collaboration with Eltron Research (2011) showed a broad effectiveness of hydrogen peroxide to kill algae, fish, and invertebrates, planktonic and bacterial organisms. The dose was carried out at 5-7 mg/L and provided effective treatment within the first 200-500 mins of contact time.

# CHAPTER 3: Materials and Methods

Several seawater and fish samples were collected along the seawater distribution systems and fish processing lines respectively, from two fish processing factory A and B. This study aimed at validating hydrogen peroxide, a disinfectant that is specific for seawater disinfection, to optimize seafood safety and shelf life. Furthermore, this study is intended to make a positive contribution to the fishing industry in Namibia by determining whether *V. alginolyticus* strains isolated from the seawater and hake fish are of health concern to humans or not.

## 3.1. Research Design

Randomized experimental design was employed in sampling and collection of data. This enabled two or more sets of data to be probabilistically similar on the average (Millsap and Maydeu- Olivares, 2009). Due to inconsistent flow of work at the fish processing factories randomized experimental design was the most appropriate. Quantitative data were generated in the laboratory based on total viable colony count in seawater and in hake fish.

SPSS statistical package was used in data analysis to compare quantitative data from different stages of seawater supply. Average log values of counts and relative standard deviations were calculated using Micro soft excel. In this study, a one-way analysis of variance (ANOVA): F-Fest was used to calculate the mean, least

significant difference, and coefficient of variation to determine the significance in responses among treatments.

## 3.2. Methods

### 3.2.1. Sample collection

Two fish processing companies (A and B), situated along the coastline of Walvis Bay in Namibia, were used in this study by permitting sampling of raw and processed seawater, as well as hake products from their seawater treatment and fish processing plants respectively. The names of the fishing companies could not be reported for commercial confidentiality reasons.

At the seawater and fish processing plants, seawater samples were collected aseptically from the following points: Raw seawater, a point after chlorine dosing, a point after UV treatment, a point at the pre-processing area (raw material cleansing area), at the baader (filleting), machine as well as at the Trio-machine (filleting machine).

### 3.2.2. Preparation and handling of water sample collection bottles

The sampling bottles were prepared according to the Namibian Standards Institution Microbiology standard operating procedure (SOP/M/05). Polyethylene sampling containers were properly cleaned using sulphate free soap (Extran); 0.5 ml of 10% Sodium thiosulphate solution (used during raw seawater and chlorine disinfected seawater sampling only) was added to each 500 ml empty bottle to achieve de-

chlorination. Sampling bottles were closed loosely and a piece of autoclave indicator tape was put on each bottle to indicate that the sampling bottles are sterile or the autoclave sterilization cycle has been completed. The bottles were marked with the autoclave batch number and sterilized in the calibrated (Hirayama) autoclave at 121℃ for 25 mins; autoclave temperature was monitored by a calibrated data logger. Upon completion of the autoclave cycle, bottles were removed and left to cool at room temperature. When the sampling bottles had reached the room temperature, the lids were tightly closed and covered with a brown paper and sealed with a rubber band. A broad masking tape was placed on the side of each sampling bottle to accommodate labelling. The sampling bottles were kept in a cupboard demarcated for sterile sampling bottles until used.

### 3.2.3. Collection of water samples

Sampling of water samples was carried out according to the Namibian Standards institution, Microbiology standard operating procedure for collection of water and ice samples for microbiological analyses (SOP/M/04). Sampling bottles were marked with sample identification name, date and time of collection. Metal water taps were disinfected by heating using a blow torch, others were disinfected by wiping the outside and inside (where possible) of the tap with absolute ethanol (99.9%).

The taps were opened and left to run for at least 3 mins to allow the water that was in the pipe to run out. The bottles' caps were removed carefully without touching

the inner surface of the cap and bottle neck with hands. The bottles were filled up to the neck leaving air space of at least 3cm and caps were replaced immediately. The samples were placed in the cooler box containing ice cubes for transportation to the laboratory. Upon arrival to the laboratory, samples were placed in the refrigerator calibrated and set at 5℃ until testing. Samples were analyzed within 6 h after collection.

### 3.2.4. Collection of fish samples along the processing line

The fish samples were collected based on the precautionary measures and sampling plans described by Midura and Bryant, (2001). Sterile, clear food sampling bags were used during fish sampling. Hands were cleaned and disinfected using hand sanitizer (purell) before sampling resumed and when they became contaminated. Latex gloves were used to decrease the possibility of contamination onto samples. The sample identities were labeled directly onto the sampling plastic bags using a permanent marker pen.

Two separate fish samples with average weight of 300 g were collected aseptically from each batch of fish along the processing line. The hake and hake products were collected from the following points along the processing line: raw material discharged from the fishing vessels (these hake fish samples have been de-headed and gutted at sea, however, they have not passed the pre-processing point) in the chiller room, raw materials cleansed with seawater at pre-processing point (these hake samples have been de-iced and graded at pre-processing point), after Baader

machine (filleted hake), after Trio-machine (skinned hake), unfrozen finished hake product as well as finished frozen hake products.

The samples were placed in the cooler box containing ice cubes for transportation to the laboratory. Upon arrival to the laboratory, samples were placed in the refrigerator calibrated and set at 5℃ until testing commenced. Samples were analyzed within 6 hours after collection.

The formula used to determine the sample size at 95% confidence interval is indicated in appendix table 3. A total of 184 seawater samples were randomly collected from both fish processing companies. The samples consisted of 24 raw seawater samples, 22 chlorinated seawater samples, 22 hydrogen peroxide treated seawater samples collected before the UV sanitizing point and 116 seawater samples collected beyond the UV treatment point for both chlorine and hydrogen peroxide seawater disinfectants. Seawater samples were collected from seawater distribution points along the fish processing line, as well as other points within the fish processing factories.

The total number of 196 fish samples were collected and analyzed from the hake fish processing line at both factories (A and B). The types and number of fish samples collected along the processing lines were as follows: 68 headed and gutted hake, 32 Skin on hake fish fillets (after baader machine), 32 skinless fillets (after trio-machine), 32 unfrozen hake final products (both skin on and skin off fillets, as

well as prime cuts) and 32 frozen products (skin on and skin off fillets, as well as prime cuts).


**Figure 3.1.** Chilled headed and gutted hake fish (a), skin on hake fillet (b), skinless hake fillet (c), and skinless hake prime cuts (d) semi-fished products.

3.2.5. Bacteria strains used in the study

Reference strain of *V. parahaemolyticus* strain RM11347B was provided by Namibian Standards Institution Microbiology Laboratory that was purchased from Health Protection Agency (UK) culture collection centres. *V. parahaemolyticus*

LMG 2850 and *V. alginolyticus* Bcc 892/LMG 4409 were kindly supplied by Prof SN Venter, Department of Microbiology and Plant Pathology, University of Pretoria.

### 3.3. Microbiological examination of water and fish samples

All water samples were analyzed for *V. alginolyticus* bacteria using TCBS and TSAT as growth media. Presumptive colonies were identified using the primary identification tests including, Oxidase test, Gram stain, and API 20E.

#### 3.3.1. *V. alginolyticus* quantification in water

Water samples were analyzed according to Kobayashi *et al*., (1963). Samples (100ml) were filtered through 0.2 µm synthetic membrane filters (Pall Corporation, Michigan, USA), mounted to the filter cup holder and connected to a vacuum pump. The membrane filter was incubated in the upright position on Thiosulfate-citrate-bile-salts-sucrose (TCBS) agar (Oxoid) and tryptose soya peptone triphenyltetrazolium chloride (TSAT) agar (made in house) for 24 h. *V. alginolyticus* colonies are flat, yellow and round on TCBS. Their size ranges between 4 to 6mm in diameter, after 24 h of incubation (ISO/TS 21872-2, 2007) On the TSAT agar, the colonies are white and occasional pink centres, smooth flat surface, 1-2 mm in diameter after 24h and 2-3 mm in diameter after 48h (Kourany, 1982). Presumptive *V. alginolyticus* colonies were counted, transferred to saline nutrient agar (SNA), screened for cytochrome oxidase enzyme and Gram stain

reaction. *Vibrio* species are oxidase positive and Gram negative (ISO/TS 21872-2, 2007).

### 3.3.2. *V. alginolyticus* quantification in fish

The fish sample analyses were carried out as per Namibian Standards Institution's microbiology test method (TM/M/06/) and ISO/TS 21872-2, (2007) for the analysis of *Vibrio* species in food, with few modifications. The sample enrichment was skipped, inoculation was carried out from the first sample dilution. For each fish sample (hake unprocessed and processed products) 25 grams were mixed with 225 ml of alkaline saline peptone water (ASPW) (Oxoid) in a stomacher bag to give 1:10 dilution, using forceps and scissors sterilised by dipping in 99% ethanol (flammable) and flaming. The mixture was crushed using the stomacher machine (Seward) at 240 rpm for 1 min. The sample mixture of 0.1 ml (Kaysner *et al*., 1992) was inoculated onto TCBS gar (Oxoid) and TSAT agar (Kourany, 1982) plates dried at 37℃ for 30 mins, for the selectivity of *Vibrio* species. The aliquots were spread over the agar using sterile plastic spreader rods. The plates were allowed to dry in an upright position at room temperature for 30 mins and incubated in an inverted position at 37℃ temperature for 24 h. All the cultures confirmed to be *V. alginolyticus* were preserved in 5 ml polyethylene sample vials containing 2 ml ASPW (24 h culture) supplemented with 25% glycerol in 1:1 ratio and stored at -80℃ until further use.

## 3.4. Biochemical Identification of bacterial isolates

Bacteria isolates were subjected to screening by oxidase test and Gram staining. The oxidase positive and Gram negative rods were identified to species level using API 20E test kits (BIOMERIEUX). The sample numbers were labeled on the elongated flap of the tray. Honey combed wells were filled with sterile deionized water, to create a humid atmosphere. Pure colonies from the saline nutrient agar (SNA) were inoculated into sterile 5 ml of 0.85% sodium chloride (NaCl) solution. The samples were mixed by vortexing and eppendorf pipette (1ml) was used to aseptically transfer the bacterial suspension to each of the wells, filling only up to the tube, immediately after preparation of the cell suspension. Caution was taken to avoid formation of bubbles at the base of the tube. Manufacturer's instructions for filling the cupules of the API 20E were followed. The lids were placed on the incubation boxes and incubated at 36℃ for 18-24 h. Manufacturer's instructions were used to read and record results on worksheets provided to obtain a reaction profile that was subsequently used to search for the organisms' species identity from the apiweb$^{TM}$ cd software (BIOMERIEUX). The results were printed out and attached to the worksheets.

### 3.5. Confirmation of bacterial species identity by Polymerase Chain Reaction and 16S rRNA and *rpoB* sequencing.

The total number of 120 bacteria isolates, identified by API 20E and preserved in 25% glycerol, from the Namibian Standards Institution in Walvis bay, were sub-cultured on saline nutrient agar (SNA) at 37℃ for 24 hours. Plates were sealed with parafilm and the cultures were further analysed at the University of Pretoria.

#### 3.5.1. DNA Extraction

Genomic DNA (G-DNA) was extracted using the guidelines instruction manual for monolayer cell DNA isolation, provided by Zymo Research kit, South Africa.

#### 3.5.2. 16S rRNA amplification and sequence determination

The 16S rRNA amplification was carried out according to Coenye *et al*. (1999) and the University of Pretoria, Department of Microbiology and Plant pathology's PCR amplification and sequence protocols. The PCR reactions were run as 25 µl (total PCR reaction volume) mixtures in each PCR tube per sample on the BioRad T100 thermo cycler. The following quantities were utilized to prepare the reaction mixtures per sample: 2.5 µl PCR buffer (10 X), 2.0 µl dNTP (2.5mM), 2.0 µl $MgCl_2$ (25 mM) , 0.5 µl Forward primer (pA) (10 mM), 0.5µl Reverse primer (pH) (10 mM) (see Table 3.1), 0.15 µl Taq polymerase (5U/µl, 16.85 µl nuclease free water (NF-$H_2O$) and 0.5 µl DNA. The primer references are presented in table 3.1.

The PCR amplification conditions were as follows: Initial denaturation at 94℃ for 10 min, followed by 30 cycles of denaturation at 94℃ for 1 min, primer annealing

at 60℃ for 1 min, primer extension at 72℃ for 1 min, with a final extension at 72℃ for 5 mins.

The verification of PCR products were carried out by gel electrophoresis in 0.1% agarose as described in procedure in the case when there was a positive band, the remaining 22.5 µl PCR product were refrigerated until carried over for sequencing PCR.

For sequencing PCR 0.5 µl Exonuclease (20U/µl) and 2 µl of FastAP (Alkaline Phosphatase) (1U/µl) were added to 22.5 µl of PCR products and were placed on a 37℃ heating block for 15 mins and then on the 85℃ heating block for 15 minutes. The addition of these enzymes were for cleaning purposes, before running the sequencing PCR.

Sequencing PCR was prepared in 12 µl mixture containing 1 µl of the 3.1 short dye, 2 µl sequencing buffer (5 X), 0.3 µl undiluted primer (*PD) (100 mM), 4.7 µl NF-$H_2O$ as well as 4 µl DNA.

The sequencing PCR was run at the following conditions: 96℃ denaturation for 5 seconds, followed by 25 cycles of denaturation at 96℃ for 10 seconds, annealing at 55℃ for 5 seconds, and extension at 60℃ for 4 mins. The sequencing PCR products were further precipitated and sent to the sequencing facility at the University of Pretoria. The precipitating procedures were as follows: 12 µl of the sequencing reactions were added to 16 µl 100% ethanol (EtOH) and 2 µl 3 M sodium acetate (pH 4.8) in sequencing Eppendorf tubes. The mixtures were centrifuged at 14000

rpm for 30 mins. The supernatant was removed carefully using a 1 000 µl automatic pipette leaving behind the invisible pellet. EtOH (300 µl) of 70% was added to the pellets for washing purposes and centrifuged at 14000 rpm for 5 mins. The supernatant was again removed carefully and this time replaced with new 150 µl of the 70% EtOH and centrifuged at 14000 rmp for 5 min. The eppendorf tubes were put on a heating block set at 90℃ for 3 mins, the heating block was switched on, 20min before the last step of precipitation. The eppendorf tubes containing the pellets were stored at -20℃ until sequenced.

3.5.3. The *rpoB* gene amplification and sequencing

In this study, the *rpoB* gene master mix was prepared according to the University of Pretoria, Department of Microbiology and Plant Pathology's PCR amplification and sequence protocols for the 16S rRNA as well as per PCR amplifications and sequencing methods with modifications used by Hazen *et al*. (2009a) and Tarr *et al*. (2007). The PCR reactions were prepared as 25 µl mix in each PCR tube per sample on the BioRad T100 thermal Cycler. The amplification PCR reaction mixtures were prepared using 2.5 µl PCR buffer (10 X), 2.0 µl dNTP (2.5mM), 2.0 µl $MgCl_2$ (25mM), 0.5 µl Forward primer (rpoB1110F) (10mM), 0.5 µl Reverse primer (CM32b) (10mM) (Table 3.1), 0.15 µl Taq polymerase (5U/µl), 16.85 µl nuclease free water (NF-$H_2O$) and genomic DNA (0.5 µl).

The PCR amplification conditions were as follows: DNA initial denaturation at 94℃ for 3 min, followed by 25 cycles of denaturation at 94℃ for 1min, annealing at 55℃ for 1 min, and primer extension at 72℃ for 2min, with a final extension at 72℃ for 5 mins.

Furthermore, the PCR products were cleaned as described above under 3.2.8.2 and sequenced as described below.

The 12 µl sequencing PCR reaction mixture contained the following: 1 µl of the 3.1 short dye, 2 µl sequencing buffer (10 X), 0.3 µl of undiluted rpoB1110F forward primer (100 mM), 4.7 µl NF-$H_2O$ as well as 4 µl DNA.

The sequencing PCR was run at the following conditions: 94℃ initial denaturation for 1min, followed by 30 cycles of denaturation at 94℃ for 15 seconds, annealing at 55℃ for 5 seconds, and extension at 60℃ for 4 mins. The sequencing PCR products were further precipitated as described above under 3.8 and sent to the sequencing facility at University of Pretoria. The eppendorf tubes containing the pellets were stored at -20℃ until sequenced.

### 3.5.4. Phylogenetic analysis

The phylogenetic trees were constructed according to a tutorial review (Baldauf 2003). The 16S rRNA and *rpoB* sequence data sets were edited and assembled using the CLC Main Workbench (CLC bio QIAGEN). Multiple sequence alignments including homology obtained from the NCBI's BLAST search engine

(http://www.ncbi.nlm.nih.gov/) were aligned and trimmed using the MAFFT (Katoh *et al*., 2002) internet program. The 16S rRNA and *rpoB* sequence data sets were aligned independently of one another. The quality check of sequences was carried out to delete gaps and trimmed the overhangs. The sequences were exported in fasta file formats to notepad and saved. Aligned sequences were saved as fasta files and opened in Mega5 (Tamura *et al*., 2007) software in mas format files. The following steps were followed in Mega5 to draw the phylogenetic tree: Phylogeny, constract neibour joining tree, bacteria plasmid, test of the phylogeny (bootstrap) and the tree was computed. The trees were only computed to see how many strains of *Vibrio alginolyticus* from Namibian marine water and hake fish are evolutionally related by determining the number of clusters formed.

3.5.5. PCR analysis of virulence related genes

The PCR presence/absence analyses for virulence related genes were performed with DNA extracts from the isolates identified as *V. alginolyticus* according to *rpoB* sequencing results. For every different primer set (Table 3.1) used, the methods were used with few modifications: specific PCR for *tlh* was done according to Oberbeckman *et al*., (2011) and Taniguchi, *et al*. (1985, 1986), *toxR* (Oberbeckman *et al*., 2011; Bauer and Rorvik, 2007), *tdh* (Oberbeckman *et al*., 2011; Nishibuchi and Kaper, 1985). The following strains were used as positive controls: *V. parahaemolyticus* LMG 2850 was used as a positive control for *tlh* and *toxR* (Pang

*et al*., 2006)*;* while *V. alginolyticus* Bcc 892 was used as a positive control for the *tdh* gene (Luan *et al*., 2006).

The PCR reactions were run as 25 µl mix on the BioRad T100 thermal Cycler as per UPDMP Laboratory protocol described in 3.5.2. The master mix compositions were prepared in the same way as indicated above in 3.5.2.

The PCR amplification conditions for the virulence related genes were as follows: *tlh*, DNA initial denaturation was carried out at 94℃ for 3min, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min, and extension at 72℃ for 2 min, with a final extension at 72℃ for 7 mins.

*toxR:* DNA Initial denaturation was carried out at 95℃ for 4min, followed by 25 cycles of denaturation at 95℃ for 1 min, primer annealing at 55℃ for 1 min, and primer extension at 68℃ for 1 min, with a final extension at 68℃ for 7 mins.

For *tdh*' DNA initial denaturation was carried out at 94℃ for 3 min, followed by 25 cycles of denaturation at 94℃ for 1 min, primer annealing at 58℃ for 1 min, and primer extension at 68℃ for 2 min, with a final extension at 68℃ for 5 mins.

### 3.6. Verification of PCR products

Visualization of gene and gene fragments was carried out by gel electrophoresis. The 0.1% agarose gel (Sea Kem® LE Agarose) was run at 100 volts for 60 mins using a 300 V/400 mA/75 W Bio-RAD power pac. The DNAs and 0.1 µg/µl 50 µg

DNA ladder (Thermo Scientific) were stained with GX Orange DNA loading dye (Thermo Scientific) and visualized using the Gel Doc EZ Imager (Bio-rad).

**Table 3.1.** Primer sets, primer sequences, target genes fragment sizes and references

| Primer | Sequence (5'-3') | Target gene | Fragment (bp) | Reference |
|---|---|---|---|---|
| pA | AGAGTTTGATCCTGGCTGAG | 16S rRNA | - | Coenye *et al*. (1999) |
| pH | AAGGAGGTGATCCAGCCGCA | 16S rRNA | - | Coenye *et al*. (1999) |
| *PD | CAGCAGCCGCGGTAATAC | 16S rRNA | - | Coenye *et al*. (1999) |
| rpoB1110F | GTAGAAATCTACCGCATGATG | *ropB* | 1600 | Tarr *et al*. (2007) |
| CM32b | CGGAACGGCCTGACGTTGCAT | *rpoB* | 1089 | Tarr *et al* (2007) |
| tl-L | AAAGCGGATTATGCAGAAGCACTG | *tlh* | 450 | Tanigushi *et al*. (1985 and |
| tl-R | GCTACTTTCTAGCATTTTCTCTGC | *tlh* | 450 | 1986) |
| UtoxF | GASTTTGTTTGGCGYGARCAAGGTT | *toxR* | 300 | Tanigushi *et al*. (1985 and |
| vptoxR | GGTTCAACGATTGCGTCAGAAG | *toxR* | 300 | 1986) |
| tdh-L | GTAAAGGTCTCTGACTTTTGGAC | *tdh* | 270 | Bauer and Rorvik (2007) |
| tdh-R | TGGAATAGAACCTTCATCTTCACC | *tdh* | 270 | Bauer and Rorvik (2007) |
| | | | | Nishibuchi and Kasper (1985) |
| | | | | Nishibuchi and Kasper (1985) |

## 3.7. Seawater Disinfection

The experiment was carried out at the seawater treatment plant for fish processing factory B. Sampling of seawater was carried out prior to and after treatment with chlorine and hydrogen peroxide. Two tanks (tank 1 and 2) each with the volume of 100 $m^3$ were used in this study, whereby one tank was disinfected with either chlorine or $H_2O_2$. One tank (tank 1) was filled with seawater treated with chlorine gas (automated dosage) according to the usual procedure for seawater disinfection

used at fish processing factory B. Tank 2 was emptied of all chorine treated Seawater, and filled to a sixth with sand filtered seawater. Thereafter 143 L of 35% Food grade $H_2O_2$ was added and the tank was finally filled with sand filtered seawater to give (0.05%) final concentration of hydrogen peroxide ($H_2O_2$), previously proven effective to eliminate bacterial growth (Shikongo-Nambabi *et al*., 2010). The disinfectant ($H_2O_2$) was left to act for 4 hours before the first set of samples was taken after treatment. The concentration of hydrogen peroxide was measured at each sampling point by the use of hydrogen peroxide test strips known as quantofix (Macherey-nagel, Germany). The sampling for both types of treated seawater ($Cl_2$ and $H_2O_2$) were carried out in triplicates for each investigational point. The formula used in calculating the concentration of hydrogen peroxide is indicated on the appendix Table 8. The water samples from tanks were analysed for total viable *V. alginolyticus* counts and *Vibrio* like species as described in procedure 3.3.1 above. The bacterial counts obtained from the two tanks were compared.

# CHAPTER 4: RESULTS

## 4.1. Detection of *V. alginolyticus* on TCBS, TSAT and Biochemical analyses

The colony morphologies for *V. alginolyticus* on TCBS media were described as follows; smooth and round, yellow in colour (sucrose positive) and 2 mm to 3mm in diameter (Figure 4.1). Colonies showing a typical *V. alginolyticus* appearance according to shape, color and size were detected on both TCBS and TSAT. However colonies with smaller sizes were also observed. Apart from small colonies (Figure 4.1), there was no definite dissimilarities in colony appearance on TCBS agar between *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* species. Primary identification tests showed that all isolates were Gram negative and oxidase positive, except *Klebsiella* species (Gram negative and oxidase negative), hence belonging to the family *Vibrionaceae*, *Shiwanellaceae* and *Aeromonaceae*.

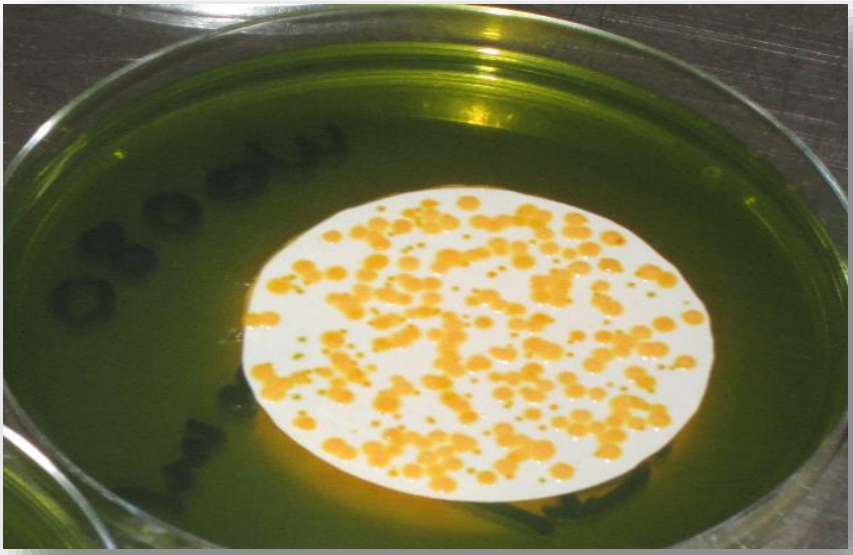
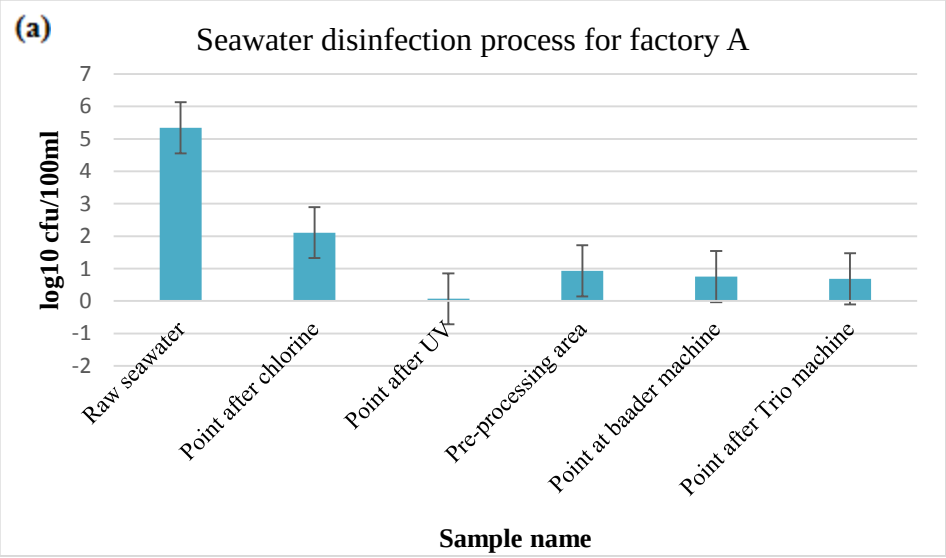


**Figure 4.1.** The colony appearance and morphology of *V. alginolyticus* (yellow) on TCBS medium. Similar characteristics have been assigned to *Aeromonas*, *Shiwanella* and *Klebsiella* species on TCBS.

### 4.2. Total counts of *V. alginolyticus* and *Vibrio* like species in seawater samples

The *Vibrio alginolyticus* and *Vibrio* like species culturable counts were determined for the raw and treated seawater along the seawater distribution systems in the Namibian fish processing factories (A and B), see Table: 4.1. The average total counts for the raw seawater at plant A and B were 2.1 $\times 10^5$ cfu/100 mℓ and 2.3 $\times 10^5$ cfu/100 mℓ respectively. Despite of seawater disinfection with chlorine gas, the bacteria counts persisted but drastically reduced to 129 cfu/100 ml and 116 cfu/100 ml, both at factory A and B respectively. The water leaving the plant after UV disinfection at factory A had average total counts for only 1 cfu/100 ml and no bacteria count on TCBS was detected for factory B.

Inside the fish processing factory A and B, the number of *V. alginolyticus* and *Vibrio* like species slightly increased at the pre-processing areas with average total counts of 9 cfu/100ml (factory A), 6 cfu/100 ml ( factory B, pre-processing area 1), 6 cfu/100 ml ( factory B, pre-processing area 2), 3 cfu/100 ml at the de-icing point (factory B), 6 cfu/100 ml baader machine (factory A) and 5 cfu/100 ml at trio-machine (factory A) along the fish processing line. At the new line 6 and final product area, total average counts were 2 cfu/100 ml and zero (factory B) respectively (Figure 4.2(a) and (b)).



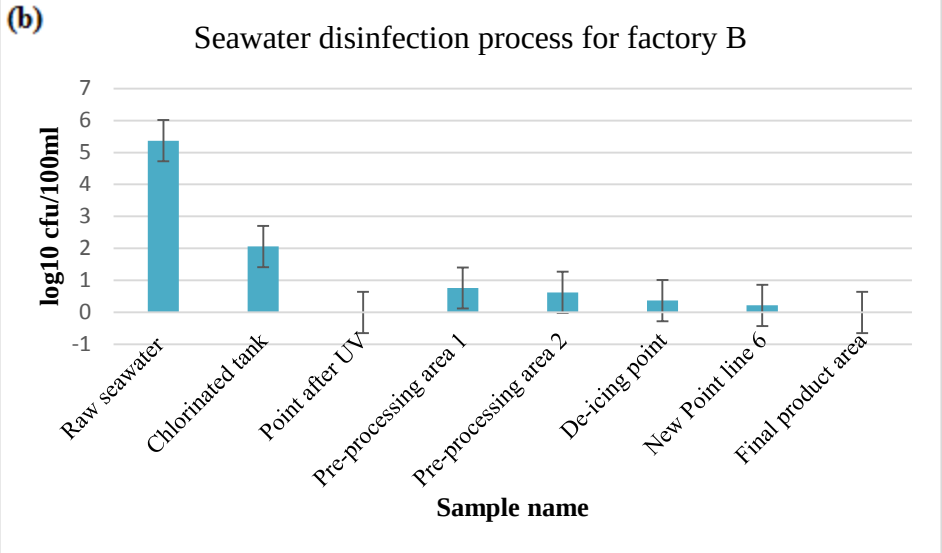


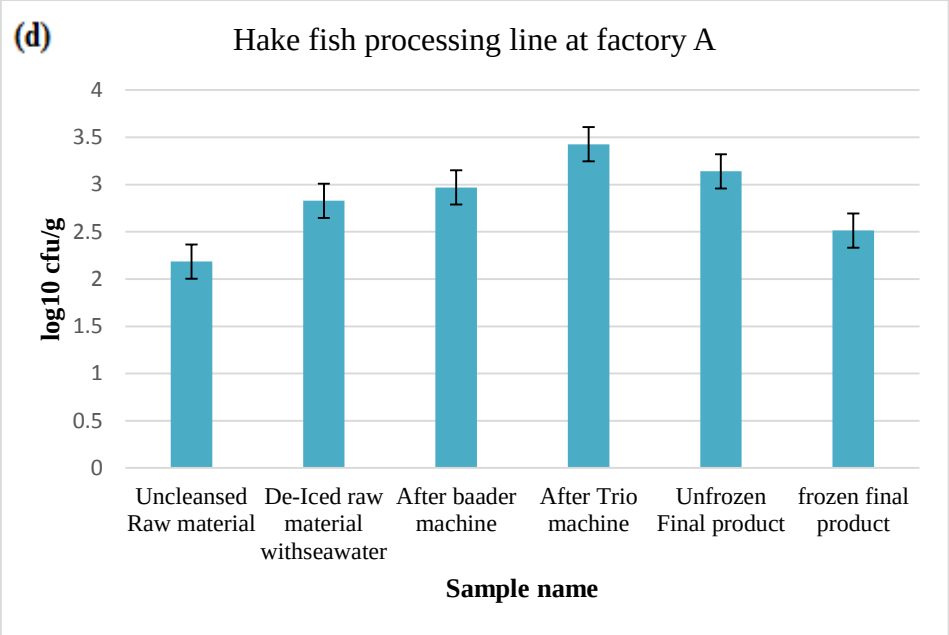

**Figure 4.2.** Total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* counts on TCBS, samples taken at points along the chlorine ($Cl_2$) disinfected seawater distribution system. Chart (a) and (b) represent fish processing factory A and B respectively.

### 4.3. *V. alginolyticus* and *Vibrio* like species quantification in hake fish

Fish processing factory A made use of seawater throughout its entire processing operation. Whereas, Fish processing factory B made use of seawater from the pre-processing point until de-icing point along the fish processing line. Further than that, factory B uses fresh water at the baader and trio-machines. However, seawater is used to clean the entire factory floors, conveyor belts and fish basins.

The *Vibrio alginolyticus* and *Vibrio* like species culturable counts on TCBS were determined on hake fish raw material and hake fish products along the processing lines in the Namibian fish processing factories (A and B), see table 4.2 below. The untreated

hake (H and G) contained least bacterial counts of 153 cfu/g at fish factory A and 0 cfu/g at factory B. However, when the hake were exposed to chlorine disinfected seawater for the pre-treatment (de-icing of raw material) purposes, the bacteria counts raised to 673 cfu/g at factory A and remained at 0 cfu/g at factory B. The bacterial counts in fish products escalated to 930 cfu/g (factory A) and 117 cfu/g (factory B) after the baader machine. The bacteria counts were highest (2 663 cfu/g at factory A and 492cfu/g at factory B) immediately after the trio-machine, when the skins were removed and fillets' flesh exposed to seawater (chlorinated). At the fish processing factory A by the packaging stations, the total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* counts were lower for the unfrozen and frozen hake products that showed 1 379 cfu/g and 327 cfu/g respectively. However, bacterial counts in the unfrozen and frozen hake fish products at similar packaging stations at fish factory B, increased and decreased to 533 cfu/g and 492 cfu/g respectively. These trends are depicted in Figure 4.3 (d) and (e).



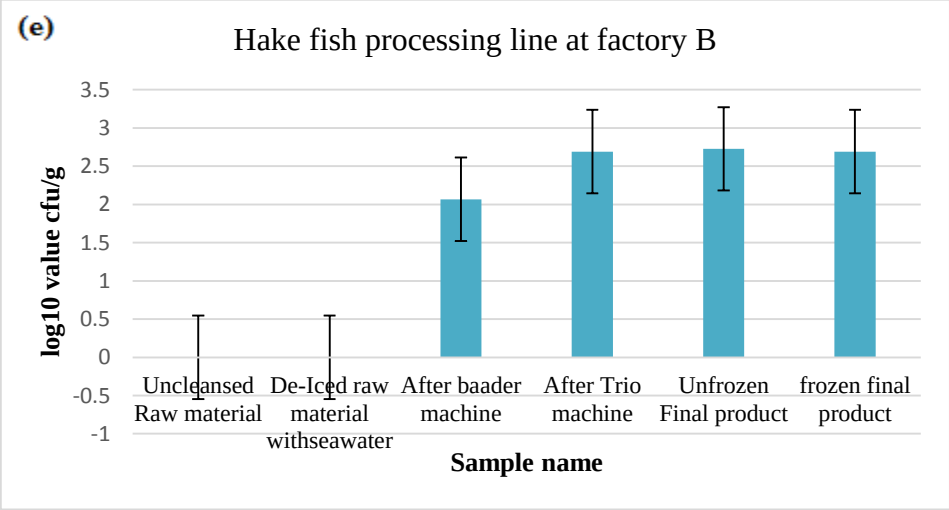




**Figure 4.3.** Total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* species counts on TCBS, isolated from hake fish, sampled along the Hake fish processing line for (d) fish processing factory A and (e) fish processing factory B.

### 4.4. Oxidase test

Bacteria isolates on TCBS were streaked onto Saline Nutrient Agar and subjected to oxidase test, to screen out unnecessary bacteria isolates and to save cost on API 20E. Two colonies were selected and tested from each sample that contained growth on TCBS. The total number of 226 bacteria isolates in seawater were counted and recorded as oxidase positive and Gram negative. Moreover, a total of 414 bacteria isolates in fish samples tested oxidase positive and Gram negative.

### 4.5. Biochemical test (API 20E)

Biochemical test profiles representing 127 selected *V. alginolyticus* strains were divided into 7 distinct groups (table 4.5) according to their biochemical percentage identity. *V. alginolyticus* LMG 4492 was used as a positive control for the biochemical tests. The API 20E results indicated that all strains were negative for the following biochemical, ortho-Nitrophenyl-β-galactoside (ONPG), alcohol dehydrogenase (ADH), CIT, sulphide ($H_2O$), tryptophan deaminase (TDA), Voges-Proskauer (VP), SOR, PHA, melibiose (MEL) and arabinose (ARA). All strains gave positive reaction to INDOLE hydrolysis and fermentation of glucose (GLUC), mannitol (MAN) and sacurose (SAC). All strains were oxidase (OXID) positive. The percentage identity differences (Table 4.5) were caused by differences in lysine decarboxylase (LDC), ornithine decarboxylase (ODC), UREA, gelatin (GEL) hydrolysis, INO and amygdalin (AMY) fermentation. The two biochemical profiles which contained a large number of strains were *V.alginolyticus* 97.7% and *V.alginolyticus* 98.6%. Their differences in percentage identity were due to ODC metabolism. Isolates 119, 78.1, 67.1, 66.1, 64.1 and 62.2, varied significantly from the other *V. alginolyticus* strains as they were able to break down UREA. Whereas 123,115, 80, 82, 85.1, 50.2, 51.2, 51.1 52.1, 92, 90 strains were unable to utilize GEL.

**Table 4.1.** API 20E Biochemical profiles for *V. alginolyticus* species isolated from seawater and Hake fish samples

| Identity Profile | *V.alginolyticus* 97.7% | *V.alginolyticus* 99.5% | *V.alginolyticus* 99.7% | *V.alginolyticus* 98.6% | *V.alginolyticus* 99.8% | *V.alginolyticus* 96.2% | *V. alginolyticus* 80.1% |
|---|---|---|---|---|---|---|---|
| ONPG | - | - | - | - | - | - | - |
| ADH | - | - | - | - | - | - | - |
| LDC | + | + | + | + | + | - | + |
| ODC | + | - | - | - | + | + | - |
| CIT | - | - | - | - | - | - | - |
| $H_2S$ | - | - | - | - | - | - | - |
| UREA | - | - | + | - | - | - | - |
| TDA | - | - | - | - | - | - | - |
| INDOLE | + | + | + | + | + | + | + |
| VP | - | - | - | - | - | - | - |
| GEL | + | - | - | + | - | + | + |
| GLUCOSE | + | + | + | + | + | + | + |
| MAN | + | + | + | + | + | + | + |
| INO | - | - | - | - | - | - | + |
| SOR | - | - | - | - | - | - | - |
| PHA | - | - | - | - | - | - | - |
| SACUROSE | + | + | + | + | + | + | + |
| MEL | - | - | - | - | - | - | - |
| AMY | + | + | + | + | - | + | + |
| ARA | - | - | - | - | - | - | - |
| OXIDASE | + | + | + | + | + | + | + |
| Strains | 125,117,116, 87,107, 108, 105, 104, 110, 112.2, 113, 76.1,73.1,73.2, 72.2, 72.1,71.1, 70.1,70.2, 52.2, 53.1, 53.2, 54.2, 54.1, 55.1,55.2, 57.1, 57.2, 58.1, 58.2, 69.2,69.1, 67.2, 64.2, 63.1, 234, 215, 219, 222, 224, 240, 241, 192,195, 197,169, 172, 173, 174, 176, 178, 179, 180, 376.1 Bcc892 | 123,115, 80, 82,85.1, 50.2, 51.2, 51.1 52.1, 92, 90 | 119, 78.1, 67.1, 66.1, 64.1, 62.2, 91, | 113,86,88,89, 79.1,79.2, 79.3, 78.1, 78.2, 77.2,77.1, 76.2, 75.2, 75.1, 74.2,74.1, 50.1, 56.1, 56.2, 65.1, 63.2, 60.1,62.1,61.2, 61.1, 60.2, 95, 94, 93, 96, 262, 235, 236, 237, 231,232, 216, 218, 220, 221, 223,239, 242, 243, 193, 194,196, 198, 170, 171,175, 177, | 111 | 70.2 | 65.2 |
| Total | 54 | 11 | 7 | 52 | 1 | 1 | 1 |

## 4.6. Confirmation of bacteria species identity by Polymerase Chain Reaction

The following molecular methods were used to further identify bacteria strains isolated from seawater and hake fish in Namibia.

### 4.6.1. 16S rRNA amplification and sequence determination

A total number of 99 isolates identified by biochemical method (API 20E) as being potential *Vibrio* species and those identified as *Aeromonas* were subjected to 16S rRNA amplification and sequencing, 78.8% (78) isolates were identified as *Vibrio* species, 20% (20) isolates were identified as *Aeromonas*, and 1.0% (1) isolate belonged to *Shewanella* group. Out of 78 *Vibrio* isolates, 75.6% (59) isolates were identified as *V. alginolyticus* species, 24.4% (19) isolates belonged to other *Vibrio* species, see pie chart, Figure 4.7.

The following strains 51.1, 272.1, 273, 65.2, 66.1, 68.1, 68.2, 74.1, 112.2, 123 and 314 were initially identified as *V. alginolyticus strains* by API 20E, however the 16S rRNA, identified them as *V. natriegens* (51.1), *V. anguillarum* (272.1; 273; 314), *V. parahaemolyticus* (65.2; 66.1) *V. metschnikovii* (68.1; 68.2) *Shewanella algae* (74.1) and *V. harveyi* (112.2). When strain 51.1 was analysed further by *rpoB* sequence, it was however identified as *Vibrio cincinnatiensis*. The strain 68.1 was identified as *V. metschnikovii,* and it was not included in the dendrogram based on the 16S rRNA sequences. The dendrogram was constructed for *V. alginolyticus* strains only. The *V. parahaemolyticus* LMG 2850 was used as a positive control that yielded a ~500 bp. The blank sample yielded no positive band and all 99 isolates produced positive bands for

the 16S rRNA amplification. The molecular weight marker of the ladder signified 100 bp (Figure 4.5).

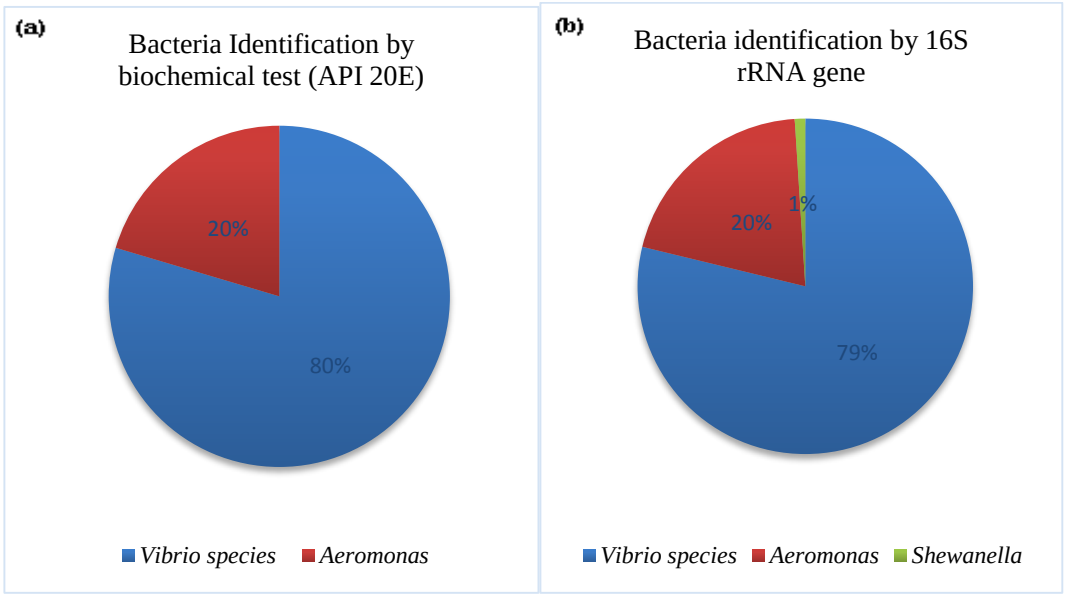



**Figure 4.4.** The percentage (%) species identified by (a) biochemical tests (API 20E) and (b) molecular tests (16S rRNA).

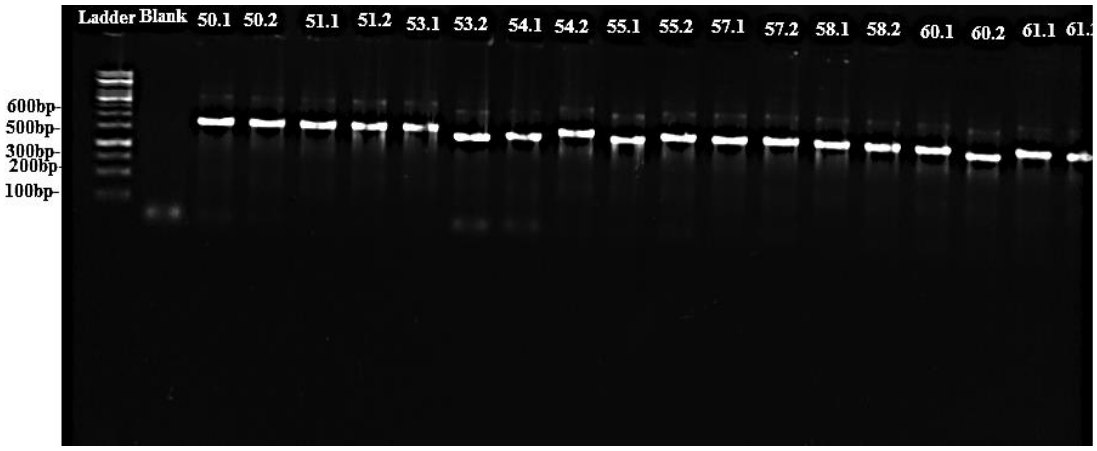



**Figure 4.5.** Agarose gel electrophoresis for the 16S rRNA amplicons from 78 *Vibrio* isolates and 21 other bacterial species. The LMG 2850 and Bcc 892, were also included.

4.6.2. Phylogenetic analysis of the 16S rRNA

Roughly 500 bp of the 16S rRNA sequences of the 59 *V. alginolyticus* strains, 2 control cultures (LMG 2850 and Bcc 892), outside group (51.1) were aligned using MAFFT program. The neighbor-joining tree was computed including 20 closely related 16S rRNA from GenBank (Figure 4.9). According to the phylogenetic tree, all the *V. alginolyticus* strains from seawater and hake fish products were identical to some known strains in the GenBank database based on 16S rRNA sequences. The tree was differentiated into five distinct groups. The 58 *V. alginolyticus* strains were assigned to one group including LMG 2850 reference strain as well as 18 known strains from the genbank database. The outside group (51.1), the reference strain Bcc892 and strain 73.1 were each grouped separately. The fourth group contained other 2 GenBank strains. Three groups contained the bootstrap values of less than 50%, which means their node

was found in less than half of the bootstrap replicates and were not reliable groupings. The group containing 2 GenBank strains was supported with high bootstrap value greater than 50%.

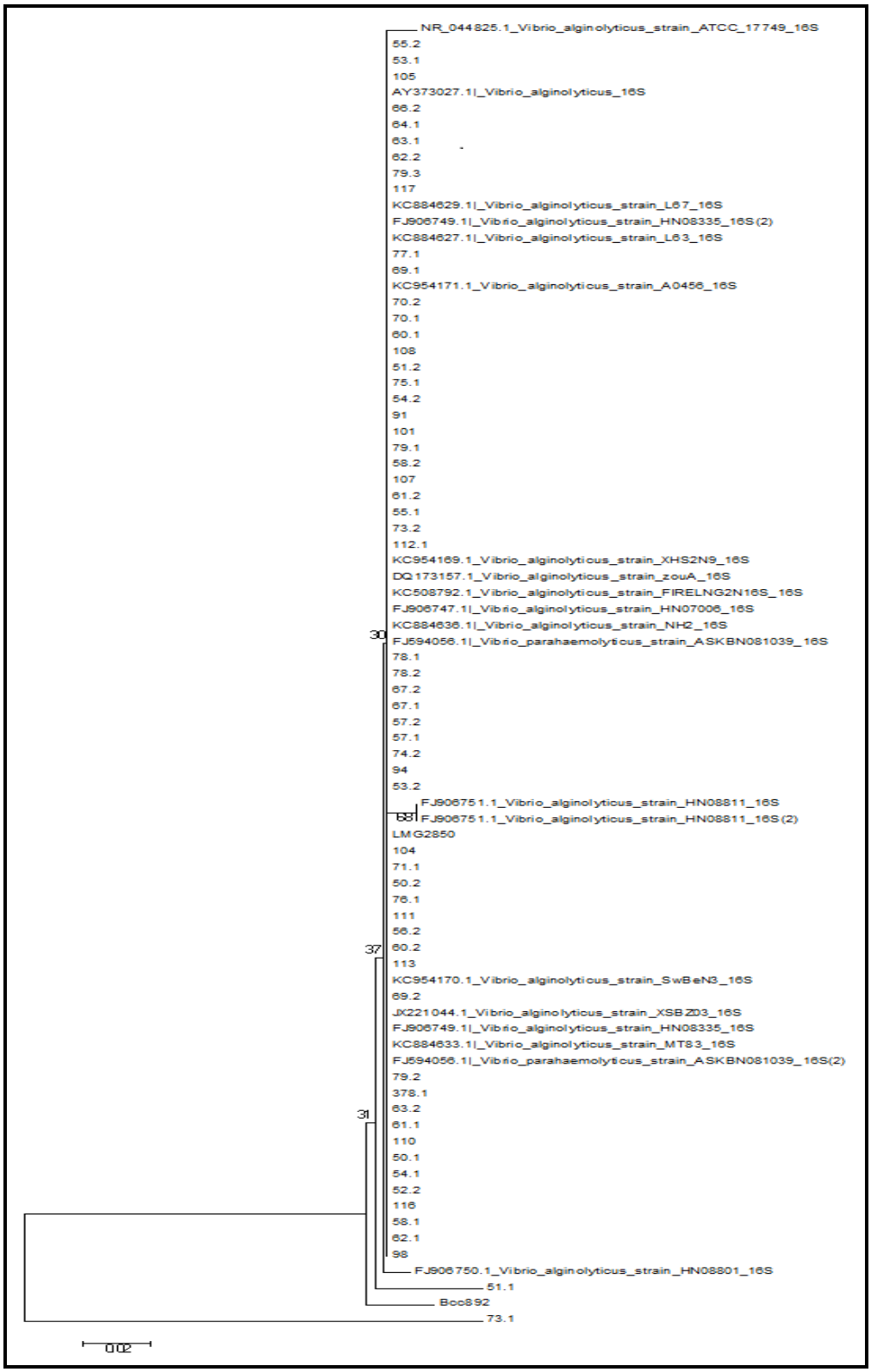

NR_044825.1_Vibrio_alginolyticus_strain_ATCC_17749_16S
55.2
53.1
105
AY373027.1|_Vibrio_alginolyticus_16S
66.2
64.1
63.1
62.2
79.3
117
KC884629.1|_Vibrio_alginolyticus_strain_L67_16S
FJ906749.1|_Vibrio_alginolyticus_strain_HN08335_16S(2)
KC884627.1|_Vibrio_alginolyticus_strain_L63_16S
77.1
69.1
KC954171.1_Vibrio_alginolyticus_strain_A0456_16S
70.2
70.1
60.1
108
51.2
75.1
54.2
91
101
79.1
58.2
107
61.2
55.1
73.2
112.1
KC954169.1_Vibrio_alginolyticus_strain_XHS2N9_16S
DQ173157.1_Vibrio_alginolyticus_strain_zouA_16S
KC508792.1_Vibrio_alginolyticus_strain_FIRELNG2N16S_16S
FJ906747.1|_Vibrio_alginolyticus_strain_HN07006_16S
KC884636.1|_Vibrio_alginolyticus_strain_NH2_16S
FJ594056.1|_Vibrio_parahaemolyticus_strain_ASKBN081039_16S
30
78.1
78.2
67.2
67.1
57.2
57.1
74.2
94
53.2
FJ906751.1_Vibrio_alginolyticus_strain_HN08811_16S
68
FJ906751.1_Vibrio_alginolyticus_strain_HN08811_16S(2)
LMG2850
104
71.1
50.2
76.1
111
56.2
37
60.2
113
KC954170.1_Vibrio_alginolyticus_strain_SwBeN3_16S
69.2
JX221044.1_Vibrio_alginolyticus_strain_XSBZD3_16S
FJ906749.1|_Vibrio_alginolyticus_strain_HN08335_16S
KC884633.1|_Vibrio_alginolyticus_strain_MT83_16S
FJ594056.1|_Vibrio_parahaemolyticus_strain_ASKBN081039_16S(2)
79.2
378.1
31
63.2
61.1
110
50.1
54.1
52.2
116
58.1
62.1
98
FJ906750.1_Vibrio_alginolyticus_strain_HN08801_16S
51.1
Boc892
73.1
0.02

**Figure 4.6.** Midpoint routed Neighbor joining tree demonstrating *V. alginolyticus* strains based on 16SrRNA sequences. Fifty nine strains of *V. alginolyticus* isolated from seawater and Hake fish in Namibia, are assigned to one group.

4.6.3. The *rpo*B gene amplification and sequencing

The 59 *V. alginolyticus* isolates were further classified by *rpoB* gene amplification and sequencing, however only 56 sequences were successfully sequenced. The 54 isolates were identified as *V. alginolyticus* strains (96.4%), other 2 isolates were classified as *Vibrio cincinnatiensis* strain (1.8%) and *Vibrio metschnikovii* (1.8%), (see pie chart, figure 4.10).

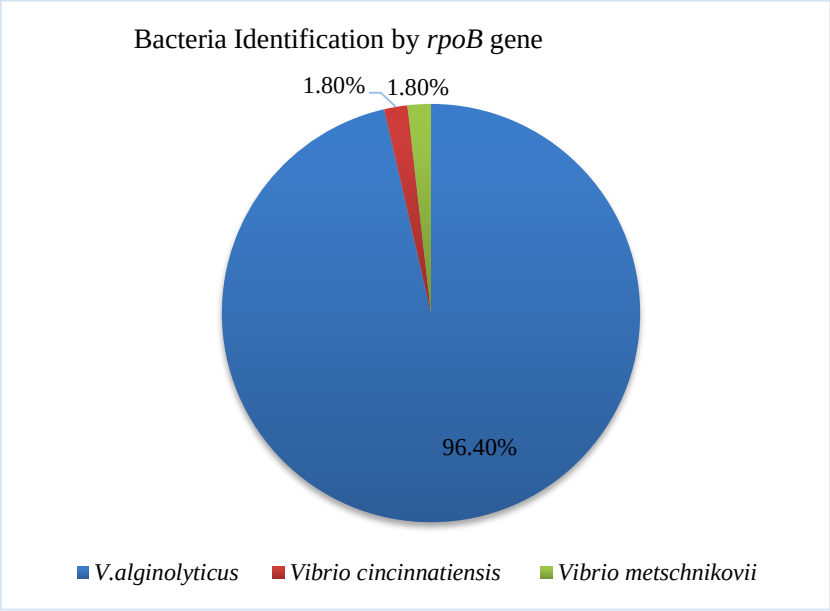



**Figure 4.7.** Percentage (%) bacterial species identification by molecular tests (*rpoB* gene).

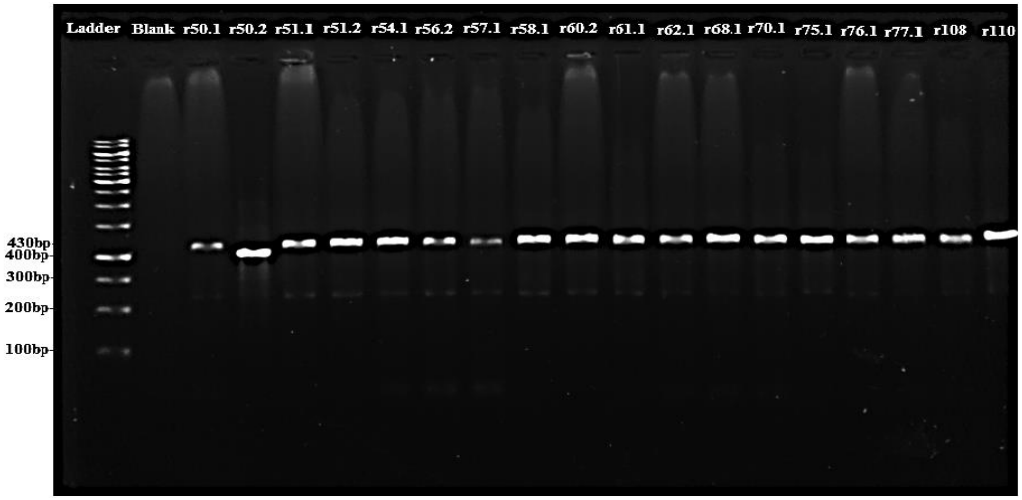



**Figure 4.8.** Agarose gel electrophoresis for the *rpoB* amplification products of the *Vibrio* (isolates) and other species (21 isolates).

4.6.4. Phylogenetic analysis of the *rpoB* gene

Approximately, 430 bp of the *rpoB* sequences of the 44 *V. alginolyticus* strains from the seawater and fish products in Namibia, outside groups (51.1 and 68.1), 9 closely related *rpoB* sequences (*V.alginolyticus*) from GenBank were aligned using MAFFT program. The neighbor-joining tree (Figure 4.12) was computed and based on the tree, 54 *V. alginolyticus* strains were grouped into 8 different groups based on the *rpoB* sequences. The largest group contained 21 and 4 *V. alginolyticus* strains from seawater and hake fish products in Namibia and GenBank database respectively. The second large group contained 18 and 1 *V. alginolyticus* strain from seawater and the GenBank database respectively. Other groups contained 2 (rp112.1, r111, EF064404) and 1 (EF064404) *V. alginolyticus* strains, 3 (51.2, r75.1, r56.2) and 1 (EF064400) *V. alginolyticus* strains (seawater and fish products as well as GenBank database respectively), 1 (r94) *V. alginolyticus* (seawater and fish products), 1 (EF064399) *V. alginolyticus* strain (GenBank database), 1 (EU652307) *V. parahaemolyticus* strain (GenBank data base), and the The other 2 *Vibrio* outside groups (51.1 and 68.1) were grouped into two distinct clusters. The six groups in the phylogenetic tree contained bootstrap values of greater than 50% which indicated that these groups are supported. Two groups in this tree have the bootstrap value of less than 50%, which indicated that these groups are not supported.

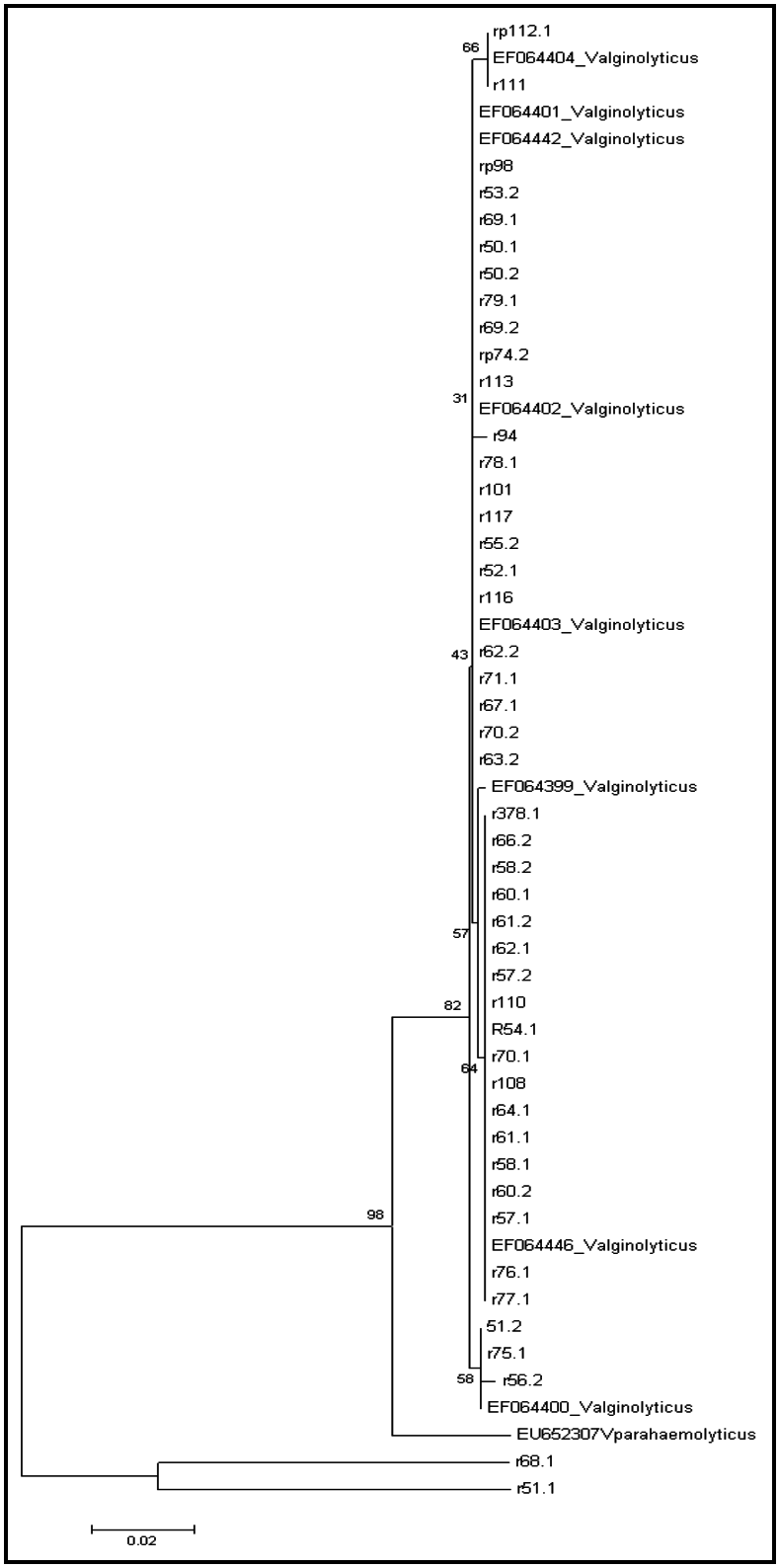

rp112.1
66
EF064404_Valginolyticus
r111
EF064401_Valginolyticus
EF064442_Valginolyticus
rp98
r53.2
r69.1
r50.1
r50.2
r79.1
r69.2
rp74.2
r113
31
EF064402_Valginolyticus
r94
r78.1
r101
r117
r55.2
r52.1
r116
EF064403_Valginolyticus
43
r62.2
r71.1
r67.1
r70.2
r63.2
EF064399_Valginolyticus
r378.1
r66.2
r58.2
r60.1
r61.2
57
r62.1
r57.2
82
r110
R54.1
r70.1
64
r108
r64.1
r61.1
r58.1
r60.2
98
r57.1
EF064446_Valginolyticus
r76.1
r77.1
51.2
r75.1
58
r56.2
EF064400_Valginolyticus
EU652307Vparahaemolyticus
r68.1
r51.1
0.02

**Figure 4.9.** Midpoint routed Neighbor joining tree demonstrating 44 *V. alginolyticus* stains based on *rpoB* sequences, strains were assigned to 8 different clusters using *Vibrio cincinnatiensis* (r68.1), *Vibrio metschnikovii* (r51.1) as outliers.

## 4.7. PCR analysis of virulence related genes

### 4.7.1. Presence/Absence of Virulent genes

The total number of 54 *V. alginolyticus* strains identified according to *rpoB* sequencing were subjected to screening of virulent genes by PCR amplification. The strains were screened for *tdh*, *tlh* and *toxR* virulent factors. Out of 54 *V. alginolyticus* strains, only1 strain tested *tlh* positive. All the necessary positive controls yielded positive bands and all the blank samples yielded no positive bands.

### 4.7.2. Detection of Thermostable Direct Hemolysin (*tdh*)

The total number of 54 *V. alginolyticus* isolates were scanned by PCR amplification of *tdh*, however there were no bands yielded on any of the strains. The *V. alginolyticus* Bcc 892 was used as a positive control and formed positive amplicons with ~ 500 bp. The positive control was repeated twice and yielded the same amplification product. The blank sample yielded no amplicons. The results are presented in Figure 4.13 below.


**Figure 4.10.** Agarose gel (1%Sea kem® Le agar) electrophoresis for *the tdh* amplification products for the present/absent in *V. alginolyticus* strains. All *V. alginolyticus* strains were *tdh* negative. Bcc 892 was used as a positive control for *tdh* virulent gene detection. The molecular weight marker represented 100 bp ladder (Thermo scientific).

4.7.3. Thermolabile hemolysin (*tlh*)

The 54 *V. alginolyticus* strains were also analyzed for the presence/absence of the *tlh* gene, however only one sample produced a *tlh* positive band with about 200 bp. The *Vibrio parahaemoyticus* was used as a positive control that yielded a very bright band. The blank sample did not yield any positive band and the molecular weight marker represented 100 bp ladder (Thermo scientific). The results are indicated in Figure 4.14.

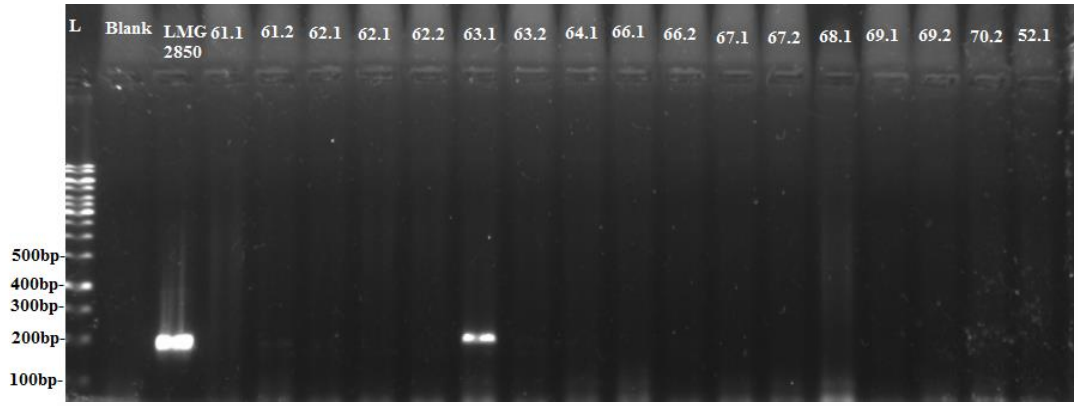



**Figure 4.11.** Agarose gel electrophoresis for the *tlh* amplification products for the present/absent in *V. alginolyticus* strains. Apart from *V. parahaemolyticus* LMG 2850, only *V. alginolyticus* strain 63.1 tested *tlh* positive. LMG 2850 was used as a positive control.

4.7.4. Toxin regulator (*toxR*)

The 54 *V. alginolyticus* isolates were further analysed for *toxR* by PCR amplification. There was no single *V. alginolyticus* strain that yielded *toxR* positive band. *V. parahaemolyticus* LMG 2850 was used as a positive control that yielded a 200 bp band. The blank sample yielded no positive band. The molecular weight marker of the ladder also represented 100 bp.The results are given in Figure 4.15.

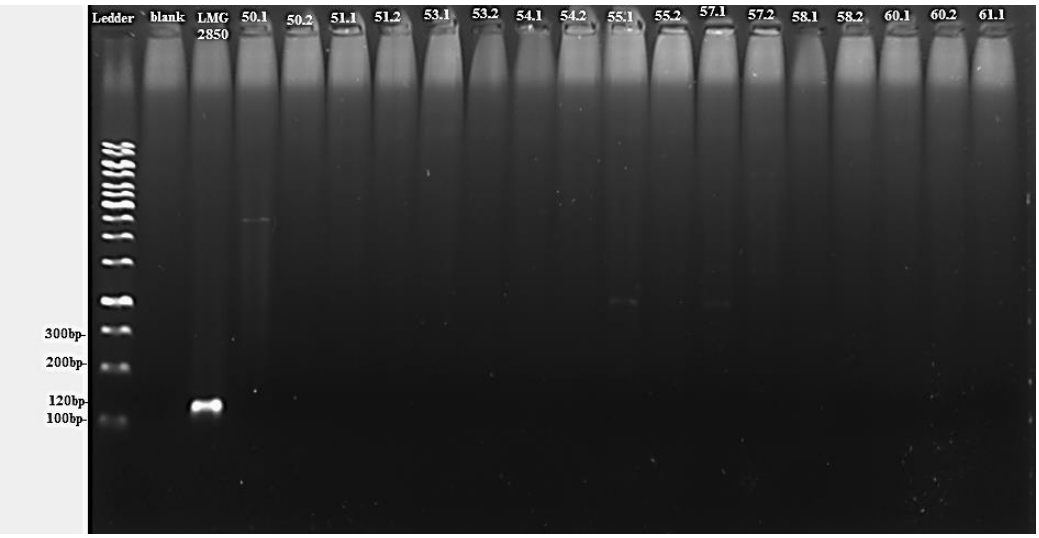



**Figure 4.12.** Agarose gel (1%Sea kem® Le agar) electrophoresis for *the toxR* amplification products for the present/absent in *V. alginolyticus* strains. All *V. alginolyticus* strain are *toxR* negative. *V. parahaemolyticus* LMG 2850 was used as a positive control for the *toxR* virulent factor.

### 4.9. Seawater Disinfection

During validation of 0.05% of hydrogen peroxide in disinfection of seawater at seawater treatment plant (fish processing factory B) in accordance to Shikongo-Nambabi *et al*., (2010), there were no detections of *V. alginolyticus*, and *Vibrio* like species in seawater after treatment. Hydrogen peroxide eradicated all bacterial cells from the seawater distribution system. The results are indicated in Figure 4.16.

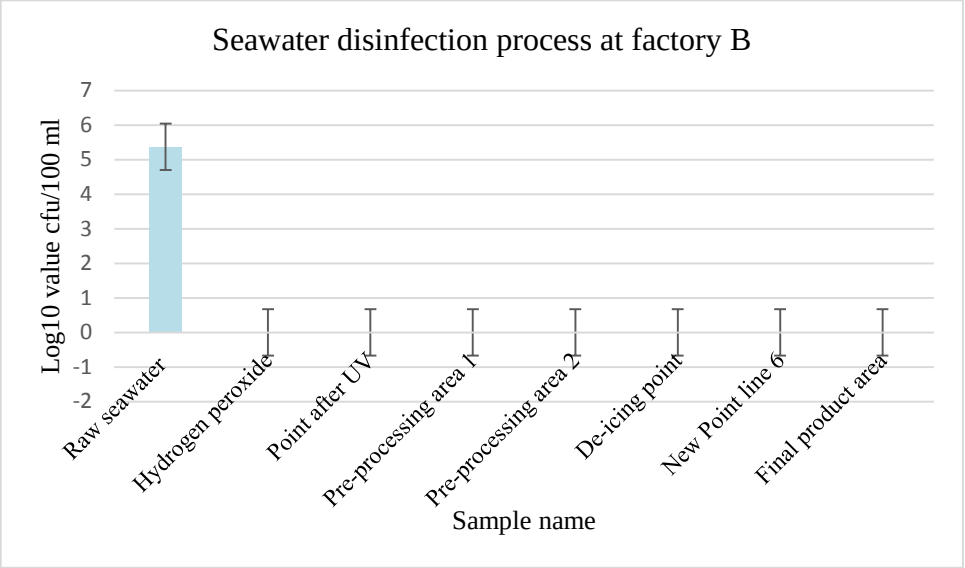



**Figure 4.13.** Average Total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* species counts on TCBS, samples taken at points along the $H_2O_2$ disinfected seawater distribution system.

### 4.10. Data analysis

In this study, a one-way ANOVA: F-Fest was used during the analysis of variance for the independent samples (chlorine treated seawater and hydrogen peroxide treated seawater). Independent samples were from seawater (K populations) and each received a different treatment. We assumed further that the response from each of the seawater treatments is a random variable. Here the null hypothesis is that the mean count of CFU is the same for both (chlorine and hydrogen peroxide disinfectants) the treatment groups. In other words, the mean CFU in Seawater Treatment with Chlorine is equal to the mean CFU in Seawater Treatment with Hydrogen Peroxide. Since this probability ($p = 0.003$) is low (less than 0.05), we would reject the null hypothesis and conclude that there are significant differences between the two disinfectants with retard to bacterial growth at 5% ($P < 0.05$) (Appendix Table 11).

# Chapter 5: Discussion

In this study, TCBS and TSAT media were used during the analysis of *Vibrio* species as per TM/M/06 and ISO/TS 21872-2 (2007) test and standard method of the Namibian Standards Institution microbiology department with few modifications. Although, TSAT can clearly differentiate colony morphologies between *Vibrio* species, the results were not considered in this study due to high spreading ability of some *V. alginolyticus* strains on this media. On the other hand, TCBS gave good colonies without spreading of any bacteria strain. Nevertheless, TCBS cannot differentiate between sucrose fermenting *Vibrio* species.

In this study, oxidase and Gram's stain test methods were used to screen out oxidase negative and Gram's positive bacteria before biochemical test (API 20E). Both test methods were very useful during this study. Furthermore, biochemical test (API 20E) method was used to identify strains to species level, however this method is not entirely dependable. During confirmations, some strains were identified as *V. alginolyticus* by the API 20E, however, further confirmations by molecular methods (16S rRNA and *rpoB* analyses) classified this strains as other *Vibrio* species and some as *Shiwanella*. It is therefore imperative that when there is a positive test on API 20E, further confirmations should be carried out.

According to Abbort *et al*. (1997), there were two occasions whereby, *Aeromonas* was misidentified as *Vibrio damsella* and *Vibrio cholerae* by the Vitek and API 20E system.

The results of the *rpoB* gene sequence analyses represented a strong and a reliable identification and classification tool for *Vibrio* species. These results matched those of Ki *et al*. (2009), as well as Oberbeckmann *et al*. (2011). In this study, the *rpoB* sequences and the phylogenetic tree analyses, displayed better resolution than 16S rRNA sequences, in the phylogenetic analyses. According to Khamis *et al*. (2004), the 16S rRNA contain low intragenus polymorphism limits, to ensure dependable identification. Furthermore, complete sequencing of 16S rRNA (approximately 1 500 bp) gene is required for accurate identification of bacteria. However, on the other hand, *rpoB* gene has a long sequence (~ 4.2 kb), and has high polymorphism level (Mollet *et al*., 1997; Walsh *et al*., 2004).

Klein *et al*. (2013), explained that due to close relatedness between *Vibrio* species, specific virulent genes are sometimes used to identify *Vibrio* species pathogenic strains. Other studies such as Xie *et al*. (2005) and Oberbeckmann *et al*. (2011), also used virulent genes (*tlh, trh, tdh, toxR, toxRS, ctxA, VPI*) to investigate pathogenicity of *V. alginolyticus* strains using aquatic animals as experimental animals. In this study, the virulent genes originating from known human pathogens such as *V. cholera* and *V. parahaemolyticus* were used to determine whether *V. alginolyticus* strains isolated from seawater and hake fish in Namibia, contain virulent genes to human or not.

During the present/absent PCR amplification of virulent genes, all process controls for the respective virulent genes *tdh* (500bp), *tlh* (200bp), and *toxR* (120bp), tested positive

with nonspecific PCR products (expected bands for PCR amplification) on the gel electrophoresis reader machine. However, none of these isolates tested positive on *tdh* and *toxR*. Similar study was carried out by Xie *et al*. (2005) and some *V. alginolyticus* strains were positive (with nonspecific PCR products) on *toxR* (658bp) and *tlh* (448bp). There was also no positive detection for *tdh* gene in all the *V. alginolyticus* strains tested. In this study only one *V. alginolyticus* strain (63.1) isolated from raw hake after the pre-processing area that tested positive for the *tlh* gene. Xie *et al*. (2008), highlighted that *V. alginolyticus* strains often retain homologues of *V. parahaemolyticus* and *V. cholera* pathogenic genes such as *toxR*, *tlh* and VPI. In this study, *tlh* was confirmed present in one *V. alginolyticus* strain.

In this study, the virulence genes were chosen on the basis of historical results explicated in the past studies. Oberbeckmann *et al*. (2011), investigated pathogenicity in *V. alginolyticus* and *V. parahaemolyticus* at Helgoland Roads whereby *tdh* was considered as one of the virulent associated genes under investigation. In addition, the detection of the virulent associated genes (*tdh* and its homolog *trh*) is regarded to be the modest method and universally used as an indicative of pathogenicity in *Vibrio* species (Parveen *et al*., 2008; Broberg *et al*., 2011; Paranjype *et al*., 2012; West, 2012). About 90% of clinical strains and 1% environmental isolates of *V. parahaemolyticus* carry *tdh* virulence factor (Miyamoto *et al*., 1968; Nishibuchi and Kaper 1985; Nishibuchi *et al*., 1992; Okuda *et al*., 1997, cited by Mclaughlin *et al*, 2005). Based on this information,

*tdh* was considered as one of the factors whose detection would determine the pathogenicity of *V. alginolyticus* in this study.

With regard to *tlh* virulent gene, Xie *et al*. (2005), reported that this gene is distributed among *V. alginolyticus* species. According to Taniguchi *et al*. (1986); Zhang and Austin, (2005), *tlh* encodes for thermolabile hemolysin, which is a phospholipase; detection of this gene was thus included as one of the means to determine *V. alginolyticus* isolates pathogenicity in this study. Despite of the fact that in this study, eight groups of *V. alginolyticus* strains were identified to species level using the *rpoB* gene sequences and phylogenetic tree analyses, there were no evidence to proof that these strains are pathogenic to humans.

The water quality plays a direct role on food quality (Kirby *et al*., 2002; Wujie *et al*., 2011). The results in this study concurred with findings made by Shikongo-Nambabi *et al*. (2010), which point out that bacteria persisted in the seawater distribution systems regardless of chlorine and UV light application in seawater disinfection. Furthermore, the ability of *V. alginolyticus* to survive and grow in treated and disinfected seawater, confirmed uninterrupted contamination of hake products. Other studies also demonstrated ineffectiveness of chlorine in eradication of entire bacteria counts in seawater distribution systems (Wong and Davidson, 1977; Goldman *et al*., 1978). The persistence of bacteria including *V. alginolyticus* in seawater distribution systems has been reported to be accompanying bacterial biofilms which forms along the seawater distribution pipelines (Shikongo-Nambabi, 2010).

In this study, the results reveal that at the fish processing factory A and B, when total *V. alginolyticus* and *Vibrio* like species counts (218667 cfu/ 100 ml and 234834 cfu/ 100 ml respectively) in untreated sea water were taken into consideration, most of the total bacteria cells were killed by chlorine, but not whole. The results showed that the remained few bacteria cells were eliminated by UV light sanitizer at factory B. At the fish processing factory A, there was a detection of 1cfu/100 ml after UV point sanitizer (seawater treatment plant). The detection of bacteria cells immediately after UV light could mean sterilization was not effective. This could result from expired UV light bulbs, insufficient light bulbs and high turbidity of seawater that could limit emission of light into seawater. The microbiological quality of clean seawater, declined after UV point of sterilization due to the following possible reasons: depreciation of free chlorine residues and formation of resistant biofilms to disinfectants along the seawater distribution system. The seawater quality decreased at the pre-processing area (9 cfu/100 ml), baader machine (6 cfu/100 ml) and trio-machine (5 cfu/100 ml) at Factory A). At the fish processing factory B, the seawater quality declined at the pre-processing area (6cfu/100 ml), de-icing point inside the processing plant (3cfu/100 ml) and at the new processing line 6 in the processing plant (2 cfu/100 ml).

George *et al*. (2001), affirmed that the presence and growth of bacteria in food processing environments could host contamination to finished products that prompt reduction of microbiological safety and quality. According to Shikongo-Nambabi *et al*., (2010), the hake fish microbial quality depreciated along the processing line and *V.*

*alginolyticus* was frequently detected and most likely to have been introduced by treated seawater. Based on high microbiological counts in hake fish and hake fish products at fish processing factory A and B, this study concurred with the findings by Shikongo-Nambabi *et al*. (2010).

In this study, the totals *V. alginolyticus* and *Vibrio* like species at the fish processing factory B were not detected in the untreated and treated hake raw material. However, the skinned hake fillet products after the trio-machine contained the highest total bacteria count on TCBS agar. Although, fish processing factory B do not use seawater directly onto fish products at the baader and trio-machine along the fish processing line, the total bacteria counts in unfrozen and frozen products did not vary so much from that detected in the semi-finished products after the trio-machine.

Apart from bacteria contamination from chlorine treated seawater, the high *V. alginolyticus* and *Vibrio* species like counts in hake fish could have been also caused by contamination and proliferation of bacteria cells along the processing line, triggered by temperature change during fish processing, time range between de-icing of raw material and freezing of finished products, as well as contamination from seawater used during cleaning of floors, conveyor belts and fish processing equipment.

Neither *V. alginolyticus* nor *Vibrio* like species were detected in seawater after disinfection with 0.05% hydrogen peroxide. Hydrogen peroxide eliminated all bacterial cells from the seawater distribution system. The results in this study matched those of

Da Pozzo *et al*. (2008) and Shikongo-Nambabi *et al*. (2010), which demonstrated biocidal effect of hydrogen peroxide in fully destroying living cells including biofilms on its path.

Shikongo-Nambabi *et al*. (2010), also reported that initiation of new biofilms and inhibition of matured biofilms generated by strains of *V. alginolyticus* were achieved at the same concentration (0.05 %/) used in this study. Linley *et al*. (2012), stated that hydrogen peroxide extinguishes living cells by producing increased oxidation of their genetic materials (DNA), proteins and membrane lipids. The concentration (300mg/L) used by Da Pozzo *et al*., 2008, was lower than the concentration (500mg/L) used by Shikongo-Nambabi *et al*., 2010 and in this study. The two mentioned studies were carried out using synthetic (artificial) seawater (*in vitro*), however, in this study, the experiment was carried out at industrial level (*in situ*). The statistical results of this study demonstrated that there was significant difference ($P<0.05$) between hydrogen peroxide ($H_2O_2$) and chlorine ($Cl_2$) gas in disinfection of seawater.

In this study, when hydrogen peroxide was introduced into the seawater distribution system at fish processing factory B in Namibia, Fizzing of seawater was observed, dead organic matter were flushed out from the tank and pipe walls through tap outlets. The water looked fizzy due to strong aeration. There were no signs of instant signs of corrosion. The system was designed to resist corrosion from seawater.

# Chapter 6: Conclusion and Recommendation

The entire bacteria load (*V. alginolyticus* and *Vibrio* like species) were eliminated by 0.05% of hydrogen peroxide ($H_2O_2$), food grade. Organic matter was flushed out of the system by the sparkling force of seawater, through the outlet points. The statistical analysis of data indicated that there was a significant difference between seawater treatment with hydrogen peroxide and chlorine in conjunction with UV light ($P<0.05$).

However, due to the fact that hydrogen peroxide is costly as compared to chlorine gas and fish processing factories use large volume of seawater at high pace during their operations. Considering the economic prestige of the fishing industry, this study recommend 0.05% of hydrogen peroxide as a periodic cleaning disinfectant, preferably twice a month or when need arise. This cleaning will eradicate any possibility of biofilm building up in the seawater distribution systems. The seawater distribution systems should be flushed with hydrogen peroxide treated seawater. However, it is up to the discreet of individual companies to decide whether to use $H_2O_2$ as a routine seawater disinfectant or as a cleaning agent. Both choices are recommended by this study. It is also important to consider the materials which are non-corrosive to hydrogen peroxide such as polypropylene plastic pipes when constructing the system. This study has

therefore concluded that food grade hydrogen peroxide ($H_2O_2$) is an excellent alternative disinfectant for seawater.

*V. alginolyticus* species are abundantly occurring in Namibian marine coastal waters. Pathogenicity results showed that all common virulent genes (*tdh*, *toxR* and *tlh*) in *V. alginolyticus* strains, which were investigated in this study tested negative, except one (1.9%) out of 54 strains that tested *tlh* positive. This study has confirmed that these strains do not contain specific virulence genes known in other *Vibrio* species and more work should be done to clarify the pathogenicity of these specific *V. alginolyticus strains*.

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# APPENDICES

## 1. General Introduction

### 1.1. Contribution of Fishery sector to Gross Domestic Product (GDP)

Table 1. Indicates the contribution of the fisheries subsector to the Gross Domestic Product (GDP) in Namibia. (Source: NPC, 2011, cited by MFMR, 2010/2011; MFRF, 2009).

| Year | 2006 | 2007 | 2008 | 2009 | 2010 |
|---|---|---|---|---|---|
| % of GDP | 4.8 | 5.3 | 4.7 | 4.6 | 3.7 |

### 1.2. Namibia landings of quota Species

Table 2. Namibia landings of quota Species (metric tons) in 2006 up until 2010 (statistics obtained from the MFMR, 2010/11, p. 21)

| Species | 2006 | 2007 | 2008 | 2009 | 2010 |
|---|---|---|---|---|---|
| Pilchards | 2 314 | 23 522 | 18 755 | 20 137 | 20 229 |
| Hake | 137 771 | 125 534 | 117 286 | 137 312 | 127 196 |
| Horse Mackerel | 309 980 | 201 660 | 186 996 | 215 996 | 185 673 |
| Monk | 9 816 | 8 932 | 7 270 | 6 922 | 7 904 |
| Crab | 2 228 | 3 245 | 2 100 | 1 577 | 766 |
| Rock Lobster | 285 | 1153 | 195 | 43 | 82 |
| Orange Roughy | 545 | 255 | 0 | 0 | 0 |
| Tuna | 2 903 | 4 596 | 3 281 | 4 241 | 2 024 |
| Seals(Number) | 83 045 | 34 728 | 47 603 | 41 145 | 47 821 |

## 2. Method

Table 3: The formula used to determine the sample size at 95% confidence interval.

$$n = \left[ \frac{Z_{\alpha/2}\sigma}{E} \right]^2$$

Whereby, $Z_{\alpha/2}$ = critical value, $E$ = margin of error, $\sigma$ = the standard deviation and $n$ = sample size (http://www.isixsigma.com/tools-templates/sampling-data/how-determine-sample-size-determining-sample-size/).

## 3. Results

Table 4. Average Total *V. alginolyticus, Aeromonas, Shewanella* and *Klebsiella* species counts on TCBS, samples taken at points along the chlorine ($Cl_2$) disinfected seawater distribution system at fish processing factory A

| **Sampling point** | **Log10 Value Cfu/100ml on TCBS** |
|---|---|
| Raw seawater | 5.339783 |
| Point after chlorine | 2.109466 |
| Point after UV | 0.066947 |
| Pre-processing area | 0.929419 |
| Point at baader machine | 0.753328 |
| Point after Trio machine | 0.684247 |

Table 5. Average Total *V. alginolyticus, Aeromonas, Shewanella* and *Klebsiella* species counts on TCBS, samples taken at points along the chlorine ($Cl_2$) disinfected seawater distribution system at fish processing factory B

| **Sampling point** | **Log10 Value Cfu/100ml on TCBS** |
|---|---|
| Raw seawater | 5.370440079 |
| Chlorinated tank | 2.06045107 |
| Point after UV | 0 |
| Pre-processing area 1 | 0.76482021 |
| Pre-processing area 2 | 0.626719407 |
| De-icing point | 0.367748093 |
| New Point line 6 | 0.220369882 |
| Final product area | 0 |

Table 6. Average Total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* species counts on TCBS, samples taken at points along the hydrogen peroxide ($H_2O_2$) disinfected seawater distribution system at fish processing factory B

| **Sampling point** | **Log10 Value Cfu/100ml on TCBS** |
|---|---|
| Raw seawater | 5.373257033 |
| Chlorinated tank | 0 |
| Point after UV | 0 |
| Pre-processing area 1 | 0 |
| Pre-processing area 2 | 0 |
| De-icing point | 0 |
| New Point line 6 | 0 |
| Final product area | 0 |

Table 7. Average Total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* species counts on TCBS, isolated from hake fish, sampled along the Hake fish processing line for fish processing factory A.

| **Sample ID** | **Aver log10 value cfu/g** |
|---|---|
| Uncleansed Raw material | 2.185636577 |
| De-Iced raw material with seawater | 2.828230115 |
| After baader machine | 2.968482949 |
| After Trio machine | 3.425425525 |
| Unfrozen Final product | 3.139879086 |
| frozen final product | 2.514104821 |

Table 8. Average Total *V. alginolyticus*, *Aeromonas*, *Shewanella* and *Klebsiella* species counts on TCBS, isolated from hake fish, sampled along the Hake fish processing line for fish processing factory B.

| **Sample ID** | **Aver log10 value cfu/g** |
|---|---|
| Uncleansed Raw material | 0 |
| De-Iced raw material with seawater | 0 |
| After baader machine | 2.066947 |
| After Trio machine | 2.691671 |
| Unfrozen Final product | 2.726999 |
| frozen final product | 2.691671 |

Table 9. Volume calculation of hydrogen peroxide (35%) required to disinfect specific volume of seawater.

The following formula was utilized to calculate the required volume of 35% hydrogen peroxide to disinfect a given volume of static or closed system of seawater: $V_1\ C_1 = V_2\ C_2$ (Richards, 2012).

Whereby: $V_1$= Initial Volume (e.g. Unknown) $C_1$= Initial concentration (eg. 35% hydrogen peroxide), $V_2$ = Final Volume (e.g. size of seawater tank) $C_1$ = Final concentration (0.05%)

According to Yanong (2008), the following formula is applied during calculations of how much volume of 35% PEROX-AID® is necessary for a certain volume of seawater disinfection. However, this product cannot be used for any other type or brand of hydrogen peroxide calculations:

$$\frac{\text{Treatment concentration (mg/L) x system volume (L) x 1000ml/L}}{396{,}100\text{mg/L}} = \text{Volume (ml) of 35\% PEROX- AID®}$$

Whereby, 396,100 is a constant that represent a number of hydrogen peroxide per liter of 35% PEROX- AID®.

## 4. Statistical Analysis

Table 10. Mean and their standard deviations of CFU for each treatment.

| Final CFU/100ml | | | |
|---|---|---|---|
| Treatment | Mean | N | Std. Deviation |
| Seawater Treatment with chlorine | 18.60 | 42 | 39.943 |
| Seawater Treatment with Hydrogen peroxide | 0.00 | 42 | 0.000 |
| Total | 9.30 | 84 | 29.590 |

Table 11. The sum of squares, degrees of freedom, mean squares, the F-value and the observed significance value.

| Analysis of variance (ANOVA) | | | | | | |
|---|---|---|---|---|---|---|
| Sources of variations | | Sum of Squares | df | Mean Squares | F or Variance Ratio | Sig. |
| Final CFU/100ml * Treatment | Between Groups (Combined) | 7261.440 | 1 | 7261.440 | 9.103 | 0.003 |
| | Within Groups | 65411.619 | 82 | 797.703 | | |
| | Total | 72673.060 | 83 | | | |

| Measures of Association | | |
|---|---|---|
| Final CFU/100ml * Treatment | Eta | Eta Squared |
| | 0.316 | 0.100 |

Table 10 shows the mean count of CFU for each of the treatments and their standard deviations. The Analysis of Variance table, Table 11 shows the sum of squares, degrees of freedom, mean squares, the F-value and the observed significance value. It can be

observed from Table 11 that the significance value for this experiment is the probability of getting an F-value of 9.103 or higher if the null hypothesis is true.

## 3. Bacterial Identification

Table 12. 16S rRNA gene sequences for *V. alginolyticus* strains identification and classification

| > LMG2850 |
| --- |
| ACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCG<br>GGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC<br>ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG<br>CCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTAGTGTTAATAGCACTATCATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAG<br>CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACC<br>TCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCG<br>AAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGG<br>AGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGG |
| >101 |
| GTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAAC<br>CATTGGAAACGATGGCTAATACCGCATRATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG<br>AGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG<br>GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTCGTGAGGAAGGTNGTGTAGTT<br>AATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG<br>GCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGG<br>GTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTG<br>GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTT |
| >104 |
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| >105 |
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>107

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>53.1

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>53.2

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ACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
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>54.1

GAGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCAT
TGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAG
GTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
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>54.2

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>55.1

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>55.2

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>56.2

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GAGGTGTGGCCTTGAGCC

>57.1

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>57.2

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| GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGTCTACTTGGA |
| >58.1 |
| TGCAAGTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGG<br>ATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAG<br>TTGGTGAGGTAAGGGCTCACCA |
| >58.2 |
| GAGCGGAAACGAGTTATCTGAACCTTCGGGGGACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCAT<br>TGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAG<br>GTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG<br>AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGTTAA<br>TAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGC<br>GTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGT<br>AGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGG<br>GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGA |
| >60.1 |
| CGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGG<br>GCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC<br>TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCC<br>TTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCA<br>GCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTC<br>GGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAA<br>GGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT |
| >60.2 |
| ATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAG<br>TTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG<br>CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAG<br>TGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAA<br>TTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAG<br>AGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGA<br>AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGG |
| >61.1 |
| GTCGAGCGGAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGACCGGTGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACC<br>ATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG<br>AGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG<br>GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTT<br>AATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG<br>GCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGG<br>GTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTG<br>GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAG |
| >61.2 |
| ACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCG<br>GGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC |

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| ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG<br>CCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAG<br>CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACC<br>TCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCG<br>AAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT |
| >62.1 |
| GCGGTCGAGCGGAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGACCGGTGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATA<br>ACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTG<br>GTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG<br>TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTCGTGAGGAAGGTGGTGT<br>AGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTA<br>CTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAG<br>GGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAA<br>AGCGTGGGGAGCAAA |
| >62.2 |
| GTTATCTGAACCTTCGGGGAACGATAACGGCGTCGACCGGTGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGG<br>CTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACC<br>AAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT<br>GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATT<br>TGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGC<br>AGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTG<br>TAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGA<br>TTAGATACCCTGGTAGTCCACGCCGTA |
| >63.1 |
| GAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGAT<br>GGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTC<br>ACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC<br>AATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGTTAATAGCTGCATT<br>ATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCA<br>TGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAG<br>GTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACA<br>GGATTAGATACCCTGGTAGTCCACGCCGTA |
| >63.2 |
| GTCGAGCGGAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACC<br>ATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG<br>AGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG<br>GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTT<br>AATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG<br>GCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGG<br>GTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTG<br>GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA |
| >64.1 |

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>66.2

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>67.1

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>67.2

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ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAA
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CCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGG
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>69.1

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GAGCATGTGGTT

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|---|
| >69.2 |
| GGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGG<br>CCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT<br>GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT<br>TCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG<br>CCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCG<br>GAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAG<br>GCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGG<br>TTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCAC<br>AAGCGGTGGAGCATGTGGTTTAATTCGATG |
| >70.1 |
| GACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTC<br>GGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCA<br>CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAG<br>GCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCA<br>GCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAAC<br>CTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGC<br>GAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTG<br>GAGGTTGTGGCCTTGAGCC |
| >70.2 |
| ACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCG<br>GGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC<br>ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG<br>CCTTCGGGTTGTAAAGTACTTTCAGTCGTGAGGAAGGTGGTGTCGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAG<br>CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACC<br>TCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCG<br>AAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGG<br>AGGTTGTGG |
| >71.1 |
| GTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGGACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAAC<br>CATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG<br>AGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG<br>GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGTT<br>AATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG<br>GCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGG<br>GTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTG<br>GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGG |
| >73.1 |
| GGGGATCTGCCCGGTCGAGGGGGATAACCTTTGGAAACCACCGCTAATACCGCATACCCCCTACGGGGGAAAGCGGGGGACCTTCGGGCCTTGCGCGATTGGA<br>TGAACCCAAGTGAGATTATCTTGTTGGTGAGGTAACGGCTCACCAGGGCCACTATCTCTATCTGGTCTGAGAGGATGACCAGTCACACTGGGACTGACACACGG<br>CCCACACTCCTACGGGACGCAGCAGTGGGGAATATTGCACAGTGGGGGAAACCCTGATGCATCCGTGCCGCGTGTGTGAAGAAGGTCTTCGGGTTGTAAAGCA<br>CTTTCAGTGGTGAGGAAAGGAGGTTGGCTAATACCTGACCTCTGTGACGTTCACCACAAAAGAAGCACCGGCTAACTCCGTG |

>73.2

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>74.2

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>75.1

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>76.1

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>77.1

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>78.1

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>79.1

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>78.2

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>79.2

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>79.3

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CATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG

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|---|
| AGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG<br>GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTT<br>AATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG<br>GCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGG<br>GTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTG<br>GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGTCTACTTGGAGG |
| >91 |
| CGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGG<br>GCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC<br>TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCC<br>TTCGGGTTGTAAAGYACTTTCAGCCGTGAGGAAGGTGGGTGTGTTAATAGCACATTCATTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA<br>GCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTC<br>GGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAA<br>GGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGTCTACTTGGAGGT<br>TGTGGCCTTGAGCCGTGGCTTTCG |
| >94 |
| GAGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCAT<br>TGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAG<br>GTAAGGGCTCACCAAGGCAACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG<br>AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGYACTTTCAGTCGTGAGGAAGGCGGTGTCGTTA<br>ATAGCGGCATCGTTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGG<br>CGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGG<br>TAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGG<br>GGAGCAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGTCTACTTGG |
| >98 |
| AGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGACCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATT<br>GGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGG<br>TAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCC |
| >Bcc892 |
| GGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATACCTTCGGGTCAAAGAGGGGGACCTTCGGGC<br>CTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGGTCTGAGAGGGATGATC |

Table 13. *rpoB* gene sequences for *V. alginolyticus* strains identification and classification.

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| >rLMG2850 |

GCTGCAGAAGCACTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCGACTGTTGGTCGTATGAAGTTTAATAGCTCTATCGGTCGTGAAGATGCT
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GACGCCATACCGTCGCGTTGTAGATGGTGTTGTAACAGACGAAGTAGATTACCTATCTGCAATCGAAGAAGGGCCAATTCGTTATCGCTCAGGCGAACGCTAAG
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>r101

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GACACCATACCGTCGCGTTGTAGATGGCGTAGTAACAGACGAAGTAGATTACCTATCTGCAATCGAAGAAGGCCAATTCGTTATCGCACAGGCGAACGCTAAG
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>r104

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CACTACGGTCGTCTATGTCCTATCGAAACGCCTGAAGGGTCCAAACATCGGTCTGATCAACTCTCTATCTGCGTTTGCGCGTTGTAACGAGTA

>r105

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>r108

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>r110

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>r113

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>r116

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>r117

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>r378.1

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>r50.1

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>r50.2

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>r51.1

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>51.2

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>r53.2

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>R54.1

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>r55.2

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>r56.2

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>r57.1

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>r57.2

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>r58.1

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>r58.2

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>r60.1

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>r60.2

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>r61.1

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>r61.2

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>r62.1

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>r62.2

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>r70.1

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>r71.1

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>r75.1

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>r76.1

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>r77.1

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>r78.1

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>r79.1

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>r91

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>r94

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>r98

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>r52.1

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>r55.1

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>r63.1

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>r63.2

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>r64.1

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>r66.2

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>r68.1

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>r69.1

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>r69.2

CTCTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCCCCTGTTGGTCGTATGAAGTTCAACAGCTCTATCGGCCGTGAAGATGCTCAAGAGCAAG
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TATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATCTTGACGCAA
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>r70.2

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| CAACCGTCGTATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATC<br>TTGACGCAATCATGCCTCAAGACTTGATCAACGCGAAGCCAATTTCTGCGGCAGTTAAAGAATTCTTTGGCTCTTCACAGCTTTCACAGTTCATGGACCAAAACA<br>ACCCGCTATCAGAAGTAACGCACAAGCGTCGTATTTCTGCATTGGGTCCT |
| >rBcc892 |
| CTGCAGAAGCACTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCGCCTGTTGGTCGTATGAAGTTCAACAGCTCTATCGGTCGTGAAGATGCTC<br>AAGAGCAAGGCACACTTGACGAGCTAGATATCGTTGAAGTGATGAAGAAGCTTATCGCGATTCGTAACGGTAAAGGCGAAGTGGACGATATCGACCACCTAGG<br>TAACCGTCGTATCCGCTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATC<br>TTGACGCAATCATGCCTCAAGACTTGATCAACGCGAAACCAATCTCTGCAGCTGTTAAAGAATTCTTTGGCTCTTCACAGCTTTCACAGTTCATGGACCAAAACA<br>ACCCGCTATCAGAAGTAACGCATAAGCGTCGTATTTCTGCATTGGGTCCTGGGGGGTCTAACTC |
| >rp107 |
| CTGCAGAAGCTCTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCGACTGTTGGTCGTATGAAGTTCAACAGCTCTATCGGCCGTGAAGATGCTC<br>AAGAGCAAGGCACACTTGATGAAACTGATATCATCGAAGTGATGAAGAAGCTGATCGCGATCCGTAACGGTAAGGGCGAAGTGGACGATATCGACCACCTAGG<br>CAACCGTCGTATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATC<br>TTGACGCAATCATGCCTCAAGACTTGATCAACGCGAAGCCAATTTCTGCGGCAGTTAAAGAATTCTTTGGCTCTTCACAGCTTTCACAGTTCATGGACCAAAACA<br>ACCCGCTATCAGAAGTAACGCACAAGCGTCGTATTTCTGCGTTGGGTCCTGGCGGTCTAACTCGTGAACGTGCTGGCTTCGAAGTACGTGACGTACACGTAACT<br>CACTACGGTCGTCTATGTCCTATCGAAACGCCTGAAGGTCCAAACATCGGTCTGATCAACTCTCTATCTGCGTTTGCGCGTTGTAACGAGTACGGTTTCCTAGAG<br>ACACCATACCGTCGCGTTGTAGATGGCGTAGTAACAGACGAAGTAGATTACCTATCTGCAATCGAAGAAGGCCAATTCGTTATCGCACAGGGCGAACGCTAAG<br>CTAAACGAAGATGGTACTTTTGCAGA |
| >rp112.1 |
| GCAGAAGCTCTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCCCCTGTTGGTCGTATGAAGTTCAACAGCTCTATCGGCCGTGAAGATGCTCAA<br>GAGCAAGGCACGCTTGATGAAACTGATATCATCGAAGTGATGAAGAAGCTGATCGCGATCCGTAACGGTAAGGGCGAAGTGGACGATATCGACCACCTAGGCA<br>ACCGTCGTATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATCTT<br>GACGCAATCATGCCTCAAGACTTGATCAACGCGAAGCCAATTTCTGCGGCAGTTAAAGAATTCTTTGGCTCTTCACAGCTTTCACAGTTCATGGACCAAAACAA<br>CCCGCTATCAGAAGTAACGCACAAGCGTCGTATTTCTGCATTGGGTCCTGGCGGTCTAACTCGTGAACGTGCTGGCTTCGAAGTACGTGACGTACACGTAACTC<br>ACTACGGTCGTCTATGTCCTATCGAAACGCCTGAAGGTCCAAACATCGGTCTGATCAACTCTCTATCTGCGTTTGCGCGTTGTAACGAGTACGGTTTCCTAGAGA<br>CACCATACCGTCGCGTTGTAGATGGCGTAGTAACAGACGAAGTAGATTACCTATCTGCAATCGAAGAAGGCCAATTCGTTATCGCACAGGCGAACGCTAAGCTA<br>AACGAAGATGGTACTTTTGCAGATGA |
| >rp54.2 |
| CTGCAGAAGCTCTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCGACTGTTGGTCGTATGAAGTTCAACAGCTCTATCGGCCGTGAAGATGCTC<br>AAGAGCAAGGCACACTTGATGAAACTGATATCATCGAAGTGATGAAGAAGCTGATCGCGATCCGTAACGGTAAGGGCGAAGTGGACGATATCGACCACCTAGG<br>CAACCGTCGTATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATC<br>TTGACGCAATCATGCCTCAAGACTTGATCAACGCGAAGCCAATTTCTGCGGCAGTTAA |
| >rp74.2 |

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| GAGCAAGGCACACTTGATGAAACTGATATCATCGAAGTGATGAAGAAGCTGATCGCGATCCGTAACGGTAAGGGCGAAGTGGACGATATCGACCACCTAGGCA<br>ACCGTCGTATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATCTT<br>GACGCAATCATGCCTCAAGACTTGATCAACGCGAAGCCAATTTCTGCGGCAGTTAAAGAATTCTTTGGCTCTTCACAGCTTTCACAGTTCATGGACCAAAACAA<br>CCCGCTATCAGAAGTAACGCACAAGCGTCGTATTTCTGCATTGGGTCCTGGCGGTCTAACTCGTGAACGTGCTGGCTTCGAAGTACGTGACGTACACGTAACGC<br>ACTACGGTCGTCTATGTCCTATCGAAACGCCTGAAGGTCCAAACATCGGTCTGATCAACTCTCTATCTGCGTTTGCGCGTTGTAACGAGTACGGTTTCCTAGAGA<br>CACCATACCGTCGCGTTGTAGATGGCGTAGTAACAGACGAAGTAGATTACCTGTCTGCAATCGAAGAAGGGCAATTTGTTATCGCTCAGGCGAACGCTAAGCTA<br>AACGAAGATGGTACTTTTGCAGATGAGCTGATCACAGCTCGTCAGAAAGGTGAATCTGGTCTACACCCTCGTGAGCACGCTCAGTACATGG |
| >rp98 |
| CTGCAGAAGCTCTATTCGAAAGCCTATTCTTCTCTGAAGAGCGTTACGATCTATCGACTGTTGGTCGTATGAAGTTCAACAGCTCTATCGGCCGTGAAGATGCTC<br>AAGAGCAAGGCACACTTGATGAAACTGATATCATCGAAGTGATGAAGAAGCTGATCGCGATCCGTAACGGTAAGGGCGAAGTGGACGATATCGACCACCTAGG<br>CAACCGTCGTATCCGTTCTGTAGGCGAAATGGCTGAGAACCAGTTCCGTGTAGGTCTAGTACGTGTTGAGCGTGCAGTTAAAGAGCGTCTAAGCCTAGGCGATC<br>TTGACGCAATCATGCCTCAAGACTTGATCAACGCGAAGCCAATTTCTGCGGCAGTTAAAGAATTCTTTGGCTCTTCACAGCTTTCACAGTTCATGGACCAAAACA<br>ACCCGCTATCAGAAGTAACGCACAAGCGTCGTATTTCTGCATTGGGTCCTGGCGGTCTAACTCGTGAACGTGCTGGCTTCGAAGTACGTGACGTACACGTAACG<br>CACTACGGTCGTCTATGTCCTATCGAAACGCCTGAAGGTCCAAACATCGGTCTGATCAACTCTCTATCTGCGTTTGCGCGTTGTAACGAGTACGGTTTCCTAGAG<br>ACACCATACCGTCGCGTTGTAGATGGCGTAGTAACAGACGAAGTAGATTACCTGTCTGCAATCGAAGAAAGCCAATTTGTTATCGCTCAGGCGAACGCTAAGCT<br>AAACGAAGATGGTACTTTTGCA |