ANALYSIS OF MARINE BIOTOXINS: PARALYTIC AND LIPOPHILIC SHELLFISH TOXINS IN MUSSELS (*MYTILUS GALLOPROVINCIALIS)* ALONG THE NAMIBIAN COASTLINE

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Abstract

The study was carried out along the Namibian coastline, this includes Henties Bay, Swakopmund, Bird Island, Walvis Bay and Lüderitz to assess presence of shellfish marine biotoxins in mussels, *Mytilus galloprovincialis* which are filter feeders and feed on some of the algal species that produces phycotoxins, that can negatively affect the mariculture industry and human health. Samples were analysed for Paralytic and lipophilic shellfish toxins; including environmental parameters and phytoplankton species composition. Samples were collected using randomized sampling techniques for the period of May 2012 to April 2013. Mussel toxin content was measured by liquid chromatography-mass spectrometry (LC-MS/MS) for lipophilic toxins and paralytic shellfish poisoning Mouse bioassay (PSP MBA). The diarrhetic shellfish poisoning (DSP) toxin profile was found to be primarily composed of Okadaic acid (OA) and dinophysistoxins-1 (DTX-1), with the highest concentration recorded at Walvis Bay area, each toxin accounted for at least 50% of the total toxin content, this is probably due to the *Dinophysis* species reported in this area during the sampling period. In addition, Yessotoxin (YTX) and its analogues 1a-Homoyessotoxin (homo-YTX) and 45- hydroxy-yessotoxin (45-OH-YTX) were all detected at concentrations below the regulatory limit of 1 mg YTX equivalents/kg with homo-YTX dominating at Swakopmund and Bird Island stations with the highest toxin content of 0.14 mg YTX equ./kg recorded at both stations. Furthermore, PSP toxin contents in mussels were only detected at Walvis Bay and Lüderitz, with highest toxin concentrations of 80 and 903 µg STX equivalents/100g respectively. This coincided with the time at which *Alexandrium* species cell concentration at Lüderitz were at maximum, and could be a source of the paralytic shellfish toxins (PST). Although the toxin content in mussels were generally low, it still highlights the

importance of a continued monitoring of both shellfish toxicity and their causative phytoplankton species, in order to produce safe shellfish for both local and international consumers.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASP: Amnesic Shellfish Poisoning

AZA: Azaspiracids

CSIR: Council of Scientific Industrial Research

CA: Competent Authority

DSP: Diarrhetic Shellfish Poisoning

DTX: Dinophysistoxins

DTX-1: Dinophysistoxins-1

DTX-2: Dinophysistoxins-2

EU: European Union

EC: European Commission

Equ: Equivalents

FAO: Food and Agricultural Organization

HABs: Harmful Algal Blooms

HPLC: High Performance Liquid Chromatograph

LC-MS/MS: Liquid Chromatograph Mass Spectrophotometry

MBA: Mouse Bioassay

MFMR: Ministry of Fisheries and Marine Resources

NSI: Namibian Standards Institute

OA: Okadaic Acid

PP2: Phosphatase

PTX: Pectenotoxin

PSP: Paralytic Shellfish Poisoning

PST: Paralytic Shellfish Toxins

STX: Saxitoxin

YTX: Yessotoxin

YTXequ.: Yessotoxin equivalents

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Dedication

I dedicate my thesis to my late father who left us on 31 March 2013. He has always encouraged me by all means to work hard; your spiritual guidance and support will always be missed.

Revelation 3:8

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Declarations

I, Dijerenge Kahe Johanna, 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.

……. Date:……..

Dijerenge Kahe Johanna

CHAPTER ONE

1. INTRODUCTION

1.1. General background and scope of research

Shellfish mariculture in Walvis Bay, Swakopmund and Lüderitz is a developing industry with great potential, the growth and survival of shellfish species are being affected by natural occurrences of Harmful Algal Blooms (HABs) (Aquafact, 2012). The shellfish industry offers great opportunities for Namibians such as improving food security, reducing poverty, generating income and creating employment and it can also provide economic opportunities without depleting the marine resources (MFMR, 2003).

Bivalve shellfish are filter feeders and they are highly sensitive to the quality of their marine environment. They concentrate bacteria, viruses and other potentially dangerous biological contaminants that threaten consumer's health (Currie, Louw, Anderson, Fernandez, McMahon, Rangel, 2005). Bivalve shellfish also feed on microscopic plants that can produce potent biotoxins. These are compounds which are not essential to the basic metabolism and growth of the organism which are present in restricted taxonomic groups, which build up in their tissue and may cause various seafood poisoning symptoms in humans if the shellfish are consumed. These potent toxins may cause illnesses and death in marine mammals and fish that consume the shellfish (Botana, 2000). The phycotoxins most commonly detected in the Northern Benguela are lipophilic toxins (Aquafact, 2012).

Seafood poisoning have been a concern for shellfish farmers and the public at large. Shellfish toxins are stimulated by multiple environmental factors, such as nutrient increase in the water column, the augmented utilization of coastal waters for aquaculture, eutrophication and unusual climatological changes (Hallegraeff, 1993). Excess level of nutrients accelerates the formation of blooms of phytoplankton and consequently depletes oxygen in the water column, which induces reduction in marine species (Svensson, 2003).

Phytoplankton species are the primary producers that make up the base of both marine and freshwater food webs. They contain chlorophyll-a to capture sunlight, consume carbon dioxide and produce oxygen and carbohydrates in a process called photosynthesis. However, some phytoplankton species gets additional energy by consuming other phytoplankton organisms. Dinoflagellates are unicellular eukaryotes and most are autotrophic but large fractions are mixotrophy, combining photosynthesis with ingestion of prey (Stoecker, 1999). Diatoms are a major group of algae and are unicellular eukaryotes encased with a cell wall made up of silica (hydrated silicon dioxide) (Horner, 2002).

Attempts to evaluate Diarrhetic shellfish poisoning (DSP) and Paralytic Shellfish Poisoning (PSP) toxins in Namibia have been limited to the use of mouse bioassay (MBA). These analyses have been performed by the competent authority, the Namibian Standards Institute (NSI) and Council of Scientific Industrial Research (CSIR) responsible for ensuring regulatory compliance shellfish. In particular, this includes meeting European Union requirements, European Commission (EC) Council Directive 91/492/EEC (EC 1998), and EC Commission Decision 93/25/EEC (EC 1997) which regulate the production and marketing of bivalves molluscs, in the European Union.

Due to the dual threat from algal toxins, microbial and other contaminants, specific regulations and procedures have been developed internationally to ensure that shellfish are harvested, handled, processed and shipped under appropriate conditions to ensure consumer safety.

This research study will therefore focus mainly on the use of liquid chromatography - mass spectrophotometry (LC-MS/MS) to analyze *Mytilus galloprovincialis* samples for Paralytic shellfish toxins (PST) and lipophilic toxins. This method provides a rapid quantitative means for the determination of various toxins in very small biological samples. Phytoplankton species diversity and abundance were also studied to provide the baseline information for biotoxins accumulation.

1.1.1. *Mytilus galloprovincialis* ecology and life history

The mussel, *M. galloprovincialis* (Lamarck, 1819) is a marine mollusc in the family Mytilidae. It is commonly known as the Mediterranean or blue mussel. It is a filter feeder, and therefore accumulates contaminants from the environment in which they grow (Currie *et al*., 2005; Fernandez *et al*., 2004). It has succeeded in establishing itself at widely distributed points around the globe, with nearly all introductions occurring in temperate regions and at localities where there are large shipping ports (Branch & Stephanni, 2004). Ship hull fouling and transport of ballast water have been implicated in its spread and its impact on native communities and native mussels has been suggested by a number of studies and observations (Carlton, 1992; Geller, 1999).

In its native range, *M. galloprovincialis* can be found from exposed rocky outer coasts to sandy bottoms (Ceccherelli & Rossi, 1984). Although this species is cultivated as food for humans in some parts Asia, in most of its non-native range it has become a nuisance species, displacing

natives. As an invader it typically requires rocky coastlines with a high rate of water flow. This animal grows up to 140 mm in length (Branch *et al*., 1994). It is a smooth-shelled mussel with a slightly broader base than that of the black mussel, with which it is often confused. Its shell is blue-violet or black, but may shade to light brown; on one side the rim of the shell ends with a pointed and slightly bent umbo while the other side is rounded, although shell shape varies by region. It can survive up to the optimum temperature of 17^{o}C and dissolved oxygen of 9.3 ml/l (Karayucel *et al*., 2003).

In the wild, *M. galloprovincialis* are virtually absent from the subtidal areas, however in aquaculture, its growth in the subtidal areas is far superior to that in the intertidal areas. There is no known reason for its absence in the subtidal, but possible causes are believed to be selective settlement, siltation and predation on juveniles (Branch & Steffani, 2004).

Figure 1: Mussels, *Mytilus galloprovincialis*

M. galloprovincialis grows prolifically along the southwest coast of Africa (Viladomiu, 2005). It has been grown commercially in South Africa since the 1970's although currently China and Spain are the primary producers of Mediterranean mussels (FAO, 2012). Currently there is only one mussel farm that uses the long line method in Walvis Bay (Aquafact, 2012) and the upwelling conditions off the Namibian coast are ideal for mussel aquaculture because of the incredible abundance of food (Pitcher & Pillar, 2010).

1.1.2. Harmful algal blooms (HABs)

Toxic harmful algal blooms (HABs) arises due to the exponential growth or proliferation of dinoflagellates or diatoms (microscopic planktonic algae) up to millions of cells per liter that produces toxins, but do not always form blooms. Most of the toxic microalgae in the marine environment belong to the dinoflagellate group (Landsberg, 2002). However, some species occasionally occur in such large numbers, or produce toxic compounds, that eventually cause harm to other organisms (Hoppenrath *et al*., 2009).

The concentration of algae may reach levels that can kill fish or shellfish directly or indirectly by causing oxygen depletion (Hallegraeff, 1993). Contamination of shellfish, predominantly bivalve molluscs, by algal toxins is one of the most serious problems for the aquaculture and fisheries industries worldwide today, causing major economic losses and bad publicity for seafood as a food resource (Shumway, 1990; Bricelj & Shumway, 1998; Vieites & Leira, 2000). Fortunately, most countries where bivalves are exploited for food production have ongoing monitoring programs to detect toxins in both algae and shellfish, including in Namibia. These programs have markedly reduced the incidences of human intoxication in recent years (Andersen, 1996). Nevertheless, the negative effects of HAB have created a great demand for management and

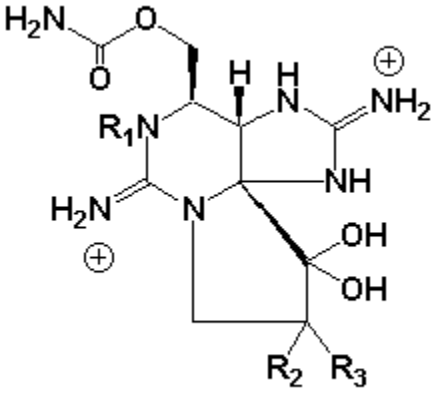
mitigation strategies to reduce the occurrence of toxic shellfish. These microscopic planktonic algae however are critical food for larvae of commercially important crustaceans and finfish.

Outbreaks of phytoplankton species associated with DSP, PSP and Amnesic shellfish Poisoning (ASP) have been detected in oysters, mussels and abalone of Walvis Bay and Lüderitz Bay shellfish farms, with mostly PSP toxins detected in abalones (Aquafact, 2012). A brief summary of these toxins will be discussed in more details due to their relevance for the current research.

1.1.2.1. Paralytic Shellfish Poisoning (PSP) toxins

Paralytic Shellfish Poisoning (PSP) is an illness caused by ingesting contaminated shellfish and may have serious and potentially fatal effects for some people. The toxins produced are Paralytic Shellfish toxins (PSTs) with saxitoxin (STX) as the parent compound. They act by binding to voltage-dependent sodium channels, thus inhibiting channel conductance and thereby blocking neuronal activity in the peripheral nervous system (Kao, 1966; Strichartz, 1984; Long *et al*., 1990); and this is the most dangerous forms of food poisoning, intoxicating consumers via mussels (Bricelj & Shumway, 1998). Until 1970, some 1600 cases of human intoxication had been recorded worldwide (Prakash *et al*; 1971). Clinical symptoms of PSP include initial numbness of the lips and face, mild headache and dizziness, in extreme cases, respiratory paralysis and death (EFSA, 2009). The EU have established a permitted level of 80 μg STX equivalents/100g. Characteristically, poisoning is sporadic and entirely unpredictable in both timing and place of occurrence, hence better professional as well as public awareness is required. In particular, genera responsible for several PSP syndromes have been observed, including *Alexandrium, Gymnodium* and *Pyrodinium* (Zhou *et al*., 1998; Liu *et al.*, 2006).

Saxitoxin and its analogues (STXs) are potent neurotoxins that block voltage-gated sodium channels on excitable cells (Kao, 1966). Many studies have been carried out on the toxin-producing dinoflagellates and the toxins they produce. There is much known about the taxonomy and ecology of the toxin-producing dinoflagellates (Steidinger, 1993). The chemical structures and pharmacological properties of the toxins have been well defined (Schantz & Kao 1986); however, little is known about the biosynthetic pathway for STXs in the STX-producing organisms. Shimizu *et al*., (1984) proved that the skeleton of STXs is synthesized from arginine and acetate. However, the process of synthesis, the enzymes involved and the biochemical function of the toxins in the toxin-producing dinoflagellates have not been defined (Botana, 2000).



STX	R_1	R_2	R_3
STX	H	H	H
GTX-II	H	H	OSO_3^-
GTX-III	H	OSO_3^-	H
NeoSTX	OH	H	H
GTX-I	OH	H	OSO_3^-
GTX-IV	OH	OSO_3^-	H

Figure 2: Basic structure of Saxitoxin (STX) (Shimizu *et al*., 1984).

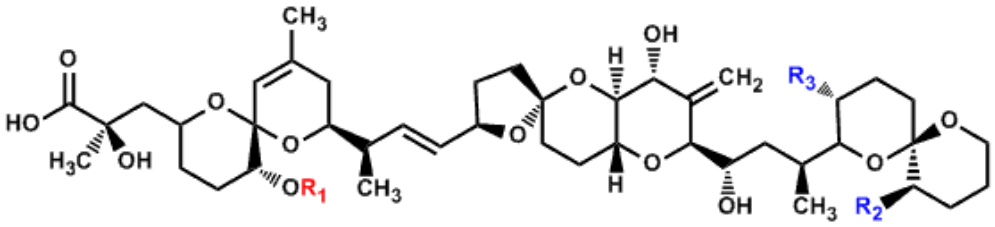
Differences in toxin profiles have been observed among strains of the same dinoflagellate species collected from different geographical locations. Based on these findings, some researchers have attempted to use the toxin profile as a biochemical marker to distinguish strains

of the same species (Oshima *et al*., 1990). Toxin profiles are reported to be influenced by physiological conditions such as nutrient conditions, the stage of the cell cycle, and other factors (Anderson *et al*., 1990).

1.1.2.2. Diarrhetic Shellfish Poisoning (DSP) toxins

This group of polyether toxins and has currently been refered to as lipophilic toxins which includes; Okadaic acid (OA), Dinophysistoxin-1 (DTX-1), -2 (DTX-2) and Pectenotoxin (PTX) (Yasumoto *et al*., 1985). DSP is a gastrointestinal illness which is caused by the consumption of contaminated shellfish. The first known incidences of this illness associated with consumption of mussels exposed to dinoflagellates were in Netherlands in the 1960s (Kat, 1979) similar symptoms were described in Japan in the 1970s (Yasumoto *et al*., 1978). The dominating symptoms are diarrhea, nausea, vomiting, and abdominal pain (EFSA, 2009). The EU has established a permissible level of 160 μg OA-equivalents/kg. Very little is known about the toxicokinetics concerning the toxins in the DSP complex, both in animals and man. Due to their lipophilicity, they might be expected to be easily absorbed and distributed in the human body (Falconer, 1993). The causative genus organisms are *Dinophysis* and *Prorocentrum* species, although there is an uneven distribution among species and location of toxin production, these dinoflagellates are widely distributed, but do not always form blooms. The associated toxins produced by dinoflagellates are Okadaic acid and its derivatives; of which at least 9 toxins produced (Lagos & Andrinolo, 2000). The common toxins are DTX-1, DTX-2 and DTX-3, all of which accumulate predominantly in the digestive organs of filter-feeding organisms (Yasumoto *et al*., 1978).

The recently discovered azaspiracids (AZA) in mussels from Ireland, UK and Norway produce a gastrointestinal intoxication in humans with similar symptoms as DSP (James *et al*., 2000). AZA has unique structural features and it has been named azapiracid poisoning (AZP). Thus in this work, only OA and DTXs will be covered as DSP toxins, which are collectively known as OA-group toxins (McNabb, 2008).



	R_1	R_2	R_3
OA	H	H	CH_3
DTX1	H	CH_3	CH_3
DTX2	H	CH_3	H
7-O-Acyl	Ac	same as above	

Figure 3: Chemical Structure of the causative agents of DSP, Okadaic acid (OA) and its analogues (www.fda.gov).

Figure 3 indicates Okadaic acid (OA) and its analogues, the dinophysistoxins (DTX-1, DTX-2, and DTX-3), together form the group of OA-toxins. OA and DTX-2 only differ by the position of one methyl group in the molecule, DTX-1 has one additional methyl group and DTX-3 represents a wide range of derivatives of OA, DTX-1 and DTX-2 esterified with saturated and unsaturated fatty acids (Hu *et al*., 1995).

Algal toxins are structurally and functionally diverse, and many are derived from unique biosynthesis pathways. Dinoflagellates, for example, synthesize a variety of polyether toxins, which fall into two structural groups, linear or fused (Yasumoto, 1990).

The relationship between the chemical structure of OA and its inhibitory effect on the protein phosphatases has been studied to some extent by experiments with OA derivatives (Nishiwaki *et al*., 1990).

1.1.2.3. Yessotoxin (YTX)

Yessotoxin (YTX) is a group of lipophilic sulfated polyether compounds. They are produced by a number of planktonic algal species particularly the dinoflagellates, *Lingulodinium polyedrum, Gonyaulax spinifera* and *Protoceratium reticulatum* (Gerssen *et al*., 2010; Álvarez *et al*., 2011).

YTXs have been associated with DSP because they are often simultaneously extracted with DSP toxins. However, recent evidence suggests that YTXs should be excluded from the DSP toxins group, because unlike OA and DTX-1, YTXs do not cause either diarrhea or inhibition of protein phosphatases (Paz *et al*., 2008). YTXs have also been shown to have cardiotoxic effects in mice but have not been shown to be toxic to humans, though the mode of action is still being investigated (Trainer et al., 2010; Gerssen et al., 2010). To date, 90 different analogues have been identified (Miles *et al*., 2005). The regulatory limit is 1 mg/kg.

Table 1: A summary of algal toxin poisoning and biological effects (Hallegraeff, 2003).

Human Syndrome	**Toxin group**	**Symptoms**		**Biological effects**
		Mild	**Severe**	
Paralytic shellfish poisoning, PSP	**Paralytic Shellfish Toxins (PST); Saxitoxin**	Within 30 minutes: tingling sensation or numbness around lips gradually spreading to face and neck, prickly sensation in fingertips and toes, headache, dizziness, nausea, vomiting, diarrhoea.	Muscular paralysis, pronounced respiratory difficulty, choking sensation, death through respiratory paralysis may occur within 2-24 hours.	Neurotoxic, block the Na+ channels of neuronal and muscular cells, preventing depolarization and propagation of the action potential
Diarrhetic shellfish poisoning, DSP	**Lipophilic toxins:** **Okadaic Acid, Dinophysistoxins and Pectenotoxins**	After 30 min.- a few hours (seldom more than 12 hours): diarrhoea, nausea, Vomiting, abdominal pain.	Chronic exposure may promote tumor Formation in the digestive system.	Inhibitors of protein phosphatase enzymes, OA presumably a tumor promoter

Table 2: Marine algal toxins, associated syndromes and producing species discussed in the study (modified from Van Dolah, 2000).

Syndrome	**Causative organism**	**Toxin**	**Regulatory limit**
Paralytic Shellfish Poisoning (PSP)	*Alexandrium* spp. *Gymnodinium* spp. *Pyrodinium* spp.	Saxitoxin (STX)	80 µg STX equivalents/ 100g
Diarrhetic Shellfish Poisoning (DSP)	*Prorocentrum lima* *Dinophysis* spp.	Okadaic acid (OA) Dinophysistoxins (DTX-1 & DTX-2) Pectenotoxin (PTX)	160 µg OA equivalents/Kg
Yessotoxins	*Gonyaulax spinifera*	Yessotoxin (YTX) Homo-YTX 45-OH- YTX	1 mg YTX equivalents/Kg

1.2. Problem statement and justification

Aquaculture is one of the fastest growing industries in the world (MFMR, 2003). The growing of shellfish species takes place in the Benguela upwelling system, and it provides nutrient-rich deep water to the euphotic zone (Pitcher & Pillar, 2010). This enhances the growth of phytoplankton and thus facilitate the frequent proliferation of harmful algal blooms (Hallegraeff, 1995). These phycotoxins producing phytoplankton accumulate in the shellfish flesh and may cause harm to consumers due to seafood poisoning (Pitcher, 1998). The implication of consuming contaminated shellfish may be devastating; it may lead to loss of life and subsequently loss of revenue due to farm closures and loss of markets. Therefore, the growing aquaculture industry needs a practical approach to assure the safety as well as the quality of farmed shellfish is within the recommended level.

Various incidents of HABs have been recorded in Namibian waters with the presence toxic dinoflagellates and as well as diatoms (MFMR, database). Previously, in Namibia, a food safety scare from the public eating mussels from a mariculture farm was reported from a restaurant in August 2011, analysis for biotoxins and *Escherichia coli* were done, and a DSP positive test result was recorded (Russell, 2006). Mouse bioassay (MBA) method (Yasumoto *et al*., 1985) was used for analysis which gives a qualitative result. As this method is very unspecific, other toxins can be detected.

Since the beginning of the Namibian shellfish sanitation monitoring programme, positive results for lipophilic toxins have been inconclusive for some major reasons: they have not been associated with their specific toxic or potentially toxic phytoplankton species; the results were obtained using MBA method which does not give information on the types of lipophilic toxins present.

1.3. Main Objectives

This study investigates the presence of marine biotoxins with their potential producers of the toxic phytoplankton species which aims mainly at assisting the Namibian shellfish sanitation monitoring programme.

1. To determine and compare presence of marine biotoxins in *M. galloprovincialis* collected from different locations along the Namibian shoreline (Henties Bay, Swakopmund, Bird Island, Walvis Bay & Lüderitz).

2. To assess the diversity and abundance of phytoplankton species (toxic and non-toxic) collected from the selected locations along the Namibian shoreline, and if they associate with the toxicity in *M. galloprovincialis* flesh.
3. To assess if marine biotoxins present in *M. galloprovincialis* fall within the European Union regulatory level for human consumption.

1.3.1. Hypotheses:

1. There are no significance differences in mean toxin concentration in *M. galloprovincialis* samples between stations.
2. There is no relationship between the abundance of the causative algal species and the presence of toxin in *M. galloprovincialis* samples.
3. The mean toxin concentrations in *M. galloprovincialis* are not within the regulatory limit set by the European Union

1.4. Significance of the study

It is envisaged that this study would be able to assist research institutes in the development of a practical strategy to reduce hazards that may be present in farmed shellfish to acceptable levels as required by markets. In addition, the research information would be vital to shellfish farmers as this information would give them an indication to improve on a practical quality assurance system. Moreover, this study will contribute to the scientific knowledge in the field of shellfish safety through publication of papers. It is also anticipated that the data obtained from this investigation will enable policy makers in governments and other stakeholders to develop

policies on the safety of farmed shellfish, hence the necessity for consumers to eat uncontaminated shellfish products.

1.5. Limitation of the study

Every scientific study investigation most probably has challenges to some extent and this study was not an exception. Another limitation was the samples collected from Lüderitz were not collected on a monthly basis as the central coast stations due to limited resources both financial and time; however the samples were collected during bloom and after bloom periods. In addition, this investigation was only conical to one shellfish species but there are other shellfish species such as oysters and abalone which are also to prone to marine biotoxins.

CHAPTER TWO

2. LITERATURE REVIEW

Diatoms and dinoflagellates are microalgae that make up the base of the marine food webs that support marine organisms. However, certain species produce potent toxins that are responsible for extensive fish kills, shellfish and human illnesses. Some algae can adversely affect growth and survival of larvae or adults of commercially important shellfish populations (Hallegraeff, 1993). Diatoms will dominate under high nutrient; high turbulent conditions while dinoflagellates dominate under low nutrient, low turbulent conditions. Historically, the identification of an algal toxin as the causative agent in shellfish events has been difficult due to inadequate detection methods for these toxins (Joseph *et al*., 2003).

Algal toxins that impact human health may be functionally categorized as neurotoxins or hepatototoxins (Cembella *et al*., 1987). Neurotoxicity of algal toxins is mediated by diverse, highly specific interactions with ion channels involved in neurotransmission. Such specificity may reflect their role in anti-predation. Alternatively, it may suggest the presence of conserved structures on primitive targets present in eukaryotic microbes, or it may support the hypothesis of primitive regulatory roles for these compounds (Botana, 2000).

Cultured mussels from Lüderitz tested positive for PSP in the mid-1990, which might have been caused by *Alexandrium catenella* (Pitcher, 1998). A number of times abalone and oysters in Lüderitz Bay tested positive for DSP and PSP toxins (Aquafact, 2012).

Since 2005, toxic species of phytoplankton have been monitored in both the Central and Lüderitz coastline. And closures of these sites for harvesting shellfish are usually recommended when cell numbers exceed 500 cells/L of *Alexandrium* spp. (Gudfinnsson *et al*., 2010).

It has been shown that there could be a high heterogeneity of DSP toxins in mussels grown at different depths along the raft-cultivated mussels and great differences between the different mussels growing sites in closely located areas (Edebo *et al*. 1988). It can also be argued that wild mussels have conditions different from the raft-cultivated mussels because the interactions between algae and mussels could be quite different (Hickman, 1992).

Several studies on shellfish toxins have been documented worldwide although more needs to be done. In Namibia, despite potentially toxin species and cysts having been sampled in Namibian waters, there are no records of human mortality that implicate micro algal biotoxins as the causative factor (Tejedor *et al*., 2004). Admittedly, the coastal community is small and harvesting of shellfish is minimal, so toxic incidents could pass undetected.

On the South African west coast, blooms of *Ceratium furca, Ceratium lineatum, Prorocentrum micans* and to a lesser extent *Alexandrium catenella* dominate (Pitcher and Calder, 2000). In the northern Benguela, *Gonyaulax tamarensis* (now *Alexandrium tamarense*), a recognized PSP – producing dinoflagellate was reported to be one of the species regularly responsible for an algal bloom in the Walvis Bay Region. Despite frequent blooms of this species, mussel poisoning was never reported on the Namibian coast (Pietrse & Van Der Post, 1967), until recent attempts to market mussels grown in the Lüderitz region failed as mouse bioassays for PSP regularly tested positive. The responsible dinoflagellate, *Alexandrium catenella* has been identified in mussel samples collected in the Lüderitz region (Sea Fisheries Research Institute, Unpublished data). It

is known from the Lüderitz area that mussels typically are continuously toxic with PSP during the winter seasons (MFMR, unpublished data) due to their low depuration rate.

In the study by Carlos *et al*., (2003), *Mytilus chilensis* from Chile concentrated PSP and DSP toxins in amounts above the international regulatory limits, after using an HPLC analysis with pre-column derivatization method for DSP toxins and post-column derivatization methods for PSP toxins, both with fluorescent detections.

Shellfish toxins is of equal importance to consumers and producers of shellfish, as well as to regulators of food safety. Human poisoning resulting from ingestion of seafood contained by phycotoxins has occurred in the past, and historical records as well as the habits of some populations in coastal and tropical areas show that harmful algal blooms (HABs) are naturally occurring events Hallegraeff *et al*., 2003). On a global basis, almost 2 000 cases of human poisonings are reported per year, with a 15 % mortality rate (Hallegraeff, 1993). PSP-producing algal blooms occur worldwide in both temperate and tropical waters.

Mussels, clams, cockles and scallops that have fed on toxic dinoflagellates retain the toxin for varying periods of time depending on the shellfish. Some clear the toxin very quickly and are only toxic during the actual bloom, others retain the toxin for a long time, even years (Schantz, 1984).

Molecules of STX and related compounds act as potent neurotoxin sodium-channel blockers and are some of the most dangerous forms of food poisoning, intoxicating consumers via mussels. HPLC methods must therefore be used if detailed information is required about the production, transformation, and different toxicity of PSP toxins (Hummert *et. al*., 1996).

The study conducted by Prakash *et al*., (1971) indicated that little is known about the factors that regulate the detoxification rate and showed that PSP detoxification varied according to the season along the eastern coast of Canada; it is frequently assumed that low water temperatures retard toxin loss (Shumway *et al*., 1995).

There have been various reports of DSP toxins in Chinese shellfish and an investigation into DSP along the Chinese coast showed that DSP toxins were more frequent than PSP, although levels were relatively low (Zhou *et al*., 1999). A more recent investigation of lipophilic toxins along the Chinese coast showed that less than 10% of samples were positive when analyzed by LC-MS (Liu *et al*., 2011). *Dinophysis acuminata* was found in the same area in the summer of 2006.

Indicated in the study by Li *et al*., (2012), shellfish poisoning incident occurred near the coast of the East China Sea in May 2011. More than 200 people fell ill after consuming mussels (*Mytilus galloprovincialis*), and reported symptoms including diarrhea and abdominal pain, typical of DSP. LC-MS/MS analysis showed that high concentrations of OA, DTX-1 and their acyl esters were responsible for the incidents.

Certain toxin producing species, significant impacts occur at population densities of only several hundred cells per liter. For example, *Dinophysis* need only be present at 100s of cells 1^{-1} to induce diarrhetic symptoms, as they are concentrated by shellfish and then ingested by human consumers (Grattan *et al*., 2001). No effect on the feeding behavior by DSP-producing species has been found to date, neither in natural episodes nor in experimental intoxication (Botana, 2000).

Differences in toxin profiles have been observed among the species belonging to the genus *Alexandrium.* These differences have also been observed among strains of the same species collected from different geographical locations. Based on these findings, some researchers have attempted to use the toxin profile as a biochemical marker to distinguish strains of the same species (Boyer *et al*., 1986; Cembella *et al*., 1987; Oshima *et al*., 1990). Toxin profiles are reported to be influenced by physiological conditions such as nutritional conditions, the stage of the cell cycle, and other factors (Anderson *et al*., 1990).

The importance of phosphatase (PP2) in a cellular homeostasis is underscored by the fact that several organisms produce PP2A-inhibiting toxins, presumably as a self-defense mechanism. It has been demonstrated that OA inhibits the growth of a variety of microalgae in a concentration-dependent manner, but not that of a DSP-producing dinoflagellates, *Prorocentrum lima* (Windust *et al*., 1996). OA is extremely potent and enhances overall levels of protein phosphorylation when present at nanomolar levels within the cell (Haystead *et al*., 1989).

The effects of different toxins vary greatly among different species and large interspecific differences in both accumulation and elimination of specific toxins are documented. The most documented examples concern the response on nerve cells to paralytic shellfish toxins (Bricelj & Shumway, 1998). So far, there is no documentation from the field or laboratory which suggests that the survival of mussels is negatively affected by the DSP toxins (Landsberg, 2002). Hence, in Namibia it is very important to perform quantitative analysis of DSP toxins in Shellfish samples. Firstly, because DSP toxins are endemic in shellfish in the central and southern regions; and as well as the mouse bioassay performed in Namibia is qualitative, and there's no information on the types of lipophilic toxins, so the level of toxins is never known.

Various species of *Dinophysis* have been recorded on the Namibian coast, and shellfish in the northern Benguela tested positive DSP as mentioned earlier (Pitcher *et al*., 1993). The widespread occurrence of *Dinophysis* on the Namibian and South African coasts, their presence throughout the upwelling months and the ability of low cell concentrations to render shellfish toxic demonstrate the potential for this shellfish poisoning (DSP) syndrome to severely restrict growth of the shellfish industry in the Benguela region (Pitcher & Pillar, 2010).

In the Benguela current system, YTX has only been detected in Southern Benguela from *Protoceratium reticulatum* cultures (Trainer *et al*., 2010). In recent studies by Chikwililwa *et al*., 2013, YTX and its analogues were detected in Northern Benguela in *Gonyaulax spinifera* and shellfish in 2011. The values measured in the shellfish exceeded the regulatory limit, and the dominant YTX analogues present were 45-OH YTX and homo-YTX.

Reasons for the increasing interest in HABs include not only public safety concerns associated with protecting human health, but also adverse effects on living resources of many coastal systems, economic losses attributed to reduced tourism, recreation, or seafood related industries, and costs required to maintain public advisory services and monitoring programs for shellfish toxins, water quality, and plankton composition.

CHAPTER THREE

3. METHODOLOGY

3.1. Study Area

Figure 4: Map of the study Area

Source: Google Maps

The study was conducted along the central coastline of Namibia, Henties bay (S 22^0 08' 55.1", E 014^o 17' 22.3"); Swakopmund (S 22^o 41' 57.0", E 014^o 31' 16.8"), Long Beach (Bird Island, S 22^o 52' 33.2", E 014 32' 22.3"), Walvis Bay (S 22^o 52' 33.2", E 014^o 32' 22.3") and Lüderitz (S 26^o 38' 45", E 15^o 9' 14") in figure 4. The coordinates of the sampling stations were marked using a portable Global Positioning System Device (Garmin GPS, Model Etrex 30). The

selection of sites was based on the Namibian shellfish and biotoxin monitoring programme, due to the shellfish farming taking place and potential upcoming farming of shellfish species. Presently, oyster, mussels and abalone is farmed in Swakopmund, Walvis Bay and Lüderitz, while Henties Bay and Bird Island are the proposed areas for upcoming aquaculture farming. Prior to sample collection, an observational visit was undertaken to roughly estimate the availability of mussels, and *M. galloprovincialis* were found to be abundant on rocky shores.

3.2. Collection and Analysis of phytoplankton species

3.2.1. Collection Technique

Phytoplankton samples were collected from the specified locations at a depth of about 1 m, with a net of 10.4 cm diameter mouth opening and a mesh size of 20 µm. Nets are commonly used to concentrate phytoplankton and usually a quantitative sample is collected. The phytoplankton net was towed horizontally, gently to avoid rupture of cells. The identification was done using an inverted microscope in a contrast phase by 20x magnification for species identification.

3.2.2. Species composition and abundance

Toxic species were then counted according to the standard of UtermÖhl technique (Utermöhl, 1958). Utermöhl method is used for bio-diversity, identification of harmful species and quantitative analysis. Therefore phytoplankton analysis is an essential part in the process of understanding and predicting changes in our marine environment. The Utermöhl method involves the microscopic examination of a preserved water sample through the process of sedimentation of an aliquot of water sample in a chamber (Edler and Elbrachter, 1979). After the samples have been preserved in 20% formalin solution, the cell counts was done by settling 20 ml of water on a settling chamber for 24 hours. Gravity causes the phytoplankton cells to settle

on the bottom of the chamber. The settled phytoplankton cells were then identified and taxonomically named using an inverted microscope. This method was only used for the Lüderitz sites.

The results of the count were expressed in cells per liter, thus establishing the numerical density of toxic species and total phytoplankton.

$$Cell\ Concentration = Cells\ Count * \frac{Area\ Chamber}{Area\ Counted} * \frac{1000\ (ml)}{Volume\ Settled(ml)}$$

(Source: Edler and Elbrachter, 1979)

3.4. Analysis of Marine Biotoxins

3.4.1. Research Design

This study was conducted using a quantitative research design. Shellfish toxins analyzed were; paralytic shellfish toxins, diarrhetic shellfish toxins and other lipophilic toxins. Occurrence of phytoplankton species diversity, seasons (winter and summer), temperature and dissolved oxygen of the samples were investigated. Analysis of variance (ANOVA) showed significant differences at 0.05 significance level was used to determine the variation of means for temperature and dissolved oxygen. All mean levels have been reported as mean± of standard error (SE).

3.5. Sampling

3.5.1. Population

The study populations are mussels, *Mytilus galloprovincialis* which were collected from the specified locations along the Namibian coastline.

3.5.2. Sampling Techniques

Simple random sampling (probability) technique was used when collecting mussel samples as this method allowed each and every element of the population to have an equal chance of being selected to be part of the sample. All water parameters measurements were done on site during each sampling period and it was determined by the YSI 550-12 model DO meter.

3.5.3. Sample size selection

Samples in this study were collected along the Namibian coastal areas from May 2012 to April 2013. They were five sampling stations (Henties Bay, Swakopmund, Bird Island, Walvis Bay and Lüderitz). Lüderitz samples were only collected during bloom and after the bloom period. They were 30 mussels collected from each station during every sampling period. The 30 mussels represented one sample per station as these mussels were homogenized and sub-sampled for PSP, DSP and other lipophilic toxin analysis. Therefore, from this population a total of 70 samples been taken into consideration for testing, 37 for PSP toxins by MBA and 33 samples for DSP and lipophilic toxin analysis by LC-MS/MS method, and whereby ± 2g was needed for the LC-MS/MS and 100 g for PSP analysis.

3.5.4. Sampling procedure

When samples were collected, the sampling date, location/station, and species name were recorded and all sampling containers were labeled for identification. All mussel collected were

within a similar range of length and similar depth. Mussel samples were collected during low tide, except those collected from Walvis Bay and Lüderitz stations, as they were collected from the suspended long oyster lines.

Research ethics were considered and observed to make sure the whole investigation is affected. The experiment was conducted in such a way that proper care for both the environment and of animal welfare is measured. Trampling when sampling mostly occurred in dense mussel beds, it was avoided as much as possible. In addition, extraction of these mussels causes direct decreases in abundances and often alters the size structure of the population, at the same time they provide food and shelter for other organisms. Therefore, proper handling of the animal was followed in returning them back into the sea if not needed for research rather than just discarding them. This also included sacrificing them before analysis by employing slow death technique under controlled freezer conditions. The study also complied with the regulations implemented by Ministry of Fisheries and Marine Resources on mussel's collection in terms of limited size and number, and as well as during transportation to the laboratory.

All samples collected were put on ice in a cooler box to minimize spoilage during transportation. Samples were stored in polythene bags in a freezer at -20 ^{0}C in the National Marine Information and Research Center (NatMIRC) laboratory till extraction and analysis processes were done at food and beverage laboratory in Cape Town, Council for Scientific and Industrial Research (CSIR).

3.5.5. Research equipment

A chromatography data processing workstation, an AB-Sciex QTrap 4000 Liquid Chromatography-Mass Spectrophotometer was used to analyze DSP toxins. PSP toxin analyses

were conducted using the Mouse Bioassay protocols, as per AOAC method 959.08, which is summarized under the heading of test method for the determination of PSP toxins by Mouse Bioassay. The results were calculated using a computer-based version of Sommer's table and correction table for mouse weight (Sommer & Meyer, 1937).

3.5.6. Preparation of mussel sample tissues for PSP and DSP toxins

After the mussels were harvested, they were sacrificed, thawed and prepared by dissecting and removing the whole flesh from the shell, after cleaning the outside of shellfish with fresh water, open shellfish, after removal from shellfish, drain tissues in a sieve to remove salt water. Then transferred the sample into a waring commercial blender, model HGBWTS3. Blended until homogenous, subsampling from this homogenate was done immediately after blending while still well mixed, or it could have been done later after mixing again. A weight of 50 - 150g of ground tissue was weighed directly into the extraction tube for PSP and DSP extraction, whereby a 100g of tissue homogenate was required for Mouse bioassay, and ±2 g was needed for the LC-MS/MS analyses. Containers containing tissues homogenates were tightly sealed and stored for several weeks at -20^{o}C in a freezer before extraction and analysis.

3.5.7. Extraction of PSP toxin analysis samples

100g of the tissue homogenate is weighed out into a 600ml beaker. Using a measuring cylinder 100ml of 0.1 N HCL is added and stir and check pH (pH should be <4.0). Heat mixture to boiling with constant stirring (5-7 minutes) and boil gently for 5 minutes then cool to room temperature. Adjust pH to 2.0-4.0 with 6N HCL or 0.1N NaOH. Add distilled water to make up weight to 200g with constant stirring to prevent local alkalinization and consequent destruction or conversion of PSP toxins. Allow homogenate to settle and centrifuge 5ml of supernatant at

4^{o}C for 5 minutes at 3500rpm. Of the centrifuged supernatant, 1ml was used to inject into the mice.

3.5.8. Extraction of Lipophilic Toxins

A 2.00 g portion of homogenized tissue sample was accurately weighed into a 50 ml plastic centrifuge tube. 9.00 ml of 100% methanol was added and homogenized the sample via vortex mixing for 3 min at maximum speed level. Centrifuged at 2000 *g* or higher for 10 min at approximately 20 ^{o}C and transferred the supernatant to a 20 ml volumetric flask. Repeated the extraction of the residual tissue pellet with another 9.0 ml of methanol 100% and homogenized for 1 min in Ultra Turrax^{TM}. After centrifugation, combined the supernatant with the first extract and make up the extract to 20 ml with 100% methanol.

DSP toxins were analyzed based on pure Certified Reference Material (CRM) for each toxin standard according to the EU- harmonized standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS, the methods were based on Gerssen *et al*., 2009.

LC-MS/MS is the most powerful chemical method today to identify and characterize toxins, especially previously unknown compounds (Marr *et al.*, 1992; Quilliam, 1995; Draisci *et al.*, 1995).

3.5.9. Mouse Bioassay (MBA) analysis for PSP toxins

PSP toxin analyses were conducted using the Mouse Bioassay protocols, as per AOAC method 959.08 (2005). This method is based on the acute toxic effect of a Hydrochloric acid extraction of the mussel tissue sample on mice. 1 ml of this extract is injected intraperitoneally into an

albino mice weighing (20±1g). This 1ml of extract corresponds to 100g of original tissue. The calculation of the toxicity is based upon the death time of the mice injected. This is followed by converting the time of death in mouse units (MU) using Sommer's table (Appendix 4.8). The method is based on the dose of PSP toxins (equivalent amount of Saxitoxin) that provokes death in mice within 1 hour after intraperitoneal injection of the shellfish extract.

3.5.10. Procedural quality control for LC-MS method

The presence of each toxin was identified, with the reference standards available, by comparing the retention time of the analytes in the sample with those of the standards.

The quantification of each toxin is determined using the external standard calibration method. Evaluation is based on the linear equation of the regression line of the individual toxins with standards available. Therefore, from the calibration curve, the concentration of the individual toxins in each analyzed sample is calculated using the following equation:

$$\text{Concentration (µg toxin/kg)} = \left(\frac{y-b}{a}\right) x \frac{\frac{V_F(ml)}{V_H(ml)} \times V_T(ml)}{W(g)} \times D$$

Source: (EURLMB, 2011)

Where:

y = Area of the chromatographic peak

b = intercept of the regression linear

a = slope of the calibration curve

V_T = Total volume of crude extract (20 mL)

V_H = Volume of extract used for performing the hydrolysis

VF = Final volume of extract after hydrolysis (and clean-up /concentration)

W = Sample tissue weigh (2 g)

D = Dilution Factor (if extract has been diluted)

Certificate of Analysis

The results of the sample analyzed were expressed in a report form, which showed free, and Total OA, DTX-1, DTX-2, PTX-1, PTX-2, AZA-1, AZA-2, YTX, 45-OH YTX, and homo-YTX. The free concentration of each Toxin is referring to the initial measurement that was made of the sample after extraction with methanol. But in order to be able to measure all the toxin content an alkaline hydrolysis on the portion of the same sample extract in order to convert any acylated esters of OA or DTX if its present, into the parent compound (OA). Therefore if the free and total values of toxin are the same, there were no OA esters in the sample. This report concentrates only on OA and YTX and its analogues.

3.6. Data Analysis

Concentrations in this study have been expressed based on the regulatory limit for each toxin (mg and μg); and cells/L for phytoplankton cell counts. GENSTAT Discovery Edition 4 software for statistical analysis was used to analyze physicochemical parameter data. Microsoft Office excel 2010 data analysis was used for toxin concentration. Data were tested for normality at 0.05 level of significance. Water parameters data was assessed using one way parametric Analysis of Variance (ANOVA) of completely randomized design (CRD). The student *t*-test was used to

determine and compare the mean differences of the water parameters between two seasons and location.

The mean value for water temperature and dissolved oxygen were measured as the mean of the standard error (± SE).

CHAPTER FOUR

4. RESULTS

4.1. Sea surface temperature (SST) and dissolved oxygen (DO)

The results from the study showed some variations in both temperature and dissolved oxygen mean values. The maximum mean temperature recorded during the experiment at 0.5 m depth is 16.8±0.27 ^{o}C and 24.3 ±1.13 ^{o}C during winter and summer, respectively for central coast (Table 3). The highest temperature in winter season was recorded in Swakopmund station and whiles the highest temperature in summer, which showed an increase was recorded in Henties Bay. The minimum mean temperature value of 12.5±0.13 ^{o}C and 12.2 ±0.71 ^{o}C in seawater was recorded for winter and summer seasons respectively. However, both minimum values were recorded in Walvis Bay station. These results conclude the temperature change between winter and a summer season was an average of 0.49 ^{o}C.

Table 3: Mean temperature and dissolved oxygen for all stations.

Station	Mean±SE			
	Temperature (^{o}C)		**Dissolved oxygen (mg/L)**	
	Winter	Summer	Winter	Summer
Henties Bay	15.21±0.23	18.18±1.13	6.44±0.34	8.13±0.86
Swakopmund	15.44±0.27	17.54±0.81	9.61±0.60	7.81±0.42
Bird Island	15.59±0.29	17.74±0.65	6.48±0.32	6.26±0.39
Walvis Bay	13.13±0.13	14.74±0.71	3.51±0.54	4.80±0.15
Lüderitz	12.35±0.078	13.05±0.18	-	-

(-) No records taken

Walvis Bay recorded the lowest mean temperature of 13.13^{o}C and Henties Bay had the highest temperature 18.18 ^{o}C. The lowest dissolved oxygen was 3.51 mg/L, and no dissolved oxygen was recorded at Lüderitz stations due to equipment availability. All the stations showed an increase in temperatures from winter to summer, while for Bird Island and Swakopmund stations decreased in dissolved oxygen in summer.

Two sample independent *t* – test analysis showed that there were significant differences in mean temperature value between winter and summer seasons across the stations at 95 % confidence interval ($P<0.05$).

Two sample independent *t* - test analysis showed that there were no significant differences in mean dissolved oxygen value between winter and summer seasons across the stations at 95 % ($P>0.05$).

4.2. Phytoplankton cell counts and shellfish toxin analysis during Lüderitz bloom

During the period of June-July 2012, a remarkable concentrations consisting of *Alexandrium* spp. cells were detected along the Lüderitz lagoon area. A maximum of 111 500 cells L^{-1} was reached on 21 June 2012 (Figure 5). In the same period, low cell concentrations of *Dinophysis acuminata* and *Gyrodinium* sp. (500 cells L^{-1}) were as well identified together with cells belonging to the *Prorocentrum* genus. These are some of the toxic species which are known to produce DSP toxins. The most abundant specie was identified to be composed primarily of *Alexandrium catenella.*

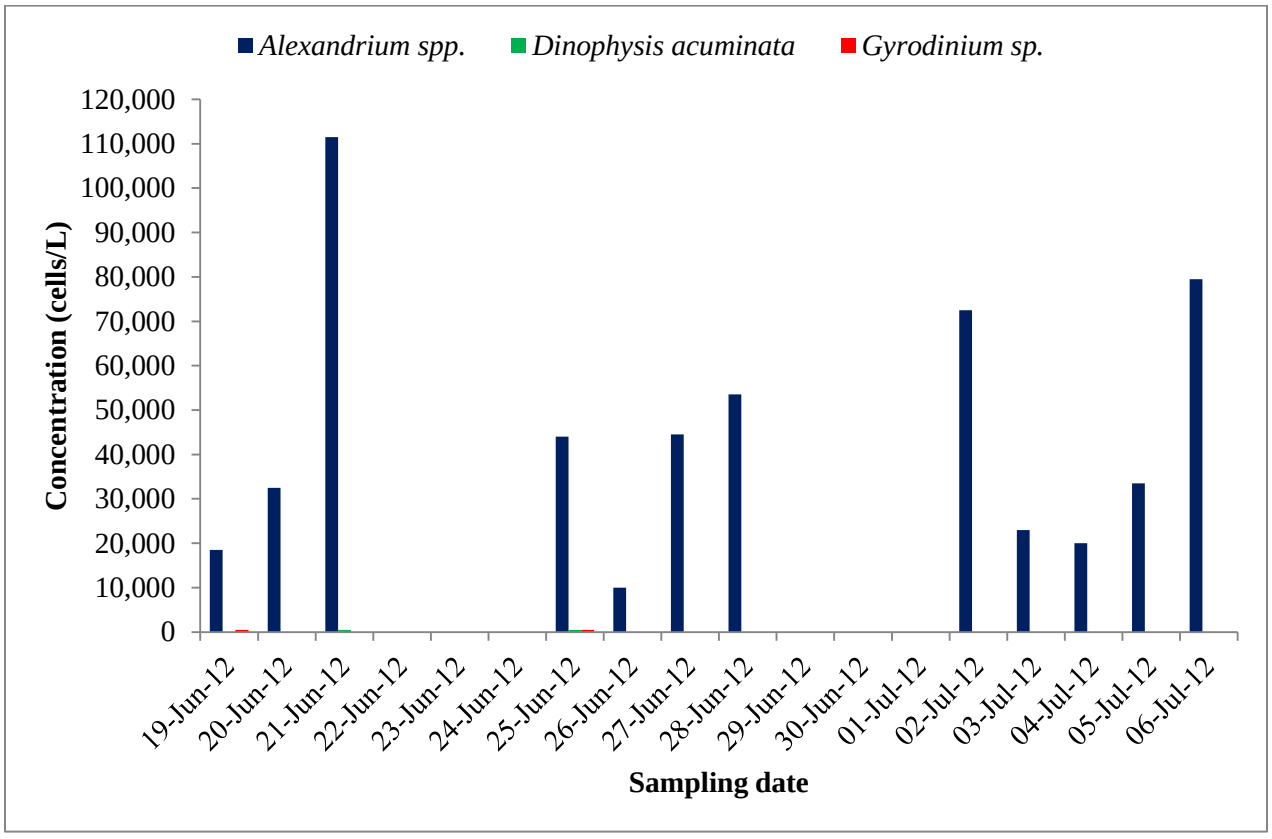


Figure 5: *Alexandrium* spp. cell counts (cells/L) during the bloom of June/July 2012 from Lüderitz lagoon area.

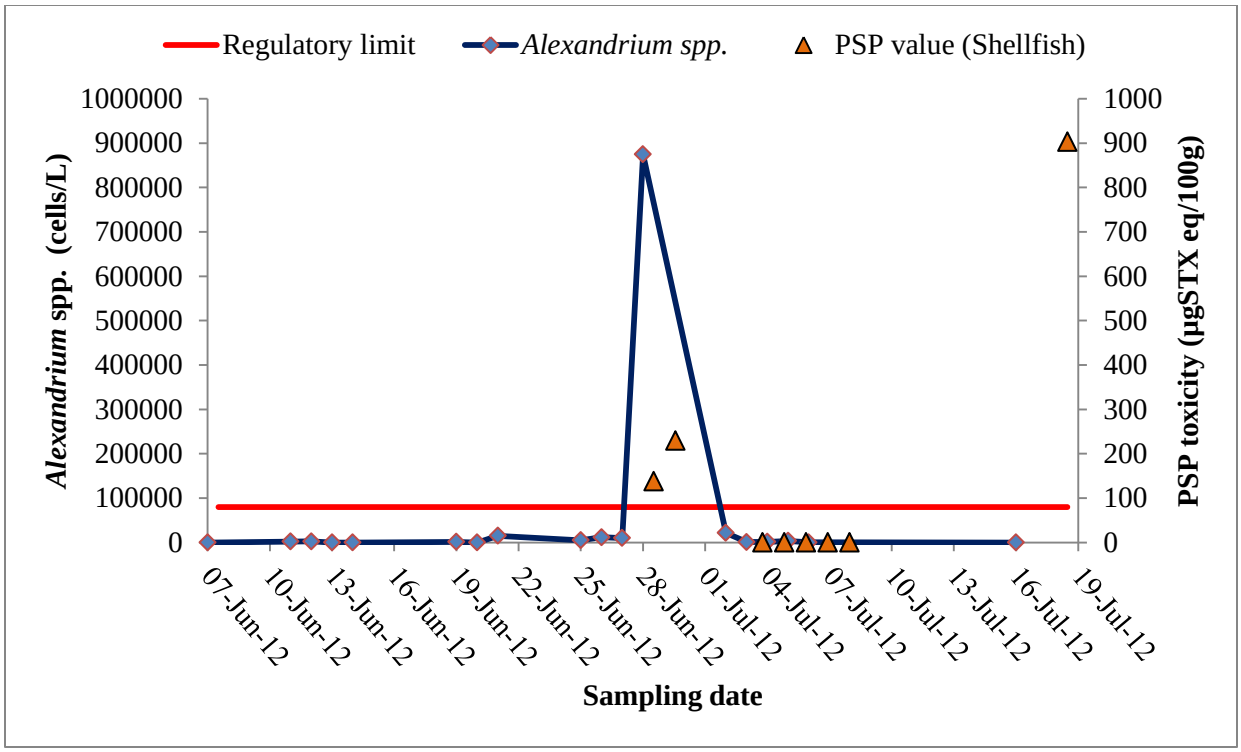


Figure 6: *Alexandrium* spp. cell counts in water samples (cells/L) and total PSP toxicity of the harvested mussels (µg STX eq./100g) by MBA at Lüderitz Bay area.

Phytoplankton cell counts recorded during June-July 2012 for *Alexandrium* bloom from Lüderitz coast (Appendix 2.2). A comparison of the results obtained from both the algal cell counts and the toxicity tests are shown on the graph for Lüderitz Bay area (Figure 6). The sample collected on 16th July had the highest PSP concentration of 903 µg STX eq./100g. This coincided with the period at which *Alexandrium* cells was at a maximum, although this detection was after the bloom. Mussel samples collected on the 27-28th June also detected PSP concentrations 138 and 229.59 µg STX eq./100g respectively, and exceeded the European regulatory limit of 80 µg STX eq./100g. *Alexandrium* spp. cells were detected with a maximum of 874, 500 cells/ L. Therefore, *Alexandrium catenella* might have been the source of the PSTs, as this specie is known to be a

producer of PSP toxins. These results were obtained using the mouse bioassay; and there is no information on the PST profile.

4.3. Species diversity: Central coastal areas

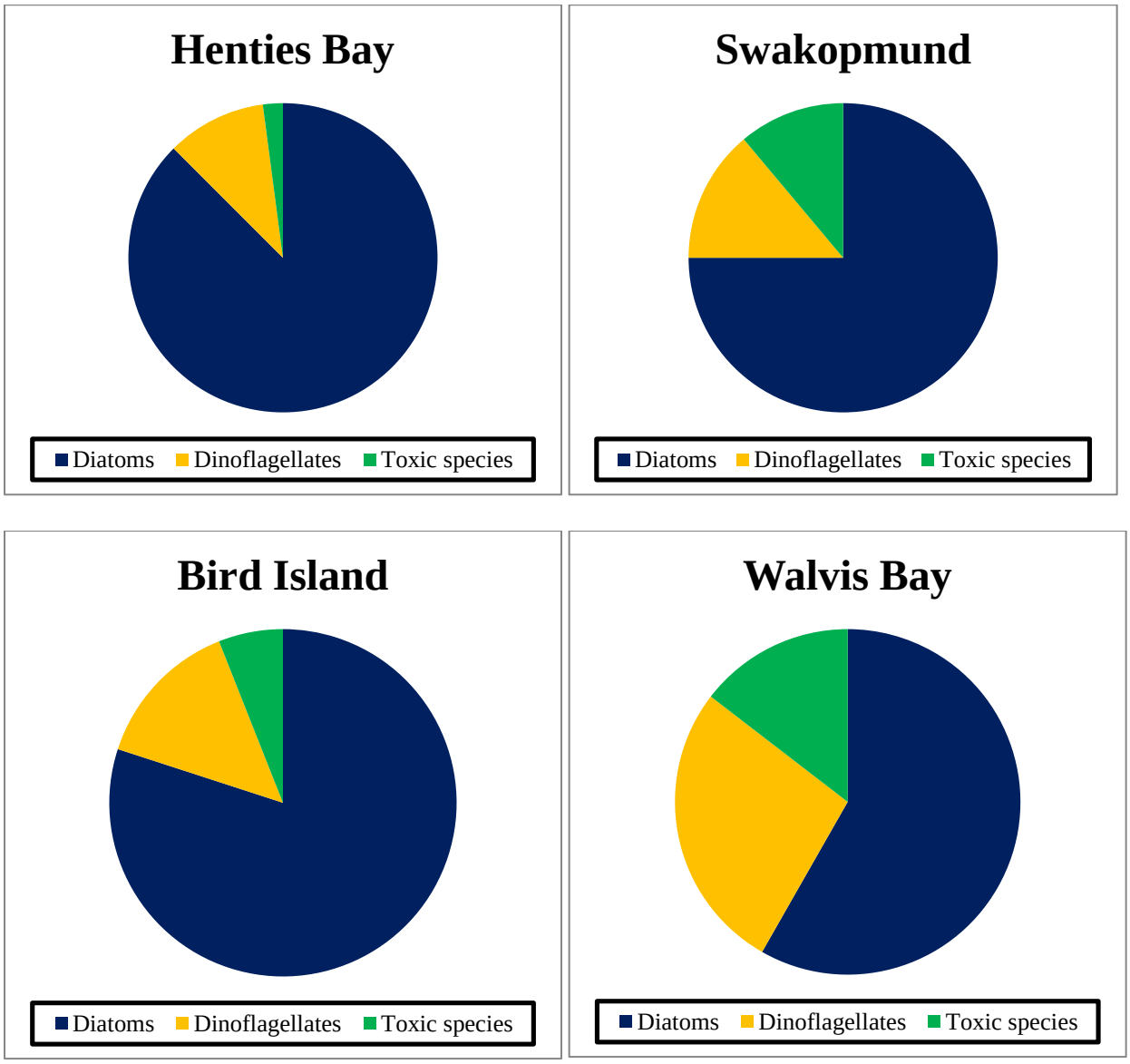


Figure 7: Total number of Phytoplankton species for diatoms, dinoflagellates and Toxic species for the sampling period of May 2012 - February 2013.

Figure 7 indicates the distribution of phytoplankton species diversity recorded during the sampling period of May 2012 to February 2013. The phytoplankton assemblages were dominated by diatoms, which constituted 71% of total phytoplankton population. The most dominant species was *Rhizoslenia spp.* at that time. Very few toxic species were recorded, however; *Dinophysis*, *Alexandrium* and *Gonyaulax* species were sampled during summer season. During the summer months species diversity was higher compared to winter months. In general most of the diatom species occurred all year round, while most of the toxic dinoflagellates species were found at Walvis Bay area. The results show that the number of species differ from station to station, Moreover *Alexandrium* spp. were sampled in November 2012 from Walvis Bay area which is unusual for central coast.

4.4. Mussel samples marine biotoxin analysis

The study investigated two types of shellfish poisoning, PSP and DSP toxins whereby OA and DTX1 were the main lipophilic toxins found; which are collectively known as OA-group toxins (McNabb, 2008). The mussels were collected from central stations and Lüderitz coastal areas during different sampling time span. There were also some variations observed in the size of mussels depending on the location they were collected from. At Walvis Bay area mussels were collected from Nam Oysters Company suspended lines in the offshore area known as the Aqua Park 1. These samples had more flesh compared to other stations while Swakopmund station had mussels with hard shells collected near the river mouth while Henties Bay mussel flesh weight was relatively low.

4.4.1. Paralytic shellfish poisoning (PSP) toxicity levels for Lüderitz and Walvis Bay.

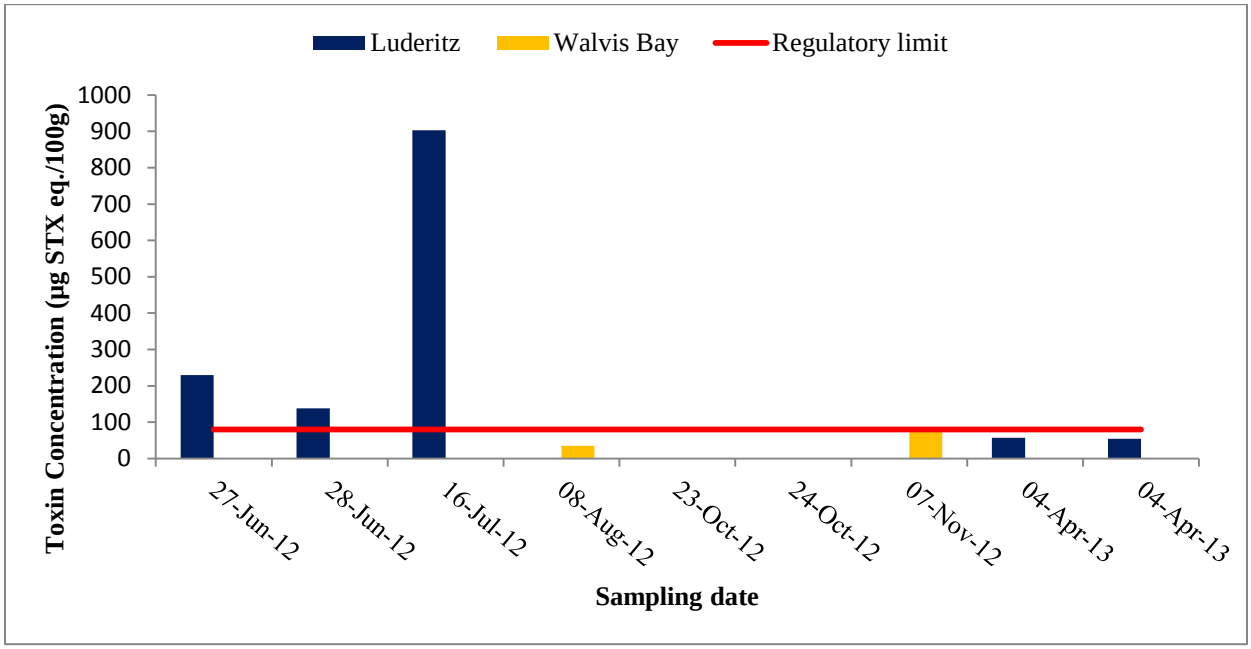


Figure 8: Total PSP toxicity (µg STX eq./100g) mussel (*M. galloprovincialis*) samples for Lüderitz and Walvis Bay stations, regulatory limit 80 µg STX eq./100g, marked in red.

Figure 8 shows PST concentrations in mussels from Lüderitz and Walvis Bay, whereby on three occasions the toxins detected at Lüderitz were found to be above the regulatory limit. These were recorded during the occurrence of blooms, whereby the first bloom occurred in June 2012 and mussel samples collected on 27 and 28 June 2012 tested positive, exceeded the regulatory limit (Figure 8). The highest concentration recorded was more than 10 times the regulatory limit and recorded 903 STX eq/100g. In October 2012, mussel samples were collected in order to verify if the toxicity accumulated were still present, which resulted in no presence of PSP concentrations. The third collection was done during the second bloom which occurred in April 2013. This was identified as an *Alexandrium* specie, and samples were suspected to contain PSTs. The MBA

method showed the presence of PSTs but did not exceed the regulatory limit (Appendix 4.7). Overally; PST concentrations for Lüderitz were mostly detected in winter compared to summer. The highest PST concentration recorded for Walvis Bay was 80 µg STX eq./100g and this was the first time the concentration of PSP toxicity reached the regulatory limit since 2005 (Appendix 2), thus most samples tested negative. *Alexandrium* spp. cells were identified in the water samples during this period but not in abundant. Furthermore, since they were only two samples that detected PSTs, there is no pattern to indicate seasonal changes, but during the past years PSTs have been detected mostly in winter but never exceeded the regulatory limit.

4.4.2. Lipophilic toxin concentrations

DSP analysis revealed lipophilic toxins (OA, DTX-1, DTX-2, PTX, and AZA), while for Yessotoxin group Toxins were YTX, homo-YTX and 45-OH YTX. All samples tested negative for PTX's and AZA's. At the time of the analysis only YTX standard was available so all the other analogues were calculated using the YTX calibration curve as per EU method. Because the YTX analogues all have an identical response on the instrument and can all be calculated from the YTX calibration curve, and the same detection limit applies to all of them, which is 0.04 mg/kg while the OA-group toxins have different detection limits.

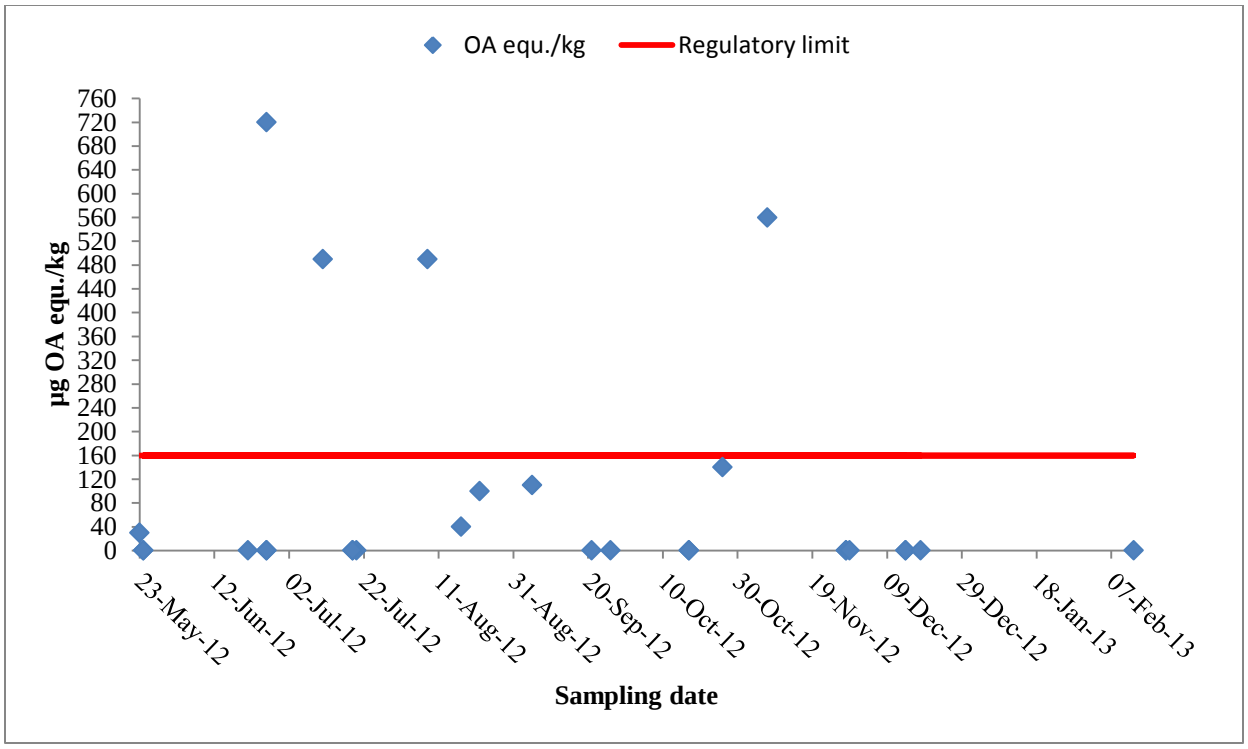


Figure 9: Total Okadaic acid toxicity in *M. galloprovincialis* samples for all central coast stations, regulatory limit 160 µg equivalents/Kg, marked in red.

Figure 9 represents Okadaic acid concentrations in mussels for all stations (Henties Bay, Swakopmund, Bird Island and Walvis Bay), four samples tested positive with the highest concentration of 720 µg/kg recorded from Walvis Bay. This station is located in the lagoon area where there is higher primary production.

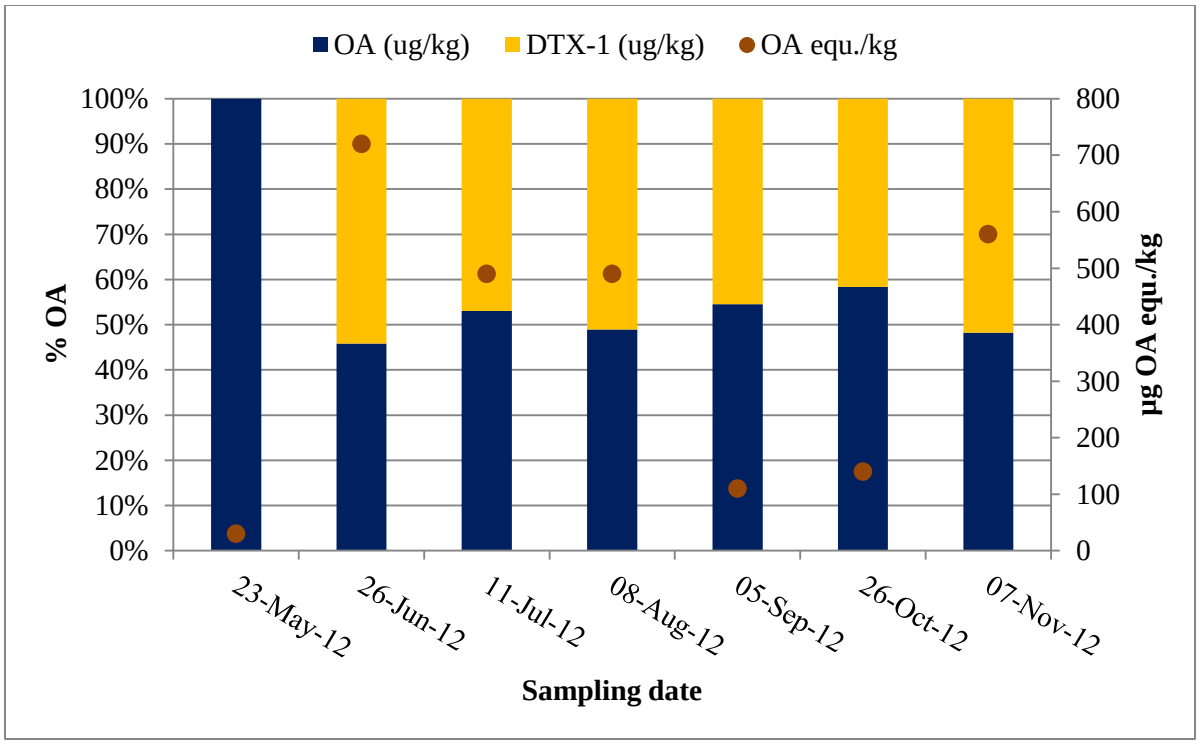


Figure 10: Concentration of OA and DTX-1 in *M. galloprovincialis* samples from Walvis Bay Area and relative composition of analogues.

Walvis Bay area is one of the areas where oysters/mussels are being farmed. Nevertheless, the highest mean concentration value was recorded during winter months. Through analysis the toxin profile found were OA and DTX-1, while DTX-2 was not detected (Figure 10). These were the most dominant toxin present in *M. galloprovincialis* samples and an overall of about 50% of each toxin was accumulated. The lowest concentration of OA was recorded in May and the only sample with no detection of DTX-1. On the 26th June the highest concentration of OA detected which is 720 µg OA equivalents/Kg. There were *Dinophysis* spp. recorded during this sampling time (Figure 11). Overally, four samples (720, 490, 490 And 560 µg OA equivalents/Kg) detected positive which were above the regulatory limit of 160 µg OA equivalents/Kg.

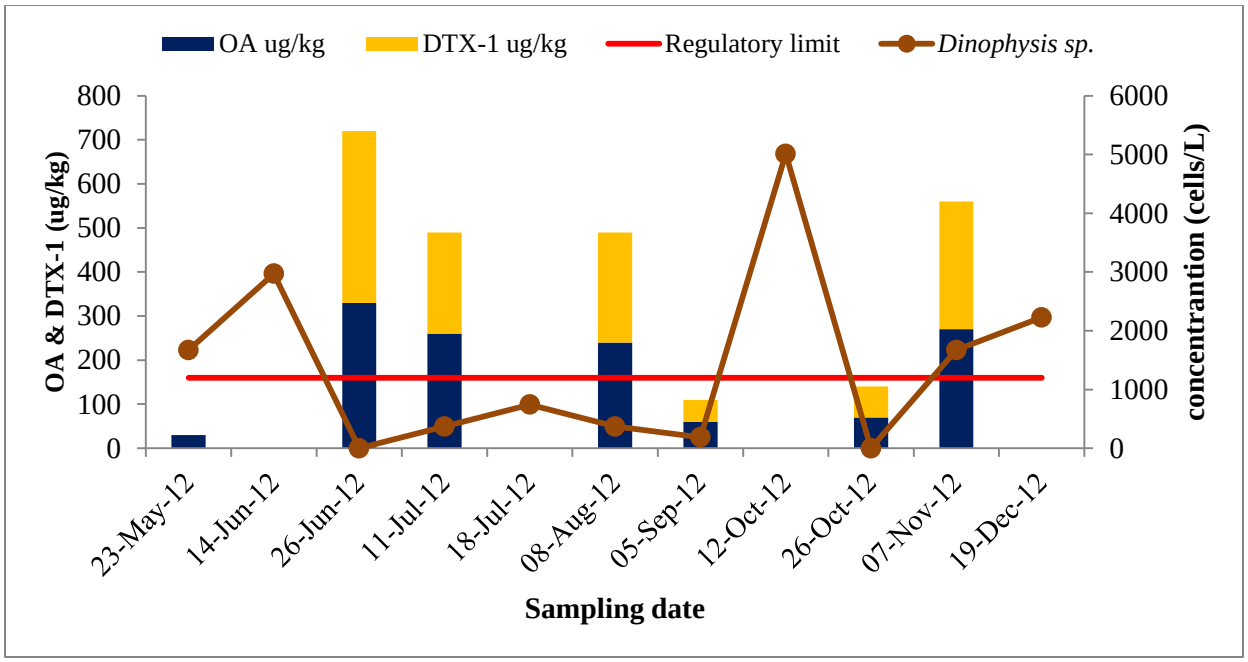


Figure 11: *Dinophysis* spp. cell counts in water samples (Cells/L) and Okadaic acid concentration of the harvested mussels at Walvis Bay.

The graph (Figure 11) represents the microalgal source of OA and DTX1 toxins in the contaminated mussels which was *Dinophysis* species, although the toxin profile in these species was not observed as part of this work. Indeed widespread of *Dinophysis* species (*Dinophysis acuminata, Dinophysis fortii, Dinophysis acuta* and *Dinophysis rotundata*) have been reported in Namibian waters. The most dominant species found was *Dinophysis acuminata* and was recorded from Winter to Autumn. OA equ. values were above the regulatory limit, though with slight disconnect with the *Dinophysis* spp. concentrations.

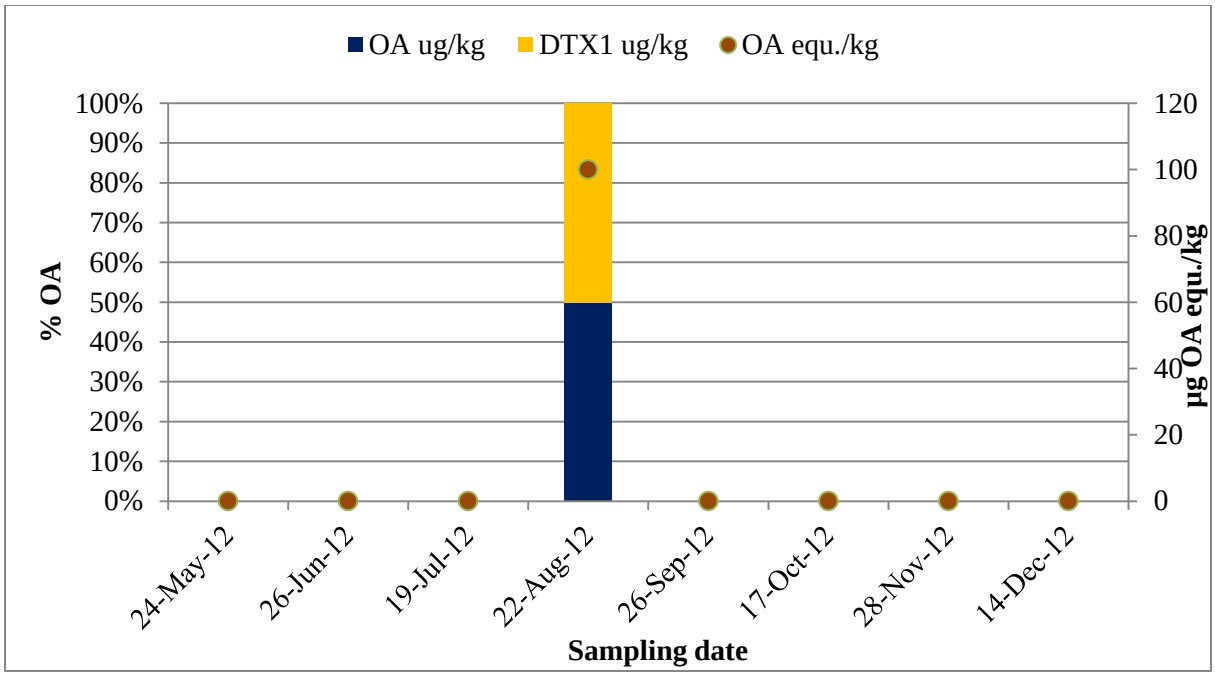


Figure 12: Concentration of OA and DTX-1 in *M. galloprovincialis* samples from Swakopmund Area

The sample from Swakopmund area detected OA and DTX-1 on the 22th August, which accounted for 50 % of each toxin content. The concentration recorded was 100 µg OAequ./Kg. All the other samples were below the detection limit. These mussel samples had hard shells and thin flesh. Henties Bay, Lüderitz and Bird Island stations tested negative for OA, DTX-1 and DTX-2.

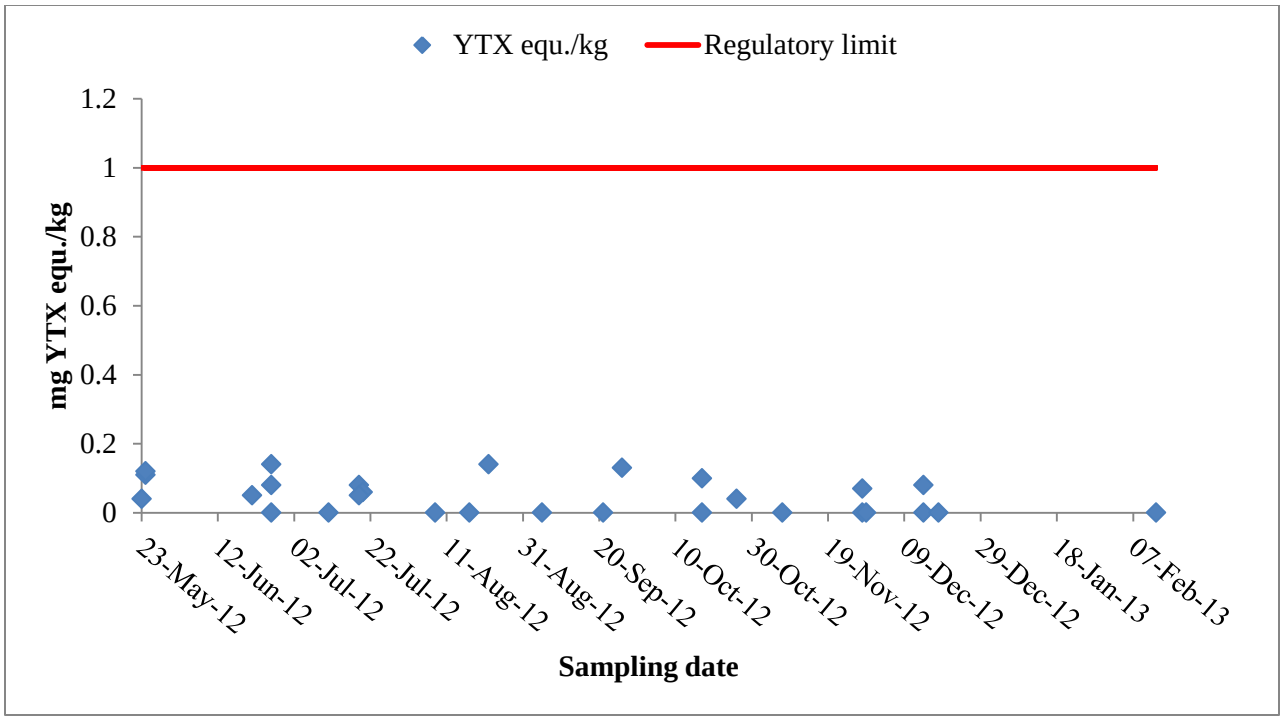


Figure 13: Total YTX toxicity (mg/Kg) from mussel (*M. galloprovincialis*) samples for all central coast stations, regulatory limit 1 mg YTX equivalents/Kg, marked in red.

The mussel samples showed some trace concentrations of YTXs which did not exceed the regulatory limit (1mg YTX eq./Kg) with the highest concentration recorded of 0.14 mg YTX eq./Kg obtained by the LC-MS. All stations detected YTX in very small concentrations, with some stations showing concentrations below the detection limit (<0.04 mg YTX/Kg). Therefore, the total YTX group toxins were all below the regulatory limit of 1mg YTX eq./Kg shellfish meat (Gonzales and Rodriguez, 2011) for both winter and summer.

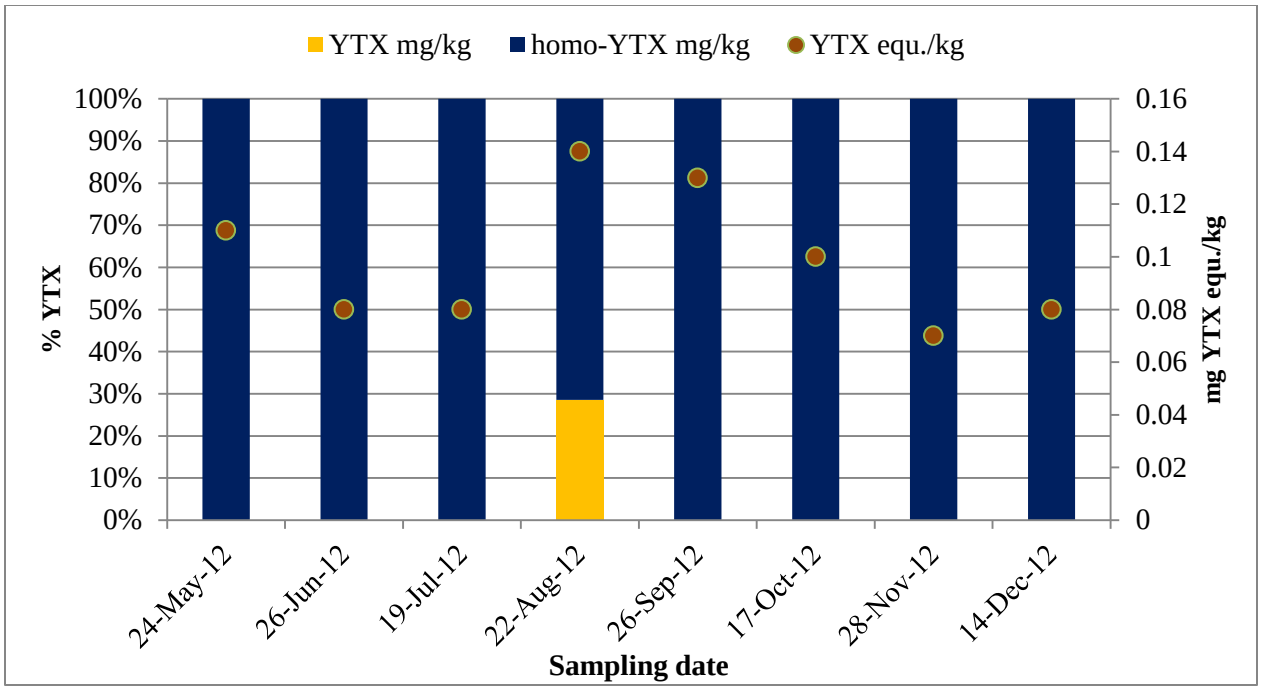


Figure 14: Concentration of YTX in mussel (*M. galloprovincialis*) samples from Swakopmund Area and relative composition of analogues.

YTX, homo-YTX and 45-OHYTX were analysed in mussel samples collected from Swakopmund station (Figure 14). First detection of Homo-YTX as the main YTX analogue that revealed an estimate of about 80 % of its concentrations, and 45-OHYTX values were below the detection limit. The highest concentration of YTX was 0.14 mg YTX equ. / kg detected in one of the samples collected on the 22th August (20 - 25%).

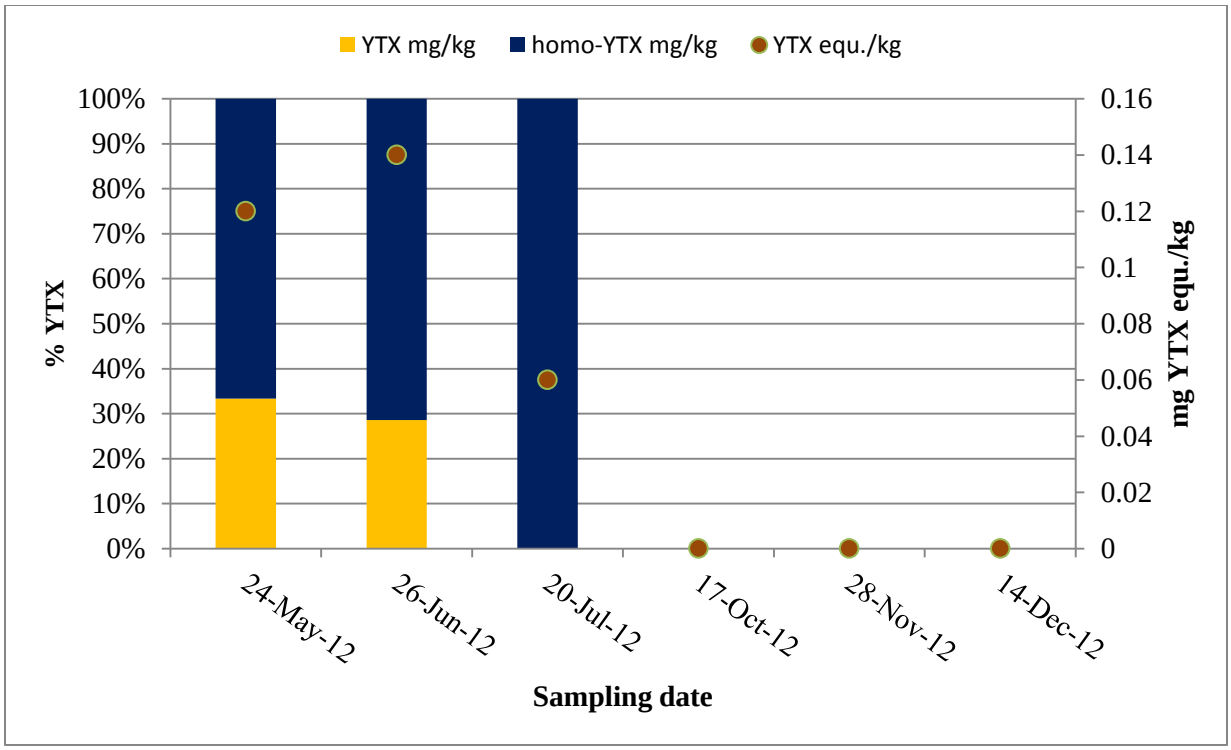


Figure 15: Concentration of YTX in mussel samples (*M. galloprovincialis*) from Bird Island and relative composition of analogues.

Homo-YTX was the most dominant toxin found with the highest concentration value of 0.14 mg/kg (Figure 15). During the last three sampling months (summer) no YTX or either homo-YTX was recorded, and 45-hydroxy YTX tested negative for all stations.

All Lüderitz mussel samples did not detect any concentrations of YTX and OA group-toxins, thus they were all below the detection limit.

4.4.3. Chromatograms of Okadaic acid and Yessotoxin group Toxins

This section outlines chromatograms obtained from the analysis of samples using an AB-Sciex 4000 QTrap mass spectrometer linked to an Agilent 1200 HPLC. These chromatograms illustrate peaks of each toxin standards, it includes traces only for the available standards. Toxin peaks can be identified by examining the ion ratios. Thus, the chromagrams shows OA and YTX with positive sample, hydrolyzed sample and a negative sample. As is it shown in Figure 18 of a negative sample, the chromatogram peaks are not smooth and differ from the positive and hydrolyzed samples. Hydrolysis is necessary to release any OA and DTX toxins which are bound up in the sample matrix (the EU method protocol explains the process in more detail) and while YTX and its analogues do not require hydrolysis. Thus, the x-axis represents the time (minutes) and on the y-axis is the intensity.

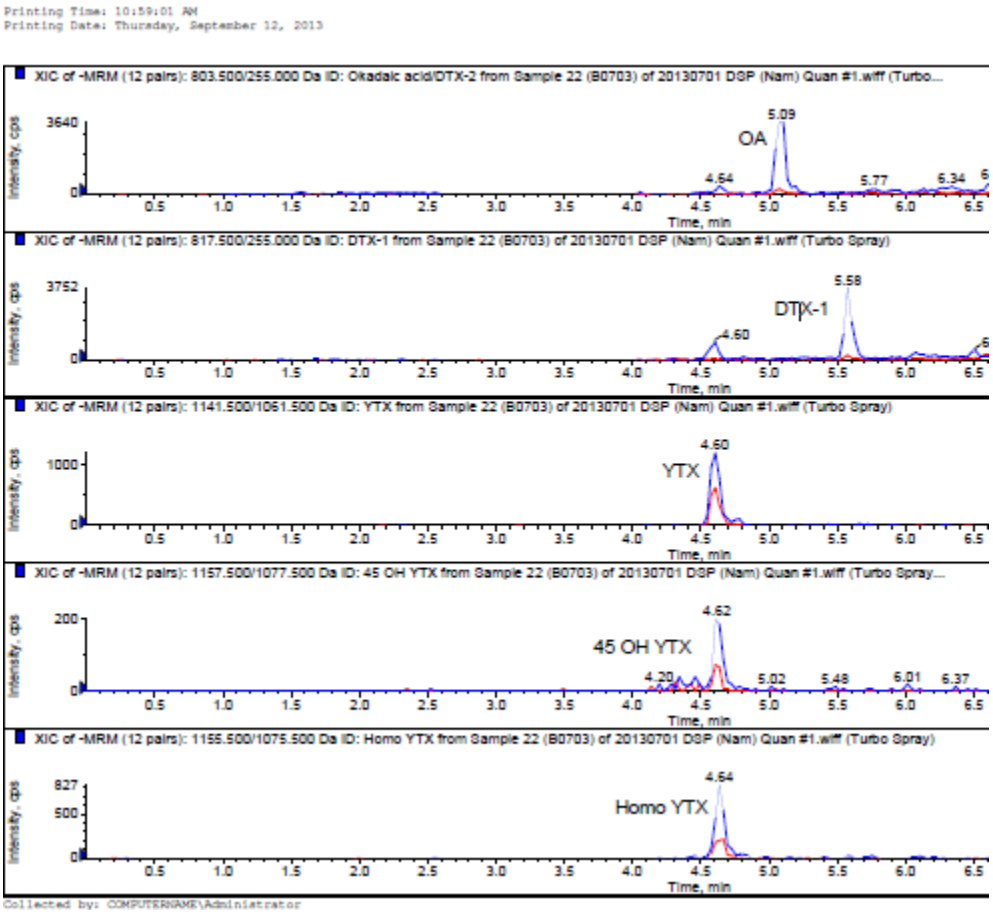


Figure 16: LC-MS chromatograms for OA and YTX-group toxins in *M. galloprovincialis* for a positive sample.

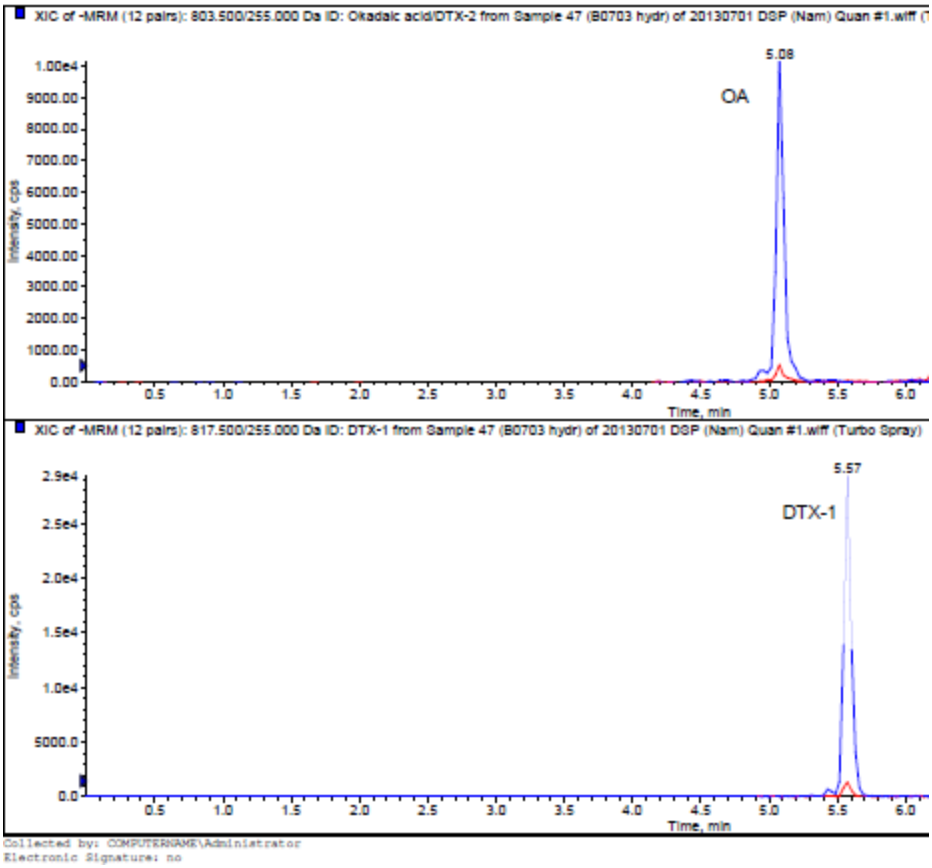


Figure 17: OA and DTX-1 chromatograms - hydrolyzed sample

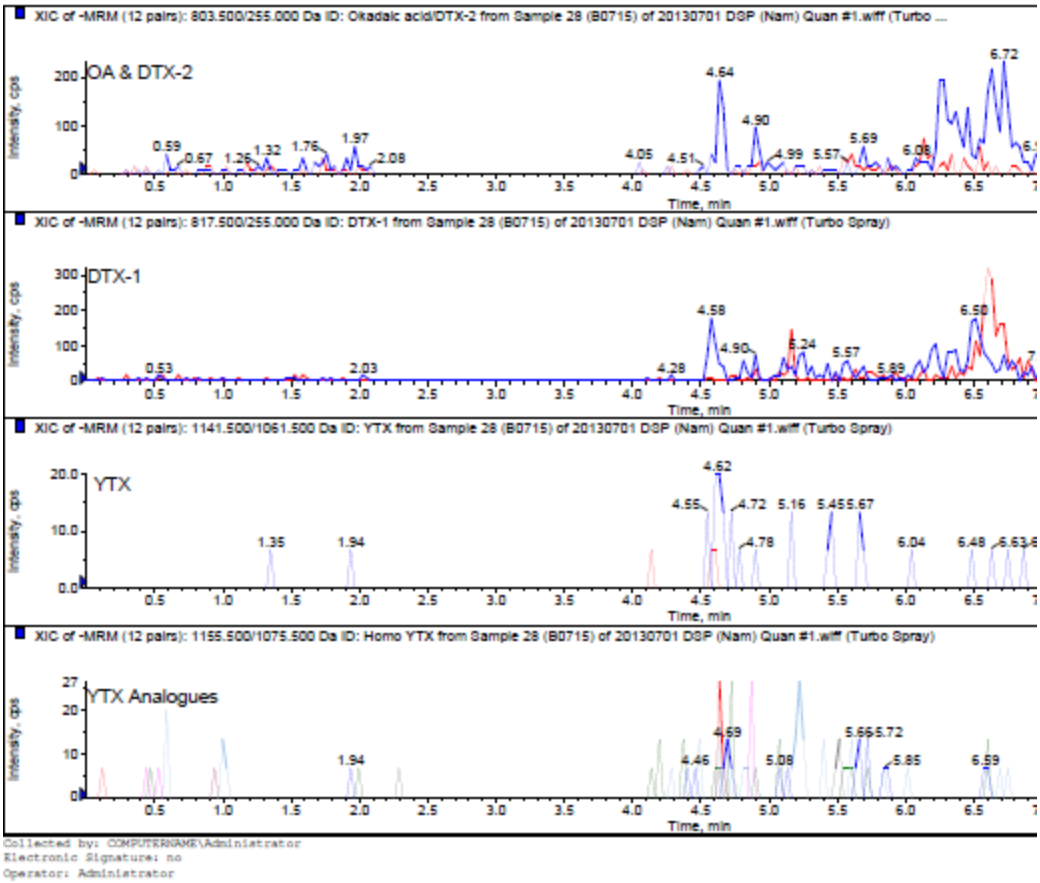


Figure 18: LC-MS chromatograms for OA and YTX-group toxins in mussels *M. galloprovincialis* for a negative sample.

CHAPTER FIVE

5. DISCUSSION

5.1. Sea surface temperature and Dissolved Oxygen

Natural communities of phytoplankton have been and will continue to be influenced by increases in temperature as algal growth rates are strongly, but differentially, temperature dependent (Eppley, 1972). Higher temperatures will decrease surface water viscosity and increase nutrient diffusion towards the cell surface, an important process when competition for nutrients between species occurs (Vogel, 1996; Peperzak, 2003).

In previous studies, it was reported that temperature not only affects the survival and distribution of a species, but also growth, rate of development, activity and reproduction as well as susceptibility to diseases. The resistance or reaction of the species depends on the species acclimation temperature, species size, salinity level, state of health and duration of the extreme temperatures (Popma and Masser, 1999).

Therefore this study obtained the highest temperature of 24.3 ^{o}C which was the normal temperature that had been recorded along the coast during summer seasons for the past years. However, there was a significant difference in mean values between winter and summer seasons across stations ($P<0.05$).

Temperature exerts a major influence on the growth and seasonal bloom cycle of most phytoplankton species. The role of temperature in regulating *Alexandrium* blooms may also be site-specific. *Alexandrium* species are commonly sampled in Lüderitz area than in Walvis Bay, and the temperatures of Walvis Bay were slightly higher than of Lüderitz area. Generally, in the

cold Benguela current, during periods of reduced upwelling (relaxed southerly winds), sea surface temperatures increases.

One of the studies done by Prakash *et al*., (1971) showed that PSP detoxification varied according to the season along the eastern coast of Canada; it is frequently assumed that low water temperatures retard toxin loss (Shumway *et al*, 1995). Notwithstanding this, temperature seems to affect different toxins and species in different ways, and slower loss of DSP from *Mytilus galloprovincialis* (Blanco *et al*., 1999).

Dissolved oxygen is one of the key environmental variables influencing habitat suitability in biologically productive systems such as the northern Benguela. Oxygen status in the water column could be a limiting factor to successful habitat conditions for the survival and growth of phytoplankton and filter feeders. The degree to which the toxicants are bioaccumulated depends upon both the physicochemical properties of the compound and biotic factors such as the pumping activity, biochemical composition, reproductive condition, metabolic activities and elimination in the mussels (Widdows & Donkin, 1992). Both the abiotic and biotic factors are in turn affected by environmental variables such as temperature and salinity which influence the dynamic processes concerned with uptake and depuration of toxic agents (Hoppenrath *et al*., 2009). The highest mean dissolved oxygen in this study was recorded at Swakopmund station, and this station is at the river month where discharges of the effluent may have affected the concentration of dissolved oxygen. Walvis Bay recorded the lowest dissolved oxygen for both summer and winter, however there was no significant difference between summer and winter across stations at 95% confidence interval ($P>0.05$). This results might have been influenced by the higher temperature recorded.

5.2. Phytoplankton species biomass and diversity

Primary production is one of the most important ecological aspects of the phytoplankton. The biomass built through photosynthesis is the nutritional basis for all higher trophic levels of consumers. The phototrophic phytoplankton biomass production is controlled by at least two factors: physical-chemical conditions and feeding pressure by the predators (Hoppenrath, *et al*., 2009).

The presence of phytoplankton toxic species has been endemic in the Namibian coastal waters during winter and summer periods. Diatoms normally dominate the northern Benguela current system, thus it is appropriate to investigate possible dominant species within the system. During the sample collection it was observed that diversity of species showed presence of diatoms as the most abundant during summer months, of which the majority of *Rhizoslenia* sp. and *Pseudo-nitzchia* blooms were also observed. As for the Walvis Bay area where shellfish farms are situated, based on the data from the mariculture database a high percentage of dinoflagellates were recorded compared to the diatoms. However, as for the combination of all stations of Aquapark 1, during winter season more dinoflagellates were recorded and while during summer season more diatoms were recorded. Therefore the presence of phytoplankton species clearly depends on seasonal changes and location.

Alexandrium species have dominated the Lüderitz coast but no studies have been done to determine the toxin profiles and this makes comparison considerably difficult. Conditions within the Lüderitz Bay area during the sampling periods were favorable for phytoplankton growth. The exact causes of the high cell number observed is unknown and could be due to a number of factors. Temperature could be one of the factors as it kept on fluctuating. It is unclear from the

results obtained to date whether these trends are related in any way to the effects of climate change or relate to the natural upwelling system since Lüderitz is a center of the upwelling in the Benguela, and it is the most intense cell found in any upwelling regime (O'Toole *et al*., 2001).

Furthermore, calm weather favors water stratification and a fast intensive phytoplankton growth commonly dominated by dinoflagellates. Intense upwelling destroys stratification and favors the diatoms. The time, intensity and composition of blooms vary from year to year whereby the conditions of physical stability and sufficient nutrients in the photic layer favor bloom development (Shannon, 1985).

The phytoplankton growth goes on as long as nutrients are available. Decreased silicate levels will limit diatom growth and for the tropical waters, phytoplankton populations in the summer tend to be small and dominated by dinoflagellates, however this is a contrary to our water system. In 1992, mussels from the Atlantic coast contained saxitoxins with no toxin producing algae detected in the water. Álvarez *et al*., (2011) reported an outbreak of *Alexandrium* species in May 2006, in Chile where the authors have not detected saxitoxins in the wild and cultured dinoflagellate; at the same time shellfish samples revealed the presence of toxin profiles.

This can be argued as the FAO (2004) suggest the possibility of non-toxic algal species to acquire ability of toxin production when exposed to a typical nutrient regime. Certain known non-toxic dinoflagellate can be able to cause PSP toxicity in some parts of the countries or that certain non-monitored toxin producing algae may be responsible for present shellfish contamination.

Diatoms were the majority species found in the waters of central coast for all stations (Figure 7), and as mentioned earlier on, diatoms always respond to upwelling. The only dinoflagellates identified were *Prorocentrum micans*, *Ceratium furca,* and some *Dinophysis* species and this are harmful and potentially harmful species. *Dinophysis acuminata* was the most dominant species found in the Walvis Bay area, whereby mussel samples tested positive for OA and DTX-1 toxins. There was a slight disconnect on the number of *Dinophysis* cells and the toxin values, this is because the same cell concentrations may cause shellfish toxicity in some circumstances but not in others. The amount of phytoplankton available in the water have an influence on how much water the shellfish can filter per day.

High cell concentration of *Alexandrium* population was only found in the Lüderitz waters. The Lüderitz *Alexandrium* bloom population mainly composed of *Alexandrium catenella* and in many cases it forms red tides and therefore this species produces STX which is a highly potent neurotoxin that causes PSP. Walvis Bay was the only station which recorded a large diversity of species of dinoflagellate and an uncommon species of *Gonyaulax* sp. was only recorded at this station as well and this is the species which is associated with YTX-group toxins.

5.3. Paralytic shellfish toxicity concentrations for central and Lüderitz coasts.

Paralytic shellfish poisoning have been reported worldwide. In Namibia, during the past years PSP toxins have been commonly detected in abalones and lately in mussels (MFMR, database). Mussel farming is relatively new; however, *M. galloprovincialis* have been grown. Currently, the Mouse Bioassay (MBA) is the reference method. The standards and requirement written in the final report for establishment of a shellfish sanitation monitoring programme in Namibia, states

that the international safe limit concentration of marine biotoxins in bivalve molluscs should not exceed 80 µg STXeq./100g for PSP toxins and 160 µg/kg for OA.

During the study, PST contamination mostly affected mussels sampled from the Lüderitz coastline, due to the bloom that occurred during June/ July 2012. It caused the mussel samples collected on 16 July 2012 to make a record of the highest PST concentrations in mussels of 903 µg STX eq./100g, more than ten times the regulatory limit. Short mouse survival time interval when testing the sample may be the reason for calculating the high content of STX eq./100g by biological method, as it would require dilution of acidic extract for testing of these samples.

Lüderitz detected the highest PST concentration during winter season and this result agrees with Adriatic Sea report of PSP toxicity being high in winter season. Another possible origin of PSP toxicity recorded in winter might be the cysts of toxic dinoflagellates and in the report of Ujevic *et al.,* (2011) of the Adriatic Sea that suggested that mussels located over areas of strong winds had persistently high toxin levels. Therefore, Lüderitz being situated in an area of strong wind might have an influence on the higher PST concentration.

The first detection of PST for central coast areas was observed during this sampling period (figure 8), a concentration value of 80 µg STX eq./100g was recored at Walvis Bay station on 07 November 2012. The causative species, *Alexandrium* spp. were also sampled during this sampling day. Shellfish accumulate dinoflagellates toxins via food webs, and thus the toxin profiles of bivalves seem to reflect those of dinoflagellates. Available evidence suggests that STXs in dinoflagellates are transported to absorbed, and accumulated in the digestive gland (Maruyama *et al*., 1983). Some of the toxin absorbed is released from the shellfish; however, the pattern of toxins in shellfish is not always similar to that of the causative dinoflagellates,

indicating that toxin components are transformed in shellfish (Oshima *et al*., 1990), due to chemical properties of toxins. This study did not test for toxin profiles in dinoflagellates to confirm this hypothesis and either the method used to detect the toxin in shellfish does not specify the different types of profiles or analogues.

Enzymatic conversion of toxin components is reported to occur also in dinoflagellates, toxin components can be attributed to enzymatic reactions as well as non-enzymatic processes (Botana, 2000). Various toxin components found in dinoflagellates and shellfish seem to be products of these reactions. Ishida *et al.,* (1993) suggested that the enzymes involved in the conversation of toxin components may be those involved in biosynthesis of the toxins. However, it should be noted that at least one of the toxin components found in toxin-producing dinoflagellates could be a product of biosynthesis of STXs. Thus, the enzyme which produces this component is the one involved in biosynthesis of STXs.

Rates of toxin accumulation and depuration depend on the species of shellfish. Mussels accumulate and eliminate the algal toxins fast (Peperzak *et al.,* 1995), while scallops may retain the algal toxins in their tissue for several years (Fernandez *et al*., 2004). This can however be associated with the highest results recorded for mussel which exceeded the legal limit for PSP of 80 µg STX eq./100g.

Shumway *et al*., (1995) suggested that, in Europe dinoflagellate develops at relatively high temperatures and abundant sunlight which results in cases of intoxications mainly between May and November. This is unlike the Namibian waters where intoxications take place commonly during winter period when the temperature is normally low.

PSTs were commonly detected in Lüderitz compared to other stations, the results revealed winter to be the season whereby consumers should be cautions of the poisoning and thel standards institutes to consider increase on the number of sampling.

5.4. Lipophilic toxicity for central and Lüderitz coasts.

Mussels being suspension-feeders and pumping large volumes of water, accumulate and concentrate a wide variety of lipophilic organic contaminants and algal toxins in their tissue. In general, bioaccumulation is a function of the uptake rate of the toxicant and the elimination rate of the same (Spacie and Hamelink, 1985; Phillips, 1993).

Shellfish poisoning have been a concern in the Namibian waters, with DSP toxins being tested with Mouse Bioassay which only provides a positive or negative result and do not indicate the concentration value. Lüderitz samples were only collected during bloom and after bloom as stated earlier, and all DSP toxins analyzed OA, DTX-1, DTX-2, YTX, PTX and AZA were below the detection limit. DSP toxins have been reported in Lüderitz Bay areas during the past years, these results were obtained using the mouse bioassay, and therefore there is no information on the types of lipophilic toxins present.

Among the toxins that were observed with LC-MS/MS, OA was commonly detected in mussels of the Walvis Bay station; and four samples detected toxin concentration above the regulatory limit, and *Dinophysis* species were observed in the Walvis Bay area. OA content per cell in the causative species (*Dinophysis* sp.) may vary considerably both temporally and spatially and is one of the factors affecting the rate of uptake of this toxin (Lee *et al*., 1987 Andersen *et al*., 1996). The rate of assimilation of OA in the digestive system is likely to be affected by the total

amounts of ingested food since this affects the gut passage time and probability for assimilation through the gut wall (Hawkins and Bayne, 1992). Apart from all aspects regarding the food resource, any other factor which affects the feeding physiology of mussels are likely to influence the uptake of toxicants including OA in mussels.

During sampling it was observed that mussels collected from Walvis Bay area had more flesh compared to mussels collected from other stations. The toxicity of the mussels depends on the toxin burden and the weight of the tissues and on the potency of each toxin. Rates of toxin accumulation of filter-feeding shellfish from toxic algae are toxin – and species – specific, both because the food ingestion of some mollusc species differs substantially in their retention capability for each group of toxins (Hallegraeff, *et al*., 2003).

The data collected for phytoplankton species diversity also proves that Walvis Bay area had higher phytoplankton productivity; therefore it can be referred to the feeding physiology and availability of the toxin-producing dinoflagellates in the plankton community. Uptake of lipophilic algal toxins such as OA is assumed to occur exclusively by feeding on the toxin-producing algae and hence the resulting assimilation of the toxins into the tissue takes place within the digestive system (Landsberg, 2002).

In this context both the absolute and relative abundance of toxic algae, together with total amount of food available for the mussels are important variables affecting general filtration and ingestion rates (Sampayo *et al*., 1990; Hawkins and Bayne, 1992).

As for the analogues, Walvis Bay still detected the highest DTX-1 in the mussel samples and they were samples above the regulatory limit, while DTX-2 tested negative. The toxin profiles determined in these samples are similar to those found in the Chinese mussel, *M.*

galloprovincialis with high levels of OA and DTX-1 and based on these profiles a *Dinophysis* species seemed likely to be responsible for the toxins (Liu *et al*., 2011).

The analogues detected in mussels for YTX analysis were mainly YTX and homo-YTX. The concentration profile recorded for total YTX group toxins for all the stations is completely different from OA. *Gonyaulax* species has been found in the Namibian water which is known to cause YTX. In the study done by Chikwililwa *et al.,* (2013), *Gonyaulax spinifera* was detected in the Benguela current upwelling system. It was found that the YTX analogues profile in the mussel samples during the bloom correlated with the YTX profile observed in the phytoplankton samples. This YTX profile also showed the dominant production of 45-OH YTX and homo-YTX. This indicates similarity for this present study of homo-YTX being the main dominant analogue, and whereas for 45-OH YTX was below the detection limit (>0.04 mg/kg). During the time of the analysis, there was no standard for 45-OH YTX. Although a good number of phycotoxin studies have been carried out and, a clearer pattern of the mode of action of YTX has emerged recently, the precise mechanism of action is not yet known. However, it seems clear that YTXs do not inhibit protein phosphatases PP1 and PP2A, as opposed to other lipophilic toxins, such as OA (Ogino *et al*., 1997).

The effects of different toxins vary greatly among different species, although this study focused only at one type of species. In this context, *Mytilus sp*. is generally regarded as a species insensitive to most toxins and these species therefore readily feed and accumulate high toxin levels, but also detoxify the toxins more rapidly compared to other marine shellfish species. Oysters have lower filter-feeding rate in comparison to mussels leading to reduced accumulation of phycotoxins.

5.5. Okadaic Acid and Yessotoxin group toxins

The concentrations of both OA and DTX-1 was observed to increase almost two-fold following base hydrolysis, indicating that significant quantities of OA and DTX-1 esters were present in the sample. These esters are formed by metabolism of the toxins in the mussels (Marr *et al*., 1992).

These results clearly show that the elevated concentrations of OA and DTX-1, as well as their esters, were responsible for mussel flesh to detect the toxins. These toxin profiles are similar to that reported for *M. galloprovincialis* from China (Li *et al*., 2012), although they had high levels of toxins compared to the present study.

The analyses showed that the toxin profile of the mussels related the 2012 DSP toxin concentrations composed primarily of OA and YTX-group compounds. The correlation coefficient used to quantify the toxins showed a strong relationship that exceeded 0.99 in all cases as shown in Appendix 5 for the calibration curves.

Toxic history and genetic differences also affect the incorporation of toxins into molluscs and the toxicity of individual shellfish in any given area is highly variable, probably mainly as a consequence of the also high inter-individual variability in the rates of food acquisition of most mollusc as confirmed by Roeder *et al*., (2011).

CHAPTER SIX

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusions

Most of the harmful algal blooms are caused by species producing toxin, and some do not discolour the water in an obvious way, for example; *Dinophysis* and *Pseudo-nitzchia* species (Horner, 2002). Some species can be toxic even at very low cell concentrations and therefore monitoring these species is essential to prevent harm to humans. The Namibian shellfish market is most commonly farmed with oysters, thus mussels are often used as sentinel species on biotoxins monitoring as they accumulate phycotoxins much more readily than oysters. They can thus be used as a warning for potential oyster toxicity. With the increasing economic importance placed upon the shellfish industry in Namibia, this highlights the importance of continued monitoring of both shellfish toxicity and their causative species, in order to produce a full and thorough risk assessment so as to provide the necessary information to ensure an appropriate biotoxins monitoring programme is continued. In addition, taking into consideration of a continued monitoring of DSP toxins, which needs to be analysed separately by Namibian standards Institute (NSI) as a routine method, this is to determine the toxin profile for each toxin and a warning system for shellfish farmers.

Detectable concentrations of OA-group toxins were higher at Walvis Bay area compared to other stations, although all stations reported below the regulatory limits. This is the first study to detect the presence of homo-YTX in the northern Benguela specifically for Swakopmund and Bird Island stations. These results of OA and YTX-group toxin profiles highlight the importance of knowing which toxins are responsible for the positive results as they all have different

regulatory limits. Thus the phytoplankton species responsible for the DSP positive results can be easily determined or identified.

Walvis Bay and Lüderitz were the only two stations that recorded PST and this was the first time PSP tested positive at Walvis Bay. Due to the natural occurrence of the *Alexandrium* blooms of Lüderitz, it recorded the highest concentration of PSP and <20 % of the total samples tested positive exceeding the regulatory limit of 80 µg STX eq./100g. These were mostly detected in winter season.

Finally, it is important to notice that the algal toxins do not give bad smell or taste to the seafood, and therefore cannot immediately be detected by the consumer, but only through bioassays or chemical analyses. It is also very important to emphasize that the algal toxins are not destroyed by cooking and thus may be present in canned, frozen or other processed seafood products. Although these algal toxins are unpredictable and shellfish products may seem safe for consumption, there is a need for continued monitoring of both phytoplankton species and biotoxins to ensure that period of potentially harmful phycotoxins events are not missed. Hence, further investigations in marine biotoxins needs to be done for further understanding.

6.2. Recommendations

Insight into physiology and bloom dynamics must be addressed for each toxic species in order to determine the variation. Further research needs to be done on the toxicity levels on both the toxic algal species and in shellfish at a molecular basis as well as in-depth research into the historical distributions of toxic species through cyst analysis. This may help distinguish between changes in geographic range of organisms and proliferation due to changes in local environmental conditions favoring bloom formation.

The ongoing established algal and toxin monitoring program should assist in providing time series needed to assess interannual and long term variability in algal and toxin occurrence, therefore frequent sampling should be considered and testing toxins using LC-MS/MS or HPLC instruments as it gives out detailed toxin profiles for research purposes.

HABs species exist in marine and brackish-water ecosystems worldwide. Their spatial distribution is dependent on hydrodynamics and maritime transport through ballast water. These processes are transboundary making HABs problem an international issue that can only be comprehensively and effectively addressed through international/ regional interdisciplinary and comparative research.

Development of a regional database and communication strategy to enhance regional collaboration and to ensure that the research finding reach a wider audience including policy makers and marine resources managers.

6.2.1. Market trends due to occurrence of Harmful Algal Blooms (HABs): Namibia Perspective

Mussel industry is new in Namibia; hence it has not received much exposure overseas, due to nearly all of the production to date being consumed mostly in South Africa and Namibia. Nevertheless, a permanent programme to study mussel mortalities, 'the mussel watch programme' that used to operate under the mariculture section should be re-established.

The production of mussels have progressively been on the increase and as its consumption rate is becoming more acceptable globally (Russell, 2006). The markets for oysters have targeted overseas countries that give mussels an opportunity to expand to such areas. Therefore, formal communication should be implemented regarding the occurrence of biotoxins to avoid serious

illnesses for the public. A joint industry and government approach is required to overcome this issue.

Finally, most of the farms are sea-based, therefore, there is not much that can be done to avoid farmed mussels from being contaminated, the only alternative is for farmers to halt exports during HABs periods when it exceeds the regulatory levels, until such a time NSI grant them the right for harvesting again after doing re-tests. To date there is no effective method for eliminating toxins from mussels and other bivalves in an economical and fast way that can be commercially profitable and free of hazards. The only solution is self-purifying or natural detoxification through metabolizing the toxins. The detoxifying process depends on various factors sometimes related to the shellfish and sometimes to its environment (Svensson, 2003). Therefore, the farmers can as well be advised to relocate their shellfish to areas of clean water before contamination occurs.

6.2.2. Risk evaluation and management of shellfish toxins

Red tides occur off the Namibian coast and can reach toxic levels; this is a significant management issue for mariculture at large. The Namibian government has actively sought to create an ‘enabling environment’ for aquaculture development which has required specific policy, legislative and institutional interventions. Lack of technology access and technical support of new aquaculture ventures is undoubtedly a constraint to mariculture development in Namibia.

Shellfish toxins pose particular problems to public health protection in particular; the lack of prediction capability of the occurrence of shellfish toxins is a major complicating factor.

One of the main difficulties in the monitoring and management of HAB is the lack of a direct and simple relationship between the occurrence of potentially toxic algal species and toxicity in

shellfish. This may be due to insufficient sampling frequencies and as well as inadequate detection methods for these toxins.

The number one factor influencing productivity and profitability of the mussels farming sector are marine biotoxins. This also means that in years of high biotoxins occurrence, some producers will invariably be in financial difficulties and mussels/oysters exceeding the regulatory limit can create a challenge in producing safe shellfish for human consumption and as well as for exporting to local and international markets.

6.3. Future Research focus

Based on these findings of this study, my future research will be focused on the following:

- ✓ Detailed investigation of the distribution and growth of toxic phytoplankton species.Temperature effect on toxin loss (depuration) under controlled laboratory conditions.
- ✓ Isolation of toxic Phytoplankton species and growing them for identification especially at molecular basis as this is the fundamental to our understanding of harmful effects.
- ✓ Threshold experiments on the uptake and retention of phycotoxins by shellfish
- ✓ Determine PSP toxin profiles by using an HPLC: (Present results were obtained by mouse bioassay)
- ✓ In general, Further research required to broaden knowledge on the occurrence of lipophilic toxins in Namibian waters.

CHAPTER SEVEN

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CHAPTER EIGHT

APPENDICES

APPENDIX 1

This section shows all observations of raw data recorded for temperature and dissolved oxygen, and its ANOVA tables.

Appendix 1.1: Mean values and standard errors (±) for temperature and Dissolved Oxygen of seawater per station.

Stations	**GPS coordinates**	**Temp (^{o}C)**		**DO (mg/L)**	
		winter	**Summer**	**Winter**	**Summer**
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	16.40	14.90	6.54	13.01
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	16.00	14.80	6.50	12.89
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	16.20	15.10	6.45	12.80
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	15.60	15.00	7.84	7.53
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	15.70	15.10	7.82	7.87
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	15.60	15.00	7.83	7.50
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	14.10	18.70	6.34	5.40
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	14.10	18.50	7.01	5.36
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	14.30	18.60	6.98	5.75
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	14.80	24.30	4.65	6.50
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	14.90	24.00	4.66	6.45
Henties Bay	S 22^{o}08' 55.1" E 014^{o}17'22.3"	14.80	24.20	4.66	6.53

Mean		**15.21**	**18.18**	**6.44**	**8.13**
Swakopmund	S 22°41'57.0" E 014°31'16.8"	16.80	15.30	11.34	7.47
Swakopmund	S 22°41'57.0" E 014°31'16.8"	16.50	15.30	11.18	7.46
Swakopmund	S 22°41'57.0" E 014°31'16.8"	16.30	15.40	10.56	7.40
Swakopmund	S 22°41'57.0" E 014°31'16.8"	15.90	14.80	11.94	10.00
Swakopmund	S 22°41'57.0" E 014°31'16.8"	16.00	14.80	11.80	10.13
Swakopmund	S 22°41'57.0" E 014°31'16.8"	16.10	14.90	11.86	10.12
Swakopmund	S 22°41'57.0" E 014°31'16.8"	14.20	18.40	8.74	7.50
Swakopmund	S 22°41'57.0" E 014°31'16.8"	14.20	18.50	8.75	7.59
Swakopmund	S 22°41'57.0" E 014°31'16.8"	14.10	18.40	8.74	7.20
Swakopmund	S 22°41'57.0" E 014°31'16.8"	15.00	21.50	6.89	6.22
Swakopmund	S 22°41'57.0" E 014°31'16.8"	15.10	21.60	6.80	6.23
Swakopmund	S 22°41'57.0" E 014°31'16.8"	15.10	21.60	6.76	6.44
Mean		**15.44**	**17.54**	**9.61**	**7.81**
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	16.00	16.20	7.23	6.23
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	16.50	16.40	7.20	6.20
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	16.23	16.30	7.25	6.37
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	16.30	15.50	7.32	7.25
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	16.30	15.70	7.40	7.16
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	16.20	15.60	7.44	7.20
Bird Island	S 22° 52' 33.2" E 014° 32' 22.3"	14.10	17.90	4.74	7.50
Bird Island	S 22° 52' 33.2"	13.80	17.80	4.73	7.48

	E 014^{o} 32' 22.3"				
Bird Island	S 22^{o} 52' 33.2" E 014^{o} 32' 22.3"	14.00	17.90	4.74	7.35
Bird Island	S 22^{o} 52' 33.2" E 014^{o} 32' 22.3"	15.90	21.30	6.45	4.20
Bird Island	S 22^{o} 52' 33.2" E 014^{o} 32' 22.3"	15.70	21.00	6.56	4.23
Bird Island	S 22^{o} 52' 33.2" E 014^{o} 32' 22.3"	16.00	21.30	6.67	3.99
Mean		**15.59**	**17.74**	**6.48**	**6.26**
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.00	12.20	0.70	4.48
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.50	12.50	0.69	4.59
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.50	12.90	0.62	5.56
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	14.00	15.00	4.43	4.35
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.00	15.00	3.78	3.89
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.00	14.50	2.81	5.04
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.00	14.00	3.90	5.47
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.00	13.50	4.41	4.49
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.50	13.00	4.47	4.40
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	12.50	15.50	5.72	5.56
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	13.00	18.00	5.36	4.98
Walvis bay	S 22 55' 38.1" E 014 27' 76.5"	12.50	20.80	5.24	4.74
Mean		**13.13**	**14.74**	**3.51**	**4.80**
S.E		**0.58**	**0.83**	**1.24**	**0.77**

Appendix 1.2: Summary of temperature and Dissolved oxygen for both summer and winter

	Temp (^{o}C)		DO (mg/L)	
	winter	**summer**	**Winter**	**Summer**
Henties Bay				
Mean	15.21	18.18	6.44	8.13
Min	14.1	14.8	4.65	5.36
Max	16.4	24.3	7.84	13.01
S.D	0.81	3.92	1.2	2.98
Se	0.23	1.13	0.34	0.86
Swakopmund				
Mean	15.44	17.54	9.61	7.81
Min	14.1	14.8	6.76	6.22
Max	16.8	21.6	11.94	10.13
S.D	0.95	2.82	2.07	1.46
Se	0.27	0.81	0.6	0.42
Bird Island				
Mean	15.59	17.74	6.48	6.26
Min	13.8	15.5	4.73	3.99
Max	16.5	21.3	7.44	7.5
S.D	1	2.26	1.1	1.36
Se	0.289	0.65	0.32	0.39
Walvis Bay				
Mean	13.13	14.74	3.51	4.8
Min	12.5	12.2	0.62	3.89
Max	14	20.8	5.72	5.56
S.D	0.43	2.49	1.8	0.53
Se	0.13	0.71	0.54	0.15
Lüderitz				
Mean	12.35	13.05		
Min	11.1	10.9		
Max	13.9	17.8		
S.D	0.075	0.18		

Appendix 1.3: Analysis of Variance (ANOVA) for water parameters

Variate: Temp_winter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stations	3	47.942	15.9807	23.18	<.001
Residual	*44*	*30.3353*	*0.6894*		
Total	47	78.2773			

Variate: Temp_Summer

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stations	3	87.996	29.332	3.39	0.026
Residual	44	381.264	8.665		
Total	47	469.26			

Variate: Do_Summer

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stations	3	85.171	28.39	8.63	<.001
Residual	44	144.826	3.291		
Total	47	229.997			

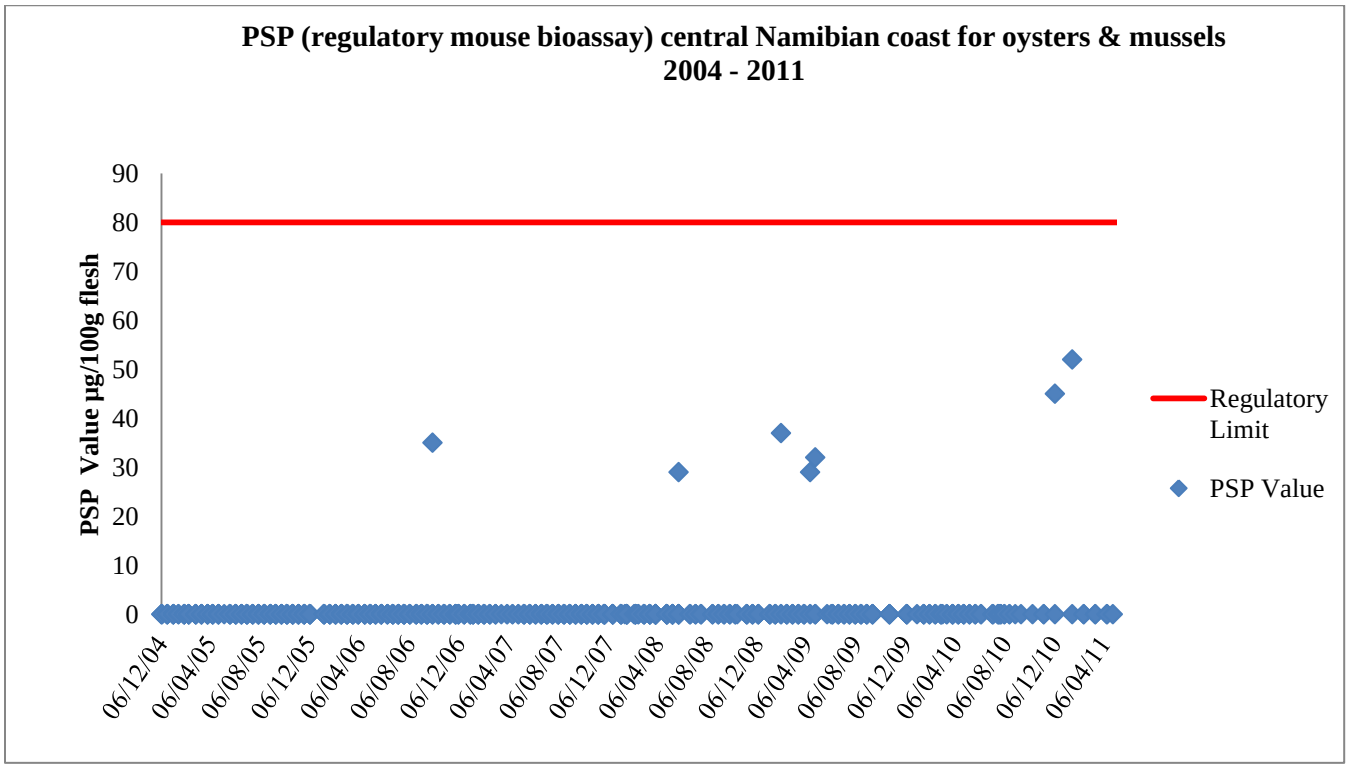
Variate:Do_Winter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stations	3	223.58	74.527	28.42	<.001
Residual	44	115.397	2.623		
Total	47	338.977			

APPENDIX 2

The data shown in the tables below are for the chemical biotoxins analysis in mussels and oysters from NSI to indicate that PSP toxins were not detected above the regulatory limit for central coast since 2004: Source: Mariculture database, MFMR

Appendix 2.1 (a):



(b):

Sampling Date	Station	Laboratory	Medium	PSP	Analyst	PSP Value
10/08/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
25/08/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
05/09/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Detected	Namibia Standards Institute	40.08
03/10/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
17/10/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
31/10/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
16/11/11	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
16/01/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
30/01/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
13/02/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
26/03/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
23/04/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
21/05/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
18/06/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
02/07/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
16/07/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
30/07/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
27/08/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
24/09/12	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0
14/01/13	Kuiseb Fishing	Namibia Standards Institute	Mussels	Not detected	Namibia Standards Institute	0

APPENDIX 3

Appendix 3.1: Phytoplankton species for Lüderitz water samples collected during bloom for June and July 2012 period

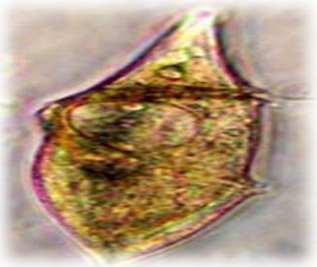
		Cell counts (Alexandrium spp.) cells/L	
Sample	**Sampling date**	**Station 1(Lagoon area)**	**Station 2 (Bay area)**
1	07/06/2012	500	0
2	11/06/2012	5000	2000
3	12/06/2012	2000	2500
4	13/06/2012	2000	0
5	14/06/2012	2000	0
6	19/06/2012	18500	1500
7	20/06/2012	32500	0
8	21/06/2012	111500	15000
9	25/06/2012	44000	5000
10	26/06/2012	10000	12000
11	27/06/2012	44500	10000
12	28/06/2012	53500	874500
13	02/07/2012	72500	21500
14	03/07/2012	23000	1000
15	04/07/2012	20000	1500
16	05/07/2012	33500	3500
17	06/07/2012	79500	500

(0) – No species recorded

Appendix 3.2: Potential harmful and harmful phytoplankton species found in the water samples

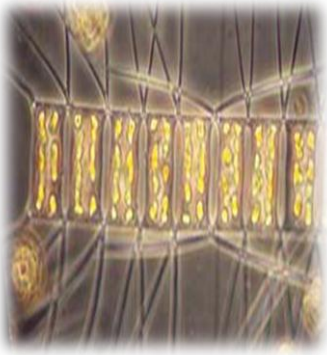
A.

B.



C.

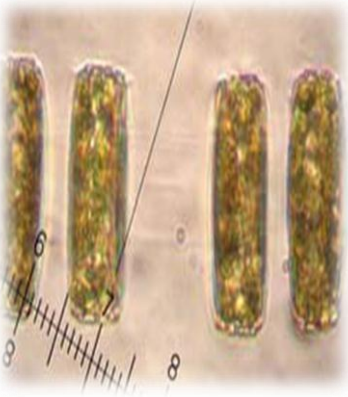
D.



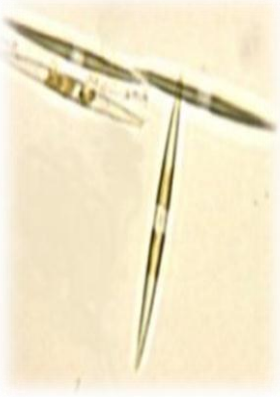
E.

F.

G.



H.



I.

A- *Prorocentrum sp.* **B-** *Dinophysis sp*. **C-** *Gyrodinium sp.* **D-** *Alexandrium sp*. **E-** *Chaetoceros* sp. **F-** *prorocentrum sp*. **G-** *Heterosigma sp*. **H-** *Thalassiosiora sp*. **I-** *Pseudo-nitzchia sp.*

Photos are a representation of species found and not taken the time they were identified.

Appendix 3.3: Phytoplankton species identified in the seawater samples per station (May 2012 - February 2013).

Month	Species	Henties Bay	Swakopmund	Bird Island	Walvis Bay
May	Diatoms Dinoflagellates Toxic species	2 2 0	2 2 1	2 3 2	6 3 2
June	Diatoms Dinoflagellates Toxic species	3 1 0	1 0 1	1 1 0	7 4 1
July	Diatoms Dinoflagellates Toxic species	7 0 0	1 0 0	4 0 0	3 1 1
August	Diatoms Dinoflagellates Toxic species	9 1 0	6 0 0	2 0 0	6 1 1
September	Diatoms Dinoflagellates Toxic species	6 0 1	2 0 1	2 0 0	3 2 1
October	Diatoms Dinoflagellates Toxic species	2 0 0	2 0 1	2 0 0	7 3 1
November	Diatoms Dinoflagellates Toxic species	6 0 0	0 0 0	5 0 0	6 3 3
December	Diatoms Dinoflagellates Toxic species	0 0 0	8 0 0	8 0 0	7 3 2
January	Diatoms Dinoflagellates Toxic species	2 1 0	1 1 0	3 0 0	3 3 1
February	Diatoms Dinoflagellates Toxic species	5 0 0	6 0 0	11 2 0	12 5 2

APPENDIX 4

Appendix 4.1: Data collection sheets for Biotoxin Analysis in *Mytilus galloprovincialis* throughout the sampling period.

Sample no.	Code No.	Date collected	Station	Homogenate date	Whole weight (g)	Test
1	05/C1	24/05/2012	Swakopmund	22/04/2013	69.02	PSP + DSP
2	05/C2	22/05/2012	Henties Bay	22/04/2013	49.32	PSP
3	05/C3	24/05/2012	Bird Island	22/04/2013	65.09	PSP+DSP
4	05/C4	23/05/2012	Walvis Bay	22/04/2013	126.11	PSP + DSP
5	06/C	26/06/2012	Walvis Bay	19/04/2013	145.69	PSP +DSP
6	06/C	26/06/2012	Swakopmund	19/04/2013	81.66	PSP+DSP
7	06/C	26/06/2012	Bird Island	19/04/2013	84.01	PSP+DSP
8	06/C	21/06/2012	Henties Bay	19/04/2013	92.92	PSP+DSP
9	07/C	11/07/2012	Walvis Bay	17/04/2013	166.55	PSP+DSP
10	07/C	19/07/2012	Henties Bay	17/04/2013	66.35	PSP+DSP
11	07/C	19/07/2012	Swakopmund	17/04/2013	80.92	PSP+DSP
12	07/C	20/07/2012	Bird Island	17/04/2013	61.96	PSP+DSP
13	08/C	08/08/2012	Walvis Bay	18/04/2013	130.91	PSP+DSP
14	08/C	17/08/2012	Henties Bay	18/04/2013	68.57	PSP+DSP
15	08/C	22/08/2012	Swakopmund	18/04/2013	70.83	PSP+DSP
16	08/C	22/08/2012	Bird Island	18/04/2013	54.6	PSP
17	09/C	05/09/2012	Walvis Bay	16/04/2013	168.78	PSP+DSP
18	09/C	26/09/2012	Swakopmund	16/04/2013	79.99	PSP+DSP
19	09/C	21/09/2013	Henties Bay	16/04/2013	71.96	PSP+DSP
20	10/C	31/10/2012	Henties Bay	22/03/2013	49.72	
21	10/C	17/10/2012	Bird Island	22/03/2013	69.36	PSP+DSP
22	10/C	26/10/2012	Walvis Bay	22/03/2013	145.35	PSP+DSP
23	10/C	17/10/2012	Swakopmund	22/03/2013	84.23	PSP+DSP
24	11/C	29/11/2012	Henties Bay	16/04/2013	80.9	PSP+DSP
25	11/C	07/11/2012	Walvis Bay	16/04/2013	68.96	PSP+DSP
26	11/C	28/11/2012	Swakopmund	16/04/2013	72.38	PSP+DSP

27	11/C	28/11/2012	Bird Island	18/04/2013	86.41	PSP+DSP
28	12/C	14/12/2013	Bird Island	19/04/2013	74.73	PSP+DSP
29	12/C	14/12/2013	Swakopmund	19/04/2013	84.55	PSP+DSP
30	12/C	18/12/2013	Henties Bay	19/04/2013	90.31	PSP+DSP
31	02/ C13	13/02/2013	Walvis Bay	18/04/2013	63.03	PSP+DSP

Appendix 4.2: Data collection sheet for Biotoxin Analysis in *M. galloprovincialis* for Lüderitz during bloom and after bloom period.

Sample no.	**Code No.**	**Date collected**	**Station**	**Homogenate date**	**Whole weight (g)**	**Test**
1	06/L12	28/06/2012	Lüderitz	17/04/2013	20.0	PSP
2	07/L12	16/07/2012	Lüderitz	17/04/2013	38.3	PSP
3	07/L12	16/07/2012	Lüderitz	17/04/2013	69.75	PSP+DSP
4	10/L12	24/10/2012	Lüderitz	22/03/2013	85.96	PSP+DSP
5	10/L12	23/10/2012	Lüderitz	18/04/2013	110.71	PSP+DSP
6	04/L13	04/04/2013	Lüderitz	19/04/2013	111.84	PSP+DSP
7	04/L13	04/04/2013	Lüderitz	19/04/2013	63.03	PSP+DSP

Appendix 4.3: Lipophilic toxin concentrations in mussel *M. galloprovincialis* samples collected from central coast in (µg/kg) using LC-MS/MS.

Station	Sampling date	OA	DTX1	DTX2	Total OA Group toxins	Mean OA Group Toxins	SE
Walvis Bay	23/05/2012	30	0.00	0.00	30	10	10
Walvis Bay	26/06/2012	330	390	0.00	720	240	120
Walvis Bay	11/07/2012	260	230	0.00	490	160	80
Walvis Bay	08/08/2012	240	250	0.00	490	160	80
Walvis Bay	05/09/2012	60	50	0.00	110	40	20
Walvis Bay	26/10/2012	70	70	0.00	140	50	20
Walvis Bay	07/11/2012	270	290	0.00	560	190	90
Walvis Bay	13/02/2013	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	24/05/2012	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	26/06/2012	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	19/07/2012	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	22/08/2012	50	50	0.00	100	30	16
Swakopmund	26/09/2012	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	17/10/2012	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	28/11/2012	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	14/12/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	26/06/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	24/05/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	20/07/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	17/10/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	28/11/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	14/12/2013	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	21/06/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	19/07/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	17/08/2012	0.00	40	0.00	40	0.00	0.00
Henties Bay	21/09/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	29/11/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	18/12/2012	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 4.4: YTX toxin in mussel *Mytilus galloprovincialis* samples collected from central coast in (mg/kg) using LC-MS.

Station	Sampling date	YTX	homo-YTX	45-OH YTX	Total YTX Group toxins	Mean YTX	SE
Walvis Bay	23/05/2012	0.04	0.00	0.00	0.04	0.00	0.00
Walvis Bay	26/06/2012	0.00	0.00	0.00	0.00	0.00	0.00
Walvis Bay	11/07/2012	0.00	0.00	0.00	0.00	0.00	0.00
Walvis Bay	08/08/2012	0.00	0.00	0.00	0.00	0.00	0.00
Walvis Bay	05/09/2012	0.00	0.00	0.00	0.00	0.00	0.00
Walvis Bay	26/10/2012	0.00	0.00	0.00	0.04	0.00	0.00
Walvis Bay	07/11/2012	0.00	0.00	0.00	0.00	0.00	0.00
Walvis Bay	13/02/2013	0.00	0.00	0.00	0.00	0.00	0.00
Swakopmund	24/05/2012	0.00	0.11	0.00	0.11	0.04	0.03
Swakopmund	26/06/2012	0.00	0.08	0.00	0.08	0.03	0.03
Swakopmund	19/07/2012	0.00	0.08	0.00	0.08	0.03	0.03
Swakopmund	22/08/2012	0.04	0.1	0.00	0.14	0.05	0.03
Swakopmund	26/09/2012	0.00	0.13	0.00	0.13	0.04	0.04
Swakopmund	17/10/2012	0.00	0.1	0.00	0.1	0.03	0.03
Swakopmund	28/11/2012	0.00	0.07	0.00	0.07	0.02	0.02
Swakopmund	14/12/2012	0.00	0.08	0.00	0.08	0.03	0.03
Bird Island	26/06/2012	0.04	0.1	0.00	0.14	0.05	0.03
Bird Island	24/05/2012	0.04	0.08	0.00	0.12	0.04	0.02
Bird Island	20/07/2012	0.00	0.06	0.00	0.06	0.02	0.02
Bird Island	17/10/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	28/11/2012	0.00	0.00	0.00	0.00	0.00	0.00
Bird Island	14/12/2013	0.00	0.00	0.00	0.00	0.00	0.00
Henties bay	21/06/2012	0.05	0.00	0.00	0.05	0.02	0.00
Henties Bay	19/07/2012	0.05	0.00	0.00	0.05	0.02	0.00
Henties Bay	17/08/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	21/09/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	29/11/2012	0.00	0.00	0.00	0.00	0.00	0.00
Henties Bay	18/12/2012	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 4.5: DSP and lipophilic Toxin concentrations in mussel (*Mytilus galloprovincialis*) samples collected during bloom and after bloom period from Lüderitz coast in (mg/kg) using LC-MS.

Station	**Sampling date**	**Total OA**	**Total DTX-1**	**Total DTX -2**	**PTX**	**Total OA Group Toxins**	**Total YTX**	**45-OH YTX**	**homo-YTX**	**Total YTX Group toxin**
Lüderitz	16/07/2012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lüderitz	24/10/2012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lüderitz	23/10/2012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lüderitz	04/04/2013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lüderitz	04/04/2013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 4.6: Paralytic Shellfish Poisoning (PSP) concentrations in mussels, *Mytilus galloprovincialis* collected from central coast, regulatory limit 80 µg STX eq./100g using Mouse Bioassay Method.

Station	sampling date	PSP value
Henties bay	22/05/2012	0.00
Henties Bay	21/06/2012	0.00
Henties Bay	19/07/2012	0.00
Henties Bay	21/09/2012	0.00
Henties Bay	29/11/2012	0.00
Henties Bay	18/12/2012	0.00
Swakopmund	24/05/2012	0.00
Swakopmund	26/06/2012	0.00
Swakopmund	19/07/2012	0.00
Swakopmund	22/08/2012	0.00
Swakopmund	26/09/2012	0.00
Swakopmund	17/10/2012	0.00
Swakopmund	28/11/2012	0.00
Swakopmund	14/12/2012	0.00
Bird Island	24/05/2012	0.00
Bird Island	26/06/2012	0.00
Bird Island	20/07/2012	0.00
Bird Island	22/08/2012	0.00
Bird Island	17/10/2012	0.00
Bird Island	28/11/2012	0.00
Bird Island	14/12/2012	0.00
Walvis Bay	23/05/2012	0.00
Walvis Bay	26/06/2012	0.00
Walvis Bay	11/07/2012	0.00
Walvis Bay	08/08/2012	35
Walvis Bay	05/09/2012	0.00
Walvis Bay	26/10/2012	0.00
Walvis Bay	07/11/2012	80
Walvis Bay	13/02/2013	0.00

Appendix 4.7: Paralytic Shellfish Poisoning (PSP) values in mussels, *Mytilus galloprovincialis* collected from Lüderitz coast, regulatory limit 80 µg STX eq./100g using Mouse Bioassay Method.

Sample no.	**Station**	**Sampling date**	**PSP value**
1	Lüderitz	27/06/2012	138.00
2	Lüderitz	28/06/2012	229.59
3	Lüderitz	16/07/2012	903.00
4	Lüderitz	16/07/2012	0
5	Lüderitz	24/10/2012	0
6	Lüderitz	23/10/2012	0
7	Lüderitz	04/04/2013	57
8	Lüderitz	04/04/2013	54

Appendix 4.8: Sommer's table relating mouse death times to toxicity units.

Death times (min:s)	Mouse units (MU)		Death times (min:s)	Mouse units (MU)
1:00	100		5:00	1.92
1:10	66.2		5:05	1.89
1:15	38.3		5:10	1.86
1:20	26.4		5:15	1.83
1:25	20.7		5:20	1.80
1:30	16.5		5:30	1.74
1:35	13.9		5:40	1.69
1:40	11.9		5:45	1.67
1:45	10.4		5:50	1.64
1:50	9.33			
1:55	8.42		6:00	1.60
			6:15	1.54
2:00	7.67		6:30	1.48
2:05	7.04		6:45	1.43
2:10	6.52			
2:15	6.06		7:00	1.39
2:20	5.66		7:15	1.35
2:25	5.32		7:30	1.31
2:30	5.00		7:45	1.28
2:35	4.73			
2:40	4.48		8:00	1.25
2:45	4.26		8:15	1.22
2:50	4.06		8:30	1.20

2:55	3.88		8:45	1.18
3:00	3.70		9:00	1.16
3:05	3.57		9:30	1.13
3:10	3.43			
3:15	3.31		10:00	1.11
3:20	3.19		10:30	1.09
3:25	3.08			
3:30	2.98		11:00	1.075
3:35	2.88		11:30	1.06
3:40	2.79			
3:45	2.71		12:00	1.05
3:50	2.63		13:00	1.03
3:55	2.56		14:00	1.015
			15:00	1.00
4:00	2.50		16:00	0.99
4:05	2.44		17:00	0.98
4:10	2.38		18:00	0.972
4:15	2.32		19:00	0.965
4:20	2.26		20:00	0.96
4:25	2.21		21:00	0.954
4:30	2.16		22:00	0.948
4:35	2.12		23:00	0.942
4:40	2.08		24:00	0.937
4:45	2.04		25:00	0.934
4:50	2.00		30:00	0.917

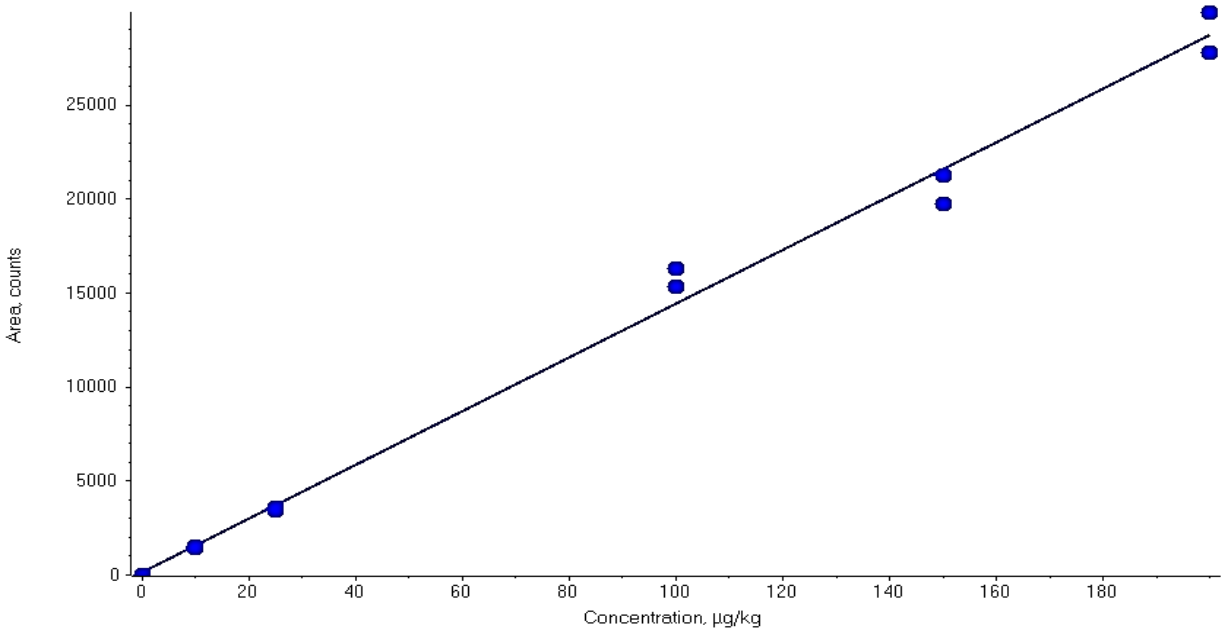
4:55	1.96		40:00 60:00	0.898 0.875

APPENDIX 5

Calibration curves used for quantification with an AB-Sciex 4000 QTrap mass spectrometer linked to an Agilent 1200 HPLC. The correlation coefficient indicated by exceeded 0.99 in all cases which shows a good precision of data.

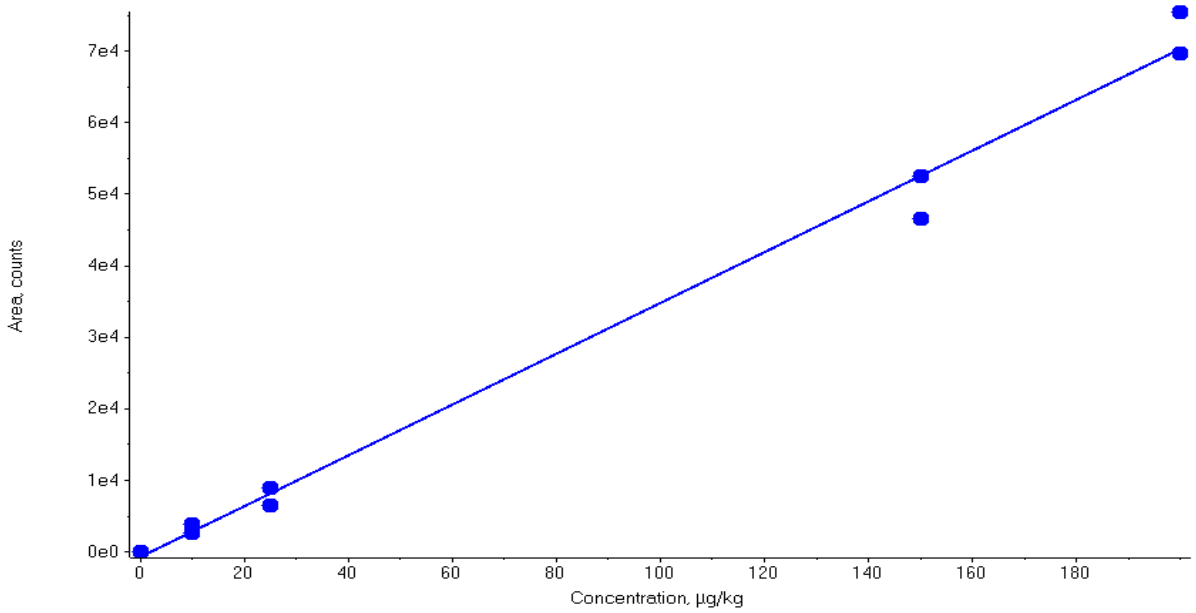
First batch:

Okadaic acid

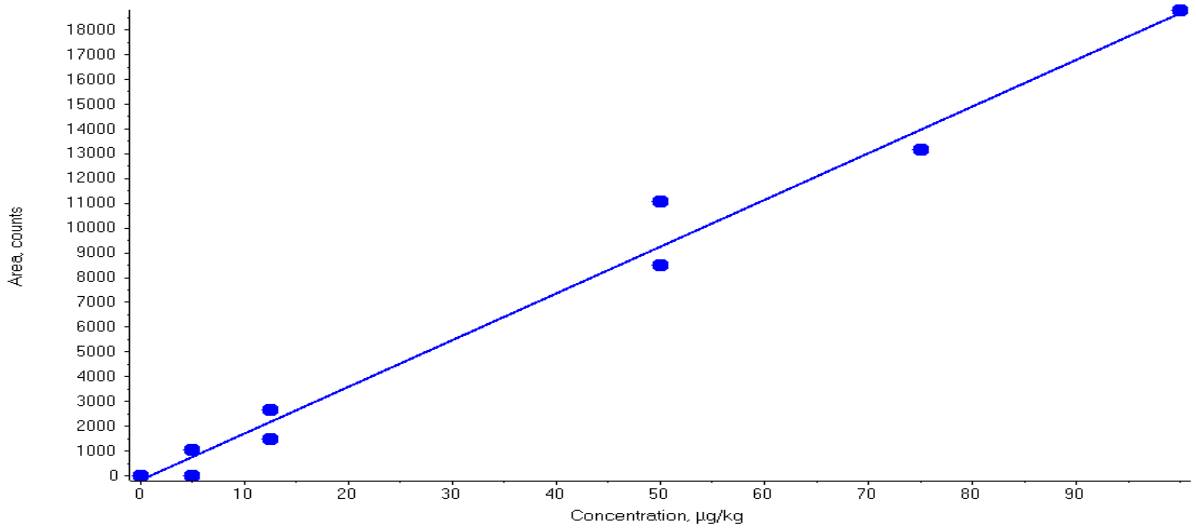


Regression Equation:
y = 143 x + 154 (r = 0.9963)

DTX-1



YTX

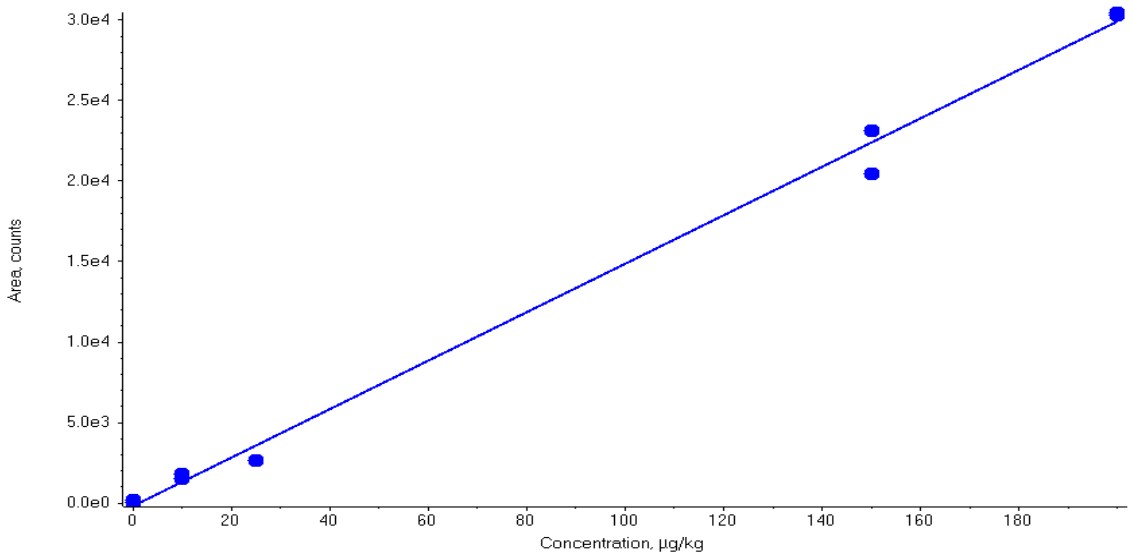


Regression Equation:

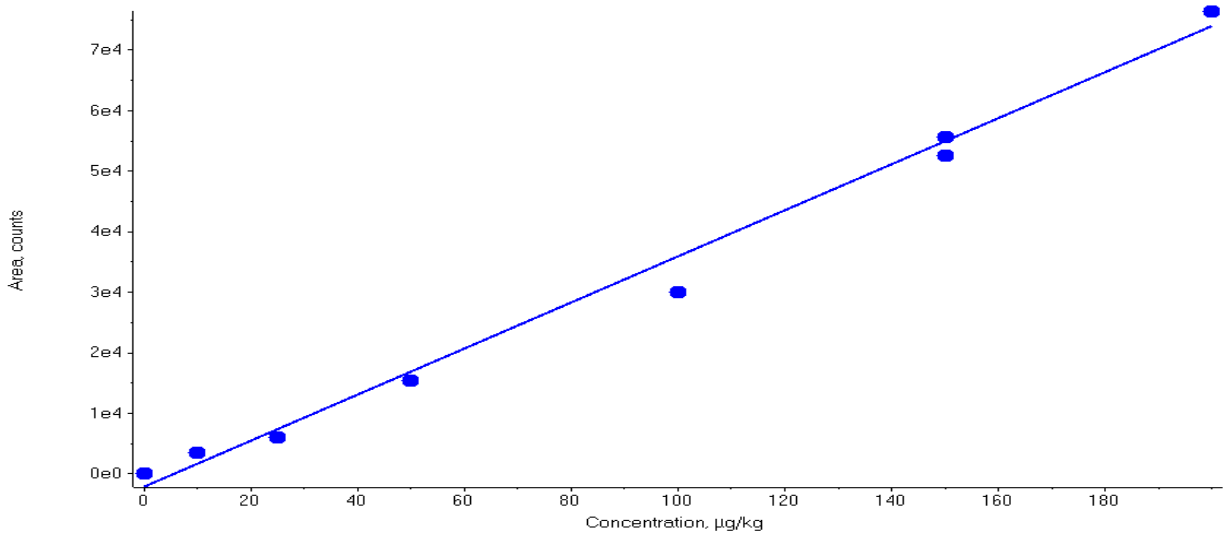
y = 189 x + -174 (r = 0.9927)

Second Batch:

Okadaic Acid



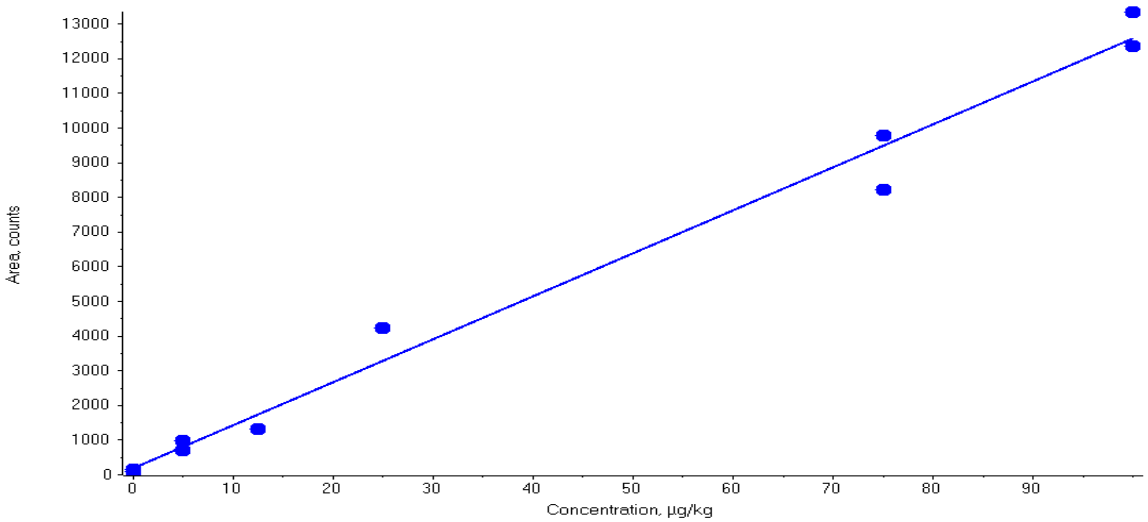
DTX-1



Regression Equation:

y = 380 x + -2.09e+003 (r = 0.9960)

YTX



Regression Equation:

y = 124 x + 194 (r = 0.9933)

APPENDIX 6

Appendix 6.1: AOAC method 959.08 for sample preparation and extraction methods (PSP) of shellfish Flesh for the mouse bioassay (MBA).