Marine phycotoxins in the northern Benguela region: biological and chemical parameters promoting the production of harmful algal blooms

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Abstract

The Benguela current is one of the four major coastal upwelling currents on the eastern boundary of the ocean basins. This upwelling system provides nutrient-rich deep water to the euphotic zone, thus facilitate the proliferation of harmful algal blooms. Namibia's mariculture sector benefits from the high cell numbers as their shellfish grow at a rate much higher than their international counterparts. Unfortunately the negative aspect of this is the fact that some of these blooms consist of algal species that produce phycotoxins. One such bloom consisting predominantly of dinoflagellates was detected during late summer of 2011 in the coastal embayment of Walvis Bay, Namibia. The phytoplankton species composition during this bloom included potentially toxic *Gonyaulax spinifera*. Environmental parameters as well as biological samples were taken during this event, and the toxin content measured by Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) and diarrhetic shellfish poisoning mouse bioassay (DSP MBA) for the oysters. Yessotoxin (YTX) and its analogues 45-hydroxy-yessotoxin (45-OH-YTX) and 1a-Homoyessotoxin (homo-YTX) were the dominant toxins present in the mussel samples at concentrations of up to 5.4 mg YTX equivalents kg^{-1} shellfish tissue. Phytoplankton YTX profile was dominated by homo-YTX and 45-OH-YTX. The highest levels produced were 156.0 pg $cell^{-1}$ with homo-YTX being produced at 96.0 pg $cell^{-1}$. This was the first time YTX and its analogues were detected in shellfish samples as well as *G. spinifera* in the Benguela current upwelling system. It was also the first detection of *G. spinifera* that produced 45-OH-YTX as one of the dominant YTX analogues. In March 2012, cultures of three *G. spinifera* strains were established from the Walvis Bay region. The toxin analysis revealed homo-YTX (82-92%) as the dominant toxin in all three strains, followed by YTX (5-14%). One strain produced 151.83 ng mL^{-1} YTXeq, which accounted for cellular homo-YTX production of 143.47 pg $cell^{-1}$. This is the highest concentration of homo-YTX produced per cell to date. All three cultures formed monophyletic group with the other YTX producing *G. spinifera* from Italy and New Zealand distinct from nontoxic *G. spinifera*. Both phosphate and nitrate reduction affected all three *G. spinifera* strains with respect to their growth and toxin production to varying degrees. Total YTX equivalents and homo-YTX increased under low phosphate conditions, while low nitrate enhanced cell growth. As a recurring HABs species in the Walvis Bay region, *G. spinifera* will have to be monitored effectively as the cells produce a high amount of YTX toxins. This will ensure continual safety for both domestic and international Shellfish consumers.

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List of abbreviations

ASP: Amnesic shellfish poisoning

AST: Amnesic shellfish toxins

AZA: azaspiracids

bp: base pairs

CSIR: Council for Scientific and Industrial Research

DA: Domoic acid

DO: Dissolved Oxygen

DNA: Deoxyribonucleic acid

dNTP: deoxyribonucleotide

DSP: Diarrhetic shellfish poisoning

DST: Diarrhetic shellfish toxins

DTX: Dinophysistoxins

DTX1: Dinophysistoxin 1

DTX2: Dinophysistoxin 2

EC: European commission

ELISA: enzyme-linked immunosorbent assay

Eq: equivalents

EU: European union

FISH: Fluorescence in situ Hybridisation

GF/F: Glass Fiber Filter

GYM: Gymnodimines

HABs: Harmful algal blooms

HCL: hydrochloric acid

H_2S: hydrogen sulphide

IOW: Leibniz Institute for Baltic Sea Research

ITS: internal transcribed spacer

1a homoyessotoxin: homo-YTX

LC-MS/MS: Liquid chromatography-Mass spectrometry

L:D: light: dark

LSU: Large sub unit of ribosomal DNA

MBA: Mouse bioassay

MCM: Marine and Coastal Management

MERIS: Marine environment and Medium Resolution Imaging Spectrometer

MFMR: Ministry of Fisheries and Marine Resources

MRSU: Marine Remote Sensing Unit

m/z: mass to charge ratio

N: cell density

NJ: Neighbourhood joining

NO_3^-: nitrate

NSP: Neurotoxic Shellfish Poisoning

OA: Okadaic Acid

P- phosphate

PbTX: Brevetoxins

p-distance: pairwise differences

PCR: polymerase chain reaction

PKS: polyketide synthetase

PO_4^{3-}: dissolved inorganic phosphate

PSP: Paralytic shellfish poisoning

PST: Paralytic shellfish toxins

PTX: Pectenotoxins

QC: quality control

rDNA: ribosomal deoxyribonucleic acid

RSD: relative standard deviation

RT: retention time

SE: Standard Error

SEM: Scanning Electron Micrograph

SiO_4: dissolved inorganic silicate (silicic acid)

SPATT: Solid Phase Adsorption Toxin Tracking

SPX: Spirolides

SSU: small sub unit of ribosomal DNA

STX: saxitoxin

TEF: toxic equivalent factor

UCT: University of Cape Town

YTX- Yessotoxin

YTXeq- yessotoxin equivalents

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I dedicate this thesis to the Namibian women pioneering scientific research/knowledge in Namibia namely: Bronwen Currie, Nadine Moroff-Kohlstaedt, Margit Wilhelm, Paloma Ellitson, Annethea Muller, Elsabe Julies, Heidi Skryzek, Anja Kriener, Anja van der Plaas, Martha Ntinda, Chishimba Kantu (Zambia is close enough, you're an honorary Namibian for this purpose) Ndawedapo Mbangula and Hilkka Ndjaula.

Declaration of the doctoral candidate according to § 4 (1) letters g and h
Of the doctoral degree regulations
Of the Faculty of Mathematics and Natural Sciences
of the University of Rostock

Name ..... .

(Name, First name)

Address.....

(Street, Postal code, Place of Residence)

I intend to elaborate a dissertation on the topic

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at the Faculty of Mathematics and Natural Sciences at the University of Rostock. My supervisor is Mr/Ms

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2. I hereby declare under oath that I have completed the work submitted here independently and have composed it without outside assistance. Furthermore, I have not used anything other than the resources and sources stated and where I have taken sections from these works in terms of content or text, I have identified this appropriately.

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Chapter 1: Introduction

1.1 Harmful Algal Blooms

Of the 5000 identified marine phytoplankton species, about 300 are known to produce Harmful Algal Blooms (HABs) due to their high proliferation rates (Hallegraeff, 1993; Smayda, 1997). The conditions under which this happens are still not fully understood.

HABs are caused by the proliferation of phytoplankton cells forming high biomasses. Some high biomasses cause discoloration of the water, which led to them, being previously known as 'red tides' (figure 1). These blooms can be dominated by a specific species and are classified into three groups according to their effect in their environment by Hallegraeff (1995) namely:

(i) Causing dissolved oxygen depletion in the water column from microbial activity during bloom decay, therefore killing oxygen dependent organisms indiscriminately;

(ii) Not producing toxins that can adversely affect humans but causes chemical and mechanical damage to fish gills. e.g. *Chaetoceros convolutes*;

(iii) Producing potent toxins causing gastrointestinal and neurological illness for ingesting contaminated shellfish.

HABs are predominantly a coastal phenomenon, whose frequency is increased in coastal embayments (Cembella et al., 2005). In upwelling regimes, they are more likely to occur towards the end of the upwelling season (late summer). The short term alternations between upwelling-relaxation periods lead to the formation of specific habitats which may lead to bloom formation if:

(i) the cellular growth rate is fast enough to take advantage of the relaxation period,

(ii) growth rate is faster than advective dispersal and mortality losses,

(iii) growth is not impaired by turbulence.

Thus this complexity and rapid fluctuations that occur make it extremely difficult to predict the occurrence of HABs (Smayda, 2008). These factors in turn determine which phytoplankton species will form the bloom. Of the 300 species mentioned above only 80 species produce phycotoxins (Smayda, 1997; Hallegraef, 2003).

Figure 1: Lingulodinium *polyedrum* bloom at La Jolla, San Diego causing water discoloration (Kai Schumann, NOAA).

1.2 Factors aiding in harmful algal bloom formation

There are very few unifying principles that explain and thus predict bloom formation in all environments (Anderson et al., 2012). The exact of interaction of the circumstances needed for bloom formation are not fully understood, though specific climatic and hydrographic conditions have been shown to play a role in this formation (Moore et al., 2009). Thus the bloom formation is primarily driven by the coupling of upwelling-relaxation-downwelling cycles, changes in nutrient availability, temperature and stratification (Kudela et al., 2005). Margalef (1978) pioneered a concept by formulating a graphical representation of bloom forming factors (figure 2). Diatoms will dominate under high nutrient; high turbulent conditions while dinoflagellates dominate under low nutrient, low turbulent conditions. The dinoflagellates that form algal blooms thrive under high nutrient, low turbulent conditions.

With respect to nutrient availability, nitrogen is normally the limiting factor for phytoplankton growth in coastal upwelling systems (Wilkerson and Dugdale, 2008). Thus in order to understand the bloom dynamics, physical (i.e. turbulence, shear and advection) and biological behaviour (migration and physiological adaptation) should be studied in unison (Gentien et al., 2005).

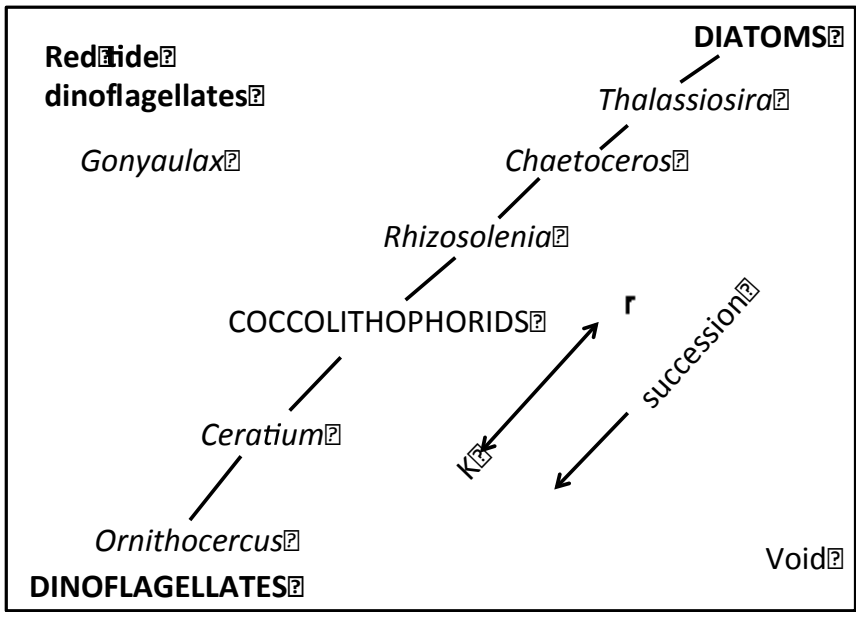


Figure 2: Modified Margalef's Mandala depicting annual cycle of phytoplankton abundance in the Rias Baixas of Galicia with respect to nutrient load and turbulence with r : small cell, high growth rate and K: large cell, low growth rate (Estrada and Berdalet, 1997).

1.3 Harmful algal blooms in the Northern Benguela

The Benguela Current extends from the southern tip of Southern Africa to the Angola-Benguela front of Angola (figure 3). This current is one of the four major coastal upwelling currents on the eastern boundary of the ocean basins (Trainer et al., 2010). The principal upwelling cell is around Luderitz, thus the area is divided into north (encompassing Walvis Bay) and southern components (Trainer et al., 2010). As an upwelling system, it provides nutrient-rich deep water to the euphotic zone (Pitcher et al., 2010). This enhances the growth of phytoplankton and can thus facilitate the frequent proliferation of HABs. Upwelling-favourable winds typically reach its maximum during spring and summer. Thus, thermal stratification is weakest in spring and strongest during late summer/early autumn (Pitcher et al., 2010; Lass and Mohrholz, 2005). As the response of phytoplankton to seasonal upwelling is lagged, this leads to the highest phytoplankton biomass during summer and autumn. Thus HAB dominated by dinoflagellates are frequent during late summer and early autumn (Pitcher et al., 2010). As Walvis Bay lies in the central upwelling cell of the Benguela Current (Huhn et al., 2007) it is an ideal area for shellfish cultivation as the phytoplankton rich water and warmer temperatures enable the shellfish to grow at rates higher than its global counterparts (Aquafact, 2012). This phytoplankton rich water can have adverse effects on the shellfish industry by aiding in the spread of hydrogen sulphide in the water column. This adversely affects the shellfish and leads to massive mortalities if these events extend over

more than two days (Emeis et al., 2002; Currie et al., 2007). Phycotoxin producing HABs are also of concern. However in the Benguela region, the southern component is more extensively documented than the north (Trainer et al., 2010). The levels of phycotoxins detected will be detailed in the subsection 1.5 of this thesis.

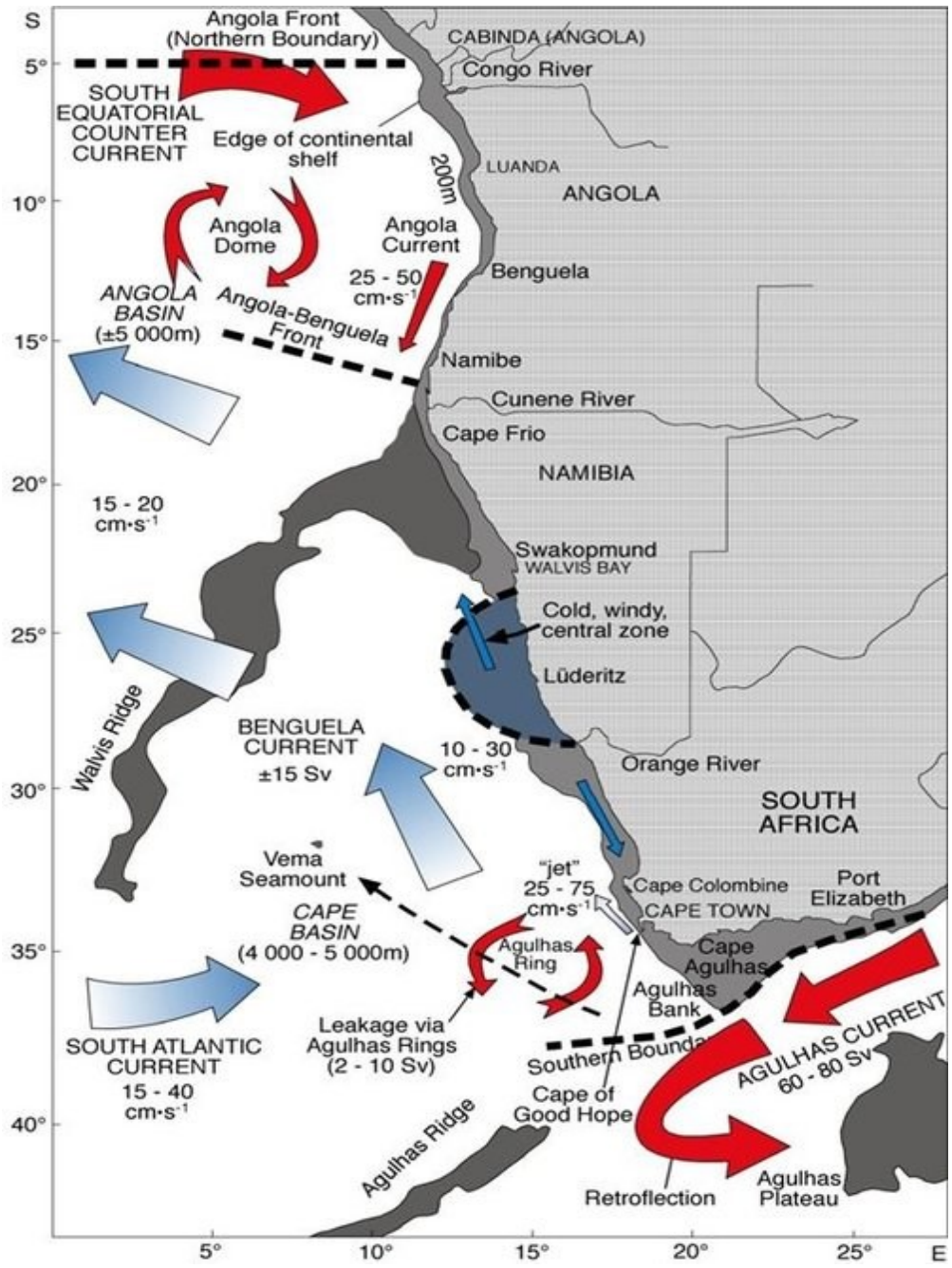


Figure 3: The Benguela system with corresponding currents (Shannon, L.V. and O'Toole, M.J., 2003).

1.4 Mariculture activities in the Northern Benguela and the impact of HABs

The mariculture industry in Namibia is becoming an increasingly important economic sector. This importance is further emphasised, as this renewable resource will greatly benefit a nation that relies heavily on non-renewable resources. This industry is predominantly concentrated in the coastal towns of Walvis Bay, Swakopmund and Luderitz due to the deep-water bays as well as their proximity to infrastructure (Silke, 2011). It is an oyster dominated industry with two species of bivalve molluscs being farmed, namely the oyster species *Crassostrea gigas* and *Ostrea edulis* with farmers producing 450 tonnes in 2008 for export to the rest of Africa and the far east (MFMR, 2012). Shellfish are a high-risk product with respect to human consumption as they filter-feed on phytoplankton which are potential producers of phycotoxins. These phycotoxin producing phytoplankton accumulate in the shellfish flesh and may cause harm to the consumer. Thus shellfish sanitation programs are implemented and regulated internationally in countries involved in mariculture activities. One such international regulation laid down by the European Union describes specific hygiene rules establishes maximum permissible levels for phycotoxins in live bivalve molluscs (Regulation (EC) No 853/2004). In order to comply with these stringent requirements that aid in the safe consumption of shellfish, the Namibian Ministry of Fisheries and Marine Resources (MFMR) drafted a Shellfish Sanitation monitoring program. This program has been running since 2004 and samples are taken for phycotoxin and microbiological analysis as well as phytoplankton counts (Anderson et al., 2004; Currie et al., 2004). Due to the detection of the causative dinoflagellate and diatom species, only lipophilic shellfish toxins (previously Diarrheic Shellfish Poisoning toxins), Paralytic Shellfish Toxins (PST) and Amnesic shellfish Poisoning Toxin Domoic acid (DA) are tested for in this region (Anderson et al., 2004). The methods used to test for these toxins are regulatory, thus they are not necessarily the most accurate. The lipophilic toxins have been detected the most regularly of the toxins tested (Aquafact, 2012).

1.5 Poisoning syndromes and the corresponding causative phycotoxins

Marine phycotoxins are generally characterized by the syndrome observed from the human consumption of contaminated shellfish. Currently the more popular characterization is based on their chemical properties. They can be divided into two different classes, namely hydrophilic and lipophilic toxins (Gerssen et al., 2010). DA and PST toxins are hydrophilic and have a molecular weight bellow 500 Da, while the Diarrheic Shellfish Poisoning Toxins (DST) and yessotoxins (YTX) are lipophilic and have a molecular weight above 600 Da (table 1).

Table 1: Marine phycotoxin groups and the causative algal species (Gerrisen et al., 2010).

	Toxin group	**Syndrome**	**Genus**	**Species**
Hydrophilic toxins	PSTs	PSP	*Alexandrium*	*angustitabulatum, catenella, fundyense, lusitanicum, minutum, tamarense, tamiyavanichii, ostenfeldii*
			Gymnodinium	*catenatum*
			Pyrodinium	*bahamense*
	Domoic acid	ASP	*Pseudo-nitzschia*	*australis, calliantha, cuspidata, delicatissima, fraudulenta, galaxiae, multiseries, multistriata, pseudodelicatissima, pungens, seriata, turgidula*
Lipophilic toxins	Okadaic acid and dinophysistoxins and pectenotoxins	DSP	*Phalacroma*	*rotundatum, arenarium, belizeanum, concavem, lima*
			Prorocentrum	*acuminata, acuta, arenarium, caudate, fortii, mitra, norvegica, ovum, rotundata, sacculus, tripos*
			Dinophysis	
	Yessotoxin		*Protoceratium*	*reticulatum*
			Lingulodinium	*polyedrum*
			Gonyaulax	*spinifera*
	Gymnodimines	-	*Karenia*	*selliforme*
			Gymnodium	*mikimotoi*
	Azaspiracids	AZP	*Azadinium*	*spinosum*
	Spirolides	–	*Alexandrium*	ostenfeldii, peruvianum
	Brevetoxins	NSP	*Karenia*	*brevis brevisulcata, mikimotoi, selliformis, papilionacea*
			Chatonella	*cf. verruculosa*

1.5.1 Hydrophilic phycotoxins

1.5.1.1 Paralytic Shellfish Poisoning toxins

This phycotoxin is predominantly produced by dinoflagellates from the *Alexandrium* genus (table 1). The toxins produced are Paralytic Shellfish Toxins (PSTs) with saxitoxin (STX) as the parent compound. There are approximately 30 different analogues within this group with variations in toxicity. It was first detected in California in 1920 and has since been detected globally with the widest distribution of all the phycotoxins (Trainer et al., 2010).

Their mode of action in mammalian cells is to cause inhibition of the voltage-gated sodium channel (blocking the excitation current in muscle and nerve cells) resulting in a reduced action potential. This leads to respiratory difficulties as well as muscle paralysis. The symptoms observed are dependent on the concentration of the toxins ingested. These can be as mild as tingling and numbness around the lips, prickly sensation of fingertips, headache, dizziness, nausea, vomiting and diarrhea. Severe cases affect motor nerves, resulting in respiratory difficulties and other muscular paralytic effects, which can lead to death (Gerssen et al., 2010). The EU has established a permitted level of 800 µg STX 2-HCl equivalents kg^{-1} shellfish.

For the Benguela region, the southern Benguela blooms of *Alexandrium catenella* are common, as well as the presence of *Alexandrium minutum*. These species have been attributed to the causing toxin concentrations higher than the regulatory limit in tested shellfish. In the northern Benguela, *Alexandrium tamarense* was identified as a regular bloom forming species in Walvis Bay in 1967, but blooms from this species have not been observed with the HABs monitoring from 2004 to present. From regulatory testing in this area oyster samples have shown low levels of PSTs on a few occasions (the method used has a limit of detection of 350 µg STX eq kg^{-1} flesh) as well as low levels of *Alexandrium* species in the water column (557 cells L^{-1}). Only abalone has consistently tested with levels of PSTs higher than the limit of detection (figure 4). Abalone do not filter feed so it is unclear as to what the vector for their toxin content is (Deeds et al., 2008).

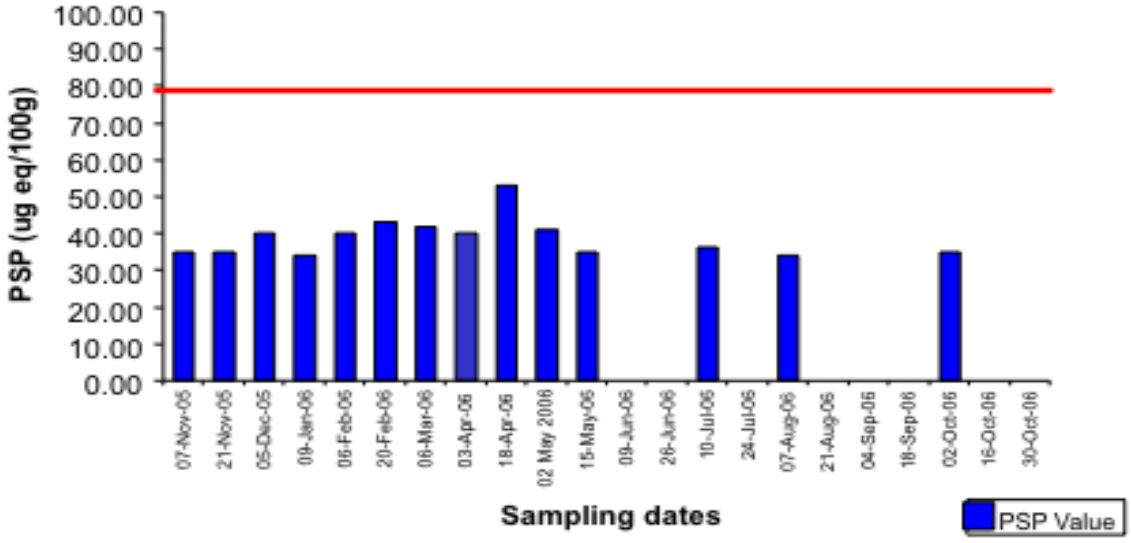


Figure 4: PSP toxins detected in *Abalone* from November 2005 - October 2006. The red line denotes the regulatory limit of 80 µg STX eq 100 g^{-1} flesh (Currie et al., 2007).

1.5.1.2 Amnesic Shellfish Poisoning (ASP) toxins

This syndrome is caused by domoic acid (DA), which is produced by some species of the diatom from the *Pseudo-nitzschia* genus (table 1). The toxin is a rare amino acid that was first detected in 1987 on Prince Edward Island after blue mussels (*Mytilus edulis*) with high levels of DA were consumed (James et al., 2005). The mode of action of this neurotoxin in mammals is to bind to glutamate receptors in the hippocampus with a high affinity. This opens the membrane channels causing an increased sodium influx and membrane depolarization. The syndromes observed are gastrointestinal disorder, nausea, vomiting, abdominal cramps, diarrhea, headache, dizziness and short-term memory loss. The EU has established a permitted level of 20 mg DA kg^{-1}.

DA has not been detected in shellfish in the Benguela region even though several of the causative *Pseudo-nitzschia* species (including *P. seriata* and *P. delicatissma*) are known to occur (Pitcher and Calder, 2000). Seawater samples containing *Pseudo-nitzschia* cells from the southern Benguela were found to contain DA at the range of 0.1-3.0 µg L^{-1} (Fawcett et al., 2007). In the northern Benguela, the Shellfish Sanitation survey conducted by Aquafact (2012) on the Namibian Shellfish Sanitation Program proposes ASP as a potential threat. This is highly unlikely due to the ASP testing that has been done

since 2004. No DA has ever been detected even though high biomasses (34000 cells L^{-1}) of *Pseudo-nitzschia species* have been found in the oyster growing areas (figure 5).

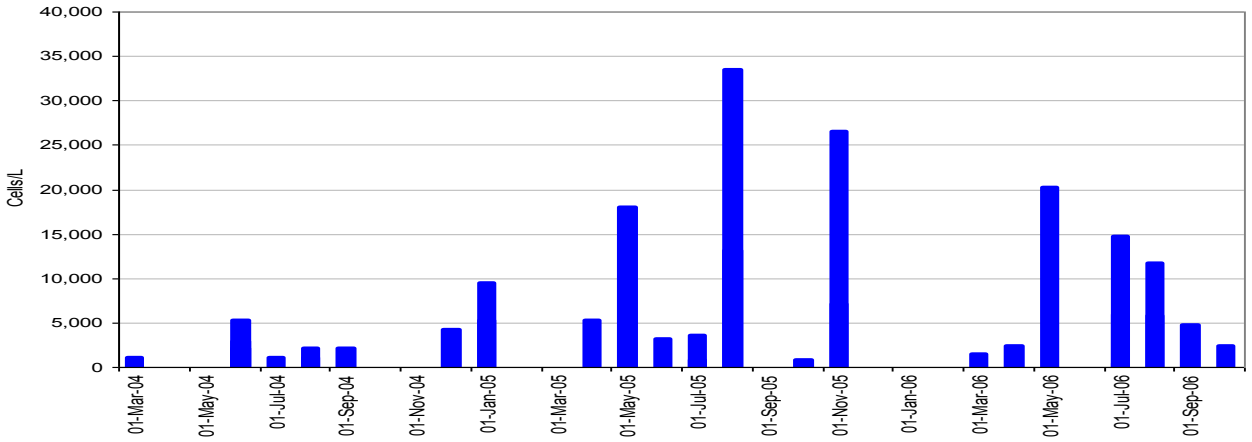


Figure 5: *Pseudo-nitschia* spp biomass from the Walvis Bay area for March 2004 - October 2006 (Currie et al., 2007).

1.5.2 Lipophilic toxins

This group of polyether toxins includes okadaic acid (OA), dinophysistoxins (DTX), pectenotoxins (PTX) and YTX. Though this group was previously known as the DSP complex (Yasumoto et al., 1985) it had to be regrouped due to the fact that PTX and YTX have different modes of action even though they generally co-occur (Gerrisen et al., 2010; Trainer et al., 2010).

1.5.2.1 Diarrhetic Shellfish Poisoning (DSP) toxins

These acid polyethers are produced by dinoflagellates from the *Dinophysis* and *Prorocentrum* genus (table 1). The toxins produced include Okadaic acid (OA) dinophysistoxin-1 (DTX1) and -2 (DTX2) as well as the esterified forms of OA, DTX1 and DTX2. The first human intoxication from these toxins was in the Netherlands in 1961 (Gerissen et al., 2010). These toxins inhibit protein phosphatase. This inhibition leads to hyperphosphorylation of proteins involved in the cytoskeletal junctions that regulate the permeability of the cell, resulting in a loss of cellular fluids (Vale and Botana, 2008). This leads to gastrointestinal distress, diarrhea, abdominal cramps, nausea and vomiting (Gerrisen et al., 2010). The EU has established a permitted level of 160 µg OA-equivalents kg^{-1} shellfish.

In the Benguela region, DSP toxins were detected in shellfish from the southern Benguela in 1991 with the causative species being *D. acuminata* (Pitcher et al., 1993). The blooms of this species were observed in autumn and coincided with periods of upwelling relaxation, which lead to the warming of inshore waters (Fawcett et al., 2007). Subsequent monitoring revealed that DSP events are frequent in this area and are usually attributed to *D. acuminata* and *D. fortii* (Trainer et al., 2010). Okadaic acid was identified as the primary toxin of Dinophysis species with low amounts of PTX2 been observed in field samples, consistent with the presence of *D. acuminata* and *D. fortii* (Fawcett et al., 2007). In the northern Benguela, positive DSP/lipophilic toxins results were observed on 26 separate occasions over a four-year period (figure 7). Unfortunately the mouse bioassay (MBA) method (Yassumoto, 1985) was used for analysis which gives a qualitative result. As this method is very unspecific, other acetone soluble toxins such as Domoic acid, azaspiracids, brevetoxins, gymnodimine, spirolides, pinnatoxin, ciguatoxin, palytoxin, aplysiatoxins, lyngyatoxin can also be detected. Thus the causative toxin cannot be discerned. Previous data from the Walvis Bay area shows that there is a discrepancy between the phytoplankton composition and the positive results obtained as the high *Dinophysis* species cell counts do not correlate with positive DSP results (Currie et al., 2004; figure 6).

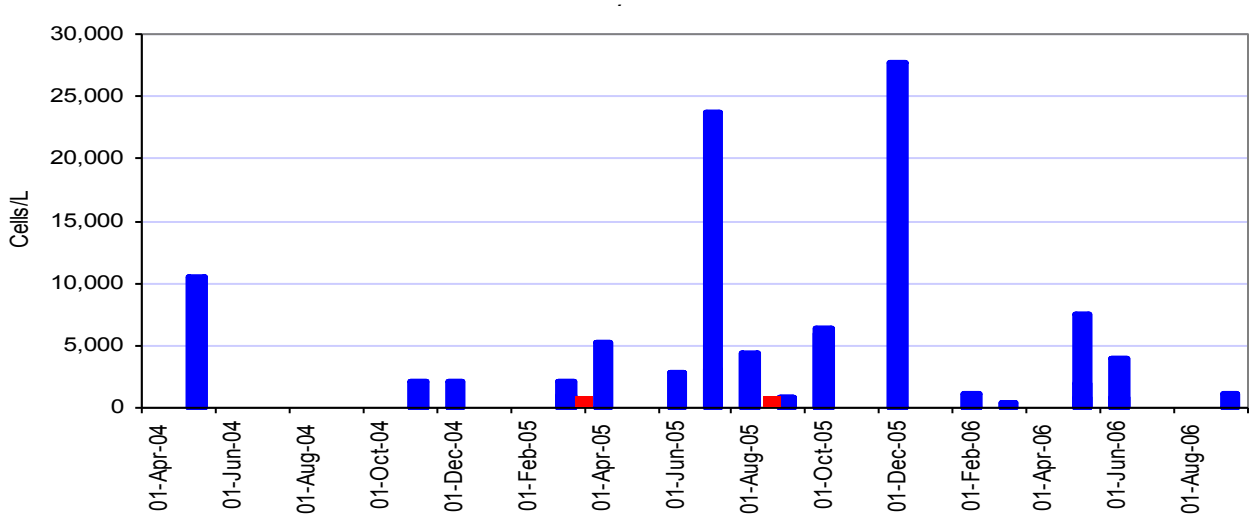


Figure 6: *Dinophysis* spp biomass (*D. fortii*, *D. acuminata* and *D. acuta*) in Aquapark 1 from April 2004 - September 2006. The red blocks denote the times when oyster samples tested positive for DSP toxins (Currie et al., 2007).

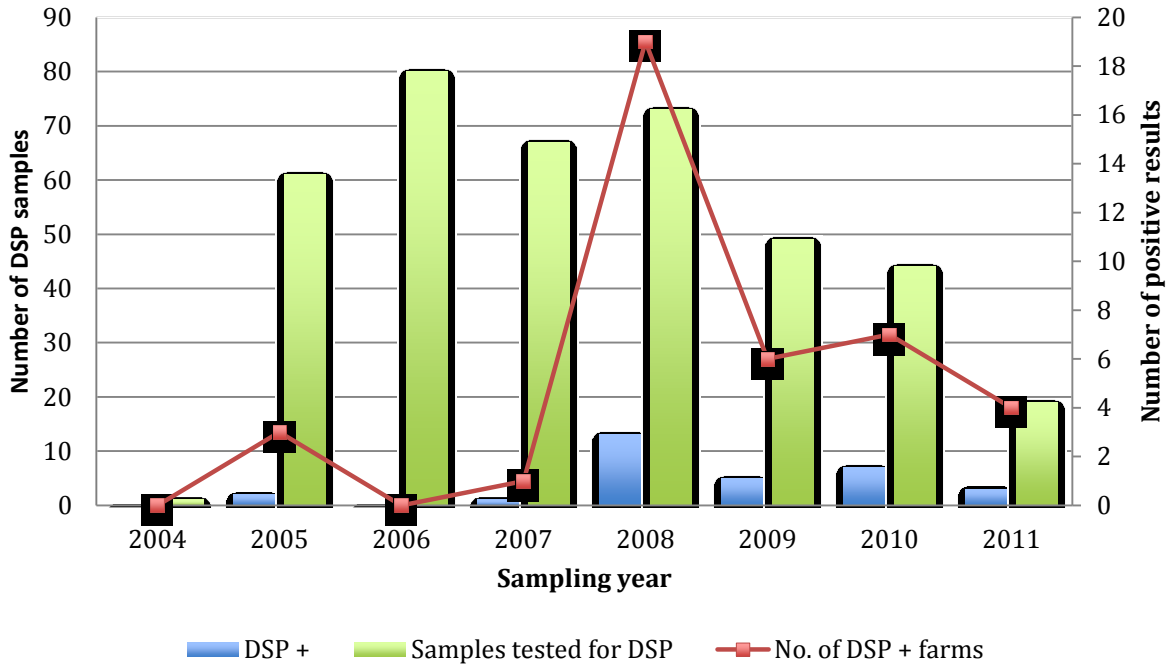


Figure 7: DSP positives in Aquapark 1 from 2004 to April 2011 for the Shellfish Sanitation program. Samples were analysed using the qualitative Mouse Bioassay (data from MFMR database).

1.5.2.2 Yessotoxin

YTX and its analogues (figure 10; Gerssen et al., 2010) are polyether toxins produced by the dinoflagellates *Lingulodinium polyedrum* (Stein) Dodge, *Protoceratium reticulatum* (Claparede and Lachmann) Buetschli and *Gonyaulax spinifera* (Claparede and Lachmann 1857) Diesing (Alvarez et al., 2011). YTX was first isolated in Japan from the scallop *Patinopecten yessoensis* (Murata et al., 1987). To date, 90 different analogues have been identified (Miles et al., 2005). Testing of shellfish samples lead to observed toxic symptoms when tested on mice by intraperitoneal injection, thus they were classified as DSP toxins. 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). The regulatory limit is 1 mg kg^{-1}.

Globally, only mussels from Norway, Italy, New Zealand, Russia and the USA have tested positive for YTX at varying concentrations (Howard et al., 2008; Paz et al., 2008). The highest concentrations detected were in Norway and the lowest in Russia (table 2). The YTX detected in dinoflagellate cells ranged from 0.005-14.0 pg YTX $cell^{-1}$ (Howard et al., 2008; table 3). *G. spinifera* was first conclusively shown to produce YTX using an ELISA test on single cells from New Zealand (Rhodes et al., 2006).

As this test uses antibody recognition, it was unable to provide any information on the YTX analogues present. The toxin content of the cells detected was 176 and 200 pg YTX cell^{-1}. *G. spinifera* was known to predominantly produce YTX until Ciminiello et al. (2003) and Riccardi et al. (2009) found varying concentrations of homo-YTX, carboxy-YTX and 45-OH-YTX in algal samples. This is controversial, as these analogues are generally believed to be produced as metabolites of YTX and homo-YTX from shellfish. Riccardi et al (2009) had cells that produced homo-YTX at levels of 33.4 pg cell^{-1}.

In the Benguela Current System, YTX has only been detected in southern Benguela from *P. reticulatum* cultures (Trainer et al., 2010, Krock et al., 2008). These cultures contained 75 fg YTX cell^{-1} with YTX being the dominant analogue produced. As the DSP samples were analysed with the MBA, YTX has never been detected in Northern Benguela. Though a survey in 2004 was conducted in the Walvis Bay area to determine the dinoflagellate cyst concentration (Joyce, 2004). Samples were taken from 32 sites using a grab to analyse the surface cyst composition. *L. polydrum* and *P. reticulatum* cysts were identified. The *L. polydrum* had a wider distribution with fewer numbers concentrated just out of the bay area. The *P. reticulatum* was relatively abundant in the bay area. A vertical distribution of cysts on a core was done, though it was based on one core which was taken in the bay area. *P. reticulatum* was the most prominent on the surface (213 cysts mL^{-1}) and could be found right through to 15 cm depth. *L. polydrum* had its maximum concentration at 0-2 cm (13 cells mL^{-1}). To date blooms of these species have not been observed in Walvis Bay (MFMR database).

1.5.2.3 Gymnodimines

Gymnodimines (GYMs) are cyclic imine phycotoxins produced by the dinoflagellates *Karenia selliforme* and *Gymnodium mikimotoi* (Seki, 1995; Miles et al., 2003; table 1). They also fall under the grouping of cyclic imines. GYMs were detected in the 1990s in shellfish from New Zealand (Mackenzi et al., 1996). The mode of action of SPX is not fully understood and no human intoxication has been reported yet (Gerrsen et al., 2010). In Namibia, none of these causative species have been detected (MFMR phytoplankton database: 2004- present), thus the presence of GYMs is unlikely.

1.5.2.4 Brevetoxins

Brevetoxins (PbTxs) are produced by *Karenia brevis* and multiple dinoflagellate species listed in table 1. This toxins mode of action is to bind to the sodium channels, thus opening them and causing a persistent activation of neural, skeletal muscle and cardiac cells (Baden and Adams, 2000). It is thus

referred to as Neurotoxic Shellfish Poisoning (NSP). The symptoms observed after ingesting contaminated shellfish are: gastrointestinal distress, nausea, vomiting, dizziness and slurred speech (Heil, 2009; Watkins et al., 2008).

For Namibia, Pitcher (1999) stated that the presence of *Gymnodinium* species strongly suggests that NSP is a potential problem in the Benguela as well. This genus has undergone redefinition and has been split into four groups namely, *Gymnodinium*, *Akashiwo*, *Karenia* and *Karlodinium* (Daugbjerg et al., 2000). Thus the PbTxs production is confined to the Karenia genus. Therefore NSP being a potential problem is highly doubtful as no *Karenia* species have been observed from the phytoplankton data collected from the Shellfish monitoring program (MFMR phytoplankton database: 2004 – present). Only *Gymnodinium* species have been observed.

1.5.2.5 Azaspiracids

Polyether azaspiracids (AZA) are produced by *Azadinium spinosum* (Tillmann et al., 2009; Krock et al., 2009, table 1). This is the smallest known phycotoxin producer. AZA were first detected in mussel samples from Galway in 1995 (McMahon and Silke, 1996). The mode of action is not yet fully understood though gastrointestinal disorder, diarrhea and abdominal cramps have been observed during AZP intoxication (James et al., 2002; 2004). The regulatory limit is 160 µg/kg AZA1-equivalents.For Namibia, the causative species has not been detected, thus this toxin is unlikely to be found in this region (MFMR phytoplankton database: 2004-present).

1.5.2.6 Spirolides

Spirolides (SPX) are cyclic imines produced by *Alexandrium ostenfeldii* and *Alexandrium peruvianum* (Cembella et al., 2000; Mackinnon et al., 2006, table 1). This toxin was identified in extracts from the digestive glands of mussels and scallops from Nova Scotia, Canada in the early 1990's (Hu et al., 195). There are 15 different SPXs found in both algae and shellfish (Aasen et al., 2005; Aasen et al., 2006; Ciminiello et al., 2007). The mode of action of SPX is not fully understood and no human intoxication has been reported yet (Gerrsen et al., 2010). Though MBA testing of contaminated shellfish extracts caused death within minutes in all test animals (Richard et al., 2000). SPXs are currently not regulated, as there is no conclusive evidence of human intoxication from contaminated shellfish (Mundy et al., 2012).

For the northern Benguela, the causative species has not been detected, thus this toxin is unlikely to be found in this region (MFMR phytoplankton database: 2004-present).

Table 2: Summary of published concentrations of yessotoxin in shellfish globally.

Shellfish	YTX range (µg YTX g^{-1})	Country	Reference
Blue mussels (*Mytilus edulis*)	0.5-14.8	Norway	Lee et al.,1988; Ramstad et al., 2001; Aasen et al., 2005
Blue mussels (*Mytilus galloprovincialis*)	0.1-9.62	Italy	Ciminiello et al., 1997, Draisci et al., 1999a Riccardi et al., 2009 Visciano et al., 2013
Greenshell mussels (*Perna canaliculus*)	1.6-3.2	New Zealand	Yasumoto and Takizawa, 1997 MacKenzie et al., 2001 MacKenzie et al., 2002
Blue mussels (*Mytilus chilensis*)	No quantities published	Chile	Yasumoto and Takizawa, 1997
Blue mussels (*Mytilus edulis*)	No quantities published	Japan	Yasumoto and Takizawa, 1997
Blue mussels (*Mytilus edulis*)	0.053	Russia	Vershinin et al., 2006
Scallops (*Patinopecten yessoensis*)	No quantities published	Japan	Murata et al., 1987, Yasumoto and Takizawa, 1997
Scallops (*Patinopecten yessoensis*)	0.44-0.79	Japan	Koike et al., 2006
Shellfish (unspecified)	No quantities published	Ireland	Howard et al., 2008
California sea mussels (*Mytilus californicus*)	0-0.1	USA	Howard et al., 2008
Blue mussels (*Mytilus galloprovincialis*)	No quantities published	Russia	Morton et al., 2007
Mussel, oyster and clams	2.0	Spain	Arevalo et al., 2006

Table 3: Summary of published studies quantifying the amount of yessotoxin per cell in *Prorocentrum reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera*.

Dinoflagellate	YTX (pg cell^{-1})	Country	Reference
P. reticulatum	5.0	Canada	Stobo et al., 2003
	5.83	Italy	Boni et al., 2002
	18-79	Norway	Samdal et al., 2004
	0.9-14.0	Japan	Satake et al., 1999, Eiki et al., 2005
	3.0-13.0	New Zealand	Satake et al., 1996, 1999), MacKenzie et al., 2002, Mitrovic et al., 2005, Rhodes et al., 2006
	0-2.6	Spain	Paz et al., 2004, 2007
	0.3	United Kingdom	Stobo et al., 2003
	0-2.1	United States	Paz et al., 2004, 2007, Cassis, 2005
	0.2-0.4	Chile	Alvarez et al., 2011
	0.075	South Africa	Krock et al., 2008, Trainer et al., 2010
L. polyedrum	0.3	Ireland	Howard et al., 2008
	1.5	Italy	Tubaro et al., 1998, Draisci et al., 1999
	0	Norway	Ramstad et al., 2001
	0, 0.3	Spain	Riobo et al., 2002, Paz et al., 2004
	0-0.02	United Kingdom	Stobo et al., 2003
	0-0.005	United states	Armstrong and Kudela, 2006 Howard et al., 2008
G. spinifera	0-200	New Zealand	Rhodes et al., 2006
	0	United Kingdom	Stobo et al., 2003
	0	United States	Howard et al., 2008
	3.6-33.4	Italy	Riccardi et al., 2009

1.6 Aims and objectives

This thesis investigates the occurrence of phycotoxin producing HABs in the northern Benguela and aims to provide guidelines for an effective warning system.

From regulatory testing done since 2004, DSTs have caused the only farm closures. Looking at the phytoplankton samplings during this time doesn't show the causative *Dinophysis* species at elevated levels. Thus this discrepancy needs to be resolved, as the regulatory test method used is indiscriminate and refined chemical analysis is needed. The answer will be critical to the shellfish industry as the results lead to closure of farms. Thus it is important to know which toxins are responsible for the positive results as they all have different regulatory limits. Once this has been assessed the phytoplankton species that is actually responsible for the DSP positive results will be determined. Culturing of HABs species for characterisation as well as determining potential toxicity, level of genetic variation and the effects of nutrient variation on toxin production will be an additional advantage. This assessment of potential toxicity will allow for the development of phytoplankton specific toxicity thresholds that can be incorporated into the Namibian Shellfish Sanitation Program and act as an early warning toxicity indicator. Examining satellite images of the Walvis Bay area can also form a component in the generation of an early warning system. The comparison of blooms with the phytoplankton biomasses of the collected samples can be looked at to determine whether the satellite chlorophyll maxima coincide with elevated abundance of the different species. This could aid in the detection of HABs.

The culmination of the data produced can be used to develop an effective early warning system for the farmers by:

1) Resolving the discrepancy between the instances of DST positive results leading to farm closures and lack of DSP toxin producers in the water column.

2) Possibly determine the physical and chemical factors that lead to bloom formation in this area. Looking at the algal bloom dynamics and species succession naturally as well as during and after H_2S eruptions.

3) Determining the HAB cell concentration trigger level that could lead to potential toxicity of shellfish in field and cultured samples.

Chapter 2 Materials and method

2.1 Study area

The four sampling sites are situated in the northern sector of the Walvis Bay in an area known as Aquapark 1 (figure 8). These sampling sites have depths that range from 8-11 m. The deep-sea waves originate from SSE to SW and the waves progressively decrease in magnitude. A shadow zone is formed from the positioning of pelican point (Figure 8). This results in a southwards setting long shore current in the harbour (Voges and Morant, 2009). In the harbour, the water circulation is driven by predominantly southerly winds, which enable a clockwise water flow. Thus the farm experiencing the influx of offshore water is Namaqua, while the outgoing water flows past Beira. The tidal range at the port is 1.4 m during spring tide and 0.6 m during a neap tide. As it is a fishing and commercial harbour, fish processing factories discharge large amounts of organic matter. Thus the predominantly southerly winds transport this matter into the Aquapark 1 area. These high organic loads coupled with decaying blooms contribute to the propagation of anoxic bottom water in this area. The wind during autumn, winter and spring is predominantly in a SW direction, and NW during summer. Blooms are most likely to occur during late summer and early autumn when there are light predominantly on-shore winds (Voges and Morant, 2009).

Figure 8: Location of the four sampling sites in Aquapark 1, namely: Beira aquaculture, Namibia Aquaculture, Joe's oyster company (now Shoreline producers) and Tetelestai Mariculture (denoted as Seatet sampling site).

2.2 Environmental variables

Seawater samples for nutrient and dissolved oxygen analysis were taken from Aqaupark 1 for the surface, middle and near-bottom with a Niskin bottle. Temperature readings were taken at the corresponding depths with a WTW cond 3110-meter kit and tetraCon probe with a 10 m cable. Samples for dissolved oxygen were collected in glass oxygen bottles and fixed with manganese chloride and alkaline iodide and analysed using the Winkler method (Grasshoff et al., 1999). The thiosulfate used in the titration was standardised weekly as per laboratory QC control.

2.3 Nutrient analysis

Inorganic nutrient concentrations (phosphate, silicate, nitrate and nitrite) taken from selected depths were filtered and collected in 250 mL soft low-density dark polyethylene acid washed bottles. Samples were frozen until analysis using the standard colorimetric manual methods (Grasshoff et al., 1999). The sample volumes were scaled down to 5 mL. All samples were analysed in duplicate.

2.4 Biological samples and phytoplankton quantification

2.4.1 Phytoplankton sampling and toxin extraction

Phytoplankton samples were taken for the first 5 meters of the water column using a pipe to obtain an integrated sample of the photic zone. These samples were preserved with formaldehyde and were used for species identification and quantification using the Utermöhl method (Hasle, 1978) with 20 mL setting chambers with an inverted microscope at 400x magnification. Vertical net haul samples were also taken at each station using a 20 μm mesh phytoplankton net. These samples were used for screening for phycotoxic species. Scanning electron microscopy was used to verify the identity of phycotoxic species.

If phycotoxic species were found, seawater was filtered onto GF/F filters and stored at -80 °C. Lipopholic toxins were extracted from filters using 4 mL 80% methanol, sonicated in an ice-bath for 10 minutes and centrifuging at 14000 rpm for 10 minutes. This was repeated once more combining supernatants and adjusting the final volume to 10 mL.

2.4.2 Shellfish sampling and toxin extraction

Shellfish samples consisting of the mussel species *Perna perna*, *Mytilus galloprovincialis* and *Semi-mytilus galloprovincialis* were collected during the *G. spinifera* bloom (15^{th}-18^{th} March 2011) as well as on three other occasions after this bloom. Only samples collected from Beira consisted of mature *Perna perna* shellfish. All shellfish samples were stored at -20°C. Lipophilic toxins were extracted from mussels using 9 mL 80% methanol, sonication in an ice-bath for 5 minutes and centrifugation at 14000 rpm for 5 minutes. This was repeated once more combining supernatants and adjusting the final volume to 20 mL. This extracted all lipophilic toxins and was analysed using Liquid Chromatography-Mass Spectrometry (LC-MS/MS).

The individual YTX and its analogues were converted to YTX equivalents kg^{-1} using the toxic equivalent factor (TEF) of YTX=1, homo-YTX=1, 45-OH-YTX=1 and homo-YTX-OH=0.5 (Roeder et al., 2011; European Food Safety Authority, 2008). As there is currently no TEF for carboxyl-YTX, it was omitted from the calculation. This enables the calculation of a regulatory limit, which has been set at 1 mg YTX equivalents kg^{-1} by the European commission in 2002.

2.5 LC-MS/MS analysis of lipophilic toxins

LC-MS/MS analyses were conducted using a Thermo Accela HPLC coupled to a Thermo TSQ Vantage quadrupole MS. The methods used were based on Gerssen et al. (2009).

2.5.1 Acidic chromatographic conditions

Chromatographic separation was done on a Kinetex 2.6 µm C18 column (50 mm x 2.1 mm) at 20°C with a flow rate of 0.2 mL min^{-1}. Two mobile phases were used, namely, A (water) and B (acetonitrile). Both mobile phases contained 50 mM formic acid and 20 mM ammonium formate (all MS grade). The injection volume was 50 µL. A gradient elution was applied using the conditions specified in table 4.

Certified reference standards used for tuning and calibration the MS were purchased from the Canadian National Research Council (primary standard) and Cifga Laboratorio s.a. (secondary standard). Extraction of shellfish homogenates (2 g) and phytoplankton filters (from 100-800 mL filtered water sample) was accomplished two times with 80 % methanol by sonicating. Final extract volume was

added up to 20 and 10 mL respectively. The mass spectrometer was tuned for domoic acid (+312.0/+266.1 m/z), okadaic acid (-803.3/-255.0 m/z), dinophysistoxin-1 (-841.4/-737.5 m/z) and -2 (-803.3/-255.0 m/z), yessotoxin (-1141.5/-1061.6 m/z), pectenotoxin-2 (+876.4/+823.6 m/z), azaspiracid-1 (+842.4/+824.7 m/z), -2 (+856.5/+838.6 m/z) and -3 (+828.5/+810.6 m/z) in selected reaction monitoring (SRM) mode. Yessotoxin was found and quantified with an external calibration function (r^2: 0.999, limit of detection: 1 ng YTX mL^{-1} respectively 10 µg kg^{-1} shellfish). The standard deviation V_{xo} was ± 2.6 %. Four metabolic products of YTX were identified in the extracts by neutral loss experiments (offset of 80 amu, SO_3 loss): homo-YTX, 45-OH-YTX, homo-YTX-OH and carboxy YTX. The transitions of the yessotoxin analogues were: -1155.5/-1075.5 (homo-YTX), -1157.5/-1077.5 m/z (45-OH-YTX), -1171.50/-1091.5 m/z (homo-YTX-OH) and -1173.5/1093.5 m/z (carboxy-YTX). They were estimated as YTX equivalents by applying the calibration function of yessotoxin.

2.5.2 Alkaline chromatographic conditions

Chromatographic separation was done on a Gemini 3.0 µm C18 column (150 mm x 2.0 mm) at 40°C with a flow rate of 0.350 mL min^{-1}. Both mobile phases, A (water) and B (acetonitrile/water, 90:10, v/v), contained 6.7 mM ammonium hydroxide (pH=11). The injection volume was 10 µL. A gradient elution was applied using the conditions specified in table 5. Certified reference standards used for tuning and calibration the MS were purchased from the Canadian National Research Council**.** The YTX and its analogues were analysed as described under basic chromatographic conditions (table 6). Under pH 11, the double charged precursor ion ($[M-2H]^{2-}$) was used.

Table 4: Liquid chromatographic gradient conditions for YTX LC-MS/MS analysis under acidic chromatographic conditions.

	Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**
Gradient	0	90	10
	6.0	10	90
	14.5	10	90
	19.0	90	10
	21.0	90	10

Table 5: Liquid chromatographic gradient conditions for YTX LC-MS/MS analysis under basic chromatographic conditions.

	Time (min)	Mobile phase A (%)	Mobile phase B (%)
Gradient	0	90	10
	1	90	10
	10	10	90
	13	10	90
	15	90	10
	19	90	10

Table 6: MS/MS conditions used for the detection of YTX and its analogues under basic chromatographic conditions.

Toxin	Precursor ion (m/z)	Product ion (m/z) 1	Product ion (m/z) 2
YTX	570.4	396.4	467.4
homo-YTX	577.4	403.4	474.4
45-OH-YTX	578.4	396.4	467.4
45-OH-homo-YTX	585.4	403.4	474.4

2.6 Batch culture experiments for growth rate, YTX profile and genetic composition

2.6.1 *Gonyaulax spinifera* cultures

Several monoclonal strains were established from the initial field sample, an area adjacent to the Aquapark 1 sampling site (22^{o} 54' 41.75'' S, 14^{o} 30' 33.17'' E) on the 1st March 2012. Examination for the species composition of the surface water net sample taken consisted of *Prorocentrum micans* (dominant), *Ceratium furca* and some *G. spinifera* cells. Cultures were established in natural seawater (salinity of 35) fortified with ½ (f/2) nutrients without silica (Guillard 1975). *G. spinifera* cells were later isolated using the capillary pipette method (Hoshaw and Rosowski, 1973) into 0.5 mL wells containing ½ (f/2) media to make monoclonal non-auxenic cultures. Cultures were maintained in an incubator at 17^{o} C with a 14:10 h L:D cycle.

2.6.2 Toxin extraction of *Gonyaulax spinifera* cultures

Batch cultures were established for each strain by inoculate 10 mL into 500 mL culture flask. Samples were taken when cells reached the exponential growth phase. 300 mL were filtered onto GF/F filters to determine the toxin profile in triplicate (100 ml x3). The YTX toxins were extracted as specified is section 2.4.2. Culture filter samples were analysed on the LC-MS/MS under the conditions specified in section 2.5.2.

2.6.3 Molecular analysis

2.6.3.1 DNA extraction, PCR amplification and sequencing

Aliquots (100 mL) of *G. spinifera* cultures were gravity filtered gently on 0.45 µm polycarbonate filters. Once the volume was less than 2 ml, the cells were collected and centrifuged at 6000 xg for 10 minutes, supernatant removed and added to the initial media filtered. The resulting pellet was collected, frozen in liquid nitrogen and stored at -80°C until analysis. The genomic DNA was extracted from the pellet using a QIAGEN kit (Hitachi Koki) for plant tissue, according to the manufacturer's instructions. Approximately 1 ng of genomic DNA was amplified using 20 µL PCR master mix containing Taq buffer (2.0 µL) two gene specific primers (0.4 µL), dNTP (0.2 µL) and DNA polymerase (0.2 µL). All amplifications were carried out using an Eppendorf master cycler with the following cycling conditions: initial template denaturation for 5 minutes at 94°C, followed by 33 cycles at 94°C for 30 seconds, 56°C for 30 seconds and extension at 72°C for 1 minute and 15 seconds, followed by a final extension at 72°C for 7 minutes. The PCR products quality and specificity were assessed by agarose gel electrophoresis. The PCR product was then purified using a Qiagens MinElute PCR purification kit in accordance with the manufacturer's instructions. The purified PCR product (1 µL) was sequenced using the following master mix with the primers specified in table 5: 5x Big dye buffer- containing polymerase dNTP and ddNTP (1.5 µL), big dye (1 µL), primer (1 µL), DNase free water (6.5 µL). The sequence cycle used was: 96°C for 1 minute, 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. This was done 25 times. The sequencing reaction product was purified up using AGENCOURT CleanSEQ kit according to the manufacturer's instructions. The purified reaction product was then transferred into MicroAmp Optical 96 well reaction plates and loaded into the sequencer. A 3130xl genetic analyser (Applied Biosystems, Darmstadt, Germany) with a 16 cappilary array was used for Sanger sequencing.

DNA fragments were separated in POP 7 polymer within the capillary with length of 80 cm. This allows for sequence reads of up to 1000 bp.

Table 7: Oligonucleotide primers used for amplification and sequencing of the small and large subunits (18S and 28S) ribosomal DNA of *G. spinifera* cultures.

Primer	**Application**	**Sequence**	**Region amplified**
1 F	PCR & sequencing of SSU rDNA—forward primer	5'GTGGACTCTTGTTCCAAACTGG'	Small subunit (SSU)
1528 R	PCR & sequencing of SSU rDNA—reverse primer	5'AAGGCCTACATGGCCGGACCG3'	
16S1N	PCR & sequencing of SSU rDNA—forward primer	5'TCCTGCCAGTAGTCATATGC3'	
16S2N	PCR & sequencing of SSU rDNA—reverse primer	5'TGATCCTCT/CGCAGGTTCAC3'	
D1R	PCR & sequencing of LSU rDNA—forward primer	5'ACCCGCTGAATTTAAGCATA3'	Large subunit (LSU)
D2C	PCR & sequencing of LSU rDNA—reverse primer	5'CCTTGGTCCGTGTTTCAAGA3'	

2.6.3.2 Sequence alignment and phylogenetic analysis

Sequences were aligned using clusteralX, version 8 and adjusted manually. The resulting sequences were aligned with the existing SSU and LSU sequences available from GenBank using Molecular Evolutionary Genetics Analysis (Mega) version 5.05. The phylogenetic relationships were inferred based on pairwise differences (p-distance) of nucleotides derived from the alignment, ignoring gaps with the species listed in tables 8. The p-distance represents the number of nucleotide differences divided by the total number of nucleotides compared. Maximum parsimony estimation was used for the phylogenetic analysis and subsequent phylogenetic tree. This was done for both the LSU and SSU of the rDNA.

Table 8: List of sequences used in the SSU and LSU region rDNA phylogenetic analysis from GenBank.

SSU		LSU	
Species	**GenBank Accession no.**	**Species**	**GenBank Accession no.**
Alexandrium minutum	AJ535381	*Alexandrium catenella*	AF200667
Alexandrium minutum	U27499	*Alexandrium margalefii*	AY154957
Alexandrium ostenfeldii	AJ535381	*Alexandrium minutum*	AF033532
Alexandrium ostenfeldii	AJ535384	*Alexandrium ostenfeldii*	AF033533
Alexandrium tamarense	AF022191	*Alexandrium pseudogoniaulax*	AY154958
Alexandrium tamarense	AJ535391	*Alexandrium tamarense*	AY438021
Alexandrium tamarense	AJ415510	*Cochlodinium polykrikoides*	AF067861
Alexandrium taylori	AJ535390	*Gonyaulax baltica*	AY154962
Gonyaulax cochlea	AF274258	*Gonyaulax digitale*	AY154963
Gonyaulax fragilis	56384899	*Gonyaulax elongata*	AY154964
Gonyaulax polyedra	AF377944	*Gonyaulax membranacea*	AY154965
Gonyaulax polyedra	AJ415511	*Gonyaulax polygramma*	DQ162802
Gonyaulax polygramma	AJ833631	*Gonyaulax cf. spinifera*	AY154960
Gonyaulax spinifera	AF022155	*Gonyaulax spinifera*	DQ151557
Gonyaulax spinifera	AF052190	*Gonyaulax spinifera*	DQ151558
Gonyaulax spinifera	DQ867107	Gonyaulax spinifera	EF416284
Gonyaulax spinifera	EU805590	*Gonyaulax spinifera*	EU532478
Gonyaulax verior	AY443013	*Gonyaulax spinifera*	EU805591
Heterocapsa triquetra	AY421787	*Heterocapsa triquetra*	AF260401
Lingulodinium polyedrum	AF274269	*Lingulodinium polyedrum*	EF613357
Lingulodinium polyedrum	AY421788	*Lingulodinium polyedrum strain*	EU532472
Prorocentrum lima	Y16235	*Lingulodinium polyedrum strain 104A*	EU532471
Prorocentrum micans	AY585526	*Prorocentrum lima*	AJ567459
Prorocentrum minimum	Y16238	*Prorocentrum micans*	AF260377
Protoceratium reticulatum	AF274273	*Prorocentrum minimum*	DQ662402
Protoceratium reticulatum	AY421790	*Protoceratium reticulatum*	EF613362
Protoceratium reticulatum	DQ217789	*Protoceratium reticulatum*	EU532476
		Protoceratium reticulatum	EU532477
		Pyrodinium bahamense var. compressum	AY154959

2.6.4 Growth rate determination

Ten cells mL^{-1} were inoculated into 50 mL culture flask (noting the number of cells inoculated) containing ½ (f/2) media for each culture. 2 ml sample were taken every third day and fixed with Lugols iodine solution. The cells were then counted using a counting chamber under a light microscope (40x magnification). These results were used to determine the specific growth rate (μ d^{-1}) of each strain using the equation with N= cell density at a given time (t): $\mu = (\ln N_1 - \ln N_0)/ (t_1 - t_0)$ (Guillard, 1973).

2.7 Experiments on macronutrient effects on toxin production in *Gonyaulax spinifera*

To determine the effects of nutrient limitation on *G. spinifera* cells, 50 mL culture flasks with varying concentrations listed in table 9 were inoculated with 4 mL of the strain in late- exponential growth phase (with a known cellular concentration) in duplicate. Two control samples were used as strain NamWB012 was grown in f/2 media with lower NO_3^- and PO_4^{3-} concentrations. The N:P ratio was adjusted for each treatment accordingly.

A sub sample of 10 mL was extracted at least every fourth day (for cell counts, PO_4^{3-} and NO_3^- concentrations) and replenished with new growth media to maintain the specified N/P ratio. Cells for each treatment were harvested after 21 days by filtering onto 0.45 µm GF/F for YTX toxin analysis after 2 ml was taken for cell counts. Filters were stored at -80°C till analysis as detailed in 2.4.1. The filtrate was retained to determine the YTX toxin content as specified in section 2.6.3.

Table 9: Nitrate, phosphate and N/P ratio in the various growth media used for *G. spinifera* cultures.

Sample name	Phosphate (µM)	Nitrate (µM)	N/P ratio	*G. spinifera* strains used in experiment
Control 1	15.78	418.70	26.53	NamWB 002 and NamWB 011
1/10 PO_4^{3-}	1.58	418.7	265.00	
1/10 NO_3^-	15.78	41.87	2.65	
Control 2	3.21	246	76.64	NamWB: 012
1/10 PO_4^{3-}	0.321	246	766.40	
1/10 NO_3^-	3.21	24.6	7.66	

Chapter 3

The first detection of YTX in *G. spinifera* in shellfish and phytoplankton samples in the northern Benguela.

3.1 Introduction

The cold Benguela Current extends from the southern tip of Southern Africa to the Angola-Benguela front of Angola. This current is one of the four major coastal upwelling currents on the eastern boundary of the ocean basins (Trainer et al., 2010). The principal upwelling cell is around Luderitz, the area is divided into north (encompassing Walvis Bay) and southern components (Trainer et al., 2010). As an upwelling system, it provides nutrient-rich deep water to the euphotic zone (Pitcher et al., 2010). This enhances the growth of phytoplankton and can thus facilitate the frequent proliferation of harmful algal blooms (Hallegraeff, 1995). These blooms can be caused by algal species that produce phycotoxins which accumulate in filter feeding shellfish, thus affecting mariculture activities. The phycotoxins most commonly detected in the northern Benguela are lipophilic toxins (Aquafact, 2012). Since these results were obtained using the mouse bioassay, there is no information on the types of lipophilic toxins present. During the week of 15th March 2011, a dinoflagellate bloom consisting of *Gonyaulax spinifera* was detected in the Walvis Bay area. This dinoflagelate has been known to produce yessotoxin (YTX) (Rhodes et al., 2006; Riccardi et al., 2009). YTX and its analogues are polyether toxins produced by the dinoflagellates *L. polyedrum*, *P. reticulatum* and *G. spinifera* (Alvarez et al., 2011, Gerssen et al., 2010). YTX was first isolated in Japan from the scallop *Patinopecten yessoensis* (Murata et al., 1987). To date, 90 different analogues have been identified (Miles et al., 2005). Globally, shellfish from Norway, Italy, New Zealand, Russia and the USA have tested for YTX at varying concentrations from *P. reticulatum* and *L. polydrum* (Howard et al., 2008; Paz et al., 2008; table 2). YTX in shellfish from *G. spinifera* has only been detected in New Zealand and Italy (Rhodes et al., 2006; Riccardi et al., 2009, figure 9). YTX was thought of to be the predominant toxin until Riccardi et al (2009) found homo-YTX and minor concentrations of carboxy-YTX and 45-OH-YTX in algal samples from the Adriatic Sea. These analogues had been believed to be shellfish metabolites of YTX and homo-YTX (figure 10; Miles et al., 2005; Roeder et al., 2011). In the Benguela Current system, YTX has only been detected in Southern Benguela *P. reticulatum* cultures (Krock et al., 2008; Trainer et al., 2010).

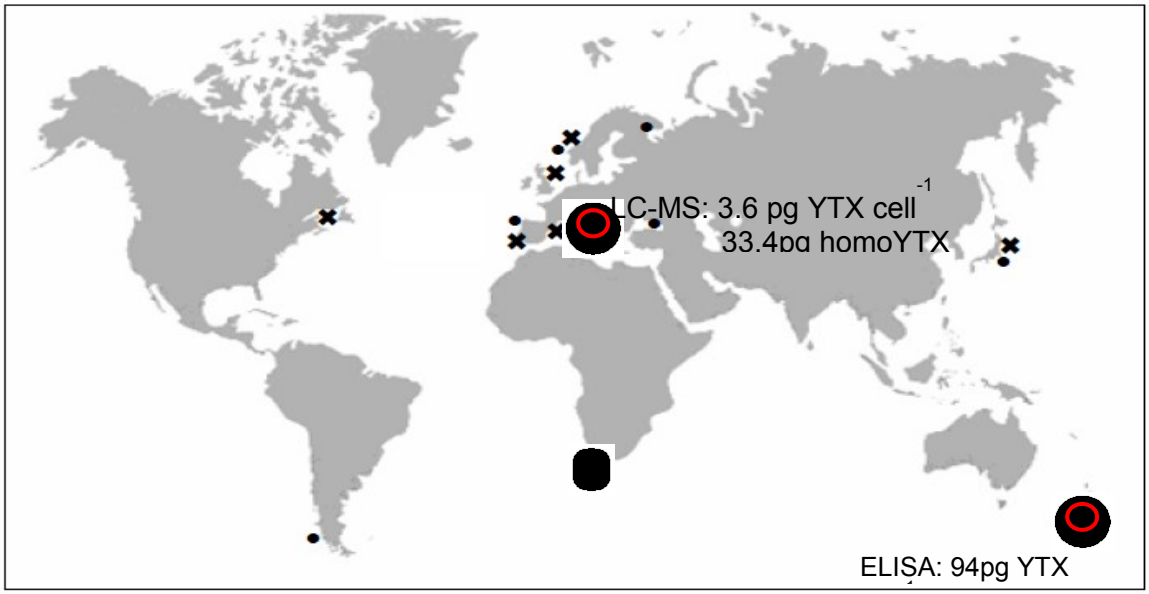


Figure 9: Global distribution of YTX from in *P. reticulatum* (x) in shellfish (●). The areas where YTX has been detected in Shellfish and phytoplankton samples from *G. spinifera* are denoted by a red circle (updated from Paz et al., 2008).

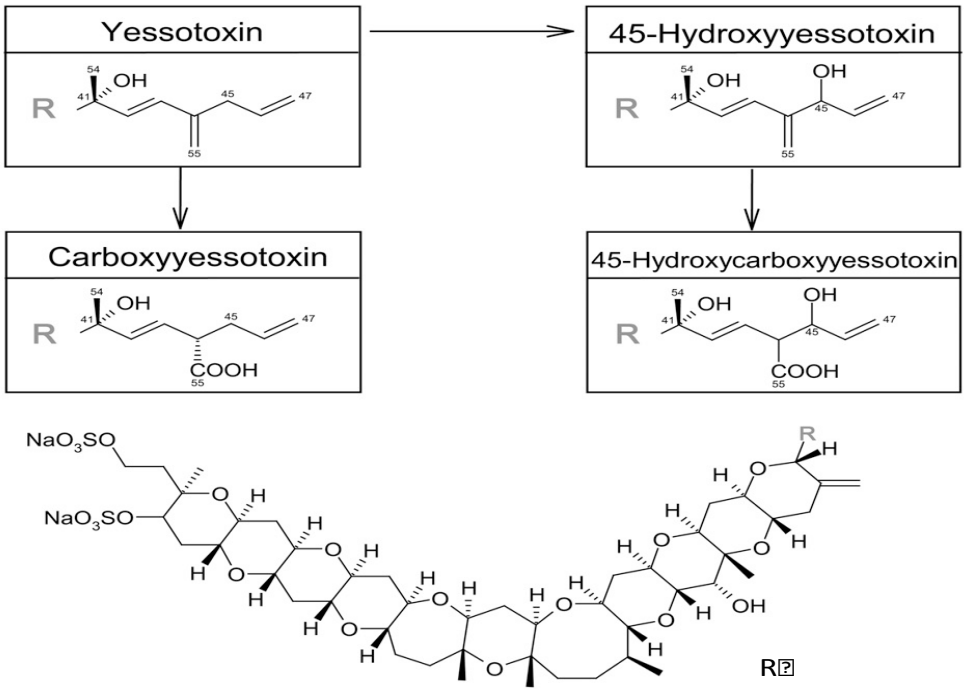


Figure 10: YTX and analogues most commonly found in shellfish. The arrows denote the possible metabolic pathway of yessotoxin in *M. edulis* (Roeder et al., 2011).

3.2 Results:

3.2.1 Phytoplankton quantitative and taxonomic analysis

The bloom during the 15-18 March 2011 was dominated by dinoflagellates (figure 11). The highest cell counts were reached on the 17-18th March with value of 1.54 x 10^6 cells L^{-1}. The dominant species during this period were *Prorocentrum micans, Prorocentrum triestinum* and *G. spinifera*. The *G. spinifera* cells increased gradually untill they reached a maximum of 6.74 x 10^5 cells L^{-1} on the 17th March 2011. *P. micans* and *P. triestinum* cell numbers increased rapidly on the 18th March while the cell numbers of *G. spinifera* reduced drastically. This might have been due to changes in favourable conditions for the *G. spinifera* cells (i.e. nutrient limitations).

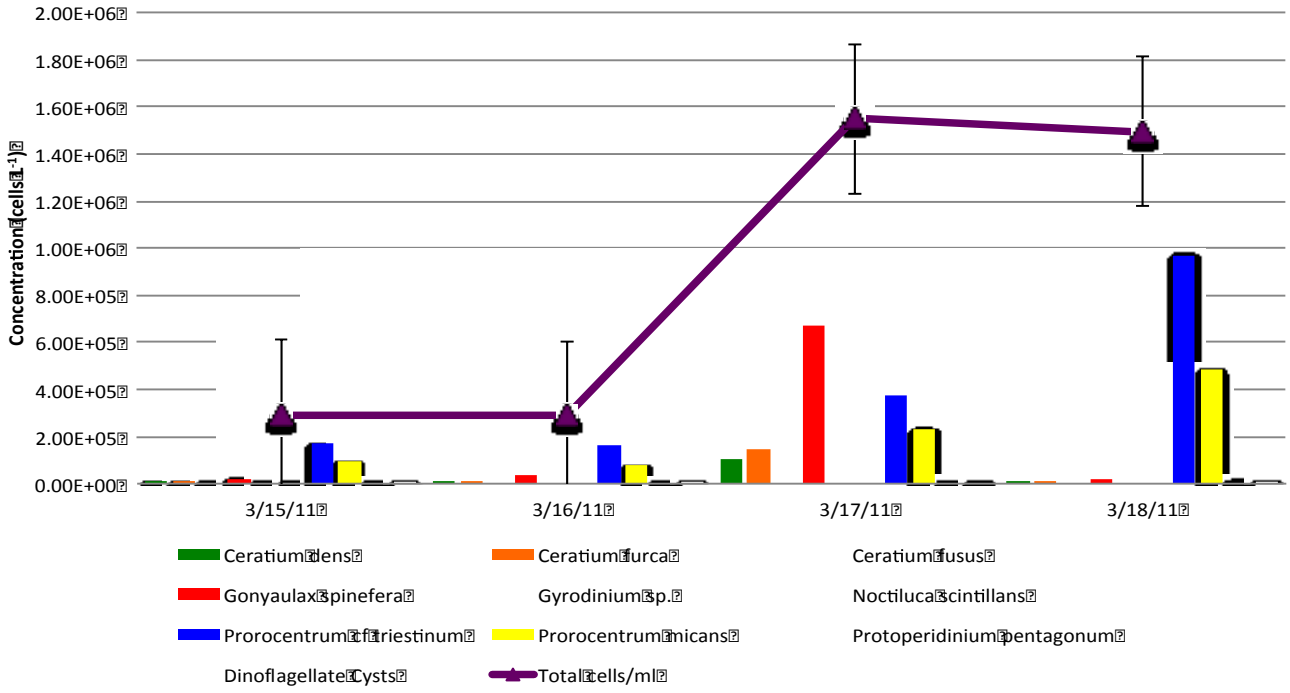
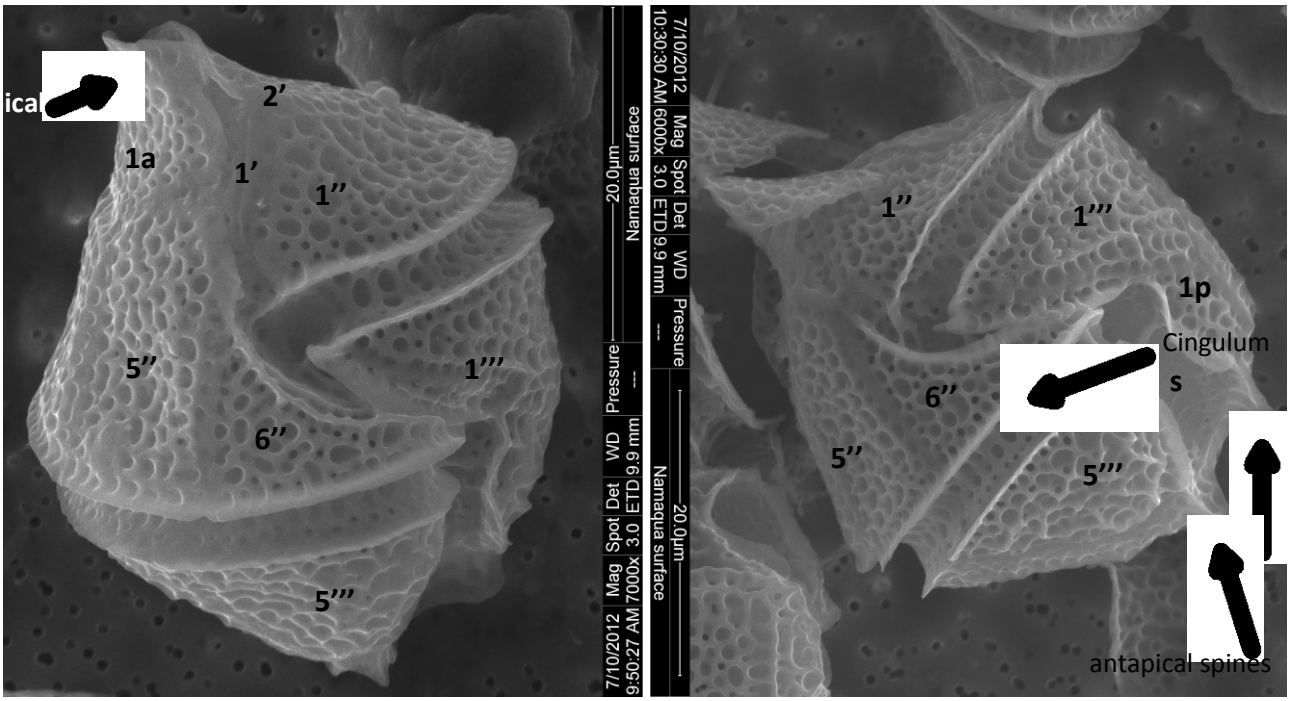


Figure 11: Phytoplankton species diversity during the dinoflagellate bloom in Aquapark 1 (15th-18th March 2011).

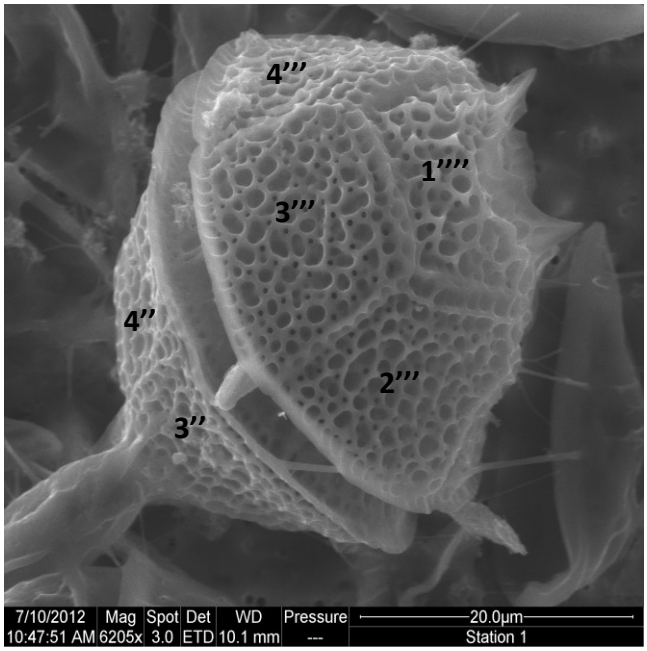
3.2.2 *Gonyaulax spinifera* identification:

Scanning Electron Micrograph (SEM) photographs enabled the verification of *G. spinifera* (Claparede and Lachmann) Diesing. The following attributes specific to *G. spinifera* were observed: a prominent and descending cingulum, two short antapical spines (figure 12 b) and a short apical horn (figure 12a). Cell surface is reticulated with the basic thecal arrangement of *G. spinifera* detailed by Dodge, 1985

(figure 12 a-c). The 6" plate on the epitheca has a distinctive triangular shape (figure 12a). The cell size varies between 24-40 μm in length and 20-30 μm in width.



a) b)



c)

Figure 12: Scanning Electron Micrographs of *G. spinifera* from field samples taken on the 16th March 2011 from Namaqua. a) Apical dorsal view b) Apical dorsal view with cingulum and antapical spines c) Dorsal antapical view. All plates are labelled according to the Kofoidean numerical notation (IOW).

3.2.3 Environmental variables and Nutrient analysis

3.2.3.1 Temperature

Sampling began during spring. All four stations had near bottom water temperatures ranging from 12.5 -14.5°C and surface waters temperatures from 14.5-15.0°C until mid-summer (28th December 2010; figures 13 a-d). Temperatures during summer increased to 17.0°C for the bottom water, with the shallower stations recording temperatures of up to 19.0°C. The surface water reached temperatures of up to 20.0°C. Late summer bottom water reached 16.0°C, while the surface temperatures reduced to 19.0°C. During autumn, the water column was stratified with temperature ranges from the surface to the bottom of 20.0-18°C. These warm conditions and the season aided in the formation of a dinoflagellate dominated bloom. These warm water conditions persisted till the beginning of April 2011. The water column temperature ranged from 12.0 -14.0°C during the winter months.

3.2.3.2 Dissolved oxygen

Sampling began during spring (figures 14 a-d). The water column was well oxygenated during spring with values ranging from 3.0-6.0 mL L^{-1}. The bottom water during late spring to early summer decrease to < 2.0 mL L^{-1}. During mid-summer, the water column had high oxygen concentrations ranging from 3.0-6.0 mL L^{-1}. This was followed by low oxygen concentrations during mid-autumn with bottom water anoxia (<1.0 mL L^{-1}) and this extended throughout the water column during 14th-22nd March, coinciding with the period when H_2S erupted in the lagoon area and spread to Aquapark 1. The decaying phytoplankton aided in the anoxic conditions observed in the bottom water. Hyperoxic conditions were measured in the surface water (>7 mL L^{-1}) during mid-autumn. This was due to the high phytoplankton biomass during this period. The water column had low oxygen concentrations during early winter (<2.5 mL L^{-1}) until late winter, when the concentration increased (>4.0 mL L^{-1}).

3.2.3.3 Dissolved inorganic phosphate

Sampling began during spring. During spring, PO_4^{3-} concentrations <2 µM were measured throughout the water column, followed by a marked increase with the highest concentration measured in the bottom water (>6.0 µM) during late summer (figures 15 a-d). The shallower stations had the lowest concentration difference between the surface and bottom water. This coincides with a period when the

bottom oxygen was <1.0 µM, making the release of PO_4^{3-} at the turn of anoxia from the sediment due to the reduction of Fe^{3+} to Fe^{2+} probable (Grasshoff et al., 1983). PO_4^{3-} concentrations were low during early autumn with near bottom water with the highest concentrations (2.5 µM) followed by low concentrations in the surface water (<1.0 µM). This was followed by an increase in PO_4^{3-} in the water column (2.0-4.0 µM). The PO_4^{3-} concentration in the water column increased during early winter with values up to 4.0 µM. This was succeeded by a decrease during the remaining winter to values < 1.5 µM.

3.2.3.4 Dissolved inorganic silicate

Sampling began during spring. All stations exhibit a cyclic pattern in the concentration of silicate (figure 16 a-d). During mid-spring, the water column at all stations had high silicate concentrations reaching 70 µM. This was followed by a decrease from late spring to early summer to <30 µM, with surface water having the lowest concentration of <5 µM. There was an increase until mid-summer with values ranging from 35-70 µM in the water column. Followed by a late summer decrease with surface water values decreasing to <10 µM and bottom water values reaching 50 µM. Autumn began with low silicate concentrations in the water column followed by an increase of 35-50 µM in mid-Autumn. This was followed by an increase of up to 55-70 µM until the end of autumn. The water column in early winter had values between 40-75 µM and decreased to <40 µM until the end of winter.

3.2.3.5 Dissolved nitrate

Beira had higher concentrations of NO_3^- from the beginning of October until the end of November with maximum values found in the bottom waters (1.2-4.0 µM, figure 17b). From the beginning of December till the beginning of February, the water column had lower concentrations (< 2.0 µM). During the periods of the 6th to 28th February and the 15th-22 March, the entire water column had very low concentrations of NO_3^- (<0.6 µM). This was during the H_2S eruption period. This could have been due to the fact that the dinoflagellate bloom utilised the nitrate. This was seen at all stations. For Namaqua, the water column had predominantly low concentrations (<2.0 µM), with high bottom water concentrations during 29th October-18th November and 22nd -30th March (3.5-5.0 µM) seen in figure 17a. Seatet had predominantly low concentrations (<1.5 µM); with high bottom water concentrations during October–November (3.0-7.0 µM) seen in figure 17c. At Shoreline, the first 5 meters showed low concentrations <10.0 µM; while the bottom water had a cyclic pattern of higher concentrations (3.0-7.0

μM) during 29 October- 18 November, 8-18 December, 26 February- 15 March and 30th March-7th April (figure 17d).

3.2.3.6 Environmental data during the dinoflagellate bloom in March 2011

Daily samples were taken from the 15th to the 18th March 2011 during the dinoflagellate bloom. This period coincided with a sulphide eruption that originated near the lagoon.

On the 17th March 2011 the bloom reached its maximum (figure 11). The temperature variation during the bloom period was low (18-19°C), suggesting a stratified water column. The highest temperature coincided with the date at which the bloom was at its maximum, with surface values of 20.0 °C and bottom water temperatures of 18.6°C (figure 18). The highest dissolved oxygen concentration was during the bloom maximum (7.5 mL L^{-1}, figure 19), with anoxic conditions in the mid to bottom water (<1.5 mL L^{-1}, figure 19). The PO_4^{3-} in the surface water decreased from 1.8-0.4 μM (figure 20). This decrease coincides with the increase in the bloom biomass. The near bottom water had high PO_4^{3-} of 3.6 μM. This suggests that the sediment is one of the sources of PO_4^{3-} to the water column. This value declined from 4 μM to 2 μM by the end of the bloom. The silicate in the water column during this period ranged from 20-40 μM, with the maximum values in the near bottom water (figure 21). The nitrate values measured were low (<1.0 μM).

3.2.4 Shellfish samples toxin analysis:

YTX, homo-YTX, 45-OH-YTX, carboxy-YTX and homo-YTX-OH were detected in mussel samples from the shellfish farms during the bloom period (figure 22 and 24). The sample from Namaqua on the 17th March 2011 had the highest concentration of YTX (5.4 mg YTX eq kg^{-1}). This coincided with the date at which the *G. spinifera* cells were at a maximum (figure 11). The second highest concentrations detected were from the 16th March 2011 at Beira followed by Seatet samples from the 30th March and 27th April 2011 (4.00, 2.40 and 3.02 mg YTX equivalents kg^{-1}) respectively. Thus these samples taken after the bloom (30th March 2011 and 27th April 2011) still had YTX values above the regulatory limit. The dominant YTX analogue in all samples was 45-OH-YTX (32-45%), followed by homo-YTX (20-35%) in the samples from Namaqua and Seatet. These samples contained a mixture of both mussel species. Samples taken at Beira consisting of mature *Perna perna* mussels had YTX as the second most abundant analogue (25-30%). The sample taken four months after the bloom (6th July 2011) has a YTX value lower than the regulatory limit (0.20 YTX equ kg^{-1}). This sample also had the highest ratio of

carboxy-YTX measured for all samples (13%), similar to the value measured at the same site on the 17th March 2011.

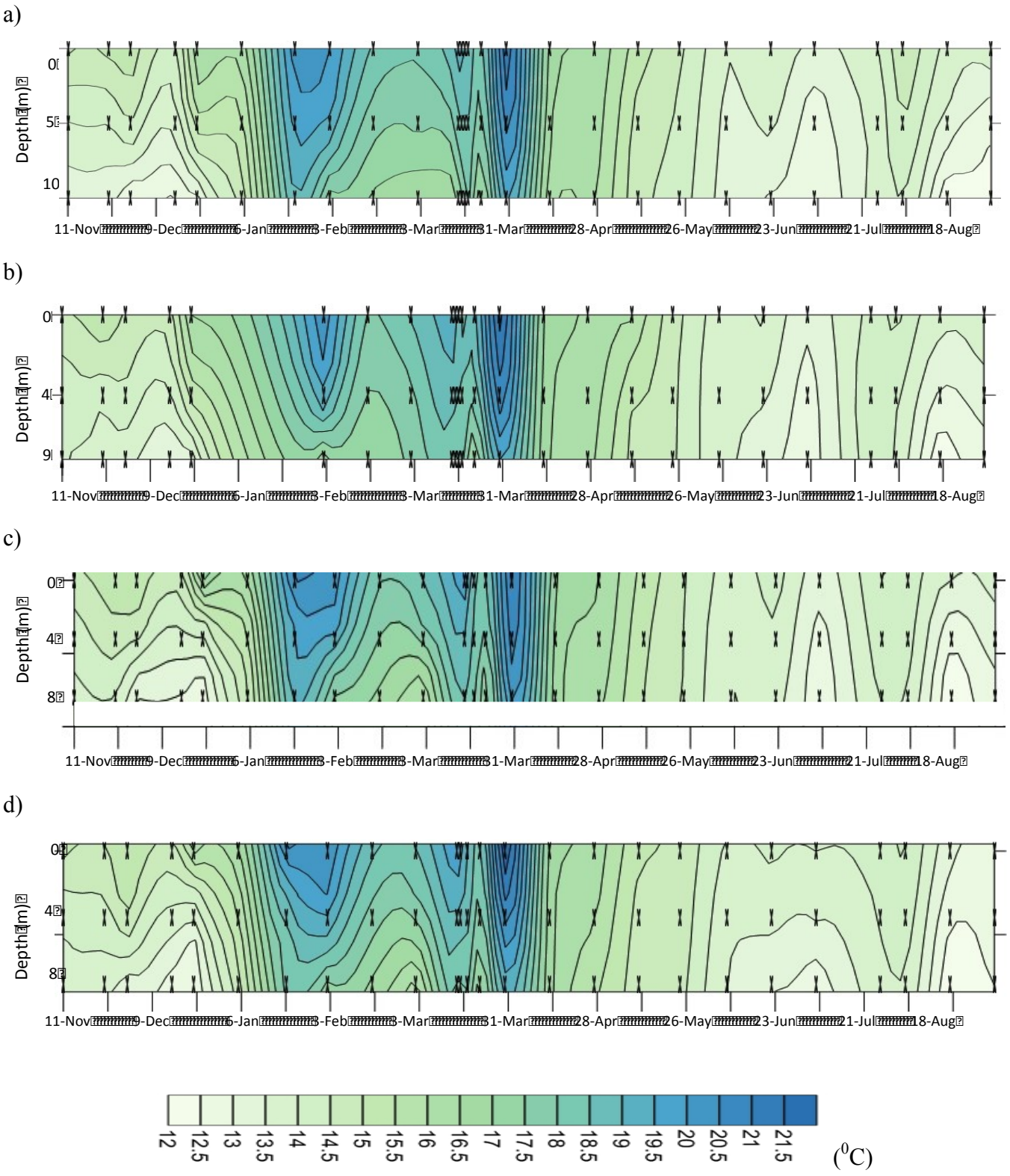


Figure 13: Temporal variation of temperature (°C) measured every 14 days at three depths at a) Namaqua, b) Beira, c) Seatet, d) Shoreline producers. Symbols indicate data points.

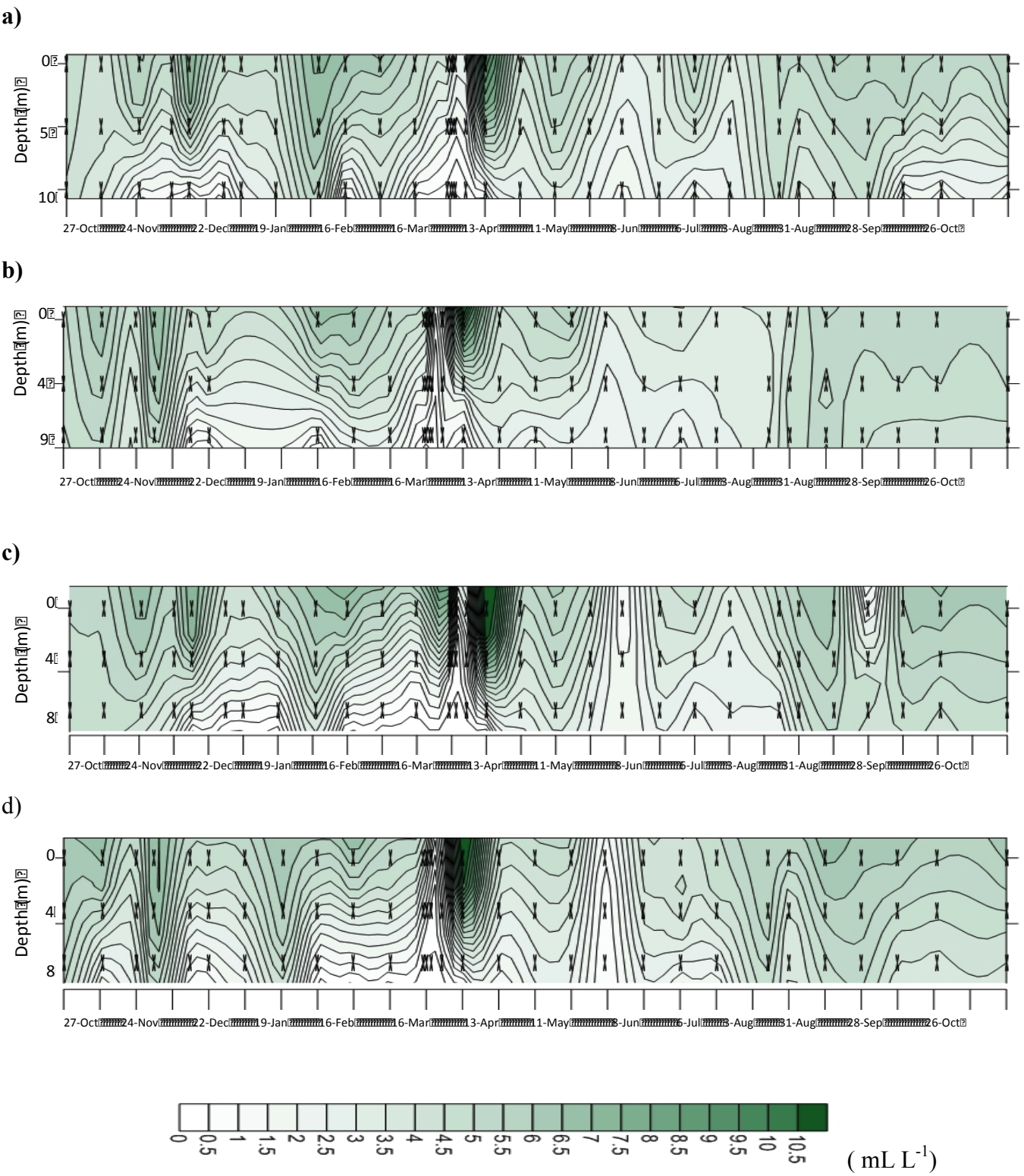


Figure 14 a-d: Temporal variation of dissolved oxygen concentrations (mL L^{-1}) measured every 14 days at three depths at the sites a) Namaqua, b) Beira, c) Seatet, d) Shoreline producers. Symbols indicate data points.

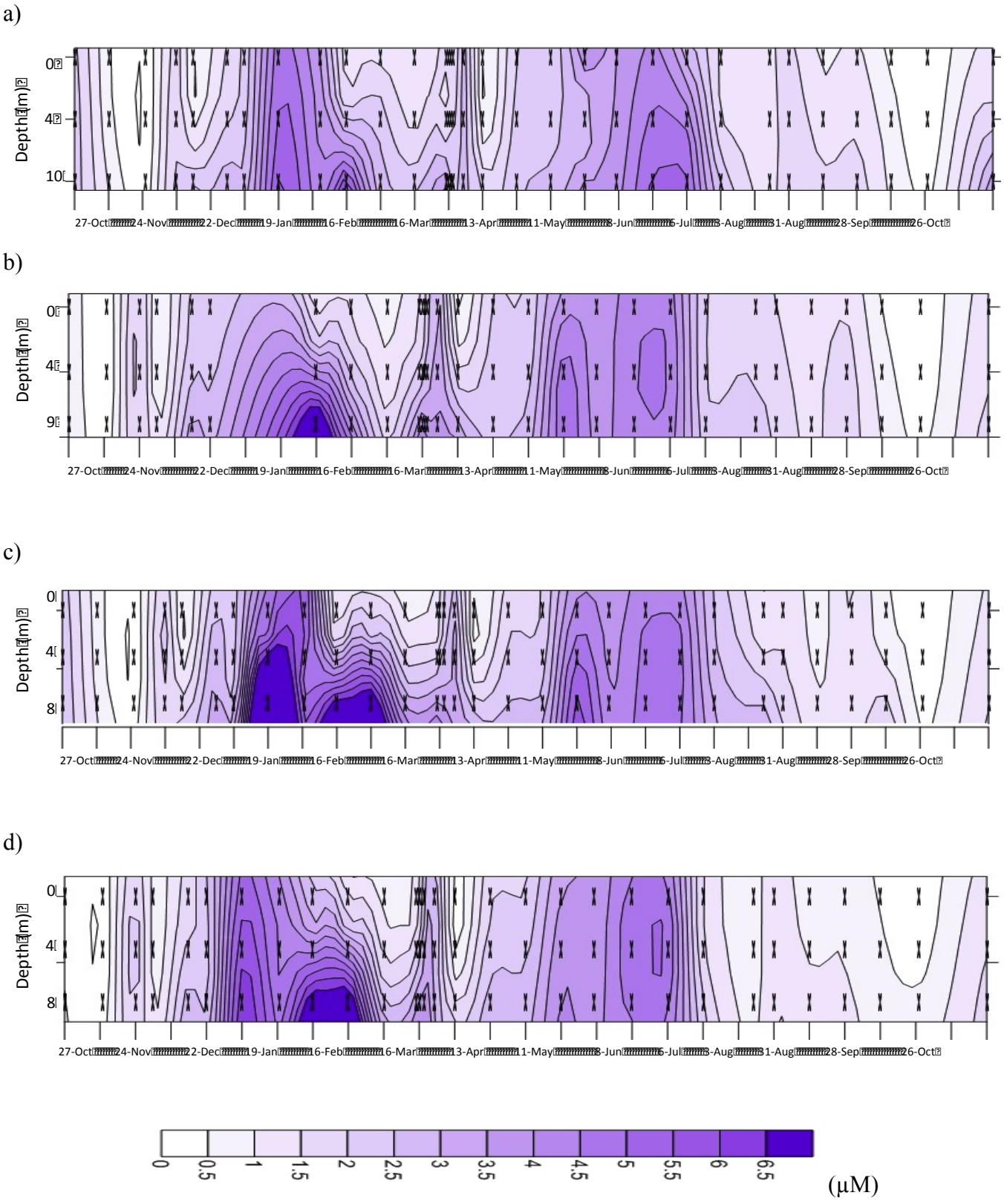


Figure 15: Temporal variation of PO_4^{3-} concentrations (µM) measured every 14 days at three depths at the sites a) Namaqua, b) Beira, c) Seatet, d) Shoreline producers. Symbols indicate data points.

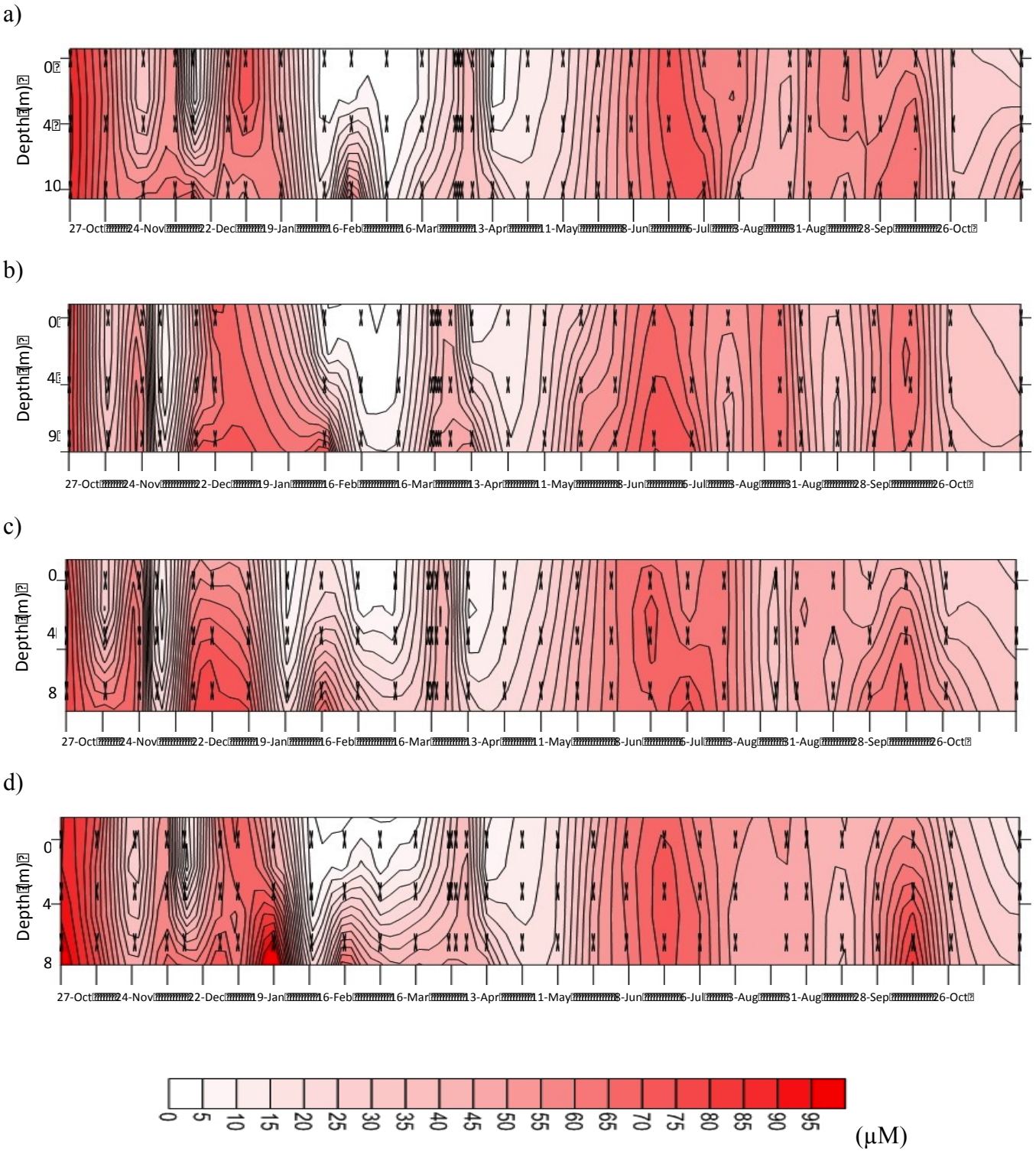


Figure 16: Temporal variation of SO_4 concentrations (µM) measured every 14 days at three depths from sampling sites a) Namaqua, b) Beira, c) Seatet, d) Shoreline producers. Symbols indicate data points.

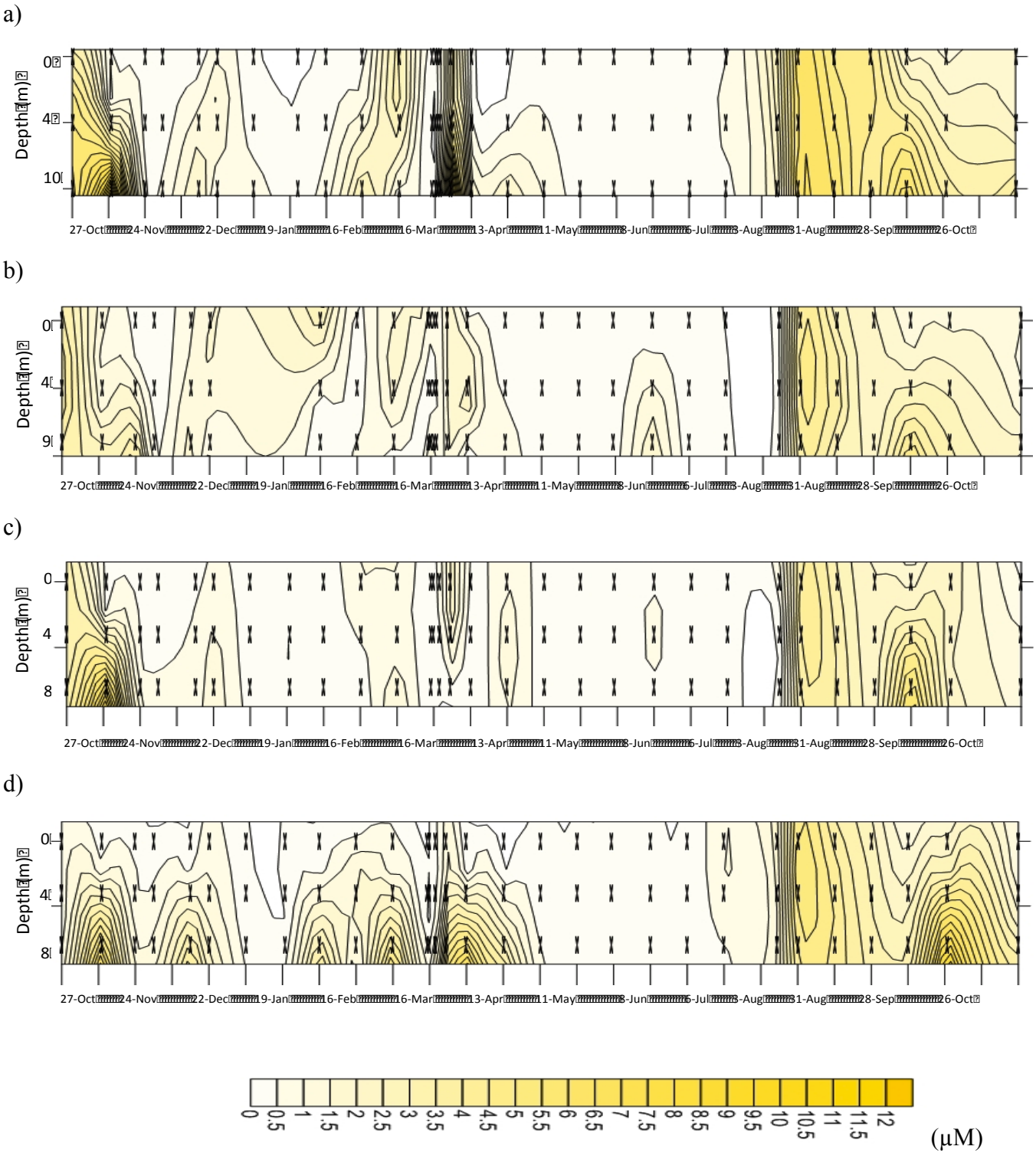


Figure 17: Temporal variation of nitrate concentrations (µM) measured every 14 days at three depths at sites a) Namaqua, b) Beira, c) Seatet, d) Shoreline producers. Symbols indicate data points.

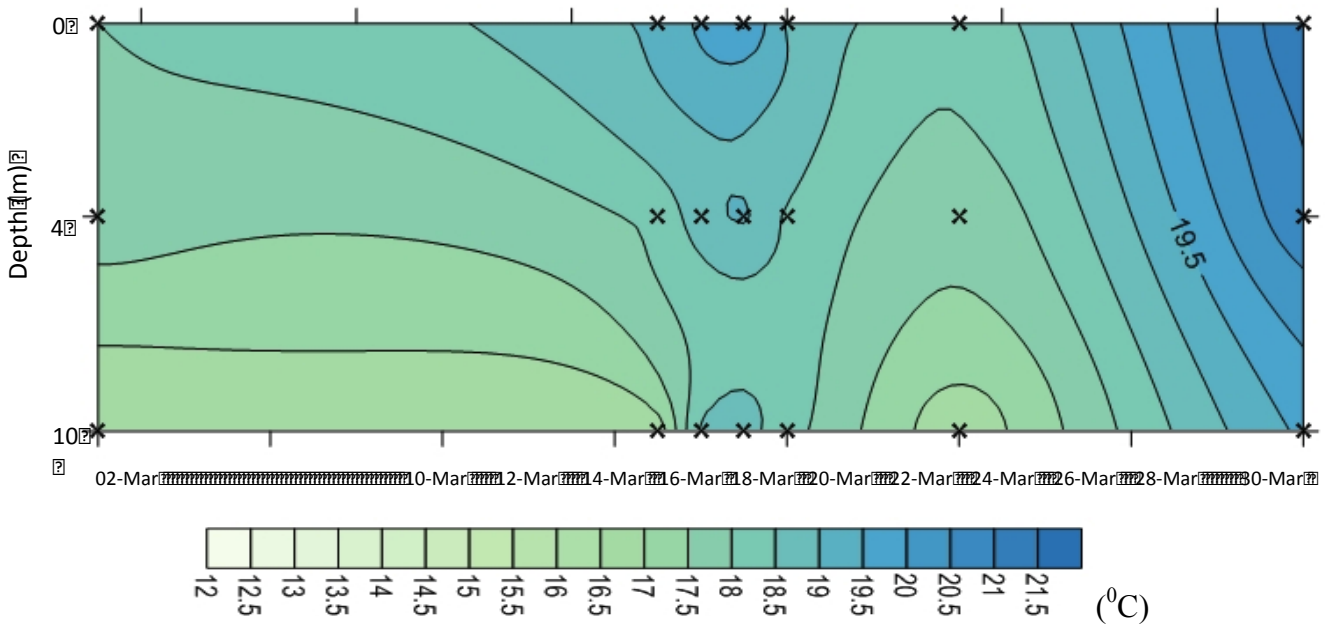


Figure 18: Temporal variation of temperature (°C) before, during and after the *G. spinifera* bloom from Namaqua sampling station.

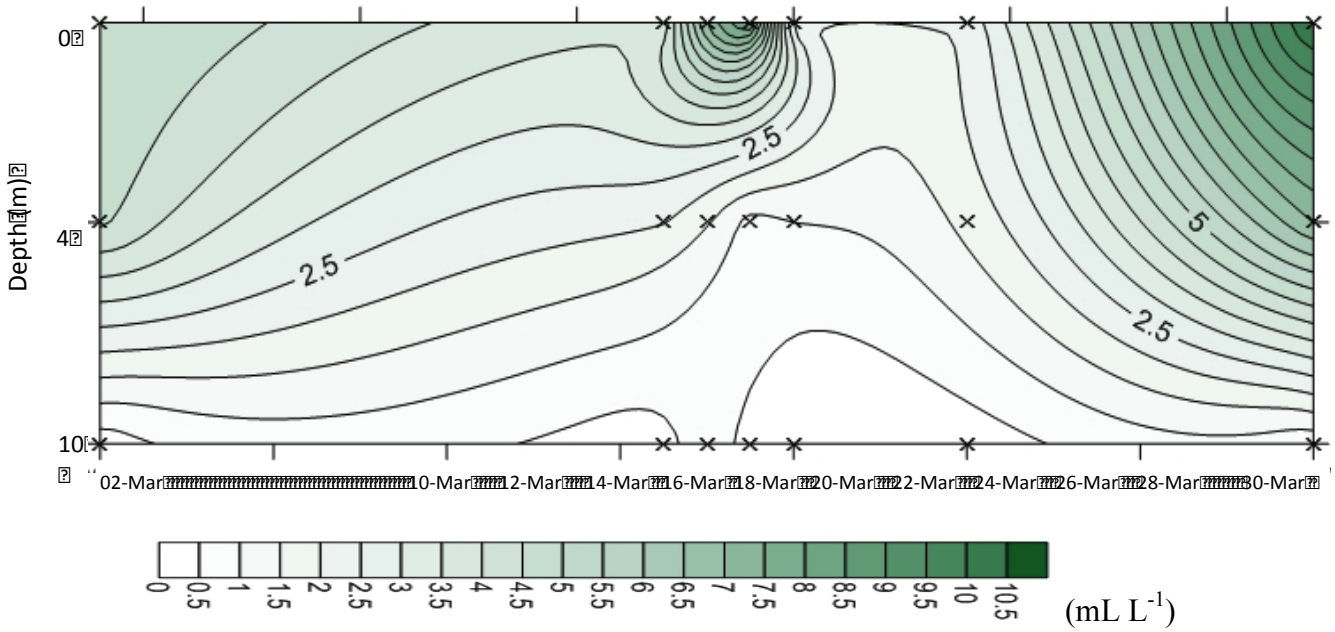


Figure 19: Temporal variation of dissolved oxygen (mL L^{-1}) before, during and after the bloom.

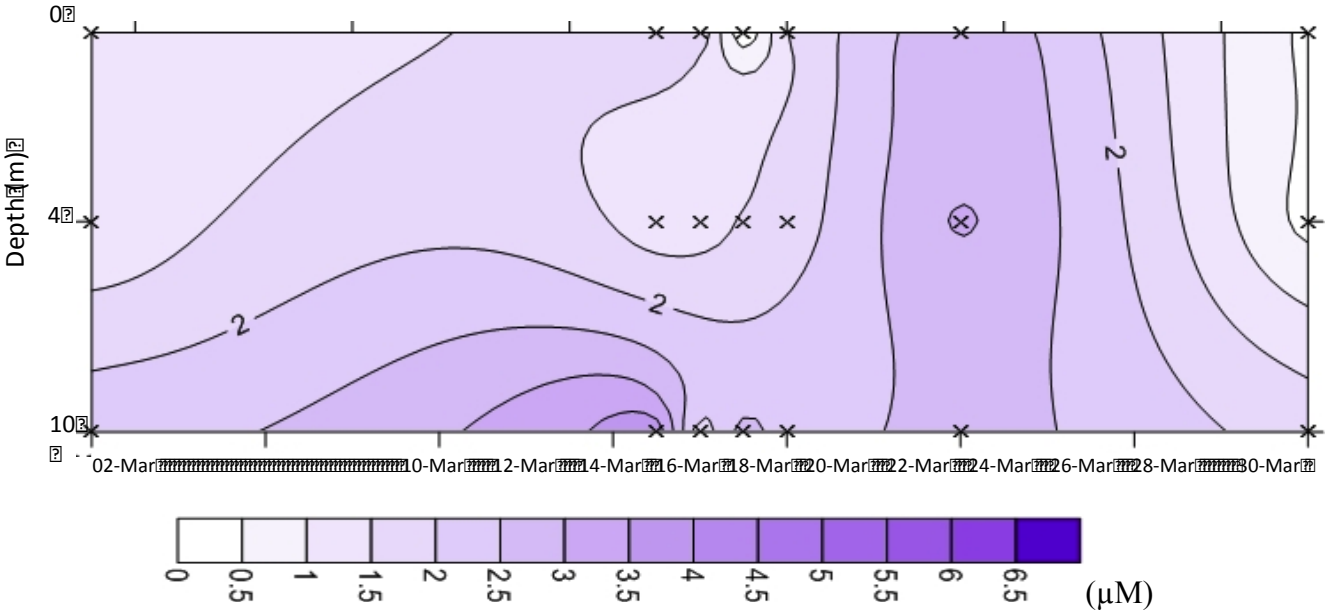


Figure 20: Temporal variation of dissolved inorganic phosphate (µM) before, during and after the bloom.

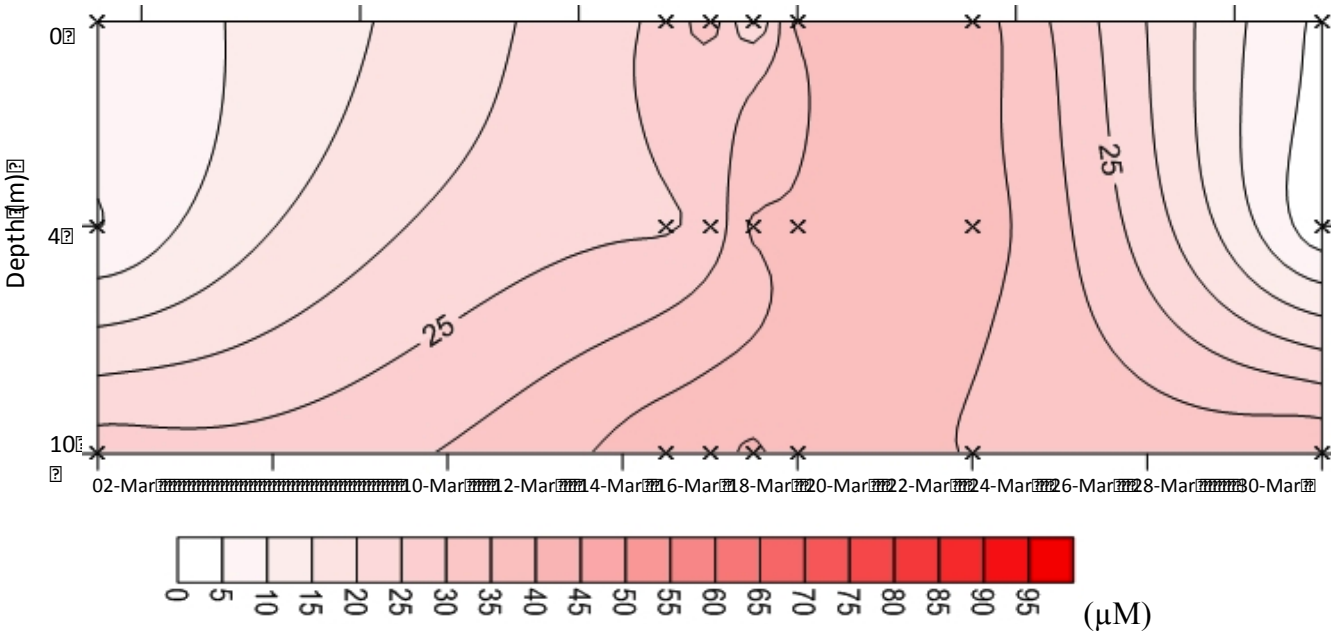


Figure 21: Temporal variation of dissolved inorganic silicate (µM) before, during and after the bloom.

3.2.5 Phytoplankton samples toxin analysis

The phytoplankton samples tested, from 17 March 2011 had the highest concentration of YTX equivalents 59.2 ng mL^{-1} (figure 23 and 25). The vertical net haul sample taken on the 15th March 2011 contained 118.4 ng mL^{-1} of YTX eq. The YTX profiles of all samples were dominated by 45-OH-YTX and homo-YTX, which accounted for > 90% of the total toxin content. This was observed in both net and composite samples. YTX was produced by the cells at 156.0 pg $cell^{-1}$ with 45-OH-YTX and homo-YTX being produced at 78.6 and 65.4 pg $cell^{-1}$, respectively.

3.3 Discussion

The YTX profile in the mussel samples during the bloom correlated with the YTX profile observed in the phytoplankton samples. Toxin concentrations in bivalves are within the range detected in the Adriatic sea (0.17-9.62 mg YTX eq kg^{-1}) for *M. galloprovincialis*. YTX, homo-YTX, 45-OH-YTX and 45-OH-homo-YTX were detected in the shellfish samples. The ratio of YTX and homo-YTX in the shellfish and *G. spinifera* samples was similar to that of *G. spinifera* from the Adriatic Sea (Ricardi et al., 2009; Ciminiello et al., 2000). The Adriatic Sea result is similar to the samples collected from Aquapark 1 with the exception of 45-OH-YTX being the dominant YTX analogue present that is similar to the profile observed in *M. galloprovincialis* from the Black Sea (Morton et al., 2007).

Feeding and depuration experiments with *Mytilus edulis* demonstrated that YTX, 45-OH-YTX and carboxy-YTX were the dominant toxins present after 16 days of feeding of *P. reticultatum* (Roeder et al., 2011). Thus the likely metabolic pathway of YTX in shellfish was that 45-OH-YTX and carboxy-YTX were produced from YTX since the *P. reticulatum* only produced YTX (Roeder et al., 2011; Aasen et al., 2005). This pathway was postulated since the *P. reticulatum* culture used for feeding had a dominant profile of YTX with trace amounts of carboxy-YTX and keto-YTX (<0.3 pg YTX eq $cell^{-1}$). The 45-OH-YTX dominant profile from *G. spinifera* suggests that the source of 45-OH-YTX in the mussels is *G. spinifera*. The low level of carboxy-YTX (7-12%) after the bloom is irregular as it is generally the second most abundant analogues found in shellfish as its depuration rate is slower than that of YTX and 45-OH-YTX (Aasen et al., 2005; Samdal et al., 2005). The low levels of carboxy-YTX might have been caused by low YTX content in *G. spinifera* cells by the mussels ingested initially or a low biotransformation rate in the mussels. The correlation between the 45-OH-YTX in the mussel and

phytoplankton samples suggests that this metabolic pathway needs to be re-examined and clarified using a YTX producing species with a more complex YTX profile.

For *M. edulis* the depuration rate was determined once the feeding was halted after 16 days. The YTX, 45-OH-YTX and carboxy-YTX concentrations measured four days later were reduced. While, with *C. gigas*, the YTX and 45-OH-YTX was almost eliminated during this period. The mussel data from Aquapark 1 only shows a reduction in the total toxicity from a sample taken four months after the bloom. The toxin profile remains relatively unchanged. This is probably due to the fact that the detoxification process is dependent on the shellfish species. Though, since these are field samples, *G. spinifera* cells might have been present during the non-sampling dates thus leading to further exposure of the mussels to YTX. The detoxification rates would have to be assessed ex situ.

The environmental data suggests that the *G. spinifera* from the Benguela region is best adapted to grow in low turbulent, warm (20°C) phosphate rich and nitrate poor water. This complements the modified Margalef's Mandala, which suggests that, the dominant phytoplankton life forms in nutrient rich, low turbulent waters are HAB producing dinoflagellates such as *Gonyaulax* species (figure 2). This is further emphasised by the sharp decrease in *G. spinifera* cells on the 18th March 2011, which coincided with a decrease in PO_4^{3-}. *P. triestunim* and *P. micans* maintaining the high cell counts during this period as they are species well known to be well adapted to nutrient poor waters. They can survive in environments with PO_4^{3-} and NO_3^- concentrations of 0.08 and 0.35 µM respectively (Guiry, 2013).

G. spinifera was first conclusively shown to produce YTX using an ELISA test on single cells from New Zealand (Rhodes et al., 2006). As this test uses antibody recognition, it was unable to provide any information on the analogues present. The toxin content of the cells detected was 176 and 200 pg YTX cell^{-1}. *G. spinifera* from the Adriatic Sea had homo YTX (8.6 pg cell^{-1}) present as a major component in the YTX profile (Riccardi et al., 2009). Thus the YTX profile from the *G. spinifera* bloom shows clear dominant production of 45-OH-YTX and homo-YTX. This contradicts the previous knowledge of 45-OH-YTX being produced from the direct oxidative metabolism of YTX in shellfish (Miles et al., 2006). As it was the most dominant YTX analogue present in field samples, this suggests that it can be directly synthesised by *G. spinifera*. Thus this is the first report of 45-OH-YTX being produced as the dominant YTX analogue on *G. spinifera* cells, the first detection of YTX in mussel and phytoplankton samples from a bloom of *G. spinifera* in the Benguela region.

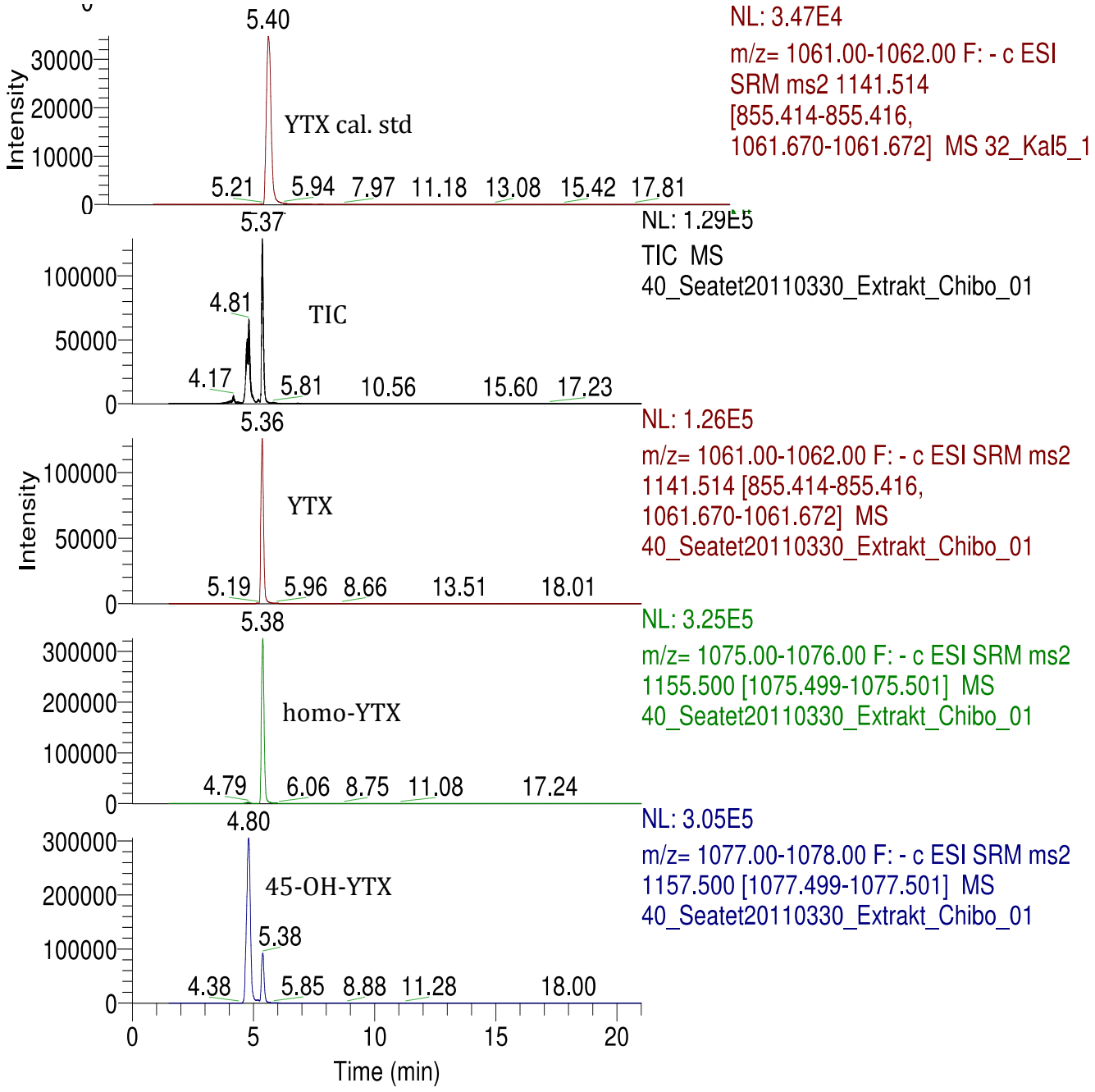


Figure 22: Chromatograms of the YTX calibration standard (YTX cal. Std) and shellfish sample extract from Seatet from the 30th March 2011. Acidic chromatographic conditions were used. YTX standard calibration curve had an r^2=0.999 and was used to determine the concentration of the other YTX analogues.

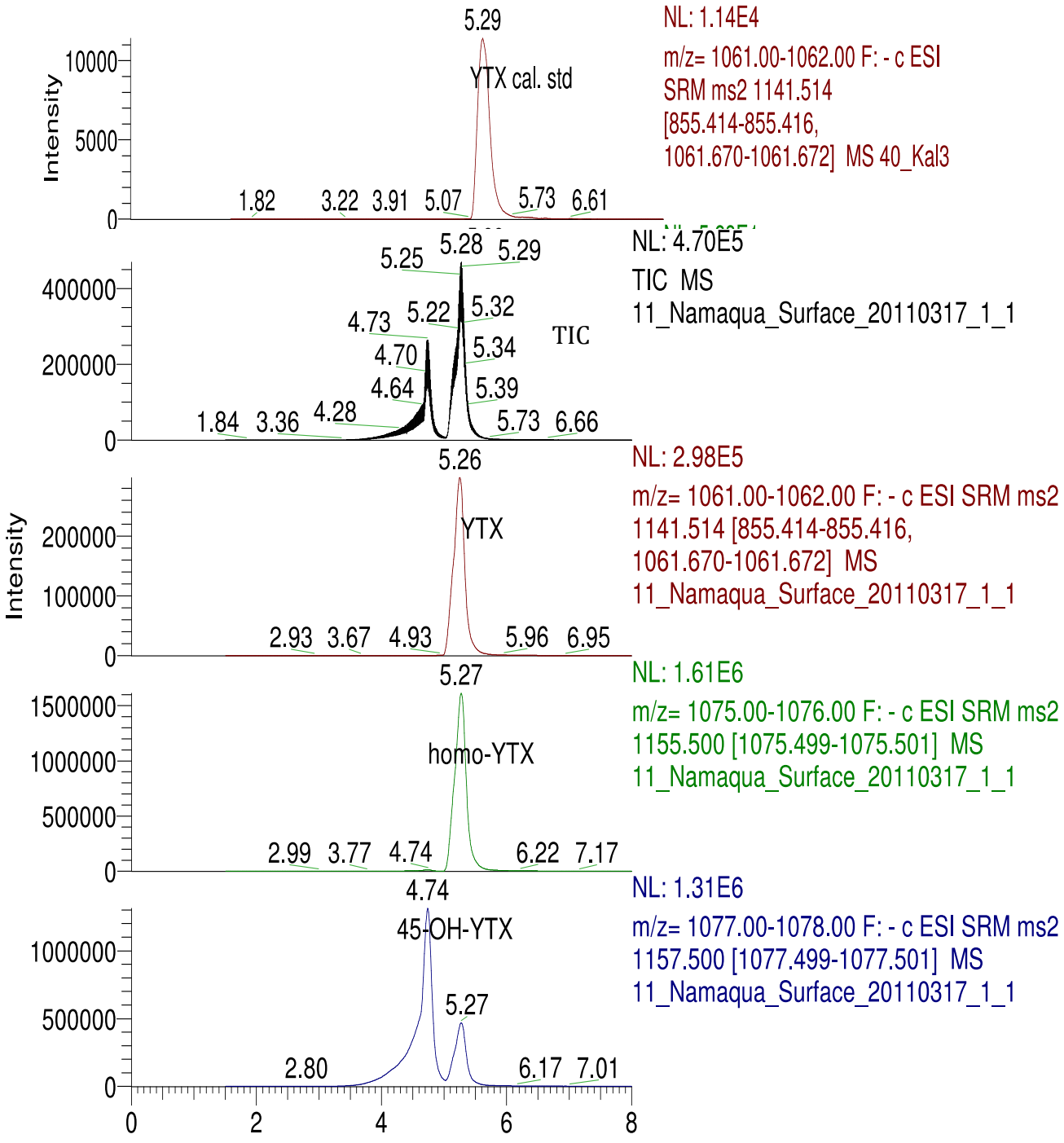


Figure 23: Chromatograms of the YTX calibration standard (YTX cal. std) as well as phytoplankton sample extract from the Namaqua surface from the 17th March 2011. Acidic chromatographic conditions were used. YTX standard calibration curve had an r^2=0.999 and was used to determine the concentration of the other YTX analogues.

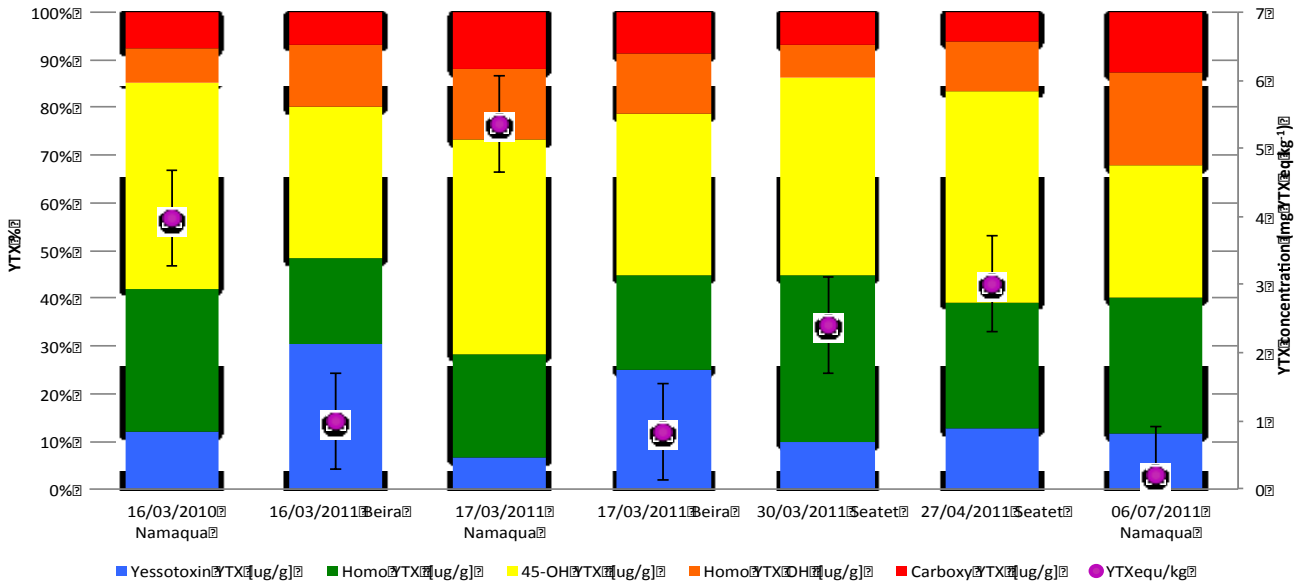


Figure 24: Concentration of YTX in mussel samples from sampling sites in Aquapark 1.

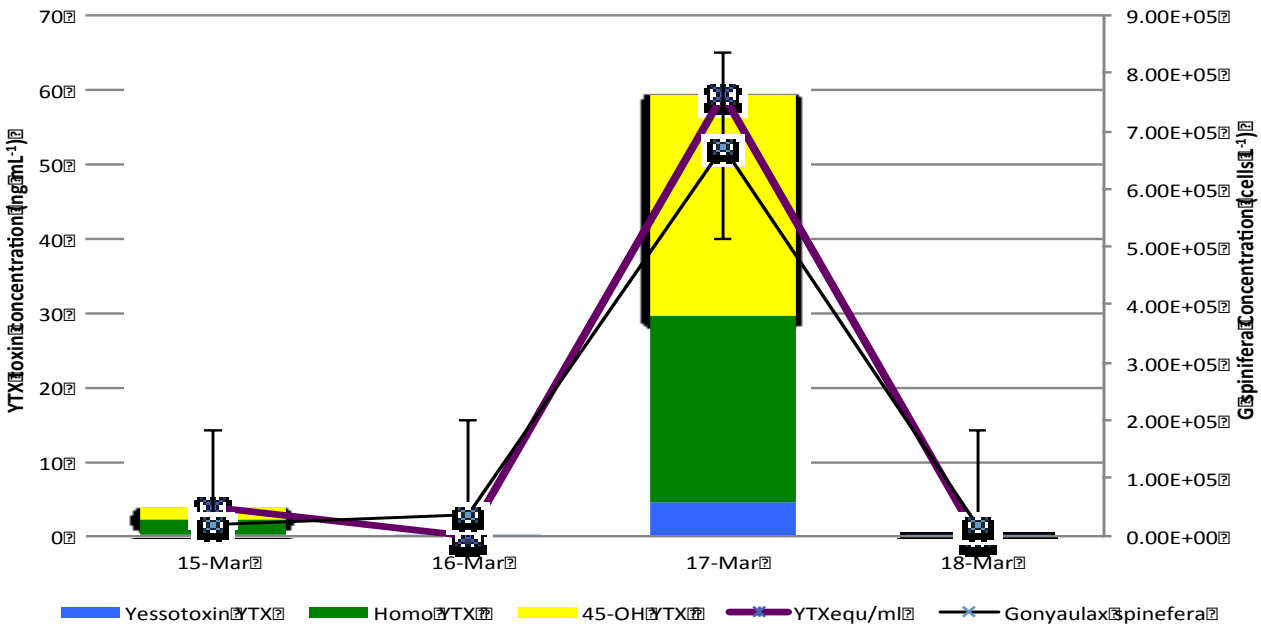


Figure 25: Concentration of YTX analogues in phytoplankton samples and *G. spinifera* cell concentration during the bloom (15-18 March 2011)

Chapter 4: Phylogeny and toxin production in *G. spinifera* cultures from the Benguela Current upwelling system

4.1 Introduction

Gonyaulax spinifera (Claparede and Lachmann) Diesing is a dinoflagellate in the order Gonyaulacales (Dodge, 1989). This cosmopolitan species has an extensive global distribution owing to its adaptability to various environments. Its distribution is described of predominantly in the northern hemisphere with no observations around the African continent (figure 26). However this distribution might be an overestimation as the taxonomy of this species is still unclear and has not been fully resolved (Rochon et al., 2009). The *G. spinifera* complex includes three species with similar morphological features, namely: *G. spinifera*, *G. digitale* and *G. diegensis* (Dodge, 1989; Lewis et al., 1999; Rochon et al., 2009). This confusion arose from the original description of this species being based on vegetative cells. However, the thecate stages hatched from various morphologically different cysts were all assigned to *G. spinifera* (Dodge, 1989; Ellegaard et al., 2002; Rochon et al., 2009). Thus this group is referred to as a 'complex'. In the northern Benguela, *G. spinifera* was observed and conclusively identified using Dodge's (1989) classification in the Walvis Bay area in March of 2011. This was of great importance to this area as it is one of the two regions where shellfish cultivation takes place and the *G. spinifera* was shown to be a potent YTX producer (detailed in chapter 3). Even though YTX is catered for in the Namibian Shellfish Sanitation program (with the relevant EU regulatory limit), it has not been explicitly tested for as the DSTs MBA method used extracts lipophilic toxins in general. Thus there is a need to effectively address this omission as toxic *G. spinifera* is found in the Benguela region.

YTX production in *G. spinifera* is relatively recent with only two other known areas of occurrence, namely Italy and New Zealand (Rhodes et al., 2006; Riccardi et al., 2009). The *G. spinifera* from these areas was shown to be the most toxic of the three genera of YTX producers to date with *G. spinifera* YTX production being 20x higher than *P. reticulatum* and 600x higher than *L. polyedrum* (Paz et al., 2004; Caron et al., 2010). The northern Benguela *G. spinifera* from 2011 had similar results with the addition of 45-OH-YTX and homo-YTX as the dominant YTX

derivatives. The origin of 45-OH-YTX has been attributed to shellfish metabolism of YTX, thus this result was of significance. Unfortunately, no cultures were established during this period, thus unambiguous evidence of the biogenic origin of 45-OH-YTX could not be verified as well as the phylogenetic relationship and extent of toxin production. In the following year (March 2012), *G. spinifera* cells were identified in Walvis Bay. These cells were isolated and three monoclonal cultures were established. Consequently, the confirmation of the biogenic origin of 45-OH-YTX could be accomplished using these cultures if they had similar toxin profiles to the 2011 Benguela *G. spinifera*. The YTX production in the New Zealand and Adriatic strains were highly variable. The New Zealand strain had a total YTX concentration of 188 pg YTXeq $cell^{-1}$ using the ELISA method (Rhodes et al., 2006). Thus there was no information on the YTX analogues present. The two Adriatic Sea strains had two distinct YTX profiles with varying YTX concentrations. The strain isolated in 2005 (DQ867107, table 10) only produced YTX at a concentration of 5.4 pg $cell^{-1}$, while the 2006 strain (EU805590, table 11) produced both homo-YTX and YTX with concentrations of 33.4 and 3.6 pg $cell^{-1}$, respectively. To date no linkage has been made in determining if each strains YTX production is also reflected in their genetic diversity. Thus this can be done with the Benguela *G. spinifera* strains due to their complex YTX profiles.

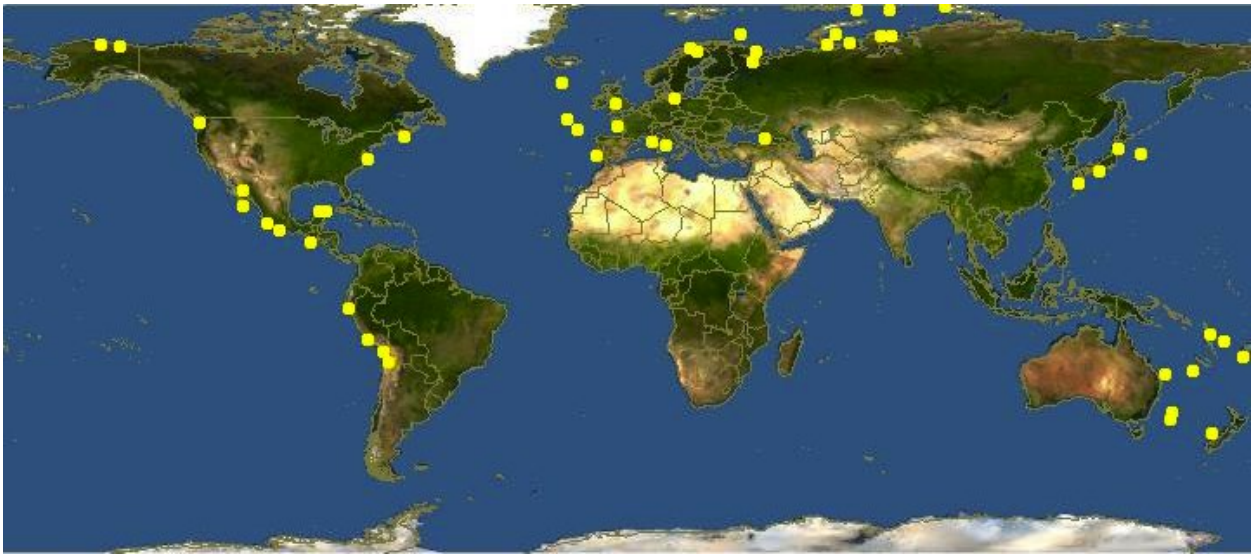


Figure 26: The global distribution of vegetative cells of *G. spinifera* (Guiry, 2013).

For phytoplankton, molecular phylogeny has become of great importance. This is further emphasised for a species like *G. spinifera* due to the species 'complex'. Previous studies using genetic characteristics of *G. spinifera* have suggested that there is a high level of diversity emphasising the *G. spinifera* complex seen from a morphological perspective (Pistocchi et al., 2012; Howard et al., 2009; Riccardi et al., 2009). For molecular phylogeny, the ribosome is generally targeted due to its universal presence in living organisms. The ribosome consists of the large subunit (LSU); the small subunit (SSU) and the 5.8S bound by internal transcribed spacer regions 1 and 2 (ITS1 and ITS2). The genes in these regions are also targeted due to their functional constraints resulting in high sequence conservation. The LSU is useful in evaluating the phylogenetic relationships of closely related species. While the SSU has a higher degree of conserved regions than the LSU, thus the resolution in phylogenetic trees from this can be poor with closely related species. The phylogenetic data available for *G. spinifera* and other species from the same genus from GenBank are not as extensive as the global vegetative cell observations, thus any inference is based on this small subset of data (see table 10 and 11). The SSU sequences in GenBank were derived from three different areas, namely Adriatic Sea (two strains EU805590 and DQ867107), Malaysia (AF052190) and the North Atlantic (AF022155). Unfortunately, only YTX production in the Adriatic strains is known, thus the remaining strains are assumed to be non-toxic. The LSU sequences were derived from three areas namely, Adriatic Sea (EU805591 and EF416284), New Zealand (DQ151557 and DQ151558) and North Atlantic (EU532478 and AY154960) with the North Atlantic strains being nontoxic.

Thus the phylogenetic relationship can be categorised for the Benguela *G. spinifera* not only to determine its relationship with other strains from various geographical areas but also add to the existing knowledge of this species. Therefore it would also be of interest to determine if the dissimilar YTX profiles in the different strains are indicative of their phylogenetic positioning. In addition, verifying the biogenic origin of 45-OH-YTX can be accomplished with these cultures.

Table 10: List of *Gonyaulax* species and YTX toxicity used in the phylogenetic analysis of the SSU. Toxic and non-toxic cultures are denoted by (+) and (-), respectively.

Species	Genbank accession number	Origin	YTX toxicity	Reference
Gonyaulax cochlea	AF274258	Rhodes Island, USA	-	Saldarriaga et al., 2001
Gonyaulax fragilis	56384899	Adriatic Sea, Italy	-	Riccardi et al., 2007
Gonyaulax polyedra	AF377944	South Korea	-	Lee et al., 2001 unpublished
Gonyaulax polyedra	AJ415511	Norway	-	Shalchian, 2001
Gonyaulax polygramma	AJ833631	South Korea	-	Jeong et al., 2005
Gonyaulax spinifera	AF022155	Maine, USA	-	Saunders et al., 1997
Gonyaulax spinifera	AF052190	Malaysia	-	Usup et al., 1998 unpublished
Gonyaulax spinifera	DQ867107	Adriatic Sea, Italy	+	Riccardi et al., 2009
Gonyaulax spinifera	EU805590	Adriatic Sea, Italy	+	Riccardi et al., 2009
Gonyaulax verior	AY443013	-	-	Saldarriaga et al., 2004

Table 11: List of *Gonyaulax* species and YTX toxicity used in the phylogenetic analysis of the LSU. Toxic and non-toxic cultures are denoted by (+) and (-), respectively.

Species	Genbank accession number	Origin	YTX toxicity	Reference
Gonyaulax baltica	AY154962	Sweden	-	Ellegard et al., 2003
Gonyaulax digitale	AY154963	Canada	-	Ellegard et al., 2003
Gonyaulax elongata	AY154964	United Kingdom	-	Ellegard et al., 2003
Gonyaulax membranacea	AY154965	Ireland	-	Ellegard et al., 2003
Gonyaulax polygramma	DQ162802	South Korea	-	Kim et al., 2006
Gonyaulax cf. spinifera	AY154960	-	-	Ellegard et al., 2003
Gonyaulax spinifera	DQ151557	New Zealand	+	Rhodes et al., 2006
Gonyaulax spinifera	DQ151558	New Zealand	+	Rhodes et al., 2006
Gonyaulax spinifera	EF416284	Adriatic Sea, Italy	+	Riccardi et al., 2009
Gonyaulax spinifera	EU532478	Massachusetts, USA	-	Howard et al., 2009
Gonyaulax spinifera	EU805591	Adriatic Sea, Italy	+	Riccardi et al., 2009

4.2 Results

4.2.1 Yessotoxin production

All three *G. spinifera* strains produced varying concentrations of total cellular YTXeq (figure 27 and 28). NamWB012 produced the highest total cellular YTX (151.8 ng mL^{-1}, 155.9 pg $cell^{-1}$). NamWB002 and NamWB011 contained a total cellular YTXeq concentration of 38.7 and 14.9 ng mL^{-1}, respectively (table 12). Homo-YTX was the dominant toxin produced by all three strains (figure 28). It accounted for 82-92% of the total cellular YTX content with NamWB012 producing the highest concentration of 143.7 pg $cell^{-1}$ (table 12). YTX was the second most abundant toxin present accounting for 5-14% of the total cellular content. NamWB012 had the lowest YTXeq content of the three strains. 45-OH-YTX was detected but accounted for less than 3% in all three strains. NamWB012 produced 1.2 pg $cell^{-1}$.

A duplicate sample of the toxin extract from NamWB012 was sent to the Institute for Marine Biosciences, Canadian National Research Council to confirm the identity of 45-OH-YTX. This was due to the fact that there is currently no calibration standard available. The sample was analysed using a neutral chromatographic LC-MS/MS method (McCarron et al., 2011). The resultant toxin profile was significantly different from the one obtained using the basic chromatographic LC-MS/MS method. It was confirmed that a hydroxyYTX (OH-YTX) was present in the sample in the same size range as 45-OH-YTX (figure 29). Further testing is needed to confirm its identity as the retention time of the peak from a confirmed 45-OH-YTX mussel sample was slightly earlier than of the NamWB 012 culture sample (2.58 and 2.64 min, respectively). The discrepancy in the results showed a considerably higher estimation of the presumed 45-OH-YTX with McCarron's method. This culminated in a 45-OH-YTX proportion of 34% of the total YTX produced with homo-YTX still being the dominant YTX derivative produced. The total toxin profile was: homo-YTX (62%), followed by 45-OH-YTX (34%) and YTX (4%).

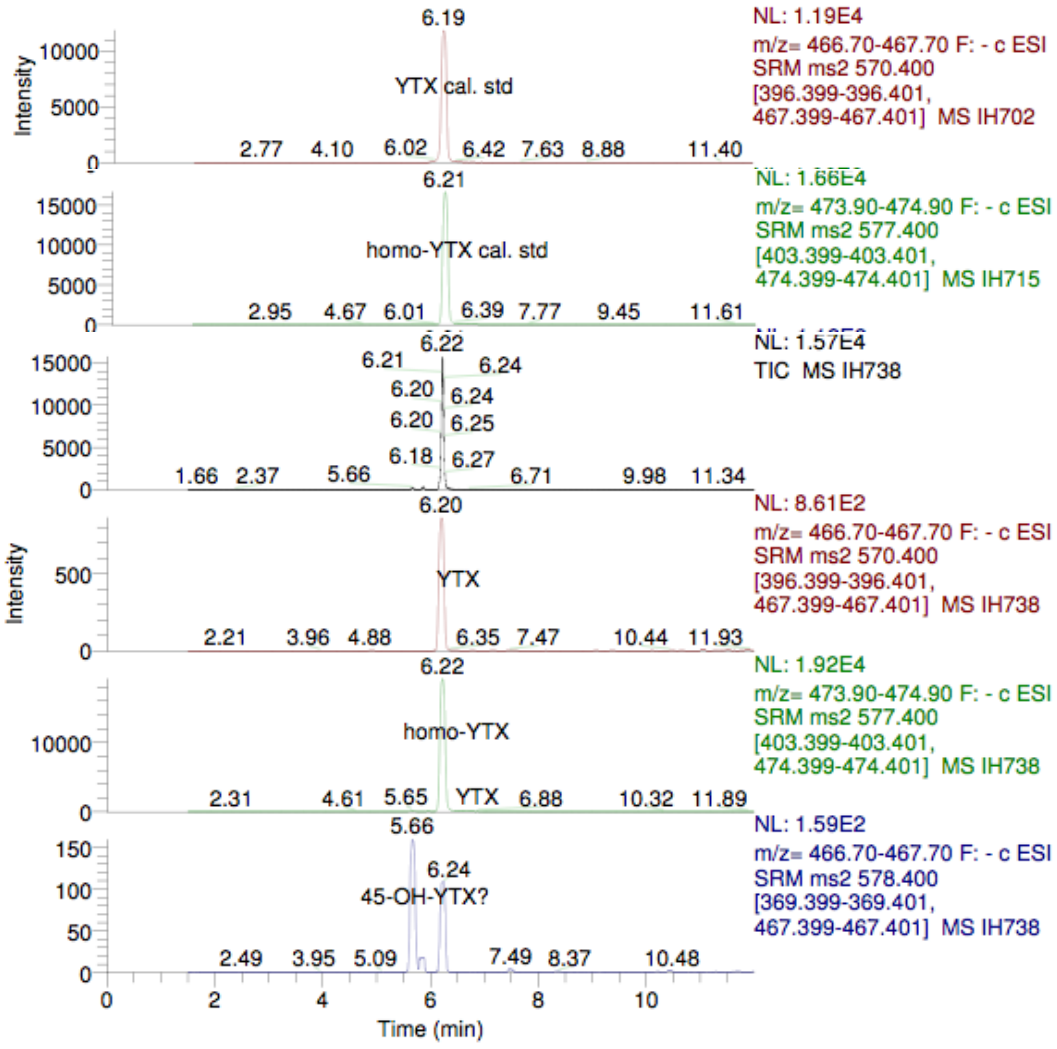


Figure 27: Chromatograms of the YTX and homo-YTX calibration standards as well as *G. spinifera* culture NamWB011. Basic chromatographic conditions were used. The quality control measures followed: $r^2 > 0.9980$, RT= $\pm$ 0.05 minutes of both standards.

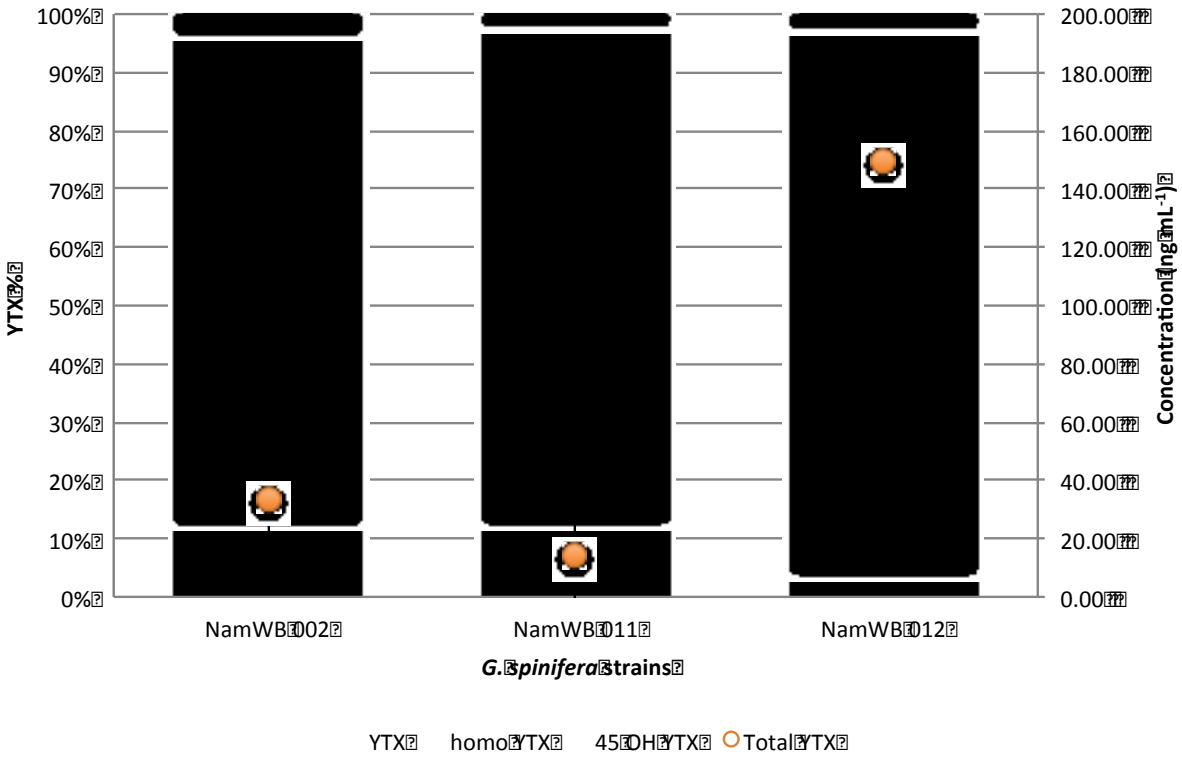


Figure 28: Total cellular YTX concentration (ng mL^{-1}) and toxin profile of the *G. spinifera* strains NamWB002, NamWB011 and NamWB012 (n=3).

Table 12: Percentage and cellular concentrations of YTX and analogues in of *G. spinifera* strains NamWB002, NamWB011 and NamWB012.

	% of total YTX content				
***G. spinifera* Strain**	**YTX**	**homo-YTX**	**45-OH-YTX**	**Total YTX (ng mL^{-1})**	**Total YTX (pg $cell^{-1}$)**
NamWB002	14	82	2	34.04	38.73
NamWB011	14	86	0	14.58	14.88
NamWB012	5	94	1	151.83	155.88

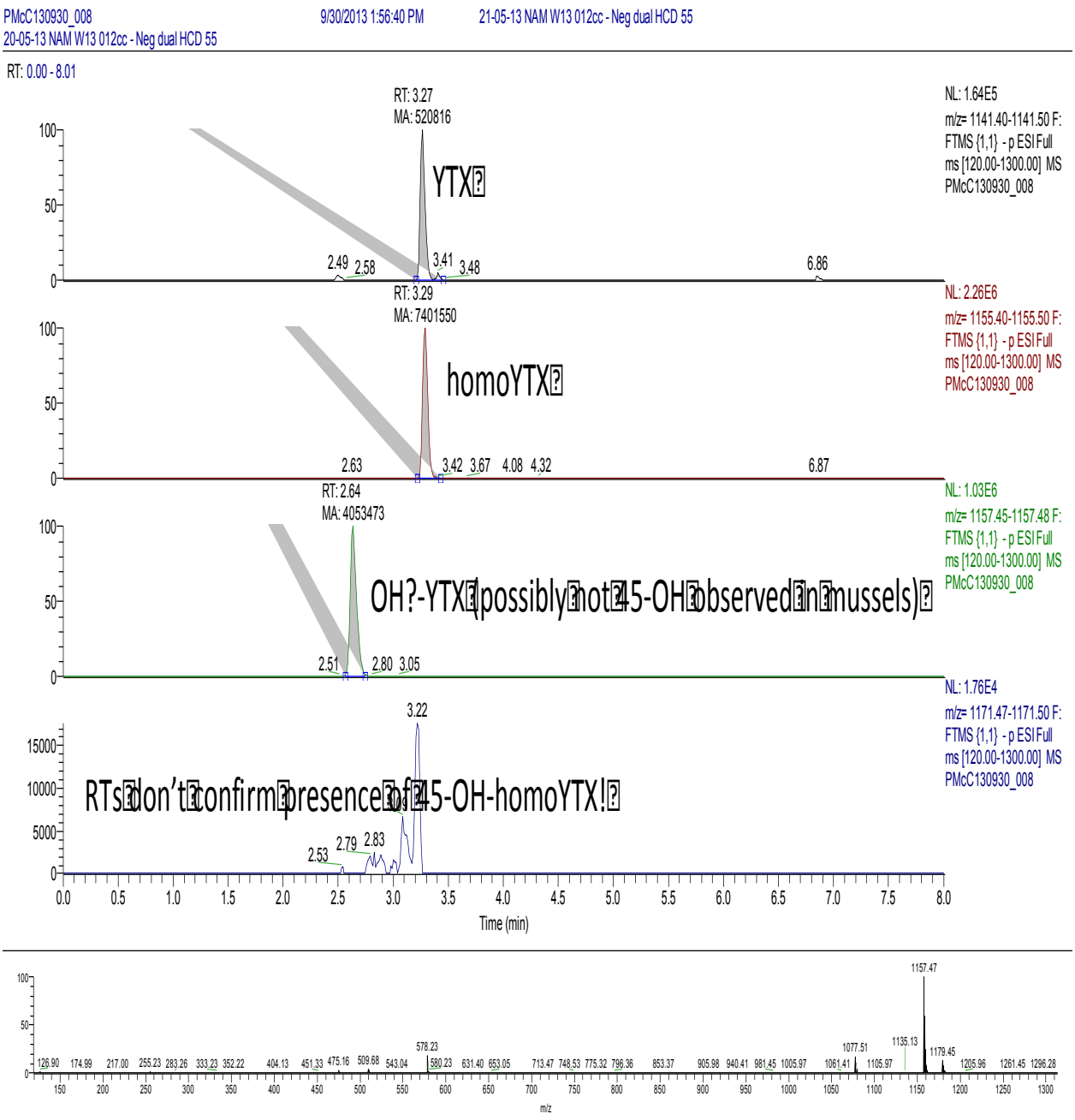


Figure 29: LC-MS/MS analysis of *G. spinifera* culture NamWB012 using a neutral chromatographic method (P Macarron, Biotoxin Certified Reference Materials Program, Canadian National Research Council).

4.2.2 Phylogeny

All three Benguela strains had identical SSU and LSU sequences with a genetic divergence (p-distance) of 0.00, thus they were denoted collectively as NamWB *G. spinifera* in the resulting phylogenetic trees. The SSU was 1673 bp long and the resulting phylogenetic trees are shown in figures 30 and 31. All three Benguela strains clustered with YTX producing strains EU805590 and DQ867107 isolated from the Adriatic Sea, Italy with high bootstrap values of 100 and 99 % in both Neighbourhood joining (NJ) (figure 30) and maximum likelihood analysis (figure 31). The p-distance between NamWB and EU805590 and DQ867107 was low (p-distance values of 0.007 and 0.092, respectively). All known YTX producing *G. spinifera* species formed a cluster and were distinct and highly divergent from the nontoxic Malaysian (p-distance of 0.253) and North Atlantic (p-distance of 0.206) *G. spinifera*. These nontoxic strains grouped with other species within the genus *Gonyaulax*.

The partial LSU was 793 bp long with the resulting phylogenetic trees shown in figures 32 and 33. As with the SSU analysis, all three Benguela *G. spinifera* strains clustered with YTX producing strains EU805591 (Adriatic Sea strain) and DQ151558/DQ151557 (New Zealand strains) with bootstrap values of 100% in both NJ (figure 32) and maximum likelihood (figure 33) trees. All five known *G. spinifera* YTX producers formed a distinct clade. The genetic divergence between NamWB and EU805591 was low (p-distance 0.002). The p-distance between NamWB and both New Zealand strains was 0.005. NamWB was highly divergent from the second Adriatic strain (EF416284) with a p-distance of 0.211. The non-toxic *G. spinifera* from the North Atlantic, namely AY154960 and EU532478 were highly divergent from NamWB with p-distances of 0.500 and 0.563, respectively.

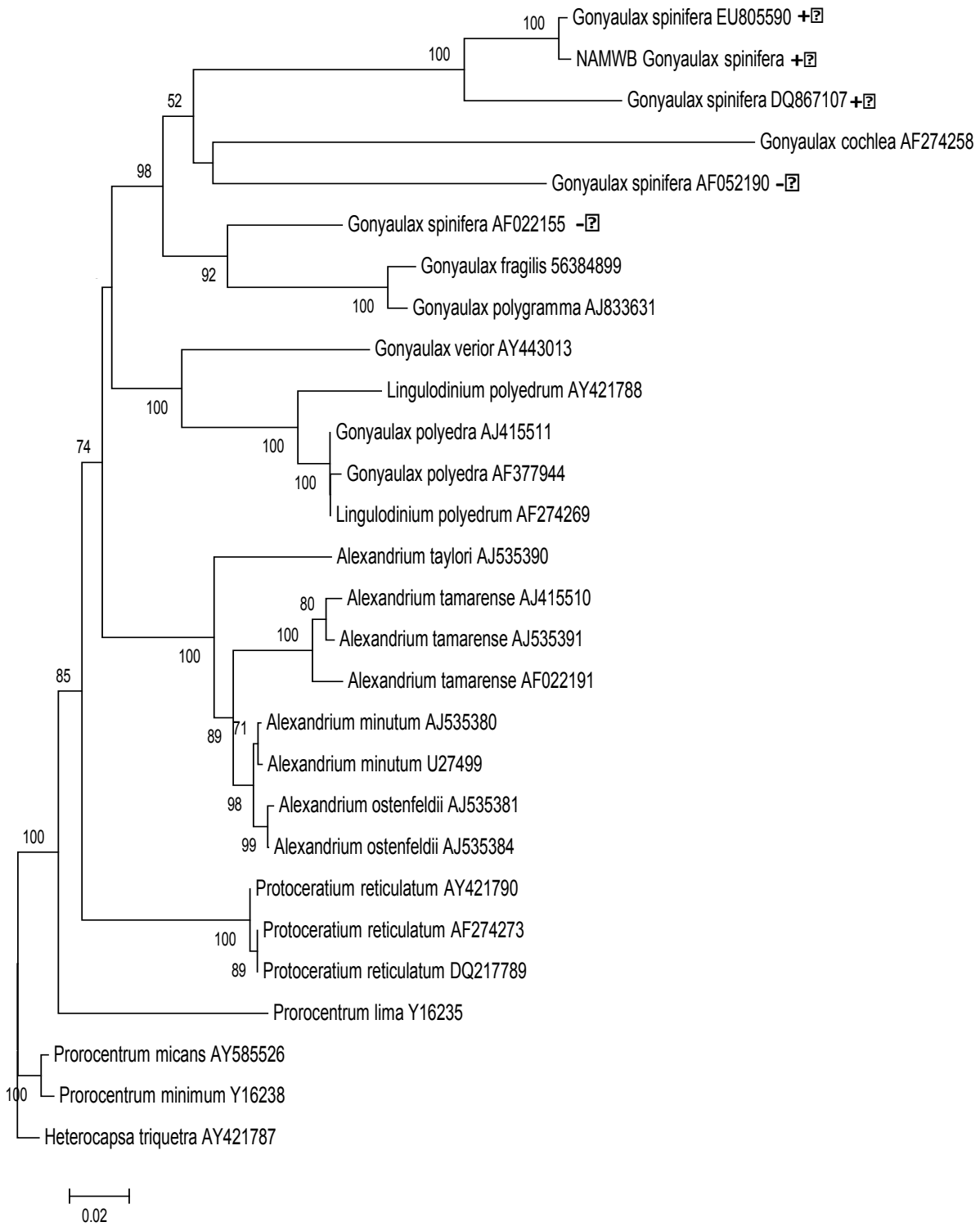


Figure 30: Neighbourhood joining (NJ) analysis of the SSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown. YTX producers are denoted by (+) and nontoxic *G. spinifera* strains are denoted by (-).

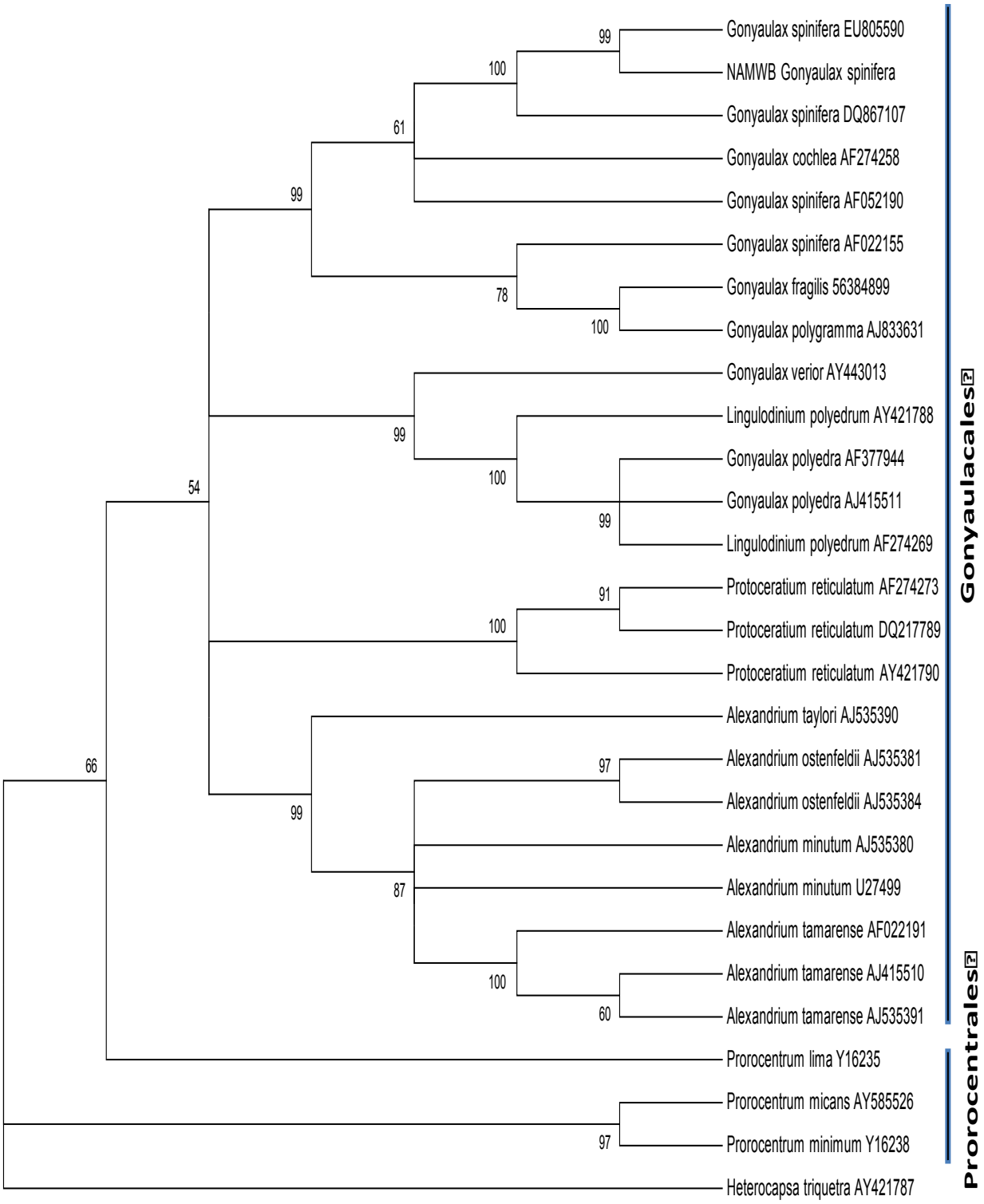


Figure 31: Maximum likelihood analysis of the SSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown.

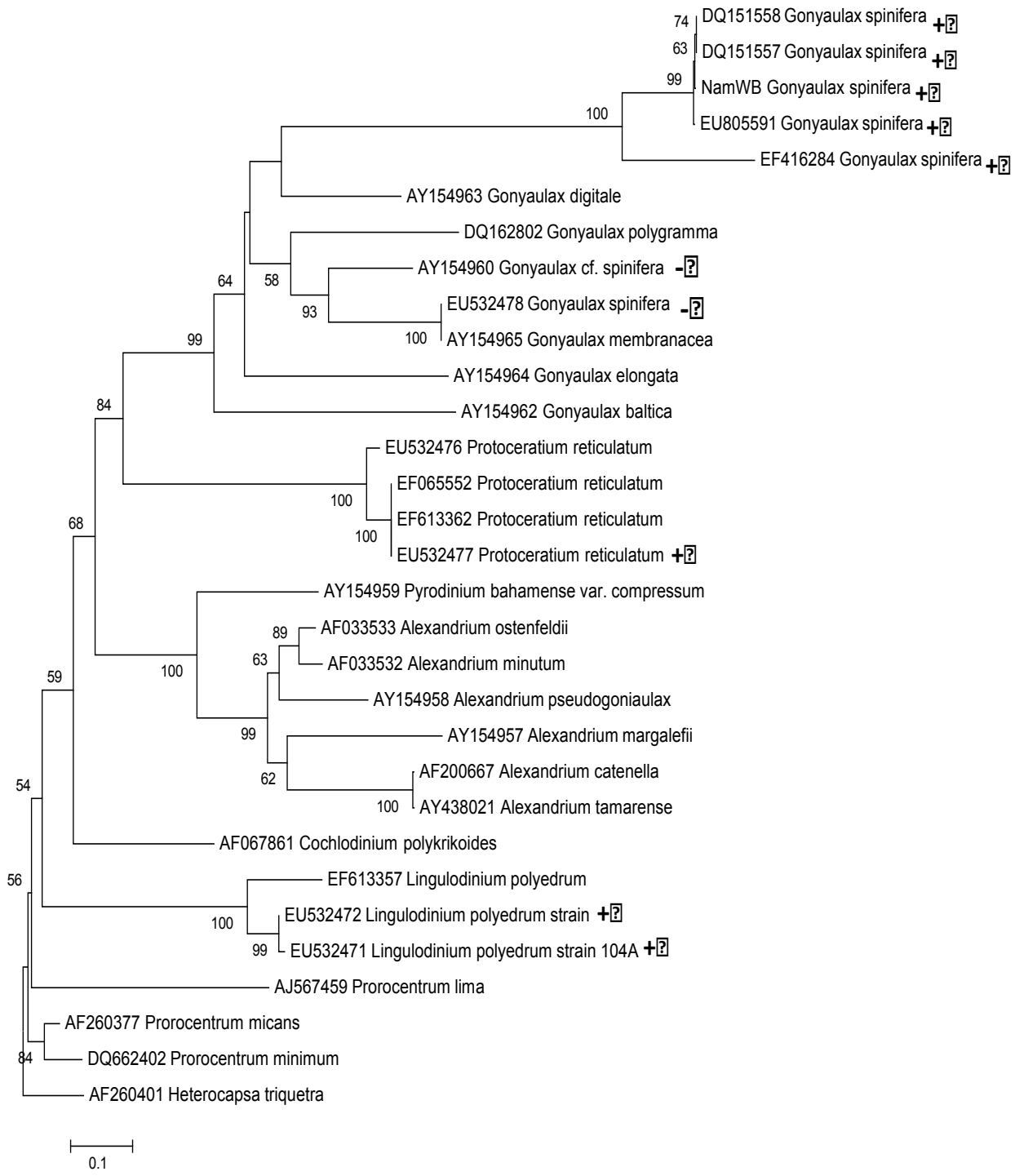


Figure 32: Neighbourhood joining (NJ) analysis of the LSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown. YTX producers are denoted by (+) and nontoxic *G. spinifera* strains are denoted by (-).

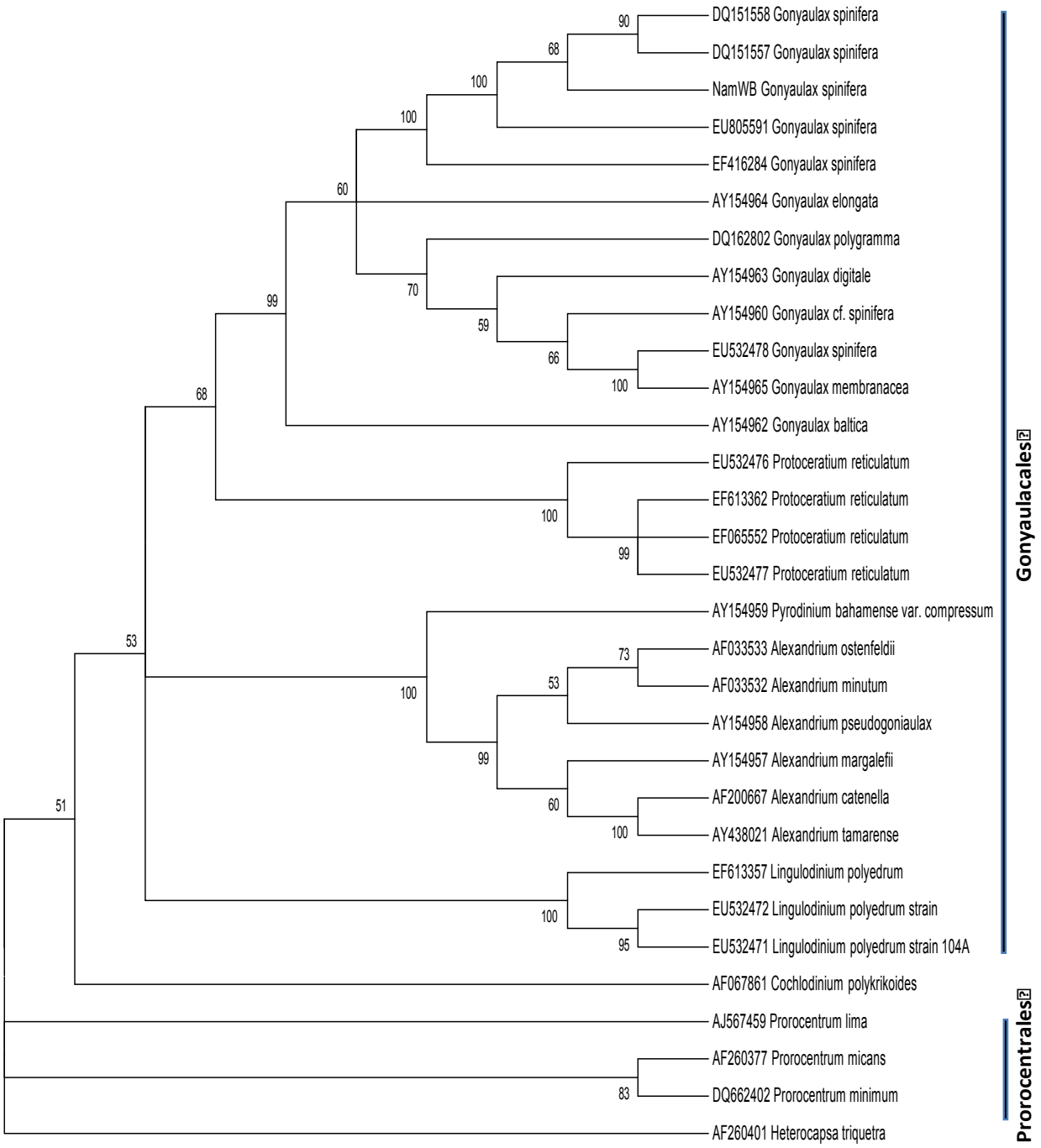


Figure 33: Maximum likelihood analysis of the LSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown.

4.2.3 Growth rate

The growth rate for all strains ranged from 0.16- 0.17 days^{-1} (figure 34). NamWB012's cells numbers reached a maximum of 3200 cells mL^{-1} and reached the stationary phase on day 35. NamWB011 and 002 reached maximum cell concentrations of 2307 and 2377 cells mL^{-1} and the stationary phase between days 31-35.

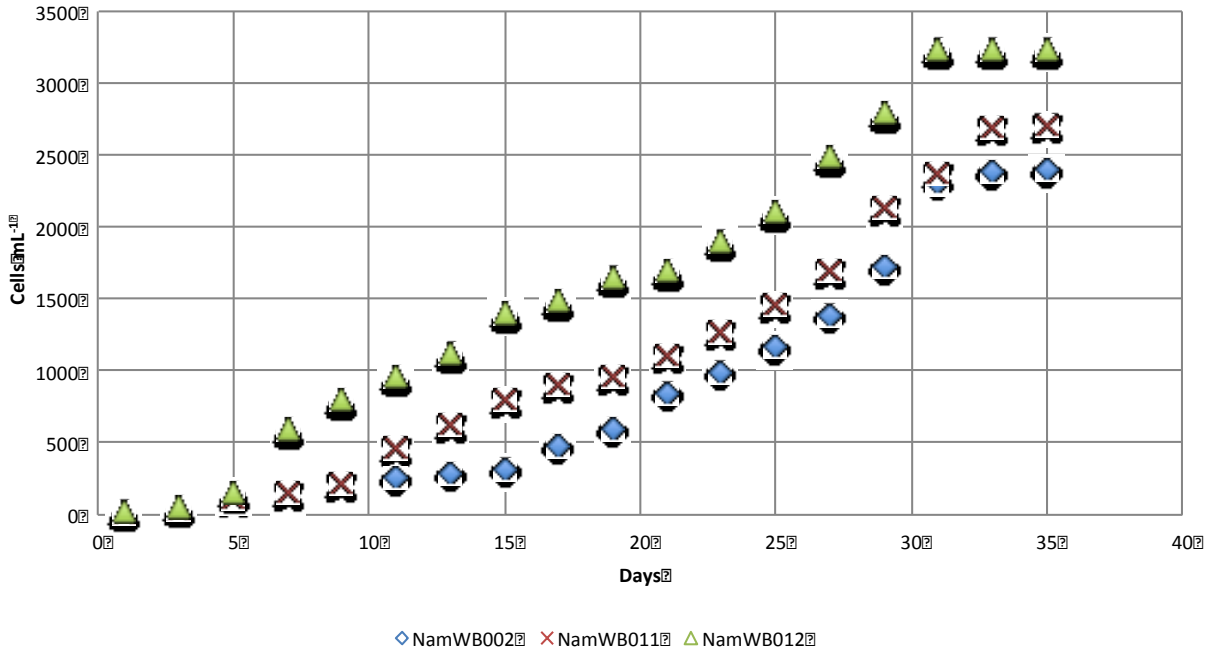


Figure 34: Growth curves of three *G. spinifera* strains NamWB002, NamWB011 and NamWB012 grown in ½ F/2 media under the same conditions.

4.3 Discussion

The recent identification and confirmation of YTX producing *G. spinifera* in the Benguela region show that it is a new, recurring and prominent concern to the Namibian shellfish mariculture industry, as *G. spinifera* cells have been detected in consecutive years around Walvis Bay in 2011 (chapter 3), 2012 and 2013 (MFMR phytoplankton database). Thus the culturing of *G. spinifera* has allowed for the unambiguous confirmation of this specie's toxicity and toxin profile. The range of toxicity in natural populations could also be assessed. All three Benguela *G.*

spinifera isolates were toxic and had highly variable toxin production even though the cells were isolated from the same sampling site during the same period as well as having similar growth rates. There was a 10-fold difference in YTXeq production between the highest (NamWB012, 155.9 pg YTX eq. cell^{-1}) and lowest (NamWB011, 14.9 pg YTX eq. cell^{-1}) *G. spinifera* isolates. This suggests that the quantity of toxin production is varied even within populations under the same environmental conditions, though this inference is hindered by the small sample size. From a global perspective, these YTXeq values are within the ranges measured in the New Zealand and Adriatic Sea strains. The highest concentration of total cellular YTXeq measured in the cultures was also relatively similar to the amount measured in field samples during the 2011 *G. spinifera* bloom (156.0 pg YTX eq. cell^{-1}). The toxin profiles were relatively similar between isolates with the dominant toxin produced being homo-YTX, accounted for 82-92% of the total YTXeq cellular content. This finding is relatively similar to the *G. spinifera* strain isolated in 2006 from the Adriatic Sea, though the homo-YTX ratio to YTX was greater (Riccardi et al., 2009). This differs from the field samples from 2011, as homo-YTX was the second most dominant toxin present. All three cultures had low concentrations of presumed 45-OH-YTX. This was in contrast with the *G. spinifera* field samples analysed during the 2011 bloom where 45-OH-YTX was the dominant YTX derivative present. Of the three isolates, NamWB012 had the highest homo-YTX production (140 ng homo-YTX mL^{-1}). Thus this isolate has the highest homo-YTX concentration of any YTX producing species known to date. The discrepancy in results with the different methods is of concern as the YTX basic chromatographic method used in this thesis gave a sizeable underestimation of the total concentration of presumed 45-OH-YTX (2%). This not only affects the toxin profile, but it also underestimates the final YTXeq result.

All three Benguela *G. spinifera* isolates has identical SSU and LSU sequences. These isolates (NamWB) formed a distinct clade with the other known toxic *G. spinifera* strains. This grouping was more defined with the LSU phylogeny, as it is used to discern relationships between closely related species. An added advantage was that there were more sequences to analyse. The Benguela *G. spinifera* was most closely related to the 2006 Adriatic Sea isolate (EU805591) that also produced homo-YTX as its dominant toxin (Riccardi et al., 2009). There was a low diversity distance between the Benguela, Adriatic Sea and the New Zealand isolates and these isolates formed a distinct group separated from the second Adriatic Sea isolate that only produced YTX

(EF16284). This distinct separation suggests that the New Zealand isolate probably not only produce YTX, but possibly homo-YTX as well since it is grouped with the other homo-YTX producing isolates. The mechanism of the production of YTX and its derivatives is of interest due to the distinct separation between the homo-YTX and the YTX producers (p-distance of 0.211 between the Benguela *G. spinifera* and Adriatic Sea isolate, EF16284). This divergence is recent and it would be of interest to determine the controls governing the production of other YTX derivatives as they contribute to the total toxicity of the cell. As there is a distinct separation between the toxic and non-toxic *G. spinifera*, the ITS region should be assessed to determine if this area could provide any information regarding YTX production or lack thereof in the various strains. The Gonyaulacales group formed a clade in both the SSU and LSU phylogenetic trees. This was consistent with a previous study (Howard et al., 2009). Though YTX production is distributed within three different genera, all these species fell under the Gonyaulacales order and were widely distributed throughout the trees. Thus the occurrence of YTX producers in different distinct genera supports the hypothesis that YTX biosynthetic capacity arose early in the divergence of this order and consequently later in the evolutionary history of the Dinophyceae. This is the largest phylogenetic assessment of *G. spinifera* sequences to date. There is a high level of intrespecific variability in *G. spinifera*. This was also observed by Howard et al. (2009) and Riccardi et al. (2009), however their analysis was conducted using a smaller data set. This and the emergence of new biogenic YTX derivatives suggest that *G. spinifera* is undergoing rapid diversification.

This diversification and probable biogenic origin of 45-OH-YTX gives rise to questions on how these toxins are produced as well as which controls dictate the production of YTX and its derivatives. YTX and its derivatives are polyketide compounds. These molecules are synthesised by a wide range of organisms such as bacteria, fungi, lichens and higher plants and vary in their molecular structure (Rein and Snyder, 2006). Their production is encoded for in polyketide synthetase (PKS) genes, but little is known about the polyketide biosynthesis in dinoflagellates (Kellmann et al., 2010; Eichholz et al., 2012). This is due to dinoflagellates large genome that lacks normal chromosomal organisation and experimental problems such as the difficulty in maintain axenic cultures (Kellmann et al., 2010). Since these genes would control the production and type of YTX produced, it would be of significance to categorise them for the *Gonyaulax* genus and use this information to understand the evolution and mechanism of YTX production.

The fact that the YTX producing *G. spinifera* aggregate into a clade suggests that there is a distinct ribotypes for the toxic species. This information can be used to develop probes specific to this grouping allowing testing on field samples to conclusively identified toxic *G. spinifera*. This would be of great use, as toxic and non-toxic *G. spinifera* cannot be distinguished from each other or the other species in the complex using light microscopy. Consequently for the Namibian Shellfish Sanitation program a threshold limit can be put in place for *G. spinifera* as a means of warning for when shellfish are most likely to be toxic, thus unsuitable for human consumption. If *G. spinifera* cells are identified incorrectly or a non-toxic strain is identified, this would limit the effectiveness of having a cell threshold limit. As the toxic *G. spinifera* is a recent addition to the Benguela region, there is currently no knowledge on the probability of the occurrence of non-toxic species or the ratio of the various species included in the *G. spinifera* 'complex'. In the areas in the world where phytoplankton threshold limits are used for phycotoxic species, the growing area is closed until further testing can be conducted on the shellfish if the cell counts limit is exceeded. If this procedure was followed in the Benguela system, the toxicity inferred and the actions taken could be incorrect and lead to unnecessary growing area closures and additional toxin testing. A method called whole cell hybridization could solve this potential problem. Initially, a probe that targets a specific genetic sequences/region shared by the toxin producers will need to be developed. This probe could then be coupled to a fluorescent marker thus a specifically targeted organism can be identified using Fluorescence in situ Hybridisation (FISH) (Amann, 1995). This method would be a faster means with which to detect toxic species or strains and could be used on field samples. It has also been successfully applied for harmful algae field samples (Scholin et al., 1997; John et al., 2003; Anderson et al., 2005). In one instance it was used with the Alexadrium tamarense 'complex' (John et al., 2005). The rRNA probes were able to distinguish between toxin and non-toxic species in field samples as they can co-occurre. This could be a viable option in an area such as Namibia as the rapid identification and enumeration of HABs species is needed in order for the information generated to be used effectively.

Thus, *G. spinifera* should thus be put on the list of toxin producing HABs species for the phytoplankton monitoring aspects in the Namibian Shellfish Sanitation program. Actions following its identification should be clarified and followed effectively.

Chapter 5

The effects of macronutrients on toxin production in *Gonyaulax spinifera* cultures from the Benguela upwelling system

5.1 Introduction

Macronutrients (i.e. phosphate and nitrate) along with light, salinity and temperature are the primary factors that influence phytoplankton growth (Kudela et al., 2010). These factors not only affect cell growth in species that produce phycotoxins, but also influence the toxin production. Some shellfish monitoring programs have threshold limits for these phycotoxin producing species as a mitigating factor/ early warning. This allows for additional consumer protection by addressing the possible danger in consuming potentially contaminated shellfish in a given area before testing is conducted. As these threshold limits are based on total cellular toxicity it is of importance to determine which factors would affect the cellular toxin content. Thus understanding the factors that could potentially influence toxin production is of importance. Of the abiotic factors, macronutrients of primary importance for Walvis Bay as this area does not experience fluctuating salinity (no river runoff as it lies in the Namib desert) and *G. spinifera* blooms are most likely to occur in the summer/early autumn months when the light and temperature are relatively constant. Macronutrient data from this area show that phosphate and nitrate concentrations can fluctuate quite extensively from 0.5-6.5 and 0.2-12 μM, respectively. Consequently this wide macronutrient variation could potentially affect the overall cellular toxicity of *G. spinifera* cells and in turn affect the toxicity of shellfish feeding on these cells. The nature of this influence needs to be clarified to support the effectiveness of any proposed threshold ranges for the Benguela *G. spinifera*.

Macronutrient studies have shown definitive influences on the phycotoxin production in various algal species (Kudela et al., 2010). Granéli and Flynn (2006) stated ‘‘it seems more the rule that limitation by either Nitrate (NO_3^-) or phosphate (PO_4^{3-}) increases toxin content” for ichthyotoxic harmful algae. This trend can also be seen across multiple algal groups (diatoms, dinoflagellates, raphidophytes, cyanobacteria) and thus suggests that this is an adaptive strategy by HAB organisms (Kudela, 2010). In order to asses this most experiments generally deal with the effects

of macronutrient limitations as well as some of the other abiotic factors mentioned above. With respect to the hydrophilic phycotoxins, studies with *Alexandrium* species have shown that a deficiency in inorganic phosphate and an increase in inorganic nitrogen enhance the PST cell content (Boyer et al., 1985, 1987; Matsuda et al., 1996; Bechemin et al., 1999; Guisande et al., 2002; Anderson et al., 2009). Experiments with low salinity also showed an increase in the PST content (Hwang and Lu, 2000). This tendency is not only seen for dinoflagellates. Studies have shown that a depletion of phosphate and silicate also increase DA production in *Pseudos-nitchia* (Bates et al., 1988, Pan et al., 1996 a, b). Additionally, *Prorocentrum lima* and *Dinophysis acuminata* were shown to release more toxins under both NO_3^- and phosphate limited conditions (McLachlan et al., 1994; Sohet et al., 1995; Johansson and Graneli, 1996). Of the three known YTX producing dinoflagellate species, macronutrient effects on YTX production have only been studied with *P. reticulatum* and *L. polyedrum (*Roeder et al., 2012*;* Pistocchi et al., 2012; Gallardo Rodriguez et al., 2009; Guerrini et al., 2007; Paz et al., 2004; Mitrovic et al., 2004; Seamer, 2000). *P. reticulatum* is the most extensively studied due to its wide geographical distribution. Seamer (2000) studied the effects of nutrients, light, salinity and temperature on a New Zealand strain. Mitrovic et al. (2004) studied the effects of selenium, iron and cobalt on growth and YTX production on a New Zealand strain. Paz et al. (2006) looked at the effects of temperature, irradiance and salinity on YTX production in a Spanish strain. Even though all these studies were conducted on *P. reticulatum*, there were still some contradictions. The New Zealand strain had much lower optimal light intensity than the Spanish strain. Additionally the response to salinity and temperature was vastly variable amongst the different strains. This revealed the wide variation amongst *P. reticulatum* strains. Guerrini et al. (2007) found that NO_3^- and PO_4^{3-} limitations affected *P. reticulatum* growth rate, with the PO_4^{3-} limitation having the strongest effect by decreasing the growth rate. YTX production was also enhanced under these conditions, while an increase in nitrogen in turn increased the YTX concentration per cell. However, no clear influence could be discerned from the varying N:P ratio on the YTX production. Results showed that nitrate affected YTX production in *P. reticulatum.* Gallardo Rodriguez et al. (2009) reported that PO_4^{3-} had no influence on the YTX content per cell. Roeder et al. (2012) observed similar results to Guerrini et al. (2007) with respect to the increase in YTX production in phosphorus-reduced treatment. It was additionally observed that growth and toxin production was the lowest in the nitrogen-reduced treatment. Pistocchi et al. (2012) conducted

nutrient experiments with *L. polyedrum* that produced low levels of homo-YTX (0.013 pg homo-YTX cell^{-1}). The same pattern was observed with low PO_4^{3-} concentrations increasing homo-YTX production in the cells. There is a variation in the effects of macronutrients on YTX production in *P. reticulatum* and *L. polyedrum*. Thus it would be of relevance to determine which trend *G. spinifera* follows. This would include determining the nature and extent of these effects, as this has not been done for *G. spinifera* to date. Since the *P. reticulatum* and *L. polyedrum* used in the above mentioned studies only produced YTX, there is very little knowledge on the effects of macronutrients on other YTX derivatives. This is of importance as these derivatives are components that add to the final cellular toxicity. The resulting information can also be used to bolster the trigger levels proposed for *G. spinifera*.

5.2 Results

5.2.1 *G. spinifera* cell growth

All three strains depicted the same tendency of higher cell abundance under both of the nutrient reduced conditions (figure 35). Of these, the strain with significant differences in cell abundance under 1/10 PO_4^{3-} and 1/10 NO_3^- media were NamWB012 and NamWB002. Of these two, NamWB012 had the lowest nitrate concentration in the 1/10 NO_3^- media. The greatest cell abundance was observed with NamWB012 with cells reaching 441 cells mL^{-1} under the 1/10 PO_4^{3-} media. All three control cultures had lower cell abundances in comparison to the 1/10 PO_4^{3-} and 1/10 NO_3^- media.

5.2.2 YTX production

All three strains produced the highest concentrations of YTX per cell under the 1/10 PO_4^{3-} media (figure 36). NamWB011 and NamWB012 had the highest concentrations with total cellular YTX of 35.8 and 31.0 pg YTX eq. cell^{-1}, respectively (table 13). The 1/10 PO_4^{3-} media was statistically significant at $p<0.1$. For NamWB 002 and NamWB 012, there was no statistically significant difference in the total YTX produced in the control and 1/10 NO_3^- media (figure 36). The control cultures of NamWB 002 and NamWB011 had varying YTX profiles compared to the initial toxin profile (table 12, chapter 4) as well as the ones under the nutrient reduced conditions.

NamWB012 was the only culture with YTX profile percentages similar to those observed when the profile of NamWB012 was established. All three cultures had significantly higher homo-YTX production per cell in the 1/10 PO_4^{3-} media (figure 38). NamWB 012 and NamWB 011 produced the highest concentrations of 27.4 and 28.9 pg homo-YTX $cell^{-1}$, respectively. NamWB011 was the only strain of the three that produced a significantly higher amount of YTX in the 1/10 PO_4^{3-} media (figure 37). Control cultures of NamWB002 and NamWB012 had significantly higher production of 45-OH-YTX (figure 39). With respect to the N:P ratio, the NamWB012 1/10 PO_4^{3-} media had the highest N:P ratio (table 9), but no trend was observed. Thus the general trend seen in the cultures was 1/10 PO_4^{3-} media lead to high YTX production, while 1/10 NO_3^- media had high cell abundance with lower total YTX eq cellular content.

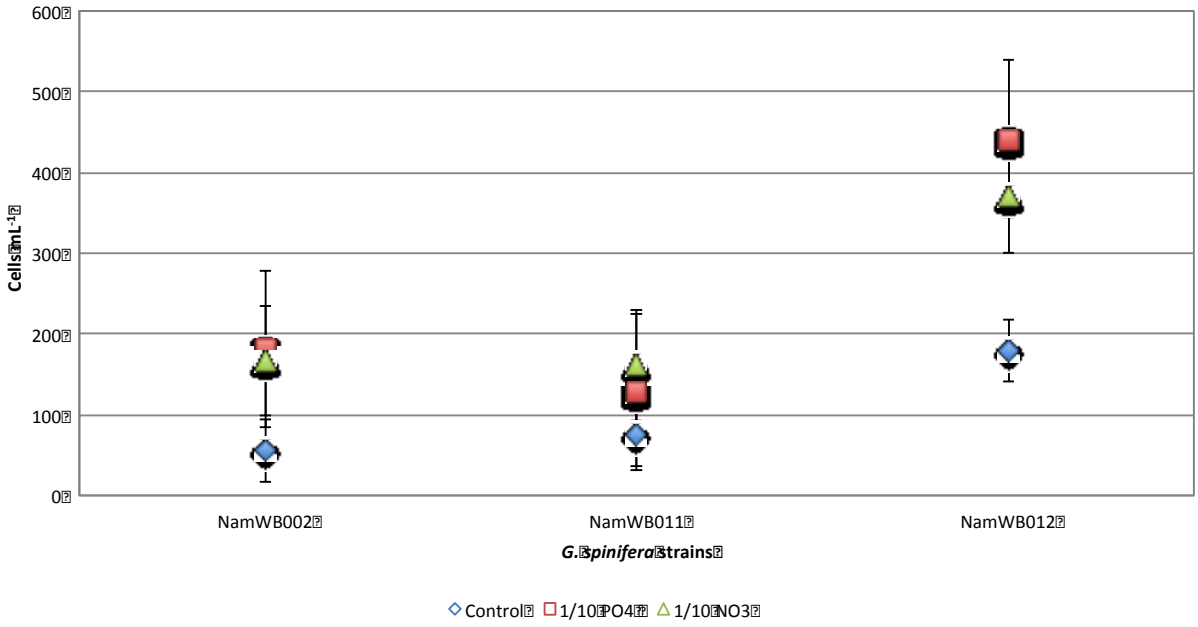


Figure 35: Cell abundance of *G. spinifera* strains NamWB002, 011 and 012 under various nutrient conditions.

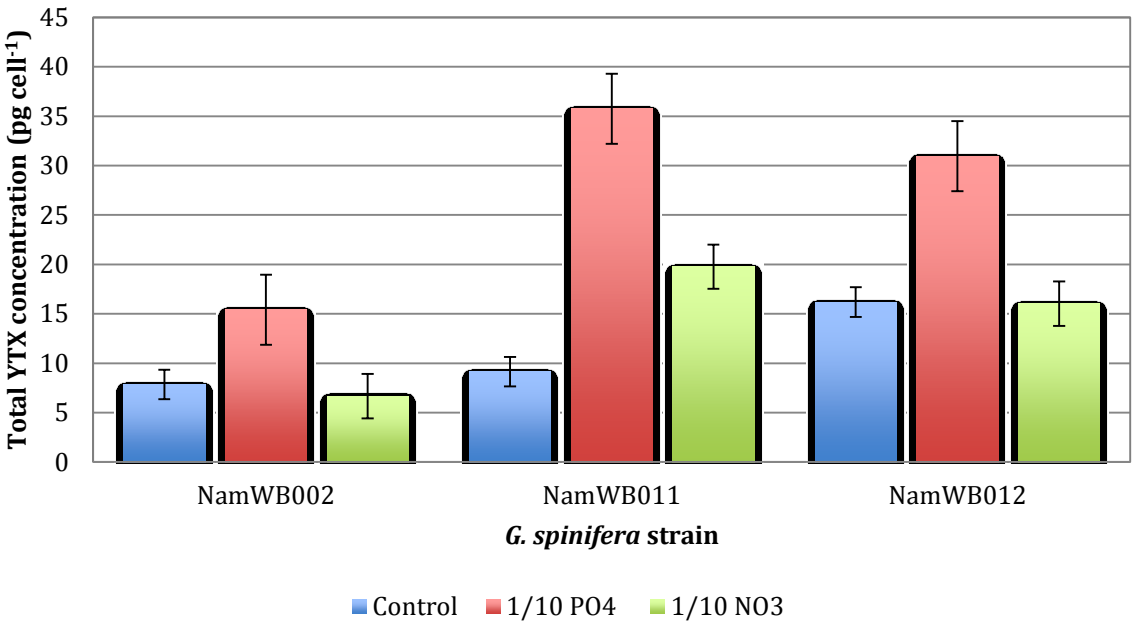


Figure 36: Concentration of total cellular YTX of *G. spinifera* strains NamWB002, 011 and 012 under various nutrient conditions. F (2; 15)=2.752, thus the treatment of 1/10 PO_4^{3-} is statistically significant at $p<0.1$.

Table 13: Variation in percentage of the YTX and its derivatives under the various nutrient limitations in *G. spinifera* strains NamWB002, 011 and 012.

	% of total YTX content			
	YTX	**homo-YTX**	**45-OH-YTX**	**Total YTX eq (pg YTX eq. cell^{-1})**
***G. spinifera* Strain NamWB002**				
Control	17.16	35.74	15.81	7.84
1/10 PO_4^{3-}	12.79	76.83	3.43	15.45
1/10 NO_3^-	12.67	79.02	2.42	6.68
***G. spinifera* Strain NamWB011**				
Control	17.51	57.08	8.67	9.15
1/10 PO_4^{3-}	13.46	80.84	1.74	35.75
1/10 NO_3^-	14.38	78.19	2.46	19.77
***G. spinifera* Strain NamWB012**				
Control	6.08	86.73	1.85	16.18
1/10 PO_4^{3-}	5.83	88.64	1.68	30.95
1/10 NO_3^-	6.10	86.01	2.22	16.03

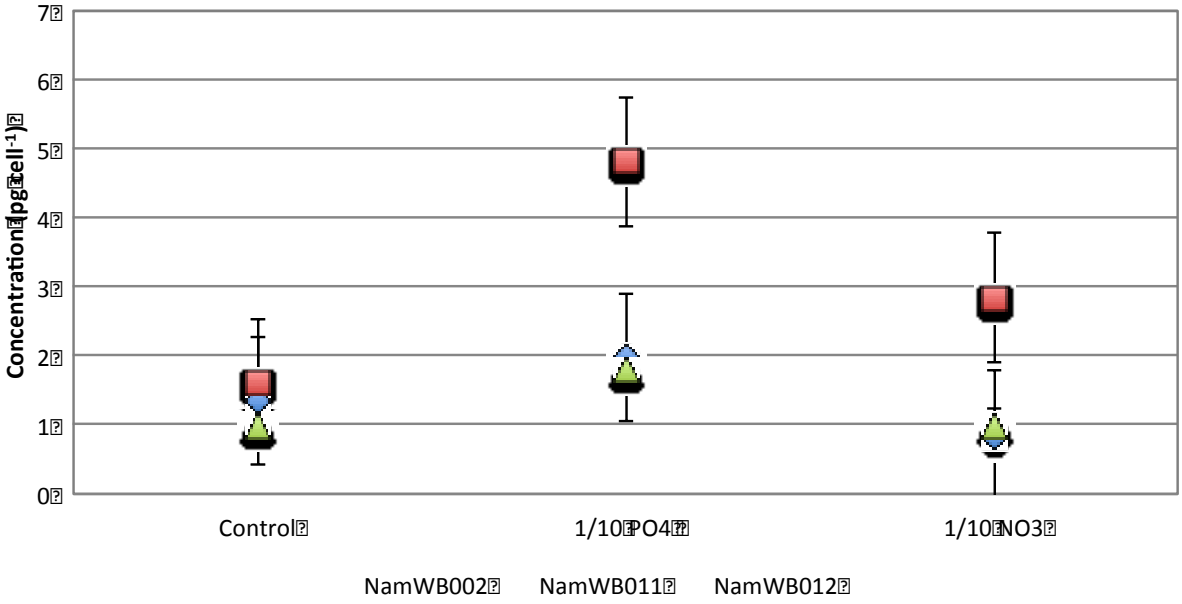


Figure 37: Cellular production of YTX in NamWB002, 011 and 012 under various nutrient conditions.

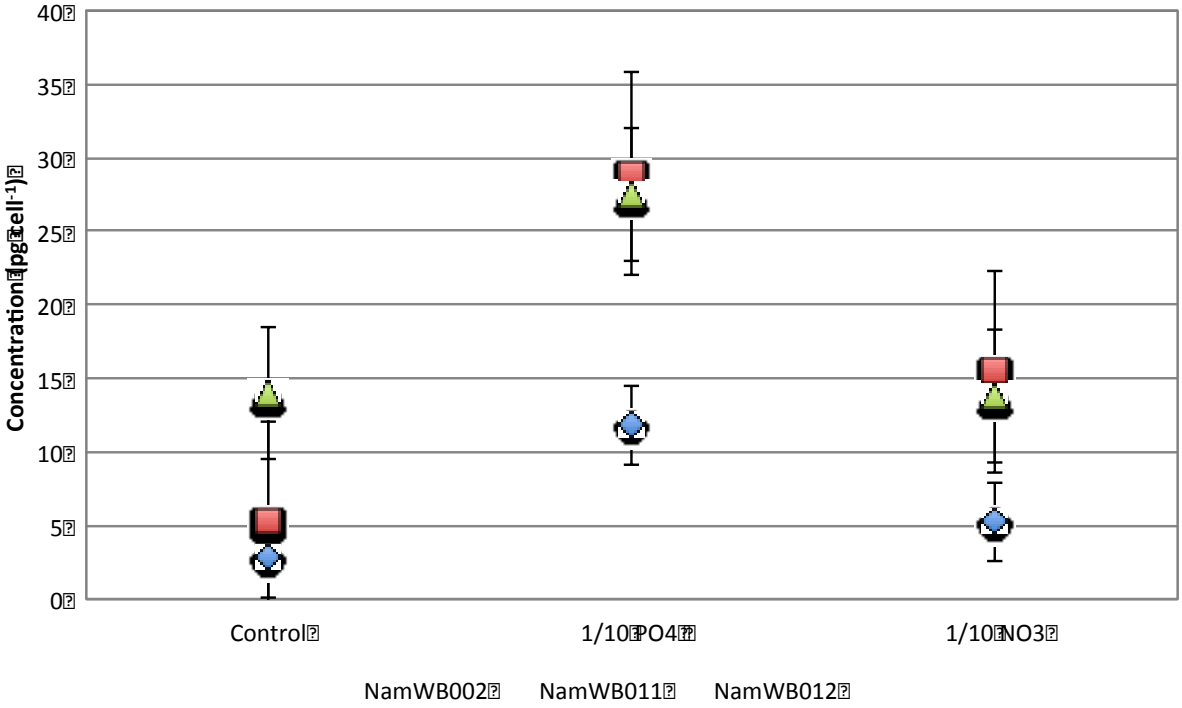


Figure 38: Cellular production of homo-YTX in NamWB002, 011 and 012 under various nutrient conditions.

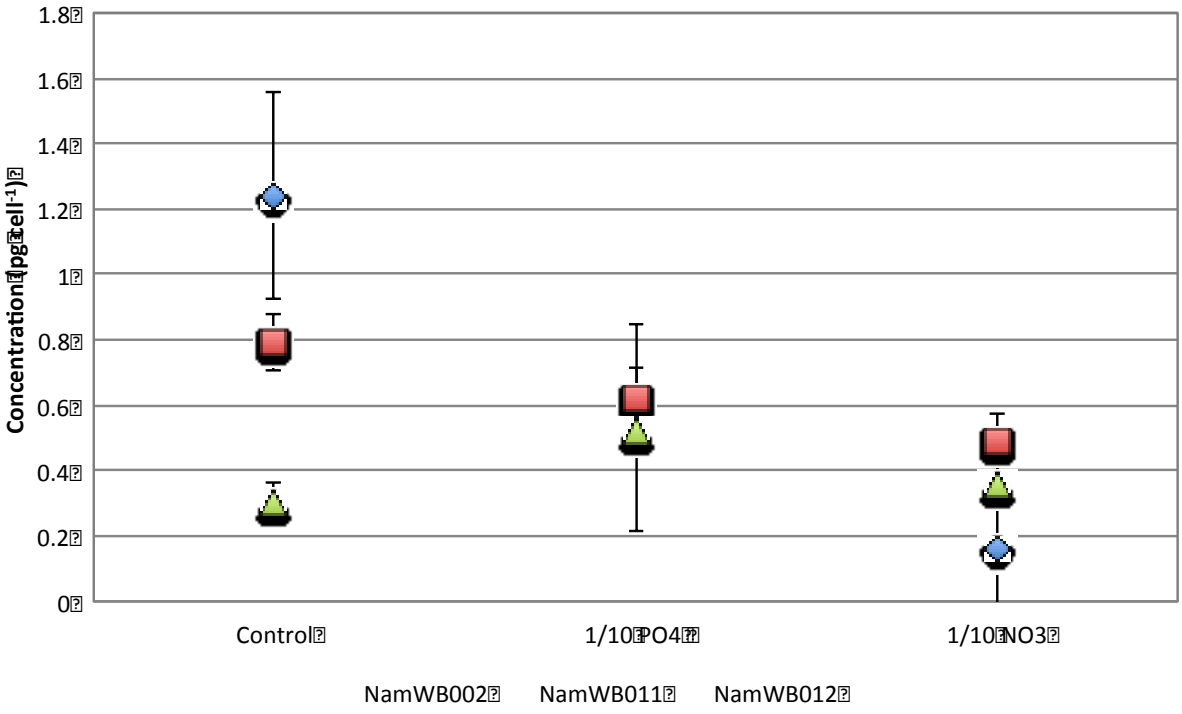


Figure 39: Cellular production of 45-OH-YTX in NamWB002, 011 and 012 under various nutrient conditions.

5.3 Discussion

Globally only two cases of YTX production in *G. spinifera* have been documented even though this species has a wide distribution (Rhodes et al., 2006; Riccardi et al., 2009). Since this species was only recently shown to be a YTX producer (Rhodes et al., 2006), it has not been as extensively studied as the other YTX producers, namely *P. reticulatum* and *L. polyedrum*. These strains were isolated from Walvis Bay, which lies in the Benguela Upwelling system. Thus it is an area that has known macronutrient fluctuations. Thus assessing the influence of macronutrient concentration fluctuations have on *G. spinifera* growth and toxin production can ultimately lead to a better understanding of the potential threat of toxic *G. spinifera*. This is of importance as a factors increasing cellular toxin production could in turn lead to shellfish becoming toxic after feeding on these cells. This toxicity could lead to concentrations above the regulatory limit in shellfish, hindering sale and consumption of shellfish. The macronutrient experiments were conducted on laboratory cultures. This limits the level of inference that can be made with respect to natural populations, but general trends observed can still be of use.

Both 1/10 PO_4^{3-} and 1/10 NO_3^- media affected all three Benguela *G. spinifera* isolates with respect to their growth and toxin production to varying degrees. YTX production increased in 1/10 PO_4^{3-} media for all three cultures, with the highest production in NamWB011 and 012 (approximately 31-36 pg YTX eq. $cell^{-1}$). This is consistent with other nutrient experiments conducted on *P. reticulatum* (Guerrini et al., 2007; Roeder et al., 2012). With these cultures producing approximately 24.0 pg YTX $cell^{-1}$ in both cases. The low-phosphate to high YTX production trend was contradicted by Guerrini et al. (2007) and Gallardo Rodriguez et al. (2009). They reported that the total YTX per cell volume was higher in the control than in the PO_4^{3-} and nitrate limited media. They attributed this to the consequence of the highest cell concentrations reached in the control cultures. Though, in the case of Gallardo Rodriguez et al. (2009) this difference was probably due to the fact that the lowest concentrations used during the PO_4^{3-} limitation experiments were quite high (36 µM) in comparison the other experiments (0.75-3.6 µM). This trend is consistent with other dinoflagellate species in the *Alexandrium* genus, where PO_4^{3-} limitation also increased the total toxin produced (Boyer et al., 1987; Anderson et al., 1990; Bechemin et al., 1999). This trend was also observed in diatoms from the genus *Pseudo-nitzschia* with DA production increasing with PO_4^{3-} limitations (Bates et al., 1988, Pan et al., 1996 a, b). Homo-YTX followed the same trend of having the highest production in 1/10 PO_4^{3-} media. This was expected, as it was the dominant toxin produced thus accounting for the largest component of the total YTX cellular content. This is consistent with Pistocchi et al. (2012) comments for homo-YTX producing *L. polyedrum*.

There was no effect on the 1/10 NO_3^- media on the YTX eq. production in two of the three strains (NamWB002 and 012). While NamWB011 had an increase in YTX production compared to the control. This is in contradiction to the *P. reticulaltum* nutrient experiments conducted by Roeder (2012) where the 1/10 NO_3^- media had a lower YTX production than the control media. The nitrate concentration used in their experiment was 88.2 µM, which is more than twice the concentration that was used in this study. These discrepancies as well as the fact that the Benguela *G. spinifera* cultures had higher growth in the 1/10 PO_4^{3-} and 1/10 NO_3^- than the control are of interest, as it is in contradiction to the other studies (Guerrini et al., 2007; Roeder et al., 2012). This is also contradictory to what is generally accepted for most dinoflagellate species as they are generally seen as having a low affinity for NO_3^- in comparison to diatoms (Smayda, 1997; 2000). Affinity in this context is defined as their ability to sequester

nutrients at low ambient concentrations. Though upwelling HABs do not seem to follow this pattern and have a high affinity for NO_3^- (Kudela et al., 2010). This suggests that they are well adapted for low or pulsed nitrate supplies as an adaptive strategy to their environment. Thus, the Benguela *G. spinifera* might be so adapted to the extent that it is able to grow readily in 1/10 NO_3^- media with a surplus of phosphate. Though this pattern for upwelling dinoflagellates is based on a small data set, it would be of interest to asses this pattern with the *G. spinifera* cultures. This can be assessed by conducting experiments with a series of nitrate and phosphate concentrations that mimic environmental concentrations in order to determine which nutrient concentrations give rise to the best growth rates in the isolates. Since nitrate and phosphate are not incorporated in the YTX molecule, the effects of macronutrient reduction most probably affect the Polyketide synthetase enzymes responsible for or involved in the YTX synthesis. This synthesis is controlled by Polyketide synthetase (PKS) genes. Unfortunately there is still no knowledge on why polyether toxins are produced by numerous dinoflagellate species so this response to macronutrients cannot be assigned to the specific function of the toxin in the cell. Though this can be used to identify the PKS genes involved in YTX production as varying the phosphate concentration can control the expression of these genes to some extent.

This data thus suggests that *G. spinifera* toxicity increases under low PO_4^{3-} conditions. The ramifications of this in the field is of importance as this could lead to low cells number of *G. spinifera* producing higher YTX concentrations in PO_4^{3-} reduced surface water. Thus since the cells produce more YTX eq. low cell numbers could potentially lead to shellfish toxicity values over the regulatory limit. Or, the competition for macronutrients with other species within a bloom could lead to a decrease in the surface water phosphate that could cause an increase in the cellular YTX production. This situation is similar to what occurred in the *G. spinifera* bloom in 2011. This bloom included a mixture of dinoflagellate species. The highest toxin production coincided with the period in which the PO_4^{3-} levels in the surface water were at their lowest (to <1.0 μM). This also coincided with the period when the *G. spinifera* cell numbers were at their highest, thus it would be impossible to determine which factor had the greatest effect. PO_4^{3-} levels should still be monitored when *G. spinifera* cells are detected as a precautionary measure. The effects of reduced PO_4^{3-} should be factored in when assessing the effectiveness of the estimated trigger levels for *G. spinifera* in a given area.

Chapter 6

Overall summary and conclusion

Development of an effective early warning system for the Namibian shellfish farmers

The culmination of the data generated in this thesis can be used for the initial construction of a practical and effective early warning system for shellfish farmers in the Walvis Bay region. The viability of these suggestions and their relation to my thesis objectives are discussed below.

1) Resolving the discrepancy between the DSP positive farm closures and lack of DST producers in the water column.

During the 20-month sampling period (October 2010-May 2012) of this study, the only phycotoxins detected were YTX and its analogues from *G. spinifera*. This was unexpected as DST have been the only phycotoxin consistently detected in shellfish tested for the Namibian Shellfish Sanitation program in the Walvis Bay region. As one of the positive DSP results coincided with the *G. spinifera* bloom in March 2011, this instance highlights one of the greatest problems with using the mouse bioassay for DST detection in this area and in general. The results from this indiscriminate test were the basis of a week-long closure of two shellfish farms in the Walvis Bay area. The lack of DST in the oyster samples can be inferred as the mussel samples taken from the same growing area were also tested for DST. Trace amounts of OA were detected (<0.02 mg kg^{-1}) which suggests that negligible amounts would be found in oysters. This is due to oysters lower filter-feeding rate in comparison to mussels leading to reduced accumulation of phycotoxins. There were at least four additional tests conducted on oyster samples after the *G. spinifera* bloom, because the Namibian Shellfish Sanitation monitoring program requires two consecutive negative results in order for a shellfish farms growing area to be opened again. These costs for retesting were unavoidable even though the initial positive result was due to YTX. As the ability of shellfish farms to sell their product is based on these results there has to be a more rigorous coupling of both the phytoplankton and phycotoxin data. This will aid in determining the possible origin and identification of the phycotoxin being tested

for until LC-MS/MS method for lipophilic phycotoxins becomes a routine method at the end of 2014. As the phytoplankton enumeration and phycotoxin testing and regulation of closures are controlled by a government agency (MFMR) and a parastatal (the Namibian Standards Institute), formal agreements and coordination needs to be clarified.

Mussels are often used as sentinel species in biotoxins monitoring as they accumulate phycotoxins much more readily than oysters. They can thus be used as a warning for potential oyster toxicity. The mussel samples tested for DSTs had trace amounts of OA (<0.02 mg kg^{-1}). This suggests that in this instance the fortnightly sampling period for phytoplankton identification and enumeration is effective. Though this still has to be further investigated to ensure that potentially harmful phycotoxic events are not missed during the non-sampling periods. Using Solid Phase Adsorption Toxin Tracking (SPATT) bags over a defined period can do this, by determining if the phycotoxins found in SPATT bags are consistent with the phytoplankton species diversity samples collected during the fortnightly sampling period, since its possible to identify spatial and temporal variability of phycotoxins.

2) **Looking at the natural algal bloom dynamics and species succession as well as during and after H_2S eruptions. Possibly determine the physical and chemical factors that lead to bloom formation in this area.**

During the sampling period, there was a H_2S incident in the lagoon area adjacent to the sampling site. The *G. spinifera* bloom collapsed aiding in the maintenance of anoxic conditions and the spread of H_2S. During the course of the next day strong winds aided in the mixing of the water column in Aquapark 1 dissipating the H_2S. Thus this brief H_2S episode was not assessed for species successions. From the environmental data, the *G. spinifera* bloom occurred during the late summer period. This coincided with a warm and well-stratified water column, which establishes the desired conditions for dinoflagellates bloom formation, as they are the dominant phytoplankton species in the water column during summer/autumn. As there are more dinoflagellate species producing phycotoxin producers than diatoms, this period of time is of great importance. Before the bloom the NO_3^- vales were very low (<1.0 μM) and this continued throughout the bloom period. The variation in phosphate during this period suggests that it is the limiting factor as the *G. spinifera* bloom collapsed once phosphate levels had decreased to <1.0

µM. Thus the *G. spinifera* from the Benguela region is best adapted to grow in low turbulent, warm (20°C) PO_4^{3-} rich and NO_3^- poor water. In field samples from 2011, *G. spinifera* cells reached up to 6.7 x 10^5 cells L^{-1}. As the subsequent *G. spinifera* cultures had very low growth rates, it is not possible to infer the time needed for the *G. spinifera* to reach the cell concentrations observed in the field in 2011. Thus temperature and PO_4^{3-} concentrations should be monitored in conjunction with the phytoplankton species identification during the late summer/early autumn period. Since the cellular YTX toxicity in *G. spinifera* cultures increase under low PO_4^{3-} conditions, this would be an additional motivation to monitor the PO_4^{3-} levels. This could lead to cell numbers with increased toxin production that leads to shellfish toxicity above regulatory limits, and the cell numbers need not reach bloom concentrations.

3) Determining the HAB cell concentration trigger level that could lead to potential toxicity of shellfish in field and cultured samples.

As the *G. spinifera* in the Benguela region are highly toxic it is necessary to determine the cell trigger levels that will lead to toxic shellfish exceeding the regulatory limit. Calculating cell trigger levels based on the *G. spinifera* cultures in this thesis turned out to be problematic as there is a large variation in toxin production even within cells isolated from the same area. However, the cell levels measured during the 2011 bloom can be used for cautionary purposes as the correlation of toxic accumulation was based on mussel samples, which accumulate toxins much more readily than oysters. The mussel samples during this bloom period exceeded the regulatory limit in some cases by a factor of 5. Thus the 6.7 x 10^5 cells L^{-1} of *G. spinifera* can be used as the upper limit for a conservative trigger level range. The YTX LC-MS/MS method used will need to be clarified for the Shellfish Sanitation program in Namibia in order to ensure that farm closures occur for the correct toxins as there are varying regulatory limits for each phycotoxin group. Though not to be ignored is the possibility that the regulatory method used for the YTX toxin content is not suitable for the Benguela region. This was discovered when a duplicate sample extract of NamWB012 was analysed by the Institute for Marine Biosciences in the National Research Council Canada. This neutral chromatographic method used gave a higher estimation of the 45-OH-YTX. The resultant toxin profile had homo-YTX (62%) as the dominant toxin, followed by 45-OH-YTX (34%) and YTX (4%). Therefore, the YTX alkaline chromatographic method used in this study gave a sizeable underestimation of the total

concentration of 45-OH-YTX (2%). This would significantly affect the final YTX eq result as 45-OH-YTX has a TEF of 1 in comparison to YTX. The alkaline chromatographic method was used, as it is the current reference method laid down by the EU for the detection of lipophilic phycotoxins using LC-MS/MS (EC regulation 15/2011). It was based on Gerssen et al. (2009) method as it allowed for the separation and detection of the major lipophilic phycotoxins in one run. The alkaline chromatographic conditions were of better use with respect to YTX and its derivatives as the peak shape improved (i.e. narrower peaks) allowing for a limit of detection three times lower than the acidic method. The method was validated in the EU with interlaboratory collaborations. Unfortunately only one sample was analysed for YTX and had reproducibility RSD of 35%. Garcia-Altares et al. (2013) also found the analysis of YTX challenging even under alkaline conditions with overestimations of YTX concentrations in validation samples. This discrepancy in result will have to be further investigated with extensive collaborative studies with shellfish and phytoplankton samples from the Benguela region, as the current regulatory method for YTX is not suitable.

As the YTX measured in shellfish in 2011 was of mussel samples, it is necessary for the Shellfish Sanitation program to determine the accumulation and depuration rates of YTX and its derivatives in the oyster species that are cultured in Namibia as the feeding rates differ with mussels and oysters. The correlation between the 45-OH-YTX in the mussel and phytoplankton samples suggests that this metabolic pathway also needs to be re-examined/clarified using an YTX producing species with a more complex YTX profile. The experiment should determine the accumulation, metabolism and depuration rates of YTXs in shellfish species that are important to the mariculture industry in the Benguela region as the *G. spinifera* in this region produces not only YTX, but two other derivatives. This will also clarify the metabolic pathway of YTX and its analogues in different shellfish species and determine if the analysis method used is effective should new metabolites be discovered. TEF will have to be quantified for these new metabolites, as 45-OH-YTX is a major component of the *G. spinifera* YTX profile.

Additional options

Examining satellite images of the Walvis Bay area to compare blooms with the phytoplankton biomasses of the collected samples to determine if any of the chlorophyll maxima coincide with elevated abundance of the different species.

Shellfish farmers in Namibia acquire access to satellite imagery (chlorophyll and sea surface temperature) from the Mariculture section in the Aquaculture Directorate of the Ministry of Fisheries and Marine Resources (MFMR). They in turn obtain these images daily from the Marine Remote Sensing Unit (MRSU). This unit uses resources from the University of Cape Town (UCT), the Council for Scientific and Industrial Research (CSIR) and Marine and Coastal Management (MCM). Their focus is on the sub-Saharan Africa marine environment and Medium Resolution Imaging Spectrometer (MERIS) data is used exclusively. The chlorophyll measurements are calculated using two algorithms depending on the area being looked at (i.e. clear waters verses coastal waters). As cloud cover tends to be the greatest problem with using satellite imagery in the Benguela region, on numerous occasions no chlorophyll data can be derived from the images due to cloud cover. This was the situation during the *G. spinifera* bloom in 2011 with cloud cover over the region from the 13^{th}-18^{th} March 2011 (figure 40). What has recently been tried in numerous publications is to combine all available images from sensors over time and/or space. Unfortunately this approach degrades the spatial and temporal resolution of the data proportionally to the number of images needed to remove the clouds. This leads to filtering small-scale variability in an uncontrolled manner, and the Walvis Bay is a small area, the needed spatial resolution is lost. Thus this would not be a reliable means with which to assess the potential threat of an algal bloom and therefore cannot be put forward as an adequate tool to monitor HABs in this region. It would be of better use to monitor the nutrient concentrations closely and increase the frequency of phytoplankton monitoring during the late summer/early autumn periods, as this is when blooms are more likely to occur. This information can then be used to predict which species would proliferate the best under those conditions if known phycotoxin producers are present in the water column.

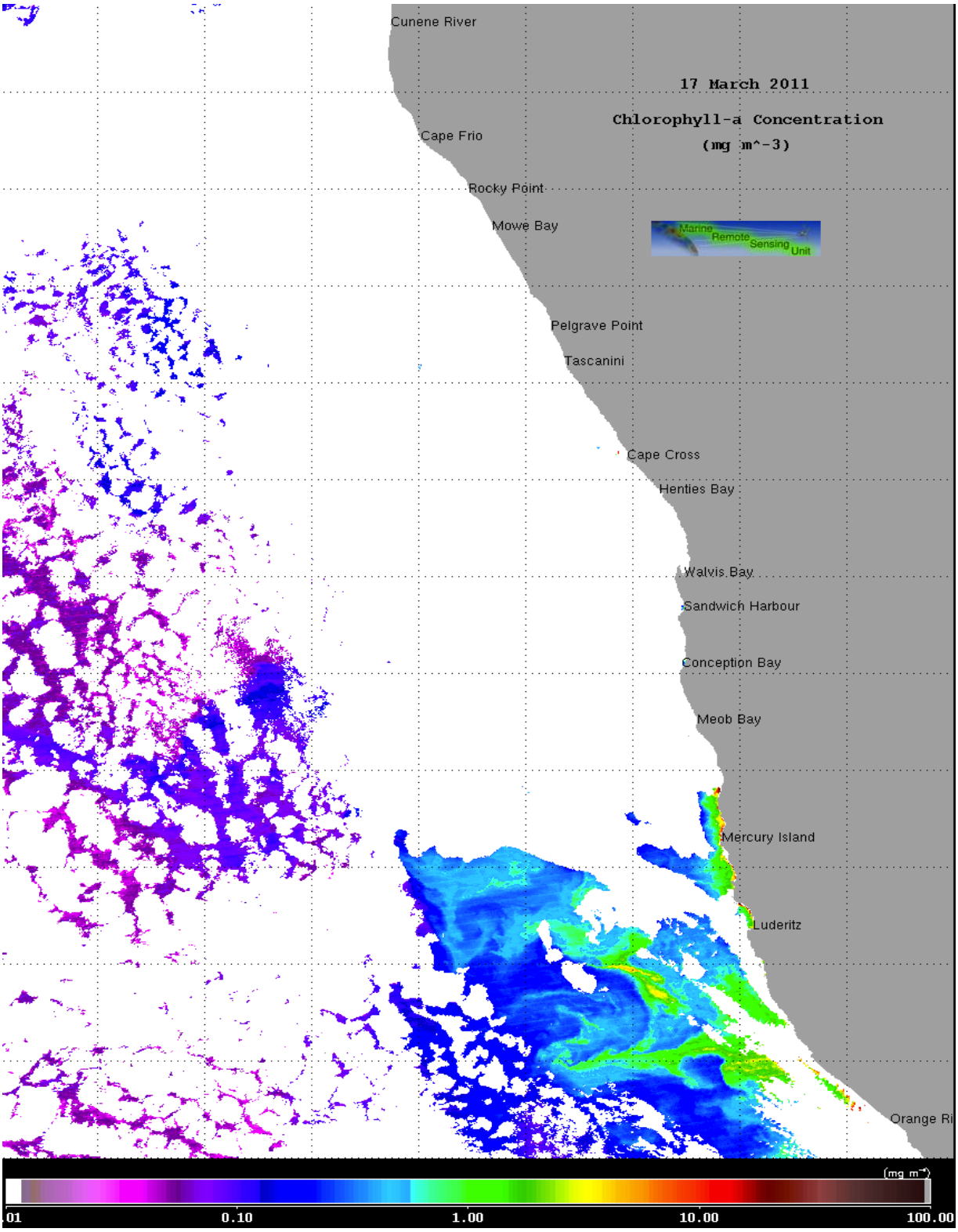


Figure 40: Sateliet image of chlorophyll (mg m^{-3}) over the northern Benguela region during the *G. spinifera* bloom on 17th March 2011 (MERIS).

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