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IDENTIFICATION OF HARMFUL ALGAL BLOOMS (HABs) TOXINS IN SEAWATER AND SHELLFISH ALONG THE COAST OF GHANA

This dissertation is submitted to the:

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In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

NUCLEAR AND RADIOCHEMISTRY

JULY 2019

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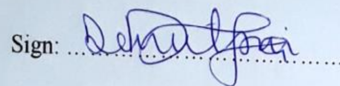
DECLARATION

This dissertation is the result of research work undertaken by DZIFA DENUTSUI in the Department of Nuclear Sciences and Applications, Graduate School of Nuclear and Allied Sciences, University of Ghana, under the supervision of Prof. Yaw Serfor-Armah, Dr. Dennis Kpakpo Adotey and Prof. Marina Cabrini.

Sign:  Date: 8/10/2020

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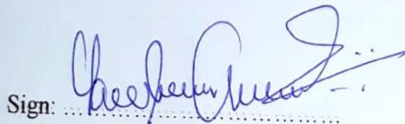
Sign:  Date: 08/10/2020

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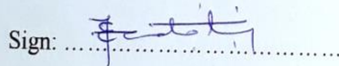
Sign:  Date: 8/10/2020

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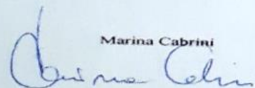

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(Co-Supervisor)

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DEDICATION

This work is dedicated to my loving husband, Mr Francis Appiah-Kubi Banson. Your love and support are immeasurable. With you, my life has been luxuriantly fruitful and full of glorious fortunes and increase. I love you with passion.

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ACKNOWLEDGEMENT

I am grateful to the Almighty God for His Wisdom and Grace on my life, and the successful completion of this research.

I acknowledge the immense contribution and support of my supervisors throughout this work: Prof. Yaw Serfor-Armah, Dr Dennis Kpakpo Adotey and Prof. Marina Cabrini.

I appreciate the role of the Ghana Atomic Energy Commission, in granting me the opportunity to undertake this study through the ICTP/IAEA PhD Sandwich programme.

Special gratitude to the International Atomic Energy Agency for their financial support throughout my study, as well as sponsoring my IAEA-IOC Advance Training Course in Marine Phytoplankton at Copenhagen, Denmark.

I am thankful to the Scientists and Technicians at the National Oceanography and Experimental Geophysics, Trieste, Italy; the Marine Research Foundation, Cesenatico, Italy; and, the Molecular Biology Laboratory, University of Trieste, Italy, for their skilled technical assistance.

Finally, I would like to express my profound gratitude to my dear husband, Mr Francis Banson and my beautiful children (Seyram, Selasi, Steve-Othniel and Sam-Ehud Banson). The critical roles played by my extended family, especially my parents, colleagues at NCERC-NNRI, and Lecturers and Auxiliary Staff of SNAS deserves commendation.

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometry
ALX	Alexandrium spp
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BOD	Biological Oxygen Demand
BTX	Brevetoxin
CFU	Ceratium furca
CHLa	Chlorophyll ‘a’
CI	Cyclic Imine
COD	Chemical Oxygen Demand
COND	Conductivity
CPT	Ceratium Protoperidinium
CTR	Ceratium Tripos
CTX	Ciguatoxin
DA	Domoic Acid
DO	Dissolved Oxygen
DPCT	Dinophysis caudata
DPF	Dinophysis fortii
DSP	Diarrhetic Shellfish Poisoning
DTX	Dinophysis toxins
EDTA	Ethylenediaminetetraacetic acid
GNSP	Gonyaulax spinifera
GPS	Global Positioning System
GTX	Gonyautoxins
LNPD	Lingulodinium polyedrum
LOD	Limit of Detection
LOQ	Limit of Quantification
NO_3^-	Nitrate
NTSC	Noctiluca scintillans

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OA	Okadaic acid
PO_4^{3-}	Phosphate
PRGC	Prorocentrum micans
PRMC	Prorocentrum gracile
PRMX	Prorocentrum mexicanum
PSP	Paralytic Shellfish Poisoning
PTX	Pectenotoxin
QA/QC	Quality Assurance/Quality Control
S/N	Signal-to-Noise Ratio
SAL	Salinity
SCT	Scrisppsiella-trochoidea
SiO_4	Silicate
STX	Saxitoxin
TDS	Total Dissolved Solids
TSS	Total Suspended Solids
UN	United Nations
EU	European Union
YTX	Yessotoxin groups

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ABSTRACT

Algal toxins can accumulate in fish and shellfish in the marine environment and thus cause these essential seafood products to be poisoned. The toxins and the algae containing them are routinely surveyed in Europe, North America, Japan and other developing countries. Interestingly, there is scarce or no information on the occurrence of toxin-causing algae in most African nations, including Ghana, apart from the North African countries and South Africa. The study identified and evaluated the distribution of harmful and potentially toxic phytoplankton causing harmful blooms of algae; the distribution of harmful phytoplankton along the coast of Ghana was evaluated. This was achieved through: **(i)** identification and characterization of harmful and potentially toxic phytoplankton species along the Central Coastline of Ghana; **(ii)** assessment of the distribution of the harmful phytoplankton; **(iii)** evaluation of the influence of the environmental driving factors {(Temperature, DO, Nutrients [PO_4^{3-}, NO_3^-, SiO_4] and Trace Elements [Zn & Fe]} on the distribution of HABs in Ghanaian Waters; **(v)** evaluation of the levels of iron (Fe) and zinc (Zn) in seawater and Hg, Mn, Cu, Zn, Fe and Cd in algae and establish the correlation between the levels of the essential elements and the presence of algae; **(vi)** isolation of the potentially Harmful Algal Bloom(HABs) species and assessment the level of toxicity of successful cultures and; **(vii)** investigation of the toxin effects of HABs in selected shellfish (Oysters, Bloody cockle and Clams) [Profiling of Toxins]. Seawater samples from five beaches (Tema, Accra Lighthouse, Bortianor-Tsokomey, Gomoa Nyanyanor and Ekumfi-Narkwa) were collected monthly between January 2014 to February 2018. Diatoms generally dominated the phytoplankton community; however, twelve (12) harmful species which are mostly thecate dinoflagellates. Five of the identified species (*Lingulodinium polyedra, Gonyaulax*

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spinifera, Dinophysis caudata, Dinophysis fortii, Alexandrium spp) were toxin producers (potentially causing diarrheic and paralytic shellfish poisoning). With the aid of the Ocean Data View and SPSS software, it was observed that temperature, pH, dissolved oxygen, phosphate, nitrate, zinc and Fe were the environmental driving forces influencing the availabilities of the harmful algal species. The densities of species varied seasonally (wet and dry). Critical densities (6200 cells/L) of *Lingulodinium polyedra* were recorded in December 2016 at Gomoa Nyanyanor; indicating its abundance in the five study areas. Preliminary observations from the study revealed the possible presence of *Alexandrium* spp, one of the most toxic genera among the HABs species. Principal Component Analysis (PCA) used to establish possible associations between algae and the physicochemical parameters revealed two distinct clusters corresponding to the sampling seasons (wet and dry seasons). The wet season (upwelling), established proliferation of diverse species whilst in the dry season less diverse species proliferated, but with high densities. Successful isolation and cultures of *Alexandrium sp, Prorocentrum sp, Prorocentrum micans, Levanderina fissa* and *Coolia sp* were achieved. *Prorocentrum micans, Levanderina fissa and Coolia canarensis* were successfully identified to species level by DNA sequencing. Toxin profile of successful cultures of Alexandrium sp, Prorocentrum sp, and Coolia sp, as well as of shellfish (Oysters, Bloody cockle and Clams) were ascertained. Three toxins [two (2) Paralytic Shellfish Poisoning) toxins, i.e. GTX2,3 GTX1,4; and one (1) Diarrheic Shellfish Poisoning toxin (an okadaic acid)] were identified in algae. A strain of Alexandrium spp was found to contain two gonyautoxins (GTX2,3) with 1.875×10^{-6} ng/cell and GTX1,4 with 1.502×10^{-4} ng/cell; while Prorocentrum spp were found to have toxicity of 2.65×10^{-4} ng/cell okadaic acids. Seven (7) bloody cockle samples tested positive

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to lipophilic toxins, Okadaic acids (OA) and Dinophysistoxins (DTX2). The levels of okadaic acids and dinophysistoxins ranges between 16 to 19 µgOA/kg and 3-6 µg OA/kg respectively. Three Bloody Cockle samples tested positive for traces of Paralytic Shellfish Poisoning toxins, Gonyautoxins (GTX2,3) and decarbamoylsaxitoxin (dcSTX). Interestingly, lipophilic toxins were identified in Ghanaian coastal waters. Okadaic acid and Dinophysistoxin (DTX2) quantified in bloody cockles may be attributed to Dinophysis species, although a partial contribution by Prorocentrum species cannot be ignored. The average concentration of dissolved Zn and Fe in studied coastal waters ranged from 0.021 to 0.23 mg/L and 0.05 to 0.204 mg/L, respectively. The highest concentrations were recorded at Gomoa Nyanyanor and Tema, while Accra Light House had the lowest levels. Studied coastal waters with high Zn and Fe concentrations showed corresponding high concentrations of phytoplankton species. The coastal water of Accra Light House with low levels of Zn and Fe showed low counts of phytoplankton. From the results, among the metals analysed for, the concentration of Cd was found to be below the detection limit (Cd<0.006). Mercury concentration detected from the total microalgae ranged from 10.05 to 18.29 (µg/kg). Other metals concentrations in the microalgae were in the ranges of 162.00 to 4418.90 (µg/kg) for Zn, 4533 to 24567 (µg/kg) for Fe and 225.10 to 2121.46 (µg/kg) for Mn. and 154.50 to 497.90 (µg/kg) respectively. Overall, the study identified the presence species of harmful algal blooms and biotoxins in the studied portion of the Ghanaian coastal waters. Apart from Accra Light House were just minute traces of harmful algal blooms species were present, the rest of the studied coastal waters had HABs well distributed. Generally, biotoxins were not identified in shellfish from the studied waters, except Ekumfi Narkwa were biotoxins were identified in only bloody cockles.

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CHAPTER ONE

INTRODUCTION

1.1 Background to Study

The ocean produces over half of the world's oxygen (for Breathing) and absorbs 50 times more Carbon Dioxide (mitigating Global Warming) than the atmosphere (primarily CO2 from human-made activities). Covering about 70 percent of the Earth's surface, the ocean transports heat from the equator to the poles, regulating climate and weather patterns.

Consequently, the explosion of plankton algae (multiplication of algae up to approximately millions of cells per litre) is beneficial for the aquaculture and wild fisheries sectors. Algal blooms, however, may have a detrimental impact, causing significant economic setbacks for aquaculture, tourism, and fisheries operations. It has crucial effects on climate and human health. Around 300 species may often occur in such high numbers within the 5,000 species of established marine phytoplankton. They decolourize the surface sea (so-called 'red tides') (Sournia et al., 1991; Gilbert et al., 2005). Only 80 or so species can create potent toxins that can find their way to humans via the food chain. The first written reference (1000 B.C.) to blooming harmful algae is documented in the Bible: "*all the waters that were in the river were turned to blood. And the fish that was in the river died, and the river stank and the Egyptians could not drink of the water of the river" (Exodus 7: 20–1)*.

Toxic blooms could aggregate and produce anoxic conditions (or oxygen depletion), thereby leading to both fish and invertebrates' death and significant impact on the ecosystem. A typical oceanic bloom in Ghana is depicted in Figure 1.1.

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Figure 1.1: Typical Oceanic Bloom in Ghanaian Waters

The low oxygen levels in the waters usually result from increased algal respiration (in dim light during the day or night) or decay of algal bloom caused by bacteria respiration. Unappealing dead fish, glop, and fluff may prevent tourism and recreational activities. Other phytoplankton themselves produce toxins that significant results in the death of aquatic resources. Shellfish and finfishes can bioaccumulate the produced algal toxins that pose a significant threat to humans via consumption. When such instances are not controlled, the contaminated shellfish and finfishes may have an overwhelming effect on the seafood industry.

Among the recorded intoxication of humans due to the consumption of shellfish contaminated with dinoflagellate toxins was 1793 when Captain George Vancouver and his crew arrived in British Columbia's area now known as Poison Cove. Captain Vancouver realized that it was off-limits for the indigenous Indian tribes to eat shellfish when the seawater turns into bioluminescent due to dinoflagellate.

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Globally, there about 2,000 cases of human poisoning (15% mortality) from contaminated seafood reported annually, and if such trends are not controlled, there will be economic damage through reducing local use.

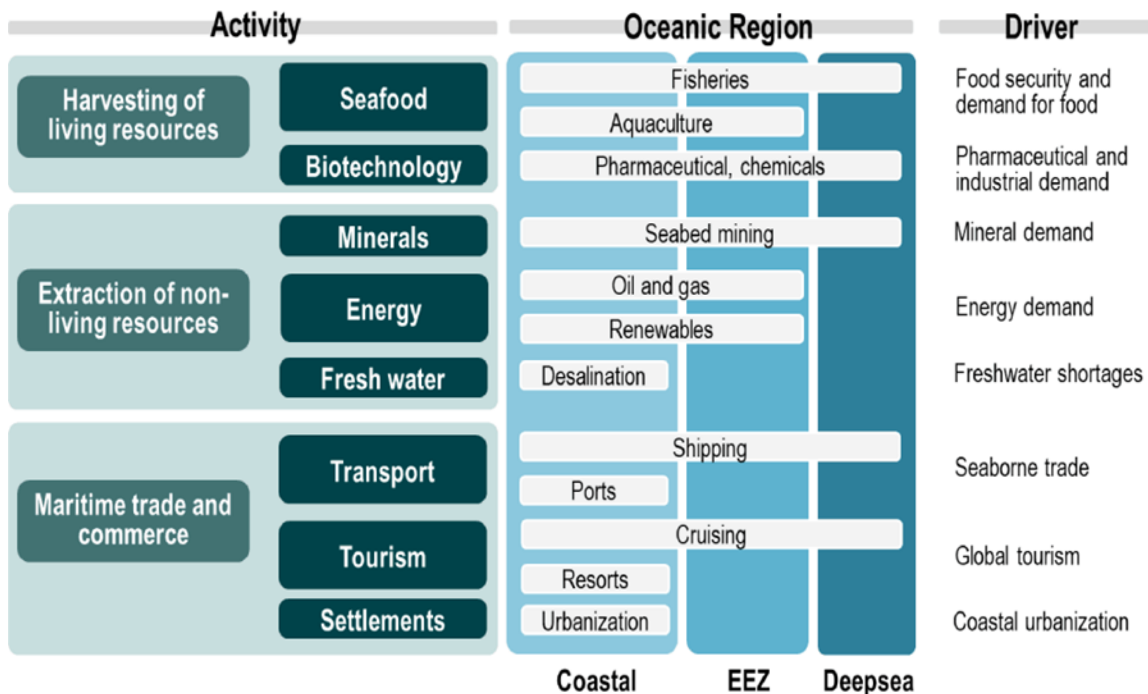

Figure 1.2: Economic importance of Oceans (Economist Intelligence Unit, 2015)

The services and stocks of natural capital provided by ecological systems are critical earth's life support system (Figure1.2). They contribute significantly to human welfare, both directly and indirectly, and therefore represent a significant portion of the planet's total economic importance. Costanza et al. (1997) assessed the current economic value of 17 ecosystem services for 16 biomasses, based on a synthesis of Studies, and some original calculations have been written. The value (most out of the market) for the entire biosphere is estimated to be in the range of 16–54 trillion US$/year, with an average of 33 trillion US/year. This must be considered a minimum estimate due to the complexities of the

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uncertainties. There is a disproportionately high importance in marine ecosystems such as estuaries, coastal wetlands, maritime waters and algae, coral reefs and continental racks. They cover only 6.3% of the world's surface but are responsible for 43% of the world's ecosystem services' estimated value. These environments are incredibly valuable in regulating the cycling of nutrients, which control plants' productivity on land and in the sea.

In addition, this may also result in a significant reduction in exports of seafood products. It would not only have a detrimental effect on seafood, but it may also affect species in the food chain such as whales and porpoises by infected plankton or fish (Geraci et al., 1989). Poisoning of manatees by dinoflagellate-producing brevetoxins confined to seagrass-attached fluids has been documented (Hitchcock et al., 2004). The coastal waters of a rising number of countries covering several parts of the world have been affected by micro-algae blooms for many years. The critical role played by marine microalgae in the control of carbon dioxide concentrations in the atmosphere is of vital importance for knowing food in the first stage of the trophic chain and providing a useful index for assessing the effects of climate change in coastal areas (Cabrini et al., 2012).

Increased understanding of the occurrence of and distribution of causative organisms, dynamic populations (rates of growth, nutrient requirements, cycles of life, etc.), ecophysiology (why and whenever toxic), better toxin detection, and toxicity testing methods are required to enhance the ability to predict and minimize events and thereby develop better mana. These measures can minimize the effects of harmful algal blooms (HABs) on human health and the economy. HABs and the potentially devastating, widespread impacts on marine and freshwater ecosystems are now a well-established

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threat. HABS's activities have increased in frequency, length, and dissemination over the past decades (Glibert et al., 2005; Anderson et al., 2019). In the case of toxic HABs, mainly dinoflagellates, are the causative organisms. Other causative organisms are diatoms, raphidophytes, and haptophytes. In the coastal marine environments of the world, these groups of microalgae are abundant. Although these toxic species only represent a small proportion of approximately 2 percent of the total number described. There are still new species that produce toxins (Smayda et al., 1997; Tillmann et al., 2009). Established taxa continue to be distributed more regularly (Aligizaki and Nikolaidis, 2006, 2008; Aligizaki et al., 2009; Verity, 2002). Factors such as anthropogenic nutrient enrichment, ballast water implementation, enhanced aquaculture practices, and, more recently, global climate change or variability, are numerous reasons for this pattern (Anderson et al., 2016; 2019). Algae or phytoplankton mostly contain algal toxins (commonly referred to as shellfish or marine biotoxins). The Joint Ad Hoc Consultants for FAO/IOC/WHO Chemical Structures in the year 2004, which have eight significant biotoxin forms in shellfish, namely cyclic imine (CI), azaspiracid (AZA), brevetoxin (BTX), okadaic acid (OA), domoic acid (DA), pectenotoxin (PTX) and saxitoxin (STX) and yessotoxin (YTX). STX and its derivatives trigger Paralytic Shellfish Poisoning (PSP) and DA trigger Amnesiac Shellfish Poisoning (ASP); OA group toxins are caused by Diarrhetic Shellfish Poisoning (DSP) [OA and DTX]; and AZA group toxins cause aseptic shellfish poisoning (AZP) [EFSA, 2010]. Ciguatoxins (CTX) are marine biotoxins found in fish from the biotransformation of Gambierdiscus toxicus (EFSA, 2010), a precursor of gambiertoxins formed by benthic dinoflagellate.

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In the digestive gland (hepatopancreas) of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams, and scallops, all the biotoxins addressed accumulate. The behaviours pose a health risk if humans eat infected shellfish and fish. Neurological issues with marine biotoxins can vary from headaches, vomits, and diarrhoea. In severe instances, this can lead to death (Vilarino, 2018). In several countries, marine biotoxin surveillance programs have been initiated by public health nutritionists, marine scientists, and other interest groups due to marine biotoxins' negative impacts.

1.2 Research Problem

Algal Bloom has been reported along portions (Western Coast; Jomoro, Ellembelle, and parts of Nzema East District) of the Coastal Zones in Ghana (Myjoyonline, April 2010; Ghanaweb, October 2010), impacting negatively on the Food Web, Fishing and Tourism;

It also causes oxygen depletion resulting in "Fish Kills;" and Toxins that Poisons Seafood; threatening Seafood Safety and Security. Nevertheless, Algal Bloom Surveys are scarcely carried out along the Coasts of Ghana, resulting in scarce data on the occurrence of Toxin-causing Algae; and rare information on prevailing conditions in Ghanaian Coastal Waters.

Systematic studies along Ghana's Coast; to ascertain the cause of these marine events has become imperative. The study will also help improve the health of the Ocean, Fisheries, Tourism and enhance Marine Biodiversity; and advance knowledge in Ecology, Nutrition, Health and Economic Risks.

Biotoxins are synthesized by phytoplankton and phytobenthos, and their effects can be seen via metabolic changes that may occur in the different stages of the food chain. Algae-produced biotoxins are numerically more significant. Unicellular algae can proliferate and

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aggregate under some favourable circumstances in their environment to constitute dense cell aggregations called 'blooms' (Morabito et al., 2012). With the growing demand for fish and shellfish-derived products around the world, shellfish aquaculture is expanding. In several systems across the globe, a vast number and species of shellfish are grown. Increased human activities and pollution are causing harm to the environmental quality of near-shore waters in the coastal regions around the world. The quality and quantity of coastal shellfish cultured are also affected by this increasing anthropogenic degradation of the coastal

Ghana also depends on tourism as a foreign exchange earner, and a threat to public safety from harmful algae needs to be evaluated. Reports of the prevalence of harmful algal blooms along the western coast of Ghana have made it possible to research the distribution of HABs systematically and to establish the effects of marine biotoxins on seafood (because these biotoxins are typically developed as a result of the presence of HABs) [CSIR STEPRI, 2012]. Data from such studies are essential and form the basis for government guidance on nutrition and marine ecosystem management.

Moreover, the export of seafood to other countries makes seafood a primary foreign exchange earner for Ghana (FAO, 1996). The EU, the United States of America and some African countries require adequate information on the safety of seafood before allowing imports into their country.

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1.3 Research Objectives and Hypothesis

1.3.1 Overall Objective

The overall objective of the study to identify and assess the distribution of harmful and potentially toxic phytoplankton causing Harmful Algal Blooms(HABs); and evaluate the densities (cell/L) of harmful phytoplankton along selected portions of the Central Coastline of Ghana

1.3.2 Specific Objectives

- **a.** To identify and characterize harmful and potentially toxic phytoplankton species along the Central Coastline of Ghana;
- **b.** To assess the distribution of the harmful phytoplankton;
- **c.** To evaluate the influence of the environmental driving factors {(pH, Temperature, DO, Nutrients [PO_4^{3-}, NO_3^-, SiO_4] and Trace Elements [Zn & Fe]} on the distribution of HABs in Ghanaian Waters;
- **d.** To evaluate of the levels of iron (Fe) and zinc (Zn) in seawater and Hg, Mn, Cu, Zn, Fe and Cd in algae and establish the correlation between the levels of the essential elements and the presence of algae;
- **e.** Isolate potentially Harmful Algal Bloom (HABs) species and assess the level of toxicity of successful cultures; and,
- **f.** To investigate the toxin effects of HABs in selected shellfish (Oysters, Bloody cockle and Clams) [Profiling of Toxins].

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1.3.3 Hypothesis

It has been hypothesized that elevated temperatures and concentrations of nutrients may have played a role in fostering the emergence of algal blooms and their increased incidence in specific areas, indicating that the recent spread of this species in international waters may have been caused by global warming (Hallegraeff, 2010).

The validity of this theory, however, has not been shown to date. In fact, the highest intensity of the blooms does not correlate positively with temperature values in the Mediterranean region (Mangialajo et al., 2011). It has not wholly elucidated even the function of nutrients.

1.4 Study Justification

During the past 10-30 years, red tides, as well as toxic phytoplankton events, have apparently been expanding in number, geographical extent, frequency, number of species responsible, intensity and damages (Anderson,1989; Smayda,1990; Sournia et al., 1991; Hallegraeff, 1993; Lassus et al., 1995; Lassus et al., 2016). Studies on harmful algae have been on-going since the last half of the century, but Africa for that matter West Africa and Ghana not much been studied or reported on the status HABs.

Another question which is sometimes raised is why harmful algae and seafood toxicity must be regularly monitored?

Climate change and increasing nutrient pollution are potentially causing HABs to occur more often, and in locations which were not previously affected. We need to learn about how and why they form and find out where they are so that we can lessen their harmful effects.

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Ghanaians depend heavily on marine resources for sustenance, primarily fish and shellfish, which provide more than 10 % of the population of the country with a livelihood (Nunoo et al., 2014). The marine subsector is the most critical source of local fish production in Ghana among catch fisheries, contributing approximately 80 % of the total fish supply, with an annual average catch estimated at 300 000 metric tonnes (FAO, 2016). While fish consumption is a delicacy for young and old across the country's demographic spectrum, access to the levels of marine algal toxin in these items is not monitored. It is widely appreciated that fish and shellfish are well able during feeding to bioaccumulate algal toxins, posing health risks to humans and animals upon consumption. Owing to human-induced activities and climate change, these microorganisms have become abundant in our coastal waters, typically benefiting from the growth of many species of harmful algae. Owing to the lack of surveillance systems for the presence of these toxic organisms in our coastal waters, much less information is available on human exposure to harmful algal blooms in Ghana. So there is an urgent need for a large scale surveillance monitoring to be placed in place to identify primary HABS exposure sources for the Ghanaian community. The presence and distribution of causative organisms, population dynamics, more profound methodologies for toxin identification and toxicity testing must be understood in greater detail.

The aquaculture industry is developing rapidly, and seafood products are becoming increasingly crucial for trade export. Hence the need for Ghana to prepare adequately to meet the global demand for safe seafood. At a recent Fisheries Conference (Accra, 2017) (https://ccm.ucc.edu.gh) all stakeholders concluded that fish catch has dwindled over the years so soon there will be the need as a nation to go into marine fish and shellfish farming.

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To protect consumers from toxic seafood, to protect the budding industry from major economic losses, and to protect the market against loss of trust in seafood products, we need to control the incidence of harmful algae and seafood toxicity regularly. Experience from many countries has shown that the best way to protect both customers, marine living resources and the market is to provide scientifically based knowledge on harmful algae in the region, combined with an efficient and consistent system for monitoring harmful algae and shellfish toxicity. Effective monitoring creates trust among authorities and consumers on export markets that seafood products from a given country are healthy. Major markets, e.g. the European Union, also include inspection systems, including surveillance of harmful algae and toxicity, to regulate and track the safety of both domestically produced and imported seafood products.

1.5 Scope of the Study

The research mainly used various analytical methods, such as the Utermöhl Method (1958), to estimate the harmful phytoplankton. Evaluation of marine water parameters that may be applied to distribution by in-situ analysis, chemical analysis and neutron activation analysis.

Identification of harmful algal species at the lowest taxonomic level (genus, species) using a wide variety of identification keys. Species names and higher taxonomic categories were reviewed for validity against the Algae Database (http/www.algaebase.org); the United Nations Educational Science and Cultural Organization (IOC-UNESCO) Taxonomic Reference List of Toxic Microalgae (http/www.marinespecies.org / HABs) [Moestrup et al., 2007; Brodie et al., 2007)]; and the most recent review of the HABs. Invariably licensed methods from the Association of Analytical Chemists (AOAC, 2015) were used to evaluate

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toxins using instruments such as LC-MS-MS, HPLC-FLD and HPLC- UV, as well as other recognized methods used to determine seawater quality both in situ and in the laboratory.

Harmful algae samples were concentrated in the Greater Accra Region (Tema, Jamestown (Lighthouse), Tsokomey-Bortianor) and the Central Region (Gomoa-Nyanyanor and Ekumfi-Narkwa) from June 2016 to February 2018. At the same time, shellfish samples were also sampled at Tsokomey-Bortianor and Ekumfi Narkwa.

The studies were categorized into three parts:

First, the monitoring of harmful algae species in sampling areas to measure and classify species using the Uthermohl Process, the evaluation of the marine water parameters that may be applied to the distribution. Samples were gathered every month, and species identities and quantities were calculated. At the same time, sampling for phytoplankton physical parameters of seawater was measured in-situ, while chemical parameters such as nutrients and essential elements (Zn and Fe) were calculated in the laboratories of the Ghana Atomic Energy Commission.

Part 2 included the culturing of individual phytoplankton species in order to isolate and culture certain harmful species and to determine their identity and toxicity. This part of the research was carried out at the Laboratory of National Oceanography and Experimental Geophysics in Trieste, Italy.

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The third part of the analysis will include the development of the toxin profile of species cultures of phytoplankton (i.e., dinoflagellates cultivated from Part Two).

They are:

a. Three Coolia spp strains, two Prorocentrum sp strains, two Alexandrium sp strains and one Gymnodinium sp strain;

b. Toxin profile of selected Ghanaian shellfish. Various European Union (EU)-Harmonized Extraction and Analysis Methods (2015) using LC-MS/MS, HPLC/FLD and HPLC/UV were used to create toxin profiles. Marine biotoxins (commonly known as shellfish toxins) are primarily produced by algae or phytoplankton.

Based on their chemical structure, the biotoxins found mainly in shellfish have been categorized into three classes, namely, Paralytic Shellfish Poisoning (PSP)-STX and its derivatives), Diarrheic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and diaphysis toxins (DTX)). Domoic acid (DA) causes Amnesic Shellfish Poisoning (ASP) and is examined.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Marine Phytoplankton and Harmful Algae

Marine microalgae play a vital role in the regulation of atmospheric carbon dioxide concentrations. Identification of these is essential for understanding the feeding of habits lower levels of the food chain. They are also valuable markers for defining the impacts of coastal climate change (Cabrini et al., 2012).

Single unicellular algae are almost always present in seawater, even if the water is clear. Events of eutrophication or red tide develop when microalgae are abundant.

The term red tide refers to various forms of algal blooms, which may vary according to their species and their photosynthetic pigments (Anderson 1989;1994).

Harmful algal blooms or HABs occur when algae colonies — simple plants living in the sea and freshwater — are out of control and produce toxic or harmful effects on people, fish, shellfish, marine mammals and birds. Human diseases caused by HABs, though rare, may be debilitating or even fatal (NOAA, 2020).

As algae multiply rapidly to become concentrated in high numbers, dense concentrations of algae are referred to as blooms. Algae blossoms in the water habitats are therefore preceded by rapid cell growth, which continuously increases the concentration of algae cells from tens of thousands to hundreds of millions per litre. Thus, a blossom in a litre of seawater could contain millions of cells. The concentration of the algae cells determines the intensity of the bloom.

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Although most blooms are not harmful, they are dangerous because of the resulting decrease in oxygen concentrations from their respiration in the environment. Some of the blooms can be extremely toxic, either by natural biotoxins (poisons) produced by certain species of microalgae or by the process of oxygen depletion initiated after death and subsequent decay of high algae concentrations.

Governments spend millions of dollars to clean up major fish kills, mark beaches off-limits, close fishing and shellfishing for harvesting, and provide medical care for people infected by marine biotoxins in the seafood they consume as a result of toxic algal blooms, or HABs. Many researchers agree that toxic algal blooms are becoming more widespread, but they point out that further surveillance has helped to identify more occurrences.

In marine waters, an apparent global rise in HABs has been recorded by several researchers (Anderson, 1998; Smayda, 1990; Van Dolah, 2000). This implies that HABs face significant global health and economic risks, combined with a growing demand for seafood products (Davidson et al., 2014).

2.2 Phytoplankton Involved in Harmful Algal Blooms

2.2.1 The Diversity of Causative Organisms

There are about 80 toxic species and about 200 noxious species in the list of micro-algal species that are potentially implicated in HABs detected so far from an estimated 4000 marine planktonic microalgae (Sournia, 1995). Owing to new cases of adverse incidents, the advancement of scientific studies and strengthened human experiences with the coastal zone, this list has risen remarkably over the last few years. Before the first instance of amnesic shellfish poisoning (ASP) in 1987, diatoms were not thought to be toxic. However,

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since then, about 10 species have been shown to produce a domoic acid a toxic compound. The total number of possibly lethal species is considered largely underestimated (Zingone and Enevoldsen, 2000), partially because three to four species are added to the list annually. This finding is hardly surprising, considering that several thousand phytoplankton species are still undescribed (Andersen, 1992).

As shown in the case of *Protoceratium reticulatum* (a widely distributed species which in 1966 was associated with death of mass white mussel on the West shore of southern Africa), it is not easy to link a known species with a harmful phenomenon. The new species, Gonyaulax grindleyi, was then erroneously named and described subsequently. Similar incidents occurred in the same region subsequently, but the cause of toxicity remained unknown. About two decades later, *P. reticulatum* was shown to produce yessotoxin a toxin known for 10 years (Zingone and Enevoldsen, 2000).

Toxicity and other detrimental effects from harmful algae are not limited to one or more classes of algae, but spread through multiple taxonomic groups. This is not surprising: weeds, plagues and poisonous organisms belong to a wide range of species from fungi to flowering plants in the terrestrial world. The diversity and ecological effects of secondary terrestrial plants are incredible. The high taxonomic diversity of hazardous algae often results in a number of toxins with relatively different modes of action at different trophic chain levels. Ecological requirements and flora dynamics often range dramatically from one species to another and, as in the case of non-hazardous species, different organisms can be harmful or non-toxic, exhibit different growth rates under the same conditions and vary from one species to another.

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Another consequence of the taxonomic diversity of HABs species is that more than once during their evolution, the development of harmful substances or other offensive or upsetting features was selected as a beneficial character. However, the value of these characteristics in terms of improved fitness or competitiveness, is not known.

2.3 Mechanism of Harm

How do certain types of phytoplankton cause harm to fish, shellfish, marine mammals, seabirds, and people?

The basis of the aquatic food chain created by phytoplankton. There are approximately 4000 phytoplankton species in marine waters (Sournia, 1995), most of which are benign to humans. However, some species are considered dangerous, affecting human and animal health through the development of a variety of potent natural biotoxins and/or causing economic losses through their adverse effects on human use of ecosystem services (Anderson et al., 2002; Davidson et al., 2011; Hallegraeff, 1993).

The smothering of gills may also kill farmed (and wild) fish due to phytoplankton mucus production or from gill abrasion by spines on the cell walls of some phytoplankton (Bruno et al. 1989). In contrast, low biomass HABs (i.e. few hundred to thousands of cells/L) threaten human health (and the health of other animals) because of the biotoxins produced by this phytoplankton being concentrated by filter-feeding shellfish and other organisms that may subsequently be ingested by humans or transferred through the marine food web (Davidson et al., 2011). Humans may also be exposed to, and affected by, biotoxin-contaminated water or aerosols (Bean et al., 2011).

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Basically, there are four ways in which microalgae are harmful first, by clogging or irritating the gills; the physical presence of too many cells can suffocate fish. Second, the decay process, aided by bacteria, will deplete oxygen water when the tightly condensed algal cells die off, which can contribute to the death of oxygen-dependent marine organisms in turn. (Algae, being plants, need the growth of nutrients including nitrogen and phosphorus. They appear to die from all at once, when they have used up the nutrients). In shallow bays, inlets, or oceans, such oxygen-related impacts are most noticeable. Third, certain microalgal organisms create deadly toxins that destroy the animals that ingest the poisons directly. Mussels, abalone and fish were killed by Dinoflagellate toxins (FAO, 2004). Airborne toxins (i.e., aerosolized toxins) have caused respiratory problems and inflammation of the eyes and skin in individuals along beaches with toxic algal blooms (Kirkpatrick et al., 2004). There are different toxins per dangerousness for human health.

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2.4 Global Distribution of Harmful Algae

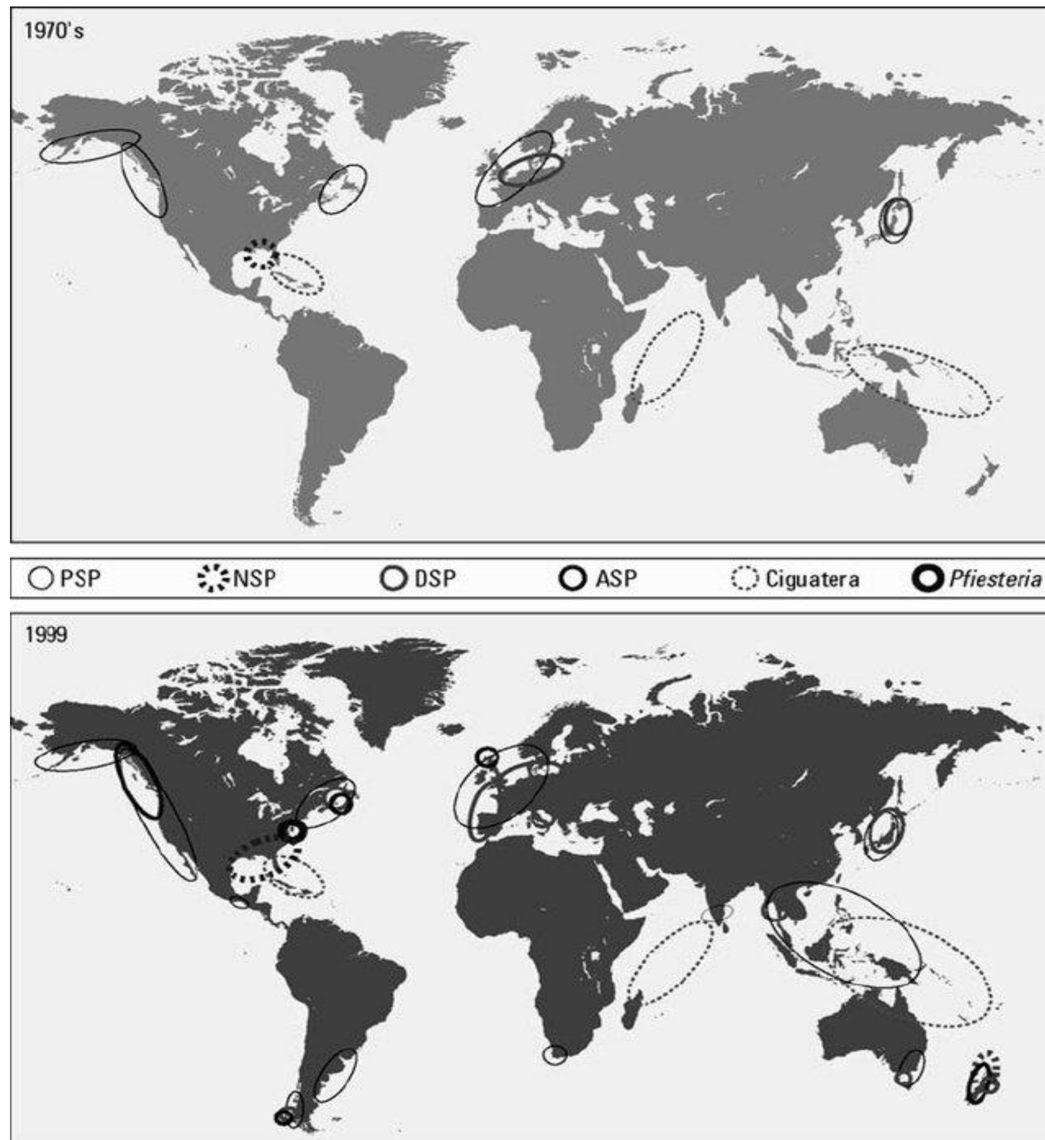

Figure 2.1: Global distribution of HABSs toxins **[Adapted from: Sellner et al., 2003]**

The global distribution HABs from the 1970s to the 1990s is presented in Figure 2.1. Global distribution of HABs has been indicated elliptically on the map (Figure 2.1). Interestingly, HABs were nonexistent on the African continent. However, HABs were identified in South Africa in 1990. This may not actually be due to the lack of HABs in African waters but may be due to the absence of regional studies.

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2.5 Transport of Harmful Algae via Ship's Ballast Water

The very first argument of cargo vessel ballast water was made over 100 years ago as a route referred to a microalga in the spread of foreign marine species (Ostenfeld, 1908). The Odentella sinensis diatom was not seen in European waters until 1903 and is well known from the tropical and subtropical coasts of the Indo-Pacific. When in the North Sea it caused dense plankton blooms. Since, in these well-studied seas, it was less likely that this massive diatom would have been previously overlooked, and it was unbelievable that tides from distant oceans might have carried it. Ostenfeld (1908) suggested that it was carried by ship for this species. Such evidence-based solely on historical absence will, to date, be well-thought-out as incomplete and inconclusive.

At the end of the trip, the first study of ballast water was not carried out until the 1970s [Medcof, 1975; Gollasch et al., 2000], when it was eventually known that the diatom species Odontella sinensis was present in the ballast water of a vessel travelling from Singapore to Germany.

Ballast tank studies mainly created lists of species from preserved samples, but subsequent reviews refined their emphasis on the occurrence of viable organisms, using ballast water algae cultivation techniques and ultimately genetic analyses. 80 percent of ships contained up to 30 culturable diatom species, including some Pseudonitzschia, possible producers of amnesiac shellfish toxins, in comprehensive Australian ballast water examinations (Forbes and Hallegraeff, 2001). More seriously, viable Paralytic Shellfish toxin cultures that contain Alexandrium catenella and some dinoflagellates. Tamarense was collected from Japan and Korea from ballast water entering Australia (Hallegraeff and Bolch, 1992; in 5%

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of ships), from ships entering British ports (A. minutum, A. catenella / tamarense: Hamer et al., 2001; 17%) and also from ships entering Canadian ports (Roy et al., 2014; 19-32% A. tamarense; 8-32% Pyrodinium bahamense). In the distribution of the brown tide Aureococcus anophagefferens into the North American Great Lakes (Doblin et al . , 2004b) and the potentially fish-killing dinoflagellate Pfiesteria piscicida were involved in the distribution of the brown tide Aureococcus anophagefferens into the North American Great Lakes (Doblin et al . , 2004b) and molecular probes and cultured from ballast water entering the US and Australian ports respectively (Doblin et al . , 2004a; Parke Therefore, by looking at the samples from Tema Port, it is appropriate to research the availability of HABS species transported to our coast.

2.6 Types of Shellfish Poisonings- Species and Toxins that Cause Them

2.6.1 Lipophilic toxins

2.6.1.1 Diarrhetic Shellfish Poisonings (DSP)

The serious gut disease caused by ingestion of shellfish infested with DSP toxins (Quilliam,1995) is diarrhetic shellfish poisoning (DSP). DSP toxins were initially categorized into three classes, okadaic acid (OA)/dinophysistoxin (DTX) analogues,

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pectenotoxins (PTXs) and yessotoxins (YTXs), on the basis of their structures.

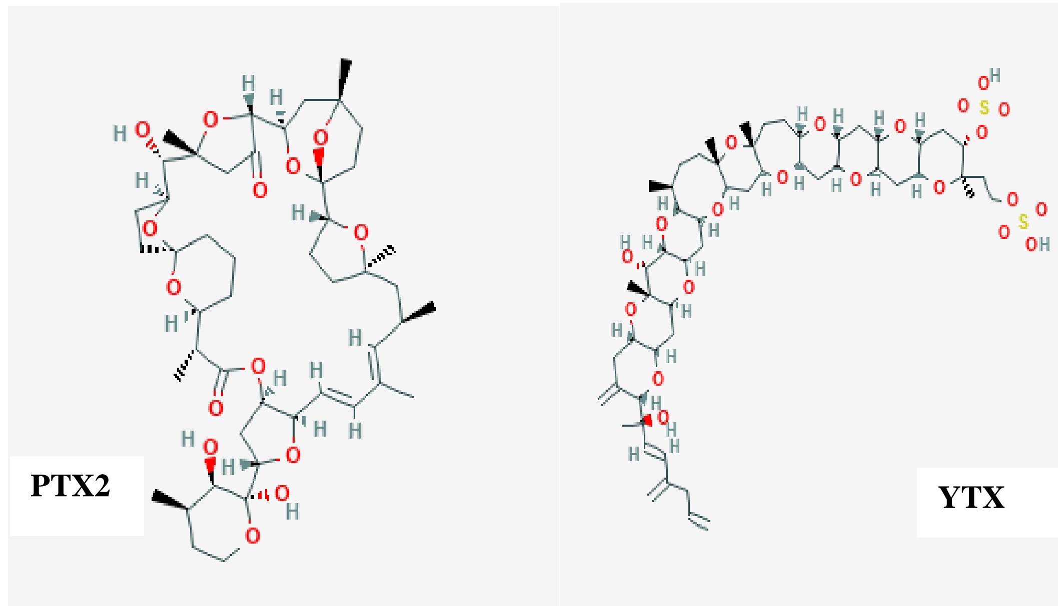

Figure 2.2: Structures of (PTX2); (YTX) [Adapted from PubChem]

The most important toxins for OA's heavy diarrheal activity and its analogues, dinophysistoxin-1 and -2 (DTX1 and DTX2). Figure 2.2 illustrates the structure above. It is reported that YTXs in human lymphocytes modulate calcium homeostatic toxicity in in-vitro studies (Miles et al., 2006). These toxins are a potent phosphatase inhibitor. YTX also causes a selective disruption in epithelial cells, including MCF-7 breast cancer cells, of the E-cadherin-catenin system in vitro studies (Ronzitti et al., 2004). Although there are several reported toxicological data for PTX and YTX, the symptoms of human PTX and YTX poisoning are relatively unknown, as no human poisoning has been reported to date. PTXs and YTXs are shown to be less toxic and do not cause diarrhoea by oral routes through animal studies. The concept of DSP toxins for PTXs and YTXs was recently eliminated. In addition to PTX and YTX, the Mouse bioassay (MBA), which is the official DSP test tool in Japan and many countries, leads to false-positive values for analogues in OA and

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DTX. For the selective detection of OA / DTX analogues only, alternative test methods are therefore required. For the three toxin classes, the different functional toxicities require shellfish testing methods, which can measure each category of toxins independently.

The method used to detect, distinguishing and quantification of marine toxins was one of the most important techniques used in electrospray ionization (ESI) liquid chromatography-mass spectrometry (LC-MS). OA / DTX and other lipophilic toxins determination. LC-MS is typically performed on LC-MS's C8 or C-18 silica column with reversed-phase chromatography and isocratic or gradient elution using mobile phases of acetonitrile/water containing volatile modifiers such as acetic acid, formic acid, ammonium format or ammonium acetate. One of the most commonly used columns is a short narrow, boring column packed with three µm Hypersil-BDS-C8, which can separate a wide variety of toxins with fast gradients. Analogues OA and its similar DTX1 and DTX2 are known to be the three potent toxins of the groups due to their high diarrhoea activity. LC-MS / MS of okadaic (OA) and Dinophysistoxin (DTX). Several naturally occurring derivatives of OA and DTX toxins have also been found in dinoflagellates and bivalves in addition to these dominant toxins. The so-called OA diol-esters are one group of OA products in which the Carboxyle OA group is combined to form allylic diol-esters into many separate C7- C9 diols. Several of these diol esters in Prorocentrum lima, P. concavum, and Dinophysis hasle have been described. The other category is a complex combination of OA, DTX1 and DTX2 (also known as DTX3 Complex) 7-O-acyl ester derivatives, according to Reguera et al. (2014). It may develop in bivalves that consume toxic dinoflagellates as metabolites of Free OA, DTX1 and DTX2.

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The LC-MS / MS techniques Larsen et al. (2007) have recently explained detailed profiles for OA diol esters and 7-O-acyl ester derivatives (DTX3) for toxic dinoflagellates and bivalves. The most useful mobile step for an analogue of OA LC-MS is composed of 50 mM of formic acid acetic acetonitrile and 2 mM of ammonium. Acidic conditions (pH 2.3). Favour good chromatography of analogue acidic OA by eliminating carboxyl ionization and preventing deleterious interactions in the stationary phase between ion exchange and residual silanols groups. However, ionization in the electrospray ion source is still encouraged, with $[M+NH4]^+$ and $[M+H]^+$ in positive, intensive, and [M-H]- ions producer acidic analogue in negative mode. [M-H] is mainly ammonium adductions. MS / MS can also be beneficial in gathering knowledge about the structure. Neutral analogues also allow mobile phase ions: $[M+NH_4]^+$ ions in the positive mode and formate attachment ions, $[M+HCO_2]^-$, in the negative mode.

DSP is caused by toxins that are produced by some species of marine microalgae and is often short-lived and not life-threatening. Nevertheless, for specific individuals, especially young children, the elderly, pregnant women and those with compromised immune systems, DSP may be essential. Diarrhoea, nausea, vomiting, headache, stomach cramps, and chills can be the symptoms of DSP.

Symptoms typically occur between 30 minutes and six hours after ingestion (James et al., 2010).

Members of the marine algal toxins class of okadaic acid (OA) and dinophysistoxin (DTX) pose a health risk due to edible seafood pollution (Aligizaki et., 2011). Diarrhetic shellfish poisoning (DSP) is a human syndrome caused by the ingestion of OA and DTX contaminated shellfish developed in the genera Dinophysis and Prorocentrum by

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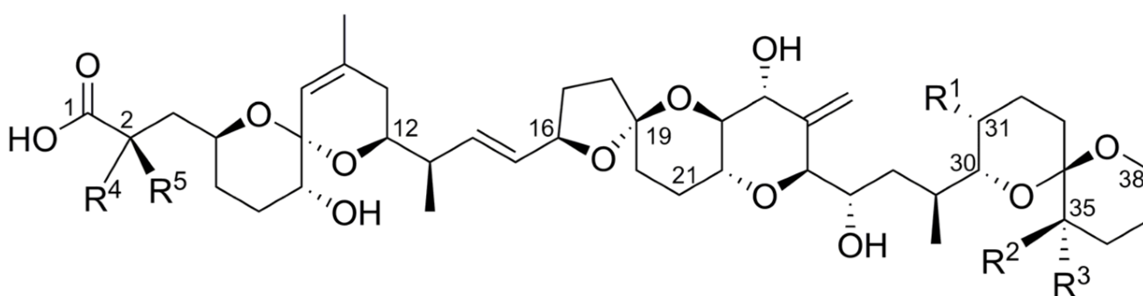
dinoflagellates (Alvarez et al., 2015; Anderson et al., 2002). The OA / DTX toxins, with thousands of individuals exposed annually, are spread worldwide. In the United States' Pacific Northwest area in 2011, the first evidence of human illness due to the ingestion of shellfish containing high levels of DTX1 was in 2011 (Bacchiocchi et al., 2015; Bean et al., 2011).

Mutagenic, carcinogenic, and immunosuppressive effects have been associated with OA and DTX (figure 2.3) (Bernd and Bernd 2008). This form of poisoning, which affects seafood and fishing activities, has been reported worldwide. (Yasumoto et al . , 1980; Kat, 1983; Raine et al., 2010; Reguera et al., 2014; Reguera & Pizarro, 2008).



	R^1	R^2	R^3	R^4	R^5
Okadaic acid	CH_3	H	H	CH_3	OH
DTX1	CH_3	CH_3	H	CH_3	OH
DTX2	H	H	CH_3	CH_3	OH
2-*epi*-DTX2	H	H	CH_3	OH	CH_3

Figure 2.3. Structures of the OA [Adapted: The IOC Manual 2003]

A series of regulations have been issued by the European Union (EU) related to marine biotoxins. One such regulation, EC No 853/2004 establishes maximum levels for lipophilic toxins in bivalve molluscs destined for human consumption as follows:

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(i) For okadaic acid, pectenotoxins and dinophysistoxins together – 160 micrograms of okadaic acid equivalents per kilogram of mollusc.

(ii) For yessotoxins – 3.75 milligrams of yessotoxin equivalent per kilogram [Reg. (EU) No 786/2013].

(iii) 160 micrograms of azaspiracid equivalents per kilogram – For azaspiracids

2.6.1.2 Neurotoxic Shellfish Poisoning (NSP)

Brevetoxins are the causative agents of neurotoxic shellfish poisoning (NSP), that may ensue after both inhaling aerosol containing the toxins and ingestion of contaminated seafood.

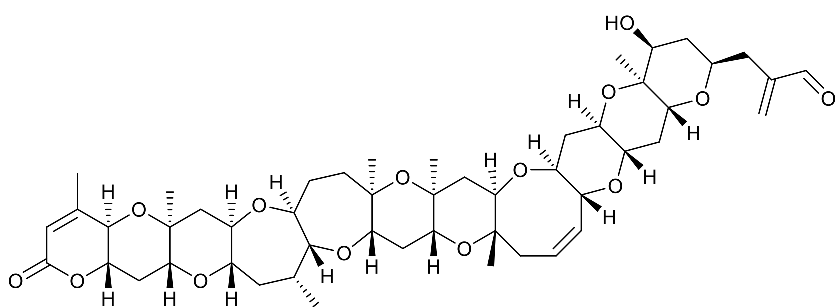

Figure 2.4: Chemical Structure of Pb-TX, paralytic shellfish poisoning (PSP) toxins

The IUPAC name is 2[[(1R,6S,5R,7S,9R,11S,12S,14R,16R,18S,20R,21Z,24S,26R,28S, 30R,31R,33S,35R,37S,42R,44S,46R,48S)-12-hydroxy-1,3,11,24,31,41,44-heptamethyl-39-oxo-2,6,10,15,19,25,29,34,38,43,47-undecaoxaundecacyclo [26.22.0.03,26.05,24.07,20.09,18.011,16.030,48.033,46.035,44.037,42] pentaconta-21,40-dien-14-yl] methyl] prop-2-enal.

When poisoning is through the respiratory tract, the exposure usually occurs on or near the waters where a bloom of PbTX producers has developed. NSP has been recorded primarily

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in the south-eastern coast of the United States, the Gulf of Mexico, and New Zealand (FAO, 2004a; Gessner and McLaughlin, 2008; Ishida et al., 1996). The symptoms due to contaminated shellfish appear after minutes/hours from its ingestion and are more severe than those found when contaminated aerosol is involved. In the former case, symptoms are both gastrointestinal (nausea, diarrhoea, and abdominal pain) and neurological (circumoral paraesthesia and hot/cold temperature reversal). In more severe cases, the muscular system (altered heart contractions, convulsions, and respiratory difficulties) may be affected. Death from NSP has never been reported in humans and, symptoms resolve within a few days after exposure to the toxins (FAO, 2004a; Gessner and McLaughlin, 2008). Brevetoxin (PbTx), or brevetoxins (Figure 2.5), are produced naturally by a species of dinoflagellate known as *Karenia brevis*

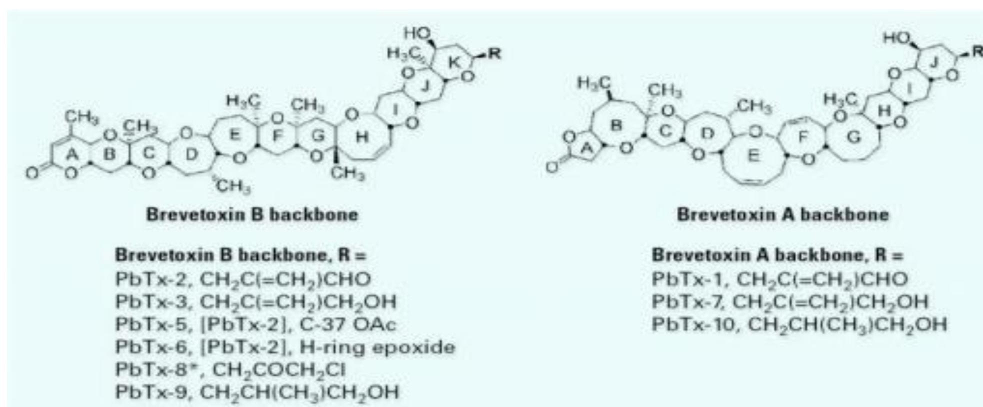

Figure 2.5: Structure of Brevetoxin (Adapted from Pierce et al., 2005)

2.6.1.3 Azaspiracid Shellfish Poisoning (AZP):

Azaspiracid Shellfish Poisoning (AZP) is caused by the azaspiracid toxin and its derivatives and is responsible for the development of the following phytoplankton toxins: amphidomataceae (Amphidoma languida, Azadinium spinosum, Azadinium poporum, Azadinium dexteroporum). Nausea, vomiting, severe diarrhoea, abdominal cramps are

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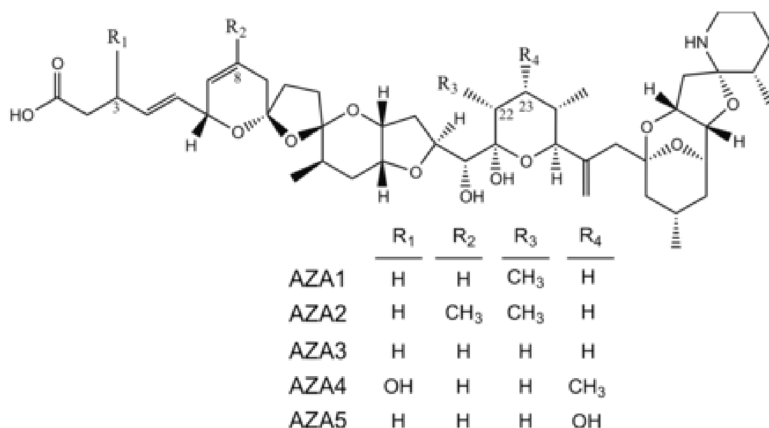
indicators; in animal tests, impacts Mouse studies require significant damage to the tissues of the stomach, spleen, and liver. This is mainly seen after ingestion of shellfish (Figure 2.4). [Twiner et al., 2008, 2012a, b, 2014); Klontz et al. (2009); Tillmann et al. (2009, 2014); Hess et al. .are the primary geographic areas of confirmed cases of AZP poisoning.



	R_1	R_2	R_3	R_4
AZA1	H	H	CH_3	H
AZA2	H	CH_3	CH_3	H
AZA3	H	H	H	H
AZA4	OH	H	H	CH_3
AZA5	H	H	H	OH

Figure 2.4: Structure of AZA and its Derivatives (Pierce et al., 2005)

2.6.2 Amnesic Shellfish Poisoning (ASP)

Amnesic Shellfish Poisoning (ASP) is caused by domoic acid, a naturally occurring acid which is produced by some microalgae (Trainer et al., 2012). Domoic acid is a potent neuro-toxic amino acid and a natural product of some marine phytoplankton (Furey et al., 2001; Morabito et al., 2018). The symptoms of ASP may include nausea, vomiting and diarrhoea, muscle weakness, disorientation, and loss memory. Signs occur 30 minutes to 6 hours, usually after consumption. If the poisoning is not severe, the symptoms disappear entirely within a few days in an otherwise healthy person. However, in extreme cases, death may occur (Clark et al., 1999). During the winter of 1987, 153 people suffered from acute intoxication after consuming cultured blue mussels (Mytilus edulis) originating from Prince Edward Island. The cause of the intoxication was ascertained to be domoic acid

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(DA) (Hallegraeff, 2004). Marine biotoxins that cause amnestic shellfish poisoning (ASP) in humans are domoic acid (DA) and its isomers. DA is a cyclic amino acid that is water-soluble, provided mainly by marine red algae of the genus Chondria and diatoms of the genus Pseudo-nitzschia. In 1987, the first confirmed outbreak of ASP occurred in Canada and was associated with mussels infected by the Pseudonitzschia f. multiseries. Multi-serial. In the United States shellfish and many European countries, DA isomers have also been found. Although several DA isomers (epi-domoic acid diastereoisomer (epi-DA) and isodomoic acid (iso-DAs)) were identified, only infections of DA and epi-DA (expressed as sum DA) have been reported. Regulation (EC) No 853/2004 sets the limit for domoic acid in shellfish at 20 mg/kg of edible tissue. Methods used for detection in this study is mass spectrometric (MS).

2.6.3 Paralytic Shellfish Poisoning (PSP)

Paralytic Shellfish Poisoning is caused by consumption of bivalve shellfish and other molluscan shellfish that have been contaminated by toxins produced by some species of marine microalgae found in coastal waters (Figure 2.2). Indications of PSP could begin within a few minutes, and up to 10 hours after consumption, They include a tingling sensation or numbness around the lips that Spreads steadily to the face and neck, prickly feeling in the fingertips and toes, drowsiness, headache and dizziness, and difficulty swallowing (Grattan et al., 2016; Morabito et al., 2017). In more severe cases, one may also experience incoherent speech, prickly sensation in the arms and legs, stiffness and non-coordination of limbs, weakness, and rapid pulse. Respiratory difficulty, salivation, temporary blindness, nausea and vomiting may also occur. In extreme cases, paralysis of

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respiratory muscles may lead to respiratory arrest and death within two to twelve hours after consumption (Clark et al., 1999).

One of the most well-studied toxicants with serious human symptoms is paralytic shellfish poisoning due to the 58 closely related tetrahydropurin-based compounds (Burrell et al., 2013). It is the product of saxitoxin (STX) and gonyautoxin (GTX) exposure. In 1957, in clams (Saxidomus giganteus) living in coastal areas of Alaska, a PSP toxin was isolated, and STX has given the chemical structure in 1975 (Figure 2.5).

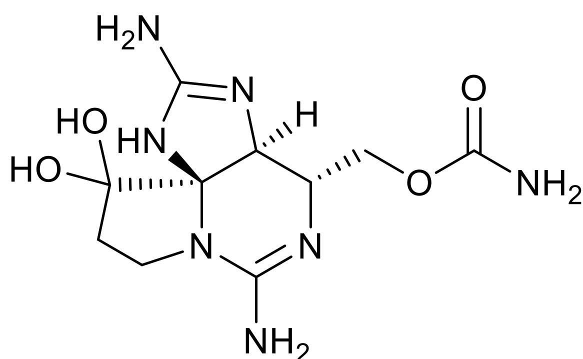

Figure 2.5: Chemical Structure of STX, paralytic shellfish poisoning (PSP) toxins

The key producers of PSP toxins on the Atlantic and Pacific coasts are dinoflagellates of the genus Alexandrium (Visciano et al ., 2016), but also in the Mediterranean Sea, where other species may be present, such as Gymnodinium catenatum (Berti and Milandri, 2014). A standard structure is given, as shown in Figure 2.5. More than 30 STX analogues and clusters in four subgroups were presented: carbamate, N-sulphocarbamoyl, dicarbamoyl and hydroxylatedsaxitoxins(EFSA,2009a).

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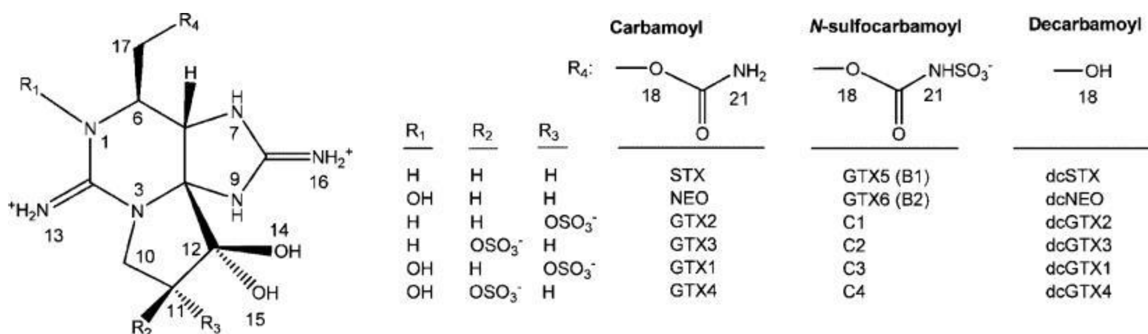

Figure 2.6: Structures of several known paralytic shellfish poisoning (PSP) toxins

Paralytic shellfish poisoning (PSP) with structure, as shown in Figure 2.6 is caused by the ingestion of shellfish containing neurotoxins formed by naturally occurring phytoplankton groups such as Alexandrium spp. Gymnodinium spp. Notably, traditional processing, including cooking, cannot kill these toxins (Diener et al., 2006). One example of such neurotoxin is saxitoxin (STX), 57 of which were recently recorded (Wiese et al., 2010).

PSP toxins are biosynthesized by Alexandrium, Gymnodium and Pyridinium marine dinoflagellates (Steidinger,1993). Some freshwater cyanobacteria such as Aphanizomenonflos-aquae, Cylindrospermopsis raciborskii and Anabaena circinalis may also generate PSP toxins. So marine algae and cyanobacteria, for example. Aphanizomenon flos-aquae (Afa) algae from Klamath Lake, Canada, and dried marine organisms used as dietary supplements or nutraceuticals can contain PSP toxins. Methods for analyzing PSP must be available to monitor this novel food [Mahmood and Carmichael, 1986; Ferreira et al., 2001; Carmichael et al., 2000]. The AOAC mouse bioassay is usually used for routine analysis of PSP toxins (AOAC, 1990), whereby the biological test only allows the determination of total sample substance toxicity.

Additionally, a loss of sensitivity occurs when PSP toxins are present in low concentrations. For this purpose, chemical methods based on onion-pair chromatographic

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separation and post-column derivation of toxins before fluorescence detection are often used to analyze PSP toxins. These HPLC / FD methods are either time consuming or separation efficiency inadequate. Botana LM (2000) reviewed HPLC methods with ion-pair reagents and phosphate buffer before post-column derivation. The Oshima et al. (1995) method requires three different isocratic runs to evaluate most PSP toxins which take much time which labour ability. The methods based on Sullivan et al. (1985) and gradient elution, on the other hand, allow the determination by one chromatographic run of NEO, DCSTX, STX and GTX 2/3, but the separation of GTX 1/4 is difficult. Creation of a new system for the complete isolation of all PSP toxins using one.

2.6.4 Ciguatera Fish Poisoning (CFP)

The Ciguatoxin toxin and its derivatives induce this. Gambierdiscus spp is the phytoplankton responsible for producing this toxin. Fukuyo spp, and (Figure 2.5). Nausea, vomiting, diarrhoea, mouth and limb numbness are typical symptoms of CFP infection. For several months, neurological symptoms may persist. Usually, infection occurs after the ingestion of coral reef fish. They are endemic, spreading to temperate latitudes in the tropics and subtropics [Friedman et al., (2008); Litaker et al. (2010); Chinain et al. (2010a, b); Tester et al., (2014)]. In figure 2.7, a typical structure is shown.

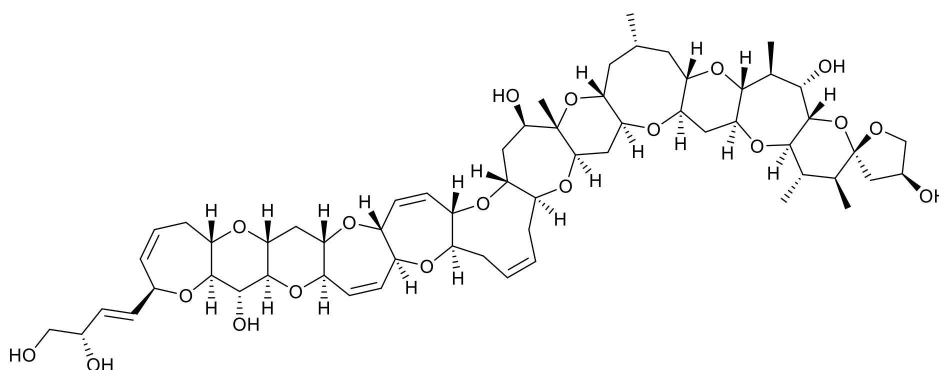

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Figure 2.7: Structure of ciguatoxin (Pierce et al., 2005)

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2.4 Classification of Marine Biotoxins

TABLE 2.1: Classification of marine biotoxins and effects in humans

TOXIN GROUP	SYMPTOMS	CAUSATIVE SPECIES
Saxitoxin (STX)	Paralytic Shellfish Poisoning (PSP)	Several species of *Alexandrium, Gymnodinium catenatum, Pyrodinium bahamense*
Domoic acid (DA)	Amnesic Shellfish Poisoning (ASP)	Several species of *Pseudo-nitzschia sp, Nitzchia navis-varingica, Halamphora coffeaeformis*
Okadaic acid (OA), Dinophysistoxins (DTXs)	Diarrhetic Shellfish Poisoning (DSP)	Several species of *Dinophysis, Phalacroma and Prorocentrum*
Pectenotoxins (PTXs)	*	Several species of *Dinophysis*
Yessotoxin (YTXs)	*	*Gonyaulax spinifera, Lingulodinium polyedrum, Protoceratium reticulatum*
Azaspiracids (AZAs)	Azaspiracid Shellfish Poisoning (AZA)	*Azadinium spinosum*
Brevetoxins	Neurotoxic Shellfish Poisoning (NSP)	Several species of *Karenia*
Ciguatoxins (CTXs)	Ciguatera Fish Poisoning (CFP)	Several species of Gambierdiscus
Spirolides-SPXs, gymnodimines- GYM (part of cyclic imines group	*Fast-acting toxins	Alexandrium ostenfeldii (SPXs), Karenia selliformis (GYM)
Palytoxins (PLTs)		Several species of Ostreopsis

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TABLE 2.2: Regulatory limits(modified by FAO/IOC/WHO, 2004; EFSA, 2009b).

Marine biotoxins	Regulatory limits	Exposure after consumption of 400g portion at the EU limit	LOAEL (1) NOAEL (2) µg/kg b.w.	Safety factors Human data (H) Animal data (A)	Acute reference dose (ARfD)	The maximum concentration in shellfish meat(400g portion) not exceeding ARfD
Okadaic acid	160 µg OA eq'/kg SM	64 µg OA eq/kg person	1 (1)	3 (H)	0.3 µg OA eq/kg b.w.	45 µg OA eq/kg SM
Azaspiracid	160 µg AZA eq/kg SM	64 µg AZA1 eq/kg person	0.4 (1)	10 (H)	0.2 µg AZA1 eq/kg b.w.	30 µg AZA1 eq/kg SM
Pectenotoxin	160 µg PTX eq/kg SM	64 µg PTX2 eq/kg person	-	-	0.8 µg PTX2 eq/kg b.w.	120 µg PTX2 eq/kg SM
Yessotoxin	3.75 mg YTX eq/kg SM	400 µg YTX eq/kg person	5000 (2)	100 (A)	25 µg YTX eq/kg b.w.	3.75 mg YTX eq/kg SM
Saxitoxin	800 µg PSP/kg SM	320 µg STX eq/kg person	2 (1)	3 (H)	0.5 µg STX eq/kg b.w	75 µg STX eq/kg SM
Domoic acid	20 mg DA/kg SM	8 mg DA/kg person	1000 (1)	10 (H)	30 µg DA/kg b.w.	4.5 mg DA/kg SM

'eq = equivalent ''SM = shellfish meat b.w. = body weight - = not reported

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2.4 Nutrients and Harmful Algal Blooms

Both land and water nutrient enrichment is the result of increased human population growth and much-related food and energy production practices, as well as the discharge of associated sewage and waste. Eutrophication is the nutrient loading mechanism in inland and coastal waters that often leads to an increase in algal biomass, often dominated by one or more classes of organisms or organisms (GEOHABS, 2006). The increased rate of harmful algal blooms (HABs) that produce high biomass kill fish, intoxicate seafood, lead to oxygen depletion, and alter trophic interactions is a significant consequence of eutrophication (Heisler et al., 2008). Nutrient enrichment can stimulate HABs not only directly through growth and biomass stimulation, but indirectly through changes in the food web and ecosystem dynamics in subtle but still important ways. The connections between these shifts and the proliferation of HABS are just beginning to be understood.

The production and persistence of many HABs are facilitated by degraded water quality from increased nutrient contamination and is one of the reasons for their expansion in the United States and other nations (Heisler et al., 2008).

In coastal waters worldwide, toxic algal blooms (HABs) are thought to be growing. As a critical causative factor of this rise, anthropogenic nutrient enrichment has been suggested through elevated concentrations of inorganic and organic nutrients and adjusted nutrient ratios. The relation between anthropogenic nutrients and HABs has been hypothesized; the correlation between anthropogenic nutrients-HABS is far from universal, and where shown, it is most often associated with high biomass rather than low biomass (biotoxin-producing) HABs. Although organic nutrients have shown support for the growth of a

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variety of HABS organisms, there is not enough evidence to make it clear whether these nutrients benefit the growth of harmful organisms directly, rather than benign, or whether or not they impact on the toxicity of harmful species.

It is widely agreed that phytoplankton proliferation in most coastal waters is likely to be mediated by the availability of dissolved inorganic nutrients (Howarth and Marino, 2006). As the supply of nitrogen (N) and phosphorus (P) to coastal waters has increased in human coastal communities, industrialization and agriculture intensification (Ferreira et al., 2011), the role of anthropogenic nutrient enrichment and associated changes in nutrient ratios are among the most frequently proposed and debated hypotheses relating to increased HABs in coastal waters.

Globally, there are multiple reports of increases in HABs associated with increased nutrient loading, a few of which are illustrated here. Although strong associations between phosphorus loads and harmful cyanobacteria blooms have been seen for many years in freshwater environments (Schindler, 1977; Burkholder, 2002), the interactions between nutrient loading and estuarine/coastal marine HABs have been recognized more recently (Smayda, 1990, 1997; Anderson et al., 2002; Glibert et al., 2005a, b; Burkholder and Glibert, 2006). In the Mexican Gulf, for example, the sedimentary record of the concentration of potentially toxic diatoms, Pseudo-nitzschia spp., has increased over the past several decades in parallel to increased nitrate loading (Turner and Rabalais, 1991; Parsons et al., 2002). In some regions, high concentrations in nutrients have led to high nutrient levels in coastal waters such as fertilizer, manure, livestock waste, atmospheric inputs and coastal aquaculture (Anderson et al., 2008; Gowen et al., 2012; Heisler et al., 2008; Smayda, 1990). It should certainly be considered that the existence of HABs is an

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undesirable condition, so eutrophication was diagnosed by HABs and high seafood toxicity occurrences (Foden et al., 2010).

Although it is often presumed that the connection between HABs and anthropogenic nutrient enhancements is widespread, the relationship is complicated since HABs are no new phenomenon and can occur naturally in the case of a broad geographical distribution preceding coastal waters, which clearly show that enrichment is anthropogenic and does not inherently occur (Richardson, That is why HABs do not necessarily imply eutrophication because other pressures like climate change will impact HABs (Moore et al., 2008; Hallegraeff, 2010). Karenia mikimotoi blooms in NE Atlantic (Davidson et al. 2009) and Alexandrium fodeyeyense in the Gulf of Maine (Anderson et al. 2008) are new examples of HABs with no known anthropogenic connection. Nevertheless, research studies have strongly supported the hypothesis that anthropogenic nutrients have increased the incidence of HABs in certain coastal regions. Tolo Harbor in Hong Kong and the Seto Inland Sea in Japan (Gowen et al., 2012) were especially prominent.

2.5 Economic effects of HABs linked to food security and seafood-related activities

Some HABs pose a threat to food production from aquaculture, which has become a significant source of protein (linked to the decrease in wild fish stocks) for human population expansion, especially in the coastal communities of developing countries. Like forestry, and also in conjunction with it, aquaculture helps sustain many local and regional economies (FAO, 2006). In fact, and concerning the production (and wild harvest) of shellfish, the most effective way to protect humans from HABS-related contamination of seafood is to track the existence of HABS species or biotoxins and to enforce periodic

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closures of commercial and recreational fishing or growing areas (Figure 2.6). Contamination of seafood products can result in economic losses in the processing and cultivation of shellfish and the aquaculture of some finfish. There may also be declines in ancillary industries, including seafood processing, transportation, wholesale and retailing (Larkin & Adams, 2007; Morgan et al., 2010).

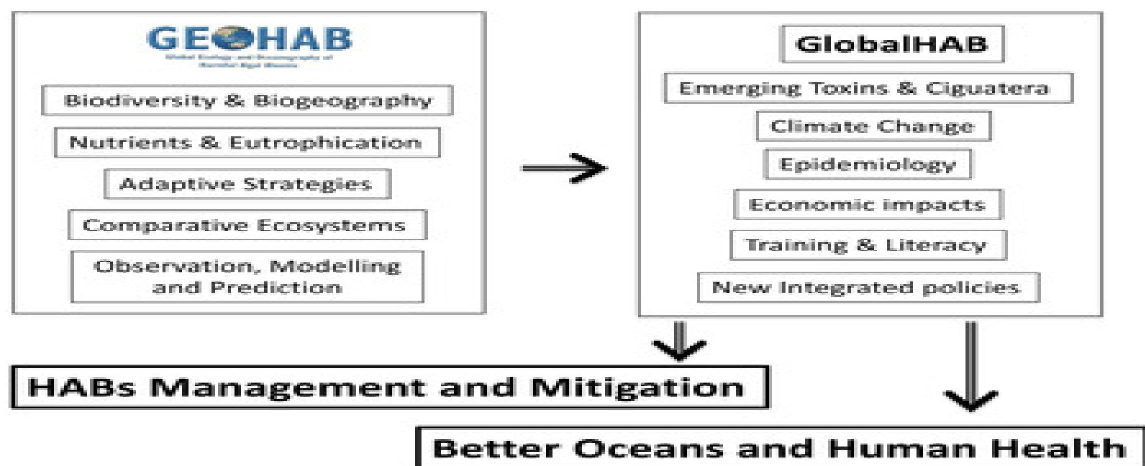

Figure 2.8: Elements GEOHABS (2000–2014) and Global HABS (2015–2018)

2.5.1 Direct Impacts of HABs On Human Health

As noted above, contamination (as mentioned above) associated with consuming contaminated seafood, skin contact with contaminated water, and inhalation of aerosolized biotoxins are related to the direct effect of HABs in marine waters on human health. There are emerging phycotoxins and risks of poisoning by biotoxin-contaminated desalinated drinking water, in addition to human health conditions associated with known toxins created by microalgae. The words' biotoxin ',' phycotoxin 'or' toxin 'will be used in this analysis to refer to toxic compounds synthesized by the microalgae of the sea.

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2.6 Scope---- World, Europe, Africa, West Africa, Ghana

There has been a global growth in recent years. In the intensity of red tides, toxic algal blooms and other harmful algal blooms (HABs), which appear to be spreading in a geographical area (Del Negro et al., 1993). The first is the emergence and growing frequency of novel flora of the phytoplankton, and the second is the spread in new areas of toxicity (Honsell et al. 1992). This is a phenomenon that includes two distinct aspects. Most of the toxic organisms belong in the group dinoflagellate in phytoplankton taxonomic information. The tissues of shellfish and fish, which are the key source of exposure to people, marine mammals and seabirds are accumulating toxins of some dinoflagellate or diatom organisms. Because of its dangerous nature, use in America, Europe and Asia has studied a significant potential threat to the protection of marine products. In Africa, South Africa, and North Africa (Morocco, Tunisia and Egypt) has been known to be monitoring harmful algae and the effect of Shellfish toxins.

As the study site for this thesis, the Gulf of Guinea, located along the west coast of Africa in the equatorial region, was selected. Other regions have been studied even more intensively, such as the North Atlantic shelf mesosystems, which would have provided a higher amount of background data for this analysis. Human exploitation of biological resources in these regions, however, has escalated to such an extent that it may well be almost impossible to distinguish natural environmental variations from anthropogenic induced ones without a strong understanding of the underlying dynamics.

On the other side, in the Gulf of Guinea, biological resources, at least until the end of the 1980s, were subject to relatively light fishing pressure (Shatz, 1998). Therefore, any association between natural variations in the climate and fluctuations in the population of

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fish should be more evident. In addition, over time, the Gulf of Guinea region has shown a high degree of physical and biological environmental variability. Many of these modifications have been adequately recorded, especially during the 1970s and 80s. Nevertheless, although many researchers have investigated the changes in this field, such studies have mostly been focused on data collected over just one or two years from only a few point sources and not much on the issue of harmful algal blooms. The complex nature of the region's dynamics has meant that few conclusions about the causes of these changes have been drawn.

There was a review by Biney (1990) on some characteristics of freshwater and coastal ecosystems in Ghana. Unlike other parts of the world, where extensive studies have been carried out on harmful algal blooms (HABs) on the marine ecosystem, knowledge of this phenomena in Ghana is rather scanty. There are no qualitative or quantitative studies on harmful phytoplankton off the Ghanaian coast, except for Hendey (1958), who reported non-toxin-producing diatoms from Takoradi Harbour, and Anang (1979), who also researched the distribution of seasonal phytoplankton variability off the coasts of Tema, Ghana. Studies on marine biotoxins produced by these harmful algae in seafood in Ghana is yet to clarify.

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2.7 Experimental Procedures for Sampling and Analysis

2.7.1 Sampling Methodologies

The Niskin bottles were used to sample phytoplankton. The samples were preserved with formalin or Lugol's solution. They have been cooled or stored and shielded from light at room temperature. Using the inverted microscope as an optical instrument, cells were regularly counted using the Utermöhl method; a stereoscopic microscope was sometimes used for the big dinoflagellate, Noctiluca scintillans (Hallegraeff et al., 1995; Throndsen, 1997).

2.7.2 Estimating Cell Numbers and Taxonomy

One of the fundamental processes in HABS control systems is the calculation of cell numbers of microalgae. Qualitative surveys may show which organisms to take into account. However, accurate knowledge of the abundance of harmful organisms is important when determining risks (Throndsen, 1997). Overall, the possibility of harmful effects is specified by high concentrations of harmful algae, while the same species may occur with no harmful effects at low densities. The HABS organism's negative concentration is species-specific (Hallegraeff et al., 1995). Some algae, such as species of the genus Alexandrium, are harmful at low concentrations and can cause PSP toxins to accumulate in shellfish at abundances of a few hundred cells per litre, while other species, such as Karenia mikimotoi, can kill millions of cells per litre of fish (Zingone and Enevoldsen, 2000). At the species/genus level, there are many microscope-based methods for quantifying microalgae. Guidelines for sampling and preservation of samples are also given for the cultivation method for estimating selected HABS species (Hallegraeff et al., 1995; Throndsen, 1997).

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Many detailed nomenclatural and other taxonomic reviews of phytoplankton taxa have been carried out over the past decades based on advances in microscopy and molecular phylogenetic research (Throndsen, 1997). For this purpose, prior to inclusion in the database, species names needed to be referenced. This was achieved using the taxon match choice available in the World Marine Species Registry (www.marinespecies.org), a universally accepted and authoritative open-access reference system maintained by VLIZ for marine species and edited by more than 240 taxonomic publishers worldwide (Hallegraeff et al., 1995). Each name of a species has a unique identifier called AphiaID20. This identifier allows the species name to be related to a generic name and associated taxonomic information that is globally recognized but also redirects to the most reliable species taxonomy information, such as recognized names and synonyms (Hallegraeff et al., 1995).

2.7.3 Phytoplankton and Harmful Algae

Phytoplankton affects almost all higher trophic levels, from copepod herbivores to zooplankton carnivores, pelagic fish, seabirds and marine mammals, because of its position as the primary producer in the ocean (Turner et al., 1997). The phytoplankton is susceptible to anthropogenic stresses. Not only can its development and composition change due to eutrophication and changes in temperature, but also due to the top-down effects of changes in higher trophic levels (e.g. through fisheries, zooplankton composition shifts). Long-term data on the structure of the phytoplankton community offers a unique opportunity to study the effect on the phytoplankton of different anthropogenic pressures and how phytoplankton can react to future changes (Hallegraeff et al., 1995).

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Phytoplankton is single-cell marine algae, some of which use flagella to travel, while others float with currents. These microscopic plants vary in size from 1/1000 mm to 2 mm and float or swim in the top 100 m of the ocean, where photosynthesis relies on sunlight. In total, basic simple inorganic chemical nutrients such as phosphate (PO_4) and nitrate (NO_3) are needed for light and oxygen (O_2). In the form of carbon dioxide (CO_2), they need carbon as well. Some phytoplanktons need a type of silicon (silicate, SiO4) as they have a glass-like shell, such as diatoms (Hallegraeff et al., 1995). While others from chains or colonies, others live as single cells.

For life on earth, marine algae are very imperative, perhaps the main living species on the planet. In at least three ways, they affect us. Second, they tend to be a major factor in regulating the greenhouse gas atmospheric carbon dioxide (CO_2), which in turn will impact heat retention in the atmosphere of the Earth. Furthermore, phytoplankton and bacteria are the base of the marine food web. Nutrients such as inorganic phosphate, nitrate, and carbon dioxide are converted at this stage (Hallegraeff et al., 1995) into more extensive, more complex organic molecules essential for life. These microscopic organisms, in turn, provide the food in the food web for the higher trophic levels or larger organisms in the food web, such as zooplankton, fish and mammals (Hallegraeff et al., 1995). Bivalve shellfish, for instance, almost exclusively eat phytoplankton for their food (oysters, mussels, scallops, clams). Finally, marine algae are important because they can produce several marine biotoxins that are highly toxic compounds. These compounds can enter the food chain and accumulate in fish and shellfish, some of which, while others are contained in the phytoplankton, can be released to the surrounding water. In most cases, these potent compounds do not appear to affect fish and shellfish, but higher species in the food web

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may become ill or even die, such as marine mammals and humans, (Hallegraeff et al., 1995). It is this very lack of impact on the fish and shellfish that we consume that makes marine biotoxins so dangerous because an external sign cannot forewarn the consumer.

In virtually all cases, only 2 can be detected by laboratory analysis of marine biotoxins produced by this phytoplankton. If conditions are right, phytoplankton can often grow and reproduce at such a high rate that they create thick, highly coloured patches in the water. They deplete the required nutrients from the water when this happens, mainly dissolved oxygen (O_2), because the growth rate is so high. Fish can suffocate when this occurs. In aquaculture, this sudden depletion in a small contained area can be a serious problem as the fish are restricted in the pens and cannot escape into more oxygenated waters.

Algal Blooms: Coastal waters are usually blue or green most of the time and relatively clear (Hallegraeff et al., 1995). Water is rarely as clear in the temperate waters of the northern latitudes as seen in tropical areas, where visibility can reach 50-75 feet. In temperate waters, visibility or turbidity limits are usually caused by algae in the water. However, in some unusual cases, until they dominate the microscopic plant community and reach such high concentrations that they decolourize the water with their pigments, a single microalgal species can increase in abundance, often referred to as a red tide. Blooms are not only red, but maybe brown, yellow, green, or milky in colour, although they are known as Red tides. High concentrations of toxic algal species can cause these blooms and are referred to as a Harmful Algal Bloom (abbreviated as HABS), but non-toxic species can also bloom and discolour the water harmlessly (Hallegraeff et al., 1995).

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Similarly, adverse effects can occur when algal cell concentrations are low, and shellfish such as clams, mussels, oysters, scallops, or small fish filter these cells from the water. Many species are affected by toxic algal blooms at higher levels of the marine food chain. Toxins, often having lethal effects, maybe passed via successive stages of the food chain. The collection of water bottles includes all but the fewest species in the water mass to achieve accurate representation of the phytoplankton's quantitative composition.

Methods of sampling of phytoplankton can be categorised into three, these are:

I. Bottle samples
II. Plankton pumps
III. Plankton nets

2.7.3.1. Bottle samplers

The recommended method is water sampling by water sampler (Sournia, 1978). The water bottle sample contains all but the fewest organisms in the sampled water mass and includes the full spectrum from the largest entities, such as colonies of diatoms in the smallest single cells (Tomas, 1997). These are suitable for quantitative collections of phytoplankton, as the appropriate amount of water from the desired depth can be obtained. Samples of water from vessels, ships or fish trawlers are generally used. As usually used for collecting water samples from any desired depth of shallow system such as the nearshore water, estuaries and mangroves, the bottle sample method is the simplest method.

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2.7.3.2 Meyers water sampler

It consists of ordinary glass or maybe bottles with a capacity of about 1-2 litres and is surrounded by a metal band. With a lead weight, it is weighted below, and two strong nylon graduated ropes are available. One was bound to the bottle's neck and the other to the cork. The closed bottle is let down to the desired depth during service, where the stopper is jerked open by a powerful cork rope pull. To hold the cork closed, water flows into the bottles, and then the cork rope is released. Afterwards, the container containing the water sample is removed from the water columns using the neck cord. These kinds of water samples could be used up to a depth of just 20 m (Hallegraeff et al., 1995).

2.7.3.3 Friedinger's Water Sampler

With two hinged covers, it is made of Plexiglas or Perspex. The sampler is sent down to the desired depth in an open state during utility, and a drop weight messenger will close it, which comes down on the sliding rail inside and seals the covers and makes the container watertight. The water is thus trapped within, along with the planktonic organisms of the stated column.

2.7.3.4 Niskin water sampler

It is used for taking water samples from subsurface levels to different depths for phytoplankton enumeration. These bottles are recommended for general purpose water sampling as a non-metallic, free-flushing sampler. These samplers can be connected to a hydro cable individually or serially and triggered by a messenger or put in any form of multisampling device (such as G.O., Sea-Bird, Falmouth Science and Small Multi sampler)

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and triggered by remote or pre-programmed commands. The Standard PVC Niskin style Sampler is made of grey PVC (RAL 7011), latex tubing spring closure with an optional stainless steel spring closure, cable attachment clamp bolts, and Multisampling System attachment mounting blocks. On both cable and multisampling systems, distribution is done with lanyards for loading. The metal components are all made of unique V4A-stainless steel. Specially designed V-LIP seal rings prevent the sampler from leaking. When the sampler is lowered, the clamp at the lower end and the plug valves are in the open position so that water can flow through the sampler. The wire rope holds the sampler in this location. It hits the release when the messenger is lowered down the chain, shutting down the valves closed by a locking mechanism. In a closed state, the water sample of the appropriate depth can then be pulled up onto the vessel so that it is sealed in the container. A group of water samplers are suspended one above the other from a wire rope for the collection of water samples from various depths 4 simultaneously and are lowered into the depths in the open state. In this scenario, before downgrading, the messenger releases another messenger that was connected to the wire clamp. The next lower sampler closes the second messenger, opening a third messenger and so on.

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2.7.3.5 Plankton pumps

Plankton pumps combine samplers which can then quickly focus on the surface through continuous filtration and inject a continuous stream of water into phytoplankton. Due to the constant collection of the pumps as the tube falls from the water column, the samples are integrated at the desired depth from the surface. This technique has its drawbacks, however, e.g. colonies are split, the big chaetoceros trees break and long cells such as Thalassiothrix spp. are split.

2.7.3.5 Plankton nets

Nets permit quantitative studies because the mesh size will select the type of phytoplankton collected. The mesh, net towing speed, and species in the water depend on gauze size, sampling via nets is highly selective. For example, Chaetoceros setae may form a good network within the gauze and single microscopic cells are retained, which move through the meshes in other cases. On the other hand, to have a sufficient diatom sample, net with very fine meshes (5 or 10 µm) sometimes filters too little water. The best mesh size is 25 µm for the collection of diatoms. Net hauls have the benefit of a simultaneous selection and plankton concentration that provides enough for the identification of species.

A typical plankton net is conical in form and has the following constituents that can be used in the surface layers (Fig 4). There is an anterior net ring consisting of stainless steel and wrapped and sealed with polythene tubing. To this, using a button and hole device, a non-filtering portion made of a coarse khaki cloth is attached. As mentioned earlier, the filtering portion is made of monofilament nylon material and is accompanied again by a

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non-filtering portion of the khaki cloth. A metal net bucket supplied with a stop cock is attached to the latter with a tight twine.

For the estimation of the standing crop, the determination of the volume of water filtered through any plankton net is crucial. The water volume traversed by the net is defined by the formula $v = r^2 d$ as an estimated value. Where V is the quantity of water pumped through the net; r is the radius at the mouth of the net; d is the distance through which the net is towed.

To separate the plankton present in it, the water collected through the various water samplers is either centrifuged or passed through fine-mesh nylon or filter paper. The smaller the sub-sample, the less unusual species can be collected. On the other hand, the concentration of large quantities of sample rich in one or a few species is pointless. The methods most used are concentration by settling, centrifugation and filtration.

Plankton concentration is generally used to resolve the damage caused by vacuum filtration, centrifugation, to specific groups of phytoplankton, especially setoid diatoms and dinoflagellates. The simple plankton concentrator, which is quite gentle in its action, consists of a stiff tube of Perspex or PVC to the end of which a filter is attached (1.2 cm diameter: 10 cm height). A filter paper (Whatman No. 42) or a membrane filter backed by a nylon monofilament netting, which acts as the filter, is glued with the aid of ethylene dichloride at the bottom of the tube. The tubes are slowly dipped into a beaker containing the phytoplankton sample during use. Water flows slowly upwards through the tube through the filter and is removed by a wide pipette. The degree of flow through the filter can be increased by forcing the tube downward.

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Centrifugation: 5-20 mL of water sample is centrifuged for over 10-20 mins at 1500-2000 rpm with the aid of an electric centrifuge. By decanting, the supernatant water is eliminated. By applying a few drops of 1 % Potassium aluminium sulphate or fixed weak neutralized formalin or Lugol's solution, the plankton is precipitated.

2.7.4. Fixation and Preservation

If samples obtained are kept alive, they should be preserved or refrigerated in an ice chest and then preserved for just a few hours. The collected plankton should be stored in fixatives and preservatives for long term study. Formalin is a very commonly used fixative and preservative for a number of species, including plankton. Commercial formalin is obtained in water dissolved as a 40 percent (saturation limit) formaldehyde. Formalin must be contained in containers of inert glass or plastic and not in containers of metal, as formalin reacts with the latter. Dissolved contaminants such as iron and formic acid, which disintegrate shells of certain planktonic species, can also contain commercial formalin. However, by adding excess calcium carbonate, the acid content of industrial formalin can be neutralized. 2 percent neutralized formaldehyde (i.e. formalin) can be used to preserve the net and other phytoplankton samples.

2.7.4.1 Lugol’s Solution

In particular, it is a healthy preservative to preserve the flagella and cilia for flagellated and ciliated phytoplankton. It consists of 10 g of iodine dissolved in 200 mL of distilled water and 20 g of glacial acetic acid and 20 g of potassium iodine. The solution can be prepared a few days in advance and placed for convenience in a dark bottle. A ratio of 1 component to 100 parts of the seawater sample is applied to Lugol's solution. Around five drops of this

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preservative is adequate for around 250 mL of a water sample containing nanophytoplankton.

2.7.4.2 Bottling

In particular, phytoplankton diatoms are favoured for storage in bottles made of soft glass. Since surface water is typically silicate saturated, storage in high-quality glass bottles such as Pyrex that does not release much silicate or plastic bottles may result in a slow solution of delicate frustules or diatom spines. This may occur in one to a few years in plastic bottles. Further use of very low-quality glass for phytoplankton storage can result in precipitates. A leak-proof cork seals the bottles. After the plankton study, the contents of the bottles and the permanent preservation of the plankton or wax coating are placed around the cork of the container after the closure of the latter. It will prevent loss of formalin by evaporation in the long term.

2.7.4.3 Labelling

Proper labelling of the plankton samples collected and bottled is essential. All sorts of information about the processing of plankton should be written on the labels in order to be able to classify the plankton samples reliably. To ensure proper identification of the sample, the label should provide relevant details about the sample obtained. A light coloured waterproof marker is used to write the name.

Traditional techniques for estimating the richness of marine phytoplankton organisms are the selection of a small amount of seawater that is examined under the microscope (Lund et al., 1958; Utermöhl, 1958; Sournia, 1978). Generally, depending on the photosynthetic biomass concentration, the sample volume varies between 5 and 100 mL. This approach is

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based on the premise that with the community's biomass, the number of phytoplankton species and thus, the chance of sampling them increases. A strong body of research, however, indicates that both prokaryotic and eukaryotic microbial plankton populations contain a large pool of unusual species with low population abundances (Pedrós-Alió, 2006; Sogin et al., 2006; Caron and Countway, 2009).

2.7.5 Phytoplankton Identification and Counting

2.7.5.1 Estimating Cell Concentration

Using the 'drop on slide' method is a simple method of quickly estimating the microalgae concentration. One drop in seawater is around 0.02 mL or 20 µl. The drop is put on a slide, mounted with a coverslip and counted to observe the species of interest at sufficient magnification. This is equivalent to approximately 50 cells mL-1 = 50,000 cells L-1 if one cell is contained in the drop. This approach is only useful for high-concentration screening for algae. L-1 counting using a compound microscope and a counting cell is easy and fast with concentrations of harmful algae > 104 cells. If, on the other hand, cell concentration is < 10^2–10^4 cells / L, before counting, the cells must be concentrated. This is a process that takes time. Counting using either inverted microscopy or epifluorescence microscopy is favoured in this situation. In many different quantities, counting cells are available. The Sedgewick-Rafter cell, with a volume of 1 mL, is a well-known form. The Sedgewick-Rafter cell's bottom plate is broken into 1,000 squares, each representing 1/1,000 of the volume of the cell. As the weight differential between the empty counting cell (including the coverslip) and the filled counting cell (including the coverslip), carefully dried out on

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the outside of the chamber using a tissue to extract excess water, the exact volume of the home-made counting cell can be determined. You may choose to count the microalgae in the whole bottom of the chamber or just a fraction of the whole bottom using a counting cell. The total number of microalgae in the counting cell can be determined by knowing the fraction of the whole bottom count and the number of microalgae encountered. When measuring the concentration of the microalgae (cells L-1), note to add the dilution/concentration factor of the sample.

2.7.5.2 Quantification of Algae

According to Utermöhl (1958), quantification of harmful algae using inverted microscopy and sedimentation chambers is useful for counting algae at rather low concentrations (< 102–104 cells / L) (Sournia, 1978). The samples may be diluted using filtered seawater before counting if the concentrations are higher. An inverted microscope, equipped with suitable slide holders, is also ideal for qualitative examination of normal slide preparations or quantitative analysis using counting cells. Sedimentation chambers are available in a variety of volumes from 2.5-50 mL from various companies. The bottom of the 50 mL settling cylinder may be greased with a thin layer of Vaseline until it is placed on the plating chamber to hold the sedimentation chambers secure. Similarly, to retain the coverslip in place and to keep the settling cylinder properly sealed and airtight, the top of the settling cylinder may be greased. The sedimentation chambers should be positioned on a horizontal surface during filling and sedimentation to facilitate a random distribution of the settling-out microalgae. In addition, the supporting surface is significant to be vibration-free, as vibration can cause cells to accumulate in ridges (HELCOM, 2000). Depending on the

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sample volume (the height of the chamber), the fixative used and the longitudinal dimension of the cells, the settling of cells in the sedimentation chamber lasts from a few hours to several days. Small cells, in general, have much longer sedimentation times than big cells. Large cells ($L > 10$ µm) must usually be allowed to settle for at least 12 hours before counting, while smaller cells must be allowed to settle for around 24 hours before counting. It is sufficient to discard deposited cells not counted within a week. 100 mL sedimentation chambers should be used with caution because it has been documented that convection currents interfere with microalgae sedimentation in chambers taller than five times their diameter (Nauwerck, 1963; Hasle, 1978). At different magnifications, the bottom of the sedimentation chamber is scanned, and a preliminary species list is produced. You need to know the concentration (cells L-1) of the various species in the counting chamber to assess

V = volume of chamber (mL);

Ba = area of the bottom of the chamber (mm^2);

Bc = area of the part of bottom counted (mm2);

N = number of cells scored for species of interest;

conversion factor (CF) = Ba÷Bc

The concentration C of species (cells mL^{-1}) is then

$$\mathbf{C = N \times (Ba/Bc)/V}$$

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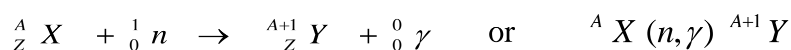
2.8 Neutron Activation Analysis

Neutron activation analysis (NAA) is a nuclear analytical technique for the detection and determination of the contents of elements in a given material. NAA is based on bombarding the stable isotope (or one of its stable isotopes, if several exist) of the element of interest in the material with neutrons, resulting in the production of a radioactive isotope of the element of interest. γ-rays emitted by the radioactive isotope produced with suitable properties for measurement (most frequently by γ-ray spectrometry) are then analysed (Kučera et al., 2000). The general nuclei equation can represent the (n,γ) reaction:



$$^{A}_{Z}X + ^{1}_{0}n \rightarrow ^{A+1}_{Z}Y + ^{0}_{0}\gamma \quad \text{or} \quad ^{A}X\,(n,\gamma)\,^{A+1}Y$$

Where Y is the daughter nuclide; n is neutrons and X is the parent nuclide.

NAA is free from reagent blank and has a high sensitivity; this makes NAA a reliable and excellent analytical tool (Kučera et al., 2001). There are several approaches to the use of NAA for the determination of arsenic, iodine and selenium. These are instrumental neutron activation analysis (INAA), epithermal neutron activation analysis-Compton suppression counting (ENAA-CSC), pre-concentration neutron activation analysis (PCNAA), and radiochemical neutron activation analysis (RNAA).

The possibility of using INAA is limited because of the high background formed by neutron activation products of Na, K, Cl, Mn and Br, which are present in large amounts in many biological materials including food (Bhagat et al., 2007; Serfor-Armah et al., 2003).

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Activation with epithermal neutrons (ENAA) followed by Compton suppression counting improves the signal-to-background ratio significantly (Serfor-Armah et al., 2003; Kučera et al., 2001); better possibilities are offered by the application of preconcentration neutron activation analysis (PCNAA) and radiochemical neutron activation analysis (RNAA) (Kučera et al., 2001). PCNAA procedures are, however, prone to contamination before irradiation thereby affecting levels of the element determined (Kučera et al., 2001). The use of PCNAA also results in the introduction of an analytical blank (like in other trace analytical techniques) which is virtually absent in RNAA (Kučera et al., 2004a).

General NAA equation

In NAA, the element to be determined is assayed using a nuclear reaction of its stable isotope (or one of its stable isotopes, if several exist) by conversion, mostly by the (n, γ) reaction, to the radioactive indicator with suitable properties for measurement (most frequently by γ-ray spectrometry) (Kucera, 2000). In (n, γ) activation analysis, when the sample is irradiated in the whole reactor spectrum, the mass fraction, C_m, of the element to be determined is given by the equation (Kucera, 2000):

$$C_m = \frac{M_a \left(\frac{N_p / t_c}{SDCW} \right)_a}{N_A\, \theta_a\, \gamma_a\, \langle (G_{th,a}\, \Phi_0\, \sigma_{0,a} + G_{e,a}\, \Phi_e\, I_{0,a}\, (\alpha) \rangle\, \varepsilon_{p,a}\, \eta_a} \quad (1)$$

where:

M	-	molar mass
N_A	-	Avogrado's number
θ	-	relative isotopic abundance of target isotope
γ	-	absolute gamma-intensity (emission probability) of the radioisotope
N_p	-	net number of counts in the full-energy peak corrected for pulse losses
t_c	-	counting time
W	-	sample mass
G_{th}	-	correction factor for thermal neutron self-shielding
G_e	-	correction factor for epithermal neutron self-shielding

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Φ_0 - conventional thermal neutron fluence rate
σ_0 - activation cross-section for neutrons with the energy of 0.025 eV
Φ_e - conventional epithermal neutron fluence rate
I_0 - resonance integral including the 1/v tail
α - measure for the deviation of the epithermal neutron fluence rate distribution from the $1/E^{1+\alpha}$ function
ε_p - full-energy peak detection efficiency, including correction for gamma attenuation
η - chemical yield of separation (if pre-irradiation or radiochemical separation is carried out)

S, D, and C are saturation, decay and counting factors, respectively, and are given as

$$S = 1 - e^{-\lambda t_i}$$

$$D = e^{-\lambda t_d}$$

$$C = (1 - e^{-\lambda t_i}) / \lambda t_c$$

Where λ = decay constant ($\lambda = \ln 2/T_{½}$)
t_i, t_d and t_c are irradiation, decay and counting times respectively
$T_{½}$ is the half-life of the radionuclide (Kucera, 2000).

2.8.1 Standardization methods in NAA

Three standardization methods can be used for quantification of the mass fraction of the analyte in RNAA (Kučera et al., 2000). The methods are: **(a)** Relative standardization (using synthetic element standards or primary matrix reference materials); **(b)** k_0-standardization; and, **(c)** Absolute standardization (parametric).

Absolute standardization is associated with unacceptable uncertainties concerning some nuclear parameters (Kučera et al., 2000). The k_0-standardization and relative standardization methods are mostly used. In this study, the relative standardization method of quantification of the concentration of analyte was used.

2.8.2 Relative standardization

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In this method, a standard of known mass, M_{STD}, is irradiated together with the sample of mass, M_{Sam}, under the same conditions. The standard is typically placed very close to the sample (to eliminate errors due to inhomogeneity in the neutron flux). γ-activity measurements are performed with γ-spectrometry under practically identical conditions, as any variation can remarkably affect the precision. Both sample and standard are counted with the same HPGe γ-ray detector using the same geometrical arrangements. Calculation of the mass fraction of analyte, $\mathbf{C_{analyte_sam}}$, is based on comparison of the activities of the sample and the standard and it is given by the expression:

$$C_{analyte_sam} = \frac{C_{analyte_STD} \quad M_{STD} \quad A_{sam}}{M_{sam} \quad A_{STD}} \tag{4}$$

Where $\mathbf{C_{analyte_STD}}$ is the mass fraction of the analyte in the standard.

$\mathbf{A_{Sam}}$ and $\mathbf{A_{STD}}$ are the activities of the sample and standard, respectively.

2.9 Biotoxin Detection: Analytical Methods for PSP

2.9.1 Fluorometric Assay

There is no intense ultraviolet (UV) absorbance or native fluorescence in the PSP toxins. Based on the oxidation of the toxins in alkaline solution to fluorescent aminopurine derivatives, a fluorometric assay method was developed (Bates and Rapoport, 1975). The fluorescence strength of the oxidation products in the solution is calculated by fluorometry in the cup after acidification. As individual PSP toxins vary both in toxicity and fluorescence intensity after oxidation, the interpretation of the results of this fluorometric assay method is complicated. The carbamate toxins [saxitoxin (STX), neosaxitoxin (NEO)

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and gonyautoxins (GTX1-GTX4)] are the most toxic, the least toxic to N-sulfocarbamoyl toxins (B1, B2, C1-C4) and intermediate toxicity to dicarbamoyl (dc-) toxins.

The N-1 hydroxylated toxins (e.g. NEO, GTX1, GTX4) typically display a lower molar fluorescence yield than their respective analogues lacking the N-1 hydroxyl moiety (STX, GTX2, GTX3); however, there is no association between toxicity and strength of fluorescence (Franco and Fernández, 1993). This fluorometric assay may be adequate for a rapid screening method, but the independent chromatographic separation of the PSP toxins, followed by fluorescence detection of the oxidation products, is recommended if the assay is intended for quantitative purposes.

2.9.2 Chromatographic methods for PSP Determination

2.9.2.1 Liquid chromatography

Knowing which PSP toxin components are present in a sample of interest is always imperative. As the oxidative fluorescence assay gives no knowledge about individual toxins, more advanced analytical methods have been established to overcome these components. In early studies, open-column (low-pressure) chromatography was used to fractionate various PSP toxin components from shellfish and dinoflagellate matrices, but now, when combined with fluorescence detection, high-performance liquid chromatography (HPLC) is the most commonly used analytical technique (Van Egmond et al., 1992), offering excellent peak resolution and high sensitivity for each toxin. In the literature, there are several alternative HPLC methods listed for PSP toxin determination. Most are based on the identification of oxidized PSP toxins by fluorescence. Before or after chromatographic separation, the formation of fluorescent aminopurine derivatives can be

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achieved. In an intercalibration exercise, the value of full chromatographic separation of all PSP toxins that lead to total PSP toxicity prior to quantification with a fluorescence detector has been confirmed. The results of this intercalibration clearly showed that the HPLC methods for evaluating PSP toxins were superior to the enzyme-linked immunosorbent assay (ELISA) initially developed for the detection of STX and for which there was an inadequate description of cross-reactivity with other PSP toxin analogues (Van Egmond et al., 1994). In addition, the quantitative and qualitative data from the intercalibration exercise showed that alkaline oxidation after chromatographic separation of underivatized PSP toxins (post-column oxidation) was most advantageous. However, different findings obtained with different chromatographic methods for the determination of PSP toxins indicated that it was important to view the conclusions to be drawn from this intercalibration exercise with caution (Quilliam, 1995). For the separation and identification of the various PSP toxins, the HPLC techniques with fluorescence detection give strong sensitivity and dynamic range. In order to obtain reliable performance, this sensitivity depends on parameters such as reagent concentrations, reaction time, pH, and oxidation reaction temperature, all variables that need to be carefully regulated.

2.9.2.2 Pre-column derivatization with fluorescence detection

Centered on pre-chromatographic oxidation, a liquid chromatographic approach has been produced using both hydrogen peroxide and periodic acid (Lawrence and Ménard, 1991; Lawrence et al., 1991, 1996). After periodate oxidation at around pH 8.7, the N-1 hydroxylated toxins, NEO, B2, GTX1 and C3, form fluorescent products, but do not form fluorescent derivatives with peroxide oxidation under the conditions specified. With both peroxide and periodate oxidation, the non-N-1 hydroxylated toxins, STX, B1, GTX2,

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GTX3, C1 and C2, form strongly fluorescent derivatives. The addition of ammonium formate to the periodate oxidation reaction significantly enhances the N-1 hydroxylated toxins' fluorescence yield (Lawrence and Ménard, 1991). This approach does not isolate the oxidation products of NEO and B2, so the parent compounds are isolated before oxidation using an ion-exchange cartridge. Two fluorescent products for GTXs, dcGTXs, dcSTX and dcNEO, are provided by both periodate and hydrogen peroxide oxidation. Neosaxitoxin cannot, under defined conditions, be determined by the application of hydrogen peroxide oxidation. Why decarbamoyl toxins function is still unknown and whether or not they interfere with the quantification of other PSP toxins (Figure 2.7)

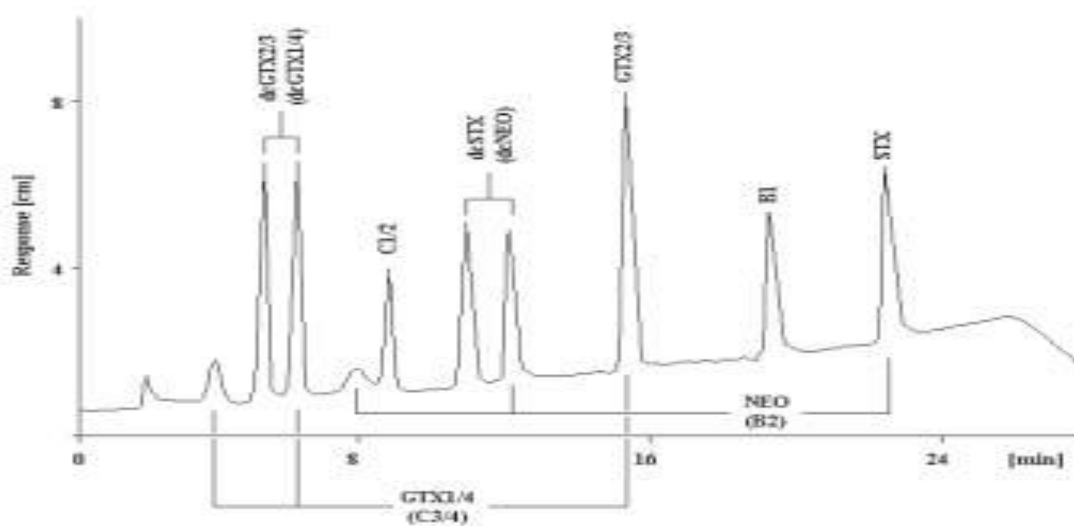

Figure 2.8: Oxidation Products of PSP toxins (Lawrence et al., 1996)

2.9.2.3 Post-column derivatization with fluorescence detection

With the effective isolation of underivatized PSP toxins by ion-pair chromatography with alkyl sulfonic acids, using a polymeric stationary step (polystyrene divinylbenzene) in the analytical column (Sullivan and Wekell, 1984), a breakthrough was made in the use of HPLC methods for PSP toxin determination. In this process, with a phosphate buffer

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containing n-hexane sulfonic acid and n-heptane sulfonic acid as ion-pair reagents, a solvent gradient is used. In a flow-through post-column derivatization unit with separate channels for oxidant and neutralizing, post-column derivatization is carried out with periodic acid as the oxidizing reagent.

Uh, acid. This approach offers a strong separation in a single chromatographic run of most of the high-potency carbamate toxins (Fig. 2.8).

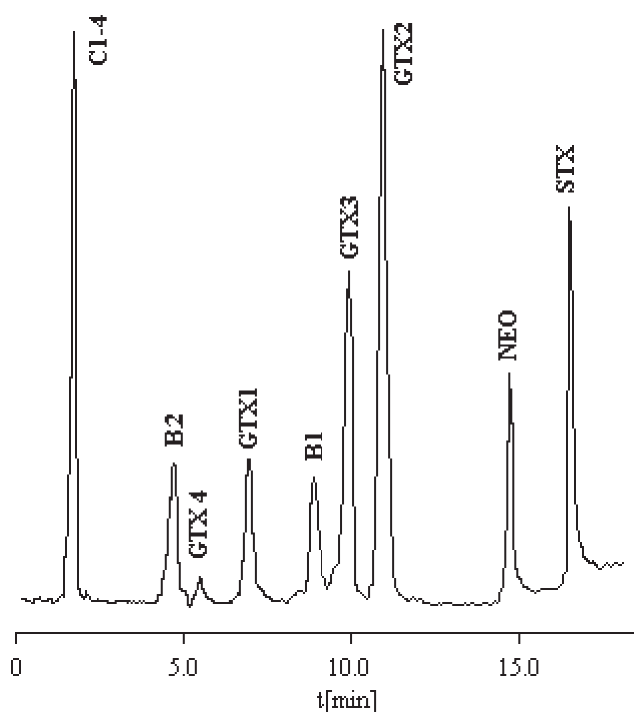

Figure 2.9: HPLC separation of PSP toxins (Sullivan and Wekell, 1984)

This method of polymer-columned separation is relatively stable but has some disadvantages. Toxins of N-sulfocarbamoyl (C1-C4) are not isolated and are hard to distinguish from fluorescent artefacts that co-elute on the front of the solvent and toxins of decarbamoyl (dc-) are not resolved from their respective analogues. The coelution of STX and dcSTX in the study, for example, would create an inaccurate estimation of total PSP

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toxicity, as STX is molarly twice as toxic as dcSTX (Fig. 2.8). Therefore, resolution and separate quantification of STX and dcSTX is needed for comparison of HPLC findings with mouse bioassessments, at least where the presence of decarbamoyl derivatives is known or strongly suspected (Luckas et al., 1990). A total of 3 different chromatographic states, separated by isocratic HPLC into the whole spectrum of known PSP toxins (Group 1: C1-C4; Group 2: GTX1-GTX4, dcGTX1-dcGTX4, B1, B2; Group 3: NEO, dcSTX, STX). A reverse-phase(RP) octyl silica (C8) column is used as a stationary phase. N-heptane sulfonic acid is developed as the ion pair forming agent for carbamate separation and decarbamoyl toxins to isolate the N-sulfocarbamoyl-11-hydroxyzide toxins C1–C4, butyl-ammonium phosphate (Eluant A), and eluents B and C. This approach suffers from relatively high time and material costs per test since three separate chromatographic runs are needed for the quantity of all PSP toxins in a sample. Figure 2.9 demonstrates the HPLC distinction, as shown by Oshima et al., (1989), of the Eluent B carbamate toxins and the Eluent C toxins. After the system's implementation, numerous changes were made, including those suggested by the creator (Oshima, 1995).

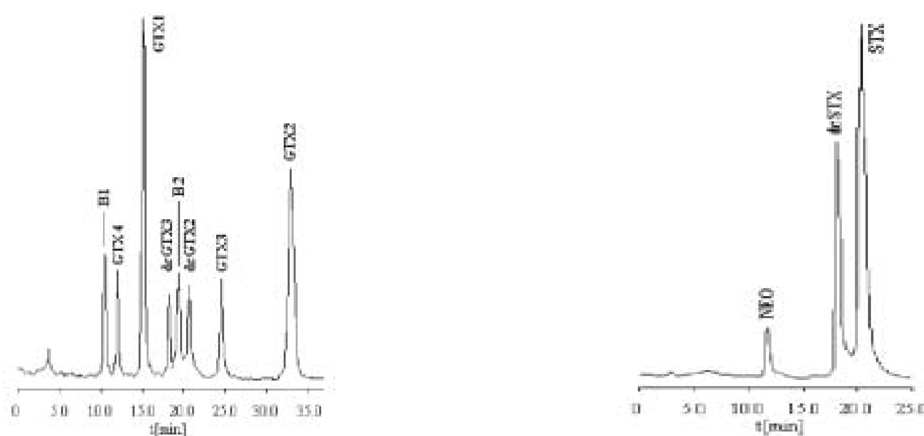

Figure 2.10: HPLC separation of PSP toxins (Oshima et al., 1989)

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Thielert et al. (1991) implemented ion-pair chromatography on an RP-C18 stationary step in an alternative post-column oxidation strategy, using n-octane sulfonic acid and ammonium phosphate in the eluent. Isocratic elution allows the separation of STX and dcSTX with this procedure, but issues have emerged with the separation of GTX components. To address this problem, a gradient with two buffers was later implemented. The two eluents contain n-octane sulfonic acid, phosphate, and tetrahydrofuran in the current process. In addition, the second eluent produces acetonitrile. This method of HPLC allows the carbamate, dicarbamoyl and N-sulfocarbamoyl toxins to be isolated extensively. Unfortunately, this technique is used by the GTX1 and GTX4 (Fig. 2.10)

It is advantageous to apply two different HPLC methods to exclude inconsistencies in the determination of PSP toxins, especially in a complex matrix with a broad spectrum of toxin analogues, and where total quantification is needed. Co-application of the HPLC techniques of Thielert et al. (1991) and Oshima et al. (1989), for instance, is an effective (though costly and time-consuming) solution to these problems. Fortunately, when using octane sulfonic acid (Thielert et al., 1991) instead of n-heptane sulfonic acid, the elution order of GTX2 and GTX3 is reversed (Oshima et al., 1989). It is possible to adjust the HPLC method of Oshima et al. (1989) using Eluents B and C with a two-step elution. No complex gradient is needed, unlike the method of Sullivan et al. (1985), and the analysis of PSP toxins is done by only moving from the first to the second eluent, which is consistent with the method of Thielert et al. (1991). The joint assessment of the chromatograms obtained using the various methods results in a precise quantification of all specific PSP toxins for the estimation of the total toxicity of PSP in the sample.

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High phosphate and ion-pair reagent concentrations in the eluent stabilize the chromatographic system, especially after extracts containing complex components of the matrix have been injected. Consequently, the application of HPLC methods with high concentrations of ammonium phosphate in the eluents does not detect any baseline drift or differences in retention periods (Hummert et al., 1997). In experiments aimed at resolving the process deficiencies, i.e. co-elution of GTX1 and GTX4, maintenance of a concentration of 40-50 mM ammonium phosphate in the buffers used as eluents in the process of Thielert et al. (1991) was therefore considered. Hummert et al. (1998) developed an efficient alternative method of HPLC based on the eluents used by Thielert et al. (1991) to overcome this limitation. A different column of HPLC was used, and a third mobile process (Eluent C) was implemented. The pH of the eluents (to pH 6.9) was also optimized, and a four-step elution was proposed. For the determination of PSP toxins in microalgae and mussels, this approach has been successfully applied (Yu et al., 1998). In comparison to the Thielert (1991) process, toxin separation, especially for GTXs, has been improved. In addition, the extraction process, using both acetic acid and hydrochloric acid, enables the findings to be compared with those obtained by the bioassay of the mouse.

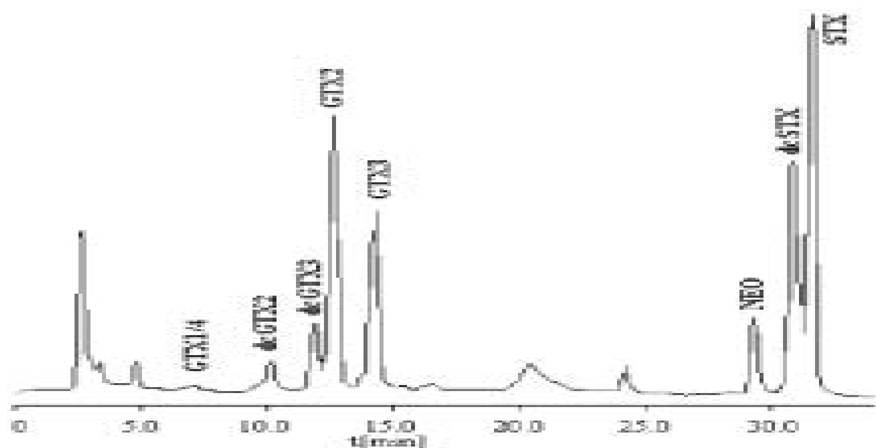

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Figure 2.11: HPLC separation of PSP toxins from mussels (*Mytilus edulis*)

During the post-column chemical reaction (PCRS), the chemical oxidation of the STX ring system to form a corresponding fluorescent derivative is very sensitive to changes in temperature, flow rate and age of the post-column reagents. STX ring system oxidation can also be achieved electrochemically, and the use of a coulometric electrochemical cell provides both advantages and disadvantages for liquid chromatography of PSP toxins over more conventional chemical oxidation (Boyer and Goddard, 1999). Electrochemical cells need fewer pumps and are less susceptible than the PCRS system to changes in flow rate. Electrochemical oxidation also enables the rapid detection of impurities that are naturally fluorescent and can co-chromatograph with PSP toxins. Real PSP toxins are not naturally fluorescent, but when the oxidizing voltage is shut off, their signal disappears. As a drawback, it is simple to plug the small pores in the electrochemical cells, and special care must be taken in the preparation of the sample. Excessive oxidation of NEO to several components, some of which are non-fluorescent, as with the pre-column oxidation system, also causes the electrochemical system to be less sensitive to N1-hydroxy compounds, such as NEO, GTX1 and GTX4. Electrochemical oxidation is consistent with the previously mentioned mobile phases (Sullivan and Wekell, 1984; Oshima et al., 1989; Hummert et al., 1998), except for the Oshima Eluant C procedure (1989). As there is no post-column buffer to regulate the oxidation pH, this function must be served by the mobile step. Under acidic conditions, oxidation of the STX ring system does not occur, so acidic mobile phases such as Eluent C (pH 4.5) (Oshima et al., 1989) require alkaline buffer post-column addition for oxidation. A typical separation and detection chromatogram of NEO and STX following electrochemical oxidation is shown in Fig. 2.12.

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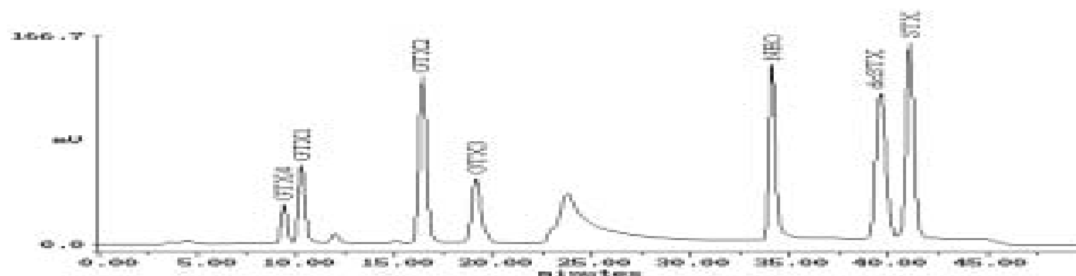

Figure 2.12: HPLC separation of PSP toxins [Hummert et al., 1998]

Representative HPLC trace of the PSP carbonate toxin components of the Alexandrium tamarense PSP-4 dinoflagellate strain; Conditions: column of Inertsil C-18, 4.6 mm ?? 150 mm. Mobile phase: A, Eluent C and B, 0.8 mL min-1 of Eluent B (Oshima et al., 1995); 800 mV ECOS voltage. Detector: excitation at 330 nm; emission at 390 nm.

2.9.2.4 Fluorescence and mass spectrometric detection

For confirmative analysis of samples containing PSP toxins, the use of mass spectrometry (MS) as a detection method is desirable. However, efficient LC / MS coupling is prevented by mobile-phase components such as phosphate, ion-pair formers, etc., as well as periodic acid from the post-column derivatization unit. An alternative HPLC method was therefore developed, enabling the chromatograph to be directly coupled with the mass spectrometer (Kirschbaum et al., 1995). STX 's mass range of HPLC-Atmospheric Pressure Ion Source / Electrospray Ionization (API / ESI) (Figure 2.13).

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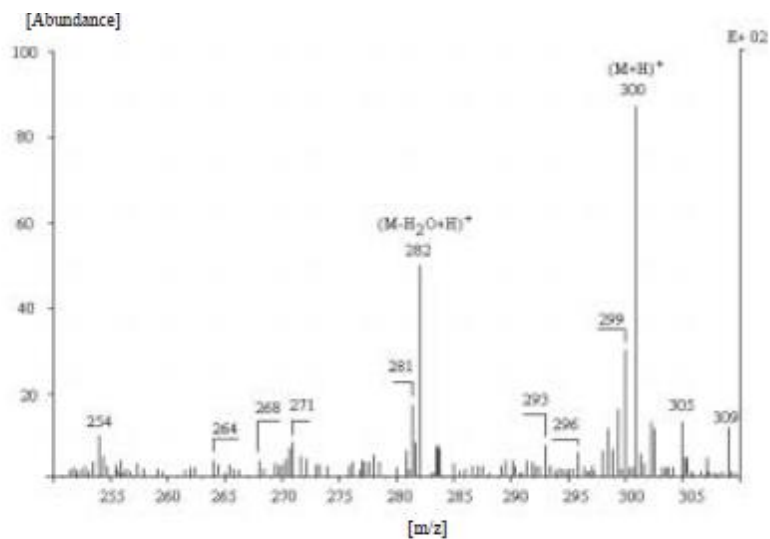

Figure 2.13: HPLC- API/ESI mass spectrum of STX [Kirschbaum et al., 1995]

The separation of PSP toxins is achieved on a weak cation exchange resin using an aqueous eluent with ammonium acetate as the only additive. In case of a parallel application of fluorescence (FLD) and MS detection, electrochemical post-column derivatization is suggested. Such an LC/FLD/MS system not only allows the unambiguous determination of PSP toxins but is also well suited for the analysis and certification of PSP toxin standards (Figure 2.13).

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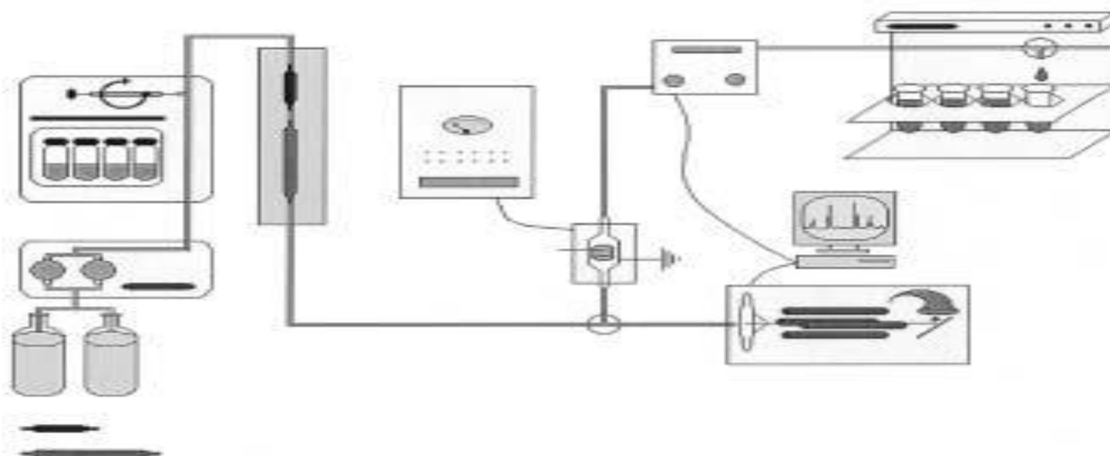

Figure 2.14: Automated HPLC system

Automated ion-exchange column HPLC system, fluorescence detector electrochemical cell, and mass spectrometer (fraction collection as an additional option). Figure 2.14 is shown (Jaime et al., 2000). Recently, by adding a combination of strong anion- and cation-exchangers, modification of the HPLC / FLD / MS method allowed the ion chromatographic separation within a single chromatographic run of all PSP toxins applicable to seafood regulation (Jaime et al., 2000). This method is well suited for the determination of PSP toxins in biological materials, both by fluorescence and MS detection, as it is possible to inject relatively large volumes into the HPLC / FLD / MS system (Figure 2.14).

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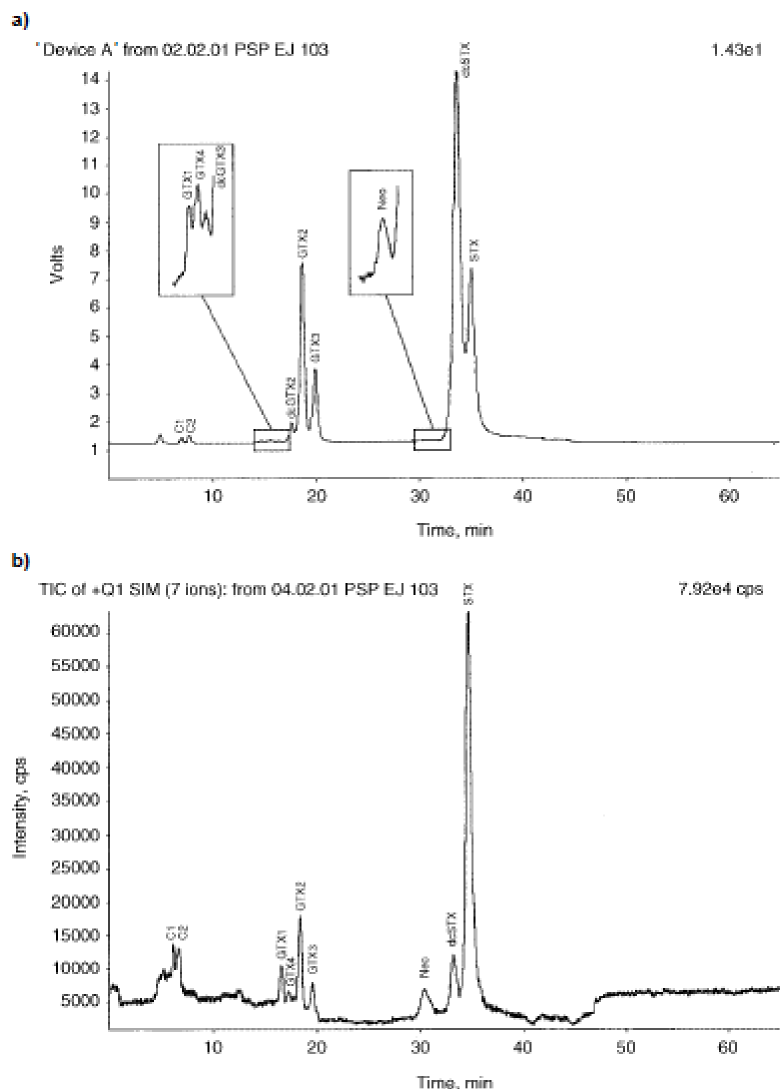

Figure 2.15: HPLC/FLD/MS determination of PSP toxins [Jaime et al., 2001]

Figure 2.15: HPLC/FLD/MS determination of PSP toxins: C1–C2 (3.5 ng each), GTX1 (17.2 ng), GTX4 (8.9 ng), dcGTX3, dcGTX2, GTX2 (28.7 ng), GTX3 (10.4 ng), NEO (34.8 ng), dcSTX (25.0 ng), STX (39.9 ng). A, fluorescence chromatogram; B, TIC chromatogram.

Short-run elution is possible through the application of ion-exchange chromatography with MS detection. In combination with MS detection, isocratic elution enables all PSP

toxins that are critical for regulatory regimes to be quantified, but detection limits are higher than with fluorescence detection. However, the sensitivity of MS detection is adequate to monitor the content of PSP toxin (800 μgSTXequivalents kg-1 wet weight of soft tissue) at the regulatory limit for seafood (Figure 2.15).

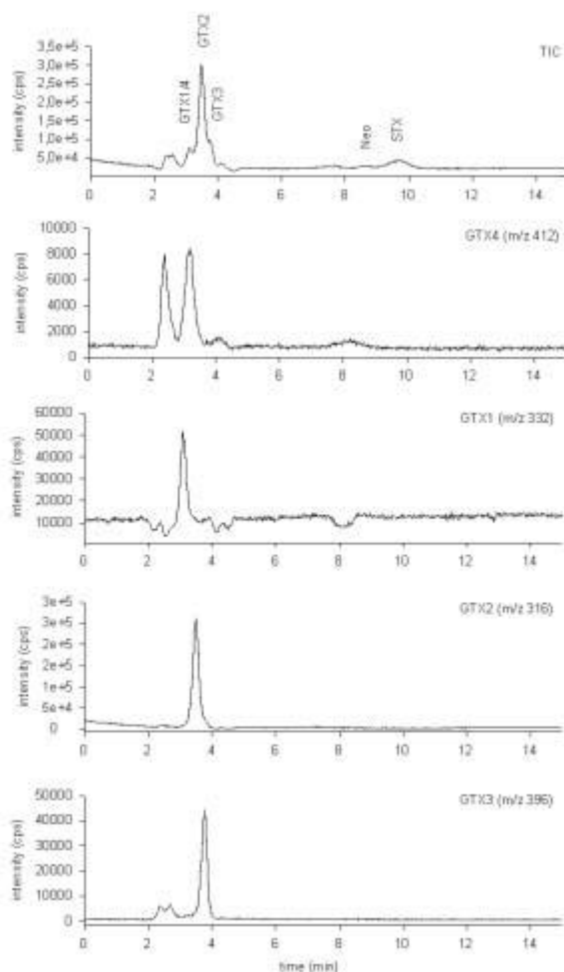

Figure 2.16: Ion-exchange HPLC/MS (Sim mode) [Jaime et al., 2001]

Figure 2.16 shows Ion-exchange HPLC/MS (Sim mode) determination of PSP toxins in mussels (Mytilus chilensis).

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2.9.2.5 Capillary electrophoresis with UV and mass spectrometric detection

For analysis of PSP toxins, many options, other than liquid chromatography, are available. Capillary electrophoresis (CE) has shown some promise among these alternatives, though this method is not in widespread use. For the separation and determination of underivatized PSP toxins, a capillary electrophoresis approach with UV detection was established (Thibault et al., 1991). Differential migration of solutes in an electric field is achieved by separation by CE. Electrophoresis is performed in CE in narrow-bore capillaries, which are typically buffer-filled. Using high electrical fields results in fast study times (a couple of minutes) and high efficiency and resolution separation. The electro-osmotic flow (EOF) also enables all solutes, irrespective of charge, to be analyzed simultaneously.

CE's flexibility is partly derived from its various operating modes. Each mode's separation mechanisms are different and can thus provide additional details. Fundamentally, the simplest type of CE is capillary zone electrophoresis (CZE). The capillary is loaded with a buffer in this procedure, and separation occurs because solutes migrate at various speeds and in distinct zones. Selectivity in CZE can most easily be altered by changes in the pH of the running buffer or by using buffer additives. A combination of two buffer systems is used in capillary isotachophoresis (CITP) to create a state in which the divided zones all travel at the same velocity. Sandwiched between so-called leading and terminating electrolytes, the zones remain. It is possible to analyze either cations or anions in a single CITP experiment (Heiger, 1992). For the study of PSP toxins, the implementation of on-column sample pre-concentration with CITP and discontinuous buffer systems before CZE has been investigated.

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(Locke and Thibault, 1994). Figure 2.17 presents the separation by CITP / CZE of a mixture of eight PSP toxin standards. The application of such a pre-concentration ('stacking') technique prior to CZE for the study of decarbamoyl toxins was demonstrated by Buzy et al. (1994). It was found that the separation conditions developed were entirely compatible with electrospray MS. This allowed crude enzyme digests to analyze PSP toxins and their decarbamoyl derivatives. Using CE combined with tandem MS, the products released during the enzymatic digestion were identified. CE separations, however, require highly purified extracts to obtain a reproducible separation. In addition, the detection limit of this process is roughly an order of magnitude greater than for HPLC with fluorescence or MS detection, given the minimal volumes injected.

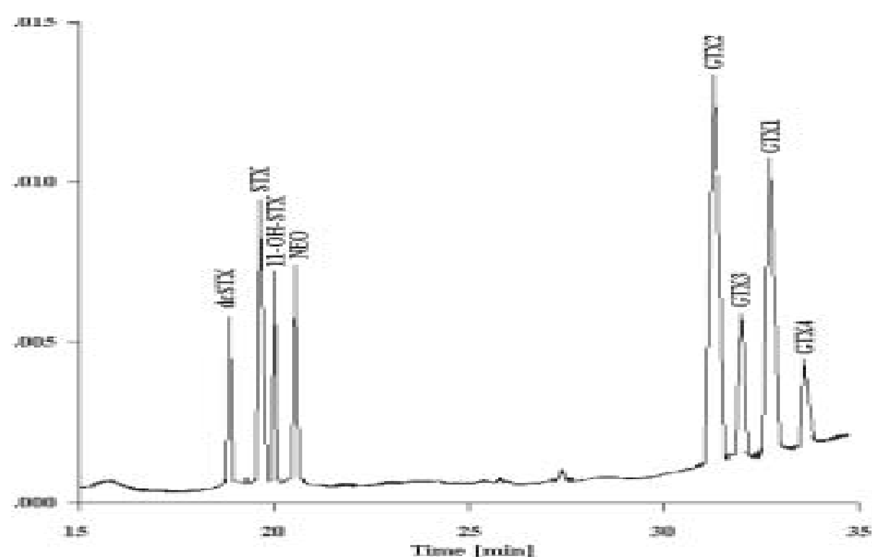

Figure 2.17: CITP/CEZ/UV analysis of a mixture of eight PSP toxin standards

Figure 2.17 shows CITP/CEZ/UV analysis of a mixture of eight PSP toxin standards. Injection of 2.2 µL of a solution containing 500 ng mL-1 GTX1 and GTX2 and 200 ng mL-1 for all other PSP toxins. Source: Locke and Thibault (1994).

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For the determination and quantification of most PSP toxin types, the HPLC chemistry process with fluorescence detection and pre-column oxidation is used. More chemical methods are still being developed for evaluating PSP toxins, such as LC-MS, for example.

Biomolecular methods are other methods capable of detecting STX-group toxins, but they are only suitable for screening purposes. Using receptors, cytotoxicity characteristics and antibodies, the biomolecular methodologies for STX community toxins are focused on three distinct strategies. With regard to the above approach, it should be noted that, while the antibodies are very sensitive, the lack of good cross-reactivity for all members of the group is their key problem in detecting toxins from the STX group. Since the toxicity variations between the compounds in such a group can also be very high, the toxicity does not always correlate to the levels of toxin quantified by the antibody.

The official tests for toxins in the DA community are based on LC with UV detection. A fully intra-laboratory-validated LC-MS approach can also be used as a screening device for that category of toxins. The use of the 2006.02 ASP system based on the Enzyme-Linked Immunosorbent Assay (ELISA) was suggested by Regulation (EC) No 1244/2007 as a screening technique for toxins in the DA community.

This demonstrates some benefits because it is responsive and quick, can be automated, requires a minimum of training and does not require comprehensive equipment. However, it must be considered that the HPLC chemical method with a UV detector is the only reliable reference method in the event of conflicting findings. The Standard Operating Procedure was validated in an inter-laboratory validation analysis performed by the

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National Reference Laboratories of Belgium, France, Germany, Ireland, Italy, the Netherlands, Sweden and the United Kingdom under the coordination of the European Reference Laboratory for Marine Biotoxins (EURL-MB). This approach resulted in a highly specific and sensitive use of the approved reference materials for each compound to be investigated for the direct quantitative identification of all four classes of controlled lipophilic toxins by the LC-MS / MS. By gradient elution, the chromatographic separation of toxins is carried out. As requested by EU legislation, findings are stated per toxin group, and the total toxicity is measured using the toxicity equivalence factors (TEF) suggested by the EFSA CONTAM group.

The identification of marine toxins as referred to in Regulation (EC) No 2074/2005 and other amendments may be performed in a series of procedures, differing in the portion (hepatopancreas or whole-body) to be checked and in the solvents used for extraction and purification. In particular, two different analytical procedures have been optimized: for species of molluscs with large-scale digestive glands (mussels, oysters, raspberries, scallops, etc.), the hepatopancreas procedure (20 g) should be used, whereas for species of molluscs with small-scale digestive glands (clams, tellins, etc.) or absent (tunicates, echinoderms, marine gastropods, etc.

A further protocol is provided by the standard operating procedure, the implementation of which is suggested when the presence of YTX in the sample is suspected. However, the lack of precision and inadequacy of the biological test for DSP to meet the regulatory limits led to the prohibition of the MBA and its replacement with validated alternative methods. The LC-MS / MS was established as the reference tool for lipophilic biotoxins in

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Regulation (EU) No 15/2011, even though the MBA may be used as a screening method for new and unknown toxins.

2.10 Trace Metals in Marine Ecosystem

The majority of trace metals through evolutionary history has become a vital part of the cellular components of the living organism. These constitute essential micronutrients required in sufficient quantities in body tissues for diverse metabolic and respiratory functions (Silva and Williams, 2001). The metals persistent and continues to contaminate water, sediments and subsequent bioaccumulation in the tissues of biota inhabiting these environments (Huang et al., 2008). Exposure to environmentally relevant metal concentration may not be acutely lethal. However, their potential to bioaccumulate through the food chain has been implicated in a wide range of sublethal effects in aquatic organisms and humans through consumption of contaminated seafood (Kalogeropoulos et al., 2012; Rahman et al., 2012).

The term trace metals are sometimes used synonymously with heavy metals. These two terms, according to Rainbow (2006), could be used equivalently to the same group of metals differentiated only based on their chemical properties. Metals with a weight higher than a specific gravity of 5 are referred to as heavy metals whereas biologically, trace metals occur in minimal quantities (less than 0.01% of mass) in organisms (Rainbow, 2006). In the ocean, however, trace metals are defined as metals that occur in concentrations less than 10^{-5} M (Salbu and Steinnes, 1995). Throughout this thesis, trace metals are used.

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In order to maintain ideal concentration within the tissues of organisms, majority of essential trace metals are carefully regulated by biochemical mechanisms of uptake, sequestration, accumulation, utilization and elimination (Newman, 1998; 2015). Some essential trace metals, including Zn, Cu, Fe, Mn and Co, occur in small quantities in nature but yet facilitate metabolic actions often as cofactors in enzymes and structural elements in proteins. The deficiency of essential metals in some cases may be far more dangerous to organisms with increased exposure (Fairbrother et al., 2007).

Meanwhile, a separate class of trace metals occur which are nonessential metals. Among these classes of metals is Hg, Cd, As, Pb and Ag, which plays no biological function and are highly toxic even at low concentrations (Castro-González and Méndez-Armenta, 2008; Sarkar et al., 2016). The disruption of biochemical pathways manifests Their toxicity in exposed organisms, and some instances tend to accumulate to greater extents than essential trace metals resulting from relatively efficient detoxification strategies and short elimination pathways (Silva and Williams, 2001). Eventually, all trace metals have the potential to exert eco-toxicological effects above certain thresholds which could be manifested in serious health problems including reduced growth and development, nervous system damage, cancers and in extreme cases death (Martin and Griswold, 2009; Jaishankar et al., 2014). Details of trace metal accumulation and biological effects in aquatic organisms are summarized below:

Zinc (Zn): Zinc is an essential trace metal with concentrations similar to iron. It is a good nutrient source for fungi, bacteria and microalgae. Despite its nutritional qualities, excesses could be potentially toxic to, and thus its releases in seawater need to be moderated. Some

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studies show toxicity in sediment and organism. According to a study by Stauber and Florence (1990), the growth of diatoms cultured under laboratory conditions was inhibited by 20 μg L^{-1} of zinc. Since Zn and Cadmium (Cd) is a similar structure, its toxicity is often linked to that of cadmium.

Zinc is a principal constituent of different biological functions in the human body. It forms part of over 100 enzymes fundamental for a multitude of metabolic processes.

Copper (Cu): Copper is an essential metal a major component of most cells with the highest concentration found decapod crustaceans, gastropods and cephalopods. Studies show that some aquatic organisms are sensitive to copper in concentrations ranging from 1 to 100 μg L^{-1}. Nelson et al. (1988) observed that 2 μg L^{-1} had significant effects on young bay scallops and surf clams. In areas with sediment copper greater than 100, most sensitive species were missing implying exposure to higher concentrations are not favourable to aquatic species (Schaanning et al., 2019) Copper is essential in mammalian physiology which has been demonstrated by several observations, especially in animals which were made copper-deficient, either experimentally or naturally as a result of their environment. Copper is fundamental for numerous proteins and enzymes including cerebrocuprein, ceruloplasmin and erythrocuprein. Despite its essential functions, its deficiency is manifested in anaemia and changes in ossification in mammals (He et al., 2019).

Manganese (Mn): Manganese is fundamental for a number of plants and animals mostly involved in enzymatic functions. Mn is an essential element for plants and animals. The

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release of Mn in aquatic ecosystems primarily occurs via industrial discharge or leachate from landfills and soils. Mn may be soluble in water or adsorbed to sediment and settle at the bottom of water bodies (Šaric and Lucchini, 2007). The transport and partitioning of manganese in water are controlled by the solubility of the specific chemical form present, which in turn is determined by pH, oxidation-reduction potential, and the characteristics of the available anions.

Manganese in water may significantly bioconcentrate at lower trophic levels. In general, the lower organisms such as algae have broader bioconcentration factors than higher organisms (Šaric and Lucchini, 2007). To protect consumers from the risk of Mn bioconcentration in marine molluscs, the US-EPA has set a criterion for manganese at 0.1 mg L^{-1} for marine waters (US-EPA, 1993).

Iron (Fe): Iron is an essential trace metal necessary for normal survival of plant and animal life (Lin et al., 2018). Iron is most prevalent in the earth crust (5%), although its concentration in seawater is very low at 0.061 to 0.18 µg/L (Coale et al., 2003). Iron is significant in photosynthesis, making it an essential trace element for phytoplankton growth in marine ecosystems. The low bioavailability of iron is a limiting factor for primary productivity in approximately 40% of the world's oceans (Lin et al., 2018). Like other trace metals, concentrations exceeding threshold values are potentially toxic to other organisms. The biogeochemistry of iron in the marine environment is primarily influenced by its low solubility, redox speciation (Abualhaija and Berg, 2014).

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Mercury (Hg): Mercury is considered as a non-essential trace metal for living organisms in which even lower concentrations have been implicated in fish and human poisoning ((Pohl et al., 1993)). The organic forms of mercury are considered the most toxic form to aquatic biotas such as toxicity to the red algae *Plumeria elegans*, larvae of the barnacle *Elminius* and brine shrimp *Artemia*. It has been revealed that marine mammals have a higher potential of accumulating a greater concentration of mercury from their food. Selenium antagonises the toxic effects of mercury, and in seals, sea lions and dolphins, the selenium keeps pace with the amount of mercury. Mercury selenide found in the connective tissue of the liver of dolphins is a product of a detoxifying mechanism for the methyl mercury acquired from their food (Roberts et al., 2008).

Cadmium (Cd): Cadmium, a soft, bluish-white metal has chemical properties similar to Zn. It is found in rare ores and as a by-product during the production of zinc, copper and lead. The primary source of Cd in the environment is mining, smelting, coal combustion and oil production (Nordberg et al., 2007). Cadmium mainly composed of 8 isotopes with ^{109}Cd being the most long-lived. It has a half-life of 462.6 days followed by ^{115}Cd with a half-life of 53.4 days. All other radioisotopes have half-lives less than 2.5 hours (Oliveira, 2011).

Until recently, Cadmium (Cd) was not known to have any biological role in molluscs and other living organisms (Lekhi et al., 2008). However, some studies have reported that Cd

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can have a nutritional role by substituting zinc in the metalloenzyme, carbonic anhydrase in some species of phytoplankton (Cullen et al., 1999).

Cadmium can, however, be extremely toxic to organisms living in Cd contaminated environment. Due to its extreme toxicity, it is regarded with high environmental concern and thus, regulated by various statutory agencies such as the European Food Safety Authorities and the United States Food and Drug Administration. For instance, an allowable Cd limit varying between 1 μg g^{-1} (European Union) and 3.7 μg g^{-1} (USDA recommended guideline) in the diet is proposed for human health protection (Lekhi et al., 2008).

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CHAPTER THREE

MATERIALS AND METHODS

This Chapter is a detailed description of the Marine Environment, the Sampling Protocols used for the study, the analytical methods used for sample analysis as well as, quality control/quality assurance techniques used.

3.1 Marine Environment Used for the Study

3.1.1 Geographical Location (General)

The Ghanaian marine coastline is approximately five hundred and fifty (550) kilometres stretching from Aflao (Volta region) in the East to Half Assini in the Western region.

The importance of the fisheries sector in the socio-economic development of Ghana cannot be overemphasized. With the fishing industry playing a significant role in sustainable livelihoods and poverty reduction in several households and communities. The sector is estimated to contribute about 3.9 per cent of the nation ‘s Gross Domestic Product (GDP) and eleven (11) percent of the Agriculture GDP (GSS, 2008). For a long time, fish has remained the preferred and cheapest source of animal protein with about seventy-five (75) per cent of total annual production being consumed locally. In Ghana, the average per capita fish consumption is said to be around 20-25 kg, which is higher than the world average of 13 kg. Importantly, as much as 60 per cent of animal protein in the Ghanaian diet countrywide is thought to be from fish, which accounts for 22.4 per cent of household food expenditures.

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Algal Bloom has been reported along portions (Western Coast; Jomoro, Ellembelle, and parts of Nzema East District) of the Coastal Zones in Ghana (Myjoyonline, April 2010; Ghanaweb, October 2010), impacting negatively on the Food Web, Fishing and Tourism;

Oxygen depletion resulting in "Fish Kills;'' and Toxins that Poisons Seafood; threatening Seafood Safety and Security.

Nevertheless, Algal Bloom Surveys are scarcely carried out along the Coasts of Ghana, resulting in scarce data on the occurrence of Toxin-causing Algae and rare information on prevailing conditions in Ghanaian Coastal Waters. Systematic studies along Ghana's Coast; to ascertain the cause of these marine events has become imperative.

This study will help improve the health of the Ocean, Fisheries, Tourism and enhance Marine Biodiversity; and advance knowledge in Ecology, Nutrition, Health and Economic Risks.

3.1.2 Tema Metropolis

Tema serves as the administrative capital of the Tema Metropolitan Assembly and is situated 25 kilometres east of Accra, the national capital. The metropolis shares common boundaries with the Accra Metropolis on the west, the Ga Municipality on the North West and the Dangme West District on the northern and eastern borders respectively. The main occupation of inhabitants varies from commerce, tourism, hoteliers and fishing. Within the metropolis, Tema fishing port lands annually over 4,000 mt in the past five years. This includes catches from the artisanal canoe fishermen. Over 40 fish species of fish belonging to various families such as the Anchovy (*Engraulis encrasicolus*), Sardinellas (Sardinella

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spp.), Bumper (*Chloroscombrus chrysurus*), Frigate mackerel (*Caranx hippos*), and Chub mackerel (*Scomber colias*) are landed in Tema due to its major infrastructure and proximity to the capital.

3.1.3 Bortianor-Tsokomey (Ga South Municipal)

The Ga South Municipality has several fishing villages including Bortianor-Tsokomey, Oshie, Kokrobite, Faana and Lanma. Fishing is one of the occupations of the people residing in the municipality. Mean fish production in the district in the past five years is around 6,600mt. Some of the most important fish species caught are Moonfish (Selene dorsalis), Cassava fish (*Pseudotolithussenegalensis),* Burrito (*Brachydeuterus auritus),* Sea breams (*Sparus spp.* and *Dentex spp*.) and Round sardinella (*Sardinella aurita*).

3.1.4 Gomoa Nyanyanor (Gomoa East Municipal)

Gomoa Nyanyanor belongs in the Gomoa East District. This district was carved from the Gomoa West district in 2012. In 2018, the Gomoa East district was divided into Gomoa East and Gomoa Central districts. The Gomoa East district has Gomoa Potsin as the capital. The Districts are bounded by Gomoa West to the South, to the North by Agona West Municipal, Asikuma-Odoben-Brakwa District to the West and the East by Awutu-Senya East District. The population of the district is 102,449. The main occupation of the people in this district is subsistence agriculture and fishing. The sardinellas are the primary fish species landed.

3.1.5 Ekumfi-Narkwa (Ekumfi District)

Narkwa is located in the Ekumfi District. This new district was carved out of the Mfantseman Municipality, and it stretches from Ekumfi Otuam to Ekumfi Srafa; with a

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population of fewer than 50,000 people. Fishing as the predominant occupation. Sardinellas are the dominant pelagic species landed. This occurs during the peak fishing season of August / September.

3.2 Study Area- Gulf of Guinea (Ghana)

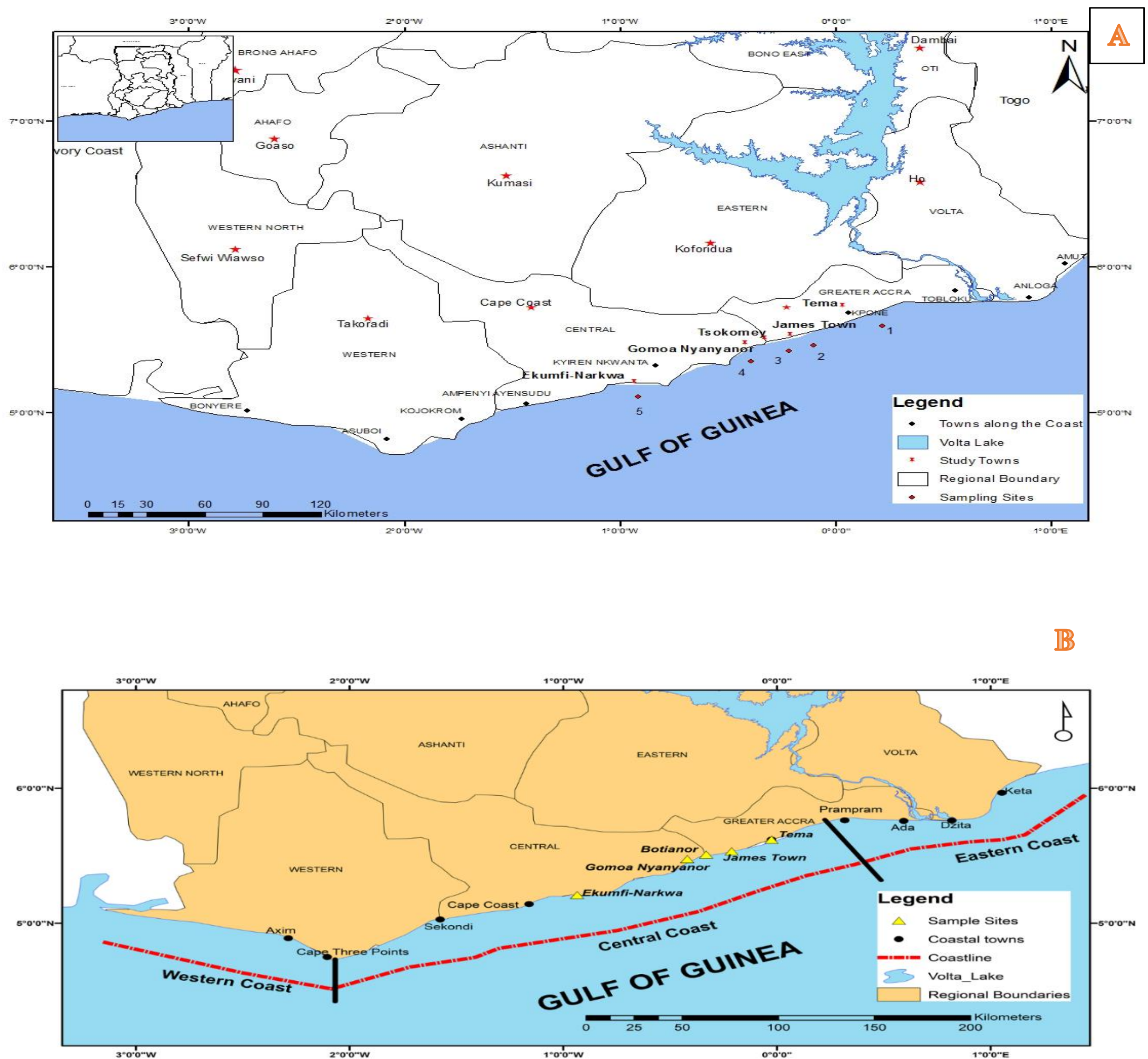

Fig. 3.1 (A & B): Map of Ghana showing the studied areas

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The study area [the Ghanaian coastal area] (Fig 3.1 [a & b]), is situated in the Guinea Current Large Marine Ecosystem, in the Gulf of Guinea. Ghana is bounded to the South by the Gulf of Guinea (Atlantic Ocean) stretching from Aflao in the East to Half Assini in the West. The total coastline of Ghana is about 550km with a continental shelf area of about 23,700 km^2 *(Tamakloe 2009; FASD, 2011).*

Ghana's Exclusive Economic Zone (EEZ) covers 225,000km^2 (Marine water area) *[MFAD, 2008]*. Ghana's coastal zone is divided into THREE Geomorphologic zones:

(i) West Coast, 95 km, [fine sand, gentle beaches, coastal lagoons].

(ii) Central Coast, 321 km, [embayed coast of rocky headlands, rocky shores, littoral sand barriers, coastal lagoons].

(iii) East Coast, 149 km, [sandy beaches, the deltaic estuary of Volta River situated halfway in-between].

There are two main seasons; the rainy (wet) season spanning from May to October and the dry season from November to April. The area is influenced by coastal upwelling which occurs seasonally along the western and eastern coasts. There are two (major and minor) upwelling seasons. Those seasons occur annually with differing duration and intensities off Ghana and Cote d'Ivoire, in the central part of the large marine ecosystem. The major upwelling season occurs from June to September, and transient upwelling events are from December to January (Anang, 1979). For this study, samples were collected from a total of five (5) sites from the eastern to the western coast with coordinates spanning latitude 5.52471°N, longitude 0.933827°E and latitude 5.23240°N, longitude 0.216478°W for the

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analysis. The locations are Tema Port (TMA), Accra Lighthouse Beach Accra (LH), Gomoa Nyanyanor (GN), Bortianor-Tsokomey (TSO) and Ekumfi-Narkwa (EN).

The general scheme for field and laboratory analysis is presented in Figure 3.2.

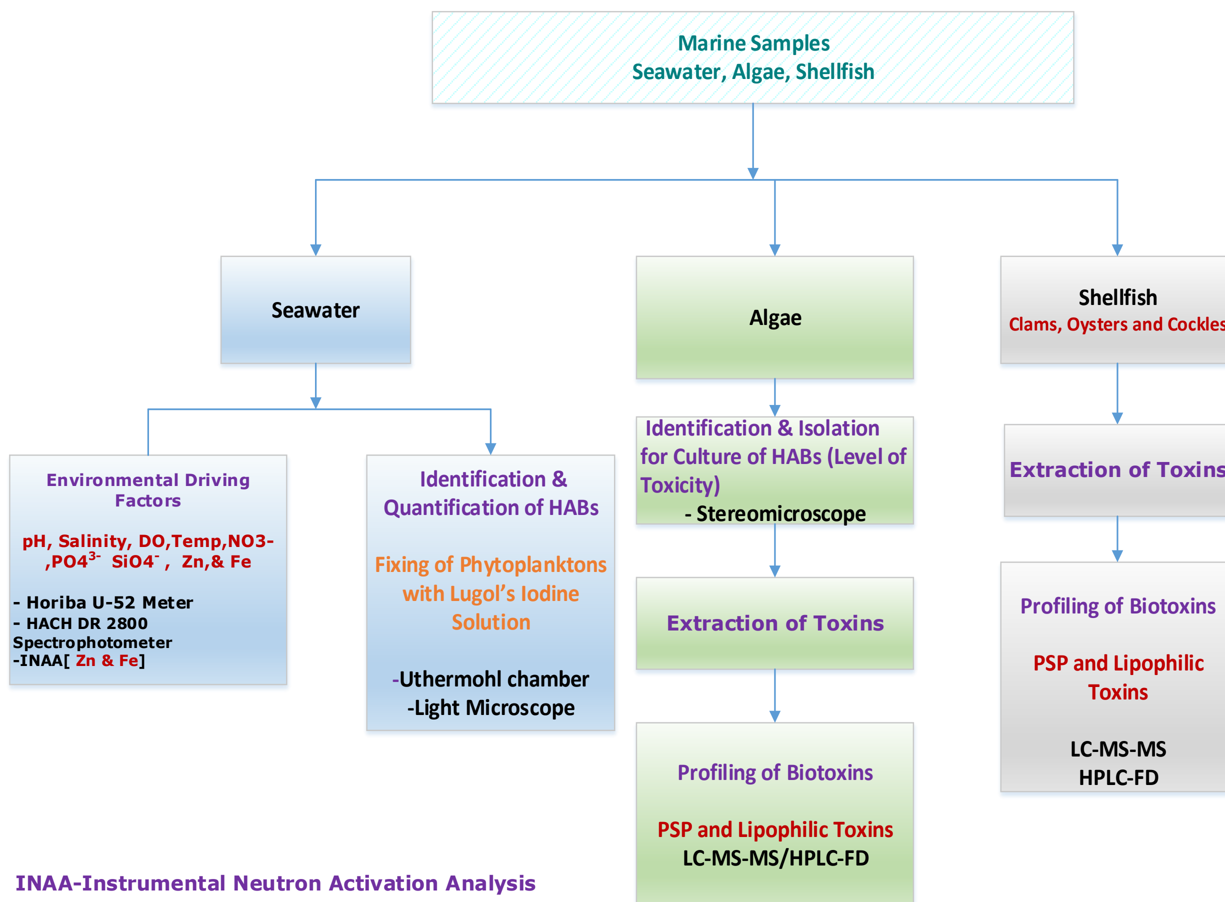

Figure 3.2: General Scheme for Field/Laboratory Analysis

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3.2.1 Collection of Seawater, Algae, and Shellfish

3.2.1.1 Collection of Seawater Samples

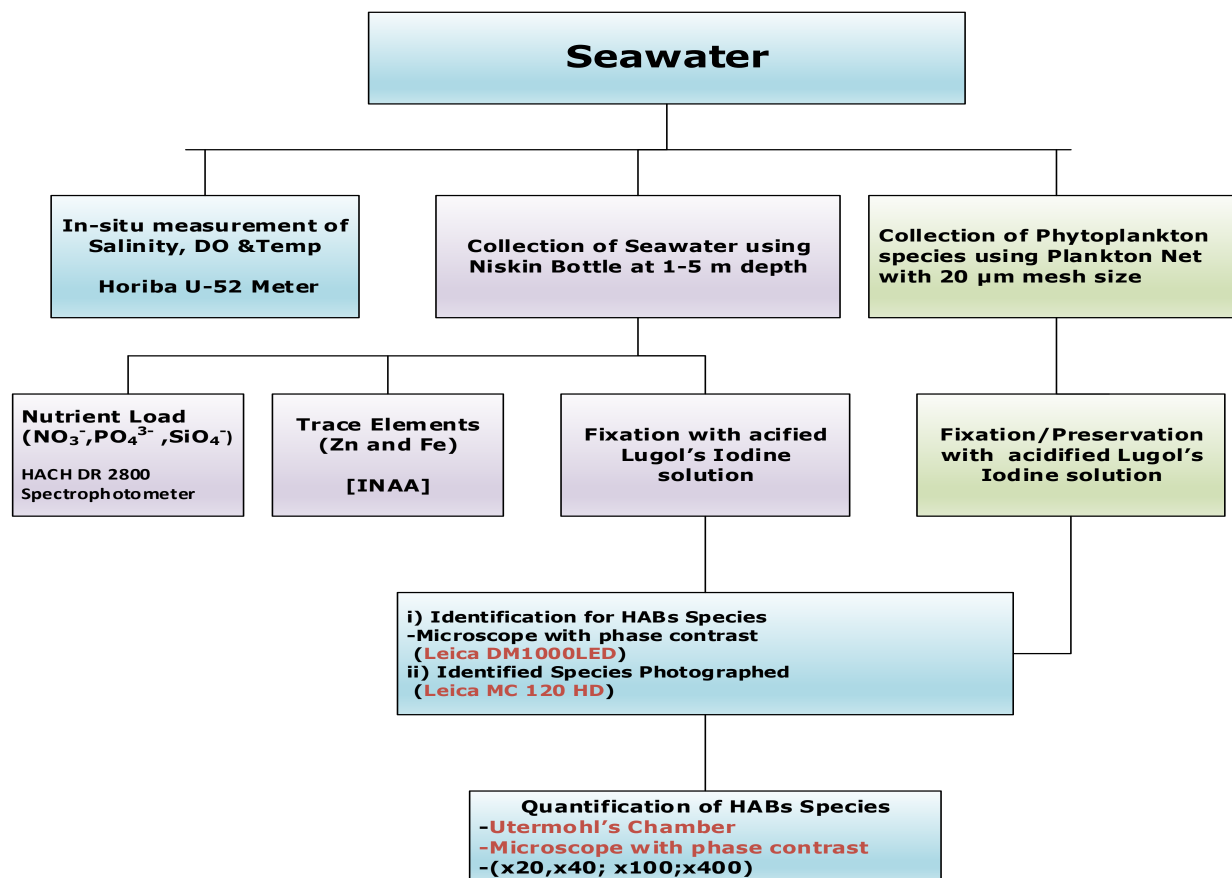

Figure 3.3: Schematic diagram for Analysis in Seawater (Hallegraeff et al., 2004)

Samples were collected for various analysis. Sample collection spanned the period of January 2014 to February 2018. Samples of seawater were collected onboard a fishing vessel from within the water column using the Niskin water sampler with open ends at both sides. The open bottle is lowered into the ocean on wire support from a Fishing Vessel until it reaches a maximum depth of depth 10 m; at this depth, the bottle is closed by a weighted trigger (called a messenger) that is sent down the cable from the surface. The water

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collected into the Niskin bottles were used for studies of microalgae which is referred to sometimes in this document as phytoplankton, HABs species and algae.

Physical Parameters like Temperature, pH, electrical conductivity (EC), total dissolved solids (TDS), Dissolved Oxygen (DO) and Salinity were measures in-*situ* using Horiba U-52 water quality meter. Scheme for analysis of seawater is presented in Figure 3.3.

For nutrient load assessment, a 60 mL syringe was rinsed with sampled water (from the Niskin Bottle), followed by in-line filtering through a 0.2 µm pore. Some quantity of water was pushed through the acrodisc syringe filter. A polyethene bottle was rinsed three times (shake the bottle when rinsing) with ~10 mL filtered water. The filtered water was transferred into fresh amber glass bottles to about ¾th full. Samples were stored on ice in a chiller and transported to the laboratory at the Ghana Atomic Energy Commission, Kwabenya-Accra.

For trace element analysis, seawater samples were collected in 1 litre pre-clean amber glass bottles (2 M hydrochloric acid cleaned) in the field. The seawater was sampled with Teflon -coated Niskin bottles, and the samples were acidified in a solution of nitric acid (1%) to ensure metals are kept in the dissolved state. Some water samples and net samples were also fixed with Lugol for a preliminary survey for phytoplankton.

For phytoplankton analysis seawater was collected in 1 Litre pre-clean amber glass bottles (2 M hydrochloric acid cleaned). Samples were preserved with Lugol solution.

Samples meant for trace elements in seawater and phytoplankton were kept on ice and transported to the laboratory.

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3.2.2 Collection of Algae (Microalgae, Phytoplankton and HABs species)

Water samples were collected with a 5-L Niskin bottle at the surface (0.5 m depth). Using 20 µm mesh net samples were horizontally collected and fixed with $Ca(HCO_3)_2$-buffered formaldehyde (0.8% final concentration) (Throndsen, 1978) or preserved with Lugol's iodine solution according to the Utermöhl's method (1958) in the field.

Phytoplankton samples were collected by horizontal and vertical tows using a plankton net made from fine bolting silk (10 µm mesh, length: 107 cm and Diameter: 29 cm). Samples were drained into the plankton bucket and preserved with Lugol's iodine in sample bottles. Light microscopy (LM) observations were carried out from 50 µL of fixed net samples deposited on a glass slide, using an inverted microscope (Leica DM 1000LED) and light microscope (Leica DM 1000LED) equipped with phase contrast at 400 final magnifications. Cells were photographed, either directly or after isolation with a micropipette, depending on the concentration of organisms and particles.

3.2.3 Collection of Shellfish

Wild species of clams (*Trivela tripla*), bloody cockles (*Anadara Senilis*) and Oysters (*Crassostrea tulipa*) were harvested by handpicking alive between August 2017 to February 2018. The clams were sampled from Bortianor-Tsokomey (TSO) whiles cockles and oysters were collected from Ekumfi-Narkwa (EN). Sampled shellfish was kept on ice in hermitically closed polyethene bags and eventually stored in thermally insulated containers. The samples were transported to the Ghana Atomic Energy Commission Laboratories (for homogenization of the shellfish) and later sent by courier to the National

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Oceanography and Experimental Geophysics Laboratory in Trieste, Italy; and subsequently to the Marine Research Foundation Laboratory in Cesenatico, Italy.

3.3 Laboratory Analysis of Samples

3.3.1 Seawater Analysis

3.3.1.1 Nitrate Determination by Cadmium Reduction Method

Nitrate analysis was done at a wavelength of 500 nm using the HACH nitrate HR PP programme. An aliquot 10 mL of the sample was measured into a reaction tube. One content of NitrateVer5 reagent powder pillow was added to the content of the reaction bottle stooped and shaken vigorously for 30 s for complete dissolution. A sample blank was also prepared. Both samples and blanks were treated following procedures of HACH (2005). The spectrophotometer was zeroed using the blank samples after which the actual samples were transferred into a cuvette and measured.

3.3.1.2 Determination of Silicate

The spectrophotometer was set to 656 silica HR programme with a wavelength of 550 nm. Ten (10 mL) of the samples were transferred into HDPE bottles with dilution. The content of one molybdate reagent powder pillow for high range silica was added to each sample cell. They were swirled until complete dissolution was obtained. The content of one Acid reagent powder pillow for high range silica was then added to the various samples cell and again swirled. A yellow colouration develops, indicating the presents of silicate. The timer was set to ten minutes, during which a reaction took place in the bottles. When the timer

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expired, the content of one citric acid powder pillow was added to the sample and swirled to dissolve. A sample blank was also prepared following procedures of HACH (2005). The spectrophotometer was zeroed using the blank samples after which the actual samples were transferred into a cuvette and measured.

3.3.1.3 Phosphate Determination using Ascorbic Acid

Phosphate analysis was done at a wavelength of 880 nm using the HACH 490p React PV programme. An aliquot of 10 mL of the sample was measured into a reaction tube. One phosver 3 phosphate powder pillow was added to the content of the reaction bottle, stopped and shaken vigorously for complete dissolution. A sample blank was also prepared. Both the sample and blank were treated following the procedure of (APHA, 2005). The spectrophotometer was zeroed using the blank sample after which the samples were measured. The appropriate dilution factors were incorporated into the programme of the spectrophotometer to obtain the final concentration of phosphate in mg/L.

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3.3.2 Seawater and microalgae sample preparation and analysis using INAA

Seawater

↓

Preparation of Sample for Neutron Irradiation

20 mL Seawater Sample in Irradiation Ampoule

↓

Irradiation of Sample in Ghana's MNSR Reactor for 4 hrs (t_{irr})

$^{64}Zn(n, \gamma)^{65}Zn$ $^{58}Fe(n, \gamma)^{59}Fe$

Irradiation Neutron Flux: **5×10^{11} neutrons $cm^{-2}s^{-1}$**

↓

Decay (Cooling) of Sample
Activities to reach levels for handling and to allow activities of Na, Cl, K, Br to reduce
[$t_d \geq 14$ days]

↓

Counting of γ-radiation Intensity on HPGe γ-ray semi conductor detector for 15 hrs (t_c)
^{65}Zn ($t_{½}$=244 d; E_γ =1115.6 keV)
^{59}Fe ($t_{½}$ =44.5 d; E_γ =1099 keV and 1291.6 keV)

↓

Evaluation of γ-Spectra
ORTEC Maestro-32 γ-ray accumulation software

↓

Calcualtion of Concentration of Zn and Fe
Comparator Method of NAA

Figure 3.4 Seawater and microalgae sample preparation and analysis using INAA

3.3.2.1 Sample Preparation

At the laboratory, all apparatus used was acid rinsed and dried in an oven at 50 °C overnight. Phytoplankton samples were sedimented for 24 h by placing in Utermohl chamber. After sedimentation, the sample was filtered with 0.2 µm cellulose acetate filters.

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The filtered samples containing the phytoplankton was frozen at -20 ^{o}C and subsequently freeze-dried in a Christ freeze dryer (Beta 1-16, LMC-1, Germany) at a temperature of -30ºC (corresponding to vapour pressure of 0.370 mbar). The phytoplankton was identified using a light microscope. The phytoplankton was weighed (in replicates of 4) and wrapped in a well cleaned transparent polyethene film, encapsulated into irradiation capsules and heat sealed.

About 20mL of seawater was weighed into clean polyethene capsule and the open-end heat sealed. The samples were subsequently placed in two's in bigger polyethene capsules and heat-sealed, as indicated in Figure 3.4.

All samples were then irradiated for 4 hours as indicated figure 3.4 using the miniature neutron source reactor Ghana Research Reactor-1 (GHARR-1) and irradiated for four hours at a neutron flux of 5.0×10^{11} neutrons/cm^{2}s at half power of 15 kW. The irradiation scheme (Table 3.1) was chosen based on the half-lives of the elements. The samples were sent into the reactor using a pneumatic transfer system operating at a pressure of 1,292.88 mmHg. At the end of their radiation, the capsules were returned and allow to 'cool' for 14-21 days. Measurement of gamma (γ)-radiation intensity was done using a high purity Germanium γ-ray semi-conductor detector. Acquisition of gamma (γ)-spectra was done with the ORTEC-Maestro-32 gamma (γ)-ray Accumulation Spectroscopic Software).

Calculation of concentration was based on the Comparator Method of NAA.

The (n, γ) reactions, half-lives and the gamma energies of elements are presented Table 3.1

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Table 3.1: Information on nuclear data for element of interest

Element	Isotope (% Abundance)	Nuclide	Cross-section (b)	Half-life	Gamma-ray energy used (KeV)
Zn	^{65}Zn (18.6)	^{65}Zn	0.072±0.004	13.76 h	438.6
Fe	^{56}Fe (0.28)	^{59}Fe	1.15±0.02	44.5 d	192.3,1099.3
Mn	^{55}Mn (100)	^{56}Mn	13.3±0.2	2.58 h	1115.5, 1481.8
Hg	^{202}Hg (29.9)	^{203}Hg	4.9±0.1	46.6 d	279.2
Cu	^{65}Cu (30.9)	^{66}Cu	2.17±0.003	5.09 min	1039.2
Cd	^{119}Cd (28.73)	^{115}Cd	0.300±0.015	53.3 h	336.2

Over the past two decades there has been increasing evidence to suggest that such low dissolved zinc concentrations may limit phytoplankton growth and their ability to fix CO_2 from seawater via the enzyme carbonic anhydrase Anderson et al., 1978; Ž Sunda and Huntsman, 1992; Morel et al., 1994.

Trace metals play important roles in ocean biogeochemistry by acting as critical micronutrients for primary producers (Sunda, 2012). For instance, iron availability limits primary productivity and controls phytoplankton species assemblage over substantial portions of the ocean (Boyd et al., 2007), modulating the biological uptake of CO_2 in the ocean.

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3.3.2.2 Method Evaluation

Standard Reference Material, NIST SRM-1547 (Peach Leaves) and procedural blanks were also prepared in triplicates and irradiated (Table 3.2).

Table 3.2: Analysis of NIST SRM-1547 (Peach Leaves) using NAA

Trace metals	This Study	Certified value
Zn	18.3±0.04	17.9±0.4
Fe	20.5±0.05	21.8±1.4
Mn	97.1±2.3	98±3
Hg	0.032±0.005	0.031±0.007
Cu	3.2±0.6	3.7±0.4
Cd	0.027±0.004	0.026±0.003

3.4.1 Characterization of Algal Species

3.4.1.1 Algal Quantification

Quantitative analysis was performed on 50 mL preserved subsample in Ghana Atomic Energy Commission's marine Laboratory. Toxic microalgae species composition of phytoplankton was estimated on samples per Utermöhl's method (1958), using an inverted microscope. Qualitative analysis was conducted using a light microscope (Leica DM 1000LED) equipped with phase contrast at 400 final magnifications. All microscopically identified phytoplankton taxa were recorded and photographed using a high-quality digital camera (Leica MC 120 HD). Identification of harmful algae specimens was made to the lowest taxonomic level (genus, species) making use of multiple identification keys. Species names and higher taxonomic categories were checked for validity against Algae Base (http//www.algaebase.org), Intergovernmental Oceanographic Commission- United Nations Educational Scientific and Cultural Organization (IOC-UNESCO)'s Taxonomic

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Reference List of Toxic Microalgae (http//www.marinespecies.org/HABs) (Moestrup et al., 2009) and the most recent review of HABs of the World Ocean (Lassus et al., 2016) supervised by the Intergovernmental Panel of Harmful Algal Blooms of IOC.

3.4.1.2 Algal Culture

Isolation and culture of harmful or potentially toxic species was carried out in the culture laboratory of National Oceanography and Experimental Geophysics, Trieste, Italy

A. Laboratory Conditions for thriving Culture of microalgae

a. Sterile lab and glassware: wash glassware with weak acid and distilled water and dry in an oven.

b. Before drying conical flask and beakers should be covered with aluminium foil

3.4.1.2.1 Preparation of Medium B

The culture medium was generally prepared with seawater taken at the bottom in the same sampling station. This water was brought to the desired salinity (about 33), filtered and sterilized according to the following procedure:

Filtration was with Durapore GVWP filters (Millipore) with a porosity of 0.22 µm and a diameter of 142 mm; then the addition of 10%(0.1 mL) trace elements with EDTA (Ethylenediaminetetraacetic acid), to keep in solution heavy metals, and autoclaving in 1L Teflon bottles for 15 mins, then it was left to cool for at least 24 hours, after that the following sterilized solutions were added. Details of nutrients used are found in Table 3.1 to 3.3:

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i. 0.9 mL stock trace elements with EDTA (minus 10% added before)

ii. 1 mL stock nutrients (nitrates + phosphates)

iii. 0.5 mL of vitamins

1 mL stock Silicates was added if Medium BS is required in the case of culturing diatoms.

The final concentrations of each component are reported as follows:

a. 1 L of seawater (33 PSU) filtered through 0.22 µm:

b. 1 mL stock trace elements with EDTA

c. mL stock nutrients

d. 0.5 mL stock vitamins

Preparation of Stock Solutions using compositions from Table 3.2-3.4

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Table 3.3 Composition of nutrients for Algal Culture

Trace elements with EDTA	**Final stock 100 mgL^{-1}**	**Stock 1 25 mgL^{-1}**	**Stock 2 25 mgL^{-1}**
Na_2EDTA	0.372		
Fe-II-citrate	0.131		
$MnCl_2$ x4H_2O		0.495	
$ZnSO_4$ x7H_2O		0.072	
$NaMoO_4$ x2H_2O		0.060	
$CoCl_2$ x6H_2O		0.030	
$CuSO_4$ x5H_2O			0.062
H_2SeO_3			0.032
$NaVO_3$			0.030
$NiSO_4$ x6H_2O			0.066

Individual solutions were prepared for each trace elements of the series of stocks 1 and 2 while 1 mL of each stock 1 and 0.1 mL of each stock 2 were added in 100 mL of final stock (working stock). The final stock was filtered 0.2 µm sterile, non-pyrogenic syringe filter using and stored at 4 ^{o}C. Filtered stocks 1 and 2 was also stored at 4 ^{o}C.

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Table 3.4: Composition of Nutrients in Medium B used for Algal Culture

Nutrients	**Stock**
	50 mgL^{-1}
$NaNO_3$	3.75
$Na_2glyceroPO_4$	0.153
NaH_2PO_4	0.25
Silicates	
	50 mg/L
Na_2SiO_3x9H2O	1.5

The filtered stock of Nutrients was also kept at 4 ^oC

Table 3.5: Composition of Vitamins used for Algal Culture

Vitamins	**Final stock**	**Stock 1**	**Stock 2**
	100 mgL^{-1}	50 mgL^{-1}	50 mgL^{-1}
Thiamine-HCl	20 mg		
Biotin		1.04	
Vitamin B_{12}			11.1

In 100 mL of final stock (vitamin working stock) 0.5 mL of stock 1 and stock 2 was added, and the final stock was filtered with 0.2 μm sterile non-pyrogenic syringe filter and kept at -20 ^oC. Filtered stock 1 and stock 2 were stored at -20 ^oC.

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Note: medium BS (for diatoms) without the silicate becomes medium B which works for dinoflagellates.

3.4.1.2.2 Isolation and Culture of Algae

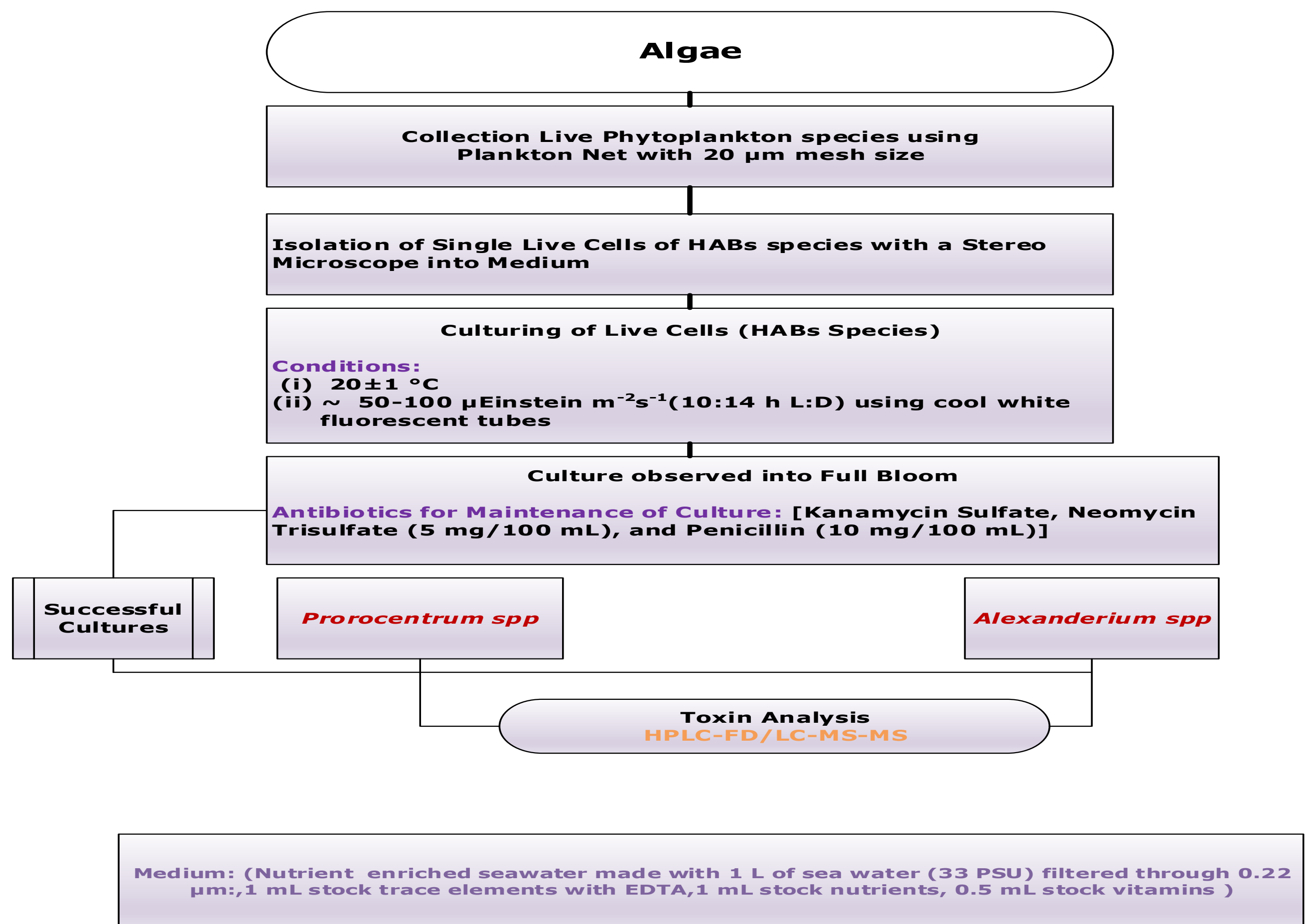

Figure 3.5: Schematic diagram for Culture of Algae

Algal cultures are essential when conducting competition studies, bioassays, assessment of phytoplankton food preferences, and determination of algal life histories. They are also necessary for molecular systematic, work in this case toxin analysis. Algal cultures may be unialgal, which means they contain only one kind of alga. Usually, a clonal population (but which may contain bacteria, fungi, or protozoa), or cultures may be axenic, meaning that

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they contain only one alga and no bacteria, fungi or protozoa, this is an objective in this culture. The major technique used to obtain this unialgal isolate is single-cell isolations, which was applied in this study, as indicated in Figure 3.5.

Procedure of Isolation

a. Several micropipettes (very fine-tipped pipettes) were prepared from glass Pasteur pipettes.

b. The tip end was held so that the glass near the tip is within the flame of a Bunsen burner (gas flame). The pipette was held in the flame until the glass becomes slightly soft. This was determined by testing for flexibility by moving the tip. Then the pipette was removed from the flame and pulled out straight. The pipette was not pulled while it was still in the flame, to seal up. The diameter of the finely pulled tip was varied by changing the speed of pulling; the diameter of a slowly pulled tip will be greater than that of a rapidly pulled tip. A narrow diameter tip if you are trying to isolate microscopic algae, but a larger diameter tip is required for large cells.

c. A multi-well plate was prepared with sterilized media in each well. Multiple drops of sterilized media were placed onto the inside surface of a sterile plate.

d. A micropipette was attached to a length of rubber bulb for suction.

e. A culture dish of algae was placed on the stage of a dissecting microscope to locate the single cell/colony/filament to be isolated, then the tip of the micropipette was moved move to the vicinity of the alga, then sucked it up into the pipette tip, then stop the suction.

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f. The micropipette was used to transfer the isolated alga from the first drop into a series of fresh drops into a fresh dish of media, then transfer the algae into another culture dish of media before the final isolation into the multi-well plate, i.e. washing procedure that helps remove contaminants. The multi-well plate holding liquid growth medium suitable for that species.

g. The procedure was repeated. As not all isolated algae can continue to grow, or some may be infected with other algal cells, several attempts are typically made.

3.4.1.2.3 Isolation of Dinoflagellates

Net samples collected were put in a thermos flask to maintain the temperature at which it was taken and brought to the OGS laboratory for isolation of and culture of harmful and potentially toxic microalgal species. Samples were identified TSO, which is from Tsokomey, BORT from Bortianor and NYA from Gomoa Nyanyanor. Upon arrival in the laboratory after 24 hours, most cells were dead. A few isolations were done from net samples for possible harmful dinoflagellates. Nutrients were added to net samples and stone with seawater samples left for 24 hours.

Samples re-examined after the 24 hours, and some potentially harmful dinoflagellates were found in net samples and samples from Tsokomey (TSO) which were then isolated for culturing.

Cells that were living and isolated for culture after the travel were *Gynmodinium spp* (Levanderina fissa) which is a naked dinoflagellate, while *Alexandrium sp, Coolia sp*, and *Prorocentrum sp*. are dinoflagellates with cellulosic theca.

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a. Net samples brought from Ghana, then cells were isolated with a stereo microscope into medium B using the single-cell isolation procedure to be cultured at about $20^{O}C \pm 1$ and approximately 50-100 µEinstein m-2 s -1 (10:14 h L: D) using cool white fluorescent tubes

b. The culture was observed and maintained every day. Cells cultured was observed to go into full bloom.

c. On the 11th day of culturing the cells, the cultures were treated with antibiotics because there were bacteria in the culture. Antibiotics used were Kanamycin sulfate, Neomycin trisulfate (5mg/100mL), and Penicillin (10mg/100mL) all from SIGMA.

d. Successful cultures were observed and maintained until the culture of various species were observed to be clean. Then clean culture in multi-well plates were transferred into test tubes containing about 15mL of medium B to grow.

e. Cultures of the three dinoflagellates were observed until they become dense.

f. Successfully established cultures were grown in B medium at 20˚C cultures of *Alexandrium sp*, *Coolia* sp, *Gynmodinium sp (Levanderina fissa)*, and *Prorocentrum sp*. established were observed under the inverted microscope.

3.4.2 Genetic Identification of Isolated Dinoflagellates

The DNA was extracted from dinoflagellates cultures, centrifuged and pelleted, using the Quick-DNA ™ Fungal / Bacterial Miniprep Kit (Zymo Research) extraction protocol, suitable for treating both fresh and frozen tissues of plants, bacteria and fungi. It includes

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a mechanical lysis passage through the stirring with marbles to guarantee the breaking of the cell walls and the solubilization of the DNA.

3.4.2.1 Preparation of samples for DNA extraction

About 10-15 mL of cultures of two strains of *Prorocentrum sp* (P2K, P2K1), *Gymnodinium sp* (GY3K, GY5K), *Coolia sp* (CO3K, CO4K, CO5K) were centrifuged at 3000 rpm for 10 minutes. The resulting pellets were transferred into Eppendorf tubes and frozen at 18°C for DNA extraction.

With the help of Quick DNA (fungal and Bacterial Miniprep Kit) from Zymo Research (California, USA) was used for DNA sequencing based on the protocol outlined:

The pellets were resuspended in 150 µL of lysis solution and transferred to a ZR BashingBead™ Lysis tube and shaken with a benchtop vortex for 2:30 minutes to destroy cells. The mixture in the ZR BashingBead™ Lysis Tube(0.1mm) was centrifuge in a microcentrifuge at 10,000 xg for 1 minute. After that 400 µL supernatant was transferred to a Zymo-Spin™ IV Spin Filter (with Orange Top) fit in a collection tube to be centrifuged at 7000 x g for 1 minute, then 1200 uL of Genome Lysis Buffer to the filtrate in the Collection Tube from the previous step. 800 µL of the mixture (filtrate+ genome lysis buffer) was then transferred into a Zymo-Spin™ IIC Column fit in a collection tube and centrifuged at 10,000 x g for 1 minute. The filtrate from this step is then discarded and the last step repeated for remaining (filtrate+ genome lysis buffer) and the second filtrate also discarded. 200 µL of DNA Pre-Wash Buffer to the Zymo-Spin™ IIC in a new collection tube, which was centrifuged for 1 minute at 10,000 x g then gDNA Wash-Buffer is also added to the Zymo-Spin™ IIC and centrifuged again for 10,000 x g for 1 minute. For DNA

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elution the Zymo-Spin™ IIC Column was transferred to a clean 1.5 mL microcentrifuge tube and 100 uL of (35 µL minimum) added directly to the column matrix, it was centrifuged for 30 seconds at 10,000 x g for DNA to elute. The successful extraction was confirmed through the quantization of DNA using the NanoDrop 2000 spectrophotometer. Ultra-pure DNA was now ready for genetic analysis.

3.4.2.2 Amplification of the barcode region and sequencing

The DNA-barcoding is a molecular technique that allows the identification of biological identities.

For the systematic attribution of the species; Gymnodinium, Prorocentrum and Coolia spp, the ITS (Internal Transcribed Spacer) (ITS1) marker were used with the primer system ITSU3-ITSp4 (White, 1990), a sequence of about 600 nucleotides, highly variable, surrounding the sequence which encodes the 5.8S, which is between the sequence encoding the SSU (Small Subunit) and the LSU (Large Subunit) of the ribosomal operon. The thermal profile for PCR amplification of the region includes an initial activation step of the polymerase (PCRBIO HS Taq DNA polymerase) at 95 ° C for 1 minute, followed by 35 cycles comprising a denaturation step for 30 seconds at 95 °C, an annealing step of the primers at 55 °C and an extension phase at 72 °C for 1 minute. The amplified was controlled on 2% agarose gel.

The ITS sequences of the analyzed Gymnodinium, Prorocentrum and Coolia sp strains were obtained by Sanger sequencing and aligned with BLASTn against the nucleotide

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database available in GenBank. Furthermore, they were aligned with 54 sequences from GenBank using MAFFT (Katoh, 2013).

3.4.3 Analysis of Algae and Shellfish Samples

3.4.3.1 Preparation of Cultures for Toxin Analysis

Dense cultures (10 mL) of *Coolia spp* (CO3K), *Coolia spp*(TSO), *Prorocentrum sp*(PL1T), *Alexandrium sp* (AL9T)) were filtered on 0.22 µm mixed cellulose esters filters (MF-Millipore Membrane Filters, Merck KGaA, Darmstadt, Germany), and residue of cells on filter papers were wrapped in aluminium foil and frozen.

Cell population were estimated by taking 2 mL of the culture for counting with Sedgewick Rafter counter after fixing them with about 80 uL of 37% formaldehyde and used to estimate toxicity.

3.4.3.2 Toxin Analysis

Toxin analysis was conducted on both microalgae and shellfish. The details of the analysis are outlined in the following sections. Three main methods were adapted for analysis of the various toxins that are found in shellfish and microalgae. Firstly, according to European-Union harmonized methods (2015), lipophilic toxins like Okadaic acid (OA) and its analogues, the dinophysis toxins (DTX1, DTX2, and DTX3), pectenotoxins (PTX1, PTX2), yessotoxins (YTX), homoYTX, 45-OH YTX, 45-OH homoYTX) Moreover, azaspiracids (AZA1, AZA2, AZA3) in shellfish are all extracted and analyzed by the same method using the LC/MS. Secondly, a procedure of analysis which applies to Paralytic Shellfish Poisoning(PSP) toxins in shellfish and microalgae was used for the quantification

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of neosaxitoxin (NEO). Saxitoxin (STX), gonyautoxins 2 and 3 (GTX 2, 3), gonyautoxins 1 and 4 (GTX 1, 4), decarbamoyl saxitoxin (dcSTX), GTX5, C-1 and C-2 together, and C-3 and C-4. This method was modified to include dcGTX2,3, dcNEO, GTX6 in this study. At the same time, the last method for this study was a procedure applied for the detection of domoic acids.

3.4.3.2.1 Principle for Analysis of Lipophilic Toxins

The process is based on the extraction from homogenized tissue of toxins of the OA, PTX, AZA, and YTX groups with 100 percent methanol. In order to examine the presence of free OA, free DTX1 and free DTX2, PTX1, PTX2, AZA1, AZA2, AZA3, YTX, homo YTX, 45 OH YTX and 45 OH homo YTX (McNabb et al., 2005), the extracts are then purified and directly analysed by liquid chromatography with tandem mass spectrometric detection (LC-MS / MS). Alkaline hydrolysis of the methanolic extract is needed to determine the total content of OA group toxins before LC / MS / MS analysis in order to convert any acylated OA and DTX esters to parent OA and DTX1 or DTX2 toxins (Mounfort et al., 2001). Extracts are purified and analysed by LC-MS / MS after hydrolysis. By gradient elution, chromatographic separation is carried out.

3.4.3.2.2 Sample Preparation for Lipophilic Toxins in Shellfish

Chemicals for Lipophilic Toxin Analysis

HPLC grade methanol and acetonitrile were acquired from VWR International Srl (Milan, Italy). Glacial acetic acid, sodium hydroxide, hydrochloric acid (37%) and citric acid (all

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of the analytical grade) were purchased from VWR International Srl (Milan, Italy). Ammonium acetate (Carlo Erba, Milan, Italy) and ammonium citrate tribasic anhydrous (Merck KGaA, Darmstadt, Germany) were of reagent grade. Ultrapure water was produced in-house using a Milli-Q Direct-8 system (Merck KGaA, Darmstadt, Germany). For analyses with the mass spectrometry instrument, methanol and ammonium hydroxide (25%) of LC/MS grade were obtained from Scharlab Italia Srl (Riozzo di Cerro al Lambro, Milan, Italy). Mobile phases consisted of (A) 2 mM ammonium acetate and 18 mM glacial acetic acid in 5.2% methanol and (B) 1mM ammonium acetate in 100% methanol.

Certified standard solutions of okadaic acid (OA), dinophysistoxins (DTX1, DTX2), pectenotoxin 2 (PTX2), azaspiracids (AZA1, -2 and -3), yessotoxin (YTX) and homo-yessotoxin (homo-YTX) were obtained from the National Research Council in Halifax, Canada.

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General scheme for Lipophilic Toxins Analysis in Shellfish is presented in Figure 3.6.

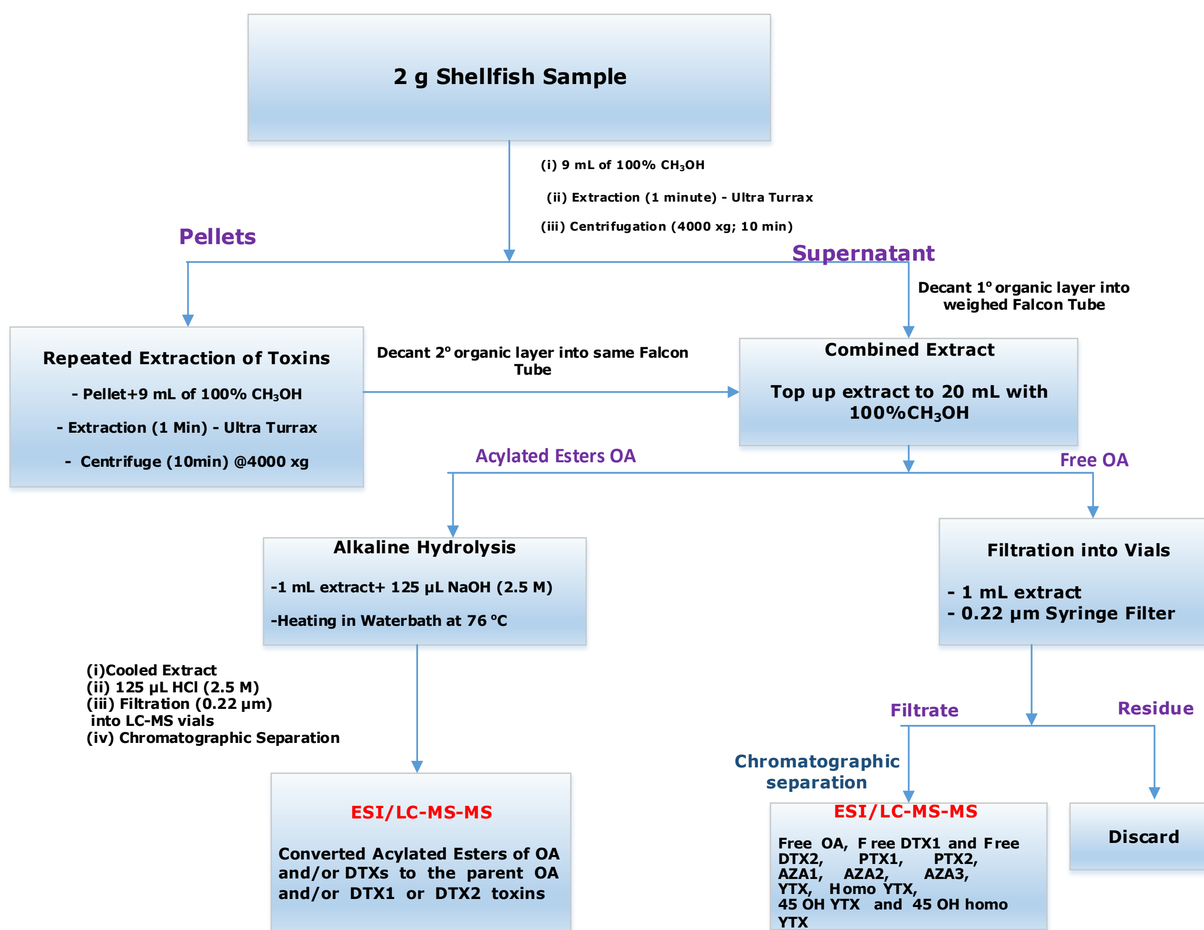

Figure 3.6: Flowchart for Lipophilic Toxins Analysis in Shellfish

3.4.3.2.3 Extraction, Clean-up and Quantification of toxins in Shellfish

About 1 kg of bivalve molluscs (Trivela tripla, Oysters and Bloody cockles) were cleaned with water. The molluscs were washed with fresh water after opening, their flesh removed and placed on a net of stainless steel, and washed again with deionized water. All the raw tissue obtained, not less than 150 g, was chopped and combined with a mixer.

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Procedure of extraction: As shown in the flow chart (Figure 3.6), 9 mL of 100 % methanol was applied to 2.00 ± 0.05 g of blended tissue, placed in a centrifuge tube, homogenized for 1 minute with Ultra-turrax ® (Ultra-Turrax T 25 Basic, IKA ® -Werke GmbH & Co. KG, Staufen, Germany) at 10,000 rpm and centrifuged for 10 minutes at 2,000 x g (Universal 16, Hettich Zentrifugen, Andreas Hett GmbH & Co. KG, Staufen, Germany). The supernatant solution was transferred into a 50mL plastic falcon tube.

Furthermore, a second 9 mL aliquot of 100 percent methanol was applied to the residual tissue pellet, and Ultra-turrax ® was homogenized for 1 minute at 10,000 rpm, and the mixture was centrifuged for 10 minutes at 2,000 xg. The supernatant solution was then moved into the same falcon tube and mixed with the first extract, producing up to 20 mL of 100 % methanol. When the solution was not tested immediately, it was kept at -20 ° C. A 0.22 µm RC syringe filter (Kinesis, Cole Parmer Instrument company Ltd., Cambridgeshire, UK) was used to filter 1mL into vials for LC/MS analysis.

3.4.3.2.4 Hydrolysis

About 1 mL of extract was transferred into a 15 mL plastic falcon tube. Approximately 125µL of sodium hydroxide solution (NaOH) (2.5 M) was added to the extract and sealed tightly and vortexed for a minute. The tube was then placed in a water-bath for 40 mins at 76 ^{o}C. The sample was then cooled to room temperature and neutralized with 125 µL of hydrochloric acid (2.5 M) then vortexed. The hydrolysed extract was then filtered into a 2 mL HPLC vial with a screw cap with PTFE/silicone septum for LC/MS/MS.

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3.4.3.2.5 Sample Preparation - Frozen dense cultures

Frozen dense cultures (10 mL) of *Prorocentrum sp* (PL1T) and *Coolia* sp. (TSO, CO5, CO6) were obtained using a 0.22 µm mixed cellulose esters filters; figure 3.7 depicts the process. Filters were extracted with 3 mL of 100% methanol using a sonicator probe (Ultrasonic® Liquid Processor Model XL2020, Heat Systems Inc., New York, NY, USA) in pulse mode for 20 minutes. The extract was further purified using Solid Phase Extraction clean-up.

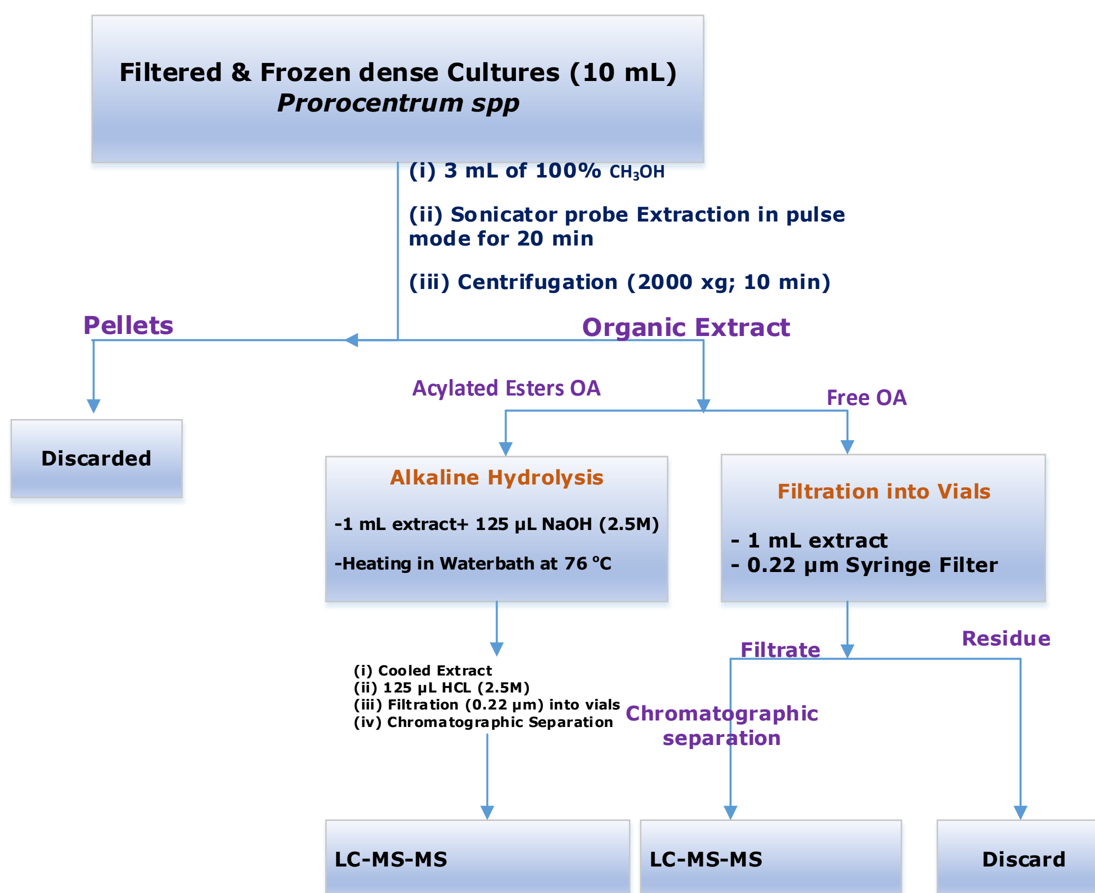

Figure 3.7: Flowchart for Lipophilic Toxins Analysis in Algae

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3.4.3.2.6 Clean-up

An SPE vacuum manifold system (Supelco, Merck KGaA, Darmstadt, Germany) was used to perform the clean up using Strata-X cartridges (33 µm, 60 mg/3 mL, Phenomenex, Torrance, CA, USA). Cartridges were activated with 3 mL of methanol. Prior to application of the sample, the cartridge was equilibrated using 3 mL 30% v v^{-1} methanol. Crude methanolic microalgae extract (2 mL) was diluted with 4.7 mL water and applied on the cartridge. The cartridge was washed with 3 mL 20% v v^{-1} methanol to remove polar compounds. Finally, the toxins were eluted from the cartridge using 3 mL 30% methanol containing 0.3% v v^{-1} ammonium hydroxide (25%). The purified extracts were transferred to an HPLC vial and analyzed by LC-MS/MS. The method was adapted from Gerssen and collaborators (2009).

3.4.3 2.7 Quantification of Toxins

Using the external standard calibration process, the quantification of each toxin is calculated. The calibration curve constructed for PTX2 is also to be used for PTX1, and the calibration curve for YTX and homoYTX is also to be used for the quantification of 45 OH YTX and 45 OH homo YTX, according to the procedure followed in the inter-laboratory validation analysis of the procedure and assuming an equimolar response. The evaluation is based on the linear equation of the line of regression of the individual toxins with available criteria. If the toxin signal is greater than the signal of the highest calibration level in the analyzed sample, the extract must be diluted with methanol to achieve a signal inside the calibration curve, and the dilution factor (D) should be taken into account for

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calculations. Therefore, from the calibration curve, the concentration of the particular toxins in each analysed sample is calculated using the following equation:

$$\textbf{Concentration}(\mu g/kg) = \frac{\left(\frac{y-a}{a}\right)x\left\{\left[\frac{VF(ml)}{VH(mL)}\right]\times VT(MmL)\right\}}{W(g)} \cdot D \quad \text{...... Equation 1}$$

where: y = Area of the chromatographic peak

b=intercept of the regression linear

a = slope of the calibration curve

V_T= Total volume of crude extract (20 mL)

V_H=Volume of extract used for performing the hydrolysis.

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

W= Sample tissue weigh (2 g)

D= Dilution factor (if extract has been diluted)

Corrected concentration (with recovery) from Equation 1:

$$\mu g\frac{toxin}{kg} = \left(\frac{\mu g}{kg}\right)_{EXTERNAL\ CALIBRATION} \cdot \frac{100}{\%R_{CRM\ or\ spiked\ extract}} \text{- ....Equation 2}$$

Where:

$(\mu g/kg)_{EXTERNAL\ CALIBRATION}$: Concentration calculated by external calibration according to equation 1

$\%R_{CRM}$: Recovery obtained in the analysis of reference material spiked extract ⇒

$$\%R = (ng/mL)\frac{(ng/ml)_{CALCULATED}}{(ng/ml)_{THEORETICAL}} \cdot 100 \text{ ....Equation 3}$$

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Modified Equation for algae

$$\textbf{Concentration (µg/cell)} = \frac{\left(\frac{y-a}{a}\right)x\left\{\left[\frac{VHF(mL)}{VHI(mL)}\right]\times\left[\frac{VSPEF(mL)}{VSPEI(mL)}\right]\times VT(mL)\right\}}{n(cells)} \cdot D \quad \text{……… Equation 4}$$

where:

y = Area of the chromatographic peak

b =intercept of the regression linear

a = slope of the calibration curve

V_T= Total volume of crude extract (3 mL)

V_{HI}=Volume of extract used for performing the hydrolysis

V_{HF}= Final volume of extract after hydrolysis

V_{SPEI}=Volume of extract used for performing the SPE clean-up

V_{SPEF}= Final volume of extract after SPE clean-up

n= number of cells

D= Dilution factor (if extract has been diluted)

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3.4.3 2.8 Expression of Results

The use of the Toxicity Equivalent Factors (TEFs) referred to in Table 3.6 as adopted by the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA-2009) is needed to convey the findings of the toxin groups as µg equivalents/kg or mg equivalents/kg in compliance with European legislation.

Table 3.6: List of Toxic Equivalent Factors (TEFs) adopted by EFSA

Toxin group	Analogue	TEF	Results expression
OA group	OA	1	µg OA equivalents/kg
	DTX1	1	
	DTX2	0.6	
PTX group	PTX2	1	µg PTXequivalents/kg
	PTX1	1	
AZA group	AZA1	1	µg AZA equivalents/kg
	AZA2	1.8	
	AZA3	1.4	
YTX group	YTX	1	mg YTX equivalents/kg
	homo YTX	1	
	45 OH YTX	1	
	45 OH homo YTX	0.5	

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3.4.3 2.9 LC/MS/MS for quantitative analysis

For quantitation, the mass spectrometer was operated in MRM mode, scanning two transitions for each toxin. Q1 and Q3 resolutions of the instrument were set at Unit (arbitrary terms). Peak area was acquired in MRM, in separate chromatographic runs, using positive (ESI^+) and negative (ESI^-) ionization modes, respectively with a scan time of 1 s. In ESI^+, the following source parameters were used: curtain gas set at 30 psi, ion spray at 5500 V, a turbogas temperature of 450 °C, gas 1 and 2 both sets at 50 psi, and an entrance potential of 10 V. In ESI^-, the curtain gas was set at 20 psi, the ion spray at -4500 V, the turbogas temperature at 550 °C, gas 1 and 2 at 40 and 50 psi, respectively, and finally the entrance potential at -13 V. MRM transitions used for each toxin is displayed in Table 3.5. Data acquisition was carried out with Agilent Mass Hunter Software.

Table 3.7: Multiple Reaction Monitoring (MRM) transitions

Toxin	Q1	Q3 quantifier	CE	Q3 Qualifier	CE
DA	312.1	266.1	23	161.1	35
GYM-A	508.4	490.2	33	392.3	49
13-desmeSPX-C	692.5	164.2	69	444.3	53
PnTX-G	694.5	164.1	75	458.3	75
AZA1	842.5	672.4	69	654.4	69
AZA2	856.5	672.4	69	654.4	69
AZA3	828.5	658.4	69	640.4	69
PTX2	876.5	823.5	31	805.6	37
PTX2sa	894.6	823.5	31	805.6	37
OA, DTX2	803.5	255.1	-62	113.1	-92
DTX1	817.5	254.9	-68	112.9	-92
YTX	1141.4	1061.6	-48	855.5	-98
homo-YTX	1155.6	1075.6	-48	869.4	-98

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NB: Using two transitions per toxin, MS detection was carried out. For quantification, the transition with the highest intensity is used, while the transition with the lowest intensity is used for confirmatory purposes.3.4.3.3 PSP Toxins by HPLC-FLD

The AOAC Official Method 2005.06 for Paralytic Shellfish Poisoning Toxins was used for the determination of saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 2 and 3 (GTX 2, 3; together), gonyautoxins 1 and 4 (GTX 1, 4; together), decarbamoyl saxitoxin (dcSTX), GTX5, C-1 and C-2 together, and C-3 and C-4. This method was however modified to include dcGTX2,3, dcNEO, GTX6.

3.4.3.3.1 Principle

Test portions are extracted by heating with acetic acid solution. Extracts are cleaned up using solid-phase extraction (SPE) C18 cartridges. After periodate and peroxide oxidation, they are analyzed by high-performance liquid chromatography (HPLC) with fluorescence detector. Most toxins (STX, C1,2, GTX5, dcSTX, GTX2,3 and dcGTX2,3) can be quantified after simple SPE-C18 cleanup. Extracts containing the toxins NEO, dcNEO, GTX1,4, C3,4, and GTX6 were further purified by using SPE-COOH cleanup/separation. PSP toxin concentrations are calculated by comparison of peak areas or heights in the test samples with those of the standards.

3.4.3.3.2 Chemicals Used for PSP toxin analysis

Reagents and chemicals used were of analytical grade. The following solutions were prepared in ultrapure water (Merck KGaA, Darmstadt, Germany): glacial acetic acid (1% and 0.1M aq, VWR International Srl, Milan, Italy), ammonium acetate (0.01M aq, Carlo Erba. Milan, Italy), ammonium formate (0.3M aq, VWR International Srl, Milan, Italy),

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dipotassium hydrogen phosphate (K_2HPO_4, 0.3M aq, Merck KGaA, Darmstadt, Germany), hydrogen peroxide (10% aq, Merck KGaA, Darmstadt, Germany), sodium chloride (0.05M and 0.3M aq, LabM, Heywood, UK), sodium hydroxide (0.1M and 1M aq, VWR International Srl, Milan, Italy), periodic acid (0.03M aq, Merck KGaA, Darmstadt, Germany). Mobile phases consisted of (A) 0.1M ammonium formate and (B) 0.1M ammonium formate in 10% methanol, and 0.1M acetic acid was used to adjust pH to 6.0. Certified standard calibration solutions for the analysis of PSP toxins were obtained from the Certified Reference Materials Program (CRMP) of the Institute for Marine Bioscience (IMB), National Research Council of Canada (Halifax, Canada) and included NRC CRM: STX, dcSTX, NEO, dcNEO, GTX1,4, GTX2,3, dcGTX2,3, GTX5 and C1,2.

3.4.3.3.3 Extraction, Cleanup (SPE C18; SPE-COOH) and Oxidation

Procedure for shellfish

Samples of shellfish with 1 percent of acetic acid were harvested. Extracts have been washed up using C18 cartridges of solid-phase extraction (SPE). Extracts were further purified using SPE-COOH ion exchange cleanup to establish neosaxitoxin (NeoSTX), gonyautoxins 1 and 4 (GTX 1,4) and decarbamoylneosaxitoxin (dcNeoSTX). Peroxide and periodate oxidative conversions of PSP toxins (in sample extracts and standard solutions) to fluorescent derivatives were required for HPLC detection. The procedure applied was as similar to that of the original method of Lawrence as possible and performed according to the scheme shown in Fig.3.3. Using Agilent 1290 Infinity II HPLC (multi sampler, pumps and column) analytical device coupled with Agilent 1290 Infinity II fluorescence detector (excitation wavelength set to 340 nm and emission to 395 nm), and Agilent

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autosampler, the PSP extracts were analyzed based on the HPLC prechromatographic oxidation method with fluorescence detection (Lawrence et al., 2005).

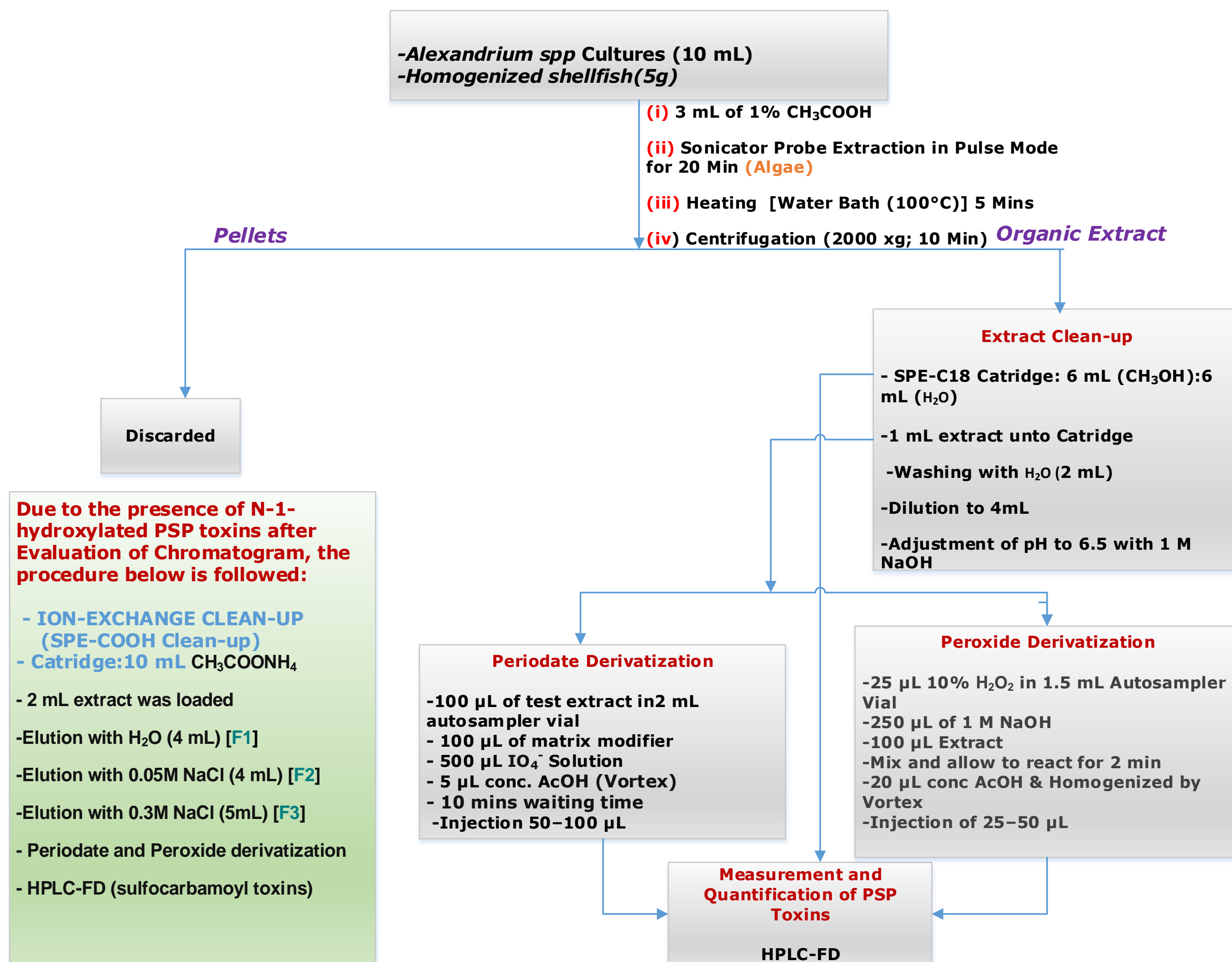

Figure 3.8: Scheme of the Lawrence Method for analysis of PSP toxins

Separation (Figure 3.8) of toxin oxidation products was carried on a reversed-phase C18 column (inertsil column ODS-3 5 µm, 150x4.6 mm) protected by a guard cartridge Phenomenex 3.2 to 8.0 mm internal diameters Column temperature was kept at 35 °C and run time was set to 25minutes. Toxin profile of PSP was identified by comparing

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chromatograms of standard solutions: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NeoSTX), decarbamoylneosaxitoxin (dcNeoSTX), gonyautoxins 1 and 4 (GTX 1,4), gonyautoxins 2 and 3 (GTX 2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX 2,3), gonyautoxin 5 (GTX 5) and N-sulfocarbamoylgonyautoxin 1 and 2 (C 1,2); NRC, Halifax, Canada. All reagents used were of HPLC or analytical grade.

HPLC was calibrated by injections of 25 or 50 mL of all individual toxin standard solutions of three mixtures with a different combination of toxin standards and one mixture with all the PSP analytical standards available.

The four mixtures were prepared as follows:

Mix I: NeoSTX, dcNeoSTX and GTX 1,4 (periodate oxidation)

Mix II: STX, dcSTX, GTX 2,3, dcGTX 2,3 and GTX 5 (peroxide oxidation)

Mix III: C 1,2 (periodate and peroxide oxidation)

Mix IV: STX, dcSTX, NeoSTX, dcNeoSTX, GTX 1,4, GTX 2,3, dcGTX 2,3, GTX 5,C 1,2 (periodate oxidation).

Retention times (chromatographic peaks) obtained after periodate and peroxide oxidations of PSP standard solutions. A few of the PSP toxins (GTX 1,4, GTX 2,3, C 1,2 and dcGTX 2,3) consist of an isomeric toxin pair that are detected together during the HPLC analysis. Consequently, these toxins are quantified together by calculating the highest toxicity factor of the two coeluted compounds. As a result, the proportion of each toxin in the pair remains unknown. Chromatographic separation was used to identify individual toxins which were

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quantitatively determined by direct comparison with calibration solutions of analytical standards.

3.4.3.3.4 PSP Toxin Extraction, Clean up and Oxidation

Procedure for Cultured Algae

PSP Toxins were extracted from frozen *Alexandrium (AL1T and AL9T)* cells by sonicating in pulse mode for 20 minutes, using 3 mL of 1% (v/v) Acetic acid solution. After sonicating, the solution was then centrifuged for 10 minutes at 2000 xg. The supernatant was then taken through clean-up. Clean-up (clean-up was performed using a Gilson Aspec XL Solid Phase Extraction Autosampler, Gilson, Merck).

SPE C18 Cleanup

SPE C18 Cleanup was done using Supelclean™ LC-18 SPE Tube bed wt. 500 mg, volume 3 mL (Supelco). A 3 mL (500 mg sorbent) SPE C18 cartridge was conditioned with 6 mL methanol followed by 6 mL water, then 1 mL of the crude extract was loaded to the cartridge keeping the flow rate between 2 and 3 mL/min for all elution. The effluent was collected into a 10 mL graduated conical test tube. The cartridge was then washed with 2 mL water and combine washings with the effluent. The pH 6.5 of the extract was adjusted with 1M NaOH using a calibrated pH meter and then diluted to exactly 4.0 mL with water. Aliquots of this extract were used for oxidation with periodate and peroxide.

SPE-COOH Clean up

SPE-COOH was done using BAKERBOND™ spe Carboxylic Acid (COOH) Disposal Extraction Columns Tube bed wt. 500 mg, volume 3 mL (J.T. Baker, Fisher Scientific

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Italia, Rodano, MI, Italy). SPE-COOH ion exchange cleanup was used only for extracts that are found to contain N-1-hydroxylated PSP toxins after C18 cleanup.

About 3 mL SPE-COOH cartridge was conditioned by passing 10 mL of 0.01 M ammonium acetate solution through it and keeping flow rate between 2 and 3 mL/min for all elutions. 2 mL aliquot of extract from the SPE C18 cleanup was passed through the cartridge and the effluent collected in a 10 mL graduated conical test tube and marked as Fraction No. 1. Then 4.0 mL water was passed through the cartridge and collected into the same tube. The final volume was adjusted to 6.0 mL in total. This fraction contains the C toxins. Then pass 4.0 mL 0.05 M NaCl solution through the same cartridge and collect in a 10mL graduated conical test tube marked as Fraction No. 2; Which was also adjusted to a final volume is 4.0 mL. This fraction may contain the toxins GTX1,4, GTX2,3, GTX5, GTX6, and dcGTX2,3. 5.0 mL 0.3 M NaCl solution was then passed through the cartridge and collected in a 10 mL graduated conical test tube marked as Fraction No. 3. The final volume was also made up to 5.0 mL. This fraction contains STX, NEO, dcNEO and dcSTX. These fractions are then ready for oxidation and HPLC analyses.

3.4.3.3.5 Oxidation Reactions

An aliquot [1 mL] of the extract and matrix modifier with water without oxidation to verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP, and not to naturally fluorescent extractives.

Periodate oxidation

All reagents and solutions to be used in the oxidation reactions are dispensed with auto-pipettes with disposable plastic tips. A 100 µL of test extract after SPE C18 cleanup was

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added to 100 µL of matrix modifier solution in a 2 mL auto-sampler vial. Then 500 µL periodate oxidant is added and mixed well on a Vortex mixer; then the solution is allowed to react at room temperature for 1 min; then 5 µL concentrated acetic acid was added mixed. The mixture was allowed to cool for 10 minutes at room temperature before injecting 50 µL into the LC system.

Peroxide oxidation

About 25 µL 10% (w/v) aqueous H_2O_2 was added to 250 µL of 1M NaOH in a 2 mL plastic auto-sampler vial and mixed. Then 100 µL of test extract after SPE C18 cleanup was also added. This was then allowed to react for 2 min at room temperature, then added 20 µL concentrated acetic acid and vortexed. 25mL of this solution was then into the LC system.

3.4.3.3.6 Quantification of PSP

Each toxin is quantitatively determined by direct comparison to external standard and linear regression method. For convenience, 4 analytical standard mixtures were used for quantitating the toxins. STX, GTX2,3, GTX5, and C1,2 produce single oxidation products with both oxidation reactions, while dcSTX produces 2 oxidation products with both reactions. However, NEO, GTX6, GTX1,4, and C3,4 each produce 3 peaks after periodate oxidation but only the second eluting peaks are used for quantitation. Because some PSP toxins (NEO and B-2; GTX1,4, and C3,4) give the same oxidation products, their quantitative determination can be made only after separation by COOH ion-exchange chromatography.

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Calculation of PSP concentration (µg/kg).

$$\textbf{Concentration (µg/kg)} = \frac{\left(\frac{y-a}{a}\right)\cdot\left\{\left[\frac{\text{VSPEC18F(mL)}}{\text{VSPEC18I(mL)}}\right]\cdot\left[\frac{\text{VSPECOOHF(mL)}}{\text{VSPECOOHI(mL)}}\right]\cdot \text{VT(mL)}\right\}}{W(\text{g})}\cdot D$$

where:

y = Area of the chromatographic peak

b = intercept of the regression linear

a = slope of the calibration curve

$\mathbf{V_T}$= Total volume of crude extract (3 mL)

$\mathbf{V_{SPEC18I}}$=Volume of extract used for performing the SPEC18 clean-up

$\mathbf{V_{SPEC18F}}$= Final volume of extract after SPEC18 clean-up

$\mathbf{V_{SPECOOHI}}$=Volume of extract used for performing the SPE-COOH clean-up

$\mathbf{V_{SPECOOHF}}$= Final volume of extract after SPECOOH clean-up

W= weight of shellfish

D= Dilution factor (if extract has been diluted).

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3.4.3.3.7 Method Evaluation

(Recovery, Precision and Accuracy, and Quality Control (QC)

Table 3.8 Quality Control

QC PARAMETERS	CRITERION
Chromatographic resolution	Peak resolution OA/DTX2 > 1.0
Sensitivity	S/N of the product ion with the lowest intensity ≥ 3
Calibration curve	Correlation coefficient $r^2 \geq 0.98$ derived from at least five calibration points and either constructed as the mean of the first and second set of the calibration curve injected.
Response drift	25% slope variation between the two sets of the calibration curve
Blank QC	Injected after high standard of the calibration curve and after sample injection No signal for lipophilic toxins (< LOD or < 10% of the lowest calibration point)
Retention time (RT) drift	< 3%

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Validation Study

For the validation of the procedure, the requirements laid down in Regulation (European Community, 2004c) No 882/2004 were used. Five-point calibration curves in the 1.5-50 µg / kg range for the OA-group, 1.5-40 µg / kg for the AZA- and PTX-group, and 3.75-250 µg / kg for the YTX-group measured linearity. As stated in Table 3.7, the correlation coefficient demonstrated a good fit for all the analytes. By examining 20 blank samples of various mollusc species (mussels, clams and oysters), the specificity was checked. Both blank samples display no interfering peaks for all the analytes in the retention period of interest. These blank samples were used to test the detection limit (LOD) and the quantitation limit (LOQ) of the system below the target concentration set by the harmonized EU SOP (2015). The LOD was 8 µg / kg for the OA, PTX, DTX, and AZA-group and 0.013 mg/kg for the YTX-group, while the LOQ was 40 µg / kg for the OA, PTX, DTX, and AZA-group and 0.060 mg/kg for the YTX-group. Cleaning of extracts.

Table 3.9: Method Evaluation: Correlation for the lipophilic toxins

Biotoxin	**R^2**
OA	0.9994
DTX1	0.9992
DTX2	0.9954
PTX2	0.9995
AZA1	0.9998
AZA2	0.9992
AZA3	0.9998
YTX	0.9994
1-Homo YTX	0.9998

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Table 4.0: Method Evaluation: Repeatability Data

Biotoxin	First level (n = 6)			Second level (n = 6)			Third level (n = 6)		
	R%		**CV%**	**R%**	**SD%**	**CV%**	**R%**	**SD%**	**CV%**
OA	93.4	9.5	9.8	94.3	6.7	8.1	88.1	6.9	8.6
DTX1	97.2	7.3	9.3	101.2	10	7.8	91.2	9.2	8.2
DTX2	100.4	6.8	8.8	102.4	4.9	5.7	94.1	10	10.2
PTX2	94.3	4.4	7.5	98.3	6.2	6.2	95.2	5.5	5.9
AZA1	90.1	6.2	8.9	92.3	4.5	5.2	87.2	3.2	4.6
AZA2	101.1	5.5	5.5	100.5	4.4	4.3	96.9	3.1	3.2
AZA3	88.9	5.6	6.3	88.4	3.5	4.2	88.9	7.4	8.4
YTX	86.2	9.2	10.2	89.9	7.5	5.5	92.7	5.1	7.3
1-Homo YTX	88.5	4.5	7.6	91.4	6.5	7	94.6	5.3	6.2

R, Recovery; SD, Standard Deviation; CV, Coefficient Variation

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CHAPTER FOUR

RESULTS AND DISCUSSION

This Chapter is a detailed presentation of the results obtained. The results are presented in the form of tables and figures. Presentation of the results is followed by a detailed discussion of trends using Ocean Data View and SPSS (version 21). Principal Component Analysis (PCA) was used to visualize similarities in water quality among locations and to describe different sources of variation in diversity of algal species.

4.1 Physico-Chemicals Parameters

4.1.1 Temperature

The seawater temperature range recorded for all stations during the period of sampling was 22.7-30.6 ºC with a mean of 27.2 ºC. This is high as Global circulation models predict warmer conditions of additional ca. 3°C in some areas of the global ocean by the end of the 21st century, [from a mean sea surface temperature (SST) of 18 °C to 21.5 °C (McNeil and Matear 2006, IPCC 2007)]. Rising SST enhances the water column stratification and decreases nutrient supply in the euphotic zone (Behrenfeld et al., 2006; Doney, 2006) which can affect the blooms of certain marine harmful phytoplankton or algae.

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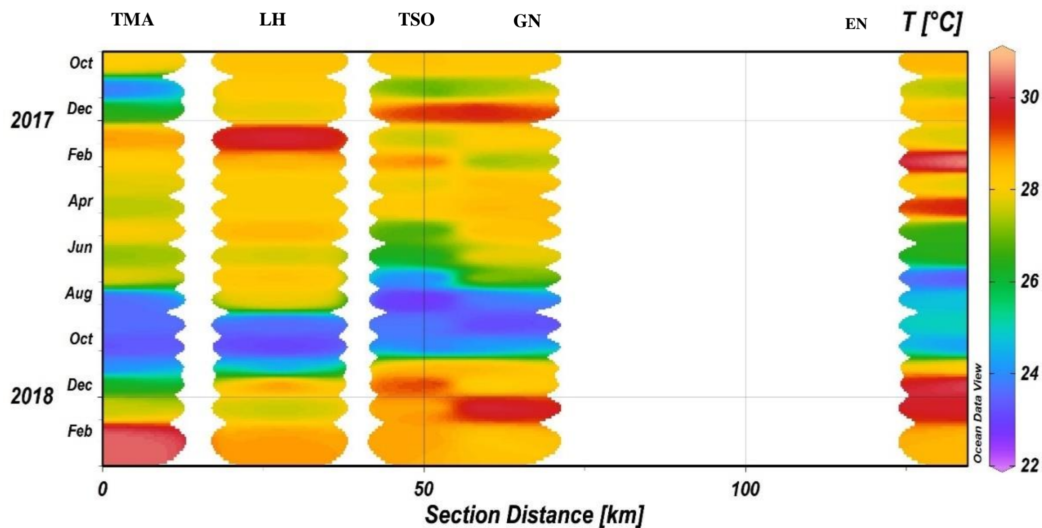

Figure 4.1: Temporal and spatial temperature over the period of sampling

All five stations recorded a minimum temperature range of 22.7-23.4 °C in the rainy season which is around (July-October; major upwelling period) temperatures below 24 ºC are consistent with Anang et al. (1979). A maximum range (29.5-30.6 °C). of surface temperature values was recorded from the end of October to the end of June. Besides the rapid drop in water temperature in July, there was also a small drop between December and January in Tema. However, all other stations did not have any drop, and this could be due to thermal expansion enhances water column stratification and a deepening of the thermocline preventing cold, nutrient-rich waters from being upwelled (Roemmich and Mc Gowan, 1995). Slight variation in temperature measurement was observed from the study (Fig. 4.1). For instance, Ekumfi-Narkwa, Tsokomey, Nyanyanor, Accra Lighthouse, and Tema recorded high mean surface temperatures. The fact that coastal waters are more susceptible to temperature fluctuations and changes can occur by both natural and anthropogenic processes can explain this finding. The coastal waters of Ghana form part of the Guinea current upwelling system of the Gulf of Guinea (Binet and Marchal, 1993).

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Two seasonal upwellings occur within the Guinea current ecosystem with a major upwelling phenomenon occurring in July to September and a minor one in January to march each year (Wiafe et al., 2008). It has been predicted that high surface temperatures are most likely to cause upwelling which introduces high concentrations of dissolved CO_2 along the continental shelf of many countries (Talmage and Gobler, 2011); and thus potential occurrence of ocean acidification. In addition, temperatures are typically known to influence physiological characteristics of aquatic organisms and primary productivity in the marine ecosystem. Warming oceans may cause thermal stress and therefore impact on survival of many invertebrates when their tolerance limits are exhausted.

Temperature is a key parameter that directly impacts multi-scale physiological rates of marine biota, e.g. enzymatic reactions, respiration, body size, time of generation, ecological interactions, metabolism of the population (Peters, 1983). Phytoplankton is experiencing an increase in enzymatic activity and growth rates over a modest temperature range with an average increase of $Q10 = 1.88$ (Eppley, 1972), suggesting that an increase in SST from today's 18 ° C to 21.5 ° C in 2100 (McNeil and Matear, 2006) may lead to an increase in the growth rate of ~25 percent assuming no other factors are present (Finkel et al., 2010). Nevertheless, given the phytoplankton community's polyphyletic complexity, the effect of temperature on metabolic rates is complicated by the susceptibility of individual organisms to warming (Huertas et al., 2011). The germination of resting spores in sediments is correlated with further implications of rising temperatures (Shikata et al., 2008). Hence temperatures recorded could be a factor to which availability of harmful algae like *Alexandrium spp, Ceratium furca, Ceratium Protoperidinium, Ceratium tripos, Dinophysis caudata, Dinophysis fortii, Gonyaulax spinifera, Lingulodinium polyedrum,*

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Noctiluca scintillans, Prorocentrum mexicanum, Prorocentrum gracile, Prorocentrum micans, Scrisppsiella-trochoidea, could be attributed to. However, sea surface temperature alone is not enough to determine the HABs presence.

4.1.2 Salinity Pertaining Along the Coast

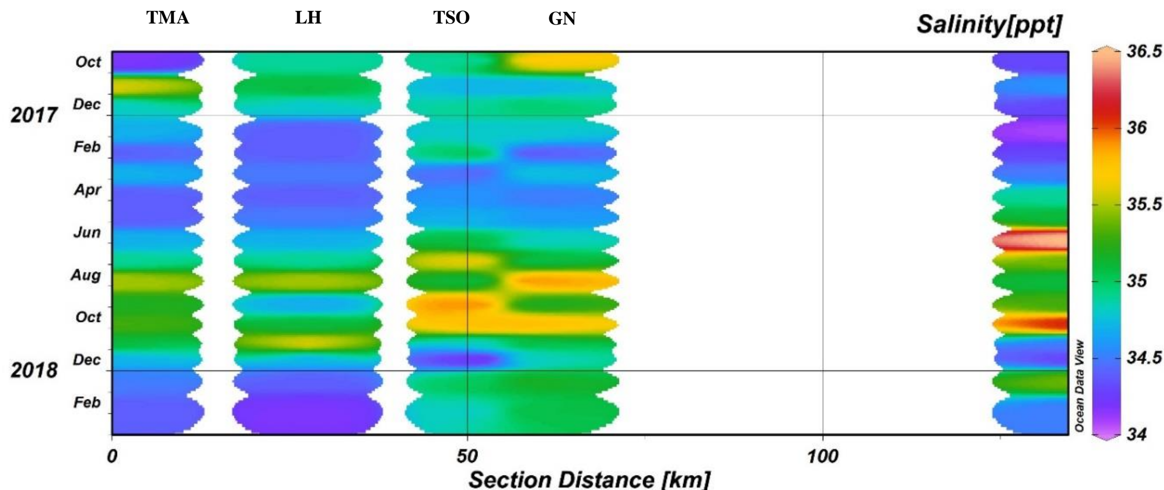

Figure 4.2: Temporal and spatial salinity over the period of sampling

Salinity measurements were within the range of 34.1-36.5 ‰ with a mean 34.8 ‰ for all five stations sampled. Salinity fluctuations were small. The salinity was below 35 ‰ from the middle of May to the end of June. Between July and the end of October, salinity values were above 35 ‰ and decreased again as temperature increased. Salinity was relatively constant among all individual sampling site studied within a range of 34.00 to 36.50 ‰ (Fig. 4.2). This may be ascribed to the fact that the sampled locations share the same open coastline. The average salinity value for all five locations, which is 34.88 ‰ is consistent with the salinity of the Atlantic Ocean areas along the equator, which is about 35 ‰. The areas with salinity above 35 ‰ were experienced around October-November, which was in the dry season when temperatures were high; this could have caused evaporation making

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the sea more saline. Along with the growing temperatures, global circulation models forecast a potential freshening of mean sea surface salinity, probably as a result of increased precipitation and ice-melt in the poles offsetting increased evaporation from the surface of the ocean in low latitudes (McNeil and Matear, 2006).

In a more limited scale, changes in atmospheric circulation also affect storm frequency and wind and precipitation patterns, which eventually may produce changes in coastal salinity, turbidity, light attenuation and inputs of terrestrial and bottom sediments-derived nutrients and pollutants (Nixon et al., 2009; Noyes et al., 2009; Wetz et al., 2011).

4.1.3 pH and Dissolved Oxygen

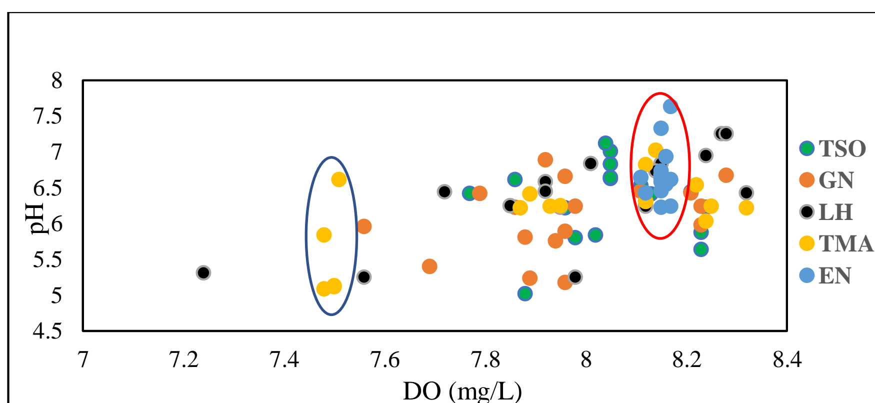

Figure 4.3: Correlation plot of pH and DO over the period of sampling

According to the general rule, high phytoplankton activity within the water gives rise to higher DO. It was observed that as pH increases, DO is also increased; DO is about 70% correlated to the pH. The pH range recorded for the period of sampling was 7.24-8.32 whiles DO was 5.02-7.63 mg/L. Dissolved oxygen positively correlated with high-density species. It has been reported that low levels of DO below 0.07 mg/L causes decline germination of algal spores whiles higher 2mg/L are known to be conducive for the growth and well-being of phytoplankton for that matter HABs.

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From Figure 4.3, a positive relationship between pH and DO was observed. In contrast, the pH values were in the range of 7.8 to 8.3. The DO was tagging along between 6- 7 mg/L. This was very explicit at the EN site where all values clustered together. The seawater around Tema was a bit acidic, and this may be due to discharge from industries.

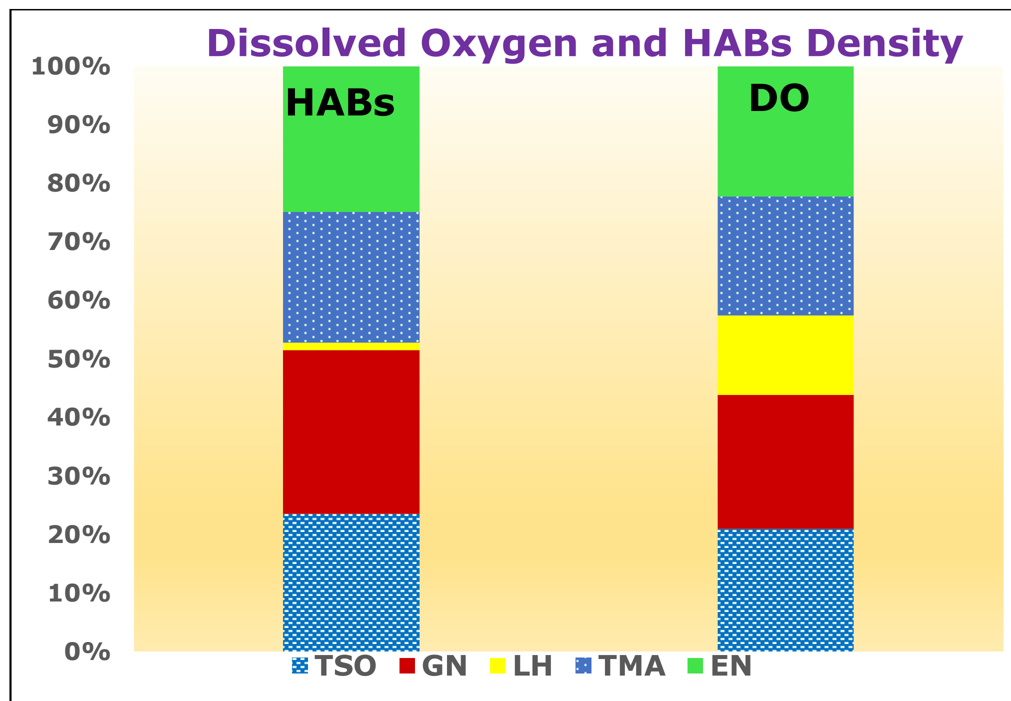

Figure 4.4: Correlation plot of HABs and DO over the period of sampling

Dissolved oxygen positively correlated with high-density species, indicating the level of DO is conducive for phytoplankton bloom.

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4.1.4 Nitrate and Phosphate Along the Coast

Figure 4.5 reveals the temporal and spatial distribution of phosphate and nitrate recorded at all five stations over the period of sampling. Generally, nitrate concentrations were higher than that of phosphates in the seawater from all sampling sites.

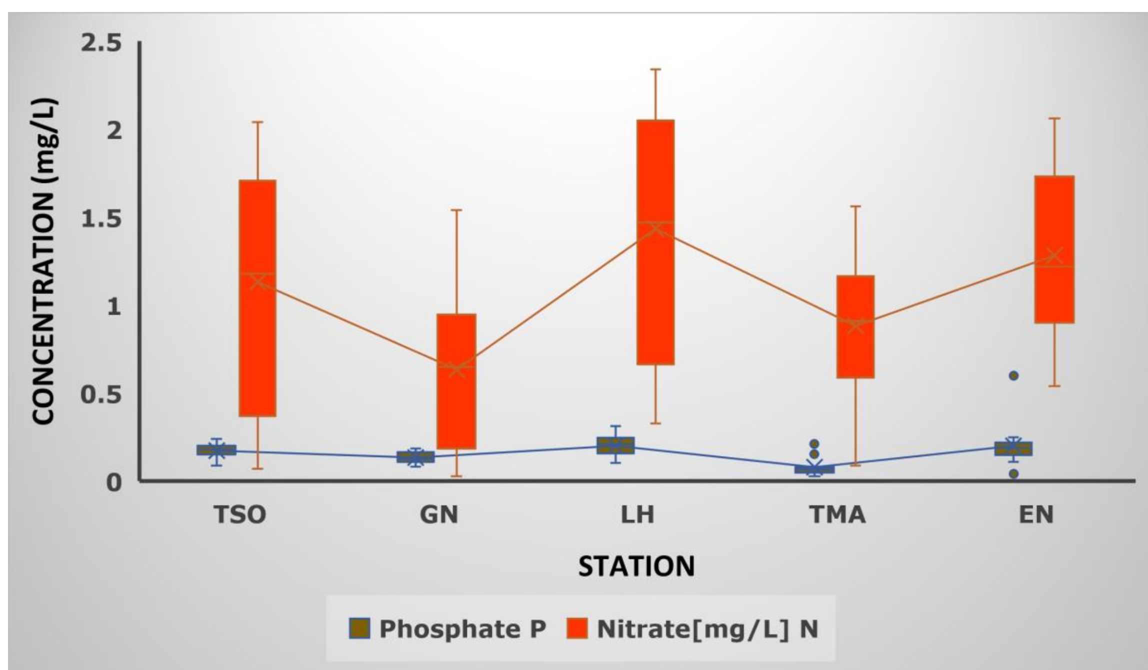

Figure 4.5: Temporal and spatial distribution of phosphate and nitrate

Figure 4.5 shows the temporal and spatial distribution of phosphate and nitrate recorded at all five stations over the period of sampling.

Nitrates

The concentrations of NO_3-N in the study areas during the sampling period were in the range of 0.038 to 2.34 mg/L (Figure 4.6). No spatial significant difference ($p > 0.05$) in nitrate concentration was observed during the dry season in spatial distribution. However, the concentration of 0.030 ±0.003 mg/L nitrate at Accra Lighthouse was significantly

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higher ($p < 0.05$) than the concentration obtained at all other stations at the wet season. Comparatively high concentrations of NO_3-N observed at all stations during the sampling period. Accra Lighthouse was observed to have the highest nitrate concentrations during sampling. NO_3-N is reported to be a limiting factor for the growth of dinoflagellates. That means the presence of NO_3-N causes growth of phytoplankton. Dinoflagellates have been documented to respond significantly to nitrate treatment whiles PO_4^{3-} treatment alone does not induce any response from phytoplankton. It has been reported that N: P of 16:1 is most optimal for the growth of phytoplankton in the aquatic environment.

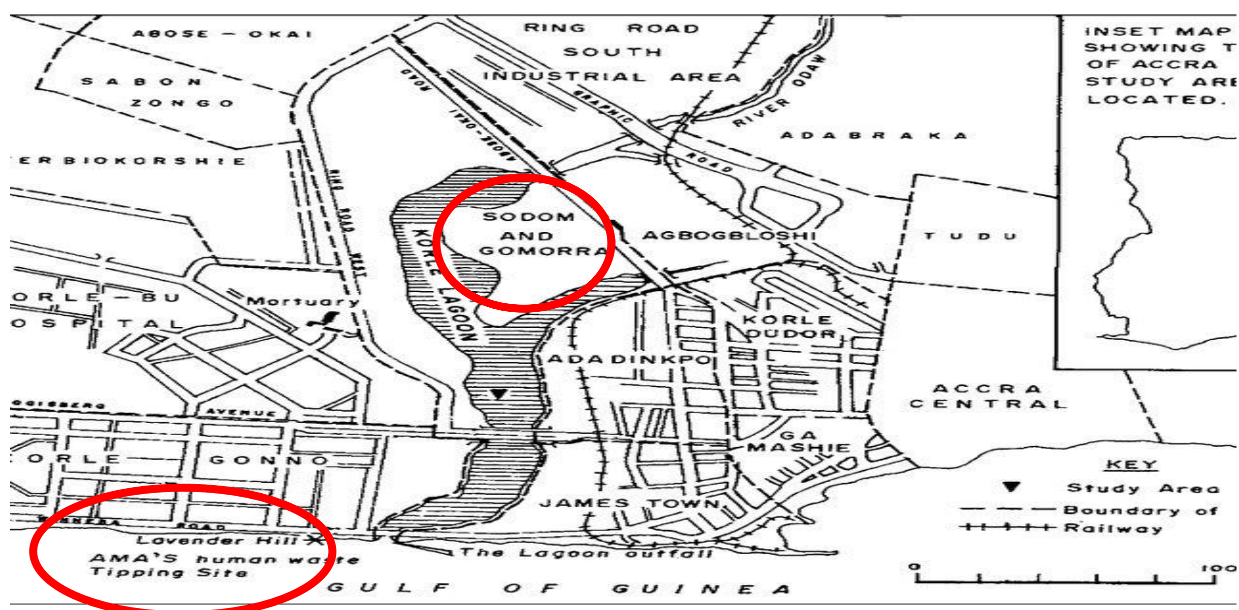

Figure 4.6: Map of Korle Lagoon (Accra) and its Catchment (Boadi & Kuitunen, 2002)

Indicating that a sudden influx of nitrate and phosphate can cause a bloom to occur. High level of nitrate at Light House could be a result of the AMA's human waste Tipping Site and the Sodom and Gomorra settlements near the sampling site (Figure 4.6).

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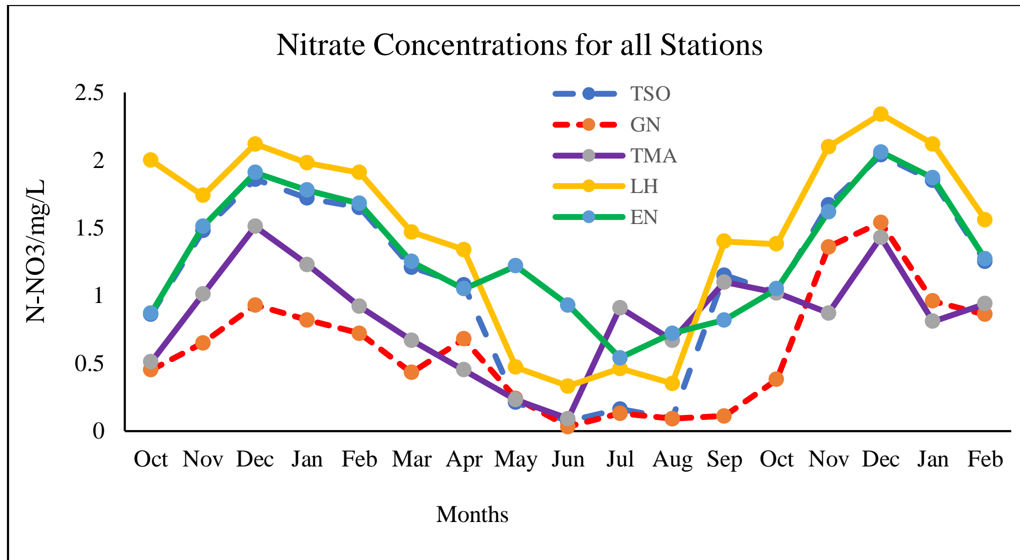

Figure 4.7: Nitrates distribution across all five stations

Generally, there was a decrease in nitrate concentrations from May-August except for Ekumfi-Narkwa. The average nitrate concentration saw a gradual increase from September to October; then increased again to a peak of over 2.34 mg/L by December (Figure 4.7).

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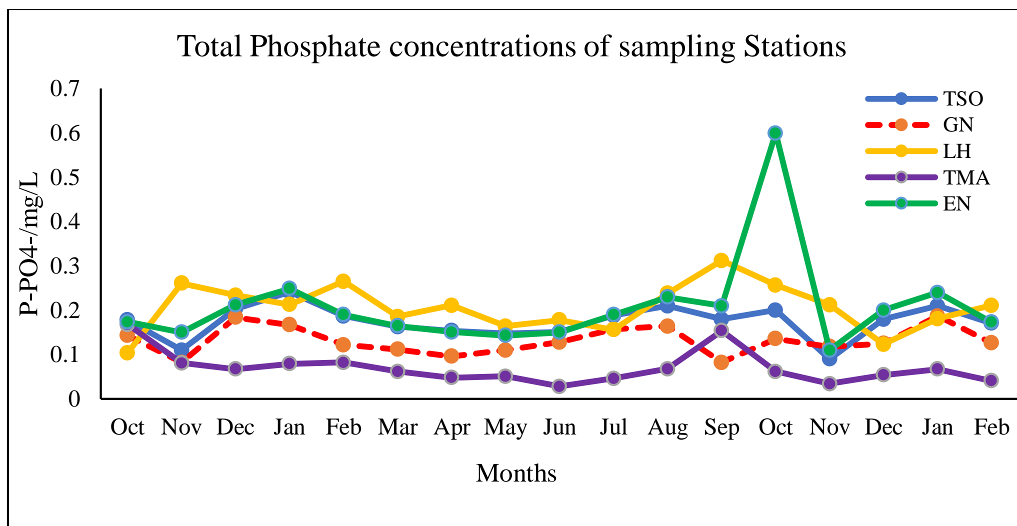

Figure 4.8: Phosphate distribution across all five stations

The concentration of phosphate in study area coastal waters ranged from 0.028 to 0.600 mg/L and an average of 0.15 mg/L, respectively (Figure 4.8). No significant difference ($p > 0.05$) was observed in the spatial distribution of phosphate concentration among all the stations. During the wet season (August, September), the phosphate concentration at all stations was relatively higher than the phosphate concentration for all other months during the sampling.

Coastal areas constitute bio-geochemically active environment with a host of high bioactivity fuelled by nutrient inputs river depositions and other natural sources (Cai et al., 2011). The concentration of phosphate recorded in the study was similar for all the sampling locations. The levels of phosphates are less than the recommended limits (5 mg/L) set for Fisheries and Aquatic life (Chapman, 1996) and therefore can say the Ghanaian coast is safe to aquatic life. The phenomenon of upwelling in addition to waste discharges from rivers is largely the source of nutrient enrichment in coastal waters of Ghana. Meanwhile, it is worth noting that high levels of nutrients in coastal systems could

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lead to excessive production of algae, some of which may be toxic to humans and aquatic life. Additionally, respiration of microalgae produces carbon dioxide, which subsequently increases the acidity of coastal waters (Cai et al., 2011).

Chlorophyll ‘a’

The concentration of Chl ‘‘a’’ showed large variability with time. From September-December, when high densities of dinoflagellates were observed. Surface Chl ‘‘a’’ ranged in all locations from 0.004 to 3.561 mg/L (mean 0.9878 mg/L) [Figure 4.9].

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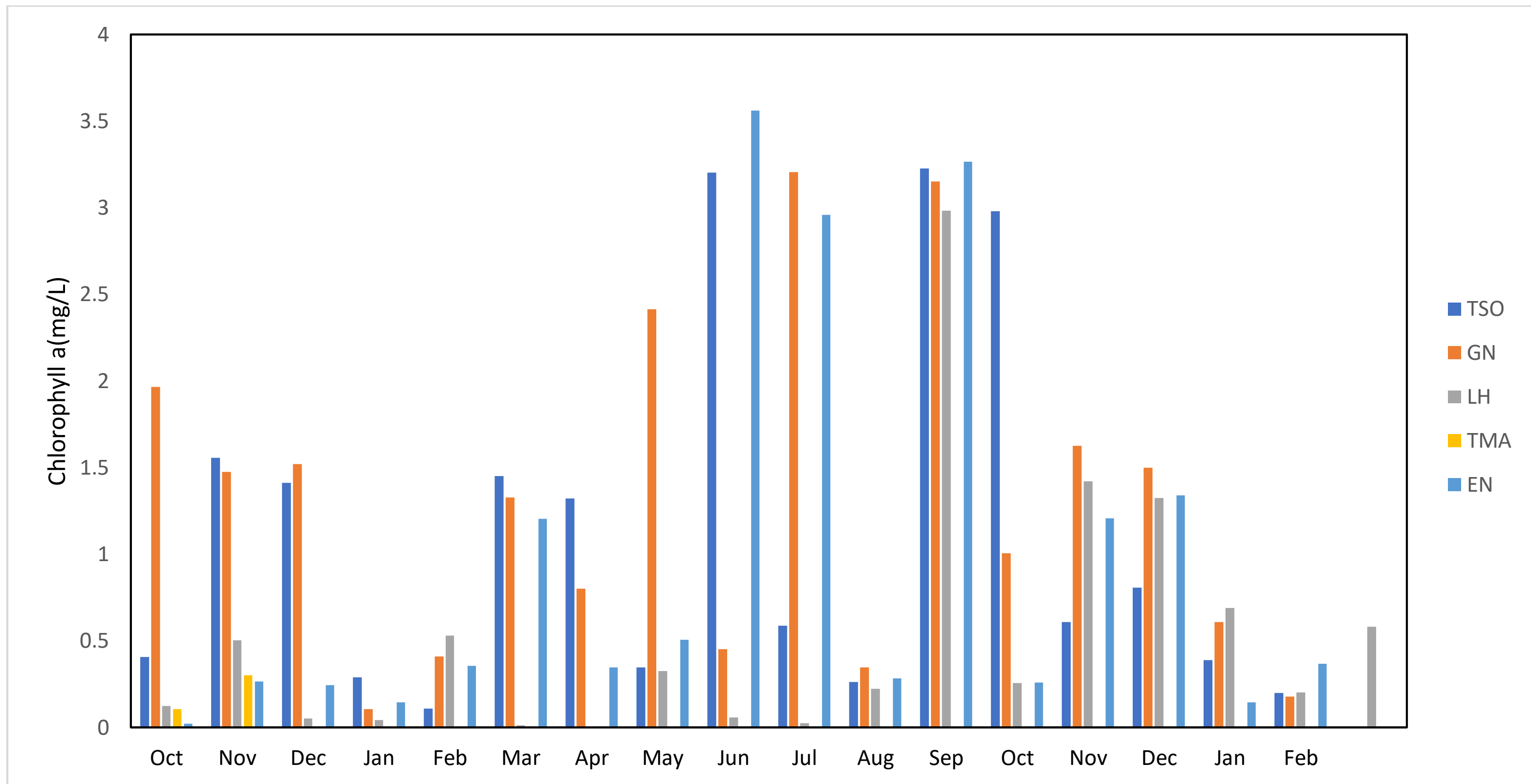

Figure 4.9: Chlorophyll ‘a’ variability over the sampling period

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Table 4.1: Correlation of Physico-Chemical Parameters with Chlorophyll 'a'

	pH	*DO*	*SAL*	*COND*	*P*	*N*	*SiO_4*	*Chl''a''*
pH	1							
DO	**0.547**	1						
SAL	-0.053	-0.224	1					
COND	0.021	-0.105	**0.631**	1				
P	0.176	0.180	0.200	0.136	1			
N	0.226	0.179	-0.252	-0.178	0.275	1		
SiO4	0.203	0.074	-0.167	-0.043	0.021	0.354	1	
Chl''a''	-0.132	-0.161	**0.402**	**0.493**	-0.115	-0.160	-0.131	1

Seawater water Chl ''a'' revealed a weak positive correlation with salinity concentrations ($R \leq 0.402$) [Table 4.1]. No significant correlation was found between Chl ''a'' concentrations and macronutrients nitrate, phosphate and silicate concentrations. The overall lack of strong correlations points toward a complex combination of factors affecting the HABs community.

4.2 Identification and Quantification (Distribution) of Algal Species

4.2.1 Harmful Algae Species Identified at All the Five Sampling Stations

HABs species composition was assessed both in water and net samples. Net samples were used for isolation. Seawater sampled per litre was used for quantification.

A total of thirteen taxa were identified and 12 at the species level. Most of the HABs species identified belong to the family of Dinoflagellates. Nine major orders (Gonyaulacales, Peridiniales, Gymnodiniales, Suessiales, Prorocentrales, Dinophysiales, Blastodiniales, Phytodiniales and Noctilucales) are recognized within the dinoflagellates

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Past studies in Ghana was only in Tema, which dates to 1979, and it was on phytoplankton distribution and not HABs. This quantitative and qualitative data estimate is the first update.

Six (6) species of potentially toxic thecate dinoflagellates [*Alexandrium sp, Dinophysis caudata, Dinophysis fortii, Gonyaulax spinifera, Lingulodinium polyedra, and Prorocentrum spp (mexicanum)*] were present in the sample. Species are described as potentially toxic when they have been identified as biotoxin producers. Those described as harmful do not produce toxin but have been known to cause oxygen depletion or fish kills by clogging the gills of fish. Not only related to its toxicity, but the absolute concentrations of alga also can cause significant functioning issues (e.g., clogging) to power and desalinization plants (Caron et al. 2010) and may lead to oxygen reduction and fish kills when blooms persist over long periods (Hallegraeff, 1993; Lewitus et al., 2016).

Data on HABs species identified from similar studies in other parts of the world including Africa is presented in Table 4.2. All species identified were dinoflagellates.

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Table 4.2: HABs species identified in the Study Area

Class	Order	Genus	This study Species		Other studies Countries	Reference
Dinoflagellates	Dinophyceae	Dinophysis	caudata	D.caudata	Spain, Kenya	Fernandez et al. (2006); UNESCO (2001)
			fortii		Italy, South Africa	Della-loggia et al. (1993); Pitcher and Calder, (2000)
	Prorocentrale	Prorocentrum	mican	Prorocentrum spp	Nigeria	Zendong et al., 2016
			gracile		Nigeria	Zendong et al., 2016
			mexicanum		Lebanon	Lassus et al., 2016
	Gymnodiniale	Ceratium	furca tripos protoperidinium	Ceratium spp	Nigeria	To et al., 2015
	Peridiniale	Scripsiella	trochoidea		Nigeria	Zendong et al., 2016
	Gonyaulacale	Gonyaulax	spinifera	G.spinifera	Ivory coast, Italy, New Zealand	Wall et al., 1977; Rhodes et al., 2006; Riccardi et al., 2009
		Lingulodinium	polyedra	Lingulodinium spp	Kenya, SouthAfrica, Sierra Leone	UNESCO, 2001; Wall et al., 1977
			Alexandrium spp	Alexandrium spp	Kenya	UNESCO, 2001
	Noctilucales	Noctiluca	scintilans			To et al., 2015

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4.2.1. Gonyaulacales

4.2.1.1 Lingulodinium polyedra (*Gonyaulax polyedra*)

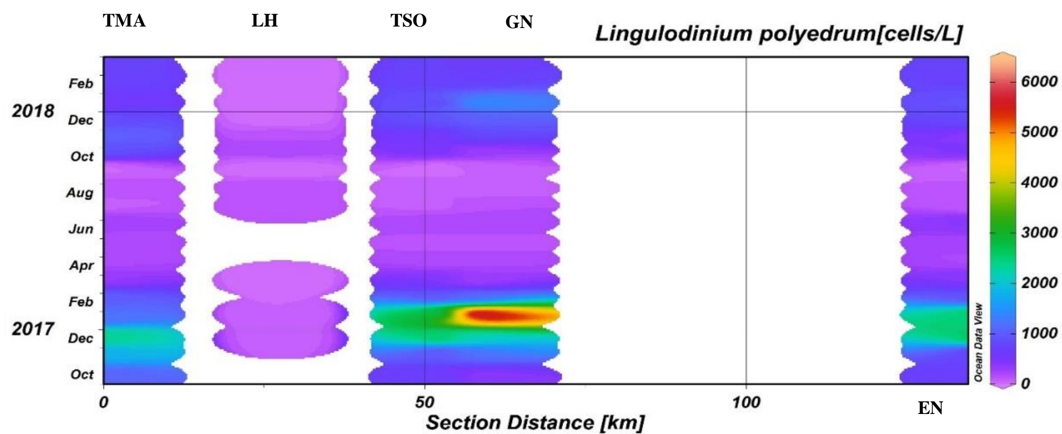

Figure 4.10: Temporal and spatial distribution of Lingulodinium polyedra

Figure 4.10 shows the temporal and spatial distribution of *Lingulodinium polyedra* at all five stations over the period of sampling. *Lingulodinium polyedra* (Stein) Dodge 1989 is bloom-forming and widely distributed species which has been documented as a yessotoxin producer and has been recently described in Nigeria (Zendong et al., 2016). In this study, it was detected in high quantities in December and January with a peak of 55416 cells/L for all stations and the highest concentration (6200 cells/L) in Gomoa Nyanyanor indicating that it is very abundant in the study area whiles in LH (Accra Lighthouse) few species of *L. polyedra* were seen.

It was observed in the dry season, a non-upwelling period (November–April) when water temperatures are high, salinity also shows higher values. The peak concentrations were recorded in December-January the small unpredictable upwelling season (minor upwelling), this confirms Anang (1979) record of high phytoplankton levels. During this

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dry months, there was an increase of 3-6 °C in the surface water temperature, associated with the onset of the seasonal thermocline which was conducive for *L.polyedra* growth

4.2.1.2 Gonyaulax spinifera

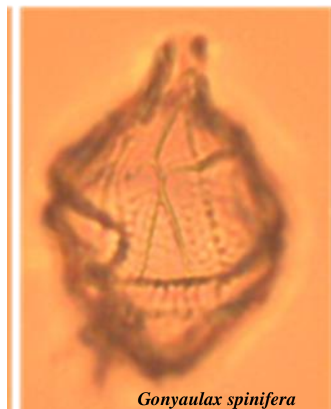

Figure 4.11: Image of Gonyaulax spinifera

Gonyaulax spinifera (Claparède and Lachmann) Diesing 1866: 382 (Figure 4.11) is a widely distributed yessotoxin producing specie (Rhodes et al., 2006). It has been reported Europe, North America and Asia and North Africa (Mauritania) but not in West Africa (Lassus et al., 2016). *G. spinifera* was the second most abundant HABs species identified during the monitoring in the study areas.

It occurred in high concentrations to a peak of 1400 cells/L in the dry season (minor upwelling and non-upwelling season) than the rainy season (major upwelling season), but it was present throughout the study period; an average of 406 cells/L was recorded for all sampling stations. In LH (Accra Lighthouse), no specie of *G. spinifera* was observed (Figure 4.12).

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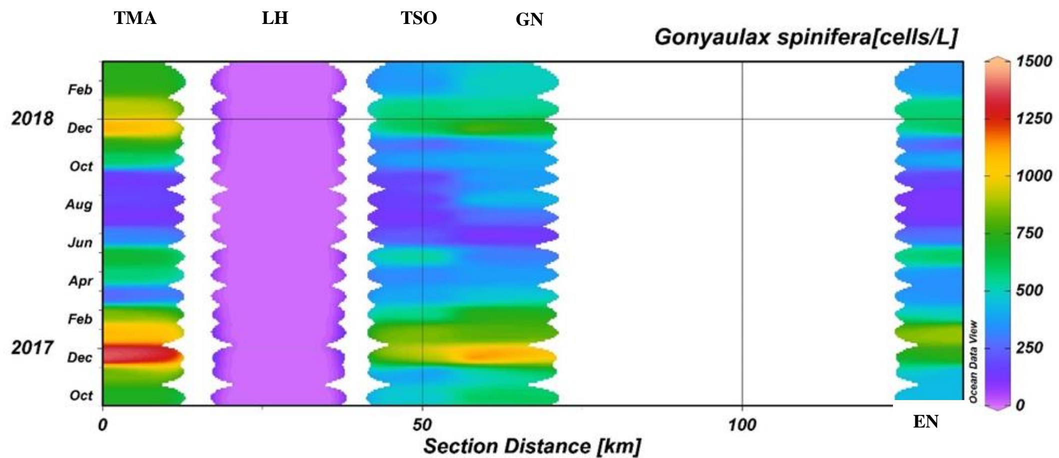

Figure 4.12: Temporal and spatial distribution of Gonyaulax spinifera

4.2.1.3 Alexandrium spp

***Alexandrium spp*:**

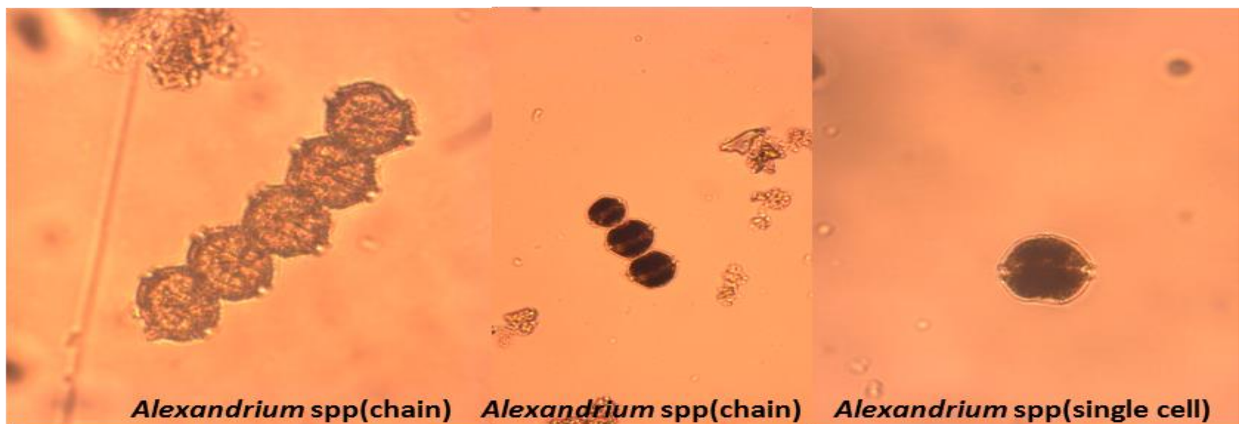

Figure 4.13: Image of *Alexanderium spp*

The genus has the most species producing paralytic shellfish poisons. Several species produce saxitoxin and related congeners from dinoflagellate Alexandrium. Although a variety of Alexandrium species has been recorded worldwide in temperate waters, potentially toxic species were recorded along the northeastern and western coasts of North

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America and in the Canadian marine provinces. Alexanderium catenella has been attributed to saxitoxin production (STX) on the west coast of South Africa (2000 Pitcher and Calder, 2001 Pitcher et al.), as well as Alexandrium sp., reported in Angola and Tunisia, but in West Africa, it was never documented.

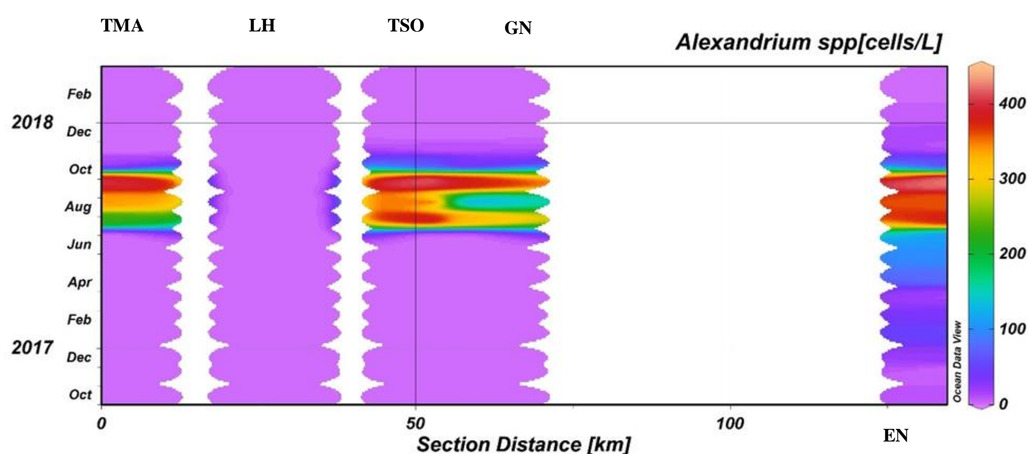

Figure 4.14: Temporal and spatial distribution of *Alexandrium sp*

Figure 4.14 shows the temporal and spatial distribution of Alexandrium sp at all five stations over the period of sampling. This species was not present until July, August, September which is part of the major upwelling season and probably due to low temperatures recorded in the season. Concentrations were between 0 - 425 cells/L. *Alexandrium sp* cells were lowest of all HABs species identified.

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4.2.2 Dinophysiales

The dinoflagellate genus Dinophysis includes species known to produce lipophilic shellfish toxins (Kazuhiko et al., 2007). *Dinophysis* blooms have been well documented in the western Mediterranean Sea (Lassus et al., 1991; Boni et al., 1993; Cabrini et al., 1993; Marasovic et al., 1998; Aubry et al., 2000; Giacobbe et al., 2000; Caroppo, 2001; Vila et al., 2001) in contrast to the Gulf of Guinea (i.e. Ghana where documented *Dinophysis* blooms did not exist or have not been documented).

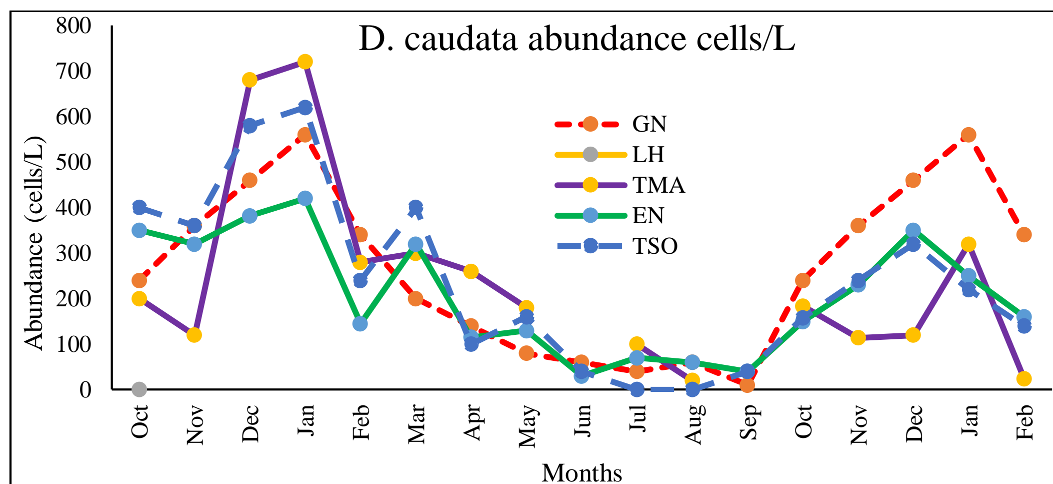

Figure 4.15: Seasonal abundance *D.caudata* (cells/L) from sampling stations

Dinophysis caudata (Saville-Kent, 1881), a producer of Okadaic acid (OA) and its analogues, the dinophysis toxins (DTX1, DTX2, and DTX3). Recently been documented to produce pectenotoxin (PTX2) as well (Lassus et al., 2016). These species have been reported in Europe, the Americas, Asia, Oceania and Nigeria. In this study, D. caudata was detected in high quantities in December and January with a peak of 15672 cells/L for all

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stations and the highest concentration (4510 cells/L) in Gomoa Nyanyanor indicating that it is very abundant in the study area. It was observed in the dry season; a non-upwelling period (November–April) when water temperatures are higher, which also influences salinity and nutrients. The peak concentrations were recorded in December-January (Figure 4.15) which happened to be a minor upwelling season (usually unpredictable).

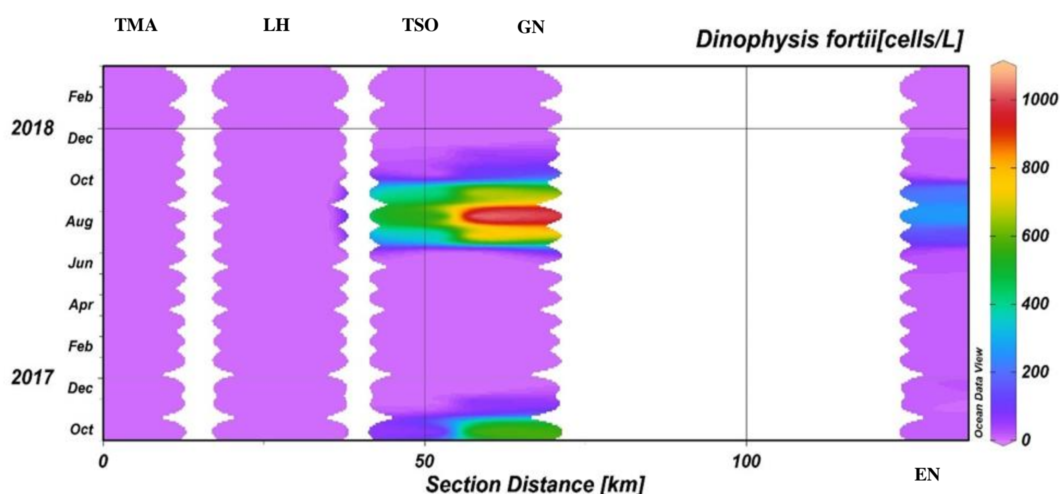

Figure 4.16: Seasonal abundance D. fortii (cells/L) from sampling stations

Dinophysis fortii (Pavillard, 1923) is a photosynthetic dinoflagellate which reproduces asexually by binary fission (Montagnes, 2006; Smithsonian, 2012). Length: 62 - 66 µm and Width: 41 - 58 µm. Like its analogue specie caudata, it produces OA, DTX2 and PTX2 (Hoshial et al., 2003). In this study, *D. fortii* were not found in Tema (TMA) and Accra Lighthouse (LH) as seen in figure 4.16. The highest concentration was recorded in Gomoa Nyanyanor with a peak of 1100 cells/L. The presence of Dinophysis fortii is an indicator of possible contamination of shellfish with OA, DTX2 and PTX2 in the coast of Ghana.

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4.2.3 S. trochoidea

S. trochoidea *(*F. Stein) A.R. Loeblich III, 1976) is a photosynthetic dinoflagellate which reproduces asexually by binary fission (Montagnes, 2006; Smithsonian, 2012). A recent report described *Scrippsiella trochoidea* in Lagos, Cross Rivers and Delta States (Ajuzie and Houvenaghel, 2009).

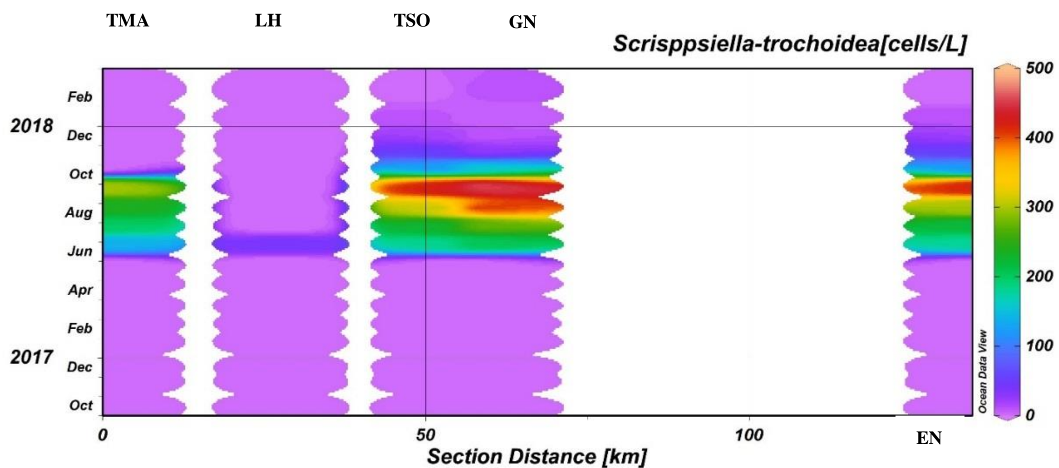

Figure 4.17: Seasonal abundance *S. trochoidea* (cells/L) from sampling stations

In this study, *S. trochoidea* had less density in Tema (TMA), i.e. 300 cells/L and LH (40 cells/L). T TMA est LH ati TSO s re GN d in Gomoa Nyanyanor with a peak of 460 cells/L, as indicated in Figure 4.17. The presence of *S. trochoidea* is an indicator of possible non-toxic bloom in the coast of Ghana. Scrippsiella trochoidea is a non-toxic marine dinoflagellate that has been revealed in both cold and tropical waters where red tide events are known to occur (Cooper et al., 2016).

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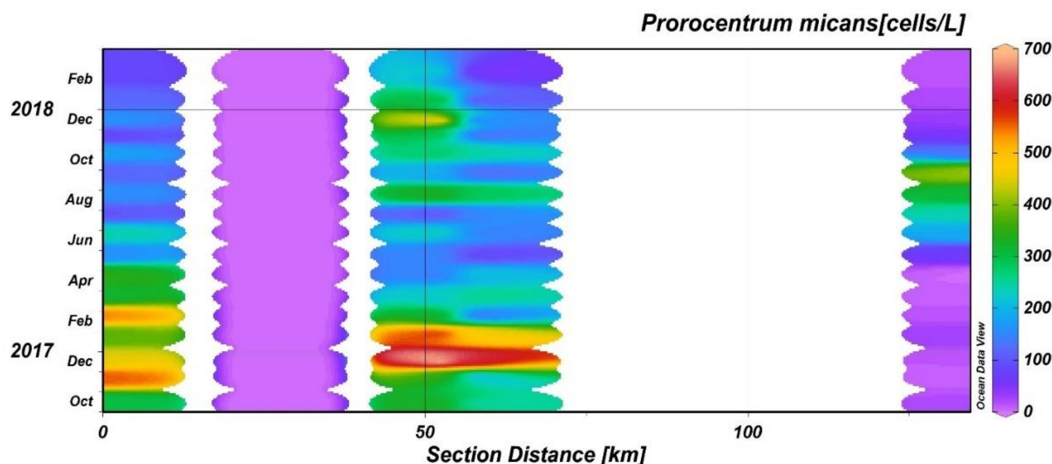

Figure 4.18: Seasonal abundance P. micans (cells/L) from sampling stations

P. micans belong in family Prorocentraceae. All species of this family are in the genus Prorocentrum. *P. micans* are associated with high biomass harmful blooms. Cosmopolitan and euryhaline, P. micans flourishes in neritic and brackish waters in the Northern Hemisphere in late summer (Ignatiades and Gotsis-Skretas, 2010). Abundance was generally between a range of 0-700 cells/L and a mean density of 173.34 cells/L for all sampling stations. With TSO (100-700 cells/L) and TMA (84-560 cells/L) recording the highest abundance in the dry season, as shown in Figure 4.18. This indicates the species might have an affinity to high temperature and high salinity as revealed by Ignatiades and Gotsis-Skretas, 2010.

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4.2.4 Noctilucaceae

Noctiluca scintillans is the single species of this family. It is a non-toxic bloom-forming species responsible for harmful outbursts (water discolouration, anoxic events) (Lelong et al., 2012).

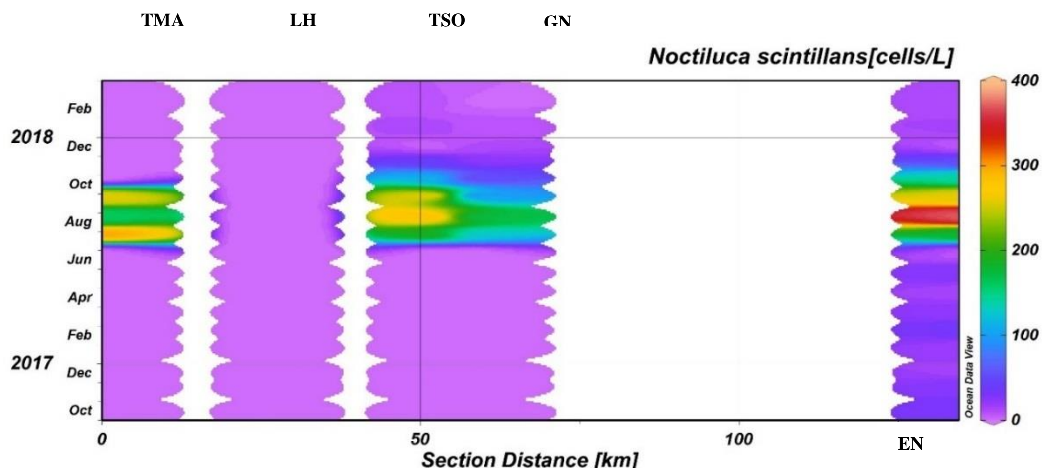

Figure 4.19: Seasonal abundance N. scintillans (cells/L) from sampling stations

Cell abundance of Noctiluca scintillans was generally between a range of 0-370 cells/L and a mean density of 39 cells/L for all sampling stations. With EN (8-370 cells/L) and TMA and TSO recording (300 cells/L) as indicated in Figure 4.19 above, in the wet season indicating the species might have a likeness to low temperature and low salinity occurs over a wide temperature range of about 10 to 25 ° C and at higher salinities that are compatible with our environment's conditions. In high productivity areas such as upwelling or eutrophic areas where diatoms dominate, it is especially abundant, since they are its preferred source of food (Türkoğlu, 2013). The existence in the upwelling season of Noctiluca scintillans strongly coincides with the Turkoğlu (2013) inference. Toxic N. blooms. Scintillans have been concerned with massive killings of fish and aquatic

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invertebrates. While this species does not contain a toxin, toxic amounts of ammonia that are then ex TMA ıto t LH un TSO ate GN ve been found to accumulate, likely acting as the killing agent in blooms (Okaichi and Nishio, 1976; Fukuyo et al., 1990). Therefore, the need to track HABs continuously.

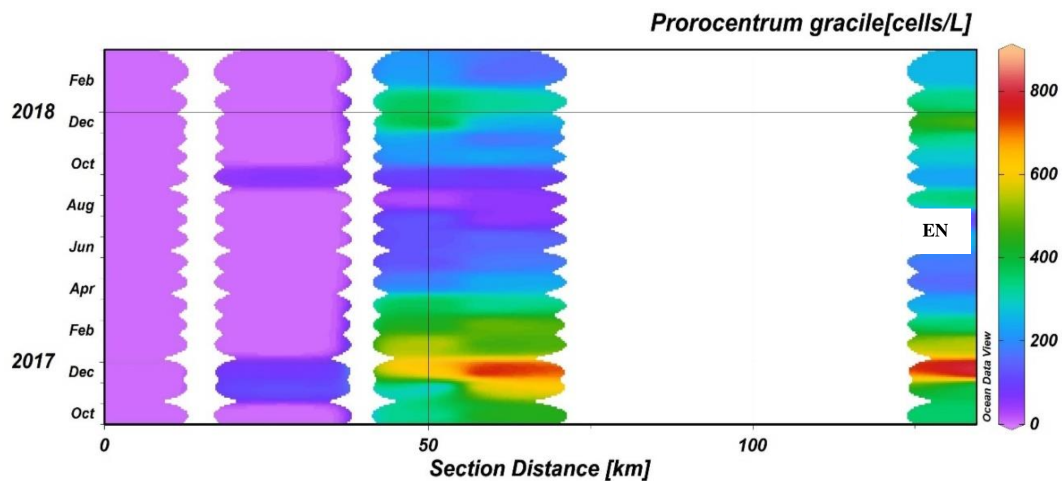

Figure 4.20: Seasonal abundance P. gracile (cells/L) from all sampling station

In cool, temperate to tropical waters, it is cosmopolitan (Steidinger and Tangen, 1996). There have been records from Chile of red tides triggered by Prorocentrum gracile (Alvial and Garcia, 1986). This species was also present in Japan (Toriumi,1980) in a red tide bloom dominated by P. minimum. Toxins are not known to be produced by this plant. In the Atlantic and Pacific Oceans and the Mediterranean Sea (Dodge,1975), P. gracile is found.In this study (figure 4.20), *P. gracile* cell abundance was mostly between a range of 0-810 cells/L and a mean density of 183 cells/L for all sampling stations. Recording high densities at EN (810 cells/L), GN (780 cells/L) and TSO (620 cells/L) in the dry season

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indicating the species might have a likeness to high temperature and high salinity.

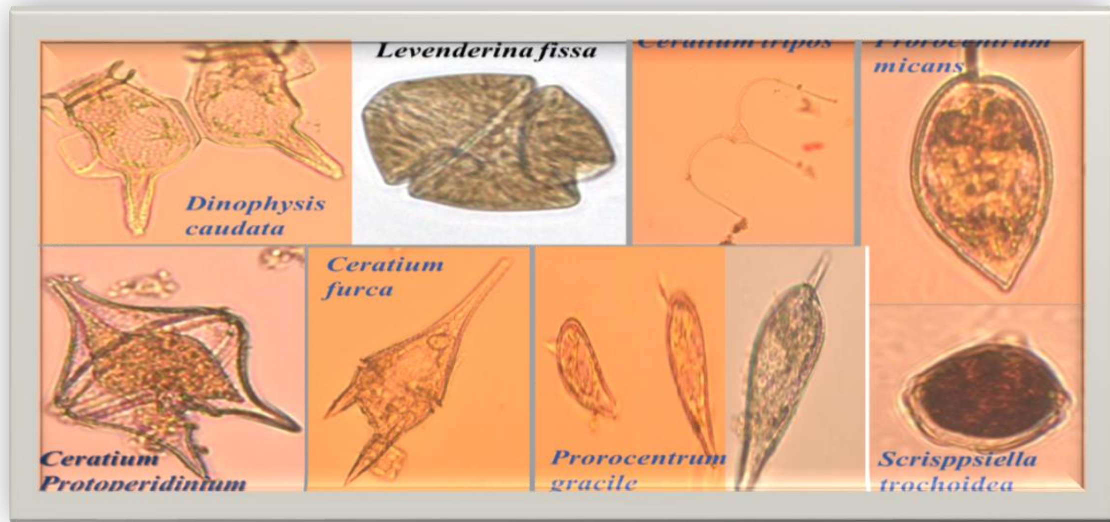

Figure 4.21: Photos of HABs species identified B

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Distribution of Harmful Algal Bloom(HABs) Species (Cells/L)

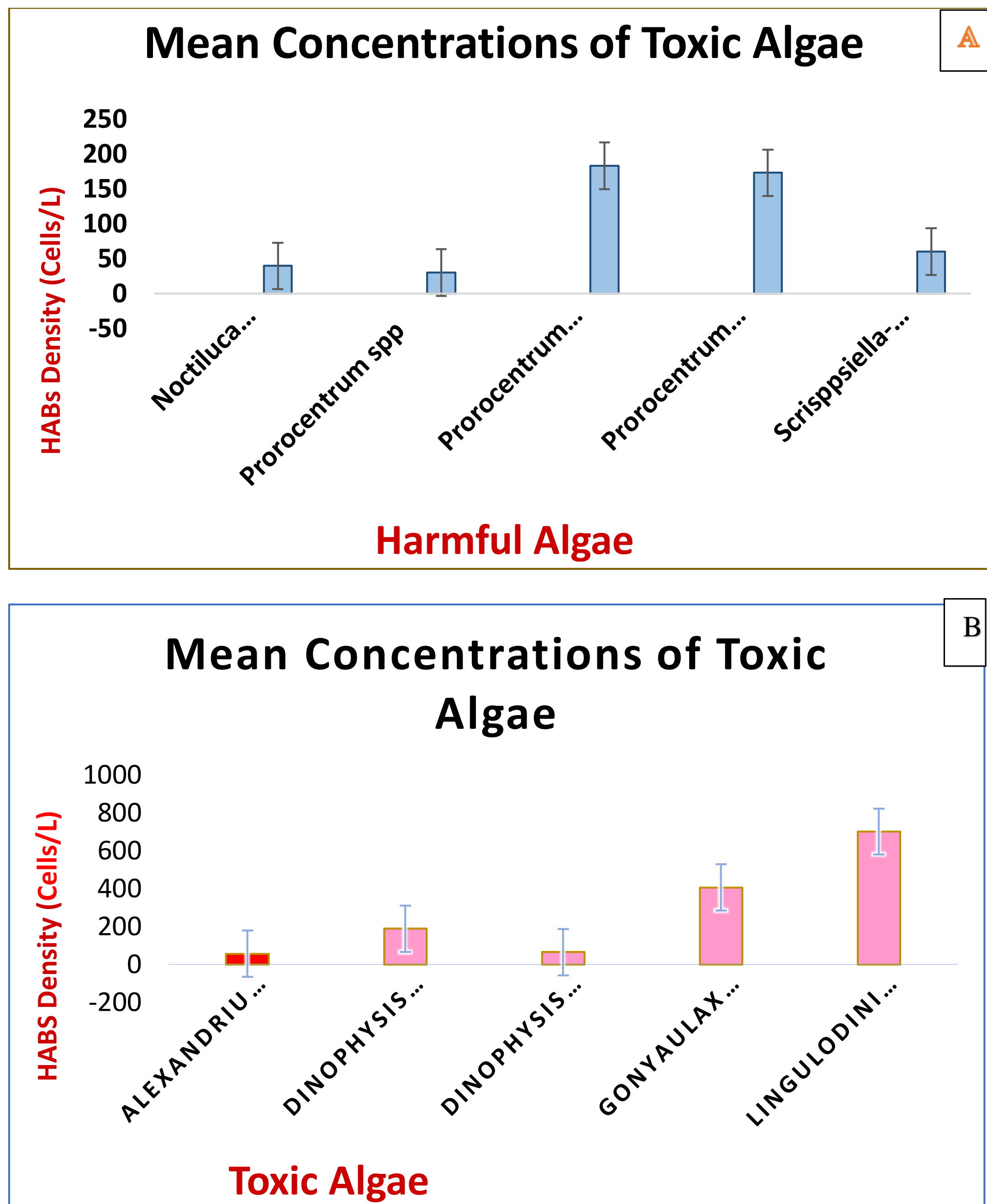

Figure 4.22 (A & B): Distribution of Harmful Algal Bloom(HABs) Species (Cells/L)

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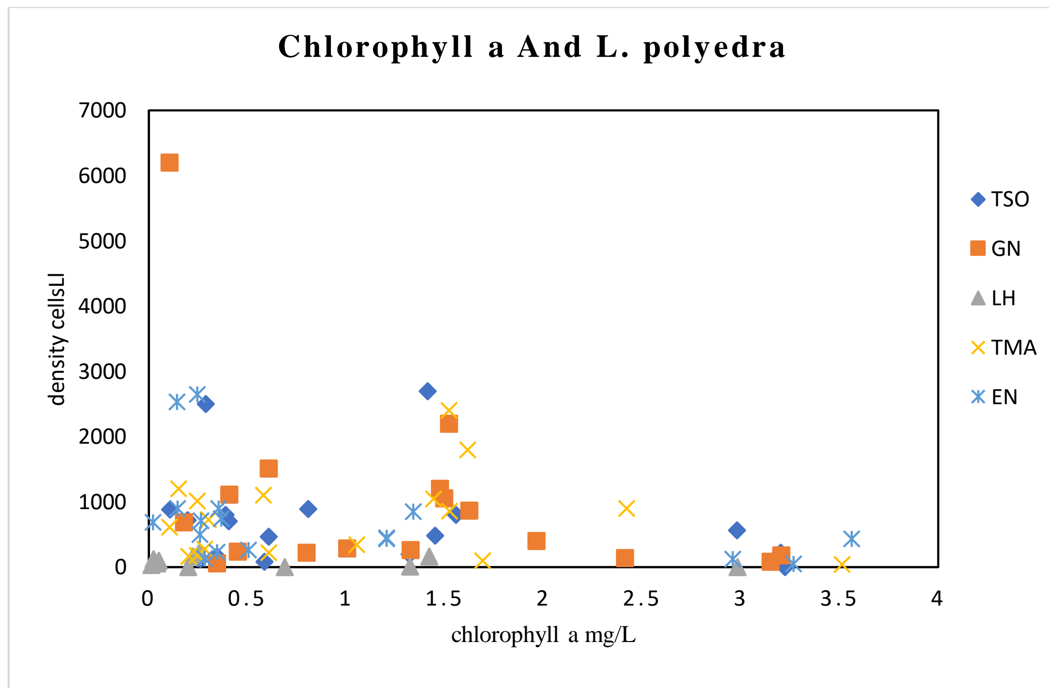

Figure 4.23: Chlorophyll 'a' and L. polyedra relationship

To test the relationship of the density of HABs species to the concentrations of chlorophyll. It was observed that chlorophyll a is not correlated or does not influence any species since L. polyedra, which is the HABs species with the highest density during the period of study. From Figure 4.23, the HABs species contributed a little of concentrations of Chlorophyll-a detected.

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4.3 Principal Component Analysis

Principal Component Analysis(PCA) was employed to visualize association that exists among various variables considered in this work. In the PCA, Varimax rotation with Kaiser Normalization was used to maximize the sum of the variance of the factor coefficients, which better explained the possible Physico-chemical parameters that influenced the abundance of the harmful algae identified.

4.3.1 Principal Component Analysis (HABs and Driving Factors)

4.3.2 Seasonal occurrence and their driving factors

Calculated factor loadings, cumulative percent and percentages of variance explained by each factor in R-mode PCA. The factor scores for all physicochemical factors and harmful algal. In the analysis, four factors with Eigenvalues >1 were extracted from the varimax-rotated factor analysis of all analytes in the data set. Factor scores represent the cumulate contribution of all analytes loaded on a factor/principal component. Positive scores in the PCA is an indication that the presence of HABs species is affected by the incidence of the physicochemical parameters that are significantly loaded on that specific factor/component. In contrast, negative scores suggest that the presence of HABs species is virtually unaffected by those Physico-chemical parameters. The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.791, with a significance of 0.000.

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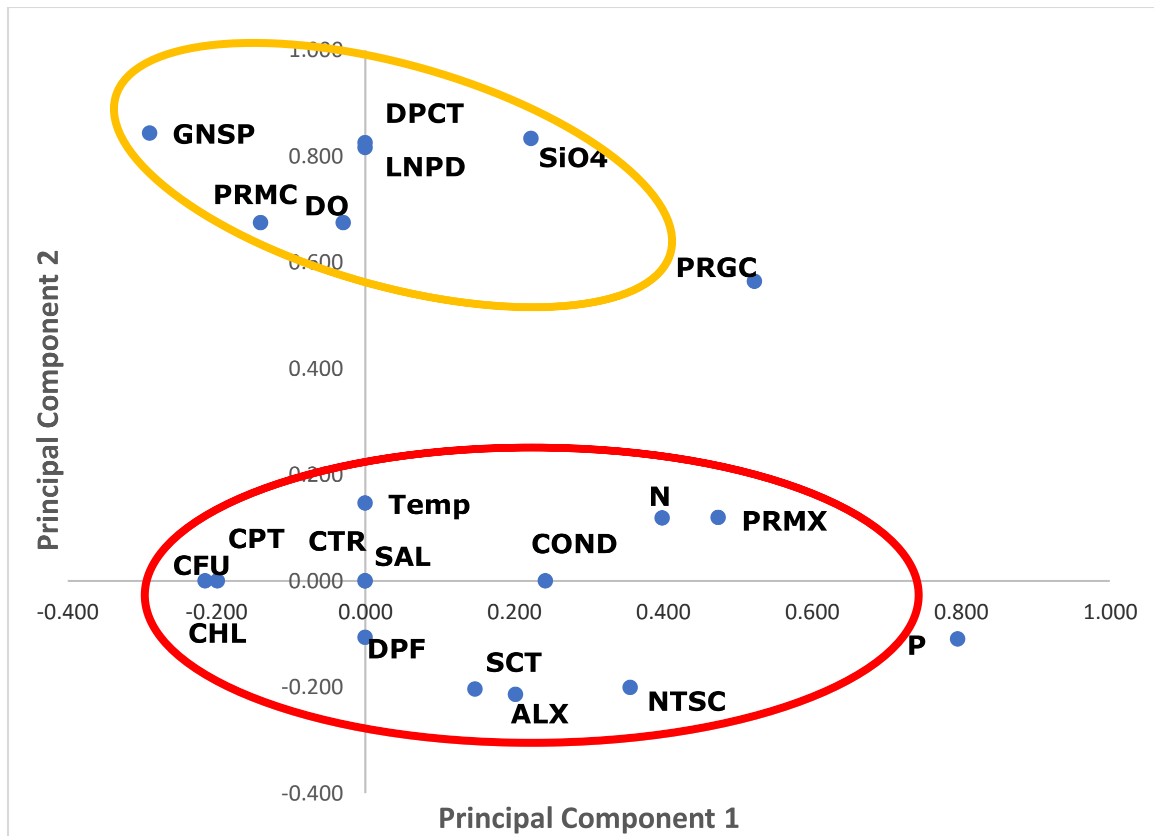

Figure 4.24: Plot of principal component 2 versus principal component 3

From Figures 4.24 and 4.25, two major components were obtained. Component 1 comprised the physical and chemicals parameters analysed for in the work. It was loaded on, Nitrate (N), Salinity, Conductivity and Chlorophyll 'a' as well as algae [such as *Alexandrium spp* (ALX), *Dinophysis fortii,* (DPF)*, Prorocentrum mexicanum* cf. (PRMX), *Ceratium furca* (CFU)*, Ceratium tripos* (CTR)*, Ceratium protoperidinium* (CPT), *Scrippsella trochoidea* (SCT) and *Noctiluca scintillans* (NTSC)].

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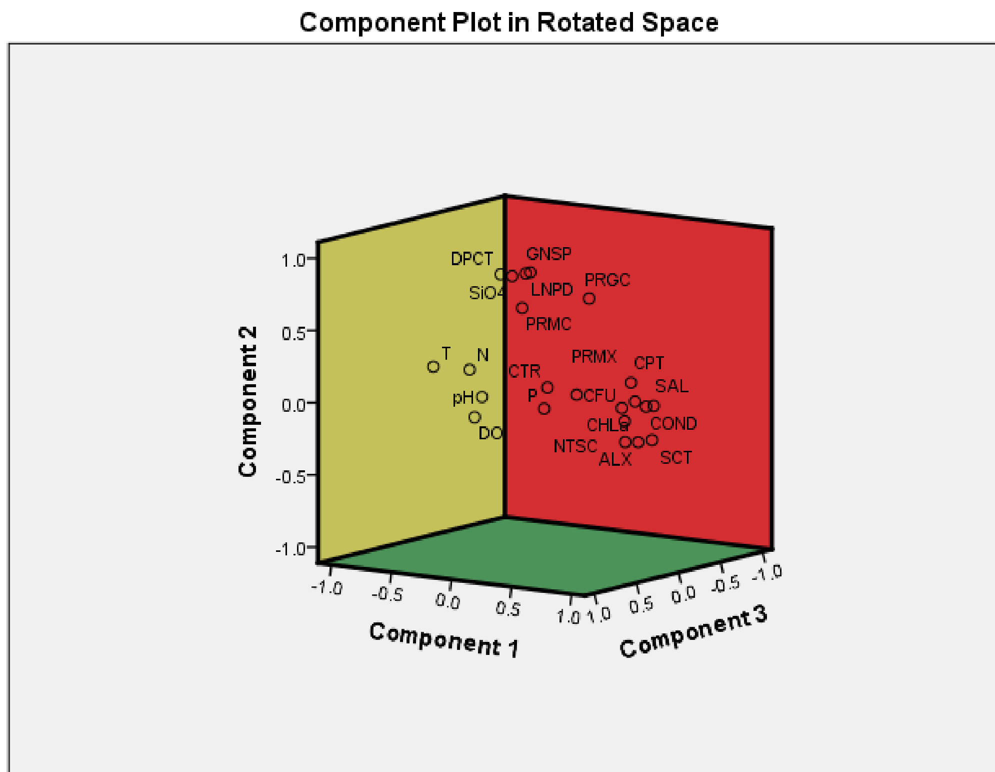

Figure 4.25: Plot of principal components showing two distinct sampling seasons

Component 2 was also loaded on silicate and the following algal species: *Alexandrium sp, (ALX), Dinophysis caudata (DPC), Gonyaulax spinifera (GSN), Lingulodinium polyedra* (LNPD)*, Prorocentrum gracile* (PRGC) *and Prorocentrum micans* (PRMC).

Gonyaulax spinifera (GSN), and Lingulodinium polyedra (LNPD) loading together agrees with Steidinger and Tangen, (1996) study that revealed that G. spinifera has close affinities to two other gonyaulacoid dinoflagellates, which are known to produce yessotoxin, P. reticulatum and Lingulodinium polyedra.

PCA analysis carried out to establish a possible relationship between HABs and Environmental Factors. The PCA also showed decoupling of Wet and Dry season

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assemblages. Low temperature is conducive for proliferation diverse of HABs species. Dissolved oxygen positively correlated with high-density species. PO_4^{3-} may not have a definite influence of HABs assemblages.

Remarkably all the cases (Figure 4.19 and Figure 4.20) which can be distinguished by the two distinct clusters. These two clusters represent the different sampling seasons (wet and dry season). During the wet season (upwelling), there was the observation revealed proliferation of diversity of species.

The second cluster revealed the species high densities but less diversity during the dry season. In the dry season, the only silicate was seen to associate with the abundance of algae found during the dry season, though silicate does not have any effect on the abundance of dinoflagellates, the presence of many diatoms in that season may explain why silicate was loading with them.

Loading of SiO_4 with high densities of dinoflagellate may be explained that at the end of diatom blooms dinoflagellates usually bloom because they do not require silicate and because, through vertical migration; they can migrate down into deep nutrient-rich waters to assimilate nitrate, then return to the surface layers to photosynthesize. Results from a real-time monitoring system in the Southern sea of Korea (Kim et al., 2006) also supported the idea that dinoflagellate blooms are associated with nutrient-limited growth of diatoms. Kudela et al. (2010) indicated differences within HABS species and their different responses to multiple ambient nutrient forms make it difficult to estimate to what extent that the role nutrients played during the bloom period. Thus, even though a chain of events

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leading to bloom can be suggested, the physical/biological mechanisms that lead to bloom formation have not been discovered.

4.3.3 Correlation between Temperature and Salinity

Temperature fluctuations have an important effect on species composition. At the same time, salinity is the main physical parameter that can be attributed to the plankton diversity and acts as a limiting factor that influences the distribution of plankton community as reported by Sridhar et al. (2006).

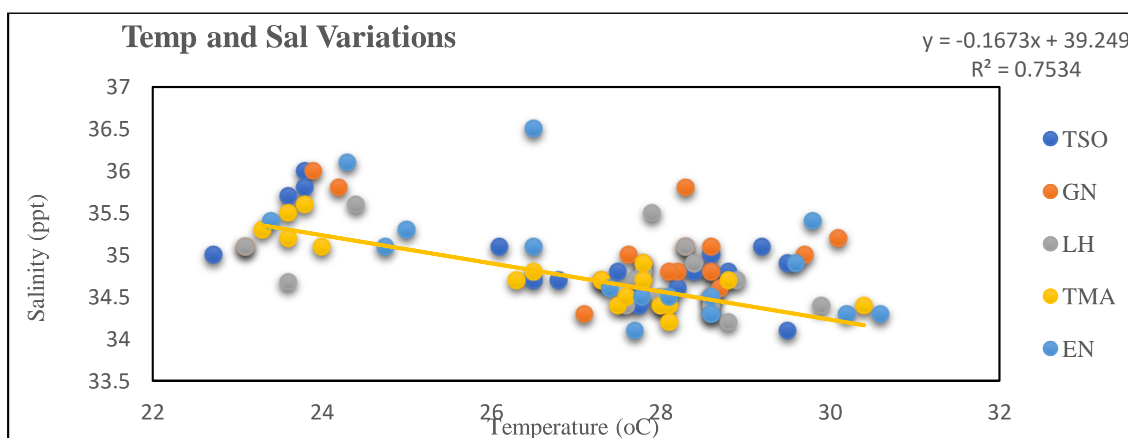

Figure 4.26: Correlation between Temperature and Salinity

Small salinity oscillations in the study areas were recorded spatially and temporally, ranging from 34.1 PSU (TSO) to 36.5 PSU (EN). The temperature was about 70% correlated to salinity from Figure 4.26 and revealed that the average temperature of sea surface temperature is about 28 ºC is favourable for phytoplankton for that matter HABs species to grow.

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4.4 Analysis of trace metals in seawater and Phytoplankton

It is usually common to find dissolved organic and inorganic trace metals in the marine environment stemming from both anthropogenic and natural sources. The presence of trace metals could play a significant role in the physiology of organisms found in the water body. In this section, Zn, Fe, Mn, Hg, Cu, and Cd were determined in sedimented samples that contained the thirteen (13) identified microalgae. The microalgae or phytoplanktons included *Alexandrium spp* (ALX), *Dinophysis fortii,* (DPF)*, Dinophysis caudata* (DPC)*, Prorocentrum mexicanum* cf. (PRMX), *Prorocentrum gracile* (PRGC)*, Prorocentrum micans* (PRMC)*, Ceratium furca* (CFU)*, Ceratium tripos* (CTR)*, Ceratium protoperidinium* (CPT)*, Scrippsella trochoidea* (SCT), *Noctiluca scintillans* (NTSC) *Gonyaulax spinifera* (GSN) and *Lingulodinium polyedra* (LNPD).

4.4.1 Concentration of dissolved trace elements (Zn and Fe) in seawater

Concentrations of Zn and Fe determined in surface waters of the Gulf of Guinea sampled from Ekumfi Narkwa lagoon in the Central Region of Ghana and seawater along the Gulf of Guinea from Bortianor-Tsokomey, Tema Port, Accra Lighthouse and Gomoa Nyanyanor are presented in Table 4.3. These are the same locations where phytoplankton was sampled for characterization and analysis. The average concentration of dissolved Zn and Fe in surface water samples ranged from 0.021 to 0.23 and 0.05 to 0.204 µg/L, respectively.

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The highest mean concentration of both dissolved Zn and Fe were observed in seawater samples at Gomoa Nyanyanor (0.23±0.05 µg/L for Zn) and (0.204±0.03 µg /L for Fe). The values obtained in the current study is in accordance with previously reported literature values from other geographical location around the world (Table 4.3). For instance, Pohl et al. (1993) reported Zn concentrations in the ranges of 0.19 to 1.30 µg /L in surface waters of Eastern Atlantic. Additionally, Sinoir et al. (2016) reported Zn concentration ranging from 0.15 to 0.96 µg /L from the Tasman Sea. Likewise, in the North Pacific ocean, the average concentration of dissolved Fe was found to be 0.056±0.005 µg /L similar to what has been summarized in Table 4.3 (Lin et al., 2018).

Table 4.3: Comparison of dissolved Zn and Fe with other surface waters of the world

Location	Depth (m)	Zn	Fe	Reference
Narkwa Lagoon	Surface	0.15±0.09	0.13±0.04	This Study
Gulf of Guinea				
Bortianor-Tsokomey	Surface	0.17± 0.04	0.05±0.03	This Study
Tema Port	Surface	0.19±0.06	0.18±0.07	This Study
Accra Light House	Surface	0.012±0.08	0.09±0.002	This Study
Gomoa Nyanoyano	Surface	0.23±0.05	0.204±0.03	This Study
Other Studies				
Gulf Stream	Surface	n/a	0.006	Liu and Millero, 2002
North-East Atlantic	Surface	0.02	n/a	Ellwood and van den Berg, 2000
Rose Sea	25	1.32-0.140	0.18-0.061	Coale et al., 2003
Antarctic Circumpolar Current	20-50	0.10-0.156	0.016-0.002	Coale et al., 2003
	Surface	0.03-0.042	n/a	Lohan et al., 2002
Ross Sea, Antarctica	Surface	2.43	0.64	Lagerström et al., 2013

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The concentrations of zinc and iron (Fe) in oceanic waters were low compared to macronutrients such as nitrates, phosphates and silicates which control the productivity and growth of most phytoplankton communities in the world's ocean (Coale et al., 2003; Sunda, 2012). The low levels of micronutrients present in the surface oceans are known to limit the growth of phytoplankton in most of the world's oceans (Coale et al., 2003). Despite its important significance in the species assemblage, trace micronutrients such as Zn over the last two decades have been shown to play a significant role in a multiple of biological and structural functions in the growth of phytoplankton (Sinoir et al., 2016; Sinoir et al., 2017).

Fe is an essential micronutrient needed for a variety of biochemical processes for phytoplankton in the world's ocean despite its low concentrations in dissolved seawater and thus considered a limiting nutrient for primary productivity in the most surface ocean (Abualhaija and Berg, 2014; Lin et al., 2018). Fe is essential for nitrate uptake, electron transport, and chlorophyll synthesis. The sources of micronutrients into oceanic waters primarily occurs through hydrothermal sources, atmospheric deposition and fluvial discharges (Coale et al., 2003; Conway et al., 2013).

The measured concentration of Zn and Iron in the present study is not entirely surprising but in accordance with other publications that reported low concentration of Zn and Fe in near-surface oceans which has been attributable to depleted nutrient caused by the uptake by biological organisms. In contrast, in deep waters, its concentration is usually increased

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(Conway et al., 2013). As previously observed, in the photic zone of oceanic waters, trace metals are used up by biological activities and scavengers (Sunda, 2012; Lagerström et al., 2013).

It has been established that iron (Fe) is a necessary micronutrient for phytoplankton, although significantly low dissolved levels are observed in surface waters that eventually limit primary productivity over much of the surface ocean. Similar to Zn, the vertical distribution of Fe in oceanic waters indicated low values in the surface to deep waters (Sunda, 2012). This has been ascribed to the fact that Fe has low solubility in coastal waters mainly introduced into seawater via atmospheric deposition rather than riverine discharges (Abualhaija and Berg, 2014). Direct interpretation on depth profiling is being made with caution as in present study; depth profile was not carried out and therefore cannot be directly compared to another previous study. What is of interest however is that the measured concentration in the surface waters falls within the levels of previous publications that conducted depth profiling of the trace metals (Sinoir et al., 2016; Pohl et al., 1993; Sunda, 2012; Sinoir et al., 2016). Although data from the current study area is lacking, the present study suggests that the low levels of the trace metals could limit the production of phytoplankton in this region.

4.4.2 Concentration of trace elements in phytoplankton

The presence of trace metals (Zn, Fe, Mn, Hg, Cu, Cd) in marine phytoplankton from the studied locations are presented in Table 4.4. From the results, the concentration of Cd was found to be below the detection limit (Cd<0.006 µg/kg). Mercury concentration detected from the total microalgae ranged from 10.05 to 18.29 (µg/kg). Other metals concentrations

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in the microalgae were in the ranges of 162.00 to 4418.90 (µg/kg) for Zn, 4533 to 24567 (µg/kg) for Fe and 225.10 to 2121.46 (µg/kg) for Mn. and 154.50 to 497.90 (µg/kg) respectively (Table 4.4). The presence of trace metals could play a significant role in the physiology of phytoplankton in the water body.

Table 4. 4: Total heavy metal concentration (µg/kg) in algae

Sampling Site/Metals	Zn	Fe	Mn	Hg	Cu	Cd
Tema Port	4418.9	24567	2121.46	18.29	406.70	<0.006
Accra Light House	162.0	4533	225.10	10.23	154.50	<0.006
Bortianor-Tsokomey	3163.0	17500	2033.00	14.66	202.30	<0.006
Narkwa lagoon	448.40	20334	2054.05	10.11	450.10	<0.006
Gomoa Nyanyanor	3395.0	22632	1911.22	10.05	497.90	<0.006

The presence of trace metals such as Mn, Fe, Cu and Zn are considered essential to marine organisms as has been previously described. They are required for basic metabolic processes and essential components of various enzymatic functions. These trace metals play a significant role in the formation of respiratory pigments and enzymes such as metallothioneins. In the present study, the high concentration of essential trace metals recorded may be a contributing factor to the high cell densities of the phytoplankton population (2155 to 50208 cells/L). The phytoplankton abundance from the sampling sites based on the results can be ranked in increasing order of Accra Lighthouse (2155 cells/L) <Tsokomey (40148 cell/L) < Ekumfi-Narkwa (42618 cell/L) <Tema Port (44242 cells/L) < Gomoa Nyanyanor (50208 cells/L). This indicates that areas with high essential trace metal concentrations (Table 4.4) at Gomoa Nyanyanor and Tema Port showed increased concentrations of phytoplankton species.

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Majority of these nutrients are considered limiting for algal growth, and thus the considerable presence of these metals in the algal species from the present study shows increased growth of the species. However, the presence of Cd and Hg with no known biological functions are considered non-essential to marine phytoplankton. These metals were either not detected (in the case of Cd) or present in very low concentrations (in the case of Hg). These are reasonable indications since their increased concentrations could be toxic to both the algae and other organisms progressing up the food chain.

4.5 Results from Genetic Identification

Amplification was controlled on 2% agarose gel before sequencing in Fig. 4.27

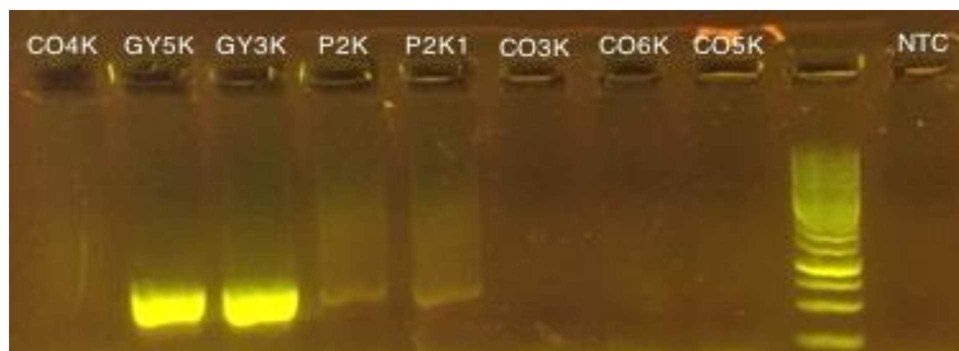

Figure 4.27: Amplification map of DNA on agarose gel

From the analysis, it was observed that one strain of Prorocentrum spp culture samples (P2K, P2K1, P2Kb, P2K1_ITS2, P2K1_ITS2) were *Prorocentrum micans* in sequence, as shown below:

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P2K

NNNNNNNNNNNNNNNNNNNNNNNNNNNATTCGGANTGCAGCAGNGNTCAG
TTCCTNANGTTGCAGCGGANGTTTAGTGNACCTTATCACTTAGAGGAAGGAG
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTCGCACGC
ATCNNATTGATTCACTGTGAACTGAANTTCGTGAGGGTCTGTGTGGTGGTGG
AGATAGCATCAATACCTCTATGCAGGCGCTCGAGGGCAGTAAGCCAGGTTTG
GACTGTCTTCCATTCCTGCCCCTGCTGCCACCTCTCTCATTTGCTTCTCAGCAT
TTATTGCATCGTTTCTTCTCGAGTGGTTATCCACTTATCCNNNCNNNNACAAC
TTTCAGCGACGGANGTCTCGGCTCGAACAACGATGAAGGGCGCAGCGAAGTG
TGATAAGCATTGNGAATTGCAGAATTCCGTGAACCAATAGGGNCTTGAACGT
ATACTGCGCTTTCGGGATATCCCTGAAAGCATGCCTTCTTCAGTGTCTATTCTT
TCTCATTCCAGCGATCTGGGTTTCCAGCGTCGCTTGGNAGTCTTTGTGCGTTA
GGGCGCTCGCCTCGTGNAGCCCNTNGACGCATTCAGCTCACNNGGGGCCTCT
CGCANNAGCNACTANGAANAGCTTCNANGCGTTTCCNTTGNNGTCTTGCTTG
CGAGTTGGGTNCNNGNCATCCNGNGNCCTANNNGNNNNNCNACCAAGNANA
TGANNNTTANGTCANCNANNNCCNNCTGAANTTNNNCANNNNNNNNN

P2K1

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNANNNNNNGNNNNNNNNNNNNGGNNNTCNNNGNNAGANANC
NGNNAACGNNTTTNNNTCTTCTAGTTGCTNGTGCGANAGGCCCCTGTGANCT
GAATGCGTCAAGGGCTACNCGANGCGAGCGCCCTAACGCACAAAGACTCCC
AAGCGACGCTGNANNNNNNGATCGCTGGAATGAGAAAGAATAGACACTGAA
GAAGGCATGCTTTCAGGGATATCCCGAAAGCGCANTATACGTTCAAGTCCCT
ATTGGTTCACGGAATTCTGCAATTCACAATGCTTATCACACTTCNCTGCGCCC
TTCATCGTTGTTCGAGCCGAGACATCCNNCGNTGAAAGTTGNNNNANAATGG
ATAAGTGGATAACCACTCGNGAAGAAACGATGCAATAAATGCTGAGAAGCA
NNNGNNNNANGTGGCANCAGGGGCNGGANTGGAANACAGTCCAAAACCTGG
GCTTACTGCCCTCGANNNCCNGCATANAGGTATTGATGCTATCTCCNCCNCC
ACACANNACCCTCACGAAATTCANTTCACAGTGAATCANTTGGGANGCNTGC
GAATGATCCCTTCCCNCAGGTNNNCCTANCGGAAACCTTGGTTNNNACTTCTC
CTTCCTCTAAGTGATAANGNTTCNNTANACTTTNNNGCTNNNNNNNNNNNAGG
AANNTGGAACCNCNNGNNNNGNCNNNGCNNNNANTTATTTNCCCCGGNAAA
NNANNNCNNNNNCGGNNNNNNNNANNCNNNNGGGGNNNGGGTGNNNNGNNN
NNNNAANGGGGNNNNNNNNGGNAANANNANNN

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P2K_b

NNNNNNNNNNNNNNNNNNNGNNNNNNNNNNNAGTANGGCNCTGGANGGCAGA
ANNNACTCGCAGCAAGACAACAAGGAAACGCNNNNNNCTCTTCTAGTTGCTT
GTGCGAGAGGCCCCTGTGAGCTGAATGCGTCAAGGGNTNCNCGAGGCGAGC
GCCCTAACGCACAAAGACTCCCAAGCGACGCTGGAAACCCAGATCGCTGGA
ATGAGAAAGAATAGACACTGAAGAAGGCATGCTTTCAGGGATATCCCGAAA
GCGCAGTATACGTTCAAGTCCCTATTGGTTCACGGAATTCTGCAATTCACAAT
GCTTATCACACTTCGCTGCGCCCTTCATCGTTGTTCGAGCCGAGACATCCGNN
CGCTGAAAGTTGTANTAGAATGGATAAGTGGATAACCACTCGAGAAGAAAC
GATGCAATAAATGCTGAGAAGCANATGAGAGAGGTGGCAGCAGGGGCAGGA
ATGGAAGACAGTCCAAACCTGGCTTACTGCCCTCGAGCGCCTGCATAGAGGT
ATTGATGCTATCTCCACCNCCACACAGACCCTCACGAAATTCAGTTCACAGN
GANTCAATTGGNATGCNNNNNNNNGATCCTTCCGCNNGNNTCACCTACGGAA
ACCTTGTTACNACTTCTCCTTCCTCTAANTGANAAGGTTCACTAAAACTTTCC
GCTGCAACGTTCNNGAACTGNAACACTGCTTGCAGTNNCGANTTANTTCNNC
NNNNANNNNCTTCANNCCGGTAGNNGCNNACGGGNNGGGNNGNTGNNNNN
NANGNGNNNGGGANNNNNN

PK2_ITS2

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNCNGNNNNNNNNNGNGNGCANNANGNCNGNNNGGC
ANNANCCAACTCGCAAGCNAGANAACAAGGNAANGCTNGAAGCTCTTCTAG
TTGCTTGTGCGANAGGCCCCTGTGAGCTGAATGCGTCNAGGGCTACNCGAGG
CGAGCGCCNNNNNNNNNCAAAGACTCCCAAGCGACGCTGGAAACCCAGATCG
CTGGAATGANAAAGAATAGACACTGAAGAAGGCATGCTTTCAGGGATATCCC
GAAAGCGCAGTATACGTTCAAGTCCCTATTGGTTCACGGAATTCTGCAATTCA
NNATGCTTATCACACTTCGCTGCGTTNNTCATCNNTGNNNN

P2K1_ITS2

NNNNNNNNNNNNANANNNNGACATNNGANTTGACGTATACTGCGCTTTCGG
GANATCCCTGAAGCATGCCTTCTTCAGTGTCTATTCTTTCTCATTCCAGCGATC
TGGGTTTCCAGCGTCGCTTGGGAGTCTTTGTGCGTTAGGGCGCTCGCCTCGTG
NAGCCCTTGACGCATTCAGCTCACAGGGGCCTCTCGCACAAGCAACTAGAAG
AGCTTCNAGCGTTTCCTTGTTGTCTTGCTTGCGAGTTGGTTCTTGCCATCCAGT
GCCTACTGCACTCCAACCAAGACATGAAGTTAGGTCAGCAAACCCGCTGAAT
TTAAGCATATCANTAAGCGGCACCGTACCCTGGTCCTGN

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P2K1_ITS2 2

NNNNNNNNNTNNNNNNCTAACTTCNTGTCTTGGTTGGAGTGCAGTAGGCNCT
GGATGGCAAGAACCAACTCGCNNGCAAGACAACAAGGAAACGCTTGAAGCT
CTTCTAGTTGCTTGTGCGAGAGGCCCCTGTGAGCTGAATGCGTCAAGGNCTN
NNNGAGGCGAGCGCCCTAACGCACAAAGACTCCCAAGCGACGCTGGAAACC
CAGATCGCTGGAATGAGAAAGAATAGACACTGAAGAAGGCATGCTTTCAGG
GATATCCCGAAAGCGCAGTATACGTTCAAGTCCCTATTGGTTCACGGAATTCT
GCAATTCACAATGCTTATCACACTTCGCTGCGTTCTTCATCGNNGN

From the DNA sequence, it was also observed that three (3) cultures *Coolia spp* samples (CO3K, CO4K, CO5K) [Appendix L] might be *Coolia canariensis* sp. nov. (Dinophyceae). A new nontoxic epiphytic benthic dinoflagellate from the observed sequence as shown.

4.6: Toxin Analysis

4.6.1 Quantification of toxins microalgae cultures

4.6.1.1 Quantification of Okadaic Acids in *Prorocentrum* spp culture

From the chromatogram (Fig. 4.28), OA peaks were observed at MRM 723 and MRM 827 at the 6.2 and 6.35 minutes of the retention times respectively for the Prorocentrum spp culture.

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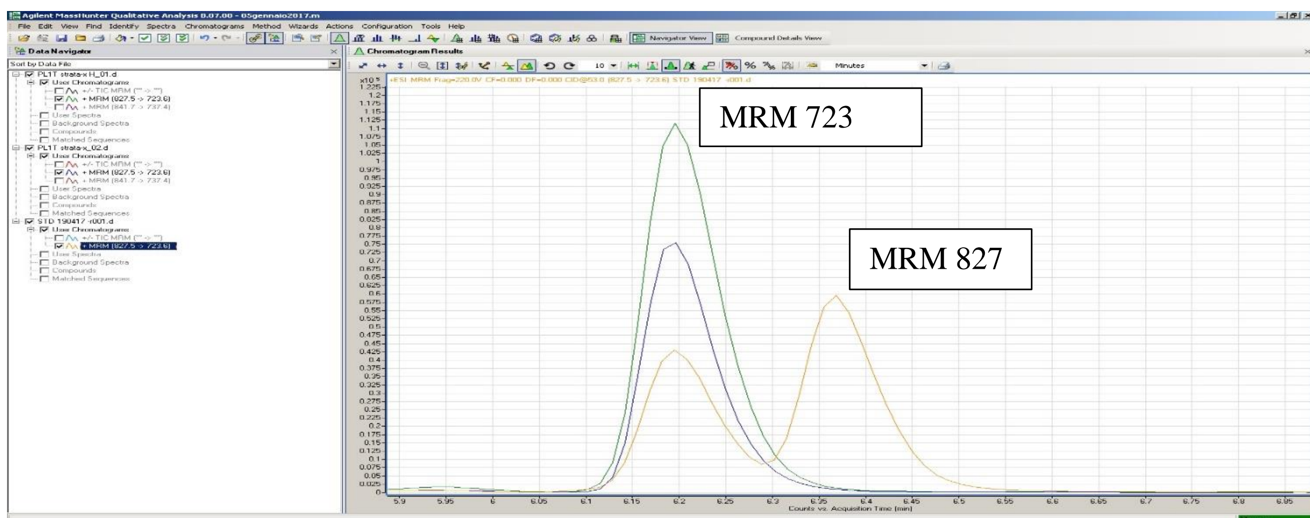

Fig 4.28: Lipophilic toxin analysis in PL1

Table 4. 5: Calibration data for okadaic acids in *Prorocentrum spp*

Quantification of okadaic acids		
Transitions 827.5-723.6		
CRM	8.372	
STOCK	0.9616	
dil 0	0.7153	212,767
dil 2	0.3575	103,521
dil 4	0.1779	46,710
dil 8	0.0883	22,090
dil 16	0.0436	11285
dil 32	0.0215	5521
dil 64	0.0106	2754
dil 128	0.0052	1170
dil 256	0.0027	724
Slope	297554.4809	
Intercept	-1862.111614	

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Table 4.5 shows the data for the calibration curve for okadaic acids in Prorocentrum spp. Calibration curves are plotted using concentrations of stock solutions and various dilutions prepared from certified reference materials.

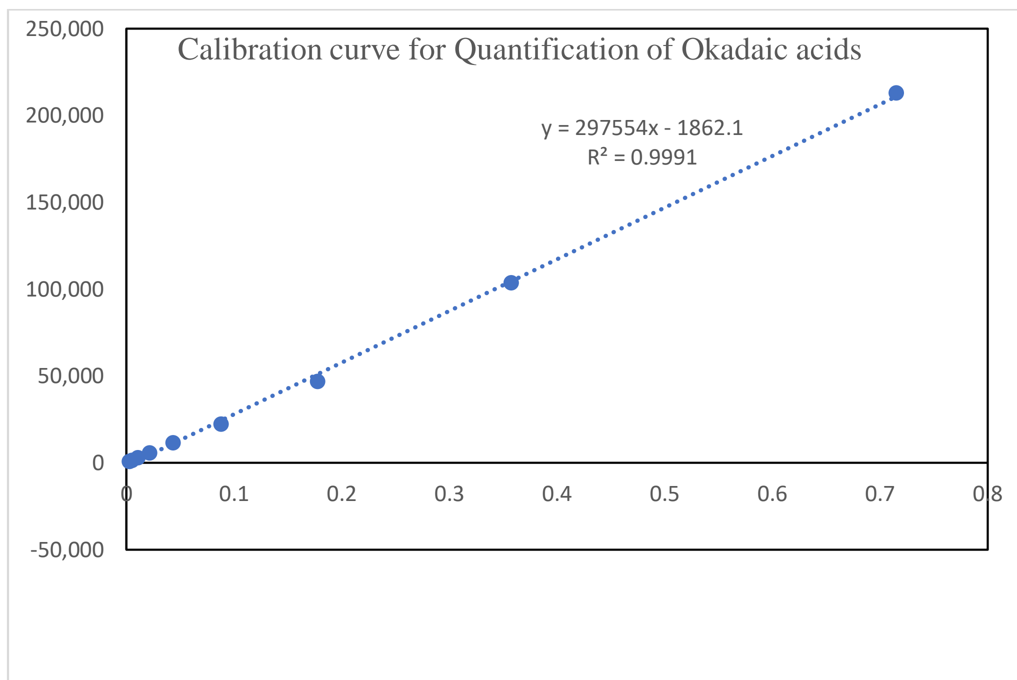

Figure 4.29: Calibration curves for quantification of OA in *Prorocentrum spp*

Table 4.6: Quantification of okadaic acids in *Prorocentrum spp*

	Peak Area	ng/5 µL	ng/3 mL extract
PL1T	259209	0.88	0.53

Concentration of Okadaic acids (2000 cells) = 0.53 ng

= 0.000265 ng/cell

Prorocentrum spp (PL1T) contains 2.65×10^{-4}ng/cell okadaic acids

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4.6.1.2 Chromatographic patterns

The oxidation products formed after periodate and peroxide oxidations of the toxins included in this study.

Toxins were quantified against linear calibrations of all currently available PST (Paralytic shellfish Toxin) certified reference standards. No ion exchange fractionation was undertaken prior to the quantification of the N-hydroxylated toxins, such as GTX1 and 4. The limit of quantification (LOQ) of the analytical method for each toxin (STX, GTX1 and 4, GTX2 and 3, GTX5, C1/2, neoSTX, dcSTX, dcGTX2 and 3). The limit of detection (LOD) was assumed to be one-third of the limit of quantification.

From the chromatograms: Alexandrium spp (AL1T) was seen not have any peak that corresponds to a toxin.

After Periodate oxidation, the chromatograms obtained are presented in Fig 4.30.

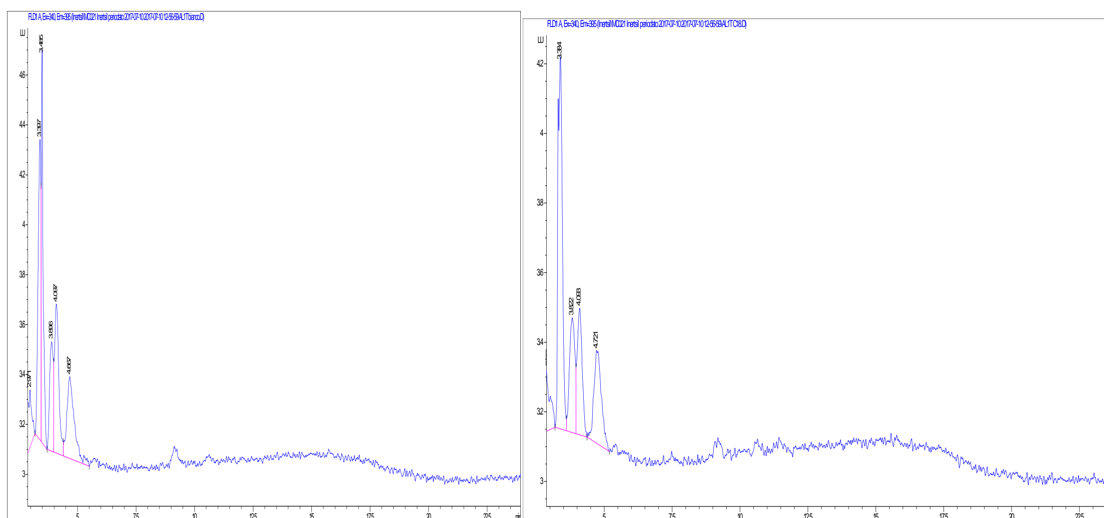

Figure 4.30: AL1T blank and C18(Sample) is not showing any peak

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AL1T blank and C18(Sample) are not showing any peak for any PSP toxin. Confirmation from Fractions 1,2, and 3 as shown in Fig. 4.31.

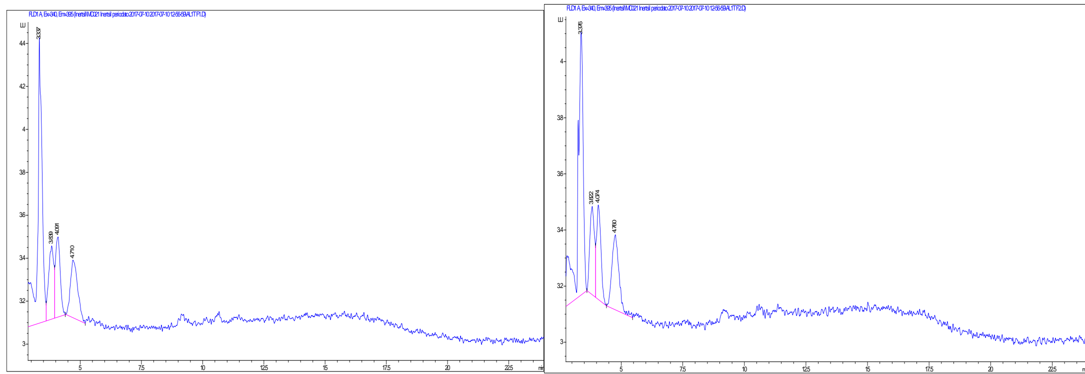

Figure 4.31: Fraction 1(AL1T F1) and Fraction 2 (AL1T F2) revealing no toxin

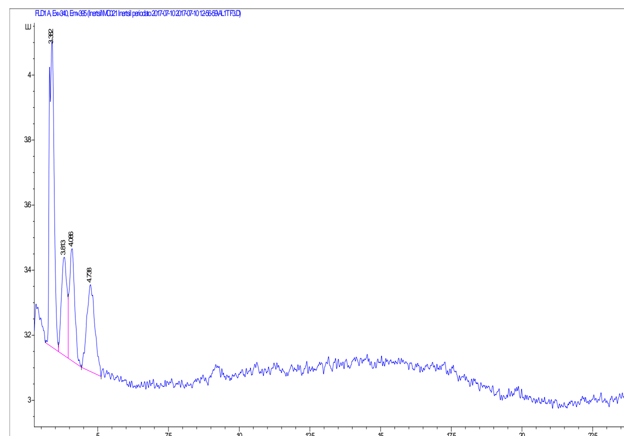

Figure 4.32: Fraction 3(AL1T F3) showing no Peak

Fractions 1, 2 and 3 also not showing any Peak for any PSP toxin indicating that Alexandrium spp strain AL1T is not toxic as observed in Figure 4.32.

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AL9T blank

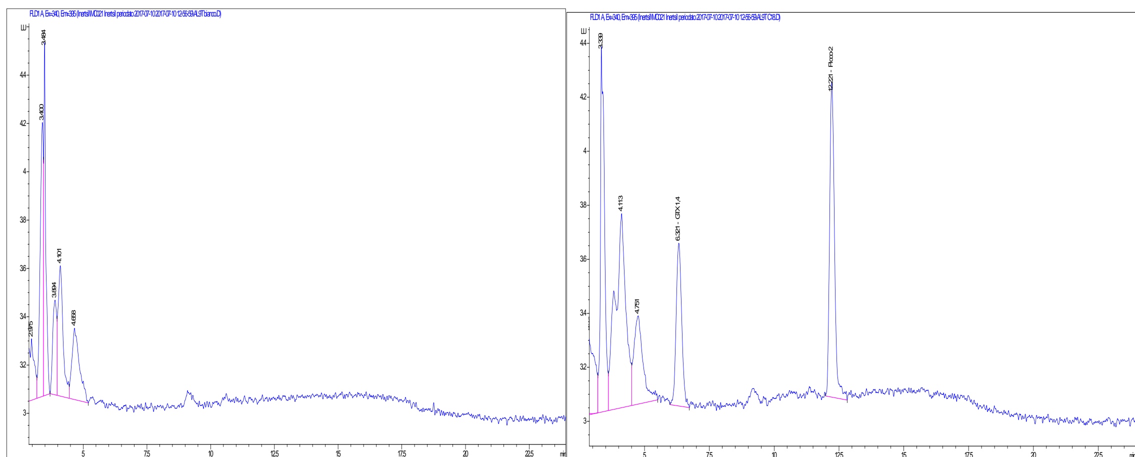

Figure 4.33: AL9T blank compared to C18 (Sample)

A Peak at the retention times 6.321 and 12.22 minutes were observed, indicating that the toxin GTX1,4 may be present, to confirm various chromatograms for Fraction 2 and 3 were reviewed in chromatograms of Figure 4.33.

AL9T F1

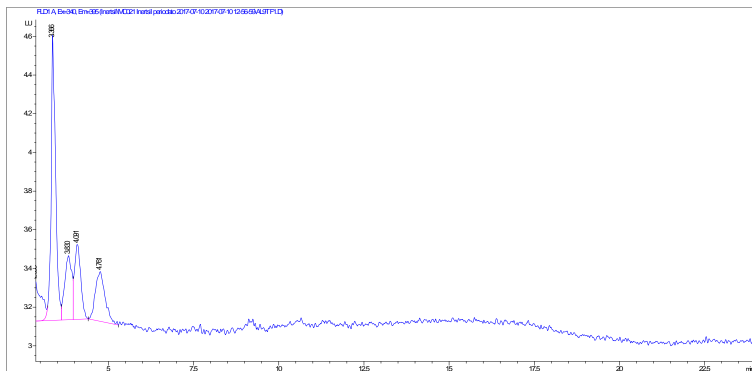

Figure 4.34: AL9T fraction1

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From Fig. 4.34, Inference: AL9T F2- GTX1,4 Peak at 6.334 and 12.231RT

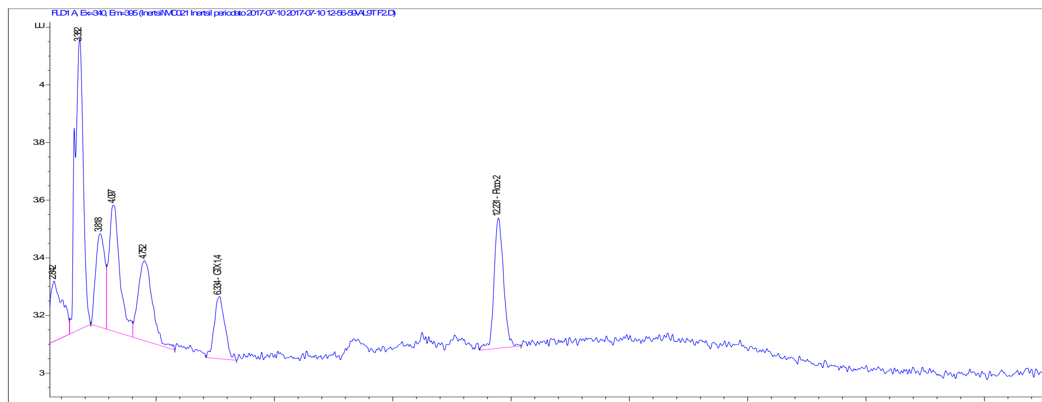

Figure 4. 35 AL9T fraction 2

From Figure 4.35, Inference: AL9T F3 GTX1,4 Peak at 6.301 and 12.1911RT

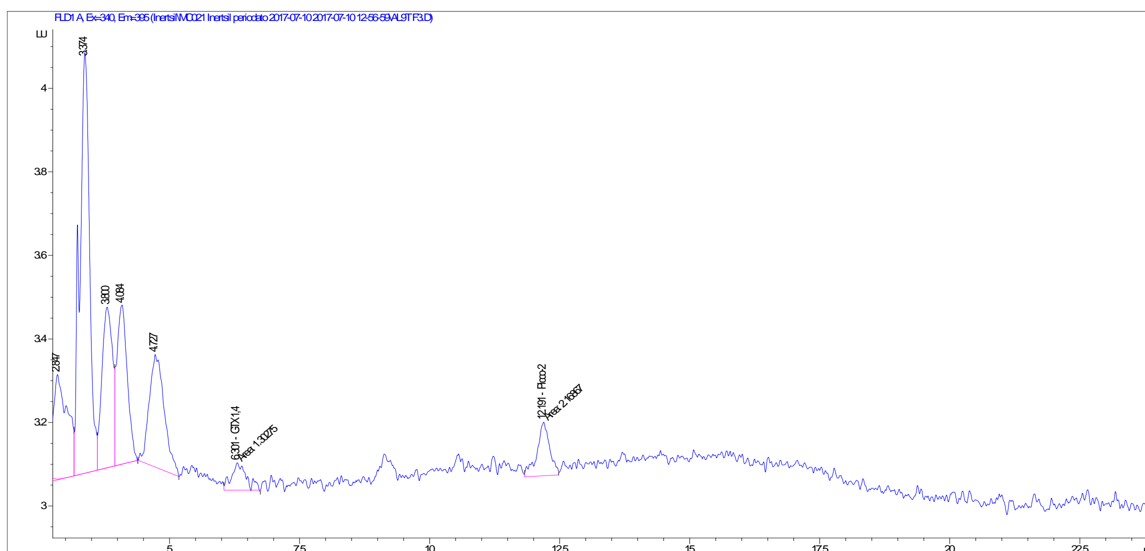

Figure 4.36: AL9T fraction 3

Figure 4.36 shows Chromatograms for Fraction 2 and 3 were indicating the presence of GTX1,4

Periodate oxidation

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From Fig. 4.37, Inference: AL9T C18 Peak of GTX2,3 observed

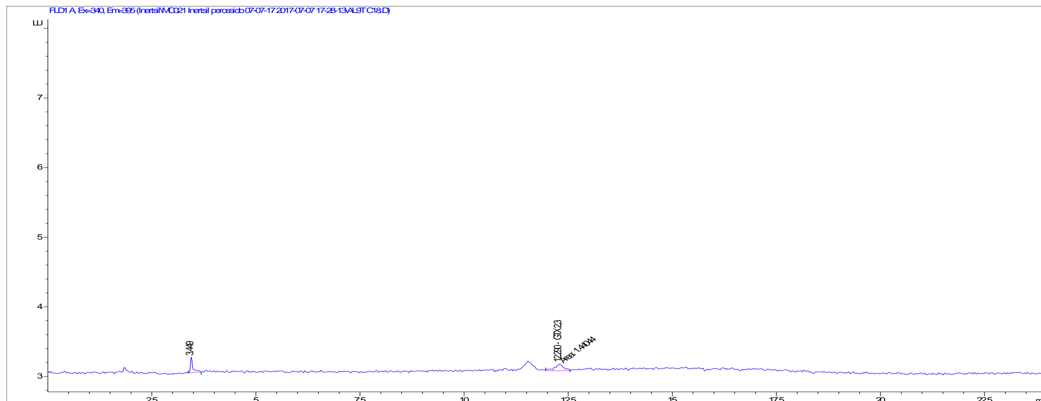

Figure 4.37: AL9T fraction 3

AL9T F1

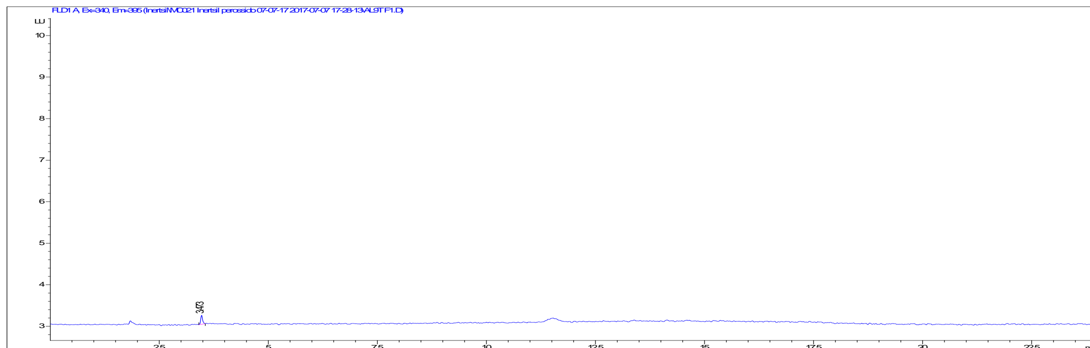

Figure 4.38: AL9T fraction 3

4.6.1.3 Quantification of PSP toxins in Alexandrium spp AL9T culture.

Quantification of GTX2,3 toxin in Alexandrium spp (AL9T)

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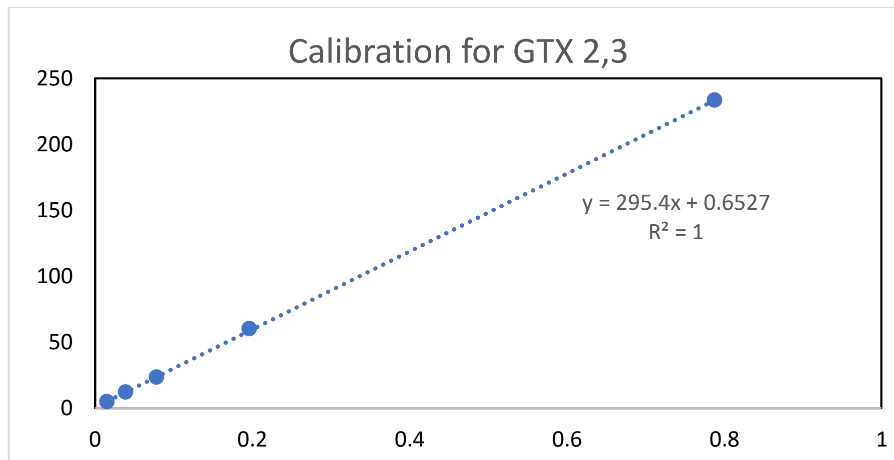

Figure 4.39: Calibration used for quantification of GTX2,3

Table 4.7: Calculation of amount of GTX2,3 toxin in *Alexandrium spp* culture

	AREA	µM C18	µM extract	µmol/3mL	µg/3mL	µgSTXdiHCL/100g	corrTEF
AL9T C18	1.4	0.0025	0.01	0.0000	0.0075	0.0071	0.0042

Concentration of GTX2,3(4000 cells) = 0075µg

$=1.875x10^{-6}$µg/cell

Alexandrium spp (AL9T) contains 1.875×10^{-6}ng/cell GTX2,3

Quantification of GTX1,4 toxin in AL9T

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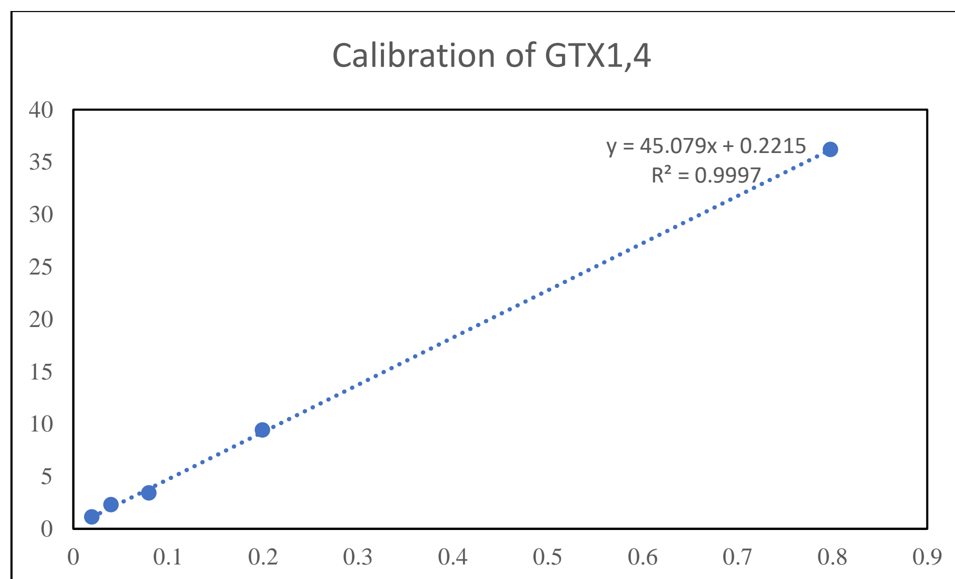

Figure 4.40: Calibration curve for quantification of GTX1,4 toxin in AL9T

Table 4.8: Calculation of amount of GTX1,4 toxin in Alexandrium spp (AL9T)

	AREA	**µM C18**	**µM extract**	**µmol/3mL**	**µg/3mL**	**µgSTXdiHCL/100g**	**corrTEF**
AL9T C18	9	0.1947	0.4868	0.0014	0.6008	0.5436	0.5436
	AREA	µM C18	µM extract	µmol/3mL	µg/3mL	µgSTXdiHCL/100g	corrTEF
AL9T F2	3.4	0.0705	0.3525	0.0010	0.4351	0.3936	0.3936

Concentration of GTX1,4(4000 cells) = 0.600856µg

$= 1.502\text{x}10^{-4}$ µg/cell

Alexandrium spp (AL9T) contains 1.502×10^{-4}ng/cell GTX1,4

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4.6.1.4 PSP Toxins in Shellfish

A complete study of the toxin profiles was carried out using the AOAC Official Method 2005.06 (Lawrence Method) for PSP and a study of them with HPLC-FLD in order to classify the toxin profiles of shellfish from Ghanaian coastal waters. Three (3) types of wild shellfish were handpicked and analyzed between August 2017 and February 2018 (Oysters, cockles and clams). The following hydrophilic toxins have been analyzed: dcGTX2,3, dcSTX, GTX2,3, GTX5, STX, GTX1,4 and NEO.

Using both periodate and peroxide oxidation, samples were oxidized and were run alongside instrumental calibrants. To assess toxin oxidation peak retention periods, peak area responses and measured toxin concentrations, chromatographic results from standards and samples were evaluated.

Peroxide Analysis

The worklist had with three standard curves (Mix II which includes all the toxins visible to peroxide oxidation that are dcGTX2,3; dcSTX; GTX2,3; GTX5 and STX the control samples (CC), the blank samples and the oxidized samples (C18). At the same time, the fraction F1, F2, F3 were made only for the control sample.

Results

Each sample was compared the fraction C18 with a standard curve in order to determine if there was some peak that had the same retention time of toxin or if the same peak was in the C18 and blank chromatogram.

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It was observed that from 1986 to 1991 (Oyster samples), the C18 and blank were similar, and no toxin was found; while from 1992 to 1999 (Cockles samples) some peaks were found. The chromatograms Figure 4.41 standard (*MixII dilution 10)* and (Fig. 4.42) presents in sample 1995/18

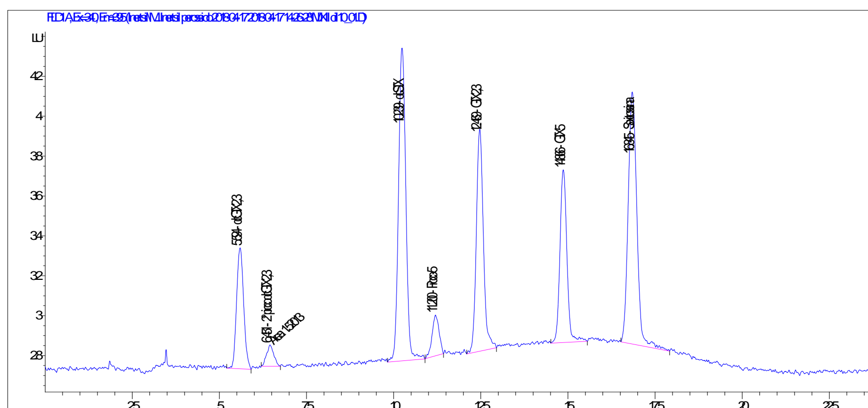

Figure 4.41: Chromatogram for standard/ samples (Mix II dilution 10)

1995-C18

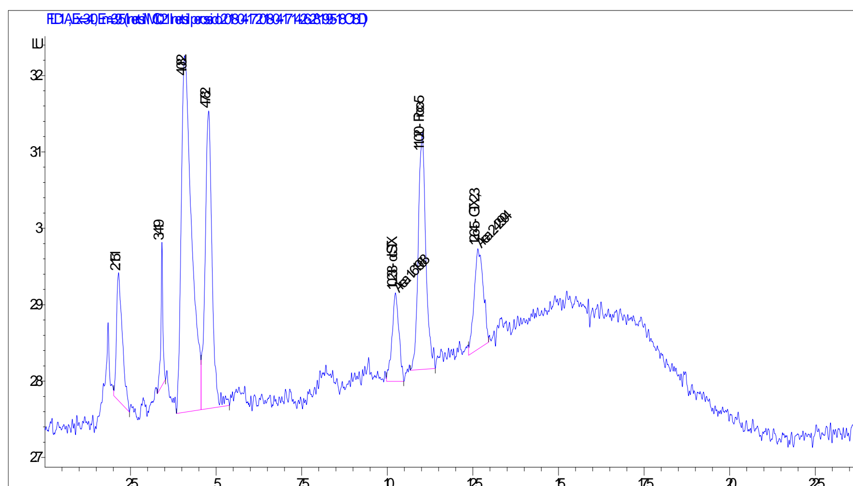

Figure 4.42: Chromatogram for sample1995(C18)

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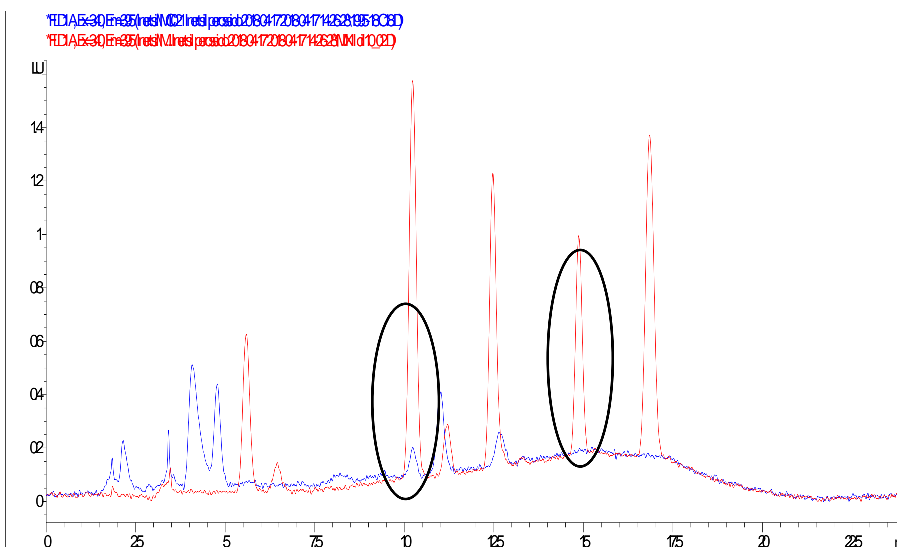

Figure 4.43: Comparison Mix II (dil 10)/1995-C18

From the comparison Figure 4.43, it can be observed that probably there were two toxins [the dcSTX and the GTX2,3 (1° and 2° ring)] because the RT of the peaks is the same. While for peak 5 (RT 11.020), it be can observe that the RT is not perfectly similar between the two chromatograms and peak observe is higher than the peak 5 of the standards. To eliminate any doubt, the fraction C18 as compared to blank of the sample in order to check if the same peak is also in the blank.

1995-Blank

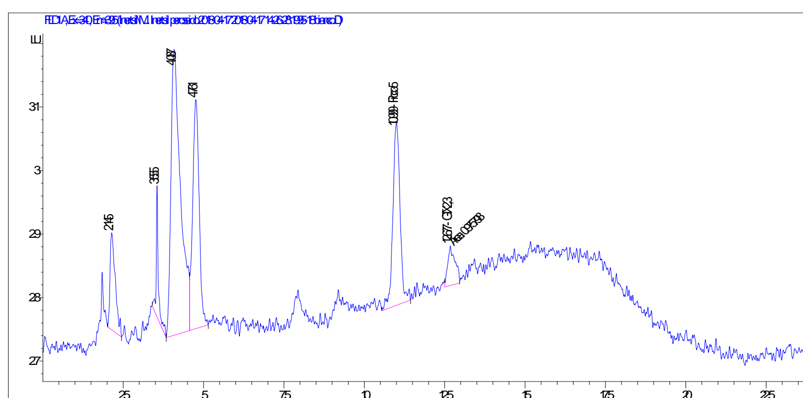

Figure 4.44: Chromatogram of Blank

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Comparison blank-C18

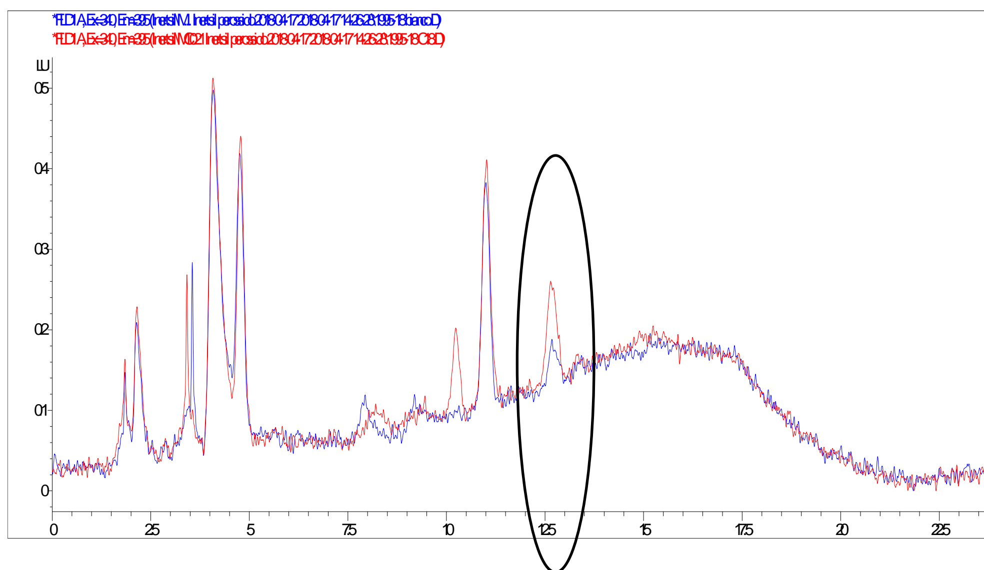

Figure 4.45: Comparison Blank/1995-C18

From this comparison, it can be concluded that:

a. The dcSTX is only in the C18 fraction. It is a small amount, and the concentration is below the EU regulatory limit (<200ug STXdiHCle.q/Kg p.e)

b. Peak 5 of blank and of C18 is perfectly similar and can be inferred to be an interference

c. Finally, for the GTX2,3 it was observed that the peak is present in the fraction C18, but in the fraction C18 (red line) is higher than the blank, there is a small amount of GTX2,3, and in this case, the concentration is <200ug STXdiHCle.q/Kg p.e

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<u>Oxidation-Periodate Analysis</u>

Three standard curves (Mix I include the periodate toxins GTX1,4 and NEO), the control samples (CC), the blank samples and the real samples (C18). While the fraction F1, F2, F3 are made only for the control sample.

Results

In this analysis, all chromatograms for cockles considered had traces of PSP toxins below the Limit of quantification the same, so sample 1995/18 is analysed below:

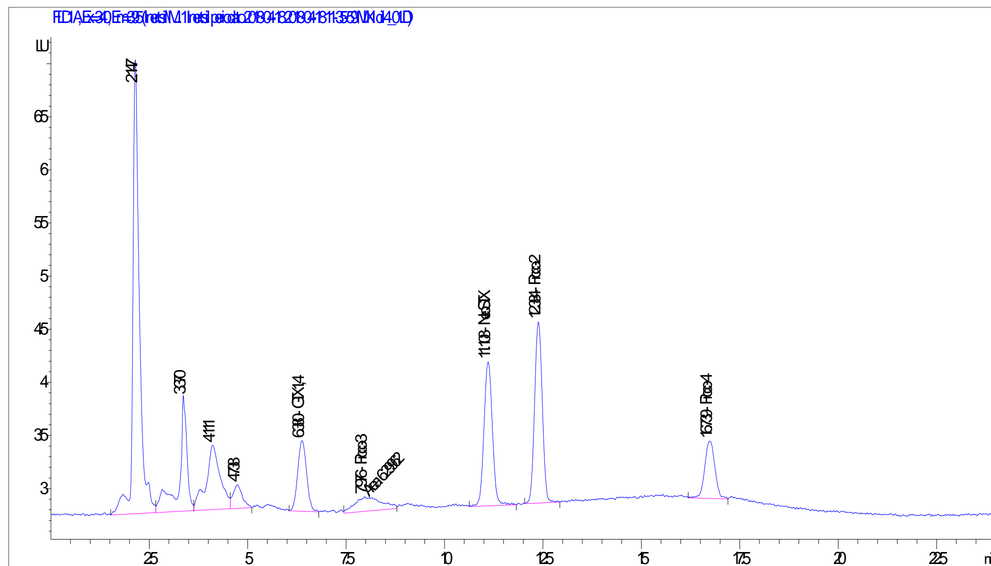

Figure 4.46: Chromatogram of Standard: Mix I dilution 4

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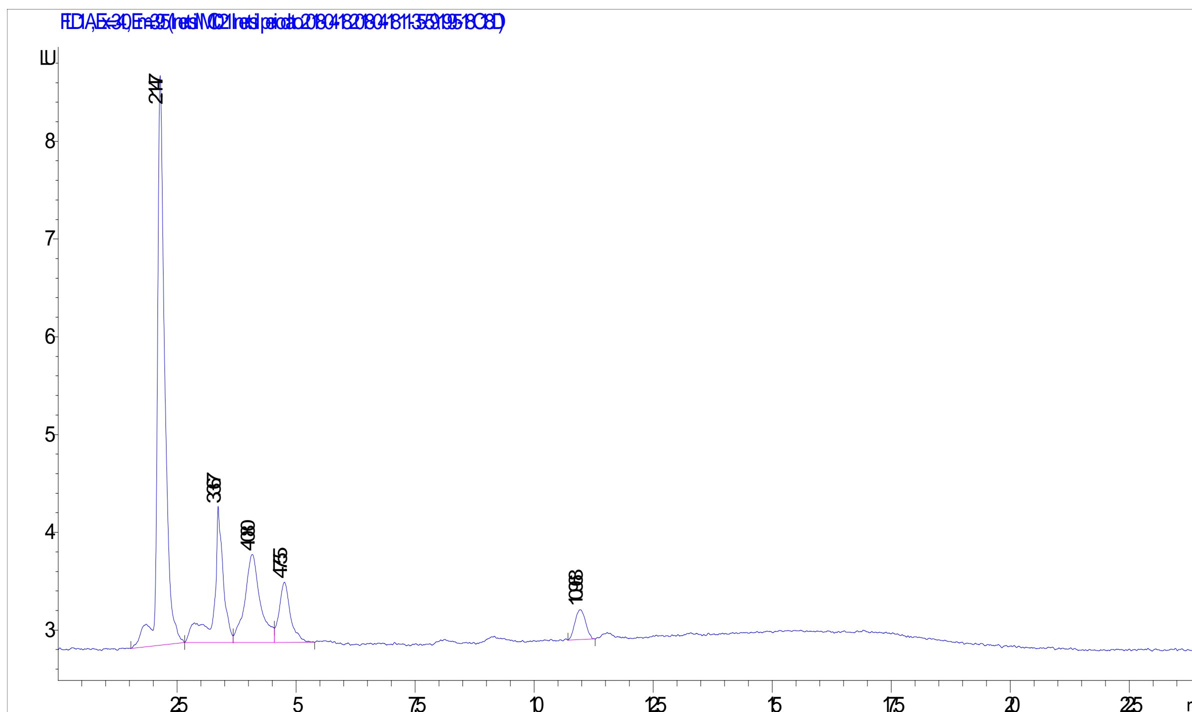

Figure 4.47 Chromatogram of 1995-C18-Sample

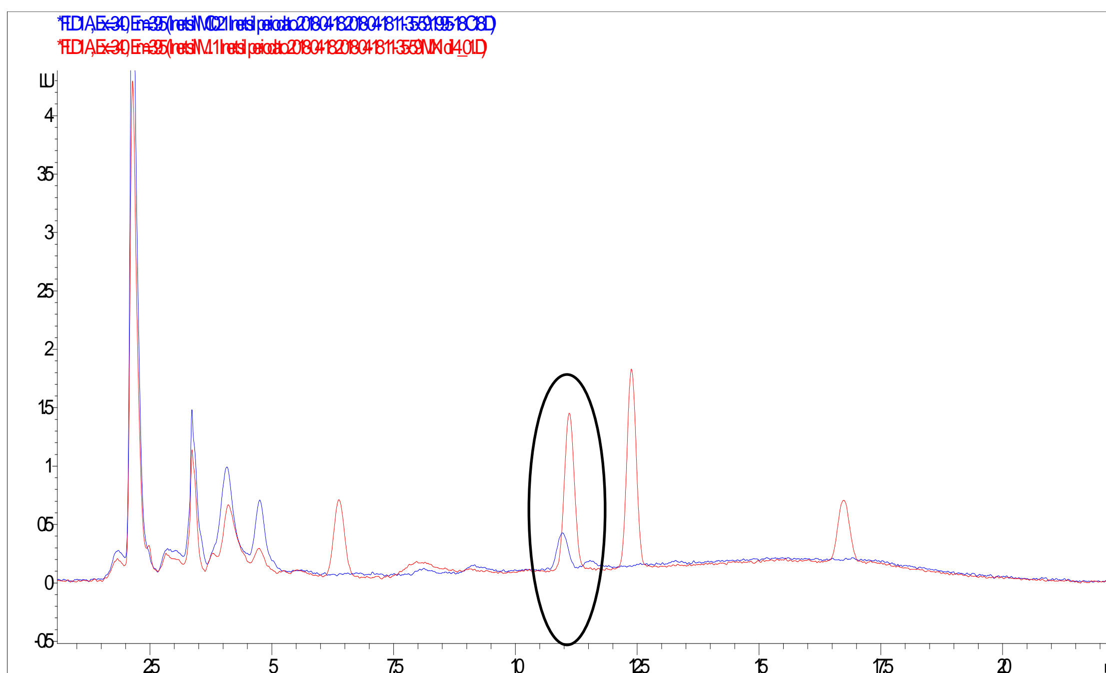

Figure 4.48: Comparison Mix I (dil4)-C18(Standard/Sample)

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From the comparison (Fig 4.44) it is suspected that probably the PSP toxin NEO is present, in concentrations below the limit of quantification.

The presence of traces of the PSP toxins dcSTX, NEO and GTX2,3 in all samples but below the limit of quantification. This may be due to co-occurrence with the PSP toxin producer *Alexandrium spp*.

4.6.1.5 Lipophilic (OA and analogues) Shellfish Toxin Profile

To characterize the lipophilic shellfish toxin profiles in shellfish, a complete analysis of the toxin profiles was carried out using the LC-MS/MS technique. Three (3) types of wild shellfish (Oysters, cockles and clams) were handpicked and analyzed during August 2017–February 2018. All lipophilic toxins that are currently legislated in the EU (OA, PTXs, YTXs, and AZAs group toxins) were analysed.

Toxin positive results have been presented:

Chromatograms of toxins present in samples are in Figures 4.45. to Figures 4.52

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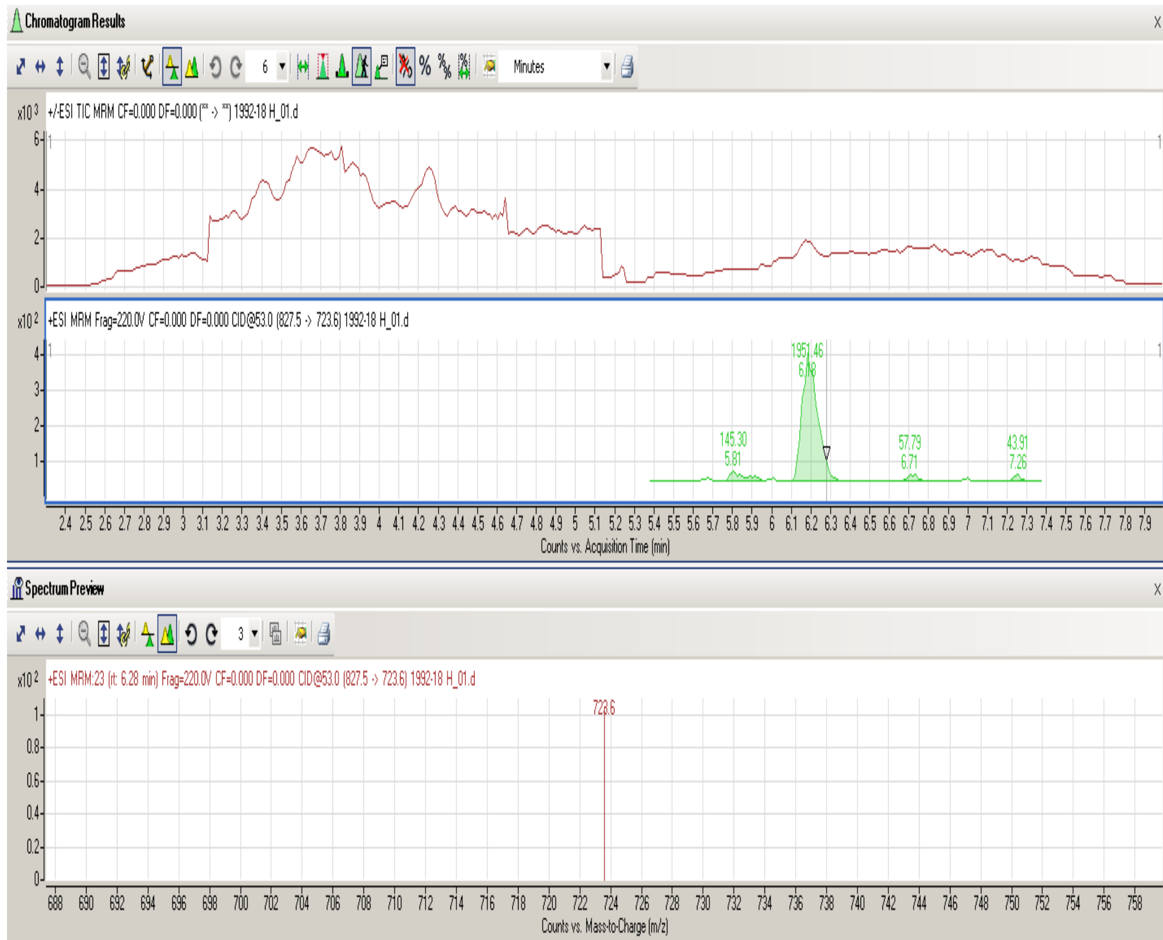

Figure 4.49: Chromatogram for sample 1992/18 showing OA toxins present

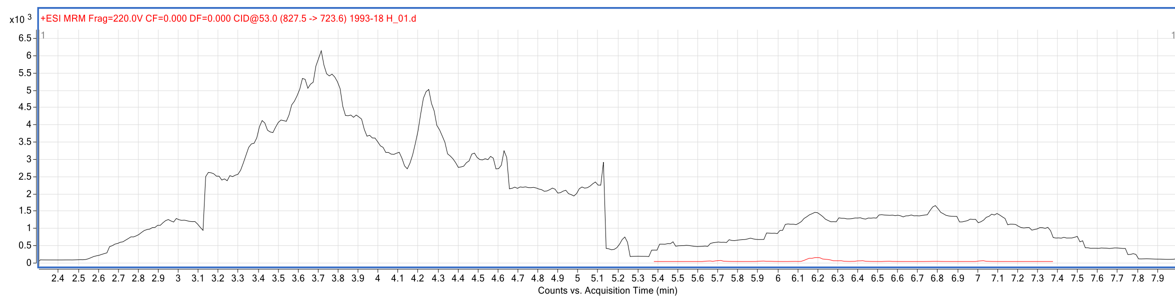

Figure 4.50: Chromatogram for sample 1993/18 showing no peak for any toxin

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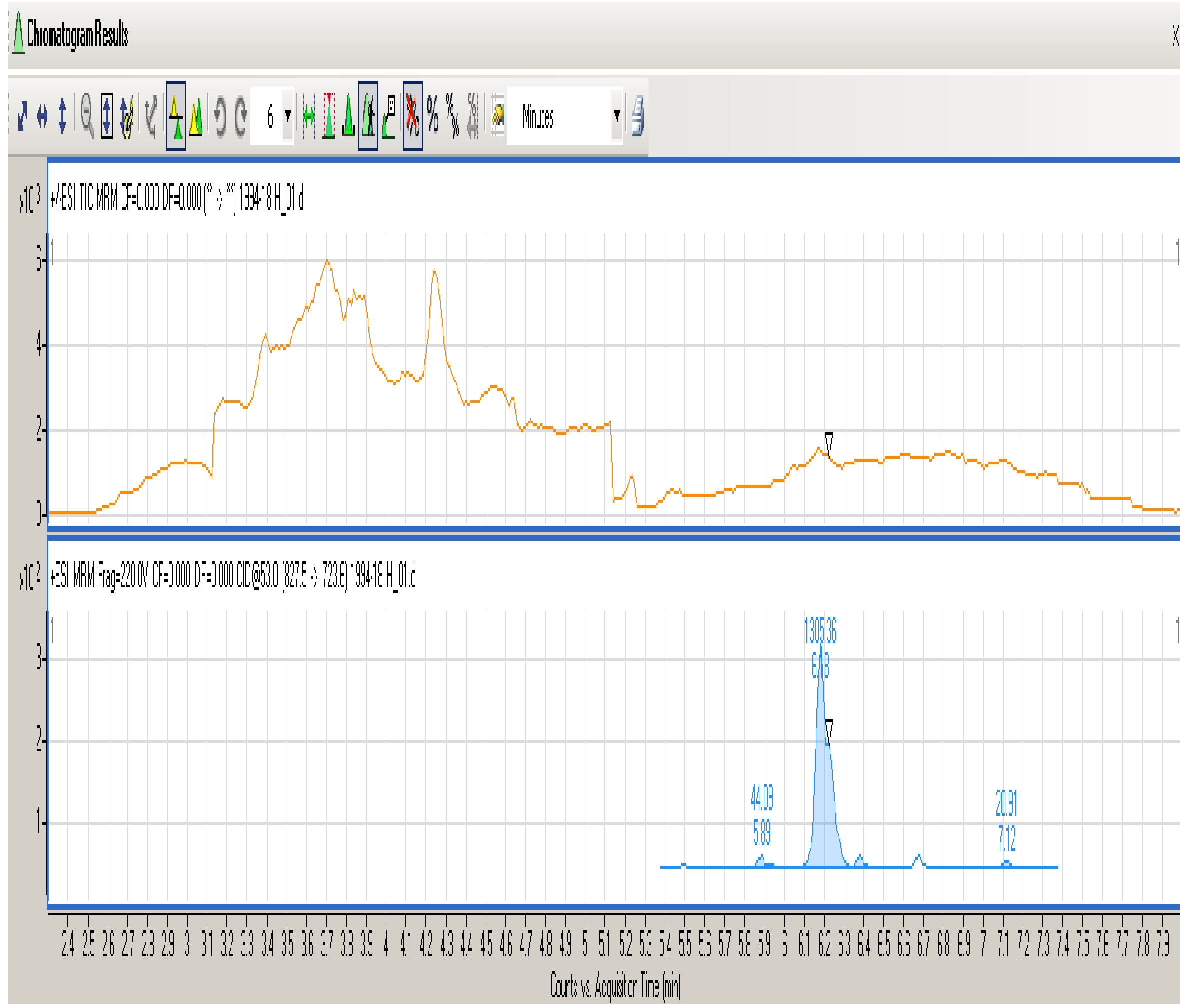

Figure 4.51: Chromatogram for sample 1994/18 showing OA toxins present

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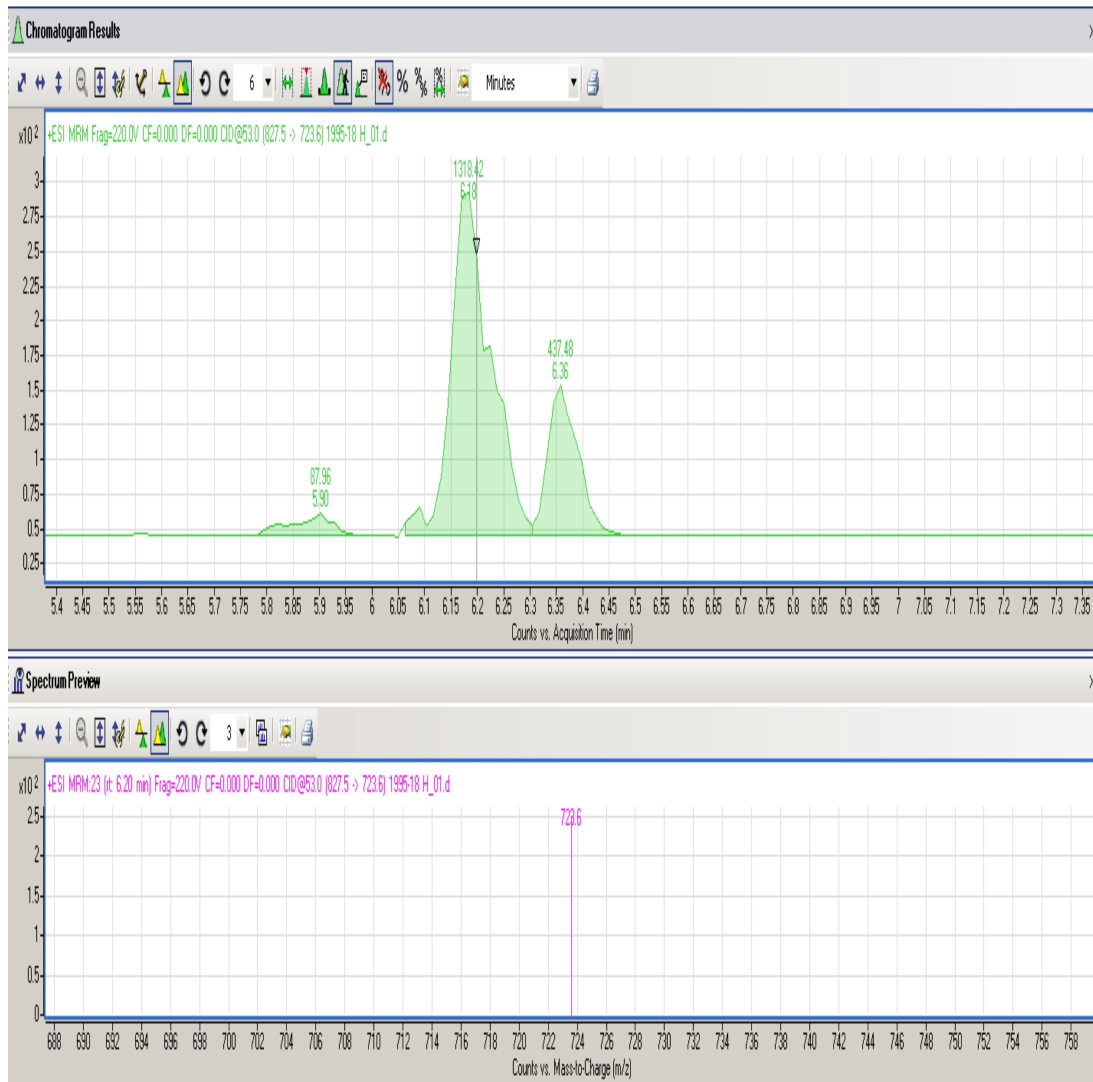

Figure 4.52: Chromatogram for sample 1995/18 showing OA toxins present

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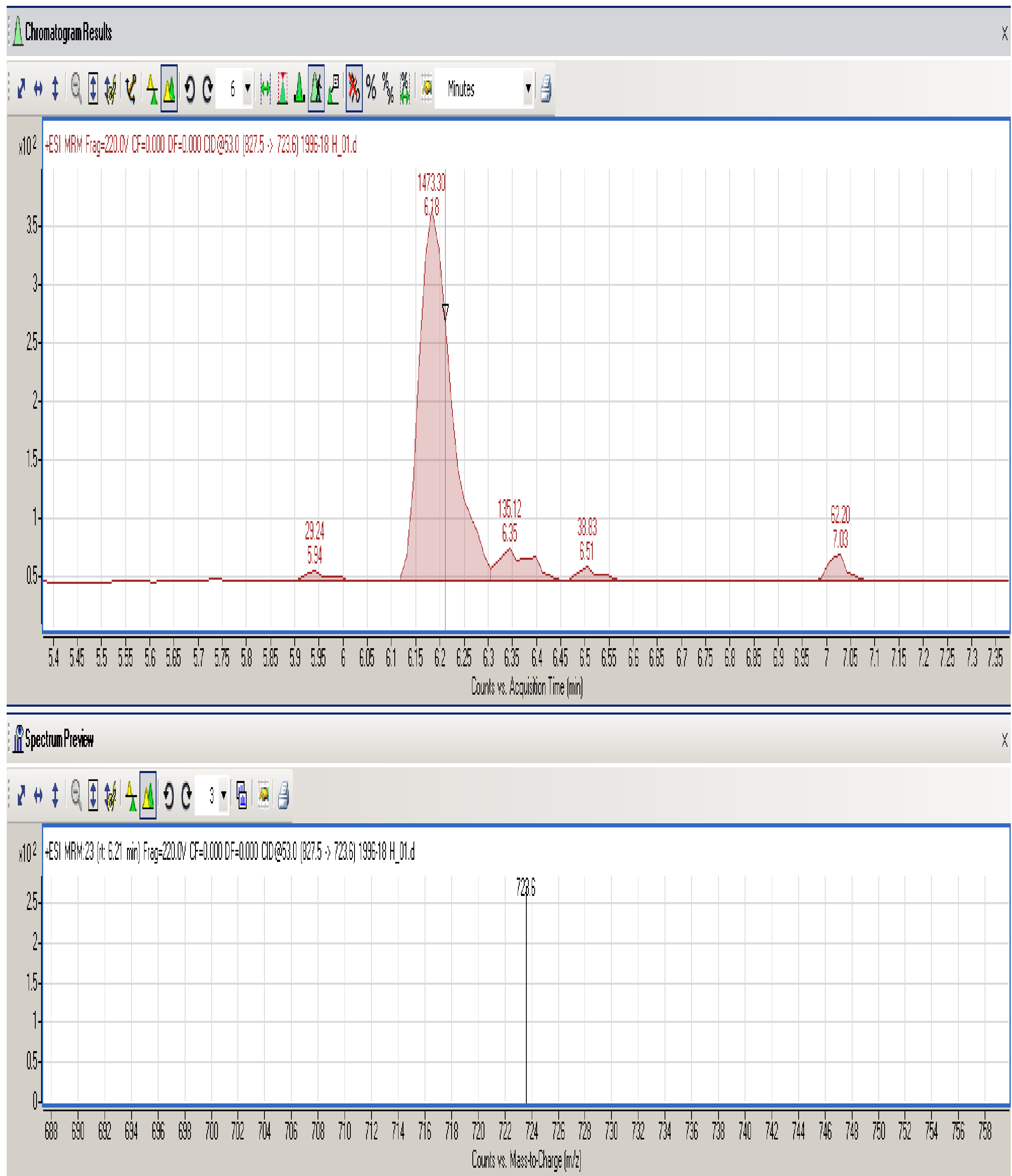

Figure 4.53: Chromatogram for sample 1996/18 showing OA toxins present

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Figure 4.54: Chromatogram for sample 1997/18 showing OA toxins present

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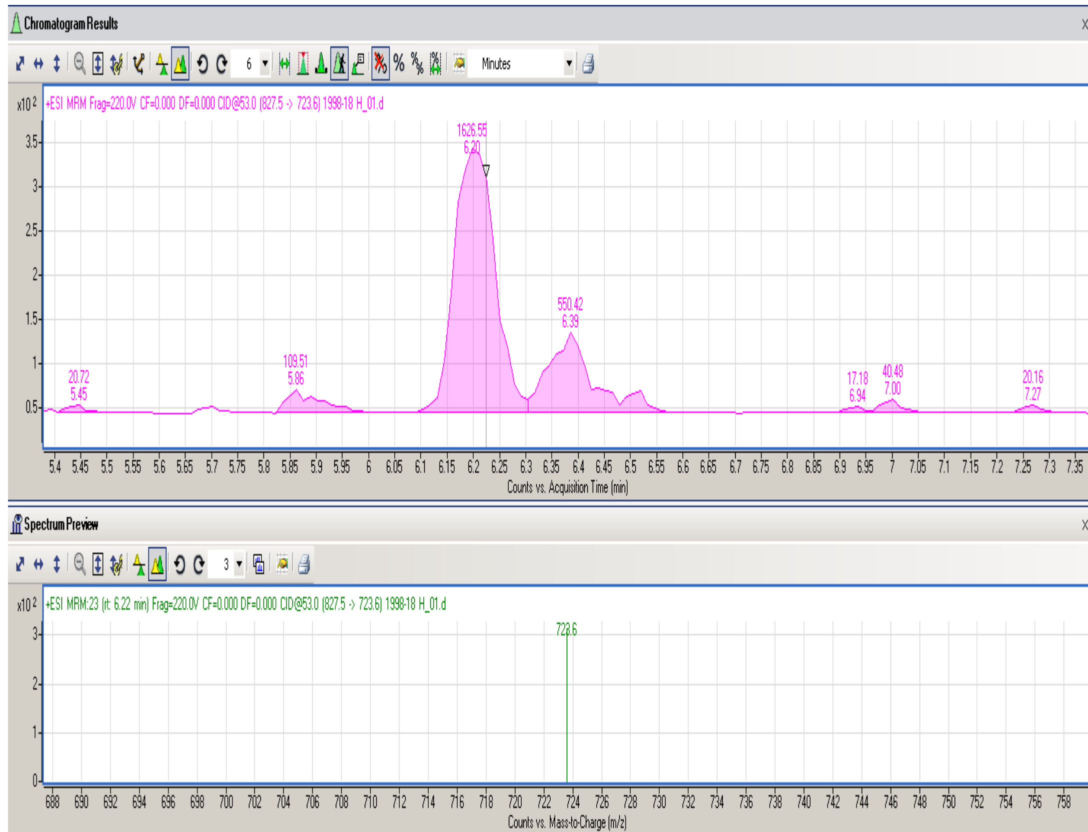

Figure 4.55: Chromatogram for sample 1998/18 showing OA toxins present

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Figure 4. 56: Chromatogram for sample 1999/18 showing OA toxins present

It was observed that from clams and oyster samples, contains no toxin while the bloody cockle samples were positive for some toxins.

Figure 4.49 to 4.56 shows the toxins indicated in a cockle sample from Ekumfi-Narkwa. Results showed that the regulated toxins OA (RT = 6.18 min), and DTX-2 (RT = 6.31 min), were found in the analyzed samples.

In the cockle samples (1992 to 1999), small concentrations of Okadaic acid and DTX2 were observed concentrations. In the chromatograms above we can observe the peak of

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Okadaic acid in samples: 1992, 1994 and 1997, Okadaic acid and DTX2 in 1995, 1996, 1998, no toxin was found in 1993.

4.6.1.6 Lipophilic Toxins found in Shellfish

From the toxin profile; the Diarrheic Shellfish Poisoning (DSP) toxin Okadaic acids(OA) and its derivative Dinophysistoxin (DTX2) were found whiles dinophysis toxins (DTX1, and DTX3), pectenotoxins (PTX1, PTX2), yessotoxins (YTX), homoYTX, 45-OH YTX, 45-OH homoYTX) Moreover, azaspiracids (AZA1, AZA2, AZA3) were not available.

The OA and its derivatives have been reported in shellfish internationally in most of Europe, the United Kingdom, South Africa and Canada (FAO, 2004). Different approaches are used to regulate DSP toxins in humans which is either through cell counts in the water column or using biotoxin meat concentrations. Using cell counts, nationals with DSP monitoring programs in place use various DSP producing *Dinophysis* and *Prorocentrum spp*. as indicators to increase monitoring or initiate restriction on harvesting. An action is initiated when cell concentrations of 100 to 1000 cells/L are exceeded (Anderson et al., 2001). In the present study, levels of *Dinophysis Caudata* in seawater from all the sampling stations using light microscope were in the range of 0-720 cells/L with an average of 188 cells/L. Water samples containing *Dinophysis fortii* was found to contain 0 to 1100 cells/L for all stations with an average of 65.06 cells/L. The range of values in seawater samples falls within the standard limits, which indicates that the Ghanaian waters may not be entirely safe for seafood production.

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Using biotoxin meat concentrations, countries with monitoring programs have standard limits to minimize exposure to its nationals and safeguard human health. For instance, the European Commission recommends guideline levels not exceeding 16 mg OA equivalents/100 g tissue for OA, DTX and PTX (EC, 2002).

Regulatory guideline limits in the range of 16 to 20 mg OA equivalents/ 100 g tissue have been set to manage DSP toxins in other nations in Central and South America, Asia, and Oceania (FAO, 2004). In the current study, levels of OA and DTX 2 in bloody cockles sampled from the Ekumfi-Narkwa lagoon in the Central Region were averagely found to contain 16.875 ug/kg of OA and 2.875ug/kg DTX 2 which is less than the EU limit of 160 µg of okadaic acid equivalents per kilogram for okadaic acid, dinophysistoxins and pectenotoxins in combination.

It should be emphasized that OA and DTX are known to promote tumour in humans, although the effect of chronic low exposure doses is not yet known (Deeds et al., 2010). This indicates that although low levels were recorded in the present study since the bloody cockles serve as a major source of food for inhabitants of Ekumfi-Narkwa and its environs, necessary precaution should be taken to minimize frequent exposures.

Even at low densities, such as 200 cells per litre, the presence of Dinophysis can result in shellfish toxification that is sufficient to affect humans (Botana et al., 1996). Although only blooms of more than 20 000 cells per litre were associated in the Dutch Wadden Sea with DSP. In Portuguese waters, a study of Dinophysis found that the time needed for shellfish to become toxic depends not only on the presence of toxic algae but also on the relative abundance of other non-toxic organisms (Aune and Yndestad, 1993). DSP outbreaks are

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not all accompanied by Dinophysis spp. Macroscopic blooms. Prorocentrum spp. or (Viviani, 1992). Toxicity of specific Dinophysis species varies spatially and temporally, and the number of cells per litre needed to intoxicate shellfish is highly variable. Substantial cell growth in blue mussels (20 000 to 30 000 cells per digestive gland) was observed for 1 000 to 2 000 cells of Dinophysis spp., resulting in high toxicity in Norway's mussels. (Aune and Yndestad, 1993) per litre of seawater.

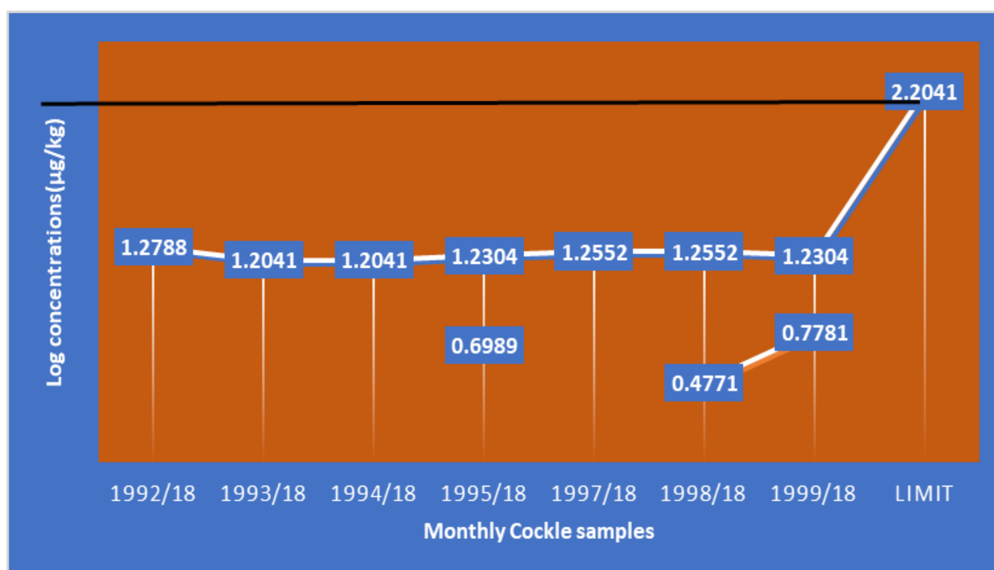

Figure 4.57: Levels of OA and DTX2 in bloody cockle samples

These levels are all below the EU regulatory limit of 160 micrograms of okadaic acid equivalents per kilogram for okadaic acid and dinophysistoxins. Though levels are less than the EU limits, regular monitoring needs to be put in place to protect the General public, and the aquatic life (Figure 4.57).

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CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The overall objective of the study was to identify and assess the dispersion of harmful and potentially toxic phytoplankton that causes harmful algae blooms and evaluate the distribution of harmful phytoplankton along a section on the coast of Ghana. This was achieved through: **[a]** Identification and characterization of harmful and potentially toxic phytoplankton species along the Central Coastline of Ghana; **[b]** Assessment of the distribution of the harmful phytoplankton; **[c]** Evaluation of the influence of environmental driving factors {(Temperature, DO, Nutrients [PO43-, NO3-, SiO4] and Trace Elements [Zn & Fe]} on the distribution of HABs in Ghanaian Waters; **[d]** Evaluation of the levels of iron (Fe) and zinc (Zn) in seawater and Hg, Mn, Cu, Zn, Fe and Cd in algae and establish the correlation between the levels of the essential elements and the presence of algae; [**e**] Isolation of potentially Harmful Algal Bloom(HABs) species and assess the level of toxicity of successful cultures and; **[f]** Investigation of toxin effects of HABs in selected shellfish (Oysters, Bloody cockle and Clams) [Profiling of Toxins]

This study established that harmful algal bloom (HABs) species were present in various concentrations ranges that can cause a bloom event with the favourable conditions in the section of coastline studied. Species identified are *Alexandrium spp, Ceratium furca, Ceratium Protoperidinium, Ceratium tripos, Dinophysis caudata, Dinophysis fortii, Gonyaulax spinifera, Lingulodinium polyedrum, Noctiluca scintillans, Prorocentrum mexicanum, Prorocentrum gracile, Prorocentrum micans, Scrisppsiella-trochoidea,*

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Levenderina fissa. Most of the species have been documented to cause bloom events (Lassus et al.,2016). These results are in agreement with various reviews in Nigeria where authors have reported potentially toxic algae and bloom-forming algae like *Prorocentrum micans, Protoperidinium depressum, Prorocentrum mite, Dinophysis caudata, Peridinium gatunense, P. cinctum, Gymnodinium fuscum* and an array of *Ceratium* species (Kadiri, 1999, 2001, 2002, 2006a, b, 2011). Earlier studies by other authors also showed irregular occurrences of *D. caudata, Protoperidinium depressum, P. diabolus, Prorocentrum micans, Noctiluca scintillans* in Lagos Lagoon (Nwankwo, 1991, 1997). *Lingulodinium polyedrum, Prorocentrum minimum, P. sigmoides and Scrippsiella trochoidea* in Lagos, Cross Rivers and Delta States (Ajuzie and Houvenaghel, 2009) with the most recent report of *Dinophysis caudata, Lingulodinium polyedrum,* and *Prorocentrum* spp by Zendong et al., (2016).

The harmful phytoplankton distribution in the coastal section studied had only small variations in cell counts with the density of cells were in the following order; Gomoa Nyanyanor (50208 cell/L) >Bortianor-Tsokomey (44242 cells/L) >Tema (4334 cells/L)>Ekumfi-Narkwa (42618 cells/L)>Accra Lighthouse (2155 cells/L). The distribution of HABs species found in the study area indicates that concentrations are high in enough to cause a bloom event that may be detrimental to Public health and marine organisms

The seawater parameters: temperature, salinity, DO, nitrates, phosphate, Zn, Fe and chlorophyll a is playing important roles as limiting factors to phytoplankton diversity. The variation in the phytoplankton groups reflects the seasonal dynamics and the impact

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of water quality. This indicates that physical-parameters of the seawater from the study area revealed that high temperature and salinities might increase densities of HABs species. Nutrients like NO_3^{2-} and PO_4^{3-} may be driving factors for a bloom to occur. Chlorophyll a concentration is not dependent on the HABs species in the study areas since another phytoplankton present may also cause the concentrations chlorophyll a to increase.

The study established that among the metals analysed for by INAA, the concentration of Cd was found to be below the detection limit (Cd<0.006). Hg concentration detected from the total microalgae ranged from 10.05 to 18.29 (µg/kg). Other metals concentrations in the microalgae were in the ranges of 162.00 to 4418.90 (µg/kg) for Zn, 4533 to 24567 (µg/kg) for Fe and 225.10 to 2121.46 (µg/kg) for Mn and 154.50 to 497.90 (µg/kg) for Cu respectively.

Ballast water influences were not indicated as, species found in Tema waters does not designate ballast water intrusion, all HABs species found currently are typical Atlantic coast and cosmopolitan source; Though analysis can be taken further offshore and also study ballast waters brought to port by ships since harmful microalgae are a severe threat to the world seas. Furthermore, they can be introduced through ballast water discharges that may cause adverse impacts to aquatic ecosystems, human health and economic activities.

The potentially toxic phytoplankton species confirmed by the study are *Dinophysis caudata, Dinophysis fortii, Prorocentrum sp and Alexandrium sp*. Paralytic Shellfish Poisoning(PSP) toxins; (GTX1,4 and GTX2,3) was detected in one strain of Alexandrium

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spp whiles one strain of *Prorocentrum sp* was intoxicated with Diarrheic Shellfish Poisoning (DSP) toxin OA.

The harmful phytoplankton this study has successfully isolated, cultured and identified are *Levanderina fissa, Prorocentrum micans, Coolia canariensis, Prorocentrum sp,* two strains *Alexandrium* spp two of which are toxic.

Shellfish toxin profiles (clams, oysters and bloody cockles) have shown that lipophilic toxins [Okadaic acid (OA) and *Dinophysistoxin* (DTX2)] have been identified for the first time in bloody cockles and can most likely be traced back to *Dinophysis* species. However, it is not possible to exclude a partial contribution by *Prorocentrum* species.

DSP and PSP may threaten our society, as Blood cockles have been shown to contain traces of PSP toxins GTX2, 3; NEO and dcSTX below the quantification limit whereby the only compounds identified below the EU Regulatory Limits of 160 μg OA equivalent of kg^{-1} in cockle samples originating from Ekumfi-Narkwa also contains lipophilic marine biotoxins belonging to the OA-group.

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5.2 Recommendations

Based on the results of the study conducted, the following recommendations are proposed:

(i) In order to ensure Seafood Safety and Quality, the MoFAD/FDA should establish well equipped Culture and Toxin Analysis Laboratories in the country, for monitoring of Paralytic Shellfish Poisoning and Diarrheic Shellfish Poisoning toxins in Ghanaian coastal waters.

(ii) To ensure Seafood Security and promote export of Seafood to major International markets, the Ministry of Trade and Industries (MoTI), and the Ghana Export Promotion Council (GEPC) must liaise with the Ministry of Fisheries and Aquaculture Development (MoFAD) to revise existing policies based on current information to guide Seafood exports from Ghana.

(iii) Further studies should be conducted on the Speciation of Fe and Zn in algae.

(iv) A study involving the determination of toxic elements like As, Hg, Cd, and Pb in algae, as well as their speciation, should be carried out in future.

(v) Universities/Research Institutions/MoFAD should collaborate to set up HABs Monitoring Laboratories.

(vi) Clinicians or Public Health Practitioners should investigate sources of Food Poisoning to help monitor HABs and Biotoxin ingestion.

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(vii) Further studies should be conducted to cover the entire coastal waters of Ghana; with modelling of the HABs situation.

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PUBLICATION

Denutsui D., Cabrini M., Adotey K. D., Gyingiri A., Kuranchie-Mensah H. Palm L., Beran A., Serfor-Armah Y **(2020)**. Diversity and distribution of Harmful Algal Bloom (HAB) species in Coastal Waters of Ghana. In: Ph. Hess [Ed]. 2020. Harmful Algae 2018 – from ecosystems to socio-ecosystems. *Proceedings of the 18th Intl. Conf. on Harmful Algae. Nantes. International Society for the Study of Harmful Algae. 214(96-99) pages. ISBN: 978-87-990827-7-3.*

Conference Presentations

- ***18th International Conference on Harmful Algae (ICHA 2018); Nantes, France (21st – 26th Oct. 2018)***

  Denutsui Dzifa, Marina Cabrini, Dennis K. Adotey, Yaw Serfor-Armah. *The Status of Harmful Algal Blooms (HABs) in the Coastal Waters of Ghana* **[POSTER]**

- ***Conference on Fisheries and Coastal Environment; Accra (25-27 September 2017)***

  Denutsui Dzifa, Marina Cabrini, Adotey K. Dennis, Serfor-Armah Yaw. *Potentially Toxic and Harmful Phytoplankton Species along the Coast of Ghana* **[ORAL]**

- ***19th Annual Ghana Chemical Society Conference; Cape Coast (28-30 September 2017)***

  Denutsui Dzifa, Marina Cabrini, Adotey K. Dennis, Serfor-Armah Yaw. *Harmful Algal Blooms (HAB's) in the Coast of Ghana, the Past, Present and Future* **[ORAL]**

- ***University of Ghana Maiden Doctoral Conference; Legon-Accra (5–6 November 2015)***

  Denutsui Dzifa, Adotey K. Dennis, Serfor-Armah Yaw. *Identification of Harmful Algal (HAB's) Toxins in Marine Fish and Shellfish along the Coast of Ghana*. **[ORAL]**

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Training and Fellowship

(i) **IAEA- IOC Advanced Training Course in Marine Phytoplankton**: - organized by the International Atomic Energy Agency (IAEA) and Intergovernmental Oceanographic Commission (IOC) in Denmark- Hillerod, University of Copenhagen from November 29th to December 12th, 2015.

(ii) **PhD Sandwich Programme Fellowship awarded by The Abdus Salam International Centre for Theoretical Physics(ICTP), and The International Atomic Energy Agency(IAEA**); Trieste, Italy [2017-2018]; (training carried out at National Institute of Oceanography and Experimental Geophysics (OGS) in Trieste, in co-operation with the Marine Research Centre Foundation (FCRM) in Cesenatico; Italy)

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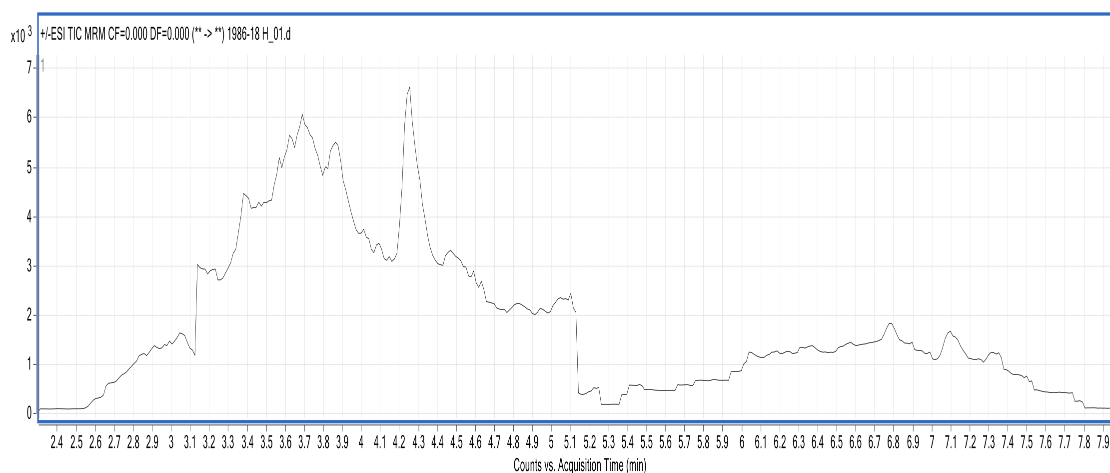
Appendices

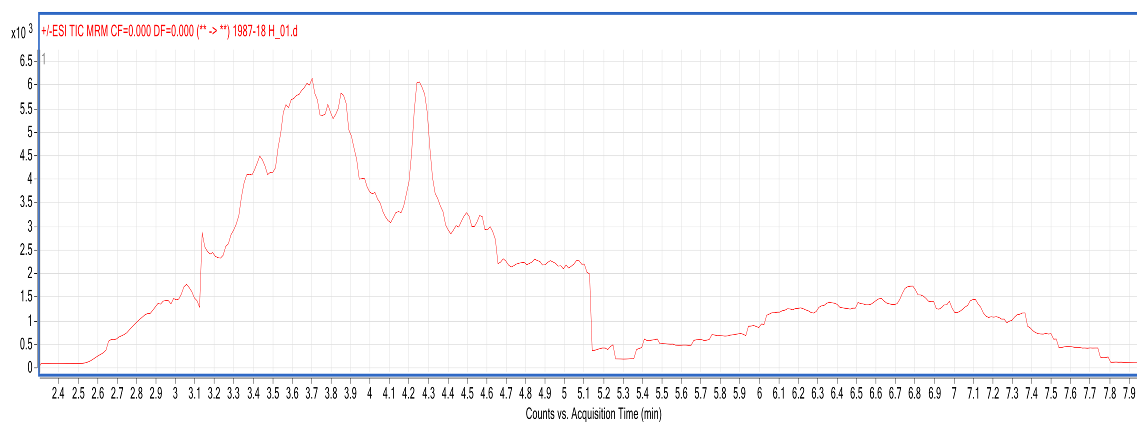
Chromatograms of Okadaic acids analysis of Shellfish

OYSTERS

OA	No	827.5 Unit/Enh (6490)	809.5 Unit/Enh (6490)	220	48	7	6.24	1	Positive
OA	No	827.5 Unit/Enh (6490)	723.6 Unit/Enh (6490)	220	53	7	6.24	1	Positive



Appendix A: Chromatogram for Sample 1986



Appendix B: Chromatogram for Sample 1987

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Appendix C: Chromatogram for Sample 1988

Appendix D: Chromatogram for Sample 1989

Appendix E: Chromatogram for Sample 1990

Cockles

Appendix F: Chromatogram for Sample 1994

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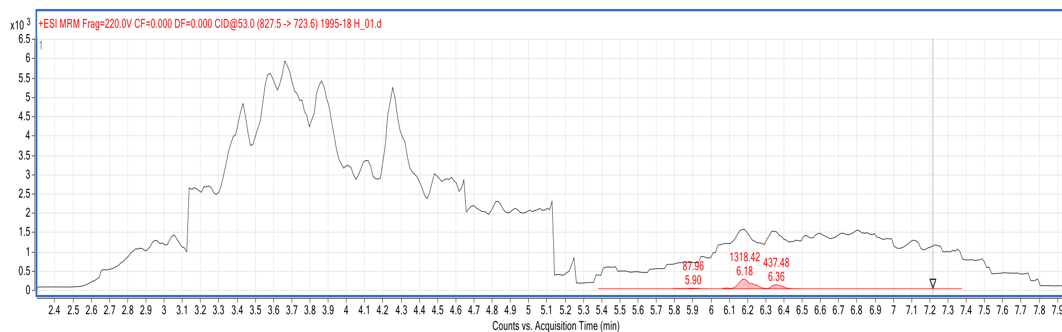
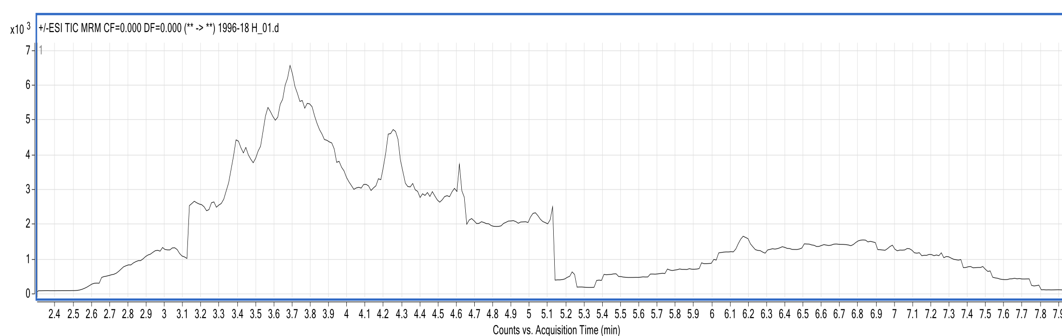
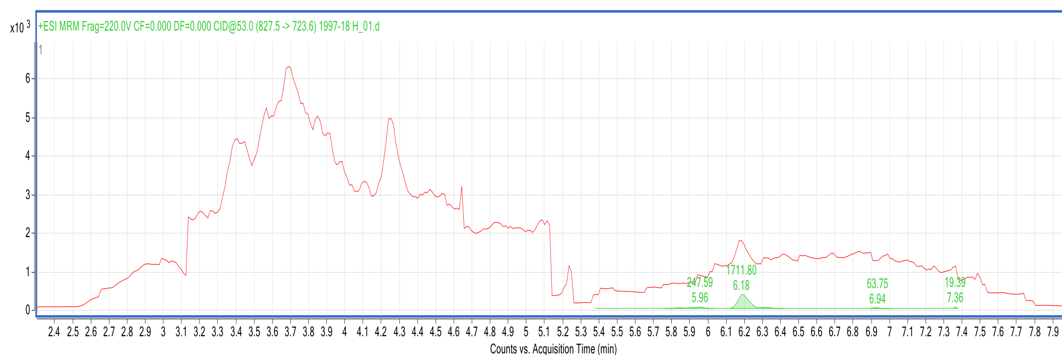


Appendix G: Chromatogram for Sample 1995



Appendix H: Chromatogram for Sample 1996



Appendix I: Chromatogram for Sample 1997

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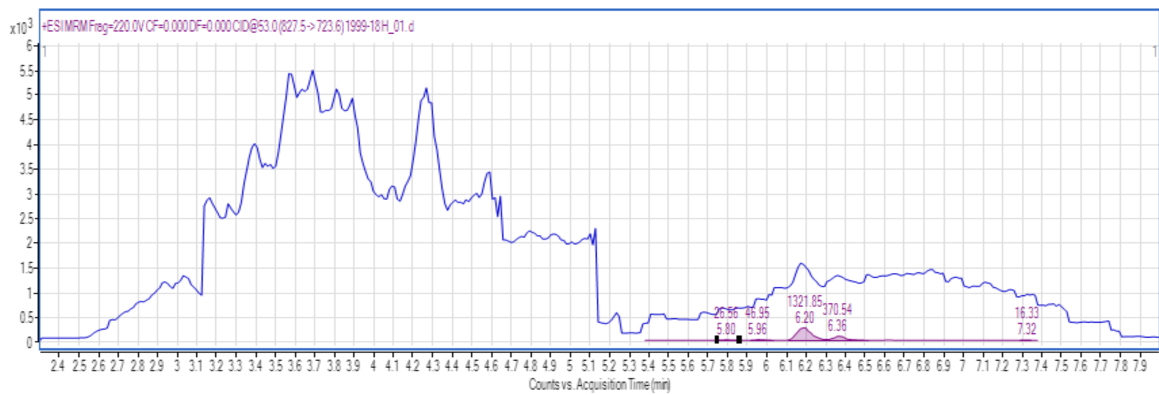
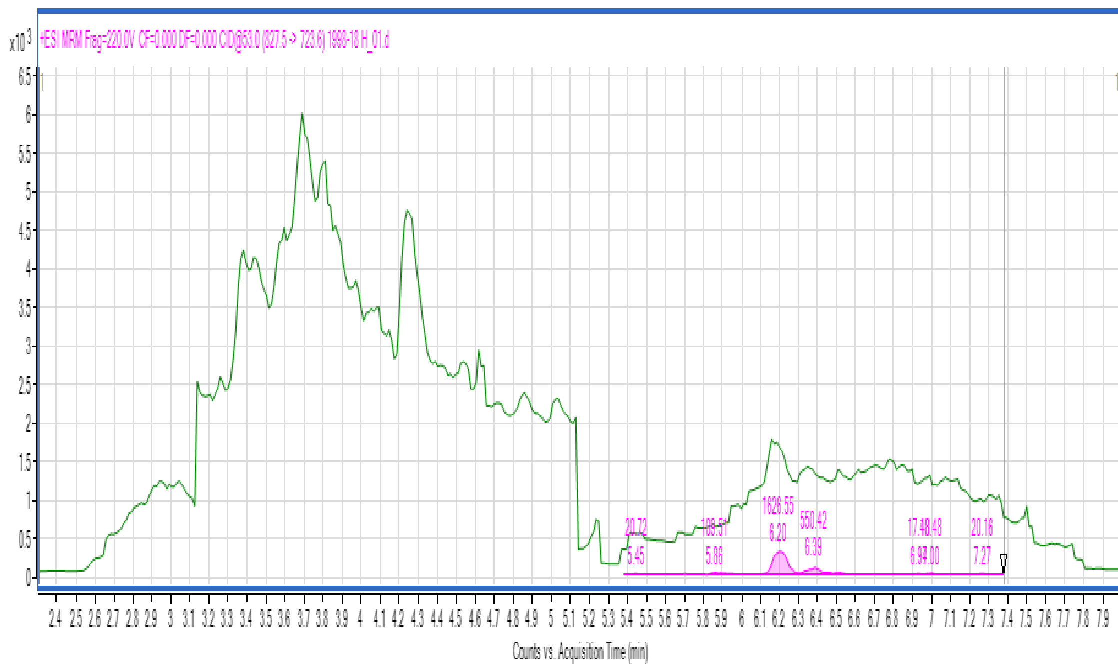


Appendix J: Chromatogram for Sample 1998



Appendix K: Chromatogram for Sample 1999

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Appendix L: DNA Sequence for *Coolia spp* identification

Co3K_30.4
CTTTCCATTGCATAAATCAAGAACTGACCAATGCTGCAGTCCAAATTATTCACCG
GATCACTCAATCGGTAGGAGCGACGGGCGGTGTGTACAAAGGGCAGGGACGT
AATCAGCACAAGCTGATGAATCATGCTTACTAGGAATTCCTCGTTTAAGATTAAT
AATTGCAATAATCAATCCCCATCACGATATGTGTTCAAAGATTACCCGGACCTTG
CCAGGCAAGGTTGTAAACTCGTTGAGCACACCAGTGTAGCGCGCGTGCAGCCC
AGAACATCTAAGGGCATCACAGACCTGTTATTGCCTCAAACTTCCTTATGTTGTA
CACACAAAGTCTCTCTAAGAAGTTGCCCACACAACAAAATACATGTAACTATTTA
GCAGATTAAGGTCTCGTTCGTTAACGGAATTAACCAGACAAATCATTCCACCAA
CTAAGAACGGCCATGCACCACCACCCATAGAATCCAAGAAAAAGCTATCAATCT
GTCAATCCATATTATGTCTGGACCTGGTAAGTTTCCCCGTGTTGAATCAAATTAA
GCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGC
CTTGCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCATAAGGTGCT
GAAAAAGTCATTCAAATAACA

Co4k_29.3
GACTGACCAATGCTGCAGTCCAAATTATTCACCGGATCACTCAATCGGTAGGAG
CGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAGCACAAGCTGATGAAT
CATGCTTACTAGGAATTCCTCGTTTAAGATTAATAATTGCAATAATCAATCCCCA
TCACGATATGTGTTCAAAGATTACCCGACCTTGCCAGGCAAGGTTGTAAACTCG
TTGAGCACACCAGTGTAGCGCGCGTGCAGCCCAGAACATCTAAGGGCATCACA
GACCTGTTATTGCCTCAAACTTCCTTATGTTGTACACACAAAGTCTCTCTAAGAA
GTTGCCCACACAACGAAATACATGTAACTATTTAGCAGATTAAGGTCTCGTTCGT
TAACGGAATTAACCAGACAAATCATTCCACCAACTAAGAACGGCCATGCACCAC
CACCCATAGAATCAAGAAAAAGCTATCAATCTGTCAATCCATATTATGTCTGGAC
CTGGTAAGTTTCCCCGTGTTGAATCAAATTAAGCCGCAGGCTCCACTCCTGGTG
GTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCGG
AACCCAAAAACTTTGATTTCTCATAAGGTGCTGAAAAA

Co4K_12.4
ACTGACCAATGCTGCAGTCCAAATTATTCACCGGATCACTCAATCGGTAGGAGC
GACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAGCACAAGCTGATGAATC
ATGCTTACTAGGAATTCCTCGTTTAAGATTAATAATTGCAATAATCAATCCCCAT
CACGATATGTGTTCAAAGATTACCCGACCTTGCCAGGCAAGGTTGTAAAGTCGT
TGAGCACACCAGTGTAGCGCGCGTGCAGCCCAGAACATCTAAGGGCATCACAG
ACCTGTTATTGCCTCAAACTTCCTTATGTTGTACACACAAAGTCTCTCTAAGAAG
TTGCCCACACAACAAAATACATGTAACTATTTAGCAGATTAAGGTCTCGTTCGTT
AACGGAATTAACCAGACAAATCATTCCACCAACTAAGAACGGCCATGCACCACC
ACCCATAGAATCAAGAAAAAGCTATCAATCTGTCAATCCATATTATGTCTGGACC
TGGTAAGTTTCCCCGTGTTGAATCAAATTAAGCCGCAGGCTCCACTCCTGGTGG
TGCCCTTCCGTC

Co4K_30.4
TAACTTTCCATTGCATAAATCAAGAACTGACCAATGCTGCAGTCCAAATTATTCA
CCGGATCACTCAATCGGTAGGAGCGACGGGCGGTGTGTACAAAGGGCAGGGA
CGTAATCAGCACAAGCTGATGAATCATGCTTACTAGGAATTCCTCGTTTAAGATT
AATAATTGCAATAATCAATCCCCATCACGATATGTGTTCAAAGATTACCCGACCT
TGCCAGGCAAGGTTGTAAACTCGTTGAGCACACCAGTGTAGCGCGCGTGCAGC
CCAGAACATCTAAGGGCATCACAGACCTGTTATTGCCTCAAACTTCCTTATGTT
GTACACACAAAGTCTCTCTAAGAAGTTGCCCACACAACAAAATACATGTAACTAT

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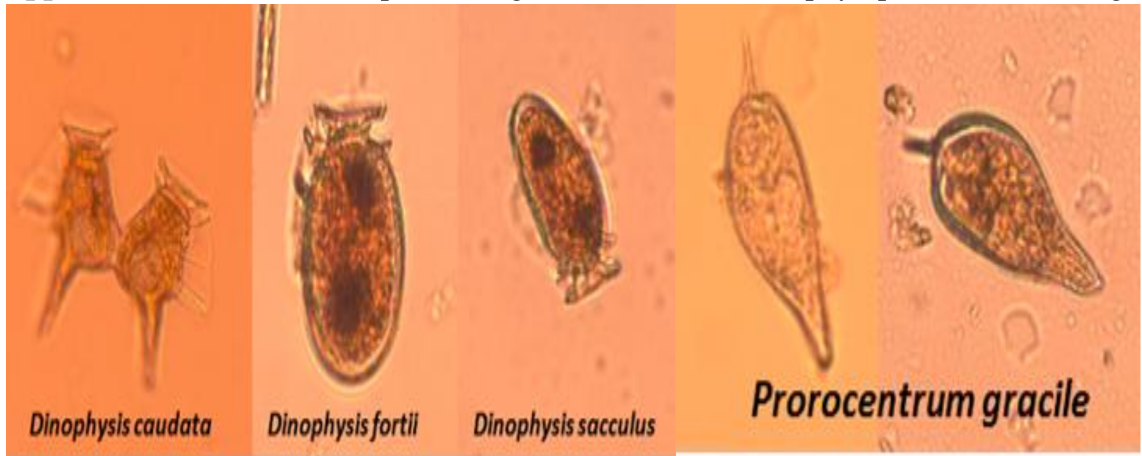
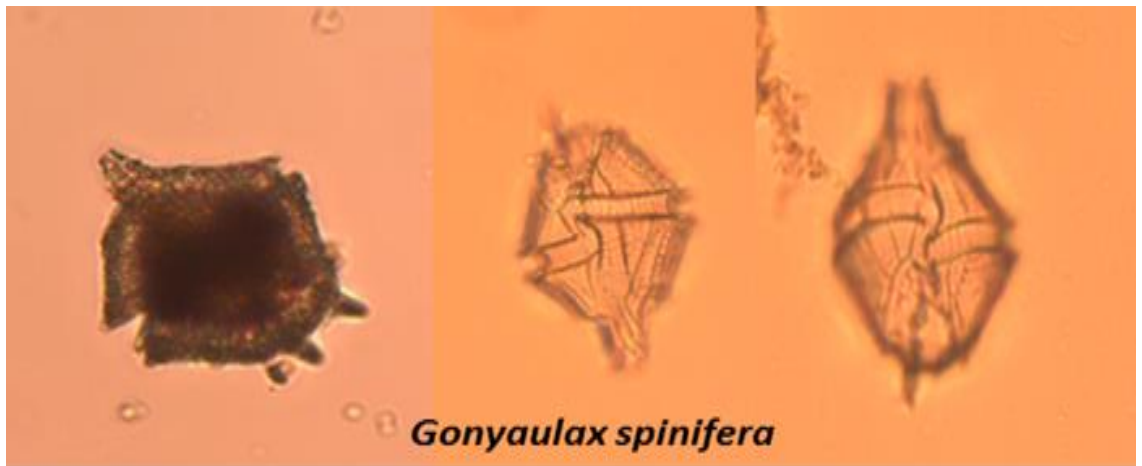
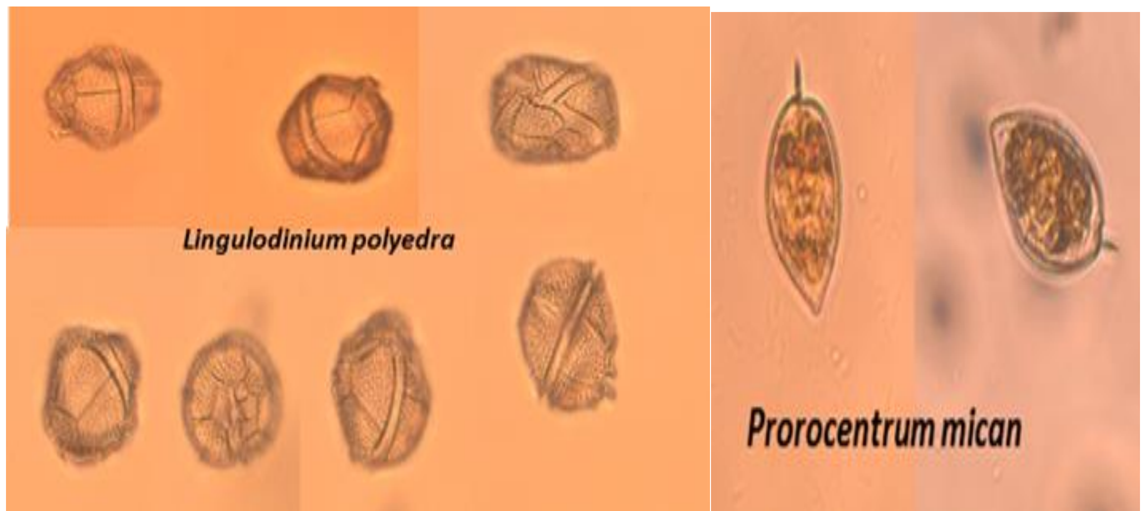
Appendix M: Microscopic images of harmful phytoplanktons/microalgae







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Appendix N: Classification and Consequences of HABs