OPTIMISATION OF HIGH VALUE METABOLITE PRODUCTION FROM BENTHIC MARINE DINOFLAGELLATE *PROROCENTRUM LIMA*

RADISTI AYU PRAPTIWI

A thesis submitted in partial fulfilment of the requirement of the Robert Gordon University, Aberdeen for the award of Doctor of Philosophy

March 2014

TABLE OF CONTENT

DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise acknowledged, and has not been submitted in any form for another degree or qualification at any other academic institution.

Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

Radisti A. Praptiwi

ACKNOWLEDGEMENT

I am sincerely grateful for the constant support, guidance and encouragement of my supervisory team, Dr Christine Edwards and Professor Linda Lawton. They have enabled me to complete this PhD. I would also like to extend my thanks to Institute for Innovation, Design and Sustainability (IDEAS), Robert Gordon University for their financial assistance.

I would also like to thank Dr Campbell Tang from Centre for Process Innovation (CPI) for the collaborative works. I would also like to thank Ulrike Worms and Erin Temple for their assistance in several parts of data acquisitions. Special thanks to Dr Jeanette Robertson and Professor Claudia Wiegand for their roles as viva examiners and for their invaluable inputs during the process. I would also like to thank Mr Leonard Montgomery for his proofreading of my thesis.

My parents, Bambang Wahyudi and Wiwik S. W. Praptiwi, to whom I dedicate this work. To my family: Radityo, Ami, Ibu, Bapak, and to Audrie and Sarah who never failed to amuse me.

I would also like to thank members of Cyanosol research group: Aakash, Mahalaksmi, Kostas, Arif, Olu, Shaista, Vijith, Carlos, Luca, Julia and Dorothy for their help and friendship throughout the years. To Evie, Abhishek and Chamaka for their generosity and supports throughout my time in Aberdeen.

Finally, to Carya Maharja.

LIST OF ABBREVIATION

ASW	: Artificial seawater
ASP	: Amnesic shellfish poisoning
ATP	: Adenosine triphospgate
AZAs	: Azaspiracids
AZP	: Azaspiracid shellfish poisoning
BPI	: Base peak ion
CBL	: Column bubble-lift
CCAP	: Culture collection for algae and protozoa
CFP	: Ciguatera fish poisoning
CPI	: Centre for process innovation
CTXs	: Ciguatoxins
DA	: Domoic acid
DCW	: Dry cells weight
DSP	: Diarrhetic shellfish poisoning
DTXs	: Dinophysis toxins
FC	: Flow cytometer
FL	: Fluorescence
FS	: Forward scatter
GTXs	: Gonyautoxins
HABs	: Harmful algal blooms
HE	: High energy
IP	: Intra-peritonial
L/D	: Light/dark
LE	: Low energy
LEDs	: Light emitting diodes
MT C	: Metric tons of carbon
NCMA	: National Center for Marine Algae and Microbiota
NSP	: Neurotoxic shellfish poisoning
NSW	: Natural seawater
OA	: Okadaic acid
PBR	: Photobioreactor
PDA	: Photodiode array

PP	: Protein Phosphatases
PSP	: Paralytic shellfish poisoning
PTXs	: Pectenotoxins
PVC	: Polyvinyl chloride
QTOF	: Quadrupole time of flight
ROS	: Reactive oxygen species
SR	: Sedgewick-Rafter
SS	: Side scatter
StW	: Salt water
STX	: Saxitoxin
TIC	: Total ion chromatogram
TPBL	: Thin-plate bubble-lift
UPLC	: Ultra Performance Liquid Chromatography
VGSC	: Voltage gated sodium channel
YTXs	: Yessotoxins

LIST OF TABLE

LIST OF FIGURE

ABSTRACT

Toxins produced by harmful algal blooms (HABs) are known to pose contamination risks to seafood products (e.g. fish and shellfish) consumed by human. In order to control contamination risks, monitoring regimes have to be performed rigorously. The effort to monitor the amount of toxins in consumable products has to rely on continuous supply to analytical standards. The current work presents the strategy in optimising the production of major diarrhetic shellfish poisoning (DSP) toxins, OA and DTX1, from *Prorocentrum lima*. The organism is also known to produce peridinin, a carotenoid pigment that has been found to have pharmaceutical potential. Results from this study showed that cultivation of *P. lima* CCAP 1136/11 was still, although not completely, reliant on supply of natural seawater. Characterisation of compounds produced by *P. lima* CCAP 1136/11 in batch culture identified three major bioactive compounds (OA, DTX1 and peridinin) and two minor OA-related compounds. Recovery of these major compounds was further optimised with two-stage extraction procedure. Several important considerations for the cultivation process include standardisation of inoculum age and initial cell density. These and several other growth parameters such as temperature, light and CO_2 supplementation have been shown to affect the growth and production of DSP toxins and peridinin in the culture. One of the main highlights in this study revealed that providing culture with light and dark cycle at frequency of 0.5 hour benefit in the enhancement of OA, DTX1 and peridinin yield from *P. lima* CCAP 1136/11. As the last part of this study, a simple and scalable design of reactor has been proposed. Contrary to common observations for dinoflagellate culture, *P. lima* CCAP 1136/11 was found to be able to withstand increased sparging within the culture system, resulting in concomitant increased of growth and production of OA, DTX1 and peridinin. Future works have been suggested to focus on: (1) exploitation of different cultivation system, such as continuous or semi-continuous systems, and (2) exploration on genetic modification to enable commercial scale production of DSP toxins and peridinin.

Keywords: Dinoflagellate, *Prorocentrum lima*, okadaic acid, dinophysistoxin-1, photobioreactor.

CHAPTER 1

Introduction

1.1 Microalgae and their ecological importance

Microalgae encompass a wide range of photosynthetic microorganisms, including prokaryotic cyanobacteria and eukaryotic members of the Protista (Table 1.1) that may exist as free-floating (phytoplankton), benthic, epiphytic or symbiotic organisms. Microalgae play an essential role within the biogeochemical cycles on Earth. They are essential for the existence and survival of life on the planet, especially as they account for most of the oxygen present in the atmosphere. Consequently, they serve as a key food source for higher animals in the food chain, such as fish and shellfish, mainly due to their lipid and starch content (Julius 2007). This is primarily due to their capability for oxygenic photosynthesis, rendering them as significant primary producers that initiate energy and material flows in Earth's ecosystem. This global importance of microalgae is highlighted by their contribution to global primary production. Due to their abundance in the ocean, microalgae are accountable for half of the global net primary production that has been estimated at a level of 48.5 petagrams carbon per annum (Pg C Y^{-1}), compared to the 56.4 Pg C Y^{-1} produced from terrestrial areas (Field *et al.* 1998, Kirchman 2012).

Microalgae exist in a diverse range of habitats, including terrestrial aquatic habitats, hot springs and Antarctic mats. In aquatic systems, distribution and variability of microalgae is largely influenced by the water turbulence that is induced by winds and other climatic factors controlling environmental stability (Holligan *et al.* 1980, Webster and Hutchinson 1994). Winds and currents can also regulate the dispersal of nutrients in water bodies, with subsequent impact on growth and populations of microalgae.

Besides the aforementioned physical factors, microalgal growth and populations in aquatic systems are also influenced by other environmental factors such as temperature, salinity and light. Under optimum environmental conditions and nutrient availability, rapid and massive proliferation of microalgae can occur. This excessive proliferation of microalgae is often referred to as blooms.

Table 1.1 Classification of microalgal groups (Adapted from Glycomar 2013).

Domain	Group
Prokaryote	Cyanobacteria
Eukaryote	Chrysophytes Cryptomonads Diatoms Dinoflagellates Eustigmatophytes Glaucophytes Haptophytes Phaeothamniophytes Phaeophytes Raphidophytes Rhodophytes Synurophytes Xanthophytes

Microalgal blooms can occur as a natural process during ecosystem succession, following seasonal changes (Kirchman 2012). For instance, it has been observed that blooms often occur in temperate waters during the spring (Gamier *et al*. 1995, Kirchman 2012). In the North Sea, spring blooms can typically be found in the month of April to early June and are dominated by diatom and haptophyta (Barlow *et al.* 1993, Peperzak *et al.* 1998). In early spring, nutrients are non-limiting and the increase of sunlight stimulates the vertical movement of planktonic microalgae that prompts higher biomass accumulation at the surface of water bodies, initiating a bloom (Legendre 1990).

The natural occurrence of blooms is a part of the perpetual seasonal succession of microalgae. The increasing and decreasing of microalgal growth rates throughout the seasons have been observed and modelled in a variety of aquatic ecosystems in the world, ranging from freshwater lakes to coastal lagoons (Admiraal *et al*. 1984, Sommer 1985, de Emiliani 1993, Fong and Zedler 1993). A constellation of natural factors, particular to the observed habitats, are known to influence the growth patterns, such as light and temperature changes, and competition for nutrients with other co-existing organisms (Sterner 1989, Fong

and Zedler 1993, Richardson et al 2000). Such systems are made even more complex by the introduction of anthropogenic factors, which makes it very difficult to model and predict the occurrence of algal blooms with high accuracy (Van Straten and Keesman 1991, Cloern 2001).

Algal blooms induced by human factors, for instance through excessive nutrient loading or emissions of greenhouse gases, have been reported to be increasingly frequent (Hallegraeff 1993, Zingone and Enevoldsen 2000). The increasing atmospheric concentration of greenhouse gases, such as carbon dioxide, emitted from human activities is capable of changing the community dynamics of both freshwater and marine algae by altering the physical properties of their environment. For instance, it has been claimed that these gases may increase algal bloom occurrence by changing the ecosystems pH, surface temperature, and mixing and evaporation patterns (Moore et al. 2008). The results of a laboratory experiment, investigating the future impacts that such environmental changes have on algal blooms, highlighted the risk of such blooms becoming more globally widespread (Peperzak 2003). The risk of the globally-felt climate change, particularly the predicted 4^{o}C temperature rise reached in 2100, was shown to be capable of increasing the growth rate of harmful dinoflagellate and raphidophyte (Peperzak 2003).

Eutrophication, associated with increased nutrient loading in water bodies, is also known to be a responsible for enhanced formation of algal blooms. Nutrients may originate from diverse sources, such as sewage, and runoff and discharge from farms and fisheries (Anderson *et al.* 2002). The availability of such nutrients, especially in the forms of ammonium, nitrate and phosphate, allow microalgae to significantly increase their growth, leading to bloom formation (Heisler *et al.* 2008). Cases of algal blooms linked with eutrophication have been increasingly reported across the globe, such as those occurring in several coastal regions in the USA, the Mediterranean Sea, the Black Sea, and lakes in Japan and China (Havens *et al.* 2001, Moncheva *et al.* 2001, Anderson *et al.* 2008).

The nature of the blooms demands that management measures are applied to control their occurrence. However, the efforts to control algal blooms induced by eutrophication must not be done by solely focusing on reducing the loads of a

particular nitrogen or phosphorus chemical species into an ecosystem. Such strategy might fail as it may instead encourage the growth of another group of harmful microorganisms (Havens and Schelske 2001). For instance, management strategies favouring only phosphorus reduction in shallow eutrophic lakes, such as Lake Okeechobee in USA and Lake Donghu in China, were predicted to promote instead the emergence of the toxic *Microcystis* bloom (Havens *et al.* 2001, Conley *et al*. 2009). As such, it has been commented that the approach to prevent and mitigate blooms caused by eutrophication should recognise that the problem is multi-faceted and thus needs comprehensive management strategy incorporating protection and restoration of the ecological community of the water bodies (Schindler 2006).

1.2 Harmful Algal Blooms (HABs)

Blooms of algae may cause major concern primarily due to their negative impacts to surrounding environment and risk to human health (Fig. 1.1). Considering such impacts, they are often termed as harmful algal blooms (HABs). Their detrimental effects on the environment are due to high biomass accumulation that can cause changes in water chemistry, for instance the depletion of dissolved oxygen (hypoxia) and reduced light penetration at the bottom layer of aquatic system (Landsberg 2002, Glibert *et al*. 2005, Anderson 2007). Prolonged oxygen depletion and reduced irradiance in aquatic systems would eventually cause the loss of animals and vegetation living within (Diaz and Rosenberg 2008, Seitz *et al.* 2009). It was predicted that hypoxia occurrences in the Baltic Sea and Chesapeake Bay, USA had caused the loss of approximately 112,000 metric tons of carbon (MT C) of potential food for fisheries (Diaz and Rosenberg 2008).

Whilst proliferation of biomass can have a severe impact on aquatic ecosystems, the production of toxins has a far more serious impact on aquatic ecosystems and human health. Many species of dinoflagellates, cyanobacteria and diatoms are known to produce metabolites that are toxic (Glibert *et al*. 2005). Toxic compounds produced by HABs can cause poisoning in humans through direct and indirect exposure to those toxins (Van Dolah *et al.* 2001). Direct exposure may

come through inhalation of HABs toxic aerosols. In contrast, the indirect exposure of the toxins is a result of the consumption of animals feeding on toxic microalgae such as fish and shellfish, which will then be consumed by other organisms in the food chain, resulting in bioaccumulation.

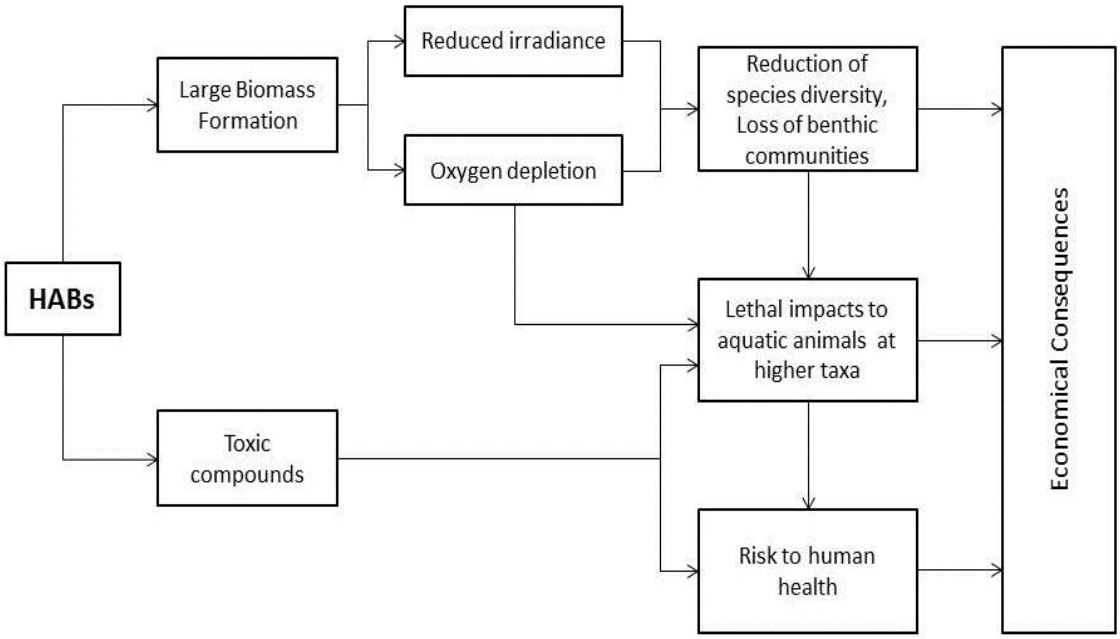


Figure 1.1 Diagram illustrating the impact of harmful algal blooms (HABs).

In addition to the health risks, HABs can also threaten human livelihood by damaging fisheries and other coastal industries (Burkholder 1998). The toxins from HABs have been reported to cause neurotoxic signs and death to the fish population (Burkholder 1998). The harmful effects of these toxins to fish populations can be both acute and chronic. Several common chronic symptoms include gill impairment and loss of swimming ability (Landsberg 2002). Considering such toxic effects, the occurrence of HABs may act as a significant regional financial burden, particularly for areas where economic activities are highly reliant on those industries. The occurrence of HABs in Oman during the year of 2008 to 2009 has been reported to have caused mortality of 200 tonnes of fish and shellfish, representing considerable financial loss (Al-Gheilani 2012). Furthermore, Anderson and co-workers (2000) reported that the economic

impact of HABs in the USA could reach $82 million annually, signifying their cumulative impacts on various sectors, such as commercial fisheries and recreational activities.

1.3 Toxins produced by HABs

Microalgae produce a diverse array of secondary metabolites, many of which are potent toxins and able to present risk to human health. In marine ecosystems, HABs toxins are produced predominantly by dinoflagellates. Human exposure to toxins produced by HABs can result in several diseases categorised according to the occurrence of their symptoms in humans. Such diseases include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), ciguatera fish poisoning (CFP), neurotoxic shellfish poisoning (NSP) and amnesic shellfish poisoning (ASP) (Table 1.2). More recently, the occurrence of another poisoning syndrome similar to DSP has been identified and termed as azaspiracid shellfish poisoning (AZP) (Twiner *et al*. 2008). Occurrences of these symptoms are the result of targeted biochemical mechanism of specific HABs toxins in mammal cells, which can be briefly classified into neurotoxins and lipophilic toxins. Neurotoxins, such as those that cause PSP, NSP, ASP, CFP and AZP, specifically target the voltage gated ion channels in mammal nerve cells, disrupting neurotransmissions (Wang 2008). Lipophilic toxins are characteristics of DSP syndrome that cause gastrointestinal illness without any apparent neurologic signs (Bialojan and Takai 1988, Haystead *et al.* 1989).

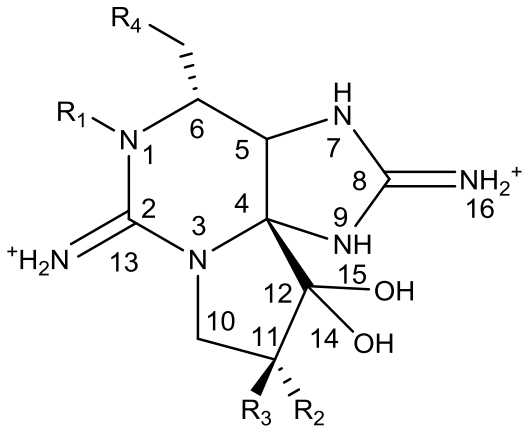
Table 1.2 Types of shellfish poisoning, their causative toxin and main producer, symptoms and mechanism of actions.

Type of shellfish poisoning	Major causative toxin (main producer)	Symptoms	Mechanism of action	References
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxins (*Karenia brevis*)	*Neurological*: numbness, tingling, pupil dilation, slurred speech, loss of coordination, partial limb paralysis. *Gastrointestinal*: nausea, vomiting, diarrhoea.	Binding of toxin on voltage-gated sodium channel (VGSC), resulting in depolarisation of nerve membrane.	Watkins *et al*. 2008.
Paralytic Shellfish Poisoning (PSP)	Saxitoxin (*Gymnodinium* sp.), Tetrodoxin (*Alexandrium* sp.)	Numbness, neurological dysfunction, complete paralysis, difficulty in breathing, respiratory arrest, cardiovascular shock or death.	Similar to NSP, toxins bind on VGSC, blocking the transmissions of nerve impulse which result on neuromuscular paralysis.	Popkiss *et al*. 1979, Andrinolo *et al*. 1999, Wang 2008.
Diarrhetic Shellfish Poisoning (DSP)	Okadaic acid (*Prorocentrum* sp., *Dinophysis* sp.)	Nausea, vomiting, diarrhoea, abdominal pain. Long term symptoms may cause promotion of tumour growth.	Inhibition of protein phosphatase 1 and 2A	Yasumoto *et al*. 1985, Cruz *et al*. 2013.
Amnesic Shellfish Poisoning (ASP)	Domoic acid (DA) (diatom *Pseudo-nitzschia* sp.)	Gastrointestinal upset, neurological dysfunction, seizures and loss of memory.	DA permeates though blood brain barrier and binds to kainite receptors. This stimulates neuronal firing which eventually cause damage to brain.	Jeffery *et al*. 2004.
Ciguatera Fish Poisoning (CFP)	Ciguatoxin, Maitotoxin (*Gambierdiscus toxicus*),	Gastrointestinal upset, neurological dysfunction, and *Dysesthesia*: abnormal sensation of temperature.	Selectively bind to VGSC.	Friedman *et al*. 2008, Louzao *et al*. 2006.
Azaspiracid Shellfish Poisoning (AZP)	Azaspiracids (AZAs) (*Azadinium spinosum*)	Gastrointestinal upset similar to DSP	The mechanism is still not fully known.	Twiner *et al*. 2008

1.3.1 Paralytic shellfish poisoning (PSP) toxins

Of all the shellfish poisoning syndromes, paralytic shellfish poisoning (PSP) appear to have the largest global distribution and subsequently most well-characterised algal toxin syndrome (Van Dolah *et al.* 2001). The occurrence of PSP was initially recognised to be endemic only in North America, Europe and Canada, however, similar cases have also been documented in areas such as South Africa, Southern Asia, Australia and New Zealand (Hallegraeff 2003). Several major causative organisms of PSP are the dinoflagellate genera *Gymnodinium*, *Gonyaulax*, *Alexandrium* and *Pyrodinium* (Shumway 1990).

The PSP toxin group consists of several tetrahydropurine compounds (Fig. 1.2) (FAO 2004). Saxitoxin (STX) has been reported to have the highest toxicity level, with the LD_{50} in mice ranging between 3-10 µg/kg body weight by intra-peritoneal (IP) injection and 236 µg/kg body weight by oral injection (Wang 2008). The production of saxitoxin has been linked to not only marine dinoflagellates, but also several species of freshwater cyanobacteria (Pearson *et al.* 2010). When ingested by mammals, PSP toxins can cause several neurological symptoms by binding to a specific site (P-loops) at the voltage gated sodium channel (VGSC) (Wang 2008). This manifests in neurological dysfunction commonly perceived as a tingling sensation on the mouth, speech disorder and muscle weakness (Franquelo *et al.* 2009).



PSP toxins	R_1	R_2	R_3	R_4
STX	H	H	H	$OCONH_2$
neoSTX	OH	H	H	$OCONH_2$
Mono-sulfated				
GTX1	OH	OSO_3^-	H	$OCONH_2$
GTX2	H	OSO_3^-	H	$OCONH_2$
GTX3	H	H	OSO_3^-	$OCONH_2$
GTX4	OH	H	OSO_3^-	$OCONH_2$
GTX5	H	H	H	$OCONHSO_3^-$
GTX6	OH	H	H	$OCONHSO_3^-$
Disulfated				
C1	H	OSO_3^-	H	$OCONHSO_3^-$
C2	H	H	OSO_3^-	$OCONHSO_3^-$
C3	OH	OSO_3^-	H	$OCONHSO_3^-$
C4	OH	H	OSO_3^-	$OCONHSO_3^-$
Decarbamoylated				
dcSTX	H	H	H	OH
dcneoSTX	OH	H	H	OH
dcGTX1	OH	OSO_3^-	H	OH
dcGTX2	H	OSO_3^-	H	OH
dcGTX3	H	H	OSO_3^-	OH
dcGTX4	OH	H	OSO_3^-	OH

Figure 1.2 Chemical structures and related variants of paralytic shellfish poisoning toxins commonly produced by dinoflagellates in marine environment (Wiese *et al.* 2010)

1.3.2 Neurotoxic shellfish poisoning (NSP) toxins

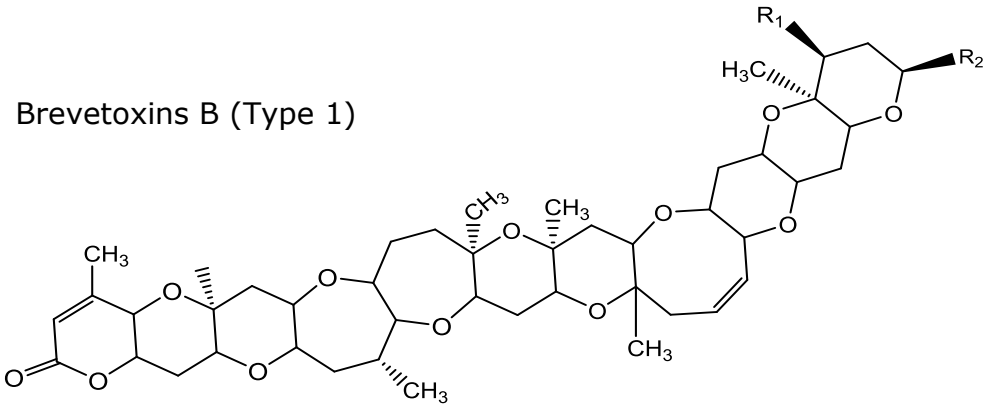
Neurotoxic shellfish poisoning (NSP) are endemic in the Gulf of Mexico, where its occurrence has been reported since 1844 (Hallegraef 2003). NSP is a disease caused by the indigestion of brevetoxins, a group of natural toxins produced by

Karenia brevis (Steidinger *et al.* 2008). Chemical structures of brevetoxins are characterised by ladder-like polycyclic ether toxins with two types of backbone structures, brevetoxin-B and brevetoxin-A (Fig. 1.3) (Wang 2008). The LD_{50} of brevetoxin in mice was reported at the levels of 0.17 mg/kg body weight by IP and 0.52 mg/kg body weight orally (Baden and Mende 1982, Kirkpatrick *et al.* 2004). Intoxication by NSP toxins results in a range of neurological and gastrointestinal symptoms and signs similar to PSP, such as headache, vomiting, pupil dilation and speech difficulty (Watkins *et al*. 2008). The neurological symptoms are caused by the binding of brevetoxins to high affinity receptors on the VGSC, resulting in enhanced influx of sodium ions (Na^+) in excitable cells (FAO 2004, Watkins *et al.* 2008). Although NSP symptoms are rarely severe and long lasting in humans, the perceived risk of NSP has led to the establishment of monitoring regimes in the coastal area of Gulf of Mexico and more recently North Carolina, USA (Morris *et al.* 1991).

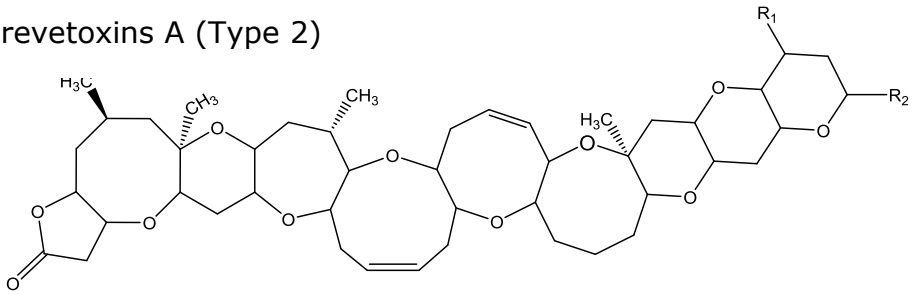
1.3.3 Ciguatera fish poisoning (CFP) toxins

The marine toxins ciguatoxins (CTXs) (Fig. 1.4) are commonly found in the liver of herbivorous fish, where they are transformed from their precursor gambiertoxins, which are produced by dinoflagellates (EFSA 2010, Louisiana Office of Public Health 2007). The disease caused by human ingestion of CTXs is commonly observed in the regions of Pacific, Caribbean and Indian oceans (EFSA 2010). However, in some of these regions, CTXs related disease is not considered to be a primary threat to the public, due to the existence of more serious health issues such as malaria, measles and malnutrition (Dalzell 2008). The ingestion of the toxins results in intermittent and recurring symptoms, such as intense itching, abnormal sensation of temperature, lethargy, and muscle and joint pain (Ting and Brown 2001, Friedman *et al.* 2008). CTXs are heat stable polyether compounds, thus their toxicity cannot be mitigated by cooking preparation (FAO 2004). The lipid-soluble compounds have LD_{50} (IP) in mice of 0.45 µg/kg and can cause illnesses in humans with oral consumption as little as 0.1 µg ciguatoxin (Wang 2008).

Brevetoxins B (Type 1)

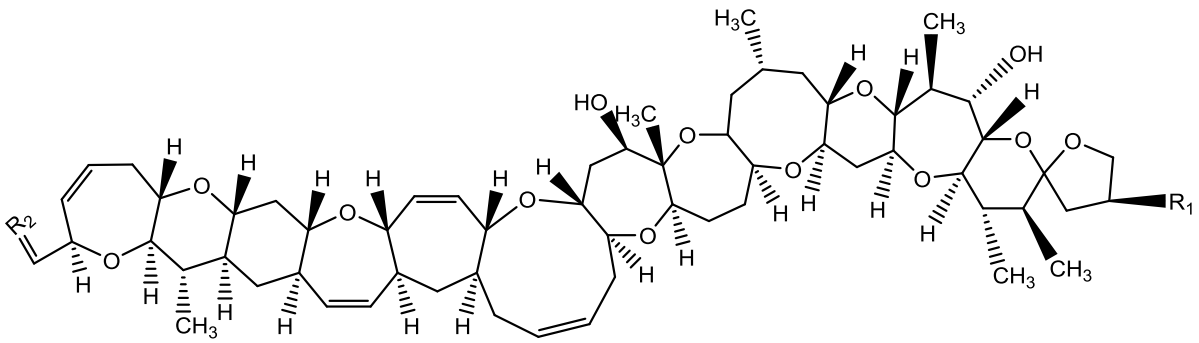


Brevetoxins A (Type 2)



NSP toxins	Type	R_1	R_2
PbTx-1	2	H	$CH_2C(CH_2)CHO$
PbTx-2	1	H	$CH_2C(CH_2)CHO$
PbTx-3	1	H	$CH_2C(CH_2)CH_2OH$
PbTx-5	1	$COCH_3$	K-ring acetate PbTx-2
PbTx-6	1	H	H-ring epoxide PbTx-2
PbTx-7	2	H	$CH_2C(CH_2)CH_2OH$
PbTx-8	1	H	CH_2COCH_2Cl
PbTx-9	1	H	$CH_2CH(CH_3)CH_2OH$
PbTx-10	2	H	$CH_2CH(CH_3)CH_2OH$

Figure 1.3 Chemical structures and species variant of brevetoxins (Van Dolah 2000 in Wang 2008).



CFP toxins	R_1	R_2
CTX-1	$HOCH_2CHOH$	OH
CTX-2/3	$HOCH_2CHOH$	H
CTX-4A/4B	$CH_2=CH$	H

Figure 1.4 Chemical structures and species variant of ciguatoxins (FAO 2004).

1.3.4 Amnesic shellfish poisoning (ASP) toxins

The amnesic shellfish poisoning is so-called due to the resulting amnesia in humans following consumption of domoic acid (Fig. 1.5) and its derivatives (FAO 2004). Besides amnesia, the ingestion of the toxins by human is also known to cause stomach pain, vomiting and disorientation (FAO 2004, Quilliam and Wright 1989). During exposure, domoic acid permeates through the blood brain barrier and selectively binds to kainite receptors, resulting in stimulation of neuronal firing and brain damage (Jeffery *et al.* 2004). The toxins effect on human memory and perception can be explained by the fact that, upon consumption, they will specifically attack the hippocampus and amygdala part of the brain within minutes to hours (Kizer 1994). Domoic acid itself, which can be categorised into an excitatory amino acid, has an LD_{50} in mice of 3.6 mg/kg (IP) (Ravn 1995).

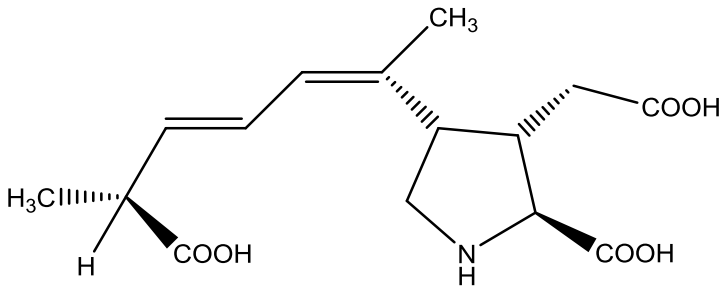


Figure 1.5 Chemical structure of domoic acid, an amnesic shellfish poisoning causative toxin (Jeffrey *et al.* 2004).

The first occurrence of ASP, associated with the consumption of restaurant meals containing blue mussels, was recorded in Canada in late 1987, where it affected more than 100 people (Quilliam and Wright 1989). Some casualties of the incident were observed to still experience severe memory loss 5 years after (Todd 1993). Although poisoning incidents at such scale have not been repeated, the analysis of seafood products from around the world has consistently identified the presence of domoic acid (Hess *et al*. 2001, Costa *et al*. 2005, James *et al*. 2005, Smith *et al*. 2006). Such findings highlight the importance of monitoring, which may be conducted in accordance to existing regulatory limits, to ensure the safety of edible marine products.

1.3.5 Azaspiracid shellfish poisoning (AZP) toxins

Azaspiracid poisoning was first reported during an outbreak occurring in 1995 in the Netherlands, associated with consumption of mussels of the species *Mytilus edulis* (James *et al*. 2003). The toxins primarily affect the gastrointestinal tract of humans and share similar symptoms with diarrhetic shellfish poisoning (DSP) (Furey et al 2010, Twiner *et al*. 2008). Although it is widely accepted that AZP toxins (Fig. 1.6) are mainly produced by the dinoflagellate *Azadinium spinosum*, the mechanisms explaining the accumulation of the toxins in shellfish are not well studied (Salas *et al*. 2011). There are approximately a dozen azaspiracid variants, which are identified as polyether containing cyclic amine and carboxylic acid groups with an LD_{50} in mice ranging of 100-700 μg/kg bodyweight (IP)

(Wang 2008, Twiner *et al.* 2008). The toxins and their derivatives have been primarily detected in mussels, with a wide ranging distribution, from the coastal areas of Africa to Europe (Magdalena *et al*. 2003, Taleb *et al*. 2006, Vale *et al*. 2008). The perceived threat of outbreaks in Europe had led the EU to establish a regulatory limit of 160 µg azaspiracid/kg of whole shellfish flesh (Twiner *et al*. 2008). However, it was commented that monitoring trends of azaspiracid toxin levels in some European regions seemed to indicate that the risks for such outbreaks were lower than diarrhetic shellfish poisoning (DSP) and paralytic shellfish poisoning outbreaks (PSP) (Vale 2004).

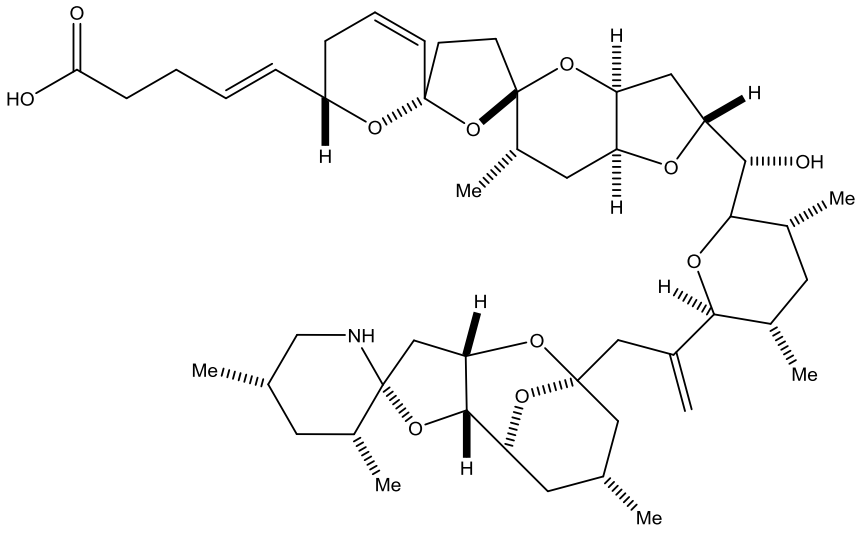


Figure 1.6 Chemical structure of a marine toxin Azaspiracid (Satake *et al.* 1998).

1.3.6 Diarrhetic shellfish poisoning (DSP) toxins

The first documentation of DSP endemic was reported in Japan in 1976 (Yasumoto *et al*. 1978 cited in Hallegraeff 2003). Compared to other shellfish poisoning, DSP is considered to have a milder effect. Gastrointestinal symptoms of DSP may occur almost immediately (3 hours) after ingestion of toxins with self-limitation of approximately 2 to 3 days (Van Dolah 2000). The primary

causative toxin of DSP is okadaic acid (OA). OA is a polyether compound mainly produced by dinoflagellates genera of *Dinophysis* and *Prorocentrum* (Figure 1.7).

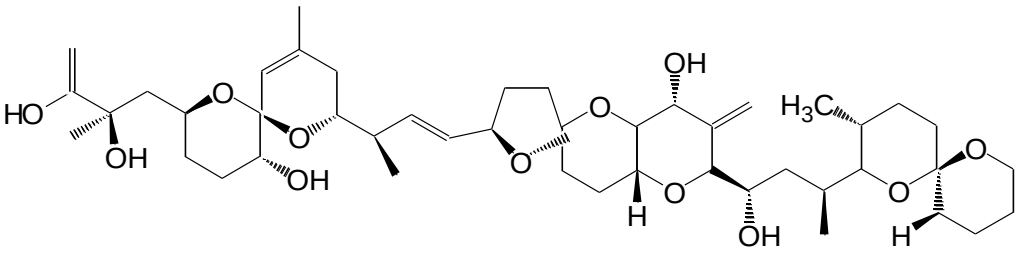


Figure 1.7 Molecular structure of main diarrhetic shellfish poisoning (DSP) toxin, okadaic acid.

Although the immediate effect of DSP is non-fatal and non-damaging to the neurological system, long term exposure to the causative toxin OA has been reported to enhance formation of tumours in mammals (Van Dolah 2000). The tumour promotion is associated with the inhibition of protein phosphatases 1 and 2A (PP1 and PP2A), with a 50% inhibitory (IC_{50}) at concentration of 20 and 0.2 nM of OA for PP1 and PP2A respectively (Suganuma *et al*. 1988, Vieytes *et al.* 2000). The transport mechanism of OA to the catalytic subunit of PP (Fig. 1.8) was demonstrated to be instigated by the dimerisation of the compound, which was facilitated by the presence of potassium ion (Cruz *et al*. 2013). Hydrophobicity of the OA dimer allows the transport through biological membranes to the cell cytoplasm, where it will be separated again to form monomers and bind PP.

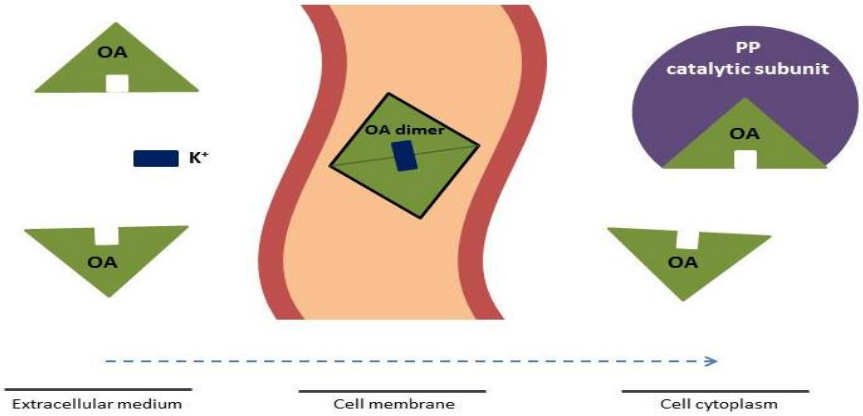
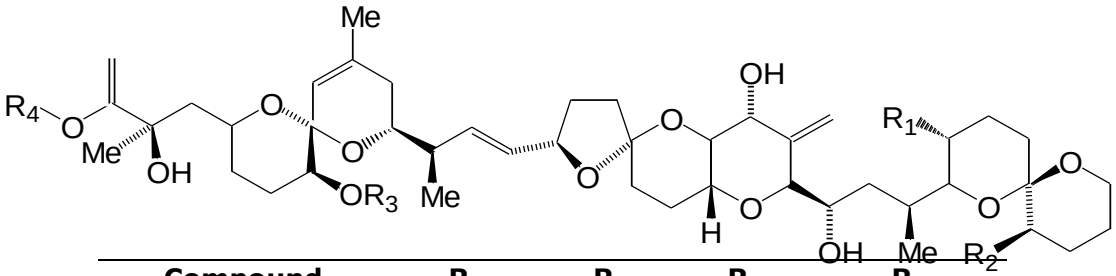
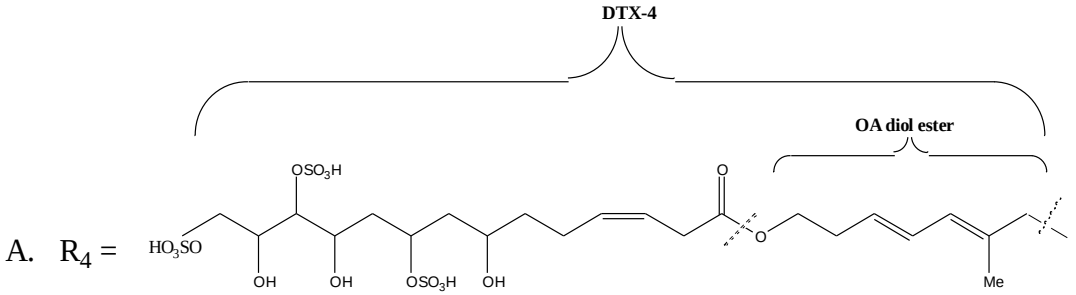


Figure 1.8 Okadaic acid transport mechanism. Two monomers bind with K^+ ion, to form a dimer with a hydrophobic surface enabling them to pass through cell membrane and bind with PP (Adapted from Cruz *et al*. 2013).

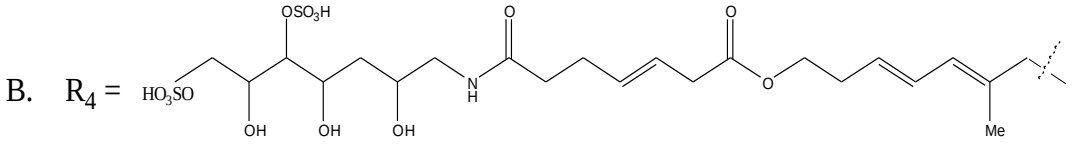
In addition to OA, DSP dinoflagellates also produce OA-relative compounds, such as dinophysistoxins (DTXs), which have also been reported to have inhibitory properties to PP1 and 2A (Hu *et al*. 1995, Larsen *et al*. 2007, Aune *et al*. 2007). DTXs are polyether ladders and were identified as derivatives of OA by comparison of their mass-spectral characteristics and molecular structures (Fig. 1.9) (Vieytes *et al.* 2000). OA and DTX1 are the two commonly identified toxins from DSP dinoflagellates, while detection of other DTXs has only been reported for specific strains. For example, DTX2 was only found for a particular *P. lima* strain isolated from Galicia, Spain (Bravo *et al.* 2001).

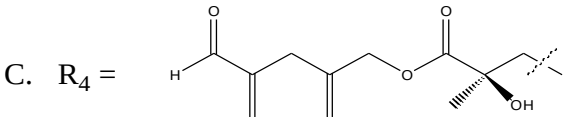


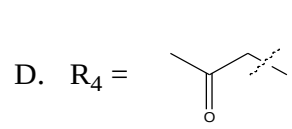
Compound	R_1	R_2	R_3	R_4
DTX-1[a]	CH_3	CH_3	H	H
DTX-2[a]	H	CH_3	H	H
DTX-3[a]	CH_3	CH_3	Acyl	H
DTX-4[a]	CH_3	H	H	A (below)
DTX-5a[a]	CH_3	H	H	B (below)
DTX-6[b]	CH_3	H	H	C (below)
Norokadanone[b]	CH_3	H	H	D (below)
OA diol-ester[b]	CH_3	H	H	E (below)



DTX-5a







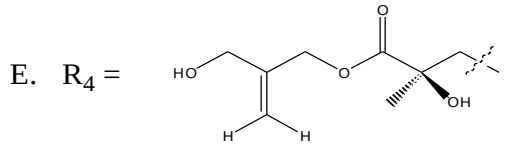
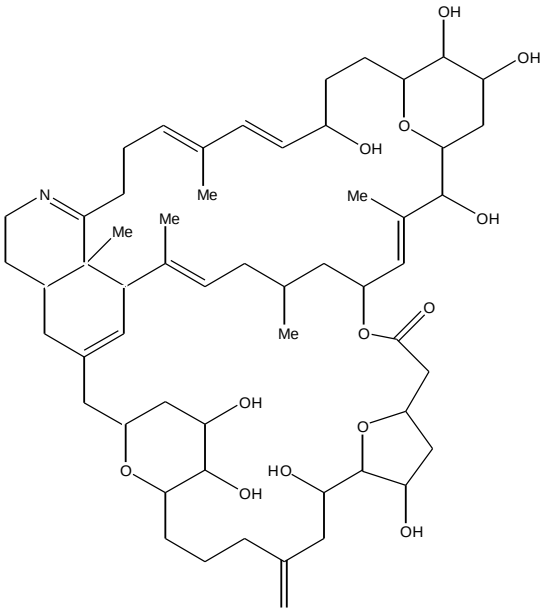
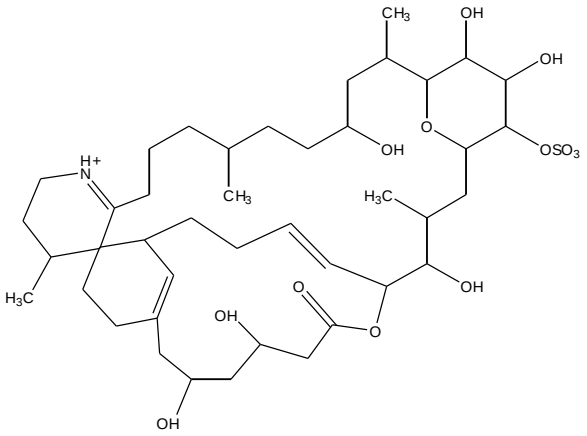


Figure 1.9 Structures of okadaic-acid-relative compounds (Source: [a]Rein and Borrone 1999; [b]Fernandez *et al.* 2003).

Apart from OA and DTXs, the dinoflagellate genera *Prorocentrum* are also known to produce several other toxic compounds, of which their functional activities are still unexplored. These include: Prorocentrolide (Fig. 1.10), a toxic nitrogenous macrocycle, reported in a study by Torigoe and co-workers (1988) using *P. lima* strain isolated from Okinawa, Japan; Spiro-prorocentrimine, a novel macrocyclic lactone found in a Taiwanese strain of *Prorocentrum sp.* (PM08) and reported in Lu *et al.* (2001); the novel 17-membered ring macolides Formosalides A and Formosalides B (Fig. 1.11) which showed cytotoxicity against cancer cells (CCRF-CEM human T-cell acute lyphoblastic leukemia cells and/or DLD-1 human colon adenocarcinoma cells) *in vitro*, were found from Taiwan strain of *Prorocentrum* sp. (Lu *et al.* 2009); and Prorocentin, isolated from *P. lima* and first described by Lu and co-workers (2005).

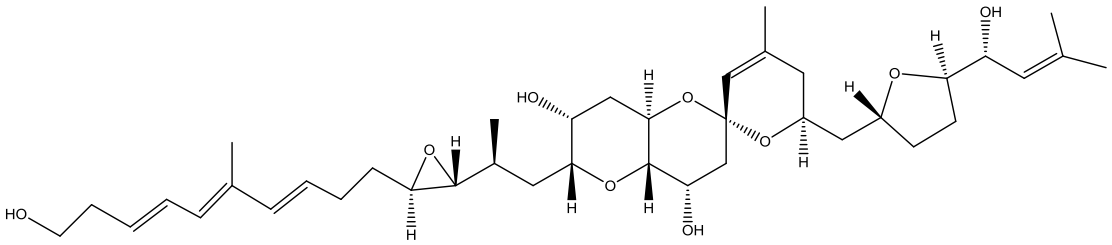


Prorocentrolide A [a]

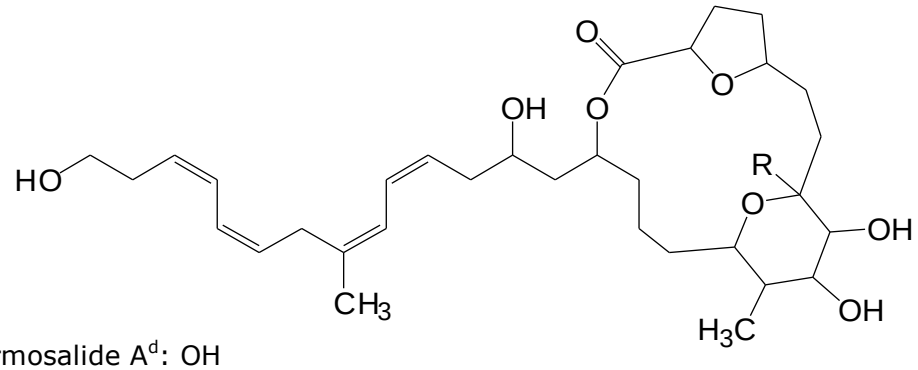


Spiro-prorocentrimine[b]

Figure 1.10 Chemical structures of prorocentrolide and spiro-prorocentrimine isolated from dinoflagellate genera of *Prorocentrum* ([a] Torigoe *et al.* 1988, [b] Lu *et al.* 2001).



Prorocentin[c]



Formosalide A[d]: OH
Formosalide B[d]: OCH_3

Figure 1.11 Chemical structures of Prorocentin and Formasalides A and B. ([c]Lu *et al*. 2005, [d]Lu *et al.* 2009).

1.4 Regulation of shellfish toxins

Shellfish poisoning is considered to be a major issue mainly due to persistent and very stable toxins associated with it. To date, the preparation procedures that can reduce the toxicity or remedy that can eliminate the symptoms do not exist (Glibert *et al*. 2005). Therefore, in order to control the widespread occurrence of these shellfish poisoning events, constant monitoring of HABs and potential toxin contamination of shellfish and fish products for human consumption is essential. As a part of the monitoring process, various regulation agencies have established limits for HABs toxin content in shellfish products, such as EU Regulation 853/2004 (Table 1.3). Such monitoring efforts require a continuous supply of authentic standards. However, maintaining continuous supply of these standards

remains a challenge, particularly due to the difficulty in obtaining a single purified standard. Further research optimising production of toxin standards is required to ensure constant monitoring effort, which eventually assists in minimising the human health risks of HABs.

Table 1.3 Maximum permitted levels of biotoxins in seafood products (EU Regulation 853/2004).

Biotoxin	**Limit per kilogram of seafood products**
PSP	800 µg saxitoxin equivalent
ASP	20 mg DA equivalent
DSP	160 µg OA equivalent
Pectenotoxins (PTXs)	160 µg OA equivalent
Yessotoxins (YTXs)	1 mg
AZP	160 µg Azaspiracid equivalent

1.5 Biotechnological significance of dinoflagellate

Despite their toxicity to humans, some of the compounds produced by dinoflagellates are observed to exhibit novel bioactivities against fungal, bacterial and animal cells, hence their potential benefits for pharmaceutical and therapeutic applications as well as research tools (Camacho *et al.* 2007). Some of the well identified applications of these bioactive compounds include anti-cancer/anti-tumour, antifungal and medical research tool (Table 1.4).

The first example is amphidinolides, the secondary metabolites of dinoflagellate *Amphidinium* sp. that have been found to have strong bioactivity against various cancer and tumour cells in mammals (Kobayashi and Tsuda 2004). Amongst amphidinolides, type H and N of amphidinolide are considered to be the lead compounds of new anticancer drugs due to their remarkable cytotoxicity on

human tumour cell lines, with IC_{50} at concentration of 0.00052 μg mL^{-1} for type H and 0.00006 μg mL^{-1} for type N (Kobayashi and Tsuda 2004). The second example is gambieric acid, produced by *Gambierdiscus toxicus*, which has been shown to have potent antifungal toxicity (Nagai *et al*. 1992, Morohashi *et al*. 2000). Similarly, antifungal properties were also found for amphidinol from *A. klebsii* (Satake *et al*. 1991).

Table 1.4 Potential applications of bioactive compounds produced by dinoflagellates.

Bioactive compounds	Main Producer	Study
Anti-cancer/Anti-tumour		
Amphidinolides	*Amphidinium* sp.	Kobayashi and Tsuda 2004
Antifungal		
Gambieric acid	*Gambierdiscus toxicus*	Nagai *et al*. 1992 Morohashi *et al*. 2000
Amphidinol	*Amphidinium* sp.	Satake *et al*. 1991
Research tool		
Okadaic acid	*Prorocentrum lima*	Mestrovic and Pavela-Vrancic 2003 Fernandez *et al.* 2002
Maitotoxin	*G. toxicus*	Gusovsky *et al*. 1990 cited in Murata and Yasumoto 2000
Yessotoxin	*Prorocentrum reticulatum, Gonyaulax spinifera, Lingolodinium polyedrum.*	Korsnes 2012

Several other compounds that are toxic to human have also been shown to be useful for medical research in understanding human cells organisations. An example of this is the potent PP1 and 2A inhibitor, okadaic acid. The inhibitory activity of OA renders it an important research tool in understanding various

cellular processes related to protein phosphatases (PP) (Mestrovic and Pavela-Vrancic 2003, Fernandez *et al.* 2002). Another example is the neurotoxic compound maitotoxins produced by *G. toxicus*. Maitotoxin is capable of causing neurological dysfunction by stimulating the increase of calcium influx in neuronal cells (Takahashi *et al*. 1982). This toxic property can then be utilised as a tool in understanding various calcium-dependent mechanisms in animal cells (Gusovsky *et al*. 1990 cited in Murata and Yasumoto 2000).

In addition to the secondary metabolites, dinoflagellates produce several characteristic carotenoid pigments that have been revealed to have pharmacological significance. Several novel properties that have been identified from these pigments include antioxidant and anti-cancer/anti-tumour properties (Nishino 1998, Naguib 2000, Nagendraprabhu and Sudhandhiran 2011). An example of this is peridinin (Fig. 1.4) and fucoxanthin that have been shown to have high efficacy in suppressing the development of skin tumours in mice (Nishino 1998, Hosokawa *et al*. 2004). This suppression mechanism was further explained by the ability of pigments to induce apoptosis (cell death) in cancer cells (Yoshida *et al*. 2007). While peridinin has not been commercially produced, astaxanthin, another dinoflagellate pigment exhibiting similar properties, is now commercially available (Kurihara *et al*. 2002).

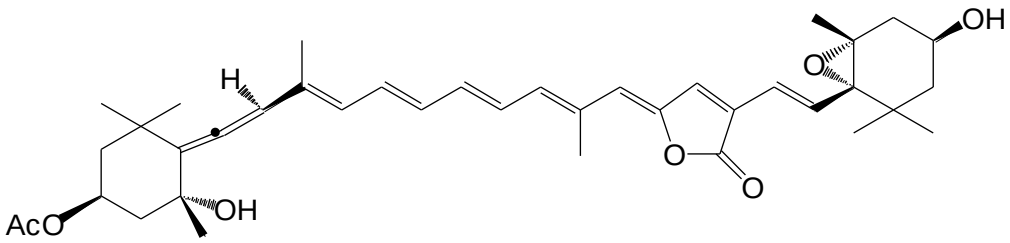


Figure 1.12 Molecular structure of peridinin (Nishino 1998), a major carotenoid pigment from dinoflagellate.

Despite the revelations of potential benefits, only a few of those bioactive compounds have been successfully developed for research and commercial applications. The main limitation, preventing the realisation of the applications of

dinoflagellate compounds, is their lack of availability (Minh *et al*. 2005, Camacho *et al.* 2007). This is also reflected in their current market price, for example okadaic acid is priced at approximately £800 per milligram (Sigma-Aldrich, UK, 2014). Therefore, further development for the application of dinoflagellate compounds would have to rely on a suitable production method that can ensure adequate and consistent supply of products.

Several methods are available to obtain purified compounds. These include chemical synthesis, natural bloom extraction and cultivation of producer organism. Chemical synthesis procedures for bioactive compounds from dinoflagellate have been demonstrated widely in previous studies (e.g. Forsyth *et al*. 1997, Ley *et al.* 1998, Kadota *et al*. 2003, Furuichi *et al*. 2004, Olpp and Brückner 2006, Vaz *et al*. 2007). This approach, however, can be unsuitable as it often involves many steps with low gain of yield (Nicholas and Phillips 2007), and many of the compounds have extremely complex structures that make synthesis unfeasible (Minh *et al*. 2005). The second alternative is collecting natural communities of toxic microalgae as demonstrated in studies by James and co-workers (1999). However, utilisation of this approach may provide unpredictable yield with inconsistent toxin variants and amounts of compounds. The third approach is *in-situ* large-scale harvesting and extraction by pumping method as exampled by Rundberget and co-workers (2007). Nevertheless, considering the small quantity of purified compound obtained (2.7 mg OA, 1.3 DTX2, 1.8 PTX2 from 18 hours pumping of 8000 L seawater), this method can be unsustainable. The limiting factors of these methods signify the potential convenience and practicality of laboratory cultivation methods for compound production.

1.6 Cultivation of dinoflagellate

Attempts have been made in laboratory scale studies to find a robust system for the cultivation of dinoflagellate and production of their bioactive compounds. Several examples of this have been demonstrated for AZAs production from *A. spinosum* (Jauffrais *et al*. 2012), and PSP toxins production from *A. tamerense* and from *A. minutum* (Hsieh *et al.* 2001, Parker *et al.* 2002, Hu *et al.* 2006). However, only a few systems are suitable to be deployed at large scale or readily

scalable. Scale up of culture system for dinoflagellate still faces difficulty in obtaining optimum production, mainly caused by cell fragility and slow growth (Camacho *et al*. 2007 and 2007b). Sound understanding of parameters influencing the cultivation process is required, which will assist with the design of robust, appropriate and scalable reactors.

Several parameters, such as nutrition and environmental conditions (e.g. light, temperature and gas supply), are known to govern the growth and metabolic processes in algal cell (Fig 1.13), and influence the optimum production of desired compounds. Manipulating these parameters can affect the whole cellular function, hence their utilisation to ensure that the metabolic pathways synthesise the preferred compound (Rosenberg *et al*. 2008). Manipulation of nutritional and environmental parameters can be performed simply by modifying the composition of culture medium and controlling culture conditions.

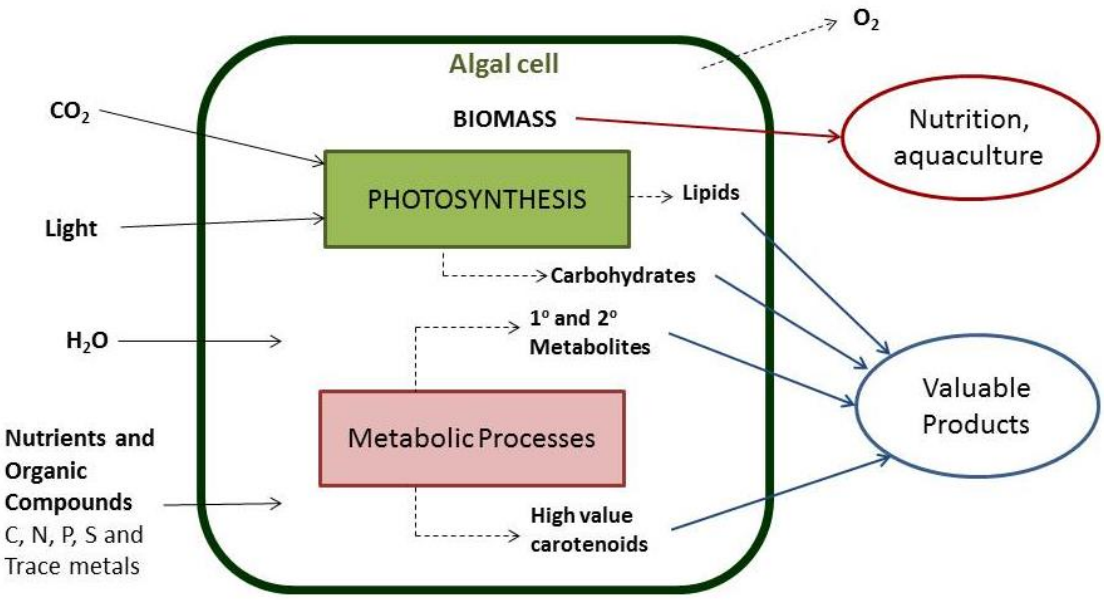


Figure 1.13 Synthesis of novel products from microalgal cell and their potential applications (Modified from Rosenberg *et al*. 2008).

1.6.1 Medium composition

Several macro nutrients such as nitrogen (N), phosphorous (P) and carbon (C) are essential for the growth of any microalgae. They are utilised as the building blocks in the metabolism of the microalgae. Limitations of these elements were reported to affect both growth and secondary metabolites production from many dinoflagellate cultures (McLachlan *et al*. 1994, Lim *et al.* 2010; Vanucci *et al.* 2010, Varkitzi *et al.* 2010).

In respect to growth in a culture system, many studies tend to agree that dinoflagellate are sensitive to limited availability of N and P. The addition of these elements below the typical level suggested in commonly used growth media, appeared to suppress division rates (McLachlan *et al*. 1994, Vanucci *et al.* 2010, Rodriguez *et al.* 2009, Lim *et al.* 2010). However, N and P can be toxic to dinoflagellate when they exist in excessive amount (Rodriguez *et al.* 2009, Varkitzi *et al*. 2010). It appears that the level of tolerance to limitations and supplementations of N and P is species (and probably strain) specific. Therefore, prior investigation of media modification is essential. Moreover, the type of the chemical species that these elements form has also been observed to affect growth and nutrient intake in the culture. For example, nitrogen in the form of NO_3^- is more preferred by *A. catanella* (Armi *et al.* 2011) while NH_4^+ is preferred by *P. lima* and *A. minutum* (Pan *et al.* 1999, Maguer *et al.* 2007, Varkitzi *et al.* 2010).

With regard to the effects of N and P on bioactive metabolites production, dinoflagellate appears to be sensitive to limitations and changes in compositions (ratio). Several studies reported the increase of intracellular secondary metabolites is commonly observed with both limitations and changes in ratio of N and P in medium (Lim *et al.* 2010, Vanucci *et al.* 2010, Varkitzi *et al.* 2010). For example, in a study by Vanucci *et al*. (2010) it was clearly shown that both N and P limitations significantly enhanced the production of secondary metabolites OA and DTX1 from *P. lima* culture by 2-3 fold. A similar finding was also reported by Varkitzi and co-workers (2010) (Table 1.5).

The mechanism underlying how N and P limitations affect the synthesis of these compounds is still unclear. Nevertheless, it can be assumed that this may be caused by the alternation of cell metabolism that occurred during culture adaptation process to stress conditions (Lim *et al.* 2010, Vanucci *et al.* 2010, Varkitzi *et al.* 2010). In contrasts to N and P, the effect of carbon (C) and trace metals availability – with the possible exception of iron (Fe) – on the cultivation of dinoflagellate and its production of bioactive compounds has not been studied extensively. Such deficiency indicates a clear requirement for further research to enable the optimisation of production of bioactive compounds.

Table 1.5 Bioactive compound production under N- and P- limitations. Illustrated by taking example of OA production in *P. lima* culture.

Culture and medium	Nutrient limitation	Toxin production	Reference
P. lima (Spain), f/2 medium	**Control (N:P=24**) $N(NO_3^-)$=883 µM $P(PO_4^{3-})$=36.2 µM	> 6.00 pg OA/cell	Varkitzi *et al.* 2010
	P-limited (N:P=80) $N(NO_3^-)$=1450 µM $P(PO_4^{3-})$=18.1 µM	11.27±3.3 pg OA/cell	
P. lima (Italy), f/2 medium	**Control (N:P=24)** $N(NO_3^-)$=883 µM $P(PO_4^{3-})$=36.3 µM	6.87-6.69 pg OA/cell 0.12-0.13 pg DTX-1/cell	Vanucci *et al.* 2010
	P-limited (N:P=488) $N(NO_3^-)$=883µM $P(PO_4^{3-})$=1.81µM	15.80 pg OA/cell 0.32 pg DTX-1/cell	
	N-limited (N:P=0.49) $N(NO_3^-)$=17.7µM $P(PO_4^{3-})$=36.3µM	12.50 pg OA/cell 0.39 pg DTX-1/cell	

1.6.2 Culturing conditions

The effects of environmental factors on growth and production of bioactive compounds have been evaluated in several studies (Siu *et al.* 1997, Parkhill and Cembella 1999, Lim and Ogata 2005, Lim *et al.* 2006). Key factors influencing growth and adaptation of dinoflagellates include light and temperature conditions.

Similar to other phototrophic organisms, dinoflagellates require energy from light for their biosynthesis processes. Light may influence the biochemical composition in an algal cell through a process termed as photoacclimation (Hu 2004). Photoacclimation is a response to stimuli or change in light conditions, according to which microalgal cells may alter its metabolic processes as part of their adaptation process (Vonshak and Torzillo 2004). Different species may acclimatise to different light level. For example, species that inhabit the bottom layer of aquatic system (benthic) are well adapted to very low light level (<10 μmol photons m^{-2} s^{-1}) compared to those that live free-floating (Gomez *et al.* 2009). Light intensity provided at below or above normal growth conditions has been demonstrated to be capable of altering cellular compositions of pigment, carbohydrate and lipid (Hu 2004). Sufficient light energy is also important in secondary metabolites production from dinoflagellate, as the compounds produced from photosynthesis (e.g. amino acid, acetate, ATP) would affect the production of secondary metabolites (Hsieh *et al.*, 2001).

Light and dark cycle is also an important factor influencing biochemical composition. Photosynthesis in microalgae occurs in both 1) light phase, the transformation of light energy into chemical energy, and 2) dark phase, the carbon fixation reactions (Iverson 2006 cited in Jacob-Lopes *et al*. 2009). It has been observed that exploitation of light and dark cycles for the cultivation of microalgae results in the increase of photosynthetic rate, hence the higher productivity of the culture (Grobbelaar 1994, Janssen *et al*. 2000 and 2001, Jacob-Lopes *et al*. 2009).

In addition to light conditions, temperature has also been shown to influence the growth and production of compounds from algal cells. Similar with the adaptation to light, dinoflagellate tolerance and acclimatisation to different temperatures are strain specific, and may depend on their original habitat. For example, the growth of tropical dinoflagellates was found to have lower tolerance to low temperature level (below 20^{o}C) compared to those isolated from temperate regions (McLachlan *et al.* 1994, Etheridge and Roesler 2005, Lim *et al*. 2006).

Temperature was also found to affect photosynthetic processes in microalgae by altering the light harvesting efficiency (Davidson 1991). Changes in photosynthesis would result in modification of cellular membrane lipid composition and content (Hu 2004). Several common changes observed during alteration of temperature level include: 1) higher accumulation of lipid content per cell at temperature below optimal range, and 2) higher production of reactive oxygen species (ROS), hence the higher accumulation of carotenoid pigments at temperature above optimum range (Tjahjono *et al.* 1994, Hu 2004). All of these suggest that production of a particular compound of interest may be enhanced by the modification of temperature in culturing system. This approach has been demonstrated previously for the production of carotenoid pigments, for which elevation of temperature above normal growth level can be exploited to stimulate higher astaxanthin production (Tjahjono *et al.* 1994).

1.6.3 Culture system

Designing a suitable culture system for dinoflagellate can be particularly difficult in ensuring that the cell viability is maintained. Dinoflagellates are known to be more vulnerable to fluid turbulence than other microalgae (Hu *et al.* 2006). Excessive shear forces may cause physical damage to cell such as flagella impairment that result in loss of swimming ability (Thomas & Gibson 1990, Camacho *et al.* 2007b). Cell tolerance to fluid turbulence is not only influenced by the intensity of shear force, but also its period and interval (Gibson and Thomas 1995). Shear force that occurs intermittently may alleviate the adverse effect of fluid turbulence as it allows cells to recover when turbulent motion ceased (Thomas and Gibson 1990).

The mechanism of shear-induced damage on cells has been hypothesised in several studies (Han *et al*. 2004 and Jeffers *et al*. 2007). It was explained that hydrodynamic shear forces may alter metabolic processes and induce higher production of intracellular reactive oxygen species (ROS). This leads to damage on lipid membranes which are important for maintaining integrity of cellular organelles (Rodriguez *et al.* 2009). Cell damage induced by ROS due to shear forces can be alleviated by addition of antioxidant agents (e.g. ascorbic acid,

carboxymethyl cellulose and Pluronic F68) in the culture medium (Rodriguez *et al*. 2009 and 2011).

Despite this, fluid turbulence is an important aspect in a bioreactor to maintain mass transfer within the system. It ensures good mixing of nutrients and gas exchange, and prevents deposition of cells at the bottom of the reactor. Therefore, selecting the cultivation system should reflect on a design that is able to provide sufficient mass transfer whilst maintaining cell viability of the culture. Various configurations of culturing system for dinoflagellates have been described previously (Table 1.6). Each system has limitations and advantages, and selection would depend on the characteristics of culture organism and other parameters such as capital and maintenance costs.

Table 1.6 Designs of photobioreactor utilised for dinoflagellate cultivation

System	Descriptions	Reference
Couette flow	Culture volume is placed in between two plates which move at different velocities resulting in the steady flow of the fluid and constant shear stress distribution. *Advantage*: uniform shear stress. *Disadvantage*: difficulties in supplying gas (aeration) without disturbing the flow field, settling and flotation in the long term operation.	Thomas and Gibson 1990, Gibson and Thomas 1995, Camacho *et al.* 2007b.
Shaken tank/flask	Tanks/vessels are placed in a rotating table or attached to a device that allows the culture to be shaken orbitally. *Advantage*: aeration can be supplied without disturbing the field flow, quantifiable hydrodynamic shear stress, easy to determine gas/liquid mass transfer area and foaming free. *Disadvantage*: may be difficult to scale-up the system.	Various sources cited in Camacho *et al*. 2007b.
Stirred tank	Hydrodynamics within the system is maintained by rotating a blade propeller at a defined and controllable speed. *Advantage*: Fluid turbulence can be monitored and controlled. *Disadvantage*: Efficiency may be reduced during scale-up process.	Rodriguez *et al*. 2007 and 2010, Camacho *et al.* 2011.
Aerated flask	Fluid turbulence is created by sparging with air (often enriched with CO_2). The level of turbulence in the culture can be controlled by the rate of air and CO_2 supply. *Advantage*: CO_2 depletion in medium can be manipulated without adding shear force to the culture. *Disadvantage*: rupture of bubbles at the surface of liquid may cause additional shear force that may affect cell viability.	Camacho *et al.* 2007b, Hu *et al.* 2006.
Airlift system	Turbulence is created by vertically circulating air across the height of the tube with the use of air duct and draft tube. *Advantage*: higher mass transfer and smaller bubbles compared to aerated flask, simple design and low cost.	Xu *et al*. 2002, Hu *et al.* 2006.

1.7 Aim and objectives of thesis

Despite their biotechnological significance, dinoflagellates are still one of the least explored groups of microalgae. Most existing studies tend to focus on the identification of the compounds and the elucidation of their potential for pharmaceutical and research applications. The realisation of these applications is currently hampered by the limited availability of these compounds. Production of dinoflagellate bioactive compounds can be sourced through several methods such as chemical synthesis and bloom collection. However, as previously discussed (section 1.5), these approaches are unreliable due to low yield and inconsistent compound profiles. A cost effective and reliable method of bulk culturing could be an important development in this field.

Studies on the optimisation of high value compounds production from cultured dinoflagellate have been described only for several species, such as *Alexandrium* spp., *Protoceratium* sp.*,* and *Azadinium* sp. (Hsieh *et al*. 2001, Camacho *et al*. 2011, Jauffrais *et al*. 2012). These studies demonstrated that scale up of cultivation process (ranging between 15 to 100 L) for dinoflagellates could be feasibly done. However, as indicated by those studies, optimum cultivation conditions can be strain specific. Therefore, efforts to optimise the production of any high value compounds from dinoflagellate have to rely on research specifically designed to identify how a particular producer organism can be cultivated optimally.

This study specifically focuses on investigations to optimise cultivation conditions of the dinoflagellate species *P. lima* (strain CCAP 1136/11), a main producer of OA and its relative compounds. As previously described, OA poses a serious threat to human health due to its contamination on seafood products, hence routine monitoring is required. The availability of high quality standards are needed to enable calibration and ensure the accuracy of the monitoring, which assist in the management of public health. Besides this, the potent inhibitors properties of OA and DTX1 on protein phosphatase 1 and 2 make them strong candidates for their exploitation as research tool in medical studies. In addition, *P. lima* is also a good source of the carotenoid pigment peridinin valued for its

anti-tumour and anti-cancer properties. These indicate the commercialisation potential of cultivating *P. lima*.

This study aims to optimise the cultivation conditions for *P. lima* to enhance the production of OA and other high value compounds. Several key objectives were included:

1. Establishment of suitable analytical methods to quantify *P. lima* growth through manual and automated cell counting methods. An appropriate LC/MS method to allow the detection and quantification of OA, DTX1 and peridinin will also be developed.

2. Determination of optimum cultivation conditions to promote optimum growth and yields of OA, DTX1 and peridinin from *P. lima*. Several growth parameters (nutrients, light, temperature and carbon sources) were selected according to literature studies and manipulated in laboratory settings to observe growth behaviour and compound production.

3. The subsequent research objective focused on evaluation of culture physiological state such as age and density, both of which were deemed to be important in determining cell *fitness* during the cultivation process.

4. Ultimately, several types of cultivation systems, consisting of flow and bubble lift reactors, were identified from existing literature and further modified to suit the aim of the study.

1.8 References

Admiraal, W., Peletier, H. and Brouwer, T., 1984. The seasonal succession patterns of diatom species on an intertidal mudflat: an experimental analysis. **Oikos**, 42 (1), 30-40

Al-Gheilani, Hameed. 2012, February 8th. *Harmful algal blooms (HABs) monitoring programmes in Omani waters*. [PowerPoint slides]. Presented at Expert Workshop on Red Tides and HABs: Impact on Desalination Plants, Oman. Available from: http://www.medrc.org/download/red_tide_workshop/12.%20Dr.%20Hamed%20Al%20Gheilani.pdf

Anderson, D. M., Hoagland, P., Kaoru, Y., White, A. W. 2000. Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. *Woods Hole Oceanog. Inst. Tech. Rept*., WHOI-2000-11.

Anderson, D. M., Glibert, P. M., Burkholder, J. M. 2002. Harmful algal blooms and eutrophication: Nutrient sources, composition and consequences. **Estuaries**, 25 (4b), 704-726.

Anderson, D. M. 2007. The Ecology and Oceanography of Harmful Algal Blooms: Multidisciplinary Approaches to Research and Management. IOC Technical Series 74, UNESCO. IOC/2007/TS/74

Anderson, D. M., Burkholder, J. M., Cochlan, W. P., Glibert, P. M., Gobler, C. J., Heil, C. A., Kudela, R. M., Parsons, M. L., Jack Rensel, J. E., Townsend, D. W., Trainer, V. L., Vargo, G. A. 2008. Harmful algal blooms and eutrophication: examining linkages from selected coastal regions of the United States. **Harmful Algae**, 8 (1), 39-53.

Andrinolo, D., Michea, L. F., Lagos, N. 1999. Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfish poison (PSP), in cats. **Toxicon**, 37 (3), 447-464.

Armi, Z., Milandri, A., Turki, S., Hajjem, B. 2011. *Alexandrium catenella* and *Alexandrium tamarense* in the North Lake of Tunis: bloom characteristics and the occurrence of paralytic shellfish toxin. **African Journal of Aquatic Science**, 36 (1), 47-56.

Aune, T., Larsen, S., Aasen, J. A., Rehmann, N., Satake, M., Hess, P. 2007. Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. **Toxicon**, 49 (1), 1-7.

Baden, D. G., Mende, T. J. 1982. Toxicity of two toxins from the Florida red tide marine dinoflagellate, *Ptychodiscus brevis*. **Toxicon**, 20 (2), 457-461

Bialojan, C., Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. **Biochemical Journal**, 256, 283-290.

Bravo, I., Fernandez, M. L., Ramilo, I., Martinez, A. 2001. Toxin composition of the toxic dinoflagellate *Prorocentrum lima* isolated from different locations along the Galician coast (NW Spain). **Toxicon,** 39: 1537-1545.

Burkholder, J. M. 1998. Implications of harmful microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. **Ecological applications**, 8 (sp1), S37-S62.

Camacho, F. G., Rodriguez, J.J.G., Miron, A. S., Garcia, M. C. C., Belarbi, E. H., Chisti, Y., Molina Grima, E. 2007. Biotechnological significance of toxic marine dinoflagellates. **Biotechnology Advances**, 25 (2), 176-194

Camacho, F. G., Rodriguez, J.J.G., Miron, A. S., Garcia, M. C. C., Belarbi, E. H., Molina Grima, E. 2007b. Determination of shear stress thresholds in toxic dinoflagellates cultured in shaken flasks: Implications in bioprocess engineering. **Process Biochemistry**, 42(11), 1506-1515.

Camacho, F. G., Rodriguez, J.J.G., Miron, A. S., Belarbi, E. H., Chisti, Y., Molina Grima, E. 2011. Photobioreactor scale-up for a shear-sensitive dinoflagellate microalga. **Process Biochemistry**, 46, 936-944.

Cloern, James E., 2001. Our evolving conceptual model of the coastal eutrophication problem. **Marine Ecology Progress Series**, 210, 223-253

Conley, D. J., Paerl, H. W., Howarth, R. W., Boesch, D. F., Seitzinger, S. P., Havens, K. E., Lancelot, C., Likens, G. E. 2009. Controlling eutrophication: nitrogen and phosphorus. **Science**, 323 (5917), 1014-1015.

Costa, P.R., Rosa, R., Duarte-Silva, A. Brotas, V., Sampayo, M. A. M . 2005. Accumulation, transformation and tissue distribution of domoic acid, the amnesic shellfish poisoning toxin, in the common cuttlefish, *Sepia officinalis*. **Aquatic Toxicology**, 74 (1) 82-91

Cruz, P. G., Norte, M., Creus, A. H., Fernández, J. J., Daranas, A. H. 2013. Self-Association of Okadaic Acid: Structural and Pharmacological Significance. **Marine drugs**, 11 (6), 1866-1877.

Dalzell, P. 2008. Management of ciguatera fish poisoning in the South Pacific. New Caledonia: South Pacific Commission.

Davison, I. R. 1991. Environmental effects on algal photosynthesis: temperature. **Journal of Phycology**, 27 (1), 2-8.

de Emiliani, Maria O.G., 1993. Seasonal succession of phytoplankton in a lake of the Parana river floodplain, Argentina. **Hydrobiologia,** 264, 101-114

Diaz, R. J., Rosenberg, R. 2008. Spreading dead zones and consequences for marine ecosystems. **Science**, 321 (5891), 926-929.

EFSA Panel on Contaminants in the Food Chain. 2010. Scientific opinion on marine biotoxins in shellfish – emerging toxins: ciguatoxin group. **European Food Safety Authority Journal**, 8 (6), 1627-1665.

Etheridge, S. M., Roesler, C. S. 2005. Effects of temperature, irradiance, and salinity on photosynthesis, growth rates, total toxicity, and toxin composition for *Alexandrium fundyense* isolates from the Gulf of Maine and Bay of Fundy. **Deep Sea Research Part II: Topical Studies in Oceanography**, 52 (19), 2491-2500.

FAO. 2004. Marine Biotoxins. Rome: Food and Agriculture Organization of the United Nations. Available from: http://www.fao.org/docrep/007/y5486e/y5486e00.HTM.

Fernandez, J. J., Candenas, M. L., Souto, M. L., Trujillo, M. M., Norte, M. 2002. Okadaic acid, useful tool for studying cellular processes. **Current medicinal chemistry**, 9 (2), 229-262.

Fernandez, J. J., Suarez-Gomez, B., Souto, M. L., Norte, M. 2003. Identification of new okadaic acid derivatives from laboratory cultures of *Prorocentrum lima*. **Journal of Natural Products**, 66, 1294-1296.

Field, C. B., Behrenfeld, M. J., Randerson, J. T., Falkowski, P. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. **Science**, 281, (5374), 237-240.

Fong, P., and Zedler, J. B., 1993. Temperature and light effects on the seasonal succesion of algal communities in shallow coastal lagoons. **Journal of Experimental Marine Biology and Ecology** 171 (2), 259-272

Forsyth, C. J., Sabes, S. F., Urbanek, R. A. 1997. An efficient total synthesis of okadaic acid. **Journal of the American Chemical Society**, 119 (35), 8381-8382.

Franquelo, F. C., Picón, F. D., De Boom, R. C. C., Andrade, A. T., García, G. Q. 2009. Paralytic shellfish poisoning. **Emergencias**, 21, 306-308.

Friedman, M. A., Fleming, L. E., Fernandez, M., Bienfang, P., Schrank, K., Dickey, R., Bottein, M. Y., Backer, L., Ayyar, R., Weisman, R., Watkins, S., Granade, R., Reich, A. 2008. Ciguatera fish poisoning: treatment, prevention and management**. Marine Drugs**, 6 (3), 456-479.

Furey, A., O'Doherty, S., O'Callaghan, K., Lehane, M., James, K. J. 2010. Azaspiracid poisoning (AZP) toxins in shellfish: Toxicological and health considerations. **Toxicon**, 56 (2), 173-190

Furuichi, N., Hara, H., Osaki, T., Nakano, M., Mori, H., Katsumura., S. 2004. Stereocontrolled total synthesis of a polyfunctional carotenoid, peridinin. **Journal of Organic Chemistry**, 69, 7949-7959.

Gamier, J., Billen, G., Coste, M. 1995. Seasonal succession of diatoms and Chlorophyceae in the drainage network of the Seine River: Observations and modeling. **Limnology and Oceanography**, 40 (4), 750-765.

Gibson, C. H., Thomas, W. H. 1995. Effects of turbulence intermittency on growth inhibition of a red tide dinoflagellate, *Gonyaulaz polyedra* Stein. **Journal of Geophysical Research,** 100 (C12), 24, 841-24,846.

Glibert, P. M., Anderson, D. M., Gentien, P. Granéli, E., Sellner, K. G. 2005. The global, complex phenomena of harmful algal blooms. **Oceanography**, 18 (2), 130-141.

Glycomar. 2013. Microalgae in drug discovery [Online]. Available from: http://www.glycomar.com/marine_natural_products_microalgae.htm

Grobbelaar, J. U. 1994. Turbulence in mass algal cultures and the role of light/dark fluctuations. **Journal of Applied Phycology**, 6 (3), 331-335.

Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase. **Phycologia**, 32 (2), 79-99.

Hallegraeff, G. M. 2003. Harmful algal blooms: a global overview. In: G. M. Hallegraef, D. M. Anderson, A. D. Cembella (Eds), *Manual of Harmful Marine Microalgae*. Paris: UNESCO, pp. 25-49.

Han, R. B., Yuan, Y. J. 2004. Oxidative burst in suspension culture of *Taxus cuspidate* induced by a laminar shear stress in short-term. **Biotechnology Progress**, 20 (2), 507-513.

Havens, K. E., Fukushima, T., Xie, P., Iwakuma, T., James, R. T., Takamura, N., Hanazato, T., Yamamoto, T. 2001. Nutrient dynamics and the eutrophication of shallow lakes Kasumigaura (Japan), Donghu (PR China), and Okeechobee (USA). **Environmental Pollution**, 111 (2), 263-272.

Havens, K. E., Schelske, C. L. 2001. The importance of considering biological processes when setting total maximum daily loads (TMDL) for phosphorus in shallow lakes and reservoirs. **Environmental Pollution**, 113 (1), 1-9.

Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P., Hardie, D. G. 1989. Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. **Nature**, 337 (6202), 78-81.

Heisler, J., Glibert, P. M., Burkholder, J. M., Anderson, D. M., Cochlan, W., Dennison, W. C., Dortch, Q., Gobler, C. J., Heil, C. A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H. G., Sellner, K., Stockwell, D. A., Stoecker, D. K., Suddleson, M. 2008. Eutrophication and harmful algal blooms: A scientific concencus. **Harmful Algae**, 8, 3-13.

Hess, P., Gallacher, S., Bates, L. A., Brown, N., Quilliam, M. A. 2001. Determination and confirmation of the amnesic shellfish poisoning toxin, domoic acid, in shellfish from Scotland by liquid chromatography and mass spectrometry. **Journal of AOAC International**, 84 (5), 1657-1667.

Holligan, P. M., Maddock, Linda., Dodge, J. D. 1980. The distribution of dinoflagellates around the British Isles in July 1977: A multivariate analysis. **Journal of the Marine Biological Association of the UK**, 60, 851-867.

Hosokawa, M., Kudo, M., Maeda, H., Kohno, H., Tanaka, T., Miyashita, K. 2004. Fucoxanthin induces apoptosis and enhances the antiproliferative effect of the PPARγ ligand, troglitazone, on colon cancer cells. **Biochimica et Biophysica Acta (BBA)-General Subjects**, 1675 (1), 113-119.

Hsieh, D. P. H., Wang, D., Chang, G. H. 2001. Laboratory bioproduction of paralytic shellfish toxins in dinoflagellates. **Advances in Applied Microbiology,** 49, 85-110.

Hu, T., Curtis, J. M., Walter, J. A., Wright, J. L. C. 1995. Identification of DTX-4, a new water soluble phosphatase inhibitor from the toxic dinoflagellate *Prorocentrum lima*. **Journal of the Chemical Society, Chemical Communication**, 5, 597-595.

Hu, Q. 2004. Environmental effects on cell composition. In: Amos Richmond (Ed), *Handbook of Microalgal Culture, Biotechnology and Applied Phycology*. Oxford, UK: Blackwell Publishing. pp. 83-93.

Hu, H., Shi, Y., Cong, W. 2006. Improvement in growth and toxin production of *Alexandrium tamarense* by two-step culture method. **Journal of Applied Phycology,** 18, 119-126.

Jacob-Lopes, E., Scoparo, C. H. G., Lacerda, L. M. C. F., Franco, T. T. 2009. Effect of light cycles (night/day) on CO_2 fixation and biomass production by microalgae in photobioreactors. **Chemical engineering and Processing: Process Intensification**, 48 (1), 306-310.

James, K. J., Bishop, A. G., Healy, B. M., Roden, C., Sherlock, I. R., Twohig, M., Draischi, R., Giannetti, L., Lucentini, L. 1999. Efficient isolation of the rare diarrhoeic shellfish toxin, dinophysistoxin-2, from marine phytoplankton. **Toxicon**, 37 (2), 343-357.

James, K. J., Moroney, C., Roden, C., Satake, M., Yasumoto, T., Lehane, M., Furey, A.. 2003. Ubiquitous 'benign' alga emerges as the cause of shellfish contamination responsible for the human toxic syndrome, azaspiracid poisoning. **Toxicon**, 41(2), 145-151.

James, K.J., Gillman, M., Amandi, M.F. 2005. Amnesic shellfish poisoning toxins in bivalve molluscs in Ireland. **Toxicon**, 46(8), 852-858.

Janssen, M., Janssen, M., de Winter, M., Tramper, J., Mur, L. R., Snel, J., Wijffels, R. H. 2000. Efficiency of light utilization of *Chlamydomonas reinhardtii* under medium-duration light/dark cycles. **Journal of biotechnology**, 78 (2), 123-137.

Janssen, M., Slenders, P., Tramper, J., Mur, L. R., Wijffels, R. 2001. Photosynthetic efficiency of *Dunaliella tertiolecta* under short light/dark cycles. **Enzyme and microbial technology**, 29 (4), 298-305.

Jauffrais, T., Kilcoyne, J., Séchet, V., Herrenknecht, C., Truquet, P., Hervé, F., Bérard, J. B., Nulty, C., Taylor, S., Tillmann, U., Miles, C. O., Hess, P. 2012. Production and isolation of azaspiracid-1 and-2 from *Azadinium spinosum* culture in pilot scale photobioreactors. **Marine drugs**, 10 (6), 1360-1382.

Jeffers, P., Kerins, S., Baker, C. J., Kieran, P. M. 2007. Generation of reactive oxygen and antioxidant species by hydrodynamically stressed suspensions of *Morinda citrifolia*. **Biotechnology Progress**, 23 (1), 138-145.

Jeffery, B., Barlow, T., Moizer, K., Paul, S., Boyle, C. 2004. Amnesic shellfish poison. **Food and chemical toxicology**, 42 (4), 545-557.

Julius, M. L. 2007. Why sweat the small stuff: the importance of microalgae in Hawaiian stream ecosystems. **Biology of Hawaiian Streams and Estuaries**, 3, 183-193.

Kadota, I., Takamura, H., Sato, K., Ohno, A., Matsuda, K., Yamamoto, Y. 2003. Total synthesis of gambierol. **Journal of the American Chemical Society**, 125 (1), 46-47.

Kirchman, D. L. 2012. *Processes in microbial ecology*. New York: Oxford University Press.

Kirkpatrick, B., Fleming, L. E., Squicciarini, D., Backer, L. C., Clark, R., Abraham, W., Benson, J., Cheng, Y. S., Johnson, D., Pierce, R., Zaias, J., Bossart, G. D., Baden, D. G. 2004. Literature review of Florida red tide: implications for human health effects. **Harmful Algae**, 3 (2), 99-115.

Kizer, K.W. 1994. Domoic acid poisoning. **Western Journal of Medicine,** 161(1): 59-60.

Kobayashi, J. I., Tsuda, M. 2004. Amphidinolides, bioactive macrolides from symbiotic marine dinoflagellates. **Natural product reports**, 21 (1), 77-93.

Korsnes, M. S. 2012. Yessotoxin as a tool to study induction of multiple cell death pathways. **Toxins**, 4 (7), 568-579.

Kurihara, H., Koda, H., Asami, S., Kiso, Y., Tanaka, T. 2002. Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. **Life sciences**, 70 (21), 2509-2520.

Landsberg, J. H. 2002. The effects of harmful algal blooms on aquatic organisms. **Reviews in Fisheries Science**, 10 (2), 113-390.

Larsen, K., Petersen, D., Wilkins, A. L., Samdal, I. A., Sandvik, M., Rundberget, T., Goldstone, D., Arcus, V., Hovgaard, P., Rise, F., Rehmann, N., Hess, P., Miles, C. O. 2007. Clarification of the C-35 stereochemistries of dinophysistoxin-1 and dinophysistoxin-2 and its consequences for binding to protein phosphatase. **Chemical research in toxicology**, 20 (6), 868-875.

Legendre, L. 1990. The significance of microalgal blooms for fisheries and for the export of particulate organic carbon in oceans. **Journal of Plankton Research**, 12 (4), 681-699.

Ley, S. V., Humphries, A. C., Eick, H., Downhamn, R., Ross, A. R., Boyce, R. J., Pavey, J. B., Pietruszka, J. 1998. Total synthesis of the protein phosphatase inhibitor okadaic acid. **Journal of the Chemical Society, Perkin Transaction 1**, 23, 3907-3911.

Lim, P. T., Ogata, T. 2005. Salinity effect on growth and toxin production of four tropical *Alexandrium species*. **Toxicon**, 45, 699-710.

Lim, P. T., Leaw, C. P., Usup, G., Kobiyama, A., Koike, K., Ogata, T. 2006. Effects of light and temperature on growth, nitrate uptake, and toxin production of two tropical dinoflagellates: *Alexandrium tamiyavanichii* and *Alexandrium* minutum (Dinophyceae). **Journal of Phycology**, 42 (4), 786-799.

Lim, P. T., Leaw, C. P., Kobiyama, A., Ogata, T. 2010. Growth and toxin production of tropical *Alexandrium minutum* Halim (Dinophyceae) under various nitrogen to phosphorous ratios. **Journal of Applied Phycology**, 22, 203-210.

Louisiana Office of Public Health. 2007. Ciguatera poisoning. Louisiana, USA: Department of Health and Hospitals.

Louzao, M., Cagide, E., Vieytes, M. R., Sasaki, M., Fuwa, H., Yasumoto, T., Botana, L. M. 2006. The sodium channel of human excitable cells is a target for gambierol. **Cellular Physiology and Biochemistry**, 17 (5-6), 257-268.

Lu, C. K., Lee, G. H., Huang, R., Chou, H. N. 2001. Spiro-prorocentrimine, a novel macrocyclic lactone from a benthic *Prorocentrum sp.* of Taiwan. **Tetrahedron Letters**, 42, pp. 1713-1716.

Lu, C. K., Chou, H. N., Lee, C. K., Lee, T. H. 2005. Prorocentrin, a new polyketide from the marine dinoflagellate *Prorocentrum lima*. **Organic Letters**, 7 (18), pp. 3893-3896.

Lu, C. K., Chen, Y. M., Wang, S. H., Wu, Y. Y., Cheng, Y. M. 2009. Formosalides A and B, cytotoxic 17-membered ring macrolides from a marine dinoflagellate *Prorocentrum* sp. **Tetrahedron Letters**, 50, pp. 1825-1827.

Magdalena, A.B., Lehane, M., Krys, S., Fernández, M. L., Furey, A., James, K. J. 2003. The first identification of azaspiracids in shellfish from France and Spain. **Toxicon** 42 (1): 105-108.

Maguer, J. F., L'Helguen, S., Madec, C., Labry, C., Le Corre, P. 2007. Nitrogen uptake and assimilation kinetics in *Alexandrium minutum* (Dynophyceae): Effect of N-limited growth rate on nitrate and ammonium interactions. **Journal of Phycology**, 43 (2), 295-303.

McLachlan, J. L., Marr, J. C., Conlon-Keily, A., Adamson, A. 1994. Effects of nitrogen concentration and cold temperature on DSP-toxin concentrations in the dinoflagellate *Prorocentrum lima* (prorocentrales, dinophyceae). **Natural toxins**, 2 (5), 263-270.

Mestrovic, V., Pavela-Vrancic, M. 2003. Inhibition of alkaline phosphatase activity by okadaic acid, a protein phophatase inhibitor. **Biochimie**, 85, 647-650.

Minh, C. V., Kiem, P. V., Dang, N. H. 2005. Marine natural products and their potential application in the future. **ASEAN Journal on Science and Technology for Development**, 22 (4), 297-311.

Moncheva, S., Gotsis-Skretas, O., Pagou, K., Krastev, A. 2001. Phytoplankton blooms in Black Sea and Mediterranean coastal ecosystems subjected to anthropogenic eutrophication: similarities and differences. **Estuarine, Coastal and Shelf Science**, 53 (3), 281-295.

Moore, S. K., Trainer, V. L., Mantua, N. J., Parker, M. S., Laws, E. A., Backer, L. C., Fleming, L. E. 2008. Impacts of climate variability and future climate change on harmful algal blooms and human health. **Environmental Health**, *7* (2), S4.

Morohashi, A., Satake, M., Nagai, H., Oshima, Y., Yasumoto, T. 2000. The absolute configuration of Gambieric Acids A–D, potent antifungal polyethers, isolated from the marine dinoflagellate *Gambierdiscus toxicus*. **Tetrahedron**, 56 (46), 8995-9001.

Morris, P. D., Campbell, D. S., Taylor, T. J., Freeman, J. I. 1991. Clinical and epidemiological features of neurotoxic shellfish poisoning in North Carolina. **American Journal of Public Health**, 81 (4), 471-474.

Murata, M., Yasumoto, T. 2000. The structure elucidation and biological activities of high molecular weight algal toxins: maitotoxin, prymnesins and zooxanthellatoxins. **Natural product reports**, 17 (3), 293-314.

Nagai, H., Murata, M., Torigoe, K., Satake, M., Yasumoto, T. 1992. Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus toxicus*. **The Journal of Organic Chemistry**, 57 (20), 5448-5453.

Nagendraprabhu, P., Sudhandiran, G. 2011. Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFkB and COX-2. **Investigational New Drugs**, 29 (2), 207-224.

Naguib, Y. M. 2000. Antioxidant activities of astaxanthin and related carotenoids. **Journal of agricultural and food chemistry**, 48(4), 1150-1154.

Nicholas, G. M., Phillips, A. J. 2007. Marine natural products: synthetic aspects. **Natural product reports**, 24 (1), 87-108.

Nishino, H. 1998. Cancer prevention by carotenoids. **Mutation research**, 402, 159-163.

Olpp, T., Brückner, R. 2006. Total synthesis of the light-harvesting carotenoid peridinin. **Angewandte Chemie International Edition**, 45, 4023-4027.

Parker, N.S., Negri, A. P., Frampton, D. M. F., Rodolfi, L., Tredici, M. R., Blackburn, S. I. 2002. Growth of the toxic dinoflagellate *Alexandrium minutum* (Dynophyceae) using high biomass culture system. **Journal of Applied Phycology,** 14, 313-324.

Parkhill, J.P., Cembella, A. D. 1999. Effects of salinity, light and inorganic nitrogen on growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. **Journal of Plankton Research**, 21 (5), 939-955.

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B. 2010. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. **Marine Drugs**, 8 (5), 1650-1680.

Peperzak, L., Colijn, F., Gieskes, W. W. C., Peeters, J. C. H. 1998. Development of the diatom-Phaeocystis spring bloom in the Dutch coastal zone of the North Sea: the silicon depletion versus the daily irradiance threshold hypothesis. **Journal of Plankton Research**, 20(3), 517-537.

Peperzak, L. 2003. Climate change and harmful algal blooms in the North Sea. **Acta Oecologica**, 24, S139-S144.

Popkiss, M. E. E., Horstman, D. A., Harpur, D. 1979. Paralytic shellfish poisoning. **South African Medical Journal**, 55, 1017-1023.

Quilliam, M. A. and Wright, J. L.C , 1989. The amnesic shellfish poisoning mystery. **Analytical Chemistry**, 61(18), 1053A - 1060A

Rein, K. S., Borrone, J. 1999. Polyketides from dinoflagellates: origins, pharmacology and biosynthesis. **Comparative Biochemistry and Physiology Part B,** 124, 117-131.

Ravn, H. 1995. HAB Publication Series Vol 1: Amnesic Shellfish Poisoning (ASP). **IOC Manuals and**

Guides, 31 (1), 15pp

Richardson, T.L, Gibson, C.E., Heaney, I. 2000. Temperature, growth and seasonal sucession of phytoplankton in Lake Baikal, Siberia. **Freshwater Biology,** 44, 431-440

Rodriguez, J. J. G., Miron, A. S., Garcia, M. del C. C., Belarbi, E. H., Camacho, F. G., Chisti, Y., Grima, M. G. 2009. Macronutrients requirements of the dinoflagellate *Protoceratium reticulatum*. **Harmful Algae,** 8, 239-246.

Rosenberg, J. N., Oyler, G. A., Wilkinson, L., Baterbaugh, M. J. 2008. A green light for engineered algae: redirecting metabolism to fuel a biotechnology production. **Current Opinion in Biotechnology**, 19, 430-436.

Rundberget, T., Sandvik, M., Larsen, K., Pizzaro, G. M., Reguera, B., Castberg, T., Gusad, E., Loader, J. I., Rise, F., Wilkins, A. L., Miles, C. O. 2007. Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. **Toxicon,** 50 (7), 960-970

Salas, R., Tillmann, U., John, U., 2011. The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels. **Harmful Algae**, 10(6), 774-783.

Satake, M., Murata, M., Yasumoto, T., Fujita, T., Naoki, H. 1991. Amphidinol, a polyhydroxy-polyene antifungal agent with an unprecedented structure, from a marine dinoflagellate, *Amphidinium klebsii*. **Journal of the American Chemical Society**, 113 (26), 9859-9861.

Satake, M., Ofuji, K., Naoki, H., James, K. J., Furey, A., McMahon, T., Silke, J., Yasumoto, T. 1998. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, Mytilus edulis. **Journal of the American Chemical Society**, *120*(38), 9967-9968.

Schindler, D. W. 2006. Recent advances in the understanding and management of eutrophication. **Limnology and Oceanography**, 51 (1), 356-363.

Seitz, R. D., Dauer, D. M., Llansó, R. J., Long, W. C. 2009. Broad-scale effects of hypoxia on benthic community structure in Chesapeake Bay, USA. **Journal of Experimental Marine Biology and Ecology**, 381, S4-S12.

Shumway, S. E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. **Journal of the World Aquaculture Society**, 21 (2), 65-104.

Siu, G. K. Y., Young, M. L. C., Chan, D. K. O. 1997. Environmental and nutritional factors which regulate population dynamics and toxin production in the dinoflagellate *Alexandrium catanella*. **Hydrobiologia**, 352, 117-140.

Smith, E. A., Papapanagiotou, E. P., Brown, N. A., Stobo, L. A., Gallacher, S., Shanks, A. M. 2006. Effect of storage on amnesic shellfish poisoning (ASP) toxins in king scallops (*Pecten maximus*). **Harmful Algae**, 5 (1), 9-19.

Sommer, U. 1985. Seasonal succession of phytoplankton in Lake Constance. **BioScience,** 35 (6), 351-357

Steidinger, K. A., Landsberg, J. H., Flewelling, L. J., Kirkpatrick, B. A. 2008. Toxic dinoflagellate. In: P. J. Walsh, S. Smith, L. Fleming, H. Solo-Gabriele, W. H. Gerwick (Eds*). Oceans and human health, risk and remedies from the seas*. USA: Academic Press. pp. 239-256.

Sterner, Robert W., 1989. Resource competition during seasonal succesion toward dominance by cyanobacteria. **Ecology**, 70(1), 229-245

Suganuma, M., Fujiki, H., Suguri, H., *et al.* 1988. Okadaic acid: An additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. **Proceeding of the National Academy of Sciences USA**, 85, 1768-1771.

Takahashi, M., Ohizumi, Y., Yasumoto, T. 1982. Maitotoxin, a Ca^{2+} channel activator candidate. **Journal of Biological Chemistry**, 257 (13), 7287-7289.

Taleb, H., Vale, P., Amanhir, A., Benhadouch, A., Sagou, R., Chafik, A. 2006. First detection of

azaspiracids in mussels in North West Africa. **Journal of Shellfish Research,** 25 (3), 1067-1070.

Thomas, W. H., Gibson, C. H. 1990. Quantified small-scale turbulence inhibits a red tide dinoflagellates, *Gonyaulaz polyedra* Stein. **Deep-Sea Research,** 37 (10), 1583-1593.

Ting, J. Y. S., Brown, A. F. T. 2001. Ciguatera poisoning: A global issue with common management problems. **European Journal of Emergency Medicine**, 8, 295-300.

Todd, E. C. D. 1993. Domoic acid and amnesic shellfish poisoning - a review. **Journal of Food Protection,** 56 (1), 69-83.

Torigoe, K., Murata, M., Yasumoto, T., Iwashita, T. 1988. Prorocentrolide, a toxic nitrogenous macrocycle from a marine dinoflagellate, Prorocentrum lima. **Journal of the American Chemical Society**, 110 (23), 7876-7877.

Twiner, M. J., Rehmann, N., Hess, P., Doucette, G. J. 2008. Azaspiracid shellfish poisoning: a review on the chemistry, ecology, and toxicology with an emphasis on human health impacts. **Marine drugs**, 6 (2), 39-72.

Vale, P. 2004. Is there a risk of human poisoning by azaspiracids from shellfish harvested at the Portuguese coast?. **Toxicon,** 44 (8), 943-947.

Vale, P., Bire, R., Hess, P. 2008. Confirmation by LC-MS/MS of azaspiracids in shellfish from the Portuguese north-western coast. **Toxicon** 51 (8), 1449-1456

Van Dolah, F. M. 2000. Marine algal toxins: origins, health effects, and their increased occurrence. **Environmental health perspectives**, 108 (Suppl 1), 133-141.

Van Dolah, F. M., Roelke, D., Greene, R. M. 2001. Health and ecological impacts of harmful algal blooms: risk assessment needs. **Human and Ecological Risk Assessment: An International Journal**, 7 (5), 1329-1345.

Van Straten, G. and Keesman, K. J., 1991. Uncertainty propagation and speculation in projective forecasts of environmental change: a Lake-Eutrophication Example. **Journal of Forecasting,** 10: 163-190.

Vaz, B., Domínguez, M., Alvarez, R., de Lera, A. R. 2007. Total synthesis of peridinin and related C_{37}-Norcarotenoid butenolides. **Chemistry A European Journal**, 13, 1273-1290.

Vanucci, S., Guerrini, F., Milandri, A., Pistocchi, R. 2010. Effects of different levels of N- and P-deficiency on cell yield, okadaic acid, DTX-1, protein and carbohydrate dynamics in the benthic dinoflagellate *Prorocentrum lima*. **Harmful Algae**, 9, 590-599.

Varkitzi, I., Pagou, K., Granéli, E., Hatzianestis, I., Pyrgaki, C., Pavlidou, A., Montesano, B., Economou-Amili, A. 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. **Harmful Algae,** 9, 304-311.

Vieytes, M. R., Louzao, M. C., Alfonso, A., *et al.* 2000. Mechanism of Action and Toxicology. In: Luis M. Botana (Ed.), *Seafood and Freshwater Toxin: Pharmacology, Physiology, and Detection.* NY: Marcel Dekker Inc, pp. 281-301.

Vonshak, A., Torzillo, G. 2004. Environmental stress physiology. In: Amos Richmond (Ed), *Handbook of Microalgal Culture, Biotechnology and Applied Phycology*. Oxford, UK: Blackwell Publishing. pp. 57-82.

Wang, D. Z. 2008. Neurotoxins from marine dinoflagellates: A brief review. **Marine Drugs**, 6 (2), 349-371.

Watkins, S. M., Reich, A., Fleming, L. E., Hammond, R. 2008. Neurotoxic shellfish poisoning. **Marine Drugs**, 6 (3), 431-455.

Webster, I. T., Hutchinson, P. A. 1994. Effect of wind on the distribution of phytoplankton cells in lakes revisited. **Limnology and Oceanography**, 39 (2), 365-373.

Wiese, M., D'Agostino, P. M., Mihali, T. K., Moffitt, M. C., Neilan, B. A. (2010). Neurotoxic alkaloids: saxitoxin and its analogs. **Marine drugs**, 8 (7), 2185-2211.

Xu, Z., Baicheng, Z., Yiping, Z., Zhaoling, C., Wei, C., Fan, O. 2002. A simple and low-cost airlift photobioreactor for microalgal mass culture. **Biotechnology Letters**, 24, 1767-1771.

Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G. K., Clardy, J. 1985. Diarrhetic shellfish poisoning. **Tetrahedron,** 41 (6), 1019-1025.

Yoshida, T., Maoka T., Das, S. K., Kanazawa, K., Horinaka, M., Wakada, M., Satomi, Y., Nishino, H., Sakai, T. 2007. Halocynthiaxanthin and Peridinin Sensitize Colon Cancer Cell Lines to Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand. **Molecular Cancer Research,** 5 (6), 615-625.

Zingone, A., Enevoldsen, H. O. 2000. The diversity of harmful algal blooms: a challenge for science and management. **Ocean and Coastal Management**, 43 (8), 725-748.

CHAPTER 2

Development of Methods for the Monitoring of Growth and Metabolite Production from *Prorocentrum lima* in Batch Culture

2.1 Introduction

Prorocentrum lima is a benthic dinoflagellate that has been found to have a world-wide distribution, from temperate to tropical waters (Bouaïcha *et al.* 2001, Bravo *et al*. 2001, Heredia-Tapia *et al.* 2002, Nascimento *et al.* 2005, Vale *et al*. 2009). The cells of *P. lima* are typically oblong to ovate in shape with an average size of 30-40 µm in length and 20-30 µm in width (Bouaïcha *et al.* 2001, Nascimento *et al.* 2005). *P. lima* is a slow growing organism (typical cell division of 0.10 to 0.15 per day) that is often found attached to sediments and macroalgae (Pan *et al.* 1999, Lawrence *et al.* 2000). *P. lima* is known to produce okadaic acid (OA) and dinophysistoxins (DTXs) that are the causative agents of diarrhetic shellfish poisoning (DSP). It was initially believed that these toxins were mainly produced only by the dinoflagellate species *Dinophysis* spp., until the occurrence of blooms on the Canadian coast confirmed another major causative organism, *P. lima* (Bravo *et al.* 2001). The organism *P. lima* is commonly used not only for studies related to DSP toxins but also as a reliable source of OA and DTX1 standards as it is difficult to obtain pure laboratory culture of *Dinophysis* spp. (Bravo *et al.* 2001).

In order to ensure the sustainable production of DSP toxins, which are needed for monitoring of DSP, continual production of *P. lima* biomass has to be performed and consistently monitored. Growth monitoring of culture is essential for optimising production and useful to determine the time for sub-culturing and harvesting. Knowledge on this should facilitate the optimum and consistent production of desired compounds between batches of culture. Besides this, growth monitoring can also be the first measure to indicate problems in the culturing process.

Growth can be easily determined by counting the number of cells in the culture. Several methods available to do so include manual counting by microscope and automated counting by cell counter or flow cytometer. Manual counting with microscope offers advantages of simplicity in procedure and inexpensive equipment (LeGresley and McDermott 2010). The method, however, is very laborious and time consuming for analysis of a large number of samples. On the other hand, automated counting provides time-efficient analysis but requires

much higher costs compared to microscopy counting. As such, either of these methods should be selected in respect to the purpose of analysis and budget constraints.

In the existing counting methods, it is essential to ensure sample homogeneity in order to count the cells individually (Lee and Shen 2004). Flow cytometry obtains the number of counted cells based on the number of light incidents detected. Each light incident is measured every time a single cell passes through the beam (Marie *et al*. 2005). Nevertheless, an aggregate of cells passing through the beam will still be automatically translated by the detector as a single cell. Therefore, the presence of cell aggregates in a sample can underestimate the actual number of cells, potentially producing inaccurate data. This problem can be avoided with pre-treatment of samples to dis-aggregate the cell clump by sonication, trypsinisation or sample dilution (Lee and Shen 2004, Marie *et al*. 2005). Evaluation of appropriate methods for both sample treatment and cell count analysis is required prior to study, and this will assist in selection of procedure suitable for handling large quantity of samples.

Another important consideration in the maintenance of marine cultures is the consistent provision of natural seawater (NSW). However, its continuous supply may be considered to be inconvenient for areas located away from the coasts. Artificially prepared marine medium (ASW) can provide a practical alternative to ensure sustainable cultivation and production. Its composition can also be controlled and easily modified (Harrison and Berges 2005). However, many compounds contained in NSW, such as trace organics, are difficult to quantify and reproduce in ASW. Thus, the direct replacement of NSW with ASW is not always a straightforward process as some microalgal species may have specific requirements of trace nutrients. This is significant as cultures poorly adapted to medium are prone to morphological changes, and in some cases the alterations are irreversible (Lorenz *et al*. 2005). It is therefore prudent to test and compare the performance of species of interest grown with ASW and NSW.

The production of high value metabolites not only relies on the perpetual maintenance of producer organisms but also on the processing of harvested cells. Appropriate handling and processing of harvested cells will ensure good

quality products. One of the important factors for this process is the extraction procedure. The efficacy of several extraction procedures for the recovery of compounds from dinoflagellate cells has been demonstrated widely in many studies (e.g. Quilliam and Ross 1996, Frassanito *et al*. 2005, Paz *et al*. 2007). Several common solvents used for extraction include methanol and acetone. The recovery efficiency of these solvents appeared to depend largely on characteristics of each compound and the addition of a cell disruption procedure prior to extraction, for example freeze-thawing and sonication. Nevertheless, few of the existing procedures can be easily adopted for regular and large scale processing.

Considering the aforementioned challenges, the investigation described in this chapter was specifically aimed at developing cultivation and monitoring methods suitable for *P. lima.* The development of standardised methods will ensure that the approach for the production of DSP toxins and peridinin from this organism will be consistent, controllable and efficient. Moreover, this will enable comparison of results between studies. Currently such comparisons cannot be robustly performed because the methods used vary widely or are impractical for handling a large quantity of samples. In addition to this, there have been no concerted attempts to develop consistent cultivation methods particularly for *P. lima*.

2.2 Materials and Methods

2.2.1 Chemicals

Chemicals used in this study were analytical-reagent grade unless otherwise stated and obtained from Fisher Scientific (Loughborough, UK). HPLC grade acetonitrile and methanol, and formic acid were supplied from Fisher Scientific and Rathburn (Walkerburn, UK), respectively. Sodium iodide and leucine-enkephalin were obtained from Sigma. Standards of DSP toxins and peridinin were purchased from: Enzo Life Science (Lausen, Switzerland) for okadaic acid, National Research Council Canada (Nova Scotia, Canada) for DTX1 and DTX2, and DHI Lab Products (Hørsholm, Denmark) for peridinin.

2.2.2 Maintenance of *Prorocentrum lima* CCAP 1136/11 culture

Marine algae *Prorocentrum lima* strain 1136/11 was obtained from Culture Collection for Algae and Protozoa (CCAP) (Oban, Scotland). This strain, also known as PL2V (Bravo *et al*. 2001), was isolated from Vigo, Spain. Compared to other strains isolated from colder waters, this particular strain was better suited to the typical room temperature (21-22^{o}C) that would be used in the laboratory cultivation. Cultures were maintained by sub-culturing every 5 weeks in natural seawater enriched according to f/2 - without silica (f/2-Si) medium (Guillard 1975, Guillard and Ryther 1962; Table 2.1). Natural seawater (NSW) was collected from Stonehaven Harbour, Scotland (Latitude 56° 58′ N, Longitude 2° 12′ W). Media was sterilised by autoclave at 121^{o}C for 50 minutes (10 L) or 15 minutes (1 L or less) (Astell Scientific, UK).

Table 2.1 Composition of f/2-Si medium (Guillard 1975, Guillard and Ryther 1962.

Component	**Concentration in Final Medium**
$NaNO_3$	0.075 g L^{-1}
$NaH_2PO_4.H_2O$	0.005 g L^{-1}
Trace metals solution	1.0 mL
Vitamins solution	0.5 mL
Trace Metals Solution (concentrations were final in solution; g L^{-1})	
$FeCl_3.6H_2O$	3.1500
$Na_2EDTA.2H_2O$	4.3600
$MnCl_2.4H_2O$	0.1800
$ZnSO_4.7H_2O$	0.0220
$CoCl_2.6H_2O$	0.0100
$CuSO_4.5H_2O$	0.0098
$NaMoO_4.2H_2O$	0.0068
Vitamins Solution (concentrations were final in solution; g L^{-1})	
Thiamine HCl (vitamin B_1)	0.200
Biotin (vitamin H)	0.001
Cyanocobalamin (vitamin B_{12})	0.001

Routine cultivation of *P. lima* CCAP 1136/11 was performed either in 100 mL or 5 L volumes. The 100 mL maintenance culture was prepared by inoculating 10 mL stock culture into 90 mL sterile freshly prepared f/2-Si medium contained in 250 mL Erlenmeyer flask. This was then used for experiments in small scale (1 L or less). The 5 L scale maintenance culture was prepared by inoculating 500 mL stock culture into 4.5 L of sterile f/2-Si medium contained in 10 L round flask (Fig. 2.1). This culture was used for large scale experiments (more than 1 L). The 5 L cultures were sparged with filtered ambient air (0.22 µm; Millipore, UK) using an air-pump (230V, 50Hz; Fisherbrand, UK). Cultures were exposed to artificial light at 20±3 µmol m^{-2} s^{-1} (Li-Cor Intelligent Light Meter Li-250) provided by cool white fluorescence tubes (58 W, Osram) and maintained in a temperature controlled room at 22±2°C.

Figure 2.1 *Prorocentrum lima* CCAP 1136/11 grown in 10 L round flask under continuous exposure to light and filtered sterile air sparging.

2.2.3 Monitoring of growth

2.2.3.1 Cell counting with Sedgewick-Rafter slide

Cover slip was placed on a counting slide perpendicularly to the long axis of the slide to allow a slight opening on the top-right and bottom-left corners. One mL aliquots of a homogenous sample were dispensed carefully into the slide through

one of the opening corners. Subsequent to this, the cover slip was re-aligned to close the two opening corners. Counting of cells was performed under a light microscope at 100x magnification. A Sedgewick-Rafter counting slide contains a chamber that holds a 1 mL volume divided into 1000 squares (20 rows by 50 columns). Therefore, determination of cell number can be performed using the following formula:

$$N_{cell}(cells.mL^{-1}) = \left(\frac{CC(cells)}{SC(squares)} \right) \times 1000(squares.mL^{-1})$$

N_{cell} = number of cell (cells mL^{-1}), CC = total number of cells counted, SC = total number of squares counted.

The amount of time required for analysis per sample typically range between 5 to 10 minutes depending on the density of sample. Performing this method was often observed to be difficult for handling large number of samples. Therefore evaluation on suitability of automated counting method was subsequently performed.

2.2.3.2 Automated cell count analysis with flow cytometer

Coulter® EPICS® XL™ flow cytometry with XL-MCL™ software (Beckman Coulter Ltd, High Wycombe UK) was used for automated analysis of cell number in culture samples. The machine was equipped with 488 nm argon laser. Light fluorescence resulted from the excitation with argon laser was detected and collected in different photomulitiplier channels; forward scatter (FS), side scatter (SS), green 525 nm (FL1), orange 575 nm (FL2), red 620 nm (FL3) and long-red 675 nm (FL4). The channels were set at different voltages: FSC =E00, SSC = 400, FL1 = 550, FL2 = 600, FL3 = 600 and FL4 = 600. Cell discrimination and events counting were analysed at FL1 and FL3 density plot. Aliquots of sample (1 ml) were loaded for measurement which lasted for 60 seconds at a flow rate of 15 µL min^{-1}. The cell number (N_{cell}) was determined using the following formula*:

$$N_{cell}(cells.mL^{-1}) = \left(\frac{E(cells)}{q(\mu L.\min^{-1})} \right) \times 1000 \times t(\min)$$

N_{cell} = number of cell (cells mL^{-1}), E = total events of live cells, q = injection flow rate (15 µL min^{-1}), t = running time per sample (1 min).

Use of flow cytometer has a number of advantages over manual counting with Sedgewick-Rafter, especially in providing rapid analysis for samples in large quantity (1 minute per sample compared to 5-10 minutes with Sedgewick-Rafter counting). Preliminary tests on the flow cytometer, however, showed inconsistent measurement of cell number for untreated culture samples. Direct observation with light microscope suggested that the problem was caused by clumping of cells in the samples, which caused underestimation of the number of cells counted. Therefore, tests on several treatments that are able to disaggregate the clumps in sample were conducted.

2.2.3.3 Evaluation of clump disaggregation

A number of disaggregation techniques have been reported in the literature, including sonication, alkaline hydrolysis and heat treatment at 90°C (Reynolds and Jaworski 1978, Humphries and Widjaja 1979, Lawton *et al.* 1999). In this set of experiments, several disaggregation procedures were tested; probe sonication, bath sonication, alkaline hydrolysis at room temperature and at 80°C, and heat treatment at 80°C. Once the most appropriate method selected, it was fully evaluated over a range of cell densities and viabilities (section 2.2.3.4) using both flow cytometry and Sedgewick-Rafter counting, and compared to dry cell weight (section 2.2.3.5). All analyses were conducted in three replicates per sample.

Clump disaggregation with probe sonication was performed by subjecting the sample to ultrasonic vibration (XL2007 Sonicator®/Microston™ Ultrasonic Processors/Cell Disruptors, Labcaire System Ltd., Avon, UK). The system was

equipped with a titanium disruptor horn at 20 kHz. Aliquots of culture (5 ml) were sampled and placed in a universal plastic tube, and sonicated for one minute with four active pulses of 5 seconds. During the sonication cycle, the sample tube was maintained in a container filled with ice to avoid localised heat generation. Sonicated samples were then vortexed for 30 seconds prior to analysis.

Disaggregation with bath sonication was performed by sampling 5 mL culture and transferring it into universal plastic tube. The tube was then placed in a sonication bath (Mechanical Ultrasonic Cleaners, Fisher Scientific, UK) at 120 V, 50/60 Hz, 1A, 130 W for 2 minutes. Subsequent to this, sample was vortexed for 30 seconds prior to analysis.

Alkaline hydrolysis was performed by adopting the method described in Reynolds and Jaworski (1978). The procedure was conducted for both non-heated (room temperature) and heated (80^{o}C). Aliquots of sample (5 mL) were transferred to universal plastic tube, to which a single pellet (~0.27 g) of sodium hydroxide (NaOH) was added. The sample was then incubated for 30 minutes, either at room temperature or at 80^{o}C, and vortexed for 30 seconds before being used for analysis.

The procedure for disaggregation with heat treatment was performed according to the method described in Humphries and Widjaja (1979). Aliquots of sample (5 mL) were placed in a 25 mL test tube. The top opening of the tube was loosely covered with aluminium foil. The tubes were immersed to the sample level in a water bath (80^{o}C) for 5 minutes. Subsequent to this, samples were vortexed for 30 seconds before being directly analysed.

2.2.3.4 Evaluation of cell count methods over a range of cell viabilities

Samples at different viability levels were prepared by mixing live cells with non-viable (killed) cells. Non-viable cells were prepared by boiling culture sample for 5 minutes. The boiled sample was then centrifuged (10 min; 10,800 g) and had the supernatant removed and replaced with freshly sterilised f/2-Si medium.

Samples were directly analysed by flow cytometry. For Sedgewick-Rafter analysis, mixed sample was stained with trypan blue (Sigma Aldrich, UK) and immediately counted under the microscope. Use of trypan blue allowed discrimination of viable and non-viable cells. Cells that were non-viable were stained with blue colour, while viable cells remained unstained.

2.2.3.5 Analysis of dry cell weight (DCW)

Measurement of dry cell weight (DCW) was achieved by using the filtration method. Filter papers (Whatman® Grade 1, Sigma Aldrich, UK) were weighed to constant weight (W_o) prior to being used for filtration of a known volume of sample. Subsequent to filtration, the filter paper was freeze-dried overnight then placed inside desiccator before the final weight (W_t) was measured. The amount of dry cells weight was calculated using the following equation:

$$DCW = \frac{Wt - Wo}{V}$$

DCW= dry cells weight (mg mL^{-1}); Wt=final weight of filter (mg); Wo= initial weight of filter (mg); V=volume of filtered sample (mL).

2.2.4 Evaluation of the use of synthetic media for *P. lima* CCAP 1136/11

Two media bases for substitution of natural seawater were tested; artificial seawater (ASW) and salt water (StW). StW base was prepared by adding sodium chloride (NaCl) into water (Milli-RO) at concentration of 35 g L^{-1}, and enriched with nutrients according to f/2-Si recipe. ASW medium base was prepared according to the recipe by Keller *et al.* (1987) with modifications described in Guillard and Morton (2003). For this, several salts were added into distilled water according to Table 2.2. ASW base was then enriched according to f/2-Si recipe. Evaluation of the use of ASW was conducted for two sequential batch cycles. The second batch of ASW culture (ASW-2^{nd}) was prepared by inoculating cells that were previously maintained in ASW medium.

Three replicates of experimental cultures were prepared by inoculating 100 mL of stock culture of *P. lima* CCAP 1136/11 into 900 mL of medium contained in a 2 L Erlenmeyer flask. Aeration and light were provided as described in section 2.2.2. Cultures were maintained in a temperature controlled room at 22±2°C.

Table 2.2 Composition of salts for artificial seawater (ASW) (Keller *et al* (1987) with modifications described in Guillard and Morton (2003).

Component	Grams per L media	Stock solution	Quantity added per L media
NaCl	24.00 g	-	24 g
KCl	0.60 g	-	0.6 g
NaBr	1.03 mg	2.0 g L^{-1}	0.515 mL
H_3BO_3	6.00 mg	6.0 g L^{-1}	1.00 mL
NaF	0.42 mg	2.0 g L^{-1}	0.21 mL
KI	33.00 µg	0.4 g L^{-1}	82.50 µL
$NaHCO_3$	0.20 g	50.0 g L^{-1}	1.00 mL
$MgCl_2.6H_2O$	3.00 g	600.00 g L^{-1}	5 mL
$MgSO_4.7H_2O$	5.00 g	1 g mL^{-1}	5 mL
$CaCl_2.2H_2O$	0.40 g	400.00 g L^{-1}	1 mL
$SrCl_2.2H_2O$	26.60 mg	26.6 g L^{-1}	1 mL

2.2.5 Analytical UPLC-PDA-QTOF for the identification of DSP toxins and peridinin from *Prorocentrum lima* CCAP 1136/11 extract

Analysis of bioactive compounds from *P. lima* CCAP 1136/11 was performed using a Waters (Elstree, UK) Acquity Ultra Performance Liquid Chromatography (UPLC) coupled to a photodiode array (PDA) and Xevo quadrupole time of flight (Qtof) mass spectrometer. Extracts were separated using a BEH C18 column (100 x 2.1 mm; 1.7 µm particle size) which was maintained at 40°C. Mobile phase consisted of Milli-Q (A) and acetonitrile (B) both containing 0.1% formic acid. Separation was achieved using a gradient from 30% B to 70% B over 10 minutes, followed by 100% solvent (B) wash step and re-equilibration. The autosampler was maintained at 6°C at all times. Eluent was monitored from 200-750 nm with a resolution of 1.2 nm and by positive ion electrospray (ESI+) in

series, scanning from m/z 50 to 2000 with a scan time of 0.25 s and inter-scan delay of 0.025 s. Parent MS surveys were conducted in low (6-10 V) and high (10-30 V) energies. Ion source parameters; capillary and sampling cone were 3.0 kV and 25 V respectively; desolvation temperature of 300^{o}C; and source temperature of 80^{o}C. Cone gas and desolvation gas flows were 50 L/h and 400 L/h respectively. Sodium iodide (2 µg/µL in 50% (v/v) aqueous propan-2-ol) was used as the calibrant with leucine-enkephaline (0.5 mg/L in 50% (v/v) aqueous methanol) as the lockspray. Instrument control, data acquisition (centroid) and processing were achieved using MassLynx v4.1. DSP toxins were identified and quantified on the basis of retention time and mass spectra compared to calibration curves of external standards (Appendix I) with extracted mass chromatograms at at *m/z* 805.5 for OA, *m/z* 819.5 for DTX1. Peridinin was identified by comparing retention time and UV-VIS absorbance at 474 nm against an authentic standard.

2.2.6 Optimisation of extraction procedure

Screening of extraction solvents was performed in order to evaluate optimum recovery of okadaic acid (OA) from *P. lima* CCAP 1136/11 cells. Pellets from 10 mL of culture (age 5 weeks, ~5.73x10^{4} cells mL^{-1}) which had been cultivated as described in section 2.2.2 were prepared in two ways, freeze-thawed and freeze-dried. Preparation of freeze-thawed pellets was performed by storage of samples in -20^{o}C for at least 24 hours and thawed at room temperature prior to extraction. Freeze-dried pellets were prepared by storage of samples at -20^{o}C for several hours and freeze-dried overnight prior to extraction. Both prepared pellets were then extracted with different solvents as listed in Table 2.3 at a volume of 2 mL. Extracted samples were incubated for 1 hour in the dark. Following this, samples were centrifuged (10 min; 10,800 g) and the supernatant was transferred into HPLC vials for further analysis by UPLC-PDA-QTOF as described in section 2.2.5. All extractions were performed in triplicate.

Table 2.3 Solvents used for extraction of okadaic acid from *P. lima* CCAP 1136/11.

Solvent	**Concentration (v/v)**	**Description**
Methanol (MeOH)	100% 75% 50% +TFA (0.01%)	100% MeOH 75% MeOH + 25% H_2O 50% MeOH + 50% H_2O MeOH + 0.01% trifluoroacetic acid (TFA)
Ethanol (EtOH)	100% 75%	100% EtOH 75% MeOH + 25% H_2O
Acetone	100% 75%	100% Acetone 75% Acetone + 25% H_2O
Acetonitrile (CH_3CN)	100% 75%	100% Acetone 75% Acetone + 25% H_2O

Based on the results obtained in the screening extraction with different solvents, optimisation of the extraction procedure was subsequently performed in order to evaluate optimum recovery of DSP toxins (OA and DTX1) and peridinin. For this, two steps of methanolic extraction procedure were performed. Step-1 of the procedure used MeOH at a concentration of 50% (v/v), and Step-2 used 80% MeOH (v/v). Three replicates of freeze-thawed harvest cells (0.5 mL; $2x10^6$ cells mL^{-1}) were extracted sequentially three times with 1 mL of 50% MeOH in the Step-1. Following this, the same cells were extracted with 80% MeOH (1 mL) for a further three times in the Step-2.

2.3 Results

2.3.1 Optimum clump disaggregation for automated cell counting analysis with flow cytometer

Evaluation of the effect of disaggregation treatments was performed by comparing consistency of measurement at different dilutions using flow cytometer and DCW. Analysis of linearity for each treatments showed that only samples treated with sonications (both probe and bath) gave good correlation between cell number and dry biomass, with R^2 value of of 0.9943 for probe and 0.9731 for bath (Fig. 2.2). It was noted, however, that despite the high R^2 value, both probe and bath sonications gave dissimilar readings of cell number in flow cytometry analysis. At the same dry biomass, cell numbers observed in samples treated by probe sonication were almost three times higher than those counted in bath sonication samples. Further observation of samples by microscopy indicated that complete disaggregation of clumps was found only for samples treated with bath sonication (Fig. 2.3), and that separation with probe sonication resulted in damage to cell membrane (Fig. 2.4). This indicated that disaggregation of clumps was best achieved by bath sonication.

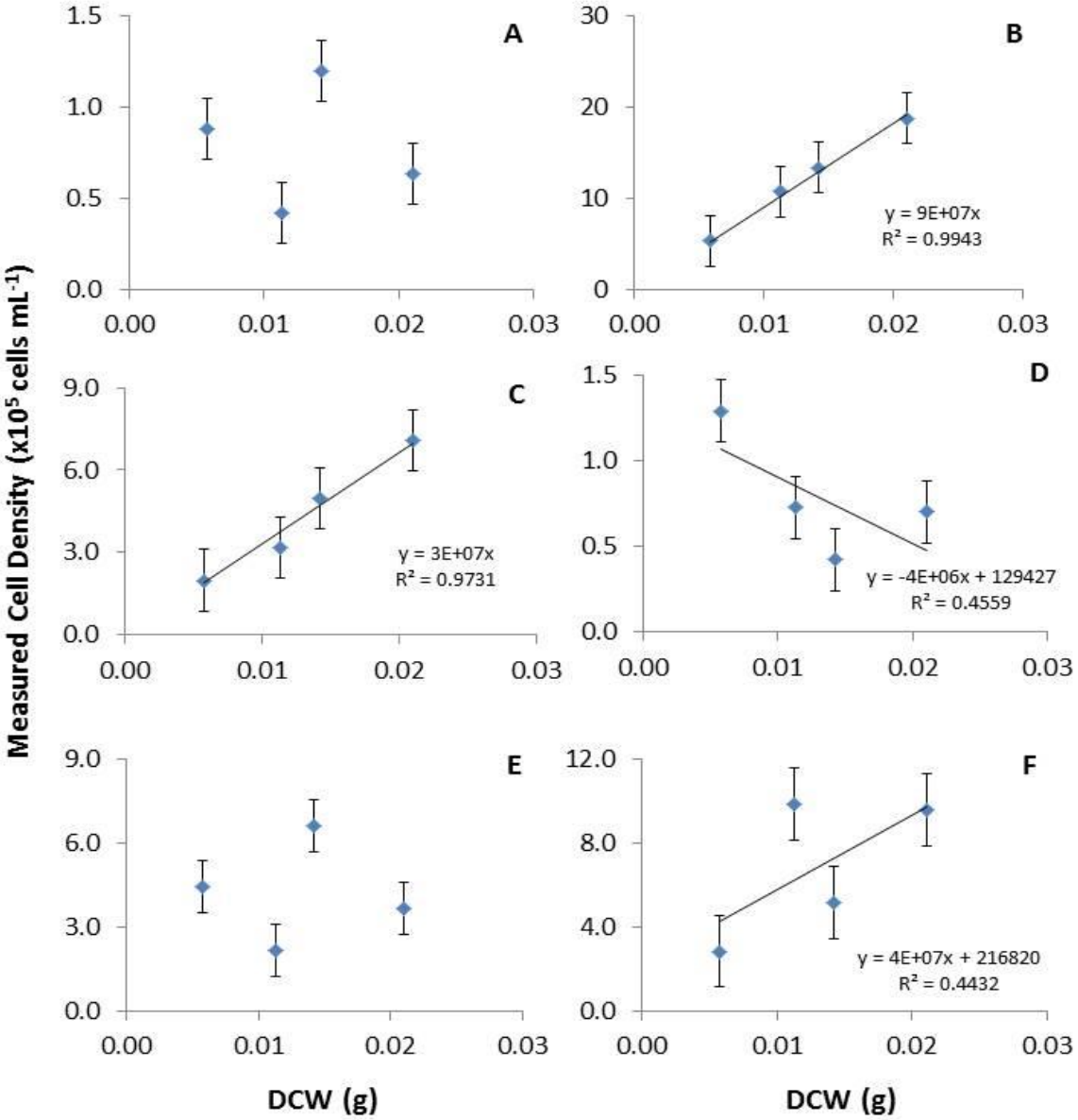


Figure 2.2 Analysis of linearity between dry biomass analysis (DCW) and predicted cell number counted by flow cytometer for samples treated with different procedure of clump disaggregation; untreated sample (A), probe sonication (B), bath sonication (C), heated alkaline hydrolysis (D), non-heated alkaline hydrolysis (E) and heat treatment at 80°C (F) (n=3, error bars denote standard deviation).

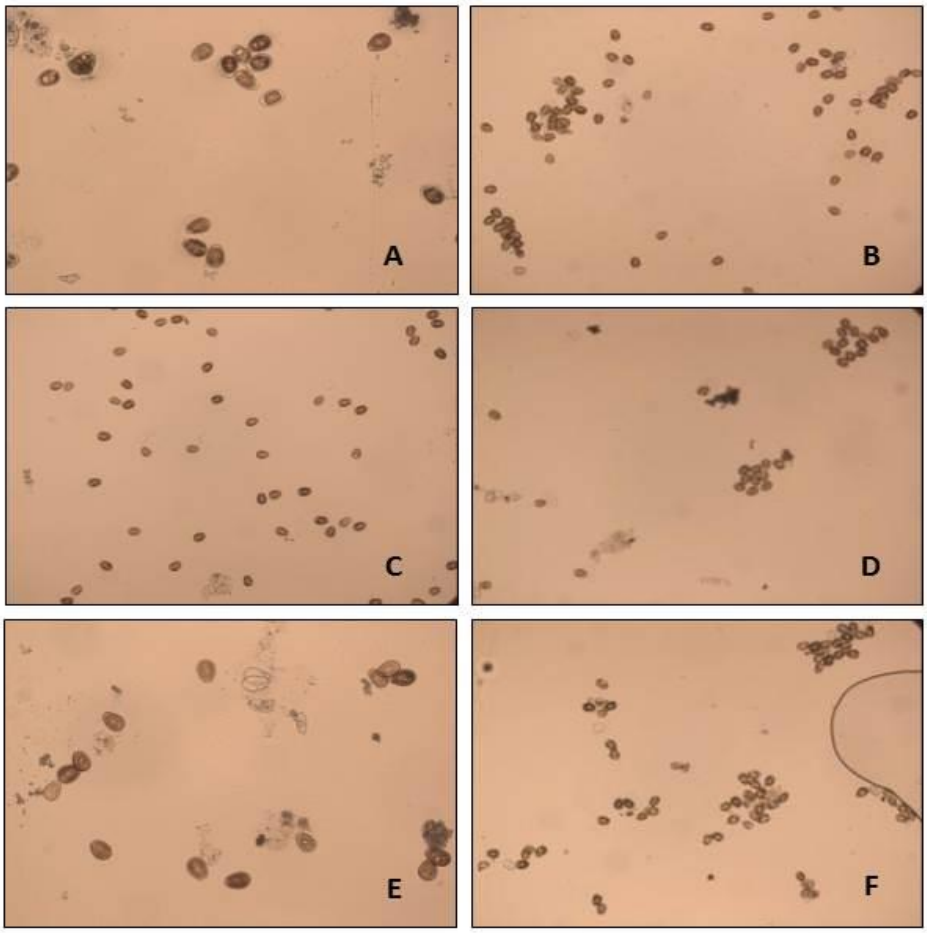


Figure 2.3 Microscopic images of cell clumps in *P. lima* CCAP 1136/11 samples after separation treatment; raw sample (A), probe sonication (B), bath sonication (C), heated alkaline hydrolysis (D), unheated alkaline hydrolysis (E) and heat treatment (F).

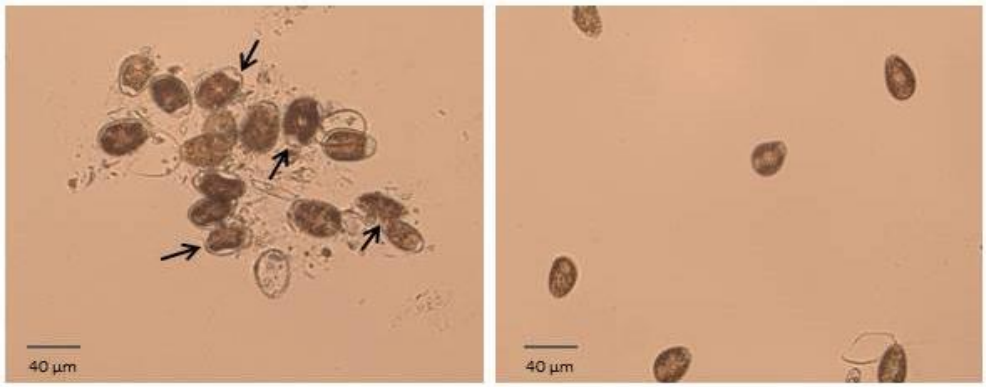


Figure 2.4 Microscopic observation on the effect of clump separation with probe (left) and bath (right) sonications on cell membrane. Arrows indicate damaged cell-membrane.

Clump disaggregation with bath sonication was further assessed by comparing two cell count methods, Sedgewick-Rafter and flow cytometer, at different dilution of culture sample. Dilution was made by adding a known volume of sterilised f/2-Si medium into the sample. It appeared that the data produced by flow cytometer at different dilutions correlated well with those produced by Sedgewick-Rafter (Table 2.4), and thus confirmed the suitability of bath sonication for clump disaggregation treatment prior to flow cytometer analysis.

Table 2.4 Comparison of Sedgewick-Rafter (SR) and flow cytometer (FC) cell counting for *P. lima* CCAP 1136/11 samples following bath sonication treatment (n=3, ± denotes SD).

% Original Sample	Measured Cell Number (x10^3 cells mL^{-1})	
	SR	FC
100.0	17.6±4.8	17.5±4.8
50.0	11.1±1.1	8.2±0.5
25.0	3.9±0.2	3.3±0.4
12.5	1.8±0.4	2.0±0.2

Further evaluation of bath sonication pre-treatment and flow cytometer counting performance was performed for samples with different levels of viability. It was shown that flow cytometer performed satisfactorily for all percentages of viability (Table 2.5). It was noted, however, that deviation of counting for viable and non-viable cells was higher for flow cytometer counting compared to Sedgewick-Rafter, which was shown by the number of viable cells counted at 0% viability samples (Table 2.5). This may be caused by higher sensitivity of light sensors/detectors on flow cytometer, which may have discriminated other particulate impurities as live cells.

Table 2.5 Comparison of Sedgewick-Rafter (SR) and flow cytometer (FC) counting for *P. lima* CCAP 1136/11 samples at different viability level following bath sonication treatment (n=3, ± denotes SD).

Predicted viability (%)	No. of cells per mL(x10^3)			
	SR		FC	
	Viable	Non-viable	Viable	Non-viable
100	12.9±1.9	1.9±0.9	15.6±5.3	0.4±0.0
50	7.8±0.0	5.2±0.5	6.9±1.4	6.3±0.5
0	0.0±0.0	9.6±5.3	1.2±0.0	14.9±1.0

2.3.2 Evaluation of artificial seawater (ASW) and salt water (StW) for the growth and DSP toxin production from *P. lima* CCAP 1136/11 in batch culture.

Evaluation of different cultivation media was performed to allow selection of artificially prepared medium (ASW or StW) as a substitute for naturally collected seawater (NSW). In addition, the long term effect of ASW medium on the cultivation of *P. lima* CCAP 1136/11 was also evaluated by comparison between the first and second transfer of culture to ASW medium (ASW and ASW-2^{nd}).

Comparison between NSW, ASW and StW showed that, whilst cell density was relatively similar at all medium types (Fig. 2.5A), use of StW caused reduction of intracellular okadaic acid (OA) detected in the culture. The reduction of intracellular OA in StW cultures was observed to be approximately 68% lower than those in NSW (Fig. 2.5B). Further analysis of extracellular OA concentration revealed that despite the low intracellular concentration, StW cultures did produce increased amounts of extracellular OA. About 63% (0.95 μg mL^{-1}) of the total OA produced in StW was excreted in to the medium (Fig. 2.5C). Release of OA to medium by *P. lima* CCAP 1136/11 culture was commonly observed as a sign of impaired cell viability, thus indicating unsuitability of NSW replacement with StW. Final intracellular concentrations of OA in the cultures were detected at 1.78, 1.56 and 0.57 μg mL^{-1} for NSW, ASW and StW, respectively.

Concentrations of extracellular OA detected at the final cultivation period were 0.44, 0.31 and 0.95 μg mL^{-1} for NSW, ASW and StW, respectively.

In addition to the analytical data, visual observation of culture samples also revealed that StW cultures exhibited abnormal pigmentation compared to NSW and ASW (Fig. 2.6), which may also indicate the detrimental effect of StW for the cultivation of *P. lima* CCAP 1136/11. Meanwhile, growth and production of OA in ASW were comparable to those observed in NSW. Cells grown in both ASW and NSW appeared to remain viable throughout the cultivation period, indicated by the amount of extracellular OA which was almost negligible for the first four weeks of cultivation. Visual observation on the samples also showed that no apparent change was observed as a result of NSW substitution with ASW. This may indicate that direct replacement of NSW was possible with the use of ASW.

Further sub-culturing was carried out to evaluate the performance of ASW for the continued cultivation of *P. lima* CCAP 1136/11. Analysis of cell number revealed that growth was enhanced by 3-fold following the second transfer to ASW medium (ASW-2^{nd}) compared to the first transfer (ASW) (Fig. 2.5D). However, similar enhancement was not reflected on the intra- and extracellular production of OA. It was noted that intracellular OA production was reduced by approximately 74% at the second transfer to ASW (Fig. 2.5E and 2.7), while extracellular OA production were comparable for both cultures. Further observation on visual appearance of the culture indicated abnormality on the growth of *P. lima* CCAP 1136/11 in ASW-2^{nd} (Fig. 2.6). It appeared that culture did not form typical brown/red pigmentation at the second transfer to ASW when compared to those in the first transfer and in NSW.

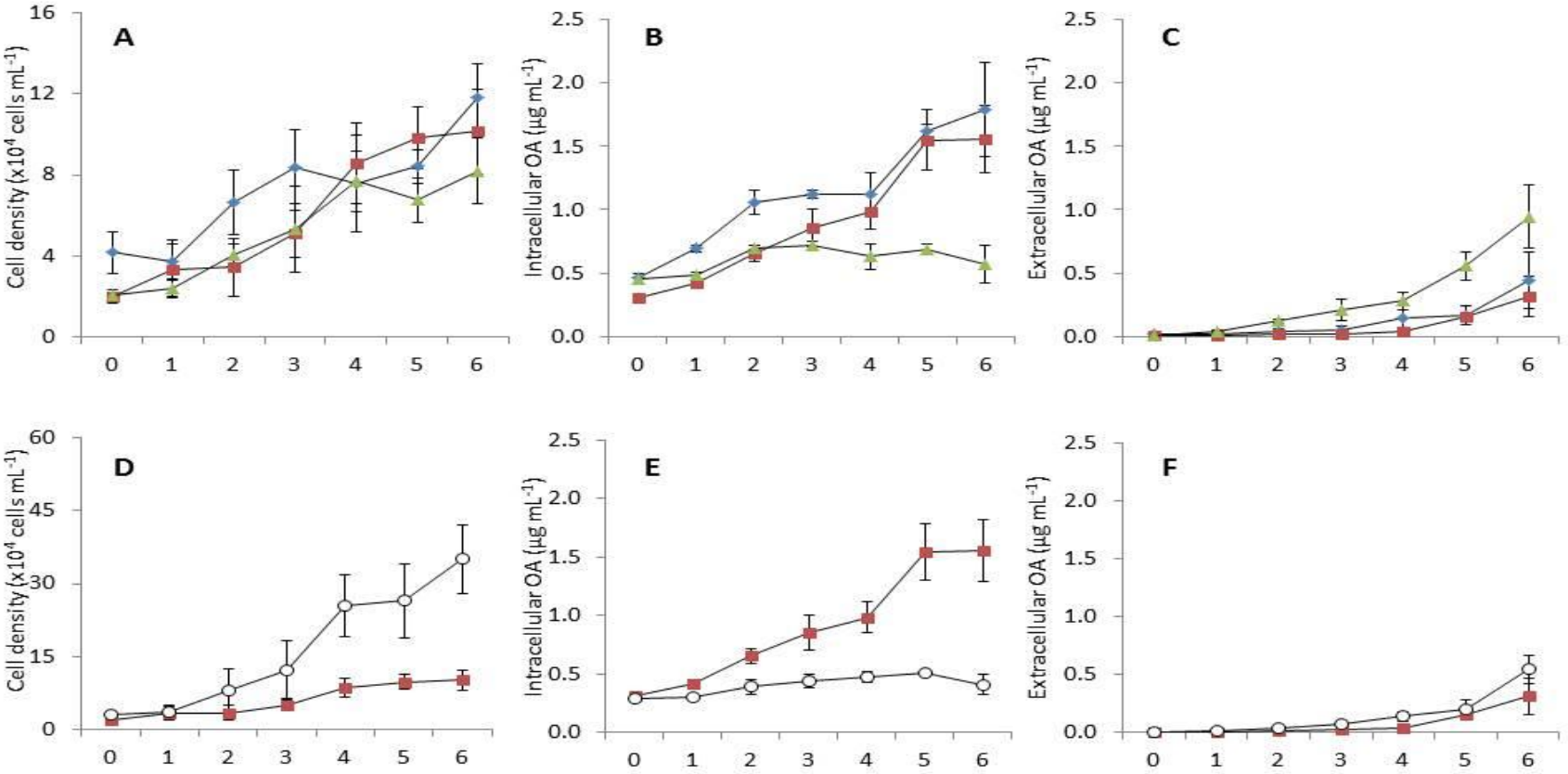


Figure 2.5 Comparison of growth and okadaic acid (OA) production from *P. lima* CCAP 1136/11 culture in different types of media (A-C) and at sequential transfers to artificial seawater (D-F) (n=3); natural seawater (NSW) (◆), artificial seawater (ASW) (■), salt water (StW) (▲) and second transfer to ASW (ASW-2nd) (○).

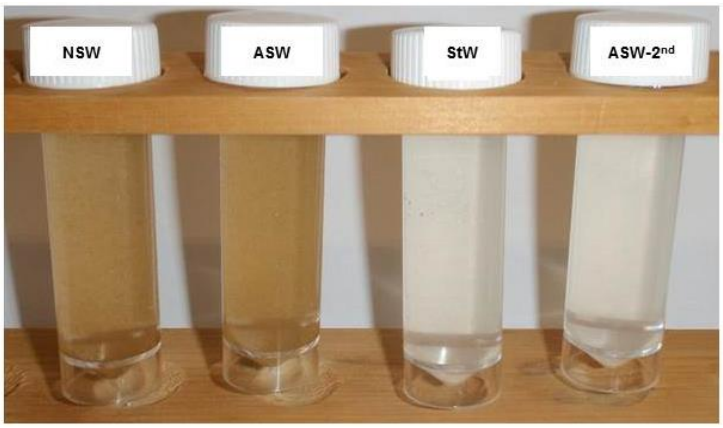


Figure 2.6 Visual appearances of *P. lima* CCAP 1136/11 cultures after five weeks of cultivation with different medium types. *Left to right*: natural seawater (NSW), artificial seawater (ASW), salt water (StW) and second transfer to ASW (ASW-2nd).

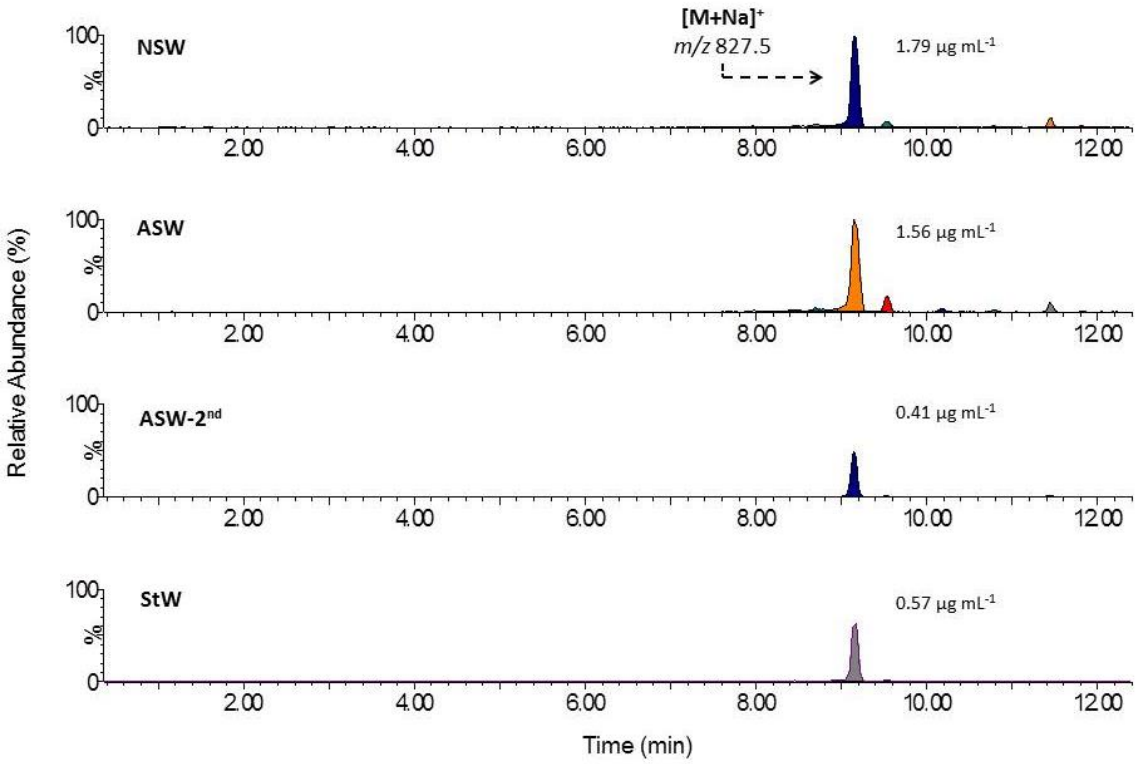


Figure 2.7 UPLC-MS analysis for intracellular okadaic acid (OA) levels in *Prorocentrum lima* CCAP 1136/11 cultures after six weeks cultivation in natural seawater (NSW), artificial seawater (ASW), salt water (StW) and second transfer to ASW (ASW-2nd).

2.3.3 Identification of bioactive compounds from *P. lima* CCAP 1136/11

2.3.3.1 Analysis of standards

Identification of compounds produced by laboratory culture of *P. lima* CCAP 1136/11 was performed by UPLC-MS-PDA analysis. The ability to generate low and high energy spectra simultaneously facilitated rapid identification of parent ions in low energy (mainly formed as $[M+H]^+$ and $[M+Na]^+$) and characterisation of fragments in high energy.

Chromatographic elution of OA and DTX1 standards showed characteristic retention time at 8.46 minutes for OA and 11.42 minutes for DTX1 (Fig. 2.8). Mass fragmentation of OA showed $[M+H]^+$ ion at *m/*z 805.47 in low energy (LE) and $[M+Na]^+$ ion at *m/*z 827.45 in high energy (HE). OA was also fragmented with consecutive losses of water (H_2O) molecules resulting in predominant ions at *m/*z 787 $[M-H_2O+H]^+$ and 769 $[M-2H_2O+H]^+$ (Fig. 2.9). In addition, several characteristic fragments were identified for OA such as *m/z* 305, 323 and 429.

Similar to OA, DTX1 was also ionised mainly as $[M+H]^+$ ion at LE (*m/z* 819.49) and $[M+Na]^+$ ion at HE (*m/z* 841.45). This was followed by consecutive losses of H_2O molecules resulted in the formation of ions at *m/*z 801 $[M-H_2O+H]^+$ and 765 $[M-3H_2O+H]^+$ (Fig. 2.10). Several characteristic fragments were also identified for DTX1, such as *m/z* 319, 429 and 535.

In contrast to OA and DTX1, peridinin was not clearly identified in mass chromatograms, but it could be readily detected by UV-Vis absorbance. It was shown that peridinin was eluted with typical retention time of 12.64 minutes (Fig. 2.8). Maximum absorbance wavelength of peridinin standard was recorded at 474 nm (Fig. 2.11).

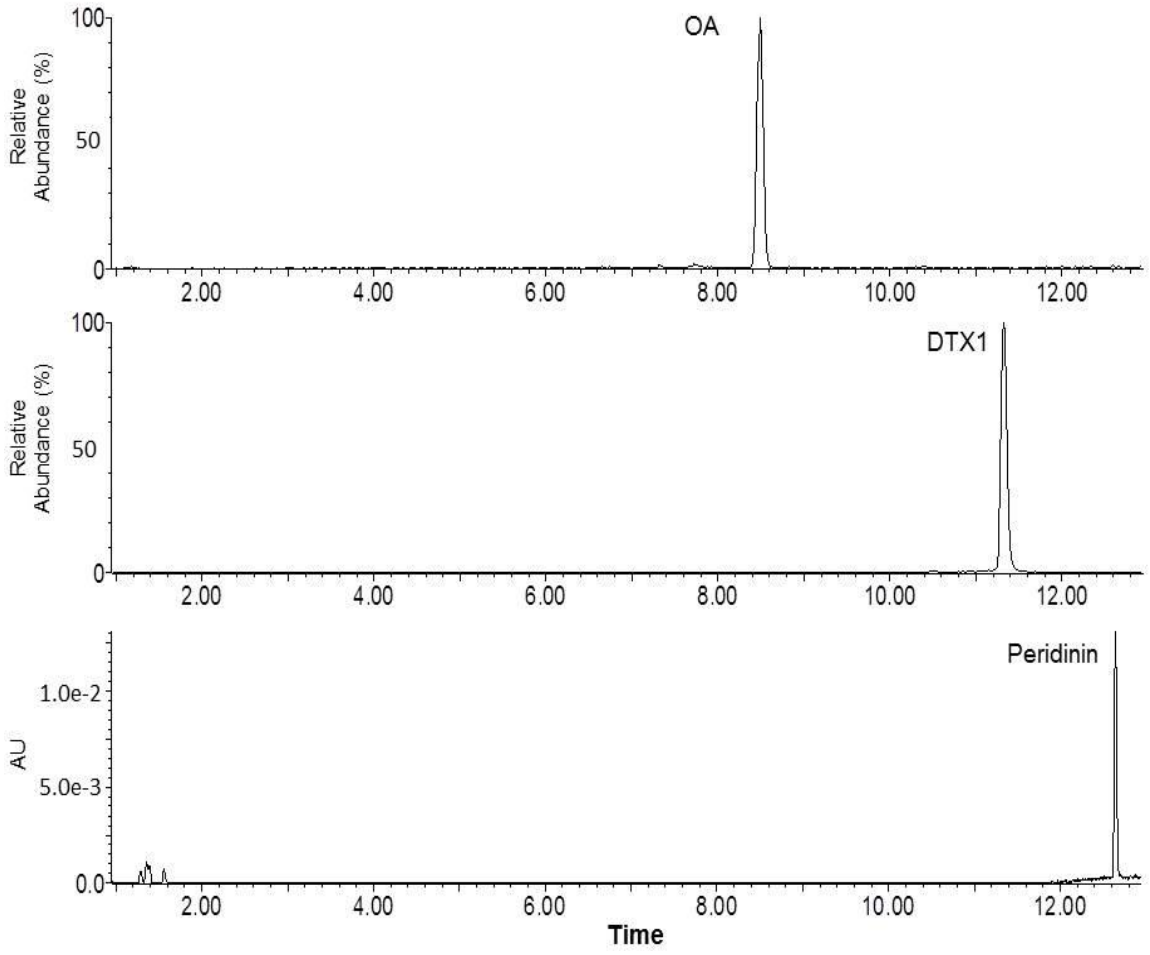


Figure 2.8 Extracted UPLC-MS-PDA chromatogram for standards of okadaic acid (OA) at *m/z* 805.47, dinophysistoxin-1 (DTX1) at *m/z* 819.49 and peridinin at 474 nm.

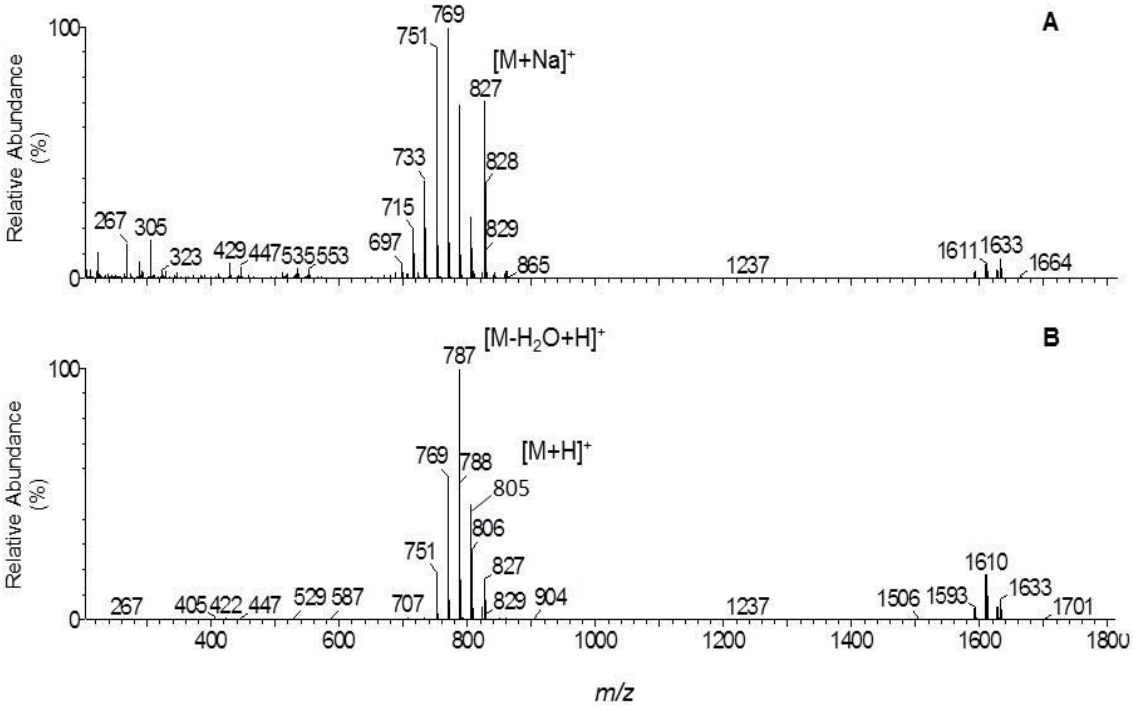


Figure 2.9 Mass spectra of okadaic acid standard in ESI+ mode at high (A) and low (B) energy levels.

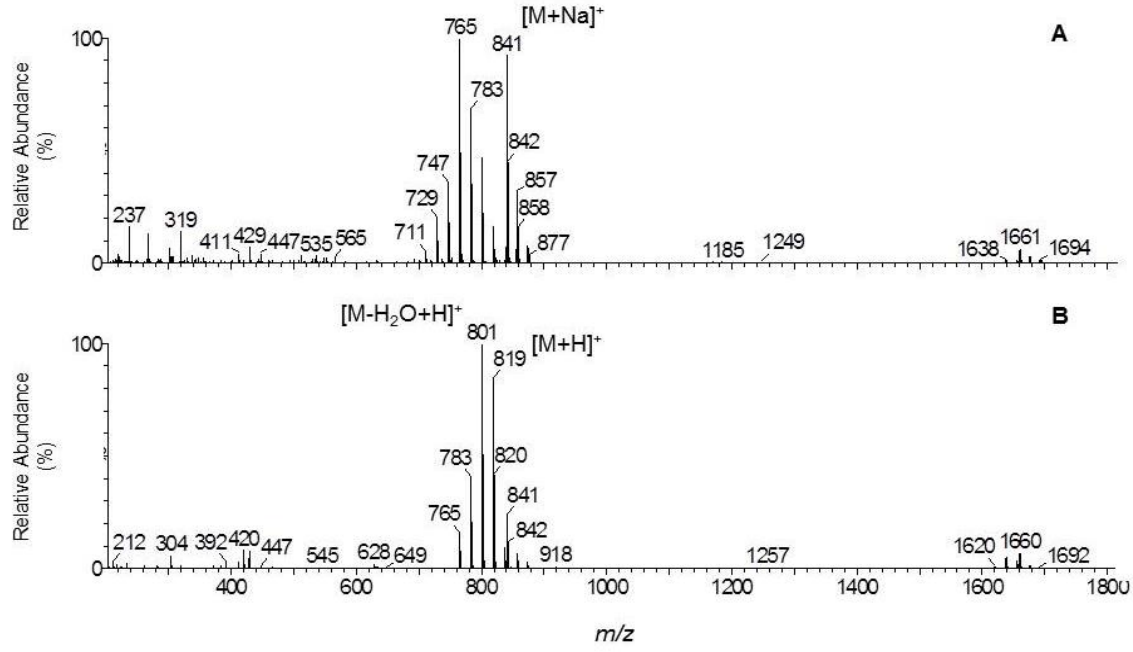


Figure 2.10 Mass spectra of DTX1 standard in ESI+ mode at high (A) and low (B) energy levels.

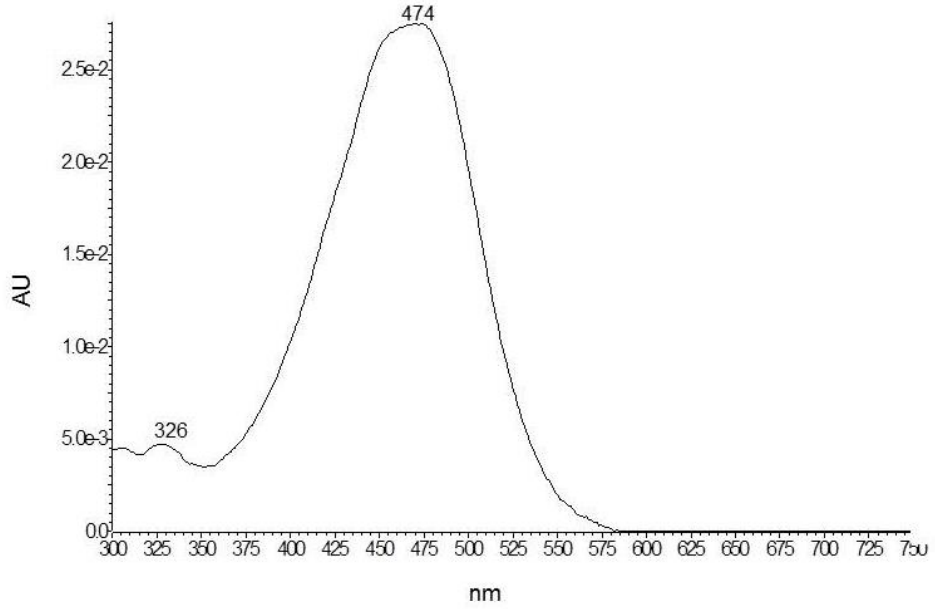


Figure 2.11 Maximum absorbance wavelength of peridinin standard (474 nm) detected by UPLC- PDA.

2.3.3.1 Identification of analytes in extract samples of *P. lima* CCAP 1136/11

Analysis of compounds with UPLC-PDA-MS for the methanolic extract of *P. lima* CCAP 1136/11 revealed the presence of nine major compounds (Fig. 2.12). Five compounds were identified as DSP toxins, their related compounds, and a carotenoid pigment peridinin (Table 2.6), while four other compounds detected were still unidentified (Table 2.7). Identified compounds were categorised based on their similarities in retention time, mass fragmentation or light absorbance characteristics to standards and published data. Existence of compounds that have the same molecular mass with those unidentified here have not been reported in the literature for dinoflagellates genus of *Prorocentrum*. Further investigation of mass spectra for these unidentified compounds also showed that they shared no similarity to OA and its relative DTX1 (Appendix III).

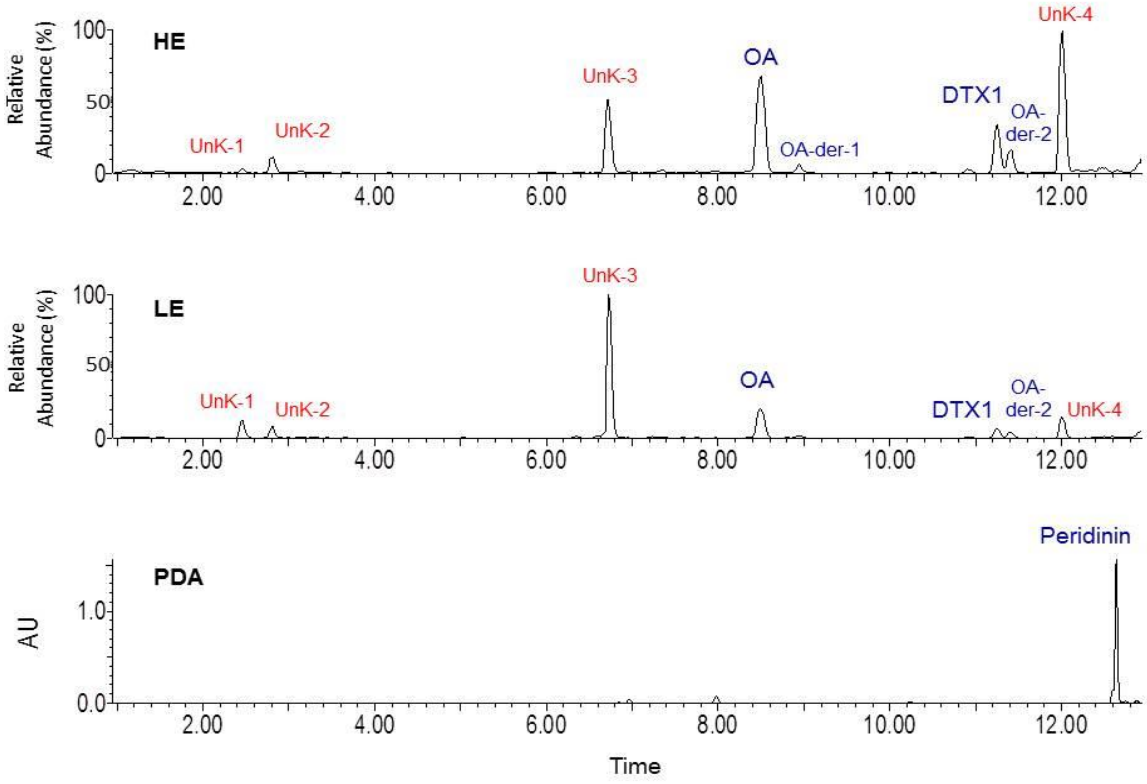


Figure 2.12 Characterisation of identified (blue letters) and unidentified (UnK-1 to UnK-4, in red letters) compounds from methanolic (100%) extract of *Prorocentrum lima* CCAP 1136/11 analysed by UPLC-MS-PDA at high energy (HE), low energy (LE) and UV-Vis absorbance (PDA).

Table 2.6 Retention time, parent ions (*m/z*) in ESI+ mode and maximum absorbance wavelength for DSP toxins, their related compounds and carotenoid pigment from *Prorocentrum lima* CCAP 1136/11.

Compound	Retention time	$[M+H]^+$	$[M+Na]^+$
Okadaic acid	8.46	805.47	827.45
OA-derivative-1	8.94	805.47	827.45
DTX1	11.21	819.49	841.47
OA-derivative-2	11.37	943.57	965.56
Compound	**Retention time**	**Maximum absorbance**	
Peridinin	12.64	474 nm	

Table 2.7 Retention time and parent ions (*m/z*) in ESI+ mode for unidentified compounds from methanolic (100%) extract of *Prorocentrum lima* CCAP 1136/11.

Compound	Retention time	$[M+H]^+$	$[M+Na]^+$
Unknown-1	2.46	742.51	764.49
Unknown-2	2.81	984.66	1006.65
Unknown-3	6.71	688.44	710.42
Unknown-4	12.01	780.51	802.49

Okadaic acid (OA) was identified due to the identical retention time and characteristic of mass spectra to the standard, with $[M+H]^+$ (*m/z* 805) in low energy and as $[M+Na]^+$ (*m/z* 827) in high energy level (Fig. 2.13). Further fragmentation was identified as consecutive losses of H_2O molecules, giving predominant ions identified at *m/z* 787 and 769. Other characteristic fragments of OA were found at *m/z* 305, 323 and 429. Furthermore, analysis of elemental composition using MassLynx v4.1 for $[M+H]^+$ ion confirmed characterisation as OA with chemical formula of $C_{44}H_{68}O_{13}$ at mass accuracy (Δppm) of 1.0.

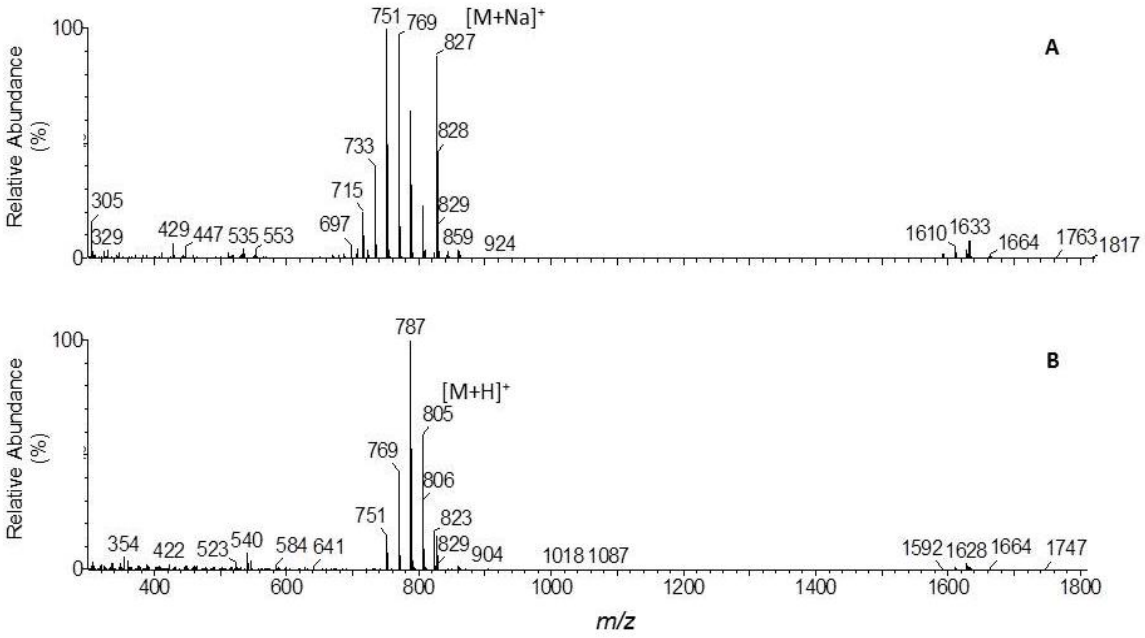


Figure 2.13 UPLC-MS fragmentation in ESI+ mode for okadaic acid from methanolic extract of *P. lima* CCAP 1136/11 at high (A) and low (B) energy levels.

OA-derivative-1 was eluted at approximately 0.48 minutes later than OA. Analysis of mass spectra indicated similar fragmentation of parent ions to OA at *m/z* 805 and 827 (Fig. 2.14). Elemental composition analysis using MassLynx v4.1 on $[M+H]^+$ indicated identical chemical composition to OA ($C_{44}H_{68}O_{13}$) with mass accuracy (Δppm) recorded at 3.1. To confirm its identification as OA related compound, spiking of sample with DTX2 standard that is known to share similar molecular mass to OA was performed (Fig. 2.15). This confirmed that OA-derivative-1 was neither OA nor DTX2, but possibly another related compound with the same chemical formula of $C_{44}H_{68}O_{13}$. Further investigation of MS fragmentation of this compound observed characteristic ion fragments that are also similar to OA, such as *m/z* 305 and 429. This suggests that OA-derivative-1 is not only isobaric but also shares similar molecular structure with OA.

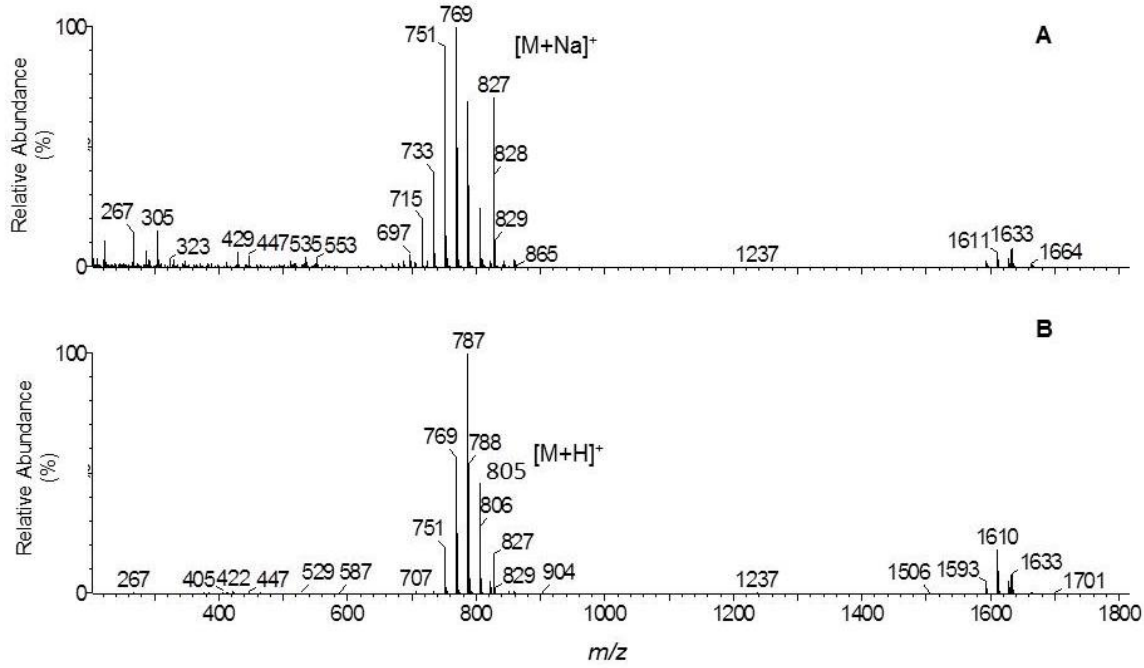


Figure 2.14 Positive ion mass spectra of OA-derivative-1 from methanolic (100%) extract of *P. lima* CCAP 1136/11 at high (A) and low (B) energy levels.

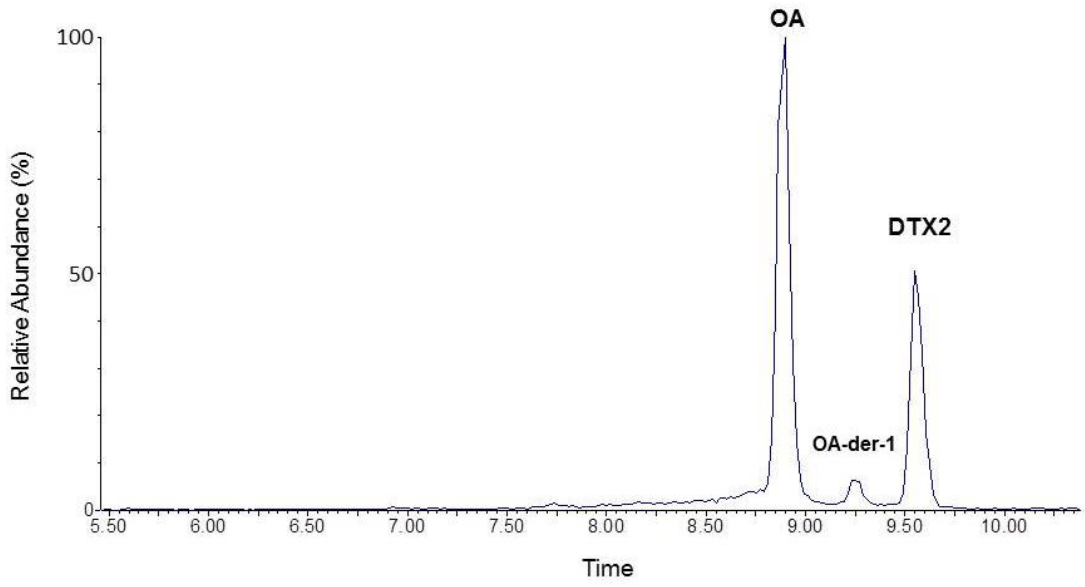


Figure 2.15 Spiking of DTX2 standard in methanolic (100%) extract of *P. lima* CCAP 1136/11 sample confirmed the difference in retention time between OA, OA-derivative-1 and DTX2 (extracted chromatogram at *m/z* 805.47).

The peak at 11.21 minutes was found to have identical retention time to the standard of DTX1 (Fig. 2.12). Evaluation on the mass spectra revealed that this peak shared the same mass fragmentation to DTX1, which formed $[M+H]^+$ ion (*m/z* 819) in low energy and as $[M+Na]^+$ ion (*m/z* 841) in high energy level (Fig. 2.16). Several other characteristic ion fragments of DTX1 (e.g. *m/z* 319, 429, 447 and 565) were also identified. Furthermore, analysis of elemental composition confirmed its identification as DTX1 ($C_{45}H_{70}O_{13}$) (Δppm 0.1).

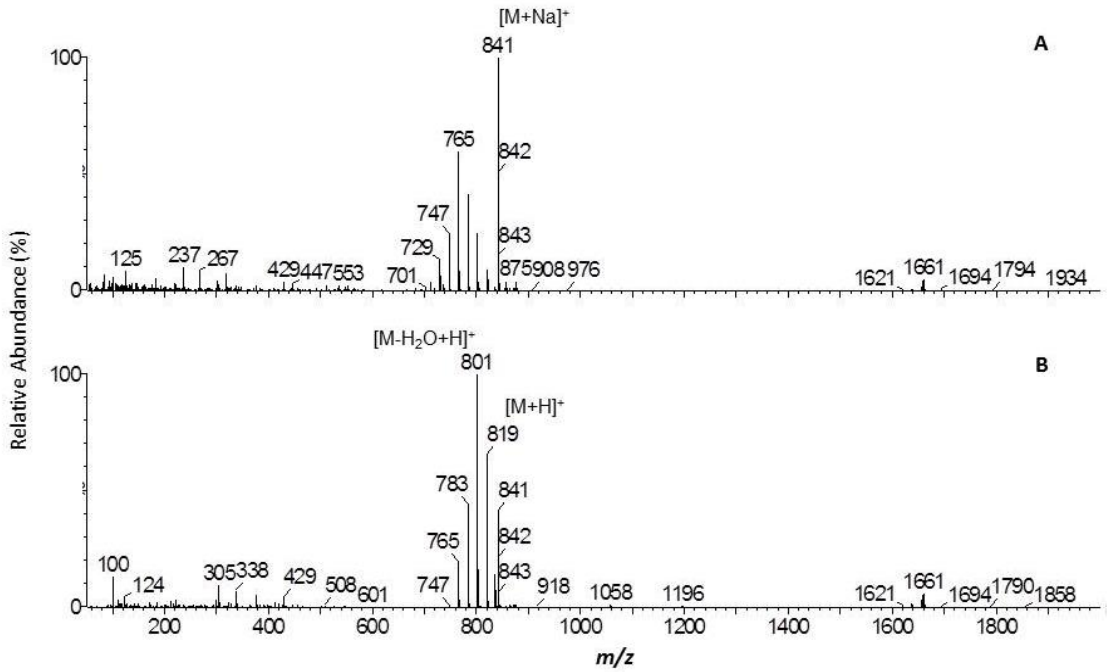


Figure 2.16 UPLC-MS fragmentation in ESI+ mode for DTX1 from methanolic (100%) extract of *P. lima* CCAP 1136/11 at high (A) and low (B) energy levels.

Mass spectra of OA-derivative-2 showed clear fragmentation of $[M+Na]^+$ ion at *m/*z 965.6 at high energy, and $[M+H]^+$ ion at *m/*z 943.6 at low energy (Fig. 2.17). This compound eluted slightly later than DTX1 at 11.37 minutes. One cluster of ion fragments showed similarity with that of OA, suggesting their possible relation (Fig 2.18). Accurate mass calculation with MassLynx v4.1 indicated identification as an OA related compound with chemical formula of $C_{53}H_{82}O_{14}$ (Δppm 0.8).

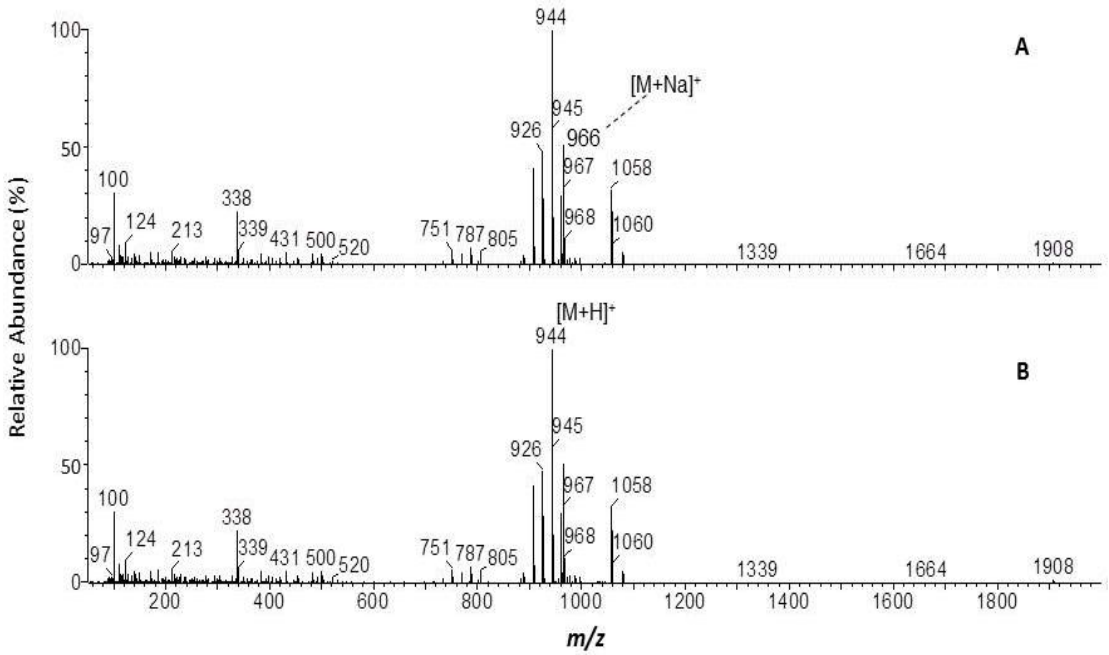


Figure 2.17 Positive ion mass spectra of OA-derivative-2 from methanolic (100%) extract of *P. lima* CCAP 1136/11 at high (A) and low (B) energy levels.

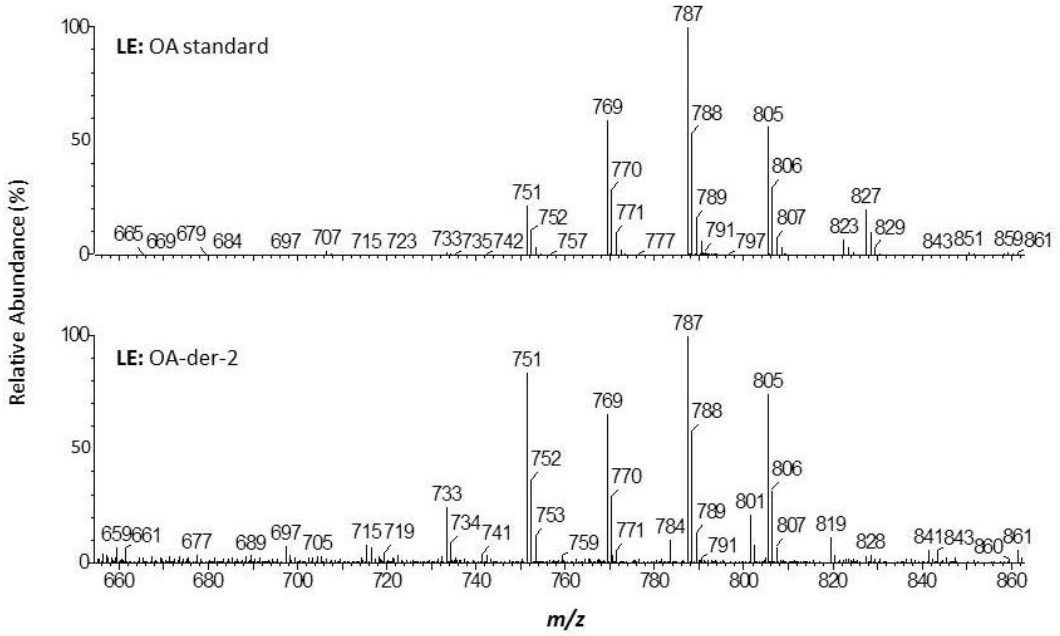


Figure 2.18 Comparison of ion fragmentation cluster in OA-derivative-2 and OA standard analysed by UPLC-MS at low energy level.

Similar to what has been observed for authentic standard (Fig. 2.8), analysis with PDA detector was used to identify the pigment content in methanolic (100%) extract of *P. lima* CCAP 1136/11. Chromatogram of UV-Vis absorbance showed indication of one major pigment peridinin at retention time of 12.64 minutes (Fig. 2.12). The maximum absorbance wavelength of this compound was recorded at 474.5 nm (Fig. 2.19), the same as peridinin standard. This along with the identical retention time to standard confirmed its identification as peridinin.

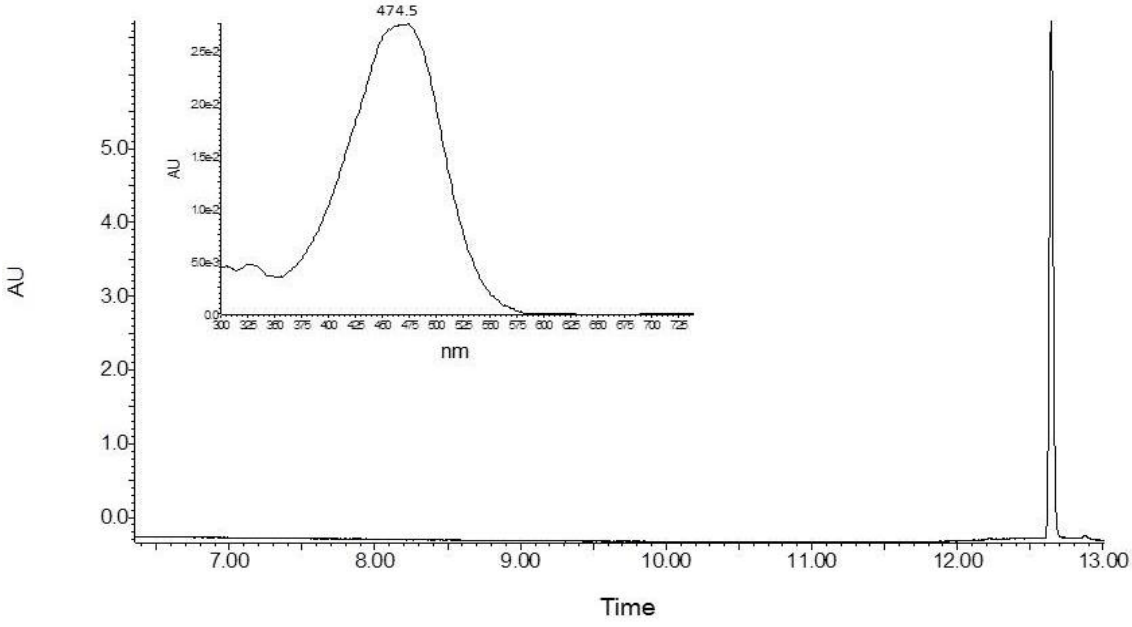


Figure 2.19 Extracted chromatogram of UV-Vis absorbance showing elution of peridinin from methanolic (100%) extract of *P. lima* CCAP 1136/11 with retention time at 12.64 minutes, and maximum absorbance wavelength at 474.5 nm (inserted).

Further research of published literatures indicated possible identification of OA related compounds (OA-derivatives 1 and 2), although no authentic standards were available to confirm this. Several compounds that have been reported to have similar molecular formula and mass to OA-derivative-1 were DTX-2b, DTX-2c and 19-epi-OA (James *et al*. 1997, Draisci *et al*. 1998, Paz *et al*. 2008) (Table 2.8). Meanwhile, OA-derivative-2 was found to share similarities with OA-D9a or

OA-D9b reported in Suzuki and Quilliam (2011), and with 7-hydroxy-2,4-dimethyl-2,4-heptadienyl-OA-ester reported by Paz and co-workers (2007).

Table 2.8 Summary of analytes identified in methanolic (100%) extract of *Prorocentrum lima* CCAP 1136/11.

Compound Possible Identification	Calculated Mass $[M+H]^+$	Observed Mass $[M+H]^+$ (Δppm)
Okadaic acid	$C_{44}H_{68}O_{13}$ (805.4738)	805.4730 (-1.0)
OA-derivative-1 (19-epi-OA, DTX-2b or DTX2c)	$C_{44}H_{68}O_{13}$ (805.4738)	802.4763 (+3.1)
DTX-1	$C_{45}H_{70}O_{13}$ (819.4895)	819.4896 (+0.1)
OA-derivative-2 (OA-D9a, OA-D9b, or 7-hydroxy-2,4-dimethyl-2,4-heptadienyl-OA-ester)	$C_{53}H_{82}O_{14}$ (943.5783)	943.5775 (-0.8)

Possible Identification	Maximum Absorbance Wavelength
Peridinin	474 nm

2.3.4 Optimisation of extraction procedure for the recovery of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Investigation of optimum extraction procedure was first performed by screening different extraction solvent for recovery of okadaic acid (OA) produced by *P. lima* CCAP 1136/11. It appeared that efficiency of recovery of several solvents was influenced by the presence of water, both in the pellets or as a mixture in the solvent. For example 100% concentration of ethanol (EtOH), acetone and

acetonitrile (CH_3CN) were optimum in freeze-thawed pellets, and the addition of water in the solvent at concentration of 25% was shown to improve the recovery in freeze-dried pellets (Fig. 2.20).

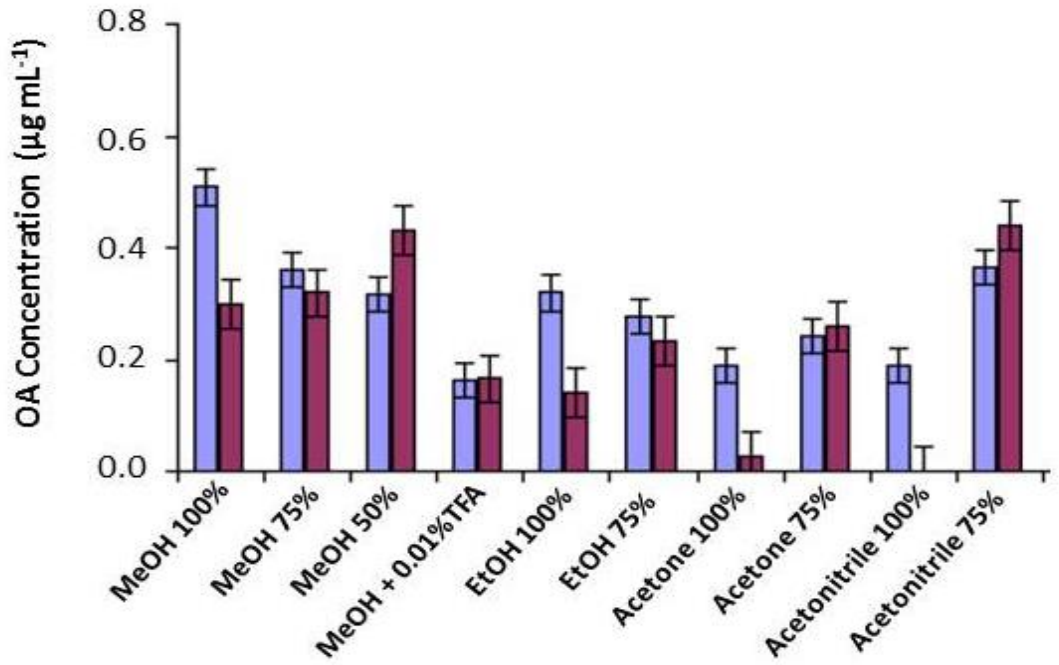


Figure 2.20 Performance of different extraction solvent on okadaic acid recovery from *P. lima* CCAP 1136/11 prepared as freeze-thawed pellets (■) and freeze-dried pellets (■) (n=3, error bars indicate standard deviation).

Good recovery of OA was obtained from three solvents; 100% methanol (MeOH) on freeze-thawed pellets, 50% (v/v) aqueous-MeOH on freeze-dried pellets, and 75% (v/v) aqueous-Acetonitrile (CH_3CN) on freeze-dried pellets. Concentrations of extracted OA were recorded at 0.51, 0.43 and 0.44 µg mL^{-1} for 100% MeOH, 50% MeOH and 75% CH_3CN, respectively (Fig. 2.20). Meanwhile, the lowest concentration of OA recovery from samples was recorded for 100% CH_3CN and acetone, both on freeze-dried pellets. Based on these results, methanol was selected for the subsequent extraction protocol development.

A two-step extraction procedure (illustrated in Fig. 2.21) was designed so that the procedure was able to interface with actual conditions during culture harvesting. During harvesting of cultures, complete removal of water was rarely

achieved. Therefore, the solvent selected should contain water. It was found that Step-1 process, which utilised a lower percentage of MeOH (50%), was optimum for the recovery of DSP toxins (OA and DTX1). During this step, total recovery efficiency for both compounds was observed at 89% for OA and 77% for DTX1. Efficiency of recovery for peridinin at Step-1 was only 34%. In contrast to Step-1, use of higher percentage of MeOH (80%) in Step-2 appeared to increase recovery efficiency for pigment, with 66% of extracted peridinin obtained during this step (Fig. 2.21). Total yield of DSP toxins and peridinin extracted from harvest culture ($2x10^6$ cells mL^{-1}) after two-steps procedure was recorded at approximately 83 $\mu g\ mL^{-1}$ for OA, 20 $\mu g\ mL^{-1}$ for DTX1 and 79 $\mu g\ mL^{-1}$ for peridinin.

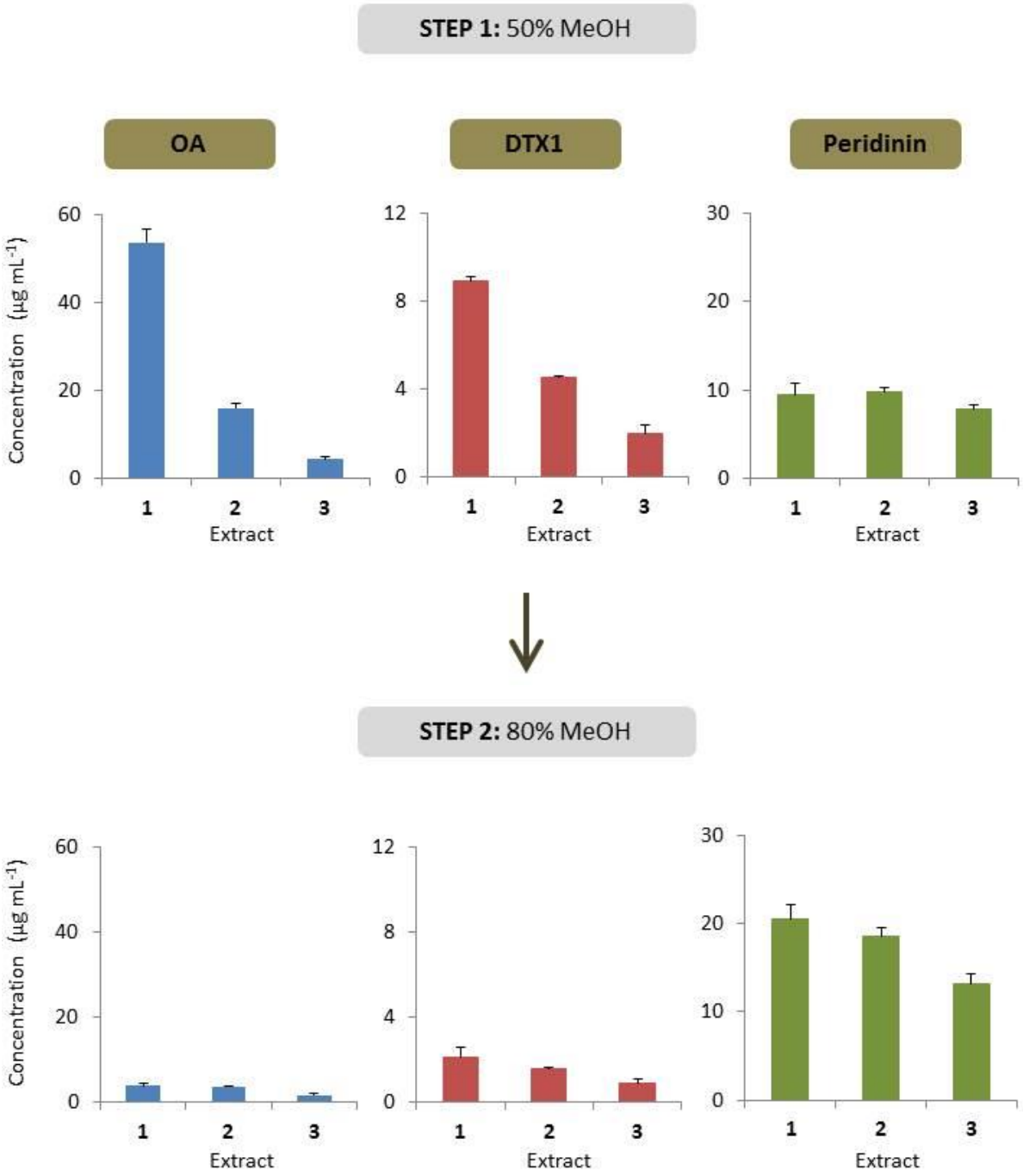


Figure 2.21 Extraction efficiency of 2-stage extraction procedure for the recovery of DSP toxins and peridinin from harvest culture of *Prorocentrum lima* CCAP 1136/11.

2.4 Discussions

This investigation developed routine culturing methods, cell number monitoring, and identification and extraction techniques for DSP toxins and peridinin produced by *P. lima* CCAP 1136/11. These methods are essential to support investigations into optimum culturing conditions for *P. lima* CCAP 1136/11.

2.4.1 Optimisation of automated cell counting analysis for *P. lima* CCAP 1136/11

Flow cytometer is a robust, time efficient and powerful tool for microalgal cell counting analysis. The method is based on the light scattering properties of a single cell entrained in a flow stream (Davey and Kell 1996). The scattered light is detected by photomultipliers or photodiodes and subsequently processed to provide a digital signal representing the number of cells passing through detectors at a certain intake volume (Marie *et al*. 2005). Despite its robustness, its accuracy is rather limited, particularly when used to analyse samples with non-homogenous particles, such as cell aggregates. The formation of cell aggregates is normally observed during the culture of *P. lima* CCAP 1136/11, particularly those having higher cell density. Cell aggregation can be caused by the formation of mucilage, consisting mainly of polysaccharides, at the surface of cell membrane triggered by nutrient stress conditions (Glibert *et al*. 2012). The occurrence of these aggregates during analysis with a flow cytometer often caused underestimation of the actual cell number in the sample. This problem can be prevented by performing cell disaggregation procedures prior to analysis.

Of all the disaggregation procedures tested, it was observed that only sonication produced satisfactory results. The method employs physical forces in the form of ultrasonic waves to separate cells aggregate. It achieves this by generating sound energy transmitted to the treated sample either directly via a probe, or indirectly through water contained in a chamber. As indicated in the results, direct sonication with probe may cause cells to disintegrate (Fig 2.4). Such conditions had also been suggested in previous studies (Reynold and Jaworski 1978, Box 1981). This disintegration of cells resulted in the overestimation of the

actual population size (Fig. 2.2). In contrasts, transmission of energy through medium (water) in bath-sonicator permitted sufficient energy to disrupt the cell aggregate whilst maintaining integrity of cells. Therefore, the use of this procedure can be considered appropriate for further applications for *P. lima* CCAP 1136/11.

Alkaline hydrolysis in both room temperature and 80^{o}C were found to be ineffective for disrupting *P. lima* CCAP 1136/11 aggregates. Alkaline hydrolysis is a denaturation process of complex molecules such as carbohydrates. It has been reported that the process can be used to degrade effectively the main constituent of outer cell wall mucilage, polysaccharide, causing cells to disaggregate (Reynolds and Jaworski 1978). It has also been reported that the effectiveness of this method may be further improved by increasing working temperature to 80^{o}C (Box 1981). However, evaluation on *P. lima* CCAP 1136/11 samples observed that elevating the temperature did not improve the disaggregation of clump. This was possibly caused by the insufficient concentration of alkaline used to disrupt cells aggregate. Nevertheless, increasing the concentration of alkaline may not provide a solution as it can result in cell loss within the sample (Box 1981).

Similar to alkaline hydrolysis, heating treatment at 80^{o}C did not result in reduced cell aggregates within the *P. lima* CCAP 1136/11 sample. Previous studies demonstrated that heating treatment was only effective for limited number of microalgal strains (Humphries and Widjaja 1979, Box 1981, Zohary and Madeira 2001, Benard *et al.* 2004). Yet, the efficacy of heat treatment for these particular strains has never been fully explained. Nevertheless, it may be deduced from the results of some studies (Box 1981, Benard *et al.* 2004) that heat treatment could be rendered less effective if the mucilage had higher heat stability. The results obtained in this study showed that heat treatment is not suitable for *P. lima* CCAP 1136/11, possibly due to higher heat tolerance of mucilage produced by this species.

The use of bath sonication for homogenous cell aggregate separation has improved the reliability of flow cytometer cell counting for *P. lima* CCAP 1136/11. Comparison between flow cytometer and Sedgewick-Rafter counting methods

revealed that both provided similar results (Table 2.4 and 2.5). It may therefore be concluded that either of these methods could be feasibly used for this strain. Nevertheless, the use of flow cytometer is considered more convenient due to it being more time-efficient for handling large quantity of samples.

2.4.2 Evaluation of the use of artificial seawater (ASW) and salt water (StW) for the maintenance of *Prorocentrum lima* CCAP 1136/11 in batch culture

Artificially prepared medium may be preferred over naturally sourced medium as its procurement is more practical and its continuous supply more feasible. The compositions of artificial seawater (ASW) suitable for growing microalgae have been continuously developed and modified. In its early development, ASW was prepared differently to those of natural seawater (NSW). Some basal salts, e.g. calcium and magnesium salts, were not added during the preparation of ASW, as they were deemed to cause precipitation problems (Harrison and Berges 2005). Since the 1980s, studies have developed several procedures (e.g. addition of pH buffer) to avoid the occurrence of such problems in artificial medium (Harrison *et al*. 1980, Keller *et al*. 1987), improving the composition of basal salts added to mimic those contained in natural sources. The use of this improved ASW has been shown to enhance growth of microalgal species that were previously difficult to grow with artificial medium (Berges *et al*. 2001).

The work carried out in this study demonstrated that *P. lima* CCAP 1136/11 was able to grow in all types of prepared medium; NSW, ASW and salt water (StW). However, the use of StW appeared to affect cell integrity in the culture, indicated both by the lack of pigmentation (Fig 2.6) and the high level of OA excretion to the medium (Fig. 2.5). The excretion of OA to medium by this organism is a common indicator of ageing cells (Pan *et al.* 1999) or impaired viability. The omission of several essential ions (e.g. Mg^{2+}, SO_4^{2-} and Ca^{2+}) in StW, which are present in NSW, might have influenced the photosynthesis and other cellular processes in the culture. In contrasts, cultures cultivated in ASW medium displayed growth behaviour commonly observed for healthy cells (i.e. pigmentation and low excretion of OA), confirming that the existence of these

ions within the culture medium is necessary for the photosynthesis and growth of *P. lima* CCAP 1136/11.

The use of ASW as a potential substitute for NSW in long term cultivation was further assessed. The results indicated that despite the significant increase in growth, long term cultivation with ASW decreased the culture pigmentation and OA production (Fig. 2.5 and 2.6). The yield of OA from *P. lima* CCAP 1136/11 grown in ASW was reduced to approximately 70% from the first to second transfers of batch culture. This finding was in agreement to a previous study that reported the reduction of paralytic shellfish poisoning (PSP) toxins production from *Alexandrium tamarense* maintained in ASW (Hsieh *et al.* 2001). The influence of long term use of ASW on the reduction of secondary metabolites production has not been clearly explained. ASW may lack in several trace elements or nutrients that occur in NSW. It has been commented that many of trace elements in NSW are still difficult to measure and identify (Berges *et al.* 2001). Some species may actually be susceptible to omission of those elements. Thus, the comparatively higher OA yield observed in the first transfer to ASW may be attributed to the intracellular retention of these elements, which were later consumed and became depleted during the second transfer (ASW-2nd).

2.4.3 Identification of bioactive compounds from batch culture of *Prorocentrum lima* CCAP 1136/11

The analytical method of LC-MS coupled to UV-Vis photodiode array detection was deployed to examine the composition of metabolites produced by batch culture of *P. lima* CCAP 1136/11. The use of the combined method (UPLC -MS-PDA) in this study has enabled effective parallel detection of DSP toxins and pigment in the sample. A similar approach was also reported by Frassanito and co-workers (2005) for the detection of a wide range of secondary metabolites from different taxa of freshwater microalgae and cyanobacteria. The authors commented that UV-PDA detection was particularly useful to classify different taxonomic composition in mixed population sample by providing information of characteristic accessory pigments (e.g. carotenoid, chlorophylls). In this study, it was found that utilisation of UV-Vis-PDA facilitated the identification and

quantification of major carotenoid pigment, peridinin, that was not easily detected by LC-MS.

Investigation of pigment composition revealed than only peridinin could be identified from *P. lima* CCAP 1136/11 extract. Identification of peridinin in other *P. lima* cultures has been reported for strains isolated from Australia and United Kingdom (Morton and Tindall 1995, Nascimento *et al*. 2005). The presence of other major carotenoids (diadinoxanthin and dinoxanthin) identified in those studies was not detected by the analytical LC-MS-PDA method performed in this study, most likely due to the combination of mobile phase used. Water based mobile phase is not commonly used for identification of pigments due to their hydrophobicity. This was also reflected in the retention time of the pigment, peridinin (12.65 minutes, Fig. 2.8 and 2.12), which was eluted near the gradient wash, i.e. the column was eluted with 100% acetonitrile. It was conceivable that other pigments were washed out of the column during this step therefore they could not be identified from the chromatogram.

Analysis of methanolic (100%) cell extract revealed the existence of two major DSP toxins. Their identities – OA and DTX1 – were confirmed by comparing retention time and spectra to that of standards. OA and DTX1 have been widely reported to be the major DSP toxin produced by *P. lima* species isolated worldwide (Heredia-Tapia *et al*. 2002, Foden *et al.* 2005, Nascimento *et al.* 2005, Vale *et al.* 2009). The presence of another form of dinophysistoxin (DTX2), previously reported from *P. lima* strain isolated from Spain (Hu *et al.* 1992), was not detected in this study. This is in agreement with other studies using the same strain (Bravo *et al*. 2001) and several other strains isolated from Portugal and United Kingdom (Nascimento *et al*. 2005, Vale *et al*. 2009). Thus, it may be surmised that the production of DTX2 by *P. lima* is specific to several strains. Further examination of DSP related compounds discovered an OA-isomer, which was characterised based on the similarity of mass fragmentation to OA (OA-der-1, Fig. 2.12). In addition to DTX2, several other compounds have also been reported to have identical molecular mass with OA, these include: DTX-2b, DTX-2c *and* 19-epi-OA *(*James *et al*. 1997, Draisci *et al*. 199*8,* Paz *et al*. 2008). The presence of these compounds in *P. lima* CCAP 1136/11 culture has not yet been reported in literatures. Moreover, the limited availability of standards has made it

difficult to identify exactly the OA-isomer found in this study. Further research is still required to do so, for instance by purifying the compound to analyse it with nuclear magnetic resonance (NMR) spectroscopy.

In addition to the OA-isomer, identification of compounds from methanolic extract of *P. lima* CCAP 1136/11 revealed another compound similar to OA (OA-der-2, Fig. 2.18). The compound that formed $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 943 and 965 was identified to have the chemical composition of $C_{56}H_{82}O_{14}$ (Δppm -0.8). Its characteristics of molecular mass and composition were found to be very similar to an ester of OA discovered in past studies that used extracts of *Prorocentrum* spp and blue mussel (Hu *et al.* 1992, Paz *et al.* 2007 and Torgensen *et al.* 2008). The possible identity of this compound could be: OA-D9a, OA-D9b or 7-hydroxy-2,4-dimethyl-2,4-heptadienyl-OA-ester (Paz *et al.* 2007, Suzuki and Quilliam 2011). However, analytical standards for these compounds are not available. As such, it was not possible to fully identify the compound. Nevertheless, further investigation into the nature of the compound and its potential uses might be worthwhile.

The existence of many other DSP-toxin related compounds, such as DTX6, norokadanone, OA ester and OA methyl ester, reported in studies using the same strain (Suárez-Gómez *et al.* 2001, Bravo *et al.* 2001, Fernandéz *et al.* 2003, Paz *et al.* 2007), could not be detected in this study. This was potentially caused by the differences in extraction procedures and the solvent used. The applied freeze-thawing process may have allowed enzymatic transformation of OA and DTX1 derivatives into the parent compounds OA and DTX1 (Quilliam and Ross 1996), preventing their detection. Quilliam and co-workers (1996) suggested that this transformation can be prevented by boiling the cells prior to extraction with solvent. The efficacy of boiling procedure for the detection of OA-, DTX1- and DTX2-esters was also demonstrated by Bravo and co-workers (2001). However, the addition of boiling procedure during extraction may be unsuitable when handling large volumes of samples. Moreover, the enzymatic action may actually provide advantage as the transformation of derivative compounds would result in higher yield of the main compounds of interest, OA and DTX1 (Quilliam and Ross 1996). The aforementioned considerations support freeze-thawing as

the preferred method for extraction of DSP toxins and peridinin from *P. lima* CCAP 1136/11 for a large number of sample.

2.4.4 Optimising extraction procedure

The characterisation of metabolites produced from batch culture of *P. lima* CCAP 1136/11 discussed in section 2.4.3 has identified three significant compounds: OA, DTX1 and peridinin. The effective extraction procedure for the recovery of those three compounds was subsequently developed. The procedure was designed with the emphasis on practicality and optimum yield.

Screening of solvents showed that methanolic extraction of freeze-thawed cells was most suitable for the extraction of OA from *P. lima* CCAP 1136/11 sample. Despite the high recovery efficiency of some solvents, extraction of freeze-dried cells was not preferred as the process could be impractical as it requires additional handling step and can be more time consuming than the simple freeze-thawing process. In addition, freeze-dry process produced an inhalable dried form of toxic sample, posing a particular human health risk.

Cell disruption prior to extraction is another important factor to consider to ensure that good recovery is achieved. Effective disruption of cells would ensure that the intracellular compounds become readily extractable and dissolved in the solvent. Disruption of cells can be performed using non-mechanical (e.g. addition of chemicals) or mechanical method (e.g. sonication and bead mill) (Chisti and Moo-Young 1986). It has been demonstrated that mechanical disruption using ultrasonication and French-press were very effective for the recovery of DSP-toxins from *P. lima* cells (Pleasance *et al.* 1990, Quilliam and Ross 1996, Paz *et al.* 2007, Paz *et al.* 2008). However, utilising mechanical disruptor equipment may provide additional costs on the process. Some of these techniques (e.g. French-press) can also be difficult for sample with large volume. This study observed that *P. lima* CCAP 1136/11 cells were sufficiently disrupted by freeze-thawing to successfully recover OA. This process requires minimal sample handling compared to mechanical techniques, thus it is more convenient to perform.

In carrying out a screening for solvents, it was found that application of 100% methanol provided the highest recovery of OA. However, providing 100% concentration of solvent can be impractical as complete removal of water from harvested cells is rarely achieved without further processing. Therefore, incorporation of the water content in final sample should be performed when using methanol extraction. Preliminary evaluation showed that aqueous-methanol at 75 and 50% (v/v) provided efficient OA recovery (Fig. 2.21). However, the 50% methanol may be more cost-efficient due to its lower solvent requirement. Therefore, 50% aqueous-methanol was selected as the primary solvent for the recovery of DSP-toxins from *P. lima* CCAP 1136/11 sample.

Nonetheless, the 50% aqueous-methanol (v/v) mixture would not be suitable for peridinin extraction due to its hydrophobicity. It was thus necessary to select a secondary solvent using methanol at higher percentage (80% v/v). A two-step extraction process was devised that incorporated the use of both solvents. The results in this study demonstrated that optimum recovery of the three major compounds could be achieved by utilising such procedure. The process may also enable early separation of the compounds, as most of DSP-toxins (OA and DTX1) will be collected in a primary solvent (Step-1) while peridinin will be collected in a secondary solvent (Step-2) (Fig. 2.21). The cost and time efficiency of the process can be further improved by utilising only two sequential extractions at each step. Such modification is justifiable as the inability to recover useful amounts of compounds from a third extraction process can be compensated by lower costs and shorter period of extraction.

2.5 Conclusion

The procedures for the maintenance and monitoring of *P. lima* CCAP 1136/11, has been successfully devised. It is concluded that maintenance of *P. lima* CCAP 1136/11 is best performed using naturally sourced seawater (NSW). Artificial seawater (ASW) may only be used temporarily to substitute NSW as its long term replacement would reduce the yield of DSP toxins. The growth of *P. lima* CCAP 1136/11 maintained in batch culture can be conveniently monitored using

flow cytometer. However, the accuracy of flow cytometer reading can be deviated occasionally by the presence of cell clumps in sample, which can be prevented effectively using bath sonication as a disaggregation treatment. The optimum recovery of compounds from *P. lima* CCAP 1136/11 can be achieved by a two-step methanolic extraction process of freeze-thawed cells. The process also enables early separation of DSP toxins and peridinin, which may benefit further purification processes.

The procedures reported here can be considered to be the first attempt to provide a standardised method in the cultivation of *P. lima* CCAP 1136/11 for production of DSP toxins and peridinin. The application of these methods in further optimisation studies highlighted their versatility. Furthermore, these procedures may also be adapted for the production of other high value compounds from other marine dinoflagellates.

2.6 References

Berges, J. A., Franklin, D. J. Harrison, P. J. 2001. Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. **Journal of Phycology**, 37, 1138-1145.

Bernard, C., Monis, P., Baker, P. 2004. Disaggregation of colonies of Microcystis (Cyanobacteria): efficiency of two techniques assessed using an image analysis system. **Journal of applied phycology**, 16 (2), 117-125.

Bravo, I., Fernandez, M. L., Ramilo, I., Martinez, A. 2001. Toxin composition of the toxic dinoflagellate *Prorocentrum lima* isolated from different locations along the Galician coast (NW Spain). **Toxicon,** 39, 1537-1545.

Bouaıcha, N., Chézeau, A., Turquet, J., Quod, J. P., Puiseux-Dao, S. 2001. Morphological and toxicological variability of *Prorocentrum lima* clones isolated from four locations in the south-west Indian Ocean. **Toxicon**, *39*(8), 1195-1202.

Box, J. D. 1981. Enumeration of cell concentrations in suspensions of colonial freshwater microalgae, with particular reference to *Microcystis aeruginosa*. **British Phycological Journal**, 16 (2), 153-164.

Chisti, Y., Moo-Young, M. 1986. Disruption of microbial cells for intracellular products. **Enzyme and Microbial Technology**, 8(4), 194-204.

Davey, H. M., Kell, D. B. 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: The impotance of single-cell analyses. **Microbiological Reviews,** 60 (4), 641-696.

Draisci, R., Giannetti, L., Lucentini, L., Marchiafava, C., James, K. J., Bishop, A. G., Healy, B. M., Kelly, S. S. 1998. Isolation of a new okadaic acid analogue from phytoplankton implicated in diarrhetic shellfish poisoning. **Journal of Chromatography A**, 798, 137-145.

Fernández, J. J., Suárez-Goméz, B., Souto, M. L., Norte, M. 2003. Identification of new okadaic acid derivatives from laboratory cultures of *Prorocentrum lima*. **Journal of Natural Products**, 66, 1294-1296.

Foden, J., Purdie, D. A., Morris, S., Nascimento, S. 2005. Epiphytic abundance and toxicity of *Prorocentrum lima* populations in the Fleet Lagoon, UK. **Harmful Algae**, 4 (6), 1063-1074.

Frassanito, R., Cantonati, M., Tardío, M., Mancini, I., Guella, G. 2005. On-line identification of secondary metabolites in freshwater microalgae and cyanobacteria by combined liquid chromatography-photodiode array detection-mass spectrometric techniques. **Journal of Chromatography A**, 1082, 33-42.

Guillard, R. R L., Morton, S. L., 2003. Culture Methods. In: G.M. Hallegraef, D. M. Anderson, A. D. Cembella, (Eds.). *Manual on Harmful Marine Microalgae.* France: UNESCO Publishing.

Glibert, P. M., Burkholder, J. M., Kana, T. M. 2012. Recent insights about relationships between nutrient availability, forms, and stoichiometry, and the distribution, ecophysiology, and food web effects of pelagic and benthic *Prorocentrum* species. **Harmful Algae**, 14, 231-259.

Harrison, P. J., Waters, R. E., Taylor, F. J. R. 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. **Journal of Phycology**, 16, 28-35.

Harrison, P. J., Berges, J. A. 2005. Marine culture media. In: R. A. ANDERSEN (Ed.), *Algal Culturing Techniques,* London, UK: Elsevier Academic Press. pp. 21-60.

Heredia-Tapia, A., Arredondo-Vega, B. O., Nunez-Vazquez, E. J., Yasumoto, T., Yasuda, M., Ochoa, J. L. 2002. Isolation of *Prorocentrum lima* (Syn. *Exuviaella lima*) and diarrhetic shellfish poisoning (DSP) risk assessment in the Gulf of California, Mexico. **Toxicon**, 40 (8), 1121-1127.

Hsieh, D. P. H., Wang, D., Chang, G. H. 2001. Laboratory bioproduction of paralytic shellfish toxins in dinoflagellates. **Advances in Applied Microbiology,** 49, pp. 85-110.

Hu , T., Marr, J., de Freitas, A. S. W., Quilliam, M. A., Walter, J. A., Wright, J. L. C., Pleasance, S. 1992. New diol esters isolated from cultures of the dinoflagellates *Prorocentrum lima* and *Prorocentrum concavum*. **Journal of Natural Products**, 55 (11), 1631-1637.

Humphries, S. E., Widjaja, F. 1979. A simple method for separating cells of *Microsystis aeruginosa* for counting. **British Phycological Journal**, 14, pp. 313-316.

James, K. J., Carmody, E. P., Gillman, M., Kelly, S. S., Draisci, R., Lucentini, L., Gianneti, L. 1997. Identification of a new diarrhoetic toxin in shellfish using liquid chromatography and mass spectrometric detection. **Toxicon**, 35 (6), 973-978.

Keller, M. D., Selvin, R. C., Claus, W., Guillard, R. R. L. 1987. Media for the culture of oceanic ultraphytoplankton. **Journal of Phycology**, 23, 633-638.

Lawrence, J. E., Grant, J., Quilliam, M. A., Bauder, A. G., Cembella, A. D. 2000. Colonization and growth of the toxic dinoflagellate *Prorocentrum lima* and associated fouling macroalgae on mussels in suspended culture. **Marine Ecology Progress Series**, 201 (9), 147-154.

Lawton, L., Marsalek, B., Padisak, J., Chorus, I. 1999. Determination of cyanobacteria in laboratory. In: CHORUS, I. and BARTRAM, J. *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management.* London: E&FN Spoon on behalf of WHO. pp. 347-367

Lee, Y. K., Shen, H. 2004. Basic culturing techniques. In: A RICHMOND (Ed.), *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*, Oxford, UK: Blackwell Publishing, pp. 40-56.

LeGresley, M., McDermott, G. 2010. Counting chamber methods for quantitative phytoplankton analysis—haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell. *Microscopic and molecular methods for quantitative phytoplankton analysis. UNESCO (IOC Manuals and Guides, nº 55.) (IOC/2010/MG/55), Paris: United Nations Educational, Scientific and Cultural Organization*, 25-30.

Lorenz, M., Friedl, T., Day, J. G. 2005. Perpetual maintenance of actively metabolizing microalgal culture. In: R. A. ANDERSEN (Ed.), *Algal Culturing Techniques,* London, UK: Elsevier Academic Press. pp. 253-267.

Marie, D., Simon, N., Vaulot, D. 2005. Phytoplankton cell counting by flow cytometry. In: R. A. ANDERSEN (Ed.), *Algal Culturing Techniques,* London, UK: Elsevier Academic Press. pp. 253-267.

Morton, S. L., Tindall, D. R. 1995. Morphological and biochemical variability of the toxic dinoflagellate *Prorocentrum lima* isolated from three locations at Heron Island, Australia. **Journal of Phycology**, 31 (6), 914-921.

Nascimento, S. M., Purdie, D. A., Morris, S. 2005. Morphology, toxin composition and pigment content of *Prorocentrum lima* strains isolated from a coastal lagoon in southern UK. **Toxicon**, 45 (5), 633-649.

Pan, Y., Cembella, A. D., Quilliam, M. A. 1999. Cell cycle and toxin production in the benthic dinoflagellate *Prorocentrum lima*. **Marine Biology,** 134, pp. 541-549.

Paz, B., Daranas, A. H., Cruz, P. G., Franco, J. M., Pizzaro, G., Souto, M. L., Norte, M., Fernández, J. J. 2007. Characterisation of okadaic acid related toxins by liquid chromatography coupled with mass spectrometry. **Toxicon**, 50, 225-235.

Paz, B., Daranas, A. H., Cruz, P. G., Franco, J. M., Norte, M., Fernández, J. J. 2008. Identification of 19-*epi*-okadaic acid, a new diarrhetic shellfish poisoning toxin, by liquid chromatography with mass spectrometry detection. **Marine Drugs**, 6, 489-495.

Pleasance, S., Quilliam, M. A., de Freitas, A. S. W., Marr, J. C., Cembella, A. D. 1990. Ion-spray mass spectrometry of marine toxins II. Analysis of diarrhetic shellfish toxins in plankton by liquid chromatography/mass spectrometry. **Rapid Communications in Mass Spectometry**, 4 (6), 206-213.

Quilliam, M. A., Ross, N. W. 1996. Analysis of diarrhetic shellfish poisoning toxins and metabolites in plankton and shellfish by ionspray liquid chromatography-mass spectrometry. In: A. P. Snyder (Ed.), *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectometry*, Washington DC: American Chemical Society, ACS Symposium Series 619, pp. 351-364.

Reynolds, C. S., Jaworski, G. H. M. 1978. Enumeration of natural *Microsystis* populations. **British Phycological Journal**, 13, pp. 269-277.

Torgensen, T., Miles, C. O., Rundberget, T., Wilkins, A. L. 2008. New esters of okadaic acid in seawater and blue mussels (*Mytilus edulis*). **Journal of Agricultural and Food Chemistry**, 56, 9628-9635.

Vale, P., Veloso, V., Amorim, A. 2009. Toxin composition of a *Prorocentrum lima* strain isolated from the Portuguese coast. **Toxicon**, 54 (2), 145-152.

Zohary, T., Madeira, A. M. P. 1987. Counting natural populations of *Microcystis aeruginosa*: a simple method for colony disruption into single cells and its effect on cell counts of other species. **Journal of the Limnological Society of Southern Africa**, 13 (2), 75-77.

CHAPTER 3

Effect of Carbon and Trace Metal Composition on the Growth and Production of DSP toxins and Peridinin in *Prorocentrum lima*

3.1 Introduction

The ability of microalgae to grow under phototrophic or heterotrophic conditions determines the selection of cultivation system, which, in turn, affects the economic valuation of the marketable products. Many novel products from algae that have been identified failed to compete in the marketplace because the high production costs were not perceived to be economically viable (Behrens 2005). Large scale cultivation of phototrophs can be performed in either a photobioreactor (PBR), which receives light from an artificial source, or in an open pond, which uses natural solar light. Compared to an open pond, PBR offers advantages of low requirement of space, and protection from contamination (Pulz 2001, Tredici 2004). However, cultivating with PBR can sometimes be limited by the difficulties in maintaining light and CO_2 distribution, particularly in cultures with high cell density (Javanmardian and Palsson 1991, Carvalho *et al*. 2006). Moreover, the measures applied to address these problems can be expensive (Lee 2001).

Heterotroph cultivation with fermenters is often preferred due to its cost efficiency, well established technology, more controllable system and extensive availability (Apt and Behrens 1999, Behrens 2005, Brennan and Owende 2009). Moreover, its potential for parallel application with large volume wastewater treatment adds to the novelty of the system (Perez-Garcia *et al.* 2011). However, most commercial algae are obligate phototrophs that cannot be grown heterotrophically.

The growth of algae does not only depend on essential macronutrients but also on the availability of micronutrient metals, such as iron, manganese, zinc, cobalt, copper and molybdenum. In microalgae, trace metals hold a significant role in numerous metabolic functions necessary for the utilisation of essential resources such as light and macronutrients (Sunda *et al*. 2005). Moreover, the use of metals in a culture medium at different trace concentrations has been shown to affect both growth and secondary metabolite production in dinoflagellate cultures, in which a species can have its own particular requirement for trace metals (Rabsch *et al.* 1984, Doucette and Harrison 1990, Mitrovic *et al.* 2004, Rhodes *et al.* 2006, He *et al.* 2010). For instance, a study from Rhodes and co-

workers (2006) showed that addition of selenium to at concentration of 0.1 μmol L^{-1} produced an increase of OA ester quota per cell in *P. lima* culture (isolated from New Zealand) by approximately 50%. Nevertheless, the study did not discuss further how modification of this trace metal could affect the strategy for mass production of these compounds. The effects of trace metals on dinoflagellate culture and their implications for mass cultivation and bioactive metabolite production strategies still require further exploration. The research presented in this chapter aims both to explore the possibility of growing *P. lima* CCAP 1136/11 in a heterotrophic cultivation system, and to evaluate the potential of enhancing growth and production of DSP toxins and peridinin by alteration of trace metal levels. Their implications for large scale cultivation are also discussed.

3.2 Materials and Methods

3.2.1 Elucidation of growth characteristics of *Prorocentrum lima* CCAP 1136/11 in batch culture

Three replicate batch cultures were prepared by adding 100 mL of f/2-Si medium into 250 mL Erlenmeyer flask. The medium was then autoclave-sterilised (121^{o}C, 20 min). Under aseptic conditions, flasks containing sterile medium were inoculated with stock culture of *P. lima* CCAP 1136/11 to achieve starting cell density of approximately $1x10^4$ cells mL^{-1}. Sampling of cultures was performed every week for the period of six weeks. During each sampling, aliquots of sample (2 mL) were taken, with 1 mL used for the analysis of cell number and 1 mL was used for determination of DSP toxins concentration. Cell counting was performed using Sedgewick-Rafter counting slides as described in Chapter 2 (section 2.2.3.1). Production of OA, DTX1 and peridinin was monitored using UPLC-MS-PDA analysis as described in Chapter 2 (section 2.2.5).

3.2.2 Evaluation of heterotrophic cultivation for *Prorocentrum lima* CCAP 1136/11 in batch culture.

Unialgal cultures of *P. lima* CCAP 1136/11, maintained as previously described (Chapter 2, section 2.2.2), were used as a stock culture for all experimental studies described here. Based on the typical growth curve established previously (section 3.2.1), actively growing stock culture (100 mL) of 28-30 days-old was inoculated into 900 mL sterile f/2-Si medium contained in 2 L Erlenmeyer flask to provide starting cell density of approximately 1-2 $x10^4$ cells mL^{-1}. Inoculation was performed aseptically in a laminar flow cabinet (Microflow® Biological Safety Cabinet Class I, Astec, UK). A sterilised sampling tube and a sterile syringe were prepared in each flask to allow withdrawal of samples during sampling.

Two sets of experimental cultures – referred as *phototroph* and *heterotroph* cultures – were prepared. Under phototrophic conditions, energy was supplied by means of artificial light to allow the fixation of inorganic carbon available in air supplied to the culture. For this, triplicate experimental flasks were continuously exposed to 25±2 µmol m^{-2} s^{-1} light provided by cool white fluorescent tubes. Cultures were grown at constant temperature (23±2°C) and continuously sparged with filtered (0.22 µm; Millipore, UK) ambient air provided by a pump (Fisherbrand, Fisher Scientific, UK). Under heterotrophic conditions, carbon was supplied by adding organic stock solution (modified from Guillard 1960; Table 3.1) to f/2-Si enriched natural seawater medium at a final concentration of 10% (v/v), as suggested by National Center for Marine Algae and Microbiota (NCMA) in their f/2+NPM medium recipe for growing heterotrophic microalgae. Chemicals for organic stock solution were supplied from Fisher Scientific (Loughborough, UK) and Sigma-Aldrich (Poole, UK). Triplicate heterotrophic cultures were maintained under continuous dark cycle at 23±2°C for period of two weeks, with aeration provided as described previously. However, due to the observed continuous decline of cell density, the cultures were then transferred into phototrophic condition in order to revive their growth. For this, cells from heterotrophic flasks were transferred into new flasks containing freshly sterilised f/2-Si medium as prepared for phototrophic culture. These cultures were then maintained under continuous light cycle (25±2 µmol m^{-2} s^{-1}) with cultivation conditions and aeration as described previously.

Table 3.1 Organic stock solution for f/2+NPM medium (Guillard 1960, with modification suggested by National Center for Marine Algae and Microbiota (NCMA)).

Component	Concentration in stock solution
Sodium acetate	1.00 g L^{-1}
Glucose	6.00 g L^{-1}
(di-) sodium succinate · $6H_2O$	3.00 g L^{-1}
Neopeptone	4.00 g L^{-1}
Bacto-tryptone	1.00 g L^{-1}
Yeast extract	0.10 g L^{-1}

Growth was monitored by sampling the culture every week during the cultivation period. Growth was expressed as the number of cells per volume of culture (cell mL^{-1}). Aliquot of sample (1 mL) was taken for flow cytometer analysis using the method established and described in Chapter 2 (section 2.2.3.2). Throughout the experiment, the sensitivity of flow cytometer was regularly monitored by random checking of the sample with Sedgewick-Rafter counting (Chapter 2, section 2.2.3.1).

3.2.3 Evaluation of the influence of trace metal content on growth and production of DSP toxins and peridinin in *P. lima* CCAP 1136/11

3.2.3.1 Modification of trace metal content

Evaluation of the effects of different trace metal content was performed by modifying the composition of trace metal stock solution of common f/2-Si medium. In the original recipe, trace metal stock solution was prepared by adding six elements (iron (Fe), copper (Cu), zinc (Zn), cobalt (Co), manganese (Mn), molybdenum (Mo)) at varying concentration (Table 3.2). Each element was modified to provide concentration at 25%, 50%, 125%, 150% and 200% of the level suggested in recipe (Table 3.2). In addition to these elements, another

metal, selenium (Se), which is not a normal constituent in f/2-Si medium recipe, was also tested. Selenium was added at 50%, 100%, 150% and 200% of the level suggested in another common type of medium for growing *P. lima*; K-min (Keller *et al.* 1987 with modification suggested by Culture Collection of Algae and Protozoa (CCAP), Oban UK). During each experiment, only one element was modified at a time (refer to detailed composition presented in Appendix IV). Control culture was prepared using trace metal stock solution as suggested for f/2-Si medium.

Table 3.2 Experimental concentration of trace metals in culture medium

Element	Final concentration in culture medium (µmol L^{-1})					
	Control	Modification				
		25%	50%	125%	150%	200%
Iron $FeCl_3.6H_2O$	11.7000	2.9250	5.8500	14.6250	17.5500	23.4000
Copper $CuSO_4.5H_2O$	0.0393	0.0098	0.0197	0.4910	0.0590	0.0786
Zinc $ZnSO_4.7H_2O$	0.0765	0.0191	0.0383	0.0956	0.1148	0.1530
Cobalt $CoCl_2.4H_2O$	0.0420	0.0105	0.0210	0.0525	0.0630	0.0840
Manganese $MnCl_2.4H_2O$	0.9100	0.2275	0.4550	1.1375	1.3650	1.8200
Molybdenum $NaMoO_4.2H_2O$	0.0260	0.0065	0.0130	0.0325	0.0390	0.0520
	Control	**50%**	**100%**	**150%**	**200%**	-
Selenium Na_2SeO_3	-	0.0058	0.0116	0.0173	0.0231	-

Note: Modification for all elements was made according to suggested level in f/2-Si medium recipe, except for selenium (Se) which was modified according to suggested level in K-min medium.

3.2.3.2 Culture set-up for rapid screening of trace metal content

Experimental cultures were first prepared by inoculating 5 mL *P. lima* CCAP 1136/11 stock culture (described in section 3.2.2) into 43 mL sterile medium contained in a 100 mL Erlenmeyer flask, to achieve final cell density of 1-2 $x10^4$ cells mL^{-1}. The cultures were then transferred to a Corning® Costar® 24-well cell culture plate (Sigma-Aldrich, UK), with each well contained 2 mL of culture. The plates were then maintained at constant temperature (21±2°C) with continuous light exposure at 23±2 µmol m^{-2} s^{-1} provided by cool-white fluorescence lamp.

Growth and metabolite production were monitored by sampling the culture every week for the period of five weeks. Three wells were sacrificed for each sampling, representing triplicate samples. The culture volume taken for sampling was 1.5 mL per well, of which 0.5 mL was used for cell density analysis with flow cytometer (as described in Chapter 2, section 2.2.3.2) and 1 mL was used for the analysis of DSP toxins and peridinin.

Based on the previous result of cultures grown with natural seawater (Chapter 2, Fig. 2.5) and consistent retention of OA within cells (approximately 99% OA retained during the first 5 weeks of cultivation), all subsequent analyses of DSP toxins and peridinin contents in the culture were based on intracellular extract. For this, 1 mL of sample was centrifuged (10 min; 10,800 g) and then the supernatant was removed from the cells. Freeze-thawed pellets were extracted with 200 µL of MeOH (100%). Extraction was performed in the dark for the duration of at least one hour. The extracts were then centrifuged (10 min; 10,800 g) and the supernatant was analysed using UPLC-PDA-MS method described previously (Chapter 2, Section 2.2.5).

3.2.4 Further investigation on iron (Fe) supplementations

Further investigation was carried out, based on the results obtained from the evaluation of trace metal modification described in the previous section, to test the effect of iron supplementation on the enhancement of DSP toxins and peridinin yields from *P. lima* CCAP 1136/11 culture. Six trace metal stock

solutions were prepared by adding iron at final concentration of 150%, 200%, 250%, 300%, 350% and 400% of the original iron level suggested in f/2-Si medium recipe (Table 3.2). The trace metal stock solutions were then used to enrich the culture medium already containing nutrients and vitamins according to f/2-Si medium recipe.

Triplicate cultures were prepared by inoculating 10 mL stock culture (maintained as described in Chapter 2 section 2.2.2) into 90 mL of freshly sterilised medium contained in 250 mL Erlenmeyer flask, to provide a starting cell density of 1-2 $x10^4$ cells mL^{-1}. Cultures were grown for a period of five weeks in a temperature controlled room at 21±2°C with continuous light exposure at 23±2 µmol m^{-2} s^{-1}. After 5 weeks of cultivation, the cultures were then re-inoculated into second batch of sterile medium containing the same iron level as the first batch. This was performed in order to test the long term adaptation to excess iron. Starting cell density and cultivation conditions for the second batch were consistent with the first batch.

3.2.5 Statistical Analysis

Results are expressed as means of triplicate samples ± standard deviation. Error bars in figures also denote standard deviation. Statistical analysis for data was performed using SPSS 17.0 software. Distribution test for normality was performed using Kolmogorov-Smirnov and Shapiro-Wilk tests. Significant differences within and between treatments were performed using parametric and non-parametric tests (one-way ANOVA and post-hoc Tukey's test, or Kruskal-Wallis test).

3.3 Results

3.3.1 Elucidation of growth characteristics of *Prorocentrum lima* CCAP 1136/11 in batch culture

In order to investigate the growth characteristics of *P. lima* CCAP 1136/11, a growth experiment was performed with weekly monitoring of cell number using

Sedgewick-Rafter analysis (Fig 3.1). The culture was examined to ensure that only actively growing cells were used in future experiment. In addition, production of secondary metabolites (OA and DTX1) was also monitored.

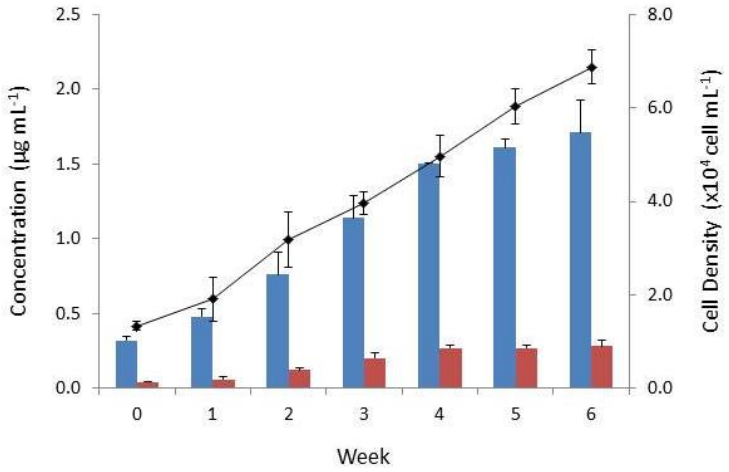


Figure 3.1 Growth and secondary metabolites production from *Prorocentrum lima* CCAP 1136/11 in batch culture (n=3); cell density (◆) and concentration of OA (■) and DTX1 (■).

It was observed that the *P. lima* CCAP 1136/11 showed slow but steadily increasing cell number throughout the six weeks of cultivation, with average cell division of 0.13 per day. There was no indication of stationary growth or decreasing cell numbers throughout the experimental period. Production of OA and DTX1 were observed to steadily increase in the first four weeks, after which the concentrations were observed to be consistent throughout the rest of cultivation period. Typical cell quota of OA and DTX1 at the end of cultivation period were observed at approximately 26 and 4 pg cell^{-1}, respectively.

3.3.2 Evaluation of heterotrophic growth of *Prorocentrum lima* CCAP 1136/11

Evaluation of carbon utilisation revealed that *P. lima* CCAP 1136/11 was not able to grow under heterotrophic condition. This was indicated by the continuous decline of cell density (Fig. 3.2). After two weeks of cultivation in the dark, cell number in the heterotrophic cultures was significantly ($p<0.05$) reduced by approximately 70%. Due to this significant reduction, heterotrophic cultures were then transferred into phototrophic conditions in order to test their capability to

recover. It was further revealed that cells, originally cultured in heterotrophic conditions, were not able to revive after transition to light. Sampling on the third week of cultivation showed that cell yield in this culture was 95% lower than those in phototrophic culture. Average cell densities after three weeks of cultivation were noted at $3.6x10^4$ and $0.19x10^4$ cells mL^{-1} for phototrophic and heterotrophic conditions, respectively.

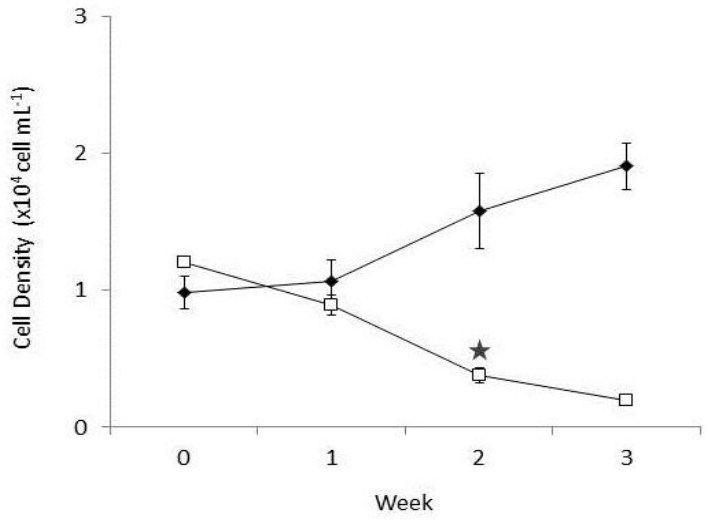


Figure 3.2 *P. lima* CCAP 1136/11 growth under phototrophic (◆) and heterotrophic (□) conditions (n=3). Star sign denotes the point where heterotrophic cultures were transferred into conditions similar to phototrophic.

3.3.2 Effects of trace metals on the growth and production of DSP toxins and peridinin in *P. lima* CCAP 1136/11.

Investigation of cell number revealed that modification of trace metal content in the medium has generally resulted in a reduction of growth of *P. lima* CCAP 1136/11 in batch culture (Fig. 3.3). However, cultures with supplementations of iron (150%, 200%) and manganese (125%, 200%), and limitation of cobalt (25%, 50%) did not produce significant difference ($p>0.05$) of cell density to those measured in control. The inhibition of growth in excess cobalt and any modifications of zinc were observed to occur faster than those observed in selenium, copper and molybdenum. This may indicate that growth of *P. lima* CCAP 1136/11 was particularly sensitive to changes in the two metal concentrations.

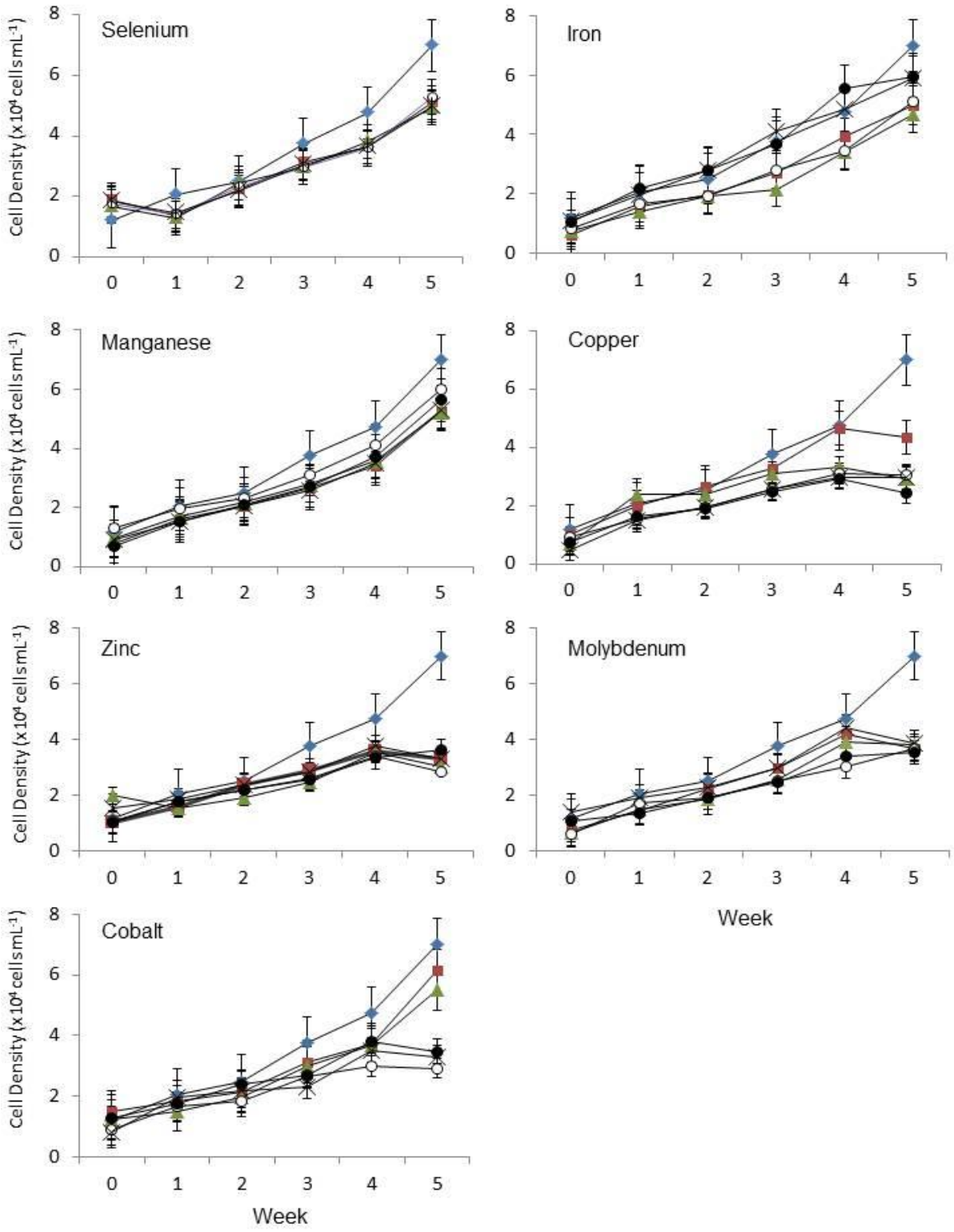


Figure 3.3 Growth of *Prorocentrum lima* CCAP 1136/11 (n=3) under varying concentrations of trace metals; control (f/2-Si medium) (◆) and modification at 25% (■), 50% (▲), 125% (○), 150% (**X**) and 200% (●). Except for selenium which was modified at 50% (■), 100% (▲), 150% (○) and 200% (**X**) from the level in K-min medium.

Despite the severe reduction in cell yields, modifications of copper, zinc, molybdenum and cobalt did not produce substantial difference on DSP toxins production (Fig. 3.4 and 3.5). The exceptions to this were: 1) limitation of copper that produced significantly higher ($p<0.05$) OA (by 18%) and DTX1 (by 57%), and 2) modification of cobalt at 125%, which produced significantly lower ($p<0.05$) DTX1 (by 42%) than the control culture (Fig. 3.5). These patterns clearly identified that suppression of cell growth with modifications of the four metals (Cu, Zn, Mo and Co) induced higher toxins quota per cell in the culture, by approximately 88% to 130% higher than control cultures (Table 3.3). Selenium and manganese treatments had little effect on the yield of OA and DTX1 in *P. lima* CCAP 1136/11 culture, corresponding to the values observed in cell yields. In contrast, supplementation of iron (150%, and 200%), which had no significant effect on growth, appeared to enhance the concentrations of OA and DTX1 in the culture by approximately 26% for OA (Fig. 3.4) and 22-34% for DTX1 (Fig. 3.5).

Production of peridinin (Fig. 3.6) generally reflected the effects of trace metal on growth, for which the modification of several metals such as manganese, copper, zinc, molybdenum and cobalt generally resulted in lower yield of peridinin in *P. lima* CCAP 1136/11 culture. Reduction of peridinin yield for these metals was observed to be approximately 22-46% for Mn, 10-60% for Cu, 18-28% for Zn, 20-31% for Mo and 11-60% for Co. These reductions were generally apparent only during the last one to two weeks of cultivation period. Moreover, there was no observable significant difference of peridinin yield observed in the modification of selenium and iron, with the exception of iron supplementation at 125% that produced 11% lower peridinin yield than those in control.

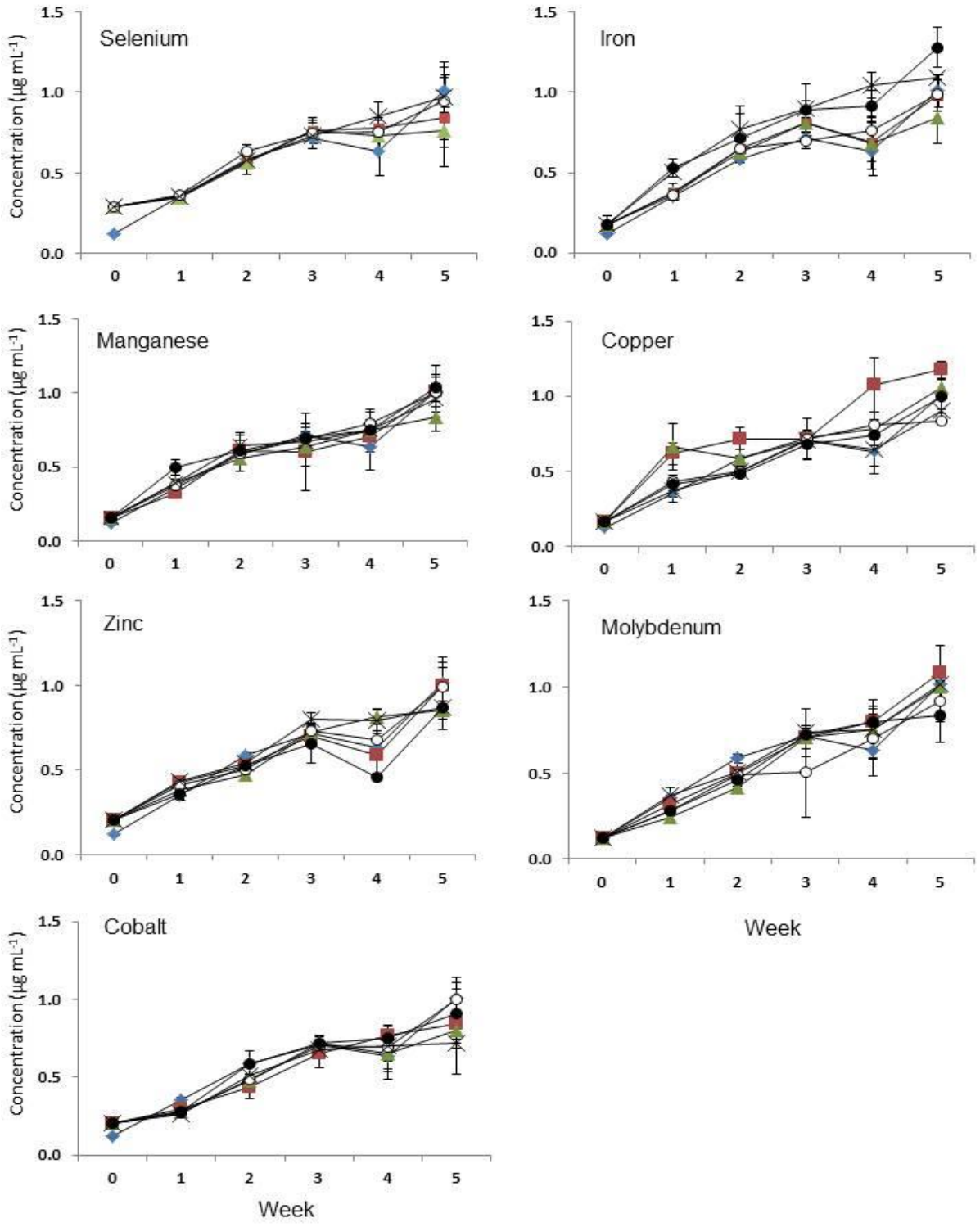


Figure 3.4 Okadaic acid production from *Prorocentrum lima* CCAP 1136/11 (n=3) under varying concentrations of trace metals; control (f/2-Si medium) (◆) and modification at 25% (■), 50% (▲), 125% (○), 150% (**X**) and 200% (●). Except for selenium which was modified at 50% (■), 100% (▲), 150% (○) and 200% (**X**) from the level in K-min medium.

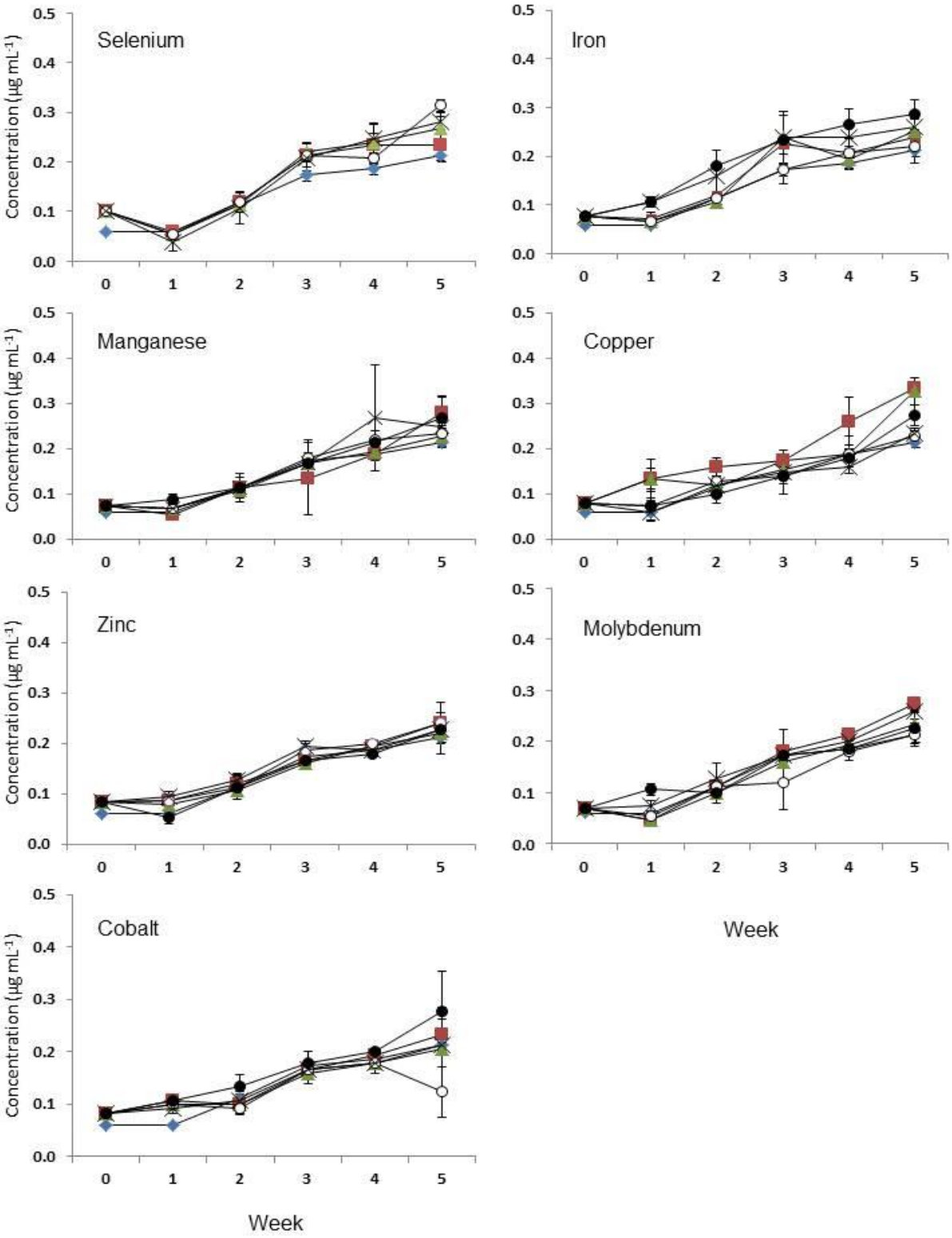


Figure 3.5 DTX1 production from *Prorocentrum lima* CCAP 1136/11 (n=3) under varying concentrations of trace metals; control (f/2-Si medium) (◆) and modification at 25% (■), 50% (▲), 125% (○), 150% (**X**) and 200% (●). Except for selenium which was modified at 50% (■), 100% (▲), 150% (○) and 200% (**X**) from the level in K-min medium.

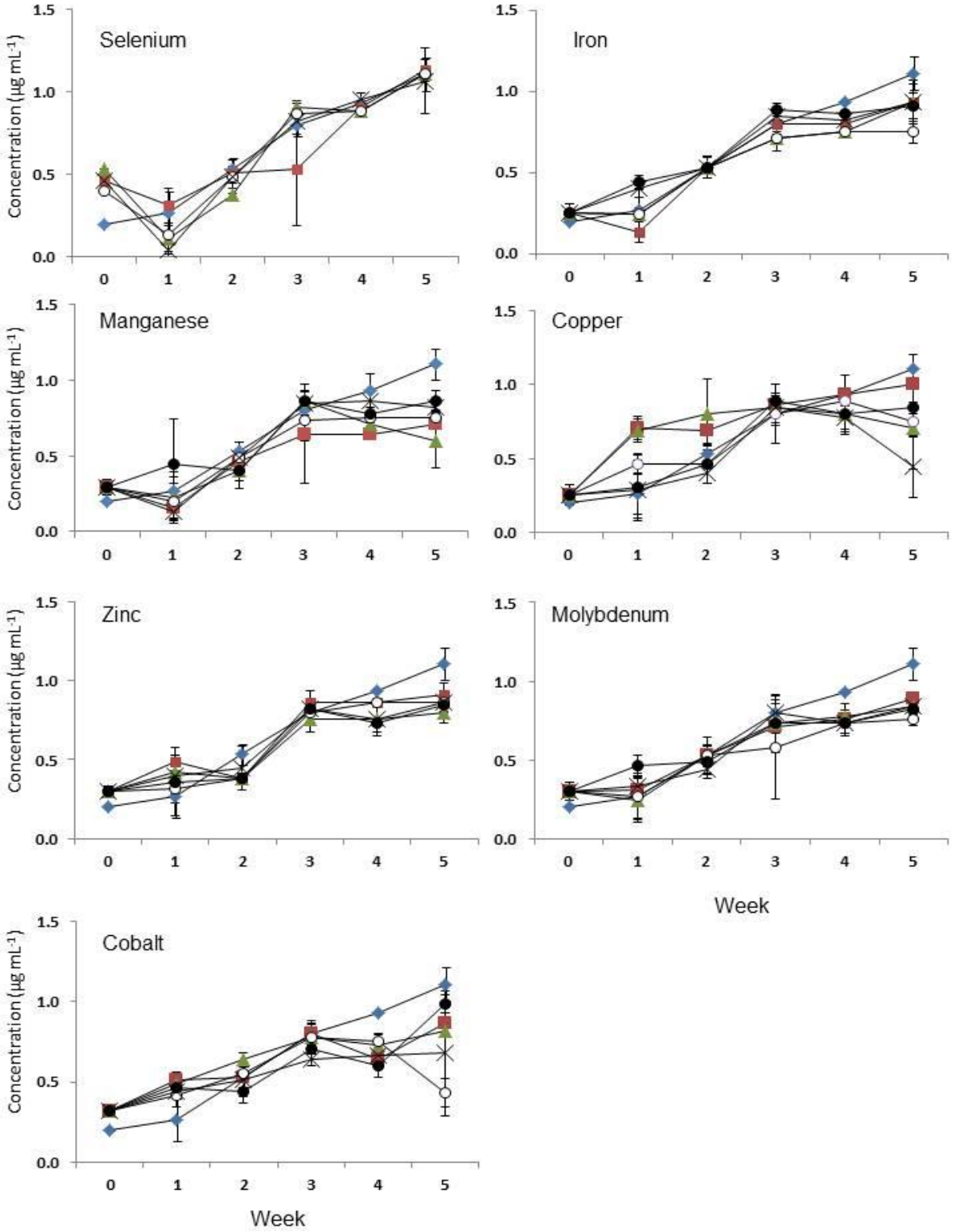


Figure 3.6 Peridinin production from *Prorocentrum lima* CCAP 1136/11 (n=3) under varying concentrations of trace metals; control (f/2-Si medium) (◆) and modification at 25% (■), 50% (▲), 125% (○), 150% (**X**) and 200% (●). Except for selenium which was modified at 50% (■), 100% (▲), 150% (○) and 200% (**X**) from the level in K-min medium.

Table 3.3 Cell quota (pg cell^{-1}) for OA, DTX1 and peridinin after 5 weeks of cultivation at different modification of trace metals (n=3). Numbers in bold denotes values that are significantly different ($p<0.05$) than control.

Metal	Control (0%)	50%	100%	150%	200%	
Selenium						
OA	14.49 ± 3.05	16.57 ± 6.08	15.52 ± 3.58	17.92 ± 4.55	19.53 ± 3.24	
DTX-1	3.05± 0.57	**4.57 ± 0.62**	**5.40 ± 0.80**	**5.93 ± 0.27**	**5.62 ± 0.68**	
Peridinin	12.63±1.34	14.16±2.07	14.71±0.86	14.80±2.33	14.40±2.25	

Metal	25%	50%	Control (100%)	125%	150%	200%
Iron						
OA	17.89 ± 3.42	18.14 ± 4.92	14.49 ± 3.05	**19.33 ± 1.14**	18.59 ±2.20	**21.48 ± 1.79**
DTX1	4.81 ± 3.05	**5.43 ± 0.41**	3.05 ± 0.57	4.31 ± 0.82	**4.42 ± 0.52**	**4.81 ± 0.16**
Peridinin	13.24±1.11	12.72±1.93	12.63±1.34	11.28±0.73	11.73±0.58	12.74±1.14
Manganese						
OA	19.35 ± 3.79	15.96 ± 2.18	14.49 ± 3.05	16.62 ± 3.56	18.05 ± 0.97	18.35 ± 1.52
DTX1	**5.35 ± 0.77**	**4.31 ± 0.41**	3.05 ± 0.57	3.88 ± 0.72	**4.64 ± 0.63**	**4.47 ± 0.55**
Peridinin	10.67±1.25	10.63±0.86	12.63±1.34	9.03±1.35	11.43±0.79	11.85±1.35
Copper						
OA	**27.23 ± 1.88**	**34.10 ± 5.17**	14.49 ± 3.05	16.62 ± 3.55	18.05 ± 4.69	18.35 ± 10.85
DTX1	**7.69 ± 0.44**	**11.21 ± 0.16**	3.05 ± 0.57	3.88 ± 1.36	**4.64 ± 0.58**	4.74 ± 1.95
Peridinin	**19.13±0.69**	**25.95±3.25**	12.63±1.34	19.88±5.57	**22.71±2.78**	**29.25±3.19**
Zinc						
OA	**30.32 ± 3.74**	**28.21 ± 1.35**	14.49 ± 3.05	**34.92 ± 5.38**	**26.42 ± 1.40**	**24.04 ± 3.82**
DTX1	**7.28 ± 1.34**	**7.27 ± 0.58**	3.05 ± 0.57	**8.44 ± 1.25**	**6.91 ± 0.43**	**6.29 ± 1.01**
Peridinin	**19.88±2.82**	**19.45±2.38**	12.63±1.34	**22.37±1.32**	**19.40±3.44**	**17.20±2.10**
Molybdenum						
OA	**29.54 ± 5.48**	**26.39 ± 1.63**	14.49 ± 3.05	**24.95 ± 4.21**	**26.21 ± 2.43**	**23.53 ± 5.68**
DTX1	**7.48 ± 0.64**	**6.16 ± 0.02**	3.05 ± 0.57	**5.83 ± 0.93**	**6.72 ± 0.15**	**6.40 ± 0.66**
Peridinin	**18.91±0.84**	**15.32±0.41**	12.63±1.34	**14.00±0.98**	**16.55±1.43**	**17.65±0.62**
Cobalt						
OA	13.76 ± 1.66	14.55 ± 2.99	14.49 ± 3.05	11.23 ± 3.72	**21.27 ± 6.67**	**31.45 ± 8.06**
DTX1	3.79 ± 0.35	3.76 ± 0.57	3.05 ± 0.57	2.81 ± 0.60	**6.52 ± 4.16**	**8.00 ± 0.93**
Peridinin	**9.65±1.22**	10.26±0.70	12.63±1.34	**26.05±1.64**	**22.82±5.22**	**19.82±2.94**

*Numbers are means of triplicate; (±) denotes standard deviation (SD).

3.3.3 Effect of adaptation to excess iron conditions in *P. lima* CCAP 1136/11 culture

In order to investigate the effect of adaptation to excess iron conditions, cultivation of *P. lima* CCAP 1136/11 was performed in two sequential batches (Fig. 3.7). It appeared that, within the same batch, growth of *P. lima* CCAP 1136/11 was not affected by addition of iron up to 400% of the control concentration (f/2-Si medium). The cell yield differences during the last two weeks of cultivation in both the first batch and second batch were observed to be statistically insignificant ($p>0.05$). However, the growth pattern of culture in the two batches was observed to be different. Cultures in the second batch exhibited slower growth during the first weeks of cultivation, which was observed to be consistent for all modifications (Fig. 3.7). Average final cell density observed in the two batches were $5.3x10^4$ cells mL^{-1} for the first batch and $4.3x10^4$ cells mL^{-1} for the second batch. It was noted that during the inoculation of the second batch, the cells were one week older than when they were inoculated to the first batch. This may explain the longer lag-phase of growth for cells in the second batch.

The insignificant effects of excess iron on growth of *P. lima* CCAP 1136/11 was also reflected in their production of DSP toxins and peridinin. It was observed that, within the same batch, yield of OA, DTX1 and peridinin after five weeks of cultivation in all iron modifications were not significantly different ($p>0.05$) to those in control culture (Fig. 3.8). However, OA yield in the second batch of culture appeared to be lower (by approximately 35%) than those measured in the first batch (Fig. 3.8). The reduction of OA between sequential batches may be associated with the lower cell density measured previously (Fig. 3.7). In contrast, the yields of DTX1 and peridinin were similar between the first and second batches.

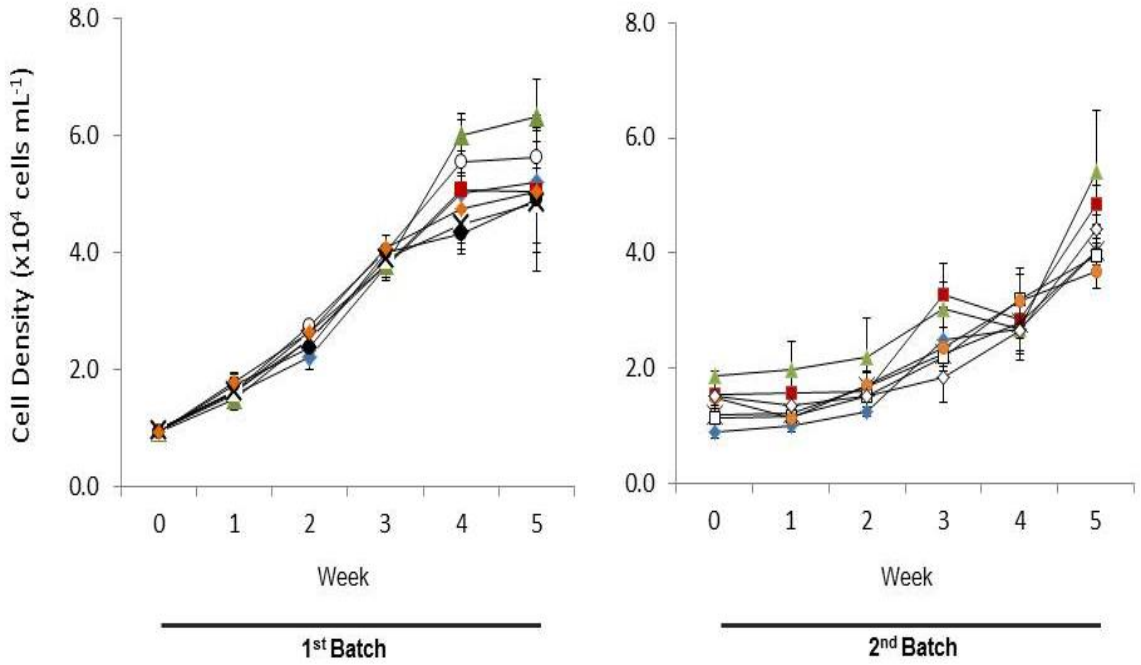


Figure 3.7 Growth of *Prorocentrum lima* CCAP 1136/11 under excess supplementation of iron (n=3, error bars denotes standard deviation); control (f/2-Si medium) (◆) and modification at 150% (■), 200% (▲), 250% (○), 300% (**X**), 350% (●) and 400% (◆).

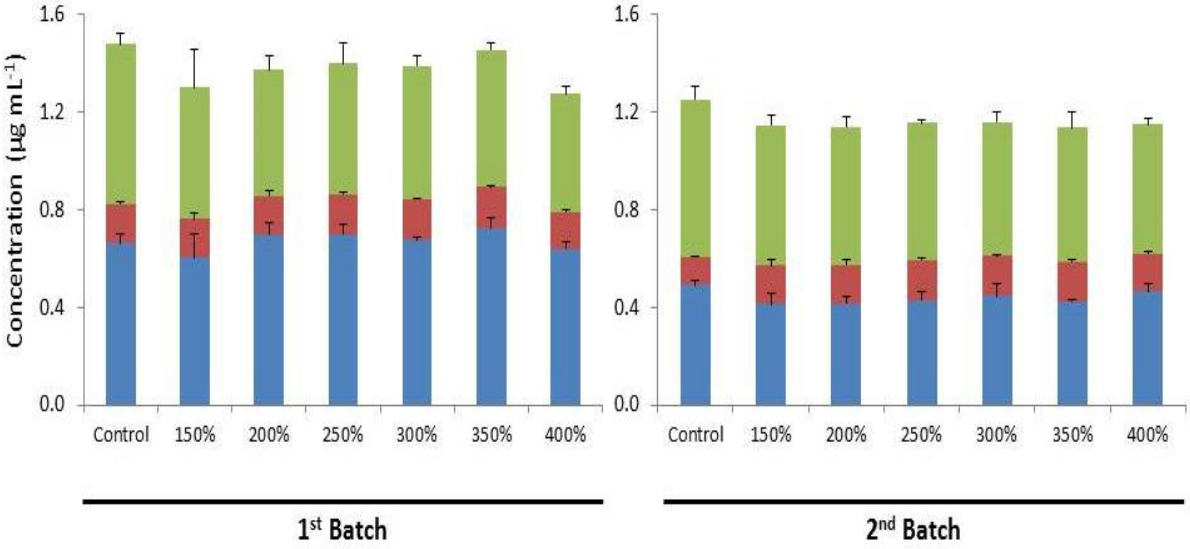


Figure 3.8 Effects of iron supplementations on the yield of OA (■), DTX1 (■) and peridinin (■) from *Prorocentrum lima* CCAP 1136/11 culture after five weeks of cultivation in two sequential batches (n=3, error bars denotes standard deviation).

3.4 Discussion

3.4.1 Evaluation of heterotrophic growth of *Prorocentrum lima* CCAP 1136/11

The obligate requirement of *P. lima* CCAP 1136/11 for phototrophic conditions was conclusively demonstrated by the reduction of cell yield during the two weeks of cultivation in heterotrophic conditions. The inability of culture to revive its growth when transferred to phototrophic conditions provided further evidence of the adverse effects of heterotrophic cultivation of *P. lima* CCAP 1136/11. For this type of organism, growth is very restricted by availability of light (Stoecker 1999), and direct transition to dark cultivation may be considered almost impossible.

Obligate-phototrophy behaviour of microalgae may be explained by the incapability, or at least inefficiency, of the organism to assimilate organic substrates (Smith *et al.* 1967, Chen and Chen 2006). This may be caused by absence of enzymes responsible for the metabolism of organic carbon, such as $NADH_2$ oxidase and α-Ketoglutarate dehydrogenase (Smith *et al.* 1967, Wood *et al.* 2004). The lack of these enzymes has been further explained to prevent the utilisation of organic carbon in generation of adenosine triphosphate (ATP), which is used by the microalgae to fulfil their energy requirement (Smith *et al.* 1967, Chen and Chen 2006).

The conversion of phototrophic to heterotrophic growth conditions for obligate-phototroph organism is possible only through genetic modification, for instance by expression of gene encoding the assimilation of organic carbon. Such technique has been applied in previous work by Zaslavskaia and co-workers (2001), who used a strain of obligate-phototroph diatom, *Phaeodactylum tricornutum*, as model organism. The study demonstrated that expression of a gene that facilitated the intracellular transport of glucose (*Glut1*) had made the obligate-phototroph able to survive and grow under heterotrophic conditions.

Despite the possibility to conduct similar method of genetic modification, the benefit of heterotrophic conversion for *P. lima* CCAP 1136/11 must be fully

evaluated first, especially considering the huge effort required to achieve this. There are possibilities that the modified organism may not be able to efficiently use inexpensive and common carbon sources (e.g. glucose), resulting in higher cultivation costs and lower economical worth of produced metabolites (Perez-Garcia *et al.* 2011). Nonetheless, the results obtained from the present work indicated that, within the scope of this study, the effort to optimise the production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 should be focused instead for the modification of cultivation parameters under phototrophic conditions.

3.4.2 Effects of trace metal modification in culture medium

It is well known that several metals that exist in trace concentrations also have important roles in various metabolic processes (Brand *et al.* 1983, Bruland *et al.* 1991, Raven *et al*. 1999). Iron, copper, manganese and zinc are directly involved in the electron transport chain of photosynthesis (Raven *et al.* 1999), while molybdenum is used for the metabolism of nitrogen (Vega *et al.* 1971). Selenium is essential for cell division and helps in maintaining the integrity of internal cell structures (Price *et al.* 1987, Douchette *et al.* 1987). While the deficiency of these metals may limit the growth and alter cell morphology, their excessive availabilities have also been found to induce cell death and cyst formation (Lindstörm 1985, Coale 1991, Lage *et al.* 1994, Sunda and Huntsman 1995, Okamoto *et al.* 1999, Mitrovic *et al.* 2004, Doblin *et al.* 2000).

In common medium recipes, the composition of trace metals has been formulised to sufficiently meet the growth requirement of most microalgal culture. Yet, in some cases, modification of its composition is necessary to enhance growth and productivity of some species. For instance, the dinoflagellates *Protoceratium reticulatum* and *Karenia selliformis*, were observed to benefit from higher concentrations of selenium, iron and copper than those composed in f/2 and GP medium (Mitrovic *et al.* 2004, Rhodes *et al.* 2006). Furthermore, modification of trace metal composition has also been known to impact on higher production of secondary metabolites from many dinoflagellate cultures (Maldonado *et al.* 2002, Mitrovic *et al.* 2004, Rhodes *et al.* 2006, He *et al.* 2010). For example, Rhodes

and co-workers (2006) found that addition of selenium at concentration of 0.1 μmol L^{-1} in GP medium increased the OA quota per cell in *P. lima* (isolated from New Zealand) by 50%. Considering this, it is likely that such modifications may also provide simple means to enhance DSP toxin production by *P. lima* CCAP 1136/11. This investigation attempted to exploit the possibility to optimise growth and production of DSP toxins and peridinin through modification of trace metals.

Five modifications of each of the seven trace metals composition in f/2-Si and K-min media were screened to enhance the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11. Considering the extensive sample number it would require, the screening test was conducted in a multi-well cell culture plate. The use of multi-well plates for efficient screening of multiple parameters in dinoflagellate cultures has been demonstrated in several studies (Lovejoy *et al.* 1998, Olli and Anderson 2002). However, it has been commented that the small volume of the well may provide limitations during analysis (Olli and Anderson 2002). It was observed in this study that samples more readily evaporated in multi-well plates compared to traditional glass flasks. Considering the small volume that the well contains, evaporation could present challenges for the analysis (i.e. by significantly altering the cell concentration). Excessive evaporation can be reduced by maintaining the humidity surrounding the plates. Nonetheless, the use of 24-well cell culture plates still provided an efficient screening tool for testing various parameters influencing growth and metabolites production of *P. lima* CCAP 1136/11. Such application of multi-well cell culture plates has never been demonstrated previously for this organism.

The screening test revealed that altering the trace metal composition may not necessarily enhance growth. Both the increase and reduction of the concentration of copper, zinc, molybdenum and cobalt from the level in original f/2-Si recipe produced growth suppression. This indicates that the original composition of f/2-Si medium was well optimised for the growth of *P. lima* CCAP 1136/11. Addition of selenium, which is not included in f/2-Si medium, provided no benefit for enhancement of either growth or production of DSP toxins and peridinin. It is reasonable to expect that the background abundance of selenium in natural seawater has sufficed for the growth requirement of *P. lima* CCAP

1136/11. It was shown that increasing selenium concentration in the medium had actually reduced the growth. Such response towards the addition of selenium indicates that the organism has a very low requirement/tolerance for the metal.

Notwithstanding the general trend of reduced growth, the modification of copper, zinc, molybdenum and cobalt still resulted in similar yields of DSP toxins and peridinin to those obtained from control cultures. This shows that the productivity of compounds per cell (cell quota) was actually enhanced (Table 3.3). While the concurrent increase of growth stress and secondary metabolites production is relatively common in dinoflagellate culture, its mechanism has rarely been fully explained (Morton *et al.* 1994, He *et al.* 2010, Varkitzi *et al.* 2010, Vannuci *et al.* 2010). Due to such lack of knowledge, the long term modification of those metals poses the risk of reducing the compound production possibly caused by cell morphological changes or death in the long term - in spite of the enhanced cell productivity in the short term. Therefore, the attempts to optimise production of compounds by means of trace metal modification should be applied for metals that do not significantly affect the growth.

Of all the treatments tested, three types of modification were found to have no detrimental effect on the growth of *P. lima* CCAP 1136/11; those were: limitation of cobalt, and supplementation of iron and manganese. Nevertheless, only iron supplementation provided the indication of increased quota per cell (pg $cell^{-1}$) that was particularly observed for OA and DTX1 (Table 3.3). It was found that cell quota of both compounds increased in parallel with the increase of iron addition, up to twice the concentration suggested in original medium recipe. Based on this result, the study progressed to investigate possibility to further enhance cell productivity through addition of iron in excess of this concentration, as discussed in subsequent section.

3.4.3 Effect of excess iron concentrations in *P. lima* CCAP 1136/11 culture

Although the screening test results gave the indication that iron supplementation could be utilised to enhance cell productivity, further evaluation of this revealed

that the addition of trace iron at concentration of up to four fold did not produce any significant effects on both growth and yield of DSP toxins and peridinin. Such results were also observed after transferring the culture to the second batch. The discrepancy between the screening results and further supplementation test might have been attributed to the different cultivation scales and vessel used. Screening with multi-well plates, which used much smaller culture volume, appeared to give higher yield of compounds (by approximately 20-40%) than those cultivated using Erlenmeyer flask. This was possibly caused by variations in several other growth factors, such as gas exchange within the vessels and light penetration.

The insignificant change of growth in excess iron indicated that *P. lima* CCAP 1136/11 is readily adapted to high iron level. The ability to grow under relatively high concentration of iron is a characteristic of microalgae found in coastal areas, from where the strain used in this study was isolated (Galician coast, Spain). Iron is naturally available more abundantly in coastal zone than in oceanic area, which explains the ability of coastal inhabiting species to survive in high iron conditions (Brand *et al.* 1983, Douchette and Harrison 1990, Sunda and Huntsman 1995). Thus, the response of *P. lima* CCAP 1136/11 towards enhanced concentrations of iron may reflect the organism adaption in its original habitat, which contain high concentration of iron.

The ability of *P. lima* CCAP 1136/11 to adapt to high level of iron might explain the relatively consistent yield of compounds it produced throughout this experiment. As indicated in many studies, secondary metabolite production is directly correlated to growth stress conditions (Morton *et al.* 1994, He *et al.* 2010, Varkitzi *et al.* 2010, Vannuci *et al.* 2010). Therefore, since the addition of iron at the tested concentration did not produce conditions that promote growth stress, it is reasonable to expect consistent production of bioactive compounds.

3.5 Conclusion

The modification of nutrient and trace metals in culture media was assessed in order to optimise growth of *P. lima* in culture. This work concludes that *P. lima* CCAP 1136/11 is an obligate-phototroph, hence the unfeasibility of its direct transition to heterotrophic cultivation. Therefore, effort to optimise the production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 should focus on the modification of cultivation parameters under phototrophic conditions.

Furthermore, the screening test of different concentrations of metals revealed that *P. lima* CCAP 1136/11 growth was very sensitive to changes in concentration of several metals, such as selenium, copper, zinc, molybdenum and cobalt. The modification of their levels in trace concentration was found to limit growth. This limitation of growth resulted in the increase of OA, DTX1 and peridinin quota per cell. Despite this, modification of metal composition in the medium was deemed to be unsuitable for large scale and long term maintenance as the suppressed cell yield may affect sustainable production of these compounds. Subsequent investigation of iron supplementation revealed that excess iron up to the level tested in this study had little effect on both growth and production of OA, DTX1 and peridinin. It was further concluded that production of bioactive compounds from this organism was seen as a function of its growth adaptation in the cultivation system. Thus, provided that the changes of cultivation conditions still enable the culture to adapt and grow, the synthesis of secondary metabolites is expected to be unchanged.

The work carried out in this chapter concluded that optimising large scale production of bioactive metabolites through metal stress is deemed to be inadequate due to the relatively low increase of compounds yield as well as the problematic growth suppression. Exploration of feasible means for large scale production should focus instead on the investigation of other growth parameters, such as abiotic factors and cultivation vessel.

3.6 References

Apt, K. E., Behrens, P. W. 1999. Commercial developments in microalgal biotechnology. **Journal of Phycology**, 35, 215-226.

Behrens, P. W. 2005. Photobioreactors and fermentors: The light and dark sides of growing algae. In: Algal Culturing Techniques. R. A. ANDERSEN (Ed), London, UK: Elsevier Academic Press. pp. 189-203.

Brand, L. E., Sunda, W. G., Guillard, R. R. L. 1983. Limitation of marine phytoplankton reproductive rates by zinc, manganese and iron. **Limnology and Oceanography**, 28 (6), 1182-1198.

Brennan, L., Owende, P. 2009. Biofuels from microalgae – A review of technologies for production, processing and extractions of biofuels and co-products. **Renewable and Sustainable Energy Reviews**, 14 (2), 557-577.

Bruland, K. W., Donut, J. R., Hutchins, D. A. 1991. Interactive influences of bioactive trace metals on biological production in oceanic waters. **Limnology and Oceanography**, 36 (8), 1555-1577.

Carvalho, A. P., Meireles, L. A., Malcata, F. X. 2006. Microalgal reactors: A review of enclosed system design and performances. **Biotechnology Progress**, 22 (6), 1490-1506.

Chen, G., Q., Chen, F. 2006. Growing phototrophic cells without light (Review). **Biotechnology Letters**, 28, 607-616.

Coale, K. H. 1991. Effects of iron, manganese, copper, and zinc enrichments on productivity and biomass in the subarctic Pacific. **Limnology and Oceanography**, 36 (8), 1851-1864.

Doblin, M. A., Blackburn, S. I., Hallegraeff, G. M. 2000. Intraspecific variation in the selenium requirement of different geographic strains of the toxic dinoflagellate *Gymnodinium catenatum*. **Journal of Plankton Research**, 22 (3), 421-432.

Doucette, G. J., Harrison, P. J. 1990. Some effects of iron and nitrogen stress on the red tide dinoflagellate *Gymnodinium sanguineum*. **Marine Ecology Progress Series**, 62, 293-306.

Guillard, R. R. L. 1960. A mutant of *Chlamydomonas moewusii* lacking contractile vacuoles. **Journal of Protozoology**, 7, 262-268.

He, H., Chen, F., Li, H., Xiang, W., Li, Y., Jiang, Y. 2010. Effect of iron on growth, biochemical composition and paralytic shellfish poisoning toxins production of *Alexandrium tamarense*. Harmful Algae, 9, 98-104.

Javanmardian, M., Palsson, B. O. 1991. High-density photoautotrophic algal cultures: Design, construction, and operation of a novel photobioreactor system. **Biotechnology and Bioengineering**, 38, 1182-1189

Lage, O. M., Parente, A. M., Soares, H. M. V. M., Vasconcelos, M. T. S. D., Salema, R. 1994. Some effects of copper on the dinoflagellates *Amphidinium certerae* and *Prorocentrum micans* in batch culture. **European Journal of Phycology**, 29, 253-260.

Lee, Y. K. 2001. Microalgal mass culture systems and methods: Their limitation and potential. **Journal of Applied Phycology**, 13, 307-315.

Lindström, K. 1985. Selenium requirement of the dinoflagellate *Peridinopsis borgei* (Lemm). **International Review on Hydrobiology**, 70 (1), 77-85.

Keller, D. K., Selvin, R. C., Claus, W., Guillard, R. R. L. 1987. Media for the culture of oceanic ultraphytoplankton. **Journal of Phycology**, 23, 633-638.

Maldonado, M. T., Hughes, M. P., Rue, E. L., Wells, M. L. 2002. The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. **Limnology and Oceanography**, 47 (2), 515-526.

Mitrovic, S. M., Amandi, M. F., McKenzie, L., Furey, A., James, K. J. 2004. Effects of selenium, iron and cobalt addition to growth and yessotoxin production of the toxic marine dinoflagellate *Protoceratium reticulatum* in culture. **Journal of Experimental Marine Biology and Ecology**, 313, 337-351.

Okamoto, O. K., Shao, L., Hastings, J. W., Colepicolo, P. 1999. Acute and chronic effects of toxic metals on viability, encystment and bioluminescence in the dinoflagellate *Gonyaulax polyedra*. **Comparative Biochemistry and Physiology**, Part C 123, 75-83.

Olli, K., Anderson, D. M. 2002. High encystment success of the dinoflagellate *Scrippsiella cf. Lachrimosa* in culture experiment. **Journal of Phycology**, 38 (1), 145-156.

Perez-Garcia, O. Escalante, F. M. E., de-Bashan, L. E., Bashan, Y. 2011. Heterotrophic cultures of microalgae: Metabolism and potential products (Review). **Water Research**, 45, 11-36.

Price, N. M., Thompson, P. A., Harrison, P. J. 1987. Selenium: An essential element for growth of the coastal marine diatom *Thalassiosira pseudonana* (bacillariophyceae). **Journal of Phycology**, 23 (1), 1-9.

Pulz, O. 2001. Photobioreactors: Production system for phototrophic microorganisms. **Applied Microbiology and Biotechnology**, 57, 287-293.

Rabsch, U., Wolter, K., Krischker, P. 1984. Influence of low cadmium and zinc concentrations on batch culture *Prorocentrum micans* (Dinophyta) containing low levels of dissolved organic carbon. **Marine Ecology Progress Series**, 14, 275-285.

Raven, J. A., Evans, M. C., Korb, R. E. 1999. The role of trace metals in photosynthetic electron transport in O2-evolving organisms. **Photosynthesis Research***,* 60 (2-3), 111-150.

Rhodes, L., Selwood, A., McNabb, P., Broggs, L., Adamson, J., van Ginkel, R., Laczka. 2006. Trace metal effects on the production of biotoxins by microalgae. **African Journal of Marine Science**, 28 (2), 393-397.

Smith, A. J., London, J., Stanier, R. Y. 1967. Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. **Journal of Bacteriology**, 94 (4), 972-983.

Stoecker, D. K. 1999. Mixotrophy among Dinoflagellates. **Journal of Eukaryotic Microbiology**, 46(4), 397-401.

Sunda, W. G., Huntsman, S. A. 1995. Iron uptake and growth limitation in oceanic and coastal phytoplankton. **Marine Chemistry**, 50 (1), 189-206.

Sunda, W. G., Price, N. M., Morel, F. M. M. 2005. Trace metal ion buffers and their use in culture studies. In: Algal Culturing Techniques. R. A. ANDERSEN (Ed)*,* London, UK: Elsevier Academic Press. pp. 35-63.

Tredici, M. R. 2004. Mass production of microalgae: Photobioreactors. In: Handbook of Microalgal Culture: Biotechnology and Applied Phicology. AMOS RICHMOND (Ed). Iowa, USA: Blackwell Publishing, pp. 178-214.

Vanucci, S., Franca, G., Milandri, A., Pistocchi, R. 2010. Effects of different level of N- and P-deficiency on cell yield, okadaic acid, DTX-1, protein and carbohydrate dynamics in the benthic dinoflagellate *Prorocentrum lima*. **Harmful Algae**, 9, 590-599.

Varkitzi, I., Pagou, K., Granéli, E., Hatzianestis, I., Pyrgaki, C., Pavlidou, A., Montesano, B., Economou-Amilli, A. 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. **Harmful Algae**, 9, 304-311.

Vega, J. M., Herrera, J., Aparicio, P. J., Paneque, A., Losada, M. 1971. Role of molybdenum in nitrate reduction by *Chlorella*. **Plant Physiology**, 48 (3), 294-299.

Wood, A. P., Aurikko, J. P., Kelly, D. P. 2004. A challenge for 21st century molecular biology and biochemistry: what are the causes of obligate autotrophy and methanotrophy?. **FEMS Microbiology Reviews**, 28(3), 335-352.

Zaslavskaia, L. A., Lippmeier, J. C., Shih, C., Ehrhardt, D., Grossman, A. R., Apt, K. E. 2001. Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. **Science**, 292 (5524), 2073-2075.

CHAPTER 4

Effect of Cell Density and Age of Inoculum on the Production of DSP Toxins and Peridinin from *Prorocentrum lima*

4.1 Introduction

The physiological state of microalgal cells influences their growth and production of metabolites during cultivation, as it affects their ability to survive and thrive under various growth stress conditions. For example, growth of inoculum cells that have reached late stationary phase may be hampered in such conditions (Agrawal and Manisha 2007, Mattos *et al.* 2012). The physiological state of the cells can also shape the dynamics of metabolite synthesis in algal cells. It has been observed, for instance, that imbalance physiology of microalgal cells is often correlated with the enhancement of their synthesis of toxic secondary metabolites (Flynn *et al.* 1994).

Both initial cell density and age of inoculum have been found to influence the culture physiology during cultivation. They are thus often manipulated to improve the production of valuable compounds. Initial cell density is observed to be important in this context as it determines cells competition to nutrient and light (Qiang *et al.* 1998, Grobbelaar 2004, Richmond 2004). For instance, in cultures with low cell density, excess of nutrient and light may lead to toxicity and/or photoinhibition. In contrast, too high cell density can reduce nutrient and light availability within photobioreactor. Both conditions may potentially inhibit the growth and alter cell metabolism (Frangópulos *et al.* 2004, Flynn *et al.* 1996, Henriksen *et al.* 2002, Richmond 2004). Furthermore, increased cell density has been demonstrated to positively correlate with the increase of toxic secondary metabolite production, which has been hypothesised to be related with intra-species cell communication (Wood *et al*. 2011; 2012). However, this hypothesis has yet to be confirmed and conclusively warrant further investigation.

In addition to cell density, inoculum age also determines the adaptability of microalgal cells in a new environment. For example, inocula that have reached stationary growth phase tend to require longer adaptation process in beginning of cultivation (Becker 1994). The adaptation process influences the time required to reach maximum growth and compounds production. It is thus desirable to reduce the length of the adaptation period so that the production process can be rendered efficient. An efficient process will require less labour and other

pertinent resources (such as energy requirement), which will reduce the cost of production and increase prospect of profitability.

Whilst the influence of cell physiological state on the production of various compounds from other species of microalgae has been widely studied (Casadeval *et al.* 1985, Alonso *et al.* 2000, Mansour *et al.* 2003), their effects on production of high value DSP toxins and peridinin from *P. lima* CCAP 1136/11 have never been investigated. Manipulation of culture physiology in the cultivation of several microalgal classes (Dinophyceae, Cryptophyceae, Bacillariophyceae and Prymnesiophyceae) has been shown to benefit the enhancement of lipids production (Mansour *et al.* 2003; 2005). Therefore, considering the benefit of this manipulation, research was carried out to investigate the possibility of optimising the production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 by manipulating inoculum age and initial cell density in batch culture.

4.2 Materials and Methods

4.2.1 Influence of cell density

To evaluate the effect of initial cell density on the growth and production of DSP toxins and peridinin in *P. lima* CCAP 1136/11, triplicate cultures were prepared using 100 mL of sterile f/2-Si medium (Chapter 2 section 2.2.2) contained in 250 mL Erlenmeyer flask. Under aseptic conditions, the media were inoculated with stock cultures (28 days old, maintained as described in chapter 2 section 2.2.2) at different volume to achieve a range of cell density from 10^2 to $3x10^5$ cells mL^{-1} (Table 4.1).

Table 4.1 Initial cell densities of *P. lima* CCAP 1136/11 determined by flow cytometry.

Intended cell density (cells mL^{-1})	Actual initial cell density achieved (cells mL^{-1})
$1x10^2$	0.11±0.0
$1x10^3$	1.06±0.0
$1x10^4$	14.56±3.7
$1x10^5$	98.00±1.3
$3x10^5$	231.10±16.8

Cultures were maintained in a temperature controlled room at 21±2°C and continuously exposed to light at 23±2 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Illumination was monitored frequently using LI250A light meter (LI-COR Environmental, UK). Sampling was performed every week for the period of five weeks. Cell counting was conducted using flow cytometer (Chapter 2 section 2.2.3.2). The sensitivity and accuracy of flow cytometer was regularly monitored throughout the experiment with random checks using Sedgewick-Rafter (Chapter 2, section 2.2.3.1). The level of pH in the culture was monitored during each sampling using pH analyser (Jenway 3305 pH Bench Meter, Jenway Limited, Essex, UK). Prior to the measurement, standard buffers of pH 4.0 and 7.0 (at room temperature) were used for calibration of the analyser. The monitoring of DSP toxins and peridinin production was performed by analysing sample extract (Chapter 3, section 3.2.3.2) with UPLC-PDA-MS using method described in Chapter 2, section 2.2.5. Statistical analysis was performed using method described in Chapter 3 (section 3.2.5) to allow analysis of significant difference between treatments.

4.2.2 Influence of inoculum age

Investigation of the effect of inoculum age was performed by inoculating actively growing cultures that had been maintained for 3, 4, 5 and 6 weeks. Cultures were inoculated under aseptic conditions into 100 mL sterile f/2-Si medium contained in 250 mL Erlenmeyer flask, to achieve final cell density of

approximately $1x10^4$ cells mL^{-1}. Each experimental culture was prepared in triplicate. Cultures were maintained in conditions as described in section 4.2.1. Growth and compounds production were monitored every week for the period of five weeks with procedures as described in Chapter 2 (section 2.2.3) and Chapter 3 (section 3.2.3.2).

4.3 Results

4.3.1 Effect of initial cell density on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Growth of *P. lima* CCAP 1136/11 over a range of initial cell densities demonstrated a clear difference on the rate of growth (Fig. 4.1). All cultures, excluding culture with initial cell density of $3x10^5$ cells mL^{-1}, exhibited an initial lag-phase during the first one-to-two weeks, followed by increasing cell density for the remaining cultivation period. With the exception of culture started with 10^3 cells mL^{-1}, all cultures showed no indication of decreasing cell number during the cultivation period.

Furthermore, determination of cell yield was performed (Table 4.2). This would allow direct comparison for cultures with varying initial cell density. Yield is defined as the increase of cell number during the five-week cultivation period, and is expressed as fold change from week-0 (the beginning of cultivation) to week-5 (the termination of cultivation period). It appeared that the initial cell density had an effect on the increase of cell number in the culture, as it was observed that the lower the number of cells deployed, the higher the cell yield it produced. The two highest yields were obtained from culture with initial cell density of 10^2 and 10^3 cells mL^{-1}, with increased of cells within 5 weeks of cultivation recorded at approximately 67 and 29 folds, respectively.

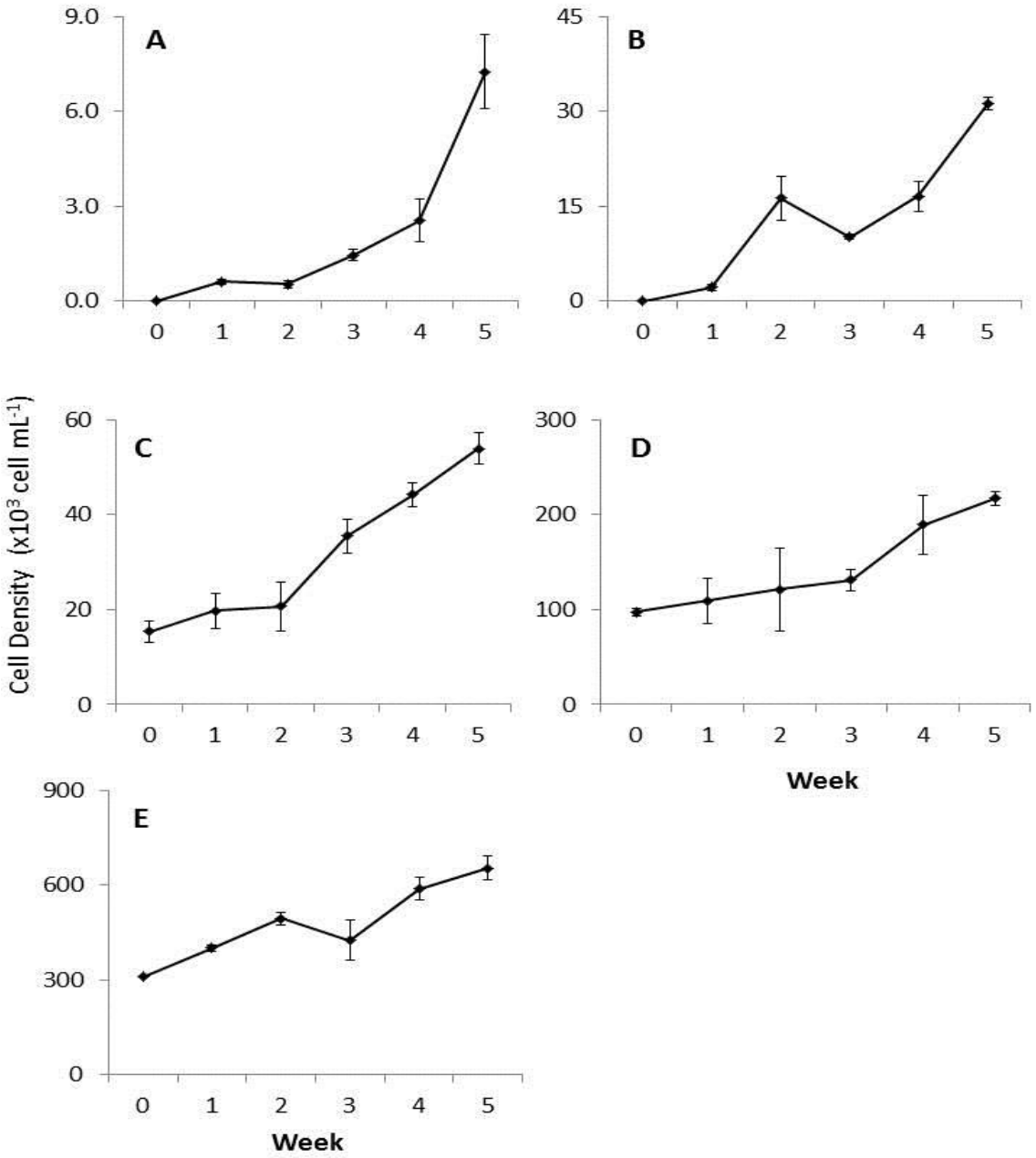


Figure 4.1 Growth of *P. lima* CCAP 1136/11 in culture at different starting cell densities; 10^2 (A), 10^3 (B), 10^4 (C), 10^5 (D) and $3x10^5$ (E) cells mL^{-1} (n=3, error bars indicate standard deviation).

Table 4.2 Yield of cells from *P. lima* CCAP 1136/11 cultures with varying initial cell density after five weeks of cultivation (n=3, ± denotes standard deviation). Different letters indicate significant difference at $p<0.05$.

Culture	Cell density ($x10^3$ cells mL^{-1})		Yield (fold change)
	Initial (actual)	Final	
10^2	0.11±0.0	7.25±1.6	67.27±10.74[a]
10^3	1.06±0.0	31.2±0.9	29.44±0.93[b]
10^4	14.56±3.7	53.9±3.2	3.86±0.99[c]
10^5	98.00±1.3	217.0±7.1	2.21±0.10[c]
$3x10^5$	231.10±16.8	653.9±38.2	2.83±0.07[c]

The influence of initial cell density to the growth of *P. lima* CCAP 1136/11 in batch culture was also reflected by the variation of pH levels during the cultivation period (Fig. 4.2). The culture pH may provide an indication of photosynthetic activity within, since the increase in pH indicates high rates of CO_2 uptake. It appeared that the pH in the three cultures with the highest initial cell density (10^4, 10^5 and $3x10^5$) increased significantly by 1 to 1.5 units during the first week of cultivation period. In contrasts, cultures with two lowest cell densities (10^2 and 10^3) remained relatively constant at the beginning of the cultivation period. This clearly indicated that the large number of cells present in the culture drove the CO_2 uptake, with the resultant elevation in pH.

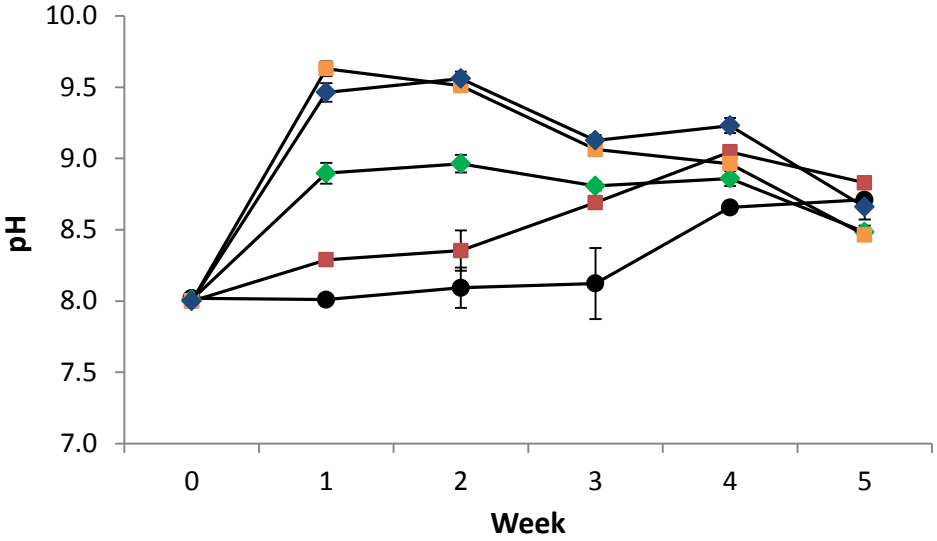


Figure 4.2 Effects of different starting cell densities on the variation of pH values during cultivation period (n=3, error bars indicate standard deviation); 10^2 cells mL^{-1} (●), 10^3 cells mL^{-1} (■), 10^4 cells mL^{-1} (◆), 10^5 cells mL^{-1} (■), and $3x10^5$ cells mL^{-1} (◆).

The production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 cultures with varying cell densities was monitored weekly (Fig. 4.3). It was revealed that all cultures showed increase of OA, DTX1 and peridinin production during the cultivation period. Cultures with high cell densities (10^4, 10^5 and $3x10^5$) appeared to reach the peak of DSP toxins and peridinin production within three weeks, after which the concentrations remained relatively constant until the end of the cultivation period. In contrast, the amount of OA, DTX1 and peridinin from cultures with the two lowest cell densities (10^2 and 10^3) peaked at the end of cultivation period, with significant increase observed during the last two weeks.

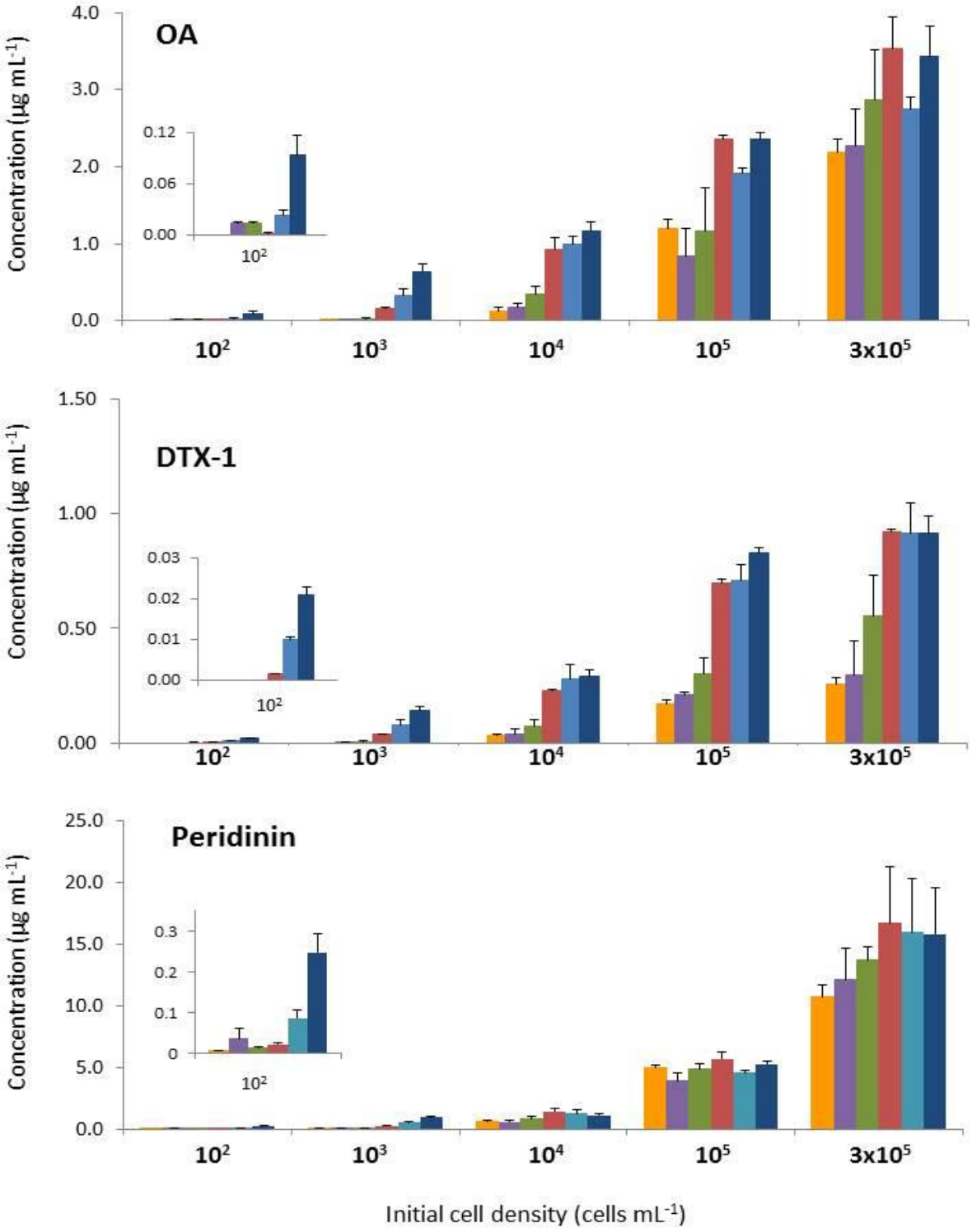


Figure 4.3 Production of DSP toxins and peridinin from batch cultures of *P. lima* CCAP 1136/1 with different initial cell densities measured at week-0 (■), week-1 (■), week-2 (■), week-3 (■), week-4 (■) and week-5 (■) (n=3, error bars indicate standard deviation).

Further determination of DSP toxins and peridinin yield (Table 4.3) showed that the rate of production in the culture was affected by the level of initial cell

density utilised. It was evident that the increase of initial cell density generally resulted in the decrease of OA, DTX1 and peridinin yields. OA yield was found to be optimum when inoculum was deployed at a level of 10^2 cells mL^{-1}. Increasing the cell density by 10 fold produced of approximately 40% decrease in OA yield. A further increase of cell density from 10^3 to 10^4 cells mL^{-1} saw an even higher decrease of yield by 80%. For DTX1 and peridinin, there were no significant differences on the yield of both compounds between cultures with initial cell density 10^2 and 10^3 cells mL^{-1}. However, increasing the density by 10^4 cells mL^{-1} was found to reduce the yield by approximately 80 to 90%.

Table 4.3 Yield of OA, DTX1 and peridinin from *P. lima* CCAP 1136/11 with varying initial cell density after five weeks of cultivation (n=3, ± standard deviation). Different letters indicate significant difference at $p<0.05$.

Initial cell density (cells mL^{-1})	Concentration (µg mL^{-1})		Yield (fold change)
	Initial	Final	
Okadaic acid			
10^2	0.001±0.00	0.10±0.01	86.70±17.40[a]
10^3	0.01±0.00	0.63±0.11	50.21±4.24[b]
10^4	0.13±0.04	1.17±0.12	9.54±3.50[c]
10^5	1.19±0.09	2.35±0.12	1.97±0.05[d]
$3x10^5$	2.19±3.44	3.44±0.39	1.58±0.26[d]
DTX1			
10^2	$15x10^{-4}$±0.00	0.02±0.00	28.33±30.06[ab]
10^3	0.002±0.00	0.14±0.04	60.59±4.45[a]
10^4	0.03±0.01	0.29±0.03	9.56±2.14[b]
10^5	0.17±0.02	0.83±0.02	4.93±0.56[c]
$3x10^5$	0.13±0.01	0.46±0.04	3.62±0.65[c]
Peridinin			
10^2	0.01±0.00	0.25±0.05	36.51±8.79[a]
10^3	0.05±0.02	0.99±0.10	22.35±13.91[a]
10^4	0.60±0.16	1.12±0.10	1.98±0.71[b]
10^5	4.99±0.02	5.26±0.24	1.05±0.03[b]
$3x10^5$	10.72±1.02	15.85±3.74	1.50±0.44[b]

4.3.2 Effect of inoculum age on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

To assess the effect of inoculum age on the growth of *P. lima* CCAP 1136/11, cultures were inoculated with stock cultures that had been maintained for different periods (3, 4, 5 and 6 weeks), and had their cell density monitored every week for the period of five weeks (Fig. 4.4). It was observed that all inocula showed growth throughout the experimental period. Cultures with 3-week-old inoculum reached the peak of its growth within four weeks, after which its cell number began to decline significantly with approximately 34% reduction observed in the fifth week. For the three other experimental cultures (4, 5 and 6 weeks), growth was observed to steadily increased throughout the cultivation period.

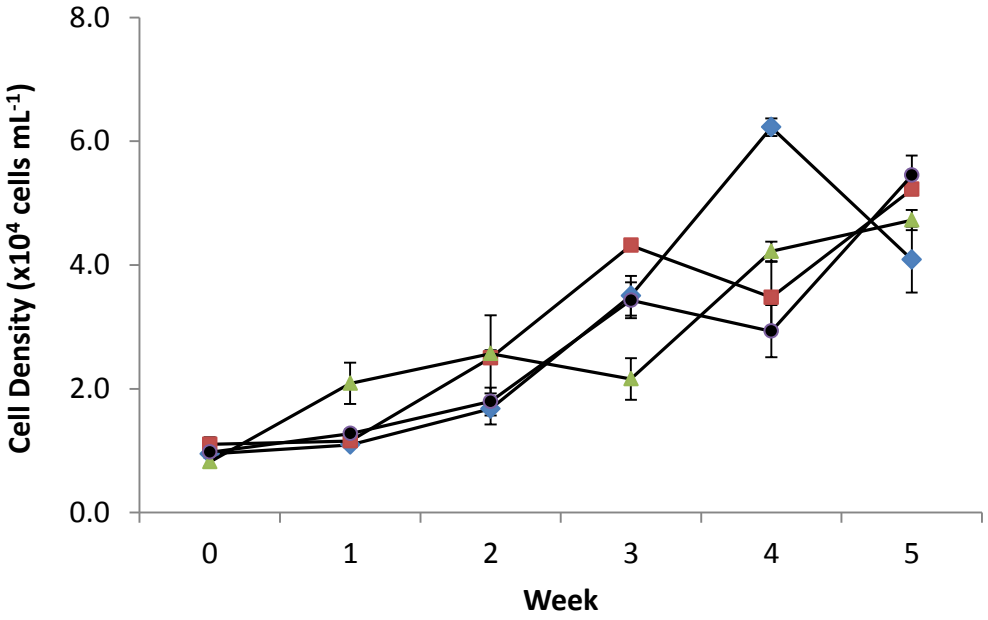


Figure 4.4 Growth of *P. lima* CCAP 1136/11 cultures with different inoculum age; 3-weeks (◆), 4-weeks (■), 5-weeks (▲) and 6-weeks (●) old (n=3, error bars denote standard deviation).

The highest cell density (6.23×10^4 cell mL^{-1}) was achieved for cultures with 3-week-old inoculum and observed after 4 weeks of cultivation (Table 4.4). Within the same week, cell density in the three other cultures were observed at 3.5×10^4, 4.2×10^4 and 2.9×10^4 cells mL^{-1} for 4-, 5- and 6-week-old inoculum,

respectively. Furthermore, the 3-week-old inoculum produced the highest cell yield with 6.6 folds increase within four weeks. The lowest cell yield was found for 4- and 6-week-old inocula, which was recorded at only approximately 3 fold.

Table 4.4 Average increase of cell number in cultures with varying inoculum age cultivated for four weeks (n=3, ± denotes standard deviation). Different letters indicate significant difference at $p<0.05$.

Inoculum Age (weeks)	Cell density ($x10^3$ cells mL^{-1})		Yield (fold change)
	Initial	Final	
3	9.4±0.3	62.3±1.4	$6.6±0.2^a$
4	11.1±1.8	34.8±5.7	$3.2±0.5^b$
5	8.2±0.7	42.2±1.6	$5.2±0.4^c$
6	9.8±0.9	29.3±4.2	$3.0±0.2^b$

Analysis of DSP toxins (Fig. 4.5) showed that, with the exception of OA in 5-week-old inocula, maximum production of compounds in all cultures was achieved within four weeks of cultivation, after which the level of concentrations remained constant. Peridinin concentration was generally peaked after 3 weeks of cultivation, after which it remained constant. The highest concentrations of OA, DTX1 and peridinin in all cultures were found for 3-weeks-old inocula on week-4 at the level of 0.83 µg mL^{-1} of OA, 0.36 µg mL^{-1} of DTX1 and 1.33 µg mL^{-1} of peridinin.

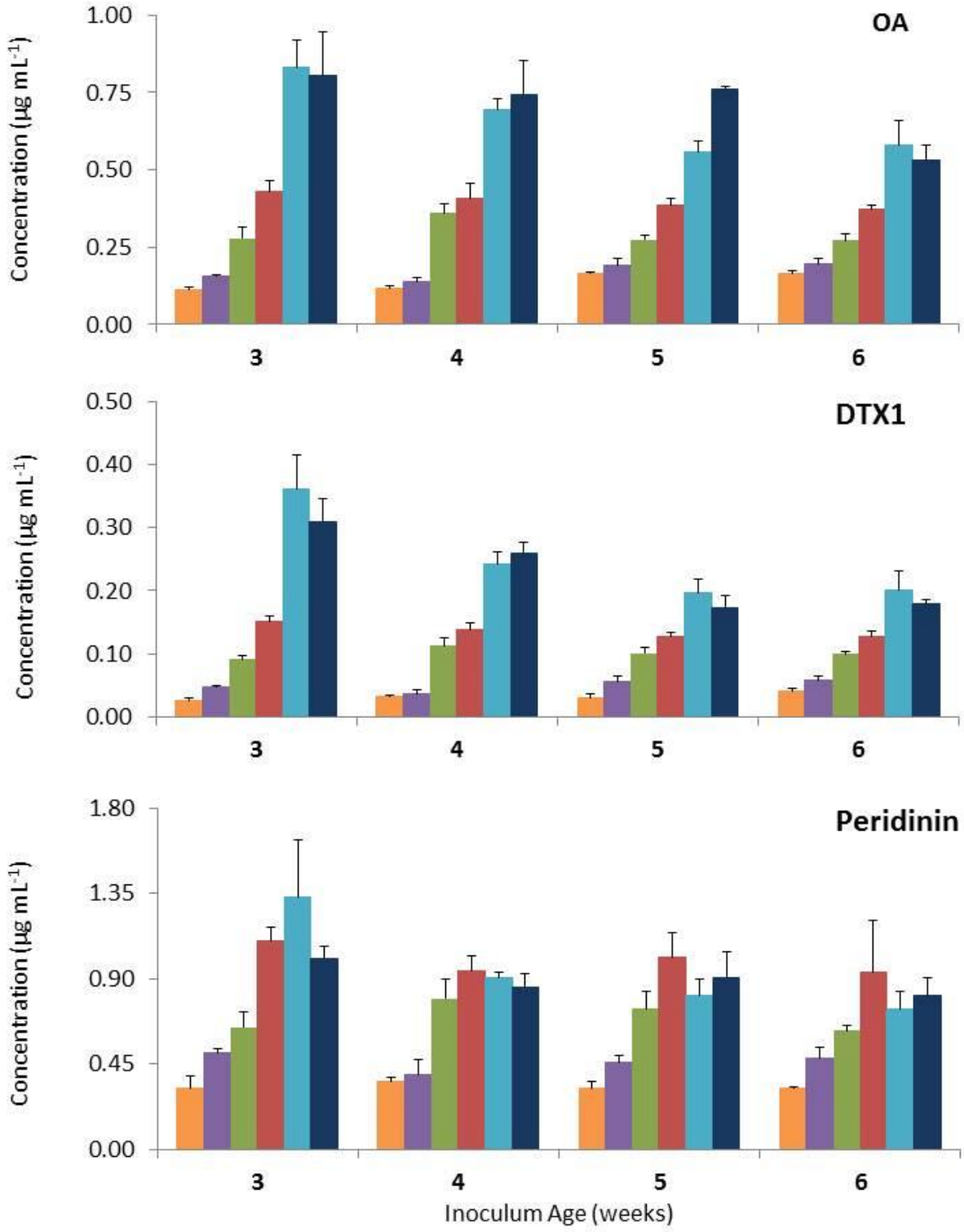


Figure 4.5 Production of OA, DTX1 and peridinin from *P. lima* CCAP 1136/11 cultures with different inoculum age measured at week-0 (■), week-1 (■), week-2 (■), week-3 (■), week-4 (■) and week-5 (■) (n=3, error bars denote standard deviation).

Table 4.5 Yield of OA, DTX1 and peridinin from *P. lima* CCAP 1136/11 cultures with varying inoculum age after four weeks of cultivation (n=3, ± denotes standard deviation). Different letters indicate significant difference to other cultures ($p<0.05$).

Culture Age (week)	Concentration (μg mL^{-1})		Yield (fold change)
	Initial	Final	
Okadaic acid			
3	0.11±0.01	0.83±0.14	7.42±0.82^{a}
4	0.12±0.01	0.70±0.04	5.96±0.41^{a}
5	0.17±0.00	0.56±0.04	3.37±0.21^{b}
6	0.17±0.01	0.58±0.08	3.53±0.57^{b}
DTX1			
3	0.03±0.00	0.31±0.14	13.64±3.03^{a}
4	0.03±0.00	0.24±0.04	7.59±0.16^{b}
5	0.03±0.01	0.20±0.04	6.60±1.18^{bc}
6	0.04±0.00	0.20±0.08	4.93±0.90^{c}
Peridinin			
3	0.32±0.07	1.01±0.07	4.24±1.25^{a}
4	0.36±0.02	0.90±0.03	2.54±0.21^{b}
5	0.33±0.03	0.81±0.09	2.52±0.54^{ab}
6	0.32±0.01	0.74±0.09	2.31±0.22^{b}

Further analysis of OA, DTX1 and peridinin yields (Table 4.5) provided additional evidence of the influence of inoculum age on the production of DSP toxins and peridinin from *P. lima* CCAP 1136/11. Consistent with what was observed for cell yield, the use of 3-week-old inoculum provided the highest yields of DSP toxins and peridinin. The increase of inoculum age by one week resulted in reduction of yield by approximately 20% for OA, 44% for DTX1 and 40% for peridinin. Furthermore, it was also observed that, despite the similar level of initial cell density used, the initial concentration of OA in the two oldest cultures (5 and 6 weeks old) was significantly higher (by approximately 30%) than the two other cultures, indicating that toxin quota per cell was higher in the older inocula. Nonetheless, the high level of OA quota per cell seemed to have no effect in increasing the production of the compound, as the yield observed at the end of cultivation period was considerably lower than the two youngest cultures (3 and 4 weeks old).

4.4 Discussion

4.4.1 Effect of initial cell density on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Cell density is one of the important factors influencing growth and productivity of microalgae in cultivation system (Richmond 1996, Su *et al.* 2011). It determines the utilisation of resources, such as nutrition and light, by the culture (Chrismadha and Borowitzka 1994, Mandalam and Palsson 1996, Richmond 2004). The results obtained in this study indicated that growth of *P. lima* CCAP 1136/11 was affected by the initial cell density used in the cultivation system. For instance, the utilisation of higher cell density significantly reduced cell yield. Such effect may have been the result of the competition of resources within the cultivation system, as cultures with high cell density would have had less access to nutrients and other growth parameters, such as light and carbon dioxide (CO_2), than those with lower cell density.

Further observation of a particular aspect of resource competition, the limitation of CO_2 availability in culture medium, was performed by analysing the variation of pH (Fig. 4.2). It was observed that cultures with high cell density experienced rapid increase in pH within the first week of cultivation. The increase of pH in the medium is recognised as a response to the depletion of CO_2 due to disproportionate CO_2 supply and uptake (Sunda *et al.* 2005). This limitation, however, did not continue in the following weeks as the pH level in all cultures eventually reached a similar level. This may indicate that cultures in all cell densities were able to adapt and compensate their requirement to continue growing, and this compensation may have partly contributed to the low cell divisions observed in high cell density cultures.

Besides the limitation of CO_2 supply, the increase of resources competition may also be caused by the depletion of nutrients availability and limitation to light exposure. Several studies (Varkitzi *et al.* 2010, Vanucci *et al*. 2010) have demonstrated that growth of *P. lima* is very sensitive to limitation in essential nutrients such as nitrogen and phosphorous. Considering that all experimental

cultures were enriched with the same level of nutrients, it was possible to suggest that these nutrients depleted in a faster rate within cultures with high cell number. In addition, high cell number might have also provided self-shading of the culture, which limited the light access for cells at deeper or lower layer of culture (Qiang *et* al. 1998, Richmond 2004). It has been observed previously that low light availability has a direct impact on lowering cell division in *P. lima* (Morton *et al.* 1992). This may explain the low cell yield observed in cultures with highest cell density.

Cell density has also been shown to influence toxin production not only through its effect on resources competition but also on inter-species cell communication. It was observed - for another species of microalgae – that higher cell density would facilitate higher production microcystins hypothesised to act as a signalling compound (Wood *et al*. 2011). However, such relation was not observed in this study. Instead, it showed the opposite effect, as high cell density produced low yield of toxins. The simultaneous occurrence of low cell yield and low toxins yield in cultures with high initial cell density may suggest that reduced level of toxins production during cultivation period was attributed to the low increase of cell numbers, which was caused by limitation of resources.

4.4.1.1 Identification of optimum cell density

In order to identify the optimum initial cell density, production of DSP toxins and peridinin were compared between cultures. However, this could not be performed by comparing the amount detected in each culture (Fig. 4.3), as this would inevitably show that the lowest number of cells would produce the lowest concentration of compounds. Instead, yield, or the fold change of increase during the cultivation period (Table 4.3), was considered to allow more appropriate comparison on cultures productivity in producing OA, DTX1 and peridinin.

Using these measurements, it was shown that cultures with lowest initial cell density were actually more productive than those with higher cell density, as indicated by the high yield of compounds obtained. Utilisation of such low initial cell density may provide benefit in terms of low requirement of stock culture. Considering the time, labour and costs required for maintaining stock cultures,

its efficient usage may impact on low investment required, hence the potential of obtaining higher profit.

Nevertheless, utilisation of culture with low cell density will normally require a huge amount of medium to be prepared. For instance, in order to produce 100 mg of OA, a culture with the initial cell density of 10^2 cells mL^{-1} would require almost 1000 litre of medium (Table 4.6 below). This high volume of medium requirement will increase the production costs, which will lower the profit and render the whole process inefficient.

Table 4.6 Overview on the potential requirement of medium for the production of 100 mg okadaic acid from cultures with different initial cell density.

Initial cell density (cells mL^{-1})	Yield of OA (fold change)	Final concentration of OA in culture (µg mL^{-1})	Volume (in Litre) of culture required to produce 100 mg OA
10^2	86.70	0.10	982.5
10^3	50.21	0.63	159.0
10^4	9.54	1.17	85.5
10^5	1.97	2.35	42.5
$3x10^5$	1.58	3.44	29.1

A closer inspection of the requirement of culture volume revealed that cultures with initial cell density of 10^3 cells mL^{-1} could still produce considerably higher amount of OA with a relatively low requirement for medium. It was shown that the 10^3 cells mL^{-1} cultures required only 15% of media volume required in 10^2 cells mL^{-1} cultures to achieve similar amount of OA (Table 4.6). This indicates that the optimum usage of inoculum may be achieved by utilising cultures with initial cell density of 10^3 cells mL^{-1}.

4.4.2 Effect of inoculum age on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Several studies have been conducted to investigate the role of inoculum growth phase in the cultivation of fast-growing microalgae (Pelizer *et al.* 2003, Agrawal

and Manisha 2007, Mattos *et al*. 2012). Inoculum age was found to be important as it represents physiological fitness of starter cells, particularly as the growth cycle gets slower as the cells aged (Agrawal and Manisha 2007). Such effect was also observed in this study. It was discovered that the utilisation of older inocula resulted in lowering growth of *P. lima* CCAP 1136/11. Of all the ages of inocula tested, cultivation with 3 weeks-old inoculum produced the highest cell yield in the shortest period. The use of such young inoculum may also provide the additional benefit of a shorter time required for the preparation of stock culture. This will significantly impact on lowering the overall production time.

A similar reduction of yield in older inocula was also observed for OA, DTX1 and peridinin. This was consistent with what has been observed previously during investigation on initial cell density (section 4.4.1). It may thus be suggested that the lower production of DSP toxins and peridinin observed was most likely to be attributed to the lower increase of cell number during the cultivation process.

Moreover, a distinct pattern was observed for the production of OA. The older cells, which had higher initial OA concentration, produced a lower increase of OA production during the cultivation period (Table 4.5). This low rate of increase may be explained by the hypothesis that OA serves a yet-to-be-elaborated biological function for the producer organism (Cembella and John 2006, Granéli and Flynn 2006). It is possible that the older cells had accumulated sufficient OA to meet their physiological needs prior to their inoculation. Thus, they may not need to synthesise the compound as much as the younger cells do in the cultivation system. Such low rate of OA production by older cells may indicate that utilisation of older inoculum should be avoided in order to achieve optimum cultivation.

4.5 Conclusions

Initial cell density and inoculum age were found to affect growth and production of DSP toxins and peridinin in *P. lima* CCAP 1136/11 culture. It was revealed that culture with 3-weeks old inoculum produced the highest yield of cells. The utilisation of early growth phase inoculum may also reduce the overall production time. This approach was observed to require the least amount of time to reach maximum yields. It was also perceived to offer an additional benefit in terms of the faster preparation of stock culture, which would be used as inoculants.

Moreover, cultures with the lowest cell density generated the highest cell and compound yields compared to all other densities tested. Nevertheless, cultivation utilising such low cell density could be argued to be impractical due to the exceedingly large medium required to achieve the intended production target. It was found instead that optimum cultivation was achieved by inoculating the culture to attain an initial cell density of 10^3 cells mL^{-1}. Furthermore, the lower yield of compounds obtained in higher cell density was predicted to be the result of competition for resources, including nutrients and light within the culture. Possible cell communication as a response to population size was not demonstrated in this study but merits further investigation.

4.6 References

Agrawal, S. C., Manisha. 2007. Growth, survival and reproduction in *Chlorella culgaris* and *C. variegata* with respect to culture age and under different chemical factors. **Folia Microbiologica**, 52 (4), 399-406.

Alonso, D. L., Belarbi, E. H., Fernández-Sevilla, J. M., Rodríguez-Ruiz, J., Grima, E. M. 2000. Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga *Phaeodactylum tricornutum*. **Phytochemistry**, 54 (5), 461-471

Becker, E. W. 1994. *Microalgae: biotechnology and microbiology*. Cambridge, UK: Cambridge University Press.

Casadevall, E., Dif, D., Largeau, C., Gudin, C., Chaumont, D., Desanti, O. 1985. Studies on batch and continuous cultures of *Botryococcus braunii*: hydrocarbon production in relation to physiological state, cell ultrastructure, and phosphate nutrition. **Biotechnology and Bioengineering**, 27 (3), 286-295.

Cembella, A., John, U. 2006. Molecular physiology of toxin production and growth regulation in harmful algae. In: E. Granéli, J. T. Turner (Eds) *Ecology of Harmful Algae.* Berlin, Germany: Springer-Verlag, pp. 215-227.

Chrismadha, T., Borowitzka, M. A. 1994. Effect of cell density and irradiance on growth, proximate composition and eicosapentaenoic acid production of *Phaeodactylum tricornutum* grown in a tubular photobioreactor. **Journal of Applied Phycology**, 6 (1), 67-74

Flynn, K., Franco, J. M., Fernández, P., Reguera, B., Zapata, M., Wood, G., Flynn, K. J. 1994. Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorus stress. **Marine Ecology Progress Series**, 111, 99-109.

Flynn, K. J., Fynn, K., John, E. H., Reguera, B., Reyero, I., Franco, J. M. 1996. Changes in toxins, intracellular and dissolved free amino acids of the toxic dinoflagellate *Gymnodinium catenatum* in response to changes in inorganic nutrients and salinity. **Journal of plankton research**, 18 (11), 2093-2111.

Frangópulos, M., Guisande, C., DeBlas, E., Maneiro, I. 2004. Toxin production and competitive abilities under phosphorus limitation of *Alexandrium* species. **Harmful Algae**, 3 (2), 131-139.

Graneli and Flynn 2006, Chemical and physical factors influencing toxin content. In: E. Granéli, J. T. Turner (Eds) *Ecology of Harmful Algae.* Berlin, Germany: Springer-Verlag, pp. 229-241.

Henriksen, P., Riemann, B., Kaas, H., Sørensen, H. M., Sørensen, H. L. 2002. Effects of nutrient-limitation and irradiance on marine phytoplankton pigments. **Journal of Plankton Research**, 24 (9), 835-858.

Mandalam, R. K., Palsson, B. Ø. 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. **Biotechnology and Bioengineering**, 59 (5), 605-611.

Mansour, M. P., Volkman, J. K., Blackburn, S. I. 2003. The effect of growth phase on the lipid class, fatty acid and sterol composition in the marine dinoflagellate *Gymnodinium* sp. in batch culture. **Phytochemistry**, 63 (2), 145-153.

Mansour, M. P., Frampton, D. M., Nichols, P. D., Volkman, J. K., Blackburn, S. I. 2005. Lipid and fatty acid yield of nine stationary-phase microalgae: applications and unusual C24–C28 polyunsaturated fatty acids. **Journal of Applied Phycology**, 17 (4), 287-300.

Mattos, E. R., Singh, M., Cabrera, M. L., Das, K. C. 2012. Effects of inoculum physiological stage on the growth characteristics of *Chlorella sorokiniana* cultivated under different CO_2 concentrations. **Applied Biochemical and Biotechnology**, 168 (3), 519-530.

McLachlan, J. L., Marr, J. C., Conlon-Keily, A., Adamson, A. 1994. Effects of nitrogen concentration and cold temperature on DSP-toxin concentrations in the dinoflagellate *Prorocentrum lima* (prorocentrales, dinophyceae). **Natural Toxins**, 2 (5), 263-270.

Morton, S. L., Norris, D. R., Bomber, J. W. 1992. Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. **Journal of Experimental Marine Biology and Ecology**, 157 (1), 79-90.

Pelizer, L. H., Danesi, E. D., Rangel, C. de O., Sassano, C. E. N., Carvalho, J. C. M., Sato, S., Moraes, I. O. 2003. Influence of inoculum age and concentration in *Spirulina platensis* cultivation. **Journal of Food Engineering**, 56, 371-375.

Qiang, H., Zarmi, Y., Richmond, A. 1998. Combined effects of light intensity, light-path and culture density on output rate of *Spirulina platensis* (Cyanobacteria). **European Journal of Phycology**, 33, 165-171.

Richmond, A. 1996. Efficient utilization of high irradiance for production of photoautotropic cell mass: A survey. **Journal of Applied Phycology**, 8 (4-5), 381-387

Richmond, A. 2004. Biological principles of mass cultivation. In: Handbook of microalgal culture, Biotechnology and applied phycology. A Richmond (Ed). Cornwall, UK: Blackwell Science Ltd.

Su, C. H., Chien, L. J., Gomes, J., Lin, Y. S., Yu, Y. K., Liou, J. S., Syu, R. J. 2011. Factors affecting lipid accumulation by *Nannochloropsis oculata* in a two-stage cultivation process. **Journal of Applied Phycology**, 23 (5), 903-908.

Sunda, W. G., Price, N. M., Morel, F. M. M. 2005. Trace metal ion buffers and their use in culture studies. In: Algal Culturing Techniques , R. A. ANDERSEN (Ed), Oxford, UK: Academic Press.

Vanucci, S., Guerrini, F., Milandri, A., Pistocchi, R. 2010. Effects of different levels of N- and P-deficiency on cell yield, okadaic acid, DTX-1, protein and carbohydrate dynamics in the benthic dinoflagellate *Prorocentrum lima*. **Harmful Algae**, 9, 590-599.

Varkitzi, I., Pagou, K., Graneli, E., Hatzianestis, I., Pyrgaki, C., Pavlidou, A., Montesanto, B., Economou-Amilli, A. 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. **Harmful Algae,** 9, 304-311.

Wood, S. A., Rueckert, A., Hamilton, D. P., Cary, S. G., Dietrich, D. R. 2011. Switching toxin production on and off: Intermitten microcystin synthesis in a *Microcystis* bloom. **Environmental Microbiology Reports**, 3(1), 118-124.

Wood, S.A., Rueckert, A., Cary, S.C., Hamilton, D.P., Dietrich, D.R. 2012. Increasing *Microcystis* cell density enhances microcystin synthesis: a mesocosm study. **Inland Waters***,* 2, 17-22.

CHAPTER 5

Effect of Light, Temperature and CO_2 on Growth and Production of DSP Toxins and Peridinin in *Prorocentrum lima*

5.1 Introduction

The determination of optimum operating conditions to ensure an efficient production system is important for the development of a simple and scalable bioreactor. In phototrophic cultivation systems, productivity is mainly controlled by the photosynthetic rate in the culture. Several parameters governing photosynthesis include utilisation of light energy, carbon dioxide (CO_2) supply and optimum operating temperature.

By definition, photosynthesis is a process that utilises light energy to produce organic matter (Masojídek *et* al. 2004). As such, investigation of light energy was considered to be essential. Several parameters that influence the delivery of light energy in cultivation systems (photobioreactor) include intensity and occurrence of light and dark cycle (Qiang and Richmond 1996, Jacob-Lopes *et al.* 2009, Posten 2009). In addition, the types of artificial light source also have to be considered when designing photobioreactors. The selection of light source is particularly important, as large scale cultivation relying on natural light source may not always be possible, especially if the organism is sensitive to fluctuation in temperature. Moreover, the utilisation of a particular light source will also impact on the overall economic performance of a cultivation system. For instance, it has been argued that the application of light emitting diodes (LEDs) as a light source offers an advantage of being economically feasible due to their efficient electric to light energy conversion compared to tubular fluorescent lamp (Yeh and Chung 2009, Yam and Hassan 2005).

In addition to light, the supply of carbon dioxide (CO_2) is also essential for the photosynthesis. CO_2 is used as the source of carbon that will be converted to biomass during photosynthesis. In high yielding photobioreactor, the supply of CO_2 at atmospheric level is deemed to be insufficient to satisfy carbon requirement of algal culture with high growth rates (Grobbelaar 2004). Thus, aeration with CO_2 enriched air is commonly used for large scale cultivation systems. Microalgal cultures may typically have tolerance to elevated CO_2 level of up to 18% (v/v CO_2–Air mixture), although a specific strain (marine green algae *Chlorococcum littorale*) has also been found to thrive at a much higher level (40% v/v) (Wang *et al*. 2008). Such capability is indicative for the potential of

exploiting microalgal cultures as a carbon sequestration technique (Wang *et al*. 2008, Pandit *et al*. 2012). It has been demonstrated that they can be used for the removal of CO_2 from flue gas produced from a steel power plant, approximately reaching the level of 60% rate of removal (Chiu *et al.* 2011).

Temperature is another important factor governing optimum growth and productivity of microalgae in cultivation system. Changes in the growth temperature of microalgae can have a significant effect on altering its cellular biochemical composition (Hu 2004). Due to this reason, manipulation of operating temperature has been utilised as one of the means to enhance the production of valuable compounds by a variety of microalgal species (De Oliveira *et al.* 1999, Converti *et al.* 2009, Xin *et al.* 2011). Nevertheless, to date, there have been no published reports of the effect of temperature to the production of DSP toxins and peridinin from *P. lima*.

In the case of *P. lima*, investigation of the influence of light conditions and temperature has never been conducted in the context of optimising metabolites production within photobioreactor. Instead, previous studies tend to focus more on the organism's ecological adaptation process (Morton *et al.* 1992, McLachlan *et al.* 1994, Pan *et al*. 1999). As such, this study aimed to evaluate several environmental factors that support the optimum growth and productivity of *P. lima* CCAP 1136/11 in a cultivation system. These include investigations of various light conditions and temperature levels. In addition, assessment of the possibility to utilise *P. lima* CCAP 1136/11 cultures as a means of CO_2 sequestration, which may potentially enhance their growth, was also conducted.

5.2 Materials and Methods

5.2.1 Evaluation of the effect of different light conditions on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Investigations of three distinct aspects of light configurations, consisting of light source, intensity and light/dark cycle, were performed in order to evaluate the effects of different light conditions on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11.

5.2.1.1 Influence of light source type

Two types of light sources were tested for the cultivation of *P. lima* CCAP 1136/11: fluorescent light and light emitting diodes (LEDs). For this, two sets of experimental cultures were prepared (in triplicates) by inoculating a stock culture of *P. lima* CCAP 1136/11 (28 days old, maintained as described in Chapter 2, section 2.2.2) into a 1 L of sterile f/2-Si medium contained in 2 L Erlenmeyer flask to give a starting density of approximately 1-2 $x10^4$ cells mL^{-1}. The cultures were then sparged with filtered ambient air (0.22 µm; Millipore, UK) using an air pump and incubated in a temperature controlled room (21±1°C). The first set of cultures were exposed to red and blue LEDs tape (Simple Lighting Company, UK), with a proportion of 50:50 red/blue (by the number of point source). The wavelengths for both LEDs were: 625-630 nm for red LED and 465-470 nm for blue LED. Simultaneously, the second set of cultures was exposed to cool-white fluorescent lamp (58 Watts, Phillips). Both types of light were arranged to provide effective illumination for the cultures at intensity of 25 µmol m^{-2} s^{-1} with continuous light cycle.

Aliquots (2 mL) were removed during sampling, which was conducted weekly for the period of five weeks, and used for the monitoring of cell number and DSP toxins and peridinin contents. Counting of cells was performed by using flow cytometer with the method already established and described in Chapter 2 (section 2.2.3.2). Determination of DSP toxins and peridinin contents was

performed by analysing extract of the samples (prepared as described in Chapter 3 section 3.2.3.2) with UPLC-PDA-MS using method described in Chapter 2 (section 2.2.5). Statistical analysis for data was performed according to method described in Chapter 3 (section 3.2.5).

5.2.1.2 Influence of light intensity

Investigation of different light intensity was conducted in an environmental chamber (Weiss Technik, UK), which was set at operating temperature of 22°C. Inside the chamber, three different light intensities (25, 50 and 95 µmol m^{-2} s^{-1}) were arranged, each with two light cycles (continuous light and 12:12 hours of light/dark cycle). Light was provided by cool-white fluorescent lamps (58 Watts, Phillips). Experimental cultures were prepared (in triplicates) by inoculating *P. lima* CCAP 1136/11 stock culture (28 days old, maintained as described in Chapter 2, section 2.2.2) into 100 mL sterile f/2-Si medium contained in 250 mL Erlenmeyer flask to provide starting cell density of approximately 1-2 x10^4 cells mL^{-1}. Sampling was performed weekly for the period of five weeks for the monitoring of cell number and DSP toxins and peridinin contents with procedure as described in previous section (Chapter 2 section 2.2.3 and Chapter 3 section 3.2.3.2). Statistical analysis for data was performed according to method described in Chapter 3 (section 3.2.5).

5.2.1.3 Investigation of different frequencies of light/dark cycle

To investigate the influence of different light/dark frequency, six sets of experimental cultures were prepared in triplicates by inoculating a stock culture of *P. lima* CCAP 1136/11 (28 days old, maintained as described in Chapter 2, section 2.2.2) into a 1 L of sterile f/2-Si medium contained in 2 L Erlenmeyer flask to provide a starting density of approximately 1-2 x10^4 cells mL^{-1}. The first set of cultures was incubated under continuous light exposure at intensity of 25 µmol m^{-2} s^{-1} provided by blue and red LEDs (section 5.2.1.1). The other five sets of cultures were exposed to blue and red LEDs which was connected to light switch timer, to give illumination with changing conditions of light and dark at frequencies of every 12, 6, 1, 0.5 and 0.25 hours. The light intensity selected for these five sets of cultures were 50 µmol m^{-2} s^{-1}, in order to provide the same

amount of total light energy with those in continuous light cycle. Aeration was provided by sparging the culture with filtered (0.22 µm; Millipore, UK) ambient air using an air-pump (230V, 50Hz; Fisherbrand, UK). Sampling was performed weekly for the period of five weeks for the monitoring of cell number, DSP toxins and peridinin using procedure as described in Chapter 2 (section 2.2.3) and Chapter 3 (3.2.3.2). Statistical analysis for data was performed according to method described in Chapter 3 (section 3.2.5).

5.2.2 Evaluation of the effects of CO_2 supplementation on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Experimental cultures were prepared (in triplicate) by inoculating a stock culture of *P. lima* CCAP 1136/11 (28 days old, maintained as described in Chapter 2, section 2.2.2) into a 1 L of sterile f/2-Si medium contained in 2 L Erlenmeyer flask to provide a starting density of approximately 1-2 $x10^4$ cells mL^{-1}. The cultures were then aerated with a mixture of CO_2 gas and air at a concentration of 5.6±0.2% (v/v) CO_2:Air. To achieve this, a silicone tube carrying 100% CO_2 gas from a cylinder (BOC, Aberdeen, UK) was passed through a rotameter (flow rate = 250 cm^3 min^{-1}) and connected to one of the two manifold inlets. The other manifold inlet was connected to an air pump (12V, 6W) allowing ambient air to flow (passed through a 0.22 µm filter) with maximum capacity of 4200 cm^3 min^{-1}. Ambient air and CO_2 gas were mixed inside a manifold and distributed to three experimental flasks (Figure 5.1). Simultaneously, control cultures were aerated with ambient air at the same flow rate, which was also split between triplicates of flasks.

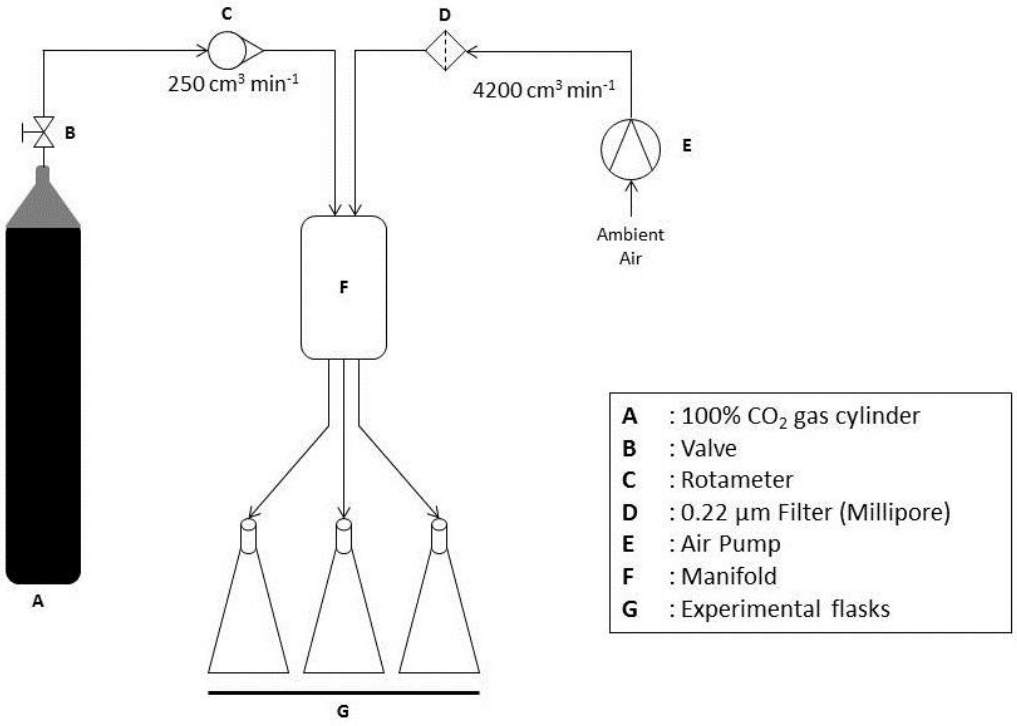


Figure 5.1 Schematic diagrams illustrating experimental set-up for the investigation of the effects of CO_2 supplementation.

All cultures were incubated in a temperature controlled room (21±1°C) with continuous illumination at 25 µmol m^{-2} s^{-1} provided by cool-white fluorescence lamps (58 Watts, Phillips). Sampling was performed weekly for the period of five weeks for the monitoring of cell number, DSP toxins and peridinin as described in Chapter 2 (section 2.2.3) and Chapter 3 (3.2.3.2). Level of pH was monitored every week using pH probe and analyser (Five Easy™ FE20, Mettler Toledo, Leicester UK). Statistical analysis for data was performed according to method described in Chapter 3 (section 3.2.5).

5.2.3 Evaluation of the effects of different levels of temperature on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

To evaluate the effect of temperature on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11, triplicates of experimental

cultures were prepared. The preparation of experimental cultures was performed by inoculating stock culture (28 days old, maintained as described in Chapter 2, section 2.2.2) into 100 mL sterile f/2-Si medium contained in 250 mL Erlenmeyer flask to provide starting cell density of approximately 1-2 $x10^4$ cells mL^{-1}. Cultures were placed in water baths that were set at three different temperatures; 22, 27 and 32°C. Cultures were maintained under constant illumination at 23±2 µmol m^{-2} s^{-1} provided with cool-white fluorescent lamps (58 Watts, Phillips). Cultures were sampled every week for the period of five weeks for cell number, DSP toxin and peridinin analysis, as per Chapter 2 (section 2.2.3) and Chapter 3 (3.2.3.2). Statistical analysis for data was performed according to method described in Chapter 3 (section 3.2.5).

5.3 Results

5.3.1 Investigation of the use of LEDs for the cultivation of *P. lima* CCAP 1136/11

Tests on two types of light source, the LEDs and fluorescence lamps, showed that growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 were not affected by the utilisation of different types of light source (Fig. 5.2). It was shown that the trends of increase in cell number and concentration of DSP toxins and peridinin during the five weeks cultivation were very similar for both LEDs and fluorescent lamps. Cell counting analysis during the harvesting point (on the fifth week) noted cell density levels of $4.6x10^4$ cells mL^{-1} for cultures exposed to LEDs and $4.4x10^4$ cells mL^{-1} for cultures exposed to fluorescent lamps. Final concentration of compounds observed in cultures with LEDs were 0.47 µg ml^{-1} OA, 0.08 µg ml^{-1} DTX1, and 0.67 µg ml^{-1} peridinin. Similarly, final concentration of compounds detected in cultures with fluorescent lamps were 0.45 µg ml^{-1} OA, 0.11 µg ml^{-1} DTX1, and 0.73 µg ml^{-1} peridinin.

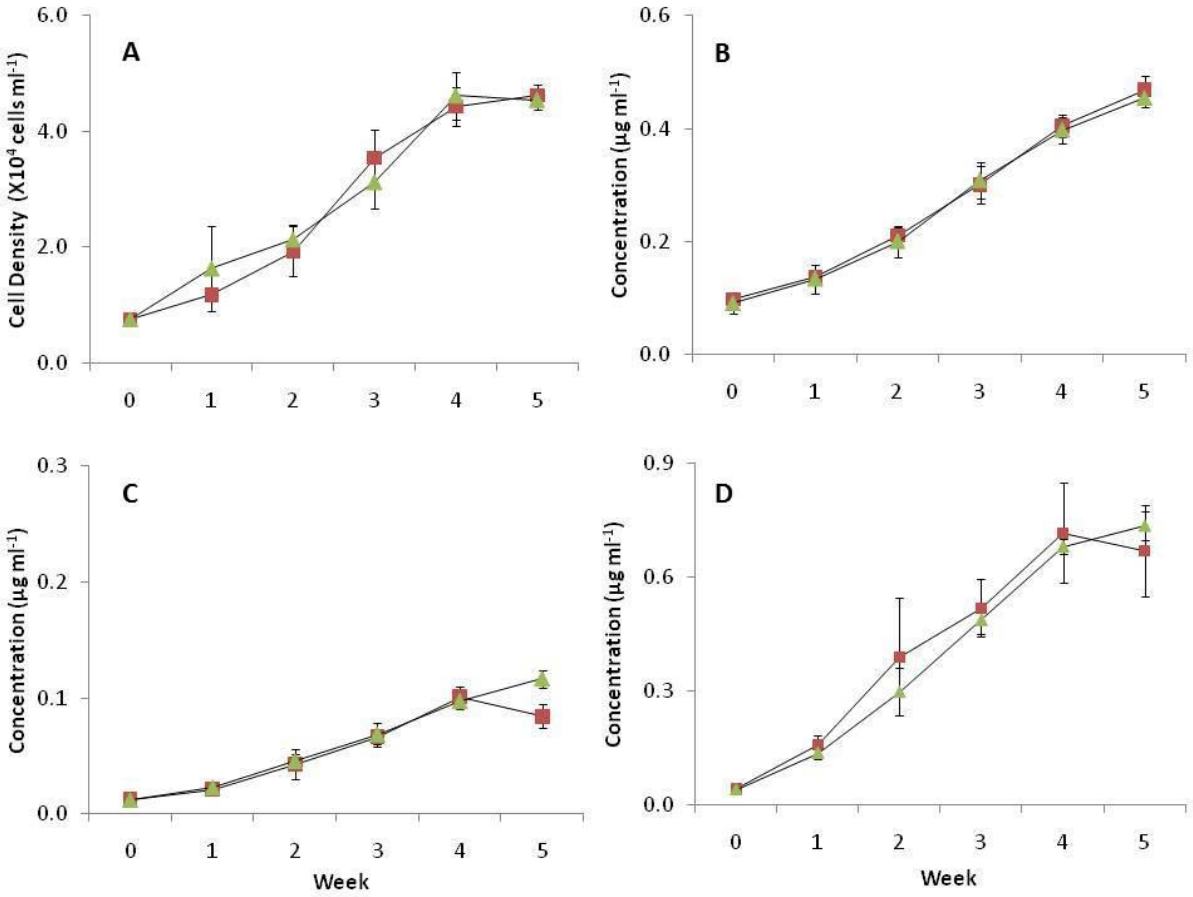


Figure 5.2 Growth (A) and production of okadaic acid (B), DTX1 (C) and peridinin (D) from *P. lima* CCAP 1136/11 cultivated under continuous exposure to red/blue LEDs (■) lamps and cool-white fluorescent lamps (▲) (n=3, error bars denotes standard deviation).

5.3.2 Influence of photoperiod at selected light intensity on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

During the investigation of the effects of light intensity, it was found that cultures of *P. lima* CCAP 1136/11 were able to grow optimally under continuous light exposure between 25 to 50 µmol m^{-2} s^{-1}, and that exposure to light above this range (i.e. 95 µmol m^{-2} s^{-1}) resulted in the suppression of growth by approximately 40% (Fig. 5.3A). Final cell densities after five weeks of cultivation were measured at the levels of $4.7x10^4$, $4.3x10^4$ and $2.7x10^4$ cells mL^{-1} for cultures with light intensity of 25, 50 and 95 µmol m^{-2} s^{-1}, respectively.

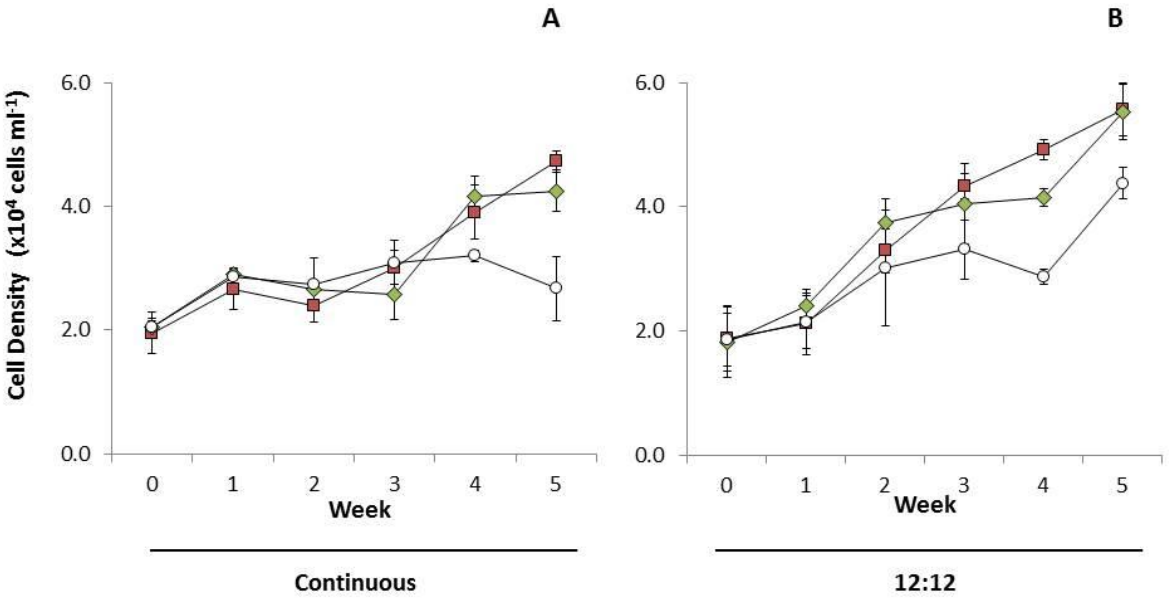


Figure 5.3 Growth of *P. lima* CCAP 1136/11 under different light intensities without (A) and with (B) photoperiod (n=3, error bars denotes standard deviation); 25 (■), 50 (◆) and 95 (○) µmol m^{-2} s^{-1}.

Further investigation of the influence of photoperiod (12:12 hour of light/dark cycle) revealed that growth at all intensities was improved with introduction of light cycle (Fig. 5.3), although the difference in final cell number observed for cultures at 25 µmol m^{-2} s^{-1} as a result of photoperiod introduction was found to be insignificant ($p>0.05$). Increase in the final cell density levels observed for the other two intensities was noted at approximately 30% for cultures at 50 µmol m^{-2} s^{-1} and 64% for cultures at 95 µmol m^{-2} s^{-1}.

Evaluation of the production of OA, DTX1 and peridinin from *P. lima* CCAP 1136/11 cultures showed that, similar to what was observed for growth, under continuous light cycle the production of DSP toxins was significantly reduced with light intensity above 50 µmol m^{-2} s^{-1} (Fig. 5.4). Cultures grown under 95 µmol m^{-2} s^{-1} were found to produce approximately 55% lower OA and 54% lower DTX1 than those incubated under 25 µmol m^{-2} s^{-1}. While the yields observed for 25 and 50 µmol m^{-2} s^{-1} were similar, introduction of a photoperiod appeared to improve DSP toxins concentration in 95 µmol m^{-2} s^{-1} cultures by approximately 36% for OA and 66% for DTX1. The final concentration of DSP toxins measured for

cultures with 12:12 light/dark cycle at 25 µmol m^{-2} s^{-1} were 0.64 µg mL^{-1} OA and 0.18 µg mL^{-1} DTX1, and similarly the 50 µmol m^{-2} s^{-1} cultures produced 0.69 µg mL^{-1} OA and 0.19 µg mL^{-1} DTX1.

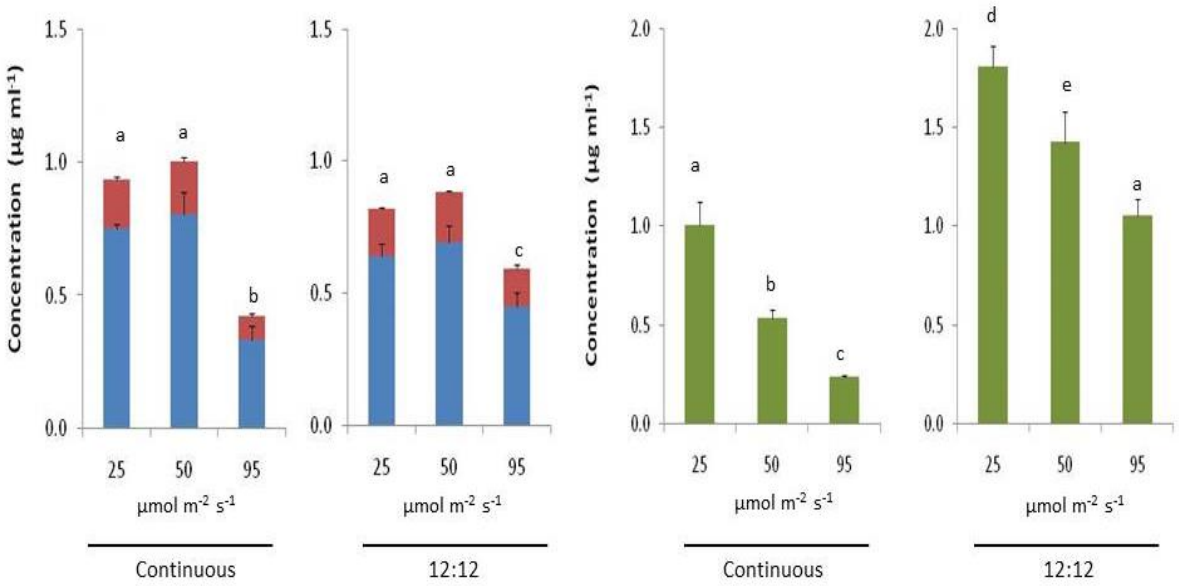


Figure 5.4 Concentrations of OA (■), DTX1 (■), and peridinin (■) from *P. lima* CCAP 1136/11 cultures after five weeks of cultivation under different light intensities and photoperiods (n=3, error bars = standard deviation). Different letters above bars indicate significant difference of cultures between and within the same photoperiods ($p<0.05$).

Moreover, both light intensity and photoperiod were found to significantly affect peridinin production from *P. lima* CCAP 1136/11 cultures (Fig. 5.4). It was observed that the increase of light intensity positively correlated with reduced peridinin production in the cultures. The introduction of light/and dark appeared to significantly improve the peridinin production from cultures at all light intensities. Highest peridinin concentration was 1.8 µg mL^{-1} for cultures grown under 12:12 light/dark cycle at 25 µmol m^{-2} s^{-1}, whereas the lowest concentration was 0.24 µg mL^{-1} for cultures grown under continuous light at 95 µmol m^{-2} s^{-1}.

5.3.3 Influence of different frequencies of light/dark cycle on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Investigation of the effect of frequencies of light/dark cycle showed that all cultures under different frequencies showed growth over the experimental period (Fig. 5.5). Cell count analysis during the final week of cultivation showed that there was no significant difference ($p>0.05$) on cell density for all cultures, except for those grown under light/dark cycle at 12 and 1 hour frequency, which were found to have lower cell density than cultures at 0.25 hour frequency. However, calculation of the cell yield, or the increase of cell number within 5 weeks of cultivation, indicated that all cultures produced similar increase of cells (Table 5.1). This showed that the growth of *P. lima* CCAP 1136/11 was not affected by the frequency of light/dark cycle.

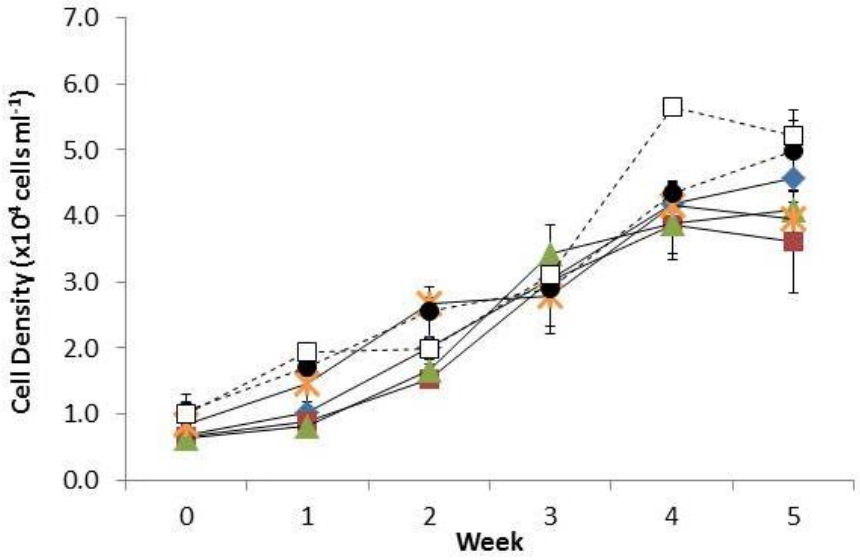


Figure 5.5 Growth of *P. lima* under various frequencies of light/dark (L/D) cycle (n=3, error bars denotes standard deviation); Continuous light (◆) and L/D frequency at 12 (■), 6 (▲), 1 (✱), 0.5 (●) and 0.25 (□) hours.

Table 5.1 Final cell density and cell yield from *P. lima* CCAP 1136/11 cultures after five weeks of cultivation under varying frequencies of light/dark cycle (n=3, ± denotes standard deviation). Different letters indicate significant difference between different treatments ($p<0.05$).

Light/Dark Frequency (hour)	Final cell density ($x10^4$ cells mL^{-1})	Cell Yield (fold change)
Continuous light	4.56±0.47	6.7 ± 0.6[a]
12	3.62±0.78	5.5 ± 1.5[a]
6	4.03±0.20	6.3 ± 0.1[a]
1	3.95±0.42	4.9 ± 1.1[a]
0.5	4.99±0.61	4.9 ± 1.0[a]
0.25	5.21±0.22	5.3 ± 0.8[a]

Analysis of UPLC-PDA-MS revealed that despite the similar cell yield observed, production of OA, DTX1 and peridinin were influenced by the frequency of light/dark cycle (Fig. 5.6). It appeared that the highest concentrations of compounds were produced by cultures exposed to the three highest frequencies (1, 0.5 and 0.25 hours). The difference in the level of concentrations for all compounds became apparent from the first week of cultivation period.

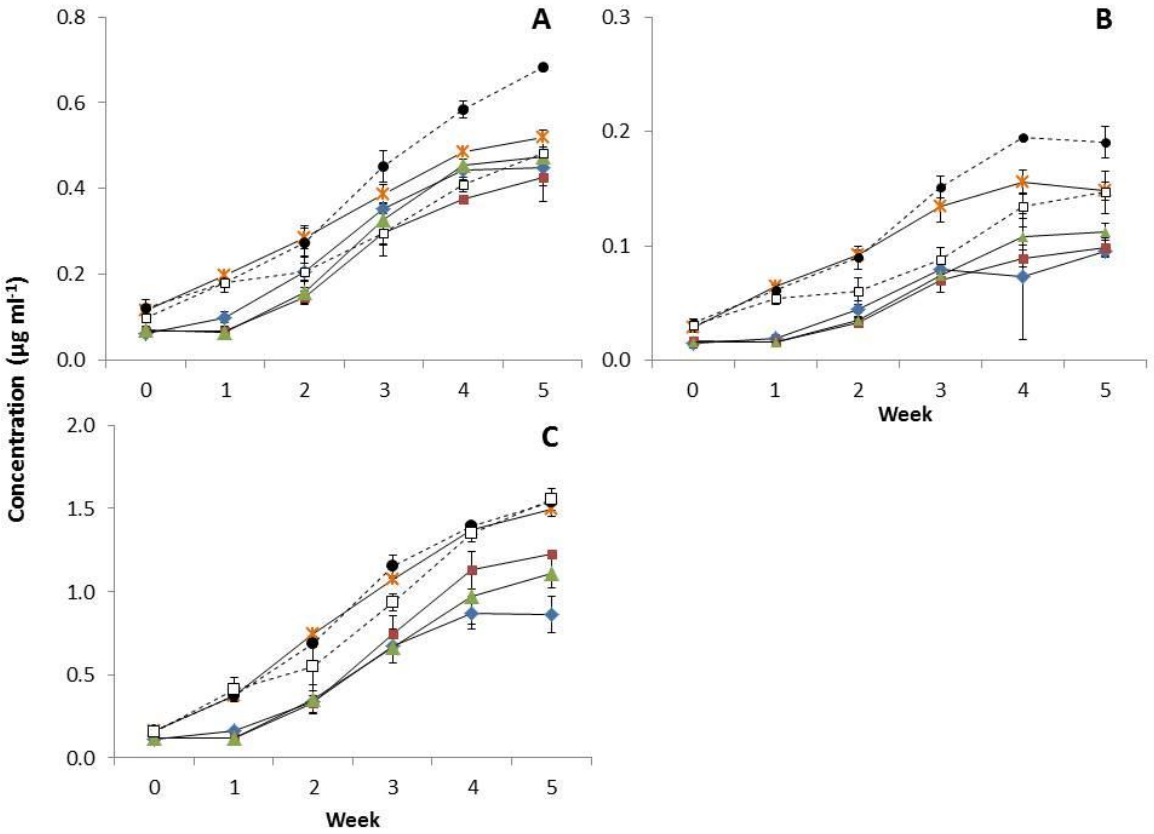


Figure 5.6 Production of OA (A), DTX1 (B) and peridinin (C) during growth under various frequencies of light/dark cycle (n=3, error bars denotes standard deviation); Continuous light (◆) and light/dark frequencies at 12 (■), 6 (▲), 1 (✱), 0.5 (●) and 0.25 (□) hours.

Determination of compound concentration at the final week of cultivation further revealed that yield of DSP toxins and peridinin were optimum when utilising light/dark cycle at frequency of 0.5 hour (Fig 5.7). Cultures with 0.5 hour frequency produced approximately 45% higher OA yield than in continuous light cycle and other light/dark frequencies. For DTX1, there was a clear pattern of increased concentration with the frequency increase of up to 0.5 hour, whilst higher frequency (0.25 hour) resulted in reduced DTX1 concentration. Providing light/dark cycle at 0.5 hour frequency was observed to increase the DTX1 concentration in culture of almost twice the level observed in continuous light cycle. Similarly, peridinin also showed increase of concentration with increase of light/dark frequency. Optimum peridinin concentration was found when utilising light/dark cycle at a frequency of 1 hour and above (up to 0.25 hour). At these frequencies peridinin content in the culture was observed at approximately 70 to

80% higher than those in continuous culture (Fig. 5.7). In total, highest concentration of compounds was observed for culture with 0.5 hour light/dark frequency, which produced 0.68 µg mL^{-1} OA, 0.19 µg mL^{-1} DTX1 and 1.54 µg mL^{-1} peridinin at the end of cultivation period.

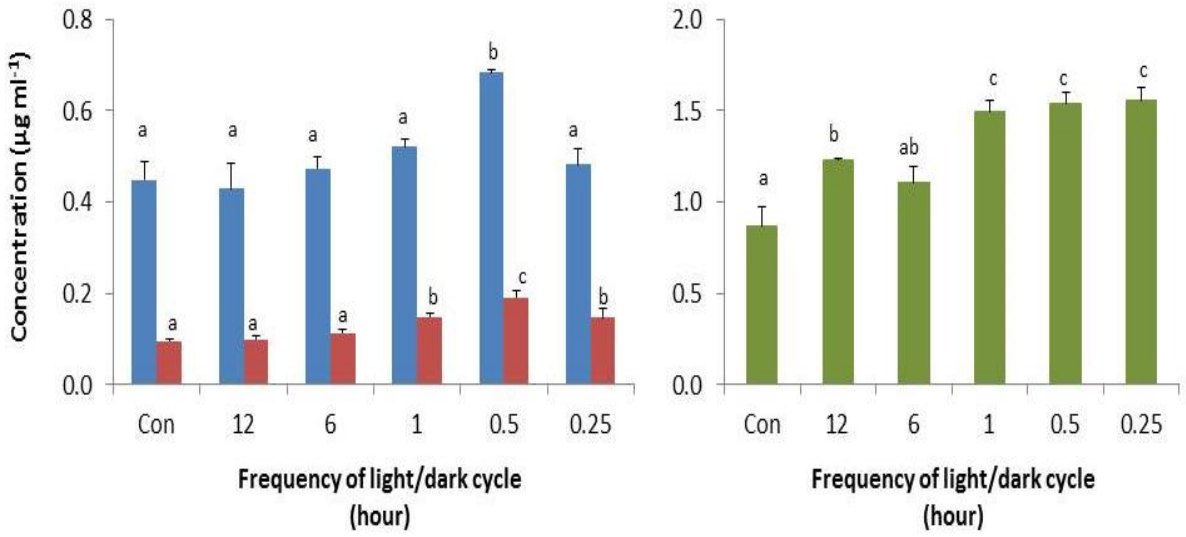


Figure 5.7 Concentrations of OA (■) and DTX1 (■) and peridinin (■) from *P. lima* CCAP 1136/11 cultures after five weeks cultivation under varying light/dark frequencies (n=3, error bars denotes standard deviation). Different letters above bars indicate significant difference between treatments ($p<0.05$).

5.3.4 Effects of CO_2 supplementation on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Investigation of the effect of CO_2 supplementation revealed that growth of *P. lima* CCAP 1136/11 was significantly affected by the increased concentration of CO_2 (Fig. 5.8A). Measurement of cell number throughout the experimental period revealed that growth was not observed in cultures with supplemented CO_2 at 5% (v/v) concentration. The detrimental effect of CO_2 supplementation for *P. lima* CCAP 1136/11 culture was also illustrated with variation in pH (Fig. 5.8B). Whilst the pH level in cultures grown with 100% air was relatively constant, cultures supplemented with CO_2 showed decrease in pH level by 1.5 units over the five

weeks of cultivation. Final cell densities were observed at $0.37x10^4$ for CO_2-supplemented culture and $6.50x10^4$ cells mL^{-1} for those grown with 100% air.

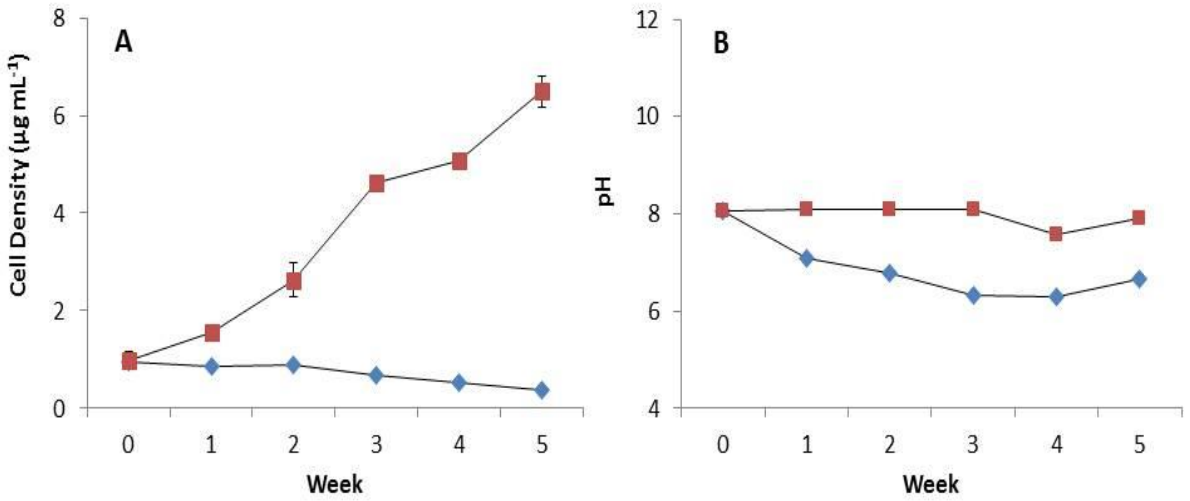


Figure 5.8 Growth (A) and variations of pH levels (B) of *P. lima* CCAP 1136/11 cultures grown with Air (■) and Air+5% CO_2 (◆) (n=3, error bars denotes standard deviation).

Further analysis with UPLC-PDA-MS also revealed similar pattern of reduction on the production of DSP toxins and peridinin (Fig. 5.9). It was clearly shown that cultures supplemented with CO_2 did not produce OA, DTX1 and peridinin during the incubation period, indicated by their steadily decreasing concentrations. Final concentration of compounds in CO_2 supplemented cultures was measured at 0.05 µg mL^{-1} OA, 0.02 µg mL^{-1} DTX1 and 0.05 µg mL^{-1} peridinin. In contrast, final concentration of compounds in cultures sparged with 100% air was noted at 0.54 µg mL^{-1} OA, 0.11 µg mL^{-1} DTX1 and 1.86 µg mL^{-1} peridinin.

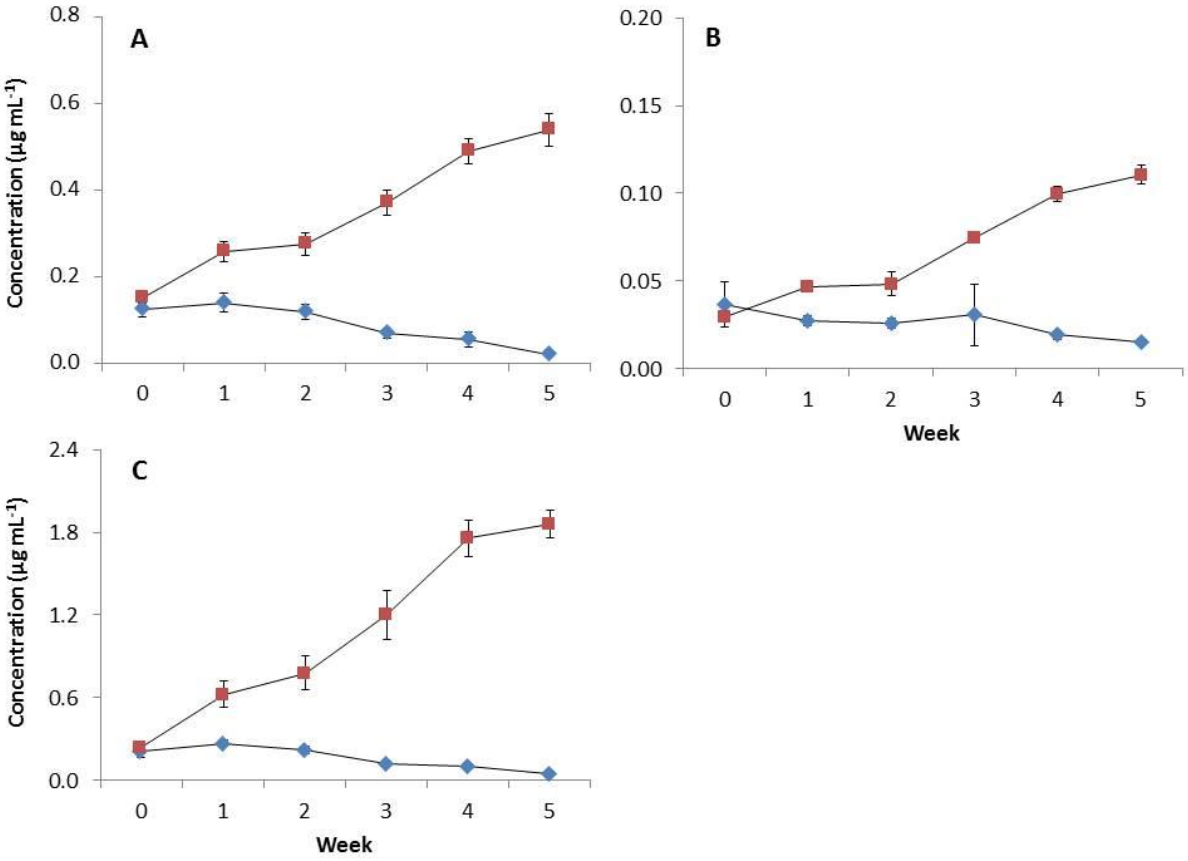


Figure 5.9 Production of OA (A), DTX1 (B) and peridinin (C) from *P. lima* CCAP 1136/11 cultures grown with 100% Air (■) and Air+5% CO_2 (◆) (n=3, error bars denotes standard deviation).

5.3.5 Effects of different temperatures on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

Investigation of the effect of different temperatures showed that growth of *P. lima* CCAP 1136/11 was highly influenced by increasing temperature (Fig. 5.10A). Analysis of cell density for cultures maintained in 22°C showed steadily increasing cell number over the cultivation period. Elevation of temperature to 27°C was found to severely inhibit the growth of *P. lima* CCAP 1136/11. Cultures were found to perish almost instantaneously after a further elevation of temperature to 32°C. It has to be noted that the stock cultures used in this experiment were routinely maintained at an average temperature of 22°C. Final cell densities after five weeks of cultivation were measured at 6.7×10^4 cells mL^{-1} for 22°C cultures and 0.6×10^4 cells mL^{-1} for 27°C cultures.

Similar to what has been observed for cell density, production of DSP toxins and peridinin was also shown to be affected by the elevation of temperature level (Fig. 5.10B, 5.10C and 5.10D). There was no production of OA, DTX1 and peridinin observed from cultures incubated at 27°C, indicated by the similar concentrations observed between beginning and end of cultivation period. For cultures maintained at 32°C, no compounds could be detected at the end of cultivation period, as all cells had died at this point. Final concentration of compounds for cultures in 22°C was measured at 0.75 µg mL^{-1} OA, 0.15 µg mL^{-1} DTX1 and 0.96 µg mL^{-1} peridinin, whereas final concentration of compounds measured for cultures maintained at 27°C was approximately at 75-80% lower than from those observed in 22°C.

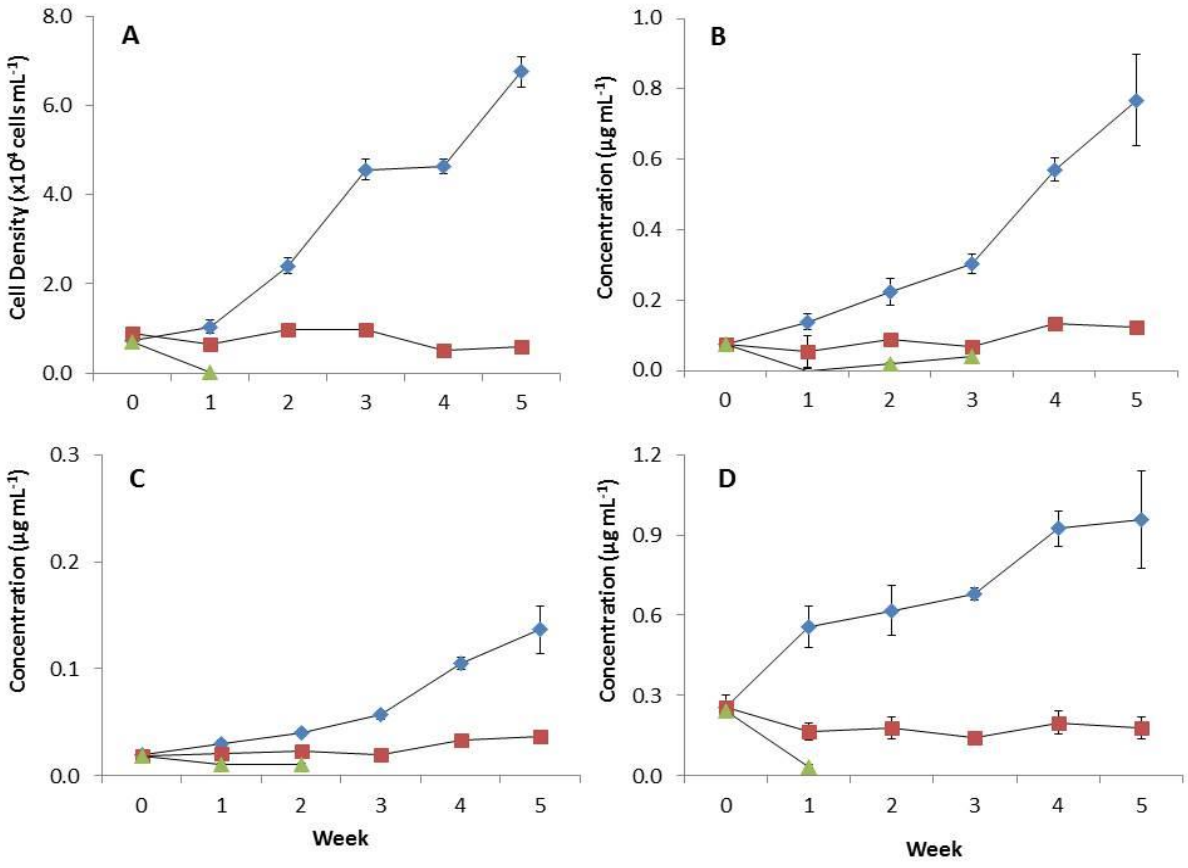


Figure 5.10 Growth (A) and production of okadaic acid (B), DTX1 (C) and peridinin (D) from *P. lima* CCAP 1136/11 cultures maintained at different level of temperatures; 22 (◆), 27 (■) and 32 (▲) degree Celsius (n=3, error bars denotes standard deviation).

5.4 Discussions

5.4.1 Influence of different light conditions on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

5.4.1.1 The use of LEDs for the cultivation of *P. lima* CCAP 1136/11

The type of light source is a critical factor in the design of photobioreactor for the cultivation of microalgae. It determines the optimum provision of light energy within the system (Matthijs *et al.* 1996, Chen *et al.* 2011). Optimum delivery of light energy ensures that the organism can photosynthesis effectively, enabling them to gain maximum productivity (Lee and Palsson 1994, Suh and Lee 2003). The selection of the type of light source will also contribute to the overall production costs of the cultivation, especially as it determines the design of the system and its operational costs, for instance in terms of energy consumption and replacement expenses (Chen *et al.* 2011).

Evaluation on two types of light sources, LEDs and fluorescence lamps, showed that both can be suitably used for cultivation of *P. lima* CCAP 1136/11. Indeed, the growth and production of DSP toxins and peridinin from this organism was not affected by changing the types of the lamps from one to another. Fluorescent lamps have been in common use within laboratory-scale cultivation system (Xu *et al.* 2009). Although they are perceived to provide high illumination area and low installation costs, they tend to have high operating costs and energy consumption (Chen *et al.* 2011). In contrasts, LEDs have the potential for higher economic efficiency for a long-term large-scale cultivation than fluorescent lamp (Lee and Palsson 1994, Matthijs *et al.* 1996, Wang *et al*. 2007, Chen *et al.* 2011). Such efficiency is the result of their lower energy consumption, potentially due to their narrow wavelength spectrum and smaller heat loss (Matthijs *et al*. 1996, Yeh *et al*. 2009).

The blue and red LEDs selected for this study were observed to possess emission spectrum that aligned with the absorption bands of the main photosynthetic pigments of the organism, chlorophyll-*a* and peridinin. The LEDs emitted light at

wavelengths of 465-470 nm (blue LEDs) and 625-630 nm (red LEDs), similar to the maximum absorbance spectrum of chlorophyll-*a* and peridinin (Masojídek *et al.* 2004). Selection of appropriate wavelengths is important to ensure efficient energy transfer for photosynthesis (Matthijs et al. 1996). The good growth observed in this study might thus indicate that the wavelengths had been selected to appropriately fit the requirement of the organism.

Furthermore, whilst not investigated in this study, there is the possibility of further enhancing the growth and production of compounds by utilising different combinations of LEDs. For instance, changing the proportion of red and blue lights to promote higher growth or utilising only blue light to target higher carotenoid (i.e peridinin) production. Such approach has been previously shown in studies optimising growth of *Spirulina platensis*, and enhancing production of carotenoid astaxanthin from *Haematococcus pluvialis* (Wang *et al.* 2007, Katsuda *et al.* 2004, Lababpour *et al.* 2004; 2005), and thus could possibly be performed for *P. lima* CCAP 1136/11.

5.4.1.2 Influence of photoperiod at selected light intensity on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

In addition to the types of light source, the intensity and occurrence of photoperiod may also influence the optimum utilisation of light energy within cultivation system. Determination of optimum light intensity is required to ensure that the provision of photon energy is sufficient for the growth requirement of microalgae (Lee and Palsson 1994). However, excessive provision of light should be avoided as it would induce photoinhibition in the culture (Vonshak and Torzillo 2004). The detrimental effect of excessive light may be prevented by providing light periodically (photoperiod) (Lorenz *et al.* 2005). Photoperiod has also been demonstrated to influence microalgal growth by increasing photosynthetic efficiency and nutrient uptake (Meseck *et al*. 2005, Liu *et al*. 2007, Jacob-Lopes *et al*. 2009).

This study revealed that the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 culture was affected by both light intensity and occurrence of photoperiod. It was shown that, under continuous light exposure, this organism grew optimally under light intensity of 25 to 50 µmol m^{-2} s^{-1}, and that light intensity above this level inhibited the growth of *P. lima* CCAP 1136/11 (Fig. 5.3A). This inhibition of growth might indicate that *P. lima* CCAP 1136/11 has low tolerance to high light intensity. Subsequent evaluation of the influence of photoperiod provided further evidence of the preference of *P. lima* CCAP 1136/11 to low light conditions, as indicated by the improved growth in high light exposure when using photoperiod (Fig. 5.3B).

The adaptability of *P. lima* CCAP 1136/11 to low light conditions may be explained by the benthic nature of the organism. As a benthic organism, *P. lima* are very well adapted to shades (Pan *et al.* 1999). They often found in habitats with poor light penetration, such as sediments and rocky substrates at the sea beds, and mussel growing farms (Rhodes and Syhre 1995, Heredia-Tapia *et al.* 2002, Levasseur *et al.* 2003, Nascimento *et al.* 2005). As such, the organism can tolerate and thrive in low light cultivation conditions.

In regards to the production of metabolites, it was found that DSP production was not affected by both light intensity between 25-50 µmol m^{-2} s^{-1} and the occurrence of photoperiod at respective intensities. The production of DSP toxins was observed to be suppressed only for the highest light intensity tested. However, the introduction of photoperiod improved both growth, and the production of OA and DTX1 at the highest light intensity. As such, this may indicate that production of DSP toxins are very much related to growth conditions of the organism. Under good growth conditions, production of DSP toxins appeared to be unaffected by changes in external factors, such as light, provided that optimum growth can be achieved. The result obtained was also consistent to what was observed during the modification of other growth factors (see iron modification in Chapter 3 section 3.4.3, and initial cell density modification in Chapter 4 section 4.4.1). In contrast, photoperiod and light intensity was found to directly influence the peridinin production in the culture. It was observed that lower light conditions induced higher peridinin production. This relationship can be reasonably anticipated as peridinin is known to be an accessory

photosynthetic pigment that supports the harvesting of light energy (Masojídek *et al*. 2004). Therefore, the increased peridinin production, as observed in this study, could be perceived as a response of cells adaptation to low light irradiance.

5.4.1.3 Influence of different frequencies of light/dark cycle on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

The increase of cell density in the mass cultivation of microalgae is known to reduce light penetration within the culture vessel. Under such condition, the mixing and turbulence within the reactor is considered to cause cells to experience frequent changing of light and dark conditions, as cell rapidly move across areas with varying degrees of light exposure (Grobbelaar 1989). Such fluctuating light/dark cycle, or flashing light effect, is known to be beneficial as it increases the photosynthetic efficiency of fast growing green microalgae (e.g. *Chlorella* spp, *Scenedesmus obliquus*) within a cultivation system, thus optimising the growth and productivity of the culture (Grobbelaar 1994, Grobbelaar *et al*. 1996, Nedbal *et al.* 1996, Park and Lee 2000, Janssen *et al.* 2001). Based upon these findings, this study assumed that the productivity of *P. lima* CCAP 1136/11 might be enhanced by utilising a similar approach. This study investigated the effects that frequent changing of light/dark cycle has on growth and production of DSP toxins and peridinin from the organism.

It was found that the utilisation of different frequency of light and dark cycle did not produce any significant effects on the growth of *P. lima* CCAP 1136/11, as indicated by the similar level of cell yield obtained after five weeks of cultivation (Table 5.1). Throughout the experiment, cultures under fluctuating light/dark cycle were exposed to light source with double the intensity of those under continuous light cycle, with the result that both cultures received the same amount of light energy in total. The findings from this experiment might indicate that the rate of cell division was more influenced by the amount of light energy they received than the occurrence of light/dark conditions. This agrees with a previous observation, stipulating that the organism was evolving with cell cycle

events independent from photoperiod, as, in their natural habitat, they were often required to survive in environments with irregular light occurrence (Pan *et al.* 1999).

Although the intermittent changing of light and dark conditions did not produce any apparent changes in cell yield, it affected the production of DSP toxins and peridinin *P. lima* CCAP 1136/11. It was observed that the organism produced higher DSP toxins and peridinin at higher frequency of light/dark cycle. However, although many studies associated the increase of these compounds with growth stress (Morton *et al*. 1994, Nascimento *et al*. 2005, Vanucci *et al.* 2010, Varkitzi *et al*. 2010), such increase, as observed in this study, could not be directly attributed to stress conditions since growth was not suppressed in all treatments.

The observed increase of these compounds may be explained by other factors. The role of peridinin as an accessory photosynthetic pigment might be associated with the increase of its production. A previous study showed that, as a response to intermittent light conditions, microalgae in culture system increased its photosynthetic pigment (chlorophyll-*a*) to enable them to harvest higher photon flux (Janssen *et al.* 1999). Such adaptation process may explain the higher peridinin concentration obtained in cultures with higher frequency of light/dark cycle.

In regards to the observed increase of DSP toxins (OA and DTX1), several factors can be hypothesised as the potential cause. One explanation may be derived from the increase of peridinin, a light harvesting pigment, that resulted in higher energy being harvested. This may have provided higher energy available for metabolism, including the production of secondary metabolites OA and DTX1. Another alternative explanation is that the frequent dark cycle might have effectively stimulated the production of enzymes responsible for the biosynthesis processes of these compounds, resulting in their increase. It has been reported that both production compounds during the cell cycle are restricted to light period (Pan *et al.* 1999). As such, the manipulation of light conditions performed in this study might have triggered the enzymatic response as an adaptation mechanism.

Overall, results in this study indicated that optimum production of compounds can be obtained by providing fluctuating light/dark conditions at a frequency of 0.5 hour. However, such light configurations in cultivation system may not be feasibly applied by utilising fluorescent lamps as it will significantly reduce the life span of lamps. In contrast, such frequent changing of light/dark cycle can be best achieved using light emitting diodes (LEDs), suggesting that the application of this lighting source should be more fully explored.

5.4.2 Effect of CO_2 supplementation on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

There has been an increasing recognition that microalgae capable of producing valuable compounds can possibly be utilised for CO_2 sequestration purposes due to their ability to perform photosynthesis (Benemann 1997, Wang *et al.* 2008). Considering such possibility, the works carried out in this part of study attempted to assess the suitability of integrating *P. lima* CCAP 1136/11 cultivation for the production of valuable compounds with CO_2 sequestration.

It appeared, however, that such integration is not a straight forward process. Increasing the CO_2 level at a concentration of approximately 5% (v/v) proved to inhibit the growth of *P. lima* CCAP 1136/11, which led to the observed low production of DSP toxins and peridinin from the culture. The increased level of CO_2 caused the gradual decrease of pH in culture medium (Fig. 5.8B). This increasingly acidic medium was accountable for the growth limitations observed. Similar observation has also been reported for other species of marine dinoflagellates, *Amphidinium carterae* and *Heterocapsa oceanica* Stein (Dason and Colman 2004), which explained that rapid decrease of pH in medium due to increased CO_2 availability impacted on the reduced pH within cell, hence impairment in photosynthetic capability.

The mechanism of pH reduction in culture medium may be explained by the carbonate chemistry in water (Kleypas and Langdon 2000):

$$CO_{2\,(gas)} \leftrightarrows CO_{2\,(aq)} + H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^- \leftrightarrows 2H^+ + CO_3^{2-}$$

Most eukaryotic microalgae consume inorganic carbon in the form of aqueous CO_2 and bicarbonate (HCO_3^-) (Colman *et al.* 2002). However, the increase in CO_2 concentration drives the equilibrium towards the increase of hydrogen ion, thereby not only increasing the aqueous CO_2 and bicarbonate but also decreasing the pH level (Doney *et al.* 2009). The results obtained in this study, which showed rapid decrease of pH with increasing CO_2 concentration, might indicate that *P. lima* CCAP 1136/11 grew too slowly and their density in the culture was not sufficiently increase to produce CO_2 fixation rate that could compensate the increased CO_2 provision.

Several alternatives that may be utilised to improve *P. lima* performance under elevated CO_2 level include pre-adaptation to low elevation of CO_2 and increasing cell density in the culture. It has been commented that both could allow microalgae to thrive under high CO_2 level conditions (Lee *et al.* 2002, Yoo *et al.* 2010). Besides those two methods, another solution may be devised by supplying the CO_2 at pulse cycle, which may improve the culture tolerance to elevated CO_2 level. It was observed that intermittent supply of CO_2 allowed some time for microalgal culture (*Microcystis aeruginosa*) to recover their pH to its normal level (Graham 2007). Such pH re-adjustment would prevent the medium from becoming too acidic, which would enable the culture to grow.

Nevertheless, it is possible that *P. lima* might not be a suitable strain candidate for CO_2 sequestration, particularly as most candidate strains utilised in CO_2 sequestration are those that can grow very rapidly (Wang *et al*. 2008). Such traits are not shared by the dinoflagellate *P. lima*. As such, genetic modification, for instance by introducing gene responsible for DSP toxins synthesis to another species of fast growing microorganism, may provide a better alternative for the purpose, provided it is feasibly done.

5.4.3 Effects of different temperatures on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11

The efficacy of manipulating growth temperature in directing microalgal culture towards the production of preferred compounds has been widely demonstrated (Tjahjono *et al*. 1994, De Oliveira *et al.* 1999, Converti *et al.* 2009, Liu and Lee 2000, Xin *et al.* 2011). The works carried out in this part of the study attempted to explore the possibility to optimise growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 culture by manipulating its growth temperature. Several temperatures (22, 27 and 32^{o}C) were selected based on previous reports studying the growth and okadaic acid production from *P. lima* and other species of *Prorocentrum* at varying temperatures (Xu *et al*. 2010, Grzebyk and Berland 1996, Morton *et al*. 1992; 1994). Those studies found that growth and OA production from this genus of dinoflagellate was optimum at temperature range higher than the level used for routine maintenance of the stock culture utilised in this study (24^{o}C to 28^{o}C).

Besides the potential effects on growth and OA production, growing the organism at high temperature might be beneficial in increasing the production of major carotenoid pigment of *P. lima* CCAP 1136/11, peridinin. This approach has been demonstrated previously for the production of another type of carotenoid pigment, astaxanthin, from other species of microalgae (Tjahjono *et al*. 1994, Liu and Lee 2000). Carotenoids in microalgae are used by the organism to protect themselves against reactive oxygen species (ROS) (Raven and Geider 2003, Masojídek *et al*. 2004). Thus, increasing the cultivation temperature above the normal growth level that promotes ROS formation in the culture would allow the stimulation of higher concentrations of carotenoid pigments in microalgal culture (Tjahjono *et al*. 1994, Li *et al*. 2008).

However, results of present study found that *P. lima* CCAP 1136/11 was very sensitive to elevation of temperature level. They grew optimally under 22^{o}C, and elevation of temperature by 5^{o}C resulted in severe inhibition of growth. Further elevation to 32^{o}C showed death of culture at almost instantaneously. The ability of dinoflagellates to grow only in a particular range of temperature reflects the conditions that they encounter in their original habitat, to which they have been

accustomed to (Koike *et al*. 1998, Kobiyama *et al*. 2010). Strain of *P. lima* CCAP 1136/11 used in this study was isolated from Vigo, Spain (Bravo *et al*. 2001), of which seasonal variations of surface seawater temperature ranged between 12°C during winter to 24°C during summer (Dumousseaud *et al*. 2010, Somavilla *et al*. 2013). This may support the findings in this research indicating the inability of *P. lima* CCAP 1136/11 to sustain growth at higher temperatures as they may not be suitably grown beyond the normal temperature range of their habitat. However, it must be noted that it was possible that the perpetual maintenance of the stock culture at a relatively constant temperature of 22°C had lowered the tolerance of this strain, hence their inability to withstand the increase in growth temperature.

Pre-adapting the stock culture under gradually increased temperature level is a possible means to improve the performance of *P. lima* CCAP 1136/11 at high temperature. Pre-adaptation process may allow the organism to increase their growth temperature, thus enabling them to withstand high temperature level. However, this may not be beneficial for the stimulation of higher carotenoids accumulation, as pre-adaptation will reduce the vulnerability of the organism towards temperature stress, which may hinder the production of stress related compounds such as carotenoid pigments.

Overall, the works presented in this study indicated that manipulation of temperature to optimise growth and production of DSP toxins and peridinin may not be suitably performed for *P. lima* CCAP 1136/11, as this organism was proved to have high sensitivity to changes in growth temperature. It is also possible to suggest that the margin of temperature increase used in this study (5°C) was too high for the organism to adapt. Therefore, future attempts to manipulate growth temperature should focus on utilising smaller temperature increase.

5.5 Conclusions

The influence of several abiotic factors, consisting of light, temperature and CO_2 supplementation, on the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 were evaluated. Results indicated that the production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 can be enhanced through the manipulation of light conditions in cultivation system. Providing fluctuating light/dark conditions at a frequency of 0.5 hour has been shown to significantly increase the production of compounds by approximately 30% for OA, 95% for DTX1 and 80% for peridinin than the continuous light cycle. To provide these fluctuating light conditions, LEDs can be conveniently used to replace fluorescent lamps without affecting the growth and production of compounds from *P. lima* CCAP 1136/11.

Furthermore, *P. lima* CCAP 1136/11 was found to be very sensitive to change in growth temperature and CO_2 levels. They grew optimally when maintained at 22^{o}C with aeration of ambient air. Elevating the level of temperature and CO_2 resulted in severe inhibition of growth, which in returns hampering the production of DSP toxins and peridinin. Therefore, manipulation of these two parameters in the cultivation system was not recommended.

5.6 References

Benemann, J. R. 1997. CO_2 mitigation with microalgae systems. **Energy Conversion and Management**, 38, S475-S479.

Bravo, I., Fernandez, M. L., Ramilo, I., Martinez, A. 2001. Toxin composition of the toxic dinoflagellate *Prorocentrum lima* isolated from different locations along the Galician coast (NW Spain). **Toxicon,** 39, 1537-1545.

Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., Chang, J. S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. **Bioresource Technology**, 102 (1), 71-81.

Chiu, S. Y., Kao, C. Y., Huang, T. T., Lin, C. J., Ong, S. C., Chen, C. D., Chang, J. S., Lin, C. S. 2011. Microalgal biomass production and on-site bioremediation of carbon dioxide, nitrogen oxide and sulfur dioxide from flue gas using *Chlorella* sp. cultures. **Bioresource Technology**, 102 (19), 9135-9142.

Colman, B., Huertas, I. E., Bhatti, S., Dason, J. S. 2002. The diversity of inorganic carbon acquisition mechanisms in eukaryotic microalgae. **Functional Plant Biology**, 29 (3), 261-270.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., Del Borghi, M. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. **Chemical Engineering and Processing: Process Intensification**, 48 (6), 1146-1151.

Dason, J. S., Colman, B. 2004. Inhibition of growth in two dinoflagellates by rapid changes in external pH. **Canadian Journal of Botany**, 82 (4), 515-520.

De Oliveira, M. A. C. L., Monteiro, M. P. C., Robbs, P. G., Leite, S. G. F. 1999. Growth and chemical composition of *Spirulina maxima* and *Spirulina platensis* biomass at different temperatures. **Aquaculture International**, 7 (4), 261-275.

Doney, S. C., Fabry, V. J., Feely, R. A., Kleypas, J. A. 2009. Ocean acidification: the other CO_2 problem. **Annual Review on Marine Science**, 1, 169-192.

Dumosseaud, C., Achterberg, E. P., T. Tyrell, Chlaralampopoulou, A., Schuster, U., Hartman, M., Hydes, D. J. 2010. Contrasting effects of temperature and winter mixing on the seasonal and inter-annual variability of the carbonate system in the Northeast Atlantic Ocean. **Biogeosciences**, 7, 1481-1492.

Graham, D. J. L. 2007. 'An Investigation into factors influencing the production and degradation of microcystins'. PhD Thesis. Robert Gordon University, Aberdeen, UK.

Grobbelaar, J. U. 1989. Do light/dark cycles of medium frequency enhance phytoplankton productivity?. **Journal of Applied Phycology**, 1 (4), 333-340.

Grobbelaar, J. U. 1994. Turbulence in mass algal cultures and the role of light/dark fluctuations. **Journal of Applied Phycology**, 6 (3), 331-335.

Grobbelaar, J. U., Nedbal, N., Tichý, V. 1996. Influence of high frequency light/dark fluctuations on photosynthetic characteristics of microalgae photoacclimated to different light intensities and implications for mass algal cultivation. **Journal of Applied Phycology***, 8,* 335-343*.*

Grobbelaar, J. U. 2004. Algal nutrition, mineral nutrition. In: Handbook of Microalgal Culture, Biotechnology and Applied Phycology. AMOS RICHMOND (Ed). Oxford, UK: Blackwell Publishing. pp. 97-115.

Grzebyk, D., Berland, B. 1996. Influences of temperature, salinity and irradiance on growth of *Prorocentrum minimum* (Dinophyceae) from the Mediterranean Sea. **Journal of Plankton Research**, 18 (10), 1837-1849.

Heredia-Tapia, A., Arredondo-Vega, B. O., Nunez-Vazquez, E. J., Yasumoto, T., Yasuda, M., Ochoa, J. L. 2002. Isolation of *Prorocentrum lima* (Syn. *Exuviaella lima*) and diarrhetic shellfish poisoning (DSP) risk assessment in the Gulf of California, Mexico. **Toxicon**, 40 (8), 1121-1127.

Hu, Q. 2004. Environmental effects on cell composition. In: Handbook of Microalgal Culture, Biotechnology and Applied Phycology. AMOS RICHMOND (Ed). Oxford, UK: Blackwell Publishing. pp. 83-93.

Jacob-Lopes, E., Scoparo, C. H. G., Lacerda, L. M. C. F., Franco, T. T. 2009. Effect of light cycles (night/day) on CO_2 fixation and biomass production by microalgae in photobioreactors. **Chemical Engineering and Processing**, 48, 306-310.

Janssen, M., Kuijpers, T. C., Veldhoen, B., Ternbach, M. B., Tramper, J., Mur, L. R., Wijffels, R. H. 1999. Specific growth rate of *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* under medium duration light/dark cycles: 13–87 s. **Progress in Industrial Microbiology**, 35, 323-333.

Janssen, M., Slenders, P., Tramper, J., Mur, L. R., Wijffels, R. 2001. Photosynthetic efficiency of *Dunaliella tertiolecta* under short light/dark cycles. **Enzyme and Microbial Technology**, 29 (4), 298-305.

Katsuda, T., Lababpour, A., Shimahara, K., Katoh, S. 2004. Astaxanthin production by *Haematococcus pluvialis* under illumination with LEDs.**Enzyme and Microbial Technology**, 35 (1), 81-86.

Kleypas, J., Langdon, C. 2000. Overview of CO_2-induced changes in seawater chemistry. *Proceedings of the 9th International Coral Reef Symposium, Bali, Indonesia*, 2, 1085-1089.

Kobiyama, A., Tanaka, S., Kaneko, Y., Lim, P. T., Ogata, T. 2010. Temperature tolerance and expression of heat shock protein 70 in the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae). **Harmful Algae**, 9, 180-185.

Koike, K., Sato, S., Yamaji, M., *et al*. 1998. Occurrence of okadaic acid-producing *Prorocentrum lima* on the Sanriku Coast, Northern Japan. **Toxicon**, 36 (12), pp. 2039-2042.

Lababpour, A., Hada, K., Shimahara, K., Katsuda, T., Katoh, S. 2004. Effects of nutrient supply methods and illumination with blue light emitting diodes (LEDs) on astaxanthin production by *Haematococcus pluvialis*. **Journal of Bioscience and Bioengineering**, 98 (6), 452-456.

Lababpour,A., Shimahara, K., Hada, K., Kyoui, Y., Katsuda, T., Katoh, S. 2005. Fed-batch culture under illumination with blue light emitting diodes (LEDs) for astaxanthin production by *Haematococcus pluvialis*. **Journal of Bioscience and Bioengineering**, 100 (3), 339-342.

Lee, C. G., Palsson, B. Ø. 1994. High-density algal photobioreactor using light-emitting diodes. **Biotechnology and Bioengineering**, 44, 1161-1167.

Lee, J. S., Kim, D. K., Lee, J. P., Park, S. C., Koh, J. H., Cho, H. S., Kim, S. W. 2002. Effects of SO_2 and NO on growth of *Chlorella* sp. KR-1. **Bioresource Technology**, 82 (1), 1-4.

Levasseur, M., Couture, J. Y., Weise, A. M., Michaud, S., Elbrächter, M., Sauvé, G., Bonneau, E. 2003. Pelagic and epiphytic summer distributions of *Prorocentrum lima* and *P. mexicanum* at two mussel farms in the Gulf of St. Lawrence, Canada. **Aquatic Microbial Ecology**, 30(3), 283-293.

Li, Y., Sommerfeld, M., Chen, F., Hu, Q. 2008. Consumption of oxygen by astaxanthin biosynthesis: A protective mechanism against oxidative stress in *Haematococcus pluvialis* (Chlorophyceae). **Journal of Plant Physiology**, 165, 1783-1797.

Liu, W., Au, D. W. T., Anderson, D. M., Lam, P. K. S., Wu, R. S. S. 2007. Effects of nutrients, salinity, pH and light:dark cycle on the production of reactive oxygen species in the alga *Chattonella marina*. **Journal of Experimental Marine Biology and Ecology**, 346, 76-86.

Liu, B. H., Lee, Y. K. 2000. Secondary carotenoids formation by the green alga *Chlorococcum* sp. **Journal of Applied Phycology**, 12 (3-5), 301-307.

Lorenz, M., Friedl, T., Day, J. G. 2005. Perpetual maintenance of actively metabolizing microalgal cultures. In: RA Andersen (Ed), *Algal Culturing Techniques*. London, UK: Elsevier Academic Press. pp. 145-156.

Masojídek, J., Koblížek, M., Torzillo, G. 2004. Photosynthesis in microalgae. In: AMOS RICHMOND (Ed). *Handbook of Microalgal Culture, Biotechnology and Applied Phycology*. Oxford, UK: Blackwell Publishing. pp. 20-39.

Matthijs, H. C. P., Balke, H., van Hes, U, M., Kroon, B. M. A., Mur, L. R., Binot, R. A. 1996. Application of light emitting diodes in bioreactors: Flashing light effects and energy economy in algal culture (*Chlorella pyrenoidosa*). **Biotechnology and Bioengineering**, 50*,* 98-107.

McLachlan, J. L., Marr, J. C., Conlon-Keily, A., Adamson, A. 1994. Effects of nitrogen concentration and cold temperature on DSP-toxin concentrations in the dinoflagellate *Prorocentrum lima* (prorocentrales, dinophyceae). **Natural Toxins**, 2 (5), 263-270.

Meseck, S. L., Alix, J. H., Wikfors, G. H. 2005. Photoperiod and light intensity effects on growth and utilization of nutrients by the aquaculture feed microalga, *Tetraselmis chui* (PLY429). **Aquaculture**, 246 (1), 393-404.

Morton, S. L., Norris, D. R., & Bomber, J. W. 1992. Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. **Journal of Experimental Marine Biology and Ecology**,157 (1), 79-90.

Morton, S. L., Bomber, J. W., Tindall, P. M. 1994. Environmental effects on the production of okadaic acid from *Prorocentrum hoffmanium* Faust I. temperature, light and salinity **Journal of Experimental Marine Biology and Ecology**, 178, 67-77.

Nascimento, S. M., Purdie, D. A., Morris, S. 2005. Morphology, toxin composition and pigment content of *Prorocentrum lima* strains isolated from a coastal lagoon in southern UK. **Toxicon,** 45, 633-649.

Nedbal, L., Tichý, V., Xiong, F., Grobbelaar, J. U. 1996. Microscopic green algae and cyanobacteria in high-frequency intermittent light. **Journal of Applied Phycology**, 8 (4-5), 325-333.

Pan, Y., Cembella, A. D., Quilliam, M. A. 1999. Cell cycle and toxin production in the benthic dinoflagellate *Prorocentrum lima*. **Marine Biology**, 134*,* 541-549.

Park, K. H., Lee, C. G. 2000. Optimization of algal photobioreactors using flashing lights. **Biotechnology and Bioprocess Engineering***, 5,* 186-190.

Posten, C. 2009. Design principles of photo-bioreactors for cultivation of microalgae. **Engineering in Life Sciences**, 9 (3), 165-177.

Raven, J. A., Geider, R. J. 2003. Adaptation, acclimation and regulation in algal photosynthesis. In: A. W. D. LARKUM, S.E. DOUGLAS, J. A. RAVEN (Eds). *Photosynthesis in Algae*. London, UK: Kluwer Academic Publishers.

Rhodes, L. L., Syhre, M. 1995. Okadaic acid production by a New Zealand Prorocentrum lima isolate.

Somavilla, R., González-Pola, C., Lavín, A., Rodriguez, C. 2013. Temperature and salinity variability in the south-eastern corner of the Bay of Biscay (NE Atlantic). **Journal of Marine System**, 109-110, S105-S120.

Suh, I. S., Lee, C. G. 2003. Photobioreactor engineering: design and performance. **Biotechnology and Bioprocess Engineering**, 8 (6), 313-321.

Tjahjono, A. E., Hayama, Y., Kakizono, T., Terada, Y., Nishio, N., Nagai, S. 1994. Hyper-accumulation of astaxanthin in green alga *Haematococcus pluvialis* at elevated temperatures. **Biotechnology Letters**, 16 (2), 133-138.

Vanucci, Silvana., Guerrini, Franca, Milandri, Anna., Pistocchi, Rosella. 2010. Effects of different levels of N- and P- deficiency on cell yield, okadaic acid, DTX-1, protein and carbohydrate dynamics in the benthic dinoflagellate *Prorocentrum lima*. **Harmful Algae**, 9, 590-599.

Varkitzi, Ionna., Pagou, Kalliopi., Graneli, Edna., et al. 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. **Harmful Algae,** 9, 304-311.

Vonshak, A., Torzillo, G. 2004. Environmental stress physiology. In: A Richmond (Ed), *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*. Oxford, UK: Blackwell Science Ltd. pp. 57-82.

Wang, C. Y., Fu, C. C., Liu, Y. C. 2007. Effects of using light-emitting diodes on the cultivation of *Spirulina platensis*. ***Biochemical Engineering Journal,*** 37*,* 21-25.

Wang, B., Li, Y., Wu, N., Lan, C. Q. 2008. CO_2 bio-mitigation using microalgae. **Applied Microbiology and Biotechnology**, 79, 707-718.

Xin, L., Hong-Ying, H., Yu-Ping, Z. 2011. Growth and lipid accumulation properties of a freshwater microalga *Scenedesmus* sp. under different cultivation temperature. **Bioresource Technology**, 102 (3), 3098-3102.

Xu, L., Weathers, P. J., Xiong, X. R., Liu, C. Z. 2009. Microalgal bioreactors: challenges and opportunities. **Engineering in Life Sciences**, 9 (3), 178-189.

Xu, N., Duan, S., Li, A., Zhang, C., Cai, Z., Hu, Z. 2010. Effects of temperature, salinity and irradiance on the growth of the harmful dinoflagellate *Prorocentrum donghaiense* Lu. **Harmful Algae**, 9, 13-17.

Yam, F. K., Hassan, Z. 2005. Innovative advances in LED technology. **Microelectronics Journal**, 36*,* 129-137.

Yeh, N., Chung, J. P. 2009. High-brightness LEDs – Energy efficient lighting sources and their potential in indoor plant cultivation. **Renewable and Sustainable Energy Review***,* 13, 2175-2180.

Yoo, C., Jun, S. Y., Lee, J. Y., Ahn, C. Y., Oh, H. M. 2010. Selection of microalgae for lipid production under high levels carbon dioxide. **Bioresource Technology**, 101, 571-574.

CHAPTER 6

Development of Photobioreactor for Large Scale Cultivation of Benthic *Prorocentrum lima*

6.1 Introduction

Potential applications of microalgal products have been repeatedly identified over the last few decades (Radmer and Parker 1994, Apt and Behrens 1999, Camacho *et al*. 2007a). Nevertheless, only a few of these compounds have been successfully commercialised, especially as the production of valuable compounds from phototrophic microalgae at industrial scale has often been hampered by the lack of practical and cost efficient culturing methods. Such hindrances have arguably steered the need to develop culture systems towards reactors that are not only controllable but also able to sustainably produce a high yield of biomass and compounds. This system is commonly termed 'photobioreactor' (PBR), which is phototrophic production in a closed vessel in which an electrical light serves as supply energy (Behrens 2005).

Continuous developments of PBR systems have enabled the commercialisation of several microalgal products (Richmond *et al.* 1993, Olaizola 2000, Kaewpintong *et al.* 2007). However, the potential utilisation of PBR system for mass production of valuable compounds from slow growing microalgae, such as group of dinoflagellates, or benthic organism is yet to be fully exploited. Currently, the applications of PBR systems are still limited to planktonic (or suspended) and fast growing microalgae, for instance those used for the production of biofuels or food products.

It has been observed that the direct application of advanced bioreactor design is not suitable for dinoflagellates as they are known to be more vulnerable to fluid turbulence than other groups of microalgae (Thomas and Gibson 1990, Juhl *et al.* 2001, Camacho *et al.* 2007b). As such, current development of photobioreactors for dinoflagellates has rarely exceeded a capacity of few hundred litres (Wang and Hsieh 2001, Camacho *et al*. 2010; 2011). In contrast, reactors developed for fast growing planktonic microalgae, which are better adapted to fluid turbulence, are able to operate at capacity of several thousands of litres (Olaizola 2000, Molina-Grima *et al.* 2001, Cheng-Wu *et al.* 2001, Kaewpintong *et al.* 2007).

Besides the vulnerability to fluid turbulence, the benthic characteristics of some species of dinoflagellates are known to pose an additional challenge during the

scale-up process of their cultivation. Benthic microalgae are traditionally cultured in simple vessels such as carboys, bags and flasks. However, the scaling up of such systems requires excessive volume of media, which are perceived to be neither practical nor safe. Furthermore, the tendency of benthic cells to settle on surfaces is known to render the application of well-developed conventional PBR system difficult for these organisms, particularly since the settled cells would not receive light, nutrients and air consistently. Considering such challenges, further development of scalable benthic PBR is needed to exploit the potential of benthic dinoflagellates.

The development of benthic PBR has been attempted for some species of microalgae (Fig. 6.1). Several reactor designs focus on providing enhanced surface area for algal attachment (Fig. 6.1 A-C), for instance by utilising a biofilm (Bruno *et al.* 2012, Ozkan *et al.* 2012, Liu *et al*. 2013), glass beads column (Fukami *et al.* 1997, Raniello *et al.* 2007) or bristles PBR (Silva-Aciares and Riquelme 2008). These systems are commonly used for either filamentous microalgae or those that readily attach onto surfaces (Silva-Aciares and Riquelme 2008). Their application may enable highly efficient energy and minimal medium consumption during cultivation (Ozkan *et al.* 2012, Liu *et al*. 2013). They are also claimed to be practical and cost efficient during harvesting process due its filament-forming ability (Esson *et al.* 2011). Another type of benthic PBR is the bubble column system (Fig. 6.1D), which has been demonstrated to be suitable for growing benthic microalgae with low surface-attachment ability (Silva-Aciares and Riquelme (2008). Such system utilises pneumatic mixing that could simultaneously provide uniform liquid circulation and CO_2 supply at low power requirement (Sánchez-Mirón *et al.* 2000).

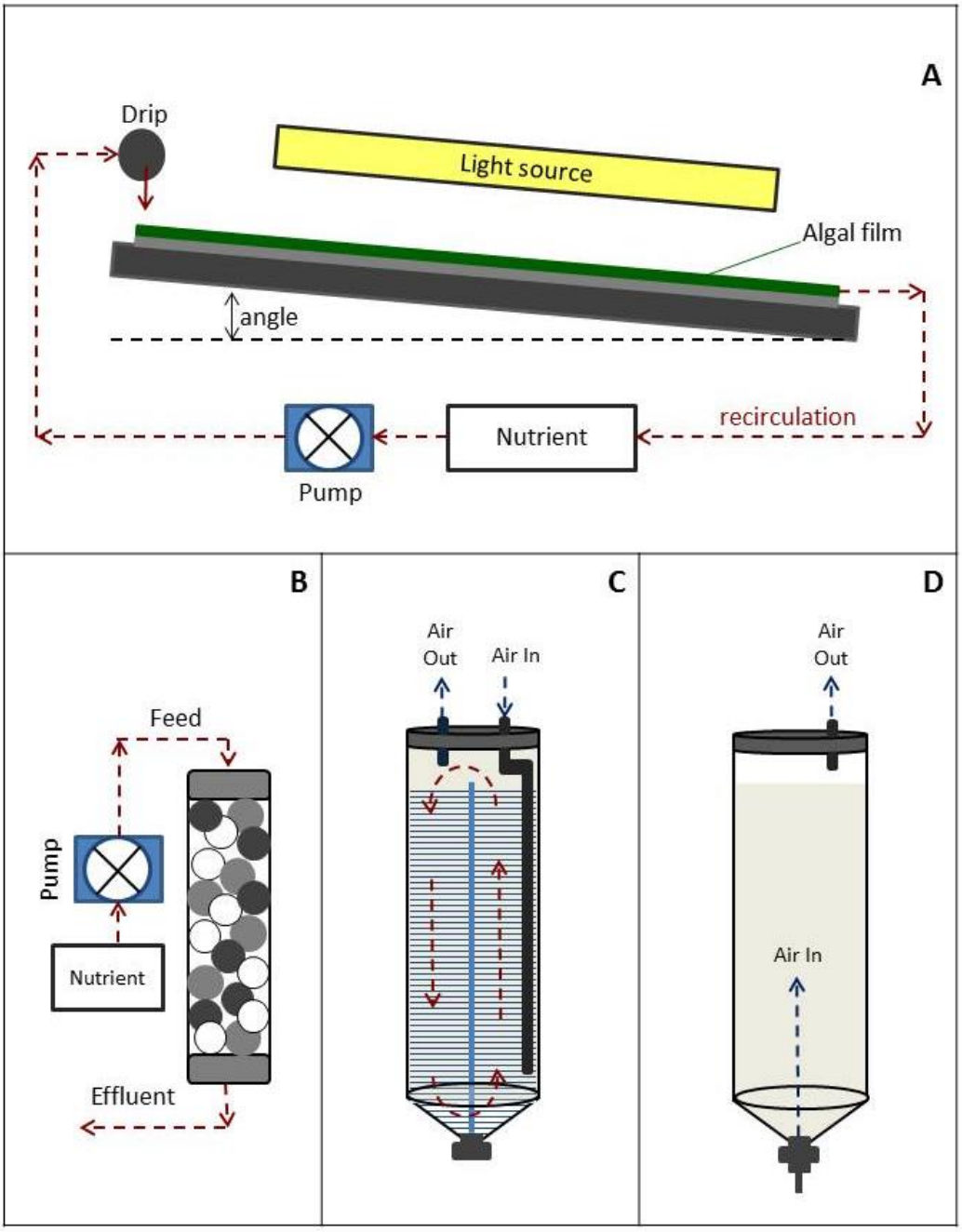


Figure 6.1 Example of developed PBR designs for benthic microalgae: (A) Biofilm (Ozkan *et al.* 2012), (B) glass beads column (Fukami *et al.* 1997), and (C) bristles PBR and (D) bubble column (Silva-Aciares and Riquelme 2008). Figures are adaptation from respective sources. Dashed red arrows show medium flow within the reactor.

Despite the growing interest in the development of benthic PBR systems, their applications for slow growing dinoflagellates are yet to be fully exploited. Investigations into the feasibility of growing benthic dinoflagellates in large scale

PBR systems are essential to enable their exploitation. As such, the works carried out in this chapter attempted to develop a scalable design of cultivation system that could be appropriately used for benthic and slow growing dinoflagellate. This study particularly focused on the development of a system for large scale production of DSP toxins and peridinin from *P. lima* CCAP 1136/11. Preliminary studies evaluating growth behaviour of this organism within various cultivation systems were also performed to determine the essential design parameters. This study represents the first attempt to optimise and develop cultivation system for the benthic dinoflagellate *P. lima*.

6.2 Materials and Methods

6.2.1 Culture and medium

Stock culture of *P. lima* CCAP 1136/11 (age 28 days, maintained as described in Chapter 2 section 2.2.2) was used throughout the experiments. Culture media were prepared according to f2-Si medium and sterilised by autoclave at 121^{o}C for 50 minutes (for 10 L volume) or 15 minutes (for volume of 1 L or less) (Astell Scientific, UK). Stock culture was inoculated into sterile medium to give a starting cell density of approximately 1-2 x10^4 cells mL^{-1}. For the monitoring of growth, cell counting was conducted using flow cytometer with method described in Chapter 2 section 2.2.3.2. The sensitivity and accuracy of flow cytometer was regularly monitored throughout the experiment with random samples checked by Sedgewick-Rafter (method described in section 2.2.3.1). The monitoring of DSP toxins and peridinin production was performed by analysing sample extract (prepared as described in Chapter 3, section 3.2.3.2) with UPLC-PDA-MS using method described in Chapter 2, section 2.2.5.

6.2.2 Evaluation of simple scale-up process

Cultures of *P. lima* CCAP 1136/11 were maintained in different scales of phototrophic cultivation in order to evaluate their growth and production of DSP toxin during scale-up process (Fig. 6.2). The experiments with 1 and 8 L scales were performed in Robert Gordon University laboratory, while the 30 L scale

experiment was conducted in collaboration with Centre for Process Innovation (CPI) (Wilton, Redcar, UK). Diagram of pipe and instrumentations for the 30 L scale reactor is presented in Appendix V.

All cultures were inoculated with stock culture of *P. lima* CCAP 1136/11 (maintained as described in Chapter 2 section 2.2.2) to give initial cell density of approximately $1x10^5$ cells mL^{-1}. Incubation of cultures was performed in a temperature controlled room at 22±1 ^oC with continuous light exposure at 20±2 µmol m^{-2} s^{-1}. Filtered (Filter disk Hepa-Vent, 0.3 µm, Whatman) ambient air was drawn into the flasks through an air pump to maintain aeration inside culture vessels. Sampling was conducted weekly throughout the cultivation period. During each sampling, aliquots of samples (10 mL) were taken and processed for analysis of cell number and OA production as described in section 6.2.1. Monitoring of cell number for the largest cultivation scale (30 L) was not feasible to perform due to the absence of appropriate facility.

Figure 6.2 Cultivation of *Prorocentrum lima* CCAP 1136/11 at 1 L, 8 L and 30 L.

6.2.3 Evaluation of surface area availability

Evaluation of the cultivation at different surface area was performed in order to investigate whether the availability of cells settlement area would affect the growth and productivity of benthic *P. lima* CCAP 1136/11. Triplicate of cultures were prepared by inoculating stock culture of *P. lima* CCAP 1136/11 into 200 mL sterile medium to provide starting cell density of $1x10^4$ cells mL^{-1}. The cultures were then placed into two types of flasks: 1) 500 mL Erlenmeyer flask with base surface area (growth area) of approximately 84 cm^2, and 2) 600 mL Corning® tissue culture flask with growth area of 150 cm^2. Both cultures were incubated in a temperature controlled room at 22±1 ^oC with continuous light exposure at 21±1 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Sampling of cultures was conducted every week for the period of five weeks. During each sampling, aliquots (2 mL) were taken and processed for analysis of growth and OA production as described in section 6.2.1.

6.2.4 Evaluation of the influence of aeration

Preliminary study of the influence of aeration in cultivation vessel was performed in order to evaluate the role of aeration to the growth and production of valuable compound from benthic *P. lima* CCAP 1136/11. The experiment was conducted by providing 4 L and 8 L cultures, which were contained in flasks of identical volumes, with the same flow rate of aeration (Fig. 6.3). Air pumps were used to provide aeration for both cultures by withdrawing ambient air, passed through a filter (0.22 µm; Millipore, UK) at flow rate of approximately 2.3 L min^{-1} for each flask. Both cultures were maintained in a temperature controlled room at 22±2 ^oC with continuous light exposure at 20±2 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Sampling of cultures was conducted every week for the period of five weeks. During each sampling, aliquots (10 mL) were taken and processed for analysis of growth and OA production as described in section 6.2.1.

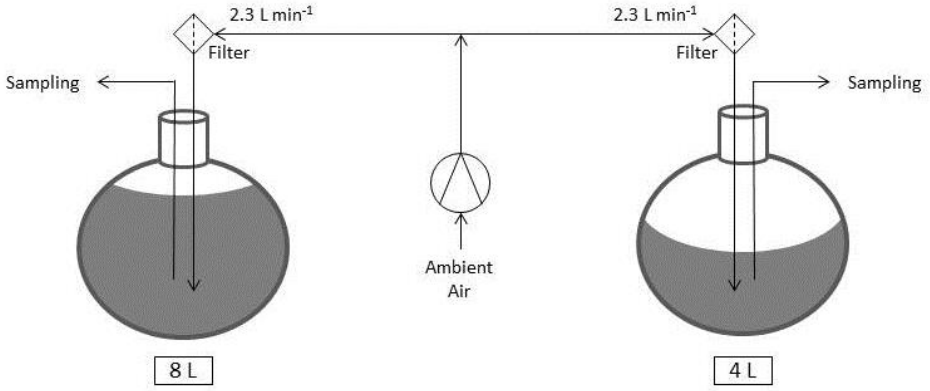


Figure 6.3 Schematic diagrams for the preliminary evaluation on the influence of culture volume and aeration for the cultivation of *P. lima* CCAP 1136/11.

6.2.5 Development of photobioreactor (PBR) with novel illumination system

The efforts to devise efficient illumination in large scale cultivation system have been beset by the requirement to provide sufficient light to a large volume of culture. However, a PBR still has to be designed to enable efficient illumination of large volume of culture. A potentially efficient novel illumination system was devised in this study by circulating the culture between dark vessel and light vessel. Such circulation would also enable the utilisation of intermittent light/dark cycle, which has been demonstrated to be beneficial for the production of DSP toxins and peridinin by *P. lima* CCAP 1136/11 (see Chapter 5 section 5.3.3). In addition, the flow dynamics utilised in this type of reactor would also enhance gas transfer and culture mixing within the system, potentially providing optimum conditions for growth for this benthic organism.

The PBR (Fig. 6.4) was constructed using two types of vessels. The first vessel, an Erlenmeyer flask containing 3 L culture, was covered with thick sheet of aluminium foil sheet to maintain dark condition. The second vessel was a clear thin-plate Plexiglas® that was exposed continuously to LEDs (Blue/Red 50:50) at an intensity of 60 µmol m^{-2} s^{-1}. The thin-plate vessel was designed to allow the culture to flow through the illuminated plate and the dark flask. The culture was

circulated between the two vessels using a peristaltic pump operated at flow rate of 1 L min^{-1}, providing effective illumination for 1 out of every 3 minutes required for the complete circulation of the culture (total illumination time of 8 hours per day for the whole culture). Figure 6.5 illustrates the cultivation of *P. lima* CCAP 1136/11 using PBR with novel illumination system. Simultaneously, a control culture was prepared and continuously exposed to light (Blue/Red LEDs, 50:50) at 20 µmol m^{-2} s^{-1}. The intensity used in the reference culture was 1:3 of that used in PBR in order to provide proportional light energy for both cultures.

All cultures were maintained in a temperature controlled room at 22±1 °C. Sampling of cultures was conducted every week for the period of five weeks. During each sampling, aliquots of samples (10 mL) were taken and processed for analysis of growth and OA production as described in section 6.2.1.

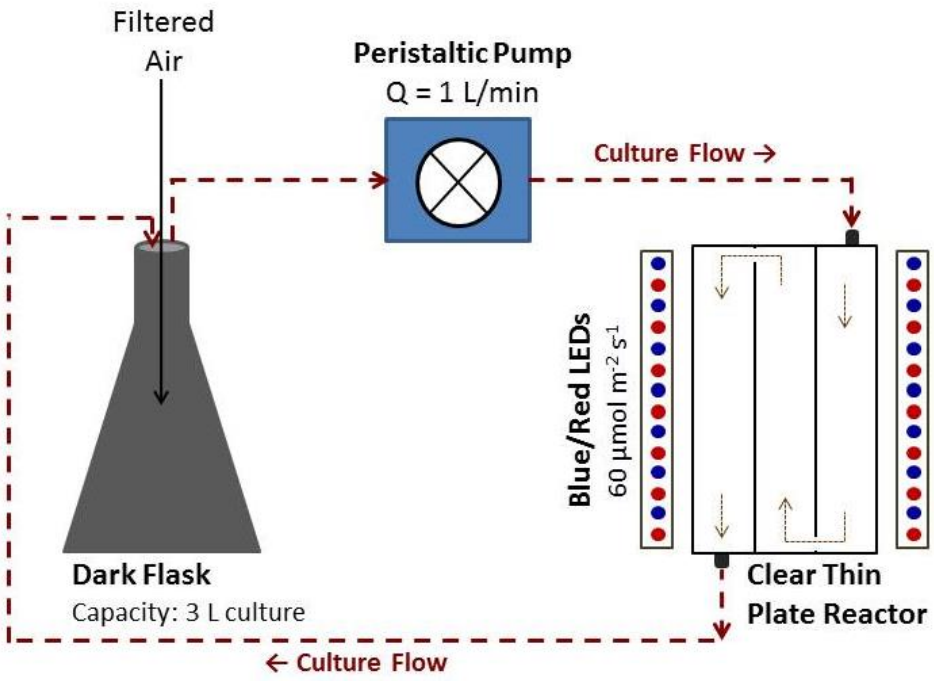


Figure 6.4 Schematic diagram showing photobioreactor (PBR) design with novel illumination system and the culture movement. Red arrows indicate culture movement within the system.

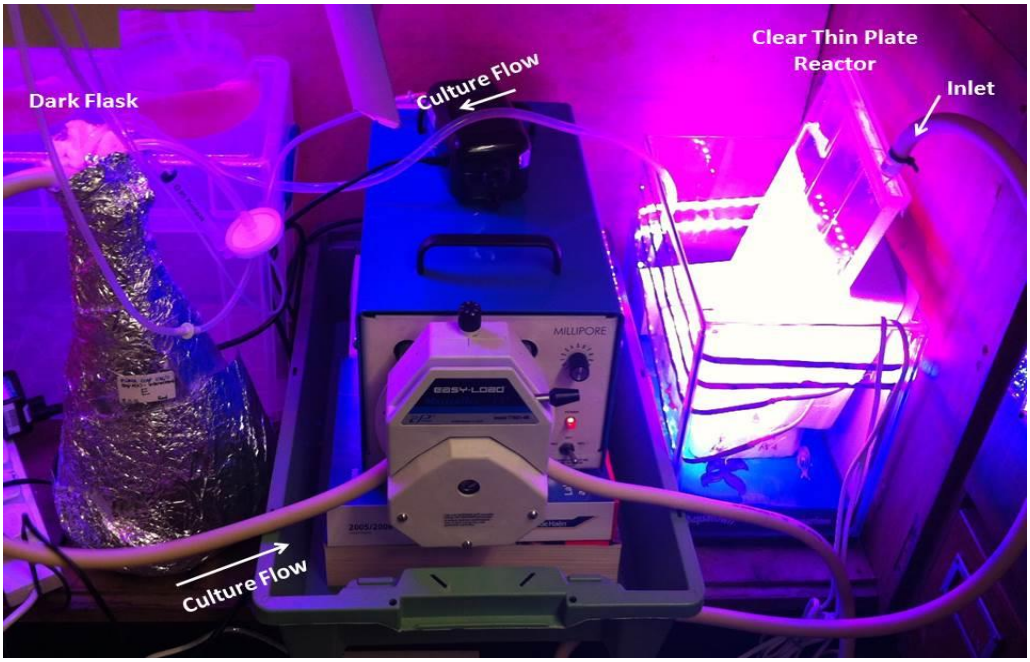


Figure 6.5 Cultivation of *P. lima* CCAP 1136/11 using photobioreactor (PBR) with novel illumination system.

6.2.6 Development of bubble-lift system for benthic *P. lima* CCAP 1136/11

Subsequent investigation focused on the development of PBR that enabled aeration over of the bottom of the reactor, on which cells tend to settle. Such aeration system may optimise the movement of cells within the reactor. The better facilitated movement of cells would ensure homogenous mixing of the culture.

Two bubble-lift systems were designed for the experiment. The first reactor, the thin-plate bubble-lift (TPBL), was constructed using a Plexiglas® sheet of approximately 1 cm thickness (Fig. 6.6). The sheet (90 x 110 cm) contained several columns that provided an even distribution of light and air mixing across the plate. A rubber aquarium bubble curtain of 90 cm length (All Pond Solution, UK) was attached at the bottom of the sheet to provide aeration across the base area on which cells might settle. At the top of the sheets, filling and harvesting

pipes were attached to facilitate inoculation, sampling and harvesting of the culture. This was constructed using a polyvinyl chloride (PVC) pipe (d=1 inch, L=1.2 m) with two ball valves attached at both ends (RS Components, UK). All attachments were provided by using marine transparent silicon sealant (Geocel Limited, Plymouth, UK). The TPBL had a capacity to operate with maximum of 8 litres batch culture. Filtered air was drawn into the reactor through an air pump providing aeration with maximum flow rate of ± 3 L min^{-1}.

The second reactor, the column bubble-lift (CBL) (Fig. 6.6), was constructed using clear acrylic tube of 1 metre length (d_i= 104 mm; d_o=110 mm); (Clear Plastic Supplies, UK). Air flow across the base area was delivered through an aquarium stone bubble disk (d=100 mm, thickness=18 mm) that was attached at the base of the reactor and supported by an acrylic sheet. All attachments were provided by using marine transparent silicon sealant. Aeration was performed using an air pump, providing air with maximum flow rate of ± 3 L min^{-1}. A sterile sponge was used to cover the top of reactor to avoid culture contamination whilst allowing air passage. CBL reactor has a capacity to operate with 7 litres of batch culture. Simultaneously, a control culture was prepared using 8 litres medium contained in 10 L round flask (section 6.2.3.1).

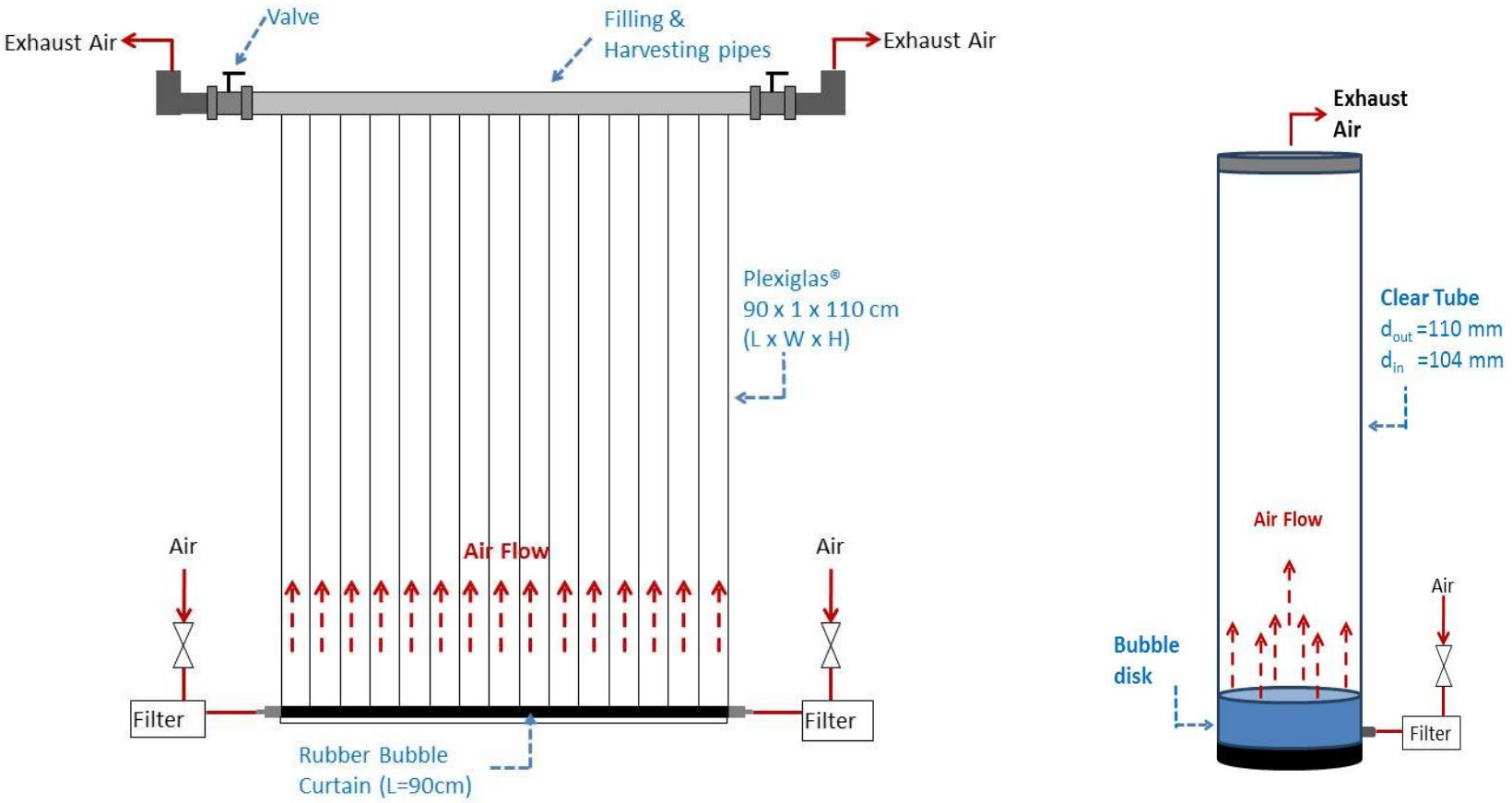


Figure 6.6 Schematic diagrams illustrating air flow inside thin-plate bubble-lift (TPBL) reactor (left) and column bubble-lift (CBL) reactor (right). Dashed red arrows demonstrate air flow within the reactor.

6.3 Results

6.3.1 Evaluation of simple scale-up process

Comparison of growth in 1 L and 8 L scales of cultures (Fig. 6.7A) revealed that cultivation in smaller scale provided much higher cell yield than medium scale. Little growth was observed for cultures in 8 L scale throughout the cultivation period. The increase of cell number within the cultivation period (yield) for the 1 L culture was noted at 100% (2 folds), while only 10% increase was observed for the 8 L culture. Final cell density measured in the 1 L culture was recorded at $2.9x10^5$ cells mL^{-1} in and in the 8 L culture was $1.79x10^5$ cell mL^{-1}.

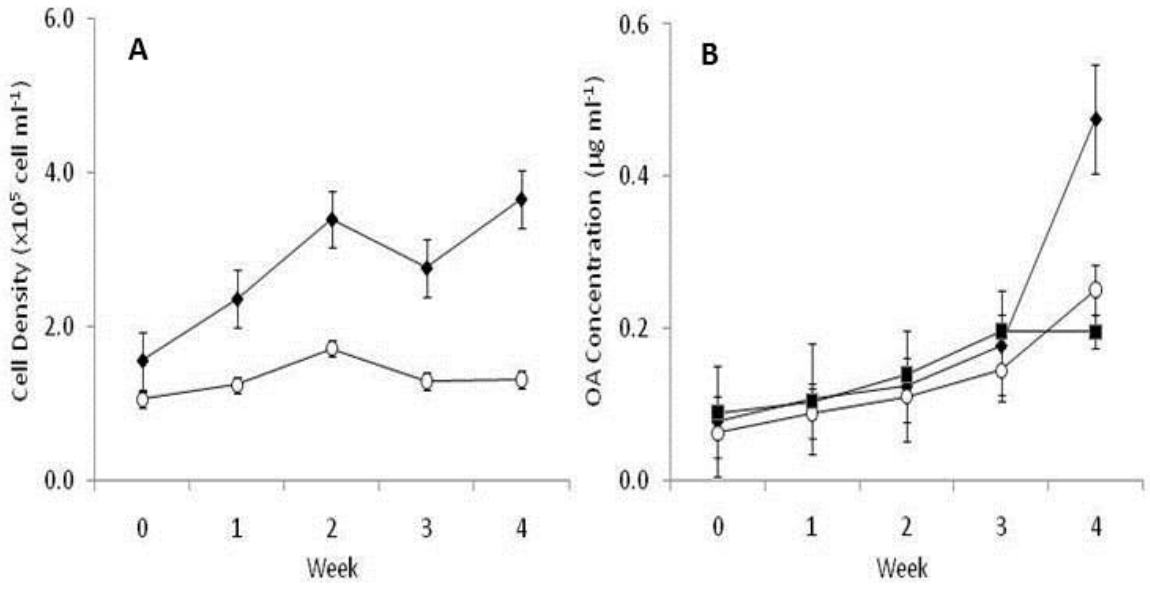


Figure 6.7 Growth (A) and okadaic acid production (B) from *P. lima* CCAP 1136/11 at different cultivation scales (n=3, error bars denote standard deviation); 1 L (♦), 8 L (O) and 30 L (■) scales.

Similar to the cell density, production of OA from *P. lima* CCAP 1136/11 was found to be higher at smaller scale. Cultures in 1 L scale produced the highest OA yield with more than 6 folds increase in concentration within the four weeks cultivation period, whereas yield for the other two scales was recorded at approximately 4 folds of 8 L culture and 2.2 folds for 30 L cultures (Fig. 6.7B).

The final OA concentration for the three cultures were observed at 0.195, 0. 250 and 0.475 µg mL^{-1} for 30 L, 10 L and 1 L respectively.

6.3.2 Evaluation of surface area availability

To evaluate the influence of surface area availability to the growth and production of OA from *P. lima* CCAP 1136/11, cultures of this benthic organism were grown inside two types of vessels; 500 mL Erlenmeyer flask with ~84 cm^2 growth area (base circumference: 32.5 cm), and 600 mL Corning® tissue culture flask with 150 cm^2 growth area. Analysis of cell density throughout the cultivation period showed similar cell density for the two vessels within the first four weeks of cultivation (Fig. 6.8A). However, the final week analysis showed that cultures with higher growth area (150 cm^2) produced higher cell number than those in 84 cm^2 vessel. The final cell density for the two types of vessels were measured at $2.1x10^4$ cells mL^{-1} for cultures in 84 cm^2 vessel, and $3.6x10^4$ cells mL^{-1} for cultures in 150 cm^2 vessel. Despite the higher cell density observed, cultures in vessel with 150 cm^2 growth area appeared to produce significantly lower OA than those in vessel with 84 cm^2 growth area (Fig. 6.8B). The final OA concentrations in both vessels were recorded at 0.12 µg mL^{-1} for the 150 cm^2 vessel and 0.24 µg mL^{-1} for the 84 cm^2 vessel.

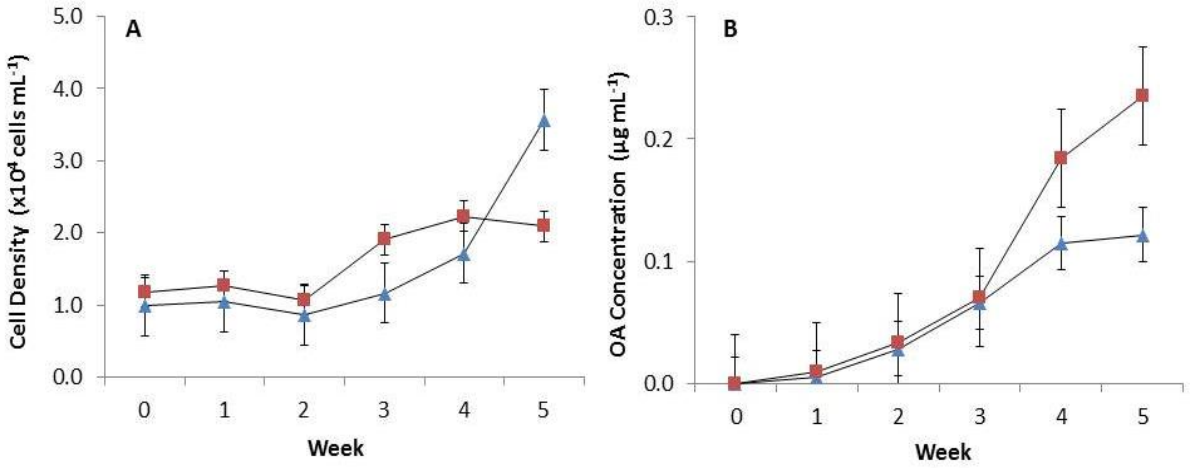


Figure 6.8 Growth (A) and okadaic acid production (B) from *P. lima* CCAP 1136/11 at vessels with different growth area (n=3, error bars denote standard deviation); 500 mL Erlenmeyer flask with growth area of 84 cm^2 (■) and 600 mL tissue culture flask with growth area of 150 cm^2 (▲).

6.3.3 Evaluation of the influence of culture volume and aeration

To evaluate the influence of culture volume and aeration to the growth and production of OA from *P. lima* CCAP 1136/11, two scales of cultures (8 L and 4 L) were grown in vessels with identical size using the same rate aeration (2.3 L min^{-1} air for each flask). Such configuration of culture volumes and aeration rate provided two cultures with different air-to-liquid ratios, resulting in the 4 L cultures having twice the ratio than the 8 L cultures (Table 6.1). In a given time of aeration, the air-to-liquid ratio in the 8 L cultures was 0.2875, whereas the ratio for the 4 L cultures was 0.5750.

Table 6.1 Calculated air-to-liquid ratio for two different culture volumes grown within identical vessels and aeration rate.

Culture Volume (Litre)	Aeration rate (Litre per minute)	Calculated air-to-liquid ratio (per minute)
8	2.3	0.2875
4	2.3	0.5750

Monitoring of cell density with flow cytometer revealed that growth of *P. lima* CCAP 1136/11 appeared to be affected by the culture volume and aeration within the cultivation vessel (Fig. 6.9). It was found that the 4 L cultures, which had higher air-to-liquid ratio, produced higher cell yield than the 8 L cultures. Cell yields after five weeks of cultivation were recorded at 3.8 folds increase for the 4 L culture and 2.0 folds increase for 8 L cultures. The final cell density for both cultures were $5.1x10^4$ and $2.4x10^4$ cells mL^{-1} for 4 L and 8 L cultures, respectively. Similarly, monitoring of OA concentration also found that cultures at lower volume (4 L) produced much higher OA than the 8 L cultures. Final concentrations of OA in both cultures were observed at 0.42 and 0.21 µg mL^{-1} for 4 and 8 L cultures, respectively.

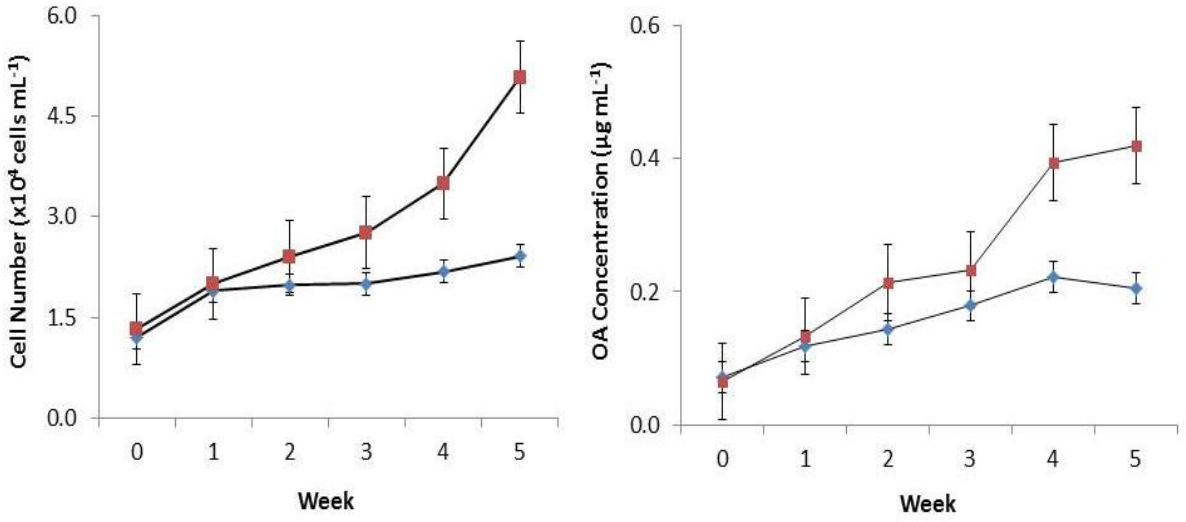


Figure 6.9 Growth (left) and okadaic acid production (right) from *P. lima* CCAP 1136/11 grown as 8 L (◆) and 4 L (■) cultures that were placed in identical size of vessels with the same rate of aeration (2.3 L min^{-1} air for each flask); (n=6, error bars denotes standard deviation).

6.3.4 Development of photobioreactor (PBR) with novel illumination system

Growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 were monitored for five weeks of cultivation inside the PBR with novel illumination system (Fig. 6.10). Although the culture did not die during the

cultivation period, it appeared that *P. lima* CCAP 1136/11 did not grow at all within the system (Fig. 6.10A). During the experiment, it was observed that cells of this benthic organism were not sufficiently circulated between the dark and the light vessels. The cells were found to settle mainly inside the dark vessel, and a small number were found attached to the bottom corners of the light vessel.

Similarly, any signs of increase in the production of OA, DTX1 and peridinin from the culture (Fig. 6.10B; 6.10C; 6.10D) were not observed. It has to be noted that the majority of OA and DTX1 detected from the samples were obtained as intracellular concentration. This indicates that the cells remained viable and intact throughout the five weeks of cultivation, as the release of OA and DTX1 to medium would normally be associated with ageing or non-viable cells.

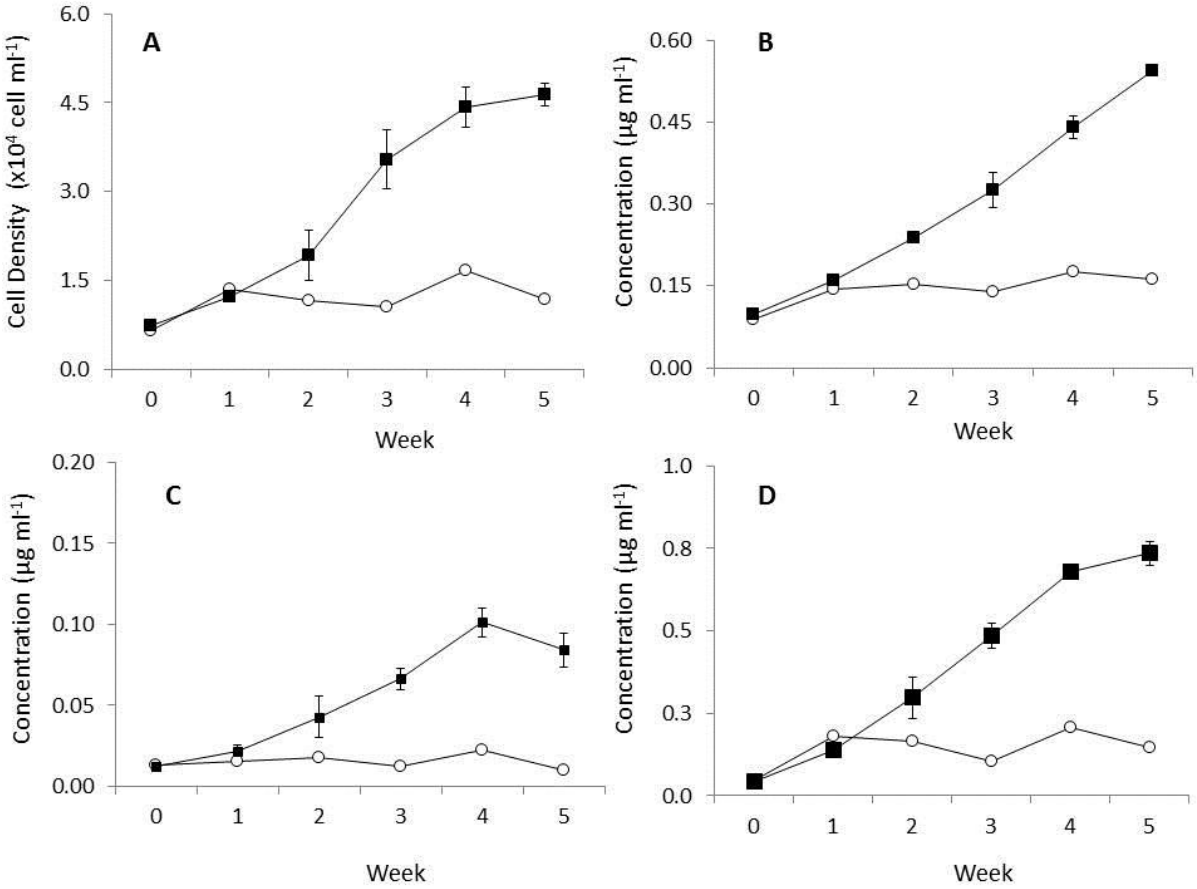


Figure 6.10 Growth expressed in cell density (A), and production of okadaic acid (B), DTX1 (C) and peridinin (D) from *Prorocentrum lima* culture cultivated inside PBR with novel illumination system (○) and conventional flask (control culture) (■); (n=1 for PBR with novel illumination system; n=2 for reference; error bars denote standard deviation).

6.3.5 Development of bubble-lift system for benthic *P. lima* CCAP 1136/11

Two designs of photobioreactor (PBR), thin-plate bubble-lift (TPBL) and column bubble-lift (CBL), were configured for benthic *P. lima* CCAP 1136/11. In both systems, the aeration facilitated good dispersion of the benthic cells of *P. lima* CCAP 1136/11 within the vessel. During the experimental period, it was observed that the use of both reactors significantly minimised the settling of cells at the reactor base. The cells of benthic *P. lima* CCAP 1136/11 were able to move freely within the system, following the liquid turbulence created by the air flow. Despite this, it was still difficult to completely eliminate cell settling within the reactor due to the difficulties in maintaining uniform air distribution across the length of bubble curtain (Fig. 6.11A) and the whole surface of bubble disk (Fig. 6.11B). In the CBL system, cell settling could be easily minimised by regularly increasing the air flow for a short period of time to create turbulence at the surface of bubble disk. This increase of turbulence caused the cells to rise from the base and float inside the reactor.

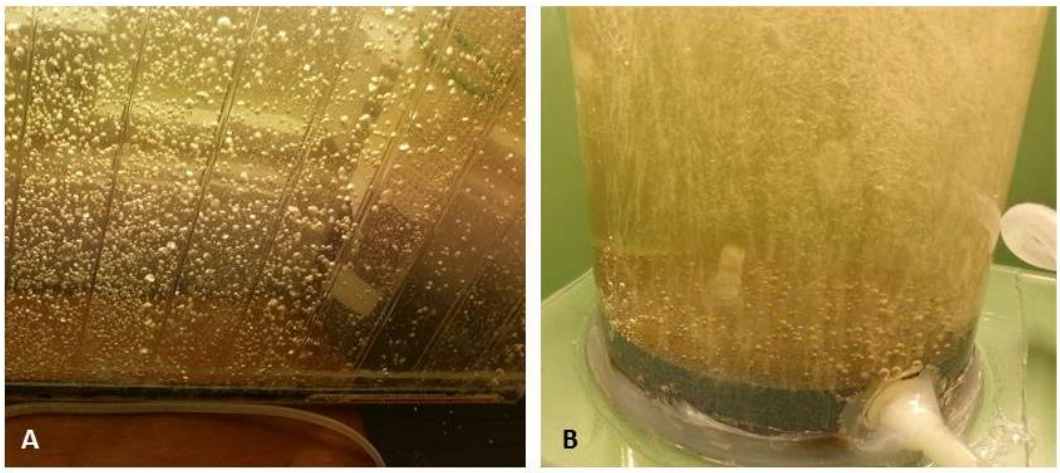


Figure 6.11 Air distributions across the length of bubble-curtain in thin plate bubble-lift reactor (A) and across the surface area of bubble-disk in column bubble-lift reactor (B).

The analysis of cell density during the final week of cultivation revealed that the TPBL system produced lower cell yield than those grown in conventional vessel (control culture) (Fig. 6.12A). It was also observed that cultures in TPBL system

showed excessive formation of foam at the top of culture, indicating the high shear tension within the system. In contrasts, growth of *P. lima* CCAP 1136/11 appeared to be enhanced when cultivated with CBL system. This was indicated by the 52% increase in cell number compared to those grown in conventional vessels. The increase in cell numbers after five weeks of cultivation were observed at 1.4 folds for TPBL, 3.9 folds for CBL and 2.0 folds for conventional vessel (control). The final cell densities obtained from all systems were recorded at $1.09x10^4$ for TPBL, $2.83x10^4$ for CBL and $1.89x10^4$ for control culture. Similarly, analysis by UPLC-PDA-MS also showed that production of DSP toxins and peridinin were lowest in TPBL and highest in CBL (Table 6.2).

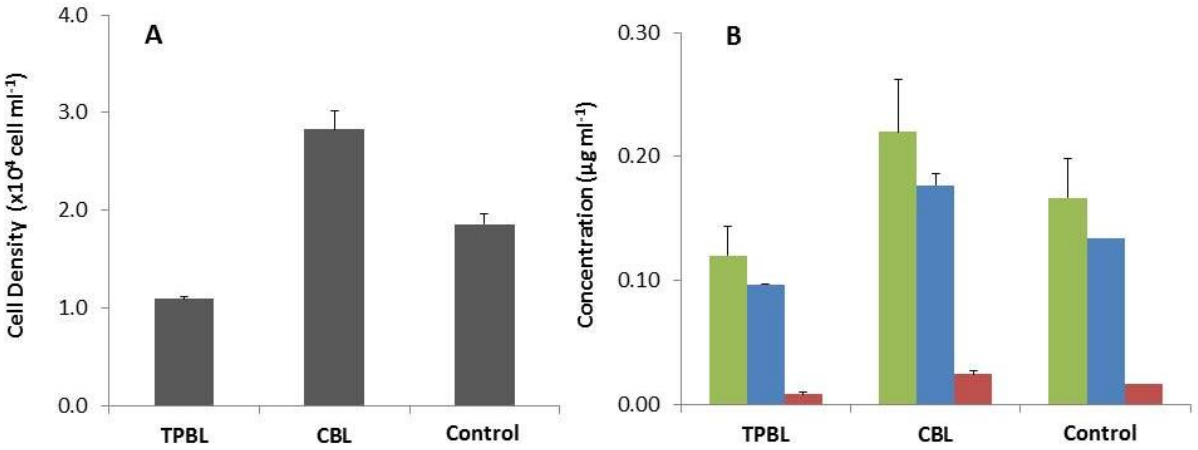


Figure 6.12 Final cell density (A) and concentration of peridinin (■), OA (■) and DTX1 (■) (B) from *P. lima* CCAP 1136/11 culture grown in thin-plate bubble-lift (TPBL) and column bubble-lift (CBL) reactors compared to conventional vessel (control) (n=2, error bars denote standard deviation).

Table 6.2 Final cell density and concentration of OA, DTX1 and peridinin from *P. lima* CCAP 1136/11 culture grown with different configurations of photobioreactor (n=2, ± standard deviation).

System	Final cell density (cell mL^{-1})	Final concentration (µg mL^{-1})		
		OA	DTX1	Peridinin
TPBL	1.09×10^4	0.097±0.00	0.008±0.00	0.120±0.02
CBL	2.83×10^4	0.177±0.01	0.024±0.00	0.220±0.04
Control	1.86×10^4	0.134±0.01	0.017±0.00	0.166±0.03

6.4 Discussion

6.4.1 Evaluation of growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 during scale-up process

In order to evaluate the growth performance and production of DSP toxin from *P. lima* CCAP 1136/11 during the scale-up process, preliminary tests on three different scales (1 L, 8 L and 30 L) of phototrophic cultivation were performed. Results from the simple scale-up process showed that this benthic organism preferred to grow within a small scale system, as indicated by the significantly higher cell and OA yields observed in 1 L scale (Fig. 6.7). Increasing the cultivation scale to 8 L was shown to lower cell density by approximately 40% and OA concentration by almost 50% in the culture.

There are several factors that may explain the difference of the growth performance of *P. lima* CCAP 1136/11 between cultivation scales. Considering their tendency to settle on surfaces, it was possible to suggest that the availability of surface area may influence the growth of this benthic organism. Providing large settlement or attachment area in a cultivation system has been shown to effectively promote the growth of benthic cyanobacteria *Phormidium autumnale* (Esson *et al.* 2011).

However, it was observed in this study that despite the higher cell yield obtained, production of OA from *P. lima* CCAP 1136/11 did not benefit from large growth area available in the cultivation system (Fig 6.8). Providing additional growth area by 1.78 folds resulted in increase of cell yield by approximately 20%, but lowered the OA yield by 48%. The lower cell yield obtained from cultures grown in flasks with small growth area may be caused by self-shading of the culture. These findings indicate that adding larger surface area for settlement or attachment may not be needed in the PBR design for this benthic organism to enhance their production of DSP toxins. Therefore, it was necessary to investigate other factors that might influence the performance of *P. lima* CCAP 1136/11 during scale-up process.

Another factor that might influence the poor performance of *P. lima* CCAP 1136/11 in larger scales was gas transfer (aeration) within the cultivation system. Providing sufficient aeration during scale-up process is important to improve mixing, medium circulation and gas exchange within the system (Ugwu *et al.* 2008). Thus, it was possible that the sparging provided for higher cultivation scales were insufficient to ensure optimal mixing and gas transfer within the system. However, during the simple scale-up process, aeration was provided at varying levels for different scales and monitoring effort was not performed to control consistent aeration level for all scales. Therefore, direct comparison between scales was impossible during this preliminary study.

The subsequent experiment was conducted to investigate the role of culture volume and aeration within the cultivation system. For this, two cultures at different scale of volume (4 and 8 L) were placed at identical size of vessel and aerated with the same rate of air flow, in order to give the cultures with different level of air-to-liquid volume ratio. It was evident from the results that higher air-to-liquid ratio within the vessel proportionally correlated with both cell density and OA concentration in *P. lima* CCAP 1136/11 culture. It was shown that the 4 L cultures, which had double the air-to-liquid volume ratio, produced twice higher cell density and OA concentration (Fig. 6.9), resulting in the same mass weight of OA produced per batch with the 8 L. This suggests that, in a regular laboratory-scale production of OA, the 4 L culture can be used to obtain the same target of production instead of the 8 L culture. The utilisation of 4 L culture would require significantly less seawater requirement than the 8 L culture, resulting in more time and costs efficient production. Overall, findings from this experiment indicate that the higher ratio of air-to-liquid in the cultivation vessel may have facilitated better mass transfer between the gas and the medium, hence higher productivity of the culture. Subsequent development on the reactor design focuses on the means to improve culture mixing and gas transfer through systems developed and discussed in section 6.4.2 and 6.4.3 below.

6.4.2 Development of photobioreactor (PBR) with novel illumination system

An important design objective of mass cultivation of microalgae with photobioreactor (PBR) is ensuring that the provision of light energy fulfils the requirement of large volume of culture. Providing high amount of light energy for large scale PBR system often requires substantial costs (Lee 2001). High energy consumptions of the system would increase the overall production costs and potentially reduce the profitability of the products. A PBR with novel illumination system was developed in this study with the aim of reducing the energy requirement of light provision.

In the proposed design of PBR, microalgal culture was allowed to circulate between a dark and a light vessel by using a peristaltic pump (Fig 6.2). As the culture was only illuminated when it reached the light vessel, the system would require significantly lower light energy compared to conventional settings that utilised full-scale illumination for the whole culture. Furthermore, utilising such circulation system would also enhance mixing and homogeneity of the culture. Sufficient mixing is important to ensure optimum mass transfer in the PBR, which may enable higher productivity of the culture (Ugwu *et al.* 2008).

However, the system developed was observed to be unsuitable for the cultivation of benthic organism. Throughout the cultivation period, *P. lima* CCAP 1136/11 culture was observed to be unable to grow inside the PBR (Fig. 6.10). It was observed that cells found settling at the base of the vessels were unable to constantly follow the flow stream within the system. The tendency to remain static might have caused the cells to be unable to circulate between the dark and the light vessels, resulting in growth limitations.

Nevertheless, despite the observed growth suppression, the culture did not perish in the system, as shown by the constant cell density throughout the cultivation period (Fig. 6.10). The survival of the culture might indicate that they were still able to intermittently move within the system, allowing them to utilise limited amount of light energy for their metabolism. Their ability to survive under

such limited light exposure is a natural characteristic of benthic organism (Pan *et al.* 1999).

Furthermore, it was observed that the cells were still possibly intact, as most of OA was extracted from intracellular samples. This indicated that *P. lima* CCAP 1136/11 was able to withstand relatively vigorous flow stream resulted from circulation of the culture. Such tolerance to vigorous hydrodynamics is uncommon for dinoflagellates as they are known to have fragile cells (Camacho *et al.* 2007a). It may also indicate that further development of PBR for *P. lima* CCAP 1136/11 could be flexibly performed due to the physical robustness of this organism.

Overall, the proposed illumination design may be more suited for planktonic microalgae than benthic organism. The ability of planktonic organism to float within liquid media would ensure consistent delivery of light energy in the system. Therefore, tests using planktonic microalgae as model organism may warrant further investigation in the future.

6.4.3 Development of bubble-lift system for benthic *P. lima* CCAP 1136/11

In order to improve gas transfer within the reactor, two reactors were designed; thin plate bubble-lift (TPBL) and column bubble-lift (CBL). Both reactors utilised pneumatic agitation that would simultaneously provide aeration and mixing for the culture. Pneumatic agitation was selected due to the advantage of potentially providing efficient CO_2 utilisation and ensures sufficient mixing of culture with relatively low energy requirement (Sánchez-Mirón *et al.* 2000, Wang *et al.* 2012).

Both reactors were designed based on an initial trial on cultivation of *P. lima* CCAP 1136/11 using conventional bubble column PBR that had its aeration provided from one-point nozzle at the base of the reactor (Fig. 6.13). During the initial trial, this benthic organism was not able to grow within the system since the air sparging did not successfully create sufficient mixing for the culture (data

not shown). The air stream that was produced from one-point source created un-aerated areas surrounding the nozzle (Fig. 6.13, white area). Although the air stream generated sufficient turbulence within the aerated areas (Fig. 6.13, red shades), the hydrodynamics within the reactor would eventually bring the cells down to the un-aerated area, resulting in complete settling of cells at the base of the reactor.

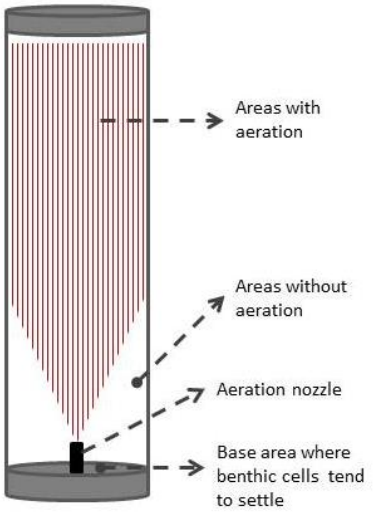


Figure 6.13 Diagram of the cultivation of benthic *P. lima* CCAP 1136/11 in a conventional bubble column photobioreactor with one-point air nozzle. Air stream within the reactor is indicated by red shades.

Subsequently, the TPBL and CBL reactor were constructed using the same principle as the conventional bubble column. However the aeration point was designed to ensure that cells would not settle at the base of the reactor by providing uniform air stream across the whole volume of the vessel (Fig. 6.6). Such aeration system was expected to provide better culture mixing within the system.

After five weeks of cultivation using both reactors, it was found that the utilisation of TPBL was proved to be unsuitable for this benthic organism, as indicated by the supressed growth and low production of compounds (Fig. 6.12). Excessive hydrodynamic stress was assumed to be the main cause of the poor performance in TPBL. An apparent indication of hydrodynamic stress was the observed foam formation at the top of the culture. Inside the TPBL, vigorous air flow that was distributed across the thin plane might have built up tension on the

liquid media and damaged the cells grown within, causing suppression of cell yield. Controlling the foam formation in this reactor was found to be difficult. Foaming inside TPBL might be reduced by reducing the air flow from the pump. However, a lower aeration rate than the operating rate used in this study was insufficient to maintain homogenous air distribution across the length of bubble curtain, resulted in significantly high cell settling.

In contrasts, the CBL reactor provided highest cell, DSP toxins and peridinin yields compared to those cultivated with TPBL and conventional vessel (control). It was found that cultivation with CBL produced increase in cell density by approximately 52% and concentration of DSP toxins and peridinin by 30-45%, than those grown in conventional vessel. Further analysis by microscope (data not shown) observed that, despite the vigorous aeration provided, *P. lima* CCAP 1136/11 were able to maintain their cells integrity inside CBL. This was also indicated by the analysis of DSP toxins that showed that majority of extracted OA and DTX1 were derived from intracellular. These findings further confirmed the suitability of CBL system for the cultivation of *P. lima* CCAP 1136/11.

In summary, tests on several designs of scalable PBR developed in this study showed that the utilisation of the CBL system could optimise the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 culture during the scale up. Furthermore, it has to be noted that the substitution of traditional vessels with CBL can be performed without requiring extensive additional space (Table 6.3), indicating its convenient applications.

Table 6.3 Summary of benthic *P. lima* CCAP 1136/11 cultivation with different types of system developed in this study.

Vessel type	Description	Scenario for 80 L cultivation	
		Space requirement (m^3)	Compounds yield (mg)*
Column Bubble-lift (CBL) – 8 L	Fairly good biomass yield, easy to maintain, low fabrication and installation cost	0.48 [(a)]	33.67
Thin plate Bubble-lift (TPBL) – 8 L	large illumination surface area, short light path, low fabrication cost, Low biomass yield due to high liquid tensions	0.66 [(b)]	17.99
Traditional 10 L vessel (with capacity of 8 L)	Easy installation and maintenance, low biomass yield, difficult in providing uniform mixing	0.44 [(c)]	25.38

Note:
*Typical yield of total OA, DTX1 and Peridinin
[(a)] Based on effective reactor dimension of 0.2m x 0.2m x 1.2m (LxWxH)
[(b)] Effective reactor dimension: 1.1m x 0.05m x 1.2m (LxWxH)
[(c)] Effective vessel dimension: 0.27m x 0.27m x 0.6m (LxWxH)

6.4.4 Mass production of DSP toxins and peridinin

Despite the improvements that the development of CBL has made possible, the mass production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 is still hampered by the slow growth of the organism. Cultivating this organism would normally take a period of weeks, in contrast to fast growing green algae cultures that would only require days. The prolonged cultivation time and very low yield of compounds, typically in order of less than a milligram per litre, render the overall production to be far less efficient than many other microalgal products.

Furthermore, throughout this study, it was consistently observed that obtaining substantial amount of compound yields was difficult in large scale (Fig. 6.14). The highest productivity observed, so far, was obtained from those in smallest

cultivation scale tested (100 mL with the modification of various growth parameters described in Chapters 3, 4 and 5). The attempts to optimise production made at a particular scale of cultivation, for example 8 L with CBL (section 6.4.3) or 1 L with flashing light (Chapter 5, section 5.3.3), were not capable of exceeding the OA concentrations obtained at smaller scales.

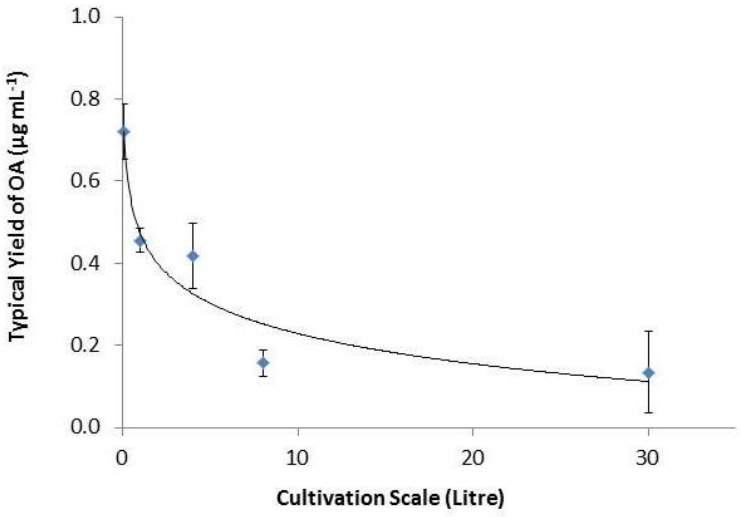


Figure 6.14 Illustration of typical yield of okadaic acid from *P. lima* CCAP 1136/11 growth at varying cultivation scales. Values are obtained from findings throughout this study.

The trend illustrated in Figure 6.14 may be explained by several possible underlying factors, such as gas exchange and localised high cell density within cultivation system. Growth limitations during scale-up process as a result of inadequate gas exchange due to insufficient aeration and culture mixing have been widely observed in high density microalgal cultures (Molina-Grima *et al.* 1999, Ugwu *et al.* 2008, Posten 2009). Moreover, as demonstrated in this chapter, the growth of *P. lima* CCAP 1136/11 was very much influenced by their access to CO_2, which might be enhanced through the provision of a high aeration rate (section 6.3.3) and/or improving culture mixing (section 6.3.5). Thus, it was possible to suggest that the difficulties in maintaining uniform gas exchange and culture mixing at larger scales have restricted the productivity of this benthic organism.

Another possible cause for the poor performance of benthic *P. lima* CCAP 1136/11 at large cultivation scales is the localised high cell density within the system. With the exception of cultures grown within bubble-lift reactors (TPBL and CBL), cells of benthic *P. lima* CCAP 1136/11 were consistently found to settle at the base of cultivation vessels. Thus, increasing the cultivation volume would result in higher number of cells occupying the bottom area of flasks. This would have limited the access of cells at the deeper layer to various growth resources, such as light, nutrients and CO_2. Limited availability of these resources has been shown to inhibit the growth of this organism (Morton *et al.* 1992, Vannuci *et al.* 2010, Varkitzi *et al.* 2010).

Considering the inefficient performance at large scale, cultivation of *P. lima* CCAP 1136/11 for the optimum production of DSP toxins and peridinin can only be currently performed in laboratory scale. Laboratory scale production is still the most commonly performed method for the production of other toxins from other dinoflagellate species (Hsieh *et al.* 2001, Camacho *et al.* 2011). The inefficient performance at large scale demonstrates that the efforts to achieve mass/commercial production of dinoflagellates products cannot rely only on conventional cultivation of the producer organism. Further works are needed to enable commercial scale production, akin to what has been achieved for the production of biofuels and other food products from microalgae (e.g. as demonstrated in Olaizola 2000). A possible means to achieve this is through research on molecular engineering, for instance through expressing gene responsible for the synthesis these toxins to other microalgal species that have high growth rates and are relatively easier to cultivate.

6.5 Conclusion

This study has demonstrated that reactor design can clearly influence yield of DSP toxins and peridinin from *P. lima* CCAP 1136/11 inside cultivation vessels. Preliminary test revealed that scale-up of this benthic organism was hindered due to their poor performance at higher cultivation scales. Low level of aeration and poor mixing were subsequently identified to be the main cause of the low productivity at larger scales of cultivation. Based on these findings, two cultivation systems were designed to improve mixing and gas transfer by utilising pneumatic agitations. The results concluded that optimising the growth and production of DSP toxins and peridinin from *P. lima* CCAP 1136/11 culture can be suitably performed by the column bubble-lift (CBL) reactor.

However, comparison of performance between scales still consistently showed that, despite the improvements made for a particular scale of cultivation, the productivity of *P. lima* CCAP 1136/11 culture was still the highest for the smallest cultivation scale. This suggests that efficient and optimum production of compounds from this organism is currently viable only at laboratory scale. Further research is still required to achieve mass/commercial scale production of DSP toxins and peridinin, especially since it cannot solely rely on conventional culturing method of *P. lima* CCAP 1136/11. Nonetheless, despite the challenges that have yet to be overcome for the mass production of DSP toxins and peridinin from *P. lima* CCAP 1136/11, this research, on the whole, has successfully identified the design parameters required to optimise the production of these compounds at a laboratory scale, as described in Chapter 7.

6.6 References

Apt, K. E., Behrens, P. W. 1999. Commercial developments in microalgal biotechnology. **Journal of Phycology**, 35, 215-226.

Behrens, P. W. 2005. Photobioreactors and fermenters: the light and dark sides of growing algae. In: R. A. ANDERSEN, ed. *Algal Culturing Techniques*, London, UK: Elsevier Academic Press. pp. 189-203.

Bruno, L., Di Pippo, F., Antonaroli, S., Gismondi, A., Valentini, C., Albertano, P. 2012. Characterization of biofilm-forming cyanobacteria for biomass and lipid production. **Journal of Applied Microbiology**, 113, 1052-1064.

Camacho, F. G., Rodriguez, J.J.G., Miron, A. S., Garcia, M. C. C., Belarbi, E. H., Chisti, Y., Molina Grima, E. 2007a. Biotechnological significance of toxic marine dinoflagellates. **Biotechnology Advances**, 25 (2), 176-194.

Camacho, F. G., Rodriguez, J.J.G., Miron, A. S., Garcia, M. C. C., Belarbi, E. H., Molina Grima, E. 2007b. Determination of shear stress thresholds in toxic dinoflagellates cultured in shaken flasks: Implications in bioprocess engineering. **Process Biochemistry**, 42(11), 1506-1515.

Camacho, G. F., Rodríguez, J. G., Mirón, A. S., Belarbi, E. H., Chisti, Y., Molina Grima, E. 2010. Photobioreactor scale-up for a shear-sensitive dinoflagellate microalga. **Process Biochemistry**, 46, 936-944.

Camacho, F. G., Rodriguez, J.J.G., Miron, A. S., Belarbi, E. H., Chisti, Y., Molina Grima, E. 2011. Photobioreactor scale-up for a shear-sensitive dinoflagellate microalga. **Process Biochemistry**, 46, 936-944.

Cheng-Wu, Z., Zmora, O., Kopel, R., Richmond, A. 2001. An industrial-size flat plate glass reactor for mass production of *Nannochloropsis* sp. (Eustigmatophyceae). **Aquaculture**, 195, 35-49.

Esson, D., Wood, S. A., Packer, M. A. 2011. Harnessing the self-harvesting capability of benthic cyanobacteria for use in benthic photobioreactors. **AMB Express**, 1:19.

Fukami, K., Nishimura, S., Ogusa, M., Asada, M., Nishijima, T. 1997. Continuous culture with deep seawater of a benthic food diatom *Nitzschia* sp. **Hydrobiologia**, 358, 245-249.

Hsieh, Dennis P. H., Wang, Dahzi., Chang, Garry H. 2001. Laboratory bioproduction of paralytic shellfish toxins in dinoflagellates. **Advances in Applied Microbiology**, 49, 85-110.

Juhl, A. R., Trainer, V. L., Latz, M. I. 2001. Effect of fluid shear and irradiance on population growth and cellular toxin content of the dinoflagellate *Alexandrium fundyense.* **Limnology and Oceanography**, 46(4), 758-764.

Kaewpintong, K., Shotipruk, A., Powtongsook, S., Pavasant, P. 2007. Photoautotrophic high-density cultivation of vegetative cells of *Haematococcus pluvialis* in airlift bioreactor. **Bioresource Technology**. 98, 288-295.

Lee, Y. K. 2001. Microalgal mass culture systems and methods: Their limitation and potential. **Journal of Applied Phycology**, 13, 307-315.

Liu, T., Wang, J., Hu, Q., Cheng, P., Ji, B., Liu, J., Chen, Y., Zhang, W., Chen, X., Chen, L., Gao, L., Ji, C., Wang, H. 2013. Attached cultivation technology of microalgae for efficient biomass feedstock production. **Bioresource Technology**, 127, 216-222.

Molina-Grima, E., Fernández, J., Acién, F. G., Chisti, Y. 2001. Tubular photobioreactor design for algal cultures. **Journal of Biotechnology**, 92, 113-131.

Molina-Grima, E., Acién Fernández, F. G., García Camacho, F., Chisti, Y. 1999. Photobioreactors: light regime, mass transfer, and scale-up. **Progress in Industrial Microbiology**, 35, 231-247.

Sánchez-Mirón, A., Camacho, F. G., Gómez, A. C., Molina Grima, E., Chisti, Y. 2000. Bubble-column and airlift photobioreactors for algal culture. **American Institute of Chemical Engineers Journal**, 46 (9), 1872-1887.

Morton, S. L., Norris, D. R., Bomber, J. W. 1992. Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. **Journal of Experimental Marine Biology and Ecology**, 157 (1), 79-90.

Olaizola, M. 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25000-litre outdoor photobioreactor. **Journal of Applied Phycology**, 12: 499-506.

Ozkan, A., Kinney, K., Katz, L., Berberoglu, H. 2012. Reduction of water and energy requirement of algae cultivation using an algae biofilm photobioreactor. **Bioresource Technology**, 114, 542-548.

Pan, Y., Cembella, A. D., Quilliam, M. A. 1999. Cell cycle and toxin production in the benthic dinoflagellate *Prorocentrum lima*. **Marine Biology,** 134, 541-549.

Posten, C. 2009. Design principles of photo-bioreactors for cultivation of microalgae. **Engineering in Life Sciences**, 9 (3), 165-177.

Radmer, R. J., Parker, B. C. 1994. Commercial applications of algae: opportunities and constraints. **Journal of applied phycology**, 6, 93-98.

Raniello, R., Iannicelli, M. M., Nappo, M., Avila, C., Zupo, V. 2007. Production of *Cocconeis neothumensis* (Bachillariophyceae) biomass in batch cultures and bioreactors for biotechnological applications: light and nutrient requirements. **Journal of Applied Phycology**, 19, 383-391.

Richmond, A., Boussiba, S., Vonshak, A., Kopel, R. 1993. A new tubular reactor for mass production of microalgae outdoors. **Journal of Applied Phycology**, 5 (3), 327-332..

Silva-Aciares, F. R., Riquelme, C. E. 2008. Comparisons of the growth of six diatom species between two configurations of photobioreactors. **Aquaculture Engineering**, 38, 26-35.

Thomas, W. H., Gibson, C. H. 1990. Effects of small-scale turbulence on microalgae. . **Journal of Applied Phycology**, 2, 71-77.

Ugwu, C. U., Aoyagi, H., Uchiyama, H. 2008. Photobioreactors for mass cultivation of algae. **Bioresource Technology**, 99, 4021-4028.

Vanucci, Silvana., Guerrini, Franca, Milandri, Anna., Pistocchi, Rosella. 2010. Effects of different levels of N- and P- deficiency on cell yield, okadaic acid, DTX-1, protein and carbohydrate dynamics in the benthic dinoflagellate *Prorocentrum lima*. **Harmful Algae**, 9, 590-599.

Varkitzi, I., Pagou, K., Granéli, E., Hatzianestis, I., Pyrgaki, C., Pavlidou, A., Montesano, B., Economou-Amili, A. 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. **Harmful Algae,** 9, pp. 304-311.

Wang, D., Hsieh, D. P. 2001. Dynamics of C2 toxin and chlorophyll-*a* formation in the dinoflagellate *Alexandrium tamarense* during large scale cultivation. **Toxicon**, 39 (10), 1533-1536.

Wang, B., Lan, C. Q., Horsman, M. 2012. Closed photobioreactors for production of microalgal biomasses. **Biotechnology Advances**, 30 (4), 904-912.

CHAPTER 7

Conclusion and Future Work

7.1 Conclusion

The adverse impacts of HABs to public health and surrounding ecosystem necessitate monitoring efforts to ensure that the risks of contaminations to products consumed by human (e.g. fish and shellfish products) are controlled. Such monitoring efforts require continuous supply of analytical standards. Of all the available methods, purification from cultured producer organism is deemed to provide the most practical and reliable means to produce toxin standards.

This study aimed to optimise the production of DSP toxins from dinoflagellate *P. lima* CCAP 1136/11. This benthic organism also produces a carotenoid pigment, peridinin, which has been found to have potential pharmaceutical uses due to its anti-cancer/anti-tumour properties. This further signifies the biotechnological significance of *P. lima* CCAP 1136/11. Several investigations have been performed in order to address issues related to the cultivation process: (1) evaluation of nutritional and environmental parameters in laboratory settings to enhance growth and production of DSP toxins and peridinin, (2) evaluation of factors, such as cell density and inoculum age, influencing physiological conditions of culture, and (3) development in the design of cultivation systems.

One of the main findings of this study showed that cultivation of *P. lima* CCAP 1136/11 still had to rely on continuous availability of natural seawater. The replacement of naturally sourced medium base with more practical options such as artificial seawater (ASW) was shown to affect the long term sustainability of high value metabolite production. Therefore, ASW medium was considered to be appropriate only as a temporary substitute to natural seawater. Besides this, the study results have also conclusively confirmed the obligate-behaviour of *P. lima* CCAP 1136/11, of which cultivation relies exclusively on the use of photobioreactor (PBR). Subsequent investigations on the optimum cultivation conditions on PBR revealed that production of DSP toxins and peridinin from this organism could be enhanced by the modification of several growth parameters (i.e. light, cell density and inoculum age) and design of cultivation vessel.

Nonetheless, despite the efforts made to optimise the cultivation conditions, the production of valuable compounds from this organism was perceived to be

currently feasible only at a laboratory scale. This was shown by the low yield obtained at larger scale cultivation (Chapter 6, Fig. 6.14). Every optimisation made for a particular scale still could not produce the same yields with those obtained at smaller scales. This problem was hypothesised to be caused by several factors such as gas exchange within cultivation system and localised high cell density that resulted in self-shading and light limitation. As such, further research is still required to enable commercial-scale production of DSP toxins and peridinin.

Despite these limitations, *P. lima* currently still serves as the most viable option of DSP toxin producer. Therefore, it is essential to persevere with this organism. In general, the research project has successfully identified the operating conditions, including reactor system, required to optimise DSP toxins and peridinin production by the benthic *P. lima* CCAP 1136/11. The works performed in the study represent the first coherent effort to devise a feasible large laboratory-scale cultivation strategy to allow optimum production and extraction of these compounds. As a final conclusion, this study proposes a strategy for the optimum cultivation and extraction process that can be easily adapted for regular laboratory scale production for high value compounds from *P. lima*. The proposed strategy was formulated based on the main findings derived from a series of investigations performed here, and is illustrated in Figure 7.1 below.

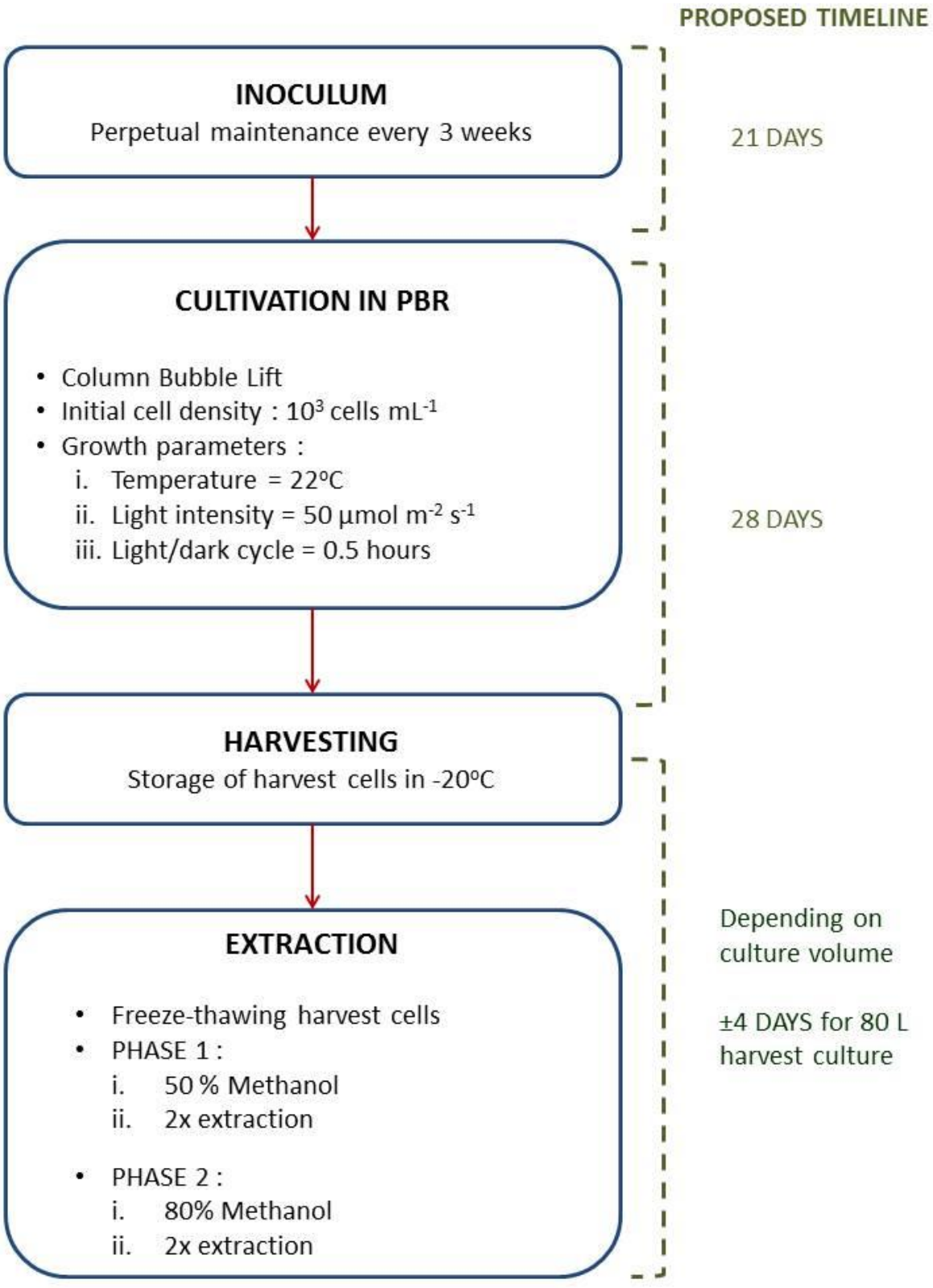


Figure 7.1 Proposed strategy for optimum cultivation conditions and processing of DSP toxins and peridinin from *Prorocentrum lima* CCAP 1136/11.

7.2 Future work

Future works on the optimisation of production of high value compounds from *P. lima* can be focused on the scale up of CBL reactor and exploration of different culture system, such as continuous or semi-continuous, as has been demonstrated in the production of paralytic shellfish poisoning (PST) toxins by Hsieh *et al*. (2001). The advantage of continuous system is that the nutrients can be replenished continuously, eliminating growth inhibitions caused by nutrient limitation.

In addition to this, further work is still required to explore the possibility to produce DSP toxins and peridinin at mass or commercial scale. This may be achieved with genetic modification of the culture. One suggestion that can be proposed is the expression of gene responsible for the synthesis of compound of interest in other fast growing microorganism. Exploitation of other fast growing microorganisms, such as yeast, for similar purposes has been attempted for the production of lipids (Huang *et al.* 2010).

7.3 References

Hsieh, Dennis P. H., Wang, Dahzi., Chang, Garry H. 2001. Laboratory bioproduction of paralytic shellfish toxins in dinoflagellates. **Advances in Applied Microbiology**, 49, 85-110.

Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G. 2010. Biodiesel production by microalgal biotechnology. **Applied Energy**, 87 (1), 38-46.

I. CALIBRATION CURVE OF STANDARDS

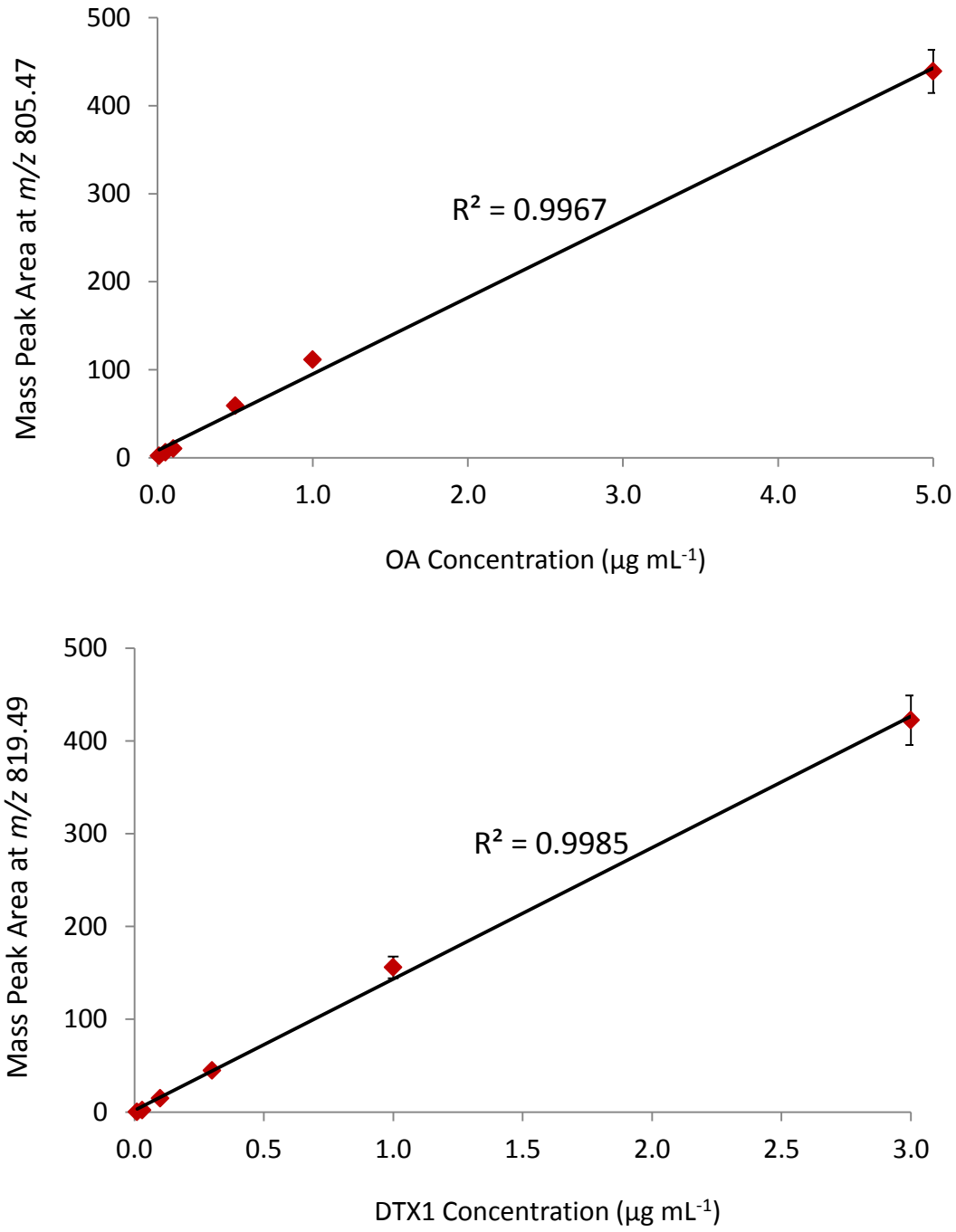


Figure 1A Calibration curves for OA (top) and DTX1 (bottom). Error bars represents standard deviation (n=3).

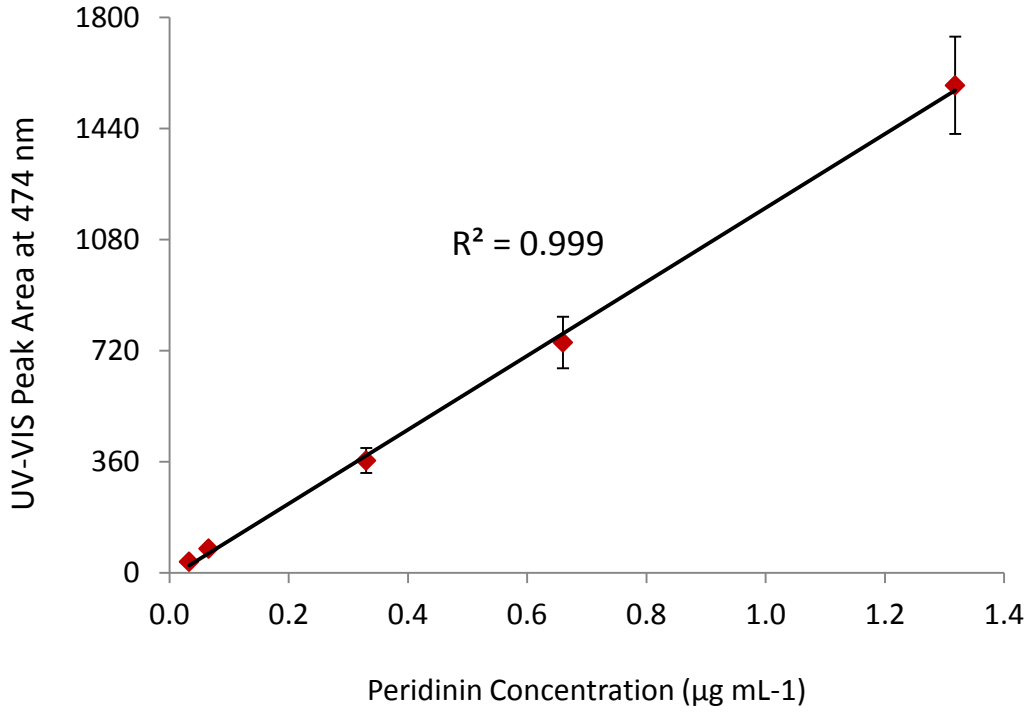


Figure 1B Calibration curve for peridinin. Error bars represents standard deviation (n=3).

Appendix II. UPLC-PDA-MS CHARACTERISATION OF AUTHENTIC STANDARDS

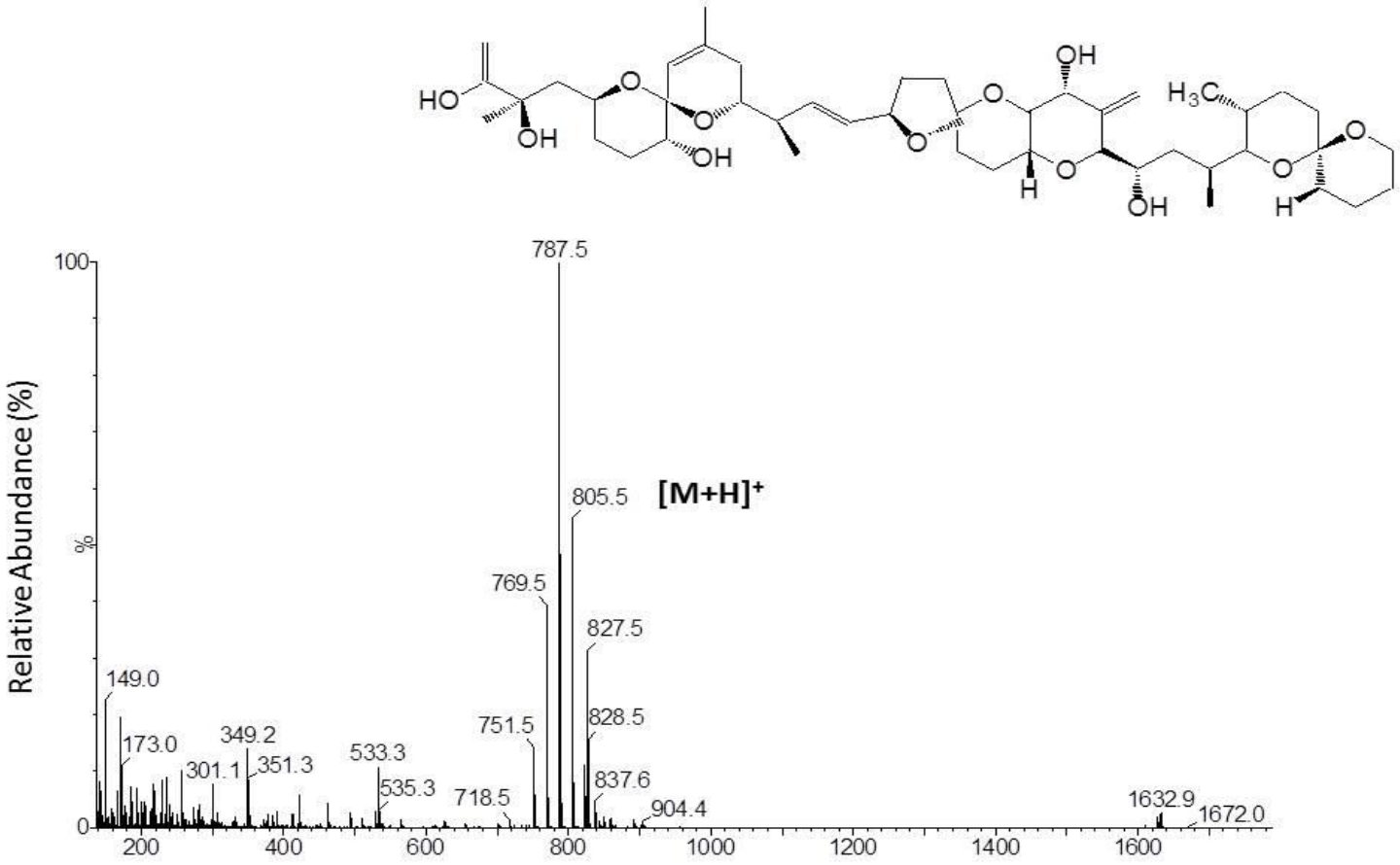


Figure 2A MS spectra at ESI+ for Okadaic Acid

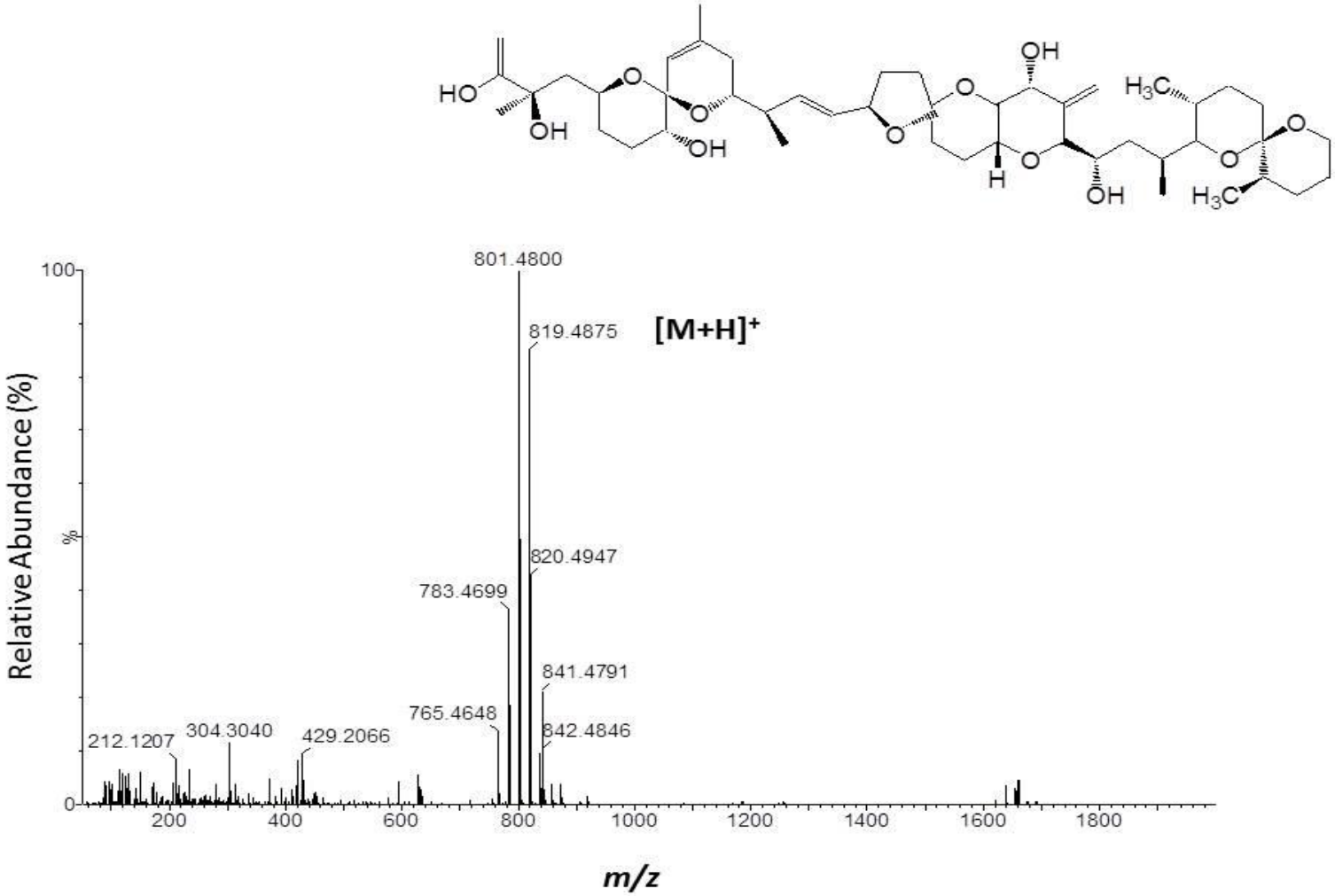


Figure 2B MS spectra at ESI+ mode for Dinophysistoxin-1 (DTX1)

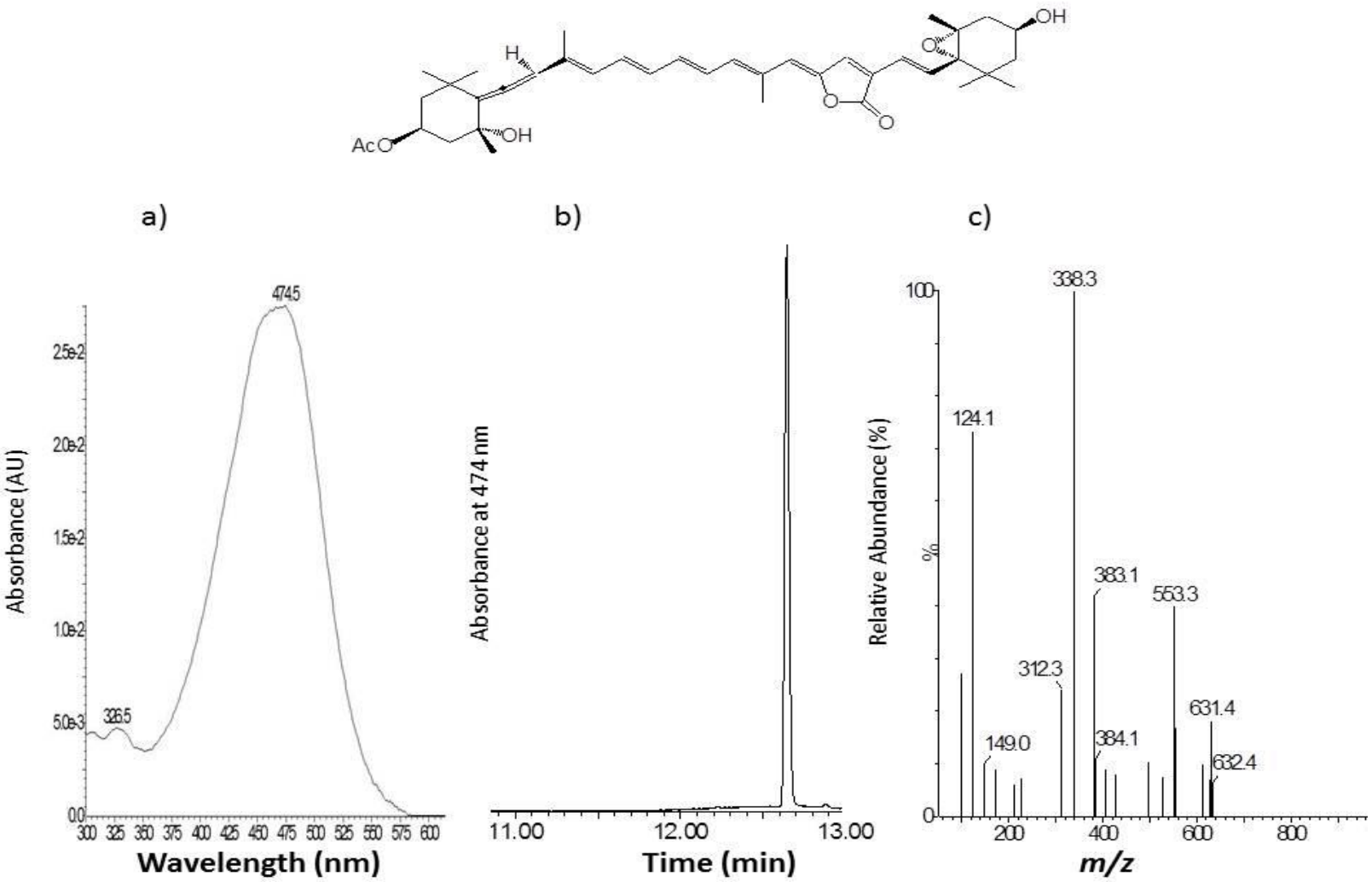


Figure 2C (a) UV Spectrum (300-600 nm), (b) UV-VIS chromatogram at 474 nm, and (c) MS ESI+ spectra for peridinin

Appendix III. Mass Fragmentation of Unidentified Compounds

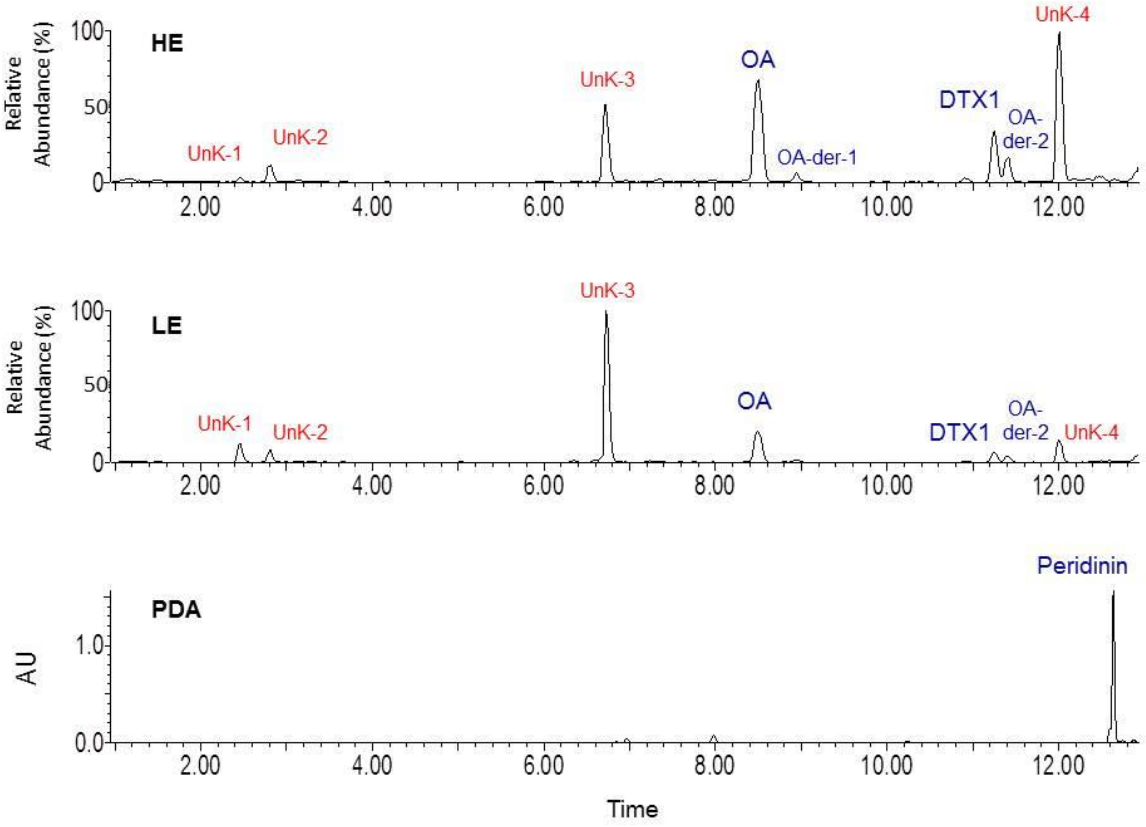


Figure 3A Characterisation of identified and unidentified (UnK-1 to UnK-4, in red letters) compounds from methanolic (100%) extract of *Prorocentrum lima* CCAP 1136/11 analysed by UPLC-MS-PDA at high energy (HE), low energy (LE) and UV-Vis absorbance (PDA).

Table 3A Retention time and parent ions (*m/z*) in ESI+ mode for unidentified compounds from methanolic (100%) extract of *Prorocentrum lima* CCAP 1136/11.

Compound	Retention time	$[M+H]^+$	$[M+Na]^+$
Unknown-1	2.46	742.51	764.49
Unknown-2	2.81	984.66	1006.65
Unknown-3	6.71	688.44	710.42
Unknown-4	12.01	780.51	802.49

III/1. Compound Unknown-1

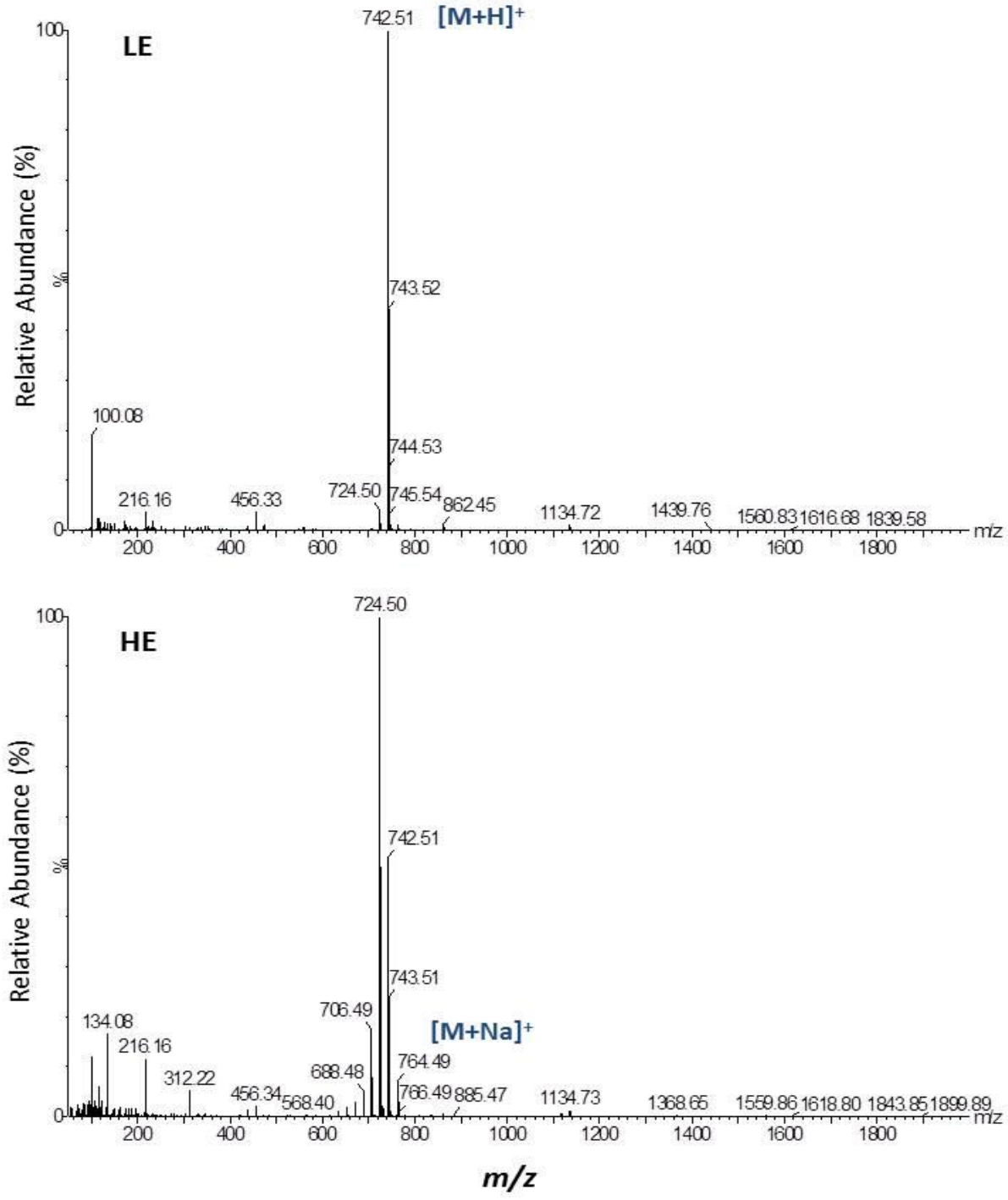


Figure 3B UPLC-MS fragmentation in ESI+ mode for Unknown-1 compound from methanolic extract of *P. lima* CCAP 1136/11 at low energy (LE) and high energy (HE) levels.

III/2. Compound Unknown-2

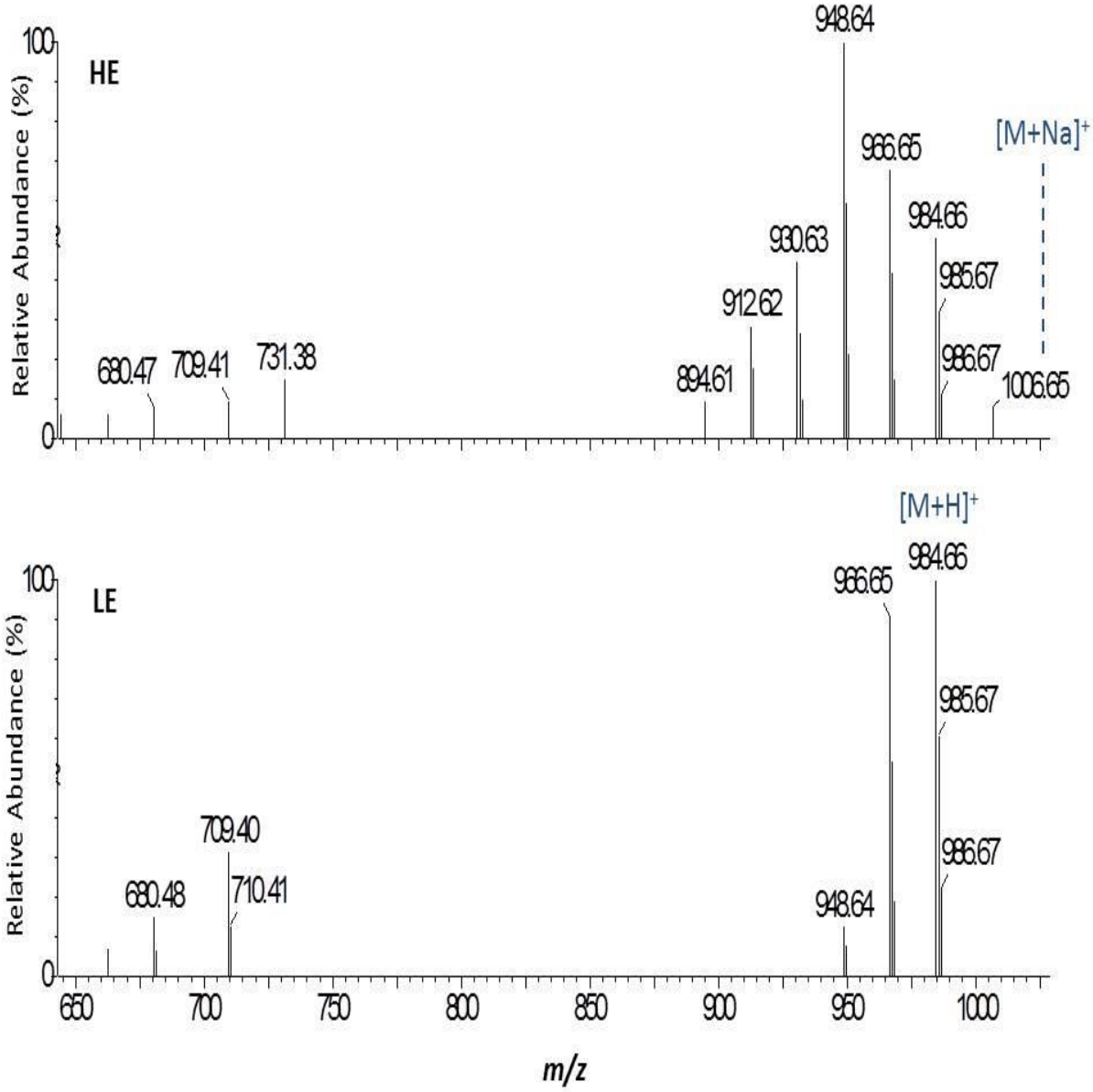


Figure 3C UPLC-MS fragmentation in ESI+ mode for Unknown-2 compound from methanolic extract of *P. lima* CCAP 1136/11 at high energy (HE) and low energy (LE) levels.

III/3. Compound Unknown-3

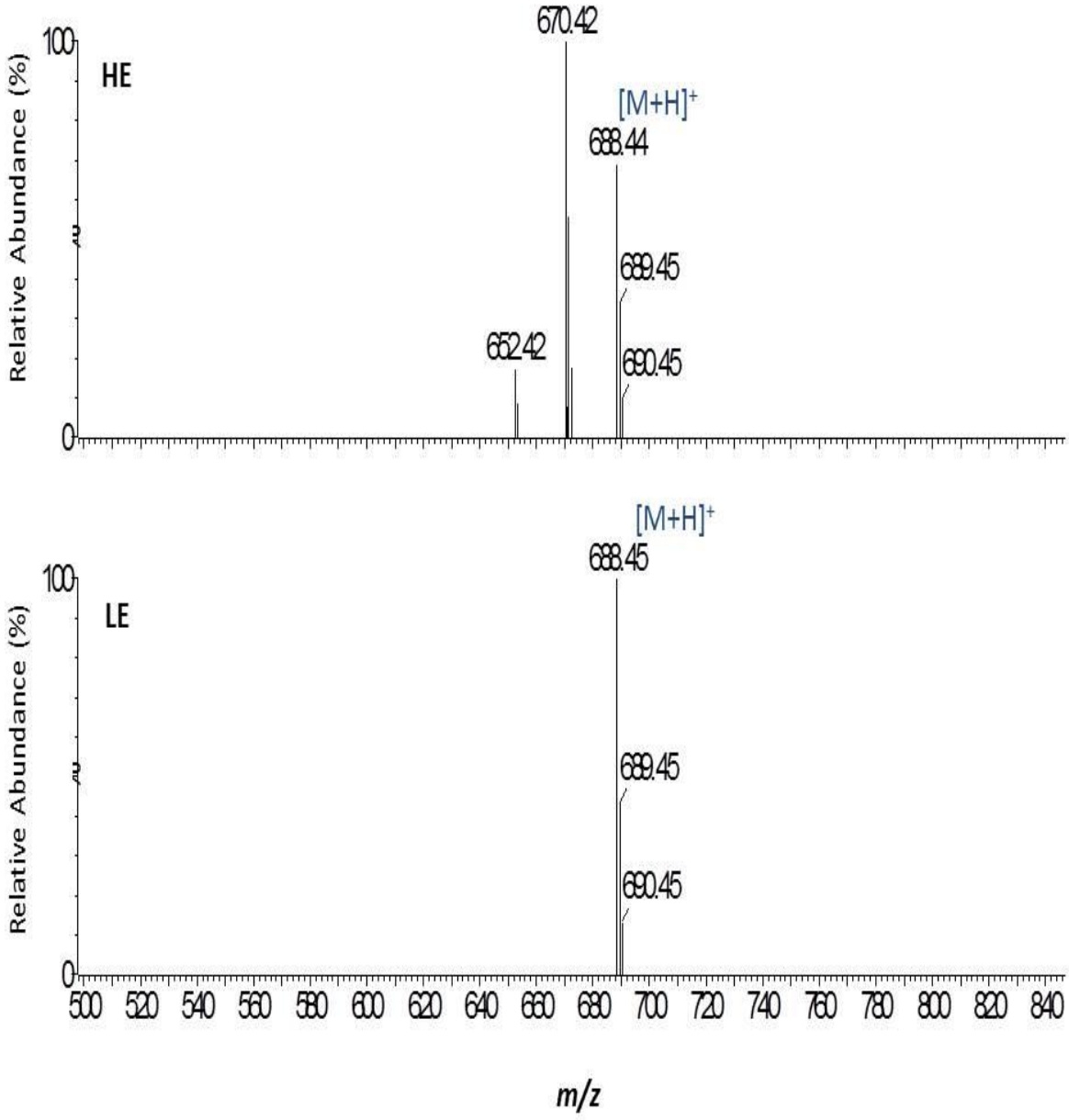


Figure 3D UPLC-MS fragmentation in ESI+ mode for Unknown-3 compound from methanolic extract of *P. lima* CCAP 1136/11 at high energy (HE) and low energy (LE) levels.

III/4. Compound Unknown-4

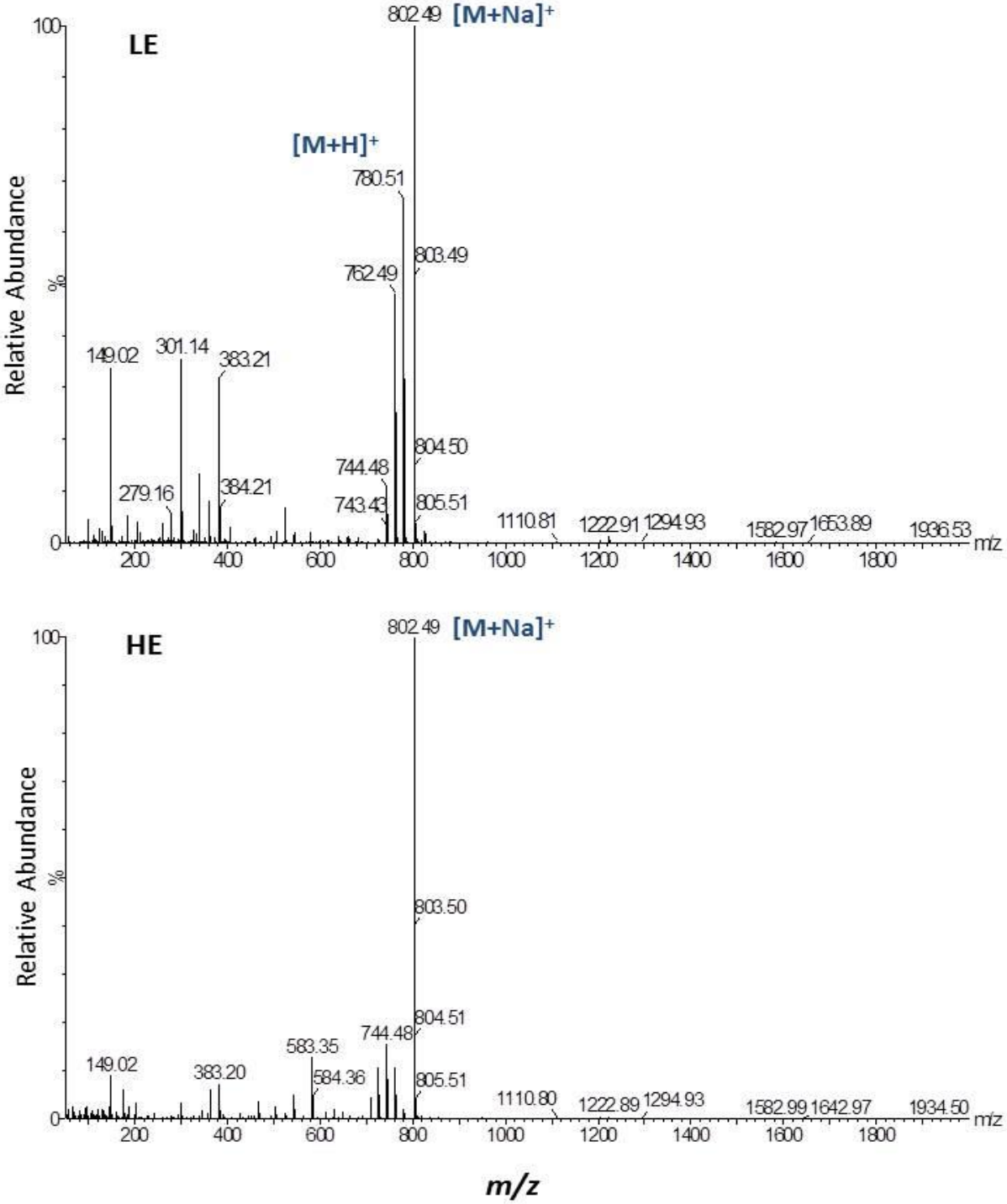


Figure 3E UPLC-MS fragmentation in ESI+ mode for Unknown-4 compound from methanolic extract of *P. lima* CCAP 1136/11 at low energy (LE) and high energy (HE) levels.

Appendix IV. Concentration of elements used for trace metal modification

Table 4A Details of experimental concentrations utilised for evaluation of trace metal content (Chapter 3, section 3.2.3).

Experimental Parameter		Concentration of trace metal in 1 L medium (µmol)						
		$FeCl_3.6H_2O$	$CuSO_4.5H_2O$	$ZnSO_4.7H_2O$	$CoCl_2.6H_2O$	$MnCl_2.4H_2O$	$NaMoO_4.2H_2O$	Na_2SeO_3
Control (f/2 medium)		11.7000	0.0393	0.0765	0.0420	0.9100	0.0260	-
Iron ($FeCl_3.6H_2O$)	C_1:25%	2.9250	0.0393	0.0765	0.0420	0.9100	0.0260	-
	C_2:50%	5.8500	0.0393	0.0765	0.0420	0.9100	0.0260	-
	C_3:125%	14.6250	0.0393	0.0765	0.0420	0.9100	0.0260	-
	C_4:150%	17.5500	0.0393	0.0765	0.0420	0.9100	0.0260	-
	C_5:200%	23.4000	0.0393	0.0765	0.0420	0.9100	0.0260	-
Copper ($CuSO_4.5H_2O$)	C_1:25%	11.7000	0.0098	0.0765	0.0420	0.9100	0.0260	-
	C_2:50%	11.7000	0.0197	0.0765	0.0420	0.9100	0.0260	-
	C_3:125%	11.7000	0.0491	0.0765	0.0420	0.9100	0.0260	-
	C_4:150%	11.7000	0.0590	0.0765	0.0420	0.9100	0.0260	-
	C_5:200%	11.7000	0.0786	0.0765	0.0420	0.9100	0.0260	-
Zinc ($ZnSO_4.7H_2O$)	C_1:25%	11.7000	0.0393	0.0191	0.0420	0.9100	0.0260	-
	C_2:50%	11.7000	0.0393	0.0383	0.0420	0.9100	0.0260	-
	C_3:125%	11.7000	0.0393	0.0956	0.0420	0.9100	0.0260	-
	C_4:150%	11.7000	0.0393	0.1148	0.0420	0.9100	0.0260	-
	C_5:200%	11.7000	0.0393	0.1530	0.0420	0.9100	0.0260	-
Cobalt ($CoCl_2.4H_2O$)	C_1:25%	11.7000	0.0393	0.0765	0.0105	0.9100	0.0260	-
	C_2:50%	11.7000	0.0393	0.0765	0.0210	0.9100	0.0260	-
	C_3:125%	11.7000	0.0393	0.0765	0.0525	0.9100	0.0260	-
	C_4:150%	11.7000	0.0393	0.0765	0.0630	0.9100	0.0260	-
	C_5:200%	11.7000	0.0393	0.0765	0.0840	0.9100	0.0260	-

Table 4A Continue.

Experimental Parameter		Concentration of trace metal in 1 L medium (µmol)						
		$FeCl_3.6H_2O$	$CuSO_4.5H_2O$	$ZnSO_4.7H_2O$	$CoCl_2.6H_2O$	$MnCl_2.4H_2O$	$NaMoO_4.2H_2O$	Na_2SeO_3
Manganese ($MnCl_2.4H_2O$)	C_1:25%	11.7000	0.0393	0.0765	0.0420	0.2275	0.0260	-
	C_2:50%	11.7000	0.0393	0.0765	0.0420	0.4550	0.0260	-
	C_3:125%	11.7000	0.0393	0.0765	0.0420	1.1375	0.0260	-
	C_4:150%	11.7000	0.0393	0.0765	0.0420	1.3650	0.0260	-
	C_5:200%	11.7000	0.0393	0.0765	0.0420	1.8200	0.0260	-
Molybdenum ($Na_2MoO_4.2H_2O$)	C_1:25%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0065	-
	C_2:50%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0130	-
	C_3:125%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0325	-
	C_4:150%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0390	-
	C_5:200%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0520	-
Selenium (Na_2SeO_3)	C_1:50%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0260	0.0058
	C_2:100%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0260	0.0116
	C_3:150%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0260	0.0173
	C_4:200%	11.7000	0.0393	0.0765	0.0420	0.9100	0.0260	0.0231

Appendix V. Large scale (30 L) photobioreactor

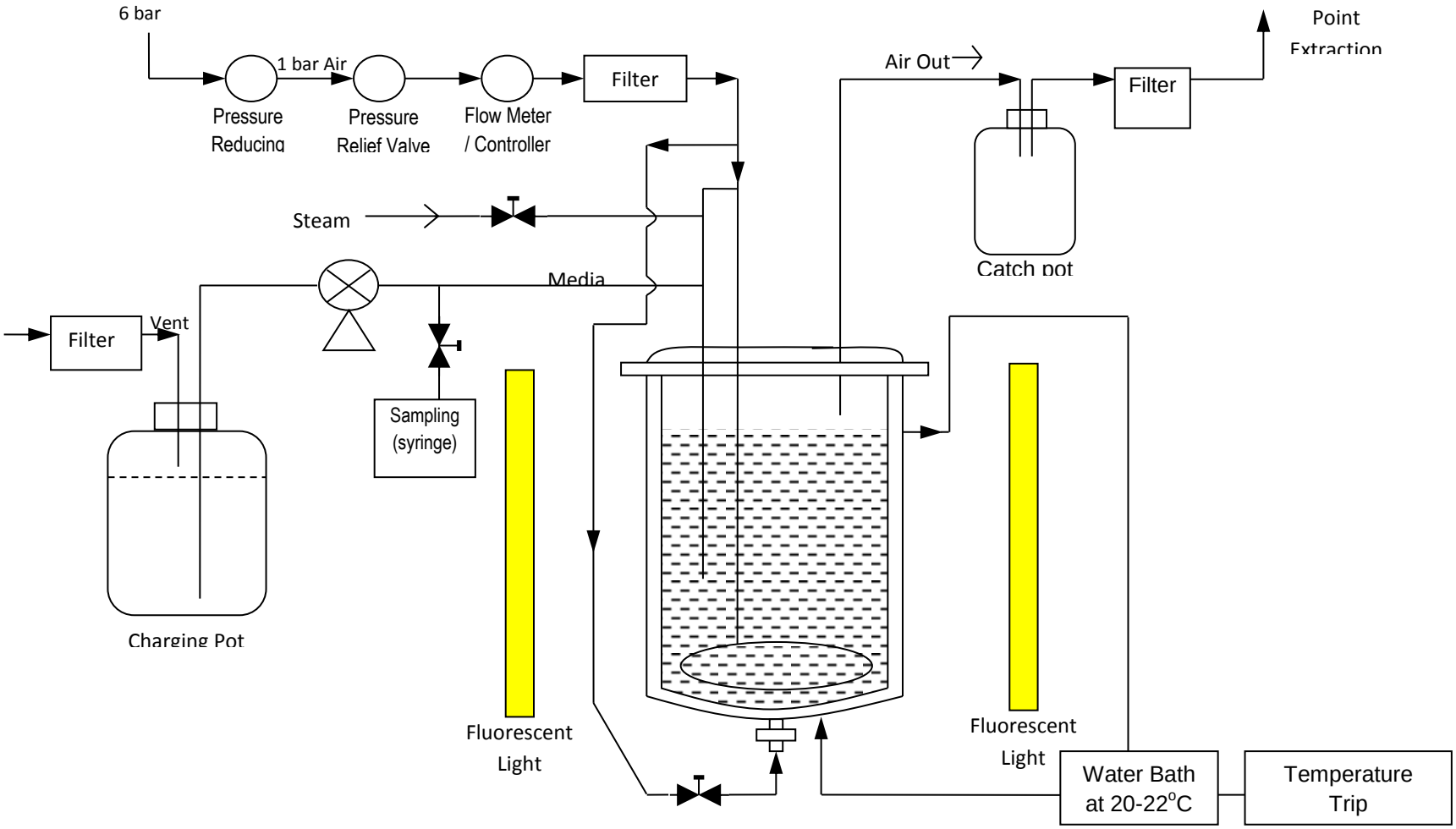


Figure 5A Diagram of pipe and instrumentation of 30 L photobioreactor.