UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCE

School of Ocean and Earth Science

Effect of increasing sea water temperature on the growth and toxin production of three harmful benthic dinoflagellates isolated from the Fleet Lagoon, Dorset, UK.

by

Aldo Aquino-Cruz

Thesis for the degree of Doctor of Philosophy

March 2012

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF NATURAL & ENVIRONMENTAL SCIENCES

Doctor of Philosophy

EFFECT OF INCREASING SEA WATER TEMPERATURE ON THE GROWTH AND TOXIN PRODUCTION OF THREE HARMFUL BENTHIC DINOFLAGELLATES ISOLATED FROM THE FLEET LAGOON, DORSET, UK.

By Aldo Aquino-Cruz

Coastal marine waters are experiencing significant environmental changes (e.g. increasing water temperature) that are influencing the dynamics and increased occurrence of harmful microalgal blooms (HABs) worldwide. Harmful blooms from benthic microalgae (e.g. dinoflagellates) are annually reported in some UK waters but little research has been conducted to date regarding the impact of a global warming scenario on their abundance and toxin production Therefore, this study aimed to investigate the effect of increasing water temperature on the growth and toxin production of three toxigenic/benthic dinoflagellates isolated from the Fleet Lagoon, Dorset, UK.

The harmful/epibenthic dinoflagellates *Amphidinium carterae*, *Prorocentrum lima*, and *Coolia monotis* were isolated from the Fleet Lagoon and grown in clonal batch non axenic monocultures between 5 and 30 °C using Guillard's F/2 medium. Cultures were maintained at irradiances between 35 and 65 µmol m^{-2} s^{-1} and under a 12h L:12h D photoperiod. A single strain was used of each microalgal species and samples for cell abundance, photosynthetic efficiency (*Fv*/*Fm*), inorganic nutrient concentration (nitrate, NO_3+NO_2 and phosphate, PO_4), chlorophyll *a* and toxin measurements were collected every few days over periods of up to 80 days. Toxins were detected and measured by LC-MS/MS from *P. lima* samples while copepod and brine shrimp bioassays and haemolytic assays were used to estimate the potential toxicity of *A. carterae* and *C. monotis*.

A. carterae cell growth rate produced a strong linear relationship (r^2= 0.97, *p*= 0.001) with temperature, with higher growth rates (µ=0.14-0.55 d^{-1}) at increasing growth temperatures ranging between 5 and 25 °C. Photosynthetic efficiency (*Fv*/*Fm* values) increased with temperature from 5 to 25 °C but showed a strong decrease at 30 °C. *A. carterae* growth produced higher NO_3+NO_2 and PO_4 consumption at increasing temperatures resulting in PO_4 limitation at temperatures between 15-25°C. Toxin assays confirmed that *A. carterae* produced hemolysins causing deleterious effects on red blood cells and mortality in harpacticoid copepods.

P. lima cell growth rate was linearly related (r^2= 0.97, *p*= <0.001) to temperature between 5 °C (µ= 0.05 d^{-1}) and 15 °C (µ= 0.17 d^{-1}) with similar growth rates at 15 and 25 °C. Maximum *Fv*/*Fm* values increased at higher temperatures, but there was a lack of coincidence between the highest *Fv*/*Fm* value (at 20 °C) and highest growth rate. NO_3+NO_2 and PO_4 uptake ratios showed that *P. lima* cells removed nutrients more efficiently at increasing temperatures, causing PO_4 limitation at 20-30 °C. NO_3+NO_2 and PO_4 uptake in cultures, particularly PO_4 depletion during the stationary growth phase, was linked with higher concentrations of the toxins okadaic acid (OA) and dinophysistoxin1 (DTX1). Furthermore, toxin production was influenced by increasing growth temperatures, Total OA varied between 2.0-10.99 pg $cell^{-1}$ while Total DTX1 ranged between 0.82-5.96 pg $cell^{-1}$ in *P. lima* cells, with OA/DTX1 ratios between 1.7 and 2.9. *P. lima* was highly toxic to *Artemia salina* with toxins causing a rapid rate of mortality (< 24h).

C. monotis cells growth rates also increased linearly (µ = 0.003 to 0.24 d^{-1}; r^2= 0.69, *p*= <0.001) with increasing growth temperatures between 5 and 20 °C. The highest *Fv*/*Fm* value was determined in *C. monotis* cells at 15 °C and higher or lower temperatures caused a reduction of *Fv*/*Fm* values. NO_3+NO_2 and PO_4 were substantially removed from culture flasks at increasing temperatures between 10 and 25 °C and PO_4 limitation occurred at these temperatures towards the end of the stationary growth phase. Haemolytic compounds were detected in *C. monotis* cells and bioassays confirmed a toxic effect of cell extracts on red blood cells.

This study provides evidence that the toxigenic dinoflagellates *A. carterae*, *C. monotis*, and *P. lima* can tolerate and grow at a range of temperatures suggesting that increasing sea water temperatures will have a positive effect on the growth of these toxigenic/epibenthic dinoflagellates, particularly under NO_3+NO_2 and PO_4 replete conditions. Theses toxic dinoflagellates can therefore be considered a threat to the Fleet Lagoon which will become a particular problem as water temperatures increase.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DECLARATION OF AUTHORSHIP

I, Aldo Aquino-Cruz, declare that the thesis entitled "Effect of increasing sea water temperature on the growth and toxic production of three harmful benthic dinoflagellates isolated from the Fleet Lagoon, Dorset, UK" and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission.

Signed: ……..

Date:…….

Acknowledgements

First and foremost I would like to thank my supervisor, Dr Duncan A. Purdie (University of Southampton), for his continued guidance and support throughout my PhD thesis. I appreciate his willingness to share knowledge during my PhD and valuable comments with the thesis writing.

I am very grateful to Dr Steve Morris, from the Centre for Environment, Fisheries and Aquaculture Science, for his invaluable effort, assistance and recommendations before, during, and after all toxin analyses carried out by LC-MS/MS at CEFAS.

I offer my gratitude to Consejo Nacional de Ciencia y Tecnología (Mexico) for providing me a PhD scholarship and funding my PhD project at the National Oceanography Centre of Southampton.

I thank Matt O'Shaughnessy for his assistance to travel and collect water samples at the Fleet Lagoon, Weymouth.

I thank Dr Mark C. Moore and Dr Martha Gledhill (National Oceanography Centre Southampton) for all facilities provided to use the FRRF and Coulter Counter respectively.

I thank the microbiology lab (National Oceanography Centre) for all facilities provided during the lab work to manipulate the algae cultures under sterile conditions.

I thank Mark Stinchcombe for devoting his time to analyze all nutrient samples collected from the microalgae cultures.

I also thank many colleagues and many international friends (Pornsri, Anna, Ibrahim, Adam, Maria, Holger and many others) that I made and supported me throughout my PhD.

I am very grateful to my mother for her selflessly devotion to bring comfort and wise support throughout my PhD.

Thanks to everyone I met at the National Oceanography Centre for the formidable experience and those happy times out from the NOC!

Abbreviations

ASP – Amnesic shellfish poisoning
AZP – Azaspiradic shellfish poisoning
$CaCl_2$ – Calcium chloride
Chlor *a* – Chlorophyll *a*
C_i – inorganic carbon
CFP – Ciguatera fish poisoning
CO_2 – carbon dioxide
CTXs – Ciguatoxins
DSP – Diarrhetic shellfish poisoning
DTX1 – Dinophysistoxin1
ELA – Erythrocyte lysis assay
Free DTX1– Free dinophysistoxin1 (native or parent toxin)
Free OA– Free okadaic acid (native or parent toxin)
Fo – Initial fluorescence
Fm – Maximum Fluorescence
FRRF – Fast repetition rate fluorometer
Fv – Variable fluorescence
Fv/*Fm* – Photosynthetic efficiency of photosystem II
HA – Harmful algae
HABs – Harmful algae blooms
HCl – Hydrogen chloride
IC_{50} – Half maximal inhibitory concentration
KCl – Potassium chloride
L _ Litres
LC-MS/MS – Liquid chromatography-tandem mass spectrometry
Ln – Natural logarithm
$MgSO_4$ – Magnesium sulphate
ml – millilitres
MTXs –Maitotoxins
NBS – National Bureau of Standards
NaCL – Sodium chloride
N-limitation – Nitrogen limitation
Nm – nanometres
NO_3+NO_2 – Nitrate+Nitrite
NSP – Neurotoxic shellfish poisoning
OA – Okadaic acid
pCO_2 – Carbon dioxide partial pressure
pg – picograms
P-limitation – Phosphate limitation
PO_4 – Phosphate
PSII – Photosystem II
PSP – Paralytic shellfish poisoning
PP-2A – Protein phosphatase 2
ppmv – parts per million in volume
PTXs – Pectenotoxins
Q_{10} – temperature coefficient
rDNA – ribosomal deoxyribonucleic acid

RBC – Red blood cells
RUBISCO – Ribulose -1,5-bisphosphate carboxylase oxygenase
Rpm – Revolutions per minute
SD – Standard deviation
SE – Standard error
SEM – Scanning electron microscopy
T – Temperature
Total DTX1– Total dinophysistoxin1
Total OA – Total okadaic acid
TRIS – Tris(hydroxymethyl)aminomethane
V – Volume
YTXs – Yessotoxins
°C – Degree Celsius
μ – Specific growth rate
μg –micrograms
μ_{max} – maximum specific growth rate
μm – micrometres

Thesis structure

CHAPTER 1 Provides a general review of toxigenic benthic dinoflagellates causative of harmful algae blooms (HABs) in coastal areas. The influence of environmental conditions, particularly the increase of sea water temperature, on the growth and toxin production of dinoflagellates is discussed in this chapter.

CHAPTER 2 Describes the methods and analysis carried out during this research to investigate the effect of temperature on the growth and toxin production of three toxic benthic dinoflagellates isolated from the Fleet Lagoon, UK.

CHAPTER 3 Describes the effect of increasing sea water temperature (5-30° C) on the growth of the toxic benthic dinoflagellate *Amphidinium carterae* and includes evidence of the effect of biotoxins from *A. carterae* using bioassays.

CHAPTER 4 Describes the effect of increasing growth temperatures (5-30° C) on the growth and toxin production of *Prorocentrum lima*. The effect of nutrient conditions on the physiological response of toxin production by *P. lima* cells grown in culture is described and discussed.

CHAPTER 5 Describes the influence of increasing temperature (5-30° C) on the growth of *Coolia monotis*. The detection of toxins from *C. monotis* cells is described and discussed based on bioassays.

CHAPTER 6 Summarizes the main finding from the investigation of the strains *A. carterae*, *P. lima*, and *C. monotis*. The increase of temperature on these microalgae is analyzed and the potential effect of climate change (warming) on toxigenic benthic dinoflagellates is discussed.

CHAPTER 1

1.1 Introduction

1.1.1 Harmful algae

The term "algal bloom" refers to one of the most common biological phenomenon caused by photosynthetic microorganisms in aquatic environments. Phytoplanktonic organisms, such as dinoflagellates, have caused microalgae blooms in diverse aquatic ecosystems and records can be certainly dated throughout several eras of the Earth's history (Hallegraeff, 2010). Algae blooms are characterized as dynamic episodes where unicellular algae produce very high cell concentrations influenced by environmental conditions. Whereas some algae blooms are known to produce substantial benefits for ecosystem functioning, others cause detrimental effects. A number of phytoplanktonic species are able to produce harm *in situ* and affect higher trophic levels. These microorganisms are known as harmful algae (HA) and at high densities HA produce a phenomenon known as harmful algae blooms (HABs).

HA can be divided in two categories according to the causative organism. These are high-biomass producers and toxin producers (van den Bergh *et al.*, 2002, Bravo *et al.*, 2001). High-biomass producers are species characterized by high production of organic matter which decompose *in situ* and, as a consequence, cause a substantial oxygen removal from the water column (hypoxia) with serious repercussions on biota. Toxin producers synthetise diverse secondary metabolites (biotoxins) that may ascend and bioaccumulate at different trophic levels in the environment causing diverse cellular malfunction in biota (Bravo *et al.*, 2001).

The existence of algae blooms may generate water discoloration (yellowish, reddish, brownish) as high cell concentrations produce high accumulation of photosynthetic pigments suspended in the water column. Some phytoplanktonic organisms produce only small amounts of pigments per cell naturally, and therefore they do not cause water discoloration despite substantial concentration of cells in the environment. Taylor *et al*. (2008) has suggested that only half of all living dinoflagellate species are photosynthetic. Furthermore, not all microalgae produce a substantial biomass in the environment under optimum bloom conditions, for instance, dinoflagellate species from the genera *Alexandrium* and *Dinophysis* are considered low biomass harmful algae (GEOHAB, 2001).

Many HABs species are distributed globally because they possess specialized mechanisms that have enabled them to adapt and survive diverse environmental conditions around the world. The biodiversity of HA comprises several phytoplankton groups of which the most important taxa, due to the number of deleterious species, are dinoflagellates, diatoms, and cyanobacteria (Moore *et al.*, 2008). Around 200 phytoplankton species have been considered as HA, of which 80 are associated with toxin producing microalgae (Hallegraeff, 2003).

In recent decades, a number of studies on HABs have comprehensibly demonstrated that this phenomenon is experiencing a global increase in aquatic ecosystems in terms of occurrence, intensity, and distribution (Peperzak, 2003, Moore *et al.*, 2008, Hallegraeff, 2010, Bravo *et al.*, 2001). In addition, there has been an increasing number of studies on harmful algae worldwide and it is argued that this has caused an impact on an increasing number of reports regarding HABs. Since HABs have produced numerous repercussions in the environment, from massive mortality of filter-feeding organisms (mussels, oysters, cockles, and crustaceans) to death of vertebrates (fish, marine mammals and humans), a global concern is shared between the scientific community regarding factors that can intensify the occurrence of algal blooms. Climate change (Hallegraeff, 2010), the increase of CO_2 (Cheung *et al.*, 2011), warmer sea water temperatures (Moore *et al.*, 2008, Laabir *et al.*, 2011, Lassen *et al.*, 2010), eutrophication of waters (Glibert *et al.*, 2010), and biogeographical changes of HA to new regions (Pearson and Dawson, 2003, Edwards *et al.*, 2006, Taylor *et al.*, 2008) have been suggested and are expected to intensify future impacts of HA worldwide.

Some epibenthic dinoflagellates have been shown to be responsible for HABs in the environment and in the last few decades these microalgae have received increased attention as many species from different genera (e.g. *Amphidinium*, *Coolia*, *Ostreopsis*, *Prorocentrum*) are known to produce potent biotoxins (see Table 1.1). Although a number of toxin-producing dinoflagellates have been characterized to date, the dynamic and ecophysiological response is poorly comprehended for many aquatic habitats of epibenthic dinoflagellates. In addition, many chemical compounds (biotoxins) and the mechanisms whereby benthic HA synthetise toxins are still unknown, not to mention the lack of analytical methods to accurately quantify and analyse routinely environmental fluctuations of biotoxins.

Table 1.0 Toxigenic benthic dinoflagellates, natural biotoxins, and syndromes associated due to ingestion of contaminated seafood.

Benthic dinoflagellate specie	Main toxin	Syndromes associated	Organisms containing toxins	Reference
Amphidinium carterae, A. klebsii	Amphidinols (A-T), amphidinim A, haemolytic and antifungal compounds	Unknown (possibly ciguatera fish poisoning)	ND	Daranas *et al*. 2001
Coolia monotis	Cooliatoxins (Yessotoxins)	Diarrhetic shellfish poisoning	Mussels, cockles, gastropods	Holmes *et al*. 1995
Gambierdiscus toxicus	Lipid soluble compounds (ciguatoxins, CTX) and water soluble toxins (maitotoxins, gambierol, gambieric acids), antifungal agents	Ciguatera fish poisoning	Fish, snails, shrimps, crabs	Dickey and Plakas 2010
Ostreopsis lenticularis, O. mascarenensis	Palytoxins (PTX),	Ciguatera fish poisoning	Finfish, crustacean	Ramos and Vasconcelos 2010
O. ovata	Palytoxins (PTX), ovatoxin-a (Ostreocin)	ciguatera fish poisoning	Fish and crustacean	Granéli and Flynn 2006
O. siamensis	Palytoxin (PTX)	Ciguatera fish poisoning	Fish and crustacean	Usami *et al*. 1995
Prorocentrum arenarium, P. belizeanum, P. cassubicum, P. concavum, P. faustidae, P. hoffmannianum, P. levis, P. maculosum, P. mexicanum	Okadaic acid (OA), dinophysistoxins (DTX), haemolytic and allelopathic compounds	Diarrhetic shellfish poisoning	Mussels, scallops, clams, gastropods	Maso and Garcés 2006
Prorocentrum micans, P.minimum, P. obtusidens, P. redfeldii, *P. dentatum*	High biomass producer (non-toxic)	-	Mussels, gastropods	Gotsis-Skretas and Friligos 1990, 2005; Nikolaidis *et al*. 2006; Ignatiades and Gotsis-Skretas 2010

1.1.2 Harmful epibenthic dinoflagellates

Numerous ecological studies on benthic dinoflagellates have found that a number of harmful dinoflagellate species share the same habitat. Okolodkov *et al*. (2007), Pistocchi *et al*. (2010), and Richlen and Lobel (2011) have suggested that the benthic harmful genera *Amphidinium*, *Coolia*, *Gambierdiscus*, *Prorocentrum*,

Ostreopsis constitute natural assemblages in coastal areas. In fact, it is understood that two toxic dinoflagellate blooms can co-exist in the environment when suitable conditions prevail (Nakajima *et al* 1981). Some studies have shown that a number of benthic dinoflagellates are particularly confined to tropical waters (Taylor *et al.*, 2008). This may suggest that temperature is importantly associated with the growth of several epibenthic HA. Rhodes and Thomas (1996) found that the benthic dinoflagellate *Coolia monotis* reached its optimum growth at 25 °C (subtropical temperature) rather than at 20 °C (temperate temperature), while Armi *et al*. (2010) observed a bloom of *C. monotis* (5×10^5 cell L^{-1}) in the environment at 22 °C. Granéli *et al*. (2011) determined that the epibenthic dinoflagellate *Ostreopsis ovata* showed the highest growth when cultured between 26-30 °C (tropical temperatures), but cell toxicity was highest in a range of 20-22 °C. Shears and Ross (2009) have indicated that blooms of *Ostreopsis siamensis* are an increasingly common phenomenon in temperate regions during the warmest summer months. Similarly, Okolodkov *et al*. (2007) found that the assemblage of *Amphidinium* cf. *carterae*, *Prorocentrum lima*, *C. monotis*, and *Ostreopsis heptagona* reached their highest abundance in the Veracruz reef zone at temperatures between 28.5 and 32 °C. Although a number of benthic dinoflagellates are generally confined to tropical waters, recent reports have suggested that some benthic HA (ciguatera producers) are increasing their biogeographical distribution to temperate waters where some have caused deleterious consequences (Luckas *et al.*, 2005, Aligizaki and Nikolaidis, 2006).

1.1.3 Temperature and algae growth

In photosynthetic organisms, temperature is a key factor regulating biological reactions, e.g. photosynthesis and respiration. Water temperature is considered one of the most important environmental parameters that determine seasonal trends and densities of microalgae communities (Edwards *et al*. 2006; Raven and Geider 1988). For example, the germination process and cell survival of the noxious raphidophyte *Heterosigma akashiwo* is strongly affected by water temperature (Shikata *et al.*, 2007). Similarly, specific growth rate and maximum cell concentration in the harmful algae *Alexandrium catenella* are predominantly influenced by temperature (Laabir *et al*. 2011). Algal responses to temperature are biogeographically linked to environmental regimes (van den Bergh *et al* 2002), but both temperature and physiological responses might not be linear during microalgal growth (Sterner and Grover, 1998).

Specific growth rates of microalgae and many other biological processes (e.g. respiration) are commonly assumed to increase exponentially with temperature (Duarte, 1990, Montagnes *et al.*, 2003). However, Montagnes *et al*. (2003) argued that microalgae growth rate is the sum of a combination of processes which might not yield an exponential growth response in many cases. These authors found that a number of autotrophic (flagellates, dinoflagellates, and diatoms) and heterotrophic organisms (ciliates) increased their specific growth rates linearly rather than exponentially with increasing temperatures, within a defined temperature range (excluding thermal extremes). Q_{10} (the rate of increase of a biological reaction for every 10 °C rise in temperature) has been used for some processes relevant to the growth of microalgae (e.g. photochemistry, catalyzed and uncatalysed chemical reactions, membrane permeation, photosynthesis, enzyme activity, thylakoid reactions) at increasing temperatures (Raven and Geider, 1988). Nevertheless, Montagnes *et al*. (2003) suggested that Q_{10} may be inappropriate applied to growth rates of autotrophic organisms that follow a linear growth response with temperature. These authors proposed that growth data should be adequately analyzed to decide whether (exponential growth data) or not (linear growth data) Q_{10} can be a suitable descriptor of the effect of temperature on the algae growth.

Algae have developed a temperature adaptation in the environment as a result of evolutionary processes over history (Hanelt *et al* 2003). Due to temperature acclimation over short term periods, algae are able to optimize photosynthesis in environments with temperature fluctuations. Raven & Geider (1988) pointed out that adaptation to high temperatures for growth involves changes in the degree of thylakoid membrane kinetics and quantity of enzymes and the ratio of light-harvesting pigments, whereas adaptation to low temperatures involves less-saturated fatty acids in the thylakoid membrane. Algal acclimation is an advantage for algae to increase growth rates under suboptimal conditions (Geider *et al* 1998). For instance, when nutrient uptake by phytoplankton becomes strongly limiting at warmer temperatures (Sterner and Grover 1998), some studies have postulated that the increased activity of the enzyme RUBISCO might be a strategy that enables different algae to increase growth rates under suboptimal conditions (Geider *et al*. 1998).

Algal growth and nutrient uptake by microalgae are generally temperature dependant. Sterner and Grover (1998) determined that nutrient uptake by phytoplankton

became strongly and consistently limiting at higher temperatures. Some studies have postulated that the increase of RUBISCO might be a strategy that enables different algae to offset the decrease of enzyme activity or protein loss as a result of heat denaturation or cold temperatures (Devos *et al* 1998). Photosynthesis becomes highly dependent on temperature when light saturation is included producing a photosynthetic rate limitation as a result of carbon fixation rates (Schofield *et al.*, 1998).

Although algal growth might respond as a function of temperature, there are many uncertainties as to how HABs can be triggered by increasing temperatures in coastal areas. Reports on mechanisms of toxin production have provided evidence that elevated temperatures (30-31 °C) were able to increase the cellular toxicity of some strains of the dinoflagellate *Ostreopsis lenticularis* due to the metabolism of bacterial symbionts (Asthon *et al* 2003). It has been suggested that as a result of global warming the growth rate of harmful algae might increase (Vale *et al.*, 2009) particularly in eutrophic coastal areas (Honjo, 1993). However, high temperature is also known to produce algal stress and is considered a damaging factor of the photosynthetic performance despite phytoplanktonic strategies for optimizing photosynthesis.

1.1.4 Global warming and ocean acidification

Environmental perturbations, such as climate change (increasing sea water temperatures), eutrophication, enhanced vertical stratification, stimulation of photosynthesis by elevated dissolution of CO_2 in water, changes of phytoplanktonic community composition, hydrologic changes, and increased frequencies of atmospheric events to name a few, are expected to influence directly the occurrence, intensity and distribution of HABs worldwide (Peperzak, 2003, Moore *et al.*, 2008, Hallegraeff, 2010, Lassen *et al.*, 2010, Paerl and Paul, 2011). With the advent of new technologies to monitor the distribution patterns of HABs, for instance the continuous plankton recorder or satellites from the space, the dynamics of HABs species over time in coastal environments has been observed to be changing in recent decades (Edwards *et al.*, 2006, Bravo *et al.*, 2001), particularly in UK waters (Bresnan et al., 2011). Shears and Ross (2009) found evidence that the genus *Ostreopsis* will increase the number of deleterious blooms as warming of surface water and water stratification increase in intensity. Similar predictions were suggested by Peperzak (2003) based on a model to predict increasing sea water temperature on the growth of HABs. He determined that four

noxious dinoflagellates (*Prorocentrum micans*, *P. minimum*, *Fibrocapsa japonica* and *Chattonella antiqua*) are more likely to increase rather than decrease their occurrence and threat in the environment due to climate change.

There is some evidence that the rise in seawater temperature (Beardall and Stojkovic, 2006) has affected the distribution and growth rate of HA (Yamaguchi *et al.*, 2010, Paerl and Paul, 2011), as in the case of the potent toxin-producing *Ostreopsis ovata* (Graneli *et al.*, 2011), *Gambierdiscus toxicu*s, and some species of *Prorocentrum* (Aligizaki *et al.*, 2009, Graneli *et al.*, 2011) . In addition, reports on mechanisms of toxin production have provided evidence that elevated temperatures were able to increase the cellular toxicity of some strains of the dinoflagellate *Ostreopsis lenticularis* (Asthon *et al* 2003), *O. ovata* (Graneli *et al.*, 2011, Pistocchi *et al.*, 2010). Furthermore, it has been suggested that as a result of global warming the growth rate of harmful algae might increase (Vale *et al*., 2009), particularly in eutrophic coastal areas (Honjo, 1993; Peperzak, 2003; Vale *et al*., 2009). Therefore, shallow coastal areas are predictably vulnerable targets to primarily experience future effects of climate change (Hallegraeff, 2010).

Since the atmospheric greenhouse gas carbon dioxide (CO_2) is increasing globally (Beardall and Stojkovic 2006) and HABs can intensify their occurrence as the availability of CO_2 increases in the water (Moore *et al.*, 2008, Hallegraeff, 2010, Low-Decarie *et al.*, 2011), an increase of HA biomass is expected to occur in many coastal areas with negative consequences in the environment (Paerl and Paul, 2011, Hallegraeff, 2010). For example, Fu *et al*. (2010) found that at high pCO_2 the cellular toxicity and growth rate of the harmful dinoflagellate *Karlodinium veneficum* increased substantially, particularly in phosphate limited cultures. Beardall and Stojkovic (2006) suggested that elevated CO_2 concentration in the environment will also bring substantial chemical changes in the composition of the sea water that have been far ignored for HABs outbreaks. Besides, elemental and biochemical changes in microalgae composition will be experienced as some components of global environment change (Gienapp *et al.*, 2008). Nevertheless, Moore *et al*. (2008) has emphasized the lack of comprehensive knowledge and rigorous statistical analyses to confirm linkages between climate and HABs, due to the paucity of long term plankton records and the difficulty to extrapolate information worldwide (Dale *et al*. 2006). Therefore, although increasing levels of CO_2, temperature, and nutrient load in aquatic ecosystems will have potential

consequences in the dynamics of HA (Bravo *et al.*, 2001, Lassen *et al.*, 2010, Paerl and Paul, 2011), ecological implications and predictions of the impact of global climate change and ocean acidification on marine HABs are not straightforward (van den Bergh *et al.*, 2002, Moore *et al.*, 2008, Shi *et al.*, 2009, Glibert *et al.*, 2010, Hallegraeff, 2010). Due to the complexity of natural systems, Edwards *et al*. (2006), Balch and Fabry (2008), Moore *et al* (2008), and Low-Décarie *et al*. (2011) suggested that future research efforts should address a new type of combined oceanographic strategy whereby very long time spans and extensive geographic scales are considered.

1.1.5 Marine toxins and syndromes produces by HABs

Hallegraeff (2003) suggested that about 80 HA are widely recognized as toxin-producing organisms of which marine dinoflagellates are the most important group responsible for human poisonings (Kellmann *et al.*, 2010). These toxic syndromes include diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), neurotoxin shellfish poisoning (NSP), amnesic shellfish poisoning (ASP), ciguatera fish poisoning (CFP), and azaspiracid poisoning (AZP). Toxins are chemically diverse and include macrolides, cyclic polyethers, spirolides, and purine alkaloids (Plumley, 1997, Kellmann *et al.*, 2010). A review of the chemical structures of HA biotoxins can be found in Daranas *et al*. (2001b) and Blanco *et al*. (2005). On the other hand, although a considerable progress has been achieved to understand HAB-toxins, many important unknowns remain (Plumley, 1997).

DSP and CFP toxins are generally produced by benthic dinoflagellates (Daranas *et al.*, 2001b, Camacho *et al.*, 2007, Dickey and Plakas, 2010) associated with the genera *Amphidinium*, *Coolia*, *Prorocentrum*, *Gambierdiscus*, and *Ostreopsis* (Hallegraeff, 2003). The main DSP toxins are okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs) and pectenotoxins (PTXs), while CFP (Ciguatera Fish Poisoning) consist of ciguatoxins (CTXs), maitotoxins (MTXs), palytoxin, and gambierol (Daranas *et al.*, 2001b, Camacho *et al.*, 2007). The genus *Prorocentrum*, considered globally distributed in temperate and tropical waters, is possibly best known for the production of OA and DTX. OA and DTXs are lipophilic compounds highly soluble in organic solvents and are known powerful inhibitors of protein phosphatases-1 and 2A (Mountfort *et al.*, 2001) and potent tumour promoters (Blanco *et al.*, 2005). Ciguatoxins and gambierol, considered some of the most potent neurotoxins, are

ascribed to epibenthic HA *Gambierdiscus toxicus* which affects more than 50,000 people annually around the world.

In some HA such as the genera *Amphidinium* and *Coolia,* antifungal and haemolytic compounds have been determined (Nayak *et al.*, 1997, Echigoya *et al.*, 2005, Meng *et al.*, 2010). Mandal *et al*. (2011) suggested that extracellular polymeric substances produced by *Amphidinium carterae* might play an important role in the formation of HABs and they may possess allelopathic implication in the environment. A vast review of bioactive macrolides (amphidinolides) as to structures, biosynthesis, and bioactivity from the noxious genus *Amphidinium* is found in Kobayashi and Tsuda (2004).

Although the epibenthic dinoflagellate *Coolia monotis* is considered a ciguatera-producer (Richlen and Lobel, 2011) and some isolates cultured produce toxicity (Fernandez *et al.*, 1996, Bravo *et al.*, 2001, Ignatiades and Gotsis-Skretas, 2010), a number of studies agreed that a better understanding of toxins and synthesis of secondary metabolites from *C. monotis* is required to comprehend why some strains of this species are not toxic and what mechanism of toxicity are involved in the environment (Armi *et al.*, 2010, Laza-Martinez *et al.*, 2011).

1.1.6 Mechanisms of toxicity

Algal toxins have been regarded as a natural defense whereby algae trigger a biological mechanism that promotes the production of compounds which counteract repercussions caused in a given algal population, for example, as a result of grazing or autotrophic competition (Van den Bergh *et al*., 2002). The mechanisms underlying activation or toxin production, however, remain obscure (Plumley, 1997; Edvardsen and Imai 2006). In fact, there are still many uncertainties why some dinoflagellate species (Lakeman *et al.*, 2009) can produce toxins in some areas whereas in others they cannot (Granéli and Flynn, 2006, Masó and Garcés, 2006). One example is toxin profile of the dinoflagellate *Gambierdiscus toxicus*, a ciguatoxin-producer, that have shown significant variation worldwide and genetic studies on speciation (Taylor *et al.*, 2008) have suggested that it is not a cosmopolitan algae, but rather a multiple cryptic species (Dickey and Plakas, 2010). Differences in toxin profiles have also been reported for populations of the epibenthic dinoflagellate *Prorocentrum lima* (Morton and Tindall, 1995).

Nutrient stress (Vanucci *et al.*, 2010, Varkitzi *et al.*, 2010), temperature (Guerrini *et al.*, 2007), allelopathic effects (Granéli and Hansen, 2006), bacteria (Tosteson *et al.*, 1989, Kopp *et al.*, 1997, Ashton *et al.*, 2003), grazing (Turner, 2006), high pH in the water, and environmental stress are acknowledged as promoting factors responsible for the increase of toxin production in a number of toxic dinoflagellates (Granéli and Flynn, 2006). However, since toxin production varies so greatly amongst strains and little is known of their metabolic pathways, a number of difficulties have been encountered to understand comprehensibly the mechanisms of toxin production of HABs.

In terms of toxic benthic dinoflagellates, a range of ecological studies on nutrient depletion emphasize that *Prorocentrum lima* significantly increase its toxicity under N-limitation or P-limitation (McLachlan *et al.*, 1994, Vanucci *et al.*, 2010, Varkitzi *et al.*, 2010). However, Morton and Tindall (1995), Bravo *et al*. (2001) and Nascimento *et al*. (2005) found that toxin production and the toxin profile of *P. lima* varied depending on the isolate. In addition, Windust *et al*. (1996) and Sugg (1999) determined that *P. lima* cells inhibited the growth of a number of microalgae (allelopathic effect), whereas Nakajima *et al*. (1981) showed that extracts from *P. lima*, *Amphidinium carterae* and *Coolia monotis* caused haemolysis in blood cells of mice.

Some groups of bacteria (*Proteobacteria*) have been reported on the phycosphere of the toxin-producing *P. lima*, but these bacterial symbionts have not been found to be associated with toxin production of *P. lima* (Lafay *et al.*, 1995). However, the growth and toxicity of the benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus* was higher when associated bacterial flora was present in cultures (Sakami *et al.*, 1999), although bacteria grown in pure cultures were not toxic (Tosteson *et al.*, 1989, Ashton *et al.*, 2003, Perez-Guzman *et al.*, 2008). Nonetheless, Mayali and Doucette (2002), Jeong *et al*. (2003), and Hare *et al*. (2005) found that bacteria in the natural environment play a role in controlling the growth and abundance of HA.

Toxin production and temperature are still poorly comprehended for many HABs and there are gaps in knowledge of the biological implications of increasing sea water temperature on marine HABs. Based on laboratory experiments, however, some findings have confirmed that elevated temperatures (>28 °C) will not only influence the algal growth (Armi *et al.*, 2010, Laabir *et al.*, 2011), but also the synthesis of biotoxins,

as found for the benthic HA *O. lenticularis* (Ashton *et al.*, 2003). This assumption, however, did not correspond with Granéli *et al*. (2011) who reported a decrease of cell toxicity in *O. ovata* cells as both growth temperature (26-30 °C) and algal growth increased. On the other hand, at low temperature (6 °C) light intensity was suggested to be a determinant for toxin production (okadaic acid and dinophysistoxin) in cultures of the HA *Dinophysis acuminata* (Tong *et al.*, 2011).

1.2 Thesis aim and objectives

HABs worldwide are envisaged to increase as water warming intensifies in many ecosystems due to climate change. Increasing water temperatures can provide a stimulatory-effect on phytoplankton communities, but also these organisms can experience significant changes in biochemical composition, biogeographical distribution, in algal physiology at intracellular level, and in populations as temperature increases. Predicting the impact of climate change on HABs in the environment is difficult and many uncertainties still persist to predict the influence of climate change (warming) on HABs. Thus, this thesis aims at extending the knowledge of the effect of increasing water temperatures on the growth and toxin production of the toxic/epibenthic dinoflagellates *Amphidinium carterae*, *Coolia monotis*, and *Prorocentrum lima* isolated from the Fleet Lagoon, Dorset. In particular, this thesis provides knowledge of the autoecology of the above-mentioned dinoflagellates grown in monocultures (non-axenic) under a range of temperatures.

The specific objectives of this investigation were:

- To determine the growth rate and physiological state of harmful epibenthic microalgal isolates (*Amphidinium carterae*, *Coolia monotis*, and *Prorocentrum lima*) grown in monocultures in nutrient replete medium at growth temperatures between 5 and 30 °C.
- To determine the influence of growth temperature on the potential biosynthesis of secondary metabolites (toxins) during the cell growth of the dinoflagellate strains in incubators maintained at temperatures between 5 and 30 °C.
- To determine whether nutrient depletion is concomitantly associated with toxin production during the cell growth of epibenthic algae cultures grown at temperatures between 5 and 30 °C.
- To estimate the potential toxicity effect of algal compounds on eukaryotic organisms using bioassays.

CHAPTER 2

2.1 Material and methods

2.1.1 Collection of biological material

Toxic dinoflagellates cells were collected during 2007 and 2008 from brackish waters of The Fleet Lagoon, Dorset, UK, localised between 50° 36’ 40’’N latitude and 02° 31’ 10’’W longitude (Figure 2.1). The stations Chickerell Point, Moonfleet, and Langton Herring, comprising the middle section along the Fleet Lagoon (Figure 2.1), were selected to collect native macroalgae (*Chaetomorpha linum*, *Ulva lactuca*) and seagrass (*Ruppia maritima*, *Zostera noltii*, *Z. marina*) with associated epibenthic microflora. The epibenthic toxic dinoflagellate *Prorocentrum lima* was present in the Fleet and isolated in December 2007 while both *Amphidinium carterae* and *Coolia monotis* were both encountered and isolated from the Fleet in May 2008. Table 2.1 shows environmental conditions (temperature, salinity, dissolved oxygen, pH, and chlorophyll *a*) determined in the water using a YSI multiprobe during the cell collection of dinoflagellates in the Fleet. Macroflora and associated microalgae were collected manually from subtidal waters (depth <1 m) at all stations using waders.

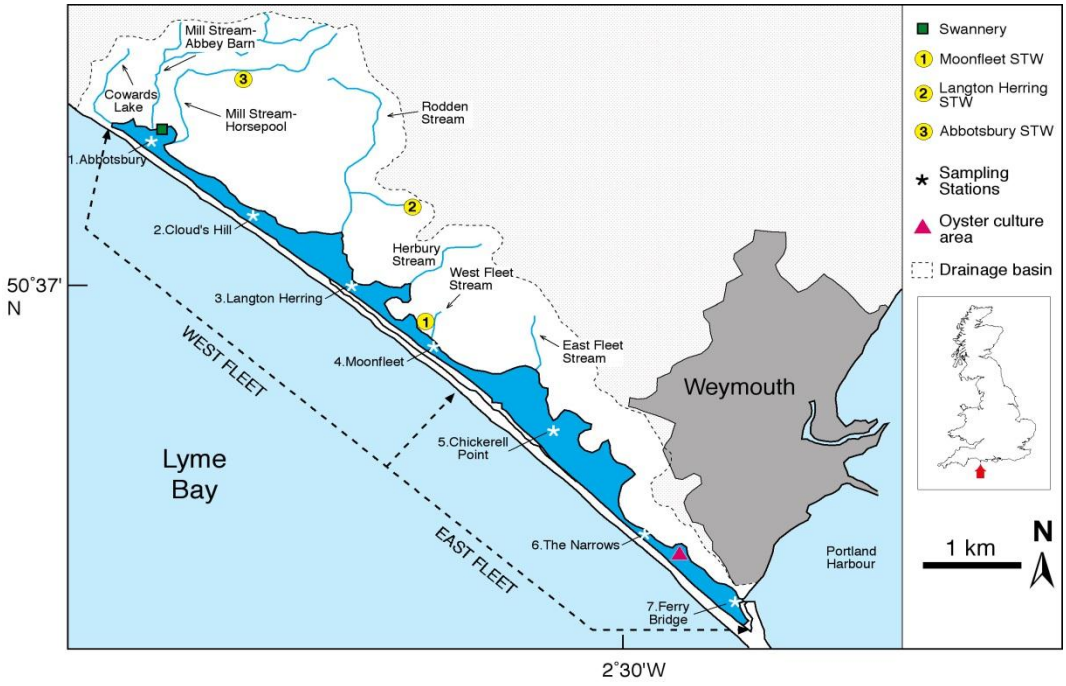

Figure 2.1 Map of the Fleet Lagoon, Dorset, United Kingdom. Sampling stations in the Fleet are enumerated and Chicherell Point (3), Moonfleet (4), and Langton Herring (5) were selected to isolate epibenthic toxigenic dinoflagellates in 2007 and 2008.

Table 2.1 Sampling stations, dates and environmental conditions recorded in the Fleet Lagoon during the collection of toxic dinoflagellates between 2007-2008. Dinoflagellate cultures were grown using natural filtered seawater (0.2 µm) from the environment collected between 2007 and 2009.

Station	Date	Year	Water temp. (° C)	Salinity	Depth (m)	pH	Chlor. *a* (µg L^{-1})	O_2
Chickerell Point	December 13th	2007	5.05	27.4	< 1	7.5	3.2	93.0
Moonfleet	December 13th	2007	4.64	22.4	< 1	7.7	3.3	94.4
Langton Herring	December 13th	2007	4.49	20.6	< 1	7.6	3.2	98.2
Moonfleet	June 27th	2008	19.6	29.4	< 1	8.9	0.1	139
Langton Herring	June 27th	2008	18.6	26.8	< 1	9.3	7.7	122
Chickerell Point	May 15th	2008	17.7	32.9	< 1	8.7	1.4	140.3
Moonfleet	May 15th	2008	19.5	31.7	< 1	8.9	1.6	172.6
Langton Herring	May 15th	2008	19.9	30.5	< 1	9.0	1.5	175
Moonfleet	August 4th	2009	18.1	32.0	< 1	8.3	304.7	-
Langton Herring	August 4th	2009	18.2	31.3	< 1	8.6	484.6	-
Ferry Bridge	January 19th	2009	6.98	33.9	< 1	10.1	-	93.6

In the sampling stations, macroalgae was gently collected by hand and placed into plastic bags underwater preventing the entrance of suspended material from the sediment. Algae specimens were collected close to the water's edge at depths between 0.2-0.6 m. At these depths, algae were sampled randomly from 2-3 different sections at each sampling station. Sampling bags contained 1-3 species of the macroalgae mentioned above. Seawater (1-1.5L) from the collection site was added to the bags to maintain the biological material. Bags were kept fresh and transferred in the dark inside a cool box prior to microscopy analysis. Plankton net samples (64 µm mesh size) were collected in the Fleet to identify potential toxic dinoflagellates suspended in low numbers in the water column. Trawls were done in the surface of the water column and all planktonic material concentrated was transferred to ~200 ml white plastic bottles. After collection, sampled material was transported in a cool box to the laboratory within 3-4 hours. Biological material was maintained in the dark in a cold room at 5° C for up to 3 days. Surface seawater from the Fleet Lagoon collected between 2007 and 2009

(Table 2.1) was used to carry out all growth experiments *in vitro*. Seawater was particularly collected from Langton Herring in 5 and 10 L plastic carboys and it was stored in a dark cool room (5 °C) before the initiation of the growth experiments.

2.1.2 Isolation of toxic epibenthic dinoflagellates

Macroalgae maintained in plastic bags where gently shaken for 30 seconds with water from the Fleet in order to detach epiphytic/benthic dinoflagellates. Large particles were left to settle inside the bags for less than 1 minute before an aliquot of 50ml of water was transferred into a beaker. A volume of ~0.5ml was pipetted in triplicate from the beaker to examine and determine the presence of dinoflagellates under a light microscope (Nikon Eclipse E200). Single cells of benthic dinoflagellates were then transferred through several drops of filtered sea water (0.2µm) on a microscope slide to eliminate debris and other autotrophs. Single cells were transferred into a 96-well plate (Termo Scientific Nunc) with F/2 media by micropipetting. Monocultures started from isolated single cells of dinoflagellates maintained in wells and regular checks of the plates were carried out to verify dinoflagellate growth or the appearance of contaminating organisms. Plates were kept at a growth temperature of ~25 °C and a photoperiod of 12L:12D, with irradiance of 35-70 µmol m^{-2} s^{-1}. Irradiance was measured in the incubator with a Li-Cor irradiance metre although in dense cultures irradiance will have been considerably reduced. Cultures that reached a high density of cells in the wells were later transferred into 250 ml conical flasks with F/2 media. All algal monocultures were non-axenic.

Illuminated (cool white lamps) temperature controlled incubation cabinets (Mercia Scientific, Figure 2.2) were used to maintaine all cultures in this study. Incubators were set up to maintain a constant temperature with a thermal variation <1 °C and 12h:12h light/dark photoperiod. Temperature variation in the incubators was monitored using a glass thermometer (Fisherbrand) with a temperature range between -10 and 110 °C. On-Off programmed timer control for white light lamps (20 watts) was assembled in the incubators. *A. carterae* and *C. monotis* cells maintained at 20 °C were grown in a LMS Ltd Cooled incubator (model 400W) programmed with a photoperiod of 12L:12D, with culture flasks placed within an irradiance of 35-70 µmol m^{-2} s^{-1}.

Figure 2.2 Cultures of benthic dinoflagellates maintained in incubators (Mercia Scientific) using white light illumination at constant growth temperatures (5-30° C). Cells were grown in incubators in non-aeraeted and static medium culture conditions as shown in the image.

2.1.3 Microalgae growth experiments from 5 to 30° C

The HAB species *Amphidinium carterae*, *Coolia monotis*, and *Prorocentrum lima* were successfully isolated and grown in monocultures in the laboratory under a regime of growth temperatures (5 to 30 °C) as mentioned below. The harmful epibenthic microalgae *A. carterae* and *P. lima* were identified by microscopy observations following the morphological descriptions of harmful dinoflagellates in Taylor *et al*. (2003), while *C. monotis* was determined according to the description of Faust (1992).

2.1.3.1 *Prorocentrum lima*

Despite the isolation and growth of different strains of *P. lima* from the Fleet, all growth experiments were carried out using the same strain to minimize the possibility of ambiguous physiological responses between different *P. lima* strains as has been reported in some studies (Calbet *et al.*, 2011). *P. lima* was grown at growth temperatures of 5, 10, 15, 20, 25 and 30 °C (± 1 °C). 1 L polycarbonate flasks (Corning) were used for the growth of *P. lima* cultures to minimize adhesion of *P. lima* cells to the wall of culture vessel. All *P. lima* cultures were capped with sterile polypropylene lids and maintained in non-aerated conditions, except when cultures were sampled under sterile conditions in a laminar flow fume hood. Algal culture media were based on autoclaved seawater from the Fleet enriched with Guillard (F/2) marine media (Sigma G0154). Guillards (F/2) marine water enrichment solution is composed of major nutrients (basal NO_3+NO_2 882 µmol L^{-1}; PO_4 36.2µmol L^{-1}), trace metals and vitamins for the growth of eutrophic algae. The F/2 media was prepared by adding 20 ml of the

Guillards solution per 1 L of autoclaved seawater maintained at room temperature. Initial nutrient concentrations of NO_3+NO_2 and PO_4 in *P. lima* cultures are shown in Table 2.2.

Between two and three replicate cultures were maintained at each growth temperature. Cultures were grown at different times of the year as shown in Table 2.2. In the *P. lima* cultures maintained for ~80 days at 5-15 °C, the final volume of culture media after sampling remained between 0.2-0.3 L in the flasks, while *P. lima* cultures incubated at 20-30 °C maintained a final volume higher than 0.5 L at the end of the growth experiments.

2.1.3.2 Amphidinium carterae and *Coolia monotis*

As in *P. lima* cultures, single strains of *A. carterae* and *C. monotis* were used throughout the growth experiments at temperatures of 5, 10, 15, 20, 25 and 30 °C (± 1 °C). *A. carterae* and *C. monotis* experiments were conducted in 1 L borosilicate (Duran) glass bottles and maintained horizontally during culture incubation. Between two and three technical replicates were maintained at each growth temperature and cultures were incubated under non-aerated condition. Dates of culture experiments and initial nutrient concentrations (NO_3+NO_2 and PO_4) for *C. monotis* and *A. carterae* cells are given in Tables 2.3 and 2.4. After samples were taken from the flasks throughout the growth experiments in the *C. monotis* and *A. carterae* cultures, the final volume of culture media remaining was higher than 0.5 L.

2.1.4 Samples for cell enumeration

Phytoplankton samples for cell enumeration were collected from each 1 L culture flask (2-3 replicates) between 10:00 and 16:00 h. Replicate cultures were transferred from the incubator to a sterile laminar flow fume hood for less than 1 hour while samples were taken; then cultures were returned to the incubator. Microalgae cultures were always manipulated under sterile conditions provided by a sterile hood and the flame from a Bunsen burner. Due to the nature of some benthic dinoflagellates being able to form clumps in culture or their exudates aggregate a number of cells in a given volume, samples from culture flasks were taken at fixed time intervals (every 2-5 days) considering the cell growth stages of the species, e.g. lag, exponential and stationary phase.

Table 2.2 Growth temperatures, initial nutrient concentration (dissolved inorganic nitrogen, NO_3+NO_2, and phosphate, PO4), and period of growth for the toxic strain of *P. lima* isolated from the Fleet Lagoon, UK.

Period	Growth temperature (° C)	Length of experiment	Initial NO_3+NO_2 (µmol L^{-1})	Initial PO_4 (µmol L^{-1})
21 Feb 2009-13 May 2009	5	80 days	1000	36
21 Feb 2009-13 May 2009	10	80 days	1000	36
21 Feb 2009-13 May 2009	15	80 days	1000	36
1 Feb 2010-18 Mar 2010	20	45 days	928.3	32.7
1 Feb 2010-18 Mar 2010	25	45 days	928.3	32.7
29 Oct 2010-12 Dic 2010	30	45 days	978.48	34.4

Table 2.3 Growth temperatures, initial nutrient concentration, and period of growth for the benthic dinoflagellate *Coolia monotis* isolated from the Fleet Lagoon, UK.

Period	Growth temperature (° C)	Length of experiment	Initial NO_3+NO_2 (µmol L^{-1})	Initial PO_4 (µmol L^{-1})
4 Oct 2010-22 Nov 2010	5	49 days	NA	NA
19 Jan 2011-5 Mar 2011	10	47 days	869.4	29.1
1 Feb 2010-13 May 2010	15	32 days	928.3	32.7
1 Feb 2010-13 Mar 2010	20	32 days	928.3	32.7
1 Feb 2010-13 Mar 2010	25	32 days	928.3	32.7
24 Nov 2009-14 Dic 2009	30	20 days	978.4	34.4

Table 2.4 Growth temperatures, initial nutrient concentration, and period of growth for the benthic dinoflagellate *Amphidinium carterae* isolated from the Fleet Lagoon, UK.

Period	Growth temperature (° C)	Length of experiment	Initial NO_3+NO_2 (µmol L^{-1})	Initial PO_4 (µmol L^{-1})
4 Oct 2010-22 Nov 2010	5	49 days	980.5	37.8
19 Jan 2011-5 Mar 2011	10	45 days	980.5	37.8
1 Feb 2010-13 May 2010	15	40 days	980.5	37.8
1 Feb 2010-13 Mar 2010	20	40 days	850.9	29.2
1 Feb 2010-13 Mar 2010	25	40 days	850.9	29.2
28 Oct 2009-19 Nov 2009	30	22 days	869.1	29.9

2.1.4.1 *Prorocentrum lima*

Cell collection from *P. lima* cultures was carried out every 5 days for all growth temperatures as *P. lima* usually exhibits low growth rates. 1L polycarbonate flasks (Corning) were gently rotated by hand for less than 30 seconds to homogenize the distribution of cells in the media. *P. lima* tends to form clumps of cells that stick to the wall and bottom of flasks leading to an uneven distribution of cells in the media. A 10 ml sterile pipette connected to a syringe was introduced in the culture whereby a volume of media was taken into the pipette and then flushed out over clumps attached to the polycarbonate flask to disaggregate cells. This technique was considered more efficient and less violent for cellular homogenization than a strong agitation of the flask whereby cells can be damaged. After cell homogenization, 10 ml of culture media was removed with a pipette and added to a glass tube containing 0.4 ml of glutaraldehyde (1% final concentration). After fixation for over 1 h, samples were shaken for some seconds and little evidence of clump formations was noted. Samples were stored at room temperature and analyzed within 2 weeks of collection.

2.1.4.2 *Amphidinium carterae* and *Coolia monotis*

A. carterae and *C. monotis* did not form clumps in culture. Therefore, a gentle rotation for less than 30 seconds was enough to produce a homogeneous cellular suspension in the culture media. An aliquot of 10ml of sample was measured with a measuring cylinder and transferred to a glass tube where glutaraldehyde (1% final concentration) was added to preserve the cells. Cells were enumerated as describe below by a Coulter Counter within three weeks of sampling.

2.1.5 Quantification of the cell abundance

Cell counts from each culture (2-3 replicate flasks) were performed in triplicate using a Coulter Counter Multisizer III. Culture samples were diluted (1/10) with 3% NaCl in 25 ml plastic cuvettes and 1 ml from each sample was injected and analyzed three times (total volume: 3 ml per flask) using the Coulter Counter with a 70 µm aperture tube. The number of particles suspended in each diluted sample (3 measurements) was enumerated and the average recorded using the Multisizer 32 software. Cell counts derived from the Coulter Counter were compared with cell counts from microscopy (Figure 2.3). Coulter and microscope quantifications showed a high correlation (>95%) for all strains. Average cell counts were plotted for all species to illustrate changes of algal growth. The growth rate of the microalgal population was

computed from the slope of a linear plot of Ln-transformed cell counts over the exponential growth phase.

2.1.6 Q_{10} (temperature-growth rate)

The factor by which the rate of a metabolic process increases for every 10-degree rise in temperature is called the Q_{10} relationship. Q_{10} was calculated based on the growth rates estimated between 5 and 30 °C to determine the effect of temperature dependency on the growth rates.

$$Q_{10} = (\mu_2 / \mu_1)^{10/(T2-T1)} \qquad \textbf{eq. 1}$$

Where μ_1 and μ_2 show the algal growth rate at temperature 1 ($T1$) and 2 ($T2$) respectively ($T2>T1$). If the growth rate doubles, Q_{10} is ~2 (Raven and Geider, 1988).

2.1.7 Photosynthetic efficiency (*Fv/Fm*)

The photosynthetic efficiency (*Fv/Fm*) of algal cells can provide a measure of the physiological state of phytoplankton cells particularly when under nutrient stress (Moore *et al.*, 2006). In this study measurements of quantum yield of photosynthesis (F_v/F_m) were carried out with a Fast Repetition Rate Fluorometer (FRRF, Chelsea II) enabled for a saturation of PSII of 100 flashlets 1.1 µs at 1.1 µs intervals. Values of *Fv/Fm* were acquired on a computer attached to the FRRF based on the following equations:

$$Fv = Fm - Fo \qquad \textbf{eq. 2}$$

$$Fv/Fm = \frac{(Fm - Fo)}{Fm} \qquad \textbf{eq. 3}$$

Where *Fv* is the variable chlorophyll-*a* fluorescence; *Fo* and *Fm* are the minimum and maximal *in vivo* chlorophyll-*a* fluorescence yield (relative) in a dark-adapted state respectively. 10ml of culture media was placed in the dark for at least 25 min to relax the reaction centres of photosystem II before a number of blue light flashes at 440nm permitted the determination of F_o and F_m using a 4.5ml quartz cuvette.

Fv/Fm was measured in the cells using 5 ml of culture media collected from each technical replicate (2-3 flasks). In this volume, six single FRRF measurements were performed per replicate and *Fv/Fm* values were averaged using the FRRF software FastInP (Chelsea Technologies Group Ltd). *Fv/Fm* values in replicate cultures were plotted at each growth temperature. In addition, average *Fv/Fm* data at each growth temperature were computed and plotted based on the number of replicates per growth

temperature. *Fv*/*Fm* in the cells was analysed during the lag, exponential and stationary growth phases based on samples taken for cell counts. In general, *Fv/Fm* was analysed every 2-4 days for *A. carterae*; every 4 days for *C. monotis*; every 5 days for *P. lima*. A blank prepared with 5 ml of culture media filtered through 0.2µm pore size (Whatman) did not show a significant influence on the *Fv*/*Fm* correction values at all growth stages.

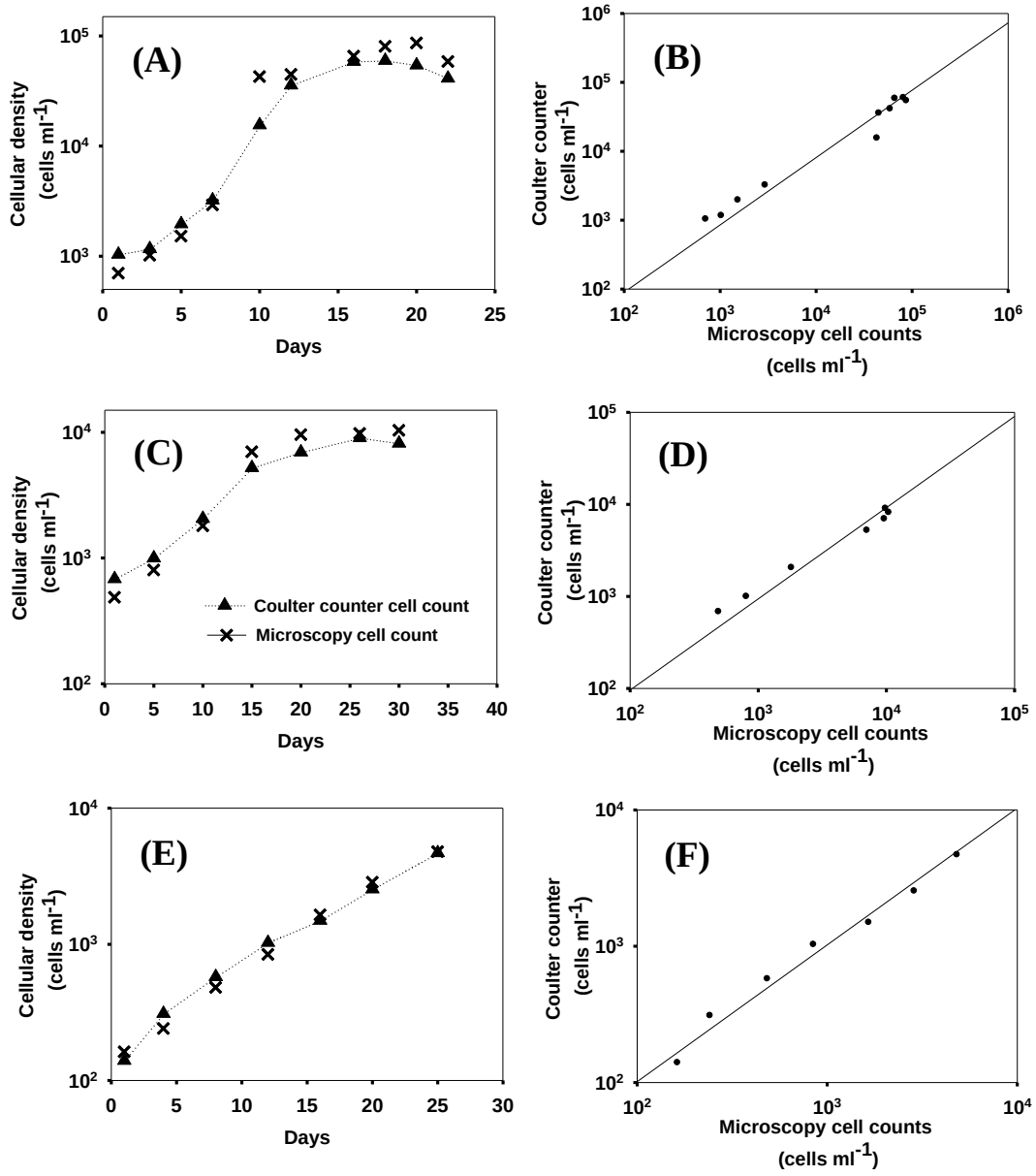

Figure 2.3 Comparison of two methods of cell quantification (Coulter Counter and microscopy) for *A. carterae* (A-B), *P. lima* (C-D), and *C. monotis* (E-F) samples (left plots). Relationships determined between Coulter Coulter and microscopy counts for the benthic dinoflagellates mentioned above (right plots).

2.1.8 Chlorophyll *a* measurements

Chlorophyll *a* concentration was measured in cultures on the same days that samples were collected for cell counts. This pigment was extracted from dinoflagellate cells overnight following a passive extraction method (MacIntyre and Cullen 2005). From a well-mixed culture, a volume of 10ml of culture media was gently filtered through a GF/F filter (25mm diameter) using a syringe attached to a filter holder. Filters were then folded in half and stored in plastic bags at -20° C prior to analysis. Filters were removed from the freezer and placed in a plastic centrifuge tube with 6 ml of 90% acetone. Plastic tubes were mixed with a vortex for 10 seconds followed by chlorophyll *a* extraction at 4° C in the dark. The acetone extracts were poured into a borosilicate glass cuvette and placed into a 10AU Turner fluorometer. The system was equipped with a blue mercury vapour lamp and an excitation (436nm) and emission (680nm) filters according to the Welschmeyer method (Welschmeyer 1994). Pheophytin correction was not applied to the chlorophyll *a* measurements. Chlorophyll *a* concentration was calculated using the following equation:

$$\text{Chlorophyll } a \text{ concentration } (\mu g\ ml^{-1}) = C \times \left(\frac{v}{V}\right) = \qquad \textbf{\textit{eq}. 4}$$

Where C= concentration of chlorophyll *a* measured by the fluorometer.

v= volume (6 ml) of acetone extract used for pigment extraction.

V= volume of culture media filtered (10 ml).

Chlorophyll *a* $cell^{-1}$ was calculated by dividing the concentration of chlorophyll *a* ml^{-1} by the cellular density ml^{-1}.

2.1.9 Nutrient analysis (NO_3+NO_2 and PO_4)

NO_3+NO_2 (dissolved inorganic nitrogen as the sum of nitrate+nitrite) and PO_4 (phosphate) were measured every 4-6 days in both *A. carterae* and *C. monotis* cultures; and every 5-10 days in *P. lima* cultures considering sampling dates as for cell counts. Plastic bottles (vol. 50-70 ml) were used to store nutrient samples. Before sampling, bottles were maintained in acid bath (HCL 10%) for 24 h to remove organic matter. Bottles were then rinsed with tap water and maintained for 24 h in distilled water before bottles were dried at room temperature in a fume hood. Nutrient samples (NO_3+NO_2 and PO_4) were collected from culture flasks by filtering 30 ml of media through GF/F

filters (Whatman). Water sampling was carried out under a sterile hood and samples were frozen and maintained at -20 °C prior to analysis.

Samples were defrosted at room temperature and diluted 1/100 using a solution of NaCl+MilliQ water (40%). NO_3+NO_2 and PO_4 were determined in filtered water samples with a QuAAtro segmented flow autoanalyser and standard colorimetric techniques described by Grasshoff (1976) and Kirkwood (1996). Each sample was analysed in duplicate and average values were considered for each sampling point in replicate cultures. The detection limit of NO_3+NO_2 and PO_4 was 0.03 µmol L^{-1} and 0.01 µmol L^{-1} respectively. Nutrient analysis was carried out by Mark Stinchcombe at the National Oceanography Centre Southampton.

2.1.10 Toxin analysis of *P. lima* cells

The epibenthic dinoflagellate *P. lima* is a common microalgae and produces marine lipophilic toxins including okadaic acid (OA) and dinophysistoxins (DTXs). These natural products can be responsible for the syndrome known as diarrhetic shellfish poisoning (DSP) following the consumption of OA/DTX contaminated edible shellfish. Lipophilic marine toxins from toxic dinoflagellates are classified in four main groups namely, 1) OA and its analogues dinophysistoxin1 (DTX1) and dinophysistoxin2 (DTX2), 2) pectenotoxins (PTXs), 3) yessotoxins (YTXs), and 4) azaspiracids (AZAs). Between 2008 and 2009, extracts of *P. lima* were analysed for OA and DTX1 by liquid chromatography coupled to tandem mass spectrometric detection (LC-MS/MS). The instrument was operated in negative ion mode with electrospray ionisation. It has been reported that a particular strain of *Coolia monotis* produces a natural product similar in chemical structure to YTX (Holmes *et al*. 1995), but LC-MS/MS analyses of *C. monotis* samples obtained from the Fleet Lagoon, Dorset, UK did not indicate the presence of these compounds. Unfortunately and due to the unavailability of the LC-MS/MS instrumentation at the time of the research, chemical analyses of extracts from *Amphidinium carterae* were not performed. Therefore, of the benthic dinoflagellates isolated from the Fleet Lagoon, only toxins from *P. lima* cells could be identified and quantified by LC-MS/MS. This study, however, opted for an alternative method of toxin detection in *C. monotis* and *A. carterae* cells by detecting haemolitic activity of chicken red blood cells and feeding assays where the mortality of crustacean due to toxin ingestion from dinoflagellate cells was analysed (see method below).

From the extracts of the *P. lima* cells, OA and DTX1 were measured as Free OA and DTX1 (known as parent toxins) and Total OA and DTX1. Free OA or Free DTX1 represents the native lipophilic toxin synthesized and concentrated in *P. lima* cells. Total OA and Total DTX1 constitute a spectrum of lipophilic toxins which includes the native OA or DTX1 toxins together with a range of OA or DTX1 esterified derivatives associated with the native lipophilic toxin OA or DTX1. Table 2.5 lists a number of OA and DTX1 derivatives that potentially contributed with the toxic burden due to OA and DTX1 in *P. lima* cells.

2.1.11 Sampling of *P. lima* cells

P. lima cell samples for toxin analysis were collected every five days from culture flasks under a sterile hood to minimize contamination. *P. lima* cells in culture flasks were gently suspended in the media by rotation of the culture flask or, at high cell abundance, a volume of culture media (<3 mL) was flushed out over the cells attached to the walls. Media was collected from the culture with a 10 ml pipette attached to a mechanical piston pipette. This technique proved to be less violent than a strong agitation of the flask to suspend cells in the media, which otherwise would have caused damage to cells. Once cells were suspended in the water, 30 ml of culture media was measured using a measuring cylinder and this volume was gently filtered through GF/F (25 mm diameter) filters using a 50 ml syringe attached to a plastic filter holder. Filters were removed from the holders, folded and placed into 2 ml vials.

Esterified derivatives of OA and DTX1 toxins in *P. lima* cells are known to modify their chemical structure and nature in a matter of minutes unless enzymatic conversion is stopped (Bravo *et al*. 2001). To inhibit enzymatic conversion of OA and DTX1 esters, vials containing the filters with cells were placed in boiling water for 5 min. Samples were then kept at 4°C for <50 min before they were transferred and stored at -20° C prior to toxin extraction and analysis.

Table 2.5 Selected ion monitoring (SIM) and multiple reactions monitoring (MRM) LC-MS/MS negative ion mode analysis of OA and DTX1 and their ester derivatives in *P. lima* extracts.

Compound	EMW	$[M-H]^-$	$[M-H]^{2-}$	$[M-3H]^{3-}$
OA and DTX2, OA and/or DTX2 diol ester, DTX4 and sulphated and carboxylic esters				
OA and/or DTX2 (SIM)	804.5	803.4		
1) OA-D8 (OA diol ester)	928.6	927.5	463.5	308.5
2) OA-D7a & b (OA diol ester)	914.6	913.5	456.3	303.8
3) OA-D9a & b [OA diol ester (unconjugated)	942.5	941.5	470.3	313.2
OA Methyl ester	818.5	817.2	408.3	271.8
OA Ethyl ester	832.5	831.5	414.3	276.5
Norokadanone	757.5	754.5	377.8	251.5
DTX4 (as OA)	1472.6	1471.6	735.3	489.9
DTX4 + SO3 (as OA)	1552.5		775.3	516.5
DTX4 + SO3 + O (as OA)	1568.7		783.3	521.9
DTX4 + SO3 + 2O (as OA)	1586.1		791.3	527.7
DTX4 + SO3 + CH2 + 2O (as OA)	1598.6		798.3	531.9
DTX4 + O (as OA)	1488.6		743.3	495.2
DTX4 + 2O (as OA)	1504.5		751.3	500.5
DTX4 + CH2 (as OA)	1486.6		742.3	494.5
DTX4 + CH2 + 2O (as OA)	1518.6		758.3	505.2
-7-deoxy-OA (minor metabolite of *P. lima*)	788.5	787.3>239.2		
DTX1, DTX1 diol ester, DTX4 & sulphated &carboxylic esters				
DTX1 (SIM)	818.5	817.4		
1) DTX1-D7 (DTX1 diol ester)	928.6	927.5	463.5	308.5
2) DTX1-D8 (DTX1 diol ester)	942.6	941.5	470.3	313.2
3) DTX1-D9 (DTX1 diol ester)	956.6	955.5	477.3	317.9
DTX4 (as DTX1)	1486.6	1485.6	742.3	494.5
DTX4 + SO3 (as DTX1)	1565.5		782.3	520.8
DTX4 + SO3 + O (as DTX1)	1582.7		790.4	526.6
DTX4 + SO3 +2O (as DTX1)	1600.1		799.1	532.4
DTX4 + SO3 + CH2 + 2O (as DTX1)	1612.6		805.3	536.5
DTX4 + O (as DTX1)	1502.6		750.3	499.9
DTX4 + 20 (as DTX1)	1518.5		758.3	505.2
DTX4 + CH2 (as DTX1)	1500.6		749.3	499.2
DTX4 + CH2 +2O (as DTX1)	1532.6		765.3	509.9
OA AND/OR DTX2 (MRM)	803.4>113.0 & 803.4>255.3 [cone=75 CE=61]			
DTX1 (MRM)	817.4>113.0 & 817.4>255.3 [cone=75 CE=61]			
DTX5a (C65 H99 N O27 S2 Na3	1392.6	1391.6	695.3	463.2
DTX5b (C66 H101 N O27 S2 Na3)	1406.6	1405.6	702.3	467.9
DTX5c (C68 H103 N O 27 S2 Na2)	1475.9	1474.9	737.0	491.0
DTX6	912.5	911.5	455.3	303.2

EMW = estimated molecular weight

2.1.12 Preparation of *P. lima* extracts for toxin analysis

Samples were removed from the -20 °C freezer and left to thaw for at least 5 min. Filters were placed in a clean glass Petri dish and cut into small strips with a methanol cleaned scalpel. Strips were carefully taken with forceps into a 15 ml plastic tube containing 3 ml of methanol/water (90:10 v:v). Samples were homogenized in the solvent using a vortex mixer for 2 min. With a clean Pasteur pipette, the supernatant was transferred into a 3 ml syringe and filtered through 0.2 µm pore size filters (25 mm Whatman) to eliminate particulate matter before LC-MS/MS analyses. 1.5 ml of sample extract was used to determine total potential toxicity (i.e., Total OA and Total DTX1), whereas the remaining 1.5 ml was used to measure the Free OA and Free DTX1 (parent toxins). Samples for total and free toxin determination were reduced to 500 µL and 200 µL respectively using a Turbo-Vap evaporator and oxygen-free nitrogen gas. To determine total OA and DTX1 concentrations, extracts were hydrolysed to transform esterified OA and DTX1 compounds to OA and DTX1 parent analytes (OA and DTX1). The 500 µL extract was placed into a 2 ml glass vial and then 63 µl of 2.5 M NaOH were added. The vial was capped and vortexed for 5 s. The bottle was then maintained at 76 °C for 40 min in a heater block. Samples were allowed to cool (5 min) and then 63 µl of 2.5 M HCL was added. Samples were then stored at -20 °C before analysis by LC-MS/MS. After hydrolysis, samples were injected on to the LC-MS/MS to determine Total OA and DTX1. Free OA and DTX1 concentrations were determined by LC-MS/MS analysis of the unhydrolysed (200 µL) extract.

2.1.13 Determination of toxins in *P. lima* by LC-MS/MS

Analyte separation from unhydrolysed and (alkaline) hydrolysed methanolic extracts was undertaken using an *Agilent 1100* LC (Agilent Technologies UK Ltd., United Kingdom), and an *XBridge* C_{18} reversed-phase analytical column (150 x 2.0 mm; 3.5 µm; Waters Ltd., Herts., United Kingdom; held at 35 °C) with a guard column and applying LC gradient modified after Gerssen *et al*. (2007). The following gradient was used: $T_{0\ min}$ 75% A; $T_{1\ min}$ 75% A; $T_{11.4\ min}$ 0% A; $T_{16.7\ min}$ 0% A; $T_{17\ min}$ 75% A; $T_{22.5\ min}$ 75% A. Mobile phase A consisted of 100% deionised water + 2 mM ammonium hydrogencarbonate (pH 11), whereas mobile phase B was 90% acetonitrile:10% deionised water + 2 mM ammonium hydrogencarbonate (pH 11). The flow rate was 0.3 mL/min. The LC was coupled to a *Quattro Micro* triple quadruple mass spectrometer (MS/MS; Waters Ltd., UK) and operated in negative ion mode and using electrospray

ionisation. Respectively, capillary and cone voltages were 60 kV and 77 V, and source and desolvation temperatures were 500 and 120 °C. Multiple reaction monitoring (MRM) was applied to acquire MS/MS data for the toxins. For OA and DTX1 respectively, the $[M-H]^-$ pseudo molecular (precursor) ions were *m/z* 803.4 and 817.4. Two transition (fragment) ions were acquired for both toxins; these were the same for OA and DTX1 and included >113.1 and >255.3. The chromatographic retention times of OA and DTX1 were 7.9 and 9.0 min, respectively.

Where OA and DTX1 were identified by the presence of the two transition ions per analyte, concentrations were determined externally using five points, linear calibration plots of OA and DTX1 obtained from certified reference materials (Marine Biosciences, National Research Council Canada, Nova Scotia, Canada). Calibration solutions were prepared in 100% methanol (Rathburns Chemicals Ltd., Scotland) and 5 μL of both calibration solutions and *P. lima* extracts were injected on to the LC column. Quantitation involved the deployment of the 803.4>113.1and 817.4>113.1 transition ions, and concentrations were determined on a pg/cell basis.

OA and DTX1 (total and free) in *P. lima* cells were plotted in relation to the cell growth of *P. lima* (i.e. lag, exponential, and stationary growth phases). Maximum OA and DTX1 in *P. lima* cells was plotted *vs* growth temperatures to determine the effect of increasing sea water temperature on toxin production in *P. lima* cells. Esterified forms of OA and DTX1 were considered within the total burden of OA and DTX1, but their molecular structure or identity was not established.

2.1.14 pCO_2 measurements

pCO_2 in the culture bottles was calculated from pH_{NBS} and temperature measurements using a Mettler Delta 350 pH meter connected to an ATC (automatic temperature compensation) sensor probe reading to 0.001 pH units. To calculate pCO_2, alkalinity was assumed to remain constant throughout the growth experiments. The instrument and probe were calibrated with fresh NBS buffers (Sigma Aldrich) at 4.0, 7.0, and 10.0 prior to pH measurements. 8ml of culture media (sample) was removed from the cultures and immediately placed in a beaker where the pH and temperature was measured. Samples were acidified with HCl (0.001 M) until the pH reached a value between 3.5 and 4. pH and temperature and volume of HCl added during acidification were recorded. Data were put in an Excel spreadsheet and pCO_2 calculations were carried out following an algorithm developed by Crawford and Harrison (1997).

2.1.15 Brine shrimp bioassays

Nauplii of the brine shrimp *Artemia salina* were used in feeding assays to determine active toxic compounds produced by *P. lima* and *C. monotis*. *P lima* has been shown to be extremely toxic to *A. salina* in other studies. *Artemia* assays were performed following the recommendation of Mayer *et al*. (1982). Briefly, nauplii were hatched after 48-72 hours from brine shrimp eggs maintained in a 7 L aquarium with filtered seawater (0.2 µm, salinity 32) at ~28 °C. *A. salina* eggs were obtained commercially from Tropi-Quaria, UK. *A. salina* eggs were maintained in aerated seawater and illuminated with incandescent light (40 watts) during the cultivation of *A. salina*. After hatching, nauplii were left to grow for 2-3 days in the aquarium before they were exposed to the harmful microalgae *P. lima* and *C. monotis*. Nauplii were fed on a mixture of microalgae (diatoms, chlorophyta and cyanobacteria). Hatched nauplii were separated from the aquarium using a net, rinsed in filtered seawater (0.2µm), and starved in 0.5 L of seawater (0.2µm) for 24 h. Nauplii were placed individually in culture wells (3×4 wells). 3.5 ml of algal culture was used per well and triplicates were performed for each microalgal concentration tested. *P. lima* replicates used 200 and 500 cell ml^{-1} while 2.5 and 2.8×10^{3} cell ml^{-1} were applied in *C. monotis* replicates. Controls were inoculated with non-toxic microalgae (diatoms, chlorophyta, cyanobacteria) and triplicates were performed in all assays. The percentage of mortality was recorded every 24 h under a stereoscopic microscope. Nauplii in a necrotic state or with a poor lack of movement were considered as dead bodies. Additionally, ingestion of *P. lima* and *C. monotis* cells was confirmed in dead nauplii by epifluorescence microscopy. Since *A. salina* ingested a high number of both *P. lima* and *C. monotis* cells and the effect of toxic compounds on the performance of *A. salina* was the main purpose of the assays, the total number of cells remaining in the wells was not quantified at the end of the feeding assays.

2.1.16 Copepod bioassays for determination of algal toxicity

As toxins were not determined in *A. carterae* and *C. monotis* cells through LC-MS/MS analyses, copepod bioassays were applied as an alternative method to investigate potential algal toxicity. Feeding experiments were carried out where the harpacticoid copepod *Tigriopus californicus* was fed on *A. carterae* and *C. monotis* cells for 8-12 days. Copepod mortality was recorded and related to *A. carterae* and *C. monotis* at different cell concentrations.

T. californicus (obtained from Reefphyto Ltd, Bristol, UK) was cultured in a 10 L flask with filtered (0.22 µm) seawater (salinity 28-31) at room temperature for a period of 3-4 weeks. Copepods were supplied a mixture of green and brown microalgae (copepod feed provided by Reefphyto Ltd, Bristol) for growth. Copepods were isolated by filtering a volume of copepod culture through a 100 µm mesh and then rinsing with distilled water for ~30 seconds to remove debris or food material attached to copepods. There was no evidence that copepods were damaged or affected during or after rinsing. Copepods were placed in filtered seawater (0.2 µm) before being picked by a broad plastic tip attached to a 1000 µl pipette. Feeding experiments used mainly adult female copepods as they were larger and easily ingest more microalgae.

Copepods were left in filtered seawater to starve for 3-5 days before the toxic dinoflagellates *A. carterae* or *C. monotis* were introduced. Cells of *A. carterae* and *C. monotis* in exponential growth were used at different concentrations to assess the potential toxicity of the benthic dinoflagellates. Well plates (8 × 12 wells) were used with 1 copepod added per well (1.5 ml) under a range of algae concentrations. Six replicates were assessed at each cell concentration (treatment). Controls were carried out using 6 replicates where starved copepods were fed a non-toxic microalgae mixture (diatoms, chlorophyta, and cyanobacteria) for the term of the feeding experiment. The plate lid was sealed with parafilm tape to reduce evaporation. Bioassays were maintained in the dark at room temperature and lasted 8-12 days. Copepods can remain motionless for several minutes on the bottom of a plate and determining the mortality of organisms by eye can be uncertain, therefore a dissection needle was submerged gently in the wells by hand to verify both the activity and/or mortality of the copepod.

2.1.17 Haemolytic bioassays

The molecular structure of many haemolytic compounds produced by harmful microalgae is still unknown. The true detection and quantification of such substances is dependent on bioassays that provide sensitive information on, for example, haemolytic impairment (Eschbach *et al* 2001). In this study the lytic response of Chicken Red Blood Cells (RBCs) was assessed when different concentrations of algal extracts were inoculated with RBCs. The method applied in this study was similar the used by Eschbach *et al* (2001) and Neely and Campbell (2006), although these authors determined the haemolytic response on Fish RBCs rather than Chicken RBCs.

2.1.17.1 Preparation of algal extracts

Cells of *Amphidinium carterae* and *Coolia monotis* were collected during the exponential growth phase to prepare algal extracts. 10ml of algal culture was centrifuged at 4° C (10 min at 3500 rpm) in 15ml plastic tube and a cell pellet was formed. Culture media was decanted from the tube and then assay buffer (150 nM NaCL, 3.2 mM KCL, 1.25 mM MgSO4, 3.75 mM $CaCl_2$, and 12.2 mM TRIS base; pH adjusted to 7.4 with HCL) was added to yield between 10^5- 10^6 cells. The cell suspension was sonicated for 2 min in buffer using a pulse sonicator with a 70% duty cycle. Algal extracts were maintained in ice during and after sonication.

2.1.17.2 Erythrocyte lysis assay

Chicken Red Blood Cells (Seralab, UK) were preserved in 10% Alsevers solution and used in bioassays within 2 weeks after delivery. 250µl of RBC were inoculated in 50 ml of assay buffer which yielded ~2.5×10^6 cells ml^{-1}. RBCs were centrifuged (10 min at 3500 rpm at 4° C) and re-suspended two times in assay buffer (50ml) to remove the anticoagulant prior to bioassays. From this suspension 5ml was used separately to prepare positive and negative controls. Positive controls were prepared by sonication (2min in a 70% duty cycle) to produce 100% lysis whereas erythrocytes in assay buffer alone were considered negative controls. RBC bioassays were carried out in triplicate in 15ml centrifuge tubes with a final volume of 5 ml. Both RBCs and the algal extract were titrated to produce different concentrations of the potential haemolytic compounds. Following algal-extract inoculation, erythrocytes were incubated in the dark at 20° C and after 18 h they were removed and centrifuged at 4° C for 10min (3000 rpm). 3ml were taken from the tube and placed into a cuvette for absorption measurements by spectrophotometry at 415nm. Photometric scans of Chicken RBCs (100% lysed cells, Figure 2.4 (A) between 350 and 600nm indicated that the peak of maximum absorption was attained at 415nm.

2.1.17.3 Reference curve of erythrocyte lysis induced by saponin

Saponin (Sigma-Aldrich 84510) was used in this study as a chemically defined haemolytic agent (Eschbach *et al* 2001). The haemolytic activity of algal compounds produced by *A. carterae* and *C. monotis* was determined based on a reference curve of RBCs lysis induced at different concentrations of saponin. Saponin concentrations ranged from 1 to 50µg ml^{-1} and complete lysis (100%) of Chicken RBCs was determined at 6µg ml^{-1} as shown in Figure 2.4 (B).

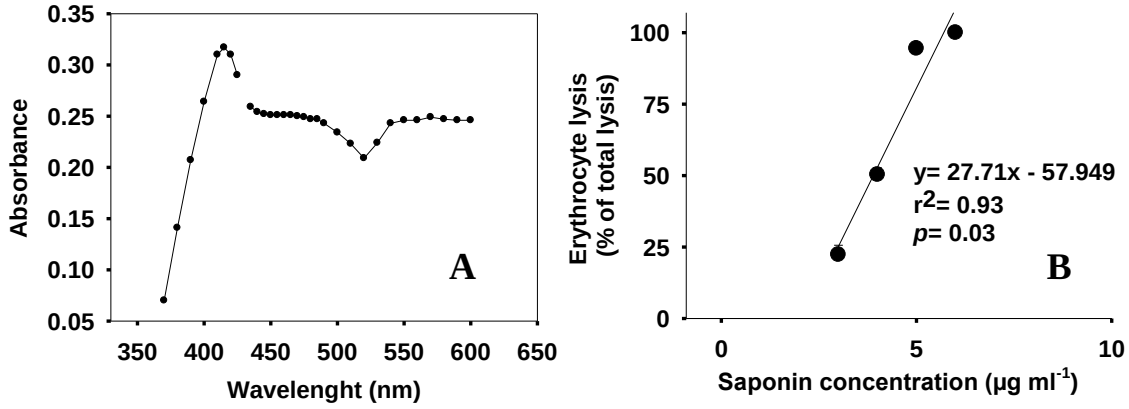

Figure 2.4 (A) Photometric scan of lysed Red Blood Cells suspended in assay buffer; **(B)** Lysis (%) of erythrocyte suspension at different concentrations of saponin. Lysis values show the average of three measurements (± SE).

2.1.18 Scanning Electron Microscopy (SEM)

Cells were collected from dinoflagellate monocultures at an early stage of the stationary phase. Cell pellets were made using the following aliquots from cultures: 50 ml of *A. carterae* (10^5 cells ml^{-1}), 100 ml of *P. lima* (10^4 cells ml^{-1}), and 100 ml of *C. monotis* (10^4 cells ml^{-1}). Cells were concentrated by centrifugation for 15 min at 950 rpm. Supernatant (culture medium) was removed and cell pellets were fixed in 1.5 ml of 0.1 M PIPES buffer (pH 7.2) combined with glutaraldehyde (3%) and formaldehyde (4%). Cells were maintained at 4 °C during fixation. Fixed cells were transferred to silicate coated cover slips by pipetting. Cells were left to settle on the bottom of cover slips for 20 min. Cover slips were then rinsed two times in 0.1 M PIPES buffer. Biological samples were dehydrated in series of ethanol concentrations (30, 50, 70, 95, and100%) for 10 min. Samples were freeze/dried in a critical point dryer, mounted on stubs, and taken to a sputter coater for 10-15 min before SEM observations were carried out using a FEI Quanta 2000 SEM. Microscopy work was carried out at the Biomedical Imagining Unit at the University of Southampton.

CHAPTER 3

3.1 Introduction

3.1.1 The epibenthic dinoflagellate *Amphidinium carterae*

A. carterae is known to produce yellow-brown water discolorations when high cell concentrations of this organism are present in the environment. Baig *et al*. (2006), for example, reported high concentrations of *A. carterae* (1.2×10^4 cells ml^{-1}) in the northern Arabian Sea during the spring season comprising as much as 85% of the total phytoplankton. In the Veracruz reef zone, Gulf of Mexico, *A. carterae* has been observed to dominate the phytoplankton community in assemblages with the potent toxigenic dinoflagellates *Prorocentrum lima, Coolia monotis*, and *Ostreopsis heptagona* (Okolodkov *et al*., 2007). Quantitative data obtained by Okolodkov *et al*. (2007) reported *A. carterae* to be present in the Veracruz reef zone for most of the year (May-December), with highest abundance (4.11×10^4 cells g^{-1} wet weight) between May and June in close association with seagrass, mainly *Thalassia testudinum*.

Amphidinium carterae has been reported to produce harmful effects and to date several haemolytic and antifungal compounds have been identified from algal extracts of this organism (Kobayashi and Tsuda 2004; Echigoya *et al*., 2005; Ignatiades and Gotsis-Skretas, 2010; Meng *et al*., 2010). *A. carterae* can produce secondary metabolites known as amphidinols (Kobayashi and Tsuda, 2004, de Vicente *et al*., 2006) which are a diverse group of biosynthetic compounds classified as poliketides (Chang, 2006). Poliketides encompass a very diverse family of natural products produced by bacteria, algae, plants and animals. They are biosynthesized by successive condensations of carboxylic acid extender units to a growing acyl chain as occurs in fatty acid biosynthesis (Kellmann *et al*., 2010). Polyketides from dinoflagellates, however, are still poorly understood (Chang, 2006; Kellmann *et al*., 2010).

Chang (2006) reported that 25 dinoflagellates are able to produce amphidinols and syndromes such as CFP (ciguatera fish poisoning), DSP (diarrhetic shellfish poisoning), and NSP (neurotoxic shellfish poisoning) are associated with poliketides. To date amphidinol-3 is considered the most active secondary metabolite produced by *Amphidinium carterae* and it exhibits a potent haemolytic activity against human

erythrocytes and antifungal activity against *Aspergillus niger* (de Vicente *et al*., 2006). Moreover, Kubota *et al*. (2005) isolated a polyhydroxyl linear carbon-chain metabolite from a culture of *Amphidinium* sp., called luteophanol D, which demonstrated antibacterial activity against *Micrococcus luteus* (33 µg ml^{-1}). Amongst amphidinols, amphidinol H and N have exhibited remarkable cytotoxicity against human tumour lines and these compounds are expected to have the potential for new anticancer drugs (Kobayashi and Tsuda, 2004). Table 3.1 describes the cytotoxic activity of several amphidinols isolated from *Amphidinium* strains from which Kobayashi and Tsuda (2004) detected high cytotoxicity of some amphidinols (1, 3, 7, 8, 13) as little amounts of these compounds produced remarkable effects on human cells.

Table 3.1 Cytotoxic activity of amphidinols reported by Kobayashi and Tsuda (2004) from 7 *Amphidinium* sp strains. (a) 50% inhibition concentration, (b) murine lymphoma L1210 cells, (c) human epidermoid carcinoma KB cells.

Amphidinolides	**Cytotoxicity (IC50,[a] µg ml^{-1})**		**Amphidinolides**	**Cytotoxicity (IC50, µg ml^{-1})**	
	L1210[b]	**KB[c]**		**L1210[b]**	**KB[c]**
1	2.0	5.7	**14**	1.7	3.6
2	0.00014	0.0042	**15**	1.6	5.8
3	0.0058	0.0046	**16**	6.4	>10
4	0.019	0.08	**17**	1.4	0.67
5	2.0	10	**18**	4.0	6.5
6	1.5	3.2	**19**	18	>20
7	0.0054	0.0059	**20**	12	>20
8	0.00048	0.00052	**21**	3.2	7
9	2.7	3.9	**22**	3.9	>10
10	1.65	2.9	**23**	0.6	7.5
11	0.092	0.1	**24**	0.8	8.0
12	1.1	0.44	**35**	3.6	3.0
13	0.00005	0.00006			

Echigoya *et al*. (2005) suggested that the terminal hydrophilic segment in the chemical structure of amphidinols appears to play an important role in the biological activity of these compounds. However, as seen in other toxigenic dinoflagellates,

amphidinol production may differ from strain to strain (Jeong *et al.*, 2003; Echigoya *et al.*, 2005; Meng *et al.*, 2010).

Jeong *et al.* (2003) determined that the minimum lethal dose of *A. carterae* to mice was a crude whole-cell extract obtained from 1.3×10^8 cells (1.3×10^7 ng C), whilst Baig *et al*. (2006) reported low mortalities in mice at doses of 7.2×10^4 (13% of mortality) and 2.5×10^5 cells ml^{-1} (16% of mortality), respectively. Furthermore, both Jeong *et al*. (2003) and Baig *et al*. (2006) found no evidence that *A. carterae* was noxious to the crustacean and predator *Artemia salina* when fed on *A. carterae*. Contrasting findings were previously reported by Ismael *et al.* (1999) as toxicological tests showed that *A. carterae*, during exponential growth, proved to be extremely toxic when grazed by the brine shrimp *A. salina*.

The growth rate of *Amphidinium carterae* (μ_{max}= 2.7 d^{-1}) was suggested by Furnas (1990) to be one of the fastest amongst many benthic and planktonic dinoflagellates. Morton *et al*. (1992) analyzed the effect of temperature on the growth of *Amphidinium klebsii* and showed that growth rates increased linearly between 16 and 28 °C, with a Q_{10} around 2 (Montagnes *et al*., 2003). Optimum growth of *A. carterae* has been suggested to be a function of light intensity, temperature (Morton *et al*., 1992) and nutrients, while growth rate increases with increasing nitrate and phosphate concentrations (Ismael *et al*. 1999).

Lee *et al*. (2003) has suggested that *A. carterae* is able to outcompete other autotrophic microalgae as it has the ability to store nitrate and phosphate for several generations. In addition, *A. carterae* has a remarkable tolerance to temperature when grown in an artificial medium (ASP7) between 20 and 33 °C, where the maximum growth rate recorded was 1.0 day^{-1} between 28-33 °C (Lee *et al*., 2003) and 2.7 day^{-1} at 25 °C (Furnas, 1990) in GPM medium (Ismael *et al*., 1999).

3.1.2 Taxonomical description

Amphidinium carterae is a small unarmoured dinoflagellate that inhabits marine and brackish waters and is commonly found in temperate and tropical areas. This unicellular photosynthetic organism varies from 11-17 µm in length and width of 9-13 µm (Murray and Patterson, 2002). Cells are oval and dorsoventrally compressed, with or without ridges or ribs on the body (Figure 3.1). This species is characterized by a finger-like epicone in ventral view, notably deviated towards the left (Figure 3.1). *A. carterae* has an epicone forming a V-shape in ventral view where a girdle is encircled (Steidinger and Tangen, 1996). The epicone shape and size is generally considered a morphological character that unites the *Amphidinium sensu stricto* species (Janson and Hayes, 2006).

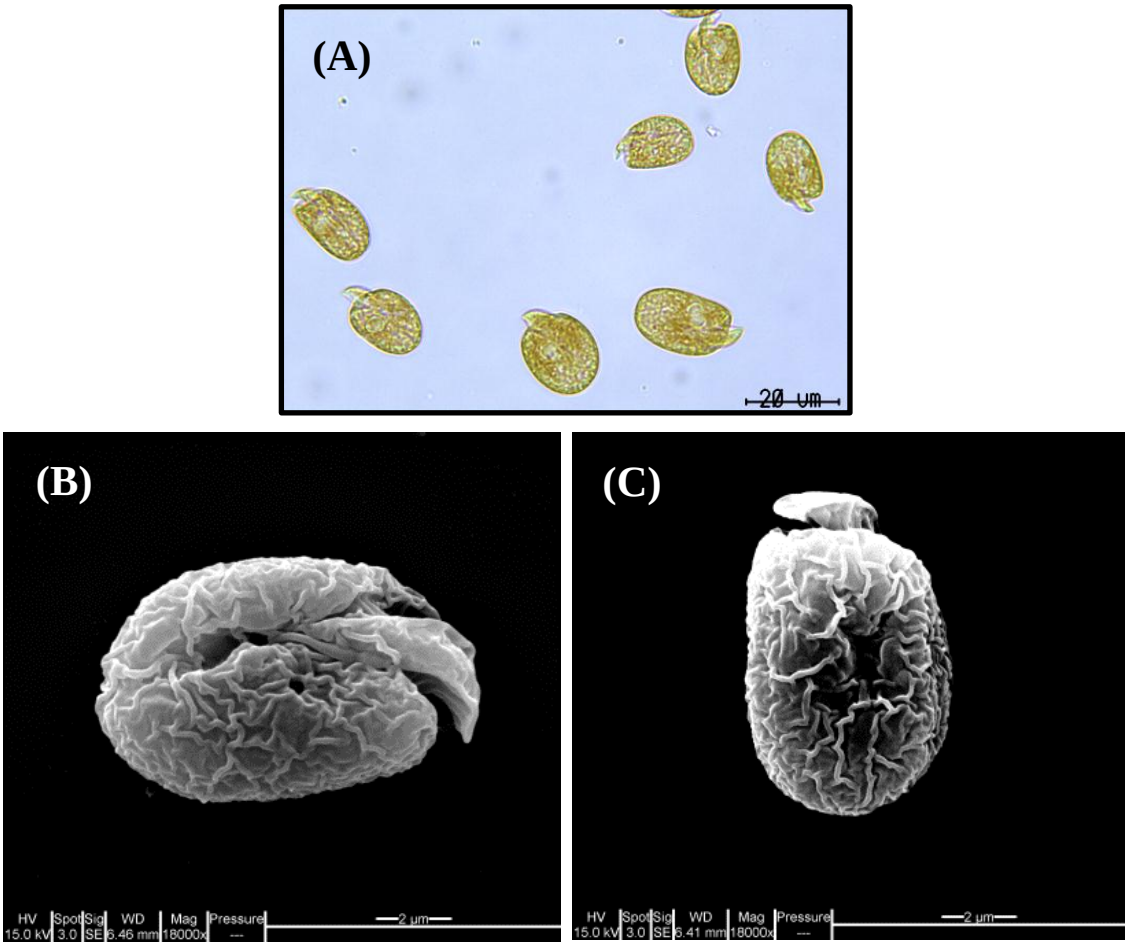

Figure 3.1 Cells of the benthic dinoflagellate *Amphidinium carterae* Hulburt isolated from the Fleet Lagoon. (A) Photo of live cells of *A. carterae* taken under a light microscope (40x). White area in cells shows the nucleus; (B) ventral view of *A. carterae*, denoting the distinctive epicone, under scanning electron microscopy (SEM); and (C) dorsal view of a cell of *A. carterae* by SEM.

3.2 Results

3.2.1 Cell growth and *Fv*/*Fm* in *A. carterae* cultures

The benthic phototrophic microalgae *Amphidinium carterae* was successfully isolated from water samples collected from the Fleet Lagoon in 2008 and clonal cultures were established in Guillards F/2 medium. Experimental cultures were incubated at a range of growth temperatures (5 to 30 °C) and samples collected at regular intervals for determination of cell counts, photosynthetic efficiency (*Fv*/*Fm*), chlorophyll *a*, and nutrient (nitrate+nitrite and phosphate) concentration. Figure 3.2 shows changes in cell density and *Fv*/*Fm* fluctuations in replicate cultures of *A. carterae*. Cultures maintained at 5, 10, and 30 °C had an initial cell concentration between 2.5 and 3.8 × 10^3 cells ml^{-1}, whereas the initial cell density in cultures from 15 to 25 °C ranged from 4.4 to 6.2 × 10^3 cells ml^{-1}.

The period of the lag phase in *A. carterae* cultures was influenced by temperature and lasted *ca*. 18 days at 5 °C, 9 days at 10 °C, and < 2 days in cultures maintained between 15 and 30 °C (Figure 3.2). *Fv*/*Fm* decreased during the lag phase when *A. carterae* cells were grown between 5 and 25 °C (Figure 3.2). The largest change from 0.30 (day 1) to 0.16 (day 5) occurred at both 5 and 10 °C. In some replicate cultures maintained between 15 and 25 °C, *A. carterae* cells displayed a minor decrease in *Fv/Fm* between day 1 and day 2, from 0.33 to 0.30 respectively, immediately before initiation of the exponential growth phase.

Table 3.2 lists the growth kinetics of the *A. carterae* strain in terms of the length of exponential growth (days), changes in cell density, and growth rates encountered in each replicate culture grown at temperatures from 5 to 30 °C. The interval considered as exponential growth showed consistency in terms of the growth period (in days) for the growth temperatures in the range of 10 to 25 °C, with only growth differences amongst replicates of 1 or 2 days at these temperatures (Table 3.2). In this study the longest exponential growth period was found at 30 °C and lasted 9 days.

Growth temperature affected cell increase and, as a result, growth rates as shown in Table 3.2. Growth rates proved to be very similar amongst replicate cultures maintained at each of the growth temperatures, except for one replicate at 15 °C which had a growth rate similar to the values calculated at 20 °C (Table 3.2). Moreover,

similarity was found in terms of the fluctuation pattern of cell density and *Fv*/*Fm* amongst replicate cultures maintained at the same growth temperatures.

During exponential growth, cell increase was always accompanied by an increase in *Fv*/*Fm*. On commencement of stationary phase, *Fv*/*Fm* gradually decreased, with the strongest reduction of *Fv*/*Fm* determined at 30 °C (Figure 3.2).

Considering the increase of sea water temperature on the growth of *A. carterae*, this study determined that the rise of 5 °C in *A. carterae* cultures caused an increase in the growth rate of up to 0.1 day^{-1} when growth temperature changed from 5 to 25 °C (Table 3.2).

Table 3.2 Growth kinetics of *Amphidinium carterae* during the exponential growth in cultures maintained at growth temperatures from 5 to 30 °C.

Temperature (°C)	Time of exp. growth (days)	Exp. change in cell density ($\times 10^4$cells ml^{-1})	Growth rate (μ, d^{-1})
5	5	1.31-2.77	0.13
	4	1.41-2.51	0.15
10	6	1.93-13.26	0.29
	7	1.43-12.13	0.28
15	6	0.68-7.82	0.40
	6	0.78-7.72	0.37
	6	0.61-4.66	0.35
20	6	0.49-7.32	0.47
	6	0.56-7.01	0.42
	7	0.49-8.01	0.40
25	6	0.57-15.17	0.55
	5	0.47-7.67	0.56
	5	0.62-8.20	0.55
30	9	0.25-6.94	0.57
	9	0.33-8.06	0.53

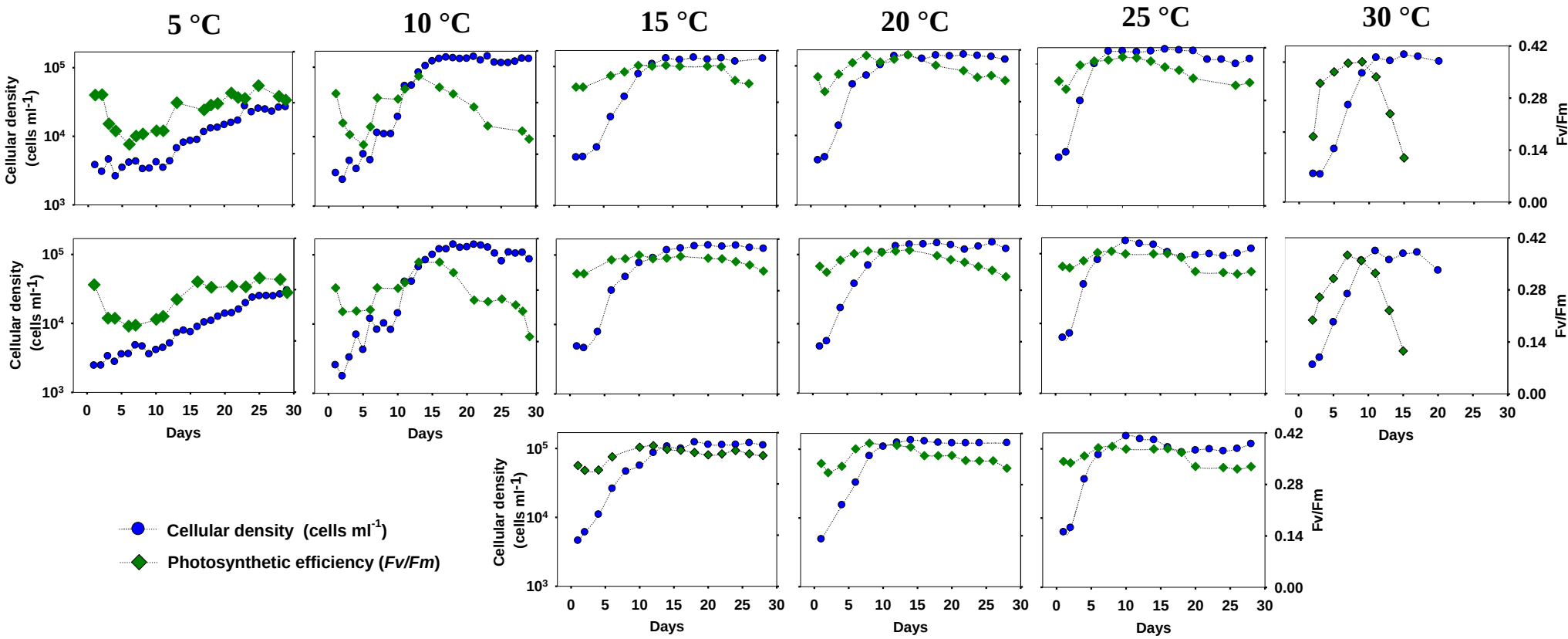

Figure 3.2 Changes in cell abundance and photosynthetic efficiency (*Fv*/*Fm*) during the growth of *Amphidinium carterae* in cultures maintained in F/2 media at temperatures between 5 and 30 °C. Two replicates are shown individually for the growth temperatures 5, 10, and 30 °C and 3 replicates are displayed from 15 to 25 °C.

Figure 3.3 illustrates both the average cell growth rates and *Fv/Fm* variation from replicate cultures of *A. carterae* maintained at growth temperatures from 5 to 30 °C. Overall, the exponential growth in *A. carterae* cultures was shorter than the stationary phase and the death phase was hardly detected. From 10 to 30 °C, maximum cell abundance remained in the same order of magnitude (10^5 cells ml^{-1}) throughout the stationary phase, while cells grown at the lowest growth temperature (5 °C) reached maxima cell concentrations in the order of 10^4 cells ml^{-1} on day 23 during the stationary phase (Figure 3.3).

Cell growth rate was notably reduced when *A. carterae* was grown at the lowest growth temperature (5 °C). However, only at this temperature *A. carterae* continued to divide up to the end of the experiment on day 29. This can be observed in Figure 3.3 where *A. carterae* demonstrated a very slow growth between day 20 and day 29.

As growth temperature increased from 5 to 30 °C, the slope of the exponential phase tended to increase and shortly after the end of the exponential growth, cell density remained with no major changes throughout the stationary phase, particularly at temperatures between 10 and 25 °C (Figure 3.3).The Q_{10} values, based on the average growth rates of *A. carterae* between 5 and 30 °C, were 2.6 (5-15 °C), 1.5 (10-20 °C), 1.5 (15-25 °C), and 1.3 (20-30 °C).

Changes in chlorophyll *a* concentration were measured in *A. carterae* cultures grown between 15 and 30 °C (Figure 3.4). Maxima concentrations in the batch cultures were produced at 20 °C ranging from 470 to 517 µg ml^{-1}, followed by concentrations determined at 25 °C (463-484 µg L^{-1}), at 15 °C (397.8-428 µg L^{-1}), and at 30 °C (386-416 µg L^{-1}) all of which were associated with the stationary phase (Figure 3.4). Replicate cultures showed similar concentrations of chlorophyll *a*, although in some samples low concentrations were found between some peaks of chlorophyll *a* during the stationary phase (Figure 3.4). Figure 3.5 (top row) shows the average concentration of chlorophyll *a* in the replicate cultures.

When *A. carterae* was grown at 30 °C, the maximum of chlorophyll *a* (401 µg ml^{-1}) occurred immediately after the exponential growth and then slightly decreased (Figure 3.4 and 3.5). This reduction of chlorophyll *a* at 30 °C was determined, however, based on few measurements of the pigment during the stationary phase. *A. carterae* chlorophyll *a* content per cell increased during the exponential growth phase between

15 and 25 °C (Figure 3.5). However, decreasing concentrations of this pigment were found in cells throughout the experimental growth in samples collected at 30 °C, even though an extended exponential growth period was determined at this growth temperature.

Chlorophyll *a* in cells maintained from 15 to 25 °C presented an intracellular increase, with maxima concentration from 3.3 to 4.1 pg chlor *a* cell^{-1}, towards the end of the stationary phase (Figure 3.5). This intracellular increase of chlorophyll *a* was in disagreement with the pattern of *Fv*/*Fm* observed during the stationary phase, as *Fv*/Fm decreased when chlorophyll a per cell increased.

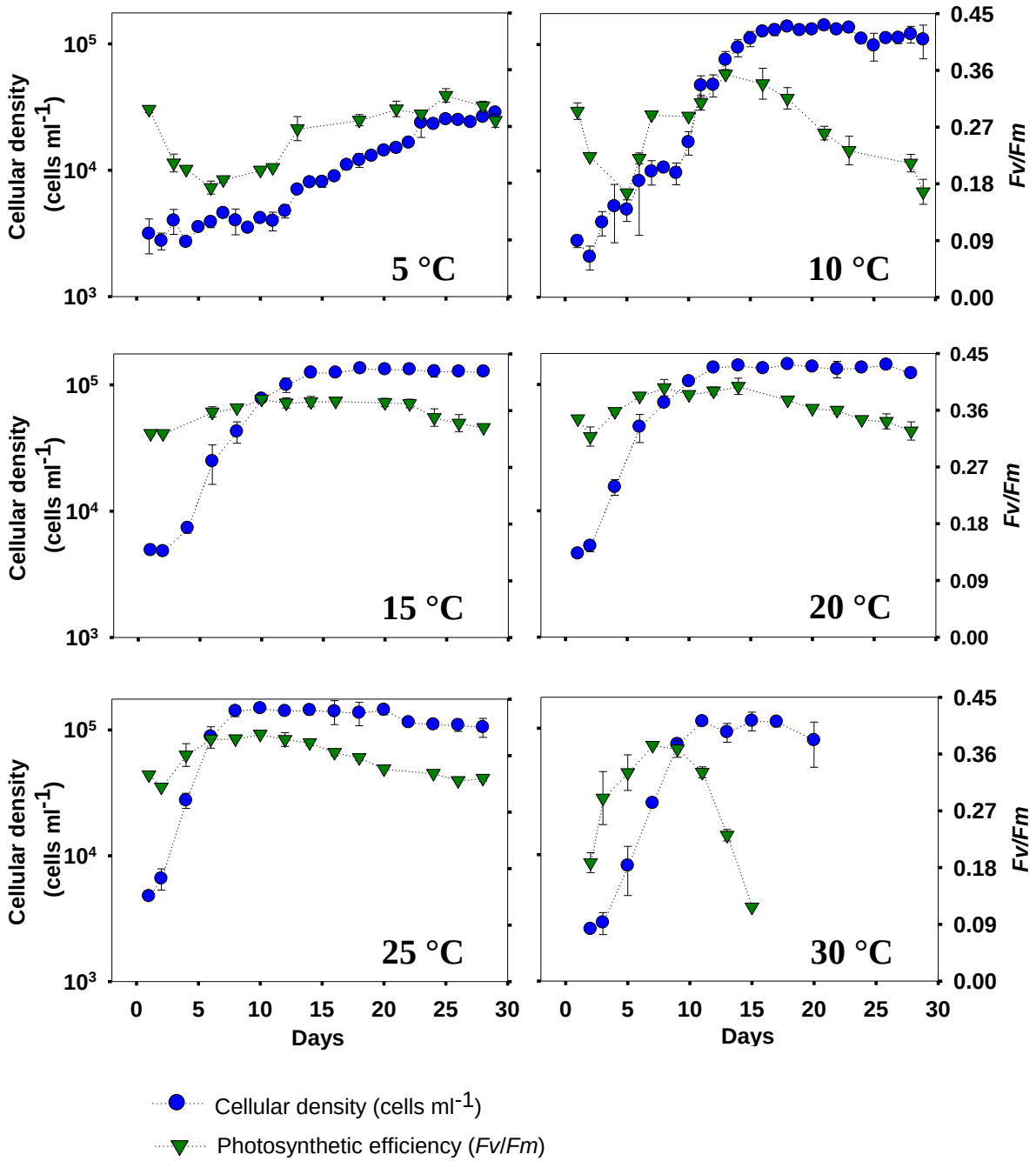

Figure 3.3 Average cell growth and variation of photosynthetic efficiency (*Fv*/*Fm*) during the growth of *Amphidinium carterae* at growth temperatures between 5 and 30 °C. Bars shows ±1standard difference (5, 10, 30 °C) and standard deviation (15-25 °C).

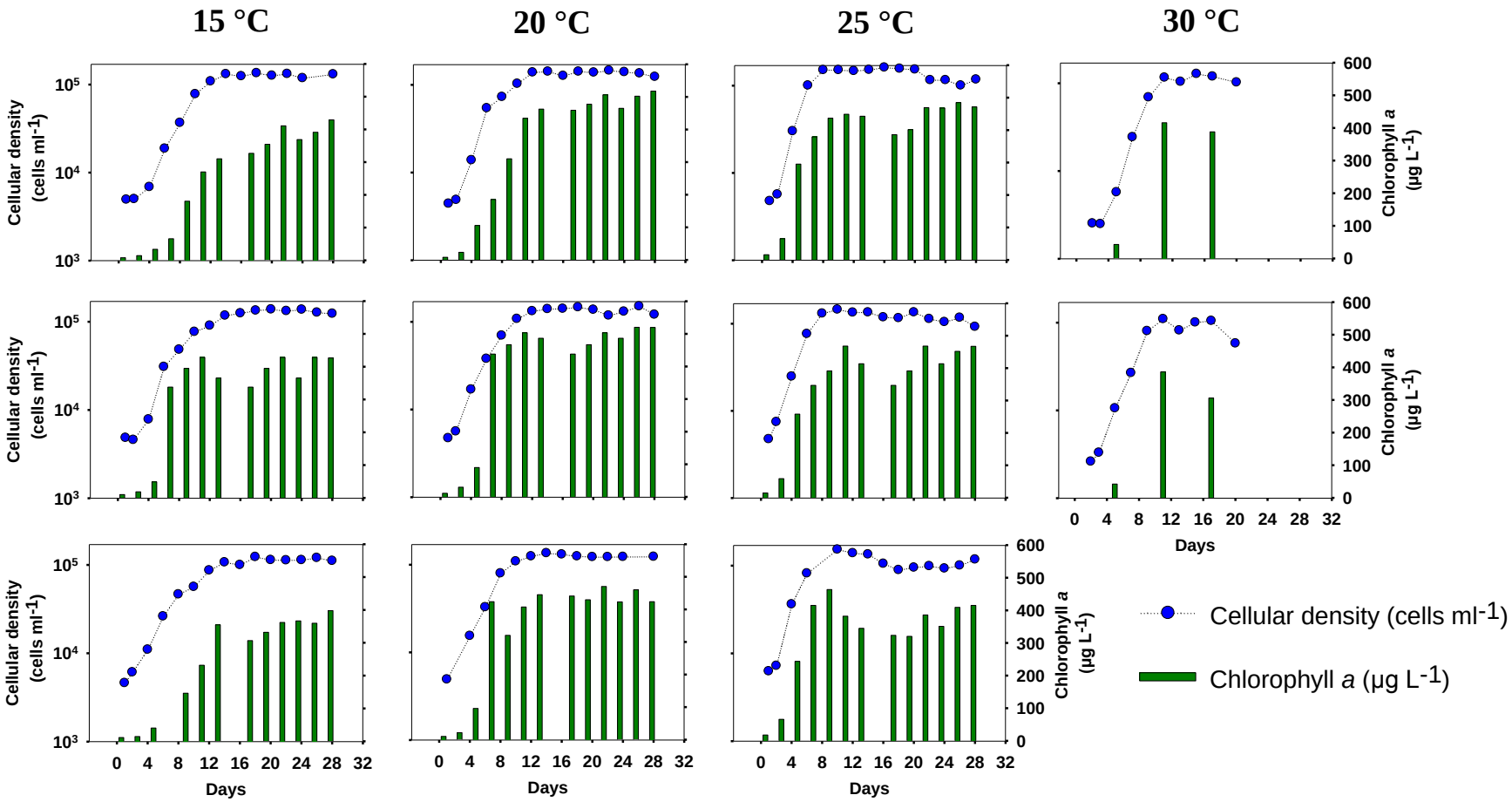

Figure 3.4 Increase of chlorophyll a (µg ml^{-1}) during the cellular growth of *Amphidinium carterae* in replicate cultures maintained at growth temperatures from 15 to 30 °C. 3 replicate cultures are shown from 15 to 25 °C and 2 replicates are shown at 30 °C.

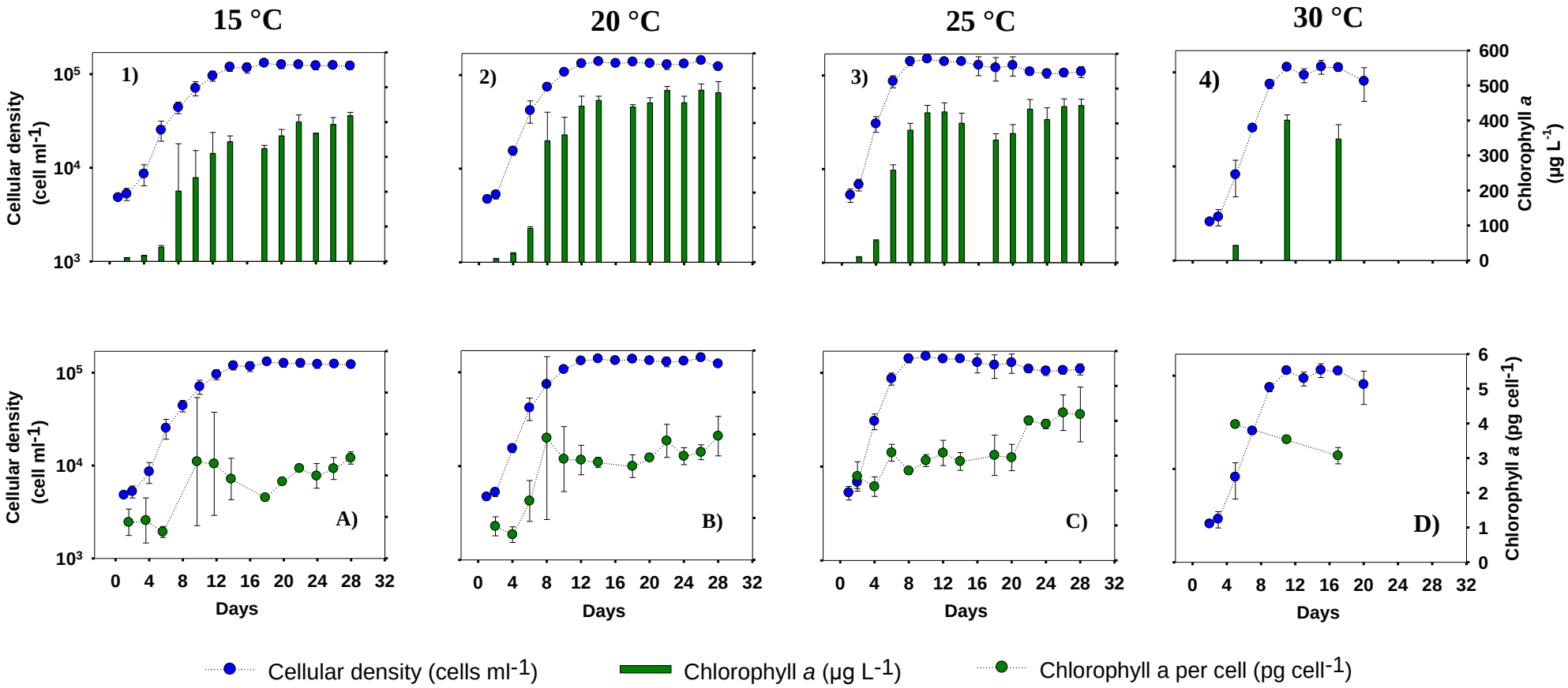

Figure 3.5 Top row shows the average of the increase of chlorophyll a (µg L^{-1}) produced during cell growth (blue dots) of *Amphidinium carterae* cultures maintained between 15 and 30 °C. Bottom row shows changes of chlorophyll *a* content (picograms $cell^{-1}$) in *A. carterae* cells from replicates cultures maintained from 15 to 30 °C. Bars show ± 1 standard difference (30 °C) and standard deviation (15-25 °C) between replicate cultures.

The effect of the increase of sea water temperature, from 5 to 30 °C, on the growth rate and maximum *Fv/Fm* reached in *A. carterae* cultures is compared in Figure 3.6 (A). This figure clearly shows the extent of the increasing growth rate when *A. carterae* was cultured under nutrient replete conditions (F/2 media) with a 12h light:12h dark photoperiod at an irradiance between 35-65 µmoles m^{-2} s^{-1}. Growth rate and *Fv*/*Fm* both increased notably as a result of the rise of growth temperature from 5 to 25 °C, although at temperatures higher than 25 °C *Fv/Fm* decreased while cell growth remained constant (Figure 3.6). The growth rates of *A. carterae* increased linearly (n= 13, r^2= 0.96) from 5 to 25 °C with increasing sea water temperature as shown in Figure 3.6 (B).

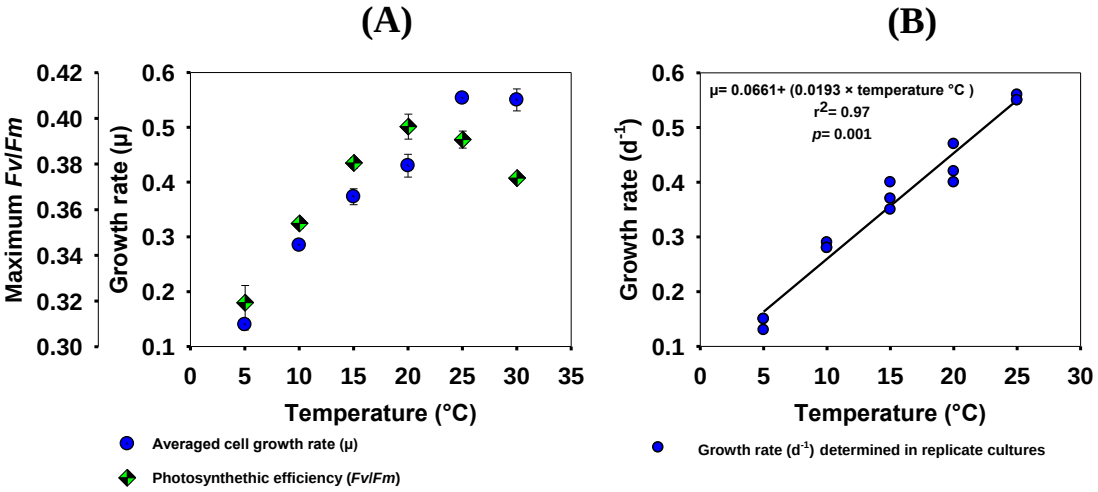

Figure 3.6 (A) Changes in cell growth rate and maximum photosynthetic efficiency (*Fv*/*Fm*) determined in *A. carterae* cultures maintained at growth temperatures between 5 and 30 °C. (B) Relationship between all growth rates and temperature in *A. carterae* cultures grown between 5 and 25 °C.

3.2.2 NO_3+NO_2 and PO_4 uptake

Samples for NO_3+NO_2 and PO_4 measurements were collected every 4-5 days from *A. carterae* cultures and initial concentration were as follows: 980.5 µmol L^{-1} of NO_3+NO_2 and 37.8 µmol L^{-1} of PO_4 (at 5-10 °C); 850.9 µmol L^{-1} of NO_3+NO_2 and 29.9 µmol L^{-1} of PO_4 (at 15-25 °C); and 955.3 µmol L^{-1} of NO_3+NO_2 and 29.4 µmol L^{-1} of PO_4 (at 30 °C). Figure 3.7 shows changes in both NO_3+NO_2 and PO_4 in replicate cultures of *A. carterae* maintained between 5 and 30 °C. Increasing NO_3+NO_2 and PO_4 uptake was observed in replete cultures as *A. carterae* was exposed to elevated temperatures (5-30 °C).

Since the growth of *A. carterae* was drastically reduced at 5 °C, verified as the reduction in cellular division plus low values of *Fv*/*Fm*, low removal rates of NO_3+NO_2 and PO_4 from the media was detected throughout the algal culture, with no evidence of neither NO_3+NO_2 or PO_4 limitation at this temperature (Figure 3.7). In comparison, cells cultured between 10 and 30 °C showed a substantial removal of NO_3+NO_2 and PO_4 from the media (Figure 3.7). Nitrate was non limiting and did not decrease below 500 µmol L^{-1} in any of the cultures. However, PO_4 concentration particularly decreased rapidly in cultures maintained between 15 and 25 °C and it became depleted during the stationary phase.

Maxima cell growth rates at 25 °C coincided with the earliest PO_4 depletion (on day 11 at 25 °C) in *A. carterae* cultures. Even though cells grown between 25 and 30 °C shared similar growth rates (μ= 0.5 d^{-1}), PO_4 depletion was not detected at 30 °C (Figure 3.7).

Figure 3.8 illustrates average plots of cell growth, NO_3+NO_2 and PO_4 changes based on the data from replicate measurements in cultures grown between 5 and 30 °C. Cells cultured between 10 and 30 °C presented lower NO_3+NO_2/PO_4 ratios in comparison to the Redfield ratio, whereas NO_3+NO_2/PO_4 ratios determined at 5 °C were more in agreement with the Redfield ratio (Table 3.3). During the period of maximum nutrient uptake, NO_3+NO_2 was linearly related to PO_4 uptake at 5, 10, 20, and 30 °C (r^2= >0.9, Figure 3.9). The lowest r^2= 0.55 however was from cultures maintained at 25 °C, where both the earliest PO_4 depletion (25 °C) and major NO_3+NO_2 variability (day 11) occurred (Figure 3.8 and 3.9).

Table 3.3 Average NO_3+NO_2/PO_4 uptake ratios determined over the exponential growth in *A. carterae* replicate cultures grown from 5 to 30 °C.

Growth Temperature (°C)	**NO_3+NO_2/PO_4 ratios**
5	16.9 (±2.33)+
10	10.0 (±0.70)+
15	8.2 (±0.35)*
20	7.0 (±0.78)*
25	4.3 (±3.09)*
30	9.7 (±1.48)+

+ =standard difference

*** =standard deviation**

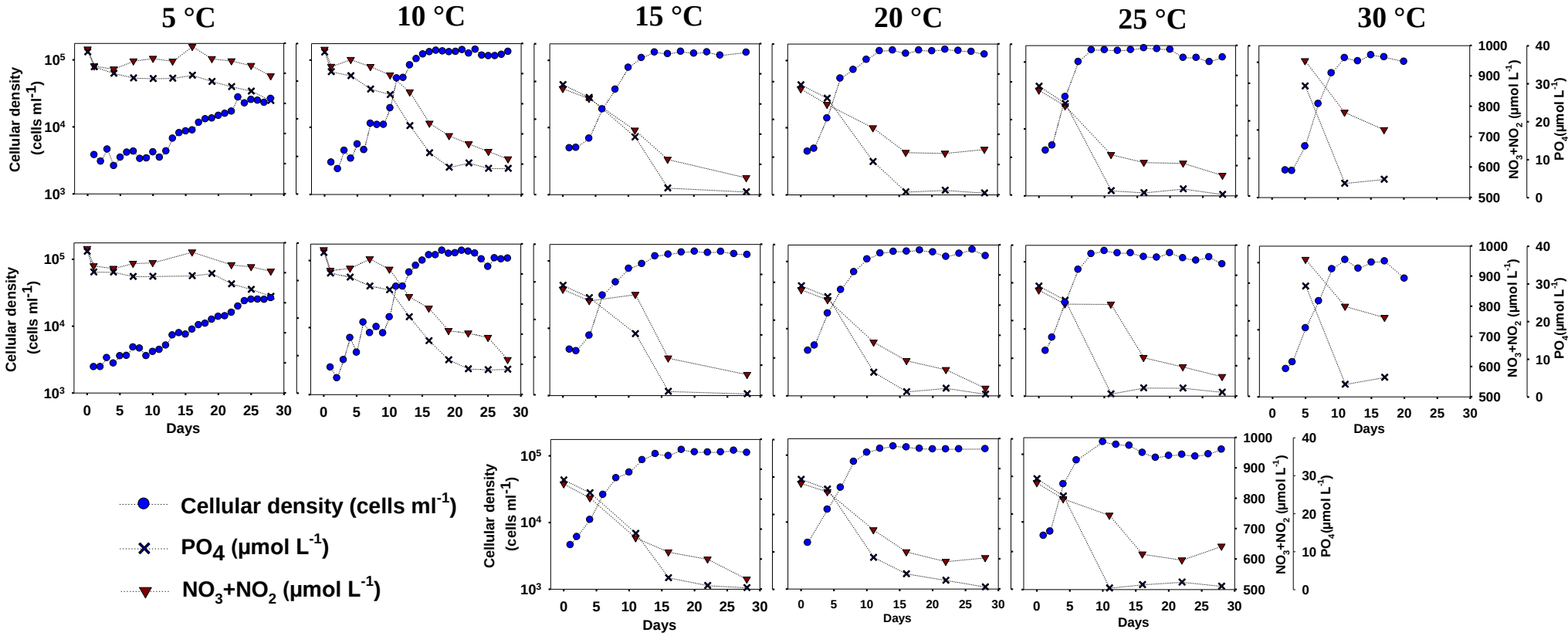

Figure 3.7 NO_3+NO_2 (dissolved inorganic nitrogen as the sum of nitrate+nitrite in µmol L^{-1}) and PO_4 (µmol L^{-1}) uptake during the cellular growth of *Amphidinium carterae* in cultures maintained in F/2 media from 5 to 30 °C. 2 replicates are shown individually for the growth temperatures 5, 10, and 30 °C and 3 replicates are displayed from 15-25 °C.

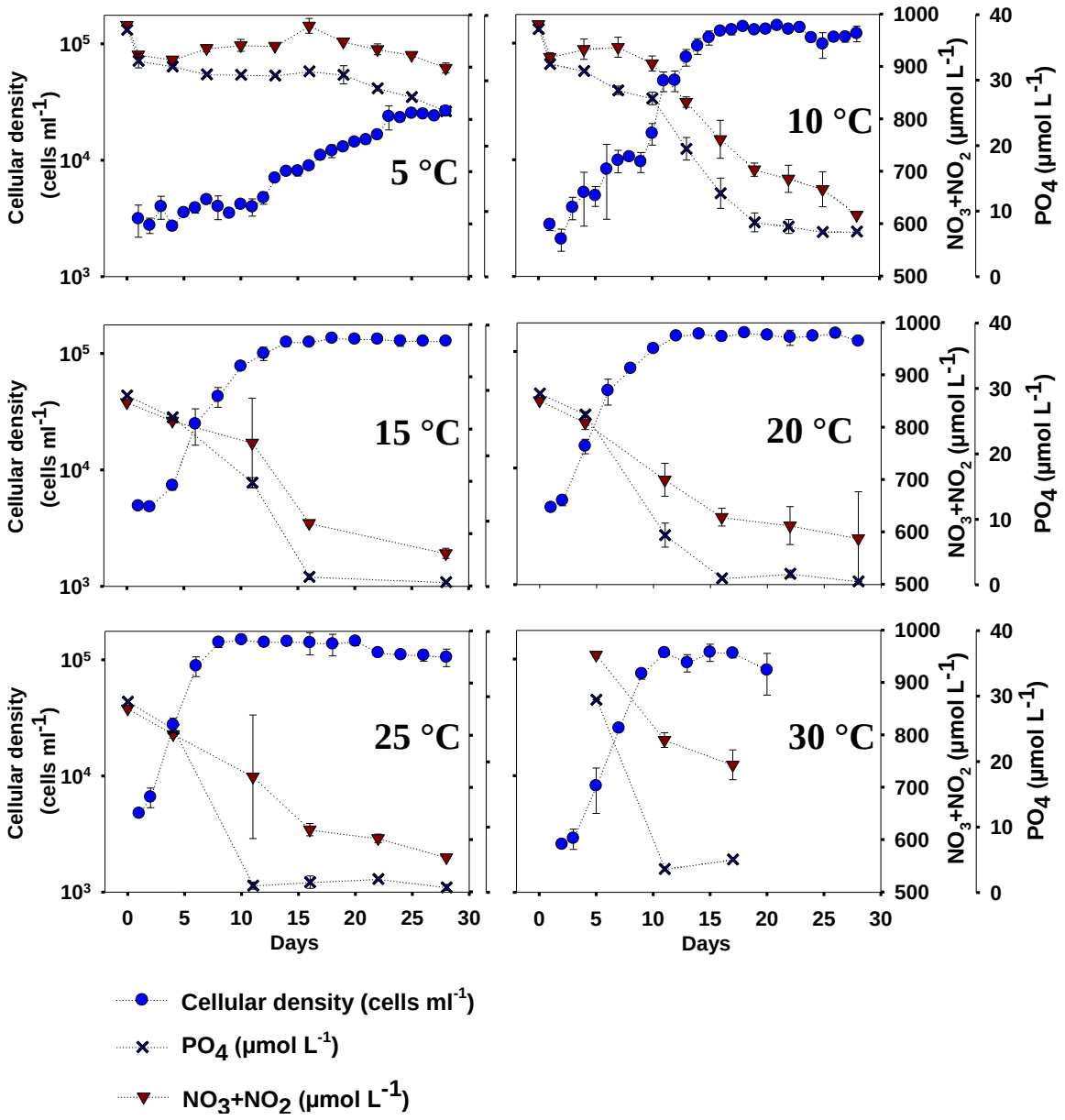

Figure 3.8 Cell abundance and nutrient (NO_3+NO_2 and PO_4) changes in *A. carterae* cultures maintained between 5 and 30 °C. Each plot shows the average of 2-3 replicates per growth temperature. Bars shows ±1standard difference (5, 10, 30 °C) and standard deviation (15-25 °C)..

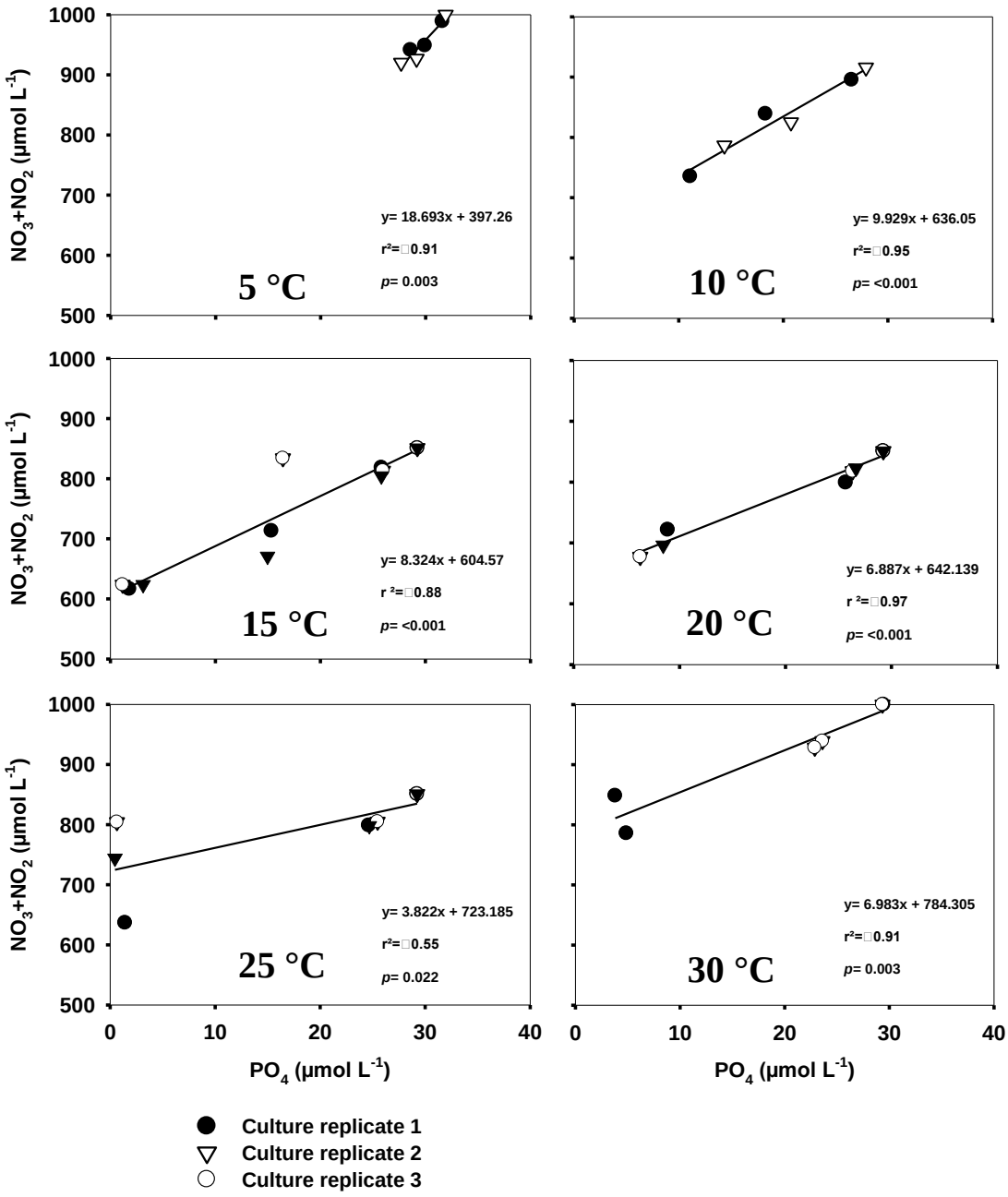

Figure 3.9 Comparison of changes in NO_3+NO_2 and PO_4 during the exponential growth of *A. carterae* at temperatures between 5 and 30 °C. Combined data from replicate cultures were considered to determine the slope and equation for NO_3+NO_2 and PO_4 relationship by linear regression.

3.2.3 Potential toxicity of *A. carterae*

Feeding assays using harpacticoid copepods and the haemolytic activity of chicken red blood cells were used in this study to investigate the potential toxicity of *A. carterae*. In the first bioassay, harpacticoid copepods (*Trigriopus californicus*) were fed on *A. carterae* cells for 9-12 days and copepod mortality at different cell concentrations is shown in Figure 3.10. In the second bioassay, extracts of *A. carterae* cells were inoculated at different concentrations to chicken red blood cells and the haemolytic effect after 18 h was determined by spectrometry (Figure 3.12). Harpacticoid copepods (*Trigriopus californicus*) were able to ingest *A. carterae* cells after being starved for 2-4 days. The mortality of harpacticoid copepods fed on different concentrations of *A. carterae* is shown in Figure 3.10. Three grazing assays were conducted and *A. carterae* concentrations range from 0.56×10^3 to 2.82×10^4 cells ml^{-1} in assay 1; 24.5×10^2 to 122.5×10^3 cells ml^{-1} in assay 2; and $2.2\text{-}111\times10^3$ cells ml^{-1} in assay 3. Six replicates were used at each cell concentration (treatments) and each replicate consisted of 1 harpacticoid copepod per well plus a given number of *A. carterae* cells (Figure 3.10). Control replicates (6) were inoculated with a mixture or non-toxic microalgae (chlorophytes, cyanophytes and diatoms) and mortality at the end of all experiment never surpassed 40% (Figure 3.10).

Chlorophyll *a* fluorescence from copepod guts confirmed that copepods ingested *A. carterae* cells, but copepod samples analyzed under fluorescent light (440nm) did not show evidence of whole cells in copepod guts. Despite a range of cell densities of *A. carterae* applied during the bioassays, maximum mortality of copepods was not necessarily associated with the highest cell density in any of the 3 feeding experiments. Copepod mortality differed between assays. In assay 1 (CB1), overall, copepod mortality fluctuated from 50-100% between day 7 and day 9 amongst cell treatments. In assay 2 (CB2), however, mortality increased towards less concentrated replicates where 83-100% of copepods died between day 4 and day 5, while at the two highest cell concentrations copepod mortality remained between 50 and 83%. Copepod mortality in assay 3 (CB3) reached 50% of dead copepods between day 6 and day 8 with the highest mortality (83%) on day 10 at the second highest cell concentration.

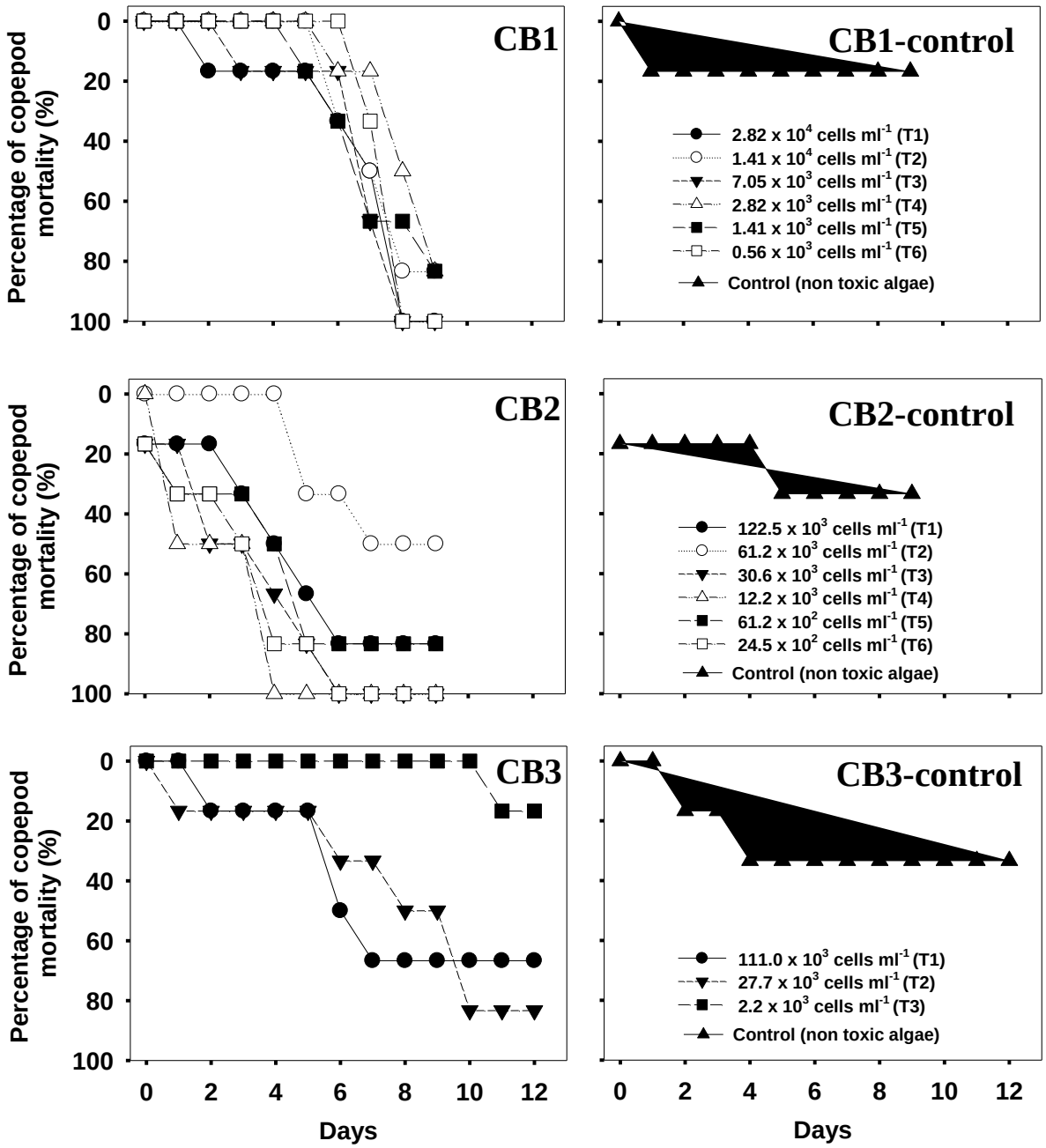

Figure 3.10 Mortality of harpacticoid copepods fed on *Amphidinium carterae* cells at different concentrations. Bioassays CB1, CB2, and CB3 and controls (copepods fed on non-toxic algae). T1-T6 represents different concentrations (treatments) of *A. carterae* cells inoculated. Six replicates (with 1 copepod well^{-1}) were tested for each treatment and data were standardized to percentage of mortality.

A. carterae cells were used to determine haemolytic effects on chicken red blood cells and the haemolytic activity at different cell concentrations is demonstrated in Figure 3.12. The method applied to chicken erythrocytes was calibrated using a known haemolytic compound (Saponin) and the calibration curve obtained by spectrophotometric analysis is presented in Figure 3.11. The spectrophotometric method of analysis showed a significant linear slope between absorbance and saponin concentration between 3 and 6 µg ml^{-1} (r^2= 0.93). Figure 3.11 (A) shows the increase of haemolytic activity as increasing concentration of the standard (Saponin) was added to the erythrocyte suspension, while Figure 3.11 (B) shows a standardized curve of the optical density recorded in Figure 3.11 (A). Algal extracts made with cellular concentrations higher than 0.5 × 10^6 cells ml^{-1} showed >95% erythrocyte cellular lysis after an 18 h incubation period (Figure 3.12 B), whereas 42% cellular lysis was produced by algal extracts made with 0.25 × 10^6 cells ml^{-1}. These high cell concentrations used in the extracts, however, were never observed in *A. carterae* cultures when grown in F/2 at any of the temperatures used during the growth experiments.

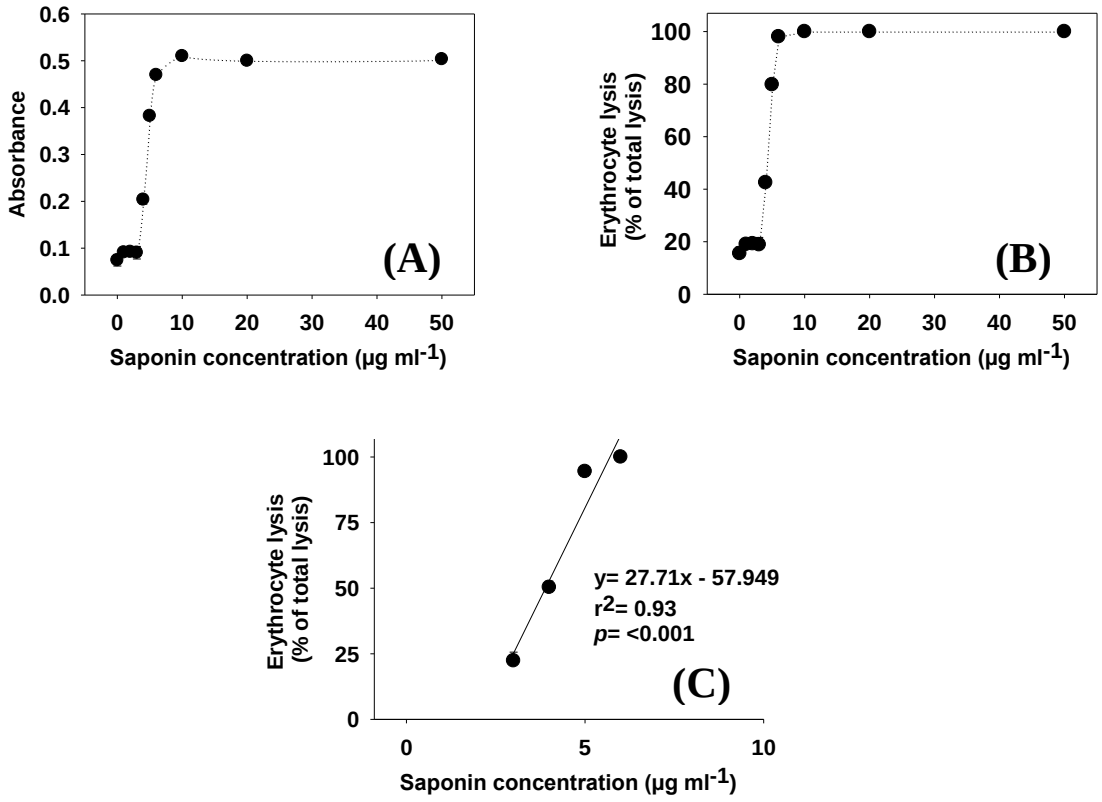

Figure 3.11 Calibration curve for haemolytic assay using Chicken Red Blood Cells. (A) Optical density of lysed erythrocytes quantified at 415nm using different concentrations of the haemolytic compound saponin. (B) Haemolytic activity standardized to percentage of cell lysis at different concentrations of saponin. (C) Lineal regression based on the haemolytic activity of saponin on Chicken Red Blood Cells.

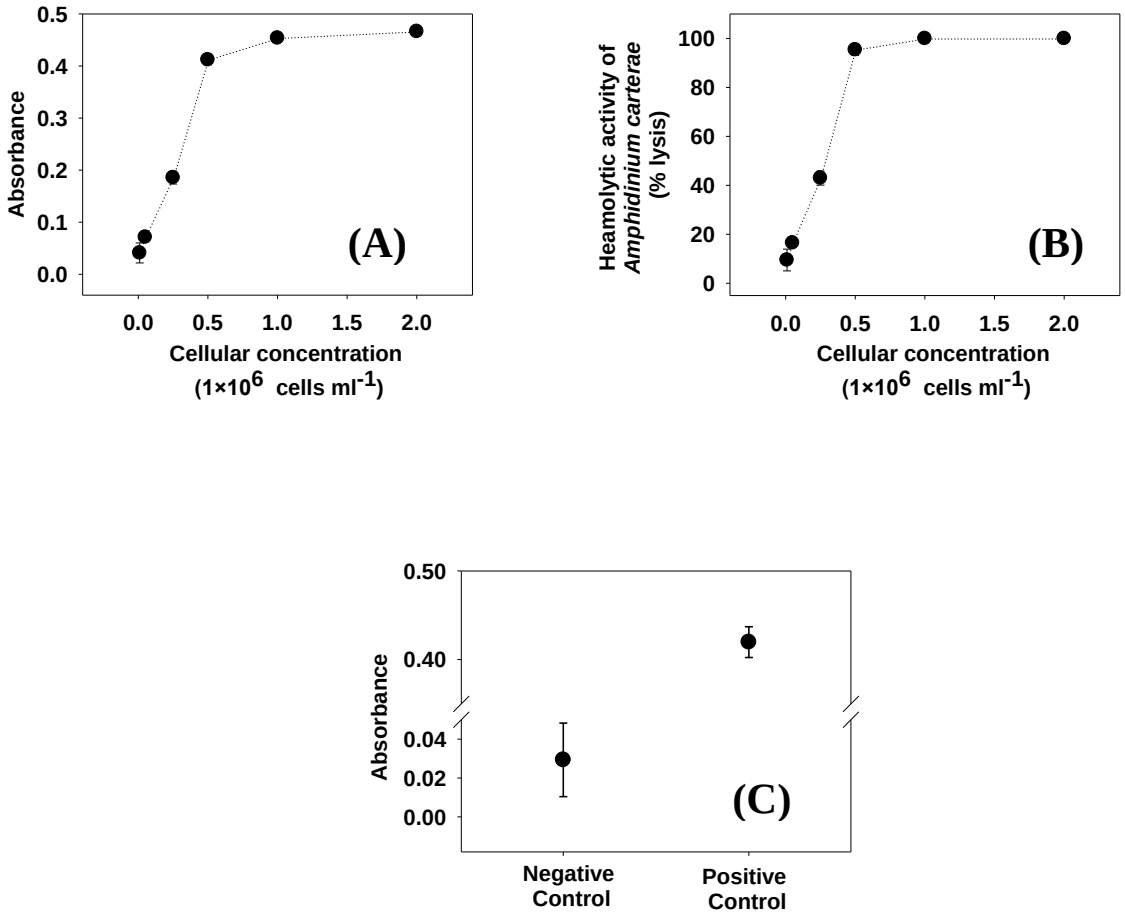

Figure 3.12 Haemolytic activity of Chicken Red Blood Cells produced at different cell concentrations of *Amphidinium carterae*. (A) Optical density of lysed erythrocytes in relation to cell concentration (haemolytic compounds) of *A. carterae*. (B) standardized haemolytic activity (%) of lysed erythrocytes. (C) Comparison of negative and positive control (100% lysis) used to estimate haemolytic activity from *A. carterae*. Bars shows standard deviation.

3.3 Discussion

3.3.1 Effect of temperature on growth rates and *Fv*/*Fm*

Amphidiniuim carterae was successfully isolated from the Fleet Lagoon and grown in monocultures (non-axenic) at a range of temperature from 5 to 25 °C. Growth rates increased with temperature in a linear relationship as has been shown in other studies (Raven and Geider, 1988, Sterner and Grover, 1998, Morton *et al.*, 1992). Table 3.4 shows culture conditions reported in the literature where isolates of *A. carterae* have been grown in cultures and maxima growth rates reported (>0.5-1 d^{-1}) have been found in F/2 medium. In this study, although growth rates (~0.55 d^{-1}) did not show substantial differences between 25 and 30 °C, the former was assumed the optimal growth temperature for *A. carterae* as little variability was encountered in growth rates between replicates.

Morton *et al*. (1992)) showed that a temperature increase above 27 °C produced decreasing growth rates (<0.45 d^{-1}) in *A. carterae*. However, results from the cultures showed that *A. carterae* maintained growth rates of ~0.55 d^{-1} between 25 and 30 °C. These results agree with other reports (Ismael *et al.*, 1999, Morton *et al.*, 1992) that *A. carterae* has an optimum growth at warm sea water conditions (> 25 °C), although Gerath and Chisholm (1989) determined that *A. carterae* exhibited a growth rate of 1 d^{-1} when grown at 20 °C with irradiances of 150-260 µmol m^{-2} s^{-1} in F/2 medium. In contrast to Gerath and Chisholm (1989), lower growth rates (0.40-0.47 d^{-1}) were detected at 20 °C in this and other studies using F/2 medium (Strom and Morello 1998), suggesting that not only temperature, but also light and nutrients influence the optimal growth of *A. carterae*.

Other species of the genus *Amphidinium* have demonstrated high tolerance to temperature, for example, Morton *et al*. (1992) found increasing growth rates of *A. klebsii* at temperatures between 19 and 28 °C, whereas Ismael *et al*. (1999) showed that *A. carterae* grew satisfactorily at a range of temperatures between 20 and 30 °C. Results from the present study showed that *A. carterae* can tolerate a wide range of temperature and proved that the isolate *A. carterae* can grow at lower temperatures (e.g. 5 °C) than those reported in the literature.

Table 3.4 Growth conditions applied during the growth of different strains of *A. carterae*.

Species	Growth rate (μ d^{-1})	Temperature (°C)	Light	Salinity	Max. cell density (cells ml^{-1})	Culture medium	Location of isolation	Reference
Amphidinium carterae	nd	25	56-112 µmol m^{-2} s^{-1}	nd	1.9×10^5	SW plus Provasoli's ES supplement	Okinawa, Japan	Nakajima *et al*., 1981
A. carterae	0.39-0.44	15	70 µmol m^{-2} s^{-1}	30	nd	F/10	Tjøme, outer Oslofjord	Sakshaug *et al*., 1983
A. carterae	(27h generation time)	21	10-70 µmol m^{-2} s^{-1}	nd	nd	F/2	nd	Olson and Chisholm 1986
A. carterae	1	20	150-260 µmol m^{-2} s^{-1}	nd	2×10^5	F/2	nd	Gerath and Chisholm 1989
Amphidinium klebsii	0.45	27	207 µmol m^{-2} s^{-1} (10% sunlight)	33	nd	K	Knight Key, Florida	Morton *et al*., 1992
A. carterae	nd	nd	28 µmol m^{-2} s^{-1}	nd	5.17×10^5	Erdschreiber's soil extract	Mangalore, India	Nayak *et al*., 1997
A. carterae	0.47	nd	nd	30	nd	F/2	nd	Strom and Morello 1998
A. carterae	2.7	25	41-48 µmol m^{-2} s^{-1}	nd	nd	GPM (2.5X)	Alexandria, Egypt	Ismael *et al*., 1999
A. carterae	0.5	20	100 µmol m^{-2} s^{-1}	nd	nd	F/2	nd	Jeong *et al*., 2003
Amphidinium sp.	1	13.6-32.9	100 µmol m^{-2} s^{-1}	38	5×10^5	ASP 7	nd	Lee *et al*., 2003
A. carterae	0.4	16	200 µmol m^{-2} s^{-1}	nd	$4\text{-}5\times10^5$	L1 with boric acid, TRIS buffer, and Na_2CO_3	CCAP, UK	Franklin and Berges 2004
A. carterae	0.13-0.55	5-30	35-70 µmol m^{-2} s^{-1}	28-33	1.5×10^5	F/2	Fleet Lagoon, UK	This study

The isolate of *A. carterae* showed a strong linear relationship with temperature when grown between 5 and 25 °C (0.1 d^{-1} increase every 5 °C), suggesting important biological implications regarding the prediction of the growth response of *A. carterae* in aquatic ecosystems. Montagnes *et al*. (2003) has shown that several epibenthic dinoflagellates including *Amphidinium klebsii* exhibited a linear growth response to temperature.

This study supports Geider *et al.*(1998) and Ismael *et al*. (1999) in that the division rate was a function of temperature, but additionally they emphasized a growth dependence in combination with light intensity and increasing concentrations of nitrate and phosphate. Light deprivation (<3 days) has been shown to inactivate PSII and produce a chlorotic state (loss of autofluorescence) in *A. carterae* cells (Franklin and Berges, 2004). Using continuous light (150 µmol m^{-2} s^{-1}) and a 10h light:14h dark photoperiod with irradiance of 260 µmol m^{-2} s^{-1}, Gerath and Chisholm (1989) reported that *A. carterae* had a growth rate of 1 d^{-1} under both illumination regimes. Here a 12h light:12h dark photoperiod with irradiances of 35-70 µmol m^{-2} s^{-1} was used and maximal growth rates reached 0.57 d^{-1}. This suggests, therefore, that light substantially influences the optimum growth rate of *A. carterae* and it is thought that the isolate used from the Fleet Lagoon might reach higher growth rates at irradiances higher than those used in this study (35-70 µmol m^{-2} s^{-1}). Although a combination of temperature, nutrients, light, and salinity are required for the optimal growth of *A. carterae*, Lakeman *et al*.(2009) has suggested that laboratory strains only represent a single trajectory to understand the ecophysiology of a species and phytoplankton strains should not be considered snapshots of natural algal populations in the environment. Therefore, this study recognizes the importance of aiming future studies at determining the influence of environmental conditions on the growth of natural population of *A. carterae* in the Fleet Lagoon and other shallow marine ecosystems.

Although temperature below 25 °C produced lower growth rates in this study (Geider *et al.*, 1998), there was no evidence of impairment in the growth of *A. carterae* at the lowest temperature (5 °C) as cell yield and *Fv*/*Fm* increased gradually from the onset of exponential growth to the end of the growth experiment (about 1 month later). This suggested that the *A. carterae* strain isolated from the Fleet Lagoon could survive and grow at temperatures below 5 °C. Therefore, as long as nutrients are available and light is not a limiting growth condition, *A. carterae* can grow over a wide range of

temperatures (from 5 to 30 °C). It is worth mentioning that maxima growth rates at 25 and 30 °C were similar despite the differences of *Fv*/*Fm* values recorded in *A. carterae* cells at these temperatures. At 30 °C a strong reduction in *Fv*/*Fm* occurred before the commencement of the stationary phase, while at temperatures < 25 °C, *Fv/Fm* showed a slow decrease throughout the stationary phase. Since the photosynthetic process is highly thermosensitive (Morgan-Kiss *et al.*, 2006), this study confirms that elevated temperatures (>30 °C) produce substantial deficiencies in the photosynthetic apparatus of the *A. carterae* isolate from the Fleet Lagoon. It is thought moreover that the adaptation period of *A. carterae* (< 2 days) in the incubator at 30 °C was not satisfactory and, therefore, a rapid decrease of *Fv*/*Fm* was triggered in the stationary phase despite the temporary and rapid cellular growth of *A. carterae* at elevated temperatures. Therefore, this study suggests that *A. carterae* might reach high growth rates in the Fleet Lagoon if the time and environmental conditions permit the development of biological adaptations in *A. carterae* for growth at elevated temperatures.

Cyst formation is known to occur as a result of stressful growth conditions in the life cycle of some dinoflagellates (Jensen and Moestrup 1997) and it is linked to a sexual stage where two gametes fuse to form a temporary cyst (Laabir *et al.*, 2011). Temporary cysts of *A. carterae* were not found in this study either at low/high temperatures or under nutrient depletion (PO_4 only). However, Barlow and Triemer (1988) found encysted cells of *Amphidinium klebsii* linked to unfavourable growth conditions in a salt water marsh. Thus, it is believed that the growth of the isolated *A. carterae* was particularly linked to an asexual cycle and the lack of gametes in cultures restrained the organism to produce resting forms.

Photosynthetic efficiency (*Fv/Fm*) is widely used for the assessment of the physiological state of phytoplankton (Parkhill *et al.*, 2001, Suggett *et al.*, 2009). Franklin and Berges (2004) reported that *A. carterae* showed optimal growth rate (μ=0.4 d^{-1}) with *Fv/Fm* ranging between 0.5 and 0.6 in cultures maintained at 16 °C under 200 µmol m^{-2} s^{-1} and 18h L:6h D photocycle. However, in the current study higher growth rates were measured despite lower values of *Fv/Fm* at 20 and 25 °C (optimum growth temperature), with lower irradiances. Gerath and Chisholm (1989) determined conclusively that *A. carterae* increased its photosynthetic efficiency, chlorophyll *a* content, and cell volume in response to the light period at increasing irradiance (150-800 µmol m^{-2} s^{-1}), while the opposite occurred during the dark period.

Although they found the same growth rates under different illumination periods (constant light and 10h L:14h D photoperiod), they could not explain what physiological changes allowed *A. carterae* to reach identical growth rates under different light conditions. Since temperature is known to affect photosynthesis and nitrogen assimilation (Geider *et al.*, 1998), Gerath and Chisholm (1989) emphasized the importance of conducting further analysis to comprehend and interpret the physiological response of *A. carterae* under different light regimes and nutrient conditions.

In this study, *A. carterae* was characterized by increasing *Fv/Fm* values with a slight decrease of cell size (data not included) during exponential growth as reported by Ismael *et al* (1999). As expected, this occurred under favorable growth conditions which Gerath and Chisholm (1989) pointed out as an enhancement of *Fv/Fm* while cell division increased. Maximal *Fv*/*Fm* values were linked to the termination of the exponential growth period at all growth temperatures, thus the drop of *Fv/Fm* was indicative of limiting growth conditons (PO_4 limitation, and possibly a CO_2+PO_4 colimitation) regardless of the temperature (Suggett *et al.*, 2009). However, Suggett *et al*. (2009) has suggested that phytoplankton are able to acclimate over long periods to nutrient limitation, so they might overcome photosynthetic growth deficiencies under limiting nutrients.

In tropical waters of the Veracruz reef zone in Mexico, *A. carterae* has been shown to be present throughout the year at temperatures ranging from 24-32 °C and the highest abundance (41,172 cells g^{-1} substrate wet weight) appeared associated with the peak of water temperature (32 °C) during spring. Although maximum cell yield is strongly influenced by water temperature (e.g. between 25 and 30 °C), Lee *et al*. (2003) reported that sustained temperatures above 34 °C resulted in death (lysed cells) of *A. carterae*. On the other hand, *A. carterae* has been negatively correlated with water motion in Johnston Atoll (Pacific Ocean) producing low abundances in lagoon and channel habitats (Richlen and Lobel 2011).

3.3.2 Nutrient consumption and growth temperature

Temperature has a significant influence on nutrient uptake (Geider *et al.*, 1998, Sterner and Grover, 1998, Berges *et al.*, 2002) in phytoplankton cells. *A. carterae* increased NO_3+NO_2 (dissolved inorganic nitrogen) and PO_4 (phosphate) uptake as cells

experienced increasing growth temperatures ranging from 5 to 25 °C. Although average growth rates at both 25 and 30 °C proved to be similar (0.5 d^{-1}), NO_3+NO_2 and PO_4 uptake rates differed at these growth temperatures, with highest uptake rates at 25 °C (optimal growth temperature). Initial cell density at 25 °C (2 times higher than at 30 °C) may possibly explain why at this temperature a higher nutrient uptake occurred in comparison to the highest growth temperature (30 °C).

Several reports have shown that *A. carterae* is a microalgae well-adapted to eutrophic conditions where cell production can change abruptly (up to 10^5 cell ml^{-1}) in a short period (<12 days) during exponential growth (Ismael *et al.*, 1999, Lee *et al.*, 2003, Franklin and Berges, 2004). In this study *A. carterae* showed a rapid cell growth when grown in F/2 medium (NO_3+NO_2: 882 µmol L^{-1} and PO_4: 36 µmol L^{-1}) and exponential growth phase lasted up to 9 days. Initial NO_3+NO_2/PO_4 ratio (24-27) in culture medium (enriched seawater based on F/2 medium) was higher than that of the Redfield ratio and estimations of NO_3+NO_2 and PO4 uptake always showed significantly higher removal of PO_4 than NO_3+NO_2. During the growth of *A. carterae* in F/2 medium, NO_3+NO_2 concentrations never decreased below 500 µmol L^{-1} whereas high PO_4 uptake rates occurred at temperatures > 10 °C, with PO_4 depletion recorded at 15-25 °C. NO_3+NO_2/PO_4 ratios confirmed that *A. carterae* cells produced higher removal of PO_4 over NO_3+NO_2 during exponential growth. Likewise, Li *et al.* (2009) reported dinoflagellate blooms with high NO_3+NO_2:PO_4 ratios in the East China Sea and indicated that the bloom progression caused the development of phosphate limitation, although high NO_3+NO_2 concentrations remained in the water column. In this study, evidently NO_3+NO_2 uptake increased at increasing growth temperatures, but the F/2 media had surplus NO_3+NO_2 that may be excessive to determine the effect of limiting nitrogen conditions on the algal growth of benthic dinoflagellates. In fact, NO_3+NO_2 uptake continued in the cultures even during the stationary phase at all growth temperatures.

High phosphate uptake by *A. carterae* and *A. klebsii* was found to be linked to the light period rather than the dark period by Deane and Obrien (1981). Although phosphate concentration was not measured at cellular level, results from this study agrees with Powell *et al*. (2009) in that temperature facilitated the accumulation of PO_4 in *A. carterae* cells. On the other hand, whereas *A. carterae* increased PO_4 uptake and produced PO_4 depletion in cultures maintained from 15 to 25 °C, PO_4 measurement

from cultures at 30 °C did not indicate depletion despite high growth rates. Since NO_3+NO_2 and PO_4 did show evidence of being limiting nutrients at 30 °C, presumably the availability of inorganic carbon (C_i) or a lack of adaptation of *A. carterae* at high temperatures might have restricted the growth of *A. carterae* (Spijkerman, 2010).

Q_{10} assumes that biological reactions (such as growth rates) respond to temperature based on a range of optimal growth temperatures (Raven and Geider, 1988). Theoretically, under optimal conditions Q_{10} ranges in phytoplankton from 1.8-2.4 considering a 10 °C increase, although some studies assume maximum values up to 3 (Montagnes *et al.*, 2003). This study found maximum Q_{10} values of 2.6 when *A. carterae* grew from 5 to 15 °C whereas lower values (~1.5) were found at higher temperatures, where maxima growth rates were determined. Based on this value, one might expect that *A. carterae* reduced its growth rate at temperatures higher than 15 °C, however, this did not occur. Montagnes *et al* (2003) has explained that the use of Q_{10} - might be inappropriate particularly when algal growth responses are linear. To elude incorrect interpretations of the effect of temperature on the growth of benthic dinoflagellates, this study considers that Q_{10} is not an accurate predictor of temperature-growth responses for phytoplankton.

3.3.3 Toxicity of *A. carterae*

A. carterae has been recognized as a toxin-producing microalgae in several studies (Mandal *et al.*, 2011, Kobayashi and Tsuda, 2004, Ismael *et al.*, 1999, Ignatiades and Gotsis-Skretas, 2010) and the structure of about 20 potential toxins (amphidinolides) linked to the genus *Amphidinium* are described by Daranas *et al*. (2001a) and Kobayashi and Tsuda (2004). Due to a lack of an optimized method to determine *Amphidinium* toxins by LC/MS-MS being available, secondary metabolites synthesized by the isolate *A. carterae* could not be quantitatively measured. However, bioassays using harpacticoid copepods and chicken red blood cells demonstrated that *A. carterae* produced mortality and haemolysis, respectively, and this suggested a potential toxicity of the strain.

Copepods fed on *A. carterae* showed some mortality but results did not produce a clear relationship between mortality and cell concentration. Likewise, Baig *et al*. (2006) using wild (2.5×10^2, 10^3, 10^5 cells ml^{-1}) and cultured (7.2×10^2, 10^3, 10^4 cells ml^{-1}) *A. carterae* cells failed to demonstrated a significant mortality effect on the brine shrimp

A. salina in feeding experiments. However, a number of studies have shown the opposite. For example, Ismael *et al*. (1999) has shown that *A. carterae* produced toxins that killed the brine shrimp *Artemia salina* after *A. carterae* cells collected from exponential growth period were ingested by this crustacean, although these authors commented that the senescence period produced loss of toxicity in *A. carterae* cells. Rhodes *et al*. (2010) determined a potent toxicity from *A. carterae* which at high doses killed mice within minutes, but they did not provide evidence of the chemical compound involved in the mortality. Likewise, Nakajima (1981) demonstrated that *A. carterae* caused ichthyotoxicity with concentrations of 100 ppm. In this study, *A. carterae* cells were sampled towards the end of the exponential phase and possibly this may have influenced the production of chemical compounds from *A. carterae*, particularly in feeding experiments with copepods.

Increases in cell concentration of *A. carterae* did not provide evidence of increasing copepod mortality rates. Crustacean exhibited different mortality rates between experiments even when exposed to fairly similar cell concentration. Although copepods were starved for 3-5 days, 100% of copepod mortality did not occur immediately after copepods were exposed to *A. carterae*. Feeding experiments determined that 100% of mortality occurred from day 4-6 in assays lasting less than 10 days. However, in the third bioassay 100% of copepod mortality was not detected despite using the second greatest cell concentration (111×10^3 cells ml^{-1}) during all copepod experiments. Considering that controls recorded the lowest mortality, this study provided evidence that the isolate *A. carterae* used in the feeding experiment might have produced potential toxins causing copepod mortality.

It is known that copepods possess highly sensitive and specific chemoreceptive and selective abilities that influence their grazing behavior (Teegarden and Cembella, 1996) while some might exhibit a substantial tolerance to ingestion of toxic algae (Senft *et al.*, 2011). Although chlorophyll *a* fluorescence from copepod guts confirmed ingestion of *A. carterae* cells, it is possible that copepods might have ingested a low number of *A. carterae* cells or they tolerated the potential toxins produced by *A. carterae*.

Haemolytic assays were conclusive in that *A. carterae* did produce hemolysins as reported by Nakajima *et* al. (1981) and Echigoya *et al*. (2005), however it is

important to mention that cell concentrations that produced >50% of lysed cells are rarely detected in the environment.

3.4 Conclusions

Increasing growth temperatures caused a proportional increase in the growth rates of the epibenthic dinoflagellate *Amphidinium carterae*. The effect of temperature on growth rates showed a strong linear relationship when growth temperature increased from 5-25 °C. In addition, this study showed that *A. carterae* reached optimum growth rates at elevated temperatures (25-30 °C). These results are in agreement with predictions where possibly the effect of climate change will increase the growth of HABs as determined in this study for the harmful algae *A. carterae*.

This study has shown that *A. carterae* is a fast growing dinoflagellate at a wide range of temperatures, when exposed to high nutrient concentrations (F/2 medium) and optimum irradiances. Therefore, results from this study suggest that *A. carterae* could produce a HAB in a wide range of environmental conditions encountered in many coastal ecosystems, most likely in eutrophic waters.

Due to the effect of increasing growth temperatures, high removal of nutrients caused increasing cell concentrations of *A. carterae* in cultures. NO_3+NO_2/PO_4 uptake ratio demonstrated that *A. carterae* showed a high removal and preference of PO_4 over NO_3+NO_2 for growth. Despite PO_4 became a limiting growth factor, *A. carterae* continued to take up NO_3+NO_2 throughout the stationary phase of growth.

A. carterae cell growth did not appear to produce NO_3+NO_2 limitation in culture media despite both higher nutrient uptake (NO_3+NO_2 and PO_4) and higher cell concentrations at increasing growth temperatures (>10 °C). Since there was a lack of coincidence between the initiation of the stationary phase and PO_4 limitation (possibly ascribed to the frequency of sampling), this study estimates that the availability of inorganic carbon (CO_2) influenced in first case nutrient stress in *A. carterae* cells followed by PO_4 limitation.

A. carterae cells applied in bioassays caused mortality in harpacticoid copepods and haemolysis in chicken red blood cells. There was, however, little evidence that the concentration of *A. carterae* was proportional to mortality rates in harpacticoid copepods. However, this study confirmed that *A. carterae* produced hemolysins causing a haemolytic effect dependent on cell abundance of *A. carterae*.

CHAPTER 4

4.1 Introduction

4.1.1 The epibenthic dinoflagellate *Prorocentrum lima*

The epibenthic dinoflagellate *Prorocenturm lima* is widely recognized as a toxin-producing microalgae and is commonly encountered in the euphotic zone of tropical and temperate coastal waters associated with seaweeds and sediments (Maranda *et al.*, 1999, Foden *et al.*, 2005, Maranda *et al.*, 2007b, Faust *et al.*, 2008, Rhodes *et al.*, 2010). Furthermore, *P. lima* can be encountered in digestive glands of filtering organisms such as mussels (Lawrence *et al.*, 2000, Levasseur *et al.*, 2003), oysters, and clams (Vale and de Sampayo, 2002, Naves *et al.*, 2006). This microalga develops resting forms (cysts) that settle in marine sediments where they excyst as a result of suitable growth conditions that permit the growth of *P. lima* cells in the environment. *P. lima* has been reported to reach high cell abundance and become a dominant species amongst other benthic dinoflagellates (Marasigan *et al*., 2001).

Numerous studies have demonstrated that benthic microalgae communities, under certain conditions, can increase the primary productivity of aquatic systems (Nadaoka *et al.*, 2008) with benefits for higher trophic levels. *P. lima* has caused noxious effects (e.g. intoxication, mortality) in coastal regions worldwide due to the accumulation of cellular biotoxins in many different organisms (FAO 2004, Hallegraeff, 2006).

Amongst toxigenic dinoflagellates from the marine environment, *P. lima* is a contributor to the human illnesses related to the syndrome ciguatera fish poisoning (CFP) (FAO 2004). The CFP name was formerly introduced to describe an intoxication caused by the ingestion of coral reef fish that accumulated ciguatoxins from toxic microalgae. A diverse range of phycotoxins are also known to directly interact and bioaccumulate in the environment in a number of shellfish organisms (Katırcıoğlu et al 2004). Toxicity in molluscs depends on both the concentration of the different toxins accumulated in soft tissues and the potency of the toxins (Fernández *et al.* 2003).

Phycotoxins are natural products that vary in nature in terms of chemical structure and sometimes they are ascribed to various human syndromes after toxin ingestion.

DSP-toxins are produced by some species from the genera *Prorocentrum* and *Dinophysis* (Windust *et al.*, 1996, Suzuki *et al.*, 2004). In the last few decades, diverse studies have provided evidence that the toxin-producing *P. lima* is able to synthesize various DSP-toxins under diverse environmental conditions (Bravo *et al.*, 2001, Nascimento *et al.*, 2005, Pistocchi *et al.*, 2010, Varkitzi *et al.*, 2010). Bravo et al. (2001) demonstrated significant variability in nineteen *P. lima* strains in terms of both toxin production and toxin profiles. Likewise, Morton and Tindall (1995) compared seventeen *P. lima* strains isolated from Heron Island, New Zealand, and found high variability in toxin content (okadaic acid and methyl-okadaic acid) between strains.

DSP-toxins are divided into 4 main categories, namely okadaic acid (OA) and dinophysistoxins (DTX1, DTX2), yessotoxin (YTX), pectenotoxins (PTX), and azaspiracids (AZAs), toxin groups in which there are a number of derivatives although DTX-toxins are known to derive from OA (Quilliam 2003). The most common toxins reported from *P. lima* cells are OA and DTX toxins (Bravo *et al.*, 2001). The molecular structure of DSP toxins has been revised by Blanco et al. (2005) and Quilliam (2003). OA and DTX are potent phosphatase inhibitors that can alter a diverse range of cellular processes in eukaryotic cells. For example OA and DTX can interfere with serine/threonine protein phosphatases (Windust *et al.*, 1996, Mountfort *et al.*, 2001) which are regulatory enzymes affecting growth and replication of vertebrate cells (Sugg and VanDolah, 1999). OA and DTX toxins are known to cause diarrhoea in humans and the triggering mechanisms include: 1) hyperphosphorylation of proteins that control sodium secretion by intestinal cells, or 2) increased phosphorylation of cytoskeletal or junctional moieties that regulate solute permeability, resulting in passive loss of fluids (FAO 2004). *P. lima* toxins (OA and DTX) have also demonstrated allelopathic (Granéli and Hansen, 2006) and tumor promoting activity (Suganuma *et al.*, 1992, Fujiki and Suganuma, 1999).

Several methods have been developed to determine DSP-toxins from biological samples. The advent of LC-MS/MS has permitted accurate determination and

quantification of several molecules related to *P. lima* toxins (Vale *et al.*, 2009). Some *P. lima* toxins can undergo enzymatic conversion in nature (Vale and de Sampayo, 2002), therefore a procedure is applied to restrict chemical changes in toxins. Fernández *et al.* (2003) commented that many toxins undergo transformation in organism in which they accumulate due to varying conditions of pH and redox potential. More recently methods have been introduced to hydrolyse toxins that experience chemical conversion (Mountfort *et al.*, 2001, Vale *et al.*, 2009).

Climate change effects on the marine environment are predicted to cause increases in sea water temperature. This has prompted the hypothesis that some benthic dinoflagellates such as the genera *Prorocentrum* will increase their growth rates and possibly become more toxic (Vale *et al.*, 2009). However, Richlen and Lobel (2011) suggested that the influence of environmental parameters on population dynamics is not well comprehended for many toxigenic benthic dinoflagellates (e.g. *Gambierdiscus*, *Prorocentrum*, *Coolia*). Recent reports have suggested that harmful blooms are increasing worldwide (Hallegraeff, 2006) and also suggested that the occurrence of deleterious episodes from benthic toxic microalgae (*Prorocentrum* and *Ostreopsis*) has increased compared to planktonic species (Aligizaki and Nikolaidis, 2006). As for the effect of temperature on phytoplankton dynamics, Aligizaki *et al.* (2009) investigated the distribution of *Prorocentrum* species in the North Aegean waters and found several new species from a tropical and subtropical origin suggesting that the biogeographical distribution of some dinoflagellates is expanding, particularly towards northern regions (Aligizaki *et al.*, 2009).

Both toxic and non-toxic dinoflagellates are known to share the same niche in the marine environment. In coastal areas around the world, *Prorocentrum lima* have been found in assemblages with other harmful benthic microalgae (Okolodkov *et al.*, 2007, Aligizaki *et al.*, 2009, Rhodes *et al.*, 2010, Richlen and Lobel, 2011). Aligizaki et al. (2009) reported 5 epiphytic *Prorocentrum* species (*P. borbonicum*, *P. emarginatum*, *P. levis*, *P. lima*, and *P. rhathymum*) co-inhabitants of coastal Greek waters; and Ignatiades and Gotsis-Skretas (2010) found 16 harmful microalgae associated with the occurrence of harmful blooms in Greek waters, of which *P. borbonicum*, *P. levis*, *P. lima*, and *P. rhathynamum* are toxin-producing species while *P. arcuatum*, *P. obtusidens*,

P. redfeldii, *P. micans*, *P. minimum*, *P. dentatum*, and *P. emarginatum* are high biomass producers. Rhodes *et al*. (2010) isolated the toxigenic *Gambierdiscus australes*, *Coolia monotis*, *Amphidinium carterae*, *P. lima*, *P.* cf. *maculosum*, and some *Ostreopsis* species from calcareous seaweed in the Cook Islands, Australia; Laza-Martinez et al. (2011) isolated 16 strains of the genera *Coolia* (*C. monotis*, *C. canariensis*, and *C. malayensis*) , *Ostreopsis*, and *Prorocentrum* (*P. rhathymum*, *P. mexicanum*, *P. emarginatum*, *P. fukuyoi*, and *P. lima*) from the south-eastern Bay of Biscay. This is particularly relevant given that during HAB outbreaks several harmful algae can contribute to the toxin burden with severe consequences in the environment.

4.1.2 Taxonomical description of *P. lima* (Ehrenberg, 1860) Stein, 1878

Prorocentrum lima is a thecate oblong to ovate cell, composed of two smooth valves attached to each other, with the broadest part situated at the middle of the posterior end and then narrowing gradually towards the anterior end (Laza-Martinez *et al.*, 2011). Figure 4.1 shows light microscopy and SEM microphotographs of cultured cells of the *P. lima* strain isolated from the Fleet Lagoon. In lateral view, cells are lenticulate to ellipsoidal (Taylor *et al*. 2003), and compressed (Nagahama *et al.*, 2011). Each valve possesses from 90 to 100 round to oblong pores and, in the cell periphery, marginal pores are typically all around the cell (Nagahama *et al.*, 2011). *P. lima* valves vary in length from 35 to 57 µm and width from 21 to 46 µm, with a length/width ratio between 1.03-2.05 (Nagahama *et al.*, 2011). Under light microscopy, a round and large pyrenoid can be seen in the middle of the body, and numerous chloroplasts are also present and vary from cell to cell (Figure 4.1, Aligizaki *et al.*, 2009).

P. lima resembles to some *Prorocentrum* species (*P. marinum* Dodge & Bibby; 1973; *P.hoffmannianum* Faust, 1990; *P. foraminosum* Faust, 1993b) and its identification can be complicated (Faust, 1991) when light microscopy is used. Based on the world register of marine species (WORMS database, 2012), the benthic dinoflagellate *P. marinum* (Dodge and Bibby, 1973) is recognized as a synonym of *P. lima*. However, while the scientific name of *P. lima* has been accepted (WORMS database) and its morphological characterization has been explain in detail in different studies (Taylor *et al*. 2003; Aligizaki *et al*. 2009), *P. marinum* has not been confirmed as a species within the genus *Prorocentrum* (WORMS database, 2012). *P. foraminosum* is incorrectly identified as *P. lima* but the former can be distinguished from *P. lima* by

its oblong to oval cell shape, larger cell size (46-66 µm long and 31-42 µm wide), and different sized flagella and auxiliary pores (Faust 1993). In addition, unlike *P. lima*, *P. foraminosum* has not been recognized as a toxin-producing species. In terms of distribution and habitat, *P. marinum*, *P.hoffmannianum*, and *P. foraminosum* are mainly encountered in tropical coastal areas and cells are commonly associated with floating detritus, while *P. lima* cells can be widely found in diverse benthic habitats (e.g. sediments, macroalgae) from tropical to temperate coastal regions (Marine Species Identification of Harmful Marine Dinoflagellates, 2012).

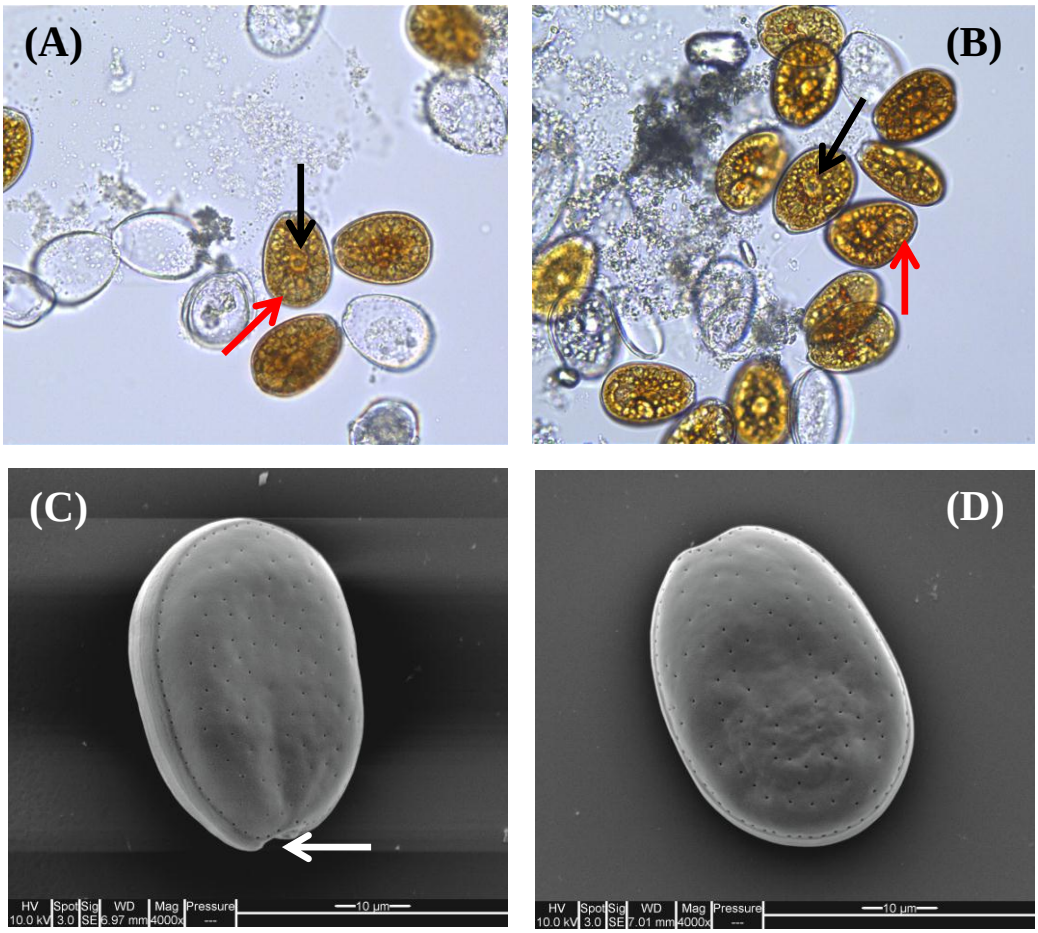

Figure 4.1 Vegetative cells of *Prorocentrum lima* (yellow-brown) from a strain isolated from the Fleet Lagoon, Dorset. (A-B) Live cells (yellow-brown) and empty valves (clear theca) of *P. lima* viewed under light microscopy. Black arrows indicate the large pyrenoid while red arrows point to the nucleus. (C-D) Microphotographs of *P. lima* cell taken by SEM (scanning electron microscopy), with distinctive pore arrangement on theca. White row indicates the V-shape anterior indentation (C).

4.2 Results

4.2.1 Temperature and cell growth of *Prorocentrum lima*

The toxigenic dinoflagellate *Prorocentrum lima*, isolated from shallow waters from the Fleet Lagoon, Dorset, was cultured *in vitro* in F/2 media and changes in cell abundance, growth rate (μ, day^{-1}), photosynthetic efficiency (*Fv*/*Fm*), chlorophyll *a*, nutrient uptake (both dissolved inorganic nitrogen (NO_3+NO_2) and phosphate (PO_4), and toxin production (both okadaic acid, OA, and dinophysistoxin1, DTX1) were monitored at growth temperatures between 5 and 30 °C. Additionally, changes in pCO_2 were inferred from *P. lima* cultures incubated at 15 and 20 °C. Finally, bioassays were used to estimate both the effect of grazing on toxin production and the potency of *P. lima* toxins towards brine shrimp (*Artemia salina*) and harpacticoid copepods (*Tigriopus californicus*).

P. lima was successfully grown in the laboratory at a range of growth temperatures using clonal cultures with F/2 media where a single *P. lima* strain was used for all growth experiments. Cultures were grown at different times of the year (see methods chapter). Growth experiments were initiated in 1 L glass flasks with the following cell densities: 0.8-1.4×10^2 cells ml^{-1} (cultured in 2 replicate cultures both kept independently at 5, 10, and 15 °C), 5.8-9.3×10^2 cells ml^{-1} (incubated in 2 replicates both kept independently at 20 and 25 °C), and finally ~2.2 ×10^2 cells ml^{-1} (grown in 2 replicates at 30 °C).

Temperature produced a regulatory effect on the growth rates and growth phases in *P. lima* cultures. Figure 4.2 shows the increase of cells and changes of *Fv/Fm* in *P. lima* cultures grown at temperatures between 5 and 30 °C. The growth of *P. lima* was substantially reduced at 5 °C, however *P. lima* cells exhibited an increasing cell abundance when cultured from 10 to 30 °C (Figure 4.2). *P. lima* cultures incubated between 5 and 15 °C had a lag phase which shortened as growth temperature increased. Consequently, the longest lag phase occurred at 5 °C (lasting between 35-40 days), followed by 10 °C (~25 days) and then 15 °C (~15days, Figure 4.2). Cells grown in cultures at temperatures higher than 20 °C did not exhibit a detectable lag phase given the sampling interval in this study (Figure 4.2).

Fv/*Fm* was notably affected at 5 °C as at this temperature *P. lima* showed the lowest values of *Fv/Fm* in comparison to higher growth temperatures (Figure 4.2). However, some increase in values of *Fv*/*Fm* were determined at 5 °C as soon as the exponential phase commenced, while at higher temperatures (10-30 °C) *Fv*/*Fm* increased constantly until the end of the exponential phase (Figure 4.2). The positive response of *Fv*/*Fm* during the exponential growth was notably in accordance with the increase in cell density at all growth temperatures. However, a substantial decrease of *Fv*/*Fm* occurred as soon as the exponential cell growth finished which, as a result, delimited both the termination and beginning of the exponential and stationary phase, respectively (Figure 4.2). Table 4.1 lists the growth kinetics of the *P. lima* strain including the exponential growth period (in days), changes in cell density, and growth rates determined in replicate cultures maintained at temperatures between 5 and 30 °C.

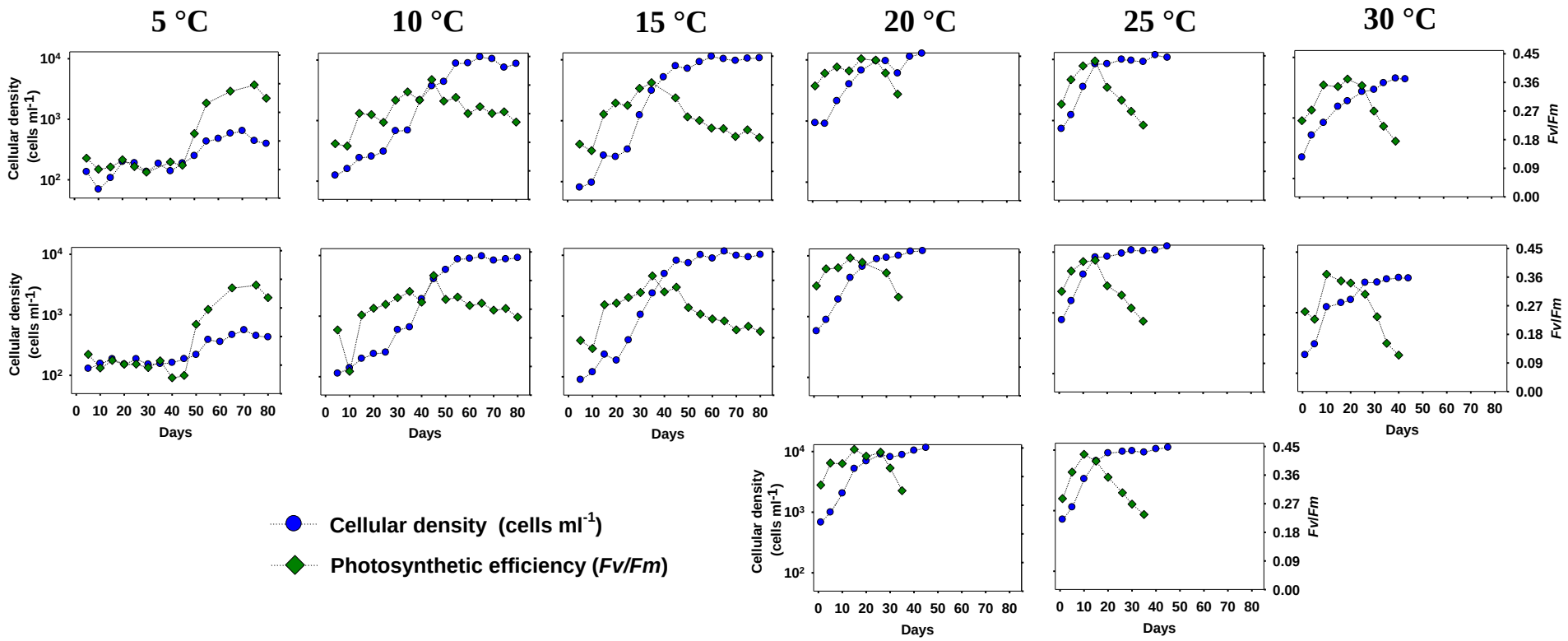

Figure 4.2 Changes in cellular density and photosynthetic efficiency (*Fv*/*Fm*) during the cellular growth of *Prorocentrum lima* in cultures maintained in F/2 media from 5 to 30 °C. 2 replicates are shown individually for the growth temperatures 5-15 and 30 °C while 3 replicates are displayed at 20 and 25 °C.

Table 4.1 Growth kinetics of *Prorocentrum lima* during exponential growth in cultures maintained at growth temperatures between 5 and 30 °C.

Temperature (°C)	Time of exp. growth (days)	Exp. change in cell density ($\times 10^2$ cells ml^{-1})	Growth rate (μ, d^{-1})
5	25	0.14-0.58	0.06
	25	0.16-0.47	0.04
10	20	0.69-88.3	0.12
	20	0.66-87.4	0.13
15	15	0.34-52.1	0.18
	15	0.40-49.7	0.17
20	15	0.91-67.6	0.13
	15	0.58-43.6	0.14
	15	0.68-52.2	0.14
25	15	0.73-84.3	0.18
	15	0.76-82.4	0.17
	15	0.71-66.4	0.17
30	15	0.22-15.4	0.15
	15	0.20-14.6	0.12

Although cell production differed over the exponential phase in cultures maintained between 15 and 30 °C, similar periods of exponential growth (~15 days) in *P. lima* cultures were found at growth temperatures between 15-30 °C (Table 4.1). The effect of temperature on cell production suggested increasing growth rates from 5 to 15 °C (Table 4.1). Measured growth rates at both 15 and 25 °C were similar (average μ=0.17 d^{-1}), but the growth rate determined at 20 °C proved to be unexpectedly lower (average μ=0.13 d^{-1}) in comparison to the rates cited previously (Table 4.1). *P. lima* showed both decreasing growth rates and an earlier drop of *Fv/Fm* at the highest growth temperature (30 °C, Figure 4.2-4.3).

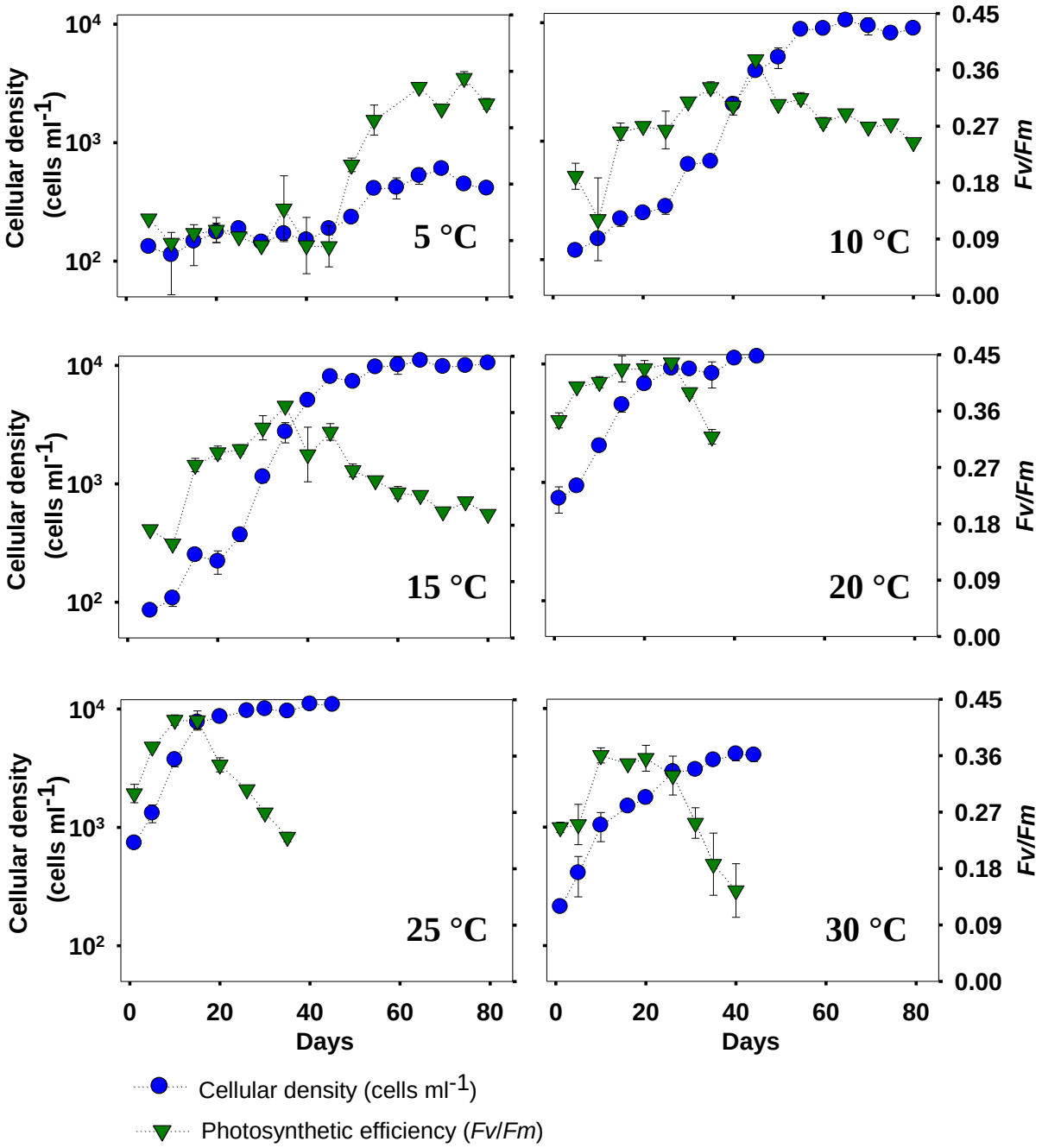

Figure 4.3 Changes in average cell density and fluctuation of *Fv*/*Fm* in *P. lima* cultures maintained in F/2 media at growth temperatures between 5 and 30 °C. Bars shows ±1 standard difference (at 5, 10, 15, and 30 °C) and standard deviation (at 20 and 25 °C).

At the end of the stationary phase, maxima cell abundance averaged in growth cultures differed by an order of magnitude as follows: 5×10^2 cells ml^{-1} (at 5 °C), ~10^4 cells ml^{-1} (at 10-25 °C), and ~4×10^3 cells ml^{-1} (at 25 °C, Figure 4.3). Figure 4.3 presents a summary of average growth and *Fv*/*Fm* for each growth temperature after averaging data shown in replicate cultures from Figure 4.2. Figure 4.4 compares both cell growth

rates (μ) and maximum values of *Fv*/*Fm* against temperature to illustrate the effect of rising sea water temperature on these parameters during the growth of *P. lima* cells. Growth rates of *P. lima* and temperature showed a significant linear relationship (r^2= 0.97) between 5 and 15 °C (Figure 4.4). The highest Q_{10} value was 4.3 (5-15 °C) followed by1.2 (10-20 °C) and 1.0 (15-25 °C and 20-30 °C). Maximum values of *Fv*/*Fm* increased in cultures incubated from 5 to 20 °C, then slightly decreased at 25 °C with a further substantial decrease in maxima *Fv*/*Fm* at 30 °C (Figure 4.4). Although both growth and maximum values of *Fv/Fm* increased with temperature, a lack of coincidence occurred between both the highest growth rate (at15°C) and highest *Fv*/*Fm* (at 20 °C, Figure 4.4).

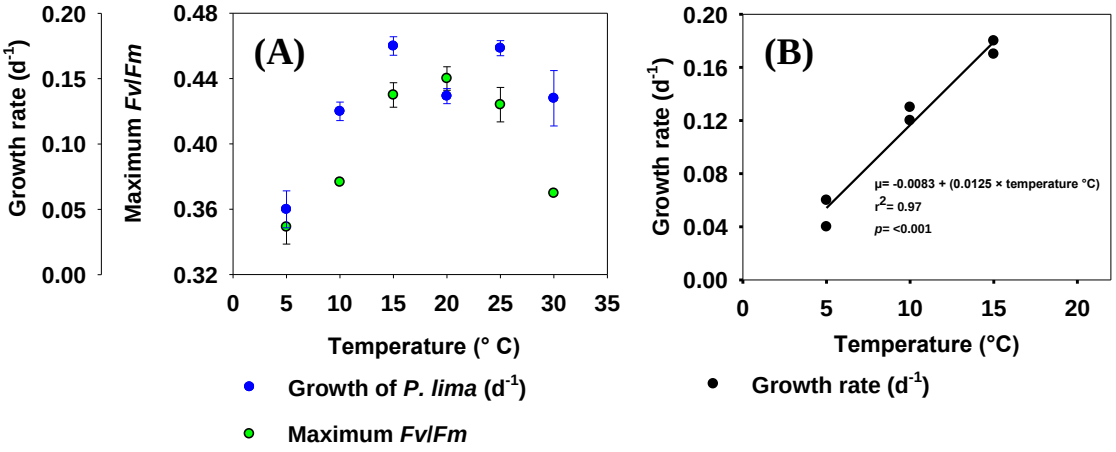

Figure 4.4 (A) Average cell growth rates and maximum photosynthetic efficiency (*Fv*/*Fm*) determined in *P. lima* cultures incubated at growth temperatures between 5 and 30 °C. **(B)** Linear relationship between temperature and cell growth rate between 5 and 15 °C.

Chlorophyll *a* concentration in cultures of *P. lima* increased during the exponential growth phase (Figure 4.5). The increase in growth temperature from 5 to 20 °C, caused both the increase and maximum production of chlorophyll *a* as follows: 1.2 μg L^{-1} (±0.24, at 5 °C), 112.7 μg L^{-1} (±3.25 at 10 °C), 123.8 μg L^{-1} (±5.94 at 15 °C), 332.7 μg L^{-1} (±19.6 at 20 °C). However, at higher temperatures (>20 °C) maxima chlorophyll *a* concentration were reduced, i.e. 288.6μg L^{-1} (±14.6) at 25 °C and 182 μg L^{-1} (±82.0) at 30 °C (Figure 4.5).

Chlorophyll *a* per cell showed some fluctuation in *P. lima* cultures (Figure 4.6) with values generally between 20 and 40 pg $cell^{-1}$ but showing no obvious pattern with growth temperature.

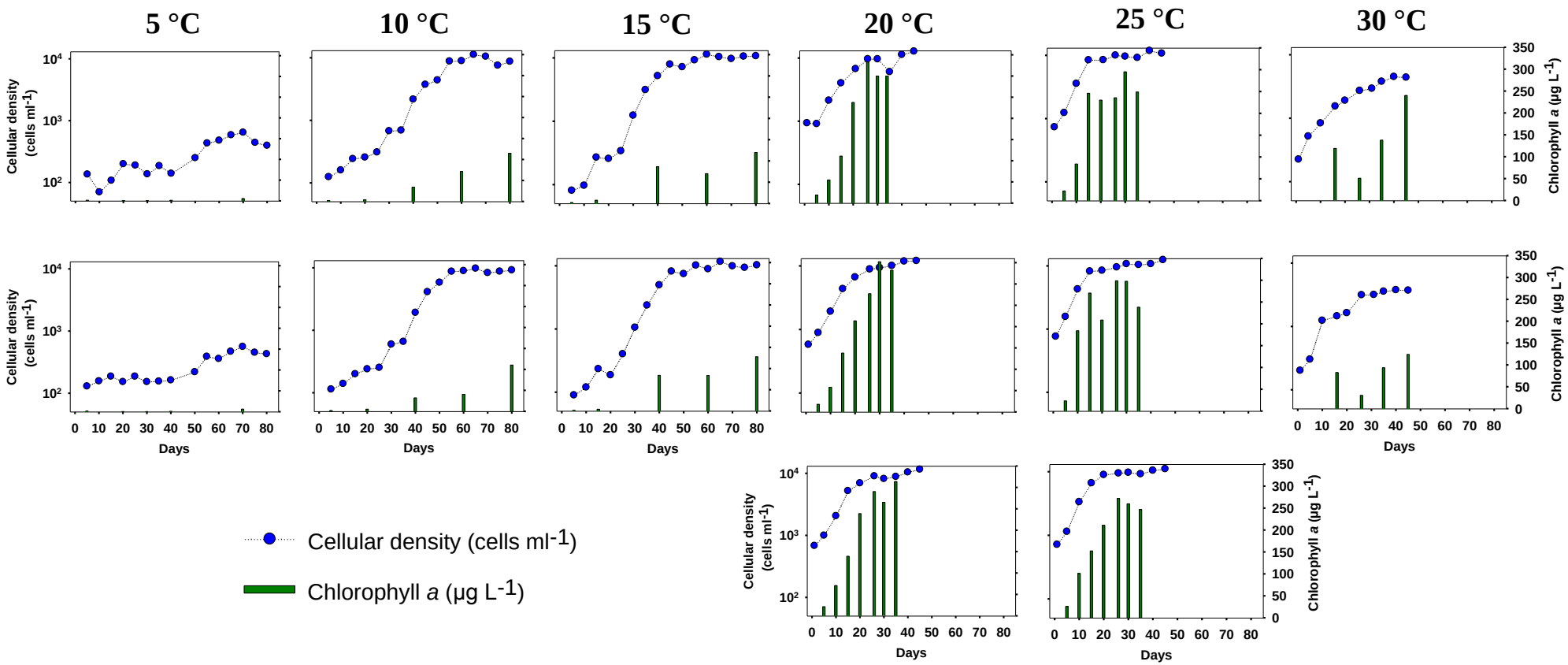

Figure 4.5 Changes in chlorophyll *a* concentration (µg L^{-1}) during the cellular growth of *Prorocentrum lima* in replicate cultures maintained at growth temperatures between 5 and 30 °C. 2 replicate cultures are shown at 5, 10, 15, and 30 °C while 3 replicates are shown at 20 and 25 °C.

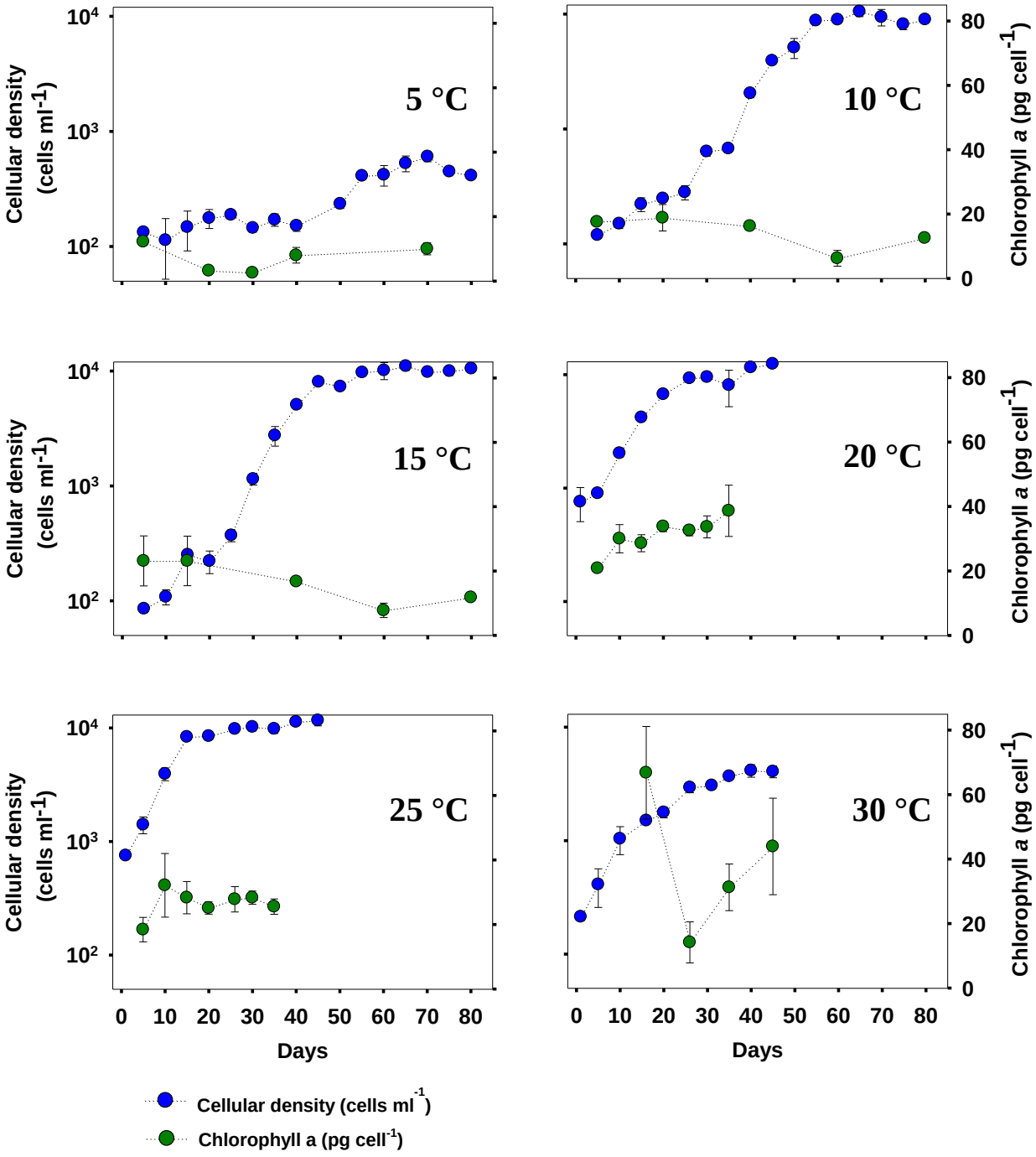

Figure 4.6 Average cell increase and chlorophyll *a* concentration per cell in *P. lima* cultures maintained between 5 and 30 °C. Chlorophyll content per cell was determined based on cell numbers and fluorometric measurements of chlorophyll *a*. Error bars shows ±1 standard difference (at 5, 10, 15, and 30 °C) and standard deviation (at 20 and 25 °C).

4.2.2 NO_3+NO_2 and PO_4 uptake

Figure 4.7 shows changes in NO_3+NO_2 and PO_4 consumption in *P. lima* cultures grown in incubators at growth temperatures ranging between 5 and 30 °C. Cell growth experiments were carried out with an initial concentration of 1000 µmol L^{-1} NO_3+NO_2 and 40 µmol L^{-1} PO_4 in cultures maintained at 5, 10, and 15 °C, while the initial NO_3+NO_2 (928 to 978 µmol L^{-1}) and PO_4 (32.7 to 34.4) were slightly lower for cultures grown between 20 and 30 °C.

In accordance with cell growth, NO_3+NO_2 and PO_4 uptake by *P. lima* cells was slow during the lag phase (seen at temperatures below 15 °C), but maximum changes of both nutrients occurred throughout the exponential growth phase regardless of the growth temperature (Figure 4.7). *P. lima* cultures incubated at 5 °C showed minimal removal of nutrients with a maximum uptake of 56 to 63 µmol L^{-1} NO_3+NO_2 and 4 to 9 µmol L^{-1} PO_4 after 80 days of incubation. Conversely, the highest uptake of PO_4 occurred in replicate cultures maintained at 25 °C, where *P. lima* growth caused PO_4 depletion after 15 days of incubation (stationary phase) and the lowest NO_3+NO_2 concentration recorded in the media was between 632 and 695 µmol L^{-1} towards the end of the stationary phase (day 40).

NO_3+NO_2 concentration was not depleted below about 600 µmol L^{-1} (Figure 4.7) whereas PO_4 depletion occurred at 20 °C with PO_4 undetectable in the cultures after day 25, in the stationary phase. There was no evidence of PO_4 depletion at 30 °C with little PO_4 uptake from day 20 onwards (stationary phase). Likewise, cultures grown at 10 and 15 °C reduced PO_4 concentration only to 9-10 µmol L^{-1} following exponential cell growth. Therefore, *P. lima* growth did not cause PO_4 depletion other than at 20 and 25 °C and NO_3+NO_2 concentration in the culture media was never reduced below 600 µmol L^{-1} at any of the growth temperatures.

Data of cell density, NO_3+NO_2, and PO_4 concentration changes were averaged and data presented in Figure 4.8. A comparison of changes in NO_3+NO_2 with changes in PO_4 (Figure 4.9) during the growth of *P. lima* showed linear relationships (r^2= >0.78, Figure 4.8). NO_3+NO_2/PO_4 uptake ratios were lower than the Redfield ratio by almost two-fold except for a higher NO_3+NO_2/PO_4 ratio found at 5 °C (Table 4.2).

Table 4.2 Average NO_3+NO_2/PO4 uptake ratios determined over the exponential growth phase in *P. lima* replicate cultures grown from 5 to 30 °C.

Growth Temperature (°C)	**NO_3+NO_2/PO_4 ratios**
5	20 (±2.82)+
10	6.3 (±1.27)+
15	8.5 (±1.83)+
20	7.1 (±0.65)*
25	5.5 (±0.35)*
30	9.1 (±3.67)+

+ =standard difference

* =standard deviation

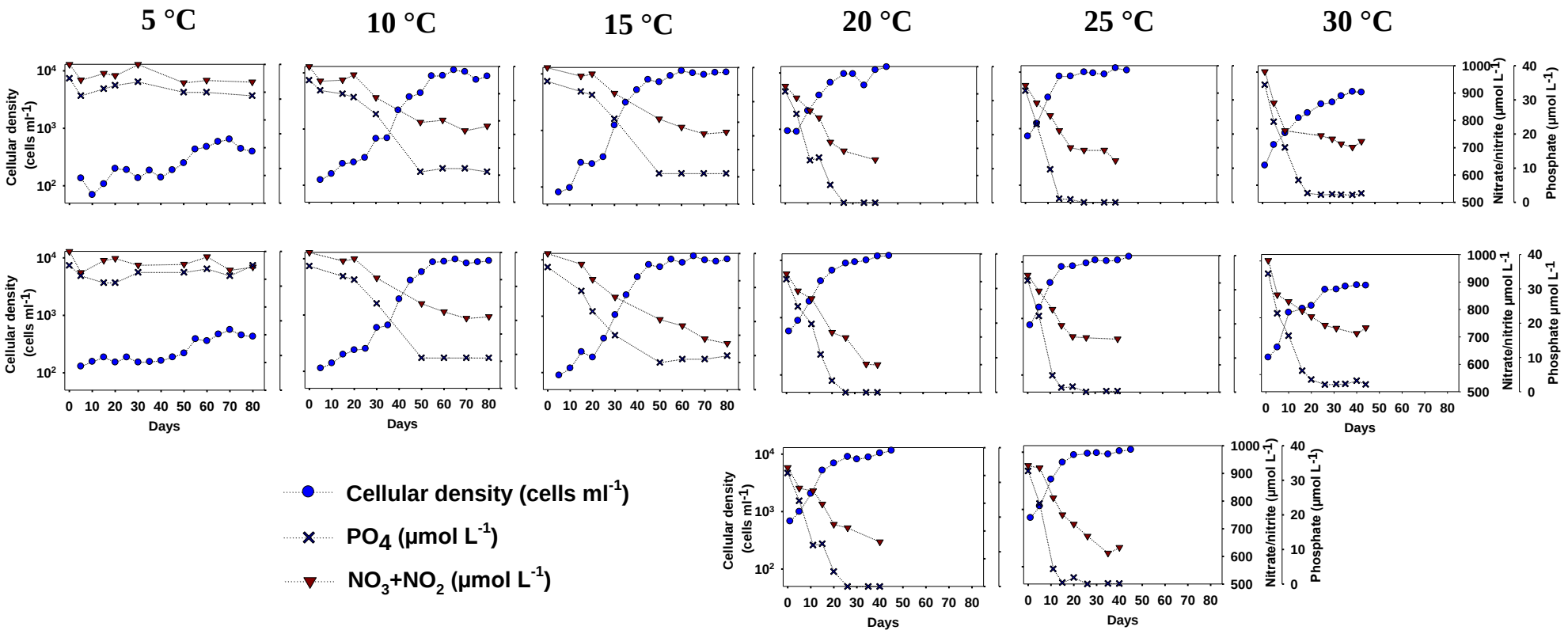

Figure 4.7 Changes in NO_3+NO_2 and PO_4 in cultures of *Prorocentrum lima* grown in F/2 media at growth temperatures between 5 and 30 °C. 2 replicate cultures were maintained at 5, 10, 15 and 30 °C while 3 replicate cultures were incubated at 20 and 25 °C.

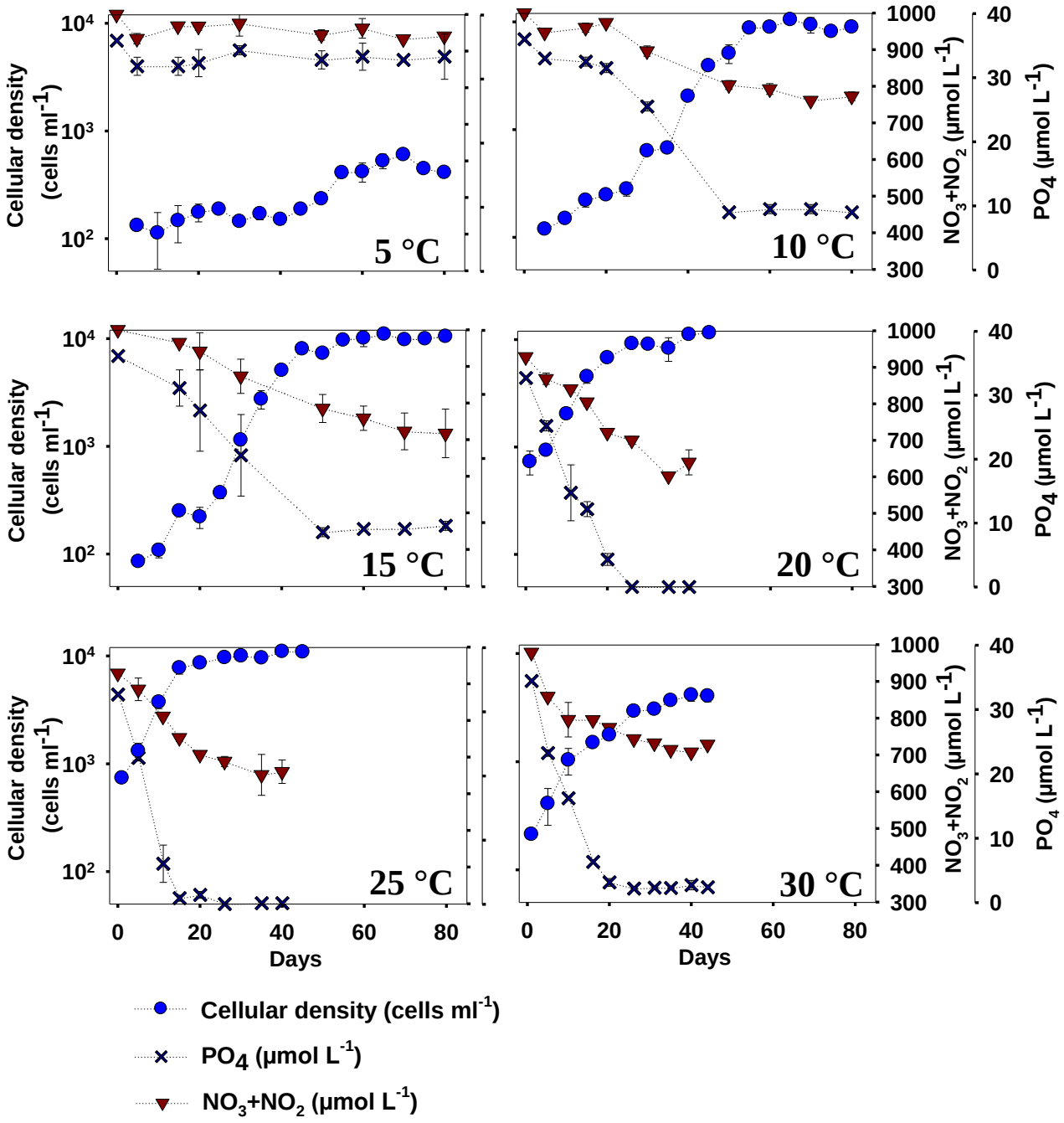

Figure 4.8 Changes in NO_3+NO_2 and PO4 concentration during the growth of *Prorocentrum lima* in cultures incubated between 5 and 30 °C. Error bars shows ±1 standard difference (at 5, 10, 15, and 30 °C) and standard deviation (at 20 and 25 °C).

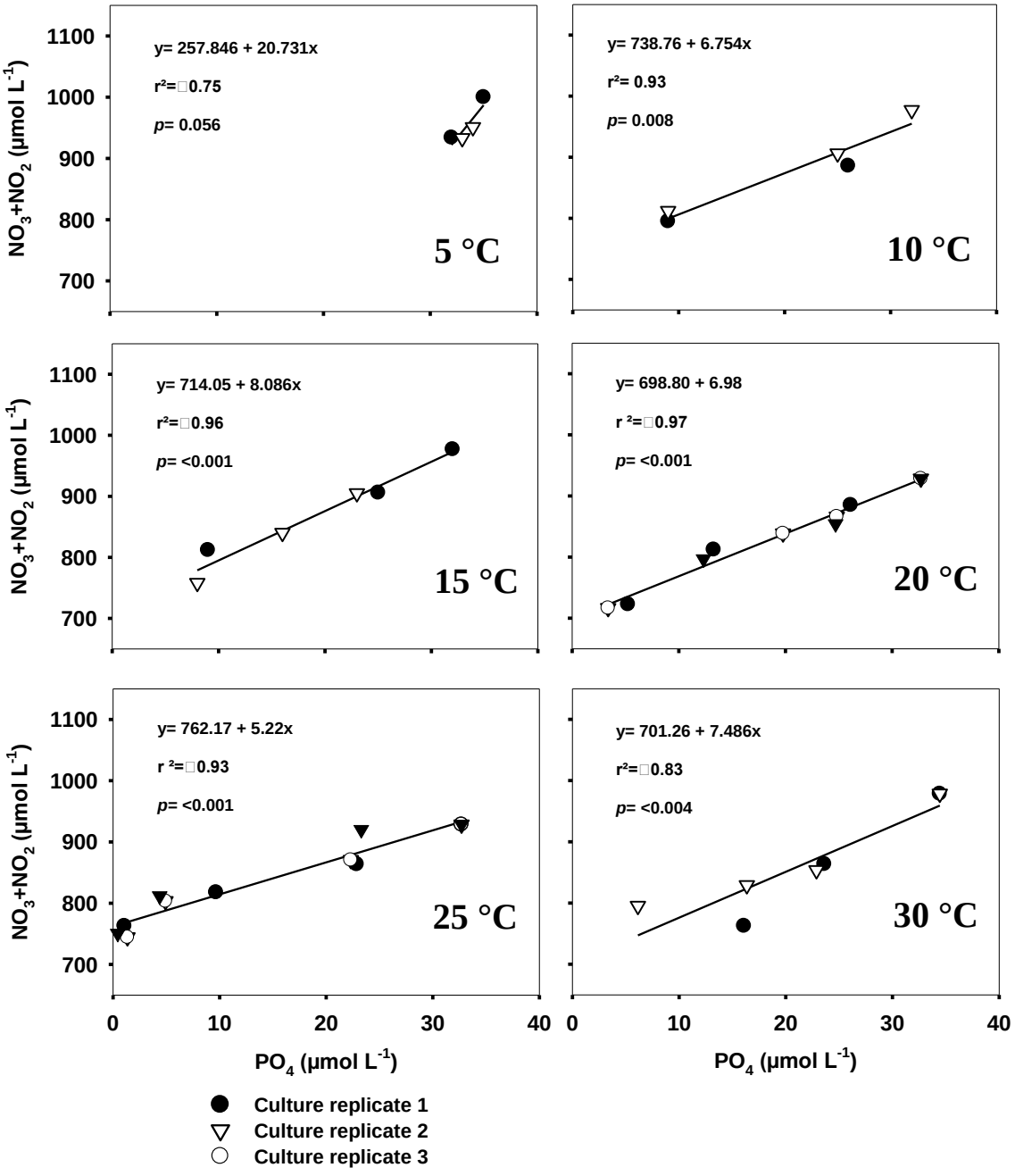

Figure 4.9 NO_3+NO_2/PO4 ratios over the exponential phase in *P. lima* cultures grown between 5 and 30 °C. Symbols represent data of two (5, 10, 15, 30 °C) or three (20 and 25 °C) replicate cultures.

4.2.3 DSP-toxin analysis by LC-MS/MS

LC-MS/MS analyses of cell extracts confirmed that the dinoflagellate *Prorocentrum lima*, isolated from the Fleet Lagoon, synthesized the biotoxins okadaic acid (OA) and dinophysistoxin1 (DTX1) in cultures maintained at growth temperatures between 5 and 30 °C. Regardless of the growth temperature, *P. lima* analyses confirmed an intracellular toxin-content (OA and DTX1) in all samples collected at all growth stages. Esterified compounds of OA were detected in higher amounts while only small amounts of esterified DTX1 compounds were detected in *P. lima* cells. Esterified compounds of both OA and DTX1 were not chemically characterized by LC-MS/MS, but quantities measured were considered within the total toxin burden of Total OA or Total DTX1. OA and DTX1 concentrations were generally low during the lag growth, but cellular synthesis of both compounds increased during the stationary phase (Figure 4.10 and 4.11). Toxin concentration in *P. lima* cells is reported in this study as Free OA, Total OA, Free DTX1, and Total DTX1, although OA and DTX1 will be also used in general terms.

Toxins released from *P. lima* cells and, therefore, dissolved in the culture media, either from *P. lima* exudates or cell disintegration, were not analyzed in this study. Free OA and Free DTX1 represent specific quantifications of OA and DTX1 (parent toxins) based on its molecular structure. Total toxicity (Total OA or Total DTX1), however, includes the parent toxin (OA or DTX1) plus esterified compounds metabolized or chemically transformed in *P. lima* cells.

Figure 4.10 and 4.11 illustrates changes in OA and DTX1 (total and free) concentrations in relation to *P. lima* growth in replicate cultures of F/2 media at growth temperatures between 5 and 30 °C. *P. lima* showed increasing concentrations of both OA and DTX1 (total and free) over the growth temperature range from 5 to 15 °C (Figure 4.10-4.11; Table 4.3-4.4), with fairly similar maximum concentrations (OA and DTX) between 10 and 15 °C (Figure 4.12). As expected, total OA reached higher concentrations than free OA regardless of the growth temperature (Figure 4.10; Table 4.3-4.4). However, total DTX1 presented almost similar concentrations to free DTX1, apart from cultures grown at 10 and 15 °C (Figure 4.11; Table 4.3-4.4).

Table 4.3 and 4.4 present OA/DTX1 ratios and ranges of OA and DTX1 measured in *P. lima* cells. Cellular concentration of Total OA was higher than total

DTX1 concentrations in *P. lima* cells regardless of the growth temperature (Table 4.3). Therefore, total OA was more concentrated in *P. lima* cells than total DTX1 (see ranges and ratios in Table 4.3). However, free OA and free DTX1 did not differ substantially in *P. lima* cells when compared at a single growth temperature (Table 4.4). For this reason, free OA and DTX1 ratios were around 1 (Table 4.4).

P. lima cultures incubated in the range of 20-30 °C showed a decline in the cellular concentration of both OA and DTX1 (total and free) in contrast to the concentrations observed in incubations at 10 or 15 °C. Little difference was determined in *P. lima* cells in terms of OA and DTX (total and free) at 20 and 25 °C, although a considerable increase of both OA and DTX1 was later observed at 30 °C, but with lower concentrations than those determined at 10 and 15 °C. Maxima concentration of OA and DTX (total and free) are contrasted in Figure 4.12 where notably the highest cellular concentration of OA and DTX (total and free) was found at 10 and 15 °C. Average plots of toxin data during the cell growth of *P. lima* are shown in Figure 4.13 (OA) and Figure 4.14 (DTX1).

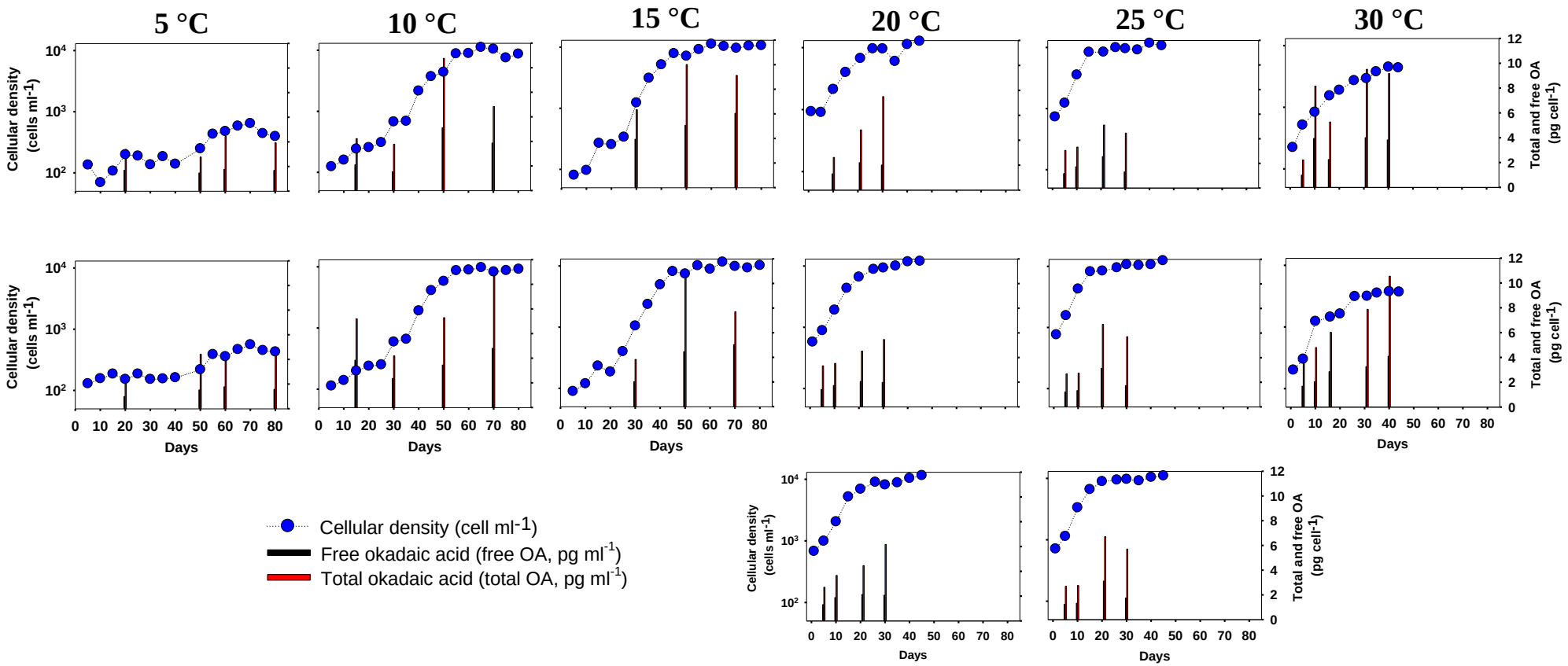

Figure 4.10 Increase of total and free OA (okadaic acid) during the cellular growth of *Prorocentrum lima* in cultures maintained in F/2 media at growth temperatures between 5 and 30 °C. 2 replicates are shown individually for the growth temperatures 5, 10, 15, and 30 °C, while 3 replicates are shown at 20 and 25 °C.

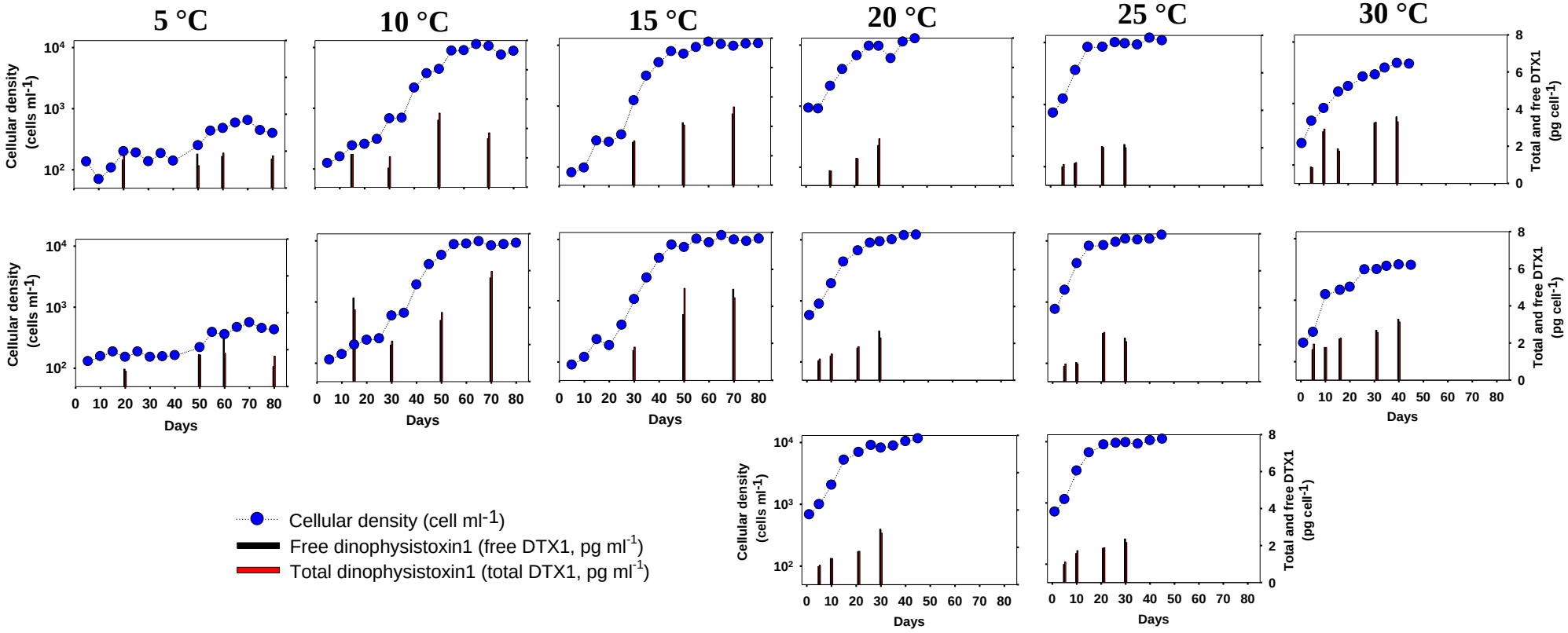

Figure 4.11 Increase of total and free DTX1 (dinophysistoxin1) during the cellular growth of *Prorocentrum lima* in cultures maintained in F/2 media at growth temperatures between 5 and 30 °C. 2 replicates are shown individually for the growth temperatures 5, 10, 15, and 30 °C, while 3 replicates are shown at 20-25 °C.

Table 4.3 Range of Total OA (okadaic acid) and Total DTX1 (dinophysistoxin1) determined by LC-MS/MS from *P. lima* cells grown at growth temperatures between 5 and 30 °C. Ranges consider toxin concentrations found during the lag, exponential and stationary growth phases. Mean of Total OA and DTX1 was based on ranges of these toxins found in replicate cultures. Ranges for Total OA/DTX1 ratios are also shown at each growth temperature.

Temperature (° C)	**Total OA (pg cell^{-1})**		**Total DTX-1 (pg cell^{-1})**		**Ratio OA/DTX-1**
	range	**mean (SE)**	**range**	**mean(SE)**	**range**
5	2.05-4.52	3.67 (0.31)	0.82-1.99	1.6 (0.14)	1.7-2.8
10	3.77-10.99	6.9 (1.0)	2.07-5.96	3.6 (0.50)	1.8-2.1
15	2.78-10.69	7.21 (1.15)	1.43-5.32	3.58 (0.58)	1.7-2.5
20	2.62-7.5	4.44 (0.45)	0.97-3.16	1.78 (0.21)	2.2-2.9
25	2.68-6.71	4.3 (0.38)	0.95-2.66	1.67 (0.17)	2.2-2.9
30	2.2-10.54	2.9 (0.35)	0.84-3.31	2.38 (0.26)	2.6 -3.4

Table 4.4 Range of Free OA (okadaic acid) and Free DTX1 (dinophysistoxin1) determined by LC-MS/MS from *P. lima* cells grown at growth temperatures between 5 and 30 °C. Ranges are shown considering toxin concentrations during the lag, exponential and stationary growth phases. Mean of Free OA and Free DTX1 was based on ranges of these toxins found in replicate cultures. Ranges for Free OA/DTX1 ratios are also shown at each growth temperature.

Temperature (° C)	**Free OA (pg cell^{-1})**		**Free DTX-1 (pg cell^{-1})**		**Ratio OA/DTX-1**
	range	**mean (SE)**	**range**	**mean(SE)**	**range**
5	0.99-1.78	1.56 (0.09)	0.94-2.60	1.63 (0.18)	0.8-1.5
10	1.51-5.12	3.38 (0.46)	1.31-5.61	3.36 (0.52)	0.9-1.2
15	1.41-6.04	3.99 (0.64)	1.18-4.92	3.33 (0.57)	1.0-1.3
20	1.30-2.20	1.83 (0.10)	0.97-2.97	1.77 (0.22)	0.7-1.4
25	1.16-3.11	1.83 (0.18)	0.82-2.60	1.67 (0.19)	0.6-1.5
30	0.98-4.09	2.89 (0.35)	0.88-3.58	2.40 (0.27)	1.0-1.4

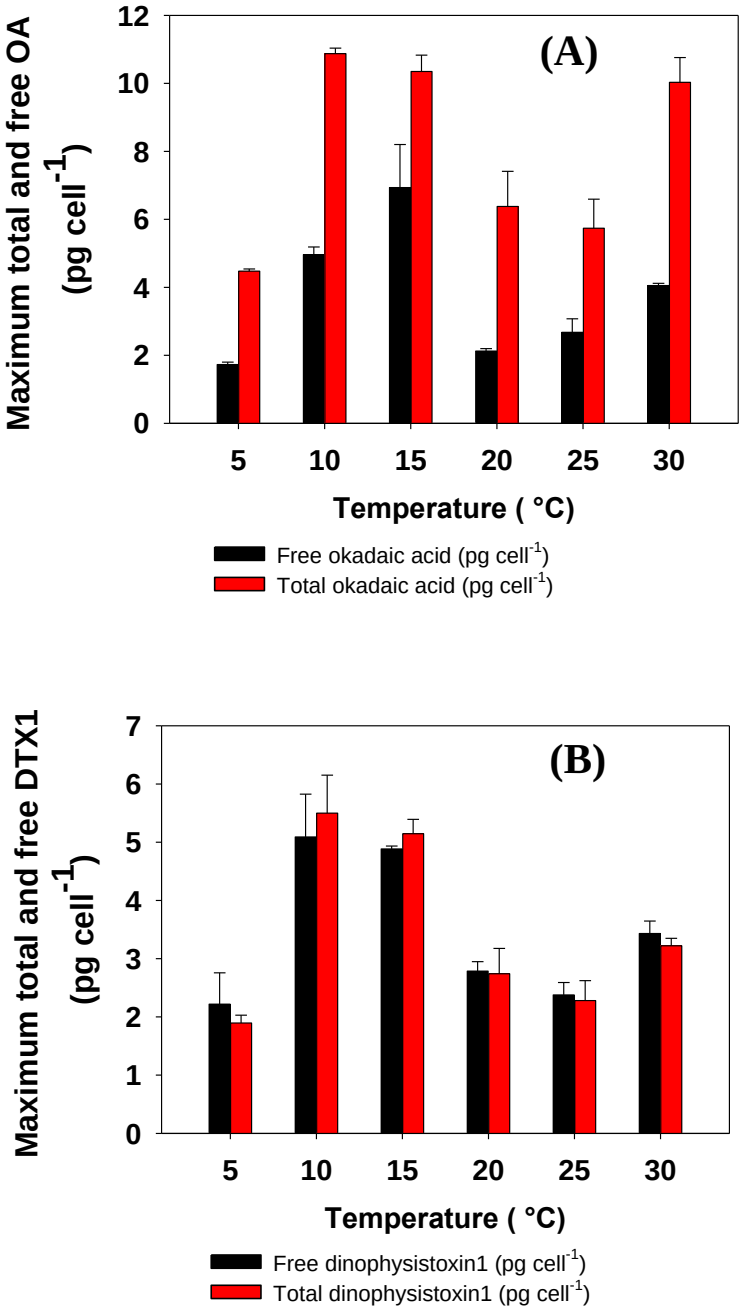

Figure 4.12 (A) Maximum concentration of total and free OA (okadaic acid) in *P. lima* cultures maintained in F/2 at growth temperatures between 5 and 30 °C. **(B)** Maximum concentration of DTX1 (dinophysistoxin1) found in *P. lima* cultures maintained at temperatures between 5 and 30 °C. Bars show ±1 SD considering maxima concentrations found in replicate cultures.

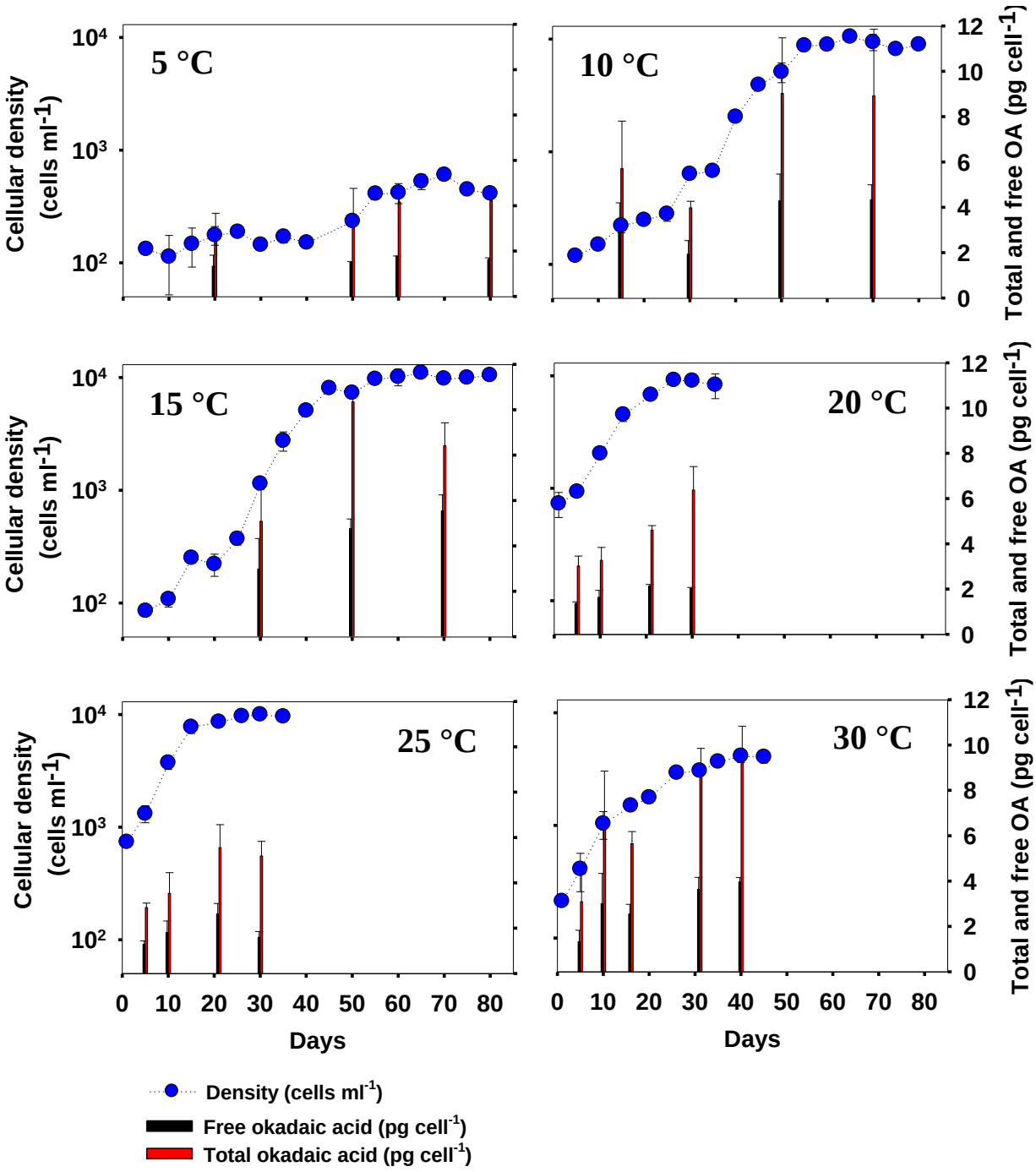

Figure 4.13 Average total and free OA (okadaic acid) during the cell growth of *P. lima* in cultures maintained in F/2 media at grown temperatures between 5 and 30 °C. Error bars shows ±1 standard difference (at 5, 10, 15, and 30 °C) and standard deviation (at 20 and 25 °C)..

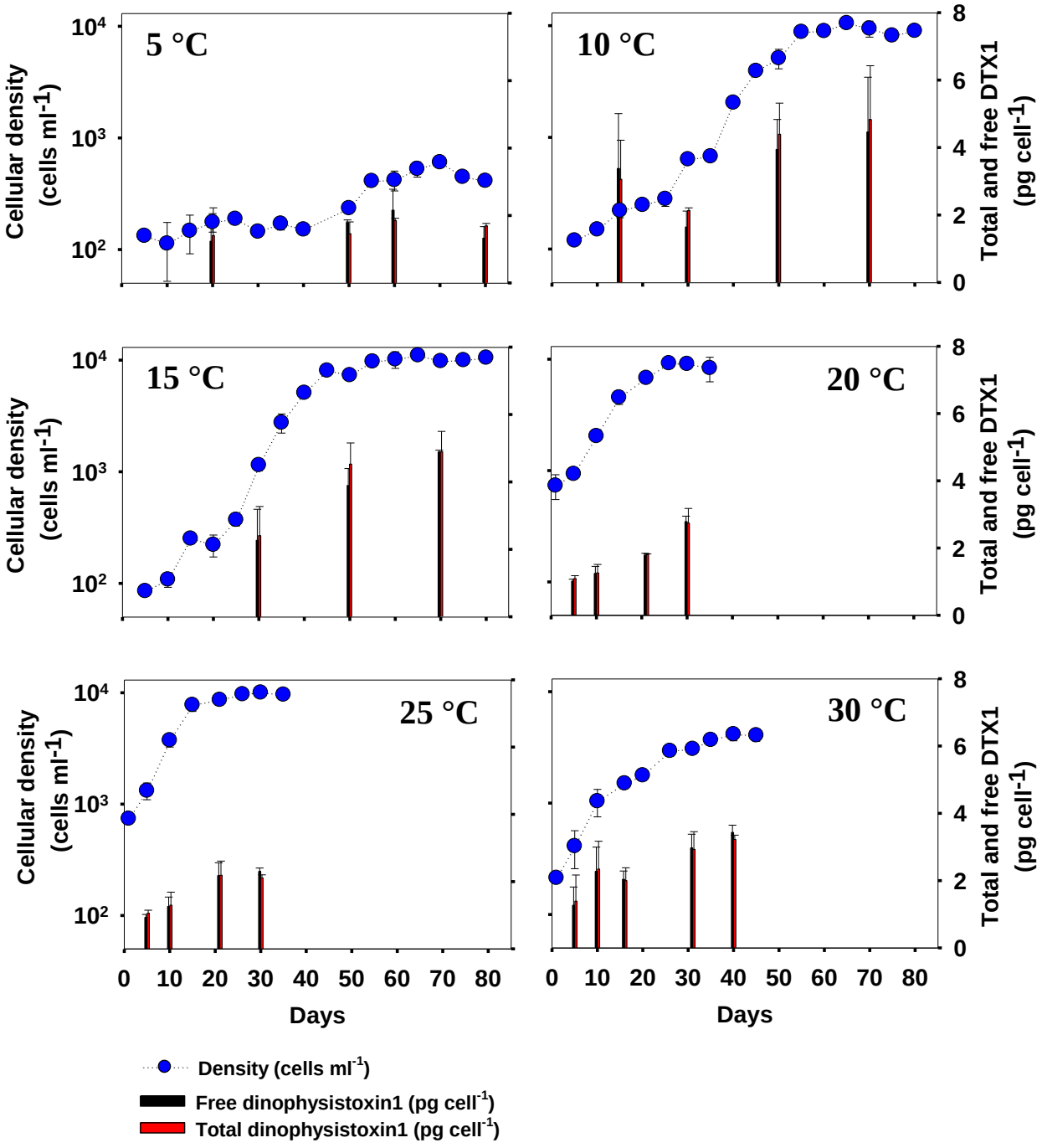

Figure 4.14 Average total and free DTX1 (dinophysistoxin1) during the cell growth of *P. lima* in cultures maintained in F/2 media at grown temperatures between 5 and 30 °C. Error bars shows ±1 standard difference (at 5, 10, 15, and 30 °C) and standard deviation (at 20 and 25 °C)..

4.2.4 pCO_2 in *P. lima* cultures

Changes in the pH of *P. lima* cultures grown at 15 and 20 °C was determined and pCO_2 derived using the equations of Crawford and Harrison (1997). Figure 4.15 illustrates pH, pCO_2 and chlorophyll *a* fluctuations during the cell growth of *P. lima* at 15 and 20 °C. Initial cell concentrations were in the order of 10^3 cell ml^{-1} at both 15 and 20 °C. NO_3+NO_2, PO_4, and DSP-toxins were not analyzed in this experiment. A concurrent increase of cell density and pH values occurred over the exponential growth in cultures at both 15 and 20 °C (Figure 4.15). The highest pH values were recorded at 20 °C and ranged between 9.3 (6.67×10^3 cells ml^{-1}) to 9.5 (8.2×10^3 cells ml^{-1}). Additionally, the highest cell abundance was detected at 20 °C with 1.08×10^4 cells to 1.09×10^4 cells ml^{-1} over the stationary phase. pH declined sharply over the stationary phase at 20 °C (lowest pH 7.4 on day 40) while *P. lima* cells showed a small increase of cell density (Figure 4.15). Maxima pH values occurred at the end of the exponential growth phase. Cultures at 15 °C showed maxima pH values from 8.8 (6.95×10^3 cells ml^{-1}) to 9.5 (6.02×10^3 cells ml^{-1}) on day 20 and day 30, respectively. Cultures at 15 °C reached maxima cell numbers between 8.8 $\times10^3$ cells ml^{-1} and 9.4×10^3 cells ml^{-1} at the termination of the experiment. Minimal pH value recorded at 15 °C was 8.4 on day 40 (Figure 4.15).

Initial pCO_2 values (on day 5) were 41-81 ppmv in replicate cultures maintained at 15 °C while 30-31 ppmv were recorded in replicates kept at 20 °C (Figure 4.15). pCO_2 decreased rapidly during the exponential growth phase which caused pCO_2 depletion before the stationary phase at both 15 and 20 °C. Despite pCO_2 depletion (day 20), *P. lima* cells increased in number during the stationary phase. Chlorophyll *a* increased and maxima concentration (day 25) reached 405-438 µg L^{-1} at 15 and 431-458 µg L^{-1} at 20 °C during the stationary phase (Figure 4.15). Growth rates at 15 °C (0.11-0.12 d^{-1}) were slightly lower than at 20 °C (0.13 d^{-1}).

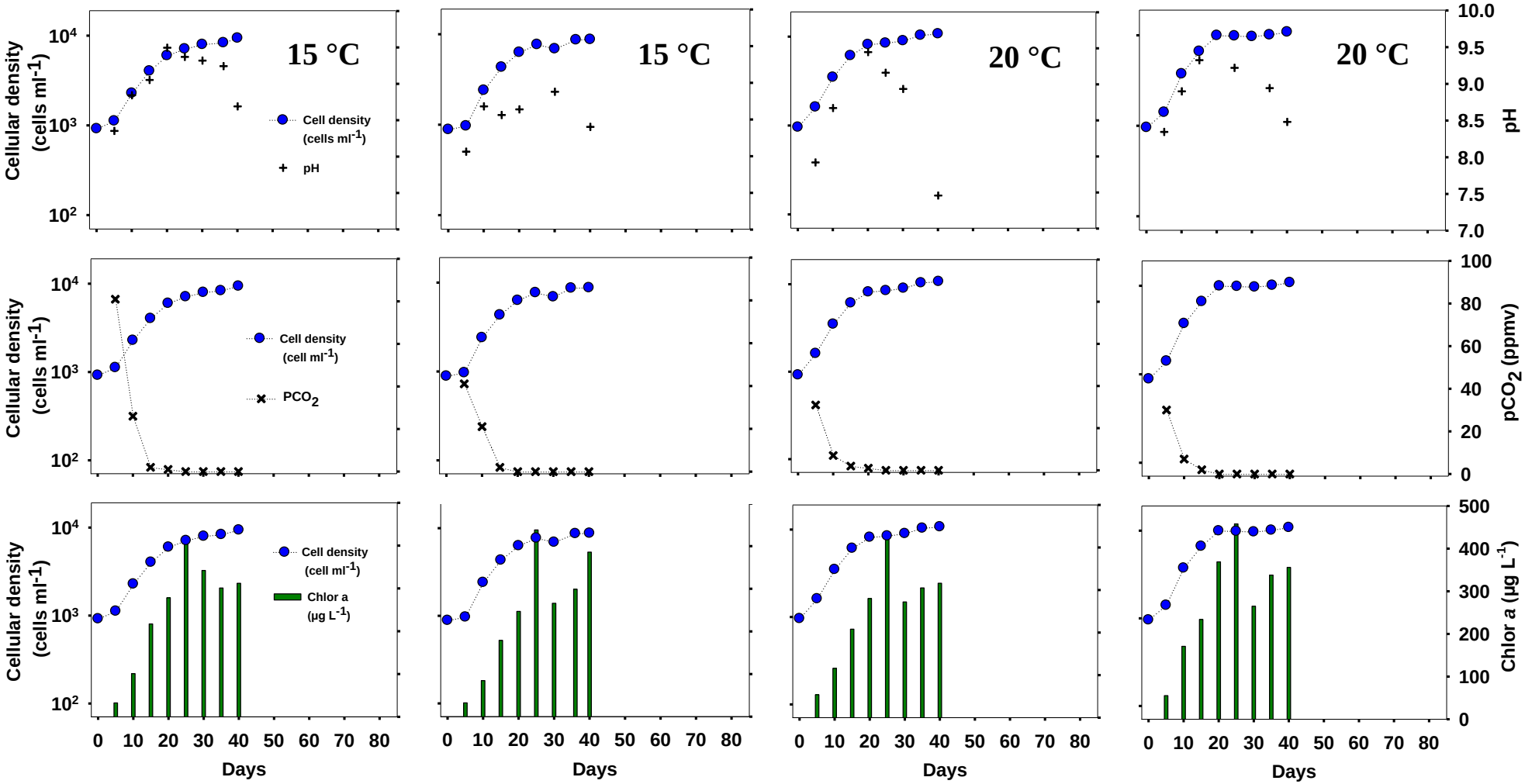

Figure 4.15 Changes in pH, pCO2 (ppvm) and chlorophyll a (µg chlor. a L^{-1}) in cultures incubated at 15 and 20 °C during the cell growth of *P. lima*. 2 replicates (each column one replicate) were carried out at both 15 and 20 °C.

4.2.5 Grazing and toxin production

A number of harpacticoid copepods (*Tigriopus californicus*) were inoculated in enriched *P. lima* cultures (F/2 media) in order to determine whether or not grazing may accelerate cellular synthesis of OA (okadaic acid) and DTX1 (dinophysistoxin1) in *P. lima* cells under nutrient replete conditions. Copepods consumed and digested *P. lima* cells in cultures maintained for 33 days under a 12h:12h light/dark photoperiod at 15 °C. DSP-toxin analyses were carried out for 10 days only in order for *P. lima* growth to restrain the effect of major nutrient deficiencies on toxic production. *P. lima* was able to sustain cell grow despite being grazed by copepods throughout the 33-day experiment (Figure 4.16). *P. lima* cells incubated without grazers (control) showed a higher net growth rate (μ= 0.15) than cells incubated with copepods (μ= 0.12 (2 replicates), Figure 4.16).

P. lima cultures were all initiated with almost the same initial cellular density of 603 cell ml^{-1} (± 54 cells ml^{-1} SD). Grazing produced a notable decrease of cells and chlorophyll *a* in treated cultures (replicates) between day 10 and day 33 (Figure 4.16). Cell density and DSP-toxins (OA and DTX1) showed a concomitant increase from day 0 to day 10, but toxin production (OA and DTX1) did not differ substantially between cultures with or without grazers (Table 4.5-4.6). Table 4.5 and 4.6 presents ranges of OA and DTX1 detected in *P. lima* cultures with or without added harpacticoid copepods. Despite the similarity in toxin content between cultures with and without grazers, the control culture (no grazers) produced cells with a slightly higher concentration of OA and DTX1, as shown in Table 4.5. OA was predominantly synthetized over DTX1 in *P. lima* cells with maximum difference of 4.5 times in the control culture.

Table 4.5 Range of total okadaic acid (OA) and total dinophysistoxin1 (DTX1) synthetized in *P. lima* cultures with (replicate culture 1 and 2) and without copepods (control).

	Total OA	**Total DTX1**	**Ratio total OA/DTX1**
Control	1.10-3.0	0.30-0.67	3.6-4.5
Replicate 1	1.07-2.0	0.40-0.61	2.7-3.3
Replicate 2	1.43-1.79	0.38-0.74	2.4-3.8

Table 4.6 Range of free okadaic acid (OA) and free dinophysistoxin1 (DTX1) synthetized in *P. lima* cultures maintained with (replicate culture 1 and 2) and without copepods (control).

	Free OA	Free DTX1	Ratio free OA/DTX1
Control	0.17-0.49	0.15-0.45	1.1
Replicate 1	0.23-0.38	0.21-0.28	1.1-1.4
Replicate 2	0.30-0.30	0.19-0.36	1.0-1.6

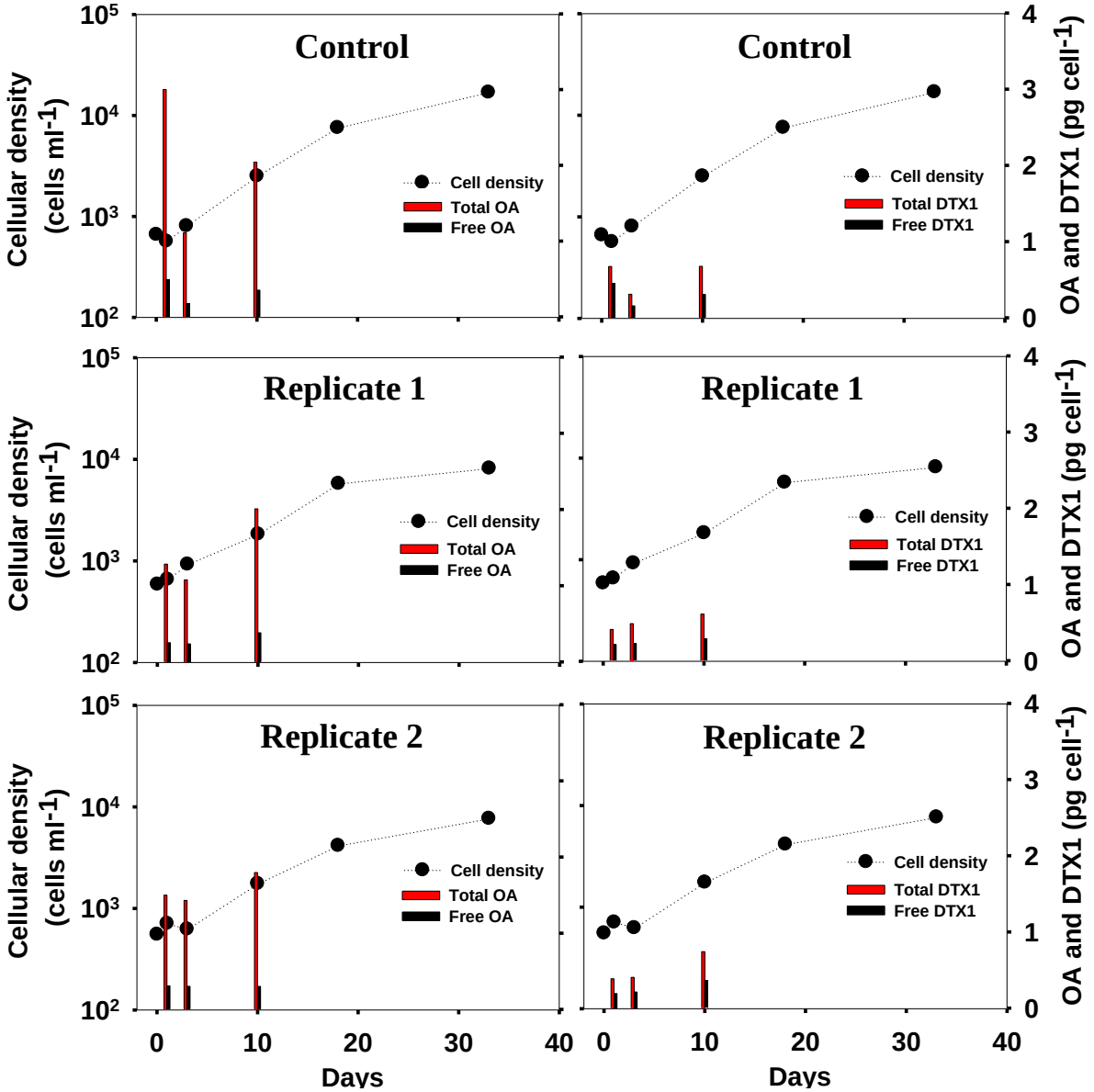

Figure 4.16 Changes in cell abundance of *Prorocentrum lima* and toxic production of okadaic acid (OA) and dinophysistoxin1 (DTX1) concentration in cultures subjected to grazing by harpacticoid copepods. Control plots (*P. lima* without grazers) show cellular growth and toxin synthesis by *P.lima*. Replicate 1 and 2 (*P. lima* cultures inoculated with grazers) show *P. lima* growth and toxin synthesis under the effect of copepod grazing.

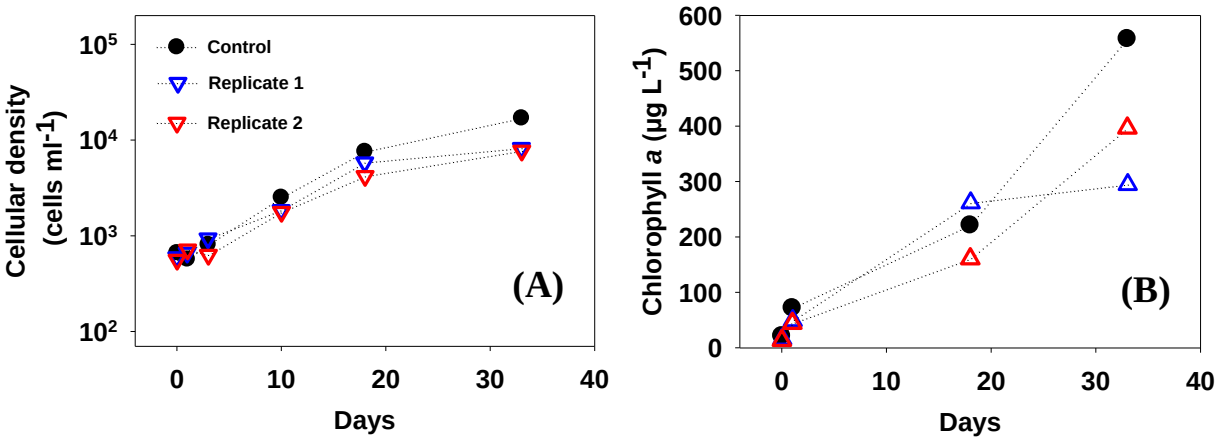

Figure 4.17 Effect of grazers (harpacticoid copepods) on the growth of *P. lima* incubated at 15 °C. (A) Cell growth and (B) chlorophyll *a* increase in *P. lima* cultures without grazers (control) and with grazers (replicate 1 and 2).

4.2.6 Bioassays

Brine shrimp nauplii (*Artemia salina*) and harpacticoid copepods (*Tigriopus californicus*) were used in toxicity assays where both crustaceans were fed on exponentially growing *P. lima* cells isolated from cultures incubated at 5, 15 and 30 °C. Figure 4.18 shows ingested *P. lima* cells by both brine shrimp and copepods after grazing on *P. lima*. *Artemia* bioassays were conducted using between 200 and 500 *P. lima* cells ml^{-1}. *Artemia* nauplii showed high sensitivity to *P. lima* and died in less than 12 h (overnight) following ingestion of cells regardless of the growth temperature. Copepods however exhibited high survival rates when fed on *P. lima*. Figure 4.19 illustrates cell density changes and mortality rates in bioassays using copepods fed on *P. lima*. The highest mortality occurred unexpectedly in copepods treated with the lowest cell density (0.50×10^2 cells ml^{-1}) while at high density of *P. lima* cells (2.64×10^3 cells ml^{-1}) copepods showed the lowest mortality (Figure 4.19).

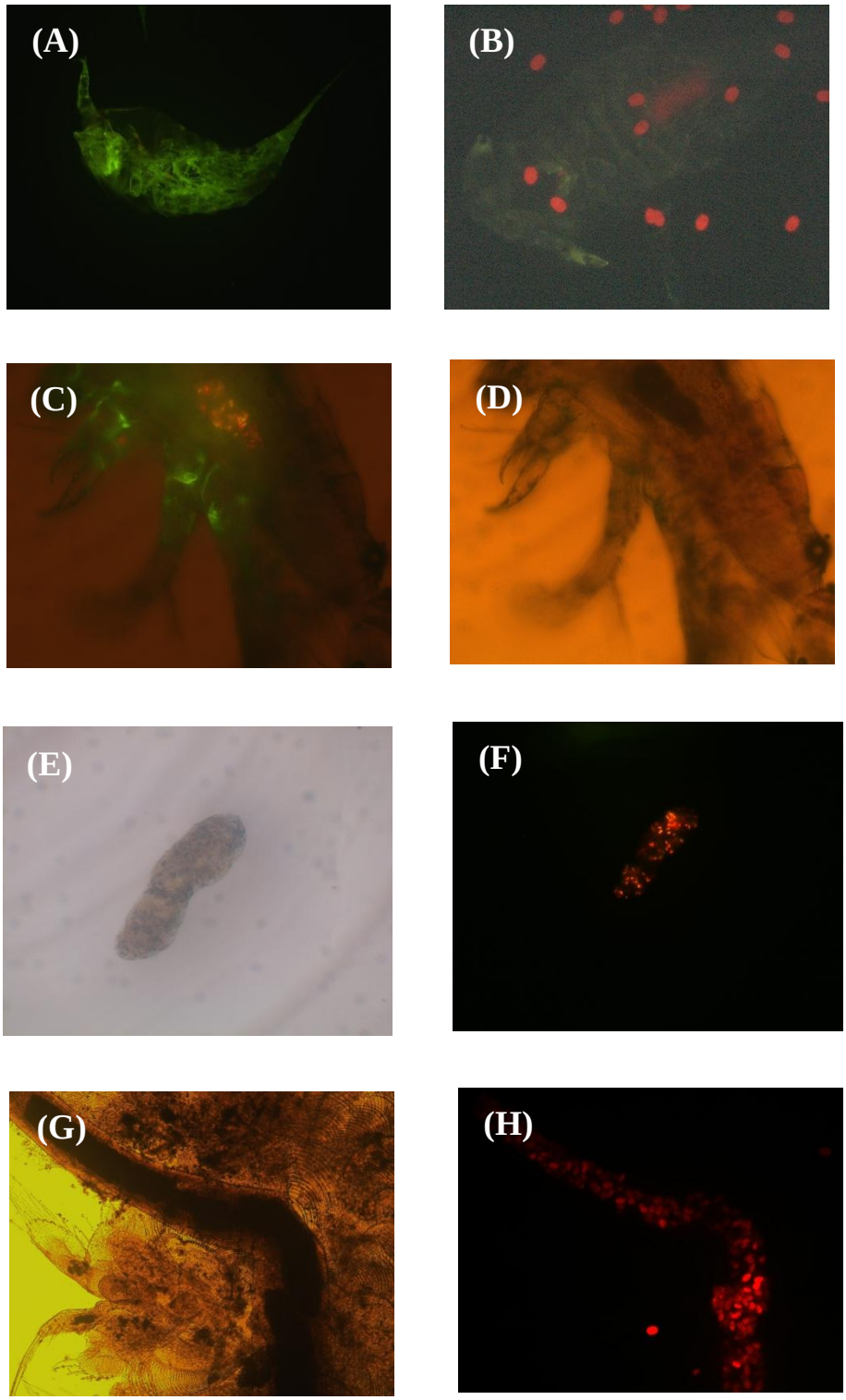

Figure 4.18 Harpacticoid copepods (A-D) and brine shrimp (G-H) viewed under light (D and G) and fluorescence microscopy (A-C, F, H) after ingesting *P. lima* cells. (A) Starved copepod maintained in filtered seawater (0.22µm) for 3-4 days. Faecal copepod pellets under light (E) and fluorescent microscopy (F).

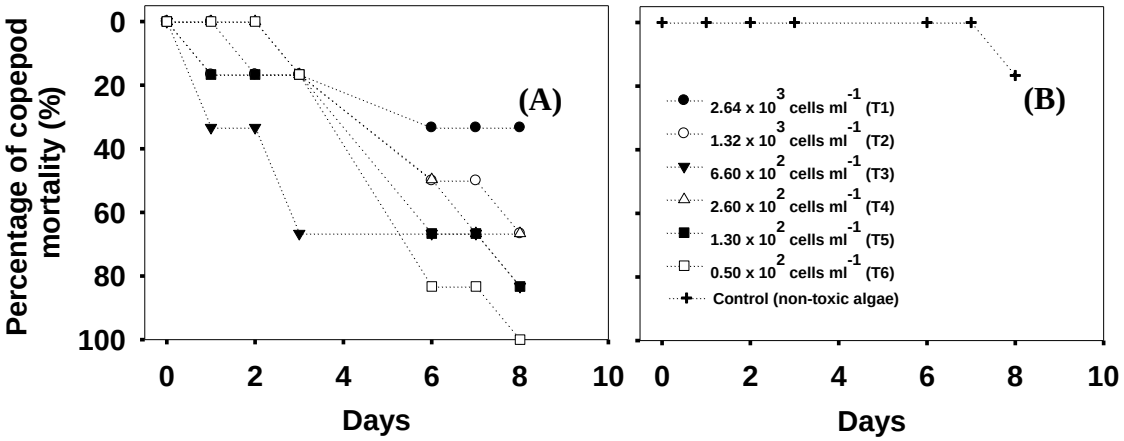

Figure 4.19 Mortality of harpacticoid copepods fed on different concentrations of *P. lima* cells during a 8-days experiment. (A) Mortality numbers are deemed a 6 copepod basis per treatment (T1-T6); (B) shows control where copepods (6 organisms) were fed on non-toxic algae.

4.3 Discussion

4.3.1 Effect of temperature on growth rates and *Fv/Fm*

Prorocentrum lima is a toxigenic microalgae with a history of causing deleterious effects in aquatic ecosystems documented from tropical to temperate marine waters (FAO 2004). The Fleet Lagoon, Dorset, UK, is a shallow coastal ecosystem where the noxious *P. lima* is present throughout the year and has been responsible for serious shellfish contamination (Nascimento 2003). In this study, *Prorocentrum lima* was isolated from the Fleet Lagoon and grown in monocultures (non-axenic) between 5 and 30 °C to determine the implication of increasing sea water temperature on the growth and toxin production of the isolate. Although predictions of the impact of climate change on HABs are not straight forward (Hallegraeff, 2010), a number of studies have concluded that substantial changes in the environment (e.g. warming) may result in an increase rather than decrease of the occurrence and impact of HABs worldwide (Peperzak, 2003, Bravo *et al.*, 2001, Hallegraeff, 2010).

In comparison to other benthic dinoflagellates, *P. lima* is considered a slow growing dinoflagellate (Varkitzi *et al.*, 2010), with maximum specific growth rates generally below 0.35 d^{-1} (see Table 4.7), although a few studies have reported higher growth rates of up to 0.5 d^{-1} (Tomas and Baden 1993). Table 4.7 compares culture conditions and growth rates for different isolates of *P. lima* reported in the literature. Both temperature and nutrient concentrations fundamentally influence the optimal growth of *P. lima* (McLachlan *et al.*, 1994, Vanucci *et al.*, 2010, Varkitzi *et al.*, 2010), and both have been implicated in the production and increase of biotoxins, particularly okadaic acid (OA) and dinophysistoxin1 (DTX1) in *P. lima* cells (Nascimento *et al.*, 2005, Vanucci *et al.*, 2010, Varkitzi *et al.*, 2010).

Table 4.7 Growth rates and culture conditions where *P. lima* strains from different regions have been grown under controlled conditions.

Species	Growth rate (d^{-1})	Temperature (°C)	Culture medium	Light intensity (photoperiod light: dark)	Salinity	Maximum cell density (cell ml^{-1})	Location of isolation	Reference
P. lima	0.11-0.22	20	F/2 different N:P ratios	35 µmol m^{-2} s^{-1} (16h:8h)	nd	10^4-2×10^4	Ria de Vigo, Spain	Varkitzi *et al.* 2010
P. lima	0.22-0.23	20	F/2 medium	90 µmol m^{-2} s^{-1} (16h:8h)	25	3.31×10^4-7.04×10^4	Adriatic Sea, Goro, Italy	Vanussi *et al.* 2010
P. lima	0.49	19	F/2 medium	40 µmol m^{-2} s^{-1} (14h:10h)	35	nd	Lisbon Bay, Portugal	Vale *et al.* 2009
P. lima	0.11	17	L-2 medium	90 µmol m^{-2} s^{-1} (16h:8h)	nd	3.05×10^4-4.83×10^4	Fleet Lagoon, Dorset, UK	Nascimento *et al.* 2005
P. lima	0.11	22	ES-Si medium	4×20Watts fluorescent lamps (12h:12h)	nd	nd	Baja California Sur, Mexico	Heredia-Tapia *et al.* 2002
P. lima	0.06	19	K medium	60-70 µmol m^{-2} s^{-1} (14h:10h)	Natural Seawater	1.5×10^4	Ria de Vigo, Spain	Bravo *et al.* (2001)
P. lima	0.08	19	K medium	60-70 µmol m^{-2} s^{-1} (14h:10h)	Natural Seawater	7.0×10^3	Ria de Pontevedra, Spain	Bravo *et al.* (2001)
P. lima	0.13	19	K medium	60-70 µmol m^{-2} s^{-1} (14h:10h)	Natural Seawater	1.5×10^4	Cíes Lagoon, Spain	Bravo *et al.* (2001)
P. lima	0.14	19	K medium	60-70 µmol m^{-2} s^{-1} (14h:10h)	Natural Seawater	1.5×10^4	Ria de Pontevedra, Spain	Bravo *et al.* (2001)
P. lima	0.1-0.15	18	L-1 medium	90 µmol m^{-2} s^{-1} (14h:10h)	32	nd	Nova Scotia, Canada	Pan *et al.* 1999
P. lima	>0.20	15-25	T1 medium	170 µmol m^{-2} s^{-1} (14h:10h)	nd	nd	Sanriku, Japan	Koike *et al.* 1998
P. lima	0.20-0.35	28	K medium	30-50 µmol m^{-2} s^{-1} (16h:8h)	nd	nd	Heron Island, Australia	Morton and Tindall, 1995
P. lima	0.5	26	Modified K medium	150 µmol m^{-2} s^{-1} (16h:8h)	nd	1.34×10^5	Florida, US	Tomas and Baden, 1993
P. lima	0.06-0.3	23-31	K medium	92 µmol m^{-2} s^{-1} (14h:10h)	36	nd	Knight Key, Florida	Morton *et al.* 1992
P. lima	0.05-0.18	5-30	F/2	30-70 µmol m^{-2} s^{-1} (12h:12h)	28-31	0.6-88.3×10^2	Fleet Lagoon, Dorset, UK	This study

Results from the current study show that *P. lima* can tolerate a wide range of temperatures (5-30 °C) when grown in nutrient rich F/2 medium. Growth rates reported in this study (0.05-0.18 d^{-1}) fit within the values (0.01-0.49 d^{-1}) documented in the literature using F/2 medium (Vale *et al.,* 2009, Vanucci *et al.,* 2010, Varkitzi *et al.,* 2010). Although F/2 medium is particularly designed for the growth of coastal marine algae, a number of studies have used K medium (designed for growing more oligotrophic marine phytoplankters, with 3-fold lower PO_4 concentration than F/2 medium) and found *P. lima* growth rates within the same range reported as for F/2 medium (see Table 4.7).

Although increasing growth rates of *P. lima* can be explained as a function of temperature from 0.05 d^{-1} (at 5 °C) to ~0.17 d^{-1} (at 25 °C), an unexpected growth decrease occurred in replicate cultures maintained at 20 °C (0.14 d^{-1}). This unexpected decrease could be explained as the interaction of both physical (light, cell densities) and physiological changes in *P. lima* cultures at this temperature and physiological changes in phytoplankton strains have been reported for short-term experiments (Lakeman *et al.*, 2009). In this study, a single clonal strain of *P. lima* was used in all growth experiments. However, *P. lima* experiments (cultures) had to be carried out at different times of the year, with a maximum of three incubation temperatures possible at one time which have led to small changes in light and nutrient concentrations. Therefore, it is considered that slight physical and physiological changes in clonal cultures could have impacted on growth variation in the short-term experiments, particularly at 20 °C.

Algal growth rates are well known to be temperature dependant (Raven and Geider, 1988, Montagnes *et al.,* 2003). In this study, a strong linear relationship (r^2= 0.97) existed between temperature and cell growth in *P. lima* cultures maintained from 5 to 15 °C. Morton *et al*. (1992) reported a significant linear relationship between growth of *P. lima* and temperature from 23-27 °C with the latter being the optimum growth temperature, whereas several other studies have found the optimal growth rate of *P. lima* to be at ~20 °C (Morton and Tindall, 1995, Vanucci *et al.,* 2010,). In the environment, maximum cell concentrations of *P. lima* have been reported at temperatures above 25 °C (Okolodkov *et al.*, 2007). A comparison of the range of growth rates at different temperatures between this study and Morton *et al*. (1992) is

provided in Figure 4.20. In this study, the highest growth rates were fairly similar at both 15 and 25 °C (0.17-0.18 d^{-1}), however this study failed to show a linear relationship from 5-25 °C, on one hand due to the decline of growth rate at 20 °C. Since cell yield and maximum *Fv/Fm* values were higher at 25 °C compared to 15 °C, results from this study suggests that the optimum growth temperature of *P. lima* might be associated with higher (25 °C) rather than lower temperatures (15 °C). This implies, consequently, that increasing cell abundances of *P. lima* in the environment is strongly regulated seasonally.

Results from this study agreed with McLachlan *et al*. (1994) in that *P. lima* is able to survive and produce toxins at 5 °C, although they suggested that *P. lima* can do the same at sub-zero water temperatures, with a potential decrease in DSP-toxins when cells experience low temperatures for long periods. This adaptation to low temperature could also imply a biological strategy of *P. lima* to expand its distribution in a number of marine habitats and, additionally, in combination with the potential increase of sea water temperature (climate change), it is thought that the biogeographical distribution of *P. lima* can be expanded to new marine habitats, as has been suggested for other toxigenic *Prorocentrum* species (Edwards *et al.*, 2006), such as *P. borbonicum* and *P. levis* (Aligizaki et al., 2009).

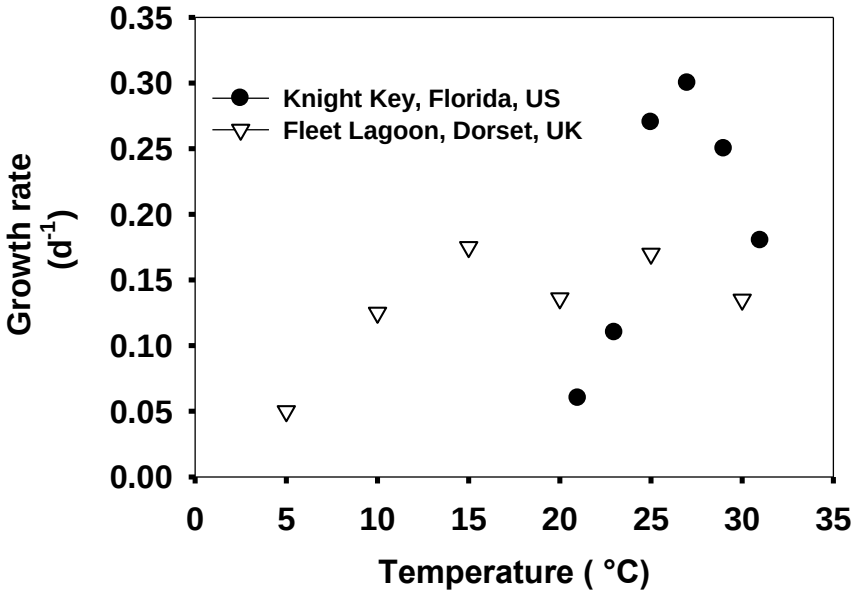

Figure 4.20 Comparison of growth rates determined over a range of growth temperatures by Morton et al. (1992) and this study.

Few studies from the literature have provided evidence of extreme suboptimal growth temperatures of *P. lima* under experimental conditions. Therefore, there might still be a lack of evidence to ascertain how *P. lima* populations may respond to a rise of sea water temperature in the environment (Moore *et al.*, 2008). As for this work, high temperatures (~25 °C) can benefit the growth of *P. lima*, but temperatures above 30 °C caused a rapid decrease of *Fv*/*Fm* and, therefore, cell yield. Morton *et al*. (1992) found similar results in terms of the immediate decrease of growth rate at temperatures higher than 30 °C. Conversely, Okolodkov *et al*. (2007) determined high abundance of *P. lima* (29,756 cell g^{-1} of substrate wet weight) in the environment at high temperatures (30-31 °C). Consequently, more studies are required to understand the physiological functioning of *P. lima* at high temperature (Moore *et al.*, 2008) as different clones can cause conflicting results (Lakeman *et al.*, 2009). For example, Morton and Tindall (1992) reported an isolate from the north of Heron Island Australia with $\mu = 0.35$ d^{-1} whilst an isolate from the same area, but from the south, reached a maximum of 0.20 d^{-1} using the same growth conditions.

Many benthic dinoflagellates are known to secrete mucilaginous material which allows them to attach to different substrates. The growth habit of *P. lima*, with high mucilaginous production and cell clumping (Foden *et al.*, 2005) in experimental assays, has caused major problems in cell quantification as homogeneous samples are difficult to obtain (McLachlan *et al.*, 1994). In addition, cell clumping might lead to considerable variation between replicates (McLachlan *et al.*, 1994). In this study, cell clumping was overcome by careful flushing the culture media from which *P. lima* cells were sampled and in general little variability was encountered between replicates. Clumps of cells, however, have been speculated to experience different physical-chemical conditions that those outside of clumps (McLachlan *et al.*, 1994).

In photosynthetic organisms, photosynthetic efficiency (*Fv*/*Fm*) provides a rapid assessment of the functionality of the photosynthetic apparatus (i.e. the efficiency by which absorbed light is utilized by photosynthesis); whereby physiological deficiencies in phytoplankton (such as nutrient limitation) can be determined (Suggett *et al.*, 2009, Schofield *et al.*, 1998). In this study, *P. lima* cells presented three distinctive *Fv*/*Fm* patterns during cultivation: 1) low and constant values of *Fv*/*Fm* associated with the lag phase at low temperatures, particularly at 5 °C (physiological adaptation), 2) increasing

Fv/*Fm* values throughout exponential growth (with maximum values toward the end of the exponential phase), and 3) much reduced *Fv/Fm* values linked to both the stationary phase (PO_4 limitation) and temperature increase. *P. lima* cultures grown at a single growth temperature (15 °C) at different times, produced significant differences in terms of maximum *Fv*/*Fm*. The first batch culture had a specific growth rate of 0.18 d^{-1}, with average maximum *Fv*/*Fm* values of 0.37, whereas the second culture reached 0.12 d^{-1}, with average maximum *Fv*/*Fm* values of 0.43. Surprisingly, this shows that low *Fv/Fm* values were associated with a higher growth rate and vice versa. This disparity may have been caused by using two different incubators where possibly the range of irradiance affected the cultures differently producing notable differences in both *Fv/Fm* and growth rates. Cell shading during algal growth has been linked to changes of *Fv/Fm*, but unfortunately this effect was not determined. Irradiances used in this study should not have caused damage to the photosynthetic apparatus of *P. lima*. In other studies *P. lima* has been grown at high irradiance (e.g. 170 µmol m^{-2} s^{-1}) (Koike *et al* . 1998) with growth rates comparable to those obtained using relatively lower irradiances, between 30- 70 µmol m^{-2} s^{-1} (Morton *et al.*, 1992, Vale *et al.*, 2009). Consequently, *P. lima* not only tolerates a wide range of growth temperatures (5-30 °C), but also this microalgae can survive under a wide range of light conditions (30-170 µmol m^{-2} s^{-1}). Results from this study agree with Varkitzi *et al*. (2010) in that irradiances of ~35 µmol m^{-2} s^{-1} were used to emulate light conditions from a benthic environment. In addition, it is worth mentioning that the photoperiod (12h Light: 12h Dark) used in this study produced growth rates comparable to those with longer photoperiods (16h Light: 8h Dark) as in the study by Varkitzi *et al.* (2010)

4.3.2 Nutrient uptake and toxin production

Okadaic acid (OA) and dinophysistoxin1 (DTX1) are lipophilic compounds classified as DSP toxins (FAO 2004). These toxins are particularly produced by a number of microalgae species belonging to the genera *Dinophysis* and *Prorocentrum* (Lee et al., 1989). OA and DTX1 are a threat to marine biota particularly when high cell concentrations of some *Prorocentrum* species are present in the environment. Human consumption of contaminated marine organisms containing high concentration of DSP toxins may provoke serious health consequences (see chapter 1). In terms of cell

concentration of toxic algae in the environment, UK and EU regulations have set a threshold limit for monitoring shellfish biotoxins of 100 cell L^{-1} of *Dinophysis* and 100 cell L^{-1} of *Prorocentrum*.

OA and DTX1 are parent toxins widely recognized as the main DSP toxins synthesized in all *P.* lima strains worldwide (Lee *et al.*, 1989). In this study, OA and DTX1 concentrations (picograms $cell^{-1}$) determined in *P. lima* cells corresponded with previous reports of isolates from the Fleet Lagoon (Foden *et al.*, 2005, Nascimento *et al.*, 2005). However, high interspecies variation in DSP toxins has been reported amongst *P. lima* strains worldwide (Morton and Tindall, 1995, Bravo *et al.*, 2001). Table 4.8 shows DSP toxin concentrations determined in different *P. lima* isolates based on published data. It is worth mentioning that Table 4.8 only includes DSP toxins reported as picograms $cell^{-1}$ as this enabled data comparison between different toxin profiles. However, several reports have documented the toxic burden of DSP toxins in shellfish and on macrophytes using *P. lima* cells g^{-1} dry weight of epibiont (Lawrence *et al.*, 2000, Levasseur *et al.*, 2003, Maranda *et al.*, 2007a).

Table 4.8 Ranges of DSP toxins (okadaic acid and dinophysistoxin1) encountered in *P. lima* strains from different locations.

Species	Okadaic acid (OA)		Dinophysistoxin1 (DTX1)		Other DSP-toxins	Method of analysis	Location of isolation	Reference
	Total	Free	Total	Free				
P. lima	80-108 pg cell^{-1}	8.4-11 pg cell^{-1}	nd	nd	nd	LC-MS	Rangaunu Harbour, New Zealand	MacKenzie *et al*. 2011
P. lima	11.27 pg cell^{-1}	nd	nd	nd	nd	HPLC-FD	Ria de Vigo, Spain	Varkitzi *et al*. 2010
P. lima	6.69-15.80 pg cell^{-1}	nd	0.12-0.39 pg cell^{-1}	nd	nd	LC-MS/MS	Adriatic Sea, Goro, Italy	Vanussi *et al*. 2010
P. lima	8.8-41 pg cell^{-1}	0.7-2.6 pg cell^{-1}	2.5-12 pg cell^{-1}	0.2-1.1 pg cell^{-1}	OA-D6, OA-D8, OA-D9, DTX1-D8, DTX1-D9	LC-MS/MS	Lisbon Bay, Portugal	Vale *et al*. 2009
P. lima	0.4-17.1 pg cell^{-1}	nd	0.4-11.3 pg cell^{-1}	nd	Diol esters derivatives of OA & DTX4	LC-MS	Fleet Lagoon, Dorset, UK	Nascimento *et al*. 2005
P. lima	0.1-1.8 pg cell^{-1}	nd	0.2-6.3 pg cell^{-1}	nd	nd	LC-MS	Fleet Lagoon, Dorset, UK	Foden *et al*. 2005
P. lima	nd	nd	nd	nd	19.0 pg cell^{-1} OA+DTX1	Mouse bioassay	Baja California Sur, Mexico	Heredia-Tapia *et al*. 2002
P. lima	0.19-12.87 pg cell^{-1}	nd	0-12.45 pg cell^{-1}	nd	0-1.14 DTX2	HPLC-FD	Galician coast, North West of Spain	Bravo *et al*. (2001)
P. lima	0.37-6.6 fmol cell^{-1}	nd	0.04-2.6 fmol cell^{-1}	nd	0.02-1.5 fmol cell^{-1} OA C8-diol-ester (OA-D8) and 1.8-7.8 fmol cell^{-1} dinophysistoxin4 (DTX4)	LC-MS & HPLC with LC-UV	Nova Scotia, Canada	Pan *et al*. 1999
P, lima	0.3-1.3 pg cell^{-1}	nd	nd	nd	nd	HPLC-FD	Sanriku, Japan	Koike *et al*. 1998
P. lima	2.33-7.06 pg cell^{-1}	nd	4.47-12.47 pg cell^{-1}	nd	6.8-19.15 pg cell^{-1} OA+DTX1	HPLC	Virgin Islands, US	Morton and Tindall 1995
P. lima	1.31-5.88 pg cell^{-1}	nd	4-12 pg cell^{-1}	nd	nd	HPLC-FD	Heron Island, Australia	Morton and Tindall, 1995
P. lima	nd	nd	nd	nd	3-24 pg cell^{-1} OA+DTX1; 140 ng ml^{-1} OA+DTX1 (cell+medium)	HPLC-FD	Mahone Bay, Nova Scotia	McLachlan *et al*. 1994
P. lima	5.0-26 pg cell^{-1}	nd	6.0-14.3 pg cell^{-1}	nd	nd	HLPC-FD	Vigo, Spain and Okinawa, Japan	Lee *et al*. 1989
P. lima	2.05-10.99 pg cell^{-1}	0.98-6.04 pg cell^{-1}	0.82-5.32 pg cell^{-1}	0.82-5.61 pg cell^{-1}	nd	LC-MS/MS	Fleet Lagoon, Dorset, UK	This study

During the cell growth of *P. lima*, several studies have demonstrated that cells can accumulate a remarkable intracellular concentration of OA and DTX during nutrient limitation (nutrient stress) (McLachlan *et al.*, 1994, Bravo *et al.*, 2001, Vanucci *et al.*, 2010, Varkitzi *et al.*, 2010). Several studies have reported that the highest toxin production (OA and DTX1) in *P. lima* cells is consistently linked to the stationary growth phase (McLachlan *et al.*, 1994, Vanucci *et al.*, 2010, Varkitzi *et al.*, 2010). This was similarly shown in this current study at all growth temperatures (5-30 °C), however at temperatures lower than 15 °C there was little evidence that NO_3+NO_2 and PO_4 removal had caused the initiation of the stationary phase (nutrient stress). Since neither NO_3+NO_2 nor PO_4 showed evidence of significant depletion at temperatures below 15 °C, it is suggested that a lack of dissolved inorganic carbon (CO_2) could have been involved in the initiation of the stationary phase and, therefore, possibly increasing concentrations of OA and DTX1. pCO_2 measurements however were not carried out in cultures where samples for toxin analyses were collected. Furthermore, there is a lack of knowledge of how CO_2 limitation affects the toxin production in *P. lima*.

To provide evidence of CO_2-limitation in *P. lima* cultures and the potential relationship with toxin increase, a pCO_2 method (Morton and Tindall, 1995) was applied in cultures maintained at 15 and 20 °C to infer CO_2 removal by *P. lima* cells. This study determined that the increase of pH was inversely proportional to CO_2 decrease and on day 20 CO_2 became a limiting growth factor for *P. lima* at both 15 and 20 °C. When comparing the beginning of CO_2 depletion (CO_2 experiments) with the beginning of the stationary phase at 15 (day 40) and 20 °C (day 25) from growth experiments, unfortunately there is a mismatch at 15 °C between CO_2 limitation (day 20) and the initiation of the stationary phase (~45); thus this study was unable to relate carbon limitation (C stress) to toxin production using an independent experiment despite the fact cultures were initiated with similar culture conditions. However, the increase of pH in *P.* lima cultures was importantly linked to the rapid decrease of CO_2 influenced by the growth temperature as found in other studies (Vardi *et al.*, 1999). Contrastingly, Fu *et al*. (2010) determined that increasing concentrations of CO_2 (230-745 ppm, pH 8.37-7.94 respectively) caused a substantial intracellular increase of toxicity (karlotoxins) in the harmful algae *Karlodinium veneficum*, suggesting ecological implications with regard to ocean acidification.

P. lima cultures grown in F/2 medium have shown contrasting results in terms of nutrient uptake (NO_3+NO_2 and PO_4) among strains, possibly due to the combined influence of initial cell concentrations and light conditions in the cultures. For example, in this study NO_3+NO_2 concentration in cultures incubated at all growth temperatures (5-30 °C) was never depleted below about 500 µmol L^{-1} at the end of the exponential phase, whereas PO_4 depletion occurred particularly at 25 °C (day 15) and then at 20 °C (day 25). Nascimento *et al*. (2005) reported that a *P. lima* strain isolated from the Fleet Lagoon caused both NO_3+NO_2 and PO_4 depletion (day 25) when grown in F/2 at 17 °C with irradiance of 90 µmol m^{-2} s^{-1}. In other studies, Varkitzi *et al*. (2010) reported that *P. lima* cells cultured in F/2 medium at 20 °C showed no evidence of nutrient limitation although a considerable decrease of NO_3+NO_2 (53.4 µmol L^{-1}) and PO_4 (<0.2 µmol L^{-1}) concentrations occurred towards the end of the cultivation. Likewise, Vanussi *et al*. (2010) reported high concentrations of NO_3+NO_2 (341 µmol L^{-1}) and low concentration of PO_4 (0.22 µmol L^{-1}) in senescent *P. lima* cultures incubated in F/2 at 20 °C and irradiance of 90 µmol m^{-2} s^{-1}. Although it is clear from above that different *P. lima* strains reduced nutrient concentrations in differing proportions, OA concentrations between this study and the aforementioned authors did not differed significantly (see table 2), with OA concentrations (0.4-17.1 pg $cell^{-1}$) within the range reported by Nascimento *et al*. (2005). However, recently MacKenzie *et al*. (2011) has reported significantly higher OA concentrations (80-108 pg $cell^{-1}$) than those within the range reported by Nascimento *et al*. (2005).

Results from this study did not show an increase of OA and DTX1 in *P. lima* cells with an increase in growth temperature (between 5 and 30 °C). In general, higher OA and DTX1 concentrations were determined in cultures at temperatures lower than 15 °C than those maintained from 20-30 °C. Total OA was generally higher than free OA (parent toxin) meaning that some OA toxins (derivatives) were importantly accumulated in cells in an esterified form. However, total DTX1 did not differ substantially from free DTX1 (parent toxin) suggesting that little amounts of esterified DTX1 was presented in *P. lima* cells. Total OA/DTX1 ratios were slightly higher than free OA/DTX1 as a result of the presence of esterified forms. Total OA/DTX ratios (1.7-3.4) reported in this study coincided well with ratios documented previously for isolates from the Fleet Lagoon (OA/DTX1= 0.14-3.20) (Nascimento *et al*. 2005).

However, other studies have reported OA/DTX1 ratios from 3-10 (McLachlan *et al*. 1994); 3.2-3.5 (Vale *et al*. 2009); 40.7-57.5 (Vanucci *et al*. 2010). These ratios evidently indicate that the biotoxin OA contributes substantially to the total toxin burden of *P. lima* cells.

In this study, total OA concentration at 10, 15 and 30 °C was high and did not differ substantially among these temperatures. By contrast, free okadaic acid (parent toxin) was the only toxin with a proportional increase in relation to temperature, but only from 5-15 °C and 20-30 °C. In addition, the highest total and free DTX1 concentration were found at 10 and 15 °C with reduced concentrations at 5 °C and at temperatures higher than 15 °C. It is not clear why *P. lima* reached high OA and DTX1 concentrations at growth temperatures of 10 and 15 °C where there was no apparent nutrient limitation (NO_3+NO_2 and PO_4). Also, the highest concentrations of OA and DTX1 were not associated with the cultures showing PO_4 limitation at 25 °C or 20 °C. It is possible therefore that some other factors (e.g. salinity of seawater, irradiance, nutrients) might have influenced physiological changes in *P. lima* over time and between growth experiments. The gap between growth experiments (months of difference) possibly affected the physiology of the strain, but also, the sampling technique and analytical methods could have influenced the result of intracellular toxin content.

P. lima is not only known to accumulate intracellular toxins, but it has also been found to release considerable amounts of toxins to the water. For example, Vale *et al*. (2009) reported a toxin content in cell free media from 3.2-33 ng ml^{-1} of OA and 0.6-8.3 ng ml^{-1} of DTX1, whereas Nascimento *et al*. (2005) found a maximum of 59.5 ng OA ml^{-1} and 94.6 ng DTX1 ml^{-1} in culture medium. In this study, toxins in the medium were not measured but it is estimated that quantities of OA and DTX1 could have been released from *P. lima* cells during sampling (cell detachment and cell homogenization in medium). In addition, *P. lima* cell sampling on GF/F filters by filtration is thought to have produced enough pressure on cells to release intracellular toxins to the medium. It is possible, therefore, that total toxin burden from *P. lima* cells may have been underestimated.

4.3.3 Bioassays

Results from this study clearly demonstrated that *P. lima* was highly toxic to the brine shrimp *Artemia salina*. During feeding experiments, there was no evidence that *P. lima* avoided the ingestion of *P. lima* cells as high number of cells were visualized in *Artemia* guts by epifluorescence microscopy (~50 cells). This study recorded 100% of mortality of the brine shrimp in less than 12 h at *P. lima* cell concentrations between 200-500 cell ml^{-1}, however, Ajuzie (2007) determined that lower concentrations, between 20 and 120 cell ml^{-1}, caused between 98-100% of mortality in *A. salina* in 24 hr. Although *P. lima* and *Artemia salina* do not occur together in nature, this study provides evidence that *P. lima* can deter grazers.

There is evidence that copepods can tolerate the ingestion of algal biotoxins (Colin and Dam, 2004, Senft *et al.*, 2011) and in some cases can promote toxin production in some dinoflagellates (Selander *et al.*, 2006). In this study, the effect of grazing on the toxin production of *P. lima* was evaluated under nutrient replete conditions, but results did not indicate that grazing promoted an increase of toxin production in *P. lima* cells. Since toxin-induction experiments lasted 10 days, it may well be that this time was not long enough to affect the cellular production of toxins.

4.4 Conclusions

In the last decade, a vast number of studies have confirmed that increasing sea water temperatures (climate change) will influence positively rather than negatively the occurrence and distribution of many HAB species worldwide. In this study, not only the growth but also the toxicity of *Prorocentrum lima* increased at increasing growth temperatures (5-15 °C). However, the effect of temperature on growth rates and toxin production did not show a strong relationship across the complete range of growth temperatures used (5-30 °C) in this study. The highest temperature response on growth and toxin production occurred as temperature increased from 5 to 15 °C.

Although *P. lima* removed nutrients (NO_3+NO_2 and PO_4) more efficiently as temperature increased and should have produced higher growth rates at elevated temperatures, some difficulties were encountered in identifying the optimum growth temperature of *P. lima*. At growth temperatures of 15 and 25 °C similar growth rates were determined. In addition, high toxin concentrations were expected to be recorded in cultures showing nutrient stress (PO_4) associated with the stationary growth phase, however the highest OA and DTX1 concentrations were determined in cultures (10 & 15 °C) where NO_3+NO_2 and PO_4 remained replete. Therefore, the availability of inorganic carbon (CO_2 limitation) was suggested as a potential factor that might have limited the growth of *P. lima* when grown in F/2 medium. Although there was evidence that PO_4 was associated with the initiation of the stationary phase, further studies are indeed required to determine the influence of inorganic carbon (CO_2 limitation) on the growth and toxic production of *P. lima*, particularly in nutrient rich media such as F/2.

The lack of agreement between nutrient removal and growth rates/toxin production was believed to be derived from physiological changes experienced by *P. lima* during both culture maintenance and the gap between growth experiments under controlled conditions. Although some high temperatures did not coincide with high toxin production, ranges encountered in this study were in accordance with ranges reported in some previous studies.

Despite the high nutrient concentrations used in *P. lima* cultures, this benthic dinoflagellate demonstrated a slow growth rate at all temperatures. Since *P. lima* tolerated a wide range of conditions (low-high temperatures and high-low nutrient

concentrations), with variations in the intracellular toxin content, aquatic systems such as the Fleet Lagoon remain under permanent threat particularly when the dynamic of this microalgae could trigger a major toxic outbreak.

Bioassays demonstrated that *P. lima* toxins were fatal to the brine shrimp *Artemia salina* within a few of hours. On the other hand, there was no indication that *P. lima* increased its cellular toxin production due to grazing from harpacticoid copepods.

CHAPTER 5

5.1 Introduction

5.1.1 The epibenthic dinoflagellate *Coolia monotis*

Coolia monotis is an epibenthic and sand-dwelling dinoflagellate commonly encountered in tropical and temperate marine waters. Holmes *et al*. (2001), Rhodes and Thomas (1996), Rhodes *et al*. (2000), Hallegraeff (2003), Taylor (2003), and Liang *et al*. (2008) have reported that *C. monotis* is a toxin-producing dinoflagellate, related to ciguatera fish poisoning (CFP), which synthesizes secondary metabolites that correspond to the mono-sulphated form of yessotoxin (Bravo *et al.*, 2001, Ignatiades and Gotsis-Skretas, 2010). Yessotoxins (YTX) are lipophilic compounds belonging to DSP (diarrhetic shellfish poisoning) toxins, although given that yessotoxins differ in chemical structure and toxicological properties, Daranas *et al*. (2001) and Ogino (1997) have suggested that YTX toxins should be reclassified. Although *C. monotis* has been linked with the mortality of juvenile fish in the environment (Armi et al., 2010), different reports have demonstrated that some strains of *C. monotis* may not be toxic (Rhodes *et al.*, 2000, Penna *et al.*, 2005). On the other hand, several reports have concluded that *C. monotis* is usually encountered in assemblages with other toxigenic epibenthic dinoflagellates such as *Ostreopsis* cf *siamensis*, *Prorocentrum lima* (Pin *et al.*, 2001, Aligizaki and Nikolaidis, 2006, Laza-Martinez *et al.*, 2011), and *Amphidinium carterae* (Faust *et al.*, 1996, Okolodkov *et al.*, 2007, Rhodes *et al.*, 2010).

Although *C. monotis* has been regarded as a harmful microalgae, information on blooms in the environment of this species is scarce and not well understood. In the Veracruz reef zone of Mexico, *C. monotis* was found throughout the year, with highest abundance (3.0×10^3 cells g^{-1} substrate's seagrass *Thalassia testudinium* wet weight) in July (Okolodkov *et al.*, 2007), while Armi *et al*. (2010) reported a bloom of *C. monotis* in the North Lake of Tunis (Mediterranean waters) with cell concentrations of up to 5×10^5 cells L^{-1}, where *C. monotis* contributed 49% of the total phytoplankton community between May-July. Furthermore, in the water column of the North Aegean Sea (Mediterranean waters of Greece), *C. monotis* has been found with 0.5×10^3 cells L^{-1}

in August, but maximum concentration (1.6×10^3 cells g^{-1} fresh weight of macrophyte) occurred predominantly on macrophytes during winter (Aligizaki and Nikolaidis, 2006). Elsewhere, in the south-eastern of the Cantabrian Sea (Bay of Biscay) characterized by warm sea temperatures (10-25° C) than the rest of the Atlantic coast of Spain, Laza-Martinez *et al.* (2011) found that *C. monotis* is widely distributed along the coast, but maximum concentrations never exceeded 100 cells L^{-1}.

C. monotis has been found to tolerate a wide range of temperatures in the environment (Aligizaki *et al*., 2009), including high temperatures and salinities (Rhodes *et al.*, 2000). For instance, Okolodkov *et al*. (2007) recorded high abundance of *C. monotis* at 28 °C and salinity of 29 in the coral reef of Veracruz, Mexico, whereas Armi *et al*. (2010) recorded a bloom of *C. monotis* during spring and summer at temperatures higher than 22 °C and salinities over 38.6 in Tunisian waters. Likewise, Rhodes and Thomas (1996) confirmed that *C. monotis* grew preferentially at 25 °C (subtropical waters) rather than at 20 °C (temperate), with salinities >28, but later Rhodes *et al*. (2000) observed that *Coolia* grew optimally at 30 °C.

In cultures, *C. monotis* reached abundances of $0.36\text{-}3.3\times10^3$ cells ml^{-1} when grown in ES media at 25 °C (Nakajima *et al.*, 1981), whereas a maximum of 2.5×10^3 cells ml^{-1} was encountered in Erdschreiber's media at 23 °C and salinity of 36 after 15 days of growth (Faust, 1992), with a maximum growth rate (μ) of 0.3 d^{-1} (Rhodes *et al.*, 2000). Rhodes *et al*. (2000) noted that *C. monotis* was unable to grow at 35 °C.

Large intraspecific genetic variability and minimal morphological differentiation (similar phenotypes) amongst *C. monotis* isolates were reported by Dolapsakis *et al.* (2006). By characterising nuclear-encoded partial LSU rDNA and internal transcribed spacer (ITS) regions of several *Coolia* strains, it has been concluded that morphological features of *Coolia* species could be misleading when the identification of the species is a concern (Leaw *et al.*, 2010). For example, a strain from New Zealand, considered as *C. monotis* (Fernandez *et al.*, 1996), has been suggested to be *C. malayensis* (Leaw *et al.*, 2010). In addition, phylogenetic analysis carried out by Leaw *et al*. (2010) suggested that the putative *C. monotis* from Belize and Indonesia may correspond to a novel taxon, which highlights the large intraspecific variability of the genus *Coolia*. This high genetic variability of *Coolia* strains was observed by Penna *et al*. (2005), who suggested that Mediterranean strains are differed from those encountered in Asia and Florida.

Benthic dinoflagellates have been suggested to be rich in toxins (Nakajima *et al.*, 1981). Nakajima *et al*. (1981) and Rhodes *et al*. (2000) determined the activity of *Coolia* toxins by bioassays whereby organic compounds extracted from a number of *Coolia* cells caused an haemolytic effect on mouse blood cells and mortality of *Artemia*. The first toxin identified from *C. monotis* was cooliatoxin (suggested to be a mono-sulphated analogue of yessotoxin) which Holmes *et al*. (2001) referred to as a potent cardiac stimulant causing death in mice. On the other hand, Rhodes *et al.* (2000) showed that the strain *C. monotis* from New Zealand did not produce cooliatoxin, but they identified two unknown analogues of a polyether compound. Moreover, Liang *et al*. (2008) determined a secondary metabolite (toxin) produced by *Coolia*, namely cooliatin, considered an unprecedented dioxocyclononane obtained from the chloroform extract from *C. monotis* cells and its chemical structure was identified by spectroscopic methods.

Richlen and Lobel (2011) suggested that population dynamics and the influence of environmental parameters on the benthic habitat of ciguatera-producing organisms are not well characterized. In UK waters, there are still many uncertainties regarding environmental factors that might trigger the proliferation of blooms of *C. monotis*. In addition, there is a poor understanding to date of chemical compounds synthesized by *Coolia* isolates worldwide. Therefore, Ignatiades and Gotsis-Sketas (2010) has proposed that *C. monotis* remains a potentially toxic microalgae despite the lack of evidence of toxic blooms in the environment and any notable incident of toxicity to marine organisms. Since *C. monotis* is able to grow optimally at high temperature (>25 °C), there is a concern that climate change may increase the distribution and occurrence of the harmful algae.

5.1.2 Taxonomical description

Coolia monotis is a round cell in apical (Figure 5.1B-C) and antapical view (Figure 5.1A). In ventral view, the anterior-posterior axis is oblique and compressed (Aligizaki and Nikolaidis, 2006), where both the sulcus and cingulum are excavated (Figure 5.1B-C). *C. monotis* possesses an equatorial and narrow cingulum where a number of pores are lined around the cingulum (Vale *et al.*, 2009). The epitheca is oblong and bigger than the hypotheca (Aligizaki and Nikolaidis, 2006). Cells are composed by an arrangement of unequal thecal plates with sparsely thecal pores on the

surface (Aligizaki and Nikolaidis, 2006). The sulcus of *C. monotis* is indented, narrow, and with left and right sulcal lists in ventral view (Laza-Martinez *et al.*, 2011). Faust (2009) found that the vegetative cells of *C. monotis* varied from 25 to 45µm in diameter and 30 to 50µm in length. Cells exhibit a nucleus situated dorsoventrally in the hypotheca (Vale *et al.*, 2009). Cells cultured in F/2 (this study) reproduced by cellular division whereby daughter cells might be smaller in size during the exponential growth phase.

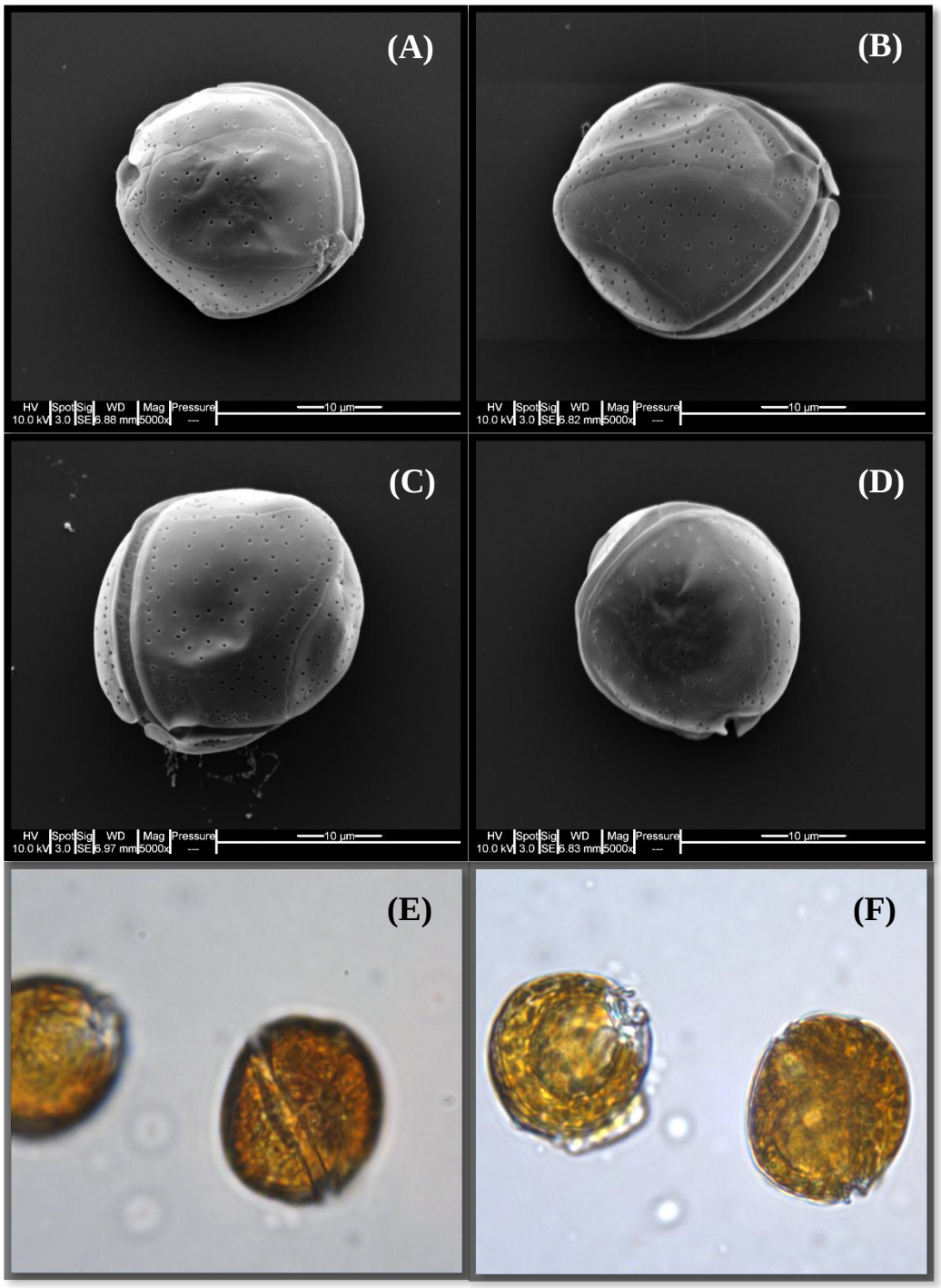

Figure 5.1 SEM (A-D) and LM (E-F) microphotographs of the isolate *Coolia monotis* grown in cultures with F/2 media. Antiapical view of a single cell of *C. monotis* (A). Apical view of *C. monotis* cells (B-D). *C. monotis* cell viewed under LM showing a deep cingulum (E). Cells viewed under LM (F).

5.2 Results

Clonal cultures of the epibenthic dinoflagellate *Coolia monotis* were maintained at growth temperatures between 5 and 30 °C where the growth rate (μ), photosynthetic efficiency (*Fv*/*Fm*), nutrient uptake (NO_3+NO_2 and PO_4,), and chlorophyll *a* content were measured. Additionally, the toxicity of the microalgae *C. monotis* was investigated in bioassays using the brine shrimp *Artemia salina*, harpacticoid copepods *Tigriopus californicus*, and chicken red blood cells.

5.2.1 Temperature and cell growth in *C. monotis* cultures

The initial cell concentration in replicate cultures was as follow: 0.79×10^3 to 1.15×10^3 at 5 °C; 0.94×10^3 to 1.13×10^3 at 10°C; 0.06×10^3 to 0.16×10^3 at 15 °C; 0.13×10^3 to 0.22×10^3 at 20 °C; 0.15×10^3 to 0.19×10^3 at 25 °C; 0.24×10^3 to 0.28×10^3 at 30 °C. *C. monotis* was successfully grown in F/2 media where both maximum growth rates and cell production in cultures were influenced by growth temperature (5 to 30 °C). Figure 5.2 shows the increase of cell density and fluctuations of *Fv*/*Fm* during the cellular growth of *C. monotis* in replicate cultures maintained under a range of growth temperatures (5 to 30 °C). Cells inoculated in cultures at temperatures higher than 10 °C commenced exponential growth after day 3 while cells incubated at 5 °C grew exponentially only two weeks after being inoculated in the media.

Table 5.1 includes both the period (in days) and change of cell density (cells ml^{-1}) during the exponential growth experienced by cells of *C. monotis* in cultures incubated between 5 and 30 °C. Additionally, Table 5.1 compares the growth rates determined between replicate cultures and growth temperatures. As similar growth rates were determined between 15 and 25 °C (Table 5.1), it was decided to grow three additional cultures at 20 °C to ensure that the maximum growth rate determined at this temperature was consistent with values found in the first growth experiment. However, growth rates determined additionally at 20 °C were almost 2-fold higher in comparison to rates found in the first growth experiment (Table 5.1), despite similar exponential growth period in both experiments. Apart from measuring cell density in the additional

cultures incubated at 20 °C, no other parameter was monitored in these cultures (plots not shown).

The majority of cultures exhibited an exponential growth phase that lasted between 10 and 16 days, except for one case at 30 °C which showed the shortest exponential growth (6 days, Table 5.1 and Figure 5.2). The growth of *C. monotis* was notably impaired at 5 and 30 °C since cell increase did not produce a substantial cellular change, in fact the initial cell density inoculated at both 5 and 30 °C hardly doubled by the end of the growth period. In addition, cells grown at the lowest (5 °C) growth temperature were characterized by a decreasing of *Fv/Fm* throughout the growth of *C. monotis*, and by a rapid decrease of *Fv/Fm* noted at 30 °C (Figure 5.2).

Table 5.1 Growth kinetics of *Coolia monotis* during the cell growth in F/2 media cultures maintained at growth temperatures between 5 and 30 C.

Temperature (°C)	Time of exp. growth (days)	Exp. change in cell density ($\times 10^3$ cells ml^{-1})	Growth rate (μ, d^{-1})
5	11	0.66-0.73	0.01
	11	0.69-1.28	0.06
	14	0.76-1.14	0.03
10	12	2.43-4.80	0.06
	12	2.30-5.06	0.06
	12	2.05-5.24	0.07
15	16	0.31-2.54	0.13
	16	0.43-3.60	0.14
	12	0.32-3.14	0.17
20	12 (13)	0.30-2.11 (0.13-9.24)	0.15 (0.32)
	12 (13)	0.28-2.20 (0.13-9.10)	0.15 (0.32)
	12 (13)	0.27-2.44 (0.13-10.0)	0.15 (0.32)
25	16	0.38-4.58	0.15
	16	0.47-5.53	0.15
	16	0.35-4.46	0.15
30	10	0.28-0.82	0.04
	6	0.28-0.63	0.06

Coolia cells showed increasing values of *Fv*/*Fm* throughout the exponential growth phase at temperatures from 10 to 25 °C, with *Fv*/*Fm* peaking towards the end of the

exponential phase. Then, the higher the culture temperature (10 to 30 °C), the faster *Fv*/*Fm* dropped particularly during the stationary phase (Figure 5.2).

Cell growth and *Fv*/*Fm* data from replicate cultures shown in Figure 5.2 were averaged and Figure 5.3 shows averaged data of both parameters. Error bars of cell growth and *Fv*/*Fm* shown in Figure 5.3 suggested that cell growth and *Fv*/*Fm* followed a similar trend in replicate cultures. It is worth to noting that at growth temperatures between 10 and 25 °C, *C. monotis* maintained an exponential growth for up to 3 weeks and *Fv/Fm* paralleled the increase of exponentially growing cells in general.

Average cell abundance in the stationary phase showed the same order of magnitude at 5 °C (0.95×10^3 ±140 cells ml^{-1}) and 30 °C (0.64×10^3 ±51 cells ml^{-1}), while an increased abundance was determined for cells at 10 °C (4.71×10^3 ±435 cells ml^{-1}), 15 °C (5.01×10^3 ±403 cells ml^{-1}), 20 °C (5.40×10^3 ±434 cells ml^{-1}), and 25 °C (5.05×10^3 ±200 cells ml^{-1}) during the same growth phase.

Figure 5.4 (A) compares averaged growth rates and maximum *Fv*/*Fm* values where the rise of sea water temperature (ranging from 5-20 °C) produced an increase of the growth rate and *Fv/Fm* values. The increase of growth rates from 5 to 20 °C was tested for linearity using growth data from replicate cultures, but the r^2 (0.69 p=<0.001) did not suggest a strong relationship between growth rate and temperature (Figure 5.4 B). Maximum *Fv*/*Fm* in *C. monotis* cells was at 15 °C with decreasing values gradually at higher or lower temperatures, although data at 30 °C suggested a drastic impairment of *Fv*/*Fm* in *C. monotis* cells.

As cell numbers increased in cultures of *C. monotis*, chlorophyll *a* production showed increasing concentrations with maximum values attained at an early point of the stationary phase, regardless of the growth temperature (Figure 5.5). Moreover, growth temperature influenced maximum chlorophyll *a* production in culture flasks and concentrations ranged from 30-35 µg L^{-1} at 5 °C; 133-145.8 µg L^{-1} at 10 °C; 108-153 µg L^{-1} at 15 °C; 120-183 µg L^{-1} at 20 °C (the highest chlorophyll *a* concentration produced by *C. monotis*), 130-145.8 µg L^{-1} at 25 °C, and 57.7-58.3 µg L^{-1} at 30 °C (Figure 5.5-5.6).

C. monotis produced variations of chlorophyll *a* cellular content (pg cell^{-1}) (Figure 5.7) with, in general, chlorophyll *a* content decreasing towards the stationary phase, except for cells maintained at 30 C in which chlorophyll *a* per cell particularly increased, with maximum concentration of up to 102 pg cell^{-1} (Figure 5.7).

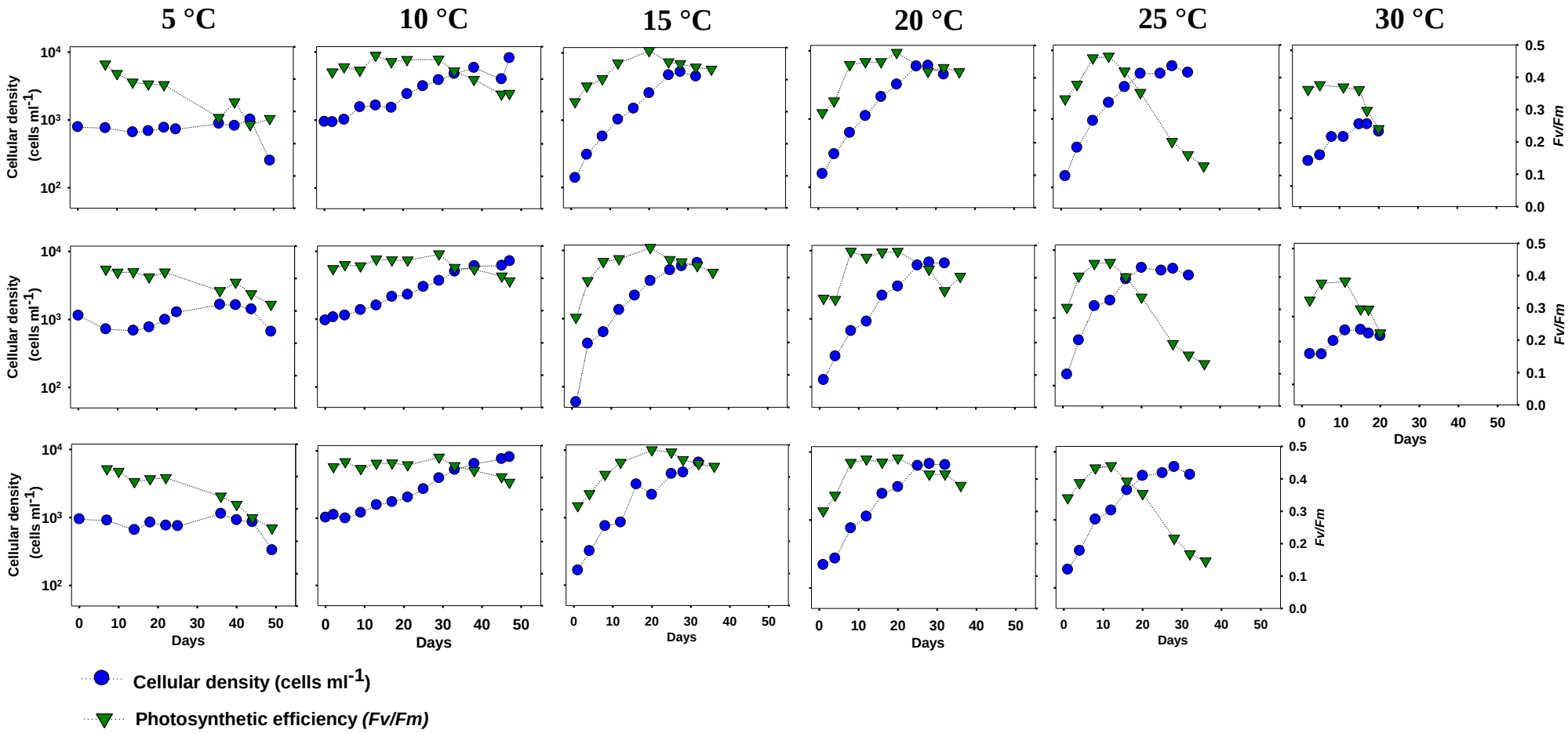

Figure 5.2 Cell growth and changes of photosynthetic efficiency (*Fv*/*Fm*) in replicate cultures of *Coolia monotis* maintained in F/2 media at growth temperatures between 5 and 30 °C. 3 replicate cultures are showed for the growth temperatures 5-25 °C and 2 replicates are shown at 30 °C.

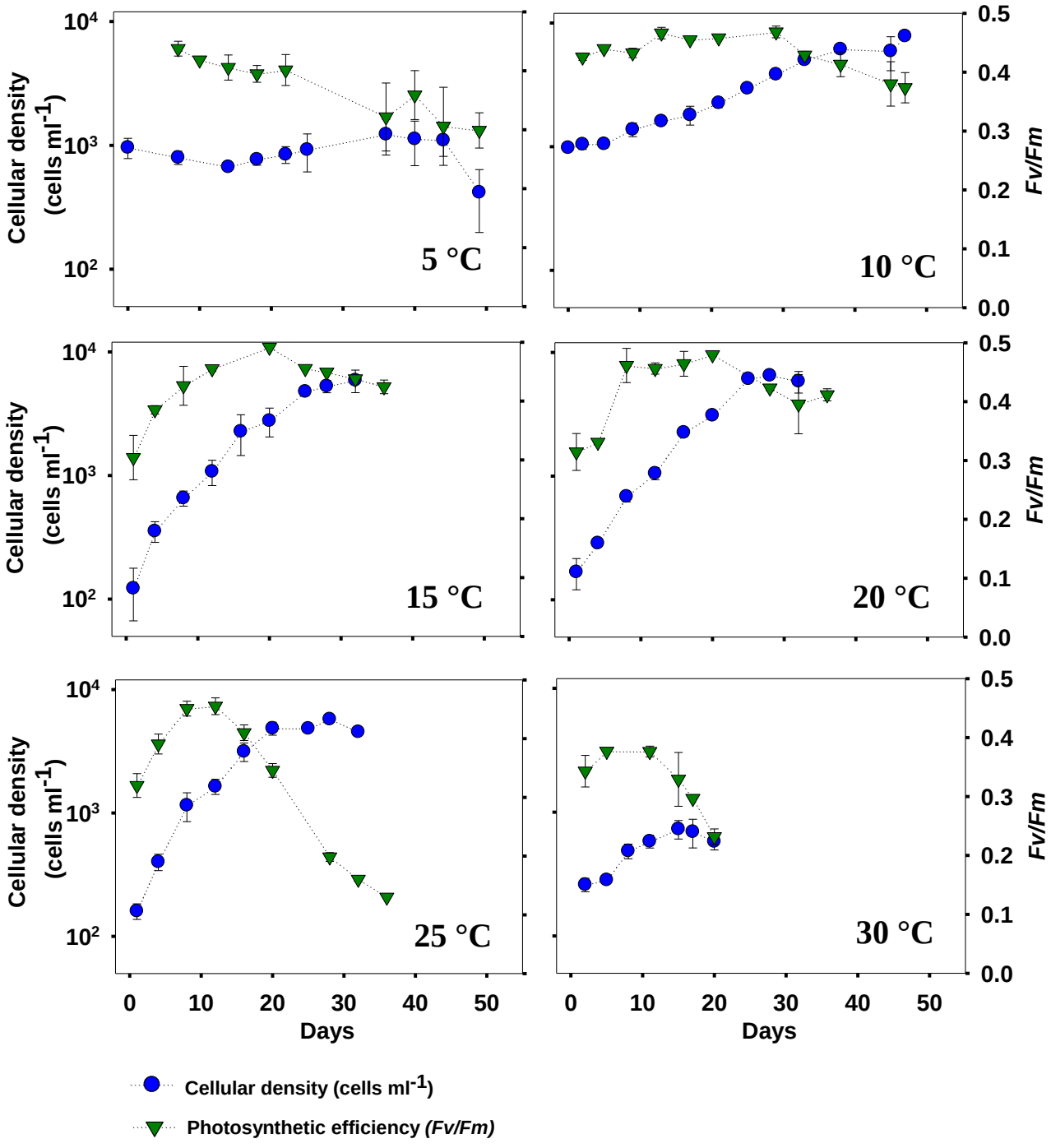

Figure 5.3 Average cell density and *Fv*/*Fm* during the cell growth of *Coolia monotis* grown in F/2 media maintained at growth temperatures between 5 and 30 °C. Bars show ± standard deviation (5-25 °C) and standard difference (30 °C).

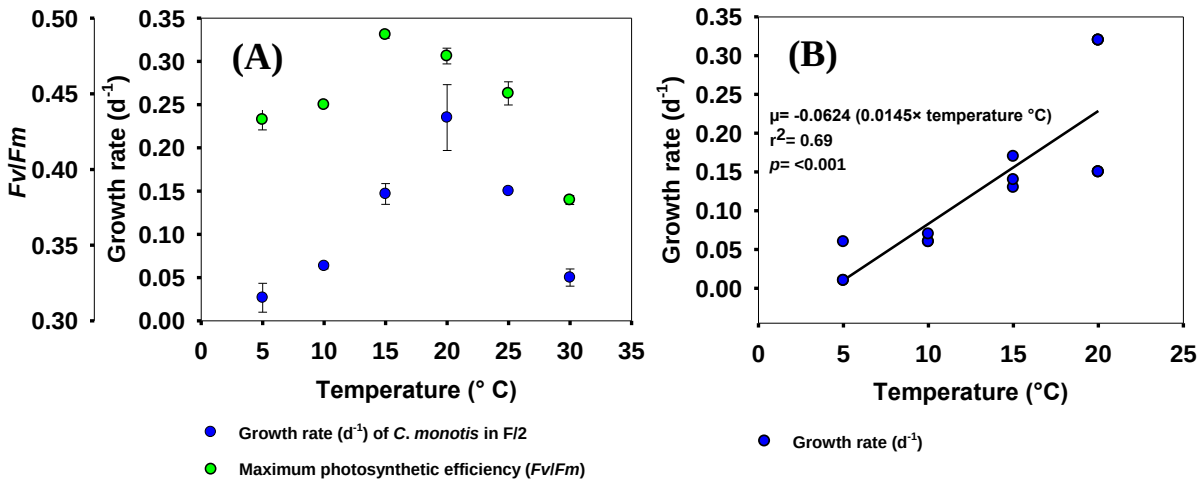

Figure 5.4 (A) Average cell growth rates and maximum photosynthetic efficiency (*Fv*/*Fm*) determined in *C. monotis* cultures incubated at growth temperatures between 5 and 30 °C. **(B)** Relationship between all growth rates and temperatures indicating a linear trend. Bars show ± standard deviation (5-25 °C) and standard difference (30 °C).

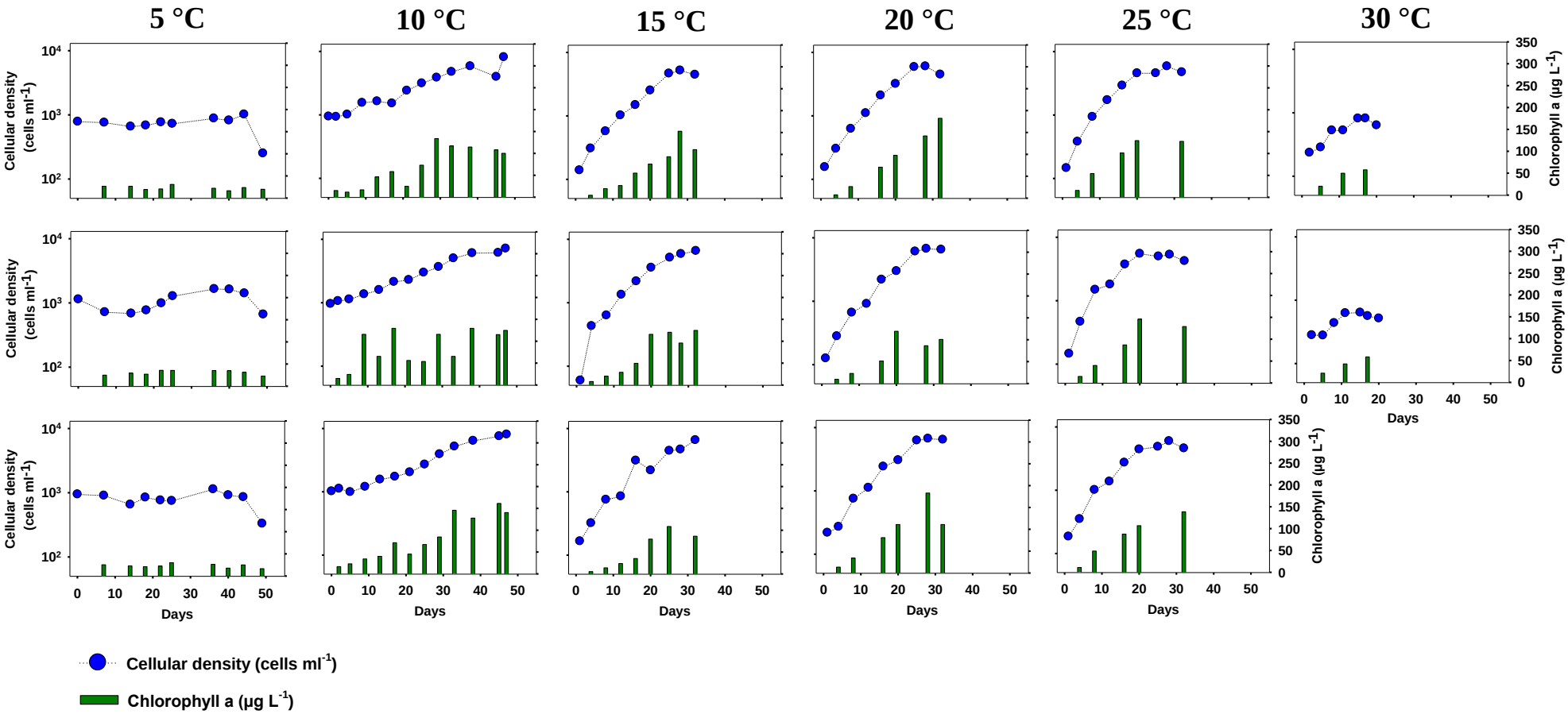

Figure 5.5 Increase of chlorophyll a (µg L^{-1}) during the cellular growth of *Coolia monotis* in replicate cultures maintained at growth temperatures between 5 and 30 °C. 3 replicate cultures are shown from 5-25 °C while 2 replicates are shown at 30 °C.

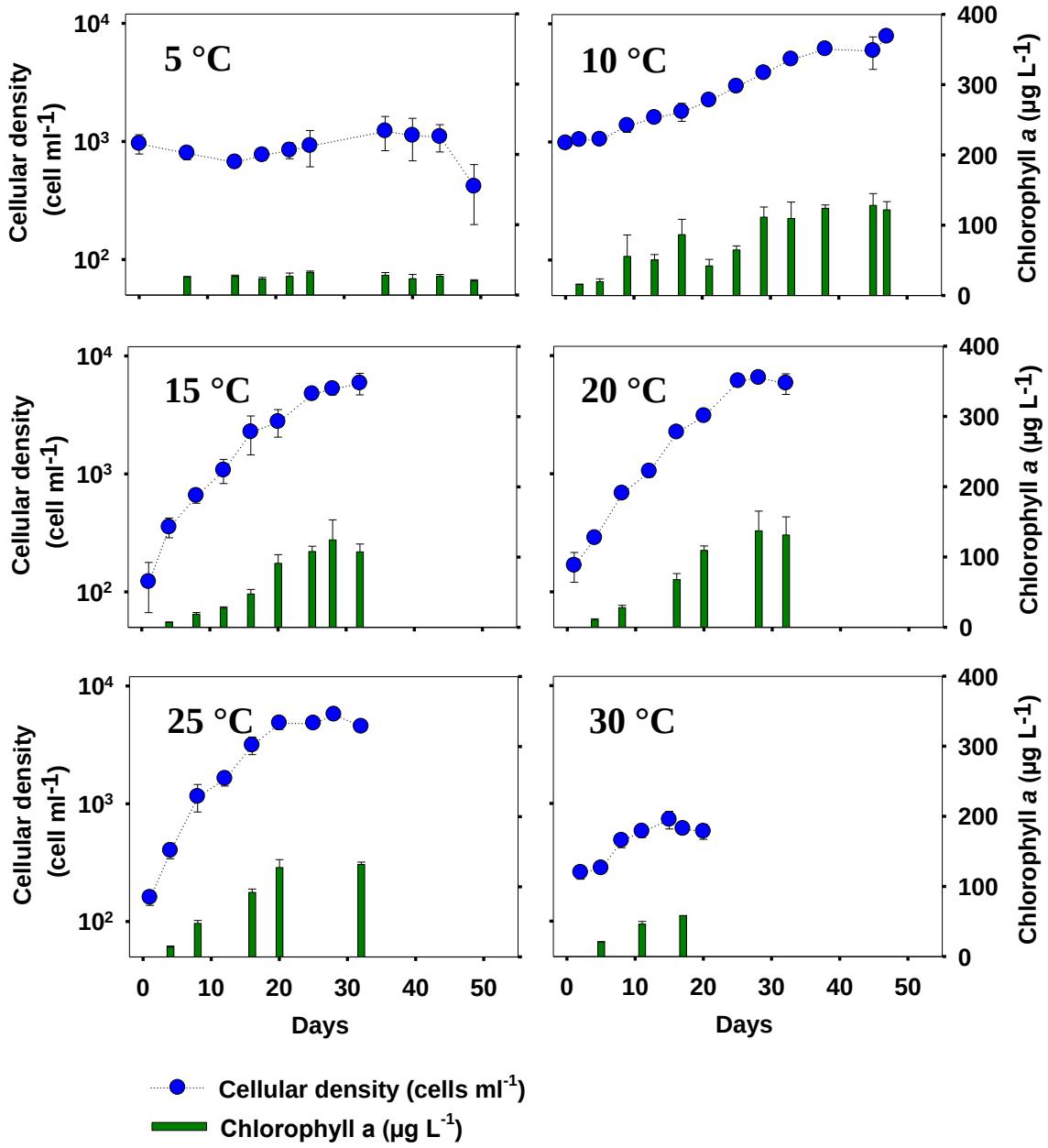

Figure 5.6 Average chlorophyll *a* (µg L^{-1}) during the cell growth of *C. monotis* in cultures incubated at growth temperatures between 5 and 30 °C. Bars show ± standard deviation (5-25 °C) and standard difference (30 °C).

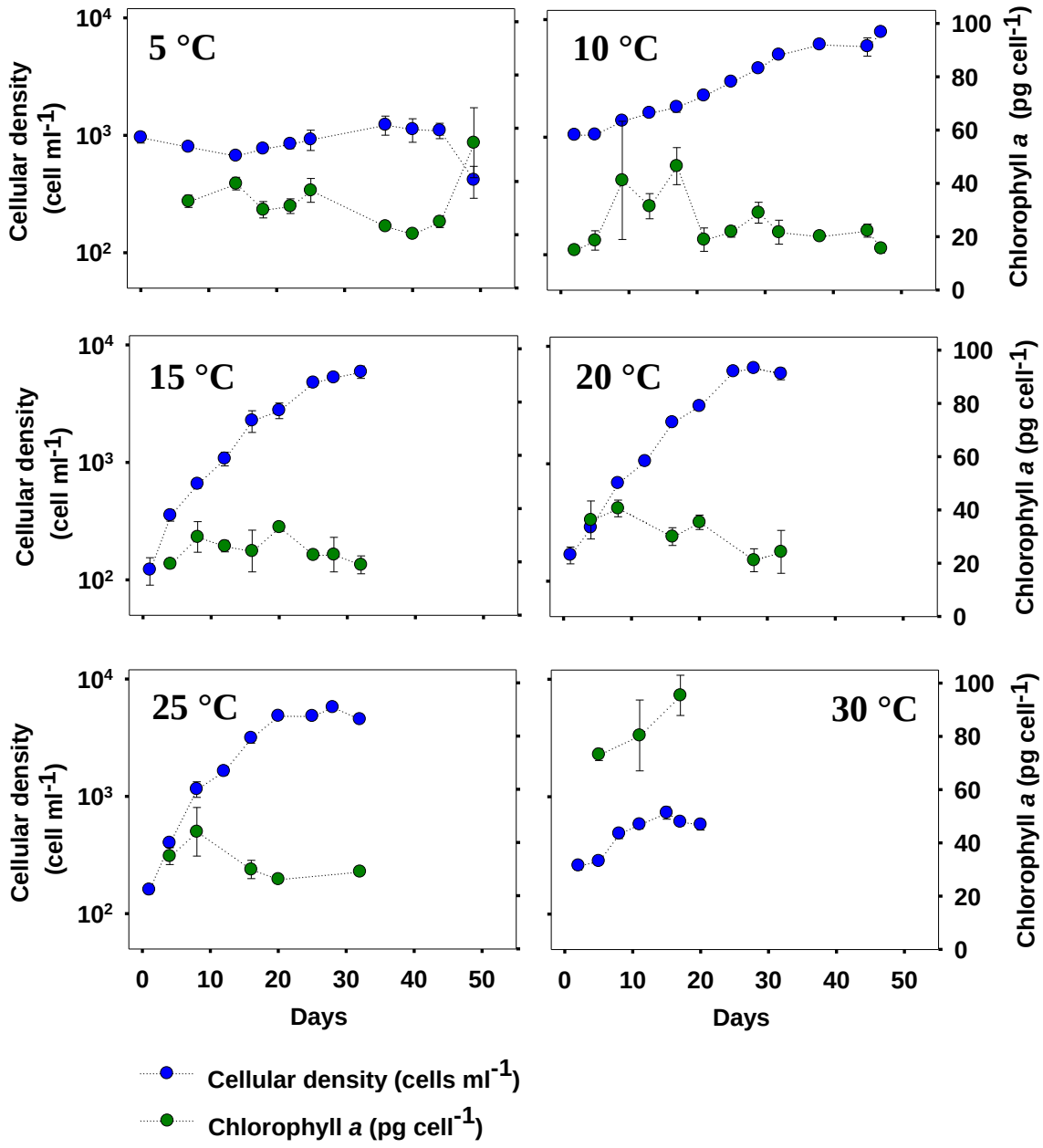

Figure 5.7 Average chlorophyll a per cell (pg cell^{-1}) during growth of *C. monotis* in cultures incubated at growth temperatures between 5 and 30 °C. Bars show ± standard deviation (5-25 °C) and standard difference (30 °C).

5.2.2 NO_3+NO_2 and PO_4 uptake

Figure 5.8 shows changes in nitrate+nitrite (NO_3+NO_2) and phosphate (PO_4) concentration during the growth of *C. monotis* cells in cultures incubated at 10-30 °C. NO_3+NO_2 and PO_4 analysis from cultures maintained at 5 °C showed high variability and unfortunately proved to be unreliable; therefore, these data have been omitted. Cell increase and growth temperature were notably associated with the decline of both NO_3+NO_2 and PO_4 in the media, particularly during exponential growth where NO_3+NO_2 and PO_4 content rapidly decreased (Figure 5.8). *Coolia* cells cultured at 10 °C were initiated in media containing 869.4 µmol NO_3+NO_2 L^{-1} and 29.1 µmol PO_4 L^{-1}, whereas cells cultured from 15-30 °C initially contained higher concentration of NO_3+NO_2 (928.3-978.4 µmol L^{-1}) and PO_4 (32.7-34.4 µmol L^{-1}). During growth experiments *C. monotis*, cells were never limited by NO_3+NO_2 concentration in the media which never decreased below 500 µmol L^{-1} in any of the growth experiments. Conversely, PO_4 was rapidly utilized and reached low concentration in the media as growth conditions for *C. monotis* experienced an increase of temperature from 10 to 25 °C. Thus, cultures at 20 and 25 °C were characterized by highest rates of PO_4 removal (Figure 5.8).

After exponential growth at 10 °C, PO_4 removal was considerably reduced and fairly constant concentrations remained throughout the stationary phase (approximately 5 µmol L^{-1}), while NO_3+NO_2 at this temperature showed unexpectedly increasing concentrations towards the initiation of the stationary phase. Although there was no indication of PO_4 depletion in *C. monotis* cultures, low PO_4 concentrations remained in cell cultures maintained at 15 °C (1.2-1.4 µmol L^{-1}), 20 °C (0.36-1.1 µmol L^{-1}), and 25 °C (1.0 µmol L^{-1}) in association with the stationary phase (Figure 5.8). NO_3+NO_2 and PO_4 data recorded from replicate cultures were averaged at each temperature and mean values of NO_3+NO_2 and PO_4 were consistent with the general pattern observed for most replicates, although some measurements suggested notable variability between replicates (Figure 5.9).

NO_3+NO_2 and PO_4 concentration showed a linear decrease in replicate cultures maintained at 30 °C, but as the growth experiment was short (<20 days) in comparison to cultures at lower temperatures, NO_3+NO_2 (>930 µmol L^{-1}) and PO_4 (>26.8 µmol L^{-1}) remained at high concentrations in the cultures at the end of the incubation period (2

weeks later, Figure 5.8-5.9). Figure 5.10 shows NO_3+NO_2 versus PO_4 concentrations changing in the media from the exponential growth phase. NO_3+NO_2/PO_4 ratio varied from 5.3 to 10.3 with lower values at higher temperatures, but all showed ratios less than Redfield ratio of 16:1 (Table 5.2)

Table 5.2 NO_3+NO_2/PO4 ratios determined during the exponential growth of *Coolia monotis* in cultures maintained at growth temperatures between 10 and 30 °C.

Growth Temperature (°C)	**NO_3+NO_2/PO_4 ratios**
10	10.3 (±3.86)*
15	9.1 (±0.95)*
20	7.0 (±0.21)*
25	5.3 (±0.24)*
30	5.4 (±0.02)+
* =standard deviation + =standard difference	

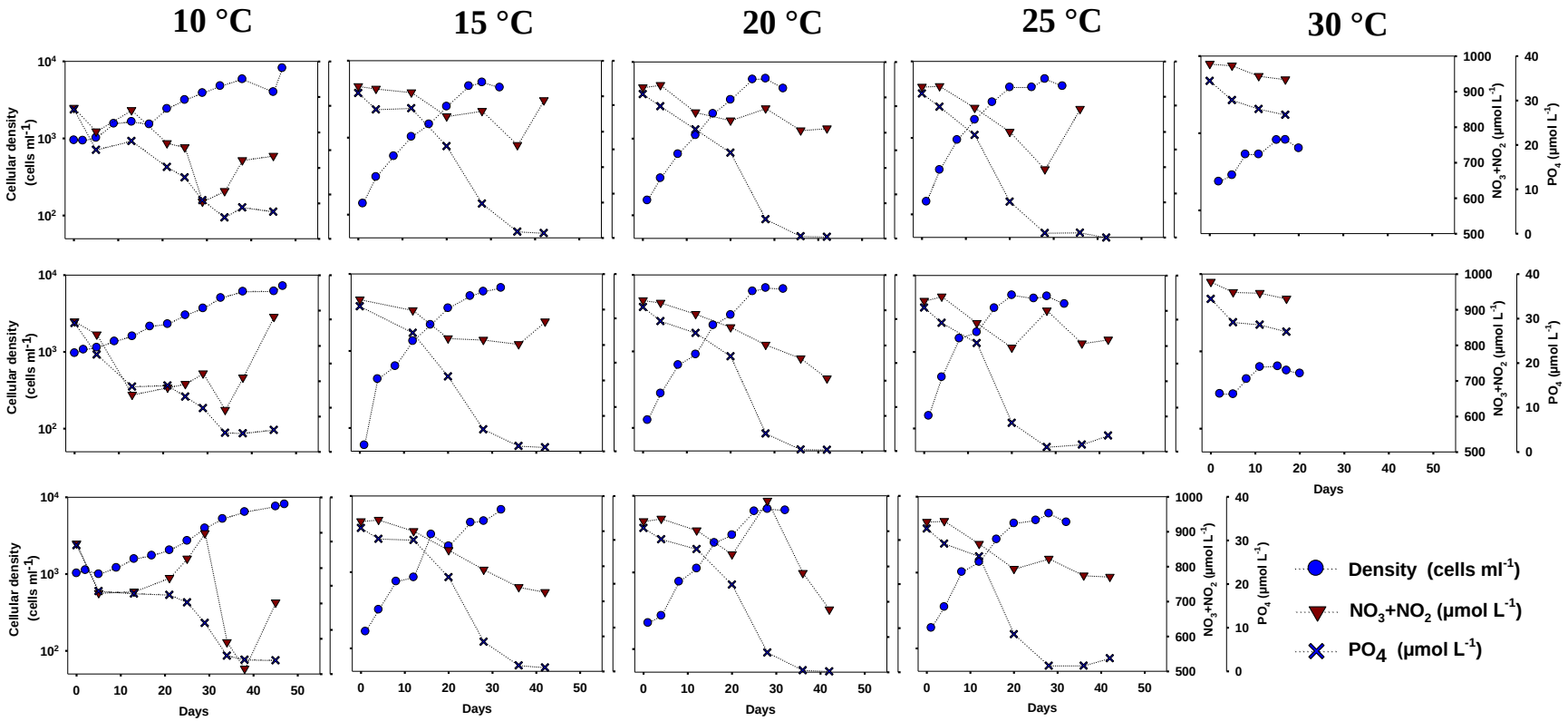

Figure 5.8 NO_3+NO_2 and PO_4 acquisition during the cellular growth of *Coolia monotis* in cultures maintained in F/2 media from 10 to 30 °C. 3 replicates are shown for the growth temperatures 10 to 25 °C and 2 replicates are depicted at 30 °C.

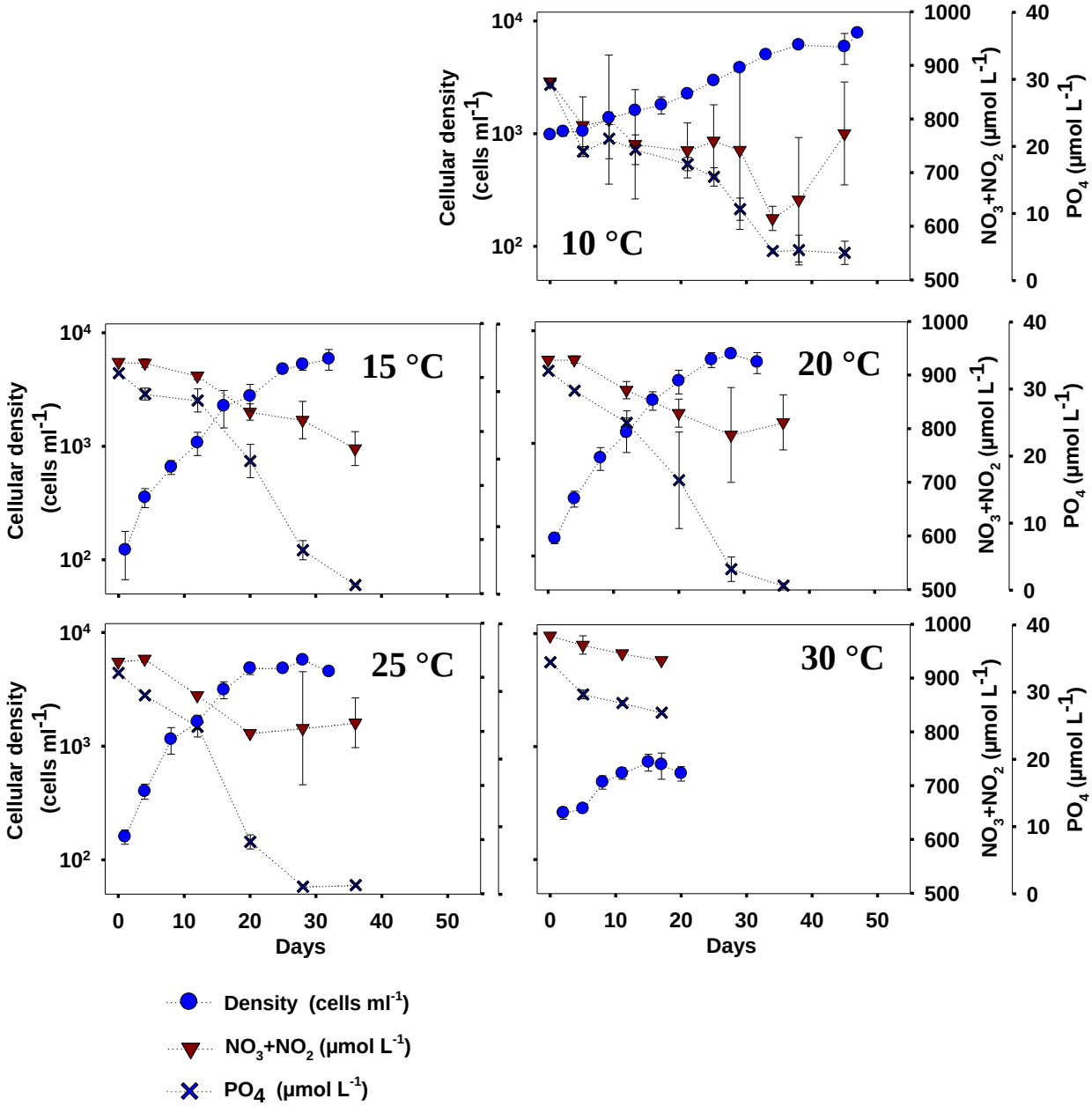

Figure 5.9 Average NO_3+NO_2 and PO4 uptake during the cell growth of *Coolia monotis* grown in cultures between 10 and 30 °C. Nutrient data at 5 °C proved to be unreliable and they are not shown. Bars show ± standard deviation (10-25 °C) and standard difference (30 °C).

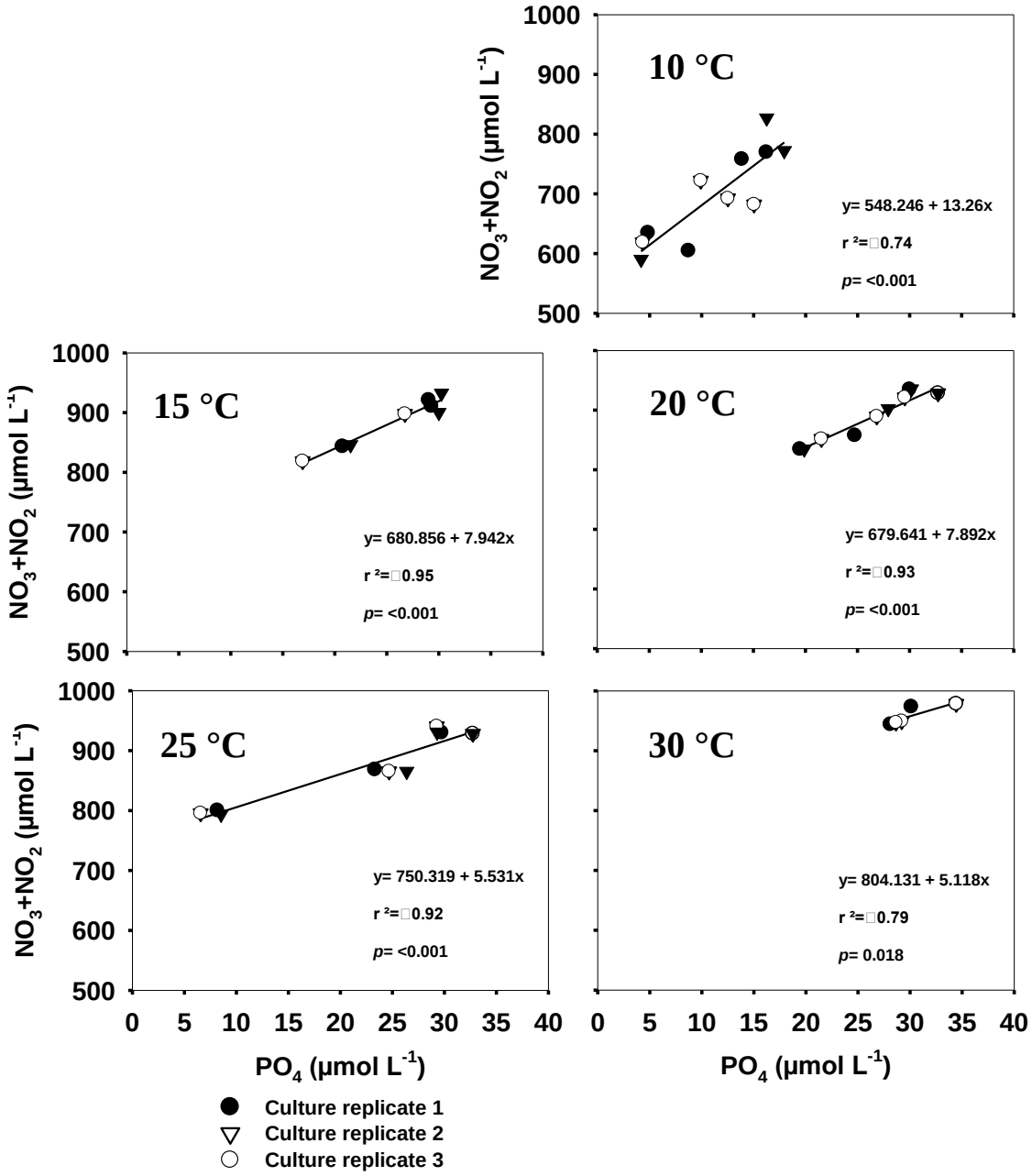

Figure 5.10 NO_3+NO_2/PO_4 relationships during the exponential growth of *Coolia monotis* in cultures grown at a range of growth temperatures from 10 to 30 °C. Symbols represent data from replicate cultures. Symbols at each growth temperature represent one replicate flask (3 replicates from 5-25 °C; 2 replicate flasks at 30 °C).

5.2.3 Potential toxicity of *Coolia monotis*

The potential toxicity of the epibenthic *C. monotis* strain was analysed using bioassays where juvenile nauplii of the brine shrimp *Artemia salina* and adults of harpacticoid copepod *Tigriopus californicus* were fed *C. monotis* cells. *A. salina* and *T. californicus* were able to digested *C. monotis* cells during the bioassays carried out in

the dark (Figure 5.11). *A. salina* was fed in triplicate with an initial concentration of 2.5×10^3 and 2.8×10^3 *C. monotis* cells ml^{-1}. Control tests were conducted in triplicate where *A. salina* individuals were fed on a mixture of non-toxic algae (particularly chlorophytes, cyanophytes and diatoms).

A. salina ingested a high number of *C. monotis* cells in the first few hours of the experiments (Figure 5.11), but towards the end of day 1, brine shrimps were generally weak and inactive or slowly moving on the bottom of the wells. *A. salina* assays inoculated with 2.8×10^3 *C. monotis* cells ml^{-1} showed more than 95% mortality on day 2, whereas 100% mortality was encountered on day 3 at concentrations of 2.5×10^3 *C. monotis* cells ml^{-1}. Controls did not show mortality within the period of the experimental assays (<4 days). The number of cells ingested by *A. salina* and cells remaining in the wells with media were not determined at the end of the experiments.

Figure 5.12 shows mortality of harpacticoid copepods when fed on different concentrations of *C. monotis*. Three experiments were carried out using different cell concentrations ranging from $0.10\text{-}5.60\times10^3$ cells *C. monotis* ml^{-1} (Figure 5.12). Six harpacticoid copepods were used at each cell concentration (treatment) and data was standardized to percentage of mortality. 100% mortality (6 copepods dead) was found between day 5 and day 9 when harpacticoid copepods experienced cellular concentrations higher than 1.37×10^3 cells *C. monotis* ml^{-1}. Copepods showed high mortality rates (>50%) at all cell concentrations from day 10 onwards, except when copepods were fed with 0.11×10^3 cells *C. monotis* ml^{-1} (Figure 5.12E). Maximum mortality encountered in control tests never surpassed 40%.

Potential toxins from *C. monotis* (yessotoxins) were analysed by LC-MS/MS, but unfortunately analysis did not provide evidence of known chemical compounds associated with yessotoxins. In addition, the LC-MS/MS method to determine potential toxins from *C. monotis* was not optimized for the whole range of toxins known from *C. monotis*.

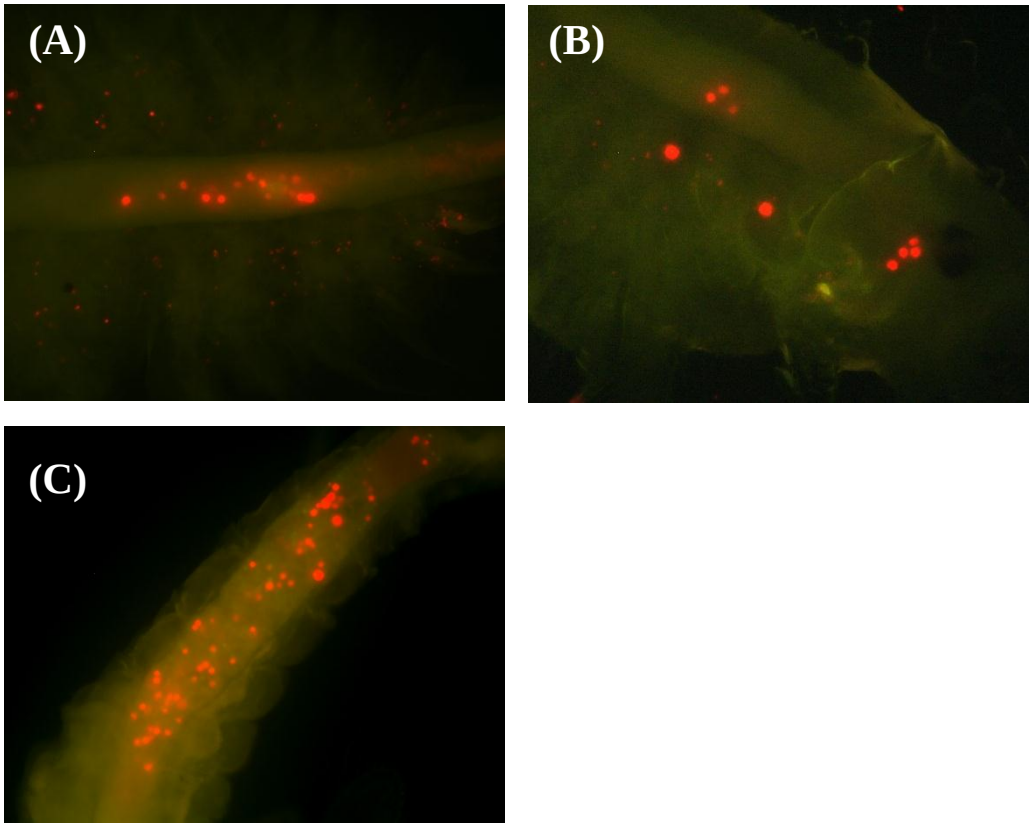

Figure 5.11 Ingestion of *C. monotis* cells by the brine shrimp *Artemia salina*. (A) Digestive tract of *A. salina* showing chlorophyll a fluorescence from *C. monotis* cells after ingestion (large red dots). (B) *A. salina* ingesting *C. monotis* cells (red dots) and small microalgae (small dots). (C) *C. monotis* cells ingested by *A. salina* during the bioassays.

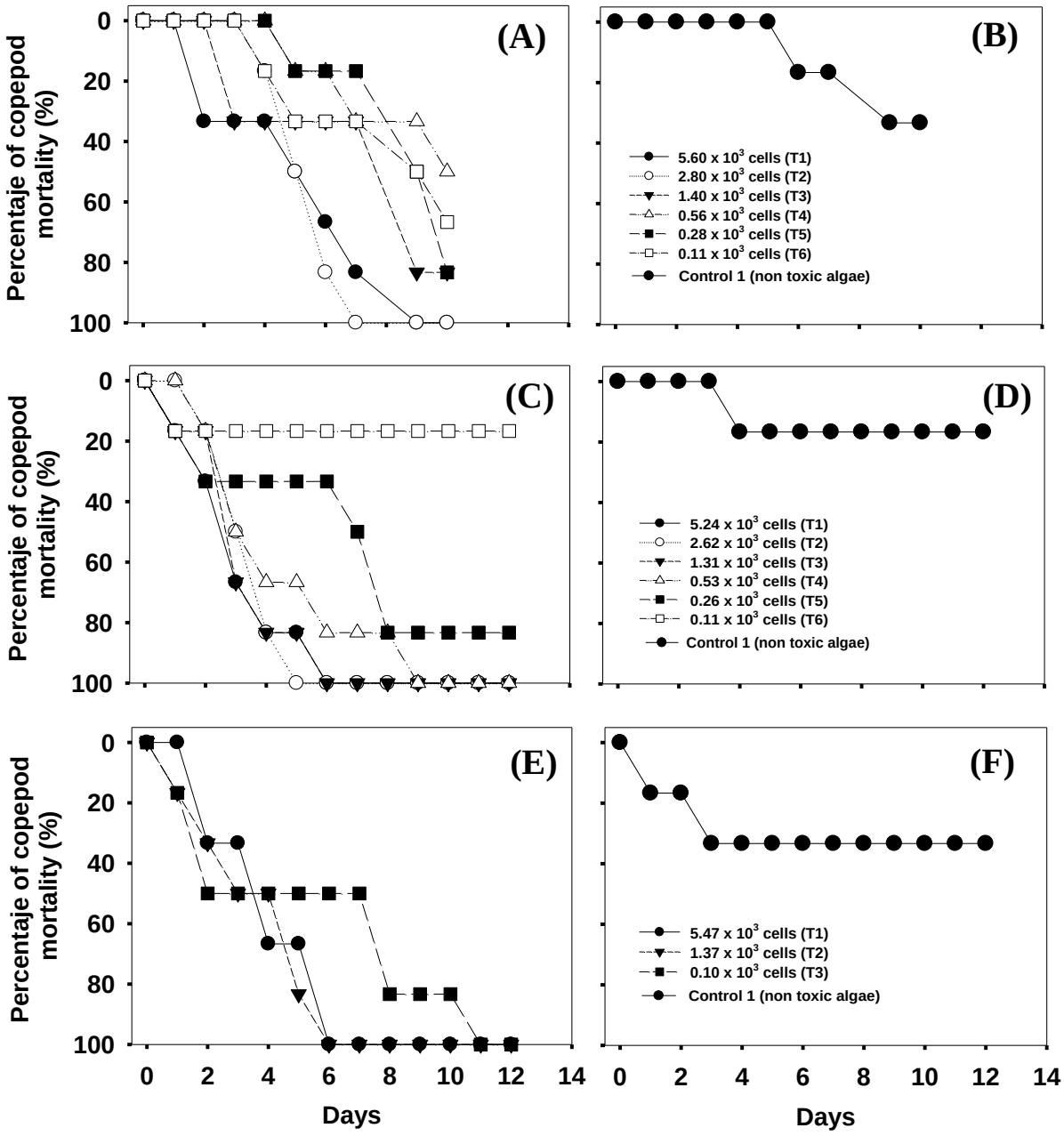

Figure 5.12 Mortality of harpacticoid copepods fed on *Coolia monotis* cells at different concentrations. T1-T6 represents different treatments where a number of *C. monotis* cells were inoculated. Six replicates (1 copepod well^{-1}) were tested for each treatment and data were standardized to percentage of mortality.

5.2.4 Haemolytic compounds from *C. monotis*

Erythrocyte Lysis Assay (ELA) was conducted to determine haemolytic compounds synthetized by the dinoflagellate *C. monotis*. Chicken red blood cells incubated with algal extracts from *C. monotis* proved that the epibenthic dinoflagellate synthetized haemolytic compounds (Figure 5.14). Algal extracts were prepared from *C. monotis* cells ranged in total from 0.30-30.0×10^3 cells ml^{-1}. ELA was tested using saponin, considered a widely known haemolytic compound, and the calibration curve is shown in Figure 5.13. Algal extracts with cell concentrations >4.0×10^3 cells ml^{-1} produced 50% of haemolysis in chicken erythrocytes (Figure 5.14). The highest cell concentration of *C. monotis* cells (30.0×10^3 cells ml^{-1}) used in the extracts produced a maximum of 77% of lysis in chicken erythrocytes (Figure 5.14).

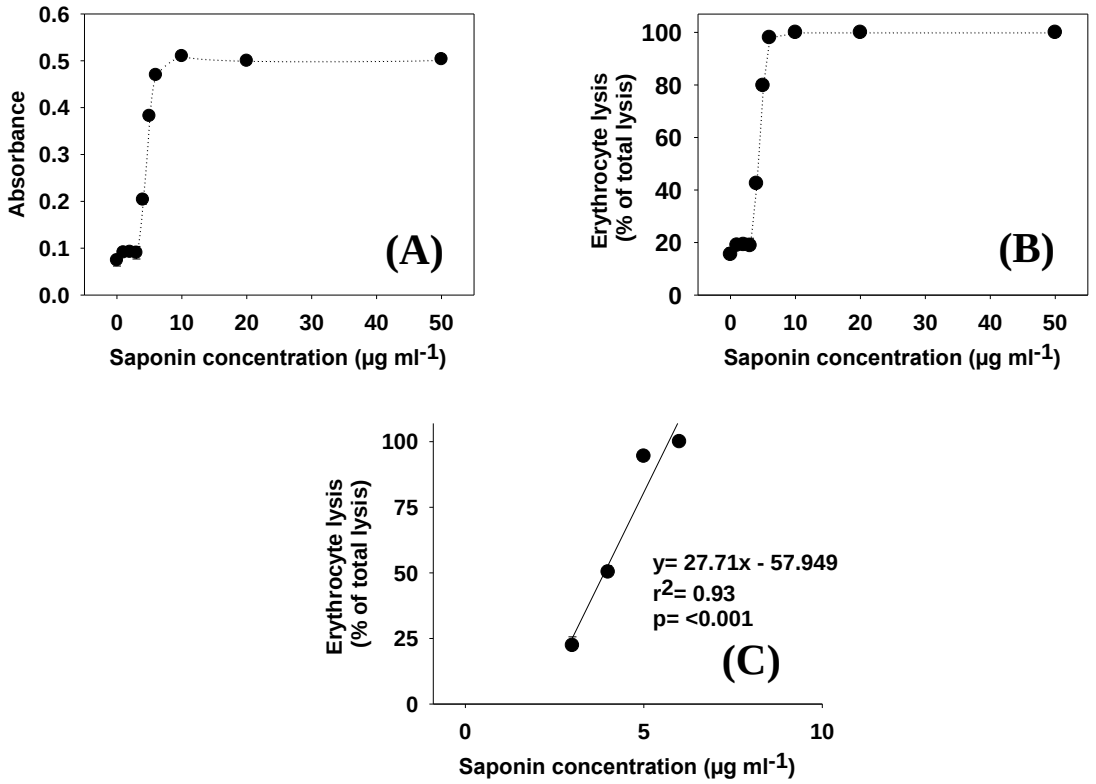

Figure 5.13 Calibration curve to test the ELA method analysed by spectrometry. **(A)** Lysis of erythrocytes (y-axis) produced at different concentrations of saponin (x-axis) determined by absorbance of haemoglobin released in the assay. **(B)** Standardized lysis of erythrocytes to percentage. **(C)** Significance of the calibration curve of ELA.

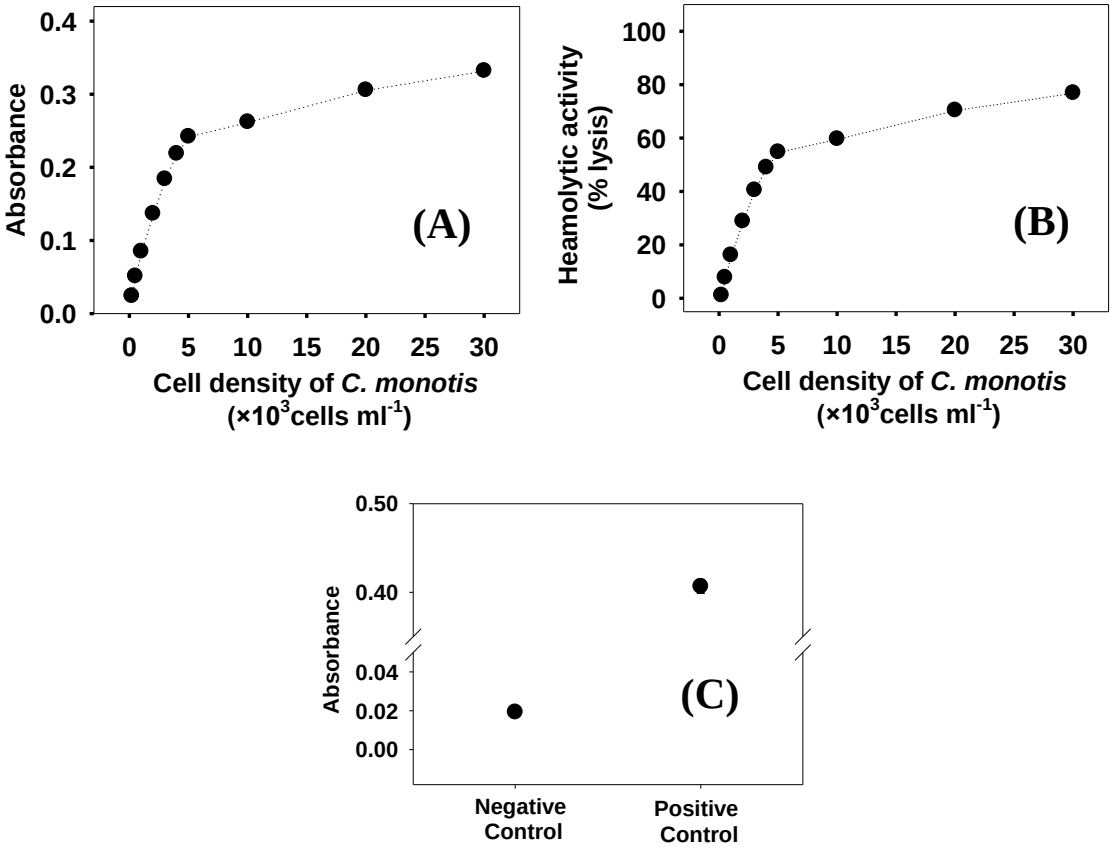

Figure 5.14 Lysis of chicken red blood cells incubated with algal extracts prepared from different concentrations of *C. monotis*. (A) Absorbance determined by lysis of erythrocytes after 18h of incubation with algal extracts (±SD). (B) Percentage of lysis produced by *C. monotis* cells. (C) Difference in absorbance between lysed (sonicated cells) and non-lysed cells (negative control) during the ELA experiment.

5.3 Discussion

5.3.1 Effect of temperature on growth rates and *Fv/Fm*

Monocultures (non-axenic) of the epibenthic dinoflagellate *C. monotis* were grown in F/2 medium at incubation temperatures between 5 and 30 °C, with the main aim of determining the effect of growth temperature on growth rates, photosynthetic efficiency, nutrient uptake (dissolved inorganic nitrogen and phosphate), and toxin production of this microalgae. *C. monotis* is considered a toxigenic dinoflagellate and is widely distributed in tropical and temperate waters sharing the same habitat with other toxin-producing microalgae (Aligizaki and Nikolaidis, 2006, Okolodkov *et al.*, 2007, Rhodes *et al.*, 2010). Although there is evidence that *C. monotis* can produce noxious effects in the environment (Nakajima *et al.*, 1981, Rhodes and Thomas, 1997, Rhodes *et al.*, 2000, Armi *et al.*, 2010) other studies have questioned whether *C. monotis* is a toxin producing species (Penna *et al.*, 2005, Laza-Martinez *et al.*, 2011). Additionally, little is known of the effects of environmental conditions on the growth and noxious toxin production of this epibenthic dinoflagellate (Armi *et al.*, 2010, Hallegraeff, 2010).

Few studies have investigated the effect of temperature, salinity, and light intensity on the growth of *C. monotis* (Morton *et al*. 1992). Algal blooms are stimulated by a complex interaction of environmental factors (Armi *et al.*, 2010) of which temperature is widely considered a determining factor in growth kinetics, physiological reactions, and population dynamics of phytoplanktonic organisms (Goldman and Carpenter, 1974, Raven and Geider, 1988, Laabir *et al.*, 2011). Table 5.3 compares previous reports where *C. monotis* growth rates have been determined in relation to a range of growth conditions. In the current study the optimal growth temperature of *C. monotis* (0.32 d^{-1}) was determined at 20 °C in F/2 medium with irradiances from 35-70 µmol m^{-2} s^{-1}, however Morton *et al* (1992) reported µ= 0.30 d^{-1} (highest growth rate) for *C. monotis* grown at 29 °C, with a light intensity of 243 µmol m^{-2} s^{-1}; Rhodes *et al*. (2000) recorded µ= 0.30 d^{-1} at 25 °C.

Table 5.3 Growth rates of *C. monotis* determined under different experimental conditions.

Species	Growth rate (μ d^{-1})	Temperature (°C)	Light	Salinity	Max. cell density (cells ml^{-1})	Culture medium	Location of isolation	Reference
Coolia monotis	0.15	33	243 µmol m^{-2} s^{-1}	33	nd	K medium (omitting Tris, copper and silica)	nd	Morton *et al*. 1992
C. monotis	0.20	31	243 µmol m^{-2} s^{-1}	33	nd	K medium	nd	Morton *et al*. 1992
C. monotis	0.30	29	243 µmol m^{-2} s^{-1}	33	nd	K medium	nd	Morton *et al*. 1992
C. monotis	0.14	27	243 µmol m^{-2} s^{-1}	33	nd	K medium	nd	Morton *et al*. 1992
C. monotis	0.10	25	243 µmol m^{-2} s^{-1}	33	nd	K medium	nd	Morton *et al*. 1992
C. monotis	0.08	23	243 µmol m^{-2} s^{-1}	33	nd	K medium	nd	Morton *et al*. 1992
C. monotis	0.3	25	100 µmol m^{-2} s^{-1}	35	nd	GP medium	Northland, New Zeland	Rhodes *et al*. 2000
C. monotis	(10 days doubling time)	15	100 µmol m^{-2} s^{-1}	27	nd	GP medium	Northland, New Zeland	Rhodes *et al*. 2000
C. monotis	(3-4 days doubling time)	23	30-90 µmol m^{-2} s^{-1}	36	2.5×10^{-3}	Erdschreiber's medium	Twin Cays, Belize	Faust 1992
C. monotis	0.01-0.32	5-30	35-70 µmol m^{-2} s^{-1}	28-33	10.0×10^{-3}	F/2	Fleet Lagoon, UK	This study

C. monotis is apparently well adapted to environmental conditions since it has been reported throughout the year in warm (Rhodes *et al.*, 2000, Okolodkov *et al.*, 2007) and temperate waters (Aligizaki and Nikolaidis, 2006, Rhodes *et al.*, 2000). In this study, *C. monotis* grew well across a wide range of temperatures and it is thought that the strain used in this study can survive even a higher range of suboptimal temperatures (<5 and >30 °C). Some members of the epibenthic taxocoenosis have been suggested to survive sub-zero temperatures, e.g. *Prorocentrum lima* (McLachlan *et al.*, 1994). Hence, it is believed that *C. monotis* may occur in the Fleet Lagoon and other aquatic environments at temperatures below 5 °C. Although most HABs do not occur at low temperatures, the result of biological adaptations at low temperatures might suggest substantial biological implications on the geographical distribution of HA in different ecosystems. This study supports that *C. monotis* may tolerate elevated temperatures (>30 °C) as this species has been found in experiments with μ= 0.15 d^{-1} at 33 °C (Morton *et al*., 1992). Despite the fact that *C. monotis* tolerates a wide range of temperatures, e.g. 5-33 °C, Rhodes *et al*. (2000) documented that a number of isolates of *C. monotis* died when cultured at temperatures higher than 35 °C.

In the marine environment, *C. monotis* has been reported to occur at high abundances associated with a range of high temperatures (29.7-32 °C) recorded in the water column (Armi *et al.*, 2010, Okolodkov *et al.*, 2007). Likewise, high temperatures (26-30 °C) have stimulated the growth of the potent toxigenic/epibenthic dinoflagellate *Ostreopsis ovata*, although the highest cellular toxicities were determined at low temperatures, between 20 and 22 °C (Graneli *et al.*, 2011). A temperature change of 2 °C caused an early initiation of blooms of *C. monotis* in Tunisian waters, but also a drop of 2 °C in the water column has been ascribed to a delay of one week as to the initiation of the bloom (Armi *et al.*, 2010). This highlights the importance of temperature, in addition to other parameters (irradiance, nutrients, water motion), on the proliferation of *C. monotis* in the environment.

Benthic dinoflagellates have been documented with high abundances in shallow areas. Richlen and Lobel (2011) demonstrated that habitat, depth, and water motion were significantly associated with both dinoflagellate abundance and community composition of epibenthic species. Since the Fleet Lagoon is a shallow aquatic ecosystem, with protected and calm sites where a number of potentially toxigenic dinoflagellates exist (*Amphidinium carterae*, *C. monotis*, and *Prorocentrum lima*),

future changes in water temperature, e.g. from climate change, may benefit rather than reduce the growth rates of toxigenic dinoflagellates.

During the growth experiments with *C. monotis*, *Fv/Fm* was diagnostic of the effect of both nutrient stress and temperature on cell growth (Parkhill *et al.*, 2001). Low (5 °C) and high (30 °C) suboptimal temperatures caused substantial decreases of *Fv/Fm* associated with reduced increase in abundance in late exponential phase. Maximum *Fv*/*Fm* at 5 and 30 °C were the lowest values recorded at all growth temperatures despite nutrients being replete. This impairment was likely caused by a lack of acclimation of *C. monotis* to these suboptimal temperatures as cells inoculated at the initiation of the growth experiments had been maintained at temperatures between 20 and 25 °C. Hallegraeff (2010) has stressed the importance of algal acclimation, whereby algae are grown for several generations at a given temperature, before isolates are exposed to experimental growth conditions. In addition, rapid evolutionary changes are known to occur when isolates are exposed to short-term experiments (Morgan-Kiss *et al.*, 2006, Lakeman *et al.*, 2009). Therefore, since *C. monotis* exhibited a low cell growth rate at 30 °C with reduced *Fv/Fm* values, it is possible that this species grows less efficiently at temperatures >30 °C. Rapid decreases of *Fv*/*Fm*, in addition, have been suggested as an inability to adjust quickly and maximize *Fv/Fm* while avoiding damaging effects of photochemical apparatus involved in regulatory processes (Morgan-Kiss *et al.*, 2006). Therefore, more evidence is required to determine the acclimation spectrum of *C. monotis* to high temperatures, with emphasis on species that might respond to the increase of sea water temperatures.

5.3.2 Nutrient consumption and growth temperature

The macronutrients nitrate and phosphate are important for dinoflagellate growth and the development of algal blooms (Gallardo-Rodríguez *et al.*, 2009). Nitrogen assimilation, for example, is essential for chlorophyll *a* synthesis (Geider *et al.*, 1998) while phosphate is required to maintain the production of amino acid and protein molecules in phytoplankton (Flynn *et al.*, 2010). However, although increasing concentration of NO_3+NO_2 and PO_4 usually enhance the growth of dinoflagellates, inhibitory effects on growth rates of HA have occurred at high nutrient concentrations (Gallardo Rodríguez *et al.*, 2009). In this study, *C. monotis* was grown in F/2 media, containing high nitrogen (NO_3+NO_2) and phosphate (PO_4) concentrations, with high

demand of NO_3+NO_2 and PO_4 as growth rate increased at increasing growth temperatures (Sterner and Grover, 1998). Since increasing temperatures (5-25° C) and high concentration of NO_3+NO_2 and PO_4 (F/2 medium) enhanced the growth of *C. monotis*, this study supports Armi *et al*. (2010) in that eutrophic waters and elevated temperatures produce suitable conditions for the optimum growth of *C. monotis* in the environment.

High cell abundances have been reported in dinoflagellate cultures maintained in F/2 medium in which the N/P ratio is 24 (Guerrini *et al.*, 2007). In this study, high nutrient uptake rate (NO_3+NO_2 and PO_4) in *C. monotis* cultures occurred as temperature increased from 15 to 25° C. Although NO_3+NO_2 showed decreasing concentrations as cell abundance increased, NO_3+NO_2 was not a limiting macronutrient in the growth of *C. monotis* at any of the growth temperatures as its concentration did not decrease below 500 µmol L^{-1}. Surplus NO_3+NO_2 in the growth of other harmful microalgae (*Protoceratium reticulatum*) cultured in F/2 medium has been reported before (Gallardo Rodríguez *et al.*, 2009). In addition, a 10-fold decrease in NO_3+NO_2 concentration, considering a basal F/2 medium, inhibited the growth by 56% of the toxic dinoflagellate *Protoceratium reticulatum* (Guerrini *et al.*, 2007).

The NO_3+NO_2/PO_4 uptake ratios encountered in this study were below the Redfield ratio and suggested an imbalance of NO_3+NO_2 and PO_4 uptake. High PO_4 uptake compared to NO_3+NO_2 was recorded at all temperatures despite the lack of depletion of these nutrients in *C. monotis* cultures.

PO_4 and CO_2 limitation in algal cultures are known to lower *Fv*/*Fm* and growth rates (Spijkerman, 2010). Despite the high PO_4 uptake rates it was not completely depleted from *C. monotis* cultures, but rather low concentrations (0.36-1.4µmol L^{-1}) were determined throughout the stationary phase at temperatures from 15-25 °C. Regardless of the lowest (5° C) and maximum (30° C) growth temperatures, *Fv/Fm* decreased before PO_4 reached low concentration, suggesting that low PO_4 concentration may induce growth limitation of *C. monotis*, additionally possible low-CO_2 conditions might have diminished the growth of *C. monotis*. Spijkerman (2010) has suggested that a colimitation of PO_4 and CO_2 occurred and limited the growth in non-aerated microalgae (*Chlamydomonas acidophila*). Since gas exchange (CO_2) in *C. monotis* cultures was limited and PO_4 reached low levels during the algal cultivation, it is

possible that CO_2 was low enough to restrain the growth of *C. monotis* before PO_4 depletion occurred. Furthermore, although *Fv*/*Fm* can indicate nutrient limitation, unfortunately this parameter is not nutrient specific (Kromkamp and Peene, 1999), hence biological interpretations of *Fv/Fm* must be made cautiously when dealing with unstable algal growth conditions.

In general, the highest rate of PO_4 uptake occurred at 25 °C (final concentration of 1-1.3 µmol L^{-1}, on Day 29). However, maximum growth rates were measured at 20 °C. In addition, the highest *Fv/Fm* values (0.48-0.49) determined at 15 °C were expected to be linked to the maximum growth rates (both in relation to the optimal growth temperature) but the latter was found at 20 °C, with average µ= 0.23 d^{-1}. However, Kromkamp and Peene (1999) have suggested that C-fixation rates and PSII electron transport rates are not a linear function at high irradiance, thus it is not possible to estimate primary production from variable fluorescence measurements. This might explain why maxima *Fv*/*Fm* values did not fit with maxima growth rates, however it is worth mentioning that difference in *Fv/Fm* values in relation to the optimum growth temperature (20 °C) were negligible, with a range from 0.48 at 15 °C to 0.49 at 20 °C. On the other hand, average growth rates of *C. monotis* did not show significant differences in the first cultures maintained between 15-25 °C (average µ=0.15 d^{-1}, see table of growth kinetics), despite the fact that temperature influenced nutrient uptake and maxima *Fv/Fm*. Considering cell abundance was enhanced at high growth temperatures, possibly a shading effect occurred more quickly at elevated temperatures decreasing *Fv/Fm* values in cells. Kolber and Falkowski (1992) have shown that *Fv/Fm* is affected by environmental factors such as light and nutrient availability. As for benthic dinoflagellates, the effect of light on *Fv/Fm* and nutrient uptake rates is unexplored and more studies are required to determine the interaction of environmental parameters on the dynamics of benthic microalgae communities. For example, solar UV radiation greatly affects phosphate uptake and little understanding exists as to UV impact on benthic ecosystem communities (Aubriot *et al.*, 2004).

5.3.3 Toxicity of *C. monotis*

A number of studies have demonstrated that *C. monotis* is a toxin-producing microalgae (Nakajima *et al.*, 1981, Holmes *et al.*, 1995, Rhodes and Thomas, 1997, Sugg and VanDolah, 1999). Holmes *et al*. (1995) characterized the first toxin of *C. monotis*, named cooliatoxin (a mono-sulphated polyether toxin), purified from cultures of a strain isolated from Australia. However, some studies have documented that not all strains are toxic (Penna *et al.*, 2005, Laza-Martinez *et al.*, 2011). Although *C. monotis* is considered as a ciguatera producer, difficulties have been encountered to identify and characterize the structure of compounds produced by this harmful algae (Daranas *et al.*, 2001b). As a result, a bioassays have been used to determine quantitative data of potential toxins produce by some microalgae (Sugg and VanDolah, 1999).

In this study, feeding experiments (using the brine shrimp *Artemia salina* and harpacticoid copepod *Tigriopus californicus*) and an erythrocyte lysis assay (ELA) indicated that the *C. monotis* isolate is a toxin-producing microalgae. Toxicity assays carried out by Rhodes and Thomas (1997) demonstrated that *C. monotis* caused morbidity (62%) and mortality (16%) in *A. salina* after 8-12 h. This study encountered 95% of morbidity after 24 h and 100% of mortality after 48 h at concentrations higher than 2.8×10^3 cell ml^{-1}, whereas at lower cell concentrations (2.5×10^3 cell ml^{-1}) *C. monotis* produced 100% mortality on day 3. *C. monotis* was shown to be ingested by *A. salina* as microphotographs showed high concentration of algae cells in *A. salina* guts. Rhodes *et al*. (2000) utilized a supernatant (3.3 litres) from a *C. monotis* culture with 2.1×10^7 cell ml^{-1} and determined rapid and high mortality of mice (< 4 min) after injecting 100µl of the algal extract intraperitoneally.

Copepods have previously been fed on toxic dinoflagellates and results showed lower somatic growth, size at maturity, egg production, reproduction, and survival (Ianora *et al.*, 1999, Colin and Dam, 2004, Kozlowsky-Suzuki *et al.*, 2009). Although this study determined mortality of copepods when fed on *C. monotis* cells, results were inconclusive in that cell concentrations did not show a relationship with mortality rates. Previous reports have shown that copepods did not experience incapacitation or adverse effects from ingested toxins of most HA (Teegarden and Cembella, 1996, Senft *et al.*, 2011). In addition, it is been suggested that some toxins can undergo metabolic

transformation in the guts of grazers whereby toxins become less harmful after ingestion (Teegarden and Cembella, 1996). As for this study, it is argued that discrepancies found in mortality rates may well be related to the combination of the toxin burden ingested and potential toxin resistance of harpacticoid copepods. However, since LC/MS-MS analyses did not detect any toxin synthetized by the isolate *C. monotis*, this study is somewhat limited to confirm that *C. monoti*s was toxic to harpacticoid copepods.

Haemolytic activity measurements have been widely applied using HA to detect and quantify the potency of ichthyotoxins from microalgae (Eschbach *et al.*, 2001). In this study, haemolytic assays determined that *C. monotis* extracts produced >50% of erythrocyte lysis when using cell concentrations higher than 4.0×10^{3} cells ml^{-1}. This result agrees with Nakajima *et al*. (1981) in terms of detection of haemolytic compounds from *C. monotis* cells, although these authors applied higher cell concentrations (10^{8} cells ml^{-1}). Although there is evidence that *C. monotis* is a potentially toxin-producing microalgae, there are still uncertainties to determine to what extent *C. monotis* biotoxins can affect the environment. Furthermore, a better understanding of the mechanisms of toxin production is required as well as the applicability of experimental results to aquatic ecosystem.

5.4 Conclusions

In this study the effect of increasing sea water temperature resulted in increasing growth rates of the isolated *Coolia monotis* cells. Maximum influence of temperature on the growth was determined from 5 to 20 °C. However, this study did not encounter a strong linear relationship between temperature and growth rates. The isolate *C. monotis* from the Fleet Lagoon was characterized by low growth rates and the optimum growth temperature was determined to be 20 °C, although several reports have shown that *C. monotis* reached high growth rates at temperatures >25 °C. Unlike other reports, in this study *C. monotis* showed low growth rates at elevated temperatures (25-30 °C) with decreasing values of *Fv/Fm* in comparison to values at lower temperatures (25-30 °C).

Despite the slow growth rate of *C. monotis* and the lack of a linearity relationship between temperature and growth rate, *C. monotis* was shown to tolerate a wide range of growth temperatures and potentially this strain might increase its growth rate under elevated temperatures.

C. monotis demonstrated a high NO_3+NO_2 and PO_4 uptake at increasing growth temperatures. Despite its low growth rates, *C. monotis* was characterized by long exponential growth periods (up to 3 weeks), with a rapid removal of PO_4 over NO_3+NO_2 when cultures were grown in F/2 medium. NO_3+NO_2 remained at high concentrations throughout the stationary phase at all growth temperatures, meaning surplus of nitrogen content in F/2 media for the growth of benthic dinoflagellates such as *C. monotis*. In addition, cell growth did not produce PO_4 limitation and there was little evidence that PO_4 had caused the initiation of the stationary phase. Therefore, this study estimates that *C. monotis* cells were deprived from a source of inorganic carbon (CO_2 limitation). Otherwise, this study suggests that *C. monotis* did not tolerate pH changes experience during the cell growth of this species under controlled conditions.

Since *C. monotis* showed a broad tolerance to a range of temperatures and nutrient conditions, it is thought that this harmful algae will more likely produce high cell concentrations in coastal waters circumscribed to eutrophic conditions.

Unfortunately this investigation was not able to analyze potential toxins of *C. monotis* by LC-MS/MS. Despite some *C.monotis* strains have been recognized as non-toxic, this study found that *C. monotis* caused mortality of harpacticoid copepods and the brine shrimp *Artemia salina*. However, this study was limited in relating copepod

mortality with cell concentration. The potential toxicity of *C. monotis* was confirmed, however, based on the effect of the haemolytic activity that *C. monotis* cells produced on chicken red blood cells.

CHAPTER 6

6.1 Review of benthic dinoflagellate strains (temperature-growth)

This aim of this study was to determine the effect of increasing growth temperature on the growth and toxin production of the epibenthic toxigenic dinoflagellates *A. carterae*, *C. monotis*, and *P. lima* isolated from the Fleet Lagoon. Increasing growth temperatures between 5 and 30 °C were used in cultures of *A. carterae*, *C. monotis*, and *P. lima* to estimate the likelihood of higher microalgal growth rates due to warmer seawater temperatures in the Fleet Lagoon. Seawater warming is considered a major factor that may lead to the increase of future outbreaks of HABs worldwide (Peperzak, 2003, Hallegraeff, 2010). In addition, some studies have reported that seawater warming can stimulate an increase in toxin production of benthic microalgae (Graneli *et al*., 2011, Ashton *et al*., 2003). Table 6.1 compares the effect of increasing growth temperature on the specific growth rate of the three benthic dinoflagellates isolated from the Fleet Lagoon. The range of temperature produced differences in the growth rates of the isolated *A. carterae*, *C. monotis*, and *P. lima*. *A. carterae* exhibited rapid growth rates at elevated temperatures and this species was considered a fast growing dinoflagellate in comparison to the rates encountered for *C. monotis* and *P. lima* (Table 6.1). Growth rates determined in this investigation corresponded with previous reports (see Tables 3.4; 4.7; 5.3 in chapters 3-5) and this study highlights the adaptability of the benthic dinoflagellates for growth at a wide range of temperatures. Therefore, *A. carterae*, *C. monotis*, and *P. lima* possess a remarkable plasticity that has enabled a wide distribution of these organisms (Gienapp *et* al. 2008) in diverse ecosystems from tropical to temperate waters. This could be considered indicative of the high genetic diversity that assemblages of epibenthic dinoflagellates exhibit in the environment (Lakeman *et al*. 2009).

Figure 6.1 depicts the influence of increasing growth temperature on the growth of *A. carterae*, *C. monotis*, and *P. lima* in non-aerated cultures at growth temperatures between 5 and 30 °C. Montagnes *et al*. (2003) determined that many planktonic and benthic microalgae show linear growth rates at increasing growth temperatures. Results from this study have shown that *A. carterae*, *C. monotis*, and *P. lima* exhibited linear

growth rates in a range of temperatures. Table 6.2 presents the temperature range of linear growth of the benthic dinoflagellate cultures in relation to temperature by the coefficient of determination (r^2). *A. carterae* was the only dinoflagellate that showed the highest growth rates at the highest growth temperatures (25 and 30 °C), while temperatures higher than 25 °C produced decreasing growth rates in both *C. monotis* and *P. lima*. The latter exhibited the highest growth rates with almost similar values at 15 and 25 °C, while 20 °C was recorded as the optimum growth temperature of *C. monotis*. The notable difference of growth rate between *A. carterae* and both *C. monotis* and *P. lima* is in agreement with the assumption that small cell sized dinoflagellates exhibit higher growth rates than large cells. Cell yield in cultures proved that *A. carterae* reached higher cell abundance than *C. monotis* and *P. lima* when grown in nutrient replete conditions between 5 and 30 °C and maximum cell numbers are shown in Table 6.2.

Table 6.1 Average growth rates (μ, div^{-1}) determined in culture flasks of *A. carterae*, *C. monotis*, and *P. lima* maintained at growth temperatures between 5 and 30 °C. Standard deviation in replicate cultures is shown in brackets.

Growth temperature (°C)	*A. carterae* μ= d^{-1} (SD)	*C. monotis* μ= d^{-1} (SD)	*P. lima* μ= d^{-1} (SD)
5	0.14 (0.01)	0.03 (0.03)	0.05 (0.01)
10	0.29 (0.01)	0.06 (0.01)	0.13 (0.01)
15	0.37 (0.03)	0.15 (0.02)	0.18 (0.01)
20	0.43 (0.04)	0.24 (0.09)	0.14 (0.01)
25	0.55 (0.01)	0.15 (0.00)	0.17 (0.01)
30	0.55 (0.03)	0.05 (0.01)	0.14 (0.02)

As the microalgae cultures were grown at different times of the year, it is possible the cells exhibited some different physiological adaptations. In addition, some initial conditions in the cultures (e.g. initial cell abundance, NO_3+NO_2, PO_4 concentrations) were not always the same; therefore, it is thought that the growth response of the strains might have varied and hence physiological responses of the cells may have produced variation in the maximum growth rates. This occurred in the

measured growth rates of *P. lima* at 20 °C in comparison to those found at 15 and 25 °C. Lakeman *et al*. (2009) argued that culture conditions select strains in differ ways and genotypes isolated from the environment will exhibit different physiological responses (growth) in comparison to natural populations.

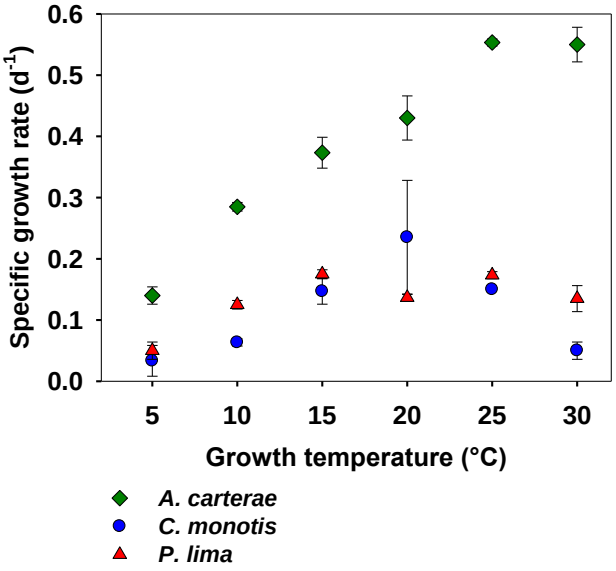

Figure 6.1 Comparison of growth rates determimed in the isolates *A. carterae*, *C. monotis*, and *P. lima* grown in cultures at growth temperatures between 5 and 30 °C.

Table 6.2 Temperature range of linear growth determined in *A. carterae*, *C. monotis*, and *P. lima* cultures and maximum cell abundance measured in culture flasks.

Species	Temperature range of linear growth (°C)	Coefficient of determination (r^2); p <0.001	Maximum cell abundance in culture
A. carterae	5-25	0.97	1.62×10^5
P. lima	5-15	0.97	1.25×10^4
C. monotis	5-20	0.69	8.2×10^3

6.2 Q_{10} (temperature effects on growth)

Berges *et al*. (2002) suggested that Q_{10} implies that biological processes (e.g growth rate) follow an Arrhenius-type relationship (this is a linearity of a log reaction in relation to an absolute temperature). Classical temperature models suggest that growth responds to temperature with a Q_{10} approximately 2 if a biological reaction doubles (Raven and Geider, 1988). However, Montagnes *et* al. (2003) has shown that the use of

Q_{10} to model microalgae growth rate (e.g. benthic dinoflagellates) is inappropriate given that many microalgae exhibit linear growth rates. Some microalgae studies have shown that Q_{10} can be much higher than 2 when the growth temperature was raised ~10 °C (Berges *et al*., 2002, Montagnes *et al*., 2003), which suggested a limitation of the concept Q_{10}. Berges *et al*. (2002) and Montagnes *et* al. (2003) concluded that Q_{10} values will be around 10 depending on the temperature range applied. Figure 6.2 shows Q_{10} values determined for *A. carterae*, *C. monotis*, and *P. lima* cultures grown at temperature between 5-30 °C. In agreement with Berges *et* al. (2002) and Montagnes *et* al. (2003), this study found that Q_{10} values were higher than 2 in *A. carterae* (Q_{10}=2.6) and *P. lima* (Q_{10}=3.5) cultures in the range 5-15 °C; *C. monotis* cultures grown in a range of 5-15 °C (Q_{10}=4.4) and 10-20 °C (Q_{10}=3.7). Temperature ranges above 10 °C produced Q_{10} values lower than 2 in cultures except for Q_{10} values of *C. monotis* in a range of 10-20 °C (Figure 6.2). Therefore, this study also indicates that the use of Q_{10} should be carefully considered particularly when microalgae exhibit linear growth rates vs temperature. Despite the limitation of Q_{10}, Berges *et al*. (2002) suggested that rather than abandoning the use of Q_{10} as a means to describe and predict temperature effects on metabolic rates, Q_{10} could be used in a more fundamental way to provide information about the temperatures to which organisms are adapted.

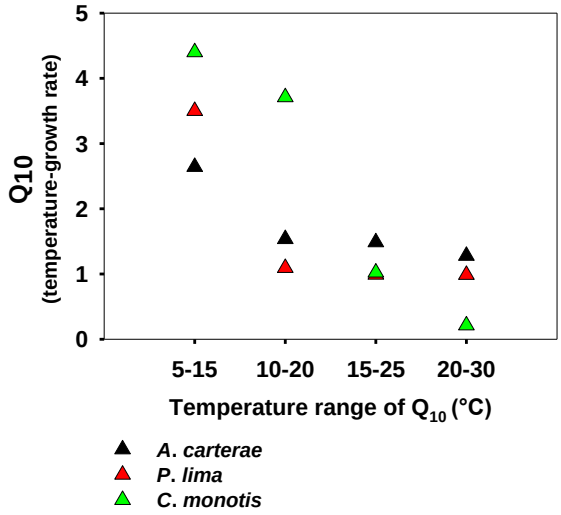

Figure 6.2 Values of Q_{10}) determined between water temperature and cell growth rate when temperature is raised 10 °C in *A. carterae*, *P. lima*, and *C. monotis* cultures. Q_{10} is equal to 2 if the growth rate doubles (Raven & Geider, 1988).

6.3 Prediction of benthic dinoflagellate growth in the Fleet Lagoon

Nascimento (2003) surveyed the temperature of the Fleet Lagoon during 2 years (May 2000 to October 2000 and April 2001 to November 2001) at several positions along the length of Fleet Lagoon (data presented in Figure 6.3). Although the growth rate of natural population of dinoflagellates is subjected to complex environmental conditions, results from this study estimates that water temperature and nutrient concentration (NO_3+NO_2, PO_4) can be used as suitable predictors of the potential increase of both growth rate and abundance of benthic dinoflagellates in the Fleet Lagoon. Based on the study of Nascimento (2003), the Fleet reached temperatures higher than 15 °C between May and the end of August (Figure 6.3). This study found that temperatures >15 °C can produce increasing growth rates of *A. carterae*, *C. monotis*, and *P. lima*. Therefore, high abundance of these dinoflagellates might be expected in the Fleet between May and August, particularly if nutrients (NO_3+NO_2, PO_4) are in replete concentrations for the growth of these toxic microalgae. Nascimento (2003) reported that in the middle of July 2000 and 2001 the Fleet reached its highest seawater temperature ranging from 20 to ~25 °C. According to this author, there was no evidence that the seawater in the Fleet Lagoon surpassed temperatures above 25 °C. Since *A. carterae* showed its highest growth rates at 25 and 30 °C in this study, it can be speculated that this species might exhibit its highest growth rate in the Fleet in association with warm water condition during July. In addition, this could possibly be the time when *C. monotis* and *P. lima* reach their optimum growth and produce their highest growth rates in the Fleet. However, due to the structure and abundance of the community of benthic dinoflagellates in the Fleet has not been monitored enough in the last decades (Steve Morris, personal communication,), it is thought that this study is somehow limited to establish the influence of increasing seawater temperature in the Fleet and the occurrence of potential blooms of toxic epibenthic dinoflagellates. *C. monotis* and *P. lima* decreased their growth rates at elevated temperatures (>25 °C), but this study cannot discard that environmental conditions could induce adaptations that may enable both *C. monotis* and *P. lima* to future selection pressures due to climate change.

Nascimento (2003) reported warmer seawater temperatures in the Fleet associated with shallow areas in the mid Fleet (e.g. Langton Herring, Moonfleet, Figure 6.3) than areas close to mouth of the estuary (southeast). As these shallow areas receive higher

inputs of nutrients (run-off from agricultural activities, Langston *et al*. 2003; Nascimento 2003) and receive reduced mixing, it is possible that this region in the Fleet possesses suitable conditions for the development of HABs of benthic dinoflagellates, particularly during summer months. In shallow waters of the Fleet (e.g. Abbotsbury which is a Swannery area) influenced by water discharges nearby (Cowards Lake, Mill Stream-Abber Barn, Mill Stream-Horsepool), blooms of cryptophytes and *Prorocentrum micans* have been reported in September and mid June, respectively (Nascimento 2003).

Langston *et al*. (2003) documented that high concentration of nitrate (0.08-0.35 mg L^{-1}) in shallow regions of the Fleet (Abbotsbury, Langton Herring, Clouds Hill) coincided with the occurrence of dinoflagellate blooms of *Glenodinium foliaceum* and *Oxyrrhis* sp. in August, 1995; and *P. micans* in Chickerell and Moonfleet stations in November, 2000. Algal monitoring at Ferrybridge (the mouth) conducted by CEFAS between 1996 and 2000 reported that *P. lima* concentrations were below 100 cell L^{-1} during this time. Over the years, the occurrence of harmful blooms in the Fleet has also been associated with *Alexandrium tamarense* (PSP producer) during 1996, 1999, and 2000; *Pseudo-nitzchia* sp. (ASP) in 1996; an unidentified DSP-producer in contaminated mussels in 2000 (Langston *et al*. 2003).

Langston *et al*. (2003) reported that NO_3+NO_2/PO_4 ratios in the Fleet (14.6 in Abbotsbury Swannery and 10.3 at the estuary mouth) are greater than 10 suggesting that PO_4 may theoretically limit algal growth. The Fleet is considered a system subjected to eutrophication and agricultural activities can contribute importantly with a maximum of 80% of nitrogen and between 56-69% of phosphate of the nutrient load (Langston *et al*. 2003). In contrast to NO_3+NO_2 reported by Langston *et al*. (2003), Nascimento (2003) determined a gradient of PO_4 and NO_3+NO_2 along the Fleet with lower concentrations of these nutrients: average PO_4 4.2 µM (in 2000) and 6.2 µM (in 2001) in Abbotsbury (shallow waters, station 1) and 0.4 µM (in 2000) and 0.5 µM (in 2001) at the narrows (station 6); average NO_3+NO_2 13.7 µM (in 2000) and 47.5 µM (in 2001) at Abbotsbury. In this study, there was evidence that *A. carterae, C. monotis* and *P. lima* cultures could become PO_4 limited. Based on the PO_4 concentrations reported in the Fleet by Nascimento (2003), it is possible that the growth of the benthic dinoflagellates reported in this study may be limited by PO_4 availability, particularly as macroalgae and planktonic phytoplankton will also compete for this nutrient. Therefore, this might

explain why the occurrence of *A. carterae*, *C. monotis* and *P. lima* in the Fleet has not reached high cell concentrations as found for other bloom-forming dinoflagellates, such as *P. micans* and *A. tamarense*. In addition, it should be considered that benthic dinoflagellates usually exhibit lower growth rates compared to planktonic species.

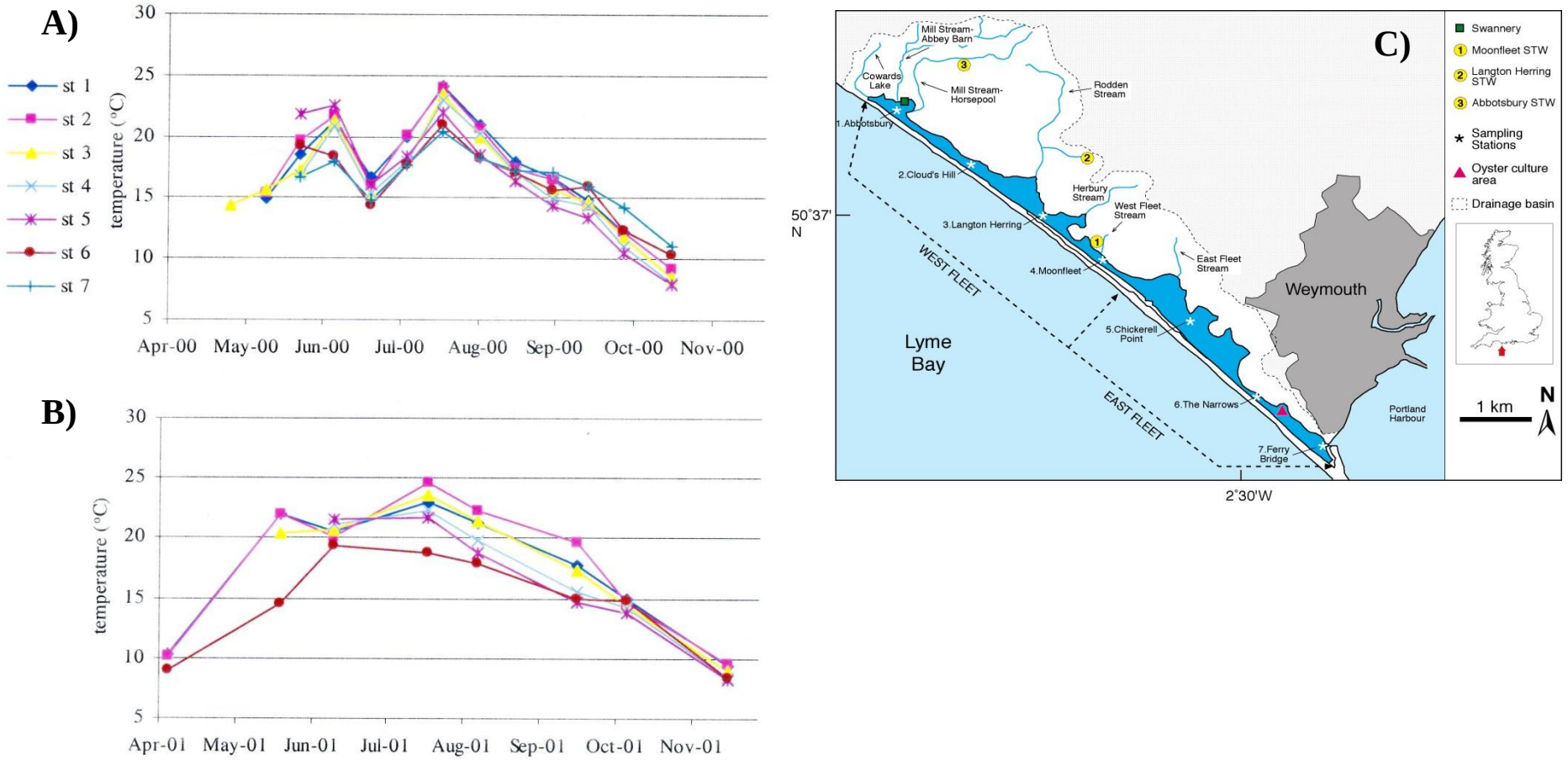

Figure 6.3 Varibility of seawater temperature determined by Nascimento (2003) along the Fleet Lagoon from May to October 2000 (A) and April to November 2001 (B). C) shows the map of sampling stations where Nascimento (2003) recorded the seawater temperature.

This study found that the effect of increasing temperature produced higher nutrient (NO_3+NO_2, PO_4) uptake in the three benthic dinoflagellate cultured between 5 and 30 °C. Figure 6.4 shows NO_3+NO_2/PO_4 uptake ratios versus growth temperature in relation to the exponential cell growth of *A. carterae*, *C. monotis*, and *P. lima*. Dinoflagellate cells exposed to increasing temperatures (>15 °C) reduced rapidly PO_4 concentration in the culture media and it became a limiting growth factor for *A. carterae, C. monotis*, and *P. lima* cells towards the stationary phase.

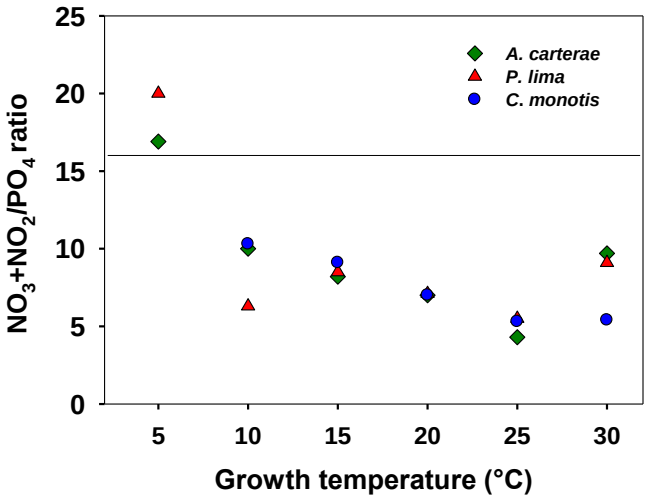

Figure 6.4 Changes of NO_3+NO_2/PO_4 uptake ratios in *A. carterae*, *C. monotis*, and *P. lima* cells during the exponential growth at increasing growth temperatures between 5 and 30 °C. Horizontal line indicates the Redfield ratio.

In this study, NO_3+NO_2 concentration in F/2 media for the growth of benthic dinoflagellates (see Table 2.2-2.4 in methods section) was considered to be too high as NO_3+NO_2 was never reduced below 500 µmols L^{-1} in any of the dinoflagellate cultures. However, Nascimento *et al*. (2005) reported that *P. lima* cells grown at 17 °C produced nutrient depletion in L2 media with basal concentrations of NO_3+NO_2 and PO_4 similar to those in F/2 media.

Despite the high PO_4 uptake during exponential growth and very low PO_4 concentrations associated with the stationary growth phase in *A. carterae*, *C. monotis*, and *P. lima* cultures, it is possible that the availability of dissolved CO_2 in the media might have influenced the growth of the benthic dinoflagellates. Since non-aerated cultures were used in the growth of *A. carterae*, *C. monotis*, and *P. lima* cells, it is possible that CO_2 limitation occurred in cultures before NO_3+NO_2 and PO_4 were considered a limiting growth factor for the cells. However, further studies are needed to

determine the CO_2/PO_4 uptake ratios in benthic dinoflagellates and its potential co-limitation in dinoflagellate cells.

6.4 Toxicity determined in benthic dinoflagellates

Table 6.3 summarizes assays for toxin detection and lipophilic toxins confirmed and measured by LC-MS/MS from *A. carterae*, *C. monotis* and *P. lima* cells. This study confirmed that *P. lima* is a toxigenic epibenthic dinoflagellate and produced the lipophilic intracellular toxins okadaic acid (OA) and dinophysistoxin1 (DTX1). Furthermore, *A. carterae* and *C. monotis* are deemed as potentially toxic epibenthic dinoflagellates as these microalgae caused haemolytic activity in chicken red blood cells and mortality in both harpacticoid copepods and nauplii of *Artemia salina* after dinoflagellate cell ingestion.

Table 6.3 Summary of assays and toxins detected that confirmed the presence and production of secondary metabolites in *A. carterae*, *C. monotis*, and *P. lima* cells.

A. carterae	*C. monotis*	*P. lima*
Haemolytic activity	Haemolytic activity	Okadaic acid and dinophysistoxin1
Mortality of copepods	Mortality of copepods	Esters of OA and DTX1
	Mortality of *A. salina*	Mortality of *A. salina*
		Mortality of copepods

This study presented independent measurements of OA and DTX1 quantified by LC-MS/MS as Free OA and Free DTX1 (parent toxins) from *P. lima* cells. Additionally, LC-MS/MS analyses determined the total burden of lipophilic toxins (as the sum of either OA or DTX1 (parent toxins) plus its OA or DTX1 esters) quantified as Total OA and Total DTX1 produced by *P. lima* cells. Increasing growth temperatures were generally associated with higher toxin production in *P. lima* cells, but this study found that the toxin increase at increasing growth temperatures varied in relation to the time the *P. lima* batch cultures were performed in the laboratory between 2009 and 2010 (see Table 2.1 in method section). Therefore, this study found that *P. lima* produced increasing concentrations of the parent toxin OA when cells were firstly cultured from 5 to 15 °C (2009). Lipophilic analyses of Free DTX1 and Total DTX1

showed little differences in *P. lima* cells at each growth temperature across the range 5 to 30 °C and the highest intracellular DTX1 production was linked to 10 and 15 °C. Batch cultures of *P. lima* cells grown between 20 and 30 °C (2010 *P. lima* culture batch) produced lower concentrations of the parent toxins than cells grown between 10 and 15 °C (2009 culture batch). However, increasing growth temperatures in *P. lima* cells between 20 and 30 °C (2010 culture batch) were also associated with higher intracellular synthesis of the Free OA, but this did not occur in Free DTX1, Total OA, Total DTX1 in *P. lima* cultures grown over this temperature range (20-30 °C).

Total OA measurements were, as expected, higher than the concentration of the Free OA meaning the presence of ester compounds of OA and DTX1 in *P. lima* cells. Results of Total DTX1 suggested that DTX1 esters were generally produced in little amounts in *P. lima* cells in comparison to the production of OA esters. Therefore, the main toxic burden in *P. lima* cells was related to the parent toxins (OA and DTX1) and OA esters. Esters of OA and DTX1 however were not chemically identified and characterized by LC-MS/MS in this study. Unlike the relationship of higher Free OA production at increasing growth temperatures (5-15 °C), Total OA and Total DTX1 were not generally associated with increasing growth temperatures across the range of growth temperatures. In fact, Total OA showed similar concentration in *P. lima* cells at 10, 15 and 30 °C, while Total DTX1 reached similar concentrations at 10 and 15 °C (see Figure 4.12 in Chapter 4).

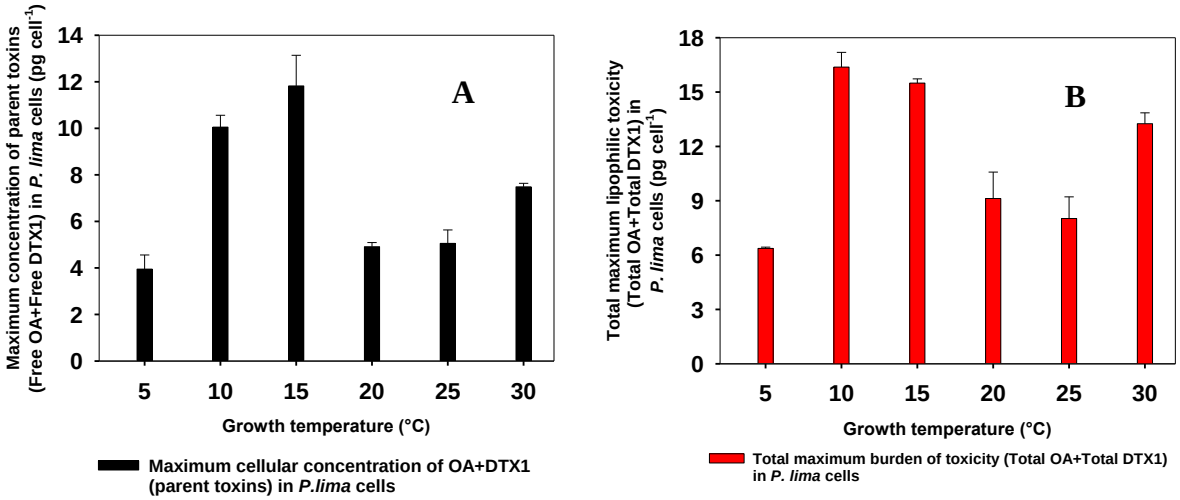

Figure 6.5 Maximum concentration of additive parent toxins (OA and DTX1) in *P. lima* cells (A) and additive total toxicity (Total OA and Total DTX1) in *P. lima* cells grown in cultures between 5 and 30 C. Error bars show the standard deviation (15-25 °C) and standard difference (5, 10, and 30 °C) between replicate cultures.

To visualize and compared the sum of toxin production across the range of growth temperature, Figure 6.5 presents the cumulative toxicity of parent toxins reached in *P. lima* cells and the maximum total burden of toxicity of lipophilic toxins in *P. lima* cells grown between 5 and 30 °C. The cumulative toxins (parent OA+DTX1 and total lipophilic toxins) in Figure 6.5 followed a similar pattern of toxin increase at increasing temperatures as those determined independently for Free OA and Total OA shown in Chapter 4 (Figure 4.12).

The potential toxicity of *A. carterae* and *C. monotis* was confirmed in haemolytic assays using chicken red blood cells and Figure 6.6 contrasts the activity of potential heamolytic compounds of *A. carterae* versus *C. monotis* at different cell concentrations. High cell concentrations of *A. carterae* and *C. monotis* were needed in the assays to produce more than 50% of haemolytic activity on chicken erythrocytes (Figure 6.6) and those cell numbers were not quantified in the dinoflagellate culture flasks despite cells being at high NO_3+NO_2 and PO_4 concentrations plus a wide range of growth temperatures. Also, no records have been reported to date from the Fleet Lagoon where *A. carterae* or *C. monotis* had caused noxious effects in the environment due to high cell abundance of these microalgae. *A. carterae* and *C. monotis* however proved to be harmful when ingested by harpacticoid copepods, but increasing concentrations of these benthic microalgae did not prove to be associated with higher mortality of copepods. In addition, feeding assays evidenced that *C. monotis* was toxic to *A. salina* and results showed a faster mortality of the brine shrimp in the assays than those performed with harpacticoid copepods. Therefore, this study concludes that the effect of increasing seawater temperature can increase the growth of *A. carterae* and *C. monotis* but their toxins and toxin effects in the environment need to be further investigated.

Many biotoxins from dinoflagellates have been characterized and quantified in the last few decades, however, a number of secondary metabolites still remain unknown in terms of the chemical structure, synthesis and biological interaction in marine trophic food webs. LC-MS/MS is the optimal analytical instrument to determine a range of DSP-toxins, however, DSP analyses by LC-MS/MS are still expensive and alternative methods to quantify biotoxins in a cost-effective manner are required. In addition, determining the dynamic (biological synthesis, transference, accumulation through the trophic web, and degradation) of DSP toxins in the environment demands a substantial

human effort that must be considered in order to comprehend how natural populations will perform under various changing environmental scenarios.

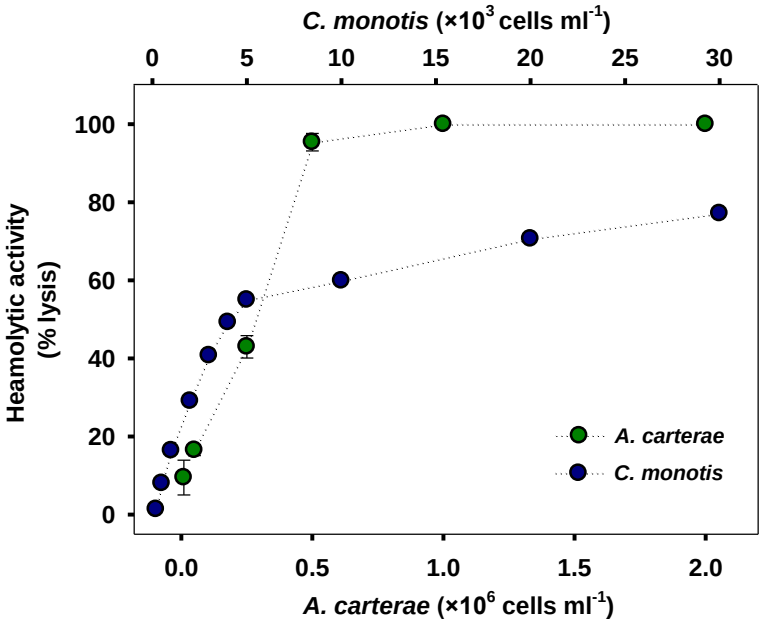

Figure 6.6 Comparison of haemolytic activity (percentage of lysis) produced by *A. carterae* and *C. monotis* cells at different cell concentrations in assays using Chicken Red Blood Cells.

References

AJUZIE, C. C. 2007. Palatability and fatality of the dinoflagellate *Prorocentrum lima* to Artemia salina. *Journal of Applied Phycology*, 19, 513-519.

ALIGIZAKI, K. & NIKOLAIDIS, G. 2006. The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the north Aegean sea, Greece. *Harmful Algae*, 5, 717-730.

ALIGIZAKI, K., NIKOLAIDIS, G., KATIKOU, P., BAXEVANIS, A. D. & ABATZOPOULOS, T. J. 2009. Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. *Harmful Algae*, 8, 299-311.

ARMI, Z., TURKI, S., TRABELSI, E. & BEN MAIZ, N. 2010. First recorded proliferation of *Coolia monotis* (Meunier, 1919) in the North Lake of Tunis (Tunisia) correlation with environmental factors. *Environmental Monitoring and Assessment*, 164, 423-433.

ASHTON, M., ROSADO, W., GOVIND, N. S. & TOSTESON, T. R. 2003. Culturable and nonculturable bacterial symbionts in the toxic benthic dinoflagellate *Ostreopsis lenticularis*. *Toxicon*, 42, 419-424.

AUBRIOT, L., CONDE, D., BONILLA, S. & SOMMARUGA, R. 2004. Phosphate uptake behavior of natural phytoplankton during exposure to solar ultraviolet radiation in a shallow coastal lagoon. *Marine Biology*, 144, 623-631.

BAIG, H. S., SAIFULLAH, S. M. & DAR, A. 2006. Occurrence and toxicity of *Amphidinium carterae* Hulburt in the North Arabian Sea. *Harmful Algae*, 5, 133-140.

BALCH, W. M. & FABRY, V. J. 2008. Ocean acidification: documenting its impact on calcifying phytoplankton at basin scales. *Marine Ecology-Progress Series*, 373, 239-247.

BARLOW, S. B. & TRIEMER, R. E. 1988. Alternate Life-History Stages in *Amphidinium klebsii* (Dinophyceae, Pyrrophyta). *Phycologia*, 27, 413-420.

BEARDALL, J., STOJKOVIC, S. 2006. Microalgae under global environmental change: implications for growth and productivity populations and trophic flow. *ScienceAsia*, 32, 1-10.

BERGES, J. A., VARELA, D. E. & HARRISON, P. J. 2002. Effects of temperature on growth rate, cell composition and nitrogen metabolism in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Marine Ecology Progress Series*, 225, 139-146.

BLANCO, J., MOROÑO, A., FERNÁNDEZ, M.L. (2005) Toxic episodes in shellfish, produced by lipophilic phycotoxins: An overview. Revista Galega de Recursos Mariños (Monog.):1, 1-70 pp.

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.

BRESNAN, E., L. FERNAND, K. DAVIDSON, M. EDWARDS, S MILLIGAN, R GOWAN, J SILKE, S KROGER & R RAINE (2010) Climate Change impacts on Harmful Algal Blooms (HABs) *in* MCCIP Annual Report Card 2010-11, MCCIP Science Review, 10pp. www.mccip.org.uk/arc

CALBET, A., BERTOS, M., FUENTES-GRÜNEWALD, C., ALACID, E., FIGUEROA, R., RENOM, B. & GARCÉS, E. 2011. Intraspecific variability in

Karlodinium veneficum: Growth rates, mixotrophy, and lipid composition. *Harmful Algae,* 10, 654-667.

CAMACHO, F. G., RODRIGUEZ, J. G., MIRON, A. S., GARCIA, M. C. C., BELARBI, E. H., CHISTI, Y. & GRIMA, E. M. 2007. Biotechnological significance of toxic marine dinoflagellates. *Biotechnology Advances,* 25, 176-194.

CHANG, S.-K. 2006. Studies toward the total synthesis of amphidinol 3. PhD thesis. The Ohio State University, USA.

CHEUNG, W. W. L., DUNNE, J., SARMIENTO, J. L. & PAULY, D. 2011. Integrating ecophysiology and plankton dynamics into projected maximum fisheries catch potential under climate change in the Northeast Atlantic. *Ices Journal of Marine Science,* 68, 1008-1018.

COLIN, S. P. & DAM, H. G. 2004. Testing for resistance of pelagic marine copepods to a toxic dinoflagellate. *Evolutionary Ecology,* 18, 355-377.

CRAWFORD, D. W. & HARRISON, P. J. 1997. Direct measurement of pCO2 in cultures of marine phytoplankton: how good is the estimate from pHNBS and single point titration of alkalinity? *Marine Ecology Progress Series,* 158, 61-74.

DALE, B., EDWARDS, M., & REID, P. C. 2006. Climate Change and Harmful Algal Blooms. In: GRANELI, E. & TURNER, J. (Eds). Ecology of Harmful Algae. Germany, Springer, pp. 367-378.

DARANAS, A. H., NORTE, M. & FERNANDEZ, J. J. 2001. Toxic marine microalgae. *Toxicon,* 39, 1101-1132.

DEANE, E. M. & OBRIEN, R. W. 1981. Uptake of Phosphate by Symbiotic and Free-Living Dinoflagellates. *Archives of Microbiology,* 128, 307-310.

de VICENTE, J., HUCKINS, J. R., & RYCHNOVSKY, S. D. 2006. Angewandte chemie. 118, 7416–7420

DICKEY, R. W. & PLAKAS, S. M. 2010. Ciguatera: a public health perspective. *Toxicon,* 56, 123-36.

DOLAPSAKIS, N. P., KILPATRICK, M. W., ECONOMOU-AMILLI, A. & TAFAS, T. 2006. Morphology and rDNA phylogeny of a Mediterranean *Coolia monotis* (Dinophyceae) strain from Greece. *Scientia Marina,* 70, 67-76.

DUARTE, C. M. 1990. Time lags in algal growth - generality, causes and consequences. *Journal of Plankton Research,* 12, 873-883.

ECHIGOYA, R., RHODES, L., OSHIMA, Y. & SATAKE, M. 2005. The structures of five new antifungal and hemolytic amphidinol analogs from *Amphidinium carterae* collected in New Zealand. *Harmful Algae,* 4, 383-389.

EDVARDSEN, B., & IMAI, I. 2006 The ecology of harmful flagellates within prymnesiophyceae and raphidophyceae. In: GRANELI, E. & TURNER, J. (Eds). Ecology of Harmful Algae. Germany, Springer, pp. 67-79.

EDWARDS, M., JOHNS, D. G., LETERME, S. C., SVENDSEN, E. & RICHARDSON, A. J. 2006. Regional climate change and harmful algal blooms in the northeast Atlantic. *Limnology and Oceanography,* 51, 820-829.

ESCHBACH, E., SCHARSACK, J. P., JOHN, U. & MEDLIN, L. K. 2001. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic alga. *Journal of Applied Toxicology,* 21, 513-519.

FAO (Food and Agriculture Organization of the United Nations), 2004. Marine biotoxins. FAO Food and Nutrition Paper 80. Food and Agriculture Organization, Rome, Italy.

FAUST, M. A. 1991. Morphology of ciguatera-causing *Prorocentrum lima* (Pyrrophyta) from widely differing sites. *Journal of Phycology*. 27: 642-648.

FAUST, M. A. 1992. Observations on the Morphology and Sexual Reproduction of *Coolia monotis* (Dinophyceae). *Journal of Phycology,* 28, 94-104.

FAUST, M. A. 1993. Three new benthic species of *Prorocentrum* (Dinophyceae) from Twin Cays, Belize: *P. maculosum* sp. nov., *P. foraminosum* sp. nov. and *P. formosum* sp. nov. *Phycologia* 32: 410-418.

FAUST, M. A., MORTON, S. L. & QUOD, J. P. 1996. Further SEM study of marine dinoflagellates: The genus *Ostreopsis* (Dinophyceae). *Journal of Phycology,* 32, 1053-1065.

FAUST, M. A., VANDERSEA, M. W., KIBLER, S. R., TESTER, P. A. & LITAKER, R. W. 2008. *Prorocentrum levis*, a new benthic species (dinophyceae) from a mangrove island, twin cays, belize. *Journal of Phycology,* 44, 232-240.

FERNÁNDEZ, M. L., SHUMWAY, S., & BLANCO, J. 2003. Management of shellfish resources. In G. M. Hallegraeff, D. M. Anderson, and A. D. Cembella (Eds), Manual on harmful marine microalgae. UNESCO. France, pp. 657-692

FLYNN, K. J., RAVEN, J. A., REES, T. A. V., FINKEL, Z., QUIGG, A. & BEARDALL, J. 2010. Is the Growth Rate Hypothesis Applicable to Microalgae? *Journal of Phycology,* 46, 1-12.

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, 1063-1074.

FRANKLIN, D. J. & BERGES, J. A. 2004. Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. *Proc Biol Sci,* 271, 2099-107.

FU, F. X., PLACE, A. R., GARCIA, N. S. & HUTCHINS, D. A. 2010. CO(2) and phosphate availability control the toxicity of the harmful bloom dinoflagellate *Karlodinium veneficum*. *Aquatic Microbial Ecology,* 59, 55-65.

FUJIKI, H. & SUGANUMA, M. 1999. Unique features of the okadaic acid activity class of tumor promoters. *Journal of Cancer Research and Clinical Oncology,* 125, 150-155.

FURNAS, M. J. 1990. *In situ* growth rates of marine phytoplankton: approaches to measurements, community and species growth rates. *Journal of Plankton Research.* 12, 1117-1151

GALLARDO-RODRÍGUEZ, J. J., SÁNCHEZ MIRÓN, A., CERÓN GARCÍA, M. D. C., BELARBI, E. H., GARCÍA CAMACHO, F., CHISTI, Y. & MOLINA GRIMA, E. 2009. Macronutrients requirements of the dinoflagellate *Protoceratium reticulatum*. *Harmful Algae,* 8, 239-246.

GEIDER, R. J., MACINTYRE, H. L. & KANA, T. M. 1998. A dynamic regulatory model of phytoplanktonic acclimation to light, nutrients, and temperature. *Limnology and Oceanography,* 43, 679-694.

GERATH, M. W. & CHISHOLM, S. W. 1989. Change in photosynthetic capacity over the cell cycle in light dark synchronized *amphidinium carteri* is due solely to the photocycle. *Plant Physiol,* 91, 999-1005.

GERSEEN, A., MULDER, P. & DE BOER, J. 2007. A novel LC method for the separation of marine lipophiliC biotoxins. *Third International Symposium on Recent Advances in Food Analysis*. 7 to 9 November 2007, Prague, Czech Republic. 249 pp.

GEOHAB, 2001. Global Ecology and Oceanography of Harmful Algal Blooms,

Science Plan. P. Glibert and G. Pitcher (eds). SCOR and IOC, Baltimore and Paris. 87 pp.

GIENAPP, P., TEPLITSKY, C., ALHO, J. S., MILLS, J. A. & MERILA, J. 2008. Climate change and evolution: disentangling environmental and genetic responses. *Molecular Ecology,* 17, 167-178.

GLIBERT, P. M., ALLEN, J. I., BOUWMAN, A. F., BROWN, C. W., FLYNN, K. J., LEWITUS, A. J. & MADDEN, C. J. 2010. Modeling of HABs and eutrophication Status, advances, challenges. *Journal of Marine Systems,* 83, 262-275.

GOLDMAN, J. C. & CARPENTER, E. J. 1974. A kinetic approach to the effect of temperature on algal growth. *Limnology and Oceanography,* 19, 756-766.

GRANELI, E., VIDYARATHNA, N. K., FUNARI, E., CUMARANATUNGA, P. R. T. & SCENATI, R. 2011. Can increases in temperature stimulate blooms of the toxic benthic dinoflagellate *Ostreopsis ovata*? *Harmful Algae,* 10, 165-172.

GRANELI, E. & FLYNN, K. 2006. Chemical and physical influencing toxin content. In: GRANELI, E. & TURNER, J. (Eds). Ecology of Harmful Algae. Germany, Springer, pp. 229-241.

GRANELI, E. & HANSEN, P. J. 2006. Allelopathy in harmful algae: a mechanism to compete for resources. In: GRANELI, E. & TURNER, J. (Eds). Ecology of Harmful Algae. Germany, Springer, pp. 189-201.

GRASSHOFF, K. 1976. Methods of seawater analysis. Verlag Chemie, NewYork, 317 p.

GUERRINI, F., CIMINIELLO, P., DELL'AVERSANO, C., TARTAGLIONE, L., FATTORUSSO, E., BONI, L. & PISTOCCHI, R. 2007. Influence of temperature, salinity and nutrient limitation on yessotoxin production and release by the dinoflagellate *Protoceratium reticulatum* in batch-cultures. *Harmful Algae,* 6, 707-717.

HALLEGRAEFF, G. M. 2003. Harmful algal blooms: a global overview. In G. M. Hallegraeff, D. M. Anderson, and A. D. Cembella (Eds), Manual on harmful marine microalgae. UNESCO. France, pp. 25-49

HALLEGRAEFF, G. M. 2010. Ocean climate change, phytoplankton community responses, and harmful algal blooms: A formidable predictive challenge. *Journal of Phycology,* 46, 220-235.

HANELT D., WIENCKE, C., BISCHOF, K. 2003 Photosynthesis in marine macroalgae. In: Larkum AW, Douglas SE, Raven JA (eds) Photosynthesis in algae. Kluwer Academic Publishers, Amsterdam, p 413–435.

HARE, C. E., DEMIR, E., COYNE, K. J., CARY, S. C., KIRCHMAN, D. L. & HUTCHINS, D. A. 2005. A bacterium that inhibits the growth of *Pfiesteria piscicida* and other dinoflagellates. *Harmful Algae,* 4, 221-234.

HEREDIA-TAPIA, A., ARREDONDO-VEGA, B. O., NUÑES-VÁZQUEZ, 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, 1121–7.

HISCOCK, M. R., LANCE, V. P., APPRILL, A., BIDIGARE, R .R., JOHNSON, Z. I., MITCHELL, B. G., SMITH, W. O., and BARBER, R. T. 2008. Photosynthetic maximum quantum yield increases are an essential componentof the Southern Ocean phytoplankton response to iron, Proc. Natl. Acad. Sci., 105, 4775–4780.

HOLMES, M. J., LEWIS, R. J., JONES, A. & HOY, A. W. 1995. Cooliatoxin, the first toxin from *Coolia monotis* (Dinophyceae). *Nat Toxins,* 3, 355-62.

HONJO, T. 1993. Overview on bloom dynamics and physiological ecology of *Heterosigma akashiwo*. *Toxic Phytoplankton Blooms in the Sea,* 3, 33-41.

IANORA, A., MIRALTO, A. & POULET, S. A. 1999. Are diatoms good or toxic for copepods? Reply to comment by Jonasdottir *et al. Marine Ecology-Progress Series,* 177, 305-308.

IGNATIADES, L. & GOTSIS-SKRETAS, O. Year. A review on toxic and harmful algae in greek coastal waters (E. Mediterranean Sea). *In:* Toxins, 2010. 1019-1037.

ISMAEL, A. A. H., HALIM, Y. & KHALIL, A. G. 1999. Optimum growth conditions for *Amphidinium carterae* Hulburt from eutrophic waters in Alexandria (Egypt) and its toxicity to the brine shrimp *Artemia salina*. *Grana,* 38, 179-185.

JANSON, S. & HAYES, P. K. 2006 Molecular taxonomy of harmful algae. In: GRANELI, E. & TURNER, J. (Eds). Ecology of Harmful Algae. Germany, Springer, pp. 9-21.

JENSEN, M. O. & MOESTRUP, O. (1997) Autecology of the toxic dinoflagellate *Alexandrium ostenfeldii*: Life history and growth at different temperatures and salinities. *Eur. J. Phycol., 32*, *9*–18.

JEONG, S. Y., ISHIDA, K., ITO, Y., OKADA, S. & MURAKAMI, M. 2003. Bacillamide, a novel algicide from the marine bacterium, *Bacillus* sp SY-1, against the harmful dinoflagellate, *Cochlodinium polykrikoides*. *Tetrahedron Letters,* 44, 8005-8007.

Katırcıoğlu, H., Akın, B., & Atıcı, T., 2004. Microalgal toxin(s): characteristics and importance. African J. Biotechnol. 3, 667–674.

KELLMANN, R., STUKEN, A., ORR, R. J. S., SVENDSEN, H. M. & JAKOBSEN, K. S. 2010. Biosynthesis and molecular genetics of polyketides in marine dinoflagellates. *Marine Drugs,* 8, 1011-1048.

KIRKWOOD, D. 1996. Nutrients: practical notes on their determination in sea water. 25 pp

KOBAYASHI, J. & TSUDA, M. 2004. Amphidinolides, bioactive macrolides from symbiotic marine dinoflagellates. *Natural Product Reports,* 21, 77-93.

KOLBER, Z. S. & FALKOWSKI, P. G. 1992. Fast repetition rate (FRR) fluorometer for making in situ measurements of primary productivity. *Oceans 92: Proceedings, Vol 1 and 2*, 637-641.

KOIKE, K., SATO, S., YAMAJI, M., NAGAHAMA, Y., KOTAKI, Y., OGATA, T. & KODAMA, M. 1998. Occurrence of okadaic acid-producing *Prorocentrum lima* on the Sanriku Coast, Northern Japan. Toxicon, 36, 2039–42.

KOPP, M., DOUCETTE, G. J., KODAMA, M., GERDTS, G., SCHUTT, C. & MEDLIN, L. K. 1997. Phylogenetic analysis of selected toxic and non-toxic bacterial strains isolated from the toxic dinoflagellate *Alexandrium tamarense*. *Fems Microbiology Ecology,* 24, 251-257.

KOZLOWSKY-SUZUKI, B., KOSKI, M., HALLBERG, E., WALLEN, R. & CARLSSON, P. 2009. Glutathione transferase activity and oocyte development in copepods exposed to toxic phytoplankton. *Harmful Algae,* 8, 395-406.

KROMKAMP, J. & PEENE, J. 1999. Estimation of phytoplankton photosynthesis and nutrient limitation in the Eastern Scheldt estuary using variable fluorescence. *Aquatic Ecology,* 33, 101-104.

KUBOTA, T., Takahashi, A., Tsuda, M., & Kobayashi, J. 2005. Luteophanol D, New Polyhydroxyl Metabolite from MarineDinoflagellate *Amphidinium* sp. *Mar. Drugs*, 3, 113-118.

LAABIR, M., JAUZEIN, C., GENOVESI, B., MASSERET, E., GRZEBYK, D., CECCHI, P., VAQUER, A., PERRIN, Y. & COLLOS, Y. 2011. Influence of temperature, salinity and irradiance on the growth and cell yield of the harmful red tide dinoflagellate *Alexandrium catenella* colonizing Mediterranean waters. *Journal of Plankton Research,* 33, 1550-1563.

LAFAY, B., RUIMY, R., DETRAUBENBERG, C. R., BREITTMAYER, V., GAUTHIER, M. J. & CHRISTEN, R. 1995. *Roseobacter algicola* sp nov, a New Marine Bacterium Isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. *International Journal of Systematic Bacteriology,* 45, 290-296.

LAKEMAN, M. B., VON DASSOW, P. & CATTOLICO, R. A. 2009. The strain concept in phytoplankton ecology. *Harmful Algae,* 8, 746-758.

LASSEN, M. K., NIELSEN, K. D., RICHARDSON, K., GARDE, K. & SCHLUTER, L. 2010. The effects of temperature increases on a temperate phytoplankton community - A mesocosm climate change scenario. *Journal of Experimental Marine Biology and Ecology,* 383, 79-88.

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, 147-154.

LAZA-MARTINEZ, A., ORIVE, E. & MIGUEL, I. 2011. Morphological and genetic characterization of benthic dinoflagellates of the genera *Coolia, Ostreopsis* and *Prorocentrum* from the south-eastern Bay of Biscay. *European Journal of Phycology,* 46, 45-65.

LEAW, C. P., LIM, P. T., CHENG, K. W., NG, B. K. & USUP, G. 2010. Morphology and molecular characterization of a new species of thecate benthic dinoflagellate, *Coolia malayensis* sp nov (Dinophyceae). *Journal of Phycology,* 46, 162-171.

LEE, J.-S., IGARASHI, T., FRAGA, S., DAHL, E., HOVGAARD, P. & YASUMOTO, T. 1989. Determination of diarrhetic shellfish toxins in various dinoflagellate species. *Journal of Applied Phycology,* 1, 147-152.

LEE, J. J., SHPIGEL, M., FREEMAN, S., ZMORA, O., MCLEOD, S., BOWEN, S., PEARSON, M. & SZOSTEK, A. 2003. Physiological ecology and possible control strategy of a toxic marine dinoflagellate, *Amphidinium* sp., from the benthos of a mariculture pond. *Aquaculture,* 217, 351-371.

LEVASSEUR, M., COUTURE, J. Y., WEISE, A. M., MICHAUD, S., ELBRACHTER, M., SAUVE, 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, 283-293.

LI, J., GLIBERT, P. M., ZHOU, M. J., LU, S. H. & LU, D. D. 2009. Relationships between nitrogen and phosphorus forms and ratios and the development of dinoflagellate blooms in the East China Sea. *Marine Ecology-Progress Series,* 383, 11-26.

LIANG, J. L., LONG, L. J., ZHANG, S. & WU, J. 2009. Cooliatin, an unprecedented natural dioxocyclononane from dinoflagellate *Coolia monotis* from South China Sea. *Chemical & Pharmaceutical Bulletin,* 57, 888-889.

LOW-DECARIE, E., FUSSMANN, G. F. & BELL, G. 2011. The effect of elevated CO_2 on growth and competition in experimental phytoplankton communities. *Global Change Biology,* 17, 2525-2535.

LUCKAS, B., DAHLMANN, J., ERLER, K., GERDTS, G., WASMUND, N., HUMMERT, C. & HANSEN, P. D. 2005. Overview of key phytoplankton toxins and their recent occurrence in the North and Baltic seas. *Environmental Toxicology,* 20, 1-17.

MACINTYRE, H.L. & CULLEN, J. J. 2005 Using cultures to investigate the physiological ecology of microalgae. In: ANDERSON, R. A. (ed.) *Algal Culturing Techniques,* Academic Press, pp 287-326.

MACKENZIE, L. A., SELWOOD, A. I., MCNABB, P., & RHODES, L. 2011. Benthic dinoflagellate toxins in two warm-temperate estuaries: Rangaunu and Parengarenga Harbours, Northland, New Zealand. *Harmful Algae,* 6 , 559-566.

MANDAL, S. K., SINGH, R. P. & PATEL, V. 2011. Isolation and characterization of exopolysaccharide secreted by a toxic dinoflagellate *Amphidinium carterae* Hulburt 1957 and its probable role in harmful algal blooms (HABs). *Microbial Ecology,* 62, 518-527.

MARINE SPECIES IDENTIFICATION PORTAL OF HARMFUL MARINE DINOFLAGELLATES. 2012. http://species-identification.org/index.php

MARANDA, L., CHAN, C. & MARTIN, C. 1999. *Prorocentrum lima* (Dinophyceae) in waters of the Great South Channel near Georges Bank. *Journal of Phycology,* 35, 1158-1161.

MARANDA, L., CORWIN, S., DOVER, S. & MORTON, S. L. 2007a. *Prorocentrum lima* (Dinophyceae) in northeastern USA coastal waters - II: Toxin load in the epibiota and in shellfish. *Harmful Algae,* 6, 632-641.

MARANDA, L., CORWIN, S. & HARGRAVES, P. E. 2007b. *Prorocentrum lima* (Dinophyceae) in northeastern USA coastal waters - I. Abundance and distribution. *Harmful Algae,* 6, 623-631.

MARASIGAN, A. N., TAMSE, A. F., & FUKUYO Y. 2001. *Prorocentrum* (Prorocentrales: Dinophyceae) populationson seagrass-blade surface in Taklong Island, Guimaras Province, Philippines. Plankton Biol. Ecol, 48, 79–84.

MASO, M. & GARCES, E. 2006. Harmful microalgae blooms (HAB); problematic and conditions that induce them. *Marine Pollution Bulletin,* 53, 620-630.

MAYALI , X. & DOUCETTE, G. J. 2002. Microbial community interactions and population dynamics of an algicidal bacterium active against *Karenia brevis* (Dinophyceae). *Harmful Algae* 1: 277-293.

MCLACHLAN, J. L., MARR, J. C., CONLON-KELLY, A. & ADAMSON, A. 1994. Effects of nitrogen concentration and cold temperature on DSP-toxin concentrations in the dinoflagellate *Prorocentrum lima* (Prorocentrales, Dinophyceae). *Nat Toxins,* 2, 263-70.

MENG, Y. H., VAN WAGONER, R. M., MISNER, I., TOMAS, C. & WRIGHT, J. L. C. 2010. Structure and Biosynthesis of Amphidinol 17, a Hemolytic Compound from *Amphidinium carterae*. *Journal of Natural Products,* 73, 409-415.

MONTAGNES, D. J. S., KIMMANCE, S. A. & ATKINSON, D. 2003. Using Q_{10}: Can growth rates increase linearly with temperature? *Aquatic Microbial Ecology,* 32, 307-313.

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.

MORGAN-KISS, R. M., PRISCU, J. C., POCOCK, T., GUDYNAITE-SAVITCH, L. & HUNER, N. P. A. 2006. Adaptation and acclimation of photosynthetic

microorganisms to permanently cold environments. *Microbiology and Molecular Biology Reviews,* 70, 222.

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, 79-90.

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, 914-921.

MOUNTFORT, D. O., SUZUKI, T. & TRUMAN, P. 2001. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. *Toxicon,* 39, 383-390.

MURRAY, S. & PATTERSON, D.J. 2002. The benthic dinoflagellate genus *Amphidinium* in south-eastern Australian waters, including three new species. *British Phycological Journal* 37: 279-298.

NADAOKA, K., BLANCO, A. C. & YAMAMOTO, T. 2008. Planktonic and benthic microalgal community composition as indicators of terrestrial influence on a fringing reef in Ishigaki Island, Southwest Japan. *Marine Environmental Research,* 66, 520-535.

NEELY, T. & CAMPBELL, L. 2006. A modified assay to determine hemolytictoxin variability among *Karenia clones* isolated from the Gulf of Mexico. Harmful Algae, 5, 592–598.

NAGAHAMA, Y., MURRAY, S., TOMARU, A. & FUKUYO, Y. 2011. Species boundaries in the toxic dinoflagellate *Prorocentrum lima* (Dinophyceae, Prorocentrales), based on morphological and phylogenetic characters. *Journal of Phycology,* 47, 178-189.

NAKAJIMA, I., OSHIMA, Y. & YASUMOTO, T. 1981. Toxicity of benthic dinoflagellates in okinawa. *Bulletin of the Japanese Society of Scientific Fisheries,* 47, 1029-1033.

NASCIMENTO, S. M. 2003. Phytoplankton blooms and water quality of the Fleet Lagoon, Dorset, UK, including studies of isolated toxic strains of *Alexandrium minutum* and *Prorocentrum lima*. PhD thesis. University of Southampton, United Kingdom.

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, 633-49.

NAVES, J. L., PRADO, M. P., RANGEL, M., DE SANCTIS, B., MACHADO-SANTELLI, G. & FREITAS, J. C. 2006. Cytotoxicity in the marine dinoflagellate *Prorocentrum mexicanum* from Brazil. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 143, 73-77.

NAYAK, B. B., KARUNASAGAR, I. & KARUNASAGAR, I. 1997. Influence of bacteria on growth and hemolysin production by the marine dinoflagellate *Amphidinium carterae*. *Marine Biology,* 130, 35-39.

OGINO, H., KUMAGAI, M. & YASUMOTO, T. 1997. Toxicologic evaluation of yessotoxin. *Nat Toxins,* 5, 255-9.

OKOLODKOV, Y. B., CAMPOS-BAUTISTA, G., GARATE-LIZARRAGA, I., GONZALEZ-GONZALEZ, J. A. G., HOPPENRATH, M. & ARENAS, V. 2007. Seasonal changes of benthic and epiphytic dinoflagellates in the Veracruz reef zone, Gulf of Mexico. *Aquatic Microbial Ecology,* 47, 223-237.

OLSON, R. J. & CHISHOLM, S. W. 1986. Effects of light and nitrogen limitation on the cell cycle of the dinoflagellate *Amphidinium carteri*. *Journal of Plankton Research* 8(4):785-793.

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.

PAERL, H. W. & PAUL, V. J. 2011. Climate change: Links to global expansion of harmful cyanobacteria. *Water Research*. *In press*

PARKHILL, J. P., MAILLET, G. & CULLEN, J. J. 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. *Journal of Phycology*, 37, 517-529.

PEARSON, R. G. & DAWSON, T. P. 2003. Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? *Global Ecology and Biogeography*, 12, 361-371.

PENNA, A., VILA, M., FRAGA, S., GIACOBBE, M. G., ANDREONI, F., RIOBO, P. & VERNESI, C. 2005. Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8s rDNA sequences. *Journal of Phycology*, 41, 212-225.

PEPERZAK, L. 2003. Climate change and harmful algal blooms in the North Sea. *Acta Oecologica-International Journal of Ecology*, 24, S139-S144.

PEREZ-GUZMAN, L., PEREZ-MATOS, A. E., ROSADO, W., TOSTESON, T. R. & GOVIND, N. S. 2008. Bacteria associated with toxic clonal cultures of the dinoflagellate *Ostreopsis lenticularis*. *Marine Biotechnology*, 10, 492-496.

PIN, L. C., TEEN, L. P., AHMAD, A. & USUP, G. 2001. Genetic diversity of *Ostreopsis ovata* (Dinophyceae) from Malaysia. *Marine Biotechnology*, 3, 246-255.

PISTOCCHI, R., VANUCCI, S., GUERRINI, F. & MILANDRI, A. 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.

PLUMLEY, F. G. 1997. Marine algal toxins: biochemistry, genetics, and molecular biology. *Limnology and Oceanography*, 42, 1252-1264.

POWELL, N., SHILTON, A., CHISTI, Y. & PRATT, S. 2009. Towards a luxury uptake process via microalgae defining the polyphosphate dynamics. *Water Research*, 43, 4207-4213.

QUILLIAM, M. A. 2003.Chemical methods for lipophilic shellfish. In G. M. Hallegraeff, D. M. Anderson, and A. D. Cembella (Eds), Manual on harmful marine microalgae. UNESCO. France, pp. 211-245.

RAATEOJA, M. P. 2004. Fast repetition rate fluorometry (FRRF) measuring phytoplankton productivity: A case study at the entrance to the Gulf of Finland, Baltic Sea. Boreal Environ. Res. 2004;9:263-276.

RAVEN, J. A. & GEIDER, R. J. 1988. Temperature and Algal Growth. *New Phytologist*, 110, 441-461.

RHODES, L., ADAMSON, J., SUZUKI, T., BRIGGS, L. & GARTHWAITE, I. 2000. Toxic marine epiphytic dinoflagellates, *Ostreopsis siamensis* and *Coolia monotis* (Dinophyceae), in New Zealand. *New Zealand Journal of Marine and Freshwater Research*, 34, 371-383.

RHODES, L. L., SMITH, K. F., MUNDAY, R., SELWOOD, A. I., MCNABB, P. S., HOLLAND, P. T. & BOTTEIN, M. Y. 2010. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. *Toxicon,* 56, 751-758.

RHODES, L. L. & THOMAS, A. E. 1997. *Coolia monotis* (Dinophyceae): A toxic epiphytic microalgal species in New Zealand (vol 31, pg 139, 1997). *New Zealand Journal of Marine and Freshwater Research,* 31, 286-286.

RICHLEN, M. L. & LOBEL, P. S. 2011. Effects of depth, habitat, and water motion on the abundance and distribution of ciguatera dinoflagellates at Johnston Atoll, Pacific Ocean. *Marine Ecology-Progress Series,* 421, 51-66.

SAKAMI, T., NAKAHARA, H., CHINAIN, M. & ISHIDA, Y. 1999. Effects of epiphytic bacteria on the growth of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). *Journal of Experimental Marine Biology and Ecology,* 233, 231-246.

SAKSHAUG, E., ANDRESEN, K., MYKLESTAD, S., & OLSEN, Y. 1983. Nutrient status of phytoplankton communities in Norwegian waters (marine, brackish and fresh) as revealed by their chemical composition. *Plankton Research,* 5, 175-196.

SCHOFIELD, O., GRZYMSKI, J., MOLINE, M. M. A. & JOVINE, R. V. M. 1998. Impact of temperature acclimation on photosynthesis in the toxic red-tide dinoflagellate *Alexandrium fundyense* (Ca28). *Journal of Plankton Research,* 20, 1241-1258.

SELANDER, E., THOR, P., TOTH, G. & PAVIA, H. 2006. Copepods induce paralytic shellfish toxin production in marine dinoflagellates. *Proceedings of the Royal Society B-Biological Sciences,* 273, 1673-1680.

SENFT, C., AVERY, D. E. & DAM, H. G. 2011. A novel approach to identifying PST tolerant copepods: An individual ingestion assay. *Harmful Algae,* 10, 804-810.

SHEARS, N. T. & ROSS, P. M. 2009. Blooms of benthic dinoflagellates of the genus *Ostreopsis*; an increasing and ecologically important phenomenon on temperate reefs in New Zealand and worldwide. *Harmful Algae,* 8, 916-925.

SHI, D., XU, Y. & MOREL, F. M. M. 2009. Effects of the pH/pCO_2 control method on medium chemistry and phytoplankton growth. *Biogeosciences,* 6, 1199-1207.

SHIKATA, T., NAGASOE, S., MATSUBARA, T., YAMASAKI, Y., SHIMASAKI, Y., OSHIMA, Y. & HONJO, T. 2007. Effects of temperature and light on cyst germination and germinated cell survival of the noxious raphidophyte *Heterosigma akashiwo*. *Harmful Algae,* 6, 700-706.

SPIJKERMAN, E. 2010. High photosynthetic rates under a colimitation for inorganic phosphorus and carbon dioxide. *Journal of Phycology,* 46, 658-664.

STEINDINGER, K.A., & TANGEN, K. 1996. Dinoflagellates. In Tomas, C. (Ed.). Identifying marine diatoms and dinoflagellates. Academic Press, San Diego, pp. 387-584.

STERNER, R. W. & GROVER, J. P. 1998. Algal growth in warm temperate reservoirs: Kinetic examination of nitrogen, temperature, light, and other nutrients. *Water Research,* 32, 3539-3548.

STROM, S. L. & MORELLO, T. A. 1998. Comparative growth rates and yields of ciliates and heterotrophic dinoflagellates. *Plankton Res.,* 20, 571-584.

SUGANUMA, M., TATEMATSU, M., YATSUNAMI, J., YOSHIZAWA, S., OKABE, S., UEMURA, D. & FUJIKI, H. 1992. An alternative theory of tissue-specificity by tumor promotion of okadaic acid in glandular stomach of rats. *Carcinogenesis,* 13, 1841-1845.

SUGG, L. M. & VANDOLAH, F. M. 1999. No evidence for an allelopathic role of okadaic acid among ciguatera-associated dinoflagellates. *Journal of Phycology,* 35, 93-103.

SUGGETT, D. J., MOORE, C. M., HICKMAN, A. E. & GEIDER, R. J. 2009. Interpretation of fast repetition rate (FRR) fluorescence: signatures of phytoplankton community structure versus physiological state. *Marine Ecology-Progress Series,* 376, 1-19.

SUZUKI, T., QUILLIAM, M. A., BEUZENBERG, V. & MACKENZIE, L. 2004. Discovery of okadaic acid esters in the toxic dinoflagellate *Dinophysis acuta* from New Zealand using liquid chromatography tandem mass spectrometry. *Rapid Communications in Mass Spectrometry,* 18, 1131-1138.

TAYLOR, F. J. R, FUKUYO, Y., LARSEN, J. & HALLEGRAEFF, G. M. 2003. Taxonomy of harmful dinoflagellates. *In*: Hallegraeff, GM, Anderson, DM & AC Cembella (Eds.). *Manual on Harmful Marine Microalgae*. UNESCO, Paris.

TAYLOR, F. J. R., HOPPENRATH, M. & SALDARRIAGA, J. F. 2008. Dinoflagellate diversity and distribution. *Biodiversity and Conservation,* 17, 407-418.

TEEGARDEN, G. J. & CEMBELLA, A. D. 1996. Grazing of toxic dinoflagellates, *Alexandrium* spp, by adult copepods of coastal Maine: Implications for the fate of paralytic shellfish toxins in marine food webs. *Journal of Experimental Marine Biology and Ecology,* 196, 145-176.

TOMAS, C. R. & BADEN, D. G. 1993. The influence of phosphorus on the growth and cellular toxin content of the benthic dinoflagellate *Prorocentrum lima. In* Smayda, T. J. & Shimizu, Y. (Eds.) Toxic Phytoplankton Blooms in the Sea. Elsevier Scientific, New York, *pp.* 565–70.

TONG, M. M., KULIS, D. M., FUX, E., SMITH, J. L., HESS, P., ZHOU, Q. X. & ANDERSON, D. M. 2011. The effects of growth phase and light intensity on toxin production by *Dinophysis acuminata* from the northeastern United States. *Harmful Algae,* 10, 254-264.

TOSTESON, T. R., BALLANTINE, D. L., TOSTESON, C. G., HENSLEY, V. & BARDALES, A. T. 1989. Associated bacterial flora, growth, and toxicity of cultured benthic dinoflagellates *Ostreopsis leticularis* and *Gambierdiscus toxicus*. *Applied and Environmental Microbiology,* 55, 137-141.

TURNER, J. T. 2006. Harmful algae interactions with marine planktonic grazers. In: GRANELI, E. & TURNER, J. (Eds). Ecology of Harmful Algae. Germany, Springer, pp. 259-270.

VALE, P., VELOSO, V. & AMORIM, A. 2009. Toxin composition of a *Prorocentrum lima* strain isolated from the Portuguese coast. *Toxicon,* 54, 145-152.

VALE, P., & SAMPAYO, M.A.M. 2002. Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determined by LC-MS – a widespread phenomenon. Toxicon, 40 (1), 33-42.

VAN DEN BERGH, J. C. J. M., NUNES, P. A. L. D., DOTINGA, H. M., KOOISTRA, W. H. C. F., VRIELING, E. G. & PEPERZAK, L. 2002. Exotic harmful algae in marine ecosystems: an integrated biological-economic-legal analysis of impacts and policies. *Marine Policy,* 26, 59-74.

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.

VARDI, A., BERMAN-FRANK, I., ROZENBERG, T., HADAS, O., KAPLAN, A. & LEVINE, A. 1999. Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO2 limitation and oxidative stress. *Current Biology*, 9, 1061-1064.

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.

WELSCHMEYER, N. A. 1994. Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b* and pheopigments. *Limnology and Oceanography*, 39, 1985-1992.

WINDUST, A. J., WRIGHT, J. L. C. & MCLACHLAN, J. L. 1996. The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin1, on the growth of microalgae. *Marine Biology*, 126, 19-25.

WORLD REGISTER OF MARINE SPECIES. 2012. http://www.marinespecies.org/aphia.php?p=taxdetails&id=110301

YAMAGUCHI, H., MIZUSHIMA, K., SAKAMOTO, S. & YAMAGUCHI, M. 2010. Effects of temperature, salinity and irradiance on growth of the novel red tide flagellate *Chattonella ovata* (Raphidophyceae). *Harmful Algae*, 9, 398-401.