THE USE OF WATER- AND POCKET-PULSE AMPLITUDE MODULATION IN INVESTIGATING PHOTOPHYSIOLOGY AND NUTRIENT LIMITATIONS IN MICROALGAE

NUR AQILAH BINTI MUHAMAD DARIF

UNIVERSITI SAINS MALAYSIA

2018

THE USE OF WATER- AND POCKET-PULSE AMPLITUDE MODULATION IN INVESTIGATING PHOTOPHYSIOLOGY AND NUTRIENT LIMITATIONS IN MICROALGAE

by

NUR AQILAH BINTI MUHAMAD DARIF

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

May 2018

ACKNOWLEDGEMENT

Alhamdulillah, all praise be to Allah S.W.T. for His blessing and for giving me strength in completing my masters' studies. My postgraduate years have been one long journey filled with many obstacles and challenges. However, I am glad and relieved to have finally completed my studies with a feeling of rejoice and great satisfaction. This would not have been possible without the guidance and support of those around and very dear to me.

A very special appreciation goes to my supervisor, Dr. Sazlina Md Salleh for her kindness, moral support and patience, and for sharing her wide knowledge and extending invaluable assistance. My special thanks to Prof. Andrew McMinn from the Institute for Marine and Antarctic Studies (IMAS), Australia for his guidance, ideas and interesting suggestions on my research. My gratitude to Dr. Mahadi Mohammad and Dr. Leaw Chui Pin for allowing me to use their laboratory facilities to conduct tropical microalgae experiment.

I am also very grateful to Dr. Sarah Ugalde for her guidance in conducting acid and bicarbonate experiment; Lee Shi Hong for PAM assistance; Dr. Marius Müller for his help in carbonate chemistry manipulations through e-mails and IMAS' staff for helping and allowing me to use the facilities and equipment to analyse cultured samples throughout my three months' research in IMAS. I would like to aknowledge MOSTI Flagship Grant (304.CDASAR.650724.P131) and USM-RUI Grant (1001.PBIOLOGI.811245) for funding my research. In addition, I would like to thank the staff from the Centre for Marine and Coastal Studies (CEMACS) and the School of Biological Sciences for accommodating workspace and facilities that I needed.

My deepest gratitude goes to my dearest and beloved family for their unwavering support, words of encouragement and prayers that have helped motivate me in carrying out my studies. Last but not least, my thanks are also extended to all my friends and laboratory mates especially Shakila, Aysha, Nadthikphorn, Alia, Hana, Ain, Hilal, Michelle, Firdaus, Ayesha and Linda for their understanding, ideas and assistance throughout my research.

To those who have directly or indirectly contributed in my studies, I would like to convey my heartfelt gratitude. Only Allah S.W.T. can repay your kindness. Thank you!

Aqilah Darif

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
[ ]	concentration
±	plus minus
ΔF%	percentage change of maximal or minimal change in fluorescence output
ΔF_d	NIFT drop
ΔF_p	NIFT peak
°C	degree Celsius
µ	specific growth rate
µg	microgram
µg L^{-1}	microgram per litre
µm	micrometre
µM	micro molar
µmol kg^{-1}	micromole per kilogram
µmol photons m^{-2} s^{-1}	micromole photons per metre squared per second
A.aff	*Alexandrium affine*
A.cat	*Alexandrium catenella*
A.min	*Alexandrium minutum*
A.tam	*Alexandrium tamiyavanichi*
Act	*Actinocyclus octonarius*
Amp	*Amphidinium massartii*
ANOVA	Analysis of Variance
ATP	adenosine triphosphate
Bys	*Bysmatrum* sp.
C.neg	*Chaetoceros neglectus*
CCM	carbon concentrating mechanism
cells mL^{-1}	cells per millilitre
CEMACS	Centre for Marine and Coastal Studies

Chae	*Chaetoceros* sp.
Chl	*Chloropsis* sp.
Chl *a*	chlorophyll *a*
cm	centimetre
CMAR	CSIRO Marine and Atmospheric Research
CO_2	carbon dioxide
Coo	*Coolia malayensis*
CRM	Certified Reference Material
CSIRO	Commonwealth Scientific and Industrial Research Organisation
d^{-1}	per day
df	degree of freedom
DIC	dissolved inorganic carbon
DO	dissolved oxygen
E.hux	*Emiliana huxleyi*
E_k	photoacclimation index or minimal saturating irradiance
Fe	iron
F_o	minimal fluorescence
F_q'/F_v'	effective quantum yield
F_t	steady state fluorescence under actinic light
F_v/F_m	maximum quantum yield of PS II
g	gram
Gam	*Gambierdiscus* sp.
HCl	hydrochloric acid
HCO_3^-	bicarbonate
HNLC	high nutrient low chlorophyll
IMAS	Institute for Marine and Antarctic Studies
IOES	Institute of Ocean and Earth Sciences
IPCC	Intergovernmental Panel on Climate Change

Iso	*Isochrysis* sp.
K	Kelvin
Kar	*Karlodinium veneficum*
L	litre
LM	light microscope
m	metre
mg	milligram
mg L^{-1}	milligram per litre
mL	millilitre
N	nitrogen
N.dir	*Navicula directa*
N.gla	*Navicula glaciei*
NADPH	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate
Nan	*Nannochloropsis* sp.
NaOH	sodium hydroxide
NH_3	ammonia
NH_4^+	ammonium
NIFT	nutrient-induced fluorescence transient
Nitz	*Nitzschia* sp.
nm	nanometre
NO_2^-	nitrite
NO_3^-	nitrate
NPQ	non-photochemical quenching
O_2	oxygen
OA	ocean acidification
P	p value or phosphorus
PAM	Pulse Amplitude Modulation
pCO_2	partial carbon dioxide

P-E	photosynthesis-irradiance
PFZ	Polar Frontal Zone
PO_4^{3-}	phosphate
ppt	parts per thousand
Pro	*Prorocentrum* sp.
r	Pearson's correlation coefficient
R^2	regression coefficient
$rETR_{max}$	maximal electron transport rate
RLC	rapid light curve
rpm	revolutions per minute
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
s	second
SD	standard deviation
SEM	scanning electron microscope
Si	silica
$Si(OH)_4$	silicic acid
SO	Southern Ocean
SPSS	Statistical Package for Social Science
T	temperature
T.sue	*Tetraselmis suecica*
TA	total alkalinity
Tab	*Tabellaria flocculosa*
Tet	*Tetraselmis* sp.
UTas	University of Tasmania
α	photosynthetic efficiency
β	photoinhibition or photodamage

PENGGUNAAN WATER- DAN POCKET-PULSE AMPLITUDE MODULATION DALAM PENYELIDIKAN FOTOFISIOLOGI DAN BATASAN NUTRIEN DALAM MIKROALGA

ABSTRAK

Penilaian batasan nutrien dan fotosintesis dapat memberikan kefahaman tentang tindak balas sel-sel mikroalga terhadap perbezaan pengaruh alam sekitar atau fizikal terutamanya nutrien. Kemajuan dalam teknik pendarfluor klorofil menyediakan cara pengukuran ekofisiologi yang pantas dan tidak invasif kepada mikroalga. Tambahan pula, Pulse Amplitude Modulation (PAM) fluorometer di pasaran mempunyai ciri-ciri teknikal dan konfigurasi yang berbeza dan menyebabkan perbezaan dalam output fotosintesis. Di dalam kajian ini, aktiviti fotosintesis mikroalga dilihat menggunakan Water-PAM dan Pocket-PAM untuk mengesahkan perbezaan antara dua fluorometer tersebut. Sampel komuniti mikroalga telah dikumpul dari Teluk Bahang dan Pantai Jerejak manakala sampel kultur spesies individu diperoleh dari Pusat Kajian Samudera dan Pantai (CEMACS), Pulau Pinang. Untuk kedua-dua analisa tersebut, hasil kuantum maksimum (F_v/F_m), kadar pengangkutan elektron relatif maksimum ($rETR_{max}$) dan indeks penyesuaian cahaya (E_k) Water-PAM mempunyai perbezaan yang ketara ($P < 0.05$) iaitu kira-kira 50% daripada nilai Pocket-PAM. Sebagai contoh, dalam sampel komuniti mikroalga air laut di Pantai Jerejak, kadar fotosintesis ($rETR_{max}$) meningkat sebanyak empat kali ganda dengan penggunaan Water-PAM (67.5 ± 3.7) berbanding Pocket-PAM (16.6 ± 2.1). Tindak balas Nutrient-Induced Fluorescence Transient (NIFT) terhadap mikroalga turut berbeza dengan penggunaan Water-PAM dan Pocket-PAM. Kajian ini menunjukkan bahawa langkah berjaga-jaga perlu dititikberatkan ketika mentafsirkan data menggunakan fluorometer yang berbeza kerana mereka adalah berbeza dari segi ciri-

ciri teknikal seperti had pengesanan anggaran, konfigurasi dan warna cahaya aktinik. Untuk memahami lebih lanjut kesan silika pada fotosintesis mikroalga, tahap kepekatan silika telah dimanipulasikan dalam dua spesies diatom: *Actinocyclus octonarius* (spesies tropika) dan *Chaetoceros neglectus* (spesies kutub). Kedua-dua diatom ini tidak menunjukkan apa-apa perubahan yang signifikan ($P > 0.05$) dalam biomas klorofil tetapi menunjukkan perbezaan yang signifikan ($P < 0.05$) dalam nilai F_v/F_m. Penemuan ini menunjukkan bahawa *A. octonarius* dan *C. neglectus* dapat hidup dengan baik tanpa mengira kepekatan silika namun fotosintesis mereka terjejas. Selain itu, batasan nutrien dalam mikroalga ditentukan dengan menggunakan pendekatan yang mudah dan cepat iaitu NIFT. Untuk menguji kesesuaian NIFT dalam kajian mikroalga, NIFT diukur dalam 28 spesies kultur di makmal dari pelbagai taxa (diatom sehingga alga hijau) dan lokasi yang berbeza (spesies kutub sehingga tropika). Semua kultur dibiarkan hidup dalam media f/2 sekurang-kurangnya empat minggu. Hal ini menyebabkan mereka mengalami kekurangan nutrien atau kehabisan nutrien dan seterusnya mereka diuji untuk NIFT. Nitrat menunjukkan respon NIFT yang positif dalam *Alexandrium affine* dan *Coolia malayensis*; fosfat menyebabkan respon NIFT positif dalam *C. neglectus*, *Euglena gracilis*, *Alexandrium minutum* dan *Gambierdiscus* sp.; manakala silika memberi pengaruh NIFT yang positif dalam *Alexandrium tamiyavanichi*, *C. malayensis* dan *Gambierdiscus* sp. Mikroalga lain mengalami sama ada negatif, tiada respon atau tidak dapat dikesan dengan NIFT. Keputusan ini menunjukkan bahawa sebahagian besar kumpulan yang terdiri daripada dinoflagellate dan alga hijau dapat dikesan manakala lima daripada sembilan diatom spesies kutub tidak dapat dikesan dengan menggunakan NIFT. Hal ini menunjukkan bahawa NIFT kurang memberi faedah dalam kajian melibatkan Lautan Selatan di mana didominasi diatom. Kajian ini telah memberikan cadangan dan

maklumat yang berharga tentang penggunaan PAM fluorometer dengan menyiasat fotosintesis mikroalga di kawasan pasang surut Pulau Pinang. Objektif utama telah berjaya dicapai dan pengetahuan tentang mikroalga menggunakan pendekatan yang lebih baru (iaitu NIFT dan asid-bikarbonat) dapat dikembangkan. Hal ini sudah pasti dapat menambah pemahaman tentang aplikasi dan kaedah yang berguna menggunakan pendekatan klorofil pendarfluor dalam mengkaji tindak balas mikroalga.

THE USE OF WATER- AND POCKET-PULSE AMPLITUDE MODULATION IN INVESTIGATING PHOTOPHYSIOLOGY AND NUTRIENT LIMITATIONS IN MICROALGAE

ABSTRACT

Investigating photosynthesis and nutrient limitations of microalgae allow for insight into how cells will respond to different environmental or physical stressors particularly nutrients. Advances in chlorophyll fluorescence techniques have provided a means for rapid and non-invasive measurement of microalgal ecophysiology. Furthermore, available Pulse Amplitude Modulation (PAM) fluorometers in the market present different technical characteristics and configuration which may lead to different photosynthetic parameters output. Here the photosynthetic activities of microalgae were observed using both Water-PAM and Pocket-PAM to validate the differences between these two fluorometers. Microalgal community samples were collected from Teluk Bahang and Pantai Jerejak while single species laboratory cultures were obtained from the Centre for Marine and Coastal Studies (CEMACS), Penang. For both field and laboratory studies, maximum quantum yield (F_v/F_m), maximum relative electron transport rate ($rETR_{max}$) and photoacclimation index (E_k) of Water-PAM were significantly different ($P < 0.05$), about 50% to those of Pocket-PAM. For example, in Pantai Jerejak community sample, photosynthetic rates ($rETR_{max}$) increased by four-fold when using Water-PAM (67.5 ± 3.7) by comparison to Pocket-PAM (16.6 ± 2.1). Similarly, nutrient-induced fluorescence transient (NIFT) using Water-PAM and Pocket-PAM were different in terms of the response output. This study showed that care should be taken when interpreting results using different PAM fluorometers because they are different in terms of technical characteristics such as approximate detection limit, configuration and actinic light colour. To further

understand the effect of silica on photosynthesis of microalgae, silica concentrations were manipulated in two species of diatoms: tropical *Actinocyclus octonarius* and polar *Chaetoceros neglectus*. Both diatoms did not show any significant changes ($P > 0.05$) in chlorophyll *a* biomass but showed significant differences ($P < 0.05$) in F_v/F_m. These findings suggest that *A. octonarius* and *C. neglectus* were able to thrive irrespective of low or high silica concentrations but their photosynthesis would be affected. Apart from that, nutrient limitations in microalgae were determined using a simple and rapid approach called NIFT. To test the suitability of NIFT in microalgal studies, NIFTs were measured on 28 species in laboratory cultures which had a broad range of microalgal taxa (from diatoms to green algae) and different regions (polar to tropical species). All aged cultures (at least four weeks-old) were either nutrient-limited or nutrient-starved as they were tested for NIFT. Nitrate was found to induce positive NIFT responses in *Alexandrium affine* and *Coolia malayensis;* phosphate addition resulted in positive NIFT responses in *C. neglectus*, *Euglena gracilis*, *Alexandrium minutum*, and *Gambierdiscus* sp.; whereas silica induced positive NIFT responses in *Alexandrium tamiyavanichi*, *C. malayensis* and *Gambierdiscus* sp. Other microalgae either experienced negative, no NIFT responses or undetectable. These results showed that mostly dinoflagellates and green alga could be detected while five out of nine polar diatoms were undetectable with NIFT, suggesting that NIFT would have little use in Southern Ocean studies where they dominated. This study has provided valuable information and recommendation on the use of PAM fluometers by investigating photosynthesis of microalgae in field at Penang intertidal areas. These key aims have been successfully addressed and knowledge on microalgae has broadened using relatively new approaches (i.e. NIFT and acid-bicarbonate). This

definitely will further add to our understanding of useful applications and approaches of chlorophyll fluorescence in studying microalgal responses.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Marine microalgae contribute approximately 50% of the primary productivity in the ocean (Befrenfeld et al., 2001; Field et al., 1998) and aquatic primary productivity is often limited by the nutrients availability (Falkowski et al., 1992; Howarth, 1988). Due to the importance of microalgae in food web, nutrient cycling and environmental indicators, it is important to assess the biomass and nutrients that may lead to changes in their growth rates. In the past, primary productivity and biomass were measured using bell jars or benthic chambers, oxygen microelectrodes and ^{14}C radioisotopes. With the advancement of technology, the usage of chlorophyll fluorescence has now been widely used in microalgal studies. Fluorescence measurements provide a non-destructive, rapid and valuable information on the photosynthetic activities and nutrient limitations of microalgae. Most research undertaken using PAM is usually conducted in polar (McMinn et al., 2007, 2012; Salleh and McMinn, 2011) and temperate regions (Lee and McMinn, 2013; McMinn et al., 2005a) but less in the tropical region (McMinn et al., 2005b; Wong et al. 2015). In addition, different fluorometers in the market present different photosynthetic output. Understanding the fundamentals of microalgae coupled with effective technique for their assessment would be feasible in water management, monitoring of microalgae and optimisation of production in commercial microalgae.

1.2 Nutrients and microalgae

Nutrients play an important role in primary productivity and growth rate of microalgae (Hecky and Kilham, 1988; Juneja et al, 2013; Mackey et al., 2008; Roberts et al., 2008; Stoń-Egiert, 2010). The most important macronutrients are nitrogen and phosphorus which are taken up to an extent that their growth is inhibited (Riley, 1971). In the past, nitrogen has been the limiting factor in the marine environment (Birch et al., 1981; Dodds et al., 1993; Elser et al., 1990; Hecky and Kilham, 1988; Lean and Pick, 1981). Generally, diatoms require silica (Egge and Aksnes, 1992; Nelson and Dortch, 1996), green algae use up phosphate whereas dinoflagellates use up nitrogen sources from ammonia or inorganic nitrogen (Glibert and Terlizzi, 1999; Lomas and Glibert, 1999; Iriarte et al., 2005). Inorganic nutrients such as nitrate, phosphate and silicic acid are required by microalgae for their cellular processes (Fields et al., 2014).

Typically in marine ecosystems, microalgae require an average molar N:P ratio of 16:1 (Redfield, 1958). Changes from this optimal ratio can be used to infer nutrient limitation of microalgal growth. If the N:P ratio is more than 16:1, it indicates most likely phosphorus is limited and if the ratio is less than 16:1, nitrogen is limited (Howarth, 1988). However, species are different in terms of their N and P requirements and nutrient kinetic uptake and thus may have different optimal N:P ratio (Hecky and Kilham, 1988). The nitrogen limitation has been confirmed repeatedly using bioassays, mesocosm experiment and in marine temperate waters, while phosphorus limitation is often found in tropical waters where it binds tightly in rocks.

Microalgal productivity in most of the open-ocean is concentrated in the Polar Regions (i.e. Arctic and Southern Oceans), where nutrient upwelling from ocean currents brings nutrients from the bottom of oceans up to the surface. In these regions, macronutrients such as nitrogen, phosphorous and carbon are seldom used up by

microalgae, although they have higher productivity because iron is far more limiting (Croot et al., 2004). In Southern Ocean, despite having the highest macronutrients such as N, P and Si in marine environment across the globe, Si and Fe are spatially and temporally variable (Martin, 1990; Coale et al., 2003, 2004; Hiscock et al., 2003; de Baar et al., 2005). The factors operating in polar and tropical regions are different (Table 1.1).

Table 1.1 Comparison between tropical and polar regions in open waters of various relative factors operating in each system (modified from Smith and Lancelot, 2004).

Factor	Tropical Regions	Polar Regions
Biological control of microalgae	High	Low
Maximum microalgae biomass	Low	High
Ambient macronutrient concentrations	Low	High
Abundance of diatoms	Low	Often high
Main source of iron	Atmospheric	Upwelling
Irradiance limitation	Rare	Frequent

1.3 Pulse Amplitude Modulation (PAM)

Pulse Amplitude Modulation (PAM) has been developed to assess photosynthetic physiology in higher plants and later developed in macroalgae and microalgae (Figueroa et al., 2003; Flameling and Kromkamp, 1998; Hanelt, 1992; Schreiber et al., 1995; Suggett et al., 2011; Wilhelm et al., 2004). It has become one of the most common, non-invasive and rapid techniques to measure variable chlorophyll fluorescence and photosynthetic performance in microalgae (Baker, 2008). There are many types of Pulse Amplitude Modulation (PAM) fluorometers available in the market. These PAM fluorometers may present different characteristics in terms of approximate detection limit, measuring light colour, actinic light colour and configuration (Table 1.2; Figueroa et al., 2013). These differences may lead to

inconsistencies and variations in outputs, making interpretations quite different from one equipment to another. Figueroa et al. (2013) compared different types of PAM concerning their characteristics and how they were manufactured. From the early type of PAM flurometers such as PAM 2000 and later developed to Water-PAM and Pocket-PAM and until recently, Multicolor-PAM. They also presented data on Water-PAM in microalga *Chlorella* sp. and Junior-PAM. Figueroa et al. (2013) were the first to compare Water-PAM (fiber configuration) and Pocket-PAM in microalga culture *Chlorella fusca* from urban sewage. They found significant difference in the effective quantum yield using Water-PAM and Pocket-PAM. In addition, when they compared Water-PAM fiber version with Water-PAM cuvette version, no significant differences ($P > 0.01$) were found in the effective quantum yield. This might suggest possible artifacts by scattering or reabsorption of fluorescence seem not to be significant (Figueroa et al., 2013).

Table 1.2 Technical characteristics of PAM (modified from Figueroa et al., 2013)

PAM Fluorometer	Measurement of algal suspensions	Approximate detection limit, µg Chl-*a* L^{-1}	Measuring light color	Actinic light color	Configuration
WATER-PAM	Immersion of fiber optics in suspension or aliquot in cuvette	0.025	Blue or red (LEDs)	Blue or red (LEDs)	Cuvette or fiber optics version
POCKET-PAM	Emitter-Detector Unit (ED) optimized to look through a glass window into an algae suspension cultivators	500	Blue (LEDs)	Blue (LEDs)	Optical head

1.4 Problem statements

1. Available Pulse Amplitude Modulation (PAM) fluorometers in the market present different technical characteristics and configuration which may lead to different photosynthetic parameters output. Usage of PAM in microalgal studies is widely used. However, photosynthetic activities of microalgae using chlorophyll fluorescence are lacking in Malaysia.

2. Nutrients and pH are important for growth and photosynthesis of microalgae. However, studies on the effect of silica on diatoms are lacking. As for pH, carbonate chemistry manipulation is understudied.

3. Conventional methods of assessing nutrient limitations in microalgae usually expensive and time-consuming. Previous studies have summarised nutrient limitations in microalgae using nutrient-induced fluorescence transients (NIFTs) but these studies use different species of microalgae and protocol. Hence, NIFT protocol is not standardised and this may lead to different results.

1.5 Objectives

The main objectives of this study were to investigate the photosynthetic activities and nutrient limitations in microalgae using chlorophyll fluorescence. While, the specific objectives with respect to using chlorophyll fluorescence were:

1. To validate differences of photosynthetic parameter outputs and NIFTs responses between Water-PAM and Pocket-PAM in field and laboratory studies of microalgae.

2. To investigate the effect of silica concentrations on photosynthesis of diatoms, tropical *Actinocyclus octonarius* and polar *Chaetoceros neglectus*.

3. To assess nutrient limitations in microalgae using NIFT and to determine the suitability of NIFT in a broad range of microalgal taxa.

1.6 Hypotheses

The hypotheses being tested in this study were:

1. Water-PAM would produce higher photosynthetic parameter outputs than Pocket-PAM when tested in both microalgal community and single species in laboratory studies.
2. Silica concentrations would affect the photosynthesis of tropical and polar diatom cultures such that higher silica concentrations would enhance photosynthetic efficiency by producing higher maximum quantum yield (F_v/F_m) than lower silica concentrations.
3. The nutrient limitations observed based on steady state under actinic light (F_t) would vary across broad microalgal taxa using NIFT.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of microalgae

Microalgae are unicellular and photosynthetic microorganisms that can be commonly found in marine and freshwater. Their size typically ranges from 2 – 200 μm (Heimann and Huerlimann, 2015; Venkatesan et al. 2015). Sometimes they can grow in colonies large enough to be seen by the naked eye. Microalgae can be divided into eukaryotic photoautotrophic protists and prokaryotic cyanobacteria (blue-green algae; Malapascua et al., 2014; Singh and Saxena, 2015). These algae can photosynthesise although they have different physical structures (Raven and Giordano, 2014). Despite their differences, all microalgae possess at least one form of chlorophyll (chlorophyll *a*) and hence can undergo photosynthesis for energy. Marine microalgae mainly comprise microalgae known as dinoflagellates and diatoms, though cyanobacteria and other algae can also be present. Examples of microalgae are cyanobacteria, diatoms, dinoflagellates, green algae and coccolithophores (Figure 2.1).

Figure 2.1 Microalgae come in many different structures, but all except for cyanobacteria are algae. Collage adapted from drawings and micrographs by Sally Bensusen, NASA EOS Project Science Office. Source from Lindsey and Scott (2010).

Diatoms have been classified under the group Bacillariophyta as they are composed of transparent cell walls made of silicon dioxide (Julius and Theriot, 2010) hydrated with a small amount of water ($SiO_2 + H_2O$). Silica is the major component of the cell walls and it resembles a glass house. The cell wall is called frustule and it consists of two valves that fit into each other to look like a little pill box (Hallegraeff, 1988; Julius and Theriot, 2010). Diatoms grow as single cells or form filaments and simple colonies. Most diatoms are microscopic and their size ranges from 2 – 500 (0.5 mm) μm. Two major groups of diatoms are centric (Coscinodiscophyceae) and pennate (Bacillariophyceae) diatoms (Round et al., 1990). Centric diatoms have cells with radial symmetry that cut about a point and they are not able to move (Round et al., 1990). Pennate diatoms have cells with bilateral symmetry that cut about a line and some of them are able to move horizontally or vertically within sediments (Round et al., 1990).

Dinoflagellates have two flagella and they can be divided into armoured (presence) and unarmoured (absence) of a theca (rigid outer cell covering), a characteristic of the cell wall composition and structure (Taylor et al., 2003). They are three-fold smaller than diatoms rendering them difficult to be identified under light microscope. They can be found in surface waters and play a key role as the symbiotic zooxanthellae in corals (Hallegraeff, 1988). All zooxanthellae are dinoflagellates and mostly they are from genus *Symbiodinium* (Thornhill et al., 2017).

Other classes of microalgae are green algae (Chloropyhceae), red algae (Rhodophyceae), golden algae (Chrysophyceae), brown algae (Phaeophyceae) and Eustigmatophyceae (Andersen, 2013). Different algae group have different body types, cell wall components, storage materials and accessory pigments. Smaller microalgae (2 – 20 μm) can have higher growth rates compared to bigger microalgae

(20 – 200 μm). For instance, cyanobacteria have a relatively higher growth rate, capable of doubling twice a day compared to diatoms, usually doubling in a week or two.

Microalgae are important primary producers as they account for 30% of primary productivity and they form the basis of food chain in marine environment (Malapascua et al., 2014). Due to their short life cycles and metabolic flexibility, it is possible to modify their cellular composition and biochemical pathways by manipulating culture conditions (Malapascua et al., 2014). They contribute in aquaculture, food and pharmaceutical industry (Hu et al., 2008). Marine microalgae such as green algae *Chloropsis* sp., *Tetraselmis* sp. and *Nannochloropsis* sp., diatom *Chaetoceros* sp. (Milione and Zeng, 2007) and flagellate *Isochrysis* sp., have been produced commercially as food source for crustacean, fish and bivalve larvae (Arkronrat and Oniam, 2012; Arkronrat et al., 2016).

2.2 Photosynthesis

Photosynthesis is derived from the Greek words "phos" and "synthesis" which mean light and placing together respectively. Microalgae carry out photosynthesis and they are responsible for significant primary productivity in aquatic environments. Generally, microalgae take up carbon dioxide and water by utilising light energy, then converting into chemical energy in a form of carbohydrate. The photosynthetic equation proposed by van Niel and Muller (1931) was:

$$6CO_2 + 12H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O \quad (2.1)$$

Photosynthesis occurs in the chloroplast and can be divided into light reactions (the photo part), in which light energy is stored as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH; reduced NADP), and dark

reactions (the synthesis part), in which the products of the light reactions are used to reduce CO_2.

Light reactions also known as light-dependent reactions occur in thylakoid membrane of the chloroplast and have been characterised as the Z-scheme (Figure 2.2). The process of light reaction begins when energy strips electrons from suitable substance such as water and converting into oxygen. The hydrogen from the splitting water produces ATP and NADPH. Photosynthetic light reactions consist of light absorption and energy conversion. With regard to light absorption, photopigments absorb light energy in which chlorophyll *a* is the major pigment absorbing red (650 - 700 nm) and blue (400 - 450 nm) wavelength. As for energy conversion, when all electrons are in excited state during photosystem II, the energy is lost in de-excitation processes such as heat. Diatoms in particular, use chlorophyll *a* and *c* pigments to obtain energy from sunlight through photosynthesis.

In the light reactions, chlorophyll *a* molecule absorbs light energy and the electron gains energy in which pigment molecule is said to be in an excited state. The electron is transferred from the excited P680 to another molecule called primary electron acceptor. The chlorophyll molecule is oxidised and has a positive charge called $P680^+$ (Krause and Weiss, 1991). Each photoexcited electron passes from the primary electron acceptor of PSII to PSI through an electron transport chain. The electron transport chain between PSII and PSI is made up of the electron carrier plastoquinone (Pq), a cytochrome complex and a plastocyanin (Pc). The electrons enter lower and lower energy state and this provides energy for the synthesis of ATP. Meanwhile, light energy is transferred via light harvesting complex pigments to the PSI reaction centre complex, exciting an electron of the P700 pair of chlorophyll *a* molecules located there. The photoexcited electron is then transferred to primary

electron acceptor of PSI, creating an electron hole in the P700 which is called $P700^{+}$ and is ready to accept electron.

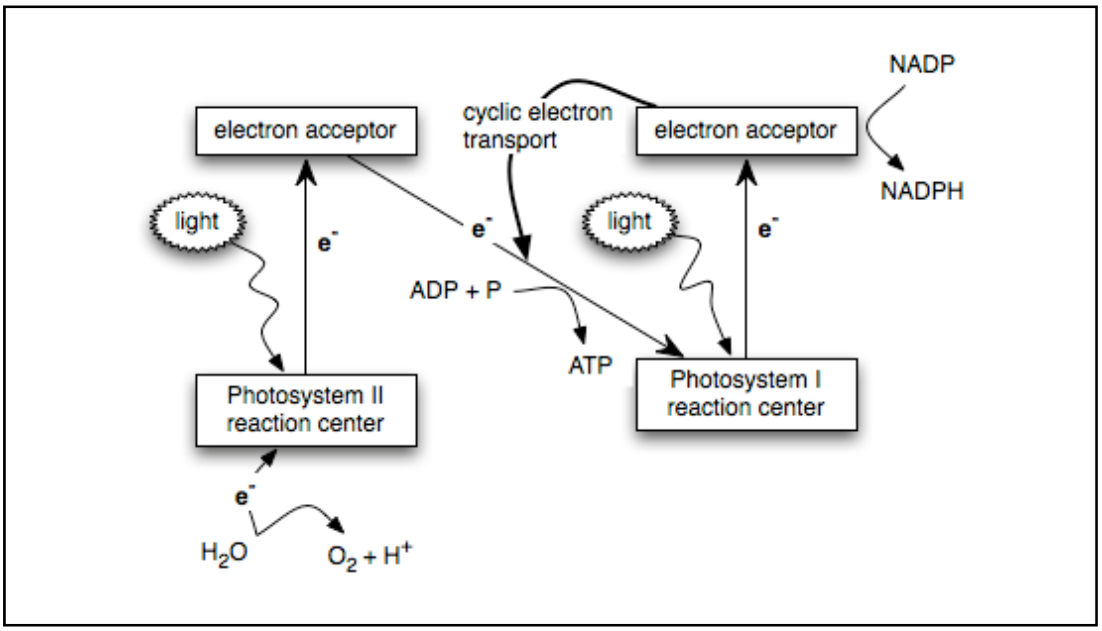

Figure 2.2 The light reactions of photosynthesis known as the Z-scheme. The linear electron flow generates ATP and NADPH. Source from Tiftickjian (2014).

Dark reactions also known as light-independent reactions occur in the stroma. They are not directly driven by light although they are activated by and require the formation of products from the light reactions to produce energy molecules. These reactions include the steps of the Calvin cycle (Figure 2.3), in which the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) catalyses the incorporation of CO_2 into simple three carbon sugar phosphate molecules using the reducing energy stored in ATP and NADPH generated by the light reactions of photosynthesis (Bassham et al., 1950). This process is called carbon fixation because CO_2 becomes incorporated, or fixed, into organic molecules that can be used for generating energy through respiration or to produce biomass (new cells, storage compounds).

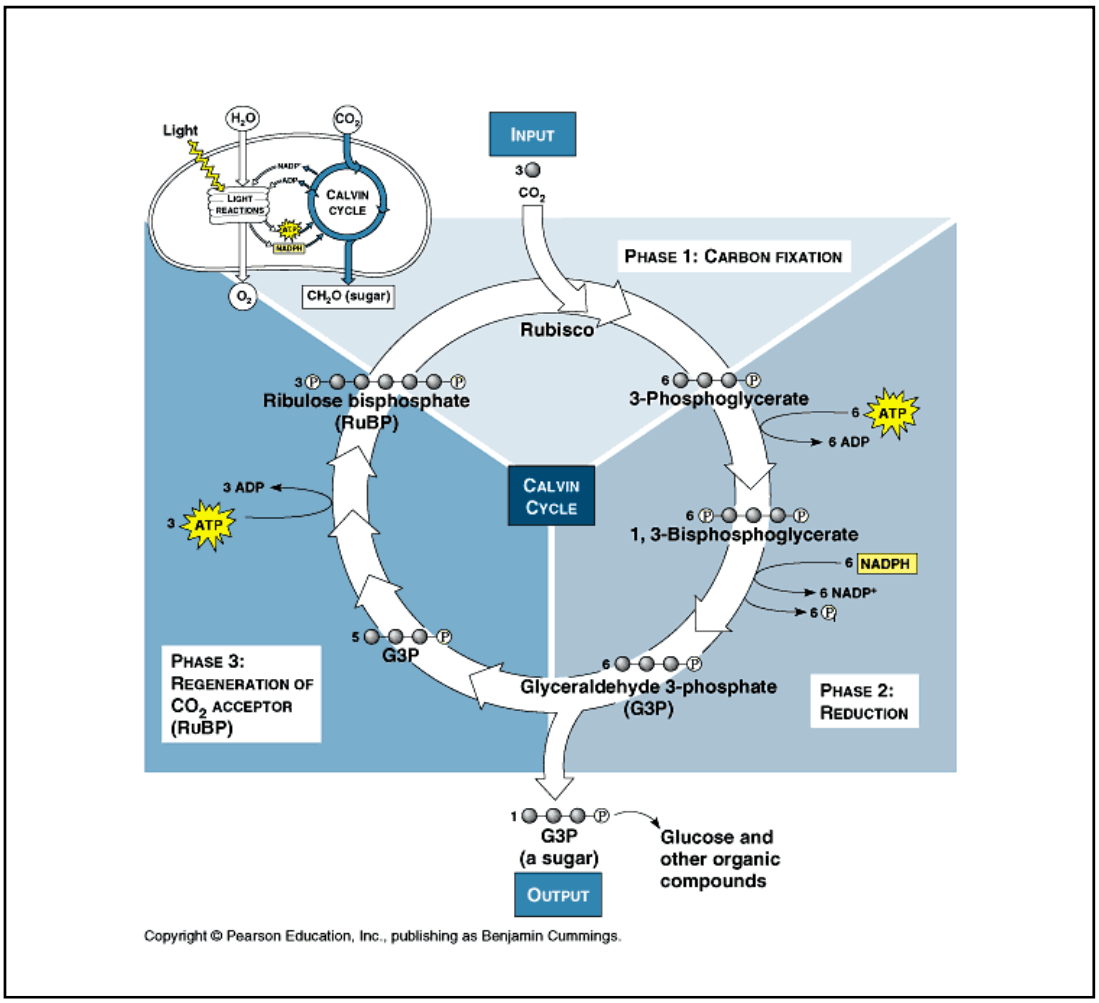

Figure 2.3 The Calvin cycle. This diagram tracks carbon atoms (grey circles) through the cycle. The three phases of the cycle correspond to the phases discussed in the text. For every three molecules of CO_2 that enter the cycle, the net output is one molecule of glyceraldehyde 3-phosphate (G3P), a three-carbon sugar. The light reactions sustain the Calvin cycle by regenerating ATP and NADPH (adopted from Campbell et al., 2008).

2.1 Introduction to chlorophyll fluorescence

The basic principle of fluorescence is the re-emission of energy (light) at a longer wavelength in the form of photon as electrons bouncing back from an excited state to a ground state (Cosgrove and Borowitzka, 2010). For example, chlorophyll *a* absorbs blue light at 430 nm and re-emits it at a red wavelength 662 - 669 nm. When chlorophyll molecules absorb light, they either undergo (1) photochemistry, (2) re-emitted as fluorescence (Huot and Babin, 2010) or dissipated as heat (Consalvey et al., 2005; Huot and Babin, 2010; Murchie and Lawson, 2013; White et al., 2011). Baker

(2008) assumed that at room temperature, the changes in the fluorescence signal arise from PSII only and ignore emission from PSI due to insignificant contribution of florescence signal below 700 nm. However, when the fluorescence signal to total F_o fluorescence at wavelengths greater than 700 nm, significant contribution of PSI were estimated approximately 30% and 50% for C3 and C4 leaves, respectively (Pfündel, 1998; Franck et al., 2002). Most commercial instruments measure a significant amount of fluorescence at wavelengths above 700 nm. Hence, significant PSI contribution to F_o at wavelengths above 700 nm has to be taken into consideration to limit source of errors when measuring quantum yields.

2.1.1 *In vivo* chlorophyll fluorescence

In vivo chlorophyll fluorescence is a valuable tool in photophysiological and ecological studies of microalgae (Baker, 2008). Chlorophyll fluorescence was first used in vascular plants (Björkman and Demmig, 1987) and later used in microalgae (Duysens and Sweers, 1963). This technique gained attention in past decades due to its advantages as rapid and non-destructive to the system under study (Baker, 2008; Consalvey et al., 2005; Schreiber, 1986). However, the underlying concepts of fluorescence technique were not straight forward and sometimes the interpretations could be difficult (Baker, 2008). Kautsky (1931) was the first to discover an association between fluorescence and photosynthesis. Good understanding between fluorescence parameters and photosynthetic electron transport *in vivo* led to a range of affordable and easy to use portable fluorometers (Baker, 2008). According to Govindjee (2004), chlorophyll fluorescence rose rapidly to a maximum then declined and reached a steady level within a few minutes.

In 1966, *in vivo* chlorophyll fluorescence technique was introduced to biological oceanography by Carl Lorenzen. Since then many fluorometers have been developed to measure variable chlorophyll *a* fluorescence under a broad range of conditions and for various applications. Each of these fluorometers is based on one of a few basic operational principals and can be classified as one of the following:

1. Pulse Amplitude Modulation fluorometer (PAM)
2. Fast Repetition Rate Fluorometer (FRRf)
3. Fluorescence Induction and Relaxation System (FIRe)
4. Pump and Probe Fluorometer (PandP)
5. Induction Fluorometer/ Continuous Excitation Fluorometer

2.1.2 Chlorophyll *a* fluorescence parameters

Chlorophyll *a* fluorescence parameters allow us to quantify the fluorescence emissions from chlorophyll of PSII after excitation by light conditions. The parameters are presented as rapid descriptors of photosynthetic processes in microalgae. Chlorophyll *a* fluorescence parameters are obtained after *in vivo* chlorophyll *a* fluorescence measurements by pulse amplitude modulation (PAM) fluorometers. The fluorescence terminologies are presented in Table 2.1.

Table 2.1 Fluorescence terminologies (modified from Consalvey et al., 2005).

	Fluorescence term	Description
Dark	F_o	Minimum fluorescence yield
	F_m	Maximum fluorescence yield during the saturating flash
	F_v	Variable fluorescence
	F_v/F_m	Maximum light utilisation efficiency of PS II
Light	F'	Fluorescence yield in the light-adapted state (used as an approximation of F_s
	F_t	Fluorescence yield at steady state
	F_m'	Maximum fluorescence in the light-adapted state during the saturating flash
	F_q'	Fluorescence quenched
	F_q'/F_m'	Light utilisation efficiency
	F_o'	Minimum light-adapted fluorescence yield
	F_v'	Variable fluorescence in the light-adapted state
	NPQ	Non-photochemical quenching

2.1.2(a) The minimal fluorescence yield (F_o)

F_o may be used as approximate of biomass (Consalvey, 2005; Serôdio et al., 1997). In dark-adapted state, the minimal fluorescence yield (F_o) presents chlorophyll *a* fluorescence emission of open reaction centres in a non-excited state. During this stage, measuring light is switched on and this light is of very low intensity to induce photosynthesis (Figure 2.4). Two assumptions are made (1) there must be a linear relationship between biomass and fluorescence and (2) the light depth must not be less than the depth of cells migrated (Consalvey et al., 2005). The value F_o is taken into account. Samples are normally dark adapted for 15 minutes (Consalvey et al., 2005). However, vertical migration has been observed in 8 minutes (Perkins et al., 2002) and within 15 minutes based on personal observation by Consalvey et al. (2005). Therefore, any fluorescence detected after that might not accurately represent the biomass of microalgae.

2.1.2(b) Maximum quantum yield (F_v/F_m)

F_v/F_m is a good indicator of maximum quantum yield of PSII chemistry (Butler, 1978; Genty et al., 1992) and photoinhibition of microalgae (Bergmann et al., 2002; Consalvey et al., 2005; Cavender-Bares and Bazzaz, 2004). To achieve maximum quantum yield, samples are kept in the dark in which the reaction centres are said to be "opened" (Baker, 2008). These cells are relaxed during dark adaptation period. The F_v/F_m value of healthy microalgae is between 0.6 and 0.7 (Kromkamp and Peene, 1999; McMinn and Hegseth, 2004; White et al., 2011), usually less than that of vascular plants. However, this depends on the type of fluorometer used as well. The decrease of F_v/F_m is often observed when microalgae are exposed to harsh conditions.

2.1.2(c) Effective quantum yield (F_q'/F_m')

In light-adapted state, the effective quantum yield denoted by F_q'/F_m' is used to determine the PSII operating efficiency under different light and other environmental conditions (Baker, 2008; Genty et al., 1989). A prime notation (') indicates sample is exposed to light (e.g. natural light and actinic light) that will drive photosynthesis. When actinic light is introduced in microalgae, the fluorescence is termed as F' which rises to maximum fluorescence, F_m' (Figure 2.4).

2.1.2(d) Rapid light curves (RLCs)

In the past, photosynthetic rates were measured using bell jars or benthic chambers (Barranguet et al., 1994), oxygen microelectrodes (Kromkamp et al., 1995) or ^{14}C radiotracers (Table 2.2; Perkins et al., 2001). Photosynthetic parameters involving ambient light intensity and photosynthesis are normally studied using

photosynthesis-irradiance (P-E) curves. The P-E curves show the relationship between irradiance and photosynthetic rate (Figure 2.5). They show characteristically linear slope, whereby the initial slope is denoted by α, until it reaches a certain point of light, it will become maximal light-saturated displayed by P_{max}. E_k is the light saturation coefficient equivalent to P_{max}/α (Talling, 1957), whereby α intercepts with P_{max} (Iriarte et al., 2013). E_s indicated the light at which it is saturated. Beyond this point, given higher light exposure, the cells will experience photoinhibition or photodamage (β). The cells will either recover or damage permanently depending on the species.

In comparison to traditional light curves, a rapid light curve (RLC) measures effective quantum yield as a function of irradiance (Ralph and Gademann, 2005) and can be obtained using the PAM fluorescence. Photosynthetic activity measurements involve calculations of electron transport rate (ETR) and relative electron transport rate (rETR) (Kromkamp et al., 1998). If rETR is plotted against irradiance, the RLC resembles traditional P-E curve but it should not be interpreted as one (Ralph and Gademann, 2005). Under low light level, the photosynthesis increases with increasing irradiance (Figure 2.5).

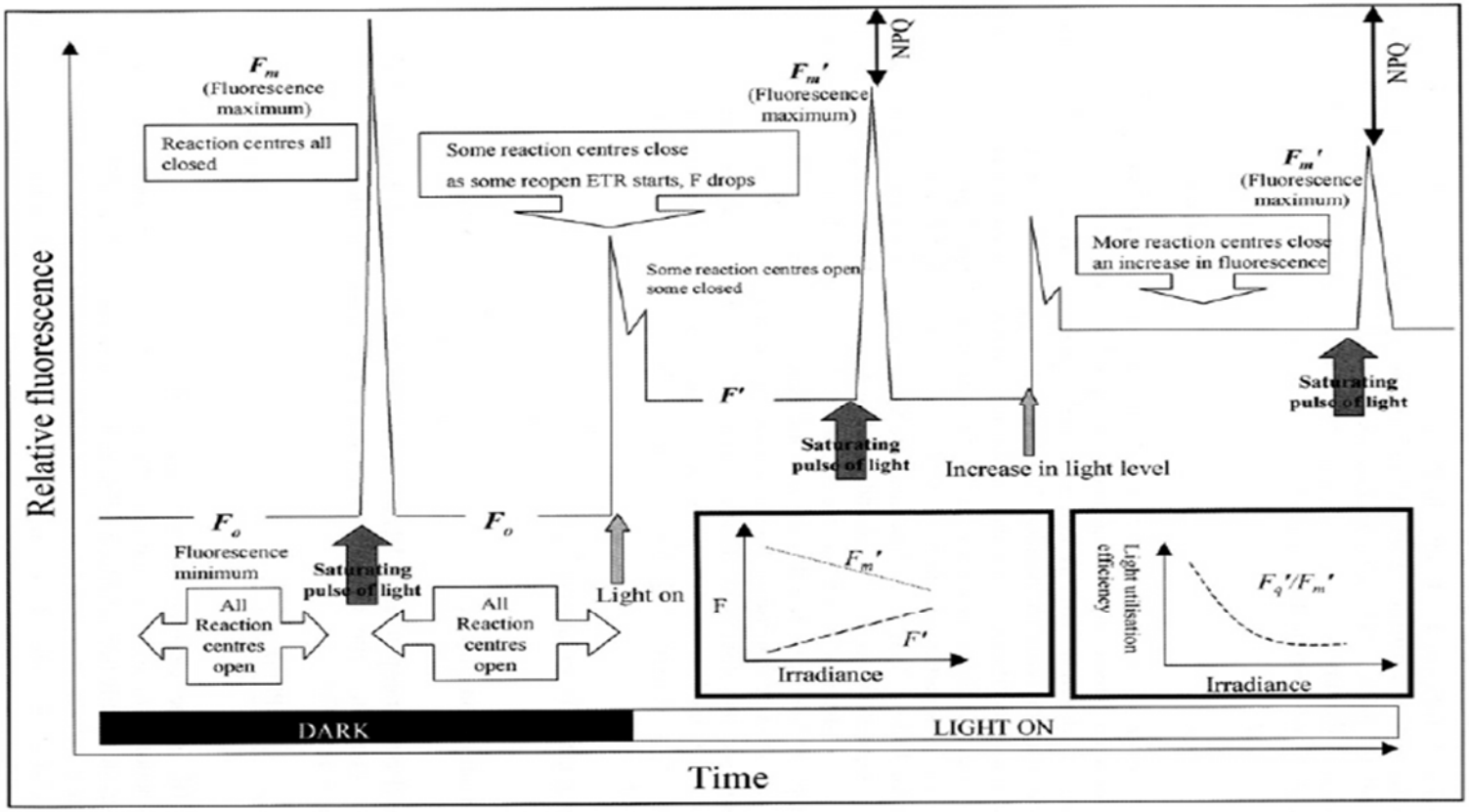

Figure 2.4 Theoretical trace of fluorescence output (adopted from Consalvey et al., 2005).

Table 2.2 Techniques used to measure photosynthesis in microalgae (modified from (Consalvey et al., 2005).

Technique	Photosynthetic measurement	References
Bell jars/ benthic chambers	CO_2 uptake/ O_2 evolution	Rasmussen et al. (1983); Colijn and de Jonje (1984)
Oxygen microelectrodes	Changes in oxygen concentration within the biofilm and into water column/ air	Kromkamp and Peene (1995); Kühl et al. (1996)
^{14}C radiotracers	C entering photosynthesis in slurries and intact biofilms	McIntyre and Cullen (1995); Perkins et al. (2001)
Fluorescence	Fluorescence levels emitted from the light harvesting pigments of PSII	McMinn et al. (2004, 2005a, 2005b, 2007, 2012); Figueroa et al. (2013)

In comparison to traditional light curves, a rapid light curve (RLC) measures effective quantum yield as a function of irradiance (Ralph and Gademann, 2005) and can be obtained using the PAM fluorescence. Photosynthetic activity measurements involve calculations of electron transport rate (ETR) and relative electron transport rate (rETR) (Kromkamp et al., 1998). If rETR is plotted against irradiance, the RLC resembles traditional P-E curve but it should not be interpreted as one (Ralph and Gademann, 2005). Under low light level, the photosynthesis increases with increasing irradiance (Figure 2.5).

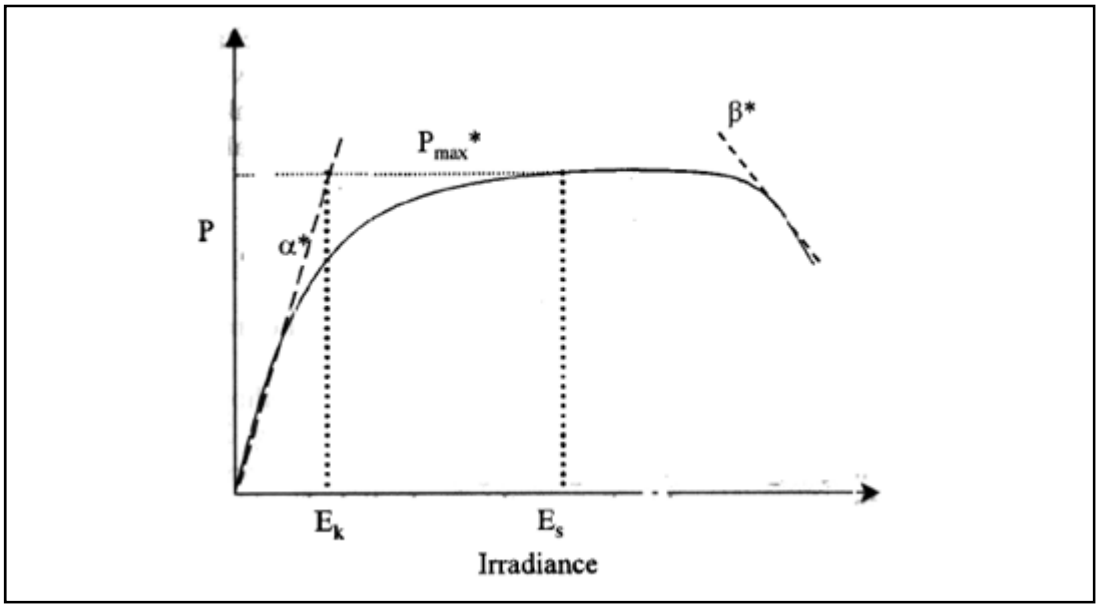

Figure 2.5 P-E curve which shows the response of a photosynthetic parameter to light (PPFD; adopted from Consalvey et al., 2005).

2.2 Usage of chlorophyll *a* fluorescence in microalgae

Microalgal studies in field and laboratory have been conducted mainly using PAM fluorometers. According to Honeywill et al. (2002), blue light is said to be optimum for green algae and diatoms meanwhile red light is suitable for cyanobacteria. Kühl et al. (2001) were the first to measure *in-situ* photosynthesis on sea-ice algae using Diving-PAM in an ice-covered Arctic fjord. Most photosynthetic studies focused on bottom-ice algae probably due to their high biomass (McMinn et al., 2007; McMinn and Hattori, 2006; Ryan et al., 2004). Surface brine algae may tolerate different surface irradiances up to 350 µmol photons m^{-2} s^{-1}. However, bottom communities are more likely to experience photoinhibition. Ryan et al. (2009) conducted a short term irradiance study to investigate photosynthetic response of surface brine and bottom-ice algae by applying irradiances from 7 to 262 µmol photons m^{-2} s^{-1} in Southern

Ocean. They found out that algae had consistent E_k to which they were normally exposed, suggesting they might survive when the ice melted in Southern Ocean.

Photosynthetic activities on microalgae in tropical region are scarce. McMinn et al. (2005b) were the first to study photosynthetic parameters in tropical marine benthic microalgae in coastal Penang, Malaysia. They investigated photosynthetic parameters in benthic microalgae at two locations, Muka Head (near jetty) and Songsong Islands (clearer water and further offshore). They found out that the microalgal composition (*Cocconeis*, *Fragilaria*, *Palaria* and *Pleurosigma*) produced low maximum quantum yield using Water-PAM. McMinn et al. (2005b) observed weak significant changes between *in-situ* measurements (salinity, pH and temperature) and photosynthetic parameters, but since the changes were small, they might not contribute to the photosynthetic parameters of benthic microalgae community.

In addition, the PAM fluorometer is a useful tool in measuring nutrient limitations in microalgae. Previous NIFT studies used Phyto-PAM (Holland et al., 2004; Majarreis et al., 2014) and Water-PAM (Majarreis et al., 2014). With some precautions, F_v/F_m could be used to assess 'stress' and nutrient status in microalgal populations (Consalvey et al., 2005; Greene et al., 1991; White et al., 2011; Young and Beardall, 2003). However, a few studies (Dijkman and Kromkamp, 2006; Napoléon et al., 2013; Parkhill et al., 2001) found out that F_v/F_m was not a good indicator of nutrient stress. For example, Napoléon et al. (2013) observed high F_v/F_m values in balanced growth of microalgae under N-limited and Si-limited cultures.

2.3 Nutrient limitations

Nutrient limitation may inhibit several biological and ecological processes (Cullen, 1991). Since the term nutrient limitations can be somewhat confusing, it is essential to recognise a particular process being 'limited' and it is worth considering the different conceptual frameworks and terms that have been used when discussing nutrient limitation (Blackman, 1905; Cullen, 1991; Liebig 1840; Shelly et al., 2010). According to Von Liebig's (1840) "Law of the Minimum", the growth of organisms is controlled by the resource that is most limiting. Hecky and Kilham (1988) assumed that all species in a community are limited by a single factor and they simplified that when the cell growth rate was increasing, it would be limited by something. Generally, nutrient enrichment or bioassay studies focuses on the total microalgal biomass produced (Hecky and Kilham, 1988) and does not measure *in-situ* nutrient limitation (Holland et al. 2004). Meanwhile, Blackman limitation's model states that when two or more factors involved in a process, its rate is limited by the pace of the slowest factor and this model determines the rate of photosynthesis (Singh and Lal, 1935; Shelly et al., 2010).

The effect of silica limitations is often studied in diatoms (Valenzuela et al., 2012; Chu et al., 2013; Schnurr et al., 2013). According to Bienfang et al. (1982), silica-depletion causes higher sinking rates compared to silica-replete cultures in diatoms *Skeletonema costatum*, *Chaetoceros gracile*, *Ditylum brightwellii* and *Coscinodiscus wailesii*. In eutrophic waters, excess nitrogen and phosphorus usually increase diatom growth and sedimentation. This may enhance silica build-up in the sediments and thus limiting the silica concentration in the water (Papush and Danielsson, 2006). This condition may affect diatom species composition (Olli et al.,

2008) as silica limitation will promote the growth of flagellates rather than diatoms (Smayda, 1990; Wasmund and Uhlig, 2003; Conley et al., 2008). Eventually, changes in microalgal composition may lead to major shifts in the entire food web and may cause harmful algal blooms (Smayda, 1990; Conley et al., 1993, Conley et al., 2008).

2.3.1 Nutrient-induced fluorescence transients (NIFTs)

Traditional methods of assessing nutrient limitations in microalgae are relatively expensive and time-consuming (Beardall et al., 2001b). They include nutrient enrichment bioassays, elemental ratio analysis, nutrient uptake kinetics, cellular protein profiling, ambient concentration analysis and measuring the growth rate of microalgae (Beardall et al., 2001a; den Haan et al., 2013; Shelly et al., 2010; Wood and Oliver, 1995). Thus, an approach called nutrient-induced fluorescence transients (NIFTs) was suggested as it was fast and cost-effective in the long run (Beardall et al., 2001a). It has the potential of assessing nutrient limitations but not many have undertaken this approach. Identifying the limiting nutrient is crucial to better understand the ecology and physiology of microalgae and may serve as a guideline in water management practices.

NIFT is an approach whereby when limiting nutrient is added into microalgae, transient changes can be observed within minutes (Beardall et al., 2001a; Lippemeier et al., 1999; Shelly et al., 2010). Several NIFT studies have been focusing on specific organisms and their physiology (Table 2.3). Recent studies on marine microalgae both in field and laboratory cultures have shown that steady state chlorophyll *a* fluorescence (F_t) may serve as a promising tool for assessing nutrient limitation in microalgae

(Shelly et al., 2010). Turpin and Weger (1988) were the first to report rapid transient changes (within minutes) in chlorophyll *a* fluorescence when nitrogen was added to N-limited microalgae cultures. This change in chlorophyll *a* fluorescence is called nutrient-induced fluorescence transient, or NIFT (Wood and Oliver, 1995).

Table 2.3 Past research on NIFT using cyanobacteria, green algae, diatoms and dinoflagellate (modified from Shelly et al., 2010).

Species	Presence/ Absence of nutrient limitation	Nutrient limitation	Reference
Green algae			
Chlorella emersonii	Present	Phosphorus	Holland et al. (2004)
Dunaliella tertiolecta	Present	Phosphorus	Roberts et al. (2008); Young and Beardall (2003)
Selenastrum minutum	Present	Nitrogen	Turpin and Weger (1988)
Red algae			
Porphyridium	Present	Nitrogen	Shelly, unpublished data
Cyanobacteria	Present	Nitrogen	Shelly, unpublished data
Oscillatoria sp.	Present	Phosphorus and nitrogen	Holland et al. (2004)
Synechococcus sp.	Absent	Phosphorus and nitrogen	Shelly, unpublished data
Diatoms			
Phaeodactylum tricornutum	Absent	Phosphorus, nitrogen, silica	Shelly, unpublished data
Cylindrotheca fusiformis	Present	Silica	van der Merwe and McMinn, unpublished data
Dinoflagellate			
Woloszynskia	Absent	Phosphorus, nitrogen	Shelly, unpublished data
Dunaliella salina	???		Lippemeier et al. (2001)

Note: ??? = unclear