MARINE PHYTOPLANKTON PRIMARY PRODUCTION AND ECOPHYSIOLOGY USING CHLOROPHYLL-A FLUORESCENCE

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BSc (Hons)

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

2007
I declare that this thesis is my own account of my research and contains work which has not previously been submitted for a degree in any tertiary institution

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(Jeffrey J. Cosgrove)
ABSTRACT

Marine phytoplankton ecophysiological state and primary production measurements have typically been controversial due to potential impacts of measurement techniques. Advances in chl-a fluorescence techniques have provided a means for rapid, non-invasive measurement of electron transport through photosystem 2 (PSII) in dilute phytoplankton suspensions. While studies on higher plants have outlined a close relationship between PSII electron transport and carbon fixation, results from studies on microalgae reveal significant variations in the relationship.

Three species of phytoplankton representing three major taxonomic groups of the marine phytoplankton were used in this study: (1) *Chaetoceros muelleri* CS176 Lemmermann (Bacillariophyta), (2) *Isochrysis galbana* CS177 Parke (Haptophyta) and, (3) *Nannochloropsis oculata* CS179 (Droop) Hibberd (Ochrophyta, eustigmatophyte). Each species was cultured in semicontinuous culture and primary production was estimated using oxygen evolution and carbon fixation techniques and compared against predictions based on chl-a fluorescence measurements. It was found that predicted values of primary production both under-estimated and over-estimated actual carbon fixation measured via radioisotope (\(^{14}\)C) techniques. This variation was primarily explained by probable errors in the assumed values for PSII density. The relationship between oxygen evolution or carbon fixation with chl-a fluorescence-derived measures was commonly linear below the light saturation parameter, with a departure from linearity occurring at higher irradiances. This departure from linearity was greatest in cultures adapted to low light conditions. At higher light intensities alternative electron pathways such as the Mehler reaction
and/or chlororespiration are likely to be more active in low light-adapted cultures, leading to this greater non-linearity.

Chl-\(a\) fluorescence measurements were also found to be a useful in characterising ecophysiology using photosynthesis-versus irradiance curves. However, an important caveat on this is the measurement of PSII density (\(\eta_{\text{PSII}}\)) rather than use of an assumed value as changes in \(\eta_{\text{PSII}}\) can have a profound impact on light curve parameters.

A field study in Fremantle Harbour found a healthy (negligible nutrient starvation), diatom dominated, phytoplankton community. Results suggest that phytoplankton are able to begin boosting photosynthetic capability just prior to morning twilight. Waters in the harbour were well mixed via tidal motion and substantial midday photoinhibition was not observed. Data suggest levels of primary production at the mouth of the harbour are similar to those of coastal waters in the plume of the Ocean Reef wastewater outfall.
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Firstly, I would like to thank my principal supervisor Prof. Michael Borowitzka for his guidance and support throughout the course of my PhD studies. His ability to cut through to the heart of an issue, or question, was a great balance on numerous occasions. His efforts to facilitate dialogue with other researchers in Australasia were profoundly helpful.

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**Abbreviations**

Please note that:

- Chl-α fluorescence parameters are defined in Table 1.1 (pg. 45)
- Biophysical parameters are defined in Table 1.3 (pg. 65)

**A** Absorptance

**ASC** Ascorbate

**CET** Cyclic Electron Transport

**DCMU** 3-(3,4-dichlorophenyl)-1,1-dimethylurea

**ΔH⁺** Transthylakoid electrochemical gradient

**ΔΨ** Electrical component of ΔH⁺

**ΔpH** Transthylakoid proton gradient (chemical component of ΔH⁺)

**E** Irradiance (W m⁻²)

**E_{PAR}** Photosynthetically Active Radiation (400 – 700 nm, µmol quanta m⁻² s⁻¹)

**E_{PSII}** Photosynthetically Active Radiation available for charge separation at PSII (350 – 400 nm, µmol quanta m⁻² s⁻¹)

**E_{k}** Saturation irradiance (350 – 400 nm, µmol quanta m⁻² s⁻¹)

**ED unit** Emittor-Detector unit (component of the Water-PAM fluorometer)

**ETC** Electron Transport Chain

**ETR** Electron Transport Rate (µmol electrons [mg chl-α]⁻¹ s⁻¹)

**ETR_{max}** Maximum Electron Transport Rate (µmol e⁻ [mg chl-α]⁻¹ s⁻¹)

**rETR** Relative Electron Transport Rate (relative units)

**rETR_{max}** Maximum Relative Electron Transport Rate (relative units)

**Φ_{CO2}** Quantum yield of carbon fixation

**Φ_{O2}** Quantum yield of oxygen evolution

**Φ_{PSII}** Quantum yield of electron transport through PSII

**f_{II}** Fraction of absorbed radiation directed to PSII

**Fd** Ferredoxin
FNR  Ferredoxin-NADP$^+$ reductase
FQR  Ferredoxin-(plasto)quinone reductase
FR  Far-red (light)
FRR  Fast Repetition Rate
$\Gamma_{O_2}$  Stoichiometric ratio of O$_2$ evolved per electron generated at PSII
GOE$_f$  Gross O$_2$-evolution predicted from chl fluorescence measurements ($\mu$mol O$_2$ m$^{-2}$ s$^{-1}$)
GOE$_f^{chl}$  Chl-specific gross O$_2$-evolution predicted from chl fluorescence measurements ($\mu$mol O$_2$ ($\mu$g chl-$a$)$^{-1}$ h$^{-1}$)
HL  High Light
LC  Light Curve
LC(3)  Light Curve with 3 min at each irradiance
LC(20)  Light Curve with 20 min at each irradiance
LED  Light-Emitting Diode
LHCI  Light Harvesting Centre (antennae) of PSI
LHClII  Light Harvesting Centre (antennae) of PSII
LL  Low Light
MAP  Mehler Ascorbate Peroxidase
MDA  Monodehydroascorbate
MDAR  Monodehydroascorbate Reductase
ndh  NAD(P)H-dehydrogenase
NIFT  Nutrient Induced Fluorescence Transient
NPQ  Non-photochemical Quenching
NPQ$_{max}$  Maximum Non-photochemical Quenching
OEC  Oxygen Evolving Complex (attached to PSII)
$P_{chl}$  Chl-specific rate of primary production ($\mu$mol C ($\mu$g chl-$a$)$^{-1}$ h$^{-1}$)
$P_f^{chl}$  Chl-specific rate of primary production predicted from chl fluorescence measurements ($\mu$mol C ($\mu$g chl-$a$)$^{-1}$ h$^{-1}$)
$P_{O_2}$  Photosynthetic rate derived from O$_2$-evolution measurements ($\mu$mol O$_2$ m$^{-2}$ s$^{-1}$)
$P_{chl}^{O_2}$  Chl-specific photosynthetic rate derived from O$_2$-evolution measurements ($\mu$mol O$_2$ ($\mu$g chl-$a$)$^{-1}$ h$^{-1}$)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PAM</td>
<td>Pulse Amplitude Modulated</td>
</tr>
<tr>
<td>PCOC</td>
<td>Photorespiratory Carbon Oxidation Cycle</td>
</tr>
<tr>
<td>Ph</td>
<td>Pheophytin</td>
</tr>
<tr>
<td>PPFD</td>
<td>Photosynthetic Photon Flux Density (µmol quanta m⁻² s⁻¹)</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
</tr>
<tr>
<td>PQH₂</td>
<td>Plastoquinol</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>PSU</td>
<td>Photosynthetic Unit</td>
</tr>
<tr>
<td>PSU₅₀₂</td>
<td>Photosynthetic Unit size of oxygen production</td>
</tr>
<tr>
<td>PTOX</td>
<td>Plastid Terminal Oxidase</td>
</tr>
<tr>
<td>qₑ</td>
<td>Energy dependent component of NPQ</td>
</tr>
<tr>
<td>qᵢ</td>
<td>Photoinhibition component of NPQ</td>
</tr>
<tr>
<td>qₚ</td>
<td>Photochemical quenching</td>
</tr>
<tr>
<td>qₜ</td>
<td>State-transition component of NPQ</td>
</tr>
<tr>
<td>Qₚₚₜₑ</td>
<td>Absorbed photosynthetically usable radiation (µmol quanta m⁻² s⁻¹)</td>
</tr>
<tr>
<td>QR₅₀₂</td>
<td>Quantum requirement for oxygen evolution</td>
</tr>
<tr>
<td>RCII</td>
<td>Photosystem II Reaction Centre (also known as P₆₈₀)</td>
</tr>
<tr>
<td>RLC</td>
<td>Rapid Light Curve</td>
</tr>
<tr>
<td>Rubisco</td>
<td>Ribulose-1,5-bisphosphate carboxylase oxygenase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
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CHAPTER 1
General Introduction

1.1 PHYTOPLANKTON PRODUCTION

Phytoplankton biomass in the world’s oceans amounts to only ~1-2% of the total global plant carbon, yet it is estimated that these organisms fix between $30 - 50 \times 10^{15}$ g of carbon annually, which is about 40% of the total global carbon fixation (Falkowski 1994; Sakshaug et al. 1997). This constitutes one of the principal processes in the Earth’s biosphere and forms a major portion of the first link in the trophic chain of marine organisms (Wozniak and Dera 2000). This contribution to the Earth’s biogeochemical cycles is significant enough to influence the global atmosphere, including processes such as the Greenhouse Effect (Wozniak and Dera 2000).


Thus, it is important to consider the factors limiting and otherwise influencing both the distribution of biomass and rate of primary production of phytoplankton (Falkowski 1994) and to have effective means for measuring their physiological status. Characterising the physiological response of phytoplankton to interacting environmental variables is essential. This is fundamental to phytoplankton management whether it be
the formation of effective coastal nutrient management practices (Bergmann et al. 2002), management of nuisance phytoplankton species in drinking water supplies (Oliver et al. 2003) or optimisation of production in commercial microalgal culturing facilities (Vonshak and Guy 1992; Vonshak et al. 2001).

Shortly after the method of $^{14}$C-uptake for primary production measurements was initially described (Steemann Nielsen 1952), Ryther's (1956) essay on the measurement of primary production illustrated that the full complexity of variations in primary production and the interaction of this process with the surrounding environment was far from being understood. While our understanding of photosynthesis and rates of primary production has advanced since the time of Ryther's publication in 1956, further understanding of the physiological processes involved and their interaction with the environment is required before accurate predictions of primary production (in nature) can be made.

1.1.1 Controlling factors

The distribution of phytoplankton in the oceans is highly variable in both time and space and is largely governed by geophysical factors affecting the depth of the upper mixed layer (currents, wind stress and isolation) as well as vertical fluxes of essential nutrients and the availability of photosynthetically active radiation (Falkowski and Kolber 1995).

Throughout most of the central ocean basins, between latitudes 30° N and 30° S, phytoplankton biomass is extremely low, averaging 0.1 to 0.2 $\mu$gL$^{-1}$ at the sea surface. In the same region chlorophyll biomass maxima are commonly associated with the thermocline which limits the vertical flux of nutrients to extremely low levels (Falkowski 1994). Additionally, if the upper mixed layer is deeper than the euphotic zone, phytoplankton will spend, on average, a lot of time at irradiances too low to
sustain net growth. As a result of this physical limitation phytoplankton blooms can only occur if the mixed layer depth is shallower than the critical depth (Falkowski 1994).

The major regulators of phytoplankton primary production are light, nutrients and temperature (Perry et al. 1981; Falkowski 1994; Babin et al. 1995; Bouterfas et al. 2002). Little is known about the reactions of phytoplankton productivity to fluctuations in nutrient supply (Lippemeier et al. 2001). The collection rate of physicochemical data has traditionally been substantially faster than for data on the photosynthetic rate of phytoplankton; thus such biological measurements have often lacked the temporal (and spatial) resolution of physicochemical data. Although the re-emission of red-shifted light by chlorophyll (fluorescence) has long been linked to photochemistry (see Govindjee (1995) for a background to the history of chl fluorescence measures), recent advances in our knowledge of the origins of chl fluorescence and suitable, portable equipment for ultrasensitive, ultrarapid assessment of this phenomenon now allow photosynthetic data to be collected at rates comparable to physicochemical data.

1.2 PRIMARY PRODUCTION AND PHOTOSYNTHESIS

1.2.1 General

The accurate and precise estimation of the rate of primary production by natural populations of phytoplankton, combined with an understanding of the factors that regulate this production, is one of the major goals of aquatic ecologists (Bates and Platt 1984; Pennock and Sharp 1994).

Photosynthesis is the process leading to primary production and can be described as a composite chain of cascading events starting with photon capture by the photosynthetic pigments and extending through O$_2$-evolution to C-fixation (Kroon et al. 1993). Antennae pigments catch photon energy and funnel this towards a
transmembrane structure in the thylakoid, called Photosystem II (PSII), via resonance transfer. It is at PSII that primary charge separation occurs and electron transport continues from PSII to Photosystem I (PSI) via the Cytochrome $b_{6}/f$ complex (Figure 1.1a). Photons are absorbed at PSI also, to provide the reducing power needed to create NADPH. During these ‘light reactions’ of photosynthesis hydroxyl ions are released into the thylakoid lumen resulting in the development of an electrochemical gradient. ATPsynthase complexes bound within the thylakoid membrane utilise this electrochemical gradient to synthesize ATP in the stroma (Figure 1.1a). Thus, simple reductants are the product of the light reactions of photosynthesis, with NADPH potentially yielding one molecule of CH$_2$O (Jumars 1993). For a more detailed account of this process the reader is referred to the books edited by Aro and Andersson (2001) and Papageorgiou and Govindjee (2004).

The fixation of inorganic carbon into organic carbon skeletons by plants is called primary production and traditionally has been estimated by the carbon radioisotope ($^{14}$C) assimilation method (Strickland and Parsons 1972). The evolution of oxygen in oxygenic photosynthesis has also commonly been utilised as an effective proxy measure of primary production. The $^{14}$C-assimilation method is time-consuming and always involves complex handling of samples and long incubation periods which may result in experimental errors (Babin et al. 1995). Despite efforts to minimise incubation time (Lewis and Smith 1983) both $^{14}$C and oxygen methods are considered with caution since the sample must first be removed from the natural environment and incubated in bottles for a given period before the photosynthetic rate can be determined. Changes in biotic processes that undoubtedly occur during incubation may result in merely a crude estimation of actual primary production (Boyd et al. 1997; Gilbert et al. 2000a; Glud et al. 2002) and operators must carefully clean incubation bottles to ensure no substances potentially toxic to metabolic activity are present.
The stoichiometry of carbon fixation is not as clearly fixed as the simple equation normally produced (i.e. \( \text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{CH}_2\text{O} + \text{O}_2 \)) might lead one to believe (Jumars 1993). Carbon fixation leading to the construction of carbon skeletons is essential for growth and energy storage to be used for nocturnal metabolism. In the light, however, reductants formed by photosynthesis are distributed between carbon fixation, nitrogen assimilation, photorespiration, inorganic carbon accumulation, chlororespiration, pseudocyclic electron transport (e.g. Mehler reaction), and respiratory phosphorylation (Figure 1.1b) (Björkman and Demmig-Adams 1995; Behrenfeld et al. 2004). Temporal separation of metabolic events govern the prominence of any particular pathway and the fraction of photosynthate allocated to carbon fixation changes with growth conditions and on time scales from seconds to generations (Behrenfeld et al. 2004). With our current understanding of photosynthetic mechanisms and processes and increased use of non-carbon-based measures of primary production (e.g. chl-\(\alpha\) fluorescence) the paradigm that perceives phototrophic life as “carbon-centric” is shifting to one where simple reductants, not carbon products, act as the fundamental currency of plant life (Behrenfeld et al. 2004). Despite this, carbon fixation is a biologically important parameter and a key issue is the reconciliation of various non-carbon-based measures of primary production into reliable empirical estimates of carbon fixation.
Figure 1.1: Photosynthetic and metabolic electron transport pathways. (A) A “cyanobacterial-type” thylakoid membrane. Light absorption by PSII results in oxygen production and electron transport (red arrows) from water to the plastoquinone pool (PQ $\rightarrow$ PQH$_2$). With potentially some cycling around cytochrome b$_6$/f, electrons are passed to cytochrome c$_{553}$ and then can either continue through to PSI, ferredoxin (Fd$_x$), and NADP$^+$ to produce the reductant, NADPH, or they can be transferred through a membrane-bound oxidase (aa$_3$-type cytochrome) back to oxygen. This latter pathway (1) to oxygen is proposed here to play a critical role in the expression of E$_k$-independent variability in prokaryotes. Its corollary in eukaryotes is shown in B as the sequence (5-6-7). In the light, NADPH is used in a variety of downstream metabolic pathways (2), with some of the resultant products leading back to the PQ pool at night in prokaryotes (3). At very high light, electrons may also pass from Fd$_x$ back to water via the Mehler reaction (4). Pathways 1 through 4 result in a transmembrane proton gradient that is used to generate ATP by proton (H) transport from the lumen to the stroma via the ATPase complex. (B) Downstream metabolic pathways from NADPH include nitrogen reduction and glutamate (glu) synthesis via the GS-GOGAT cycle, carbon reduction and triose phosphate (GAP) export, and ATP synthesis through oxygen reduction (7) via the mitochondrial electron transport chain (ETC) and substrate shuttles (6) linking the chloroplast, cytosol, and mitochondria. The two shuttles shown here are the oxaloacetate-malate (OAA-Malate) and dihydroxyacetone phosphate-phosphoglyceric acid (DHAP-PGA) shuttles. GAP produced by the Calvin-Benson cycle can be used for long-term carbon storage (which is generally a one-way path in the light), carbon skeletons for amino acid biosynthesis, or mitochondrial ATP synthesis via the citric acid cycle. Electron pathways illustrated in A and B are not comprehensive and have neglected multiple alternatives, including cyclic electron flow around PSII and PSI, photorespiration, and metabolic pathways common to multiple cellular compartments (e.g. carbohydrate formation). Purple arrows, transport across membranes; Ndh, prokaryotic dehydrogenase complex; PTOX, stromal terminal oxidase; RubBP, ribulose-1, 5 bisphosphate; gln, glutamine; PEP, phosphoenolpyruvate; BPGA, glycerate-1, 3-bisphosphate. Membrane-imbedded ovals with grey bar indicate decarboxylate translocators for the OAA-Malate shuttle and a phosphate translocator for the DHAP-PGA shuttle.

* Taken from Behrenfeld et al. (2004)
1.2.2 Photosystem II (PSII)

Given that the vast majority of chl-\(a\) fluorescence measured by most fluorometers originates from chl-\(a\) bound to PSII (Björkman and Demmig-Adams 1995), the structure and function of PSII will be briefly described. Further information on the light harvesting systems of photosynthesis can be obtained in many texts (Staehelin and Arntzen 1986; Critchley 1997; Bricker and Frankel 2002).

PSII is a transmembrane protein complex that binds the components of initial charge separation and electron acceptors/donors in the conformation required for the process to continue. This complex binds the specialised catalytic chlorophyll called chl_{680} to form the PSII Reaction Centre (RCII), also known as P_{680} (the “680” subscript refers to the wavelength of the absorption band of this pigment centre). The RCII is surrounded by a bed of light harvesting pigments but, rather than being catalytic, these pigments are involved in energy transduction and typically funnel the collected energy towards the catalytic RCII, effectively acting as an antenna (Bullerjahn 1997). The absorption bands of these antennae pigments are at a shorter wavelength than that of the RCII, since excitation energy is preferentially transferred “downhill”, and the red peak for the lowest singlet excited state of chl-\(a\) in most antenna systems is around 675 nm. This achieves energy transfer to the RCII, however the absorption band difference is so small that energy can escape from the reaction centre back into the pigment bed and consequently the RCII is known as a “shallow trap” (Falkowski and Raven 1997).

The most commonly cited proteins in the PSII complex include the core reaction centre proteins D1, D2 and cytochrome \(b_{559}\) (cyt-\(b_{559}\); see page 35); plus the core chlorophyll antenna proteins CP43 and CP47 (Kolber et al. 1988, 1990, 1994; Greene et al. 1991; Geider et al. 1993b; Falkowski 1994; Bergmann et al. 2002). D1 and D2 hold all of the primary acceptors and donors of the photosystem together with the RCII. D1 is
known to have a fast light-induced turnover rate (Carpentier 1997). Studies on higher plants have found that PSII is preferentially located in the stacked grana regions of the thylakoid, while PSI and ATPsynthase are more common in the unstacked stromal regions. Cells with other thylakoid arrangements, especially cyanobacteria and chl-c containing algae, tend to have fewer thylakoid layers than higher plants (Büchel and Wilhelm 1993) and therefore one might expect a reduced spatial separation of PSII from PSI and ATPsynthase. Excitation energy reaching PSII is received by the RCII, which enters an excited state (RCII\(^+\)). Within picoseconds of RCII\(^+\) formation primary charge separation occurs, in which an electron is donated from RCII\(^+\) to pheophytin (Ph\(\rightarrow\)Ph\(^-\)), and the reaction centre becomes a cation radical (RCII\(^+\)) (Stryer 1988). RCII\(^+\), a strong oxidant, returns to RCII by accepting an electron from a manganese cluster located on the lumen-side of PSII, via a tyrosine residue Y\(_z\). Ph\(^-\) continues electron transport by reducing a quinone (QA\(\rightarrow\)QA\(^-\)) which in turn reduces a plastoquinone (QB\(\rightarrow\)QB\(^-\)). It is only after this process has taken place that the energy of another absorbed photon can by accepted by the PSII reaction centre (Kolber and Falkowski 1993). With the repeat of this process QB\(^-\) accepts a second electron and removes a proton from each of two stroma-based water molecules (leaving two hydroxyl ions) to become plastoquinol (PQH\(_2\)) and is released into the thylakoid membrane to become part of the plastoquinone (PQ) pool (Kolber and Falkowski 1993). Since the concentration of PQ exceeds that of QA by a factor of 5-30, the PQ pool is capable of acting as a temporary store for electrons between PSII and PSI (Kolber and Falkowski 1993). After 4 photons have been absorbed resulting in 4 primary charge separations, 2 reduced PQ molecules are transferred to the thylakoid membrane and 4 positive charges have accumulated on manganese complex. This positively charged complex extracts electrons from 2 water molecules leading to the formation of oxygen and 4 hydrogen ions. The primary
molecular processes of photon trapping, charge separation, and charge stabilisation in
PSII have been described by an exciton-radical pair model (Trissl et al. 1993).

Photochemical energy conversion efficiency depends on the processes of (i) light harvesting, (ii) trapping of excitation energy and primary charge separation, (iii) stabilisation of charge separation on secondary acceptors, and (iv) the repopulation of the manganese complex leading to the oxidation of water (Kolber et al. 1988; Greene et al. 1992). Kulandaivelu and Daniell (1980) suggest that under optimal conditions 85% of energy contained in the excited RCII* is used in photochemistry, the rest dissipated as heat or fluorescence.

Whereas the stabilisation of charge separation on secondary acceptors (Q_A oxidation) occurs with a time constant of ~0.6 ms, PQH_2 reoxidation rate is controlled by the dark reactions of photosynthesis (2-15 ms). Thus, as irradiance increases and the rate limiting steps of the dark reactions reach their full processing capacity, the PQ pool becomes progressively reduced and re-oxidation of Q_A slows, resulting in closure of reaction centres and a reduction in the quantum yield of photosynthesis (Kolber et al. 1988; Kolber and Falkowski 1993).

1.2.3 Light and Absorption

Radiant energy, or irradiance, is expressed as energy incident per unit time and area and in photosynthetic studies has previously been denoted with the symbol ‘I’. However I is also used for radiant intensity (W sr^{-1}) and the symbol E is now preferred to denote irradiance (W m^{-2}). Incident radiance in the 400 – 700 nm waveband is generally considered the photosynthetically active component of total spectral irradiance (E_{(\lambda)}) and is termed Photosynthetically Active Radiation (PAR or E_{PAR}). Algae can photosynthesise at wavelengths as low as 350 nm, however, difficulties measuring the 350 – 400 nm waveband and its small contribution to total irradiance
(solar or from commonly used emission sources) means this fact is usually ignored (Geider and Osborne 1991; Sakshaug et al. 1997). Since a photon of any wavelength between 400 nm and 700 nm is equally competent at generating charge separation it is common for $E_{\text{PAR}}$ to be given units of mol quanta m$^{-2}$ s$^{-1}$. Phytoplankton are considered to absorb radiant energy from all directions and hence photosynthetically active scalar irradiance ($\dot{E}_{\text{PAR}}$), measured with a $4\pi$ sensor is considered the most appropriate measure (Sakshaug et al. 1997).

In order to obtain accurate estimates of the quantum yields (efficiency) of electron transport through PSII ($\Phi_{\text{PSII}}$), oxygen evolution ($\Phi_{\text{O}_2}$) or carbon fixation ($\Phi_{\text{CO}_2}$) one must know the amount of $E_{\text{PAR}}$ that is absorbed by the study organism. Difficulties associated with accounting for the scattering component of attenuation in optical measurements have limited the determination of absorbed irradiance as distinct from incident irradiance (Geider and Osborne 1991). Spectrophotometric techniques are used for the determination of optical absorption coefficients and a number of techniques have been developed to estimate absorption of light by phytoplankton cells. Absorption ($\alpha$) is defined as the fraction of total incident flux that is absorbed (Equation 1):

$$\alpha = \frac{\phi_a}{\phi_o} \quad (1)$$

where $\phi_a$ equals absorbed flux and $\phi_o$ is total incident flux.

Conventional spectrophotometers generally provide results as transmittance and/or absorbance (A). Techniques that have negligible losses due to scattering, such as the opal glass method (Shibata et al. 1954), use of a diffusing plate and minimal sample-detector distance (Bricaud et al. 1983), or an integrating sphere (Bricaud et al. 1983; Maske and Haardt 1987) allow absorptance (A) to be calculated as follows:

$$A = -\log_{10}(1-\alpha) \quad (2)$$
This parameter used extensively for characterising the absorption of a cellular suspension is the absorption coefficient ($a$) with units of m$^{-1}$. The absorption coefficient is expressed as an exponential function of the absorptance ($\alpha$) and the pathlength in metres ($l$) through the suspension:

$$a = -(l/l)\log_e(1-\alpha)$$  \hspace{1cm} (3)

Rearranging above equations allows $a$ to be calculated directly from $A$:

$$a = 2.303 \cdot A/l$$  \hspace{1cm} (4)

When expressed per unit mass of chlorophyll-$a$, the chlorophyll-$a$ specific absorption coefficient $a^{chl}$ is:

$$a^{chl} = a/[Chl - a]$$  \hspace{1cm} (5)

where [chl-$a$] is the chlorophyll-$a$ concentration (mg.m$^{-3}$). $a^{chl}$ has been found to vary from 0.004 - 0.043 m$^2$(mg chl-$a$)$^{-1}$ (Geider and Osborne 1991).

The $E_{PAR}$ that is absorbed and photosynthetically useable can be estimated via a number of different methods: two of the more commonly applied methods will be described here. Calculation of the absorbed, chlorophyll-specific, photosynthetically usable radiation ($PUR^{chl}$) to produce a result with units of $\mu$mol quanta-(mg chl-$a$)$^{-1}$s$^{-1}$ can be achieved via Equation 6. However, the parameter $Q_{phar}$ (Equation 7), which provides an estimate of absorbed photosynthetically usable radiation in units of $\mu$mol quanta m$^{-2}$s$^{-1}$ has been used preferentially for the calculation of absolute ETR (Gilbert et al. 2000a, 2000b; Toepel et al. 2004; Wilhelm et al. 2004; Jakob et al. 2005). Estimation of either $PUR^{chl}$ or $Q_{phar}$ requires a knowledge of emission and absorption spectra.

$$PUR^{chl} = \sum_{\lambda=400}^{\lambda=700} E(\lambda) a^{chl}(\lambda)$$  \hspace{1cm} (6)
\[ Q_{\text{phar}} = \int_{400\text{nm}}^{700\text{nm}} \left( E(\lambda) - E(\lambda) \cdot e^{-a^{\text{chl}}(\lambda)[\text{chl-a}]d} \right) d\lambda \] (7)

where \( E(\lambda) \) = photosynthetically available (incident) spectral radiation (\( \mu\text{mol m}^{-2} \text{ nm}^{-1} \text{ s}^{-1} \)); \( a^{\text{chl}}(\lambda) = \text{chl-a specific in vivo absorption coefficient of the cell suspension at wavelength } \lambda \text{ in } [\text{m}^2 \text{ mg}^{-1}(\text{chl-a})] \); \([\text{chl-a}] = \text{chl-a concentration in } [\text{mg (chl-a) m}^{-3}] \); \( d = \text{optical pathlength (m)} \).

Not all of the absorbed photosynthetically usable radiation is necessarily actively used in photosynthesis. Absorption by photosynthetic pigments is described by the photosynthetic cross-section (Suggett et al. 2003, 2004; MacIntyre and Cullen 2005).

The functional absorption cross-section for PSII \( (\sigma_{\text{PSII}}; \text{Å}^2 \text{ quanta}^{-1}) \) is the product of the light-harvesting capability (optical absorption coefficient) of the photosynthetic pigments and the efficiency of excitation transfer to the reaction centre and provides a measure of the effectiveness of incident light capture and conversion to electron transfer in PSII (Mauzerall and Greenbaum 1989; Kolber and Falkowski 1993; Wood and Oliver 1995). Increases in \( \sigma_{\text{PSII}} \) have been associated with the onset of nutrient starvation and may be a useful indicator of nutrient limitation (Kolber et al. 1988; Geider et al. 1993b; Greene et al. 1994; Falkowski and Kolber 1995; Wood and Oliver 1995). Geider et al. (1993a) observed higher \( \sigma_{\text{PSII}} \) in surface waters compared to at the deep chlorophyll maximum and suggested that this supported the hypothesis of surface nutrient limitation of phytoplankton photosynthesis. Decreasing growth irradiance has also been shown to result in an increase in \( \sigma_{\text{PSII}} \), the magnitude of which is species specific (Kolber et al. 1988, 1990).

As PSII reaction centres become inactive, energy transfer via the antennae pigments is rerouted to serve the remaining reaction centres, leading to an increase in \( \sigma_{\text{PSII}} \) (Falkowski and Kolber 1995). This is thought to be the mode by which \( \sigma_{\text{PSII}} \)
increases during nutrient limitation (Berges et al. 1996). On the other hand, non-photochemical dissipation of excitation energy, such as heat dissipation by the xanthophyll cycle, will decrease $\sigma_{PSII}$ (Olaizola et al. 1994; Babin et al. 1996; Barranguet and Kromkamp 2000a). Another regulatory mechanism, state transitions (discussed below) can also result in significant reductions in measured $\sigma_{PSII}$ (Falkowski and Raven 1997; Behrenfeld and Kolber 1999). Masojidek et al. (2000) expressed surprise when stressed cultures (-nutrients, +NaCl) of the green alga *Chlorococcum* sp. were measured to have a higher $\sigma_{PSII}$ than non-stressed cultures at the same (high) irradiance level. They had expected the higher non-photochemical quenching (NPQ) exhibited by the stressed culture to result in a smaller $\sigma_{PSII}$, however their data on maximum quantum yield of PSII suggests significantly greater PSII reaction centre inactivity (damage) in the stressed cultures which would lead to a larger $\sigma_{PSII}$ (due to exciton spillover) (Falkowski and Kolber 1995). Therefore, increased incident irradiance may induce a reduction of $\sigma_{PSII}$ as a result of various energy dissipation and distribution mechanisms, however this may be offset if radiant intensity is sufficient to cause significant loss of PSII functionality (Suggett et al. 2004).

Algae may experience 3-fold changes in magnitude of $\sigma_{PSII}$ without any changes in quantum efficiency of PSII as they adapt to different growth irradiances, although the quantum yield of $\text{O}_2$-evolution may be altered (Olaizola et al. 1994). All in all, measurements of $\sigma_{PSII}$ in natural phytoplankton communities are highly variable, with data from Kolber & Falkowski (1993) suggesting a range from 250 - 1000 Å$^2$ quanta$^{-1}$ and an average of ~500 Å$^2$ quanta$^{-1}$. However, it still remains that relatively few measurements of $\sigma_{PSII}$ have been obtained from individual taxa under controlled conditions (Suggett et al. 2004).
As $\sigma_{\text{PSII}}$ reflects light harvesting capacity of the PSII antenna and exciton transfer efficiency, the time interval between exciton arrivals at the PSII reaction centre can be calculated by:

$$\tau = (\sigma_{\text{PSII}} \times E_{\text{PAR}})^{-1}$$  \hspace{1cm} (8)

where $\tau$ is the PSII turnover time (s). $\tau$ can increase markedly as cells adapt to lower growth irradiance and the ability of the dark carbon fixation pathways to match the pace of the light-driven Electron Transport Chain (ETC) (when in saturating irradiances) is reduced (Kolber and Falkowski 1993).

Importantly, for those studying chlorophyll fluorescence, $\sigma_{\text{PSII}}$ multiplied by incident irradiance and the density of functional PS II centres, $\eta_{\text{PSII}}$ (mol PSII (mol chl $a$)$^{-1}$), provides a measure of total photon flow available for charge separation at PSII ($E_{\text{PSII}}$) (Kromkamp and Forster 2003).

$$E_{\text{PSII}} = E_{\text{PAR}} \times \sigma_{\text{PSII}} \times \eta_{\text{PSII}}$$  \hspace{1cm} (9)

We can therefore say that the parameters $\sigma_{\text{PSII}}$ and $\eta_{\text{PSII}}$ combine to provide the chl $a$–specific rate of light absorption for PSII photochemistry ($a_{\text{PSII}}^{\text{chl}}$, m$^2$ (mg chl $a$)$^{-1}$) (Suggett et al. 2004):

$$a_{\text{PSII}}^{\text{chl}} = \sigma_{\text{PSII}} \times \eta_{\text{PSII}} \times 0.00674$$  \hspace{1cm} (10)

where the constant 0.00674 is a conversion factor based on conversion of $\sigma_{\text{PSII}}$ units to m$^2$ (mol PSII)$^{-1}$ and $\eta_{\text{PSII}}$ to mol PSII (mg chl-$a$)$^{-1}$ ($\sigma_{\text{PSII}} \times 6023$ and $\eta_{\text{PSII}}/893490$).

Unfortunately $\eta_{\text{PSII}}$ is difficult or impractical to measure and assumed values are common (Suggett et al. 2004). Values of $1.6 \times 10^{-3}$ – $2 \times 10^{-3}$ mol PSII (mol chl $a$)$^{-1}$ based on the measurements of total photosynthetic unit (PSU) size by Emerson and Arnold (1932) and Gaffron and Wohl (1936) are common. These authors measured between
2000 and 2500 chlorophyll molecules associated with each PSU and $\eta_{\text{PSII}}^{-1}$ was considered to be one quarter of the PSU size due to the requirement for 4 electrons to pass through PSII for each $O_2$ molecule evolved (Ley and Mauzerall 1982). However, $\eta_{\text{PSII}}^{-1}$ values ranging from 260 to 800 have been used (Kromkamp and Forster 2003 and references therein).

Adopting an assumed value for $\eta_{\text{PSII}}$ may produce inaccurate results since $\eta_{\text{PSII}}$ is known to change as a result of photoacclimation, photoinhibition and nutrient limitation (Suggett et al. 2004).

1.2.4 State Transitions

To maintain efficient electron transport through and between the photosystems energy must be balanced to maintain re-oxidation of plastocyanin (or cytochrome $c_{553}$) and plastoquinone pools (through the action of PSI) while maintaining maximal provision of reducing equivalent from PSII. Adjusting the amount of photon energy funnelled into either photosystem is of great importance since over-excitation of a reaction centre in excess light can result in severe damage (Björkman and Demmig-Adams 1995). PSII is particularly vulnerable to photodamage (Oxborough and Baker 2000) and, in conditions of moderate to high light, transfer of excitation energy away from PSII would be advantageous.

The light-harvesting antennae for PSII and PSI are thylakoid membrane-bound protein-pigment complexes and are generally termed Light Harvesting Centre II (LHCII) and Light Harvesting Centre I (LHCI) respectively (Bullerjahn 1997). The PSII / LHCII complexes are located in the appressed granal regions of the membrane, whereas the PSI / LHCI complexes are present in the unstacked stromal regions (Bullerjahn 1997). Studies, mainly on higher plants, have shown that a portion (CP25) of the LHCII complex is mobile and can reversibly uncouple from PSII (Figure 1.2).
This uncoupled portion of LHCII, termed LHCII_{b}, becomes associated with PSI instead (Nixon and Mullineaux 2001), thus PSII antenna size decreases while PSI antenna size increases (Bullerjahn 1997) in a transition that is called a state transition. In higher plants 15 – 20% of the LHCII complex is transferred during state transition while measurements in the green alga Chlamydomonas have reported an ~80% change in antenna size (Finazzi et al. 1999).

Figure 1.2: Model for the arrangement of the PSII antenna system as seen from above. A PSII centre is surrounded by an internal antenna of CP24, 26, and 29, a bound LHC II composed largely of CP27, and a peripheral, mobile LHCII that is composed of the major substrate for the thylakoid kinase (CP25) [taken from Bullerjahn, 1997].

State transitions aid in managing energy partitioning between the photosystems (Fork and Satoh 1986). In state 1 LHCII_{b} is coupled with PSII and the absorption cross-section of PSII is maximal, allowing more excitation energy to reach or remain longer in PSII and thus increasing the probability of its being re-emitted as fluorescence. In state 2 LHCII_{b} dissociates from PSII and moves laterally to become more closely associated with PSI and energy transfer from PSII to PSI is enhanced. Both of these outcomes reduce the probability of fluorescence being emitted by PSII (Fork and Satoh 1986; Büchel and Wilhelm 1993). Readers are referred to the review by Allen and
State transitions are thought to be regulated by membrane-bound enzymes, with both kinase and phosphatase activities found in isolated thylakoid preparations (Bullerjahn 1997). Two protein kinases, when activated by reduction of the PQ pool, phosphorylate the PSII core proteins and the LHCII (Fork and Satoh 1986; Bullerjahn 1997; Rintamäki and Aro 2001). The repulsion between the bound, negatively charged phosphate groups is thought to induce the dissociation of LCHII \( b \) and its movement away from the RCII (Fork and Satoh 1986).

As the PQ pool returns to a more oxidised state the activity of these kinases is reduced and a continuously active phosphatase dephosphorylates LHCII. This results in a return to state 1 as PSII and LHCII \( b \) reassociate (Bullerjahn 1997).

Light with wavelengths below about 670 nm is preferentially absorbed by PSII in higher plants and green algae and is, for this reason, termed “Light 2”. At light with longer wavelengths, “Light 1”, absorption by PSI is dominant (Fork and Satoh, 1986). State 1 is generally considered to occur when exposed to Light 1 while Light 2 will yield state 2. Using the chlorophyte alga Chlorella sp., Bullerjahn et al. (1997) illustrated that red light (645 nm) caused a decrease in PSII fluorescence, indicating transition to state 2, while far-red light (710 nm) combined with modulated red light elicited a return to state 1 accompanied by an increase in PSII fluorescence. Chlorophyll fluorescence techniques, combined with the application of inhibitors (eg. DCMU, NaF) and/or manipulation of incident light wavelength and intensity, has proven important in the research of state transitions (Schreiber et al. 1995b).

The condition that occurs in the dark can be somewhat variable depending on conditions and is, to an extent, taxon-specific (Fork and Satoh 1986). Cyclic electron
pathways that promote reduction of the PQ in the dark, such as chlororespiration, may trigger kinase activity and transition to state 2.

As well as increasing the efficiency of linear electron transport between PSII and PSI a transition to state 2 can act to enhance cyclic electron transport around PSI (Fork and Satoh 1986; Wagner et al. 2006). Under such circumstances ATP:NADPH production ratio would increase, which may be advantageous when ATP requirement is enhanced due to photodamage-repair and other metabolic demands.

The model for state transitions presented above has been based on studies of higher plants (especially spinach, tobacco, maize and barley) and chlorophyte algae such as Chlorella. There is a paucity of data available on management of excitation distribution in the Chromophyta (Heterokontophyta) despite them comprising a major portion of the marine phytoplankton (Gibbs and Biggins 1989). Most studies of chl-c containing algae have found only a single LHC that serves both PSII and PSI and fewer, smaller grana (lamellae usually have three thylakoid layers) (Büchel and Wilhelm 1993), hence one might expect some differences to be evident. A series of studies by Gibbs & Biggins (1989; 1991) on the chrysophyte Ochromonas danica (chl-a/c/fucoxanthin) described the presence of wavelength-dependent state transitions despite no changes in phosphorylation of the LHC. While some evidence of state transitions in diatoms has been described (Büchel and Wilhelm 1993), others suggest there is no evidence that diatoms exhibit such a mechanism (Jakob et al. 2005; Wagner et al. 2006). Wagner (2006) dismisses the proposition that direct energy transfer from PSII to PSI (“spillover”) could act as an alternative mechanism for energy redirection based on the separation of PSII/LHCII (grana) from PSI (stroma). This overarching comment ignores the occurrence of different thylakoid arrangements among the chromophytes and cyanobacteria where evidence from picosecond time-resolved fluorescence indicates that state transition may be a function of changes in spillover
between PSII and PSI (Gibbs and Biggins 1989). State 1 - state 2 transitions from spillover rather than changes in absorption cross-section have also been measured with photoacoustics in mutant tobacco plants deficient in grana partitions and less stacking, although LHCII phosphorylation still had a role in this process (Canaani 1990). Given the extent of experimental evidence supporting the validity of the spillover model and that phosphorylation of LHCs has been found to be almost universal (Falkowski and Raven 1997) it appears likely that the properties of the negatively charged (phosphorylated) LHCs enhance spillover in a state transition-like redistribution of energy.

In general, elucidation of the exact mechanism(s) for state transition in algae is a work in progress and the involvement of protein phosphorylation remains an open question (Büchel and Wilhelm 1993; Wollman 2001; Wagner et al. 2006). Other mechanisms may exist to redirect excitation energy away from PSII and toward PS I (Bullerjahn 1997).

### 1.2.5 Photoinhibition

Although the predominant proportion of marine phytoplankters are light limited, those at or near the surface or in shallow coastal environs may experience significant periods of excess light energy reaching the photosynthetic apparatus. Along with state transitions an array of mechanisms have evolved to minimise the physical damage that can be caused by such an excess of energy (Magnusson 1997). While a number of these mechanisms will be discussed in Chapter 1.3.2, the actual process of photoinhibition, inhibition of photosynthesis due to photodamage, will be covered here. Photoinhibition is not to be confused with ‘downregulation’ of photosynthesis, a reduction in photosynthetic efficiency reflecting mechanistic changes to enhance energy dissipation through non-radiative decay (heat) (see Chapter 5.1).
In circumstances where incident quantum flux is higher than the achievable rate of electron flux through the photosystems, the excess energy must be dissipated as heat or fluorescence for photoinhibitory damage to be avoided (Krall and Edwards 1990). The critical photon fluence rate capable of initiating photoinhibition varies depending on the efficiency of these energy dissipation mechanisms (downregulation), the efficiency of carbon fixation and the action of defence mechanisms (Hideg et al. 2000; Oxborough and Baker 2000). The capacity for photochemistry and downregulation can be monitored via chlorophyll fluorescence and hence this technique is useful for the study and detection of photoinhibition and its relation with environmental and biological variables (Magnusson 1997). Any condition that inhibits the functioning of the photosynthetic electron transport chain or exhausts excess energy relaxation mechanisms promotes photoinhibition at lower $E_{\text{PAR}}$ (Hideg et al. 2000). For example, studies have indicated that phytoplankton in nutrient poor ocean waters are more prone to photoinhibition due to the nutritional requirements for the repair of the D1 protein in PSII (Falkowski 1994; Underwood 2002). Understandably, due to its impact on enzyme activity, temperature has been shown to impact the level of PAR algae can tolerate before the onset of photoinhibition (Bouterfas et al. 2002). In general, those conditions that suppress photochemical electron transport or the efficacy of downregulation processes enhance the potential for photodamage leading to photoinhibition (Hideg et al. 2000).

There remains some contention as to the specific target site(s) for photodamage. It is generally accepted that most sites are within PSII (Bergmann et al. 2002), however there also is evidence that damage may occur at PSI in algae (Hihara and Sonoike 2001). When the PQ pool is fully reduced the $Q_b$ binding site within D1 remains unoccupied and $Q_A^-$ cannot pass on its electron. With the continuation of charge separation the long-lived $Q_A^-$ becomes protonated and doubly reduced ($Q_{A\text{H}_2}$) and will
not accept further electrons from Ph\(^{-}\). This blockage forces recombination of RCII\(^{+}/Ph\(^{-}\) and formation of a triplet excited state (\(3^{3}\)RCII\(^{*}\)). Chlorophyll triplets are non-destructive, however, they readily react with molecular oxygen to form reactive singlet oxygen (\(1^{1}O_{2}\)) (Equation 11) (Oxborough and Baker 2000; Andersson and Aro 2001). It is these reactive \(1^{1}O_{2}\) species which has been shown to result in preferential destruction of RCII and D1 (Krall and Edwards 1991; Vass et al. 1992; Carpentier 1997).

Bergmann et al. (2002) found that D1 protein damage caused a significant fraction of the midday depression in photochemical efficiency of phytoplankton in 55 L outdoor mesocosm tanks. The primary role of D1 in photoinhibition is indicated by the good correlation between its degradation and the maximum photochemical efficiency of PS II (\(\Phi_{\text{PSII max}}\)) as measured by chl-a fluorescence (\(F_v/F_m\)) (Fracheboud 2001). The high propensity of PSII for photodamage is due in part to its close association with the O\(_2\)-evolving complex. This is the dominant process for acceptor-side photoinhibition since chlorophyll triplets formed in LHCII through intersystem crossing are normally quenched by carotenoids (Andersson and Aro 2001). However, even superoxide and hydrogen peroxide formed at PSI have been shown to induce damage at D1 (Andersson and Aro 2001).

\[
3^{3}\text{Chl}^{3}O_{2} \rightarrow ^{1}\text{Chl}^{1}O_{2}
\]  

(11)

Damage to the D1 protein may also occur in the absence of oxygen due to donor-side photoinhibition. This occurs when a lack of electrons donated from the Mn cluster cause RCII\(^{+}\) to be long-lived. In such circumstances RCII\(^{+}\) can cause oxidative damage to D1 in the area between Tyr\(_{73}\), a tyrosine in D1 that participates in electron transport, and RCII (Andersson and Aro 2001). It has been shown that UVB radiation can damage the Mn cluster (Bergmann et al. 2002) and this, in turn, may enhance donor-side inhibition of RCII and D1 (Hideg et al. 2000; Andersson and Aro 2001).
Even at low irradiance D1 becomes irreversibly damaged, however its turnover rate is low and rapid proteolytic degradation and replacement of D1 results in no measurable photoinhibition (Andersson and Aro 2001). When the rate of damaging reactions exceeds the rate of repair photoinhibition will be observed.

1.2.6 Other electron consuming processes

Although studies on higher plants have indicated that electron transport to electron acceptors not associated with photosynthetic CO$_2$ assimilation is small (von Caemmerer 2000), significant transfer of electrons to O$_2$ before PSI can occur in cyanobacteria due to the co-location of respiratory and photosynthetic electron transport chains (ETC) (Cournac et al. 2002). A respiratory electron transport pathway is now known to exist in the chloroplast of eukaryotic photoautotrophs (Bennoun 1982, 2002; Peltier and Cournac 2002), allowing the potential for recombination of electrons with O$_2$ (eg. via PQH$_2$, PSI FeS, Fd and MDAR) (Badger et al. 2000; Behrenfeld et al. 2004).

When under stress, such as when the Calvin-Benson cycle is rate-limiting and energy within the photosynthetic ETC is in excess, electrons can be funneled into alternative sinks. Such sinks may be responsible for the major proportion of changes in $\Phi_{CO2}$ (Badger et al. 2000). Even in favourable conditions recorded $\Phi_{CO2}$ maxima are often ~0.10 mol C (mol quanta)$^{-1}$, reduced from the theoretical maximum of 0.125 mol C (mol quanta)$^{-1}$, due to redirection of reductant to N-assimilation pathways (Equation 12) (Badger et al. 2000). Alternative electron pathways can consume up to 30% of electrons gathered photosynthetically by higher plants, however, at present the lack of hard data for the chromophytic algae prevents such as estimation. Diatoms are probably the most studied organisms of the Chromista, in this respect, and indications are that alternative electron pathway activity is low, but may still play a significant role (Wilhelm et al. 2006).
The three primary alternative electron pathways appear to be cyclic electron transport around PSI, the Mehler Reaction and photorespiration. The importance of a fourth pathway, chlororespiration, remains an issue of debate. There is increasing evidence to suggest that alternative electron pathways around PSI appear to be more functionally significant than cyclic electron pathways around PSII (Bukhov and Carpentier 2004). While it is not within the scope of this dissertation to detail all of the potential electron pathways and their functional significance, an outline of the current knowledge of the major alternative electron pathways will be presented below. The reader is encouraged to refer to the quoted references for more detailed information if desired.

**Photorespiration**

In the dark reactions of photosynthesis carbon is fixed via the carboxylation of ribulose-1,5-bisphosphate to yield two molecules of 3-phosphoglycerate. This reaction is catalysed by the world’s most abundant enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase, otherwise known as Rubisco. However, as its name suggests, Rubisco can also act as an oxygenase by catalysing the addition of O$_2$ to ribulose-1,5-bisphosphate to produce phosphoglycolate and 3-phosphoglycerate (Equation 13) (Taiz and Zeiger 1991). The term Photorespiration is used to describe this oxygenase reaction and the metabolic pathways used by the cell to save some of the carbon and energy within phosphoglycolate. As the carboxylation and oxygenation reactions are directly competitive, their ratio depends on the relative concentrations of CO$_2$ and O$_2$ (Björkman and Demmig-Adams 1995). Under normal atmospheric conditions at a temperature of 25°C approximately one quarter of Rubisco activity in higher plants will be oxygenase activity, however, the oxygenation:carboxylation ratio has been observed to increase
with rising temperatures and environmental stress (Björkman and Demmig-Adams 1995). The kinetics of Rubisco are not constant between taxa and the potential for oxygenase activity in algae and cyanobacteria has been shown to be substantially lower (Badger et al. 2000). Many algae also decrease the oxygenation:carboxylation ratio by employing CO$_2$-concentrating mechanisms (Badger et al. 2000) and most data indicates only limited photorespiration in most marine microalgae (Beardall 1989).

\[
\text{Ribulose 1,5-bisphosphate} + \text{O}_2 \xrightarrow{\text{Rubisco}} \text{Phosphoglycolate} + 3\text{-Phosphoglycerate} \quad (13)
\]

\[
\text{Phosphoglycolate} + \text{H}_2\text{O} \xrightarrow{\text{Phosphoglycolate phosphatase}} \text{Glycolate} + \text{HOPO}_3^{2+} \quad (14)
\]

Phosphoglycolate must be metabolised or excreted as it is an inhibitor of Rubisco carboxylase activity (Beardall et al. 2003) and potentially damaging (Badger et al. 2000). This process typically starts with the dephosphorylation of phosphoglycolate (Equation 14). The continued metabolism of glycolate involves a chain of reactions that spans between the chloroplast, peroxisomes and mitochondria (Taiz and Zeiger 1991; Badger et al. 2000). In many algae (most of which lack peroxisomes) and cyanobacteria the cycle is shortened either by excretion of glycolate (Wilhelm et al. 2004) or by one of a number of alternative pathways for recycling energy and carbon from glycolate back into useful metabolic processes. While substantial excretion of glycolate by algae has been observed in photorespiratory conditions, it has been proposed that this may only occur when production of glycolate via Rubisco oxygenase activity is greater than the cells capacity to recycle it (Parker et al. 2004). Such excretion is energetically wasteful and modelling indicates that many phototrophic organisms could not achieve net carbon fixation if this glycolate was continuously lost from the cells (Beardall et al. 2003). Using the diatom *Thalassiosira weissflogii*, Parker et al. (2004) recently provided evidence that marine microalgae can recycle much of the glycolate produced by photorespiration.
Most marine microalgae utilise glycolate dehydrogenase to oxidise glycolate to glyoxylate (Beardall 1989). However, along with higher plants, some algal classes including the Eustigmatophyceae oxidise glycolate via glycolate oxidase. In both cases glyoxylate is metabolised in the Photorespiratory Carbon Oxidation Cycle (PCOC). The glycolate dehydrogenase PCOC occurs in the mitochondria and is coupled with the respiratory ETC, releasing CO$_2$, NH$_3$ and glycerate (Beardall et al. 2003). The glycolate oxidase PCOC occurs primarily in the peroxisomes and, not being linked to oxidative phosphorylation, is not associated with the generation of ATP or NADH (Beardall et al. 2003). Thus, cells employing glycolate dehydrogenase have an energetic advantage of about 1.5 ATP/glycolate compared to cells that use glycolate oxidase (Beardall 1989).

Many member of the Heterokonts, a subgroup of the Chromista, have a variation of the glycolate dehydrogenase PCOC that applies malate synthase to produce malate from glyoxylate and acetyl coenzyme A. Malate can then be used in the generation of NAD(P)H (which could then be used to form ATP), used in the tricarboxylic acid cycle or shuttled across to the chloroplast (Beardall et al. 2003).

Goyal and Tolbert (1996) identified a light dependent glycolate-quinone oxidoreductase associated with the thylakoid membranes of Chlorophyte algae and proposed that this enzyme was used to recycle electrons back to the PQ pool (Figure 1.3). It was proposed that this was the predominant oxidation pathway for glycolate, with NADPH-glyoxylate reductase subsequently recycling glyoxylate back to glycolate within the chloroplast. In this scenario glyoxylate dehydrogenase/oxidase and the PCOC would be activated only when glycolate-quinone oxidoreductase is saturated (Goyal and Tolbert 1996).
Photorespiration utilises energy from the linear photosynthetic ETC and in this way can act to diminish photoinhibition when photochemical energy is provided in excess. Some do not consider photorespiration to be a regulatory mechanism due to its minor role in microalgae (Behrenfeld et al. 2004), however photorespiratory enzyme levels increase in response to increasing irradiance, suggesting that photorespiration may remain important in the regulation of excess energy dissipation (Beardall et al. 2003). The cellular benefit from this photoprotection may outweigh the energetic losses that accompany photorespiration (Parker et al. 2004).

**MEHLER REACTION**

In conditions when CO\textsubscript{2} fixation is inefficient, such as when availability of inorganic carbon is poor or Calvin-cycle enzymes have not been fully activated (eg. immediately after onset of illumination), a lack of NADP\textsuperscript{+} may lead to electron transfer from reduced ferredoxin (F\textsubscript{d}\textsubscript{red}) to O\textsubscript{2} (Asada 1999; von Caemmerer 2000). Stable isotope studies have shown that the O\textsubscript{2} used in this reaction has its origins in the water molecules split at the oxygen evolving complex associated with PSII (Asada 1999).

The reduction of O\textsubscript{2} at PSI leads to the formation of reactive superoxide (O\textsubscript{2}\textsuperscript{+}). Superoxide is rapidly scavenged by the thylakoid membrane-bound enzyme superoxide...
dismutase and, with the addition of H\(^+\), hydrogen peroxide (H\(_2\)O\(_2\)) and oxygen are produced. The photoreduction of O\(_2\) and subsequent disproportionation of O\(_2^*\) to H\(_2\)O\(_2\) is known as the *Mehler reaction* (Schreiber et al. 1995a). The immediate scavenging of H\(_2\)O\(_2\) is essential as its reaction with reductants (such as transition metal ions) may lead to the production of hydroxyl radicals (•OH) which can cause significant oxidative damage within the chloroplast. As there is no •OH-specific scavenging enzyme H\(_2\)O\(_2\) must be scavenged prior to •OH production (Asada 1999). The detoxification of H\(_2\)O\(_2\) by the ascorbate-peroxidase reaction to form water is intimately related to the Mehler reaction and the whole reaction sequence is often referred to as the *Mehler ascorbate peroxidase (MAP) pathway* (Figure 1.4). In essence the electrons stripped from water at the oxygen evolving complex are returned to water, hence the MAP pathway is also known as the water-water cycle.

Despite significant research attention there are still many questions regarding the occurrence and significance of the MAP pathway. Some of the proposed functions of the MAP pathway include; (1) allowing linear electron flow to continue under limited NADPH consumption, (2) aid the development and maintenance of high transthyllakoidal ΔpH (enhanced non-photochemical quenching) and, via this ΔpH, (3) to help balance the different ATP and NADPH requirements of C-fixation and photorespiration (von Caemmerer 2000; Behrenfeld et al. 2004). Enhanced ATP production may also be useful in high light conditions as consumption of ATP by processes such as D1 protein repair and CO\(_2\)-transport can be expected to increase.
Figure 1.4: Simple representation of the Mehler reaction (solid arrows) and the ascorbate-peroxidase reaction (dashed arrows). At the oxygen-evolving complex (1.) water donates its electrons to the photosynthetic electron transport chain to produce O$_2$ and release four protons into the lumen ($\Delta$pH). These electrons are carried from PSII to PSI via Plastoquinone (PQH$_2$), the Cyt $b_6$f complex and plastocyanin (PC). At PSI light energy drives the passage of electrons from PC through $P_700$ and a number of other intermediaries to reduce ferredoxin (Fd'). In the absence of other electron acceptors O$_2$ is reduced by ferredoxin to produce superoxide (O$_2^-$). In an extremely rapid and efficient series of reactions membrane-bound superoxide dismutase (SOD) catalyses the disproportionation of O$_2^-$ to O$_2$ and H$_2$O, which is then reduced to water by ascorbate (ASC) in a reaction mediated by an ascorbate-specific peroxidase (2.). Monodehydroascorbate (MDA) is formed by the oxidation of ASC, which must be regenerated. ASC can be regenerated via the reduction of MDA by NADPH or Fd' with the aid of monodehydroascorbate reductase (3.).

The Mehler reaction may account for up to 40% of total electron flow in higher plants, however less is known about the dynamics of this pathway in algae and cyanobacteria (Wilhelm et al. 2004). Observations suggest that many algae have stromal enzymes that are resistant to damage from H$_2$O$_2$ and excretion of H$_2$O$_2$ may replace the ascorbate-peroxidase reaction (Badger et al. 2000) while other reports have indicated that electron flow to O$_2$ may be minimal in algae (Franklin and Badger 2001; Carr and Björk 2003).
**Cyclic Electron Transport Around PSI**

Cyanobacteria, eukaryotic algae and higher plants are known to cycle electrons around PSI (Bukhov and Carpentier 2004). Like the Q-cycle associated with the cyt-b\(_{6}/f\) complex (described in Joliot et al. (2004)), cyclic electron transport has been associated with increasing proton transport across the thylakoid and consequently increasing the transthylakoid electrochemical gradient, non-photochemical quenching and ATP production (Bendall and Manasse 1995). Such an activity may be of particular use during the dark-light transition of a dark adapted cell, when the concentration of ATP limits the rate of the Calvin-Benson cycle (Joliot et al. 2004). Many Calvin-Benson cycle enzymes are light activated and immediately after illumination the use of reductant provided by photosynthetic linear electron transport can be particularly slow. In these circumstances dissipation of excess energy via alternative pathways would be important and the enhancement of non-photochemical quenching via increased \(\Delta pH\) would provide such a pathway. The importance of this feature is highlighted by the observation that cells acclimated to high light conditions exhibit higher rates of cyclic electron transport around PSI (Bendall and Manasse 1995). ATP derived from cyclic electron transport provides reducing energy for other important metabolic processes, including: nitrogen fixation, protein synthesis and ion transport (Bendall and Manasse 1995).

The Calvin-Benson cycle requires ATP and NADPH to be provided in a ratio of 3:2 (Allen 2003). Current understanding is that photosynthetic linear electron transport yields an ATP:NADPH ratio of 9:7 but that the addition of a cyclic electron transport component can meet the ATP deficit (Allen 2003). PSI cyclic electron transport is also thought to make up the deficit in C\(_{4}\) photosynthesis, which is even more ATP-hungry (Bendall and Manasse 1995; Nixon and Mullineaux 2001).
Despite the important role of cyclic electron transport around PSI researchers have not yet been able to elucidate the exact pathway(s) by which electrons are transferred from PSI back into the ETC. Of the pathways proposed, there are two dominant categories: (1) NAD(P)H-dehydrogenase (ndh) mediated and, (2) ferredoxin-mediated (Figure 1.5) (Nixon and Mullineaux 2001). The ferredoxin-mediated pathways are considered to be the most likely mode of electron transport from PSI to PQ (Bendall and Manasse 1995; Nixon and Mullineaux 2001), however, the mediating enzyme has not yet been conclusively identified (Bukhov and Carpentier 2004).

Figure 1.5: Schematic representation of the major pathways proposed for cyclic electron transport around PSI in chromophytic algae. Photochemical charge separation at PSI allows the reduction of ferredoxin (Fd). In pathway 1 ferredoxin-NADP\(^+\) reductase (FNR) facilitates the reduction of NADP\(^+\) to NADPH, which is then used to reduce the PQ pool via NAD(P)H dehydrogenase (ndh). In the case of most marine microalgae this is a type 2 ndh. In pathway 2 the reduced ferredoxin from PSI acts to directly reduce the PQ pool via a putative ferredoxin-(plasto)quinone reductase (FQR). In Pathway 3 FNR is associated with Cyt-\(b_6/f\), helping to bind reduced ferredoxin and passing electrons from ferredoxin via a chain of uncharacterised intermediaries to PQ. The dotted arrow indicates a possible reverse action of FNR to reduce ferredoxin by oxidising NADPH (see text). Each pathway leads to the uptake of protons in the stroma and their release in the lumen, thus enhancing the transthylakoid electrochemical gradient.

Although ndh activity has been reported in a wide range of phototrophic organisms (Bukhov and Carpentier 2004), measurements show that its concentration is only about 1% that of other ETC components and its role in cyclic electron transport around PSI is not likely to be significant (Joliot et al. 2004). There is evidence that mutants deficient in ndh have reduced non-photochemical quenching of chl-\(a\)
fluorescence in the early stages of illumination (Nixon and Mullineaux 2001), however this may be associated with chlororespiration (see below).

A recent account of Ferredoxin-NADP reductase (FNR) co-purification with the cyt-\(b_6/f\) complex (Zhang et al. 2001) gave support to the theory that reduced ferredoxin (\(\text{Fd}_{\text{red}}\)) can donate electrons directly to the cyt-\(b_6/f\) complex (transfer process described in Joliot et al. (2004) and Bukhov and Carpentier (2004)). The previously preferred pathway saw electrons from \(\text{Fd}^-\) donated to the PQ pool via a putative ferredoxin-(plasto)quinone reductase (FQR) associated with the thylakoid membrane (Figure 1.5) (Bendall and Manasse 1995; Nixon and Mullineaux 2001).

Cyclic electron transport is generally considered to be a light driven pathway, however, FNR also has an oxidase capacity and may act to reduce ferredoxin by oxidising NADPH (Nixon and Mullineaux 2001). It is therefore conceivable that ferredoxin could be reduced and pass its electrons to cyt-\(b_6/f\) or the PQ pool in the dark.

Membrane structure and stacking has recently been receiving significant attention as to its role in facilitating cyclic electron transport. Cytochrome-\(b_6/f\) complexes located in the stroma and closely associated with PSI would have a high potential for cyclic electron flow. On the other hand PSI centres located along the periphery of the grana would associate with both the Cyt-\(b_6/f\) and PSII complexes in the grana to perform, more predominantly, linear electron transport. For a recent perspective on thylakoid membrane structure that covers this topic please refer to Dekker and Boekema (2005).

**CHLORORESPIRATION**

The presence, and a basic scheme, of a respiratory cycle in the chloroplast was outlined in the early 1980’s by Bennoun (1982) after the earlier proposal that oxygen may act to oxidise the PQ in the dark after its reduction by externally-derived reductant
(Diner and Mauzerall 1973). Initially the source of controversy, recent headway in the identification of its molecular components has seen it gain greater acceptance (Cournac et al. 2002). This section provides a general description of the present model for chlororespiration and briefly outlines some of the physiological roles proposed for such a pathway. More detailed accounts of the model and the research leading to its proposal can be found in the references quotes, particularly the recent reviews by Bennoun (2002), Nixon (2000) and Beardall et al. (2003).

The non-photochemical reduction of PQ in the dark is now accepted across a wide range of phototrophic organisms, however, there remains an active debate over the identity and location of the responsible enzymatic agents and reductants (Cournac et al. 2002; Peltier and Cournac 2002). Similarly, while it is now accepted that a terminal oxidase is responsible for PQ pool reoxidation in the dark, the identity and location of such an oxidase remains an enigma (Beardall et al. 2003; Wilhelm et al. 2006). Part of the confusion, no doubt, is the likelihood that numerous reduction/oxidation pathways exists and that these vary between taxa (Beardall et al. 2003).

Despite the confusion with regard to the agents involved in the chlororespiratory pathway, the sequence of reactions is quite simple. The reduction of PQ by exogenous reductant (R) is catalysed by enzyme ‘X’ associated with the thylakoid membrane. Di-oxygen is then used to oxidise PQ via a plastid terminal oxidase (PTOX) located on either the luminal or stromal side of the thylakoid membrane (Bennoun 2002). NAD(P)H is most likely to be the reductant (R) since a number of metabolites, such as malate, can be coupled to its formation in the stroma (Beardall et al. 2003). Consequently, by the use of reductant and consumption of O$_2$, chlororespiration acts to decrease $\Phi_{\text{CO2}}$ and $\Phi_{\text{O2}}$. Via the reduction of the PQ pool, $\Phi_{\text{PSII}}$ is decreased also.
Figure 1.6: Simplified scheme for chlororespiration (see text for details). Exogenous reductant (e.g. NAD(P)H) reduces the PQ pool in the dark via enzyme X (e.g. NAD(P)H dehydrogenase). A plastid terminal oxidase (PTOX) then oxidises PQ, consuming oxygen and producing water. Adapted from Bennoun (2002).

As was mentioned earlier, NAD(P)H-dehydrogenase (ndh) activity has been found in higher plants, algae, and cyanobacteria. A type 1 ndh is generally accepted to be the major enzyme involved in the reduction of PQ in higher plants while only type 2 ndh has been identified in the Heterokonts. Type 1 ndh is capable of proton transport while type 2 ndh is a smaller monosubunit flavoprotein and cannot transfer protons across the thylakoid membrane (Nixon 2000; Peltier and Cournac 2002).

A possible alternative for the nonphotochemical reduction of PQ is via the putative ferredoxin-(plasto)quinone reductase (FQR), with reduced ferredoxin being supplied by the FNR-mediated oxidation of NADPH (Nixon 2000; Peltier and Cournac 2002).

The reduction and oxidation of the PQ during chlororespiration was thought to result in the transport of protons across the thylakoid membrane, increasing the ΔpH component of the electrochemical gradient (ΔµH+) (Bennoun 1982). This could lead to promotion of non-photochemical quenching and the generation of ATP. However, the discovery of an ATP-drive ion pump (different from protons) (Rappaport et al. 1999) and the observation that an ΔµH+ can develop in the dark in anaerobic conditions has
lead to a general dissociation of chlororespiration from $\Delta \mu_{\text{H}^+}$ formation (Bennoun 2002; Peltier and Cournac 2002; Beardall et al. 2003). This new ion pump has been shown to be responsible for the electrical component ($\Delta \Psi$) of $\Delta \mu_{\text{H}^+}$, however, when ATPase remains operational the $\Delta p$H component accounts for at least two-thirds of $\Delta \mu_{\text{H}^+}$ (Rappaport et al. 1999). This indicates that reverse functioning of ATPase may act to shuttle protons from the stroma to the lumen. It is probable that the role of chlororespiration in formation of an electrochemical gradient varies between taxonomic groups and further investigation into a wide range of species is required (Beardall et al. 2003).

Recent experiments by Jakob et al. (1999; 2001) on diatoms found the development of $\Delta p$H in the dark which was significant enough to result in de-epoxidation of diadinoxanthin to diatoxanthin. It was suggested this dark-induced $\Delta p$H was derived from chlororespiration and, since diatoxanthin concentration is positively correlated with non-photochemical quenching, this could be seen as a photoprotective mechanism (Jakob et al. 2001). In highly fluctuating light environments, such as those experienced by phytoplankton in mixed environments, maintaining dissipative mechanisms during periods of darkness may prevent significant photodamage in the early stages of illumination.

Chlororespiratory activity has been hard to measure due to the molecular similarity of its component with the closely associated mitochondrial ETC and the action of other O$_2$-consuming processes (eg. Mehler reaction) (Beardall et al. 2003). Research suggests, however, that chlororespiration in algae may be substantial (as much as 20% of total respiration) and more developed than in higher plants (Diner and Mauzerall 1973; Bennoun 1994; Franklin and Badger 2001).
The redox state of the PQ pool is important in the regulation of both linear and cyclic photosynthetic electron transport. Even modest amounts of chlororespiration may act to ‘poise’ redox state to balance electron flow between the various pathways and influence state transition (Nixon 2000; Beardall et al. 2003). This may be especially important under highly fluctuating light intensities (Beardall et al. 2003).

**Cyclic Electron Transport around PSII**

The passage of electrons from the acceptor side of PSI back into the ETC at the PQ pool or cyt-b$_{6}$/f complex has been described above. In this process primary charge separation at PSII strips electrons from H$_{2}$O to produce O$_{2}$ and these electrons are passed into the intersystem ETC. As cyclic electron transport around PSI effectively recycles electrons, they may still play a role in the fixation of carbon. Therefore, the correlation of $\Phi_{PSII}$ with either $\Phi_{O2}$ or $\Phi_{CO2}$ should not be significantly impacted by the activity of cyclic electron transport around PSI.

Under conditions of moderate, continuous light all electrons replenishing RCII$^{+}$ originate from water. However, it has been observed that oxygen evolution may become decoupled from PSII electron transport at both low (Seaton and Walker 1990) and high (Falkowski et al. 1986b; Prášil et al. 1996; Lavaud et al. 2002c) irradiances. Such a phenomenon may indicate the presence of cyclic electron transport around PSII where electrons are recycled back to RCII$^{+}$ from the PSII acceptor side (Falkowski et al. 1986b; Prášil et al. 1996).

Cyclic electron flow around PSII may be of particular functional benefit when the oxygen evolving complex (OEC) cannot pass electrons to RCII$^{+}$ via the tyrosine residues. Prášil et al. (1996) suggested that when the PQ is highly reduced and there is a strong electrochemical potential across the thylakoid membrane ($\Delta\mu_{H^+}$), cyt-b$_{559}$ changes to a high potential form that can reduce RCII$^{+}$ after accepting electrons from
PQH$_2$ still located in the Q$_B$ pocket. It has also been hypothesised that cyt-$b_{559}$ can accept electrons from Ph$^-$ instead of PQH$_2$ (Carpentier 1997; Lazár et al. 2005 and references therein). Such a cycle would not only help to avoid donor side inhibition (see section 1.2.5) but would reduce the potential of PSII acceptor side photoinhibition as well (Carpentier 1997; Lavaud et al. 2002c). At low irradiances the time between advancement of each OEC S-state is extended and there is an increased potential for deactivation of the PSII acceptor side (Schreiber 2004).

Electrons that are passed through RCII are measured by chlorophyll fluorescence, however those electrons that are sourced from cyt-$b_{559}$ instead of water do not yield oxygen and the relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{O}_2}$ deteriorates (Falkowski et al. 1986b; Lavaud et al. 2002c; Schreiber 2004). Also, since electrons are diverted away from linear electron transport (back to the donor side of PSII) and must see another primary charge separation before having another chance to reduce CO$_2$, there is a similar breakdown in the $\Phi_{\text{PSII}}$: $\Phi_{\text{CO}_2}$ relationship (Prášil et al. 1996).

**1.3 Fluorescence**

The remainder of this chapter provides an outline of the chl-$a$ fluorescence signal, the parameters derived from the fluorescence signal and the impact of environmental variables. It is not intended to be an exhaustive overview of the topic as a number of excellent reviews on chl-$a$ fluorescence and its measurement and application have already been published and the reader is referred to these for a more detailed discussion (Krause and Weis 1984, 1991; Sivak and Walker 1985; Büchel and Wilhelm 1993; Govindjee 1995; Schreiber et al. 1995a; Samson et al. 1999; Roháèek 2002; DeEll and Toivonen 2003; Kromkamp and Forster 2003; Oxborough 2004; Lazár 2006). For a single reference item on the subject that is highly informative on all areas of chl-$a$
fluorescence I suggest the 19th volume of the Advances in Photosynthesis and Respiration series (Papageorgiou and Govindjee 2004).

For the newcomer to chl-a fluorescence one of the most confusing aspects can be the large number of terms applied in the field, many of which are duplicative. While a few attempts to standardise fluorescence notation, most notably by van Kooten & Snel (1990), have reduced the extent of this variation in more recent publications, some variation still occurs (Baker and Oxborough 2004). The nomenclature used here reflects that used in Papageorgiou & Govindjee (2004) which is founded on van Kooten & Snel (1990). Kromkamp and Forster (2003) have suggested additional notation in order to indicate whether single- or multiple-turnover fluorescence techniques were used.

Fluorescence is the re-emission of energy in the form of a photon (light) as an electron returns to ground state from an singlet excited state. In the case of chl-a fluorescence a chlorophyll molecule can become excited and achieve singlet state 1 (S$_1$) after absorbing a photon of less than 670 nm wavelength (Bolhàr-Nordenkampf and Öquist 1993). If the energy is not utilised in charge separation, heat dissipation or resonance energy transfer, fluorescence will occur as the electron drops out of the excited state. As some energy is also given off as heat the photon is red-shifted with an emission peak of ~685 nm. If the absorbed photon is of a shorter wavelength (eg. blue light at about 420 nm) the extra energy excites the chlorophyll molecule to the singlet state 2 (S$_2$) and heat is emitted as it rapidly decays to the S$_1$ state. It is commonly considered that, at ambient temperatures, nearly all fluorescence (~95%) originates from PSII (Krause and Weis 1991) and represents 0.6 to ~10% of the absorbed light (Nicklisch and Köhler 2001). As mentioned earlier, PSII is considered a shallow trap (see section 1.2.2) while the absorption maximum for PSI is sufficiently red-shifted (25 nm) relative to its antenna that there is much less chance for energy to escape (Falkowski and Raven 1997).
Light energy absorbed by a photosystem and its LHC can be used/dissipated through one of three competing pathways: (1) photochemistry (primary charge separation and photosynthetic electron transfer), (2) thermal dissipation (non-radiative decay) or, (3) fluorescence emission (Falkowski et al. 1986a; Seaton and Walker 1990; Kolber and Falkowski 1993; Nicklisch and Köhler 2001). It is assumed that the sum of the quantum yields of each of these processes is unity. Thus, changes in fluorescence yield reflect changes in the complementary pathways. In order to quantify changes in photochemistry from the chl-\(a\) fluorescence signal a measure of changes in thermal dissipation must be obtained also (Baker and Oxborough 2004).

The quantum efficiency of fluorescence (\(\Phi_f\)) is simply the ratio of quanta fluoresced to total quanta absorbed:

\[
\Phi_f = \frac{Q_f}{Q_a}
\]  \hspace{1cm} (15)

where \(Q_a\) is the quanta absorbed and \(Q_f\) the quanta fluoresced. If \(\Phi_f\) is known then this can be used to determine fluorescence emission on a per unit chlorophyll basis:

\[
F = E \times \bar{a}_chl \times \Phi_f
\]  \hspace{1cm} (16)

where \(F\) is the chlorophyll-specific fluorescence, \(E\) = incident irradiance, \(\bar{a}_chl\) = average chl-specific light absorption coefficient (\(m^{-2} (mg \text{ chl})^{-1}\)) (Estrada et al. 1996).

Higher \(\Phi_f\), and therefore higher \(F\), is correlated with a closure of RCIIIs since energy must then be dissipated to a greater extent by non-photochemical means (Ralph and Gademann 2005). As the redox state of the quinone \(Q_A\) determines whether PSII RS is open (\(Q_A^\bullet\)) or closed (\(Q_A^-\)), it is also the main controlling factor determining chl-\(a\) fluorescence yield (Schreiber et al. 1998).

Carl Lorenzen introduced the technique of \textit{in vivo} chlorophyll fluorescence analysis to biological oceanography in 1966 (Lorenzen 1966). While passive chlorophyll fluorescence is still being used for quantitative estimation of phytoplankton...
biomass, over the past 20 years variable (or “active”) fluorescence has come to serve as an important tool in photosynthesis and ecophysiological research in the marine environment. Recent advances in the sophistication and sensitivity of fluorometers and data processing equipment has seen the broadening of the application of chlorophyll fluorescence analysis both in the laboratory and in the field. The popularity of the technique lays in its non-intrusive nature, often no sample preparation or incubation is required, along with its rapidity, sensitivity and ease of use. Chl-\(a\) fluorescence analysis is now a common method for the assessment of: photosynthesis (Schreiber et al. 1995a; Barranguet and Kromkamp 2000b), physiological state (Bergmann et al. 2002) including energy metabolism and the balance between C-fixation and other electron-consuming pathways (Badger et al. 2000; Beardall et al. 2001; Bukhov and Carpentier 2004), photosystem components (White and Critchley 1999; Fracheboud 2001), ecotoxicology (Juneau and Popovic 1999; Juneau et al. 2002, 2003) and plant breeding (Khanizadeh and DeEll 2003). However, despite the now common usage of this technique the fluorescence signal is influenced by numerous factors not all of which are fully understood and, especially in vivo, the overlapping signal response to these factors can be quite complex (Schreiber et al. 1995a). Some of these complicating factors include PSII heterogeneity, spillover, non-photochemical quenching dynamics and potential contribution of PSI to the fluorescence signal. While numerous studies have found good correlations between chl-\(a\) fluorescence and other photosynthetic parameters (Weis and Berry 1987; Genty et al. 1989: refer to Chapter 4) the interpretation and use of fluorescence data should be approached with caution (Cullen and Renger 1979; Demers et al. 1985; Bolhår-Nordenkampf and Öquist 1993; Kolber and Falkowski 1993; Schreiber et al. 1995a). Given that most of our current knowledge and models for photosynthesis are based on studies of higher plants and a limited number of green algae, with much of the theoretical basis for the interpretation of
fluorescence data based on these models, using a similar basis for the interpretation of fluorescence data from chl c- or phycobilin-containing algae may prove inaccurate (Büchel and Wilhelm 1993). The key functional differences that influence fluorescence signal in such algae relate to LHC structure (Nicklisch and Köhler 2001) and the importance of alternative electron pathways (Badger et al. 2000). Despite these difficulties chl-a fluorescence provides reliable information and remains a useful tool in studies of photosynthesis and its response to environmental variables as well as toxicants (Schreiber et al. 1998).

A large number of fluorometers have been designed to measure variable chl-a fluorescence in a wide range of conditions and 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 (FRR)
3. Fluorescene Induction and Relaxation System (FIRe)
4. Pump and Probe Fluorometer (PnP)
5. Induction Fluorometer

The PAM type fluorometer is currently the most popular and various models are applicable to studies of marine phytoplankton (Water-PAM, Phyto-PAM, Microscopy-PAM and PAM-101 among them) while the FRR type fluorometer is commonly used for specialised measurements (eg. functional absorption cross-section of PSII) and, due to its great sensitivity, in biological oceanography. The PAM and FRR fluorometers have largely replaced the older PnP and induction fluorometers. It is important to note that these fluorometers measure fluorescence yield (which may vary up to a factor of 5
or 6), not fluorescence intensity (which may by a factor of several thousand), as it is the former that carries information on photosynthesis (Dau 1994; Schreiber 2004).

The Kautsky curve describes the characteristic changes in chl-α fluorescence yield upon illumination of a dark-adapted alga or leaf (Figure 1.7). This pattern was first described by Kautsky and Hirsch (1931) but has been elaborated on since then (Govindjee and Papageorgiou 1971; Govindjee 1995: for details). Upon illumination fluorescence rises rapidly, usually within a second, from the origin (O) to a peak (P) via an inflection (I) and dip (D) of variable magnitude (Figure 1.7). If the actinic light is strong another inflection (J) can be observed between O-I (Govindjee 1995; Strasser et al. 2004). This phase (OJIDP) has been labelled the fast phase (Bolhàr-Nordenkampf and Öquist 1993) and reflects changes in the redox state of the RCII coinciding with the primary processes of photosynthesis (Govindjee and Papageorgiou 1971; Büchel and Wilhelm 1993).

Following the fast phase there is a polyphasic decline in chl-α fluorescence that ends at a terminal steady state level (T). This phase of the curve is known as the slow phase and may include a secondary peak (M) (refer to Walker 1981: for a discussion on the occurrence and dynamics of the M-peak) after a trough (S) (Figure 1.7). This slow phase is primarily related to the balancing of a number of processes including the rapid build up of a proton gradient across the thylakoid membrane, and light activation of Calvin Cycle enzymes (Bolhàr-Nordenkampf and Öquist 1993; Büchel and Wilhelm 1993; White and Critchley 1999).
Figure 1.7: Stylised representation of the chl-α fluorescence induction curve. Closed arrow represents activation of non-actinic measuring light. On application of strong actinic light (open arrow) fluorescence rises from the origin (O) to a peak (P) via two inflections (J and I). A dip (D) may occur after I. This O-P rise is known as the fast phase and reflects primary photochemistry and redox state of Q$_A$. After P fluorescence declines due to formation of a transthylakoid pH gradient and associated thermal quenching. The remainder of the transient (S-M-T) is called the slow phase and is the result of induction of Calvin cycle enzyme activity and its subsequent interaction with the electron transport chain (via NADPH) and photochemical and non-photochemical quenching.

Due to the complex nature of the numerous interactions influencing the chl-α fluorescence induction curve there has been some question over the level of interpretation of the data that is possible (Holzwarth 1993; Trissl et al. 1993; Falkowski et al. 1994; Trissl 1994), however, it is generally acknowledged that analysis of the induction curve remains a useful tool that will only improve as our knowledge of photosynthetic (and interacting) processes advances (Govindjee 1995).

The fast phase of the induction curve is commonly used to estimate photochemical quantum yield. The method applied is the saturation pulse method (Figure 1.8) and involves analysis of the quenching components. One of the first to apply this method was Schreiber et al. (1986) and the technique has been further described (eg: Schreiber et al. 1995b, 1995c; Schreiber 2004). However, a brief outline will be included here due to the direct application of the method in this study. Minimum
fluorescence yield ($F_o$) will occur when all RCIIs are open ($Q_A$ in all RCIIs is oxidised) and quantum energy reaching the reaction centre has maximal chance of being utilised photochemically and negligible chance of being dissipated as heat or fluorescence. This state is generally considered to be achieved after adaptation to the dark and dissipation of any transthylakoid pH gradient (see Section 1.3.2 for qualifications). In order to measure such a state the fluorometer’s measuring light must be weak enough ($<0.5 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) so as not to induce reduction of $Q_A$ and closure of any reaction centres. When a pulse of high intensity light sufficient to close all RCIIs (reduce all $Q_A$) is applied to a sample a condition is induced where photochemistry is reduced to zero and fluorescence yield is maximal. If the sample was dark-adapted prior to application of the saturation pulse, non-photochemical quenching will be negligible and fluorescence yield will reach its true maximum ($F_m$). However, if the sample was not dark-adapted, non-photochemical quenching will act to quench the fluorescence yield and the achieved maximum value will be lower ($F_m'$). Hence a drop in $F_m$ to $F_m'$ can be used as a measure of non-photochemical quenching. These measures assume that no non-photochemical quenching is induced by the short saturation pulse (Schreiber et al. 1995a; Schreiber 2004). We can therefore use these principles to estimate photochemical quenching (see Figure 1.8 and Table 1.1). It should be noted here that there is the potential for induction of non-photochemical quenching pathways in the dark (possibly linked to chlororespiration) (Jakob et al. 2001) which would impact particularly the $F_m$ value. The potential error caused by this can be overcome by applying a short pulse of far-red light to ensure full reoxidation of the PQ pool (P. Ralph pers. comm.) or by correcting $F_o$ and $F_m$ based on measured non-photochemical quenching (Ting and Owens 1993).
Figure 1.8: Fluorescence induction kinetics including application of the saturation pulse method and associated nomenclature (refer to text). Dark arrows indicate measuring light on (up) and off (down); Grey arrows indicate application of a short pulse of saturating light; open arrows indication activation (up) and deactivation (down) of actinic light. Modified from Büchel & Wilhelm (1993).

The measured $F_m$ (or $F_{m}'$) is dependent on the measuring technique. Whereas PAM fluorometers apply an extended flash (usually between 0.4 and 1.0 s) of high intensity light that results in multiple turnovers of the reaction centre to saturate $Q_A$, $Q_B$ and PQ to close all RCIIIs, FRR fluorometers use a rapid flash (also called a “flashlet”) that is short enough (10 – 100 µs) to result in only a single reduction of $Q_A$ to close the RCII and cause a rise in fluorescence to $F_m$ (or $F_{m}'$). The new Fluorescence Induction and Relaxation (FIRE) system by Satlantic employs both single- and multiple-turnover protocols. A comprehensive comparison of these two saturation techniques and their respective advantages and disadvantages has been provided by Kromkamp and Forster (2003), however, it is fundamentally important to note that direct comparison of results
between single turnover and multiple turnover techniques cannot be made as they yield different results. Even direct comparison of results within the same fluorometer type, PAM fluorometers for example, should be done with some caution since system geometry and the position of the detector relative to the cuvette can impact results (Mouget and Tremblin 2002).

**Table 1.1:** Definition of fluorescence parameters (following Baker et al. (2001)). Note: all parameters are dimensionless.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synonym</th>
<th>Definition</th>
<th>Derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(F_o)</td>
<td>O</td>
<td>Minimum fluorescence yield (dark adapted, all RCIIIs open)</td>
<td></td>
</tr>
<tr>
<td>(F_m)</td>
<td>P</td>
<td>Maximum fluorescence yield (dark adapted, all RCIIIs closed with no NPQ)</td>
<td></td>
</tr>
<tr>
<td>(F_v/F_m)</td>
<td></td>
<td>Maximum photochemical efficiency (quantum yield) of open RCIIIs</td>
<td>((F_m - F_o)/F_m)</td>
</tr>
<tr>
<td>(F')</td>
<td>F</td>
<td>Fluorescence yield in actinic light ((F_i) or (F_t) used when at steady state)</td>
<td></td>
</tr>
<tr>
<td>(F_o')</td>
<td></td>
<td>Minimum fluorescence yield in actinic light</td>
<td></td>
</tr>
<tr>
<td>(F_m')</td>
<td></td>
<td>Maximum fluorescence yield in actinic light</td>
<td></td>
</tr>
<tr>
<td>(F_v')</td>
<td></td>
<td>Variable fluorescence yield in actinic light</td>
<td>(F_m' - F_o')</td>
</tr>
<tr>
<td>(F_q)</td>
<td>(\Delta F)</td>
<td>Difference between fluorescence yields (F_m') and (F')</td>
<td>(F_m' - F')</td>
</tr>
<tr>
<td>(F_q'/F_m')</td>
<td>(\Delta F / F_m')</td>
<td>Effective photochemical efficiency of RCIIIs in actinic light</td>
<td>((F_m' - F') / F_m')</td>
</tr>
<tr>
<td>(F_v'/F_m')</td>
<td></td>
<td>Maximum PSII photochemical efficiency or quantum yield in actinic light.</td>
<td>((F_m' - F_o') / F_m')</td>
</tr>
<tr>
<td>(q_P)</td>
<td>(F_q' / F_v')</td>
<td>Photochemical quenching</td>
<td>((F_m' - F') / (F_m' - F_o'))</td>
</tr>
<tr>
<td>NPQ</td>
<td></td>
<td>(Stern-Volmer) Non-photochemical quenching</td>
<td>((F_m - F_m') / F_m')</td>
</tr>
</tbody>
</table>

### 1.3.1 Quantum yield efficiency for PSII \((\Phi_{PSII})\)

**Definition and Measure**

The efficiency of a light-dependent process is referred to as the *quantum yield* or *quantum efficiency*. In basic terms the quantum yield \((\Phi)\) can be described as the ratio of product output to gathered quanta:

\[
\Phi = \frac{\text{mol product out}}{\text{mol quanta in}}
\]  

(17)
Saturation pulse analysis, as described above, can be used to estimate the quantum yield, or efficiency, of PSII photochemistry (Φ_{PSII}) (Genty et al. 1989; Schreiber et al. 1995c). In this circumstance the end product can be described as primary charge separation and the passage of an electron through PSII.

When in the dark, with QA in a fully oxidised state and no non-photochemical quenching, the maximum quantum yield of PSII (Φ_{PSII,max}) can be estimated by normalising the variable fluorescence (F_v) to the maximum fluorescence yield (F_m) (Table 1.1). In the light the photochemical efficiency of PSII and closure of reaction centres (reduced QA) decreases due to the induction of non-photochemical quenching (and possibly photoinhibition in high light) and the parameter F_q is normalised to the light adapted maximum fluorescence yield (F_m') (Table 1.1). Thus, by applying the saturation pulse methods the quantum yield of PSII can be rapidly and sensitively measured on a virtually real-time basis (Kolber et al. 1994).

Maximum theoretical values for F_v/F_m are ~0.65 (Kolber and Falkowski 1993) for single turnover saturation pulses and ~0.83 for multiple turnover pulses (Magnusson 1997). However, whilst the values of maximum achievable F_v/F_m are close to these maximum theoretical values across numerous species of microalgae (Falkowski 1994), some variation has been observed also (Koblížek et al. 2001). Environmental factors that impact upon PSII, directly or indirectly, will also impact measures of F_v/F_m (Greene et al. 1992). The dominant factors include light, nutrient status and temperature (Wozniak et al. 2002) and their potential impact will be discussed here. Also, Brand (1982) found that many marine phytoplankton species exhibit endogenous diel patterns in fluorescence parameters and suggested this may be the result of changes in cellular metabolism to “predict” environmental condition (Diel patterns are discussed in Chapter 6). All these factors combine to confound interpretation of F_v/F_m and other fluorescence parameters (Kroon et al. 1993).
**LIGHT**

The major proportion of the observed variability in PSII quantum yield in the marine environments can be explained by changes in the light conditions, with a significant negative correlation observed between the two variables (Magnusson 1997; Wozniak et al. 2002). Light may act to decrease \( F_v/F_m \) in a number of different ways. As irradiance increases \( Q_A \) will become increasingly reduced, resulting in an increase in the base fluorescence value from \( F_o \) to \( F' \) as described by the Kautsky curve (see above), while \( F_m \) decreases to \( F_m' \) due to the induction of non-photochemical quenching. Both of these changes result in a lowering of the PSII quantum yield from \( F_v/F_m \) to \( F_q/F_m' \).

These changes are quite rapidly reversed on return to the dark and an \( F_v/F_m \) can often be achieved again after a dark adaptation of 10 – 15 min. In fact, it has been found that in nutrient replete conditions \( F_v/F_m \) is not influenced by growth irradiance (Kolber et al. 1988; Parkhill et al. 2001).

Photodamage occurs at the detriment to functioning and efficiency of the photosystems and therefore results in a decrease in \( F_v/F_m \). The main reason for this is an increase in \( F_o \). In this context \( F_o \) increases as a result of damaged D1 protein and/or the physical dissociation of the LHC from the PSII core, both resulting in a reduction in the rate constant for energy trapping at PSII (Fracheboud 2001). The reduction of \( F_v/F_m \) due to photodamage is persistent and can remain for many hours after a return to darkness, especially if nutrient limitation is disabling repair of the photosynthetic proteins (Bergmann et al. 2002).

**TEMPERATURE**

The temperature of marine waters is relative stable compared to nutrients and light, hence relatively few studies have investigated the response of fluorescence parameters in marine microalgae to temperature changes. However, studies by Geel et
al. (1997), Longhi et al. (2003) and Morris and Komkamp (2003), all using marine diatoms, found quite a uniform influence of temperature on $F_v/F_m$. In general, optimum $F_v/F_m$ was experienced at ~5°C, with only a very gradual decline in values as temperatures increased to between 25°C and 30°C. A more substantial decline was observed in Antarctic species (Longhi et al. 2003). At temperatures higher than 30°C $F_v/F_m$ begins to decrease rapidly and is close to zero by 40°C. This response is likely due to the thermolabile properties of the various PSII components, which should be uniform for most algae from similar climatic regions (Morris and Kromkamp 2003).

**Effect of Nutrients**

The proteins of the light harvesting complexes, enzymes involved in the dark reactions and alternative electron pathways, as well as the transducers (eg. ATP) all contain substantial amounts of nitrogen (N), phosphorus (P) and iron (Fe). A limitation in any one of these nutrients consequently has a large impact on photochemical efficiency and chl-$a$ fluorescence (Beardall et al. 2001). This means that chl-$a$ fluorescence techniques can be used to assess nutrient status. One technique that may allow the identification of the specific limiting nutrient is the analysis of nutrient induced fluorescence transients (NIFTs) (Young and Beardall 2003b), however $F_v/F_m$ remains the most popular indicator of nutrient limitation (Cleveland and Perry 1987; Kolber et al. 1990; Greene et al. 1994; Falkowski and Kolber 1995; Wood and Oliver 1995; Bergmann et al. 2002). Nitrogen- and iron-limitation induce the greatest decline in $F_v/F_m$, while the impact of phosphorus-limitation is more moderate (Geider et al. 1993b; Kolber and Falkowski 1993). Silicate-limited diatoms display a similar reduction in photochemical efficiency compared to values obtained in nutrient replete conditions (Lippemeier et al. 1999). The primary cause for these observed changes in $F_v/F_m$ was an increase in $F_o$ as RCIIIs became non-functional (Lippemeier et al. 2001). Each of these authors report a rapid recovery of $F_v/F_m$ after re-supply of the limiting
nutrient, suggesting that chl-\(a\) fluorescence can be used as a useful bioassay tool for monitoring nutrient limitation. Olaizola et al. (1996) found support for the use of \(F_v/F_m\) as an indicator of nutrient status when \(F_v/F_m\) was observed to increase with natural injections of nutrient rich water into the photic zone.

Nutrient limitation also leads to a reduction in \(F_q'/F_m '\). Both \(F'\) and \(F_m '\) decrease with the onset of nutrient limitation, however the reduction of \(F_m '\) is disproportionately higher, suggesting that non-photochemical quenching is enhanced (Greene et al. 1992; Lippemeier et al. 2001). These fluorescence changes can be explained by activation of energy dissipation and regulation pathways (e.g. state transitions, cyclic electron transport) in order to limit photodamage while nutrients are in deficit.

There are occasions when a direct relationship between \(F_v/F_m\) and nutrient concentration cannot be found (Olaizola et al. 1996). Such instances may be achieved when samples are in a state of balanced, or partially balanced, growth (Parkhill et al. 2001). During balanced growth the production of compounds that utilise the limiting nutrient are limited and growth rate is matched to the available nutrient supply (Cullen et al. 1992). Thus, marine phytoplankton achieving balanced growth in nutrient limited conditions may have fewer total RCIIIs, however, more will be functional (higher \(F_v/F_m\)) than would be the case during unbalanced growth. For this reason, as well as due to the confounding effects of the other variables discussed here, a degree of caution should be taken when applying \(F_v/F_m\) as an indicator of nutrient limitation.

**Species composition**

The photosynthetic apparatus of marine phytoplankton is not of uniform design and function. While the chl-\(a/b\) containing chlorophytes are like higher plants in that they have grana-forming thylakoids and spatial separation of photosynthetic complexes, other taxa have different photosynthetic pigments and arrangements of their thylakoid
membranes (Büchel and Wilhelm 1993). Light harvesting complexes also show variations in how antennae interact with the reaction centres (Owens 1986; Gibbs and Biggins 1989). As explained earlier, differences in the location and composition of enzymes involved in alternative electron transport pathways also exist between taxonomic groups. Each of these factors has an influence on fluorescence induction kinetics and the derived fluorescence parameters (Büchel and Wilhelm 1993). For example, chl-c or phycobilin containing algae often exhibit high $F_o$ in comparison to $F_m$, resulting in lower $F_v/F_m$ values (Müller 1999). Using a Phyto-PAM (Walz), Schreiber (1998) found substantial taxonomic differences in fluorescence parameters recorded during light curve measurements on phytoplankton samples taken from Sydney Harbour.

Given that changes in the composition of phytoplankton communities are likely to co-occur with changes in physical variables any interpretation of fluorescence data would need to deconvolute the influences of multiple variables.

### 1.3.2 Quenching

Fluorescence, or radiative decay, is one of three competitive pathways for the de-activation of chl-excited states in the photosynthetic reaction centres and their antennae. Thus, the other two pathways, photochemistry and non-radiative decay (heat dissipation), act to quench the fluorescence signal. These processes are called photochemical- and non-photochemical quenching respectively.

To quantify these quenching pathways various quenching coefficients have been defined. As mentioned previously, the saturation pulse method can be used to measure each of the quenching components.

Photochemical quenching, $q_P$, estimates the percentage of RCIIIs that are open (Magnusson 1997) or the capacity for photochemistry to compete for trapped quantum
energy (Ting and Owens 1993). When all reaction centres are open \( q_P = 1 \) and when all centres are closed \( q_P \) is zero (Schreiber et al. 1986). The RCII is considered ‘open’ when \( Q_A \) is oxidised and capable of accepting an electron from RCII via pheophytin. It is important to note that energy transfer between RCIIIs, or “connectivity”, modifies the linear relationship between \( q_P \) and the fraction of RCIIIs that are open (Sugget et al. 2003; Schreiber 2004). Thus, \( q_P \) more accurately represents the redox state of \( Q_A \). The quinones \( Q_A \) and \( Q_B \) where labelled “Q” because they act to quench fluorescence, \( Q_B \) being a secondary quencher as it acts to re-oxidise \( Q_A \), thereby returning it to its quenching state. Likewise, the redox status of PQ may influence \( q_P \). For example, \( q_P \) can be increased by increasing ambient dissolved inorganic carbon (DIC) concentrations as this acts to favour the Rubisco carboxylase reaction and increase the rate of linear electron flow, resulting in partial reoxidation of the PQ pool (Carr and Björk 2003). Given that these primary components influencing \( q_P \) are highly conserved across taxa, the mechanism of \( q_P \) are likely to be similar (Ting and Owens 1993).

Based on the work of Bilger and Schreiber (1986) which found that \( F_o \) could be quenched to \( F_o' \), \( q_P \) was defined by Van Kooten and Snel (1990) with standardised nomenclature (see Table 1.1). \( F_o' \) may be hard to measure and on occasion \( F_o \) has been used for the calculation of \( q_P \) instead (eg. Weis and Berry (1987) or Ralph and Gademann (2005)). This risks overestimation of \( q_P \) and, as a consequence, an alternative method for deriving \( F_o' \) has been described (Equation 18) (Oxborough and Baker 1997).

\[
F_o'' = \frac{F_o'}{F_v/F_m + F_o'/F_m} 
\]  

(18)

The total non-photochemical quenching coefficient (\( q_N \)) is a measure of the fraction of maximum dark-adapted variable fluorescence (\( F_m - F_o \)) that is quenched in the light (Equation 19). This coefficient has two disadvantages in that; (1) it involves the estimation of \( F_o' \), and (2) there is evidence that it may be influenced directly by the
rate constant of photochemistry (Krause and Jahns 2004). Another measure of total non-photochemical quenching (NPQ) can be calculated using the Stern-Volmer equation (see Table 1.1). In this case NPQ represents the relative increase in the sum of the rate constants of the non-photochemical deactivation processes (fluorescence emission, heat dissipation and spillover of excitation energy from PSII to PSI) relative to the dark-adapted state (assuming no non-photochemical reduction of the PQ pool in the dark) (Krause and Jahns 2004). The NPQ parameter is commonly considered more robust and is often used in preference to $q_N$ (Ralph and Gademann 2005).

\[
q_N = 1 - \frac{(F_m' - F_o')}{(F_m - F_o)}
\]  

(19)

The primary site for the development of non-photochemical quenching is thought to be the light harvesting antennae and is largely independent of QA redox state (Ting and Owens 1993; Oxborough and Baker 1997). Given the diversity in composition of light harvesting complexes one may expect the mechanisms and response of non-photochemical quenching to vary between taxa (Ting and Owens 1993).

Non-photochemical quenching of chl fluorescence has three major components, each of these components can be distinguished by careful analysis of dark relaxation kinetics, as described by Horton and Hague (1988). The dominant component of non-photochemical quenching is energy dependent quenching ($q_E$) (Ting and Owens 1992). However, the relative contribution of each NPQ component is dependent upon the light history of the sample and conditions under which the measurements were taken (Ting and Owens 1992). Energy dependent quenching is also the quickest NPQ component to relax upon return to darkness and reports on $q_E$ relaxation time vary from 30 – 60 s (Ralph and Gademann 2005) to 2 – 3 min (White and Critchley 1999) or a $t_{\frac{1}{2}}$ of <1 min (Masojidek et al. 1999).
Energy dependent non-photochemical quenching is linked to the formation of a pH gradient (ΔpH) across the thylakoid membrane as the photosynthetic ETC transports protons from stroma to the lumen (Krause and Weis 1988). As well as providing the proton motive force for ATP formation, this ΔpH results in conformational changes in the reaction centres and their antennae and triggers the de-epoxidation of xanthophyll cycle pigments, each of which acts to increase non-radiative energy dissipation (Schreiber et al. 1995a). Govindjee (2002) explains that debate remains over protonation of the PSII antennae complexes and whether this (a) promotes a closer association of the excited chlorophyll molecule with the de-epoxidised xanthophyll pigment (zeaxanthin in higher plants and chlorophytes, diatoxanthin in chromophytes) which then accepts the quantum energy and dissipates it as heat, or (b) result in a conformational change in the antennae complexes that promotes deactivation by heat loss. It is possible that both mechanisms occur. Despite this continued debate it is generally recognised that epoxidation status of xanthophyll cycle pigments has a good correlation with NPQ (Ralph and Gademann 2005).

Energy dependent quenching can account for up to 90% of the decay in Fv on exposure to light (Krause and Weis 1991). This down-regulation of PSII photochemistry may act as a photoprotective mechanism by reducing the potential for 3chl in the RCII and the formation of reactive oxygen species (Krause and Jahns 2004) and there is overwhelming evidence indicating that photoinhibition is diminished by the development of a large qE (Krause and Weis 1991). An early adaptive response by plants and algae in unfavourable conditions is to increase qE (Schreiber et al. 1995a) and plants adapted to high light have been shown to have more active qE and less PSII closure than low light adapted plants (Ralph and Gademann 2005).

The next NPQ component has a relaxation half-time of ~5 – 10 min and is known as state transitional quenching, or qT (Masojidek et al. 1999). As state transitions
(state I → state II) involve the movement of LHCIIb from PSII to PSI and comparatively little fluorescence escapes from PSI, fluorescence is consequently quenched (Schreiber et al. 1995a). In this scenario $F_v$ and $F_o$ are quenched by the same proportion (Krause and Weis 1991). While $q_T$ may be of particular importance in low light environments, as light increases acidification of the lumen appears to inhibit LHCIIb phosphorylation and the role of $q_T$ may become negligible (Krause and Jahns 2004). However, as discussed in Section 1.2.4, there remain many questions as to the mechanisms and function of state transitions, especially in the Chromista, hence the role and activity of $q_T$ in many taxa remains unclear.

The slowest NPQ component to relax, taking >10 min to hours, is photoinhibitory quenching ($q_I$) which is related to photoinhibitory damage of PSII. There is general agreement that the primary source of $q_I$ is damage of the PSII core protein D1 as a result of donor- or acceptor-side photoinhibition (Hill et al. 2005). However, since $q_I$ formation may precede inactivation of D1, other mechanisms such as the presence of persistent levels of de-epoxidised xanthophyll cycle pigments in the dark (Krause and Jahns 2004) must be considered. Photoinhibitory quenching relaxes only as PSII repair mechanisms take place, such as the synthesis and replacement of damaged D1 proteins (Masojídek et al. 1999). Due to the longer recovery time $q_I$ can be indicated by a reduction in $F_v/F_m$ after exposure to high light (Krause and Weis 1991). As a rule, this reduction in $F_v/F_m$ is the result of diminished $F_v$, however the contribution of the $F_o$ component to this decrease may vary (Krause and Weis 1984; Krause and Weis 1991).

While studying state transitions in cyanobacteria Mullineaux and Allen (1986) reported quenching of fluorescence in the dark and linked this to the influence of the respiratory ETC on the photosynthetic ETC and state transitions. A little over a decade ago Ting and Owens (1993) reported the unexpected observation that DCMU revealed
quenching of fluorescence in dark adapted cultures of the diatom *Phaeodactylum tricornutum*. Since then other researchers have encountered the same phenomenon in *P. tricornutum* (Geel 1997; Jakob et al. 1999, 2001), *Thalassiosira weissflogii* (Dijkman and Kroon 2002), benthic diatom mats (McMinn et al. 2004; Serôdio et al. 2005) and the brown alga *Fucus serratus* (Mouget and Tremblin 2002). In these circumstances quenching of $F_m$ (and to a much smaller extent $F_o$) relaxes in low light (generally $<100 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$, although Serodiô et al (2005) recorded minimal quenching at 178.5 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$) and recorded $F_m'$ values are higher than $F_m$ (Mouget and Tremblin 2002; Kromkamp and Forster 2003). This may also yield $F_q'/F_m'$ measurements higher than $F_v/F_m$. When $F_m' > F_m$ Serodiô et al (2005) termed the maximum $F_m'$ value $F_m''$. The quenching is known to be induced by the build up of a $\Delta\text{pH}$ across the thylakoid membrane and therefore in the form of $q_E$. The most commonly proposed mechanism for this dark induced quenching is chlororespiration (Ting and Owens 1993; Schreiber et al. 1995b; Schreiber et al. 1997; Kromkamp and Forster 2003; McMinn et al. 2004) as it has, until recently, been linked with proton transport (Beardall et al. 2003). This theory has been questioned since dark-induced quenching can occur in anaerobic conditions, which inhibits chlororespiration (Ting and Owens 1993), and proposals that chlororespiration does not transfer protons across the thylakoid (Bennoun 2002; Peltier and Cournac 2002; Beardall et al. 2003). Another proposed mechanism for $F_m' > F_m$ has been light-induced reorganisation of the antennae (Mouget and Tremblin 2002) and in cyanobacteria it is well known that quenching in the dark is a function of transition to state 2 brought about by a reduction of the PQ pool by the respiratory ETC (Mullineaux and Allen 1986). It is clear, however, that the capacity for NPQ to develop in the dark requires further investigation across a range of taxa. This capacity may be of particular importance for phytoplankton in mixed turbid environments where light levels may
rapidly oscillate from darkness to full sunlight and an already-active NPQ could prevent photodamage.

1.4 PRIMARY PRODUCTION MODELS

A number of models of the forcing and feedbacks between phytoplankton dynamics and ocean circulation have been developed and are critical to our knowledge and understanding of how phytoplankton photosynthesis affects carbon cycles and is affected by ocean dynamics (Sakshaug et al. 1997). These models utilise satellite data of ocean chlorophyll, require numerous *in situ* observations to obtain ‘typical’ vertical profiles for different areas of the world ocean (Falkowski 1994) and rely on a number of assumptions. We now know that earlier models were inaccurate due to numerous simplifications made and the incorrect assumption that the maximum quantum yield of photosynthesis ($\Phi_{\text{max}}$) is constant (Bates and Platt 1984; Geider et al. 1993a; Kolber and Falkowski 1993; Olaizola et al. 1996; Boyd et al. 1997; Wozniak et al. 2002). Studies have indicated that this assumption of invariant $\Phi_{\text{max}}$ has been the most significant source of error in bio-optical models of primary production (Kroon et al. 1993).

A knowledge of $\Phi_{\text{max}}$ variability is therefore important for the continued refinement of these mathematical models. Recent technological advances (eg. in the area of chlorophyll fluorescence) have allowed a greater diversity of measures of photosynthetic capacity and the near-future potential for satellite-borne laser technology to provide real-time measures of $\Phi_{\text{max}}$ on an ocean basin scale (Krikke et al. 2004). This and other, currently applied, remote sensing technology will provide useful data with which to improve both the accuracy of prognostic models of primary production and our understanding of global biogeochemical cycles. Significant challenges still remain, however, one of these being the inability of satellite images of ocean chlorophyll to provide information about the vertical distribution of phytoplankton. Current satellite
observations of the ocean are restricted to the upper 30-40 m of the water column, which means that on most occasions the chlorophyll maximum is missed (Falkowski 1994).

1.4.1 Photosynthesis-Irradiance Curves

The capture of photons by the photosynthetic apparatus is the initial process and the driving force of photosynthesis. It follows that most of the variability in photosynthesis appears to come about through variations in light (Platt et al., 1977). This functional dependence is known as the Photosynthesis-Irradiance (P-E) curve and there are a number of semi-empirical models which describe it (Table 1.2). These models have the general form:

\[ P = f(E, a_1, a_2) \]  \hspace{1cm} (20)

where \( E \) = irradiance, \( a_1 \) and \( a_2 \) = constraints determining the shape of the curve, \( f \) = a saturating function (Marra et al. 1985).

The P-E curve plays a central role in both physiological investigations and predictive modelling for primary production and is fundamental for those studying (photoautotroph) ecology and the meaning of so-called adaptive responses to environmental conditions (Platt and Jassby 1976; Yentsch 1980; Marra and Heinemann 1982; Lewis and Smith 1983; Boyd et al. 1997; Gilbert et al. 2000b). The most frequently used empirical equation of the P-E curve is from Jassby & Platt (1976) (Jumars 1993):

\[ P = P_{\text{max}} \tanh(\alpha E / P_{\text{max}}) - R \]  \hspace{1cm} (21)

Where \( R \) is the intercept at zero irradiance and is commonly considered to be a measure of dark respiration (mg C [mg chl-a]^{-1}.h^{-1}).
Table 1.2: A list of the more commonly applied photosynthesis-irradiance curve models

<table>
<thead>
<tr>
<th>Equation / Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P = P_m \left[ \frac{e^{\alpha E}}{[1-(P_m P_2/a)]} - 1 \right]$</td>
<td>Chalker (1980)</td>
</tr>
<tr>
<td>$P = P_m \left( 1 - e^{-\alpha E / P_m} \right)$</td>
<td>Webb at al. (1974)</td>
</tr>
<tr>
<td>$P = P_m \tanh(\alpha E / P_m)$</td>
<td>Jassby and Platt (1976)</td>
</tr>
<tr>
<td>$P = P_m \tanh(E / E_k)$</td>
<td>Greene et al. (1991)</td>
</tr>
<tr>
<td>$P = P_m \left( \frac{\alpha E}{P_m + \alpha E} \right)$</td>
<td>e.g. Baly (1935)</td>
</tr>
<tr>
<td>$P = P_m \left( 1 - e^{-(\alpha E / P_m)} \right) \cdot e^{(-\beta E / P_m)}$</td>
<td>Platt et al. (1980)</td>
</tr>
<tr>
<td>$P = P_m \alpha E \sqrt{[P_m]^2 + (\alpha E)^2}^{1/2}$</td>
<td>Smith (1936); Talling (1957)</td>
</tr>
<tr>
<td>$P = E \left[ (\alpha E^2 + bE + c) \right]$</td>
<td>Eilers &amp; Peeters (1988)</td>
</tr>
</tbody>
</table>

Care should be taken when choosing a model to describe the P-E relationships or comparing the results from different studies with different models (Frenette et al. 1993). Most models fit P-E data adequately, but none are mathematically perfect (being based on data shape rather than functional dynamics) and different models may yield different parameter values when applied to the same data-set (Sakshaug et al. 1997). Many researchers are interested in the initial slope ($\alpha$), but few seem to realise that values of $\alpha$ are not interchangeable between the mathematical models (Frenette et al. 1993). Data collected by Gilbert et al. (2000) indicated that values of $E_k$, $\alpha$, and $P_{\text{max}}$ can be used comparatively only if the data set is fitted carefully using the same model. Differences in the application of a model, such as constraining or not constraining the P-E curve to the origin (R=0), has a major impact on the estimation of $P_{\text{max}}$ and $\alpha$ (Frenette et al. 1993).

P-E curves are constructed by measuring the rate of photosynthesis at a number of irradiances over a wide irradiance range. Each curve typically has three major regions:
(i) At the lowest irradiances, photosynthetic rates are virtually linearly proportional to irradiance. This initial slope of the curve ($\alpha$; Figure 1.9) is generally assumed to be a function of the light reactions of photosynthesis (Chalker 1980).

(ii) As irradiance increases photosynthetic rates become increasingly non-linear, in the manner of a Poisson function, and rise to a saturation level and continuing increases in light intensity yield no further increase in photosynthetic rate. This horizontal asymptote is defined as the photosynthetic capacity, photosynthetic maximum, or assimilation number (P$_{\text{max}}$; Figure 1.9)

(iii) If irradiance intensity increases beyond the point where P$_{\text{max}}$ is achieved, a reduction in the photosynthetic rate may take place (down-regulation or photoinhibition). This is dependent on both irradiance and duration of exposure (Sakshaug et al. 1997).

Figure 1.9: A stylised representation of a photosynthesis versus irradiance curve (P-E curve). The three most commonly derived parameters are shown (see text for details).
The irradiance at which the initial slope of the curve ($\alpha$) intercepts the horizontal asymptote is defined as $E_k$ (Figure 1.9; also called $I_k$) and is commonly used as a measure of the adaptation of the plant to its light environment (Chalker 1980). $E_k$ can be calculated by applying Equation 22.

$$E_k = \frac{P_{\text{max}}}{\alpha}$$  \hspace{1cm} (22)

Biophysically, $E_k$, also known as the saturation irradiance, is analogous to the irradiance at which the rate of excitons arriving at PSII begins to exceed the rate of electron transport through PSII, and as such can also be expressed using the PSII turnover rate ($\tau$):

$$E_k = (\tau \times \sigma_{\text{PSII}})^{-1}$$  \hspace{1cm} (23)

When absorbed rather than incident light is measured $\alpha$ provides a useful estimate of $\Phi_{\text{max}}$. For this reason good estimates of $\alpha$ are usually required for modelling primary production.

The P–E relationship, and hence the photosynthetic parameters, depends in part on whether data is normalised to cell abundance, total cell volume, carbon biomass, or chl-$\alpha$ concentration (MacIntyre et al., 2002). When normalised to cell abundance or volume, $\alpha$ varies with the summed light-harvesting capacity of the photosynthetic units. Because pigment content is a measure of light-harvesting capacity, a relatively constant value for the light-limited slope is anticipated upon normalisation to chl concentration ($\alpha^{\text{chl}}$), particularly when chl is the primary light-harvesting pigment (Behrenfeld et al. 2004).

The shape of the P–E curve also depends on the method being used to measure photosynthetic rate. For example, Gilbert et al. (2000) found that $E_k$ values derived from variable fluorescence, $O_2$-evolution and $^{14}$C-fixation measurements can differ significantly from each other. Due to their relative positions in the photosynthetic
reaction pathway one could expect that the quantum yield of electron transport through PSII ($\Phi_{\text{PSII}}$) would be greater than the quantum yield of O$_2$-evolution ($\Phi_{\text{O}_2}$), which would in turn be greater than the quantum yield of carbon fixation ($\Phi_{\text{CO}_2}$). This would result in the respective change in $\alpha$ depending on the method being used.

Despite large efforts devoted to measuring P-E parameters in marine waters, the environmental determinants of $P_{\text{max}}$ and $\alpha$ have proven difficult to identify (Greene et al., 1991). The $\alpha$ parameter is often used to evaluate short term responses to changes in environmental variables such as irradiance (prehistory) (Falkowski et al. 1981; Harrison and Platt 1986; Sakshaug et al. 1997), temperature (Harrison and Platt 1986; Geel et al. 1997) or nutrient levels (Cleveland and Perry 1987; Underwood 2002). Temperature often appears to have only minimal impact on values of $\alpha$, while it markedly affects $P_{\text{max}}$ (Platt and Jassby 1976; Harrison and Platt 1986; Falkowski 1994). The initial slope can be equated to the product of a chl-$a$ specific absorption coefficient and the maximum realised photon yield of photosynthesis ($\Phi_{\text{max}}$).

$$\alpha^{\text{chl}} = \alpha^* \cdot \Phi_{\text{max}}$$ (24)

Variation in $\alpha^{\text{chl}}$ is often due to variations in $\alpha^*$, however, adverse growth conditions can lead to a decline in $\Phi_{\text{max}}$ and hence a decline in $\alpha^{\text{chl}}$ (Geider and Osborne 1991).

Under most conditions $P_{\text{max}}$ appears to be limited by processes “downstream” of PSII, as photoinhibitory reduction of the number of functional PSII reaction centres appears to yield no concomitant reduction in $P_{\text{max}}$ (Behrenfeld et al. 1998). Downstream processes influence P-E variables either by channelling reductant into non-carbon sinks or by transferring electrons back to O$_2$ (Behrenfeld et al. 2004). P-E data collected by Platt et al. (1992) using the $^{14}$C-method indicated an important role of nutrients (NO$_3^-$) in variations of $\alpha$ and $P_{\text{max}}$ (Behrenfeld et al., 2004). Diversion of electrons/reductant
(NADPH) from C-fixation to nitrogen reduction (Equation 12) can cause changes in carbon-based, but not oxygen-based, determinations of $\alpha$ and $P_{\text{max}}$. The Mehler reaction, chlororespiration and photorespiration are all variable downstream processes that may influence photosynthetic parameters.

Light prehistory (photoacclimation) has a strong influence on the chl-normalised light-saturated photosynthetic rate ($P_{\text{chl}}^{\text{max}}$) (Behrenfeld et al. 2004), with most studies finding higher $P_{\text{chl}}^{\text{max}}$ with increasing light intensities (Grobbelaar et al. 1992). Despite catering for this by applying the average irradiance for the 3 days before sampling Platt and Jassby (1976) found it hard to accurately predict $P_{\text{chl}}^{\text{max}}$. Marra and Heinemann (1982) found inadequacies, but concluded that P-E curves offer the best possibility for predictive modelling of primary production. In a later study, Marra et al. (1985) found that daily predictions were good only when the maximum irradiance achieved during the day was low.

Theoretically the P-E response should refer to instantaneous light and provide information on the photosynthetic state of the sample at the moment of collection. When investigating phytoplankton communities, however, some acclimation will take place unless the incubation time is only a few minutes (Marra and Heinemann 1982; Lewis and Smith 1983; Sakshaug et al. 1997). Consequently the ecological interpretation of these data is hampered. It was such ‘incubation effects’ that prompted Lewis and Smith (1983) to develop a short incubation technique for the $^{14}$C method. Similar incubation effects need to be considered when measuring O$_2$-evolution. Chl-$a$ variable fluorescence has shown promising signs that it can be applied as a non-intrusive tool to estimate photosynthesis (Öquist et al. 1982; Delieu and Walker 1983; Walker et al. 1983; Bates and Platt 1984; Falkowski and Kiefer 1985; Falkowski et al. 1986a). A major challenge has been the development of equipment with the sensitivity to measure fluorescence in oceanic phytoplankton samples (Geel 1997). These measurements are now possible
(Schreiber et al. 1993, 1995b) and chl-a fluorescence measurement systems provide the ability to perform rapid assessments of the P-E relationship that largely avoid the complication of sample acclimation occurring within the duration of analysis. Fluorescence techniques allow the calculation of the electron transport rate (ETR) through photosystem II (PSII) and the curves produced are termed ETR-E curves and ETR$_{\text{max}}$ is the equivalent of P$_{\text{max}}$ (see next section for more information).

1.4.2 Fluorescence-based ETR-E curves

Given that the saturation pulse method allows the estimation of the quantum yield of photochemistry at PSII ($\Phi_{\text{PSII}}$) one should be able to determine the Electron Transport Rate (ETR) through PSII given a knowledge of $Q_{\text{phar}}$ and the proportion $Q_{\text{phar}}$ directed towards PSII. Thus, in generic terms:

$$ETR = \Phi_{\text{PSII}} \times Q_{\text{phar}} \times f_{\text{II}}$$  \hspace{1cm} (25)

where $f_{\text{II}}$ is the fraction of absorbed radiation directed to PSII and ETR provides a measure of gross photosynthesis ($\mu$mol e$^{-}$ [mg chl-a]$^{-1}$ s$^{-1}$).

While accurate estimation of all three parameters may be achieved in an ideal laboratory-based situation, $Q_{\text{phar}}$ and $f_{\text{II}}$ determination is often hampered due to equipment or operational restrictions (especially during field-based studies) and best available alternatives are applied. The most common outcomes are the use of incident PAR ($E_{\text{PAR}}$) and an absorption factor (A; often chl-specific absorption coefficient ($a_{\text{chl}}$)) (Sakshaug et al. 1997)) to estimate $Q_{\text{phar}}$ and/or $f_{\text{II}}$ is replaced with the constant 0.5 on the assumption that radiant energy is split equally between PSII and PSI (Equation 26: see application in Gilbert et al. (2000a; 2000b) and Wagner et al. (Wagner et al. 2006); Equation 27: see application in Underwood (2002) and Morris and Kromkamp (2003)). Although the assumption that $f_{\text{II}}$ equals 0.5 has been supported by recent data on a range of taxa (Suggett et al. 2004), significant variation in $f_{\text{II}}$ has been reported previously.
(Kroon et al. 1993) and for some taxa (eg. cyanobacteria) values of 0.25 have been recorded (Suggett et al. 2004). There is, therefore, the potential for significant errors when \( f_\text{II} \) is replaced with an assumed value of 0.5 in the calculation of the absolute ETR.

\[
ETR = \left( \frac{F'_q}{F'_m} \right) \times Q_{\text{phar}} \times 0.5 \tag{26}
\]

\[
ETR = \left( \frac{F'_q}{F'_m} \right) \times E_{\text{PAR}} \times 0.5 \times A \tag{27}
\]

Where units of measure for Equation 26 and Equation 27 are \( \mu \text{mol electrons m}^{-2} \text{s}^{-1} \).

In situations where relative changes rather than absolute values will suffice further simplification of Equation 27 is possible:

\[
r\text{ETR} = \left( \frac{F'_q}{F'_m} \right) \times E_{\text{PAR}} \tag{28}
\]

where \( r\text{ETR} \) signifies relative electron transport rate (arbitrary units). Equation 28 frequently includes the 0.5 constant (as per Equation 27) and in some cases may also include an assumed absorptance factor (A) (eg. Durako et al. 2003), but in all cases it is assumed that absorptance remains constant over the duration of measurement and across all treatments. The \( r\text{ETR} \) parameter is frequently used as a measure of photosynthetic rate (Ensminger et al. 2000, 2001; Ralph et al. 2002a; Morris and Kromkamp 2003) given that relative changes are often all that is required.

The determinations of absolute ETR indicated above (i.e. Equation 26 or Equation 27) involve measurements of optically derived absorption coefficients (eg. \( a^{\text{chl}} \), refer to Table 1.3), however it is possible to utilise parameters derived from biophysical techniques to achieve the same end. A recent assessment of optical and biophysical techniques for the determination of light absorption by Suggett et al. (2004) provides a thorough comparative background to the application of these techniques. Although biophysical techniques are most commonly associated with FRR measurements of chlorophyll fluorescence, recent advances in PAM fluorometers (Trtílek et al. 1997) have added further capabilities in the assessment of parameters such
as the functional absorption cross-section of PSII ($\sigma_{\text{PSII}}$; units of Å$^2$ quanta$^{-1}$) (Nedbal et al. 1999). The product of $\sigma_{\text{PSII}}$ and the number of RCIIs per mg chl-\(a\) can then be used to estimate chl-\(a\) specific light absorption coefficient of PSII (Equation 10), allowing a biophysically-derived alternative to Equation 28 which may provide more accurate estimation of absolute ETR as it eliminates the use of $f_{\text{II}}$ (or the assumed constant 0.5):

$$ETR = \left(\frac{F'_{q}}{F'_{m}}\right) \times E_{\text{PAR}} \times \sigma_{\text{PSII}} \times \eta_{\text{PSII}} \times 0.00675$$ (29)

The resulting ETR has the units of µmol electrons (mg chl-\(a\))$^{-1}$ s$^{-1}$. The density of PSII relative to chl-\(a\) ($\eta_{\text{PSII}}$; units of mol PSII [mol chl-\(a\)]$^{-1}$) has previously been calculated from photosynthetic unit size for oxygen production (PSU$\text{O}_2$, also known as the Emerson-Arnold unit, see Emerson and Arnold 1932), which is the ratio of chlorophyll molecules to oxygen molecules evolved, using oxygen flash yields (Ley and Mauzerall 1982; Laws et al. 2002):

$$\eta_{\text{PSU}} = \left(PSU_{\text{O}_2}\right)^{-1}$$ (30)

$$\eta_{\text{PSII}} = \eta_{\text{PSU}} \times Q_{\text{R}_{\text{O}_2}}$$ (31)

where $Q_{\text{R}_{\text{O}_2}}$ is the quantum requirement for oxygen evolution and is often assumed to be the minimum value of 4 (4 mol electrons [mol O$_2$]$^{-1}$) although some have suggested that 10 is the more appropriate value (Ley and Mauzerall 1982). The density of PSII is often assumed (0.002 mol PSII [mol chl-\(a\)]$^{-1}$), however, since oxygen flash yield measurements are not always possible to perform.
Table 1.3: Definitions of biophysical and absorption parameters.

<table>
<thead>
<tr>
<th>Sign</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \sigma_{\text{PSII}} )</td>
<td>Functional absorption cross-section of PSII</td>
<td>( \text{Å}^2 ) (quanta(^{-1} ))</td>
</tr>
<tr>
<td>( \sigma_{\text{PSU}} )</td>
<td>Functional absorption cross-section of the photosynthetic unit</td>
<td>( \text{m}^2 ) (mol O(_2))(^{-1} ))</td>
</tr>
<tr>
<td>( \eta_{\text{PSU}} )</td>
<td>PSU density</td>
<td>mol O(_2) (mol chl a(^{-1} ))</td>
</tr>
<tr>
<td>( \eta_{\text{PSII}} )</td>
<td>Density of RCII</td>
<td>mol RCII (mol chl a(^{-1} ))</td>
</tr>
<tr>
<td>( a_{\text{chl PSII}} )</td>
<td>Chl a specific absorption by PSII</td>
<td>( \text{m}^2 ) (mg chl a(^{-1} ))</td>
</tr>
<tr>
<td>( a_{\text{Chl}} )</td>
<td>Chl a specific absorption coefficient</td>
<td>( \text{m}^2 ) (mg chl a(^{-1} ))</td>
</tr>
</tbody>
</table>

ETR (or rETR) represents photosynthetic rate and hence curves analogous to P-E curves may be plotted. Due to the nature of chl-a fluorescence measurements an estimation of ETR at each irradiance can be achieved almost immediately and well before optimum or steady state is reached. Chl-a fluorescence measures can therefore be used to measure immediate adaptational/physiological state via a rapid sequence of measurements (Rapid Light Curve; RLC), or exposure length at each light level can be extended until steady state is reached (Light Curve; LC). When RLCs are applied, the period of exposure at each irradiance level (LC-Width) should be as short as possible (typically 10 s), while still allowing time for fluorescence to relax after the saturation pulse (Ralph and Gademann 2005). Changes in LC-Width, such as to measure a steady state LC rather than a RLC, will cause changes in maximum photosynthetic rate (ETR\(_{\text{max}}\)) as well as \( \alpha \) and NPQ (Ralph and Gademann 2005). One should also note that
the downturn of the RLC at supra-saturating irradiances is thought to represent active
down-regulation of PSII, rather than photoinhibition, as there is not enough time for
photodamage to accumulate (Ralph and Gademann 2005).

Investigation of photosynthesis as a function of irradiance (eg. P-E curve, RLC
or LC) requires consideration of the range of irradiances to be used. Enough points need
to be located in the light-limited portion of the curve to accurately define the $\alpha$-slope
while also allowing enough points for good extension into the light-saturated/supra-
saturated region. Insufficient points in the light-limited region can result in the
underestimation of $\alpha$, however $ETR_{\text{max}}$ does not seem to be affected as long as a
saturating irradiance is achieved (Ralph and Gademann 2005). Other changes in
measurement protocol, such as dark adaptation time, may also impact photosynthesis
parameters.

During experiments on the higher plant *Rubus fruticosus* Rascher et al. (2000)
found that RLCs showed no differences between $ETR_{\text{max}}$ with short (30 s) or long (30
min) dark adaptation periods, however the $ETR_{\text{max}}$ of steady-state measurements
(ambient) was significantly lower (Figure 1.10). There was no mention in the above
paper of the impact of the sudden decrease in $F_{\text{q}}'/F_{\text{m}}'$ (effective quantum yield) at low
PPFD observed in samples dark adapted for 30 min (Figure 1.10c) on the $\alpha$ of the
equivalent ETR versus $E_{\text{PAR}}$ (PPFD) relationship (Figure 1.10d). It follows that
decreased $F_{\text{q}}'/F_{\text{m}}'$ at low irradiances would have resulted in a lower $\alpha$-value and this can
readily been seen in Figure 1.10 and is shown by a higher $E_k$. Rascher et al. (2000)
suggested that this phenomenon was a result of dark-inactivation of photosynthesis,
with light activation during the course of measurements yielding an $ETR_{\text{max}}$ not
significantly different from samples experiencing only 30 s in the dark.
Similar data collected by the current author using a WATER-PAM fluorometer (Walz GmbH, Germany) on the phytoplankter *Pavlova lutheri* (Chrysophyta) found a sharper drop in $F_{q}^{d}/F_{m}^{d}$ at low $E_{\text{PAR}}$ in samples dark adapted for 30 min compared to those dark adapted for only 30 s (and exposed to an 8 s pulse of far-red light) (Figure 1.11). Even at high irradiance the $F_{q}^{d}/F_{m}^{d}$ was lower in samples dark adapted for 30 min than in those that were dark adapted for only 30 s and consequently $r\text{ETR}_{\text{max}}$ was affected by dark-adaptation time when studying *P. lutheri*. A possible explanation for this discrepancy in results could be the shorter LC-Width (20 s) used by the current author.
compared to the 30 s applied by Rascher et al. (2000). This is likely to have restricted the amount of induction occurring during the measuring process. It has been shown that RLCs always have an inductive effect on the photosynthetic apparatus, but that this is minimal when the sample is exposed to light before measurements commence (Ralph and Gademann 2005). Interestingly, a study by Longstaff et al. (2002) found that α was lower in light curves constructed from Fq'/Fm' measurements taken at ambient irradiance levels throughout the day than in RLCs measured on the same day. This contrasts against the observations of Rascher et al. (2000) (described above) and indicates that more investigation is required into the relationship between light curves produced from steady-state measures of Fq'/Fm' and RLCs. Any such investigation should attempt to take any potential diel affects into account.

![Figure 1.11](image)

**Figure 1.11:** Left panels (a,c): Fq'/Fm' measurements of Pavlova lutheri (a) after 30 min dark adaptation; (c), after 30 s dark adaptation and exposure to far-red light (8 s). Right panels (b,d): rETR-E curves produced from the same measurements.

Although not directly comparable with traditional P-E curves, light curves produced from ambient Fq'/Fm' measurements or through RLCs have been increasingly
applied in an attempt to measure the physiological response of phototrophic organisms to environmental changes. It had been shown that the addition of mineral nutrients to the water surrounding *U. lactuca* elicits a rapid increase in ETR (Longstaff et al. 2002). Such a response suggests a possible role for chl-$
$ fluorescence based photosynthesis versus irradiance curves as an indicator of nutrient status. Increases in $\alpha$ at low irradiance levels have been related to photoacclimation by increased chlorophyll antenna size per PSII reaction centre (Beer et al. 1998a), while decreases in $\alpha$ at high irradiance levels have been linked to the state of the xanthophyll cycle pigments (Masojídek et al. 1999).

### 1.5 Objectives

The objectives of this thesis were 3-fold. Of primary interest was further characterisation of the relationship between chl-$
$ fluorescence based estimates of photosynthesis and the more traditional measurements of O$_2$-evolution and C-fixation (Chapter 4). Particular attention was paid to the light exposure methodology during fluorescence measurements and how this may impact upon results (Chapter 2). While other researchers have employed ‘short’ and ‘long’ exposure intervals (eg. Kühl et al. 2001) it is believed that this work provides new information on fluorescence response to varying light exposure treatments. The use of both high light and low light adapted cultures allowed a secondary investigation into photoacclimation of marine phytoplankton using chl-$
$ fluorescence parameters (Chapter 5). While chl-$
$ fluorescence has recently become more popular for the study of photoacclimation (eg. Boyd et al. 1997; Fietz and Nicklisch 2002; Brunet et al. 2003; Fine et al. 2004; Küster et al. 2004; Serôdio et al. 2005), the data presented in this thesis provides unique information, particularly the quenching data, for some of the less studied taxa. Finally, a diurnal field study of shallow, well-mixed coastal waters was performed to look at diurnal patterns in photosynthesis and primary production and variations in the chl-$
$
fluorescence : O₂-evolution : C-fixation relationship (Chapter 6). While diel patterns in the photosynthetic parameters of coastal phytoplankton populations has long been recognised (MacCaull and Platt 1977) there is a paucity of data on such patterns in the coastal waters around Perth (Western Australia) and on few occasions has data been collected on such a fine temporal scale.

On the broad scale, this thesis aims to increase our understanding of chl-α fluorescence measurements and how they can applied to ecophysiological studies of the marine phytoplankton community. Chl-α fluorescence has already provided researchers with insights into many aspects of photosynthesis and physiological regulation in phototrophic organisms (Govindjee 1995). With further developments in instrumentation and analytical power, there is little doubt that chl-α fluorescence will only continue to increase in potential as a powerful research and monitoring tool.
CHAPTER 2
Method Optimisation

2.1 GENERAL

The three primary techniques utilized throughout this study were: [1] chl-α fluorescence (PAM fluorometer), [2] oxygen evolution (Clark-type oxygen electrode) and, [3] carbon fixation (14C-uptake). As with all research studies, confidence can be placed in any data obtained only if the methods applied have been previously tested and optimised. Whilst there are guidelines in the literature for each of the above techniques for specific applications, there were some components the author deemed it would be prudent to optimise for the studies in question. These are outlined below:

2.2 CHLOROPHYLL-Α FLUORESCENCE

2.2.1 Rapid Light Curve Light Width

INTRODUCTION

As a sample of photosynthetic tissue is exposed to a new irradiance there is a period of adjustment, as photosynthetic electron transport chain components and associated metabolic pathways respond to the new environment, before reaching a new steady state. This process can take some time and, due to the nature of Rapid Light Curves (RLCs), the sample is typically in non-steady state when the measurements are taken. Such measurements reflect the immediate light history of the sample and reflect the actual state of photosynthesis rather than an optimum state that may be achieved in steady state (Ralph and Gademann 2005). As a RLC is intended as a measure of the immediate photosynthetic state, the possibility for photoinduction to occur over the course of the measurement in response to the changing light environment should be minimised. For this reason the length of exposure to each actinic irradiance (described
as the “Light Width”) should be as short as possible while allowing for the proper relaxation of fluorescence after each successive saturation pulse (Ralph and Gademann 2005). While there has been some push to standardise the Light Width of RLC measurements to 10 s (Ralph and Gademann 2005), and this appears to be a relatively common setting (White and Critchley 1999), a range of Light Width settings have been used (Beer et al. 1998a: 30 s; Barranguet and Kromkamp 2000a: 120 s; Longhi et al. 2003: 60 s).

During preliminary measurements the fluorescence trace showed some photoinduction during the course of RLC measurements with 10 s Light Width (data not shown). A series of measurements were performed to determine if reducing the Light Width to 5 s was feasible. That is, does a 5 s Light Width allow sufficient time for full relaxation of the fluorescence signal from $F_\text{m}(F_\text{m}')$ to $F'$ for the new actinic irradiance?

**METHODS**

Sample material for these measurements was nutrient replete *Nannochloropsis oculata* (Eustigmatophyceae) grown in aerated semicontinuous culture at a growth irradiance of 550 µmol quanta m$^{-2}$ s$^{-1}$ on a 12:12 h day:night cycle (for full description of culture setup refer to Chapter 3). The light cycle started at 6 am each day and RLC measurements were performed at both 4 am and 6:45 am to ensure that measurements represented both relaxed and induced photokinetic states respectively.

Rapid light curves were performed using a Water-PAM fluorometer (Walz GmbH, Germany), consisting of a PAM-CONTROL unit and a WATER-ED (emitter/detector) unit. The WATER-ED was designed for ultrasensitive detection of chl-$\alpha$ fluorescence in dilute algal suspensions (eg. surface waters) by utilising a photomultiplier detector based on Hamamatsu’s Photosensor Module H-6779-01 with high red-sensitivity, collimating optics and a 710 nm long-pass filter optimized for low
background signal. Light-emitting diodes (LEDs) provide non-actinic measuring light (3 LEDs, spectral peak at 650nm), actinic light/saturation pulses (12 LEDs, spectral peak at 660nm) and far-red light (3 LEDs, spectral peak at 730nm). These LEDs are located in a ring that encircles the 15mm diameter quartz sample cuvette when in position within the ED-unit.

Rapid light curves were performed with either 5 s or 10 s exposure duration to each of eight incremental irradiances (measured with the US-SQS/WB sensor at the light distribution maxima ~3 mm above the base of the cuvette) after an initial quasi-dark measurement (~1.5 µmol quanta m\(^{-2}\) s\(^{-1}\)) to provide estimates of \(F_0\) and \(F_m\). Saturation pulses lasted 0.8 s. Maximum irradiances obtained during 4 am and 6:45 am measurements were 934 µmol quanta m\(^{-2}\) s\(^{-1}\) and 1860 µmol quanta m\(^{-2}\) s\(^{-1}\) respectively. The higher maximum irradiance was required at 6:45 am as there had been considerable induction of the photosynthetic apparatus in response to the beginning of the light cycle at 6:00 am.

Sample material was harvested from the photobioreactor and dark adapted for 10 min at 25°C in a foil-wrapped vial. Sample transfer to the ED-unit was performed in dim light and the darkening hood was then set in place. Far-red (FR) light was subsequently applied for 5 s to ensure full oxidation of QA. Once fluorescence had stabilised after applying FR light the RLC programme (within WINCONTROL software Ver. 2.08, Friedmann Schlosser, Walz GmbH) was initiated. Immediately prior to initiation of each RLC sequence, the chart function in the WINCONTROL software was activated. A fresh sample was used for each RLC measurement.

**Results**

Typical fluorescence signal traces, as produced by the chart function in WINCONTROL, are shown in Figure 2.1. The time axis for each of the Light Width
treatments has been plotted separately for an easier comparison. It is clearly evident that relaxation from the $F_m\ (F_m')$ peak to a new $F'$ value with each irradiance can occur within the limits of the shorter 5 s Light Width interval. Induction of photosynthesis, indicated by a decrease in $F'$, was more apparent at 6:45 am (Figure 2.1b). Comparison of the fluorescence traces shows that using a 5 s Light Width interval reduces the amount of photoinduction that occurs during the light curve measurement. This is most readily apparent during the first and second light levels.

**Figure 2.1:** Fluorescence traces from RLCs conducted at (a) 4 am and (b) 6:45 am. Light source (550 µmol quanta m$^{-2}$ s$^{-1}$) started at 6 am. Blue line indicates RLC with Light Width of 5 s while black line indicates 10 s Light Width measurements.

**DISCUSSION AND CONCLUSION**

RLCs are performed to assess the immediate photosynthetic state of the study organism. Therefore, by definition, the Light Width used should be as short as possible to avoid any induction of photosynthesis as a result of the measurement procedure. However, the Light Width must be long enough to allow for proper relaxation of fluorescence after each successive saturation pulse (Ralph and Gademann 2005).
While there is no standard Light Width for RLC measurements, 10 s appears to be the most commonly used. Observations during early measurements of this project suggested that significant photoinduction was occurring during the 10 s exposure to each irradiance. The data presented here indicate that, for dilute suspensions of nutrient-replete microalgae, a shorter 5 s Light Width may be more appropriate in order to obtain a measure of photosynthetic state less likely to have a measurement-induced bias.

2.2.2 Chlorophyll Concentration

**Introduction**

It is widely acknowledged that one factor that can influence chlorophyll fluorescence measurements is sample chlorophyll concentration. Ting and Owens (1992) indicated that increasing chlorophyll concentration yielded a disproportionate increase in $F_o$ compared to $F_m$, with a resultant decrease in $F_v/F_m$. The sources of this problem are light reabsorption and scattering phenomena that become significant with increasing chlorophyll concentration (Büchel and Wilhelm 1993). Before the advent of ultrasensitive photomultipliers it was common for high chlorophyll concentrations to be used (>10 µg chl a mL$^{-1}$) in order to increase signal to noise ratio (Ting and Owens 1992). Studies to quantify the impact of changing chlorophyll concentration on $F_v/F_m$ values have produced results similar in trend to those presented in Figure 2.2, however, the chlorophyll concentration at which $F_v/F_m$ reaches its peak has been variable (likely related to the different geometry of the equipment used). Ting and Owens (1992) found the $F_v/F_m$ maxima at 10 µg chl a mL$^{-1}$, while the data presented by Büchel and Wilhelm (1993) (Figure 2.2) showed that $F_v/F_m$ peaked at ~30 µg chl a mL$^{-1}$. Both indicate a rapid decline in $F_v/F_m$ from this peak with decreasing chl-a concentration and a slower decline with increasing chl-a concentration. These studies were performed in the early 1990’s and falling $F_v/F_m$ values at low chl-a concentration was probably related to poor
signal quality. The sensitive photomultiplier in the Water-PAM should eliminate this effect until much lower chl-a concentrations are encountered. A more recent comment by Geel (1997), using a PAM 101-ED (Walz, GmbH) found that $F_v/F_m$ was independent of chlorophyll concentration between 0.5 and 40 µg mL$^{-1}$.

This short study was performed to determine the potential impact of chlorophyll concentration on chlorophyll fluorescence measurements obtained with the Water-PAM fluorometer.

**METHODS**

Sample material for these measurements was nutrient replete *Isochrysis galbana* (Haptophyceae) grown in f/2 medium (Guillard and Ryther 1962) in aerated semicontinuous culture with a growth irradiance of 50 µmol quanta m$^{-2}$ s$^{-1}$ on a 12:12 day:night cycle (for full description of culture setup refer to Chapter 3). A low growth irradiance was chosen for this experiment to ensure no long-term fluorescence quenching was present (such as photoinhibitory quenching) and the highest possible $F_v/F_m$ was achieved.

Sample material was taken from the photobioreactor and dilutions were made based on $F_o$ (refer to Table 2.1). All dilutions were performed with culture filtrate.
(Whatman GF/C filters used). Triplicate subsamples were taken from the final dilutions and filtered onto Whatman GF/C filters under low pressure for chl-a analysis. Filters were immediately wrapped in foil and stored at -80°C until processed. Chl-a extraction was performed as outlined in Chapter 3.

Table 2.1: Fluorescence signal (photomultiplier gain = 9; output gain = 3) and related chlorophyll concentrations determined spectrophotometrically. 
* Insufficient culture material for chl extraction – value determined from linear regression of other data.

<table>
<thead>
<tr>
<th>F_o</th>
<th>1600</th>
<th>800</th>
<th>300</th>
<th>100</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg chl-a mL⁻¹</td>
<td>0.559±0.005</td>
<td>0.273±0.002</td>
<td>0.106±0.001</td>
<td>0.038±0.000</td>
<td>*0.007</td>
</tr>
<tr>
<td>± se (n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each chlorophyll concentration 5 replicate 15 min dark-adapted RLC measurements were performed. On each occasion, saturation pulses were 0.8 s and the Light Width at each irradiance was 5 s. An estimate of F_v/F_m was provided by the first saturation pulse of each RLC. To plot RLCs the relative electron transport rate (rETR) was calculated (rETR = F_v/F_m × E_PAR) and expressed as a function of irradiance.

RESULTS

Sample chlorophyll concentration had no significant impact on F_v/F_m (Figure 2.3) or rETR_max (Figure 2.4) between 0.559 and 0.038 µg chl-a mL⁻¹. However, with further sample dilution to approximately 0.007 µg chl-a mL⁻¹, both F_v/F_m and rETR_max increased significantly (Table 2.2).

Figure 2.3: Plot of average F_v/F_m recorded at each chlorophyll concentration. Error bars = se, n = 5.
Figure 2.4: Rapid light curves recorded from cultures of *Isochrysis galbana* at varying chlorophyll concentrations. Error bars = se, $n = 5$.

Table 2.2: One-way ANOVA results for comparison of mean maximum quantum yield ($F_v/F_m$) values between microalgal suspensions of varying chlorophyll concentration. $P << 0.05$, therefore the null hypothesis that all means are equal is rejected. Tukey post-hoc testing revealed that the mean $F_v/F_m$ at the lowest chlorophyll concentration (0.007 µg mL$^{-1}$) was significantly different from all other means.

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>$F$</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>.015</td>
<td>4</td>
<td>.004</td>
<td>39.720</td>
<td>.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>.002</td>
<td>20</td>
<td>.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>.016</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION AND CONCLUSION**

The data presented here cover substantially lower chlorophyll concentrations than those investigated by Ting and Owens (1992), Büchel and Wilhelm (1993) and Geel (1997). Both chlorophyll fluorescence parameters $F_v/F_m$ and $rETR_{max}$ showed no influence of chlorophyll concentration over the range of 0.559 – 0.038 µg chl-$a$ mL$^{-1}$, however, measurements at 0.007 µg chl-$a$ mL$^{-1}$ showed a significant increase in both parameters.
One priority in experimental design for future work was to minimise sample handling and manipulation procedures, since a number of microalgal species are known to be particularly sensitive and even minor stresses can impact chlorophyll fluorescence parameters, such as $F_v/F_m$, negatively (Peter Ralph, pers. comm.). Culturing at very low cell densities heightens the risk of culture contamination and culture failure. Identical culture material was to be used for chlorophyll fluorescence, oxygen evolution and carbon fixation measurements in later experiments. Thus, the sample chlorophyll concentration was required to be suitable for all three methods. It was therefore considered most appropriate that chlorophyll concentration of samples for further experiments would average ~0.1 µg chl-\textit{a} mL$^{-1}$ and should no be less than 0.04 µg chl-\textit{a} mL$^{-1}$. This would also be beneficial in that there would be no concern that small changes in chlorophyll concentration throughout the course of a study would have a direct impact on results. This would be harder to manage if concentrations were less than 0.04 µg chl-\textit{a} mL$^{-1}$.

2.2.3 Dark Adaptation Time

\textit{Introduction}

In order to estimate the maximum quantum efficiency of electron transport through PSII ($F_v/F_m$) all reaction centres must first be in the “open” state. That is, $Q_A$ should be in its oxidised form so that electrons can be immediately drawn away from an excited RCII$^\ast$. In the field, where a samples recent light history may not be known, samples are often dark adapted to relax NPQ and standardise results – helping to minimise variation simply due to recent light history rather than physiological changes. Many studies apply a dark-adaptation period of 15 minutes (Kromkamp and Peene 1999; Underwood 2002; Morris and Kromkamp 2003; Young and Beardall 2003a),
however, this interval can range from 5 min (Gilbert et al. 2000a) to overnight (Schreiber and Neubauer 1990).

As the majority of studies in this project were in a controlled environment with a known and constant light history, the primary reason for dark-adaptation of samples was to measure \( F_v/F_m \). To achieve maximum photochemical yield through PSII, the quenching mechanisms \( q_E \), \( q_T \) and \( q_I \) should be negligible.

**METHODS**

Culture material was collected from batch cultures in early log-phase growth, diluted to \(~0.1 \mu g \text{ chl-}a \text{ mL}^{-1}\), and uniformly exposed to 200 \( \mu \text{mol quanta m}^{-2} \text{ s}^{-1} \) for 45 min to equilibrate.

Quantum yield measurements were performed using the saturation pulse method. Five replicate measurements were taken in the light adapted state and after 5, 10 and 30 min in darkness. Far-red light was applied for 10 s prior to each measurement in an attempt to preferentially excite PSI and drain electrons away from PSII. Chlorophyll concentrations of the individual species cultures were 0.111 ± 0.001 \( \mu g \text{ mL}^{-1} \) (Isochrysis galbana), 0.101 ± 0.005 \( \mu g \text{ mL}^{-1} \) (Nannochloropsis oculata) and 0.071 ± 0.001 \( \mu g \text{ mL}^{-1} \) (Chaetoceros muelleri), as measured spectrophotometrically (error = se; methods described in Chapter 3).

A second study using *I. galbana* cultured semicontinuously under low light conditions (50 \( \mu \text{mol quanta m}^{-2} \text{ s}^{-1} \)) was performed. On this occasion the WATER-ED actinic light was activated (54 \( \mu \text{mol quanta m}^{-2} \text{ s}^{-1} \)) before the sample was transferred from the photobioreactor. Once the fluorescence signal was stable a saturation pulse to determine light-adapted effective quantum yield (\( F'_v/F'_m \)) was initiated. The actinic light was then immediately deactivated and quantum yield was measured via saturation pulses at 1 min intervals for a total of 20 min.
RESULTS

After exposure to moderate irradiance ($200 \, \mu\text{mol quanta m}^{-2} \, \text{s}^{-1}$), quantum efficiency of electron transport through PSII increased rapidly over the first 5 – 10 min in the dark (Figure 2.5). Under these conditions *N. oculata* was the only species to show a decrease in apparent $F_v/F_m$ when dark adaptation was extended to 30 min (Figure 2.5b). The recovery of PSII quantum yield is much more rapid in samples exposed to lower irradiance ($50 \, \mu\text{mol quanta m}^{-2} \, \text{s}^{-1}$) and an apparent $F_v/F_m$ value is achieved after only 30 s in the dark (Figure 2.6). Maintaining samples in the dark for longer than this resulted in a small but continuing decreasing trend in measured quantum yield of PSII electron transport.

![Figure 2.5: $F_v/F_m$ values estimated after increasing dark-adaptation periods. Batch cultures of (a) *Isochrysis galbana*, (b) *Nannochloropsis oculata* and, (c) *Chaetoceros muelleri*, all in log-phase growth, had been pre-exposed to 45 min of 200 $\mu$mol quanta m$^{-2}$ s$^{-1}$ light before dark-adaptation commenced. Error = se, $n = 5$.](image-url)
Figure 2.6: Recovery of PSII quantum yield in *Isochrysis galbana* after exposure to low light (54 µmol quanta m\(^{-2}\) s\(^{-1}\)). Open circles indicate measurements taken on samples exposed to far red light for 10 s prior to each saturation pulse. Samples represented by closed circles were not exposed to far red light. Actinic light was turned off immediately after first saturation pulse to determine light-adapted quantum yield (time = zero) and saturation pulses were then applied at 1 min intervals to track the recovery of quantum yield to its maximum (F\(_{v}/F_{m}\)). Error = se, n = 5.

**DISCUSSION AND CONCLUSION**

Studies on phytoplankton and other phototrophic organisms often use changes in the maximum quantum yield of PSII (F\(_{v}/F_{m}\)) as a physiological indicator. Measurements should be optimised for each species as fluorescence behaviour varies between taxa, potentially affecting the accurate determination of F\(_{0}\) and F\(_{m}\) (Schreiber et al. 1995b).

The measurements taken here suggest that, after exposure to moderate light (200 µmol quanta m\(^{-2}\) s\(^{-1}\)), approximately 10 min is required for full relaxation of quenching mechanisms and the achievement of F\(_{v}/F_{m}\). This state indicates full reoxidation of the PQ pool and QA and relaxation of nonphotochemical quenching components. Due to the nutrient-replete status of the cultures used and the light levels applied, it was assumed that qI would be minimal.

It is of interest to note that the eustigmatophyte *Nannochloropsis oculata* showed a decrease in measured F\(_{v}/F_{m}\) when dark adaptation was longer than 10 min (Figure 2.5b). As discussed in Chapter 1, this can result from any one of a number of
proposed mechanisms, ranging from chlororespiration to enhanced spillover of light energy from PSII to PSI. Given this result for *N. oculata* and the propensity for diatoms to show a similar pattern (Serôdio et al. 2006 and references therein), it came as some surprise that a similar decrease was not also observed in *Chaetoceros muelleri* samples (Figure 2.5c).

Results indicate (Figure 2.6) that measurements of \( F_v/F_m \) can be achieved on a much shorter time-scale if samples have been exposed to low irradiances only. \( F_v/F_m \) was maximal (0.748 ± 0.005) after just 30 s of darkness. A one-way ANOVA revealed that this value was significantly higher than the light adapted quantum yield of 0.714 ± 0.004, but not significantly different from \( F_v/F_m \) estimates after longer dark adaptation periods (although there was an apparent decreasing trend).

These results indicate that a dark adaptation time of 10 – 15 min is likely to be most appropriate in conditions where photoinhibitory quenching (\( q_I \)) is not present.

### 2.2.4 Sample Homogeneity

**INTRODUCTION**

Chlorophyll-\( a \) fluorescence serves as a valuable probe of the primary biophysical events in photosynthesis (Falkowski et al. 1986a; Hill and Ralph 2005; Lazár et al. 2005), including the efficiency of PSII photochemistry (Beardall et al. 2001). The development of highly sensitive Pulse Amplitude Modulation (PAM) fluorometers in the early 1990’s (Schreiber et al. 1993), and further refinement of the electronics since then, has resulted in a recent surge in the application of chl-\( a \) fluorescence in scientific research (Samson et al. 1999; Villareal 2004; Jakob et al. 2005). Instruments with sufficient sensitivity to measure variable chl-\( a \) fluorescence of

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1 This section has been published as: Cosgrove, J. and M. Borowitzka (2006). Applying Pulse Amplitude Modulation (PAM) fluorometry to microalgae suspensions: stirring potentially impacts fluorescence. *Photosynthesis Research* **88**: 343-350.
marine phytoplankton in coastal waters are now available (Schreiber et al. 1993; McMinn and Hegseth 2004).

The Water-PAM (Walz GmbH, Germany) was designed for the assessment of phytoplankton in natural surface waters. Light-emitting diodes (LEDs) provide non-actinic measuring light (3 LEDs, spectral peak at 650nm), actinic light/saturation pulses (12 LEDs, spectral peak at 660nm) and far-red light (3 LEDs, spectral peak at 730nm). These LEDs are contained in the ED unit and are arranged in a ring which circles the 15mm diameter quartz sample cuvette. When in place, the base of the sample cuvette rests upon the detector (Figure 2.7a).

Accurate and precise fluorescence measurement with the Water-PAM requires cell distribution to remain homogenous during sampling and that all cells experience the same irradiance. This basic principle also applies to other PAM fluorometers of similar design, such as the PHYTO-PAM (System II PHYTO-ED configuration) and Toxy-PAM (Walz GmbH, Germany). The Water-S stirring device (supplied as an optional accessory) is aimed at maintaining sample homogeneity and preventing cells from settling to the bottom of the cuvette (Figure 2.7a). Observations during use of the Water-PAM fluorometer have indicated that the act of stirring the sample can, however, have a profound effect on fluorescence readings and the derived parameters. This study presents evidence for this and suggests a design modification that the author feels could negate this effect.

**METHODS**

*Nannochloropsis oculata* CS179 (Droop) Hibberd (Eustigmatophyceae) was chosen as the study organism as it is considered hardy (not physically impacted by stirring) and has a slow sinking rate resulting in little to no cell movement over the duration of a rapid light curve (RLC) or induction curve. A vertical flat plate
photobioreactor (5.5 L, 10 cm wide) was used to grow *N. oculata* in semi-continuous culture at 25±2°C at an irradiance of 540 µmol quanta m⁻² s⁻¹ with a 12h:12h light/dark cycle provided by cool white fluorescent lamps set along both sides of the reactor. Mixing was provided by bubbling air at a rate of 1.0 L min⁻¹. Near-shore coastal seawater was collected and treated with activated charcoal overnight (1:1000 w/w charcoal to seawater mix) before being filtered sequentially through double thickness Whatman No.1 filter paper followed by a 0.45 µm MFS mixed cellulose ester filter. Culture media was prepared by enriching this ‘double-filtered’ seawater with f/2 nutrients (Guillard and Ryther 1962). The culture was maintained in logarithmic-phase by daily dilutions, with fresh culture media, based on cell density.

Samples were collected from the photobioreactor and placed into darkened vials for 10 min dark adaptation. Dark adaptation of 10 min had previously been found to yield the peak $F_v/F_m$ (see Section 2.2.3). Samples were then transferred to the sample cuvette (in a darkened room) which was immediately inserted into the ED unit and the stirrer unit fitted. Sample volume was maintained at 2.5 mL unless otherwise specified. Far-red (FR) light was subsequently applied for 5 s to ensure full oxidation of $Q_A$. Once fluorescence had stabilised after applying FR light, either the induction curve or rapid light curve (RLC) programme (within WINCONTROL software, Walz GmbH) was initiated. Two treatments were used: (1) not stirred and; (2) stirred. For both treatments the stirring device was in place, however, it was not activated during treatment 1.

Chlorophyll *a* content was calculated spectrophotometrically using the equations of Jeffrey and Humphrey (1975) after extraction of filtered samples (Whatman GF/F) in 90% acetone. All measurements were performed both at “high” (140.9 ± 11.9 µg.chl-*a* L⁻¹; mean ± se, *n* = 3) and “low” (6.3 ± 0.27 µg.chl-*a* L⁻¹; mean ± se, *n* = 3) chlorophyll concentrations.
The relative distribution of light in the Water-PAM was measured using the spherical micro-quantum sensor attachment (US-SQS/WB, Walz GmbH). The ED-unit actinic light was activated and irradiance measured at 1 mm intervals from the bottom of the cuvette. Actinic light was turned off between measurements to avoid changes in LED temperature.

Rapid light curves were performed with 10 s exposure duration to each of eight incremental irradiances (50, 76, 118, 176, 271, 406, 579 and 806 µmol quanta m\(^{-2}\) s\(^{-1}\) measured at the base of the cuvette with US-SQS/WB sensor) after an initial quasi-dark measurement (~1.5 µmol quanta m\(^{-2}\) s\(^{-1}\)) to provide estimates of F\(_{o}\) and F\(_{m}\).

Rapid light curves were constructed by calculating relative electron transport rate (rETR, Equation 32) through PSII for each level of actinic light.

\[
\text{rETR} = \left( \frac{F_{m} - F}{F_{m}'} \right) \times E_{\text{PAR}} \times 0.5
\]  

(32)

Where (\(F_{m}' - F)/F_{m}'\) estimates the effective quantum yield of PSII, \(E_{\text{PAR}}\) is the actinic irradiance in µmol quanta m\(^{-2}\) s\(^{-1}\), 0.5 is a multiplication factor based on the assumption that 50% of the absorbed quanta are distributed to PSII (Beer et al. 1998b).

RLC data were fitted to the model of Platt et al. (1980) in order to obtain values for the initial slope (\(\alpha\)), inhibition term (\(\beta\)), light saturation parameter (\(E_k\)), and maximum relative electron transport rate (\(r\text{ETR}_{\text{max}}\)). \(E_k\) was derived from \(r\text{ETR}_{\text{max}}\) and \(\alpha\) (Equation 33).

\[
E_k = \frac{r\text{ETR}_{\text{max}}}{\alpha}
\]  

(33)

Non-photochemical quenching (NPQ) was calculated following Serôdio et al. (2005) since \(F_{m}'\) values were found to be higher than \(F_m\) in stirred samples (Equation 34).
\[ NPQ = \left( F'_m - F'_m' \right) / F'_m \]  

(34)

Where \( F'_m \) is the maximum \( F'_m' \) value and was replaced by \( F_m \) when the dark-adapted value was the highest.

An additional observation was made by plotting the fluorescence trace of a light curve (LC) with a longer duration at each actinic irradiance (3 min). Sample homogeneity was maintained during the extended time period of this curve by stirring. Stirring was performed for 10 s, beginning approximately 10 s after each saturation pulse, leaving over 2.5 min for fluorescence to stabilise before the next saturation pulse.

Induction curves were measured with a delay of thirty seconds between determination of \( F_v/F_m \) and onset of actinic illumination (406 \( \mu \)mol quanta m\(^{-2}\) s\(^{-1}\) measured at the base of the cuvette). A saturation pulse measuring quantum yield and NPQ was performed immediately after the onset of actinic illumination and every 20 s thereafter until deactivation of actinic light 4 min later. A trace of the fluorescence signal was recorded for each treatment and changes in NPQ and quantum yield were calculated as previously described.

RLC and induction curve data were also obtained using a Diving-PAM (Walz, Germany) to use as a reference against Water-PAM data to assist in discerning between 'typical' and 'atypical' fluorescence patterns. *Nannochloropsis oculata* was filtered onto a Whatman GF/C filter paper under low pressure and left damp. In a darkened room the Diving-PAM's 8 mm fibre-optic probe was positioned \(~3\) mm above the filter paper and RLC and induction curve programs activated. The fibre-optic probe was repositioned to a fresh, darkened portion of the filter between each measurement.

Independent t-test’s (\( n = 5 \)) were performed, using SPSS for Windows, to detect significant differences between photosynthetic parameters measured from stirred and unstirred samples.
**RESULTS**

The impact of stirring was similar at both chlorophyll concentrations, however more background noise was evident when analysing the “low” chlorophyll samples (higher photomultiplier gain settings used). For this reason only results for “high” chlorophyll concentration samples are presented here.

Irradiance measurements indicate that the single ring of LEDs produces a narrow band of light with maximum irradiance measured just over 3 mm above the base of the cuvette (Figure 2.7b). Just ~2.1 mm further above or below this point irradiance was 50% of maximum. The use of the Water-S stirring accessory promotes mixing of the sample both horizontally and vertically (Figure 2.7a) and, as a consequence, cells move through different irradiance fields.

**Figure 2.7:** (a) Basic line drawing (not to scale) of Water-PAM cuvette with stirring device in place. Stirring is both in the horizontal plane and the vertical plane; (b) Light distribution measured in the centre of the cuvette when mounted within the ED-unit of the Water-PAM.
Figure 2.8: Fluorescence traces of 10 min dark adapted samples of *N. oculata* for the duration of a RLC. Numbers indicate actinic irradiance in µmol quanta m⁻²s⁻¹ applied for each 10 s period between saturation pulses. (a) Stirrer not activated, (b) stirrer activated.

Representative plots of the fluorescence trace for each treatment are presented in Figure 2.8. These fluorescence data indicate a substantial influence of stirring on the dynamics of fluorescence measurements. In particular, $F_{m'}$ remains high and $F$ increases with each increase in PAR in stirred samples compared to unstirred samples (Figure 2.8b). This resulted in higher effective quantum yield values, hence $\alpha$ was significantly higher in the stirred treatment compared to the unstirred treatment (Table 2.3). While $rETR_{max}$ was significantly higher in stirred samples (Table 2.3, Figure 2.9a) as a result of enhanced $F_{m'}$ values, the effect was limited by increasing $F$ at higher PAR (Figure 2.8b). NPQ in stirred samples was severely diminished by artificially high $F_{m'}$ values (Table 2.3, Figure 2.9b).

<table>
<thead>
<tr>
<th></th>
<th>*rETR_{max}</th>
<th>*$\alpha$</th>
<th>$I_k$</th>
<th>*Max. NPQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stirring</td>
<td>42.9 ± 2.0</td>
<td>0.270 ± 0.005</td>
<td>159.4 ± 8.3</td>
<td>0.876 ± 0.017</td>
</tr>
<tr>
<td>Stirring</td>
<td>52.8 ± 1.4</td>
<td>0.331 ± 0.011</td>
<td>160.0 ± 3.3</td>
<td>0.073 ± 0.003</td>
</tr>
<tr>
<td>$t$, P(.05)</td>
<td>-4.082, 0.004</td>
<td>-5.210, 0.001</td>
<td>-0.064, 0.950</td>
<td>46.165, &lt;0.001</td>
</tr>
</tbody>
</table>
The fluorescence chart from the extended LC (3 min at each irradiance) clearly illustrates an impact of stirring on the fluorescence signal (Figure 2.10). There is also an indication that the impact of stirring becomes greater at higher actinic irradiances, with negligible impact observed during the first actinic light level (50 \(\mu\text{mol quanta m}^{-2} \text{s}^{-1}\)).

Induction curve data were adversely impacted by stirring samples during chlorophyll-\(\alpha\) fluorescence.

**Figure 2.9:** RLC data illustrating the impact of stirring on (a) rETR and, (b) NPQ (n=5, error bars indicate standard error).

**Figure 2.10:** Fluorescence trace of a light curve with 3 min exposure to each successive irradiance (intensities indicated). Measurements were performed with dark-adapted *N. oculata*. Magnified region details 10 s delay after saturation pulse before stirred was turned on (up arrow), fluorescence rise during 10 s stirring and transient fluorescence peak before a rapid decline once the stirrer was turned off (down arrow).
measurements in the Water-PAM. Representative plots of the fluorescence trace for each treatment are presented in Figure 2.11. Induction curve data obtained from the unstirred sample follow the typical ‘slow phase’ kinetics of fluorescence induction (Lazár 1999). While NPQ appeared active in samples that were not stirred, data from samples which were stirred gave an impression of severely limited NPQ development (Figure 2.12a). NPQ was significantly lower in stirred samples compared to unstirred samples ($F_{0.05(1),138} = 422.29, P<0.001$). Effective quantum yield data were also impacted by stirring (Figure 2.12b) with stirred samples displaying significantly higher measures than unstirred samples ($F_{0.05(1),138} = 12.582, P = 0.001$). This increase in effective quantum yield resulted in a similar increase in rETR values (data not shown).

**Figure 2.11:** Fluorescence traces of 10 min dark-adapted samples for the duration of an induction curve. (a) Stirrer not activated, (b) stirrer activated. Up arrows and down arrows indicate activation and deactivation of actinic light (406 µmol quanta m$^{-2}$s$^{-1}$) respectively.

Diving-PAM RLC fluorescence trace data (Figure 2.13a) indicated a decline in $F_m'$ compared to $F_m$ similar to that observed in the unstirred Water-PAM samples. Similarly, induction curve chlorophyll-α fluorescence data obtained with the Diving-PAM (Figure 2.13b) resemble the Water-PAM data from the unstirred treatment (Figure 2.11a).
Figure 2.12: (a) NPQ and (b) effective quantum yield measurements, taken from successive saturation pulse data during the course of an induction curve (actinic light = 406 µmol quanta m$^{-2}$s$^{-1}$). Error bars = se, n = 5.

Figure 2.13: Diving-PAM (8mm fibre-optic probe) fluorescence traces taken from *N. oculata* filtered onto Whatman GF/C filter paper. (a) RLC trace with figures indicating actinic irradiance in µmol quanta m$^{-2}$s$^{-1}$, and (b) induction curve where up and down arrows indicate activation and deactivation of actinic light (423 µmol quanta m$^{-2}$s$^{-1}$) respectively.
**DISCUSSION AND CONCLUSION**

PAM-fluorescence measurements have become popular for assessing the photochemical status of crops (Schäfer and Björkman 1989), corals (Schreiber et al. 1997; Beer et al. 1998a; Hill et al. 2004), seagrasses (Ralph et al. 2002b; Campbell et al. 2003; Durako et al. 2003), macroalgae (Häder et al. 1998; Beach et al. 2003; Beer and Axelsson 2004) and microalgae (Kromkamp and Peene 1999; Gilbert et al. 2000a; Suggett et al. 2003). With the advent of sensitive and portable equipment such as the Water-PAM (Walz GmbH, Germany) the application of chlorophyll-α fluorescence measurements to assess phytoplankton in natural surface waters can be expected to further increase in popularity. Despite the efforts of some to detail methodological guidelines for PAM-fluorescence studies (Ralph and Gademann 2005), some points of debate still remain regarding specific procedures.

When collecting chlorophyll-α fluorescence measurements using the Water-PAM it is important that: (i) cells do not settle to the bottom of the cuvette during measurements; (ii) all cells experience the same light environment and; (iii) the cells are evenly distributed throughout the sample (homogeneous). To aid in fulfilling these criteria a stirrer is sold as an optional accessory with the Water-PAM. We have shown, however, that stirring samples during RLC or induction curve measurements may significantly impact results. The PHYTO-PAM (System II PHYTO-ED configuration) and Toxy-PAM (Walz GmbH, Germany) ED units have similar geometry and light fields to that of the Water-PAM ED unit and stirring is likely to have a similar impact in each of these fluorometers. Different results may be apparent with units varying in geometry and light environment.

Stirring samples within the ED-unit during chlorophyll-α fluorescence measurements resulted in significantly higher RLC parameters $\text{rETR}_{\text{max}}$ and $\alpha$, while
NPQ readings were significantly negatively impacted (both RLC and induction curve). Artificially high \( F_m' \) values recorded while samples were stirred appear to be the cause of these changes. Concomitant increases in F tend to moderate increases in effective quantum yield and the derived rETR values.

Comparison of fluorescence patterns observed in the Water-PAM against those obtained using a Diving-PAM revealed a close similarity between the pattern of the Diving-PAM fluorescence data and the unstirred Water-PAM data. This suggests that the unstirred data were 'typical' rather than 'atypical'. While changes in F during the course of a RLC recorded with the Diving-PAM did appear to deviate from that observed with the Water-PAM, the pattern observed (Figure 2.13a) was very similar to dark-adapted pea leaf RLC fluorescence patterns observed by White and Chritchley (1999) using a MINI-PAM (Walz GmbH, Germany).

Irradiance measurements show that there is a heterogeneous light environment within the ED-unit (Figure 2.7b). The LED array is located in a ring near the bottom of the cuvette (Figure 2.7a); providing the maximum PAR irradiance just over 3 mm above the base of the cuvette and 50% PAR ~2.1 mm above or below this point. The fluorescence signal received by the detector is likely to be predominantly from those cells within this narrow beam. It is suggested that uneven illumination of the sample within the cuvette may be the underlying cause of artificially high \( F_m' \) and F values when the sample is stirred. Cells outside the main light path would experience less quenching of fluorescence and by activating the stirrer these cells become vertically mixed and pass into the ‘high light’ zone. The relatively unquenched state of these cells would then yield an increase in measured fluorescence. This could potentially also happen (to a lesser extent) in unstirred samples if cells are motile or sink significantly during the course of a RLC or induction curve. Both of these situations would result in cells moving across a gradient of light intensities resulting in an impact on their
quenched state. Sinking rate data from Bienfang (1980) suggest that cells within a sample of natural phytoplankton may sink 0.34 – 1.66 mm within the 87 s it takes to run a RLC with a 10 s exposure at each irradiance. Our measurements indicate that a cell sinking 1.66 mm to the point of maximum light intensity would experience a 64% increase in irradiance. Actively swimming cells have the potential to experience even greater variation in irradiance. Even the moderately paced cryptophyte, *Plagioselmis nannoplanctica* (Clegg et al. 2003), could swim a distance of ~7 mm over the duration of a similar RLC. Phototactic responses of motile species could also influence quenching data simply due to accumulation (or dispersion) of cells into (or away from) the narrow beam of light.

One would expect the rise in fluorescence yield upon activation of the stirrer to become more substantial as the actinic irradiance setting is increased. The fluorescence trace recorded during the course of a LC with 3 min exposure at each irradiance displays both a rise in the stirrer-induced fluorescence peak and a greater amplitude to the recovery-wave with each successive increase in actinic irradiance. These data indicate that stirring will have a negligible influence on fluorescence data, when only the measuring light or low irradiance actinic light (approx. <150 µmol quanta m\(^{-2}\) s\(^{-1}\)) is activated.

Induction curve data obtained from unstirred samples (Figure 2.11a) and filtered samples (Diving-PAM, Figure 2.13b) follow typical fluorescence induction patterns (Lazár 1999), however, stirred samples are atypical and experience elevated F and F\(_m'\) values (Figure 2.11b). This impact on F and F\(_m'\) values is not transient and persists for the period of the induction curve. Again, the fluorescence parameter most influenced by this change is NPQ (Figure 2.12).
Significant methodological questions still remain when stirring samples to maintain sample homogeneity when using the Water-PAM ED-unit, and other similar units. Users should be mindful of the need to stir their sample (particularly if the cell sinking rate is moderate to high), the susceptibility of the study organism(s) to stirrer-generated shear and enhanced “noise” caused by stirring. Some users stir the sample by placing the entire ED unit on a modified orbital shaker, effectively eliminating stirrer-generated shear forces and reducing vertical mixing (P. Ralph, pers. comm.).

One might suggest that by using minimal sample volume, thereby reducing the proportion of sample outside the light path, the impact of stirring on the fluorescence signal could be reduced. While this approach does reduce the magnitude of the impact a substantial change in fluorescence remains (data not shown). It is proposed that providing a vertical dimension to the LED-array, such as the addition of a second ring of LEDs at a higher level relative to the cuvette, plus restriction of sample volume to 2 mL, would result in more even illumination of the whole sample and significant mitigation of the described fluorescence artefacts.
CHAPTER 3
General Materials and Methods

3.1 ALGAL CULTURE

Samples for culture inoculation originated from the CSIRO Marine culture collection in Hobart, Tasmania (Australia). The species cultured were *Chaetoceros muelleri* CS176 Lemmermann (Bacillariophyta), *Isochrysis galbana* CS177 Parke (Haptophyta) and *Nannochloropsis oculata* CS179 (Droop) Hibberd (Ochrophyta, eustigmatophyte).

Cultures to be used for experimental purposes were grown in a vertical flat plate photobioreactor (5.5 L; dimensions = H36×L25×W10 cm) in semi-continuous culture at 25±2°C with a 12h:12h light:dark cycle. Temperature stability was enhanced by placing the photobioreactor within a water filled aquarium fitted with an aquarium heater set to 25°C (Figure 3.1, Figure 3.2). Cool white fluorescent lamps set along both sides of the reactor provided growth irradiance. Each species was cultured in both High Light (HL = 500 µmol quanta m⁻² s⁻¹) and Low Light (LL = 50 µmol quanta m⁻² s⁻¹) conditions. Mixing was provided by bubbling air at a rate of ~1.0 L min⁻¹.
Figure 3.1 Line Drawing of photobioreactor setup (not to scale). An airpump (1) provided air at a rate of ~1.0 L.min⁻¹. The air passed through a humidifier (2), Whatman Vacu-Guard™ in-line filter (3) and a one-way valve (4) via silicon tubing to a diffuser (5) set longitudinally along the deepest part of the reactor. Culture was harvested via the harvesting tube (6) and air was allowed to escape at the top of the reactor (7) then passed through a second one-way valve and dilute hydrochloric acid solution (8). To help maintain culture temperature close to 25°C the photobioreactor was set in a water bath heated with an aquarium heater (9).

Figure 3.2: Photobioreactor setup. Fluorescent lamps are in position for High Light growth irradiance (500 µmol quanta m⁻² s⁻¹) and are on a 12:12 h light cycle.
Culture media consisted of double-filtered seawater (charcoal-treated then filtered through Whatman No.1 filter paper followed by a 0.45 µm MFS mixed cellulose ester filter) enriched with f/2 nutrients under sterile conditions (modified from Guillard and Ryther 1962). For culture of *N. oculata* and *I. galbana* the media did not include silicate whereas the diatom *C. muelleri* was cultured with half the normal f/2 silicate component (Table 3.1).

**Table 3.1** f/2 Medium (modified from Guillard & Ryther, 1962).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Stock Solution (g L⁻¹)</th>
<th>Vol. Stock L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>150</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>10</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>1.22</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>1.046</td>
<td></td>
</tr>
<tr>
<td>*Na₂SiO₃·9H₂O</td>
<td>30</td>
<td>0.5 mL</td>
</tr>
<tr>
<td><em>Vitamin Mix</em></td>
<td></td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Cyanobalamin</td>
<td>1.0×10⁻³</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>1.0×10⁻³</td>
<td></td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>PII Metal Solution</em></td>
<td></td>
<td>0.5 mL</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.0196</td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.013</td>
<td></td>
</tr>
</tbody>
</table>

*Dosage here is half the standard f/2 dosage

The culture was maintained in logarithmic-phase by daily dilutions based on cell density. Cell density was determined using a Neubauer haemocytometer. Culture density was maintained such that the culture was optically thin at all times in an effort to maintain homogeneity of the light environment within the photobioreactor.
3.2 CHL-a FLUORESCENCE

Determination of quantum yield of photochemistry at PSII and quenching coefficients was performed via the saturation pulse method using a Water-PAM (Walz GmbH, Germany) (See Sections 3.2.1 – 3.2.3). A separate instrument (FL-3000, Photon Systems Instruments, Czech Republic) applying a different saturation protocol was used to determine the functional absorption cross-section of PSII (Section 3.2.4).

Numerous studies have shown the presence of both endogenous rhythms and light-influenced diel cycles in photosynthetic activity (Brand 1982; Harding et al. 1982; Boyd et al. 1997). It was necessary for this study to minimise any confounding influence of these changes in photosynthetic activity on the data-set. For this reason data were collected on the normal daily changes in the major chl-a fluorescence parameters ($F_v/F_m$, rETR$_{\text{max}}$ and NPQ$_{\text{max}}$) for each of the cultured species under High Light conditions (500 µmol quanta m$^{-2}$ s$^{-1}$). Measurements were performed under High Light growth conditions only as it was assumed that under these conditions any diel cycle fluctuations would be more pronounced than in Low Light conditions. Data (not shown) indicated that, during the commencement of the day cycle (6 am – 6 pm), induction and balancing of photosynthesis could take a few hours. Each of the fluorescence parameters measured achieved relative stability by about 10 am before beginning to change again in the late afternoon. For this reason all experiments were setup in the morning and commenced at 10:00 – 10:30 am, with the aim of finishing before 3 pm. In this way the confounding effect of diurnal changes in photosynthetic parameters were minimised.

3.2.1 RLC

RLC measurements were performed on both dark adapted and light adapted samples. Ten replicate measurements were made for each condition. Dark-adaptation
was for 15 min and was performed by collecting 5 – 8 mL aliquots of fresh culture into foil-wrapped 10 mL vials and leaving them to sit in a 25°C water bath.

Prior to each series of measurements the actinic light intensities in the Water-PAM ED-unit were recalibrated using a spherical micro-quantum sensor attachment (US-SQS/WB, Walz GmbH). For this purpose the sensor head was placed in the region of light maxima (raised ~3 mm from the base position) as the dominant portion of the fluorescence signal received by the photomultiplier would come from this region (Figure 3.3).

PAM-control setting were set such that the fluorescence signal (F) in the dark was between 100 and 200 units. The light-width was set to 5 s (see Chapter 2) and the saturation pulse intensity was set to level 10. Saturation pulse width was set to 800 ms for measurements of *N. oculata* and *C. muelleri*, while the pulse width was increased to 1.2 s for studies of *I. galbana*. This was justified by the shape of the fluorescence induction curve for each species (Figure 3.4). Saturation pulse intensity was >6500 µmol quanta m\(^{-2}\) s\(^{-1}\) for all measurements. RLC irradiance levels were set such that, at maximum irradiance, down-regulation of photosynthesis could potentially be observed, while keeping the initial irradiance levels as low as possible. The WINCONTROL software does not allow individual allocation of light levels and a sequence of eight consecutive light levels is required. On account of this lack

![Figure 3.3: Image of Water-PAM showing the emitter-detector unit with spherical quantum sensor (4π) inserted for irradiance measures (foreground). The PAM control box is immediately behind the emitter-detector unit.](image-url)
of flexibility some trade-off between maximum irradiance and number of sampling points in the light limited portion of the curve is frequently required. For all RLCs the Light Width at each irradiance was set for 5 s.

![Fluorescence plots](image)

**Figure 3.4:** Plots of representative saturation pulses for (a) *Chaetoceros muelleri*, (b) *Isochrysis galbana* and, (c) *Nannochloropsis oculata*. The biphasic fluorescence rise shown here for *I. galbana* was common and for this reason the length of the saturation pulse was extended from 0.8 s to 1.2 s.

Light-adapted samples were analysed first. For these samples the ED-unit actinic light was activated prior to sample collection to an irradiance equivalent to the growth irradiance. This was done to minimise relaxation of quenching pathways before the measuring sequence could be initiated. Samples were collected from the reactor and immediately transferred to the sample cuvette, which was promptly inserted into the ED-unit and the cap put in place. Sample volume was maintained at approximately 2.5 mL.

Samples to be used for dark-adapted measurements were placed in individual foil-wrapped vials and incubated at 25°C for 15 min before being transferred to the PAM ED-unit. Far-red (FR) light was subsequently applied for 5 s in an attempt to ensure full Q_A oxidation. Once fluorescence had stabilised after applying FR light the rapid light curve (RLC) programme (within WINCONTROL software) was initiated.
Ten replicate light curves were conducted for each of the light-adapted and dark-adapted treatments. A trace of the fluorescence signal was also recorded using the chart function in WINCONTROL for 3 of these replicates and on such occasions dark recovery was also measured (“LC + Recovery” program was used).

For estimation of both qP and NPQ there was a requirement to measure $F_o$ and $F_m$. For the light-adapted samples this was not possible, so a duplicate aliquot was taken at the time of sample collection and dark-adapted for 15 min. The dark-adapted duplicates were then exposed to FR-light (5 s) followed by a single saturation pulse to obtain measures of $F_o$ and $F_m$.

### 3.2.2 LC(3)

Rapid light curves are different from traditional P-E curves since they assess immediate, non-steady state photosynthetic activity. P-E curves, as measured by $O_2$-evolution and C-fixation techniques, represent are more optimal steady state and more independent of light prehistory (Ralph and Gademann 2005). Thus, when intending to compare photosynthetic rates measured via chlorophyll fluorescence with those measured via $O_2$-evolution, it may be useful to extend the length of exposure to each irradiance to a period roughly equivalent to that used in the $O_2$-evolution measurements. This concept was applied by Kühl et al. (2001) when comparing chl fluorescence based measures of photosynthetic activity with $O_2$ measurements. These longer measurements were simply called “Light Curves” and given the abbreviation LC(3); this nomenclature will be followed here.

LC(3) measurements were essentially performed in a similar manner to RLC measurements, with replicate light curves constructed for both light- and dark-adapted treatments. However, the issues highlighted in Section 2.2.4 resulted in some significant changes. Maintaining sample homogeneity throughout a 24 min (8 actinic irradiances ×
3 min) LC(3) would pose a problem considering the impact of stirring on results (refer to Chapter 2.2.4). An extended length of time in the sample cuvette is also likely to impact photosynthetic properties. For this reason each LC(3) was constructed from saturation pulse measurements of eight individual samples each exposed to a single actinic irradiance. Culture material was transferred to the ED-unit in either the light- or dark-adapted state and a saturation pulse performed (measuring $F_q'/F_m'$ or $F_v/F_m$ respectively); the actinic light was then activated on a 3 min timer setting and another pulse was performed just before the actinic light deactivated. Due to the extended time required for measurement of each LC(3) only three replicates could be performed per treatment.

*Nannochloropsis oculata* is small and observations suggested that cells remained in suspension and relatively homogeneous over the 3 min measurement period. *Chaetoceros muelleri* on the other hand sinks rapidly, while *I. galbana* is motile, and homogeneity of these samples needed to be maintained by a activating the WATER-S (Walz, GmbH) stirring attachment for a 20 s period approximately halfway through each exposure period. At the completion of the highest irradiance measurement the sample was left in place (in the dark) and dark-recovery measurements were recorded. The stirrer was activated after the third dark-recovery pulse to maintain homogeneity (impact of stirring darkened samples is minimal).

### 3.2.3 LC(20)

Carbon-fixation measurements made throughout the duration of this study were performed using 20 min incubations following the methods of Lewis and Smith (1983) (see Section 3.4). The 20 min exposure to each irradiance would yield values of steady state photosynthesis and may even induce photoinhibition, a process that cannot be measured with RLCs as the measurements are too rapid to cause photodamage (Ralph
To minimise differences between C-fixation and chlorophyll fluorescence techniques and to gain data that represented measures of samples in the most similar state possible, chlorophyll fluorescence measurements were performed using samples incubated in a photosynthetron (refer to Chapter 3.4 for description of photosynthetron) for 20 min at irradiances as close as possible to the Water-PAM ED-unit actinic irradiances. Since C-fixation measurements are an integrated value of C-fixation that occurred over the whole 20 min, rather than an instantaneous value, chlorophyll fluorescence measurements were also taken after 5 min and 10 min to allow some form of integration across the whole 20 min time period.

Light intensities in the photosynthetron were measured with the spherical micro-quantum sensor (US-SQS/WB), with sample positions chosen to give three replicates at each irradiance. Three vials were also double-wrapped in foil to provide dark measures. Three millilitres of culture material was added to each vial and the vials were then placed in the photosynthetron sequentially at 1 min intervals and removed after 5 min. Upon removal from the photosynthetron the sample was gently pipetted (pipette aperture expanded to ~3 mm) into the PAM cuvette and transferred to the ED-unit with actinic light activated to the equivalent irradiance. This process was performed as quickly as possible to minimise quenching relaxation. A single saturation pulse to determine $F_{q}'/F_{m}'$ was then initiated within 5 s. For samples that had been in the dark (foil-wrapped vials) a 5 s exposure to FR-light preceded the saturation pulse measurement. Once all samples had been processed the whole procedure was repeated two more times, once with 10 min and another with 20 min light exposure. The results from each sequence were compiled to for a light curve and the three light curves were subsequently averaged to gain a light curve integrated over the 20 min. This light curve was termed an LC(20).
3.2.4 Functional Absorption Cross-section of PSII (σ_{PSII})

Measurements of the functional absorption cross-section of PSII ($\sigma_{PSII}$) are important for the calculation of absolute values of electron transport rate through PSII. Measurements of this parameter were determined for each of the species studied in both the light- and dark-adapted state.

To perform these measurements a double-modulated FL-3000 PAM fluorometer with a fast measuring head (Photon Systems Instruments, Brno, Czech Republic) was used (Figure 3.5). This instrument follows the same principle as that described by Nedbal et al. (2005).

Sample material was collected from the photobioreactor and either dark-adapted for 15 min or maintained at growth irradiance (either 50 µmol quanta m$^{-2}$ s$^{-1}$ or 500 µmol quanta m$^{-2}$ s$^{-1}$). Sample material was then transferred to the cuvette and rapidly placed in the measuring head. The Fast Flash Induction protocol (FluorWin v3.6 software, Photon Systems Instruments) was initiated with actinic flash intensity set to 100% (~200,000 µmol quanta m$^{-2}$ s$^{-1}$) for a duration of 50 µs. A Microsoft Excel spreadsheet (provided by M. Trtílek, Photon Systems Instruments) was used for data processing to determine $\sigma_{PSII}$ (Å$^2$ quanta$^{-1}$). Five replicate measurements were performed for each species at each growth irradiance.

**Figure 3.5:** The Photon Instruments FL-3000 dual modulated fluorometer. Main picture shows control box (left) connected to the measuring head (right). Insert is a top-view of the measuring head with actinic light activated. During operation a cap is placed over the cuvette holder to shield the sample from ambient light.
3.3 Oxygen Evolution

Oxygen evolution measurements were performed using Clark-type oxygen electrodes (Rank Brothers, United Kingdom). These were set up following Rank Brothers oxygen electrode operation manual guidelines (Issue 1a, 2002) a day or two before use to allow a small, stabilising silver chloride layer to develop on the anode. The setup used was of the glass incubation chamber type and was connected, via silicon tubing, to a thermostatted flow-through water bath maintained at 25°C (Figure 3.6). Light was provided by a slide projector fitted with a single Fuji halogen lamp (EXY, 82 volt, 250 watt). Neutral density filters were used to provide a range of irradiances for determination of the photosynthesis versus irradiance relationship. Irradiances were measured at the centre of the chamber using a spherical micro-quantum sensor (US-SQS/WB, Walz, Germany).

Figure 3.6: Rank Brothers Clark-type oxygen electrode setup. A slide projector using a halogen lamp was used as the light source and neutral density filters were used to manipulate light intensity. The electrode chamber was temperature regulated at 25°C (waterbath not shown).

Oxygen electrodes were calibrated prior to each day of experiments. A 0% saturation point was set by flooding the chamber with oxygen-free nitrogen gas and 100% oxygen saturation was set using freshly aerated deionised water (oxygen content
of water was calculated using Carpenter (1966), Table 7, pg. 270). Measurements at each irradiance utilised a new sample of microalgal culture. On each occasion 4 mL of sample was carefully pipetted into the chamber and oxygen saturation levels reduced to 35 – 40% by gentle N₂ bubbling. The sample was then sealed in the chamber with a stopper and the light activated.

Light intensities were not applied in succession, but were randomly ordered, to reduce pre-treatment effects of earlier irradiances. Thick felt cloth was used to cover the sample chamber for dark respiration measurements (EPAR = 0 µmol quanta m⁻² s⁻¹). Three replicate series of P-E curve measurements were performed for each treatment condition. Three of the samples used in each of the light curve sequences were filtered onto 25 mm Whatman GF/C filters and frozen at -80°C for later determination of chl concentration (see Section 3.6 for chl extraction methods). The rate of oxygen evolution was calculated from the slope of the chart trace and normalised to chl-a concentration.

### 3.4 Carbon Assimilation

The ¹⁴C uptake methods of Lewis and Smith (1983) were used as a general guideline for measuring carbon assimilation. These are small volume, short incubation time methods and will be described below.

The photosynthetron used was constructed on site and used an array of dichroic halogen lamps to provide irradiance to the underside of a clear Perspex®-bottomed sample tray (Figure 3.7a). A honeycombed durable rubber mat was placed in the sample tray that provided a standard matrix in which to mount glass scintillation vials (Figure 3.7b). The sample tray was connected to a temperature-controlled water bath and a small electric water pump used to cycle 25°C water around the samples. This setup provided a light gradient that allowed samples to be placed in different positions in the matrix to achieve a range of irradiances. The height of the tray was set such that the
irradiance range experienced was \( \sim 50 - 1400 \ \mu\text{mol quanta m}^{-2} \text{s}^{-1} \); approximately the same as that provided by the ED-unit of the Water-PAM.

Prior to each experiment the irradiance was measured (using the US-SQS 4\( \pi \) sensor from the Water-PAM) at each position in the matrix and sample locations allocated to give an even distribution of light intensities. Each experiment involved the processing of:

- 40 sample vials with 3 mL of culture each, including 3 “dark” vials that were wrapped in foil.
- 5 time-zero vials, also containing 3 mL of culture.
- 10 specific activity checks.

Each of the sample and time zero vials were spiked with 150 \( \mu \text{L} \) of radioactive bicarbonate solution (NaH\(^{14}\text{CO}_3\)), equating to \( \sim 3 \ \mu\text{Ci} \) per vial. They were then sealed and incubation commenced, at which time the time-zero vials were terminated with 500
µL of 0.2 M hydrochloric acid each. During the remaining 20 min incubation the specific activity checks were processed.

A source vial of 3 mL deionised water spiked with 150 µL NaH$^{14}$CO$_3$ was used for the specific activity checks. Each specific activity check consisted of a 20 µL subsample from the source vial and 10 mL of Ultima Gold XR (Perkin-Elmer, U.S.A.) scintillation fluid.

At the conclusion of the incubation period the lights were turned off and the sample vials terminated by addition of 500 µL 0.2 M hydrochloric acid. Samples were left in a ventilated fume hood overnight to degas. The samples were then neutralised with 0.2M sodium hydroxide (NaOH), shaken and left to sit for 30 min before the addition of 10 mL Ultima Gold XR scintillation fluid. Samples were kept in the dark for ~18 h and then transferred to the scintillation counter (diagrammatic outline of experimental procedure is presented in Figure 3.8).

![Figure 3.8: Schematic representation of carbon assimilation experimental procedure. 40 samples, including three darkened vials, were spiked with NaH$^{14}$CO$_3$ solution (150 µL or ~3 µCi per vial), incubated for 20 min and terminated by acidification. Five time-zero controls were acidified at the commencement of the incubation period and transferred to a fume hood to degas. After degassing overnight all samples were neutralised with NaOH. Ten specific activity checks were processed per incubation. All samples and specific activity checks were left in the dark for ~18 h after the addition of scintillant, then transferred to a scintillation counter.](image-url)
Uptake of $^{14}$C in mg C m$^{-3}$ h$^{-1}$ was calculated following Strickland and Parsons (1972) (see Equation 35: where $R_s$ is the sample counts per minute, $R_b$ is the counts per minute of the time-zero controls, $R$ is the activity added to the sample vials (from activity checks), $W$ is the weight of carbon present in the water, $N$ is the incubation time in hours and 1.05 is a factor to account for $^{12}$C/$^{14}$C differences) before being normalised to chl-$a$ concentration and converted to units of µmol C (µg chl-$a$)$^{-1}$ h$^{-1}$.

$$^{14}C - \text{uptake} = \frac{(R_s - R_b) \times W \times 1.05}{R \times N}$$ (35)

### 3.5 Curve Fitting

Photosynthesis versus irradiance data from chl-fluorescence, oxygen evolution and carbon assimilation measurements were imported into Matlab® (The Mathworks, U.S.A.) and fitted to the model of Eilers and Peeters (1988) (see Table 1.2). Rather than fitting a curve to data from each individual P-E curve replicate and obtaining descriptive statistics from the resulting parameter (eg. $P_{\text{chl}}^{\text{max}}$, $E_k$ or $\alpha_{\text{chl}}$) populations, each curve was fitted to the data from all replicates and covariance matrix data used to calculate the 95% confidence interval of each parameter.

This method is preferable as it takes into account the error of the curve fit, unlike the usual method of fitting each individual curve which ignores the inherent error surrounding each replicate measure of each of the curve parameters. The Matlab® code used to calculate the 95% confidence intervals for P-E curve parameters is included below:

```matlab
sigma_sq_hat = sum(resid.^2)/(n-3);
D = diag(wts); % needed to calculate covariance matrix for weighted parameter estimates
covar = 1./n.*sigma_sq_hat.*(1./n.*jac'*D*jac)^-1; % the covariance matrix of the estimates

Pmax = (2.*sqrt(a.*c)+b).^(-1);
de_wrt_a = -(2.*sqrt(a.*c)+b).^2.*sqrt(a.*c).^(-1);
de_wrt_b = -(2.*sqrt(a.*c)+b).^2.*sqrt(a.*c).^(-1);
for delta = [de_wrt_a de_wrt_b de_wrt_c];
```

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3.6 Chlorophyll Determination

Where determination of chl content was necessary, material was collected onto 25 mm diameter GF/C (Whatman) filter under low pressure. For most samples there was a delay between collection and extraction. On such occasions sample filters were individually wrapped in foil and stored at -80°C to prevent chlorophyll degradation (Jeffrey et al. 1997). Extraction was performed with ice-cold 90% (v/v) acetone by manual grinding using a glass mortar and pestle. The homogenate was set in ice and away from light for 30 min and then clarified by centrifugation at 2100 rpm (1000 g) for 15 min in a Beckman GPR model centrifuge at 4°C. The centrifuge vials were then returned to ice and the supernatant used to determine chl-α content spectrophotometrically using the equations of Jeffrey and Humphrey (1975) for chromophyte algae containing chlorophylls α, c1 and c2 (Equation 36).

\[
Chl \alpha (\mu g mL^{-1}) = \left(11.47 E_{664} - 0.4 E_{630}\right) \times \frac{Volume \ extracted}{Volume \ filtered} 
\]  

(36)

Where \(E_y\) equals the absorbance of the extract through a 1 cm path length of the solution at wavelength \(y\) (nm). Spectrophotometric measurements were performed using a
Beckman DU-50 (UV-VIS) series spectrophotometer. Care was taken during measurements to avoid condensation on the surface of spectrophotometer cuvettes.
CHAPTER 4
Primary Production Estimates Using Chl-a Fluorescence

4.1 INTRODUCTION

In Chapter 1 quantum yield was defined as the ratio of gathered quanta to
product output (Equation 17). Quantum yield can be determined for individual steps in
photosynthesis such as electron transport through PSII ($\Phi_{\text{PSII}}$), oxygen evolution ($\Phi_{\text{O}_2}$)
and carbon fixation ($\Phi_{\text{CO}_2}$). The quantum yield for each of these steps is directly
influenced by, and must be lower than, the previous step in the chain of photosynthetic
events (Kroon et al. 1993). With greater distance from the start of the photosynthetic
chain $\Phi$ becomes more variable as the number of alternative pathways for electron flow
increases (see Chapter 1) (Behrenfeld et al. 2004).

Genty et al. (1989) demonstrated a linear relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{CO}_2}$ in
higher plants. Given that, in the cascading chain of photosynthetic events, electron
transport through PSII is more closely associated with oxygen evolution than C-
fixation, it could be expected that the relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{O}_2}$ is stronger
(less variable) than the $\Phi_{\text{PSII}}$: $\Phi_{\text{CO}_2}$ relationship. However, experimental comparison of
$\Phi_{\text{PSII}}$, $\Phi_{\text{O}_2}$ and $\Phi_{\text{CO}_2}$ in algae has given contradictory results (Masojidek et al. 2001).

As discussed in Chapter 1, chl-$a$ fluorescence measurements have a number of
distinct advantages over traditional $\text{O}_2$-evolution and C-fixation measures of
photosynthesis. They are:

- Extended isolation of samples in chambers is not required; eliminating
  potential “bottle effects”.
- Measurement of oxygen production is limited by low sensitivity (Gilbert et al.
  2000b).
• The constant stirring required for Clark-type polarographic O₂-electrodes has the potential to influence results by causing stress to the organism studied.

• Results are unambiguously related to gross photosynthesis, unlike O₂ and ¹⁴C-fixation measurements which reflect something between net and gross photosynthesis (Kolber and Falkowski 1993; Gilbert et al. 2000a).

• Expensive consumables such as radioisotope are not required.

• Special handling requirements and precautions such as those necessary for the use of radioisotope is not required. The ¹⁴C method also is labour intensive.

• The rapid nature of chlorophyll fluorescence measurements and the ability to measure chlorophyll fluorescence from a variety of platforms allows higher intensity spatial and temporal sampling and permits better comparison with physicochemical data (Kolber and Falkowski 1993; Cunningham 1996).

This study aimed to characterise the relationship between chlorophyll fluorescence measures of photosynthesis and the more traditional measures of O₂-evolution and C-fixation in more detail. A review of the current understanding of the relationship between these parameters is presented below.

4.1.1 Chl-a Fluorescence Vs. Oxygen evolution

ETR as measured by chl fluorescence reflects gross PSII-dependent electron transport, while photosynthetic O₂-evolution represents net photosynthesis (ETR minus losses due to cyclic electron transport around PSII and oxygen consuming mechanisms such as the Mehler reaction and photorespiration; see Chapter 1) (Masojídek et al. 2001).

Four stable charge separations at RCII (RCII⁺ → RCII⁻) are required to oxidise the PSII-associated Mn-complex to the extent that it will accept an electron from each
of two water molecules and evolve oxygen (O₂) and four hydrogen ions (H⁺). The equivalent number of charge separations must take place at PSI in order to maintain this electron transport and hence a minimum of eight photons are required to evolve each molecule of O₂ on a continuing basis. As a result the minimum quantum requirement of O₂-evolution (ΦO₂⁻¹) is at least 8-fold the value of the quantum requirement of electron transport through PSII (ΦPSII⁻¹) (Kroon et al. 1993).

Based on these relationships, two similar formulae have been proposed for the prediction of photosynthetic oxygen evolution from chl-α fluorescence measurements. Equations can be specific to particular chl-α fluorescence methodologies and the paper by Kromkamp and Forster (2003) explores some of the differences encountered between single- and multiple-turnover protocols. Only those calculations relevant to multiple-turnover techniques are included here. Equation 37 describes the most commonly used function to estimate gross oxygen evolution from chl fluorescence data, where ΓO₂ is the stoichiometric ratio of O₂ evolved per electron generated at PSII (GOEf, with units of µmol O₂ m⁻² s⁻¹) (Kroon et al. 1993; Gilbert et al. 2000b; Toepel et al. 2004). Also, the parameter ΓO₂ may be replaced with ΦO₂ on the assumption that each photon delivered to the RCII successfully results in primary charge separation and the removal of an electron from the oxygen evolving complex. The estimates of PSII quantum efficiency (Fq/Fm), absorbed light (Qphar) and fraction of light directed to PSII (fII) have been defined previously (Chapter 1).

\[
GOE_f = \left(\frac{F_q}{F_m}\right) \times Q_{phar} \times f_{II} \times \Gamma_{O₂}
\]  

(37)

GOEf can then be normalised to chl-α to arrive at a theoretical chl-α specific rate of oxygen evolution (GOEf[chl]). GOE[chl] can also be calculated using biophysical variables (Equation 38) (Kromkamp and Forster 2003):
\[ GOE_{j}^{chl} = \left( \frac{F_{q}'}{F_{m}'} \right) \times E_{PAR} \times \sigma_{PSII} \times \eta_{PSII} \times 0.0243 \times \Gamma \]  \hfill (38)

This is based on Equation 29 (p. 65), however the conversion factor has been modified to arrive at units of \( \mu \text{mol O}_2 \ (\mu \text{g chl-a})^{-1} \text{ h}^{-1} \). Equations 37 and 38 can be simplified to:

\[ GOE_{j}^{chl} = ETR \times \Gamma \]  \hfill (39)

The particular set of variables used often depends on equipment available, with some of the more difficult parameters (e.g. \( f_{II} \), \( \eta_{PSII} \), \( a_{PSII}^{*} \)) applied primarily in laboratory-based studies.

As mentioned earlier, \( \text{O}_2 \)-evolution is closely coupled to PSII electron transport and, for this reason, a close relationship between these parameters could be expected. While a number of studies have shown such a correlation for a range of taxa, under non-optimal conditions variable and non-linear relations between \( \text{O}_2 \) and chl fluorescence measures have been reported (Table 4.1 and Flameling and Kromkamp 1998). Measurements on various microalgal species by Geel (1997) indicated that linearity extends to an irradiance which is 2 to 5 times higher than the growth irradiance, with non-linear patterns at higher irradiances resulting from a decrease in the comparative rate of \( \text{O}_2 \)-evolution. A decrease in the rate of \( \text{O}_2 \)-evolution without a matching decrease in PSII ETR (or \( GOE_{j} \) over-estimating \( P_{\text{O}_2} \)) appears to indicate either the presence of oxygen consuming processes such as the Mehler reaction or photorespiration, or cyclic electron flow around PSII (Badger et al. 2000; Gilbert et al. 2000a; Ruuska et al. 2000).

There has been some debate about which process is the most likely cause of non-linearity between these measures of photosynthesis (Geel et al. 1997; Flameling and Kromkamp 1998; Badger et al. 2000; Longstaff et al. 2002; Beer and Axelsson 2004). Geel (1997) excluded photorespiration as the major cause and data collected in nonphotorespiratory conditions (5% CO\(_2\)) suggest that \( \text{O}_2 \) reduction by the Mehler
reaction could play a major role in the development of a curvilinear relationship between chl-fluorescence measurements and O₂-evolution (Seaton and Walker 1990; Schreiber et al. 1995a). However, more recent measurements using mass spectrometry have pointed to cyclic electron flow around PSII or non-photochemical energy quenching within PSII centres (Weis and Berry model) as more likely causes of non-linearity than O₂-consuming processes (Weis and Berry 1987; Morris and Kromkamp 2003). Evidence for this mechanism had previously been presented by Falkowski et al. (1986b) and Prášil et al. (1996), however this was considered inconclusive by Geel et al. (1997).

Table 4.1: Summary of studies investigating the relationship between chlorophyll fluorescence- and oxygen evolution-based estimates of photosynthesis. POE = polarographic oxygen electrode, MS = mass spectrometer (¹⁸O₂), FRRF = fast repetition rate fluorometer,

<table>
<thead>
<tr>
<th>Species / Community</th>
<th>Parameters compared</th>
<th>Relationship</th>
<th>Study</th>
<th>Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₄-like seagrass</td>
<td>ETR:Pₒ₂</td>
<td>Linear (r²=0.93)</td>
<td>Beer et al. (1998)</td>
<td>PAM : POE</td>
</tr>
<tr>
<td>C₃-like seagrasses</td>
<td>ETR:Pₒ₂</td>
<td>Curvilinear</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Halodule wrightii</em></td>
<td>ETR:Pₒ₂</td>
<td>Curvilinear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulva spp.</td>
<td>ETR:Pₒ₂</td>
<td>Linear (r²&gt;0.92)</td>
<td>Beer et al. (2000)</td>
<td>PAM : POE</td>
</tr>
<tr>
<td>Ulva lactuca</td>
<td>ETR:Pₒ₂</td>
<td>Curvilinear (steady state)</td>
<td>Longstaff et al. (2002)</td>
<td>PAM : POE</td>
</tr>
<tr>
<td>Ulva spp.</td>
<td>ETR:Pₒ₂</td>
<td>*Non-linear – Linear (r²=0.69)</td>
<td>Figueroa et al. (2003)</td>
<td>PAM: POE</td>
</tr>
<tr>
<td>Porphyra leucostica</td>
<td>Φᵦᵦ/Φₒ₂</td>
<td>Exponential (r²=0.92)</td>
<td>Franklin &amp; Badger (2001)</td>
<td>PAM : MS</td>
</tr>
<tr>
<td>Porphyra columnina</td>
<td>Φᵦᵦ/Φₒ₂</td>
<td>Linear &lt; Φₒ₂=0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulva australis</td>
<td>Φᵦᵦ/Φₒ₂</td>
<td>Curvilinear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zonaria crenata</td>
<td>ETR:Pₒ₂</td>
<td>Linear (RLC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>GOE₂:Pₒ₂</td>
<td>Curvilinear (r²=0.87)</td>
<td>Toepel et al. (2004)</td>
<td>PAM : POE</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>ETR:Pₑₑ₂</td>
<td>Curvilinear</td>
<td>Masojídek et al. (2001)</td>
<td>PAM : micro-optode</td>
</tr>
<tr>
<td>Tetraselmis sp.</td>
<td>ETR:Pₒ₂</td>
<td>~Linear (low/moderate PAR)</td>
<td>Geel et al. (1997)</td>
<td>PAM : POE</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>Phaeodactylum tricornutum</td>
<td>Linear</td>
<td>Suggett et al. (2003)</td>
<td>FRRF : MS</td>
</tr>
</tbody>
</table>

* Differences based on changes in measurement protocol
Non-linearity of the ETR: $P_{O_2}^{chl}$ or $\Phi_{PSII} \Phi_{O_2}$ relationship may also be observed at low irradiance. While the roles of O$_2$-consuming processes and cyclic electron flow around PSII at high irradiance may be an issue of some contention, there is a general understanding that the most likely causes at low irradiance are either variable, light-stimulated rates of respiration or cyclic electron flow around PSII (Morris and Kromkamp 2003; Schreiber 2004).

Measurements with a PAM fluorometer are fundamentally different to those obtained using gas exchange or oxygen electrode equipment. PAM fluorometry provides an immediate measure of responses following each alteration in irradiance, while obtaining a similar O$_2$ measurement can take a few minutes. O$_2$-evolution is also more influenced by temperature changes and short-term changes in temperature may affect the relationship between $P_{O_2}^{chl}$ and ETR (Morris and Kromkamp 2003).

4.1.2 Chl-a Fluorescence Vs. Radiocarbon Uptake

The $^{14}$C incubation technique can only be regarded as a crude estimate of algal primary production (Peterson 1980). The photosynthetic activity in situ may be very different from the rates extrapolated from incubations as there will be unavoidable changes in the chemical and physical environment during measurement (Gilbert et al. 2000a; Glud et al. 2002). The $^{14}$C-technique, however, has been most widely adopted for the estimation of primary production because it is sensitive and provides a direct measure of carbon fixation.

In 1931 when Hans Kautsky and A. Hirsch published a short paper in which they related chlorophyll fluorescence to carbon assimilation (Kautsky and Hirsch 1931). However, it was not until Weis and Berry (1987) developed a semi-empirical equation of the relationship that the rate of C-fixation was predicted from chlorophyll fluorescence data. This model was based on the assumption that quenching occurs at the
PSII reaction centre. Another model, now widely used, was proposed in 1989 and based on the premise that quenching occurs in the antennae (Genty et al. 1989, 1990). Genty et al. (1989) found that the chlorophyll fluorescence parameter $F_q/F_m$ could be used to predict $\Phi_{PSII}$ and was positively related to $\Phi_{CO2}$ in a linear fashion.

Depending on the type of information required, there are a number of ways in which photosynthetic activity data from chlorophyll fluorescence measurements can be related to the estimation of primary production by carbon fixation. One of the most simple and common methods is to compare $\Phi_{PSII}$ with $\Phi_{CO2}$. $\Phi_{CO2}$ is often calculated from $^{14}$C data by following Equation 40, however, this does not take into account that not all photons incident on a cell will be absorbed and better estimates are generated when absorbed irradiance rather than incident irradiance is used (Equation 41).

$$\Phi_{CO2} = \frac{\text{Carbon assimilation}}{E_{PAR}}$$  \hspace{1cm} (40)

$$\Phi_{CO2} = \frac{\text{Carbon assimilation}}{E_{PAR} \times \alpha_{chl}} \approx \frac{\text{Carbon assimilation}}{Q_{phar}}$$  \hspace{1cm} (41)

Following the Z-scheme for electron transport, $\Phi_{CO2}$ can reach a maximum value of 0.125 mol C.(mol quanta)$^{-1}$ because each carbon molecule fixed requires the energy of eight photons to move four electrons through each of the two photosystems (assuming a perfect quantum yield of charge separation) (Seaton and Walker 1990). In favourable conditions $\Phi_{CO2}$ in higher plants is often close to 0.107 mol C.(mol quanta)$^{-1}$ (Seaton and Walker 1990), or 0.10 mol C.(mol quanta)$^{-1}$ as a result of nitrate assimilation (Badger et al. 2000), while in situ values obtained for phytoplankton assemblages have been measured at 0.011 mol C.(mol quanta)$^{-1}$ (Gilbert et al. 2000a). In suboptimal conditions a greater number of electrons are moved per carbon fixed, resulting in lower $\Phi_{CO2}$ values (Baker and Oxborough 2004).
Although CO$_2$ reduction is the major sink for photosynthetically generated electrons, and CO$_2$ reduction seems to be closely linked with PSII activity, a number of studies have found that the linear relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{CO}_2}$ falters under certain conditions. One particular example that is especially relevant for C$_3$ plants is when they are exposed to high O$_2$ or low CO$_2$ conditions and photorespiration is enhanced (Harbinson et al. 1990; Krall and Edwards 1990; Cornic and Briantais 1991; Krall and Edwards 1991; Badger et al. 2000). Given that $\Phi_{\text{PSII}}$, as estimated by $F'_q/F_m$, is a measure of all PSII activity (not only that component whose ultimate fate is linear electron transport) it can be considered an indicator of photorespiration and other forms of O$_2$-dependent electron flow as well as C-fixation (Schreiber et al. 1995a). Exposure to supraoptimal temperatures and conditions where nitrate reduction is enhanced have also been shown to cause a greater decrease in $\Phi_{\text{CO}_2}$ than $\Phi_{\text{PSII}}$ (Krall and Edwards 1991; Biehler and Fock 1995).

$\Phi_{\text{PSII}}$ has also previously been correlated with $^{14}$C-determined primary productivity on the basis that such correlations could be used to predict primary production from chlorophyll fluorescence measurements given they are performed within the same stable microalgal community as the initial calibration (Öquist et al. 1982).

Krall and Edwards (1991), while studying the relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{CO}_2}$, embarked on a somewhat trivial exercise and converted $\Phi_{\text{CO}_2}$ to a quantum yield value based on photons absorbed only at PSII ($\Phi_{\text{CO}_2(PSII)}$) (Equation 42). The theoretical minimum of $\Phi_{\text{CO}_2(PSII)}$ is therefore 0.25 (four electrons through PSII per CO$_2$ fixed). Generally, however, this introduces an assumption that half the absorbed photons go to PSII ($f_{\text{II}} = 0.5$).
Measurements of absolute ETR through PSII can be used to predict the rate of primary production normalised to chl-a ($P_{f}^{chl}$), with units of $\mu$mol C ($\mu$g chl-a)$^{-1}$ h$^{-1}$.

$$P_{f}^{chl} = \left(\frac{F_{m}'}{F_{m}}\right) \times E_{P4R} \times \sigma_{PSII} \times \eta_{PSII} \times 0.0243 \times \Phi_{CO2(PSII)} = ETR \cdot \Phi_{CO2(PSII)}$$

(43)

Where $\Phi_{CO2(PSII)}$ is often taken to be $\Phi_{O2}^{max}$ (0.25) and the inverse of the O$_2$:C photosynthetic quotient, which is commonly assumed to be either 1.1 mol O$_2$ (mol C)$^{-1}$ (Corno et al. 2005) or 1.2 mol O$_2$ (mol C)$^{-1}$ (Baker and Oxborough 2004; Melrose et al. 2006). Thus, taking the latter measure:

$$P_{f}^{chl} = ETR \times 0.208$$

(44)

While studies relating $P_{f}^{chl}$ to $P_{f}^{chl}$ have often found a linear relationship that can be quite strong (Table 4.2), many researchers have found mild to severe non-linearity in this relationship (Geel 1997; Baker and Oxborough 2004), especially in more severe environments (Glud et al. 2002). Loss of linearity has been reported to occur in extreme low- or high-light environments (Seaton and Walker 1990; Hartig et al. 1998) and Barranguet and Kromkamp (2000b) found a deterioration in the $P_{f}^{chl}$ : $P_{f}^{chl}$ ratio at irradiances above $E_k$ for C-fixation. PSII activity has been found to be more variable in algae compared to higher plants and, consequently, the use of $\Phi_{PSII}$ to derive algal productivity measurements should be treated with more caution (Franklin and Badger 2001).
Table 4.2: Summary of studies investigating the relationship between chlorophyll fluorescence- and carbon-based estimates of photosynthesis. The species or community studied is provided: where IAC = ice algae community, MPB = microphytobenthos and NPC = natural phytoplankton community. Techniques include: PAM = Pulse Amplitude Modulated fluorometer; IF = Integrated fluorometer; PPF = Pump and Probe fluorometer; FRRF = Fast Repetition Rate fluorometer and incubation times are for $^{14}$C-uptake measurements.

<table>
<thead>
<tr>
<th>Species / Community</th>
<th>Parameters compared</th>
<th>Relationship</th>
<th>Study</th>
<th>Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phaseolus sp.</td>
<td>rETR:ETR$_{CO_2}$</td>
<td>Linear</td>
<td>Weis &amp; Berry (1987)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Helianthus sp.</td>
<td>rETR:ETR$_{CO_2}$</td>
<td>Linear</td>
<td>Genty et al. (1989)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Maize</td>
<td>Φ$<em>{PSII}$:Φ$</em>{CO_2}$</td>
<td>Linear/curvilinear</td>
<td>Harbinson et al. (1990)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Barley</td>
<td>Φ$<em>{PSII}$:Φ$</em>{CO_2}$</td>
<td>Linear/curvilinear</td>
<td>Cornic &amp; Briantais (1991)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>Φ$<em>{PSII}$:Φ$</em>{CO_2}$</td>
<td>Linear/curvilinear</td>
<td>Harbinson et al. (1990)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Hedra helix</td>
<td>Φ$<em>{PSII}$:Φ$</em>{CO_2}$</td>
<td>Linear</td>
<td>Gentry et al. (1989)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Phaseolus sp.</td>
<td>Φ$<em>{PSII}$:Φ$</em>{CO_2}$</td>
<td>Linear</td>
<td>Gentry et al. (1989)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>Maize</td>
<td>ETR:P$_{chl}^{et}$</td>
<td>Linear ($r^2=0.990$)</td>
<td>Earl &amp; Tollenaar (1998)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>IAC</td>
<td>rETR:P$_{chl}^{et}$</td>
<td>Non-linear</td>
<td>Glud et al. (2002)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>MPB</td>
<td>ETR:P$_{chl}^{et}$</td>
<td>Linear ($r^2=0.886$)</td>
<td>Hartig et al. (1998)</td>
<td>PAM: Gas exchange</td>
</tr>
<tr>
<td>MPB</td>
<td>Φ$<em>{PSII}$:Φ$</em>{CO_2}$</td>
<td>Curvilinear</td>
<td>Barranguet &amp; Kromkamp (2000)</td>
<td>PAM: 1 h incubation</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>A$<em>{DCMU}$:P$</em>{chl}^{et}$</td>
<td>Linear – non-linear</td>
<td>Bates &amp; Platt (1984)</td>
<td>IF: 15 min incubation</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>rETR: growth rate</td>
<td>Linear</td>
<td>Hofstraat et al. (1994)</td>
<td>PAM: Flow Cytometer</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>Φ$_{PSII}$:P</td>
<td>Linear ($r^2=0.88 – 0.97$)</td>
<td>Öquist et al. (1982)</td>
<td>IF: 2-4 h incubation</td>
</tr>
<tr>
<td>NPC</td>
<td>P$<em>{chl}^{et}$:P$</em>{chl}^{et}$</td>
<td>Linear ($r^2=0.74$)</td>
<td>Kolber &amp; Falkowski (1993)</td>
<td>PPF: 4 h incubation</td>
</tr>
<tr>
<td>NPC</td>
<td>ETR:P$_{chl}^{et}$</td>
<td>Poor correlation</td>
<td>Gilbert et al. (2000)</td>
<td>PAM: 3 h incubation</td>
</tr>
<tr>
<td>NPC</td>
<td>P$<em>{chl}^{et}$:P$</em>{chl}^{et}$</td>
<td>Linear ($r^2=0.591$)</td>
<td>Suggett et al. (2001)</td>
<td>FRRF: 1 h incubation</td>
</tr>
<tr>
<td>NPC</td>
<td>P$<em>{chl}^{et}$:P$</em>{chl}^{et}$</td>
<td>Linear ($r^2=0.92$)</td>
<td>Moore et al. (2003)</td>
<td>FRRF: 1-2 h incubations</td>
</tr>
<tr>
<td>NPC</td>
<td>P$<em>{chl}^{et}$:P$</em>{chl}^{et}$</td>
<td>“consistent”</td>
<td>Smyth et al. (2004)</td>
<td>FRRF: 24 h incubations</td>
</tr>
<tr>
<td>NPC</td>
<td>GOE$<em>{f}$:P$</em>{chl}^{et}$</td>
<td>Linear ($r^2=0.906$)</td>
<td>Corno et al. (2006)</td>
<td>FRRF: Dawn-Dusk incubation</td>
</tr>
</tbody>
</table>

A close relationship between chlorophyll fluorescence- and carbon-based estimates of photosynthesis may be unexpected for a number of reasons:

- Differences in sample treatment (often including the spectral quality of actinic light).
- Chl fluorescence is a gross measure of electron transport through PSII whereas $^{14}$C incubations may provide a gross or net measure (or anywhere in between) of C-fixation (Harris 1980; Grobbelaar et al. 1992; Laws et al. 2002).

- High potential for cyclic electron transport (see Chapter 1.2.6).

- Reductant produced from the light reactions of photosynthesis can be used for metabolic processes other than C-fixation (Kolber and Falkowski 1993; Barranguet and Kromkamp 2000b).

Simply put, the fluorescence method gives photosynthetic potential while the $^{14}$C method is an approximate measure of the actual rate of photosynthesis (Öquist et al. 1982). Intercalibration between the methods for each study in the laboratory or in the field is one way of assessing the difference between potential and realised photosynthesis and providing a conversion factor for $P_f^{ch}$ calculation. However, this is problematic given the concerns with $^{14}$C measures of C-fixation (Öquist et al. 1982). Some researchers have preferred to use element analysis to measure carbon and nitrogen assimilation (Toepel et al. 2004).

While there has been moderate success at describing relationships between $\Phi_{PSII}:\Phi_{CO2}$, ETR:$P^{ch}$ and $P_f^{ch}:P^{ch}$; there has been much less success relating the P-E curve parameters between the different measurement techniques (Ralph Smith, pers. comm.). Barranguet and Kromkamp (2000b) found no significant correlation for $P^{ch}_{max}$, $\alpha$, or $E_k$ between PAM and C-fixation methods. However, other studies have found significant correlations, indicating that the same basic property of the resident algal population may have been measured (Boyd et al. 1997; Hartig et al. 1998; Suggett et al. 2001). Comparing P-E curve parameters between techniques must still be treated with the utmost caution.
The purpose of this study was to determine if, under controlled and favourable conditions, a linear relationship between oxygen evolution or carbon fixation and predicted values from chl-a fluorescence measurements could be achieved. Three species from common marine phytoplankton taxa were used as study organisms to gain a broad representation of the relationship between predicted (chl-a fluorescence) and observed measures of primary production. The potential for methodology, such as sample adaptation state and Light Width, to influence this relationship was also examined.

4.2 METHODS

All measurements were performed on unialgal cultures of Isochrysis galbana (Prymnesiophyceae), Nannochloropsis oculata (Eustigmatophyceae) and Chaetoceros muelleri (Bacillariophyceae). These cultures were grown as described in the General Methods chapter (Chapter 3).

Methods for chlorophyll fluorescence, O₂-evolution and C-fixation measurements are provided in Chapter 3. Data were collected at the same time of day for each experiment in order to minimise the impact of diurnal changes in photosynthetic performance on results.

Chlorophyll fluorescence RLC, LC(3) and LC(20) data were converted to absolute ETR by Equation 29 however the conversion factor of 0.00675 was changed to 0.0243 to give ETR units of \( \mu \text{mol electrons (µg chl-a)}^{-1} \text{h}^{-1} \). This method, using biophysical rather than optical parameters, was chosen as \( \sigma_{\text{PSII}} \) could be measured for each of the conditions more easily (and frequently) than \( \tilde{\zeta}_{\text{chl}} \) and it is directly related to PSII. Unfortunately the density of RCIIs (\( \eta_{\text{PSII}} \)) was not able to be estimated via flash oxygen yield measurements. This parameter has been found to be relatively constant at 0.002 mol RCII (mol chl)⁻¹ when the minimum theoretical quantum requirement at PSII
for O₂-evolution (QR_{O₂} = 4) is applied or 0.004-0.005 mol RCII (mol chl)^{-1} when a Z-
scheme quantum requirement including 25% inefficiency is applied (QR_{O₂} = 10) (Ley
and Mauzerall 1982; Suggett et al. 2004). For the purpose of this study η_{PSII} has been
assumed based on results from Dubinsky et al. (1986) and Fisher et al. (1996) (Table
4.3).

Table 4.3: Assumed values of η_{PSII} (mol RCII (mol chl)^{-1}) for each species at both growth irradiances.
Based on a quantum requirement for O₂-evolution of 10 and η_{PSII} values (taken from Dubinsky et al.
(1986) and Fisher et al. (1998))

<table>
<thead>
<tr>
<th>Growth PAR (µmol quanta m⁻² s⁻¹)</th>
<th>C. muelleri</th>
<th>I. galbana</th>
<th>N. oculata</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>4.3 × 10⁻³</td>
<td>4.6 × 10⁻³</td>
<td>3.7 × 10⁻³</td>
</tr>
<tr>
<td>500</td>
<td>7.0 × 10⁻³</td>
<td>9.5 × 10⁻³</td>
<td>3.7 × 10⁻³</td>
</tr>
</tbody>
</table>

The emission spectra of light sources were measured using an Ocean Optics
USB2000 spectrometer (100 µm entrance slit) fitted with grating No.2, the L2 detector
collection lens, an OFLV-350-1000 order-sorting detector filter and a 400 µm fibre-
optic probe. Data with a resolution on 0.34 nm was captured using the OOIBase32
program provided by Ocean Optics. Five scans were performed for each light source
and the results averaged to produce a final curve (Figure 4.1).

Figure 4.1: Relative emission spectra for each of the light sources used. PAM LEDs used for chl
fluorescence measurements have an emission peak at 666 nm. The photosynthetron halogen lamps were
those used in C-fixation measurements and the projector halogens were used in the slide projector for O₂-
evolution measurements. Curves represent an average of five measurements and area under each curve
equals 1.
Absorption spectra were obtained for each species at both Low Light (LL; 50 \mu\text{mol quanta m}^{-2}\text{s}^{-1}) and High Light (HL; 500 \mu\text{mol quanta m}^{-2}\text{s}^{-1}) growth conditions (Figure 4.2). Measurements were performed with cells in suspension using a GBC 916 UV/VIS dual beam spectrophotometer equipped with a Taylor-type integrating sphere. Quartz glass 10 mm cuvettes were used to hold the sample against the integrating sphere. The absorption of each sample was obtained, using culture media as a reference and scanned from 200-900 nm with a spectral resolution of 1.3 nm. Between sample scans, the reference cell was removed from the spectrophotometer and placed in a room temperature water bath to reduce temperature effects in the scans. After absorbance values were normalised to zero at 750 nm the chl-specific absorption coefficient ($a_{chl}^{\lambda}$) with units of m$^{-1}$ was calculated using Equation 5. These values were then used in the calculation of $Q_{phar}$ (Equation 7). Spectrally averaged (400 – 700 nm) $a_{chl}^{\lambda}$ values, $\bar{a}_{chl}$, are presented in Table 4.4

Photosystem II functional absorption cross-section ($\sigma_{PSII}$) data were collected using a FL-3000 dual modulated PAM fluorometer (Photon Systems Instruments, Czech Republic). Since $\sigma_{PSII}$ is spectrally dependent and the FL-3000 fluorometer has a different emission spectrum to the Water-PAM fluorometer an extra conversion factor was applied to the ETR component of Equations 39 ($G\text{OE}_{f}^{chl}$) and 44 ($P_{f}^{chl}$) based on the respective absorption and emission spectra (factor ranged from 2.036 – 2.134).
Figure 4.2: Chlorophyll-a specific absorption spectra for (a) Chaetoceros muelleri, (b) Isochrysis galbana and (c) Nannochloropsis oculata measured in suspension with an integrating sphere attachment. Solid line represents high light acclimated cells while dotted line represents low light acclimated cells.
Table 4.4: \( \sigma^{\text{chl}} \) values, with units of m\(^2\) (mg chl-a)\(^{-1}\), calculated from absorption and emission spectra. Due to the different spectral quality of the light sources used in the Water-PAM (red LEDs), slide projector (halogen) for O\(_2\)-evolution measurements and photosynthetron (halogen) for C-fixation measurements, three values are given for each condition.

<table>
<thead>
<tr>
<th>Growth Condition Method</th>
<th>Growth Condition</th>
<th>Method</th>
<th>C. muelleri</th>
<th>I. galbana</th>
<th>N. oculata</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Light</td>
<td></td>
<td>PAM</td>
<td>8.73 \times 10^{-3}</td>
<td>9.42 \times 10^{-3}</td>
<td>1.16 \times 10^{-2}</td>
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<tr>
<td></td>
<td></td>
<td>O(_2)</td>
<td>6.91 \times 10^{-3}</td>
<td>8.26 \times 10^{-3}</td>
<td>7.32 \times 10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-fix</td>
<td>6.94 \times 10^{-3}</td>
<td>8.24 \times 10^{-3}</td>
<td>7.37 \times 10^{-3}</td>
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<tr>
<td>Low Light</td>
<td></td>
<td>PAM</td>
<td>8.26 \times 10^{-3}</td>
<td>9.35 \times 10^{-3}</td>
<td>7.95 \times 10^{-3}</td>
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<tr>
<td></td>
<td></td>
<td>O(_2)</td>
<td>6.34 \times 10^{-3}</td>
<td>7.57 \times 10^{-3}</td>
<td>4.28 \times 10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-fix</td>
<td>6.40 \times 10^{-3}</td>
<td>7.62 \times 10^{-3}</td>
<td>4.68 \times 10^{-3}</td>
</tr>
</tbody>
</table>

Data from each method was cross-referenced to predicted values obtained from chl-fluorescence at the same \( Q_{\text{phar}} \). Linear regressions were fitted to linear portions of the relationship (usually below the saturation irradiance) or between the origin and the first data point if a linear portion was not observed with the data collected. Modelled data was also plotted.

### 4.3 Results

The relationship between measured O\(_2\)- evolution (\( P^{\text{chl}}_{O2} \)) and estimates of O\(_2\)- evolution from chl-fluorescence data (\( \text{GOE}_{f}^{\text{chl}} \)) was variable between species and growth treatment. Use of different chl-fluorescence methodologies also had some impact on the outcome, however this appeared minimal compared to species and growth treatment factors.

The \( P^{\text{chl}}_{O2}:\text{GOE}_{f}^{\text{chl}} \) relationship observed for *Chaetoceros muelleri* grown in low light conditions was non-linear (Figure 4.3a, c). Plotting the line from the origin to the first point on these graphs yields slopes greater than one (Table 4.5), however, as irradiance and photosynthetic rate increased the \( P^{\text{chl}}_{O2}:\text{GOE}_{f}^{\text{chl}} \) ratio became less than one.
Figure 4.3: Chl-specific gross oxygen evolution by the diatom *Chaetoceros muelleri* as predicted from chl-fluorescence measurements (GOE$_{chl}$) plotted as a function of measured chl-specific oxygen evolution (P$_{chl}$O$_2$) at the same $Q_{phar}$. Closed circles (●) and solid lines (—) represent results and models (Eilers and Peeters 1988), respectively, from 15 min dark adapted samples. Open circles (○) and dotted lines (·····) represent results and models obtained from light adapted samples. Results for Linear regressions through the initial data points or the linear portion of the relationship have been plotted to show the initial slope of the relationship: medium dashed lines (—–) and double-dotted lines (····) represent the linear regression for dark adapted and light adapted samples respectively. Measurements were taken after acclimation to both low light (a, c) and high light (b, d) growth conditions (50 and 500 µmol quanta m$^{-2}$ s$^{-1}$ respectively). Two chl-fluorescence methods are represented: data from rapid light curves (RLC) with 5 s at each light interval (a, b) plus data from light curves with 3 min at each light interval (LC(3)) representing a semi steady-state condition (c, d). For P$_{chl}$O$_2$ and LC(3) estimates of GOE$_{chl}$, $n = 3$ and error bars = range; $n = 7 – 10$ for RLC data and error bars = se.

In contrast to the observations for cells grown in low light, *C. muelleri* grown in high light conditions exhibited a curvilinear relationship with an initial slope less than one (Figure 4.3b,d and Table 4.5). The trend for greater overestimation of O$_2$-evolution by GOE$_{chl}$ at higher irradiance was still apparent and Figure 4.3 indicates that this overestimation was greatest when RLCs were performed using light adapted samples.
Figure 4.4: Chl-specific gross oxygen evolution by Isochrysis galbana as predicted from chl-fluorescence measurements (GOE$_{f\text{chl}}$) plotted as a function of measured chl-specific oxygen evolution (P$_{chl\text{O}_2}$) at the same Q$_{phar}$. Closed circles (●) and solid lines (—) represent results and models (Eilers and Peeters 1988), respectively, from 15 min dark adapted samples. Open circles (○) and dotted lines (····) represent results and models obtained from light adapted samples. Results for Linear regressions through the initial data points or the linear portion of the relationship have been plotted to show the initial slope of the relationship: medium dashed lines (– – –) and double-dotted lines (– · · –) represent the linear regression for dark adapted and light adapted samples respectively. Measurements were taken after acclimation to both low light (a, c) and high light (b, d) growth conditions (50 and 500 µmol quanta m$^{-2}$ s$^{-1}$ respectively). Two chl-fluorescence methods are represented: data from rapid light curves (RLC) with 5 s at each light interval (a, b) plus data from light curves with 3 min at each light interval (LC(3)) representing a semi steady-state condition (c, d). For P$_{chl\text{O}_2}$ and LC(3) estimates of GOE$_{f\text{chl}}$, $n = 3$ and error bars = range; $n = 7 – 10$ for RLC data and error bars = se.

The P$_{chl\text{O}_2}$:GOE$_{f\text{chl}}$ relationships in Isochrysis galbana (Figure 4.4) were similar to those described for C. muelleri. High light adapted I. galbana displayed, like C. muelleri, a curvilinear P$_{chl\text{O}_2}$:GOE$_{f\text{chl}}$ relationship (Figure 4.4b,d); however the deviation towards greater overestimation of P$_{chl\text{O}_2}$ at higher irradiances was not as great. For low light adapted I. galbana cultures GOE$_{f\text{chl}}$ underestimated P$_{chl\text{O}_2}$ at low irradiances and then, as P$_{chl\text{O}_2}$ reached its maxima and GOE$_{f\text{chl}}$ continued to increase, began to
overestimate $P_{chlo2}$ at higher irradiances (Figure 4.4a,c). This was more pronounced when chl-fluorescence measurements were taken in semi steady-state (Figure 4.4c) as induction of electron transport through PSII occurred.

Polarographic oxygen electrode measurements of oxygen evolution were more variable than measures of photosynthesis by other methods. This was especially the case with measurements performed on cultures of *Nannochloropsis oculata*. This high variability impacts interpretation of Figure 4.5, however, results suggest that in the case of *N. oculata* a greater departure from linearity was observed in high light adapted cultures than in low light adapted cultures (Figure 4.5b,d vs. a,c). *N. oculata* also differed from the other species measured in that $GOE_{fchl}$ almost always underestimated $P_{chlo2}$ and this discrepancy was greater in high light rather than low light adapted cells (Figure 4.5 and Table 4.5).

It was found that the relationship between $P_{chl}$ and $P_{fchl}$ was more consistent and exhibited greater linearity than $P_{chlo2}$ and $GOE_{fchl}$ (see Table 4.5 vs. Table 4.6 and Figure 4.6 - Figure 4.8). This was unexpected considering the factors outlined in the introduction of this chapter.
Figure 4.5: Chl-specific gross oxygen evolution by *Nannochloropsis oculata* as predicted from chl-fluorescence measurements (GOE*_{chl}*) plotted as a function of measured chl-specific oxygen evolution (P*_{chl}O_2*) at the same Q*_{phar}*. Closed circles (●) and solid lines (——) represent results and models (Eilers and Peeters 1988), respectively, from 15 min dark adapted samples. Open circles (○) and dotted lines (···· ·) represent results and models obtained from light adapted samples. Results for Linear regressions through the initial data points or the linear portion of the relationship have been plotted to show the initial slope of the relationship: medium dashed lines (– – –) and double-dotted lines (– · · –) represent the linear regression for dark adapted and light adapted samples respectively. Measurements were taken after acclimation to both low light (a, c) and high light (b, d) growth conditions (50 and 500 µmol quanta m\(^{-2}\) s\(^{-1}\) respectively). Two chl-fluorescence methods are represented: data from rapid light curves (RLC) with 5 s at each light interval (a, b) plus data from light curves with 3 min at each light interval (LC(3)) representing a semi steady-state condition (c, d). For P*_{chl}O_2* and LC(3) estimates of GOE*_{chl}*, n = 3 and error bars = range; n = 7 – 10 for RLC data and error bars = se.

For cultures of *C. muelleri* grown in low light conditions, all chl-fluorescence methods resulted in an underestimation of P*_{chl}*. The slope of the linear portion of the curve was highest when the LC(20) methodology was used (Table 4.6, Figure 4.6e), indicating greatest underestimation occurred when seeking to emulate the sample exposure conditions of the photosynthetron. The same pattern was evident in high light acclimated cultures however the slope of the linear portion of the curve was closer to
unity (Table 4.6, Figure 4.6). Nonlinearity was not as substantial in high light acclimated cultures as is was in those acclimated to low light.

**Table 4.5:** Slope and r-squared values (in brackets) of the linear regression fit to the relationship between gross oxygen evolution predicted from PAM data (GOE\textsubscript{chl}) and oxygen evolution measurements from chlorophyll (chl) and oxygen evolution measurements from oxygen electrode data (P\texttextsubscript{chl}O\textsubscript{2}). Measurements were performed on light adapted (LA) and dark adapted (DA) samples grown under low light (LL) and high light (HL) conditions (50 and 500 μmol quanta m\textsuperscript{-2} s\textsuperscript{-1} respectively). Linear regressions were forced through the origin and fitted only to data points where linearity was visually evident (i.e. values represent b in the equation y=bx, errors indicate standard error, no r-squared value indicates where a line from the origin was plotted through data from a single irradiance only. r\textsuperscript{2} values presented here cannot be compared to r\textsuperscript{2} for models with an intercept).

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth treatment</th>
<th>RLC</th>
<th>LA</th>
<th>DA</th>
<th>LC(3)</th>
<th>LA</th>
<th>DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaetoceros</td>
<td>LL</td>
<td>2.68 ± 0.03</td>
<td>(-)</td>
<td>1.63 ± 0.06</td>
<td>1.54 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>0.79 ± 0.03</td>
<td>(0.922)</td>
<td>0.72 ± 0.03</td>
<td>0.81 ± 0.06</td>
<td>0.75 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Isochrysis</td>
<td>LL</td>
<td>1.98 ± 0.05</td>
<td>1.66 ± 0.04</td>
<td>2.14 ± 0.11</td>
<td>1.62 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>0.75 ± 0.02</td>
<td>(0.967)</td>
<td>0.63 ± 0.01</td>
<td>0.71 ± 0.03</td>
<td>0.73 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Nannochloropsis</td>
<td>LL</td>
<td>2.13 ± 0.14</td>
<td>2.31 ± 0.13</td>
<td>2.42 ± 0.30</td>
<td>2.51 ± 0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>3.34 ± 0.08</td>
<td>(0.938)</td>
<td>3.88 ± 0.09</td>
<td>3.34 ± 0.13</td>
<td>3.41 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

*Isochrysis galbana* grown in low light conditions showed the greatest non-linearity in the P\textsubscript{chl}.P\textsubscript{f}\textsubscript{chl} relationship. While at low to moderate irradiances (typically up to ~20 - 30 μmol quanta (μg chl-a\textsuperscript{-1} h\textsuperscript{-1}) P\textsubscript{f}\textsubscript{chl} underestimated P\textsubscript{chl}, a comparatively low C-fixation P\textsubscript{chl}\textsubscript{max} resulted in overestimations of P\textsubscript{chl} as irradiance increased further (Figure 4.7a, c, e). As observed for C. muelleri the linear portion of the relationship extended to higher irradiances in high light acclimated cultures, however, in the case of I. galbana, P\textsubscript{f}\textsubscript{chl} overestimated P\textsubscript{chl} (Table 4.6, Figure 4.7b, d, f). The chl-fluorescence methodology used had no substantial impact on the P\textsubscript{chl}.P\textsubscript{f}\textsubscript{chl} relationship for high light acclimated cultures. Chl-fluorescence measurements on cultures grown in low light following the LC(3) protocol yielded higher ETRs, and therefore P\textsubscript{f}\textsubscript{chl}, than either the RLC or LC(20) methods, leading to an exacerbation of the non-linear relationship (Figure 4.7c). This illustrates the potential impact of sampling protocol on the measured...
The LC(3) procedure yielded higher $P_{chl}^{f}$ estimates as photoinduction had taken place compared to RLC data (Figure 4.7a) yet downregulation of PSII had not occurred to the extent observed in the LC(20) data (Figure 4.7c).

Figure 4.8 shows the relationship between $P_{chl}$ and $P_{f}^{chl}$ measurements taken from low and high light acclimated cultures of *N. oculata*. All conditions show a significant underestimation of carbon fixation by chl-fluorescence measurements as evidenced by the high slope coefficient of the plotted linear regressions (Table 4.6). In the case of *N. oculata* the relationship was quite similar between low light and high light adapted cultures, with an approximately linear relationship at low to moderate irradiance and an earlier peak in C-fixation compared to electron transport through PSII at moderate to high irradiance leading to non-linearity. The pattern of the $P_{chl}$:$P_{f}^{chl}$ relationship differed marked only when $P_{f}^{chl}$ was estimated from cultures grown in high light via RLC methods using dark adapted samples (Figure 4.8b). In such conditions electron transport, and hence $P_{f}^{chl}$, began to decrease slightly earlier and to a greater extent than C-fixation ($E_k$ was very low at only 142 $\mu$mol quanta m$^{-2}$ s$^{-1}$, equivalent to a $Q_{phar}$ of 22.3 $\mu$mol quanta m$^{-2}$ s$^{-1}$). Apart from slightly lower linear regression slopes from the LC(20) methods, possibly due to photoinduction over the incubation period, there were no substantial changes in the $P_{chl}$:$P_{f}^{chl}$ relationship as a result of differing chl-fluorescence methods.
Figure 4.6: Chl-specific rate of primary production by the diatom *Chaetoceros muelleri* as predicted from chl-fluorescence measurements (P\(_{\text{chl}}^f\)) plotted as a function of measured chl-specific carbon fixation (P\(_{\text{chl}}\)) at the same Q\(_{\text{phar}}\). Closed circles (●) and solid lines (—) represent results and models (Eilers and Peeters 1988), respectively, from 15 min dark adapted samples. Open circles (○) and dotted lines (·····) represent results and models obtained from light adapted samples. Results for Linear regressions through the initial data points or the linear portion of the relationship have been plotted to show the initial slope of the relationship: medium dashed lines (— —) and double-dotted lines (· · ·) represent the linear regression for dark adapted and light adapted samples respectively. Measurements were taken after acclimation to both low light (a, c, e) and high light (b, d, f) growth conditions (50 and 500 µmol quanta m\(^{-2}\) s\(^{-1}\) respectively). Three chl-fluorescence methods are represented: data from rapid light curves (RLC) with 5 s at each light interval (a, b), data from light curves with 3 min at each light interval (LC(3)) representing a semi steady-state condition (c, d) and steady-state data (e, f) from measurements integrated over a 20 min exposure period (LC(20)). For RLC data \(n = 7 - 10\) and error bars = se, otherwise \(n = 3\) and error bars = range.
Figure 4.7: Chl-specific rate of primary production by *Isochrysis galbana* as predicted from chl-fluorescence measurements ($P_{fl}^{chl}$) plotted as a function of measured chl-specific carbon fixation ($P_{chl}^{f}$) at the same $Q_{phar}$. Closed circles (●) and solid lines (—) represent results and models (Eilers and Peeters 1988), respectively, from 15 min dark adapted samples. Open circles (○) and dotted lines (·····) represent results and models obtained from light adapted samples. Results for Linear regressions through the initial data points or the linear portion of the relationship have been plotted to show the initial slope of the relationship: medium dashed lines (– – –) and double-dotted lines (– · · –) represent the linear regression for dark adapted and light adapted samples respectively. Measurements were taken after acclimation to both low light (a, c, e) and high light (b, d, f) growth conditions (50 and 500 µmol quanta m$^{-2}$ s$^{-1}$ respectively). Three chl-fluorescence methods are represented: data from rapid light curves (RLC) with 5 s at each light interval (a, b), data from light curves with 3 min at each light interval (LC(3)) representing a semi steady-state condition (c, d) and steady-state data (e, f) from measurements integrated over a 20 min exposure period (LC(20)). For RLC data $n = 7 – 10$ and error bars = se, otherwise $n = 3$ and error bars = range.
Figure 4.8: Chl-specific rate of primary production by *Nannochloropsis oculata* as predicted from chl-fluorescence measurements ($P_{\text{chl}}$) plotted as a function of measured chl-specific carbon fixation ($P_{\text{chl} \text{fix}}$) at the same $Q_{\text{ph}}$. Closed circles (●) and solid lines (—) represent results and models (Eilers and Peeters 1988), respectively, from 15 min dark adapted samples. Open circles (○) and dotted lines (·····) represent results and models obtained from light adapted samples. Results for Linear regressions through the initial data points or the linear portion of the relationship have been plotted to show the initial slope of the relationship: medium dashed lines (– – –) and double-dotted lines (– · · –) represent the linear regression for dark adapted and light adapted samples respectively. Measurements were taken after acclimation to both low light (a, c, e) and high light (b, d, f) growth conditions (50 and 500 µmol quanta m$^{-2}$ s$^{-1}$ respectively). Three chl-fluorescence methods are represented: data from rapid light curves (RLC) with 5 s at each light interval (a, b), data from light curves with 3 min at each light interval (LC(3)) representing a semi steady-state condition (c, d) and steady-state data (e, f) from measurements integrated over a 20 min exposure period (LC(20)). For RLC data $n = 7 – 10$ and error bars = se, otherwise $n = 3$ and error bars = range.
Table 4.6: Slope and r-squared values (in brackets) of the linear regression fit to the relationship between gross oxygen evolution predicted from PAM data (P_f^chl) and carbon fixation measurements from radioisotope data (P_{^4}^{14}C). Measurements were performed on light adapted (LA) and dark adapted (DA) samples grown under low light (LL) and high light (HL) conditions. Linear regressions were forced through the origin and fitted only to data points where linearity was visually evident (i.e. values represent irradiance only. r^2 to data points where linearity was visually evident (i.e. values represent irradiance only. r^2 values presented here cannot be compared to r^2 for models with an intercept).

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth treatment</th>
<th>RLC</th>
<th>LC(3)</th>
<th>LC(20)</th>
</tr>
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<td></td>
<td>LA</td>
<td>DA</td>
<td>LA</td>
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<tr>
<td>Chaetoceros</td>
<td>LL 2.34 ± 0.02</td>
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<td>2.64 ± 0.04</td>
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<td>HL 1.36 ± 0.02</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>HL 6.32 ± 0.09</td>
<td>6.75 ± 0.14</td>
<td>6.00 ± 0.16</td>
<td>6.21 ± 0.16</td>
</tr>
</tbody>
</table>

4.4 Discussion

The relationship between P_f^chl and P_{^4}^{14}C was found to be variable both within and between species. Suggett et al. (2003) indicated that direct estimates of P_{^4}^{14}C and P_f^chl may vary from fluorescence-based estimates such as GOE_f^chl and P_f^chl respectively by factors ranging from 0.067 to 4. Using the slopes of the linear portion of the relationships obtained in this study provides P_{^4}^{14}C:GOE_f^chl factors ranging from 0.63 to 3.88 (Table 4.5) and P_{^4}^{14}C:P_f^chl factors ranging from 0.38 to 8.02 (Table 4.6).

The variation in the slopes described above can be attributed to a number of possible processes. Many of the large variations between light acclimated states within a species (e.g. Isochrysis galbana high light versus low light acclimated cultures; Figure 4.7) are likely to be related to the assumption made regarding the density of RCIIIs (η_{PSII}). This parameter is difficult to measure and remains one of the major barriers to accurate estimation of PSII light absorption and absolute ETR (leading to GOE_f^chl and P_f^chl) (Suggett et al. 2006). It has been shown that η_{PSII} changes with photoacclimation (Herzig and Dubinsky 1993; Suggett et al. 2004) and an attempt to take this into account.
was made here by using values derived from the literature (refer to section 4.2) rather than using a single static value. For *Chaetoceros muelleri* and *I. galbana* $\eta_{\text{PSII}}$ was assumed to be low in low light growth conditions and increase with acclimation to higher irradiance. The high slopes obtained for all species when grown in low light conditions (Table 4.5 and Table 4.6) may be taken to indicate an underestimation of $\eta_{\text{PSII}}$ for all species.

All plots of measured versus predicted primary production had some degree of curvature. Typically curvature was toward a decreasing measured:predicted ratio and was observed to be more substantial in LL acclimated compared to HL acclimated cultures (e.g., Figure 4.3a,c vs. b,d). The $P_{\text{chlO2}}:\text{GOE}_{f_{\text{chl}}}$ data from cultures of *Nannochloropsis oculata* is an exception to this trend, with HL acclimated cultures showing the greatest deviation from linearity. Belonging to the Eustigmatophyceae, *N. oculata* lacks the chlorophyll c possessed by both *C. muelleri* and *I. galbana*, having chlorophyll a only. This difference in pigment content may contribute to the contrasting photosynthetic response.

In physiological terms, the tendency to deviate from linearity towards lower $P_{\text{chlO2}}:\text{GOE}_{f_{\text{chl}}}$ or $P_{\text{chl}}:P_{f_{\text{chl}}}$ ratios indicates that, as irradiance increased, PSII was able to continue increasing its rate of supply of electrons to the ETC, however an increasing proportion of electrons appears to have been diverted away from C-fixation in a process that involves the consumption of oxygen. Photorespiration is one such process (refer to section 1.2.6.1), however it is not considered to play a significant role in many marine phytoplankton due to their possession of Rubisco with high specificity for CO$_2$ over O$_2$ as well as efficient carbon concentrating mechanisms (Badger et al. 2000). Preliminary measurements had also indicated that addition of 1 mM NaHCO$_3$ (final concentration) to samples during oxygen evolution measurements resulted in no change in photosynthetic rate, suggesting that photorespiration was negligible (data not shown).
The Mehler reaction (cf. Chapter 1.2.6) has also been proposed as the mechanism causing non-linearity between chl-fluorescence predictions and actual measurements of $P_{\text{chl}O_2}$ and $P_{\text{chl}}$ (Geel 1997; Suggett et al. 2006), however others have suggested that cyclic electron flow around PSII (cf. section 1.2.6) is just as likely (Lavaud et al. 2002c). It has been reported that Mehler reaction activity may be minimal in algae (Franklin and Badger 2001; Carr and Björk 2003) and, considering the proposed lack of state transitions in most phytoplankton taxa, the action of cyclic electron flow around PSII could be particularly beneficial in reducing photodamage. The action of mitochondrial alternative oxidase, which consumes oxygen and has been found to have a role in the dissipation of photosynthetically-produced reducing equivalents and thus photoprotection (Padmasree and Raghavendra 1999; Bartoli et al. 2005), may also explain the tendency towards lower $P_{\text{chl}O_2}:GOE_{f\text{chl}}$ and $P_{\text{chl}}:P_{f\text{chl}}$ ratios at higher irradiances.

Studies using fast repetition rate fluorometers have found that significant reduction in $\sigma_{\text{PSII}}$ can occur within 20 min of the onset of illumination (Ralph Smith, pers. comm.). In the context of this study light-adapted and dark-adapted measures of $\sigma_{\text{PSII}}$ were used in the calculation of $GOE_{f\text{chl}}$ and $P_{f\text{chl}}$ and this value was assumed to stay constant during photosynthesis measurements. If $\sigma_{\text{PSII}}$ did decrease during measurements and this was not taken into account, an overestimation of $P_{\text{chl}O_2}$ and $P_{\text{chl}}$ would result. This hypothesis is, however, considered unlikely in this circumstance since curvature towards overestimation is observed in RLC data and $\sigma_{\text{PSII}}$ is not likely to change substantially on such a short time scale.

Results obtained from the diatom *C. muelleri* were unusual in that substantially greater curvature was recorded in the $P_{\text{chl}O_2}:GOE_{f\text{chl}}$ relationship than in the $P_{\text{chl}}:P_{f\text{chl}}$ relationship, particularly in LL-adapted cultures (Figure 4.3 vs. Figure 4.6). Given that
photorespiratory activity has been discounted, this suggests that in oxygen evolution measurements for this species the dark respiration rate was underestimated at high irradiances (and possibly overestimated at low irradiances). During oxygen evolution measurements new culture was used at each irradiance and dark respiration measurements were performed on light adapted samples recently collected from semicontinuous culture (50 µmol quanta m\(^{-2}\) s\(^{-1}\) LL; 500 µmol quanta m\(^{-2}\) s\(^{-1}\) HL).

Considering the rate of oxygen consumption has been shown to increase with increasing irradiance (Harris 1980), an underestimation of oxygen consumption in the light is thought to be the most likely explanation for this discrepancy and may explain why curvature is not so significant in HL-adapted cultures.

LC(3) data for *I. galbana* tends to show increased \(\text{GOE}^\text{chl}_f\) and \(\text{P}^\text{chl}_f\) predictions compared to RLC or LC(20) data, particularly for LL-adapted cultures (Figure 4.4c, Figure 4.7c). This indicates higher measured ETR resulting from greater photoinduction than in RLC measurements, but less downregulation of PSII than was apparent in LC(20) measurements. Thus it appears that despite attempts to take measurements after similar exposure periods to actinic light, greater downregulation of photosynthesis occurred at high irradiances in the electrode chamber than in the PAM fluorometer. High light acclimated cells tend to have more efficient mechanisms for downregulation and it is likely that both photoinduction and downregulation had taken place by the time LC(3) saturation pulse measurements were taken (Figure 4.7d).

The dark- or light-adapted state of the cells upon chl-fluorescence measurement had relatively little impact on the \(\text{P}^\text{chl}\_O_2\):\(\text{GOE}^\text{chl}_f\) and \(\text{P}^\text{chl}:\text{P}^\text{chl}_f\) relationships. In many cases, particularly in HL-adapted samples, dark-adapted chl-fluorescence measurements resulted in higher ratios of \(\text{P}^\text{chl}\_O_2\) and \(\text{P}^\text{chl}\) to \(\text{GOE}^\text{chl}_f\) and \(\text{P}^\text{chl}_f\), respectively, compared to those obtained from light-adapted samples. This is likely due to the inactivation of
Calvin-Benson cycle enzymes during the 15 min dark-adaptation period resulting in faster decreases in qP compared to light-adapted samples (Büchel and Wilhelm 1993).

Considering the experiments performed here utilised nutrient replete cultures maintained in a constant, controlled environment one would expect chl-fluorescence based predictions of primary production to be less accurate in the natural environment. However, the ability to measure chl-fluorescence simultaneously with oxygen evolution or C-fixation (eg. Delieu and Walker 1983; Kroon et al. 1993; Gilbert et al. 2000a; Küpper et al. 2004), rather than separately as performed here, would yield substantial improvement in results.

4.5 Conclusion

It remains a significant challenge to convert chl-fluorescence estimates of quantum yield to absolute values of ETR. A key factor in this challenge is the accurate estimation of light absorption by PSII, with one of the biggest hurdles here being the determination of ηPSII. The development of an efficient, field applicable method for measurement of ηPSII would act to substantially increase our ability to accurately estimate absolute ETR.

In the last 10 – 15 yrs there have been significant advancements in our understanding of photosynthesis. However, significant gaps in our understanding of cyclic and other alternative electron pathways remain (cf. Section 1.2.6). Modelling of photosynthesis and predictions of primary production from ETR through PSII will continue to improve as our knowledge of components such as the ATP-driven X-pump and pathways including cyclic electron flow around PSII and PSI and chlororespiration increases.
The data presented here, collected from three marine members of the Chromista, illustrate a number of key points when attempting to predict O₂-evolution or C-fixation from chl-fluorescence measurements:

- Photoacclimational state of the sample can have a substantial impact on $P_{\text{chl}}^{\text{O}_2}:\text{GOE}_f^{\text{chl}}$ and $P_{\text{chl}}^{\text{chl}}:P_f^{\text{chl}}$ relationships. Those samples that were acclimated to high light exhibited less curvature towards overestimation of oxygen evolution and carbon fixation, suggesting decreased activity of alternative electron transport pathways. That is, high light acclimated cells are more capable of maintaining linear electron flow resulting in carbon fixation.

- Accurate measurements of $\sigma_{\text{PSII}}$ and $\eta_{\text{PSII}}$ to estimate light absorption at PSII are essential for correct ETR determination and achieving a measured:predicted slope of close to 1:1 (In the field biophysical measurements are likely to be more popular than optical measurements).

- Understanding the photosynthetic architecture and alternative electron transport pathways and their energetics in a wide variety of algal taxa will improve our ability to account for and predict deviations from a linear relationship.
CHAPTER 5
Photoacclimation

5.1 INTRODUCTION

Photosynthetic organisms optimise the functioning of their photosynthetic apparatus to the ambient light conditions. This photoacclimation can maximise light harvesting at low irradiances and down-regulate light harvesting and energy transduction at high irradiances. Down-regulation is imperative since over-excitation can result in severe damage to reaction centres (photoinhibition – see Chapter 1.2.5) and is often referred to as photoprotection (Björkman and Demmig-Adams 1995). Features of photoacclimation occurs at the optical, biophysical, biochemical, ultrastructural, physiological, and molecular levels and include relatively large, rapid and reversible changes in the complement of the cellular pool of light-harvesting chlorophyll and carotenoid pigments (Falkowski 1994; Fisher et al. 1998). For example, a greater ratio of light-harvesting chlorophyll to stromal enzymes tends to occur in low light adapted cells compared to high light adapted cells (Björkman and Demmig-Adams 1995). Recent evidence suggests that a primary mechanism for photoacclimation in coastal phytoplankton populations may be regulation of the PSII Reaction Centre (RCII) concentration relative to Calvin-Benson cycle activity (Moore et al. 2006). Alterations to the composition and also the intracellular arrangement of pigments in relation to RCII impact $\sigma_{\text{PSII}}$ and are a dynamic way of managing energy input to the photosynthetic ETC (Suggett et al. 2004).

Studies have shown that cells increase their content of chlorophyll and other photosynthetically active pigments in responses to light-limiting conditions (Perry et al. 1981). Correspondingly, in the ocean, cellular pigments have been found to increase
with increasing depth (Falkowski 1994). Pigment content usually takes a few days to weeks for acclimation (Ralph et al. 2002b). Such changes can be made in two ways; by altering the number of photosynthetic units (PSUs), or by maintaining the same number of units and instead reducing the size of each unit (Dubinsky et al. 1986; Herzig and Dubinsky 1993). The strategy employed varies between taxa; for example Isochrysis galbana and the diatom Skeletonema costatum have been observed to change PSU size rather than number (Falkowski et al. 1981; Herzig and Dubinsky 1993), while Nannochloropsis sp. uses the reverse strategy (Fisher et al. 1996). Many higher plants utilise the former strategy and, as a result, chl:RCII ratios have been observed to increase from 220 – 480 chl RCII⁻¹ to 630 – 940 chl RCII⁻¹ after a transition from HL to LL growth conditions (Perry et al. 1981; Mauzerall and Greenbaum 1989).

The ratio of RCII:RCI can also be changed to maintain photosynthetic efficiency and the required ATP:NADPH product ratio for optimal cellular function (Mauzerall and Greenbaum 1989). In membrane preparations the RCII:RCI ratio has been observed to vary from 0.43 to 3.3, with the pattern of variation changing between taxa. For example, Falkowski et al. (1981) found that with photoacclimation to decreased growth irradiance RCII:RCI in the diatom S. costatum more than doubled as RCI density dropped; whereas RCII:RCI remained essentially unchanged in the chlorophyte Dunaliella tertiolecta. Thus, assumptions that incident photons are equally distributed between the photosystems (e.g. Beer et al. 1998b; Underwood 2002) may be erroneous, as cells shift their physiology to maintain redox poise and yield sufficient ATP to fuel their metabolic processes.

Photosynthetic organisms adapted to low light conditions often have smaller pools of carotenoid pigments which are also less active in their de-epoxidation (Demmig-Adams et al. 1989; Demmig-Adams and Adams III 1992; Masojídek et al. 1999). Carotenoids include the xanthophyll cycle pigments and have important
functions in both light harvesting and photoprotection (Masojidek et al. 2000). The light harvesting function of carotenoids involves singlet-singlet energy transfer from carotenoid to chlorophyll whereas the photo-protective role relies on triplet-triplet energy transfer in the reverse direction: triplet chlorophyll ($^3$chl*) could otherwise release its energy to $O_2$ to produce singlet oxygen ($^1$O$_2$). The rate of carotenoid de-excitation of $^3$chl* (several nanoseconds) is faster than de-excitation via $O_2$ and is therefore the preferred reaction (Young et al. 1997). The xanthophyll cycle in higher plants and some chlorophyte algae involves rapid, light-dependent, reversible de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin. The energy of the low-lying singlet state, $S_1$, of chl-$a$ has been shown to be lower than the $S_1$ state of violaxanthin but higher than the zeaxanthin $S_1$ state. This means that it is energetically possible for zeaxanthin to deactivate the $^3$chl*, while violaxanthin may only act as a light-harvesting pigment (Young et al. 1997). Among the bulk pigments of the thylakoid membrane the xanthophyll cycle pigments are the only ones that exhibit large dynamic changes in response to excessive light, both in the short term (reversible de-epoxidation) and in the longer term (pool size adjustment), providing indirect evidence that this cycle plays a role in photoprotection (eg. Figure 5.1) (Björkman and Demmig-Adams 1995). Zeaxanthin formation has often been directly related to increased heat dissipation of excess light energy in higher plants (Demmig-Adams et al. 1990; Demmig-Adams and Adams III 1992; Young et al. 1997; Masojidek et al. 2000). While the role of antheraxanthin is not as clearly defined, it too is thought to be involved in dissipation of excess light energy (Young et al. 1997; Fracheboud 2001).

A similar cycle in chromophyte algae including the diatom *Phaedactylum tricornutum* involving the carotenoid pigments diadinoxanthin and a de-epoxidised form, diatoxanthin, has been found to perform a photoprotective function comparable to violaxanthin/antheraxanthin/zeaxanthin (Young et al. 1997; Kashino et al. 2002).
Certain algae, fungi and crustaceans produce secondary carotenoids (carboxylated xanthophylls) such as astaxanthin and canthaxanthin almost exclusively under stress conditions (e.g. nutrient starvation, salinity stress, temperature extremes in synergism with high irradiance). The physiological function of secondary carotenoids has not yet been clarified, however, it is considered that they function as passive photoprotectants by reducing the amount of light which can reach the light-harvesting pigment complexes of PSII (Masojídek et al. 2000).

De-epoxidation of violaxanthin to zeaxanthin (or diadinoxanthin to diatoxanthin), leading to enhanced non-radiative energy dissipation, occurs when light-driven proton pumping lowers the thylakoid lumen pH and forms a proton gradient across the thylakoid membrane (violaxanthin de-epoxidase has optimum activity around pH 5) (Björkman and Demmig-Adams 1995). The proton gradient would supposedly increase when the number of protons pumped into the thylakoid lumen, due to the action of the photosynthetic electron transport chain, exceeds the number pumped out by ATP synthase to produce ATP (influenced by rate of ATP consumption in the carbon reduction/oxidation cycles and other ATP-consuming processes) and any leakage of protons back into the stroma. The Mehler reaction (see ‘other electron consuming processes’) may assist in maintaining a substantial and persistent proton gradient since it consumes NADPH but no ATP (Björkman and Demmig-Adams 1995).

Figure 5.1: Nonphotochemical fluorescence quenching (NPQ) and relative zeaxanthin (Z) content at steady state in relation to the PFD incident on a *Hedera canariensis* leaf. *Taken from Björkman & Demmig-Adams (1995)*
There is also evidence for the existence of an indirect mechanism by which xanthophyll cycle pigments alleviate potential photoinhibition by increasing energy dissipation. Antheraxanthin and zeaxanthin interact with protonated minor LHCII complexes in a different manner to violaxanthin. Heat dissipation from LHCII increases after conformational changes relating to xanthophyll de-epoxidation, chl-protein protonation and binding of xanthophylls to the protonated chl-proteins (Gilmore et al. 1995; Young et al. 1997). Interestingly, it was also observed that violaxanthin may actually act to inhibit the non-radiant dissipation of energy (Young et al. 1997).

This tuning of the photosynthetic machinery by the cell directly impacts the photosynthesis versus irradiance relationship. Hence, as mentioned in Chapter 1.4, P-E curves can be used to study photoacclimation (Harding et al. 1985; Blanchard and Montagna 1992; MacIntyre et al. 2002; Behrenfeld et al. 2004). It has also been shown previously that photoacclimation status can be characterised using chl-fluorescence quenching analysis techniques (McMinn et al. 2004).

In this chapter chlorophyll fluorescence techniques were used to investigate the photosynthetic performance of Chaetoceros muelleri, Isochrysis galbana and Nannochloropsis oculata acclimated to HL and LL growth conditions.

5.2 METHODS

Three species representing taxa significant in the marine phytoplankton community were chosen for study. These species were Chaetoceros muelleri (diatom), Isochrysis galbana (haptophyte) and Nannochloropsis oculata (eustigmatophyte). Species description and strain numbers are provided in Chapter 3, along with methods used for their semicontinuous culture. Each species was grown in both low light (50 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \)) and high light (500 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \)).
Chl-fluorescence methods were used to measure the quantum yield of photosynthesis in the dark and at eight successively increasing light intensities. Measurements were collected using three methods aimed at discerning photosynthetic rate of the sample in non steady-state (RLC method), semi steady-state (LC(3) method) and steady state (LC(20) method) conditions. Each of these three methods has been outlined in the General Methods chapter (Chapter 3). The absolute electron transport rate through PSII (ETR) was calculated as previously described and these data were then used to form photosynthesis versus irradiance curves (P-E curves) by plotting ETR against calculated $Q_{phar}$ (refer to Chapter 4 for absorption and emission spectra used to calculate $Q_{phar}$ via Equation 7). P-E curves were modelled following Eilers and Peeters (1988) using MatLab software. Matlab was also used to generate values for the P-E curve parameters ($\alpha$, ETR$_{max}$, $E_k$) with 95% confidence intervals (refer to Chapter 3.5 for details). Changes in P-E curve parameters (e.g. between treatments) were considered significant if the 95% confidence intervals did not overlap.

Photochemical and non-photochemical quenching parameters were also determined from the chl-fluorescence saturation pulse analysis. Photochemical quenching ($q_P$) (see Table 1.1) was calculated with the parameter $F_{o'}$ being derived (Equation 18). Non-photochemical quenching (NPQ) was determined by applying the Stern-Volmer equation (see Table 1.1), however on occasions where non-photochemical quenching in the dark was apparent this was adjusted to Equation 45 as suggested by Serôdio et al. (2005).

$$NPQ = \left( \frac{F_{m'} - F_m}{F_m} \right) / F_m'$$  \hspace{1cm} (45)

At the conclusion of the final saturation pulse measurement of each light curve measurement (after exposure to the maximum irradiance) the sample in the ED-unit returns to darkness. During each RLC and LC(3) analysis three replicates were
maintained in the ED-unit at the completion of light curve measurements to measure the recovery of quantum yield, q_P and NPQ in the dark. During RLC analysis this was performed by activating the “LC+Recovery” protocol in WinControl as opposed to the “Light Curve” protocol normally activated. This protocol performs a series of saturation pulses in the dark, at extending intervals, after the completion of the light curve. These dark saturation pulses are pre-set in WinControl and occur 10 s, 40 s, 100 s, 220 s, 520 s and 1120 s after completion of the RLC. This series of recovery measurements was performed manually during LC(3) measurements on the samples exposed to the maximum irradiance. No recovery measurements were performed after LC(20)s.

5.3 RESULTS

5.3.1 Chaetoceros muelleri (diatom)

CHLOROPHYLL CONTENT

Cellular chl-a content in LL-acclimated C. muelleri was 755.6 ± 14.8 fg chl-a cell\(^{-1}\) (±se; n = 34). Acclimation to HL resulted in a 43% reduction in chl-a to 433.5 ± 14.4 fg chl-a cell\(^{-1}\) (±se; n = 39) (Figure 5.2).

Biophysical measurements indicated that HL-acclimated cultures had marginally higher PSII functional absorption cross sections (\(\sigma_{\text{PSII}}\)) than LL-acclimated cultures (Figure 5.3). This indicates that lower chl-a content was achieved via large reduction in the number of photosynthetic units per cell, rather than reductions in antenna size.
LIGHT CURVES

HL-acclimated samples were found to have significantly higher ETR\textsubscript{\text{max}} than LL-acclimated samples (Figure 5.4, Table 5.1-5.2). The length of exposure to each irradiance had little impact on the P-E relationship of LL-acclimated samples. However, light adapted samples acclimated to HL conditions exhibited a reduction in ETR\textsubscript{\text{max}} and E\textsubscript{k} values as exposure time was increased from 5 s to 3 min (Figure 5.4a, b). Even lower values were measured when the LC(20) methodology was applied, although the lowest HL-acclimated values were recorded after 5 min of exposure to actinic light (Figure 5.4, Table 5.2). The α\textsubscript{chl}-slope was primarily impacted by growth condition (LL vs. HL) rather than light curve method or adaptation state (Figure 5.4, Table 5.1-5.2).

Quantum yield decreased more rapidly and to lower levels in cells that were grown in low light compared to those grown in high light conditions (Figure 5.5a-c). These relative changes in F\textsubscript{q}/F\textsubscript{m} help to explain the higher ETR\textsubscript{\text{max}} and α\textsubscript{chl} values achieved by HL-acclimated cultures, but are not sufficient to account for the full difference. This difference is therefore necessarily related to another of the variables in the calculation of ETR. Both the assumed values of η\textsubscript{PSII} (Table 4.3) and the measured
σ_{PSII} values (Figure 5.3) were higher in HL- than in LL-acclimated cultures, leading to higher ETR and α_{chl} estimates. η_{PSII} had the greatest relative impact in this regard. There was no direct impact on E_k since the changes in ETR and α_{chl} were proportional.

Figure 5.4: Chaetoceros muelleri (diatom). Rate of electron transport through PSII as measured following (a) RLC, (b) LC(3) and (e) LC(20) protocols for collecting light curve data. Measurements of cultures grown in High Light (HL, upwards triangles) and Low Light (LL, downwards triangles) were made in all three protocols. For RLC and LC(3) protocols samples were measured in both dark adapted (DA, closed triangles) and light adapted (LA, open triangles) states (see text for details). The three sampling periods over the duration of the LC(20) (5, 10 and 20 min) have been plotted separately to illustrate changes in photosynthetic performance over the exposure period. Lines represent models fitted following Eilers and Peeters (1988). Legend for a. applies to b. also. Error bars = range (n = 3) [in a.error bars = se (n = 7-10)].

Table 5.1: Chaetoceros muelleri (diatom). Photosynthesis versus irradiance curve parameters (ETR_{max}, α_{chl} and E_k) derived from Eilers and Peeters (1988) models of ETR data. Cultures were grown in Low Light (LL; 50 μmol quanta m^{-2} s^{-1}) and High Light (HL; 500 μmol quanta m^{-2} s^{-1}) conditions and analysis was performed both in the Light Adapted (LA) and Dark Adapted (DA) state. Values are shown for RLC and LC(3) protocols, each with n=7-10 and n=3 respectively, and represent mean ± 95% confidence interval.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Light Curve Method</th>
<th>Adaptation state</th>
<th>ETR_{max}</th>
<th>α_{chl}</th>
<th>E_k</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>RLC</td>
<td>LA</td>
<td>10.2 ± 0.14</td>
<td>0.670 ± 0.041</td>
<td>15.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>9.2 ± 0.17</td>
<td>0.760 ± 0.064</td>
<td>12.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LC(3)</td>
<td>LA</td>
<td>10.4 ± 0.15</td>
<td>0.729 ± 0.049</td>
<td>14.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>10.1 ± 0.31</td>
<td>0.789 ± 0.110</td>
<td>12.9 ± 0.5</td>
</tr>
<tr>
<td>HL</td>
<td>RLC</td>
<td>LA</td>
<td>28.0 ± 0.55</td>
<td>1.042 ± 0.069</td>
<td>26.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>19.7 ± 0.26</td>
<td>1.258 ± 0.076</td>
<td>15.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>LC(3)</td>
<td>LA</td>
<td>23.1 ± 1.23</td>
<td>1.095 ± 0.141</td>
<td>21.1 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>19.4 ± 0.41</td>
<td>1.073 ± 0.104</td>
<td>18.1 ± 0.4</td>
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</table>
Table 5.2: Chaetoceros muelleri (diatom). Photosynthesis versus irradiance curve parameters (ETR$_{\text{max}}$, $\alpha^{\text{chl}}$, and $E_k$) derived from Eilers and Peeters (1988) models fitted to LC(20) data. Cultures were grown in Low Light (LL; 50 µmol quanta m$^{-2}$ s$^{-1}$) and High Light (HL; 500 µmol quanta m$^{-2}$ s$^{-1}$) conditions and measurements were taken during the 20 min incubation period at 5 min, 10 min and 20 min (see text for more details). Values represent mean ± 95% confidence interval, $n=3$.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Incubation period (min)</th>
<th>ETR$_{\text{max}}$</th>
<th>$\alpha^{\text{chl}}$</th>
<th>$E_k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>5</td>
<td>8.6 ± 0.35</td>
<td>0.589 ± 0.098</td>
<td>14.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.8 ± 0.18</td>
<td>0.624 ± 0.057</td>
<td>14.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.4 ± 0.28</td>
<td>0.554 ± 0.068</td>
<td>16.9 ± 0.8</td>
</tr>
<tr>
<td>Integrated</td>
<td>5</td>
<td>8.9 ± 0.18</td>
<td>0.589 ± 0.049</td>
<td>15.1 ± 0.4</td>
</tr>
<tr>
<td>HL</td>
<td>5</td>
<td>14.7 ± 0.50</td>
<td>0.902 ± 0.127</td>
<td>16.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>17.3 ± 0.44</td>
<td>0.990 ± 0.109</td>
<td>17.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>17.1 ± 0.43</td>
<td>1.010 ± 0.110</td>
<td>16.9 ± 0.4</td>
</tr>
<tr>
<td>Integrated</td>
<td>16.3 ± 0.40</td>
<td>0.968 ± 0.104</td>
<td>16.9 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.5: Chaetoceros muelleri (diatom). PSII quantum yield measurements of cultures grown in High Light (HL) and Low Light (LL) conditions. Data were collected from samples in both light adapted (LA) and dark adapted (DA) states (see text for details). Quantum yield measurements were collected over the course of light curves: (a) RLC, (b) LC(3) and (c) LC(20). Recovery of quantum yield in the dark after (d) RLC and (e) LC(3) is also shown, with reference to the original dark adapted F$_{\text{Fm}}$/F$_{\text{m}}$ values. Legend for a. applies to b., d. and e also. Error bars = range ($n=3$) [in a. error bars = se ($n=7-10$)].
Changes in $\sigma_{\text{PSII}}$ can also be used to explain why the shapes of the P-E curves measured using LL-acclimated samples (RLC and LC(3) methods) were very similar between light and dark adapted samples despite differences in PSII quantum yield (Figure 5.5a, b). It was found that, in both LL- and HL-acclimated cultures, light-adapted samples had lost ~10% of the $\sigma_{\text{PSII}}$ measured in dark-adapted samples.

Non-photochemical quenching (NPQ) was influenced by growth condition, adaptation state and the light curve method (Figure 5.6). During RLC measurements (Figure 5.6a) cells grown in HL conditions were able to develop almost twice as much non-photochemical quenching data were collected over the course of light curves: (a) RLC, (b) LC(3) and (c) LC(20). Recovery of NPQ in the dark after (d) RLC and (e) LC(3) was also recorded. Legend for a. also applies to b., d. and e. Error bars = range (n = 3) [in a. error bars = se (n = 7-10)].
NPQ as LL-acclimated cells. HL-acclimated samples maintained in a light adapted state showed relaxing NPQ until $Q_{\text{phar}}$ reached 20.8 $\mu$mol quanta (µg chl-α)$^{-1}$ h$^{-1}$ ($E_{\text{PAR}} = 664$ $\mu$mol quanta m$^{-2}$ s$^{-1}$) and then increased again as actinic irradiance continued to increase. For each growth condition NPQ at moderate-to-high irradiances was highest in dark adapted samples.

NPQ values determined during the LC(3) measurements were very similar for all treatments (Figure 5.6b), unlike the NPQ values measured during RLC measurements. While a slight trend for higher NPQ in dark adapted samples remained apparent, high- and low-light acclimated cultures developed roughly equal amounts of NPQ. The extended exposure of samples to low light during the LC(3) procedure resulted in an increase in $F_{\text{m}}'$ from the dark-adapted value, indicating the presence of dark-induced quenching, particularly in the HL-acclimated cells (Figure 5.6b).

Dark-induced quenching increased further when LC(20) methods were used and $F_{\text{m}}'$ was found to continue to increase from the dark-adapted value for between 10 min (HL-acclimated cultures) and 20 min (LL-acclimated cultures) (Figure 5.6c). The development of NPQ over the course of LC(20) measurements performed using the photosynthetron as the actinic light source was similar to that observed during LC(3) measurements until $Q_{\text{phar}}$ reached 18.8 $\mu$mol quanta (µg chl-α)$^{-1}$ h$^{-1}$ ($E_{\text{PAR}} = 635$ $\mu$mol quanta m$^{-2}$ s$^{-1}$). As irradiance increased above this level NPQ increased to a much greater extent than had been observed during RLC and LC(3) measurements, reaching values of 1.55 ± 0.13 in LL-acclimated cultures and 1.06 ± 0.06 in HL-acclimated cultures (Figure 5.6c). For LL-acclimated cultures this maximum was achieved after just 5 min of exposure to maximum irradiance. In HL-acclimated cultures the highest NPQ (recorded at maximum irradiance) after 5 min was just 0.61 ± 0.01, but increased to 0.82 ± 0.01 after 10 min and to 1.06 ± 0.06 after 20 min exposure.
During RLC and LC(3) measurements light adapted samples experienced less RCI closure than their dark adapted counterparts, while HL-acclimated samples were able to maintain a greater proportion of open reaction centres than LL-acclimated samples (Figure 5.7a, b). Thus, light adapted HL-acclimated cells experienced the least closure of RCIIs, suggesting they had the fastest PSII turnover time. However, it was observed that longer exposure periods during LC(20) measurements acted to negate qP differences between LL- and HL-acclimated cultures (Figure 5.7c). The main reason for this was a substantial increase in the qP of LL-acclimated cultures compared to those measured in RLCs and LC(3)s.

![Graphs showing qP over time for different conditions](image.png)

**Figure 5.7:** Chaetoceros muelleri (diatom). Photochemical quenching (qP) of PSII fluorescence measurements taken from cultures grown in High Light (HL) and Low Light (LL) conditions. Data were collected from samples in both light adapted (LA) and dark adapted (DA) states (see text for details). Photochemical quenching data were collected over the course of light curves: (a) RLC, (b) LC(3) and (c) LC(20). Recovery of NPQ in the dark after (d) RLC and (e) LC(3) was also recorded. Legend for a. also applies to b., d. and e. Error bars = range (n = 3) [in a. error bars = se (n = 7-10)].
**Recovery**

Upon return to darkness, after the RLC protocol had been completed, the $F_{q'/m'}$ of dark adapted HL-acclimated samples recovered fully within 3 min 40 s. Light adapted HL- and LL-acclimated samples recovered to their initial $F_{q'/m'}$ within a further 5 min, while dark adapted LL-acclimated samples did not fully recover until 10 min had passed (Figure 5.5d). The light adapted HL-acclimated samples appeared to exhibit a biphasic recovery of NPQ (Figure 5.6d) that was reflected in both the $F_{q'/m'}$ and $q_p$ recovery results (Figure 5.5d and Figure 5.7d respectively). This pattern of recovery was similar to that observed when $q_T$-like quenching has occurred along with $q_E$-type quenching.

An interesting phenomenon was the *increase* in NPQ observed in light adapted samples between the final, highest light level and 10 s after return to dark (Figure 5.6a,d). This was due to a continuing decrease in $F_{m'}$ after the actinic light was deactivated. For the remainder of the recovery period, however, the NPQ of light adapted samples decreased to zero. Unlike light adapted samples, those that were dark adapted prior to RLC measurements experienced an immediate decrease in NPQ. Within the first 10 s of recovery the NPQ of dark adapted LL-acclimated samples had decreased by 37% ($0.227 \pm 0.009$ to $0.144 \pm 0.014$), while in the same period the NPQ of HL-acclimated samples dropped 69% ($0.381 \pm 0.013$ to $0.117 \pm 0.020$) and continued to decline sharply. Dark adapted samples from both growth conditions began to redevelop NPQ after roughly 4 min in the dark as $F_{m}$ began to decrease again.

The recovery of chl-fluorescence parameters in the dark after 3 min sample exposure to the highest actinic light level at the conclusion of LC(3)s was measured on HL-acclimated samples only (a technical mishap occurred during recovery phase sampling of LL-acclimated cultures). Neither light nor dark adapted samples recovered
to their initial $F_v/F_m$ value, indicating the possibility of some photoinhibition (Figure 5.5e). Long-term NPQ remained in both the light and dark adapted samples, providing a further indication that photoinhibitory quenching ($q_p$) may have been present (Figure 5.6e). Recovery of $q_p$ was slower than after RLCs and, unlike RLC measurements, the recovery of light adapted samples was more rapid than that of dark adapted samples. Approximately 2% of RCIIIs in dark adapted samples remained closed at the end of the 18 min 40 s recovery analysis (Figure 5.7e).

### 5.3.2 Isochrysis galbana (Prymnesiophyceae)

**Chlorophyll Content**

Cellular chl-α content in LL-acclimated *I. galbana* was found to be 383.1 ± 20.1 fg chl-α cell$^{-1}$ (± se; n = 36). Acclimation to HL resulted in a 51% reduction in chl-α to 189.5 ± 7.6 fg chl-α cell$^{-1}$ (± se; n = 51) (Figure 5.8).

The $\sigma_{PSII}$ of HL-acclimated cultures was marginally smaller than LL-acclimated values (Figure 5.9). When dark adapted, $\sigma_{PSII}$ of HL-acclimated cultures was 11% smaller, however, when light adapted the decline was 3% only. Such minor reduction in

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**Figure 5.8:** *Isochrysis galbana* Chl-α content (mean ± se) measured after acclimation to low light (LL; black) and high light (HL; white) growth conditions.

**Figure 5.9:** *Isochrysis galbana* functional PSII absorption cross-section ($\sigma_{PSII}$) measurements (± se; n = 5). Samples were either dark-adapted (DA) or light-adapted (LA) and taken from semi-continuous cultures grown in low light (LL) or high light (HL) growth irradiance.
photosynthetic unit size cannot account for the large decrease in cellular chl-\(a\) content, thus *I. galbana* must acclimate to high light conditions via a large reduction in the number of photosynthetic units per cell.

**Light Curves**

HL-acclimated cultures of *I. galbana* were capable of achieving substantially higher ETR\(_{\text{max}}\) than LL-acclimated cultures (Figure 5.10). This pattern was similar to that seen in *C. muelleri* (Figure 5.4). In LL-acclimated cultures it was found that dark adapted samples achieved higher ETR\(_{\text{max}}\) values than light adapted samples, however, the opposite was true for HL-acclimated cultures (Table 5.3, Figure 5.10a, b). *I. galbana* ETR\(_{\text{max}}\) did not decline as exposure length at each irradiance was increased (RLC → LC(3) → LC(20)) and photosynthesis moved towards steady state; unlike *C. muelleri* ETR\(_{\text{max}}\). In fact, for each treatment except HL-acclimated dark adapted samples, ETR\(_{\text{max}}\) was significantly higher when measured by LC(3)s rather than RLCs (Table 5.3). LC(20) ETR\(_{\text{max}}\) values were similar to the values obtained by light adapted samples during LC(3)s. During LC(20) measurements on the HL-acclimated *I. galbana* culture,

**Figure 5.10:** *Isochrysis galbana* (haptophyte). Rate of electron transport through PSII as measured following (a) RLC, (b) LC(3) and (c) LC(20) protocols for collecting light curve data. Measurements of cultures grown in High Light (HL, upwards triangles) and Low Light (LL, downwards triangles) were made in all three protocols. For RLC and LC(3) protocols samples were measured in both dark adapted (DA, closed triangles) and light adapted (LA, open triangles) states (see text for details). The three sampling periods over the duration of the LC(20) (5, 10 and 20 min) have been plotted separately to illustrate changes in photosynthetic performance over the exposure period. Lines represent models fitted following Eilers and Peeters (1988). Legend for a. applies to b. also. Error bars = range \((n=3)\) [in a. error bars = se \((n=7-10)\).
an asymptote for ETR data was not reached and while the final and maximum ETR values achieved for each of the three sampling periods were very similar (~30 μmol electrons (μg chl-a)^{-1} h^{-1}), lower convexity in the 5 min data (ie. slower transition from light limitation to light saturation) yielded a potentially higher ETR_{max} to be predicted by the Eilers and Peeters (1988) model (Figure 5.10c, Table 5.4). At the 20 min sampling periods of the LC(20) protocol the ETR_{max} of LL-acclimated cultures decreased significantly.

Table 5.3: Isochrysis galbana (haptophyte). Photosynthesis versus irradiance curve parameters (ETR_{max}, α^{hl} and E_k) derived from Eilers and Peeters (1988) models of ETR data. Cultures were grown in Low Light (LL; 50 μmol quanta m^{-2} s^{-1}) and High Light (HL; 500 μmol quanta m^{-2} s^{-1}) conditions and analysis was performed both in the Light Adapted (LA) and Dark Adapted (DA) state. Values are shown for RLC and LC(3) protocols, each with n=7-10 and n=3 respectively, and represent mean ± 95% confidence interval.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Light Curve Method</th>
<th>Adaptation state</th>
<th>ETR_{max}</th>
<th>α^{hl}</th>
<th>E_k</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>RLC</td>
<td>LA</td>
<td>8.4 ± 0.21</td>
<td>0.603 ± 0.055</td>
<td>14.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>9.9 ± 0.19</td>
<td>0.901 ± 0.086</td>
<td>10.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>LC(3)</td>
<td>LA</td>
<td>10.5 ± 0.31</td>
<td>0.663 ± 0.094</td>
<td>15.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>13.9 ± 0.57</td>
<td>0.821 ± 0.152</td>
<td>16.9 ± 0.7</td>
</tr>
<tr>
<td>HL</td>
<td>RLC</td>
<td>LA</td>
<td>30.0 ± 0.31</td>
<td>1.149 ± 0.044</td>
<td>26.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>25.4 ± 0.30</td>
<td>1.510 ± 0.084</td>
<td>16.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LC(3)</td>
<td>LA</td>
<td>32.7 ± 0.40</td>
<td>1.195 ± 0.064</td>
<td>27.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>25.8 ± 0.48</td>
<td>1.236 ± 0.104</td>
<td>20.9 ± 0.4</td>
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</tbody>
</table>

Table 5.4: Isochrysis galbana (haptophyte). Photosynthesis versus irradiance curve parameters (ETR_{max}, α^{hl} and E_k) derived from Eilers and Peeters (1988) models fitted to LC(20) data. Cultures were grown in Low Light (LL; 50 μmol quanta m^{-2} s^{-1}) and High Light (HL; 500 μmol quanta m^{-2} s^{-1}) conditions and measurements were taken during the 20 min incubation period at 5 min, 10 min and 20 min (see text for more details). Values represent mean ± 95% confidence interval, n=3.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Incubation period (min)</th>
<th>ETR_{max}</th>
<th>α^{hl}</th>
<th>E_k</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>5</td>
<td>10.6 ± 0.65</td>
<td>0.695 ± 0.114</td>
<td>15.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.1 ± 0.21</td>
<td>0.623 ± 0.058</td>
<td>16.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.1 ± 0.29</td>
<td>0.558 ± 0.070</td>
<td>16.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Integrated</td>
<td>9.7 ± 0.24</td>
<td>0.625 ± 0.070</td>
<td>15.6 ± 0.4</td>
</tr>
<tr>
<td>HL</td>
<td>5</td>
<td>38.7 ± 19.69</td>
<td>1.398 ± 0.185</td>
<td>27.7 ± 15.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>31.6 ± 1.55</td>
<td>1.256 ± 0.121</td>
<td>25.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>30.4 ± 1.42</td>
<td>1.294 ± 0.126</td>
<td>23.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Integrated</td>
<td>31.8 ± 1.67</td>
<td>1.314 ± 0.092</td>
<td>24.2 ± 1.6</td>
</tr>
</tbody>
</table>
RLCs, LC(3)s and LC(20) all gave significantly higher $\alpha_{chl}$-slopes in HL-compared to LL-acclimated cultures (Table 5.3, 5.4). The $\alpha_{chl}$ of dark adapted samples was consistently higher than in light adapted samples, except for the HL-acclimated cultures when measured by LC(3) (Table 5.3, 5.4). The light curve measurement method appeared to have no influence on observed $\alpha_{chl}$ values.

The point of light saturation ($E_k$) was higher in HL-compared to LL-acclimated cultures (Table 5.3, 5.4) due to the much higher $ETR_{max}$ achieved by those samples cultured in high light conditions. Dark adapted samples had a lower $E_k$ than light adapted samples, although this was not the case for HL-acclimated cultures when measured by LC(3) due to the substantially higher $ETR_{max}$ achieved by light adapted samples. Significantly higher $E_k$ values were calculated from LC(3) data compared to RLC data (Table 5.3). LC(20) data indicated that, for LL-acclimated cultures, further increases in irradiance exposure time did not influence $E_k$ to a measurable extent. For HL-acclimated cultures, however, $E_k$ achieved at the 5 min sampling period was similar to that obtained by light adapted samples during LC(3) measurements while any further extension of the exposure time resulted in a decreasing $E_k$ (Table 5.4).

For each of the light curve methods, HL-acclimated cultures were found to maintain higher PSII quantum yields than LL-acclimated cultures, particularly at moderate to high irradiance (Figure 5.11a-c). Although significant, this difference does not fully explain the substantially greater $ETR$s achieved by cultures that were HL-acclimated. It was the assumption that HL-acclimated cells have more than twice the density of RCIIs ($\eta_{PSII}$) than LL-acclimated cells (see Chapter 4.2) that lead to the enhancement of ETR measured in HL-acclimated cultures, although this was suppressed due to their smaller $\sigma_{PSII}$ (Figure 5.9). Figure 5.9 also shows that dark adapted cells had
a significantly higher $\sigma_{PSII}$ than light adapted cells. This can explain why, for LL-acclimated cultures, higher ETRs were calculated for dark adapted rather than light adapted samples despite the latter having equal or higher PSII quantum yields (Figure 5.11a, b).

During LC(3) measurements no differences were found between the PSII quantum yields of dark or light adapted samples of LL-acclimated cultures (Figure 5.11b). Such differences remained, however, in HL-acclimated cultures, although they were less than the differences observed in RLC data.
Quantum yield measurements taken during LC(20) analyses showed a similar pattern to those observed for *C. muelleri*, including an increase at low irradiance compared to the dark adapted value (Figure 5.11c). This was a result of $F_{m}'$ increasing above $F_m$ at low irradiances and is also reflected in the NPQ data (Figure 5.12c).

**Figure 5.12:** *Isochrysis galbana* (haptophyte). Non-photochemical quenching (NPQ) of PSII fluorescence measurements taken from cultures grown in High Light (HL) and Low Light (LL) conditions. Data were collected from samples in both light adapted (LA) and dark adapted (DA) states (see text for details). Non-photochemical quenching data were collected over the course of light curves: (a) RLC, (b) LC(3) and (c) LC(20). Recovery of NPQ in the dark after (d) RLC and (e) LC(3) was also recorded. Legend for a. applies to b., d. and e also. Error bars = range ($n = 3$) [in a. error bars = se ($n = 7-10$)].

Rapid light curve analyses of *I. galbana* found more NPQ in HL- than in LL-acclimated cultures, however, in most cases NPQ development was only minimal (Figure 5.12a). Only HL-acclimated, light adapted samples exhibited any substantial NPQ (reaching $0.299 \pm 0.003$ at maximum irradiance). Despite being taken from a high light environment ($500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) these samples showed no decrease in NPQ.
during the initial, low irradiance portion of the RLC analyses; this is in contrast to the results for the diatom *C. muelleri* (Figure 5.6a). LL-acclimated samples, on the other hand, were found to have significant dark induced quenching but less NPQ than light adapted samples.

In the LC(3) analyses NPQ reached higher levels, particularly for dark adapted samples which were able to achieve levels equivalent to those attained by light adapted samples (Figure 5.12b). The longer period at low irradiance during the analyses also yielded an increase in NPQ in the dark observed in HL-acclimated samples. With this sampling method it remained clear however, that HL-acclimated cells were able to dissipate a greater amount of excess energy via NPQ than LL-acclimated cells.

Non-photochemical quenching in both the light and dark adapted states was highest when the LC(20) technique was applied, despite a significantly lower maximum $Q_{\text{phar}}$ as a result of the lower absorption coefficient under halogen lamps (Table 4.4). At the 5 min and 10 min sampling periods cells from both growth conditions had developed similar levels of NPQ at each irradiance measured (Figure 5.12c). However, after 20 min exposure to high irradiances ($Q_{\text{phar}} > 21.4 \mu\text{mol quanta (}\mu\text{g chl-}a^{-1} \text{ h}^{-1}; E_k > 634 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) NPQ in LL-acclimated cultures had increased to levels significantly higher than those in cells acclimated to high irradiance and coincided with decreases in PSII quantum yield (Figure 5.11c) and $q_p$ (Figure 5.13c).
Photochemical quenching ($q_P$) responded to light in a fashion similar to that already described for PSII quantum yield (Figure 5.11). Cultures acclimated to HL conditions were able to maintain higher $q_P$, or a greater proportion of open RCIIIs, than LL-acclimated cultures. The only exception to this was found at the 5 min sampling period of LC(20) analyses (Figure 5.13c). Dark-adaptation of samples appeared to result in a greater reduction of $q_P$ during the course of light curve analyses, particularly when short Light Widths are applied (Figure 5.13a, b). This effect was more apparent in HL-acclimated cultures. It can be seen by comparing graphs a, b and c of Figure 5.13 that extending the exposure time at each irradiance allowed $q_P$ to recover as
closed RCIIs re-opened. However, Figure 5.13c illustrates that exposure to moderate to high irradiances for longer than 10 min may cause $q_P$ to decline again.

**Recovery**

Recovery data indicated a relatively rapid recovery of $F_{q'/F_m}$, particularly in LL-acclimated samples (Figure 5.11d, e). Previously dark adapted, LL-acclimated samples recovered to initial $F_{v/F_m}$ levels within 40 s of RLC completion, while light adapted samples took another minute. HL-acclimated samples took longer to recover, but even in the light adapted samples $F_{q'/F_m}$ recovered to 88% of the initial $F_{v/F_m}$ within 10 s. Full recovery took another 8.5 minutes. At the conclusion of RLC analyses the quantum yield of HL-acclimated samples recovered to values higher than the originally measured $F_{v/F_m}$ (Figure 5.11d).

The recovery of NPQ was not as rapid as quantum yield recovery and little change occurred within the first 10 s of darkness (Figure 5.12d). Over the duration of the recovery period there was no observed dissipation of the small amount of NPQ that had accumulated in dark adapted LL-acclimated cultures (very similar to *C. muelleri*, see Figure 5.6d). It follows that the large recovery of quantum yield within the first 10 s of recovery was due to a decrease in $F'$ as RCIIs re-opened and this was indeed reflected in the $q_P$ recovery data (Figure 5.13d).

Quantum yield recovery in HL-acclimated cells after LC(3) analyses was similar to that seen after RLCs, however, despite an equivalent recovery over the first 10 s of darkness, return to values equivalent to the initial $F_{v/F_m}$ required an extra 5 – 10 min (Figure 5.11e). Unlike RLC recovery, quantum yield values did not increase above the initial $F_{v/F_m}$. Post-LC(3) recovery of quantum yield in LL-acclimated cells was as for post-RLC recovery over the first 10 s (quantum yield achieved ~95% of $F_{v/F_m}$). However, recovery then slowed and quantum yield peaked at 0.723 or 98.4% of the
original Fv/Fm before decreasing slightly over the remainder of the recovery period. This decline was associated with both a slight increase in NPQ (Figure 5.12e) and decrease in qP (Figure 5.13e). Post-LC(3) dissipation of NPQ was minimal over the first 10 s of the recovery period. While there was no further reduction of NPQ in LL-acclimated cultures, NPQ in HL-acclimated cultures remained high for ~40 s and then underwent exponential decay and was almost fully dissipated by the end of the recovery period (Figure 5.12e).

The recovery of qP post-LC(3) was similar to that observed after RLC analyses (Figure 5.13e vs. Figure 5.13d). In both HL- and LL-acclimated cultures most qP recovery occurred within the first 10 s of darkness, although LL-acclimated cells recovered to a greater extent than HL-acclimated cultures over this period. It is interesting to note that, with the extended exposure, light adapted samples recovered faster than those that were dark adapted (Figure 5.13e); an opposite trend to that seen after RLC analyses (Figure 5.13d). However, common to both RLC and LC(3) methods was a slight decrease in qP of LL-acclimated cultures as the recovery period extended past 3 min 40 s. This was the result of a combination of slight increases in F and decreases in Fm values.

5.3.3 *Nannochloropsis oculata* (Eustigmatophyceae)

**Chlorophyll Content**

Cellular chl-a content in LL-acclimated *N. oculata* was found to be 207.9 ± 5.7 fg chl-a cell⁻¹ (n = 18; error represents standard error). Acclimation to HL resulted in a 77% reduction in chl-a to 47.4 ± 3.7 fg chl-a cell⁻¹ (n = 18) (Figure 5.14).
HL-acclimated cultures had marginally higher PSII functional absorption cross sections ($\sigma_{\text{PSII}}$) than LL-acclimated cultures (Figure 5.15). This indicates that lower chl-$a$ content was achieved via large reduction in the number of photosynthetic units per cell, rather than reductions in antenna size.

**Figure 5.14:** *Nannochloropsis oculata* Chl-$a$ content (mean ± se) measured after acclimation to low light (LL; black) and high light (HL; white) growth conditions.

**Figure 5.15:** *Nannochloropsis oculata* functional PSII absorption cross-section ($\sigma_{\text{PSII}}$) measurements (± se; $n = 5$). Samples were either dark-adapted (DA) or light-adapted (LA) and taken from semi-continuous cultures grown in low light (LL) or high light (HL) growth irradiance.

### LIGHT CURVES

The small unicellular eustigmatophyte *N. oculata* achieved a relatively low ETR$_{\text{max}}$ in comparison to the other species studied here (Figure 5.16; Table 5.5; Table 5.6). As for the other species studied, HL-acclimated cultures were able to achieve significantly higher ETR$_{\text{max}}$ than cultures acclimated to LL conditions, although the difference between HL- and LL-acclimated cultures was not as great when RLCs were applied (Figure 5.16a). Also, the ETR$_{\text{max}}$ of LL-acclimated cultures was found to be similar across all light curve methods (the only outlier being a significantly lower ETR$_{\text{max}}$ found in dark adapted samples analysed by RLC) while HL-acclimated samples were able to attain a significantly higher ETR$_{\text{max}}$ when in semi-steady state (LC(3), Figure 5.16b) or steady state (LC(20), Figure 5.16c).
Figure 5.16: *Nannochloropsis oculata* (eustigmatophyte). Rate of electron transport through PSII as measured following (a) RLC, (b) LC(3) and (c) LC(20) protocols for collecting light curve data. Measurements of cultures grown in High Light (HL, upwards triangles) and Low Light (LL, downwards triangles) were made in all three protocols. For RLC and LC(3) protocols samples were measured in both dark adapted (DA, closed triangles) and light adapted (LA, open triangles) states (see text for details). The three sampling periods over the duration of the LC(20) (5, 10 and 20 min) have been plotted separately to illustrate changes in photosynthetic performance over the exposure period. Lines represent models fitted following Eilers and Peeters (1988). Legend for a. applies to b. also. Error bars = range (n = 3) [in a. error bars = se (n = 7 – 10)].

RLCs recorded a large difference between light and dark adapted samples (Figure 5.16a; Table 5.5). As a result of a substantial increase in the ETR\(_{\text{max}}\) of dark adapted samples as Light Width was extended from 5 s to 3 min, adaptation state appeared to have little influence on the P-E relationship when the longer exposure period was applied (Figure 5.16b; Table 5.5).

Table 5.5: *Nannochloropsis oculata* (eustigmatophyte). Photosynthesis versus irradiance curve parameters (ETR\(_{\text{max}}\), \(\alpha^\text{hl}\) and \(E_k\)) derived from Eilers and Peeters (1988) models of ETR data. Cultures were grown in Low Light (LL; 50 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\)) and High Light (HL; 500 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\)) conditions and analysis was performed both in the Light Adapted (LA) and Dark Adapted (DA) state. Values are shown for RLC and LC(3) protocols, each with \(n=7-10\) and \(n=3\) respectively, and represent mean ± 95% confidence interval.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Light Curve Method</th>
<th>Adaptation state</th>
<th>(ETR_{\text{max}})</th>
<th>(\alpha^\text{hl})</th>
<th>(E_k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>RLC</td>
<td>LA</td>
<td>3.4 ± 0.04</td>
<td>0.588 ± 0.033</td>
<td>5.8 ± 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>2.1 ± 0.03</td>
<td>0.638 ± 0.054</td>
<td>3.2 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>LC(3)</td>
<td>LA</td>
<td>3.4 ± 0.16</td>
<td>0.442 ± 0.089</td>
<td>7.7 ± 0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>3.2 ± 0.14</td>
<td>0.493 ± 0.113</td>
<td>6.5 ± 0.74</td>
</tr>
<tr>
<td>HL</td>
<td>RLC</td>
<td>LA</td>
<td>4.8 ± 0.09</td>
<td>0.372 ± 0.027</td>
<td>13.0 ± 0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>2.7 ± 0.04</td>
<td>0.448 ± 0.030</td>
<td>5.9 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>LC(3)</td>
<td>LA</td>
<td>5.6 ± 0.22</td>
<td>0.433 ± 0.052</td>
<td>13.0 ± 1.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA</td>
<td>5.6 ± 0.18</td>
<td>0.400 ± 0.055</td>
<td>14.0 ± 1.04</td>
</tr>
</tbody>
</table>
Table 5.6: *Nannochloropsis oculata* (eustigmatophyte). Photosynthesis versus irradiance curve parameters (ETR$_{\text{max}}$, $\alpha^{\text{chl}}$ and $E_k$) derived from Eilers and Peeters (1988) models fitted to LC(20) data. Cultures were grown in Low Light (LL; 50 µmol quanta m$^{-2}$ s$^{-1}$) and High Light (HL; 500 µmol quanta m$^{-2}$ s$^{-1}$) conditions and measurements were taken during the 20 min incubation period at 5 min, 10 min and 20 min (see text for more details). Values represent mean ± 95% confidence interval, $n=3$.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Incubation period (min)</th>
<th>ETR$_{\text{max}}$</th>
<th>$\alpha^{\text{chl}}$</th>
<th>$E_k$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LL</strong></td>
<td>5</td>
<td>3.3 ± 0.26</td>
<td>0.500 ± 0.140</td>
<td>6.7 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.3 ± 0.05</td>
<td>0.559 ± 0.033</td>
<td>5.9 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.1 ± 0.14</td>
<td>0.571 ± 0.099</td>
<td>5.5 ± 0.22</td>
</tr>
<tr>
<td>Integrated</td>
<td></td>
<td>3.3 ± 0.08</td>
<td>0.556 ± 0.054</td>
<td>5.9 ± 0.13</td>
</tr>
<tr>
<td><strong>HL</strong></td>
<td>5</td>
<td>5.8 ± 0.10</td>
<td>0.350 ± 0.023</td>
<td>16.6 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.0 ± 0.25</td>
<td>0.366 ± 0.066</td>
<td>16.4 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.7 ± 0.24</td>
<td>0.383 ± 0.061</td>
<td>15.0 ± 0.59</td>
</tr>
<tr>
<td>Integrated</td>
<td></td>
<td>5.8 ± 0.13</td>
<td>0.368 ± 0.033</td>
<td>15.8 ± 0.33</td>
</tr>
</tbody>
</table>

Dark adaptation usually had a negligible impact on $\alpha^{\text{chl}}$, however, when RLCs were performed on HL-acclimated cultures $\alpha^{\text{chl}}$ was significantly higher when samples were dark adapted (Table 5.5). Growth condition also influenced $\alpha^{\text{chl}}$, although not to the extent seen in *C. muelleri* or *I. galbana*. RLC and LC(20) methods gave higher $\alpha^{\text{chl}}$ values in LL-acclimated cultures, while no difference was seen when LC(3)s were applied (Figure 5.16; Table 5.5 – 5.6).

In RLCs the changes in ETR$_{\text{max}}$ and $\alpha^{\text{chl}}$ than resulted from changes in adaptational state lead to significant changes in $E_k$. The $E_k$ measured from both LL- and HL-acclimated cultures was lower when samples were dark adapted (Table 5.5). No such difference was observed when LC(3)s were applied. In all light curve methods $E_k$ was significantly higher in HL-acclimated cultures than in those that were grown in low light (Table 5.5 – 5.6). The choice of light curve method also influenced $E_k$, especially if samples were dark adapted. LL-acclimated cultures achieved peak $E_k$ during LC(3) analyses, while HL-acclimated cultures maximised their $E_k$ during the longer irradiance exposure of the LC(20) method.
The decline in quantum yield during RLC analysis of *N. oculata* (Figure 5.17a) was influenced by growth condition and adaptation state in a manner similar to that observed in *C. muelleri* and *I. galbana* (Figure 5.5a and Figure 5.11a respectively). HL-acclimated and light adapted samples were able to maintain higher quantum yields than their respective opposites. However, *N. oculata* quantum yield declined faster and to lower levels than in the other species. Quantum yield of light and dark adapted samples converged with the longer (3 min) exposure time of LC(3)s, yet values remained substantially higher in HL-acclimated samples compared to LL-acclimated samples (Figure 5.17b). In LC(20)s there was no significant changes in the decline of quantum...
yield as the exposure period increased from 5 min to 20 min (Figure 5.17c). The HL-acclimated samples were able, under these steady-state conditions, to maintain higher quantum yields at low to moderate irradiances compared to those determined with the other light curve methods (Figure 5.17a-c).

The functional absorption cross-section of PSII ($\sigma_{\text{PSII}}$) in *N. oculata* (Figure 5.15) changed with growth condition and adaptation state in a pattern similar to *C. muelleri* (Figure 5.3). Photoacclimation to high light resulted in a marginally higher $\sigma_{\text{PSII}}$ while exposure to light resulted in a smaller $\sigma_{\text{PSII}}$. These changes did not appear to have a significant impact on ETR in comparison to other parameters.

*Nannochloropsis oculata* was capable of developing higher levels of NPQ than the other species examined here. The formation of NPQ was rapid, with values as high as $1.18 \pm 0.02$ (relative units) recorded during RLC analysis (Figure 5.18a). Unlike the other species, NPQ in HL-acclimated cultures of *N. oculata* measured in the light adapted state dissipated substantially within the 5 s of exposure to the first and lowest irradiance. During both RLC and LC(3) measurements dark adapted samples developed more NPQ than light adapted samples, although the difference was not as great during the longer light curve method (Figure 5.18a, b).

Interestingly, LL- and HL-acclimated cultures showed similar amounts of NPQ in RLCs. This finding was different to the observations of both *C. muelleri* and *I. galbana*, in which HL-acclimated cultures had more NPQ than those that were LL-acclimated (see Figure 5.6a and Figure 5.12a). LC(3) measurements showed little difference between the NPQ of LL- and HL-acclimated cultures, however, at moderate irradiances ($Q_{\text{phar}} = 3.65 – 18.74 \mu\text{mol quanta (µg chl-α)}^{-1} \text{h}^{-1}$; $E_{\text{PAR}} = 87 – 447 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$), particularly in dark adapted samples, cultures acclimated to high light
exhibited more NPQ (Figure 5.18b). As was observed for the other species in this study, conditions of the LC(20) method resulted in substantially higher NPQ than the other light curve methods (Figure 5.18c). LC(20)s found that in steady state conditions LL- and HL-acclimated cultures had similar NPQ at low irradiances, however they quickly diverged as irradiance increased (at about $Q_{phar} = 8.37$ µmol quanta (µg chl-a)$^{-1}$ h$^{-1}$ or $E_{PAR} = 294$ µmol quanta m$^{-2}$ s$^{-1}$) and LL-acclimated samples generated significantly more NPQ than samples from HL-acclimated culture.

![Figure 5.18: Nannochloropsis oculata (eustigmatophyte). Non-photochemical quenching (NPQ) of PSII fluorescence measurements taken from cultures grown in High Light (HL) and Low Light (LL) conditions. Data were collected from samples in both light adapted (LA) and dark adapted (DA) states (see text for details). Non-photochemical quenching data were collected over the course of light curves: (a) RLC, (b) LC(3) and (c) LC(20). Recovery of NPQ in the dark after (d) RLC and (e) LC(3) was also recorded. Legend for a. applies to b., d. and e also. Error bars = range (n = 3) [in a. error bars = se (n = 7-10)].]
Figure 5.19: Nannochloropsis oculata (eustigmatophyte). Photochemical quenching (qP) of PSII fluorescence measurements taken from cultures grown in High Light (HL) and Low Light (LL) conditions. Data were collected from samples in both light adapted (LA) and dark adapted (DA) states (see text for details). Photochemical quenching data were collected over the course of light curves: (a) RLC, (b) LC(3) and (c) LC(20). Recovery of qP in the dark after (d) RLC and (e) LC(3) was also recorded. Legend for a. applies to b., d. and e. also. Error bars = range (n = 3) [in a. error bars = se (n = 7-10)].

In N. oculata, as for the other species in this study, the qP versus $Q_{\text{phar}}$ relationship (Figure 5.19a-c) was very similar to the equivalent quantum yield relationship (Figure 5.17a-c). This is to be expected since the effective quantum yield ($F_v'/F_{m'}$) is considered the product of qP and the maximum quantum yield of PSII in the light adapted state ($F_v'/F_{m'}$) (Genty et al. 1989; Kromkamp and Forster 2003). HL-acclimated cultures were more capable at maintaining qP (open reaction centres) than LL-acclimated cultures, especially when sampled in semi-steady state (i.e. LC(3) analyses) or steady state (i.e. LC(20) analyses) conditions. In non-steady state conditions (i.e. RLC analyses) dark adapted samples experienced greater closure of
RCIs than those that were kept in the light adapted state (Figure 5.19a). This appeared to be a short-term phenomenon as it was not evident in LC(3) data (Figure 5.19b). Photochemical quenching did not change significantly between LC(20) sampling times (Figure 5.19c).

**Recovery**

Within the recorded recovery period after RLC and LC(3) analyses, HL-acclimated cultures were able to recover all measured photosynthetic parameters back to their original dark adapted levels. LL-acclimated cultures, on the other hand, showed a persistent negative impact on the light harvesting apparatus. Post-LC(3) recovery was slower and less complete than recovery after RLCs.

Quantum yield of dark and light adapted LL-acclimated cultures had reduced to just 7% and 12% of $F_v/F_m$ (adjusted to account for any quenching in the dark), respectively, by the completion of RLC analyses (Figure 5.17a). Both adaptation states recovered to a quantum yield of 0.473, or 77% of $F_v/F_m$, within 10 s of actinic light deactivation (Figure 5.17d). Rapid recovery of PSII quantum yield to 86% of $F_v/F_m$ continued over the following 1.5 min. Recovery then slowed, however, by the end of the recovery period (a total of 18 min 40 s) PSII quantum yield had recovered to within 4% of $F_v/F_m$. The pattern of quantum yield recovery was similar after LC(3) analyses, except that it was slightly slower and less complete (Figure 5.17e). Quantum yield recovered from 0.08 (Figure 5.17b) to 0.463 (78% of $F_v/F_m$ adjusted to account for quenching in the dark) over the first 1 min 40 s of the recovery period but remained 11% lower than $F_v/F_m$ at the final measurement.

Post-RLC recovery of quantum yield in HL-acclimated cultures was rapid and complete (Figure 5.17d). Within the first 10 s of recovery quantum yield increased from values as low as 0.044 (see Figure 5.17a dark adapted samples; 7% of $F_v/F_m$) to 0.550,
or ~85% of $F_v/F_m$. Full recovery was achieved within a further 1.5 min. As recovery continued, recorded quantum yield increased fractionally above the original $F_v/F_m$. In comparison, quantum yield of HL-acclimated cultures recovered to a maximum of 98.5% of $F_v/F_m$, with 75% and 93% recovery within the first 10s and 1 min 40 s respectively.

Non-photochemical quenching dissipation at the conclusion of RLC and LC(3) analyses showed an exponential decay pattern (Figure 5.18d, e). Ten seconds from the onset of post-RLC recovery >70% and >75% of NPQ had dissipated in LL- and HL-acclimated cultures respectively. While HL-acclimated cultures experienced full dissipation of NPQ within 10 min of dark recovery, LL-acclimated retained small amounts of NPQ (0.050 in dark adapted and 0.059 in light adapted samples) throughout the recovery period (Figure 5.18d). It is hypothesised that this may be persistent photoinhibitory quenching ($q_I$). In post-LC(3) recovery HL-acclimated cells were again able to dissipate >75% of the accumulated NPQ within 10 s (Figure 5.18e). However, full NPQ dissipation took longer than experienced after RLCs and required the full recovery period. The NPQ of LL-acclimated cells, particularly those that were dark adapted prior to analysis, decreased substantially within the first two min of recovery, indicating a predominance of $q_E$ type quenching. 1 min 40 s of darkness decreased NPQ by close to 81% and 85% of in light and dark adapted samples, yielding values of 0.203 and 0.179 respectively (Figure 5.18e). However, in each case NPQ dissipated by only another 2% over the remainder of the recovery period. Thus, low but persistent NPQ values of 0.183 and 0.158 were detected in light and dark adapted samples respectively, suggesting that the extended exposure to high irradiance during LC(3)s resulted in more $q_I$ type quenching than found after RLCs.

Recovery of $q_P$ was consistent with the recovery of quantum yield. Photochemical quenching of HL-acclimated *N. oculata* cultures fully recovered ($q_P$ =
1.00) within 1 min 40 s of the conclusion of RLCs (Figure 5.19d). This recovery was faster than either *C. muelleri* (Figure 5.7d) or *I. galbana* (Figure 5.13d) were able to achieve. There was a tendency for previously dark adapted samples to recover faster than light adapted samples in the initial 10 s of recovery after both RLCs and LC(3)s (Figure 5.19d, e), however, in each case the difference in $q_p$ became almost insignificant within 30 s (except for LL-acclimated samples post-RLC, when it took 3 min 40 s for light adapted samples to achieve $q_p$ equivalent to dark adapted samples). In both LL- and HL-acclimated cultures $q_p$ recovered more slowly and less completely (within the time of the recovery analysis) after LC(3) analyses than after the shorter RLC technique.

### 5.4 Discussion

Phytoplankton live in a highly variable light environment and, in order to maximise photosynthetic yield while minimising photodamage, their photosynthetic apparatus and associated metabolic pathways exhibit an inbuilt plasticity in response (Falkowski 1980; Macedo et al. 2002; Raven and Geider 2003; Walters 2005). While there are numerous short-term (seconds to minutes) responses to changes in photon fluence rate, cellular responses over the intermediate timescale (hours to days) allow *photoacclimation* and an increase in the niche width for a given genotype (Raven and Geider 2003; Colombo-Pallotta et al. 2006). Evidence gathered over the last decade suggests that photoacclimation is strongest in variable environments and is adjusted to an irradiance somewhat higher than the arithmetic mean of the daily irradiance, thus slightly favouring protection from photoinhibition over maximising photosynthetic performance in low light (Raven and Geider 2003; Walters 2005).

Several basic strategies for photoacclimation have been recognised in most phytoplankton, where the predominant changes are in (1) size of the photosynthetic unit, (2) density of the photosynthetic unit or, (3) abundance/activity of Calvin-Benson cycle enzymes (Falkowski and La Roche 1991; Raven and Geider 2003). One of the more
obvious physiological responses to increased irradiance is a reduction of cellular chl-\(a\) content (Herzig and Dubinsky 1992). Each of the species studied here conformed to this response pattern, with chl-\(a\) content of HL-acclimated cells anywhere between 43\% (\textit{C. muelleri}, Figure 5.2) and 77\% (\textit{N. oculata}, Figure 5.14) lower than in LL-acclimated cells. Data from fast flash analysis of \(\sigma_{\text{PSII}}\) suggests that, for each species, this reduction of chl-\(a\) was primarily achieved via a reduction in the number of photosynthetic units (i.e. strategy 2 as mentioned above). These results for \textit{I. galbana} are not consistent with the findings of Dubinsky et al. (1986), who found substantial reductions in estimates of both \(\sigma_{\text{PSII}}\) and chl-\(a\) per photosynthetic unit. However, studies by Suggett et al. (2004) and Fisher et al. (1996) yield a similar indication of acclimation via changes in the number photosynthetic units for \textit{C. muelleri} and \textit{Nannochloropsis sp.} respectively.

The maximum chl-specific rate of photosynthesis consistently has been shown to increase with acclimation to high light (Falkowski and La Roche 1991; Herzig and Dubinsky 1992; Colombo-Pallotta et al. 2006), however exceptions to this also have been reported (eg. Grobbelaar et al. 1992). All three species studied here were found to conform to this pattern, exhibiting substantially higher chl-specific ETR\(_{\text{max}}\) when HL-acclimated (Figures 5.04, 5.10 and 5.16). This is attributed to a combination of higher \(\eta_{\text{PSII}}\) in HL-acclimated cultures (as assumed from previous studies) as well as their ability to maintain higher \(F_{q'/F_m'}\) in saturating irradiances (Figures 5.05, 5.11 and 5.17). For \textit{N. oculata} \(\eta_{\text{PSII}}\) was assumed to be invariable and therefore increased ETR\(_{\text{max}}\) was predominantly a result of higher \(F_{q'/F_m'}\), with a small contribution from \(\sigma_{\text{PSII}}\) as it was 7\% larger in HL-acclimated cultures (Figure 5.15). Increases in photosynthetic rate with acclimation to high light have been correlated with faster turnover of the photosynthetic apparatus (\(\tau\)) (Herzig and Dubinsky 1992). Cellular changes that enable a faster \(\tau\) include increases in density of the cytochrome \(b_6/f\) complex and ATP synthase (Walters
2005), however the dominant factor is likely to be an increase in the concentration of Rubisco per active reaction centre (Herzig and Dubinsky 1992; Walters 2005; Colombo-Pallotta et al. 2006). Enhanced levels of Rubisco can explain instances where HL-acclimated or light adapted samples display lower NPQ and higher \( q_p \) relative to LL-acclimated or dark adapted samples (eg. Figure 5.18a,c and Figure 5.19c), especially when increases in ETR\(_{\text{max}}\) are accompanied by a relatively stable \( \alpha^{chl} \) (Figure 5.16c) (Fisher et al. 1996; Colombo-Pallotta et al. 2006).

In the present study samples were analysed in both the light and dark adapted states. Non-steady state RLC analyses typically found light adapted samples to have a higher ETR\(_{\text{max}}\) than dark adapted samples, although the \( \alpha \)-slope was generally lower since NPQ had not relaxed. This ability of light adapted cells to achieve a higher ETR\(_{\text{max}}\) was attributed to partial deactivation of Calvin-Benson cycle enzymes (such as Rubisco) and was more profound in HL-acclimated cultures. The tendency for this to have less of an impact on LL-acclimated cultures (both \( C.\ muelleri \) and \( I.\ galbana \) showed no difference between light and dark adapted ETR\(_{\text{max}}\) (Figure 5.4 and 5.10)), suggests that they either require a longer dark period for deactivation of Calvin-Benson cycle enzymes (ie. longer than the 15 min applied for dark adaptation) or that other electron transport chain factors are rate limiting. While the difference between adaptation states decreased or became negligible in \( C.\ muelleri \) and \( N.\ oculata \) during LC(3) analyses(Figure 5.4b and 5.16b), presumably a result of reactivation of Calvin-Benson cycle enzymes, greater differences in ETR\(_{\text{max}}\) were observed in the haptophyte \( I.\ galbana \) (Figure 5.10b). In the case of LL-acclimated cultures measured in semi-steady state (i.e. via LC(3) methodology), dark adapted samples of \( I.\ galbana \) actually achieved significantly higher ETR\(_{\text{max}}\) than light adapted samples. Given that quantum yield, NPQ and \( q_p \) were similar in both light and dark adapted samples, the difference is attributed to the higher \( \sigma_{PSII} \) in dark adapted samples (Figure 5.9).
The saturation irradiance, $E_k$, is commonly used as an indicator of the photoacclimation status of microalgae (Serôdio et al. 2006), with higher values of $E_k$ suggesting acclimation to higher irradiances. In this study the $E_k$ for HL-acclimated cultures of each species was always significantly higher than for LL-acclimated cultures. The only exception to this was during the 20 min sampling period of LC(20) analyses of *C. muelleri* cultures, which had a similar $E_k$ for both acclimational states (Table 5.2). Despite this, when the data were integrated over the whole 20 min period LL-acclimated $E_k$ ($15.1 \pm 0.3 \ \mu$mol quanta ($\mu$g chl-a)$^{-1} \ \text{h}^{-1}$; error = 95% confidence interval) was lower than HL-acclimated $E_k$ ($16.9 \pm 0.4 \ \mu$mol quanta ($\mu$g chl-a)$^{-1} \ \text{h}^{-1}$).

While RLCs provide a measure of an immediate photosynthetic response based on recent light history, steady-state LC(20)s provide a better measure of the potential photosynthetic performance over the medium term. All three species responded to the extended irradiance period differently, however only one, *N. oculata* displayed symptoms of substantial photoinhibitory damage. The chl-a/c containing *C. muelleri* and *I. galbana* exhibited no appreciable downturn of ETR at high irradiances in the LC(20) (Figure 5.4 and 5.10). At high irradiance ($Q_{\text{phar}} = 45 \ \mu$mol quanta ($\mu$g chl-a)$^{-1} \ \text{h}^{-1}$; $E_{\text{PAR}} = 1335 \ \mu$mol quanta m$^{-2} \ \text{s}^{-1}$) there was a slight depression in the ETR of LL-acclimated *I. galbana* which coincided with a sudden increase in NPQ (Figure 5.12). *Isochrysis galbana* had previously shown the vast proportion of NPQ to be of the fast recovering q$_E$ type with only small amounts (roughly equivalent to the amount of NPQ apparent in the dark (Figure 5.12b)) remaining after 3 min 40 s of recovery (Figure 5.12d, e). Since q$_P$ was not overly depressed and quantum yield remained at approximately 0.2, this depression of ETR is considered to be the result of regulatory changes enhancing non-radiative dissipation of excess energy rather than long-term photoinhibition. On the other hand, Figure 5.16c shows that the LC(20)s of LL- and HL-acclimated *N. oculata* cultures, while achieving equivalent or higher ETR$_{\text{max}}$ to
RLCs or LC(3)s, also experienced substantial downturn. Recovery data after LC(3) measurements of LL-acclimated *N. oculata* found persistent depression of quantum yield and qP while 13 – 17% of the developed NPQ remained at the end of the recovery period. This is interpreted as minor photoinhibition as, unlike the chl-a/c containing species, *N. oculata* (chl-a only) developed minimal NPQ in the dark. While a lack of recovery data post-LC(20) hinders interpretation of the downturns present in Figure 5.16c, extrapolation from the LC(3) recovery data and previous evidence from Magnusson (1997) would suggest that the extended exposure to high irradiance resulted in more substantial photoinhibition as the core D1 protein became damaged.

Each of the species studied here developed substantially more NPQ during LC(20)s than during RLCs or LC(3)s. This phenomenon does not seem to be related to a specific accessory pigment since *N. oculata* possesses the violaxanthin-asteraxanthin-zeaxanthin cycle, while *C. muelleri* and *I. galbana* possess the diadinoxanthin-diatoxanthin cycle (Raven and Geider 2003). It is also not likely to be purely time-related as the difference between maximum NPQ recorded in LC(3)s and the 5 min sampling period of LC(20)s was large and data from Olaizola and Yamamoto (1994) indicate that, in *C. muelleri*, most of the diadinoxanthin pool becomes de-epoxidised within 2 min of exposure to bright light. However, given that xanthophyll cycle carotenoids absorb predominantly in the 450 – 500 nm range (Colombo-Pallotta et al. 2006) and actinic lights that included this waveband were used during LC(20)s only, it is possible that direct energetic activation of xanthophyll cycle pigments may increase the speed of NPQ development or the binding of diatoxanthin to protonated antenna sites, an event which may be obligatory for energy dissipation (Lavaud et al. 2002a). Like Olaizola and Yamamoto (1994), Serôdio et al. (2005) found that the major component of NPQ in diatoms developed within 2 – 3 min, however this data was
collected with a PAM fluorometer fitted with an LED emitting in the 430 – 470 nm waveband.

5.5 Conclusion

The data presented here highlight the dynamics of photosynthesis and, as a consequence, the impact of sampling procedure (light adapted / dark adapted) and exposure time (RLC / LC(3) / LC(20)) on final results. Ultimately the choice of non-steady state versus steady state measurements is dependent on the question being asked, however, dark adaptation appears to understate the differences between LL- and HL-acclimated cultures. In essence, the use of ETR$_{\text{max}}$ (or rETR$_{\text{max}}$) values taken from dark adapted samples can lead to inaccurate characterisation of photosynthetic potential. While dark adaptation has been considered useful in field situations to minimise variations based on changes in recent light history such as cloud cover, maintaining samples for a uniform period in low-moderate light may avoid dark inactivation of Calvin-Benson cycle enzymes and phenomena such as dark-induced NPQ. Importantly, the consistently higher NPQ recorded in each species during LC(20)s compared to RLCs and LC(20)s implicates an impact of light quality on processes leading to the build up of non-radiative energy dissipation.

Each of the microalgae studied here responded to high light conditions by reducing chl-a content and adjusting the photosynthetic apparatus and associated metabolic pathways to enable exploitation of the enhanced photon fluence rate to achieve higher chl-specific rates of photosynthesis.

Relatively minor changes in $\sigma_{\text{PSII}}$ indicate that cells decreased the number of photosynthetic units in response to high light. A common response to high light involves an increase in Rubisco concentration per reaction centre (Herzig and Dubinsky 1992; Colombo-Pallotta et al. 2006). Fluorescence data suggest that, while this was a
feature of photoacclimation in each of the three species studied here, it was particularly important in the eustigmatophyte *N. oculata.*

All species appeared capable of photoacclimation to maximise photosynthetic efficiency while minimising the chance of photoinhibition. *Isochrysis galbana* was most capable of maintaining high rates of electron transport through PSII in high light, with only minor indications of possible photoinhibition developing in LL-acclimated cultures after 20 min exposure to $Q_{phar}$ of 46.3 µmol quanta (µg chl-a) h$^{-1}$ ($E_{PAR} = 1365$ µmol quanta m$^{-2}$ s$^{-1}$). *Chaetoceros muelleri* maintained moderate rates of electron transport while effectively modulating dissipation of excess energy to avoid photoinhibition. *Nannochloropsis oculata* appeared to be most susceptible to photoinhibition, although this was greatly minimised in HL-acclimated cultures.
CHAPTER 6
Photosynthesis in Fremantle Harbour – A Diurnal Study

6.1 INTRODUCTION

The city of Perth, Western Australia, surrounds the Swan-Canning Estuary. This estuary is the second largest estuary in southwestern Australia (Thompson 1998) and Fremantle Harbour is located at its mouth. Freshwater flow from tributaries is highly seasonal and a salt-wedge extends up the estuary from October to July/August (http://www.wrc.wa.gov.au/srt/riverscience/). Apart from during periods of high flow in winter and early spring, the near-surface waters in Fremantle Harbour can be assumed to be representative of coastal waters. There is a paucity of data on phytoplankton production and photosynthetic dynamics in the lower Swan-Canning Estuary and, to a lesser extent, the Perth coastal waters. Measurements by Thompson (1998) indicated phytoplankton primary production in the lower estuary was ~20 mg C m\(^{-3}\) h\(^{-1}\) or, based on his chlorophyll data, ~0.41 µmol C (µg chl-a)\(^{-1}\) h\(^{-1}\). Similar rates of production were later measured in the Sepia Depression, located in coastal waters south-west of Fremantle Harbour (Thompson and Waite 2003). The recent Strategic Research Fund for the Marine Environment (SRFME) programme has gone some way to filling in gaps in our knowledge regarding phytoplankton primary production in our coastal marine environment, however, ship-based resources are limited and evaluating the applicability of chlorophyll-fluorescence techniques for the rapid assessment of primary production is an important question. The use of chlorophyll fluorescence techniques for this purpose would allow greater sampling frequency and resolution and would, to a certain extent, reduce the requirement for a large vessel as a platform. A significant amount of comparative data would need to be collected before such a scenario could become reality and while some C-fixation versus rETR (Phyto-PAM) data has recently been
collected in Perth’s coastal waters as a part of the SRFME programme (F. Verspecht pers. comm.) further data collection is essential.

This Chapter aims to take the experience and knowledge gained in Chapters 4 and 5 and apply it to a natural population of phytoplankton in the field. The study was performed over a diurnal period to allow evaluation of any changes in the relationship between chl-fluorescence based measurements of primary production and O₂-evolution or C-fixation measurements. This study also allowed the collection of data on diurnal patterns of photosynthesis in a coastal phytoplankton population. To my knowledge, after a thorough literature search, such measurements have not previously been performed in the temperate, oligotrophic coastal waters near Perth.

Available data suggest that daily endogenous rhythms in P_{max} and α, that are independent of changes in chl-α content, occur in all major taxonomic groups of phytoplankton (Harding et al. 1982; Boyd et al. 1997; Behrenfeld et al. 2004). Such rhythms should be taken into account when designing experiments or interpreting results. Some taxa, such as the diatoms (Bacillariophyta), appear to display a greater diel periodicity than others (Behrenfeld et al. 2004).

Studies have found both morning (Marra et al. 1985) and afternoon (Harding et al. 1982) maxima of P_{max}. Timing differences in the pattern of diel changes have previously been explained by changes in latitudinal photoperiod and variations in nutrient assimilation and utilisation between phytoplankton size fractions (Harding et al. 1982). More generally, diel rhythms in P_{max} and α are commonly understood to result from oscillations between metabolic pathways which are influenced by light periodicity and exogenous nutrient supply (Harding et al. 1982; Behrenfeld et al. 2004). Nutrients accumulated during dark periods may help to yield a high P_{max} the following morning (Erga and Skjoldal 1990).
Boyd et al. (1997) found that the light saturation point ($E_k$) measured using PAM fluorescence techniques decreased towards evening while $E_k$ measured by the $^{14}$C-fixation method remained relatively high. While this discrepancy may be due, at least in part, to the longer incubation and light exposure required for the $^{14}$C-fixation method, it potentially supports the assertion by Behrenfeld et al. (2004) that enhanced ATP requirements during the afternoon result in the diversion of photosynthetic reductant from C-fixation. Down-regulation of PSII has been suggested to be the cause of low ETR$_{\text{max}}$ values in the afternoon (Longstaff et al. 2002).

A common measure of photosynthetic efficiency and physiological stress, the chl-fluorescence parameter $F_v/F_m$ is extensively used to describe diurnal patterns in photosynthesis. Diurnal variability in $F_v/F_m$ has been shown to be closely related to incident irradiance and midday depression of $F_v/F_m$ is frequently observed when irradiance is high (i.e. no or limited cloud cover) (e.g. Bergmann et al. 2002). Reduction of $F_v/F_m$ can result from a number of physiological processes: down-regulation of photosynthesis and closure of RCIIIs (associated with D1 dynamics) if caused by a decline in $F_m$ and increase in $F_o$ (Demmig and Björkman 1987; Magnusson 1997; Campbell et al. 2003), a disproportionate reduction of $F_o$ compared to $F_m$ may represent enhanced xanthophyll cycle activity (photoprotection) or enhanced NADPH-reductase activity leading to state-2 in the dark (Demmig and Björkman 1987; Magnusson 1997; Fracheboud 2001). However, it should not be assumed that $F_v/F_m$ maxima and minima always occur during the evening and around midday respectively. Behrenfeld and Kolber (1999) describe measurements taken in the South Pacific Ocean (prokaryote-dominated phytoplankton community in iron limited conditions) that exhibit nocturnal $F_v/F_m$ minima.

The aim of this study was to investigate diurnal changes in maximum quantum efficiency of PSII ($F_v/F_m$) and photosynthesis (ETR and O$_2$-evolution measurements).
Carbon fixation measurements were taken with the dual aims of testing the accuracy of predictions from chl-fluorescence measurements and to evaluate daily patterns in primary production.

6.2 METHODS

The study site was located at the mouth of Fremantle Harbour, with sample collection from a dinghy between the Maritime Museum of Western Australia and the OceanFarm jetty (Figure 6.1; GPS coordinates: 32°03’18.90” S 115°44’14.93” E). All sample collection and on-site measurements were performed on 7th December, 2005. Data on solar irradiance (W m$^{-2}$) incident at sea level were taken from Murdoch University meteorological station data (located 9.4 km from Fremantle Harbour; http://wwwmet.murdoch.edu.au/downloads.htm). This data was converted to photosynthetically available radiation (400 – 700 nm) in units of µmol quanta m$^{-2}$ s$^{-1}$ following the method of Thompson (1998) (Equation 46):

$$PAR = TSI \times 0.45 \times 4.151$$  \hspace{1cm} (46)

where 0.45 estimates the proportion of total solar irradiance (TSI) that is photosynthetically active and 4.151 is a unit conversion from W m$^{-2}$ to µmol quanta m$^{-2}$ s$^{-1}$. Water temperature was 18°C during the early morning and late evening and 20°C throughout the day from 10 am to 6 pm. Lower temperatures coincided with ebb tide while higher temperatures occurred during flood tide. Tidal information was collected from the Coastal Data Centre (http://www.dpi.wa.gov.au/imarine/coastaldata/1895.asp) and on-site observations.
Figure 6.1: The study site (yellow dot) on the seaward edge of Fremantle Harbour, the mouth of the Swan-Canning Estuary (image courtesy of http://maps.google.com/). The line drawing shows the Swan and Canning Rivers flowing into the estuary, with Fremantle harbour located at the lower left.

The natural phytoplankton community was not concentrated for C-fixation measurements and surface water samples were collected from the jetty. This water was immediately passed through a 180 µm mesh to remove zooplankton and 1 L set aside and preserved with Lugol’s iodine solution for later identification and enumeration of the phytoplankton species. The remaining sample was then used for short incubation measurements of $^{14}$C uptake as described in Chapter 3. However, as no facilities for degassing samples at the end of each incubation were available, samples were killed at the end of the 20 min incubation with 50 µL of buffered formalin. Once all samples were collected and returned to the laboratory the samples were degassed and processed as per Chapter 3.

Phytoplankton identification and enumeration was performed using a Sedgewick-Rafter chamber at 200X magnification under a light microscope. Samples had previously been allowed to settle and concentrated 10 – 15 fold. Cell density
calculation took concentration and dilution (due to addition of Lugols) into account. The most dominant phytoplankton were identified to species level, however in most instances cells were simply categorised into one of the following groups: Bacillariophyta (diatoms), Dinophyta (dinoflagellates), Chlorophyta (chlorophytes), prasinophytes, Cryptophyta (cryptomonads), Euglenophyta (euglenoids), Chrysophyta (chrysophytes), Raphidomonadophyceae (raphidophytes), Haptophyta (haptophytes), Silicoflagellata (silicoflagellates) and others.

For each sampling period a ~5 L near-surface (~0.5 m) grab sample was collected for chlorophyll determination. Samples were kept cool and in dim light until they could be returned to the university laboratory (no longer than 2 h) for filtration through Whatman GF/F (45 mm) filters under low pressure. Filters were patted dry, wrapped in aluminium foil and frozen at -80°C for later extraction. Chlorophyll extraction and measurements were performed as outlined in Chapter 3.

Samples for chl-fluorescence and O₂-evolution measurements were collected from a small dinghy using a 25 µm mesh-size phytoplankton net towed just below the surface until a concentration of phytoplankton material satisfactory for O₂-evolution measurement was collected. This generally took about 20 – 25 minutes. Upon return to the shore the suspension in the phytoplankton net cod-end was passed through a 180 µm mesh to remove zooplankton prior to sample measurements.

Oxygen evolution measurements were performed in triplicate using three separate polarographic oxygen electrodes following the methods as detailed in Chapter 3, except in this case the temperature of the water jackets was maintained at 20°C (similar to the water temperature at the sampling location).

All fluorescence measurements were performed with a Water-PAM fluorometer (Walz, Germany) consisting of a Water-ED emitter-detector unit and a PAM-Control
box. Data collection was via the WinControl software provided with the fluorometer. Preliminary measurements found that signal-to-noise ratio was too low using unaltered samples and as a consequence concentrated samples were used (as described above for oxygen evolution measurements). Samples were kept in dim light until used. Concentration of samples in this manner may shock phytoplankton cells and can result in substantially lowered quantum yield measurements (Peter Ralph, pers. comm.). However, the quantum yield values measured on concentrated samples were high, suggesting that during this study the procedure had no detrimental impact on the physiological state of sampled phytoplankton. Samples were collected for fluorescence analysis on an hourly basis from 3 am until 10 pm.

Rapid light curves (RLCs) were performed to measure electron transport rate (ETR). Light levels were chosen, based on preliminary measurements, to achieve a balance between maintaining as many points in the light-limited region of the curve while allowing an asymptote or down-turn to be evident. Gain levels were adjusted to maintain a dark fluorescence signal of approximately 200 units. Light width at each irradiance was 5 s and saturation pulse duration was 800 ms. For each sampling period ten replicate RLCs were performed on light adapted samples (kept in shaded conditions during collection procedure and during any delay before measurement). Ten replicate dark adapted $F_v/F_m$ measurements were also collected at each sampling period. Dark-adaptation was for 15 minutes and samples were exposed to 5 s of far-red light immediately prior to applying the saturation pulse.

Calculation of absolute ETR was performed using Equation 26 (Chapter 1, pg. 63) rather than as described in Chapter 4. This was due to the failure of the FL-3000 dual PAM fluorometer (Photon Systems Instruments, Czech Republic), resulting in a lack of $\sigma_{\text{PSII}}$ data for this study. A number of assumptions were made in order to
calculate absolute ETR. The absorption coefficient of cultured, low light acclimated *Chaetoceros muelleri* was used in the calculation of $Q_{\text{phar}}$ as is was assumed to approximate the absorption coefficient of the diatom-dominated natural phytoplankton community at the mouth of the Swan-Canning Estuary. It was also assumed that 50% of the absorbed quanta were directed to PSII. Due to a lack of $\sigma_{\text{PSII}}$ data and the reliance on the above assumptions, absolute ETR was calculated for the 3 am, 7 am, 11 am and 3 pm time periods only. This data was used to predict gross oxygen evolution ($\text{GOE}_{\text{fch}}$, refer to Equation 37; pg. 116) and carbon assimilation ($\text{P}_{\text{fch}}$, refer to Equation 44; pg. 122) to compare with measured values. Relative electron transport rate (rETR; Equation 28; pg. 64) was used to report diurnal changes in PSII photosynthetic rate.

Diurnal changes in P-E curve parameters are also reported. These parameters were determined by fitting the model of Eilers and Peeters (1988) to photosynthesis versus irradiance data as described in Chapter 3. Unlike the laboratory data, this model was able to be used for C-fixation data. The error of the parameter estimates was derived from the covariance matrix of the fitted model and expressed as standard error.

### 6.3 RESULTS

#### 6.3.1 Species composition, cell density and biomass (chl-a)

Diatoms (Bacillariophyceae) were found to be the dominant taxon throughout the sampling period. Diatoms were most dominant in the evening, representing a maximum of 88% of the phytoplankton community at 10 pm and a minimum of 46% at 3 pm (Figure 6.2). The highest percentage of diatoms occurred close to the end of the flood tide. However, the large species *Chaetoceros lorenzianus* and *C. curvisetus* were dominant at 3 pm while the small species (unidentified and referred to as *Chaetoceros* sp1.) was dominant in the evening (Table 6.1). The most commonly abundant species during the sample period were: *Chaetoceros* sp1., *Pseudonitzschia seriata*, unidentified
nannoflagellates and *C. curvisetum*. Due to the predominance of diatoms throughout the entire sampling period it was assumed that changes in species composition had negligible impact on chl-fluorescence measurements.

**Figure 6.2:** Percentage composition of phytoplankton taxa in Fremantle Harbour over the duration of the study period. Ebb tide is indicated by the thatched bar at the top of the graph while flood tide is indicated with an open bar.

**Figure 6.3:** Some examples of the dominant diatom flora taken from the sampling site. (a) *Chaetoceros* sp1., (b) *C. curvicetus*, (c) *C. cf. laciniosus* and (d) *Pseudonitzschia seriata*. Image (d) was taken from the Algaebase website ([www.algaebase.org](http://www.algaebase.org)) and no scale bar was available.
Table 6.1: Fremantle Harbour phytoplankton community composition and relative abundance at ~0.5 m depth. Grab samples were collected every second hour throughout the day. (+) present, (++) present in moderate relative abundance, (+++) present at a high abundance relative to other species.

<table>
<thead>
<tr>
<th>Time</th>
<th>Diatoms</th>
<th>Dinoflagellates</th>
<th>Cryptophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 AM</td>
<td>Bacillaria cf. paradoxa (+)</td>
<td>Amphidinium sp. (+)</td>
<td>cf. Plagioselmis spp. (+)</td>
</tr>
<tr>
<td>5 AM</td>
<td>Cerataulina sp. (+)</td>
<td>Ceratium furca (+)</td>
<td>cf. Rhodomonas sp. (+)</td>
</tr>
<tr>
<td>7 AM</td>
<td>Chaetoceros curvisetus (+++)</td>
<td>Dinophysis acuminata (+)</td>
<td>cf. Teleaulax sp. (+)</td>
</tr>
</tbody>
</table>
| 9 AM | Chaetoceros cf. lorenzianus var. forceps (+) | Diplopsalis sp. (+) | ...(continued next page)…
| 11 AM | Chaetoceros decipiens (+) | Gonyaulax sp. (+) |
| 1 PM | Chaetoceros tenuissimus (+) | Gymnodinium spp. (+) |
| 3 PM | Chaetoceros sp1. (+++) | Gyrodinium spp. (+) |
| 5 PM | Cylindrotheca closterium (+) | Katodinium glaucum (+) |
| 7 PM | Cyclotella meneghiniana (+) | Katodinium rotundata (+) |
| 10 PM | Diploneis sp. (+) | Polykrikos sp. (+) |
| 12 AM | Entomoneis sp. (+) | Prorocentrum minimum (+) |
| 2 AM | Guinardia sp. (+) | Prorocentrum micans (+) |
| 4 AM | cf. Gyrosigma sp. (+) | Prorocentrum triestinum (+) |
| 6 AM | Licmophora sp. (+) | Protoperidinium spp. (+) |
| 8 AM | Navicula spp. (+) | Scrippsiella cf. trochoidea (+) |
| 10 AM | Nitzschia spp. (+) | Stenionema costatum (+) |
| 12 PM | Pseudonitzschia seriata (+++) | cf. Striatella sp. (+) |
| 2 PM | Pseudonitzschia pseudodelicatissima (+) | Thalassionema sp. (+) |
| 4 PM | cf. Roicosigma sp. (+) | Thalassiosira sp. (+) |
| 6 PM | Skeletonema costatum (+) | \(\ldots\)(continued next page)…
Phytoplankton cell density and chl-$a$ concentration in Fremantle Harbour over the duration of the study period. Inconsistencies at 1300 hrs and 2200 hrs relate to changes in species composition.

The chl-$a$ concentration of the marine waters at the mouth of Fremantle Harbour increased throughout the day, reaching a peak of 2.54 µg chl-$a$$^{-1}$ L$^{-1}$ at 15:00 hrs before decreased in the evening towards the end of flood tide. While phytoplankton cell density
was generally positively related with chl-\(\alpha\) concentration, there were some periods where dominance of large (13:00 hrs) or small (10:00 hrs) species yielded relatively high or low chl-\(\alpha\) concentrations, respectively.

6.3.2 Diurnal variability in photosynthesis

Analysing \(F_v/F_m\) data indicated a typical diurnal cycle pattern (Figure 6.5a). \(F_v/F_m\) was quite high (~0.65) at 0300 hrs and 0400 hrs, however immediately prior to dawn \(F_v/F_m\) increased to a value of 0.81 ± 0.01 (error = se, \(n = 10\)). This value was very close to theoretically maximal values of 0.83 (Magnusson 1997). A rapid decrease in \(F_v/F_m\) occurred over the early daylight hours and, throughout most of the day, values between 0.57 and 0.60 were observed. \(F_v/F_m\) dropped below this to values of 0.51 ± 0.02 at 0900 hrs, 0.54 ± 0.01 at 1300 hrs and 0.51 ± 0.01 at 1500 hrs. Each of these minima were associated with a period of high incident irradiance during the sample collection period (Figure 6.5a).

The maximum photosynthetic rates as measured by chl-fluorescence (rETR_{\text{max}}) indicate that photosynthetic processes were substantially down-regulated during the night (Figure 6.5b). Re-induction of these processes was rapid and possibly even predictive, since photosynthetic capability, as indicated by rETR_{\text{max}}, began to increase prior to dawn. This development of photosynthetic capacity was also reflected by the sudden and large increase in \(\alpha\) (Figure 6.5c). rETR_{\text{max}} reached a peak at 0800 hrs (408 ± 10.6 relative units, error = se, \(n = 10\)) before declining again as the first periods of high irradiance (Figure 6.5a) resulted in downregulation of photosynthesis and some apparent photoinhibition. Downregulation was indicated by the earlier and proportionately greater drop in \(\alpha\) compared to rETR_{\text{max}}, leading to higher values for \(E_k\) (Figure 6.5d).
Figure 6.5: (a) Phytoplankton $F_v/F_m$ (mean ± se, $n = 10$) over the course of the day relative to incident irradiance. The diurnal changes in (b) maximum photosynthetic rate, (c) alpha and (d) $E_k$ parameters measured by each method ($●$ = chl-$a$ fluorescence; $▼$ = $O_2$ evolution and $\Delta$ = C-fixation) were determined by modelling P-E curve data with the model of Eilers & Peeters (error bars = se as determined by the model).
The depression of \( F_v/F_m \) at 0900 hrs and a concomitant decrease in \( \text{rETR}_{\text{max}} \) and \( E_k \), after a gap in the clouds resulted in the first period of high irradiance for the day, suggested photoinhibition due to damage of the photosynthetic apparatus may have occurred. A substantial recovery of \( F_v/F_m \) from 0.51 ± 0.02 (error = se) at 0900 hrs to 0.59 ± 0.01 in the following hour suggests that down-regulation rather than photoinhibition was responsible for the downturn in photosynthesis, however, a continuing slow recovery of \( F_v/F_m \) over the next 2 hours of cloud cover (low incident irradiance) suggests that some photodamage did occur. Depression of \( F_v/F_m \) was observed again at 1300 hrs and 1500 hrs after extended periods of high light, with the 1500 hrs depression being accompanied by decreases in \( \text{rETR}_{\text{max}} \) and \( \alpha \) despite slightly lower incident irradiances. By dusk \( \text{rETR}_{\text{max}} \) began to decrease substantially despite large increases in photosynthetic efficiency (as indicated by \( \alpha \)). This indicates substantial rate limitation by the Calvin-Benson cycle as its associated light activated enzymes begin to ‘switch off’. The diurnal pattern of \( \alpha \) closely reflected \( F_v/F_m \) changes, given that the initial slope is the product of the chl-specific absorption coefficient and maximum photosynthetic efficiency, this suggests that the former parameter remained relatively stable throughout the day.

Through the morning and early afternoon the P-E curve parameters determined from \( \text{O}_2 \)-evolution and C-fixation data follow similar trends to those for the chl-fluorescence data (Figure 6.5). \( \text{P}^{\text{chl}}_{\text{max}} \), like \( \text{rETR}_{\text{max}} \) had minimum values in the early morning prior to dawn, however did not increase until after dawn and did not reach its peak value \( (2.27 ± 0.25 \ \mu\text{mol C (\mu g chl-a)}^{-1} \ \text{h}^{-1}) \) until 1 pm. In general, \( \text{P}^{\text{chl}}_{\text{max}} \) and \( \text{rETR}_{\text{max}} \) exhibited a typical pattern (Henley 1993) of increased values with acclimation to higher irradiance. \( \text{O}_2 \)-evolution measurements, on the other hand, gave a relatively stable \( \text{P}_{\text{O}_2}^{\text{chl}}_{\text{max}} \) throughout the day, with a slight depression at 0900 hrs and high variances at 1300 hrs and 1500 hrs coinciding with periods of relatively high light.
Each of the measurement techniques recorded peaks in $\alpha_{chl}$ around dawn followed by a decrease for the greater part of the day and, where measured, a recovery in the evening (Figure 6.5c). This common trend is reflected in the $E_k$ value which increased at dawn from its nocturnal minimum, and then remained high throughout the day before rapidly decreasing at dusk (Figure 6.5d).

On four occasions throughout the day it was possible to attempt a prediction of oxygen evolution and C-fixation from chl-fluorescence measurements and compare the predictions with actual measurements. Results obtained in the field reflect previous laboratory results in that chl-fluorescence measures, as calculated here, tend to underestimate $P_{O_2}^{chl}$ and $P^{chl}$ (Figure 6.6). Also, apart from those samples collected at 0300 hrs, $P_{O_2}^{chl}$ had a curvilinear relationship with $GOE_f^{chl}$. The predicted values from chl-fluorescence measurements tending to give overestimates at moderate to high irradiances (Figure 6.6). The linear relationship between $P_f^{chl}$ and $P^{chl}$ was strongest ($r^2 = 0.830$) at 1100 hrs, during an extended period of cloud cover (Figure 6.6). This relationship was weakest ($r^2 = 0.181$) at 1500 hrs when a period of high light stress was occurring.
Figure 6.6: Relationships between GOE$_f$ (chl) and measured P$_{O_2}$ (chl) (○) plus P$_f$ (chl) and measured P$_{chl}$ (●) at four different times of the day. Error bars along the x-axes represent standard error (n = 7 – 10) while error bars along the y-axes represent range (n ≤ 3).

6.4 DISCUSSION

The phytoplankton community in the coastal marine waters sampled at the mouth of the Swan-Canning Estuary (this study) were found to be dominated by members of the Bacillariophyceae (diatoms) (Figure 6.2,
Dominance of diatoms in both near-shore coastal waters (Thompson and Waite 2003) and in the lower reaches of the Swan-Canning Estuary (Thompson 1998) has been described previously. The persistent dominance of diatoms throughout the entire study period was fortunate, as shifts in taxonomic composition between species with different pigments (light harvesting and accessory) and/or thylakoid arrangements can influence the signature obtained by PAM fluorometers (Büchel and Wilhelm 1993). Thus, the observed taxonomic stability gave some confidence to the assumption that changes in fluorescence signal represented changes in physiological state of the phytoplankton community rather than taxonomic shifts.

The chl-a concentrations ranged between 0.881 µg L\(^{-1}\) at 0300 hrs to 2.539 µg L\(^{-1}\) at 1500 hrs (Figure 6.4). Peaks in chl-a concentration above ~1.6 µg L\(^{-1}\) were dependent on the presence of large, chlorophyll-rich diatom species such as Chaetoceros curvicetus, C. laciniosus and Skeletonema costatum. The chl-a concentrations here were similar to those recorded at Blackwall Reach, about 6 km upstream, by Thompson (1998), although cell densities were substantially lower. These low concentrations meant that concentration of the phytoplankton community was required for both oxygen electrode and PAM chl-fluorescence measurements to achieve an acceptable signal to noise ratio. Given that \(\frac{F_v}{F_m}\) values recorded after the concentration procedure were as high as 0.807 ± 0.007 (error = se, \(n = 10\); Figure 6.5a) any negative impact on phytoplankton physiology was considered negligible.

Endogenous diel oscillations in the P-E response of marine phytoplankton have been widely reported (Harris 1980; Harding et al. 1981, 1982; Boyd et al. 1997). Henley (1993) states that maximum photosynthetic rate usually peaks in the morning or close to midday and that this pattern may be endogenous, rather than related to chl-content, photoinhibition, nutrition or feedback inhibition by accumulated photosynthate. In this study the observed patterns of \(P^{\text{chl}}_{\text{max}}\) and particularly \(r\text{ETR}_{\text{max}}\) appear to be a response...
to the changing light environment, with induction in the morning as Calvin-Benson cycle enzymes increase at dawn (with light activation) and down-regulation or inhibition during the middle part of the day as RCIs become closed or damaged (Figure 6.5a, b). However, the significant increase in rETR$_{\text{max}}$ (and $\alpha_{\text{chl}}$) prior to dawn suggests that, to some extent, an endogenous rhythm was also present.

Unlike rETR$_{\text{max}}$ and P$_{\text{chl}}^{\text{max}}$, the maximum photosynthetic rate obtained from O$_2$-evolution measurements (P$_{\text{O}_2}^{\text{chl}}_{\text{max}}$) was relatively stable throughout the day (Figure 6.5b). This can be explained by the differences in sample treatment. Each P-E curve measured using the O$_2$-electrode used only one sample. The extended illumination period (up to ~50 min) may have allowed for significant induction of photosynthesis, resulting in relatively high P$_{\text{O}_2}^{\text{chl}}_{\text{max}}$ in the morning. This may also have resulted in some negative impact on cells (for example, due to the shearing effects of the magnetic stirrer), yielding lower maximum photosynthetic rates during the day relative to the other measurements. Other possible explanations for low P$_{\text{O}_2}^{\text{chl}}_{\text{max}}$ levels during the day are enhanced Mehler reaction and/or mitochondrial alternative oxidase activity. Given that light levels were randomised during O$_2$-evolution measurements to reduce pre-treatment effects, these alternative pathways for the dissipation of redox potential may be the more likely explanation. It has previously been found that, when using oxygen electrode systems, photosynthetic rate stability is achieved more rapidly when irradiances are increased by small increments rather than randomised (Henley 1993). It is thought that the large and immediate changes in radiant intensity experienced by the samples in the oxygen electrode chambers may have promoted that activity of alternative electron transport pathways.

However, the patterns followed by P-E curve parameters were similar for all measurement techniques, suggesting that they were measuring the same basic properties or characteristics of the phytoplankton assemblage. The differences observed are likely
the result of differences in sample handling and the potential for some physiological processes to impinge on one of the measures more than another.

The PAM chl-fluorescence parameters \( F_{\text{v}}/F_{\text{m}} \), \( \text{rETR}_{\text{max}} \) and \( \alpha \) indicate possible photoinhibition of phytoplankton photosynthesis at 0900, 1300 and 1500 hrs (Figure 6.5). Measurements at 0900 hrs were taken shortly after a break in cloud cover resulted in surface irradiances of \(~1500~\mu\text{mol quanta m}^{-2}\text{s}^{-1}\) and \( F_{\text{v}}/F_{\text{m}} \) was found to be depressed \((0.513 \pm 0.023\) compared to \(0.642 \pm 0.007\) at 0800 hrs). This drop in \( F_{\text{v}}/F_{\text{m}} \) was accompanied by a lower recorded \( \text{rETR}_{\text{max}} \), while \( \alpha \) had undergone a sudden decrease at 0700 hrs and subsequently remained relatively stable.

The highest irradiances for the day were recorded between midday and 1300 hrs as the cloud cover that had persisted for most of the morning dissipated (Figure 6.5a). The \( F_{\text{v}}/F_{\text{m}} \) of the phytoplankton community decreased from \(0.614 \pm 0.007\) at midday to \(0.538 \pm 0.005\) at 1300 hrs (Figure 6.5a), however on this occasion there was no concomitant decrease in \( \text{rETR}_{\text{max}} \) (Figure 6.5b).

Data from Olaizola et al. (1994) suggest that changes in diadinoxanthin cycle pigment pool size could occur between hourly sampling periods. An increase in dissipative diatoxanthin content relative to chl-\(a\) would result in a decrease in both \( F_{\text{m}} \) and \( F_{\text{o}} \) (Lavaud et al. 2004), however \( F_{\text{v}}/F_{\text{m}} \) would also decrease as the drop in \( F_{\text{m}} \) is proportionately greater. It has been proposed that epoxidase activity is inhibited after exposure to high light (Olaizola et al. 1994). Thus the low \( F_{\text{v}}/F_{\text{m}} \) recorded during this study at 1300 hrs may be the result of increased levels of energy dissipating diatoxanthin rather than significant photodamage to RCIIIs.

At 1500 hrs, like at 0900 hrs, the drop in \( F_{\text{v}}/F_{\text{m}} \) was associated with a concomitant reduction of \( \text{rETR}_{\text{max}} \) indicating that some photoinhibition may have occurred (Figure 6.5a, b). A decline in dark adapted \( F_{\text{v}}/F_{\text{m}} \) accompanied by an increase
in \( F_o \) can be interpreted as photoinhibitory damage while, as mentioned above, \( F_v/F_m \) reduction where both \( F_m \) and \( F_o \) values decrease indicates down-regulation (Brown et al. 1999). Sampling methods did not allow for comparison of \( F_o \) and \( F_m \) between sampling periods and thus achieving a more quantitative assessment of the proportion of photoinhibition compared to photoregulatory down-regulation was not possible. The low signal to noise ratio of PAM chl-fluorescence measurements meant that quenching parameters were considered inconclusive (data not shown). However, substantial recovery of \( F_v/F_m \) was observed within the hour after each decline, indicating that that this was more likely to be an expression of dynamic regulatory mechanisms rather than photoinhibition (recovery from photoinhibition occurs on a time scale of hours to days).

Evidence of active quenching mechanisms was present in the pattern of \( E_k \). Boyd (1997) interpreted a significant decrease in \( E_k \) towards evening as relaxation of non-photochemical quenching. In this study, the substantially higher \( E_k \) values during the daylight hours compared to before dawn and after dusk (Figure 6.5d) resulted from a significant decline in \( \alpha \) (Figure 6.5c) with little or no reduction in \( r\text{ETR}_{\text{max}} \) (Figure 6.5b) and can be construed as an increase in non-photochemical quenching. Also, the diurnal pattern of the P-E curve parameters tends to represent a photoacclimation response type A, as described by Richardson et al. (1983), where the activity of accessory pigments is thought to play a major role.

Diatoms, via the diadinoxanthin cycle, are capable of efficient downregulation of photosynthesis via non-radiative dissipation of excess energy (Lavaud et al. 2002b). The data presented here suggest that the diatom-dominated marine phytoplankton community found in Perth’s coastal waters at the time of this study was capable of avoiding photoinhibitory damage by employing this tactic.
Along with the recorded high Fv/Fm values, the ability of the phytoplankton community to effectively regulate photosynthesis and avoid photodamage indicates that they are not nutrient limited. However, the nutrient concentrations of Perth’s coastal waters have been described as “in the lower part of the range reported for temperate coastal waters elsewhere” (Johannes et al. 1994) with low N:P ratios resulting in a likelihood of N-limitation (Thompson and Hosja 1996). This suggests that phytoplankton at the mouth of the Swan-Canning Estuary at the time of this study were efficiently recycling nutrients from within the plankton community, or turbulence of the water column due water movement through the mouth of estuary provided sufficient nutrients via suspension of benthic solids.

Maximum chl-specific rate of primary production ($P_{\text{chl}}^{\text{max}}$) as measured by carbon uptake increased more than ten-fold over the course of the day, from a low of $0.21 \pm 0.009$ µmol C ($\mu$g chl-a)$^{-1}$ h$^{-1}$ at 0300 hrs to a maximum of $2.23 \pm 0.250$ µmol C ($\mu$g chl-a)$^{-1}$ h$^{-1}$ (error = se of model estimate). PAM chl-fluorescence estimates of primary production ($P_{\text{f,chl}}$) persistently underestimated measured values. This underestimation is most likely due to a combination of the three major assumptions used in the calculation of $P_{\text{f,chl}}$: (1) the chl-specific absorption coefficient may have been higher than assumed, (2) greater than 50% of absorbed quanta may have been directed towards PSII and, (3) the quantum requirement for carbon fixation may have been lower than assumed. The second of these explanations is considered the most likely.

The strongest linear relationships between $P_{\text{f,chl}}$ and $P_{\text{chl}}$ were recorded at 0300 hrs ($r^2 = 0.683$) and 1100 hrs ($r^2 = 0.830$). Both of these were recorded during periods of low light while physiological conditions were relatively stable. The relationship was substantially weaker at 0700 hrs, when physiological processes would have been in a state of flux associated with dawn; and 1500 hrs, a period of high irradiance. Thus, it appears that recent light history can impact upon chl-fluorescence-based estimates of
primary production. However, the strong relationship obtained at 1100 hrs suggests that targeted sampling after proper calibration between techniques may allow accurate estimation of $P_{\text{chl}}$ via PAM chl-fluorescence techniques.

A linear relationship between chl-specific rates of gross oxygen evolution ($P_{\text{chl}O_2}$) and predictions of gross oxygen evolution from chl-fluorescence data ($\text{GOE}_{\text{chl}}$) was found at 0300 hrs only. During daylight hours all $P_{\text{chl}O_2}$ : $\text{GOE}_{\text{chl}}$ relationships were curvilinear, with deviation at higher irradiances towards increasing overestimation of oxygen evolution by $\text{GOE}_{\text{chl}}$. That is, at higher irradiances the number of electrons passing through PSII per oxygen molecule evolved increased. This phenomenon has previously been described in other studies (Masojidek et al. 2001). Given that this occurred while $P_{\text{chl}}$ : $P_{\text{chl}}$ relationships remained relatively linear the process responsible caused a reduction in measured $O_2$ without impacting linear electron transport to the dark reactions. Mechanisms such as photorespiration, the Mehler reaction, mitochondrial oxidative metabolism and cyclic electron transport around PSI would act to reduce measured oxygen evolution compared to estimates from chl-fluorescence, however carbon assimilation would be impacted also. Without inhibitor studies it is difficult to predict which alternative electron pathway is responsible for the observed non-linearity.

6.5 CONCLUSION

The marine phytoplankton community at the mouth of the Swan-Canning estuary exhibited a typical diurnal pattern of photosynthesis, with maximum rates of photosynthesis occurring during the daylight hours as photosynthetic processes became rapidly induced. High $F_v/F_m$ values, reaching close to the theoretical maximum just prior to dawn, indicated that phytoplankton were physiologically competent and not nutrient-limited. The fast recovery of $F_v/F_m$ after periods of high light while maintaining near maximal rates of photosynthesis ($\text{rETR}_{\text{max}}$, $P_{\text{O}_2\text{chl}}_{\text{max}}$ and $P_{\text{chl}}_{\text{max}}$) supports this
finding and highlights the photoprotective ability of diatoms, which were the dominant taxon. Under these apparently favourable conditions a relatively high rate of primary production was recorded.

The ability of chlorophyll measurements to predict oxygen evolution and carbon fixation was found to be variable. However, the linear relationship between $P_{f}^{chl}$ and $P_{chl}$ was found to be strong in stable conditions of relatively low light (before dawn and during cloud cover). Thus, proper calibration between techniques may allow accurate estimation of $P_{chl}$ via PAM chl-fluorescence techniques in favourable environmental conditions. Future developments in our ability to easily and rapidly measure important physiological parameters such as $\tilde{a}_{chl}^{\text{ff}}, f_{II}, \sigma_{\text{PSII}}$ and $\eta_{\text{PSII}}$, combined with our increasing knowledge of alternative electron transport pathways will allow more accurate predictions over a wider range of environmental conditions.
CHAPTER 7

General Discussion and Conclusion

Marine phytoplankton are responsible for about 40% of the total global carbon fixation and thus constitute an important component of the Earth’s biogeochemical cycles (Falkowski 1994; Sakshaug et al. 1997). Understanding of the factors that regulate this production and achieving accurate and precise measures of its rate have been long-standing goals of aquatic ecologists (Bates and Platt 1984; Pennock and Sharp 1994). Estimation of phytoplankton photosynthesis has traditionally been by the \( \text{O}_2 \) (Gaarder and Gran 1927) and \( ^{14}\text{C} \) (Steemann Nielsen 1952) methods. While the use of polarographic techniques to measure oxygen evolution increased after the late 1960s (Mountford 1969) these were not sensitive enough in most field conditions (sample concentration is an option, at the risk of introducing artefacts into the data (Harris 1978)) and the majority of the scientific community has relied on the \( ^{14}\text{C} \) method to estimate primary production (Moigis and Gocke 2003). However, it is also recognised in the literature that \( ^{14}\text{C} \) methods are prone to “bottle effects” (Falkowski and Kolber 1993), appear to underestimate primary production (Peterson 1980) and oscillate somewhere between estimating net or gross primary production rates (Harris 1980). Other issues include the cost of materials and the extended time required for in situ incubations (Falkowski and Kolber 1993).

The measurement of active chl-a fluorescence with Pulse Amplitude Modulated (PAM) fluorometers has become increasingly popular over the past 10 – 15 years. Much of this popularity stems from the rapid nature of chl-a fluorescence measurements, avoiding the need for incubations and allowing higher intensity sampling on both spatial and temporal scales (for better comparison with physicochemical data), and their non-intrusive nature (Krause and Weis 1991; Kolber and Falkowski 1993; Cunningham
Despite the complex nature of the chl-α fluorescence signal (Kolber and Falkowski 1993; Schreiber et al. 1998) a number of attempts have been made to predict primary production from chl-α fluorescence estimates of electron transport through PSII. While studies with higher plants have often found a strong linear relationship between such predictions and carbon uptake or oxygen evolution measurements (Weis and Berry 1987; Genty et al. 1989; Cornic and Briantais 1991; Beer et al. 1998b), equivalent studies utilizing algae have yielded variable results, with a greater tendency for non-linearity (Geel et al. 1997; Barranguet and Kromkamp 2000b; Gilbert et al. 2000a; Suggett et al. 2003).

This study aimed to optimise PAM measurements of chl-α fluorescence using the Water-PAM fluorometer commercially available from Walz GmbH (Germany) and apply chl-α fluorescence techniques to estimate phytoplankton primary production while photosynthesis was in non-steady state (RLC), semi-steady state (LC(3)) and steady state (LC(20)). Importantly, it is the first time steady state fluorescence measurements have been calculated from measurements integrated over the incubation period. While some studies have looked at comparing RLC’s with LC(3)s (Kühl et al. 2001) or steady state light curves (Serôdio et al. 2006), to the author’s knowledge this is the first time an extensive study has investigated how each sampling method impacts the prediction of oxygen evolution and primary production.

Rapid Light Curves measure immediate adaptational/physiological state. The length of exposure to each irradiance during RLC analysis (termed the Light Width), most commonly 10 s (Ralph and Gademann 2005), is long enough to allow fluorescence yield to recover from the previous saturation pulse but as short as possible to minimise changes in photosynthetic state (such as photoinduction) as a result of the light treatment. Results of a short study presented here (Chapter 2.2.1) indicated that the Light Width could be reduced to 5 s. The shorter Light Width reduced photoinduction
during the course of RLC measurements while allowing proper recovery from saturation pulses to a new F' level (refer to Chapter 1.3 for definition).

While it is recognised that the time taken for a pulse of saturating light to induce an increase in fluorescence yield to Fm (or Fm' under ambient light) may vary between taxa (Schreiber et al. 1995c) most studies investigating phytoplankton photosynthesis using PAM fluorescence techniques have found a saturation pulse duration of 0.6 – 0.8 s to be appropriate (Kühl et al. 2001; Rech et al. 2003; Villareal 2004; McMinn et al. 2005). Measurements described here (Chapter 3.2.1) illustrate that such durations may be suitable for many species, including C. muelleri and N. oculata, but not for others. It was found that the fluorescence rise kinetics of I. galbana were clearly biphasic and a saturation pulse longer than 1 s was required to achieve a clear peak (Fm, Figure 3.4b). Such variation between species may be an issue in field studies where phytoplankton community composition may change.

Dark adaptation of samples is undertaken for two primary reasons: (1) to allow for full re-oxidation of the reaction centres and electron transport chain, leaving all reaction centres in the “open” state and allowing for the estimation of maximum potential quantum yield of PSII and, (2) to help standardise the short term light history of samples collected in the field. Data presented here indicate that formation of NPQ in the dark may cause underestimation of Fv/Fm, with Fm' frequently increasing above recorded Fm values when samples were exposed to low irradiances. This in itself is not new, with researchers such as Jakob et al. (2005) attempting to avoid partial reduction of the PSII acceptor pool in the dark by exposing samples to low irradiance (21 µmol quanta m⁻² s⁻¹) rather than dark adapting. However, comparison of data presented in Chapter 2.2.3 and Chapter 5 suggests that the occurrence and extent of dark-induced NPQ (leading to decreases in apparent Fv/Fm) may be influenced by growth state. Batch culture samples showed evidence of reduced Fv/Fm in Nannochloropsis oculata when
dark adaptation was longer than 15 min, but no such evidence in *Chaetoceros muelleri* and *Isochrysis galbana* (Figure 2.5). On the other hand, data presented in Chapter 5 from semi-continuous cultures provide evidence of substantial NPQ in dark adapted samples of *C. muelleri* and *I. galbana*, but only small amounts in *N. oculata*.

In Chapter 5, measured P-E curves, particularly RLCs, provide evidence that dark adaptation can profoundly influence curve parameters, particularly $ETR_{\text{max}}$ and $E_k$. These changes are suggested to be associated with the deactivation of Calvin-Benson Cycle enzymes in the dark (during dark adaptation). Any reduced activity of these enzymes is likely to lead to a diminished capacity to reoxidise the electron transport chain and closure of more reaction centres. When LC(3)s rather than RLCs were applied the difference between light and dark adapted samples was usually either greatly reduced or no longer present, as the enzymes of the Calvin-Benson Cycle became reactivated under the actinic light. Researchers performing rapid, non-steady state measurements of photosynthetic performance via chl-$a$ fluorescence using dark adapted samples risk underestimation of $ETR_{\text{max}}$ and $E_k$.

Importantly, it was found that stirring the sample to maintain homogeneity during chl-$a$ fluorescence measurements (e.g. using the Water-S stirring device) significantly impacted fluorescence parameters, particularly NPQ (Chapter 2.2.4). NPQ was significantly lower in stirred samples compared to unstirred samples ($F_{0.05(1),138} = 422.29, P<0.001$). Effective quantum yield data was also impacted by stirring with stirred samples displaying significantly higher measures than unstirred samples ($F_{0.05(1),138} = 12.582, P = 0.001$). It is suggested that, even with the use of low sample volumes, the light environment that the whole sample is exposed to in the Water-PAM emitter/detector unit is not uniform and cells in stirred samples pass through darker regions and thus experience a relatively low average irradiance. This results in severely limited NPQ development and higher measured PSII quantum yield, leading to
significantly higher $\alpha$-slopes in RLCs (refer to Table 2.3). It is proposed that providing a vertical dimension to the LED-array, such as the addition of a second ring of LEDs at a higher level relative to the cuvette, plus restriction of sample volume to 2 mL, would result in providing more even illumination of the whole sample and therefore significant mitigation of the described fluorescence artefacts.

Three microalgal species representative of major marine phytoplankton taxa were chosen for the investigation of primary production prediction using chl-$\alpha$ fluorescence: *Chaetoceros muelleri* (Bacillariophyta), *Isochrysis galbana* (Haptophyta) and *Nannochloropsis oculata* (Ochrophyta). It was found that the relationship between the “traditional” estimates of primary production, oxygen evolution ($P_{\text{chl}O_2}$) and carbon fixation ($P_{\text{chl}}$), and chl-$\alpha$ fluorescence-based predictions ($GOE_{f_{\text{chl}}}$ and $P_{f_{\text{chl}}}$ respectively) varied between non-linear to mildly curvilinear, with the slope of the linear portion of the relationship varying between 0.38 and 8.02. Results were influenced by acclimation state and Light Curve technique, but adaptation state (light versus dark adapted) did not appear important in this regard. At moderate to high irradiances both $P_{\text{chl}O_2}:GOE_{f_{\text{chl}}}$ and $P_{\text{chl}}:P_{f_{\text{chl}}}$ tended toward lower values. This was particularly evident in Low Light (LL; 50 $\mu$mol quanta m$^{-2}$ s$^{-1}$) acclimated samples of the chl-$\alpha$,c/carotenoids containing *C. muelleri* and *I. galbana*. This suggests that LL-acclimated cells are less capable than High Light (HL; 500 $\mu$mol quanta m$^{-2}$ s$^{-1}$) acclimated cells at maintaining linear electron transport at moderate to high irradiances and alternative electron pathways help to reduce photodamage and maintain redox balance. However, the variable nature of the data collected here illustrates a number of key issues:

1. When calculating ETR using biophysically derived parameters (Equation 29) it is important that $\eta_{\text{PSII}}$ is measured rather than estimated as this parameter is variable and has the potential to substantially impact ETR calculation. As a consequence, overestimation of $\eta_{\text{PSII}}$ leads to an
underestimation of $P_{chl \text{O}_2}: \text{GOE}_f^{chl}$ or $P_{chl}^f : P_{chl}$. Unfortunately, direct measurement of $\eta_{PSII}$ currently requires specialised equipment and is difficult in the field. Development of techniques to rapidly measure $\eta_{PSII}$ in situ should be an important focus of future research.

2. Other major assumptions impacting $P_{chl \text{O}_2}: \text{GOE}_f^{chl}$ and $P_{chl}^f : P_{chl}$ are the stoichiometric ratio of $O_2$ evolved per electron generated at PSII and the photosynthetic quotient (mol $O_2$ mol$^{-1}$ C). In this study it was also assumed that these values remained constant across all irradiances, however data from previous studies (Dubinsky et al. 1986, Grobbelaar et al. 1992, Herzig and Dubinsky 1992, Laws et al. 2002) indicates that these values are dynamic. Thus, changes in these parameters that have not been accounted for may be (partially) responsible for the observed decreases in $P_{chl \text{O}_2}: \text{GOE}_f^{chl}$ and $P_{chl}^f : P_{chl}$ at moderate to high irradiances.

Despite these concerns results from the field (Chapter 6) found that estimation of primary production via chl-$a$ fluorescence has promise, with an $r^2$ value of 0.831 measured for the $P_{chl}^f : P_{chl}$ relationship obtained during a period of reduced light stress (cloud cover). The method used to calculate $\text{GOE}_f^{chl}$ and $P_{chl}^f$ from the field data generally resulted in an underestimation of primary production (Figure 6.6).

Due to its ability to rapidly assess a number of photosynthetic parameters, chl-$a$ fluorescence has become a popular technique for the assessment of photoacclimation. Here (Chapter 5), chl-$a$ fluorescence data were analysed to compare the photosynthetic performance of LL- and HL-acclimated cultures. Each of the three species displayed typical responses of lower levels of chl-$a$ per cell and higher $ETR_{max}$ in HL-acclimated cultures compared to those that were LL-acclimated. Changes in $\sigma_{PSII}$ were small relative to the changes in chl-$a$ content, suggesting that cells decreased the number of
photosynthetic units in response to high light. Increasing the Rubisco concentration per reaction centre was a feature of photoacclimation to High Light in each of the three species studied here, however it appeared particularly important in the Eustigmatophyte *Nannochloropsis oculata*.

The summer field study component (December 2005) of this project found that the marine waters at the mouth of the Swan-Canning Estuary (Fremantle) were dominated by diatoms. $F_v/F_m$ values ranged between 0.6 and 0.8 during the early morning and evening (Figure 6.5a), suggesting that the photophysiological capacity of the natural phytoplankton community was not impacted by nutrient limitation (Springer et al. 2005). The PAM chl-fluorescence parameters $F_v/F_m$, $r_{ETR_{max}}$ and $\alpha$ indicate possible photoinhibition of phytoplankton photosynthesis during periods of high irradiance at 0900, 1300 and 1500 hrs (Figure 6.5). However, it is also suggested that some reduction in $F_v/F_m$ may be the result of increased levels of the energy dissipating diatoxanthin pigment. This hypothesis is supported by the finding that $\alpha$ decreased during the day while $r_{ETR_{max}}$ increased.

The natural phytoplankton population also appeared to display substantial endogenous cycling of photosynthetic activity. Very low effective photosynthetic capacities were found during the night, however, this capacity began to increase just before dawn and remained high for much of the day. It is hypothesised that much of this pattern is the result of shifts in the activity of Calvin-Benson cycle enzymes, with deactivation of these enzymes occurring during the night.

Active chl-*a* fluorescence techniques have been shown to be a useful tool for assessing phytoplankton health and photosynthetic performance. However, even under favourable conditions it has been shown that prediction of O$_2$-evolution and carbon fixation is difficult. Chl-fluorescence measures essentially provide an estimate of the
gross energetic potential made available within the photosynthetic electron transport chain (Suggett et al. 2006). The numerous dynamic cellular processes that potentially divert portions of this energy away from carbon fixation (eg. cyclic electron flow, photorespiration or nitrate reduction) act to complicate the relationship between gross energetic potential and realised carbon fixation (primary production). Current and future scientific work leading to increased understanding of algal photosystem structure and alternative electron transport pathways, plus the development of new techniques to enable in situ assessment of $\eta_{\text{PSII}}$, will increase the ability of chl-$\alpha$ fluorescence measurements to provide accurate predictions of primary production.
REFERENCES


