Action spectra of oxygen production and chlorophyll *a* fluorescence in the green microalga *Nannochloropsis oculata*

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# Abstract

The first complete action spectrum of oxygen evolution and chlorophyll *a* fluorescence was measured for the biofuel candidate alga *Nannochloropsis oculata*. A novel analytical procedure was used to generate a representative and reproducible action spectrum for microalgal cultures. The action spectrum was measured at 14 discrete wavelengths across the visible spectrum, at an equivalent photon flux density of 60 µmol photons m-2 s-1. Blue light (~414 nm) was absorbed more efficiently and directed to photosystem II more effectively than red light (~679 nm) at light intensities below the photosaturation limit. Conversion of absorbed photons into photosynthetic oxygen evolution was maximised at 625 nm; however, this maximum is unstable since neighbouring wavelengths (646 nm) resulted in the lowest photosystem II operating efficiency. Identifying the wavelength-dependence of photosynthesis has clear implications to optimising growth efficiency and hence important economic implications to the algal biofuels and bioproducts industries.

Keywords: *algal biofuel; Nannochloropsis oculata; action spectrum; oxygen production; chlorophyll fluorescence*

# Abbreviations

α rate constant of gross photosynthesis

E irradiance (W m-2)

Ek minimum saturating irradiance

F0 minimal fluorescence yield in the dark-acclimated state

Fm maximal fluorescence yield in the dark-acclimated state

F’ minimal fluorescence yield in the light-acclimated state

Fm’ maximal fluorescence yield in the light-acclimated state

fAQPSII proportion of light absorption directed towards PSII

MC-PAM multi-colour pulse-amplitude-modulated fluorometer

NPQ non-photochemical quenching

P gross photosynthesis

Pmax maximum gross photosynthesis

PAR photosynthetically active radiation (400-700 nm)

PFD photon flux density (µmol photons m-2 s-1)

PI curve photosynthesis versus irradiance curve

PSI photosystem I

PSII photosystem II

ΦPSII operating efficiency of PSII

# Introduction

## 1.1 Algal biofuels produced from Nannochloropsis

Sustainable transport fuels of the future may be produced using microalgae. Algal biofuel technology exploits algal photosynthesis and biosynthesis processes to produce oils using only sunlight, carbon dioxide, water and limited nutrients. Oil production capacity from microalgae far exceeds that from any higher plant, including traditional biofuel crops such as corn, sugarcane and palm (Georgianna and Mayfield, 2012; Larkum et al., 2012). Consequently, research and pilot projects are being carried out worldwide to expand this technology to support major industrial process scaling (Wijffels and Barbosa, 2010).

The green microalga of the genus *Nannochloropsis* (class Eustigmatophyceae) is a leading candidate for biofuel production due to its ability to accumulate high oil content (28.7 % of cellular ash-free dry weight) with reported oil productivities of ~25.8 mg L-1 day-1 (Gouveia and Oliveira, 2009); thus, research has recently focussed on better understanding the fundamental attributes that regulate biomass productivity, and ultimately oil production, for species from this genus. The photosynthetic apparatus of *Nannochloropsis* is unusual in that the only chlorophyll pigment it contains is chlorophyll *a*; as such, light absorption properties are significantly different compared to other common, commercially-relevant, green microalgae such as *Spirulina*, *Chlorella* or *Dunaliella*, which also contain chlorophyll *b* (Kandilian et al., 2013). Furthermore, the species *Nannochloropsis oculata* has the capacity to grow in saline, brackish and hypersaline water, which ensures that, when grown in production facilities, it will never compete with food crops for arable land or fresh water (Bartley et al., 2013; Borowitzka and Moheimani, 2013). The biofuel productivity of *N. oculata* is now known to be affected by a number of environmental parameters, including light and temperature regimes (Sukenik et al., 2009; Tamburic et al., 2014).

## 1.2 Light environment determines productivity

Algal growth is impossible without illumination, so it is not surprising that *N. oculata* oil productivity is principally determined by its light environment, in terms of both the quantity and quality of available light (Simionato et al., 2011). Providing appropriate illumination requires an understanding of all its constituent factors: wavelength, irradiance, photo-saturation, light attenuation and light history. The photosystems of *N. oculata* are only capable of absorbing light within the 400-700 nm wavelength range, i.e. photosynthetically active radiation (PAR); this absorption is governed by the presence and concentration of photosynthetic pigments, the composition of the photosynthetic pigment-protein complexes, and the constituents of the photosynthetic electron transport chain;. As such, wavelength-specific absorption is highly variable amongst taxa with different pigment arrays (Millie et al., 2002). In *N. oculata*, carotenoids and chlorophyll *a* are responsible for absorption of blue light, while chlorophyll *a* also absorbs red light (Kandilian et al., 2013).

Irradiance is a measure of light pour incident on a surface. As a general rule, the response of photosynthesis to irradiance follows a classical and highly conserved pattern (MacIntyre et al., 2002), the photosynthesis-irradiance curve (PI curve): under relatively low irradiances, algal growth rate and irradiance increase in proportion since more photons become available for photosynthesis; however, under relatively high irradiances, photosynthesis remains constant (or declines) with increasing irradiance as the photosynthetic electron transport chain becomes saturated (and ultimately photoinhibited). Raising irradiance above the photosaturation limit should be avoided because it reduces the photosynthetic efficiency of cells (Sukenik et al., 2009), which results in energy loss through heat dissipation (such as non-photochemical quenching) and fluorescence, as well as the energy costs associated with repairing damaged photosynthetic apparatus (Raven, 2011). However, algal cells in culture rarely receive the same number of photons constantly as a result of light attenuation, in particular where cell densities are high and cultures optically thick (Lehr and Posten, 2009). Flexibility of the photosynthetic apparatus for light harvesting and light utilisation via photo-acclimation, as observed in *Nannochloropsis* (Sforza et al., 2012), is thus a key attribute for large scale culturing.

## 1.3 Action spectrum informs light optimisation

An action spectrum measures the rate of photosynthesis across different PAR wavelengths. It can be measured in terms of a proxy for photosynthetic efficiency, such as chlorophyll *a* fluorescence (Emerson and Arnold, 1942), or in terms of oxygen evolution, the tangible result of photosynthesis (Haxo and Blinks, 1950). Importantly, an action spectrum is different to an absorption spectrum since not all absorbed photons lead to photosynthetic oxygen production, which occurs at photosystem II (PSII). For example, photons may be absorbed by photosystem I (PSI), their excitation energy may be dissipated as heat, or emitted as fluorescence that can be quenched by various photochemical and non-photochemical processes (Baker, 2008; Suggett et al., 2003). Furthermore, absorbed energy may lead to oxygen evolution that is internally recycled (and hence not detected by conventional oxygen sensors) via a number of alternative photochemical reactions, such as the Mehler reaction or photorespiration (Cardol et al., 2011).

Measuring the action spectrum is important because it provides the best description of the wavelength-specific response of that algae’s photosynthesis and importantly, it can be used to identify which wavelengths are utilised most efficiently. In terms of *N. oculata* biofuel production, the impacts are significant since the spectral composition of artificial illumination in small-scale laboratory systems could be optimised to enhance photosynthetic efficiency. Specifically, it may be possible to determine the wavelength ranges that drive photosynthetic primary production with higher efficiency and reduce the energetic costs of maintaining redundant photoprotective processes (Raven, 2011). Such ‘tuning’ of the spectral nature of illumination to increase absorption efficiency could result in two beneficial effects: (i) enhanced algal growth rate, or (ii) reduced power consumption to achieve the same growth rate. Large-scale outdoor demonstration facilities could also be retrofitted with inexpensive light filters to modulate the solar spectrum incident on *N. oculata* cultures in order to enhance growth and oil productivity.

## 1.4 Aim and objective

The aim of this study is to develop and measure the first complete action spectrum for oxygen evolution and chlorophyll *a* fluorescence in *N. oculata*. The objective is to better understand photosynthetic responses at different wavelengths and develop a more effective basis for optimising the light delivery to *N. oculata* in both artificial and natural environments.

# Materials **a**nd Methods

## 2.1 Nannochloropsis strain and stock cultures

*Nannochloropsis oculata* (Droop) Green (Australian National Algae Culture Collection; strain CS-179) was grown in three separate 250 mL cultures using f/2 seawater medium at 25°C (Labec Temperature Cycling Chamber incubator, Labec Pty Ltd, Australia). Stock cultures were subjected to a 12 h/12 h light/dark cycle under fluorescent illumination with a photon flux density (PFD) of 50±5 µmol photon m-2 s-1 PAR. Cultures were diluted (1/20 v/v) with fresh media 1 week prior to experimentation; consequently, all cultures were in exponential growth phase (as verified by cell counts using a haemocytometer) and 7-12 days old at the time of experiment.

## 2.2 Absorbance measurements

Scatter-corrected *in vivo* absorption spectrum was measured using a fibre-optic spectrometer (as described previously by Petrou et al., 2013). Briefly, a 3 mL algal sample was vacuum-filtered onto a grade GF/F glass microfiber filter (Whatman, GE Healthcare Life Sciences, NSW, Australia; 20 mm diameter) and the moist filter was placed on a standard microscope slide. The slide, filter, and algal cells were clamped into position across the light-collection port of an integrating sphere (FOIS-1, Ocean Optics, Florida, USA) and in the light path of a tungsten halogen lamp (LSI, Ocean Optics, Florida, USA). The integrating sphere detection port was connected to a spectrometer (USB2000, Ocean Optics, Florida, USA). Absorbance was calculated against a reference of a medium-only moistened filter.

## 2.3 Action and fluorescence spectra experimental setup

Photobiology experiments were performed in a cuvette-based system with rectangular geometry. A 1 cm square-faced quartz cuvette with a working volume of 1.6 mL and a custom-designed gas-tight lid was used. The cuvette was housed within a temperature-controlled optical unit (ED-101US/MD, Heinz Walz GmbH, Germany) with integrated magnetic stirring to keep *N. oculata* cells in suspension; temperature was maintained at 25°C throughout the experiment. The experimental setup enabled simultaneous application of three key instruments on the same sample: (i) a programmable light source (OL 490 Agile, Gooch & Housego, Florida, USA) to provide spectrally-resolved irradiance, (ii) a multi-colour pulse-amplitude modulated fluorometer (MC PAM, Heinz Walz GmbH, Germany) to measure chlorophyll *a* fluorescence, and (iii) a fibre-optic oxygen minisensor (OXF1100-OI, PyroScience GmbH, Germany) to measure photosynthetic oxygen evolution. *N. oculata* cells were illuminated through one cuvette face with monochromatic actinic light produced using the light source (OL 490 Agile). The light source uses a digital light processor microchip (Texas Instruments, Texas, USA) to produce specific user-defined spectra at variable intensity and high-resolution spectral output and was powered by a Xenon lamp through a 150 µm slit; in this configuration the light source generated irradiance at various predefined wavelengths with a 5 nm bandwidth at a bandwidth precision of ±1 nm. The illumination with the LED source of the MC PAM to record chlorophyll *a* fluorescence parameters was applied through the opposite cuvette face and the MC PAM photodetector collected fluorescence at a 90° angle to both the actinic and measuring light. The oxygen minisensor was inserted from above through a small hole (1.2 mm diameter) in the gas-tight cuvette lid using a micromanipulator (Marzhauser Wetzlar GmbH, Germany).

## 2.4 Experimental cultures

Prior to experimentation, a small volume (approx. 30 mL) of stock culture was diluted in fresh f/2 media to a minimal fluorescence of unity (F0 = 1.0 in the dark with 440 nm measuring light) to ensure optically-thin *N. oculata* cultures of a similar cell density for all experiments. The cell density and cell size of experimental cultures was periodically tested using a cell counter (Cell and Particle Coulter Counter, Beckman Coulter GmbH, Germany). Briefly, a 0.1 mL sample was extracted from the experimental culture and diluted with f/2 media (1/100 v/v). This sample was drawn up into the cell counter through a 20 µm aperture tube. *N. oculata* cell densities of 5.33±0.25 x 106 cells·mL-1 and cell diameters of 2.12±0.22 µm were consistently measured (n = 12). Experimental cultures were placed in a water bath at 25°C and acclimated to low ambient PFD of ~4 µmol photon m-2 s-1 PAR for a minimum of 30 min. Each measurement was performed using a fresh (no wavelength-specific illumination history) 1.6 mL algal sample from the experimental culture. In total, this procedure was repeated 9 times over 6 days across the triplicate cultures to ensure full biological replication.

## 2.5 Chlorophyll fluorescence measurements

Chlorophyll *a* fluorescence was measured using the MC PAM fluorometer (see Schreiber and Klughammer, 2013 and Schreiber et al., 2012 for more details). A measuring light with a wavelength of 440 nm and a PFD of <0.5 µmol photon m-2 s-1 PAR was used to measure *F0* and *F’* because it yields the highest fluorescence response without disturbing the actual dark-acclimated or light-acclimated state in *N. oculata* and thus maximises the signal-to-noise ratio for relatively low (optically thin) cell densities. Saturating pulses provided by the MC PAM (440 nm; ~2,000 µmol photon m-2 s-1 PAR; 0.8 s pulse width) were applied 30 min after dark adaptation (to measure *Fm*) and at the end of each 8 min illumination period (to measure *Fm’*). The light source (OL 490 Agile) generated monochromatic actinic light to drive photosynthesis. Two photophysiological parameters were calculated (according to Baker, 2008): (i) the operating efficiency of photosystem II (PSII), ΦPSII ([*Fm’*-*F’*]/*Fm’*), which provides an estimate of the quantum yield of linear electron flux through PSII, and (ii) the non-photochemical quenching, NPQ (*Fm*/[*Fm’*-1]), which yields the rate constant for heat loss from PSII.

## 2.6 Oxygen evolution measurements

Changes in dissolved oxygen concentration in the cuvette were measured using a fibre-optic minisensor and used to calculate oxygen evolution (net photosynthesis). The gas-tight cuvette lid was carefully closed to ensure each sample was free of air bubbles. The underside of the lid was concave in shape to ensure that air bubbles can escape through the oxygen minisensor insertion hole in the centre of the lid. Each culture was continuously stirred to prevent the formation of oxygen gradients within the cuvette. The oxygen optode was a fixed needle-type minisensor (1.1 mm tip diameter) with optical isolation and a response time <3 s; periodic calibration was performed against air-saturated seawater (100% air saturation) and nitrogen-saturated seawater (0% air saturation) at 25°C. Data was collected every second using a *FireSting* datalogger (Fibreoptic Oxygen Meter FS02-01, PyroScience GmbH, Germany). Upon inserting the oxygen optode into the cuvette, the *N. oculata* culture was illuminated with actinic light for a period of 8 min. A linear fit of the increase in dissolved oxygen concentration was used to estimate oxygen evolution rate (adapted from Cooper et al., 2011).

## 2.7 Action spectrum measurement

An action spectrum was collected to resolve the spectrally-dependent photosynthetic response of *N. oculata*. In order to produce a complete and representative action spectrum, appropriate measurement wavelengths and PFD had to be determined. Analysis of the *in vivo* absorption spectrum of *N. oculata* was used to identify the 14 wavelengths of the overall action spectrum (400, 406, 414, 441, 459, 483, 490, 559, 582, 600, 626, 646, 679 and 700 nm), which correspond to the mathematical turning points of the spectrum. Turning points were identified using the *peakfinder* algorithm (by Nathaniel Yoder, freely available via MatLab file exchange). This algorithm looks for changes in the first and second derivatives between adjacent data points. A sensitivity parameter is defined in order to separate genuine turning points from random noise, i.e. to ensure that changes in the derivatives are consistent over a large range of data points. Oxygen evolution rates were calculated using the *linefit* algorithm (by Small Satellites, freely available via MatLab file exchange). The variation of photosynthesis versus irradiance (PI curve) at the blue and red chlorophyll *a* absorption peaks was used to determine the PFD for the action spectrum (60 µmol photon m-2 s-1 PAR). The 14 discrete data points of the action spectrum were connected and smoothed using a shape-conserving interpolant function (MatLab). The OL 490 light engine was calibrated using a 4π light sensor (US-SQS/WB Spherical Micro Quantum Sensor, Heinz-Walz GmbH, Germany) in order to generate the same PFD at all actinic wavelengths. All experiments were performed in triplicate (n = 3), and a different stock culture was used for each replicate.

# Results and Discussion

## 3.1 In-vivo absorption spectrum

In order for light to promote a photochemical reaction, it must first be absorbed; an absorption spectrum therefore provides a first order estimate for photosynthetic activity (Arnold, 1991) and is governed by preferential absorption by different types of pigments throughout the PAR waveband. In the case of *N. oculata*, pigmentation (and hence absorption, Fig. 1) is characterised by the presence of chlorophyll *a* (approx. 150 ng [106 cells]-1) and the carotenoids violaxanthin, astaxanthin, antheraxanthin, vaucheriaxanthin, zeaxanthin, canthaxanthin and *β*-carotene (Lubián et al., 2000).

Spectral absorption was smooth and highly reproducible, apart from some (maximum percentage error = 7.8%) light scattering-induced noise at low wavelengths (Fig. 1). The singlet excitation states of chlorophyll *a*, the Q bands, are clearly resolved in the 550-700 nm range, with the chlorophyll *a* red maximum, i.e. the lowest singlet excitation state *Qy*, occurring at 679 nm as expected and minor absorption bands (*Qx*) at 600-650 nm (Kandilian et al., 2013; Solovchenko et al., 2011). The 400-550 nm region of the absorption spectrum shows a convolution of chlorophyll *a* and carotenoid absorption peaks. The small peak at 490 nm coincides with the absorption maxima for astaxanthin and zeaxanthin whilst blue absorption bands of chlorophyll *a*, the Soret bands (or B bands), occur at 400-450 nm, with the blue absorption maximum *Bx* at 440 nm (Egeland et al., 2011). The Soret bands indicate the population of high energy triplet excited states that quickly decay to the singlet energy state, resulting in the emission of heat. Although the *N. oculata* absorption spectrum (Fig. 1) has a well-defined shoulder at 441 nm, the blue absorption maximum actually occurs at 414 nm, which is essentially violet light. This contrasts the findings of Solovchenko et al. (2011), who observed a blue maximum closer to 440 nm using a similar *in vivo* absorption measurement technique in another *Nannochloropsis* isolate, most likely since *N. oculata* has a higher violaxanthin concentration (Lubián et al., 2000), which absorbs strongly in violet light (Egeland et al., 2011) particularly when in combination with the chlorophyll *a By* absorption band at 414 nm.

The *in vivo* absorption spectrum (Fig. 1) was used to identify the most important wavelengths for *N. oculata* action spectrum measurements. Since light absorption is the starting point of photosynthesis, it is reasonable to assume that variations in the absorption spectrum will be reflected in variations in the action spectrum (Haxo and Blinks, 1950). Hence, the most efficient method for developing the action spectrum is to measure oxygen evolution at wavelengths corresponding to the mathematical turning points (maxima and minima) of the absorption spectrum. The *peakfinder* algorithm (with sensitivity set to 0.1) identified 14 wavelengths associated with turning points in the absorption spectrum, which are represented by red circles in Fig. 1.

## 3.2 PI curves measured using oxygen evolution

The absorption spectrum of *N. oculata* was used to identify blue and red absorption maxima at 414 nm and 678 nm, respectively (Fig. 1). The rate of photosynthesis at these wavelengths depends not only on the probability of photon absorption, but also on the quantity and the energy of photons delivered by the actinic light source to PSII, as well as the wavelength-dependent cross-sectional area of PSII (Kandilian et al., 2013). This can be quantified in terms of the photon flux density (PFD), which is essentially a photon count across a surface, or the irradiance (*E*), which defines the power incident on a surface and is thus adjusted according to photon energy. Net photosynthesis was measured in terms of the increase in dissolved oxygen concentration upon illumination. Over the first 2 minutes, oxygen concentration increased rapidly before stabilising into a slower steady linear rise. This time lag corresponds to the gradual increase in light-enhanced respiration until it reaches steady-state (Cooper et al., 2011; Weger et al., 1989). The *linefit* algorithm was used to identify and fit the most linear 6 min of data. The resulting oxygen evolution rate provides an estimate of steady-state net photosynthesis (Cooper et al., 2011) and it was used to plot PI curves at 414 and 679 nm (Fig. 2).

The PI curves (Fig. 2) follow closely the standard exponential fit of Jassby and Platt (1976).

$$P(E)= P\_{max}(1- e^{\frac{-αE}{P\_{max}}})$$

Photosynthesis (*P*) is an exponential function of irradiance (*E*), and the free-fitting parameters *Pmax* and *α* represent maximum photosynthesis and the photosynthesis rate constant, respectively (MacIntyre et al., 2002). An additional parameter, the minimum saturating irradiance *Ek*, is calculated from the intercept of α·*E* and *Pmax* (as shown in Fig. 2a).

At 414 nm, photosynthesis was observed to saturate (*Pmax* =) 63.3 µmol O2 L-1 h-1 and the minimum saturating irradiance (*Ek*) had a value of 35.6 µmol photons m-2 s-1 (Fig. 2a). In contrast at 679 nm, there was only limited evidence of photosaturation within the measured PFD range; here, rates of absorption were lower (*α* at 679 nm = 0.51, whereas *α* at 414 nm = 1.78), as expected given the lower effective absorption for these wavelengths in the red compared to the blue (Fig. 1). Once PI curves were plotted against irradiance as opposed to PFD (Figure 2b), the photosynthesis rate constant *α* becomes a proxy for the absorbance at a particular wavelength. Indeed, the ratio between *α* at 414 nm (5.97) and *α* at 679 nm (3.76) is 1.59, which is almost identical to the ratio between blue and red peaks of the absorption spectrum (1.58; Fig. 1).

## 3.3 PI curves measured using fluorescence

It is clear from PI curves at 414 and 679 nm that the PFD used to perform the action spectrum experiments must be carefully selected so as not to favour photosynthesis at one wavelength over another, and thus produce a skewed and unrepresentative action spectrum. Importantly for commercial growth of *N. oculata*, a PFD should be chosen so as not to inhibit photosynthesis under blue wavelengths, but still providing sufficient energy to saturate photosynthesis under red wavelengths. Here, *Ek* at 414 nm (35.6 µmol photons m-2 s-1) provides the lower limit for a viable action spectrum, but photosynthesis stimulated by red light at this PFD would be severely limited. However, not all of this absorbed energy is passed to PSII for O2 evolution and hence a more robust means to ascertain the PFD threshold for an action spectrum is to examine the wavelength dependency of photophysiological fluorescence parameters that directly relate to PSII photochemical activity, ΦPSII and NPQ(Fig. 3).

The operating efficiency of PSII (ΦPSII) decreased with PFD more rapidly in blue light than in red light (Fig. 3a). In order to understand this response, it is necessary to look at the behaviour of the fundamental fluorescence parameters *F’* and *Fm’*. The minimum fluorescence *F’* increases faster at 414 nm, indicating a great rate of excitation energy trapping by PSII reaction centres (Oxborough et al., 2012; Suggett et al., 2003) which is consistent with more absorbed photons carrying more energy at this wavelength. Maximum fluorescence *Fm’* stays relatively constant with increasing PFD intensity at 679 nm, but it begins to decrease with lower PFD at 414 nm. Such trends in *Fm’* correspond to the dissipation of excess energy as heat, and thus reflected in an increase in non-photochemical quenching (NPQ) with increasing PFD for 414 nm but not 679 nm (Fig. 3b). Notably, NPQ increases at PFDs in excess of 60 µmol photons m-2 s-1 at 414 nm; this increase in NPQ must be avoided to accurately measure the action spectrum since it lowers the efficiency with which absorbed light is used for PSII photochemistry and thus O2 production (Baker, 2008). Therefore, a PFD of 60 µmol photons m-2 s-1 PAR was chosen to generate subsequent action spectrum.

## 3.4 Action spectrum

The action spectrum of *N. oculata* at 60 µmol photons m-2 s-1 PAR is shown in Fig. 4; as expected, the mathematical turning points were conserved between the action spectrum of oxygen evolution (Fig. 4a) and the absorption spectrum (Fig. 1). The blue maximum was clearly evident at 414 nm, with an oxygen evolution rate of 55.2±1.9 µmol O2 L-1 h-1 (as consistent with the data from the PI curve, Fig. 2a); this value is 16.7% higher than the oxygen evolution at the presumed (Solovchenko et al., 2011) blue maximum of 441 nm (46.0±1.3 µmol O2 L-1 h-1). Similarly, the red peak at 679 nm yielded an oxygen evolution rate of 25.6±0.3 µmol O2 L-1 h-1, as expected from the corresponding PI curve, but significantly less than would have been predicted from the absorption spectrum alone.

All chlorophyll *a* Q bands and the carotenoid peak at 490 nm were clearly resolved from the action spectrum (Fig. 4a). However, there were some peculiarities in the chlorophyll *a* absorption spectrum (Fig. 4b). Generally, the blue maximum wavelength resulted in the lowest operating efficiency, and ΦPSII increased towards the red end of the spectrum (ultimately recovering to 0.649±0.003 in effective PAR darkness at 700 nm) but the data points at 459, 600, and in particular 646 nm responded in an uncharacteristic manner (Fig. 4b). Lowest values of ΦPSII (0.546±0.002) were recorded at 646 nm, a wavelength that corresponds to a local minimum in both the absorption and oxygen evolution action spectra. This wavelength was associated with the *Qx* band of chlorophyll *a*, but also with absorption by chlorophyll *b*, which is absent in *Nannochloropsis* (Egeland et al., 2011); similarly, accessory carotenoids that dissipate absorbed energy as heat rather than for photochemistry and thus lower the photochemical efficiency (e.g. Suggett et al. 2009) do not absorb here (Egeland et al., 2011). It is possible that energy absorbed at 646 nm drives a state transition of light-harvesting complexes from PSII to photosystem I (PSI) to explain the lower operational efficiency of PSII. Recent evidence in *Nannochloropsis gaditana* (Basso et al., 2014) indicates that the light harvesting complex of PSII contains a mobile antenna component that might provide the structural flexibility for energy balance regulation between PSII and PSI; this will reduce ΦPSII, as seen at 646 nm for *N. oculata* (Fig. 4b).

Different methods have been developed to evaluate the relative absorption between PSII and PSI and are all based on an action spectrum (PSII or O2) compared against the corresponding absorption spectrum (summarised in Suggett et al. (2011)). According to the “no-overshoot” approach (Johnsen and Sakshaug, 2007), oxygen production is scaled to relative absorbance so that relative oxygen production never exceeds absorbance. In this case, the action spectrum has been scaled to 0.377 at 626 nm (Fig. 5a). However, an alternative approach is to integrate and equate the areas under the two spectra, and then to scale accordingly (Suggett et al., 2004). The benefit of this latter approach is that major differences between the spectra are immediately visible (Fig. 5b). For *N. oculata*, it is apparent that more of the absorbed energy goes into oxygen evolution at 626 nm than at 679 nm.

The trends observed in Fig. 5 can be treated in a more quantitative way to assess performance at different wavelengths to calculate the proportion of light absorption directed towards PSII, or *f*AQPSII (Johnsen and Sakshaug, 2007; Suggett et al., 2011). For the no-overshoot approach, this is simply the ratio between relative oxygen production and relative absorbance. In the case of the equal-area approach, it is calculated as the same ratio multiplied by 0.5. The equal-area approach assumes that on average, half of absorbed energy is directed to each photosystem, but that wavelength-specific variations arise due to a mismatch between the absorption spectrum and the action spectrum (Suggett et al., 2011). Although the two methods result in different absolute values for *f*AQPSII, its relative value across different wavelengths remains unchanged (Fig. 5c). A high *f*AQPSII percentage indicates that absorbed light is directed towards PSII and ultimately results in oxygen evolution; this is a desirable result for algal growth and biomass production. However, although *f*AQPSII at 531 nm is high, gross photosynthesis remains low since very little light is absorbed at this wavelength. This analysis does yield two interesting results. Firstly, *f*AQPSII at 414 nm represents the local maximum and it is 27.3% higher than *f*AQPSII at 679 nm, which represents the global minimum. *N. oculata* is thus much better at using light absorbed at the *By* Soret triplet (414 nm) than at the *Qy* lowest-energy singlet for photosynthetic oxygen production (679 nm). On the other hand, red light absorbed at the *Qx* singlet at 626 nm is especially well-used for photosynthesis, with a global *f*AQPSII maximum that is 21.8% higher than *f*AQPSII at 414 nm. This preferential absorption by PSII over PSI at 459, 600 and especially at 646 nm may be driving the suspected state transition at these wavelengths, which results in lower than expected ΦPSII (Fig. 4b).

In summary, the photosynthesis maximum for *N. oculata* was established to be 414 nm and not 440 nm as previously suggested. Light at this blue maximum was absorbed more effectively and directed more efficiently towards PSII than at the red maximum of 679 nm. Blue illumination is therefore an effective option for growth of this alga, provided that irradiation is maintained below saturating intensities. This has interesting economic implications for biofuel applications: operating blue LEDs at lower power results in better energy use efficiency for *N. oculata* growth. That said, our data would suggest that the most energy-efficient solution may be to grow *N. oculata* under red light at 625 nm, where the conversion of absorbed photons into photosynthetic oxygen evolution is maximised and there is less NPQ. One concern is that this maximum may be unstable, since a closely neighbouring wavelength 646 nm appears to lower photosynthetic efficiency, possibly as a result of a state transition that would likely reduce *N. oculata* growth.

# Conclusion

The first complete action spectrum of oxygen evolution and chlorophyll *a* fluorescence for the biofuel candidate alga *N. oculata* has been measured. This technique takes advantage of state-of-the-art technology and sophisticated analytical tools to develop a representative and reproducible action spectrum at a PFD of 60 µmol photons m-2 s-1 PAR. Future research can take advantage of this technique to determine the optimal light regime for *N. oculata* growth, or to compare the action spectra of different microalgae. Understanding the wavelength-dependence of photosynthesis is an important advance towards utilising this environmentally and economically important bioresource.

Supplementary figures associated with this article can be found online.

# Figures

**Fig. 1** Scatter-corrected *in vivo* absorption spectrum of *N. oculata*. Absorption has been normalised to unity at the red chlorophyll maximum (679 nm). Shaded grey areas indicate standard error (n = 3). Red circles indicate mathematical turning points, identified using the *peakfinder* algorithm (MatLab).

**Fig. 2** Oxygen evolution by *N. oculata* across different photon flux density **(a)** and irradiance **(b)** at actinic wavelengths of 414 nm (blue, full) and 679 nm (red, empty). Error bars depict standard error (n = 3). Standard Jassby & Platt exponential curve has been fitted to data (as described in Suggett et al., 2003). Dashed lines in **(a)** show Blackman-type fitting parameters *α*, *Pmax* and *Ek*.

Unit conversion: PFD [µmol m-2 s-1] = E [W m-2] · λ [nm] · 0.00836

**Fig. 3** Photophysiological response of *N. oculata* across different photon flux density at actinic wavelengths of 414 nm (blue, full) and 679 nm (red, empty). Comparisons between the PSII operating efficiency ΦPSII **(a)** and the non-photochemical quenching NPQ **(b)** are shown. Error bars depict standard error (n = 3).

**Fig. 4** Action spectrum of *N. oculata* at 60 µmol photons m-2 s-1 PAR. Absolute oxygen evolution **(a)** and PSII operating efficiency ΦPSII **(b)** are compared at different wavelengths. Error bars depict standard error (n = 3).

**Fig. 5** Action spectrum of *N. oculata* at 60 µmol photons m-2 s-1 PAR (purple, dashed) scaled against its absorption spectrum (black, full) according to: **(a)** the no-overshoot approach (Johnsen and Sakshaug, 2007), where oxygen production at 626 nm was scaled to the corresponding absorbance so that relative oxygen production never exceeds absorbance; and **(b)** the equal-area approach (Suggett et al., 2004), where the trapezoidal integral of the area under the two curves is equalised. The proportion of light absorption directed to PSII, *f*AQPSII, **(c)** is calculated as: a ratio between relative oxygen production and relative absorbance from Fig. 5a; and the same ratio multiplied by 0.5 from Fig. 5b. Analysis modified from Suggett et al. (2011).

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# References

1. Arnold, W., 1991. Experiments. Photosynth. Res. 27, 73–82.
2. Baker, N.R., 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu. Rev. Plant Biol. 59, 89–113.
3. Bartley, M.L., Boeing, W.J., Corcoran, A.A., Holguin, F.O., Schaub, T., 2013. Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. Biomass and Bioenergy 54, 83–88.
4. Basso, S., Simionato, D., Gerotto, C., Segalla, A., Giacometti, G.M., Morosinotto, T., 2014. Characterization of the photosynthetic apparatus of the Eustigmatophycean *Nannochloropsis gaditana*: Evidence of convergent evolution in the supramolecular organization of photosystem I. Biochim. Biophys. Acta 1837, 306–314.
5. Borowitzka, M.A., Moheimani, N.R., 2013. Sustainable biofuels from algae. Mitig. Adapt. Strateg. Glob. Chang. 18, 13–25.
6. Cardol, P., Forti, G., Finazzi, G., 2011. Regulation of electron transport in microalgae. Biochim. Biophys. Acta 1807, 912–918.
7. Cooper, T.F., Ulstrup, K.E., Dandan, S.S., Heyward, A.J., Kühl, M., Muirhead, A., O’Leary, R.A., Ziersen, B.E.F., Van Oppen, M.J.H., 2011. Niche specialization of reef-building corals in the mesophotic zone: metabolic trade-offs between divergent *Symbiodinium* types. Proc. R. Soc. B Biol. Sci. 278, 1840–1850.
8. Egeland, E.S., Garrido, J.L., Clementson, L., Andresen, K., Thomas, C.S., Zapata, M., Airs, R., Llewellyn, C.A., Newman, G.L., Rodríguez, F., Roy, S., 2011. Part VII - Data sheets aiding identification of phytoplankton carotenoids and chlorophylls, in: Roy, S., Llewellyn, C.A., Egeland, E.S., Johnsen, G. (Eds.), Phytoplankton Pigments. Cambridge University Press, pp. 665–674.
9. Emerson, R., Arnold, W., 1942. The photosynthetic efficiency of phycocyanin in *Chroococcus* and the problem of carotenoid participation in photosynthesis. J. Gen. Physiol. 25, 579–595.
10. Georgianna, D.R., Mayfield, S.P., 2012. Exploiting diversity and synthetic biology for the production of algal biofuels. Nature 488, 329–35.
11. Gouveia, L., Oliveira, A.C., 2009. Microalgae as a raw material for biofuels production. J. Ind. Microbiol. Biotechnol. 36, 269–274.
12. Haxo, F., Blinks, L., 1950. Photosynthetic action spectra of marine algae. J. Gen. Physiol. 33, 389–422.
13. Jassby, A., Platt, T., 1976. Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. Limnol. Oceanogr. 21, 540–547.
14. Johnsen, G., Sakshaug, E., 2007. Biooptical characteristics of PSII and PSI in 33 species (13 pigment groups) of marine phytoplankton, and the relevance for pulse-amplitude-modulated and fast-repetition-rate fluorometry 1. J. Phycol. 43, 1236–1251.
15. Kandilian, R., Lee, E., Pilon, L., 2013. Radiation and optical properties of *Nannochloropsis oculata* grown under different irradiances and spectra. Bioresour. Technol. 137, 63–73.
16. Larkum, A.W.D., Ross, I.L., Kruse, O., Hankamer, B., 2012. Selection, breeding and engineering of microalgae for bioenergy and biofuel production. Trends Biotechnol. 30, 198–205.
17. Lehr, F., Posten, C., 2009. Closed photo-bioreactors as tools for biofuel production. Curr. Opin. Biotechnol. 20, 280–285.
18. Lubián, L., Montero, O., Moreno-Garrido, I., Huertas, I.E., Sobrino, C., González-del Valle, M., Pares, G., 2000. *Nannochloropsis* (Eustigmatophyceae) as source of commercially valuable pigments. J. Appl. Phycol. 12, 249–255.
19. MacIntyre, H., Kana, T., Anning, T., Geider, R.J., 2002. Photoacclimation of photosynthesis irradiance curves and photosynthetic pigments in microalgae and cyanobacteria. J. Phycol. 38, 17–38.
20. Millie, D.F., Schofield, O.M., Kirkpatrick, G.J., Johnsen, G., Evens, T.J., 2002. Using absorbance and fluorescence spectra to discriminate microalgae. Eur. J. Phycol. 37, 313–322.
21. Oxborough, K., Moore, C.M., Suggett, D.J., Lawson, T., Chan, H.G., Geider, R.J., 2012. Direct estimation of functional PSII reaction center concentration and PSII electron flux on a volume basis: a new approach to the analysis of Fast Repetition Rate fluorometry (FRRf) data. Limnol. Oceanogr. Methods 10, 142–154.
22. Petrou, K., Jimenez-Denness, I., Chartrand, K., McCormack, C., Rasheed, M., Ralph, P., 2013. Seasonal heterogeneity in the photophysiological response to air exposure in two tropical intertidal seagrass species. Mar. Ecol. Prog. Ser. 482, 93–106.
23. Raven, J.A., 2011. The cost of photoinhibition. Physiol. Plant. 142, 87–104.
24. Schreiber, U., Klughammer, C., 2013. Wavelength-dependent photodamage to *Chlorella* investigated with a new type of multi-color PAM chlorophyll fluorometer. Photosynth. Res. 114, 165–177.
25. Schreiber, U., Klughammer, C., Kolbowski, J., 2012. Assessment of wavelength-dependent parameters of photosynthetic electron transport with a new type of multi-color PAM chlorophyll fluorometer. Photosynth. Res. 113, 127–144.
26. Sforza, E., Simionato, D., Giacometti, G.M., Bertucco, A., Morosinotto, T., 2012. Adjusted light and dark cycles can optimize photosynthetic efficiency in algae growing in photobioreactors. PLoS One 7, e38975.
27. Simionato, D., Sforza, E., Corteggiani Carpinelli, E., Bertucco, A., Giacometti, G.M., Morosinotto, T., 2011. Acclimation of *Nannochloropsis gaditana* to different illumination regimes: effects on lipids accumulation. Bioresour. Technol. 102, 6026–6032.
28. Solovchenko, A., Khozin-Goldberg, I., Recht, L., Boussiba, S., 2011. Stress-induced changes in optical properties, pigment and fatty acid content of *Nannochloropsis* sp.: implications for non-destructive assay of total fatty acids. Mar. Biotechnol. 13, 527–535.
29. Suggett, D.J., MacIntyre, H.L., Geider, R.J., 2004. Evaluation of biophysical and optical determinations of light absorption by photosystem II in phytoplankton. Limnol. Oceanogr. Methods 2, 316–332.
30. Suggett, D.J., Moore, C.M., Geider, R.J., 2011. Chapter 6: Estimating Aquatic Productivity from Active Fluorescence Measurements, in: Suggett, D.J., Prášil, O., Borowitzka, M.A. (Eds.), Chlorophyll *a* Fluorescence in Aquatic Sciences: Methods and Applications. Springer Netherlands, Dordrecht, pp. 103–127.
31. Suggett, D.J., Oxborough, K., Baker, N.R., MacIntyre, H.L., Kana, T.M., Geider, R.J., 2003. Fast repetition rate and pulse amplitude modulation chlorophyll *a* fluorescence measurements for assessment of photosynthetic electron transport in marine phytoplankton. Eur. J. Phycol. 38, 371–384.
32. Sukenik, A., Beardall, J., Kromkamp, J., Kopecký, J., Masojídek, J., van Bergeijk, S., Gabai, S., Shaham, E., Yamshon, A., 2009. Photosynthetic performance of outdoor *Nannochloropsis* mass cultures under a wide range of environmental conditions. Aquat. Microb. Ecol. 56, 297–308.
33. Tamburic, B., Guruprasad, S., Radford, D.T., Szabó, M., Lilley, R.M., Larkum, A.W.D., Franklin, J.B., Kramer, D.M., Blackburn, S.I., Raven, J.A., Schliep, M., Ralph, P.J., 2014. The effect of diel temperature and light cycles on the growth of *Nannochloropsis oculata* in a photobioreactor matrix. PLoS One 9, e86047.
34. Weger, H., Herzig, R., Falkowski, P., Turpin, D., 1989. Respiratory losses in the light in a marine diatom: measurements by short-term mass spectrometry. Limnol. Ocean. 34, 1153–1161.
35. Wijffels, R.H., Barbosa, M.J., 2010. An outlook on microalgal biofuels. Science 329, 796–799.