Chromatic photoacclimation, photosynthetic electron transport and oxygen evolution in the Chlorophyll d-containing oxyphotobacterium Acaryochloris marina

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Received 13 June 2006; received in revised form 30 October 2006; accepted 22 November 2006
Available online 30 November 2006

Abstract
Changes in photosynthetic pigment ratios showed that the Chlorophyll d-dominated oxyphotobacterium Acaryochloris marina was able to photoacclimate to different light regimes. Chl d per cell were higher in cultures grown under low irradiance and red or green light compared to those found when grown under high white light, but phycocyanin/Chl d and carotenoid/Chl d indices under the corresponding conditions were lower. Chl a, considered an accessory pigment in this organism, decreased respective to Chl d in low irradiance and low intensity non-white light sources. Blue diode PAM (Pulse Amplitude Modulation) fluorometry was able to be used to measure photosynthesis in Acaryochloris. Light response curves for Acaryochloris were created using both PAM and O2 electrode. A linear relationship was found between electron transport rate (ETR), measured using a PAM fluorometer, and oxygen evolution (net and gross photosynthesis). Gross photosynthesis and ETR were directly proportional to one another. The optimum light for white light (quartz halogen) was about 206±51 μmol m−2 s−1 (PAR) (Photosynthetically Active Radiation), whereas for red light (red diodes) the optimum light was lower (109±27 μmol m−2 s−1 (PAR)). The maximum mean gross photosynthetic rate of Acaryochloris was 73±7 μmol mg Chl d−1 h−1. The gross photosynthesis/respiration ratio (P/R) of Acaryochloris under optimum conditions was about 4.02±1.69. The implications of our findings will be discussed in relation to how photosynthesis is regulated in Acaryochloris.

Keywords: Acaryochloris; Oxyphotobacteria; Photosynthesis; Chlorophyll d; Photo adaptation; Oxygen electrode; PAM; Light saturation curve; Induction curve

1. Introduction
While chlorophyll (Chl) a is recognised as the dominant pigment in most oxygenic phototrophs, the novel oxyphotobacterium Acaryochloris marina possesses Chl d, in excess of 90% of total chlorophyll, and Chl a [1]. Depending on growth conditions, the Chl a content of this organism ranges from 10% to as little as 1.6% [1–3], and light-harvesting-complexes are functionally and structurally distinct from those of typical cyanobacteria [2,4–6]. Chl d is not only the major pigment in the antenna proteins of both photosystem (PS) I and II, but it is also known to comprise the special pair of PSI [5]. It has not yet been confirmed that Chl d is the special pair in PS II [6–8], but even so Acaryochloris has a truly unique light harvesting apparatus [9,10]. Photosynthetic oxygen evolution based upon Chl d rather than Chl a therefore constitutes a major new light-harvesting process in oxygenic photosynthesis.

The presence of a C3-formyl group on ring 1 in Chl d instead of the vinyl group of Chl a gives Chl d an absorption peak that is shifted into the far-red region at about 691 nm in acetone solvent [11] or 708–720 nm in vivo [1]. The ability to harvest solar energy at such wavelengths is hypothesised to be an adaptation to the peculiar environmental niche of Acaryochloris [5,12,13], which is known to form biofilms on the underside of didemnid ascidians [13]. In this habitat, it is only exposed to light that has first passed through the water column (0–5 m), the ascidian body and a layer of Prochloron symbiont, a Chl a–b–MgDVP (Magnesium divinyl pheoporphyrin)-containing novel oxyphotobacterium [14], and thus Acaryochloris may exploit those wavelengths that other phototrophs cannot. Acaryochloris has been reported to occur on the similarly light deficient stalk bases of certain red algae [15,16], and most recently, an Acaryochloris-like organism was found free-living in the Salton Sea
(a turbid saline lake in the USA) [17]. *Acaryochloris* has been identified as a distinct lineage within the oxyphotobacterial (Cyanobacterial) radiation [18] and has been suggested to represent an evolutionary intermediate between cyanobacteria (and their symbiogenetically-derived chloroplast descendants), which use Chl *a* as their primary photosynthetic pigment, and the far-red/infra-red (750–900 nm) absorbing anoxygenic bacteria, which use various types of bacteriochlorophyll as their primary photosynthetic pigment [5,19].

This study investigated chromatic photoacclimation and photosynthesis in *Acaryochloris*. The ability to maintain optimal photosynthetic efficiency by altering pigment composition has important consequences for the ecology, distribution and evolution of photosynthetic organisms and occurs to varying degrees in all eukaryotic microalgae and cyanobacteria and their oxyphotobacteria relatives. The phenomenon of photoacclimation is characterised by an overall increase of pigments with decreasing irradiance and an increased ratio of accessory chlorophylls, carotenoids and phycobiliproteins (PBPs) relative to the primary chlorophyll [20]. In addition to Chl *a/d* and MgDVP [15], *Acaryochloris* possesses PBP, which exhibit atypical structural organization compared to cyanobacteria and rhodophytes [4,15,21].

Investigation of photosynthesis in *Acaryochloris* demands new techniques for this organism. For example the far-red absorption band of Chl *d* in vivo is outside the spectral range normally considered to be Photosynthetically Active Radiation (PAR, 400–700 nm) and so the light absorbed by Chl *d* cannot be reliably measured by quantum light meters conventionally used in photosynthetic research. Photosynthesis by Chl *d*-dominated oxygenic photosynthetic organisms is practically zero at such wavelengths and it was generally thought that far-red light (∼720 nm) could not provide enough energy to allow water to be used as a source of electrons for photosynthesis [20].

Pulse amplitude modulated chlorophyll fluorometry (PAM) has revolutionised photosynthesis investigation in recent decades, providing rapid and accurate measurements of keystone photosynthetic parameters. This technique employs the fluorescence emission of chlorophyll that results from a brief but strong light pulse of known intensity as an indicator of Electron Transport Rate (ETR) [22]. PAM fluorometers were designed for use on Chl *a*-dominated vascular plants, although the tenets of fluorometry might be expected to hold for a Chl *d* scheme. Verifying the integrity of PAM may have valuable consequences for future research of *Acaryochloris* physiology.

In this investigation we have compared photosynthesis in *Acaryochloris*, using both conventional oxygen electrode techniques and PAM techniques, presenting comparisons of photosynthesis vs. light intensity (P vs. J) curves using the two techniques performed under a range of light regimes.

2. Methods

2.1. Chromatic photoacclimation

*Acaryochloris marina* (MBIC11017, Marine Biology Institute Culture Collection, Kamashi, Japan) was cultured in enriched seawater C medium at the University of Sydney Marine Sciences Laboratory [11]. Details of the original isolation and pigment composition of *Acaryochloris* are given in Miyashita et al. [15]. Crompton 4100 K/33 cool white lights were used for routinely growing *Acaryochloris* at a temperature of about 25 °C. Two types of quantum light meter were used to measure light. A Li-Cor Quantum LI-189 light meter (LiCor Corp, USA) was used to measure PAR (400–700 nm). A Hansatech SKYE Instruments SKP 200 Red Quantum Sensor (RQS) meter (SKYE Instruments, Powys, UK) was used to measure light from 550 to 750 nm. The doubling time for cultures grown under white light (∼100 μmol m⁻² s⁻¹ (PAR); ∼40 μmol m⁻² s⁻¹ (RQS)) was 33±3 h (n=24; Table 1): this growth rate is considerably faster than doubling time of 48 to 60 h reported by Swingley et al. [3] who used lower light intensities of about 20 μmol m⁻² s⁻¹ (PAR). Four cultures were established and grown for at least 4 weeks under each of four light regimes; high intensity white (∼100 μmol m⁻² s⁻¹ (PAR); ∼40 μmol m⁻² s⁻¹ (RQS)), low intensity white (∼5 μmol m⁻² s⁻¹ (PAR); ∼2 μmol m⁻² s⁻¹ (RQS)), red glass (∼17 μmol m⁻² s⁻¹ (PAR); ∼14 μmol m⁻² s⁻¹ (RQS)), and green glass (∼6 μmol m⁻² s⁻¹ (PAR); ∼2 μmol m⁻² s⁻¹ (RQS)). Low intensity white light treatments used ‘tents’ of black horticultural shade cloth, over flasks in a white light lit growth room. The green and red light growth experiments were run using boxes covered by a sheet of coloured window glass (see Spectra in Supplementary material). Green window glass had a broad transmission from 460 to 620 nm with a peak at 530 nm but maximum transmission was only about 18%; red glass had virtually zero transmission below 605 nm but >30% transmission above 650 nm. *Acaryochloris* culture grew well but very slowly under low intensity white, red or green light.

Chl *a/d* ratios were determined by spectrophotometry (Shimadzu UV-2550) using pigment extract in an ethanol solvent and appropriate Chl *d* equations [11]. Phycocyanin is the major phycobiliprotein of *Acaryochloris* [21] and has a major in vivo peak at 609 nm [15]. Chl *d* has an in vivo peak at 708 to 715 nm. Thus if absorbance at 750 nm (A750) is used as a baseline, the ratio of (A609–A750)/(A708–A750) could be used as a phycocyanin index (PC index) of the relative abundance of phycocyanin to Chl *d*. Similarly, carotene and xanthophyll pigments have an absorption peak near 485 nm and so a carotenoid–xanthophyll index (CX index) could be calculated from (A485–A750)/(A708–A750).

2.2. Growth studies

Measurements were made of the growth of cultures grown under high white light, low white light, red and green glass. The exponential doubling times of

<table>
<thead>
<tr>
<th>Growth parameters for <em>Acaryochloris marina</em> cells grown under high white, low white, red and green light</th>
<th>High white light (≥100 μmol m⁻² s⁻¹ (PAR))</th>
<th>Low white light (≥5 μmol m⁻² s⁻¹ (PAR))</th>
<th>Red light (≥17 μmol m⁻² s⁻¹ (PAR))</th>
<th>Green light (≥6 μmol m⁻² s⁻¹ (PAR))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential doubling time (t₁ h)</td>
<td>33±3 (24)</td>
<td>87±9 (24)</td>
<td>99±10 (24)</td>
<td>222±43 (3, 60)</td>
</tr>
<tr>
<td>Cells ml⁻¹ A750</td>
<td>125 (±3.8)×10⁶ (168)</td>
<td>94 (±5.1)×10⁶ (48)</td>
<td>84.5 (±5.1)×10⁶ (48)</td>
<td>90.8 (±6.0)×10⁶ (48)</td>
</tr>
<tr>
<td>g Chl d cell⁻¹</td>
<td>30.9 (±0.93)×10⁻¹⁵ (168)</td>
<td>55.1 (±3.3)×10⁻¹⁵ (48)</td>
<td>58.0 (±3.7)×10⁻¹⁵ (48)</td>
<td>73.9 (±4.6)×10⁻¹⁵ (48)</td>
</tr>
<tr>
<td>g Chl a cell⁻¹</td>
<td>1.69 (±0.15)×10⁻¹⁵ (168)</td>
<td>2.28 (±0.16)×10⁻¹⁵ (48)</td>
<td>1.95 (±0.15)×10⁻¹⁵ (48)</td>
<td>1.44 (±0.14)×10⁻¹⁵ (48)</td>
</tr>
<tr>
<td>Chl a/d ratio</td>
<td>0.0552±0.00396 (168)</td>
<td>0.0393±0.00187 (48)</td>
<td>0.0346±0.00199 (48)</td>
<td>0.0186±0.000753 (48)</td>
</tr>
</tbody>
</table>

The exceptionally slow growth rate under green light was confirmed on three separate starting cultures after it became apparent that the cells took a long time to adjust to green light. The number of independent replicates is shown in brackets.
cultures were determined by measuring A750 of dilute cultures (A750 at starting time about 0.1) over at least 7 days. Doubling times were estimated using non-linear least squares fitting methods and the asymptotic errors calculated by matrix inversion [23]. To ensure that the cells were properly photoacclimated, cultures had been growing under the specified conditions for at least 1 month and subcultured at least once before being used for a growth kinetics or pigment analysis studies. The relationships between light scattering of cultures measured at 750 nm, chlorophyll content (determined as described above [11]), and cell numbers (Thoma cell hemacytometer) were determined using least squares methods [23]. To properly test for linearity between these parameters, cultures with a wide range of cell densities from about A750=0.1 to A750=1.3 were used.

2.3. Oxygen electrode studies

An illuminated oxygen electrode chamber was used to measure photosynthetic and respiratory rates (Hansatech, England, UK) used as described by Walker [24]. Four tubes of 10 ml Acaryochloris culture were centrifuged (at ~3000×g) for 10 min at 20 °C and the pellets combined to give an appropriate Acaryochloris suspension of Chl d/≈15±5 μg ml⁻¹. One ml of the suspension was used in the electrode chamber. The chamber was exposed to either eleven different intensities representing eleven different percentages of the maximum light intensity: 2, 3, 5, 7, 10, 14, 20, 30, 50, 70 and 100%; later because the two lowest light intensities gave negligible photosynthesis only eight light intensities were used (5, 10, 14, 20, 30, 50, 70 and 100%). It took several hours to complete a light response curve and so the different light intensities were given in random order to defeat error arising from possible photoadaptation during the course of the experiment. The light was turned off for 10 min between each light intensity to allow respiration rates to be measured and to allow time for a rapid PAM light saturation curve (≈3 min) to be performed if required (see Supplementary material for an example of an O₂-electrode trace).

Two different light sources, red or white, were used. The red diode light sources (Hansatech, England, UK) had a maximum output at about 665±15 nm giving a total light of about 130 μmol m⁻² s⁻¹ (PAR). The white light source was a fibroptic light source from Fibreoptic Lightguides, Waitara, NSW, Australia fitted with a 100W-EFP tungsten-halogen globe. The light intensities were adjusted directly using the attached light box. The LI-COR PAR light meter was used to determine the maximum light (dial turned up full) to which the chamber was exposed when it contained only water. To correct for diffraction caused by the organism in suspension, this was compared to the light meter reading when Acaryochloris was added into the chamber. The ratio of these values was used to adjust light meter readings appropriately as the voltmeter-dial was turned to give higher or lower intensities. The maximum light intensity was about 350 μmol m⁻² s⁻¹ (PAR). This method has the inherent disadvantage that the colour-temperature (and hence the spectrum) of the light would change as the voltage was changed.

2.4. PAM fluorometry

The optic fibre of a blue diode (485±40 nm) Junior PAM portable chlorophyll fluorometer (Gademann Instruments GmbH, Wurzburg, Germany) was fed through a small hole in the cap of the O₂-electrode chamber lid to permit simultaneous ETR and oxygen exchange. PAM parameters were calculated using WINCONTROL software using standard settings (Heinz Walz GmbH, Effeltrich, Germany). The default adsorption factor of 0.84 was used on the Junior PAM to calculate ETR (see [13]). Eleven PAM experiments were run on cell suspensions in the oxygen electrode giving 11 matched ETR/O₂-evolution measurements using red diodes as the light source for the oxygen electrode measurements. Three similar experiments were run using white light as the light source giving 53 matched ETR/O₂-evolution determinations. Sets of PAM light curve measurements took about 3 min to complete and were run during the 10 min dark periods used to measure respiration with the O₂ electrode.

Rates of gross photosynthesis (Pₚ) oxygen and ETR were plotted as light response curves (photosynthetic rate (Pₚ) vs. light intensity (I); Pₚ vs. I) and fitted to a Waiting-in-Line model (probability density function or exponential waiting time distribution), used for a system where a rate is initially directly proportional to the amount substrate but eventually saturates at a given substrate density and then is inhibited if the substrate is further increased.

\[ P = A k I e^{-k I} \]

where, P is photosynthesis (either Pₚ or ETR), A is a scaling constant for the height of the curve, k is a scaling constant for the x axis, I is the substrate, in this case light.

In the case of the Waiting-in-Line model, it can be shown that \( P_{\text{max}} \) (the maximum velocity) is equivalent to \( A / e \) and \( P_{\text{max}} \) occurs at a substrate value of 1/k. The photosynthetic efficiency (Alpha, α) is the initial slope of the curve at \( S=0 \) (α = A/k or α = \( P_{\text{max}} \cdot e \cdot k \)). Curves were fitted by non-linear least squares fitting and the asymptotic errors calculated by matrix inversion [23].

The PAM data and the oxygen electrode data were used to prepare ETR vs. O₂ evolution rate curves to find the degree of correlation between ETR and photosynthetic rate. A linear model was fitted to ETR vs. net photosynthesis (\( \gamma = mx + b \)). For ETR vs. gross photosynthesis a linear model was first fitted but it was found that the \( \gamma \)-intercept was not significantly different to zero and so a simple proportional model was demonstrated to be the simpler model consistent with the data (\( \gamma = mx \)). Since ETR should correlate linearly to gross photosynthetic oxygen evolution, a straight-line relationship between the two would be a demonstration that the PAM machine gives valid estimates of photosynthesis in Acaryochloris.

Induction curves were run using the standard settings of the WinControl software but the actinic light intensity was set to 221 μmol m⁻² s⁻¹ PAR. Twelve replicate samples of Acaryochloris cells were filtered onto Whatman GF-C glass fibre filters (Whatman International, Maidstone, England, UK) in a Millipore apparatus for 25 mm filters then dark treated in a dish of seawater for at least 10 min. Only one induction experiment was run on each filter to avoid confounding effects of multiple experimental treatments. Chlorophyll was then extracted from the filter using ethanol and Chl d and Chl a determined using the algorithms of Ritchie [11].

2.5. Statistics

All errors quoted are ±95% confidence limits. The number of replicates are quoted in brackets (n), where the brackets contain two the number (a, b) the first refers to the number of independent experiments and the second refers to the total number of data points.

3. Results

3.1. Chromatic photoacclimation

Relative Chl a content in Acaryochloris showed limited variation with light regime, ranging from approximately 2% to 6% of total chlorophyll (Table 1). Chl a/d was the highest when grown under high white light (Chl a/d=0.0552±0.00396), slightly lower when grown under low white light (Chl a/d=0.0393±0.00187), or red light (Chl a/d=0.0346±0.00199) and lowest when under green light growth conditions (Chl a/d=0.0186±0.00753) (Table 1). Both the PC and CX indices were highest in cells grown in high white light and reduced by about 40 to 70% under low light intensities (Fig. 1), regardless of the spectral quality of the light. The mean PC index varied from around 0.85±0.083 (high white) to 0.26±0.028 (green) and the CX index fell from 2.99±0.27 in high white light to a minimum of 1.82±0.050 under green light (Fig. 1). Although there are statistically significant differences between the cultures grown low white and coloured light, Fig. 1 shows that the only culture greatly different from the others are the cells grown in high white light.
Reflectance of cultures was measured using the Shimadzu Taylor-sphere attachment (ISR-240A) on standard settings to measure the in vivo light absorption properties of pigments of the cells. Chl $d$ in vivo absorbs strongly in the range 710 to 720 nm (Fig. 2). Reflectance values were standardised onto the red Chl $d$ absorption peak at 710 nm. It was noted that the major Chl $d$ peak showed a slight spectral shifts from 709 to 711 nm in different batches of cells but no consistent spectral shifts in chlorophyll-containing proteins could be found in cultures grown under different light conditions. The reflectance curves of *Acaryochloris* grown under high white, low white light, green and red glass are virtually identical. *Acaryochloris* grown under high white light have decreased reflectance at 600 to 650 nm due to decreased amounts of phycocyanin relative to chlorophyll. The carotenoid and xanthophyll content of cultures grown under red glass is lowered compared to the other cultures.

![Fig. 1. Phycocyanin to Chl $d$ (PC) and Carotenoid: Chl $d$ (CX) indices (+95% confidence limit) in *Acaryochloris marina* grown under light of different irradiances and/or spectral quality (n=16).](Image)

**Fig. 1.** Phycocyanin to Chl $d$ (PC) and Carotenoid: Chl $d$ (CX) indices (+95% confidence limit) in *Acaryochloris marina* grown under light of different irradiances and/or spectral quality (n=16).

**3.2. Growth experiments**

Table 1 shows that *Acaryochloris* grew much faster under the high light regime ($t_2 \approx 33$ h) than under low white, red light ($t_2 \approx 80–100$ h) and very slowly under green light ($t_2 \approx 222$ h). Light scattering measurements (A750) of cells grown under different conditions show that cultures grown in high white light are much less opaque than cells grown under the other growth conditions. Measurements of the amount of Chl $a/d$ per cell show that *Acaryochloris* grown under high light conditions has much less Chl $d$ per cell. Comparisons of Figs. 1, 2 and Table 1 shows that the major photoacclimation effect in *Acaryochloris* is a decrease in total chlorophyll per cell in high light rather than large changes in Chl $a/d$ ratios or changes in phycoerythrin or carotenoid content.

**3.3. Photosynthesis measured using $O_2$-electrode and PAM fluorometry**

*Acaryochloris*, despite having an in vivo Chl $d$ absorption peak at about 710 nm, is nevertheless capable of photosynthetic oxygen evolution using white or red light (see Supplementary figure of $O_2$-electrode trace). Both oxygen-derived net and gross photosynthesis were linearly correlated with electron transport rate ETR measured by blue light PAM (Figs. 3 and 4). Linear regression of ETR ($\mu$mol m$^{-2}$ s$^{-1}$) vs. net photosynthetic rate ($\mu$mol mg Chl $d$ $^{-1}$ h$^{-1}$) was calculated for *Acaryochloris* by equating PAM fluorometry and oxygen electrode measurements on the same sample of *Acaryochloris* cells ($n=8, 75$) (Fig. 3). Data fitted by linear regression ($y=mx+b$), $m=0.177 \pm 0.024, b=2.91 \pm 0.88, r=0.867$, where $y$ represents ETR and $x$ represents net photosynthesis ($P_n$). Similarly Fig. 4 shows that ETR vs. gross photosynthesis ($P_g$) are linearly related; $m=0.170 \pm 0.026, b=-0.196 \pm 1.35, r=0.835$ where $y$ represents ETR and $x$ represents gross photosynthesis.

![Fig. 2. In vivo spectra of *Acaryochloris marina* grown under different light qualities and intensities. Reflectance readings have been standardised onto the reflectance reading at 710 nm and 100% reflectance was set at 750 nm. There is very little spectral difference between the cultures grown under different conditions. Cultures grown under green glass (D) have significantly decreased relative reflectance at about 485 nm compared to the other cultures (A, B, C) whereas cultures grown under high white light (A) have decreased relative reflectance at 600 to 650 nm where phycocyanin absorbs significant amounts of light. The far-red Chl $d$ peak varies from 709 to 712 nm in different cultures but a consistent spectral shift in either the blue or red Chl $d$ peaks was not apparent in different batches of cells grown under different light conditions.](Image)

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![Fig. 3. Linear regression of Electron Transport Rate (ETR) ($\mu$mol m$^{-2}$ s$^{-1}$) vs. net photosynthetic rate ($\mu$mol mg Chl $d$ $^{-1}$ h$^{-1}$) calculated for *Acaryochloris marina* by equating PAM fluorometry and oxygen electrode measurements on the same sample of *Acaryochloris* ($n=8, 75$). Data fitted by linear regression ($y=mx+b$), $m=0.177 \pm 0.0238, b=2.906 \pm 0.878, r=0.867$, where $y$ represents ETR and $x$ represents net photosynthetic oxygen evolution.](Image)

**Fig. 3.** Linear regression of Electron Transport Rate (ETR) ($\mu$mol m$^{-2}$ s$^{-1}$) vs. net photosynthetic rate ($\mu$mol mg Chl $d$ $^{-1}$ h$^{-1}$) calculated for *Acaryochloris marina* by equating PAM fluorometry and oxygen electrode measurements on the same sample of *Acaryochloris* ($n=8, 75$). Data fitted by linear regression ($y=mx+b$), $m=0.177 \pm 0.0238, b=2.906 \pm 0.878, r=0.867$, where $y$ represents ETR and $x$ represents net photosynthetic oxygen evolution.
the $y$-intercept was not significantly different to zero, a simple proportional fit ($y=mx$) was a simpler model, giving a slope of 0.167±0.014 (or 46.4 (±4.0)×10⁻²) mg Chl d⁻¹ h⁻¹ in rationalised units. It is notable in the case of correlations of ETR with both net and gross photosynthesis that although the overall correlations are both greater than 0.8 there is considerable variation around the fitted lines.

Waiting-in-Line curves were fitted to both gross photosynthesis vs. light intensity and ETR vs. light intensity. The fitted parameters are shown in Table 2. *Acaryochloris* cells used for the experiments were grown under white fluorescent light ($I\approx 50 \mu$mol m⁻² s⁻¹ (PAR)). The data extended to higher light intensities in the case of ETR measurements ($I\approx 350 \mu$mol m⁻² s⁻¹ (PAR)) because the blue diodes of the PAM machine provided higher light intensities than the red diode lights of the Hansatech oxygen electrode could provide (maximum 180 $\mu$mol m⁻² s⁻¹ (PAR)). The optimum light intensities using the O₂ electrode and PAM machines were considerably different: 109±27 $\mu$mol m⁻² s⁻¹ for the O₂ electrode and 291±60 $\mu$mol m⁻² s⁻¹ for the PAM apparatus but the latter used a blue diode light source, whereas gross photosynthesis was determined using a red diode or white light source.

Light saturation curves were also determined using an oxygen electrode and an incandescent white light source (quartz-halogen), on *Acaryochloris* grown in white light as above. Table 2 shows that the saturating light intensities for $P_n$ vs. $I$ curves using white light and red diode light sources were not significantly different (approximate overall mean = 73±7 $\mu$mol mg Chl d⁻¹ h⁻¹ (14, 146)). On a quantum basis, however, red light was a more efficient light source than white light. The optimum light intensity for white light was about 206±51 $\mu$mol m⁻² s⁻¹ (PAR), whereas for the red diodes the optimum light was much lower (110±22 $\mu$mol m⁻² s⁻¹ (PAR)). As would be expected the photosynthetic efficiency ($\alpha$) was much higher using the red diodes (438 (±112)×10⁻⁶ m² mg Chl d⁻¹) than for the white light ($\alpha=298$ (±107)×10⁻⁶ m² mg Chl d⁻¹) because *Acaryochloris* does not absorb green and yellow light very efficiently (Fig. 2). The overall mean respiration rate based on all O₂ electrode experiments was 16.9±4.5 $\mu$mol mg Chl d⁻¹ h⁻¹.

![Fig. 4](image1.png) Fig. 4. Electron Transport Rate (ETR) vs. Gross photosynthetic oxygen evolution ($P_n$) calculated for *Acaryochloris marina* by equating PAM fluorometry and oxygen electrode measurements on the same sample of the *Acaryochloris* culture ($n=8$, 75). Linear regression gives a linear least squares model $y=mx+b$, $m=0.170±0.0262$, $b=−0.196±1.347$, $r=0.835$ where $y$ represents ETR and $x$ represents gross photosynthesis. The $y$-intercept is not significantly different to zero. A simple proportional fit ($y=mx$) gives a slope of 0.167±0.0143 (mg Chl d⁻¹ h⁻¹) or 46.4 (±4.0)×10⁻² mg Chl d⁻¹ in rationalised units.

![Fig. 5](image2.png) Fig. 5. PAM Induction curve on *Acaryochloris marina* using an actinic light PAR of 65, 136 and 552 $\mu$mol m⁻² s⁻¹ using standard settings. ETR is Electron Transport Rate and NPQ is Non-Photochemical Quenching. Three sets of 8 glass fibre disks impregnated with *Acaryochloris* cells with a known amount of Chl d (240±5.2 mg Chl d⁻¹ ($n=8$)) were used for the induction curves. Error bars are ±95% confidence limits on 8 separate glass fibre disks per light intensity.

**Table 2** Photosynthetic parameters for *Acaryochloris marina* cells measured under white light and red light calculated from light saturation curves fitted using the Waiting-in-Line model.

<table>
<thead>
<tr>
<th></th>
<th>k (mol⁻¹ m² s¹)</th>
<th>Optimum light (µmol m⁻² s⁻¹)</th>
<th>Photosynthetic efficiency (µmol Chl d⁻¹)</th>
<th>Maximum gross photosynthesis (µmol mg Chl d⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross photosynthesis in white light ($n=3$, 35; $r=0.8123$)</td>
<td>4864±1197</td>
<td>206±51</td>
<td>298 (±107)×10⁻⁶</td>
<td>81.1±21.3</td>
</tr>
<tr>
<td>Gross photosynthesis in red light ($n=11$, 111; $r=0.8052$)</td>
<td>9039±1759</td>
<td>110±22</td>
<td>438 (±112)×10⁻⁶</td>
<td>64±11</td>
</tr>
</tbody>
</table>
(n=14) which is consistent with the x-intercept in Fig. 3. The gross photosynthesis/respiration ratio (P\textsubscript{r}/R) of Acaryochloris under optimum conditions is about 4.02±1.69.

Fig. 5 shows the results of three induction experiments run using standard settings and 65, 136 and 552 μmol m\textsuperscript{-2} s\textsuperscript{-1} (PAR) actinic light. ETR and NPQ (Non Photochemical Quenching) were calculated using the WinControl software. ETR was calculated using the WinControl software as mol m\textsuperscript{-2} s\textsuperscript{-1}. A set of 24 glass fibre filters were impregnated with suspensions of Acaryochloris cells using a glass Millipore filtration apparatus (inside diameter 15.9 mm) and kept moist with seawater in the dark for at least 10 min before being used in an experiment. At each light intensity, a set of 8 runs of an induction curve were run giving means (±95% confidence limits) for ETR and NPQ (Non Photochemical Quenching) at each time. The Chl d content of the remaining 8 glass fibre discs from the same batch of cells was determined in ethanol using the equations of Ritchie[11] and the Chl d of the glass fibre discs used in the induction curve experiments could be calculated on a surface area basis (240±5.2 mg Chl d m\textsuperscript{-2} (n=8)). In this particular experiment 1 mol m\textsuperscript{-2} s\textsuperscript{-1} ETR would therefore be equivalent to an ETR of 15±0.325 mol mg Chl d\textsuperscript{-1} h\textsuperscript{-1} on a chlorophyll d basis. The ETR reaches a peak at the 60 s time interval and afterwards decreases slightly. This indicates that there was little indication of significant photo inhibition over time. The NPQ reached a near maximum after 60 s and thereafter slowly increased during the 5 min course of the experiment. Induction curves run at 552 μmol m\textsuperscript{-2} s\textsuperscript{-1} (PAR) actinic light gave high non-photochemical quenching.

4. Discussion

4.1. Chromatic photoacclimation

On various lines of evidence Acaryochloris is apparently a member of the Cyanobacteria[12,17,18], despite its adoption of Chl d for the majority of its photosynthetic reactions. Acaryochloris also possesses phycobiliproteins, although these are not present as the typical phycobilisomes of other cyanobacteria[4,12,21]. Classically, cyanobacteria show increase of phycobiliproteins under low light conditions and some exhibit marked chromatic adaptation to red and green light[25,26]. The responses to changes in light intensity and light quality found in the present study differ from those found in the classical situations where Chl a is always the primary photosynthetic pigment (cyanobacteria (Chl a only), Chl a/b and Chl a/c containing organisms).

Acaryochloris exhibited photoacclimative shifts of chlorophyll pigment ratios but in an unexpected ways. High white light increased, not decreased, the Chl a/d ratio. Growth at low light intensities, regardless of light quality, led to decreases in the proportional amount of Chl a (Table 1). The PC index was conspicuously higher in cells grown in high white light (Figs. 1 and 2). Chl a in Acaryochloris under our conditions varied, as a proportion of total chlorophyll, from 2 to 3% under low light intensities and to up to about 6% in high white light, a range similar to those noted in other studies[1–3,11]. In other words, the relative amounts of Chl a to Chl d change in a way consistent with the biochemical evidence that Chl d not Chl a is the primary photosynthetic pigment[1–3,5–8,15]. Assuming Chl a to be an accessory pigment in Acaryochloris, it would be expected to increase with low light intensity, as with phycobiliproteins (above) and other accessory pigments[20,25–28]. Chen et al.[2] found the Chl a/d ratio of isolated light-harvesting complexes to be highest in high irradiance treatments and very low Chl a/d ratios have also been reported by Swingley et al.[3] in cultures grown in low light. Low irradiance (white, red or green light) all resulted in a decrease in the Chl a/d ratio but the effect observed in the present study was small. The data presented in Figs. 1, 2 and Table 1 indicate that the major effect of low irradiance is to increase the chlorophyll content on a per cell basis without a substantial change the Chl a/d ratio. The very slow growth rate of Acaryochloris under green light (Table 1) shows that in some cases Acaryochloris cells have to be grown under constant conditions for very long periods (about 1 month) to properly allow enough time for any photoacclimation to occur.

In vivo Chl d absorption, varied from 709 nm to 712 nm in different cultures but were not consistent enough to confirm that there were spectral shifts in the absorption peak in Acaryochloris cells grown under different light conditions (Fig. 2) although Table 1 shows that there were measurable changes in chlorophyll ratios. Shifts in the semi-quantitative phycocyanin (PC) and carotenoid–xanthophyll (CX) indices also reflected some atypical chromatic trends, with the PC and CX indices both being greatest when Acaryochloris was grown in high white light and much lower under low light intensities, regardless of the light quality (Fig. 1). This is a quite different response to that typically found in classical cyanobacteria containing only Chl a and rhodophyte algae possessing phycobiliproteins[25–27]. Only two phycobilin accessory pigments, phycocyanin and allophycocyanin have been identified in Acaryochloris[21]. Many cyanobacteria, cryptophytes and rhodophytes increase their phycoerythrin content when grown under green light[20,25–32], but phycoerythrin is thought to be lacking in Acaryochloris. The response of Acaryochloris to growth under green light in the present study shows no increase in green light absorbing pigments compared to the other photosynthetic pigments (Figs. 1 and 2). Cells grown under red glass would have received no light useable by carotenoids or xanthophylls. This would account for the lowered reflectance of cultures at about 485 nm when grown under red glass.

The pigments considered to be accessory in Acaryochloris, namely Chl a, phycocyanin, carotenes and xanthophylls all seem to decrease relative to Chl d in low light intensities regardless of the spectral quality of the light (Table 1 and Fig. 1) but the effect upon the Chl a/d ratio is very small. The more logical explanation of this is that Acaryochloris produces proportionally more chlorophyll (both Chl d and a) in light-limited conditions, rather than changing the Chl a/d ratio. These results conflict with the notion of the relationship of Chl d to Chl a in Acaryochloris being a dominant/accessory
pigment situation, analogous to that with Chl a/Chl b in algae and land plants [26]. These observations call for broader investigations; in particular, more data on net changes in pigment quantity calculated on a cellular basis are needed to complete the picture of chromatic adjustment [27,30,31]. The observation that _Acaryochloris_ produces more chlorophyll on a per cell basis (but with limited change in the Chl a/d ratio) under low white, red or green light does not preclude that there may be large changes in the pigment composition of the various light-harvesting complexes (LHC) under different spectral growth conditions [32]. Changes in the size of antennae units compared to the reaction centres (PSI and PSII) will need to be examined [33].

The extent to which an alga can shift pigment ratios in response to the quality of incident light must be important in defining its niche [20,25–37]. For example, microalgae growing in the extreme spectral environment beneath Antarctic ice sheets have heightened fucoxanthin/Chl b ratios [34], field collections of the prochlorophyte _Prochlorococcus marina_ have low Chl b in surface waters and high Chl b at depth [35] and marine _Synechococcus_ species adjust their phycobilin content in response to blue light conditions at depth [27]. Kühle et al. [13] cite the apparent high photoacclimation ability of _Acaryochloris_ to be paradoxical to its perceived spectrally-defined narrow niche. However, it is very possible Chl-d organisms are more widespread than currently acknowledged [11,13,16] and may be adapted to a wider range of light climates than so far found. Further investigation of pigment versatility in _Acaryochloris_ may provide information on the likely distribution of this organism, its environmental niche and evolutionary history and provide clues where similar organisms might be found.

4.2. PAM fluorometry

A direct proportionality might be expected between data produced from ETR measurement using PAM fluorometry and photosynthetic oxygen evolution (Figs. 3 and 4). In the present study, oxygen evolution was measured on a Chl d basis for a bulk suspension of _Acaryochloris_ (mol mg Chl d−1 h−1) and ETR was measured as mol m−2 s−1 on the basis of the cross-sectional surface areas of a beam of light reaching the cell suspension. It was not possible to calculate how much chlorophyll was illuminated by the beam of light from the PAM in the experimental configuration used in the present study and so O2-evolution and ETR could not be expressed in the same units. In contrast, in their studies of the flat-sheeted green alga, _Ulva_ spp, Beer et al. [38], Franklin and Badger [39] and Longstaff et al. [40] could calculate both O2-evolution and ETR on a thallus-surface area basis leading to the calculation of ETR/O2 evolution rate as a simple pure number ratio. Nevertheless, photosynthesis measured as mol mg Chl d−1 h−1 and ETR as mol m−2 s−1 in _Acaryochloris_ should be directly proportional to each other. Indeed, this has been shown in the present study. However, the two parameters were not as tightly coupled as one might expect from previous studies such as Beer et al. [38] and Franklin and Badger [39]. Other studies on flat-sheeted eukaryotic algae have found a tight relationship between ETR and O2-evolution under low light but a poor relationship at under high light conditions [38–41] in contrast to the present findings for _Acaryochloris_ (Fig. 4). This indicates that in effect oxygen evolution and electron transport is not a tight 4e−/O2 relationship and that cyclic electron flow probably makes a substantial and variable contribution to photosynthetic electron flow in _Acaryochloris_.

The calculated optimum light intensities, based on light saturation curve fits (Table 2) are rather different with oxygen evolution seeming to saturate at a lower light intensity compared to the PAM-ETR results. This might simply reflect differences in photosynthetic efficiency using blue diode or red diode light sources or the geometry of the experimental set-up used. The light source for O2-evolution was applied horizontally to the stirred cell suspension whereas the PAM light source was inserted vertically into the vortex of the stirred cell suspension.

PAM fluorometers are often difficult to use with cyanobacteria such as _Synechococcus R-2_ (PCC 7942) [42,43]. The light source in the PAM machine and the growth conditions used to grow the cells both seem to be critical. For example, in a review, Campbell et al. [43] extensively discusses results on _Synechococcus_ obtained using PAM machines on cultures grown on 5% CO2. Campbell et al. [43] attributes problems using PAM machines on cyanobacteria to the very low fluorescence often found in cyanobacteria and the finding that phycobilisomes absorb much of the light used for photosynthesis but transfer energy to both PS I and PS II. This explanation does not seem to account for our own experience with _Synechococcus_: we have met with no success using PAM machines equipped with red or blue diode light sources on air grown or high CO2 grown cultures of _Synechococcus_. PAM techniques have been used to follow the effect of rehydration in cyanobacterial crusts of marine beach rock [44] so useful results can be obtained with at least some cyanobacterial populations. Interestingly, PAM fluorometers work quite well on _Prochloron_ both in vivo and in situ [13,45]. _Prochloron_ is an oxyphotobacterium that uses a similar light-harvesting protein to _Acaryochloris_, albeit one that binds Chl a and b instead of Chl d [46].

PAM light saturation and induction curves carried out with _Acaryochloris_ appear to yield results similar to those for most other oxygenic photosynthetic organisms. Thus the use of the PAM fluorometer here has great potential for future investigation of the photosynthetic physiology of _Acaryochloris_. For example, it may aid further enquiry of photoacclimative ability and induced responses to photoinhibition and various other stresses, such as iron-stress [3,9,46,47]. Use of PAM on various eukaryotic macroalgae however has been noted to have some limitations which preliminary evidence suggests may apply to _Acaryochloris_ e.g. imprecision at high irradiances [38–41]. Comparison of light saturation curves obtained using a red diode light source for gross photosynthesis with ETR curves determined using a PAM machine using blue diodes shows that the quantum efficiency of the photosynthetic apparatus is lower using blue than red light (Table 2) indicating...
that the choice of diode is critical for photosynthetic analyses using PAM.

Table 2 compares gross photosynthesis vs. I curves obtained using white light with those obtained using red diode light sources. The saturating light intensity is much higher when assayed under white light regime than when a red light source is used; this result is what one would expect in comparing a broad spectrum light source to a narrower bandwidth red diode light source. This is also what would be expected from the reflectance measurements made using the Taylor-sphere (Fig. 2). Determining the limitations of PAM technology is critical when examining organisms with unusual photopigment signatures but incorporating PAM procedures into Acaryochloris studies will open new avenues of photosynthetic research on this unique phototroph.

The fluorescence induction curves at low, medium and high light intensity (Fig. 5) indicate that the rate of photosynthesis is extremely efficient at low light intensities. This is in line with previous in situ findings [13] and demonstrates that cultures of Acaryochloris have similar properties to the in situ organism. The drift downwards of ETR over a period of several minutes is clearly associated with a non-photochemical mechanism, as shown by the curves for non-photochemical quenching (NPQ).

As expected, here, the highest NPQ was observed at the highest light intensity. Although Prochloron didemni has been documented to have substantial NPQ at moderate light intensities [45]. Because so very little is known about the mechanism of non-photochemical quenching in cyanobacteria, especially the prochlorophytes, and especially in prochlorophytes and Acaryochloris, more investigations are warranted.

Acknowledgements

Many thanks to Cecilia Phu for participating in the cultivating and light adaptation experiments as part of an Advanced Student Project in the School of Biological Sciences, The University of Sydney. We thank the School of Biological Sciences for partially funding Rosalyn Gloag’s participation in this project as part of the Advanced Student Scheme at The University of Sydney. Dr. Min Chen holds an Australian Postdoctoral Research Fellow supported by the ARC (Australian Research Council).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2006.11.014.

References


