



RESEARCH ARTICLE

UPDATE Reactive oxygen production induced by near-infrared radiation in three strains of the Chl *d*-containing cyanobacterium *Acaryochloris marina* [version 2; peer review: 2 approved, 1 approved with reservations]

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Abstract

Cyanobacteria in the genus *Acaryochloris* have largely exchanged Chl *a* with Chl *d*, enabling them to harvest near-infrared-radiation (NIR) for oxygenic photosynthesis, a biochemical pathway prone to generate reactive oxygen species (ROS). In this study, ROS production under different light conditions was quantified in three *Acaryochloris* strains (MBIC11017, HICR111A and the novel strain CRS) using a real-time ethylene detector in conjunction with addition of 2-keto-4-thiomethylbutyric acid, a substrate that is converted to ethylene when reacting with certain types of ROS. In all strains, NIR was found to generate less ROS than visible light (VIS). More ROS was generated if strains MBIC11017 and HICR111A were adapted to NIR and then exposed to VIS, while strain CRS demonstrated the opposite behavior. This is the very first study of ROS generation and suggests that *Acaryochloris* can avoid a considerable amount of light-induced stress by using NIR instead of VIS for its photosynthesis, adding further evolutionary arguments to their widespread appearance.

Open Peer Review

Reviewer Status

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	1	2	3
UPDATE			
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Any reports and responses or comments on the article can be found at the end of the article.

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UPDATED Changes from Version 1

The manuscript has been edited with the help of the reviewer's comments and should now address the most critical points. We have augmented parts of the discussion section, which now provides additional information concerning the potential role of carotenoids in the quenching of ROS. A sentence was added to clarify which radicals are detected with the KMBA assay. Additionally, we have tried to tone down our claims of the significance of this study and acknowledged the fact that some measurements could be uncertain due to lack of replication or biological variation. All minor points given by the three referees were addressed. We hope that the changes made are in agreement with the reviewers comments and improve the manuscript.

See referee reports

Introduction

Most oxyphototrophs use visible light (VIS, 400–700 nm) for chlorophyll (Chl) *a*-driven photosynthesis, while cyanobacteria in the genus *Acaryochloris* largely employ Chl *d*, thereby enabling them to use near-infrared radiation (NIR, >700 nm) for oxygenic photosynthesis^{1,2}. Two of the strains are well described in their growth and photopigment composition: The type strain *Acaryochloris marina* MBIC11017 was isolated from the didemnid ascidian *Lissoclinum patella* from coral reefs habitats in Palau^{2,3} and was later genome sequenced⁴. The other strain, HICR111A, originates from swipes of coral substrate collected on Heron Island, Australia and was also genome sequenced⁵. Since its first discovery, other *Acaryochloris* strains have been obtained from Japanese macroalgae (strain Awaji⁶), from surfaces in a hypertrophic lake in the US (strain CCMEE5410⁷), and most recently from Australian mangroves⁸ (strain MPGRS1) and stromatolites in Shark Bay, Western Australia⁹ (ssball1).

The light microenvironment in natural habitats occupied by *Acaryochloris* spp. has a high contribution of NIR relative to visible light^{10–12} and such habitats appear to create similar niche differentiation with bacteria carrying specialized photopigments such as Chl *dlf* or bacteriochlorophylls^{10,13}. The notion of a global distribution of Chl *d* and cyanobacteria in the genus *Acaryochloris*^{11,14} further reinforces the need to obtain information on the photobiology of Chl *d*-containing oxyphototrophs. Understanding the adaptive mechanisms in oxyphototrophs capable of using wavelengths beyond VIS is of interest as it provides information concerning the usability, stress levels and limitations associated with NIR-driven oxygenic photosynthesis.

Of all biological pathways, photosynthetic electron transport is particularly prone to produce reactive oxygen species (ROS) due to the very high (positive) redox potential of the primary donor of photosystem II, needed to oxidize water, and the low redox potential of the primary electron acceptor of photosystem I, needed to reduce ferredoxin; here, singlet oxygen (¹O₂), is produced by PSII and superoxide anions (O₂⁻/hydrogen peroxide (H₂O₂)) in the Mehler, ascorbate peroxidase (MAP) pathway of PSI^{15–17}. ROS encompasses the production of singlet oxygen, superoxide anions, hydrogen peroxide and hydroxyl radicals (OH[•]), all of which are derived through the local energization of O₂. If not properly quenched by protective mechanisms, ROS can damage proteins,

DNA and other cellular macromolecules, and this damage can ultimately lead to cell death. Known quenching mechanisms encompass enzymes such as superoxide dismutase and catalase or non-enzymatic antioxidants like glutathione, carotenoids and α-tocopherol (vitamin E)¹⁸. In plants, ROS and in particular ¹O₂ production has been shown to occur at photosystem II upon illumination with visible light^{19,20}. In cyanobacteria, shorter wavelengths such as ultraviolet radiation (UVR, <400 nm), are known to induce ROS, causing DNA damage, lipid-peroxidation and overall decreased photosynthetic efficiency^{21,22}. To our knowledge no study has investigated the effect of NIR on ROS production in cyanobacteria.

Relative levels of ROS can be estimated through measurements of e.g. gene expression²³, ROS sensitive fluorescence probes²⁴ and enzyme activity²⁵. These methods provide integrated values of ROS production over incubation time intervals ranging from minutes to hours. In this study we used a fast and sensitive laser photo-acoustic gas detector²⁶ that can measure the ethylene produced from the reaction of certain types of ROS with the substrate 2-keto-4-thiomethylbutyric acid (KMBA), previously added to the samples. Such near real-time ROS detection is valuable in determining the immediate effect of treatments on the physiological state and stress level within living organisms. KMBA is thought to diffuse into intact cells²⁷ and, when supplied at saturating concentrations outcompetes other radical scavenging mechanisms. In the KMBA assay, the butyric acid moiety is known to react with the ROS peroxynitrite, hydroxyl radicals and peroxy radicals²⁸, resulting in the formation of ethylene, which can then be quantified. Whether singlet oxygen and superoxide anions react with KMBA and subsequently form ethylene has not been investigated yet. In other studies, KMBA has been used to test the antioxidant capacity of radical scavengers via their ability to inhibit ethylene formation relative to a control reaction (total oxyradical scavenging capacity, TOSC)^{28,29}.

In this study, we report the effect of light intensity and spectral composition on ROS generation, as measured in real-time using a laser-photoacoustic gas detector in three different strains of NIR utilizing cyanobacteria belonging to the genus *Acaryochloris*, including a new strain (named *Acaryochloris* CRS), isolated from phototrophic biofilms growing on dead coral branches collected on Heron Island, Australia.

Results and discussion

We aimed to determine the stress levels associated with Chl *d*-driven oxygenic photosynthesis and tested NIR, VIS and more narrow wavebands for their capacity to induce ROS in three strains of *Acaryochloris*: i) The *Acaryochloris marina* type strain MBIC11017^{2,3}, ii) strain HICR111A⁵ and, iii) a novel strain, named CRS, isolated from dead coral branches which, based on *16S rRNA* gene sequencing, grouped within the genus *Acaryochloris* (Figure 1). *Acaryochloris* strains MBIC11017 and HICR111A are both well described in terms of their photopigmentation, genomic content, ultrastructure and their capability to perform photoacclimation^{4,5,30}. To test whether photoacclimation, i.e., light-dependent change in pigment levels, was associated with increased resistance or sensitivity towards ROS, we acclimated the strains to NIR or VIS prior to experiments.

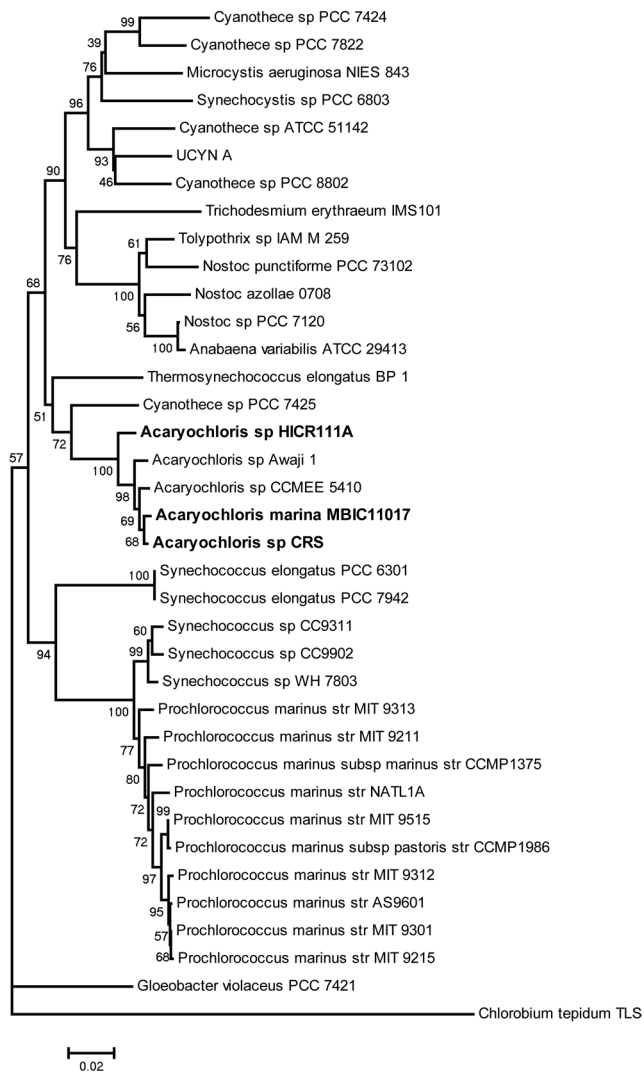


Figure 1. Phylogenetic tree indicating the position of the novel *Acaryochloris* strain CRS based on full-length 16S rRNA gene sequences. Sequences from other cyanobacteria (35 in total) were obtained from the SILVA database while CRS-specific sequences were obtained through PCR amplification and subsequent sequencing. Phylogeny was calculated using Neighbor-joining methods and Jukes-Cantor substitution models as implemented in MEGA5. Tree stability was assessed using bootstrapping at 10000 replications. Only bootstrap values >50% are displayed within the tree. The scale represents 0.02 substitutions per nucleotide position. The green-sulphur bacterium *Chlorobium tepidum* TLS was chosen as an outgroup.

By taking advantage of the real-time ROS detection method, we could for the first time demonstrate the immediate effects of NIR, VIS and other wavelengths on the generation of certain ROS within living cyanobacteria. Specifically, we found that:

(i) Depending on strain and previous adaptation, ROS levels were lower in cells exposed to NIR than in those exposed to VIS (Figure 2A);

(ii) Exposure to shorter wavelengths such as blue and cyan, generated the most ROS in strain MBIC11017 and HICR111A, while less ROS was produced upon exposure to longer wavelengths (green, amber and red) (Figure 2C).

(iii) Strain CRS generated less ROS upon exposure to VIS when previously acclimated to NIR, while strain MBIC11017 and HICR111A appear more sensitive to VIS when adapted to NIR (Figure 2A).

In *Acaryochloris*, VIS irradiance is primarily absorbed by the photopigments Chl *d* (with maximum absorption occurring at 440–470/710 nm), Chl *a* (440–470/675 nm), carotenoids (440–520 nm) and if present, phycobiliproteins (560–650 nm). NIR provides a more targeted stimulation of photosynthesis and is almost exclusively absorbed by Chl *d*. At comparable photon irradiances (VIS = 340–480 $\mu\text{mol m}^{-2} \text{s}^{-1}$ versus NIR = 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$), we found that, depending on strain and previous adaptation, ROS levels were lower in cells exposed to NIR than in those exposed to VIS (Figure 2A). Based on pulse-amplitude modulated (PAM) fluorometry measurements the light intensities used in our experiments are known to saturate relative-electron transport rates in the type strain MBIC11017¹². Photoinhibition is not observed even at higher, photon irradiance, but we hypothesize that prolonged exposure to relatively high-irradiance (10–20 fold more irradiance than during culturing for 15–20 min) could result in the over-reduction of the primary acceptors on the PSI and PSII side resulting in the production of ROS^{15,23}. This over-reduction could lead to the transfer of electrons to O₂, the subsequent generation of superoxide radical, followed by their conversion to hydrogen peroxide as well as hydroxyl radicals^{23,31}. Alternatively, ROS generation could occur via photosensitized light-harvesting pigments³²; however, in intact light-harvesting complexes the efficiency of electron transfer towards the reaction centers is usually outcompeting the formation of long-lived (ROS-forming) chlorophyll triplet states³³. This appears particularly true for the unique phycobiliprotein antenna rods in *A. marina* MBIC11017, in which excitation electron transfers to PSII were found to be significantly faster than in Chl *a*-containing cyanobacteria³⁴. Additionally, it is known that within light-harvesting complexes carotenoids are outcompeting O₂ in the de-excitation of triplet chlorophyll states³⁵. All three strains used in the current study were found to contain zeaxanthin (Table 1) as well as α -carotene, these carotenoids will bring about rapid quenching of excited Chl states and if necessary can also quench singlet oxygen and aid in general non-photochemical quenching^{33,36}.

Exposure to shorter wavelengths, such as blue (470 nm) and cyan (495 nm) light generated the most ROS in strain MBIC11017 and HICR111A, while less ROS was produced upon exposure to longer wavelengths (green, amber and red) (Figure 2C). Blue and cyan light-induced ROS production in MBIC11017 and HICR111A is probably due to spectral overlap with the Soret-band absorption of

ROS-induced ethylene production in the three *Acaryochloris* sp. strains MBIC11017, HICR111A and the novel strain CRS.

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.155780>

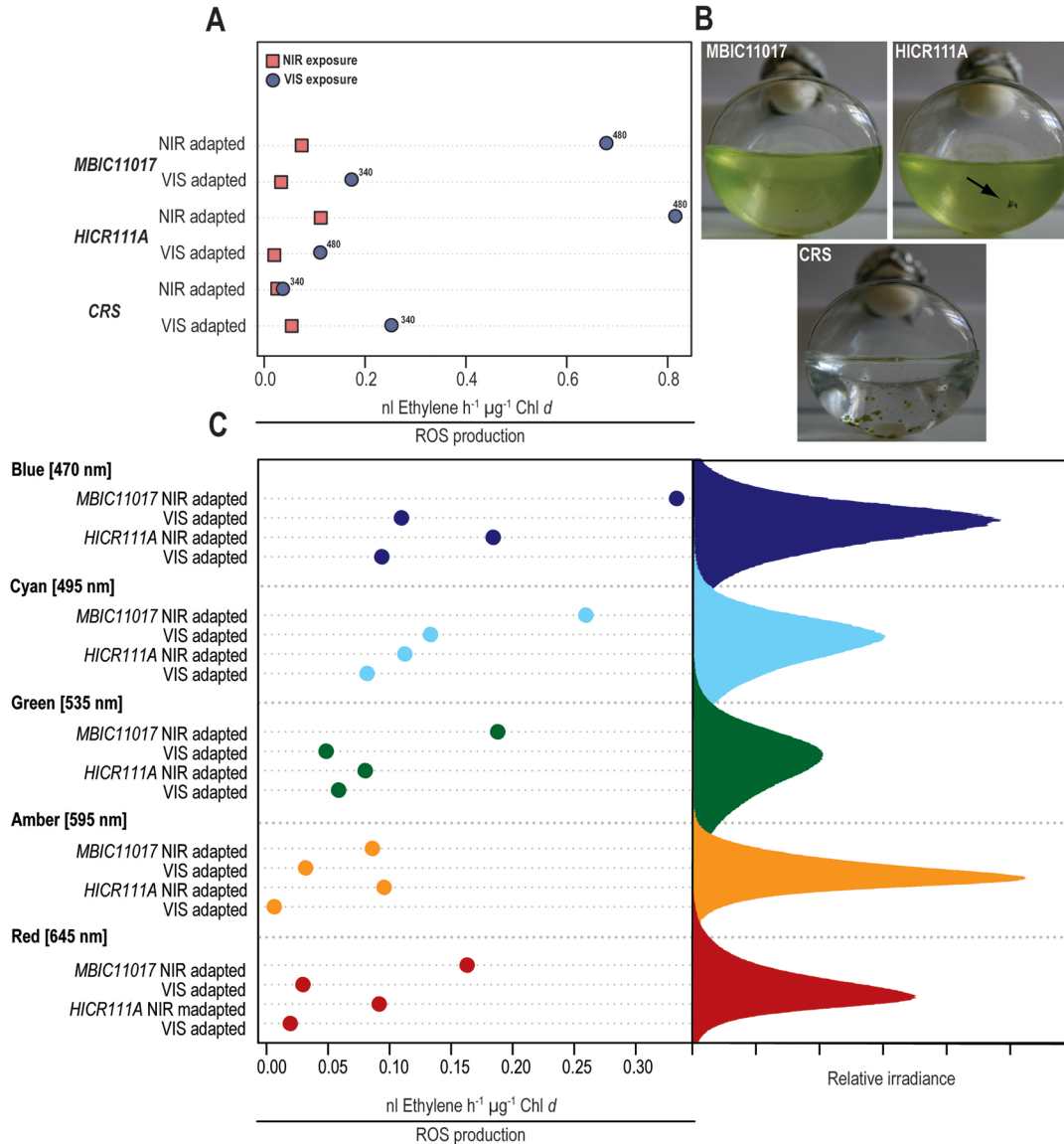


Figure 2. ROS induced ethylene production in the three *Acaryochloris* sp. strains MBIC11017, HICR111A and the novel strain CRS. All values were normalized to Chl *d* concentration as determined by spectrophotometry. All cultures were grown under either near-infrared radiation (NIR, 720 nm) or visible light (VIS, 400–700 nm) before subsequent light exposure. **(A)** Cleveland dot-plot of ROS levels measured during exposure of VIS or NIR adapted *Acaryochloris* cells to either VIS (blue dots, 340–480 μmol photons m⁻² s⁻¹, as denoted on the graph) or NIR (red squares, 400 μmol photons m⁻² s⁻¹). **(B)** Action spectrum of ROS-induced ethylene production in VIS or NIR adapted strains MBIC11017 and HICR111A. Due to lack of sufficient culture material, action spectra were not determined for *Acaryochloris* strain CRS. Peak emissions of the monochromatic LEDs used for illumination were: red (645 nm), amber (595 nm), green (535 nm), cyan (495 nm) and blue (470 nm). The irradiance in this experiment was adjusted to 300 μmol photons m⁻² s⁻¹. **(C)** Growth forms of the different *Acaryochloris* strains MBIC11017, HICR111A and CRS. All strains displayed were grown under NIR and are approximately one week old. Please note the natural formation of biofilms in *Acaryochloris* strain HICR111A (arrow) and CRS.

Chl *ald* (440–470 nm) (Figure 2B) and the above-mentioned mechanisms in ROS generation. Red (645 nm), amber (595 nm) and green (535 nm) light overlaps with the absorption spectra of phycobiliproteins which, if present, aid in light harvesting and excitation energy transfer towards the photosystems³⁴. Strain MBIC11017 is known to express the phycobiliproteins phycocyanin and allophycocyanin³⁷, while strain HICR111A reportedly lacks phycobiliproteins⁵.

Comparable ROS levels were observed in strain HICR111A and MBIC11017 under yellow and green light, suggesting the presence of pigments absorbing these wavelengths or the possibility of other light-induced ROS production mechanisms. Spectrophotometric analysis of the strains showed weak absorption in the phycobiliprotein-specific region within all three strains (Figure 3). This would corroborate the excitation energy transfer to PSI and II

Table 1. Photopigment content of the three *Acaryochloris* strains MBIC11017, HICR111A and CRS. The strains were adapted to either visible light (VIS) or far-red light (NIR). Photopigments were identified manually from HPLC chromatograms and ratios calculated based on the derived peak areas. Average values and standard error from the mean from two independent growth experiments are displayed.

Pigment ratio	<i>Acaryochloris</i> strain					
	MBIC11017		HICR111A		CRS	
	NIR adapted	VIS adapted	NIR adapted	VIS adapted	NIR adapted	VIS adapted
Chl <i>a/d</i>	0.035 ± 0.002	0.032 ± 0.005	0.047 ± 0.007	0.034 ± 0.001	0.055 ± 0.022	0.069 ± 0.039
Chl <i>d/zeaxanthin</i>	2.229 ± 0.845	3.169 ± 0.224	2.422 ± 0.691	3.438 ± 0.037	2.209 ± 0.123	NA
Chl <i>d/α-carotene</i>	1.694 ± 0.304	2.248 ± 0.394	1.907 ± 0.384	2.262 ± 0.065	2.484 ± 0.387	2.750 ± 0.333
Zeaxanthin/ <i>α-carotene</i>	0.827 ± 0.177	0.704 ± 0.075	0.808 ± 0.072	0.658 ± 0.026	1.138 ± 0.238	0.507 ± 0.341

in strain HICR111A and could explain the observed ROS production under yellow and green light. However this would also refute previous reports on the absence of phycobiliproteins in this strain⁵. Given that phycobiliproteins were not purposely extracted, and further analyzed, in the present study, we can at this point only speculate about their presence and relative expression under different growth conditions.

There is a long history of associating pigment compositions within phototrophs with the spectral composition of ambient light and exposure history: for recent work see³⁸ and²³, respectively. These two factors are likely to determine the sensitivity of phototrophs to irradiance and their capability to cope with ROS levels generated upon irradiation. Interestingly, we found that strain CRS generated very little ROS upon exposure to VIS when previously acclimated to NIR, while strain MBIC11017 and HICR111A appear to be more sensitive to VIS when previously adapted to NIR (Figure 2A). HPLC analysis revealed a higher ratio of zeaxanthin/*α-carotene* in NIR-acclimated CRS cells than in the other two strains (Table 1), albeit with a relatively large standard error. In *A. marina* MBIC11017 *α-carotene* was found to be an integral part of both photosystem reaction center cores^{39,40} and zeaxanthin is predominantly found in the periphery of light-harvesting complexes³⁶.

Based on the higher zeaxanthin/*α-carotene* ratios in NIR-adapted cells of all three strains we hypothesize that there are slightly more antenna complexes (zeaxanthin and Chl *d*) in NIR-adapted cells than in those pre-adapted to VIS, thus providing more potential for ROS production/quenching. Given that the antenna complexes are predominantly composed of Chl *d* and thus absorb in the NIR part of the light spectrum, this chromatic photoacclimation is expected and further corroborated by higher Chl *d/zeaxanthin* ratios in NIR-adapted strains in this study. We hypothesize that the higher contribution of zeaxanthin in the NIR-adapted strain CRS could aid in effectively capturing NIR but potentially also in quenching ROS produced during illumination with either VIS or NIR. However, we acknowledge that this hypothesis is based on HPLC analysis of pigment levels with a relatively high variability and one individual ROS measurement.

Besides their light harvesting capability carotenoids (including zeaxanthin) are also known for their antioxidative abilities^{15,35}. It is known

that zeaxanthin mostly operates outside of the reaction centers (RC) and is predominantly found within the peripheral light-harvesting complexes³⁵. Here, zeaxanthin was shown to play a crucial role in non-photochemical quenching and energy dissipation from sensitized chlorophyll molecules or singlet oxygen³⁵. Singlet-oxygen formation is often inevitable and is believed to necessitate the rapid turnover of the photosystem II-D1 polypeptide⁴¹. It was found that the PSII-D2 protein acquires a certain photoprotection against singlet-oxygen by close association with *β-carotene* molecules in the RC of PSII^{42,43} and hence has a lower turnover than the D1 protein. We thus hypothesize that the presence of *α-carotene* in the reaction centers of strain MBIC11017^{40,44} could provide photoprotection in certain strains of *A. marina*. Whether carotenoids are directly involved in ROS quenching remains unknown and thus we can only speculate about the exact levels of ROS being produced at PSI/PSII and within light-harvesting complexes of *A. marina*. Nonetheless, our measurements indicate lower levels of ROS during exposure to NIR when compared to VIS; whether this is related to the replacement of Chl *a* with Chl *d* in *A. marina* remains to be investigated.

Lastly, it is possible that strain specific differences in ROS production are associated with dissimilarities in growth forms: Strain HICR111A forms cell aggregates⁵, and so does strain CRS (Figure 2B), whereas strain MBIC11017 usually grows as dispersed cells⁵ but can be immobilized into biofilms⁴⁵. The formation of aggregates in strain HICR111A and CRS might provide photoprotection through self-shading, a behavior reportedly less pronounced in strain MBIC11017⁵. Both HICR111A and CRS originate from shallow reef flats, a high irradiance habitat. In contrast, strain MBIC11017 was isolated from a didemnid ascidian², a light environment depleted of VIS but with sufficient NIR¹⁰⁻¹².

Based on these first, preliminary, measurements, we suggest that through utilization of NIR, *Acaryochloris* can avoid a considerable amount of light stress, while harvesting a portion of the electromagnetic radiation spectrum not used by other oxyphototrophs. Additionally, aggregation of certain strains could protect against excess amounts of ROS generated during high irradiance exposure. Overall, this could add further arguments as to why *Acaryochloris* is a successful and apparently globally widespread oxy phototroph¹¹.

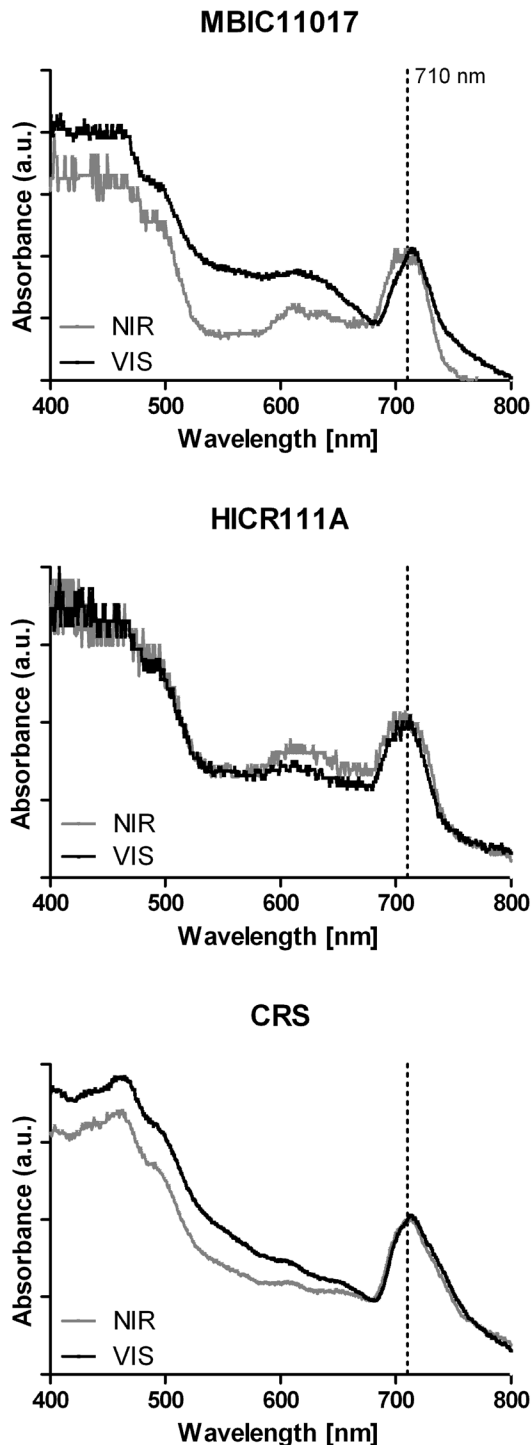


Figure 3. *In vivo* absorption spectra of the three *Acaryochloris* strains MBIC11017, HICR111A and CRS. All strains were adapted to either visible light (VIS) or near infrared radiation (NIR) prior to measurements. All spectra were normalized to the maximal absorbance of Chl *d* at 710 nm.

Materials and methods

Isolation of the novel *Acaryochloris* strain CRS

Dead coral branches with patches of faint yellow-greenish pigmentation were collected during low tide from coral patches on the inner reef flat off Heron Island, Queensland, Australia (see more details on the sampling site in¹⁰). The samples were transported back to the lab in a container with seawater and immediately placed into outdoor aquaria that were continuously flushed with aerated ambient seawater pumped in from the reef flat. Bacterial cells found on the dead coral branch were removed using a sterile scalpel and immediately placed into KESM media and kept under dim visible light for three days. After transportation to Sydney the cells were kept in KESM media under NIR LEDs (centered at 720 nm, Cat. No. L720-04AU, Epitex Inc., Japan). NIR irradiance was set to $\sim 5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ using a SKP200 light meter equipped with a SKP216ER irradiance sensor with a 550 to 750 nm light sensitivity range (Skye Instruments, United Kingdom). After three weeks, the growing cells were diluted into aliquots of fresh KESM medium. After additional incubation, the pigmentation of the cells was inspected by measuring their absorption characteristics using a spectrophotometer (UV-2550, Shimadzu, Japan). The cells were hereafter maintained in KESM media under NIR.

Acaryochloris growth conditions

Acaryochloris strains MBIC110771, HICR111A and the newly isolated *Acaryochloris* strain CRS were grown in 200 ml cell culture flasks in marine KESM media (salinity of 30) in a shaking incubator at 28°C as previously reported¹. All cultures were shaken at 100 rpm under a 12/12 h light-dark shift. Near infrared radiation (NIR) was provided by narrow band LEDs (L720-04AU, 700–740 nm, centered at 720 nm, Epitex Inc., Japan) at an irradiance of 20–40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Another set of cultures was grown under the same irradiance but using visible light delivered by a halogen lamp equipped with a heat filter (HQ Power, Brinck Elektronik, Denmark). Absolute irradiance measurements of NIR and visible light were done with a calibrated spectroradiometer (Jaz ULM-200, Ocean Optics, Dunedin, FL, USA).

DNA extraction and PCR amplification

Six ml of dense cell culture was spun down and then extracted using the FastDNA for Soil kit (MP Biomedicals, France) using the manufacturers standard protocol. The resulting DNA was quantified using the Qubit system (Invitrogen, Life Technologies Europe, USA) and diluted 1:10 using molecular grade water. The *16S rRNA* gene was amplified using the primers 16SCYfw (5'-GGCTCAGGATGAACGCTGGCGG-3') and 16SCYrv (5'-ACCTTGTTACGACTTCACCCAGTC-3') using the PCR Master (Roche, Switzerland) with 30 amplification cycles. The resulting PCR product was purified on an agarose gel and the band excised using a sterile scalpel. DNA was extracted from the excised gel using the QiaexII gel extraction kit (Qiagen Nordic, Sweden) and then cloned into the pCR4-TOPO cloning vector (Invitrogen, Life Technologies Europe, USA) and transformed into One-Shot TOP-10 chemically competent cells (Invitrogen, Life Technologies Europe, USA). Clones were subsequently grown in LB-medium, plasmids were extracted using the Qiaprep kit (Qiagen Nordic, Sweden), and checked for correct

sized inserts using gel electrophoresis. Three clones were sent off for subsequent sequencing by a commercial provider (Macrogen, Seoul, Korea).

Phylogenetic analysis

Cyanobacterial *16S rRNA* gene sequences were retrieved from the SILVA database (<http://www.arb-silva.de/>) and aligned together with sequences retrieved from clones using MUSCLE as implemented in the Molecular Genetic Analysis (MEGA) software package version 5.0. Neighbor-joining (NJ) was used to infer phylogenetic relationships among sequences; support values with Jukes-Cantor distances and 10000 bootstrap replicates are displayed next to branches displayed in the phylogenetic tree (Figure 1).

Chlorophyll extraction and spectrophotometry

Two ml of each culture were pelleted by centrifugation at $8000 \times g$. The supernatant was removed, while the resulting pellet was re-suspended in 96% ethanol and incubated at 4°C for 60 min in darkness. During the ethanol extraction, the samples were vortexed at maximal speed every 15 minutes. After one hour, the cells were pelleted by centrifugation at $8000 \times g$ and the supernatant was used to determine Chl *d* concentrations via spectrophotometry (UV-2101PC, Shimadzu, Japan) according to Ritchie⁴⁶. The same spectrophotometer was used to measure the *in vivo* absorbance spectra of the different cultures. *Acaryochloris* strains HICR111A and CRS proved very difficult to keep in suspension and were therefore sonicated (Misonix sonicator 4000, Qsonica LLC., Newtown, CT, USA) for one minute at maximum speed prior to spectrophotometric measurements. To prevent bleaching of the photopigments, all handling was done as quickly as possible and under low-light conditions.

HPLC-based pigment analysis

For HPLC analysis, 2 ml of *Acaryochloris* cultures were spun down at maximum speed (~13,000 rpm) in a bench centrifuge, the supernatant was removed and the remaining pellet resuspended in cold acetone-methanol (7:2 by vol) and the cells sonicated for 20s using a Misonix sonicator 4000 (Qsonica LLC., Newtown, CT, USA) according to⁴⁷. The cells were incubated for 2 min on ice in complete darkness, centrifuged again and the extract filtered through a Minisart 0.2- μ m pore-size filter (Sartorius, Germany). Ammonium acetate (15 μ l; 1.0M) was added to the extracts (150 μ l) to further improve pigment resolution before subsequent injection. Pigment separation was performed on an Agilent 1260 infinity HPLC machine (Agilent Technologies, Santa Clara, CA, USA) and a Nova-pak C18 column (dimensions: 3.9 \times 300 mm). A 1260 Infinity Multiple Wavelength Detector (Agilent Technologies, Santa Clara, CA, USA) was used for the detection of compound specific absorption wavelengths. *Acaryochloris* culture extracts were run with solvent A (methanol:acetonitrile:water, 42:33:25 by vol) and solvent B (methanol:acetonitrile:ethyl acetate, 39:31:30 by vol) in a gradient comprised of 40% solvent B at time of injection, a linear increase to 100% B at 60 min and back to 40% B in 3 minutes. Flow rate was kept constant at 1 ml min⁻¹ and the column at a temperature of 30°C. Photopigments were identified manually from HPLC chromatograms and ratios calculated based on the derived peak areas. Average values and standard error from the mean from two independent growth experiments were calculated and are displayed in Table 1.

ROS measurements via real-time ethylene detection

Real-time ethylene production was measured using a laser-based photo-acoustic ethylene detector (ETD-300, Sensor Sense, the Netherlands) combined with an in-line gas-flow through system (Valve Controller VC 6, Sensor Sense, the Netherlands). The system was described in reference²⁶. Custom made gas-tight incubation chambers were connected via the valve controller to the ETD, which could sequentially sample ethylene fluxes from the different incubators. The incubator was made of anodized aluminum (51ST quality) and contained a cooling/heating channel to control temperature and a glass window to supply light to the samples (see details in⁴⁸). The incubator could hold 2 ml aliquots of *Acaryochloris* culture. Culture samples were augmented with α -keto- γ -(methylthio)butyric acid sodium salt (KMBA) (<97% purity, K6000, Sigma-Aldrich) made to a final concentration of 2.8 mM in KESM media; earlier experiments on cyanobacterial cultures demonstrated saturating levels of KMBA when supplied at this concentration (Staal *et al.*, unpublished). After addition of KMBA, the cultures were mixed using a pipette to obtain a uniform distribution of the chemical. All measured ethylene concentrations were normalized to the Chl *d* concentration in the samples.

To ensure steady state ethylene fluxes at the moment a sample was connected to the ETD, we supplied a continuous flow of moisturized air (2 l h⁻¹) over every individual incubator during the experiments. The air was moisturized by flushing it through gas tight vials filled with de-ionized water; this was necessary to prevent evaporation of media in the incubator. The system was continuously controlled for gas leaks, by automated comparison of the incoming and outgoing gas flow. The outlet of the incubator was connected to a CO₂ trap (KOH pellets) and water scrubber (CaCl₂) placed before the ethylene detector. The valve controller allowed each measuring chamber to be alternately connected for 20 minutes to the ethylene detector. Steady state ETD readings from the cultures were obtained within ~4 minutes after connection to the ETD. The ETD-300 has a sample frequency of ~12 samples min⁻¹ and the concentrations of the last two minutes per treatment were averaged. Typical standard deviations were 0.15 ppbv for ethylene measurements under steady state conditions. The averaged concentrations were normalized to the amount of Chl *d* present in the cultures to correct for differences in biomass between samples.

Light experiments

Light dependent ROS production was measured using both visible light (400–700 nm) and near-infrared radiation (NIR). For visible wavelengths, we used an incubator setup (Mini-Incubator, Sensor Sense, Nijmegen, The Netherlands), fitted with an array of 11 1W cool white LEDs (Luxeon Star, 1W, Lumileds, USA) connected to a PC-driven controller. Irradiance levels were set between 340–480 μ mol photons m⁻² s⁻¹ for visible light. Different irradiance levels were adjusted by varying the electrical current of the LED array via a special software routine (Sensor Sense, Nijmegen, The Netherlands) and measuring the downwelling irradiance with a calibrated light meter (LI250, LiCOR Biosciences, Lincoln, USA).

For NIR exposure, the actinic light was provided by four collimated NIR LEDs (M3L1-720-30, 700–740 nm, centered at 720 nm,

Roithner Lasertechnik, Vienna, Austria) at an intensity of 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The absolute NIR irradiance level was measured with an irradiance sensor attached to a calibrated spectroradiometer (Jaz ULM-200, Ocean Optics, USA). Subsequently, the measured irradiance spectrum was integrated over a spectral range of 650–800 nm. All samples were incubated at 28°C using a cooler/heater bath (HD-25, Julabo, Germany).

Action spectra of ROS production were measured using three high-power LEDs (Luxeon star, 1W, Lumileds, USA) mounted onto an aluminum plate for efficient cooling. The following colors and centered wavelengths were used in the action spectra: Blue (470 nm, 25 nm spectral half-width, LXHL-MB1D), Cyan (495 nm, 30 nm spectral half-width, LXHL-ME1D), Green (535 nm, 35 nm spectral half-width, LXHL-MM1D), Amber (595 nm, 14 nm spectral half-width, LXHL-ML1D), Red (645 nm, 20 nm spectral half-width, LXHL-MD1D). The LED's were powered by LED power supplies (LED31, Velleman, Belgium). For each LED color, the incident irradiance was adjusted to 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ by adjusting the distance from the LEDs to the incubation chamber window. Photon irradiances were measured with a calibrated light meter (LI250, LiCOR Biosciences, Lincoln USA) before each measurement.

To the best of our knowledge we are the first to provide data on ROS levels associated with NIR-driven oxygenic photosynthesis. Unfortunately, due to measuring time constraints at the trace gas facility we were unable to provide replicated measurements of ROS levels under various light regimes. This emphasizes the need to perform additional measurements of NIR induced ROS production in *A. marina* and other far-red utilizing oxyphototrophs.

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Author contributions

LB, MK and AWDL conceived the study. MK, LB and MK designed the experiments. LB, MK and SMC carried out the research. FJMH and SMC contributed to the design of experiments and provided access to the trace-gas facility. AWDL and MS isolated the novel *A. marina* strain CRS. LB, AWDL and MK prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

There are no competing interests.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Helen Yap 

The Marine Science Institute, University of the Philippines, Quezon City, Philippines

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 17 January 2014

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Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

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Alison Telfer

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This paper shows experiments on several cyanobacterial strains of the *Acaryochloris* genus, in which chlorophyll *a* is replaced by Chl *d* which absorbs in the near infra red (NIR) - 25-30 nm to the red of Chl *a*. It shows that under stress levels of NIR excitation (and also adaptation to growth under NIR) less reactive oxygen species (ROS) are formed. The authors conclude that this is a strategy of this genus (more species of which are being discovered widely around the planet) to protect against photodamage during high irradiance exposure - a more and more likely occurrence these days.

The authors stress the point (page 7, left, para 3) that this is a preliminary study in which fully replicated measurements of ROS were not possible. I understand this constraint and accept that reporting measurements of ROS, using the novel technique of real time ethylene detection, from *Acaryochloris* is very interesting but I feel the paper goes too far in its claims. It also is unclear about the different types of ROS detected and the mechanism by which protection is provided by carotenoids.3, right para 2.

- The ROS detection method (real time ethylene detection RTED) described in ref 28 (Regoli and Winston) appears to only directly detect very strong oxidants hydroxyl and peroxy radicals and peroxy nitrite. It does not directly detect superoxide or singlet oxygen (1O₂). This should be made clearer in the text and there should be discussion about production of hydroxyl radicals.
- Relating to point 1.: The correlation between light-induced increase in relative amounts of the zeaxanthin containing antenna complexes and the ROS levels detected by the RTED system should be explained in more detail. Carotenoids in photosynthetic complexes mainly operate by quenching chlorophyll triplet states before they can form 1O₂ or they directly quench any 1O₂ that is formed. They do also quench oxygen radicals but these are more likely to be produced in the aqueous phase (e.g. from reduced ferredoxin in PSI) where they are usually quenched by antioxidant enzymes such as ascorbate peroxidase etc. Though it is possible alpha-Car in the PSII reaction center could quench radicals produced from reduced quinone. When the ROS levels, detected by RTED, increase it is likely that other ROS (e.g. superoxide) and perhaps 1O₂ also increase and so carotenoids would be helpful. However, the text makes it sound as if in CRS the zeaxanthin level increases relative to chlorophyll when it is simply more antenna (Zea plus Chl *d*) being produced and so there is more potential for 1O₂ production.
- Essentially I feel the text, though cautious, claims too much. The errors on the pigment levels especially for CRS (they are huge) suggest the very different values for NIR and VIS adapted cells could be a fluke. Also the single point for CRS in Fig. 2A NIR adapted under VIS exposure could be a fluke.

Minor Points

p. 4 end para beginning 'In *Acaryochloris*....': Change last sentence round so it says: zeaxanthin will bring about rapid quenching of excited chlorophyll states and if necessary can also quench singlet oxygen - or something similar to this.

p.6 end para 3: Rephrase: ...and zeaxanthin is restricted to the peripheral light-harvesting complexes

(PCB proteins).

p.6 Second sentence from bottom: I do not like the sentence emphasising that zeaxanthin quenches singlet oxygen. The α -carotene in the reaction centre is at least as likely to be quenching singlet oxygen as it is well known it cannot directly quench the radical pair triplet state as it is bound to far away from the highly oxidising primary electron donor. The reference to energy dissipation by zeaxanthin is irrelevant here.

Fig. 2C For clarity use different symbols (but same colour) for NIR adapted and VIS adapted points.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 18 February 2013

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Overall evaluation: In this manuscript, Behrendt and coworkers present some novel results about the generation of reactive oxygen species (ROS) by a cyanobacterium (*Acaryochloris marina*) that relies on chlorophyll *d* for photosynthesis. Although I am not an expert on the biochemical pathways involved in photosynthesis, the methodology chosen appears appropriate to address the question at hand and the experiments appear to have been well conducted. Additional replicates and tests are still necessary to confirm the pattern found, but I believe the subject matter addressed in this manuscript and the results obtained are of enough interest to warrant immediate publication.

Limitation of the study: As the authors point out, additional measurements of ROS levels under various light regimes are necessary. Data should also be subjected to appropriate statistical analyses to derive stronger conclusions. In addition, it will be interesting to test another strain that does not aggregate to reinforce some of the conclusions presented in the article.

Introduction: In the first paragraph you mentioned that 2 strains of *A. marina* have been well described, but then proceed to describe only one (MBIC11017). Please, add some information about the second well-described strain.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Helen Yap 

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Overall evaluation: This is a well-written paper describing potentially ground-breaking research which has universal application, considering the ubiquitous occurrence of the cyanobacteria studied, and the crucial role they play in primary production of shallow-water habitats worldwide.

Limitation of the study: The authors acknowledge that the experiment suffers from lack of replication of measurements of ROS levels under the different light regimes. They also do not include statistical analyses that would have confirmed significant differences among the different strains, and between treatments (NIR- and VIS-adapted).

Nevertheless, the results are interesting and merit further investigation. In general, the experiment appears to have been conducted following appropriate scientific standards and protocols, and using valid methods.

Results and Discussion: 3rd from last paragraph: “concretionary” is not a common term to define coral reef substrata

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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