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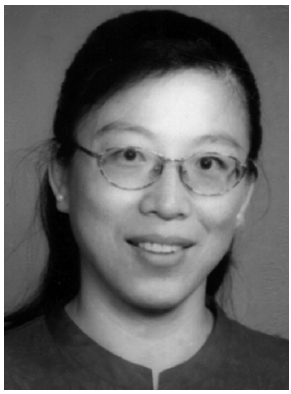

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BIOLOGY OF THE CHLOROPHYLL *D*-CONTAINING CYANOBACTERIUM *ACARYOCHLORIS MARINA*

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1. Introduction

Oxygenic phototrophs (cyanobacteria, algae and higher plants) primarily absorb solar energy in the visible spectral (400–700 nm) region by use of various chlorophylls, while anoxygenic phototrophs are bacteria, which can absorb infrared wavelengths (>700–1100 nm) by use of different bacteriochlorophylls (Overmann and Garcia-Pichel, 2004). Each of the groups also has a variety of characteristic antenna pigments and other accessory pigments that can enhance light capture and/or provide protection against excess actinic light and UV-radiation in specific habitats. However, amongst these broadly defined groups there are outlier organisms exhibiting atypical photopigmentation. Amongst the oxygenic phototrophs, the most conspicuous are:

1. The endolithic green alga *Ostreobium* sp. that inhabits coral skeletons and thrives under extreme shade below the coral tissue due to possession of a special Chl *a* antenna absorbing in the far-red region around 700–730 nm (Halldal, 1968; Fork and Larkum, 1989; Koehne et al., 1999), that is a region of the solar spectrum which is not absorbed by the overlying coral tissue (Magnusson et al., 2007);
2. The prochlorophytes present three independent lineages of cyanobacteria, that is the genera *Prochlorococcus*, *Prochlorothrix* and *Prochloron*. *Prochlorococcus* contains unique divinyl-Chl *a* and divinyl-Chl *b* photopigments and only minor amounts of phycobiliprotein (PBP) pigment, while *Prochlorothrix* and *Prochloron* are the only prokaryotes containing Chl *b* (Partensky and Garczarek, 2003).

Prochloron, which lives mainly as an exosymbiont in the outer test and exhalant canals of didemnid ascidians, has so far resisted any cultivation attempt (Kühl and Larkum, 2002). However, during one such attempt to isolate *Prochloron* from ascidians, the perhaps most unique oxygenic phototroph was cultivated by a Japanese group (Miyashita et al., 1996), viz. the Chl *d*-containing cyanobacterium *Acaryochloris marina* (Miyashita et al., 2003). In this review we summarize the

current knowledge about *A. marina* including a brief account on the discovery and properties of Chl *d* (see also Larkum and Kühl, 2005).

2. Discovery of Chl *d* and *A. marina*

Chl *d* was first found in pigment extracts from red algae (Manning and Strain, 1943). The chemical structure of Chl *d* is only different from Chl *a* by the presence of a 3-formyl group, which replaces the vinyl group on ring I (Fig. 1) (Holt and Morley, 1959). But this structural change causes a pronounced red-shift of the long-wavelength absorption maximum (Q_y) of Chl *d* by about 30 nm relative to Chl *a* (Fig. 1), that is into the near-infrared (NIR) spectral region with an in vivo absorption peak at 710–720 nm (Chen et al., 2002a). However, after its discovery the new chlorophyll could not be assigned to a specific organism and it was shown that Chl *d* could also be formed as an intermediate byproduct from other chlorophylls during pigment extraction (Holt, 1961). Consequently, the biological relevance of Chl *d* remained unresolved until the discovery of a Chl *d*-containing microorganism in 1996 (Miyashita et al., 1996).

This microbe was first isolated from didemnid ascidians, when a Japanese research group attempted to isolate the ascidian symbiont, that is the prochlorophyte *Prochloron*. While *Prochloron* resisted isolation, another pigmented microorganism was isolated, which turned out to contain large amounts of Chl *d*. The organism was named *A. marina* and analysis of its 16S rRNA gene later showed that it belonged to the cyanobacteria (Miyashita et al., 2003). This assignment has later been supported by additional phylogenetic analysis of genes encoding proteins such as the light-harvesting protein prochlorophyte Chl a/b (Pcb) (Chen et al., 2005b). Cultures of *A. marina* are easy to keep in the laboratory, and such cultures have been subject to detailed biochemical and photophysiological studies. Furthermore, the genome of *A. marina* is currently being sequenced (see http://genomes.tgen.org/index.html), and this will soon reveal a much more detailed picture of its phylogenetic position and functional characteristics.

3. Cell Biology of *A. marina*

A. marina is a unicellular non-motile cyanobacterium with a spheroidal/ellipsoidal shape, 1.8–2.1 × 1.5–1.7 μm in size (Miyashita et al., 1996). It appears dull yellow-greenish under the microscope, and TEM shows the presence of 6–12 layers of thylakoid membranes arranged peripherally in the cells (Fig. 2). In contrast to other cyanobacteria (prochlorophytes aside) it does not have phycobilisomes (Miyashita et al., 1997; Marquardt et al., 2000), but has PBPs (see Section 4.1.1).

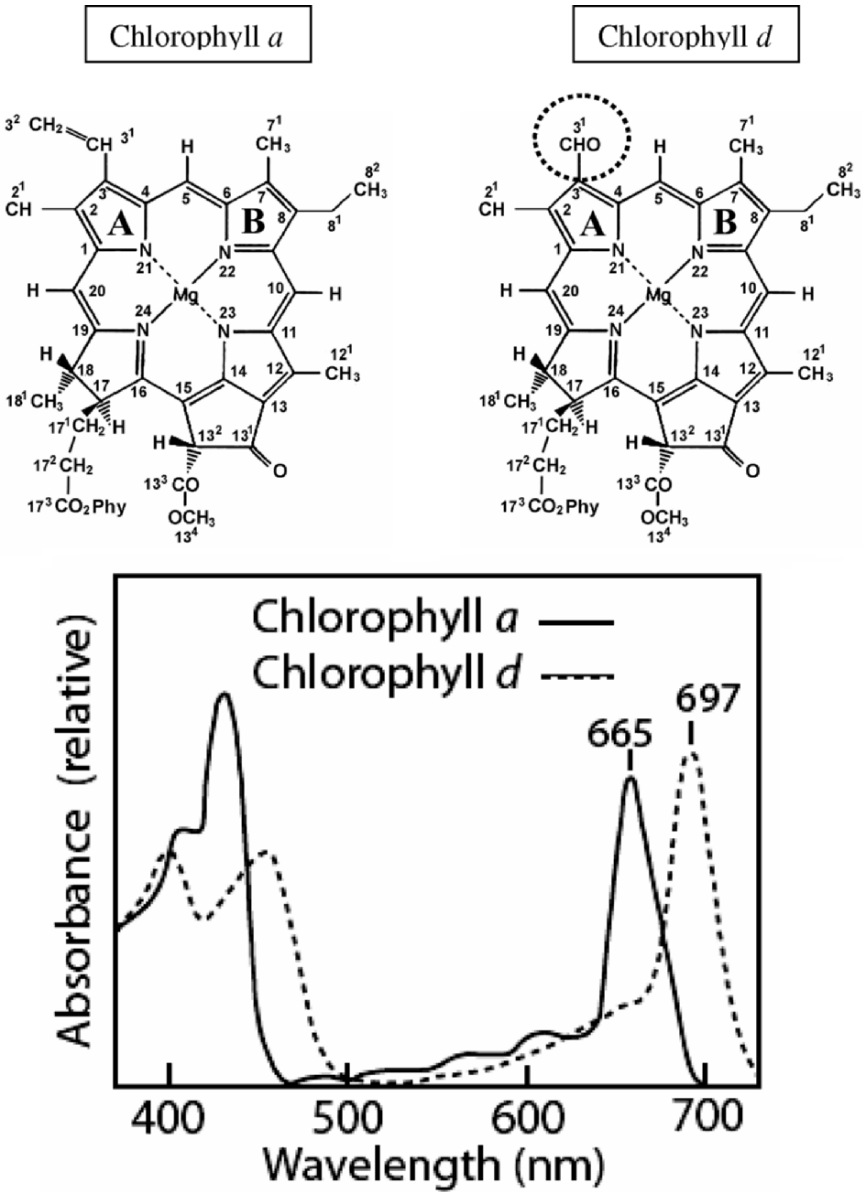

Figure 1. Structure of Chl *a* and Chl *d* and their in vitro absorbance spectra in acetone extracts. A divinyl group in ring A of Chl *a* is replaced by a formyl group in Chl *d* (dotted circle). This shifts the Q_y absorption maximum of Chl *d* about 30 nm towards the infrared relative to Chl *a*.

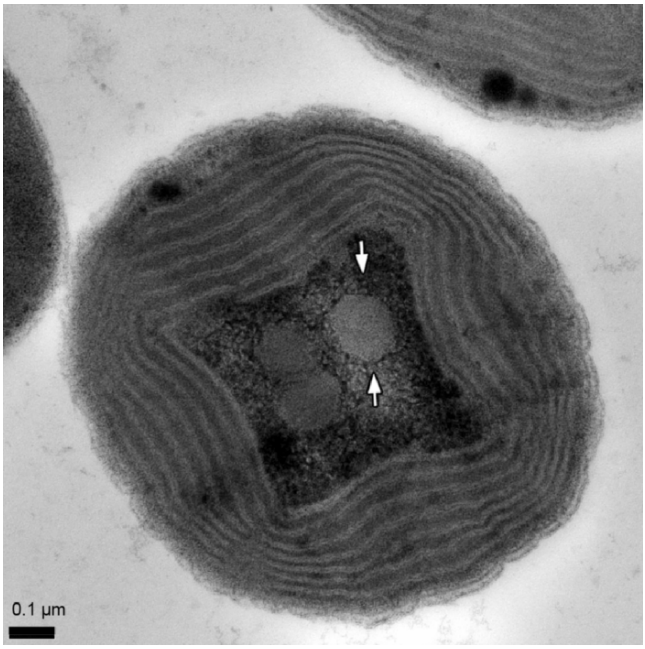

Figure 2. TEM picture of *A. marina*. Ultrathin section (~60 nm). Cells were fixed with glutaraldehyde (1%, 45 min), followed by potassium permanganate (1%, 10 min) and embedded in Spurr's resin. Arrows indicate the position of carboxysomes. Scale bar indicates 0.1 µm (provided by Dr Martin Hofman and Prof. Robert E. Blankenship, Arizona State University).

Thylakoids in *A. marina* show appressed regions predominantly with PSI trimers (Chen et al., 2005c) and more separated thylakoid regions containing PSII and small rod-shaped PBP complexes (Marquardt et al., 1997; Hu et al., 1999). This lateral heterogeneity is not present in phycobilisome-containing cyanobacteria. The thylakoid membrane stacks in *A. marina* are perforated by channel-like structures connecting central and peripheral cell portions. This ultrastructural feature has not been found in other photosynthetic organisms, and its function is unresolved (Marquardt et al., 2000).

Like most other cyanobacteria, *A. marina* contains carboxysomes (Fig. 2), that is polyhedral inclusions in the cell containing ribulose-1,5-bisphosphate carboxylase/oxygenase (*Rubisco*), the key enzyme involved in inorganic carbon fixation. Whether *A. marina* possesses similar carbon concentration metabolism as other cyanobacteria remains to be shown. The *Rubisco* large subunit gene sequence (rbcL, AB065004) of *A. marina* shows high homology (83% identity) with other cyanobacteria. Investigation on metabolic pathways in *A. marina* is still at an early stage. Besides the unique photopigmentation, so far no other unique biochemical characteristics were detected in *A. marina* based on the limited information.

4. Photosynthesis Driven by Chl *d*

For decades, it was believed that Chl *a* is an essential chlorophyll in the reaction centres for oxygenic photosynthesis because it is the only chlorophyll that can form the special pair of chlorophylls needed to generate the redox potential span to split water into molecular oxygen. Bacteriochlorophylls can also form special pairs in the reaction centre but absorb longer wavelengths, that is lower energy radiation, and therefore the excited state energy and redox potential spans generated by those photopigments are significantly less than Chl *a* and only functional in anoxygenic photosynthesis.

A. marina has exchanged most of its Chl *a* with Chl *d*, both in its antenna and the reaction centres, allowing it to harvest far-red wavelengths (700–730 nm) for oxygenic photosynthesis. The exact in vivo absorption and fluorescence emission maxima of *A. marina* are known to show some variability. Schiller et al. (1997) showed the presence of various spectral forms of Chl *d* in *A. marina*, and showed that two phenotypes (one with low Chl *a* and another with somewhat higher Chl *a*) had different in vivo absorbance spectra in the far-red. Furthermore, Chen et al. (2002a) showed that variations in fluorescence emission maxima of *A. marina* depended on iron-availability. No unusual photophysical processes are detected for isolated Chl *d* molecules in solvent (Nieuwenburg et al., 2003) and this is inconsistent with the postulate that uphill energy transfer may occur between Chl *d*-antenna and a Chl *a*-reaction centre (Mimuro et al., 2000, 2004).

Chl *d*-containing organisms have puzzled biologists and the bioenergetic features of *A. marina* have been debated (Blankenship and Hartman, 1998). How and why does *A. marina* use Chl *d* as its major photopigment? The current evidence, provided by fluorescence resonance energy transfer (FRET) analysis, demonstrated that the coordination reaction of Chl *d*, that is the chlorophyll binding to the imidazole of histidine by the fifth coordination bond of Mg, is similar to that of Chl *a*, but not to that of Chl *b* (Chen et al., 2005d). The basic functional properties of the photosynthetic apparatus in *A. marina* are similar to Chl *a*-containing oxygenic phototrophs. There are two photosystems, PSI and PSII, and preliminary protein sequence data show that the polypeptide composition of both PSI and PSII complexes of *A. marina* are similar to that of well-known Chl *a*-containing analogues (Hu et al., 1998; Miyashita and Sasaki, 2001) despite its unique light-harvesting antenna systems.

4.1. PIGMENT COMPOSITION AND ANTENNA SYSTEMS OF *A. MARINA*

A. marina contains five photopigments of which Chl *d* is the major pigment amounting to up to 99% of the total lipid-soluble pigment of the cell and more than 2% of the cell dry weight. Chl *a* is a minor constituent and its quantity varies

with culture conditions from 1% to 10% of the total chlorophylls (Miyashita et al., 1997; Akiyama et al., 2001, 2002). Under high-light conditions, the ratio of Chl *a*/*d* is increased either due to additional Chl *a* synthesis in the cells (Mimuro et al., 1999; Chen et al., 2002a), or due to inhibition of Chl *d* biosynthesis under high light. Enriched iron in the culture medium caused a low ratio of Chl *a*/Phe *a* of 0.68 in *A. marina*, which is significantly less than the ratio of 1.43 found under iron-stressed culture (Swingley et al., 2005). This recent finding is inconsistent with the previous suggestion of a minimum Chl *a*/Phe *a* ratio of 1 (Mimuro et al., 2004), used as a major argument for the proposal that PSII in *A. marina* contains two or more Chl *a* molecules along with two pheophytin (Phe) *a* molecules. Minor photopigments in *A. marina* include a Chl *c*-like pigment, zeaxanthin and α-carotene (Miyashita et al., 1997). α-carotene is an unusual carotenoid for cyanobacteria, which typically have β-carotene, and besides *A. marina*, α-carotene is otherwise reported in certain taxa of eukaryotic algae. There is only a small difference between these carotenes as α-carotene has ten conjugated double bonds while β-carotene has 11. However, there has been no investigation on the physiological significance and effects of these differences.

4.1.1. Phycobiliproteins

A. marina has two light-antenna systems, PBPs and Pcb-bound Chl *d* complexes (Chen et al., 2002b). Phycobilins are present in most cyanobacteria, as well as some eukaryotic algae, that is glaucocystophytes, rhodophytes and cryptophytes, where the bilin chromophores are attached in variable numbers to polypeptides to form PBPs. PBPs are often associated with linker polypeptides to form a supramolecular antenna-complex, the phycobilisome, on the outside surface of thylakoid membranes. Phycobilisomes are the primary light-harvesting antennae in cyanobacteria, glaucocystophytes and rhodophytes allowing these organisms to utilize the spectral region from green to orange, where only a few other light-harvesting systems are active. Cyanobacteria can exhibit ontogenetic complementary chromatic adaptation, whereby they express those PBPs best able to exploit a particular light climate (Larkum and Barrett, 1983). There are no phycobilisomes reported in *A. marina*, but some PBPs (phycocyanin and allophycocyanin) are present and organized in rods of four hexameric units that can act as antenna for light harvesting (Hu et al., 1999; Marquardt et al., 2000). The PBP content of *A. marina* is normally very low, but a recent investigation showed that replete iron-availability in the growth medium caused higher ratio of PBPs to Chl *d* (Swingley et al., 2005).

How the PBPs link with the photosystem-reaction centre in *A. marina* is uncertain. Action spectra of intact cells of *A. marina* indicate that the PBPs transfer energy to PSII with somewhat higher efficiency than to PSI (Boichenko et al., 2000). Excitation energy transfer studies with time-resolved fluorescence spectroscopy indicated that the elemental structure of PBP in *A. marina* provides efficient energy transfer from PBPs to Chl *d* in PSII with a time constant of 70 ps, which is about three times faster than energy transfer from phycobilisomes to PSII in the Chl *a*-containing cyanobacteria (Petrasek et al., 2005).

4.1.2. Pcb-bound Chl d

The main light-harvesting antenna system in *A. marina* consists of Pcb-bound Chl *d* complexes. Pcb are Chl *a*/*b*-binding proteins, which are found in the three different prochlorophyte lineages of cyanobacteria. The surprising discovery of Pcb-protein bound Chl *d* in *A. marina* (Chen et al., 2002b) showed that Pcb-bound Chl *d*-protein pigment complexes can function as the major light-harvesting protein. There are two Pcb genes in *A. marina*, *pcbA* and *pcbC*. Phylogenetic analysis supports that they may be functionally associated to PSII and PSI, respectively, under different nutrient conditions (Chen et al., 2005a–c); however, recent genomic information indicates that there are multiple copies of the Pcb gene in *A. marina* (M. Chen and R. Blankenship, unpublished data).

4.2. PHOTOSYSTEM I

Because PSI complexes are more stable and easier to isolate, the properties of PSI in *A. marina* have been resolved in more detail than the properties of PSII and its connected O_2 evolving reactions. Sequence comparisons indicated that PsaA/PsaB of *A. marina* has 85–87% homology to the typical cyanobacterium *Synechocystis* sp. PCC 6803. There are PsaA,-B,-C,-D,-E,-F,-L,-K and two unidentified polypeptides of < 6 kDa peptides in isolated PSI complexes. It was suggested that the primary electron donor of PSI in *A. marina* is a special pair of Chl *d*, and it was shown by laser spectroscopy that the oxidation of the primary donor of PSI occurs at 740 nm and this caused the *Acaryochloris* PSI reaction centre to be named P740 (Hu et al., 1998; Akiyama et al., 2001). The size of PSI and PSII and their pigment content is still not fully resolved. Hu et al. (1998) showed that isolated PSI had a Chl *d*/*a* ratio of 180, which is six times higher than in intact cells, and a Chl *d*/P740 ratio of 150. However, an antenna size of 80–90 Chl *d* per PSI reaction centre has also been suggested (Boichenko et al., 2000).

Laser spectroscopy, ENDOR and FTIR studies (Hastings, 2001) indicated that the basic structure and environment of P740 is similar to that of P700. The (P740+ to P740) IR spectrum band pattern was very similar to that of (P700+ to P700) from the cyanobacterium *Synechocystis* sp. PCC 6803. This indicates that P740 is probably a dimer consisting of a special pair of chlorophylls in the reaction centre similar to P700, but with a pair of Chl *d* molecules instead of Chl *a*. Extraction and reconstitution of the phylloquinone molecule was performed on the PSI particle, and the function of quinone was assumed to be similar to that observed in isolated PSI complexes from other organisms (Itoh et al., 2001).

The primary electron acceptor is also identified as Chl *d* (Akiyama et al., 2001, 2002). This is unique among all photosynthetic organisms including anaerobic photosynthetic bacteria that use a Chl *a*-type pigment as the primary acceptor. Chen et al. (2005a) revealed that Pcb–PSI super-complexes were formed when *A. marina* was grown under iron-limitation (Bibby et al., 2001; Boekema et al., 2001). In vivo action spectra of PSI in *A. marina* demonstrated that a low but reliable efficient energy transfer happened from the PBPs to PSI (Boichenko et al., 2000; Fig. 4).

4.3. PHOTOSYSTEM II

PSII characteristics in *A. marina* have not yet been fully resolved and since its discovery it has been debated whether Chl *d* has replaced the primary photochemical role of Chl *a* in the PSII reaction centre. PSII reaction centre polypeptides D1, D2, cytochrome b559 and CP 43 and 47 were reported for PSII in *A. marina* (Hu et al., 1999) and the sequence of D1/D2 shows high (up to 93%) homology with polypeptides in Chl *a*-containing cyanobacteria.

Action spectra of PSII activity showed an efficient and preferential energy transfer from PBP to PSII (Boichenko et al., 2000; Fig. 4), which agrees with the ultrastructural evidence for a close physical connection between PSII complexes and PBPs (Hu et al., 1999). However, isolated mega-antenna-reaction centres of the PSII super-complex showed an incompatible ultrastructure arrangement of PSII reaction centres and its antenna systems (Chen et al., 2005b). The isolated PSII-antenna super-complexes consisted of tetrameric PSII reaction centres surrounded by decamer Pcb along each side of the tetrameric reaction centre (Fig. 3). This unique subunit arrangement could explain experimental results showing that the effective optical cross-section of O_2 evolution in *A. marina* is

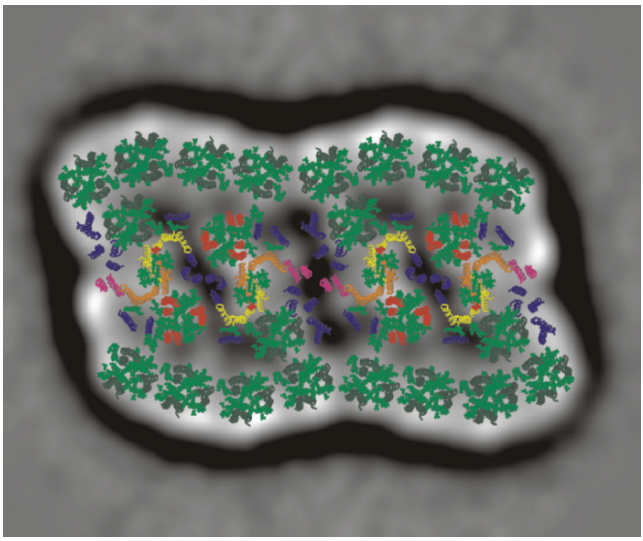

Figure 3. Modelling of the high-resolution X-ray structure of PSII and CP43 (Ferreira et al., 2004) into the Pcb–PSII super-complex isolated from *A. marina* viewed from its lumenal surface. The 36 Chls and 14 Chls of each PSII monomer and Pcb subunit, respectively, are shown in light green. Other colours represent transmembrane helices of D1 protein (yellow), D2 protein (orange), CP47 (red), CP43 and Pcb proteins (dark green), cytochrome b559 (pink), low molecular weight subunits (blue). Maximum length of the negatively stained particle is 385 Å, inclusive of the detergent shell (model by Dr J. Nield, Imperial College).

about 65 Å^2, that is two times larger than with a dimeric-reaction centre arrangement. In addition, the functional antenna size of PSII in *A. marina* indicates at least the double number of CP43/47 subunits per reaction centre as compared to other cyanobacteria, that is that one reaction centre is supported by one CP43/47 and 2–3 Pcb subunits as its antenna systems (Boichenko et al., 2000).

4.4. UPHILL ENERGY TRANSFER IN *A. MARINA*

It is conventional to consider that energy flows from a higher energy source to a lower energy sink. In terms of light this means that energy flows, pigment-wise, from the blue to the red end of the spectrum, and the final sink is the special pair of chlorophylls in either reaction centre (PSI or PSII). In *A. marina*, this question has caused some debate because the special pair in PSII may turn out to be either Chl *a* or Chl *d*. If it is Chl *a* then light absorbed by light-harvesting Chl *d* would have to flow "uphill" from as far out as 750 nm to P680 at 680 nm. If it were Chl *d* then light would still have to flow from 750 nm to the hypothetical P715 at 715 nm. However, it has been shown clearly that this does not pose a problem in terms of quantum mechanics (Nieuwenburg et al., 2003; Trissl, 2003) and there are experimental indications for uphill energy transfer in *A. marina* (Mimuro et al., 2000). In fact, this phenomenon is already known to happen in "conventional" algae; for example, it has long been known that the endolithic alga *Ostreobium queckettii*, found in the skeleton of living corals, has a light-harvesting protein, containing Chl *a* and Chl *b*, that can harvest light up to at least 750 nm (Halldal, 1968) and pass this light on to P680 at 680 nm. The mechanism for this transfer process is similar to that which operates in *A. marina* (Trissl, 2003; Wilhelm and Jakob, 2006).

4.5. ACTION SPECTRUM AND PHOTOSYNTHETIC O_2 PRODUCTION

Light intensity and quality are known to play an important role for regulating the PSI/PSII ratio and the size of the light-harvesting antenna in oxygenic phototrophs (Razeghifard et al., 2005). The function of oxygenic photosynthesis in *A. marina* is similar to other oxygenic phototrophs, that is the turnover of the O_2-evolving system under flash excitation follows the S-state cycles with a period-4 oscillation, although the O_2 release is slower than in classical Chl *a*-containing cyanobacteria (Boichenko et al., 2000). Action spectra of photosynthetic oxygen production showed that Chl *d* and PBPs act as antenna for both PSI and PSII, and that both far-red and blue wavelengths can drive photosynthesis effectively in *A. marina* (Boichenko et al., 2000; Fig. 4). Interestingly, the contribution of PBPs to photosynthesis decreased when the original culture was grown under white light or under far-red light pointing to some ability of *A. marina* to optimize its pigmentation towards ambient light conditions.

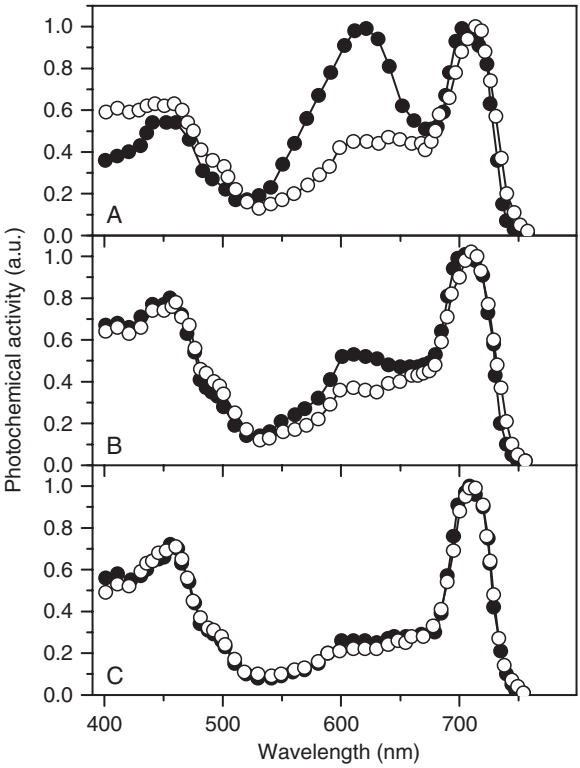

Figure 4. Action spectra of PSI (open symbols) and PSII (solid symbols) activity in a *A. marina* culture (A), and in the same culture kept under white light (B) and red light (C), respectively. Redrawn after Boichenko et al. (2000).

Oxygen production as a function of irradiance has been investigated in cultures of *A. marina* by several groups (e.g. Miyashita et al., 1997), showing a remarkable ability of *A. marina* to cope with high irradiance. The O_2 activity of cells was significantly increased compared to the typical activity of 70–80 µmol O_2 (mg of Chl)$^{-1}$ h^{-1} when *A. marina* was grown in white light at an irradiance of 60 µmol photons m^{-2} sec^{-1} provided by a metal-arc lamp. Such high-light conditions caused the PSI content to decrease to half and the light harvesting complex (LHC) component decreased, therefore, O_2 activity (based on chlorophyll concentration) was increased (Razeghifard et al., 2005). In a recent study (M. Kühl et al., unpublished data) we used a new microrespirometry system (Unisense A/S, Denmark) to measure photosynthesis and respiration as a function of irradiance (Fig. 5). In line with earlier reports, *A. marina* showed a remarkable tolerance of high irradiance up to > 700 µmol photons m^{-2} sec^{-1} without any sign of photoinhibition. Maximum rates of gross photosynthesis reached > 200 µmol O_2 (mg

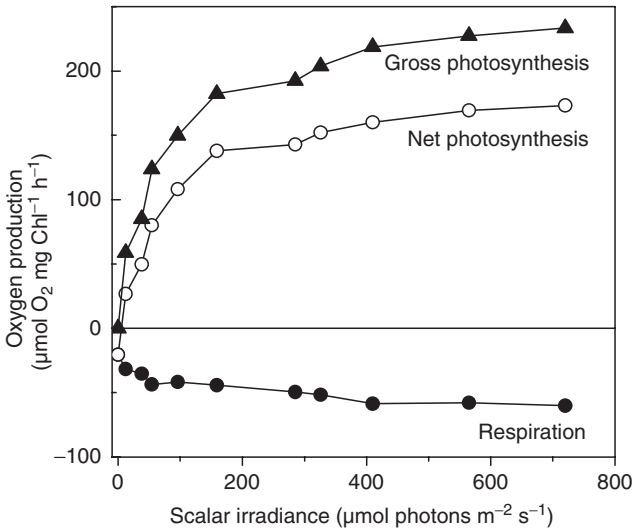

Figure 5. Photosynthesis and respiration as a function of scalar irradiance in a culture of *A. marina*, as measured with an oxygen microelectrode respirometry system (Unisense A/S, Denmark). (M. Kühl, unpublished data).

Chl)$^{-1}$ h^{-1}. Furthermore, we found a pronounced post-illumination respiration reaching 300% of the dark respiration level at the highest irradiance. The mechanism behind this needs further investigation and there evidently is a need for more detailed ecophysiological analysis of *A. marina* cultures, for example with respect to effects of pH, inorganic carbon, salinity, temperature and nutrient regimes on the photosynthetic performance and growth. Micronutrients such as iron may also play an important role (see Swingley et al., 2005).

5. Evolutionary Relationships of *A. marina*

A. marina is a cyanobacterium, according to small-subunit (SSU) rRNA analysis (Miyashita et al., 2003; Fig. 6). According to morphological and cytological classification of cyanobacteria (Castenholz, 2001), *A. marina* is accommodated in the *Chroococcales*-group of unicellular cyanobacteria, but the molecular data show that *A. marina* has no close relationship to any of the unicellular subgroups. *A. marina* is placed in the middle of the cyanobacterial lineage and diverges independently from the other cyanobacterial subgroups. The possession of Chl *d* of *A. marina* poses an interesting question concerning the presence of this unique chlorophyll. Why is it only found in *A. marina* and none of its distant relatives? The answer to these questions can be only speculative at present and would be

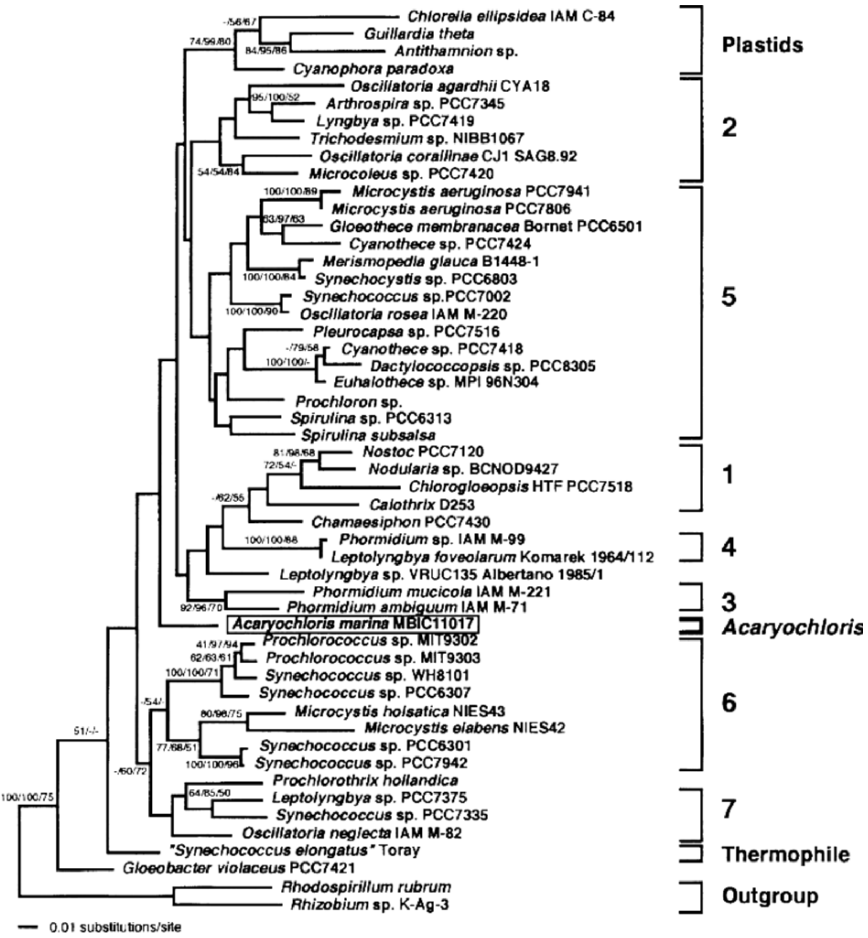

Figure 6. Evolutionary relationship of *A. marina* to other cyanobacteria based on SSU rRNA analysis (from Miyashita et al., 2003).

much less so if we had: (i) the whole genome of *A. marina*, which is currently being sequenced at the University of Arizona (Blankenship, see above), and (ii) the biosynthetic pathway for Chl *d*.

In the absence of these vital pieces of evidence, we can fall back on the weaker evidence from the light-harvesting protein that binds Chl *d* (Chen et al., 2005c). It is clear that *A. marina* has the same or similar light-harvesting chlorophyll protein as prochlorophytes and some classical cyanobacteria. In some classical cyanobacteria an iron-stress induced protein A (isiA protein) is inducible under low iron-conditions (Ting et al., 2002). In prochlorophytes Chl *b* is present, in addition to Chl *a*, and attached to a LHC, the so-called pcb protein, which is, phylogenetically, closely related to isiA proteins. It is clear that the LHC of

A. marina belongs to the same class as those of the isiA and pcb LHCs (Chen et al., 2005e).

In those *Prochlorococcus* species, for which a whole genome analysis has been done (Dufresne et al., 2003; Rocap et al., 2003), it is clear that there are wide differences in the genetic structure but these organisms all have a small number of genes allowing them to express pcb LHCs and to make Chl *b*; note that Chl *a* oxygenase, the key enzyme in forming Chl *b* in *Prochloron*, algae and plants is not present in *Prochlorococcus* species, so some other gene(s) must be responsible. These species may thus be joined in their ability to express pcb LHCs, while showing great evolutionary diversification in other characters (Ting et al., 2002); although other evidence suggests that *Prochlorococcus* species may be a real taxonomic group (A.W.D. Larkum et al., unpublished data).

The other prochlorophytes, *Prochloron* and *Prochlorothrix* may have gained entry to this group of organisms merely by the lateral transfer of the suite of Chl *b* synthesis and *pcb* genes (Chen et al., 2005e) and a similar reasoning may be put forward for *A. marina*, although we know nothing of the biosynthetic mechanism of Chl *d*. Reinforcing this view is the fact that in phylogenetic analyses of *Prochlorococcus* species, three "classical" cyanobacteria, with no Chl *b* but possessing PBPs, that is *Synechococcus* CC9605, *Synechococcus* CC9902 and *Synechococcus* WH8102 are found within the *Prochlorococcus* branch (Rocap et al., 2003), suggesting that these are closely related. Furthermore, it is also known that a "classical" cyanobacterium without Chl *b* and with PBPs, *Synechocystis trididemni*, is closely related to *Prochloron* in terms of SSU rRNA (Shimada et al., 2003).

6. Natural Habitats and Niche of *A. marina*

A. marina was first isolated during an attempt to isolate *Prochloron* from the didemnid ascidian *Lissoclinum patella* (Miyashita et al., 1996, 2003), and it was for several years regarded a symbiont of didemnid ascidians. Cultures of *A. marina* are easy to keep in the laboratory, and such cultures have been subject to detailed biochemical and photophysiological studies, but the actual niche and habitat of this unique cyanobacterium remained unknown until 2004/2005, when it became clear that *A. marina* is more widespread than previously thought. Murakami et al. (2004) reported *A. marina* growing as small epiphytic patches on the red macroalga *Ahnfeltiopsis flabelliformis*, and they obtained several strains from such habitats. Miller et al. (2005) isolated *A. marina* in enrichments from a benthic sample taken in the eutrophic and saline Salton Sea, the largest lake in California. These two studies clearly showed that *A. marina* could be free-living, so what about the initial claim of *A. marina* being a symbiont in didemnid ascidians? A thorough investigation of several didemnid ascidians showed that *A. marina* was indeed not living as a symbiont inside the ascidian but formed dense cell patches in biofilms growing below the animal (Kühl et al., 2005; Fig. 7A). By the help of microscopy, microspectrometry, and variable chlorophyll

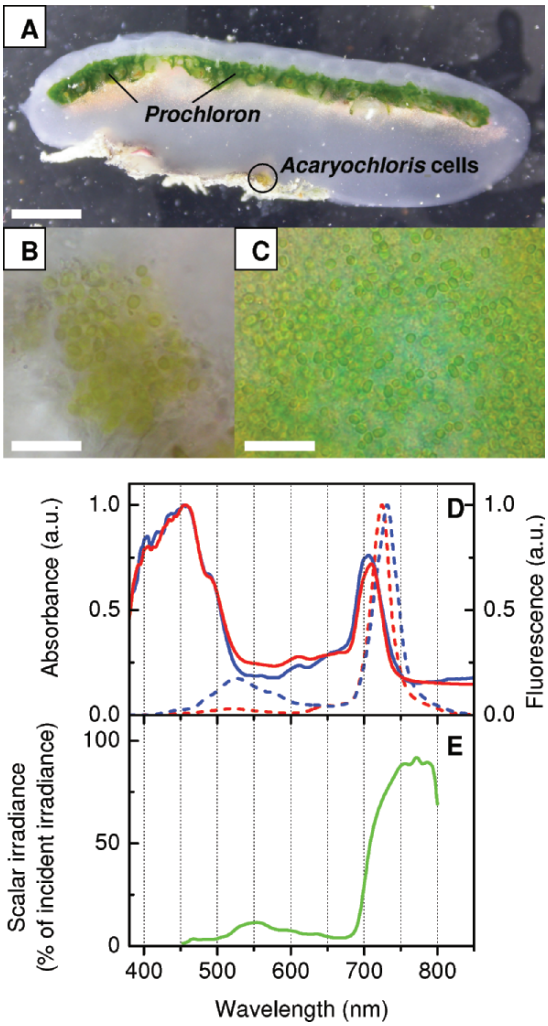

Figure 7. Microscopic observation and spectral analysis of Chl *d*-containing cells associated with the didemnid ascidian *Diplosoma virens*. A. Vertical section through the ascidian showing symbiotic *Prochloron* sp. (green colour) in the cavities of the transparent test and patches of biofilms with Chl *d*-containing cells growing on the underside of the ascidian (scale is 2 mm). B. Chl *d*-containing cells within the circled biofilm area in panel a (scale is 10 µm). C. Cells from an *A. marina* culture (scale is 10 µm). D. Spectral absorbance (solid lines) and UV-excited fluorescence (dashed lines) of cells growing in the biofilm (circled area in A, blue lines) and of an *A. marina* culture (red lines). Data were normalized to the maximal absorbance and fluorescence, respectively. E. Transmission of spectral scalar irradiance in tissue of *D. virens*, as measured 1.3 mm below the upper surface of the ascidian. Data are expressed in percent of the downwelling irradiance at the tissue surface (from Kühl et al., 2005).

fluorescence imaging both the distribution, spectral characteristics and the photosynthetic activity of *A. marina* could be studied in situ, and this enabled the first description of the niche that *A. marina* inhabits.

6.1. HABITAT CHARACTERISTICS AND IN SITU ACTIVITY OF *A. MARINA*

Contrary to the long held belief that *A. marina* is a symbiont of didemnid ascidians, we were not able to demonstrate the presence of Chl *d*-containing cells in the test or internal cavities of the ascidians. However, the underside of didemnids ascidians harbours a dense and diverse biofilm of phototrophic microorganisms and here we found patches of *A. marina*-like cells with morphology and spectral characteristics similar to cells in a *A. marina* culture (Fig. 7B–D). The spectral light field below ascidians is characterized by a strong depletion in visible wavelengths, while far-red light is, relatively, much more abundant (Fig. 7E) and can support the photosynthesis of *A. marina*. Using variable chlorophyll fluorescence imaging, we were able to perform the very first in situ photosynthetic activity measurements of *A. marina* in its natural habitat below the didemnid ascidians harbouring a dense internal population of *Prochloron* (Fig. 8), showing a high maximal quantum yield of PSII ranging from 0.7 to 0.8 and adaptation towards high irradiance.

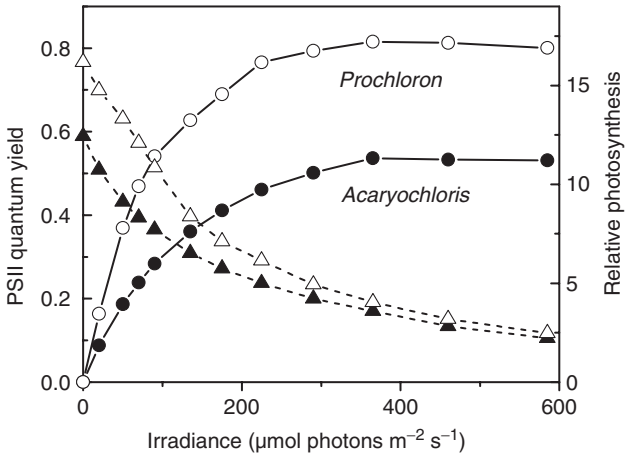

Figure 8. Effective quantum yield of PSII (dotted curves, triangles) and derived relative photosynthesis (=yield × irradiance × absorptivity) (solid curves, circles) measured as a function of irradiance with an imaging PAM fluorometer equipped with blue LED's (470 nm). Measurements were done on intact didemnid ascidians harbouring *Prochloron* symbionts (open symbols) in their internal cavities and a biofilm with *A. marina* (solid symbols) on their underside. Data are from specific areas of interest shown by microscopy and spectroscopy to be predominated by *Prochloron* and *A. marina*, respectively (from Kühl et al., 2005).

There is an apparent paradox of *A. marina* growing in extreme shade in situ and at the same time showing characteristics of high-light adaptation. However, *A. marina* is de facto adapted to high levels of far-red light that for this organism is photosynthetically active due to the presence of Chl *d* as the major photopigment. In this context it is important that: (i) far-red light absorbed by Chl *d* drives both PS I and PS II (Boichenko et al., 2000), (ii) Chl *d* also absorbs substantially in the blue, which we made use of to assess PS II quantum yield via PAM fluorometry (Schiller et al., 1997; Kühl et al., 2005) and (iii) for photoacclimation of the cells it is irrelevant whether a high electron transport rate is driven by far-red or blue light. Interestingly, a photosynthesis (measured as oxygen production) versus irradiance experiment with *A. marina* cultivated under 80 µmol photons m^{-2} sec^{-1} (Miyashita et al., 1997) showed a similar high-light adaptation, but of course such culture experiments are strongly dependent on the actual growth conditions used and the authors did not quantify far-red intensity.

While blue light is rather insignificant in the niche of *A. marina* due to the strong absorption in the overlaying ascidian tissue, the strong inherent absorption of Chl *d* in the blue spectral region allowed us to use PAM fluorometry with blue excitation light. Blue and far-red light have been shown to be almost equally efficient in the action spectra of both photosystems in *A. marina* cultures (Boichenko et al., 2000) and the equivalence of blue and far-red should be even more pronounced in situ due to the pigment flattening effect in the highly scattering biofilm. This allowed us to determine the high-light adaptation towards far-red light on the basis of PAM fluorometry with blue light (Fig. 8). In an analogous example, a Chl *b*-containing phototroph (e.g. a green leaf) will also become high-light adapted if it is irradiated with light depleted of, for example 480 nm. Once this adaptation has taken place, it would also be possible to demonstrate high-light adaptation from active fluorescence measurements using 480 nm as actinic light, although this wavelength was absent during the adaptation period.

Our laboratory analysis of the microhabitat below didemnid ascidians has clearly shown that *A. marina* can thrive here in extreme shade provided sufficient NIR radiation is available. To demonstrate the latter, we performed in situ measurements of the spectral light field within the dense patches of dead corals, which were colonized by didemnid ascidians (Fig. 9). Far-red light around 700–730 nm, that is the range of the in vivo absorption maximum of Chl *d*, and further NIR wavelengths predominated the spectral light field in the natural habitat. This indicates that the high Chl *d* content of *A. marina* may indeed be adaptive to the ambient spectral irradiance in the environment where it occurs, as hypothesized by Blankenship and Hartman (1998).

6.2. DISTRIBUTION OF *A. MARINA*

Since our initial discovery of *A. marina* growing in photosynthetic biofilms below didmenids harbouring *Prochloron* (Kühl et al., 2005), we have also found *A. marina*

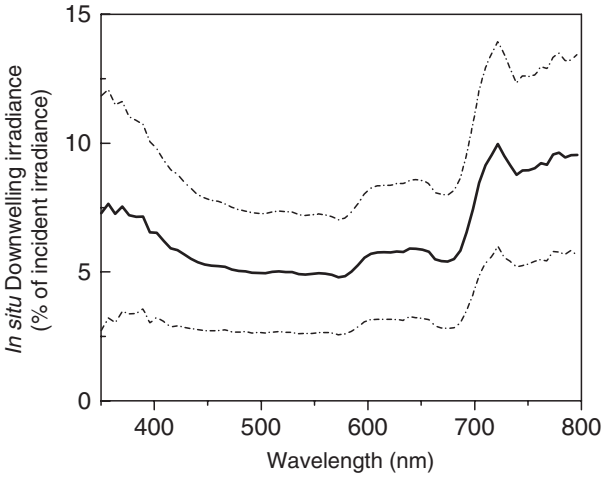

Figure 9. In situ measurements of downwelling spectral irradiance a few cm below the surface of dead coral patches on the reef flat at Heron Island (GBR) harbouring didemnid ascidians with *Prochloron* symbionts and external biofilms of *A. marina*. Solid line indicates the mean, while dotted lines indicate mean ± standard deviation, respectively (n = 10). Data were obtained with an Ocean Optics spectrometer (USB2000) equipped with a 600 µm diameter quartz fiber and a cosine collector (Ocean Optics CC-3-UV). Data were normalized to the downwelling irradiance measured just below the water surface. (M.J. Durako et al., unpublished data).

biofilms below white didemnids growing without photosynthetic symbionts on dead coral branches. Together with the finding of *A. marina* as red algal epiphytes (Murakami et al., 2004) and in enrichments from biofilm samples taken from a hypertrophic salt lake (Miller et al., 2005), our data indicate that Chl *d*-containing cyanobacteria may be more widespread in shallow-water niches, where visible wavelengths are strongly depleted and sufficient NIR prevails.

So far, all free-living *A. marina* have been found in biofilms growing on biotic or abiotic substrates, and *A. marina* seems adapted to a surface-associated lifestyle. There is now a need for a more careful screening of such habitats to assess the abundance of Chl *d*-containing phototrophs. There may also be other yet undiscovered microenvironmental conditions, besides the light microclimate, that shape the niche of *A. marina*-like organisms. For example, *A. marina* has neither been found in endolithic habitats or in microbial mats, both of which are characterized by a strong depletion of visible wavelengths by microalgae and cyanobacteria in the surface layers and efficient penetration of NIR wavelengths into deeper layers (e.g. Kühl and Fenchel, 2000; Magnusson et al., 2007).

In the coral skeleton, the spectral range where Chl *d* is absorbing is also covered by the special antenna pigments of the predominating endolithic green alga *Ostreobium*. In microbial mats, NIR is absorbed by various bacteriochlorophylls present in dense subsurface layers of anoxygenic phototrophs, but the spectral

window where Chl *d* absorbs is not affected by these pigments, and based on the light microclimate this seems an ideal niche for Chl *d*-containing phototrophs. But microbial mats are also characterized by extremely steep and strongly fluctuating light-dependent gradients of chemical variables such as oxygen (zero to almost pure oxygen), pH (<6 to >9.5) and poisonous hydrogen sulfide (zero to several mM). We know nothing about how *A. marina* can cope with such extreme conditions, or whether the habitats where it has been found exhibit similar characteristics.

Does *A. marina* have an efficient carbon concentrating mechanism allowing for efficient carbon fixation under high pH? Can *A. marina* tolerate sulfide, or even use it for anoxygenic photosynthesis as many phycobilisome-containing cyanobacteria are able to? Can *A. marina* fix nitrogen, like some other unicellular cyanobacteria? These are but a few open questions that call for more detailed in situ and in vitro physiological investigations of *A. marina* in concert with data mining of its genome for hitherto unknown metabolic capabilities.

7. Summary

The cyanobacterium *A. marina* is the only known oxygenic phototroph with Chl *d* as its major photopigment. In *A. marina* Chl *d* is the major light-harvesting pigment along with a minor amount of PBPs, and Chl *d* has replaced the primary photochemical role of Chl *a* in the PSI reaction centre (and maybe also in PSII). The cell biology and photophysiology has been studied in cultures of *A. marina* and its genome is currently being sequenced. However, the in situ habitat and ecophysiology of this unique phototroph is underexplored. Oxygen and fluorescence-based measures of photosynthesis show that *A. marina* is high-light adapted and does not suffer from photoinhibition at high-irradiance levels. While initially regarded a symbiont of didemnid ascidians, it is now clear that *A. marina* is free-living and grows in biofilms associated with biotic and abiotic surfaces (red algae, didemnid ascidians, and in the hypersaline Salton Sea), where it occupies a niche depleted of visible wavelengths but enriched in far-red light. Thus *A. marina* is probably widespread and can be found in a range of habitats yet to be explored.

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