Growth and pigmentation of marine infrared radiation (IR) absorbing phototrophs

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ABSTRACT

A common method of phototrophy for globally abundant microbes is the absorption of infrared radiation (IR) using bacteriochlorophyll (bChl) pigments. The goal of this study was to assess growth and pigment production of marine phototrophs under defined IR enrichment conditions. Aerobic seawater samples collected offshore Woods Hole, MA and seawater intertidal marsh mat samples from Sippewissett Marsh were compared for growth, pigment production, and rRNA 16S community composition under IR (940 nm), light, and dark conditions. Isolations of IR-absorbing phototrophs into axenic cultures were also attempted with successful cultivation of a *Marichromatium purpuratum* purple sulfur bacteria containing bChl *a*. Marsh mat enrichments under IR conditions in sulfide rich media resulted in the growth of the IR absorbing bacteria with bChl *b* consistent with the description of the purple sulfur bacteria *Thioflaviococcus mobilis*. IR absorbing isolates with bChl *a* were also grown in IR and light treatments using hydrogen rich media. Seawater enrichments did not result in pigment production under IR conditions and the community composition was similar to the light and dark treatments. This study did successfully isolate two IR-absorbing microbes. Future directions include examining growth and pigment production under the IR exclusive conditions to gain a better understanding their potential photophysiology and production.

INTRODUCTION

Marine photosynthetic organisms are well recognized for their contribution to global primary production and carbon dioxide fixation and past research has largely focused on the cyanobacteria and eukaryotic oxygenic photosynthesis. Bacterial phototrophs absorbing infrared radiation (IR) using bacteriochlorophyll pigments (bChl) and exhibiting phototrophic productivity were largely thought confined to unique sediment niches transmitting IR (> 800 nm). The discovery of globally abundant aerobic anoxygenic phototrophs that absorb IR energy on the ocean surface has expanded our view and potential significance of bacterial IR phototrophy.

Open ocean IR absorbers include the alpha-proteobacteria strains *Roseobacter* sp. and *Erythrobacter* sp. with bacteriochlorophyll a (bChl) absorbing at 830-890 and 850 nm. The bChl *a*-containing bacteria have been suggested to account for ~8% of the total bacterial community in

the open ocean (Kolber et al. 2001) and less is known about the abundance of other bChl containing bacteria in the oceans. Clone library surveys indicate that *Roseobacter* sp. is a globally abundant aerobic anoxygenic phototroph group (Britschgi and Giovannoni 1991), despite the fact that IR attenuates rapidly into aquatic environments.

The IR absorbing phototrophs found in benthic sediments including intertidal marsh mats are better studied because of their high densities and prominent pigmentation. IR-absorbing phototrophs in the marsh mats are specialized to this environment because 100% of the light reaching anoxygenic phototrophs in sediments is in the infrared region (Overmann 2002). In the marsh mats, the visible region light is primarily absorbed by the upper mat layer, which includes cyanobacteria and diatoms. The IR is well transmitted by the layers of sand and is able to reach the IR-absorbing purple and peach colored sulfur bacteria. Bacteria able to absorb the longest wavelengths such as the bChl *b* (1020 and 1035 nm) containing sulfur bacteria are well adapted to this environment.

There is a limited growth environment for the seawater IR absorbing microbes despite the fact that their pigment absorbance wavelengths suggest IR specific growth specialization. Since these organisms are capable of utilizing other means of electron energy for heterotrophic growth, it is unknown if growth of these microbes is optimized, or even affected, under IR exclusive conditions with limited nutrients for heterotrophic growth.

The primary goal of this study was to assess growth and pigment production of phototrophs under IR enrichment conditions. Aerobic seawater samples and anaerobic intertidal marsh mat samples were compared for growth, pigment production, and 16S rRNA community composition under IR, light, and dark conditions. Isolations of IR-absorbing phototrophs into axenic cultures were also attempted.

MATERIALS AND METHODS

Enrichments. Pink colored marsh mat was collected from the Great Sippewissett marsh in Falmouth, MA. About 5 g of sample was inoculated in a sulfide rich seawater media containing artifical seawater, NH_4Cl, K phosphate, MOPS buffer, EDTA-chelated trace metals, 12-vitamin solution, vitamin B12 solution, bicarbonate, and DCMU. Final sulfide concentration was 1 mM with a pH of 7.2. Seawater samples were collected from surface waters just offshore Woods

Hole, MA in Buzzards Bay. 100 µl of unfiltered sample was inoculated in low nutrient seawater media containing artifical seawater, NH4Cl, K phosphate, MOPS buffer, EDTA-chelated trace metals, 12-vitamin solution, vitamin B12 solution, bicarbonate, and 10 mM acetate. Replicate samples were held under an incandescent light bulb (visible spectrum), an infrared radiation LED cluster (940 nm), and dark conditions. Samples were exposed to continuous light, IR, or dark exposure at room temperature.

Cultures. A purple sulfur bacteria was isolated from purple sulfur bacteria aggregates ('purple berries') and grown in a sulfide rich artificial seawater media as described above with the modification of 2.5 mM sulfide and a more acidic pH of 6.8. A seawater sample from the Woods Hole Oceanographic Institution dock was plated on 1.5% seawater complete media. The pink colored colony was streak purified and grown in the seawater media described above with the modification of an initial growth curve experiment with different 10 mM carbon sources including succinate, acetate, citrate, glycerol, and glycerol with yeast and tryptone.

Growth and pigment production. Growth of enrichments and cultures was monitored by optical density at 600nm using a spectrophotometer. Pigment absorbance was measured using 1 ml whole cell suspensions. 3 ml of enrichments were also centrifuged and the cell pellet pigments extracted in methanol for 4 hours. The absorbance scans were conducted from 1100-400 nm on a spectrophotometer.

Taxonomic identification. Environmental DNA from approximately 1g of pink colored marsh mat sediment was extracted using the Ultra Clean Soil DNA kit (Mo Bio). A 300 ml seawater sample from the NOAA pier was also filtered and extracted using the kit with unsuccessful extraction results. A seawater DNA sample was provided courtesy of Gail Ferguson. This sample was extracted from 40 L of filtered seawater using the CTAB method with hexadecyltrimethylammonium bromide. Cultured cells were suspended in 20 µl of water and cleaned with 0.1 µl P40 detergent, and boiled at 102°C for 5 minutes. The DNA from environmental extractions or single cell isolates was then amplified using PCR with 8F and 1492R universal bacterial primers. Single isolates were cleaned with ExoSAP and sequenced. The environmental PCR products were cloned into TopoII plasmid vector and grown in *E. coli*

cells using the Topo TA cloning kit (Invitrogen) followed by plasmid preps and sequencing. All sequences were aligned using ARB software and maximum parsimony phylogenetic tree construction.

RESULTS

The seawater culture used in the IR study was initially identified as a *Roseobacter* sp. culture. During the experiments, it did not produce any pigmentation and was identified using 16S rRNA as a *Halomonas* sp., a non-phototrophic gamma-proteobacteria (Figure 1a). *Halomonas* sp. was found to grow in both light and dark conditions on a variety of substrates including acetate, succinate, citrate, glycerol, and glycerol with tryptone and yeast. The doubling time of Halomonas sp. was ~20-26 hours depending on the media with acetate and glycerol additions resulting in greater growth compared to citrate and succinate (Figure 1b). Growth was greatest under incandescent light and comparatively less under IR and dark conditions (Figure 1c). There was no detectable pigment production in any of treatments.

The environmental seawater sample did not show IR growth that was detectable from the dark treatment. The initial log growth of the light treatment sample was greatest compared to IR and dark, with no detectable difference between the treatments after 60 hours. The community composition of each treatment was examined to determine if particular microbial groups were dominant. All treatments heavily enriched for gamma-proteobacteria and *Acrobacteria* (Figure 2a). There were two groups that were found in all treatments in nearly equal clone library representation - *Marinomonas* sp. and *Acrobacter* sp. *Oceanospirrilum* sp. was also was present in all treatments, but prominent in the light treatment with 12 out of 31 light treatment clones (39%). The IR treatment did appear to enrich for microbes similar to *Neptunomonas*, a gamma Proteobacteria capable of degrading aromatic hydrocarbons. None of the enriched microbes were present in the seawater clone library (Figure 2b), however the clone library only represents 17 clones due to unidentified cloning or sequencing problems. The majority of these clones cluster into the alpha proteobacteria SAR 11 clade (11 clones), gamma proteobacteria SAR 86 clade (3 clones), and single gamma proteobacteria *Alteromonadaceae* and *Actinobacteria* clones. No known IR absorbers were identified from the seawater clone library or enrichment studies.

An axenic culture of an anaerobic IR-absorbing purple sulfur bacterium was isolated from purple sulfur bacteria 'purple berries' from Sippewissett Marsh using deep agar shakes (Figure 3a). The rod shaped cells contained sulfur globules (Figure 3b) and were found to display absorbance peaks at 850, 805, and 590 nm, typical of the IR absorbing pigment bChl *a*. The isolate was identified using 16S rRNA as *Marichromatium purpuratum* (Figure 3d). The isolate was not obtained in time for an IR growth and pigment production experiment to be conducted.

Enrichment cultures of pink colored microbial marsh mat from Sippewissett Marsh containing purple sulfur bacteria and purple non sulfur bacteria (Figure 4a) were exposed to the light, IR, and dark experimental conditions. Enrichments with H_2CO_2 media resulted in colored growth under all light conditions, with greatest pigment production in light and IR treatments (Figure 4b). The light and IR treatments were found to contain major absorbance peaks at 860, 790, 590, and 450 nm, corresponding to bChl *a*, with more pronounced peaks in the light treatment (Figure 4c). Methanol extracted pigment spectra showed peaks at 790, 660, 610, 520, 460, 440, and 360nm, which probably correspond to structural degraded/extracted bChl products and carotenoids.

The sulfide rich media for PSB resulted in greatest growth under IR conditions, with less growth in light and dark treatments (Figure 5a). The IR treatment displayed absorbance peaks at 1029, 835, 604, and 404 nm, consistent with the whole cell absorbance spectra of bChl *b* (Figure 5b). The peaks at 530, 490 and 459 nm are consistent with the carotenoid 3,4,3',4'-tetrahydrospirilloxanthin. The light treatment displayed a spectra consistent with bacteriochlorophyll *a*, and no peaks were detectable in the dark treatment that could be verified independent of cell scatter. The methanol extracted absorbance spectra shows peaks for the light and dark treatment with offsets for the IR treatment, which are consistent with the whole cell absorbance light and IR differences (Figure 5c). The IR treatment was examined under 100x oil immersion to primarily contained motile coccoid shaped cells containing sulfur globules (Figure 5d). Repeated DNA extractions using a variety of protocols were attempted with unsuccessful results. DNA extractions were successful for the pink colored Sippewissett marsh mat, but several PCR amplification attempts were all unsuccessful. Therefore, a 16S rRNA clone libraries were not constructed. However, pigment composition and microscopy evidence suggests the enrichment is dominated by *Thioflaviococcus mobilis*.

DISCUSSION

This study successfully enriched for IR absorbing microbes containing bChl *a* and *b* under both light and IR conditions using pink colored marsh mat inoculum. The growth of the bChl *b* containing microbe was only obtained under the IR conditions and sulfide rich media. The coccoid shape, mobile cells, intracellular sulfide globules, and absorbance spectra consistent with the pigments bChl *b* and 3,4,3',4'-tetrahydrospirilloxanthin suggest that the IR enrichment contains the purple sulfur bacteria (gamma-proteobacteria) *Thioflaviococcus mobilis*, first described by Imhoff and Pfenning (2001). This species was first isolated during the 1986 *Microbial Diversity* course from a peach colored layer of laminated microbial mat at the Great Sippewissett salt marsh. Imhoff and Pfenning (2001) reported that cultured cells appear peach colored. Therefore, the pink colored IR PSB enrichment was probably not a pure culture of bChl *b* containing cells and likely also contained bacteriochlorophyll a containing cells. *Thioflaviococcus mobilis* is an obligate phototroph which grows anaerobically by oxidizing sulfide to elemental sulfur and sulfate and photoassimilates acetate, pyruvate, and ascrobate. A similar bChl *b* containing species is the immobile *T. pfennigii* (Eimhjellen, 1970). I was unable to differentiate between non-mobile viable cells and non-viable mobile cells and this may be a mixed culture of the two species. A 16S clone library could be used to resolve the taxonomic identification.

The H_2CO_2 media also enriched for IR absorbing microbes containing bChl *a* with under both light and IR treatments. Throughout the summer, fellow students were also able to enrich for bChl *a* containing phototrophs under the incandescent light treatment. Growth of the bChl *a* containing microbes appears to be optimal under the incandescent light conditions, but the IR treatment and PSB media were the only conditions which resulted in a far IR absorbing microbe containing bChl *b*.

The low nutrient seawater enrichment treatment and IR conditions did not enrich for known IR absorbing microbes or growth that resulted in measurable pigment production. One interesting group that was only present in the IR treatment clone library was *Neptunomonas*, a gamma Proteobacteria capable of degrading aromatic hydrocarbons. Some marine bacteria in the *Roseobacter* clade, known IR absorbers, are also capable of degrading aromatic hydrocarbon

compounds in the same method as *Neptunomonas* (Buchan et al. 2000). It is unknown what role if any IR absorbance has in this degradation process.

Cultured growth and pigment production studies under IR conditions were not successful for either aerobic seawater or anaerobic intertidal sediment microbes. Growth response to exclusively IR conditions has previously been studied using filters that block out visible wavelengths, but has not been attempted with a defined IR wavelength. This particular type of study is interesting because IR-absorbing photosynthetic microbes contain much lower concentrations of pigment and display about an order of magnitude less CO_2 fixation compared to visible light absorbing microbes in their ambient environment (Koblizek et al. 2003). It is possible that both pigment and photosynthetic production may increase under IR exclusive conditions. Understanding this response will aid in better understanding their potential physiology and production in oligotrophic environments where IR is available for phototrophic growth but heterotrophic growth is limited.

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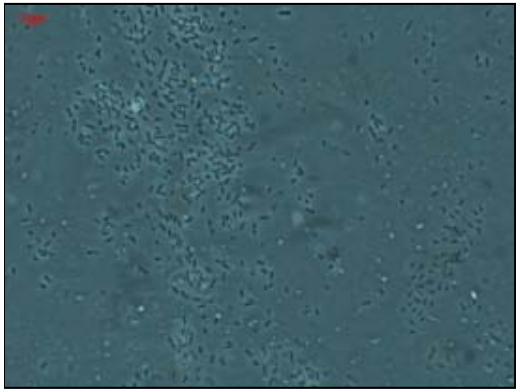

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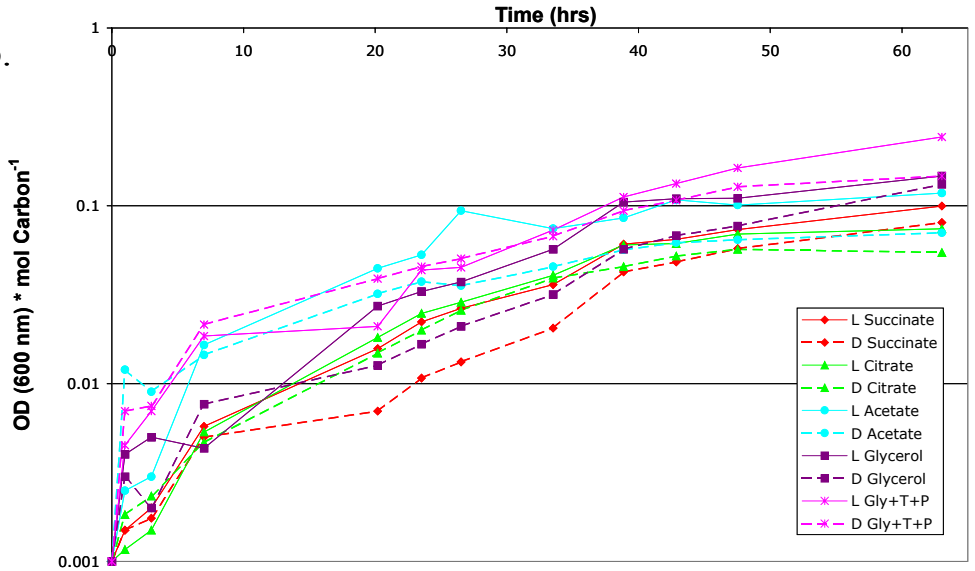

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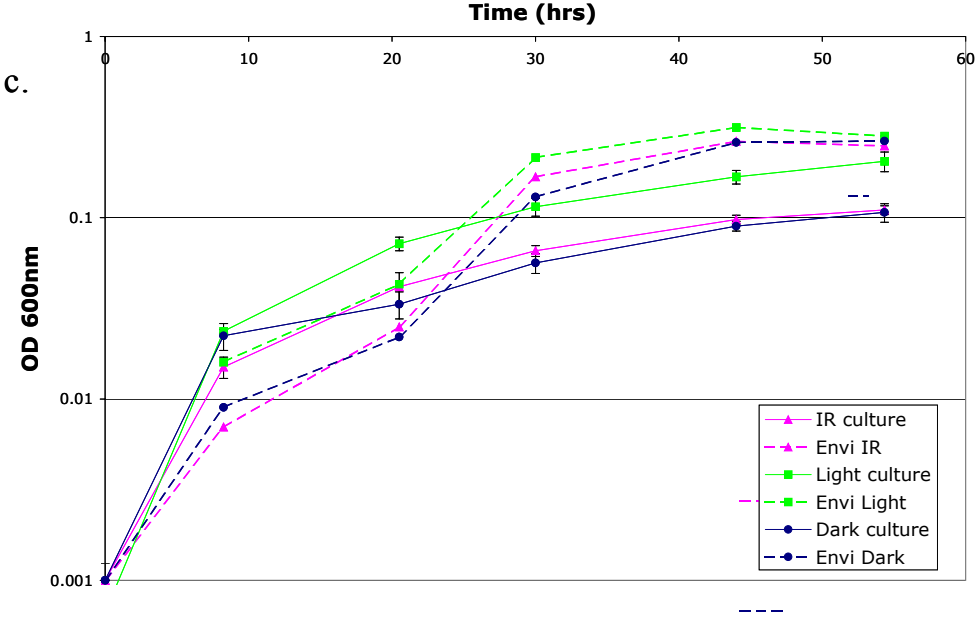

Figure 1. a, *Halomonas* sp. culture under 100x phase contrast oil immersion. b, Growth experiment on *Halomonas* sp. cultures with varying carbon substrates including succinate, citrate, acetate, glycerol, and glycerol with yeast and tryptone. Solid lines are light and dotted indicate dark held cultures. c, Growth curve of *Halomonas* sp. culture (solid line) and environmental seawater samples (dotted line) grown in infrared radiation (IR), light, and dark conditions.

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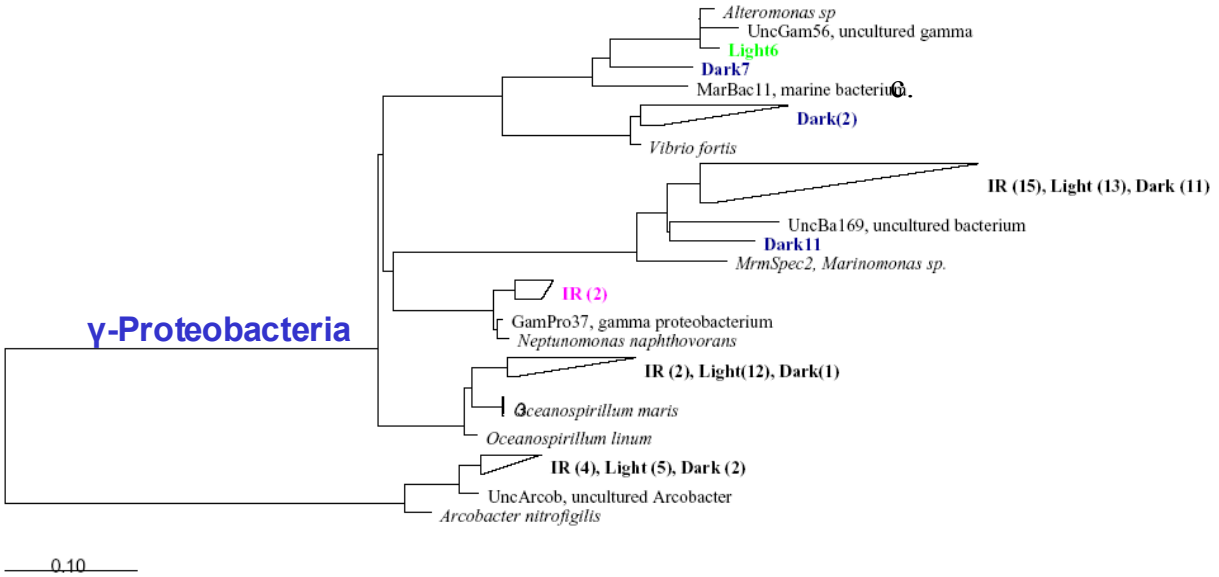

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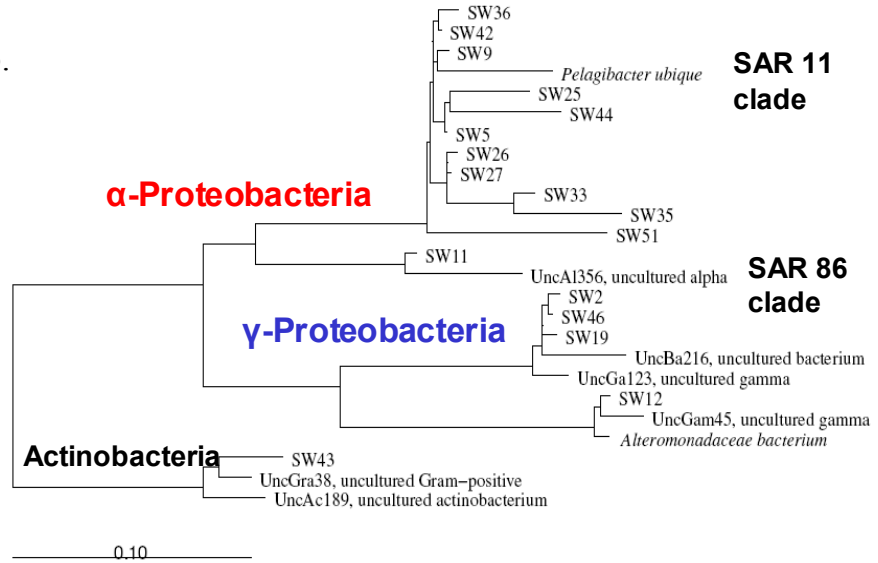

Figure 2. Comparison of clone library microbes from a, ambient seawater and b, phototroph enrichment experiment with clones designated by light source and number of clones recovered (n).

Figure 3. Characterization of *Marichromatium purpuratum* isolate from Sippewissett Marsh purple berries. a, Deep agar shakes with purple colonies, b, enrichment culture (non-pure) with sulfur-containing cells, c, whole cell absorbance spectrum with bChl *a* peaks (prominent 805 and 865nm), and d, 16S maximum parsimony tree with PSB (Sipp Berries) isolate.

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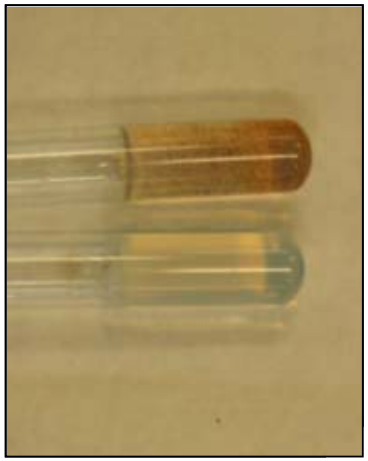

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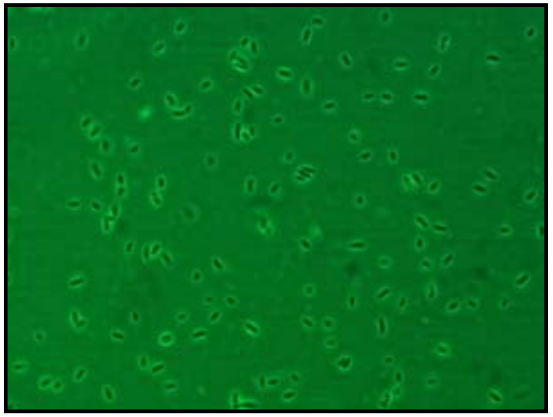

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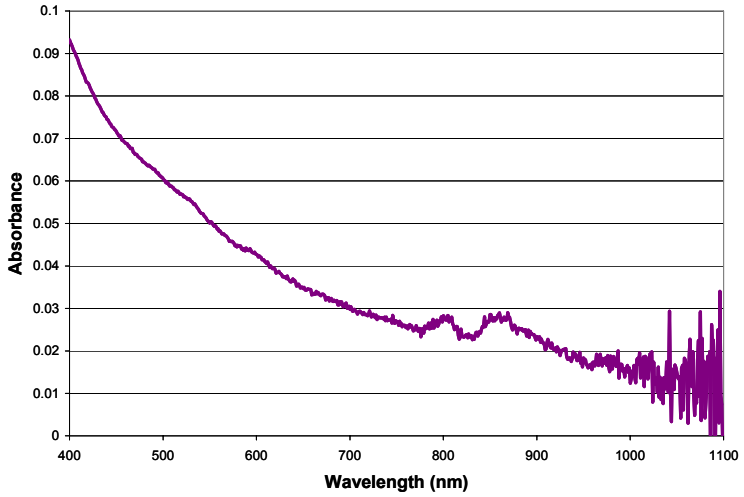

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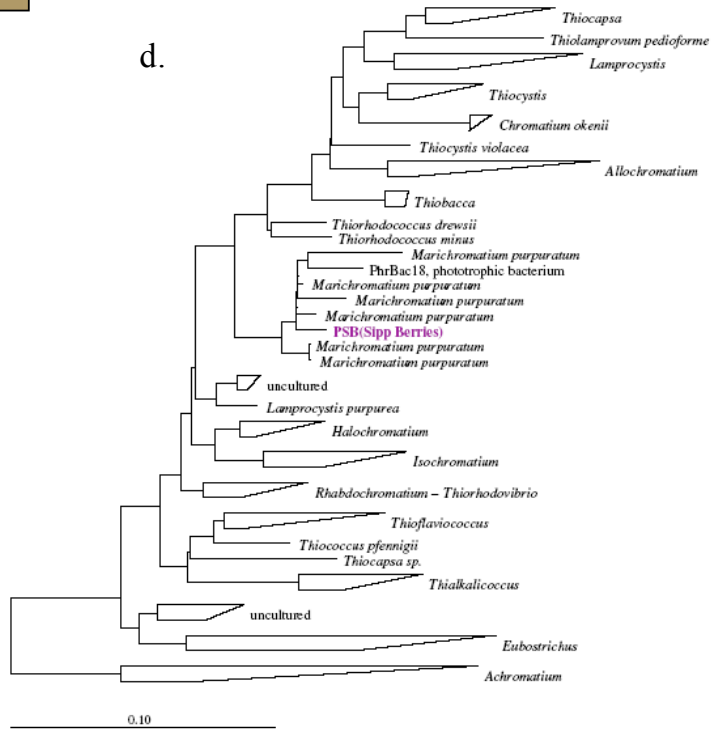

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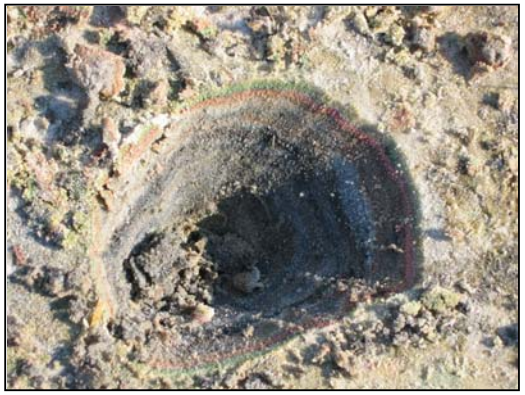
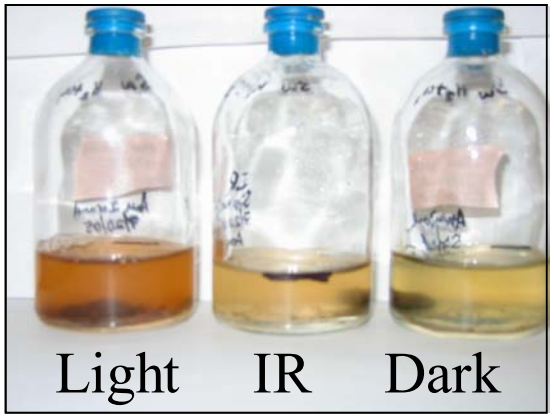


Figure 4. a, Enrichment bottles with H_2CO_2 media at day 8. b, whole cell absorbances with major bChl *b* peaks in IR and bChl *a* peaks in light enrichments. c, methanol extracted absorbance, and d, Sippewissett marsh mat displaying pigmented phototroph layers and pink/peach layers used for enrichments.

b.



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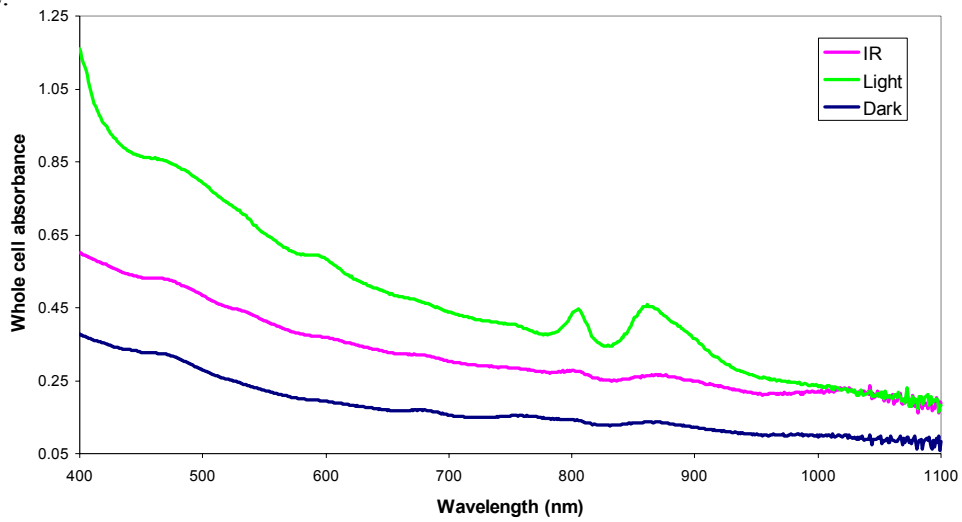

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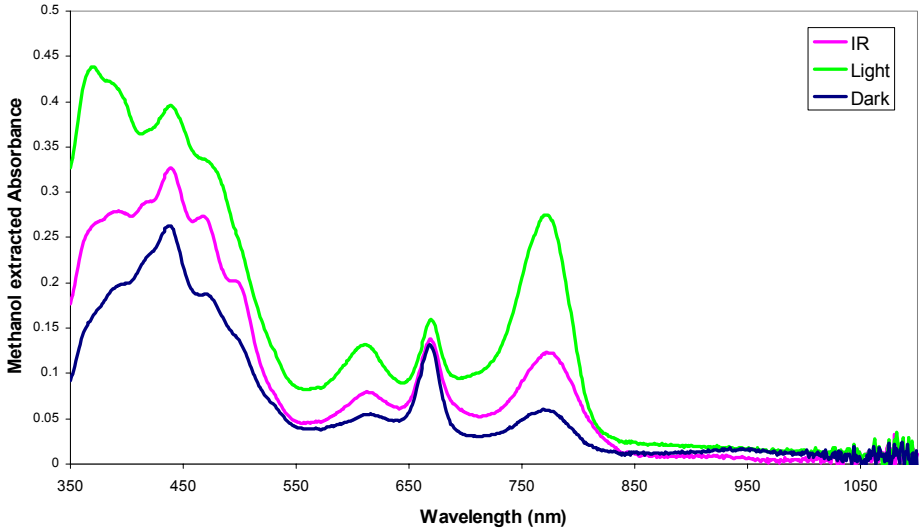

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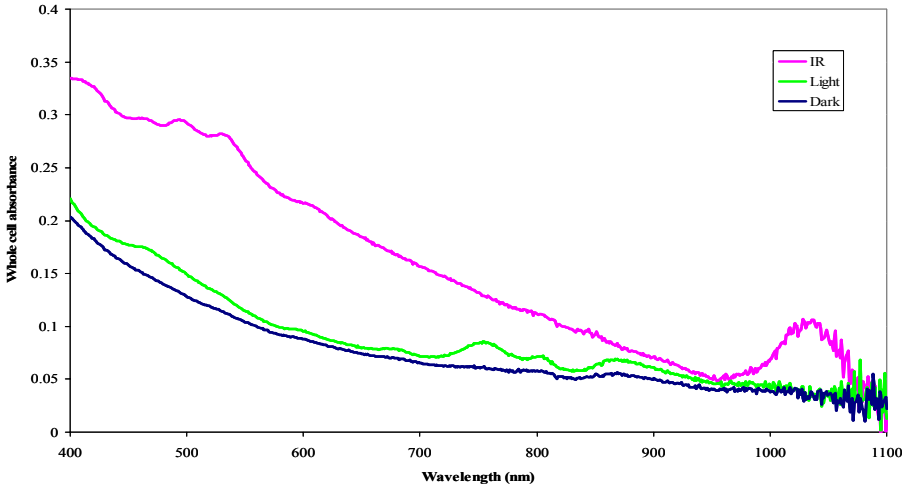
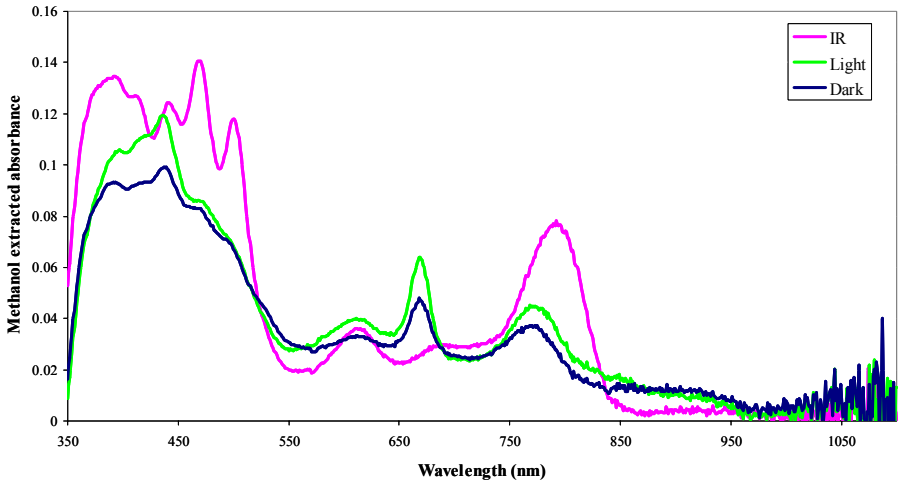


Figure 5. a, Enrichment bottles with PSB media at day 8. b, whole cell absorbances with major bChl *b* peaks in IR and bChl *a* peaks in light enrichments. c, methanol extracted absorbance, and d, 100x phase contrast image of IR enrichment cells.

c.



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