Oxidation of Ferrous(II) Iron by Phototrophic Bacteria and the Continuing Search for the Elusive Manganese(II)-Oxidizing Phototrophs: Enrichment from the Salt Pond Water Column

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Abstract

Salt Pond (Woods Hole, Massachusetts) is a brackish pond with an oxic-anoxic transition zone (OATZ) about 3.5m below the surface. Enrichment cultures for the selection of ferrous(II) iron- and manganese(II)-oxidizing phototrophs were made, and phototrophic iron-oxidation was observed in cultures that contained ferrous iron as the only electron donor. Spectral analysis indicated that iron-oxidizing enrichments contain phototrophic bacteria with bacteriochlorophyll (Bchl) a and possibly Bchl b. Although no direct evidence for phototrophic manganese-oxidation was observed, the addition of manganese as the sole electron donor stimulated phototrophic growth.

Introduction

The oxidation of ferrous iron (II) and its use as a sole electron donor for anoxigenic photosynthesis was a matter of speculation for years (Hartman, 1984), and it was recently demonstrated by Widdel et al. (1993) in phototrophic bacteria possessing photosystem I. This form of bioenergetics is not only interesting from a metabolic perspective, but is also relevant in paleoecological discussions concerning ancient (3.8×10^9 years) banded-iron formations (BIFs) deposited when there was little free oxygen in the atmosphere. Oxygenic photosynthesis can indirectly result in the oxidation of ferrous (II) to ferric (III) iron, and it is generally accepted that oxygen production is responsible for the formation of BIFs. However, oxygenic photosynthesis requires photosystem I and II: an complex biological system that may not have been present in microorganisms existing several hundred million years after the origin of life.

The physiological and ecological importance of phototrophic oxidation of ferrous iron is an open question. Heising and Schink (1998) examined type-strains related to

isolates enriched using ferrous iron as the sole electron donor, and it was found that over half were able to oxidize various Fe (II) salts. This suggests that this phenotype is distributed within many anoxigenic phototrophs, and may be an environmentally significant component of the global iron cycle. An interesting problem faced by microorganisms oxidizing soluble ferrous iron is that ferric iron is insoluble, accrues on the exterior of the cell, and eventually prevents further substrate entry. To combat this problem, iron-oxidizing phototrophs must find a way to remove the highly insoluble ferric precipitates by either possessing enzymes to reduce them, or existing within ecological niches containing iron-reducing microorganisms.

Salt Pond (Woods Hole, MA) is a brackish pond that is chemically and thermally stratified, has a maximum depth of ~4.5m, and the oxic-anoxic transition zone (OATZ) occurs at approximately 3.5m. Geochemical analysis of iron (dissolved and particulate Fe(II) and particulate Fe(III)) within the water column of Salt Pond by Bazylinski et al. (1997) revealed a peak in particulate Fe(II) concentration at the OATZ. This suggests that microbial oxidation of ferrous iron by anoxygenic phototrophic bacteria may be occurring within the OATZ. Therefore, enrichments for ferrous(II) iron-oxidizing phototrophs were constructed with the goal of culturing microorganisms capable of this process. Media was also designed to select for phototrophs capable of oxidizing the chemically related metal manganese(II).

Materials and Methods

Source of enrichments. Pond water was collected in June and July of 1999 at increasing depth from Salt Pond (Woods Hole, Massachusetts) and utilized for enrichment cultures and nutrient/elemental analysis. Profiles were constructed for

oxygen, sulfide, manganese, nitrate/nitrite, ammonia, pH, temperature, and salinity (described in Salt Pond water column group report, 1999). Samples from 3.25m, 3.5m, and 3.75m below the surface were utilized as inocula for phototrophic ferrous iron- and manganese-oxidizing enrichments. Approximately 250ml of pond water from each depth was centrifuged to concentrate microbial biomass and remove dissolved organic compounds, the pelleted material was resuspended in 5ml 1/2 strength sterile sea water, and 100µl of this suspension served as the culture inoculum.

Media and growth conditions. Enrichments were cultivated on a modified medium designed for phototrophic non-sulfur bacteria from brackish environments and contained the following per liter: 0.33g of $(NH_4)_2SO_4$, 0.493g $MgSO_4 \bullet 7H_2O$, 0.147g $CaCl_2 \bullet H_2O$, 17.53g NaCl, and 0.37g of KCl. In sulfate-free media, the magnesium source was replaced with $MgCl_2 \bullet 6H_2O$ and the nitrogen source was replaced with NH_4Cl_2. The basic salt medium was autoclaved and cooled to 45°C under N_2/CO_2 (9:1) and the following components were added aseptically from sterile stock solutions: 1ml of trace element solution (Kaiser and Hanselmann, 1982), 6 ml of 1M $NaHCO_3$, 4ml of 1M KPO_4 (pH 7.0), 1ml of 1000X vitamins solution (Kaiser and Hanselmann, 1982), 5ml of 10% yeast extract (filter sterilized - 0.2 µM), and 0.4ml $Na_2S. \bullet 9H_2O$ (100mM, pH 8.0). Acetate and pyruvate were added to final concentrations of 1mM and 0.8mM, respectively, and H_2/CO_2 was provided in an 80:20 (vol/vol) ratio. $FeSO_4$ was added to a final concentration of 2mM, and chelated with an equimolar amount of nitrilotriacetate (NTA). Cyclohexamide was added to a final concentration of 0.01% to prevent the growth of photosynthetic algae. $MgCl_2$ was supplemented to a final concentration of 4mM, which resulted in the formation and precipitation of rhodochrosite ($MnCO_3$).

Cultures were incubated in 50ml Pfennig bottles at 25°C roughly 40cm from a 40W bulb on a 12 hour light-dark cycle.

Spectral analysis of carotenoid pigments and chlorophyll. In vivo analysis of photosynthetic pigments was performed by absorbance of cell suspensions from 350-950nm on a Varian Cary 50 double-beam spectrophotometer. Pigments were extracted with 7:2 acetone/methanol. Cells were harvested by centrifuging 1.5ml of culture, and resuspending the pellet in extraction solvent. Extracts were acidified by adding 10µl of 2N HCl to 100µl of extract (final pH 2.0)

Results and Discussion

Enrichments from 3.25m, 3.5m, and 3.75 m depths were constructed that contained 2mM $FeSO_4$ and 4mM $MnCl_2$ supplemented with acetate or pyruvate and were incubated in the light. After 7 days, the formation of red-brown precipitate was observed in an enrichment from a 3.5m sample that contained 2mM $FeSO_4$ and 1mM acetate. However, when this culture was examined under the microscope, eukaryotic algae were present along with various bacteria. The observed formation of ferric iron could thus be a result of an indirect oxidation fueled by oxygenic photosynthesis. To verify that this enrichment also contained microorganisms utilizing ferrous iron for phototrophic metabolism, a subculture in the same medium was made including 0.01% cycloheximide. After 10 days incubation, ferric iron precipitation was observed in these cultures. Microscopic examination revealed that the precipitate was densely packed with bacteria, consistent with ferrous iron being utilized as an electron donor, resulting in the accumulation of iron oxides on the cell surface (Figure 1). These cultures were subsequently spiked with 2mM more $FeSO_4$, to provide additional substrate. In

enrichments containing manganese and the cosubstrates acetate or pyruvate as potential electron donors, phototrophic growth occurred, but no evidence for manganese-oxidation (formation of grayish-black precipitate) was observed.

To assess the composition of the phototrophic species that inhabit the pink layer, in vivo spectrophotometry of whole cells was performed (Figure 2). A major peak is present at 723nm, indicating the presence of bacteriochlorophyll (Bchl) e, which is present in green sulfur bacteria such as *Chlorobium phaeobacteroides*. A similar pattern was observed in Winogradsky enrichments of water from the 3.5m layer incubated in the light (Figure 3). Further analysis included extraction of carotenoids and chlorophyll from the 3.5m layer (Figure 4) and from enrichments in which iron oxidation was observed (Figure 5). The largest peak in acidified extracts from the 3.5m layer is consistent with the presence of Bchl e (~660nm). Peaks are also observed at 605nm (Bchl b or Chl a) and 545nm (Bchl a). Spectral patterns of extracted pigments from iron-oxidizing cultures that contained acetate and pyruvate were identical, with a major peak at ~770nm (Bchl a) and a smaller peak at 605nm (Bchl b or Chl a).

A major oversight in the construction of the original enrichment media was that sulfate, in the form of $MgSO_4$, $(NH_4)_2SO_4$, and $FeSO_4$, was at a concentration of 12mM. In the presence of acetate, pyruvate, and H_2/CO_2, sulfate-reducing bacteria (SRB) quickly dominated many cultures, producing sulfide (HS^-), which serves as an electron donor for anoxygenic sulfur phototrophs. Sulfide production in iron-containing cultures resulted in the formation of iron sulfide (FeS), which forms a black precipitate. The enrichment medium was therefore modified to include a sulfate-free magnesium and nitrogen source.

However, the instability of $FeCl_2$ made it necessary to use $FeSO_4$ as the ferrous iron source, and the second round of enrichments included 2mM sulfate.

A basic salt medium with decreased sulfate concentration was inoculated with fresh samples from the Salt Pond water column, and electron donors were provided as follows: 500μm acetate (half the amount used in the first enrichment) and 2mM $FeSO_4$, 500μm acetate and 2mM $MnCl_2$, 2mM $FeSO_4$, and media without an electron source. Within one week, cultures with ferrous iron and acetate turned black, indicating SRB activity. Cultures containing manganese(II) and acetate were turbid and colored indicating phototrophic growth. Since sulfate was completely excluded from the latter, growth was attributed to non-sulfur phototrophs. Of particular interest were the enrichments that contained iron as the only electron source. Two cultures turned black, denoting the formation of iron sulfide (acidification in 2N HCl confirmed that the black precipitate was not magnetite). Since no organic carbon was provided, the only way to explain such a result is through phototrophic iron oxidation. Phototrophic microorganisms using ferrous iron as the sole electron donor fix CO_2 and excrete acetate, which would be utilized by SRB and result in the formation of iron sulfide. In addition, after several days of further incubation, there appeared to be a decrease in iron sulfide, followed by the formation of a red-brown precipitate that resembled ferric iron. This suggests that iron-oxidizing phototrophs began to use iron sulfide as an electron donor, which has been reported previously (Ehrenreich and Widdel, 1994; Heising and Schink, 1998). However, it can not be excluded that the sulfur component of iron sulfide was used as an electron source, and the red-brown precipitate was merely the formation of pigmented biomass.

A series of enrichments that did not contain an electron source remained clear after 1 week of incubation. These cultures were subsequently spiked with 2mM $FeSO_4$, 2mM $MnCl_2$, and 500μM acetate. After several days, turbidity was observed in all cultures. The cultures that contained iron and manganese were both green-brown, and growth on acetate was yellowish-red. Although no black-brown precipitate was observed, which would be a positive indication of manganese oxidation, phototrophic growth initiated only after the addition of manganese.

Comments

Strategies for the enrichment of iron-oxidizing phototrophs should be designed to exclude sulfate from the culture media. The inclusion of $FeSO_4$ as a source of iron made it difficult to definitively interpret many of the results. The use of the less stable compound $FeCl_2$ is therefore recommended in future enrichments. In addition, the presence of an absorbance peak at 605nm may be due to the presence of cyanobacteria in enrichment cultures. This could be corroborated by adding DCMU (2mM final) to subcultures and using spectral analysis to observe if the 605nm peak disappears in its presence.

Although the presence of manganese-oxidizing phototrophs was not confirmed in this study, it is clear that when present as the only electron donor, anoxygenic phototrophic growth occurred. A more thorough analysis of the forms of manganese produced via oxidation by microorganisms may make it possible to identify this thermodynamically favorable process, that if exists, still eludes detection by laboratory culture.

References

Bazylinski et al. 1997. Biomineralization of magnetic iron minerals. In: Geomicrobiology: interactions between microbes and minerals, ed. Banfield, J.F. and K.H. Nealson

Ehrenreich, A. and F. Widdel. 1994. Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. Appl. and Environ. Microbiol. 60:4517-4526.

Hartman, H. 1984. The evolution of photosynthesis and microbial mats: a speculation on the banded iron formation. Microbial mats: stromatolites, pages 449-453 Alan R. Liss, Inc. New York.

Heising, S. and B. Schink. 1998. Phototrophic oxidation of ferrous iron by a *Rhodomicrobium vannielii* strain. Microbiology 144:2263-2269.

Kaiser, J-P and K.W. Hanselmann. 1982. Fermentative metabolism of substituted monoaromatic compounds by a bacterial community from anaerobic sediments. Arch. Microbiol. 133:185-194.

Widdel, F., S. Schnell, S. Heising, A. Ehrenreich, B. Assmus, and B. Schink. 1993. Ferrous iron oxidation by anoxygenic phototrophic bacteria. Nature 362:834-836.

Figure Legends

Figure 1 - Micrograph of a phototrophic iron-oxidizing culture grown in the presence of 2mM $FeSO_4$ and 1mM acetate.

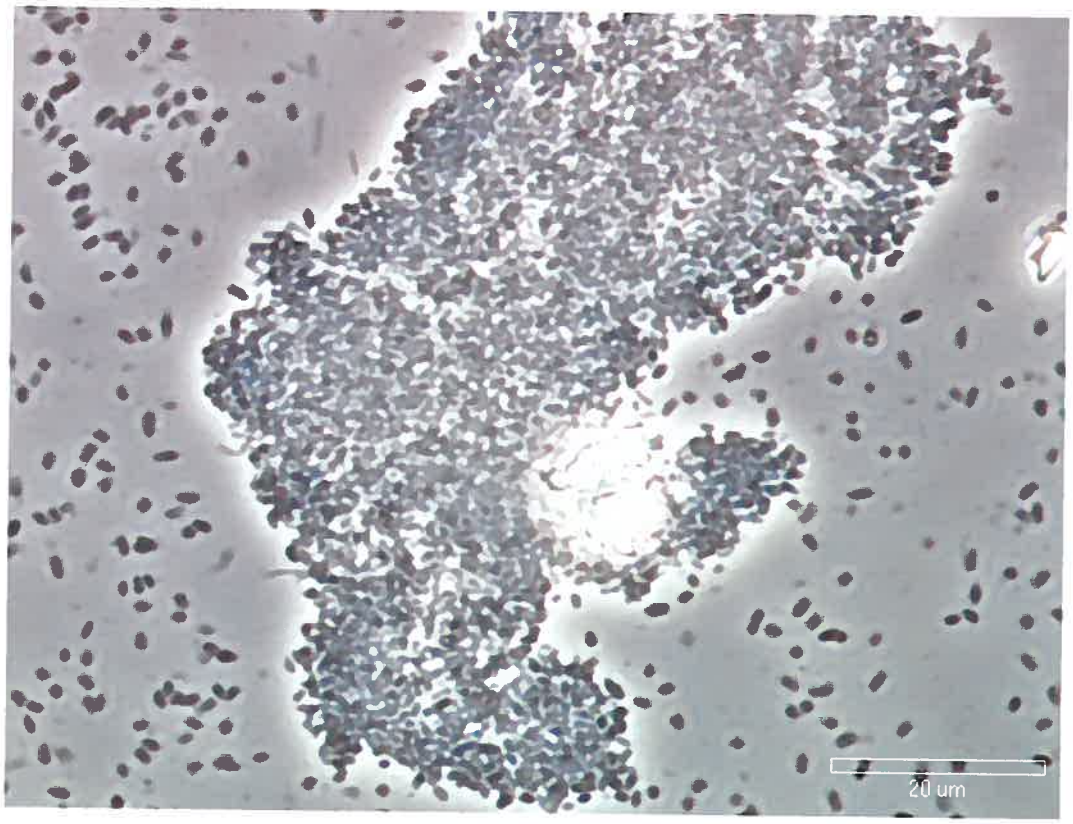
Figure 2 - In vivo spectral analysis of cells from 3.5m below the surface of Salt Pond.

Figure 3 - In vivo spectral analysis of cells from a Winogradsky enrichment of water from the 3.5m pink layer.

Figure 4 - Spectral analysis of carotenoids and chlorophyll extracted from the 3.5m pink layer. Acidified with HCl to a final concentration of 1N (pH 2.0).

Figure 5 - Spectral analysis of carotenoids and chlorophyll extracted from phototrophic iron-oxidizing cultures containing 1mM acetate and 0.8mM pyruvate.

Iron-Oxidizing Phototrophs



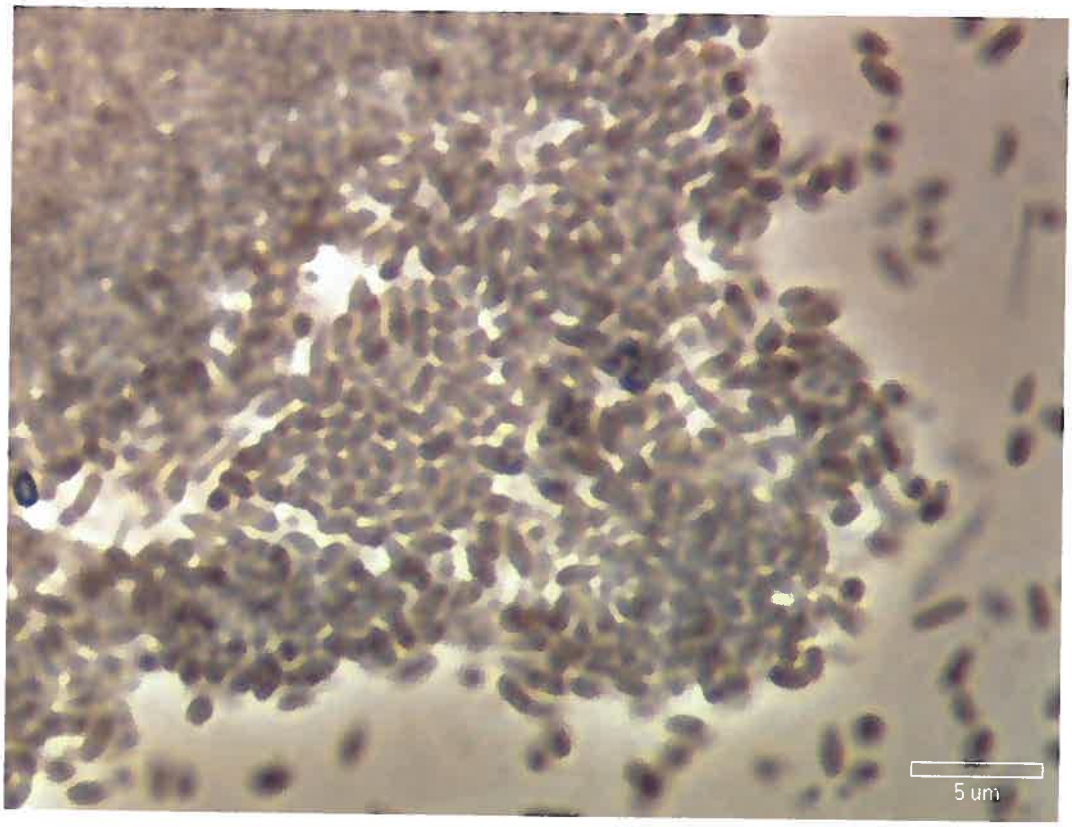


Figure 1

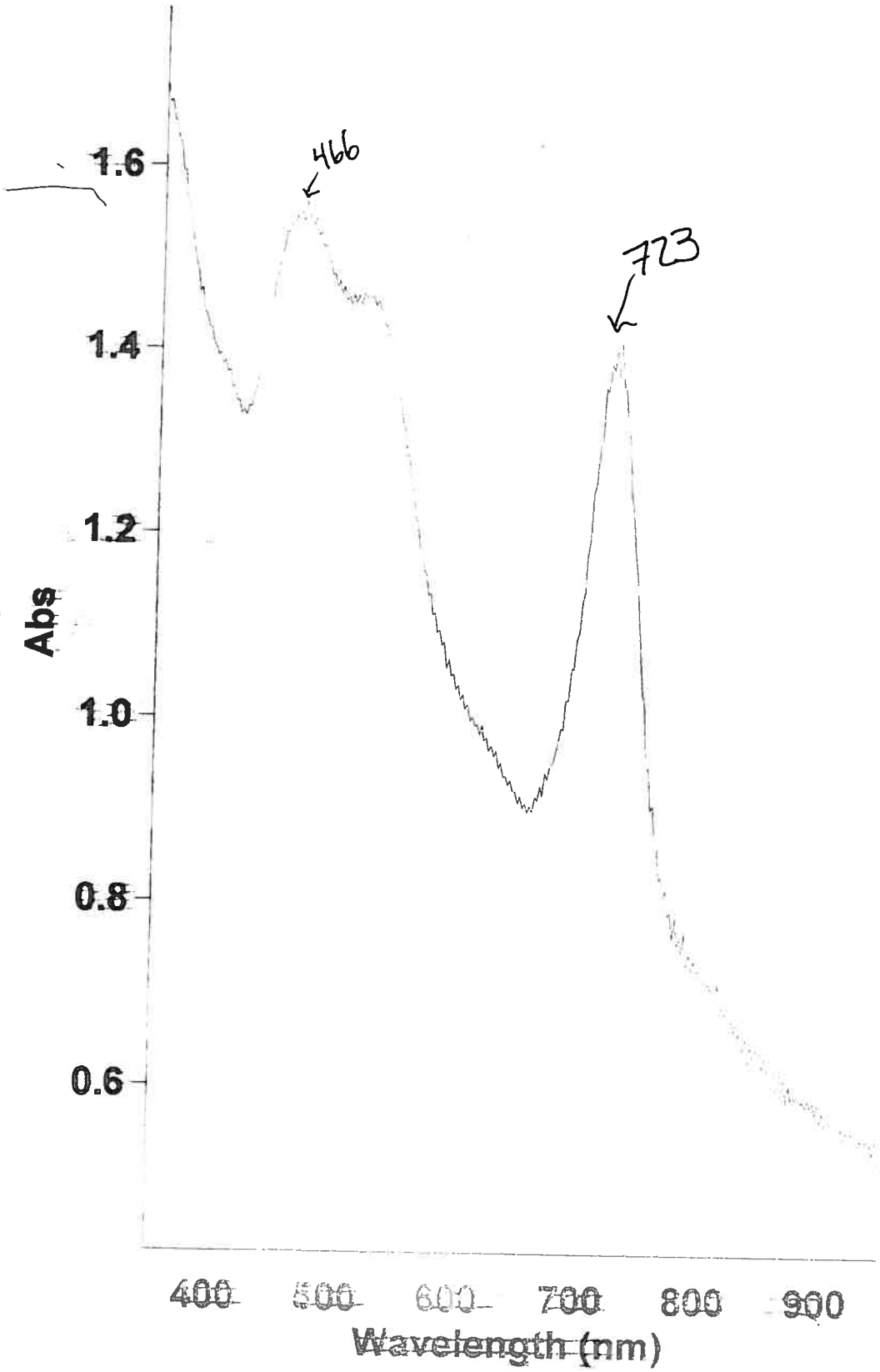

466
723
1.6
1.4
1.2
1.0
0.8
0.6
Abs
400
500
600
700
800
900
Wavelength (nm)

Figure 2

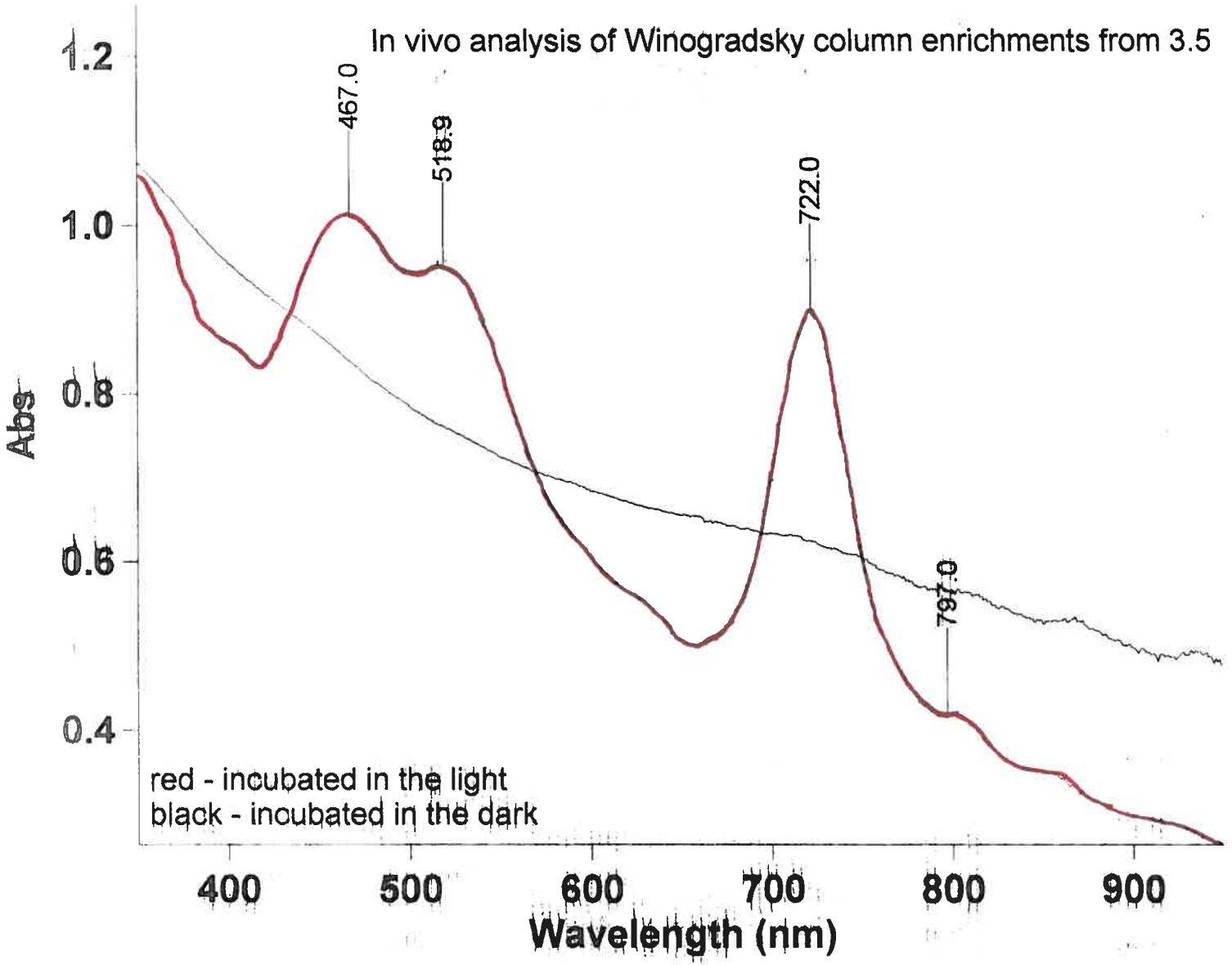

In vivo analysis of Winogradsky column enrichments from 3.5
467.0
518.9
722.0
797.0
1.2
1.0
0.8
0.6
0.4
Abs
red - incubated in the light
black - incubated in the dark
400
500
600
700
800
900
Wavelength (nm)

Figure 3

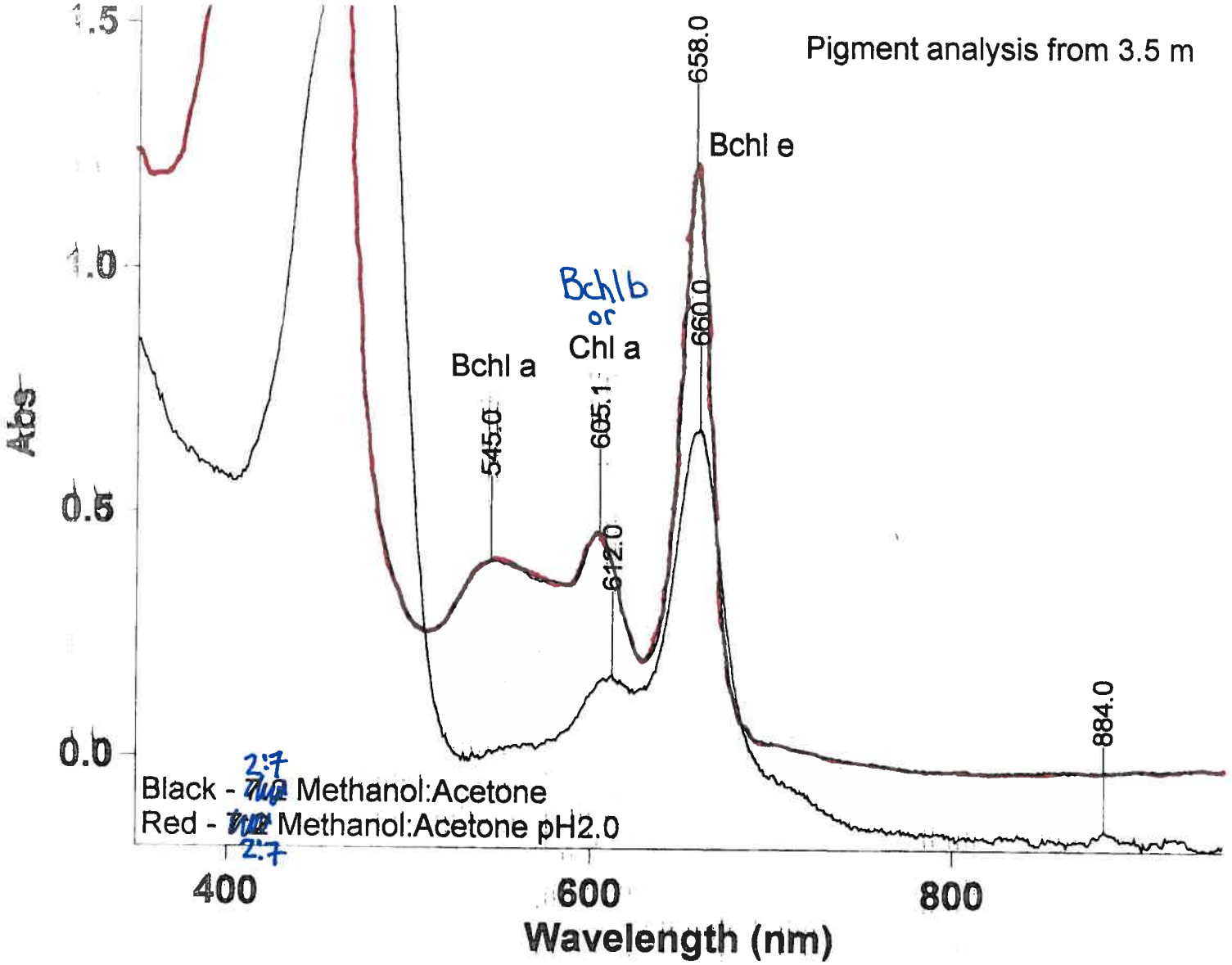

Pigment analysis from 3.5 m
658.0
Bchl e
Bchl b
or
Chl a
660.0
Bchl a
545.0
605.1
612.0
884.0
Abs
1.5
1.0
0.5
0.0
Black - 2:7 Methanol:Acetone
Red - 2:7 Methanol:Acetone pH2.0
400
600
800
Wavelength (nm)

Figure 4

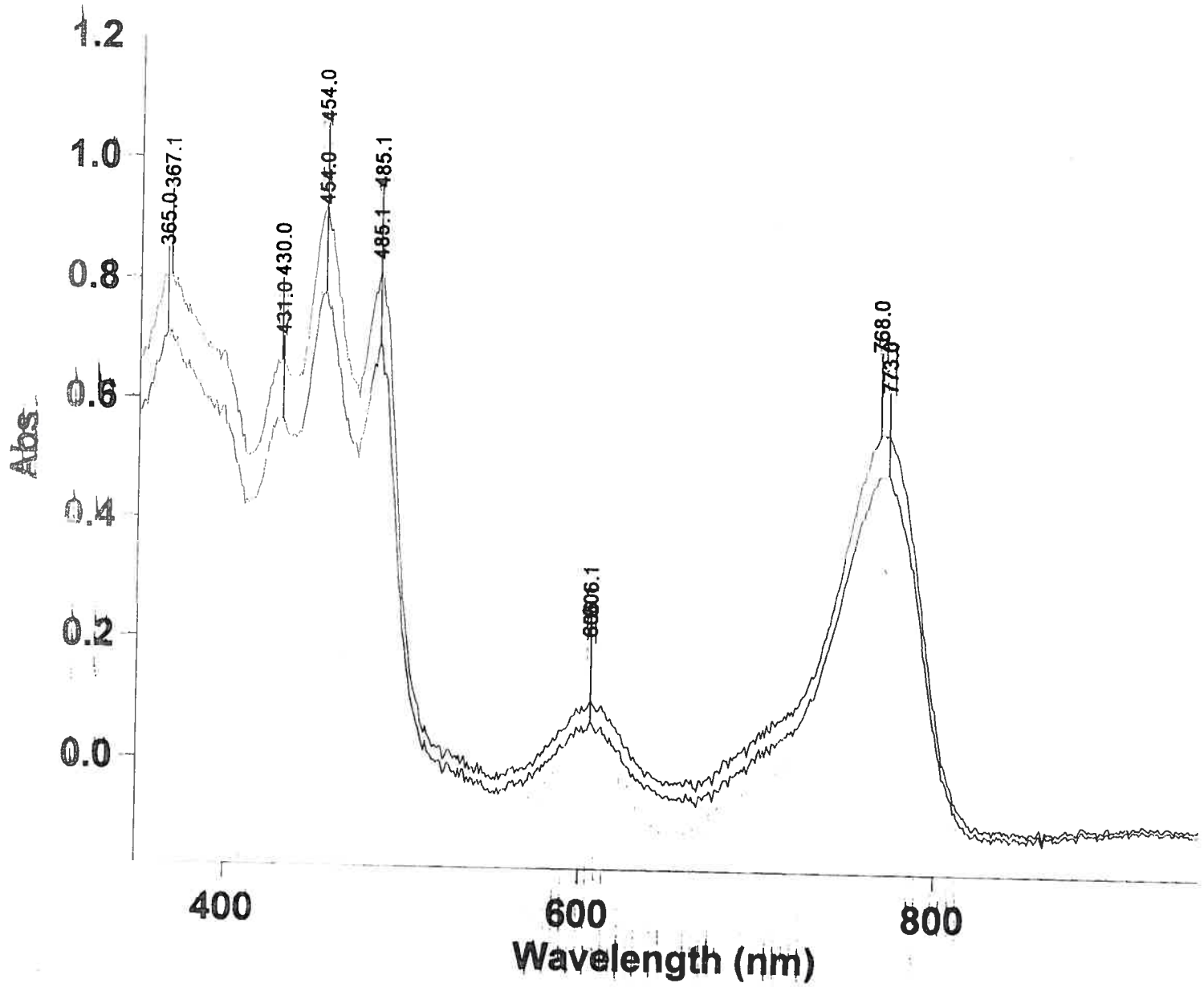

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Abs
365.0
367.1
431.0
430.0
454.0
454.0
485.1
485.1
606.1
768.0
773.0
400
600
800
Wavelength (nm)

Figure 5