Anoxygenic Phototrophy and Nitrogen Limitation in Salt Pond

James Peter Sáenz
MIT-WHOI Joint Program in Chemical Oceanography
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INTRODUCTION

Nitrogen fixation is an important pathway in the global nitrogen cycle and provides a means for productivity to persist in environments where fixed forms of nitrogen (NO3, NH4) are consumed to growth limiting levels. Traditionally it is accepted, and sometimes assumed, that in the oceans nitrogen fixation only occurs in oligotrophic environments such as the open ocean or hydrothermal vents, where allocthonous sources of fixed nitrogen are scarce. However in the past several years evidence for the occurence of nitrogen fixation in coastal upwelling zones has raised the possibility that n-limiting conditions may not be restricted to open ocean or deep sea environments. Any environment where nitrogen is drawn down to growth limiting concentrations should favor the capacity for nitrogen fixation. In particular phototrophs living in n-limiting environments where a suitable electron acceptor and light are not limited should are predicted to carry out nitrogen fixation during N-limiting conditions. This in turn should increase the total amount of organic carbon that can be fixed and potentially buried in marine sediments. Another way of putting this is that in the absence of nitrogen fixation, carbon export and burial may be subject to a biogeochemical bottleneck, which would alter the balance of the global carbon cycle, and potentially eliminate the stability of an oxygenated atmosphere.

The Black Sea is one of the world's most productive marine environments and may represent a modern analogue for oceanic conditions that are thought to have prevailed on a global scale over Earth's history. Density stratification in the upper water column of the Black Sea inhibits vertical mixing, which prevents atmospheric oxygen from penetrating into deeper waters. As a result of oxygen consumption by respiration, the upper water column is characterized by a sharp chemocline from

shallow oxic waters to deep euxinic water. The interface between oxic and euxinic water allows for the growth of a range of chemoautotrophic organisms including non-oxygenic phototrophs using sulfide as their electron donor for photosynthetic fixation of carbon dioxide. However, the presence of ammonia oxidizing bacteria and Archaea and anaerobic ammonium oxiding bacteria consuming both nitrate (via reduction to nitrite) and ammonium leads to a drawdown of fixed nitrogen just below the chemocline. This raises the question: does nitrogen fixation play an important role in maintaining photoautotrophic productivity in the euxinic waters of highly productive stratified water columns? Furthermore, in the absence of nitrogen fixation, would it even be possible for there to be burial of organic carbon? To address these questions, I have begun to study the presence and activity of nitrogen fixing non-oxygenic phototrophs in the chemocline of Salt Pond, which is a local seasonally stratified tidally influenced pond that is physically and chemically analogous to the water column of the Black Sea.

At the outset of this project I set out to answer the following questions:

1) What is the community structure of anoxygenic photoautotrophs in Salt Pond
2) Do microorganisms with the capacity for nitrogen fixation exist in the euxinic waters of Salt Pond and what is their phylogenetic diversity?
3) Does N-fixation occur in the euxinic waters of Salt Pond and how does nitrogenase activity change with depth?
4) How important is phototrophic N-fixation compared with heterotrophic N-fixation

To address these questions, I have applied both culture independent and culture-based strategies

1) extract environmental DNA from water column samples spanning the chemocline and assess the diversity of 16S and nifH genes.
2) Extract mRNA from the water column and determine if nifH is being actively expressed.

3) Perform an acetylene reduction assay (ARA) on samples spanning the chemocline in order to determine the relative activity of nitrogenase between samples. For each sample, ARA was performed on samples incubated in light and dark conditions to establish a rough estimate of the ratio of phototrophic to heterotrophic N-fixation, based on the assumption that most non-oxygenic phototrophic N-fixation is carried out in the presence of light.
4) Enrichments were conducted for non-oxygenic sulfur oxidizing phototrophs capable of growing on media without a source of fixed nitrogen.

MATERIALS AND METHODS

Field Site and Sampling

Samples were collected from salt pond from the R/V Bagel during mid-day from the northern end of the pond (where water is deepest). A YSI probe was deployed to record depth, temperature, salinity dissolved oxygen and oxygen saturation. YSI probe was fitted with a ¼" Tygon tubing, which was attached to a peristaltic pump ship-board in order to collect water from the same depth that probe measurements were made. Water was collected in ground glass stopper bottles and mixing with air was minimized to prevent oxygen exposure of sulfidic samples. Samples were collected for DNA extraction, enrichment culture inoculation, acetylene reduction assay, and flow cytometric sorting. Samples for flow sorting were fixed on site with 4% formaldehyde.

Enrichments

Enrichments for anoxygenic photoautotrophs were prepared from the recipes listed in the Microbial Diversity Hand Book, however media for nitrogen fixing bacteria were not amended with nitrate. Additionally, some enrichments were prepared without adding DCMU, in order to ensure that we were not selecting against the enrichment of as yet undiscovered anoxygenic phototrophs using photosystem II.

Nutrient Analyses

Nutrient analysis were carried out at the Woods Hole Oceanographic Institution in Dr. Karen Casciotti's lab. Ammonium were measured by the phenolhypochlorite method (Sloranzo 1969), and nitrite by addition of a pink azo dye (Pai et al., 1990), both detected colorimetrically. Nitrate+Nitrite was analyzed by vanadium reduction method (Yang, et al. 1997)(Braman and Hendrix 1989). Concentrations were determined by comparing response to a standard curve with standards ranging from 5uM to 50uM.

DNA and RNA extraction

For DNA 100mL of water was filtered on 0.2um 47mm filter and then extracted using a MoBio Power Soil kit (see kit instructions).

For RNA, 1L of fresh water was filtered on 0.2um 142mm isopore filter and extracted using a MoBio Soil RNA kit.

PCR and RT-PCR conditions

Amplification of nifH gene fragments was peformed using a nested primer set reported by Zani et al., (2000). Primers and PCR conditions are listed therein.

RT-PCR was carried out with an Invitrogen Superscript III RT-PCR kit using the 1st round primers from Zani et al. Second round amplification was carried out using standard PCR conditions (see above).

Acetylene Reduction Assay

The Acetylene reduction assay was performed on 50mL of fresh water from Salt Pond. Water was added anaerobically to a 100mL serum bottle, the head space was flushed with N2/CO2 and the bottle was sealed. 10mL of Acetylene gas (10% of total bottle volume) was added by syringe and bottles were shaken vigorously to enhance solubilization of acetylene into the fluid phase. For each sample, a dark and light treatment were prepared and left to incubate for three days. Gas samples were analyzed by GC (capillary column, 20ml/min N2 flow, 130 ºC oven temp) for the presence of ethylene, the reduction product of acetylene.

Flow Cytometric Sorting

Flow sorting was performed at MIT in Dr. Penny Chisholm's lab with generous help from MIT-WHOI JP student, Jake Waldbauer. Samples fixed for flow sorting were filtered on a 8um pre-filter to remove any large particles that could interfere with the flow cytometer, and run at room temperature. Sorted cells were kept on ice and frozen for subsequent analysis.

RESULTS AND DISCUSSION

Salt Pond is a seasonally stratified pond, which experiences the onset of water column euxinia in late June through September (Simmons & Edwards, 2007). At the time we sampled, we observed a well-defined chemocline between 1.5-3m (Fig. 1). Surface waters were characterized by high nitrite concentrations (with some ammonium), low salinity, and oxygen supersaturation. Fixed nitrogen in the surface waters is likely derived from anthropogenic inputs of fertilizers as well as ammonium from soil runoff. However, fixed nitrogen concentrations remain close to our analytical detection limit throughout the chemocline, and it is this depth range where we would expect nitrogen fixation to occur. Below 3 meters oxygen falls below detection levels, and sulfide concentrations rise to nearly 1.5mM. For comparison, sulfide concentrations in the Black Sea are on the order of tens of uM, so this is clearly a rich environment for bacteria utilizing sulfide in their metabolism. The profile of ammonium concentration closely parallels sulfide and reaches just over 100uM at 4m. Ammonium at depth is likely being supplied by organic matter remineralization in sediments, as well as influx of both fresh and saline ground waters through permeable sediments underlying the pond (Colman & Masterson, 2008). The suppression of ammonium concentrations for nearly 1.5 meters through the chemocline strongly suggests that ammonium oxidation is an important pathway of fixed nitrogen removal in this system. While archaeal and bacterial ammonium oxidation are likely prominent members of this community, it is not clear if anaerobic ammonium oxidation (anammox) would be

favored in this environment, given the absence of detectable nitrate of nitrite through most of the chemocline.

Enrichments for anoxygenic phototrophs were successful using innoculum from all depths spanning the chemocline and euxinic waters. The most successful enrichments were those in which 50mL of inocculum was concentrated on a 0.2um filter rather than simply inoculating with 1mL of unconcentrated Salt Pond water. Most of the enrichments yielded growth within two weeks of inoculation. Significantly, I observed growth in all of the nitrate-free treatments, demonstrating the ubiquity of phototrophs with the capacity for nitrogen fixation. Enrichments from most depths resulted in a community dominated by a small motile autoflourescent organism that imparted a deep purple tint to the enrichment bottle, and these are presumably purple sulfur bacteria. However, one innoculum from 2.2m depth resulted in the enrichment of dominantly green population of small non-motile autoflourescent organisms, and these are presumably green sulfur bacteria. Additionally, several enrichments yielded populations with a brownish color, which are most likely green sulfur bacteria with brown accessory pigments. While I had hoped to bring some of these organisms to isolation, the slow growth of these particular organisms and the short duration of the project made this unfeasible. So, in the absence of clonal populations, it was not possible to directly sequence 16S rDNA for phylogenetic characterization. I am continuing to pursue the isolation of organisms from these enrichments following the completion of the course.

To try and circumvent the problem of obtaining isolates for 16S sequencing, I attempted to isolate discrete populations of autoflourescent bacteria from Salt Pond water column samples and my enrichements using rapid flow cytometric sorting. Figure 2 shows a representative property-property plot of forward scatter (roughly correlated to cell size) versus absorbance at 692nm ±40nm, which is the basis by which populations were defined and sorted. Samples from three depths and two representative enrichments in the water column were analyzed and sorted for DNA extraction and direct 16S sequencing. At the deepest sample, taken below the chemocline, two distinct populations

were observed and sorted. When compared with enrichments, it appears that the dominant groups in the purple and green enrichments are present in the deep sample, and it is likely that these represent the two most dominant groups of anoxygenic phototrophs at this depth. The sample taken within the chemocline showed two distinct groups, one corresponding to the group observed in the purple enrichement and the second more abundant group being unique by virtue of forward scatter values nearly an order of magnitude higher than observed in the euxinic sample (Figure 3). Both of these groups had low phycoerytherin absorbance (not shown) and thus are not likely to be cyanobacteria. A surface sample yielded one dominant group with low phycoerytherin absorbance and high forward scatter and most likely represents a phototrophic eukaryote. While it is not possible to rule out that this might be a cyanobacterium with relatively low phycoerytherin/chlorophyll a pigment composition. The absence of a population possessing more typical cyanobacterial characteristics (low forward scatter, high phycoerytherin absorbance) strongly suggest that Eukaryotic phototrophs such as algae or diatoms dominate the oxic surface waters of Salt Pond.

I attempted extraction of DNA (boiling in NP40 for 10minutes) and amplification of 16S and nifH from all of the sorted fractions, however, subsequent 16S amplification proved problematic. While nanodrop measurements indicated that DNA had been extracted in 10-100 ng/uL concentrations, 16S amplification was poor and yielded only faint bands on gel. To establish that poor amplification was not due to chemical inhibition, I performed PCR amplification on two 10-fold dilutions of the original template, however this failed to produce any detectable product. Conversely, nifH amplification was successful, however the negative control turned up positive, indicating that a contaminant, rather than DNA from sorted cells, was being amplified. When sequenced, all of the nifH products, including the negative control, returned BLAST hits most closely related to known GSB strains, which would not be expected for the two water column samples taken above the chemocline. Given the sensitivity of nested PCR reactions, it is not surprising that nifH amplification was more successful than 16S rDNA amplification. That the negative control turned up positive and sequences

from the negative control returned BLAST hits most closely matching known GSB, it is likely that these samples were cross-contaminated by one of the enrichments or water column samples containing GSB. Using the remaining sorted cells, I plan to repeat the extractions using a more vigorous protocol and hopefully generate 16S and nifH clone libraries from these samples.

In order to assess community composition we submitted environmental DNA for 454 pyrosequencing of highly variable V6 16S regions. This method allows for the generation of large libaries (~30,000 sequences per sample) and assessment of the entire bacterial population. Using this database I surveyed the community structure of photoautotrophic bacteria with respect to depth and chemical profiles in Salt Pond. The results from this survey are illustrated in figure 4. As expected cyanobacteria are the dominant bacterial phototroph in surface waters. The top five most abundant sequences match most closely to fresh water Synechococcous sp. There are no matches to any known nitrogen fixing cyanobacteria, indicating that if nitrogen fixation is indeed occurring in the oxic portion of the water column, then it is most likely being carried out by heterotrophic bacteria. There is a clear transition to dominance by green sulfur bacteria mid-way through the chemocline at around 2.9 meters. A small population of purple sulfur bacteria is limited to 2.9 meters. In terms of total bacterial population, it is remarkable that green sulfur bacteria (Chlorobi) comprise over 50% of the population by taxon abundance. The dominance of GSB over PSB may be in part a consequence of the relatively high sulfide concentrations in Salt Pond. In general, GSB have been shown to thrive at much higher sulfide concentrations than PSB, and this is in agreement with our observation that PSB are most abundant at depths just above the transition from micoraerophilic to sulfidic water where sulfide concentrations are relatively low.

Green sulfur bacteria are well known for their participation in symbiotic consortia with colorless β-proteobacteria. It has been proposed that such aggregates facilitate syntrophic cycling of sulfur (Pfenning, 1980), presumably through the coupling of phototrophic sulfide oxidation and some as yet undetermined means of sulfate reduction, and may provide an advantage under conditions where

sulfide is a limiting substrate for GSB. Given such high abundance of GSB in Salt Pond, it is possible that, on the microscale of diffusion relevant to bacteria, sulfide could be limiting, even in the presence of high measurable levels of sulfide. Based on observations that all of the colorless symbionts known to aggregate with GSB have been characterized as belonging to β-proteobacteria, and they occur in a GSB:symbiont ratio of around 10-20 (Brock Biology of Microorganisms) I compared the abundance of all β-proteobacteria with that of GSB to determine the maximum possible percent of the GSB community that could be involved with such consortia. From this back of the envelope estimate, it appears that GSB involved in consortia could make up no more than around 1% of the total GSB population. However, it should be noted that a GSB consortia named "*Chlorochromatium aggregatum*" were enriched from pebble sediments of Salt Pond (Overmann, 2001), and in the absence of microscopic evidence, it is not possible to rule out the possibility that phototrophic consortia are present and possibly prolific in this environment. In particular, it would be interesting to investigate whether such consortia might prevail during the onset of stratification, when sulfide concentrations are lowest, and consequently, when ecological strategies for the efficient cycling of sulfur would have a selective advantage.

To determine if nitrogen fixation was actively occurring in the water column I employed both acetylene reduction assay and detection of mRNA coding for nifH as an indication of nitrogenase expression. The nitrogenase assay yielded un-interpretable results: no acetylene was detected, suggesting reduction by nitrogenase, however no ethylene was detected either, which would be the expected product of acetylene reduction by nitrogenase. Acetylene is very soluble in water, and it is possible that it partitioned nearly completely into the liquid phase, preventing detection in the gas phase. Ethylene should, however, readily partition into the gas phase, and the absence of detectable levels of ethylene provides evidence for the absence of nitrogenase activity. Unfortunately, no positive controls were available at the time the experiment was carried out, so it is not possible to establish whether the experiment failed or whether these negative results are indicative of the absence of

nitrogenase activity. There are several reasons why the nitrogenase activity assay may have failed under the chosen conditions: 1) not enough biomass was incubated to yield a detectable conversion of acetylene to ethylene. In the future it would be advisable to concentrate cells from a large volume of water (1-2L) to achieve detectable rates of acetylene reduction; 2) The liquid:gas ratio was too high, and ethylene was mostly partitioned into the liquid phase; 3) While care was taken to avoid exposing the samples to oxygen, all preparation was carried out on the bench top. In the future it would be worth doing all preparations in an anaerobic hood.

While the acetylene reduction assay was not entirely successful, some promising results were garnered from amplification of nifH mRNA. A one-step RT-PCR followed by nested PCR of nifH yielded positive for amplification products in samples from 3.9. 3.0, and 1.7m suggesting that nitrogen fixation may occur at these depths. However, a parallel nested PCR was run on the RNA extract to ensure that no DNA was present, and this resulted in positive products for both 3.9 and 3.0m samples, indicating that DNA and not RNA may have been responsible for the amplification of nifH in the one-step RT-PCR reaction. However, the sample from 1.7m yielded negative results for the nested PCR suggesting that nifH and thus nitrogenase is being actively expressed at this depth. The absence of any cyanobacterial 16S V6 sequences grouping with known nitrogen fixing cyanobacteria raises the possibility that, in oxic waters, nitrogen fixation is being carried out exclusively by heterotrophic bacteria. Until these experiments can be repeated, the role of nitrogen fixation in supporting anoxygenic photoautotrophic carbon fixation remains tantalizingly uncertain.

CONCLUSIONS

The bacterial community in Salt pond is dominated by photoautotrophy in and below the chemocline, suggesting that organic carbon burial could be very rapid in this environment. Furthermore, low fixed nitrogen concentrations in the chemocline indicate that nitrogen fixation is a requirement to sustain high levels of phototrophy. The expression of nifH mRNA in the water column is further evidence in

favor of nitrogen fixation as an important source of fixed nitrogen in the Salt Pond water column, however additional work needs to be done to clearly demonstrate the activity of nitrogenase or its expression in the euxinic waters of Salt Pond. The results of this study indicate that nitrogen fixation is likely an important process in supporting photoautotrophic communities in stratified marine and coastal environments. Future studies should attempt to quantify the rates of nitrogen fixation with depth (via acetylene reduction assay, or paired nitrogen isotope tracer experiments) and possibly the change in nitrogen fixation rates on a seasonal time scale.

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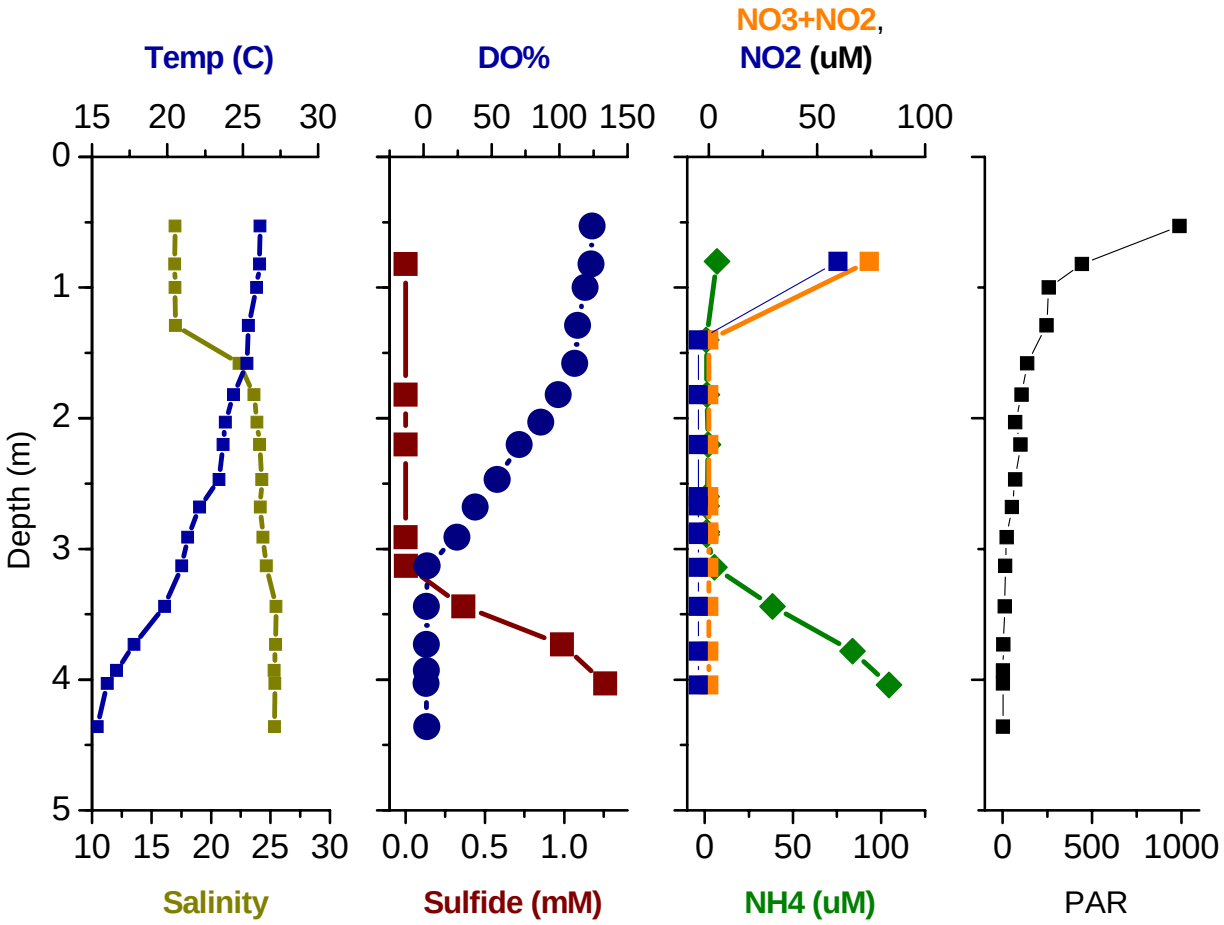

Figure 1

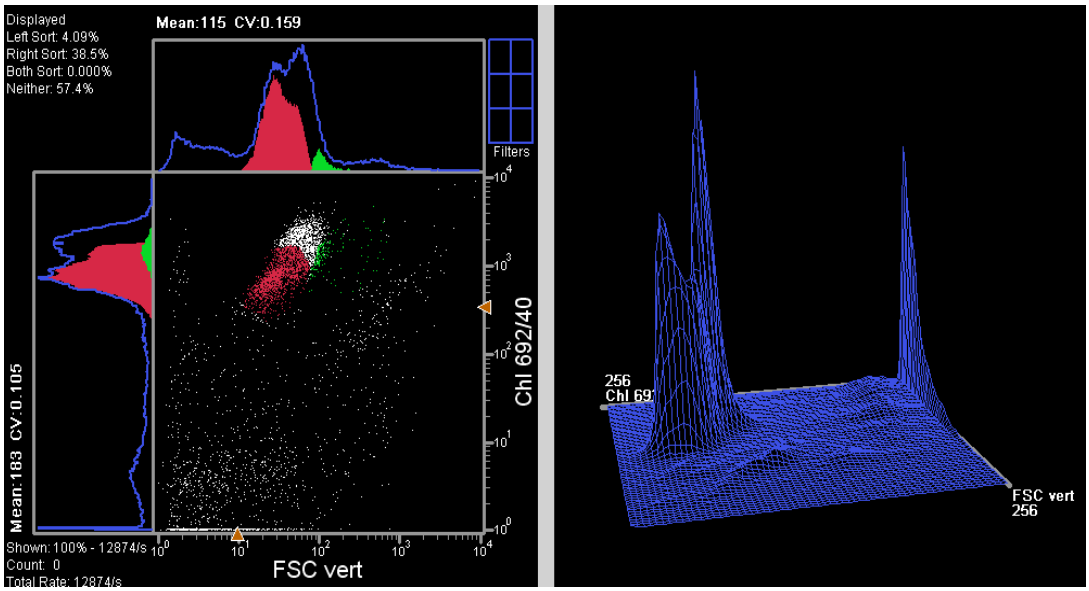

Figure 2.

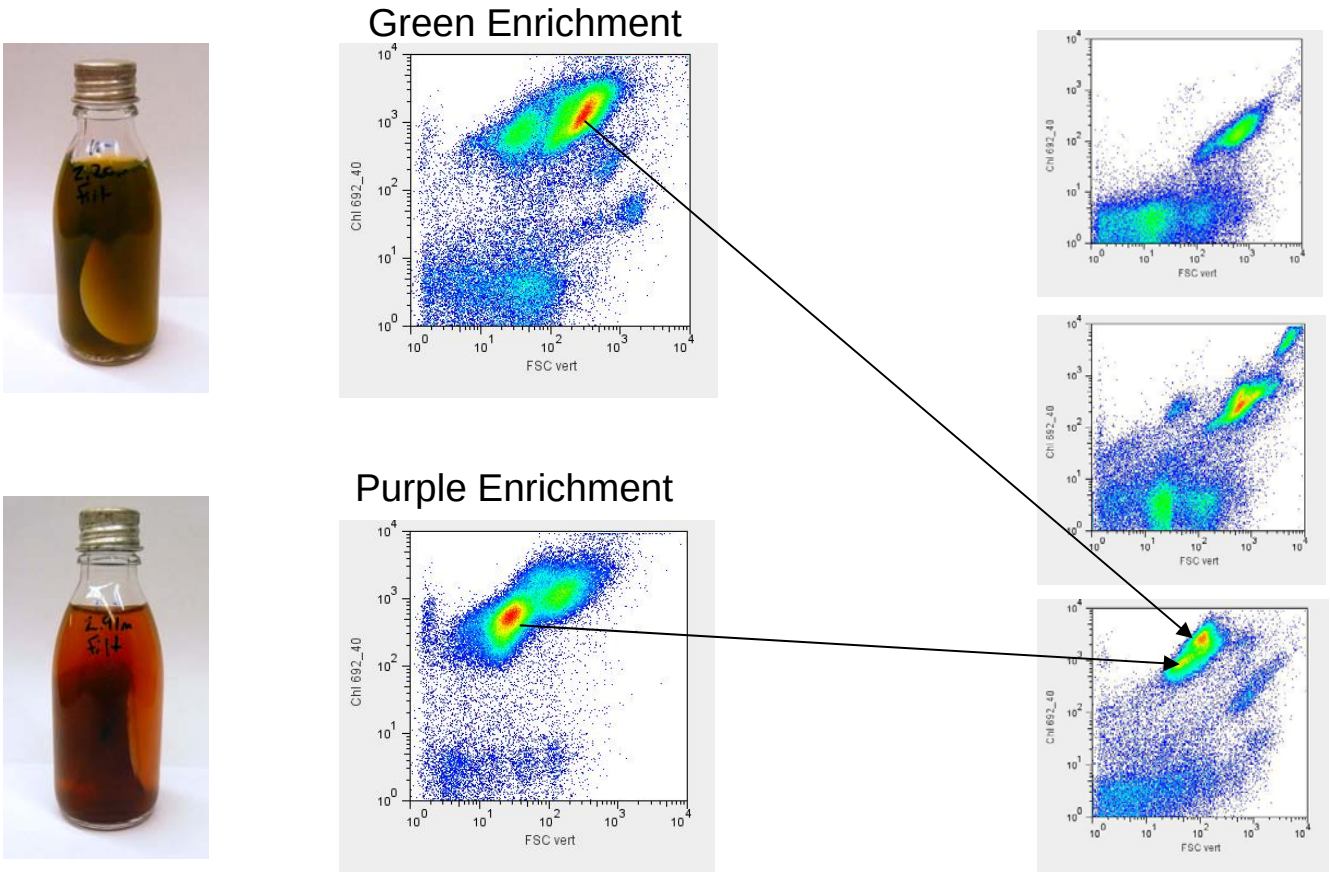

Figure 3.

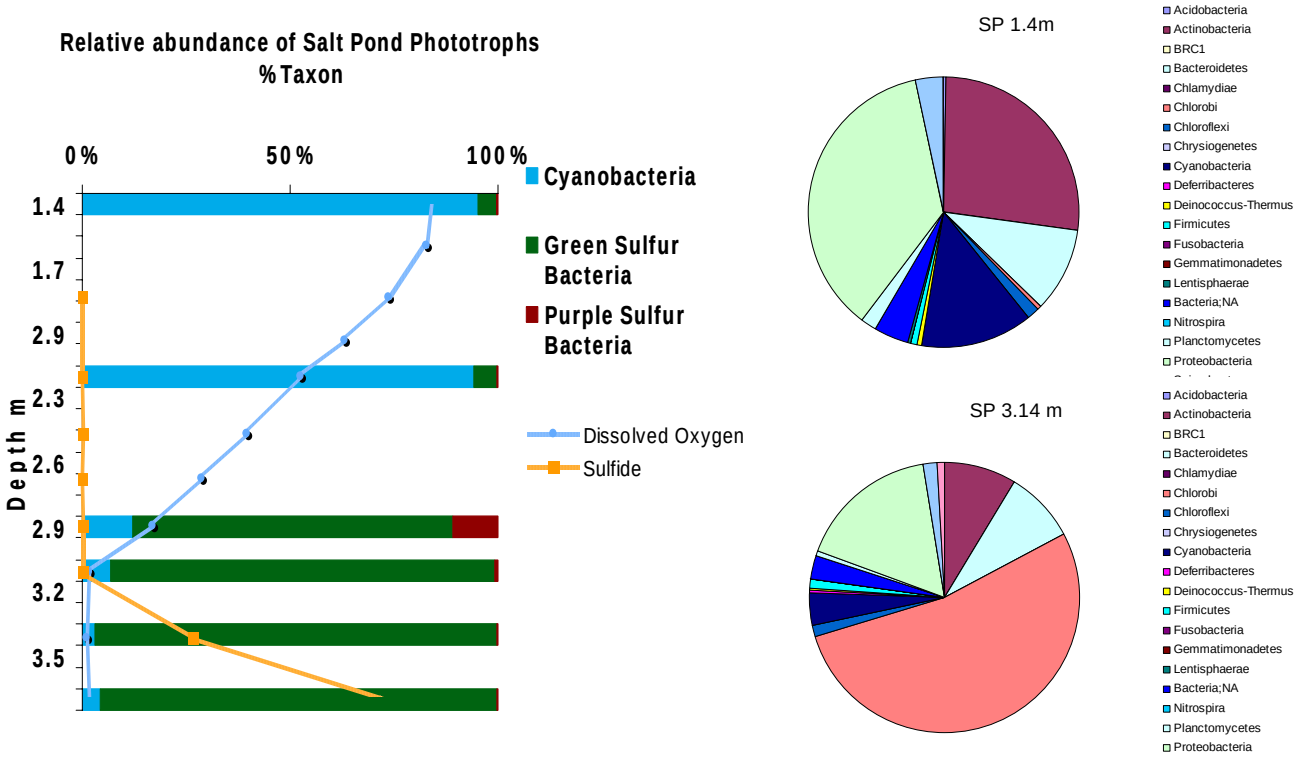
Relative abundance of Salt Pond Phototrophs
% Taxon
0 %
50 %
100 %
Depth m
1.4
1.7
2.9
2.3
2.6
2.9
3.2
3.5
Cyanobacteria
Green Sulfur Bacteria
Purple Sulfur Bacteria
Dissolved Oxygen
Sulfide
SP 1.4m
SP 3.14 m
Acidobacteria
Actinobacteria
BRC1
Bacteroidetes
Chlamydiae
Chlorobi
Chloroflexi
Chrysiogenetes
Cyanobacteria
Deferribacteres
Deinococcus-Thermus
Firmicutes
Fusobacteria
Gemmatimonadetes
Lentisphaerae
Bacteria;NA
Nitrospira
Planctomycetes
Proteobacteria

Figure 4.