An odd enrichment for purple non-sulfur bacteria from Trunk River

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INTRODUCTION

Trunk River exhibits a brackish environment with seawater inflow from the Atlantic Ocean into freshwater, and rich organics on the riverbed provides a breeding ground for anaerobic sulfur-cycling microbes. Sulfate from organics can be reduced to sulfide, which can then be re-oxidized to elemental sulfur and back to sulfate. In this environment, we found many examples of intracellular and extracellular elemental sulfur in the form of white films, as in *Thiovulum* and *Thiothrix* species. We also found instances of pink and green microbial mats, evidence of phototrophic sulfur and non-sulfur bacteria. The anoxygenic phototrophic bacteria are capable of using light energy and a variety of carbon sources for their growth. They do not produce oxygen, as they do not use H_2O as an electron donor, using instead organic compounds, reduced inorganic sulfur compounds, or hydrogen gas. The anoxygenic sulfide-oxidizing bacteria comprise the so-called purple sulfur bacteria (PSB) and green sulfur bacteria (GSB), which grow best under conditions of ~1mM sulfide in infrared light (~800nm) and up to 5mM sulfide in far red light (~650nm), respectively. The investigation in this report concerns primarily an enrichment for purple non-sulfur bacteria (PNSB), which exhibit a wide variety of metabolic strategies. These bacteria can grow photoautotrophically or photoheterotrophically, using a range of organic carbon sources as well as low levels of reduced sulfur compounds as electron donors.

MATERIALS & METHODS

Inoculation and passaging of initial enrichment

A sample of sediment from Trunk River was inoculated into a medium containing 20mM succinate as the carbon source and 250uM sulfate. The culture was grown anaerobically in ~52ml Pfennig bottles at 850nm infrared light. For passaging, 200ul of initial turbid enrichment was added to a fresh Pfennig bottle with PNSB media.

The recipe for 2L of non-sulfur media was made in a Widdel vessel as follows:

1.95L deionized water

20ml 100x FW base

11g Sodium succinate (20mM final)
10ml 1M NH4Cl solution
0.5ml 1M Sodium sulfate (250uM final)
20ml 100mM K phosphate pH 7.2
2.5ml 1M MES buffer, pH 5.5
2ml trace elements (Wolfe's minerals)
This mixture was then autoclave on a liquid cycle, and then cooled under N_2/CO_2 (80%/20%) gas while stirring. After cooling, the following was spiked into the stirring media:
2ml multivitamin solution
5ml 1M sodium bicarbonate
25mg DCMU (cyanobacteria inhibitor)
This was then dispensed using the bell attached to the Widdel vessel into sterile Pfennig bottles, ~52ml volume per bottle, leaving a small bubble for the inoculum and then capped.

Preparation of DNA for bacterial 16S sequencing

A bacterial colony of interest was picked and boiled in 20ul Alkaline PEG (ALP) reagent for 10 minutes at 95C in a PCR machine. Per sample, the following reaction mix was prepared:
3ul boiled colony sample (DNA)
25ul Promega GoTaq G2 Hot Start Green Master Mix
1ul 16S_8F (15pmol) forward primer
1ul 16S_1391R (15pmol) reverse primer
20ul Nuclease free water
The PCR program below was done:
Initial denaturation at 95C, 2 min
Denaturation at 95C, 30 sec
Annealing at 55C, 30 sec
Extension at 72C, 1.5min
Repeat the above italicized steps ~25x
Final extension at 72C, 10 min
Hold at 12C, forever
PCR products were checked using DNA agarose gel electrophoresis stained with SYBR Safe, for a band around 1400bp corresponding to 16S DNA. The verified PCR product could then be submitted for sequencing.

Preparation of new enrichment cultures

1mM sulfate (52ul of 1M sodium sulfate) or 1mM sulfide (52ul of 1M sodium sulfide) were spiked into 52ml Pfennig bottles (4 each) filled with PNSB media. Four additional bottles containing just regular PNSB media ("original") were added to the setup. To each of these twelve bottles, 200ul of inoculum from one of the passages of the original PNSB enrichment was added. Two bottles out of four of each media condition were incubated at 850nm IR light, and the remaining two were incubated in a box covered with aluminum foil inside the IR light cabinet, to control for temperature. This way, each individual medium/light condition had 2 replicates.

To enrich for sulfate reducing bacteria (SRB), anaerobic media was made with the same components as the PNSB media except for 10mM final sulfate concentration. To enrich for the sulfide oxidizing bacteria (e.g., Chromatium spp.), I used 1.5mM sulfide and 1mM acetate instead of succinate as the carbon source.

Absorption spectra

Whole cell absorption spectra of liquid cultures or colonies were taken using SR-1900 Series Spectroradiometer (Spectral Evolution) with the help of Kurt Hanselmann, and plotted using R with the help of Sebastian Kopf.

Ion chromatography to measure concentration of sulfate

200ul samples from Pfennig bottle cultures in both the light and dark were filtered through a 0.2 micron syringe filter, then combined with 1.8ml Millipore water for a 1:10 dilution. Samples were measured with the help of an autosampler on a Thermo Scientific™ Dionex™ ion chromatography system with an AS-18 column.

High performance liquid chromatography to measure concentration of organic acids

450ul samples from Pfennig bottle cultures in the light and dark were filtered through a 0.2micron syringe filter, then combined with 50ul 5N H_2SO_4 and then spun down at 16,000xg for 3 minutes. 450ul of the supernatant was placed into HPLC vials and measured with the help of an autosampler on a Shimadzu™ High Pressure Liquid Chromatography system.

Light Microscopy

Light microscopy analysis of colonies or cultures was performed using a Zeiss SteREO Discovery.V12 or a Zeiss AXIO Imager.A2 microscope, respectively.

RESULTS & DISCUSSION

An anaerobic enrichment for purple non-sulfur bacteria (PNSB) from a Trunk River sediment sample (Duck Island) yielded a community containing PNSB (possible Rhodobacter spp.), purple sulfur bacteria (possible *Chromatium* spp.), sulfate-reducing bacteria (possible *Desulfovibrio* spp.) and others (rods, spirilla, spirochetes, protozoa), purely based on morphological observations (Figure 1). The appearance of sulfide oxidizing bacteria containing elemental sulfur granules was especially intriguing, as no sulfide was added to the original medium. It seemed to imply that the small amount of sulfate in our medium was being reduced so rapidly to sulfide, which led to so much sulfur being sequestered as sulfur granules. This was unexpected, so we proposed the following hypothetical scheme as to what might be sustaining this diverse community (Figure 2).

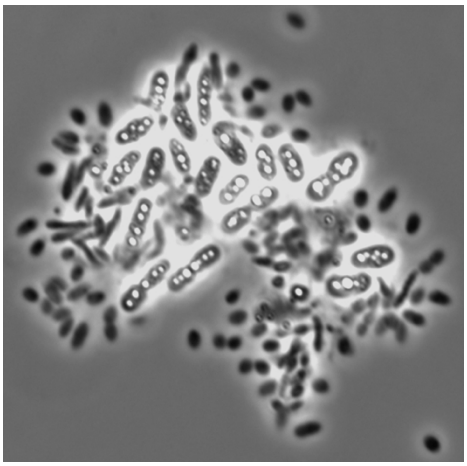

Figure 1. Original enrichment for PNSB, grown at 850nm IR light. Anaerobic culture in Pfennig was mustard yellow.

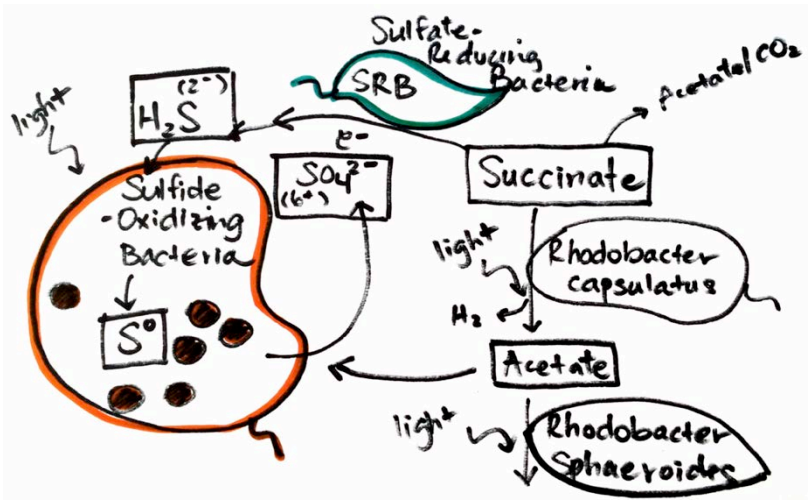

Figure 2. Hypothetical scheme for sulfur cycling and photoheterotrophic growth in PNSB enrichment. Succinate is used as a carbon source by SRB, which cause the sulfide content to increase. This causes the sulfide oxidizing bacteria to make elemental sulfur granules in the presence of light, which they may further oxidize into sulfate. Outside of this sulfur cycling, various PNSB (e.g., Rhodobacter) can use succinate to grow

photoheterotrophically, producing acetate and other breakdown products that can be used by other members of the consortium.

I endeavored to isolate as many species from this enrichment as possible, in order to separate the members and perhaps reconstitute them. To that end, I isolated two species of Rhodobacter, which are purple non-sulfur bacteria, from the original enrichment. This proved to be nontrivial as they have very similar colony morphologies. The first strain I isolated appeared to be a strain of *Rhodobacter capsulatus* from 16S sequencing (Figure 3). When grown on 5YE plates aerobically, colonies of this strain are a dark purplish-red with a thin white border. Plate colonies grew up after about 48 hours of incubation. In anaerobic shake tubes, mustard yellow, disc-shaped colonies appeared after 48 hours. Under the light microscope, cells are ovoid to rod/dumbbell shaped and motile. They are most motile and "happy" when grown anaerobically either in shake tube colonies or in liquid culture in non-sulfur media. They form chains (Bergey's and The Prokaryotes) ranging from four cells to more than 20 cells, at times zigzagging. This particular strain of R. capsulatus also formed rosettes, which appeared in both liquid or agar culture. The rosettes appear to form around a phase-bright nucleation site, and they coexist with chain formation, though relative abundance of diplococcoid, chains, and rosettes varied.

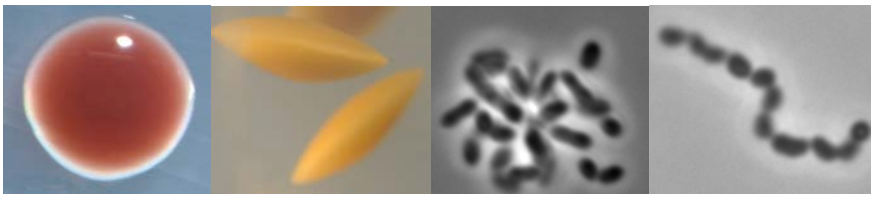

Figure 3. *Rhodobacter capsulatus.* From left to right: aerobic colony grown on 5YE, anaerobic shake tube colony grown on non-sulfur media, rosettes, and chains.

I have included here a part of the 16S sequencing result (reverse direction), and both glycerol and DMSO freezer stocks of a liquid pure culture of this strain have been stored as 15YW1G or 15YW1D, respectively.

CTTCCCCAGTGACCACTGAATGATGGCAACTGAAAGTGTGGGTTGCGCTCGTTGCCGGACTTAACCGAACATCTCACGACACGAGCTGACGACAGCC
ATGCAGCACCTGTCTGGGATCCAGCCGAACTGAAGGAAACCGTCTCCGGTAACCGCGATCCCGATGTCAAGGGTTGGTAAGGTTCTGCGCGTTGCTT
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CCGTTAGGTGTGTCACCGAATAGCATGCTACCCGACGACTGGCATTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAC
GCTTTCGCACCTCAGCGTCAGTATCGAGCCAGTGAGCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAATTC
CACTCACCTCTCTCGACCTCAAGACCAGGAGTTTCAAAGGCAGTTCCAAGGTTGAGCCCTGGGATTTCACCTCTGACTTTCTGATCCGCCTACGTGC
GCTTTACGCCCAGTAATTCCGAACAACGCTAGCCCCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTCTTCTGGTGGTACCGTCA
TTATCTTCCCACCTGAAAGAGCTTTACAACCCTAANGCCTTCATCGCTCACGCGGCATGGNTAGATCAAGGGTTTCCCCCATTGTCTAAGATTCCCC
ACTGCTGCCTCCCGTAGGAGTCTGGGNCCGTGTCTCAGTCCCNNTGTTGNTGATCATCCTCTCAAACAGCTATGGATCGTCGGCTTGGTAGGCATTA
CCCCNCAANTA

The second species of PNSB I isolated appeared to be *Rhodobacter sphaeroides* from 16S sequencing. This species was previously thought to be the same bug as the *R. capsulatus*, but upon streaking both bugs on a limited nutrient plate where acetate was the only available carbon source, only this *R. sphaeroides* survived. On acetate plates, the cells were leaf shape with prominent polyhydrobutyrate granules. On 5YE plates (Figure 4), the colonies were a bright red color with a white boundary, but with more diffuse color than the *R. capsulatus* when magnified. The colonies viewed collectively on plates appeared a more true red than did colonies of the *R. capsulatus*, which were often darker and almost maroon or purple-ish. *R. sphaeroides* cells were lemon-shaped and non-motile, appearing to be embedded in a matrix, equidistant from one another at highest density. This feature was especially prominent in liquid anaerobic cultures grown in non-sulfur media in Pfennig bottles. In addition, this culture never seemed to become pure. There was always the presence of an unidentified long filamentous bacterium also embedded in the same extracellular matrix (Figure 4), both in colonies and in liquid culture. It is quite interesting to think about whether this is a circumstantial event of something getting physically stuck in someone else's "muck" vs. a result of more specific forces leading to this interaction. This filamentous bacterium can get very long, ranging from 5-10 cell lengths of R. sphaeroides to 30+ cell lengths.

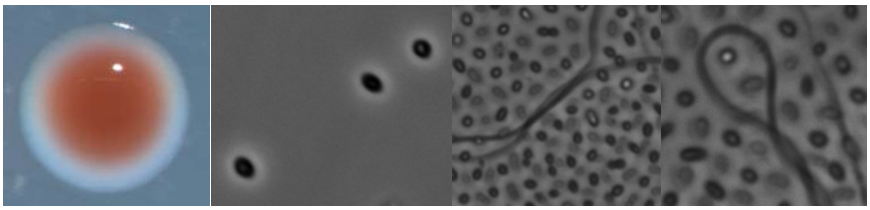

Figure 4. *Rhodobacter sphaeroides*. From left to right: colony grown on 5YE, cells in both liquid culture and plates look lemon shaped and are often embedded in matrix with a long filamentous bacteria.

I have included here a part of the 16S sequencing result (forward direction).

TGGGCGTAAGCGCACGTAGGCGGATCGGAAAGTCAGAGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTGAAACTCCCGATCTTGAGGTCGAG
AGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGTCGTCGGGCAGCATGCTGTTCGGTGAC
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TCTAGGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTTACGGGTTGGGCTACACACGTGCTACAATGGCA
GTGACAATGGGTTAATCCCAAAAAGCTGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAATCGCTAGTAATCGCGTAACAGCA
TGACGCGGTGAATACGTTCCCGGGCCTTGCAC

In addition to these two species, strains of *Shewanella putrefaciens*, *Pseudomonas fluorescens*, and *Aeromonas hydrophila* were also isolated from the original PNSB enrichment using 5YE agar plates and resolved via 16S sequencing.

To probe the hypothetical coexisting sulfur cycling and photoheterotrophic system outlined in Figure 2, I set up new enrichments with 1mM sulfate or 1mM sulfide added to compare to the original medium. In addition, replicates of these enrichments were placed in either 850nm infrared light or in the dark, for comparison. I wanted to monitor growth of these cultures via measuring metabolite concentration and observing any changes in the composition in the bacterial population using light microscopy. The dark cultures did not reach an appreciable level of turbidity in the time allowed for the experiment, but the light cultures are shown in Figure 5.

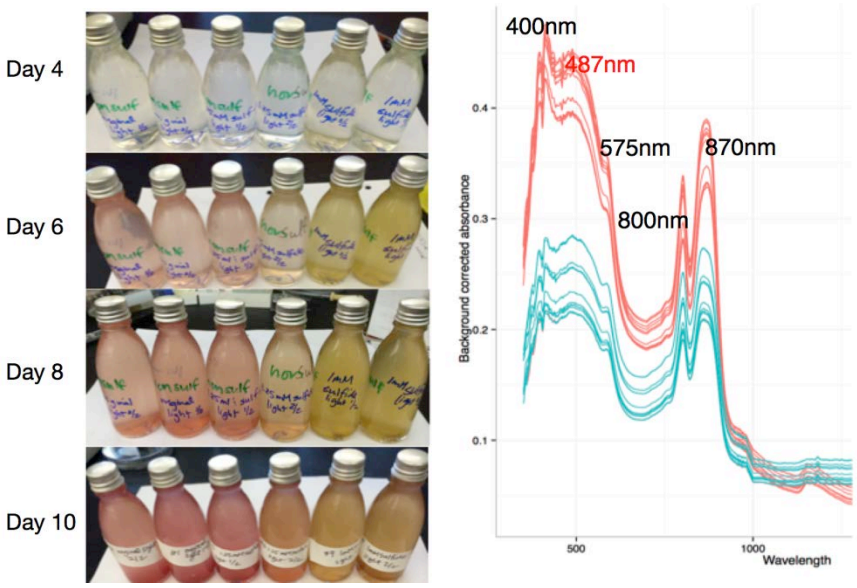


Figure 5. (Left) Bottle growth monitoring and absorption spectra. The cultures are lined up thusly from left to right: 2 replicates of original medium (pink), 2 replicates of 1.25mM sulfate medium (pink to pink/yellow), 2 replicates of 1mM sulfide medium (mustard yellow to brown). When monitored over 10 days of growth, a 1ml sample was taken every other day from each bottle, including the dark ones (samples taken on days 0, 2, 4, 6, 8). (Right) A representative absorption spectra of liquid cultures and colonies of PNSB. The indicated peaks are consistent with the presence of carotenoids and bacteriochlorophyll A. Red and blue traces are indicative of two different intensities (i.e. from a larger or smaller colony, from a more or less turbid liquid culture).

It may be of interest to describe the original enrichment that was used as an inoculum for the various experimental conditions. Just prior to starting the experiment, the original enrichment contained an abundance of *Chromatium* spp. with sulfur granules. Since this was the outstanding feature that makes the question of this investigation compelling, it seemed like a good enrichment from which to sample for the experiment. The new experimental enrichment in the original non-sulfur condition bottles (leftmost two) were colored pink, which is different from the previously observed mustard yellow to brown color of the original enrichment from Trunk River, which persisted through many passages. In fact, no previous passages from the initial enrichment have been pink until now. To investigate any changes in population and relative abundance of specific species in the new enrichments, pictures were taken using light microscopy (Figure 6).

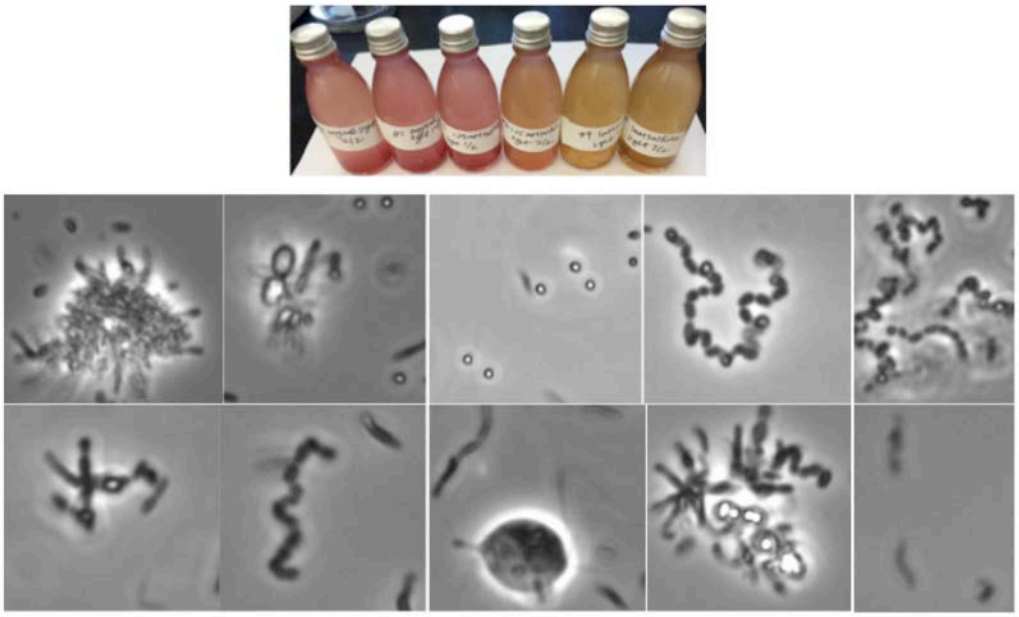


Figure 6. Population changes in new enrichments. Microscopy images in columns 1 and 2 are from the two leftmost bottles with original media. Column 3, from two center bottles, with 1mM sulfate media. Columns 4 and 5, from two rightmost bottles, with 1mM sulfide media.

In contrast to the (yellow/brown) original enrichment from Trunk River (Figure 1), the new (pink) enrichments in original non-sulfur medium exhibited mainly Rhodobacter type cells with evidence of Chromatium spp., though with depleted sulfur granules. In fact, no intact sulfur granules were observed in the sample used for microscopy. Highly motile *Rhodobacter capsulatus* cells exhibited both rosette and zigzag chain aggregates, with a few phase-bright

spots interspersed throughout the aggregations. *Rhodobacter sphaeroides* was also seen in abundance, embedded in an extracellular matrix and often phase bright if the cells are pointed into the plane of the slide. A similar population was observed in the 1.25mM sulfate bottles, with the addition of protozoa that was found in the original enrichments. There was also a slightly more enriched population of *R. sphaeroides* embedded in a matrix. The 1mM sulfate bottles were the darker yellow/brown color of the original enrichments, and they contained *Chromatium* spp. with bright sulfur granules. This culture was also very enriched for *R. capsulatus* chains, including very long zigzagged chains. These chains frequently featured a phase-bright spot every few cells, as if the cells were tethered via some type of nucleation site. This phenomenon was also observed when only a few cells were aggregated – this frequently happened in the presence of a phase-bright spot in the middle of the aggregation. Potential *Desulfovibrio* spp. (sulfate-reducers) were also observed, with their characteristic spiral shape.

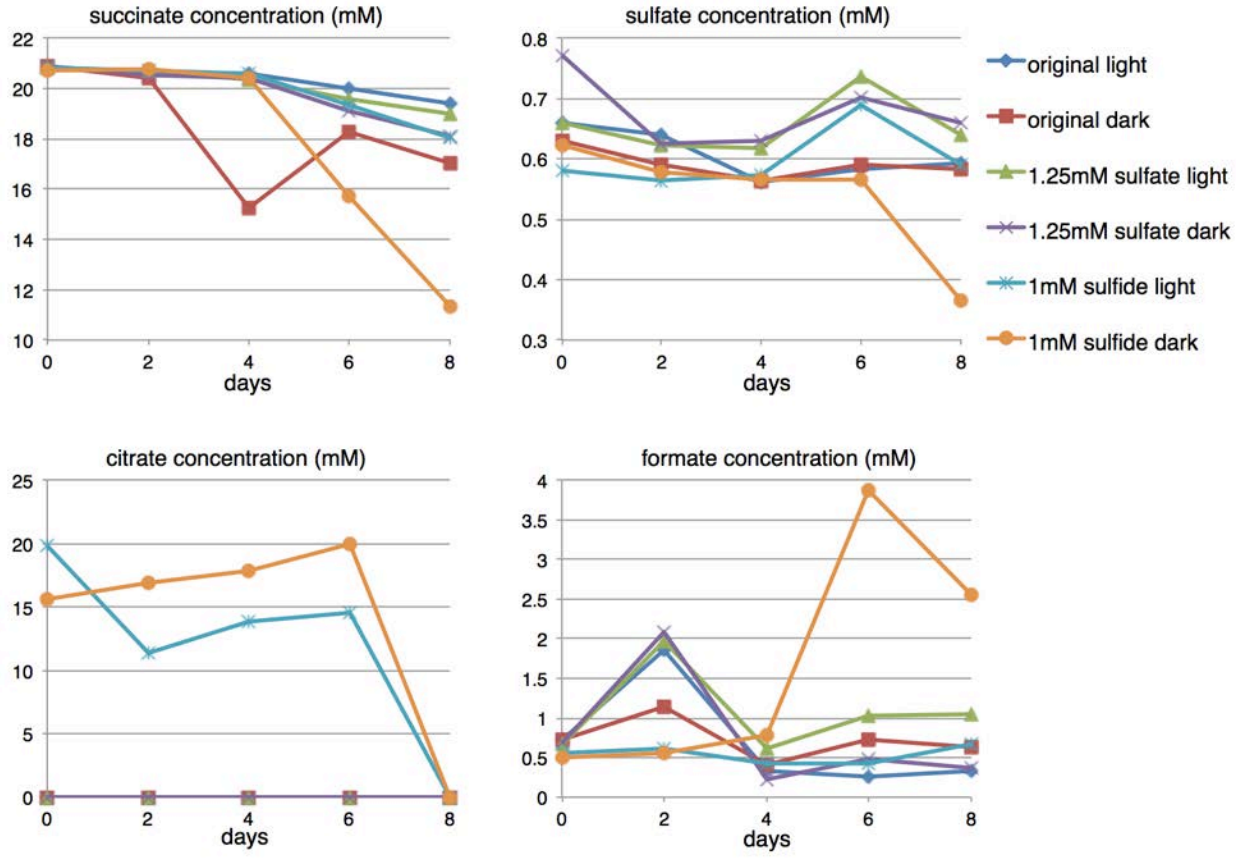


Figure 7. Metabolite and ion concentrations measured using HPLC or IC.

To investigate the fluctuations in metabolites and ions in these cultures, a 1ml sample was taken upon inoculating (day 0), and subsequently on days 2, 4, 6, and 8. This sample was syringe filtered immediately into eppendorf tubes, then separated for either HPLC or ion chromatography analysis. 1ml of the same media was used to top off the Pfennig bottles, ensuring that any bubble remaining, if any, is no more than a few millimeters in diameter. The results of this analysis are shown in Figure 7.

Notably, succinate is depleted as the sulfide cultures age, even though succinate is replenished every time samples are taken. Initial sulfate concentrations appear inconsistent with calculated amounts, which could be a result of inaccurate stock solution concentrations or error in calculation when making media. Sulfate concentrations dropped steadily in the 1mM sulfide cultures, which goes against the idea that sulfate-reducing bacteria are the first population to use the added sulfate to start making sulfide. If this were the case, it would seem that additional sulfide would push this reaction backward toward sulfate. However, the opposite is observed for unclear reasons.

Several observations stand out as somewhat unexpected. Firstly, the sulfide-oxidizing *Chromatium* members of the original enrichment all but disappeared from the new cultures with no sulfide added. Since their existence in the absence of added sulfide was an initial driving force for this investigation, this is intriguing. Secondly, the dominance of *Rhodobacter* spp. in the experimental cultures is spread across all conditions, with both sulfate and sulfide. Rhodobacter is known to be able to use low concentrations of sulfide as electron donors. The marked difference between these new cultures and the original enrichment from which they were inoculated may point to another hypothetical scheme over time (Figure 8).

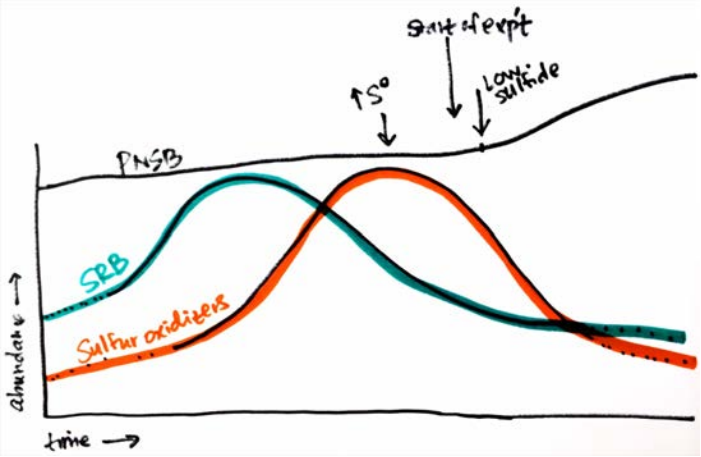


Figure 8. Model for change in relative abundance of consortium members over time.

This model suggests that at the beginning of the original enrichment, i.e. right after the sediment from Trunk River was inoculated into the first bottle, the sulfate-reducing bacteria were the first on the scene, reducing 250uM sulfate present in the medium to sulfide. As soon as the sulfide reached an appreciable level, the sulfur oxidizing bacteria (e.g., Chromatium spp.) began to oxidize this sulfide into elemental sulfur and storing it. As this happened more as time went on, more of the sulfur in the system was sequestered in sulfur granules, which takes it out of circulation. Under those circumstances, the sulfate reducers can no longer persist as well, and thus die down. When that happens, the sulfide concentration also drops, leading the Chromatium to further oxidize their sulfur granules back into sulfate – this would explain the depleted sulfur granules. By this point, sulfide is low enough for the purple non-sulfur bacteria (e.g., Rhodobacter) to bloom, as they can now use both succinate as a carbon source and sulfide as an electron donor, eventually becoming the predominant player in the consortium. Under this model, I hypothesize that the enrichment for the mini-project was started right as the sulfide levels were dropping and as the PNSB were blooming.

There are many directions for future development on this project. A more thorough identification or isolation of individual players would have benefited this analysis greatly, especially if such measurements and analyses could have begun at the very beginning of the original inoculation from Trunk River. Creation of a 16S clone library was attempted several times for this mini-project, though it was unsuccessful and likely needed optimization, since very few colonies resulted after the initial transformation. If the sulfate-reducer and sulfide-oxidizer could have been isolated, they could have been combined in the presence and absence of the isolated PNSB species in a reconstitution experiment. Measurements of other metabolites such as propionate or acetate would have aided in the understanding of nutrient cycling in this microcosm. Overall, it can be concluded that while studying organisms in isolation has many benefits and opportunity for elegant, well-controlled experiments, there are many subtleties that can be missed by overlooking naturally occurring consortia. Interspecies interactions and community dynamics over time can change drastically before any measurements or observations can be made.

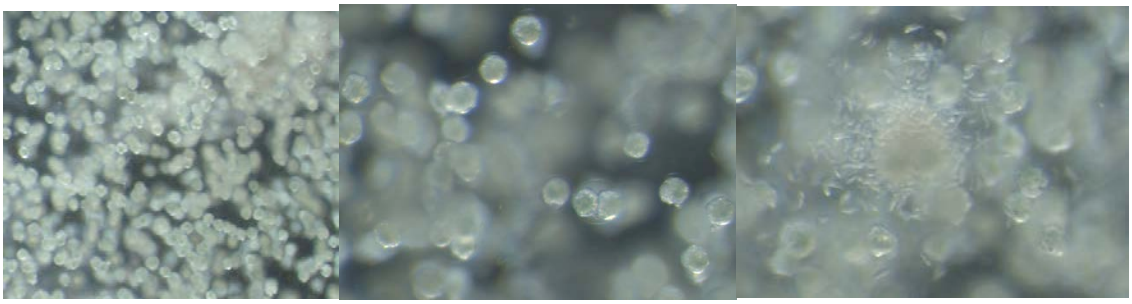
Appendix 1. Protozoa in PNSB culture

Several morphologies of single celled eukaryotes were found in the initial Trunk River PNSB enrichment, especially at the benthic zone of the Pfennig bottle where visible biomass accumulated. These were either amoeboid, ovoid, or circular flagellated protozoa capable of

“whipping” its flagella at passing bacteria. The circular protozoa were found especially frequently near microbial mats of Rhodobacter and Chromatium, as shown in Figure 1. These protozoa could be stably transferred to subsequent purple non-sulfur enrichments, as long as the inoculum was taken from the benthic layer of the original culture.

Appendix 2. Snail egg epibionts?

A floating egg sac like object was found in Little Sippewissett Salt Marsh. These sacs would at times be covered in brown or purple bacteria. The sac was brought back to the lab in its own surrounding seawater, then washed once in 1x Seawater base in a petri plate to remove sand and environmental bacteria. A piece of the sac was dissected from the eggs and was examined under the microscope. It was determined to have many egg-like suspensions, as well as a huge variety of protozoa, ciliates, cyanobacteria, diatoms, and bacteria. The sac was then “streaked” on a Seawater Complete agar plate, and many colonies of different morphologies grew over 24 hours at 30C. Follow up was not possible, since this was done with only a few days left in the course. Two days later, the egg-like suspensions had spilled out into the extra-egg-ular medium and (potentially snail?) larvae with cilia were observed. In addition, a “bloom” (or perhaps infection) of protozoa appeared to be emanating from a few eggs within the sac.



Top row from left to right: Egg sac collected at Sippewissett, egg sac in the lab being washed in 1x SW base, washed egg being rolled on Seawater Complete plate.
Bottom row from left to right: Egg suspension at low magnification, egg suspension at high magnification, protozoa blooming inside (infecting?) the egg sac.

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I would like to thank the course directors, Jared Leadbetter and Dianne Newman, for organizing what turned out to be an amazing life experience for me. I especially valued your encouragement for free exploration both in the lab and in my own mind, and the idea that I could experimentally follow whatever tugged at my interest was incredibly empowering. I'd also like to thank my fellow Group 4 members: Kate, Lorenzo, Alicja, and James – for being a constant source of support and for teaching me so many things. I could not have imagined better group members and lab mates – and now friends. Kurt Hanselmann, thank you for introducing me to the colorful world of phototrophs, for calming me with measurements of immutable things, and for our long discussions about sulfur. I will never take a nature walk the same way again. Lina and Seb, thank you for teaching me how to do anaerobic work, Elise, for teaching me to use the IC, Scott and Srijak, for helping me with 16S sequencing. To everyone at the course: I am indebted to all of you for taking time out of your busy schedules to help us gain a shockingly large body of knowledge, life-changing experiences, and long-lasting camaraderie.

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