

Polymicrobial sulfur cycling among enrichments of purple sulfur bacteria and sulfate reducing bacteria

Abstract:

Biogeochemical cycling at the micrometer-scale occurs from closely associated syntrophic taxa whereby electron donors and acceptors can be transported directly from cell to cell. We developed a hypothetical sulfur cycle model whereby sulfate reducing bacteria produce an electron acceptor for purple sulfur bacteria (PSB), which in turn produce fixed carbon used as an electron donor for sulfate reducing bacteria (SRB). The first aspect of this work involved enriching for PSB and SRB with metabolisms that coincide with the model cycle. Next, we discovered that sulfide cycling in these co-enrichments was tightly linked given often no sulfide can be detected within enrichments. The sulfide transfer between PSB and SRB appears to decouple at in the dark when purple sulfur bacteria cannot fix carbon, as evidenced by sulfide accumulation in the morning. Inhibiting SRB in co-enrichments results in little sulfide production, showing dark sulfide production is likely from sulfate reduction not sulfur dissimilation. Co-enrichments were successfully transferred to minimal media that required syntrophy for growth. Observations of the syntrophic enrichment indicate sulfur globules are present during the light cycle and are minimal following the end of a 12-hour dark cycle. Absorption spectra indicate the presence purple sulfur bacteria with two bacteriochlorophyll a absorption peaks at 800 and 850nm. MonoFISH confirmed the presence of sulfate reducing bacteria in the syntrophic enrichment. Further work is necessary to characterize sulfur cycling, isolate a pure co-culture of SRB and PSB, and compare the microbial community in this enrichment to environmental samples could further elucidate polymicrobial sulfur cycling in natural geochemical cycles.

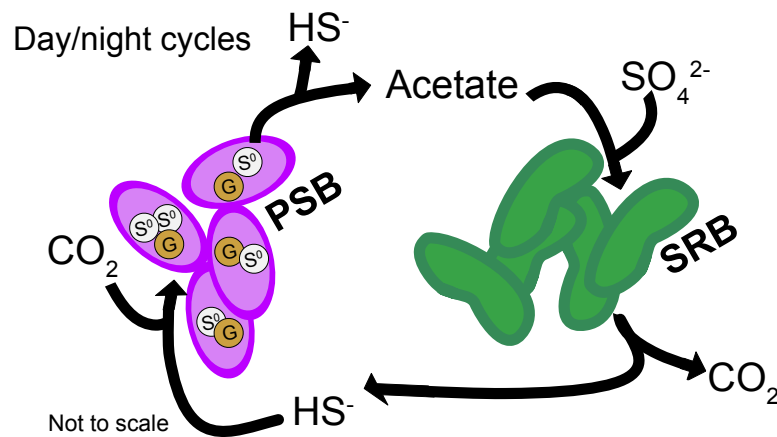


Figure 1. Model of sulfur cycling in hypothetical consortia of purple sulfur bacteria (PSB, pink) and sulfate reducing bacteria (SRB, green). PSB fixes carbon dioxide into biomass with light using sulfide as an electron acceptor. PSB forms sulfur (S⁰) and glycogen globules as a form of electron storage during light cycles. In the dark, PSB can respire sulfur (S⁰) and glycogen globules to produce acetate and sulfide. SRB can oxidize electron donors from a variety of sources including the photosynthate (acetate) produced by PSB during dissimilation.

Introduction:

Syntrophy is an obligately mutualistic interaction between two organisms that is based on the exchange of metabolites. Tightly coupled metabolic associations occur on spatiotemporal scales that often elude detection by classic geochemical analytical approaches (Boetius et al., 2000). Purple sulfur bacteria (PSB) are photosynthetic anaerobes in the phylum of Proteobacteria, which are capable of fixing carbon dioxide with sulfide as an electron donor (Figure 1). Most purple sulfur bacteria synthesize bacteriochlorophyll and carotenoids as their light-harvesting pigment complex, and thus can be selectively enriched with light emission in the infrared spectrum (Iba et al., 1988). The habitat for purple sulfur bacteria requires anoxic conditions, light, and sulfide; as such, they are often found in organic rich aquatic environments where sulfate reducing bacteria thrive. Sulfate reducing bacteria (SRB) are a phylogenetically distinct group that span many bacterial phyla including Proteobacteria, Firmicutes, as well as some archaea. The association of these microbial consortia have been studied throughout the history of the MBL course, and they often comprise major constituents of “pink berries” at the Great Sippewissett Salt Marsh (Wilbanks et al., 2014).

Syntrophy between purple sulfur bacteria and sulfur reducing bacteria depends on the limitation experienced by each organism in the selective environment (first isolation Biebel and Pfenning, 1978). In marsh environments experiencing massive fluxes in redox potential, the mutualistic interaction between purple sulfur bacteria and sulfur reducing bacteria allows these microbes to exploit metabolic niches that may otherwise be inaccessible to each independent counterpart. In environments where electron donors and acceptors are limiting, there may be a benefit to spatially anchoring with a syntrophic counterpart, which favors the formation of polymicrobial mats and/or aggregates. In co-culture this can be observed with syntrophic partners reach higher cell densities and form dual species biofilms on provided surfaces. Ecologically significant compounds exchanged between bacterial cells may comprise (1) signaling compounds as in the case of quorum sensing, (2) growth factors, and (3) compounds directly involved in energy metabolism: in the latter category the exchange of electron donors/acceptors is especially well understood for bacteria participating in the sulfur cycle (Morris et al., 2013).

Hypothesis:

- (H1) Polymicrobial sulfur cycling can be facilitated in cultures of organisms with complementary metabolisms
- (H2) Syntrophy between purple sulfur bacteria and sulfate reducing bacteria promotes growth on media where electron donors and acceptors are limiting

Results and Discussion:

We successfully enriched for purple sulfur bacteria and sulfate reducing bacteria from thiosulfate/sulfide enrichments. The thiosulfate/sulfide enrichments of purple sulfur bacteria began on 07/09/18. This produced 10 turbid/pigmented bottles that we used for further study beginning on 07/30/18. 60% of the bottles showed evidence for the presence of sulfate reducing bacteria and produced sulfide in acetate rich media (see SRB enhancement in methods).

After enhancing the amount of sulfate reducers in the co-enrichments we were able to see growth in two sample series that became the focal point for studying sulfur cycling within our enrichment strategy (towards addressing **H1**). We tested whether sulfur cycling was occurring in the two samples with growth on the energy rich media, and thus began a diel time series to

measure sulfate, sulfide, and acetate using ion chromatography and the colorimetric cline assay (mentioned above). At this stage, there was no growth on the minimal media and thus the diel study was not conducted on the one syntrophic minimal media bottle.

From our results (Figure 2) we observe that sulfide production and consumption are tightly coupled in our incubations with no quantifiable sulfide measured during the day. At night, the interactions between sulfate reducers and purple sulfur bacteria are decoupled and we are able to sulfide production from 400-40 nM). These results are concurrent with our hypothesized model, where purple sulfur bacteria consume sulfide during the day for biomass assimilation and election and energy storage (equation 1 and 2). At night, purple sulfur bacteria dissimilate glycogen and sulfur globules (according to 3) to produce acetate and sulfide. Sulfate reducing bacteria continually reduce sulfate to sulfide regardless of light cycles (according to equation 5). There is no hypothetical sulfide consumption in the dark, whereas there are two pathways for sulfide production (dissimilation by purple sulfur bacteria and sulfate reduction). The dark control in this work was an SRB enhanced enrichment that was kept in the dark for 15 days, and in this sample, we only see sulfide production. The abiotic control was co-culture media. To further test this hypothesis, we took a sample 2.4 out of the diel cycle and continued to measure the sulfide production throughout the course of the experiment (figure s4). From this, we observed that sulfide was continually produced and this likely indicates activity from the sulfate reducing bacteria. Further, at the end of the experiment we added 5 mM of sodium molybdate to sample 1.3 and 1.4 and monitored sulfide production over a night cycle. We saw no measurable production of sulfide according to the cline assay and the cupric sulfate colorimetric assay indicating purple sulfur dissimilation (equation 3) is less significant than sulfate reduction (equation 4).

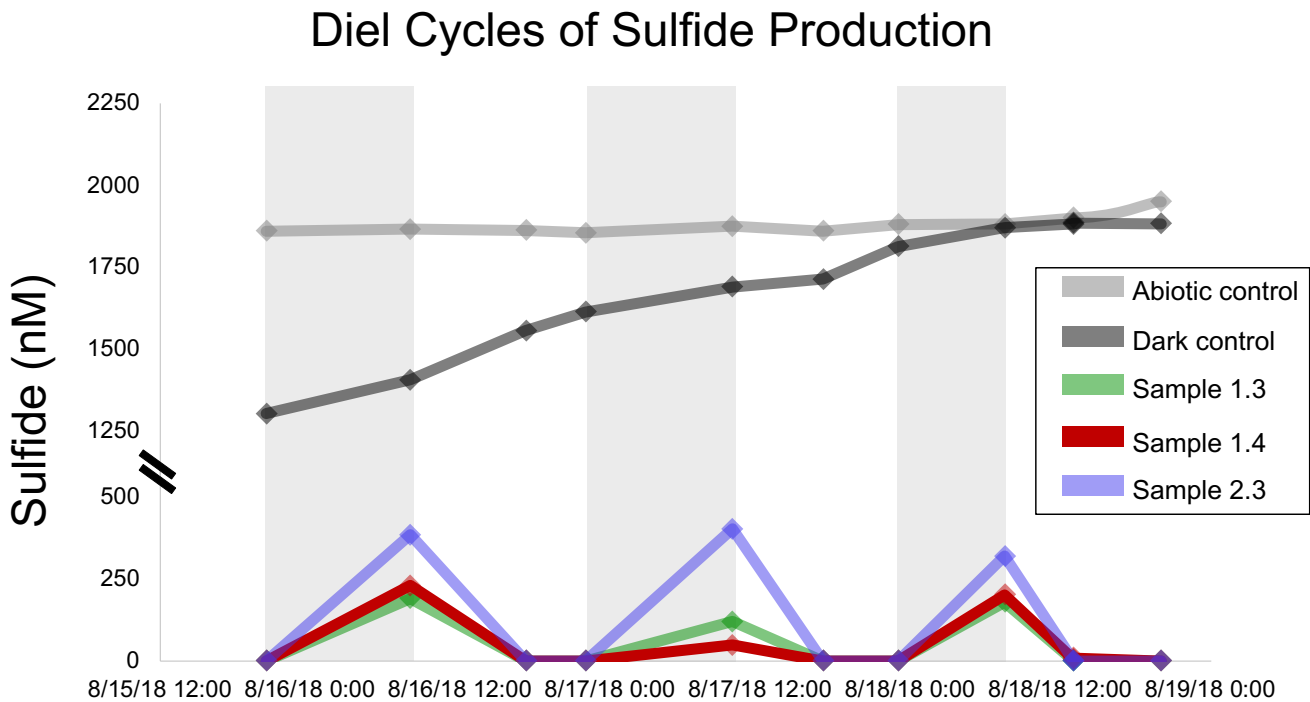


Figure 2. Sulfide concentration is minimal (not quantifiable by cline assay) during light cycles. Sulfide accumulates over the course of a night cycle across all 3 co-enrichments. Dark controls show sulfide accumulation over time. Abiotic controls are relatively stable across daily sampling efforts.

We also measured acetate over the course of the time series. Acetate concentrations show no appreciable trends related to the diel cycle (Figure 3). This likely indicates that acetate was in steady state and was not limiting to the sulfate reducers. Trends may also be related to the high concentration of acetate at the start of the diel experiment.

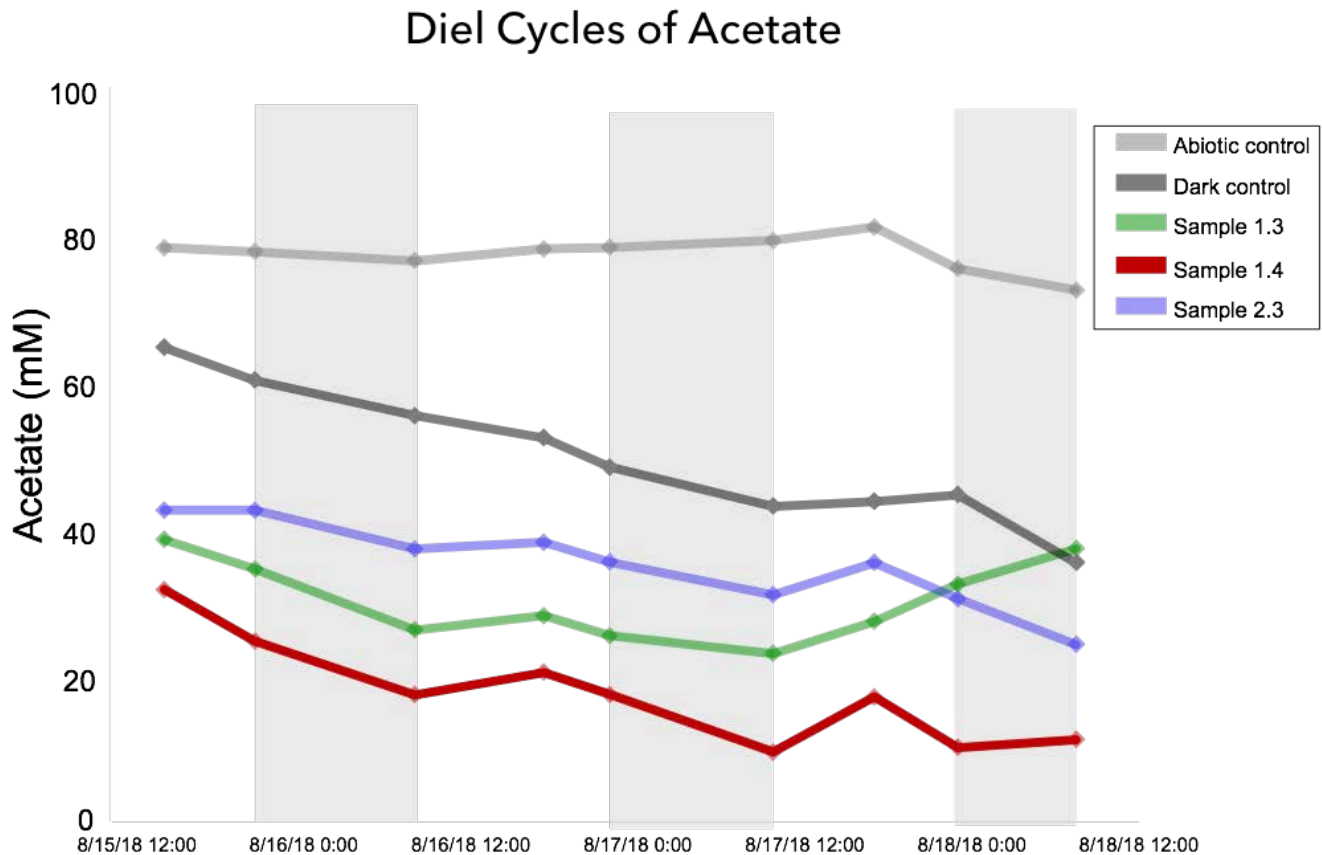
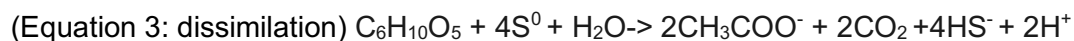
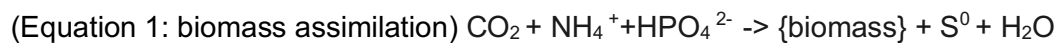
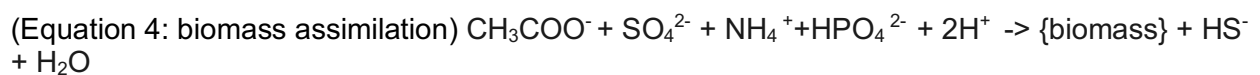


Figure 3. Sulfide concentration is minimal (not quantifiable by cline assay) during light cycles. Sulfide accumulates over the course of a night cycle across all 3 co-enrichments. Dark controls show sulfide accumulation over time. Abiotic controls are relatively stable across daily sampling efforts.

Purple sulfur bacteria:



Sulfate reducing bacteria



(Equation 5: anaerobic respiration) $\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} + 2\text{H}^+ \rightarrow 2\text{CO}_2 + \text{HS}^- + 2\text{H}_2\text{O}$

Lastly, we characterized the one sample that showed growth on the electron donor/acceptor minimal media towards our hypothesis **H2**. Turbidity in this sample alone shows evidence for syntrophy between the sulfate reducing bacteria and the purple sulfur bacteria due to the media composition. In this enrichment, the purple sulfur bacteria depend on the sulfate reducers for their electron acceptor (sulfide). In turn, the sulfate reducing bacteria depend on the purple sulfur bacteria for an electron donor in the (hypothesized as acetate). Using light microscopy, we observed the presence of sulfur globules during the day and these globules appear to be absent at night. Which supports our hypothetical sulfur model and thus our hypothesis (**H1**). We also used monoFISH to analyze the presence of Desulfobacteraceae with the probe DSS658.

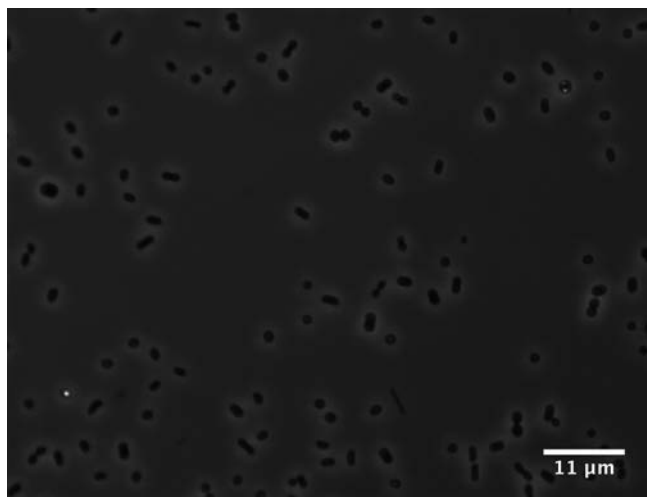
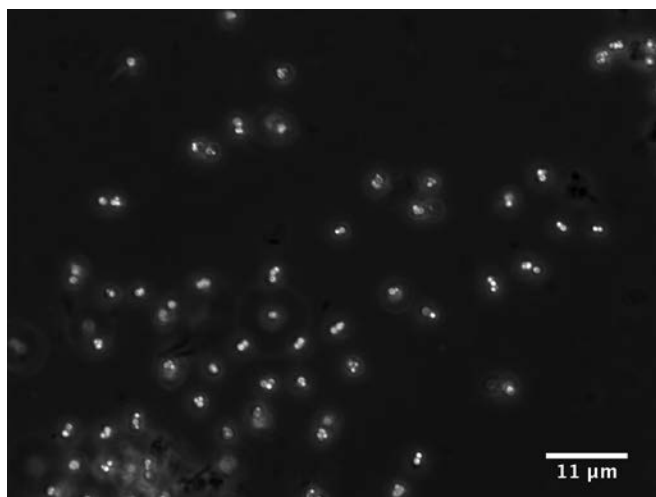


Figure 4 (top left). Sulfur globules of PSB in electron/acceptor minimal media. Samples viewed at hour 11 of day cycle.

Figure 5 (top right). Sulfur globules in PSB are mostly absent in hour 11 of night cycle. One globule can be viewed on the bottom left of the image.

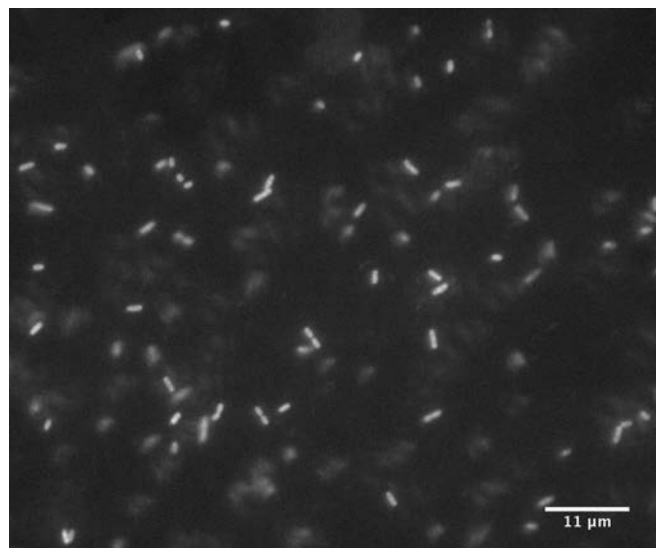


Figure 6 (bottom left). MonoFISH results indicating fluorescence in Cy3 of sulfate reducing bacteria using DSS658 probe for Desulfobacteraceae.

The absorbance spectra of the syntrophic culture were measured over the course of the experiment. Interestingly, the transition from “energy rich” media to the minimal media caused a shift in community and thus the resulting spectra. The energy rich media had a peak at 900 nm

that appeared to disappear over in the transition to minimal media. The carotenoids (wavelength 500 to 560nm) also appear to shift with the media transition.

Absorption Spectra

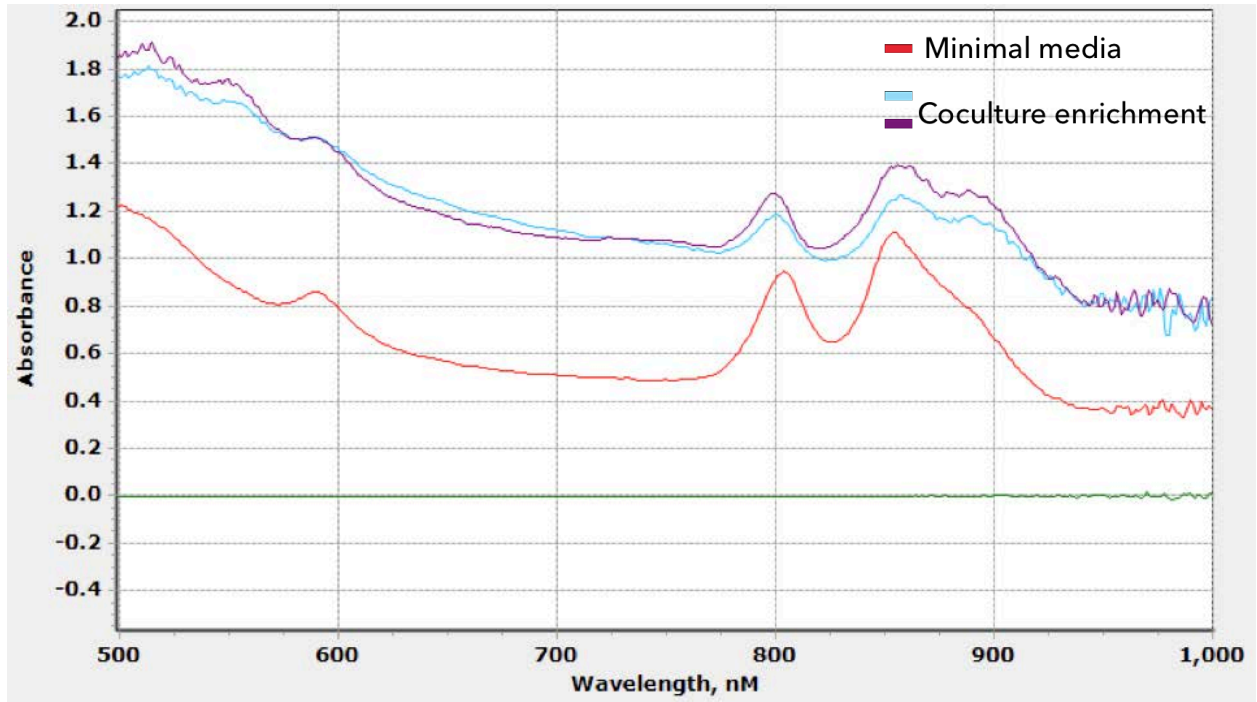


Figure 7. Absorption spectra of co-enrichment series. Spectra in red indicates the sample that showed growth on electron donor/acceptor minimal media. Purple and blue spectra correspond to enrichment on rich media. Peaks at 590, 800, and 850 correspond to bacteriochlorophyll a.

Methods:

Original PSB enrichments: Due to time constraints, we enriched for PSB and SRB syntrophs on 07/30/18 in the thiosulfate/sulfide enrichments that began when the course started on 07/09/18. Trunk River samples were specifically selected for study because the organic content of the soil provides a breeding ground for anaerobic sulfur cycling. Enrichments were inoculated with sediment ~1g or 1 mL of sediment supernatant. Purple sulfur enrichments were on brackish media (1:1 mixture of FW base and SW base) that contained thiosulfate and sulfide as electron acceptors and carbon dioxide (bicarbonate) as electron donors. Detailed media as follows, 2.9L of DI water, 30 mL of 100x FW-Base, 30 mL of 1M NH₄Cl solution, 30 mL of 100mM potassium phosphate (pH 7.2), 15 mL of MES buffer (pH 5.5), 30 mL of 1 M thiosulfate, and 3 mL of trace elements. 100X fresh water (FW) base consists of 100 g NaCl, 40 g MgCl₂·6H₂O, 10 g CaCl₂·H₂O, 50 g KCl per liter. This was autoclaved, and then stock solutions were added under a stream of N₂CO₂ (80%:20%), 3mL of multivitamin, 150 mL of 1M sodium bicarbonate, 3 mL of 1M sulfide, and 50 mg of DCMU. Media was dispensed into sterile Pfenning bottles in the

anaerobic chamber. Purple sulfur bacteria were selectively enriched using constant infrared LEDs with emission at 850 nm at room temperature.

Co-enrichment of PSB and SRB: From turbid the thiosulfate/sulfide enrichments mentioned above with absorption peaks at 800 and 850 nm (discussed in detail below) we introduced 12 hr light/dark cycles at 850 nm to promote production of acetate at night. We analyzed the production of sulfide at night using a qualitative cupric sulfate colorimetric test. For this test we created a 5 mM $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ solution in 80 mM HCl by adding 1.25 grams of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ to 4.31 mL of 80 mM HCl. This solution is mixed with the experimental sample in a 1:1 ratio and the production of brown coloration indicates the presence of sulfide. We used this to determine which PSB thiosulfate enrichments to continue with.

Large volumes (15 mL) of the thiosulfate/sulfide enrichments were transferred to Pfenning bottles containing 'energy rich' coculture media. This media contains both acetate and sulfide to enrich for SRB that use acetate as an electron donor and PSB that use sulfide as an electron acceptor. The media recipe per liter of DI water is as follows: 500 mL of SW-base, 5 mL of FW-base (same stock as mentioned above), 11 mL of 5M sodium chloride (used to balance ion strength between all media types), 5 mL of 1M NH_4Cl solution, 5 mL of 100mM potassium phosphate (pH 7.2), 5 mL of MES buffer (pH 5.5), 30 mL of Na_2SO_4 , 1.25 mL of 2M sodium acetate anhydrous, 1 mL of 1000x multivitamin, and 10 mL 1M sodium bicarbonate. SW- base consisted of 342.2 mM NaCl, 14.8 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 6.71 mM KCl. The pH was adjusted to 6.8 to reflect the pH of Trunk River. After approximately 5 days of selective light the incubations were moved to white light to facilitate faster growth rates.

Enhancement of SRB: Slow growth rates were observed on the rich co-culture media, which was likely due to the transition to sulfide as the sole electron acceptor for the purple sulfur bacteria and low abundances of sulfate reducing bacteria. To increase the amount of sulfide cycling through the system we transferred 20 mL from the coculture media incubations to new media that was identical to the 'energy rich' media but lacked sodium sulfide, had 60 mM sodium acetate (as opposed to 2.5 mM), and kept the samples in the dark. This increased the abundance of sulfate reducers and we observed turbidity as well as increased sulfide production (via the cupric sulfate colorimetric test). 35 mL of this 'SRB enhanced' media was mixed with 35 mL of the original coculture enrichment for each treatment in the anaerobic chamber. There were 10 original thiosulfate enrichments that were transferred to 'rich coculture media' and of those 6 of the SRB enhanced treatments showed turbidity and were reinoculated back to their original coculture enrichments.



Figure 8. 10 original thiosulfate/sulfide incubations used for transfers into 'rich co-culture' media.

Minimal media to facilitate syntrophy between SRB and PSB: Each of the 6 SRB enhanced enrichments were transferred to media that forced syntrophy between SRB and PSB by lacking acetate and sulfide. The media recipe per liter of DI water is as follows: 500 mL of SW-base, 5 mL of FW-base (same stock as mentioned above), 12 mL of 5M sodium chloride (need to increase to balance ionic strength), 5 mL of 1M NH_4Cl solution, 5 mL of 100mM potassium phosphate (pH 7.2), 5 mL of MES buffer (pH 5.5), 30 mL of Na_2SO_4 , 1.25 mL of 2M sodium acetate anhydrous, 1 mL of 1000x multivitamin, 10 mL 1M sodium bicarbonate. The pH was adjusted to 6.8. Large volume transfers were necessary for this minimal media due to slow growth and time limitations. Only 1 out of 6 enrichments showed turbidity on 08/17/2018.

Enrichment Strategy

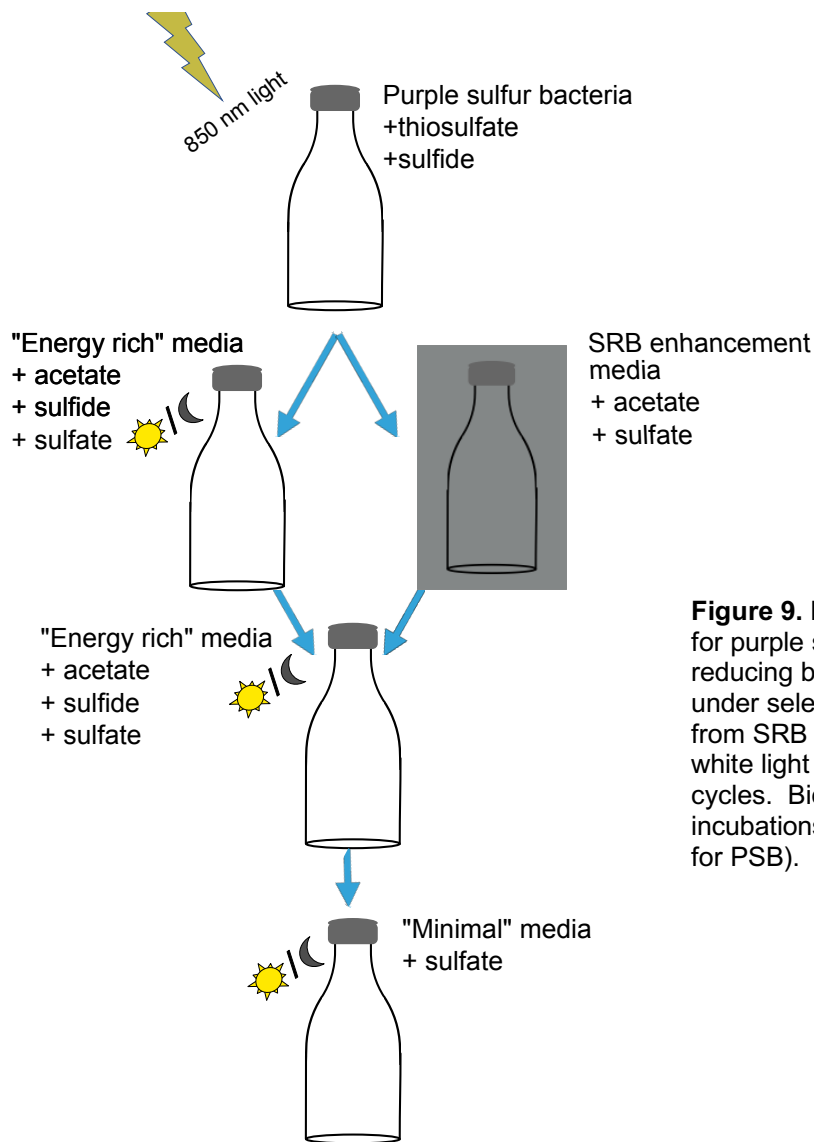


Figure 9. Basic enrichment strategy for purple sulfur bacteria and sulfate reducing bacteria. After initial growth under selective light samples (aside from SRB enhanced) were moved to white light with 12 hour light/dark cycles. Bicarbonate was added to all incubations (necessary electron donor for PSB).

Measuring absorbance spectra:

Enrichments with turbidity bottle were analyzed in Pfenning bottles using the Spectral Evolution spectroradiometer (model SR-1900) and DARWin software (Spv1.2.5093). Each media type was used for a blank reference before each measurement and 4 to 6 replicate absorbance spectra were analyzed for each bottle.

Sulfide Cline assay : Water samples were fixed for sulfide analysis according to (Cline et al., 1969). Samples (1.2 mL, 0.22 micron filtered) were fixed with 0.3 mL of 91mM zinc acetate and kept in the dark at room temperature until analysis. 91 mM zinc acetate was prepared from 20g zinc acetate dihydrate, 200 μ L of glacial acetic acid, 1000 mL milliQ water, and stirred at room temperature. Reagent 1 (0.2% DPPDS solution) and 2 (1% ferric ammonium sulfate) were mixed in a 1:1 ratio, and 0.5 mL of the mixture was added to the 1.5 mL of fixed sulfide samples and incubated for 30 minutes. Samples were then added to a 96 well plate, diluted (1:4, 1:16, 1:64) if dark in color, and measured on a spectrophotometer plate reader at 665 nm. Standard curves were made from a 0.1M Na₂S stock solution from 0-100 μ M.

Ion chromatography: Sulfate and acetate concentrations were determined from water samples on the Dionex ICS- 2000 using an IonPac AS22 Fast Column (Dionex) and IonPac Ag22 Guard Column (Dionex) at 30°C. Samples were 0.22 micron filtered and stored at 4°C for up to 3 days before analysis. Samples were taken 3x daily approximately every 5.5 hours.

MonoFISH: Water samples were fixed with 3.3% paraformaldehyde overnight at 4°C and filtered onto a 0.22 micron polycarbonate (25mm) with a cellulose nitrate backing filter. Filters were cut into 8 sections and hybridized in buffer according to probe requirements. The following probes were tested for sulfate reducer detection: DSR651, DSS658, and DSV698. Non338 was used as a non-target control and Eub338 was used to detect all bacteria. Hybridized filters were then washed with buffer according to probe requirements. Filters were washed with milliQ water and counterstained with DAPI. Samples were mounted in a 4:1 mixture of Citifluor and Vectashield and stored frozen until analysis.

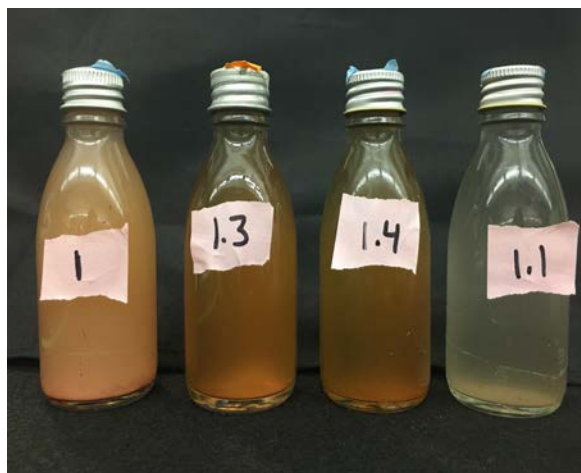


Figure S1 (left). Enrichment series that resulted in growth on energy minimal media. Bottle 2 is the original thiosulfate/sulfide incubation. Bottle 2.3 and 2.4 were passaged to coculture energy rich media from 2.2, which was a 1:1 mixture of SRB enhanced and the thiosulfate enrichment. Bottle 2.5 was passaged from bottle 2.4 into “minimal media”. **Figure S2 (right).** Other enrichment series that grew on co-culture “energy rich” media. Bottle 1 is the original thiosulfate incubation, bottle 1.3 was a 1:1 mixture of bottle 1.1 (SRB enhanced) and bottle 1. 10 mL of bottle 1.3 was transferred to co-culture “energy rich” media and grew turbid.

Supplemental Material:

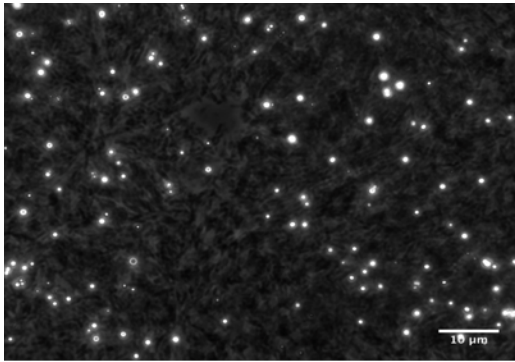
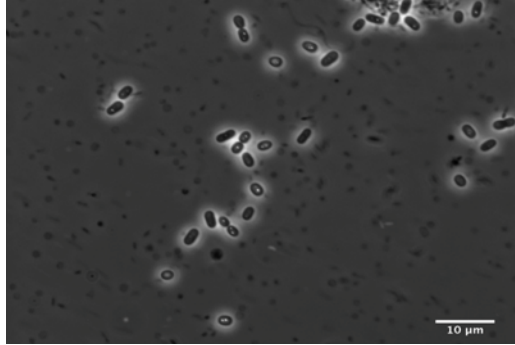
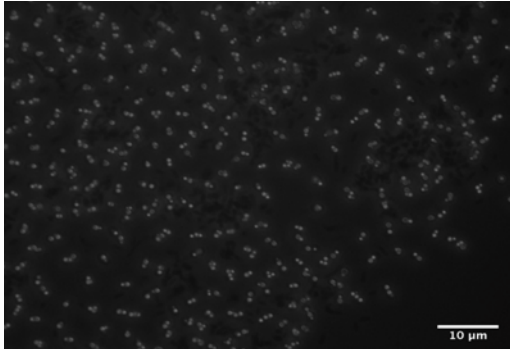


Figure S3. Morphology of purple sulfur bacteria observed in thiosulfate incubations. Globules are observed in all three images and the bottom left image appears to have extracellular sulfur globules.

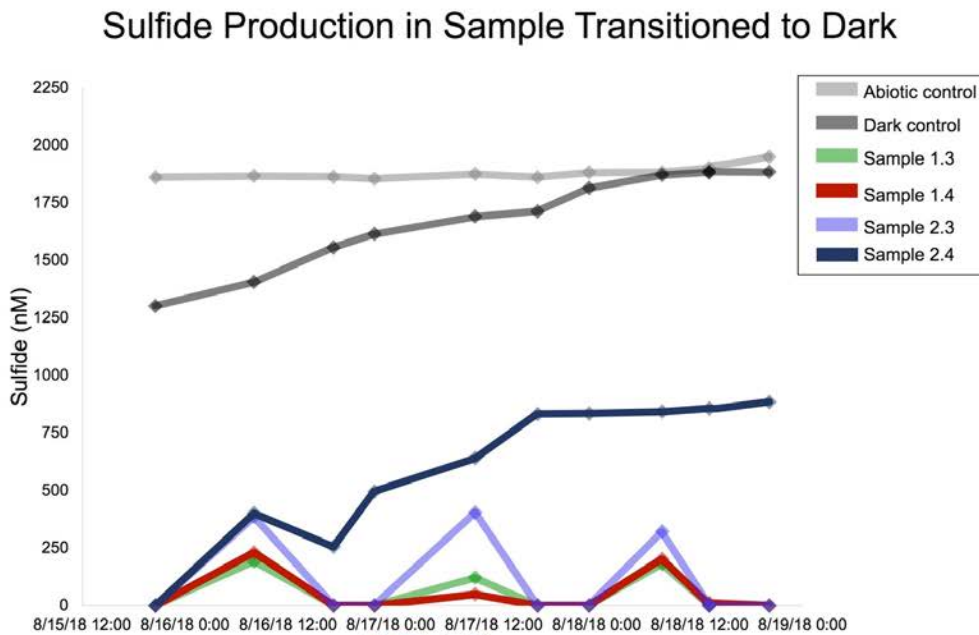


Figure S4. Basic enrichment strategy for purple sulfur bacteria and sulfate reducing bacteria. After initial growth under selective light samples (aside from SRB enhanced) were moved to white light with 12 hour light/dark cycles. Bicarbonate was added to all incubations (necessary electron donor for PSB).

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