Targeted enrichment and isolation of sulfur-oxidizing bacteria from the Skaftárkatlar subglacial lakes, Iceland

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Abstract
The Vatnajökull ice cap is known to host a series of subglacial lakes with geochemical gradients that support and suggest life. Because a clear sulfate and sulfide gradient has been recorded within the East and West Skaftárkatlar lakes, a microbially-driven sulfur cycle has been proposed to dominate this system. However, little research has been conducted on sulfur-oxidizing microbes in these subglacial lakes. In this study, Rhizobiaceae Rhizobium, Microbacteriaceae Microbacterium, Caulobacteraceae Brevundimonas, and Oxalobacteraceae Janthinobacterium have been isolated from the the East and West Skaftárkatlar lakes and support previous findings on lake depth origin. Measured increase in sulfate concentration and decrease in sulfide concentration over time in isolated enrichment samples indicates active sulfur redox; however, due to contamination of the controls, it is unclear on whether or not the observed sulfur oxidation reactions are a result of biotic or abiotic redox processes.

Introduction
The Vatnajökull ice cap harbors a story previously unknown to the scientific community. While it is known that the ice field occupies 3,127 mi$^2$ of Iceland, little is documented about the abundance of activity below the blanket of ice – especially pertaining to microbial life. The tectonic and volcanic activity that occurs beneath the ice ultimately engenders a system of subglacial lakes that have the potential to support life. The subglacial lake systems focused on within this study are the East and West Skaftárkatlar lakes. Overall, very little is known about psychrophilic organisms although over 80% of life lives in temperatures below 5°C (Priscu and Christner, 2004). This gap in biological knowledge is concerning. In this case, the microbiological community is interested in exploring the possibility of microbial life in this cold, dark, and nutrient-poor environment. This study is focused on investigating the microbial communities living within the subglacial lake systems beneath the Vatnajökull ice cap, which have yet to be explored.

In June of 2015, samples were collected from the East and West Skaftárkatlar subglacial lakes for microbial analysis – specifically looking for sulfur oxidizers, sulfur reducers, hydrogen oxidizers, and acetogens. Previous studies of the water composition found that these subglacial lakes are sulfidic, making sulfur-oxidizing microbes a particular subject of interest since they may be the dominant players in this redox system (Figure 1). By culturing and characterizing these microbes, a better understanding of the microbially-driven redox processes that govern these subglacial environments can be obtained. Increased understanding of these microbial phylogenies and their role in this subglacial lake environment will lead to a more comprehensive understanding of subsurface psychrophilic life on Earth as well as a better idea of how life may potentially behave in similar systems on Europa and Mars. Overall, these subglacial environments are characterized by low temperature, a lack of light, and low nutrient levels. It is postulated that beneath the kilometers of ice on Europa, life could potentially live in the cold, dark waters, fueled by
volcanic or hydrothermal vent activity (Gaidos et al., 1999). Indeed, studying accessible systems on Earth can lead to greater insights on other planets and moons within our solar system. Moreover, the Snowball Earth theory postulates an environment highly similar to that of these ice-covered, cold and dark environments (Vincent et al., 2000). Although this study aims to better understand current metabolic systems on Earth, these findings can also give light to new astrobiological and paleobiological perspectives of Earth and other potential life-harboring solar system bodies.

**Background**

Iceland is unique because it lies on the diverging North American and Eurasian plates as well as on a hot spot (Figure 2). As a result, the Vatnajökull ice cap is exposed to underlying heat from volcanic activity which causes glacial melting, resulting in the creation of subglacial lakes. Iceland’s most active volcano, the Grímsvötn volcano, is responsible for the creation of both the East and West Skaftárkatlar lakes as can be seen in Figure 3. Due to convective flow, driven by less dense warm water rising and cooler water sinking, the East and West Skaftárkatlar lakes are rather uniform in composition (Johannesson et al., 2007). Each lake is approximately 3.5°C and has a pH of approximately 5.0 (supplemental Figure 1). On average, each lake is approximately 90m deep, overlaid with a 300m thick ice sheet, each approximately 1 km² large (Gaidos et al., 2009).

These subglacial sites are interesting because the lake water compositions are not simply a result of glacial melting. Previous studies on the composition of the East and West Skaftárkatlar lakes indicate unusually high concentrations of CO₂, SiO₂, and H₂S. Additional cations and anions and their concentrations within each lake have been determined; this study is especially concerned with the presence of sulfide and sulfate (see Supplementary Figure 2). It has been proposed that these compounds are a result of volcanic outgassing and additional geothermal fluid inputs (Johannesson et al., 2007). Figure 4 depicts this system of the geothermal process – melting the glacier ice, creating a lake which can be accessed by creating a borehole through the ice sheet.
This supply of energy sources is crucial in establishing geochemical gradients capable of supporting microbial metabolisms within lakes. The highly similar fluid compositions and phylotypes in both subglacial water columns suggest that the lakes are connected, possibly by a crustal aquifer system (Flowers et al., 2003; Marteinsson et al., 2012). Indeed, pyrosequencing, cloning, and fluorescent in-situ hybridization (FISH) sample analysis of the East and West Skaftárkatlar lakes have shown that the two lakes are nearly identical in geochemical composition and are both dominated by the same few microbial communities — Acetobacterium, Paludibacter, Sulfuricurvum, and Sulfurospirillum (Figure 5; Supplementary Figure 2). Cell counts amount to approximately $5 \times 10^5$ cells per ml in each lake, and while bacteria dominate the microbial scene, archaea make up 0-1% of this microbial community (Gaidos et al., 2009). The highly sulfidic water composition seems to support various microbially-mediated sulfur redox reactions, making sulfur oxidizers an important target for study.

Although previous research has illuminated the geochemistry and microbial taxa that dominate the lake, most of the microbial ecology of the lake has yet to be characterized. Moreover, despite the fact that a microbially-driven sulfur cycle has been proposed, no research on sulfur-oxidizing bacteria has been done. This investigation aims to enrich, isolate, and characterize microbes from the East and West Skaftárkatlar subglacial lakes and link them back to previous findings on the lake ecology in order to determine whether bacterially-mediated sulfur oxidation is the major microbial metabolism governing these local redox systems.
3. Materials and Methods

3.1 Sample Acquisition

In order to acquire the water samples, one borehole was created in each the East and West Skaftárkatlar lakes using a hot water drill. Snow was taken from the surface of the ice and heated to 80-90°C before being pumped through the drill as it melts through the ice and creates a hole. Water samples were collected at three different depths – 338, 358m, 378m – in the East Skaftárkatlar lake and samples were collected from two depths – 318m and 338m – in the West Skaftárkatlar lake (Figure 6). An ice sample was collected at each the East and West Skaftárkatlar lakes at 310m and 292m respectively. A snow sample was also collected at the surface of the ice sheet. The water samples were filtered on the site and the filters were placed into freshwater-base media – consisting of 10 mL of 100x Freshwater base, 10 mL of 100x NH₄Cl (1M), 1 mL of 1000x Na₂SO₄ (1M), 1 mL of 1000x Na₂HPO₄ (1M), 10 mL of 100x phosphate buffer, 1 mL of 1000x trace minerals, and 1 mL of 1000x vitamins brought up to a 1 L volume.

3.2 Sulfur Oxidizer Enrichments

The samples were brought back and stored at 4°C in 120 mL serum bottles sealed with rubber stoppers and 20mm aluminum crimp caps. In order to select for the sulfur oxidizers in these bottles, these enrichment cultures were degassed bimonthly for 30 minutes and then 200 µl of either 0.5 M H₂S or 0.5 M Na₂S was added to each bottle by injecting the sulfide through the rubber stoppers using a syringe and 0.6 x 25mm needles. Every three months, the enrichment cultures were degassed and 10 mL of this culture was placed into a new serum vial with 20 mL of freshwater-base media.

3.3 Sulfur Oxidizer Isolation

Thiosulfate plates were created using the same freshwater-base media with the addition of 15 grams of bacteriological agar, 6 grams of sodium thiosulfate, and 0.02 grams of bromothymol blue. Approximately 10 µl of enrichment media was extracted from the enrichment cultures, spread onto the thiosulfate plates using inoculation loops, and stored at 4°C. The colonies were then left to grow.
3.3.1 *Sulfuricurvum* Isolation

Approximately 10 µl of enrichment media from the East lakes at the depth of 338m was extracted from the enrichment cultures, spread onto the thiosulfate plates using inoculation loops, and stored at 4°C. The colonies were then left to grow.

3.4 Sequencing Sulfur-Oxidizing Colonies

3.4.1 Colony Selection and Amplification

12 colonies were selected from the plated enrichment cultures for amplification and potential sequencing. Selected colonies were removed using a pipette tip, tapped onto another labeled thiosulfate plate, and mixed into 17.5 µl of Milli-Q water in small PCR (Polymerase Chain Reaction) tubes. These samples were then subjected to 4-5 freeze-thaw cycles – 10 minutes in an -80°C freezer and then immediately placed in boiling water until melted. Rather than adding a colony to the PCR tube for the controls, 1 µl of Milli-Q water was added to the negative control and 1 µl of pre-made *E. coli* aqueous sample was added to the positive control.

In the PCR hood, a PCR Master Mix of reagents was created – 2.5 µl of buffer, 2.5 µl of DNTPs, 0.5 µl of forward primer, 0.5 µl of reverse primer, and 0.5 µl of taq (per sample); 6.5 µl is added to each PCR tube containing the lysed colonies.

These tubes are then placed in the Veriti 96 Well Thermal Cycler and the Colony PCR protocol is run – Stage 1: 95°C for 10 minutes, Stage 2: thirty five cycles of 95°C for 1 minute, 54°C for 90 seconds, and 72°C for 1 minute, Stage 3: 72°C for 5 minutes, Stage 4: hold at 4°C until run is ended by user.

3.4.2 Gel Electrophoresis Run

A gel is created by dissolving 0.5 grams of agarose in 50 mL of 1x TAE buffer and then adding either 5 µl of SYBR safe DNA gel stain or 5 µl of ethidium bromide. This solution is then poured onto a gel tray with the comb. Once solidified, the gel tray is placed into the buffer chamber on the gel electrophoresis apparatus and 1x TAE buffer is added to the fill line. 3 µl of the Thermo Scientific O’GeneRuler 1kb Plus DNA Ladder is added to the first well using a pipette. 2 µl of each sample is mixed with 2 µl of Thermo Scientific 6X Orange DNA loading dye on a strip of parafilm, and 2 µl of this mix is loaded into each well, with a separate sample in each well. The lid with the power supply lead is placed on the top of the buffer chamber and connected to the power source at 86 volts for approximately 20 minutes. The gel is then removed and placed in the BIORAD Molecular Imager Gel Doc XR+ Imaging System and after positioning the gel on the screen, either the SYBR safe or ethidium bromide protocol is run.

3.4.3 DNA Purification and Sequencing

Based on the presence of bands on the gel run visualization, certain colonies are selected for DNA purification before sending off for sequencing. Colonies selected for sequencing were purified using a Zymo Research DNA Clean and Concentrator Kit and submitted to GeneWiz (South Plainfield, NJ) for Sanger sequencing. Sequencing results were then returned. Results with high quality scores were inputted into NCBI BLAST program to determine taxonomy. Classifications were checked against SILVA database after removing any poor-quality nucleotides sequences (‘N’ repeats) within the returned sequences.
3.5 Colony Characterization: Metabolic and Reproductive

Upon selection and inoculation of 12 colonies into 50 mL of freshwater-base media with Na₂S separately, measurements were made every other day to analyze the rate of growth, amount of sulfide reduction, and amount of sulfate production.

3.5.1 Microscopy: Cell Counts

In order to perform cell counts, 500 µl of unfiltered enrichment cultures were placed in a 1.5 mL eppendorf tube with 200 µl of acridine orange (4 µg/mL), inverted a few times, and placed in the dark for 15 minutes. Whatman membranes (25mm 0.2 um) were placed on support pads in each well on the AA Hoefer vacuum filter. Chimney weights were then placed on each well over the pad and filter membrane. Following this, the 700 µl sample mixture is transferred on each well using a pipette. The filter system is turned on and is run for approximately 2-3 minutes. Using sterile tweezers, the membrane filter is placed on a VWR microslide (3 in x 1 in x 1mm) and a drop of Type A immersion oil is added before placing a VWR micro cover glass (22mm x 40mm) on the filter. The slide is then placed on the stage of a Zeiss Microscope and visualized with the 40x/0.75 lense and filter #2 for acridine orange visualization. 20 fields of view are chosen and the number of cells counted in each field of view is recorded.

3.5.2 Sulfide Analysis

In order to run a sulfide analysis using the spectrophotometer, 15 µl of ferric chloride (FeCl₃) and 50 µl of acid sulfide reagent was placed into each cuvette. 750 µl of filtered enrichment culture was then added to each cuvette and diluted with Milli-Q water as needed to ensure an absorbance reading between 0.1 and 1 in the spectrophotometer. A blank consisting of 750 µl total volume of FeCl₃, acid sulfide reagents, and Milli-Q water served as the blank before the sample absorbance readings were measured. The dilutions and absorbance readings for each sample were recorded. For the colony characterizations, this measurement was made every other day.

3.5.3 Sulfate Analysis

In order to measure the change in sulfate concentration, ion chromatography was used. First, 500 µl of enrichment cultures were filtered through a 0.2um filter. Each sample was diluted to a total volume of 5mL and a set of sulfate standards were made – 1:1, 1:10, 1:100, 1:1000. Each sample was covered in foil and placed on the rotating rack. The Ion Chromatograph system in the Nealson Lab was operated by Pratixa Savalia and results were recorded after cycle termination.

Results

Sulfur Oxidizer Isolation

A change in plate color was visible for different sulfur oxidizer isolations. Some plates changed from green to blue, and other plates changed from green to yellow while some plates remained green.

Figure 7. Thiosulfate plate color change depending on pH change; less acidic (left) to more acidic (right)
Crystal-like dendritic growth appears on several blue plates (Figure 8). Each of these blue plates is either from the second depth of the East Skaftárketill lake – 358m – or from the second depth of the West Skaftárketill lake – 338 m.

Figure 8a. Close up image of crystal-like growth (right).

Figure 8b. Crystal-like growth in blue plates (left).

Sulfuricurvum Isolation
After inoculating the thiosulfate plates and leaving them at 20°C, there was an abundance of very very small white colonies, most of which indistinguishable from one another (Figure 9).

Sequencing Sulfur-Oxidizing Colonies
After sequencing the 12 colonies, one sequencing failed and the other 11 sequences were successful. Using SILVA database, the sequences were compared to other organisms inputted into the database. Based on the SILVA database, the colonies sequenced from the East Skaftárketill lake depth of 378m were primarily Rhizobiaceae *Rhizobium* (78-89% identity), Microbacteriaceae *Microbacterium* (85.61% identity), Caulobacteriaceae *Brevundimonas* (99.53% identity), and Oxalobacteriaceae *Janthinobacterium* (100% identity). All of these isolates came from yellow plates with the exception of the Oxalobacteriaceae *Janthinobacterium* isolate. The West Skaftárketill lake sample sequences at a depth of 338m indicated similarity to Caulobacteriaceae *Brevundimonas* (75-76% identity) and Rhizobiaceae *Rhizobium* (72-82% identity), both of which were isolated from yellow plates. Two isolates from green plates came from the East Skaftárketill lake samples at the first depth – 338m – and had a 79-84% identity with Oxalobacteriaceae *Janthinobacterium*. This is outlined in Figure 10.

Figure 9. Potential *Sulfuricurvum* isolation growth on thiosulfate plate.
Figure 10. Isolates and percent identities for the East and West Skaftárkatlar lakes based on SILVA database. Figure courtesy of Jayme Feyhl-Buska.

Figure 11. Percent composition of enrichments from the east and west skafta lakes, borehole ice sample, and snow melt sample. (Photo courtesy of Jayme Feyhl-Buska)
The isolation of Oxalobacteraceae at 338m and 378m in the East Skaftárketill lake is supported by its presence at these same depths as Figure 10 shows. The isolation of Caulobacteraceae and Rhizobiaceae at 338m in the West Skaftárketill lake is also consistent with the depths at which it was found in previous studies. Microbacteriaceae was also isolated at 378m in the East Skaftárketill lake which is consistent with the fact that it composes approximately 10% of the samples from this depth (Figure 11).

**Colony Characterization: Metabolic and Reproductive**

Upon selection and inoculation of 12 colonies into 50 mL of freshwater-base media with Na₂S separately, measurements were made every other day to analyze the rate of growth, amount of sulfide reduction, and amount of sulfate production.

**Microscopy: Cell Counts**

Cell count data was collected for samples 1-12 and the controls on day of inoculation. All samples contained an average of no cells at this point. There were 1-4 cells visible per 500 µl. Difficulties in staining methods resulted in incomplete data; however, sample 2 did show an increase in cell count, 2 cells/ µl at T = 0 days to 387 cells/ 500 µl at T = 5 days. After 5 days, the controls did appear to contain cells as well.

**Sulfide Analysis**

There is an overall decrease in sulfide concentration over time for both the samples and the control. The controls begin at a concentration of 1.25 mM on average and become stable at approximately 0.133 mM at 19 days. The samples begin on day 2 at 0.45 mM on average and become stable 0.134 mM. This can be seen in Figure 12.

![Figure 12. Decrease in average sulfide concentration (mM) over time (days) for both samples and controls. Standard deviation included.](image-url)
Sulfate Analysis

The concentration of sulfate increased as time increased for both the samples and the controls (Figure 13). The controls begin at an average concentration of 0.06 mM and reach approximately 0.19 mM after 16 days. The samples begin at an average concentration of 0.1 mM and reach approximately 0.19 mM after 16 days, nearly identical to the average control.

![Figure 13. Average increase in concentration of sulfate (mM) with increase in time (days). Standard deviation included.](image)

Discussion

The thiosulfate plates contain a pH indicator that causes the plate to change color based on change in plate pH. Plates that became blue indicated H\(^+\) consumption and sulfur oxidation as thiosulfate is converted to tetrathionate. This reaction is summed up in the following redox equation:

\[
4\text{S}_2\text{O}_3^{2-} + \text{O}_2 + 4\text{H}^+ \rightarrow 2\text{S}_4\text{O}_6^{2-} + 2\text{H}_2\text{O}
\]

Plates that remained green indicated no pH change.

Plates that became yellow indicated H\(^+\) production and sulfur oxidation as thiosulfate is converted to sulfate. This is summed up in the following reaction:

\[
\text{S}_2\text{O}_3^{2-} + \text{H}_2\text{O} + 2\text{O}_2 \rightarrow 2\text{SO}_4^{2-} + 2\text{H}^+
\]

Colonies isolated on yellow plates and green plates proved simple to amplify while colonies isolated on blue plates were difficult to lyse and amplify. Upon running the PCR and
colony amplification protocol, most yellow and green colonies amplified and were apparent in the UV visualization. A few mishaps occurred with the PCR reagents as well as DNA loading dye, DNA ladder, and SYBR Safe dye resulting in many failed attempts of amplification and visualization. After replacing old reagents and using completely new material, 6 out of 32 isolates from the blue plates amplified. These will be sent for sequencing in the coming weeks.

Initially, the 11 successfully isolated and sequenced colonies (out of the 12 sent off for sequencing) from the yellow and green plates appeared to have weak identity to any previously characterized microorganism in the SILVA database. Selection for two of these isolates for characterization was in preparation; however, the DNA sequences were re-inputted into the database excluding the poor quality nucleotides. By re-inputting only the good quality nucleotides – cutting the sequence with the appearance of an ‘N’ in the nucleotide sequence – the percent identity of the Rhizobiaceae *Rhizobium* (78-89% identity), Microbacteriaceae *Microbacterium* (85.61% identity), Caulobacteraceae *Brevundimonas* (99.53% identity), and Oxalobacteraceae *Janthinobacterium* (75-76% identity) all became 99-100% identical to previously identified and characterized organismal species.

Each of the sequenced colonies can be successfully linked back to previous studies of microbes previously isolated and sequenced from the lake. By comparing Figure 10 and Figure 11, it is clear that the isolated and sequenced family data relate back to the composition of each lake at each different depth and correlate very well with this data (i.e., there were no isolations that were not previously found to be present within the specific lake depths in each lake.

Although these 12 colonies turned out to be identical to previously isolated and characterized microorganisms, they were still inoculated in freshwater-base media with Na$_2$S samples for analysis of growth rate, sulfide reduction, and sulfate production. Results from cell counting are inconclusive. At T = 0 days (the day of inoculation) there was an average of 0 cells/ 500 µl per sample. Sample 2 is the only sample with additional data at T = 5 days. The cell count for sample 2 was 387 cells/ 500 µl. There is no quantitative data for the other samples; however, there did appear to be several cells in Control 1. Fogging of the membrane on the slides created very poor visibility and prevented accurate calculations (see Supplementary Figure 3). It is posited that the acridine orange staining agent may interact with the sulfur oxidation products. Cyto-9 live/dead staining proved much more effective, but time prevented cell count completion.

Results from IC analysis indicate that there is a rather steady increase in sulfate concentration over time for all samples (Figure 13). One issue is that there is an identical increase in sulfate concentration over time for the two negative controls as well. Although there is evidence that there is sulfur oxidation, because the controls are also experiencing the same oxidation, this may mean that either there is abiotic oxidation of sulfur occurring, or the negative controls also contain sulfur oxidizers by error of contamination. Since the cell count data is inconclusive based on the issues with the microscope and staining agents, a conclusion cannot be drawn on whether or not these microbes are sulfur oxidizers or perhaps surviving off of consuming one another (while capable of surviving in a highly sulfidic environment). Regardless
of their metabolic activity, these microbes (there is evidence that they are present through cell counts) are interesting because they are capable of growing in a 0.5M Na$_2$S environment.

Results from the sulfide assay show that there is an overall decrease in sulfide over time for all the samples. However, Figure 12 shows that the decrease in sulfide over time for the controls is nearly identical to the decrease in sulfide over time for each sample. There is a discrepancy between the times that the data was taken. This is because the control was forgotten and made two days after the first time point was taken for the samples. Since the samples gathered from the non-controls were two days older, the data for the controls lags behind by two days throughout. This may also play a role in why the amount of sulfide is initially higher in the controls. It is most likely because there was less time to oxidize the sulfide in the controls since they were made two days after the samples and analyzed that very day they were made, while the samples were analyzed after two days, allowing for oxidation of the sulfide. This is why the samples appear to have less sulfide at their starting point. Once again, the issue is that the controls are behaving in the same manner as the samples. All samples and controls level off at 0.133-0.135 mM indicating, again, that either there are sulfur oxidizers in the controls, or the sulfide is being oxidized abiotically, perhaps by the oxygen present within the vial.

**Sulfuricurvum Isolation**

Previous researchers have been unable to isolate *Sulfuricurvum* from the enrichment cultures. After testing the growth of *Sulfuricurvum* at 4°C on a thiosulfate plate, it is clear that 4°C is too low of a temperature for this organism to grow. After approximately one month on a thiosulfate plate at 20°C, there is a very small amount of *Sulfuricurvum* growing. However, its small colony growth over a span of one month indicates that 20°C is still not an optimal growth temperature. Interestingly, *Sulfuricurvum* constitutes a significant enough portion of the microbial community in the East and West Skaftárkatlar lakes which have temperatures between 3.5-5°C. Thiosulfate may be a non-ideal electron source for *Sulfuricurvum*, so perhaps a new sulfur-based media will increase the growth rate of *Sulfuricurvum* on a plate.

**Future Work**

The 6 isolates from the blue plates have amplified genetic material stored in 4°C. These products will be purified using a Zymo Research DNA Clean and Concentrator Kit and sent to GeneWiz for Sanger sequencing. Any results with high quality scores will be inputted into NCBI BLAST program to determine taxonomy, and classifications will be checked against SILVA database after cutting of any poor-quality nucleotides sequences. If percent identity indicates that any of these microbes are dissimilar to previously isolated and characterized microorganisms (< 97% identity), the isolate organism/s will be characterized. These isolated enrichments will be tested for optimal pH and temperature growth. The pHs that will be tested will be 4.5, 5.5, and 6.5; the temperatures at which the isolated cultures will be tests at will be 5°C, 10°C, and 15 °C. High temperatures may kill the microbial community as a result of too drastic of a temperature change – from 4°C to a temperature above 80°C. These isolated enrichments will also be analyzed for optimal electron donors, including H$_2$S and tetrathionate, as well as G+C content. The cell growth curve specific to this bacteria will also be determined by taking samples daily and recording cell count observations. The shape, size, and any notable physical characteristics of these bacteria will be analyzed using a scanning electron microscope (SEM).
If the DNA from the sequenced blue-plate colonies are identical or nearly identical to previously characterized organisms, the 11 returned sequences from the yellow plate isolates will be looked at. A decision will be made of which isolates to characterize. Characterizations will be done in the same manner as stated above.

Ionic analysis should also be done on the crystal-like growths on the blue plates. Further tests should be carried out to determine whether or not this growth is of biotic or abiotic origin. If these growths prove to be a result of a microbial metabolism byproduct, these microbes growing on or within this crystal-like structure should be further investigated.

The *Sulfuricurvum* isolation experiment will be continued. Alternative media with different electron sources will be used in order to assist the growth of the bacteria. Approximately 10 µl of enrichment media from the East lakes at depth 338 m will be extracted again from the enrichment cultures, spread onto the plates using inoculation loops, and stored at 20°C. Colony growth will be monitored and recorded.
References


Supplemental Figures

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Supplementary Figure 1. Temperatures and pHs outlined for each lake and lake depth.

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Supplementary Figure 2. Cation and anion concentrations outlined for each lake and lake depth.
Supplementary Figure 3. Clouding of slides upon addition of acridine orange, obstructing visibility.