The Role of Iron-oxidizing Bacteria in Aquifers of Southeastern Minnesota: An Analysis of Springs in the Cannon River Wilderness Park and Goliath's Cave

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ABSTRACT

Iron-oxidizing bacteria have long been associated with the formation of iron-oxide-coated mats at springs in southeastern Minnesota. However, no study has yet attempted to characterize the complex ecosystems involved in the production of these mats and the impacts of iron-oxidation on stream chemistry. The presence of iron-oxide-bearing mats at springs and in cave systems reflects groundwater redox processes that have yet to be fully documented. Iron-oxidizing bacteria from two circumneutral groundwater systems in SE Minnesota were cultured in FeS-O gradient tubes. Bacteria from different sites grew at different levels in the tubes, indicating differing optimum iron and oxygen concentrations. A discussion of potential redox cycles and possible future interdisciplinary research topics is presented.

INTRODUCTION

Stream and groundwater systems are host to a rich community of micro-organisms which can have a significant impact on stream chemistry and nutrient cycles. Iron-oxidizing bacteria are important members of these aqueous ecosystems, converting ferrous iron to ferric iron and often producing extensive orange mats of organic material coated with iron-oxides. In addition to impacting groundwater chemistry, the activity of iron-oxidizing bacteria can have a significant impact on manmade systems; iron-oxide formation can clog pipes, increase steel corrosion, and result in a variety of negative impacts on industry (Schiermeyer, 2000). However, bacteria can be useful in groundwater remediation due to their ability to remove a variety of contaminants from solution (eg. Sogaard, 2000).

Iron oxidation typically occurs at the anoxic-oxic interface, in waters with dissolved oxygen (DO) content of less than 10% (Liang et al., 1993). At pH levels greater than 5, $Fe2+$ will rapidly and spontaneously oxidize to $Fe3+$ (Ehrlich, 1999). In fully aerated freshwater at pH 7, the half life of $Fe2+$ oxidation is less than 15 minutes (Stumm et al., 1981). In order to thrive in these transition zones, bacteria must out-compete the rapid autoxidation of ferrous iron. The extent to which iron oxide mats result from biological or abiotic oxidation must be determined on a case-by-case basis.

The current study explores the role of iron-oxidizing bacteria in local aquifers and natural hydrologic systems, specifically at groundwater springs and cave walls. Iron-oxide stains were sampled at two main sites in Southeastern Minnesota and examined to determine whether iron-oxidation is abiotic or biologically-mediated. Ultimately, this work is intended to encourage future interdisciplinary study between biologists, chemists, and geologists by focusing on the of iron-oxidizing bacteria in southeastern Minnesota water systems.

STUDY AREA AND SAMPLING LOCATIONS

Cannon River Wilderness Park (CRWP) - Northfield, Minnesota

The Cannon River Wilderness Park, south of Northfield, MN, includes 850 acres of woodland adjacent to the Cannon River. Several springs enter the drainage at the interface between the poorly cemented St. Peter sandstone and Shakopee dolostone aquitard (Figure 1). Unpublished studies utilizing chlorofluorocarbon dating (CFC-11, CFC-12) have found spring water residence times on the order of 40-60 years (Ziller et al. unpub.). Water in the springs is affected by the surrounding agricultural activity; however two previous studies (Blue et al., 2004; Ziller et al., 2006) have both found acceptable levels of EPA regulated contaminants. The springs are active year-round and had a temperature of 8.5oC as of 15th May 2007.

Orange-red flocculent attached to rocky and organic substrates marks the emergence of each spring and extends for several meters downstream (Figure 2). An oily film and sulfur smell (indicative of H2S gas) have been observed in proximity to the springs and associated mats. Iron-coated algal streamers provide a habitat for a diverse community of micro-organisms (Figure 3).

Two locations at one of the CRWP iron springs were sampled for this study (Figure 2; Figure 4). The first sample was taken from well-attached, whitish, flocculent material at the emergence of the highest spring. Although many individual flows contribute to the spring in this location, the second sample was taken farther from where the water emerged yet still within the orange-stained area. Spiraled and straight filaments along with motile bacteria comprise the observed fauna in both CRWP sampling locations. The larger streamers observed in the CRWP are phototrophic algae coated with iron oxides (Figure 3A). Two mat samples were transported and inoculated into enrichment media within 1 hour.

Table 1 provides a summary of basic water chemistry for sampling locations

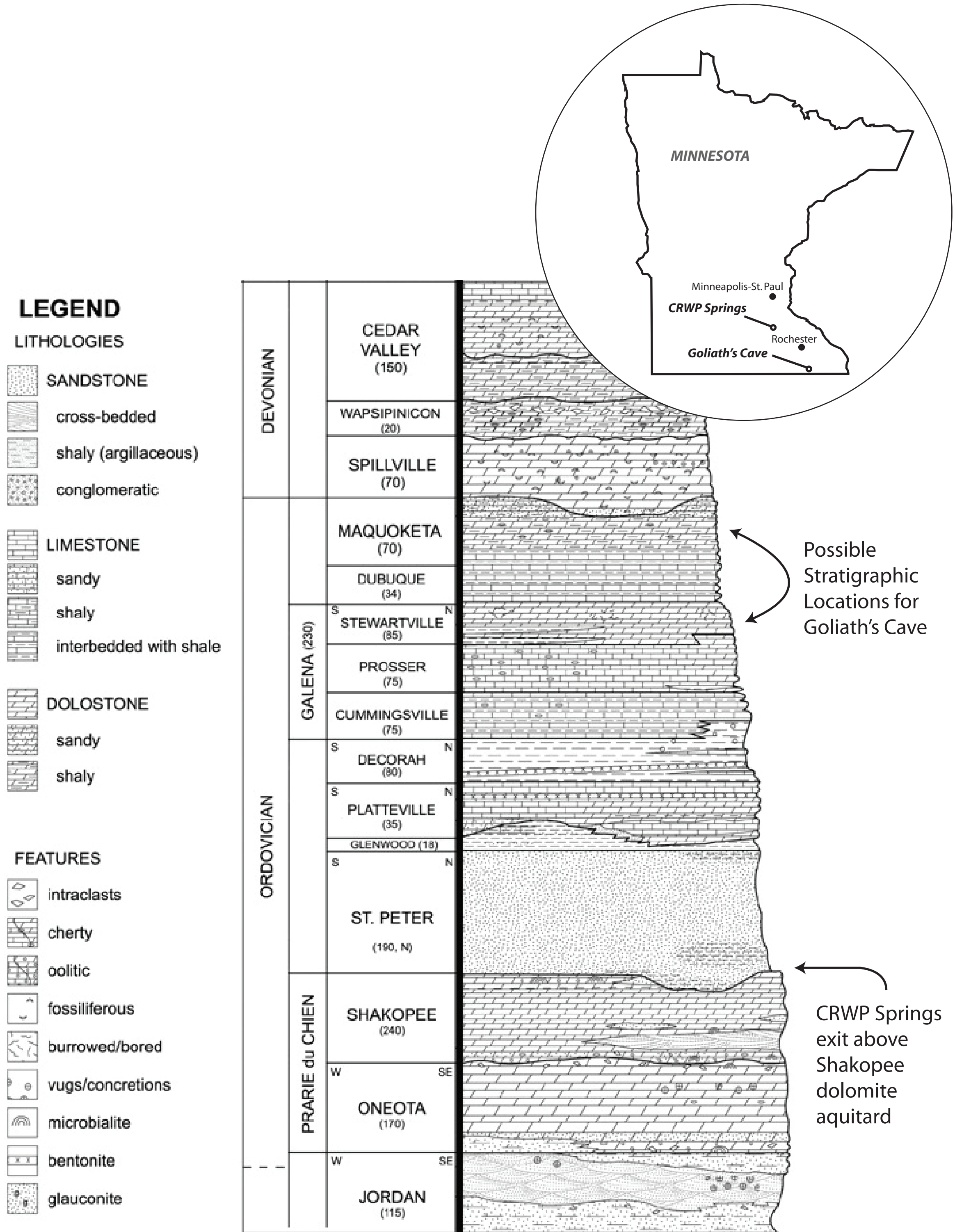
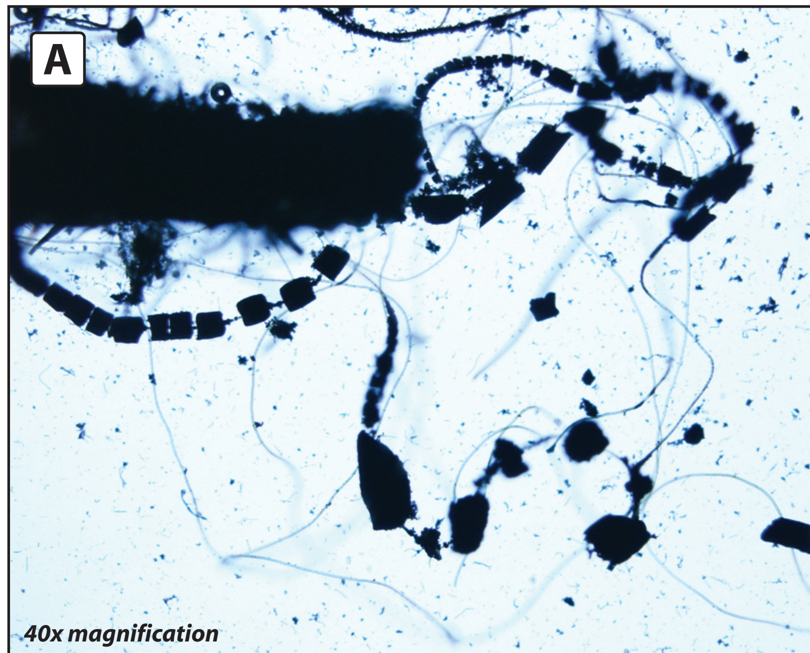
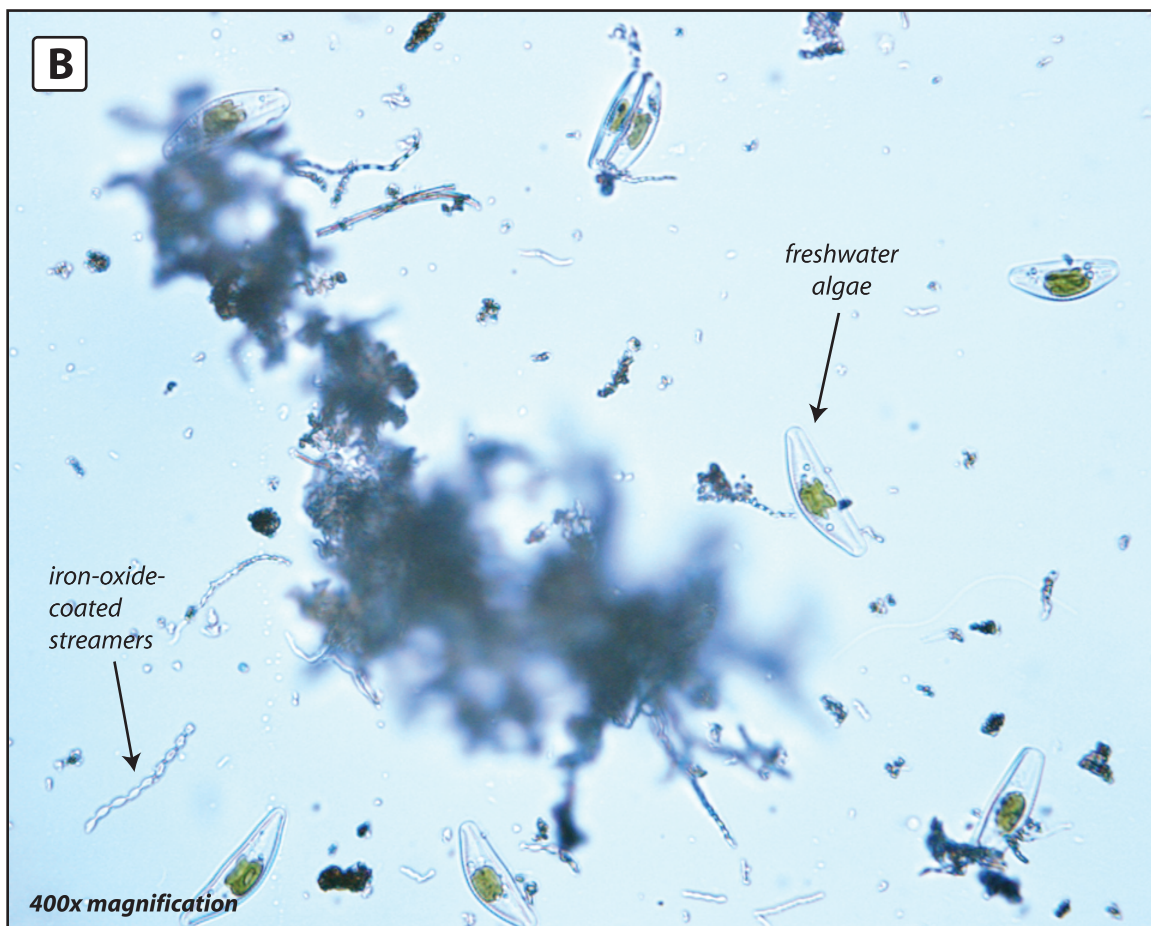


Figure 1. Upper stratigraphy of southeastern Minnesota and geographic location of study sites. The springs that support the CRWP bacteria are sourced by the St. Peter sandstone and flow over the Shakopee dolomite whereas the Goliath's Cave bacteria thrive on waters from carbonates of the stratigraphically higher Galena group (stratigraphic column and legend adapted from Mossler, 1987).

Figure 2. Photos of sampling locations in the CRWP. (A) Note that the main stream channel to the left is clear and becomes stained with orange iron-oxides only after the influence of the spring, shown on the right side of the image. (B) Larger image focusing on two specific spring outputs where samples were collected. Purple cups mark locations where spring water enters main stream. Cup covers are about 6 cm in diameter.

Figure 3. Photomicrographs of bacterial mats from CRWP. (A) Larger-scale image of iron-oxide coated streamers collected from downstream spring (CRWPDS). (B) Higher-magnification image of coiled bacterial streamers along with a freshwater community of micro-organisms.





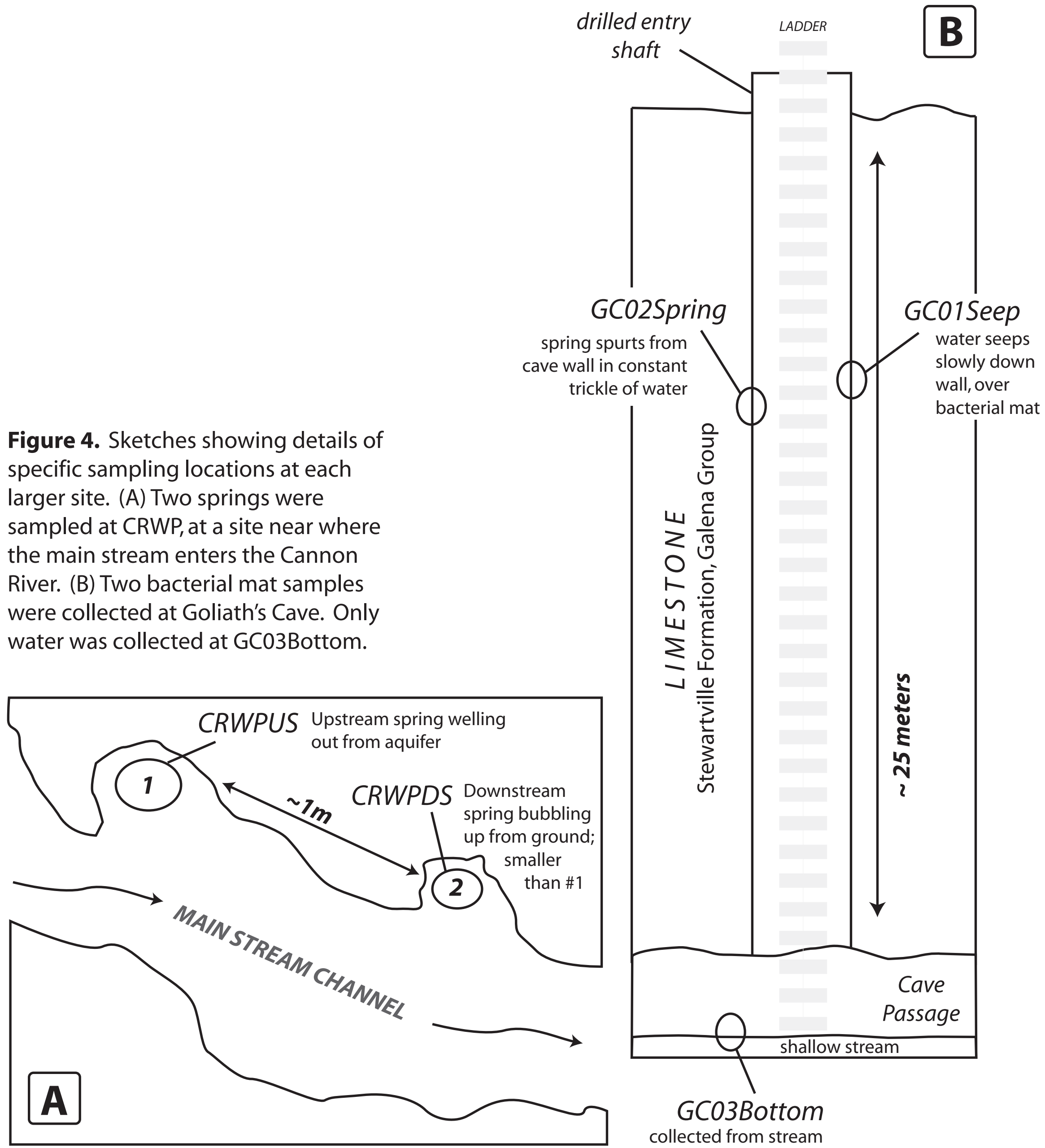


Figure 4. Sketches showing details of specific sampling locations at each larger site. (A) Two springs were sampled at CRWP, at a site near where the main stream enters the Cannon River. (B) Two bacterial mat samples were collected at Goliath's Cave. Only water was collected at GC03Bottom.

in the CRWP and Goliath's Cave (see below). Aerobic iron-oxidizing bacteria generally need at least 0.1 mg/L of ferrous iron to thrive. Samples collected directly from the CRWP spring mouths yielded concentrations of ferrous iron lower than this value, with only one sample yielding a relatively high 0.22 mg/L. However, past surveys of the St. Peter-Prairie du Chien-Jordan aquifer system report much higher Fe2+ concentrations with a mean of 6.37 mg/L (Campion and Wetzel, 1997). The mean sulfate value reported in the same study is 47 mg/L, a value above the 2.9 mg/L minimum required to support sulfate-reducers (Lovley and Klug, 1986).

Goliath's Cave - SE Minnesota

Goliath's Cave is part of southeastern Minnesota's extensive karst system. At the time of the current study, the Goliath's Cave system is thought to cut through the Stewartville formation of the upper Ordovician Galena carbonate group but may be as stratigraphically high as the Maquoketa formation (Figure 1). Dye-tracings have revealed at least two sinkhole water supplies for the cave and surface-to-cave flow times on the order of 2 hours in the main channels (C. Alexander, pers. comm., 12 May 2007). The average temperature of water in the cave is around 8.5oC, however the relatively shallow depth (about15 m below surface level) of the sites sampled for this study means that seasonal changes in surface temperature affect the water temperature at the sampling sites (C. Alexander, pers. comm., 12 May 2007). As with samples from the CRWP sites, ferrous iron is very low in the Goliath's Cave water (Table 1).

Iron mats have accumulated in the artificial entrance over the three years since the entryway was drilled (Figure 5). At the first sampling location (GC01), water seeps from a row of cracks in the carbonate bedrock, well below the metal casing at the top of the entry shaft (Figure 6). At GC01 the iron oxides form distinctive threads that hang from the wall. An enrichment sample and water were taken from the thick mat of fibrous iron oxides that coats the wall below the seep. Microscope examination revealed motile bacte-

TABLE 1: CHARACTERIZATION OF SAMPLING LOCATIONS

	GC01Spring[1]	GC02Seep	GC03Bottom	CRWPUS	CRWPDS
Temperature (°C)	-	-	-	8.5	8.5
pH	7.89	-	7.86	7.74	7.45
Dissolved Oxygen (mg/L)	-	-	-	2.53	2.25
Fe^{2+} (mg/L)	0.06	-	0.00	0.01	0.22[2]
NO^{3-} (mg/L)[3]	-	-	-	0.00	-
SO_4^{2-} (mg/L)	-	-	-	61.16	-
Physical Location	Cave wall, limestone, spurting spring directly above site	Cave wall, limestone, steady trickle of water	Shallow rocky stream at bottom of ladder, on cave floor; partially fed by trickles from above	Spring bubbling out from sandstone, surrounded by forest, organic leaf litter	2 meters dwnstream from CRWPUS, feeds directly into main stream
Bacterial Mat Appearance	Thick mat; threads are attached to wall at top end and drape down surface	Thinner orange mat, laminated with a layer of water from the seep	No bacteria evident in stream	Orange red flocculent material with streamers attached to rocky substrate	

Notes: Samples were collected during mid-afternoon, in mid-May. Temperature, DO, and Conductivity were measured in-situ with a YSI 85 probe. Iron concentration was measured using atomic absorption spectrometry.

[1] See Figure 3 for labeled sketches of sampling locations.

[2] Sample was filtered before analysis to remove bacterial mat remnants. Relatively high value may be due to dissolved iron from mat, not from water source.

[3] Nitrate and sulfate values from study conducted at same spring location by Blue et al. (2004).

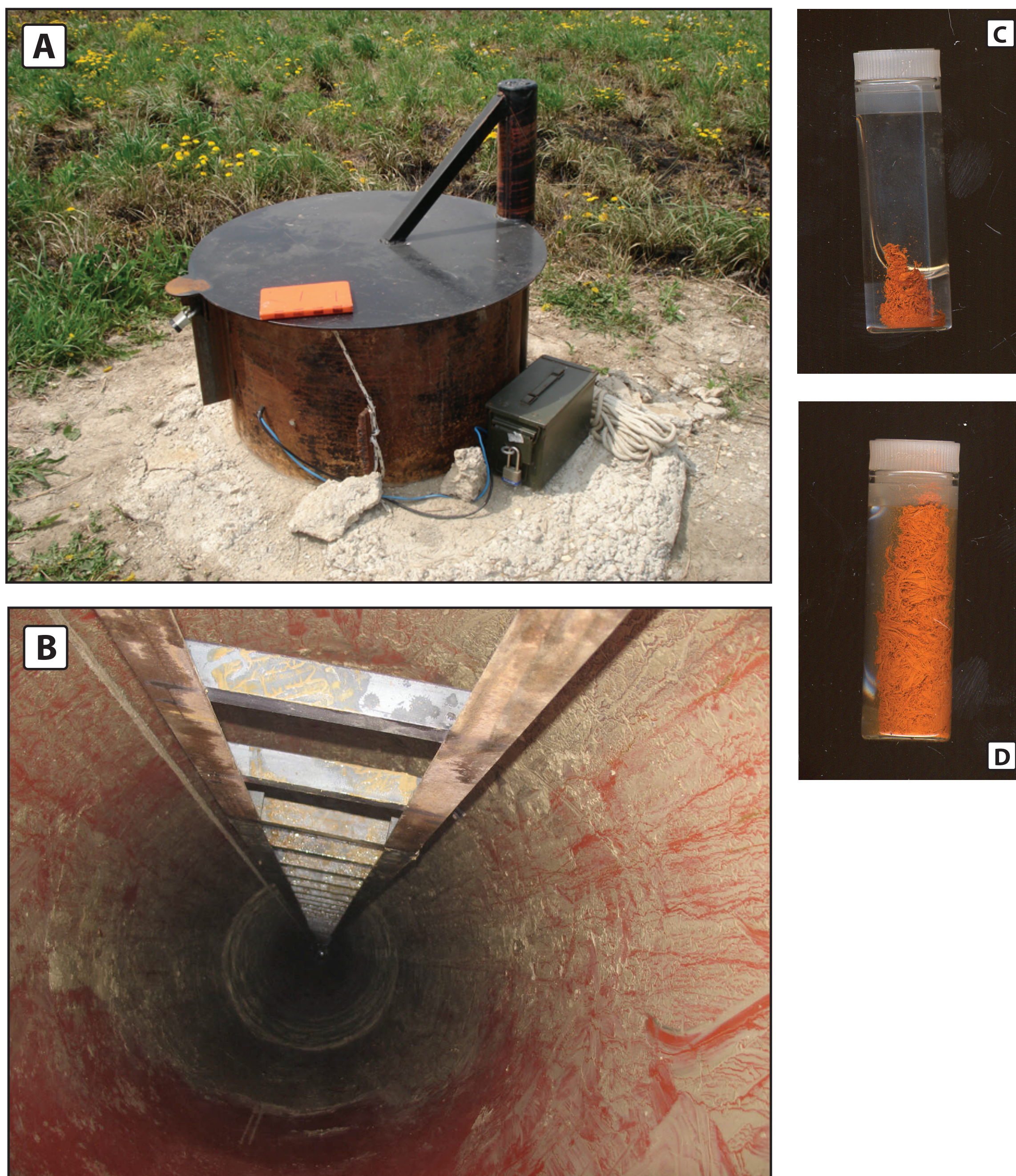
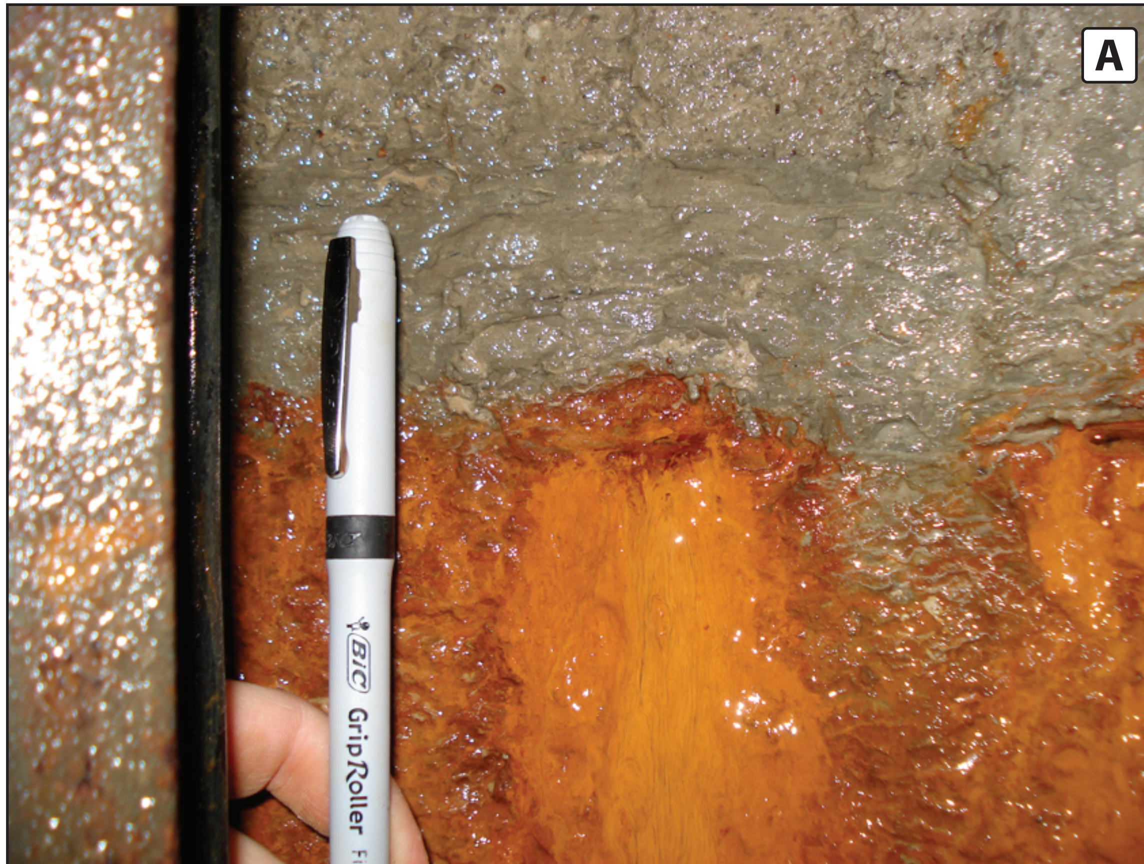


Figure 5. Goliath's Cave can be entered via an entry shaft drilled for the Minnesota Karst Preserve. Iron-oxidizing bacteria have colonized the walls of this entry shaft. (A) Lid covering the entry shaft, resulting in dark conditions for the majority of the year and (B) the 30-inch diameter entry shaft is reinforced by a steel pipe for the first few meters, after which the shaft is bound by limestone walls (photo from Minnesota Karst Preserve, http://www.karstpreserve.com/goliath.html). (C) collection tube holding orange iron-bacteria streamers from site GC02Spring and (D) from site GC01Seep.



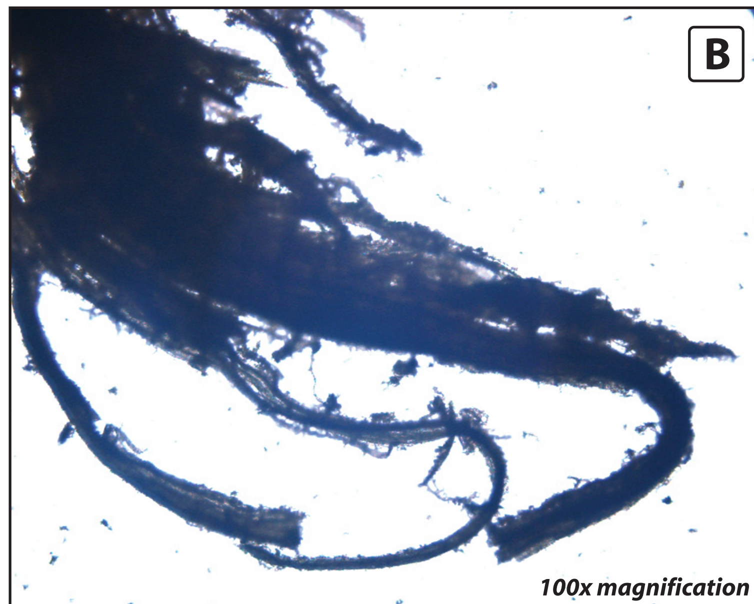


Figure 6. Photo and photomicrograph of GC01Seep site. (A) Bacterial mat appears part way down the entry shaft, where spring water seeps ou from cracks in the limestone. (B) Photomicrograph showing bacterial mat stringers collected from the mat pictured in (A).

ria and thick Fe-oxide aggregates (Figure 6).

The second sampling site (GC02) is a spring on the opposite side of the borehole, slightly below GC01. At GC02 a mat-surrounded stream of water shoots a few inches out of the wall (Figure 7). The GC02 mat is thinner than at GC01, and the sampled portion contained striking spiral structures (Figure 7) and fewer motile bacteria. At another mat, located closer to the surface, the iron oxides are covered by a black material. The blackened mat was too thin and the water flow too low for sampling at the time of this study, but would be an excellent location for future sampling.

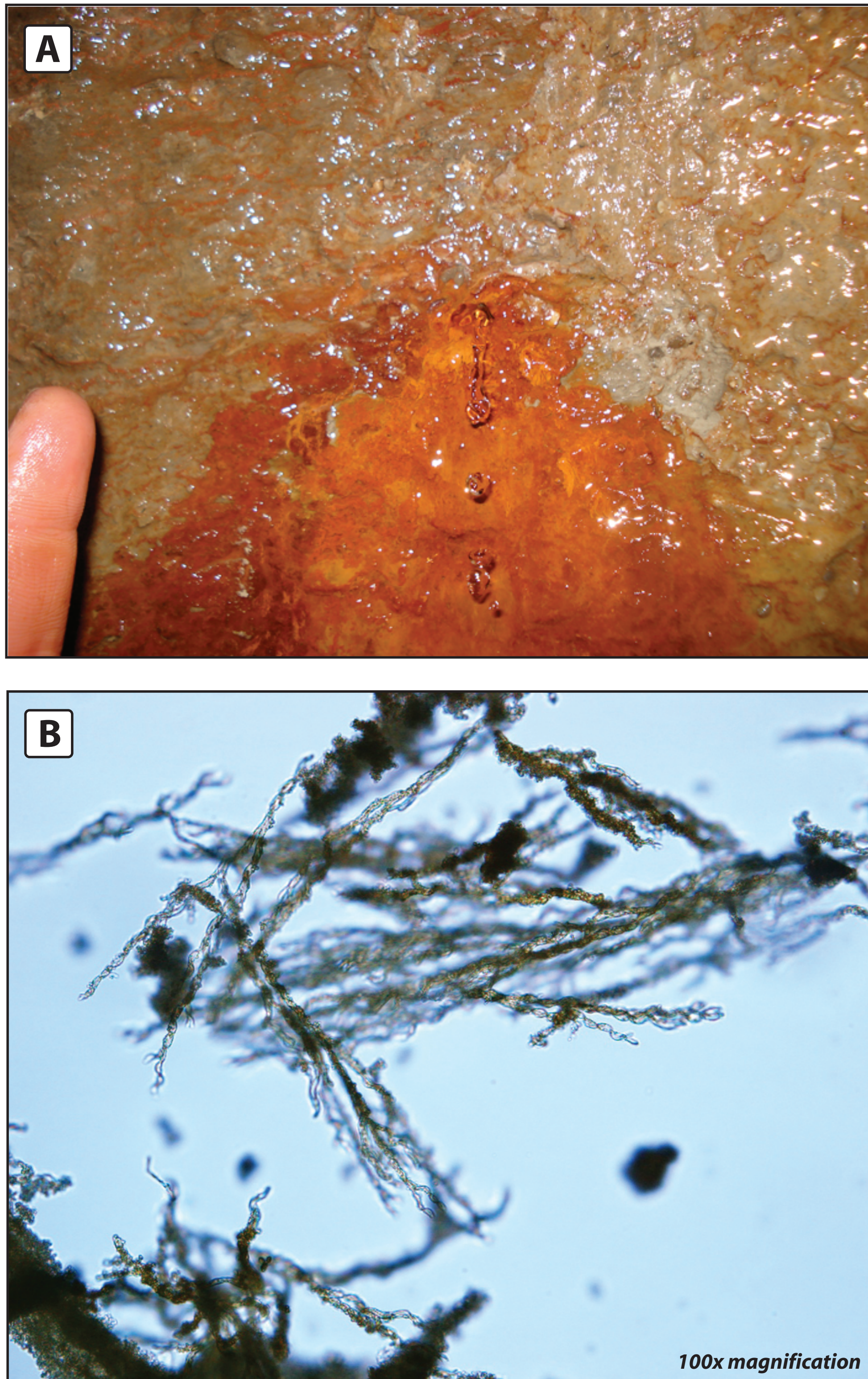


Figure 7. Images of Goliath's Cave GC02Spring site where water spurts from the wall in a constant trickle. (A) bacterial mat begins just below water input from surrounding limestone, (B) Photomicrograph of bacterial spiral streamers from orange mat pictured in (A).

Enrichment Medium – Opposed Oxygen-Iron Gradient Culturing

Bacteria were inoculated into an opposed oxygen-iron gradient medium adapted from Hanert (2006), Emerson and Moyer (1997), and Teske and Nelson (2006). In the opposed gradients method, iron diffuses upward from an iron sulfide (or steel wool) plug while oxygen diffuses downward from the headspace through a basic mineral medium, creating a continuum of iron and oxygen conditions (Figure 8). Bacteria, inoculated along a vertical column, grow most rapidly at the location in the gradient that most closely matches preferred iron and oxygen conditions. Agar in the medium prevents oxides from sinking and reduces disturbances of the gradient without hampering bacterial motility.

The iron sulfide plug was prepared according to Hanert, 2006. Iron sulfide salts from the addition of 140 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) to 120 g sodium sulfide ($Na_2S \cdot 9H_2O$) dissolved in 1 liter of type one water at ~50oC. A stir bar is necessary, as thick, black iron sulfide precipitates the instant the ferrous sulfate is added. To wash the iron sulfide, the precipitates were centrifuged, the supernatant was poured off, then the pelleted iron sulfide was remobilized in fresh type 1 water and centrifuged again. The washing process was repeated until the supernatant had neutral pH. Excess iron sulfide - which may need further rinsing - was left in centrifuge vials in the geochemistry lab freezer for future use.

After the final rinse, the iron sulfide was transferred to an autoclave-safe corning bottle and mixed with 1.5g bacteria-grade agar, 40 µL of 0.5M K_2HPO_4 stock solution, 200 µL of 1M $NaHCO_3$ stock solution, and 1ml of 1x Modified Wolfe's Mineral Medium (MWMM, per 1 L type 1 water: 1 g NH_4Cl, 0.2 g $MgSO_4 \cdot H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, and 0.05 g $K_2HPO_4 \cdot 3H_2O$). This mixture was autoclaved and kept in an oven at 60oC until gradient tubes were assembled.

The overlay solution consisted of 195.5 mL type 1 water, 0.5 g bacteria-grade

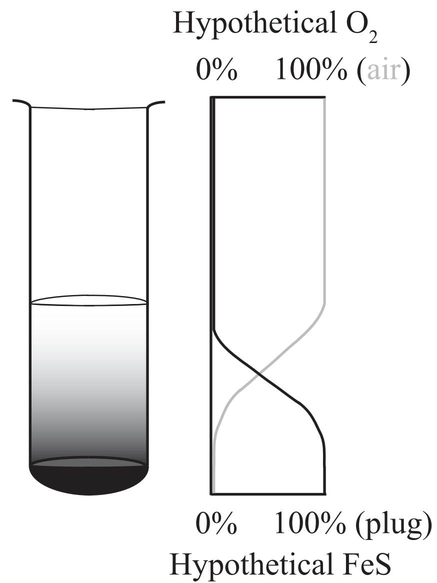


Figure 8. Hypothetical oxygen-iron gradient in enrichment culture test tubes. Bacteria innoculated in the agar column will survive and continue to multiply at the level that provides appropriate concentrations of iron and oxygen. This may help distinguish between species or simply between the different metabolic needs of multiple species within a larger bacterial community. Concentrations presented here are hypothetical, but with proper instruments it is possible to map the gradient of each specific tube and obtain precise values for iron and oxygen at any particular level.

agar, 80 µL of 0.5 M K_2HPO_4 stock solution, 400 µL of 1 M $NaHCO_3$ stock solution, and 2 mL of 1x MWMM. 1mL of filter sterilized vitamin solution (1L type 1 water: 10 mg nicotinic acid, 10 mg thiamine HCl, 5 mg vitamin B12, 10 mg Ca-pantothenate, 10 mg riboflavin, 5 mg biotin, 5 mg alpha-lipoic acid) was added to the overlay solution following sterilization in an autoclave. The overlay solution was also kept in a 60oC oven to prevent the agar from solidifying. At this point, many studies recommend bubbling the overlay with N_2 or CO_2 to remove oxygen. The vitamin mixture traditionally includes 10 mg pyridoxine HCl and 5mg folic acid. The enrichments in this study do not appear to have suffered from oxygen toxicity or the absence of two vitamins, but these deviations from standard protocol may have introduced culturing bias.

Gradient tubes were prepared by pipetting 0.75 mL of plug solution into sterilized test tubes and placing the tubes in the refrigerator for a few minutes to solidify the plug. Once the plug solidified, 3.75 mL overlay solution was carefully pipetted into the tubes without disturbing the plug. Gradient tubes were kept in a dark refrigerator prior to inoculation.

Enrichment Cultures

Mat samples were collected by scooping a small amount of mat into a pre-sterilized glass tube (Figure 5) and filling both tube and lid to the rim with seep water in order to minimize air in the tube. Mat samples were transported at constant temperature and inoculated into the prepared enrichment media within 2.5 hours. Water and mat material was collected from the two springs at the CRWP. At Goliath's cave material was collected at the two mat sampling locations and from the stream that flows along the cobbled floor of the cave (Figure 4). A total of 10 enrichment tubes were inoculated with Goliath's cave mat material and/or mat-associated water, and 9 tubes with CRWP material.

Organic material was emplaced across the oxygen-iron gradients by Pasteur pipette. Following inoculation, the enrichment tubes were incubated in the dark at room

temperature. Four of the ten Goliath's Cave replicates were inoculated from sample water alone. Eleven of the replicates from Goliath's Cave and the CRWP were kept in tightly corked enrichment tubes, while the remaining eight were capped loosely. A tightly capped, un-inoculated control was kept with the enrichment tubes throughout incubation. The inoculation process was performed quickly to reduce chance of contamination.

Note: The following steps have yet to be performed.

Initial Growth

Enrichments were allowed to grow for approximately 5 days until stable bands formed. At this time the growth bands were removed from the vials. This was done by using a sterile spatula to remove the excess agarose from on top of the band and transferring the gel containing the band to a 1.5 mL Eppendorf tubes. The tubes were incubated in a water bath at 70°C for 12 minutes to melt the agarose. They were immediately centrifuged at the maximum setting for 5 minutes at 37°C. The supernatant was removed and the pellet resuspended in 100 µL of deionized water.

Generation of Pure Colonies

According to Emerson and Moyer 1997, at dilutions of 10-7 or 10-8, cell growth began growth from individual colonies. After executing a dilution series of cells in deionized water, new gradient tubes should be inoculated with the new dilutions and incubated until bands appear. Further experimentation progressed using the cultures derived from the highest dilution while still retaining growth. After two or three passage of cells, culture purity was examined by simple visual inspection under a light microscope.

Preparation of Cells

Cells from the pure culture were isolated as done previously. After resuspending the bacterial cells and iron oxides, 200 µL of 0.5 M oxalic acid was added. This was

incubated at 30°C shaking at 150 rpm for 20 minutes. The sample turned from deep reddish brown to light yellow as the oxalic acid reduced the oxides. The sample was then spun down for 5 minutes at 37°C. The cell pellet was then washed twice with 1.5 mL of deionized water. After the final spin, the supernatant was discarded and the pellet was stored at -20°C until DNA extraction.

Extraction of DNA

Extraction of genetic material was described in Moyer et al., 1994.

Amplification of 16S rDNA

The 16S small subunit (SSU) rDNA was PCR amplified from the isolated genomic DNA. Oligonucleotide primers were designed to anneal to conserved regions of the bacterial 16S rDNA. The forward primer was 5'-TNANACATGCAAGTCGAICG-3' and the reverse primer was 5'-GGYTACCTTGTTACGACTT-3' (Moyer et al., 1994). The PCR reaction should be run for no longer than 30 cycles to reduce the chance of false positives. A sample of PCR conditions is: initial denaturing at 94ºC for 3 minutes, followed by 25 cycles of denaturation for 1 minute, primer annealation at 60ºC for 1 minute, and chain extension at 72ºC for 3 minutes. After the final cycle, the chain extension was allowed to last for an additional 7 minutes, after which the reaction mixture was kept at 4ºC indefinitely until it was transferred to the -20ºC freezer.

Phylogenetic Analysis

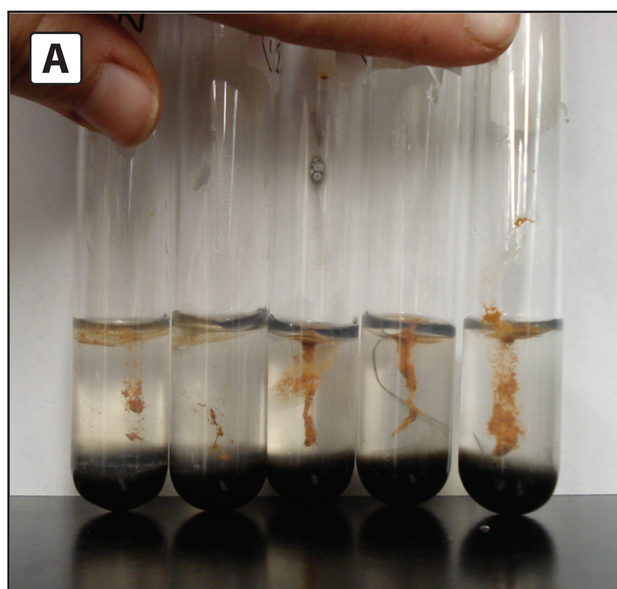
Sequence data were compiled and aligned either manually or by a software program. These sequences were compared to rDNA sequences obtained from the Ribosomal Database Project (Maidak et al., 1996).

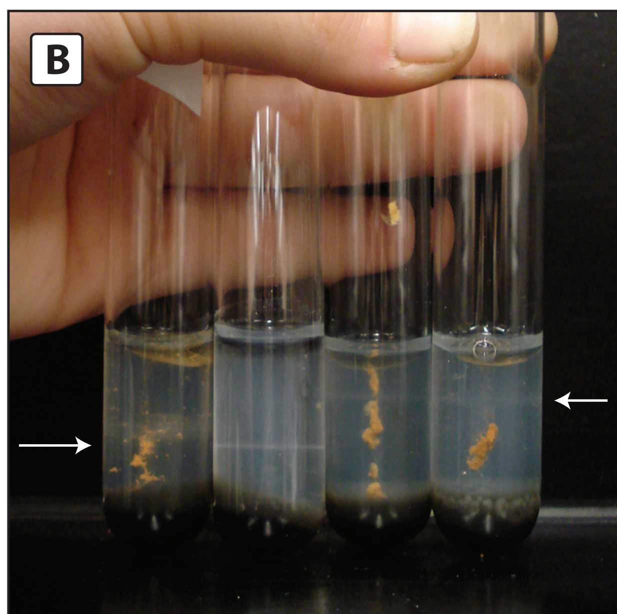
RESULTS AND DISCUSSION

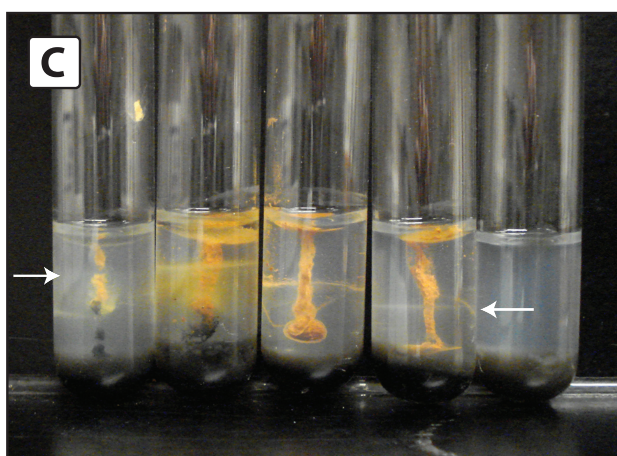
Results of Enrichment Cultures

Enrichment cultures of bacteria from Goliath's Cave and the CRWP showed evidence of growth within a very short time period. Faint white bands were noted in Goliath's Cave test tubes five days after inoculation and three days after inoculation in the CRWP test tubes (Figure 9). At ten days following inoculation, bands in the CRWP enrichments had grown from faint white lines to thick yellow layers. The bands in the Goliath's Cave enrichments became an orange color similar to that of the original mat material, but did not broaden. The control remained clear, indicating that growth was not simply a result of contamination or abiotic oxidation. The location of the band differed between CRWP and Goliath's Cave enrichments, however all bands for one site first appeared at the same level, regardless of whether caps were sealed or loose, and regardless of whether tubes had been inoculated with spring water alone or actual mat material. Bands in the enrichment samples from Goliath's Cave occurred at a lower level, closer to the iron source at the base of the tube, than the CRWP samples (Figure 9b). The bands initially grew level to the upper surface of the medium rather than parallel to the FeS plug, which lay at an angle in some tubes, with the implication that oxygen was limiting in the gradient tubes or that iron diffused evenly despite the uneven surface. Although cap tightness and the angled plug or tube made little initial difference, changes in the Fe-O gradient over time may have affected growth location (Figure 9C).

The simple methods used in this study cannot identify the exact oxygen, iron, or sulfur concentrations the bacteria from the two sites prefer. The difference in environmental or metabolic preferences between bacteria from the CRWP and Goliath's Cave may indicate that the mat communities are dominated by different species or that the same species may have adapted to the conditions at each site. Furthermore, the samples taken from each site cannot be assumed to represent the entire community at that site.







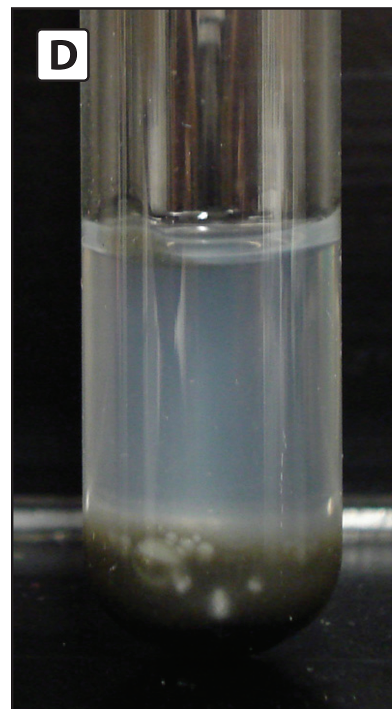


Figure 9. Test tubes showing results of enrichment cultures. (A) initial state of tubes directly after innoculation. Samples were innoculated with either stream water or orange microbial mat in a column from the iron-rich base to the oxygen-rich top. (B) tubes after several days of growth. The left two tubes are bacteria from Goliath's Cave (after five days of growth) and the right two tubes are bacteria from CRWP (after three days of growth). Note faint white lines where bacteria have begun to grow at different levels. (C) Growth in tubes after approximately ten days. Bands are more diffuse, although still located at different heights, and some are more distinct. Left two tubes are from CRWP, next two are Goliath's Cave, and the final tube is the control. Some bands have turned orange and a darkening of the base of the CRWP tubes, indicates reduction may be occuring at the contact with the FeS Plug. Note that this does not appear to be happening in the Goliath's Cave tubes. (D) A control test tube containing no bacteria after five days of growth. The lack of bacterial growth confirms a sufficiently sterile procedure.

A final observation regarding the opposed gradient enrichment media used in this study is the darkening of the inoculated mat material closest to the plug. The darkening is likely due to reduced iron, rather than to iron sulfide upwelling as photos of the tubes show little movement between inoculation and darkening. Darkening (reduction) may result from facultative iron reducers inoculated concurrently with the oxidizers or from abiotic reducing conditions in the gradient tube.

Groundwater Redox Cycling

Water geochemistry at both Goliath's Cave and the Cannon River Wilderness Park presents a unique opportunity to study the effects of groundwater processes on associated aqueous ecosystems. However, little work has been done to determine the route by which water travels through these systems, and the mechanisms that control water chemistry. Surface to groundwater path is significant for its implications regarding iron source and redox cycling in the subsurface (Figure 10A,B,C). Local geology does not include iron formations or basaltic igneous rocks as iron sources. However, galena and pyrite-bearing carbonates are sandwiched by the CRWP and Goliath's Cave stratigraphy (Figure 1). The presence of iron/lead sulfides allows for redox cycling between iron and sulfur species. Some bacteria can engage in sulfide oxidation coupled to iron reduction, dissolving pyrite and releasing both Fe(II) and SO42- into the groundwater. When this water nears the anoxic-oxic boundary, another community of bacteria may derive energy through iron oxidation and sulfate reduction, producing the iron flocculent and H2S scent observed at the CRWP (Figure 10).

Groundwater residence times are also significant to examining redox cycling in a system. Residence time may control the amount of iron solubilization (eg. through reduction or chelation), which in turn affects microbial activity when the water emerges. Mat formation may therefore corespond to flow rate in a system, with the confound of mat destruction at high flow rates.

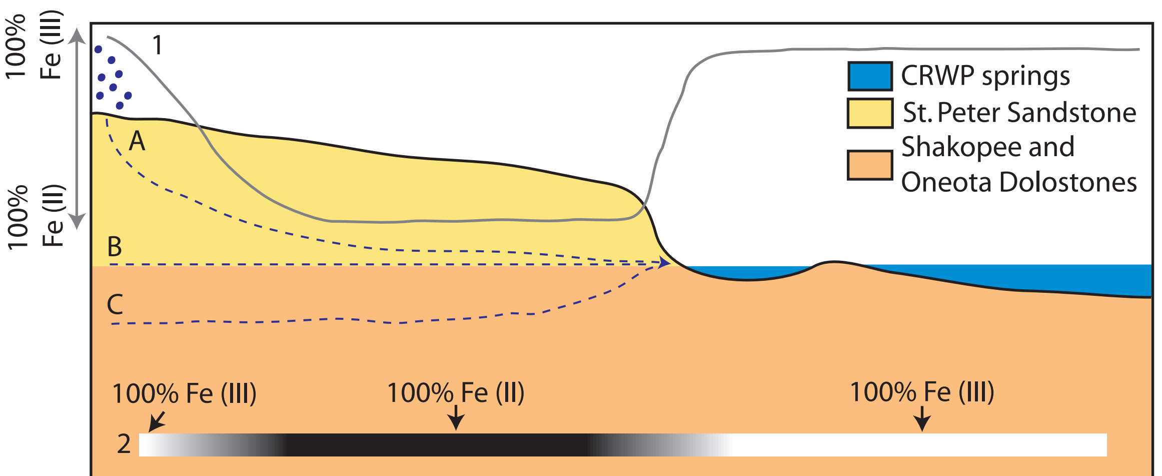


Figure 10. Hypothetical subsurface geochemistry at Cannon River Wilderness Park springs. A, B, and C represent possible paths taken by springwater. Redox gradient depicted as 1. vertical graph of iron concentrations as ferrous iron is converted to ferric iron over the transition from the anoxic zone within the aquifer to the oxic zone at the spring mouth, and 2. gray-scale gradient of iron species concentration.

Rates of Biological and Abiotic Iron Oxidation

The low values for ferric iron in samples taken from the mouth of the springs accompanied by decimeter to meter-scale mats indicates downstream transport of ferric precipitates. Biological oxidation, rapid autoxidation, or a combination of the two slightly below ground may account for the low iron concentrations in water from the mouth of the springs. If the majority of ferrous iron is oxidized immediately and transported downstream, bacteria must have growth rates or attachment mechanisms sufficient to maintain populations within the appropriate redox conditions.

FUTURE RESEARCH TOPICS

Biotic and abiotic oxidation kinetics (Ad Lab II: Kinetics)

A study of the iron oxidation kinetics would provide a more comprehensive understanding of the natural process. Students could examine rates of autoxidation and microbially driven oxidation reactions through relatively simple and tested methods (James and Ferris, 2004 and Boon et al., 1999).

Microbial oxidation at circumneutral pH, in which the bacteria would have to compete with abiotic oxidation, would be of particular interest in the study of the CRWP iron oxidation. Through this project, students would be familiarized with the concepts of enzymatic reactions, kinetic relationships and accompanying data analyses.

Iron and sulfur cycling along soil-stream interfaces (Ecosystems Ecology)

Bacteria are present directly at the mouth of the CRWP springs but little is known about oxidation and other processes occurring within the aquifer, before water is put in contact with the atmosphere. Future projects might include sampling of water and soil at points along a transect, stretching from a location on land some distance from the spring to the water just by the spring. Soil reduction-oxidation potential could be measured and water samples and soil cores could be analyzed for a variety of properties, including Fe2+, Fe3+, H2S, and SO42- concentrations. These would give some idea of the processes occurring within the soil, showing whether iron oxidation is already occurring within the spring and, if so, how rapid the gradient from ferric to ferrous iron is (Figure 10). On a more basic level, more must be learned about the path taken by the water to reach the spring. It is not clear if water entering the St. Peter over a small area close to the spring or is it flowing from much further away before pooling on the underlying dolostone and flowing downwards to the spring mouth. Knowledge of the physical path taken by water would help constrain possible inputs of iron and other compounds from local sources.

Study of cell morphology

The inspection of the cellular morphology using transmission electron microscopy could validate phylogenetic analyses. The comparison of previously uncharacterized bacteria to related species would strongly support the generated cladogram. TEM micrographs would also provide illustration of the association of iron oxides with the bacterial cells.

Characterization of bacteriogenic iron oxide precipitates

Electron microscopy has revealed that Fe-oxides are tightly associated with the cell walls of the bacteria. The Fe-oxides often appear very fine-grained and amorphous (Emerson and Moyer 1997). Fe3+ in particular is bound tenaciously by bacteria and commonly precipitates to form hydrous ferric oxide coatings on cell surfaces (Ferris et al. 1989; Fortin et al. 1993; Kennedy et al. 2003). Some common forms of oxide precipitated by iron-oxidizing bacteria are magnetite, goethite, ferrihydrite, iron phosphate, and ferric hydroxysulfate (Ehrlich, 1999). Future work could focus on analyzing and identifying the specific oxides present at the CRWP and Goliath's Cave sites use Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), and X-ray Absorption Spectroscopy (XAS) methods. This might give more clues as to the specific species of bacteria present and would give a better idea of iron cycling within the CRWP and cave hydrologic systems.

Characterization of oily substance

Stream waters at the CRWP springs at the time of sampling were covered in places by what appeared to an oily substance with a rainbow sheen. This substance appears to be seasonal as it in past years it has not been present during the fall. It is unclear whether these oil slicks are compounds secreted by the bacteria themselves or if they represent actual bacteria. No papers appear to have been written on the topic of the

chemistry of these oil sheens, indicating that this may be a good area for further research. Informal educational websites report that these sheens are bacteria, likely simply missing the characteristic iron-oxide stains and organic gelatinous material. Several sources note that one can distinguish between bacteria and hydrocarbon oil runoff by swirling the mass with a stick, and seeing whether the oil breaks into sections, indicating bacteria, or simply swirls, indicating gasoline (Arlington Dept. of Environmental Services, May 25, 2007).

Interesting projects would include collecting some of the surface water containing this oil and observing it under a microscope to see if bacteria are present. Surface waters could also be analyzed for a variety of organic and inorganic chemical compounds to determine possible origins of the oil. If the slicks are produced as a by-product of bacterial activity, it would be important to better understand the effects of this oil on surrounding aqueous ecosystems. Longer term projects might include monitoring oil presence and extent over a year to better understand seasonal changes in oil production and whether these factors and linked to seasonal fluctuations in bacterial presence and population sizes.

Whole-system diversity analysis

While the emphasis of our project is on characterizing the bacteria responsible for the geochemical transformation of iron species, researchers in other fields may be more interested in the diversity of the whole system. A culture-independent analysis of the microorganisms from the springs and cave could be performed to analyze the genetic diversity of the systems. This method would be done by extracting DNA directly from the mat material or water sample. One would perform a primary PCR reaction to amplify 16S rDNA from the total extracted DNA, then a secondary PCR amplification to attach a GC-rich clamp. You could get a visual estimate of the genetic diversity in the environment from this product by running a denaturing gel gradient electrophoresis (DGGE) and seeing the assortment of bands. You could then extract the bands from the polyacrylamide gel and sequence the DNA fragments. This would provide information necessary

for phylogenetic analysis to characterize the bacterial community.

This method could be adapted to study different type of bacteria within the community by using more selective primers to target specific genes or less conserved regions of the 16S rDNA. This culture-independent method removes any incubation bias.

PCR amplification of gene responsible for ferric iron oxidoreductase

This method is similar to PCR amplification of 16S rDNA but would use primers specific to the oxidoreductase gene. This method could be used in conjunction with whole-system analysis.

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