Geomicrobiology

of Acid Mine Drainage

from the Kempton Coal Mine Complex,

Maryland

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Abstract

Kempton Coal mine, located in Western Maryland, operated between 1914 and 1950, and has been abandoned since. The environmental effects of the closure of the mine include acidic water flowing, at approximately six to eight million gallons of water per day with high concentrations of dissolved metals, into tributaries of the Potomac River. The pH of the mine effluent is approximately 3.45, and is relatively constant. There have in the past been several remediation efforts on the state and local level to treat the acidic water as it exits the mine. The influences of microbiological organisms in acid mine drainage (AMD) situations have been researched in the past and many positive steps have been taken to identify the microbe or microbes acting as catalysts for pyrite oxidation. Cultures of samples collected were analyzed for chemical changes for the duration of the experiment. Analysis of the culture medium showed a variety of microbes present. The formation of biofilms in the culture flasks was an indicator of the comfortable environment created for the microbes in this study. This study supports that finding through the varying morphologies found in the cultures and the prevalent biofilm. The diluted AMD samples taken from Kempton when cultured at low pH did support microbes living in environments characterized by extreme conditions. The average Ph at the Kempton borehole is 3.45 and the cultures were successful at a pH between 1.0 and 1.5. This supports the adaptable nature of microorganisms. Geomicrobiology of acid mine drainage is an important part of the entire global cleanup and understanding. It has financial prospective as well as natural resource implications. In order to get a better understanding of the interaction of microbes with the terrestrial world emphasis needs to be placed on understanding the individual AMD sites and incorporating this information into the general body of knowledge

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Introduction

The environmental degradation due to acid mine drainage (AMD) and the economic potential of the microorganisms thriving in the acidic setting, have focused recent attention to this issue. Bioleaching of minerals is becoming an important field in the recovery of low-grade ores. Developing research is focusing on treating each separate situation with a better understanding of the interaction of microbes within their surroundings.

Overview

In the early part of the last century the need for fossil fuel increased dramatically due to manufacturing and population growth. This need for energy was partly met through the mining of coal in the Eastern United States, where large quantities of high grade, low volatile content, and relatively shallow coal were available. Since that era many of these underground digs have been abandoned. As a result mines located at or below the water table have filled up with significant amounts of water. This water eventually finds its way through the mine workings and tailings into local streams that feed larger bodies of water.

Water from the mines is generally high in dissolved metals such as Al, and acids, both of which can be poisonous to aquatic environments. These toxic waters make their way through tributaries and have devastating long-term effects on water quality in bays and estuaries along the coast. One of these mines, The Kempton Mine, lies at the headwaters of the Potomac River.

History

The western most counties of Maryland, Garrett and Allegany, are at the eastern extreme of a large coal-producing region that includes the states of Virginia, West Virginia, Pennsylvania, Ohio, and Kentucky. All of these states produced large quantities of coal, and

production in some states continues. In 1995, the four counties of West Virginia adjacent to Garrett and Allegany counties produced 5,853,723 tons of coal from 46 mines. Of the 46 mines, 16 are underground operations at varying depths. The estimated recoverable coal reserve for these four counties in 1995 was 2,448,823,408 tons (WVGES, 2002). Coal removed in 1995 from these four counties was ~ 0.24 % of their estimated accessible stockpile.

The mine at the focus of this study, Kempton Mine complex, is located at the very tip of the southwestern most extent of Garrett Co, Maryland. It includes the Kempton Coal Mine and the Coketon Mine.

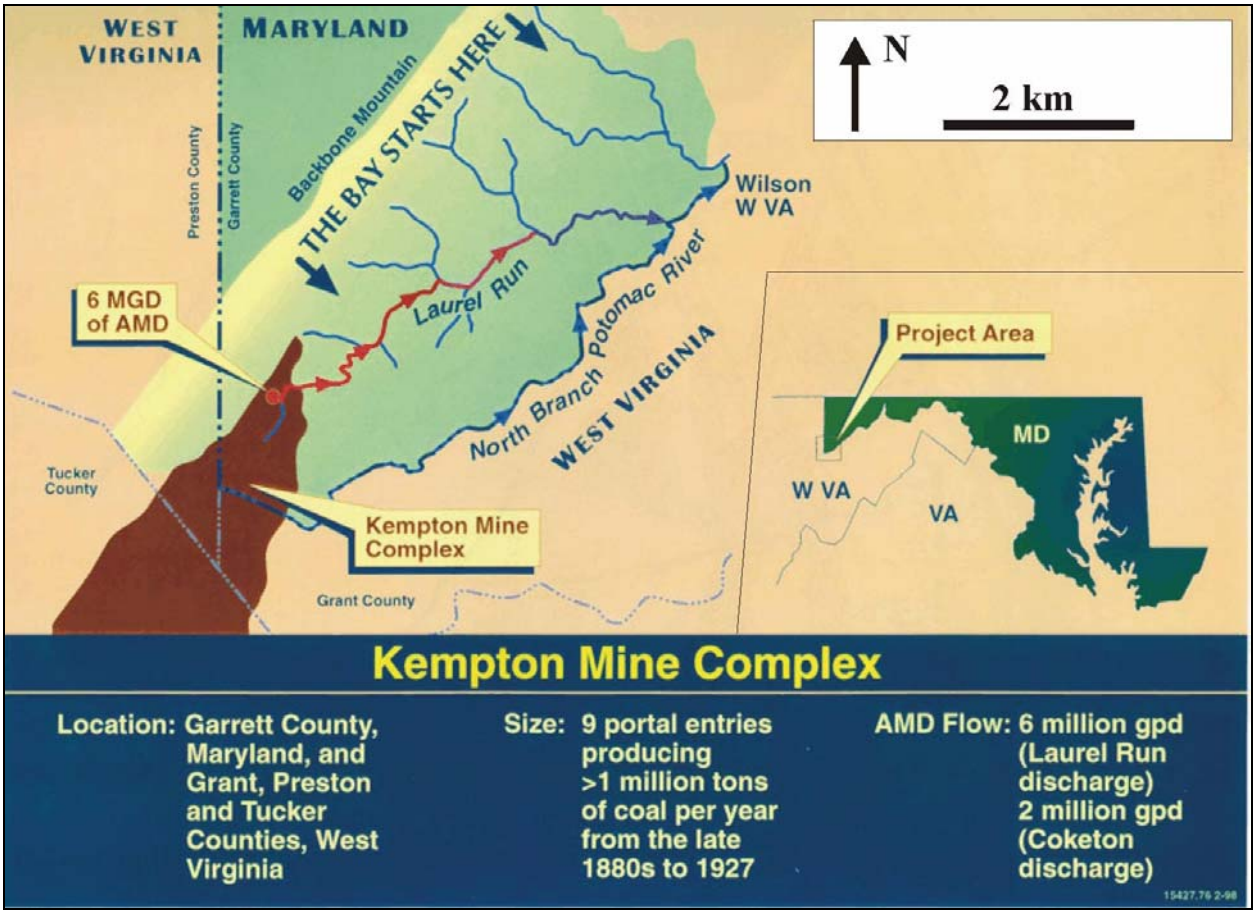


Figure 1. Location of project area, Kempton Mine Complex, and outflow into Laurel Run from the mine towards the North Branch of the Potomac River. The red dot indicates the borehole sampling location and where AMD enters Laurel Run. The Coketon location is south of the Maryland-West Virginia border in Grant County, WV. (Adapted from the Power Plant Research Project)

The town of Kempton MD, is the first settlement from the headwaters of Laurel Run, and is named after the mine. The Kempton mine was worked from 1914 to 1950 when it was shut down. During the years from 1915 to 1921 the mine produced over a million tons of coal per year (Maryland Bureau, 2002). Western Pocahontas Properties and CSX Railroad System currently own the mine. The colliery underlies portions of Garrett County, MD and Preston and Grant Counties, WV. There are two 420 ft deep main shafts into the mine. They are located in the center of the town of Kempton. The underground workings included removing the seam of coal completely, leaving only square columns at regular intervals to support the overburden. Numerous service, ventilation, and cable shafts were also dug to get the necessities to the miners. Two sampling areas for this study are from these secondary shafts. The borehole is an electric cable shaft that was dug in 1938 extending down 240 feet into the Kempton Mine, and is located approximately a mile down valley from the main shafts. It was dug for electrical conduits running from the mines electrical generating facility. The other site is a vent shaft (see Fig. 1) dug into the Coketon mine at the southern extent of the Kempton Mine complex.

Below the vertical shafts, the interior portion of the mine has filled up with water. The volume of water presently in the mine has been estimated at over a billion gallons. The source of 90% of the water source has been determined, using oxygen isotope analysis over a two-year period, to be from groundwater. The other 10% could be attributed to runoff entering the mine in several ways (D. Earnest, 2002). Decaying shaft covers and the various other portals dug, as well as fracturing from the collapse of overburden have provided pathways for water to enter. There are two schools of thought for the mine pool. These pools could also be thought of as hydraulic heads. One version has the pool as a continuous large body of water and the other has the pool

split into the south west Coketon portion, separated by a burn or raised plateau, from the north east Kempton mine. Recent mapping of the mine workings shows the separation, but whether the impermeable boundary is intact or not, is not known.

The service shafts used in this study are dug into the mine at lower elevations than the main shaft. They now lie below the hydraulic head, and water flows out in both. The Geospacial Research Group, Frostburg State University, monitored a ventilation shaft upslope from the borehole and during the recent drought water stopped flowing, while the flow from the borehole remained relatively constant. This information was used to estimate the pool size and the hydraulic head. The Geospacial Research Group also monitored dissolved cations exiting the mine in effluent from the borehole. The readings show high levels of cations hypothesized to be from sulfuric acid attacking the host rock in the mine and mobilizing these elements.

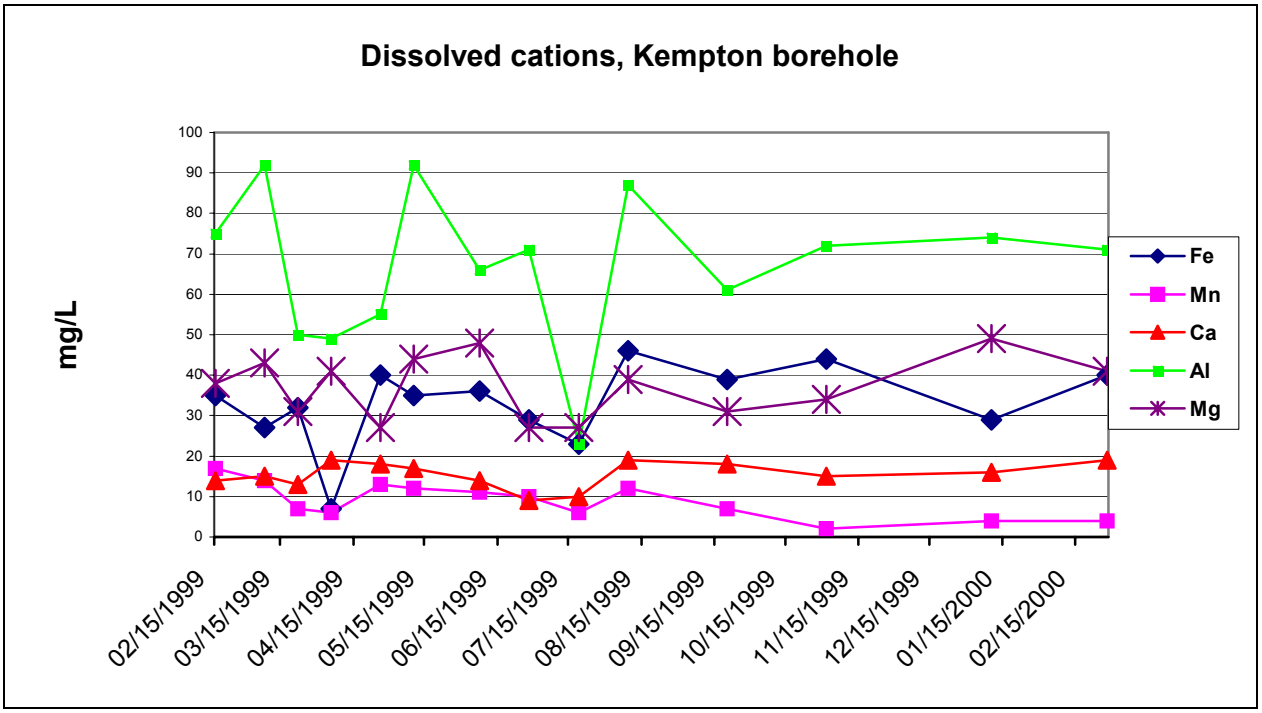


Figure 2 plots of dissolved cations in the mine effluent at the borehole sampling area in milligrams pre liter. Readings taken from 02/15/99 to 02/28/00. Adapted from the Geospacial Research Group.

Microbial oxidation of pyrite has shown a resulting propagation cycle where more sulfate is produced which would weather at the host rock. At times during the observation period Al levels rose to greater than 90 mg/L. These levels of dissolved metals can be poisonous to some aquatic life and are presumably part of the pollution affecting the Laurel Run. The Coketon shaft carries two million gallons of water per day (gpd), and the Kempton borehole carries six million gpd of acidic water. There are several issues associated with this site. The public health and safety concerns stem from the open mine workings and toxic drainage into streams. Associated with the runoff are environmental degradation concerns including, the loss of aquatic life in Laurel Run, large areas of spoilage along side the stream, and in bogs where foliage is severely reduced or nonexistent. There are 28.5 total acres of environmental disturbance, of which 4.2 acres are important wetlands (Kempton Reclamation, 2002). Mining structures litter the landscape and cave-ins are a threat as the cap over the main vertical shafts is deteriorating.

Geology

The geology of this coal region is important in that the long process of coalification has provided us with an important natural resource that provides energy for millions of the population. This coal region is also suffering from environmental degradation left over from many mining operations involved in removing the ore. Coal is deposited in Western Maryland in a series of five synclines from the Taconic orogeny that lifted the depositional environment (Maryland Bureau, 2002). These synclines are present in Garrett and Allegany counties and have a southwest to northeast trend. The synclines are filled with Pennsylvanian aged (320 – 286 Ma) swamp deposits that are interbedded with sandstones and shales. This type of deposit is typical of regression/transgression events along a relatively low-lying coastal plain. The five coal regions in Western Maryland, from west to east, are the Lower Youghiogheny, Upper Youghiogheny,

Casselman, Upper Potomac, and Georges Creek. The Kempton Mine complex is in the Upper Potomac. The Upper Potomac is made up of various coal seams of which the Upper Freeport is the most viable to mine

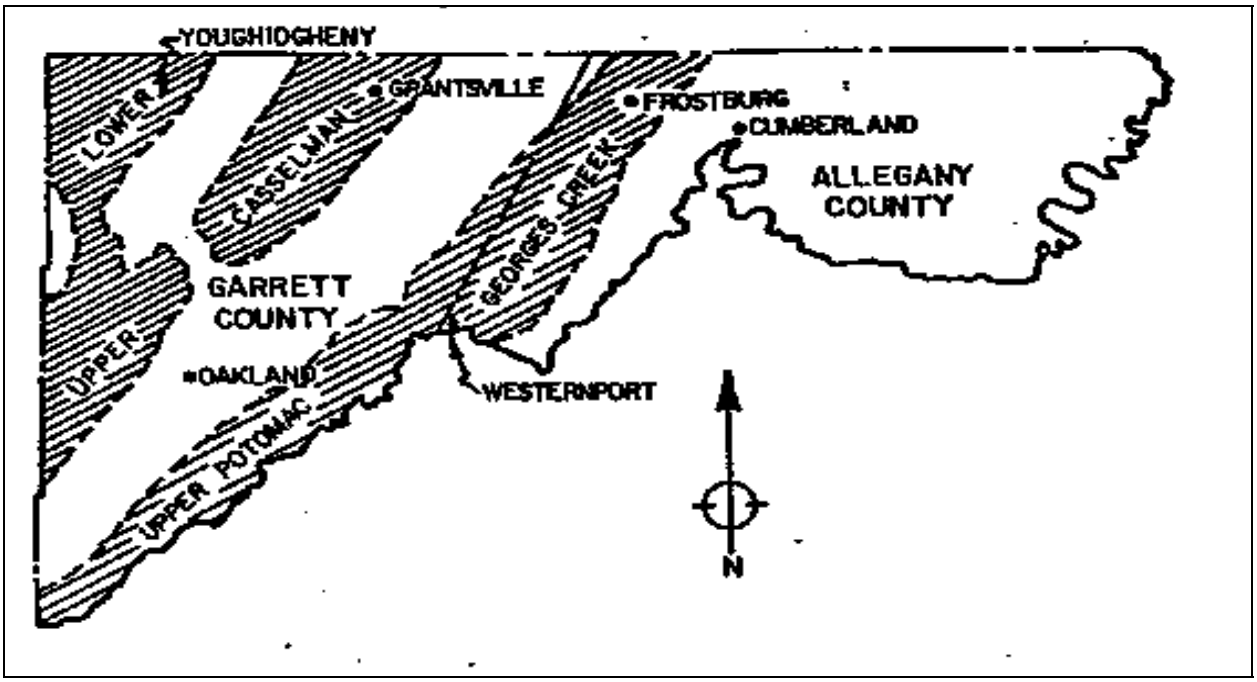


Figure 3 locations of five coal regions contained within synclines in Western Maryland. (Adapted from MDE, 2002).

The seam is deep enough at Kempton that removal of the overburden would not have been cost effective. This is why Kempton is a deep mine as opposed to a strip mine. The Upper Freeport has an average thickness of 62 inches and at the location of the Kempton mine complex has a gentle dip of approximately 6 degrees. It is composed of high quality, low volatile content, bituminous coal. The Upper Freeport extends several miles into Maryland and significantly further into West Virginia and is still mined. This mountain region receives significant rainfall and is riddled with small mountain streams that quickly carry runoff to the coastal plain. The readily available water transport system adds to the acid mine drainage issue, as well as diluting the mine effluent.

Remediation Efforts

There are currently several remediation efforts underway to correct or lesson the acid mine drainage problem. Since 1994 the Maryland Bureau of Mines has been dumping lime, using an automated system, into streams at several locations in order to neutralize the outflow before it reaches larger bodies of water. At the borehole an automatic lime doser uses an average of 580 tons of carbonate per year to neutralize the effluent and is backed up by a second doser that activates if the pH is not suitably buffered or in case of failure (KMCRP, 2002). The Lime Dosing Project has had some success in treating the acidic water, but the lime is having its own negative effect. The valley below the dosing locale is choked with carbonate sludge and stained with iron precipitate. This short-term solution treats the end result of acid mine drainage but not the cause. One benefit of the dosing project is that fish hatcheries operate at the base of Jennings Randolph Reservoir's dam and are being run with treated waters from the mine. Another sign of recovery is a naturally reproducing trout population in the North Branch of the Potomac. There is currently no lime dosing at the Coketon shaft, although the exiting water does flow through a failed lime drain from past efforts.

The Kempton Mine Complex Research Project is a joint venture between state and federal agencies to determine permanent solutions to lesson the impact of the mine waters. Their goals are to decrease the amount of water flux into the mine, or to increase the water quality exiting the site (KMCRP, 2002). This ongoing project includes detailed investigation and mapping of the mine structure and the regional hydrology.

Researchers at the University of Maryland undertook a unique and different approach to this problem as part of the Power Plant Research Program. The laboratory study was undertaken to develop a grout designed to neutralize the acidic environment, support the structure of the

overburden, and use Coal Combustion Byproducts (CCB's). Power companies typically have to pay for the disposal of CCB's. The grout had several properties including, using a significant portion of coal combustion byproduct, as well as the grout having to be able to pour into the mine and harden to carry the overburden. Fly ash, flue gas desulfurization products, and limekiln dust were used in different proportions to formulate the grouts (Piccoli et al., 2001).

Significant effort is put into remediation of acid mine drainage sites. The remediation is important to communities surrounding the mine as well as farther down stream. In its current condition the lands affected by this environmental pollution cannot be used. As land becomes more valuable with population growth it is important to return the natural beauty of these areas for future use. Part of this natural environment is aquatic life in and alongside the streams and marshes that need to be nurtured back to a healthy, productive status.

Biological Influence

Pyrite (FeS_2) is a common constituent of coal and although not necessarily present in all coal mines, its weathering produces acid mine drainage. Pyrite is the most common sulfide mineral in nature and is created by the reaction of iron and sulfur (Nordstrm and Southam, 1997). Pyrite occurs in minable bituminous coal in the Eastern United States. It is typically locked away in the earth and slowly oxidizes as part of the geochemical sulfur cycle. Exposure to water and oxygen is magnified through mining procedures that introduce the sulfide minerals to the atmosphere. Approximately 50% of the world's river sulfate transportation is mobilized through withdrawal and mining operations, totaling 150×10^{12} g of sulfur per annum (Bond et al., 2000). The oxidative dissolution of metal sulfide minerals at low pH is a slow process and has been found to be a small part of the acid mine drainage problem. Past research has shown that acid mine drainage conditions exist without the presence of pyrite (Runnels et al., 1993). With the

labor of Bacteria and Archaea involved it has been found that the microbial interaction with sulfides speeds up the oxidation rate significantly when compared with chemical oxidation alone (Edwards et al., 1999).

The phylogeny of living organisms, through 16s or 18s ribosomal RNA comparative sequencing, is divided into three major domains, Bacteria, Archaea, and Eukarya (Canfield and Raiswell, 1999). Eukarya includes animals and are multicelled organisms with a defined nucleus in each cell. Bacteria and Archaea are single celled organisms that do not have a defined nucleus and have adapted to varying extreme environments. Bacteria are separated from Archaea through a number of phenotypic traits. Bacteria typically have a cell wall that contains peptidoglycan, a polysaccharide, while Archaea do not. The lipids in the membrane of the cell are linked chemically, in Archaea they are ether-linked and in Bacteria they are ester-linked. Bacteria cells contain a single type of RNA structure with simple geometry. Archaea use several types of RNA polymerase and are more complex in than Bacteria.

Bacteria have been divided into eleven defined major groups. Archaea, who are less well understood, have been divided into two defined major groups. Their small size (~1um depending on morphology) and rapid reproducibility along with easily adaptable DNA are reasons for their success and duration on earth. Archaea have been identified in 700 degrees Celsius mid-ocean ridge thermal vents. Acidophilic (acid loving) chemolithotrophs thrive in low pH water and are able to proliferate the acid mine drainage problem. Chemolithotrophs use inorganic chemicals, such as S, Fe^{2+}, and H_2, as energy sources and HCO_3 as a carbon source (Chapelle et al., 2002). Molecular oxygen (O_2) and ferric iron (Fe^{3+}) can function as electron acceptors for this process. Initially this progression using oxygen is slow and at moderate pH is represented by the following chemical equation,

$$FeS_{2(s)} + {}^{7}/_{2}O_{2(g)} + H_2O_{(l)} \longrightarrow Fe^{2+}_{(aq)} + 2SO_4^{2-}{}_{(aq)} + 2H^{+}_{(aq)}.$$

As the initiating reaction creates acidic conditions and lowers pH, ferric iron becomes more stable and takes over as the electron acceptor to oxidize pyrite at rates up to one hundred times faster than oxygen (McKibben and Barnes, 1986). The chemical equation

$$FeS_{2(s)} + 14Fe^{3+}_{(aq)} + 8H_2O_{(l)} \longrightarrow 15Fe^{2+}_{(aq)} + 2SO_4^{2-}{}_{(aq)} + 16H^{+}_{(aq)},$$

represents conditions when acid mine drainage is catalyzed by microorganisms adding to the environmental problems. For every one-part pyrite, the result of this reaction is 2 parts sulfate and 16 parts hydrogen that combine to form sulfuric acid. This resulting acid then attacks other metal phases in the host rock, mobilizing metals such as aluminum. Bacteria and Archaea in turn use Fe^{2+} as an energy source, oxidizing it back to ferric iron, which can oxidize the pyrite. The process takes place at an ever-increasing rate creating a propagation cycle. While the water flows out of a mine it carries some of the ferrous iron with it (Candela et al., 1997). As this ferrous iron reaches oxygenated water with more moderate pH, it forms an insoluble ferric iron precipitate that stains the streambed a reddish yellow color.

Microbial catalysts of acid mine drainage were discovered as early as the 1950's (Harrison and Thode, 1958). Much research has centered on strains of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* as the microbiota responsible for a large part of generating acid mine conditions. Recent research has played down the role of the aforementioned microorganisms and is focusing on other microbiota, as it appears that at locations where optimum conditions for *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* do not exist, other microorganisms take on the same function; and in some cases could be considered the main contributors to the site specific acidic conditions (Bond et al., 2000). Since the 1980's, when Archaea were accepted as a distinct group, more and more work has been done to understand this

domain of organism. A study done at Iron Mountain, California, showed that Archaea was more prevalent than Bacteria in certain locals (Edwards et al., 1999). Much of the commercially mined iron ore in cooler parts of the world are from bog iron deposits from earlier geologic eras (Canfield and Raiswell, 1999). These large deposits were created in much the same way as bog iron deposits today. As ferrous iron leaches into water, with more neutral pHs, surrounding the bog oxidation occurs. This oxidation can be spontaneous or it is helped by iron oxidizing bacteria such as *Gallionella* and *Leptothrix* (Nordstrm and Southam, 1997). The resulting insoluble ferric hydroxide falls out of suspension and a deposit is formed.

Importance of Microbiological Study

Why study microbiology and the interaction of microorganisms with the natural environment? Generally we study and research topics for a better understanding of the complex interactions of our planet and recover processes that benefit mankind. Microbial research has led scientists to believe that life on other planets will be found as microbial communities. The study at Kempton Mine is important due to new research showing that the bacteria *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* may not play as important a role in all acid mine drainage conditions as previously hypothesized (Chapelle et al., 2002). Conditions at different sites have excluded strains of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* in favor of other microbiota who could be capable of oxidizing pyrite and other sulfide minerals. In addition Archaea and their role in the oxidation of sulfides is better understood through ongoing research. One environmental consulting and remediation company, MVTI Environmental Solutions, has developed a product that strips *Thiobacillus ferrooxidans* of its protective outer cytoplasmic membrane. This film allows *Thiobacillus ferrooxidans* to survive in highly acidic environments. With this membrane destroyed, the acid that the organism helped to create

destroys it. This would work well if all acid mine drainage sites were related to these organisms. The research however, is showing that arrays of organisms (Bacteria and Archaea) are responsible for the acid created during the oxidation of pyrite. It then makes good sense to try and determine what other microorganisms are creating acidic conditions from different environments. As more sites are observed and analyzed for different microbiota, the understanding of the processes and the diversity involved will better prepare us to solve the problem rather then treat the resulting environmental hazard.

Another technology that is gaining a forefront is the use of microorganisms for bioleaching of economically important minerals (Schrenk et al., 1998). For each mineral that is mined there is a cost associated with removal upon which it is decided whether it is economically viable to mine. Sulfides, iron, and uranium, are some of the minerals that current microbial bioleaching research is investigating. Efforts are being made to use bioleaching to recover these minerals from low-grade deposits, as well as past mine tailings that could still contain significant amounts of ore. Many countries including Spain, Brazil, India, and Pakistan are revisiting old mine workings and low-grade deposits that they could not process using conventional methods. The concept behind this is that the microorganism will devour the surrounding rock and leave behind the load. The process is slow, but may be the only cost effective way of mining these resources. The use of organism such as Archaea to aid in the clean up of contaminated sites, such as petroleum spills, could lesson the overall stress on the environment.

Specific Hypotheses

- Samples from the Kempton Mine complex are consistent with recent research that shows more widely varying microbiota play a role in the oxidation of pyrite (FeS_2) then previously hypothesized.

- Acid mine drainage samples taken from diluted mine effluent, when cultured at a lower pH, can produce microbes, characterized by more extreme environments.
- Microbial activity in cultured samples changes pH, Fe_{Total}, Fe^{2+}, Fe^{3+}, and sulfate (SO_4^{2-}) at a greater rate when compared with controls lacking microbe inoculations.

Methods

Subsequent methods were followed to test the hypotheses:

Sampling

Water samples were taken using presterilized Falcon Blue Max 15 ml polypropylene conical tubes with screw on lids, opened and closed underwater at a depth of approximately 3 cm, and transported on ice. Sediment samples were taken at a depth of approximately 8 cm using a sterile trowel, placed in a plastic sealed sample bag, and transported on ice. Replicate samples of water and sediment were taken from both the borehole and Coketon locations (see Fig. 1). At the borehole water samples were taken from two different areas.

Site Number	Sample Location	Sample Numbers	Type of Sample
1	Borehole Exit	1,2	Water
2	Borehole Exit	3,4	Sediment
3	Exit Sluice	5,6	Water
3a	Exit Sluice	Bag A	Sediment
4	Coketon Vent	7,8	Water
5	Coketon Vent	9,10	Green Material
6	Coal Seam	11	Coal

Table 1. Kempton Mine Complex Samples**.** Sampling done by Graeme Bowles, 03/04/2002. (See Fig. 1 for sample location map)

As close to the shaft exit as safely possible and again from the bottom of the exit sluice prior to the mine effluent joining the general water body. Approximately 6 meters separates the borehole exit and the end of the sluice. All samples were kept refrigerated once back at the lab in College Park. The samples collected in the field were stored in a refrigerator in the Chemistry Lab (3111). Unfortunately due to an electrical failure the samples may have been destroyed. The old samples were replaced with samples collected by Dan Earnest on the same field excursion to the Kempton mine and a later trip to the Crellin borehole. The Crellin borehole is several miles from the Kempton mine, yet possibly undergoes similar chemical and microbial activities. Two Series of cultures were done. The first in order to determine if the field samples had been destroyed. After it was established through SEM analysis that aliquots of cultures from both field and replacement samples contained microorganisms with a similar morphology as Bacteria, a second series of cultures was undertaken. The replacement samples, identified with Kempton replacement and Crellin, were stored in the Hydrology Lab in the Chemistry Building and did not undergo the same electrical failure.

Field Measurements

Temperature (^{0}C), Total Dissolved Solids (mg/L) (TDS), and Dissolved Oxygen (mg/L) (DO) measurements were taken in the field using an YSI model 600-pH meter. Measurements were taken at both locations and also included Oxidation/Reduction Potential (ORP), Dissolved Oxygen (%) (DO%), and pH (see Index 2). The YSI model 600 sensors are combined in a casing that was lowered approximately 1 meter below the surface of the water into the borehole. Fe^{total}, Fe^{2+}, Fe^{3+}, and SO_4^2 measurements were taken in situ at the borehole using a portable HACH Direct Reading Spectrophotometer Field Kit.

Preliminary Identification

To determine if samples contained viable microorganisms an optical microscope was used to observe microbe activity in the samples from the field. Samples were transferred to sterilized glass slides using a sterilized pipette and covered with a sterilized plastic cover. The Nikon microscope, in Geol 1113, is calibrated to 1.7 microns between tick marks at 600-x magnification and was used for a first order identification. Preliminary Optical Microscope observation using the Nikon Microscope determined that there were microbiota in the samples previously collected at the Kempton Mine. Motile single cells approximately 1um in diameter were observed. In addition to the single cells, when the magnification was decreased small green worms and Eukaryotes were observed moving in the sample, with diameters ranging from 10 to 100 microns.

Prior to culturing pyrite cubes were analyzed using the JEOL JXA 8900RT WD/ED (SEM) combined microanalyzer was used to image the surface topography of the cubes and also determine composition. The resolution at the time of observation was ~100 microns and no visible microbiota were observed. Salts on the surface of the cubes were analyzed and images of the surface topography were taken.

Culturing

Culturing took place in a sterilized fume hood in the Chemistry Lab (3111) in the Geology Building. The interior and exterior of the fume hood was cleaned thoroughly with soap and water, and then dried. The fume hood was activated and both the interior and exterior were wiped with ethanol on two separate occasions. The fume hood glass door will be covered with foil to keep most of the light out. Nineteen 500 ml Pyrex glass Erlenmeyer flasks were used for culturing. Each flask contained 15 grams of crushed pyrite enriched mine sediment. The

sediment was dried in a drying oven and the crushed pyrite added to it. Each flask also contained a cube of pyrite from the Navajun Mine, Logrono, Spain. The cube faces are ~1 cm across giving a surface area of 1000 mm. This pyrite was formed in limestone and sandstone during the Jurassic-Cretaceous age (125-130 Ma). Once assembled and capped with aluminum foil the flasks were autoclaved at 120 ^{0}C and 20 pounds per square inch (psi) for a period of twenty minutes. The growth media for culturing was adopted from Dr. Katrina Edwards (Schrenk et al., 1998).

Three liters of base media were made separately by adding trace salts to 1 liter of double deionized water. The water was distilled using a Barnstead/Thermolyne Mega Pure® System MP-12A. The pH of each liter was adjusted using 11.66 molar hydrochloric acid (HCl).

Salts (g/l)	**Liter 1 (pH 3.2)**	**Liter 2 (pH 3.2)**	**Liter 3 (pH 1.2)**
$(NH_4)\ SO_4$	0.803 g/L	0.806 g/L	0.801 g/L
KH_2PO_4	0.403 g/L	0.407 g/L	0.405 g/L
$MgSO_4*7H_2O$	0.164 g/L	0.161 g/L	0.161 g/L

Table 2. Base media containing salts in grams per liter (g/L) of double deionized water.

Trace Nutrients	**Grams per liter**
$CoCl_2*6H_2O$	0.115 g/L
$CuCl_2*2H_2O$	0.106 g/L
$MnCl_2*4H_2O$	0.113 g/L
$ZnCl_2$	0.114 g/L
$CaCl_2*2H_2O$	0.116 g/L

Table 3. Trace nutrient solution containing salts in grams per liter (g/L).

The liter flasks were capped with aluminum foil and autoclaved. Each liter of base media was inoculated with 20 uL of trace nutrient solution. The trace nutrient solution also contains 4.3 ml of 11.66 molar HCl. After the solution was completed the flask was wrapped in foil to prevent degradation and autoclaved. For series 2 all the media for culturing was filtered through a 0.22-micron Nucleopore filter prior to inoculation. A 2% concentrated yeast extract solution was made by adding 2g of yeast extract to 100 ml of double deionized water. This was capped with aluminum foil and autoclaved. To reach a 0.02% concentration in the 100 ml of culturing media one ml of yeast extract was added to select culturing flasks.

100 ml of base media was added to the autoclaved culturing flasks already containing the pyrite cubes and pyrite enriched mine sediment. For this culturing process the base media serves as a nutrient source for cell function and the pyrite as an energy source. A carbon source comes from CO_2 in the atmosphere. Once prepared the culturing flasks were inoculated with 1 ml of water from the samples using an Eppendorf 3130 1000 uL pipette with disposable sterilized tips.

Two replicate culturing series were performed. Both series contained samples from both the field and replacements. The first series was started on June 1, 2002, and the Crellin culture was added on July 1, 2002. The second series was started on October 9, 2002. The following tables (4 through 6) contain information on location from which the individual cultures were inoculated with and the conditions set for each culture.

Series-1 06/01/02

New 1-Kempton borehole

New 2-Kempton borehole

New 3-Kempton borehole

Old 4-Kempton borehole ramp exit

Old 5-Kempton borehole exit

Control (no inoculation)

Crellin 07/01/02-Crellin borehole collected by Dan Earnest (D.E)

Series-2 10/09/02

A-Crellin borehole

B-Crellin borehole

C-Kempton replacement from D.E.

D-Kempton replacement from D.E.

E-Coketon vent

F-Coketon vent

G-Kempton replacement from D.E.

H-Kempton borehole exit

I-Crellin borehole

J-Kempton borehole exit

Control I (no inoculation)

Control II (no inoculation)

Table 4 Sample locations used for culture inoculation separated by series.

Sample	Initial pH	Refrigerated	Yeast extract
New-1	3.23	No	No
New-2	3.23	Yes	No
New-3	3.23	No	Yes
Old-4	3.23	No	No
Old-5	1.22	Yes	Yes
Control	3.23	No	No
Crellin 07/01/02	3.17	No	No

Table 5 Culture conditions Series-1 06/01/02

A	3.08	Yes	Yes
B	3.08	No	No
C	3.08	No	Yes
D	3.08	No	No
E	3.08	No	Yes
F	3.08	No	No
G	3.08	Yes	No
H	3.08	No	No
I	1.08	No	No
J	1.08	No	No
Control I	3.08	No	No
Control II	3.08	No	No

Table 6 Culture conditions Series-2 10/09/02

SEM Analysis

Aliquots from the first run started June 1, 2002 were examined at the Laboratory for Biological Ultrastructure (LBU) on the University of Maryland campus on September 12th and 18th, 2002. The LBU uses the Denton DCP-1 Critical Point Dryer to prepare the samples for SEM analysis. Critical Point Drying (CPD) is a sensitive method of preserving the samples by avoiding the destructive effects of surface tension from water dehydration. This allows the organic matter to retain its morphology when exposed to the vacuum in the SEM. Examination was done using a JEOL JXA 8900RT WD/ED combined microanalyzer (SEM) located at the LBU.

Fixation (adapted from Tim Maugel, LBU)

1 ml samples from the cultures were taken and fixed in the following process:

1. A 4% glutaraldehyde (GA) solution was added to Falcon Blue Max tubes containing the aliquots as a preservative.
2. Samples were then filtered using a syringe onto a 0.22 um Nucleopore filter held in a Swinney filter holders.
3. The filters were left in the holders while a 4% solution of glutaraldehyde was passed through the holders.
4. The samples are then washed free of GA with double distilled (DD) water for three successive passes.
5. Samples are then fixed to the filter paper using a 1% osmium (OsO_4) solution.
6. The osmium solution is then washed free with 3 changes of DD water.
7. After the last wash the filters are removed to flat tissue culture dishes for the rest of the process. The key to this process is to not let the filters dry out.

8. Filters in the tissue culture dishes are then submitted to dehydration using 75% ethanol.
9. This is followed by 95% ethanol and finally three treatments with 100% ethanol.
10. The filters are then transferred to the critical point dryer under a 100% ethanol solution in a porous container.

Critical Point Dryer

The Critical Point Drying system uses Carbon Dioxide (CO_2) because when kept under pressure and as the temperature is lowered a meniscus does not form with enough surface tension to damage the delicate tissue of organisms such as bacteria. The critical pressure of CO_2 is 1072 psi and the critical temperature is 31^{o}C. First the chamber containing the sample is pressurized and then slowly vented as the temperature is slowly lowered allowing the sample to pass from the liquid state to the gas state while bypassing the solid and damaging state of freezing.

The filtered samples emerge from the CPD with the organisms intact except for ~10% shrinkage from the dehydration. The morphology of the organism is preserved. The filters are then mounted to stubs using sticky tabs and the edges are coated with conductive carbon paint. A Denton Vacuum Coater was then used to coat the stubs with a 20 nm thickness of platinum/palladium (Pt/Pd). The coater uses a vacuum to evenly distribute a vaporized platinum/palladium wire.

Scanning Electron Microscope (SEM)

Scanning Electron Microscopes have taken over as Transmitted Light technology has reached a barrier in resolution capabilities. The SEM can give clear concise details on a micron-sub micron scale that Transmitted Light technology does not meet. Patterned after Transmitted Light Technology, SEM's utilize a monochromatic, highly focused electron stream. Condensers focus the beam and turn away high angle electrons. The stream becomes more focused after

passing through successive condenser lenses. The stream flows through an objective aperture, which further focuses it and is then directed by computerized commands to run patterns across the surface. As the stream enters the sample it instigates interactions due to the negatively charged electrons. As electrons inside atoms, affected by the stream, fall from an outer orbital towards an inner orbital, if there is room, they release energy. This small energy released is detected from each location where the beam pauses. The more energy released and detected the brighter that pixel becomes in the image produced.

Analysis by the Scanning Electron Microscope gives several different results. Images of topography show surface features and texture down to nanometer scale. The shape, size, and scale of objects on samples are morphological features detectable by the SEM. It can analyze chemical composition of areas less than one micrometer

Backscatter Electron Images show composition as variances in degree of light to dark. Darker portions are from elements higher up on the periodic table and the opposite is true of the light areas. This grayscale shows differences in mean atomic number or means of multi-element constituents. The backscatter image is not exclusive of the Secondary Electron Image (SEI). The SEM was used to image the microbes grown in culture.

Chemical analysis

Chemical analysis was done on aliquots extracted from both runs and analyzed on November 11, 2002. The second run was then reanalyzed on November 20, 2002. So as to limit the amount of media withdrawn from the active cultures and to preserve the microbial activity a 2 ml aliquot was taken using disposable sterilized pipettes. The aliquot was then transferred to a syringe were the sample was passed through a 0.22 micron Nucleopore filter into a 10 ml graduated cylinder. A Hanna pH 211-microprocessor pH meter was used to analyze the pH of the

cultures. The probe on the pH meter is small enough to fit in the 10 ml graduated cylinder in order to get a reading. The thermometer that is part of the system was rested in a flask of double deionized water at room temperature consistent with that of the culture flasks. After analysis for pH the aliquot was diluted with 98 ml of double deionized distilled water. The ratio of dilution is 2:98.

The prepared samples were then analyzed in the Hydrology Lab in the Chemistry building for Fe_{total}, Fe^{2+}, Fe^{3+}, and sulfate (SO_4^{2-}). A Hach DR 2000 Direct Reading Photo spectrometer was used to do the analysis. A sample was also taken of growth media added to a culture flask one hour prior to sampling in order to get a reference point for the chemical conditions at the beginning of culturing. The growth media was also analyzed using the Hach DR 2000 prior to it being added to the pyrite enriched sediment to determine the chemical properties.

Controls

Sterilization of equipment and work areas was vital to the integrity of this study. All work areas were cleaned thoroughly with ethanol prior to work being started. Disposable gloves and aseptic methods were utilized including, washing experiment related equipment with antimicrobial soap, and keeping experiment vessels covered at all times to prevent contamination. During extractions the foil coverings on the flasks were lifted for a minimal time under the fume hood so the air rising out of the flasks would minimize contamination. Open flasks with the same pH media as the capped culturing flasks were in the fume hood as test controls for atmospheric contamination in culture flasks. Abiotic flasks that had not been inoculated with mine effluent were placed in the fume hood covered with aluminum foil as a culturing growth rate control. A short-term control was used to test chemical changes one hour

after growth media was added to sediment in a culture flask. All controls were identical to the set up in the experimental flasks except for inoculation.

Equipment and media for the culturing process were autoclaved at 120^0C and 20 psi with the help of a technician in the Biology Department at the University of Maryland.

Washing with soap and water, then rinsing with 0.1 molar HCl wash sterilized equipment used in the lab. The equipment was then rinsed in ethanol followed by double deionized water. Sampling equipment and filtering apparatus were presterilized and appropriately discarded after each use. Equipment used for measurements was calibrated prior to each reading according to the manufacturer's specifications.

Limitations

Limitations in this laboratory experiment exist because of the size and extent of the AMD problem. The underground mine workings are of considerable extent and the exact nature of what is taking place at depth is little understood. The surface area of available pyrite, and water quantity in the culturing flasks falls orders of magnitude short of those available in the mine. This may have limited the density of cultured cells, but these experiments could be applied to natural environments under similar chemical conditions.

Access to the inner portions of the mine is restricted, yet significant amounts of water are exiting the mine and have been sampled and analyzed. Several different studies have used the available mine effluent as a basis for their analysis. The mine effluent is diluted which may exclude certain acidophilic organisms, but it is documented that certain microbes form spores to ensure species survival until more ideal environmental conditions can be found (Barns and Nierzwicki-Bauer, 1997).

Data

Field Chemical analysis

Field measurements show the mine effluent from the abandoned coalmine contains aqueous Fe^{3+}, Fe^{2+}, and SO_4^{2-}, along with a low pH. The mean pH from Coketon was 3.29 ($^{+}$. 0.35, n=6) and for the borehole sampling area the mean pH was 3.45 ($^{+}$. 0.88, n=12) (see Index 2).

Location	pH	Temp ^{0}C	Fe_{total} mg/L	Fe^{2+} mg/L	Fe^{3+} mg/L	SO_4^{2-} mg/l	DO%
Kempton Borehole	4.33	10.58	58	22	36	600	2.5
Coketon Vent	3.28	9.74					6.7

Table 7. Field Measurements taken by Graeme Bowles from Kempton Mine Complex, 03/04/2002

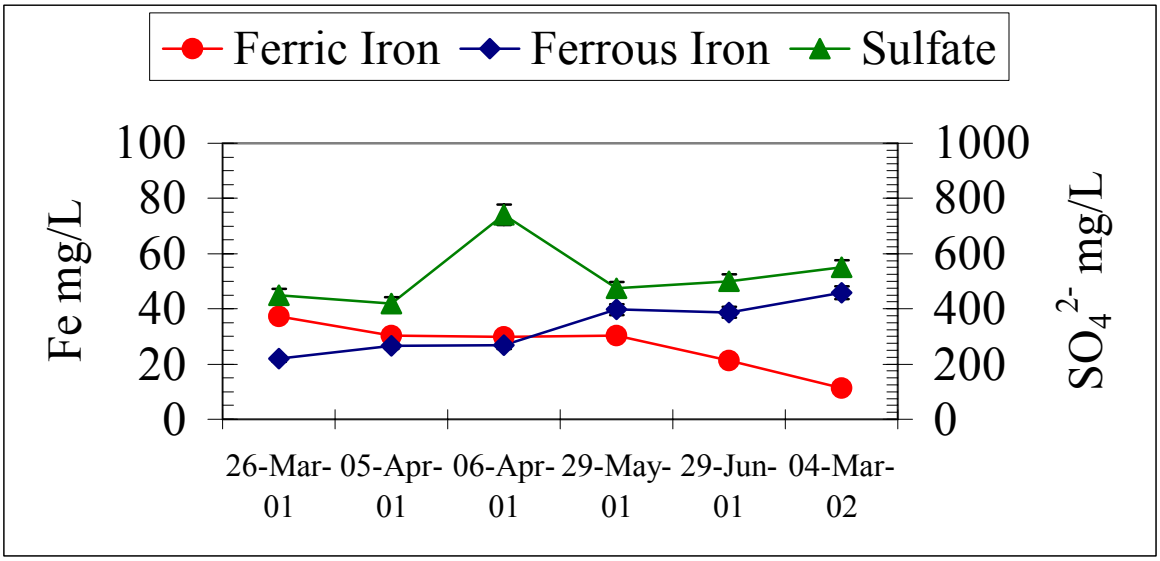


Figure 4. Plot of ferric iron, ferrous iron, and sulfate concentrations in mg/L recorded from 03/26/2001 to 03/04/2002 at the Kempton borehole by Dan Earnest and Graeme Bowles.

Elevated levels of dissolved oxygen at the Coketon sampling area are attributed to the mine effluent gaining oxygen as it flows through a shallow failed lime drain. The turbulence of

the drain introduces oxygen to the mine water. The elevated pH reading at the borehole, done on 03/04/2002 is ascribed to snow melt runoff increasing the amount of water in the system. On the day field measurements were taken it was snowing in the vicinity of the sampling area. A white precipitate was observed in the water flowing out of the borehole, and since this was not observed at the Coketon site it is attributed to the localized effect of the snowmelt raising pH levels.

Chemical Analysis of Cultures

The following pH and chemical data listed in Index 1 have been subdivided into sections based on the location that the samples were retrieved from, either in the field, or as replacement samples for those feared destroyed in the refrigeration failure.

Controls	**Initial pH**	**pH**	**Fe_{total} mg/L**	**Fe^{2+} mg/L**	**Fe^{3+} mg/L**	**SO_4^{2-} mg/l**
Initial Media	3.23		0	0	0	850
Control One hour	3.08	2.54	39	24	16	1200
Control I 10/09 Series 2	3.08	3.00	115	36	79	1900
Control II 10/09 Series 2	3.08	2.71	181	18	164	2100
Control 06/01 Series 1	3.23	2.45	93	7	86	2500

Table 8 chemical analyses of controls

Coketon Field Samples	**Yeast**	**Refrige-rated**	**Initial pH**	**pH**	**Fe_{total} mg/L**	**Fe^{2+} mg/L**	**Fe^{3+} mg/L**	**SO_4^{2-} mg/l**
E Series 2	Yes	No	3.08	2.80	141	37	104	2000
F Series 2	No	No	3.08	2.78	161	58	104	1450

Table 9 chemical analyses of Coketon field samples

Kempton Field Samples	Yeast	Refrige-rated	Initial pH	pH	Fe_{total} mg/L	Fe^{2+} mg/L	Fe^{3+} mg/L	SO_4^{2-} mg/l
Old 4 Series 1	No	No	3.23	2.63	129	33	96	2150
H Series 2	No	No	3.08	2.53	53	3	51	2150
J Series 2	No	No	1.08	1.43	596	79	517	2950
Old 5 Series 1	Yes	Yes	1.22	1.51	498	129	369	2700

Table 10 chemical analyses of Kempton field samples.

Kempton Replacement Samples	Yeast	Refrige-rated	Initial pH	pH	Fe_{total} mg/L	Fe^{2+} mg/L	Fe^{3+} mg/L	SO_4^{2-} mg/l
New 1 Series1	No	No	3.23	2.27	177	2	175	3450
D Series 2	No	No	3.08	2.73	156	27	130	1900
New 2 Series 1	No	Yes	3.23	3.11	236	32	204	2050
G Series 2	No	Yes	3.08	2.69	112	59	54	1750
New 3 Series 1	Yes	No	3.23	2.73	224	32	193	2450
C Series 2	Yes	No	3.08	2.76	229	60	169	2150

Table 11 chemical analyses of Kempton replacement samples.

Crellin Borehole Samples	Yeast	Refrige-rated	Initial pH	pH	Fe_{total} mg/L	Fe^{2+} mg/L	Fe^{3+} mg/L	SO_4^{2-} mg/l
A Series 2	Yes	Yes	3.08	2.99	124	56	68	1800
B Series 2	No	No	3.08	2.81	149	21	129	1950
I Series 2	No	No	1.08	1.43	458	115	343	2500
Crellin 07/01 Series 1	No	No	3.17	2.07	430	40	390	3800

Table 12 chemical analyses of Crellin borehole samples.

The pH values listed in the tables were taken 11/11/2002. There was a second analysis on series 2 done 11/20/2002 but due to the proximity to the initial analysis was not included in these tables, but is represented in the supporting figures and listed in Index 1. pH trends for the cultures from both series were compared with the pH changes of the controls. The average trend of the controls is consistent with those of the cultures.

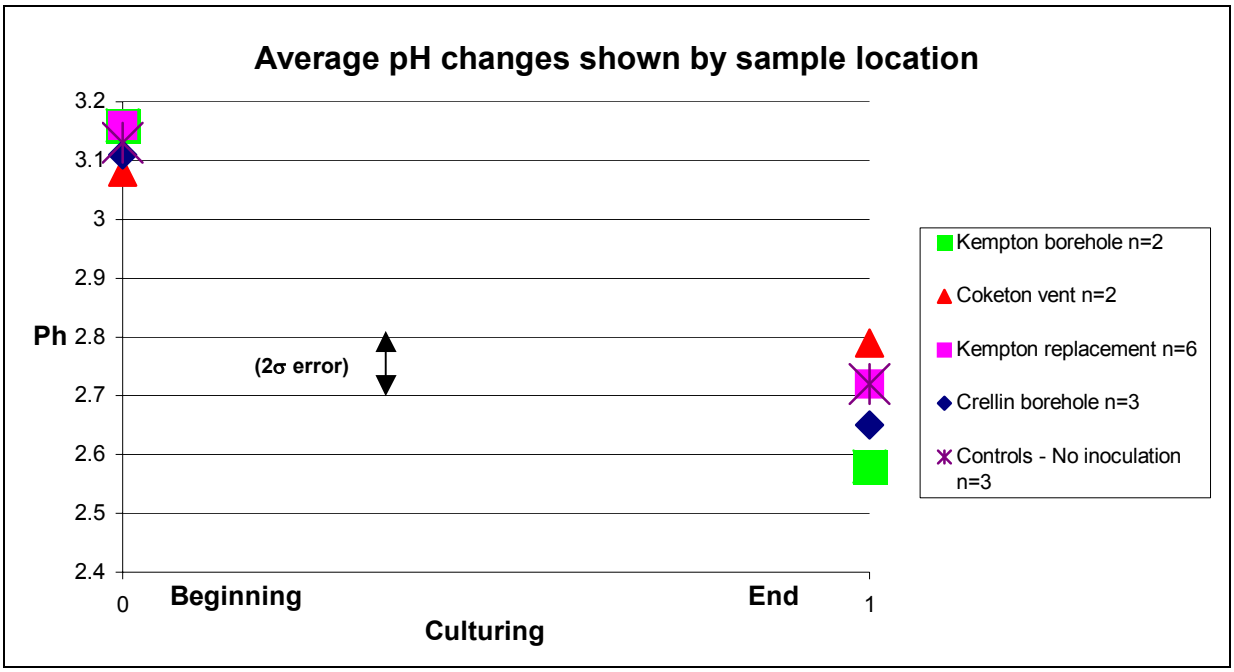


Figure 5 pH changes for the duration of culturing separated by location.

To examine the trend in pH changes for the culturing process the chemical analysis was divided into series one and two as well as high and low pH. The trend for high initial pH and low initial pH cultures for both series-1 and 2 show a trend towards a chemical equilibrium. The results from the chemical evaluation were also divided according to the initial conditions set up in each series. The addition of yeast extract was compared with the results from cultures with no yeast added. Refrigerated cultures were also compared with the results from cultures that were not refrigerated. The following three figures are graphical representations of these comparisons.

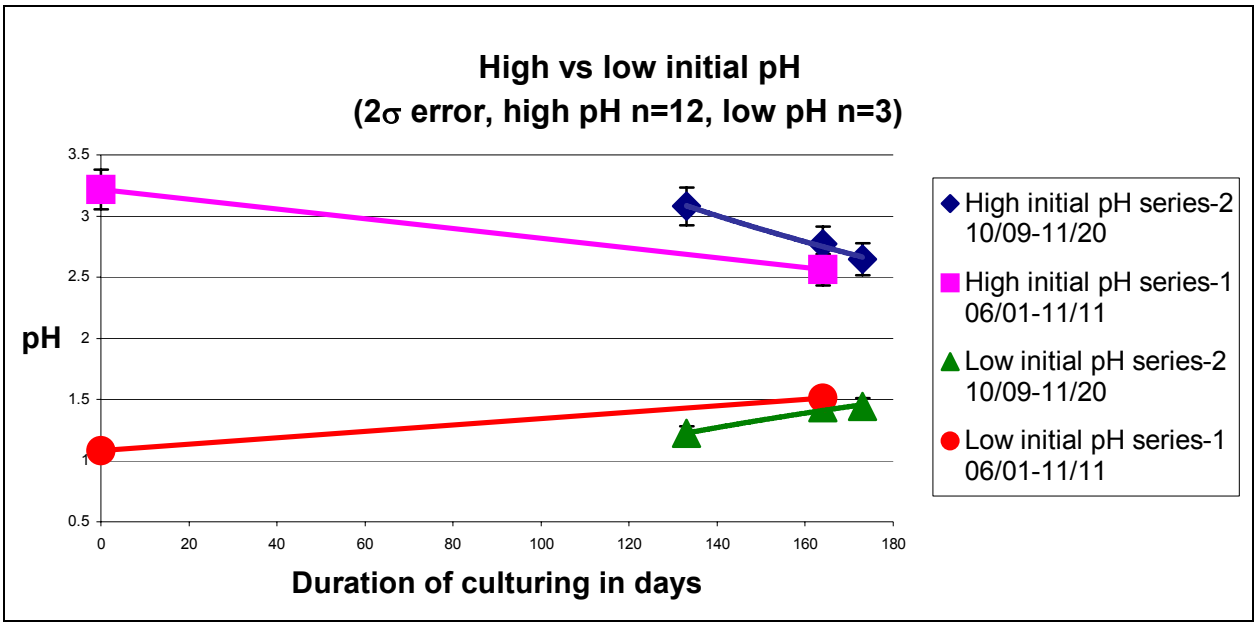


Figure 6 ph changes for the duration of culturing separated by high and low initial Ph.

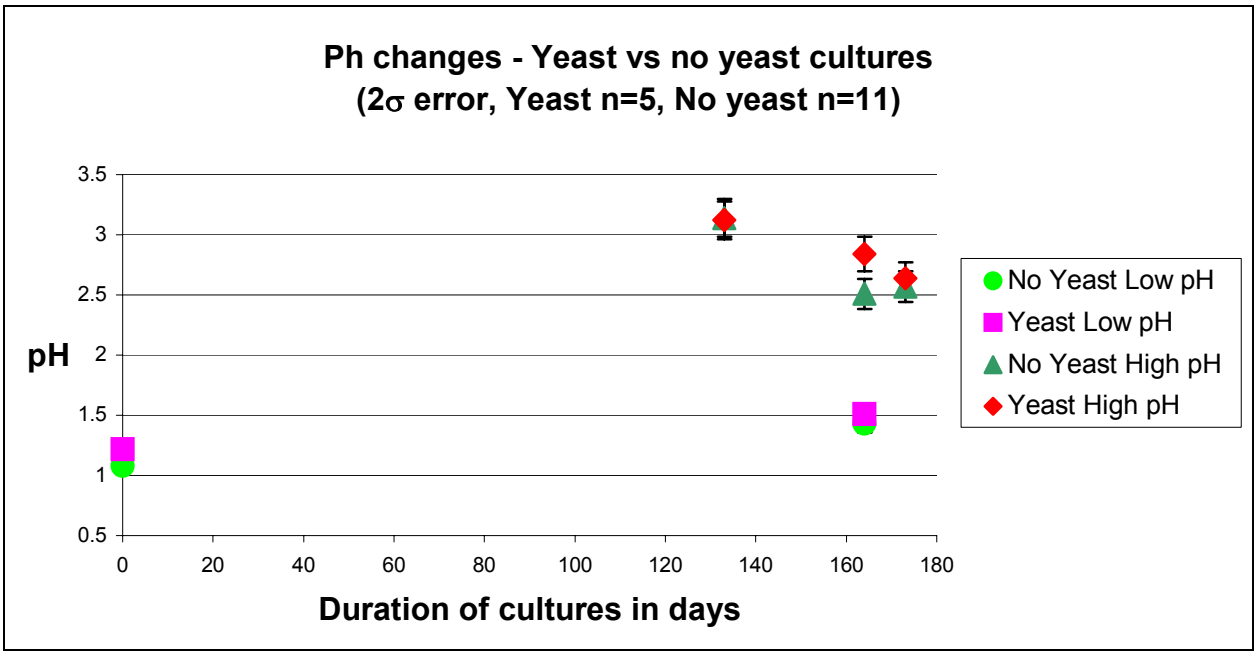


Figure 7 pH changes for the duration of culturing separated by yeast and no yeast.

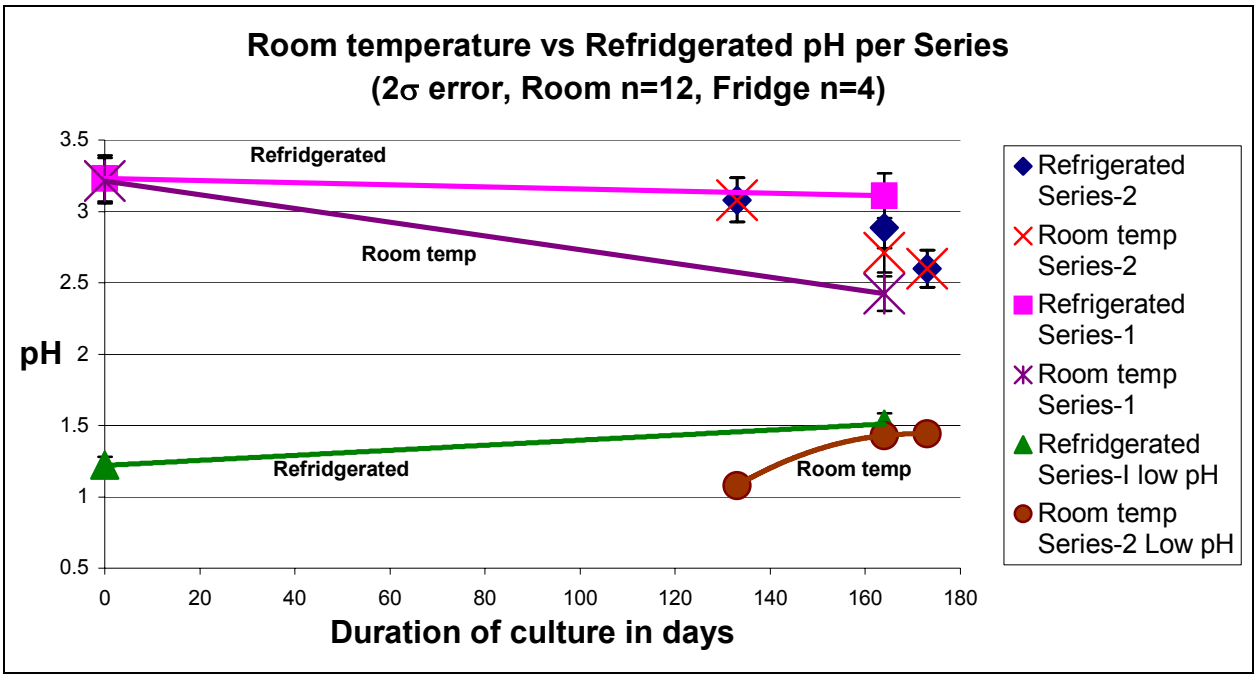


Figure 8 pH changes for the duration of culturing separated by refrigerated and room temp.

The following two figures show trends between low initial pH and high initial pH using sulfate and Fe_{total} levels.

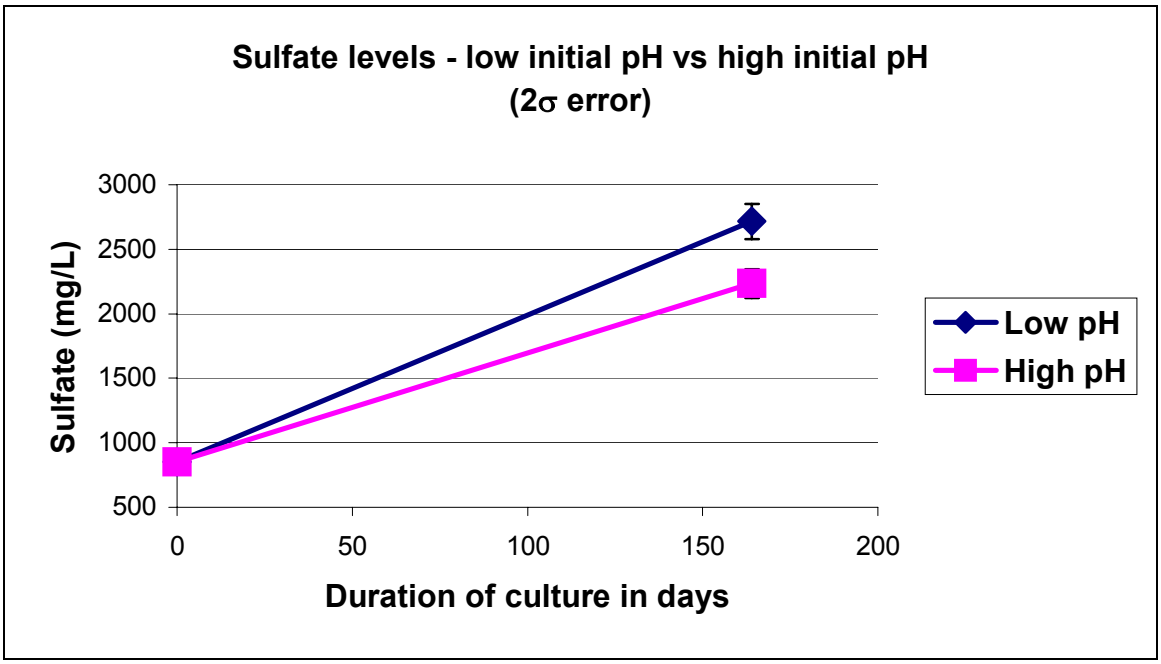


Figure 9 Sulfate levels after culture for low vs. high initial pH.

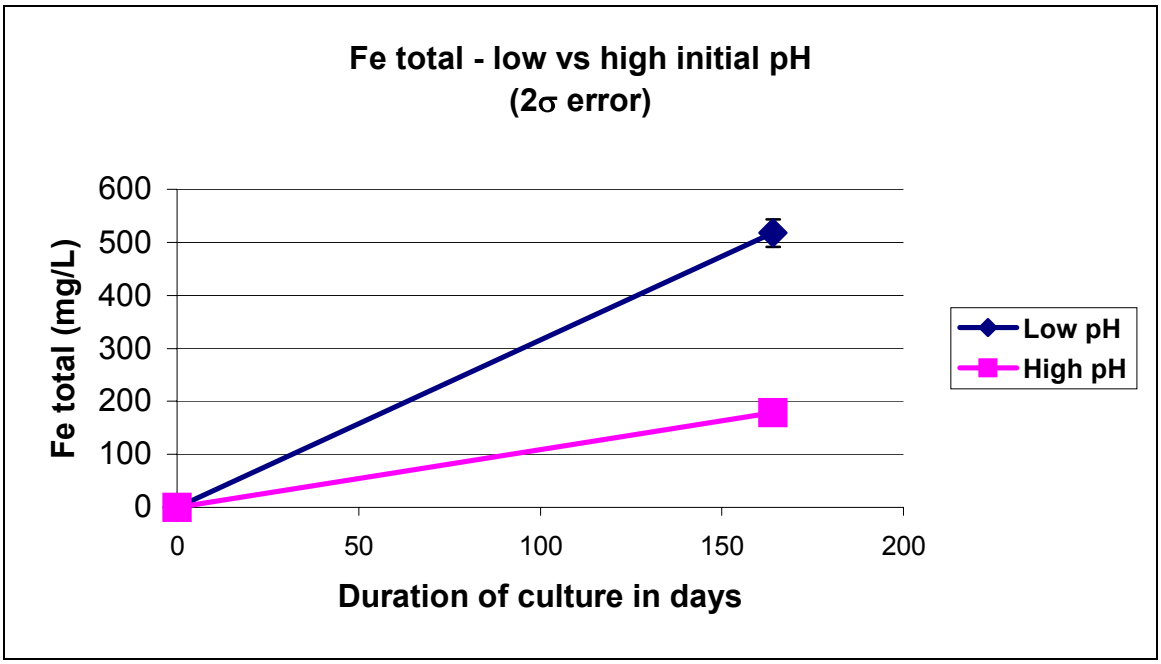


Figure 10 Fe_{total} levels after culture for low vs. high initial pH.

Preculturing SEM Analysis

Scanning Electron Microscope analysis was done on the pyrite cubes prior to autoclaving and culturing. Unusual dark areas in a backscatter electron image were compositionally analyzed and determined to be salts on or at the surface of the pyrite. A clear area of the pyrite (FeS_2) (see Fig. 12) was compositionally analyzed and found to contain the following:

Element	Weight %	Atomic %	2σ Error +/-
S	52.7	66.0	0.01
Fe	47.3	34.0	0.01

Table 13 Compositional analysis of pyrite cube prior to culturing.

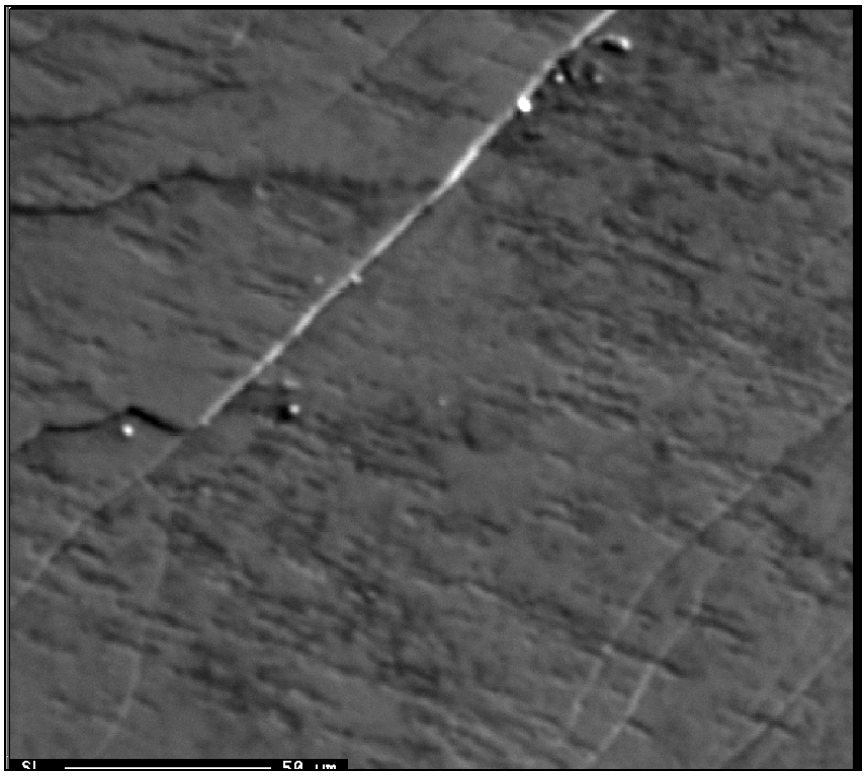
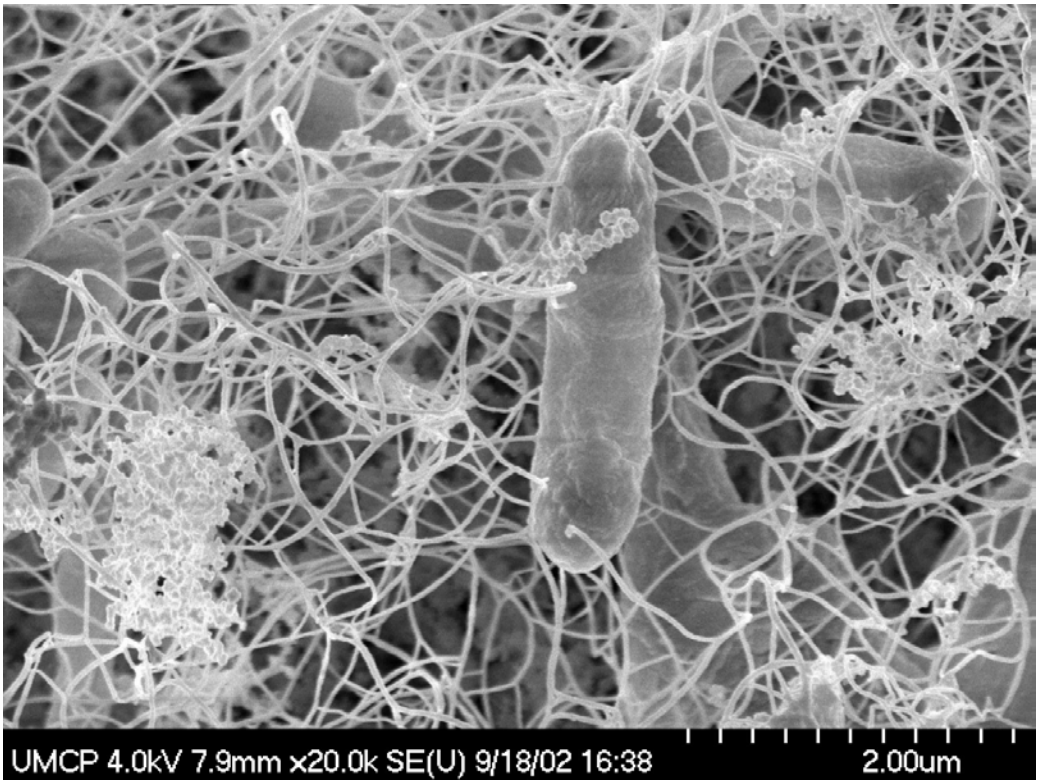


Image 1 surface of the pyrite cube where compositional analysis was done. Unaided this face of pyrite appears smooth. Under SEM analysis fine striations can be seen running perpendicular to fractures or cleavage planes. (SEI, scale bar =50 um)

SEM analysis of cultures

After allowing series-1 to culture from 06/01/2002 to 09/12/2002 1 ml aliquots were taken from the cultures and examined at the Laboratory for Biological Ultrastructure at the University of Maryland. The aliquots were fixed using osmium and prepared for analysis using a Critical Point Drying system, as previously described. Microbe images were taken from all the samples analyzed. In the culturing flasks delicate films of material could be observed. With the enhancement of the SEM these films were imaged revealing an intricate interwoven web of polysaccharides. Positioned throughout the mesh were microbes of varying morphologies and size. The biofilm and microbes appeared to be encrusted with conglomerations of unknown substance. The following images were taken at the LBU.



Images 2 and 3 showing the interwoven network of the polysaccharide biofilm and attached microbes.

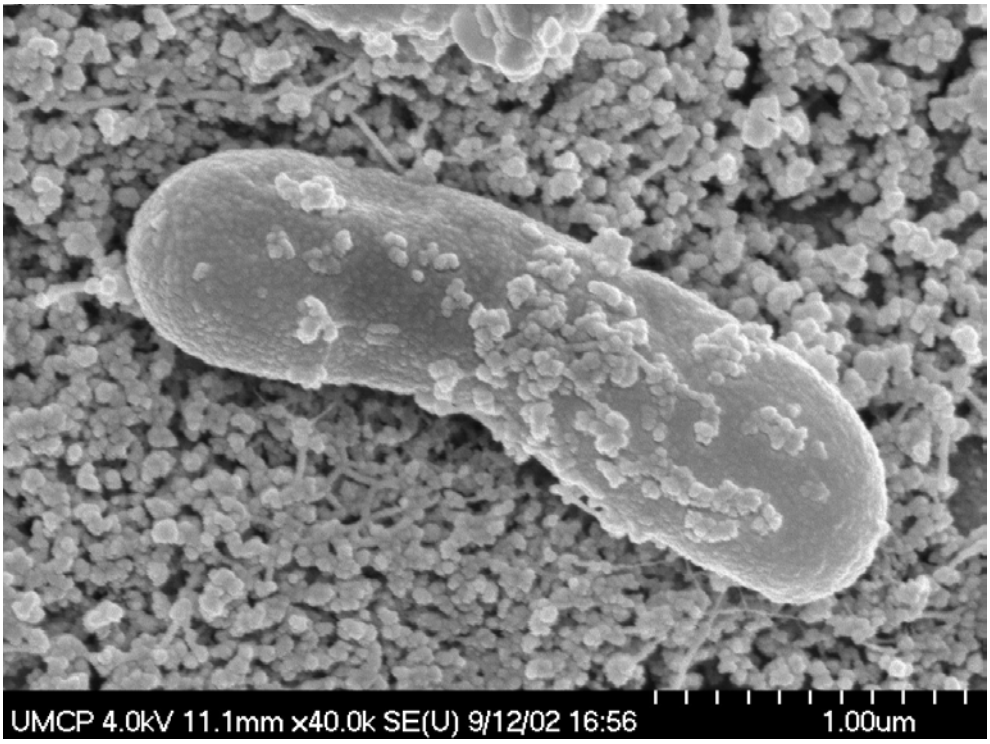


Image 4 showing an encrusted bacteria cell and underlying biofilm.

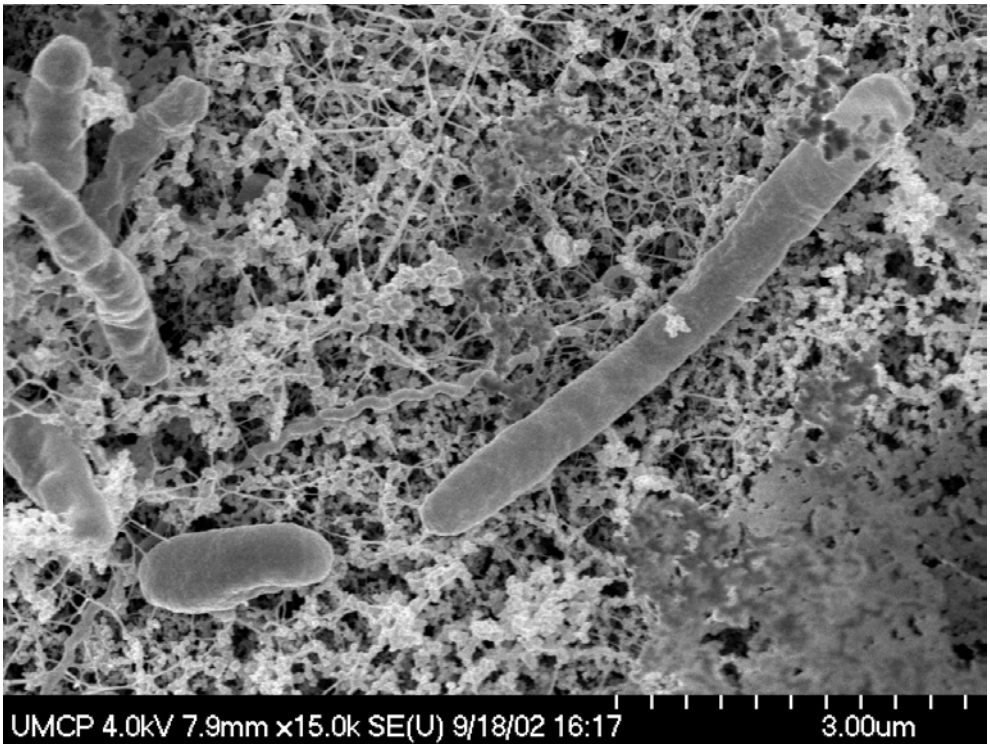


Image 5 showing three distinct microbial morphologies attached to encrusted biofilm.

Error

To minimize the uncertainty of the measurements taken in this study, the equipment used was calibrated to manufacturers specifications, where applicable. All error in the previous data was calculated and reported to two standard deviations (2σ) and were appropriate the sample size was reported as (n = sample size). To determine the uncertainty associated with the measurements and where possible the same sample was measured 15 different times and recorded. Microsoft Excel was used to calculate error out to two standard deviations. Since the parameters for sulfate measurements were different from the Fe measurements, error was calculated for each. Error for the SEM could not be calculated and was taken from literature and operation manuals. The following table contains determined error for the associated equipment.

Equipment	Analysis	Error (+/-2σ)	Sample size (n =)
Hach Direct Reading DR2000 photospectrometer	Sulfate	264 mg/L	n = 15
Hach Direct Reading DR2000 photospectrometer	Fe	2 mg/L	n = 15
YSI model 600-pH meter	pH	0.04	n = 8
Hanna pH 211-microprocessor pH meter	pH	0.04	n = 15
JEOL JXA 8900RT WD/ED combined microanalyzer (SEM)	Secondary electron imaging / EDS	0.01	n/a

Table 14 established error for equipment used in this study.

Discussion of Results

Comparisons between field measurements and laboratory results can be made when keeping in mind that in the cultures the amount of water available is orders of magnitude less than that of the mine. In addition the amount of pyrite (FeS_2) available in the culturing flasks is orders of magnitude greater than that in the mine. The less acidic conditions of the mine effluent can be attributed to the large volume of water contained in the mine. Conditions at the actual site of acid mine drainage generation may be severely underestimated when analyzing effluent from the various sampling locations. The mine has been unused since the 1950's and when compared with the cultures more time may be needed to acquire a thorough understanding of how the cultures relate to the chemical conditions in the mine. The levels of sulfate and Fe are in the same order of magnitude as those measured at the mine, but the cultures had significantly less water and more pyrite available.

The analysis of the cultures shows that the controls in the experiment show a consistent pattern with that of the inoculated cultures. The inoculated cultures were analyzed with the SEM and showed a variety of microbes actively attaching to minerals in the effluent and forming biofilms that cover the surface of the sediment in the flasks. The biofilms were also visible from the sediment to the air-water interface. This suggests a complex interaction of microbes that rely on different food sources and environments. SEM imaging of culture media showed a variety of microbe morphologies. Gram positive and negative microbes were imaged as well as spirillum, rods, elongate rods or chains, and cocci.

The consistent pattern of the controls with that of the cultures suggests two possible options. The first is that the controls were compromised, even though the strictest of controls was followed to avoid this. The low pH of the cultures would also appear to exclude contamination

except by the hardiest of microbes. The second is that the changes in the chemical analyses are not due to microbial interaction, but should rather be attributed to chemical reactions taking place in the culturing flasks. The changes in chemistry would happen regardless of the presence of microbes and this was evident in the changes that took place in the one-hour control. Sulfate levels went from 850 mg/L to 1200 mg/L and Fe_{total} went from 0 mg/L to 39 mg/L in just one hour. Generally in the cultures there was more Fe^{3+} than Fe^{2+} which is opposite of that in the one hour control. This could suggest that over time the microbes do make chemical changes to the growth media through the consumption of some of the constituents. Comparisons between the locations of the samples do not show any feasible trends except for the Crellin cultures, which appear slightly accelerated. The Crellin mine is not associated with Kempton and the samples could have contained a more representative field of AMD producing Bacteria.

There were several different conditions set up in the culture flasks to test for different results. There was no significant difference in cultures that had the yeast additive from the cultures that did not include yeast. Graphical representations of chemical levels between these two conditions do not show any differing trends. Yeast may not aid in the attachment of microbes to pyrite, proposed to increase the rate of pyrite oxidation, or the microbes isolated from the mine effluent do not respond to this addition. Prior to culturing pyrite cubes were imaged with the SEM. After culturing the same cubes were imaged to look for dissolution pits that would be the result of microbial attachment. The surface of the pyrite cubes did not show any visible alteration after being submerged in the culture media for three months.

Four of the culture flasks were refrigerated for the duration of the study. The refrigerated cultures showed less chemical change or progress than the cultures kept at room temperature. The trend was for room temperature cultures to show faster development in lowering pH and

increasing dissolved sulfates and Fe. This trend can be explained in two ways. The chemical reaction rate is expected to be less in colder conditions and microbial interaction may also be slower under these conditions. These results are not conclusive but could suggest that AMD conditions will take longer to emerge in colder climates.

One of the most conclusive trends that lend support to a chemical rather than biological motive for the changes is for the lower initial pH cultures to increase sulfate and Fe levels at a greater rate when compared with cultures with a higher initial ph. The lower pH cultures were more successful at mobilizing Fe and sulfate. This would be expected that a lower pH media would be more aggressive at mobilizing cations than a higher pH one. It could also be proposed that the microbes active in lower pH conditions are more suited than microbes in higher pH. Acidophilic microbes thrive in highly acidic conditions. The general trend of the pH from low and high cultures was that of progressing towards a chemical equilibrium.

The cultures were exposed to CO_2 from the atmosphere and this could have led to formation of carbonic acid. Carbonic acid or other acids formed would aid in the mobilization of cations regardless of the presence or absence of microbial assistance. In addition other phases available in the growth media could have precipitated out forming compounds and locking up vital nutrients needed by microbes. These phases could also have been removed through deposition on the biofilm or attachment to the microbes. Sphalerite (ZnS) deposits have been documented as forming nodules on natural biofilms made be sulfate-reducing Bacteria (Labrenz et al., 2000). The research done on biofilm characteristics for mineral attachment is limited but has profound consequences for the bioleaching of otherwise unrecoverable natural resources.

Another factor when analyzing the data is that the Bacteria isolated in this study may not be representative of the microbes active in the mine. The sampling area is some distance from

the center of the mine and the effluent may have undergone significant changes from its source. Microbes living in perfect conditions deep in the mine may not survive the proposed dilution by large amounts of water on the way out. In addition the water conditions at the time of sampling were unusual due to recent snowfall. A white precipitate was evident in the effluent leaving the mine and the pH levels were elevated from their typical stage (see Index 2).

Due to illness this study was not able to do fluorescent DNA tagging for Bacteria versus Archaea or cell density counts. The gentleman at the LBU responsible for the only fluorescent microscope on campus that had the correct resolution took seriously ill and access to the equipment was lost.

Suggestions for Future Work

Geomicrobiology of acid mine drainage is an important part of the entire global cleanup and understanding. It has financial prospective as well as natural resource implications. In order to get a better understanding of the interaction of microbes with the terrestrial world emphasis needs to be placed on understanding the individual AMD sites and incorporating this information into the general body of knowledge. To do this a more in-depth study needs to be done on the Kempton site. Samples should be taken at regular intervals and cultured. Isolating microbes from culture and identifying them through DNA analysis to be compared with microbes identified from other AMD sites would possibly reveal a common trend. Cell density counts as well as counts for Bacteria versus Archaea will help in understanding the interaction between microbes in complex communities. Maybe the answer to this question is not to isolate the microbe responsible for pyrite dissolution, but rather to isolate a microbe that helps create conditions for "the" AMD microbe to flourish in. The sediment used in this study was not analyzed for its composition and therefore the chemical reactions taking place in the cultures were not well

defined. More control should be placed on the chemical reactions in order to isolate the contribution of microbes in chemical changes.

Conclusions

Analysis of the culture medium showed a variety of microbes present. The formation of biofilms in the culture flasks was an indicator of the comfortable environment created for the microbes in this study. The media supported the culturing of microorganisms for an extended period of time and chemical analysis revealed trends for the duration of culturing. Recent research has shown that a wide variety of microbes, and the formation of biofilm, play a role in the generation of AMD. This study supports that finding through the varying morphologies found in the cultures and the prevalent biofilm, yet the microbes sampled from Kempton mine could not be isolated from chemical reactions causing the changes in chemistry, or the oxidation of pyrite. The diluted AMD samples taken from Kempton when cultured at low pH did produce microbes living in environments characterized by extreme conditions. The average Ph at the Kempton borehole is 3.45 and the cultures were successful at a pH between 1.0 and 1.5. This supports the adaptable nature of microorganisms. Monitoring of chemical changes under prescribed conditions was not able to show a consistent difference between the controls that were not inoculated and the cultures, which were. There was no significant difference in the rate of change for pH, Fe_{Total}, Fe^{2+}, Fe^{3+}, and sulfate (SO_4^{2-}) between the controls and the cultures. The changes observed in the cultures are attributed to chemical reactions rather than direct microbial activity. An extended study could reveal the influence of microbes that may have been too small to observe in this study.

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Sample	Run	Yeast	Fridge@ 6 °C	06/01/2002 Initial pH	11/11/2002 pH	Temp °C	$Fe_{Total\ Mg/L}$	$Fe^{2+}{}_{Mg/L}$	$Fe^{3+}{}_{Mg/L}$	$Sulfate_{Mg/L}$
New 1	1	no	no	3.23	2.27	23.50	177.00	2.00	175.00	3450.00
New 2	1	no	yes	3.23	3.11	29.00	236.00	32.00	204.00	2050.00
New 3	1	yes	no	3.23	2.73	23.70	224.00	31.50	192.50	2450.00
Old 4	1	no	no	3.23	2.63	28.90	128.50	33.00	95.50	2150.00
Old 5	1	yes	yes	1.22	1.51	22.80	498.00	129.00	369.00	2700.00
Control	1	no	no	3.23	2.45	23.40	92.50	6.50	86.00	2500.00
Crellin 7/1/02	1	no	no	3.17	2.07	28.90	430.00	40.00	390.00	3800.00
				10/09/2002	**11/11/2002**					
				Initial pH	**pH**	**Temp °C**	**$Fe_{Total\ Mg/L}$**	**$Fe^{2+}{}_{Mg/L}$**	**$Fe^{3+}{}_{Mg/L}$**	**$Sulfate_{Mg/L}$**
A	2	yes	yes	3.08	2.99	29.00	123.50	55.50	68.00	1800.00
B	2	no	no	3.08	2.81	28.30	149.00	20.50	128.50	1950.00
C	2	yes	no	3.08	2.76	28.20	229.00	60.00	169.00	2150.00
D	2	no	no	3.08	2.73	28.50	156.00	26.50	129.50	1900.00
E	2	yes	no	3.08	2.80	28.50	141.00	37.00	104.00	2000.00
F	2	no	no	3.08	2.78	28.20	161.00	57.50	103.50	1450.00
G	2	no	yes	3.08	2.69	22.90	112.00	58.50	53.50	1750.00
H	2	no	no	3.08	2.53	23.00	53.00	2.50	50.50	2150.00
I	2	no	no	1.08	1.43	28.20	458.00	115.00	343.00	2500.00
J	2	no	no	1.08	1.43	28.30	596.00	79.00	517.00	2950.00
Control I	2	no	no	3.08	3.00	23.20	114.50	35.50	79.00	1900.00
Control II	2	no	no	3.08	2.71	23.00	181.00	17.50	163.50	2100.00
Initial Media							0.00	0.00	0.00	17.00
Added to sed	After 1 hr			3.08	2.54	21.70	39.00	23.50	15.50	1200.00

Index 1 Chemical analysis of cultures, series 1 and series 2. Analysis done by Graeme Bowles.

	11/20/2002					
	pH	**Temp ^{o}C**	**$Fe_{Total\ Mg/L}$**	**$Fe^{2+}{}_{Mg/L}$**	**$Fe^{3+}{}_{Mg/L}$**	**$Sulfate_{Mg/L}$**
A	2.67	15.9	134.5	23.5	111	1850
B	2.58	21.8	188	37	151	2000
C	2.6	21.8	195	28.5	166.5	2100
D	2.73	21.6	168	30.5	137.5	2000
E	2.65	21.8	149.5	26	123.5	2100
F	2.55	21.7	175	64	111	1700
G	2.53	15.9	210	23	187	1650
H	2.48	21.8	137	1	136	2100
I	1.44	21.7	700	121.5	578.5	3000
J	1.44	21.8	538	136	402	2150
Control I	2.88	21.70	114.00	21.00	93.00	1750.00
Control II	2.80	21.60	193.00	52.50	140.50	2100.00

	Date	pH	TDS (mg/l)	DO%	Measurements made by
Borehole	06/08/2000	3.08	N/a	6.1	DE
	08/03/2000	3.14	0.688	2.3	DE
	08/24/2000	3.45	0.673	7.1	DE
	01/23/2001	3.45	0.649	16.2	DE
	03/26/2001	3.16	0.705	3.9	DE
	04/05/2001	3.29	0.706	3.7	DE
	04/06/2001	3.34	N/a	14	DE
	05/29/2001	3.16	0.688	4.5	DE
	06/29/2001	3.09	0.671	4	DE
	10/14/2001	3.60	0.666	6	DE
	03/04/2002	4.33	0.632	2.5	GB
	03/04/2002	4.33	0.614	2.5	GB
Mean		**3.45**	**0.6692**	**6.067**	
Standard Deviation		**0.88**	**0.060**	**9.012**	
Coketon	06/08/2000	3.08	N/a	6.1	DE
	08/24/2000	3.27	0.683	8.7	DE
	01/23/2001	3.49	0.661	15.7	DE
	02/15/2001	3.48	0.68	4	DE
	03/26/2001	3.12	0.725	8.2	DE
	03/04/2002	3.28	0.772	6.7	GB
Mean		**3.29**	**0.7042**	**8.233**	
Standard Deviation		**0.35**	**0.089**	**8.040**	

Index 2 Field samples from Kempton borehole and Coketon vent. Measurements made by Dan Earnest (D.E.) and Graeme Bowles (G.B.).