DRA-24. MICROBIAL POPULATIONS OBSERVATIONS IN GOAT HILL NORTH TRENCH SAMPLES AND SELECTED SITE SAMPLES IN RELATIONSHIP TO SITE GEOCHEMISTRY AND ROCK PILE WEATHERING AND STABILITY

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1. STATEMENT OF THE PROBLEM

What relationships were observed between microbial populations in respect to geophysical and geochemical parameters in the Goat Hill North **(GHN)** rock pile and do these observations reflect rock pile changes and potential changes in stability?

2. PREVIOUS WORK

Considerable previous work has been completed on acid drainage (AD) often referred to as acid mine drainage (AMD) and acid rock drainage (ARD) and the microorganisms responsible for acceleration of these problems. The pyrite weathering process is a series of chemical reactions that have an important microbiological component. When solid-phase pyrite in rock is exposed to oxygenated water (1,2), the following chemical reaction, that can be greatly accelerated by microbial activity, occurs:

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{\ 2-} + 2H^+$$

As a result of this oxidation, the concentration of both reduced iron and acid in drainage waters increases significantly. In addition, the simultaneous presence of reduced iron and oxygen promotes the growth of acidophilic, autotrophic iron- and sulfur-oxidizing microorganisms. These include *Acidithiobacillus species, Acidiphilium sp., Leptospirillum sp.*, and others such as *Sulfolobus acidocaldarius,* the colorless sulfur-oxidizing bacteria, and *Archaea sp*. (3,4). Although these and other microbial species may play an important role in mineral leaching, *Acidithiobacillus ferrooxidans* has often been used as a model bacterium for studying iron oxidation (4,5,6).

It is widely believed that *T. ferrooxidans* and other iron oxidizers act as a catalyst for acid production by constantly oxidizing reduced iron (Fe^{2+}) to ferric iron (Fe^{3+}) which then acts as a chemical oxidant of pyrite, thereby liberating more reduced iron, sulfate, and large amounts of acidity as shown in the following two reactions (6,7).

$$4Fe^{2+} + O2 + 4H+ \rightarrow 4Fe^{3+} + 2H_2O$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{\ 2-} + 16H^+$$

Oxidation of Fe^{2+} serves as the rate-limiting step in biologically mediated acid production (4). Because organic carbon tends to be relatively scarce in most mine rock piles, *Acidithiobacillus species* and other iron oxidizers often compete quite well with other bacterial species for oxygen and nutrients.

The term environment in this document covers macro- and micro-environments, from geological units to the cracks and pits in rocks. A rate law derived by Williamson, et al, (7) indicates the abiotic rate of pyrite oxidation increases with increasing oxygen concentration and increases slightly as pH decreases. In the environment, the rate of sulfide mineral oxidation increases as pH decreases into a range conducive to microbial mediation of ferrous iron oxidation (1,2,8). Microbial pyrite oxidation rates begin to exceed chemical oxidation rates at around pH 3.5, and at pH 2 can be several orders of magnitude greater (9). Both abiotic and biotic rates tend to increase with increasing temperature.

The activity of the microbes involved in these processes is pH dependent with optimal conditions in the range of pH 2 to 3. Thus, once pyrite oxidation and acid production has begun, conditions are favorable for microbes to further accelerate the reaction rate. At pH ~6 and above, microbial activity is thought to

be insignificant or comparable to abiotic reaction rates in pyrite mediated acid production. Sulfur bio-oxidation takes place from a pH of 8.5 to 1.9; however, the species involved vary, each in turn oxidizing the sulfur to sulfate. Therefore, a "succession" of microbial species takes place as the pH of the rock pile materials are lowered by the production of sulfate. Other microbially-formed organic and nitric acids can also play an important role in environment transformation at micro- and macro scales. (1,10).

Recent research and studies of microbial populations at Questa and in other rock pile environments show that a broad spectrum of microorganisms, microbial products, and cell components can participate in and even accelerate many geochemical reactions within the rock pile. Microbes use various minerals present in cell growth and in metabolism as electron donors or terminal electron acceptors. Microbes can accelerate natural rock pile chemistry and can be the main driving component of geochemical changes. Additionally, microbial populations present at higher pH's may play a significant role in the succession of microbial population(s) and their establishment in a regime that causes the orders of magnitude greater acid production (11-15).

A concern at the Questa, New Mexico site is the mine rock piles that extend to heights approaching 1,600 ft with slope angles in the range of 36°. Natural failures within alteration scars have produced debris flows in the vicinity of the mine rock piles and shallow failures have occurred within the Questa rock piles. Upon initiation of the microbial portion of this study, the questions asked were *(1)* Can the microbial populations present, or more correctly, their activity, cause changes within the rock piles that can lead to increased rock pile instability? *(2)* Can this instability lead to rock pile failure? and *(3)* If these changes are possible, over what time frames might they occur?

From the available literature and analysis of various other acid producing rock piles, it was expected that the site microbes involved in the oxidation of sulfide to elemental sulfur belong principally to the genera *Acidithiobacillus*, *Leptospirillum*, *Sulfolobus,* and the colorless sulfur-oxidizing bacteria, with *Acidithiobacillus sp.* most likely being the dominant genus (3,6,7). Therefore, the premise formulated on initiation of the Questa rock pile microbial study was that *Acidithiobacillus* and *Leptospirillum species* were going to be found to be the predominant acid generating species found within the rock pile environments.

3. TECHNICAL APPROACH

Samples were collected and analyzed according to SOP's 54 through 59. At the same time samples were gathered for geochemical analysis, microbial samples were collected from similar materials. It was envisioned that this paired collection of samples would allow comparison of microbial types with geological zone geochemistries. Microbes were enriched, isolated, and enumerated using site specific microbial media screened for this study, classical microbiological culture techniques were used. Selected microbes isolated directly from samples and from selected culture media were examined using microscopy, metabolic profiles, and nucleic acid analysis. Information gathered was examined to assess the microbial populations found relative to the site in general and specifically within the GHN rock pile. A generalized summation will assess trench and site microbial populations for potential impacts to rock pile geochemistry with extrapolations to rock pile changes and stability.

4. CONCEPTUAL MODEL(S)

While it is considered that knowledge is insufficient to assess the microbial interactions and actions at a detailed level, general knowledge in this area is sufficient to provide a broader scope model.

Microbial populations can greatly influence site geochemistry and in some instances, many of which are poorly defined, can greatly accelerate reactions like pyrite oxidation. They cause mineral precipitation

and deposition and form calcite cements that can form crystal structures or fill cracks in existing rock materials at the microscopic level. Microbes and microbial materials – biopolymers can act as nuclei of formation for crystal growth and form cementation structures such as ferricretes and magnocretes; jarosite and gypsum can precipitate as interstitial cements.

In most rock pile environments an organic carbon source along with phosphate are normally the limiting factors for diverse microbial growth. Without an organic carbon source, growth of microbes that can utilize inorganic carbon, including carbon dioxide, is favored with time. This can lead to successions of microbial populations in some environments, resulting in the dominance of *Acidithiobacillus/ Leptospirillum species.* However, these microbes are seldom found alone and other microbes like acidophiles and *Archaea* species that prefer acid environments in a macro-oxygenated environmental setting will also be present. In these environments, localized or micro-environments will exist where organics are present and oxygen will be limited.

As Berner (1981) indicates, site geochemical environments can be generally described as follows:

Oxic Environments
- Dissolved oxygen > 30 μM
- Mn2+ below detection

Suboxic Environments
- Dissolved oxygen > 1 μM, < 30 μM
- Fe2+ below detection
- Mn2+ detectable

Anoxic Environments
- Dissolved oxygen < 1 μM)
- Anoxic – Sulfidic
- H_2S > 1 μM
- Anoxic – Non-sulfidic
- H_2S < 1 μM

These environments are based on measured dissolved oxygen and H_2S and are strongly tied to redox reactions (8). Moisture along with oxygen, geochemistry, Eh, and pH are the major controllers of the types and extent of chemical reactions occurring, the types of microorganisms present, and the types of reactions they are participating in, mediating, or controlling. Changes in pE induce both chemical reduction sequences as well as sequences in microbial ecology. In general:

- **Aerobic microorganisms** that utilize oxygen do not function below pE of 5
- **Denitrifying bacteria** function in the pE range of +10 to 0
- **Sulfate-reducing bacteria** live at pE's below 2

Oxygen is an electron acceptor, and reducing oxygen provides energy for microorganisms; in the absence of oxygen, nitrate and other ionic species can provide this energy through numerous redox half reactions that are possible within the rock pile materials. However, the higher the redox potential, the higher the energy yield, the more negative the Gibbs free energy and the greater the energy benefit to microorganisms. The greater the energy benefit to microbes the more they will grow and contribute to reactions leading to dissolution or precipitation of rock pile materials; this is the case with all microbial populations, not just those dominated by *Acidithiobacillus/ Leptospirillum sp.*

In oxygen rich environments, aerobic microorganisms use oxidized forms of Fe, S, Mn, and some metal oxyanions. Nitrate respiration below pE 8 produces products that include NO_2^-, N_2, N_2O, NH_4^+, and other metal oxyanions with denitrification producing nitrogen gases. Solid phase manganese reductions

can occur in the presence of NO_3^-. However, solid phase iron reduction, does not occur in the presence of NO_3^- or O_2 and the Mn:Fe ratio is an indication of whether a denitrifying environment is present. Anaerobic microbes produce products that include H_2S, HS^-, $S_2O_3^-$.

Iron oxyhydroxides generated by pyrite oxidation, as well as other reaction products, can aid in the cementation of soil and sand materials. Formation of these types of aggregates would tend to increase rock pile stability. Ferricretes – represent iron transported by reducing acid waters and precipitated as hematite and goethite at higher Eh (above 0.4) and/or pH (above 6) under more oxidizing, less acidic conditions. Hematite and goethite structures have also been observed to form in bioreactor systems, operating in sulfate reducing conditions at pH 6 to 7. Physical, chemical, and microbially-influenced weathering can have counter influences on rock pile stability. A conceptual model for microbial growth and succession and influence on rock pile weathering and stability is shown in *Figure 1*.

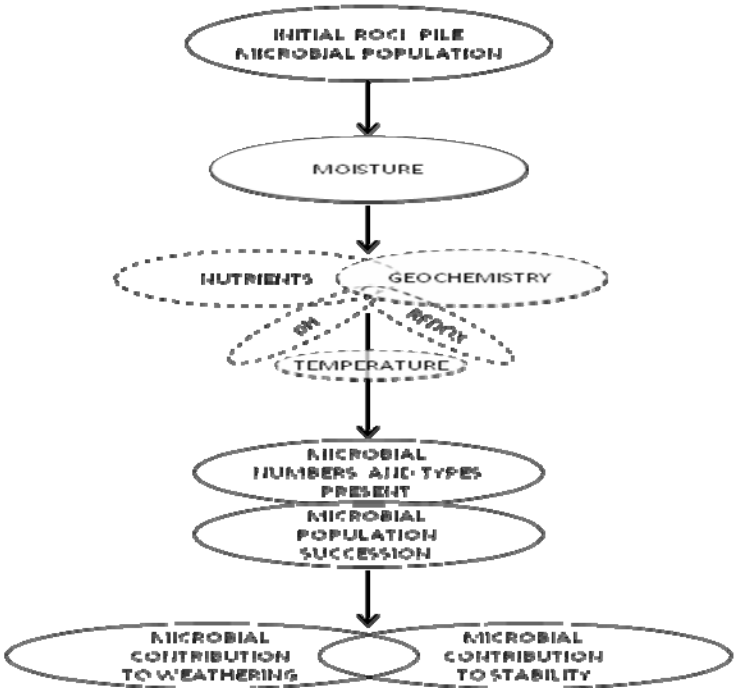


Figure 1. Conceptual model for microbial growth and succession in rock pile environment.

5. STATUS OF COMPONENT INVESTIGATION

General Status

Acidithiobacillus and *Leptospirillum species* are widely considered to be the microorganisms that control the rate of acid generation in rock piles. However, much is unknown about their natural distribution, how they interact with other microbial species over time that leads to their abundance or dominance in rock piles, at some pH's and in some environments, and their actual contribution to acid formation.

Microbial enumeration and characterizations have defined the site microbiology in both general and specific characteristics. Variations of stock media were tested over a broad pH range and numerous minor adjustments in component levels to determine the optimal pH and component concentration for use of the media with site samples. Five main metabolic groupings were used to adjust the culture media content in an attempt to cultivate as many different microbes from site samples as possible. Stock control samples of *Acidithiobacillus ferrooxidans, A. thiooxidans*, a general acidophilic microbial mix, *Leptospirillum,* a general acidophilic microbial mix, and laboratory stock cultures were cultured in the respective recommended culture media.

The media and analysis of bacteria in the Questa system was designed around bacterial metabolism rather than specific bacteria. Analysis looks at bacteria that perform specific biochemical functions or transformations rather than specific bacteria of a particular genus and species. Bacteria can be thought of as small chemical factories; under the right conditions, many different genus and species of bacteria can perform similar geochemical transformations.

Recent studies, including the Questa microbial study have shown that *Acidithiobacillus* and *Leptospirillum species* occuring within microbial communities may not be as important as previously thought in some subsurface acid-forming environments. *Acidithiobacillus species are* thought to affect the precipitation of ferric iron solids. *Figure 2* shows the relative microbial populations at the Questa site and in the GHN trench samples. *Acidithiobacillus* and *Leptospirillum species* are certainly dominant members of the site and trench sample microbial communities, but as can be seen in the GHN trench samples, they interact in most samples with acidophilic microbes, heterotrophs, and Archaea species, *Figures 3-15* and Technical Appendicies.

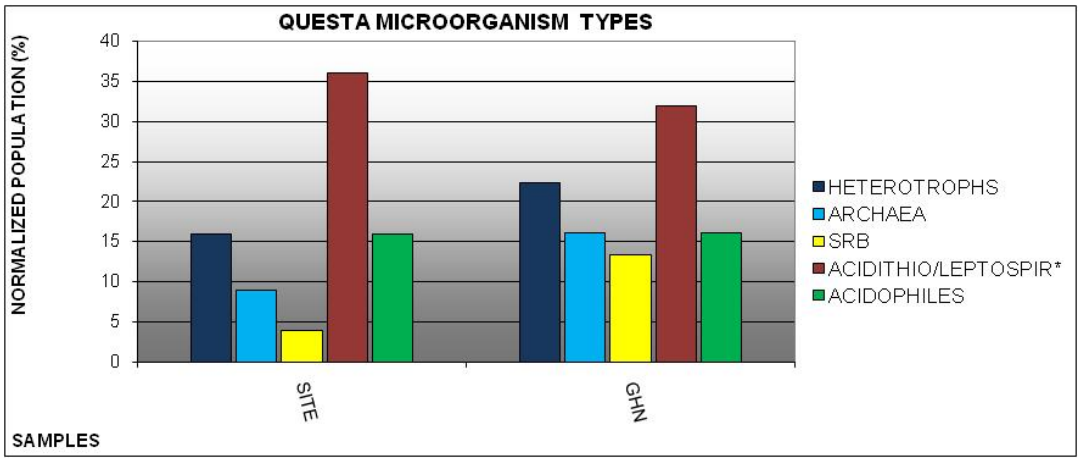
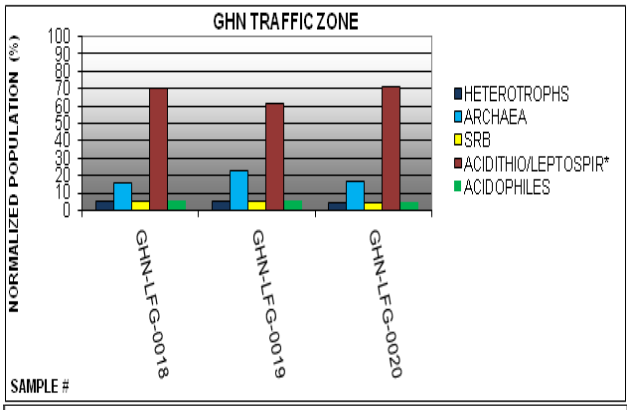
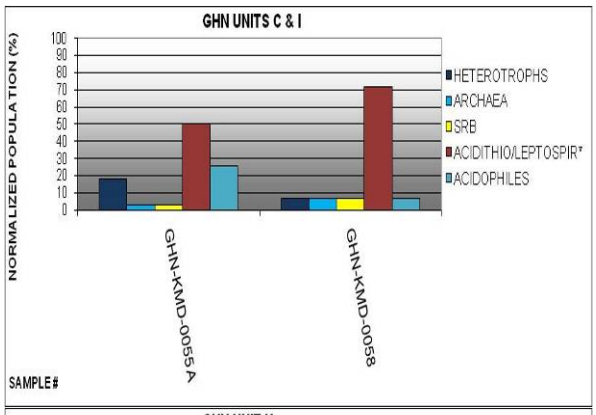


Figure 2. Distribution of microbial population at the Questa site and in the GHN trench samples.


GHN TRAFFIC ZONE
NORMALIZED POPULATION (%)
100
90
80
70
60
50
40
30
20
10
0
HETEROTROPHS
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
GHN-LFG-0018
GHN-LFG-0019
GHN-LFG-0020
SAMPLE #


GHN UNITS C & I
NORMALIZED POPULATION (%)
100
90
80
70
60
50
40
30
20
10
0
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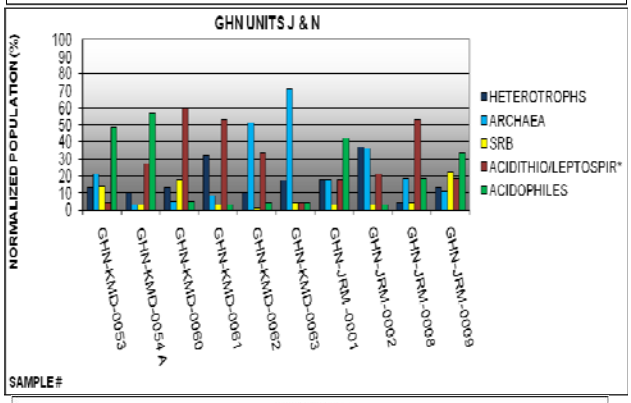
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
GHN-KMD-0055A
GHN-KMD-0058
SAMPLE#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NORMALIZED POPULATION (%)
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90
80
70
60
50
40
30
20
10
0
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ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
GHN-KMD-0053
GHN-KMD-0054 A
GHN-KMD-0060
GHN-KMD-0061
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GHN-JRM-0002
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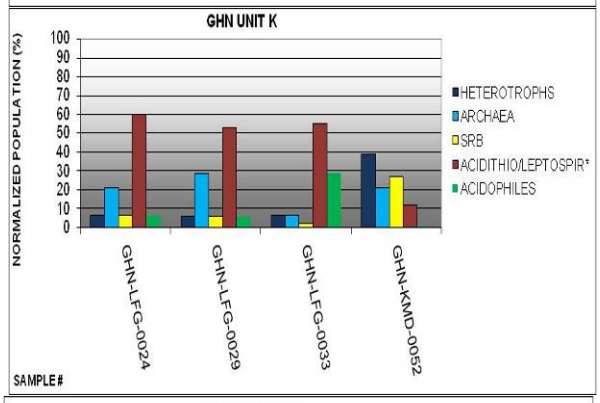
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NORMALIZED POPULATION (%)
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80
70
60
50
40
30
20
10
0
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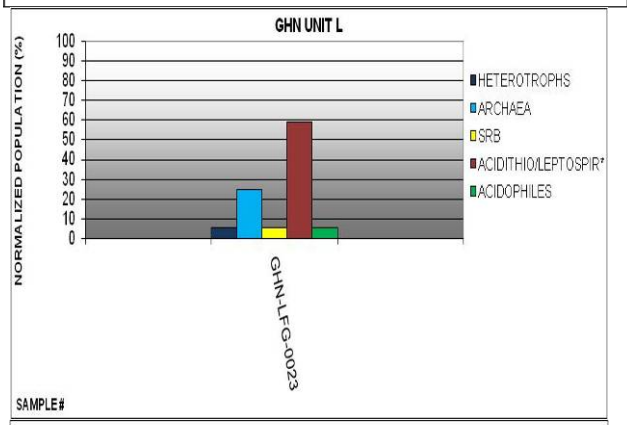
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
GHN-LFG-0024
GHN-LFG-0029
GHN-LFG-0033
GHN-KMD-0052
SAMPLE#


GHN UNIT L
NORMALIZED POPULATION (%)
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90
80
70
60
50
40
30
20
10
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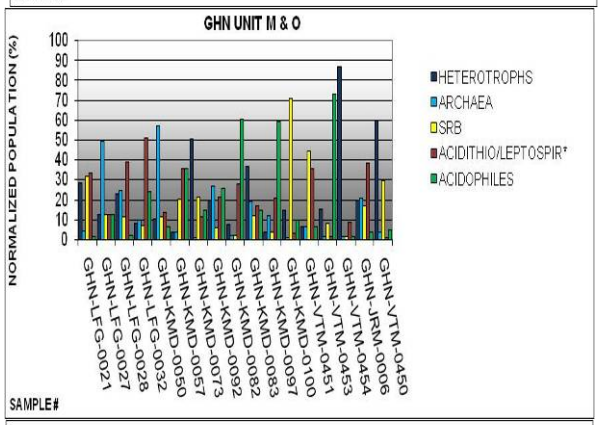
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
GHN-LFG-0023
SAMPLE#


GHN UNIT M & O
NORMALIZED POPULATION (%)
100
90
80
70
60
50
40
30
20
10
0
HETEROTROPHS
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
GHN-LFG-0021
GHN-LFG-0027
GHN-LFG-0028
GHN-LFG-0032
GHN-KMD-0050
GHN-KMD-0057
GHN-KMD-0073
GHN-KMD-0092
GHN-KMD-0082
GHN-KMD-0083
GHN-KMD-0097
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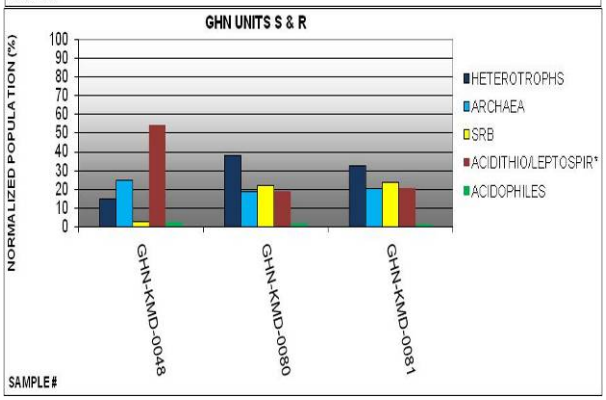
GHN UNITS S & R
NORMALIZED POPULATION (%)
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90
80
70
60
50
40
30
20
10
0
HETEROTROPHS
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
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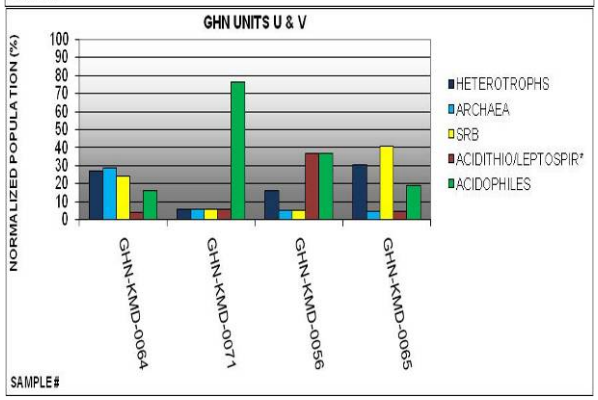
GHN-KMD-0080
GHN-KMD-0081
SAMPLE#


GHN UNITS U & V
NORMALIZED POPULATION (%)
100
90
80
70
60
50
40
30
20
10
0
HETEROTROPHS
ARCHAEA
SRB
ACIDITHIO/LEPTOSPIR*
ACIDOPHILES
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GHN-KMD-0071
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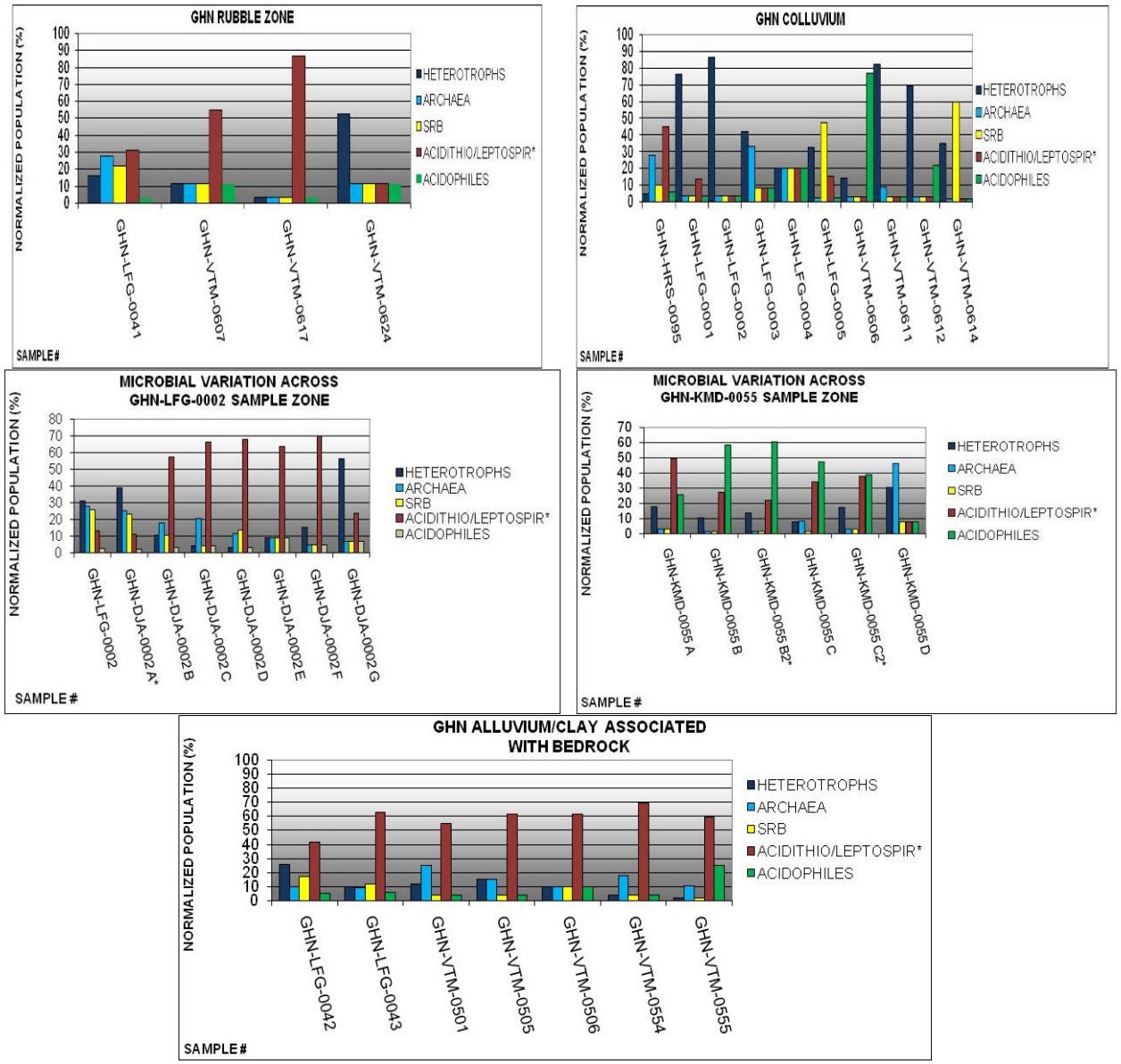


Figures 3-15. GHN microbial samples and selected sample groupings. Samples labeled with 'LETTERS' following the 10 digit sample number represent samples collected at a particular site; not split samples. * *Acidithiobacillus/Leptospirillum sp*

In about ~36% of the samples *Acidithiobacillus/Leptospirillum sp.* were the predominant microbes present; 50% or more of the microbial number present. In about 32% of the samples, either heterotrophs or acidophilic microbes were dominant. Acidophiles are organisms that can withstand and even thrive in acidic environments where the pH values range from 1 to 5 and include types of *Bacteria* and *Archaea* that are found in a variety of acidic environments. It is recently that the importance of acidophiles and their role in acid mine drainage has been recognized.

Possible *Archaea* type microbes are dominant in about 9% of the samples and are noticeably present in populations where *Acidithiobacillus/Leptospirillum sp.* dominated, but not the other way around. Sulfate reducers are dominant in ~4% of the samples, and as with all the other samples where one population was dominant, were in GHN samples; see Technical Appendices. From these results, it is believed that

the acidophilic and acidophilic heterotrophic bacterial species play an important role in both mineral leaching, dissolutions and transformations that provide opportunities for re-precipitation and crust formation on the surface and within selected rock pile environments. They probably play important roles on both sides of the mineral leaching and re-precipitation event zones within a rock pile environment.

Analysis of the rock pile microbes at the Questa site has yielded somewhat similar results in respect to the presence and abundance of *Acidithiobacillus* and *Leptospirillum* microbes. They are present but do not necessarily have a strong correlation with the more acidic environments examined. For the nine pH values available for GHN samples that corresponded with ≥50% *Acidithiobacillus/Leptospirillum species* dominance, the average pH was ~4.4 and ranged from ~2.7 to ~7.0, *Figure 16.*

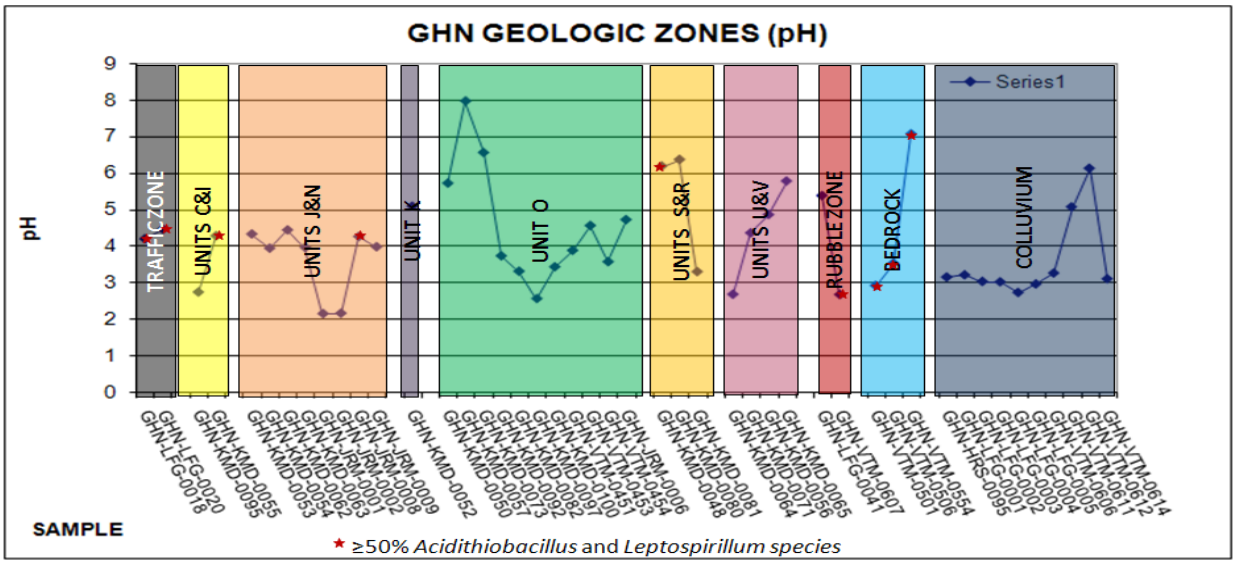


Figure 16. PAG pH of GHN geological zones with samples that have ≥50% *Acidithiobacillus* and *Leptospirillum species* highlighted (★). Samples were collected from the same geological zone; not split samples.

We have found large numbers of *Acidithiobacillus* and *Leptospirillum species* widely distributed at the Questa site which indicates that they play an important environmental role, *Figure 3*. They are present in the circumneutral rock pile materials as well as in samples with lower pH's. In some samples, denitrifyers and in some instances large numbers of sulfate reducing bacteria are present in some lower pH (~3.5) environments and other microbes like heterotrophs, acidophiles, and *Archaea sp* occur with *Acidithiobacillus* and *Leptospirillum species*.

In both the Questa site and trench samples examined, we have noted substantial variations in geochemical conditions that are accompanied by variability in microbial populations and numbers. Questa rock pile studies support evidence obtained at other sites, suggesting that the current models based on *A. ferrooxidans* should be examined more thoroughly to reflect the involvement of different species promoting sulfide weathering and rates.

With the relative small number of samples examined for correlations with geochemistry, *Acidithiobacillus* and *Leptospirillum species* are not correlated with areas of low pH. *Leptospirillum species* have a role that is classically defined as catalyzing sulfide mineral dissolution (catalyzing sulfide oxidation by aqueous ferric iron). These microbes are found in samples at low to high pH's as "a

common inhabitant" and it may be inferred that the impact of these species on pyrite oxidation reactions in the rock pile is at least somewhat restricted or acting in concert with other microbial species present, *Figure 17*.

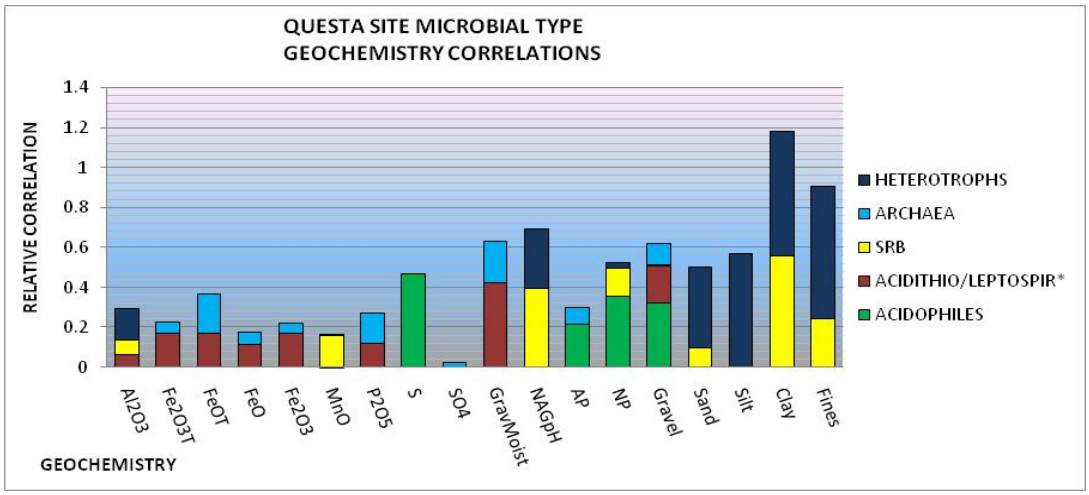


Figure 17. Relative microbial population correlation with site geochemistry parameters; based on analysis of 35 to 72 sample correlations per factor. Bar height is directly related to degree of correlation. Samples for geochemistry were samples from the same area; not sample splits.

Microbial Associations with Crustal and Cemented Materials

From the results obtained in these studies, it is believed that the *Archaea species*, acidophilic, and acidophilic heterotrophic bacterial species play an important role along with *Acidithiobacillus* and *Leptospirillum species* in both mineral leaching, dissolutions and transformations. These microbial populations and their actions and interactions provide opportunities for re-precipitation and crust formation, on the surface and within selected rock pile environments. All microbes produce some form of acids during growth cycles and require minerals present in the various samples for life functions.

Many microbes present produce organic chelators that can remove metallic ions from rocks and others can form accretion deposits. Biosorption can take place through extra cellular accumulation/ precipitation, cell surface sorption/ precipitation, and intracellular accumulation; we know that bacteria can act as precipitation nuclei. As microorganisms and microbial products participate in and accelerate many geochemical reactions within the rock pile, they use various minerals present as electron donors or terminal electron acceptors. Microbes and microbial materials can accelerate rock pile chemistry changes, respond to rock pile chemistry, and be a main driving component of rock pile chemistry changes.

We should not overlook the contribution of nanobacteria and biopolymers that have been documented to have a very high affinity for metals in general, minerals, or specific metals. Nanobacterial type structures have been found in metallic sulfide minerals such as pyrite, chalcopyrite and chalcocite and some complex silicates - clay minerals. Gypsum crystals may be initiated via microbial and or biopolymer involvement, as the crystallization nuclei. The formation of biomaterial structures is strongly influenced by physicochemical parameters such as ionic strength, pH and the concentration of competing organic and inorganic compounds, moisture cycle characteristics, and particle sizes. These

minerals and metals removed by various mechanisms can act as precipitation nuclei and inter-chelating structures for the formation of various cementing materials.

Most environments at a pH ≥3.0 contain a consortium of heterotrophic, acidophilic, sometimes sulfate reducing bacteria, acidophilic and chemolithotrophic bacteria. Crustal materials do have a dominant *Acidithiobacillus/Leptospirillum sp.* population; one sample has a population profile similar to the site samples, the other does not, *Figure 18*. The crustal material samples appear to be in two different stages of development or possibly breakdown, in that their component populations are significantly different. One sample has a strong dominant population of *Acidithiobacillus/Leptospirillum sp.* while the other has a mixture of different microbial population types. These populations could cycle during the year with different microbial populations being present as crustal materials are forming and breaking down. These cycles could also change from year to year with one being dominant during longer climate cycles.

The scar material microbial populations are dominated by acidophilic microbes, *Figure 19*. In all but one of the alteration/scar zones sampled, the acidophilic microbial populations are dominant along with the heterotrophic populations. It would be interesting to note the state of weathering in this sample. This general population profile is significantly different from that of the crustal samples shown above and indicates different microbial activities taking place in these sample zones.

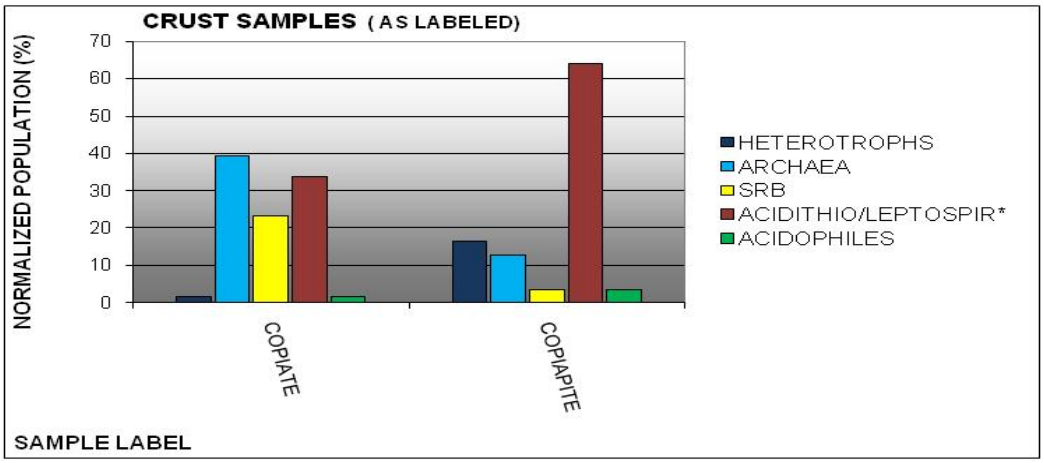


Figure 18. Population profiles of two different crustal material samples. * *Acidithiobacillus/Leptospirillum sp.*

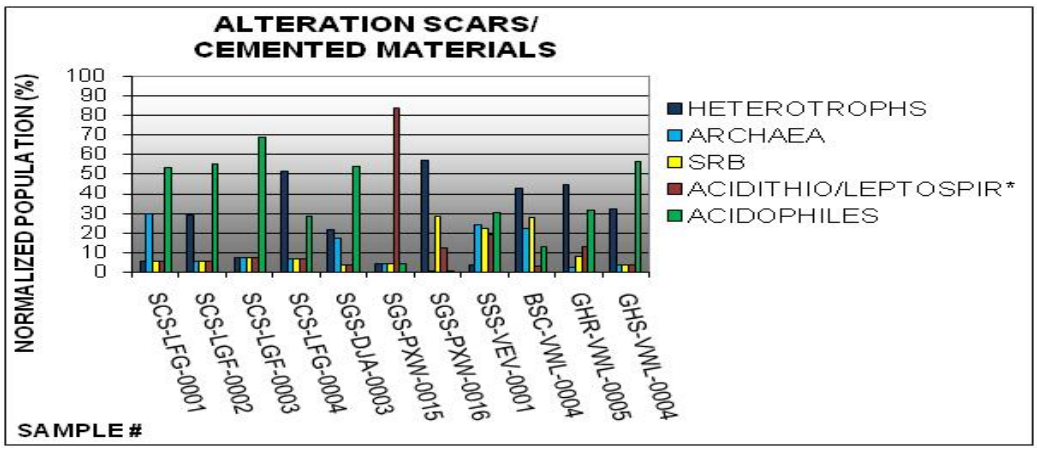
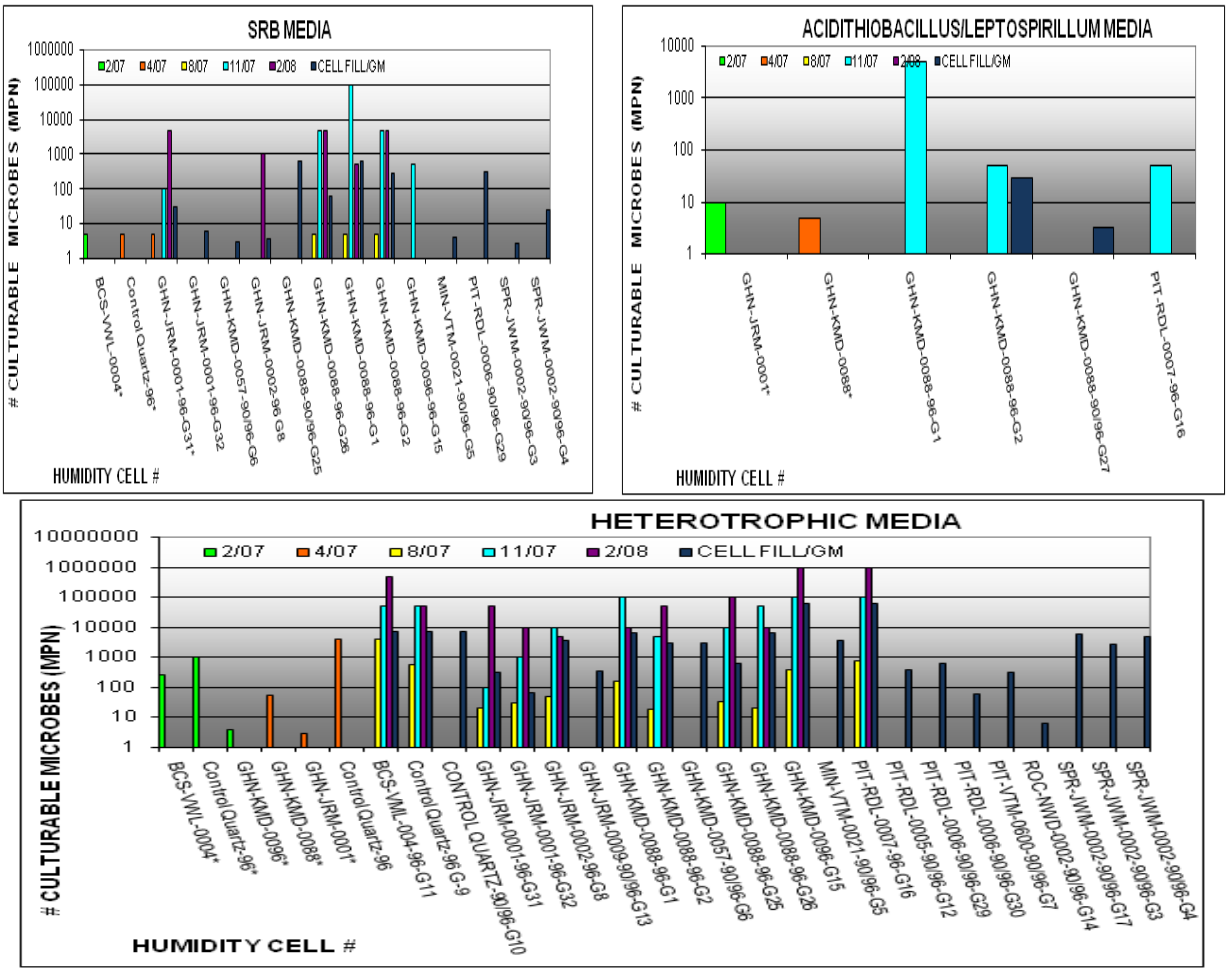


Figure 19. Population profiles of eleven scar and ferricrete samples. * *Acidithiobacillus/Leptospirillum sp.*

Humidity Cell Microbial Evaluation

Active microbes from all the groups of microbes common to the Questa site and specific for the samples in the humidity cells were inoculated into humidity cell materials that may have contained dormant microbes and some of the microorganisms introduced will respond differently in the humidity cell environments. These inoculated microbes did influence the microbial population development in the humidity cells as some microbial types were present at different levels throughout the experiment and inoculated heterotrophs and an acidophilic heterotrophic population were present in increasing numbers in the humidity cell tests over time.

Living cells require several types of nutrients. These supply carbon, energy, building blocks for biochemicals, or trace factors such as vitamins or hormones. Organic or inorganic carbon, nitrogen, sulfur, and phosphate; possibly an alternate energy source; electron donors; and appropriate electron acceptors. Nutrient components are used in a balanced C:N:P:S ratio of ~115: 15: 1.5: 1.5 plus trace elements and vitamins for optimal growth of any microbe to occur. A microbial population within a given environment will utilize the available nutrient components at different rates. If one of the components is missing or at low levels, lower amounts of the rest of the nutrient components will be utilized for microbial growth; microbial growth will slow down. As the humidity cell environments age, organics are added in the form of bacterial biomass and some of the cell's environment starts to change and so does the microbial population present, *Figures 20-22*.



Figures 20-22. Microbial populations in the humidity cells over time.

In the humidity cell leachates, no acidophilic microbes could be reisolated/recultured/assayed using the original acidophilic medium. After learning that the pH in the humidity cells was low and we were getting a large number of heterotrophs in the leachates of some cells, low pH heterotrophic media was used as a separate culture media at a pH 3.5. A number of low pH tolerant microorganisms were cultured that did not mirror the heterotroph populations cultured in the heterotrophic media at pH 7.

As the micro-environments change, different materials are added/subtracted to/from the different zones established within the cell's micro- and macro-environment. During the last six months of the humidity cell experiments, the cells with the most microbes would have micro-environments between the Oxic and SubOxic zones with very low to minor organic levels. SRB levels were just beginning to affect the micro-environments in a few cells and heterotrophs have been affecting mineral transformations or mobilizations in the humidity cells for months. However, measurable and noticeable transformations at the microbial levels shown in *Figures 5-7* are estimated to take weeks to months depending on the transformation/mobilization.

Boulder Weathering - Microbial Analysis

Microbial types present in the boulder samples were quite similar to the general microbial population found at the Questa site, with the exception that no SRB were isolated. In general, microbial numbers in the boulder samples were at low levels due to the dryness of the samples tested. When sufficient

moisture is present to penetrate the cracks and crevices and be present for several days, it is expected that microbial number would increase significantly. In the samples tested, heterotrophic microbes were detected in all but one boulder sample; CAP-DCJ-0002B; acidophilic microbes were present in this sample. Heterotrophic microbes were at low levels, but similar to other heterotrophic microbes found throughout the Questa site. Heterotrophic microbes ranged in concentration from ~1 to >200 microbes per 10 grams of sample, *Figure 23*.

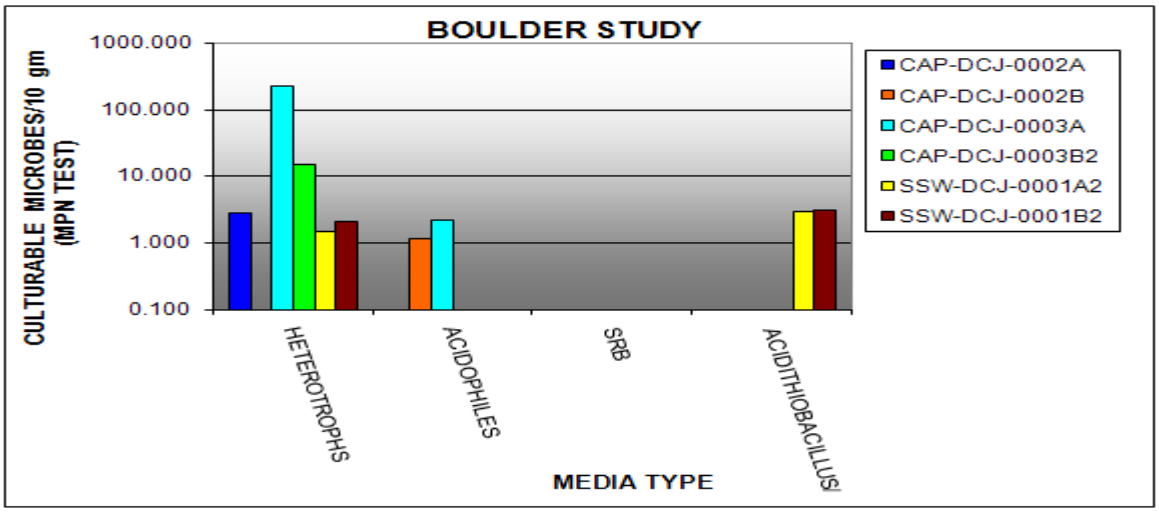


Figure 23. Results of MPN tests, conducted in triplicate, in four media types selected to enumerate specific types of culturable microorganisms present in boulder samples. Microbes are presented in numbers per 10.0 grams of sample.

Acidophilic heterotrophic microbes were found in only two samples: CAP-DCJ-0002B and CAP-DCJ-0003A ranging in concentration from ~1 to ~2 microbes per 10 grams of sample. No sulfate reducing microbes were cultured from any of the boulder samples. Acidithiobacillus/Leptospirillum sp. were found in two samples from Sugar Shack West samples; SSW-DCJ-0001A2 and SSW-DCJ-0001B2 at concentrations of ~3 microbes per 10 grams of sample. Tests with cultured microbes and boulder sample materials and controls, combined with a comprehensive chemical analysis are needed to determine what microbial reactions were possible under different conditions.

Due to the low number of samples, only generalized speculation can be made about the microbial populations found in the boulder samples. There was a relationship observed with the higher microbial counts in relationship with samples containing higher levels of key nutrient minerals; it can be speculated that in at least some samples, a nutrient component may have been limiting. It is known that all microbes utilize nutrient components, C:N:P:S:K and many others, in specific ratios to grow.

Microbial growth in micro-cracks and consumption/mobilization of materials during moisture events and growth cycles can be speculated and could be the cause of irregularities observed in boulder sample micrographs. Redox environments in micro-cracks and boulder interiors could be significantly different from on the surface and support different microbial populations. All microbes produce some form of acids during growth cycles and require minerals present in the boulder samples for life functions. Upon dying, microorganisms leave behind proteins and lipids that can act to bind and accumulate different ions than were in a crack or pit originally.

Heterotrophic and acidophilic microbes were found in the samples examined that were capable of growth over a broad pH range. Microbes and biomaterials represent organic chelators that can remove

metallic ions from rocks. Live microbes actively form microbial coatings, collect and internalize various ions, and can form accretion deposits. The types of microbes found are able to influence the pH of the micro-rock environments when present in sufficient numbers. As an example, microbes present in the thousands to tens of thousands per mL of solution can create noticeable/measurable changes in months. Microbes present in the millions to tens of millions can create noticeable/measurable changes in weeks. These actions are likely to occur during high moisture events for the boulders and would be more dynamic in micro-environments.

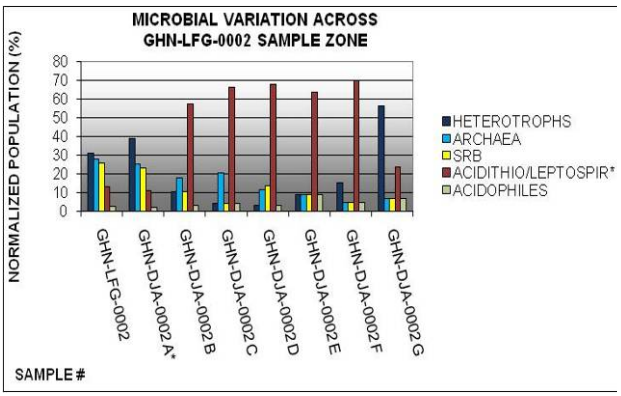
6. RELIABILITY ANALYSIS

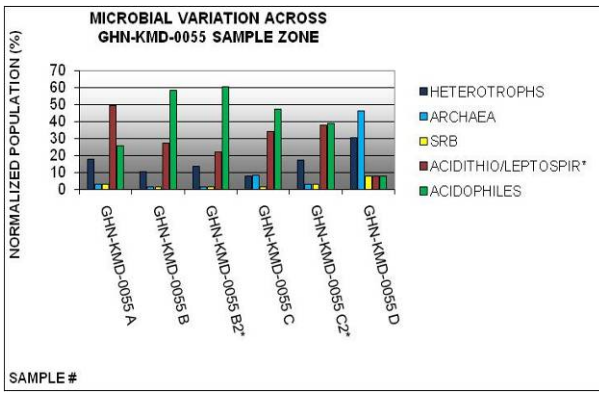
A microbial study based on only the culturable microbes can be constrained with respect to viewing the microbial diversity. This is because most microbes defy cultivation by standard methods; it is difficult to obtain a representative picture of total microbial diversity. Molecular tools and perspectives based on gene sequences were found to be problematic outside a clean room environment due to low numbers of microbes, and small amounts of extractable nucleic acid in the drier mine site environments, especially the boulder materials. In this setting and for these purposes, the culturable microbial types provide a good basis for evaluation of microbial involvement and their contribution to potential reactions involved in boulder weathering.

Past studies have indicated that *Acidithiobacillus* and *Leptospirillum sp*. would be the dominant microbes in acid generating zones and be responsible for the acid generation in these zones. In the Questa samples, *Acidithiobacillus* and *Leptospirillum* species do appear in greater numbers throughout the mine site in general and in the GHN trench samples, but their presence is not correlated with low pH samples; they are present with a number of other microbial populations that most likely interact with them in the respective environments. The Questa microbial analysis aligns with more recent studies that indicate that *Acidithiobacillus* and *Leptospirillum species* may not necessarily be the only microorganisms responsible for acid generation across the rock pile environments.

The small number of samples representing individual Questa site samples is problematic in performing any type of correlation analysis. This can be seen in samples where geological unit cross sections have been sampled, *Figures 24 & 25*. In the middle of the geological unit, the microbiology is quite consistent. However, as the edges of the geological units are approached, the microbiology varies considerably, as expected. Data and analysis reliability deficiencies are apparent with small sample sizes particularly in boulder samples, crustal materials, vent area and well samples.

An additional data area that is problematic for reliability is the way samples were collected for paired microbiology and geochemistry analysis. These samples were not samples split from one sample, but were paired samples collected in the same area. With the variation noted in *Figures 24 & 25*, this could reduce the reliability of the sample microbiology and geochemistry correlations.





Figures 24 & 25. Microbial variation in samples across geological units.

Archaea primers were obtained to attempt to confirm the presence or absence of *Archaea sp.* in selected samples; no *Archaea* were detected with the primers used. Additionally, although all commercially available extraction methods were tried, only DNA preps from cultured microbes were mostly successful. No detectable DNA was extracted from most 10 g and 20 g of soil samples directly; PCR on potentially successful extractions was unsuccessful. Some soil samples yielded substantial amounts of DNA, however, impurities were sufficient to inhibit PCR amplification of the 16S rDNA, even in the presence of the PCR inhibitor inhibitors, PVP40 and PTB. Denaturing gradient gel electrophoresis (DGGE) analysis was therefore only achieved for cultured microorganisms. Since the microbes are grown in media before the DNA is extracted, it is likely the population profile determined by the DGGE is skewed from the population found in the soil, *Figure 17*. This severely hampered the usefulness of this tool and therefore was not considered a reliable method of assessing overall microbial diversity as originally planned.

7. CURRENT CONCLUSION OF THE COMPONENT

- Microbial analysis indicates that *Acidithiobacillus* and *Leptospirillum sp*. are common across the mine site environments, but that other species can dominate specific geological zones and specific geochemical locals within these zones.
  - *Acidithiobacillus* and *Leptospirillum species* are usually accompanied by heterotrophic, acidophilic, and *Archaea species*.

- The microbial complement present is potentially capable of numerous reactions including a number of dissolution and/or cementation type reactions as well as pyrite oxidation when conditions favor these reactions.
  - Microbes present can function to contribute to slope stability under the right conditions – these conditions have not been defined.
  - Microbes present can contribute to slope instability – these conditions have been defined as being in oxygenating environments and acid production.
    - These conditions might lead to initial rock pile instability and later contribute to rock pile stability.
  - Some of the more important processes caused or influenced by microorganisms, nanobacteria, and biopolymers include:
    - Break-up of particles, by the consumption of particle materials during growth – redox reactions.

- Growth of microorganisms in micro-cracks or fractures causing particles to fracture to a greater extent.
- Chemical processes, like solution, can be enhanced by carbon dioxide produced by respiration forming carbonic acids.
- Microbially-produced organic acids and chelators that sequester minerals both on the inside and outside of their cells.
- Microbes play key roles in pyrite oxidation, resulting in sulfuric acid, which dissolves other minerals.
- Microbes can oxidize nitrogen-containing organic materials and form nitric acids
- *Acidithiobacillus sp*. can accrete protective surface crusts and the production of 2-ketogluconic acid can lead to the deposition of silica skins
- Microbes can influence the moisture regime in microcosms and therefore enhance weathering as water is a necessary component in several physical and chemical weathering processes and all known biological processes
  - Microorganisms can influence the pH of the micro rock environments through respiration and cation exchange reactions such as the exchange of basic cations for hydrogen ions.

- The GHN site has had acid generating sites in the past, there were higher acid zones at the time the trenches were sampled, the microbial profiles indicate microbial involvement in these events, and there appear to be acid generating zones within other Questa rock piles presently.
  - The GHN microbially-mediated acid producing zones were toward the outside, the oxidized portion, of the rock pile.
  - The formation of GHN acid producing zones have occurred in less than 50 years.
  - Because the general site microbial profiles are quite similar to the GHN rock pile, this indicates that the type of acid producing zones observed in GHN are occurring in other rock piles.
    - Development of microbially-mediated acid producing zones in other rock piles will require a similar pyrite/mineralogical make up, oxygen, and moisture.
  - How have the mineralogical changes within the oxidized portion of the GHN rock pile affected cohesion, shear strength, and short term stability?
  - Since there are numerous examples of shallow failures on piles is there any way to determine if these events have been associated with an acid production zone?
  - Would an acid generating zone deeper in the rock pile lead to a larger deep-seated failure event?
  - What is the size and location of an acid producing zone that would produce a 'significant' rock pile event?

- As microorganisms and microbial products participate in and accelerate many geochemical reactions within the rock pile, they use various minerals present as electron donors or terminal electron acceptors.

- Microbial materials can accelerate rock pile chemistry changes, respond to rock pile chemistry, and be a main driving component of rock pile chemistry changes.

- Biopolymers have been documented to have a very high affinity for metals in general, minerals, or specific metals.
  - Gypsum crystals may be initiated via microbial and or biopolymer involvement, as the crystallization nuclei.

- Minerals and metals removed by various mechanisms can act as precipitation nuclei and inter-chelating structures for the formation of various cementing materials.

- The formation of biomaterial structures is strongly influenced by physicochemical parameters such as ionic strength, pH and the concentration of competing organic and inorganic compounds, moisture cycle characteristics, and particle sizes.

- In rock piles, microbial populations can often be at low levels due to lack of moisture, nutrients, or other appropriate environmental conditions.

- Water is often the major limiting factor to growth and microbial activity in a rock pile environment.

- Under all circumstances, the rates of microbial activity are a direct function of individual microbial population sizes, composition, and total microbial mass.

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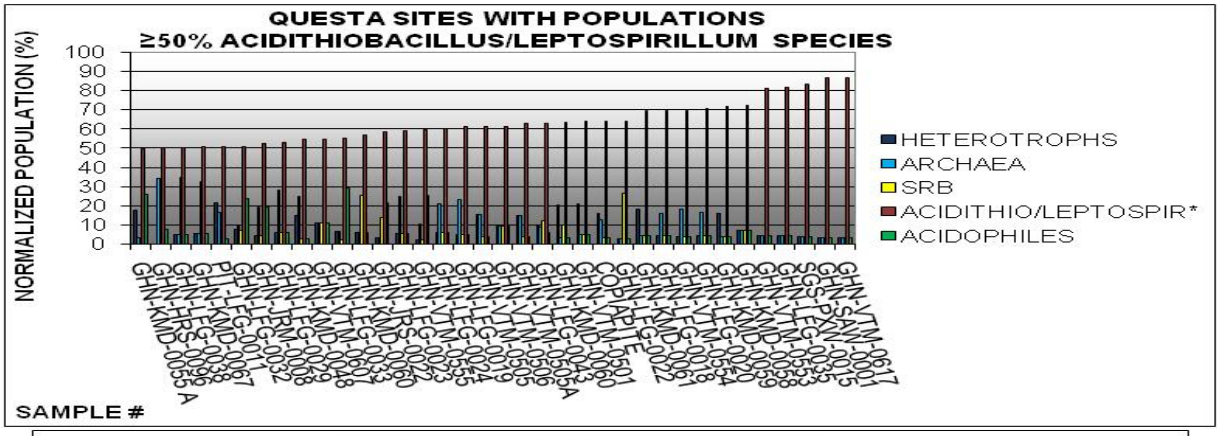
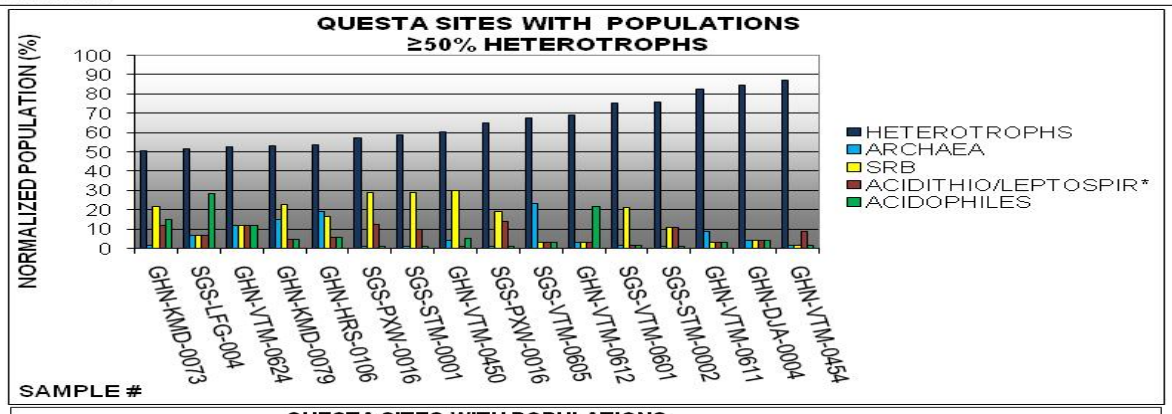
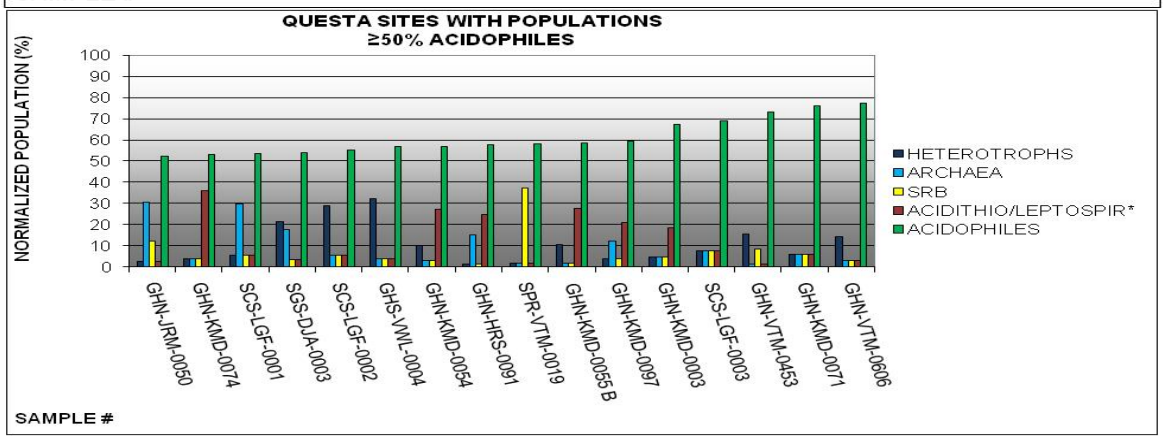
9. TECHNICAL APPENDICES

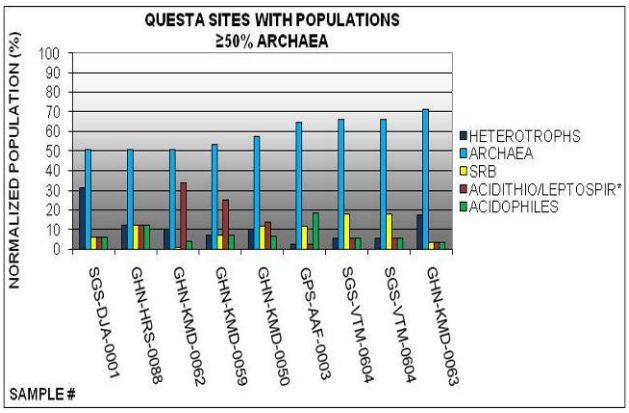
See additional site documents:

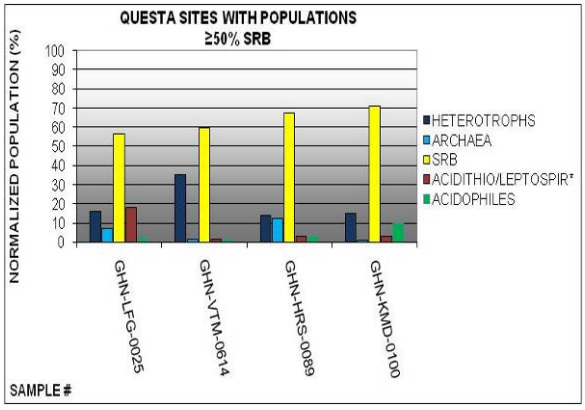
- QUESTA MINE SITE: MICROBIAL ANALYSIS
- QUESTA MINE SITE: MICROBIAL ASSOCIATIONS WITH CRUSTAL AND CEMENTED MATERIALS
- QUESTA MINE SITE: HUMIDITY CELL MICROBIAL EVALUATION
- QUESTA MINE SITE: BOULDER WEATHERING - MICROBIAL ANALYSIS



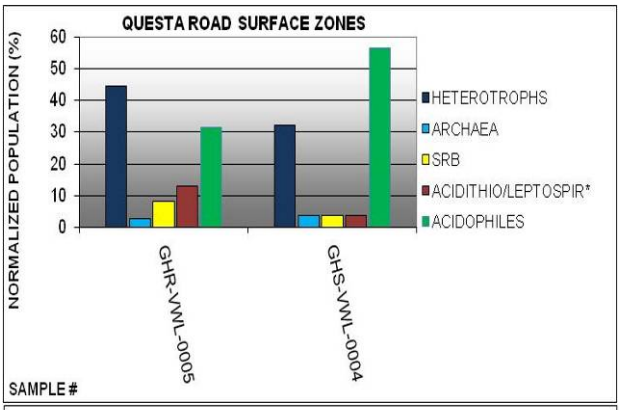


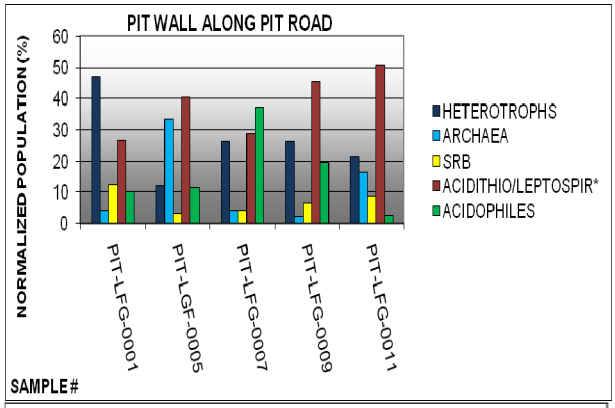


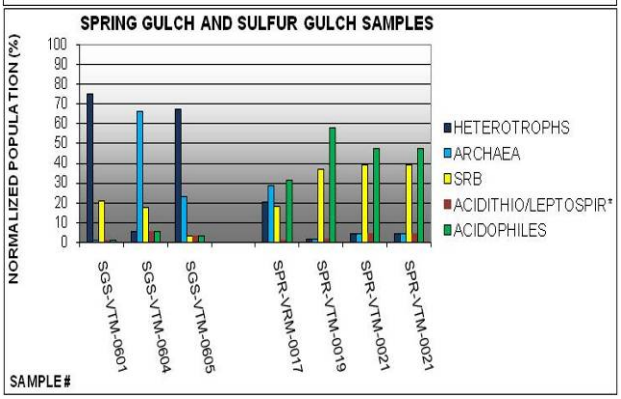


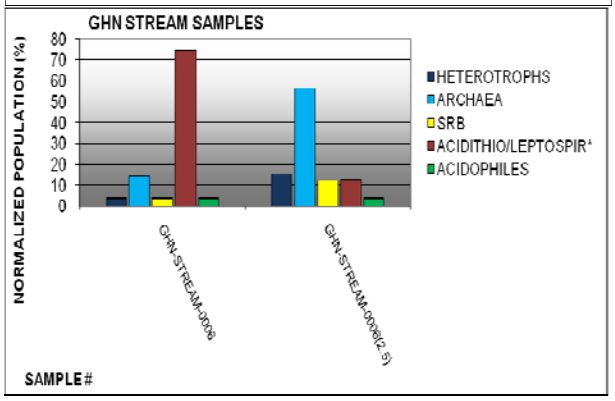


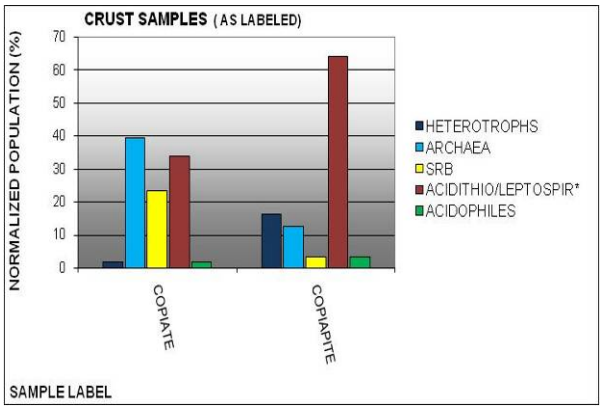
Figures 1-5. Questa site microbial profiles of samples having ≥50% of a given microbial population or metabolic type. Samples labeled with 'LETTERS' following the 10 digit sample number represent samples collected at a particular site; not split samples. * *Acidithiobacillus/Leptospirillum sp.*

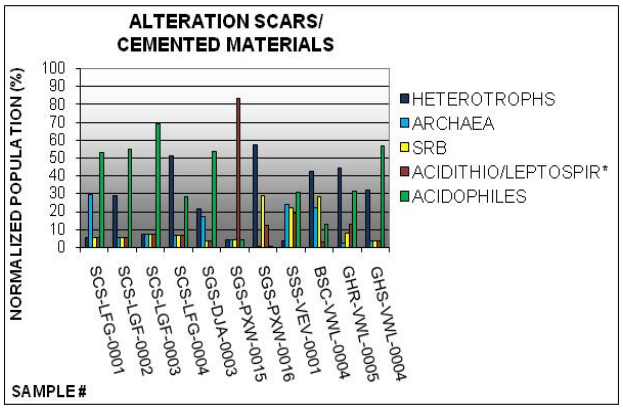












Figures 5-10. Questa site samples at various locations.