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FINAL REPORTC A BIOLOGICAL SURVEY OF THE BERKELEY PIT LAKE SYSTEM

MINE WASTE TECHNOLOGY PROGRAM ACTIVITY IV, PROJECT 10

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MINE WASTE TECHNOLOGY PROGRAM ACTIVITY IV, PROJECT 10

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Foreword

Today, industries are developing and modifying technologies to more efficiently produce their products. The waste generated by these industries, if improperly dealt with, can threaten public health and degrade the environment. The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's land, air, and water resources. Under mandate of national environmental laws, EPA strives to formulate and implement actions leading to a balance between human activities and the ability of natural systems to support and sustain life. These laws direct EPA to perform research that defines and measures the impact and search for solutions to environmental problems.

The National Risk Management Research Laboratory (NRMRL) of EPA is responsible for planning, implementing, and managing research, development, and demonstration programs to provide an authoritative, defensible engineering basis in support of the policies, programs, and regulations of EPA with respect to drinking water, wastewater, pesticides, toxic substances, solid and hazardous wastes, and Superfund-related activities. The Federal Energy Technology Center (FETC) of the U.S. Department of Energy (DOE) has responsibilities similar to NRMRL in that FETC is one of the several DOE centers responsible for planning, implementing, and managing research and development programs. In June 1991, an Interagency Agreement was signed between EPA and DOE that made funds available to support the Western Environmental Technology Office's operating contractor, MSE Technology Applications, Inc., and Montana Tech of The University of Montana for the development of the Mine Waste Technology Program (MWTP). This publication is one of the products of the research conducted by the MWTP through these two federal organizations and provides a vital communications link between the researcher and the user community.

The objectives of Activity IV, Project 10 were to determine the baseline biological community structure of the Berkeley Pit Lake and evaluate the isolated species for possible use in bioremediation. The results of this study will help in the future design of treatment processes for cleaning up large acid mine water storage lakes. This experimental test program was conducted at Montana Tech of The University of Montana and was directed by Dr. Grant Mitman (Department of Biological Sciences).

Executive Summary

Little is known about the microorganisms that are tolerant of mine waste environments in Montana. However, it is known that if heterotrophic and autotrophic organisms are properly nutrified, they can bioremediate mine waste-influenced areas as a benefit of their physiological processes. However, before any type of bioremediation of an ecosystem can begin, it is essential to gain a fundamental understanding of the components of the indigenous microbial community. Defining the baseline community structure is the first step toward understanding the interaction of the different biota and toward assessing any enhancement in biodiversity within the biotic community.

The specific research goals of this project are: 1) isolate and culture organisms from mine waste; 2) maintain the isolates in culture; 3) produce a photographic and written record of these organisms; 4) determine numerical abundance and species diversity; 5) determine the organisms' abilities to sequester metals; 6) determine the organisms' capabilities to raise pH as a result of nutrification; and 7) collect useful information that may be applied to other mine waste areas. Together, this information will begin to provide an understanding of the interactions among acid mine environments and the organisms that dwell there.

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1. Introduction

Little is known about the microorganisms found in Montana=s waters that are impacted by acid mine waste. One of the few references to algae is a simply a checklist without illustrations (Ref. 1); however, little mention is made of mine areas. The only other study of algae in Montana (mostly diatoms) was conducted by Loren Bahls,* but much of his work is unpublished (Ref. 2). Even less is known about the diversity of protistans, fungi and bacteria that inhabit mine waste sites. This area of research is quite intriguing because numerous organisms are growing in water with pH levels as low as 2 and loaded with high accumulations of dissolved metals. What is most significant is that these organisms may exhibit the potential to bioremediate contaminated sites. To begin to understand these organisms, this project investigated the species diversity, numerical importance, and the potential role of the Berkeley Pit Lake System organisms as possible solutions in bioremediation.

1.1 Background

The Berkeley Pit is part of the Mine Flooding Operable Unit of the Silver Bow Creek/Butte area Superfund site. The Berkeley Pit Lake is 542-meters deep with a lateral extent of approximately 1.8 kilometers (km) by 1.4 km across the rim. This represents roughly 1,140 billion liters of pH 2.7, metal-laden, contaminated water. It is the goal of this research to begin to gain an understanding of the microbial ecology of the Berkeley Pit Lake System, which will ultimately provide necessary data for bioremediation studies and may apply to other contaminated locales worldwide.

The primary goals of this study are to determine species diversity and numbers for organisms present in this pit lake and their potential ecological role for bioremediation of the system. Various beneficial processes occur because of algal and photosynthetic bacterial growth in aquatic habitats. These processes are important because they may affect the chemistry in a number of ways:

- These organisms are primary producers (photosynthetic organisms), and as a result of their physiology, naturally produce bicarbonate to raise the pH of acidic solutions in which they are growing.
- As algae grow, they leak excess photosynthates that, in turn, promote bacterial growth.
- These microbes play a role in the biological magnification of toxic materials (each trophic level of the food web will increase the concentration of many metals 10 times).
- Photoautotrophs oxygenate the water promoting aerobic activity.
- Photoautotrophs are important in biogeochemical cycling of carbon (C), nitrogen (N), phosphorus (P), sulfur (S), and other elements—most importantly, nitrogen fixation and sulfate reduction.
- Algal cells may directly sorb metal ions through several mechanisms that include ion exchange, complexation, and physisorption.
- Accumulation and eventual decomposition of algal biomass will increase the organic carbon component of the pit lake systems, which, in turn, promote heterotrophic growth of bacteria, fungi, and protozoans.
- Algal biotransformation or enzyme-catalyzed conversion of metals will result in less toxic organic compounds.

Heterotrophic bacteria, fungi, and protists are important because they also play key roles in microbial ecology:

- These organisms naturally raise the pH of the acidic solutions in which they are growing by various physiological processes.
- They are major consumers and decomposers in the food web.
- These microbes play a role in the biological magnification of toxic materials (each trophic level of the food web will increase the concentration of many metals 10 times).
- Heterotrophic microbes are important in biogeochemical cycling of C, N, P, S, and

*Personal communication with Loren Bahls.

other elements—most importantly in nitrogen fixation and sulfate reduction.

- Some microbes have metalothionines—detoxifying enzymes that may have a synergistic effect on the microbial community.
- Biomass accumulation and eventual decomposition will not only increase the organic carbon component of pit lake systems, but will also promote heterotrophic growth of other bacteria, fungi, and protozoans.
- Heterotrophic microbes will release bound nutrients during decomposition (the microbial loop model) (Ref. 3). These combined physiological processes of microbiota have been observed to bioremediate aquatic mine waste environments (Ref. 4). Consequently, if a mine waste site, such as the Berkeley Pit Lake System, is properly nutrified with N, P, or potassium (K), then this nutrification may cause a successional cascade of increased diversity and biomass that is coupled with an increase in pH. These beneficial processes may lead to a natural restoration process. Thus, if systems are to function correctly and to recover from pollution-induced perturbations, fundamental information both on the autotrophic and the heterotrophic components of the microbial community is essential. Defining the baseline community structure is the first step toward not only understanding the interactions of the different groups of organisms, but also assessing any improvement in biodiversity within the biotic community.

2. Objectives

This project had two principal objectives:
1) determine the baseline community structure of the Berkeley Pit Lake; and 2) evaluate the isolated species for possible use in bioremediation of the Berkeley Pit Lake System. These two goals were necessary to gain a fundamental understanding of the microbial ecology of pit lakes and evaluate the potential of these organisms as bioremediators.

3. Experimental Procedure

Integrated surface water samples were collected for phytoplankton enumeration from various locations in the Berkeley Pit Lake System. A Surface Plankton Net [10 micrometers (Fm) mesh] and a Thin Layer Water Sampler (TLWS) (Aquatic Research Instruments™) were used. Subsamples were fixed in Lugol=s Solution or 2.5% calcium–carbonate buffered glutaraldehyde and settled for enumeration. These samples were examined with a NikonJ SK-2 inverted microscope according to the method of Utermöhl (Ref. 5). Subsurface samples were collected at the photic and aphotic regions of the Berkeley Pit from the limnetic to the profundal zones by using the TLWS. Samples from as many depths as possible were examined. Profundal sediment samples were examined from samples obtained using a slide hammer coring device. In addition, subsamples at different core depths were examined from these cores. Frequencies of sampling were determined by the accessibility to the Berkeley Pit Lake System. Although sampling was sporadic, the researchers made use of whatever samples were obtained. As a part of the sampling process, measurements of pH, oxidation-reduction potential (E_H), dissolved oxygen (DO), light (Fmol@m^{-2}@s^{-1}), temperature, and chemical composition of the water were made simultaneously with the biological sampling.

3.1 Sampling Collection

The Berkeley Pit was sampled twice, once in the fall and once in the spring. The first sampling event occurred in November 1997. Water samples were collected as described below. Sediment sampling was not successful during this sampling event.

The second collection occurred from April 22 to May 28, 1998. Six samples were collected April 22, three from a shallow depth (180 feet) and three from a greater depth (890 feet). At each depth two water samples and one sediment sample were collected. From May 6B8, samples were collected with a vertical sampler at depths of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 880, 885, and 888 feet. On May 11, two grab samples were taken from a spring on the south side of the Berkeley Pit—one of spring water and one of spring/pit water. On May 28, 14 thin layer samples were collected from the surface (0B4 feet), the thermocline (10B14 feet), and near bottom (880B884 feet). On the same day, the following grab samples were collected: Northeast Spring Sediment, Northeast Spring Water, North Spring, North Side Wood Near Spring, and North Side Wood.

All sampling was conducted by the Montana Bureau of Mines and Geology. Water column samples were collected with a vertical sampler. The vertical sampler consists of a plastic tube with two doors held open at either end. When the vertical sampler is at the appropriate depth, a messenger weight is released down the line, tripping a lever allowing the two doors to securely close. Upon reaching the surface the vertical sampler contents were transferred to quart-size mason jars with no headspace. Water column samples were also collected with a TLWS. The thin layer sampler consists of a frame with 11 syringes spread over a vertical distance of 4 feet. With the syringes held closed, rubber tubing is attached to the end of each syringe. Upon reaching the chosen depth, a messenger weight is released, which triggers the release of the syringes. Syringes were capped upon return to the surface.

3.2 Media Development

A variety of media was used to culture the various organisms collected during this study. Media types are referred to in the Section 4 in connection with the particular organism cultured.

3.3 Enumeration

Enumeration of algae and bacteria and protists were made by epifluorescence microscopy. Bacteria were stained with 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI), a DNA fluorochrome, and algae were detected by use of epifluorescence of chlorophyll. All samples were prepared by passing the water through a 0.22 black polycarbonate membrane filter to concentrate them on the filter surface. Fungi were not enumerated.

4. Results and Discussion

The results and discussion are presented in three separate sections by organism types: Algae, Fungi and Yeasts, and Bacteria and Heterotrophic Protists.

4.1 Algae

4.1.1 Algal Enumeration

All algae were examined using epifluorescence and conventional light microscopy. Algae were not detectable from small samples, and thus, counts could not be made. Filtration of larger volumes of water tended to clog filters because of suspended solids [mostly iron (Fe)] in the water. These filters were placed in enrichment solutions (2X Bold=s Basal Medium) and incubated in environmental chambers at 100 FmolCm^{-2}Cs^{-2} full spectrum fluorescent light at 10EC. These enrichments yielded many of the algal isolates.

Algae were detected from plankton tows; however, since a flowmeter was unavailable, numbers could not be generated. The algae collected and identified from these samples were primarily a planktonic species of Chrysophyta-*Chromulina freiburgensis* Dofl (Figure 4-1) and *Euglena mutabilis* Schmidt (Figure 4-2). *C. freiburgensis* is certainly the dominant species of the Phytoplankton community, whereas the *E. mutabilis* is strictly benthic and was most likely stirred up from the bottom or detached from floating driftwood. As a result of this study it is difficult to speculate on the microbial ecology of the algae of the Berkeley Pit Lake System without more data (i.e., seasonal collections). Nevertheless, it can be hypothesized that *E. mutabilis* is the dominant benthic species and *C. freiburgensis* is the dominant planktonic species and together these algae are the major primary producers (organisms that use light to synthesize new organic matter from carbon dioxide) for this system.

Furthermore, these algae are likely correlated to the numbers of bacteria and other heterotrophs present in the Berkeley Pit since they may well constitute the major food source other than decomposing wood (which at best must be minimal). However, these results should be confirmed by further study.

4.1.2 Diversity of Algae

All algae in this study were derived from enrichment cultures of double strength Bold Basal Medium. In general, all algae from the Berkeley Pit were extremely fastidious; it took many months before individual species could be isolated in culture. The isolation of organisms from collected samples is an ongoing process and will continue for the course of the project. Enrichments were inoculated from photic zone waters, shoreline sediments, driftwood, plankton tows, and filtered photic zone water. Samples were subsequently incubated in PercivalJ 36L Environmental Chambers at 100FmolCm^{-2}Cs^{-2} full spectrum fluorescent light at 10°C and 20°C. The species in Figures 4-1 through 4-6 were isolated. Many have never before been isolated from acid mine waste waters. Figures 4-1 through 4-6 show illustrations of the six algae isolated from the Berkeley Pit Lake System with relevant remarks regarding these isolates.

4.1.3 Experimentation

A preliminary bioremediation experiment was completed (see Table 4-1). A set of eight 1-liter aspirator bottles containing unfiltered or unsterilized Berkeley Pit water was placed in a culture chamber at a photofluence rate of 100 Fmol@m^{-2}@s^{-1}. Four of these bottles were aerated and four were nonaerated. In each set of four, one bottle was treated with a basic nutrient addition (double strength Bold Basal Medium), one was supplied with 10 grams per liter (g/L) organic carbon (rice), one was supplied with both organic carbon and nutrients, and the final bottle had no additions (control). Consequently, *Chromulina freiburgensis* Dofl. was isolated from Berkeley Pit Lake water that was supplied with nutrients and aeration. This alga has been grown to a maximum standing crop of 1.0375 x 10^7 cells/mL (cell size. 10 Fm^3). This is an incredible biomass; cell cultures were so dense that it was impossible to see through a 1-liter aspirator bottle. This abundant production of organic carbon has the potential to stimulate heterotrophic growth, which has the potential to raise pH. Furthermore, this degree of biomass production has the potential to bioremediate metals on its own through adsorption, absorption, oxygenation, or other processes.

Explanation of Results
This was primarily an enrichment experiment; thus, it had none of the proper controls or variables. However, some interesting data were generated. Bacterial populations increased at the end of the 99-day experiment. If the overall growth rates for bacteria are observed (positive effects seem to be algal growth, bubbling, and rice), it does not appear that nutrients have an effect on growth.

Rice (Organic Carbon) Increase in Bacterial Growth
When comparing bottles 1 and 2, the only difference is the addition of rice (organic carbon in the form of starch) to bottle 2. Algal growth is roughly the same; however, with the addition of rice, bacterial growth increases by 4.67×10^7. This is confirmed when comparing bottles 7 and 8 (note, there is no algal growth); the same decrease is observed between the two specimens. In fact, without algae, bacterial counts drop below the starting population (decline).

Aeration Increase in Algal Growth Bacterial Growth—Inconclusive
When bottles 1–4 are compared with bottles 5–8, more algal growth with aeration was seen in bottle 5 ($5,416 \times 10^6$) and also bottle 6 (8.33×10^5). It appears nutrients are essential for algal growth and aeration helps considerably [most likely due to carbon dioxide (CO_2), which may explain why results from bottle 5 were better than those from bottle 6]. The bacterial and algae have a synergistic relationship (each produces what the other needs). Algae produce organic carbon and oxygen and use CO_2, whereas bacteria use oxygen and organic carbon and produce carbon dioxide.

Algal Growth-Increase in Bacterial Growth
In nearly every case (excluding bottle 6) where there is algal growth, bacterial growth also increases. Why bottle 6 does not comply cannot be readily explained and may be an anomalous result (replicates are needed).

Metal Reduction
Unfortunately, evaporation occurred during the course of this experiment; volume losses were 200 mL for nonbubbled water and 400 mL for bubbled water. Taking this fact into account, metal reductions by bacteria and algae in Berkeley Pit water may be dramatic; however, once again, a proper experiment must be conducted to explore this hypothesis. No supported conclusions can be drawn at this time. However, it can be hypothesized that metal reductions will occur, and many of the positive benefits of autotrophic and heterotrophic growth (as outlined in the introduction) did occur in these bottles.

The above experiment should be repeated using an experimental matrix with appropriate controls, replicates, and variables. The objective of such studies would be to determine minimum nutrient additions necessary for growth, growth rates, potential to activate remediation of Berkeley Pit sediment and bioremediative potential. This same baseline matrix should also be used to evaluate other algal and bacterial candidates for bioremediation of Berkeley Pit waters.

4.2 Sulfate Reducing Bacterias

The original focus of this study was the cultivation, isolation, and identification of sulfate-reducing bacteria (SRB). Thus, the first growth media used were specifically for SRB: Postgate C medium and SRB Enrichment medium. Many variations on the original Postgate C medium were used. Concentrations of carbon sources were decreased to closely resemble the low nutrient level of the Berkeley Pit waters, and different carbon sources were used. Instead of 6 milligrams (mg) of lactic acid, 1 mg was used. Acetic, propionic, and butyric acid were substituted for lactic acid; all four acids were used together at a total concentration of 1 mg/L. Postgate C medium was made both as a liquid (as directed) and as a solid with the addition of Bacto-Agar.

The Postgate E medium and the SRB enumeration medium were prepared as directed. Several media were made with filtered sterilized Berkeley Pit water to arrive at a medium more closely resembling the Berkeley Pit water. Media made with Berkeley Pit water include Bacto-Agar, Nutrient Agar, and Postgate C. A *Thiobacillus* medium (ATCC Medium 125) was made for the cultivation of the acidophilic

Thiobacillus organism.

4.2.1 Equipment

To hold liquid media, 20 mL and 40 mL U.S. Environmental Protection Agency (EPA) vials with silica septa were used. Solid media was poured into disposable petri dishes or test tubes. Inoculations or isolation streaks were made with disposable loops. Inoculation of all liquid media was done with syringes and size 18 needles. Inoculation of petri dishes was done with either syringes or pipettes. Anaerobic conditions were maintained with anaerobic jars. Jars were made anaerobic by inserting BBL Anaerobic Gas Packs or by flooding the jar with filtered nitrogen gas. Working anaerobic conditions were maintained in a custom-made glovebox under positive pressure. Autoclaving was done in the manual Market Forge Sterilmatic™ autoclave or in the computerized Amsco Scientific™ SG-120 autoclave. Microscopy was done with the Nikon™ 800 Eclipse. Fluorescent dyes used for bacterial counts of sediment samples were DAPI and Molecular Probes Live/Dead *Bac*Light.

4.2.2 Methods

Petri dishes were inoculated with liquid samples by adding 1 mL of liquid to the agar surface and tilting the dish to evenly distribute liquid over the surface of the agar. Vials were inoculated by first disinfecting the septa with alcohol and then injecting 1 mL of sample. Fluorescent dyes, to stain bacteria, were used as directed by the manufacturers.

Isolation was attempted using three approaches: streaking, dilution, and solidification/entrapment. Streaking involved streaking a loopful of culture continuously back and forth across the agar surface in a perti dish. Dilution involved adding a loopful of culture to either sterile water purged with filtered nitrogen or to a liquid medium. A fraction of the inoculated liquid was then withdrawn and added to another vial of liquid. This was repeated until sufficient dilution was achieved. A fraction of each dilution was then withdrawn and used to inoculate solid medium in a petri dish. Solidification/entrapment included allowing a solid medium to cool after being removed from the autoclave. Prior to solidification of the medium, it was poured into test tubes, inoculated, and mixed with a vortex spinner. Upon solidification of the medium, paraffin was poured on top of the wax to seal the medium, make it anaerobic, and prevent contamination.

4.2.3 Results

Bacterial Counts

Bacterial counts of the sediment samples failed because of an inability to stain the samples. However, bacterial counts of the water column proved successful. Surface water counts are from the December 1997 samples of Berkeley Pit water. Depth counts (180 and 890 feet) are from May 1998 Berkeley Pit water sampling event. Although counts varied greatly, the generalization can be made of the water column that the bacterial population is greater at the surface (see Figure 4-7).

Culture, Isolation, and Identification of Microorganisms

Bacteria and fungi cultured well. Bacteria did best in the complex SRB medium. Little success was achieved with the simplest of media—the Berkeley Pit water and Bacto-agar. No growth was evident in the *Thiobacillus* medium.

Fungi was isolated easily with the streaking method. Nineteen molds and one yeast were isolated. Bacterial isolation was attempted using all three isolation methods on all previously mentioned media. All bacterial isolation attempts failed. After an isolation attempt, the results have been one of two possibilities—no bacteria or a mixed culture.

Five samples, four molds, and one yeast were sent to Microbial ID, Inc. (MIDI) Laboratories for identification using fatty acid analysis (see Appendix A). The yeast was identified as *Candida famata*, and two of the molds as *Exophiala moniliae* and *Botrytis spp*. The other two molds could not be identified and may be new organisms.

4.2.4 Discussion

Fungi cultured and isolated easily; however, nothing can be said of their activity in the Berkeley Pit water. From the samples gathered, it was discovered that the Berkeley Pit is not the best environment for SRB.

In none of the samples collected was the E_H below 650 millivolts (mV) or the pH above 3.5. Optimum SRB growth occurs at a near-neutral pH and an E_H of roughly B200 mV. While SRB growth has not been confirmed through isolation, SRB are suspected to be present based upon inspection with a microscope. If SRB are cultured from the Berkeley Pit, they may be present as a dormant cell or as an active member of a microbial community.

While bacteria other than SRB have cultured well, they have been resistant to isolation. There are several possible reasons for this lack of isolation. Although technique may have been a problem when the project began 1 year ago, it is not seen as cause for current isolation problems.

The most likely possibility is the bacteria are simply very fastidious. While they seem to culture well, the further they are removed from their native environment during the process of isolation, the worse they do. This is tied to the possibility that the correct medium may not have been employed. SRB media were originally used because SRB are the main bacteria of interest. The use of these media was continued because it appeared to work well culturing bacteria. Different media may be needed for successful isolation of the particular type of bacteria present in culture isolated from Berkeley Pit Lake water.

Another possibility is that some of the bacteria are very motile. While viewing mixed cultures under the microscope, several bacteria have been witnessed to be highly motile. This may explain why some isolation attempts have resulted in a mixed culture.

Another possibility is that syntropy is occurring. Syntropy is when one bacteria's byproduct is used by another species to survive; thus, isolation would kill the latter bacterial strain. Again, this is unlikely, but if proven, it would be a discovery in itself.

The possible motility problem would be dealt with effectively through the solidification/entrapment method. This method was recently initiated and therefore, results are not yet available.

Bacterial counts from the water column are low. A normal count from a neutral water source is near 1 million. The low numbers found in the Berkeley Pit are most likely a result of the carbon source limited nature of the Berkeley Pit.

Bacterial counts of the sediment samples failed. The two fluorescent dyes used seemed to have a greater affinity for the sediment than the bacteria. In switching between brightfield and fluorescent microscopy, it was evident that the bacteria were not stained but that instead it was the sediment particles were fluorescing.

4.2.5 Recommendations

Continued research must take place on the isolation and identification of Berkeley Pit Lake bacteria. This should consist of continued testing of different media until one is found that isolates bacteria well. Prior to the counts, a reliable counting method must be found and tested. After a rigorous counting method has been developed, sediment bacterial counts should be performed.

4.2.6 Conclusion

Fungi have cultured and isolated well, resulting in 19 isolates. Three of the isolates have been identified: *Candida famata*, *Exophiala moniliae*, and *Botrytis spp*. Little can be said about the activity of the fungi in situ.

While bacterial counts have been successful for the water column, a method must still be found for performing a count on sediment bacteria. Research is underway to determine a methodology for staining bacteria in sediments. This first step is to use different chemical fixatives and different fluorescent dyes. Bacteria have cultured well but have resisted all attempts at isolation. The two most likely explanations for this are fastidious bacteria and motile bacteria. Bacterial counts of the Berkeley Pit water column are at least one order of magnitude below what exists in a fresh water lake; the low population is most likely a result of minimal carbon source availability.

It should be stressed that these results should be seen as a preliminary due to the difficulty of isolating bacteria. This study is involved with a topic of

which truly nothing is known—the microbiology of the Berkeley Pit Lake. The lack of previous work provides no foundation from which to build upon. Work will continue on bacterial isolation and identification until April 1999. When bacteria isolation and identification are successful, an updated report will be submitted.

4.3 Bacteria and Heterotropic Protists

A complete understanding of the dynamics of acid mine aquatic sites requires knowledge of the interaction of the physical, chemical, and biotic components of the system. The sum of biochemical mechanisms resulting in acid generation is poorly understood; however, the important microbial involvement (whereby metal sulfides are oxidized by groups of bacteria to form sulfuric acid) is readily acknowledged. Far less is known about the role of the protists, which are the only other life forms inhabiting such extreme sites.

Protists are single-celled eukaryotes that can be classed as either autotrophs (the algae) or heterotrophs (the protozoa) on the basis of their nutrition. Both types of microbes influence the activity of the bacterial community. Algae release oxygen and stimulate bacterial degradation by providing oxygen-rich zones. Consequently, algae are often used to oxygenate wastes being broken down by bacteria. Conversely, protozoa are the main consumers of bacteria. Studies on the microbial degradation of petroleum hydrocarbons have shown that breakdown rates are markedly improved in the presence of grazing protozoa, presumably because their cropping action maintains the bacteria in a healthy exponential growth phase.

Other potential biotic interactions that may apply at acid sites are less clear. The primary producers (i.e., the algae) produce bicarbonate as a result of photosynthetic activity. This may be able to raise the pH of the surrounding waters and counteract acidification. Photosynthetic organisms also leak excess photosynthate (dissolved organic carbon) into surrounding waters and fuel the heterotorophic microbial populations. This enhanced activity is important in the regeneration of nutrients and in the flow of carbon through the simple food web. Some biota have the ability to sorb metal ions while others contain special detoxifying enzymes (metalothionines) to enable them to withstand extreme conditions. Such organisms may have a beneficial effect on the entire microbial assemblage.

This project was designed to provide preliminary information about the biotic components of the Berkeley Pit Lake in terms of the abundance and types of indigenous organisms. As noted above, the activity of aquatic biota is essential for controlling ecosystem function particularly concerning the cycling of nutrients and carbon. However, their activity at anthropogenically contaminated sites, such as the Berkeley Pit, may have particular significance in terms of either maintaining acidity or in helping to define future bioremediation options.

4.3.1 Methods

Enumeration of Bacteria

All bacterial counts were conducted by epifluorescence microscopy (a direct counting method). After staining bacteria with the deoxyribonucleic acid (DNA)-specific fluorochrome DAPI, water samples were passed through a 0.22-Fm black membrane filter to capture bacteria on the filter surface. Because of the flocculent nature of the samples, only a few milliliters of water could be filtered (typically between 2 and 5 mL). Using ultraviolet (UV) illumination, the number of bacteria in each sample was estimated by counting cells in 30 random fields of view.

In addition to the total bacterial count, samples were scanned for the presence of cyanobacteria that, because of their photosynthetic pigments, fluoresce orange when excited by blue light. All samples collected (see Appendices B-E for dates) were fixed immediately in 5% glutaraldehyde. Samples were transported to the South Dakota School of Mines and Technology (SDSM&T) for subsequent processing.

Diversity of Heterotrophic Protists

The presence of heterotrophic protists and information on their diversity (i.e., number of different morphotypes) was determined by cultivation methods. No one culture method was

appropriate for all protists; hence, many different culture trials were set up and examined regularly for the appearance of protistan populations. The most appropriate medium was found to be acidified soil extract medium (SEM) made as follows:

- E (soil extract)[†]	10 mL
- K_2HPO_4, 0.1% weight/volume (w/v)	2 mL
- $MgSO_4.7H_2O$, 0.1% w/v	2 mL
- KNO_3, 1.0% w/v	2 mL
- Glass distilled water	84 mL

After autoclaving, the medium was adjusted to pH 3.0 with sulfuric acid.

Two other media used were 1) filtered water collected from the Berkeley Pit and 2) acidified amoeba saline (AS) made from the following stock solutions:

- NaCl	1.20 g in 100 mL distilled water
- $MgSO_4.7H_2O$	0.04 g in 100 mL distilled water
- $CaCl_2.2H_2O$	0.04 g in 100 mL distilled water
- Na_2HPO_4	1.42 g in 100 mL distilled water
- KH_2PO_4	1.36 g in 100 mL distilled water

The final dilution was made by adding 100 mL of each stock solution to distilled water to make 1 liter.

Samples were collected from the Berkeley Pit and sent overnight to the SDSM&T for processing. Aliquots of sample were added to culture dishes containing one of the following media:

- SEM
- SEM diluted with AS
- AS with two sterile rice grains
- Filtered Berkeley Pit water with two sterile rice grains

Locations and dates of sample collection were as follows:
November 1997

B Surface plankton sample (0B1 feet)
B Scrapings from floating log
B Shore sample (sediment)
B Bottom sediment sample (from core)
B Plankton tow

December 1997

B Pooled plankton sample (top 40 feet)

April 1998

B AShallow@ plankton sample—180 feet
B ADeep@ plankton sample—890 feet

On all sampling occasions, multiple cultures were established (at least 10 replicate dishes) and incubated at 20°C in the dark. Cultures were examined every week for up to 2 months, at which time cultures were subcultured into new petri dishes with fresh medium. All examinations were made using an OlympusJ IX70 inverted microscope at 400x or 600x magnification using either phase contrast optics or Nomarski optics.

Enumeration of Protists
Nanoplankton (cells 2 Fm – 20 Fm). Flagellates and amoebae within this range were counted by epifluorescence microscopy. After bacteria were enumerated, the filter was scanned at a lower magnification (600x) for the presence of DAPI-stained protists. In all cases, 100 random fields of view were searched.

Microplankton (cells 20 Fm – 200 Fm). Protozoa in this size range were counted in settling chambers. Volumes of water (10 to 500 mL) were settled after fixing cells in Lugol=s iodine (enough fixative was added to impart a pale yellow color to samples). Settled material was transferred to a settling chamber and examined by inverted microscopy.

4.3.2 Results

Enumeration of Bacteria

[†]Soil extract was prepared by autoclaving garden soil in tap water (1 part: 4 parts) for 1 h. After settling for at least 1 week, the supernatant was decanted and filtered through a glass fiber filter. The stock was stored frozen until needed.

Bacteria were always present in samples collected from the Berkeley Pit, regardless of the depth of water or time of year. The first samples processed (those from November 1997) showed that bacteria in the water were extremely patchy in terms of their spatial distribution. These initial samples were only shaken gently before filtering and the counts per field of view showed considerable variation. For example, in the surface sample (see data in Appendix B), bacterial numbers ranged from 0 to 121 per single field of view on the filter surface. It was evident that this spatial patchiness was due to bacteria associating with floc particles in the water. Microscopy showed that many of the aggregations were devoid of bacteria but others were heavily colonized. Samples collected thereafter were shaken vigorously before processing to obtain an improved average count on the filter surface. Tables 4-2 and 4-3 show the results for bacterial counts in the surface waters of the Berkeley Pit (raw data, Appendices 1 and 2). Overall, the mean number of bacteria in the surface waters was 116,127 bacteria mL^{-1} to a depth of 39 feet (sampling interval). There was considerable scatter in the data throughout the sampling interval (Figure 4-8). One data set, at 18 feet, was approximately 5 times higher (476,020 bacteria mL^{B1}) than the levels of bacteria seen throughout the rest of the surface water column. There was no apparent explanation for this although at this depth the pH was low at 2.75 (Figure 4-9, Table 4-4).

The data presented in Table 4-5 compares replicate samples collected at two depths—180 feet and 890 feet. At the shallower site, the mean density was 7,645 bacteria mL^{-1} (95% confidence limit 5,813). At the deeper site, the mean density was similar at 7,278 bacteria mL^{-1} (95% confidence limit 1,559). Comparing all data collected over the winter months suggests that the surface layers supported higher levels of bacteria compared to deeper sites (Figure 4-10). On the other hand, the data presented in Table 4-6 suggests uniformity in bacterial density with depth; surface waters (top 4 feet) averaged 20,479 bacteria mL^{-1} (95% confidence limit 17,172), the thermocline region averaged 18,375 bacteria mL^{-1} (95% confidence limit 18,999) and the bottom samples averaged 38,213 bacteria mL^{-1} (95% confidence limit 26,527). However, these samples were all from June 1998, after the water column had turned over.

Attempts to enumerate bacteria in the sediment samples were unsuccessful. The sediment/floc particles on the filter surface also fluoresced and masked the presence of any fluorescing bacteria.

Enumeration of Cyanobacteria and Protists

Examination of over 50 water samples failed to reveal the presence of any cyanobacteria. Likewise, heterotrophic nanoplankton were rare; cells presumed to be nanoflagellates were only seen on four occasions suggesting that small protists number less than 4 mL^{-1}. This estimate is based on the total area of filter scanned over the entire study and takes no account of location in the water column. These numbers are very low since heterotrophic flagellates typically number 1,000 mL^{-1} in undisturbed freshwater lakes and coastal waters. On slightly more occasions, cyst-like structures were observed on the filters (approximately 0.5 per 100 fields of view corresponding to approximately 25 mL^{-1} of water sample). These were not counted formally since DAPI-staining does not reliably stain cysts. However, this observation does suggests that the bulk of the protistan population was in a dormant, encysted state.

Attempts to count larger protist (microplankton) in the settling chambers were unsuccessful. The dense floc material masked the presence of any protists. Settling smaller volumes of material failed to show any protists, suggesting that like the nanoplankton, they were present (since they grew in culture) but rare in the Berkeley Pit system.

Diversity of Protists

Inoculating cultures with 2 mL of Berkeley Pit water always yielded protistan cultures. No one set of samples (i.e., from a specific location) yielded unique morphotypes. This suggests that many of the cultures were derived from cysts that were distributed throughout the water column. Likewise, no one type of media stimulated the growth of a specific isolate. Generally, most isolates appeared briefly in culture either initially (after a few days) or when cultures were old (after 2 months). In short, strains of acidophilic protozoa were fastidious and more work is needed to define appropriate methods

for the long-term cultivation of most of the strains encountered.

The November 1997 samples were also inoculated into anaerobic bottles containing the various media given above. These were examined monthly for the presence of active protozoa. Active protozoa were observed once under these culture conditions (i.e., the bodonic flagellate, morphotype 13). Since this isolate was also capable of growth in petri dishes, it was probably a microaerophilic protist. The anaerobic bottles, although capped and sealed, were not degassed before inoculation and did not provide true anaerobic conditions.

Over the entire study, 16 different morphotypes (species) were identified. At this time, these isolates are best referred to as morphotypes since species designations are not given. In the case of protozoa, the establishment of pure cultures with subsequent electron microscopy or specific staining methods for light microscopy is required for accurate identification. Where possible, the most probable genus has been indicated. However, all identifications are tentative since it is likely that most of the isolates were new to science. Extreme acidophilic protozoa (to this author's knowledge) have not previously been reported. Figures 4-11 to 4-26 show illustrations of the 16 morphotypes found in the Berkeley Pit with relevant remarks about these isolates.

Table 4-1. Aspirator Bottle Experimental Results

Date	1-B,N,R	2-B,N,nR	3-B,nN,R	4-B,nN,nR	5-nB,N,R	6-nB,N,nR	7-nB,nN,R	8-controll
pH Measurements								
20Jul98	2.57	2.57	2.69	2.67	2.59	2.58	2.68	2.62
27Jul98	1.88	1.89	2.17	2.02	1.78	1.81	2.03	2.02
03Aug98	2.25	2.45	2.56	2.55	2.41	2.40	2.57	2.55
17Aug98	2.46	2.47	2.65	2.66	2.54	2.50	2.65	2.66
24Aug98	2.84	2.70	2.78	2.75	2.55	2.55	2.69	2.69
31Aug98	2.35	2.40	2.58	2.58	2.54	2.46	2.62	2.63
08Sep98	2.43	2.45	2.61	2.60	2.40	2.43	2.59	2.62
13Sep98	2.50	2.52	2.65	2.66	2.52	2.48	2.67	2.67
21Sep98	2.08	2.10	2.22	2.22	2.06	2.04	2.25	2.24
28Sep98	1.98	2.05	2.16	2.15	1.85	1.90	2.15	2.15
05Oct98	2.06	2.01	2.10	2.08	1.97	2.05	2.25	2.24
12Oct98	2.22	2.28	2.37	2.37	2.07	2.11	2.33	2.35
19Oct98	2.33	2.39	2.45	2.45	2.23	2.28	2.48	2.48
Bacterial Counts (Number of Bacteria/mL)								
20Jul98	11.2E6	11.2E6	11.2E6	11.2E6	11.2E6	11.2E6	11.2E6	11.2E6
26Oct98	71.2E6	24.6E6	15.8E6	5.8E6	35.8E6	1.3E6	25.4E6	1.2E6
Algal Counts (Number of Algae/mL)								
26Oct98	10E6	10.4E6	0	0	5.4E6	.8E6	0	0

B – bubble; N B nutrients; R B rice; nB B no bubble; nN B no nutrients; NR B no rice

Table 4-2. Number of Bacteria (mL^{-1}) in Preliminary Samples Collected 21 November 1997[H]

Location	Mean	Standard Error (n=30)
Surface	53,436	19,028
Surface water with floating log	19,908	4,389

[H] Raw Data, See Appendix B—Counts Based on 30 Random Fields of View on Filter Surface

Table 4-3. Number of Bacteria (mL^{-1}) as a Function of Depth in the Water (Feet)[H]

Location	Mean	Standard Error (n=30)
surface	36,540	6,714
1 ft	152,765	18,234
2 ft	40,386	5,661
3 ft	205,693	11,642
4 ft	80,856	1,358
5 ft	55,771	11,489
6 ft	8,362	1,877
9 ft	99,753	6,937
12 ft	227,684	15,319
15 ft	64,634	13,381
18 ft	476,020	18,005
21 ft	71,323	7,019
24 ft	186,211	19,347
27 ft	53,764	6,515
30 ft	72,996	9,719
32 ft	23,998	4,272
33 ft	85,036	16,387
36 ft	190,057	15,258
39 ft	190,642	11,871

[H]Samples from December 1997. Raw Data, See Appendix C.

Table 4-4. Physical/Chemical Data for the Water Column in December 1997

Sample Number	Depth(ft)	pH	SC(Ms)/CM at 20EC	E_H(mV)	DO(%O_2)	T(C)
1	1	3.19	7.43		3.1	4.2
2	2	2.81	7.6	454	7	4.2
3	3	2.74	7.71	462	7	3.7
4	4	2.95	7.76	456	12	3.6
5	5	3.27	7.76	458	0	3.6
6	6	3.04	7.77	458	0	3.4
7	9	3.0	7.8	458	0	3.6
8	12	2.94	7.77	457	0	3.8
9	15	2.85	7.8	457	0	3.8
10	18	2.75	8.28	457	0	3.8
11	21	2.90	7.77	457	0	3.6
12	24	2.84	7.8	455	0	4.1
13	27	2.73	7.77	449	0	4.4
14	30	2.87	7.8	446	0	4.6
15	32	3.12	7.94	431	0	5.1
16	33	2.95	8.19	413	0	5.1
17	36	3.31	8.21	411	0	5.3
18	39	2.94	8.21	409	0	5.8

Table 4-5. Comparison of Number of Bacteria at 2 Depths—180 ft and 890 ft[H]

Location	Mean	Standard Error (n=30)
180 ft	3,779	732
180 ft	7,258	1,428
180 ft	18,981	3,967
180 ft	4,766	1,114
180 ft	3,445	873
890 ft	7,247	1,078
890 ft	5,142	1,366
890 ft	8,796	1,593
890 ft	7,927	2,026

[H]Samples are replicates at these two depths. Raw data given in Appendix 3.

Table 4-6. Comparison of Bacterial Numbers (mL^{-1}) at the Surface, Thermocline and Pit Bottom. Counts at Each Location Span a Depth of 4 Feet (i.e., Samples 1 To 11 Equally Spaced Over This Distance). Raw Data in Appendix E.

Location	Mean	Standard Error (n=30)
Water Samples:		
surface 1	7,418	2,707
surface 2	4,496	1,682
surface 3		no sample
surface 4	26,299	4,717
surface 5	20,230	3,774
surface 6	34,391	6,809
surface 7	1,573	1,025
surface 8	2,922	1,436
surface 9	83,169	2,009
surface 10	3,821	1,887
surface 11		no sample
thermocline 1	1,573	1,025
thermocline 2	20,230	3,938
thermocline 3		no sample
thermocline 4		no sample
thermocline 5	44956	8,901
thermocline 6		no sample
thermocline 7		no sample
thermocline 8		no sample
thermocline 9	6,743	2,174

Table 4-6. Comparison of Bacterial Numbers (mL^{-1}) at the Surface, Thermocline and Pit Bottom. Counts at Each Location Span a Depth of 4 Feet (i.e., Samples 1 To 11 Equally Spaced Over This Distance). Raw Data in Appendix E.

Location	Mean	Standard Error (n=30)
Water Samples:		
thermocline 10	no sample	
thermocline 11	no sample	
pit bottom 1	20,905	3,815
pit bottom 2	26,974	4,748
pit bottom 3	44,956	3,240
pit bottom 4	55,296	5,989
pit bottom 5	35,290	9,844
pit bottom 6	165,663	9,106
pit bottom 7	22,478	4,799
pit bottom 8	10,565	3,199
pit bottom 9	6,743	3,076
pit bottom 10	6,743	2,666
pit bottom 11	24,726	4,840
Grab Surface Samples:		
Wood North	2,248	1,272
Wood North Spring	4,496	1,682
North Spring	27,648	3,363
NE Spring/ sediment near shore	93,733	7,547

Figure 4-1 shows a species is a member of the Chrysophyta.

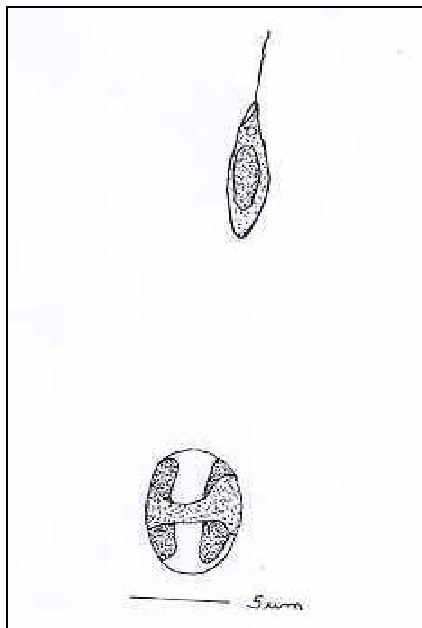

Figure 4-1. ***Chromulina freibugensis*** **Dofl.**

The cells most commonly found in our enrichment cultures were like the lower form of Figure 4-1. These cells were somewhat oval in size varying from approximately 4 to 8 Fm in width and 7 to 15 Fm in length. The chloroplast was yellow brown in color with a greenish rim at the membrane border and channel shaped with two lateral lobes connected by a bridge. No stigma was present. This cell was grown up to numbers exceeding 1 x 10^7 cells/mL in culture.

The upper form of *Chromulina freiburgensis* as seen on Figure 4-1 was seen rarely in culture, but was seen repeatedly from plankton tows. This cell was typically much smaller (2 to 3 Fm in width and 4 to 5 Fm in length), excluding flagellum. The flagellum was 3 to 5 Fm in length and originated from the apex of the cell from a small opening. The flagellum was quite active and no stigma was present. A contractile vacuole was noted at the front end of the cell near the flagellum base. Leukosin droplets appeared to be present throughout the cell.

Figure 4-2 shows a diatom species, which is a member of the Bacillariophyta. Cells were isolated by enrichment and cultures from driftwood scrapings. The diatom valve linear and rostrate are 15 to 20 Fm in length and 5 to 7 Fm in width and have cuneiform ends with a narrow axial area. Striae are convergent at the ends with a broad transverse central area-these are key characteristics for the species [valve view (left) and girdle view (right)].

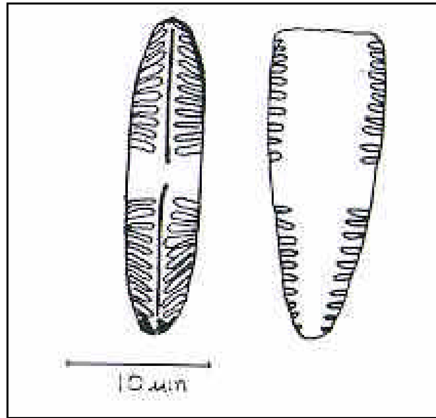

Figure 4-2. ***Pinnularia obscura*** **Krasske var. obscura.**

Figure 4-3 shows unicellular alga, which is a member of the division Chlorophyta. Cells were isolated by enrichment cultures from filtered plankton tows. Unicells occurred solitary or in aggregates in culture. The chloroplast is a parietal cup with a pyrenoid. Reproduction was observed in culture by four daughter cells produced from the protoplast of the parent cell. The cells ranged from 5 to 15 Fm in diameter.

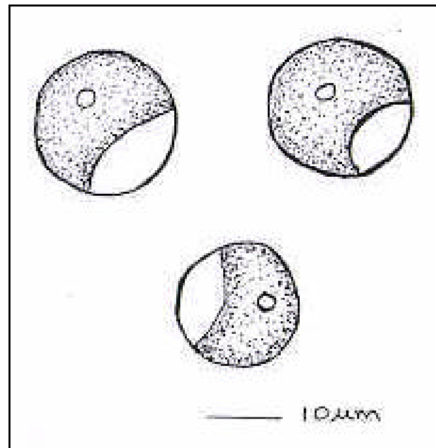

Figure 4-3. ***Chlorella vulgaris*** **Beyerinck.**

Figure 4-4 shows a unicellular alga that is a member of the division Chlorophyta. Cells were isolated by enrichment cultures from filtered plankton tows. Unicells occurred solitary or in aggregates in culture. The chloroplast is a folded plate with a pyrenoid. Reproduction was not observed. The size of the cells was approximately 6 to 8 Fm by 10 to 15 Fm in diameter.

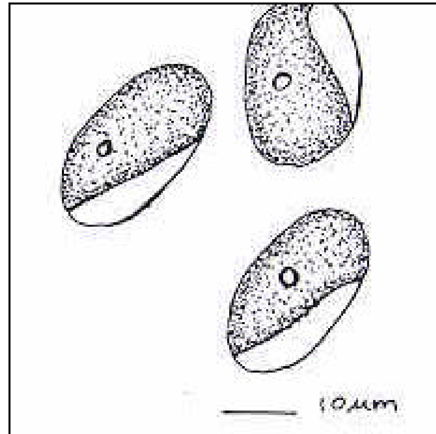

Figure 4-4. ***Chlorella ellipsoidea*** **Gerneck.**

Figure 4-5 is a unicellular alga that is a member of the Euglenophyta. Cells were found growing on any substrate; however, these algae were most commonly found on wood and rope. Occasionally they were collected from plankton tows. This benthic species is elongately cylindrical to an elongate tip. The anterior of the cell is decidedly narrowed. The cell exhibits a high degree of metaboly and no flagellum was observed. Chloroplast are highly variable, 2 to 4 in number with pyrenoids and paramylon present. Inclusions that appeared to be ferrous in nature were also present. The cells ranged in size from 7 to 10 Fm by 60 to 120 Fm, but were highly variable because of metaboly.

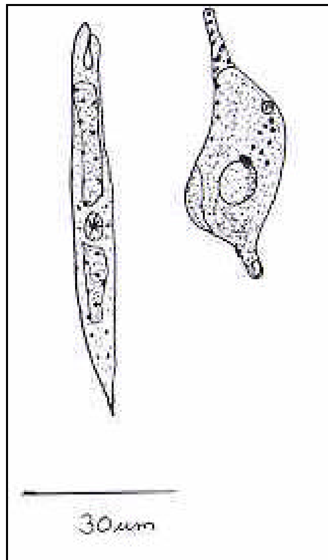

Figure 4-5. ***Euglena mutabilis*** **Schmitz.**

Figure 4-6 is of the division Bryophyta. This filamentous stage of moss was isolated from wood scrapings and grew into balls in culture. If germination into the leafy stage occurs, this organism may be identified. Presently, it can only by described as having branched multicellular filament with numerous discoid chloroplasts. The cell is approximately 25 Fm in diameter.

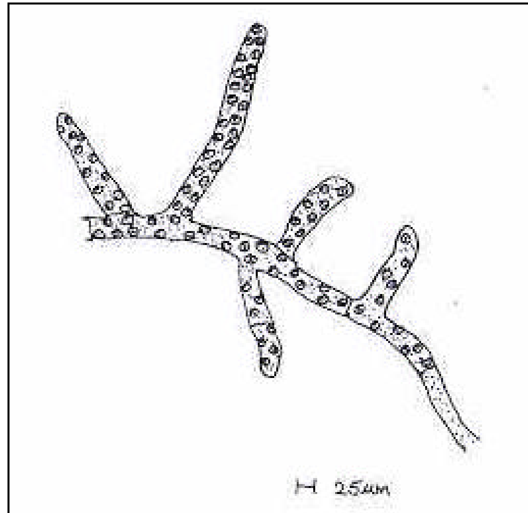

Figure 4-6. Moss protonema—species unknown.

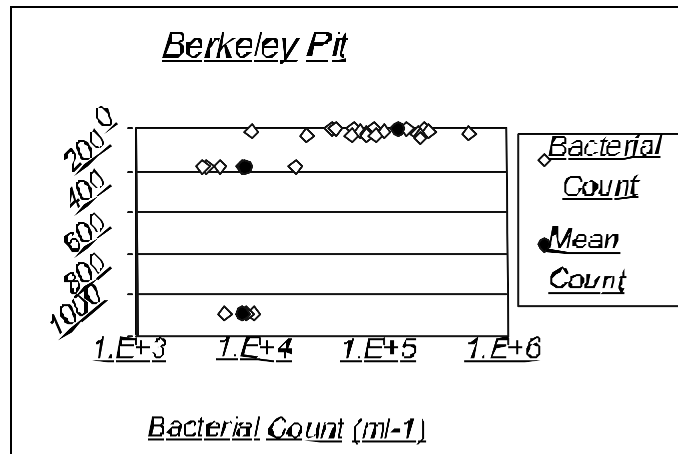

Figure 4-7. Bacterial counts of the Berkeley Pit water column. Reported counts (mL^{-1}) are mean values.

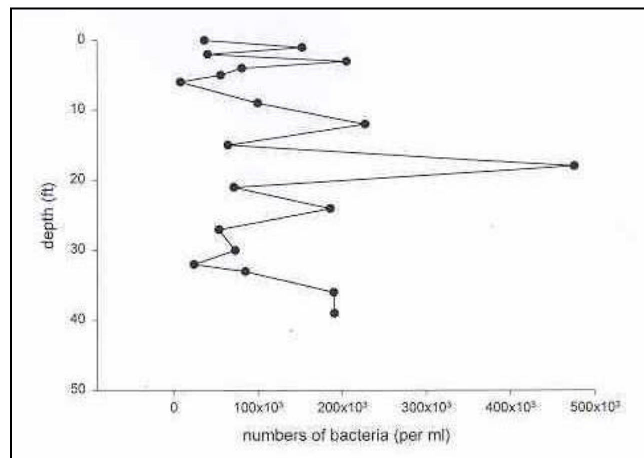

Figure 4-8. Bacterial abundance per milliliter.

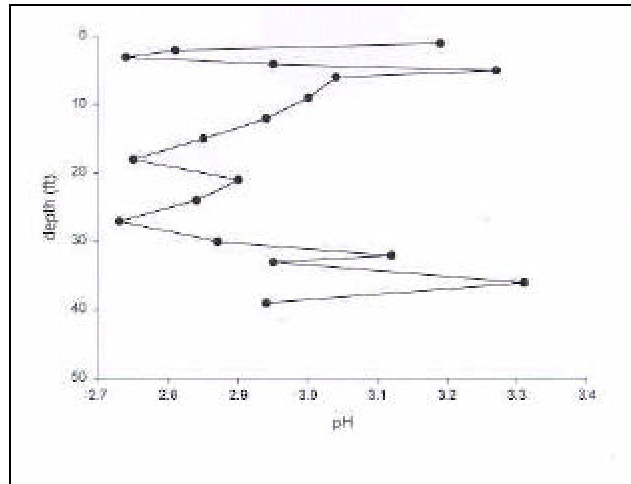

Figure 4-9. pH as a function of depth.

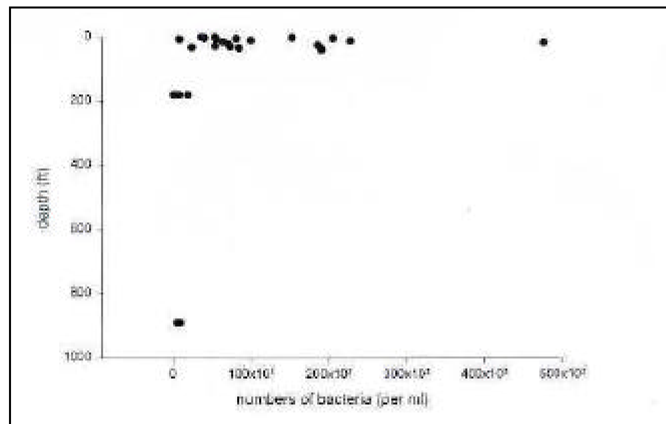

Figure 4-10. Bacterial abundance per milliliter.

Figure 4-11 show limax (slug-like) amoebae that display steady rather than eruptive locomotion. During locomotion, they may form slight bulges from their anterior edge but these are never eruptive as in the heterolobose amoebae. An anterior hyaline cap (zone) is usually present during continuous locomotion. Posterior often has few trailing filaments. The cells are uninucleate.

This isolate averaged 19.5 Fm in length during locomotion. Movement was steady, rather than eruptive. A thin hyaline zone was evident, which is narrower than that found in many *Hartmannella*. Posterior trailing filaments were common and the cells were uninucleate. Relatively common in cultures from different areas of the Berkeley Pit. The arrows indicate direction of locomotion.

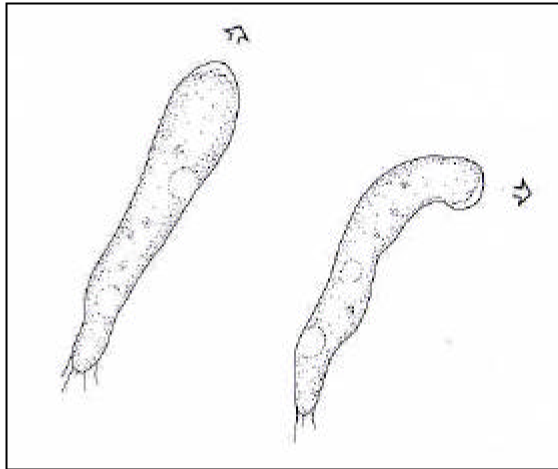

Figure 4-11. Morphotype 1, Hartmanella Alexeieff.

In general, any eruptive limax amoeba should be considered a probable vahlkampfiid (see Figure 4-12) (in the sense of Page) (Ref. 6). Many of these amoebae alternate between amoeboid and flagellated stages. Therefore, some view these amoebae as flagellated protozoa since molecular studies have shown that the flagellate stage evolved only once in flagellated organisms; thus, the ancestors of the schizoprenids (eruptive amoebae without fruiting bodies) must have been flagellated organisms. At the

ultrastructural level, mitochondria have discoid or flattened cristae rather than tubular cristae, which are found in other naked amoebae.

This isolate cannot be identified to genus without culture and ultrastructural studies. It is an eruptive amoeba, most likely a vahlkampiid in the sense of Page (Ref. 6). Within this grouping, the genus *Vahlkampfia* do not (or no longer) transform to flagellates. Other genera do transform to flagellates (e.g., *Naegleria, Willaertia, Adelphamoeba, Paratetramitus, Tetramitus, Tetramastigamoeba*) and the form of the flagellate stage is used in their identification. Morphotype 2 did not show any evidence of transformation and may be the genus *Vahlkampfia,* although this identification is at best tentative.

Movement was sluggish when undergoing directed motion but markedly eruptive when cells were changing direction. Amoebae ranged in length from 8 to 16 Fm. The trailing posterior filaments were common. Arrows indicate direction of locomotion. This isolate is relatively common in cultures from many regions of the Berkeley Pit.

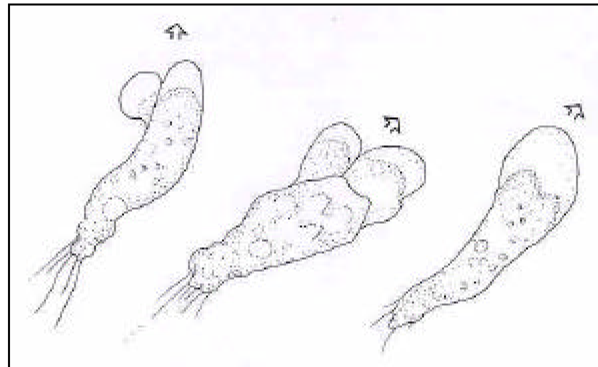

Figure 4-12. Morphotype 2, Vahlkampfiid amoeba.

The amoeba shown in Figure 4-13 was large (30 to 40 ***m***m in length) and displayed markedly eruptive locomotion. This gives it affinity with the vahlkampfiid amoebae, but it is white unlike the described genera of this grouping. The isolate had conspicuous crystalline or granular inclusions and a prominent single nucleus with a central nucleolus. Cells were observed to consume flagellates. This is a rare isolate that was only found in one culture from surface waters. This morphotype appeared briefly in the culture and could not be maintained. The arrow indicates direction of locomotion.

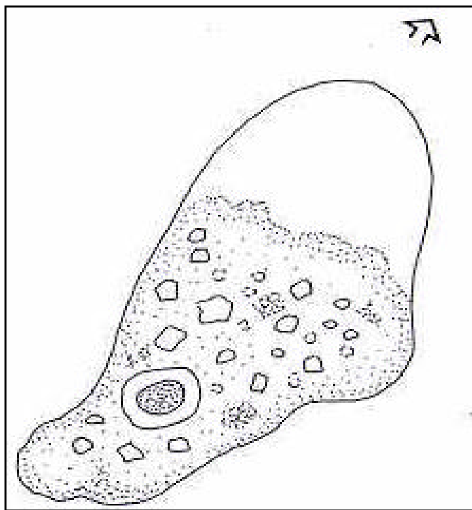

Figure 4-13. Morphotype 3, unidentified amoeba.

Figure 4-14 shows amoebae with a flattened locomotive form, more or less fan-shaped rather than tongue-shaped (linguiform). The anterior hyaloplasm usually occupies up to one half of the length. Some have a spatulate appearance with a long posterior tail. The floating form often has a long, markedly tapering pseudopodia. At the ultrastructural level, these specimens have a surface coat covered in pentagonal glycostyles. Note: this genus is easily confused with *Platyamoeba,* which is also markedly flattened although cells tend to be more oblong and the floating forms often have blunt pseudopodia.

This slow-moving, markedly fan-shaped amoeba ranged in length between 8.0 and 11.0 Fm. The cell had an extensive anterior hyaline zone and occasional posterior tail—features consistent with the vannellids. This isolate was markedly flattened and appeared uniformly dark under phase contrast microscopy. The arrow indicates the direction of locomotion. This is common in most cultures and often attain high densities.

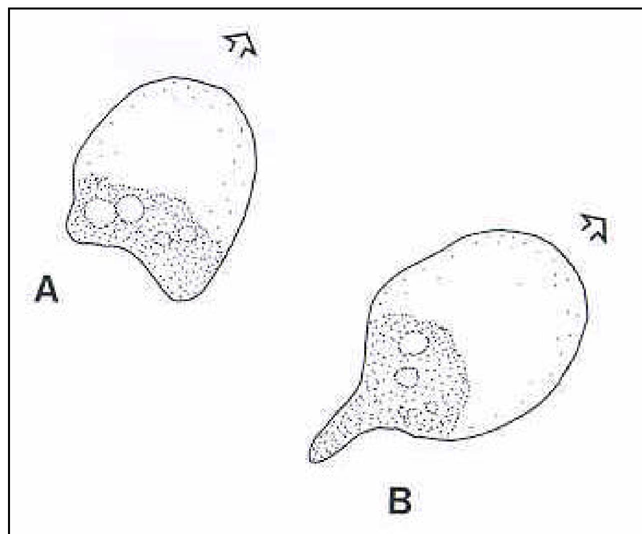

Figure 4-14. Morphotype 4, *Vannella* Bovee.

Figure 4-15 (see comments regarding morphotype 4) shows an isolate that had the characteristics of *Vannella*. Cells were flattened with an extensive hyaline zone. Overall, cells ranged between 8.0 to 11.0 Fm.

However, unlike morphotype 4, these vannellids were fast moving, displaying a characteristic rolling motion. Moreover, the posterior cytoplasmic region was more raised than in *Vannella* morphotype 4. Under phase contrast microscopy, the anterior hyaline zone was dark (i.e., thin) but the posterior cytoplasm was yellowing in appearance (hence thicker). This is common in most cultures.

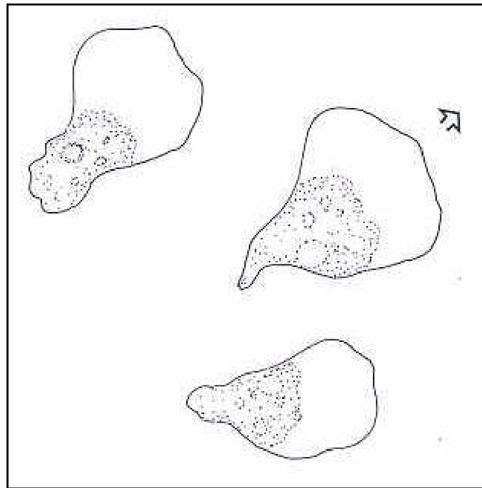

Figure 4-15. Morphotype 5, *Vannella* Bovee.

Figure 4-16 (see comments regarding morphotype 4) shows an isolate that had the characteristics of *Vannella* making it the third vannellid species indigenous to the Berkeley Pit. The cells were small with a mean length of only 8 Fm. This isolate differed from morphotypes 4 and 5 in that it had an obvious irregular leading edge with occasional waves moving up the anterior hyaline zone. Of the three vannellids, this isolate had the most obviously raised posterior cytoplasmic mass, which appeared golden under phase contrast. This slow-moving amoeba is common in most cultures.

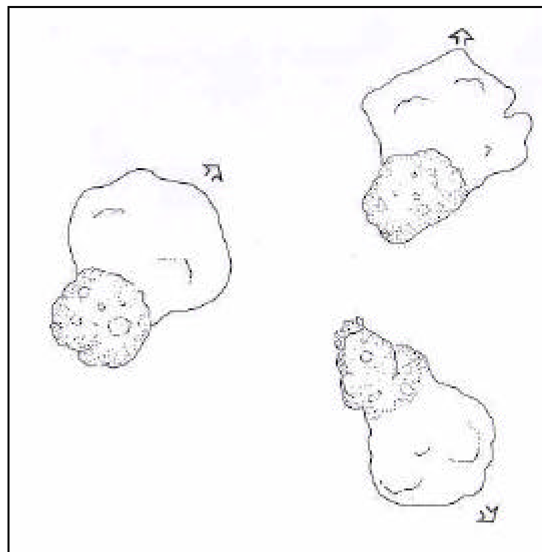

Figure 4-16. Morphotype 6, *Vannella* Bovee.

Figure 4-17 shows a discoid or globose amoebae enclosed incompletely in a tectum of delicate microscales (used to identify species). The tectum was open on the side applied to the substratum. There were attached amoebae with a flattened hyaline margin from which short subpseudopodia may emerge. The margin

surrounds a central granular, cytoplasmic mound. *Cochliopodium* is classified with the testate amoebae (subclass Testacealobosia) by Page (Ref. 6) and Bovee (Ref. 7). Other workers suggest that the genus belong with the naked amoebae (subclass Gymnamoebia) (Ref. 8).

This islolate had the characteristic morphology of *Cochliopodium* and possessed intracellular crystals and scales, barely visible in the light microscope. Arrows indicate direction of locomotion, which was slow moving. Maximum dimension was 16 Fm. This amoeba was relatively common in cultures and survived for long periods.

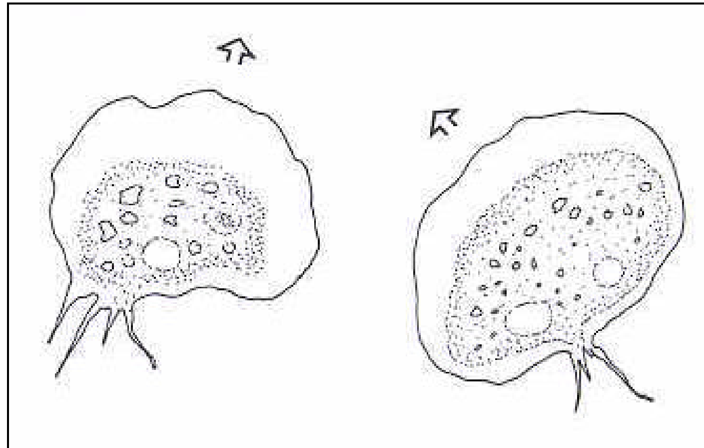

Figure 4-17. Morphotype 7, *Cochliopodium.*

Figure 4-18 shows a filose amoebae with slender clear pseudopodia that may branch, but do not regularly anastomose. Genus includes species that are flattened or spherical. Cells are colorless except for ingested prey. Some species have one or more mucus layers around the cell and pseudopodia emerge from over the entire cell surface.

The body of the cell is approximately 12.5 Fm in diameter with filose pseudopodia extending up to approximately 30 Fm. The tips of these pseuodopodia were occasionally swollen (A). Cells were either floating or attached, frequently to detrital or floc particles (B). Some cells extended a thickened pseudopodium, as shown in (C). Cells readily changed shape, especially when viewed under strong light. Many pseudopodia were branched but were never anastomosing. Some cells had considerable amounts of ingested detrital material (D), presumably some of the abundant flocculent material in these samples. This morphotype was relatively common in most cultures.

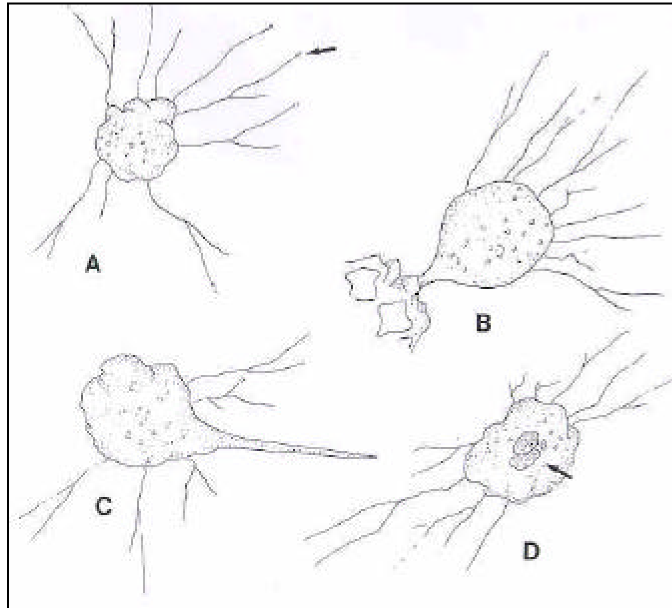

Figure 4-18. Morphotype 8, *Nuclearia.*

The isolate in Figure 4-19 was very small and flattened. Its overall length was approximately 8.0 Fm, and its body was approximately 2 Fm in diameter. This cell was difficult to observe on the base of petri culture dishes; therefore, few details can be given. Movement was restricted to the occasional waving of unattached filose pseudopodia. This may be a small, unidentified *Nuclearia*. This was relatively common but hard to see in most cultures.

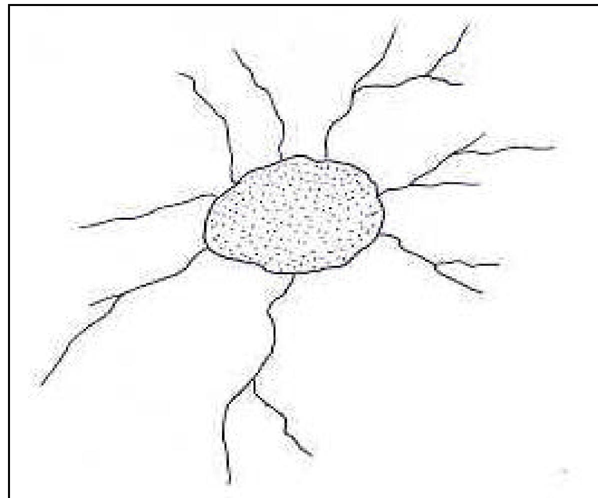

Figure 4-19. Morphotype 9, Unidentified filose amoeba.

The body of the isolate shown in Figure 4-20 was approximately 10 Fm in diameter and had straight radiating pseudopodia resembling the arms of heliozoans. Normally, small bodies (termed extrusomes) move along the pseudopodia to help in prey capture. Extrasomes were not seen in this isolate, perhaps because it was so small. The pseudopodia are barely visible in the photomicrograph. There are other freshwater protists similar to heliozoans such as *Pinaciophora* and *Pompholyxophyrs* (see Ref. 9), which have radiating pseudopodia

without the stiffening elements found in the heliozoans. Moreover, these genera do not have extrusomes (Ref. 10). This is uncommon in cultures.

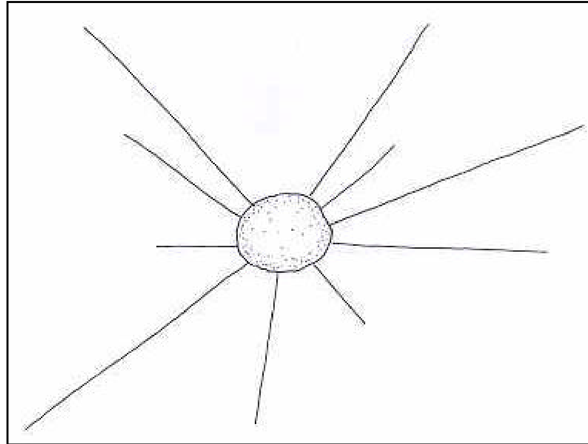

Figure 4-20. Morphotype 10, Unidentified heliozoan or heliozoan-like cell.

The flagellum of the isolate in Figure 4-21 trailed on the substratum while the anterior flagellum propelled the cell forward (direction of arrow). The body was very flexible as found in *Cercomonas*; however, at no time were pseudopodia formed, which is common in cercomonads. Consistent with *Cercomonas*, the posterior of the body of the cell was frequently drawn out along part of the gliding flagellum. The cell body ranged between 6.5 and 12.5 Fm in length. These cells were common and robust in culture.

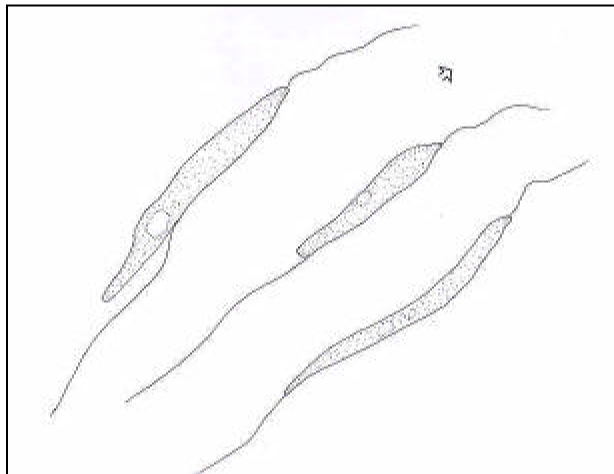

Figure 4-21. Morphotype 11, unidentified Cercomonas-like flagellate.

Chrysomonads are colorless, small cells with two unequal flagella emerging from the same region of the cell surface (see Figure 4-22). A long, undulating flagellum (held in a gentle arc) usually extends from the front of the cell. Some cells are covered in a layer of delicate spicules, which cannot be observed with the light microscope, although sometimes they can be detected as a halo around the cell (e.g., *Paraphysomonas*). Other chrysomonads are without spicules (e.g., *Spumella, Monas* and *Heterochromonas*). Colorless

chrysophytes often attach to detritus at their posterior via a thin extension of the cell or by use of a mucous stalk.

The present isolate was small (body 3.5 to 5.0 Fm in length) and was frequently attached to debris, although no attachment stalk could be observed. The body was often granular, giving the cell a “chunky” appearance.

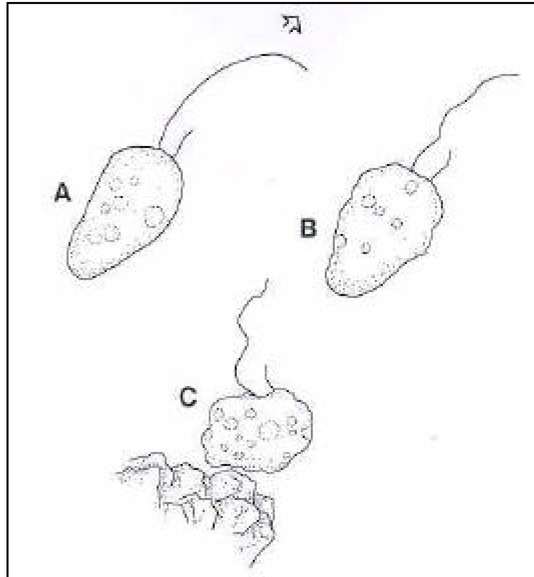

Figure 4-22. Morphotype 12, unidentified Chrysomonad flagellate.

Bodonids (as seen in Figure 4-23) are flagellates that attach to the substratum by one flagellum and frequently exhibit flicking, jerky movements. They feed on suspended material using their shorter anterior flagellum. They frequently detach and swim.

The present isolate was small, approximately 4.8 Fm in length (body only). Attached cells displayed a markedly jerky motion. The cell was filled with distinct globules or granules. Careful observation showed that both flagella inserted at the same anterior point, consistent with bodonids. Most cells were attached (A). Some cells when detached moved forward (B) or displayed a flicking motion in the water column with both flagella waving in different directions (C).

This protist was the most common in all cultures. It was universal throughout the Berkeley Pit and formed very dense cultures. It was the only protist to appear in the anaerobic (i.e., microaerophilic) treatments and was very common in the “remediation” trials with nutrient enrichment provided by rice grains (see Figure 4-24).

See comments on morphotype 13 regarding Figure 4-24. This flagellate was similar in behavior to morphotype 13, but had a distinctive anterior shape with a short “probosis”. The length of the cell body was 4.8 Fm. This isolate was relatively rare in cultures.

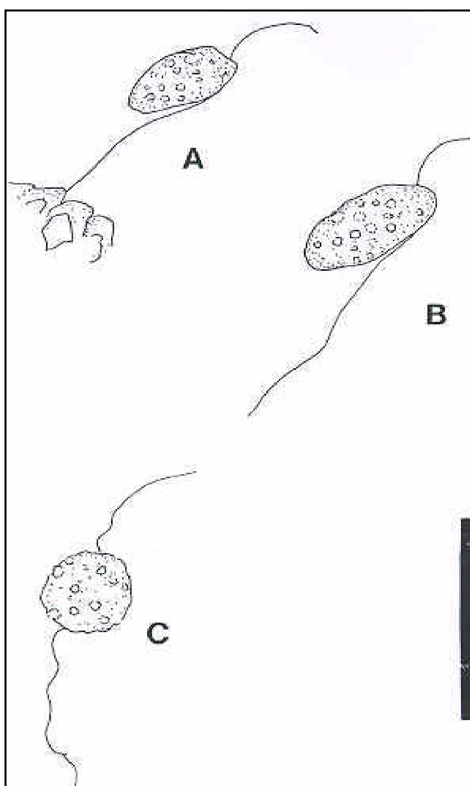

Figure 4-23. Morphotype 13, Bodonoid flagellate.

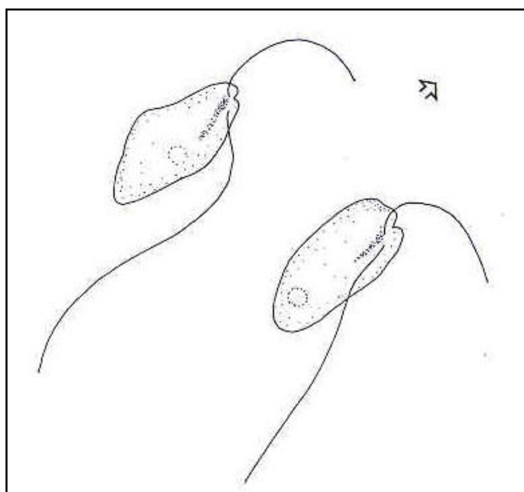

Figure 4-24. Morphotype 14, Bodonid flagellate.

The ciliate shown in Figure 4-25 was between 25 to 30 Fm in length. It appeared briefly in culture in very small numbers and then disappeared. Hence, few observations were possible.

The cell was evenly ciliated with an obvious mouth on the ventral surface (no details of associated membranelles can be given). The contractile vacuole was towards the posterior of the cell. These features make it a *Glaucoma*-like ciliate, but more observations would be required before an improved identification could be given.

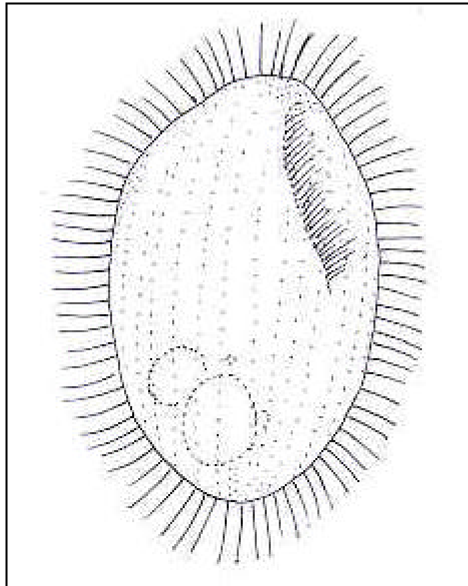

Figure 4-25. Morphotype 15, unidentified ciliate.

The ciliate in Figure 4-26 was only observed on a single occasion and few details were noted. It was 80 to 100 Fm in length. An obvious adoral zone of membranelles was noted. It bears some resemblance to the heterotrich ciliate *Metopus*, which is encountered in anoxic or reducing habitats.

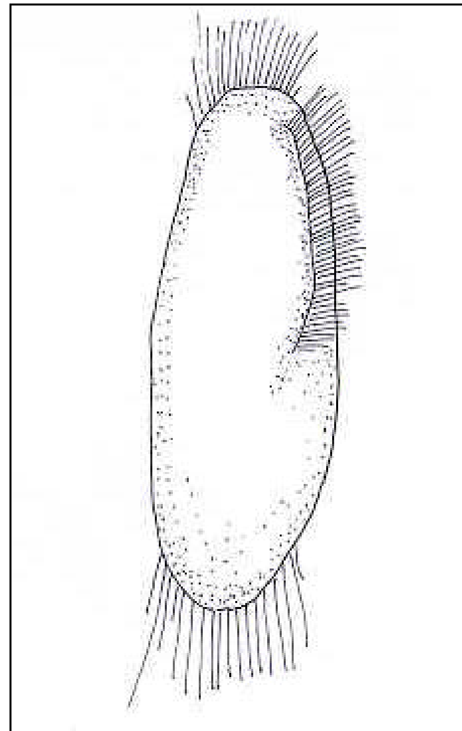

Figure 4-26. Morphotype 16, unidentified ciliate.

5. Discussion and Conclusions

There has been considerable research conducted on the biota of moderately acidic natural environments (approximately pH 4.0), which include acid lakes, pine forests, soils, and acid bogs. Such sites only superficially resemble acid mine drainage (AMD) sites. Their orange color and acidic nature is largely the result of oxidized iron compounds and the substantial amounts of dissolved organic carbon at these sites. Acid mine drainage sites, on the other hand, are extreme environments often associated with mineral extraction, drainage waters, and mining effluents. Here, waters can be as low as pH 2.0 and can contain a whole range of metal contaminants at high concentrations. At these sites, iron-sulfur pyrites and marcasites in mine waste are oxidized by chemical and microbially catalyzed reactions to sulfuric acid (Ref. 11). Very little is known about the tolerance of organisms impacted by acid mine waste, although there has been recent interest in using algae as a agents of bioremediation (Ref. 4). A few studies have reported the presence of phototrophs in acidic and metal influenced ponds (Ref 12) and Dr. Mitman identified two species of algae from the Berkeley Pit water as *Euglena mutabilis* and *Chlamydomonas acidiophila*.

This study is the first to provide information on the entire microbial assemblage of the Berkeley Pit waters. Knowledge about all the microbes is important since Gyure et al. (Ref. 11) concluded that nutrient availability may be more important than pH in limiting algal production. Given the importance of the microbial loop (Ref. 3) in regenerating nutrients in the water column, information on the heterotrophic components of the system is required if future remediation steps using algae are to be considered.

This study has shown that bacterial abundances were high throughout the surface water column of the Berkeley Pit. On average, approximately 116,000 bacterial mL^{-1} were found, which is only approximately 9 times less than levels in an noncontaminated freshwater lake. Water samples from the lower depths in the Berkeley Pit Lake contained far fewer bacteria, around 7,000 mL^{-1}. The method used gave direct counts of bacteria, which could include active, inactive (dormant) and dead bacteria. Therefore, this count gives no information about the activity (production) of the bacterial component. Further studies using radioactive labeling methods would be necessary to estimate bacterial production rates.

Sixteen morphotypes of heterotrophic protists were identified in this study. Very few live cells were found in fresh samples of water and sediments, suggesting that active populations in the Berkeley Pit were rare and that most populations may exist as cysts, particularly in the deeper anaerobic layers. Therefore, the protozoan component of the Berkeley Pit system is largely opportunistic, becoming visible when nutrients are added during aerobic cultivation. Only one isolate, the bodonid flagellate, appeared active under microaerophilic cultivation. Modest nutrient stimulation promoted temporary dense populations of some of the 16 heterotrophic protists identified in this study. These responded to the increased bacterial prey densities in the cultures. Although the dynamics of the system were not investigated, it is likely that increased heterotrophic activity resulted in the increased mobilization of nutrients. Future bioremediation steps should consider not only stimulation of the photyosynthetic populations but also the grazing populations that are important in nutrient turnover. As noted earlier, Gyure et al. (Ref. 11) believed that low photosynthetic rates of epilimnetic algae in acidic strip mine lakes (pH 3) were due to low nutrient availability rather than pH. Morever, at least one of the protists in the cultures was observed to directly ingest flocculent material (Morphotype 8). Whether this ingestion had any direct action on the particles remains to be examined.

A trial experiment to test the effectiveness of a massive increase of nutrients (in the form of sterile rice grains, w/v ratio of 1:10) showed that fungi were present in the waters (at least as spores). No attempt was made in this study to enumerate fungal spores; it is possible that some of the cyst-like bodies observed in the water column may have been spores rather than protistan cysts. Methods are available for counting fungal spores such as

epifluorescence microscopy using the cellulose/chitin specific fluorochrome Calcuofluor white. This nutrient enrichment trial stimulated high numbers of the small heterotrophic bodonid flagellate (Figures 4-11 through 4-26) and produced dense fungal mycelial mats. Interestingly, the zones around the rice particles, which were rich in flagellates, lost their orange flocculent material and the water showed a slight increase in pH, at least initially.

All attempts to enumerate protists in this study were unsuccessful because of the dense flocculent material in the samples. This meant that only a few milliliters of sample could be filtered for the epifluorescence method, a volume that proved inadequate for the detection of the low numbers of protists alive in the water. Conversely, settling 10 mL or more of sample for counting by the settling chamber method was also ineffective since the flocs again obscured any cells present. Future attempts should use an indirect counting method to estimate the size of the viable populations. One such method (the aliquot method) has been used successfully for enumerating heterotrophic protists in sediments (Ref. 13).

This study has shown that significant numbers of bacteria inhabit the Berkeley Pit waters and that there are at least 16 different heterotrophic protist most likely new to science, residing in the Berkeley Pit Lake. However, the SRB that were expected to be found did not exist. The identifications are incomplete and should be followed by more intensive studies to define culture conditions, and hence, species diagnoses. The descriptions serve as a “first atlas of the protozoa” upon which subsequent studies can be built.

6. Glossary

agar	a dried polysaccharide extract of red algae used as a solidifying agent in microbiological media
anthropogenically contaminated sites	contaminated by human activity
aphotic region	the layer in a body of water where photosynthesis does not occur
cropping action	the consumption or grazing of bacteria by protozoans
extremophiles	organisms that thrive under extreme physical conditions (e.g., temperature, acidity, salinity, pressure)
fastidious	an organism that is difficult to isolate or cultivate on ordinary culture media because of its need for special nutritional factors
limnetic zone	the surface region of a body of water away from the shore
microbial loop	the energy flow from algae in the form of excreted photosynthate to heterotrophic bacteria to protozoa to zooplankton
Montana algae	the species of algae found in Montana
motile	the property of movement of a cell under its own power
photic region	the layer in a body of water where photosynthesis occurs
photosynthates	the compounds produced as a result of photosynthesis (e.g., carbohydrates)
primary producers	an organism that uses light to synthesize new organic matter from carbon dioxide
profundal zone	the bottom and deep-water area that is beyond the depth of light penetration

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Appendix A

Appendix B

Raw bacterial count data. Samples from 21 November 1997. Samples collected from various sites in Berkeley Pit. All counts based on DAPI-staining –2 mL filtered onto a filter (black membrane, 0.2 Fm pore size). Filter surface examined using x1000 optics (magnification factor = x9111)

Sample ID	counts	mean	standard deviation
surface 1 ft.	2 2 5 53, 2 2 1 2 2 2 2 3 10, 13, 0 4 2 9 8 6 7 8,10, 23, 121, 18, 6 7 7 8	11.93	22.97
surface water with log material	10, 6 1 6 0 1 0 3 4 0 3 1 4 5 3 3 0 3 10, 2 26, 3 4 9 13, 3 0 1 1 6	5.13	6.93
plankton tow	0 4 3 2 9 10, 2 0 15, 1 3 0 0 1 3 0 3 1 1 4 8 2 3 0 9 8 1 2 7 2 3	4.26	5.59

Appendix C

Raw bacterial count data. Samples from December 1997. Samples collected in surface Berkeley Pit waters between 0 and 39 feet. All counts based on DAPI staining –2mL filtered onto a filter. Filter surface examined using x600 optics with additional magnification onto a video screen (magnification factor = x16,723)

Sample ID	count	mean	standard deviation
surface	4 2 2 0 3 0 1 5 10, 1 5 10, 2 1 10, 3 5 3 1 2 21, 10, 9 4 3 1 3 5 3 2	4.37	4.40
1 ft	35, 23, 23, 31, 34, 40, 31, 23, 23, 21 10, 5 3 0 11, 11, 10, 21, 30, 11 5 3 11, 3, 5, 30, 32, 20, 9 34	18.27	11.95
2 ft	6 10, 13, 11, 11, 6 3 2 1 3 6 8 2 1 2 1 5 2 7 4 1 2 2 1 5 11, 10, 5 3 1	4.83	3.71
3 ft	21, 31, 24, 10, 21, 23, 22, 25, 23, 15 31, 33, 38, 27, 9 11, 15, 27, 30, 31 21, 29, 17, 19, 27, 28, 36, 31, 33, 30	24.60	7.63
4 ft	2 1 2 1 0 0 3 2 1 1 0 3 1 0 1 0 0 1 1 1 2 1 0 1 0 1 2 1 0 0	9.67	0.89
5 ft	9 4 11, 0 5 4 17, 0 0 3 1 33, 10, 3 5 0 3 3 1 10, 5 11, 10, 3 3 0 0 11, 17, 18	6.67	7.53
6 ft	1 0 0 1 0 0 0 0 1 1 1 3 0 1 2 0 0 1 0 2 1 1 2 3 5 0 0 1 3 0	1.00	1.23
9 ft	17, 30, 15, 4 15, 0 2 3 36, 29, 26, 3 5 11, 3 9 20, 4 3 21, 3 2 3 3 3 8 3 34, 27, 16	11.93	11.10
12 ft	36, 35, 30, 39, 23, 15, 30, 24, 29, 21, 13, 26, 41, 43, 10, 6 20, 33, 21, 41, 23, 20, 19, 15, 41, 38, 37, 27, 30, 31	27.23	10.04
15 ft	4 3 2 3 0 2 5 2 6 4 2 20, 31, 20, 22, 15, 2 5 3 0 3 31, 3 3 9 6 3 7 15, 1	7.73	8.77
18 ft	72, 58, 50, 56, 46, 42, 40, 46, 72, 70 68, 64, 66, 56, 39, 59, 76, 70, 65, 60 53, 50, 61, 31, 73, 41, 49, 53, 56, 66	56.93	11.80

21 ft	7 11, 12, 6 6 5 3 17, 8 16, 16, 15, 9 5 3 7 8 8 15, 9 14, 10, 5 3 2 1 3 9 11, 12	8.53	4.60
24 ft	10, 11 5 26, 38, 41, 52, 15, 20, 28 27, 22, 31, 33, 31, 40, 11, 12, 17, 17 21, 20, 29, 40, 31, 3 3 5 15, 14	22.27	12.68
27 ft	9 4 5 1 13, 3 7 8 15, 1 6 18, 6 6 9 11, 8 3 3 0 9 4 5 5 11, 3 8 8 3 1	6.43	4.27
30 ft	25, 15, 12, 11, 10, 9 16, 3 15, 9 6 6 4 5 4 1 0 1 3 9 21, 20, 3 8 8 11, 10, 13, 1 3	8.73	6.37
32 ft	2 0 4 0 3 3 10, 3 2 0 0 0 1 0 3 6 5 7 3 1 11, 3 3 4 5 1 1 1 3 1	2.87	2.80
33 ft	26, 23, 20, 21, 20, 40, 0 3 1 0 3 7 2 0 12, 5 26, 4 26, 3 12, 12, 0 2 2 3 1 8 20, 3	10.17	10.74
36 ft	12, 34, 18, 18, 37, 30, 48, 31, 42, 12 11, 13, 15, 18, 18, 31, 21, 21, 31, 40 11, 15, 19, 19, 17, 21, 20, 27, 18, 14	22.73	10.00
39 ft	21, 8 16, 29, 36, 27, 33, 10, 19 21, 29, 23, 14, 19, 35, 27, 21, 22, 23 9 15, 14, 21, 27, 31, 31, 29, 34, 18	22.80	7.78

Appendix D

Raw bacterial count data. Samples from 28 April 1998. Samples from Berkeley Pit Shallow (180 feet) and from Berkeley Pit Deep (890 feet). All counts based on DAPI staining - between 2 mL and 5 mL filtered onto a filter. Filter surface examined using x600 optics with additional magnification onto a video screen (magnification factor = x16,723)

Sample ID	vol. filtered	counts	mean	standard deviation
180 feet water sample 1	5mL	0 2 0 0 0 0 2 1 0 2 3 3 1 2 1 0 0 1 2 1	1.13 1 1 0 2 2 0 5 0 1 1	1.20
180 feet water sample 2	5 mL	3 2 0 6 0 4 0 2 0 0 0 1 3 1 3 6 4 2 0 0 1 9 5 2 1 0 0 1 4 5	2.17	2.34
180 feet water sample 3	2 mL	0 0 1 1 0 0 1 2 5 0 10, 3 3 2 0 3 2 0 4 5 10, 3 3 1 0 0 1 3 3 2	2.27	2.60
180 feet water sample 4	5 mL	0 2 0 0 0 0 2 1 0 2 3 3 1 2 1 0 0 1 2 1 1 1 1 1 0 2 2 0 5 0	1.13	1.19
180 feet water sample 5	2mL	0 0 1 1 0 1 0 0 0 0 0 0 1 1 2 0 2 0 0 0 0 1 0 0 2 1 1 0 2 1	0.57	0.73
180 feet water sample 6	5 mL	3 2 0 6 0 4 2 0 0 0 0 0 1 1 2 0 2 0 0 0 0 1 0 0 2 1 1 0 2 1	1.03	1.43
890 feet water sample 1	3 mL	2 1 1 2 1 2 0 3 0 0 2 0 1 1 3 2 0 1 3 2 1 0 0 3 1 1 1 3 2 0	1.30	1.06
890 feet water sample 2	4 mL	0 1 1 1 1 2 2 0 1 1 1 1 2 1 0 5 1 0 0 0 0 2 1 0 1 0 0 1 1 2	0.97	1.03
890 feet water sample 3	5 mL	5 5 2 0 2 0 0 0 5 0 0 0 6 2 2 1 6 7 1 7 0 1 5 3 1 4 8 5 0 1	2.63	2.61
890 feet water sample 4	5 mL	2 1 1 0 1 3 7 3 2 1 0 1 2 0 3 1 2 2 2 1 2 5 1 0 1 2 2 1 4 2	1.83	1.51

Note: water/sediment samples from both shallow and deep sites were impossible to count. The particulate material obscured any fluorescing bacteria.

Appendix E

Raw bacterial count data. Samples from (i.e., sent) June 1998. Three zones were sampled; surface, thermocline and Berkeley Pit bottom with 11 sampling syringes equally spaced in a 4-foot frame.

All counts based on DAPI staining –2 mL of sample collected on a filter. Filter surface examined using x1000 optics with additional magnification via projection onto a video screen (magnification factor = x44,956)

Sample ID	counts	mean	standard deviation
Surface 1	0 0 0 0 0 1 2 0 1 0 0 0 0 0 0 0 0 0 1 0 1 2 2 0 0 0 0 0 0 1	0.17	0.46
Surface 2	0 0 0 0 1 1 0 0 1 0 0 0 0 1 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0	0.60	2.37
Surface 3	(no sample)		
Surface 4	0 0 0 1 0 1 1 1 1 2 1 0 0 0 0 0 0 3 2 3 3 3 0 1 1 3 2 3 1 2	1.13	1.17
Surface 5	2 2 2 3 0 1 0 2 2 1 1 0 0 0 0 0 2 0 0 1 1 2 1 0 1 0 0 2 1 0	0.97	1.03
Surface 6	0 6 2 5 4 3 1 2 2 2 0 1 0 1 0 0 0 0 1 3 2 1 2 4 0 1 3 0 0 0	1.53	1.66
Surface 7	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 0	0.07	0.25
Surface 8	0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1	0.13	0.35
Surface 9	0 1 1 0 1 0 1 0 1 0 0 0 1 1 0 0 0 0 0 1 0 0 0 1 1 1 0 0 0 0	0.37	0.49
Surface 10	0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 2 0 0 0 0 0	0.17	0.46
Surface 11	(no sample)		
Thermocline 1	0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0	0.07	0.25
Thermocline 2	3 3 2 0 1 1 2 0 0 0 0 0 1 1 0 0 2 1 0 0 0 1 2 1 1 0 2 1 0 2	0.90	0.96
Thermocline 3	(no sample)		
Thermocline 4	(no sample)		

Thermocline 5	6 5 5 4 0 6 2 1 7 0 0 1 1 4 3 1 1 0 0 1 3 0 4 2 0 2 1 0 0 0	2.00	2.17
Thermocline 6	(no sample)		
Thermocline 7	(no sample)		
Thermocline 8	(no sample)		
Thermocline 9	2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 1 1 1 0 0 0 1 0	0.30	0.53
Thermocline 10	(no sample)		
Thermocline 11	(no sample)		
Berkeley Pit bottom 1	1 1 0 2 1 0 0 0 1 0 0 1 0 0 1 0 0 2 3 1 2 2 1 0 1 0 3 2 1 0	0.93	0.94
Berkeley Pit bottom 2	0 0 0 1 2 1 0 2 2 2 0 3 0 4 0 2 3 1 0 2 0 1 0 1 1 2 0 3 1 2	1.20	1.16
Berkeley Pit bottom 3	2 3 2 3 2 1 3 2 0 3 2 2 2 3 2 2 1 2 3 2 2 1 3 2 2 1 1 2 3 1	2.00	0.79
Berkeley Pit bottom 4	2 3 5 3 5 1 0 0 2 3 1 1 4 2 3 2 2 6 5 3 0 2 4 3 3 2 2 1 1 3	2.46	1.46
Berkeley Pit bottom 5	2 1 0 0 1 0 0 0 1 0 1 2 1 4 4 6 4 2 3 1 3 0 1 0 1 0 0 0 0 0	1.57	2.40
Berkeley Pit bottom 6	11, 7 8 10, 5 6 8 9 7 6 6 4 4 9 10, 6 6 9 13, 6 8 5 8 6 10, 9 8 8 4 5	7.37	2.22
Berkeley Pit bottom 7	1 1 0 3 2 2 2 0 0 0 1 2 0 0 0 0 2 0 0 3 4 3 1 0 1 0 1 0	1.00	1.17
Berkeley Pit bottom 8	0 1 1 0 0 1 0 0 0 0 0 1 1 0 0 2 0 0 0 0 0 0 1 0 0 2 1 1 1 0	0.47	0.78
Berkeley Pit bottom 9	0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 3 2 2 1 0 0 0 0 0 0 0 0 0 0	0.30	0.75
Berkeley Pit bottom 10	1 0 0 0 1 0 2 0 0 0 0 0 2 2 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0	0.30	0.65
Berkeley Pit bottom 11	2 1 0 3 1 1 2 1 1 2 2 1 1 0 0 0 1 1 1 0 1 1 0 1 0 1 1 0 1 6	1.10	1.18
Grab surface samples:			
Wood North	0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 0 0	0.10	0.31

Wood North Spring	1 0 0 0 0 1 1 1 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0	0.20	0.41
N Spring	1 1 1 0 1 1 0 0 3 2 2 2 1 2 2 4 2 2 0 2 1 2 1 1 1 1 1 2 0 2	1.23	0.82
NE Spring/ sediment at shore	2 2 2 3 1 4 3 6 4 3 1 2 5 6 5 3 7 3 2 5 7 6 6 4 5 4 6 5 7 6	4.17	1.84