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Using Acidiphilium Acidiphilium to Bioleach Metals from Printed Circuit Boards

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USING ACIDIPHILIUM ACIDOPHILUM TO BIOLEACH METALS FROM PRINTED CIRCUIT BOARDS

BY

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ABSTRACT

As many as 500 million computers became obsolete between the years of 2000 and 2007 (U.S. EPA, 2007), due in large part to the increasing rate of technological advances. Printed circuit boards (PCB) are the essential components of most electronic waste. Metals that are contained within PCB are potentially hazardous to the environment. These metals include copper, aluminum, chromium, mercury, zinc, lead, and nickel (Li et. al., 2004). If not contained properly, metals can leach into soils and possibly damage the ecosystem. This problem of growing electronic waste not only exists in the USA, but it also extends overseas in countries like China, India, and Pakistan. An eco-friendly recycling technique needs to be investigated in order to deal with the exponential amount of electronic waste domestically. Bioleaching is using microbes to extract metals from material by mobilizing metal ions in solution. Bioleaching may hold the key to creating a process that is environmentally friendly and economically sound for extracting metals from PCB.

In this study, *Acidiphilium acidophilum* ATCC 27807 were grown in 9-K glucose liquid medium in a sterile environment at 26°C and plated onto 9-K glucose solid medium to insure purity. Printed circuit boards (PCB) that were involved in the study were shredded and sifted for particles greater than 0.841 mm and less than 1.680 mm. These particles were then briefly air cleaned to remove dust and autoclaved for sterility before use in experiments.

Experimental units ran under sterile conditions with 100 ml of 9-K glucose liquid solution in 250 ml baffled flasks at 26°C and 150 RPM. Experimental units that required cells were inoculated with 5ml of 4.26×10^8 cells/ml $\pm$ 1.1×10^7 cells/ml

culture into 95ml of 9-K glucose liquid solution. Printed circuit board material was added to experimental flasks once *Acidiphilium acidophilum* ATCC 27807 reached the stationary phase. Experiments ran for 8 days in total. Cell densities and pH were recorded a least daily during experimental runs. Cell densities for live cells in the lag and exponential phases were obtained using a spectrophotometer (Milton Roy Spectronic 10001 plus, Item #335005) with a wavelength of 500 nm. Cells in the stationary and death phases, where live and dead cells coexist, were measured using the drop plating and dilution techniques. pH readings were taken using a pH probe AccTupH Catalog number 13-620-183 in conjunction with an electronic meter Accumet Basic AB 15 pH meter by Fisher Scientific. Calibrations of both the probe and meter were taken every 30 minutes, before and between experimental readings, to insure proper pH readings. Metal samples were processed after experiments ran for 8 days. Samples from experimental units were filtered and acidified with 2 N Optima Nitric Acid and stored at 4°C until they were ready to be analyzed. Samples were analyzed for Cu, Ni, Zn and Al using an Electron Inductively Coupled Plasma Mass Spectrometer (ICP-MS) at the RI- INBRE Centralized Research Core Facility at the University of Rhode Island.

Levels of 0, 8, 16, 24 and 32 g/L of PCB were tested with *A. acidophilum*. Two main controls were included in experiments: flasks with just cells and flasks with just PCB. This allowed for a comparison to the normal growth curve and for a basis for just chemical/medium leaching. The maximum amount of copper that was bioleached by cells was 77.7 mg/L at 8 g/L of PCB. Total copper leached into solution yielded a 9.6% total copper recovery with 8.6% of the total copper leached

specifically by *A. acidophilum.* Zinc was also bioleached by cells with a maximum amount of 3.9 mg/L at 16 g/L of PCB. This yielded a 42.8% total recovery of zinc in solution; 36.3% of the total recovery was contributed by *A. acidophilum.* Nickel and aluminum were not bioleached. Experimental flasks with 8 g/L of PCB showed higher cell densities and lower pHs than the control curves of *A. acidophilum.*

Flasks specifically with 8 g/L of PCB and cells shows that *Acidiphilium acidophilum* ATCC 27807 found an energy source from the PCB that allowed for continued growth and production of an unidentified acid. This is also shown at levels of 16 g/L of PCB. The reason for metal specific bioleaching could be explained due to biofilm formation; this should be further investigated. And the reason for the death of cells in some of the PCB levels could be explained due to the high pH that these cells are not accustomed to living at.

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1 INTRODUCTION

As a result of rapid technological advances in the electronics and semiconductor industry, computers and electronic equipment are continuously being retired at faster rates. It has been estimated that "computer processing power roughly doubles every two years" (Carroll, 2008). The short lifetimes of computers also lead to fast turnover of peripherals, monitors, and other electronic equipment. In fact, as many as 500 million computers became obsolete between the years of 2000 and 2007 (U.S. EPA, 2007). Although the electronics in successive generations of computers and peripherals may be significantly different, printed circuit boards (PCB) remain the essential building blocks for computers and electronic products, and as such, they constitute a significant proportion of waste generated; they make up about 3% of all weight from electronic scraps (Li et al., 2004). The bulk of electronic waste can be attributed to casings, i.e. shells of computers, covers of cell phones, etc., all of which are easily removable. These high volumes of electronic waste lead to a very important issue: environmental impact. The study involved in this thesis aims to focus on the metal content treatment of PCBs.

Metals that are contained within the PCB are potentially hazardous to the environment. "Metals in PCBs consist of a large amount of base metals such as copper, iron, aluminum and tin; rare metals like tantalum, gallium (and other rare platinum groups metals); noble metals such as gold, silver, and palladium. Hazardous metals such as chromium, lead, beryllium, mercury, cadmium, zinc, nickel are also present" (Li et al., 2004). If not contained properly, these metals can leach into soils in landfills and possibly damage the ecosystem. This can cause health issues not only

to animals and plant life but also to people (Carroll, 2008; Basel Action Network, 2002).

PCB waste generated in the United States creates problems not only locally, but also globally in countries such as China, India, and Pakistan. Workers in these countries, where PCB are exported to, use "hammers, gas burners and their bare hands to extract metals, glass and other recyclables, exposing themselves and the environment to a cocktail of toxic chemicals" (Basel Action Network, 2002). In fact the Basel Action Network (2002) tabulated some of the issues that were occurring in Guiyu, China. An excerpt of that table is shown below.

Table 1: Hazardous of Disposing Electronic Waste in Guiyu, China (Basel Action Network, 2002)

Computer / E-Waste Component	Process Witnessed in Guiyu, China	Potential Occupational Hazard	Potential Environmental Hazard
Cathode ray tubes (CRTs)	Breaking, removal of copper yoke, and dumping	- Silicosis - Cuts from CRT glass in case of implosion - Inhalation or contact with phosphor containing cadmium or other metals	Lead, barium and other heavy metals leaching into groundwater, release of toxic phosphor
Printed circuit boards	De-soldering and removing computer chips	- Tin and lead inhalation - Possible brominated dioxin, beryllium, cadmium, mercury inhalation	Air emission of same substances
Dismantled printed circuit board processing	Open burning of waste boards that have had chips removed to remove final metals	- Toxicity to workers and nearby residents from tin, lead, brominated dioxin, beryllium, cadmium, and mercury inhalation - Respiratory irritation	- Tin and lead contamination of immediate environment including surface and groundwaters. - Brominated dioxins, beryllium, cadmium, and mercury emissions
Chips and other gold plated components	Chemical stripping using nitric and hydrochloric acid along riverbanks	- Acid contact with eyes, skin may result in permanent injury - Inhalation of mists and fumes of acids, chlorine and sulphur dioxide gases can cause respiratory irritation to severe effects including pulmonary edema, circulatory failure, and death.	- Hydrocarbons, heavy metals, brominated substances, etc. discharged directly into river and banks. - Acidifies the river destroying fish and flora

According to Table 1, it is apparent that exporting our waste to third world environments does not solve the problem of electronic waste disposal; it compounds

the problem since these environments are not fully equipped to protect themselves from the negative consequences. Some Americans make a conscious decision to turn in used electronic equipment to be recycled; however, many recyclers "sell it to brokers who ship it to the developing world, where environmental enforcement is weak" (Carroll, 2008). Approximately 50-80 percent of waste to be recycled from the US is shipped overseas. In fact, China has banned the import of electronic waste since 1996, but due to the vast amounts of trading it is impossible for authorities to check every shipping container (Basel Action Network, 2002). The United States needs to find a way to deal with this exponential amount of electronic waste domestically.

1.1 Current Printed Circuit Board Recycling

This research is concerned specifically with the recycling of printed circuit boards. A complete PCB recycling process consists of 3 main stages: pretreatment, separation/concentration, and mechanical/chemical refining. In the composition stage the material is usually assessed to minimize environmental impact as well as maximize any recoverable material. Since information about PCB composition is usually not available, chemical and analytical methods are commonly used.

The next step in this stage is dismantling items that are reusable or hazardous to isolate them from further processing. The most common method of dismantling is "hand picking." Items are manually removed selectively or all components attached to the PCB are removed entirely; this, of course, is labor intensive. The last step involves incineration/crushing which is completed in order to reduce the weight of material as well as make the material more consistent (Li et.al, 2004).

In the second stage, separation/concentration, PCB scraps are further crushed down to 100 μm to 300 μm. These small particles can then be separated by a variety of methods: magnetic, size, density, etc. After this separation, recovered parts might contain certain metals that can be separated from the main part of the PCB recycling route. Sometimes incineration is used to reduce the weight of the PCB; organic material such as plastic is removed by incineration in rotating furnaces at temperatures of 400-800℃ (Li et.al, 2004).

In the third stage, refining/recovering the rest of the material usually involves further chemical/mechanical treatment (Li, et. al., 2004). Chemical leaching, the method most commonly used to extract these metals from waste, is not an economical process. The cost of chemicals and the small amount of metal being obtained makes chemical leaching inefficient economically. One estimate for chemical leaching of metal contaminated soil estimates fixed costs totaling between $15,000 and $25,000 and operating costs of $135 - $450 per cubic yard (NAVFAC). In addition to the economic problems, environmental issues also come into the picture: factors such as chemical waste and hazardous byproducts become an issue. An alternative approach to chemical leaching, bioleaching may be the key to extracting these small amounts of metals economically and in an environmentally sound manner. Bioleaching saves energy, has minimum pollution potential, and yields value-added by-products such as organic acids or chelating agents (Agate, 1996; Chio, 2004).

1.2 Background Information on Bioleaching and Mechanisms

Bioleaching is the extraction of metals from materials by the use of microorganisms. It has long been recognized that select microorganisms have the ability to react preferentially with metals. As early as 166 A.D., Galen, a naturalist and physician, recorded in his journal an account describing what sounded like in-situ leaching in a Cyprus copper mine. He described the process of water percolating in these mines. Later the same water would return loaded with copper sulfate (Rossi, 1990). Copper was then recovered by iron precipitation according to the following reaction: $CuSO_4 + Fe^o \rightarrow Cu^o + FeSO_4$. Years later in 1940 commercial usage of in-situ leaching commenced in the Rio Tinto mine of Spain. These metal solubilization processes was thought to only consist of reactions with water and oxygen; it wasn't until 1947 when it was demonstrated in mine waters that iron oxidation and sulfuric acid formation was catalyzed by bacteria. Today microbial bioleaching of metals has been showing promise in a number of places from extracting metals from ores to removing metals from industrial wastes (Brombacher, 1997). There have also been studies on metal recovery/removal from sewage sludge and contaminated soils using microbes (Bosecker, 1997).

Microbes are able to interact with metals by two different processes: metal mobilization and metal immobilization. Metal mobilization can occur in four different ways: redoxolysis, acidolysis, complexolysis, and alkylation. Redoxolysis is a mechanism in which microbes oxidize/reduce metals in oxidation-reduction process causing an increase of metal in solution. Acidolysis occurs when microbes help to generate protons that ultimately bind to surfaces. As a result, the surface starts to

weaken chemical bonds causing metal ions to dissociate from their original form. Another mechanism, complexolysis, uses the formation of complexing or chelating agents which allows the solution to become less metal ion concentrated. By allowing the formation of these molecules the solution can hold more metal ions. The last mechanism in mobilization is alkylation. Alkylation happens when alkyl groups are "enzymatically transferred to the metal and covalently bound." The first three methods are more common than alkylation (Brandl and Faramarzi, 2006).

Microbes can also immobilize metals. This process also has a few different mechanisms: biosorption, bioaccumulation, redox reaction, and complex formation. Biosorption occurs on the surface of microbial biomass whether alive or dead. In this mechanism, metal becomes concentrated by chemical sites such as carboxyl functional groups. Another mechanism is bioaccumulation in which metals are taken up through microbe's cell membrane and stored within the cells. Redox reactions can also reduce metal mobility in solution, rather than increasing it like redoxolysis. Metals in this mechanism are also oxidized/reduced by microbes; the difference is metal mobility in solution is decreased rather than increased. And lastly complex formation can precipitate metal through the process of forming complexing agents such as sulfides (Brandl and Faramarzi, 2006).

Bioleaching processes, particularly metal mobilization mechanisms, depend on a variety of factors: pH, temperature, medium components/nutrients, and microorganisms. The common factor in these leaching systems is their pH values: because of the affinity for metal ions to stay in solution at low pH, pH in bioleaching processes typically ranges from 1.5 to 3 (Bosecker, 1997). There are a multitude of

microorganisms known to have bioleaching capabilities. A table listing some of these microorganisms can be found in the Appendix A.

1.3 Current Literature on Electronic Waste Bioleaching

There are a few studies that involve microbes and bioleaching electronic waste. Some of these studies in which microbes leach material similar to printed circuit board will be explained further. In at study by Brandl et. al. (2001), the dust from a recycling process of electronic equipment was bioleached by bacteria (*Thiobacillus thiooxidans* and *Thiobacillus ferroxidans)* as well as fungi *(Aspergillus niger* and *Penicillum simplicissium*). Separate experiments were preformed for bacteria and fungi. The dust that was bioleached was less than 0.5 mm in size and contained the following concentrations of metal.

Table 2: Concentrations of Selected Metals in Brandl's 2001 Study

Element	Content (g kg^{-1})
Aluminum	237
Copper	80
Lead	20
Nickel	15
Tin	23
Zinc	26

(Metals levels listed above totals 401 g/kg; this percentage of 40.1% of metal is not consistent with the percentage of metals in printed circuit boards.)

Brandl et. al. (2001) found with earlier growth experiments that if the microorganisms were grown separate from the leaching material before electronic dust is introduced, more efficient metal mobilization can occur. Thus, before electronic

dust was placed into experimental units, bacteria were grown for seven days. After this seven day period, electronic dust was added and grown for an additional 10 days.

The results for bacteria leaching with a mixed culture of *Thiobacillus thiooxidans* and *Thiobacillus ferroxidans* was positive in that the organisms grew well at concentrations less than 10 g/L of electronic dust. Metals that were monitored were aluminum, copper, nickel, lead, tin and zinc. All levels tested of electronic dust showed that these all metals monitored (except aluminum at 100 g/L, tin and lead) were mobilized to some extent. (See Figure 1.)

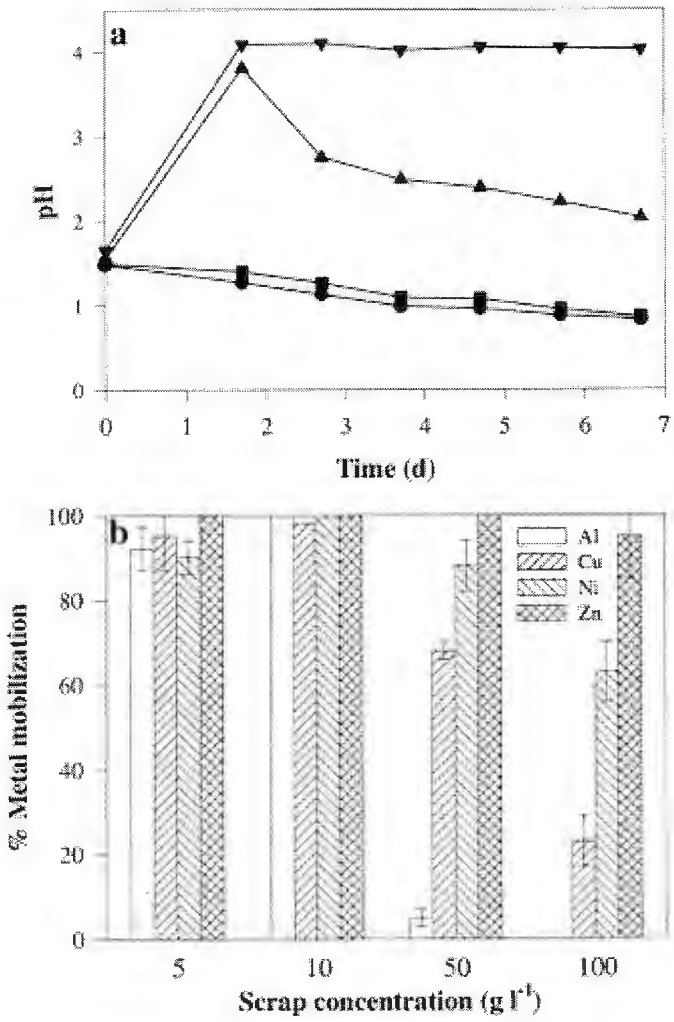

Figure 1: Results of Bacterial Studies on Electronic Dust. "(a) Growth (determined by pH decrease) of a mixed culture of *T. ferroxidans* and *T. thiooxidans* on different amounts of electronic scrap (g/L): • 0 (control); ■ 1; ▲ 5; ▼10 g/L. Points represent mean values of duplicates. Standard errors are within 0.15 pH units. (b) Mobilization of Al, Cu, Ni, and Zn from different concentrations of electronic scrap (g/L) at 30 ° C in a two-step process: A mixed culture of T. ferooxidans and T. thiooxidans were previously grown for 7 days in the absence of electronic scrap. Scrap was added and the culture was grown for an additional period of 10 days. Bars represent mean values ± standard errors of duplicate experiments" (Brandl et. al, 2001).

The growth of bacteria was signified by the decrease in pH as shown in Figure 1 part a. Aluminum, nickel, copper and zinc were metals that were successfully leached at least 90% or higher at electronic dust concentrations of 10 g/L or less. Metals that were not mobilized in solution, lead and tin, precipitated as $PbSO_4$ and SnO

respectively. The microbe mechanisms involved in the study were not presented in the journal article.

In the fungi experiments, microbes were not mixed, *Aspergillus niger* and *Penicillum simplicissum* leached metals in separate experiments. After the growth of fungi for 6 weeks, electronic dust was placed into experimental units and allowed to grow further for an additional 21 days. Results for *Aspergillus niger and Penicillum simplicissum* are shown in Figure 2. The mechanisms for these experiments were also not discussed.

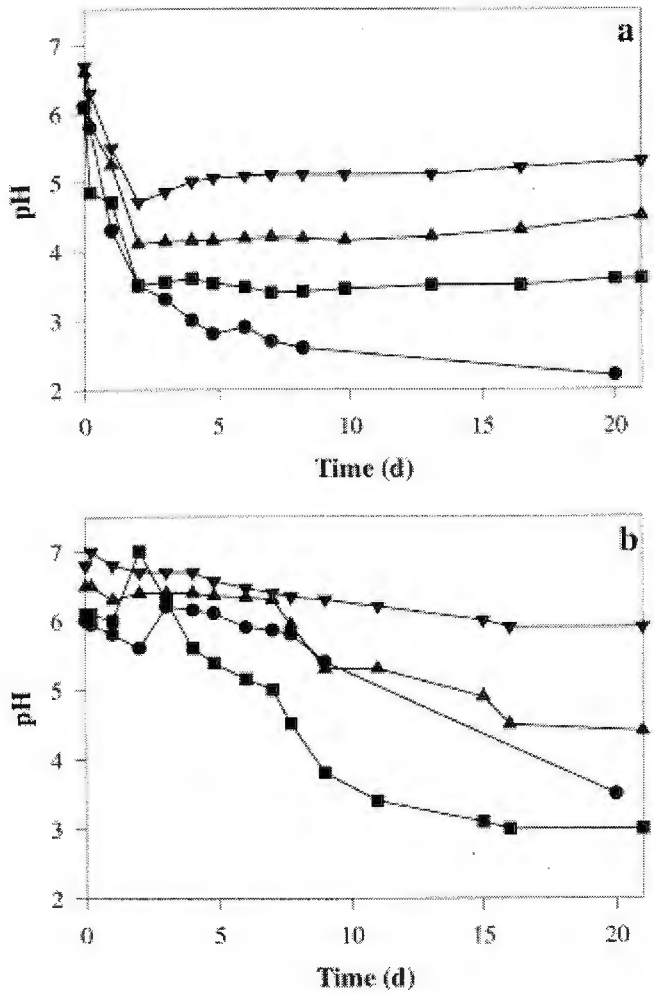

Figure 2: pH Results of Fungi Experiments. "Growth (determined as pH decrease) of *A. niger* (a) and *P. Simplicissimum* (b) on different concentrations of electronic scrap (g/L): • 0 (control); ■ 1; ▲ 5; ▼ 10. Points represent mean values of duplicates. Standard errors are within 1.2 pH units" (Brandl et. al, 2001).

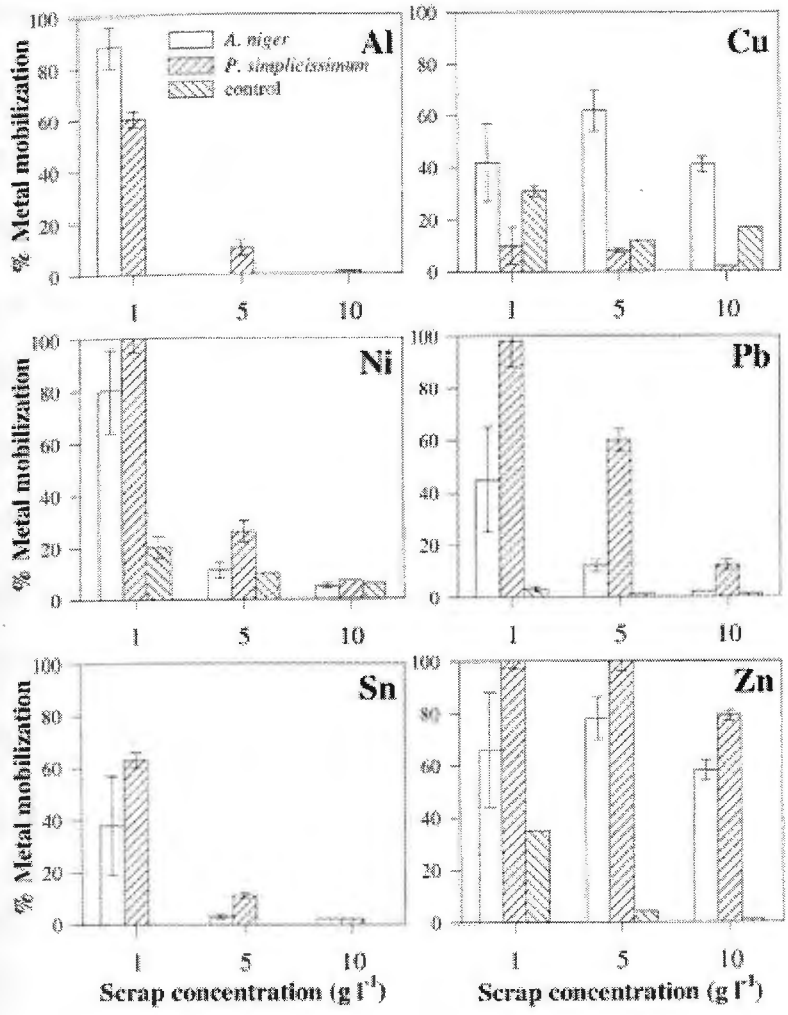

Figure 3: Percent Mobilization of Fungi Experiments. "Leaching of metals from electronic scrap by A. niger and P. simplicissimum after 21 days at 30℃ on different concentrations of electronic scrap (g/L). Sterile non-inoculated medium was used as a control. No Al and Sn were detected in controls. Bars represent mean values ± standard errors of duplicate experiments (Brandl et. al, 2001).

According to these figures *Penicillium simplicissimum* mobilized more metals compared to *Aspergillus niger* at the same conditions; on the other hand, looking at the copper bar chart in Figure 3, *Aspergillus niger* did leach more copper overall. Comparing the bacteria experiments with the fungi ones, the bacteria experiments mobilized more copper, aluminum, nickel, and zinc. Fungi, on the other hand, mobilized more lead and tin while bacteria precipitated both of these metals.

The studies of Hahn et. al. (1993) focused on the ability of bacteria and fungi (*Bacillus* sp., *Saccharomyces cerevisia, Yarrowia lipolytica*) to leach electronic waste of lead, copper, and tin. This waste is an organic polymer matrix that consists of lead, tin, and copper in a 1:2:20 ratio broken into particle size of 20 x 20 mm. A more exact composition of the material was not determined due to its heterogeneous nature. Microbes were not mixed in these studies. Experiments were performed with electronic waste pulp densities of 1%-10%. Substrates used were glucose and sulphite waste liquor (SWL); however, results for SWL experiments will not be shown since chemical extraction of SWL contributed to the results. Experiments ran for a maximum of 20 days. Below are the results for copper, tin and lead solubilization in glucose using the batch method. Mechanisms in this study were not described.

Table 3: Metal Bioleaching of Electronic Waste in the Study of Hahn et. al (1993)

	Final Metal Concentration in g/l, (Time in days)		
Microorganism, Substrate	Cu	Sn	Pb
Bacillus sp. L1, Glucose	0.72 (18)	0.13 (7)	0.41 (7)
Saccharomyces cerevisiae, Glucose	0.71 (18)	0.68 (9)	0.18 (5)
Yarrowia lipolytica, Glucose	0.82 (20)	0.14 (5)	0.11 (7)

The time in days represents the day that the maximum solubilization was met. On interesting thing to note from this table is that after 5-9 days tin and lead's maximum solubilization was met; after 18-20 days copper's maximum concentration was met. This could lead to the possibility of selective leaching. The article was not clear on which pulp densities were reported in the table above.

In the same research Hahn et. al. (1993) also performed fed batch experiments. He leached metals using a mixed culture of *Bacillus* sp. LP1 and Strain KAT4 for 35 days with pulp densities of 2.5 and 5 %. In these fed batch experiments nutrient medium and sucrose solution was continuously added; tin and lead solubilization completed at close to 2 g/L for both metals. Copper, on the other hand, was only 30% (~5g/L) dissolved. Fed batch experiments exhibited a biphasic metal solubilization like the batch experiments. The fed batch experiments were more effective in amounts of metal leached. Again, the article was not clear on the pulp density of the results reported.

In the last study discussed here, Choi et. al. (2004) used *Acidithiobacillus ferroxidans* to leach copper from printed circuit boards. The printed circuit boards were shredded and then particles smaller than 1.41 mm and larger than 0.84 mm were collected. The copper content of this material was 759.3 ± 335.9 g/kg of shreds. Experimental units contained 50 g/L of shredded PCB and different amounts of Fe^{2+} ranging from 0-9 g/L. Organic acid was not a byproduct of the microbes. Instead, *Acidithiobacillus ferroxidans* normally obtains their energy from oxidizing ferrous complexes. Therefore, concentrations of Fe^{2+} were shown to decrease as they were being oxidized. Experiments ran for approximately 72 hours. See Figure 4 below for Fe^{2+} concentrations and solubilized copper concentrations from experiments.

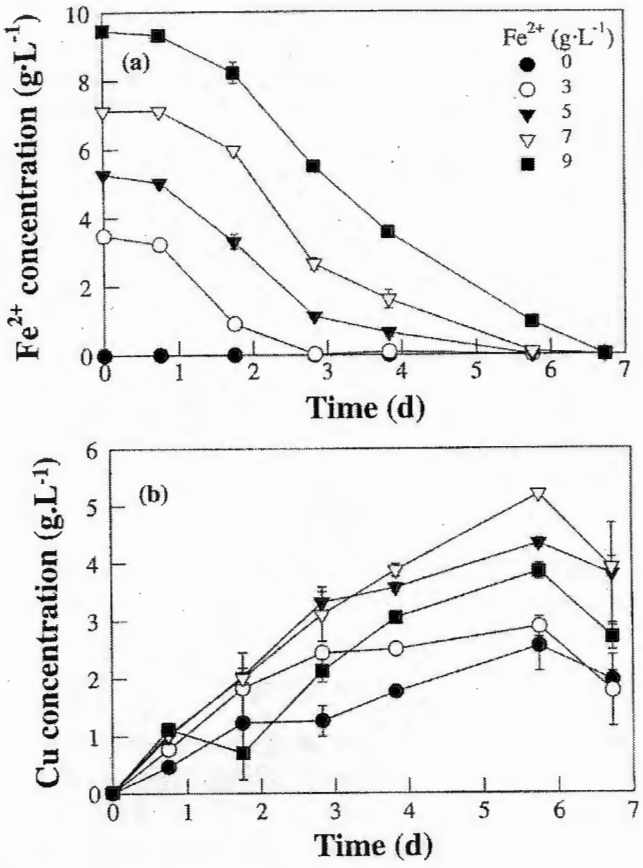

Figure 4: "Time Profiles of [Initial] Fe^{2+} (a), and Cu^{2+} Concentration in the Leachate (b) for Different Initial Amount of Ferrous Iron" (Choi et. al., 2004).

Without ferrous ions, the maximum amount of copper leached by *Acidithiobacillus ferroxidans* was ~2.550 g/L. By the addition of ferrous ions the level of copper leached increased to 5.190 g/L. As the experiments progressed, copper started to precipitate as well. Taking into account the solubilized and precipitate metal, the ferrous ion concentration that proved most effected was 7 g/L; copper recovery was 24%.

It was concluded that microbial leaching of copper from printed circuit boards was accomplished by two reactions: the oxidation of ferrous ions which then leads to the oxidation of elemental copper:

$$Fe_2(SO_4)_3 + Cu^0 \rightarrow Cu^{2+} + SO_4^{2-} + 2FeSO_4$$

In addition to this reaction copper is also believed to be partly leached out chemically by the following reaction:

$$2Cu^0 + 2H_2O + O_2 \rightarrow 2Cu^{2+} + 4OH^-$$

Choi et. al. (2004) also added a complexing agent, citric acid, to help improve the solubility of copper in solution since copper was precipitating. Using the same conditions as those in his copper leaching experiments, he added the presence of 1 g/L of citric acid as a variable. When citric acid was not added a total of 37% by weight of copper leached remained in solution; however with citric acid the total copper leached that remained in solution was 80% by weight.

These articles show it is possible to bioleach metals efficiently from electronic waste. In the study of Brandl et. al. (2001), even though PCB was not leached, showed metal solubilizations (of Cu, Al, Ni, and Zn) by bacteria of over 90%. In the study of Hahn et. al. (1993), the possibility of selective leaching in electronic waste was introduced. Copper in his study was leached up to 0.82 g/L by *Yarrowia lipolytica.* Lastly, the Chio et. al. (2004) study, copper recovery by *Acidithiobacillus ferroxidans* had a 37% copper recovery in solution; the addition of citric acid yielded 80% by weight recovery of copper in solution.

1.4 Proposed Experiment

Initially for the experiment in this thesis two bacteria were chosen to perform bioleaching, *Acidithiobacillus thiooxidans* ATCC 15494 and *Acidiphilium acidophilum* ATCC 27807, since bacteria in communities usually perform better than

individually. *Acidithiobacillus thiooxidans* ATCC 15494 are also well known in the leaching community (Agate, 1996; Rawlings, 1998). This combination of bacteria has been already tested in different bioleaching environments with success (Brandl et. al., 2001; Krebs et. al, 2001). However, this combined bacterial effort was not possible since *Acidithiobacillus thiooxidans* ATCC 15494 was difficult to isolate from the culture received by the American Type Culture Collection. Therefore, in the experiments only *Acidiphilium acidophilum* ATCC 27807 was used to bioleach metals from shredded computer printed circuit boards.

The morphology of *A. acidophilum* consists of short rods in the size of 0.5-0.8 μm by 1.0-1.5 μm. The cells can be found singly and in pairs (Guay, 1975). Their optimum living conditions of temperature and pH are 25-30°C and 3 respectively (Krebs, 1997). They grow equally well with glucose or sulfur as an energy source; hence designating them as heterotrophic and chemolithoautotrophic (Guay, 1975; Harrison, 1983).

In the proposed experiment a glucose solution will be used due to the difficulty in counting cells in a sulfur medium. (Sulfur medium is not transparent, glucose medium is and thus it is easier to quantify cell density.) It is expected that through the production of acid, probably due to the oxidation of glucose, *Acidiphilium acidophilum* ATCC 27807 will be able to bioleach metals from computer shredded printed circuit boards. A probable result for this experiment is that at lower concentrations of PCB the higher recovery of metals in solution. At high concentrations of printed circuit boards it is expected the environment will not be suitable for growth. Growth of the bacteria along with pH and final metal

concentration of Cu, Ni, Al and Zn in solution will be measured. Varying factors will be the presence of *Acidiphilium acidophilum* ATCC 27807 and amount of printed circuit board in solution.

2 MATERIALS AND METHODS

2.1 Pre-Experimental Tasks

As mentioned previously, *Acidiphilium acidophilum* ATCC 27807 will be used in this experiment to bioleach metals from printed circuit boards. The varying factors will be the presence of cells and the level of PCB in the experimental units. Before conducting main PCB experiments a few parameters were determined first: the control growth curve of *Acidiphilium acidophilum* ATCC 27807, a calibration curve for spectrophotometer for the cell quantification, determining the constant and variable factors for the experiments and figuring out which metals to measure in solution.

The temperature recommended to grow *Acidiphilium acidophilum* ATCC 27807 was used in experiments: 26°C. The shaker speed of 150 RPM was chosen because it was commonly used in related studies (Brandl et. al, 2001; Yahya, 2002; Rodriguez, 2003; Chan, 2003 and Oliazadeh, 2006). It is assumed that this shaker speed allows for the maximum amount of oxygen and carbon dioxide to the cells. To increase the mixing, 250 ml baffled flasks were used. In a similar manner, the size of the flasks along with the solution in experimental units was chosen because of its commonality among articles: 250 ml flask and 100ml solution (Brandl et. al, 2001; Choi et. al, 2004; Bacelar-Nicolau, 1999; Falco, 2003; and Wong, 2004). It is assumed that this volume ratio compared to the flask size allows for the optimum head space for air exposed to the bioleaching environments. The range of shredded PCB determined for testing was used from Brandl et. al. (2001). The size of the printed circuit boards were chosen as close as possible to the studies of Chio et. al. since his

study specifically dealt with the bioleaching of printed circuit boards (Chio, 2004). Metals Cu, Ni, Zn and Al were determined to be common among printed circuit board articles or bioleached by similar bacteria (Brandl et. al., 2001; Brombacher, 1998 and Result Technology AG).

After constants for the PCB experiments were determined (26° C, 150 RPM, and 250 ml baffled flasks with 100 ml of 9-K glucose liquid solution) the control growth curve was created using the drop plate technique to understand the relationship between time and cell density. Once the growth curve was determined, a calibration curve was created using the absorbance function of spectrophotometer and the drop plating method (Gerhardt ed., 1994). By creating the calibration curve, readings in the lag and exponential phase (when all cells are alive) can be quantified fairly easily. The drop plate method, although very accurate, is labor intensive. (See Appendix B for the drop plating method.) The calibration curve along and the growth curve can be found in the Results and Discussion section.

2.2 Organism Used in Bioleaching

Acidiphilium acidophilum ATCC 27807was obtained from American Type Culture Collection (ATCC). These cells were grown in ATCC medium: 738 9-K glucose liquid medium. The 9-K glucose liquid medium was aseptically prepared as denoted on ATCC's website (ATCC, 2006). For this medium the pH was adjusted to 3.5 ± 0.1. (See the Appendix C for the specific recipe.) Growth cultures were kept in an incubator (Precision 818 low temperature illuminated incubator) at 26°C. The organisms were initially streaked from –75°C glycerol stock onto agar plates (9-K

glucose solid medium) to ensure purity of the culture. (Note this solid medium is prepared differently from the liquid medium, further information can be found in the Appendix C.) Agar plates were also kept in an incubator at 26°C. A few colonies were picked and placed into 9-K glucose liquid medium. The resulting culture solution was back diluted to insure homogeneity and then used to inoculate experimental flasks. Subsequent inoculation cultures were made using liquid cultures of *Acidiphilium acidophilum* ATCC 27807 or from the –75°C glycerol stock.

2.3 Material to be Bioleached

A 10% sample of the entire lot of the shredded printed circuit boards (PCB) received has the following composition:

Table 4: Metal Components in Shredded Printed Circuit Board (Jim Gardner, personal communication, April 07, 2005)

Metal in Shredded Printed Circuit Board	Amount
Gold (Au)	230 ppm
Silver (Ag)	690 ppm
Palladium (Pd)	20 ppm
Copper (Cu)	21.60%
Nickel (Ni)	0.18%
Aluminum (Al)	1.62%
Iron (Fe)	0.21%
Lead (Pb)	0.04%
Tin (Sn)	0.05%
Chromium (Cr)	0.00%
Bismuth (Bi)	0.00%

Before using PCB in experiments the shredded PCB were collected in the size range of -12/+20 mesh (particles are less than 1.680 mm and greater than 0.841mm). Table 5 shows the metal analysis results of copper, zinc, nickel and aluminum for printed

circuit board shreds in this size range. This analysis was performed using an ICP-MS (inductive coupled plasma mass spectrophotometer).

Table 5: Metal Components in Shredded Printed Circuit Board (-12/+20 mesh)

	Amount in PCB
Metal	%
Cu	11.2894%
Zn	0.0672%
Ni	0.0877%
Al	0.1980%

The meshed out PCBs were then briefly air cleaned to remove the majority of the dust, mixed thoroughly and also autoclaved at 121 °C, 15 psi for 20 minutes before used in experiments.

2.4 Experimental Pre-Setup

All experimental units had the following conditions in a sterile environment: 100 ml 9-K glucose solution in a 250ml baffled flask, 26°C incubator temperature and a 150 RPM shaker speed. Experimental units with cells were inoculated with 5ml of a 4.26×10^8 cells/ml ± 1.1×10^7 culture into 95ml of 9-K glucose liquid medium. Flasks were covered with aluminum foil to prevent evaporation and contamination.

After inoculation of cells, flasks were placed on a shaker and incubated for approximately 3 days to allow cells to acclimate. Printed circuit board material was added once *Acidiphilium acidophilum* ATCC® 27807 reached the stationary phase, a growth phase in which cell density remains constant, this was signified with a cell density of 1.1×10^9 cells/ml (or a 0.20 absorbance value at 500nm). The most active bioleaching activity occurs in the exponential phase of the microorganism growth as denoted by Ballester et. al. (1989) and Liu et. al. (2003). The exponential phase

occurs before the stationary phase. (See Appendix F for information about growth phases.) In the exponential phase, cells grow the fastest because of the initial nutrient rich environment. Following the work done by Brandl et. al. (2001), Choi et. al. (2004) and Chen et. al. (2003), bacteria were allowed to acclimate before printed circuit board material was introduced into experimental units. According to Brandl et. al (2001) allowing bacteria to acclimate before the introduction of waste will help to reduce its toxic effects on the bacteria. Bacteria were allowed to acclimate to the stationary phase within the experimental units to allow for maximization of acid production and density of cells.

2.5 Recorded Experimental Factors

In addition to monitoring the growth of the cells at least daily during the experiment, pH and solubilized metal concentrations were also measured. Growth curves and cell densities for *Acidiphilium acidophilum* ATCC 27807 were obtained using two techniques. The first technique involved serial dilutions in conjunction with drop plating. This was used when some or all cells present in solution were expected to be dead. This technique is much more accurate than using the spectrophotometer since it factors in cells that are dead. (See Appendix B for details on serial dilutions and drop plating.) In the second technique a spectrophotometer (Milton Roy Spectronic 10001 plus, Item # 335005) with a wavelength of 500 nm along with a calibration curve was used when cells were expected to all be alive. (Cells start to die once they reach their stationary phase, which can be determined by creating a control growth curve.) This technique was used due to its quick counting ability using

turbidity. The first technique is more labor intensive. During experiments only cells in solution were counted; cells that were attached to shredded printed circuit boards were not accounted for.

pH readings were taken on a daily basis using a pH probe AccuTupH Catalog number 13-620-183 and an electronic meter Accumet Basic AB 15 pH meter by Fisher Scientific. Calibrations of both instruments together were taken every 30 minutes before and between experimental readings to ensure proper pH readings.

Metal samples were processed after experiments were complete using a Thermo Electron Inductively Coupled Plasma Mass Spectrometer (ICP-MS) at the RI-INBRE (Rhode Institutional Development Award (IDeA) Network of Biomedical Research Excellence) Centralized Research Core Facility at the University of Rhode Island. Before submission to RI-INBRE, experimental units were filtered through a 0.2 μm filter to insure bacteria did not remain in the sample to be analyzed. Three filtered 15 ml samples were taken from each experimental unit. 15 ml samples were then stored with ½ ml of 2N Optima Nitric Acid and placed in the refrigerator to be analyzed at a later date. "Blank" or deionized water samples were also taken to insure the metal sampling technique did not contaminate the experimental samples. Metals that were focused on in the experiments were copper, aluminum, nickel, and zinc. (For further information on how equipment was taken care of to prevent metal contamination see the Appendix D.)

2.6 Structure of Experiments

In the first experiment, designated Experiment A, there were two variables: levels of printed circuit board and the presence of cells. These variables were chosen to determine the effects of cells on printed circuit board concentration. Other conditions that were consistent among flasks are as follows: temperature 26 °C, 150 rpm shaker speed, 100 ml 9-K glucose liquid medium and a sterile environment. Initial levels of PCB to be tested were 16, 32, 48, 64, 80 and 96 g/L; 16 and 32 g/L were tested in the first experiment. Only duplicates were performed due to time constraints: 15 minutes for a plate count, 10 minutes for pHing and clean up. For the first experiment there were 10 experimental units that took a total of 4 hours daily. Below is a table of how Experiment A was set up.

Table 6: Setup of Experiment A: The First Experiment

Label on Flask	***Acidiphilium acidophilum* ATCC® 27807 present**	**Printed Circuit Board Level in g/L**
A1	Yes	0
A2	Yes	0
A3	No	16
A4	No	16
A5	No	32
A6	No	32
A7	Yes	16
A8	Yes	16
A10	Yes	32
A11	Yes	32

Two types of controls were built in: cell growth (flasks A1 and A2), and medium leaching (flasks A3-A6). These controls will allow for a comparison to experimental units if something is wrong with the control cell growth curve or if chemical medium leaching becomes a factor that affects metal bioleaching results. As stated before,

cells were first allowed to acclimate in the experimental units for approximately 3 days before the addition of PCB. After PCB was introduced into the environment, cells were allowed a further incubation period of about 5 days.

After examining at the results for the first experiment, the levels for the second experiment, designated Experiment B, were adjusted. (For more on this see the Results and Discussion section.) Instead of analyzing levels 48 and 64 g/L of PCB 8 and 24g/L were used. Also, it was noticeable that duplicates for the first experiment were not clearly similar. For the second experiment it was decided that triplicates were necessary, this tacked on an additional hour to collect data from experiments for a total of 5 hours daily. All other experimental factors were kept the same as Experiment A. Below is the experimental setup for the second experiment.

Table 7: Setup of Experiment B: The Second Experiment

Label on Flask	ATCC 27807 *Acidiphilium acidophilum* present	Printed Circuit Board Level in g/L
B1	Yes	0
B2	Yes	0
B3	No	8
B4	No	8
B5	No	24
B6	No	24
B7	Yes	8
B8	Yes	8
B9	Yes	8
B10	Yes	24
B11	Yes	24
B12	Yes	24

Controls in Experiment B are also similar to Experiment A. Chemical leaching controls are B3 through B6; this will take into account medium leaching at both levels

8 and 24 g/L. B1 and B2 were also incorporated to take into account the control growth curve of *Acidiphilium acidophilum* ATCC 27807 without PCB.

3 RESULTS AND DISCUSSION

3.1 Control Growth Curve of *Acidiphilium acidophilum* ATCC 27807

The control growth curve for *Acidiphilium acidophilum* ATCC 27807 can be found below in Figure 5. Two different growth curve data points were plotted; the curve takes into account both data sets. As shown in Figure 5, as with any batch bacterial curve, 4 distinct phases can be seen: lag, exponential, stationary and death phases.

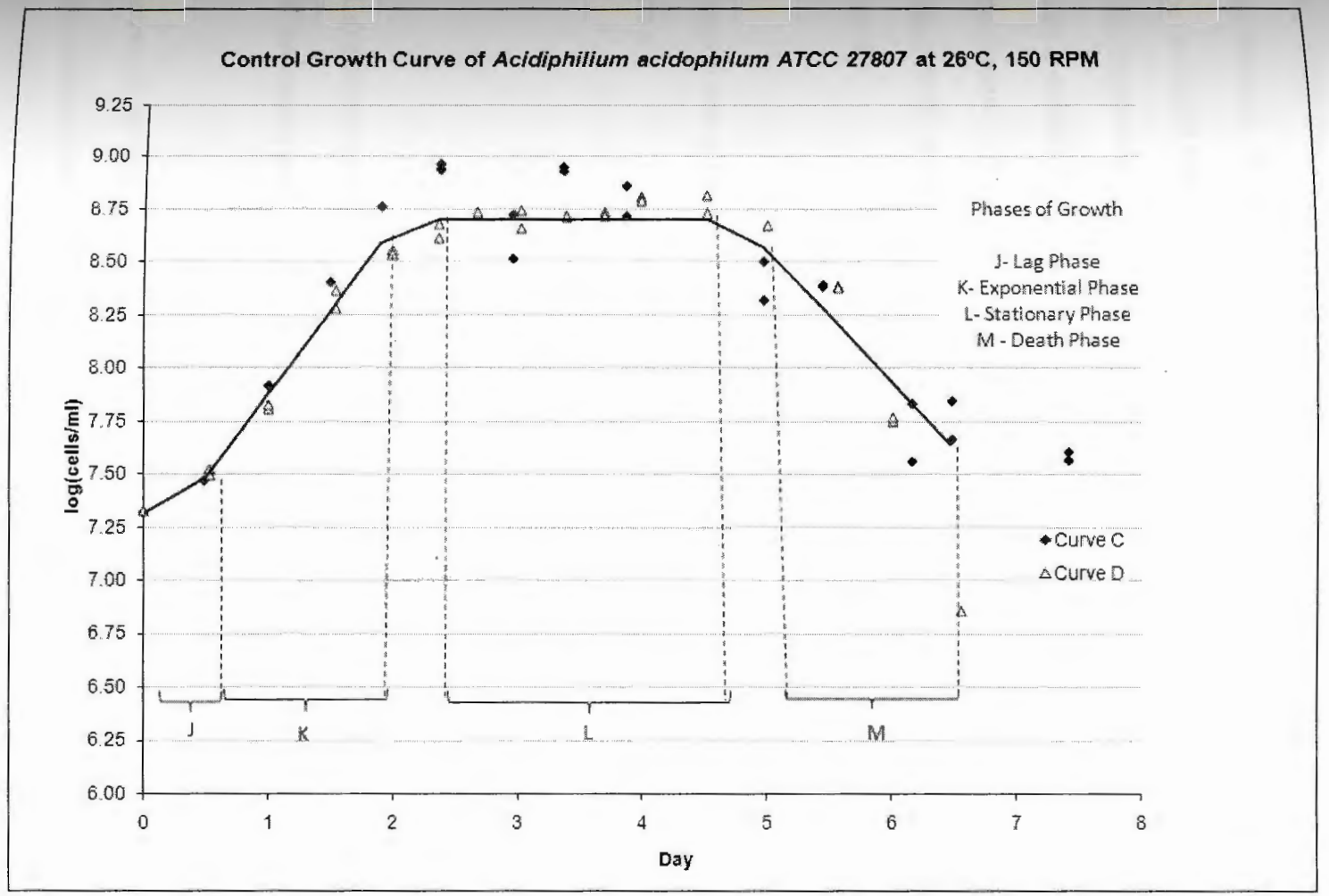

Figure 5: Control Growth Curve of *Acidiphilium acidophilum* ATCC 27807

The lag phase occurs when bacteria are adjusting to a new environment. Once adjusted to their environment, cells grow at an exponential rate due to the nutrient rich environment. At this point the time it takes for a group of cells to double in size can be calculated; this is called doubling time. According to Prescott (1996) there is a direct relationship in the exponential phase between the number of cells and time. This is possible because a population of cells doubles every generation; in other words the increase in populations is always 2^n where n is the number of generations. If we designate N_t to be final number of cells and N_0 to be an initial number of cells then we can say

$$N_t = N_0(2^n)$$

where n here is the number of generations in time t. Solving the above equation for n we get:

$$\log N_t = \log N_o + n(\log 2)$$

$$n = \frac{\log N_t - \log N_o}{\log 2} = \frac{\log N_t - \log N_o}{0.301}$$

Using this information, we can calculate the growth rate constant k; this is the number of generations per unit time.

$$k = \frac{n}{t}$$

where t is the time it took to get n generations. Plugging in the equation for n that we got previously and rearranging:

$$k = \frac{\log N_t - \log N_o}{(0.301)t} = \left(\frac{1}{0.301}\right)\left(\frac{\log N_t - \log N_o}{t}\right)$$

If we look carefully at this equation, we can see the rate of change of cells over time; this is essentially the slope of the exponential phase. A least squared regression was completed in Excel on points in Figure 5 between 0.5 and 2 days. The resulting equation was

$$\log\left(\frac{cells}{ml}\right) = 0.791\ x\ (Time\ in\ days) + 7.088$$

Plugging the slope of this line back into equation k we get

$$k = \left(\frac{1}{0.301}\right)(0.791) = 2.628\ generations\ per\ day$$

Inverting this equation we can get the doubling time, g.

$$g = \frac{1}{k} = \frac{1}{2.628} = 0.3805\ days\ per\ generation = 9.132\ hours\ per\ generation$$

During the exponential phase *Acidiphilium acidophilum* ATCC 27807 duplicates every 9.132 hours. Once the nutrients starts to become scarce cells start to die at the same rate they are producing; this stage is called the stationary phase. As nutrients become further depleted cells enter the death phase. It is important to note that bacteria growth curves only exist if nutrients are limited. (See the Appendix F for a more in depth discussion of growth phases.)

3.2 Calibration Curve for *Acidiphilium acidophilum* ATCC 27807

The relationship between absorbance and cell density was obtained by the use of a spectrophotometer with a wavelength of 500 nm in conjunction with serial dilutions and the drop plating technique. Figure 6 shows the calibration curve that was

obtained. This curve was created with the same conditions as the control growth curve. (See Appendix E for data.) All points from the four experiments were graphed, and from those points a linear trend was plotted and a linear relationship of

$$\log(\text{absorbance}) = 0.874(\log(\text{cells/ml})) - 8.357$$

was obtained and used for quantifying cells from absorbance values.

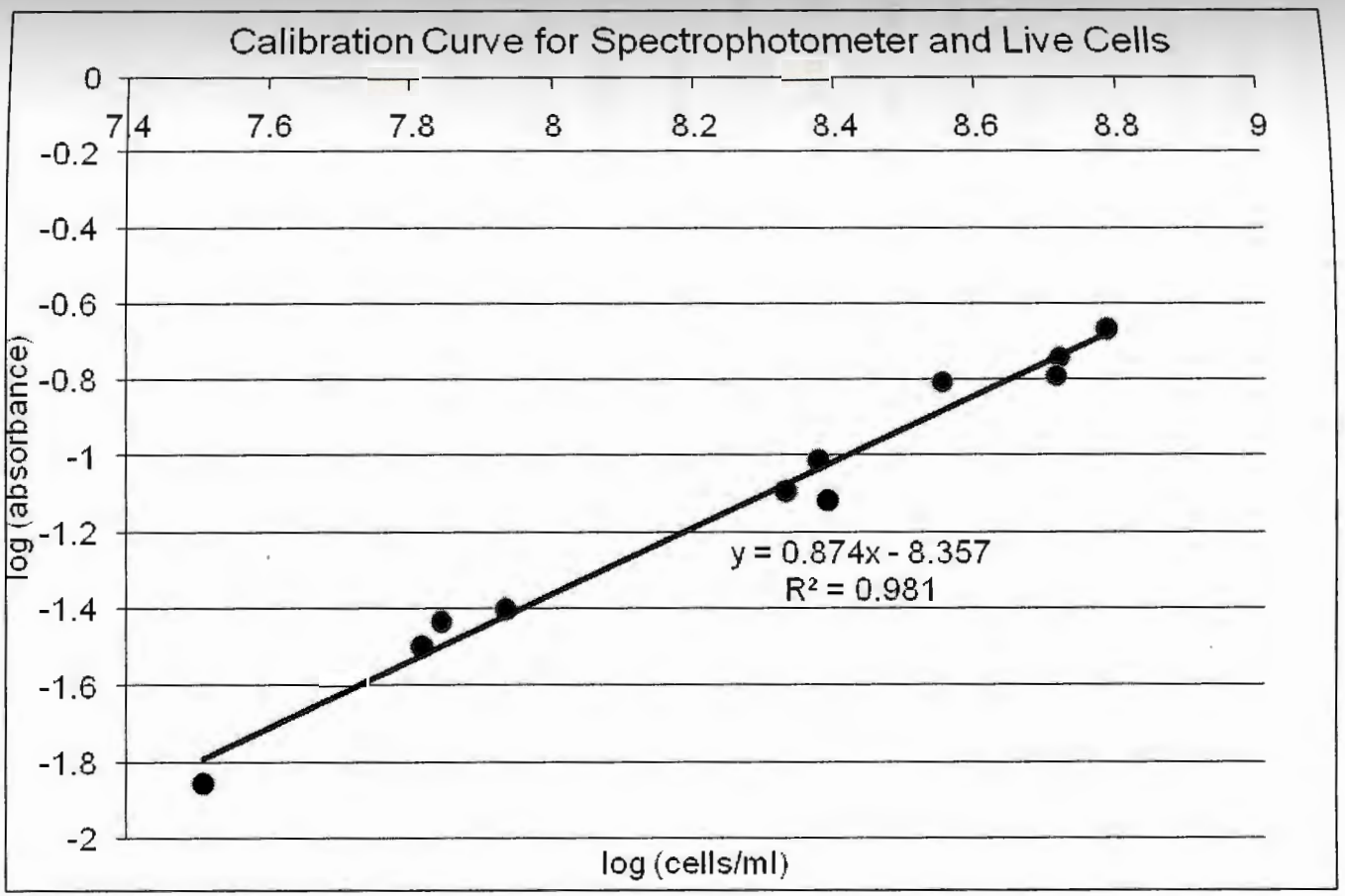

Figure 6: Calibration Curve for *Acidiphilium acidophilum* at 26°C, 150 RPM

3.3 Growth Curves During Printed Circuit Board Experiments

Growth curves for PCB experiments are in shown in Figures 7 through 9. (If cell curves go below 6.0 for the log (cells/ml), cells were no longer detected in solution.) In Figure 7, data from Experiment A, it is noticeable that flasks A8 and A7 (flasks with cells and 16 g/L of PCB) have higher cell densities than flasks A1 and A2 (cells without PCB). Flask A10 also shows a higher density than flasks A1 and A2. This relationship signifies that there is new source of energy available due to the introduction of PCB that can support further cell growth. Unfortunately, the position of flask A10 in Figure 7 cannot be confirmed since flask A11, its duplicate, did not grow more than the control growth curves of Experiment A. This variation could be due to the heterogeneous nature of PCB. Slight variations in material/metal could have caused the death of cells in one flask, while the other flask A10 had cells growing.

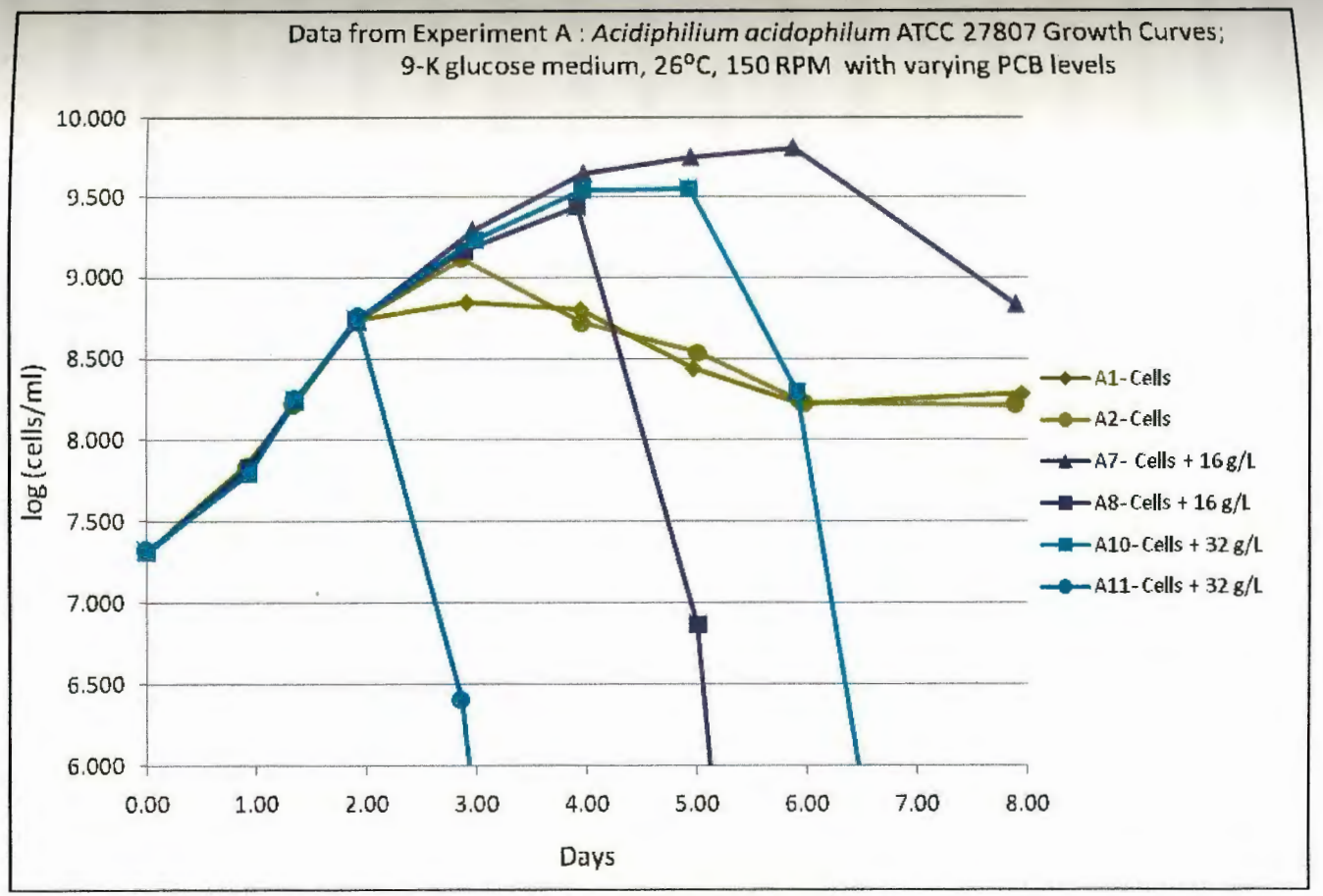

Figure 7: Growth Curves of Experiment A

Due to the sudden cell death that occurred with flask A11, the densities to be originally tested were changed. The goal in this experiment is to make the environment with PCB and cells more likable for cell growth and metal solubilization. Therefore, Experiment B involved lower levels: 8 and 24 g/L of PCB. The results of this experiment are shown in Figure 8. Similarly to Experiment A, the relationship of cell growth and presence of PCB is noticeable in data from Experiment B. Flasks B7, B8 and B9 all have cell growth well above the control growth curves, B1 and B2. Cells in flasks B10 through B12, cells and 24 g/L of PCB, started to die off immediately after the introduction of PCB.

The green lines, shown in Figure 8, represent control growth curves for *A. acidophilum*. These control growth curves lie in between the orange lines (cells + 24 g/L of PCB) and the red lines (cells + 8 g/L of PCB) because at certain levels of PCB, cells can exist in conditions that are more favorable to growth while others are unfavorable. In this case, flasks with 8 g/L of PCB and cells are providing an environment with more nutrients. In the case of flasks with 24 g/L of PCB and cells the surplus of nutrients may not be enough due to other detrimental factors, i.e. pH. Further testing needs to be completed to determine what this PCB threshold is for cell growth. This threshold value could be even smaller than 24 g/L of PCB. The highest cell density recorded from both experiments is 9.10×10^9 cells/ml from flask B9, cells and 8g/L of PCB, at 6.9 days; this is more visible in Figure 9.

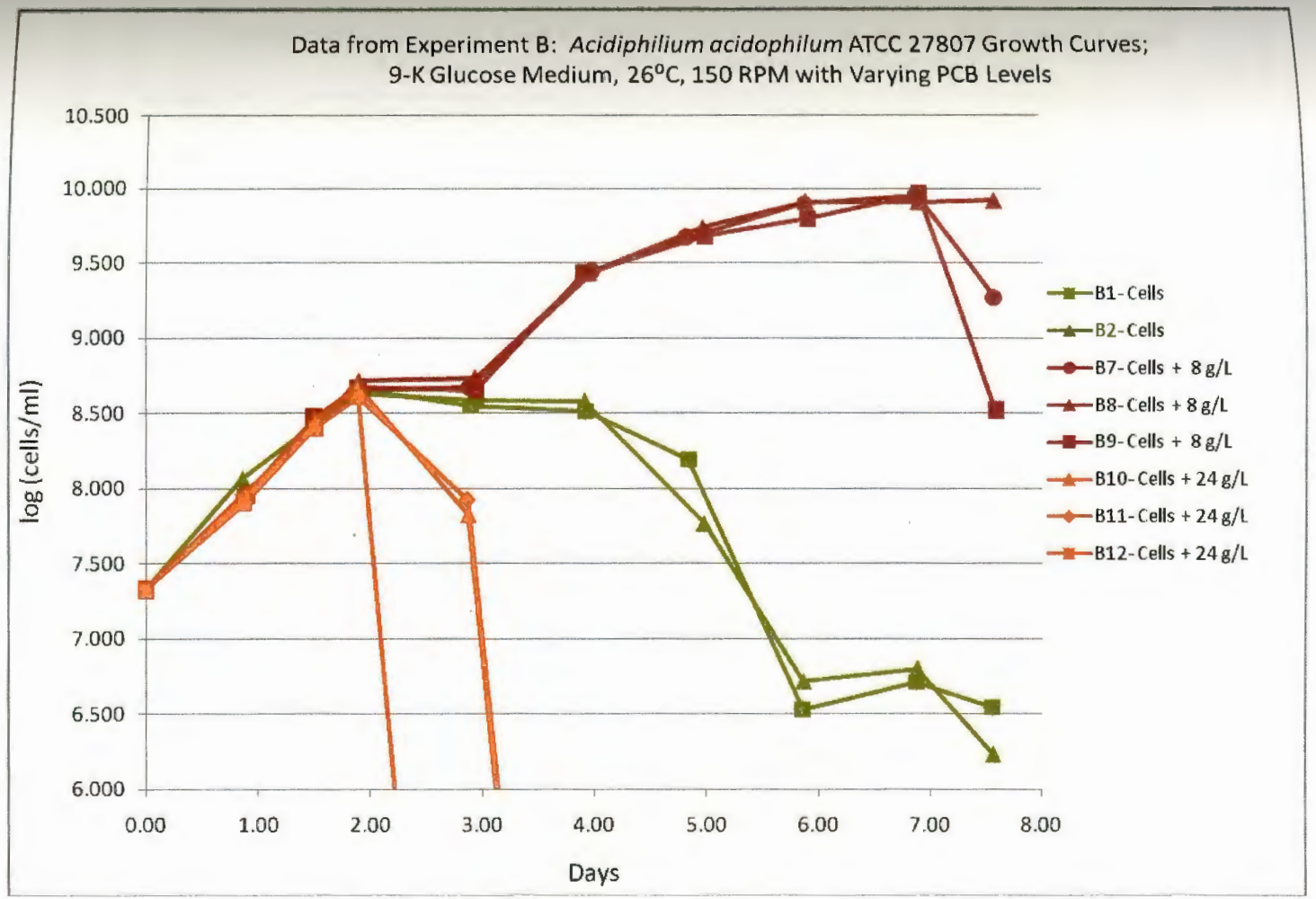

Figure 8: Growth Curves of Experiment B

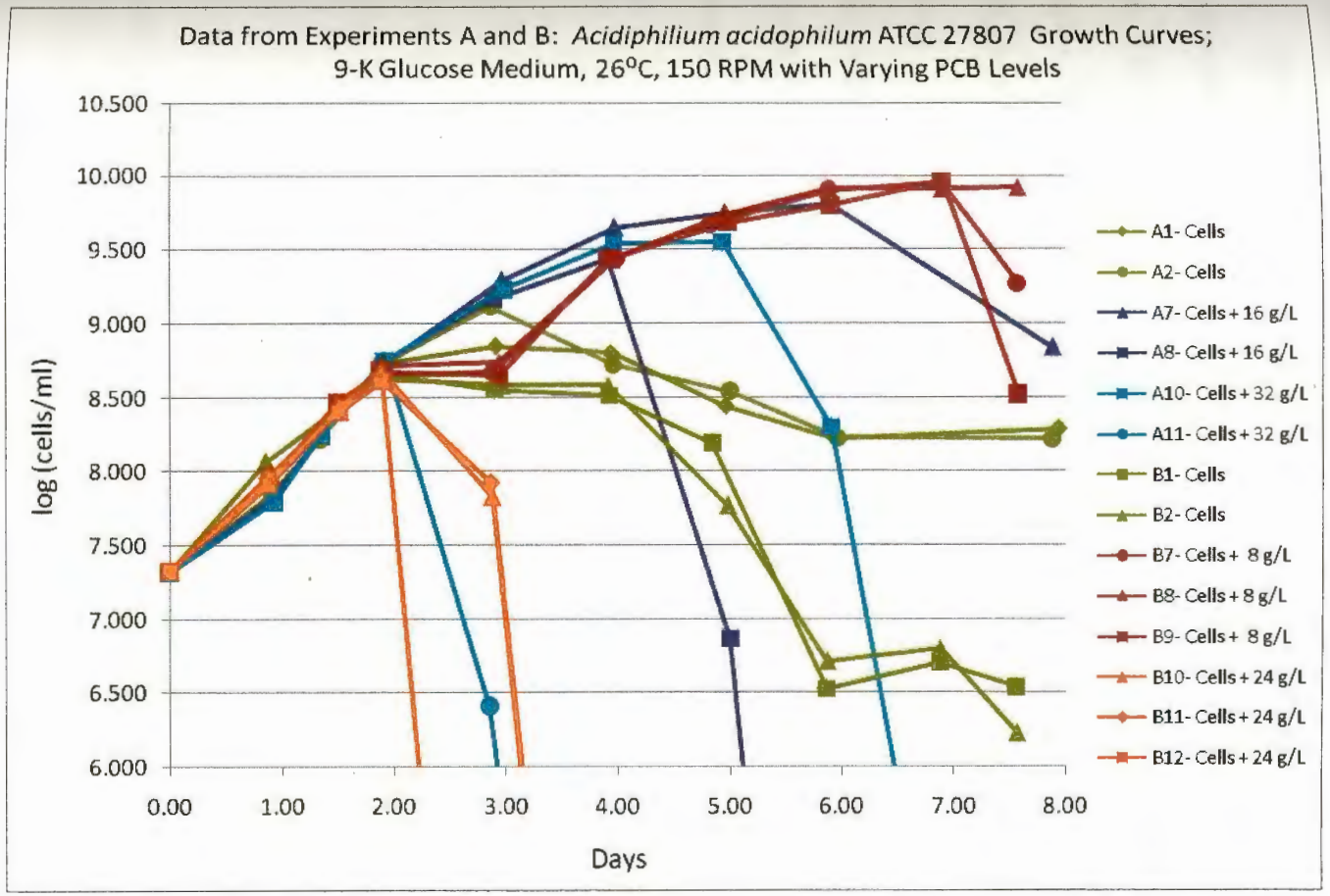

Figure 9: Growth Curves of Experiments A and B

3.4 pH During Printed Circuit Board Experiments

pH results are shown in Figures 10 through 13. In Figure 10, pH results for Experiment A are shown. As expected flasks A1 and A2 have pHs that decrease over time. This is consistent with the fact that *Acidiphilium acidophilum* are acid loving bacteria. These cells thrive in pHs that are between 1.5 and 6.0. *Acidiphilium acidophilum* ATCC 27807 is capable of chemolithoautrophic and heterotrophic growth. They oxidize "elemental sulfur, sugar, amino acids, and carboxylic acids" (Rehm and Reed ed., 1988). A hypothetical statement would be that *Acidiphilium acidophilum* ATCC 27807 are helping with the acid generation of pyruvic acid, since glucose is the main energy source. Further investigation needs to be done to confirm this.

In Figure 10, as PCB level increased so did the pH. This phenomenon holds true for experimental units with cells and without cells. One can also observe this increase in pH by looking at Figure 11, pH data from Experiment B. By combining pH data into two groups, flasks with cells (Figure 12) and flasks without cells (Figure 13), this fact is clearer. One possible reason for the increase in pH could be due to the reactions in the medium caused by the addition of PCB. For example, copper may be directly leached out by the following reaction: $Cu^{o} + 2H_2O + O_2 \rightarrow 2\ Cu^{2+} + 4\ OH^{-}$ (Choi, 2004). By releasing OH^{-} into the bioleaching environment, pH will subsequently decrease. Of course other reactions may also be occurring that could be contributing to the alkalinity of the experimental solutions. Those reactions were not further investigated. Copper as denoted in the Materials and Methods section is the dominate metal within the PCB sample that was leached.

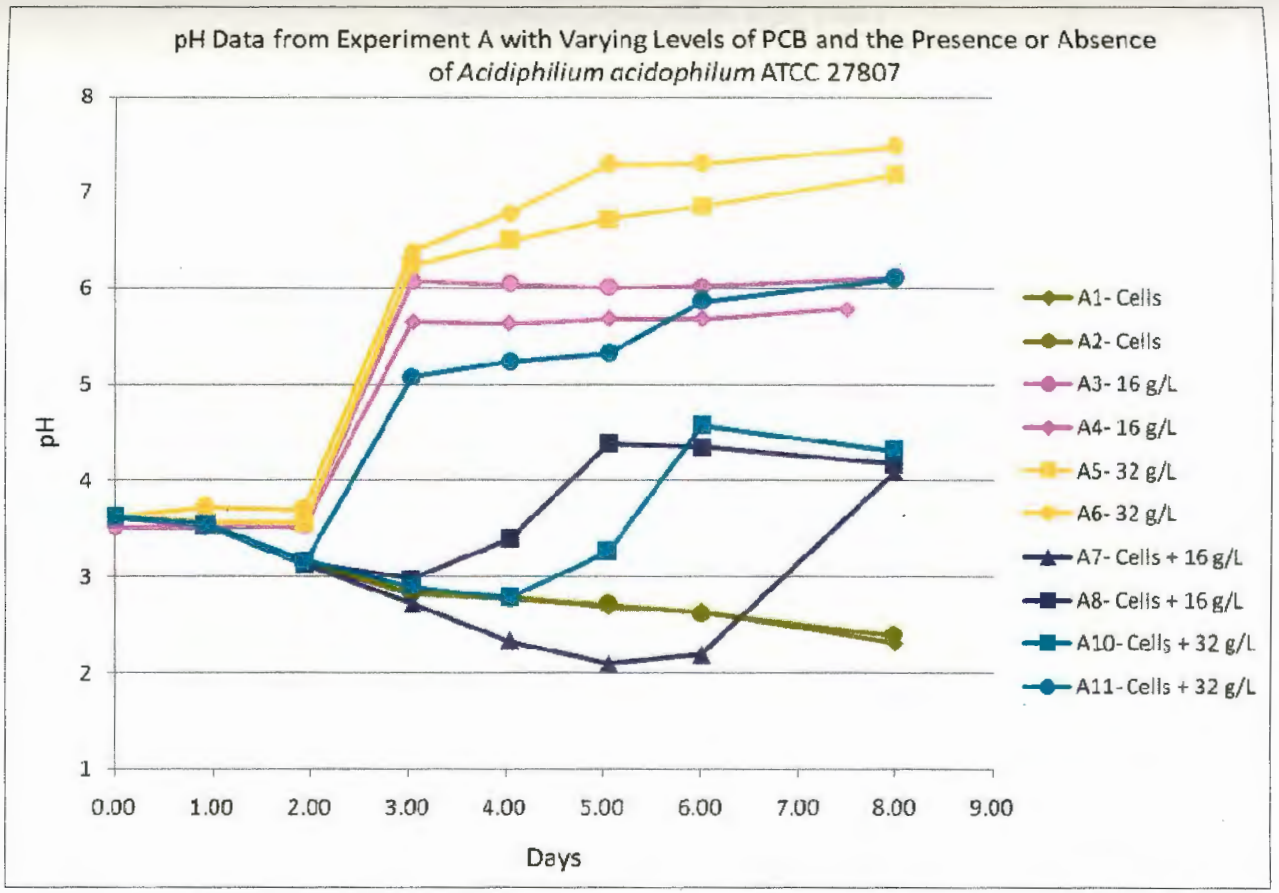

Figure 10: pH Data from Experiment A

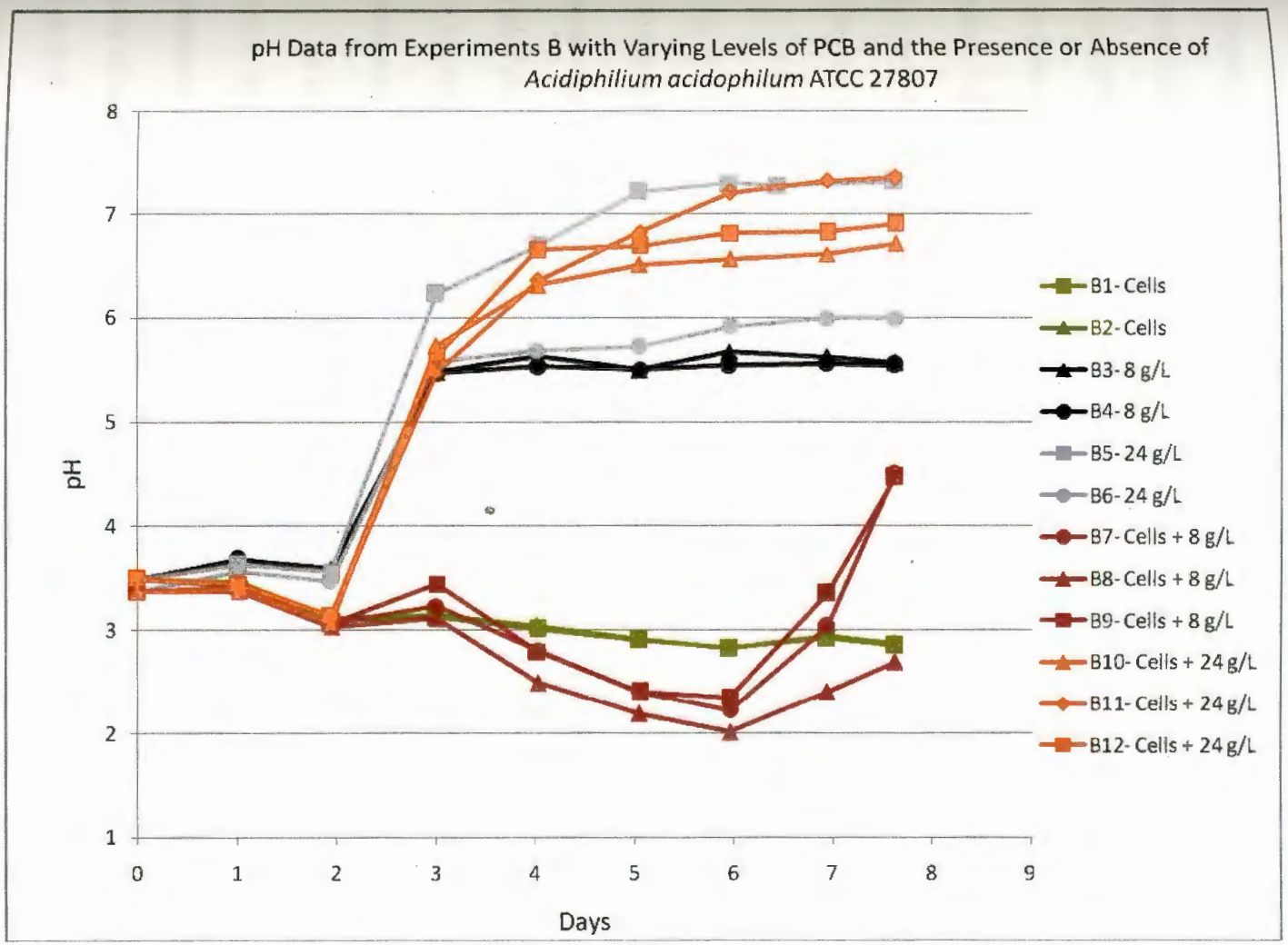

Figure 11: pH Data from Experiment B

Another important observation is in Figure 12, pH of flasks with cells. More flasks have lower pH than that of Figure 13, pH of flasks without cells. This shows that the interactions of cells at certain levels of PCB are still able to generate enough acid to decrease pH. This is shown in Figure 10 with flask A10, cells and 32 g/L of PCB, behaving differently than flasks A5 and A6, flasks with just 32g/L of PCB. Flasks A7 and A8, compared their medium leaching counterparts A3 and A4, also have lower pHs. In Figure 11, only flasks with 8 g/L show this relationship. Flasks B7, B8 and B9 all have lower pH than their counterparts without cells.

Above 16 g/L of PCB, as shown in Figure 12, almost all pH levels reach 6 or above. A pH above 6 is detrimental to cells since they have only been recorded to live in pH conditions of 1.5-6 (Krebs, 1997). One theory is that above 16 g/L of PCB experimental units behave as if cells are not present/dead. Comparing this finding to the growth curves in Figure 9, most experimental units with greater than 16 g/L of PCB significantly decreased in cell density once PCB was introduced into the environment.

In the flasks that do not go above pH 6.0 are those with PCB densities equal to or lower than 16g/L. In Figure 7 and Figure 8, the cell growth curves of both experiments A and B prove that the cells are growing at higher cell densities than their control growth curves at PCB densities equal to or less than 16 g/L. Flask A10, 32 g/L of PCB with cells also shows this trend, however it isn't validated with the duplicated flask: A11.

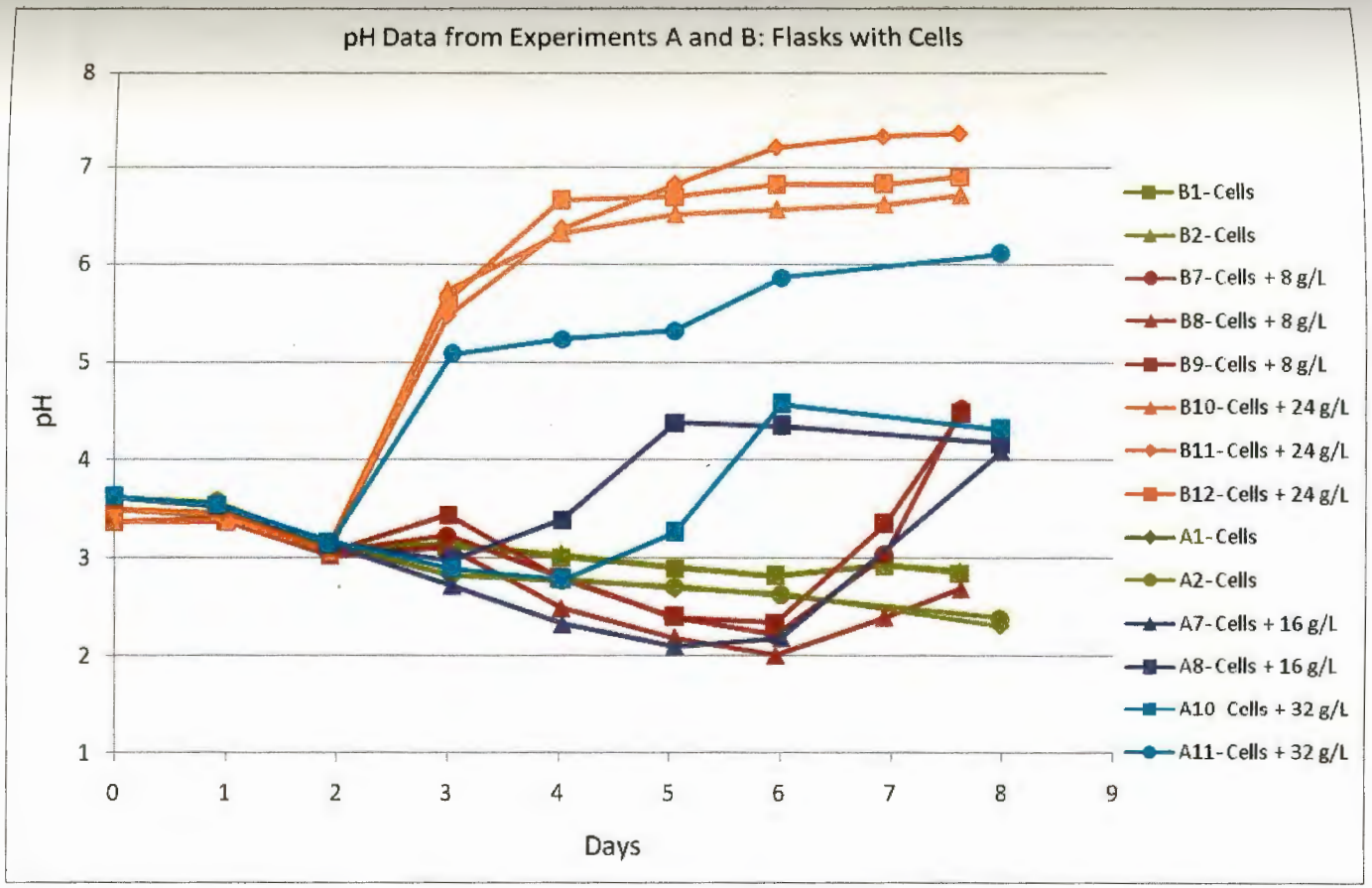

Figure 12: pH Data for Experimental Units with Cells Present

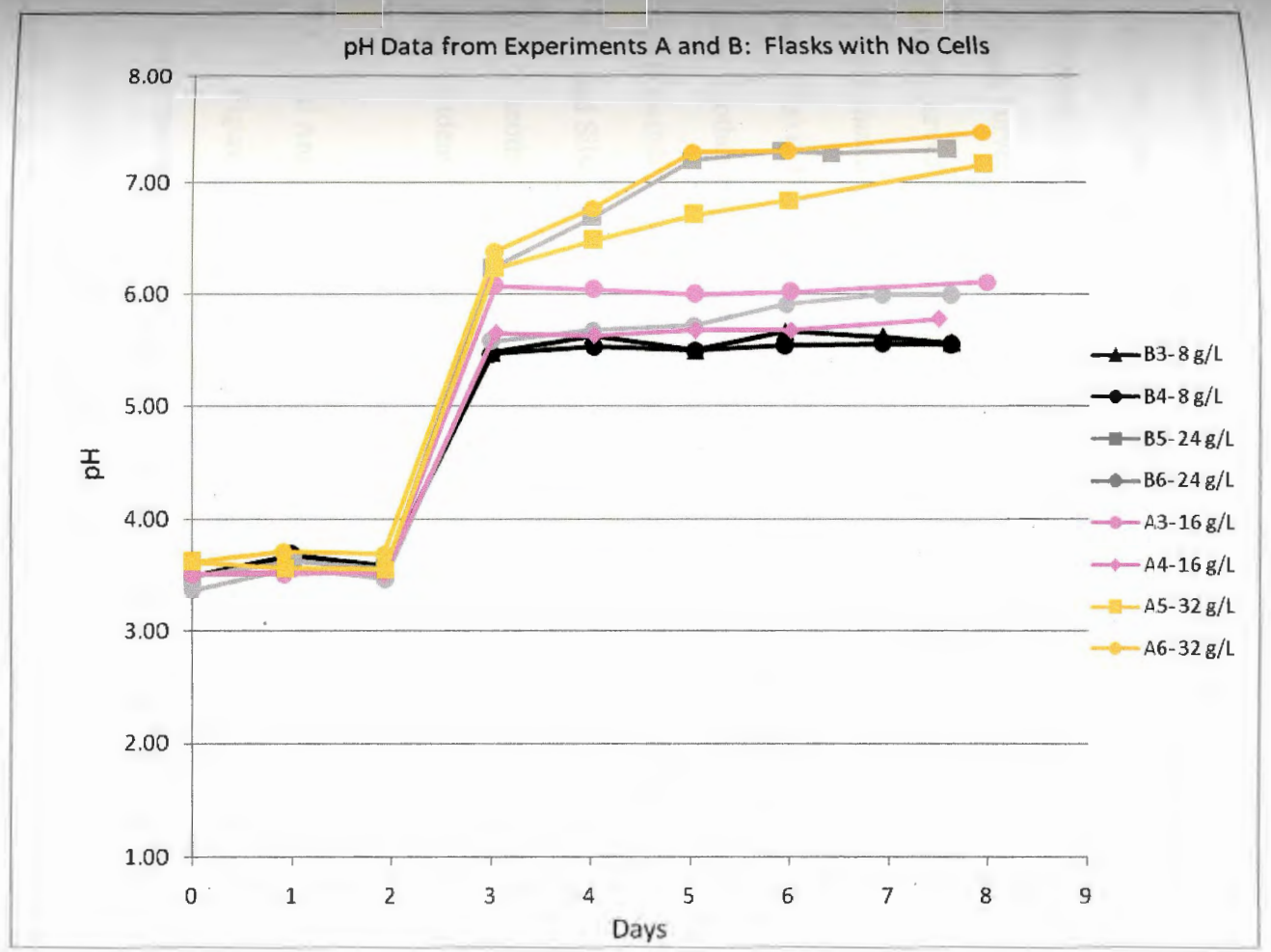

Figure 13: pH Data from Experimental Units with no Cells Present

Other than the pH of the medium, other factors may also be affecting the environment that the cells are growing in. An interesting phenomenon occurred in flasks that contained cells and 8 g/L of PCB. Revisiting Figure 8 and Figure 11, flask conditions for 8g/L and cells show pH lower than the normal pH curves: B1 and B2. Growth curves for B7, B8 and B9 also shows higher cell densities than the "control" growth curves: B1 and B2. This would signify that at specific concentrations of PCB *Acidiphilium acidophilum* ATCC 27807 grows better because of interactions with material(s) in PCB. One thought is *Acidiphilium acidophilum* ATCC 27807 may have found another energy source. This is a feasible hypothesis since this organism can readily switch between energy sources, specifically glucose and sulfur as denoted by Guay and Silver (1975). One possibility is there may be a sulfur energy source in the PCB or another energy source for *Acidiphilium acidophilum* ATCC 27807 that has not yet been identified.

3.5 Metal Analysis

Figure 14 and Figure 15 show results for amount of metal in solution for experiments A and B respectively. Each value shown is an average of 3 samples taken from the same experimental unit. The standard deviations of the three samples are shown in the "STD" column in 8 and Table 9. The "blanks" for the metal sampling technique in Table 8 and Table 9 have very low metal concentrations; therefore, it can be deduced that the metal sampling technique used for obtaining samples did not significantly the affect results. "Blanks" will be ignored in further discussion. Averaging the metal analysis readings among duplicates and triplicates was not an

option due to the heterogeneous nature of the shredded printed circuit boards. Therefore individual data from experimental units were assessed separately.

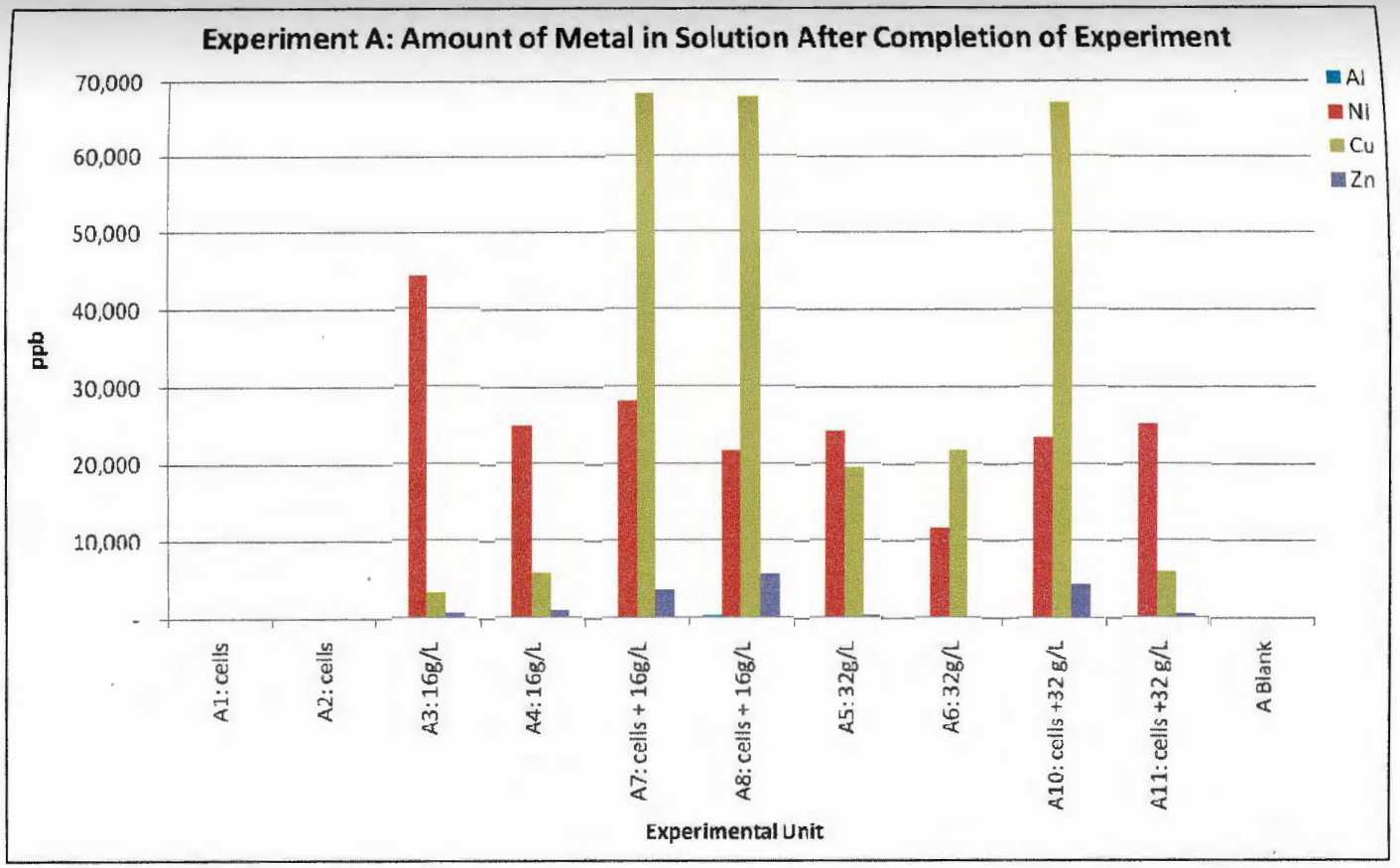

Figure 14: Metal Analysis for Experiment A

Table 8: Metal Analysis Results for Experiment A

Flask and Description	Al Average values	STD	Ni Average values	STD	Cu Average values	STD	Zn Average values	STD
				(Values are in ppb)				
A1: Cells	36.34	0.90	3.14	0.57	14.94	0.43	85.06	0.98
A2 Cells	26.45	1.06	1.78	0.15	15.15	0.26	76.75	1.05
A3: no cells + 16g/L	3.88	0.40	44,203.33	535.75	3,322.33	82.25	505.20	9.90
A4: no cells + 16g/L	2.77	0.20	24,750.00	944.09	5,695.33	176.83	918.27	17.90
A7: cells + 16g/L	134.13	4.92	27,970.00	741.82	68,280.00	91.65	3,633.00	57.56
A8: cells + 16g/L	232.50	2.55	21,590.00	17.32	67,736.67	40.41	5,514.67	57.42
A5: no cells + 32g/L	7.97	0.22	24,073.33	423.36	19,480.00	546.72	245.63	3.91
A6: no cells + 32g/L	0.98	0.10	11,560.00	533.57	21,476.67	805.25	79.44	5.80
A10: cells +32 g/L	70.77	0.62	23,366.67	175.59	66,983.33	118.46	4,245.00	32.92
A11: cells +32 g/L	0.24	0.19	24,983.33	312.14	5,783.00	129.46	384.17	10.55
A Blank for sampling technique	0.03	0.27	4.29	6.67	9.91	2.65	4.11	1.64

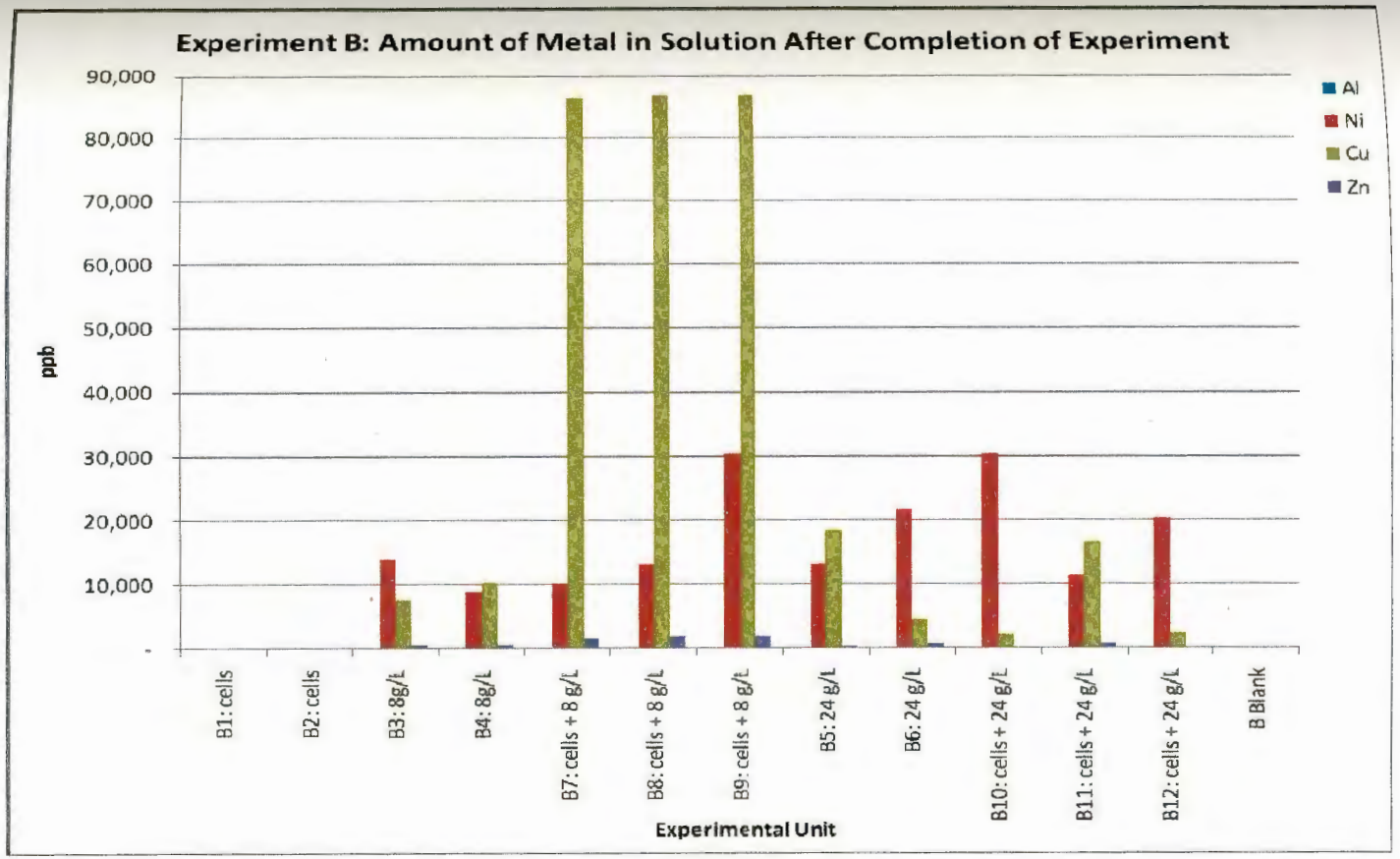

Figure 15: Metal Analysis Results for Experiment B

Table 9: Metal Analysis Results for Experiment B

Flask and Description	Al Average values	Al STD	Ni Average values	Ni STD	Cu Average values	Cu STD	Zn Average values	Zn STD
				(Values are in ppb)				
B1: cells	18.54	0.89	7.97	11.21	8.18	6.66	36.60	1.89
B2: cells	23.65	0.34	1.25	0.24	3.71	0.26	29.19	0.62
B3: no cells + 8g/L	4.96	0.02	13,900.00	52.92	7,527.67	16.44	629.10	0.78
B4: no cells + 8g/L	2.47	0.57	8,805.33	18.90	10,236.67	20.82	559.20	2.39
B7: cells + 8 g/L	19.63	0.12	10,146.67	112.40	86,463.33	51.32	1,589.33	17.62
B8: cells + 8 g/L	31.75	2.01	13,133.33	83.86	86,660.00	10.00	1,963.33	7.77
B9: cells + 8 g/L	4.56	0.02	30,393.33	237.56	86,720.00	0.00	1,950.67	12.34
B5: no cells + 24 g/L	0.62	0.62	13,163.33	125.03	18,453.33	181.75	365.63	1.77
B6: no cells + 24 g/L	2.80	0.24	21,723.33	282.90	4,628.00	66.73	779.57	6.76
B10: cells + 24 g/L	2.05	0.03	30,386.67	130.13	2,203.00	96.32	29.93	1.20
B11: cells + 24 g/L	0.78	0.62	11,350.00	81.85	16,613.33	167.43	862.10	7.15
B12: cells + 24 g/L	0.45	0.47	20,316.67	188.24	2,367.00	34.04	34.66	0.70
B Blank for the sampling technique	0.21	0.79	22.29	32.57	26.25	9.94	5.60	0.49

3.5.1 Nickel

Table 8 and Table 9 shows that nickel values are relatively similar in terms of magnitude with all flasks that had printed circuit board (PCB) material. This is shown at every level of PCB used in experiments. In 8, experimental units with just 16 g/L of PCB had levels 44 and 24 mg/L; on the other hand, experimental units with PCB at the same level and cells had levels of 27 and 21 mg/L. Looking at the data for 16 g/L with cells and without cells there is not enough data to conclude leaching at this level since 27, 21 and 24 mg/L are too similar. 32 g/L of PCB also has a similar occurrence. In flasks A5 and A6, 32 g/L of PCB, nickel levels in solution were 24 and 11 g/L and in flasks A10 and A11, cells and 32 g/L of PCB, nickel values were 23 and 25 mg/L. Once again this information is not clear enough to assume bioleaching. Looking at the other levels in Table 9 the same phenomenon occurs. For flasks B3 and B4, 8 g/L PCB, nickel levels were 13 and 8 mg/L, with cells and 8g/L of PCB values were 10, 13 and 30 mg/L. Similarly with flasks that had 24 g/L of PCB without cells had values of 13 and 21 mg/L, and flasks that had cells with 24 g/L of PCB had values of 30, 11 and 20 mg/L. The nickel values for all levels of PCB ultimately show that nickel was not leached at any level by *Acidiphilium acidophilum* ATCC 27807. This is easily visible in 16. Medium leaching definitely played a role in leaching nickel; this can be deduced because flasks with and without cells have the same nickel levels of PCB. The control experimental units with just cells shows that only a very small amount of nickel was in solution; therefore the cells individually did not affect nickel metal analysis results.

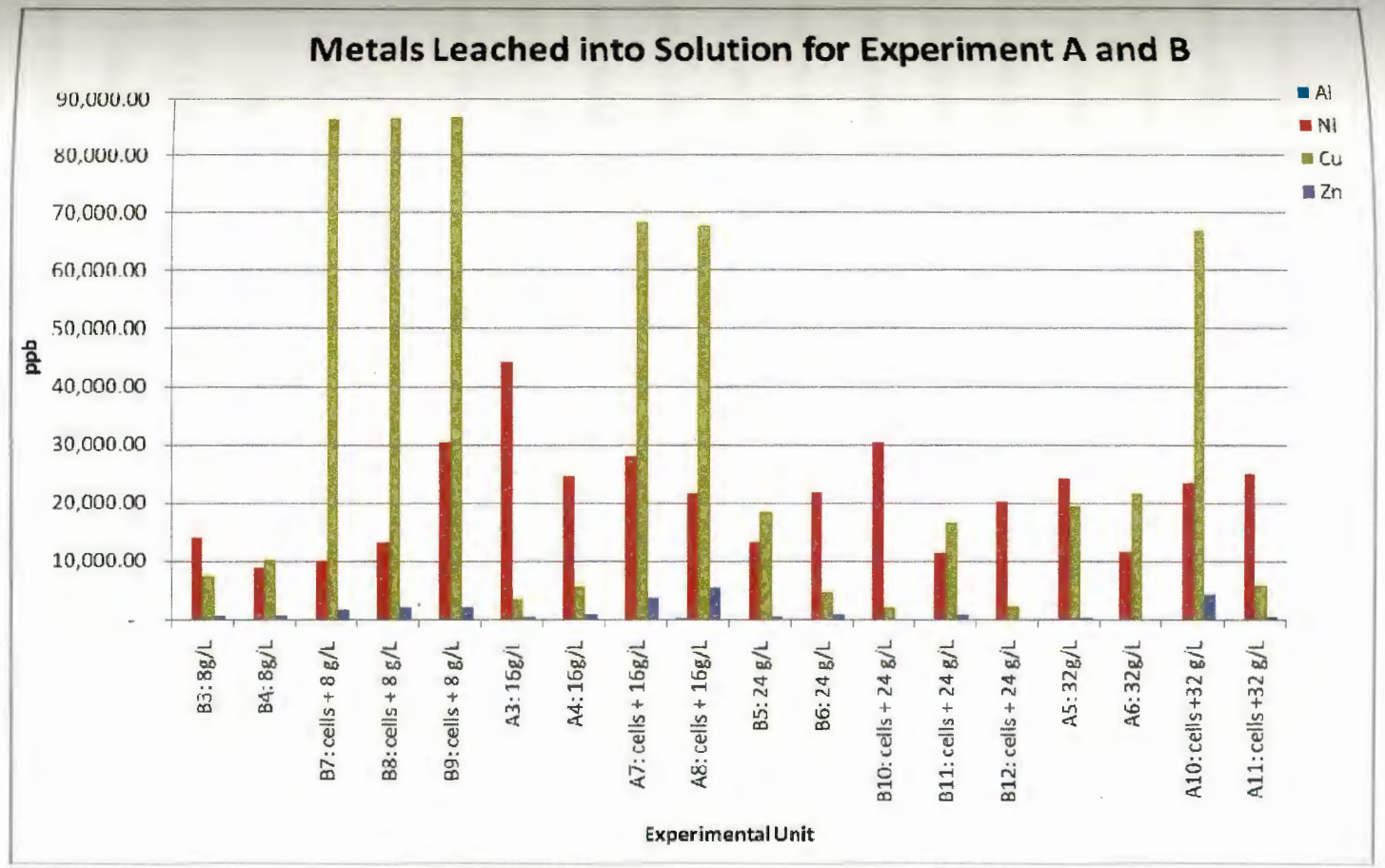

Figure 16: Metal Analysis for Experiments A and B

3.5.2 Copper

A. acidophilum showed promise in terms of bioleaching copper. Metal analysis results for both Experiments A and B are shown in Figure 16. Experimental units with cells and 8 g/L had values of copper in solution about 86 mg/L across all three experimental units. Experimental units with just cells looked normal in comparison to the normal growth curves done before PCB experiments. Medium leaching at 8 g/L was not large enough to discount cells bioleaching metal, but values were significant: 7 and 10 mg/L. Experimental units with 16 g/L had a similar phenomenon where copper in solution had a value of ~68 mg/L; in addition medium leaching at this level of PCB only had values of ~ 3 and ~6 mg/L. Bioleaching at 24 g/L of PCB were inconclusive since flasks with cells had values very similar to those without cells. And finally results for 32 g/L were also inconclusive because the values were similar in range between flasks with cells and PCB material compared to their medium leaching counterparts. Flasks A10 and A11, 32 g/L of PCB with cells, had values of 67 and 6 mg/L while flasks A5 and A6, 32 g/L of PCB, had 19 and 21 mg/L.

3.5.3 Aluminum

The bioleaching by *A. acidophilum* of aluminum was not clear. As seen in Figure 17 the levels of aluminum for flasks with just cells seemed high considering no aluminum was introduce into these flasks. An initial response to these values was that the aluminum covering the experimental units may have affected the results. However these "off" values did not appear in all experimental flasks that contained cells; for flasks with just cells values ranged from 18.54 ppb to 36.34 ppb. Some bioleaching

may be occurring at 16 g/L of PCB; values at this level reached 134.13 ppb and 232.50 ppb. These values are significantly higher than the flasks with just cells and the flasks with 16 g/L PCB only (3.88 ppb and 2.77 ppb). However, more experiments need to be performed in order to confirm this. Metal values of such small magnitude (in ppb) may also be contributed to contamination. Other aluminum values were too small or in the case of flask A10, cells and 32 g/L, didn't have duplicate data to confirm metal values in solution.

Figure 17: Metal Analysis for Aluminum in Solution

3.5.4 Zinc

For the last metal zinc, which can be seen better in Figure 18, a significant amount was present in the control flasks with just cells: 85.06, 76.75, 36.60 and 29.19 ppb. However, these control values are not large enough to discount values of soluble Zn in other samples. Flasks B7, B8 and B9 (8 g/L of PCB and cells) have soluble zinc levels that ranged from 1,589.33 ppb to 1,963.33 ppb; flasks without cells however, only had values of 559.2 ppb and 629.10 ppb. Similarly at 16 g/L of PCB with cells present values ranged from 3,633.00 ppb to 5,514.67 ppb, while flasks without cells present had values of 505.20 ppb and 918.27 ppb. At both levels of PCB, 8 g/L and 16 g/L, the cells are bioleaching zinc into solution. Other experimental units showed inconclusive data for zinc bioleaching. Flasks B5 and B6 (24 g/L of PCB) had values for experimental units that fell in the same range as flasks B10, B11 and B12 (24 g/L of PCB and cells); therefore bioleaching at this level is inconclusive. At 32 g/L values between the two duplicates were not confirmed, since the magnitude between them was over 8; this level of PCB was concluded that bioleaching of zinc was not occurring at this level.

Figure 18: Metal Analysis for Zinc in Solution

4 CONCLUSIONS

Bioleaching for recovering metal from PCBs is a promising technique that needs to be investigated further. As shown in these experiments the maximum amount of copper leached was 86 mg/L at 8 g/L of PCB. Approximately 9.6% of total copper is leached, with 8.6% of the total copper leached specifically by *A. acidophilum*. The maximum amount of zinc was leached 3.9 mg/L at 16 g/L of PCB. This yielded a 42.8% total recovery of zinc in solution; 36.3% of the total recovery was contributed by *A. acidophilum.* One main experimental limitation is the repeatability of the experiments due to the complexity and heterogeneous nature of various computer printed circuit board compositions.

4.1 Bioleaching Metals

Analyzing the overall data, the best conditions to bioleach the most copper was 8 g/L of PCB with cells. Also at 8g/L of shredded printed circuit boards cells also grew at their highest density: ~9.10 *10^9 cells/ml at 6.90 days; this can be seen clearer in Figure 9. For the death phase to begin cell nutrients within a solution start to become depleted. This can be seen in Figure 5 at around 4 days for the control growth curve of *Acidiphilium acidophilum* ATCC 27807. Because of the addition of the shredded PCB cells came out of the stationary phase and started to go into the exponential phase signifying a new found source for nutrients. (See Figure 8.) *Acidiphilium acidophilum* ATCC 27807 is known for readily switching energy sources between glucose and sulfur. A good hypothesis is that some sulfur/or alternate energy source is contained in the printed circuit boards that gave them the extra boost they

needed to outgrow their control growth patterns. At these conditions more acid was generated as seen with pHs as low as 2 in Figure 12. Considering that copper is the dominate metal in solution it wasn't surprising that it was leached the most. Further experiments at lower levels than 8 g/L should be performed, to see if 86 mg/L is the maximum amount that can be put into solution in this bioleaching environment.

A similar occurrence happened with cells with 16 g/L of PCB for copper leaching at 8 g/L of shredded PCB and cells. Levels of copper at 16 g/L and cells still yielded high levels of copper in solution, around 68 mg/L. Again cell densities surpass the normal growth curve with experimental units that had cells and 16 g/L of PCB. (See Figure 7.) pH was low as well at these conditions as expected and can be seen in Figure 10.

A. acidophilum unfortunately didn't leach nickel into solution; values were very similar across all experimental units that contained shredded PCB levels. Aluminum was also not bioleached by *A. acidophilum*. Highest aluminum levels leached were 134.13 ppb and 232.50 ppb in flasks with cells and 16 g/L. These values are so small on a magnitude level that contamination could very well explain them.

Zinc, on the other hand, does show bioleaching by *A. acidophilum* at shredded PCB levels of 8 and 16 g/L with the highest leaching occurring at 16 g/L. At 16 g/L of PCB and cells, values ranged from 3.6 to 5.5 mg/L. In terms of the zinc and copper, it is presumed that other levels were not leached due to the increase of pH by PCB.

4.2 Possible Mechanisms Occurring in this Study

As mentioned in the Introduction section, there are many different mechanisms that occur for metal mobilization or metal immobilization. In this study according to pH, growth curves and metal analysis results, *Acidiphilium acidophilum* ATCC 27807 are causing more metals to mobilize in solution. Acidolysis, a metal mobilization mechanism, occurs when microbes help to generate protons that ultimately bind to surfaces. As a result, the surface starts to weaken chemical bonds causing metal ions to dissociate from their original form (Brandl and Faramarzi, 2006). It is possible that *Acidiphilium acidophilum* ATCC 27807 first oxidizes glucose to produce pyruvic acid. Then, when PCB is introduced at a concentration less than or equal to 16 g/L, the microbe switches energy sources to a material in the PCB that is oxidized to produce even more protons. In addition, there is the possibility that these bacteria are specific to copper and zinc in these experiments. Chemical leaching is non-specific to the metal being leached. *A. acidophilum* could be only producing biofilms on copper and zinc which subsequently leads their bioleaching.

5 SUGGESTED FUTURE WORK

Other than refining these experiments to confirm inconclusive results other interesting options for expanding upon this project are listed here. Adding a complexing/chelating agent to bioleaching environment may help to leach more metals into solution. Choi et al. (2004) for example, used citric acid to aid in bioleaching process. By adding citric acid to experiments Choi et. al. increased the solubility of metals in solution from 37% to 87% by weight, thus subsequently making the bioleaching process even more efficient. Other complexing/chelating agents other than citric acid could be researched as well.

The possible addition of an acid could also be a possible extension to these experiments. Since the death of the cells most likely occurred due to the heighted pH, the addition of the acid may allow for *A. acidophilum* growth.

Leaching with more than one microorganism could also make the bioleaching environment more efficient (Agate, 1996; Rawlings, 1998). In fact the initial premise to these experiments was to use two microorganisms; however, *Acidithiobacillus thiooxidans* ATCC 15494 became problematic to enumerate. An immediate count could be established with *Acidiphilium acidophilum* ATCC 27807 using a spectrophotometer since its growth medium is transparent. *Acidithiobacillus thiooxidans* ATCC 15494, on the other hand, had sulfur floating within its growth medium which made the spectrophotometer obsolete in terms of a useful immediate enumeration technique.

In order to understand the reason for specific bioleaching, biofilms on the surface of the printed circuit board material should also be looked into. Perhaps biofilms are being formed on zinc and copper and not on any other surfaces.

Expanding this project even further, in terms of recycling, future studies could include research on how to recover the metals from solution, the disposal/reuse of the culture medium as well as what to do with the left over PCB material. More interestingly in terms of usefulness in industry, scale up and associated costs could also be researched.

APPENDIX

A. Microorganisms Known for Bioleaching

Table A1: "Selection of Microorganisms Known to Mediate Metal Bioleaching Reactions" (Krebs, 1997)

Organism	Type	Nutrition	Main leaching agent	pH range	Temperature range
Acetobacter methanolicus	bacteria	heterotrophic	gluconate	acidiphilic	
Acidianus brierleyi	bacteria	facult. heterotrophic	sulfuric acid	acidiphilic	45-75
Acidophillum cryptum	bacteria	heterotrophic	organic acids	2.0-6.0	mesophilic
Actinomucor sp.	fungi	heterotrophic	oxalate, malate, pyruvate, oxalacetate		
Alternaria sp.	fungi	heterotrophic	ocalate, malate, pyruvate, oxalacetate		
Arthrobacter sp.	bacteria	heterotrophic			
Aspergillus amstelodami	fungi	heterotrophic			
Aspergillus clavatus	fungi	heterotrophic	aspartate		
Aspergillus ficuum	fungi	heterotrophic	oxalate		
Aspergillus fumigates	fungi	heterotrophic			
Aspergillus niger	fungi	heterotrophic	oxalate, citrate, gluconate, lactate		
Aspergillus ochraceus	fungi	heterotrophic	citrate, glutamate		
Bacillus megaterium	bacteria	heterotrophic	citrate, amino acids		
Candida sp.	fungi	heterotrophic			
Cerostamella sp.	fungi	heterotrophic			
Chromobacterium violaceum	bacteria	heterotrophic	cyanide		
Cladosporium resinae	fungi	heterotrophic			
Coriolus versicolor	fungi	heterotrophic			
Corynebacterium sp.	bacteria	heterotrophic			
Crenothrix sp.	bacteria	facult. heterotrophic	ferric iron	5.5-6.2	18-24
Cunninghamiella sp.	fungi	heterotrophic			
Fusarium sp.	fungi	heterotrophic	oxalate, malate, pyruvate, oxalacetate		
Gallionella sp.	bacteria	autotrophic	ferric iron	6.4-6.8	6-25
Gleophyllum trabeum	fungi	heterotrophic	oxalate		
Leptospirillum ferrooxidans	bacteria	chemolithoautotrophic	ferric iron		30
Leptospirillum thermoferrooxidans	bacteria	chemolithoautotrophic	ferric iron		45-50
Leptothrix sp.	bacteria	facult. autotrophic	ferric iron, sulfuric acid	5.8-7.8	5-40
Metallogenium sp.	bacteria	heterotrophic	ferric iron	3.5-6.8	
Metallosphaera sedula	bacteria	chemolithoautotrophic	ferric iron, sulfuric acid	acidophilic	extr. thermophilic
Mucor sp.	fungi	heterotrophic	fumatate, gluconate		
Paecilomyces variotii	fungi	heterotrophic	malate		
Penicillium brevicompactum	fungi	heterotrophic			
Penicillium cyclopium	fungi	heterotrophic			
Penicillium funiculosum	fungi	heterotrophic	citrate, glutamate		
Penicillum notatum	fungi	heterotrophic			
Penicillium ochrochloron	fungi	heterotrophic	oxalate		
Penicillium oxalicum	fungi	heterotrophic	oxalate		
Penicilliym simplicissimum	fungi	heterotrophic	citrate, oxalate, gluconate		
Penicillium spinulosum	fungi	heterotrophic	oxalate		

Continued...

Table A1: "Selection of Microorganisms Known to Mediate Metal Bioleaching Reactions" (Krebs, 1997)

Organism	Type	Nutrition	Main leaching agent	pH range	Temperature range
Penicillium variotti	fungi	heterotrophic			
Phanerochaete chrysosporium	fungi	heterotrophic			
Pichia sp.	fungi	heterotrophic			
Pseudomonas putida	bacteria	heterotrophic	citrate, gluconate		
Rhizopus sp.	fungi	heterotrophic	lactate, fumarate, gluconate		
Schizophyllum commune	fungi	heterotrophic	malate		
Sclerotium rolfsii	fungi	heterotrophic	oxalate		
Siderocapsa sp.	bacteria	heterotrophic	ferric iron		
Sulfobacillus thermosulfidooxidans	archaea	chemolithoautotrophic	ferric iron, sulfuric acid	extremely acidophilic	50
Sulfobacillus thermosulfidooxidans sub. *thermotolerans*	archaea	chemolithoautotrophic	ferric iron, sulfuric acid	extremely acidophilic	37-42
Sulfobacillus thermosulfidooxidans	archaea	chemolithoautotrophic	ferric iron, sulfuric acid	extremely acidophilic	50
Sulfolobus acidocaldarius	archaea	chemolithoautotrophic	ferric iron, sulfuric acid	0.9-5.8	55-85
Sulfolobus ambivalens	archaea	chemolithoautotrophic	ferric iron, sulfuric acid		extremely thermophilic
Sulfolobus solfataricus	archaea	chemolithoautotrophic	ferric iron, sulfuric acid		extremely thermophilic
Sulfolobus thermosulfidooxidans	archaea	chemolithoautotrophic	ferric iron, sulfuric acid		extremely thermophilic
Sulfolobus brierleyi	archaea	chemolithoautotrophic	ferric iron, sulfuric acid		extremely thermophilic
Sulfolobus yellowstonii	archaea	chemolithoautotrophic	ferric iron, sulfuric acid		extremely thermophilic
Sulfurococcus sp.	bacteria	mixotrophic	ferric iron, sulfuric acid	acidophilic	extremely thermophilic
Thermothrix thiopara	bacteria	chemolithoautotrophic	sulfuric acid	neutral	60-75
Thiobacillus acidophilus	bacteria	chemolithoautotrophic	sulfuric acid	1.5-6.0	25-30
Thiobacillus albertis	bacteria	chemolithoautotrophic	sulfuric acid	2.0-4.5	28-30
Thiobacillus capsulatus	bacteria	chemolithoautotrophic	sulfuric acid		
Thiobacillus concretivorus	bacteria	chemolithoautotrophic	sulfuric acid	0.5-6.0	
Thiobacillus cuprinus	bacteria	chemolithoautotrophic	sulfuric acid		
Thiobacillus delicatus	bacteria	mixotrophic	sulfuric acid		25-30
Thiobacillus denitrificans	bacteria	chemolithoautotrophic	sulfuric acid	5.0-7.0	30
Thiobacillus ferrooxidans	bacteria	chemolithoautotrophic	ferric iron, sulfuric acid	1.4-6.0	28-35
Thiobacillus intermedius	bacteria	chemolithoautotrophic	sulfuric acid	1.9-7.0	30
Thiobacillus kabobis	bacteria	mixotrophic	sulfuric acid	1.8-6.0	28
Thiobacillus neoplitanus	bacteria	chemolithoautotrophic	sulfuric acid	3.0-8.5	28
Thiobacillus novellus	bacteria	chemolithoautotrophic	sulfuric acid	5.0-9.0	30
Thiobacillus organoparus	bacteria	mixotrophic	sulfuric acid	1.5-5.0	27-30
Thiobacillus perometabolis	bacteria	chemolithoheterotrophic	sulfuric acid	2.6-6.8	30
Thiobacillus prosperus	bacteria	chemolithoautotrophic	sulfuric acid		
Thiobacillus rubellus	bacteria	chemolithoautotrophic	sulfuric acid		25-30
Thiobacillus tepidarius	bacteria	chemolithoautotrophic	sulfuric acid		
Thiobacillus thiocynoxidans	bacteria	chemolithoautotrophic	sulfuric acid		
Thiobacillus thiooxidans	bacteria	chemolithoautotrophic	sulfuric acid	0.5-6.0	10-37
Thiobacillus thioparus	bacteria	chemolithoautotrophic	sulfuric acid	4.5-10.0	11-25
Thiobacillus versutus	bacteria	chemolithoautotrophic	sulfuric acid		

Continued...

Table A1: "Selection of Microorganisms Known to Mediate Metal Bioleaching Reactions" (Krebs, 1997)

Organism	Type	Nutrition	Main leaching agent	pH range	Temperature range
Trametes versicolor	fungi	heterotrophic			
Trichoderma harzianum	bacteria	heterotrophic			
Trichoderma viride	bacteria	heterotrophic			
Yarrowia lipolytica	fungi	heterotrophic	citrate		

B. Serial Dilutions and Drop Plating Protocol

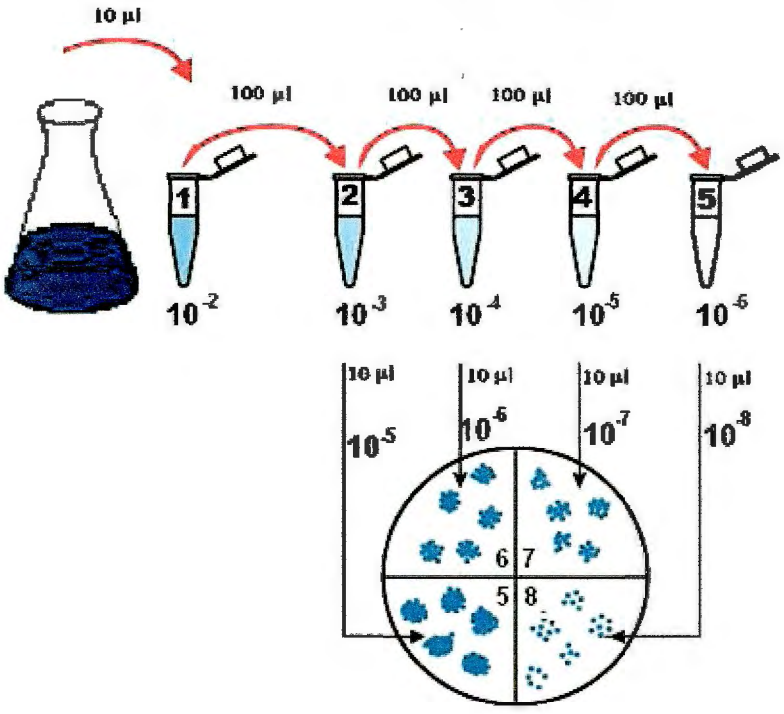

Figure A1: Schematic of Serial Dilutions and Drop Plating (Biofilms ONLINE)

Serial dilutions were completed as shown in the figure above.

1. 1.7 ml sized microtubes were filled with sterile 9-K glucose liquid medium solution in the following manner:
    a. Microtube 1: 990 µl
    b. Microtubes 2-5: 900 µl
2. The flask/tube to be tested for cell density was mixed by manually shaking or vortexing. 10 µl of culture was then placed into a microtube 1 containing 990 µl of sterile culture medium. (This solution is 10^{-2} times the dilution of the original solution.)

3. Cap microtube 1 and vortex it. Take 100 µl from the first microtube and transfer it to the second microtube that will already contain 900 µl of sterile culture medium. Cap the microtube 1 and 2. (The solution in microtube 2 is 10^{-3} times that of the original solution.)
4. The third, fourth and fifth microtubes are prepared the same as the second microtube where 100 µl of the previous microtube is placed into the next microtube that contains 900 µl of sterile culture medium. (See Figure A1.) Be sure to vortex each solution before it is transferred into the next microtube. Also change your micropipette with each transfer.
5. Once all the dilutions are finished as shown in the picture, the next step is drop plating. Take an agar plate and draw two straight lines that intersect perpendicularly on the bottom side of the petri dish. (See Figure A1.) Also label quadrants in relationship to their dilution factor. i.e. in the quadrant that will receive drops containing a dilution factor of 10^{-5}, label the corner closest to the center with 5.
6. Take microtube 5 vortex and pipette at least 4 drops, each 10 µl, onto the plate in quadrant 8 making sure that the drops are spaced out. Note that only plating 10 µl changes the dilution factor: i.e. the fifth microtube dilution goes from 10^{-6} to 10^{-8}.
7. Repeat step 6 three more times as shown in the Figure A1 working backwards with the microtubes. By working backwards you can use one pipette for the drops being placed on the plate.

8. Repeat all steps 1-7 with new microtubes and an agar plate, this will ensure that the dilutions scheme was correct by comparing the 2 resulting plates later after incubation.
9. Place plates in incubator with the temperature of 26°C.
10. Once *Acidiphilium acidophilum* ATCC 27807 starts to grow and you can see colonies forming, you can start counting the plates. Remember each drop represents a count of cells. Only count drops that have cells between 10 and 100. The following will represent an example count: Quadrant 8 had 4 drops that averaged about 10 colonies. The cell density of the original culture is $10x10^8$ cells/ml or $1x10^9$ cells/ml.

(This technique was obtained from Dr. David Nelson, professor of the Microbiology Department at the University of Rhode Island in a private discussion.)

C. Media Preparation

ATCC medium: 738 9-K glucose liquid medium (1000 mL)

1. Obtain 1 2000 Erlenmeyer ml flask and fill with 1000 ml of deionized water.
2. Place a magnetic stirrer into the flask.
3. Add the components in Table A2, in order, making sure that each component is fully dissolved.

Table A2: Chemicals Added for the Preparation of 1L of 9-K Glucose Solution (Liquid/Solid)

Chemical	Amount
0.1 g/L stock solution of $FeSO_4*7H_2O$	100 µl
17.99g/L solution of $Ca(NO_3)_2*4H_2O$ Note that this solution is clear.	1 ml
$C_6H_{12}O_6*H_2O$	11 g
$(NH_4)_2SO_4$	3 g
KH_2PO_4	0.5 g
$MgSO_4*7H_2O$	1.024 g
KCl	0.1g

4. Adjust the pH of this solution to 3.5 using H_2SO_4. If the pH goes too low then use a solution of NaOH to bring the pH higher.
5. Filter-sterilize this solution using an autoclaved filter apparatus with a 0.22 µm sterile membrane filter.
6. Place the new solution into a new autoclaved 2000 ml flask. And label with date, pH, and "filter-sterile 9-K glucose medium." This solution has a shelf life of about a month in an air tight container in the refrigerator.

Components for medium and pH adjustment data was obtained from American Type Culture Collection (2006).

ATCC medium: 738 9-K glucose solid medium (1000 mL)

1. Obtain a 1000 ml clean flask and fill it with 500 ml deionized water.
2. Place in magnetic stirrer into the flask.
3. Repeat step 3 in "ATCC medium: 738 9-K glucose liquid medium (1000 mL)."
4. Adjust the pH of this solution to 4.5 using H_2SO_4. If the pH goes too low then use a solution of NaOH.
5. Filter-sterilize this solution using an autoclaved filter apparatus with a 0.22 μm filter. This solution is called the 9-K basal glucose solution 2x concentrated.
6. Obtain a new 1000 ml cleaned flask. And aseptically place the filtered solution into it. (At this point you can save the solution in the refrigerator for later use, if doing so label it "2X 9-K glucose solution", date made, "filter-sterile" and pH of the solution.)

   To continue making plates continue to the next step.
7. If the solution has been refrigerated, equilibrate at room temperature for 12 hours before autoclaving.
8. Obtain another 1000 ml clean Erlenmeyer flask. Fill this flask with 250 ml of 0.2 μm filtered deionized water add 7.5 g of agar to it. This is 2x concentrated agar solution.
9. Obtain a 500 ml clean Erlenmeyer flask. Fill this flask with 250 of the 9-K basal glucose solution 2x concentrated.
10. Take both flasks and autoclave them both at 121°C, 15 psi for 31 mins.

11. Once the temperature of both solutions get down to about 55°C add the 9-K basal glucose solution 2x concentrated to the 1000 ml autoclaved flask with the concentrated agar. Gently mix the solution aseptically.
12. Obtain clean and sterile petri dishes and pour about 50 ml of solid medium into them. This solution should make approximately 8 glass plates/10 plastic plates. Note that it takes approximately 30 minutes for the solid medium to solidify so work quickly and efficiently.
13. Repeat steps 9-12 to make another set of plates if desired from prepared 9-K basal glucose solution 2x concentrated.

Components for medium and pH adjustment data was obtained from American Type Culture Collection (2006).

D. Metal Analysis Procedure of Obtaining Samples and Cleaning Protocol

These procedures are intended for any equipment that comes into contact with samples.

Equipment cleaning procedure for metal analysis:

Falcon Tubes/ Filtration Unit/ Experimental Units (baffled flasks)

1. Soak unit in 20% Liquid Grade Lab Detergent 80% DI water for 8 hours, and then scrub in similar solution.
2. Rinse with DI water several times.
3. Soak with 2 N nitric acid, using DI water for dilution, for at least 24 hours.
4. Rinse with DI water several times.
5. Air dry.

Sample collection procedure for samples (from baffled flasks of 100 ml)

Also through each set of samples created create a DI sample to go through the same process; designate it as the blank solution. This will allow the experimenter a reference point to see if the sampling technique itself is contaminating the samples.

1. Pour ~ 40 ml of the sample into 50 ml falcon tube.
2. Centrifuge at 4000 for 45mins.
3. Carefully pour off the supernatant into the filtration unit with 0.22 um filter or 0.2 um.

4. Carefully pour filtrate into a sample clean container. See sample storage section.
5. Carefully remove filter membrane.
6. Rinse out filter 3-4 times with DI water and shake remaining DI water out.
7. Replace filter membrane if further samples need processing and repeat steps 1 through 6.

Sample storage

1. Add 1/2ml amount of 2N Optima Nitric Acid to sample of 15 ml.
2. And store at 4°C, samples will be valid for about 6 months

(This technique was obtained from Skip Viator, professor of Civil and Environmental Engineering Department at the University of Rhode Island in a private discussion.)

Table A3: Data for Curves C and D for the Control Growth Curve

Curve C		Curve D	
Time, days	log(cells/ml)	Time, days	log(cells/ml)
0.00	7.32	0.00	7.32
0.48	7.47	0.53	7.52
1.00	7.92	0.53	7.50
1.49	8.40	1.00	7.80
1.90	8.76	1.00	7.82
2.38	8.94	1.54	8.28
2.38	8.96	1.54	8.36
2.96	8.72	2.00	8.56
2.96	8.51	2.00	8.54
3.37	8.93	2.37	8.68
3.37	8.95	2.37	8.61
3.87	8.72	2.68	8.72
3.87	8.86	2.68	8.74
4.97	8.50	3.03	8.66
4.97	8.32	3.03	8.74
5.45	8.39	3.39	8.71
5.45	8.38	3.39	8.72
6.16	7.56	3.69	8.74
6.16	7.83	3.69	8.72
6.48	7.66	4.00	8.81
6.48	7.85	4.00	8.79
7.41	7.56	4.53	8.81
7.41	7.61	4.53	8.73
		5.01	8.67
		5.57	8.38
		5.57	8.38
		6.01	7.75
		6.01	7.77
		6.56	6.86
		6.56	6.82

Table A4: Calibration Curve Data from 4 Experiments

Experiment	log (cells/ml)	log (abs)
1	7.93	-1.40
	8.71	-0.74
2	7.51	-1.85
	7.81	-1.49
	8.33	-1.09
3	8.55	-0.80
	7.84	-1.43
	8.37	-1.01
	8.78	-0.66
4	8.38	-1.11
	8.71	-0.79

Table A5: Growth Curve Data for Experiment A

Flask A1- Cells

Time, days	log(cells/ml)
0.00	7.32
0.93	7.84
1.34	8.22
1.91	8.73
2.91	8.85
3.94	8.80
4.96	8.44
5.93	8.22
7.94	8.29

Flask A2- Cells

Time, days	log(cells/ml)
0.00	7.32
0.91	7.84
1.34	8.22
1.91	8.73
2.87	9.11
3.96	8.72
5.01	8.54
5.97	8.23
7.89	8.22

Flask A7- Cells + 16 g/L of PCB

Time, days	log(cells/ml)
0.00	7.32
0.93	7.83
1.35	8.25
1.92	8.73
2.97	9.29
3.97	9.65
4.95	9.75
5.89	9.81
7.88	8.84

Flask A8- Cells + 16 g/L of PCB

Time, days	log(cells/ml)
0.00	7.32
0.93	7.83
1.35	8.24
1.91	8.74
2.89	9.16
3.91	9.44
5.00	6.87
5.92	*
7.91	*

Flask A10- Cells + 32 g/L of PCB

Time, days	log(cells/ml)
0.00	7.32
0.93	7.80
1.35	8.25
1.92	8.74
2.98	9.23
3.96	9.54
4.93	9.55
5.91	8.29
7.93	*

Flask A11- Cells + 32 g/L

Time, days	log(cells/ml)
0.00	7.32
0.93	7.80
1.35	8.25
1.92	8.75
2.86	6.41
3.92	*
4.94	*
5.96	*
7.90	*

* cell growth undetectable

Table A6: Growth Curve Data for Experiment B (continued on next page)

Flask B1- Cells

Time, days	log(cells/ml)
0.00	7.33
0.89	7.94
1.52	8.46
1.90	8.66
2.90	8.55
3.93	8.51
4.85	8.19
5.86	6.53
6.88	6.71
7.56	6.54

Flask B2- Cells

Time, days	log(cells/ml)
0.00	7.33
0.86	8.07
1.52	8.43
1.89	8.63
2.97	8.58
3.91	8.58
4.98	7.77
5.87	6.71
6.89	6.80
7.56	6.23

Flask B7- Cells + 8 g/L of PCB

Time, days	log(cells/ml)
0.00	7.33
0.87	7.92
1.52	8.44
1.88	8.65
2.89	8.67
3.99	9.44
4.83	9.67
5.88	9.90
6.89	9.95
7.57	9.27

Flask B8- Cells + 8 g/L of PCB

Time, days	log(cells/ml)
0.00	7.33
0.90	7.98
1.52	8.41
1.90	8.72
2.94	8.74
3.95	9.43
4.97	9.74
5.89	9.91
6.90	9.91
7.58	9.92

Table A6: Growth Curve Data for Experiment B (continued from previous page)

Flask B9- Cells + 8 g/L of PCB

Time, days	log(cells/ml)
0.00	7.33
0.90	7.96
1.51	8.47
1.89	8.67
2.95	8.65
3.90	9.43
4.99	9.68
5.91	9.80
6.90	9.96
7.58	8.51

Flask B10- Cells + 24 g/L

Time, days	log(cells/ml)
0.00	7.33
0.90	7.96
1.52	8.46
1.90	8.68
2.88	7.82
3.94	*
4.97	*
5.91	*
6.91	*
7.59	*

Flask B11- Cells + 24 g/L

Time, days	log(cells/ml)
0.00	7.33
0.88	7.96
1.51	8.41
1.90	8.61
2.87	7.92
3.99	*
5.01	*
5.92	*
6.92	*
7.60	*

Flask B12- Cells + 24 g/L

Time, days	log(cells/ml)
0.00	7.33
0.88	7.90
1.51	8.40
1.90	8.61
2.96	*
3.93	*
5.01	*
5.93	*
6.93	*
7.60	*

* cell growth undetectable

Table A7: pH Data for Experiment A (continued on next page)

Flask A1- Cells

Time, days	pH
0.00	3.62
0.93	3.53
1.93	3.15
3.05	2.81
4.04	2.79
5.05	2.69
6.01	2.64
7.99	2.31

Flask A2- Cells

Time, days	pH
0.00	3.62
0.93	3.57
1.93	3.14
3.04	2.83
4.04	2.77
5.06	2.71
6.01	2.62
7.99	2.39

Flask A3- 16 g/L

Time, days	pH
0.00	3.51
0.93	3.51
1.94	3.53
3.05	6.07
4.04	6.04
5.06	6.00
6.01	6.02
7.99	6.11

A4- 16 g/L

Time, days	pH
0.00	3.51
0.93	3.53
1.93	3.51
3.05	5.65
4.03	5.63
5.05	5.68
6.01	5.68
7.49	5.78

Flask A5- 32 g/L

Time, days	pH
0.00	3.62
0.93	3.56
1.94	3.55
3.04	6.23
4.04	6.48
5.06	6.71
6.01	6.84
7.99	7.17

A6- 32 g/L

Time, days	pH
0.00	3.62
0.93	3.71
1.93	3.69
3.05	6.38
4.04	6.77
5.05	7.28
6.01	7.29
7.99	7.47

Table A7: pH Data for Experiment A (continued from previous page)

Flask A7- Cells + 16 g/L of PCB

Time, days	pH
0.00	3.62
0.93	3.53
1.94	3.13
3.05	2.72
4.04	2.33
5.05	2.10
6.01	2.19
7.99	4.09

Flask A8- Cells + 16 g/L of PCB

Time, days	pH
0.00	3.62
0.93	3.54
1.94	3.15
3.04	2.98
4.04	3.39
5.06	4.38
6.01	4.35
7.99	4.17

Flask A10- Cells + 32 g/L of PCB

Time, days	pH
0.00	3.62
0.93	3.54
1.94	3.17
3.05	2.89
4.04	2.78
5.05	3.26
6.01	4.57
7.99	4.31

Flask A11- Cells + 32 g/L

Time, days	pH
0.00	3.62
0.93	3.53
1.94	3.13
3.04	5.08
4.04	5.23
5.05	5.32
6.01	5.86
7.99	6.10

Table A8: pH Data for Experiment B (continued on the next page)

Flask B1- Cells

Time, days	pH
0.00	3.49
1.02	3.42
1.95	3.09
3.01	3.12
4.03	3.01
5.05	2.90
5.96	2.82
6.94	2.92
7.62	2.85

Flask B2- Cells

Time, days	pH
0.00	3.49
1.01	3.46
1.94	3.11
3.01	3.15
4.02	3.04
5.05	2.91
5.96	2.83
6.94	2.94
7.62	2.87

B3- 8 g/L

Time, days	pH
0.00	3.49
1.01	3.68
1.95	3.59
3.01	5.48
4.03	5.63
5.05	5.50
5.96	5.67
6.94	5.62
7.62	5.57

B4- 8 g/L

Time, days	pH
0.00	3.49
1.01	3.68
1.95	3.57
3.01	5.47
4.03	5.53
5.05	5.50
5.96	5.54
6.94	5.56
7.63	5.55

Table A8: pH Data for Experiment B (continued from previous page, and continued on the next page)

B5- 24 g/L

Time, days	pH
0.00	3.49
1.02	3.63
1.95	3.56
3.00	6.23
4.03	6.69
5.05	7.21
5.96	7.29
6.44	7.27
7.63	7.31

B6- 24 g/L

Time, days	pH
0.00	3.37
1.02	3.56
1.94	3.47
3.01	5.58
4.02	5.68
5.05	5.72
5.96	5.91
6.94	5.99
7.63	5.99

Flask B7- Cells + 8 g/L of PCB

Time, days	pH
0.00	3.37
1.01	3.41
1.94	3.06
3.01	3.23
4.03	2.80
5.04	2.40
5.96	2.22
6.94	3.03
7.63	4.51

Flask B8- Cells + 8 g/L of PCB

Time, days	pH
0.00	3.37
1.02	3.37
1.95	3.03
3.01	3.11
4.03	2.49
5.05	2.19
5.96	2.01
6.94	2.40
7.63	2.69

Table A8: pH Data for Experiment B (continued from previous page)

Flask B9- Cells + 8g/L of PCB

Time, days	pH
0.00	3.37
1.02	3.38
1.94	3.06
3.01	3.44
4.02	2.79
5.05	2.40
5.96	2.34
6.94	3.35
7.63	4.47

Flask B10- Cells + 24 g/L

Time, days	pH
0.00	3.37
1.02	3.38
1.94	3.06
3.00	5.73
4.03	6.31
5.05	6.51
5.96	6.56
6.94	6.61
7.63	6.71

Flask B11- Cells + 24 g/L

Time, days	pH
0.00	3.49
1.01	3.43
1.95	3.12
3.00	5.47
4.03	6.36
5.06	6.82
5.96	7.20
6.94	7.32
7.63	7.35

Flask B12- Cells + 24 g/L

Time, days	pH
0.00	3.49
1.01	3.44
1.94	3.13
3.01	5.63
4.03	6.65
5.05	6.69
5.96	6.81
6.94	6.82
7.63	6.90

F. Bacterial Growth Curve Phases in a Batch Culture

When an innoculum of bacteria is placed into fresh growth liquid medium four distinct major stages can usually be seen in its growth curve: lag phase, exponential (or logarithmic) phase, stationary phase and death phase. This is due to the fact that no new nutrients are being introduced into the medium as they are being depleted by the growth of the bacteria. As seen in the typical standard bacterial growth curve below these phases can be seen.

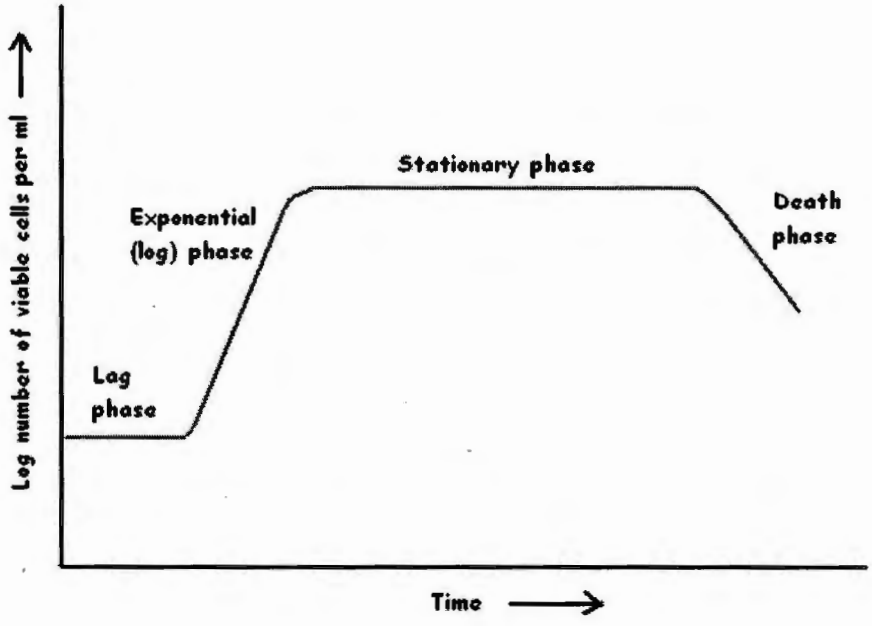

Figure A2: Typical Growth Curve of Bacteria Growing in a Batch Culture (Prescott, 1996)

Since the bacteria grow exponentially the results of the curve can be plotted as the log number of viable cells versus time (Volk & Brown, 1997).

Initially when bacteria are introduced into sterile nutrient rich medium, they usually need time to adapt to the new environment; this is called the lag phase. In this

phase, the bacteria "lag" in self-multiplication. The cells are still metabolically active; they grow in size and produce ATP, adenosine triphosphate (the energy of the cell). Depending on the conditions of the bacteria, the old medium in which the bacteria are being taken from and the new medium in which the bacteria are being inoculated determines the length of the lag phase (Black, 2002). If exponential phase bacteria are introduced into the new medium there is a possibility that no lag phase can be seen; this phenomenon is due to similar conditions of medium. The bacteria in this case will continue to grow exponentially at the same rate as the bacteria were previously growing. If the inoculated bacteria come from the stationary phase and are introduced into fresh sterile medium a lag will occur. This occurs because the cells in this stage are depleted of cell constituents (i.e. transport proteins, and enzymes) needed for multiplication, thus time is needed to resynthesize them (Brock, Madigan, Martinko & Parker, 1994).

Once resynthesis of cell constituents are complete the bacteria transitions from the lag phase into the exponential phase. In this phase the bacteria grow the fastest because they have adapted metabolically to the medium. Using the exponential phase data, a generation time (time for the existing quantity of the bacteria to duplicate) can be calculated. The generation time differs between microorganisms, but within an individual microorganism's growth curve the generation time is constant; hence, why the exponential phase is a straight line on the growth curve. Other factors in effecting generation time can be contributed to the environmental conditions in which the bacteria are growing (Brock et. al., 1994).

Once the nutrients within the medium starts to become depleted, the cells decrease their production of ATP thus subsequently decreasing their growth rate as well (Black, 2002). Another possible reason for this decrease in growth rate is "waste product buildup by the bacteria which may inhibit growth" (Brock et. al, 1994). The small convex curve after the exponential growth section signifies this decrease in growth rate. Eventually the growth levels off into what is called the stationary phase.

In the stationary phase newer cells are being produced at the same rate that older cells are dying. The horizontal line in the bacterial growth curve represents this phase. At this stage the medium still contains a minimal amount of nutrients and could be accumulating toxic waste products from the bacteria. In addition, the oxygen supply may also become inadequate for the remaining live cells (Black, 2002).

After some period of time, the conditions that the bacteria are growing become decreasingly accommodating. The rate of death is no longer equivalent to the rate of growth, it is greater. A decrease in the growth curve can be seen after the stationary phase into what is called the death phase. During the death phase many of the cells start to change shape, a process called involution. This makes it more difficult to identify the bacteria. Dead cells may lyse, or dissolve (Black, 2002).

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