# THE ROLE OF SULFATE REDUCING BACTERIA IN COPPER REMOVAL FROM AQUEOUS SULFATE SOLUTIONS

by

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B.Sc., University of British Columbia, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Chemical and Bio-Resource Engineering)

We accept this thesis as conforming

to the required standard

The University of British Columbia

September, 1998

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Date Dec-23/98

DE-6 (2/88)

# ABSTRACT

Grown waters contaminated with copper from the mining industry are matters of concern in Canada. This research investigated the feasibility of using sulfate-reducing bacteria (SRB) for treatment of copper sulfate solutions. The growth of sulfate reducing bacteria in copper sulfate solutions and their efficiency of removal of copper from those solutions were investigated by (i) varying the copper concentration from 0-200 ppm, (ii) comparing copper precipitation between SRB enriched from a mixed culture and two pure cultures: *Desulfovibrio desulfuricans*, and *Desulfovibrio vulgaris,* and (iii) the study of copper removal kinetics. Electron microscopy and subsequent image analysis of the micrographs determined the location of copper precipitates and idicated the direct role of the bacteria in copper removal.

Copper was removed effectively by the SRB enrichments to less than 0.1 ppm from copper-sulfate solutions containing 150 ppm or less initial copper concentration. All cultures having copper included showed some degree of sulfate inhibition after five days of incubation, although this inhibition was not very significant below 100 ppm initial copper concentration. Kinetic experiments indicated that precipitates of copper form very early in the incubation period. This early precipitation is most likely due to the reaction of copper ions with hydrogen sulfide present in the inocula. *Desulfovibrio desulfuricans*, and *Desulfovibrio vulgaris* removed copper from sulfate solution, but not as effectively as the SRB enrichments. Above 50-ppm initial concentration of copper, significant inhibition of sulfate reduction was observed.

Visual observations of the precipitates and solids indicated dense packing and good settling. Well-contrasted images of unstained bacteria confirmed that copper

sulfides formed when associated with the cell surface. Cells showed a high degree of capsule production in the presence of copper in solution. This phenomenon is the most likely reason for the formation of the flocculates.

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# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ARD | Acid Rock Drainage |
| ATCC | American Type Culture Collection |
| BC | British Columbia |
| CELA | Canadian Environmental Law Association |
| CSD | Crystal-size Distribution |
| EDS | Energy-dispersion Spectrometer |
| LPS | Lipopolysaccharide |
| N/A | Not Available |
| NSERC | Natural Sciences and Engineering Research Council of Canada |
| PSD | Particle-size Distribution |
| SEM | Scanning Electron Microscopy |
| SRB | Sulfate Reducing Bacteria |
| STEM | Scanning Transmission Electron Microscopy |
| TEM | Transmission Electron Microscopy |
| UBC | University of British Columbia |
| UASB | Upflow Anaerobic Sludge Blanket |
| WDS | Wavelength-dispersion Spectrometer |

# LIST OF NOMENCLATURE

| | |
|---|---|
| °C | Degrees Celsius |
| $cm^3$, ($m^3$) | Cubic centimeter, (cubic meter) |
| eV | Electron volts |
| g, (mg), (kg) | Grams, (milligram), (kilogram) |
| hrs, (h) | Hours, (hour) |
| J, (kJ) | Joules, (kilo-joules) |
| kV | Kilovolts |
| L (µl), (ml) | Litre, (microlitre), (milliliter) |
| M, (mM) | Molar, (millimolar) |
| m, (nm) | Meters, (nanometers) |
| min | Minutes |
| mole | Moles |
| ppm | Parts per million |
| rpm | Revolution per minute |
| s | Second |

# ACKNOWLEDGEMENTS

The author greatly acknowledged the contribution of many people involved in the completion of this research. First and foremost, the author offers sincere thanks to her supervisor, Dr. Susan Baldwin, for her guidance, support and encouragement. The committee members, Dr. Sheldon Duff, and Dr. William Mohn, provided helpful comments on the experimental setup of this research. Their contributions are greatly appreciated.

The author is indebted to Ping-Huang Liao (Department of Chemical and Bio-resource Engineering), as well as Paula Parkinson and Susan Harper (UBC Environmental Engineering Laboratory), who provided invaluable technical assistance. Dr. Elaine Humphary (UBC Electron Microscopy Facility, UBC), and Mary Mager (Department of Metals and Materials Engineering) patiently provided valuable insights to sample preparation for electron microscopy and various image analyses techniques. Guidance of Julie Macintosh (Department of Earth Science) is gratefully acknowledged, for helping in preparation of samples for Scanning Electron Microscopy and subsequent image analysis. Thanks are offered to the technicians in the Department of Chemical and Bio-Resource Engineering, Neil Jackson and Jurgen Pehlke, for their assistance in the laboratory and providing various self-made laboratory equipment. Sincere thanks go to all these individuals. Last, and certainly not least, the author would like to thank her parents for their moral and financial support through this research. Without their moral support and patience, this thesis would not have been possible.

This research was funded by the Natural Science and Engineering Research Council of Canada (NSERC).

# 1 INTRODUCTION

## 1.1 Scope and Rationale of Research

### 1.1.1 Metal Pollution

Metal pollution and the resulting environmental impacts have been documented as far back as 1874 in lead mining districts of the British Isles (Leland *et al,* 1986). Since then, continuous of mining and other metal polluting industries have greatly increased the amounts of metals in the biosphere. Some of the major sources of metal pollution include wastes from mines, electroplating factories, emissions from the burning fossil fuels and emissions from refuse incinerators. Metal pollution is of special concern because metals do not biodegrade, and in certain instances, they may biomagnify (Stumm *et al,* 1981).

"The mining industry generates 650 million tons of waste per year, more than 20 times generated by every household, business, institution and farm in Canada combined. The mining industry is Canada's leading source of a range of major toxic water pollution in Canada, with discharges of sulfates and heavy metals" (CIELAP, 1996). Moreover, the Canadian Environmental Law Association (CELA) estimated that there are more than 10,000 abandoned mines in Canada, and 6,000 abandoned tailing sites. According to Environment Canada, there are 857 million tons of mining waste produced per year capable of causing acid rock drainage in Canada (CIELAP, 1996).

### 1.1.2 Bioremediation of Heavy Metals

Microbial processes have been developed to remove metals from contaminated waters, particularly those due to acid rock drainage where high concentrations of heavy metals are encountered. Hence, the investigation of the possibilities offered by

microbiological methods for not only purification of the effluents but for the recovery of the metals dissolved therein is becoming a new topic of interest.

A number of investigations have shown that bacteria and their products can bind significant quantities of soluble metal cations due to electrostatic interactions with anionic carboxyl or phosphoryl groups within the constituent polymers of the bacteria (Beveridge *et al*, 1976, 1978, 1980, 1985; Brierly *et al*, 1989). Metal analyses further indicate that these cellular structures not only tenaciously bind metallic ions, but also serve as templates for the formation of minerals (Ferris *et al*, 1989).

This research focuses on one of the promising method of treating acid rock drainage (ARD), which uses biogenic sulfide produced by sulfate reducing bacteria (SRB). The use of biological sulfate reduction to treat sulfate and dissolved heavy metals in either groundwater or ARD is well documented in literature (Barnes *et al*, 1991; Dvorak, *et al* 1992; Hammack *et al*, 1992; Hammack *et al*, 1993; Rowley *et al*, 1997; Tuttle *et al*, 1969). However, due to the complex nature of the cell membrane and the cellular transport system, much of the information regarding the exact mechanisms involving precipitation and subsequent mineralization has remained unknown.

## 1.2 Objectives

There is strong evidence in the literature that anionic sites on the cellular membrane serve as a nucleation sites for metal deposition and subsequent mineralization. This suggests that aside from the production of $H_2S$, immobilization and precipitation of metal sulfides would be enhanced in the presence of the sulfate reducing bacteria. How significant this effect is, and its relevance to the design of bioreactors for metal removal

from contaminated water is uncertain. One of the goals of the work was to describe the direct role that these bacteria play in the precipitation of metal sulfide from a sulfate-rich solution. Of the metals of toxicological concern in aquatic environments, copper exhibits a great tendency to associate with organic matter. We chose to examine copper because this metal, as Table 1.1 indicates, is found in high concentrations in acid rock drainage and exhibits a great tendency to associate with organic matter (Mittleman *et al*, 1985; Rowley *et al*, 1997; Sanchez *et al*, 1973).

**Table 1.1: Mining effluent copper concentrations in different mine sites.**

| Site | Copper concentration (ppm) |
|---|---|
| Berkeley Pit influent feed, Montana (Sanchez *et al*, 1973) | 178 |
| Britannia Mine, British Columbia (Rowley *et al*, 1997) | 12-28 |
| Duluth Gabbro Waste Rock, Minnesota (Hammack *et al*, 1992) | 115 |
| Budleco Zinc Refinery, Netherlands (Barnes *et al*, 1992) | 0.8 |
| Iron Mountain Mine, California (Hammack *et al*, 1993) | 150-304 |

The following research objectives were thus established:

1. Investigate the effectiveness of copper removal using enrichments of sulfate reducing bacteria from a mix culture and two different pure cultures of SRB. The purpose of this objective is to determine whether different enrichments from the same initial mix culture and two pure cultures that were not previously adapted to grow in the presence of copper can remove copper from the solution to the same extent. The

results obtained can indicate whether the type of the inocula will have a significant effect in the copper removal process.

2. Perform image analysis to study the nature of the metal precipitates, and locate the position of the precipitates on the bacterial cell surface. This objective was established to confirm a proposed two-step mechanism for the precipitation of metals by bacteria (Beveridge *et al*, 1980, 1983, 1985). The results will hopefully show that, besides producing hydrogen sulfide, sulfate reducing bacteria play an important role in metal precipitation by providing nucleation sites on their cell surface, where metal precipitation can take place.
3. Investigation of a possible copper treatment system where the precipitation of the copper sulfides can take place in the bioreactor, in one step. As will be discussed later in Chapter Two, one of the technologies using sulfate reducing bacteria for heavy metal removal uses a two-step treatment system where the generation of biomass and sulfide is conducted in a separate reactor from the precipitation of heavy metal sulfides. This objective will explore whether the biological and the precipitation process can take place in the same reactor.

The objectives and the experiments of this research were designed to confirm the significance of the presence of bacteria in the heavy metal precipitation process. Once the objectives are met, the results obtained can contribute to bioreactor design and hence, more efficient downstream processing of heavy metal-contaminated effluents.

## 1.3 Thesis Organization

Chapter Two presents an overview of one of the major causes of metal pollution in Canada, acid rock drainage. Then, some conventional physico-chemical treatment methods and biological methods for removal of heavy metals are reviewed. Consequently, sulfate reducing bacteria (SRB) and their microbiology, biomineralization, and metal precipitation by SRB are discussed in detail. Chapter Two then emphasizes bacterial-metal interactions, and previous studies regarding bacterial metal uptake are introduced.

Chapter Three presents the methods and experiments performed to fulfill the objectives of this research. This chapter will present the methods for preparation of sulfate reducing bacterial isolates from a mixed culture and subsequent methods for metal analysis, and sulfate analysis. A detailed review of image analysis and electron microscopy will also be covered in this chapter.

Chapter Four provides a detailed discussion of the experimental results. The first part of this chapter concentrates on the results obtained for enrichment of sulfate reducing bacteria using different media suggested by the literature. The results for copper removal and sulfate reduction using different enrichments of sulfate reducing bacteria and the pure cultures will then be compared and discussed. Moreover, this chapter also provides qualitative observations made during the five-day incubations of the various cultures. This chapter ends with a presentation of electron microscopy images and detailed discussion of the results provided by image analysis.

Finally, in chapter five, the main conclusions of this study are reviewed, followed by a list of recommendations for future research in this area.

Appendix A presents the procedure for sulfate analysis, while Appendix B provides raw data for all experimental results. Appendix C provides a list of the different growth media used and their composition.

# 2 LITERATURE REVIEW

The purpose of this chapter is to review some of the literature significant in providing a background for this research. The chapter begins with a description of acid rock drainage (ARD), which is one of the major causes of metal pollution. After this, chemical and biological treatment processes for ARD are compared and contrasted. This chapter continued with a discussion of sulfate reducing bacteria (SRB) and treatment processes that use these microorganisms. Finally, literature on various bacteria-metal interactions is reviewed.

## 2.1 Acid Rock Drainage (ARD)

In British Columbia, heavy metal pollution derived from the mining industry is a great concern, especially since this province has sulfide-containing ore deposits. The generation of ARD has become a serious problem for five of the 16 metal mines currently operating in British Columbia, and ARD may develop in some of the other mines over time (Errington *et al*, 1987). The Britannia Mine located at Britannia Beach on the Squamish Highway, BC, produces flows of up to 600 $m^3/d$ of ARD, and until recently did not employ any type of treatment (McCandless 1995). ARD is formed when sulfide containing ores such as pyrite ($FeS_2$), and chalcopyrite ($CuFeS_2$), are allowed to oxidize in the presence of water. Equations 1-4 denote the sequential generation of ARD (Garrels *et al*, 1960):

$$FeS_{2(S)} + H_2O + 7/2\ O_2 \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+ \quad (1)$$

$$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O \quad (2)$$

$$Fe^{3+} + 3\ H_2O \rightarrow Fe(OH)_{3(S)} + 3\ H^+ \quad (3)$$

$$FeS_{2(S)} + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+ \quad (4)$$

The presence of *Thiobacillus ferrooxidans* can speed up the first reaction as much as $10^6$ times (Singer *et al*, 1970). Other bacteria that aid in the formation of ARD include *Thiobacillus thiooxidans*, *Leptospirillium ferrooxidans*, and *Sulfolo brieleyii* (Silver, 1989). Browning has postulated that up to 80 percent of the ARD generated in the U.S. is due to the activity of these bacteria (Browning *et al,* 1980).

Once $Fe^{3+}$ is present, it can directly oxidize pyrite which releases more acidity. The precipitated $Fe(OH)_3$ typically lines stream beds and is trapped in interstices in rock dumps where it provides a reserve of $Fe^{3+}$ that can further oxidize pyrite. The oxidation of pyrite to sulfuric acid and ferrous sulfate is exothermic and releases 1440 $kJ.mol^{-1}$ $FeS_2$ (Bennett *et al*, 1988). Because of these exothermic reactions and the direct oxidation of pyrite by dissolved ferric iron (Equation 4), the ARD generation process tends to be self-perpetuating. Once the low pH is established, metals that were initially immobile come into solution and can affect the aquatic communities (Bennett *et al*, 1988).

### 2.1.1 Technologies for Heavy Metal Treatment

Much research has and many field demonstrations have been conducted on selecting the most efficient and suitable method for a specific ARD problem. Hydrated lime, sodium hydroxide, sodium carbonate, and ammonia have been used to treat ARD. These chemicals precipitate iron and other heavy metals, allowing the water to be

discharged into streams or rivers. Each chemical has advantages and disadvantages when used for ARD treatment (Bhattacharya *et al,* 1981).

Acidic, metal-contaminated rock drainage is commonly treated by adding quicklime (CaO), hydrated lime [$Ca(OH)_2$], or caustic soda (NaOH) to precipitate metals from solution as hydroxides (Bhattacharya *et al,* 1979). Soda ash ($Na_2CO_3$) is sometimes used to precipitate metals such as Cd and Ni as insoluble carbonates. Metals can also be precipitated from wastewater as insoluble sulfides. In most existing sulfide treatment systems, an alkaline agent such as lime is first added to the water to raise its pH to between 6 and 8. Sulfide is then added in either a soluble ($Na_2S$, NaHS), slightly soluble (BaS, FeS) or gaseous ($H_2S$) form, and one or more metal sulfide precipitates form (Bhattacharya *et al,* 1979, 1981; Streeter *et al*, 1970).

The majority of metals found in ARD are in concentrations high enough to exceed the levels permitted by environmental regulations, but too low to economically justify recovery via conventional chemical or physico-chemical processes. Moreover, the physico-chemical methods are not selective enough and oftentimes require further downstream processing and detoxification. Ion-exchange process was studied by Billiton Research in Arnhem, The Netherlands. The conclusions that were reached were that: any one cation-exchange resin would only be effective for a certain metal or group of metals, and it was estimated that operating costs would be relatively high (Barnes *et al*, 1992). Results from a liquid membrane permeation process developed at the University of Graz, Austria, showed that removal was selective for some metals, but the capital cost of the plant would be rather high (Barnes *et al*, 1992).

Hence, due to the pitfalls of the physico–chemical treatment systems, microbiological methods have become more attractive. The investigation of the possibilities offered by microbiological methods suitable not only for the purification of the effluents from their pollutants, but also for the recovery of the metals dissolved therein is therefore of great environmental and economic significance (Barnes *et al*, 1992).

### 2.1.2 Biological Treatment Methods

Experts in the field explore the use of economically viable and sound biological routes for metal recovery and waste decontamination. In addition, such processes can also be used to recover precious metals, such as gold. Metal analyses and electron microscopy images from research done by Beveridge *et al* indicate that bacterial cell walls not only tenaciously bind metallic ions, but also serve as templates for the formation of distinct minerals (Beveridge *et al*, 1976, 1978, 1986).

There are five predominant mechanisms by which microorganisms facilitate removal of soluble metals from solution (Brierly *et al*, 1989; Rossi *et al*, 1990):

1. Binding to the cell surface. Many metals readily bind to cell walls as a result of the presence of specific functional groups, which readily exchange protons for divalent cations. In a similar manner to intracellular accumulation, there is a close association between the fates of the metal and of the cell.
2. Extracellular complexing. Some microorganisms generate chemicals that have a binding capacity for metals. Examples include the production of siderophore systems in bacteria and generation of metal-binding polymers. In general, siderophores have

extremely strong binding affinities for ferric ions and do not appear to react strongly with other metals. However, there is some evidence that specific siderophores bind copper and molybdenum (Lundgren *et al*, 1979). Many organisms produce extracellular polysaccharides that strongly bind metals. Physical entrapment by extracellular polymers of precipitated or insoluble metals facilitates their removal as well.

3. Intracellular accumulation. Concentrations of metals within bacterial and other microbial cells can result from interactions with surface ligands followed by slow transport into the cell. This may be an important form of detoxification or a means of incorporating specific metals into enzymes (e.g., Cu and Zn). Whatever the biochemical function, mobility of the metal is directly dependent on the physiology of the cell (Rossi *et al*, 1990).
4. Extracellular precipitation. Immobilization occurs because of formation of insoluble salts such as metal sulfides as a byproduct of SRB. Insoluble compounds can also be formed by precipitation of metal oxides.
5. Transformation and volatilization of metals. Volatilization occurs when living microorganisms methylate metals (Thayer *et al*, 1984). It is proposed that methylation processes are mechanisms that microorganisms use for detoxification of their environment. In addition, some toxic metals can be used as electron acceptors under anaerobic conditions.

Furthermore, microorganisms exhibit three important advantages that have attracted the interest of researchers involved in water purification problems (Rossi *et al*, 1990); they can:

1. Remove metal ions from aqueous solutions even in concentrations as low as a few parts per million,
2. Concentrate the metals taken up from solution to levels as high as 36-40% of dry cell weight, and
3. Drastically reduce the concentration of many organic compounds present in tailings waters to concentrations of the order of tens of ppm.

The advantage offered by biological systems compared to ion exchange or activated carbon methods is the higher loading capacity at low heavy metal concentrations. Therefore, the biological materials treat lower concentrations of heavy metals more effectively (Mattison *et al*, 1993). Moreover, biological oxidation/reduction treatments avoid addition of the toxic chemicals that are often used in chemical oxidation/reduction or leaching processes. Chemical treatments may allow more flexibility in the selection of chemicals to use but produce much more waste that requires further downstream processing and detoxification. When available, biological mechanisms can achieve leaching, oxidation, or reduction without the addition of acids, bases, or oxidation/reduction agents (Mattison *et al*, 1993). Moreover, biological treatment methods produce a more compact waste.

#### 2.1.2.1 Live Bacteria Versus Dead Bacteria

Microbial treatment processes operate by three distinct mechanisms: sorption of metals on non-growing or dead biomass, accumulation of metals by living cells and extracelluar precipitation. The first is a physico-chemical process that uses cells for chelation, adsorption and possibly precipitation of metals. The advantages of this system

include precise control of metal-removal processes in reactors or as biofilms and the use of cheap biomass produced from other industrial activities. The use of dead biomass or derived products eliminates problems of toxicity, nutrient supply, and maintenance of optimal growth and metal recovery. However, after metal loading the biomass must be separated from the solution using filtration, sedimentation, or centrifugation. Such a process scheme is neither cost effective or efficient. Living cells exhibit a wider variety of mechanisms for metal removal; this system can be used to adsorb, accumulate, and precipitate metals. These different mechanisms can lead into more selective metal uptake (Rossi *et al*, 1990). Dead biomass has a finite capacity for metal binding whereas, in living cells, cell biomass is being continuously generated as are cell metabolites, which may be involved in metal removal.

For example, the biogenically-generated sulfide by SRB can contribute to the removal of metals from solution by formation of insoluble metallic sulfide compounds (Brierly *et al*, 1989). In this case, instead of using commercial sulfide reagents, sulfides might be generated less expensively by SRB acting on waste organic materials and sulfate from the mine water. Furthermore, the use of living cells may be a viable treatment process when large volumes of contaminated waters must be treated in holding ponds.

#### 2.1.2.2 Mechanisms of Biomineralization

Biosorption and bioaccumulation are processes where metal removal is a direct consequence of the physical contact of microorganisms with metal ions. The most important feature of biosorption from the practical viewpoint is that the metal uptake

process can also occur with non-living biomass. However, metals can also be removed without the need of this physical contact; this phenomenon is encountered whenever metal ions combine with the direct or indirect products of microbial metabolism and is therefore dependent on the existence of a viable biomass (Rossi *et al*, 1990; Brierly *et al*, 1989). Organic or inorganic acid metabolites produced by microorganisms, including *Thiobacillu* sp., *Serratia* sp., *Pseudomonas* sp., *Bacillus* sp., *Desulfovibrio* sp., are able to bind metals.

Two fundamentally different processes of mineral formation can be distinguished (Lowenstam *et al*, 1981; Mohaghaghi *et al*, 1984; Westbroek *et al*, 1983). The first is an "organic matrix-mediated" process. In general, the organism constructs an organic framework or mold into which the appropriate ions are actively introduced to crystallize and grow. The mineral type, orientation of crystallographic axes, and microarchitechtures are under genetic control. The second basic process of mineral formation, exemplified by some bacterial species as well as various green and brown algae, is characterized by bulk extracellular and/or intracellular mineral formation, without the elaboration of organic matrices. This "biologically induced" mineralization results in the minerals having crystal structure similar to those produced by precipitation from inorganic solutions. The bacterial mineral precipitates form as a result of the interaction between the biogenically formed gases and metal ions present in the external medium. According to Lowenstam, the initial precipitates may differ from the form in which they are finally stabilized, or during development of the organism, one mineral may substitute for another (Lowenstam *et al*, 1981). Biominerals are generated as secondary events from interactions between metabolic end products, such as $CO_2$, $H_2S$, $H^+$, and $NH_3$ and the surrounding

environment. No organic matrices are involved. However, since these processes occur at the bacterial polysaccharide wall, they can not be strictly described as biologically-induced because of the presence and involvement of matrix components. Alternatively, they are not formally organic matrix-mediated events since there appears to be no regulation of structure or crystal orientation.

## 2.2 Sulfate Reducing Bacteria (SRB)

### 2.2.1 Interaction of SRB with Heavy Metals

Metals can be removed from a solution without the need of a physical contact with the bacterial cell-surface. One of the best examples of extracellular precipitation of metals is through the production of hydrogen sulfide by the SRB (Mohaghaghi *et al*, 1984). The key role of SRB in the formation of sulfur deposits is known from studies of the fractionation of $^{34}S/^{32}S$. As compared to a meteoritic sulfate standard, sedimentary sulfide is highly enriched in $^{32}S$. Non-biogenic sulfide does not show this bias toward the lighter isotope (Mohaghaghi *et al*, 1984). The $H_2S$ produced by the microorganisms combines with heavy metal cations to form insoluble sulfides which precipitate according to Equation 5:

$$M^{2+} + H_2S \rightarrow MS + 2H^+ \qquad (5)$$

where $M^{2+}$ is a divalent cation (Brierly *et al*, 1989). As Figure 2.1 indicates, experiments done by Mohagheghi *et al* at different environmental conditions, show considerably

greater uranium removal from solution in the presence of SRB cells than in otherwise nearly identical cell-free solutions (Mohagheghi *et al*, 1984).

SRB do not normally select and precipitate specific metals. They act as a hydrogen sulfide generator and if metals are present in an anaerobic zone, certain metallic sulfides will precipitate. Such metals react with $H_2S$ strictly based on their specific chemical affinity for $S^{2-}$ ions (Ferris *et al*, 1984; Mohagheghi *et al*, 1984). In addition, the absorption of metal ions onto the bacterial cell surface may facilitate reduction by aqueous sulfide, particularly at low metal ion concentrations. Mohagheghi *et al* concluded that the mechanism of uranyl ion adsorption to the bacterial cell walls most probably occurs by a two-step attachment mechanism (Beveridge *et al*, 1985; Mohagheghi *et al*, 1984). This mechanism will be discussed in detail, later on in the chapter.

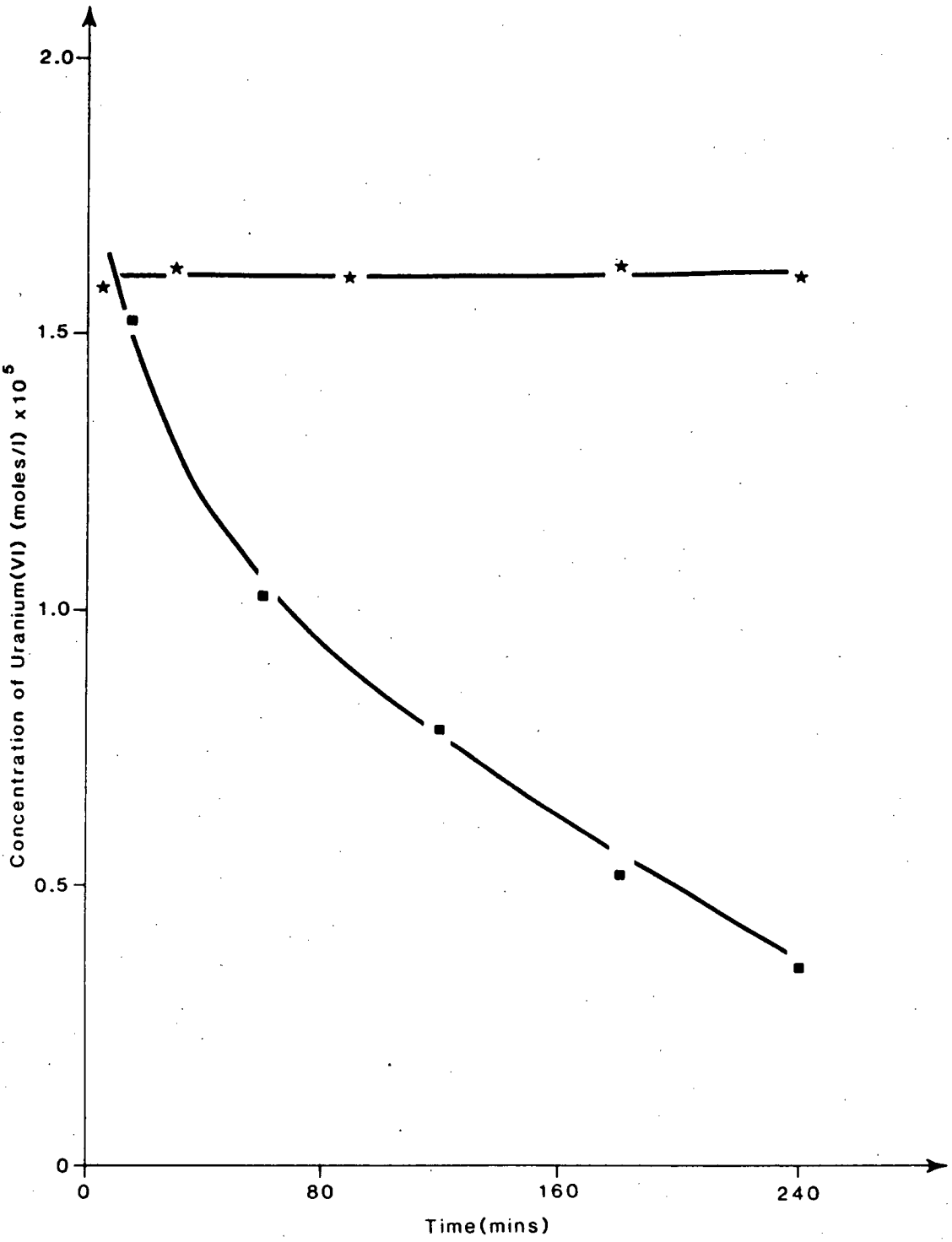


**Figure 2.1: Effect of the presence of *Desulfovibrio desulfuricans* on the removal of uranyl ion from solution at pH 8.0 ■, Uranyl ion added to grown culture; * , uranyl ion added to bacterial cell-free filtrate (Mohagheghi *et al*, 1984).**

### 2.2.2 The Sulfur Cycle

Sulfur is found in three significant forms in nature. According to oxidation states of sulfur, these three forms are $S^{2-}$ (sulfhydryl, R-SH, and sulfide, $HS^-$), $S^0$ (elemental sulfur), and $S^{6+}$ (sulfate, $SO_4^{2-}$) (Brock *et al,* 1994). The bulk of the sulfur on earth is found on sediments and rocks in the form of sulfate minerals (primarily gypsum, $CaSO_4$) and sulfide minerals (primarily pyrite, $FeS_2$). The biological sulfur transformations that take place in the environment can be adequately represented by the redox cycle for sulfur as shown in Figure 2.2 (Brock *et al*, 1994; Postgate, 1984).

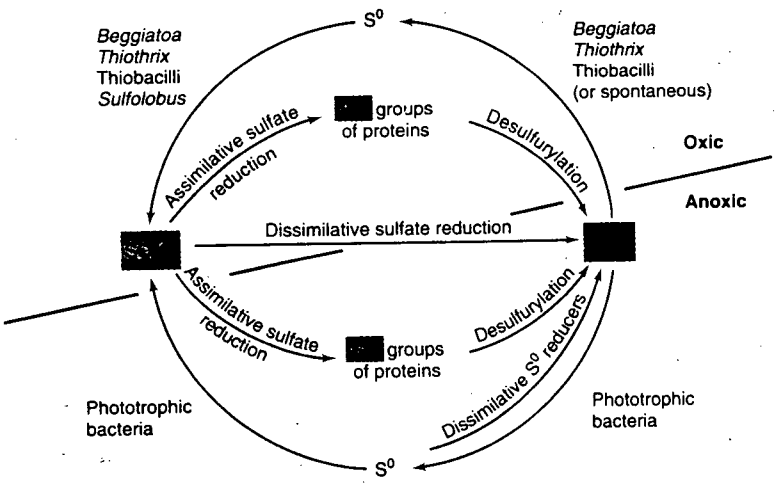


**Figure 2.2: Redox cycle for Sulfur (Brock *et al*, 1994).**

As shown in Figure 2.2, in assimilatory sulfate reduction, sulfate is enzymatically assimilated into the cell, followed by reduction to sulfite and sulfide intermediates. The

hydrogen sulfide is converted into organic sulfur (R-SH). However, in dissimilatory sulfate reduction, hydrogen sulfide is excreted from the cell as a waste product.

Desulfurylation is the process of organic sulfur degradation, which is the major source of $HS^-$ in fresh (Zehnder, 1988). Hydrogen sulfide is reversibly transformed to elemental sulfur and then to sulfate by chemolithotrophic bacteria such as *Beggiatoa* sp., *Thiothrix* sp., *Thiobacilli* sp., *Sulfolobus* sp., as well as phototrophic and cyanobacteria in anoxic environments (Brock *et al*, 1994).

### 2.2.3 Microbiology of Sulfate Reducing Bacteria

Microbiological aspects such as classification, structure, and metabolic processes are important for a thorough understanding of the process, which is required if biological sulfate reduction is to be an industrially viable technology. SRB are best known for the production of their end product, hydrogen sulfide gas, which has a "rotten egg" smell. In order to achieve the reduction of oxidized forms of sulfur, SRB require strictly anaerobic conditions, under which these microbes can utilize organic substrates for energy, with the sulfate being the terminal electron acceptor in the process. The general chemical reaction of sulfate reduction catalyzed by SRB is shown in Equation 6 (Postgate, 1984; Zehnder, 1988).

$$SO_4^{2-} + 2\{CH_2O\} + 2H^+ \rightarrow H_2S + 2H_2O + 2CO_2 \qquad (6)$$

The organic matter $\{CH_2O\}$ in this general reaction refers to the organic substrate, which provides carbon and reductant for the generation of biomass. As the general

reaction in Equation 6 shows, carbon dioxide is formed, which then provides a bicarbonate buffer when in equilibrium in water. The SRB catalyze the reduction of sulfate by oxidizing various organic compounds and directing the electrons arising from the oxidation to the sulfate reducing system.

Classification of SRB is based on nutritional and morphological characteristics. There has been some verification of this type of classification system in the literature, namely chemical criteria such as guanine plus cytosine content of DNA and the presence of special pigments. Moreover, contributions of 16S rDNA analyses are one of the key tools for classification of the microorganisms (Brock *et al*, 1994; Zehnder, 1988). Morphological characteristics are used to identify and classify bacteria, however this method may not be completely reliable since growth conditions may affect cell shape. Cell shape may in fact vary from long rod-shaped to cocci, depending on the growth conditions and species.

The electron sources known to be oxidized by SRB are always low-molecular-weight compounds or hydrogen. Nearly all these compounds are fermentation products from the anaerobic bacterial degradation of carbohydrates, proteins, and other components of dead biomass. Many different organic substrates have been examined for use by SRB as electron sources (Postgate, 1984; Zehnder, 1988). Surprisingly, SRB can use an extremely large number of organic substrates, some of which are hydrogen, lactate, pyruvate, ethanol, fumarate, malate, choline, acetate, propionate, butyrate, long-chain fatty acids, benzoate, indole, and hexadecane.

### 2.2.4 Removal of Heavy Metals Using Biogenic Sulfide

A promising method of treating ARD is with the use of SRB. The use of biological sulfate-reduction to treat sulfate and heavy metals in either groundwater or ARD is well documented in literature (Barnes *et al*, 1991, 1992; Dvorak, *et al* 1992; Hammack *et al*, 1992, 1993; Rowley *et al*, 1997; Tuttle *et al*, 1969). Besides having the available negatively charged cell surface for bonding to heavy metals, SRB produce $H_2S$ as their end product. The hydrogen sulfide generated by SRB contacts metal cations, forming insoluble metal sulfides that precipitate according to Equations 7 and 8:

$$SO_4^{2-} + \text{Nutrients} + H_2O \rightarrow H_2S + \text{biomass} + HCO_3^- \quad (7)$$

$$M^{2+} + S^{2-} \rightarrow MS_{(s)} \quad (8)$$

The precipitation of metals as sulfides has several advantages over hydroxide precipitation. Sulfides form more rapidly, create a denser sludge, and are less soluble than hydroxides. Moreover, sulfide sludges exhibit better thickening and dewatering characteristics than corresponding hydroxide sludges (Hammack *et al*, 1994; Rowley *et al*, 1997). Moreover, metal sulfides allow more complete precipitation of contaminant metal ions even at low pH (Hammack *et al*, 1994). There is additional experimental evidence that suggests that metallic ions complexed to SRB tend to be more reactive with sulfide compared to when in strictly inorganic systems (Mohagheghi *et al*, 1984). Table 2.1 shows the solubility products for different metal sulfides and hydroxides.

In summary, the application of biological sulfate reduction to treat ARD effluents may improve water quality in the following ways:

- The process consumes sulfate.
- Sulfide generated either in the form of biosulfide or hydrogen sulfide will react quickly with many dissolved metals to form insoluble precipitates and decrease dissolved metal concentration.
- Alkalinity generated in the form of bicarbonate will neutralize acidity and raise pH.

**Table 2.1: Solubility products for different metal sulfides and hydroxides (Hao *et al*, 1996).**

| Metal | $K_{sp}$* | |
|---|---|---|
| | Metal sulfide | Metal hydroxide |
| Cu | $8.5 \times 10^{-45}$ | $1.6 \times 10^{-19}$ |
| Zn | $1.2 \times 10^{-23}$ | $4.5 \times 10^{-17}$ |
| Fe | $3.7 \times 10^{-19}$ | $1.8 \times 10^{-15}$ |

*: $Ksp = \frac{[M^{2+}][S^{-2}]}{[MS]}$ M: Metal; S: Silfide

### 2.2.4.1 Current Technologies for Removal of Heavy Metals Using SRB

The application of biological sulfate reduction for the treatment of ARD requires the engineering of an organic-rich or $H_2$, anaerobic environment that can sustain an active population of SRB. Systems for treatment of ARD with SRB include anaerobic digesters, upflow activated sludge-bed reactors (USAB), or constructed wetlands. Specific technologies that have been successful in applying biological sulfate reduction to the ARD include the Paques "THIOPAQ" system and the NTBC Biosulfide Process (Barnes *et al*, 1991, 1992; Rowley *et al*, 1997).

#### 2.2.4.1.1 The Paques Process- "THIOPAQ"

Paques Environmental Technology based in the Netherlands has developed a technology, which uses biological sulfate reduction to remove sulfate and dissolved metals from mine waters. The technology is marketed under the name of THIOPAQ, consisting of two biological steps. In the first anaerobic biological step, sulfate is converted to sulfide by SRB. The second stage of the system is an aerobic reactor that houses a bio-catalyzed redox-reaction by sulfide oxidizing bacteria. The excess sulfide formed from the USAB is oxidized by the bacteria into elemental sulfur, by Equation 9:

$$HS^- + \tfrac{1}{2} O_2 \rightarrow S^0 + OH^- \qquad (9)$$

The elemental sulfur formed is separated from the water stream by sedimentation in a separate vessel that uses tiled plates to separate solids and liquids. In the final step of the treatment process, the water passes through a sand filter to remove any remaining solid particles and reduce biological oxygen demand of the effluent. In the second step, depending upon the sulfate load of the influent, hydrogen gas or ethanol may be used in the THIOPAQ process (Barnes *et al*, 1991, 1992).

The system shown in Figure 2.3 uses an UASB reactor for metal precipitation with SRB. The THIOPAQ system is very well known and has been successfully applied at the Budelco Zinc refinery in the Netherlands as a full scale operation treating heavy metal laden acidic groundwater. The process at the Budelco zinc refinery was successful in reducing zinc concentrations from 100 mg/L to less than 0.3 mg/L, and sulfate from 1000 mg/L to less than 200 mg/l (Barnes *et al*, 1991, 1992)

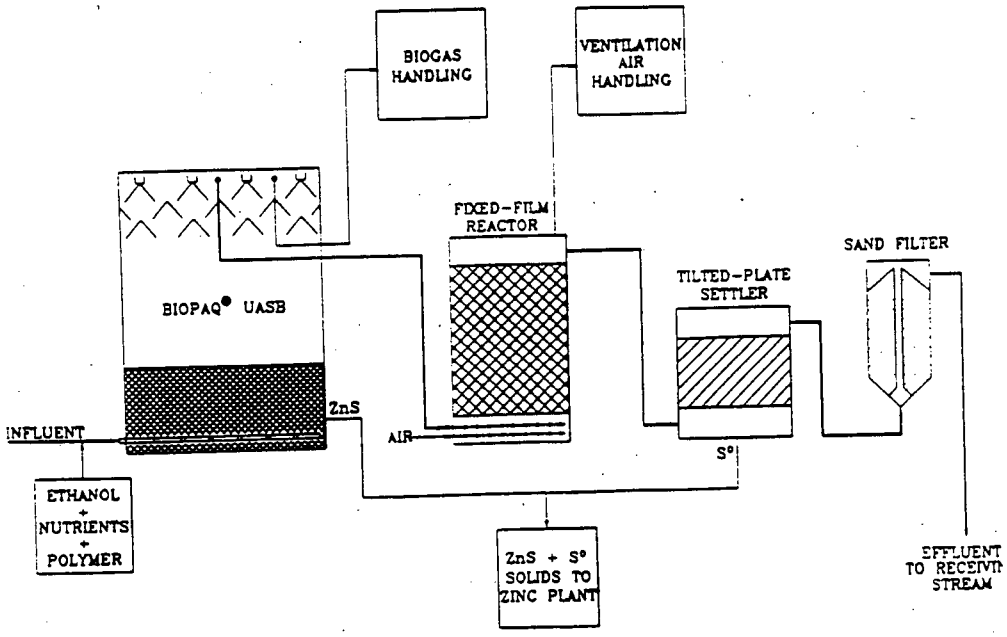


**Figure 2.3: The Paques Process (Barnes *et al*, 1992).**

### 2.2.4.1.2 The NTBC Biosulfide Process

The Canadian company, NTBC Research, has developed a technology that employs biological sulfate reduction to treat ARD with high heavy metal concentration (Rowley *et al*, 1997). The technology differs from Paques THIOPAQ system in that the biological generation of sulfide is conducted in a separate reactor from the precipitation of heavy metal sulfides. Figure 2.4 shows the process developed at NTBC research, which has been applied at a pilot plant scale at the Britannia copper mine site.

The combined chemical-biological treatment plant had a total bioreactor volume of 5 $m^3$, and was capable of treating up to 50 L/min of ARD to remove Cu, Cd, and Zn. The results from the pilot plant include the removal of copper and zinc from the 50 to 100

mg/L range to the 0.01 to 0.05 mg/L range, respectively (Rowely *et al*, 1997). Some highlights of the Biosulfide process are as follows:

- In order to eliminate the possible toxicity effects of high metal concentration, the biological component of the process is separated from the chemical precipitation/neutralization stage. Whereas, in the Paques process, biological sulfate reduction and metal precipitation take place in the same reactor vessel.
- Metal concentrates, and metal sludge can be removed selectively for sale or disposal.

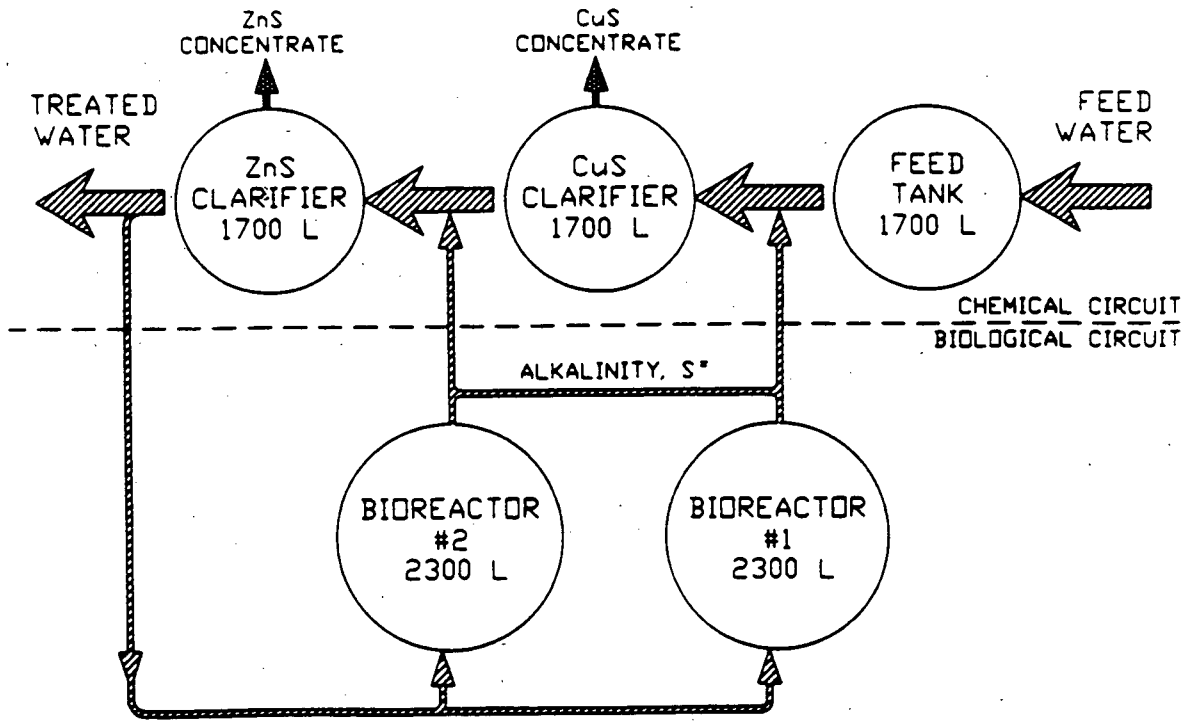


**Figure 2.4: NTBC Biosulfide Process (Rowley *et al*, 1997)**

## 2.3 Bacterial-Metal Interactions

If microbes are to be used in high technology processes for the removal of toxic heavy metals, it is important to understand in detail what cell components are involved in the metal ion binding. Most Sulfate reducing bacteria are Gram positive but an overview of both Gram positive and Gram negative cell wall structures and their relevance in heavy metal interactions will be presented in the next two sections.

### 2.3.1 Bacterial Cell Wall

#### 2.3.1.1 Gram-Positive Bacteria

In Gram-positive bacteria, the cell wall is a solvent exposed organelle that may offer the first encounter between a bacterium and a molecule in its environment. Most cell walls of the Gram-positive bacteria contain reasonably high amounts of peptidoglycan, a meshwork of linear *N*-acetylmuramyl-*N*-acetylglucosamine strands covalently bonded by peptides, and anionic polymers such as teichoic or teichuronic acid, which is a polymer of α-D-glycopyranosyl-glycerol-phosphate (Brock *et al*, 1994).

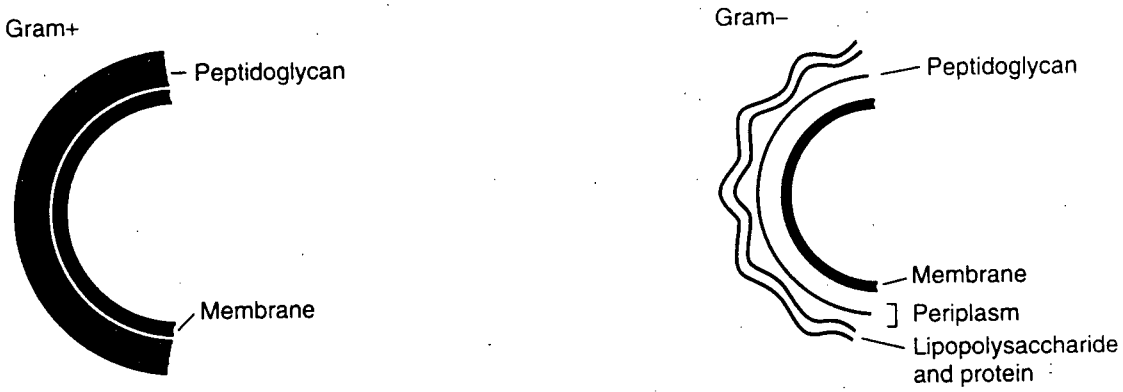


**Figure 2.5: Demonstration of Gram-Positive and Gram-Negative bacterial cell wall (Brock *et al*, 1994).**

A great deal of research has been done by Beveridge *et al*, on metal ion interaction with the Gram-positive strain, *Bacillus subtilis* (Beveridge *et al*, 1980, 1985, 1986). When *Bacillus subtilis* walls were suspended in 5m*M* metal salt solutions and washed free from the unbound metal, so much metal remained that visible electron dense aggregates could be seen by electron microscopy (Beveridge *et al*, 1976, 1978). Precipitates were not seen in the metal solutions without the addition of the walls. By extracting teichoic acid, a decrease in the extent of the metal uptake was observed (Beveridge *et al*, 1985). However, neutralization of the carboxylate group of peptidoglycan resulted in a profound decrease in binding capacity. Measuring the metal bound, it was apparent that most of the binding capacity remained associated with the peptidoglycan.

A two-step mechanism for the deposition process was proposed by Beveridge *et al* (Beveridge *et al*, 1980, 1983, 1985). In the first step, the metal ion binds to an active site on the cell wall. This interaction acts as a nucleation site for the deposition of more metal ions from the solution in the second step. The deposition product therefore increases in size within the intermolecular spaces of the fabric of the cell wall until it is physically constrained by the polymeric meshwork of the wall, unless covalent bonds are broken. The size of the mineral would depend on a number of variables, the most obvious of which are the concentration of the metallic ions in the solution and the amount of time through which the reactions proceed (Beveridge *et al*, 1985). Studies on other *Bacillus* species such as *Bacillus licheniformis*, where the wall composition is different and contains less peptidoglycan, suggested that unlike the case of *Bacillus subtilis,* it is the

teichoic acid that interact most strongly with metallic ion in solution (Beveridge *et al*, 1985, 1986).

#### 2.3.1.2 Gram-Negative Bacteria

The outer membrane and the peptidoglycan layer constitute the wall of these bacteria. Besides having a cytoplasmic membrane and a peptidoglycan layer, Gram-negative bacteria also posses an outer membrane that consists of protein, phospholipids and lipopolysaccharide (LPS) arranged in an exact format. This highly specialized structure serves as a selective permeability barrier that controls the access of solutes and other external agents to the plasma membrane. Of the two lipids (i.e. Phospholipids and LPS), LPS possesses the greatest number of electronegative sites per molecule. The hydrophobic polar head groups of the lipids, which contain anionic phosphoryl and carboxyl groups, therefore remain exposed to the external environment and effectively determine the reactivity of the cell surface (Beveridge *et al*, 1983; Brock *et al*, 1994).

To study metal-cell wall interactions in Gram-negative bacteria, Beveridge *et al* looked at the cell wall of *Escherichia coli* (Beveridge *et al*, 1981, 1983, 1985). The envelope of these bacteria did not bind as much metal from solution as their Gram-positive counterparts, but there was still enough metal to give an electron-scattering profile. Thus, the outer membrane of the Gram-negative bacteria is capable of binding a wide range of metallic ions; these metal cations are generally regarded as important accessory components that function to stabilize the molecular architecture of the outer membrane. Presumably, these metallic ions, bound by the outer membrane, reduce charge repulsion between highly anionic constituent molecules, bridge adjacent molecules of

LPS and protein, and help anchor the outer membrane to the underlying fabric of the peptidoglycan. For example, $Mg^{2+}$ and $Ca^{2+}$ formed an integral component part of the membrane since they are required for the correct packing order of the lipid constituents.

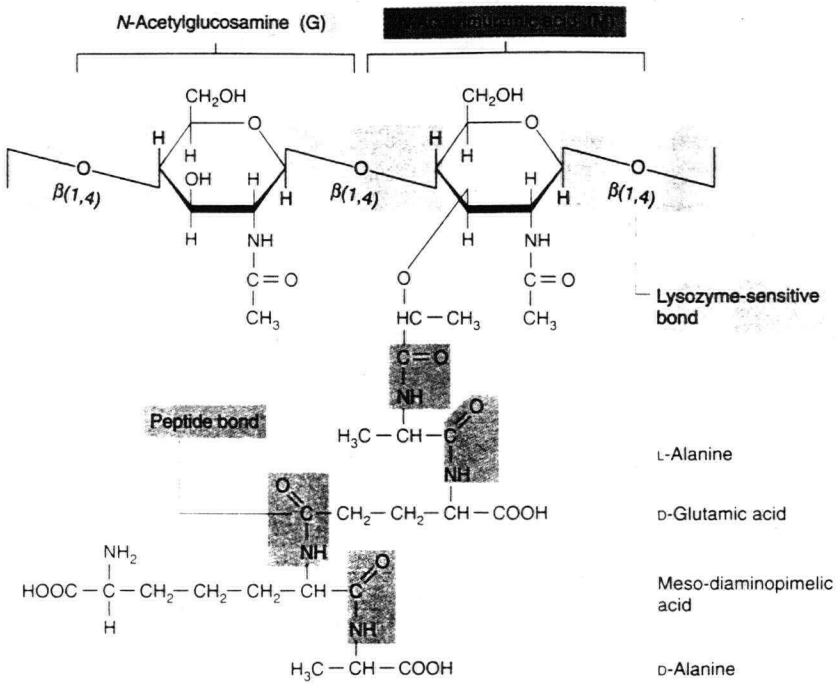


**Figure 2.6: Structure of one of the repeating units of the peptidoglycan cell-wall structure. The structure given is that found in *Escherichia coli* and most other Gram-negative bacteria (Brock *et al*, 1994).**

Studies of the metal binding capacity of native and EDTA-modified (LPS deficient) outer membranes showed that, because of its structural complexity, the outer membrane could exhibit a certain degree of selectivity with regard to the types of metal

cations that are used to stabilize the molecular architecture of the membrane (Beveridge *et al*, 1981). Beveridge *et al* (1983, 1985) proposed that the two-step deposition mechanism described earlier is also at work with *Escherichia coli* peptidoglycan, but since there is only a monolayer fabric, there are few intermolecular spaces in which metal aggregates can grow.

Due to the presence of the outer membrane, Gram-negative bacteria are more selective in metal adsorption. This selectivity can be explained by the requirement of the outer membrane for metals such as $Mg^{2+}$ and $Ca^{2+}$, which are involved in providing stability. However, other researchers have concluded that the selectivity of the metal binding is highly dependent on many other factors such as pH, temperature, and redox potential (Beveridge *et al*, 1981, 1983, 1985).

### 2.3.2 Extracellular Complexing

The capsule, which is usually comprised of polysaccharide with a repeating sequence of two to six sugar subunits, is anchored to the Gram-negative bacterial outer membrane (Brock *et al*, 1994). These naturally produced polymers bond electrostatically or physically to each other and subsequently bridge the bacterial cells and other particulates to settle as floc aggregates (Shen *et al*, 1993). Capsules possess features that suggest that they act as effective modulators of metal ion concentration at the cell surface, scavenging metals from solution when their concentrations are low and serving as impermeable barrier when metal ions exist at toxic levels in the surrounding environment. Different strains of the same bacterium may elaborate exopolymers that maintain different associations with the cell (Geesy *et al*, 1989).

As discussed before, the composition of bacterial exopolymer confers a net negative charge to the polymer and electron microscopy observations after staining with cationic dyes, as in Figure 2.7, lead to the suggestion that they are acidic in nature (Corpe *et al*, 1975). Lone pair electrons on carboxyl groups are known to interact with the charge-compensating metal ions. Weak electron donors are also present on acidic and neutral polysaccharides in the form of oxygen atoms associated with the ether bond and hydroxyl residues on the sugar subunits (Liu *et al*, 1971). On the basis of Rendelman's interpretation of ion interactions with polysaccharides, metal binding by uncharged polysaccharides occurs as a result of coordination between the metal cation and oxyanion and hydroxyl groups on the donor molecule (Rendleman *et al*, 1976).

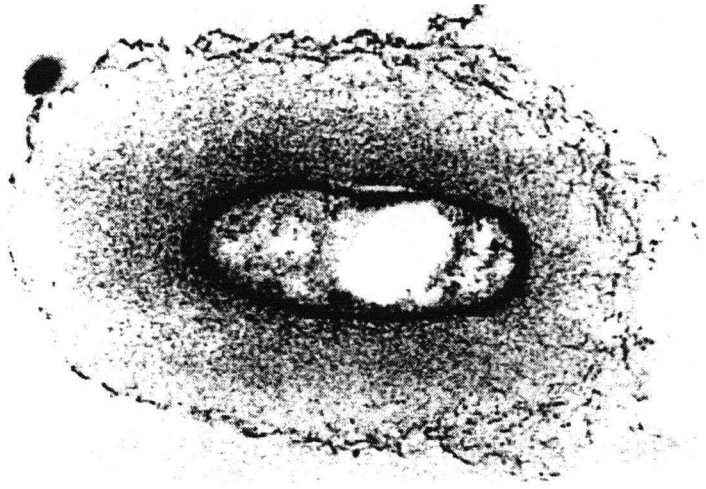

**Figure 2.7: Electron micrograph of a thin section of *a Rhizobium trifolii* cell stained with ruthenium red to reveal the capsule (Brock *et al*, 1994).**

Metal interactions with charged polysaccharides are controlled to some extent by linear charge density; these interactions are often stronger when the polymer exists as a gel than when dissolved in the solution. This is likely due to increase in charge density in

the former. In general, carboxylated polysaccharides exhibit preferential binding to cations with large ionic radii. With few exceptions, carboxylated polyanions exhibit a higher selectivity for transition metals than the alkaline earth metals (Geesy *et al*, 1989).

In another study, Corpe *et al* found that extracellular polymers produced by cells of *P. atlantica* exhibited selectivity for Mg and Cu over Ca, Zn, Pb, Co, and Ni (Corpe *et al*, 1975). It was reported that cells cultured in the presence of $5 \times 10^{-5}$ *M* copper contained no more of the metal than exopolymers from cells cultured under conditions in which no copper was added (besides that present in the nutrients added for growth). On the basis of these results, Corpe *et al* (1975) concluded that the binding of metal by bacteria is not influenced by the amount of free metal in the surrounding medium. However, when the added copper concentration was increased to $4 \times 10^{-4}$ *M*, the cell wall and the extracellular fraction contained significantly higher concentrations of the metal than wall and exopolymer preparations obtained from cells receiving no copper supplement. This is contradictory to their earlier conclusion, thus more research is required in this area.

From these studies and other studies conducted to date, it appears that bacteria that produce exopolysaccharides tolerate higher concentrations of metal than strains that produce little or no capsule or slime. Others have demonstrated that the amount of metals bound by extracellular polymers increases with increasing free ion concentration. Shen *et al* (1993) concluded that the relative concentration of each metal on the capsule strongly depends on its presence in the feed that was supplied. Thus, there is some evidence to suggest that the amount of metal bound by these envelope components is controlled by the surrounding metal concentration.

In another study, where metal uptake was compared between a capsule producing and a non-capsulated strain of *K. aerogens*, only Ni was adsorbed by both strains (Cu, Cd, Ni, Mn, and Co were tested) (Shen *et al*, 1993). All the other metals were concentrated to a great extent by the capsule-producing bacteria. Furthermore, the extraction of extracellular polymers reduced the capacity of cells to adsorb most of the metals. The majority of studies conducted to date, therefore, demonstrate that bacterial exopolymers exhibit an affinity and selectivity for metal ions. No generalizations can be made with respect to the effect metals and other cations have on bacterial capsule production. This likely reflects basic physiological differences between bacterial types. It may also reflect differences in the types and concentrations of metals in the environment in which the bacteria exist. Unfortunately, the type of quantitative information needed for comparison of affinities of different microbial exopolymers for various metals has not been thoroughly investigated yet.

# 3 METHODS AND MATERIALS

In this chapter, a description of all materials and experimental methods used in this research is presented. Section 3.1 presents the procedures for the preparation of SRB enrichments from a mixed culture and the pure cultures. Sections 3.2, 3.3, and 3.4 outline the detailed protocols of all batch tests and analytical methods. Finally, section 3.5 gives a detailed description of the various image analyses methods and sample preparation for electron microscopy.

## 3.1 Preparation of SRB Inocula

### 3.1.1 Preparation of SRB Enrichments from a Mixed Culture

The purpose of this experiment was to select different SRB enrichments from a mixed culture that had already been grown in copper solution. Since SRB are strict anaerobes, extra attention was given to their nutrient media and growth and cultivation conditions; reducing agents are added to most anaerobic media to depress and poise the redox potential at optimum levels. A mixed culture that had already been adapted to grow in copper solution was obtained from NTBC Research Corporation, Richmond, BC. To ensure that the results were reliable, growth of different isolates from the initial culture was attempted.

Since the primary enrichment of SRB from the original culture proved to be difficult, their growth was attempted using different solid nutrient media, as suggested by literature. The solid media used were as follows: Postgate's medium E, Iverson's medium, and Postgate's medium F (A.P.H.A., 1995; Postgate, 1984). Additionally, growth of SRB on Postgate's medium E, which was modified by reducing the amount of

yeast extract (i.e. from 1 g to 0.5 g) was also tested. The reason for this modification was to make this medium more minimal and prevent growth of other anaerobic microorganisms that grow on a rich media. The exact compositions of all the media are described in detail in Appendix C.

As Figure 3.1 shows, in order to select different enrichments of SRB, a set of plates and agar shake tubes containing the different media were inoculated with the initial NTBC culture. Aliquots of 0.25 ml from the NTBC cultures were transferred to different petri dishes and immediately after, 15 ml of molten medium (40 °C) was transferred to each of the petri dishes. All the solid media mentioned previously were used for all of the enrichment steps. After rotating of the petri dishes gently to enhance the mixing of the inoculum with media, and subsequent solidification of the media, the plates were incubated at 35°C under strict anaerobic conditions provided by the GasPak anaerobic jar. Another set of petri dishes was prepared where the agar was allowed to solidify prior to inoculation. Once the agar had solidified, a sterile metal inoculator was used to inoculate the surface of the agar with the original NTBC culture.

Another method for growth of individual colonies is the Agar Shake Method. The agar shake method has the advantage that it is very effective for the enumeration and isolation of anaerobes (Brock *et al*, 1994). The solid media was autoclaved and rapidly dispensed in 9 ml volumes in sterile test tubes and the tubes were closed with butyl rubber seals. The tubes were maintained in a water bath at 42-44 °C to keep the medium molten. The first tube in the series was inoculated with 1 ml of NTBC culture and after mixing and inversion, a dilution series was prepared by aseptically transferring 1.0 ml from one tube to the next using pipettes. After mixing, the tubes were rapidly bubbled

with nitrogen gas, closed by rubber stoppers, and cooled in a cold water bath. Plates and tubes were incubated at 35°C until individual black colonies, that are an indication of presence of SRB due to the formation of FeS, appeared. The colonies were then examined under the microscope and Gram stain test was applied.

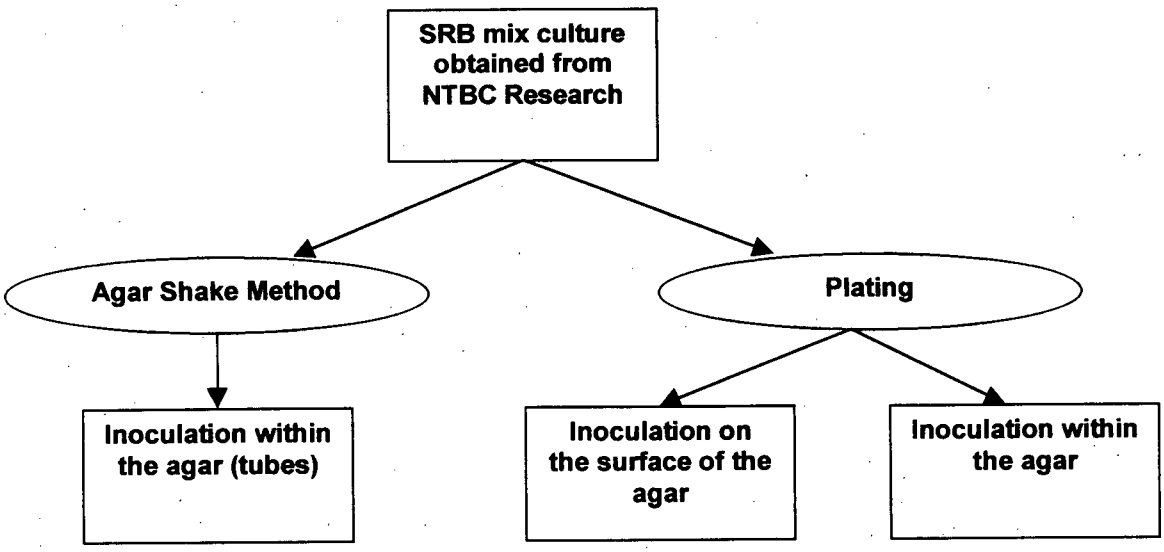


**Figure 3.1: Selection of SRB enrichments using two different inoculation methods.**

For further cultivation, once SRB colonies were identified and selected, they were transferred into a liquid medium. Postgate's medium B contains reducing reagents that provide the proper environment for the enrichment of SRB. This liquid medium contained (in grams per liter of tap water) the following: $KH_2PO_4$, 0.5; $NH_4Cl$, 1.0; $Na_2SO_4$, 1; $CaSO_4$, 1.0; $MgCl_2.7H_2O$, 2.0; sodium lactate, 3.5; yeast extract, 1.0; ascorbic acid, 0.1; thioglycollic acid, 0.1; $FeSO_4.7H_2O$, 0.5 (Postgate, 1984). The medium was adjusted to a pH of 7.5 with concentrated NaOH. Four different black colonies referred to

as enrichment A to D were transferred into 40 ml of Postgate's medium B. A more detailed description of these enrichments and their source of isolation will be discussed in the next chapter. The inoculated serum bottles were immediately bubbled with nitrogen and closed with a rubber stopper to provide the anaerobic conditions required for growth

To minimize changes in the different enrichments, seed stocks were established from early cultures. Cells were harvested in the early stationary phase (after 4 days of incubation), and centrifuged. The pellet was resuspended in $1/20^{th}$ the volume of fresh Postage's medium B. An equal volume of 20% sterile glycerol was added and small aliquots of the suspension were dispensed into small sterile vials. These vials were stored in the vapor phase of liquid nitrogen tank at –50 °C.

### 3.1.2 Preparation of Pure Cultures from American Type Culture Collection

In order to compare copper removal between SRB enrichments and pure cultures, two pure cultures known to be present in ARD conditions were purchased from American Type Culture Collection (ATCC) and cultivated. Cultures *Desulfovibrio desulfuricans* (ATCC 7757) and *Desulfovibrio vulgaris* (ATCC 29579) were received as two freeze-dried pellets in double-vial preparations. In order to rehydrate these freeze-dried cultures, the tip of the outer vial was heated in a flame and a few drops of water were squirted on to the hot tip to crack the glass. The tip was removed with a pencil. After removing the insulation and the inner vial, the cotton plug was raised with forceps. Half of the pellet was aseptically removed from the vial into a sterile test tube and about one ml of sterile Postgate's medium B was added to the test tube with a sterile Pasteur pipette. After mixing of the pellet, an additional volume of 6 ml of sterile medium was added to the test

tube. The test tube was then immediately placed in an anaerobic jar that had already been bubbled with nitrogen gas. A sterile filter ensured the sterility of the nitrogen gas. The test tube culture was then incubated in the anaerobic jar at 35 °C until the culture became turbid; this process took a period of approximately seven days. The other half of the pellet was saved as a backup.

The turbid 6 ml culture was then aseptically transferred to 20 ml of Postgate's medium B and incubated for another four days. Glycerol stocks were prepared as described previously.

## 3.2 Extent of Copper Removal and Inhibition of SRB Activity

The purpose of these experiments was to measure the extent of copper removal by the four different enrichments and the two pure cultures. Ten different glycerol stocks had been prepared for each individual enrichments and pure culture (a total of 60 vials). Each glycerol stock was thawed in a 37°C water bath and grown in 20 ml of Postgate's medium B. Once the cultures were turbid (after five days), 16 ml was transferred into 104 ml of Postgate's C medium and incubated for 3 days. Postgate's medium C was used in this part of the experiment since its defined nature is necessary for metal quantification and analyses. This medium consisted (in grams per liter of distilled water) of the following: $KH_2PO_4$, 0.5; $NH_4Cl$, 1.0; $Na_2SO_4$, 4.5; $CaCl_2.6H_20$, 0.06; $MgSO_4.7H_2O$, 0.06; sodium lactate, 6.0; yeast extract, 1.0; $FeSO_4.7H_2O$, 0.004; sodium citrate.$2H_20$, 0.3 (Postgate, 1984). The pH was adjusted to 7.5 with concentrated NaOH. After 3 days of incubation, the optical density of the culture was measured to ensure that the amount of biomass used for the different samples was constant. Since the optical density method is

able to measure both viable and non-viable cells, this method was used to measure monitor the amount of biomass. Furthermore, this method is not affected by the interference of the precipitates in the samples.

Aliquots of 8 ml from the three-day culture were transferred into 40 ml polypropylene centrifuge tubes containing Postgate's medium C with 0.0, 10, 20, 25, 50, and 100 mg of copper ion per liter of Postgate's medium C, respectively. These copper concentrations in the tubes were calculated after the addition of Postgate's Medium C, copper chloride solution, and inoculum. Since this medium was not supplemented with reducing agents, a large inoculum consisting of ~1/5 of the total volume (i.e. 8 ml of inocula was andded to 45 ml of medium) was used to provide low $E_h$ conditions for initiation of growth (mohagheghi *et al*, 1984) The copper source was solutions of 1000 mg/L and 5000 mg/L of copper as $CuCl_2.2H_20$. The experimental conditions of these batch tests are shown in Table 3.1. Enrichments B, C, D, *Desulfovibrio desulfuricans*, and *Dusilfovibrio vulgaris* were also used in these experiments. When referring to them, the samples will have their respective enrichment name name. For example, in the first experiment, a sample containing an initial copper concentration of 100 ppm and isolate B will be named as 1-B-100.

**Table 3.1: Experimental conditions for the selected enrichments and the pure cultures.**

| | 1-A-0 | 1-A-10 | 1-A-25 | 1-A-50 | 1-A-100 | 1-A-150 | 1-A-200 |
|---|---|---|---|---|---|---|---|
| Enrichment | A | A | A | A | A | A | A |
| Nutrient medium | P. C [1] | P.C | P.C | P.C | P.C | P.C | P.C |
| [Cu] (ppm) | 0 | 10 | 25 | 50 | 100 | 150 | 200 |
| Incubation time (days) | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Incubation Vessel | Tube [2] | Tubes | Tubes | Tubes | Tubes | Tubes | Tubes |

1: Postgate's medium C
2: Oak Ridge centrifuge tubes

As Figure 3.2 indicates, the tubes were incubated for 5 days at 35°C; a 5-day incubation period was chosen under the assumption that sulfate reduction would be at steady state after this time period (Mohagheghi *et al,* 1984). The tubes were then centrifuged at 750 × *g* for 40 min to separate bacterial cells together with their complement adsorbed or chelated metal precipitate from the supernatant. The centrifuge used was the *Silencer*, H103N3 series. The bacterial-free supernatant was analyzed for sulfate using the Turbidimetric method (A.P.H.A., 1995). In this procedure, sulfate ion ($SO_4^{2-}$) is precipitated in an acetic acid solution with $BaCl_2$ so as to form barium sulfate ($BaSO_4$) crystals of uniform size. Light absorbency of the $BaSO_4$ suspension is measured by a spectrophotometer and the $SO_4^{2-}$ concentration is determined by comparison of the

reading with a standard curve. A more complete description of the Turbidimetric method for sulfate analysis is given in appendix A. The purpose of sulfate analysis in these experiments was to monitor bacterial activity in the presence of different copper concentrations.

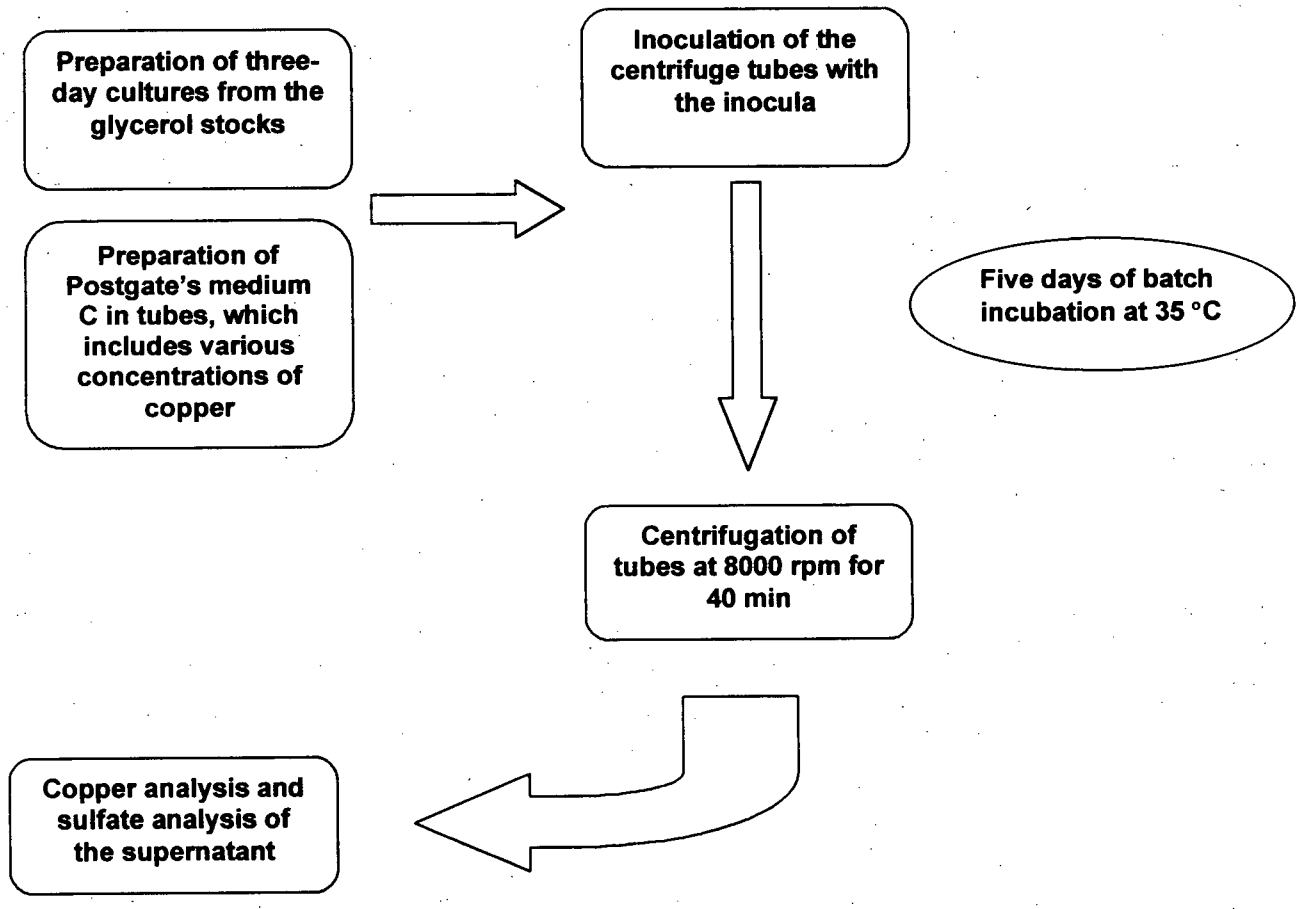


**Figure 3.2: Schematic diagram for copper removal and sulfate reduction analyses for enrichment A-D and pure cultures.**

The concentration of the copper ion in the supernatant was determined using atomic adsorption spectroscop with a Perkin-Elmer model 2380 atomic absorption

spectrophotometer. All the plasticware and glassware used in these studies were soaked in 3 M $HNO_3$ and rinsed several times with deionized water before use to avoid metal contamination. Moreover, each experiment for an individual enrichment was repeated twice, including two replicates for each sample. This experiment was repeated for all the enrichments and pure cultures. Furthermore, control samples were also included. One set of control cultures was allowed to incubate without the addition of inoculum. The purpose of these controls was to investigate whether the pH of the media would affect on the removal of copper from the copper sulfate solution. The other set of inoculum did not have any copper included and the purpose of this control was to study bacterial growth and activity when no copper was added to the sulfate solution.

### 3.3 Affect of Inoculum Age On Copper Removal

The purpose of this experiment was to investigate the effect of inoculum age on copper removal and bacterial activity. Enrichment D was used in this experiment. The growth procedure is similar to the 5-day batch experiments, as described before. Once the Postgate's medium B cultures had grown turbid after five days, 16 ml were transferred to four individual serum bottles containing 104 ml of Postgate's medium C. These four bottles were incubated for 2, 3, 4, and 5 days, respectively. After incubation, 8 ml of inoculum from each serum bottle was used to inoculate 40 ml of Postgate's Medium C in each three serum bottles with copper concentrations of 50, 100, and 200 mg/L respectively. As previously described, the cultures containing copper were incubated for five days at 35°C. The experimental conditions are summarized in Table 3.2. While referring to a sample, the age of the inoculum used will also be mentioned. The samples

were filtered through Whatman # 42 filter paper to remove bacterial cells and precipitates. The filtrate was then analyzed for copper and sulfate concentration.

**Table 3.2: Experimental conditions for enrichment D in a second batch test.**

| | 2-D-0 | 2-D-50 | 2-D-100 | 2-D-200 |
|---|---|---|---|---|
| Enrichment | D | D | D | D |
| Nutrient medium | P.C [1] | P.C | P.C | P.C |
| [Cu] (ppm) | 0 | 50 | 100 | 200 |
| Incubation time (days) | 5 | 5 | 5 | 5 |
| Incubation vessel | Serum bottles | Serum bottles | Serum bottles | Serum bottles |

1: Postgate's medium C

## 3.4 Investigation of the Kinetics of Copper Removal and Sulfate Reduction

The purpose of this experiment was to measure the kinetics of copper removal and sulfate reduction over a 5-day batch period. A volume of 40 ml of Isolate D was used to inoculate 300 ml flasks containing 150 ml of Postgate's medium C and copper in concentrations of 10, 25, 50, 100, 150, and 200 ppm, respectively. This is the same ratio of biomass to nutrient media as all the previous experiments described previously. As Table 3.3 indicates, the flasks were incubated at 35°C under anaerobic conditions using nitrogen gas and 5 ml samples were taken at specified time intervals for a period of 125

hours (~ 5 days). The samples were filtered through Whatman # 42 filter paper to remove bacterial cells and precipitates and the filtrates were analyzed for copper and sulfate as described previously.

**Table 3.3: Experimental conditions for enrichment D in a third batch test.**

| | 3-D-0 | 3-D-10 | 3-D-25 | 3-D-50 | 3-D-100 | 3-D-150 | 3-D-200 |
|---|---|---|---|---|---|---|---|
| Enrichment | D | D | D | D | D | D | D |
| Nutrient medium | P.C [1] | P.C | P.C | P.C | P.C | P.C | P.C |
| [Cu] (ppm) | 0 | 10 | 25 | 50 | 100 | 150 | 200 |
| Incubation time (days) | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Incubation Vessel | E. Flask[2] | E. Flask | E. Flask | E. Flask | E. Flask | E. Flask | E. Flask |

1: Postgate's medium C
2: Erlenmeyer flask

## 3.5 Analysis of the Copper Precipitates

Since bacteria are small organisms, their effects on metal precipitation can be difficult to visualize; bulk analytical imaging techniques, such as wet chemistry, powder X-ray diffraction and light microscopy are not adequate. Electron microscopy techniques together with subsequent image analysis were used to better visualize the location and the structure of the minerals on the bacterial walls.

The purpose of performing the image analysis techniques in this research was to visualize and confirm whether SRB have a direct role in the process of biomineralization.

Moreover, an attempt was made to compare this visual direct role when the initial copper concentrations in the media are different. The results obtained will be used to evaluate the efficiency of using biomass directly in the precipitation process, where, unlike the NTBC Biosulfide Technology, the precipitation of heavy metals will take place in the bioreactor.

It was postulated that if the metal precipitates adhere to the bacteria, then their settling will be enhanced. SRB form aggregates or flocs which settle very easily and any substance that is adsorbed to the bacterial flocs will therefore settle. This combination of flocculation and settling is therefore a mechanism by which metal removal would be more efficiently removed in biological treatment (Steiner *et al*, 1976). Hence, it would be beneficial to have more information regarding the size and the shape of the minerals and the precipitates.

### 3.5.1 Particle Shape and Size

As no two particles will be exactly the same size, the material must be characterized by particle-size distribution (PSD). Examples are the crystal-size distribution (CSD) produced in a precipitation or crystallization process. (Randolph *et al*, 1988). A characteristic dimension of a particle can be thought of as a line passing through the center of mass of the particle and intersecting two opposing surfaces. For highly irregular particles, such characteristic sizes are infinite in number and can be represented by a one-dimensional frequency distribution. Fortunately, this variation in characterization of individual sizes is averaged when a size distribution is obtained on a sample containing a large number of particles. The actual size that is measured in a particle-size distribution analysis lies between the minimum and the maximum

characteristic size (Lowenstam *et al*, 1981). It is essential to note that no definition of particle size is complete without specifying the technique of size measurement. In order to determine the particle size, a representative slurry sample is removed with care and filtered (Randolph *et al*, 1988).

The samples are then removed from the filter and spread on a watch glass, and dried at a temperature below the crystal decomposition temperature (Lowenstam *et al*, 1981). Crystals samples are usually sized by standard sieving. Three-inch standard screens are useful because of sample size. Sizes below 100 μm are best examined by some other techniques, such as zone-screening instrument. Typical instruments are Coulter counters or Particle Data counters. Laser light scattering, sedimentation, and photographic counting can also be used (Lowenstam *et al*, 1981). The techniques used in this research will be discussed later in section 3.5.3.

### 3.5.2 Electron Microscopy

As mentioned before, no chapter on methods for studying biological/mineralogical interactions would be complete without a discussion of the well-established and extensively used electron-probe microscopy techniques: scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Although considered to be bulk analytical techniques, they are invaluable for preliminary characterization of materials and processes. These techniques also can produce near-surface information (e.g., analysis of thin samples and secondary electron imaging) (Macintosh *et al*, 1997).

The TEM is one of the key tools in the study of structural features in biological specimens. It projects electrons through a very thin slice of specimen to produce a two-dimensional image on the phosphorescent screen. The brightness of a particular area of the image is proportional to the number of the electrons that are transmitted through the specimen. Structural information is acquired from transmission electron imaging. The SEM produces an image that gives the impression of three dimensions. This microscope uses a 2-3 nm spot (diameter) of electrons that scans the surface of the specimen to generate secondary electrons from the specimen that are then detected by a sensor. The image is produced over time as the entire specimen is scanned. It is possible to attach a number of different detectors to electron microscopes. For instance, the SEM may be fitted with detectors for X-rays. Electron – probe systems use either an energy-sensitive Si(Li) detector in an energy–dispersion spectrometer (EDS), or a crystal detector. The crystal detector dispersed X-rays, according to wavelength, in a wavelength-dispersion spectrometer (WDS). When an X-ray strikes the semiconductor crystal, the absorbed energy alters the ability of the crystal to conduct a charge. Since the crystal is maintained at a bias voltage of 100 to 1,000 volts, an increase in conductivity of crystal can be readily detected and quantitated. Since the energy of the X-ray is directly proportional to the increase in conductivity in the silicon crystal, it is possible to collect and measure the conducted current over a period of time and determine the intensity of the X-ray emission (Macintosh *et al*, 1997; Buseck *et al*, 1992; Bozzola *et al*, 1992).

### 3.5.2.1 Sample Preparation for Electron Microscopy

Whatever the electron-probe technique, sample preparation is an important component of microanalysis and imaging. The results of electron-probe examination are directly dependent on the integrity of the sample being studied. Since the imaging and analysis are done under vacuum, specimens must be dry. As most mineralogical materials are semi-conductors or insulators, conducting mounts are used and the sample is coated with a thin layer of carbon, gold, or platinum (Bozzola *et al*, 1992). The only other requirement for SEM equipment is that the sample fits the sample chamber (e.g. 100 x 100 x 25 nm) (Buseck *et al*, 1992; Macintosh *et al*, 1997). Mineralogical specimens are prepared either as sample blocks, thin sections, or epoxy mounts. Because TEM instruments use transmitted electrons for imaging, the material must be thin enough to allow electrons to pass through. This is achieved for mineralogical samples either by crushing the sample in methanol and mounting on special TEM micro-grid, or by ion milling a hole in a thin chip to produce electron-transparent edges (Buseck *et al*, 1992).

Preparation of biological material is more complex, principally because they are highly incompatible with the vacuum environment of the instrument (i.e., they contain much water), are prone to damage under electron irradiation, and are composed primarily of light elements (Flegler *et al*, 1993). Most biological samples are chemically fixed in a solution, dehydrated (in ethanol), critical-point dried (the dehydrated sample is infiltrated with liquid $CO_2$, then is heated until the liquid becomes gas), mounted and coated with gold (Morgan, 1985). Alternative methods include air-drying, air-drying from a solvent, vapor fixation or prefixation by using $OsO_4$, freeze drying, embedding in resin, crymicrotomy (for sectioning material), and cryofracturing (Bozzola *et al*, 1992).

Because biological tissue is made up primarily of light elements (C, H, N, and O), structural features are difficult to image in TEM system; these systems require that transmitted electrons be deflected for imaging, and deflection occurs most commonly for the heavier elements. To overcome this difficulty, samples commonly are stained with heavy-metal salts (Hayat *et al,* 1980).

However, if the microorganisms themselves concentrate heavy metals, staining is not required. Degens *et al* report that high contrast was obtained without heavy metal staining, suggesting it is solely due to natural (in situ) heavy metal staining (Degens *et al*, 1982). They have concluded that this phenomenon is due to an exchange of hydrogen or alkali and alkaline-earth ions fixed at the membrane surface for certain heavy metals present in interstitial solutions. Furthermore, metal ions coordinate to oxygen functions present in the organic matter and cause the formation of distinct metal-oxygen polyhedra.

Beveridge *et al* (1978, 1980, 1986) have done significant research regarding sample preparation and electron microscopy techniques (Brierly *et al*, 1976; Geesy *et al*, 1978). Based on Beveridge's results, different metals and different staining techniques on the same sample will yield different structural patterns (Beveridge *et al*, 1978). Since the electron-microscopic image produced by the more common staining reagents (i.e. uranyl accetate, $OsO_4$, ruthenium red) must be a response of the cell wall to their ionic form and charge, the image produced by several stains have been also compared and evaluated in parallel with the data derived with the different metal salts. Four types of staining response were seen when the wall fragments of *Bacillus subtilis* was reacted with 40 different metals: (1) diffuse stain; (2) surface stain; (3) aggregates; and (4) crystals. These

different staining responses are due to the different electron–scattering powers of the metals (Beveridge *et al*, 1978).

In previous research, the samples were most often viewed under TEM (Beveridge *et al,* 1976). In order to further investigate the exact role of cell surface of bacteria in metal deposition process, *Bacillus subtilis* cells were disrupted with French press and the cell envelopes were isolated centrifugally. After treatments with various buffers and several heating steps (to denature autolysins), the fragments were agitated with various metal salts and then fixed in 4% gluteraldehyde. The main purpose of the fixative is to preserve the structure of the living specimen with no alteration from the living state. The walls were next enrobed in Nobel agar, dehydrated through an acetone series, embedded in resin mixture (Vestopal W), sectioned on ultramicrotome, and silver sections were collected on carbon-Formvar-coated 200-mesh grids. No stains were applied to the sections for the initial detection of metal by electron microscopy. To observe and characterize the metal precipitates by EDS, uncontrasted sections were used. In several instances, ruthenium red or uranyl acetate followed by lead citrate were used to stain the sections on the grids to ascertain the "stainability" of the walls after their metal incubations (Beveridge *et al,* 1976, 1980).

Beveridge *et al* (1980) used either a Philips EM300 or EM400T electron microscope, operating at 60 kV under standard conditions to examine the sections. EDS was performed on the Philips EM400T apparatus, which was interfaced to a LINK analytical LZ-5 light elemental detector with an EXL processor. In some samples, selected-area electron diffraction was used to examine the crystallinity of the precipitates. Most metals that had atomic numbers greater than 11 and could be detected by electron

microscopy appeared to diffusely stain thin sections of the wall. Areas of the walls also acted as nucleation sites for the growth of microscopic elemental gold crystals when incubated in solutions of auric chloride.

### 3.5.3 Copper Sulfides on the Cell Surface of SRB

In this research, the purpose of electron microscopy was to obtain qualitative information on the interaction of SRB with copper. As Table 3.4 indicates, four samples were taken from five day old cultures of enrichment D growing in Postgate's medium C and various copper concentrations of 0, 25, 100 and 200 ppm.

**Table 3.4: Growth conditions for samples used for image analysis.**

| | 4-D-0 | 4-D-25 | 4-D-100 | 4-D-200 |
|---|---|---|---|---|
| Enrichment | D | D | D | D |
| Nutrient Medium | P.C [1] | P.C | P.C | P.C |
| [Cu] (ppm) | 0 | 25 | 100 | 200 |
| Incubation time (days) | 5 | 5 | 5 | 5 |
| Incubation Vessle | Serum bottles | Serum bottles | Serum bottles | Serum bottles |

1: Postgate's medium C

After five days of incubation, 15 µl of these samples were mounted on carbon coated nickel grids and stained with uranyl acetate for observation under a Philips EM 400T (operating at 100 kV) TEM equipped with EDS. Samples that came from cultures grown in the presence of copper did not need to be stained with uranyl acetate (Degens *et*

*al*, 1982). The instrument was operated with a liquid nitrogen cooled anticontamination device in place at all times. An unstained sample that initially had a copper concentration of 100 PPM was examined with a Philips EM400T (operating at 100 kV) equipped with an energy dispersive X ray spectrometer (LINK Analytical eXL/ LZ-5) for elemental analysis. EDS was conducted with a spot size of 4 nm and a beam current of 0.1 μA, and a counting time of 100s.

### 3.5.4 Precipitate Composition, Size and Shape

In this research, the precipitate composition, size and shape were determined using the following method. The solid samples were obtained by carefully filtering samples 4-D-25, 4-D-100, and 4-D-200 (Table 3.1), using Whatman filter paper, size 42. The precipitates were then well spread on the filter paper. The precipitates were carefully washed with distilled water and together with the filter paper, were put in an oven at 60°C for 2 hrs. In order to avoid oxidation of the samples, the filter papers containing the crystals were then put into a desiccator. Small sections of the filter paper were then thin-coated with carbon and observed using a Philips XL30 SEM, equipped with PGT IMIX (Integrated Microanalyzer for Imaging and X-ray) energy dispersive spectroscopy (EDS)/image analysis (IAS) system. The IAS system program is able to detect and calculate particle size of crystals and precipitates. EDS was conducted with different spot sizes at 15-20 kV and the spot size was chosen according to the magnification being used.

# 4 RESULTS AND DISCUSSION

This chapter presents a detailed discussion of the results obtained and the data collected in the experiments introduced in the previous chapter. It includes four main headings: (1) SRB enrichments from a mixed culture, (2) Extent of copper removal and inhibition of SRB activity for the enrichments and the pure cultures, (3) Copper removal versus sulfate reduction, and (4) Location and structure of copper sulfides.

## 4.1 SRB Enrichment Preparation from a Mixed Culture

The SRB cultures used in these experiments were enrichments from a mixed culture of anaerobes obtained from NTBC Research Corporation, Richmond, BC. Adjusting the pH to the optimal range (7-7.50) and ensuring a very anaerobic environment were crucial for growth of black colonies in the primary enrichment step (Postgate, 1984). Growth of distinct black colonies proved to be difficult and oftentimes, the colonies grew better within the agar. This phenomenon most likely suggests that these bacteria need very strict anaerobic environments for growth.

**Table 4.1 Evaluation of different solid media for enrichment of SRB.**

| Solid growth medium* | Extent of growth |
|---|---|
| Iverson's medium | + |
| Postgate's medium F | + |
| Postgate's medium E | ++ |
| Modified Postgate's medium E | ++++ |

* The exact composition of the media is described in Appendix C.

Table 4.1 illustrates the extent of growth of SRB using different growth media. It was concluded that SRB grew best on modified Postgate's medium E, which was modified by decreasing the amount of yeast extract by half (i.e. from 1 g to 0.5 g). Growth of black areas due to the formation of FeS was evident within the agar in the shake tubes after 24-36 hrs. The black areas started as small patches evenly distributed within the agar. Additionally, very small whitish colonies were also observed within the agar. Between the time period of 36- 48 hrs, the agar turned a uniform black color. Although there seemed to be substantial growth of creamy colonies on the other solid media, no significant growth of SRB (i.e. black areas) was observed. Most likely, the other white and creamy colonies were other microorganisms such as methane producing bacteria and yeast in the NTBC culture had the advantage to grow on the media, which had more yeast extract included.

Growth of SRB on the surface of agar proved to be more difficult. In the first set of plates, which were prepared by mixing of inoculum with molten agar, very few distinct dark patches were observed. However, in the second set of plates where the cells were spread on the surface of the agar, no black colonies were observed on the surface of the agar. The majority of colonies observed were white and creamy. As Table 4.2 indicates, four different colonies were selected from the plates and agar shake tubes, referred to as Enrichments A to D. Enrichment C and D were isolated from the plates where the inoculum was mixed with the molten agar. More than one colony was selected to test whether the different enrichments of SRB from the original mixed NTBC culture had different copper removal capabilities. The results obtained will be discussed in section 4.2.1.

**Table 4.2: SRB colonies from plates and agar shake tubes.**

| Enrichments | Source | Growth medium |
|---|---|---|
| A | Isolated from an agar shake tube | Modified Postgate's medium C |
| B | Isolated from an agar shake tube | Modified Postgate's medium C |
| C | Isolated from a plate | Modified Postgate's medium C |
| D | Isolated from a plate | Modified Postgate's medium C |

Microscopic observation indicated that the colonies were gram-negative and the individual cells were rod-shaped. The majority of the cells formed clusters, possibly due to presence of extracellular polymers. After two days of incubation, Postgate's medium B turned dark black as this medium contained a high concentration of iron. Inoculation of Postgate's medium C with SRB did not result in as intense black color, since this medium has a minimal concentration of iron. However, presence and growth of SRB was indicated due to the strong odor of hydrogen sulfide and sulfate removal as indicated in Table 4.3. In this table, enrichments A, B, and D indicated similar values for sulfate reduction; however, enrichment C does not indicate significant sulfate reduction. The pure cultures also indicate some sulfate reduction, although not as high as the enrichments.

Based on results obtained, enrichment of sulfate reducers is recommended through the agar shake tube method, which provides the necessary anaerobic environment for the growth of SRB. Moreover, as Table 4.1 suggests, Postgate's medium E, with a reduced amount of yeast extract (i.e, 0.5 g), was successful for SRB growth. This

suggests that using a medium with less yeast extract allowed for better SRB selection, perhaps by minimizing growth of competing species. The enrichments grew very well after transfer to Postgate's medium B. It is strongly recommended to transfer SRB from a solid media to Postgate's medium B, which contains the necessary reducing conditions for SRB growth (Postgate, 1984). Once SRB grow in this medium and the $H_2S$ produced provides the necessary reducing conditions, the culture can be transferred to Postgate's medium C, a defined medium, for various analyses.

**Table 4.3: Sulfate reduction of the different enrichments used as inocula.**

| Culture | Inoculation period (days) | Nutrient media | Initial $[SO_4]$ (mg/L) | Final $[SO_4]$ (mg/L) |
|---|---|---|---|---|
| Enrichment A | 3 | P.C [1] | 3067 | 1681 |
| Enrichment B | 3 | P.C | 3169 | 1573 |
| Enrichment C | 3 | P.C | 3155 | 2973 |
| Enrichment D | 3 | P.C | 3206 | 1670 |
| *D. desulfuricans* | 3 | P.C | 3208 | 2076 |
| *D. vulgaris* | 3 | P.C | 3265 | 1944 |

1:Postgate's medium C.

## 4.2 Extent of Copper Removal and Sulfate Reduction for the Enrichments and the Pure Cultures

In these experiments, sulfate reduction by the SRB enrichments and the pure cultures in presence of copper, and copper removal from the solution was investigated. The 5-day batch experiments investigated the extent of sulfate reduction and copper

removal of the enrichments and the pure cultures. Table 4.4 presents the average copper concentration of the two runs after five days. Figures 4.1-4.6 present the final copper concentration versus initial copper concentration results for all the different isolates *Desulfovibrio desulfuricans*, and *Desulfovibrio vulgaris*.

### 4.2.1 Copper Removal

**Table 4.4: Copper concentration after a 5-day batch experiment.**

| | Final [copper] (ppm) | | | | | |
|---|---|---|---|---|---|---|
| Initial [Cu] (ppm) | Enrichment A | Enrichment B | Enrichment C | Enrichment D | *Desulfovibrio desulfuricans* | *Desulfovibrio vulgaris* |
| 0 | 0.000 | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 |
| 10 | 0.070 | 0.095 | 6.00 | 0.050 | 0.080 | 0.150 |
| 25 | 0.040 | 0.078 | 23.0 | 0.040 | 0.100 | 0.655 |
| 50 | 0.040 | 0.060 | 56.3 | 0.033 | 3.98 | 9.10 |
| 100 | 0.015 | 0.040 | 103 | 0.030 | 6.70 | 13.6 |
| 150 | 0.065 | 0.033 | 158 | 0.040 | 16.6 | 29.8 |
| 200 | 10.3 | 20.2 | 206 | 10.6 | 23.9 | 43.2 |

Table 4.4 shows that copper removal below 0.1 ppm was achieved after five days for the isolates A, B, and D, grown in medium containing an initial copper concentration of 150 ppm or less. There was little or no copper removal by enrichment C. Since there was no sulfate reduction for these cultures, this enrichment probably did not have an active population of sulfate reducers. *Desulfovibrio desulfuricans* and *Desolfovibrio vulgaris* showed copper removal, although not as much as the enrichments A, B, and D. A discussion of the postulated reasons for these results follows later in the chapter. In the case of enrichments A, B. and D, it is indicated that at an initial concentration of 200 ppm, the final concentration of copper is different for the two replicate runs. For

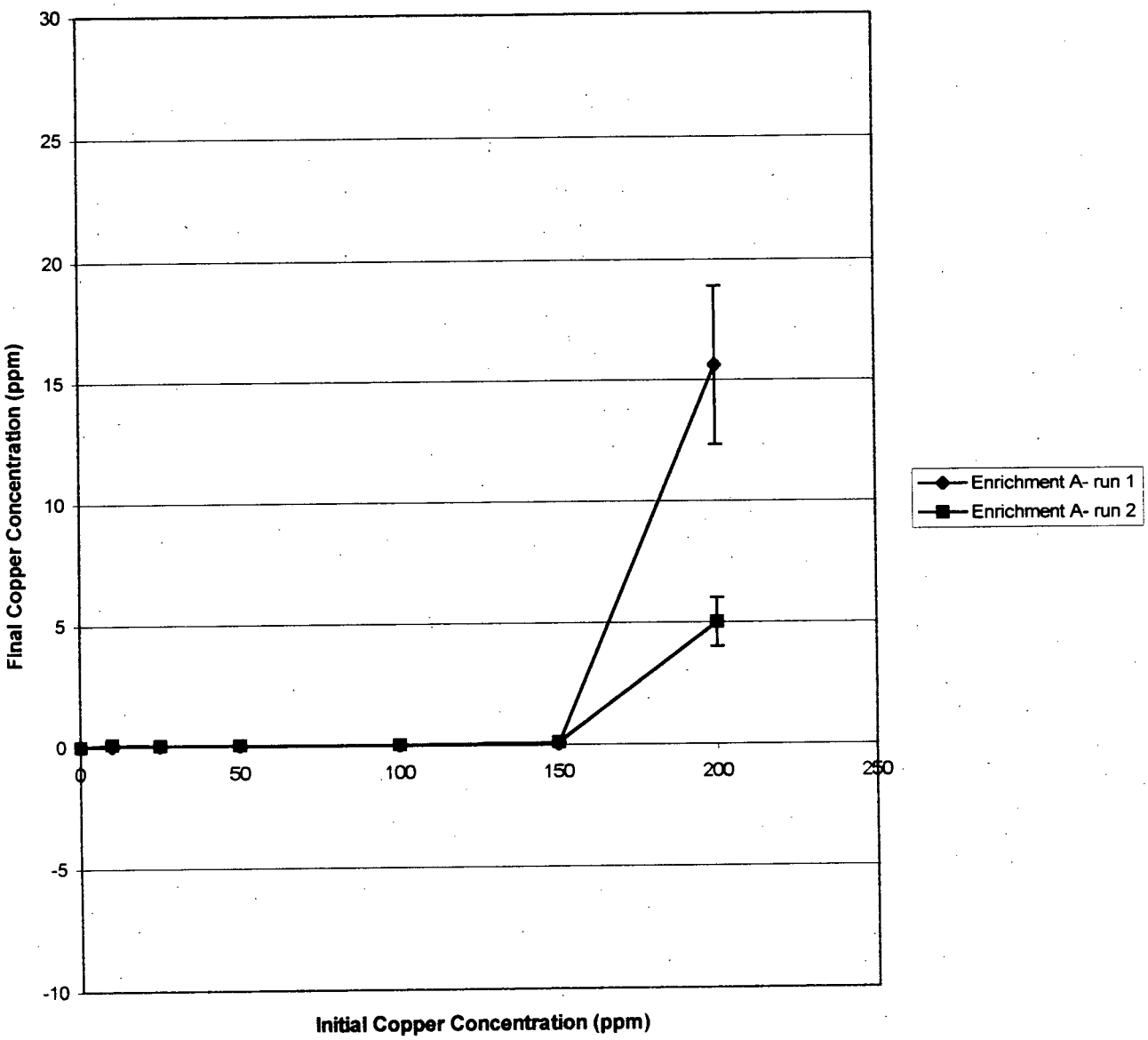


**Figure 4.1: Final copper concentration in the supernatant after five days incubation of SRB enrichment A in Postgate's medium C versus initial copper concentration.**

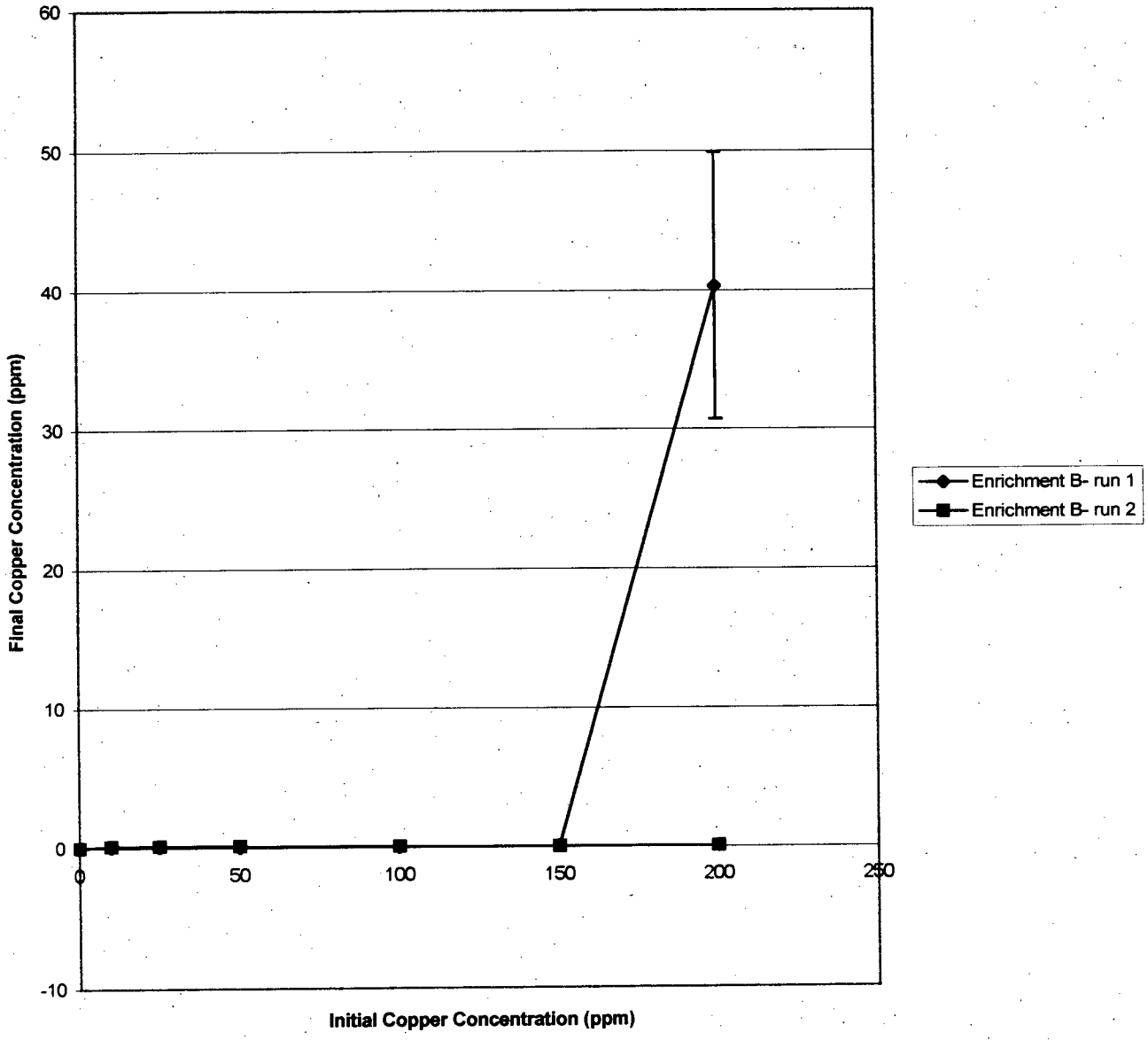


**Figure 4.2: Final copper concentration in the supernatant after five days incubation of SRB enrichment B in Postgate's medium C versus initial copper concentration.**

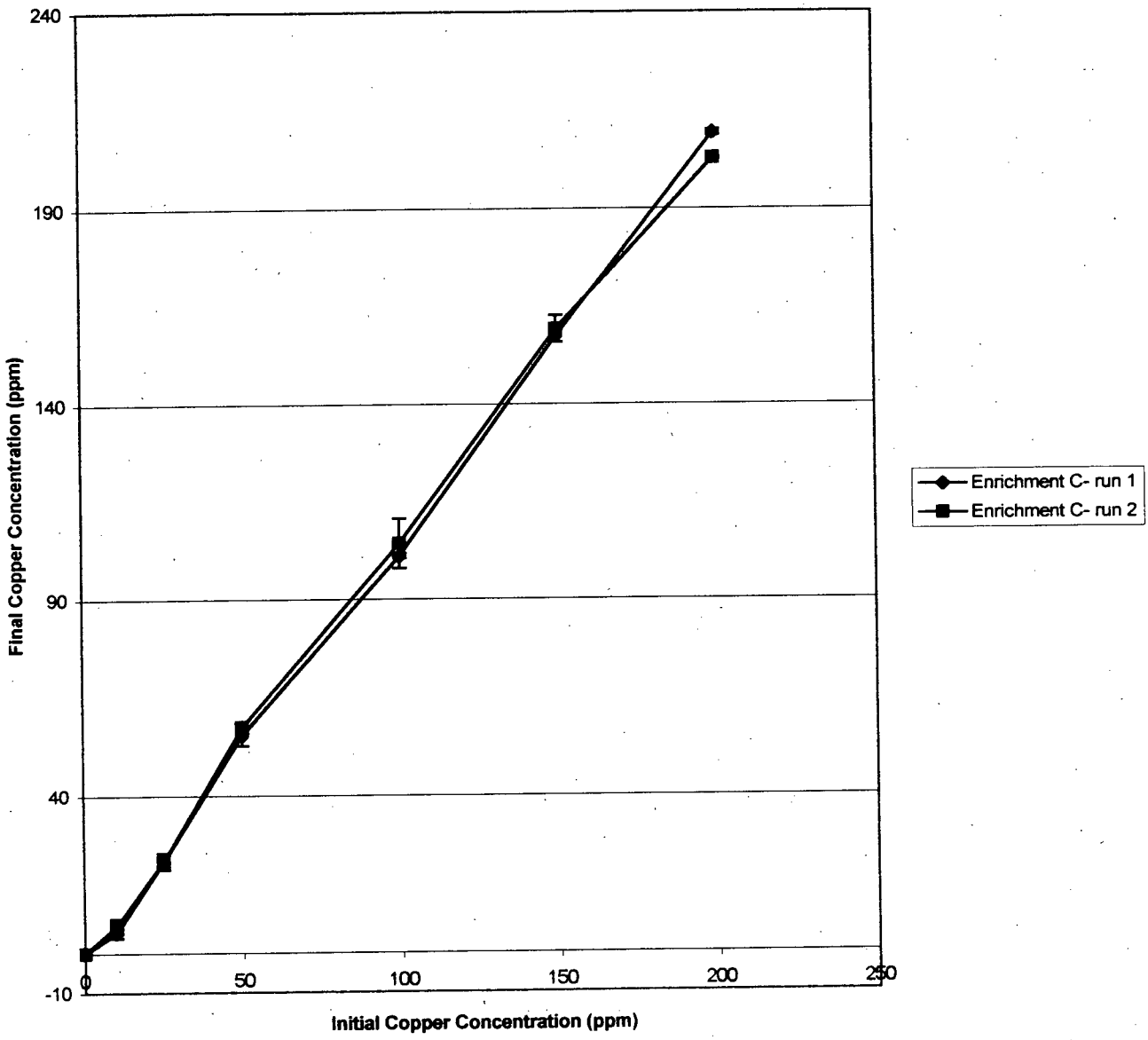


**Figure 4.3: Final copper concentration in the supernatant after five days incubation of SRB enrichment C in Postgate's medium C versus initial copper concentration.**

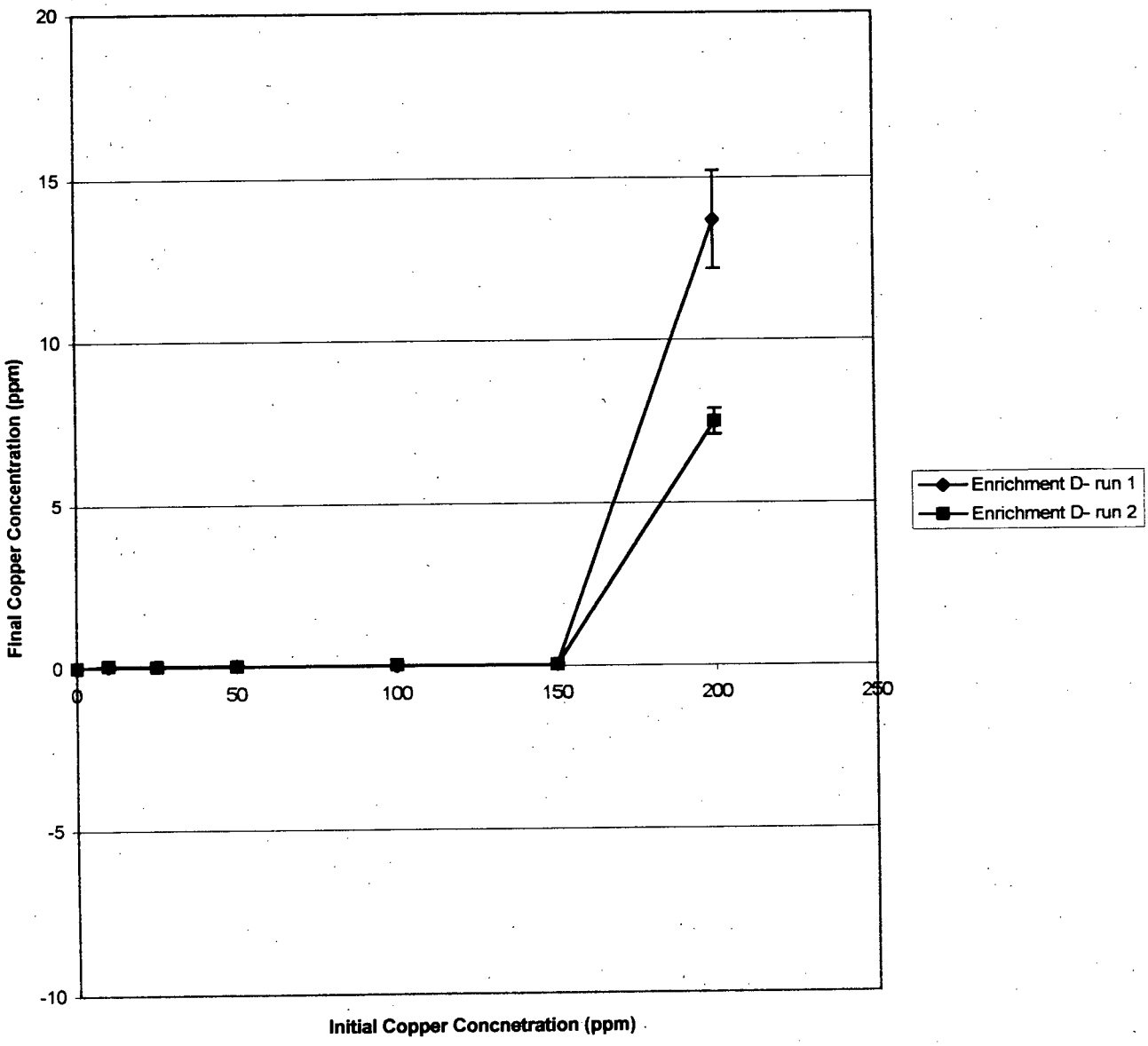


**Figure 4.4: Final copper concentration in the supernatant after five days incubation of SRB enrichment D in Postgate's medium C versus initial copper concentration.**

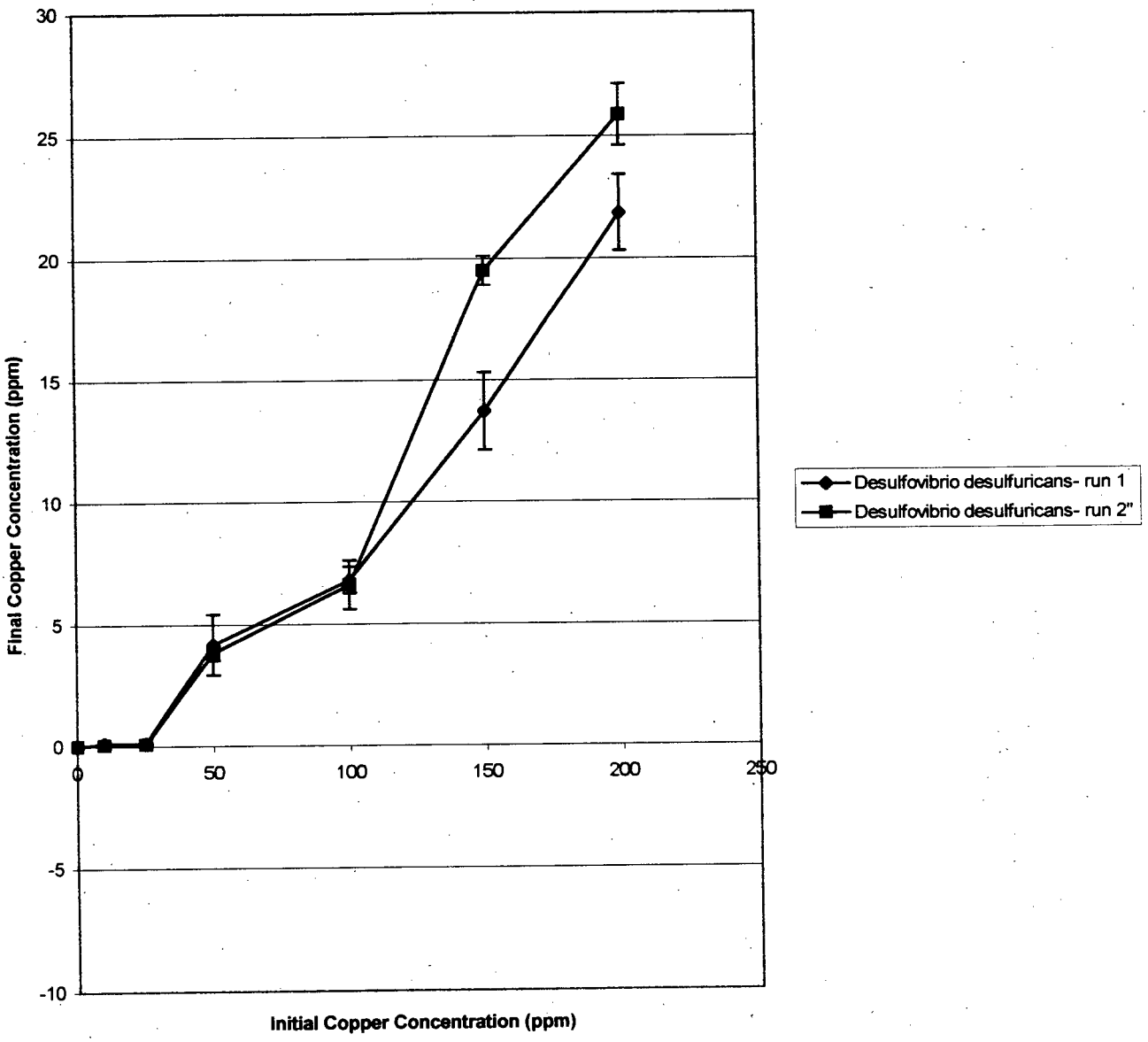


**Figure 4.5: Final copper concentration in the supernatant after five days incubation of *Desulfovibrio desulfuricans* in Postgate's medium C versus initial copper concentration.**

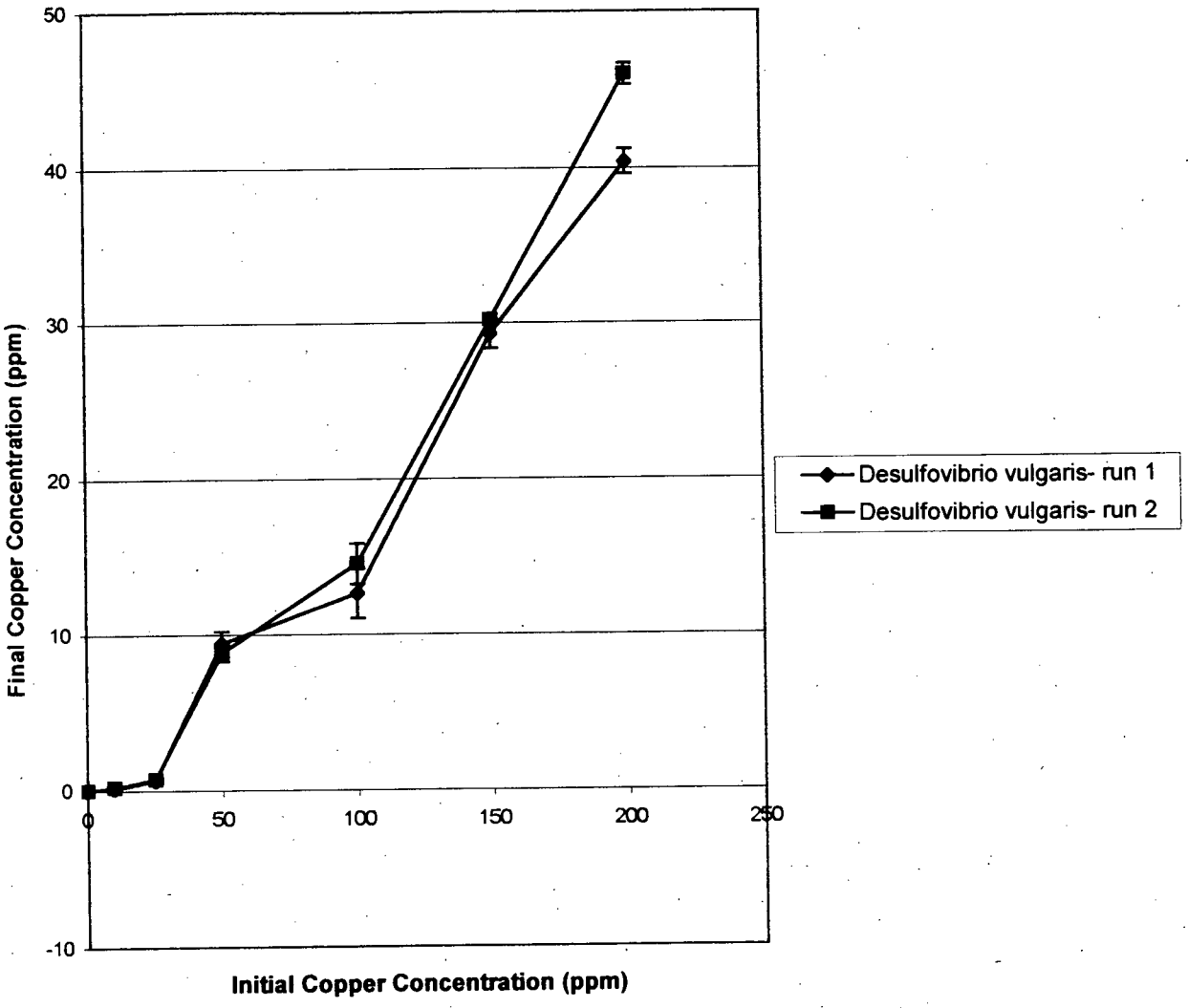


**Figure 4.6: Final copper concentration in the supernatant after five days incubation of *Desulfovibrio vulgaris* in Postgate's medium C versus initial copper concentration.**

enrichments A, B, and D, the error bars indicate that there are significant differences between the replicates having initial copper concentration of 200 ppm. This difference between the replicates might be due to different experimental conditions where the $H_2S$ concentrations in the inocula were different. The error bars indicated in Figures 4.1-4.6 were obtained using duplicates of each samples; error bars were calculated for all samples and they are not all visible due to their being very small. As mentioned earlier, optical density measurements ensured that the same amount of biomass was used in these experiments. It is believed that for lower initial copper concentrations, all the copper was removed due to sufficient hydrogen sulfide introduced in the inocula. Moreover, for cultures having a lower initial copper concentration that 200 ppm, very similar values were obtained for replicates; hence, the error bars are invisible on the plots. For the cultures having higher initial copper concentration (i.e. 200 ppm), not all the copper is removed from solution after addition of inocula. Some copper remains in the solution, where it may affect SRB growth. The original bacterial population obtained from NTBC was adapted to grow in the presence of lower concentrations of copper (50 ppm). Control samples that were not inoculated with bacteria confirm the fact that copper was not precipitated because of the pH of the media, but due to the presence of SRB. The copper removal results of these samples are not represented in Table 4.4 since all the samples confirmed no copper removal. These results are represented in Appendix B.

Figure 4.7 presents the results for copper removal of enrichment D for a time period of 125 hours, i.e. ~ five days. Table 3.3 summarizes the experimental conditions for this experiment. Figure 4.7 indicates that samples 3-D-10, 3-D-25, and 3-D-50, the final copper concentrations dropped to less than 1 ppm immediately after inoculation

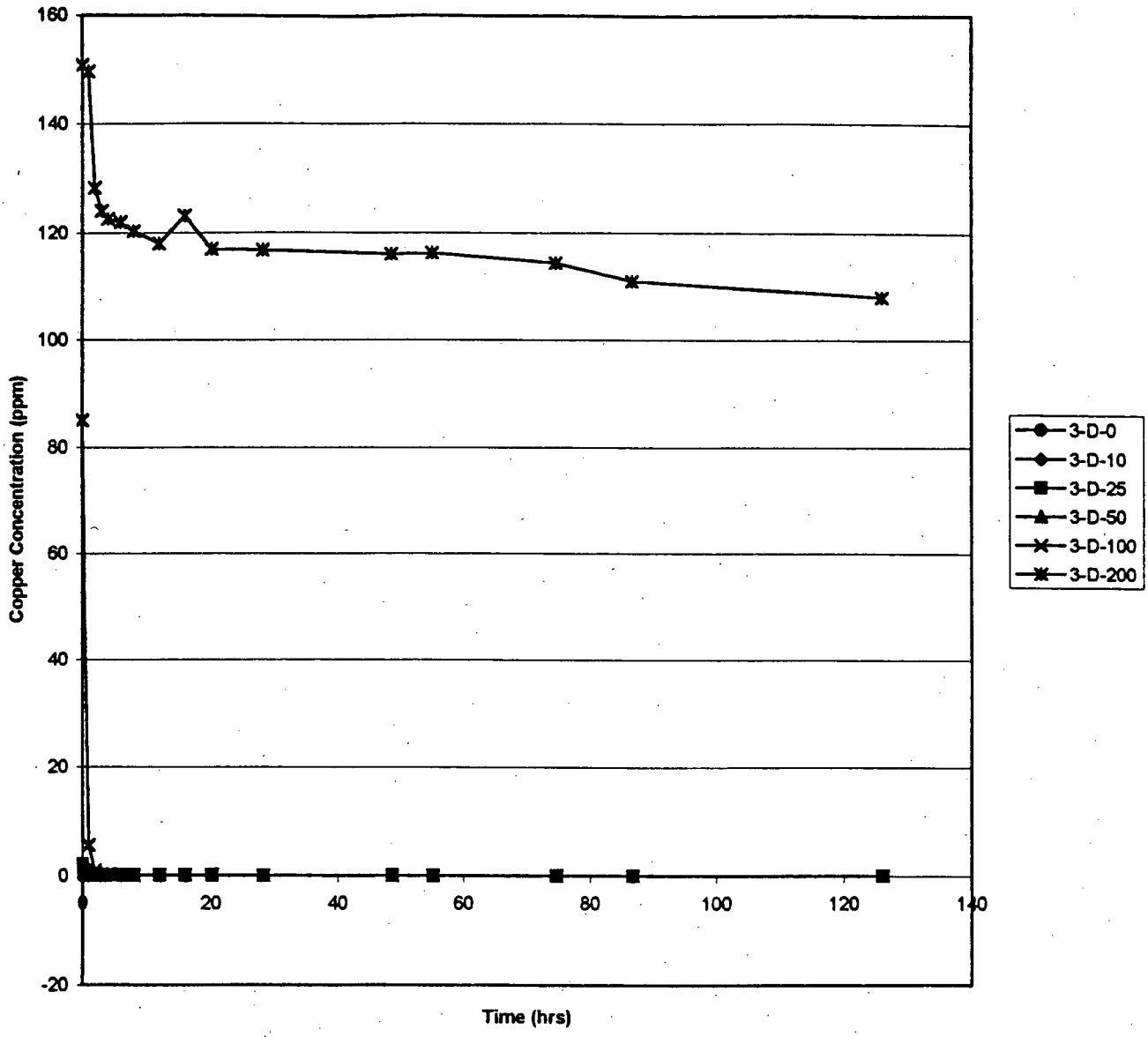


**Figure 4.7: Copper removal versus time for 125 hours of incubation of SRB isolate D in Postgate's medium C, in presence of copper of different initial concentrations.**

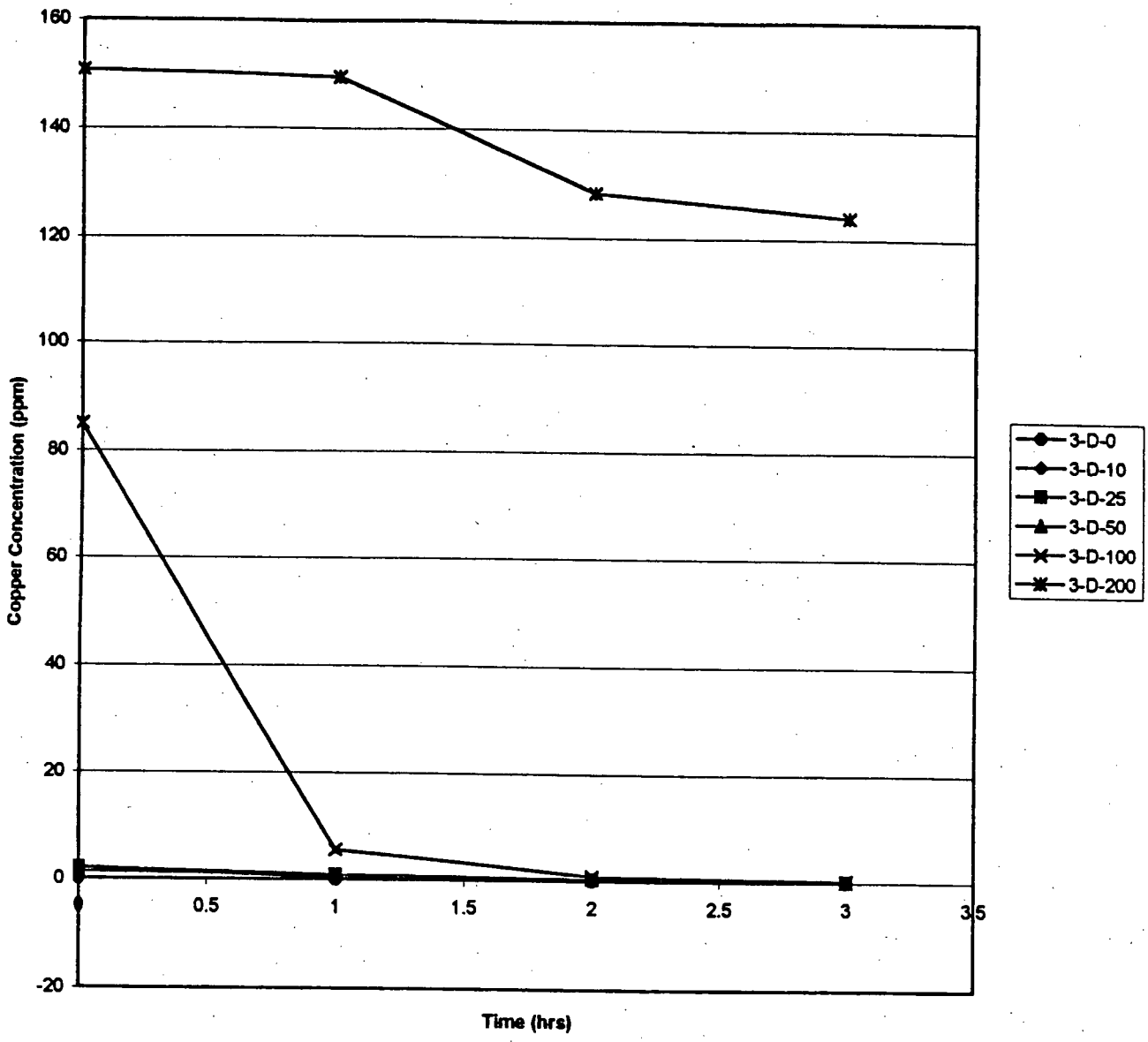


**Figure 4.8: Copper removal versus time for 3 hours of incubation of SRB isolate D in Postgate's medium C, in presence of copper of different initial concentrations.**

(i.e., t=0). For culture 3-D-100, it took about 3 hours to get all the copper precipitated in the solution. The early decrease in copper concentration in these samples is better illustrated in Figure 4.8, which is a modification of Figure 4.7; Figure 4.8 shows copper concentration over a period of 3 hours. The 3-D-200 culture also had a sharp decrease of copper concentration immediately after inoculation. However, the copper concentration did not decrease significantly after the first hour; the rapid drop in copper concentrations immediately after inoculation most likely was due to reaction of copper with $H_2S$ in the inoculum and precipitation of CuS. In order to verify this hypothesis, an experiment would have to be performed where the same amount of hydrogen sulfide is added to the copper sulfate solution, without the presence of bacteria.

### 4.2.2 Sulfate Reduction (SRB Activity)

The extents of sulfate reduced by the enrichments and the pure cultures are shown in Table 4.5 and Figures 4.9-4.10.

**Table 4.5: Sulfate concentration after 5-day batch experiment.**

| | Sulfate reduced (%) | | | | | |
|---|---|---|---|---|---|---|
| Initial [Cu] (ppm) | Enrichment A | Enrichment B | Enrichment C | Enrichment D | *Desulfovibrio desulfuricans* | *Desulfovibrio vulgaris* |
| 0 | 45.4 | 47.4 | 6.2 | 52.8 | 37.8 | 36.1 |
| 10 | 45.5 | 47.6 | 2.2 | 46.5 | 34.8 | 34.3 |
| 25 | 46.8 | 46.8 | 2.9 | 46.9 | 33.0 | 31.6 |
| 50 | 44.0 | 44.2 | 3.0 | 49.2 | 29.6 | 26.7 |
| 100 | 39.6 | 39.5 | 0.9 | 39.3 | 19.2 | 16.6 |
| 150 | 27.9 | 28.4 | 1.9 | 35.3 | 14.6 | 12.0 |
| 200 | 15.6 | 12.6 | 0.1 | 9.7 | 4.0 | 2.0 |

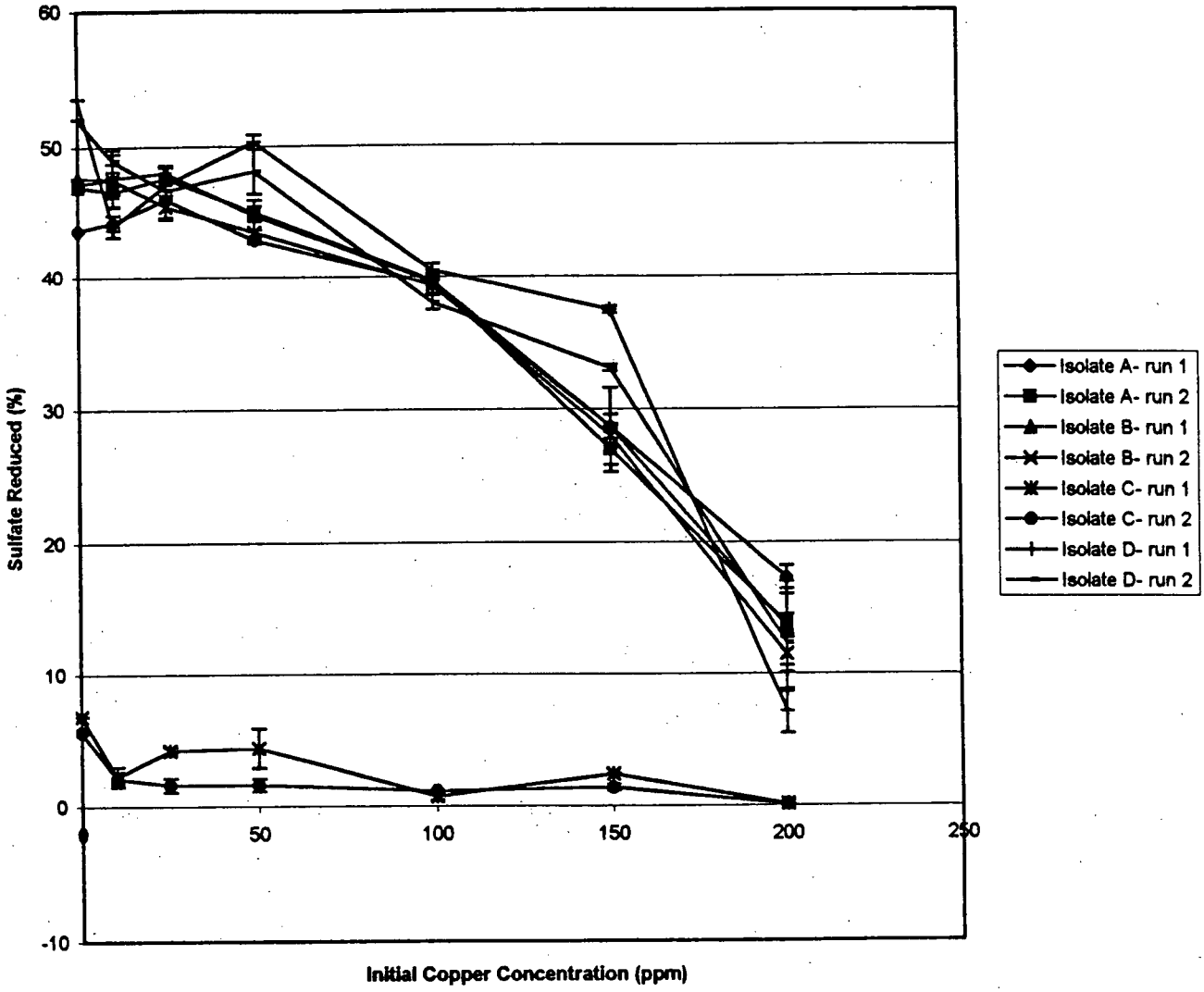


**Figure 4.9: Sulfate reduction after five days of incubation of SRB isolates A, B, C, and C and D in Postgate's medium C versus initial copper concentration.**

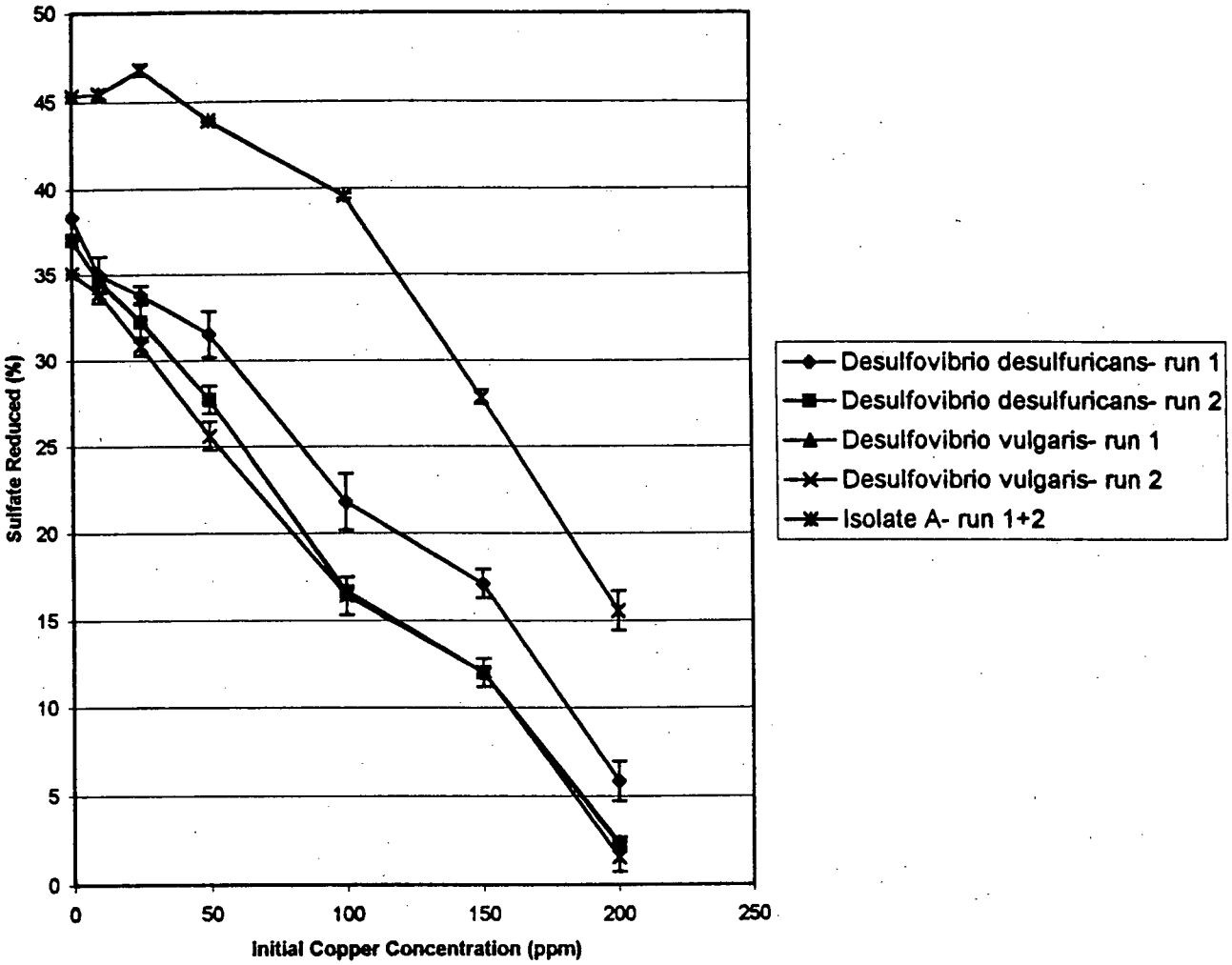


**Figure 4.10: Sulfate reduction after five days of incubation of SRB isolates A, *Desulfovibrio desulfuricans*, and *Desulfovibrio vulgaris* in Postgate's medium C versus initial copper concentration.**

As indicated in Figure 4.9, when the copper concentration was increased from 0 to 50 ppm, the degree of sulfate reduction remained more or less the same. However, for copper concentrations greater than 50 ppm, the degree of sulfate reduction declined with increasing copper concentration. At low concentrations, copper was rapidly precipitated due to the presence of available initial soluble $H_2S$; at higher initial copper concentrations than 100 ppm, sulfate reduction declined sharply, most likely due to the presence of copper in the solution. Sulfate reduction inhibition will further be discussed this section.

At lower initial copper concentrations, sulfate is reduced by approximately 50% and the reduction level and its trend seem to be similar for all the other isolates expect isolate C. As Figure 4.10 shows, compared to enrichment A, *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* reduced less sulfate as the initial copper concentration in the media was increased. Unlike Enrichment A, B, and D, sulfate reduction decreased at lower initial copper concentrations i.e. 50 ppm, versus 100 ppm for the enrichements. As Table 4.5 shows, overall, compared to the enrichments, less sulfate is reduced by the pure cultures.

Comparing the extent of copper removal of different enrichments and pure cultures was one of the objectives of this research. It was of interest to know whether different enrichments of SRB would be capable of the same levels of copper removal. Copper removal by the pure cultures was not as high as the enrichments from a mix culture grown under the same conditions. As Figures 4.5 and 4.6 suggest, copper removal ability of *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* declined at an initial copper concentration of 50 ppm. Figure 4.10 also shows that the maximum sulfate reduced by these cultures is less than 40%. One of the explanations for these observations

is that the pure cultures would be sensitive to copper in their environment and would need to grow in presence of copper for longer period of time for a physiological adaptation. Moreover, as Table 4.3 previously indicated, the pure culture inocula did not reduce as much sulfate as the SRB enrichments from the original mixed culture; hence less hydrogen sulfide was available in the inocula to remove the copper initially. The lower sulfate reduction by the pure culture may be caused by a prolonged lag phase. Furthermore, no significant difference between the two SRB species was observed.

Figure 4.11 presents the results for sulfate reduction by enrichment D, over a period of 125 hours. Culture 3-D-0 shows high levels of sulfate reduction after the first 20 hours of inoculation. After 125 hours, close to 90% of the total sulfate in the solution had been reduced. For cultures 3-D-10, 3-D-25, 3-D-50, and 3-D-100, after 125 hours of incubation, around 50% of the total sulfate in the solution was reduced. At this point, the sulfate reduction seemed become constant as the SRB reached stationary phase. Although the cultures 3-D-10, 3-D-25, 3-D-50, and 3-D-100 were able to precipitate close to 100% of the copper within the first few hours of incubation, they show less sulfate reduction compared to the control culture 3-D-0. The possible reasons for this observation will be explained later. Culture 3-D-200 had a different level of sulfate reduction compared to the other cultures. After 125 hours of incubation, close to 35% of the total sulfate was reduced. Table 4.6 summarizes the results of sulfate reduction of enrichment D over the 5-day batch period. Furthermore, the initial rates of sulfate reduction between t = 6 hrs and t = 20 hrs for all the cultures are calculated. It is indicated that the rate of sulfate reduction decreases with increased copper concentration in the media.

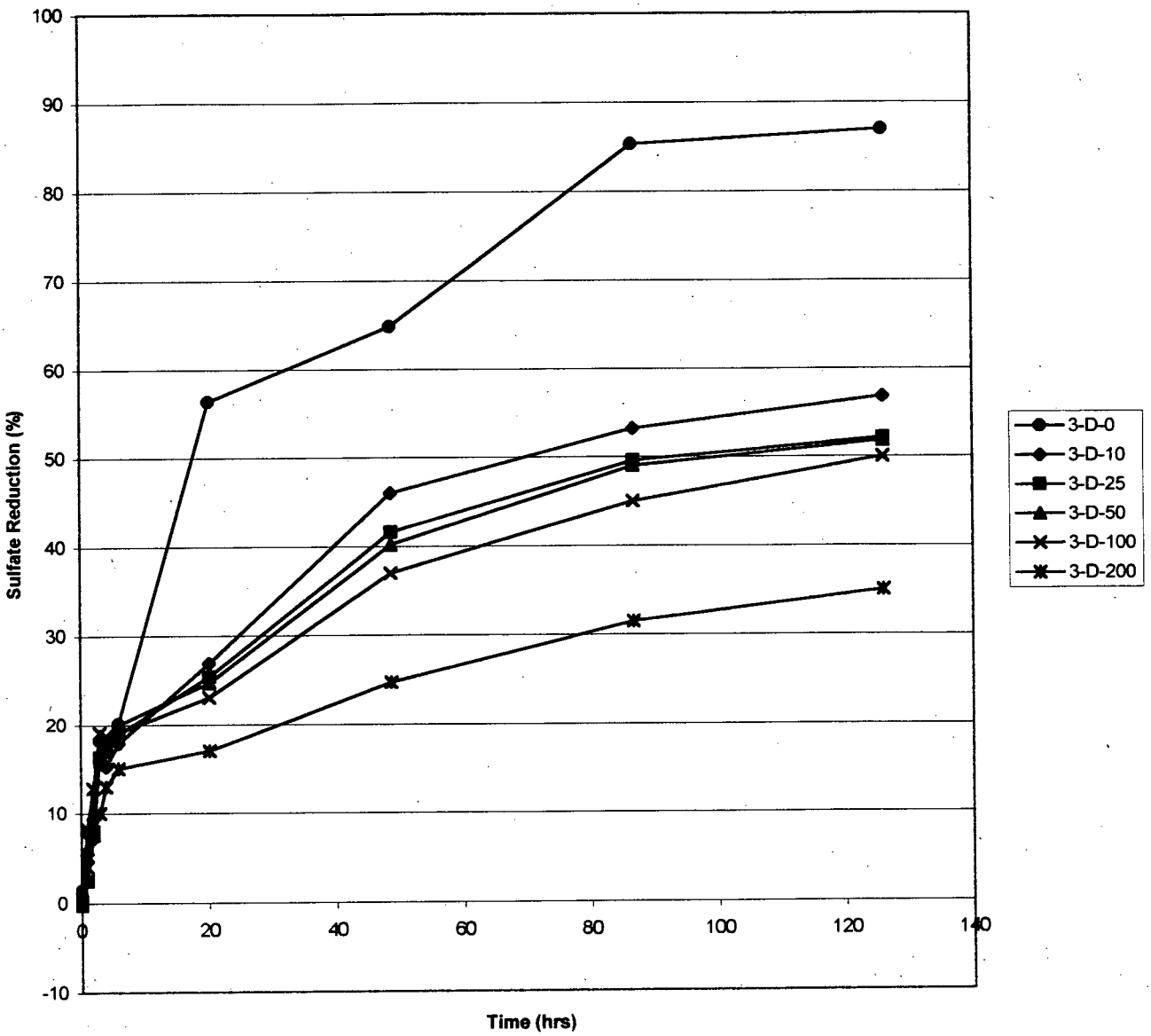


**Figure 4.11: Sulfate reduction versus time for 125 hours of incubation of SRB enrichment D in Postgate's medium C, in presence of copper of different initial concentrations.**

**Table 4.6: Sulfate reduction of enrichment D in batch cultures over a 125 hours.**

| Culture | Rate of sulfate reduction* (mmoles $L^{-1} \cdot hr^{-1}$) | Sulfate reduced after 125 hrs (%) |
|---|---|---|
| 3-D-0 | 1.45 | 87 |
| 3-D-10 | 0.352 | 57 |
| 3-D-25 | 0.255 | 52 |
| 3-D-50 | 0.184 | 52 |
| 3-D-100 | 0.153 | 57 |
| 3-D-200 | 0.078 | 35 |

*: The rate of sulfate reduction between t=6 hrs and t=20 hrs.

Figure 4.12 shows the plot of sulfate reduction inhibition versus initial copper concentration. The sulfate reduction inhibition was calculated by dividing the rate of sulfate reduction by the different cultures by the sulfate reduction rate of sample 3-D-0, between time 6 and 20 hours. The sulfate reduction inhibition was increased with the increase in the initial copper concentration. This observation appears to contradict with earlier observations that copper concentration less that 50 ppm had no effect on sulfate reduction. Hence, more experiments and research is needed regarding sulfate reduction inhibition All the cultures that had copper included showed sulfate reduction inhibition. It seems that even though copper was completely precipitated in cultures 3-D-10, 3-D-25, 3-D-50, and 3-D-100, the presence of the copper precipitates somewhat inhibited sulfate reduction. Perhaps this is due to the presence of copper precipitates on the bacterial cell surface, where they may interfere with cell metabolism.

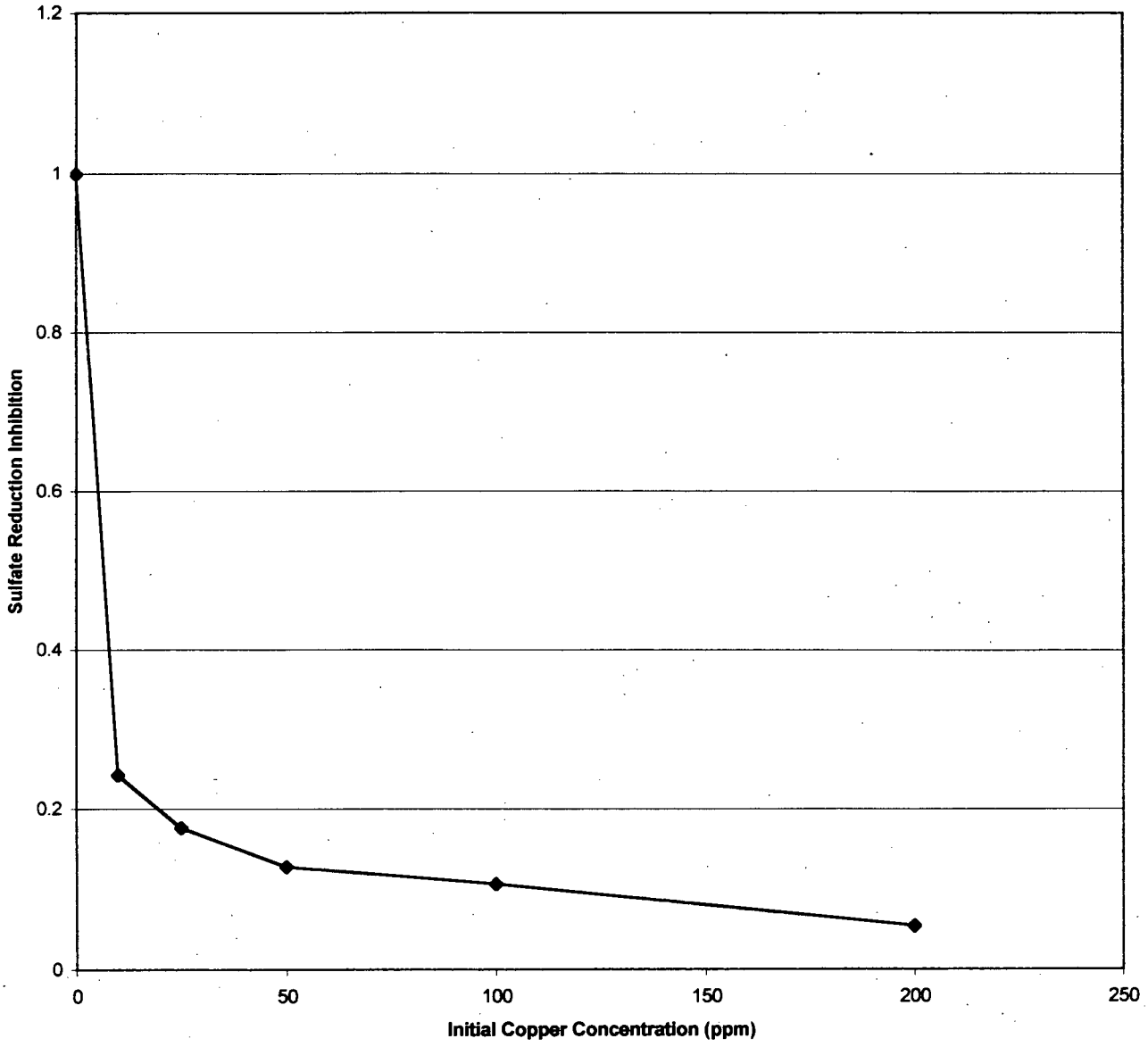


**Figure 4.12: Sulfate reduction inhibition after five days of incubation of SRB enrichment D in Postgate's medium C versus initial copper concentration.**

Figures 4.7 and 4.11 suggest that while culture 3-D-200 reduced 15% of the total sulfate from t = 20 hrs to t=125 hrs, the copper concentration did not change significantly and remained relatively constant. This observation may be due the sampling procedure used in this experiment. Since taking samples for copper and sulfate analysis required opening of the caps of the Erlenmeyer flasks, some $H_2S_{(g)}$ was lost to the air; hence, less gaseous hydrogen sulfide was trapped in the incubating flask. As the $H_2S_{(l)}$ is in equilibrium with gaseous $H_2S$ at low redox potentials, less soluble sulfide was available to precipitate copper (Brock *et al,* 1994; Zehnder, 1988). In the case of the samples with lower initial copper concentration, the loss of some hydrogen sulfide to air did not affect the results. Presumably, more than enough hydrogen sulfide was present in these samples to precipitate all of the copper present in the solution.

It is recommended to perform this experiment in bottles that include outlets for sampling on the bottom of the flasks, where only liquid may escape from the flask. In this way, much less hydrogen sulfide would be lost from the flask.

### 4.2.3 Observations of the Enrichments and the Pure Cultures During the 5-day Batch Period

Table 4.7 provides detailed observations of cultures having different initial copper concentration before and after inoculation. Note that these observations do not describe a specific culture but all the cultures having the same initial copper concentration.

After inoculation, cultures having copper included immediately turned into a brownish solution from an initial pale blue medium. The intensity of the brown color was increased as the initial copper concentration was increased. After a period of 2-3 hours

after inoculation, the cultures with initial concentration of 10-50 ppm copper showed settling of particles, most likely metal sulfides and biomass. Once all the particles were settled, i.e. after 1 day, the growth media turned into a pale yellow color, the color observed before addition of copper and inoculum. When the initial copper concentration was 150 ppm and higher, a very fine dark brown suspension was observed by the third day and the particles settled after five days of incubation. Most of the particles settled after the five days of incubation.

**Table 4.7: General qualitative observations prior and after inoculation.**

| | Observations | | | | |
|---|---|---|---|---|---|
| Initial [Cu] of the culture | Before inoculation | Immediately after inoculation | 2-3 hrs after inoculation | 3 days after inoculation | 5 days after inoculation |
| 0 | Yellow | Slightly turbid | Slightly turbid | Turbid [1] | Very turbid |
| 10 | Very pale blue | Brown and turbid | Pale brown solution with particles settled on the bottom | Yellow solution with dark brown precipitates on the bottom | Yellow solution with dark brown precipitates on the bottom |
| 25 | Very pale blue | Brown and turbid | Pale brown solution with particles settled on the bottom | Yellow solution with dark brown precipitates on the bottom | Yellow solution with dark brown precipitates on the bottom |
| 50 | Pale blue | Dark brown | Brown solution with particles settled on the bottom | Pale brown solution with dark brown precipitates on the bottom | Yellow solution with dark brown precipitates on the bottom |

| Initial [Cu] of the culture | Observations | | | | |
|---|---|---|---|---|---|
| | Before inoculation | Immediately after inoculation | 2-3 hrs after inoculation | 3 days after inoculation | 5 days after inoculation |
| 100 | Pale blue | Dark brown | Brown solution with particles settled on the bottom | Pale brown solution with dark brown precipitates on the bottom | Yellow solution with dark brown precipitates on the bottom |
| 150 | Dark blue | Very dark brown | Dark brown solution with suspended particles in the solution | Brown solution with particles settled on the bottom | Very pale brown solution with dark brown precipitates on the bottom |
| 200 | Dark blue | Very dark brown | Dark brown solution with suspended particles in the solution | Brown solution with particles settled on the bottom | Very pale brown solution with dark brown precipitates on the bottom |

1: In this table, turbid refers a bleak suspension having a high cell density.

## 4.3 Hydrogen Sulfide Present in the Inocula

Figures 4.13-4.18 present copper removal and sulfate reduction on the same plot for all isolates and the pure cultures. We were interested to see whether copper removal would be dependent on sulfate reduction and visa versa.

It is clearly indicated that in the case of the enrichments, copper removal is close to 100% when the initial copper concentration in the solution is less than 150 ppm. It appears that there was sufficient $H_2S$ in the inoculum to remove all the copper. Hence, copper ions will have been precipitated and transformed into very insoluble minerals before they come into contact with the microorganism. Hence, this initial indirect

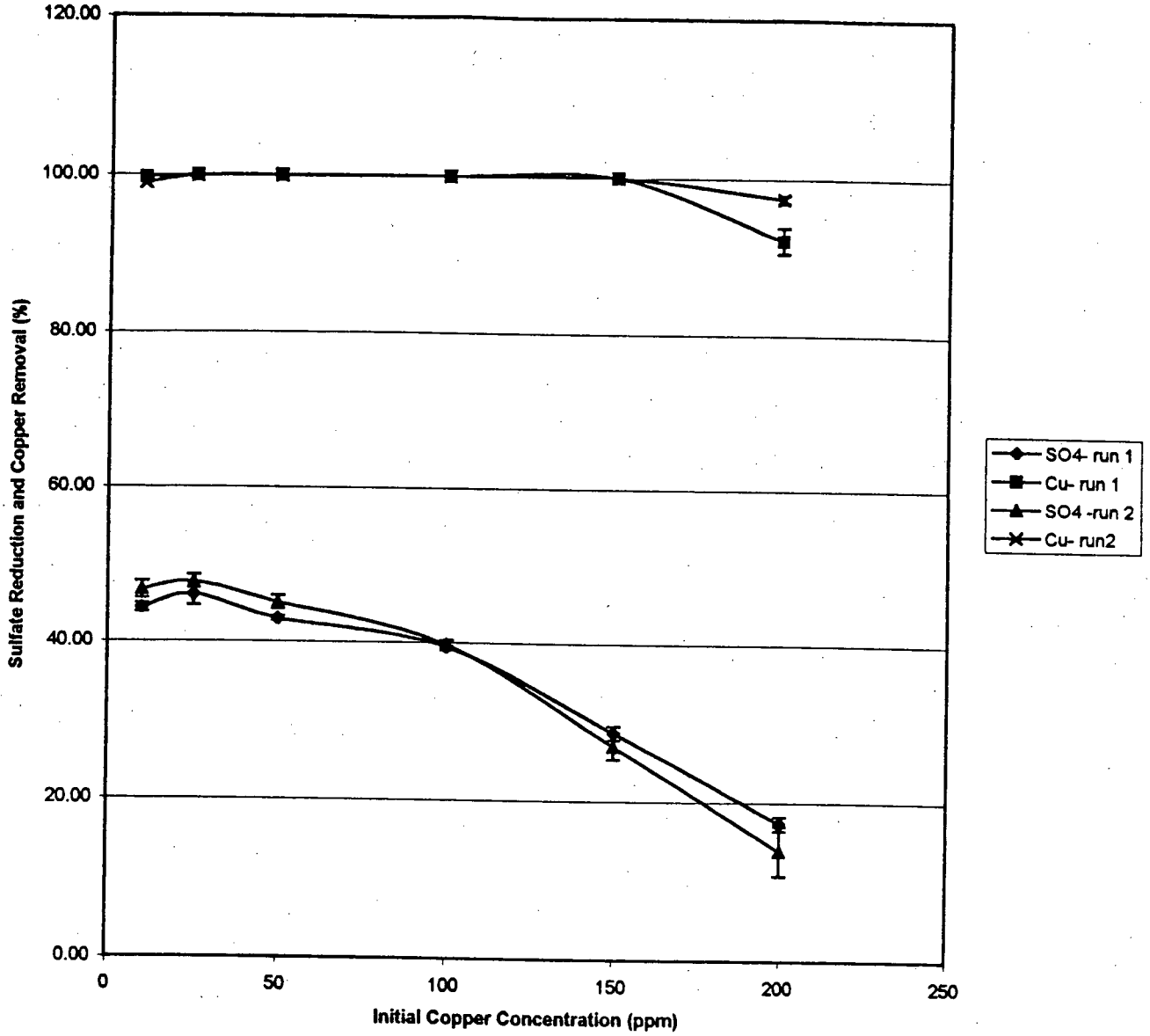


**Figure 4.13: Copper removal and sulfate reduction after five days of incubation of SRB isolate A in Postgate's medium C versus initial copper concentration.**

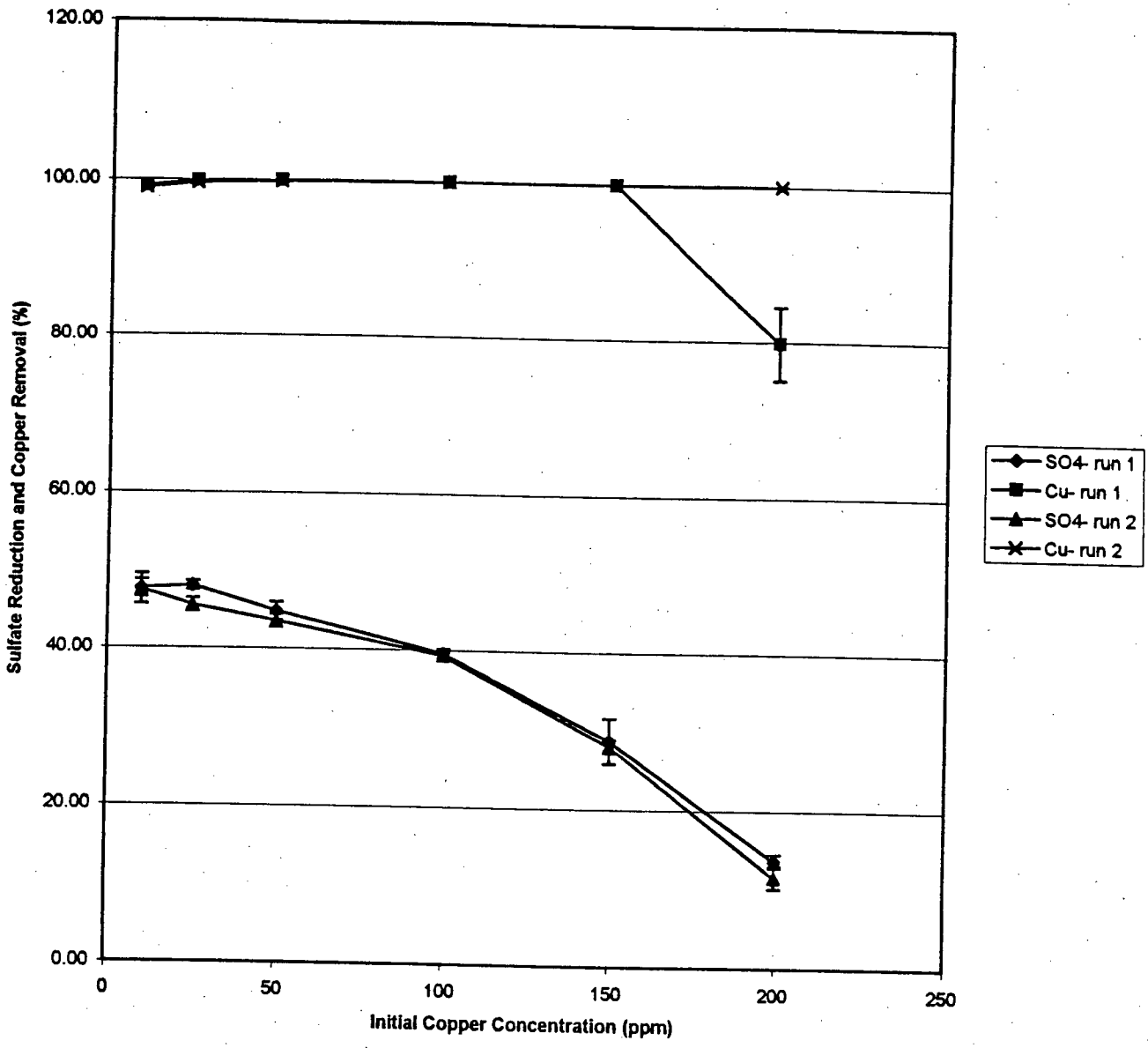


**Figure 4.14: Copper removal and sulfate reduction after five days of incubation of SRB isolate B in Postgate's medium C versus initial copper concentration.**

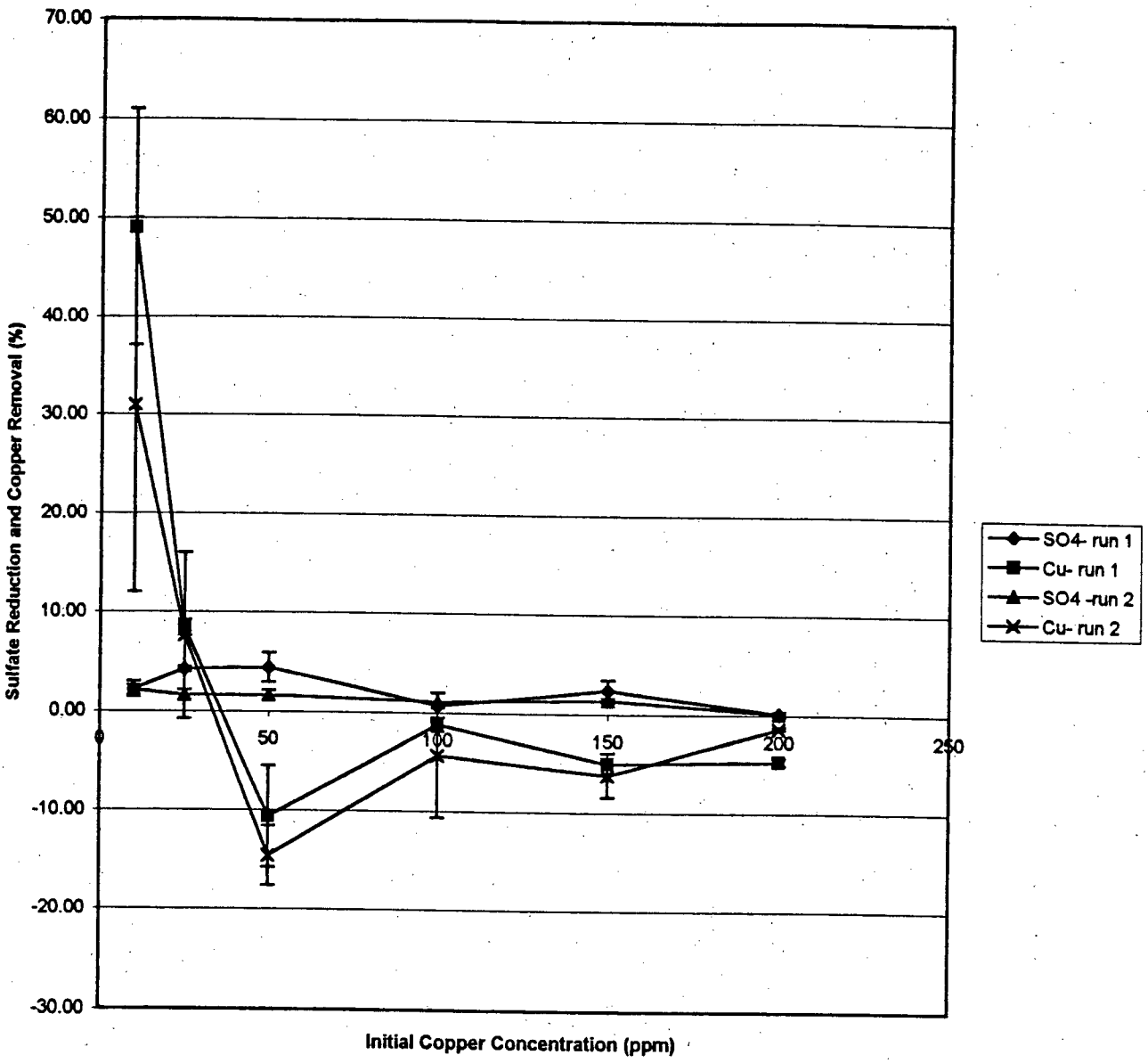


**Figure 4.15: Copper removal and sulfate reduction after five days of incubation of SRB isolate C in Postgate's medium C versus initial copper concentration.**

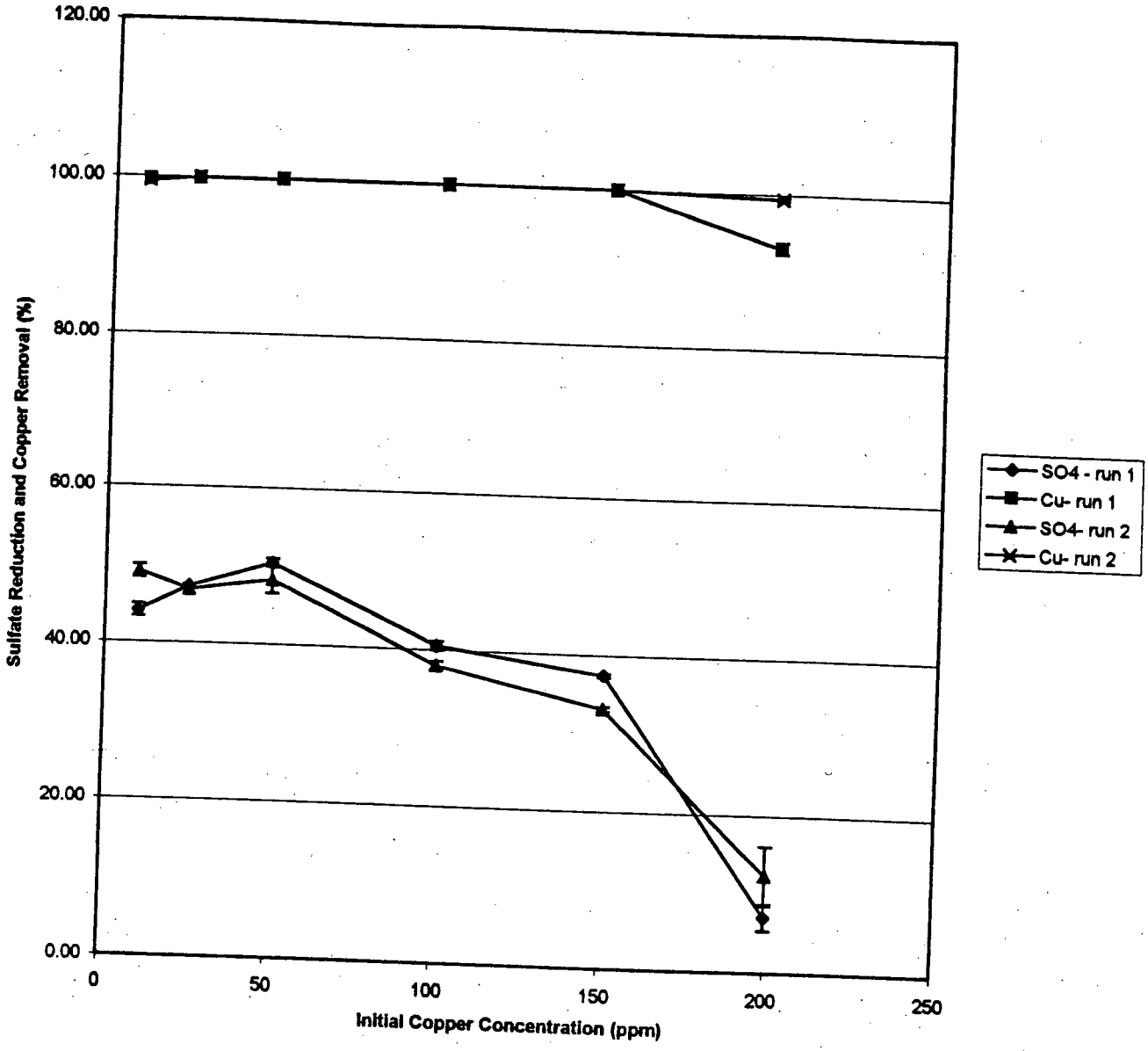


**Figure 4.16: Copper removal and sulfate reduction after five days of incubation of SRB isolate D in Postgate's medium C versus initial copper concentration.**

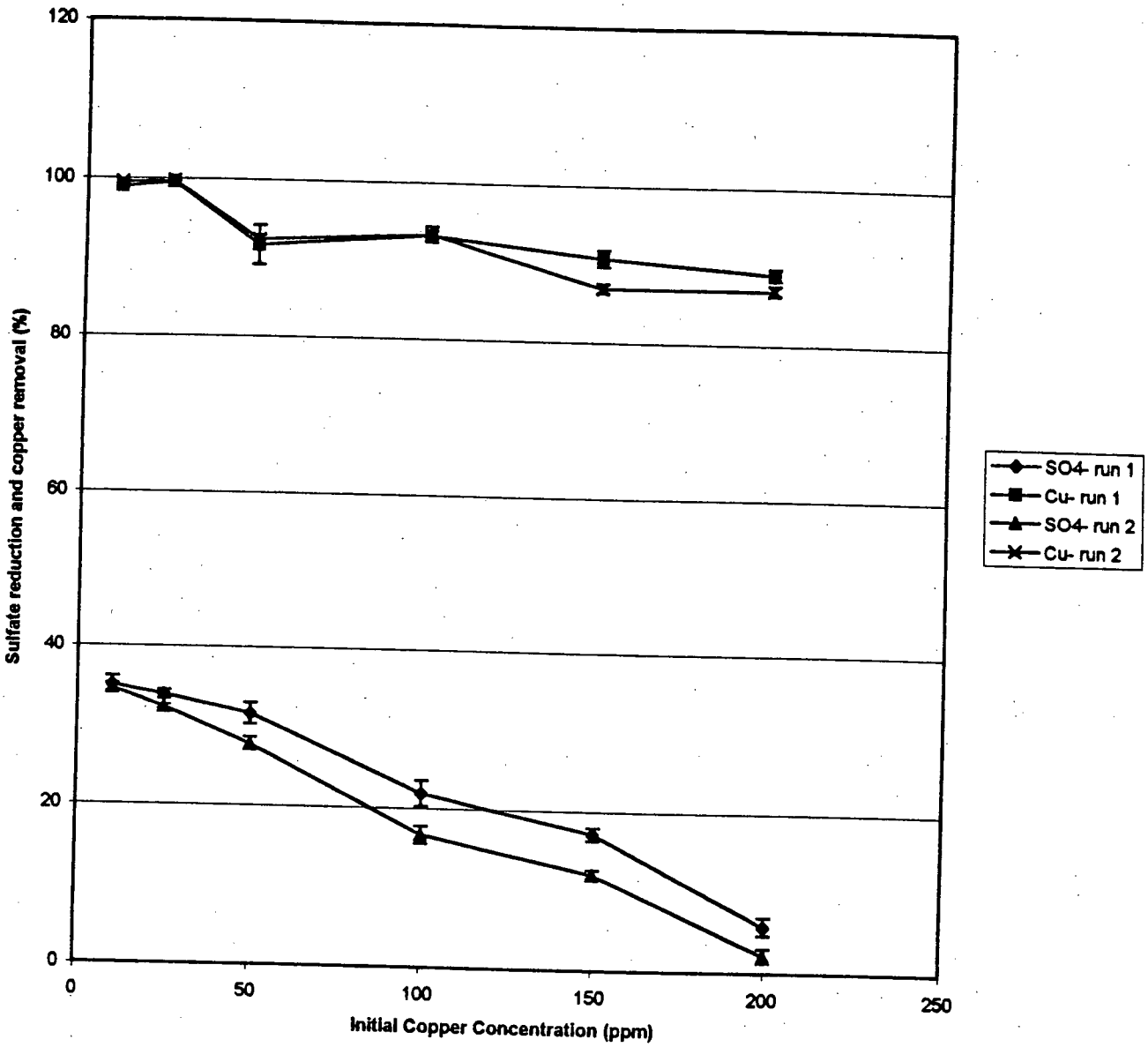


Figure 4.17: Copper removal and sulfate reduction after five days of incubation of *Desulfovibrio desulfuricans* in Postgate's medium C versus initial copper concentration.

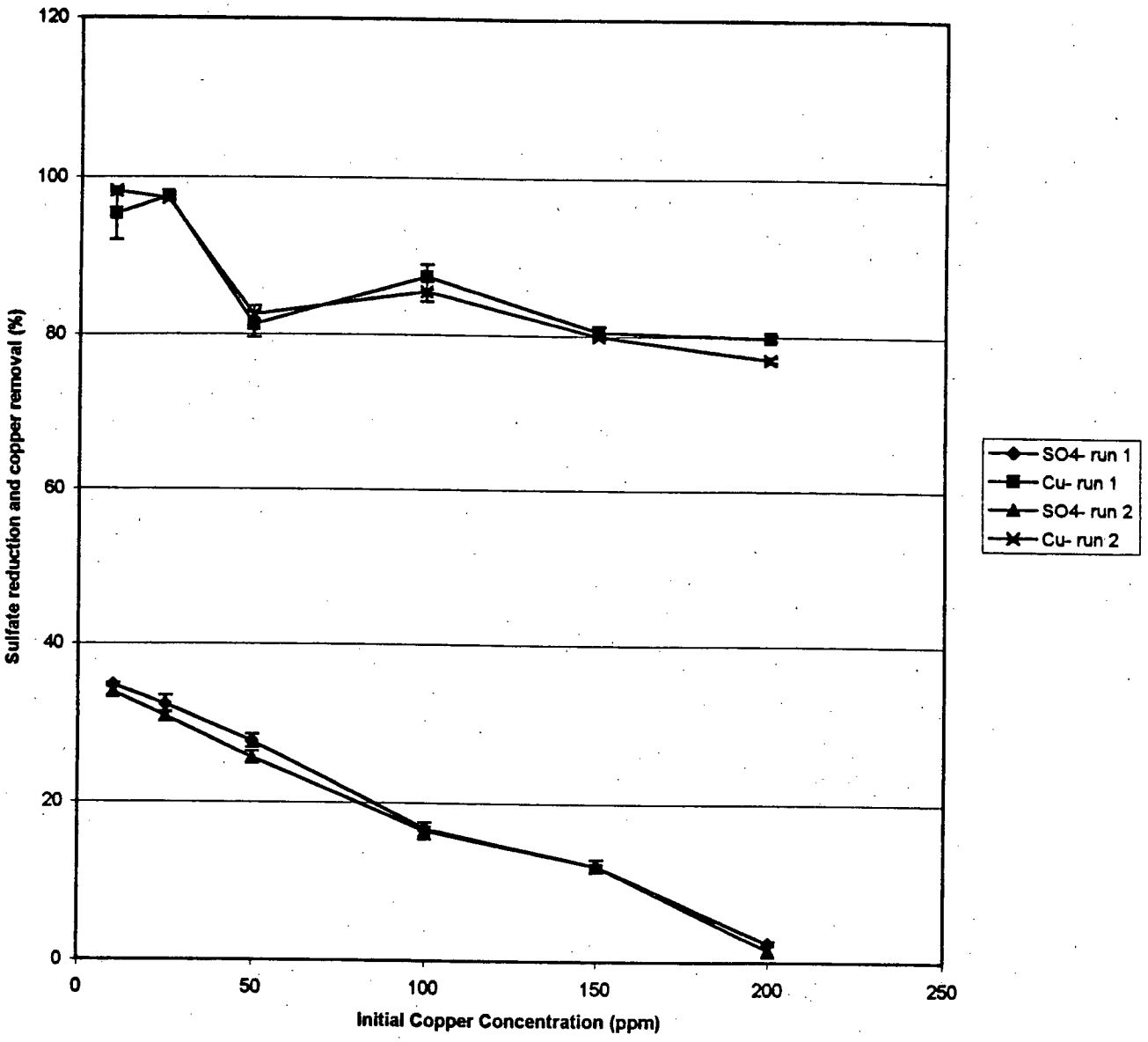


**Figure 4.18: Copper removal and sulfate reduction after five days of incubation of *Desulfovibrio vulgaris* in Postgate's medium C versus initial copper concentration.**

microbial action is a major mechanism for detoxification of metal ions that may otherwise be hazardous to the microorganism in an ionic form (Ferris *et al*, 1989; Lett *et al*, 1980; Mittman *et al*, 1985).

As the initial copper concentration exceeds 150 ppm, $H_2S$ provided by inoculum is not enough to react with all the copper ions; more sulfate is needed to be reduced to precipitate the rest of copper remaining in the solution. Thus, at higher initial copper concentrations, copper removal is dependent on sulfate reduced throughout the experiment. Most likely, SRB precipitate out copper with hydrogen sulfide available in the inoculum and since the copper concentration is still too high and toxic to the bacteria, reduction of sulfate decreases significantly after inoculation.

Another experiment was conducted where different inocula were used. Enrichment D was selected for this experiment and the inocula had been incubated for 2, 3,4, and 5 days respectively. Figure 4.19 shows the extent of copper removal versus inoculum age after five days of incubation. It was believed that the inocula incubated for a longer time would have a higher concentration of biomass and $H_2S$. The extents of sulfate reduction of the different inocula are shown in Table 4.7. The results for the culture 2-D-100 showed that 100% of copper was removed when using all inocula. For cultures 2-D-150 and 2-D-200, copper removal increased with increasing inoculum age. This phenomenon indicates that the increased initial $H_2S$ concentration in the media can remove copper close to 95% even the initial copper concentration is 200 ppm.

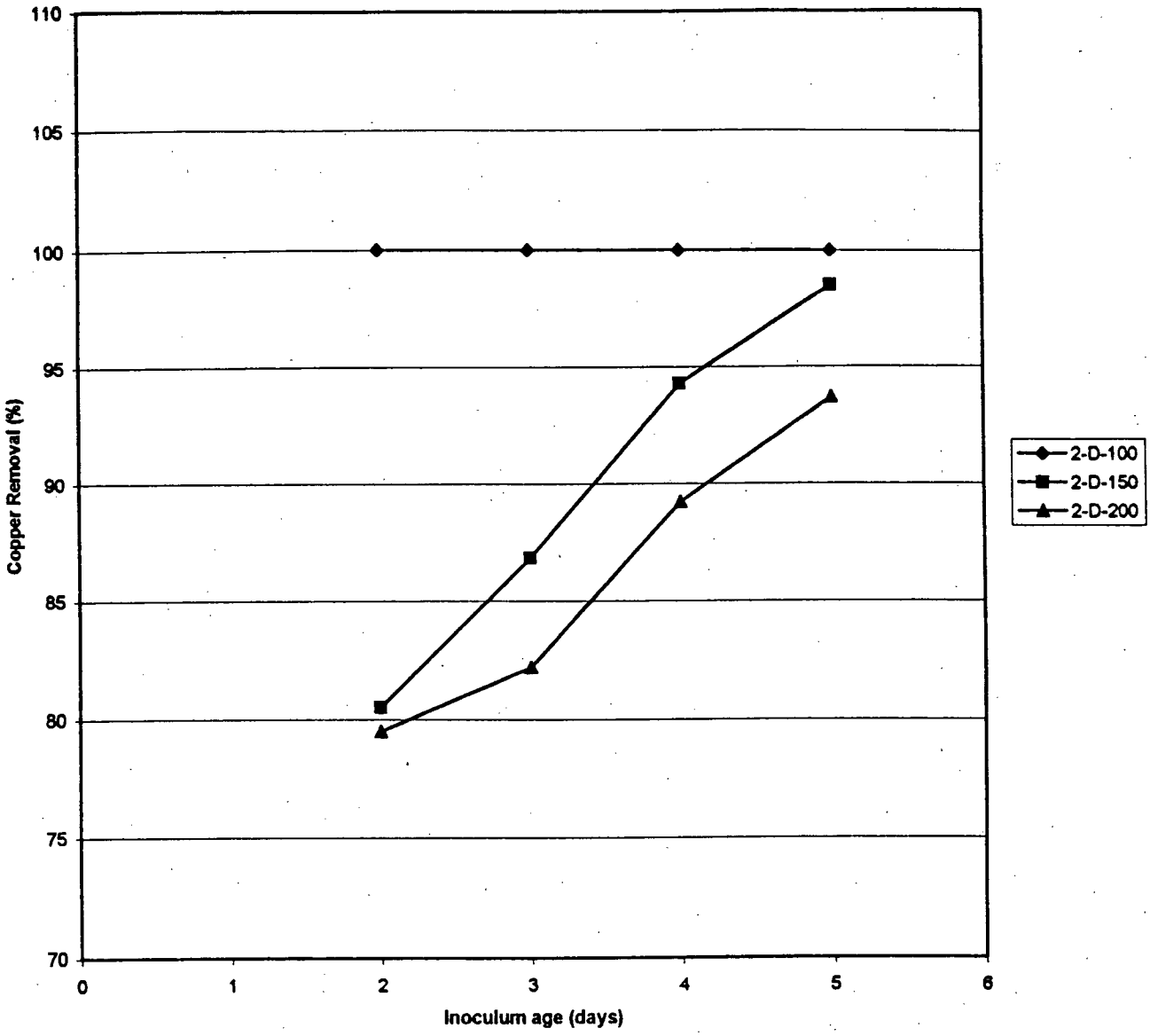


**Figure 4.19: Copper removal versus inoculum age after five days incubation of SRB isolate D in Postgate's medium C in the presence of different initial copper concentrations.**

**Table 4.7: Extent of sulfate reduction of the inocula (Enrichment D) incubated in Postgate's medium C, for different time periods**

| Incubation period of the inoculum (days) | [Sulfate] of the media before inoculation (mg $L^{-1}$) | [Sulfate] of the cultures after incubation (mg $L^{-1}$) |
|---|---|---|
| 2 | 3097 | 1896 |
| 3 | 3097 | 1681 |
| 4 | 3097 | 1589 |
| 5 | 3097 | 1368 |

Figure 4.20 shows the results for sulfate reduction versus inoculum age. Culture 2-D-0, the control, reduced sulfate between 45-50 %. Culture 2-D-100 showed a lower level of sulfate reduction compared to the control. The overall sulfate reduced did not change for culture 2-D-100 as the age of the inocula was increased. However, cultures 2-D-150 and 2-D-200 showed very significant changes in sulfate reduction as the inoculum age increased. The reduction rates increased from 10% to almost 30% of the total sulfate in the solution. This increase in sulfate reduction is probably due to the increased activity of SRB, after the increased precipitation copper in the medium.

From the results obtained, it can be concluded that copper sulfide formation takes place within the first hour of inoculation where the copper concentration is below 100 ppm. As the initial copper concentration is increased (above 100 ppm), the initial $H_2S$ provided by inoculum is not sufficient to precipitate all the copper. The copper in

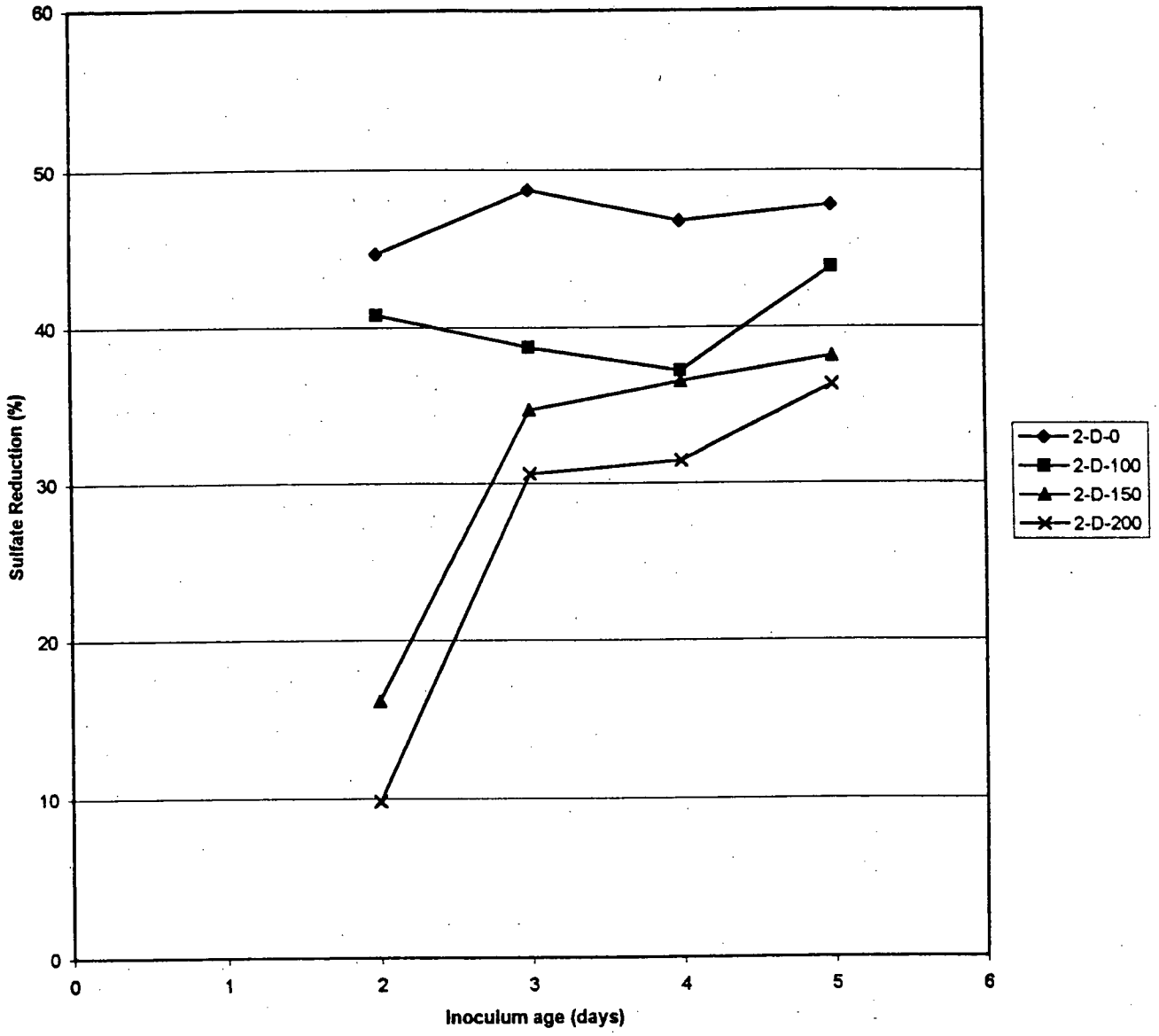


**Figure 4.20: Sulfate reduction versus inoculum age after five days incubation of SRB isolate D in Postgate's medium C in the presence of different initial copper concentrations.**

solution affects the bacteria and hence less sulfate is reduced. As the $H_2S$ concentrations and biomass in the inocula increase, more of the copper precipitates very quickly.

Observations of the cultures indicated that after five days of incubation, the solution was not turbid and most likely, most of the biomass had settled on the bottom of the tubes and bottles, regardless of the initial copper concentration. This observation implies that biomass plays an important role in the settling of the precipitates. Biomass forms aggregates or flocs and any substance that is adsorbed by the bacterial flocs will therefore settle. This combination of flocculation and settling is therefore a mechanism by which metal would more efficiently be removed in biological treatments (Shen *et al,* 1993). However, to further confirm this phenomenon, the settling of the copper sulfides in a control with hydrogen sulfide and no biomass should be calculated and compared with results from the experiments in this research. In the cultures where the initial copper concentration was high, although most of the cells can not participate in sulfate reduction due to copper inhibition, their cell surface can still act a nucleation site for the formation of minerals. In fact, it is believed that besides the higher concentration of $H_2S$ in the inoculum, the older inocula were more turbid and had a higher concentration of biomass; increase in biomass can be measured by optical density. This could be another reason for the increased metal removal, since more cells could interact with the copper ions.

The results obtained from the experiments suggest a continuous treatment system where the SRB can produce biomass and $H_2S$ in high concentrations in a bioreactor. It should be mentioned that the $H_2S$ concentration should not exceed 160 ppm, which is a toxic concentration for SRB (Hao *et al*, 1996). The microbial broth can then be transferred into another bioreactor where the copper-containing effluent would enter as

well. In this way, the $H_2S$ produced in the first bioreactor can overcome the toxicity of the high concentrations of copper in the second bioreactor; the biomass can reduce more sulfate to produce hydrogen sulfide in the second bioreactor and aid in the process of flocculation and settling of the copper sulfides.

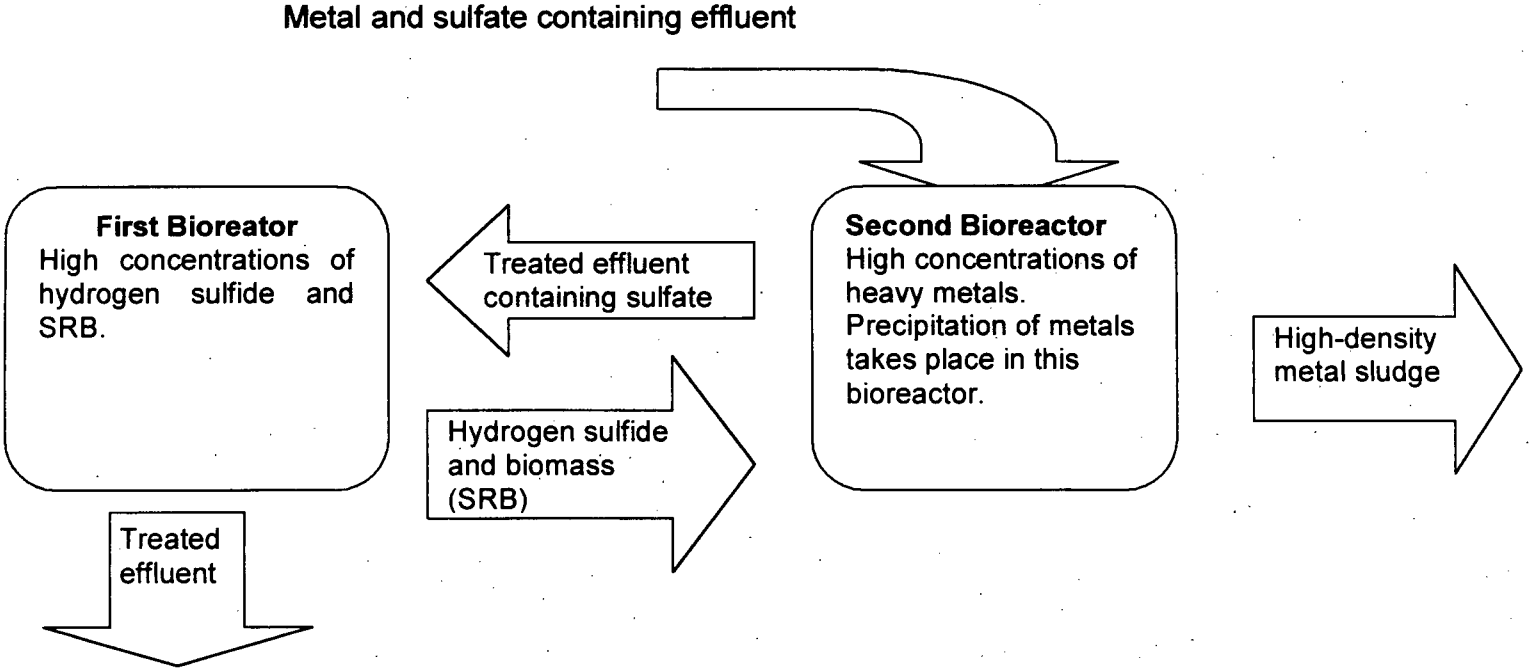


**Figure 4.21: A diagram for the proposed two-bioreactor system for treatment of heavy metals with SRB.**

## 4.4 Location. Structure, and Composition of Copper Precipitates

Electron microscopy techniques together with subsequent image analysis were used to better visualize the location and structure of copper precipitates on the bacterial walls. Electron-probe microscopy techniques such transmission electron microscopy

(TEM) and scanning electron microscopy (SEM) were used to in this part of the research. These techniques including quantitative analyses such as energy-dispersion spectroscopy (EDS) were used to investigate chemical composition of the precipitates.

### 4.4.1 Precipitate Composition, Size, and Shape

As already shown in Table 4.6, cultures having different initial copper concentrations precipitated copper at different times during the 5-day batch period. Figure 4.22 presents a SEM image of the dried solids on the filter paper from a sample obtained from culture 4-D-25, after 5 days of incubation. The precipitate was a thick layer of particles that were firmly attach together, most probably by the sticky capsule layer produced by the biomass. Unfortunately, due to sticky nature of the precipitate, no individual bacterial cells and individual particles could be observed. EDS was conducted with different spot sizes at 15-20 kV and the spot size was chosen according to the magnification being used. Figure 4.23 shows the EDS result from the same sample observed in Figure 4.22. Figure 4.23 clearly indicates one sharp peak for sulfur and two peaks for copper. The other occasional peaks were iron, magnesium, and chlorine that were present in the media. The peak for carbon is due to carbon coating and the presence of the biomass. Hence, the composition of the precipitate was verified to be copper sulfide.

Figures 4.24 and 4.25 show two additional EDS spectra obtained from samples that were taken from cultures 4-D-100 and 4-D-200, after five days of incubation. Once again, these two figures indicate a sharp peak for sulfur and two peaks for copper, emphasizing the copper sulfide composition of the precipitates. Other additional peaks

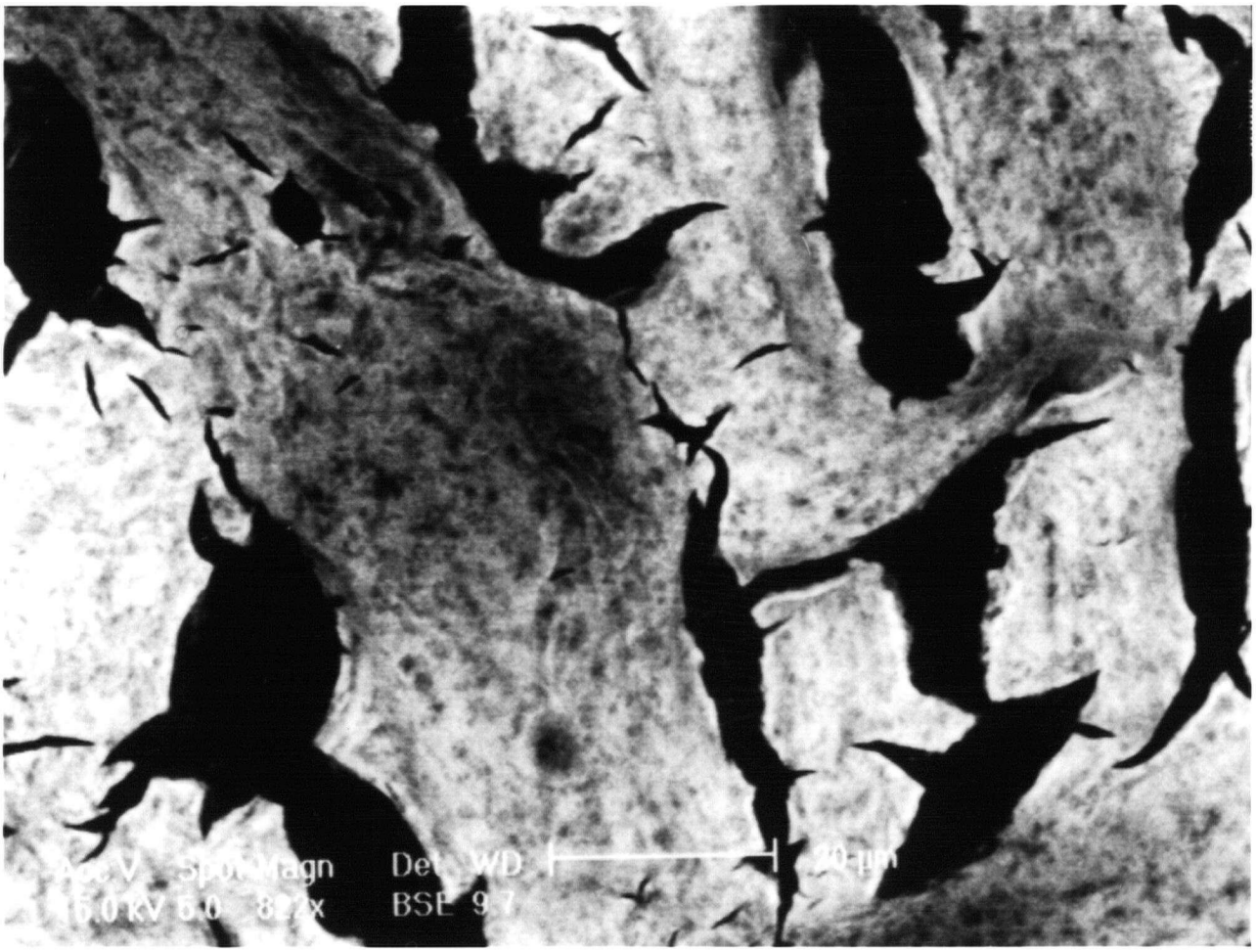


**Figure 4.22: A SEM image of the dried solids on the filter paper showing the densely packed precipitate. The precipitated was obtained from culture 4-D-25, incubated in Postgate's medium C with initial copper concentration of 25 ppm. The image was viewed with a philips XL30 SEM equipped with PGT INIX (Integrated Microanalyser for Imaging) and EDS. Under a magnification of 822X, the white section shows the dense precipitate and the black background is caused by the filter paper.**

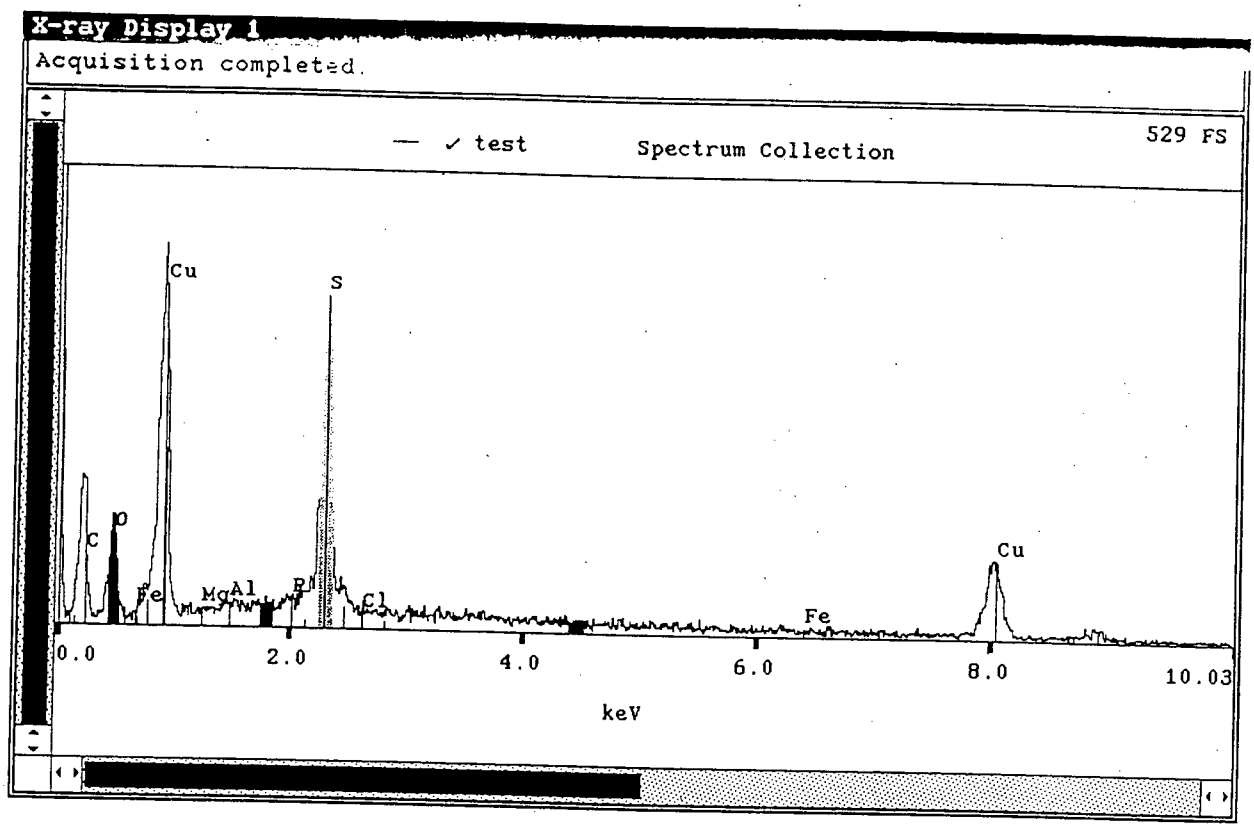


**Figure 4.23: EDX spectrum of the sample in Figure 4.22, indicating the strong copper and sulfur peaks.**

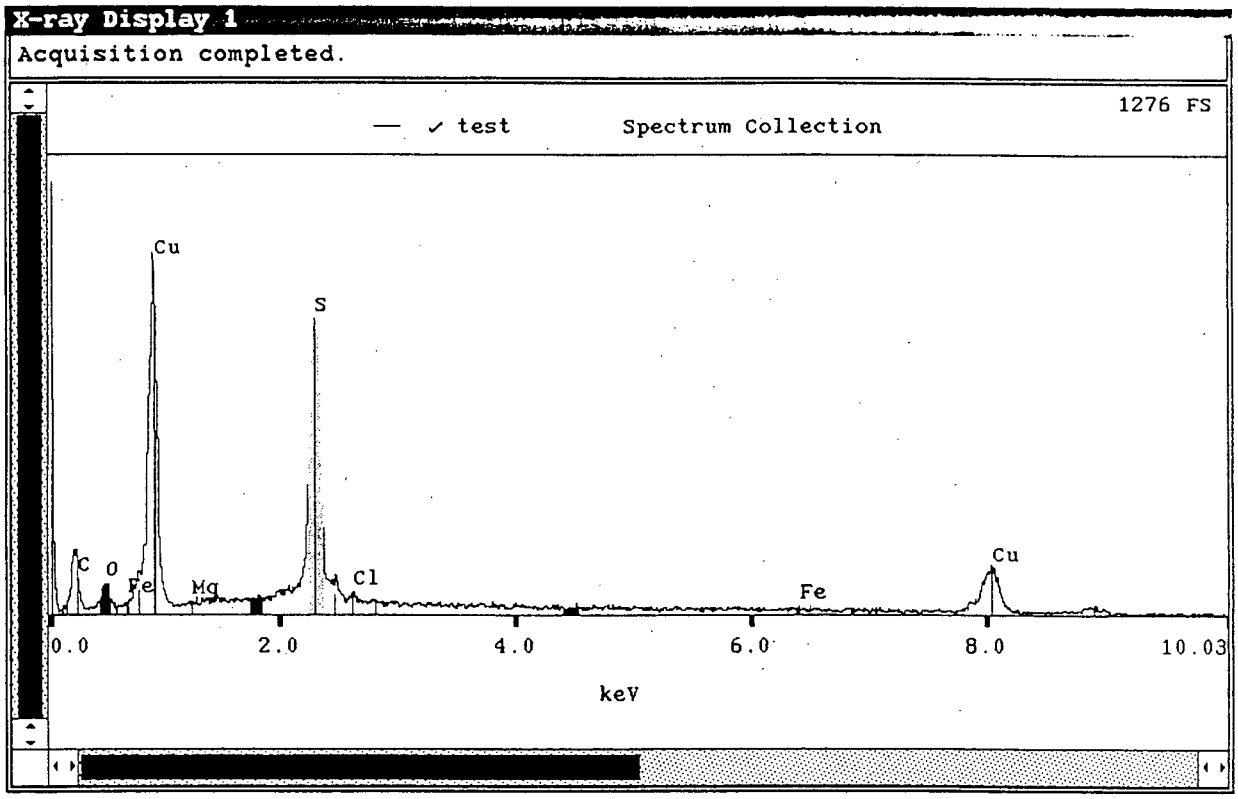


**Figure 4.24: EDX spectrum of a precipitate obtained from culture 4-D-100, incubated in Postgate's medium C and initial copper concentration of 100 ppm. The strong copper and sulfur peaks are indicated.**

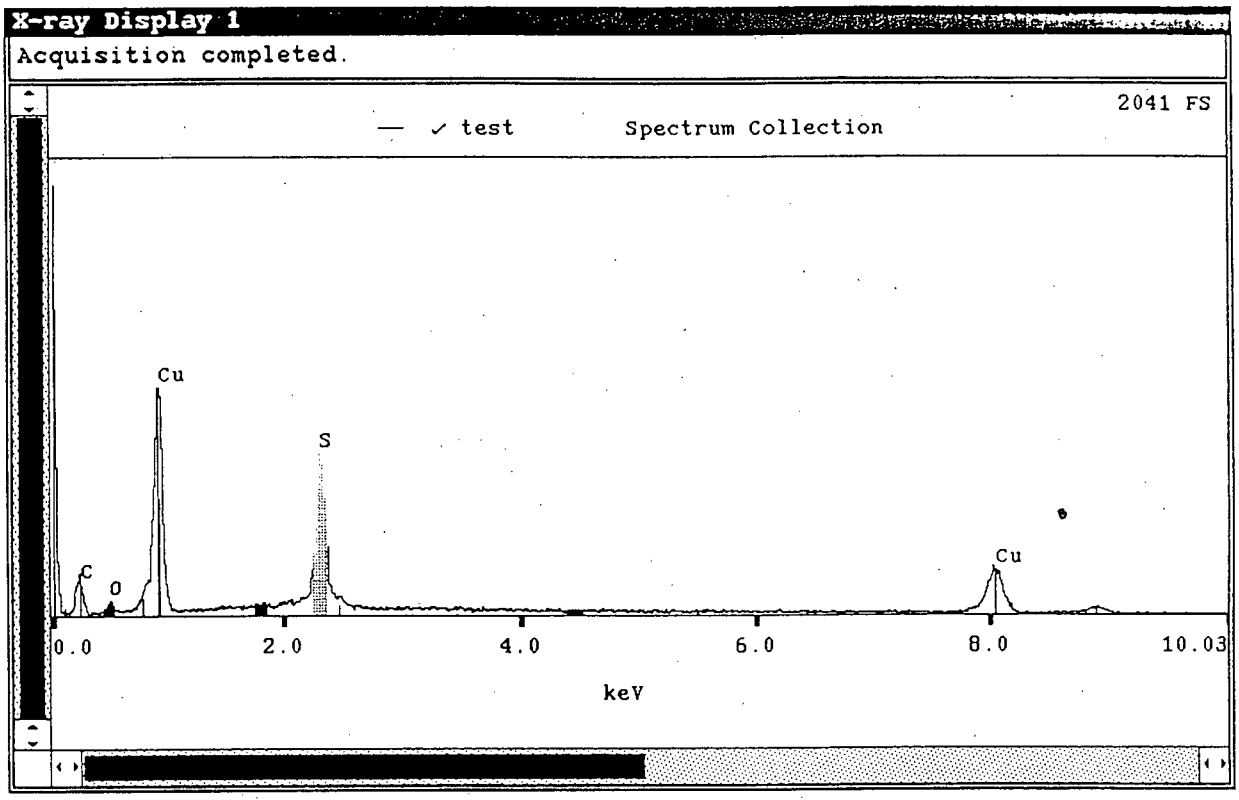


**Figure 4.25: EDX spectrum of a precipitate obtained from a culture 4-D-200, incubated in Postgate's medium C and initial copper concentration of 200 ppm. The strong copper and sulfur peaks are indicated.**

for carbon, oxygen, iron, and magnesium are also observed. Just like the sample taken from culture 4-D-25, no particle size could be calculated for samples 4-D-100 and 4-D-200. Thus, no particle size distribution could be provided for the precipitates. Perhaps dilution of the samples or using different methodologies discussed in the previous chapter could provide a particle size distribution for these samples.

### 4.4.2 Extracellular Polymer Formation by SRB in the Presence of Copper

In order to locate copper sulfides on the bacterial cell wall, samples were taken from cultures 4-D-0, 4-D-25, 4-D-100, and 4-D-200 and mounted on carbon-coated nickel grids. These grids were examined by a TEM, a Philips EM 300 at 60 KeV. Figures 4.26-4.29 show the TEM image of the different samples. Several interesting observations were noted. Firstly, it was observed that individual cells could be seen without the requirement for thin sectioning. This means that the samples were thin enough to allow electrons through. Secondly, cells grown in the presence of copper showed good contrast and could be seen without uranyl acetate staining. However, the sample from culture 4-D-0 needed to be stained with uranyl acetate before observation by TEM. This means that the copper ions were attached to the bacterial cell wall for the samples grown in the presence of copper and had enough electron-scattering power to provide a contrast to see individual cells. However, the sample from culture 4-D-0 that did not have any copper included in the growth media needed to be stained with uranyl acetate.

Thirdly, it was observed that a thick, slimy layer surrounded the cells. Cells that had grown in higher concentration of copper had a thicker capsule layer. For example, the cell in Figure 4.29 that had been incubated in the presence of 200 ppm of copper showed

**Figure 4.26: A TEM image of uranyl acetate stained bacterium from culture 4-D-0, incubated in Postgate's medium C with no copper added. The image was viewed with a Philips EM400T transmission electron microscope operating at 100 kV. Magnification was at 25,000 X. No extracellular polymer is observed around the cell.**

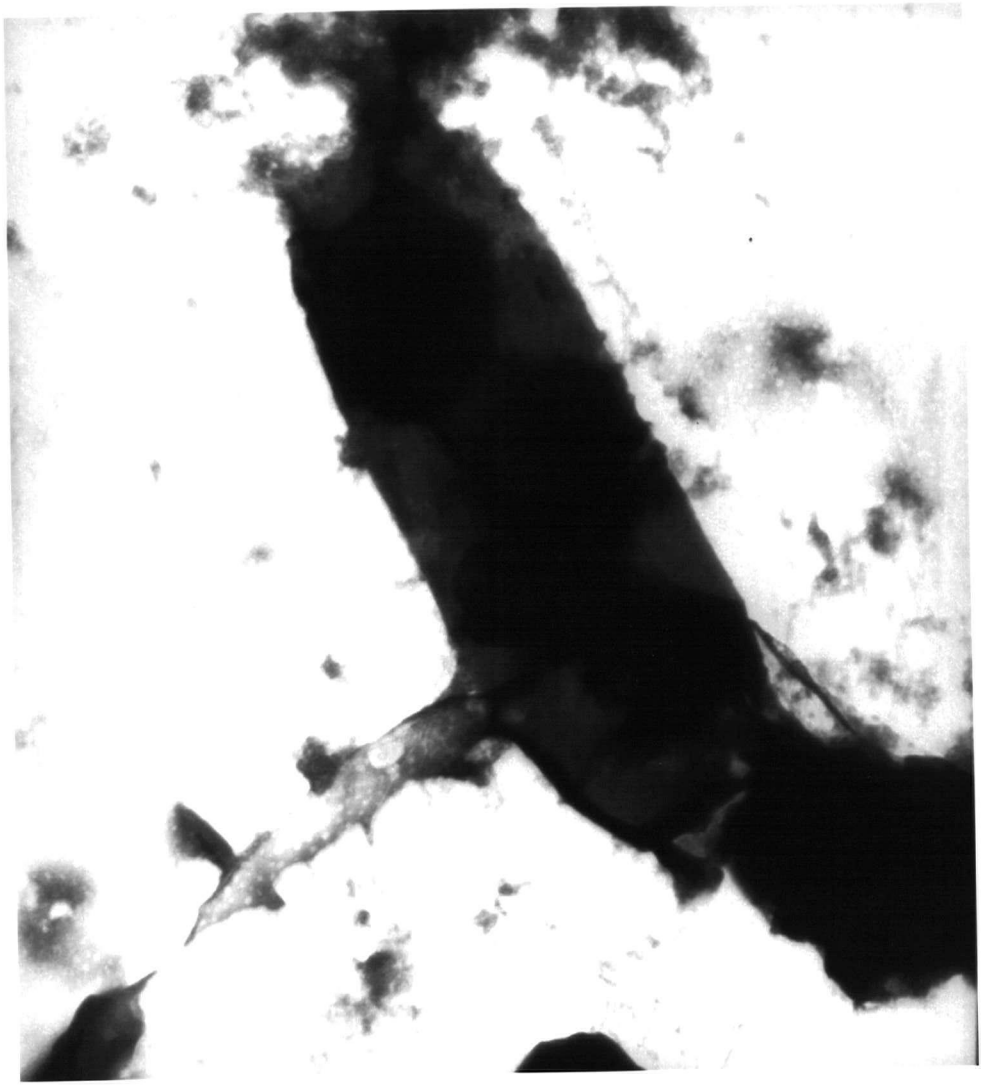

**Figure 4.27: A TEM image of unstained bacterium from culture 4-D-25, incubated in Postgate's medium C with initial copper concentration of 25 ppm. The image was viewed with a Philips EM400T transmission electron microscope operating at 100 kV. Magnification was at 25,000 X. There is evidence of some extracellular polymers around the cell.**

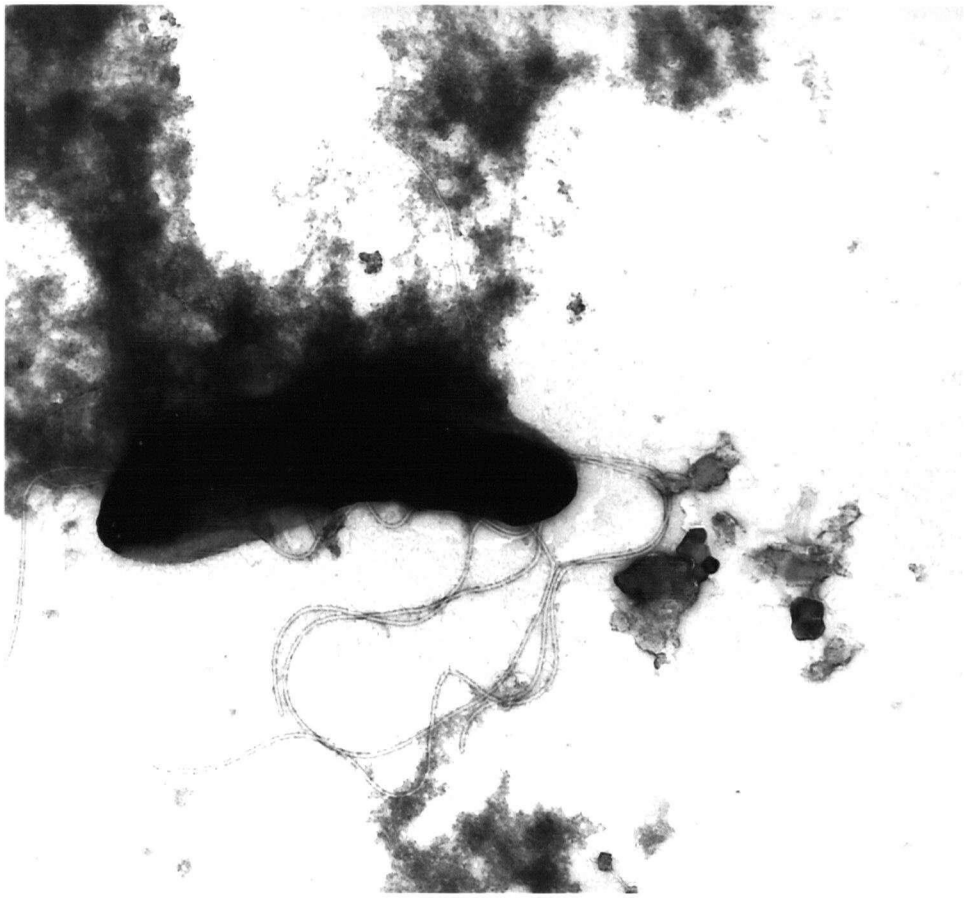

**Figure 4.28: A TEM image of an unstained bacterium from culture 4-D-100, incubated in Postgate's medium C with initial copper concentration of 100 ppm. The image was viewed with a Philips EM400T transmission electron microscope operating at 100 kV. Magnification was at 25,000 X. The extracellular polymers around the cell are very well contrasted.**

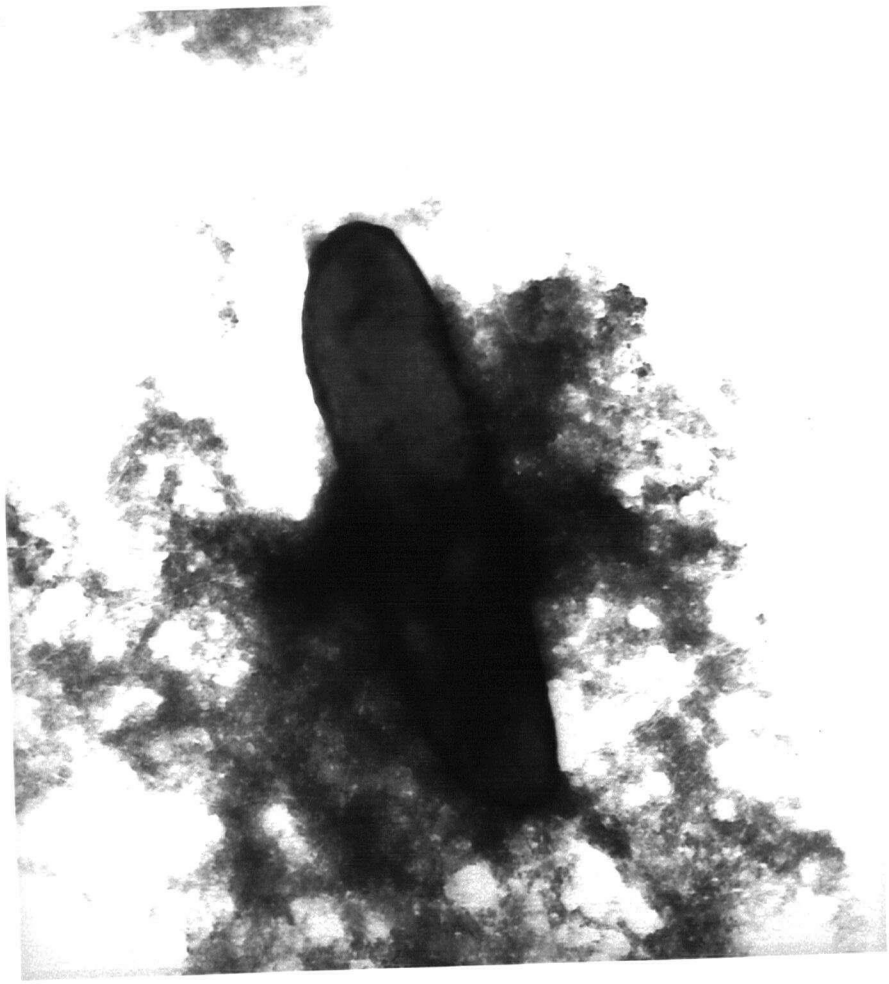

**Figure 4.29: A TEM image of an unstained bacterium culture 4-D-200, incubated in Postgate's medium C with initial copper concentration of 200 ppm. The image was viewed with a Philips EM400T transmission electron microscope operating at 100 kV. Magnification was at 25,000 X. The extracellular polymers are highly contrasted and visible.**

a thicker layer compared to the cell grown in 25 ppm of copper, as shown in Figure 4.26. Moreover, the slimy layer around the cell showed very good contrast under the microscope, implying that copper ions were attached to the extracellular polymers of the cell. This slime layer, or capsule, is usually a polysaccharide with a repeating unit of two to six sugar subunits and it is anchored to the gram-negative bacterial outer membrane (Brock *et al,* 1994). These naturally produced polymers bond electrostatically or physically and subsequently bridge the bacterial cells and other particlulates to settle as floc aggregates. It has been suggested that the extent of capsule production is a function of the free metal ions in the solution surrounding the bacteria (Corpe *et al,* 1975; Shen *et al,* 1993; Stumm *et al,* 1996). This polysaccharide layer serves both as a metal scavenger and also acts as an impermeable barrier when metal ions exist at toxic levels in the surrounding environment.

### 4.4.3 Copper Sulfide on the Cell Surface of SRB

In order to verify the presence of copper sulfides on the SRB cell surface, an unstained sample that came from a flask 4-D-100 was examined with a Philips EM400T (operating at 100 kV) equipped with an energy dispersive X ray spectrometer (LINK Analytical eXL/ LZ-5) for elemental analysis. Figure 4.30 shows the TEM image and Figure 4.31 presents the EDS spectrum provided for the spot indicated by the arrow in Figure 4.30. EDS was conducted with a spot size of 4 nm, and a beam current of 0.1 μA, and a counting time of 100s. Once again, due to the association of copper with the bacterium surface, excellent contrast is provided.

As Figure 4.31 indicates, the elemental analysis of the surface of an individual cell revealed two sharp peaks for copper and sulfur, confirming the presence of copper sulfides on the cell membrane. This observation can be explained by the two-step mechanism suggested by Beveridge *et al* (Beveridge *et al*, 1980, 1983, 1985). In the first step, the metal ion binds to an active site on the cell wall. This interaction acts as a nucleation site for the deposition of more metal ions from the solution in the second step.

**Table 4.8: Summary of TEM and SEM sample observations.**

| samples | Staining procedure (TEM) | Extent of Capsule production (TEM) | Particle size (SEM) | Elemental composition of the cell membrane (EDS) | Elemental composition of the precipitates (EDS) |
|---|---|---|---|---|---|
| 4-D-0 | Uranyl acetate | + | N/A | N/A | N/A |
| 4-D-25 | None | + | N/A | N/A | Cu, S (Mg, C, Fe, Cl, O, Al) |
| 4-D-100 | None | +++ | N/A | Cu, S (C, O, Mg, Al, P, Fe) | Cu, S (C, O, Mg, Cl, Fe) |
| 4-D-200 | None | ++++ | N/A | N/A | Cu, S (C, O) |

SEM observations of the solids indicated the dense nature of the precipitates and EDS spectra confirmed the precipitation of copper as copper sulfides. As Table 4.8 summarizes, samples that had been incubated in the present of copper did not need to be stained with uranyl acetate for visualization. Copper present in the solution provided enough electron-scattering power to provide good contrast. Furthermore, extracellular polymer production of SRB seemed to be affected by the copper concentration in the solution; increase in the copper concentration of the media seemed to increase the extent

**Figure 4.30: A TEM image of an unstained bacterium from culture 4-D-100, incubated in Postgate's medium C with initial copper concentration of 100 ppm. The image was viewed with a Philips EM400T transmission electron microscope operating at 100 kV. Magnification was at 25,000 X.**

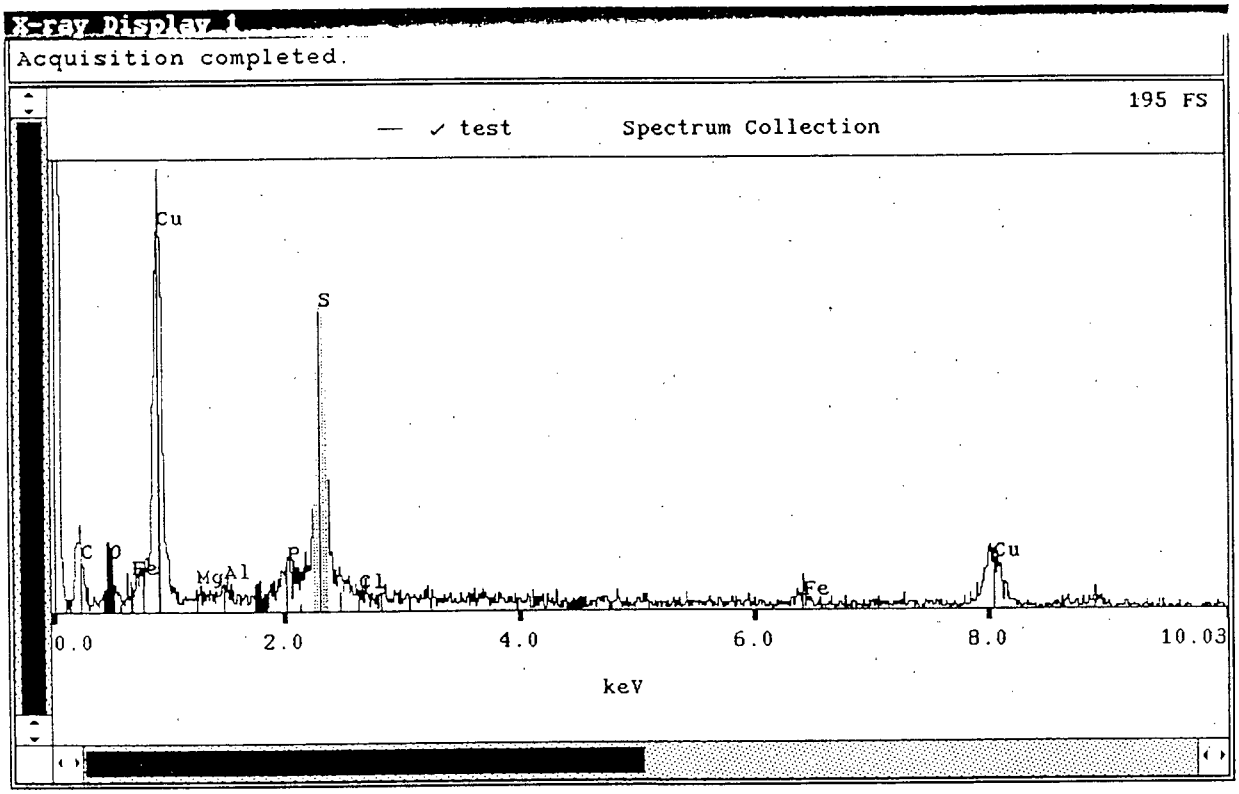


**Figure 4.31: EDS spectrum of the spot of dimension 4 μm on the membrane indicated by the arrow shown in Figure 4.30. The Philips EM400T was equipped with an energy dispersive X-ray spectrometer (LINK Analytical eXL/LZ-5) for elemental analysis. A beam current of 0.1 μA and a counting time of 100 seconds were used.**

of extracellular polymer production. Thus, copper sulfide formation and association with the bacterial cells and capsule is advantageous for copper removal from wastewater since it facilitates settling and separation of the metal precipitates from the supernatant. Moreover, well-contrasted images of the unstrained bacteria and subsequent ESD analyses confirmed the presence of copper sulfides on the cell surface.

# 5 CONCLUSIONS

This research investigated the role of sulfate reducing bacteria in copper removal from a copper sulfate solution. The experiments were designed to provide useful information regarding the design of a treatment system with SRB. It was concluded that the direct presence of the bacteria and their flocculation could enhance precipitation and subsequent settling of the precipitates. This conclusion should be confirmed by comparing copper removal in the presence of SRB with copper removal, under the same conditions, in the absence of SRB. The objectives of the research, discussed in the first chapter, were fulfilled as followed:

## 5.1 Copper Removal Efficiency Using SRB Enrichments and Pure Cultures

(a) Under the experimental conditions used, copper was removed effectively by SRB enrichments to less than 0.1 ppm from sulfate solutions containing 150 ppm or less initial copper concentration. Under the same conditions, *Desulfovibrio desulfuricans* removed copper to less than 0.1 ppm when the initial copper concentration was 25 ppm. The other pure culture, *Desulfovibrio vulgaris* was even less effective; copper was removed to 0.2 ppm when the initial copper concentration was 10 ppm. Most probably, the pure cultures removed less copper due to the lack of physiological adaptation in the presence of copper.

(b) The process of copper precipitation was highly dependent on the concentration of sulfide present in the inocula; most of the copper precipitation was observed to take

place within the first few hours of inoculation. Copper removal is also dependent on other factors such as the amount of biomass introduced to the copper solution.

## 5.2 Image Analysis and Interaction of Sulfate Reducing Bacterial Cell Surface With Copper

(a) Visual observations of the solids indicated dense packing and good settling. At higher copper concentrations, a fine suspended precipitate formed and later settled.

(b) Well-contrasted images of the unstained bacteria confirmed that the minerals formed were associated with the cell surface. This observation can be explained by the two step mechanism suggested by Beveridge (Beveridge *et al*, 1980, 1983, 1985). In the first step, the metal ion binds to active site on the cell wall. This interaction acts as a nucleation site for the deposition of more metal ions from the solution.

(c) EDS spectra confirmed the precipitation of copper as copper sulfides.

(d) Cells showed a high degree of capsule production in the presence of the copper in solution. This phenomenon is the most likely reason for the formation of flocculates, fast setting, and the dense appearance of the precipitates.

## 5.3 Precipitation of Copper in a Proposed Bioreactor

(a) Copper precipitation and subsequent settling may be improved with the presence of bacteria in a bioreactor.

(b) A two-bioreactor system is suggested for the treatment of Copper contaminated effluent with SRB. The first bioreactor will contain SRB that can produce biomass

and $H_2S$ in high concentrations. This biomass, together with $H_2S$ will be transferred to another bioreactor where the copper-containing effluent will enter as well.

# 6 RECOMMENDATIONS

This research was an attempt to investigate the interaction of sulfate reducing bacteria with heavy metals, copper specifically. A few recommendations are suggested to further enhance the applications of this research. The first suggestion is to repeat the same experiments in a larger scale; copper removal should be tested using two separate laboratory-scale bioreactors, as earlier discussed in the conclusions. In this way, valuable information regarding optimal flow rates, growth conditions for SRB, and possible pitfalls of this two-step biological precipitation system can be determined.

Secondly, precipitation of other heavy metals present in ARD such as Zn and Fe can be investigated and using the same experimental settings in this research, the results can be compared. The results can provide some information regarding the selectivity of metal precipitation by SRB.

As the results of this research indicate, physiological adaptation of SRB in the presence of copper plays a significant role in increased precipitation of copper by SRB. It is recommended to gradually adapt SRB to high concentrations of copper (i.e, 100-200 ppm). The experiments can be repeated and the results will indicate how significant the adaptation process is in removing copper from the solution.

Lastly, the particle size distribution could not be calculated in this research. It is recommended to use other methodologies described previously in Chapter Three. Furthermore, as the precipitates seemed to be very thick, the samples should be diluted for determination of the particle size. The information about the particle size of the precipitates would be beneficial for calculating the settling rate of the precipitates in a large-scale system.

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# Appendix A. Sulfate Analysis:

## A.1 Turbidimetric Method (A.P.H.A., 1995)

Principle: Sulfide ion is precipitated in a hydrochloric acid medium with barium chloride. Barium sulfate crystals are formed. The absorbance of barium sulfate is measured by a spectrophotometer and the sulfate ion concentration is determined by comparison of the reading with a standard curve.

**Materials:**
50-ml Erlenmeyer flasks
Stopwatch
Magnetic stirrer apparatus
Spectrophotometer
Buffer Solution A: Dissolve 30 g magnesium chloride, $MgCl_2 \bullet 6H_2O$, 5 g sodium acetate. $CH_3COONa \bullet 3H_2O$, 1 g potassium nitrate, $KNO_3$, and 20 ml acetic acid, CHCOOH (99%), in 500 ml distilled water and make up to 1000 ml.
Buffer Solution B: Dissolve 30 g magnesium chloride, $MgCl_2 \bullet 6H_2O$, 5 g sodium acetate. $CH_2COONa. \bullet 3H_2O$, 1 g potassium nitrate, $KNO_3$, 0.111 g sodium sulfate, $NaSO_2$ and 20 ml acetic acid, CHCOOH (99%), in 500 ml distilled water and make up to 1000 ml.
Barium chloride crystals, 20-30 mesh
Standard sulfate solution: Prepare a standard sulfate solution by dissolving 0.1479 g anhydrous $Na_2SO_4$ in distilled water and dilute to 1000 ml. 1.00 L = 100 μg $SO_4$

**Procedure**:

20 ml of sample that has been diluted with distilled water is placed in a 50-ml Erlenmeyer flask. 4.00 ml of the buffer (buffer A for concentrations >10 mg/L and buffer B otherwise) solution is added and mixed using magnetic stirrer. As the solution is being stirred, 0.145 g of barium chloride crystal is added. The mixture is then stirred for 60 s at a constant speed.
After the stirring period has ended, some of the solution is poured into the absorption cell of the spectrophotometer machine and the turbidity is measured after 5 min.
The sulfate concentration of the solution is determined by comparing the turbidity with a calibration curve. Carrying standards through the procedure above makes the calibration curve. The standards will be spaced at 5 mg/L increments in the 0 to 40 mg/L sulfate range.
A correction for the sample color and turbidity must be made; therefore, blanks from which the barium chloride is not present will be run,
The concentration of sulfate is calculated from the formula below:

$$\textbf{mg/ L } SO_4 = \textbf{mg } SO_4 * \textbf{ 1000/ml sample}$$

# Appendix B. Eperimental Data

## B.1 Batch Expeiments

Wavelengths for measuring [Cu]:
324. For more sensitive conditions
327.4 nm for less sensitive conditions

### Isolate A

### Run 1A

Incubation time: Oct. 14-19 (8:00 PM)
Media pH: 7:58
SO4 calibration curve: Oct 24/97
[SO4] of inoculum: 1698 mg/ml
[SO4] of media: 3011 mg/ml

| *Sample* | *[Cu] initial* | *vol. med* | *vol. SRB* | *vol. Cu* | *vol. total* | *[Cu] sup* | *STD* | *[SO4] initial* | *A* | *dilution factor* | *[SO4] final* | *SO4 remined* | *SO4 reduced* | *AVE* | *AVEDEV* | *Cu removed* | *AVE* | *AVEDEV* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ppm* | *ml* | *ml* | *ml* | *ml* | *ppm* | | *mg/L* | | | *mg/L* | *%* | *%* | | | *%* | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.05 | 0.01 | 2707.236 | 0.062 | 100 | 1523.438 | 56.3 | 43.7 | 44.3 | 0.58 | 99.50 | 99.60 | 0.10 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.03 | 0.01 | 2707.236 | 0.060 | 100 | 1492.188 | 55.1 | 44.9 | | | 99.70 | | |
| 1C | 10 | 38 | 8 | 0.3838 | 46.384 | 9.5 | 1.4 | 2759.627 | 0.160 | 100 | 3054.688 | 110.7 | | | | | | |
| 2A | 25 | 30 | 8 | 0.9797 | 38.980 | 0.03 | 0.01 | 2665.849 | 0.059 | 100 | 1476.563 | 55.4 | 44.6 | 46.1 | 1.47 | 99.88 | 99.88 | 0.00 |
| 2B | 25 | 30 | 8 | 0.9797 | 38.980 | 0.03 | 0.01 | 2665.849 | 0.054 | 100 | 1398.438 | 52.5 | 47.5 | | | 99.88 | | |
| 2C | 25 | 38 | 0 | 0.9797 | 38.980 | 21.6 | 2.5 | 2935.323 | 0.164 | 100 | 3117.188 | 106.2 | | | | | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 0.03 | 0.01 | 2707.236 | 0.064 | 100 | 1554.688 | 57.4 | 42.6 | 42.9 | 0.29 | 99.94 | 99.95 | 0.01 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 0.02 | 0.01 | 2707.236 | 0.063 | 100 | 1539.063 | 56.8 | 43.2 | | | 99.96 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 48.5 | 5.2 | 2980.893 | 0.162 | 100 | 3085.938 | 103.5 | | | | | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 0.01 | 0.01 | 2679.888 | 0.069 | 100 | 1632.813 | 60.9 | 39.1 | 39.4 | 0.29 | 99.99 | 99.99 | 0.00 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 0.01 | 0.01 | 2679.888 | 0.068 | 100 | 1617.188 | 60.3 | 39.7 | | | 99.99 | | |
| 4C | 100 | 40 | 0 | 0.7755 | 40.776 | 92.5 | 1.2 | 2953.734 | 0.159 | 100 | 3039.063 | 102.9 | | | | | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 0.03 | 0.01 | 2652.559 | 0.087 | 100 | 1914.063 | 72.2 | 27.8 | 28.7 | 0.88 | 99.98 | 99.98 | 0.00 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 0.03 | 0.01 | 2652.559 | 0.084 | 100 | 1867.188 | 70.4 | 29.6 | | | 99.98 | | |
| 5C | 150 | 30 | 0 | 1.175 | 31.175 | 157.1 | 1.05 | 2897.514 | 0.157 | 100 | 3007.813 | 103.8 | | | | | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 12.3 | 0.03 | 2625.218 | 0.105 | 100 | 2195.313 | 83.6 | 16.4 | 17.3 | 0.89 | 93.85 | 92.20 | 1.65 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 18.9 | 0.25 | 2625.218 | 0.102 | 100 | 2148.438 | 81.8 | 18.2 | | | 90.55 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 197.4 | 3 | 2890.584 | 0.160 | 100 | 3054.688 | 105.7 | | | | | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2734.579 | 0.063 | 100 | 1539.063 | 56.3 | 43.7 | | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0.001 | 0.02 | 3011.000 | 0.162 | 100 | 3085.938 | 102.5 | -2.5 | | | | | |

Notes:

## Isolate A

## Run 2A

Incubation time: Oct. 18-23 (6:00 PM)
Media pH: 7:6
SO4 calibration curve: Oct. 24/97
[SO4] of inoculum: 1663 mg/ml
[SO4] of media: 3123 mg/ml
OD 600 of inoculum:

| Sample | [Cu] initial ppm | vol. med ml | vol. SRB ml | vol. Cu ml | vol. total ml | [Cu] sup ppm | STD | [SO4] initial mg/L | A | dilution factor | [SO4] final mg/L | SO4 remianed % | SO4 reduced % | AVE | AVEDEV | Cu removed % | AVE | AVEDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.12 | 0.030 | 2787.478 | 0.062 | 100.0 | 1523.44 | 54.7 | 45.3 | 46.5 | 1.12 | 98.80 | 98.85 | 0.05 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.11 | 0.010 | 2787.478 | 0.058 | 100.0 | 1460.94 | 52.4 | 47.6 | | | 98.90 | | |
| 1C | 10 | 38 | 8 | 0.3838 | 46.384 | 14.9 | 0.400 | 2845.347 | 0.166 | 100.0 | 3148.44 | 110.7 | | | | | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.05 | 0.010 | 2745.238 | 0.058 | 100.0 | 1460.94 | 53.2 | 46.8 | 47.6 | 0.85 | 99.80 | 99.80 | 0.00 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.05 | 0.020 | 2745.238 | 0.055 | 100.0 | 1414.06 | 51.5 | 48.5 | | | 99.80 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 25.4 | 0.300 | 3044.922 | 0.162 | 100.0 | 3085.94 | 101.3 | | | | | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 0.06 | 0.010 | 2787.478 | 0.061 | 100.0 | 1507.81 | 54.1 | 45.9 | 45.1 | 0.84 | 99.88 | 99.89 | 0.01 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 0.05 | 0.010 | 2787.478 | 0.064 | 100.0 | 1554.69 | 55.8 | 44.2 | | | 99.90 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 51.9 | 1.000 | 3091.773 | 0.163 | 100.0 | 3101.56 | 100.3 | | | | | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 0.02 | 0.010 | 2759.320 | 0.070 | 100.0 | 1648.44 | 59.7 | 40.3 | 39.7 | 0.57 | 99.98 | 99.98 | 0.00 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 0.02 | 0.020 | 2759.320 | 0.072 | 100.0 | 1679.69 | 60.9 | 39.1 | | | 99.98 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 102.5 | 0.600 | 3060.541 | 0.165 | 100.0 | 3132.81 | 102.4 | | | | | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 0.09 | 0.050 | 2731.181 | 0.095 | 100.0 | 2039.06 | 74.7 | 25.3 | 27.1 | 1.72 | 99.94 | 99.93 | 0.01 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 0.11 | 0.010 | 2731.181 | 0.089 | 100.0 | 1945.31 | 71.2 | 28.8 | | | 99.93 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 169.9 | 1.700 | 3029.330 | 0.159 | 100.0 | 3039.06 | 100.3 | | | | | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 6 | 0.340 | 2703.029 | 0.119 | 100.0 | 2414.06 | 89.3 | 10.7 | 13.9 | 3.18 | 97.00 | 97.50 | 0.50 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 4 | 0.030 | 2703.029 | 0.108 | 100.0 | 2242.19 | 83.0 | 17.0 | | | 98.00 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 193.8 | 1.700 | 2998.105 | 0.165 | 100.0 | 3132.81 | 104.5 | | | | | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.010 | 2815.632 | 0.060 | 100.0 | 1492.19 | 53.0 | 47.0 | | | #DIV/0! | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0.001 | 0.010 | 3123.000 | 0.168 | 100.0 | 3179.69 | 101.8 | -1.8 | | | #DIV/0! | | |

Notes:
MMAT tubes



## Isolate B

## Run 1B

Incubation time: Oct. 21-26 (10:30 AM)
Media pH: 7:74
SO4 calibration curve: Nov 1/97
[SO4] of inoculum: 1794 mg/ml
[SO4] of media: 3171mg/ml
OD 600 of inoculum:

| Sample | [Cu] initial ppm | vol. med ml | vol. SRB ml | vol. Cu ml | vol. total ml | [Cu] sup ppm | STD | [SO4] initial mg/L | A | dilution factor | [SO4] final mg/L | SO4 remianed % | SO4 reduced % | AVE | AVEDEV | Cu removed % | AVE | AVEDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.1 | 0.02 | 2852.297 | 0.060 | 100.0 | 1525.806 | 53.5 | 46.5 | 47.6 | 1.13 | 99.00 | 99.20 | 0.20 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.06 | 0.01 | 2852.297 | 0.056 | 100.0 | 1461.290 | 51.2 | 48.8 | | | 99.40 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 11.9 | 0.2 | 3139.293 | 0.164 | 100.0 | 3203.226 | 102.0 | -2.0 | | | -19.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.05 | 0.01 | 2809.075 | 0.057 | 100.0 | 1477.419 | 52.6 | 47.4 | 48.0 | 0.57 | 99.80 | 99.82 | 0.02 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.04 | 0.01 | 2809.075 | 0.055 | 100.0 | 1445.161 | 51.4 | 48.6 | | | 99.84 | | |
| 2C | 25 | 38 | 0 | 0.9797 | 38.980 | 19.9 | 0.3 | 3091.301 | 0.168 | 100.0 | 3267.742 | 105.7 | -5.7 | | | 20.40 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 0.02 | 0.01 | 2852.297 | 0.061 | 100.0 | 1541.935 | 54.1 | 45.9 | 44.8 | 1.13 | 99.96 | 99.96 | 0.00 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 0.02 | 0.01 | 2852.297 | 0.065 | 100.0 | 1606.452 | 56.3 | 43.7 | | | 99.96 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 53.7 | 0.4 | 3139.293 | 0.162 | 100.0 | 3170.968 | 101.0 | -1.0 | | | -7.40 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 0.01 | 0.02 | 2823.484 | 0.070 | 100.0 | 1687.097 | 59.8 | 40.2 | 39.7 | 0.57 | 99.99 | 99.99 | 0.00 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 0.02 | 0.01 | 2823.484 | 0.072 | 100.0 | 1719.355 | 60.9 | 39.1 | | | 99.98 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 105.3 | 0.7 | 3107.581 | 0.160 | 100.0 | 3138.710 | 101.0 | -1.0 | | | -5.30 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 0.04 | 0.01 | 2794.690 | 0.084 | 100.0 | 1912.903 | 68.4 | 31.6 | 28.7 | 2.89 | 99.97 | 99.97 | 0.01 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 0.06 | 0.01 | 2794.690 | 0.094 | 100.0 | 2074.194 | 74.2 | 25.8 | | | 99.96 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 157.6 | 1 | 3075.890 | 0.161 | 100.0 | 3154.839 | 102.6 | -2.6 | | | -5.07 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 51.4 | 0.35 | 2765.884 | 0.115 | 100.0 | 2412.903 | 87.2 | 12.8 | 13.6 | 0.87 | 74.30 | 79.08 | 4.78 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 32.3 | 0.2 | 2765.884 | 0.112 | 100.0 | 2364.516 | 85.5 | 14.5 | | | 83.85 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 206.1 | 1.1 | 3044.186 | 0.164 | 100.0 | 3203.226 | 105.2 | -5.2 | | | -3.05 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2881.105 | 0.059 | 100.0 | 1509.677 | 52.4 | 47.6 | | | #DIV/0! | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0 | 0.01 | 3171.000 | 0.165 | 100.0 | 3219.355 | 101.5 | -1.5 | | | #DIV/0! | | |

Mmat tubes

## Isolate B

## Run 2B

Incubation time: Oct.26-31 (11:00 AM)
Media pH: 7:44
SO4 calibration curve : Nov 1/97
[SO4] of inoculum: 1852 mg/ml
[SO4] of media: 3167 mg/ml
OD 600 of inoculum:

| Sample | [Cu] initial | vol. med | vol. SRB | vol. Cu | vol. total | [Cu] sup | STD | [SO4] initial | A | dilution factor | [SO4] final | SO4 remianed | SO4 reduced | AVE | AVEDEV | Cu removed | AVE | AVEDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ppm | ml | ml | ml | ml | ppm | | mg/L | | | mg/L | | % | | | % | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.09 | 0.01 | 2861.26 | 0.055 | 100.0 | 1445.2 | 50.5 | 49.5 | 47.5 | 1.97 | 99.10 | 98.90 | 0.20 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.13 | 0.01 | 2861.26 | 0.062 | 100.0 | 1558.1 | 54.5 | 45.5 | | | 98.70 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 13.9 | 0.1 | 3135.33 | 0.166 | 100.0 | 3235.5 | 103.2 | -3.2 | | | -39.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.1 | 0.01 | 2817.90 | 0.059 | 100.0 | 1509.7 | 53.6 | 46.4 | 45.6 | 0.86 | 99.60 | 99.56 | 0.04 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.12 | 0.01 | 2817.90 | 0.062 | 100.0 | 1558.1 | 55.3 | 44.7 | | | 99.52 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 22.7 | 0.4 | 3087.82 | 0.162 | 100.0 | 3171.0 | 102.7 | -2.7 | | | 9.20 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 0.11 | 0.01 | 2861.26 | 0.064 | 100.0 | 1590.3 | 55.6 | 44.4 | 43.6 | 0.85 | 99.78 | 99.80 | 0.02 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 0.09 | 0.01 | 2861.26 | 0.067 | 100.0 | 1638.7 | 57.3 | 42.7 | | | 99.82 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 59.9 | 0.9 | 3135.33 | 0.165 | 100.0 | 3219.4 | 102.7 | -2.7 | | | -19.80 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 0.07 | 0.01 | 2832.36 | 0.073 | 100.0 | 1735.5 | 61.3 | 38.7 | 39.3 | 0.57 | 99.93 | 99.94 | 0.00 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 0.06 | 0.06 | 2832.36 | 0.071 | 100.0 | 1703.2 | 60.1 | 39.9 | | | 99.94 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 111.7 | 0.9 | 3103.66 | 0.160 | 100.0 | 3138.7 | 101.1 | -1.1 | | | -11.70 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 0.02 | 0.01 | 2803.47 | 0.092 | 100.0 | 2041.9 | 72.8 | 27.2 | 28.0 | 0.86 | 99.99 | 99.98 | 0.00 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 0.03 | 0.01 | 2803.47 | 0.089 | 100.0 | 1993.5 | 71.1 | 28.9 | | | 99.98 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 160.2 | 0.6 | 3072.01 | 0.164 | 100.0 | 3203.2 | 104.3 | -4.3 | | | -6.80 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 0.03 | 0.01 | 2774.57 | 0.120 | 100.0 | 2493.5 | 89.9 | 10.1 | 11.6 | 1.45 | 99.99 | 99.99 | 0.00 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | n/a | n/a | 2774.57 | 0.115 | 100.0 | 2412.9 | 87.0 | 13.0 | | | | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 190.4 | 1.3 | 3040.35 | 0.164 | 100.0 | 3203.2 | 105.4 | -5.4 | | | 4.80 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2890.16 | 0.060 | 100.0 | 1525.8 | 52.8 | 47.2 | | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | -0.01 | 0.01 | 3167.00 | 0.166 | 100.0 | 3235.5 | 102.2 | -2.2 | | | | | |

Mixed tubes
Forgot to put the sample in the incubator right away. There was an overnight delay. The samples were incubated a bit longer



## Isolate C

## Run 1C

Incubation time: Nov.19-24 (8:00 PM)
Media pH: 7:55
SO4 calibration curve: Nov 26/97
[SO4] of inoculum: 2978 mg/ml
[SO4] of media: 3147.7 mg/ml
OD 600 of inoculum:

| Sample | [Cu] initial | vol. med | vol. SRB | vol. Cu | vol. total | [Cu] sup | STD | [SO4] initial | A | dilution factor | [SO4] final | SO4 remianed | SO4 reduced | AVE | AVEDEV | Cu removed | AVE | AVEDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ppm | ml | ml | ml | ml | ppm | | mg/L | | | mg/L | % | % | | | % | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 6.3 | 0.1 | 3082.73 | 0.165 | 100.0 | 2993.8 | 97.1 | 2.9 | 2.1 | 0.75 | 37.00 | 49.00 | 12.00 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 3.9 | 0.2 | 3082.73 | 0.168 | 100.0 | 3040.0 | 98.6 | 1.4 | | | 61.00 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 12 | 0.2 | 3116.23 | 0.177 | 100.0 | 3178.5 | 102.0 | -2.0 | | | -20.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 22.7 | 0.1 | 3036.02 | 0.160 | 100.0 | 2916.9 | 96.1 | 3.9 | 4.2 | 0.25 | 9.20 | 8.40 | 0.80 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 23.1 | 0.1 | 3036.02 | 0.159 | 100.0 | 2901.5 | 95.6 | 4.4 | | | 7.60 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 24.1 | 0.2 | 3069.00 | 0.170 | 100.0 | 3070.8 | 100.1 | -0.1 | | | 3.60 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 57.9 | 0.5 | 3082.73 | 0.159 | 100.0 | 2901.5 | 94.1 | 5.9 | 4.4 | 1.50 | -15.80 | -10.60 | 5.20 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 52.7 | 0.3 | 3082.73 | 0.165 | 100.0 | 2993.8 | 97.1 | 2.9 | | | -5.40 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 59 | 0.1 | 3116.23 | 0.170 | 100.0 | 3070.8 | 98.5 | 1.5 | | | -18.00 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 100.5 | 0.6 | 3051.59 | 0.168 | 100.0 | 3040.0 | 99.6 | 0.4 | 0.6 | 0.25 | -0.50 | -1.20 | 0.70 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 101.9 | 0.6 | 3051.59 | 0.167 | 100.0 | 3024.6 | 99.1 | 0.9 | | | -1.90 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 102.5 | 1.2 | 3084.75 | 0.169 | 100.0 | 3055.4 | 99.0 | 1.0 | | | -2.50 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 155.8 | 2 | 3020.47 | 0.164 | 100.0 | 2978.5 | 98.6 | 1.4 | 2.4 | 1.02 | -3.87 | -4.93 | 1.07 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 159 | 1.2 | 3020.47 | 0.160 | 100.0 | 2916.9 | 96.6 | 3.4 | | | -6.00 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 162.2 | 0.8 | 3053.29 | 0.169 | 100.0 | 3055.4 | 100.1 | -0.1 | | | -8.13 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 208.6 | 2.1 | 2989.34 | 0.164 | 100.0 | 2978.5 | 99.6 | 0.4 | 0.1 | 0.26 | -4.30 | -4.65 | 0.35 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 210 | 1.5 | 2989.34 | 0.165 | 100.0 | 2993.8 | 100.2 | -0.2 | | | -5.00 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 213.3 | 0.6 | 3021.82 | 0.163 | 100.0 | 2963.1 | 98.1 | 1.9 | | | -6.65 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.2 | 3113.87 | 0.159 | 100.0 | 2901.5 | 93.2 | 6.8 | | | #DIV/0! | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0.03 | 0.1 | 3147.70 | 0.175 | 100.0 | 3147.7 | 100.0 | 0.0 | | | #DIV/0! | | |

## Isolate C

### Run 2C

Incubation time: Nov.20-25 (8:00 PM)
Media pH: 7:53
SO4 calibration curve: Nov 26/97
[SO4] of inoculum: 2967 mg/ml
[SO4] of the media : 3163.1mg/ml
OD 600 of inoculum:

| *Sample* | *[Cu] initial* | *vol. med* | *vol. SRB* | *vol. Cu* | *vol. total* | *[Cu] sup* | *STD* | *[SO4] initial* | *A* | *dilution factor* | *[SO4] final* | *SO4 remianed* | *SO4 reduced* | *AVE* | *AVEDEV* | *Cu removed* | *AVE* | *AVEDEV* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ppm* | *ml* | *ml* | *ml* | *ml* | *ppm* | | *mg/L* | | | *mg/L* | *%* | *%* | | | *%* | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 5 | 0.01 | 3090.60 | 0.166 | 100.0 | 3009.2 | 97.4 | 2.6 | 2.1 | 0.50 | 50.00 | 31.00 | 19.00 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 8.8 | 0.01 | 3090.60 | 0.168 | 100.0 | 3040.0 | 98.4 | 1.6 | | | 12.00 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 14.2 | 0.2 | 3131.47 | 0.174 | 100.0 | 3132.3 | 100.0 | 0.0 | | | -42.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 21 | 0.02 | 3043.77 | 0.166 | 100.0 | 3009.2 | 98.9 | 1.1 | 1.6 | 0.51 | 16.00 | 7.60 | 8.40 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 25.2 | 0.01 | 3043.77 | 0.164 | 100.0 | 2978.5 | 97.9 | 2.1 | | | -0.80 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 27.6 | 0.2 | 3084.02 | 0.174 | 100.0 | 3132.3 | 101.6 | -1.6 | | | -10.40 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 55.8 | 0.04 | 3090.60 | 0.169 | 100.0 | 3055.4 | 98.9 | 1.1 | 1.6 | 0.50 | -11.60 | -14.60 | 3.00 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 58.8 | 0.04 | 3090.60 | 0.167 | 100.0 | 3024.6 | 97.9 | 2.1 | | | -17.60 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 51.6 | 0.3 | 3131.47 | 0.175 | 100.0 | 3147.7 | 100.5 | -0.5 | | | -3.20 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 98 | 0.01 | 3059.38 | 0.167 | 100.0 | 3024.6 | 98.9 | 1.1 | 1.1 | 0.00 | 2.00 | -4.30 | 6.30 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 110.6 | 0.01 | 3059.38 | 0.167 | 100.0 | 3024.6 | 98.9 | 1.1 | | | -10.60 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 106.1 | 0.8 | 3099.84 | 0.178 | 100.0 | 3193.8 | 103.0 | -3.0 | | | -6.10 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 155.8 | 0.01 | 3028.18 | 0.164 | 100.0 | 2978.5 | 98.4 | 1.6 | 1.4 | 0.25 | -3.87 | -6.13 | 2.27 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 162.6 | 0.01 | 3028.18 | 0.165 | 100.0 | 2993.8 | 98.9 | 1.1 | | | -8.40 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 158.2 | 1.8 | 3068.23 | 0.172 | 100.0 | 3101.5 | 101.1 | -1.1 | | | -5.47 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 203 | 2.5 | 2996.97 | 0.165 | 100.0 | 2993.8 | 99.9 | 0.1 | 0.1 | 0.00 | -1.50 | -1.35 | 0.15 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 202.4 | 1.5 | 2996.97 | 0.165 | 100.0 | 2993.8 | 99.9 | 0.1 | | | -1.20 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 200.5 | 1.8 | 3036.60 | 0.169 | 100.0 | 3055.4 | 100.6 | -0.6 | | | -0.25 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0.002 | 0.02 | 3121.82 | 0.162 | 100.0 | 2947.7 | 94.4 | 5.6 | | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0.001 | 0.01 | 3163.10 | 0.176 | 100.0 | 3163.1 | 100.0 | 0.0 | | | | | |

Mixed tubes

## Isolate D

### Run 1D

Incubation time: Dec 4-9(8:00 PM)
Media pH: 7:48
SO4 calibration curve: Dec 14/97
[SO4] of inoculum: 1743 mg/ml
[SO4] of media: 3245 mg/ml
OD 600 of inoculum:

| *Sample* | *[Cu] initial* | *vol. med* | *vol. SRB* | *vol. Cu* | *vol. total* | *[Cu] sup* | *STD* | *[SO4] initial* | *A* | *dilution factor* | *[SO4] final* | *SO4 remianed* | *SO4 reduced* | *AVE* | *AVEDEV* | *Cu removed* | *AVE* | *AVEDEV* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ppm* | *ml* | *ml* | *ml* | *ml* | *ppm* | | *mg/L* | | | *mg/L* | | *%* | | | *%* | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.03 | 0.01 | 2899.50 | 0.072 | 100.0 | 1471.9 | 50.8 | 49.2 | 50.0 | 0.81 | 99.70 | 99.60 | 0.10 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.05 | 0.01 | 2899.50 | 0.069 | 100.0 | 1425.0 | 49.1 | 50.9 | | | 99.50 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 12.8 | 0.1 | 3212.55 | 0.179 | 100.0 | 3143.8 | 97.9 | 2.1 | | | -28.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.05 | 0.01 | 2855.57 | 0.074 | 100.0 | 1503.1 | 52.6 | 47.4 | 47.1 | 0.27 | 99.80 | 99.80 | 0.00 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.05 | 0.01 | 2855.57 | 0.075 | 100.0 | 1518.8 | 53.2 | 46.8 | | | 99.80 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 25.2 | 0.4 | 3163.87 | 0.185 | 100.0 | 3237.5 | 102.3 | -2.3 | | | -0.80 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 0.02 | 0.01 | 2899.50 | 0.071 | 100.0 | 1456.3 | 50.2 | 49.8 | 50.3 | 0.54 | 99.96 | 99.92 | 0.04 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 0.06 | 0.2 | 2899.50 | 0.069 | 100.0 | 1425.0 | 49.1 | 50.9 | | | 99.88 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 54.7 | 0.9 | 3212.55 | 0.182 | 100.0 | 3190.6 | 99.3 | 0.7 | | | -9.40 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 0.01 | 0.01 | 2870.21 | 0.086 | 100.0 | 1690.6 | 58.9 | 41.1 | 40.6 | 0.54 | 99.99 | 99.98 | 0.01 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 0.03 | 0.06 | 2870.21 | 0.088 | 100.0 | 1721.9 | 60.0 | 40.0 | | | 99.97 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 105.1 | 0.9 | 3180.10 | 0.185 | 100.0 | 3237.5 | 101.8 | -1.8 | | | -5.10 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 0.06 | 0.02 | 2840.94 | 0.092 | 100.0 | 1784.4 | 62.8 | 37.2 | 37.5 | 0.27 | 99.96 | 99.97 | 0.01 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 0.04 | 0.01 | 2840.94 | 0.091 | 100.0 | 1768.8 | 62.3 | 37.7 | | | 99.97 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 154.8 | 0.8 | 3147.67 | 0.186 | 100.0 | 3253.1 | 103.4 | -3.4 | | | -3.20 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 15.2 | 0.01 | 2811.66 | 0.148 | 100.0 | 2659.4 | 94.6 | 5.4 | 7.1 | 1.67 | 92.40 | 93.15 | 0.75 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 12.2 | n/a | 2811.66 | 0.142 | 100.0 | 2565.6 | 91.2 | 8.8 | | | 93.90 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 207.1 | 1.3 | 3115.23 | 0.181 | 100.0 | 3175.0 | 101.9 | -1.9 | | | -3.55 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2928.79 | 0.065 | 100.0 | 1362.5 | 46.5 | 53.5 | | | #DIV/0! | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0 | 0.01 | 3245.00 | 0.188 | 100.0 | 3284.4 | 101.2 | -1.2 | | | #DIV/0! | | |

## Isolate D

### Run 2D

Incubation time: Dec 7-12(8:00 PM)
Media pH: 7:58
SO4 calibration curve: Dec 14/97
[SO4] of inoculum: 1597 mg/ml
[SO4] of media: 3166 mg/ml
OD 600 of inoculum:

| Sample | [Cu] initial | vol. med | vol. SRB | vol. Cu | vol. total | [Cu] sup | STD | [SO4] initial | A | dilution factor | [SO4] final | SO4 remianed | SO4 reduced | AVE | AVEDEV | Cu removed | AVE | AVEDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ppm | ml | ml | ml | ml | ppm | | mg/L | | | mg/L | | % | | | % | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.07 | 0.01 | 2807.3 | 0.068 | 100.0 | 1409.4 | 50.2 | 49.8 | 49.0 | 0.83 | 99.30 | 99.40 | 0.10 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.05 | 0.01 | 2807.3 | 0.071 | 100.0 | 1456.3 | 51.9 | 48.1 | | | 99.50 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 9.8 | 0.1 | 3134.3 | 0.177 | 100.0 | 3112.5 | 99.3 | 0.7 | | | 2.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.02 | 0.01 | 2764.8 | 0.071 | 100.0 | 1456.3 | 52.7 | 47.3 | 46.8 | 0.57 | 99.92 | 99.88 | 0.04 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.04 | 0.01 | 2764.8 | 0.073 | 100.0 | 1487.5 | 53.8 | 46.2 | | | 99.84 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 27 | 0.4 | 3086.8 | 0.178 | 100.0 | 3128.1 | 101.3 | -1.3 | | | -8.00 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 0.01 | 0.01 | 2807.3 | 0.068 | 100.0 | 1409.4 | 50.2 | 49.8 | 48.1 | 1.67 | 99.98 | 99.96 | 0.02 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 0.03 | 0.2 | 2807.3 | 0.074 | 100.0 | 1503.1 | 53.5 | 46.5 | | | 99.94 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 52.7 | 0.9 | 3134.3 | 0.179 | 100.0 | 3143.8 | 100.3 | -0.3 | | | -5.40 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 0.05 | 0.01 | 2779.0 | 0.089 | 100.0 | 1737.5 | 62.5 | 37.5 | 38.0 | 0.56 | 99.95 | 99.96 | 0.01 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 0.03 | 0.06 | 2779.0 | 0.087 | 100.0 | 1706.3 | 61.4 | 38.6 | | | 99.97 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 102.7 | 0.9 | 3102.7 | 0.183 | 100.0 | 3206.3 | 103.3 | -3.3 | | | -2.70 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 0.03 | 0.02 | 2750.6 | 0.096 | 100.0 | 1846.9 | 67.1 | 32.9 | 33.1 | 0.28 | 99.98 | 99.98 | 0.00 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 0.03 | 0.01 | 2750.6 | 0.095 | 100.0 | 1831.3 | 66.6 | 33.4 | | | 99.98 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 158.8 | 0.8 | 3071.0 | 0.179 | 100.0 | 3143.8 | 102.4 | -2.4 | | | -5.87 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 0.8 | 0.01 | 2722.3 | 0.137 | 100.0 | 2487.5 | 91.4 | 8.6 | 12.4 | 3.73 | 99.60 | 99.40 | 0.20 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 1.6 | n/a | 2722.3 | 0.124 | 100.0 | 2284.4 | 83.9 | 16.1 | | | 99.20 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 200.1 | 1.3 | 3039.4 | 0.181 | 100.0 | 3175.0 | 104.5 | -4.5 | | | -0.05 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2835.7 | 0.065 | 100.0 | 1362.5 | 48.0 | 52.0 | 26.1 | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0 | 0.01 | 3166.0 | 0.180 | 100.0 | 3159.4 | 99.8 | 0.2 | | | | | |



## Isolate E

### Run 1E *(Desulfovibrio desulfericans)*

Incubation time: Feb 2-5 (8:00 PM)
Media pH: 7:52
SO4 calibration curve: Feb 10 /97
[SO4] of inoculum: 2184 mg/ml
[SO4] of media: 3200 mg/ml
OD 600 of inoculum:

| Sample | [Cu] initial | vol. med | vol. SRB | vol. Cu | vol. total | [Cu] sup | STD | [SO4] initial | A | dilution factor | [SO4] final | SO4 remianed | SO4 reduced | AVE | AVEDEV | Cu removed | AVE | AVEDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ppm | ml | ml | ml | ml | ppm | | mg/L | | | mg/L | | % | | | % | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.13 | 0.01 | 2956.2 | 0.087 | 100.0 | 1850.8 | 62.6 | 37.4 | 38.5 | 1.07 | 98.70 | 98.90 | 0.20 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.09 | 0.01 | 2956.2 | 0.083 | 100.0 | 1787.3 | 60.5 | 39.5 | | | 99.10 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 13.2 | 0.2 | 3168.0 | 0.170 | 100.0 | 3168.3 | 100.0 | 0.0 | | | -32.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.12 | 0.02 | 2911.4 | 0.092 | 100.0 | 1930.2 | 66.3 | 33.7 | 34.2 | 0.55 | 99.52 | 99.56 | 0.04 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.1 | 0.01 | 2911.4 | 0.090 | 100.0 | 1898.4 | 65.2 | 34.8 | | | 99.60 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 25.4 | 0.2 | 3120.0 | 0.168 | 100.0 | 3136.5 | 100.5 | -0.5 | | | -1.60 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 5.4 | 0.04 | 2956.2 | 0.104 | 100.0 | 2120.6 | 71.7 | 28.3 | 29.6 | 1.34 | 89.20 | 91.70 | 2.50 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 2.9 | 0.04 | 2956.2 | 0.099 | 100.0 | 2041.3 | 69.0 | 31.0 | | | 94.20 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 53.1 | 0.3 | 3168.0 | 0.172 | 100.0 | 3200.0 | 101.0 | -1.0 | | | -6.20 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 6.2 | 0.01 | 2926.4 | 0.108 | 100.0 | 2184.1 | 74.6 | 25.4 | 23.7 | 1.63 | 93.80 | 93.25 | 0.55 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 7.3 | 0.01 | 2926.4 | 0.114 | 100.0 | 2279.4 | 77.9 | 22.1 | | | 92.70 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 103.2 | 0.8 | 3136.0 | 0.170 | 100.0 | 3168.3 | 101.0 | -1.0 | | | -3.20 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 15.3 | 0.01 | 2896.5 | 0.115 | 100.0 | 2295.2 | 79.2 | 20.8 | 19.9 | 0.82 | 89.80 | 90.87 | 1.07 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 12.1 | 0.01 | 2896.5 | 0.118 | 100.0 | 2342.9 | 80.9 | 19.1 | | | 91.93 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 156.3 | 1.8 | 3104.0 | 0.169 | 100.0 | 3152.4 | 101.6 | -1.6 | | | -4.20 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 20.3 | 2.5 | 2866.7 | 0.130 | 100.0 | 2533.3 | 88.4 | 11.6 | 10.5 | 1.11 | 89.85 | 89.08 | 0.77 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 23.4 | 1.5 | 2866.7 | 0.134 | 100.0 | 2596.8 | 90.6 | 9.4 | | | 88.30 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 200.5 | 1.8 | 3072.0 | 0.164 | 100.0 | 3073.0 | 100.0 | 0.0 | | | -0.25 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.2 | 2986.1 | 0.085 | 100.0 | 1819.0 | 60.9 | 39.1 | | | #DIV/0! | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0.001 | 0.1 | 3200.0 | 0.172 | 100.0 | 3200.0 | 100.0 | 0.0 | | | #DIV/0! | | |

## Isolate E

### Run 2E (*Desulfovibrio desulfericans)*

Incubation time: Feb 4-9 (8:00 PM)
Media pH: 7:52
SO4 calibration curve: Feb 10 /97
[SO4] of inoculum: 1967 mg/ml
[SO4] of media: 3215.9 mg/ml
OD 600 of inoculum:

| *Sample* | *[Cu] initial* | *vol. med* | *vol. SRB* | *vol. Cu* | *vol. total* | *[Cu] sup* | *STD* | *[SO4] initial* | *A* | *dilution factor* | *[SO4] final* | *SO4 remianed* | *SO4 reduced* | *AVE* | *AVEDEV* | *Cu removed* | *AVE* | *AVEDEV* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ppm* | *ml* | *ml* | *ml* | *ml* | *ppm* | | *mg/L* | | | *mg/L* | | *%* | | | *%* | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.03 | 0.02 | 2923.4 | 0.091 | 100.0 | 1914.3 | 65.5 | 34.5 | 35.1 | 0.54 | 99.70 | 99.50 | 0.20 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.07 | 0.01 | 2923.4 | 0.089 | 100.0 | 1882.5 | 64.4 | 35.6 | | | 99.30 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 9.5 | 0.2 | 3183.7 | 0.169 | 100.0 | 3152.4 | 99.0 | 1.0 | | | 5.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.08 | 0.01 | 2879.1 | 0.091 | 100.0 | 1914.3 | 66.5 | 33.5 | 33.8 | 0.28 | 99.68 | 99.70 | 0.02 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.07 | 0.01 | 2879.1 | 0.090 | 100.0 | 1898.4 | 65.9 | 34.1 | | | 99.72 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 21.6 | 0.3 | 3135.5 | 0.172 | 100.0 | 3200.0 | 102.1 | -2.1 | | | 13.60 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 4.1 | 0.01 | 2923.4 | 0.095 | 100.0 | 1977.8 | 67.7 | 32.3 | 31.5 | 0.81 | 91.80 | 92.40 | 0.60 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 3.5 | 0.01 | 2923.4 | 0.098 | 100.0 | 2025.4 | 69.3 | 30.7 | | | 93.00 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 48.5 | 0.4 | 3183.7 | 0.172 | 100.0 | 3200.0 | 100.5 | -0.5 | | | 3.00 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 7.6 | 0.02 | 2893.9 | 0.115 | 100.0 | 2295.2 | 79.3 | 20.7 | 21.8 | 1.10 | 92.40 | 93.40 | 1.00 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 5.6 | 0.01 | 2893.9 | 0.111 | 100.0 | 2231.7 | 77.1 | 22.9 | | | 94.40 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 92.5 | 0.7 | 3151.6 | 0.171 | 100.0 | 3184.1 | 101.0 | -1.0 | | | 7.50 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 18.6 | 0.01 | 2864.4 | 0.119 | 100.0 | 2358.7 | 82.3 | 17.7 | 17.1 | 0.55 | 87.60 | 87.00 | 0.60 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 20.4 | 0.01 | 2864.4 | 0.121 | 100.0 | 2390.5 | 83.5 | 16.5 | | | 86.40 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 157.1 | 1 | 3119.4 | 0.174 | 100.0 | 3231.7 | 103.6 | -3.6 | | | -4.73 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 24.6 | 0.35 | 2834.9 | 0.137 | 100.0 | 2644.4 | 93.3 | 6.7 | 5.9 | 0.84 | 87.70 | 87.08 | 0.63 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 27.1 | 0.2 | 2834.9 | 0.140 | 100.0 | 2692.1 | 95.0 | 5.0 | | | 86.45 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 197.4 | 1.1 | 3087.3 | 0.176 | 100.0 | 3263.5 | 105.7 | -5.7 | | | 1.30 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2953.0 | 0.085 | 100.0 | 1819.0 | 61.6 | 38.4 | | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0.001 | 0.01 | 3215.9 | 0.173 | 100.0 | 3215.9 | 100.0 | 0.0 | | | | | |



## Isolate F

### Run 1F (*Desulfovibrio vulgaris)*

Incubation time: Feb 27- Mar 4 (2:30 PM)
Media pH: 7:54
SO4 calibration curve: Mar 6 /98
[SO4] of inoculum: 1873 mg/ml
[SO4] of media: 3296.8 mg/ml
OD 600 of inoculum:

| *Sample* | *[Cu] initial* | *vol. med* | *vol. SRB* | *vol. Cu* | *vol. total* | *[Cu] sup* | *STD* | *[SO4] initial* | *A* | *dilution factor* | *[SO4] final* | *SO4 remianed* | *SO4 reduced* | *AVE* | *AVEDEV* | *Cu removed* | *AVE* | *AVEDEV* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ppm* | *ml* | *ml* | *ml* | *ml* | *ppm* | | *mg/L* | | | *mg/L* | | *%* | | | *%* | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.8 | 0.1 | 2967.1 | 0.087 | 100.0 | 1931.7 | 65.1 | 34.9 | 34.6 | 0.27 | 92.00 | 95.30 | 3.30 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.14 | 0.2 | 2967.1 | 0.088 | 100.0 | 1947.6 | 65.6 | 34.4 | | | 98.60 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 11.3 | 0.2 | 3263.8 | 0.174 | 100.0 | 3312.7 | 101.5 | -1.5 | | | -13.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.5 | 0.1 | 2922.1 | 0.088 | 100.0 | 1947.6 | 66.7 | 33.3 | 32.3 | 1.09 | 98.00 | 97.56 | 0.44 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.72 | 0.1 | 2922.1 | 0.092 | 100.0 | 2011.1 | 68.8 | 31.2 | | | 97.12 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 23.6 | 0.2 | 3214.4 | 0.176 | 100.0 | 3344.4 | 104.0 | -4.0 | | | 5.60 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 8.6 | 0.5 | 2967.1 | 0.099 | 100.0 | 2122.2 | 71.5 | 28.5 | 27.7 | 0.80 | 82.80 | 81.20 | 1.60 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 10.2 | 0.3 | 2967.1 | 0.102 | 100.0 | 2169.8 | 73.1 | 26.9 | | | 79.60 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 54.1 | 0.1 | 3263.8 | 0.173 | 100.0 | 3296.8 | 101.0 | -1.0 | | | -8.20 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 14.2 | 0.6 | 2937.1 | 0.119 | 100.0 | 2439.7 | 83.1 | 16.9 | 16.7 | 0.27 | 85.80 | 87.40 | 1.60 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 11 | 0.6 | 2937.1 | 0.120 | 100.0 | 2455.6 | 83.6 | 16.4 | | | 89.00 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 108.3 | 1.2 | 3230.9 | 0.172 | 100.0 | 3281.0 | 101.6 | -1.6 | | | -8.30 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 28.3 | 2 | 2907.2 | 0.125 | 100.0 | 2534.9 | 87.2 | 12.8 | 12.0 | 0.82 | 81.13 | 80.50 | 0.63 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 30.2 | 1.2 | 2907.2 | 0.128 | 100.0 | 2582.5 | 88.8 | 11.2 | | | 79.87 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 155.2 | 0.8 | 3197.9 | 0.170 | 100.0 | 3249.2 | 101.6 | -1.6 | | | -3.47 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 39.6 | 2.1 | 2877.2 | 0.142 | 100.0 | 2804.8 | 97.5 | 2.5 | 2.2 | 0.28 | 80.20 | 79.80 | 0.40 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 41.2 | 1.5 | 2877.2 | 0.143 | 100.0 | 2820.6 | 98.0 | 2.0 | | | 79.40 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 208.3 | 0.6 | 3165.0 | 0.175 | 100.0 | 3328.6 | 105.2 | -5.2 | | | -4.15 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.2 | 2997.1 | 0.084 | 100.0 | 1884.1 | 62.9 | 37.1 | | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0 | 0.1 | 3296.8 | 0.173 | 100.0 | 3296.8 | 100.0 | 0.0 | | | | | |

## Isolate F

## Run 2F (*Desulfovibrio vulgaris*)

Incubation time: Mar 1-5 (4:00 PM)
Media pH: 7:49
SO4 calibration curve: Mar 6 /98
[SO4] of inoculum: 2014 mg/ml
[SO4] of media: 3233.3 mg/ml
OD 600 of inoculum:

| *Sample* | *[Cu] initial* | *vol. med* | *vol. SRB* | *vol. Cu* | *vol. total* | *[Cu] sup* | *STD* | *[SO4] initial* | *A* | *dilution factor* | *[SO4] final* | *SO4 remained* | *SO4 reduced* | *AVE* | *AVEDEV* | *Cu removed* | *AVE* | *AVEDEV* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ppm* | *ml* | *ml* | *ml* | *ml* | *ppm* | | *mg/L* | | | *mg/L* | | *%* | | | *%* | | |
| 1A | 10 | 30 | 8 | 0.3838 | 38.384 | 0.17 | 0.01 | 2947 | 0.087 | 100.0 | 1932 | 65.6 | 34.4 | 33.9 | 0.54 | 98.30 | 98.15 | 0.15 |
| 1B | 10 | 30 | 8 | 0.3838 | 38.384 | 0.2 | 0.01 | 2947 | 0.089 | 100.0 | 1963 | 66.6 | 33.4 | | | 98.00 | | |
| 1C | 10 | 38 | 0 | 0.3838 | 38.384 | 13.7 | 0.1 | 3201 | 0.174 | 100.0 | 3313 | 103.5 | -3.5 | | | -37.00 | | |
| 2A | 25 | 30 | 8 | 0.9744 | 38.974 | 0.78 | 0.01 | 2902 | 0.091 | 100.0 | 1995 | 68.7 | 31.3 | 30.7 | 0.55 | 96.88 | 97.22 | 0.34 |
| 2B | 25 | 30 | 8 | 0.9744 | 38.974 | 0.61 | 0.01 | 2902 | 0.093 | 100.0 | 2027 | 69.8 | 30.2 | | | 97.56 | | |
| 2C | 25 | 38 | 0 | 0.9744 | 38.974 | 21.3 | 0.4 | 3152 | 0.174 | 100.0 | 3313 | 105.1 | -5.1 | | | 14.80 | | |
| 3A | 50 | 30 | 8 | 0.3838 | 38.384 | 9.3 | 0.04 | 2947 | 0.102 | 100.0 | 2170 | 73.6 | 26.4 | 25.6 | 0.81 | 81.40 | 82.50 | 1.10 |
| 3B | 50 | 30 | 8 | 0.3838 | 38.384 | 8.2 | 0.04 | 2947 | 0.105 | 100.0 | 2217 | 75.2 | 24.8 | | | 83.60 | | |
| 3C | 50 | 38 | 0 | 0.3838 | 38.384 | 55.6 | 0.3 | 3201 | 0.170 | 100.0 | 3249 | 101.5 | -1.5 | | | -11.20 | | |
| 4A | 100 | 30 | 8 | 0.7755 | 38.776 | 15.8 | 0.01 | 2917 | 0.117 | 100.0 | 2408 | 82.5 | 17.5 | 16.4 | 1.09 | 84.20 | 85.50 | 1.30 |
| 4B | 100 | 30 | 8 | 0.7755 | 38.776 | 13.2 | 0.01 | 2917 | 0.121 | 100.0 | 2471 | 84.7 | 15.3 | | | 86.80 | | |
| 4C | 100 | 38 | 0 | 0.7755 | 38.776 | 110.2 | 0.8 | 3169 | 0.172 | 100.0 | 3281 | 103.5 | -3.5 | | | -10.20 | | |
| 5A | 150 | 30 | 8 | 1.175 | 39.175 | 30.5 | 0.01 | 2887 | 0.125 | 100.0 | 2535 | 87.8 | 12.2 | 11.9 | 0.27 | 79.67 | 79.90 | 0.23 |
| 5B | 150 | 30 | 8 | 1.175 | 39.175 | 29.8 | 0.01 | 2887 | 0.126 | 100.0 | 2551 | 88.3 | 11.7 | | | 80.13 | | |
| 5C | 150 | 38 | 0 | 1.175 | 39.175 | 149.2 | 0.8 | 3136 | 0.173 | 100.0 | 3297 | 105.1 | -5.1 | | | 0.53 | | |
| 6A | 200 | 30 | 8 | 1.583 | 39.583 | 45.3 | 0.01 | 2858 | 0.144 | 100.0 | 2837 | 99.3 | 0.7 | 1.6 | 0.83 | 77.35 | 77.00 | 0.35 |
| 6B | 200 | 30 | 8 | 1.583 | 39.583 | 46.7 | n/a | 2858 | 0.141 | 100.0 | 2789 | 97.6 | 2.4 | | | 76.65 | | |
| 6C | 200 | 38 | 0 | 1.583 | 39.583 | 204.3 | 1.3 | 3104 | 0.172 | 100.0 | 3281 | 105.7 | -5.7 | | | -2.15 | | |
| Cont. SRB | 0 | 30 | 8 | 0 | 38.000 | 0 | 0.01 | 2977 | 0.087 | 100.0 | 1932 | 64.9 | 35.1 | | | | | |
| Cont. med | 0 | 30 | 0 | 0 | 30.000 | 0 | 0.01 | 3233 | 0.169 | 100.0 | 3233 | 100.0 | 0.0 | | | | | |

## B.2 Inoculum age experiment

March 18/98

pH of media: 7.5

Incubation temperature: 35C

Incubation duration: 5 days

| sample | Initial [Cu] | age of inoculum | Vol. Media | vol. Inoculum | vol copper solution | total vol | final [Cu] | st dev | % removal |
|---|---|---|---|---|---|---|---|---|---|
| | ppm | days | ml | ml | ml | ml | ppm | | |
| 1 | 100 | 2 | 30 | 8 | 0.7755 | 38.78 | 0.001 | 0.02 | 100.0 |
| 2 | 150 | 2 | 30 | 8 | 1.175 | 39.18 | 29.3 | 0.2 | 80.5 |
| 3 | 200 | 2 | 30 | 8 | 1.583 | 39.58 | 41.06 | 0.21 | 79.5 |
| 4 | 0 | 2 | 30 | 8 | 0 | 38.00 | 0 | 0.01 | |
| 5 | 100 | 3 | 30 | 8 | 0.7755 | 38.78 | 0.001 | 0.02 | 100.0 |
| 6 | 150 | 3 | 30 | 8 | 1.175 | 39.18 | 19.8 | 0.01 | 86.8 |
| 7 | 200 | 3 | 30 | 8 | 1.583 | 39.58 | 35.55 | 0.24 | 82.2 |
| 8 | 0 | 3 | 30 | 8 | 0 | 38.00 | 0 | 0.34 | |
| 9 | 100 | 4 | 30 | 8 | 0.7755 | 38.78 | 0.001 | 0.01 | 100.0 |
| 10 | 150 | 4 | 30 | 8 | 1.175 | 39.18 | 8.6 | 0.1 | 94.3 |
| 11 | 200 | 4 | 30 | 8 | 1.583 | 39.58 | 21.58 | 0.15 | 89.2 |
| 12 | 0 | 4 | 30 | 8 | 0 | 38.00 | 0 | 0.01 | |
| 13 | 100 | 5 | 30 | 8 | 0.7755 | 38.78 | 0.001 | 0.02 | 100.0 |
| 14 | 150 | 5 | 30 | 8 | 1.175 | 39.18 | 2.31 | 0.1 | 98.5 |
| 15 | 200 | 5 | 30 | 8 | 1.583 | 39.58 | 12.66 | 0.13 | 93.7 |
| 16 | 0 | 5 | 30 | 8 | 0 | 38.00 | 0 | 0.02 | |

| sample | Initial [Cu] | Absorbance | dilution | final [sulfate] | initial [sulfate] | sulfate remaining | Sulfate reduced |
|---|---|---|---|---|---|---|---|
| | ppm | A | | ppm | ppm | % | % |
| 1 | 100 | 0.07 | 100 | 1650.8 | 2787.1 | 59.2 | 40.8 |
| 2 | 150 | 0.117 | 100 | 2373.8 | 2830.1 | 83.9 | 16.1 |
| 3 | 200 | 0.127 | 100 | 2527.7 | 2801.5 | 90.2 | 9.8 |
| 4 | 0 | 0.065 | 100 | 1573.8 | 2844.3 | 55.3 | 44.7 |
| 5 | 100 | 0.072 | 100 | 1681.5 | 2742.6 | 61.3 | 38.7 |
| 6 | 150 | 0.081 | 100 | 1820.0 | 2785.7 | 65.3 | 34.7 |
| 7 | 200 | 0.087 | 100 | 1912.3 | 2756.8 | 69.4 | 30.6 |
| 8 | 0 | 0.056 | 100 | 1435.4 | 2798.9 | 51.3 | 48.7 |
| 9 | 100 | 0.074 | 100 | 1712.3 | 2726.8 | 62.8 | 37.2 |
| 10 | 150 | 0.077 | 100 | 1758.5 | 2769.6 | 63.5 | 36.5 |
| 11 | 200 | 0.085 | 100 | 1881.5 | 2740.9 | 68.6 | 31.4 |
| 12 | 0 | 0.059 | 100 | 1481.5 | 2782.7 | 53.2 | 46.8 |
| 13 | 100 | 0.062 | 100 | 1527.7 | 2723.0 | 56.1 | 43.9 |
| 14 | 150 | 0.074 | 100 | 1712.3 | 2766.4 | 61.9 | 38.1 |
| 15 | 200 | 0.076 | 100 | 1743.1 | 2737.2 | 63.7 | 36.3 |
| 16 | 0 | 0.057 | 100 | 1450.8 | 2779.5 | 52.2 | 47.8 |
| media | | 0.164 | | 3096.9 | | | |
| 2day inoculum | | 0.086 | | 1896.9 | | | |
| 3day inoculum | | 0.072 | | 1681.5 | | | |
| 4day inoculum | | 0.067 | | 1604.6 | | | |
| 5day inoculum | | 0.066 | | 1589.2 | | | |

## B.3 Kinetic Study:

This experiment is designed to measure the rate of Cu removal and SO4 consumption for the different batch flasks having a concentration of 10-200

Incubation time: Dec 14-19(8:00 PM)
Media pH: 7:48
[SO4] of inoculum: mg/ml

| | *#1* | *#2* | *#3* | *#4* | *#6* | *Cont.SRB* |
|---|---|---|---|---|---|---|
| [Cu] initial | | | | | | |
| vol med. | 150 | 150 | 150 | 150 | 150 | 150 |
| vol. SRB | 40 | 40 | 40 | 40 | 40 | 40 |
| Vol. Cu | 1.9 | 4.9 | 1.9 | 3.9 | 7.9 | 0 |
| Total vol | 191.9 | 194.9 | 191.9 | 193.9 | 197.9 | 190 |
| SO4 initial | 5363.2 | 5363.2 | 5363.2 | 5363.2 | 5363.2 | 5363.2 |

| | 10-PPM Culture | 25-PPM Culture | 50-PPM Culture | 100-PPM Culture | 200-PPM Culture | Control (0-PPM ) |
|---|---|---|---|---|---|---|
| Time | [Cu 2+] | | | | | |
| (hrs) | PPM | | | | | |
| 0 | 2.1 | 2 | 1.4 | 85 | 150.8 | 0 |
| 1 | 2.06 | 0.92 | 0.93 | 5.6 | 149.6 | 0 |
| 2 | 0.2 | 0.4 | 0.4 | 0.94 | 128.3 | 0.001 |
| 3 | 0.07 | 0.11 | 0.05 | 0.23 | 124 | 0 |
| 4 | 0.1 | 0.1 | 0.12 | 0.22 | 122.6 | 0 |
| 6 | 0.13 | 0.13 | 0.11 | 0.13 | 122 | 0.001 |
| 8 | 0.15 | 0.16 | 0.13 | 0.1 | 120.3 | 0 |
| 12 | 0.11 | 0.18 | 0.13 | 0.2 | 118 | 0 |
| 16 | 0.06 | 0.1 | 0.13 | 0.12 | 123.1 | 0 |
| 20.25 | 0.05 | 0.11 | 0.19 | 0.17 | 117 | 0 |
| 28.25 | 0.07 | 0.1 | 0.09 | 0.08 | 116.8 | 0 |
| 48.75 | 0.05 | 0.12 | 0.09 | 0.1 | 116 | 0 |
| 55.25 | 0.06 | 0.09 | 0.08 | 0.08 | 116.2 | 0 |
| 74.75 | 0.06 | 0.09 | 0.09 | 0.08 | 114.4 | 0 |
| 86.75 | 0.07 | 0.09 | 0.08 | 0.07 | 110.9 | 0 |

# Appendix C. Growth Media Composition:

## C.1 Iverson's media (A.P.H.A., 1995)

| | |
|---|---|
| Tryptose Soy Agar | 40.0 g |
| Agar | 5.0 g |
| Sodium lactate , 0.4 % solution | 600 ml |
| $MgSO_4$ | 2.0 g |
| Ferrous ammonium sulfate | 0.5 g |
| Distilled water | 400 ml |

Adjust pH to 7.2 to 7.4.

## C.2 Postgate's Medium B (Postgate, 1984)

| | |
|---|---|
| $KH_2PO_4$ | 0.5 g |
| $NH_4CLl$ | 1 g |
| $CaSO_4$ | 1 g |
| $MgSO_4$ | 2 g |
| Sodium lactate | 3.5 g |
| Yeast extract | 1 g |
| Ascorbic Acid | 0.1 g |
| Thioglycollic acid | 0.1 g |
| $FeSO_4.7\ H_2O$ | 0.5 g |

Tap water 1 litre, adjust reaction to pH ~7.5.

## C.3 Postgate's medium C (Postgate, 1984)

| | |
|---|---|
| $KH_2PO_4$ | 0.5 g |
| $NH_4Cl$ | 1 g |
| $Na_2SO_4$ | 4.5 g |
| $CaCl_2 \bullet 6\ H_2O$ | 0.06 g |
| $MgSO_4 \bullet 7H_2O$ | 0.06 g |
| Sodium lactate | 6 g |
| Yeast extract | 1 g |

| | |
|---|---|
| $FeSO_4 \bullet 7\ H_2O$ | 0.004 g |
| Sodium citrate. $2\ H_2O$ | 0.3 g |

Add one liter of distilled water and adjust pH to ~7.5.

## C.4 **Postgate's Medium E** (Postgate, 1984)

| | |
|---|---|
| KH2PO4, | 0.5 g; |
| $NH_4Cl$, | 1.0 g |
| $Na_2SO_4$ | 1.0 g |
| $CaCl_2 \bullet 6H_2O$ | 1.0 g |
| $MgCl_2.7H_2O$ | 2.0 g |
| Sodium lactate | 3.5 g |
| Yeast extract | 1.0 g |
| Ascorbic acid | 0.1 g |
| Thiyoglycollic acid | 0.1 g |
| $FeSO_4.7H_2O$ | 0.5 g |
| Agar | 15 g |

Adjust pH to 7.6 with concentrated NaOH after boiling of the agar.

## C.5 **Modified Postgate's Medium E** (Postgate, 1984)

| | |
|---|---|
| KH2PO4, | 0.5 g; |
| $NH_4Cl$, | 1.0 g |
| $Na_2SO_4$ | 1.0 g |
| $CaCl_2 \bullet 6H_2O$ | 1.0 g |
| $MgCl_2.7H_2O$ | 2.0 g |
| Sodium lactate | 3.5 g |
| yeast extract | 0.5 g |
| Ascorbic acid | 0.1 g |
| Thiyoglycollic acid | 0.1 g |
| $FeSO_4.7H_2O$ | 0.5 g |
| Agar | 15 g |

Adjuste pH to 7.6 with concentrated NaOH after boiling of the agar.

## C.6 Postgate's Medium F

| | |
|---|---|
| KH2PO4, | 0.5 g; |
| $NH_4Cl$, | 1.0 g |
| $Na_2SO_4$ | 1.0 g |
| $CaCl_2 \bullet 6H_2O$ | 1.0 g |
| $MgCl_2.7H_2O$ | 2.0 g |
| Sodium lactate | 3.5 g |
| yeast extract | 0.5 g |
| Ascorbic acid | 0.1 g |
| Thiyoglycollic acid | 0.1 g |
| $FeSO_4.7H_2O$ | 0.5 g |
| Agar | 15 g |

Adjuste pH to 7.6 with concentrated NaOH after boiling of the agar.