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Microbial catalyzed acid production in Los Angeles County sewers

Price, Steven Dwight, M.S.

The University of Arizona, 1989

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MICROBIAL CATALYZED ACID PRODUCTION IN LOS ANGELES COUNTY SEWERS

by

Steven Dwight Price

A Thesis Submitted to the Faculty of the

DEPARTMENT OF CIVIL ENGINEERING AND ENGINEERING MECHANICS

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR OF CIVIL ENGINEERING

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY AUTHOR

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27 April 1989

Date

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ABSTRACT

Corrosion of concrete sewer crowns will cost the County Sanitation Districts of Los Angeles County approximately $150 million for repairs to their deteriorating sewage system. Other parts of the country are experiencing similar problems.

Crown corrosion is induced by microbial oxidation of reduced sulfur to sulfuric acid, which attacks the concrete. Bacteria, of the genus *Thiobacillus* are generally responsible for catalyzing these reactions.

Thiobacilli collected from sewers were used to establish stoichiometry and biochemical aspects of sulfide oxidation. Metals inhibition was studied among the same cultures.

Thiobacilli collected from extremely corroded sewers possess a greater tolerance for metals than those from lightly corroded areas. Acidophilic isolates grow at greater rates and oxidize sulfide more efficiently than non-acidophiles.

Chemical inhibitor studies indicated that S(-II) oxidation is tightly linked to respiration by *T. thiooxidans*. It is doubtful that initial steps in bacterially catalyzed S(-II) oxidation are linked to oxidative phosphorylation.

CHAPTER 1

INTRODUCTION

1.1 Sulfide Corrosion of Concrete Sewers

1.1.1 General Description of the Sulfide Corrosion Problem

Sulfide-induced crown corrosion of concrete sewer pipe and/or structures has caused concern among operation and maintenance agencies, including the County Sanitation Districts of Los Angeles County (LACSD), for many years. Costs associated with sulfide corrosion control and/or system replacement can be extremely high. Current estimates for replacement of LACSD sewage collection system structures are in excess of $150 million (Morton, 1989). Therefore, potential corrosion retardation systems warrant attention.

The corrosion mechanism illustrated in Figure 1-1 consists of four distinguishable steps:

1. Sulfide [S(-II)] is generated in an anaerobic slime layer by sulfate-reducing bacteria.
2. S(-II) diffuses into the sewage. Among the reduced sulfur species is gaseous hydrogen sulfide (H_2S), which eventually volatilizes and diffuses to the sewer crown.
3. At the crown, H_2S is bacterially oxidized to sulfuric acid (H_2SO_4).

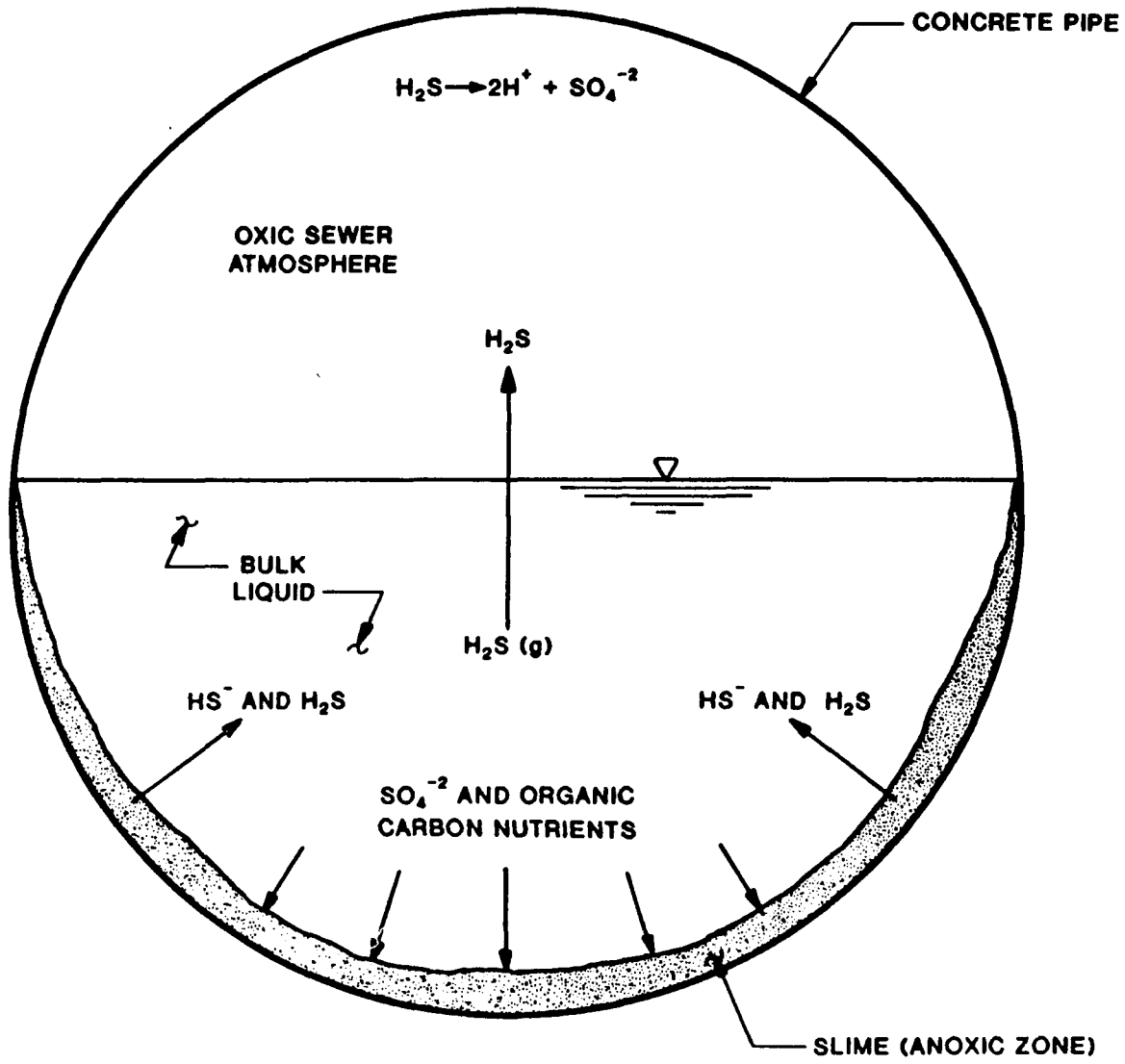


Figure 1-1. The physiological basis of hydrogen sulfide corrosion in concrete sewers.

4. The acid chemically attacks the concrete of the sewer pipe. This reaction may lead to excess crown corrosion and eventual sewer collapse (Holder, 1983; EPA, 1974; Parker, 1951).

Steps designed to impede crown corrosion may be directed at any of these processes. Potential means of mitigating crown corrosion include (i) inhibition of bacteria which reduce aqueous sulfate (SO_4^{-2}) to sulfide [S(-II)], (ii) physicochemical methods for precipitation and/or oxidation of liquid-phase sulfide, and (iii) inhibition of bacteria which oxidize H_2S to produce acid at the crown (Holder, 1983; LACSD, 1988; EPA, 1974). Efforts and processes which have been used to interrupt corrosion of concrete sewer pipe at many locations are presented later in this chapter.

1.1.2 The Basis of Sulfate Reduction in Sewage

Sulfate is present in all natural fresh waters, ranging from small amounts (1.6 mg/L in groundwater found near Oakland, California) to large concentrations exceeding 250 mg/L on the Colorado River in Arizona (Snoeyink, V.L. and D. Jenkins, 1980). Sulfate concentrations in municipal water supplies are augmented by an average of 100 mg/L as water is used and discharged to sewage collection systems (Neal, 1964).

Under anaerobic conditions, such as those which can prevail in a slime layer beneath stagnant or slow-moving sewage, sulfate [S(VI)] is dissimilatively reduced by a broad class of bacteria known collectively as sulfate-reducers. During dissimilative sulfate reduction, S(VI) is utilized as a terminal electron acceptor for microbially catalyzed

oxidation/reduction reactions. In contrast, assimilative S(VI) reduction, necessary for biosynthesis of the amino acids cysteine and methionine, is carried out by virtually all bacteria under both aerobic and anaerobic conditions.

The pathway for assimilative reduction is shown in Figure 1-2. A total of eight electrons are transferred as S(VI) is reduced to S(-II) in several enzyme-catalyzed steps. S(VI) is converted initially to adenylsulfate, and the process is completed via the catalytic reduction of sulfite (SO_3^{-2}) to S(-II). The S(VI) to S(IV) reaction utilizes energy stored in phosphate bonds, reductant and catalysis from three specific enzymes. The steps from S(IV) to S(-II), a six electron transfer, are catalyzed by a complex flavometallo-protein, sulfite reductase (Stanier, 1976).

Dissimilative reduction is accomplished via successive transfer of four pairs of electrons from organic substrate to SO_4^{-2}. The oxidation of lactate to acetate coupled with S(IV) reduction to S(-II) is schematically illustrated in Figure 1-3. Three enzymes are required for the reduction of S(VI) to S(IV), (i) ATP sulfurylase, (ii) pyrophosphorylase and (iii) APS reductase (Gottschalk, 1986). The last three electron-pairs are transferred via sulfite reductase in a three-step recycling process as illustrated in Figure 1-4 (Stanier, 1976).

Dissimilative SO_4^{-2} reduction is the major source of S(-II) production in the sewer environment (Postgate, 1959; Pfennig, 1971; Stanier, 1976; Sand, 1987). Two genera of bacteria, *Desulfotomaculum* and *Desulfovibrio*,

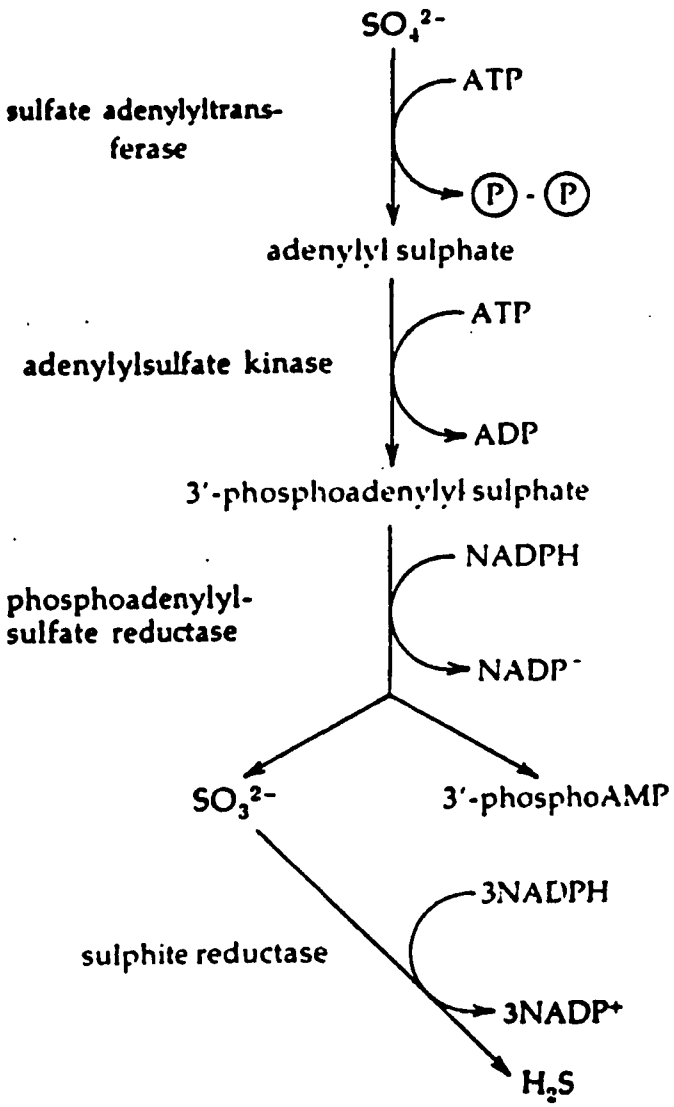


Figure 1-2. Steps in the assimilatory reduction of sulfate to H_2S for use in biosynthetic reactions (Stanier, 1976).

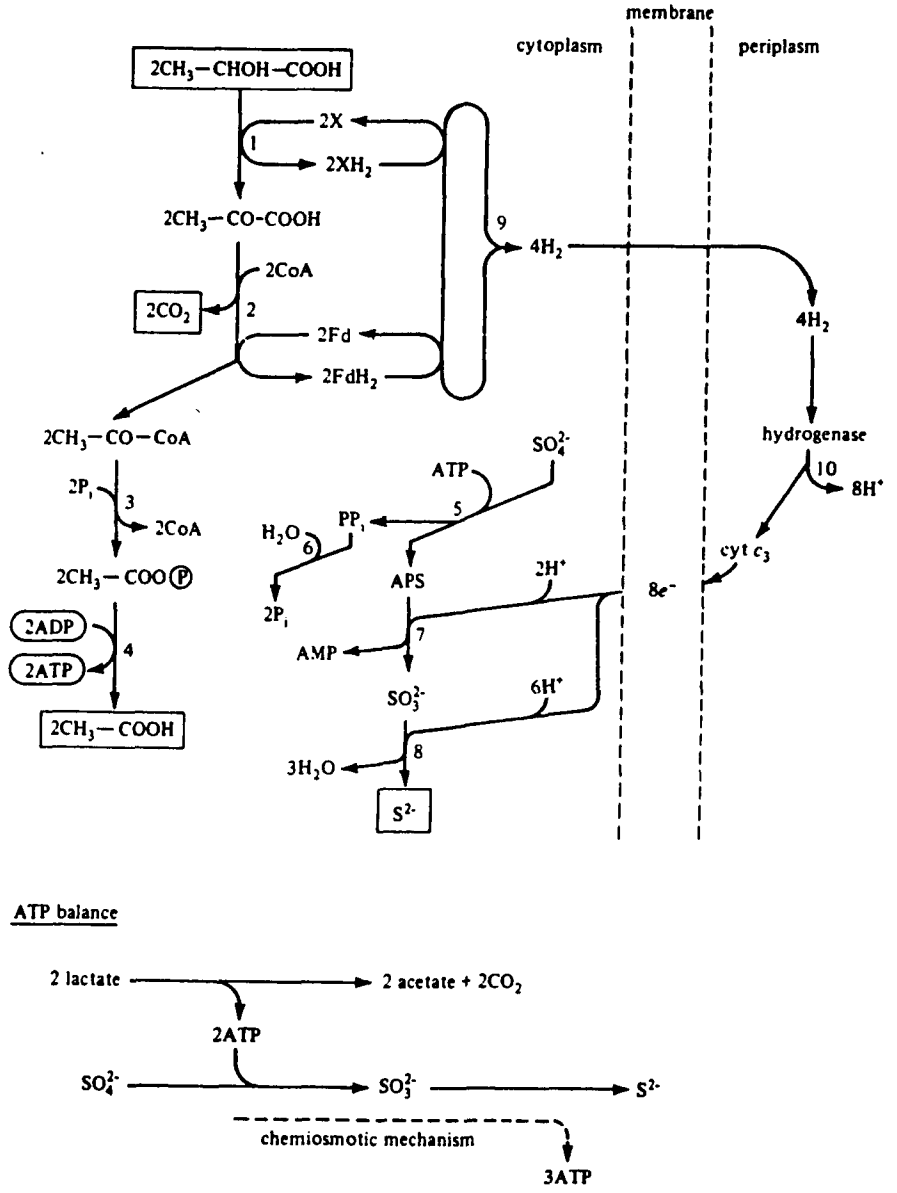


Figure 1-3. Pathway of dissimilatory sulfate reduction in *Desulfovibrio* species and "hydrogen cycling" hypothesis. 1, Lactate dehydrogenase, membrane-bound, H-acceptor not known; 2, pyruvate-ferredoxin oxidoreductase; 3, phosphotransacetylase; 4, acetate kinase; 5, ATP sulfurylase; 6, pyrophosphatase; 7, APS reductase; 8, sulfite reductase; 9, cytoplasmic hydrogenase; 10, periplasmic hydrogenase.

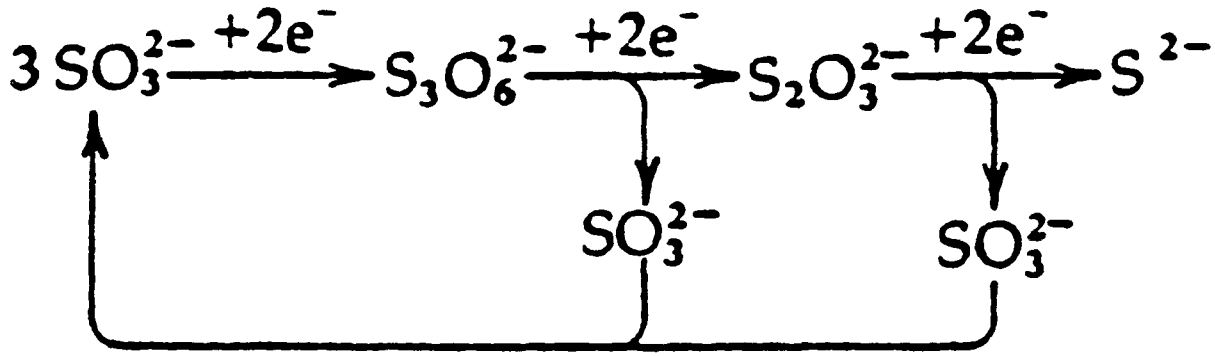


Figure 1-4. Recycling pathway for the dissimitory reduction of sulfite to sulfide in an anaerobic environment (Stanier, 1976).

are primarily responsible. They are strict anaerobes which are unique due to their massive S(-II) producing ability. There are many other species which reduce partially oxidized sulfur intermediates, but their importance to S(-II) production in sewers is considered to be relatively minor.

Figure 1-5 further illustrates the process of S(VI) reduction in gravity flow sewer systems. Molecular oxygen, normally present at low concentrations in bulk sewage, is depleted at a water-slime interface by aerobic microorganisms. Between the oxygen depletion zone and the conduit wall lies a zone in which microorganisms grow anaerobically using nitrate, SO_4^{-2} and perhaps other electron acceptors. Dissimilative SO_4^{-2} reduction produces S(-II) which reenters the wastewater stream via diffusion (EPA, 1985).

1.1.3 Prediction of Sulfide Concentrations in Gravity Flow Sewers

Empirical models have been developed for predicting the steady concentration of total sulfides in sewage as a function of specific physical and chemical properties (Holder, 1983; EPA, 1985). These models use measured values for biological oxygen demand (BOD), SO_4^{-2} concentration, temperature and fluid velocity of the sewage as independent variables. The model of Pomeroy and Parkhurst (1977) for pipes flowing less than full is:

$$\frac{d[S]}{dt} = \frac{M'\ EBOD}{R} - \frac{m\ [S](su)^{3/8}}{d_m} \qquad \text{Equation 1-1}$$

where,

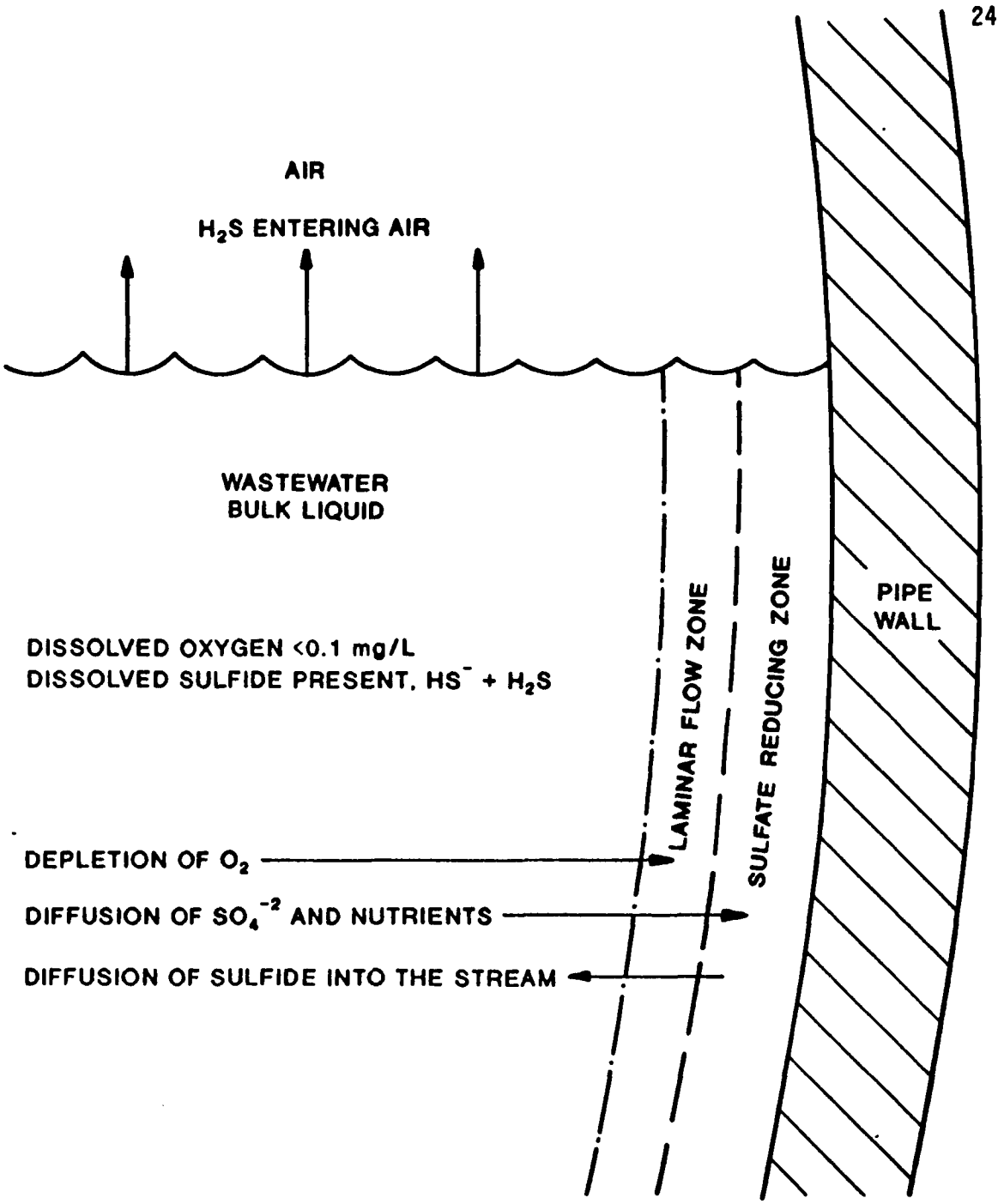


Figure 1-5. Sulfate reduction mechanism found in gravity flow sewer systems.

$\frac{d[S]}{dt}$ = rate of change of total liquid-phase sulfide concentration, mg/L-hr

M′ = effective sulfide flux coefficient for sulfide generation by the slime layer in gravity sewers (empirical constant), m/hr. A commonly suggested value is 0.32×10^{-3} m/hr

EBOD= effective BOD = $BOD_5 \times 1.07^{T-20}$, mg/L

T = wastewater temperature, °C

R = hydraulic radius (cross-sectional area of the flow divided by the wetted perimeter), m

m = empirical coefficient to account for sulfide losses by oxidation and escape to the atmosphere, dimensionless. Suggested values are 0.96 for sewage with low dissolved oxygen (O_2) concentration and 0.64 for higher O_2 concentrations.

[S] = total sulfide concentration, mg/L

d_m = mean hydraulic depth (cross-sectional area of the flow divided by surface width), m

u = mean sewage velocity, m/s

s = slope of energy grade line, m/m.

The first term on the right side of the above equation represents the generation of S(-II) within the slime layer. The form of this term reflects the assumption that no S(-II) is produced in the bulk-liquid, which is typically accurate. The second term accounts for the losses of S(-II) due to oxidation in the bulk-liquid and volatilization to the sewer head space. Therefore, at steady-state:

$d[S]/dt = 0$, and

$$\frac{M'EBOD}{R} = \frac{M[S](su)^{3/8}}{d_m} \qquad \text{Equation 1-2}$$

Solving for [S] yields the theoretical steady-state upper limit of liquid-phase sulfide concentration:

$$[S] = (M'/m)\ EBOD\ (su)^{-3/8}\ (d_m/R) \qquad \text{Equation 1-3}$$

The downstream S(-II) concentration $[S]_2$ can be predicted by using Equation 1-3 and:

$$[S]_2 = [S] - \frac{[S] - [S]_1}{\log^{-1}\ \frac{[m(su)^{3/8}t]}{[2.31\ d_m]}} \qquad \text{Equation 1-4}$$

where,

$[S]_2$= predicted downstream sulfide concentration at time t_2, mg/L

$[S]_1$= sulfide concentration at time t_1, mg/L

[S] = theoretical upper limit sulfide concentration, mg/L

t_2 = travel time in a given sewer reach with constant slope, diameter and flow, hrs

s,u,m and dm = as previously described.

The suggested values for empirical constants can be used as first estimates. Model calibration is then accomplished by adjusting the parameters to minimize the sum of squared error between model predictions and measured values.

1.1.4 Sulfide Speciation and Mechanisms Promoting Acid Production in Sewers

Aqueous inorganic S(-II) is distributed among H_2S, HS^- and S^{-2}; with the relative speciation primarily dependent on the pH of the liquid. Figure 1-6 represents the percentage of the species in water at pH values ranging from 5 to 9. The pK_a's for the deprotonation of H_2S in water are 7.0 and 13.9, respectively (Stumm, 1981). The unionized species, H_2S, the only volatile form of inorganic sulfide, is present in appreciable quantities only when the pH is below about 9.0. The extent of H_2S volatilization is a function of the sewage residence time in the collection system and local fluid turbulence as well as pH. Therefore, H_2S gas is normally present in higher concentrations at pipe intersections and system manholes.

Gaseous H_2S is completely oxidized to H_2SO_4 on the crown surface. It has been suggested H_2S is abiotically converted to elemental sulfur (S^o), which accumulates temporarily on the crown. The sulfur is then microbially oxidized to H_2SO_4 (Sand, 1984). Chemolithotrophs (predominantly thiobacilli) are responsible for the latter conversion.

1.1.5 Chemistry and Prediction of Acid Catalyzed Concrete Corrosion

Biologically produced H_2SO_4 is neutralized by concrete alkalinity as follows:

$H_2SO_4 + CaCO_3 \rightarrow CaSO_4 + H_2O + CO_2$ (Sand, 1987)

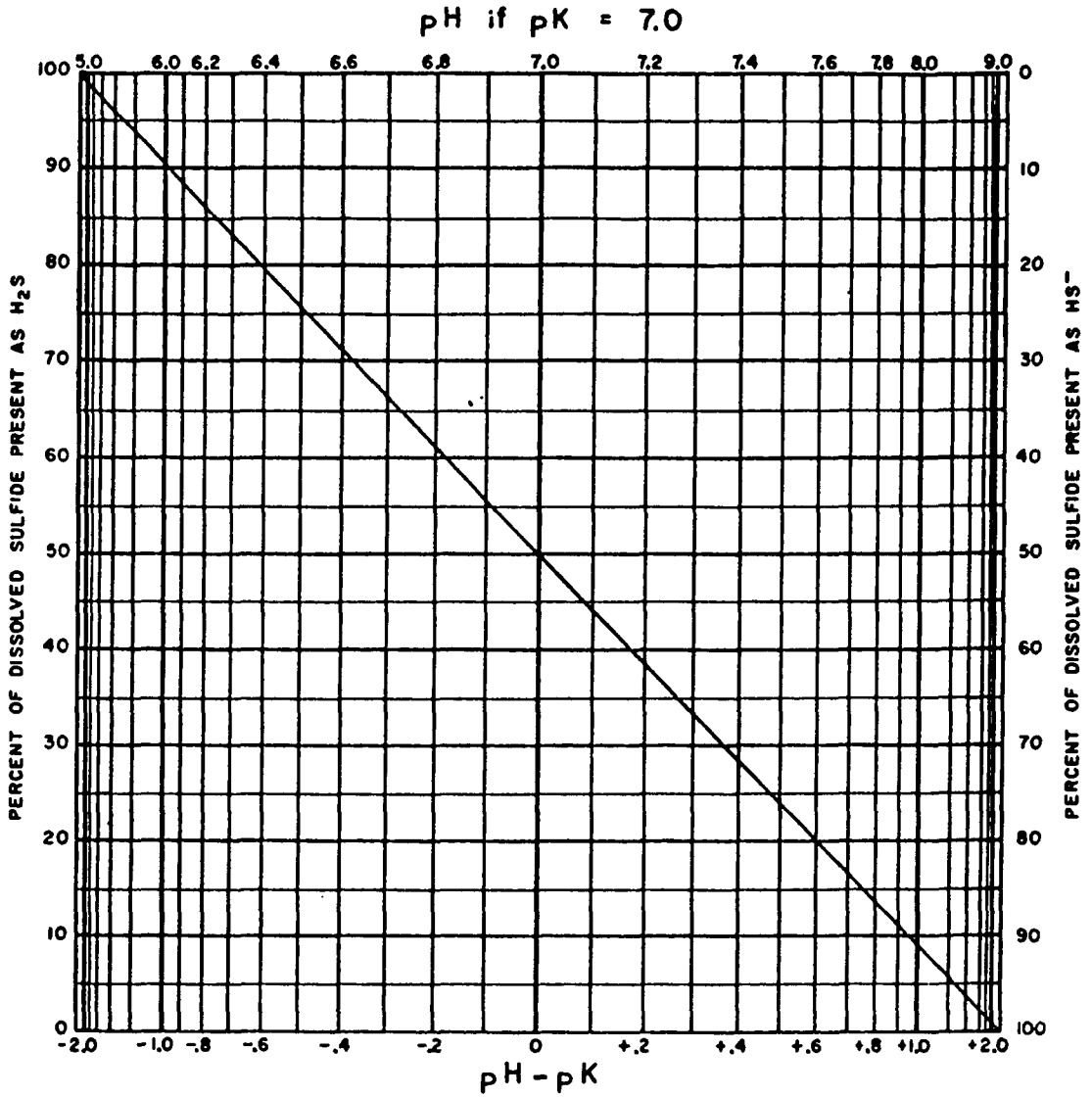


Figure 1-6. Effects of liquid phase pH on the relative concentrations of sulfide species, H_2S and HS^- (EPA, 1985).

The reaction product is gypsum, and the result is a loss of concrete integrity. Initially, the pH of the pipe crown is constant, but eventually acid accumulation decreases the crown pH. Thirty-two (32) grams of H_2S are required to produce sufficient H_2SO_4 to dissolve 100 grams of alkalinity expressed as $CaCO_3$ (EPA, 1985).

EPA (1974) developed the following empirical expression for predicting concrete corrosion rates:

$$C_{AVG} = \frac{0.31\ k(su)^{3/8}\ j[DS](b/P^1)}{A} \qquad \text{Equation 1-5}$$

where,

C_{AVG} = average rate of penetration, millimeters (mm)/yr

k = coefficient of efficiency for acid reaction considering the estimated fraction of total acid remaining at attack the concrete of the sewer structure. May be as low as 0.3 for humid areas and as high as 0.9 to 1.0 for large diameter pipes

s = energy gradient of wastewater stream, m/m

u = stream velocity, m/s

j = fraction of dissolved sulfide present as H_2S (a function of pH)

[DS] = average annual concentration of dissolved sulfide in the wastewater, mg/L

b/P^1 = ratio of width of wastewater stream at surface to exposed perimeter of the pipe wall above the water surface

A = alkalinity of the cement bonded material, expressed as $CaCO_3$ equivalents. Approximately 0.18 to 0.23 for granitic aggregate concrete, 0.9 for calcareous aggregate, 0.4 for mortar linings and 0.5 for asbestos cement.

The alkalinity of concrete pipe varies with the manufacturing process and construction material. Alkalinity also varies with depth in "spun" reinforced concrete pipe (RCP) although values remain consistent with depth in cast RCP (LACSD, 1987). Fine, high alkalinity concrete material is concentrated near the inside wall of spun pipe. Analyses of spun RCP showed an inside layer alkalinity of 25.5%, center portions 13.2%, and outside 15.3%, all as weight to weight measurements of alkalinity as $CaCO_3$. The alkalinity of cast-in-place RCP is only 7.2% as $CaCO_3$ (Morton, 1988). Therefore, spun RCP initially neutralizes more acid than cast RCP. However, after buffering capacity at the surface of spun RCP has been exhausted, corrosion rates can potentially increase, a prediction which is supported by LACSD observations. Figure 1-7 compares corrosion rates predicted by the model (Equation 1-5) with measured rates of a spun concrete pipe section in LACSD's system. Corrosion rates experienced in the past 10 to 15 years have exceeded predicted values by a factor of 3 to 4.

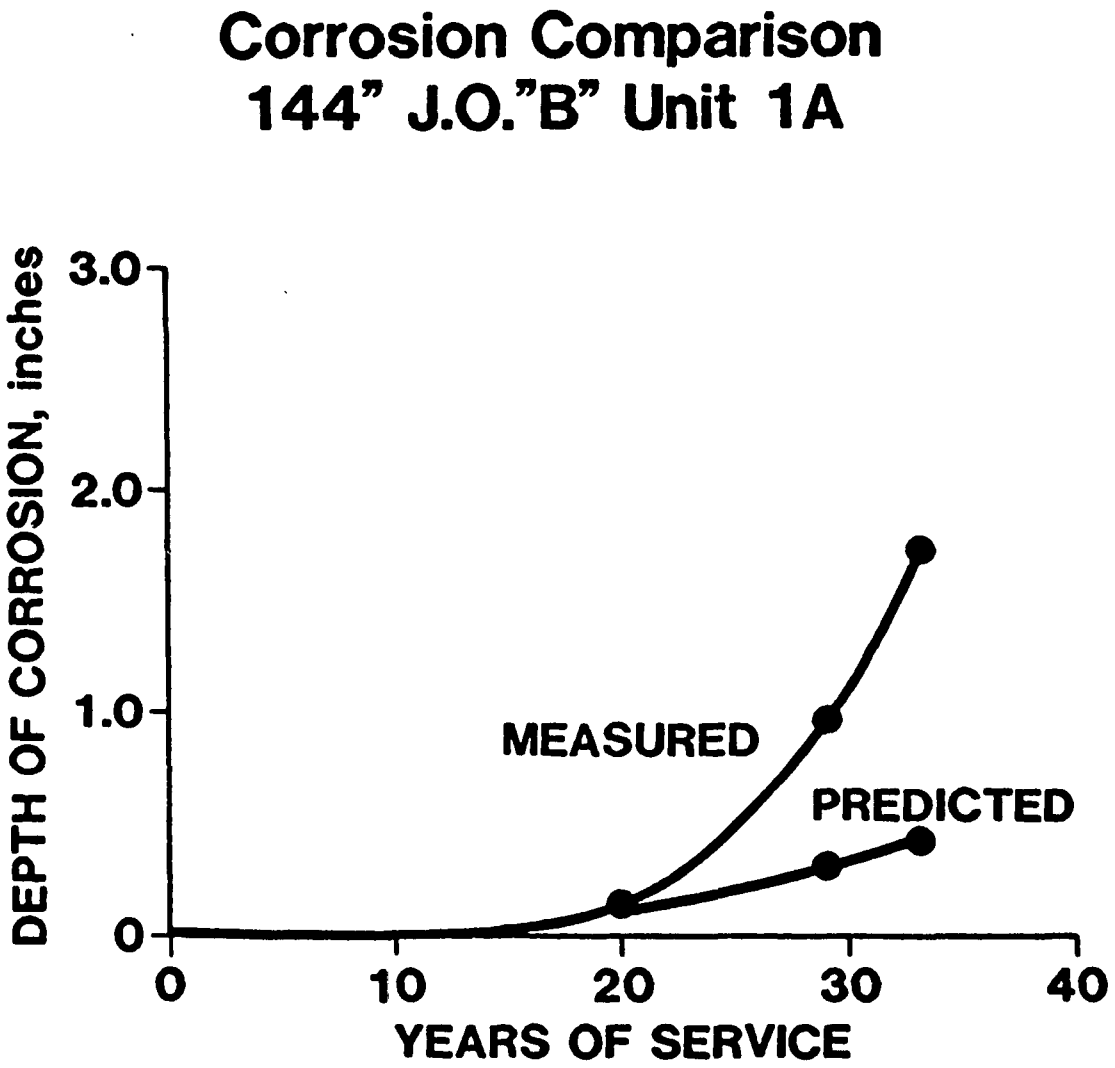


Figure 1-7. Actual and predicted depth of corrosion of a 144-inch diameter reinforced concrete sewer pipe. Pipe measured is a section of Jiont Outfall "B" of the LACSD collection system (LACSD, 1987).

1.1.6 Design of Sewers to Prevent Corrosion

The physical design of sewers is important for minimizing H_2S generation and potential concrete corrosion (EPA, 1974). Proper selection of gravity sewer slopes will effectively reduce the thickness of the anoxic slime layer in which SO_4^{-2}-reducers produce S(-II). Long sewer retention times and warm temperatures enhance the activity of sewer microflora resulting in rapid depletion of molecular O_2 and nitrate. Under such conditions, SO_4^{-2} becomes the preferred electron acceptor for microbial catabolism (EPA, 1974).

Traditional sewer pipe design allows for corrosion of concrete sewers at a controlled, predictable rate. The empirical model (Equation 1-5) has been used to predict the rate of corrosion. On that basis, pipe thickness has been specified to provide an acceptable design life for concrete pipe. Actual corrosion rates that are greater than predicted values will lead to a drastically reduced service life for the pipe designed in this manner and potentially enormous, unplanned expenses.

Alternatively, a plastic liner is frequently embedded on the vulnerable areas of the pipes and manhole sections. The liner provides a barrier to acid corrosion of the pipe crown.

1.2 The Los Angeles County Case Study

1.2.1 Nature of the Problem

Like many other warm-climate areas, southern California experiences sulfide corrosion in portions of its sewage collection system. As the operation and maintaining agency for Los Angeles County (excluding the City of Los Angeles), LACSD, has observed an acceleration of corrosion rates in concrete sewers over the past 10 to 15 years. Almost no detrimental corrosion was noticed during sewer system inspections conducted in the mid 1970's. Subsequent inspections in the early 1980's indicated approximately 40 miles of large diameter sewers corroding at a rate which would warrant repair or replacement within the next five years. Associated costs for rehabilitating these sections were estimated to exceed $150 million dollars; other sewer structures may also need repair sooner than previously predicted (Jin, 1987).

Photographs which show time-dependent crown corrosion at various points in the LACSD collection system are included in Figures 1-8 through 1-11. The pipe sections shown in Figure 1-8 is located near manholes C8. Figures 1-9 and 1-10 show the pipe near manhole C23. Joint Outfall "C" (JO-C) was constructed in 1946. Figure 1-11 (top) shows the pipe at manhole A62 on the Jo "A" trunk line which was placed into service in 1927. It is a 63-inch elliptical pipe lined with tile. The bottom photograph of Figure 1-11, near manhole E30, is a 54-inch diameter RCP

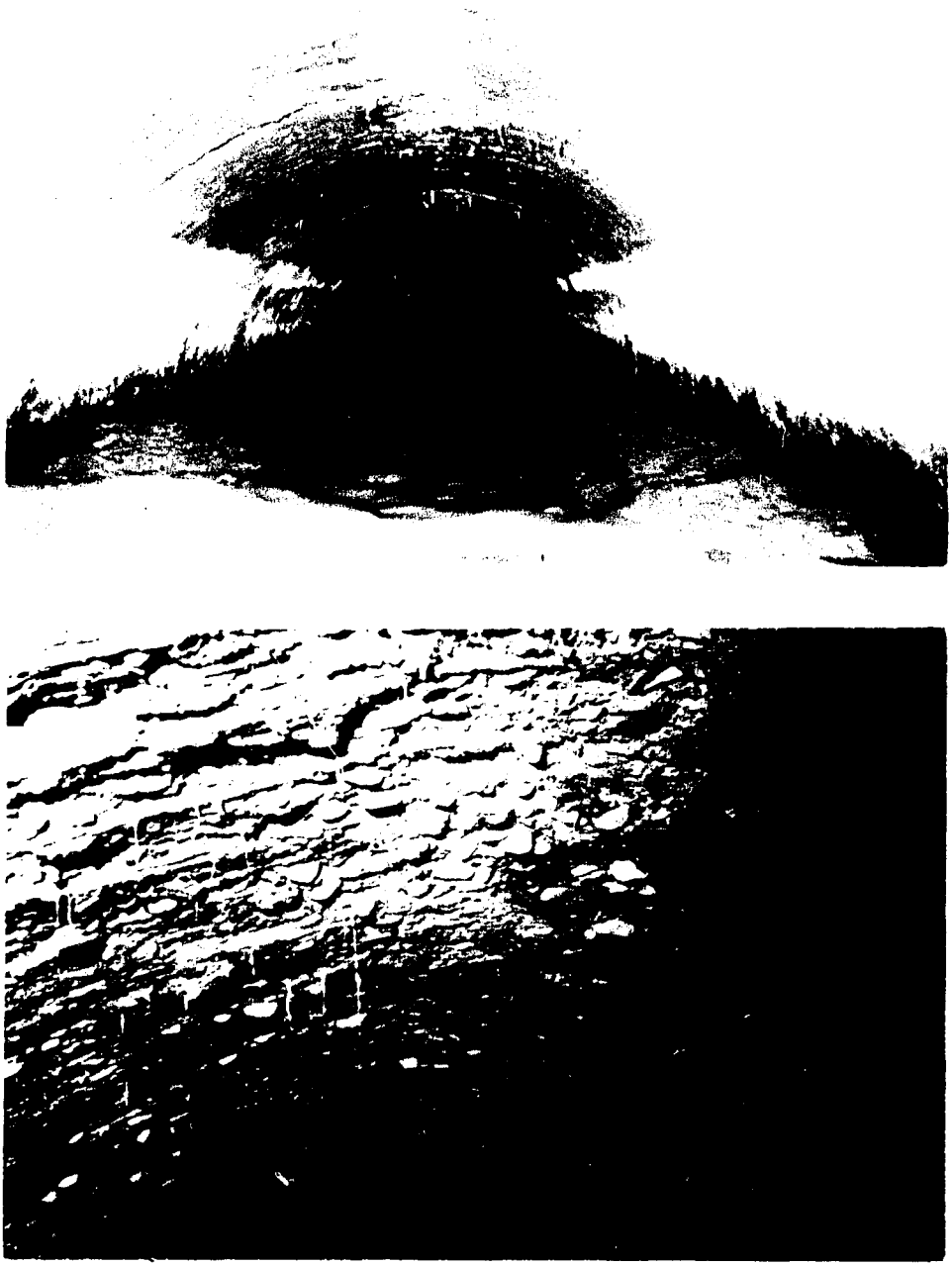


Figure 1-8. Photographs showing pipeline near manhole C8 of the LACSD sewage collection system. The top photograph was taken in October, 1982 and the bottom in January, 1987

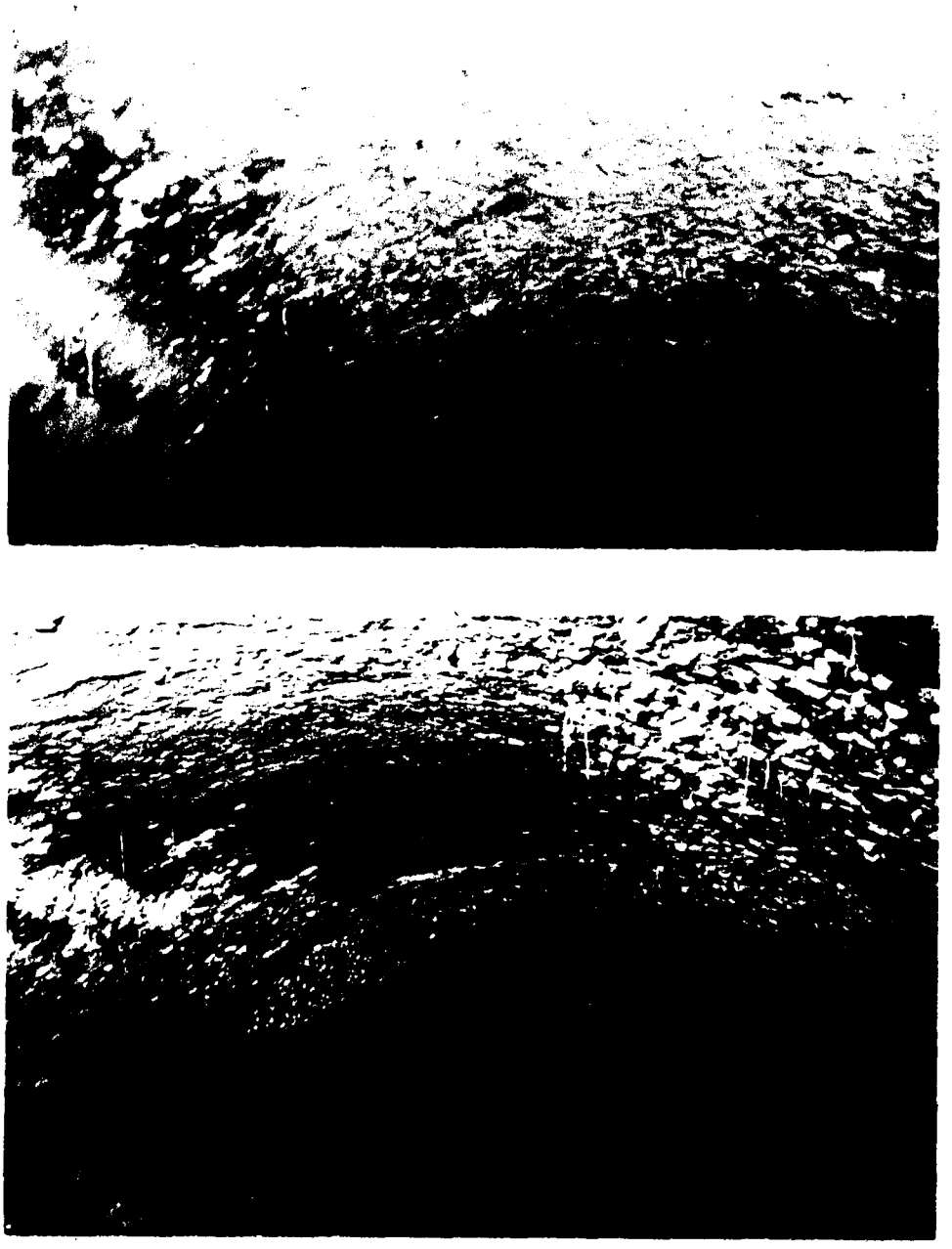


Figure 1-9. Photographs showing pipeline (looking upstream) near manhole C23 of the LACSD sewage collection system. The top photograph was taken in October, 1982 and the bottom in January, 1987

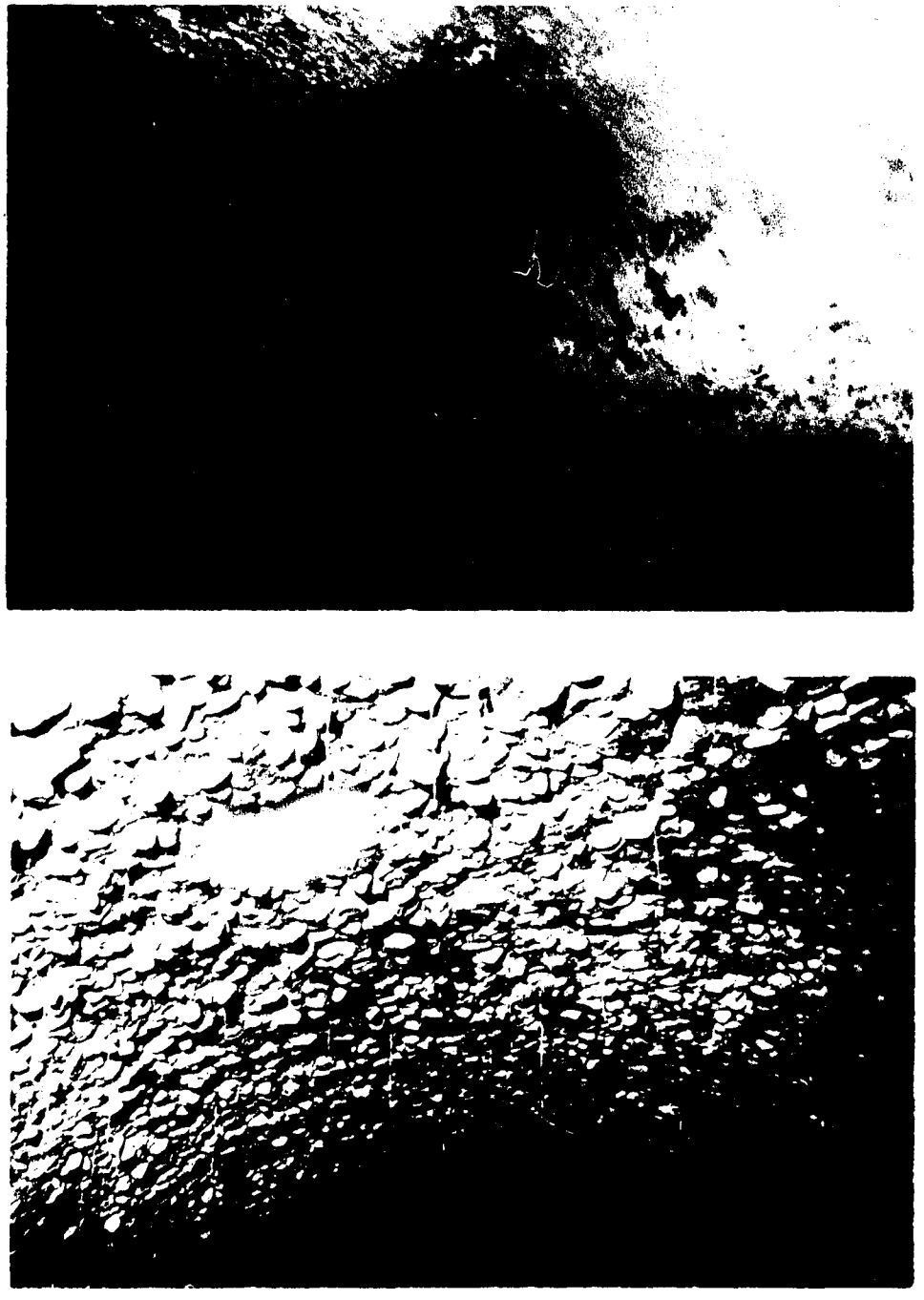


Figure 1-10. Photographs showing pipeline (looking downstream) near manhole C23 of the LACSD sewage collection system. The top photograph was taken in October, 1982 and the bottom, showing an area where a core sample has been removed for inspection and determination of corrosion depth, in January, 1987

Figure 1-11. Photographs showing pipeline near manhole A62 (top) and E30 (bottom) of the LACSD sewage collection system. The top photograph was taken in October, 1987 and the bottom in January, 1988

constructed in 1954. Figure 1-12 is a map of these trunk sewer lines within the LACSD collection system.

1.2.2 Observations/Treatments by LACSD

When the magnitude of the problem in Los Angeles County became apparent, the causes of advanced corrosion rates were investigated. Sulfide concentrations had doubled in the sewage from the late 1970's to early 1980's. This increase coincided with lower metals concentrations in the sewage stream, largely a result of implementing the United States Environmental Protection Agency's (EPA) industrial waste pretreatment regulations mandated by the Clean Water Act of 1974 (Redner, 1988). There has been speculation that lower metal concentrations in sewage permit greater growth and activity among the sulfate-reducers, resulting in enhanced production of S(-II). Additionally, in the presence of lower concentrations of metal ions, which form metal-sulfide precipitates, more H_2S is free to enter the sewer air space (LACSD, 1987). H_2S concentrations at the crown are higher, and subsequent acid generation becomes more rapid.

Attempts by LACSD to impede crown corrosion have included measures designed to (i) inhibit SO_4^{-2}-reducing bacteria, (ii) oxidize aqueous S(-II) prior to its diffusion to the sewer head space, and (iii) mechanically remove acid producing bacteria from the sewer crown surface. These processes are described in detail later in this section. In-house research by LACSD includes use of a H_2S chamber to empirically determine

research by LACSD includes use of a H_2S chamber to empirically determine rates of corrosion as a function of such factors as pH, H_2S concentration and pipe material. They have also operated pilot anaerobic digestors in order to anticipate effects which various corrosion-inhibition additives would have on downstream treatment processes. Chemical additives have generally taken the form of strong oxidants for liquid-phase S(-II) oxidation or non-toxic metals, predominately Fe(II), for the precipitation of S(-II).

Specifically, LACSD has attempted to mitigate their problem by four methods: (i) raising the crown pH above 13 by slug dosing with sodium hydroxide (NaOH); (ii) adding peroxide to the bulk liquid phase; peroxide was to serve as both a bactericide for sulfate reducers and a strong oxidant for liquid-phase sulfides; (iii) aerating sewage to suppress growth of SO_4^{-2}-reducers and chemically oxidize aqueous S(-II); and (iv) adding ferrous chloride ($FeCl_2$) to the bulk-liquid. Ferrous chloride promotes the formation of an insoluble iron-sulfide complex. Results suggest that ferrous chloride addition provides an effective, though expensive means for controlling the flux of H_2S to the sewer crown (Redner, 1988; Jin, 1987). Other strategies have proven expensive and only marginally successful. Optimal treatment required a combination of liquid-phase aeration and upstream caustic addition.

USEPA recommends similar treatments for sewage collection systems which show evidence of excessive sulfide corrosion. These consist of air/oxygen injection and chemical addition, including the addition of an

alternative electron acceptor (to SO_4^{-2}) for bacterial respiration. Air/oxygen injection to increase the dissolved oxygen concentration above 1.0 mg/L should rapidly oxidize S(-II) in the wastewater stream to thiosulfate (EPA, 1985). Effective chemical additives include chlorine, hydrogen peroxide, nitrate and metal salts. Chlorine and hydrogen peroxide oxidize aqueous S(-II) to prevent the release of H_2S into the sewer headspace. Metal salts form insoluble metal-sulfide complexes as previously described. Adding nitrate ions (NO_3^-) to the sewer stream offers a more energetic (and thus preferable) electron acceptor for some anaerobic bacteria. Sulfate reduction is usually absent under denitrifying (bacterial catalyzed NO_3^- reduction) conditions.

LACSD has also funded several academic institutions to investigate aspects of the sulfide corrosion problem. The California Institute of Technology will test a catalyst of S(-II) oxidation, cobalt tetrasulfophthalocyanine (CoTSP). This compound is capable of catalyzing the oxidation of ten thousand molecules of sulfide per one molecule of CoTSP each minute. The kinetics of the reaction are important due to the volatility of H_2S once it is formed. If CoTSP can oxidize aqueous S(-II) prior to volatilization, the gaseous H_2S concentration in the sewer atmosphere could be held below the concentrations required for generation of cell maintenance energy among acid-producing bacteria (LACSD, 1987).

The University of California-Los Angeles will investigate methods for inhibiting anaerobic sulfate-reducing bacteria without disrupting microflora which are responsible for biological waste treatment (Redner,

1988).

University of Southern California (USC) researchers will develop a corrosion-monitoring device that will enable LACSD employees to quantitatively measure corrosion rates in the field. USC will also develop a mechanical system for periodically flushing the sewer crown in order to maintain the crown pH at an acceptable level (Redner, 1988).

A portion of the work conducted at the University of Arizona is the subject of this report. Our primary objective is to develop fundamental data and information relative to the acid-producing bacteria in LACSD sewer crowns. Details are provided later in this chapter.

1.3 Other Related Research

The first investigations of H_2S-driven corrosion were reported by Parker (1945). This work included identification of acid-producing bacteria from corroded concrete (exclusively from the genus *Thiobacillus*) and development of the corrosion mechanism. In 1951, Parker summarized studies relating to the presence of S(-II) in sewage, transport of H_2S across the interface between liquid and sewer headspace, acid-production at the sewer crown, and chemistry of acid-catalyzed concrete corrosion.

Kempner (1966) studied acid production by *Thiobacillus thiooxidans* in liquid media with elemental sulfur (S^o) as the electron donor. He concluded that the species was capable of lowering the bulk-phase pH to 0.52, not to negative values as previously reported. Reported values less

than 0.5 were probably due to evaporation of liquid media and concentration of acid, not to microbial metabolism.

Numerous studies conducted in Hamburg, West Germany, were motivated by the extent of crown corrosion experienced in that City's sewer system. Milde (1983) isolated and enumerated thiobacilli from the crown and bulk-liquid phase of many sewers. *T. thiooxidans* was responsible for the bulk of the acid production in the sewer crowns. Severely corroded sewers contained a dense population of these microbes living in an acid environment (crown pH approximately 2.0). Milde also discovered that the density of *T. thiooxidans* would decrease and the crown surface pH would increase (from 2.0 to 6.0) upon oxygenation of the bulk-liquid phase. Accumulation of S° on sewer crowns led these investigators to conclude it was the energy source for thiobacilli living on the sewer crown, as opposed to H_2S, $S_2O_3^{-2}$ or organic polysulfides. The oxidation of H_2S to S° was speculated to be an abiotic process.

Pohl (1984) studied the microbial degradation of methionine, a sulfur-containing amino acid, to volatile organic polysulfides. Previous work had shown that unidentified species of thiobacilli were capable of growth on such compounds (Sivelä, 1975). Pohl found that relatively high concentrations of protein in sewage increased the availability of organic sulfides to the acid-producing bacteria responsible for crown corrosion.

Results of Sand (1987) contradicted those by Pohl (1984). H_2S was considered the primary source of electrons for the acidophilic thiobacilli in the sewer environment, and thiosulfate ($S_2O_3^{-2}$) was the primary

substrate for non-acidophilic thiobacilli. Mercaptan and other organic polysulfides did not support growth of the thiobacilli tested. Sand's work was conducted in a controlled breeding chamber into which substrate was added. Corrosion of concrete pipe core samples in the chambers could be accelerated eight-fold over corrosion rates observed in the sewer (Sand, 1984 and 1987). The presence of S^{o} on concrete pipe core samples placed in the breeding chamber was also observed in this study.

LACSD has undertaken studies similar to those conducted in Hamburg by developing a thiobacilli/H_2S breeding chamber to simulate concrete corrosion. They have undertaken tests involving different pipe materials and/or epoxy coatings and variation in gaseous H_2S concentration. Results are preliminary (LACSD, 1988).

It is possible to inhibit thiobacillus metabolism. Jack (1980) found that 1.96×10^{-4} M vanadium as vanadate (V), 5.2×10^{-4} M molybdenum (VI) and 1.9×10^{-4} M chromium (VI) inhibited growth of *T. thiooxidans*. Growth (via direct count) was used as a indication of toxicity. LACSD (1988) measured the average concentrations od various metals found in sewage before and after implementation of the industrial waste pretreatment program. Table 1-1 summarizes the average annual concentrations of metals measured in 1971 and 1988. All metals measured were reduced in quantity after pretreatment measures were enforced with the exception of silver.

Table 1-1. Molar concentrations of certain metals in the LACSD sewage collection system (LACSD, 1988)

	1971	1988	Percent Reduction
Cadmium	2.2×10^{-7} M	9.3×10^{-8} M	58
Chromium (total)	1.5×10^{-5} M	3.4×10^{-6} M	77
Copper	7.1×10^{-6} M	2.8×10^{-6} M	61
Mercury	1.1×10^{-8} M	5.0×10^{-9} M	55
Silver	1.3×10^{-7} M	1.9×10^{-7} M	(46)[1]
Zinc	3.0×10^{-5} M	9.2×10^{-6} M	69

[1] Denotes increase of metal concentration

1.4 Thiobacilli and Other Sulfide-Oxidizing Bacteria

1.4.1 General Growth Mechanisms of Thiobacilli

Chemolithotrophic bacteria use reduced inorganic compounds for energy. All thiobacilli are chemolithotrophs; they generally derive energy by oxidizing reduced or partially reduced inorganic sulfur compounds. Sulfides, elemental sulfur, thiosulfate, polythionates and sulfite are oxidized biochemically to produce a common end product -- sulfate. This process is accompanied by acid generation.

The genus includes strict autotrophs (those which assimilate carbon from carbon dioxide exclusively), facultative autotrophs (carbon assimilation from either carbon dioxide or organic carbon compounds) and a single heterotrophic species which requires both reduced sulfur and organic matter for growth (Vishniac, 1974). Carbon dioxide assimilation (or CO_2 fixation) is accomplished at the expense of stored cellular energy and reducing power. The general biochemistry of autotrophic growth and chemolithotrophic energy production using reduced sulfur compounds is summarized in subsequent sections.

1.4.2 Bacterial Respiration and Oxidative Phosphorylation

Bacterial respiration is performed via a system of membrane-bound proteins, collectively called the electron transport chain, which facilitate the transfer of electrons to a terminal electron acceptor (either O_2 or an alternate compound). The electron acceptor during aerobic

respiration is molecular oxygen (O_2). Energy stored during electron transport is converted to adenosine-5′-triphosphate (ATP) by oxidative phosphorylation (Gottschalk, 1986). The mechanism by which electron transport is coupled to oxidative phosphorylation (chemosmotic hypothesis) was first proposed by Mitchell (1961). The passage of electrons along the transport chain is thought to drive the translocation of protons from the cell's cytoplasm to the periplasmic space or cell surroundings. The process establishes an electrochemical proton-motive force which is comprised of two components, (i) a gradient in proton concentration and (ii) a difference in electric potential, or charge gradient across the cytoplasmic membrane. Retranslocated protons drive membrane-associated functions, including phosphorylation of adenosine-5′-diphosphate (ADP) to produce ATP (Hinkle, 1978).

The electron transport chain of *Escherichia coli* shown in Figure 1-13 illustrates the mechanism of ATP synthesis by oxidative phosphorylation. The proton-motive force drives hydrogen ions through a protein complex known as ATPase. Although in the figure one molecule of ADP is phosphorylated to ATP for every two protons passing through ATPase, process stoichiometry is thought to be species-dependent (Gottschalk, 1986).

1.4.3 Carbon Dioxide Fixation

During autotrophic growth, CO_2 is converted to organic material via a series of enzymatic reactions known collectively as the Calvin cycle. CO_2

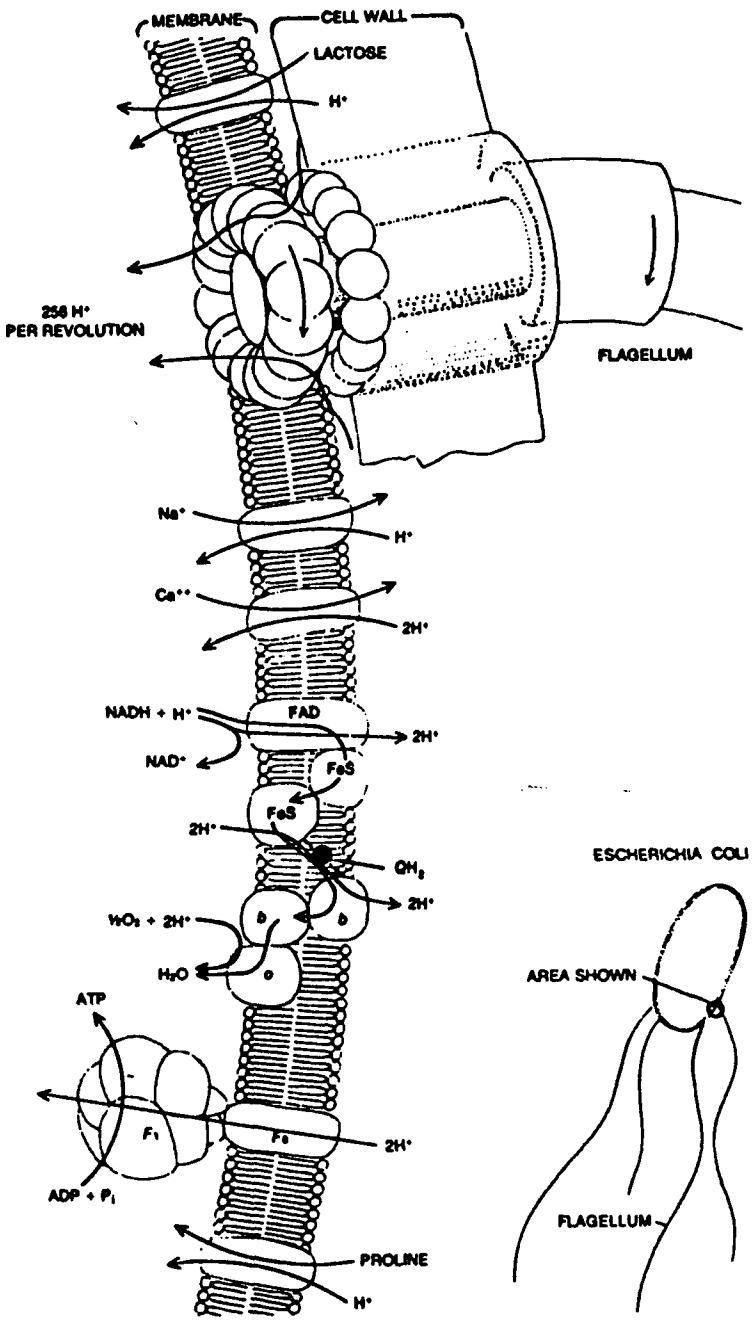


Figure 1-13. The electron transport chain and phosphorylation of ADP to ATP in E. coli (Lin, 1984).

fixation demands energy (ATP) and reducing power (NADH) produced by bacterial catabolism. Figure 1-14 summarizes the Calvin cycle reactions. Overall, 18 moles of ATP and 12 moles of NADH are required to convert 6 moles of CO_2 to one mole of glucose. Calvin cycle reactions account for at least 80% of total biosynthetic energy demand among chemolitho-autotrophs (Dawes, 1986). The primary end product of the Calvin cycle reactions is the organic compound 3-phosphoglycerate (Gottschalk, 1986).

Two enzymes, phosphoribulokinase and ribulose-1,5-biphosphate carboxylase (RuBPase) are unique to the Calvin cycle. The remaining enzymes perform related functions in other biochemical processes. Chemicals which selectively inhibit these unique Calvin cycle enzymes (and the accompanying acid production by thiobacilli) without disrupting other cellular functions, might provide a means for selectively inhibiting microbially-induced acid production in the sewer environment.

Shively and others (1973) demonstrated that in *T. neapolitanus*, RuBPase is localized in organelles called carboxysomes. The function of these inclusions is not completely clear since the presence of other Calvin cycle enzymes in carboxysomes is a matter of some debate.

1.4.4 Microbial Catalysis of Sulfur Oxidation

The biochemistry of dissimilative sulfur oxidation is incompletely understood. A recently proposed pathway of S(-II), S°, or thiosulfate oxidation is illustrated in Figure 1-15. Sulfide is oxidized to polysulfide in a reaction that requires glutathione (see Step 1 in Figure

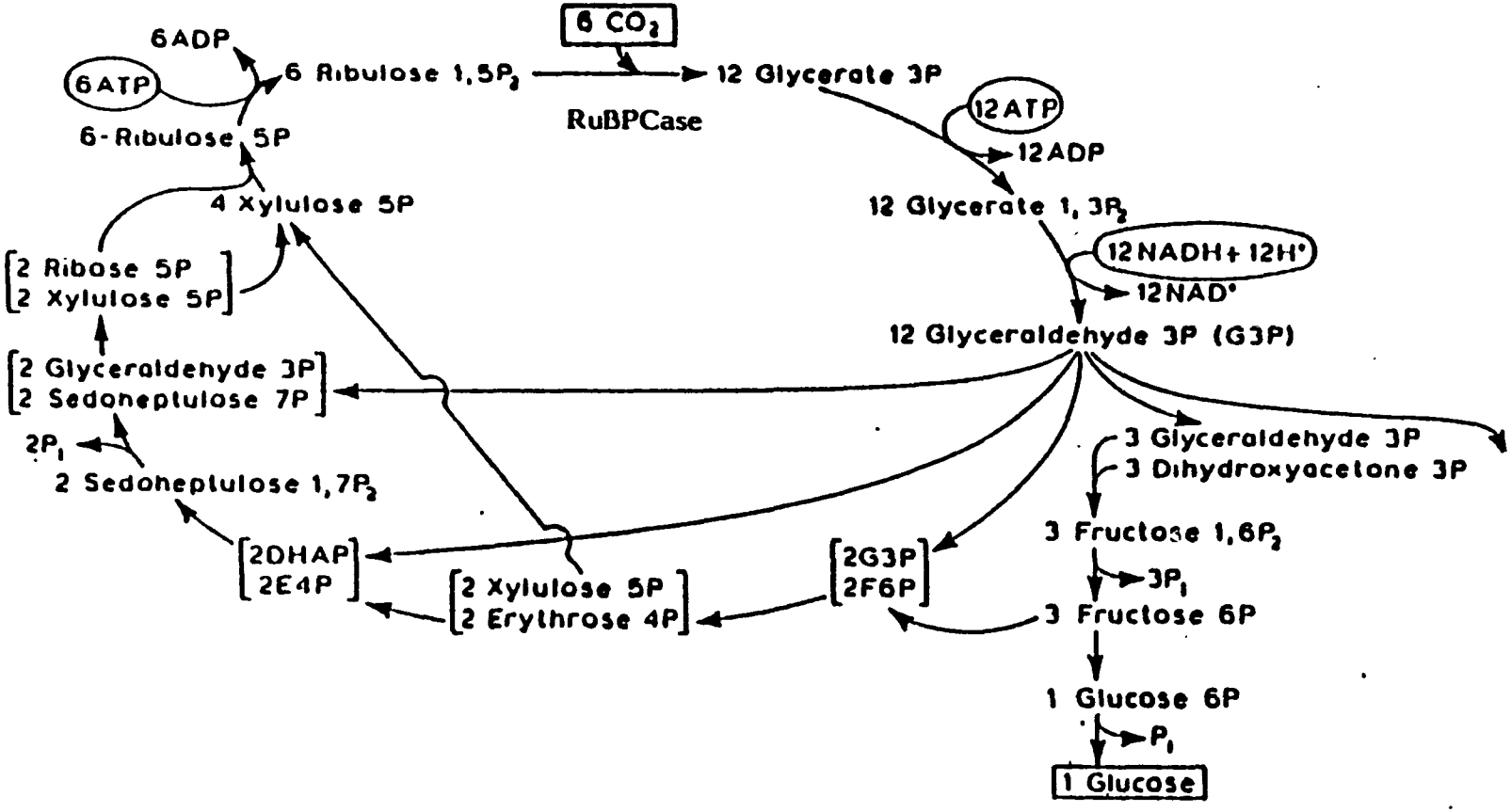


Figure 1-14. The reductive pentose phosphate or calvin cycle for the conversion of CO_2 into glucose. For each turn of the cycle, one mole of hexose is synthesized from 6 CO_2 at the expense of 18 ATP and 12 NADH, this energy being derived from the oxidation of inorganic substrate for chemolithotrophs.

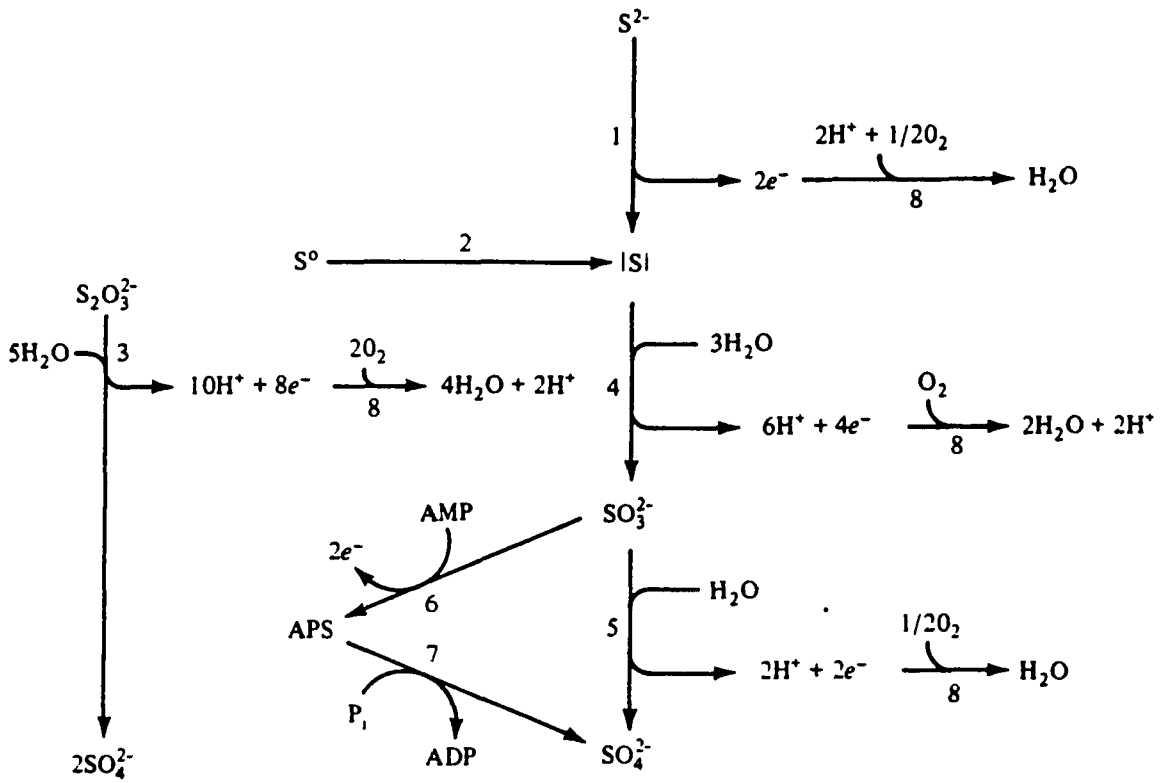


Figure 1-15. Routes of the oxidation of sulfur compounds in chemolithotrophs. 1, Oxidation of sulfide to polysulfide sulfur [S]; 2, conversion of elemental sulfur to polysulfide sulfur; 3, thiosulfate-oxidizing multi-enzyme complex; 4, sulfur oxidase; 5, sulfite oxidase; 6, APS reductase; 7, ADP-sulfurylase; 8, electrons are transferred to oxygen via components of the respiratory chain (Gottschalk, 1986).

1-15). S° is also oxidized in this manner. All electrons released from the oxidation are transferred to O_2 via respiration. The system for thiosulfate oxidation yields an end product of two sulfate molecules formed for every thiosulfate molecule oxidized (Gottschalk, 1986).

Figure 1-16 illustrates the mechanism of electron transport from reduced sulfur compounds in thiobacilli (Kelly, 1982). ATP is generated from the transfer of electrons and proton-motive force. Reducing power as nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) is produced by reversing the direction of electron transport. Reductant is required by the Calvin cycle to reduce CO_2 for cellular biosynthesis. The formation of reducing power is ATP-dependent.

1.4.5 Taxonomy of Thiobacilli

Thiobacilli are small, rod-shaped, motile, gram-negative cells which can derive energy from the oxidation of one or more reduced sulfur compounds. Eight (8) species were identified in Bergey's Manual of Determinative Bacteriology (Vishniac, 1974). Figure 1-17 illustrates the basis of taxonomic classification among the thiobacilli to the species level.

Strictly autotrophic, acidophilic species include *T. thiooxidans* and *T. ferrooxidans*; each initiates growth at pH below 4.5. Reduced sulfur compounds H_2S, S° and $S_2O_3^{-2}$ can support their growth. *T. ferrooxidans* can also oxidize reduced iron [Fe(II)] for generation of energy.

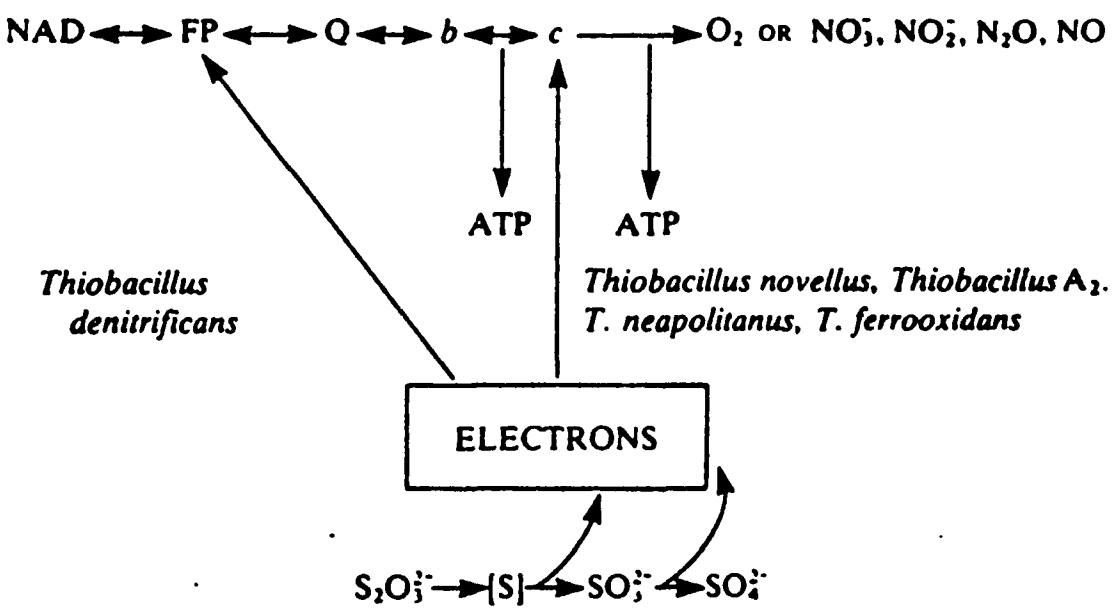


Figure 1-16. Principles of electron transport and phosphorylation in thiobacilli (Kelly, 1982).

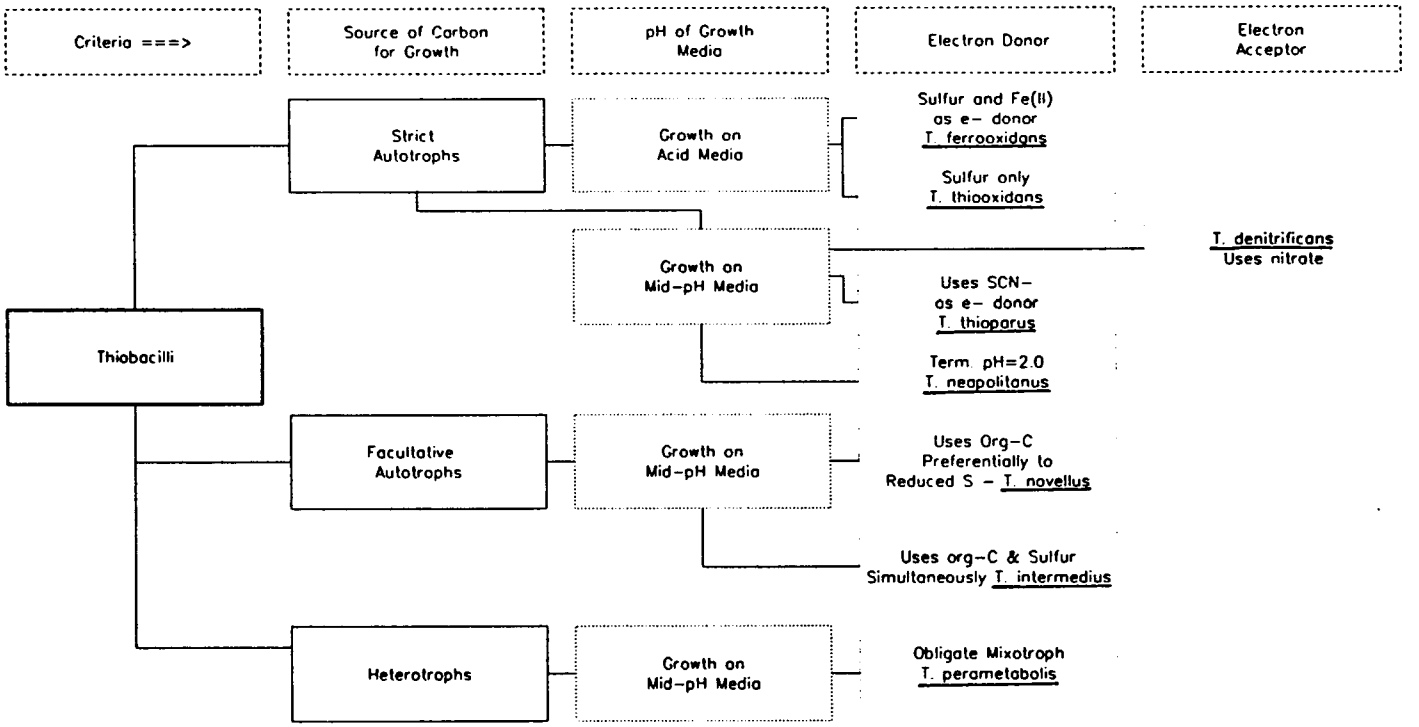


Figure 1-17. Taxonomy of the thiobacilli and unique characteristics of each species.

Non-acidophilic, strictly autotrophic thiobacilli include *T. neapolitanus* and *T. thioparus*. The latter is capable of using thiocyanate (SCN^-) as the electron donor. Both initiate growth at neutral pH and oxidize most of the reduced inorganic sulfur compounds. Metabolic acid-production ceases at pH 4.5 for *T. thioparus* and 3.0 for *T. neapolitanus*.

Another strict autotroph, *T. denitrificans*, can grow anaerobically by utilizing nitrate as the terminal electron acceptor. H_2S and S° are slowly oxidized by this species as compared to the aerobic species (Hutchinson, 1969).

Both facultative autotrophs, *T. intermedius* and *T. novellus* initiate growth at neutral pH values. The two species differ in their ability to metabolize different growth substrates. *T. intermedius* simultaneously oxidizes reduced sulfur compounds and organic nutrients. Oxidation of reduced sulfur is repressed by the presence of organic substrate when cultivating *T. novellus*. Metabolic function ceases in *T. novellus* when pH decreases to 5.0; *T. intermedius* is capable of growth in environments with pH below 2.0 (Vishniac, 1974).

The heterotrophic species, *T. perametabolis* is capable of very slow growth using either specific organic compounds or reduced sulfur. Growth is enhanced by the simultaneous presence of both substrate, suggesting the species is an obligate mixotroph. *T. perametabolis* initiates growth at neutral pH values and is viable at pH's below 3.0 (Vishniac, 1974).

Thiobacilli cultivated on solid media with $S_2O_3^{-2}$ show various colony characteristics. Of the acidophilic species, *T. thiooxidans* forms minute, transparent colonies and *T. ferrooxidans* produces colonies with irregular edges and white centers. The two non-acidophilic, strict autotrophs, *T. thioparus* and *T. neapolitanus*, yield whitish-yellow colonies. Colonies from *T. neapolitanus* changes colors with age; from pink to brown. *T. novellus produces a colorless colony which eventually forms sulfur precipitates. In contrast, the other facultative autotroph, T. intermedius* yields a yellow-opaque colony with veil-like edges (Vishniac, 1974).

1.4.6 Other Sulfur-Oxidizing Bacteria

Sulfur-oxidizing bacteria not belonging to the genus *Thiobacillus* range from strict chemolithoautotrophs to heterotrophs. Table 1-2 summarizes the different organisms which are capable of sulfur oxidation, excluding thiobacilli. These species usually live in a narrow environment in which H_2S and molecular oxygen are simultaneously present.

Sulfolobus species are spherical with lobes. They are non-motile, gram-negative, facultative autotrophs which use elemental sulfur as an energy source. The genus is unique in its ability to grow at temperatures between 55 and 85° C. Thus, these species thrives in hot, acidic solfatara (pH ranging from 2 to 3) environments (Brock, 1974).

Other genera of sulfur-oxidizing bacteria accumulate sulfur granules and do not produce acid. Many of these are found near pond and stream

Table 1-2. Microorganisms capable of oxidizing inorganic sulfur compounds (excluding *Thiobacilli*) (Kuenen, 1982)

Classification via Substrate Preference	Subclassification via Respiration Mechanisms Used	Genus or Species
Obligate Chemolithotrophs	Aerobes	*Tms. pelophia*[1]
	Facultative Anaerobes	*Tms. denitrificans*
Facultative Chemolithotrophs	Aerobes	*Sulfologus acidocadarius* *Sulfolobus brierleyi*
	Facultative Anaerobes	*Thermothrix thiopara* *Paracoccus denitrificans*
Chemolithoheterotrophs		*Pseudomonas sp.*
Heterotrophs		*Beggiatoa spp.* *Pseudomonas spp.*
Unclassified		*Thiovulum* *Thiophysa* *Thiothrix* *Thiospira* *Thioploca*

(1) *Tms.* is an abbreviation for *Thiomicrospira*

surfaces which contain H_2S. *Beggiotoa* spp. oxidize H_2S to S^o, which accumulates in the cell (Kuenen, 1982). The process is used as a detoxification mechanism as opposed to an energy source.

1.5 Chemical Inhibition of Bacterial Activity

1.5.1 Introduction

A variety of reagents are known to interfere with bacterial respiration at specific points along the electron transport chain or with oxidative phosphorylation. Consequently, model inhibitors are useful tools with which to study the composition of the electron transport chain or respiration kinetics. In such studies, dissolved oxygen utilization is a useful measure of overall electron transport kinetics.

1.5.2 Cyanide (CN^-)

Cyanide blocks electron transport by binding to membrane bound cytochrome oxidase (see Figure 1-13), interfering with the transfer of the electrons to O_2. Inhibition by CN^- results in a decrease or arrest of O_2 utilization and likewise, the rate of ATP formation. Biosynthetic activity becomes impossible in obligate aerobes upon exposure to CN^- (Gottschalk, 1986).

1.5.3 Dicyclohexylcarbodiimide (DCCD)

DCCD interferes with the function of the membrane bound ATPase by preventing oxidative phosphorylation. DCCD blocks the proton translocating channel (F^{o} subunit) of the ATPase enzyme (Haddock, 1977).

Related physiological effects include (i) acute inhibition of electron transport due to the build-up of proton-motive force and (ii) the gradual decrease in oxygen utilization (Arnold, 1988).

1.5.4 Metals

The presence of specific transition metals were shown to have inhibitory effects in batch cultures of thiobacilli (Jack, 1980). Additionally, it may be significant that acid-induced corrosion rates increased in LACSD sewers following the implementation of EPA's industrial waste pretreatment programs. Per Table 1-1, pretreatment measures generally reduced the concentration of specific metals in sewage, some of which are potentially toxic to thiobacilli.

1.6 Objectives

1.6.1 Isolation Experiments

Sample collection and isolation of thiobacilli from LACSD sewer crowns preceded all other laboratory work. Isolates were subjected to growth and biochemical tests which led toward their identification at the species level.

Isolation/identification procedures generally followed those developed during research conducted in Hamburg, W.G. (Milde, 1983). Comparison of growth characteristics exhibited by sewer-acclimated strains of thiobacilli were an essential part of the research.

1.6.2 Stoichiometry Experiments

Bench-scale tests to determine the kinetic and stoichiometric relationships between microbial O_2 and H_2S usage rates were undertaken to study substrate preference among the strains isolated from the sewer. Experiments were also conducted to determine yield coefficients associated with bacterial growth and acid production among the isolates grown on H_2S. Tests results formed the basis of comparison among strains isolated from pipe sections showing various degrees of corrosion.

1.6.3 Acute Inhibition Experiments

Oxygen utilization tests were modified to determine effects of the chemical inhibitors, cyanide and DCCD. Inhibition studies led to speculation relative to the role H_2S has in metabolism and energy generation among sewer isolates.

1.6.4 Chronic Metals Inhibition

The object of this portion of the work was to screen a variety of metals, many which were or are present in LACSD wastewaters, as inhibitors of thiobacillus metabolism and acid production.

Results of metal-inhibition tests were used to (i) compare metals tolerances among sewer-acclimated strains and (ii) speculate relative to the importance of metals as inhibitors microbial sulfide oxidation prior to the implementation of industrial waste source-control measures in Los Angeles County.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation of Thiobacilli from the LACSD Sewer Crowns

2.1.1 General

Bacteria were collected from the LACSD sewer crowns on two separate occasions - in November, 1987, and in March, 1988. The second sample set was taken after thiobacilli failed to grow in the isolation media used initially.

Samples were taken from locations within the LACSD collection system which were selected to represent conditions ranging from uncorroded to highly corroded pipe sections. Test tubes containing growth media were inoculated using techniques adopted from similar work in Hamburg, W.G. and modified for this project. The November, 1987 media were prepared per the description of others and contained several essential metals (Milde, 1983). In March, 1988, selective media were considerably simpler and less redundant.

On each occasion (November and March), pH was measured on exposed pipe surfaces. In March, samples of solid material were withdrawn from the sewer crowns and prepared for inspection via scanning electron microscopy (SEM). Isolation of bacterial strains from physical samples involved liquid-culture enrichment followed by colony development on selective solid media.

2.1.2 Design of Selective Media for the Isolation of Thiobacilli

November 1987 Samples. Selective media were designed to satisfy the metabolic requirements of specific thiobacilli or groups of thiobacilli. Media compositions are summarized in Table 2-1. Species capable of growth in each medium are also listed in the table. A medium used by Milde (1983) to support growth of *T. denitrificans* was not utilized because that species was not found to be a major contributor to acid production and crown corrosion in Hamburg sewers.

March 1988 Samples. The complexity of the media compositions and inability of sewer thiobacilli to grow cultures subjected to the November, 1987 media led to the simplification of selective media used in the March, 1988 sampling program. The number of different media was reduced from seven to four. One or more of these four media provided nutrients for all thiobacilli described by Vishniac (1974) except *T. denitrificans* which was found to be non-existent in the Hamburg, W.G. studies. Table 2-2 includes a description of each medium with a list of thiobacilli which should be capable of growth based on specific taxonomic characteristics listed in current literature.

All media except Medium #1 were sterilized by autoclaving for 15-20 minutes at 121° C. Medium #1, which contained elemental sulfur, was sterilized via tyndallization (Stanier, 1980). Iron was added from a filter-sterilized stock solution after heat-sterilization of other ingredients was completed.

Table 2-1. Selective Media Used for the November 1987 Sewer Crown Samples (Milde, 1983)

	Quantity per Liter, grams (1)	Selected Thiobacilli
Medium A		
$Na_2S_2O_3 \cdot 5H_2O$	10.0	*T. neapolitanus*
KH_2PO_4	4.0	*T. thioparus*
K_2HPO_4	4.0	
$MgSO_4 \cdot 7H_2O$	0.8	
NH_2Cl	0.4	
Trace Metal Solution	10.0 ml added to 1 liter of medium	
EDTA	50.0	
$ZnSO_4 \cdot 7H_2O$	22.0	
$CaCl_2$	5.5	
$MnCl_2 \cdot 4H_2O$	5.1	
$FeSO_4 \cdot 7H_2O$	5.0	
$(NH_4)_6Mo_7O_{24}$	1.1	
$CuSO_4 \cdot 5H_2O$	1.6	
$CoCl_2 \cdot 6H_2O$	1.6	
Distilled water	1.0 L	
Adjust trace metal solution pH to 6.0 w/KOH		
Adjust liquid medium pH to 6.6 w/NaOH		
Medium B		
$Na_2S_2O_3 \cdot 5H_2O$	10.0	*T. thiooxidans*
Na_2PO_4	1.2	*T. ferrooxidans*
KH_2PO_4	3.8	
$MgSO_4$	0.1	
$(NH_4)_2SO_4$	0.1	
$CaCl_2$	0.03	
$FeCl_3 \cdot 6H_2O$	0.02	
$MnSO_2 \cdot 4H_2O$	0.02	
NaCl	1.0	
Adjust medium pH w/HCl or NaOH to 4.5		

	Quantity per Liter, grams(1)	Selected Thiobacilli
Medium C		
$Na_2S_2O_3 \cdot 5H_2O$	5.0	*T. intermedius*
$CaCl_2$	0.1	
Biotin	10^{-4} M	
NH_4Cl	1.0	
$MgSO_4$	0.5	
KH_2PO_4	0.4	
$K_2HPO_4 \cdot 3H_2O$	0.6	
$FeCl_3 \cdot 6H_2O$	0.02	
Pfennig's Trace Salts	3.0 ml:	
Titraplex III(EDTA)	75.0 mg	
$FeSO_4 \cdot 7H_2O$	10.0 mg	
$ZnSO_4 \cdot 7H_2O$	5.0 mg	
$MnCl_2 \cdot 7H_2O$	1.0 mg	
Vitamin B_{12}	30.0 ug	
KH_2PO_4	1.0	
$(NH_4)_2SO_4$	1.0	
$MgSO_4 \cdot 7H_2O$	1.0	
KCl	0.5 mg	
$CaCO_3$	0.15 mg	
$CaSO_4 \cdot 2H_2O$	2.0	
$NaHCO_3$	4.5	
$Na_2S \cdot 9H_2O$	2.25	
Distilled Water	3.0 L	
Adjust to pH = 6.6-6.8 if required		
Medium D		
NH_4CNS	0.02	*T. thioparus*
$Na_2S_2O_3 \cdot 5H_2O$	10.0(2)	*T. ferrooxidans*
Na_2PO_4	1.2	
KH_2PO_4	3.8	
$MgSO_4$	0.1	
$(NH_4)_2SO_4$	0.1	
$CaCl_2$	0.03	
$FeCl_3 \cdot 6H_2O$	0.02	
$MnSO_2 \cdot 4H_2O$	0.02	
Adjust pH to 4.5		

	Quantity per Liter, grams(1)	Selected Thiobacilli
Medium F		
Yeast Extract	1.0	*T. novellus*
$Na_2S_2O_3 \cdot 5H_2O$	10.0	*T. intermedius*
KH_2PO_4	4.0	
K_2HPO_4	4.0	
$MgSO_4 \cdot 7H_2O$	0.8	
NH_2Cl	0.4	
Trace Metal Solution	10.0 ml	
EDTA	50.0	
$ZnSO_4 \cdot 7H_2O$	22.0	
$CaCl_2$	5.5	
$MnCl_2 \cdot 4H_2O$	5.1	
$FeSO_4 \cdot 7H_2O$	5.0	
$(NH_4)_6Mo_7O_{24}$	1.1	
$CuSO_4 \cdot 5H_2O$	1.6	
$CoCl_2 \cdot 6H_2O$	1.6	
Distilled water	1.0 L	
Adjust trace metal solution pH to 6.0 w/KOH		
Adjust liquid medium pH to 6.6 w/KOH		
Medium G		
$Na_2S \cdot 9H_2O$	0.2	*T. thiooxidans*
Na_2HPO_4	1.2	*T. ferrooxidans*
KH_2PO_4	3.8	
$MgSO_4$	0.1	
$(NH_4)_2SO_4$	0.1	
$CaCl_2$	0.03	
$FeCl_3 \cdot 6H_2O$	0.02	
$MnSO_2 \cdot 4H_2O$	0.02	
Adjust pH to 4.5		

Media H

	Quantity per Liter, grams(1)	Selected Thiobacilli
$Na_2S_2O_3 \cdot 5H_2O$	10.0	*T. neapolitanus*
Na_2HPO_4	1.2	*T. thioparus*
KH_2PO_4	1.8	
$MgSO_4$	0.1	
$(NH_4)_2SO_4$	0.1	
$CaCl_2$	0.03	
$FeCl_3 \cdot 6H_2O$	0.02	
$MnSO_4 \cdot 4H_2O$	0.02	
Adjust pH to 6.8		

Notes: (1) All quantities are in grams unless otherwise noted.

(2) This salt was inadvertently included into the media. Thiocyanate was supposed to be the only source of reduced sulfur.

Table 2-2. Media Used for the March 1988 Samples

	Quantity per Liter, grams	Thiobacilli Capable of Growth
Medium 1		
$(NH_4)_2SO_4$	0.2	*T. thiooxidans*
$MgSO_4 \cdot 7H_2O$	0.5	*T. ferrooxidans*
$CaCl_2$	0.25	
KH_2PO_4	3.0	
$FeCl_3$	0.02	
Elemental Sulfur	10.0	
Distilled Water	1.0 L	
Adjust pH to 3.5		
Medium 2		
Na_2HPO_4	1.2	*T. neapolitanus*
KH_2PO_4	1.8	*T. thioparus*
$MgSO_4 \cdot 7H_2O$	0.1	
$(NH_4)_2SO_4$	0.1	
$CaCl_2$	0.03	
$FeCl_3$	0.02	
$MnSO_4$	0.02	
$Na_2S_2O_3$	10.0	
Distilled water	1.0 L	
Adjust pH to 6.8		
Medium 3 - Low pH		
NH_4Cl	0.1	*T. thiooxidans*
KH_2PO_4	3.0	*T. ferrooxidans*
$MgCl_2$	0.1	
$CaCl_2$	0.1	
$Na_2S_2O_3 \cdot H_2O$	5.0	
Adjust pH to 4.2		

Medium 4 - Mid pH, Facultative	Quantity per Liter, grams	Thiobacilli Capable of Growth
Na_2HPO_4	1.2	*T. novellus*
KH_2PO_4	1.8	*T. intermedius*
$MgSO_4.7H_2O$	0.1	*T. perometabolis*
$(NH_4)_2SO_4$	0.1	
$CaCl_2$	0.03	
$FeCl_3$	0.02	
$MnSO_4$	0.02	
$Na_2S_2O_3$	10.0	
Yeast Extract	1.0	
Distilled water	1.0 L	
pH_o = 6.8		

Note: All quantities are in grams unless otherwise noted.

2.1.3 Sampling Methods

Safety procedures for collection of sewer cultures were those customarily followed by LACSD sewer-maintenance personnel. Sewer headspace was vented with fresh air prior to personnel entrance. Oxygen and combustible gas concentrations in the pipeline headspace were measured before personnel were lowered into the sewer on an electric winch and safety rope.

Crown samples were taken using individually wrapped, sterile cotton swabs. Swabs were handled using flame-sterilized needle-nose pliers when removing crown material. The swab was wiped across a small area of the sewer crown and placed directly into a test tube which contained selective growth media. Reasonable efforts were taken to avoid sample contamination by airborne bacteria present in the humid head space of the sewer.

The crown surface pH was measured using paper indicator sticks (American Scientific). When surface moisture of the crown concrete was inadequate for such measurements, a small volume of distilled water was sprayed onto the crown surface prior to application of the pH stick.

Biological samples were placed in an insulated container, packed with ice and transported to the laboratory. Within 24 hours of sampling, tubes were placed on a shaker table (November samples) or a tube rotator (March samples) and incubated at room temperature (23° to 25° C).

Samples of loose concrete from heavily corroded areas of the sewer crown were preserved for SEM inspection in a 5% formalin solution (equivalent to 37% formaldehyde) at pH 7.4 (Cavanaugh, 1988). Electron

micrographs were later prepared at the California Institute of Technology. Two of these micrographs are shown in Figure 2-1. Both show a large population of bacteria sized rods on the surface of mineral crystals found on the crowns. The mineral present appeared to be similar to pyrite (iron sulfide -- FeS_2).

Indications of cell growth and activity included direct counts and measurements of culture pH, respectively. A more detailed description of related methods is provided subsequently. Steps utilized for the isolation and identification of acidophilic and non-acidophilic thiobacilli are summarized in Figures 2-2 and 2-3.

2.1.4 Enrichment Cultures

Inoculated media were incubated at room temperature -- 24°C +/- 1°C - for several weeks. At approximately 7-day intervals, samples were withdrawn for inspection via light microscopy and pH measurement. After two weeks, a drop was aseptically transferred from each of the original cultures to fresh, identical media to eliminate growth which was driven by constituents present in the original sample. These "first-transfer" cultures were cultivated and analyzed in the same manner as the original cultures.

Light Microscopy and Growth Measurement. Direct counts (light microscopy) were used to monitor growth in selective media. The time dependence of species predominance (noted via such characteristics as cell morphology and motility) was also monitored in this fashion. Observations

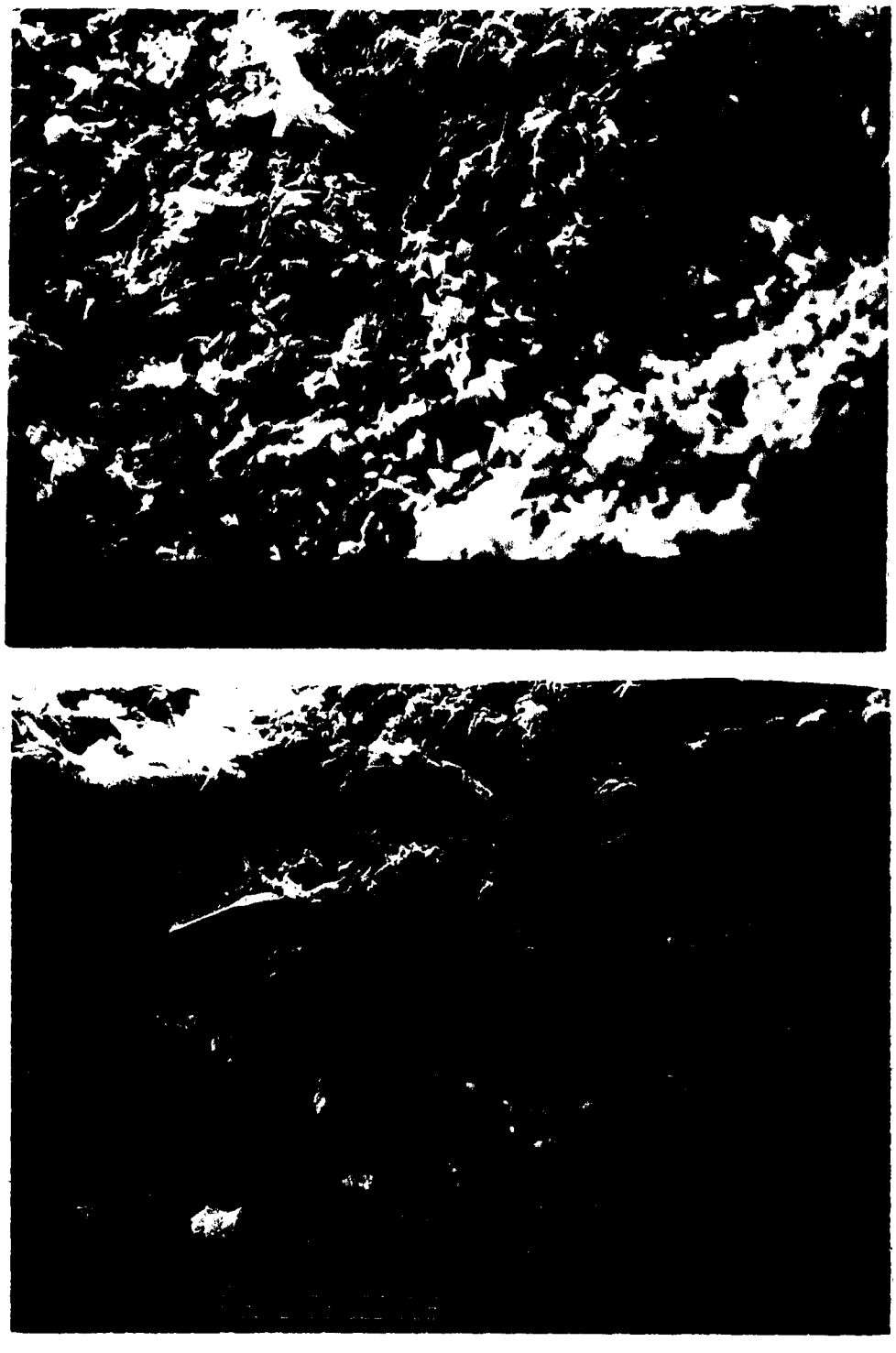


Figure 2-1. Electron micrograph of sewer particles from manholes C8 (top) and A62 (bottom) of the LACSD sewage collection system.

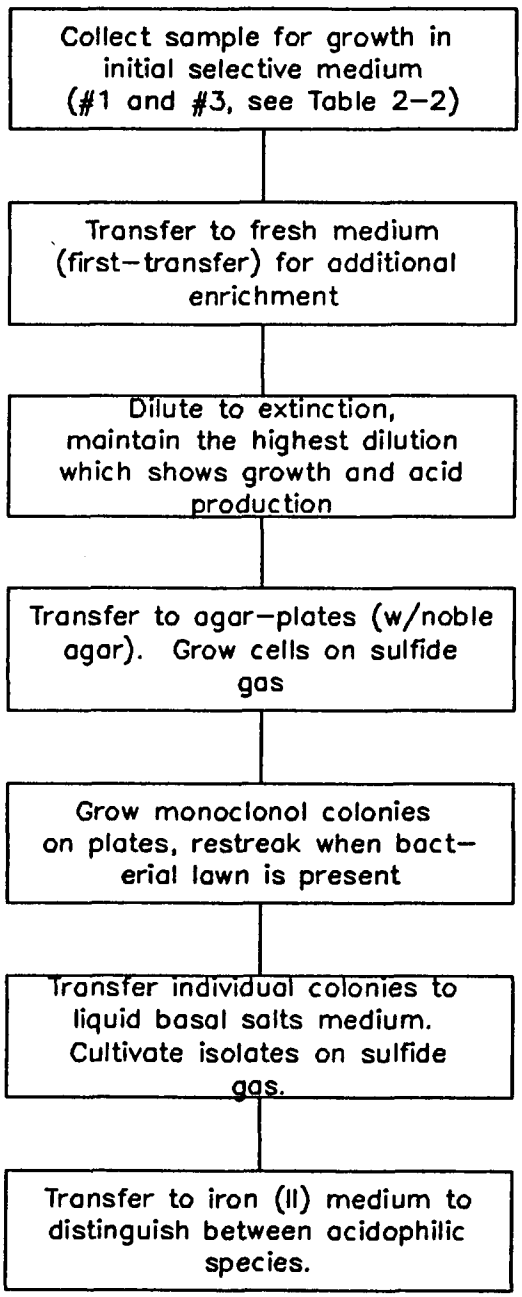


Figure 2-2. Steps used for the isolation and identification of acidophilic sewer crown samples.

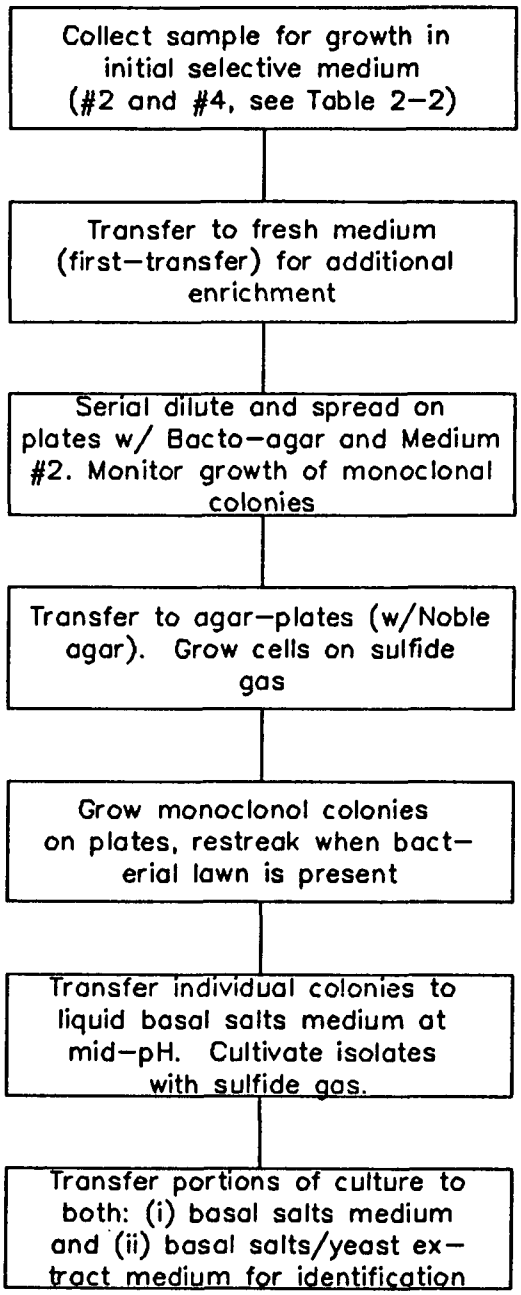


Figure 2-3. Steps used for the isolation and identification of non-acidophilic sewer crown samples.

were made with an Olympus B42 light microscope at 400X or 1000X (oil immersion) magnification.

Two cell counting methods were used throughout this research. In the first method, developed by Milner (1988), twenty microliters (20 μl) were aseptically withdrawn from the culture and placed on a microscope slide. The liquid drop was covered with a 22 x 22 mm cover slip. Liquid was assumed to coat the microscope slide to a uniform thickness, calculated at 41 μm, between the slide and cover slip. Since the area of the circular viewing field of the microscope at 400X was 0.080 mm^2, cell density could be estimated by averaging the numbers of cells in several fields and making an appropriate volume conversion. The number of cells per ml was determined by averaging cell counts from five or more fields. This quantity was multiplied by 3.05 X 10^5 field volumes/ml to determine number of cells per ml.

The second counting method employed a Petroff-Hauser counting chamber to determine cell number. This counting chamber is a framed microscope slide divided into a grid pattern etched on the glass. The dimension of squares on the grid is 0.05 mm x 0.05 mm. A cover slip placed on two raised ledges of the slide provides a uniform space, 0.02 mm thick, between the top of the chamber glass to the cover slip. A drop of sample was placed on the slide and cover slip set on the raised ledges. Excess liquid was displaced from the grid section of the slide to recessed-channels adjacent to the divided grid. The number of cells per square were determined by averaging cell counts from five or more squares.

This quantity was multiplied by 2 X 10^7 square volumes/ml to determine number of cells per ml.

Measurement of pH. Sequential pH measurements were used as an indication of general metabolic activity and acid production. Two methods of measurement, pH sticks or a pH meter/probe (Orion Research Model 881 microprocessor), were used throughout this work. The pH sticks were used to measure culture pH when concern of contamination and/or lack of liquid volume precluded other methods. The pH sticks are considered accurate within 0.2 pH units. Accuracy of the pH meter and probe were within 0.02 units.

2.1.5 Serial Dilutions

First-transfer cultures which indicated growth were used to obtain pure strains of thiobacilli via (i) dilute-to-extinction and (ii) spread-plate techniques. Dilution media were identical to the original growth media. When serial dilution immediately preceded spread-plating, reduced-sulfur and/or organic substrate were omitted.

Serial dilutions tubes were placed onto tube rotators at room temperature. Growth was determined by methods described earlier. Extinction was reached if the highest dilution tubes did not indicate growth within a few days after inoculation. Monoclonal cultures of thiobacilli were obtained from the highest dilution tube which showed growth via standard spread-plate techniques.

Solid media were generally prepared by fortifying appropriate selective media with agar prior to sterilization. For low pH media, pH was adjusted after sterilization and before solidification. The process was structured in this manner to avoid acid hydrolysis of agar at high temperature.

Initially, Bacto agar (DIFCO) was used at 1.5% (w/v) to solidify plates. This product contains organic nutrients which can be used for growth by some microorganisms. The use of Noble agar (DIFCO), a more refined product, was used to eliminate growth of heterotrophic contaminants.

When growth on solid media was too dense for selection of individual colonies, representative "colonies" were restreaked onto fresh plates of identical media in a manner designed to permit development of monoclonal colonies.

The final step in the strain-isolation procedure involved growth on solid media containing only basal salts and Noble agar. Following inoculation via streaking, plates were placed into a gas-tight chamber to which H_2S gas was periodically injected. The chamber was opened approximately every other day to provide additional molecular O_2 and CO_2 for energy and biosynthesis.

2.2 Identification of Sewer Isolates

2.2.1 Acidophilic Isolates

Media #1 and #3 (Table 2-2) were used to enrich for acidophilic chemolithotrophs, *T. thiooxidans* and *T. ferrooxidans*. To distinguish between these isolates, a portion of a dense culture was transferred to a mineral medium containing Fe(II), designed for isolation of *T. ferrooxidans* (Kelly, 1982). Medium description and sterilization method are summarized in Table 2-3. *T. thiooxidans* is unable to use Fe(II) as a substrate.

Figure 2-4 show electron micrographs (magnified 36,000 times for the top micrograph and 54,000 times for the bottom) of two different acidophilic sewer crown isolates, one from manhole A62 and the other from manhole E30 (each micrograph prepared by Sima, 1988). Each of these show dense inclusions inside the cells. These inclusions are apparently carboxysomes where the enzymes used by the Calvin cycle are produced.

2.2.2 Non-acidophilic Isolates

Preliminary species identifications were premised on colony development on solid media (basal salts, Bacto agar, with $S_2O_3^{-2}$ as substrate). The colony appearance was matched to the descriptions by Vishniac (1974) as described in Chapter 1.

Small volumes of pure cultures consisting of non-acidophilic crown isolates were split between two 55-ml tubes containing either basal salts

Table 2-3. Media Used for Identification of Acidophilic Sewer Crown Isolates. Cultures Capable of Growth are *T. ferrooxidans* (ATCC, 1984)

Solution A (1)		
	$(NH_4)_2SO_4$	0.40 g
	KH_2PO_4	0.20 g
	$MgSO_4 \cdot 7H_2O$	0.08 g
	Distilled Water	400 ml
Solution B (2)		
	$FeSO_4 \cdot 7H_2O$	10.0 g
	1 N H_2SO_4	1.0 ml
	Distilled Water	100.0 ml

Notes: (1) Solution A was autoclaved.
(2) Solution B was filter-sterilized.
(3) pH of the mixture of Solutions A and B was 2.8.

Figure 2-4. Electron micrographs of acidophilic sewer crown isolates identified as *Thiobacillus thiooxidans*. The top micrograph is from manhole A62 and the bottom from manhole E30.

alone or basal salts plus yeast extract. H_2S was injected through a teflon septum in the cap of each tube. Oxygen and CO_2 were replenished by periodically exchanging gases from the tube head space with ambient air. Cell number and pH were periodically measured to determine growth and metabolic activity. The rationale for metabolic tests leading to species identification was explained previously. Obligate autotrophs, *T. neapolitanus* and *T. thioparus*, were expected to grow at similar rates in both the inorganic and organic based media. Rates among the facultative autotrophs and heterotrophs, *T. novellus*, *T. intermedius* and *T. perometabolis*, were expected to be greater in the tubes containing yeast extract. It was assumed that *T. perometabolis* was not present because prior isolation steps used inorganic media and this species grows slowly without an organic substrate.

The terminal pH (pH_T) of batch cultures was used to distinguish between species showing either obligate or facultative autotrophic growth characteristics. The pH_T of individual species of thiobacilli were provided in the Section 1.4.5.

2.3 Stoichiometry Experiments

2.3.1 General

Stoichiometric relationships among growth, O_2 utilization, and H_2S utilization were developed using two kinds of experiments. In the first, simultaneous oxygen and sulfide utilization rates were determined (short-

term experiments). The second (long-term experiments) yielded relationships between bacterial growth or acid production and H_2S utilization for various crown isolates.

2.3.2 Dissolved Oxygen Measurement

Oxygen uptake experiments were performed using a Yellow Springs Instruments (YSI) model 3500 biological oxygen monitor and probe. The instrument was designed for continuous measurement of dissolved oxygen concentration in 3 to 8 ml batch liquid cultures under isothermal conditions. The probe assembly was designed to reduce O_2 transfer between the culture and its surroundings. Dissolved O_2 measurements, as percent of saturation, was recorded every 30 to 60 seconds and converted to molar concentrations. Tangents to curves representing O_2 concentration versus time were used to estimate O_2 utilization rates in cultures of thiobacilli.

2.3.3 Total Sulfide Measurement

A methylene blue, colorimetric assay was utilized to measure the concentration of total aqueous inorganic sulfides (Standard Methods, Part 428C, 14th Edition). A standard curve was prepared using dissolved sodium sulfide ($Na_2S \cdot 9H_2O$) in distilled/deionized water. The pH of sulfide stock solutions was maintained above 9.0 to minimize volatilization of H_2S. The concentration of dissolved H_2S above pH of 9.0 is less than 1.0 % of the total sulfide present (see Figure 1-6).

Samples for measurement of total sulfide concentration were periodically withdrawn from the YSI chamber via a syringe which was inserted through a small slot in the O_2-probe assembly. The procedure permitted simultaneous, semi-continuous measurement of dissolved oxygen and total sulfide concentrations in batch cultures of thiobacilli. Abiotic experiments confirmed significant concentrations of H_2S was not lost via either autoxidation or volatilization during these experiments.

Procedures specified in Standard Methods were altered to reduce the sample volume required for measurement of total sulfide from 0.75 ml to 0.30 ml. The standard curve was prepared using the same, modified procedure. Color development, which is proportional to total sulfide concentration was measured at a wavelength of 665 nm using a Perkin-Elmer/Hitachi 200 dual light path spectrophotometer.

2.3.4 Determination of Acid Production

To determine the quantity of microbially produced acid, a 1.0 ml aliquot of bacterial culture was back titrated with strong base to the initial pH of the culture. The volume of base added was recorded and acid production calculated by the following:

$$\text{Acid Produced, } \underline{N} = \frac{(\text{volume of base, ml})(\text{Normality of base, N})}{(\text{Volume of sample, ml})}$$

For acidophilic cultures and the final stages of non-acidophilic

culture growth, 1.0 N NaOH was used as a titrant. During the initial stages of non-acidophilic culture, 0.1 N NaOH served as the titrant to provide greater sensitivity.

2.3.5 Cell Number Determination

Cell density was measured using a Petroff-Hauser counting chamber as previously described.

2.3.6 Total Organic Carbon and Total Protein Analyses

Measurements of total (particulate) organic carbon (TOC) and total protein were considered as surrogate growth parameters. An aliquot of H_2S grown culture was taken daily and frozen until analyzed.

TOC was measured using a Dohrman carbon analyzer, Model DC-80 following sample acidification and CO_2 stripping. This system measures the CO_2 produced via the UV-catalyzed (chemical) mineralization of organic carbon.

Samples were diluted to lower the TOC concentration to the most sensitive level of the analyzer (between 0 and 15 mg TOC/liter) by mixing 0.5 ml of liquid culture with 4.5 ml of distilled/deionized water. The diluted sample was acidified to pH 2.5 by adding 3 drops of a 15% phosphoric acid solution. The CO_2 was then stripped from solution using a stream of nitrogen gas. The organic carbon present in 1.0 ml injection volumes was oxidized to CO_2 and sparged to a non-dispersive infrared detector. Calibration was accomplished using an acidified/sparged glucose

preparation of known concentration.

Total protein analyses utilized the assay developed by Biuret and Lowery and modified by Sigma Chemical (Procedure 690). Reagents were purchased in kit form and used per product instructions. Samples were concentrated by 2X via centrifugation/resuspension prior to protein measurements. Cell membranes were also solubilized via addition of a strong base (sodium hydroxide). Sample absorbance was measured at a wavelength of 755 nm on a Perkin-Elmer/Hitachi 200 spectrophotometer.

2.3.7 Bacterial Isolates Tested

Four isolated strains of thiobacilli (three acidophilic isolates and one non-acidophile) were used to determine stoichiometric relationships. The acidophiles C8/12/1.2, C22/12/1.2 and E30/12/1.2, were isolated from sewer crowns of vastly different pH and corrosion conditions. The first two from highly corroded sewers with crown pH of 2.0, and the last from a lightly corroded sewer with crown pH above 6.0. The non-acidophilic strain used was C8/12/2.3B, isolated from an extreme, low-pH environment. Isolate designation and identification is listed in Table 3-10.

2.4 Inhibition Experiments

2.4.1 General

Two types of inhibition experiments were performed: (i) acute inhibition tests using model inhibitors and oxyanionic metal compounds and (ii) chronic toxicity tests involving various metal compounds. The acute inhibition experiments were similar to microbial O_2/H_2S utilization rate studies described previously in that dissolved oxygen consumption was used as a measure of relative inhibition. These tests normally lasted less than an hour.

Chronic (metals) inhibition studies were based on measurement of relative rates of acid production over much longer periods (days to weeks).

2.4.2 Acute Inhibition Tests

Inhibitors included cyanide (CN^-), dicyclohexylcarbodiimide (DCCD), and oxyanions of vanadium and molybdenum (VO_4^- and MoO_4^-). Rates of O_2 and H_2S utilization, measured as described previously, were employed as indicators of toxicity. Twenty milliliters (20 ml) of culture and solutions containing the various inhibitors were added to 55-ml test tubes. Tubes were sealed with Dynatech Minivert gas tight valve tops, gaseous H_2S was then added before the cultures were thoroughly mixed. Samples were withdrawn periodically for measurement of dissolved O_2 and H_2S concentrations. Because DCCD was dissolved in acetone prior to addition,

control experiments involving acetone inhibition if thiobacilli were also carried out.

2.4.3 Chronic Metals Inhibition

Chronic metals tests for acidophilic isolates were based on growth and acid production in a basal-salts medium supplemented with elemental sulfur (Medium #1, pH_o = 3.75, Table 2-2). Cultures were grown in fresh media in a 250 ml flask. Acid production was monitored via pH measurements. When pH decreased to approximately 3.5, 10 ml of culture were transferred to tubes containing presterilized elemental sulfur.

Solutions of inhibitors were filter sterilized and added at various concentrations to the 10-ml of cultures. Table 2-4 lists the metals and concentrations tested. Sodium dichromate was dried in a 105° C oven for 24 hours prior to preparation of chromium solutions.

The cultures were continuously mixed on a Roto-Torque rotator (Cole-Parmer) for 6-10 days. Growth/inhibition was noted by periodic measurement of pH. Silver inhibition tests were conducted in both, (i) chloride-free (ii) standard mineral salts media. The non-chloride salts media was tested of the limited solubility of silver-chloride in water (K_{so} for AgCl(s) = 1 X 10^{-10}; Snoeyink, 1980). Constituents of the non-chloride medium are summarized in Table 2-5.

Table 2-6 lists the constituents of a "metals elixir". This mixture comprised the trace metals solution used in various media in the November, 1987 sampling (see Table 2-1). Lack of growth in the November samples led

Table 2-4. Metals and sewer isolates used for chronic toxicity tests.

Metal (valence)	Concentrations Tested (molar)	Isolate(s) Tested
Molybdate Mo(VI)	1.00E−03 1.00E−04 1.00E−05 1.00E−06	C8/12/1.2 A62/12/1.2 C22/12/1.2 LACSD
Orthovanadate V(V)	1.00E−03 1.00E−04 1.00E−05 1.00E−06	C8/12/1.2 A62/12/1.2 C22/12/1.2 LACSD
Cadmium Cd(II)	1.00E−03 1.00E−04 1.00E−05 1.00E−06	C8/12/1.2
Mercury Hg(II)	1.00E−04 1.00E−05 1.00E−06 1.00E−07	A62/12/1.2 C22/12/1.2
Dichromate Cr(VI)	1.00E−04 1.00E−05 1.00E−06 1.00E−07	A62/12/1.2 C22/12/1.2

Table 2-4. Metals and sewer isolates used for chronic toxicity tests - continued.

Metal (valence)	Concentrations Tested (molar)	Isolate(s) Tested
Zinc Zn(II)	1.00E-04 1.00E-05 1.00E-06 1.00E-07	A62/12/1.2 C22/12/1.2
Silver (1) Ag(I)	1.00E-04 1.00E-05 1.00E-06 1.00E-07	C8/12/1.2 E30/12/1.2 LACSD
Copper Cu(II)	1.00E-03 5.00E-04 1.00E-04 1.00E-05	C8/12/1.2 E30/12/1.2 LACSD
Metals Elixer (fraction of concentration listed in Table 2-6)	1.00E+00 4.00E-01 8.00E-02 1.60E-02 3.20E-03	C8/12/1.2 E30/12/1.2
Cobalt Co(II)	1.00E-02 1.00E-03 1.00E-04 1.00E-05	LACSD

(1) Medium of both chloride and chloride-free used due to the potential of forming insoluble silver chloride.
(2) See Table 3-10 for designation explanation and isolate identification.

Table 2-5. Non-Chloride Media
for the Chronic Toxicity Test of
Ag(I) on Acidophilic Thiobacilli (Milner, 1988)

Non-chloride Medium Constituents	Quantity per Liter, grams
$(NH_4)_2SO_4$	0.20
$MgSO_4 \cdot 7H_2O$	0.50
$CaSO_4 \cdot 2H_2O$	0.40
KH_2PO_4	3.0
$Fe(SO_4)_3 \cdot nH_2O$	0.013

Table 2-6. Final Concentrations
Metals-Elixer components (1X concentration)

Component	mg/l (as shown)	molar
EDTA	506	1.7×10^{-3}
$ZnSO_4 \cdot 7H_2O$	220	7.7×10^{-4}
$MnCl_2 \cdot 4H_2O$	50.6	2.6×10^{-4}
$FeSO_4 \cdot 7H_2O$	50.0	1.8×10^{-4}
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	11.0	6.2×10^{-5}
$CuSO_4 \cdot 5H_2O$	157	6.3×10^{-5}
$CoCl_2 \cdot 6H_2O$	32.2	1.4×10^{-4}

to investigation of the metals combination as a potential source of toxicity among thiobacilli tested.

2.4.4 Isolates Tested for Chronic Metals Toxicity

Table 2-4 also lists the isolates utilized in metals-toxicity tests. The "LACSD" strain was isolated by the County Sanitation Districts of Los Angeles County. It has been used for other metals inhibition work and grows well in a Fe(II) medium. Because non-acidophilic isolates would not grow on elemental sulfur or thiosulfate after acclimation to a H_2S-supplemented medium, their use in metals inhibition experiments was impractical.

CHAPTER 3

RESULTS

3.1 Field Observations

3.1.1 Crown pH and Corrosion

Crown pH and degree of deterioration were noted in specific LACSD trunk sewers. Results are summarized in Tables 3-1 and 3-2 for the November 1987 and March 1988 sampling trips, respectively. The crown surfaces with lower pH exhibited a much greater degree of concrete degradation. Manhole C23 was inaccessible during the March 1988 sampling period; therefore the manhole directly downstream, C22, was used in its place (as shown in Figure 1-12). Generally, the lowest pH of was observed at the 12:00 o'clock position (or crest) of each crown.

Photographs of several pipe sections sampled were shown previously (Figures 1-8 through 1-11). Large concrete aggregate and reinforcing steel of the RCP were visible in manholes C8,C22, and C23. Manhole A62 and the adjacent tiled pipe sections were moderately to highly corroded as tiles in the vicinity of the manhole were missing. Corrosion in manhole E30 was light.

Table 3-1. Summary of physical conditions at crown sampling locations (November, 1987).

Interceptor and Location Identification	Position on Crown[1]	pH	Crown Condition
C8	12	2.0	Highly corroded
C8	1	2.5	Highly corroded
C8	2	2.5	Moderately corroded
C8	3	3.0	Moderately corroded
C23	12	2.5	Highly corroded
A62	12	3.0	Moderately corroded
A62	3	2.8	Moderately corroded
E30	12	5.5	Slightly corroded
E30	2	6.5	Slightly corroded
E30	3	6.5	No corrosion

[1]Indicates position on clockface. The sewer itself is the clock; the clockface is to be read looking in the downstream direction.

Table 3-2. Summary of physical conditions at crown sampling locations (March, 1988).

Manhole Sampled[1]	Crown Location[2]	Crown pH	Collected for Isolation	Extent of Corrosion
C8	12:00	2.0	Yes	Heavy
C8	1:00	2.5-3.0	Yes	Heavy
C8	2:00	4.0	Yes	Moderate-Heavy
C8[4]	3:00	4.0	Yes	Light-Moderate
C22	12:00	2.0	Yes	Heavy
C22	1:00	4.5	No	Moderate
C22	2:00	6.0	No	Light
C22[4]	3:00	6.0	No	Very Light
A62[3]	12:00	2.0	Yes	Falling tile[5]
A62	1:00	3.0	No	Moderate
A62	2:00	2.5	No	Light
A62[4]	3:00	2.5	No	Light
E30	12:00	>6.6	Yes	Very Light

[1] LACSD designation
[2] Position is in terms of clock location standing in the manhole facing in the downstream direction.
[3] Tiled pipe
[4] Sample taken from slim layer on sewer wall
[5] Tile falling from crown apparently due to mortar decomposition

3.2 Growth and Identification of Sewer Crown Samples in the Laboratory

3.2.1 General

Enrichment media were designed to promote growth among specific classes of thiobacilli present in the crowns of sewers sampled in November, 1987 and March, 1988. Samples collected from in November, 1987 did not provide a sufficient quantity of growing cultures to satisfy subsequent testing protocol, therefore more samples were required.

3.2.1 November, 1987 Samples

Growth of acid-producing bacteria in the initial enrichment media and the first-transfer media containing sewer crown samples are summarized in Tables 3-3 and 3-4, respectively. Time-dependent decrease of pH in the culture media was the primary indication of microbial activity.

Samples from the first-transfer cultures which produced acid were diluted and cultivated on solid media (Bacto agar fortified with $S_2O_3^{-2}$). The plates supported growth of a variety of contaminants in addition to candidate thiobacilli. Colonies were visually analyzed, noting size, color and extent of surrounding sulfur deposits (data not shown).

In no case did the transfer of colonies from the plates back to the original liquid selective media (with $S_2O_3^{-2}$) support chemolithotrophic growth. The November, 1987 cultures were not subjected to further experiments.

Table 3-3. Summary of pH changes for initial-enrichment media via direct inoculation with sewer crown material - November, 1987 samples (pH after 10 days incubation/ after 21 days incubation).

Sampling point (sewer identifier/ crown location)	Grown Medium. See Table 2-1 for description Initial pH in parentheses						
	A (6.6)	B (4.5)	C (6.8)	D (4.5)	F (6.6)	G (4.5)	H (4.5)
C8 (12)	5.5/5.3	4.2/4.3	N.D.	3.3/3.7	5.5/5.3	4.0/3.8	4.5/4.6
C8 (1)	6.0/6.0	5.0/5.0	4.5/4.7	3.5/3.3	5.5/5.6	4.3/4.3	5.0/1.6
C8 (2)	5.5/5.5	4.5/4.7	4.5/4.6	4.0/4.1	5.5/5.5	4.0/4.1	4.5/2.7
C8 (3)	3.5/1.1	5.0/5.1	4.2/2.8	4.0/3.9	2.5/1.7	4.5/4.6	2.5/2.5
C23 (12)	5.5/1.6	5.0/5.3	5.5/5.4	4.5/5.0	6.0/7.0	4.5/4.7	1.5/1.5
A62 (12)	6.0/6.1	4.7/4.0	3.8/3.1	3.3/3.1	5.5/6.8	3.5/3.6	4.5/5.0
A62 (3)	5.5/4.4	5.0/4.7	3.5/2.5	4.0/3.6	5.7/5.2	4.0/3.6	N.D.
E30 (12)	3.5/3.2	5.0/4.0	4.7/4.6	4.3/3.2	3.0/3.3	3.5/4.1	4.5/5.0

*N.D. -- No Data

Note: Media A,C, and F contain trace metal solutions

Table 3-4. Summary of pH changes for first-transfer cultures - November, 1988 samples (cultures derived by transferring one drop of original culture after 10 days of growth to fresh enrichment media; pH after 19 days following transfer/ after 27 days following transfer).

Sampling point (sewer identifier/ crown location)	Grown Medium. See Table 2-1 Initial pH in parentheses						
	A (6.6)	B (4.5)	C (6.8)	D (4.5)	F (6.6)	G (4.5)	H (4.5)
C8 (12)	6.0/5.5	4.5/5.0	N.D.	5.0/5.3	5.5/4.2	4.5/4.7	5.0/5.4
C8 (1)	5.5/5.6	4.5/4.9	6.0/6.5	5.0/5.3	4.2/4.4	4.2/4.6	5.0/5.4
C8 (2)	6.0/6.0	4.5/4.7	6.0/6.5	4.5/5.3	5.5/5.8	4.5/4.7	5.0/5.4
C8 (3)	2.0/1.9	4.5/4.9	2.3/2.3	1.7/1.3	4.0/3.0	4.5/4.7	5.0/5.2
C23 (12)	5.5/5.3	4.2/4.9	6.0/6.5	5.0/5.2	6.0/7.2	4.5/4.6	5.0/5.2
A62 (12)	6.0/7.3	4.5/4.5	6.0/6.5	4.5/5.3	5.5/6.4	4.5/4.7	4.7/5.4
A62 (3)	5.0/4.1	4.5/4.5	6.0/6.5	5.0/5.3	5.5/5.6	4.5/4.7	N.D.
E30 (12)	3.5/3.2	4.5/4.5	3.5/3.1	1.9/1.5	2.6/3.2	4.5/4.7	4.5/5.3

*N.D. -- No Data

Note: Media A,C, and F contain trace metal solutions

3.2.2 March 1988 Samples

Tables 3-5 and 3-6 summarize growth of sewer crown microorganisms collected in March, 1988, in initial enrichments and first-transfer cultures, respectively. Growth was indicated by acid production and an increase in cell density. With the exception of Medium #3, (low pH, with $S_2O_3^{-2}$ as the electron donor) most first-transfer media supported growth of acid-producing bacteria. These tables also indicate the subsequent isolation method used for each culture. Figures 2-1 and 2-2 summarized the steps used for the isolation of acidophilic and non-acidophilic cultures, respectively.

Table 3-7 is a summary of observations in low pH cultures (Media #1 and #3), which were subjected to serial dilutions and recultivation. The low pH, elemental sulfur medium (#1) supported rapid growth. In contrast, the low pH, $S_2O_3^{-2}$ medium (#3) did not support growth of any microbes despite the fact that sampling procedures, growth and transfer conditions were otherwise identical. The transfer of Media #1 cultures onto solid media supplemented with approximately 0.2 % (v/v) H_2S (g) yielded colonies from each sample location. Subsequent transfer from colonies to a basal salts medium plus H_2S also supported growth.

Cultures used for subsequent studies were selected by their ability to form apparent axenic colonies. Also, attempts were made to use cultures from vastly different crown pH and corrosion conditions. See Table 3-10 for designation and identification of isolates selected and used for further study.

Table 3-5. Summary of growth, pH modification, and subsequent fate of original crown samples taken March, 1988.

SAMPLE SITE	MEDIA DESCRIPTION			
	Medium #1[2] S^o pH_o=3.5	Medium #2 $S_2O_3^{-2}$ pH_o=6.8	Medium #3 $S_2O_3^{-2}$ pH_o=4.0	Medium #4 $S_2O_3^{-2}$ + Yeast extract pH_o=6.8
C8/12:00[1]	pH_{14} = -2.2[3] Growth: Heavy, High Cell# Transferred[4]	pH_{17} = 0 Growth: Heavy Transferred	pH_{17} = +0.1 Growth: Diverse, Moderate Transferred	pH_{11} = -2.9 pH_{14} = -4.7 Growth: Heavy Serially Diluted
C8/1:00[1]	pH_{14} = -2.8 Growth: Heavy, High Cell# Transferred	pH_{17} = 0 Growth: Moderate Transferred	pH_{17} = -0.8 Growth: Moderate Transferred	pH_{11} = +0.4 Growth: Light To Moderate Transferred
C8/2:00[1]	pH_{14} = -3.0 Growth: Heavy, High Cell# Transferred	pH_{17} = 0 Growth: Moderate Transferred	pH_{17} = -0.5 Growth: Diverse Moderate Transferred	pH_{11} = +0.4 Growth: Moderate Transferred
C8/3:00[1]	pH_{14} = -3.0 Growth: Heavy, High Cell # Transferred	pH_{17} = 0 Growth: Heavy Transferred	pH_{17} = +0.1 Growth: Heavy Transferred	pH_{11} = -4.5 Growth: Heavy Serially Diluted
C22/12:00[1]	pH_{14} = -3.0 Growth: Heavy, High Cell# Transferred	pH_{17} = -0.9 Growth: Heavy Transferred	pH_{17} = -0.5 Growth: Heavy Transferred	pH_{11} = -3.1 Growth: Heavy Serially Diluted
A62/12:00[1]	pH_{14} = -3.0 Growth: Heavy, High Cell# Transferred	pH_{17} = -4.7 Growth: Moderate Serially Diluted[4]	pH_{17} = +0.1 Growth: Moderate Transferred	pH_{11} = -2.4 pH_{14} = -4.7 Growth: Heavy Serially Diluted
E30/12:00[1]	pH_{14} = -2.2 Growth: Heavy, High Cell# Transferred	pH_{17} = -3.2 Growth: Heavy Serially Diluted	pH_{17} = +0.1 Growth: Diverse Moderate Transferred	pH_{11} = -3.4 Growth: Heavy Serially Diluted

Notes:

[1] Sample site designation refers to LACSD manhole location and crown location of sample taken.

[2] For complete media description, see Table 2-2.

[3] pH subscript refers to number of days after inoculation pH was measured. Value given is the change of medium pH during the period indicated.

[4] Serial dilute = dilute to extinction; transferred = transferred one drop to fresh enrichment medium.

Table 3-6. Summary of growth, pH modification, and subsequent fate of first-transfer cultures of samples taken March, 1988.

SAMPLE SITE	MEDIA DESCRIPTION			
	Medium #1[2] S^{o} pH_{o}=3.5	Medium #2 $S_2O_3^{-2}$ pH_{o}=6.8	Medium #3 $S_2O_3^{-2}$ pH_{o}=4.0	Medium #4 $S_2O_3^{-2}$ + Yeast extract pH_{o}=6.8
C8/12:00[1]	pH_{8} = -2.2[3] Growth: Heavy Serial Dilute[4]	pH_{21} = 1.1 Growth: Moderate Plated	pH_{30} = -2.7 Growth: Light Serial Dilute	pH_{8} = -5.0 Growth: Moderate Plated
C8/1:00[1]	pH_{8} = -2.8 Growth: Heavy Serial Dilute	pH_{15} = -3.7 Growth: Heavy Plated	pH_{30} = -2.7 Growth: Light Serial Dilute	pH_{30} = +1.1 Growth: Moderate, Bact. Cells Watch Growth
C8/2:00[1]	pH_{8} = -2.5 Growth: Heavy Serial Dilute	pH_{21} = -0.6 Growth: Little To None Plated	pH_{30} = -2.7 Growth: Light Serial Dilute	pH_{8} = -4.6 Growth: Heavy Plated
C8/3:00[1]	pH_{8} = -2.8 Growth: Heavy Serial Dilute	pH_{15} = -4.0 Growth: Heavy Plated	pH_{30} = -2.4 Growth: Light Serial Dilute	pH_{8} = -3.5 Growth: Moderate Plated
C22/12:00[1]	pH_{8} = -2.8 Growth: Heavy Serial Dilute	pH_{15} = -3.4 Growth: Heavy Plated	pH_{30} = 0 Growth: None Wait for Growth	pH_{8} = -4.7 Growth: Moderate Plated
A62/12:00[1]	pH_{8} = -2.8 Growth: Heavy Serial Dilute	pH_{15} = -1.9 Growth: Heavy Plated	pH_{30} = 0 Growth: None Wait for Growth	pH_{10} = -1.0 Growth: Moderate Plated
E30/12:00[1]	pH_{8} = -2.8 Growth: Heavy Serial Dilute	pH_{15} = -3.4 Growth: Heavy Plated	pH_{30} = 0 Growth: None Wait for Growth	pH_{8} = -2.6 Growth: Moderate Plated

Notes:

[1] Sample site designation refers to LACSD manhole location and crown location of sample taken.

[2] For complete media description, see Table 2-2.

[3] pH subscript refers to number of days after inoculation pH was measured. Value given is the change of medium pH during the period indicated.

[4] Serial dilute = dilute to extinction; transferred = transferred one drop to fresh enrichment medium.

Table 3-7. Analysis of serial dilutions, low-pH cultures - March, 1988 samples.

SAMPLE SITE	NARRATIVE SUMMARY	
C8/12:00[1]	Media #1[2]	- pH dropped to 1.3 after 12 days at room temperature[3] in 10^{-9} dilution.[4] Isolate #C8/12/1.1.
	Media #3[2]	- Slow growth in serial dilution, 1st transfer cultures showed pH drop to 1.6. Continue to monitor.
C8/1:00	Media #1	- pH dropped to 1.0 after 12 days at room temperature in 10^{-9} dilution. Isolate #C8/1/1.1.
	Media #3	- Slow growth in serial dilution, 1st transfer cultures showed pH drop to 1.6.
C8/2:00	Media #1	- pH dropped to 1.0 after 12 days at room temperature in 10^{-9} dilution. Isolate #C8/2/1.1.
	Media #3	- Slow growth in serial dilution, 1st transfer cultures showed pH drop to 1.6.
C8/3:00	Media #1	- pH dropped to 1.0 after 12 days in 10^{-9} dilution. Isolate #C8/3/1.1
	Media #3	- Slow growth in serial dilutions, 1st transfer cultures showed pH drop to 1.3.
C22/12:00	Media #1	- pH dropped to 1.0-1.3 after 12 days in 10^{-8} dilution. Isolate #C22/12/1.1.
	Media #2	- Slow or no pH drop in serial dilutions. No pH drop in 1st transfer culture.
A62/12:00	Media #1	- pH dropped to 1.6 after 12 days in 10^{-9} dilution. Isolate #A62/12/1.1.
	Media #3	- Slow or no pH drop in serial dilution. No pH drop or visible growth in 1st transfer culture.
E30/12:00	Media #1	- pH dropped to 1.6 after 12 days in 10^{-10} dilution. diluted to 10^{-10} for the second time. pH drop ended with 10^{-9} dilution. Continue to monitor. Isolate #E30/12/1.2.
	Media #3	- Slow or no pH drop in serial dilution. No pH drop or visible growth in 1st transfer culture.

[1] Sample site designation refers to LACSD manhole location and crown location of sample (See Figure 1-12).
[2] For complete media description, see Table 2-2.
[3] Room temperature was approximately 24°C.
[4] First dilution to 10^{-10}.

Differentiation of *T. ferrooxidans* from other acidophilic chemoautotrophic thiobacilli, primarily *T. thiooxidans*, was based on growth in media containing basal salts plus Fe(II), at an initial pH of 2.8. Sewer isolates, including a pure culture provided by LACSD, were tested in this manner. Per Figure 3-1, only the LACSD culture was capable of growth and acid production on Fe(II). The pH of media containing the other isolates tested decreased only slightly relative to the abiotic control. This decrease was presumably due to residual microbial activity from the transfer of small quantities of reduced sulfur. From the figure, it is evident the isolation procedures produced four strains of *T.thiooxidans*. The LACSD culture was presumed to be *T. ferroxidans*.

Mid-pH media (#2 and #4, Table 2-2) supported growth and acid production in most of the original enrichment and first-transfer cultures as listed in Tables 3-5 and 3-6. These cultures were serially diluted and spread plated to produce (apparently) axenic colonies. Initial plates used Bacto agar fortified with $S_2O_3^{-2}$. Colony size and appearance led to the preliminary identification of cultures to the species level as summarized in Table 3-8. Colonies which showed characteristics of thiobacilli cultivated on solid media with $S_2O_3^{-2}$ (as per Vishniac, 1974) were selected for subsequent isolation, identification, and experimentation.

Cultures which initiated growth at pH 6.80 were, from this point on, considered to be non-acidophiles. Colonies were re-streaked onto solidified basal salts media supplemented with H_2S (g). This process was

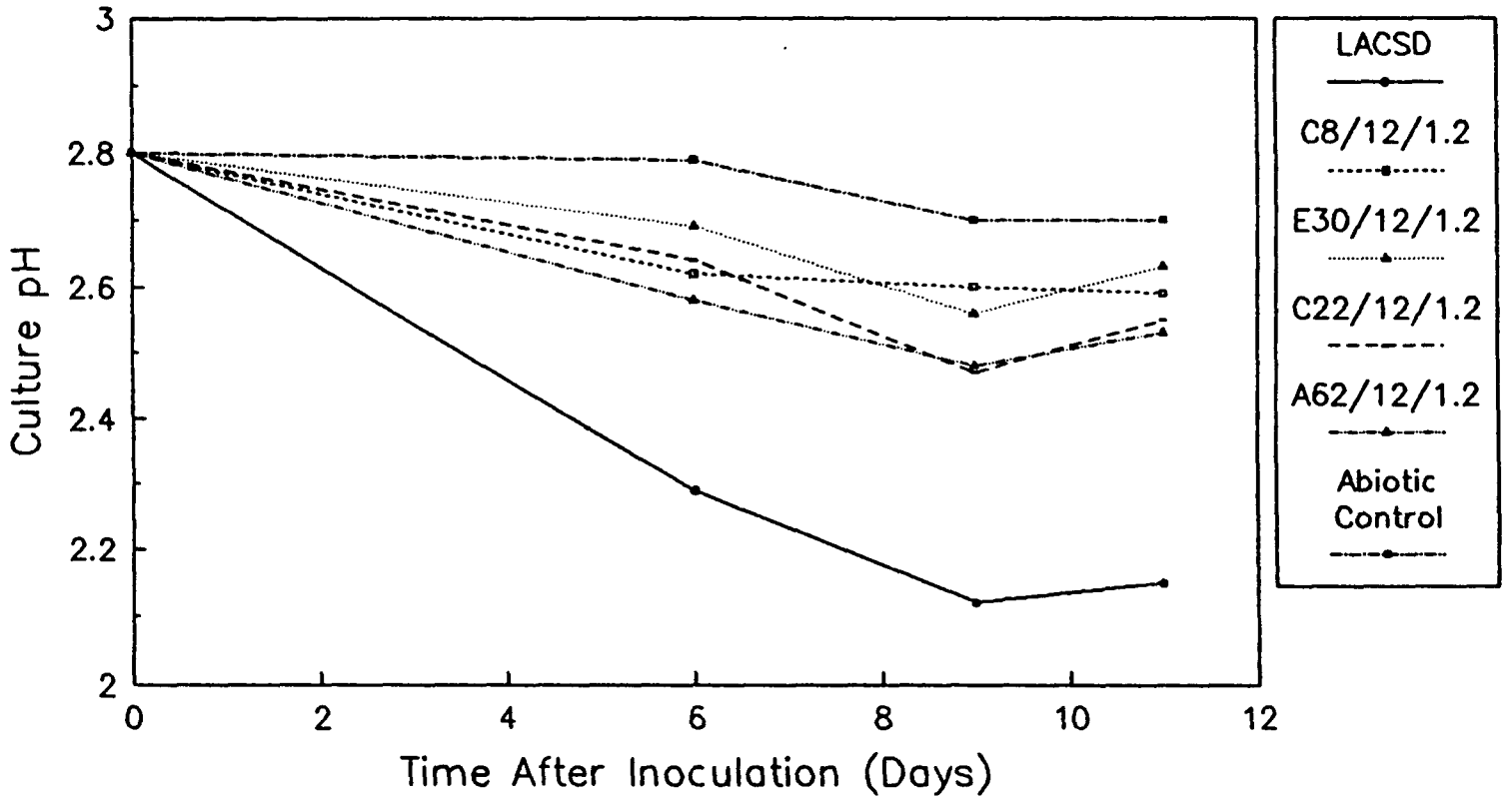


Figure 3-1. Acid production as measured by pH decrease in medium containing pure cultures of thiobacilli using Fe(II) as substrate.

Media used: ATCC #64 - Table 2-3

Table 3-8. Summary of plating results of mid-pH cultures from March, 1988 sewer crown samples.

Sample Site	Narrative Description of Colonies on Plates with Media 2 Ingredients and 1.5 Percent Agar
C8/12:00(1)	Media #2(2) - 10^{-4} Dilution - Many colonies (~150) 10^{-5} Dilution - 9 colonies - some white, possibly *T. thioparus* or *T. neapolitanus*
	Media #4(2) - No growth
C8/1:00	Media #2 - All dilutions, 100's of small, white spreading colonies From microscope analysis, appear oval (not *Thiobacillus* sp.)
	Media #4 - No growth
C8/2:00	Media #2 - No growth
	Media #4 - 10^{-4} Dilution - 100's of very small colonies 10^{-5} Dilution - 145 colonies - diverse 10^{-6} Dilution - 13 colonies-some transparent, some opaque Tranparent -> rods, probably *T. intermedius* Opaque -> rods, probably *T. thioparus* or *T. neapolitanus*
C8/3:00	Media #2 - 10^{-4} Dilution - 100's of colonies, very small 10^{-5} Dilution - 218 colonies - appear to be the same 10^{-6} Dilution - 19 colonies, semi-transparent, 1 mm-dia., possibly *T. novellus*
	Media #4 - 10^{-4} Dilution - 100's of small colonies 10^{-5} Dilution - 170 colonies - transparent edges with yellow grains 10^{-6} Dilution - 66 colonies - transparent edges with yellow grains

Table 3-8. Summary of plating results of mid-pH cultures from March, 1988 sewer crown samples - continued.

Sample Site	Narrative Description of Colonies on Plates with Media 2 Ingredients and 1.5 Percent Agar
C22/12:00	Media #2 - No growth
	Media #4 - 10^{-4} Dilution - 100's of colonies 10^{-5} Dilution - 100's of colonies 10^{-6} Dilution - 48 colonies Transparent edges with yellow grains (precipitated sulfur) Possibly: *T. novellus* Could be: *T. thioparus* or *T. neapolitanus*
A62/12:00	Media #2 - No growth
	Media #4 - No growth
E30/12:00	Media #2 - No growth
	Media #4 - 10^{-4} Dilution - 100's of colonies 10^{-5} Dilution - 100's of colonies 10^{-6} Dilution - 21 colonies - transparent Possibly: *T. novellus*

Note:

[1] Sample site designation refers to LACSD manhole location and crown location of sample (12:00 being the top of the pipe).

[2] For complete media description, see Table 2-2.

assumed to yield pure colonies which were transferred to liquid enrichment media for cultivation and further identification. Growth characteristics in two different S(-II) media: (i) an inorganic, basal-salts media and (ii) a yeast-extract/basal-salts media were used to identify these non-acidophilic isolates. Sulfide was provided as H_2S (g). Growth characteristics and subsequent identification of these isolates are summarized in Table 3-9.

Cultures containing both basal-salts and yeast extract produced a yellow precipitate upon addition of H_2S (g). Also, in the presence of organic carbon, culture pH decreased more slowly than in its absence (data not shown). Additionally, terminal pH was higher and terminal cell number was significantly lower.

Table 3-10 summarizes the origin of cultures and their final identification to the species level for both acidophilic and non-acidophilic isolates.

3.3 Stoichiometry of Sulfide Oxidation

3.3.1 General

Two methods were used to study S(-II) oxidation by thiobacilli isolates collected from LACSD sewer crowns. The methods include (i) short-term kinetic utilization of both dissolved oxygen and total sulfide and (ii) long-term, S(-II) exhaustion experiments. The short-term tests (described in Sections 3.3.2 and 3.3.3) were also used in conjunction with

Table 3-9
Growth Characteristics and Identification of
Non-acidophilic Sewer Crown Isolates

Candidate species based on autotrophic growth in mid-pH media (nos. 2 and 4): *T. thioparus*, *T. neapolitanus*, *T. novellus*, and *T. intermedius*. See Figure 1-17 for summary of thiobacillus taxonomy.

Isolate Designation: C8/2/2.2[2]
Terminal pH:
Medium #2[1]: 2.19
Medium #4: 5.81
Cell Number:
Medium #2: 260×10^6
Medium #4: 3×10^6

Isolate Identification: *T. neapolitanus*
Basis of identification: (i) Lack of growth in medium containing organics
(ii) Terminal pH in inorganic medium

Isolate Designation: C22/12/2.2
Terminal pH:
Medium #2: 4.87
Medium #4: 5.85
Cell Number:
Medium #2: 2×10^6
Medium #4: 64×10^6

Isolate Identification: *T. novellus*
Basis of identification: (i) Rapid growth in medium supplemented with organics
(ii) Terminal pH in growth medium

Table 3-9 (Continued)

Isolate Designation: C22/1/2.2
Terminal pH:
Medium #2: 2.42
Medium #4: 5.72
Cell Number:
Medium #2: 280 X 10^6
Medium #4: 36 X 10^6
Isolate Identification: *T. neapolitanus*
Basis of identification: (i) Lack of growth in medium containing organics
(ii) Terminal pH in inorganic medium

Isolate Designation: C8/12/2.3B
Preliminary identification: *T. neapolitanus*
Basis: Colony appearance (Table 3-8)
Terminal pH:
Medium #2: 2.12
Medium #4: 5.79
Cell Number:
Medium #2: 180 X 10^6
Medium #4: 48 X 10^6
Isolate Identification: *T. neapolitanus*
Basis of identification: (i) Lack of growth in medium containing organics
(ii) Terminal pH in inorganic medium

Isolate Designation: C8/12/2.3D
Preliminary identification: *T. novellus* or *T. intermedius*
Basis: Colony appearance (Table 3-8)
Terminal pH:
Medium #2: 5.16
Medium #4: 5.78
Cell Number:
Medium #2: 1.3 X 10^6
Medium #4: 56 X 10^6
Isolate Identification: *T. novellus*
Basis of identification: (i) Lack of growth in medium containing organics
(ii) Terminal pH in inorganic medium

Notes: [1] Medium #4 contains yeast extract: #2 does not. See Table 2-2 for complete media description
[2] See Table 3-10 for explanation of isolate designation.

Table 3-10. Explanation of isolate designation and summary of identification to the soecies level of thiobacilli collected from LACSD sewer crowns.

Isolate Designation	Taxonomic group enrich-ment media favored	Final Identification
C8/12/1.2	Acidophilic	T. thiooxidans
C8/1/1.2	Acidophilic	T. thiooxidans
C8/2/1.2	Acidophilic	T. thiooxidans
C8/3/1.2	Acidophilic	T. thiooxidans
C22/12/1.2	Acidophilic	T. thiooxidans
A62/12/1.2	Acidophilic	T. thiooxidans
E30/12/1.2	Acidophilic	T. thiooxidans
C8/2/2.2	Non-acidophilic	T. neapolitanus
C22/12/2.2	Non-acidophilic	T. novellus
C22/1/2.3	Non-acidophilic	T. neapolitanus
C8/12/2.3B	Non-acidophilic	T. neapolitanus
C8/12/2.3D	Non-acidophilic	T. novellus

Legend for isolate designation: A/B/C.D.E
A = LACSD manhole where original sample was collected
B = Pipe location, clock face values looking downstream
C = Number of the original enrichment media, see table 2-2
D = Number of steps taken to ascertain axenic culture
E = Optional, colony designation used for preliminary identifications

chemical inhibition tests to study the biochemical mechanisms of S(-II) oxidation. The long-term tests (described in Sections 3.3.4 through 3.3.7) were used to develop yield coefficients of both acidophilic and non-acidophilic sewer crown isolates.

3.3.2 Sulfide Standard Curve

The standard curve relating sample color (following addition of reagents -- methylene blue procedure) to total aqueous sulfide concentration is shown in Figure 3-2. Data representing concentrations between 0 and 4.0 mg/L total sulfide (0 to 0.12 m$\underline{M}$) yield a straight line relationship (R^2 = 0.997). On this basis, Equation 3-1 was developed to convert sample absorbance reading to the molar concentration of total sulfide:

$$[\text{Total sulfide (m}\underline{M}\text{)}] = [\ ABS_{665} * 0.154\ ] \qquad \text{Equation 3-1}$$

The data above 4.0 mg/L as sulfide were both non-linear and outside the range of total sulfide concentrations generally encountered in these experiments.

3.3.3 Kinetic Studies -- Oxygen and Sulfide Utilization Rates

Figures 3-3 and 3-4 represent the time-dependent molar concentrations of dissolved O_2 and H_2S, measured simultaneously in pure,

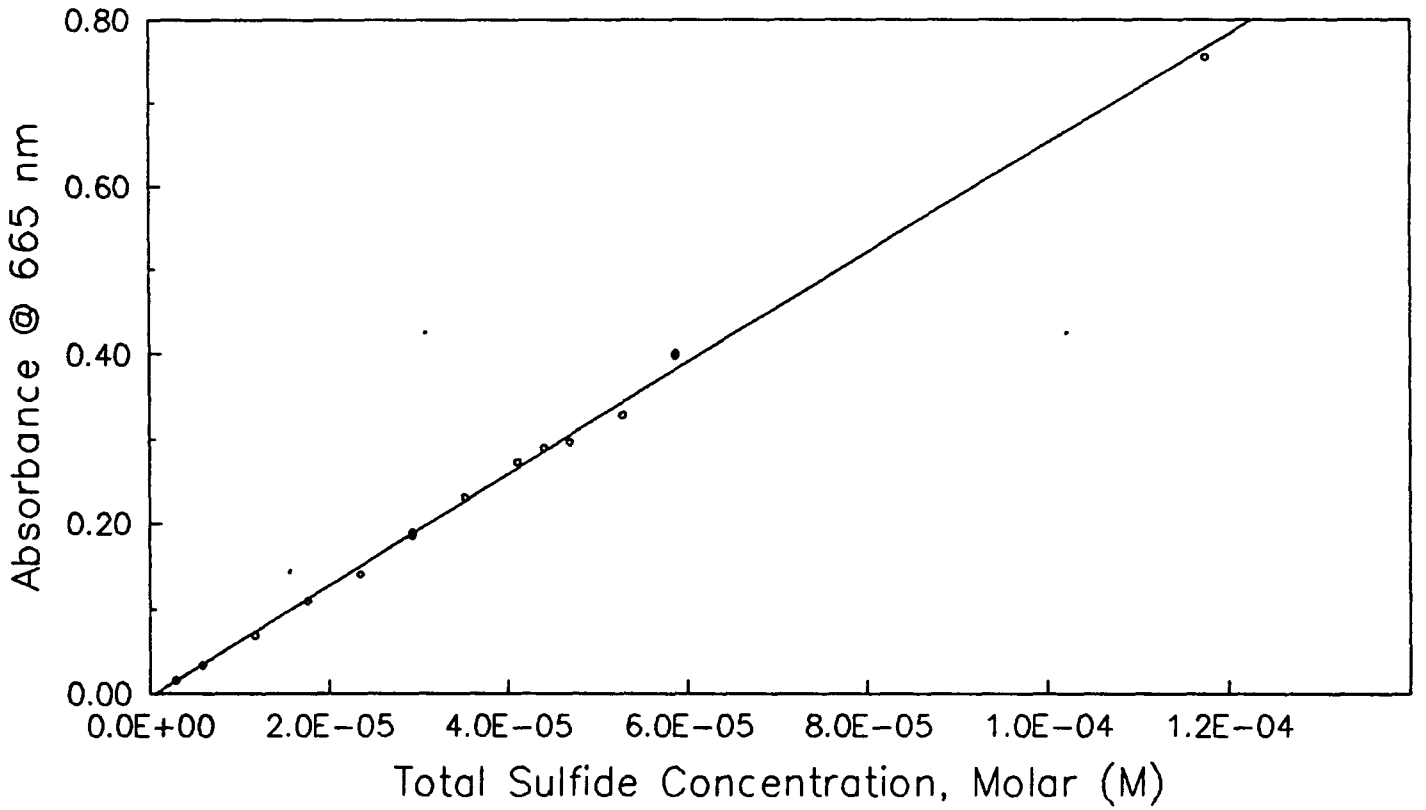


Figure 3-2. Total aqueous sulfide standard curve

Note: R-Squared = 0.997

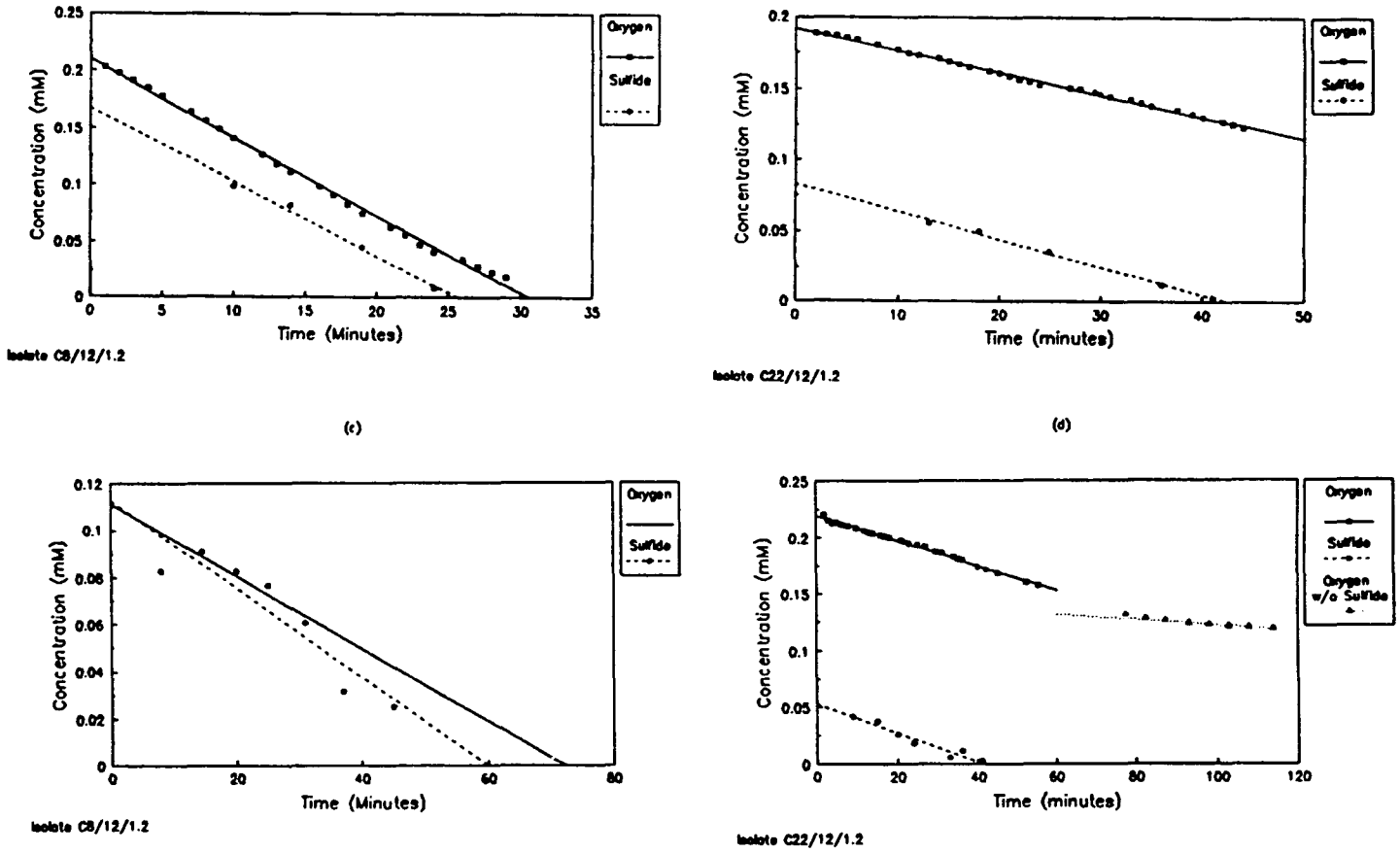


Figure 3-3. Representative simultaneous measurements of dissolved oxygen and total sulfide concentrations in cultures of *Thiobacillus thiooxidans*.

See Table 3-10 for isolate identification

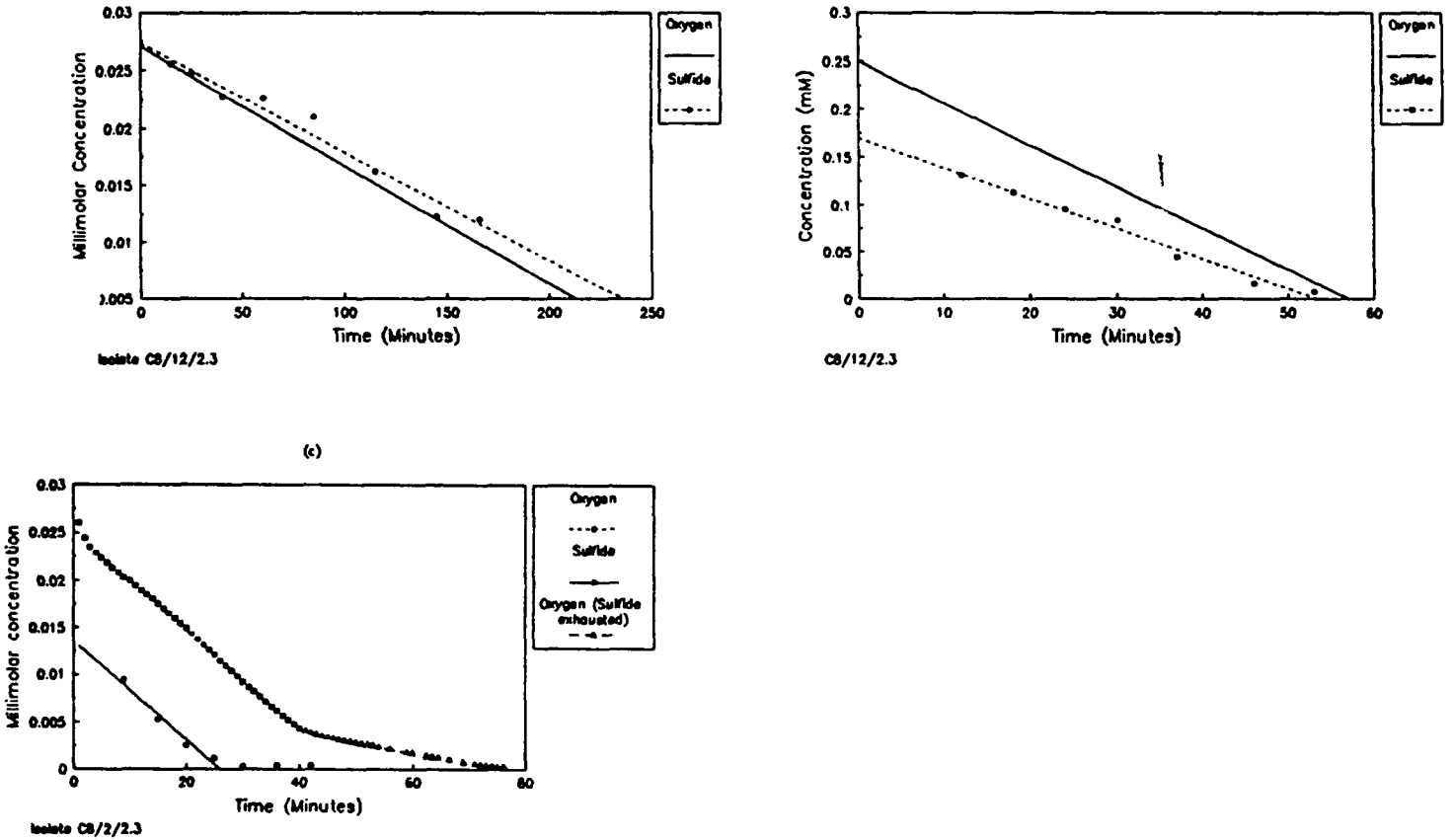


Figure 3-4. Representative simultaneous measurements of dissolved oxygen and total sulfide concentrations in cultures of non-acidophilic thiobacilli.

See Table 3-10 for isolate identification

batch cultures of acidophilic and non-acidophilic isolates, respectively. Linear regression relationships are provided on the figures. In several cases, representation of individual dissolved oxygen data points was impractical due to the frequency of such measurements. Slopes of the linear regression lines and their corresponding correlation coefficient are summarized in Table 3-11.

These experiments were originally started by adding a significant amount of H_2S (g) to a large sealed flask containing "starved" thiobacilli (See Materials and Methods Section 2.3). After a brief period for equilibration of gas/liquid-phase S(-II), an 8-ml sample was drawn for semi-continuous measurement of O_2 and S(-II) following procedures outlined previously. Residual liquid culture in the sealed flask typically became turbid after the H_2S addition/sample removal procedure, perhaps due to the development of an S° precipitate. This turbidity would disappear over a period of several hours.

The time-dependent oxygen demands of a turbid culture is illustrated in Figure 3-5. A duplicate sample was removed for simultaneous measurement of dissolved oxygen and total sulfide. The first did not receive an injection of H_2S and proved to be sulfide-free throughout the period of measurement. Despite the lack of dissolved sulfide, there was a significant oxygen demand. H_2S (g) added to the second sample produced a 73 % increase in the observed rate of O_2 utilization. In addition, the calculated molar ratio of O_2 to S(-II) oxidation was significantly higher than ratios observed in the precipitate-free or "starved" cultures

Table 3-11. Summary of simultaneous dissolved oxygen and total sulfide utilization rates in batch cultures of sewer crown isolates with no inhibitor present.

Isolate Tested	Oxygen utilization millimoles/min-L	Correlation Coeffi-cient, R-squared	Sulfide utilization millimoles/min-L	Correlation Coeffi-cient, R-squared	Moles oxygen per moles sulfide used
C8/12/1.2	0.00687	0.997	0.00654	0.989	1.05
C22/12/1.2	0.00153	0.998	0.00195	0.995	0.79
C8/12/1.2	0.00154	0.976	0.00188	0.853	0.82
C8/12/1.2	0.00400	0.989	0.00429	0.979	0.93
C8/12/1.2	0.00586	0.997	0.00675	0.963	0.87
E30/12/1.2	0.00099	0.990	0.00128	0.983	0.77
E30/12/1.2	0.00336	0.998	0.00312	0.990	1.08
E30/12/1.2	0.00435	0.997	0.00416	0.986	1.04
E30/12/1.2	0.00748	0.995	0.00616	0.963	1.21
C8/12/1.2	0.00518	0.999	0.00508	0.991	1.02
C8/12/1.2	0.00660	0.999	0.00685	0.969	0.96
C8/12/1.2	0.00865	0.998	0.00760	0.813	1.14
C8/12/1.2	0.01314	0.997	0.01181	0.938	1.12
C8/12/2.3B	0.00104	0.998	0.00086	0.928	1.20
C8/2/2.3	0.00053	0.999	0.00052	0.968	1.01
C8/12/1.2	0.00332	0.998	0.00352	0.995	0.94

See Table 3-10 for isolate designation and species level identification.

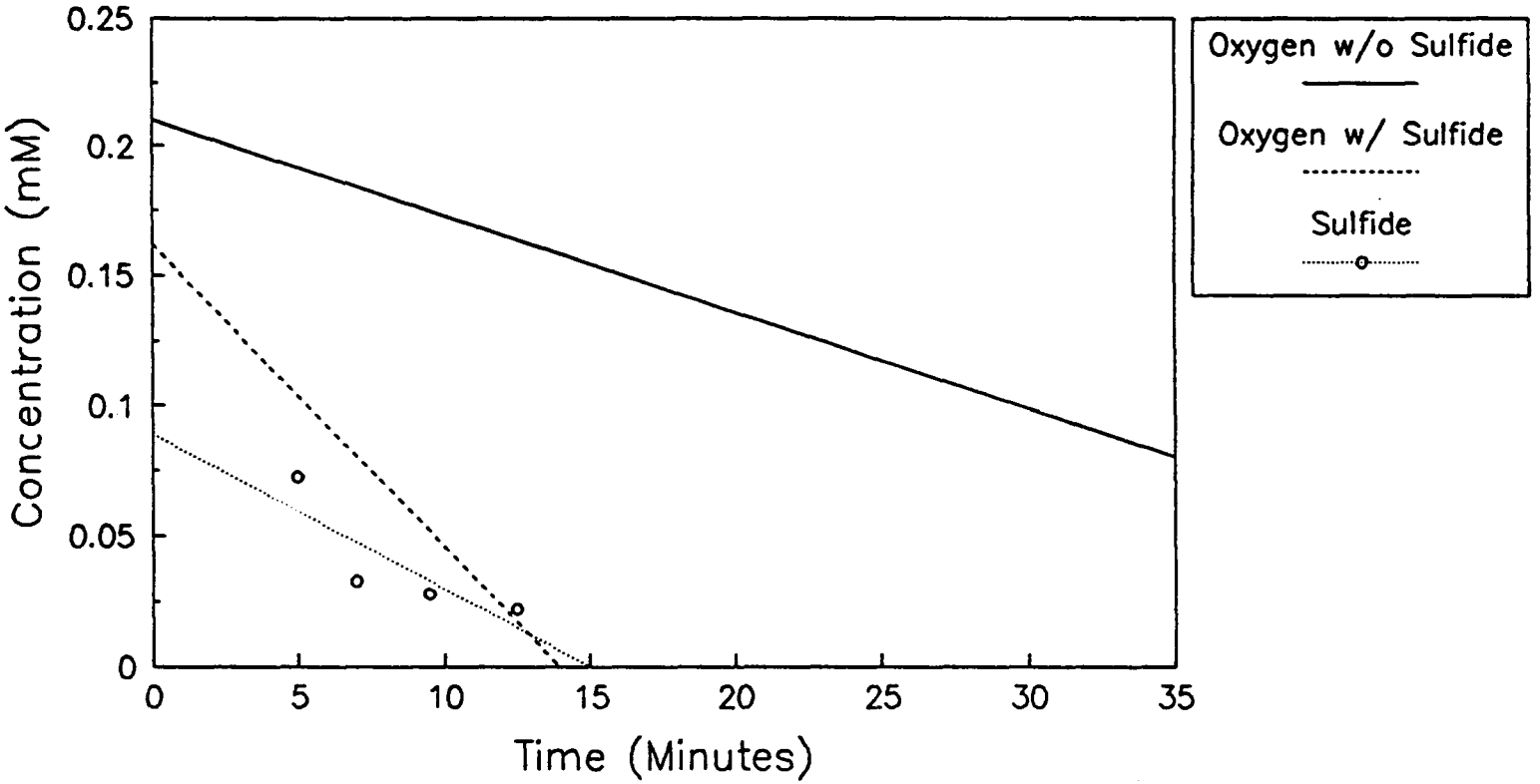


Figure 3-5. The effects of oxygen utilization with the presence of a sulfur-precititate (solid line) and the additive effect caused by the augmentation of H_2S to the batch culture (dashed line).

Isolate C8/12/1.2
Sulfur precipitate present in medium

See Table 3-10 for isolate identification

described immediately above.

Figures 3-3(d) and 3-4(c) show the effects of H_2S exhaustion on oxygen utilization rates. The straight lines for time-dependent O_2 concentration after the sulfide has been exhausted shows the decrease in O_2 utilization by 79 % and 78 %, respectively. Once again, the lines of best-fit were developed via linear regression analysis of O_2 data collected before and after exhaustion of dissolved S(-II).

3.3.4 Correlation of Cumulative Acid Production and Solution pH

Acid production as a function of pH in Medium #1 (pH_o = 6.80, Table 2-2) for various acidophilic cultures is shown in Figure 3-6. The relationship does not appear to be strain-dependent among the acidophilic isolates.

Figure 3-7 illustrates the same relationship using a non-acidophilic isolate, C8/12/2.3B (*T. neapolitanus*) in Medium #2 (see Table 2-2) at pH_o = 6.80.

3.3.5 Acid Production

Long-term experiments in which H_2S (g) was added periodically to closed, batch cultures of thiobacilli were used to investigate the stoichiometry of S(-II) oxidation/acid production among various thiobacilli isolated from LACSD sewers. Acid produced (estimated by converting pH data to cumulative acid production using Figures 3-6 and 3-7) is plotted versus cumulative H_2S addition to these cultures in Figure 3-8.

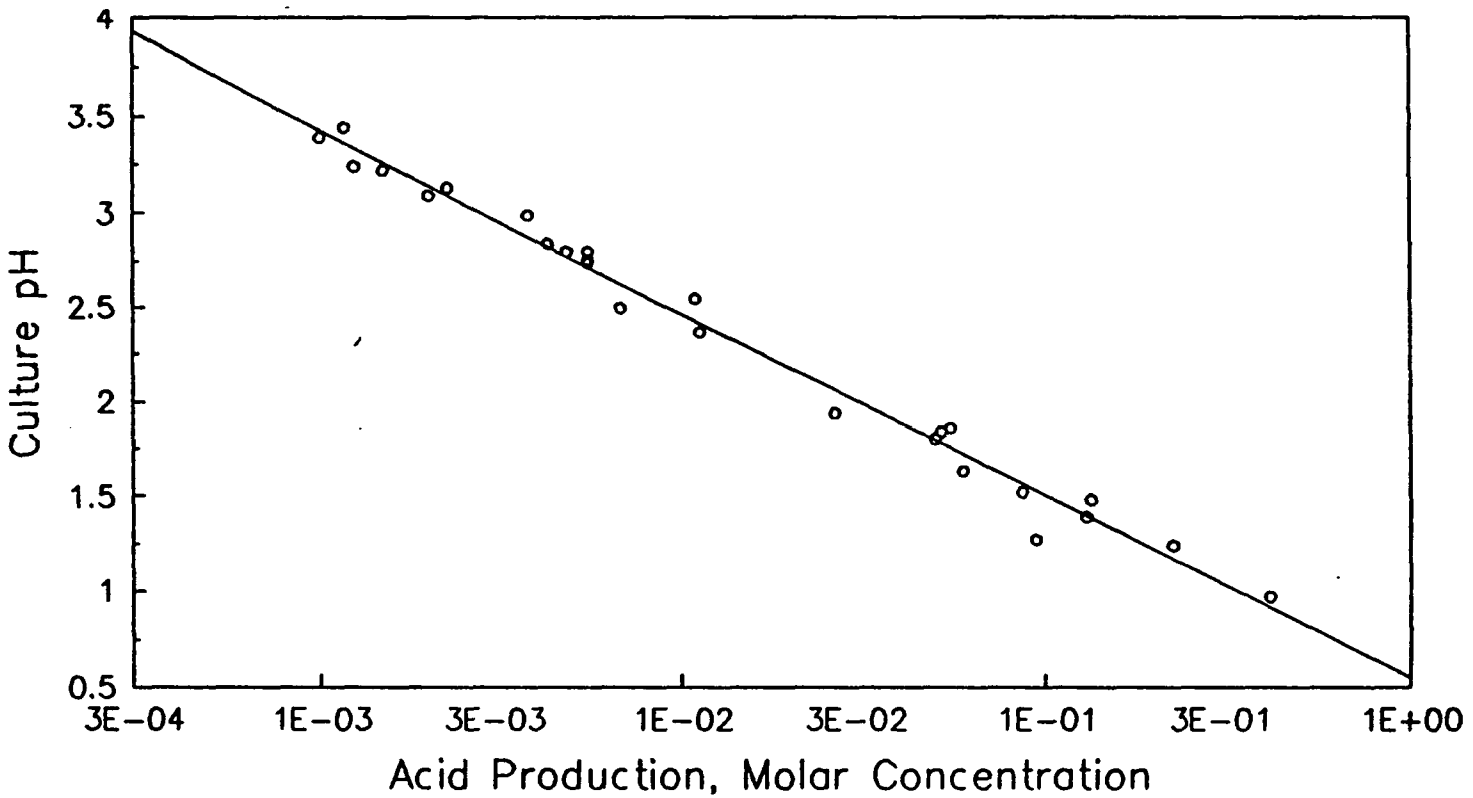


Figure 3-6. Acid production as a function of pH in several cultures of acidophilic thiobacilli cultivated in Media #1 (Table 2-2). Acid production was measured by back-titrating to the cultures original pH (3.75).

R−Squared = 0.986

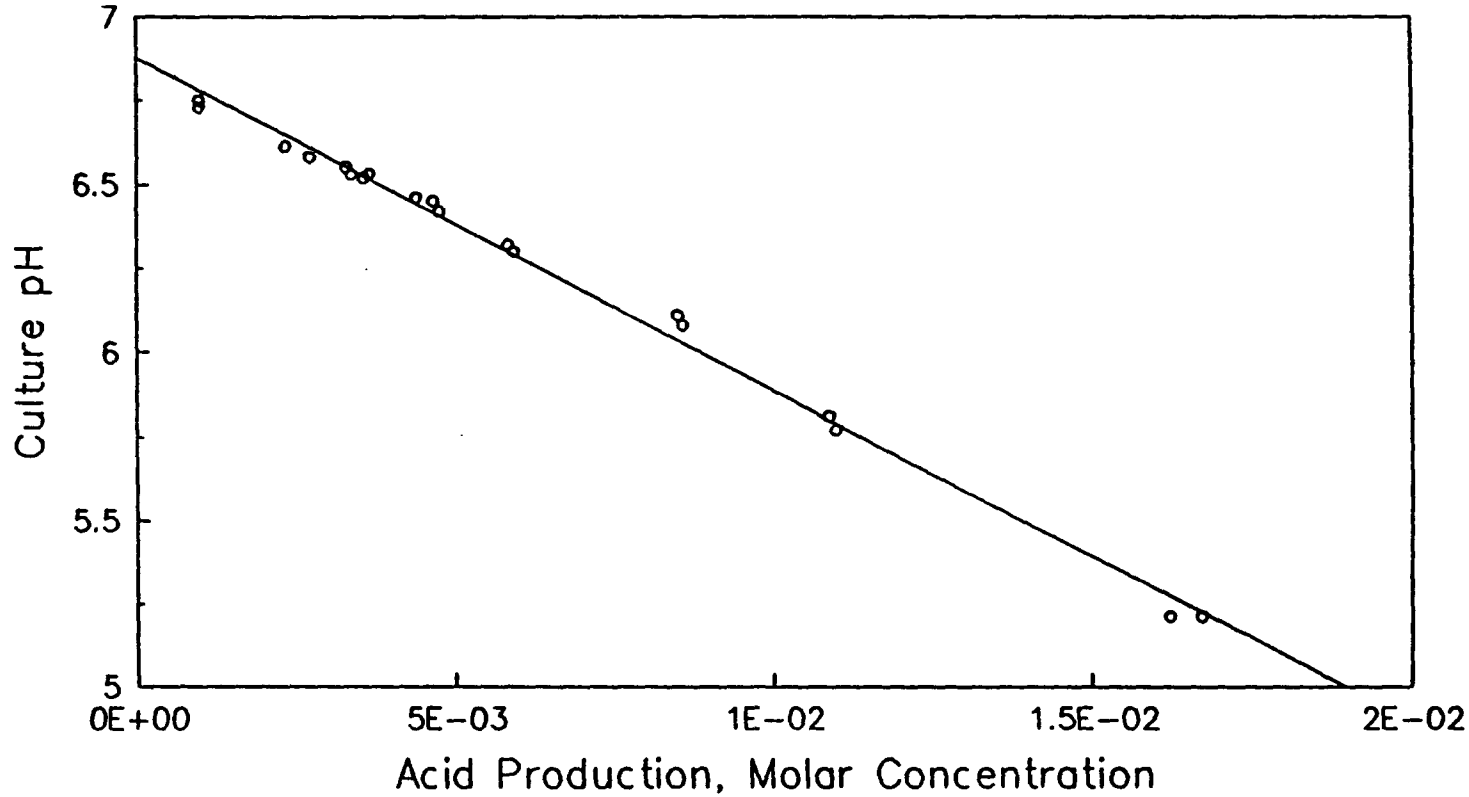


Figure 3-7. Acid production as a function of pH in a culture sewer isolate C8/12/2.3B (*Thiobacillus neapolitnus*), a non-acidophilic species. The culture was cultivated in Media #2 (Table 2-2). Acid production was measured by back-titrating to the cultures original pH (6.80).

R-Squared = 0.994

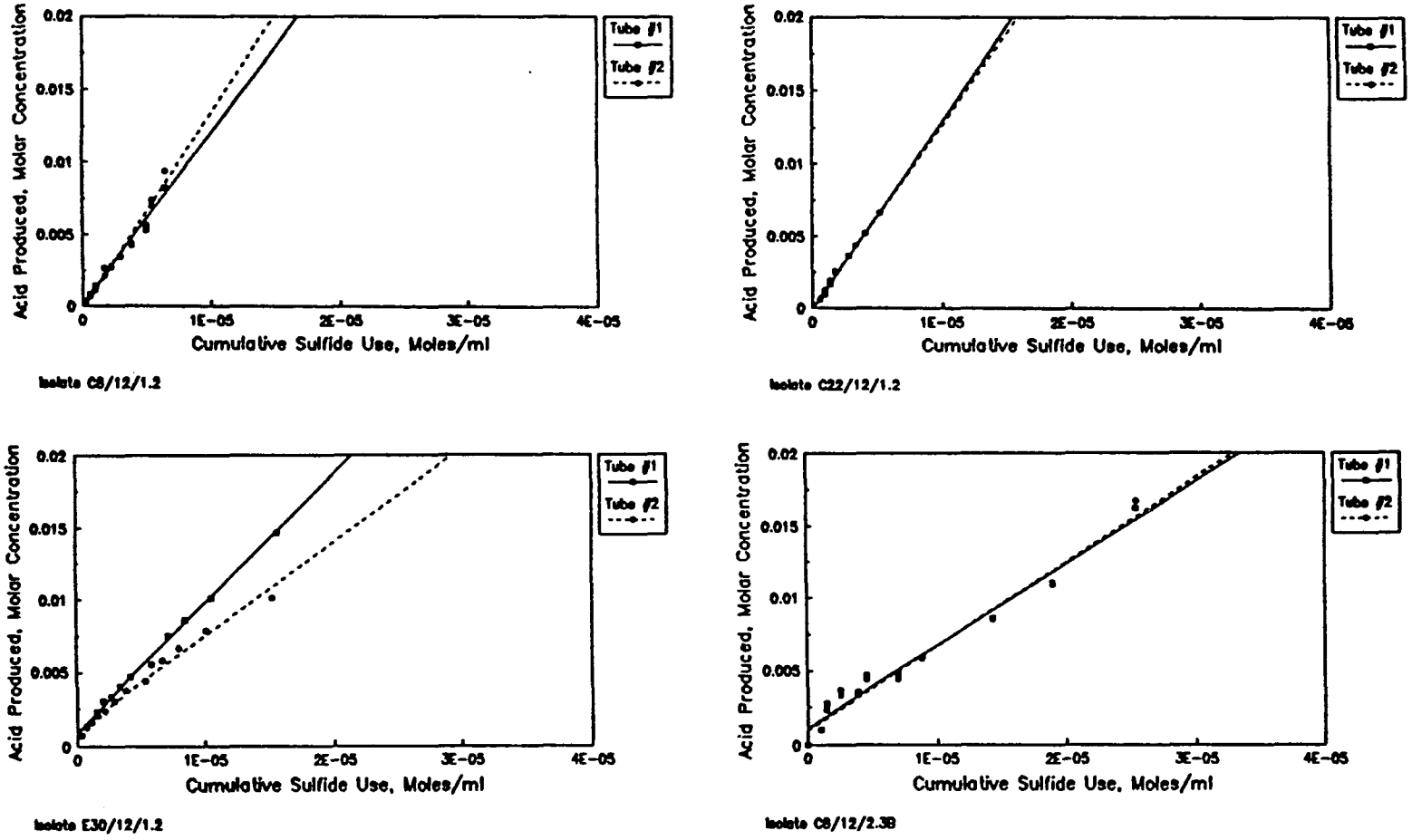


Figure 3-8. Acid production by *Thiobacillus thiooxidans* (top two and bottom left graphs) and *Thiobacillus neapolitanus* (bottom right graph) as a function of hydrogen sulfide added to closed, batch cultures of sewer crown isolates.

See Table 3-10 for isolate identification

Table 3-12 summarizes the stoichiometric relationships produced in the manner described. Acidophilic isolates collected from low-pH sewer crowns, such as C8/12/1.2 and C22/12/1.2, produced significantly more acid per mole of S(-II) oxidized than either (i) E30/12/1.2, an acidophilic strain collected from a mid-pH sewer crown (*T. thiooxidans*) or (ii) the lone non-acidophilic strain tested, C8/12/2.3B (*T. neapolitanus*).

3.3.6 The Stoichiometry of Growth

Cell number was periodically estimated via direct cell count in the same long-term experiments. Results are illustrated for the various isolates tested in Figure 3-9. Stoichiometric parameters are summarized in Table 3-12. Estimated cell yields among the acidophiles were an order of magnitude higher than the corresponding estimate for the non-acidophilic culture. The strain of *T. thiooxidans* tested, E30/12/1.2 (mid-pH crown), yielded 20-30 percent fewer new cells per mole of S(-II) oxidized than the isolates from low-pH crowns.

3.3.7 TOC and Protein Analyses

Total particulate organic carbon (TOC) and total protein were explored as surrogate growth parameters to direct bacterial counts. Only one of the three acidophilic cultures tested showed a positive correlation between TOC and H_2S utilization; results are considered inconsistent and are not

Table 3-12. Summary of stoichiometric relationships of sulfide oxidation among acid and cell production in pure, batch cultures of sewer crown isolates.

Isolate	Acid Produced per mole Sulfide (1)	Correlation Coefficient, R-squared	Cells Produced per mole Sulfide (2)	Correlation Coefficient, R-squared
C8/12/1.2				
Tube #1	1.19	0.976	2.2E+13	0.873
Tube #2	1.37	0.972	2.9E+13	0.958
E30/12/1.2				
Tube #1	0.86	0.996	2.0E+13	0.941
Tube #2	0.65	0.984	1.9E+13	0.898
C22/12/1.2				
Tube #1	1.30	0.996	3.1E+13	0.988
Tube #2	1.25	0.996	2.6E+13	0.949
C8/12/2.B				
Tube #1	0.57	0.975	2.7E+12	0.961
Tube #2	0.58	0.971	2.7E+12	0.961

See Table 3-10 for isolate designation and species level identification.

Units: (1) Equivalents acid produced per mole sulfide utilized
(2) Cells produced per mole sulfide utilized

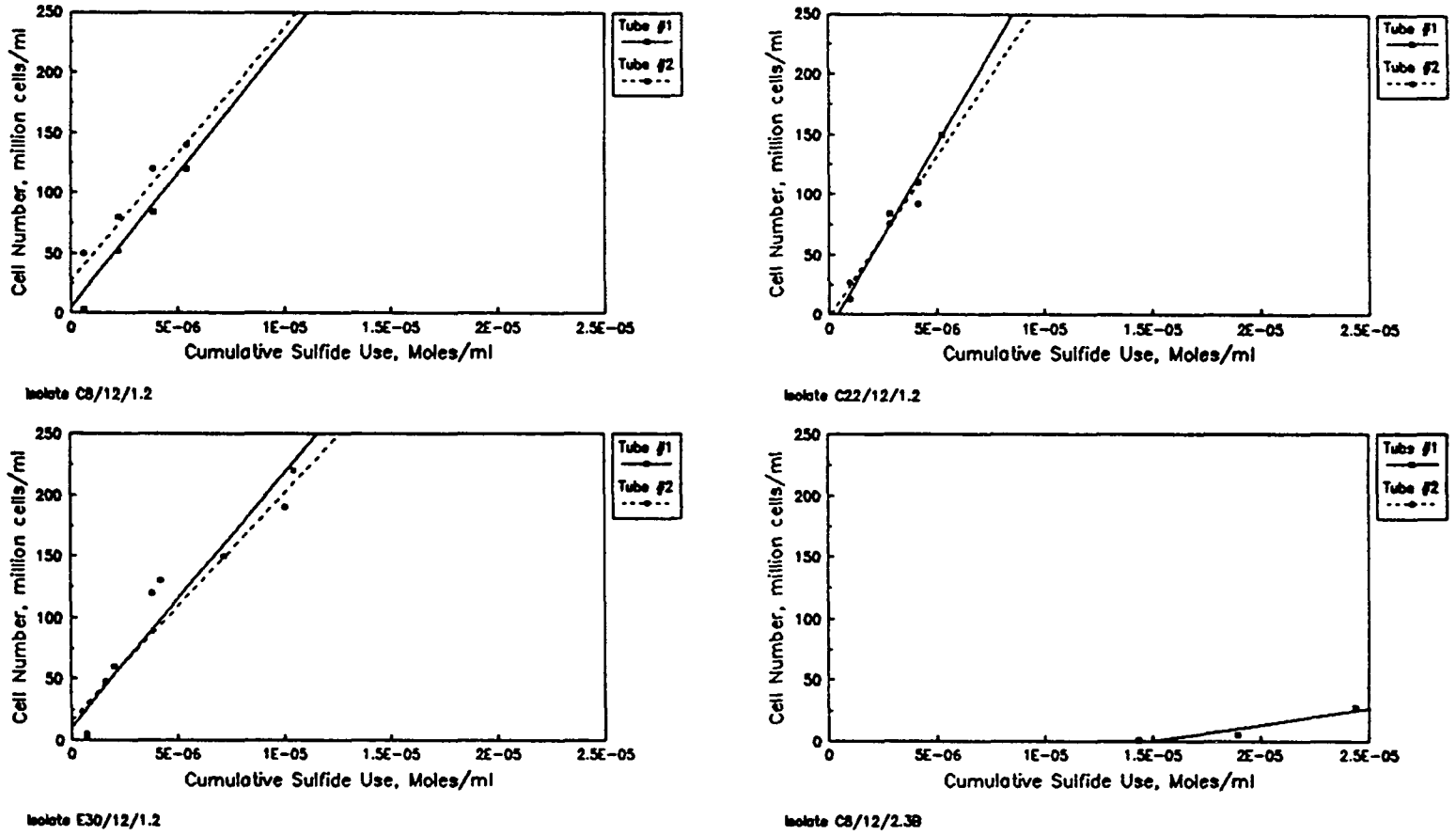


Figure 3-9. Growth of *Thiobacillus thiooxidans* (top two and bottom left graphs) and *Thiobacillus neapolitanus* (bottom right graph) as a function of hydrogen sulfide added to closed, batch cultures of sewer crown isolates. Growth was measured directly by use of a Petroff-Hauser counting chamber.

reported. Total particulate and soluble protein concentrations were not within the recommended range of the assay procedure and are not reported.

3.4 Inhibition Studies

3.4.1 General

Studies involving inhibition of thiobacillus metabolism were classified as either (i) long-term (chronic) or (ii) short-term (acute) experiments. Model inhibitors were used to explore relationships between electron transport and energy generation among the thiobacilli's mechanisms of S(-II) oxidization. Potentially toxic metals were also used in both acute and chronic toxicity experiments.

3.4.2 Cyanide Inhibition of Oxygen Demand and Sulfide Oxidation

Dissolved oxygen and total sulfide utilization rates were used to measure effects of CN^- in batch cultures of acidophilic sewer isolates. Figures representing individual tests are shown in the Appendix. Table 3-13 summarizes and Figure 3-10 illustrates the inhibitory effects of both dissolved O_2 and total sulfide utilization as a function of CN^- concentration. Cyanide concentrations of 7.5×10^{-5} M and greater inhibited oxygen utilization more than 90 %. The dependence of S(-II) oxidation rate on CN^- concentration closely followed that of culture respiration rate as shown in Figure 3-10. No difference in the CN^- tolerance of the strains of *T. thiooxidans* tested was apparent.

Table 3-13. Summary of simultaneous dissolved oxygen and total sulfide utilization rates in batch cultures of sewer crown isolates of *Thiobacillus thiooxidans* in the presence of cyanide or DCCD.

Isolate	Inhibitor (Molar concentration)	Oxygen utilization millimoles/min	Sulfide utilization Inhibited	Oxygen,mmoles/min Inhibited	Sulfide,mmoles/min ization inhibited	Percent oxygen util- utilization inhibited	Percent sulfide
C8/12/1.2	Cyanide(10E-2 M)	4.00E-03	4.25E-03	2.63E-04	1.31E-03	93	70
C8/12/1.2	Cyanide(10E-3 M)	5.86E-03	6.75E-03	0.00E+00	5.49E-04	100	92
E30/12/1.2	Cyanide (5 X 10E-4 M)	3.36E-03	3.12E-03	1.77E-04	3.45E-04	95	89
E30/12/1.2	Cyanide(10E-4 M)	9.85E-04	1.28E-03	1.01E-05	1.80E-04	99	86
C8/12/1.2	Cyanide(10E-4 M)	1.16E-02	5.95E-03	2.96E-05	9.44E-04	100	84
E30/12/1:2	Cyanide (7.5 X 10E-5 M)	7.48E-03	6.16E-03	5.55E-04	5.74E-04	93	91
E30/12/1.2	Cyanide (5 x 10E-5 M)	4.35E-03	4.16E-03	6.72E-04	8.27E-04	85	80
E30/12/1.2	Cyanide (2.5 X 10E-5 M)	1.43E-02	1.15E-02	4.71E-03	5.72E-03	67	50
E30/12/1.2	Cyanide(10E-5 M)	1.97E-03	2.88E-03	1.89E-03	2.86E-03	4	1
C8/12/1.2	DCCD (1 X 10E-4 M)	1.39E-02	1.20E-02	1.39E-02	1.19E-02	0	0
C8/12/1.2	DCCD (1 X 10E-5 M)	1.39E-02	1.18E-02	1.39E-02	8.62E-03	0	27

See Table 3-10 for isolate designation and species level identification.

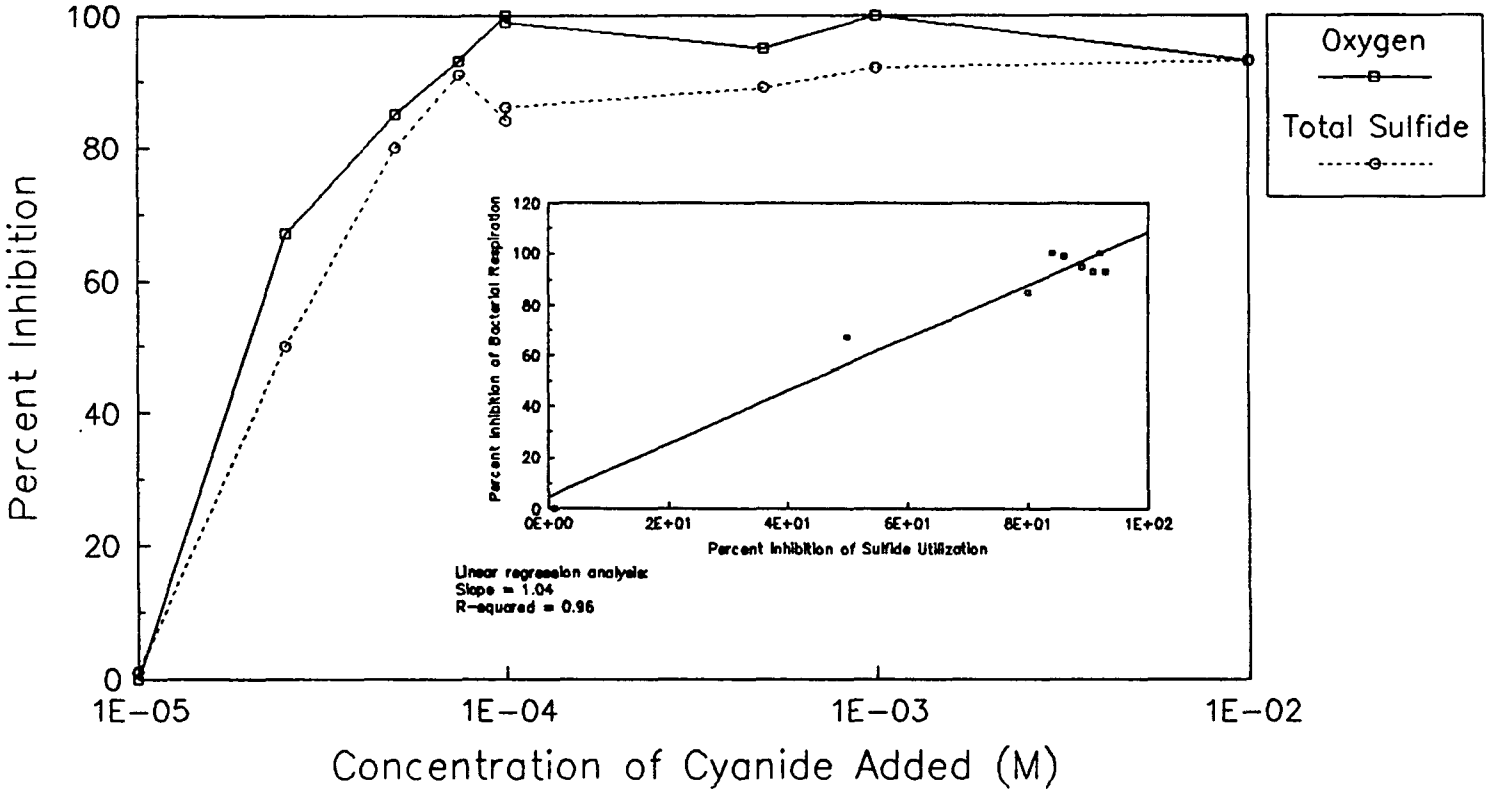


Figure 3-10. Percent of dissolved oxygen and total sulfide utilization inhibited in batch cultures of *T. thiooxidans* when exposed to various concentrations of cyanide.

3.4.3 DCCD Inhibition

DCCD prevents oxidative phosphorylation by blocking the trans(cyto)plasmic membrane transfer of protons via membrane-bound ATPase. The effects of acetone, 10^{-4} M, and 10^{-5} M DCCD on O_2 utilization by *T. thiooxidans* are illustrated in Figure 3-11 and summarized in Table 3-13. Because of its limited solubility in water, acetone was used to dissolved the chemical prior to its addition to growing cultures. Sulfide utilization rates were not reported because control sulfide data were not considered reliable. Culture respiration was essentially undisturbed by addition of DCCD in the 10^{-5} to 10^{-4} M range.

3.4.4 Acute Inhibition Metals Oxyanions

The acute effects of molybdate (MoO_4^-) and orthovanadate (VO_4^-) on respiration by *T. thiooxidans* are illustrated in Figures 3-12 and 3-13, respectively. High concentrations of these metals (10^{-2} M MoO_4^- and 10^{-3} M VO_4^-) increased the rate of oxygen utilization in these cultures. Addition of 1 X 10^{-3} M or more of either metal was generally accompanied by an immediate disappearance of dissolved sulfide and an abrupt increase in solution turbidity, suggesting that metals addition was responsible for either the formation of insoluble S(-II) complexes or the oxidation of S(-II) to S^o. Some of the lower concentrations of Mo(VI) and V(V) tested showed "delayed" inhibitory effects. The rate of O_2 utilization gradually decreased during the course the experiment. Best-fit representations of S(-II) and O_2 utilization rates are summarized in Table 3-14. Inhibition

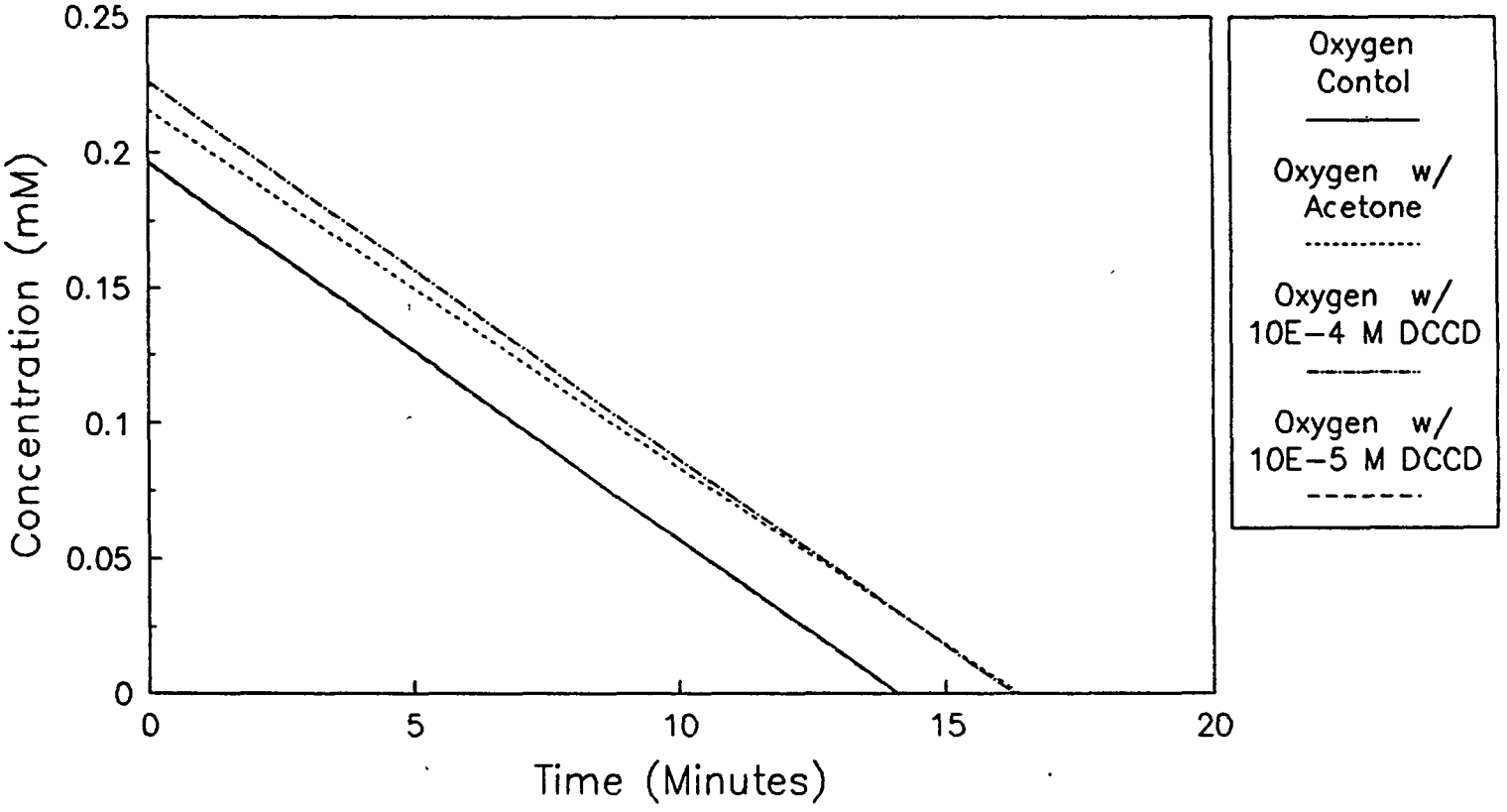


Figure 3-11. The effect of acetone, 10^{-4} M, and 10^{-5} M, DCCD on dissolved oxygen and total sulfide rates of *Thiobacillus thiooxidans*.

Isolate C8/12/1.2
See Table 3-10 for isolate identification

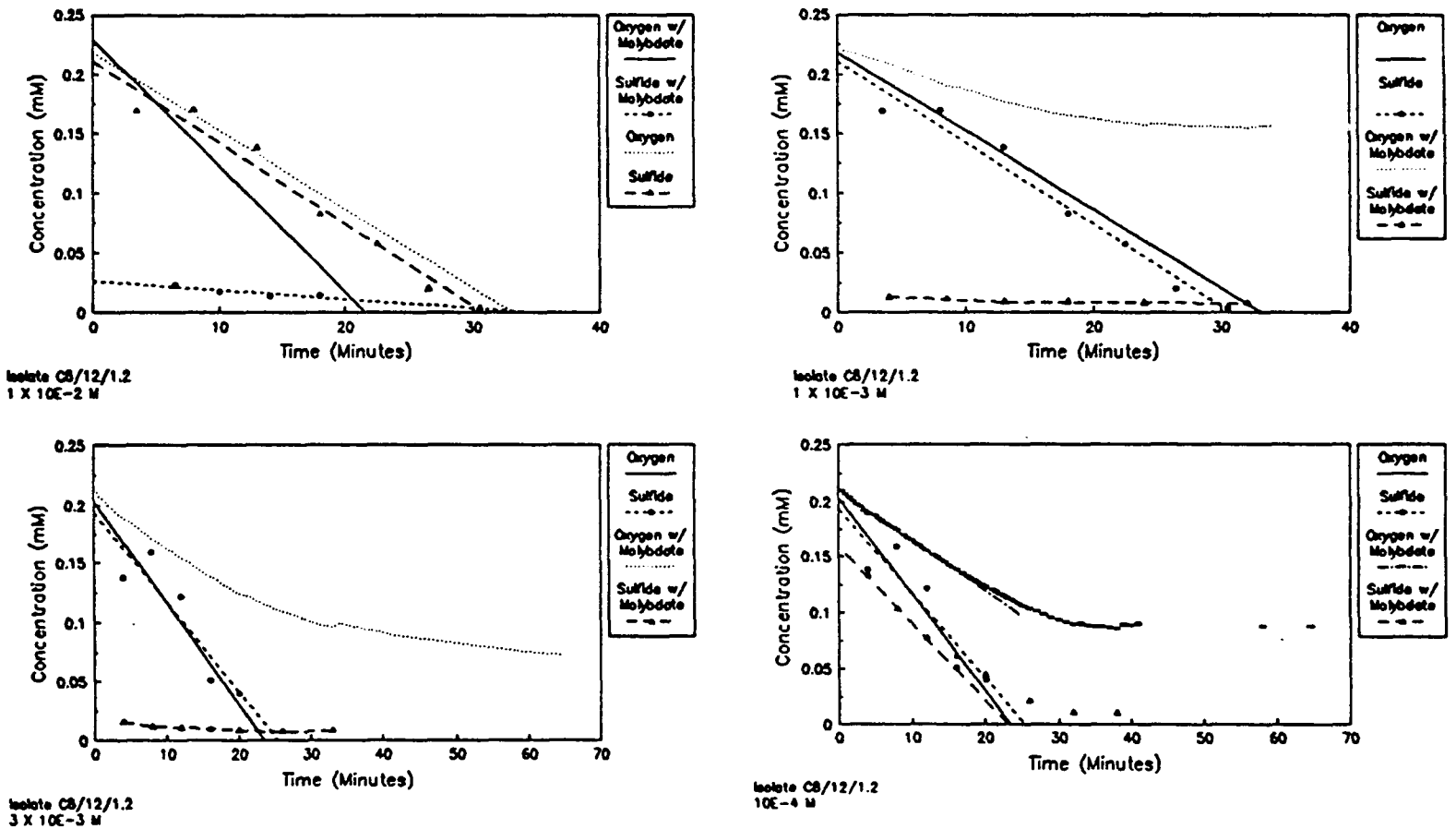


Figure 3-12. The effect of various concentrations of molybdate (MoO_4^-) addition on the dissolved oxygen and total sulfide utilization rates of *Thiobacillus thiooxidans*.

See Table 3-10 for isolate identification

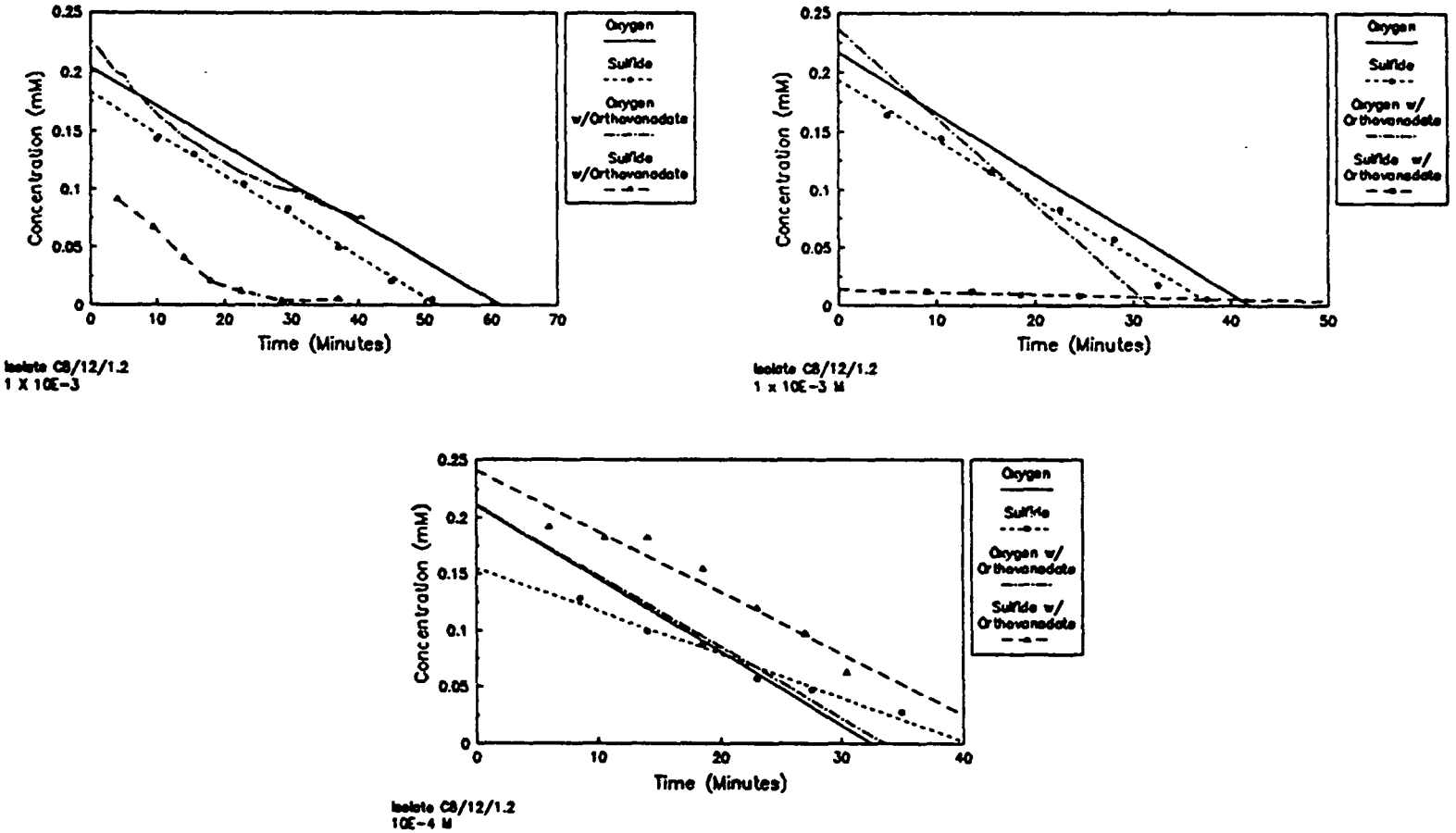


Figure 3-13. The effect of various concentrations of orthovanadate (VO_4^-) addition on the dissolved oxygen and total sulfide utilization rates of *Thiobacillus thiooxidans*.

See Table 3-10 for isolate identification

Table 3-14. Summary of simultaneous dissolved oxygen and total sulfide utilization rates in batch cultures of sewer crown isolates of *Thiobacillus thiooxidans* in the presence of molybdate or orthovanadate.

Isolate Used	Inhibitor Concentration, molar	Oxygen utilization (moles/liter-min.)			Sulfide utilization (moles/liter-min.)		
		Uninhibited	Inhibited	% Reduction	Uninhibited	Inhibited	% Reduction
C8/12/1.2	Molybdate 1 X 10E-2 M	6.597E-03	1.060E-02	(61)	6.850E-03	7.530E-04	89
C8/12/1.2	Molybdate 3 X 10E-3 M	8.652E-03			7.604E-03		
C8/12/1.2	Molybdate 1 X 10E-3 M	5.178E-03	7.474E-03	(44)	5.078E-03	1.950E-04	96
C8/12/1.2	Molybdate 1 X 10E-3 M	6.597E-03			6.850E-03		
C8/12/1.2	Molybdate 1 X 10E-4 M	8.652E-03	4.500E-03	48	7.604E-03	3.190E-03	58
C8/12/1.2	Orthovanadate 1 X 10E-3 M	3.220E-03			3.523E-03		
C8/12/1.2	Orthovanadate 1 X 10E-4 M	6.477E-03	3.827E-03	41	6.280E-03	5.373E-03	9

See Table 3-10 for explnation of isolate designation.
Blank spaces represents a nonlinear utilization rate during the period of the test.

values in parenthesis represent an increase of the respective utilization rates. Linear regression analyses were not conducted for the cultures showing the delayed inhibition effect.

3.4.5 Chronic Metals Toxicity

Elemental sulfur was provided as growth substrate in all the chronic metals toxicity tests (Medium #1, Table 2-2). In such tests, media were inoculated at pH of 3.75, and metals were added after sufficient acid had been generated to drop the pH to approximately 3.5. Table 2-5 lists the metals screened in this manner. Figures 3-14 through 3-24 illustrate the time-dependent acid production of cultures exposed to various metals.

Generally, the various metals either showed complete inhibition, no inhibition, or an acclimated (or lag in acid production) effect. Complete inhibition is shown as an approximately horizontal line, signifying no change of medium pH over the time period of the experiment. If the medium pH did not differentiate from the control medium, no inhibition was assumed. The acclimated effect was observed by a temporary suspension of acid production immediately after metals addition. Growth then recommenced as measured by the pH decrease of the medium.

Figures 3-20 and 3-21 illustrate the effects of silver on thiobacillus activity in both a standard basal salts medium (Table 2-2) and a chloride-free basal salts medium (Table 2-5), respectively. The effects on acid production by *T. thiooxidans* exposed to various concentrations of metals elixir (Table 2-6) is illustrated in Fig-

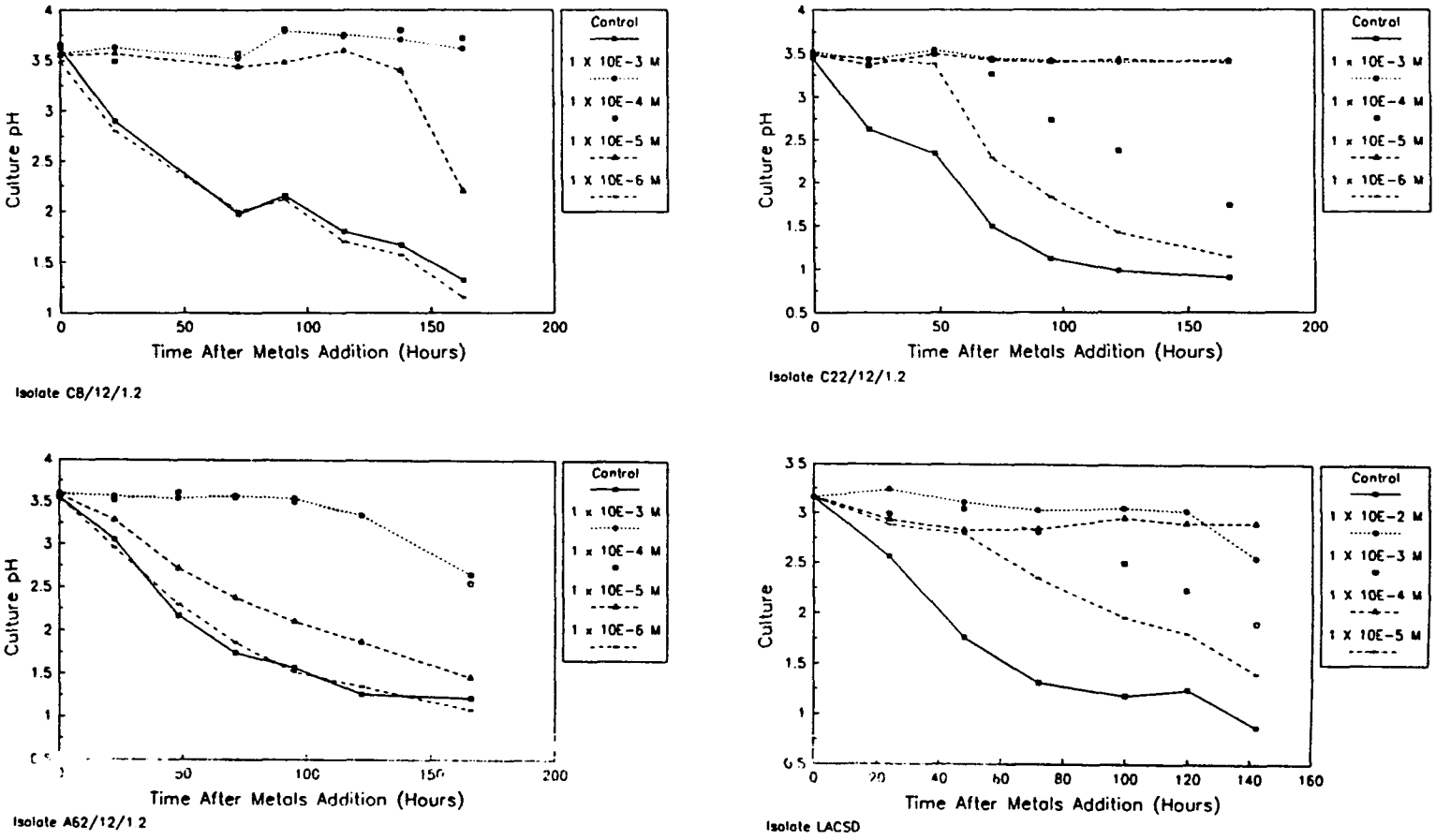
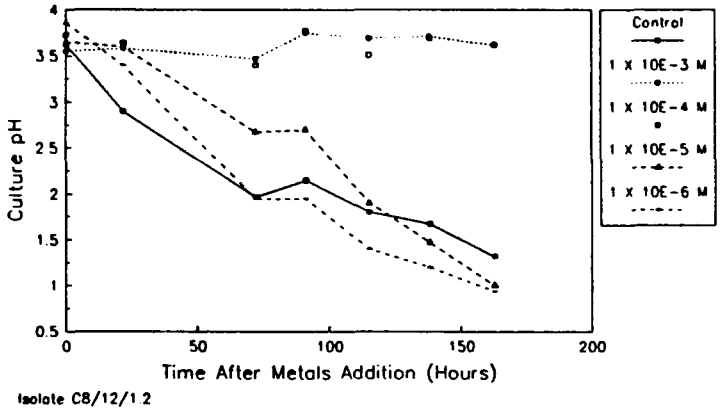
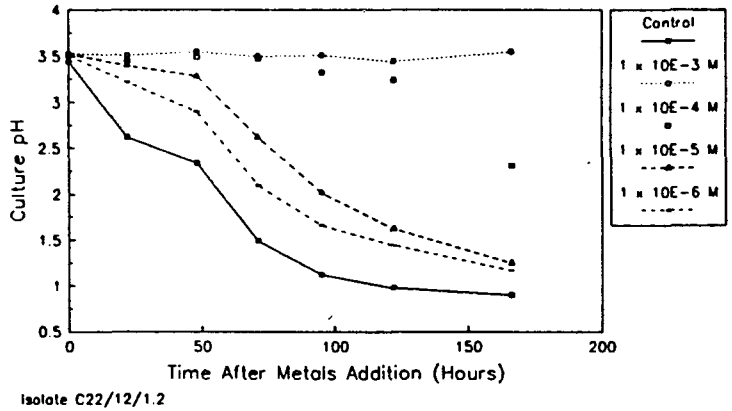


Figure 3-14. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with molybdate [Mo(VI)].





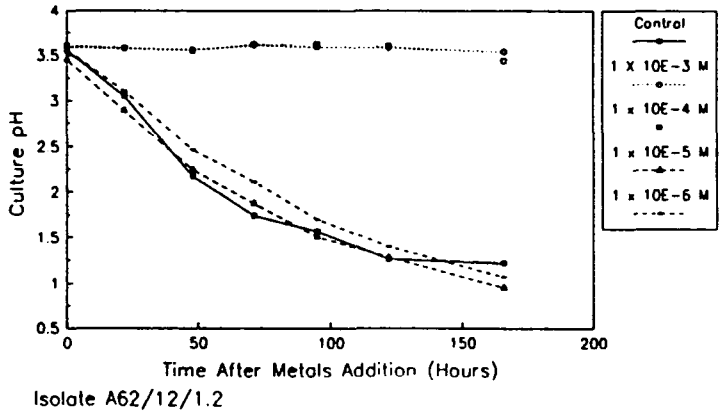


Figure 3-15. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with orthovanadate [V(V)].

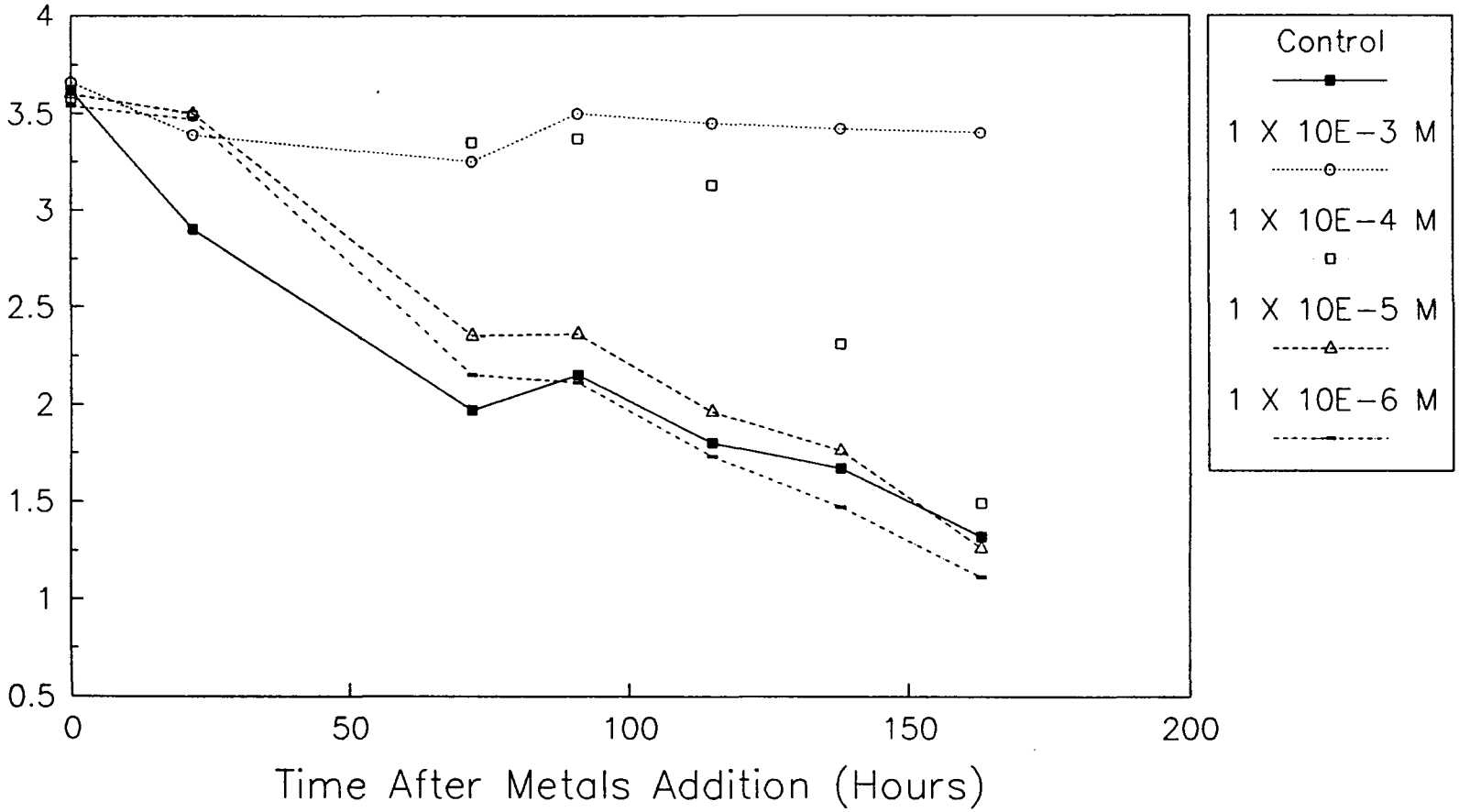
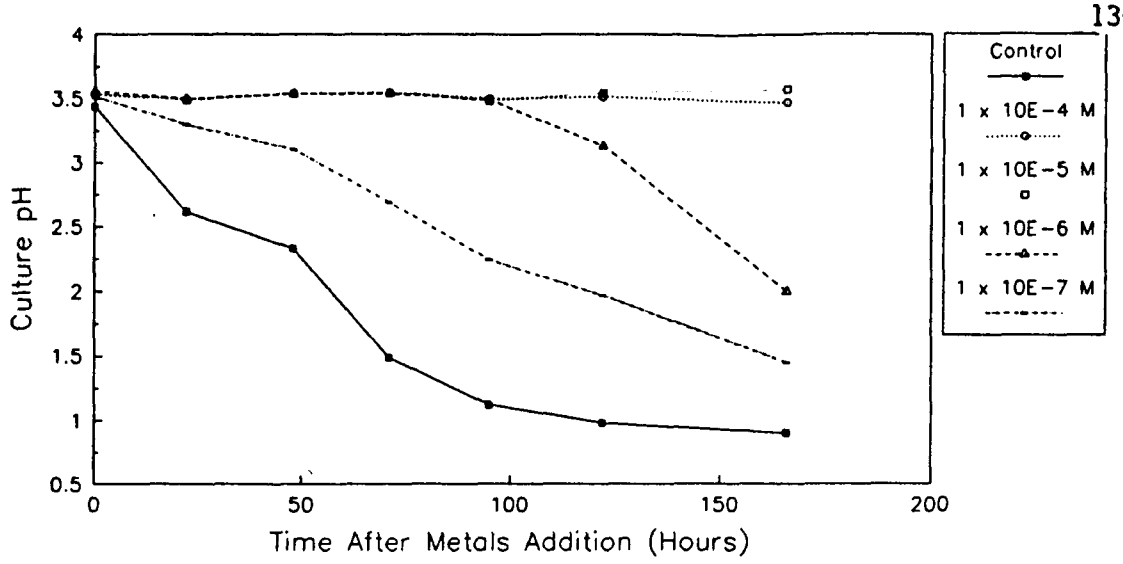
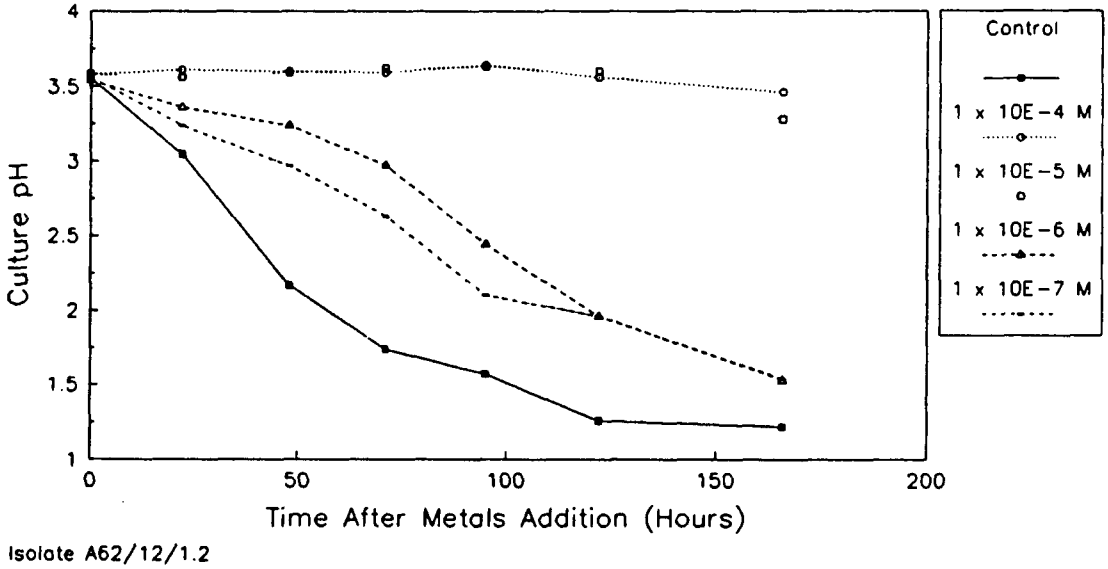


Figure 3-16. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with cadmium [Cd(II)].

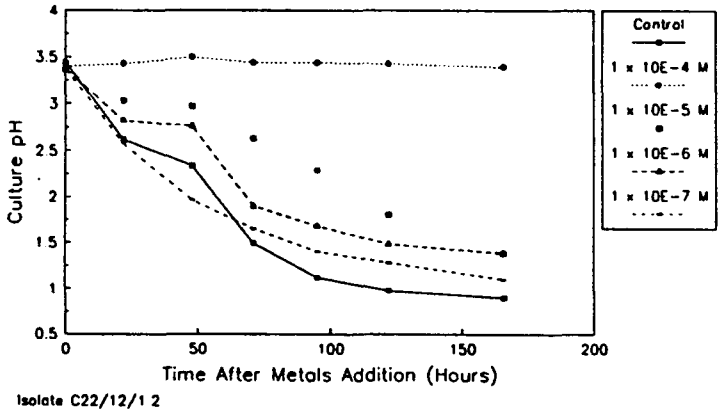


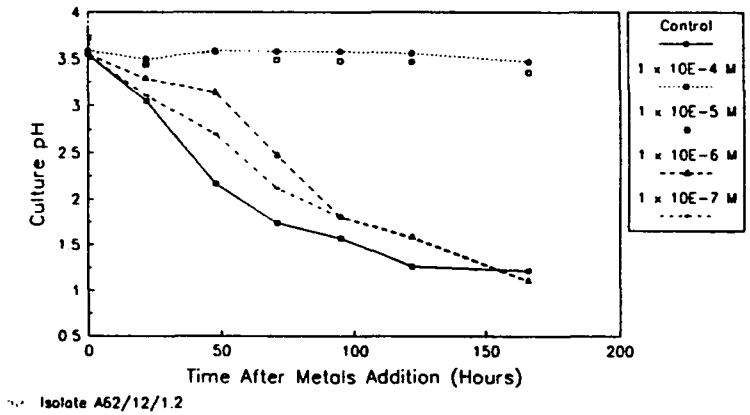
Isolate C22/12/1.2



Isolate A62/12/1.2

Figure 3-17. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with mercury [Hg(II)].





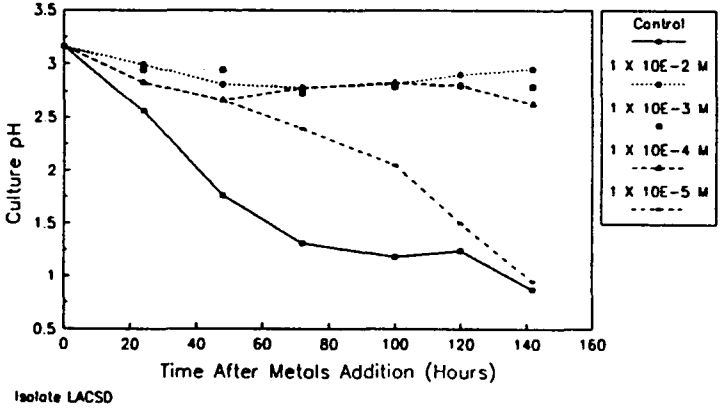
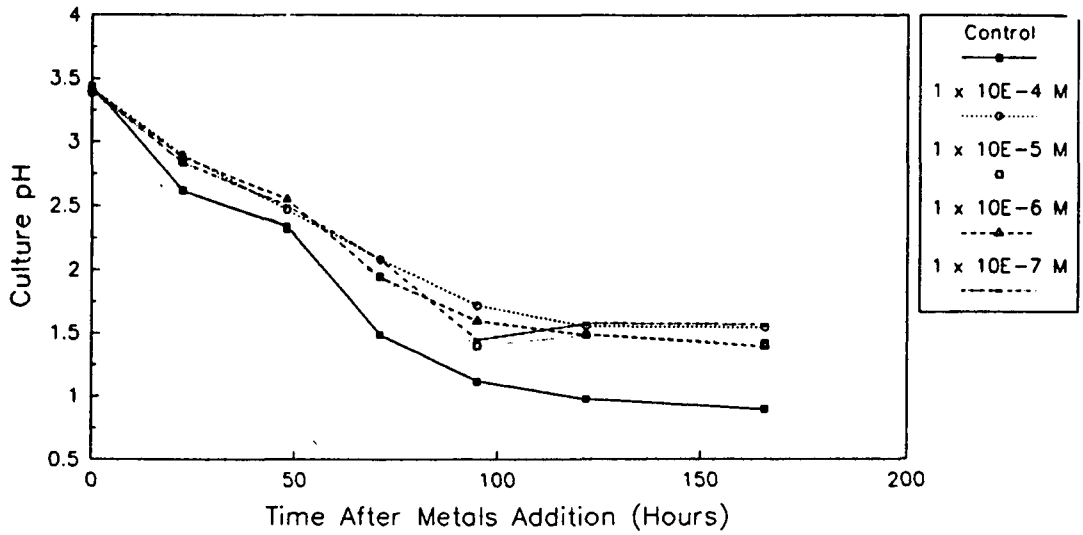
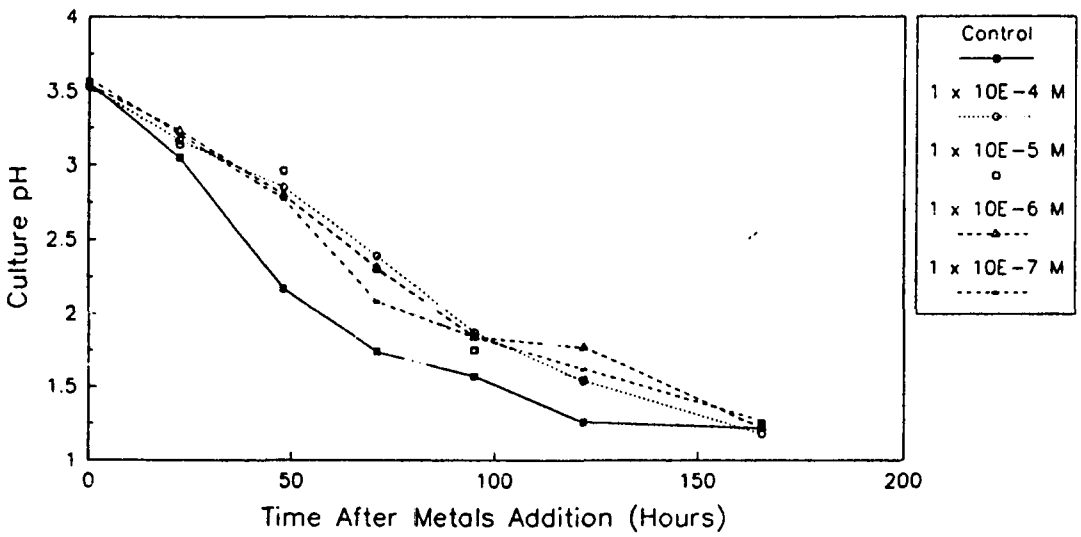


Figure 3-18. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with dichromate [Cr(VI)].

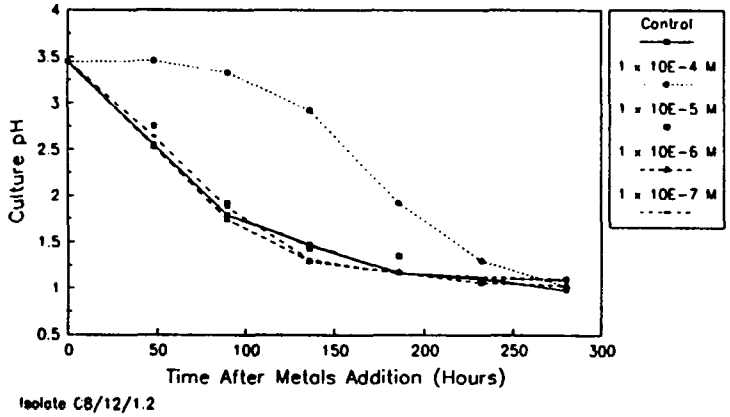


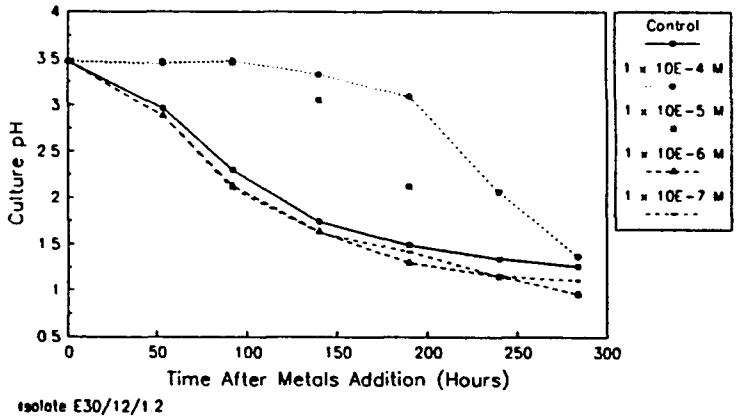
Isolate C22/12/1.2



Isolate A62/12/1.2

Figure 3-19. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with zinc
[Zn(II)].





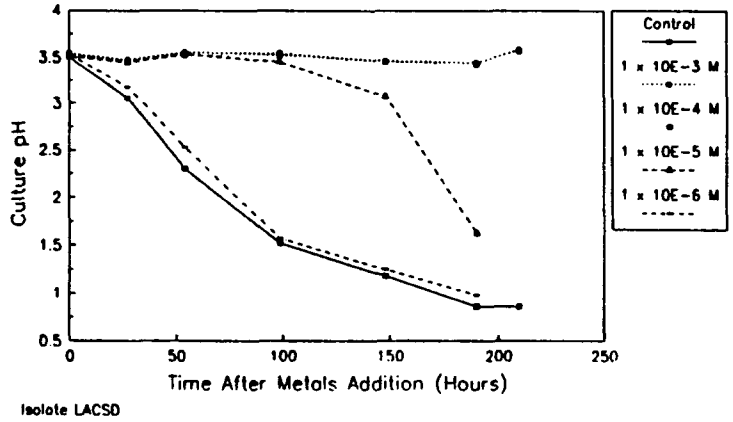
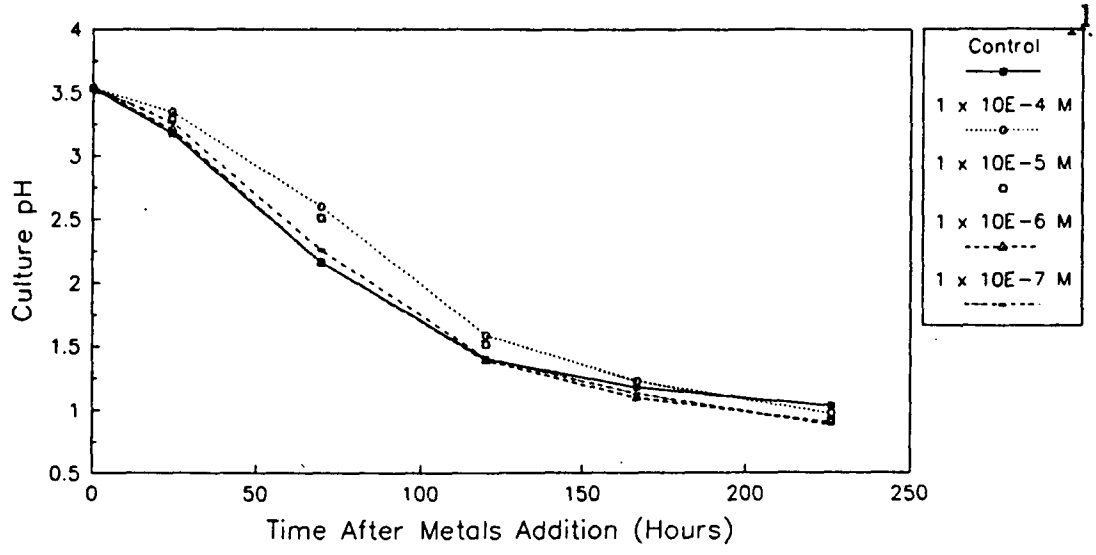
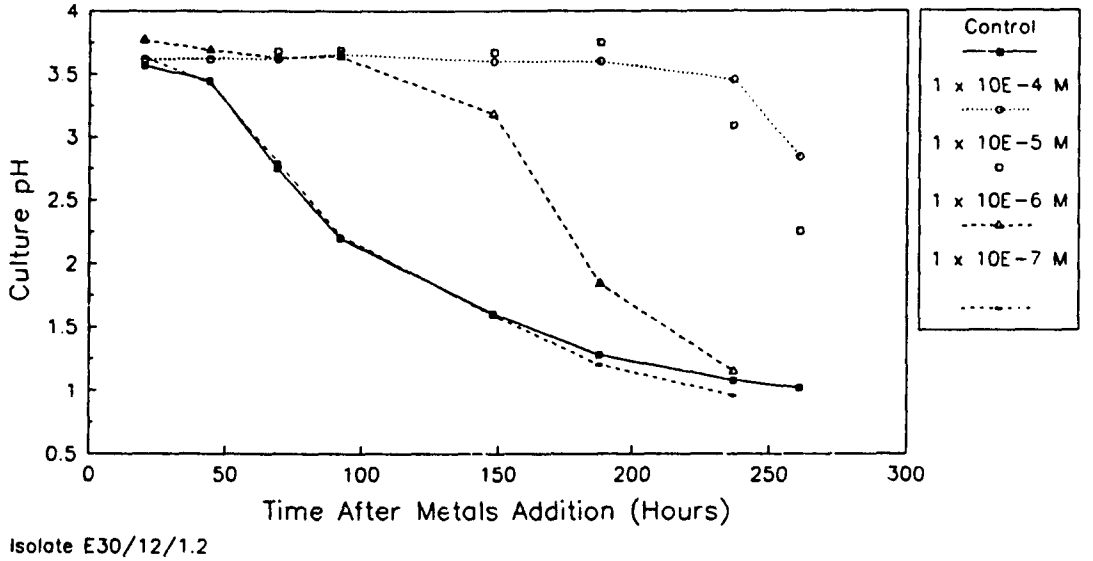


Figure 3-20. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with silver [Ag(I)] in chloride-free medium.

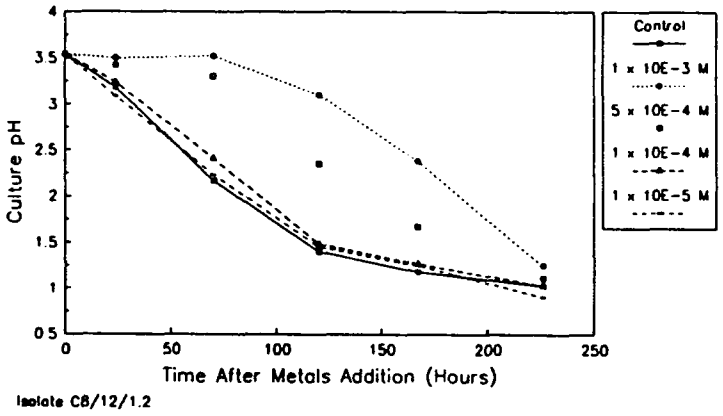


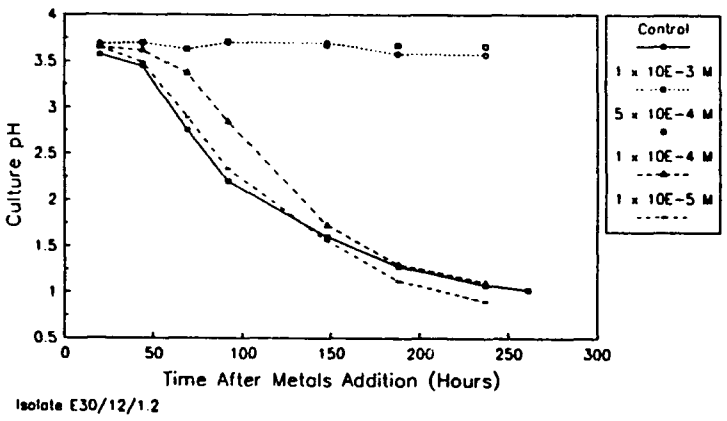
Isolate C8/12/1.2



Isolate E30/12/1.2

Figure 3-21. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with silver [Ag(I)] in medium containing chloride salts





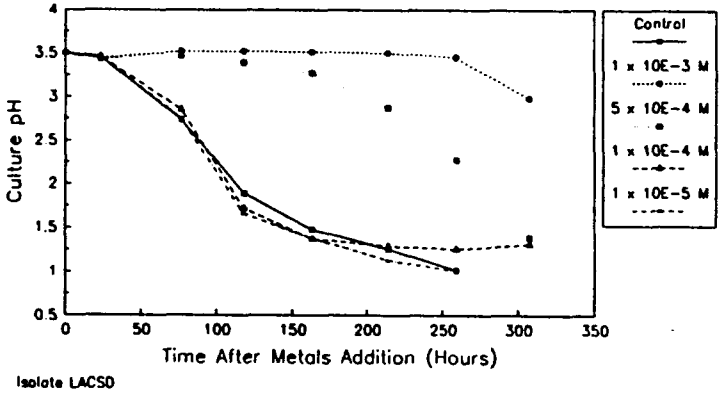
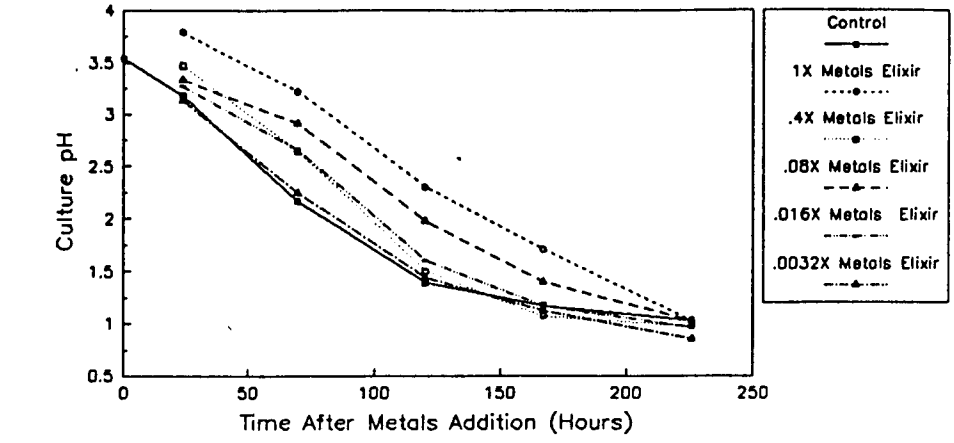
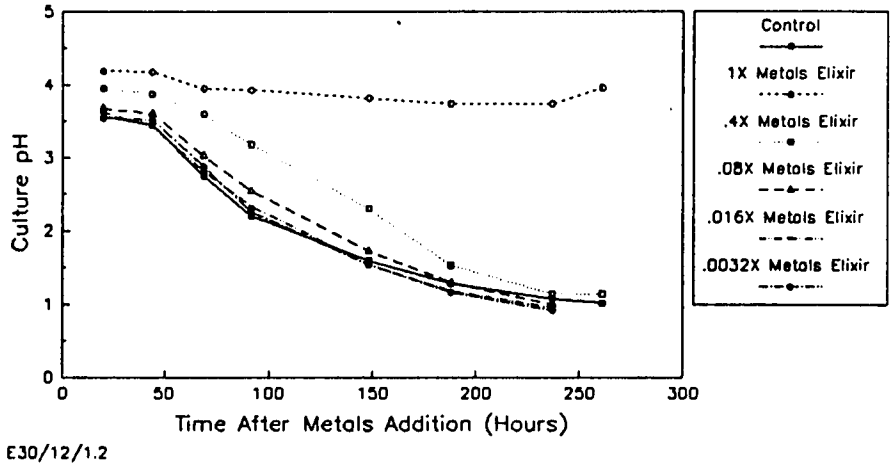


Figure 3-22. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with copper [Cu(II)].



Isolate C8/12/1.2



Isolate E30/12/1.2

Figure 3-23. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with metals elixir.

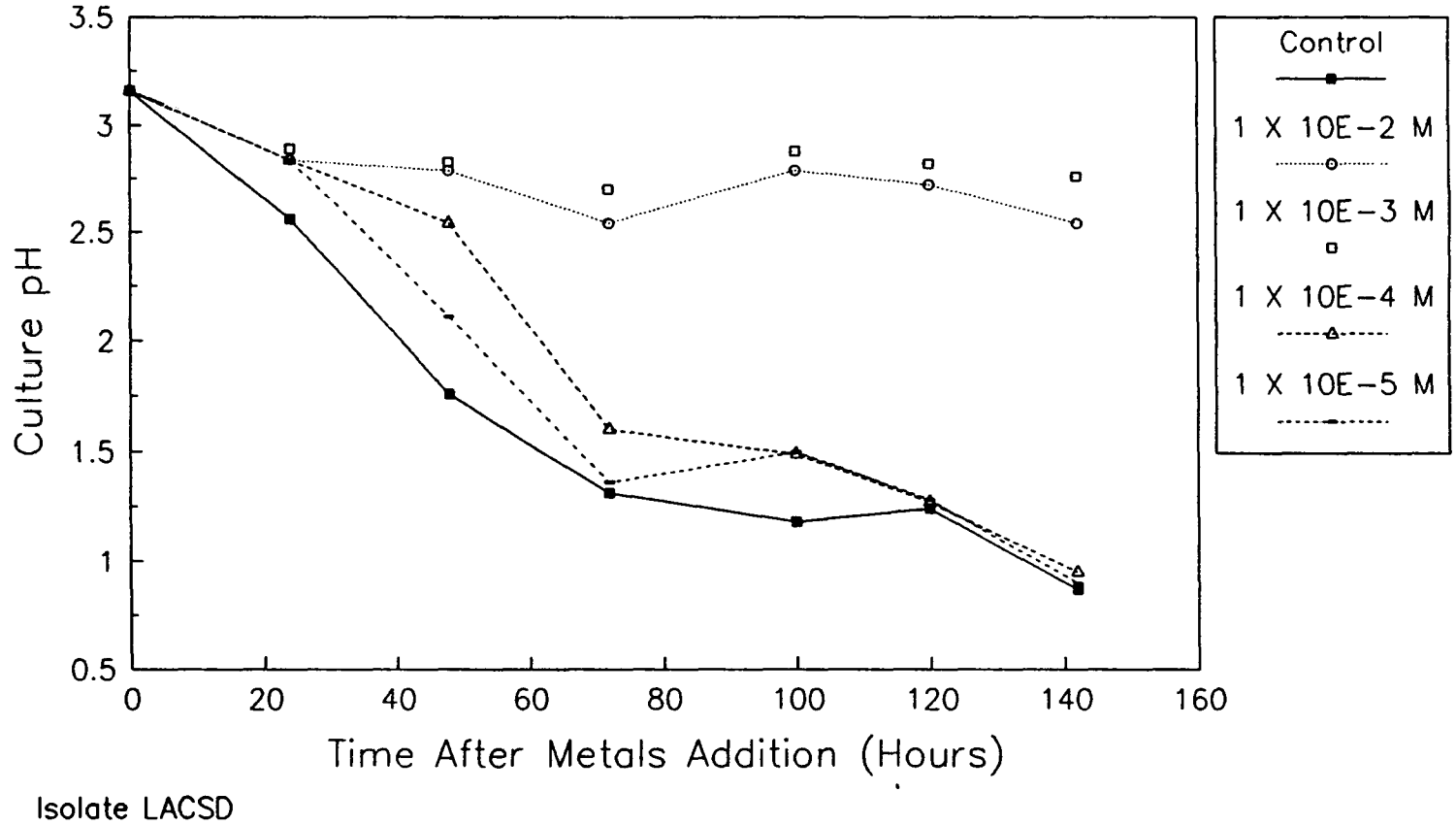


Figure 3-24. Dependence of pH on time and metals concentrations in pure, batch cultures of sewer crown isolates (as noted) amended with cobalt [Co(II)].

ure 3-23. Metals concentrations present in the elixir are given as fractions of the metals composition listed in Table 2-6.

Non-acidophilic isolates acclimated to grow on H_2S proved unable to grow on Medium #2 (mid-pH with $S_2O_3^{-2}$) in this experiment. However, the use of H_2S in metal inhibition tests was precluded due to insolubility of many metal-sulfide complexes. Therefore, toxic effects of transition metals on non-acidophilic thiobacilli isolates were not investigated.

CHAPTER 4

DISCUSSION

4.1 Sample Collection and Cultivation

4.1.1 Crown Conditions

The surface pH and corrosion of sewer crowns at sample collection locations were inversely related. This suggests that the increased rate of acid production at low-pH areas has increased the rate of H_2SO_4 conversion to gypsum, presumably during the past 10 years. The decrease of crown pH appeared to enhance the growth of acidophilic thiobacilli, the taxonomic group generally responsible for acid production.

One type of pipe used by LACSD, spun reinforced concrete pipe, has a greater buffer capacity at its inner surface. However, this buffer capacity decreases with depth into the concrete. The buffer capacity has probably been depleted at the inner surface of severely corroded pipes such as those which comprise joint outfall "C" of the LACSD system. The remaining concrete, with less alkalinity, is consumed at a faster rate in order to balance the same rate of acid production.

Measured pH were generally the lowest at the pipe crown, increasing to normal values in the slime layer close to the bulk-liquid surface. Restoration of pH was probably caused by the periodic submergence and washing of this area by sewage due to diurnal variation of the sewage flow

rate and depth. The slime layer itself might contain additional buffer capacity which can neutralize H_2SO_4 before the acid attacks the concrete, therefore, conserving the pipe's alkalinity and protecting its integrity.

The pipe crest (12:00 o'clock position) was generally dry in all sampling locations. The lack of moisture and low-pH did not appear to reduce the activity of thiobacilli present. Numerous heterotrophs were also present at the pipe crowns with low-pHs, suggesting that they could co-exist with thiobacilli in acidic environments. Heterotrophic samples from the pipe crowns grew well in neutral-pH media, without acid generation (data not shown).

Manhole A62 showed isolated areas of heavy corrosion, and the pH of the crown decreased between 0.5 and 1.0 pH units in the four months between measurements. Mortar which secures the pipe tiles has deteriorated, presumably via acid-induced corrosion. The tiles which remain intact appear to have resisted corrosion; they have been exposed to H_2S for over 60 years with little effect. Manhole E30 showed very little corrosion; its crown pH was between 6.0 and 7.0.

Since joint outfall sewer lines E and C (JO "C" and JO "E") were constructed within a few years of each other (E in 1952 and C in 1947), sewage-retention time could be a factor in the corrosion of these sewers (JO "E" is much shorter, see Figure 1-12). Long retention times result in the conversion of particulate biological oxygen demand (BOD) to soluble BOD, which is able to penetrate the anaerobic depths in the slime layer more successfully (Tchobanoglous, 1989) and promote sulfate reduction. As a result of greater anoxic activity, higher S(-II) concentrations are

produced; this could be the major factor in the accelerated corrosion rates observed in JO "C" during the past decade.

4.1.2 Culture Cultivation of November, 1987 Samples

Initial enrichment media used for the November, 1987 sampling program produced dense, mixed cultures. However, the first-transfer enrichments were less successful. Only thirteen of the 56 first-transfer cultures showed a decrease of pH and/or increase in cell density. This indicates that either (i) sewer thiobacilli could not use the nutrients provided or (ii) basic media were toxic to the species of interest. The media were identical to those used successfully in the Hamburg, W.G. study. On that basis, it was initially assumed that sewer acclimated thiobacilli were able to metabolize the enrichment substrate.

Mid-pH Samples. Many of the mid-pH media were supplemented with a trace metals solution. Metals could have provided a "synergistic" toxic effect to the culture. A few of the individual metals in solution were probably not bactericidal since metals concentrations in the medium were less than the concentrations found to be toxic to low-pH isolates by other researchers (Jack, 1980). The inability to grow non-acidophilic isolates without H_2S as the electron donor for subsequent chronic metals toxicity tests precluded the attempts to determine if trace metals inhibited growth in these initial cultures.

Low-pH Samples. The inability to grow the collected samples in low-pH medium with $S_2O_3^{-2}$ (Media B, Table 2-1) did not duplicate previous efforts in this laboratory using a purchased strain of *T. thiooxidans* ATCC #8085 (Sima, 1988). The low-pH media was initially adjusted to 4.5, which was considered to be the highest pH the acidophiles, *T. thiooxidans* and *T. ferroxidans*, would initiate growth. It is possible that cultures collected from the crowns were acclimated to a more acidic environment (that below pH 2.0) and unable to initiate growth at the pH of the medium. Therefore, more samples were collected and cultivated using modified enrichment media (as described in Table 2-2).

4.1.3 Media Modification

To avoid duplication the unsuccessful sewer sample cultivation attempts of the November, 1987 sampling program, changes were made in the media design. Possible sources of problems in the original media were the presence of trace metals and initial pH of the acidic media. Therefore, trace metals solutions were deleted from the recipe(s) used for mid-pH media and the pH for acidic media was lowered to 3.5. Growth of acidophilic thiobacilli in this laboratory also appeared optimum when elemental sulfur replaced thiosulfate as substrate in low-pH media.

4.1.4 Culture Cultivation of March 1988 Samples

Low-pH Samples. Two acidophilic media were used in the March, 1988 sampling program -- Medium #1 (S° as substrate) and Medium #3 ($S_2O_3^{-2}$ as

substrate). The S° medium supported growth of inocula and first-transfer cultures originating from all sampling points. No growth was observed in any of the first-transfer cultures using the $S_2O_3^{-2}$ medium. The lack of growth observed using Media #3 was consistent with the results from the identical medium used for the November, 1987 samples (Medium A). The pH_o of the Media A was 4.5 and 4.0 for Media #3; it appears that the pH_o of medium was not a factor effecting growth of acidophilic thiobacilli.

It is possible that sewer acclimated strains of thiobacilli could not grow on thiosulfate ion. The original enrichments probably metabolized material transferred with the original sample, and could not use the available $S_2O_3^{-2}$ after the sewer nutrients were diluted or exhausted. These strains may have lost their ability to use $S_2O_3^{-2}$ due to the consistent presence of preferable substrates including H_2S and S°.

Mid-pH Samples. Growth in the mid-pH enrichment media (#2 and #4) were positive for most original enrichments and first-transfer cultures. That is, most sampling points contained thiobacilli which were capable of initiating growth on mid-pH basal-salts media supplemented with thiosulfate or both thiosulfate and yeast extract. Media #4 showed relatively rapid pH decrease in original, first-transfer and serial-dilution cultures, suggesting that the presence of a usable organic substrate, yeast extract, increased growth and acid production rates. These cultures probably contained facultative thiobacilli (See Tables 3-5 and 3-6).

Unlike the November samples, these media were free of supplementary trace metals. The cultivation of bacteria was significantly more successful, suggesting that trace metals were toxic to mid-pH thiobacilli growth.

The Bacto agar initially used to solidify media for the cultivation of axenic colonies of thiobacilli supported growth of heterotrophic contaminants. Noble agar plates did not support heterotrophic growth. The "monoclonal" colonies from the H_2S (g) plates were not identical in appearance to those on plates fortified with $S_2O_3^{-2}$. Also, accumulation of sulfur was not observed on the solid media, which suggests S(-II) autoxidation did not precede microbial oxidation to greater oxidation states.

Attempts to enumerate thiobacilli on sewer crowns were not undertaken due to the difficulty of collecting identical samples consistently.

4.2 Identification of Isolates

4.2.1 Acidophilic Isolates

Among the acidophilic strains tested, only the LACSD isolate (*T. ferrooxidans*) was capable of producing acid using Fe(II) as substrate. Cell numbers increased for that strain during the same experiment. The other isolates subjected to the test decreased media pH slightly more than the abiotic control. This could have resulted from the transfer of reduced sulfur when inoculating the ferrous iron medium. The remainder of the acidophiles collected in this sampling program were identified as

T. thiooxidans.

Acidophiles were isolated from all of the crowns sampled, even the crown pH exceeded the optimum range for these species. This suggests that acidophiles can function in neutral pH environments or that they produce small, acidic "pockets" where acidophilic metabolism is possible.

4.2.2 Non-acidophilic Cultures

Preliminary identifications of non-acidophilic sewer crown isolates were based on the physical characteristics of colonies grown on solid media fortified with $S_2O_3^{-2}$. Subsequent tests were premised upon terminal pH and cell number in cultures enriched with two separate media, one consisting of mineral-salts plus H_2S and the other identical to the first except it was augmented with yeast extract. Four potential species could metabolize the two separate substrate provided. The two strict autotrophs, *T. neapolitanus* and *T. thioparus*, were expected to exhibit identical growth rates in the two media. The facultative autotrophs, *T. intermedius* and *T. novellus*, were expected to grow in the medium containing yeast extract at much faster rates than in the inorganic medium. The terminal pH of the media would distinguish between the two species belonging to one of the two groups.

A yellow precipitate formed in the yeast extract media. This, coupled with slow growth after exposure to H_2S (g) suggests that S(-II) was either (i) incompletely microbially oxidized to a sulfur intermediate or (ii) autoxidized it to S°. Incomplete oxidation to intermediate sulfur explains

the lack of pH change and elemental sulfur was previously found to be unusable by non-acidophilic sewer isolates. The inability of strains to use the precipitate formed suggests it was a form of S^{o}.

Apparent strict autotrophs subjected to the final non-acidophilic identification test did not support growth or acid production at the same rates in the two media. The initial growth and acid production results led to the determination between strict and facultative autotrophs. The species identification was determined by the terminal pH in the media.

In general, colony characteristics from preliminary tests led toward the same species identity as the final test (Table 3-10). Colony size, sulfur deposit, and color of thiobacilli on solid media fortified with $S_2O_3^{-2}$ provides adequate information for quick identification to the species level.

The final identification of non-acidophilic isolates should be confirmed using modified methods since the procedures used led to unexplainable results in regards to the media containing yeast extract.

4.3 Stoichiometric Studies

4.3.1 Kinetic Utilization Studies

When sulfur was provided as H_2S, both acidophilic and non-acidophilic thiobacilli consumed molecular oxygen and total S(-II) by approximately a 1:1 ratio. Based on these results, it is evident that S(-II) was initially oxidized to intermediate product(s) with an average oxidation state of +2, per Equation 4-1.

O_2 + S(-II) ==> S(II) Equation 4-1

Interestingly, thiosulfate sulfur has an oxidation state of +2. Acidophilic isolates proved unable to oxidize thiosulfate in separate experiments.

In experiments where S(-II) was exhausted prior to dissolved oxygen, the respiration rate decreased abruptly at the point of sulfide exhaustion -- by approximately 80% in both acidophilic and non-acidophilic cultures. This suggests that oxidation of some form of sulfur continues after S(-II) exhaustion -- presumably oxidation of sulfur intermediates. Further identification of steps along the S(-II) oxidation pathway and kinetics of intermediate oxidation would require measurement of expected sulfur intermediates (thiosulfate, sulfite, or sulfate ions) concentrations in these cultures.

When growing cultures for stoichiometric studies, H_2S (g) was injected into sealed flasks containing a mineral growth medium and the seed culture. After H_2S addition, a precipitate formed slowly in the flask, only to disappear, usually within 24 hours. When respiration/S(-II)-utilization tests were conducted prior to the disappearance of the precipitate, a two to one (2:1) ratio of $d[O_2]$ to d[S(-II)] disappearance rate was observed in cultures of *T. thiooxidans*. Also, the culture's respiration rate increased by 73 % following H_2S addition (a test measuring the respiration rate was conducted prior to H_2S addition). The increased

O_2 demand of the culture when supplemented with H_2S suggests either a preference for S(-II) over the sulfur precipitate or the simultaneous oxidation of both substrates. The 2:1 ratio of O_2 to S(-II) use indicates that both forms of sulfur were utilized since the initial step in S(-II) oxidation does not produce an end product with the oxidation state of sulfate as determined by prior tests. Autoxidation of S(-II) over the time period proved negligible. This experiment also suggests thiobacilli can use sulfide directly for respiration, as opposed to chemically oxidized intermediates. Further tests involving chemical inhibitors provide more insight to this observation (See Section 4.4).

4.3.2 Determination of Growth Coefficients

The results of the long-term stoichiometry experiments established relationships between either (i) microbial-catalyzed acid production or (ii) culture growth, and cumulative sulfide use. The acid production yield coefficients for isolates C8/12/1.2 and C22/12/1.2 (*T. thiooxidans*) were virtually identical (see Table 3-12). Isolate E30/12/1.2 (*T. thiooxidans* collected from a lightly corroded crown) produced 41 percent less acid via oxidation of an equivalent amount of S(-II). It appears the isolate is less efficient at completing the oxidation of S(-II) to acid. C8/12/1.2 and E30/12/1.2 could be a better-adapted strains. More work would be necessary to determine whether interstrain differences contribute to the relative acidification and corrosion rate in LACSD sewers.

The non-acidophilic culture tested, C8/12/2.3B produced 0.58 equivalents of acid per moles of H_2S added, less than one-half the yield by the acidophiles. A stable precipitate formed in the media during the experiment, suggested that the oxidation of sulfide was incomplete. Incomplete oxidation by non-acidophiles could be a self-preservation mechanism, keeping the culture pH in the optimum neutral range for a longer period. The precipitate formed during this experiment did not appear to support growth of the culture in the absence of S(-II), suggesting it was the final product of S(-II) oxidation.

Direct counts provided a measure of growth and produced additional yield coefficients (growth per S(-II) consumed) for the isolates tested. Among the strains of *T. thiooxidans*, C8/12/1.2 and C22/12/1.2, had a greater growth yield than E30/12/1.2 (the isolate from the neutral-pH crown) by 24% and 32%, respectively. This suggests the quantity of acid produced is probably proportional to the number of cells in the culture. The non-acidophilic culture produced a growth yield coefficient which was lower than any of the acidophilic isolates by a factor of two. It is apparent from both acid production and growth yield coefficients that acidophiles isolated from low-pH crowns utilizes S(-II) more efficiently than either the acidophile from the lightly corroded crown or the non-acidophilic species tested. Much more work would be necessary to establish a cause-effect relationship between species present and crown deterioration.

Total particulate organic carbon or protein analyses, used as a possible surrogate parameters for cell growth, did not produce consistent data. Modified methods would be required to make the parameters effective.

4.4 Acute Inhibition Tests

4.4.1 Cyanide Inhibition

The effects illustrated in Figure 3-10 for various concentrations of cyanide suggest that oxygen and sulfide utilization rates were jointly inhibited. S(-II) autoxidation was negligible when respiration ceased. Results suggest that the oxidation of S(-II) is tightly coupled to microbial electron transport. When respiration is blocked, so is sulfide oxidation.

It is still possible, as suggested by Milde, et al (1983), that autoxidation of S(-II) to S° precedes bacterial oxidation of sulfur in sewer crowns. The concentrations of thiobacilli in these experiments were probably much higher than of the sewer crown environment and the abiotic oxidation of S(-II) may occur on a time scale which was not investigated here. Furthermore, the sewer crown may provide surfaces or metals for chemical catalysis of the S(-II) to S° reaction which were not present in these experiments. What is conclusively established here, however, is the ability of thiobacilli to catalyze this reaction and the linkage of S(-II) oxidation to electron transport.

4.4.2 DCCD Inhibition

No inhibition of oxygen utilization rates was detected in the presence of DCCD. Blocking proton retranslocation via membrane-bound ATPase (and presumably oxidative phosphorylation) did not effect electron-transport or the oxidation of sulfide. This suggests that oxidative phosphorylation does not result from bacterial catalysis of the S(-II) oxidation reaction. The oxidation of S(-II) may be a non-energy-producing first step of catabolism involving hydrogen sulfide. ATP measurements could be used in conjunction with additional inhibition studies to test that hypothesis.

4.4.3 Acute Metals Inhibition

Inhibition tests using oxyanionic metals are summarized in Table 3-14. High concentrations of both molybdate and orthovanadate (10^{-2} M and 10^{-3} M, respectively) produced an increase in O_2-utilization rate. This effect is similar to that expected in the presence of a chemical inhibitor such as 2,4-dinitrophenol. This chemical allows respiration to continue, but uncouples it from the phosphorylation of ADP (Lehninger, 1975). Uncoupling is accomplished by interfering with the cell-membrane function. The membrane is rendered permeable to ions and respiration does not generate proton-motive force necessary for oxidative phosphorylation. Cell growth ceases, although electron transport may actually accelerate temporarily.

Total dissolved sulfide concentrations were depleted almost immediately by the addition of oxyanions to these cultures. This suggests

that sulfide is either chemically oxidized or forms complexes with metals. Some precipitates appeared after the addition of both S(-II) and the metals, suggesting the formation of insoluble complexes. This explains the exhaustion of soluble S(-II) in the cultures.

Lower concentrations metals, 10^{-3} M molybdate and 10^{-4} M orthovanadate, produced similar inhibitory effects on both O_2 and sulfide utilization rates. Actual inhibitory concentrations are questionable since the metals were tested with S(-II) present. Metals speciation and oxidation state in the presence of sulfide may be altered by metals reduction and/or precipitation reactions. Direct measurement of soluble metals concentrations, differentiating among oxidation states would data helpful in evaluating the toxic and non-toxic forms. Jack (1980) speculated that adaptation of *T. thiooxidans* to vanadium (V) by the microbial reduction of the metal to the non-toxic form, vanadium (IV).

A delayed inhibition effect was observed using respiration rates for concentrations of both oxyanions (see Figure 3-12 for 3 X 10^{-3} M molybdate and Figure 3-13 for 10^{-3} M orthovanadate). This delay of inhibition suggests that culture was either starved for reduced sulfur as substrate after the test commenced or the diffusion of the toxic metal to the cells was slow.

4.5 Chronic Metals Toxicity

4.5.1 General

Screening experiments were used to measure the toxicities of several metals to sewer isolates of thiobacilli. Typically, a lag in acid production was observed after metals addition. The length of the lag period was directly related to metals concentration, suggesting that inhibition was either bactericidal, possibly leaving a small number of cells to continue growth, or bacteriostatic, followed by events leading to culture acclimation. Acclimation might be linked to a reduction of the available metals in solution due to adsorption to the glass tube, cells, or elemental sulfur present as substrate. Alternatively, metals speciation may be altered microbially, eventually leading to more tolerable forms and apparent culture acclimation.

4.5.2 Comparison of Metals Tolerances

Silver, copper and the metals elixir (Figures 3-20 through 3-23) were used to compare metals tolerance of two isolates of *T. thiooxidans* collected from different environments; C8/12/1.2 collected from an acidic, highly corroded crown; and E30/12/1.2 isolated from a lightly corroded crown. C8/12/1.2 was generally more tolerant to metals. The LACSD isolate (*T. ferrooxidans*) showed approximately the same level of sensitivity as E30/12/1.2 to silver and copper. Results from others indicate the LACSD isolate (*T. ferrooxidans*) is more tolerant to metals than C8/12/1.2 (Milner, 1988). Other researchers previously have

generally found *T. ferroxidans* to be tolerant to environments with high metals concentrations (e.g., acid mine runoff).

4.5.3 Implications of the Industrial Waste Pretreatment Program

The enormous increase in sewer crown deterioration rates over the past decade coincided with the reduction of metals discharged to the LACSD sewage collection system as mandated by EPA's industrial waste pretreatment program. Two of the metals which average concentrations in sewage was measured before and after implementation of the pretreatment program, copper and zinc, were non-inhibitory to the time-dependent acid production of pure cultures of *T. thiooxidans*. However, there is more evidence that metals concentrations present prior to the implementation of mandated program inhibited thiobacilli via a synergistic effect. Experiments by others involved with this project will test the toxicity of combinations of metals. Table 4-1 lists the toxic concentrations of metals found in this laboratory and by Jack (1980). Also included in the table are the metals concentrations found in the LACSD sewer system in 1972 and 1987, before and after implementation of industrial waste pretreatment measures.

Table 4-1. Metals concentrations found toxic to batch cultures of acidophilic thiobacilli isolates and metals concentrations in LACSD sewage before (1972) and after (1987) implementation of the industrial waste pretreatment program.

Metals	Concentrations in Levels which have proven toxic (moles/liter)				LACSD sewage	
	T. thiooxidans isolated from low-pH crowns	*T. thiooxidans* isolated from a mid-pH crown	*T. thiooxidans* (ATCC 8085 Jack, 1980)	*T. ferrooxidans* isolated by LACSD	1972	1987
Mo (VI)	1×10^{-4}		5.2×10^{-4}			
Cu	NT[1]	5×10^{-4}		1×10^{-3}	1.1×10^{-5}	3.2×10^{-6}
Co				1×10^{-3}		
Fe					2.3×10^{-4}	1.1×10^{-4}
Zn	NT				3.4×10^{-5}	9.2×10^{-6}
Cr (VI)	1×10^{-5}		4.2×10^{-5}	1×10^{-4}		
Pb					2.2×10^{-5}	3.9×10^{-6}
Ag[2]	NT	1×10^{-4}		1×10^{-4}		
Ag[3]	NT	NT				
Ni					5.3×10^{-6}	1.7×10^{-6}
V (V)	1×10^{-4}		2.0×10^{-2}			
Cd	1×10^{-3}					
Hg	1×10^{-5}					
Metals Elixir	NT	1x[4]				

1 NT = metals were not toxic at concentrations tested
2 Without chloride salts
3 With chloride salts
4 Metal elixir toxic at concentrations of metals listed in Table 2-6

CHAPTER 5

CONCLUSIONS

Experimental results support the following conclusions:

5.1 Cultivation and Isolation of Sewer Samples

5.1.1 Cultures collected from LACSD sewer crowns were generally adapted to oxidize of either S° or S(-II). Thiosulfate-based media supported growth and acid production of the original samples collected, but only occasionally did first-transfer cultures grow in the same medium. Growth in the original enrichment cultures was probably dependent on nutrients within the inoculum.

5.1.2 Acidophilic isolates were all identified as *T. thiooxidans*, indicating the prominance of this species in corroded, acidic sewer crowns. The LACSD isolate was the only acidophilic isolate which was capable of oxidizing Fe(II).

5.1.3 The five non-acidophilic isolates were identified as either *T. neapolitanus* or *T. novellus*. Organic media used for identification of isolates formed a yellow precipitate after H_2S (g) was added. These cultures did not support growth or acid production at the same rate as the inorganic medium. This left a degree of uncertainty concerning the species identification.

5.2 Stoichiometry of *Thiobacillus* Metabolism on Sulfide

5.2.1 Starved isolates oxidized S(-II) incompletely following H_2S addition. Initial S(-II) conversion reactions demanded O_2 at a one to one (1:1) molar ratio. This reaction yields an immediate sulfur products with an average oxidation state of +2 (the oxidation state of thiosulfate sulfur). Upon exhaustion of S(-II) in batch cultures, O_2 utilization continues, although at a significantly lower rate, suggesting that the oxidation of the initial sulfur intermediate to higher oxidation states is possible.

5.2.2 Crown isolates can simultaneously use two sources of reduced sulfur. The supplementation of a batch culture containing a visible sulfur precipitate with H_2S (g) produces an additive effect on the oxygen utilization rate.

5.2.3 Yield coefficients for strains of *T. thiooxidans* collected from an acidic environment were significantly larger than strains collected from neutral-pH and less corroded crowns.

5.2.4 Representative yield coefficients for acidophiles C8/12/1.2 and C22/12/1.2 were 1.28 equivalents of acid produced per mole sulfide consumed and 2.7×10^{13} cells produced per mole of sulfide comsumed. By comparison, the strain of *T. thiooxidans* isolated from a mid-pH environment, E30/12/1.2, produced 0.76 equivalents of acid and 2.0×10^{13} cells per mole of sulfide consumed. The non-acidophilic isolate tested C8/12/2.3B (*T. neapolitanus*), yielded only 0.58 equivalents of acid and

2.7 X 10^{12} cells per mole sulfide consumed. These may be important differences from the stand point of steady-state crown pH and deterioration rates.

5.2.5 The acidophilic isolates were predominately responsible for acid production and crown corrosion of damaged sewers. The non-acidophilic cultures probably were responsible for establishing environmental conditions allowing development of acidophilic communities.

5.3 Sulfide Oxidation by *Thiobacillus thiooxidans*

5.3.1 The simultaneous inhibition of dissolved O_2 and total sulfide utilization rates in batch cultures of sewer crown isolates via cyanide addition indicates that both acidophilic and non-acidophilic isolates are capable of oxidizing S(-II). In the absence of bacterial respiration, S(-II) was not oxidized within the time frame required to complete the studies. Yellow sulfur precipitates commonly observed on sewer crowns may be the result of either chemical or partial microbial oxidation of H_2S.

5.3.2 Dissolved O_2 and sulfide utilization rates were not affected by DCCD addition in batch cultures of *T. thiooxidans*. Therefore, the oxidation of sulfide is not linked directly to oxidative phosphorylation. It is also possible that DCCD did not inhibit membrane-bound ATPase in *T. thiooxidans* at the concentrations tested.

5.4 Metals Inhibition

5.4.1 Molybdate [Mo(VI)] and orthovanadate [V(V)] were inhibitory to thiobacilli. The mechanisms of inhibition appeared to be concentration dependent. At the highest concentrations tested, electron transport and oxidative phosphorylation seemed to be uncoupled, leading to an increase in O_2-utilization rates. Lower concentrations of both Mo(VI) and V(V) retarded O_2 and total sulfide utilization rates.

5.4.2 The extent and duration of long-term (chronic) inhibition of bacterial acid production was a function of metals concentrations. None of the individual metals concentrations found in LACSD collection system prior to implementation of the industrial waste pretreatment measures significantly inhibited batch cultures of acidophilic thiobacilli. However, numerous metals retard growth or acid production in batch cultures at concentrations of 1×10^{-5} M and lower. The lag period of acid production in these tests indicate an acclimation of the culture by an unknown mechanism.

5.4.3 Isolate C8/12/1.2 (*T. thiooxidans*) was generally more tolerant to metals than the *T. thiooxidans* strain which was isolated from a non-acidic environment, (E30/12/1.2).

APPENDIX

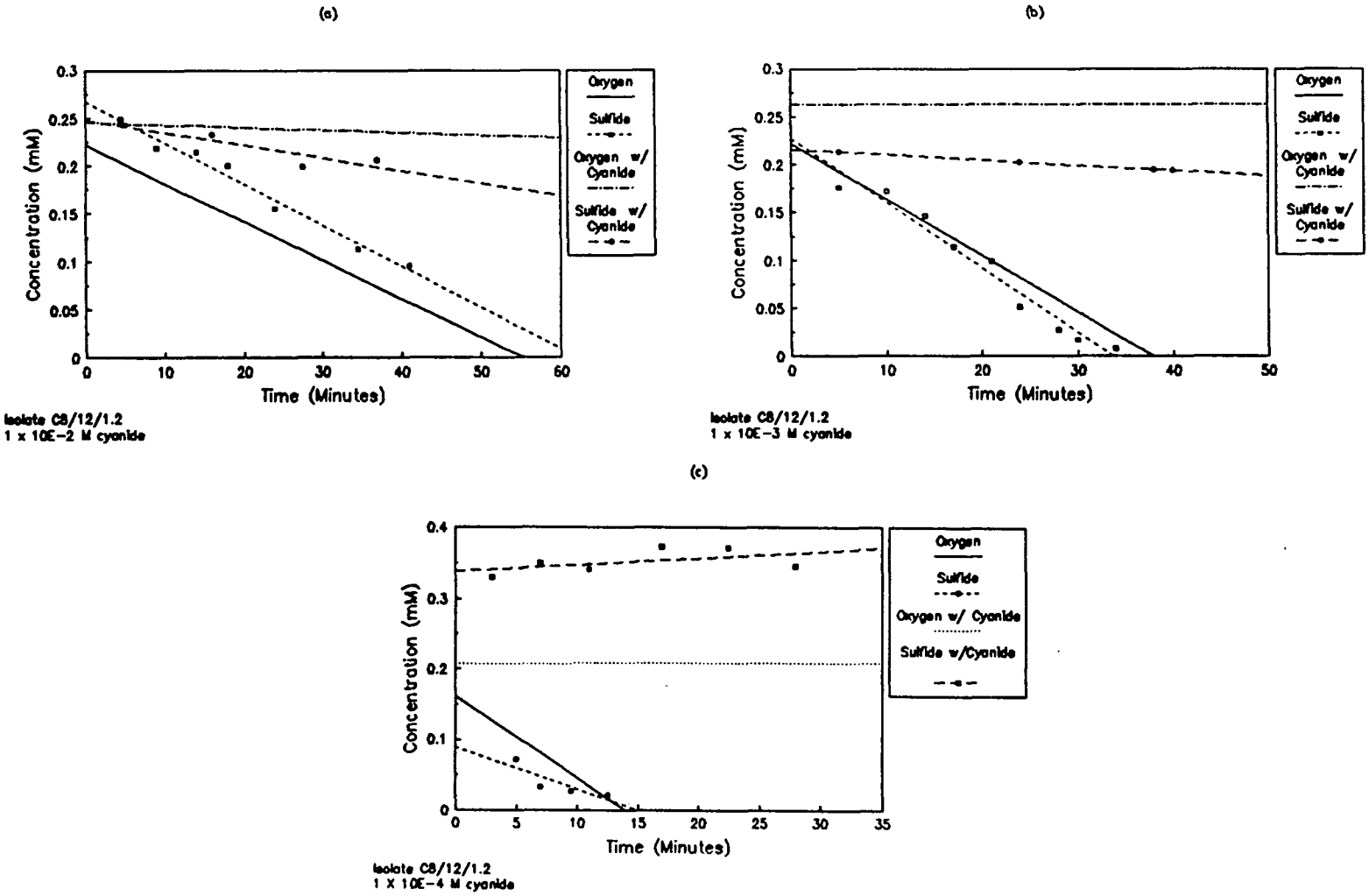


Figure A-1. The effect of (a) 10^{-2} M cyanide, (b) 10^{-3} M cyanide, and (c) 10^{-4} M cyanide on dissolved oxygen and total sulfide utilization rates of *Thiobacillus thiooxidans*.

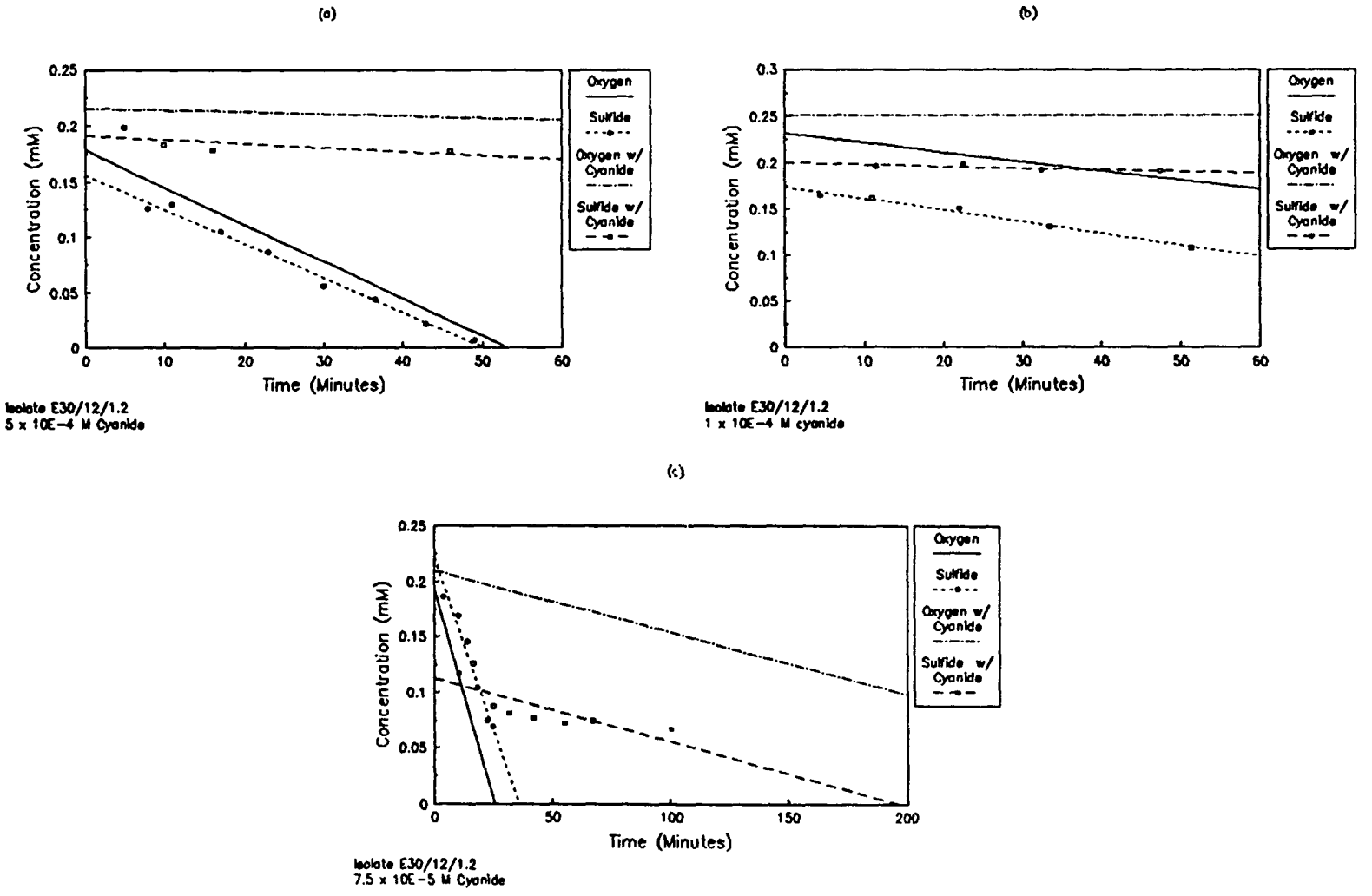


Figure A-2. The effect of (a) 5 X 10^{-4} M cyanide, (b) 10^{-4} M cyanide, and (c) 7.5 X 10^{-5} M cyanide on dissolved oxygen and total sulfide utilization rates of *Thiobacillus thiooxidans*.

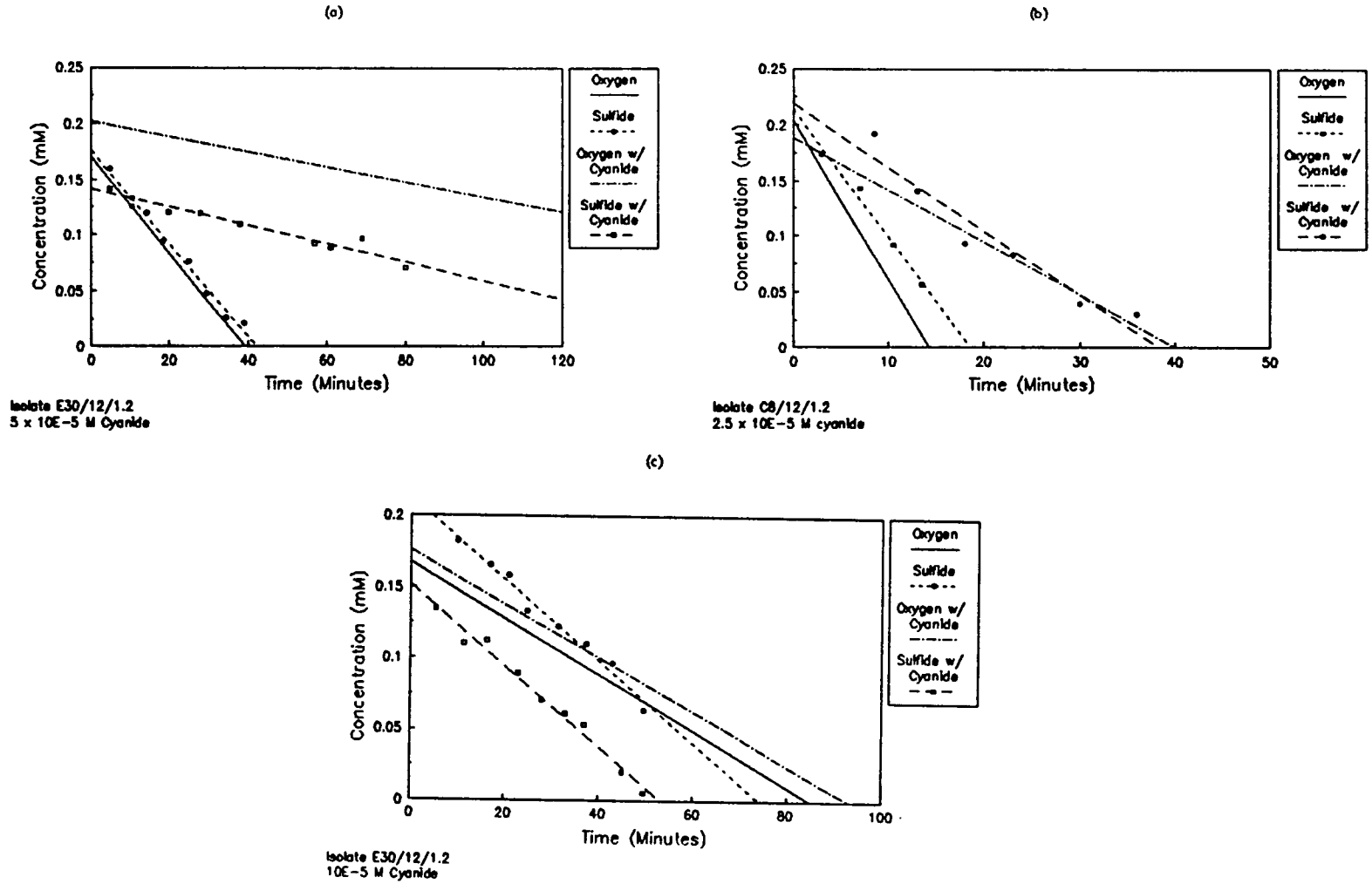


Figure A-3. The effect of (a) 5 X 10^{-5} M cyanide, (b) 2.5 X 10^{-4} M cyanide, and (c) 10^{-5} M cyanide on dissolved oxygen and total sulfide utilization rates of *Thiobacillus thiooxidans*.

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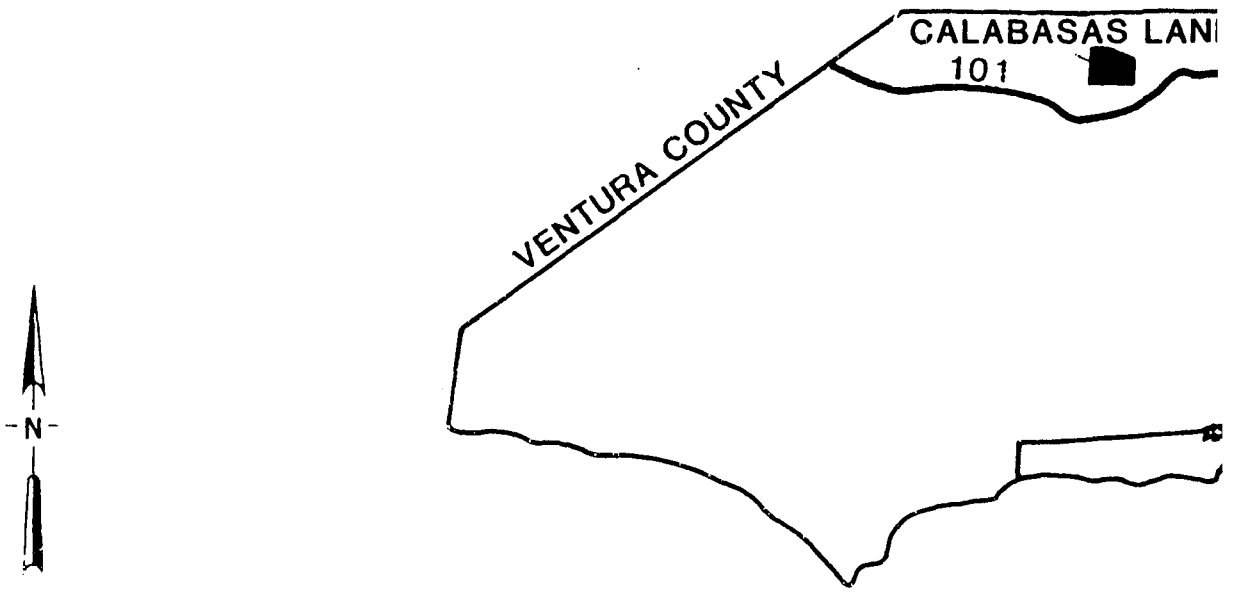
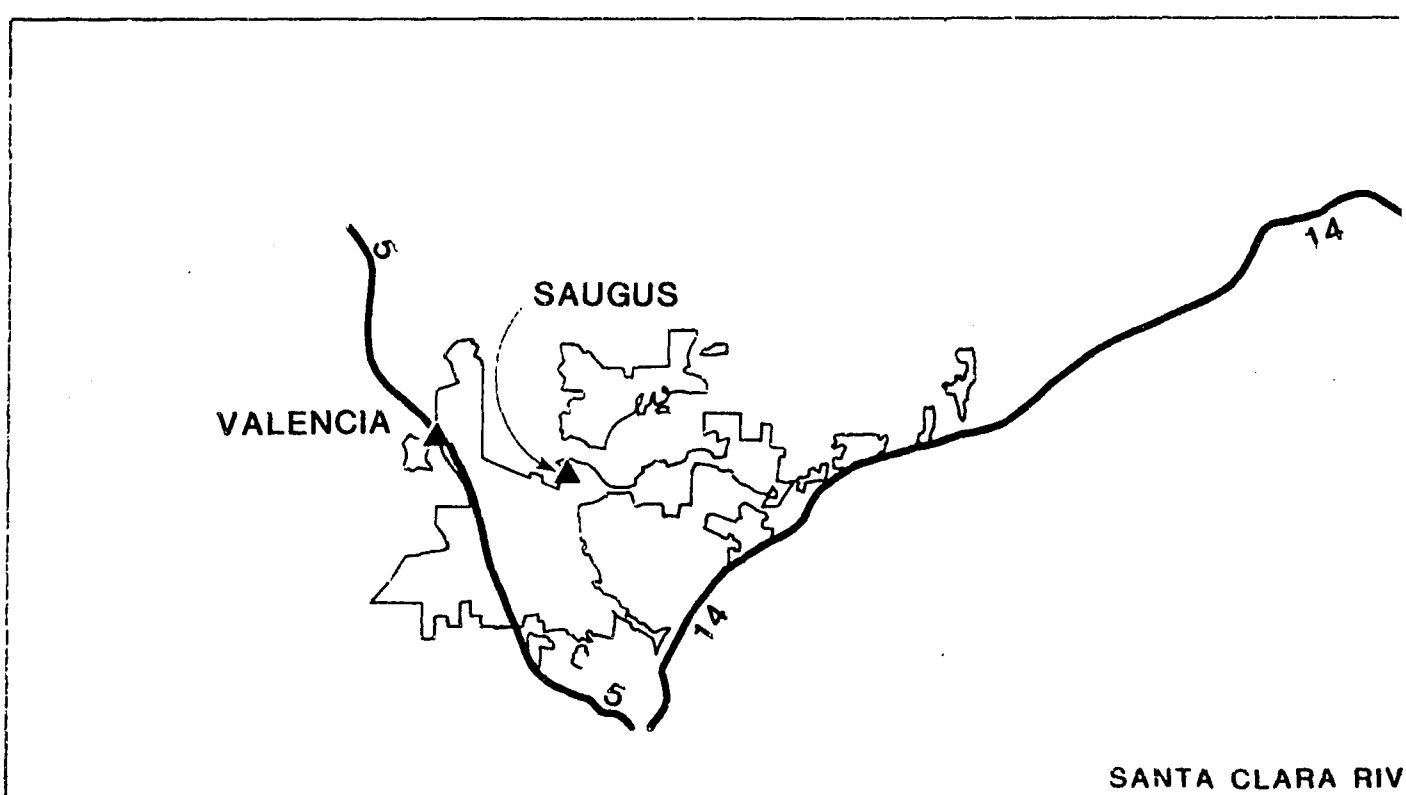
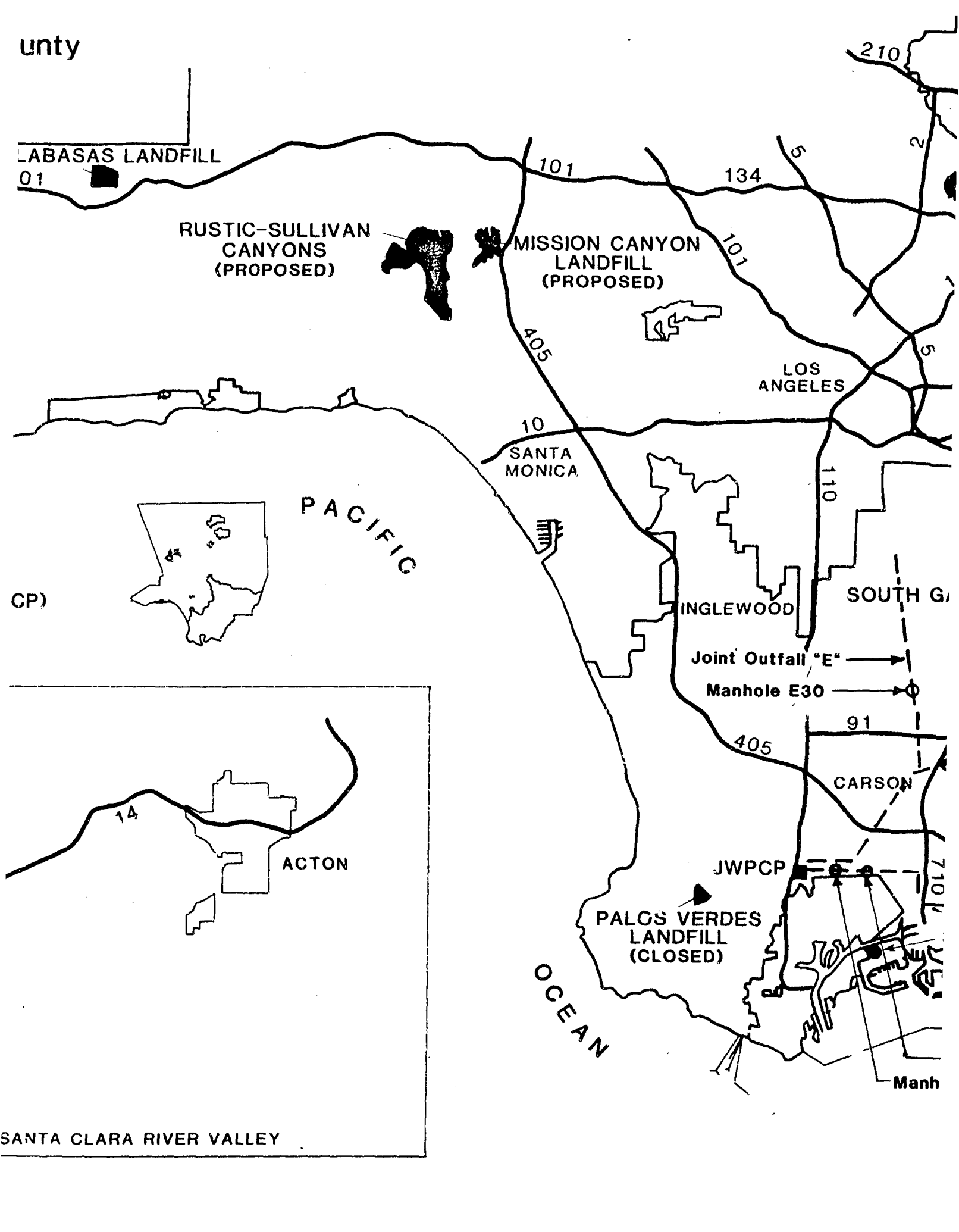
County Sanitation Districts Of Los Angeles County
FACILITIES MAP



- ▲ WATER RECLAMATION PLANT
- ● REFUSE-TO-ENERGY FACILITY
- ○ PROPOSED REFUSE-TO-ENERGY FACILITY
- □ JOINT ADMINISTRATION OFFICE (JAO)
- ■ JOINT WATER POLLUTION CONTROL PLANT (JWPCP)
- ★ TRANSFER STATION

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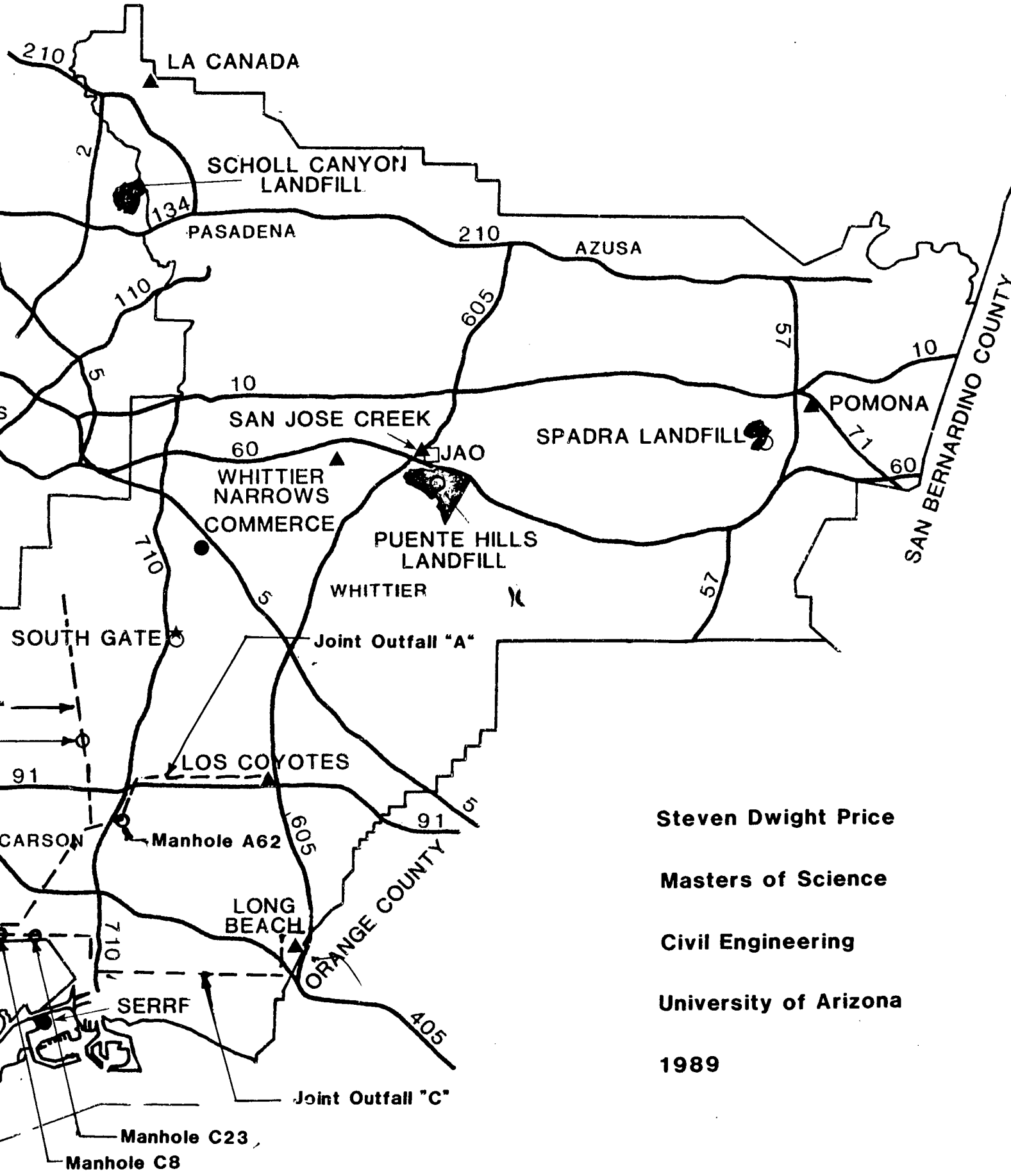

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