INHIBITION OF THIOBACILLUS FERROOXIDANS USING ANTIBIOTICS AND ANTIBACTERIAL SUBSTANCES

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Inhibition of Thiobacillus Ferrooxidans Using Antibiotics And Antibacterial Substances

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Laboratory experiments were carried out to evaluate the effectiveness of antibacterial substances and antibiotics against Thiobacillus ferrooxidans, the organisms responsible for bacterial mediated acidic mine drainage. Twenty two antibiotics (obtained from Lilly and Co.) and two antibacterial substances were added to bacterial culture ATCC 19859 grown in 9K medium. Appropriate controls were maintained. Inhibition of iron oxidizing bacteria was recorded in terms of changes in Eh of the medium treated with the compound. Seven antibiotics (A38533A$_1$, A38533B, 197506, 13780, 171541, chloramphenicol and cephalexin) and the two antibacterial substances [N-serve(nitrapyrin) and Dicyandiamide] effectively inhibited the oxidation of Fe^{2+} ions in the medium. The kinetics of Fe^{2+} oxidation with the addition of antibiotics and the antibacterial substances was studied. N-serve [2-chloro-6-(trichloromethyl) pyridine], used as a nitrification inhibitor in agriculture, was highly effective at concentrations greater than 0.1 ml/l. Iron oxidation levels were reduced to levels close to that in uninoculated controls (abiotic oxidation). The use of N-serve to inhibit acid mine drainage (AMD) causing bacteria seems to be both economical and environmentally safe.

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INTRODUCTION

Acidic mine drainage (AMD) refers to the low pH drainage originating from coal mining operations. Enormous quantities of acid are produced when pyrite (FeS_2) oxidation is catalysed by bacteria. The acid formed in the upper exposed layers of pyritic material can leach down to pollute groundwater, and the runoff and leachates drain into streams and rivers causing damage. Adverse impacts of acid mine drainage on the environment include degradation of water quality for industrial uses and human consumption, the destruction of aquatic life, and lowering of the recreational value of the receiving water body. In the U.S, there are about 7000 active mines of which about 2600 produce acidic drainage. An estimated 5000 miles of streams carry in excess of two million tons of sulfuric acid equivalents each year (Lovell, 1983).

Oxidation of pyrites to form acidity occurs by the action of abiotic and biotic agents. Initial oxidation of pyrites occurs upon their exposure to air (oxygen). The oxidation rate is greatly enhanced (10^6 times) by bacteria belonging to the species Thiobacillus ferrooxidans.

T. ferrooxidans are chemolithotrophic, gram negative, aerobic rods. They are obligate acidophiles with a pH optimum between 2.5-5.8. Growth can occur even below pH 1.4. Optimum temperature for growth is between 15-20° C, but the upper range can extend to 40° C (Bergey's Mannual of Determinative Bacteriology, 1974).

Energy is derived from the oxidation of reduced iron and sulfur compounds. Carbon dioxide is reduced to provide cellular material. Electrons generated from the oxidation of Fe^{2+} to Fe^{3+} go through the electron transport system (ETS) of the cell via cytochrome $c+c_1$ and cytochrome a.

Adenosine-triphosphate (ATP) production occurs between the cytochrome $c+c_1$ and cytochrome a. Cytochrome b is found in small amounts compared to $c+c_1$ and a. Cytochrome b is thought to be involved in reverse electron transport for the generation of reduced pyridine nucleotides (Lundgren, 1964).

The cells are also capable of oxidizing elemental sulfur and generating energy by electron transport phosphorylation. T. ferrooxidans populations adapted to growing in iron rich environments are found to oxidize sulfur at a very low rate. Cell walls are selectively

impermeable to high concentrations of H^+ ions. Certain phospholipids in the cellwall are also known to protect the intracellular components from their low pH external environment.

Pyrite is a crystalline form of iron sulfide (FeS_2), commonly occurring in bituminous coals and ores. When pyrite is exposed to air through mining operations, a slow spontaneous oxidation occurs as follows:

$$FeS_2 + 7/2\ O_2 + H_2O \text{ ---> } Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad [1]$$

This reaction produces acidity which then drives the pH down. In the next step, favored by low pH , T. ferrooxidans produces more acidity by catalyzing the oxidation of Fe^{2+} ions. In the absence of bacteria, this reaction occurs at a very slow rate.

$$Fe^{2+} + 5/2\ H_2O + 1/4\ O_2 \text{ ---> } Fe(OH)_{3\,(s)} + 2H^+ \quad [2]$$

$$Fe^{2+} + 1/4\ O_2 + H^+ \text{ ---> } Fe^{3+} + 1/2\ H_2O \quad [3]$$

The ferric ions formed react with pyrite to produce more ferrous ions.

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \text{ ---> } 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+ \quad [4]$$

The ferrous ions formed are again oxidized by T. ferrooxidans to ferric ions and the cycle continues, generating large amounts of acidity.

Control of acid mine drainage includes preventive and inhibitory methods. Some of the preventive measures include the following (Lovell, 1983) :

1. Construction of high wall diversion ditches that extend beyond the end of a cut.
2. Minimizing the exposure of disturbed strata.
3. Providing barriers of undisturbed streams to prevent lateral flow of water.
4. Use of alkaline backfill.
5. Minimizing surface infiltration by compaction of the mine spoil, revegetation etc.,.

Attempts to control acid formation by inhibitory methods include chemical treatment of the refuse. Lime is added to create alkaline conditions to retard the growth of bacteria, and also to neutralize the acid formed. Revegetation of mine lands is also being followed to encourage the formation of CO_2. The growth of heterotrophic microorganisms is also induced by the root systems of the plants. Carbon dioxide in the

subsurface layers helps dissolve compounds that contribute to alkalinity and the heterotrophs compete with the acid producing bacteria thereby inhibiting their growth.

Revegetation, however, requires initial liming of the soil to create pH conditions optimum for plant establishment. Application of lime to reduce acid formation is expensive. The drainage problem can be effectively controlled by inhibiting the bacteria that accelerate the acid formation. A review of literature pertaining to this concept is discussed in the following section.

Antibiotics, organic acids and surfactants have been used to inhibit the activity of T. ferrooxidans (Shearer et al, 1969; Everson, 1970; Tuttle and Dugan, 1976; Kleinmann et al 1981; Onysko et al, 1984). Many antibiotics are known to specifically inhibit a single species or closely related species of bacteria (Pleczar, 1986). Antibiotics that can specifically inhibit T. ferrooxidans can reduce acid production without affecting the heterotrophic population and thus aid in the establishment of vegetation.

Antibacterial substances such as dicyandiamide and N-serve [2-chloro-6-(trichloromethyl) pyridine] are known to inhibit species of Nitrosomonas (Bremner, 1974; Nuti et al, 1975; Powell and Prosser, 1986) which are chemoautotrophic bacteria. It has been suggested that the inhibition of these bacteria by dicyandiamide and N-serve, is through the inhibition of the enzyme cytochrome oxidase which is involved in the generation of ATP (Laskowski and Bidlack, 1977). Cytochrome oxidase is also involved in the production of ATP in T. ferrooxidans. Research was therefore carried out to investigate the possibility of inhibition of T. ferrooxidans using low grade antibiotics, dicyandiamide and N-serve.

Research was carried out between September, 1987 to May, 1988 with the following objectives:

1. Screen antibacterial substances and antibiotics for their capacity to inhibit the growth and activity of T. ferrooxidans.
2. Identify a candidate compound (compound found to be the most effective at a low cost of application) for field trails.

REVIEW OF LITERATURE

GENERAL

Although the role of bacteria in causing acid mine drainage has been known for about four decades, attempts to control the drainage have been mainly through the application of lime. Only in the last decade has research been directed towards controlling acid drainage by inhibiting the growth and activity of the bacteria involved in acid production. Several abiotic agents such as organic acids, antibiotics and surfactants have been found to be inhibitory to T. ferrooxidans. Bacteria capable of reducing acidity by inhibiting T. ferrooxidans have also been identified. Literature dealing with the biotic and abiotic inhibition of T. ferrooxidans is discussed below.

Biotic Inhibition

Shearer et al, (1969) observed that a stream receiving mine drainge did not show any alteration in pH level. This was attributed to factors inhibiting the activity of T. ferrooxidans. The inhibitory factor was later found to be bacteria belonging to the genus Caulobacter. Caulobacter cultures were then adapted to

grow at low pH. Laboratory experiments involving inoculating cultures of these bacteria to containers of acid producing coal, showed improvements in pH and acidity values over the uninoculated controls. Field tests using the bacterial inoculum also lowered acidity.

Actinomycetes are well known for their antibiotic production. In environments with healthy populations of certain species of actinomycetes growth and activity of T. ferrooxidans are found to be inhibited. Shearer, et al (1969) and Everson, (1970) carried out laboratory experiments to study the inhibitory effects of antibiotic producing bacteria such as Streptomyces aureofaciens and S. spheroides on T. ferrooxidans. Inoculation of cultures of these bacteria to coal refuse reduced acidity for 5-7 days beyond uninoculated controls.

Abiotic Inhibition

Shearer et al, (1969) and Everson, (1970) screened fifteen different antibiotics for their effectiveness in inhibiting T.ferrooxidans. Only three antibiotics, novobiocin, oleandomycin and chlorotetracyclin, were found to be effective. Similar experiments were carried out by Pichuantes et al, (1986). The minimum inhibitory concentrations of antibiotics chloramphenicol,

ampicillin, streptomycin, kanamycin and nalidixic acid on four different strains of T. ferrooxidans were determined at pH levels of 3.2 and 6. All the strains tested were found to be susceptible to all the antibiotics at pH 6. Whereas, at pH 3.2, ampicillin was inactivated due to low pH. Minimum inhibitory concentrations ranged from 1.9 µg/ml for ampicillin to 250 µg/ml for streptomycin, kanamycin and nalidixic acid.

The growth and activity of T. ferrooxidans was also found to be inhibited by organic acids (Tuttle and Dugan, 1976; Onysko et al, 1984). Organic acids such as pyruvic, oxaloacetic, malonic, itaconic and dihydroxyfumaric acids caused inhibition of growth at concentrations greater than 4×10^{-5} M. Concentrations greater than 4×10^{-4} M of citric, transaconitic, isocitric, malic and succinic acids were required for a similar inhibitory effect (Tuttle and Dugan, 1976).

Cells of T. ferrooxidans treated with organic acids were found to release varying amounts of DNA, RNA, sugars, phosphates and iron. The inhibition of their growth and activity was attributed to disruption of the function of the cell membrane. Inhibition may also be due to the direct inhibition of the iron oxidizing system (Tuttle and Dugan, 1976; Tuttle et al, 1977). Organic

acids such as benzoic acid and sorbic acid and a surfactant sodium lauryl sulfate (SLS), at concentrations greater than 5 mg/l, can also inhibit growth of the iron oxidizer.

The activity of T. ferrooxidans was completely inhibited by reduced sulfur compounds (Hurtado et al, 1987). A 10 mM concentration of sulfite, metabisulfite, tetrathionate, bisulfite and thiosulfite caused inhibition. Inhibition in the case of metabisulfite, bisulfite and thiosulfite was found to be due to the disruption of electron transport phosphorylation caused by the reduction of the components involved (cytochromes).

Kleinmann et al, (1981) were able to inhibit T. ferrooxidans in laboratory experiments and in field trials using sodium lauryl sulfate (SLS), alkyl benzene sulfonate and alpha olefin sulfonate. Since the surfactant SLS, is highly soluble in water, losses occur after a rainfall, limiting the period through which it can remain effective. To overcome this problem an SLS + vulcanized rubber formulation was developed which would ensure controlled release of the detergent. This formulation has been found to remain effective for a period of 2-5 years. However, the post application

monitoring of out flows is required to maintain a detergent concentration of less than 1 mg/l.

Effectiveness of anionic surfactants is found to be dependent on the amount of weathered materials in the mine waste. Application of SLS, potassium sorbate and potassium benzoate to slightly weathered pyritic rock reduced numbers of T. ferrooxidans but no reduction in acid production was observed. Application to weathered sulfite tailings however did not cause a reduction in bacterial population or acidity levels. When weathered materials were removed from the sulfide tailings and then treated with compounds, lower acidity levels and bacterial population were observed (Watzlaf, 1988). To date, SLS and alkylbenzene sulfonate have been used in field applications.

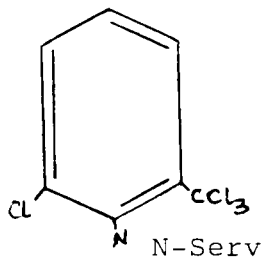
Field applications of an anionic surfactant + rubber formulation have been carried out on two coal mine refuse piles in Ohio and West Virginia (Rastogi, 1987). Long term effectiveness of this application needs to be monitored so as to prevent detergent toxicity to aquatic biota (Kleinman and Erickson, 1983). Search for compounds other than SLS capable of inhibiting acid formation is being carried out (Watzlaf, 1988). Screening of compounds other than SLS may result in

compounds that prove to be cost effective in field applications. The present research was therefore carried out to screen antibiotics and antibacterial substances capable of inhibiting T. ferrooxidans. The ultimate objective was to identify a compound capable of selectively inhibiting T. ferrooxidans at a low cost.

Dicyandiamide and N-serve are known to inhibit nitrification in soils by inhibiting species of Nitrosomonas (chemoautotrophic bacteria). N-serve was found to be highly effective in inhibiting nitrification (Bremner, 1974). Possible inhibition of T. ferrooxidans using N-serve has not been considered. In this investigation research was carried out to study the effect of N-serve on reducing acidity levels through bacterial inhibition. A brief discussion of literature pertaining to the use of N-serve is presented below.

N-SERVE

The reported active ingredient in N-serve a product of Dow Chemical Co., is 2-chloro-6 (trichloromethyl) pyridine, (common name: nitrapyrin). N-serve contains approximately 22 % of the active ingredient, 2.5 % of related pyridines and 75.5 % of inert ingredients.



N-Serve (Active Ingredient)

Ammonia fertilizers are widely used in agriculture. Significant losses of ammonia occur due to the activity of nitrifying organisms in the soil. These microbes convert the ammonia from the ammonia yielding fertilizers to nitrate which then either leaches and pollutes the ground water or is acted upon by denitrifiers and is lost as nitrogen gas. These losses can be minimized by the inhibition of nitrifying bacteria. N-serve is the most widely used compound for inhibiting nitrifying organisms. In North America, N-serve is being used on more than a million hectares annually (Rodgers and Ashworth, 1982).

N-serve is known to be more bactericidal than bacteriostatic to ammonia oxidizing bacteria (Rodgers and Ashworth, 1982). Inhibition of the activity of cytochrome oxidase has also been reported. Usually N-serve containing about 1/4 to 2 lbs of the active ingredient is coated on fertilizer and incorporated into the soil at a depth of 3 cm. Surface application can result in high amounts of N-serve volatilization (Briggs, 1975).

The effect of nitrapyrin on various soil bacteria and fungi from seven soils was investigated (Laskowski et al, 1975). The results showed that there was no decrease in the numbers of microorganisms and also no reduction in CO_2 production was noticed. Various tests conducted showed that there were no adverse effects associated with the use of N-serve in soils (Bremner, 1974). Application of N-serve increases crop yield (Parr et al, 1971) and reduces ground water pollution (Powell and Prosser 1986; Bundy and Bremner 1973). Based on the possible beneficial effects of application of N-serve to soils and its probable capacity to inhibit T. ferrooxidans the compound was used in the present research.

SUMMARY

Acid mine drainage catalyzed by bacteria can be controlled by inhibiting the growth and activity of the bacteria involved. T. ferrooxidans are found to be inhibited by antibiotics, organic acids and surfactants. Organic acids are believed to inhibit T. ferrooxidans either by disrupting the activity of cell membrane or by directly inhibiting the iron oxidation system. Field application of organic acids may not prove to be economical as these organic acids are found to be

utilized by acidophilic heterotrophs belonging to the genus Acidophilum (Shuttleworth and Unz, 1988).

Use of anionic detergents is currently the most economical method to inhibit T. ferrooxidans. Long term effectiveness of detergents needs to be monitored. Anionic surfactants have been shown to be effective on coal, coal refuse, and unweathered waste rock from a sulfide orebody.

MATERIALS AND METHODS

GENERAL

Numerous compounds have been shown to be inhibitory to T. ferrooxidans (Shearer et al, 1969; Tuttle and Dugan, 1976; Kleinmann et al, 1981; Onysko et al, 1984; Watzalf, 1988). Probable inhibition of acid generating bacteria using antibacterial substances such as dicyandiamide and N-serve which are known to be inhibitory to related chemoautotrophs has not been investigated. Laboratoy experiments were carried out to determine the minimum inhibitory concentrations of two antibacterial substances and 22 antibiotics. A list of all the compounds screened is shown in Table 1.

SUMMARY OF METHODS FOLLOWED

Varying concentrations of the antibiotics, dicyandiamide and N-serve were added to bacterial cultures grown in 9K medium (Silverman and Lundgren, 1959). Inoculated control (medium with the bacterial culture but without the test compounds) and uninoculated control (medium without the bacterial inoculum and test compounds) were maintained to quantify biotic and abiotic oxidation rates respectively.

Table 1. Antibiotics and Antibacterial Substances Evaluated.
Chloramphenicol
Cephalexin
Kasugamycin
197506
17739
A201A
156565
2-3 deoxy-o mycaminosyltylonlide
N-demethyl hygromycin B
Tropolone
Nebramycin factor 5
15658
Nebramine
Methyl aprosaminide
Althiomycin
Neomycin
Streptomycin sulfate
Hygromycin B_2
A38533B
171541
137870
A38533A_1
Dicyandiamide
N-serve [2 chloro 6 (trichloro) methylpyridine]

The inhibition of bacterial oxidation of the medium was recorded as changes in Eh (redox potential or electrical potential) of the treatments with time. Medium with compounds recording Eh values close to that of uninoculated controls were further investigated for their ability to inhibit bacterial mediated ferrous iron oxidation. The changes in ferrous iron concentrations with time as affected by the selected compounds were recorded. The possible mechanism of inhibition of T. ferrooxidans by N-serve was also investigated.

Since a large number of compounds were screened, it was necessary to obtain results with minimum loss of time. The best method was therefore found to be measurement of Eh with time. Eh has been shown to be a good measure of T. ferrooxidans activity (Dugan and Lundgren, 1964). Eh measurements vary with temperature and components in the medium that can effect the Fe^{3+}/Fe^{2+} couple. The upper range of Eh in the laboratory medium and in the field was found to be 590 and 670 mV respectively (Dugan and Lundgren, 1964; Kleinmann, 1983).

Microorganism

Thiobacillus ferrooxidans culture # 19859 was obtained from American Type Culture Collection (ATCC). The culture was mass cultured by transfering it to 9K medium (Silverman and Lundgren, 1959). The medium was composed of $(NH_4)_2SO_4$ 3.0 g, KCl 0.1 g, $MgSO_4 . 7H_2O$ 0.5 g, $Ca(NO_3)_2$ 0.01 g, H_2SO_4 (10 N) 1.0 ml, and $FeSO_4 . 7H_2O$ 14.74 % w/v in one litre of water.

Inoculum preparation

One ml of the mass culture was transferred to 100 ml of 9K medium in 250 ml flasks. Two ml of the 2 day old (cells in their log phase) culture was used in most screening experiments.

Compounds used in the experiments

Antibiotics used in the experiments were obtained from Lilly and Co. Dicyandiamide was obtained from Fisher Scientific and N-serve [2-chloro-6-(trichloro) methylpyridine] was supplied by Dow Chemical Co.

Strength of the inoculum

The number of cells/ml of the culture inoculum was determined by the MPN method (Standard Methods for Water and Waste Water, 1985).

Measurement of Eh

Eh measurements were taken using a platinum electrode. Zobell solution was used as the standard solution and appropriate temperature corrections were made (Nordstrom, 1977).

Percent Inhibition Of Various Compounds

The percent effectiveness of inhibition of the various compounds at different concentrations was calculated using the relationship :

$$\% \text{ Inhibition} = \frac{S_1 - S_2}{S_1 - S_0} \times 100 \qquad [5]$$

Where,

S_0 = Eh of the unioculated control.

S_1 = Eh of the inoculated control.

S_2 = Eh of the treatment.

SCREENING ANTIBIOTICS AND ANTIBACTERIAL SUBSTANCES

Seven different experiments were carried out to screen all the compounds at different concentrations. A concentration of 100 mg/l was used initially for screening most of the compounds. If only partial inhibition was observed, the tests were repeated with higher concentrations.

Experiment I. Screening of Cephalexin, Chloramphenicol and Dicyandiamide.

One ml of 2 day old culture was transferred to flasks containing 125 ml of sterile 9K medium. Inoculated controls, i.e., flasks receiving the bacterial inoculum, but not supplemented with the compounds being tested, and uninoculated controls, i.e., flasks without bacterial inoculum and without added compounds, were maintained in triplicates. To the rest of the flasks bacterial inoculum, dicyandiamide, cephalexin and chloramphenicol were added, again in triplicates, to obtain a final concentration of 84 mg/l, 347.2 mg/l and 323.2 mg/l respectively (simultaneous with bacterial inoculum). These concentrations corresponded to 10^{-3} M of these compounds in the medium.

The flasks were incubated at room temperature (23°-25°C), on a shaker (180 rpm). Eh readings were taken soon after inoculation and at every 24 hr interval. The average Eh value of the three replicates versus time was plotted.

Experiment II. Screening of dicyandiamide, chloramphenicol, cephalexin and kasugamycin.

The procedure followed was similar to Experiment I but with two variations. The treatments were maintained in duplicates and 2.5 ml of the bacterial inoculum was used. The concentrations of various compounds used were dicyandiamide at 840 mg/l and at 8.4 g/l, cephlexin at 3.47 g/l, chloramphenicol at 32.3 mg/l and kasugamycin at 332.6 mg/l. A graph of the average Eh value against time was plotted.

Experiment III through VII. Screening of 19 antibiotics and N-serve at different concentrations.

Procedures followed were similar to Experiment I with two variations. The quantity of the bacterial inoculum used was 2 ml and the amount of medium in the flasks was 100 ml. Duplicate samples were used. The different compounds screened and the concentrations used

are shown below. Eh measurements were recorded periodically.

Experiment III

The compounds used and their concentration were: antibiotics #197506 (100 mg/l), #17739 (100 mg/l), #A201A (100 mg/l), #15665 (100 mg/l) and 2-3-deoxy-0-mycaminosyltylonlide (100 mg/l).

Experiment IV

The following compounds were screened in this experiment : N-demethyl hygromycin B (100 mg/l), Tropolone (100 mg/l), Nebramine (100 mg/l), A 38533 A_1 (100 mg/l), and Nebramycin factor 5 (100 mg/l).

Experiment V

Six antibiotics # 156568, methyl aprosaminide, althiomycin, neomycin, streptomycin sulfate, hygromycin B_2 were tested, each at 100 mg/l concentration.

Experiment VI

Antibiotics # 171541, # A 38533 B, and # 137870 were tested at 100 and 200 mg/l each and N-serve was tested at 50 and 20 ml/l concentrations.

Experiment VII

Antibiotics # 197506 and A 38533 A_1 were both tested at 300 mg/l concentration, whereas lower concentrations (than in experiment VI) of N-serve (5 ml/l, 10 ml/l, and 15 ml/l) were used.

INHIBITION OF FERROUS IRON OXIDATION ACTIVITY OF T. FERROOXIDANS

Three experiments to observe the inhibition of iron oxidation were carried out using cephalexin, #197506, A38533A_1, dicyandiamide and N-serve at four different concentrations. The changes in ferrous iron concentrations with time were determined. Ferrous iron concentrations close to that of uninoculated controls indicated effective inhibition of T. ferrooxidans.

Experiment VIII. Inhibition by cephalexin and dicyandiamide.

One hundred ml of sterile 9K medium was transferred to sterile 250 ml flasks. A weight of 840 mg of dicyandiamide (10^{-1} m) and 347 mg of cephalexin were added to the flasks in duplicates. Simultaneously, 2 ml of the bacterial inoculum were also added. Inoculated controls and uninoculated controls were maintained in duplicates.

The flasks were kept incubated on a shaker, at room temperature, throughout the course of the experiment. Flasks were removed from the shaker for about 10 min at the time of sampling. Five ml samples were taken at the onset of the experiment and at 24 and 48 hrs after inoculation. The samples were filtered through a 0.25 μM filter and were analysed for ferrous and ferric iron concentrations using a Dionex ion chromatograph (Dionex document # 32558). Ferrous ammonium sulfate was used as the standard ferrous iron solution, and ferric iron solution (Fisher Scientific) was used as the ferric iron standard. The changes in the ferrous iron concentrations in the medium was plotted against time.

Experiment IX. Inhibition of ferrous iron oxidation by T. ferrooxidans as affected by antibiotics # 197506 and A 38533 A_1.

The procedure followed was similar to experiment VIII. Ferrous and ferric iron concentrations were measured initially and at various time intervals for 17 days. The concentrations of antibiotics # 197506 and A 38533 A_1 used were 300 mg/l.

Experiment X. Inhibition of ferrous iron oxidation by T. ferrooxidans as affected by N-serve.

N-serve at concentrations of 0.1, 1.0, 5.0 and 10 ml/l were used in the experiment. The procedure followed was similar to Experiment VIII. Ferrous and ferric iron concentrations were measured initially and at various time intervals for up to 5 weeks by ion chromatography.

Experiment XI. Effect of nitrapyrin on T. ferrooxidans.

Since nitrapyrin is the reported active ingredient in N-serve an experiment was performed to study the effect of nitrapyrin on T. ferrooxidans. As described in Experiment III, inoculated and uninoculated controls were utilized. To flasks containing 100 ml of 9K medium 0.1

ml of N-serve and a weight of .022g of nitrapyrin (the amount present in 0.1 ml of N-serve) dissolved in 1 ml of ethanol was added and duplicate samples were maintained. These treatments were maintained to study the effect of the two compounds on the medium. Two more treatments containing N-seve and nitrapyrin at the above mentioned concentrations along with 2 ml of bacterial inoculum were maintained. Since ethanol was used to dissolve nitrapyrin, in order to separate the effect of ethanol from nitrapyrin, 1 ml of ethanol was added to two flasks along with the bacterial inoculum. Eh measurements were taken periodically.

EFFECT OF N-SERVE ON BACTERIAL CELLS

Morphological changes in T. ferroxidans cells as affected by N-Serve were observed as follows. Two flasks containing 100 ml of 9K medium were inoculated with 2 ml of bacterial culture. The flasks were incubated on a shaker at room temperature (26° C). After two days of incubation, one flask was inoculated with 0.1 ml (1 ml/l) of N-serve. The flasks were incubated for 3 more days after which they were removed from the shaker and 90 ml of the culture from both the treated and untreated flask was centrifuged at 10,000 g for 15 minutes. The clear supernatant solution was siphoned and the cells in the

bottom were retained. Direct microscopic observation was carried out by staining treated and untreated cells with 1 % (v/v) acid fuchsin.

RESULTS

Twenty two different antibiotics (Table 1) were screened for their capacity to inhibit T. ferrooxidans at a reasonable cost. Dicyandiamide and N-serve [2-chloro-6(trichloromethyl) pyridine], which have been shown to be inhibitory to nitrifying bacteria, were also screened for their inhibitory effect on T. ferrooxidans.

Inhibition of bacterial oxidation of 9K medium was followed by supplementing the medium with bacterial inoculum and the test compound. Appropriate controls were also maintained. The effectiveness of inhibition was measured in terms of Eh with time. Increasing concentrations of the test compounds were added until Eh values in the treatment recorded were close to that of uninoculated controls. Complete oxidation of ferrous iron in the 9K medium by the bacteria registered Eh values in the range of 640-650 mv, whereas, the medium without the bacterial inoculum registered 270-290 mv.

Figure 1 shows the effectiveness of chloramphenicol (0.323 g/l) and Cephalexin (0.347 g/l) in inhibiting ferrous iron oxidation. Dicyandiamide at a concentration of 84 mg/l did not inhibit bacterial oxidation. Chloramphenicol and cephalexin both recorded Eh values

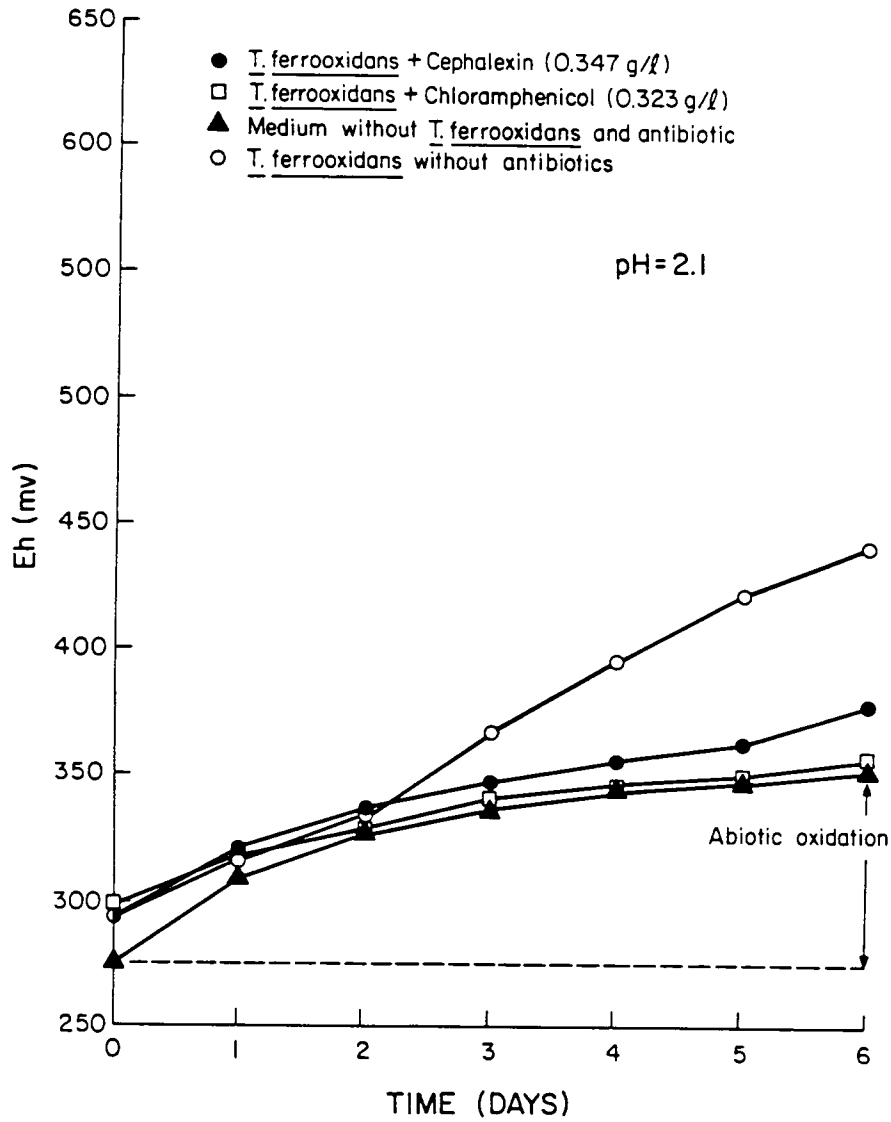


FIG 1: Oxidation of Fe^{2+} as measured by electrical potential vs time. Chloramphenicol and cephalexin are effective at high doses.

close to the uninoculated control indicating inhibiton of bacteria. Chloramphenicol was found to be more effective than cephalexin at the concentrations tested.

A lower concentration of chloramphenicol (32.3 mg/l) and higher concentrations of cephlexin (3.47 g/l) and dicyandiamide (0.84 g/l & 8.4 g/l) along with kasugamycin (0.332 g/l) were used in Experiment II. Only cephlexin (3.47 g/l) and dicyandiamide at 8.4 g/l concentration were found to be effective (Fig. 2). The minumum effective inhibitory concentration for chloramphenicol was 0.323 g/l.

The concentrations of ferrous iron with time in the medium as affected by cephlexin and dicyandiamide are shown in Figure 3 (Experiment VIII). Within 4 days all the ferrous iron in the inoculated control was oxidized whereas approximately was oxidized in flasks treated with dicyandiamide and cephlexin. This value is very close to that recorded for the unioculated control.

The percent inhibition caused by the different compounds is shown in Table 2. N-serve was found to inhibit ferrous iron oxidation at a concentration of 0.1 ml/l. It is clear from Table 2 that antibiotics require higher doses to similarly inhibit the bacterial activity.

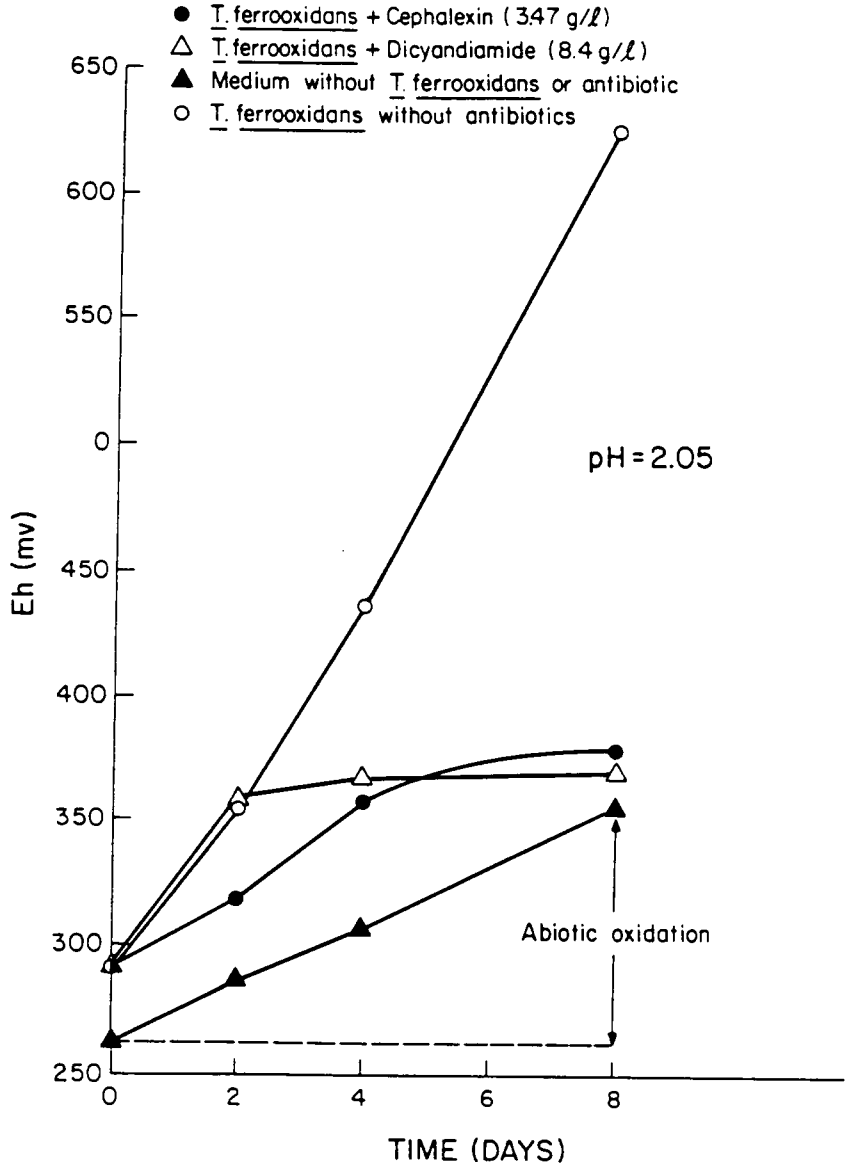


FIG 2: Oxidation of Fe^{2+} as measured by electrical potential vs time. Cephalexin and dicyandiamide are effective at high concentrations.

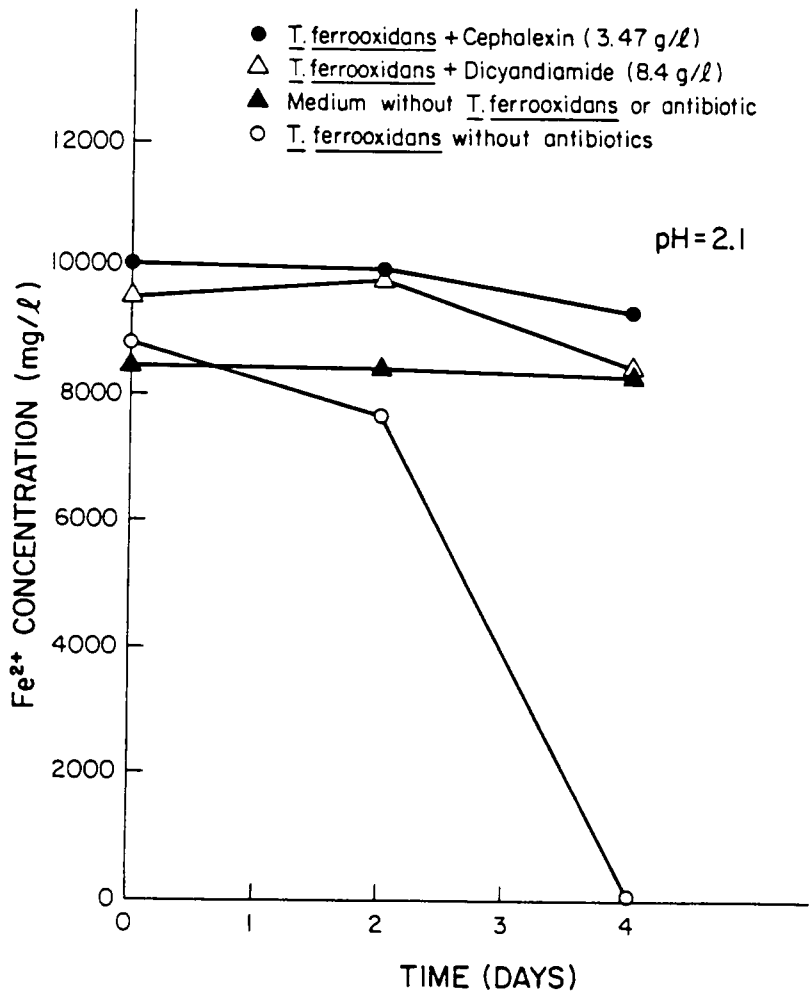


FIG 3: Change in Fe^{2+} iron concentrations as affected by cephalexin and dicyandiamide. Fe^{2+} oxidation is effectively inhibited by cephalexin and dicyandiamide.

Table 2. Percent Effectiveness of Various Antibacterial Substances Screened.

Compound	Concentration	% inhibition
Chloramphenicol	323.2 mg/l	97
Chloramphenicol	32.0 "	12
Cephalexin	347.2 mg/l	72
Cephalexin	3,472.0 "	95
Dicyandiamide	84.0 mg/l	16
Dicyandiamide	840.0 mg/l	25
Dicyandiamide	8,400.0 "	91
Kasugamycin	332.0 "	5
197506	100.0 "	82
197506	300.0 mg/l	94
17739	100.0 "	0
2,3 deoxy-o myminosyltylonide	100.0 "	0
A201A	100.0 "	0
156565	100.0 "	67
N-demethyl hygromycin B	100.0 "	0
Tropolone	100.0 "	3
Nebramine	100.0 "	0
A38533A_1	100.0 "	71
A38533A_1	300.0 "	92
Nebramycin factor 5	100.0 "	0
15658	100.0 "	0
Methyl aprosaminide	100.0 "	0
Althiomycin	100.0 "	0
Neomycin	100.0 "	0
Streptomycin sulfate	100.0 "	0
Hygromycin B_2	100.0 "	0
A38533B	100.0 "	0
A38533B	200.0 "	86
171541	100.0 "	18
171541	200.0 "	97
137870	100.0 "	0
137870	200.0 "	80
N-serve	5.0 ml/l	94
N-serve	10.0 "	93
N-serve	15.0 "	93
N-serve	20.0 ml/l	97
N-serve	50.0 ml/l	95

Five more antibiotics were found to inhibit the bacterial activity at concentrations lower than that observed for cephalexin or chloramphenicol. Antibiotics 137870, A38533B and 171541 (Experiment VI) reduced oxidation levels close to that of the uninoculated control at concentrations of 200 mg/l each (Fig. 4). Concentrations of 300 mg/l each, of the antibiotics 197506 and A38533A$_1$ (Experiment VII) were required (Fig.5) to inhibit ferrous iron oxidation levels closer to the uninoculated control. The latter two compounds also reduced ferrous iron oxidation rates (Experiment IX) close to that of the uninoculated control for 17 days (Fig.6).

Initial screening of N-serve showed no difference in the effectiveness of inhibition between doses of 5 - 15 ml/l (Fig.7). A lower range of concentrations i.e., 0.1, 1.0, 5, and 10 ml/l of the N-serve inoculum was used, and the change in the concentration of ferrous iron was measured with time (Experiment X). The concentration of ferrous iron in all the treatments was measured for 5 weeks and the changes in concentration are shown in Figure 8. There was very little variation in the ferrous iron concentration among the different strengths of N-serve inoculum. Ferrous iron concentrations were almost

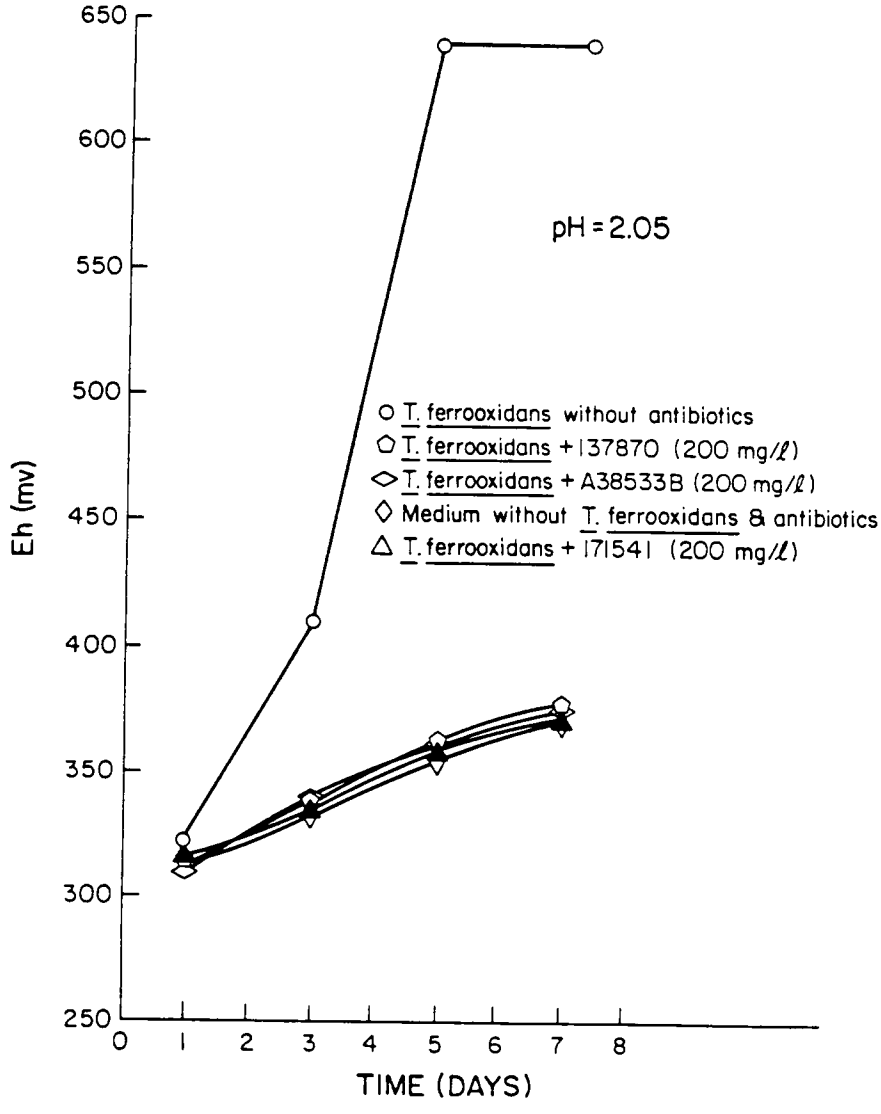


FIG 4: Oxidation of Fe^{2+} as measured by electrical potential vs time. Antibiotics A38533B, 137870 and 171541 all effective at 200 mg/l.

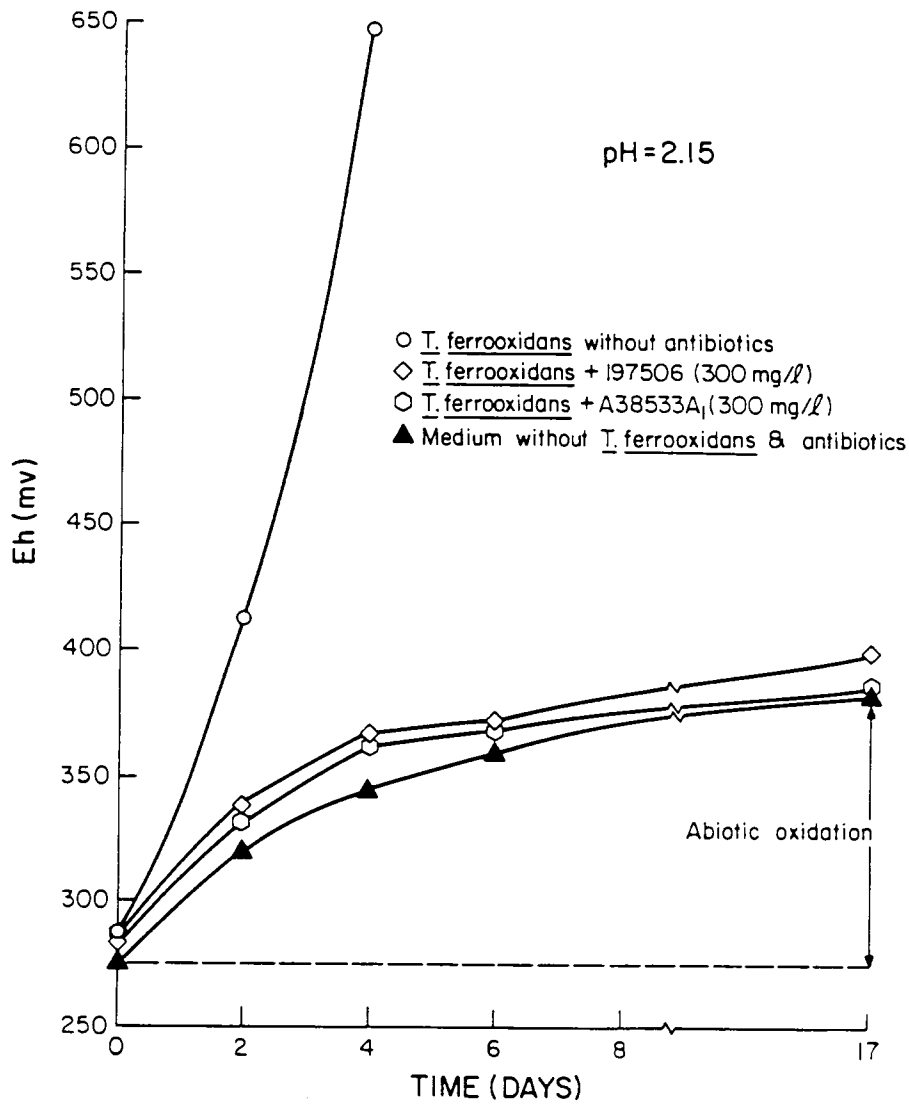


FIG 5: Oxidation of Fe^{2+} as measured by electrical potential. $A38533A_1$ and 197506 are effective at concentration of 300 mg/l.

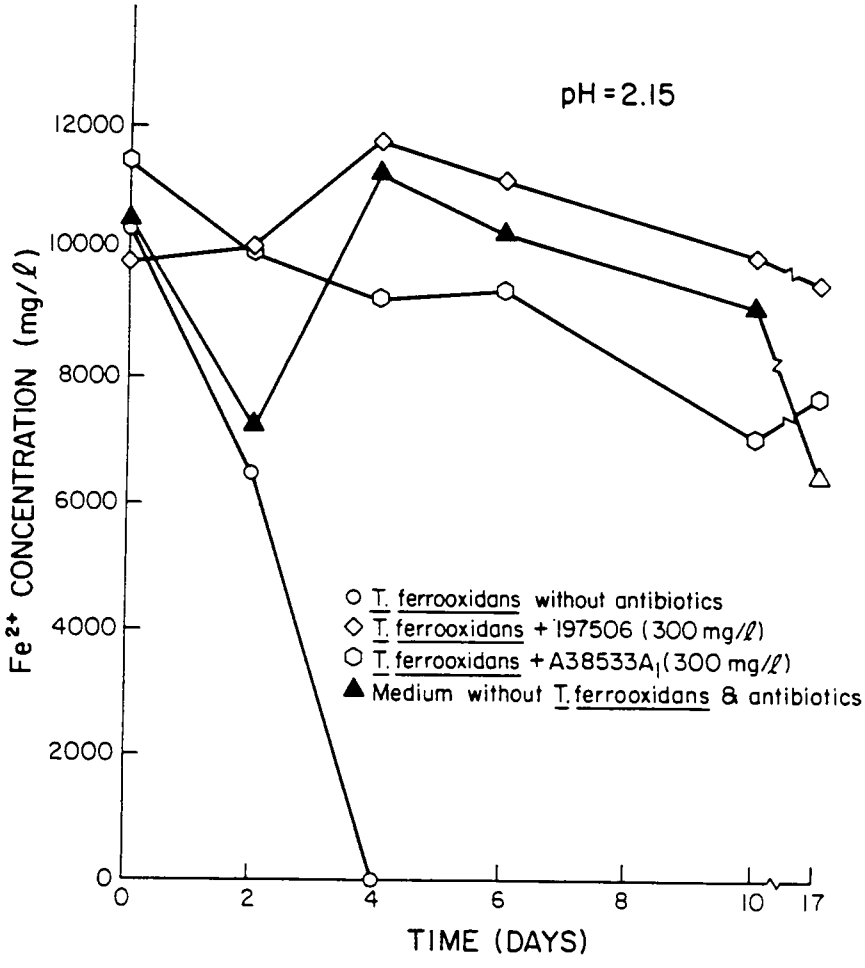


FIG 6: Change in Fe^{2+} concentration by antibiotics 197506 and A38533A$_1$. Fe^{2+} oxidation in antibiotic supplimented treatments is similar to that of medium without T. ferrooxidans and antibiotics.

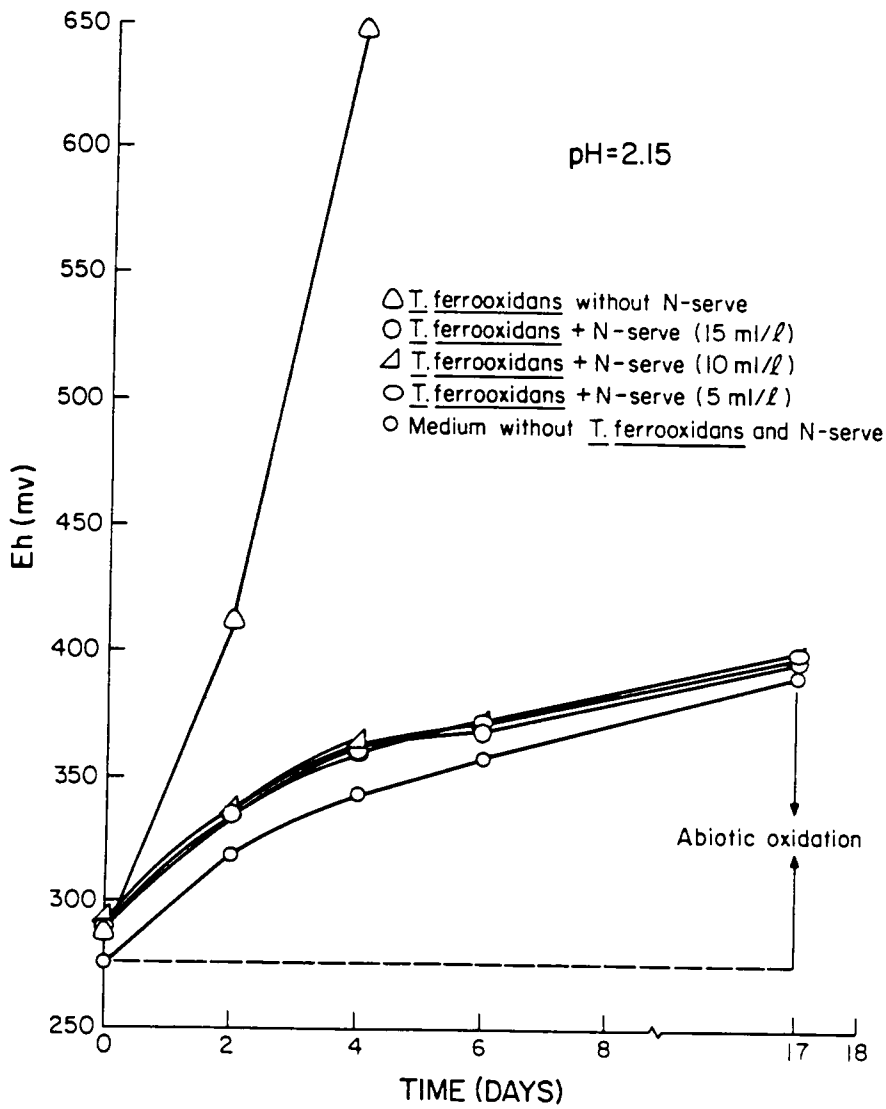


FIG. 7: Oxidation of Fe^{2+} as measured by electrical potential. Response to different doses of N-Serve show complete inhibition with 5-15 ml/l.

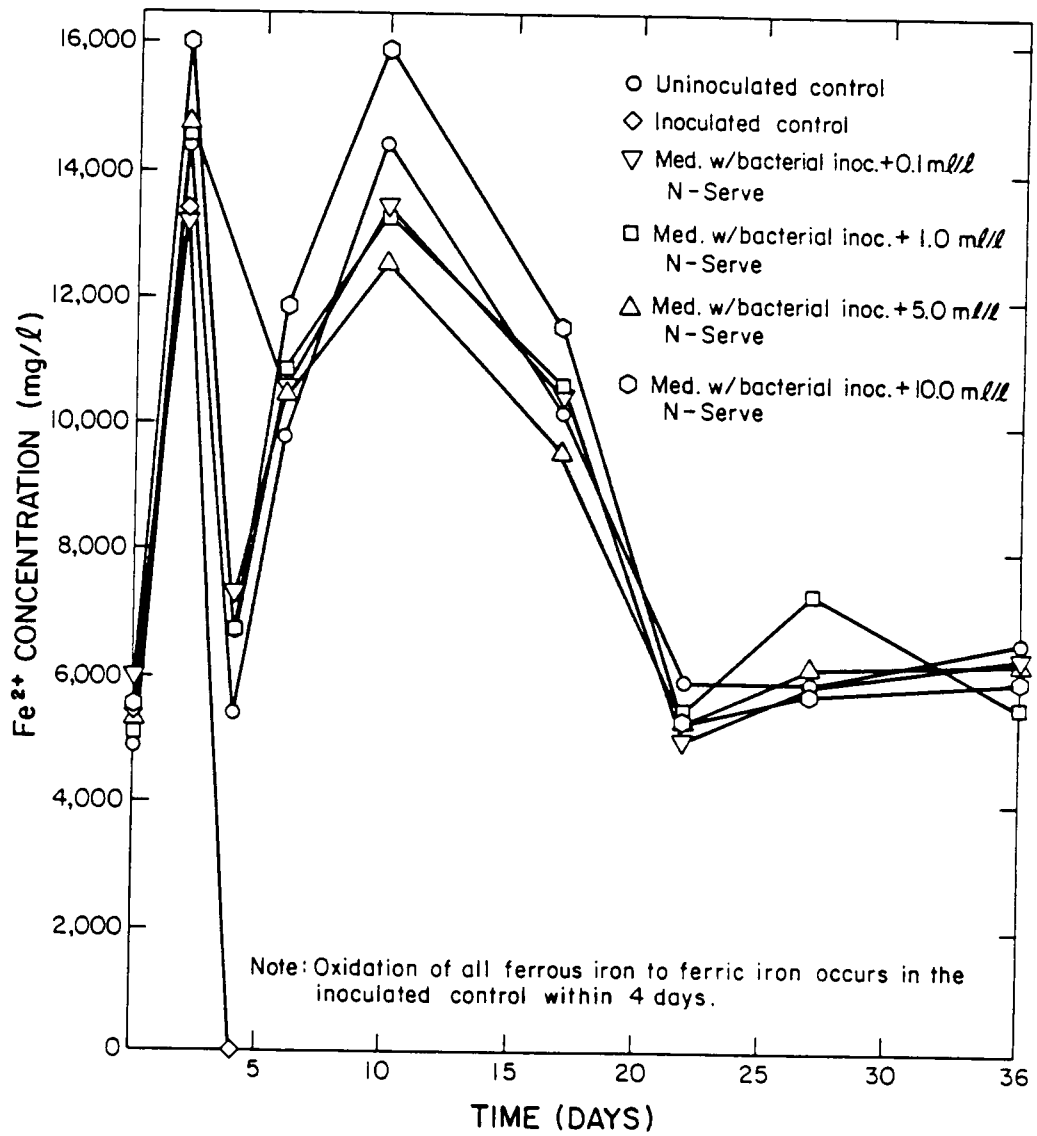


FIG. 8: Change in Fe^{2+} concentration as affected by different doses of N-Serve. Fe^{2+} oxidation rates in all treatments receiving N-Serve are similar to the uninoculated control.

identical to uninoculated controls indicating complete inhibition of baterial activity, probably due to the death of the bacteria. In the inoculated control however, all the ferrous iron was oxidized to ferric form in 4 days. Strength of the bacterial inoculum used in the experiments, based on the MPN method, was found to be approximately 3 x 10^5 cells/ml.

Figure 9 shows the effect of nitrapyrin vs N-serve on T. ferrooxidans. It can be seen that treatments receiving nitrapyrin and N-serve recorded almost identical Eh values. T. ferrooxidans was completely inhibited by nitrapyrin with Eh values close to that of the uninoculated control. It can also be seen that ethanol, used as a solvent for nitrapyrin, did not have any inhibitory effect on the bacteria. Hence, any inhibitory effect in the flasks receiving the bacterial inoculum and nitrapyrin dissolved in ethanol can be attributed to nitrapyrin only.

Photomicrographs of bacterial cells treated with N-serve and cells not receiving the treatment (control) are shown in Figure 10 and Figure 11, respectively. It can be seen that treated cells were very poorly stained and a loopful of inoculum had fewer cells than a similar loopful of untreated cells. This suggests that the

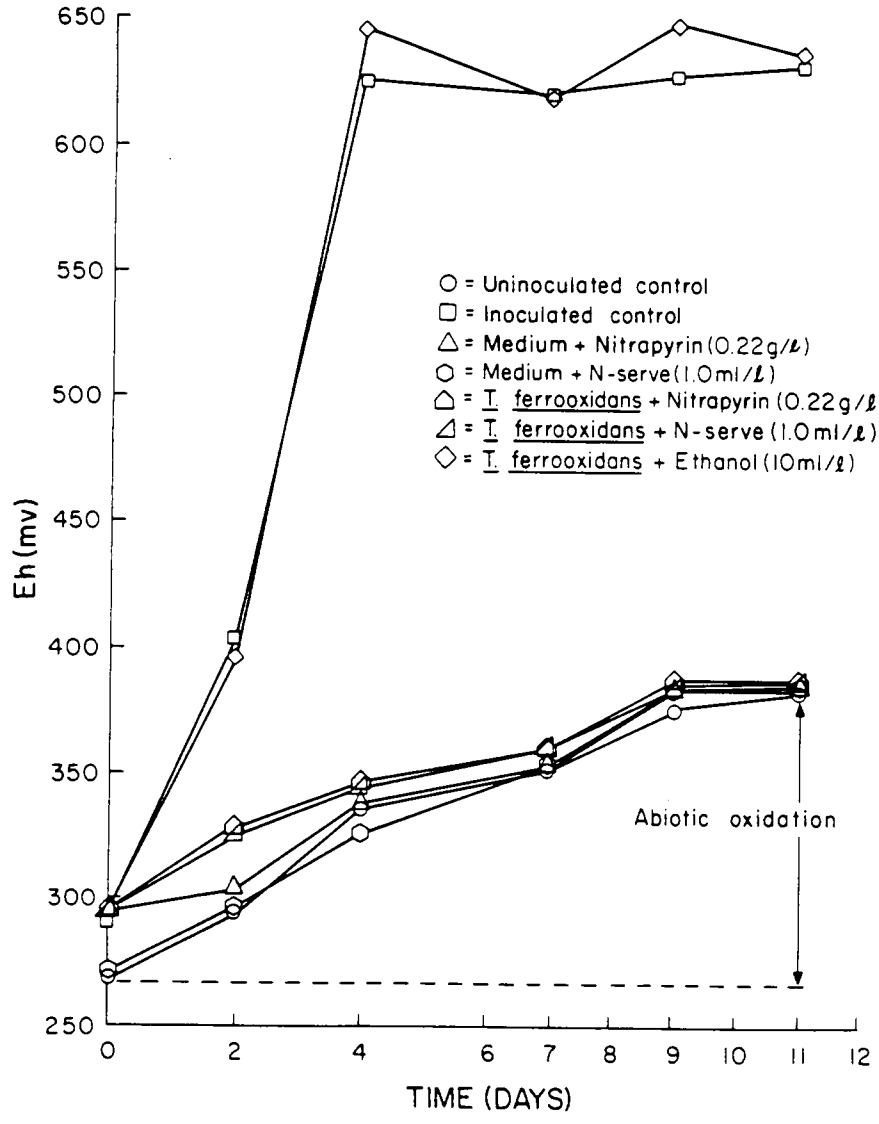
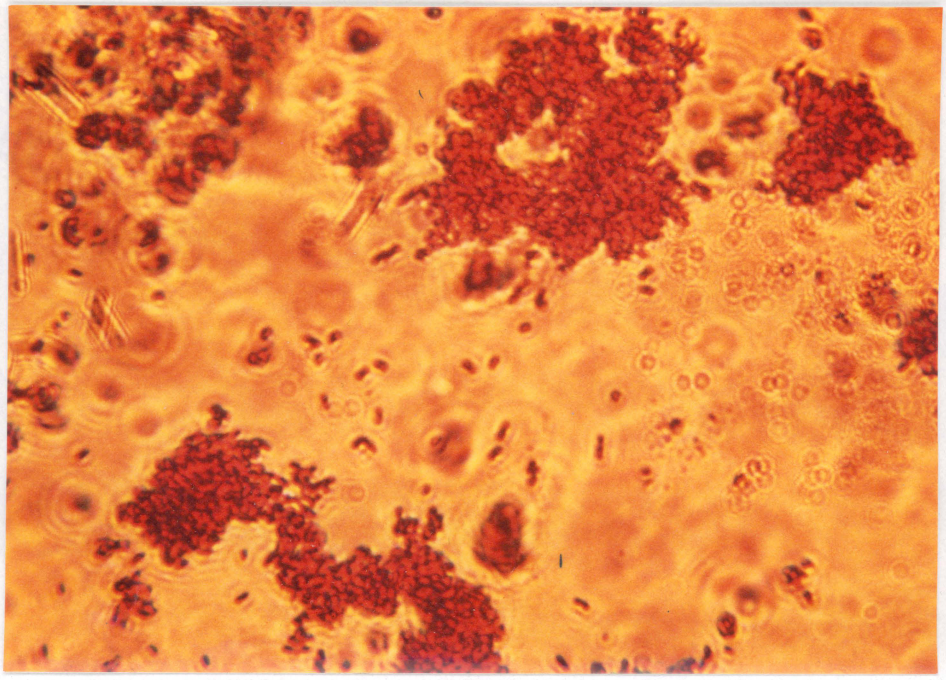
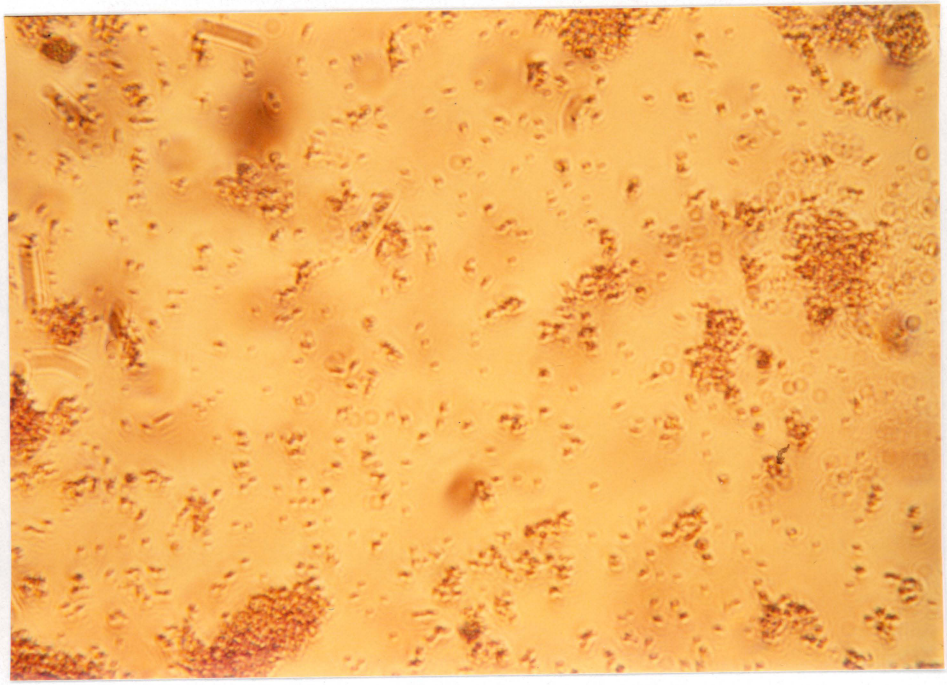


FIG. 9: Oxidation of Fe^{2+} as measured by electrical potential. Nitrapyrin, the active ingredient in N-serve, is the component inhibiting T. ferrooxidans

Figure 10. Photomicrograph of T. ferrooxidans not treated with N-Serve. Cells are poorly stained. (100X).

Figure 11. Photomicrograph of cells treated with N-Serve. Cells are poorly stained. (40X).





possible effect of N-serve on bacterial cells is the destruction of the cell wall, rendering them unable to take up the stain. By destroying the cell wall and possibly the cell membrane the ultimate effect of the compound is probably death of cells.

DISCUSSION

The role of bacteria in causing acid mine drainage is well understood. The possibility of inhibition of these bacteria using low grade antibiotics was studied. Antibiotics are known for their lethal and inhibitory effects on bacteria. Research was directed towards screening of antibiotics for their minimum inhibitory concentration to these bacteria. Further, N-serve [2-chloro-6 (trichloro) methylpyridine] and dicyandiamide, are known to inhibit nitrifying bacteria possessing enzyme systems (cytochrome oxidase) similar to T. ferrooxidans. The capacity of these compounds to inhibit acid producing bacteria was also investigated.

Twenty four different compounds were screened for their ability to inhibit the activity and growth of T. ferrooxidans. Nine antibiotics, dicyandiamide and N-serve (nitrapyin) were found to be effective. Concentrations greater than 100 mg/l of the antibiotics were required to inhibit the acid producing bacteria. A very high dose of dicyandiamide (8.4 g/l) was found to be effective, whereas, 0.01 ml/100 ml of N-serve effectively inhibited the bacteria. Nitrapyrin, which is the active ingredient in N-serve, was found to be responsible for the inhibition of T. ferrooxidans.

As observed by Pichuantes et al, (1986), low pH of the medium might have contributed to the ineffectiveness of 13 of the antibiotics . The mode of action of the antibiotics based on the determination of the ferrous iron oxidation rate seems to be the inhibition of the enzymes involved in the ferrous iron oxidation rather than direct lethality to the bacteria. Treatment of acid mine drainage causing bacteria using antibiotics does not appear to be cost effective.

Bacterial cells treated with N-serve did not take up stain indicating possible dissolution of the cell walls. Therefore, the effect of N-serve on cells may be death due to destruction of cell wall and possibily membrane components. N-serve was also found to be a potential inhibitor of ferrous iron oxidation. Ferrous iron concentrations in flasks treated with N-serve were almost identical to uninoculated controls indicating complete inhibition of bacterial activity. No recovery in bacterial activity was observed even 5 weeks after the treatment. At this concentration the cost of field application of N-serve would appear to be cost effective.

Thus, N-serve when applied on mine refuse can possibly inhibit acid producing bacteria and also inhibit

nitrification and thus aid in the establishment of vegetation. However, the application of N-serve and the of planting crops for revegetation must be carefully timed so as not to encounter any adverse effect on root establishment. The cost of applying N-serve on agricultural fields is approximately $5/acre (Dr. George Hawkins pers. comm.).

CONCLUSIONS

Twenty two different antibiotics plus dicyandiamide and N-serve (nitrapyrin) were screened for their ablity to inhibit T.ferrooxidans. Laboratory experiments were carried out to determine the rate of ferrous iron oxidation as influenced by some of the compounds found to inhibit the bacteria. Based on analysis of the data collected, the following conclusions were made :

1. Only 7 antibiotics out of 22 were found to inhibit T. ferrooxidans. Concentrations greater than 100 mg/l were required to cause noticeable inhibition.

2. Based on a cursory review of the cost of some antibiotics, treatment of acid mine drainage-causing bacteria using antibiotics is not cost effective.

3. N-serve was found to inhibit ferrous iron oxidation by T. ferrooxidans at concentrations of 0.1 ml/l. Ferrous iron concentrations in N-serve treatments was close to that of uninoculated controls suggesting complete inhibition of bacterial activity which may be

due to the death of the organisms.

4. There was no difference in the ferrous iron oxidation rates when concentrations of 0.1, 1.0, 5.0, and 10ml/l of N-serve were used.

5. Nitrapyrin the active ingredient in N-serve is the component inhibiting T. ferrooxidans.

6. The mechanism of inhibition of ferrous iron oxidation by N-serve may be death of bacteria due to the destruction of cell wall.

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