

**The Effect of Carbon Addition, pH and Fe Concentration on  
Microbial Sulfate Reduction and the Subsequent Precipitation of Fe and Mn  
from Acid Mine Drainage in Wetland Mesocosms**

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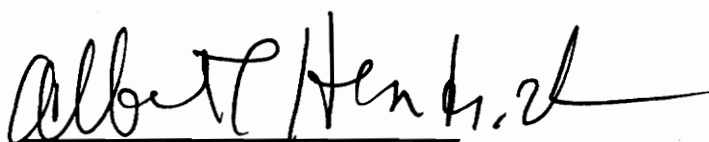
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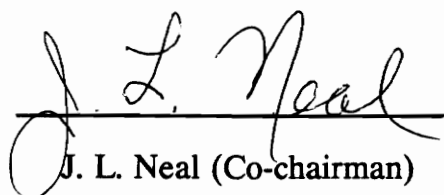
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## ABSTRACT

A wetland was constructed near Norton, VA by Westmoreland Coal Company to treat acid mine drainage (AMD) from an inactive coal refuse pile. The AMD had an average inflow pH of 7.0, and average inflow concentrations measuring 4 mg/L total Fe, 3 mg/L total Mn and 450 mg/L dissolved sulfate. An 18 month field study of water quality improvement and sulfate reducing bacterial (SRB) populations revealed that the wetland was effectively treating the AMD. Iron and Mn both met compliance standards set by the EPA requiring an instream Fe concentration of 3 mg/L and an instream Mn concentration of 2 mg/L. SRB averaged  $8.7 \times 10^4$  through the 18 month study period as determined by the Most Probable Number (MPN) method. The concentration of sulfate was decreased by an average of 360 mg/L as the AMD passed through the wetland.

In a separate laboratory study, the effect of carbon addition, pH and Fe concentration on microbial sulfate reduction and the subsequent precipitation of Fe and Mn was determined in mesocosms built to simulate a wetland. Mesocosms were constructed with plexiglass sheets and measured 6" x 6" x 24". Each mesocosm was filled with a 4 inch layer of limestone gravel beneath 17 inches of weathered pine bark mulch. A perforated PVC pipe was installed within the limestone layer to act as an underground drain. With mulch as the only source of available carbon, a 15% decrease in total sulfate concentration occurred in AMD containing initial concentrations of 1500, 750 and 375 mg/L sulfate. The population of SRB averaged  $10^5$ /g dry mulch. The addition of 300 mg/L carbon as lactate resulted in an a 3 log<sub>10</sub>

increase in SRB population. Following the addition of carbon as lactate, the concentration of sulfate decreased 95%. Total Fe decreased 90% from inflow concentrations prior to the addition of lactate, and decreased 96% following the addition of lactate to the AMD.

The effect of varying the influent pH of AMD was studied using wetland mesocosms, and a pH of 3.5 adversely affected microbial sulfate reduction and water quality improvement. Populations of SRB decreased by 3 log<sub>10</sub> from an initial population of 10<sup>8</sup> SRB/g dry mulch. Iron and Mn concentrations decreased 70 and 37% respectively. Hydrogen ion concentration increased to 7.0 and above when inflow pH was 4.5 and 6.0, but increased to an average of 6.4 when inflow pH was 3.5.

The effect of different concentrations of Fe within AMD was investigated using wetland mesocosms, and total inflow Fe concentrations of 155 and 301 mg/L resulted in a greater percent decrease in sulfate concentrations than at a lesser Fe concentration averaging 85 mg/L. Total Mn decreased 12% at an inflow Fe concentration of 85 mg/L, and decreased 43% at an inflow Fe concentration of 301 mg/L. The results generated from both the analysis of the Pine Branch wetland and the laboratory mesocosm experiments demonstrate that subsurface flow constructed wetlands are a viable form for treatment of AMD.

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## INTRODUCTION

Acid mine drainage (AMD) is a product of the coal mining industry that has come under considerable scrutiny. It is formed by the oxidation of pyritic minerals upon exposure to oxygen and water during the mining process. AMD is typically characterized by a low pH and high concentrations of ferric iron, manganic manganese, and sulfate. When untreated AMD flows into surface streams or lakes it degrades water quality. High iron content consumes oxygen upon formation of ferric hydroxide and release of acidity. Water becomes devoid of complex aquatic plants, fish, most invertebrates and all but a few species of tolerant algae and microorganisms. Over 12,000 Km of streams and rivers in the Appalachian coal region alone are contaminated with AMD (Appalachian Regional Commission, 1969).

Regulations have been established by federal and state authorities to monitor discharge quality. The Environmental Protection Agency requires a pH of 6.0-9.0, an instream iron concentration of less than 3 mg/L and instream manganese concentration of less than 2 mg/L (EPA). Traditional treatment methods involve the use of liquid caustic soda or lime to raise the pH and precipitate metals. The need for constant monitoring and maintenance, chemical costs and waste disposal make this a labor intensive and expensive treatment of AMD. The use of constructed wetlands as an alternative to traditional chemical treatment has often proven to be a cost effective and more efficient means for mitigating AMD (Hedin et al., 1988;

Wieder et al., 1988).

"Wetlands" is a general term that includes marshes, swamps, bogs, wet meadows, etc. "Constructed wetlands" refers to man made complexes of substrates, aquatic vegetation, animals and microorganisms that are built to treat contaminated water such as AMD (Hammer, 1989). They utilize both nonbiological and microbiological processes as a means for abatement of contaminated water. Microbial sulfate reduction in particular is an important process involved in treating AMD. Two products of sulfate reduction, hydrogen sulfide and bicarbonate are responsible in part for the mitigation of AMD within constructed wetlands. Bicarbonate contributes alkalinity and increases the pH, and  $H_2S$  precipitates iron and other metals as metal monosulfides. Wetlands constructed to enhance anaerobic conditions, and subsequently microbial sulfate reduction, by means of subsurface water flow have been shown to increase water quality over those wetlands with surface flow only (McIntire et al., 1990).

The objectives of this study were to 1) evaluate the success of a wetland constructed to treat AMD in southwest Virginia and 2) to determine the effect of carbon, pH and Fe concentrations on microbial sulfate reduction and water quality improvement of AMD through the use of wetland mesocosms.

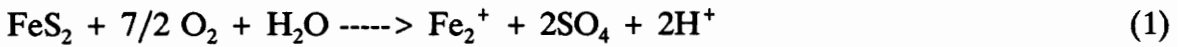
## LITERATURE REVIEW

### Acid Mine Drainage

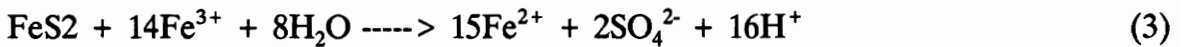
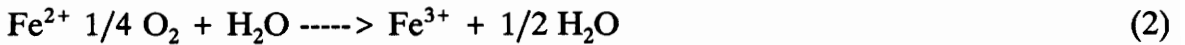
Acid mine drainage (AMD) results from the abiotic and biotic oxidation of pyrite upon exposure to oxygen and water within coal mines. When pyrite is exposed to water during mining, a slow oxidation with molecular oxygen takes place (Eq. 1). The oxidation at a neutral pH is thought to be largely abiotic (Starkey, 1966; Stumm and Morgan, 1970). However, organisms able to oxidize sulfur compounds at a neutral pH may contribute to the overall rate on a limited basis. Oxidations occurring above a pH of 5.0 result in the consumption of oxygen, the release of ferrous iron and an increase in acidity in the form of sulfuric acid (Brock, 1988). The low pH also solubilizes associated metals such as manganese into the AMD (Hammer, 1991). The ferrous ions produced during pyrite oxidation react with oxygen and hydrogen to form ferric iron and water (Eq. 2). Above pH 4.5, abiotic ferrous iron oxidation proceeds rather rapidly. Between pH 4.5 and 3.5 the abiotic oxidations slows. As acidity increases and pH decreases (Eqs. 2 and 3), abiotic oxidation of ferrous iron slows, and the amount of microbially catalyzed oxidation increases. Walsh and Mitchell (1972) suggest that a succession of different genera of iron bacteria are responsible for the continued oxidation of ferrous iron below pH 5.0. They suggest that at pH 4.0 - 3.5, a bacterium of the genera *Metallogenium* is active. Other iron oxidizing microorganisms including *Sulfolobus* sp., *Leptothrix* sp., *Gallionella* sp., and *Siderocapsa* sp. may be present (Hammer, 1990) *Thiobacillus*

*ferrooxidans*, a chemoautotrophic aerobic acidophile with a pH range of 3.5 - 1.5, is largely responsible for iron oxidation below pH 3.5 (Walsh and Mitchell, 1972, Hutchins et al., 1986). Investigations have revealed that *T. ferrooxidans* catalyzes the oxidation of ferrous iron by a factor of 300 over that of abiotic oxidation (Singer and Stumm, 1970). Studies have also suggested that microorganisms influence the rate of iron oxidation (Eq.2) because that alone is the rate limiting step (Silverman and Lundgren, 1959).

#### Pyrite Oxidation



#### Pyrite Degradation (ferrous iron oxidation) Cycle



#### Ferric Iron Deposition



The ferric ions released from the oxidation of ferrous iron (Eq. 2) may be oxidized by pyrite to create more ferrous ions and acidity in the absence of oxygen (Eq. 3), or they may react with oxygen and water, after leaving the mine and entering

receiving streams, and be deposited as ferric hydroxide (Eq. 4). When AMD enters receiving bodies of water, the precipitation of ferric hydroxide, often called "yellow boy", consumes oxygen and produces acidity in the form of hydrogen ions (Stumm and Morgan, 1970). The majority of the acidity in AMD is produced by the oxidation of pyrite by ferric iron during pyrite degradation (Eq. 3) or the formation of ferric hydroxide and release of hydrogen (Eq. 4) (Walsh and Mitchell, 1972). This entire series of events is referred to as the "Propagation Cycle" (Brock, 1988). Once the propagation cycle has been started, oxygen is no longer directly involved and the oxygenation of pyrite (Eq. 1) is no longer significant (Stumm and Morgan, 1970).

Over 17,000 Km of streams and rivers in the United States, many of which are sources for human consumption, are contaminated with AMD (Council on Environmental Quality, 1981). When untreated AMD flows into surface streams or lakes, it renders the water nonpotable and unable to support most forms of aquatic life. High ferric iron content consumes oxygen by forming yellow boy and releasing acidity as the AMD enters the receiving body of water. Acidity, low pH, and heavy metal concentrations cause the water to be devoid of complex aquatic plants, fish, most invertebrates, and all but a few species of tolerant algae. Due to the instrumental role that *T. ferrooxidans* plays in the production of AMD, treatment methods that prevent the formation of AMD by the inhibition of *Thiobacillus* have been suggested. Walsh and Mitchell (1972) proposed breaking the succession of iron oxidizing bacteria at the pH 4.0 - 4.5 range, possibly through the addition of lime.



The U.S. Bureau of Mines has tested various biodegradable detergents, such as sodium lauryl sulfate, which have been shown to successfully inhibit *T. ferrooxidans* (Kleinmann, 1985). Success has been limited however, since populations of *T. ferrooxidans* increase after several months and constant reapplication of the detergent is necessary. Gussmann et al. (1991) have attempted to treat underground mine water to remove calcium and sulfates through ion exchange processes. Another system for the control of *T. ferrooxidans* and acidity production, the anoxic limestone drain, has been proposed by the Tennessee Valley Authority (Brodie et. al., 1990). This involves sealing and backfilling the mine with limestone. Doing so maintains anaerobic conditions within the mine as well as keeping the pH above the iron oxidizers optimal range. Anaerobic conditions also keep the iron in the soluble ferrous form. The AMD flow is diverted underground to maintain anaerobic conditions and passed through a clay capped ditch filled with limestone. The limestone adds alkalinity to the water thus buffering the acidity that is produced once the AMD is aerated.

Traditional methods for treating AMD after leaving the mine include adding powdered lime or liquid caustic soda to raise the pH and precipitate metals. Constant monitoring and maintenance, chemical costs, and waste disposal make this a labor intensive and expensive means of treating AMD. Compounds such as Trapzene have been studied which raise pH, buffer, and remove metals simultaneously (Lovett et al., 1991). Studies have also been conducted that examine

the addition of phosphate refuse to coal mine refuse to prevent the formation of AMD (Hart and Stiller, 1991; Renton et al., 1988). The use of constructed wetlands as an alternative to traditional chemical treatment has often proven to be a cost effective and more efficient means for mitigating AMD (Hedin et al., 1988; Duddleston et al., 1992; Wieder et al., 1988).

### **Constructed Wetlands**

"Wetlands" is a general term that includes marshes, swamps, bogs and wet meadows. "Constructed wetlands" is a term used to identify those man made complexes of substrates, aquatic vegetation, animals and bacteria that are built to treat contaminated water such as AMD (Hammer, 1989). According to Hammer and Bastian (1989), most constructed wetlands have six principal components: Substrates such as mulch or mushroom compost, plants adapted to water saturated substrates, high acidity and metals, a water column flowing in or above the substrate, vertebrates and invertebrates, and an aerobic and anaerobic microbial population. Substrates such as soils, sands and compost provide physical support for plants and attachment surfaces and carbon for microorganisms. Plants provide oxygen transport and release organic acids which act as utilizable carbon sources for other organisms. The water column allows for transport of substances and gases to microbes as well as removal of end products. Microbes, including bacteria, fungi, algae and protozoa play a large role in AMD treatment by utilizing substances in the water as nutrients and energy sources.

Wetlands constructed to treat AMD have encompassed a wide variety of design styles. According to a study of 142 wetlands east of the Mississippi conducted by Wieder (1989), most constructed wetlands consist of an organic substrate of some type. Popular choices include mushroom compost, as well as composted manure, hay or straw, peat, or a combination of several. Vegetation is also a common design parameter, usually in the form of cattails (*Typhus* sp.) or sometimes mosses and rushes (Wieder, 1989). Very frequently, wetlands are constructed to consist of several ponds in succession. Wieder (1989) concluded in his study that wetland treatment at this point is extremely variable and unpredictable in terms of potential treatment success. In his study, Wieder (1989) found that over 11% of the wetlands he surveyed yielded greater concentrations of metals in the AMD after it passed through a constructed wetland. Although Wieder (1989) also concluded from his survey that there was no correlation between wetland design criteria and treatment efficiency, several studies have been conducted that compare designs of wetlands with regard to water quality (Hedin and Nairn, 1992; Kepler, 1990; Stark et al. 1990) Kleinmann et al. (1986) and others (Hedin and Nairn, 1992) have developed design criteria for constructed wetlands which depend on water quality and space available. However, constant change and upgrading of design criteria continue, and they are useful only for general planning (Brodie, 1990).

The Tennessee Valley Authority (TVA) has reported success using aerobic surface flow constructed wetlands to treat AMD (Brodie, 1991). TVA operates 11

wetland systems at three coal mines and a reclaimed coal preparation plant site (Brodie, 1991). Five of TVA's wetlands have produced effluent meeting all discharge parameters without additional chemical treatments. Two of these have been released from NPDES monitoring requirements (Brodie, 1991). The five systems that do not require the addition of chemicals treat AMD consisting of moderate water quality (Fe = 69 mg/L, Mn = 14 mg/L) and high treatment areas (Brodie, 1991). Four of the 11 wetland systems produced high acidity and low pH due to iron oxidation and precipitation. The inflow iron concentrations at these sites range from 40 - 170 mg/L (Brodie, 1991). One of the 11 systems has experienced a higher outflow of Mn. It was speculated that the absence of Mn oxidizing bacteria or lack of Fe-Mn co-precipitation was the cause (Brodie, 1991). Stillings et al. (1988) reported a 74% removal of the iron and an 8.3% removal of Mn in a surface flow only wetland. Similarly, Stark et al. (1988) reported a 70 - 85% reduction in iron as AMD passed through a surface flow wetland.

Wetlands utilizing underground drains, thereby enhancing both aerobic and anaerobic phases of wetland treatment have also been successful. Hendricks (1991) achieved 90% reduction in Fe (inlet averaged 326 mg/L) and an increase in pH from 3.5 to 6.1 in a wetland constructed to treat AMD from an inactive pyrite mine. A wetland constructed by Westmoreland Coal Co. to treat AMD from a coal refuse pile has met in stream compliance standards for Fe, Mn and pH without the addition of chemicals (Duddleston, et al., 1992).

Tuttle et al. (1968) showed that when AMD rich in ferric and sulfate ions passed through a porous dam composed of wood dust, the wood dust was enriched with organic nutrients and supported the growth of heterotrophic bacteria, including sulfate reducers. They found that these microbes reduced sulfate to hydrogen sulfide, and the hydrogen sulfide in turn reduced ferric to ferrous iron which was precipitated as ferrous sulfide (FeS). Herlihy et al., (1987) calculated the sulfate influx-efflux budget for the Contrary Creek arm of Lake Anna in Virginia. The budget showed that 48% of the sulfate that entered the arm of the lake did not leave through the outflow. They found rates of sulfate reduction to equal 200% of the sulfate removal, and that the generation of alkalinity by sulfate reduction was enough to account for the increase in pH in the lake. By precipitating metals as metal monosulfides and generating alkalinity which helps raise pH, the significance of microbial sulfate reduction becomes apparent as a means of treating AMD.

McIntire and Edenborn (1990) constructed a wetland called Friendship Hill in Pennsylvania utilizing both surface and subsurface flow ponds (often called cells). They found that when AMD passed through the cell incorporating an underground drain, and thus subsurface flow, an increase in water quality was seen over that of surface flow only. These findings were attributed to an increase in contact time between AMD and wetland substrate, as well as exposure of the AMD to the anaerobic zone within the wetland containing sulfate reducing bacteria (SRB). Several studies have been conducted to determine the contribution of sulfate

reduction (SR) to treatment of AMD within constructed wetlands. Hedin et al. (1988) found that where organic substrate in a wetland was black and loose, indicating SR, sulfate concentrations were 15 -59 % less than inflow, Fe 52 - 98% less and pH 2 - 3 units higher. Ninety six percent of the iron present in these areas was in the ferrous form. In areas where the substrate was blanketed with ferric hydroxide, the water quality was similar to that of inflow (Hedin et al., 1988). Hammack and Edenborn (1990) found that when adding sodium lactate to inflow AMD containing high concentrations of nickel, a 7 fold increase in nickel removal was seen over that of no added lactate. This was presumed to be due to increased SR rates. The study indicates that bacterial sulfate reduction can treat nickel concentrations up to 500 mg/L if carbon is non-limiting (Hammack and Edenborn, 1990). Dvorak et al. (1992), utilizing pilot scale reactors in which bacterial sulfate reduction was enhanced, found that Fe, Zn, Mn, Ni and Cd concentrations were lowered over 95% from total inflow concentrations, and existed in the form of insoluble sulfides.

Ferric iron reducing bacteria have also been shown to mineralize organic matter in anaerobic sediments (Lovley and Phillips, 1986). Studies indicate that under selected environmental conditions, ferric iron reducers may out compete sulfate reducers for available carbon (Lovley and Phillips, 1987). *In lieu* of these findings, the reduction of ferric iron to ferrous iron by iron oxidizing bacteria may be a contributing metabolic process occurring in wetlands constructed to treat AMD.

### **Sulfate Reducing Bacteria**

Sulfate reducing bacteria (SRB) were first isolated by Beijerinck 1895 (Postgate, 1979). Sulfate reduction had previously been attributed to algae and the filamentous microorganism *Beggiatoa* (Postgate, 1979). Although SRB were known in the late 1800's, isolation of pure cultures was not accomplished successfully until 1949 (Butlin et al.) primarily due to the lack of experience necessary to obtain anaerobic conditions (Postgate, 1979). Classification of SRB is based primarily on nutrition and morphology. Recent DNA and rRNA data have supported this classification (Widdel, 1988), with some exceptions.

Sulfate reducing bacteria are strict anaerobes utilizing sulfate as an electron acceptor (most can also use sulfite or thiosulfate) and low molecular weight carbon sources as electron donors (Brock, 1991). Some of these include lactate, formate, and acetate (Brock, 1991). Some strains have been shown to be capable of incomplete oxidation of propionate, butyrate, alcohols and various organic acids. Consequently, they depend on other microorganisms to degrade more complex carbon substrates with subsequent release more simple forms of carbon into the environment. Sulfate reducers require a low redox potential and are thus restricted to reducing environments such as sediments, muds, and water logged soils. The activity of SRB in these environments depends on the presence of sulfate and organic content. Kimata et al. (1955, in Postgate, 1979) showed that organic material was necessary as a source of carbon rather than nitrogen in the mud of Hiroshima Bay.

Many species of SRB are capable of fixing nitrogen (Postgate, 1979). Desrochers and Fredette (1960) studied the population of SRB in the Ottawa river sediment in Canada at a point downstream from paper mill effluent outfall. They found a dramatic increase in SRB population over that of upstream sites. They suggested this was probably due to increased sulfate concentrations and organic material.

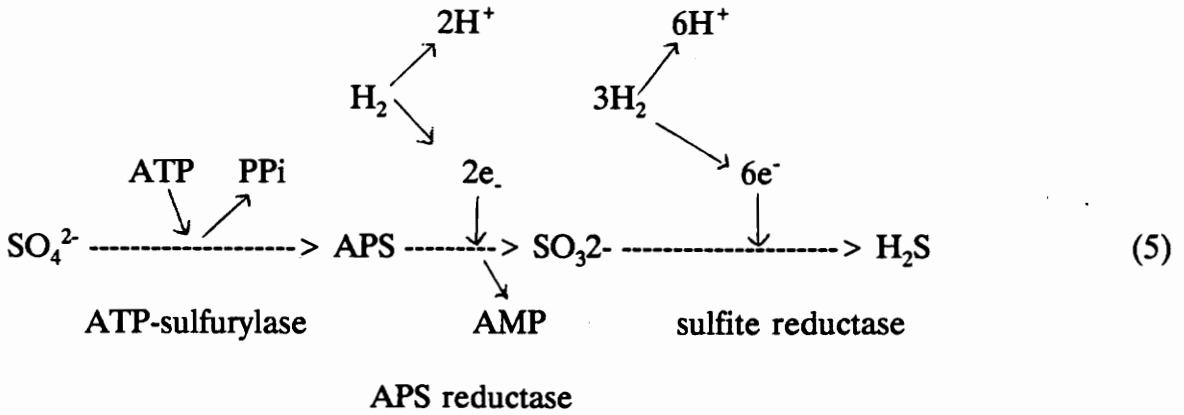
The presence of SRB in an environment can greatly change the nature of the environment (Postgate, 1979). When aerobic activity decreases, and anaerobic activity increases, much of the oxidizable carbon undergoes fermentation instead of reduction. The production of sulfide, the end product of sulfate reduction, tends to further inhibit aerobic activity at both the micro- and macro-biological level. The production of sulfide and alkalinity also have an effect on pH. During periods of active sulfate reduction, the environment tends to become more alkaline (Postgate, 1979). Sulfate reducers are considered terminal degraders, as are methanogenic bacteria that form methane and carbon dioxide under anaerobic conditions (Widdel, 1988). Under anaerobic conditions where sulfate is present, sulfate reduction is the predominant degradative process (Hoppe-Seyler, 1886). Once sulfate is depleted, methanogenesis becomes the predominant degradative process. These microorganisms appear to utilize common sources of electron donors (Widdel, 1988).

The SRB include terrestrial and aquatic, both marine and freshwater, thermophilic and psychrophilic, and spore forming and non-spore forming types



(Widdel, 1988). They have been shown to tolerate pH ranging from below 5 to 9.5, as well as live on deep ocean floors at high hydrostatic pressures (Postgate, 1979). *Desulfovibrio* is the most common sulfate reducer found in brackish and marine environments. Although strains of *Desulfotomaculum* are able to acclimate to saline conditions under laboratory conditions, they are not normally present in marine environments (Postgate, 1979). Even the rumen of ruminant mammals and the guts of certain insects have been shown to contain strains of either *Desulfovibrio* or *Desulfotomaculum* (Postgate, 1979).

Equation 5 shows the reduction of sulfate to hydrogen sulfide as carried out by SRB. ATP-sulfurylase activates sulfate to APS (adenosine phosphosulfate), splitting ATP and releasing phosphorus. APS reductase catalyses the reduction of APS to sulfite with the release of AMP. APS reductases in thiobacilli, *Desulfovibrio vulgaris*, *Desulfobulbus propionicus*, and *Chlorobium limicola* are similar in that they contain flavin and non-heme iron-sulfur clusters (Stille and Truper, 1984; Trudinger and Williams, 1982; Truper and Pfennig, 1966). Several strains of sulfate reducers have APS reductases that behave similarly within their genus but differently between genera (Widdel, 1988). Sulfite reductases vary between species depending on the optimum carbon source and temperature.



The amount of hydrogen sulfide produced far exceeds the amount of sulfide necessary for cell synthesis (Widdel, 1988). For example, the production of 1.4 grams cell mass with a sulfur content of only 1.3% requires that only 0.2 g hydrogen sulfide need be produced. However, studies have shown that a cell mass of 1.4 g produces 100 mg hydrogen sulfide with 100 mg acetate as electron donor (Widdel, 1988).

## MATERIALS AND METHODS

### Field Studies

Site description. A wetland was constructed near Norton, Virginia, by Westmoreland Coal Company in late summer 1990. The 0.7 acre wetland was constructed to treat seepage from an inactive coal refuse pile containing 4.5 million cubic yards of waste silt and rock material. Rain and spring water flowing through the refuse pile resulted in AMD flowing from its outer slopes and underground drains at a rate of 50 gpm. Prior to chemical treatment the AMD pH ranged from 3.5-9.1, the iron from 4.6-38.5 mg/L and manganese from 2.5-4.3 mg/L.

Three ponds located downstream from the refuse pile were used for containment and sedimentation of plant process water during active mining. The middle of these three ponds was converted to a wetland at a cost of \$25,880. From the wetland, the AMD flows into the third pond where additional chemicals may be added if necessary before flowing into Pine Branch, a tributary to the Powell River.

The wetland was built with a bed of limestone one foot deep beneath a one foot layer of weathered pine bark mulch. Six inch perforated pipes were placed within the limestone bed to serve as an underground drain. An outlet stand pipe was installed to allow for water level adjustment. Additional standpipes were installed within the wetland for cleaning of the underground drains (Fig 1). Cattails (*Typhus* sp.) were planted in April, 1991 (Fig 2).

Water chemistry and sulfate reducing bacteria enumeration. Inlet and outlet

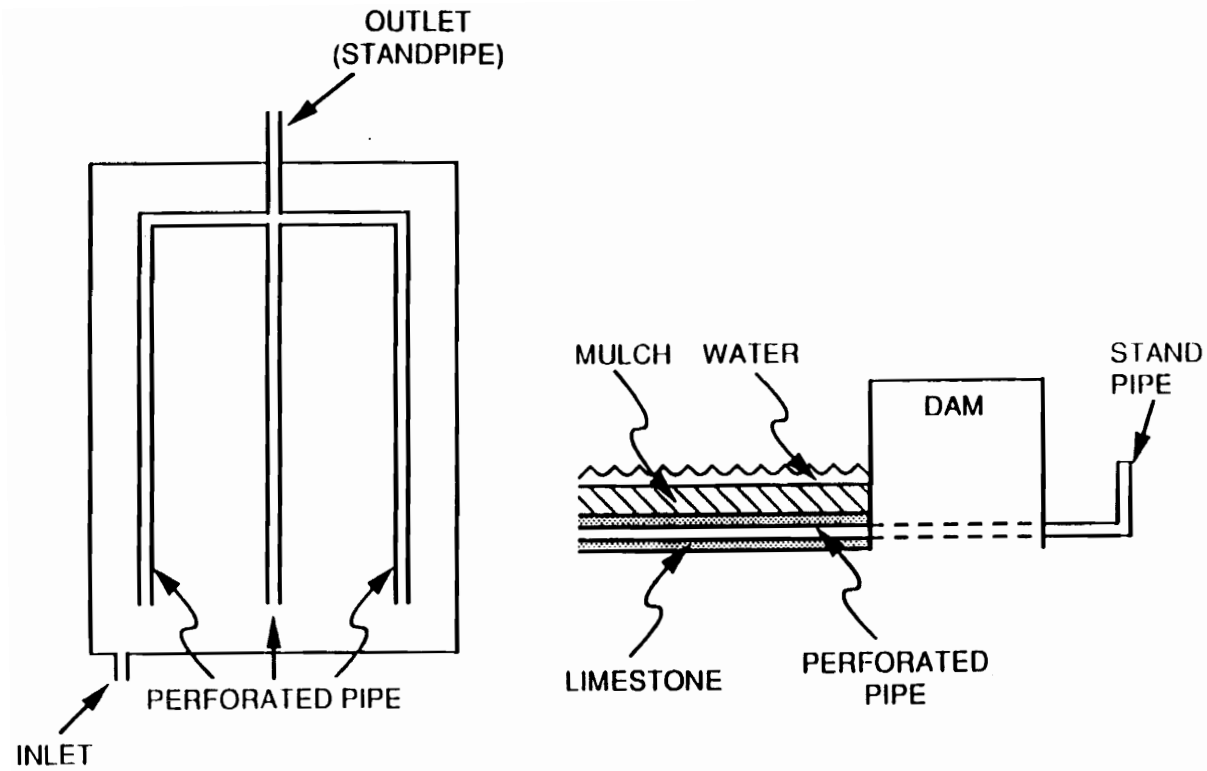


Figure 1. Diagram of underdrain arrangement. Underdrains were embedded within the limestone layer below the wetland substrate.

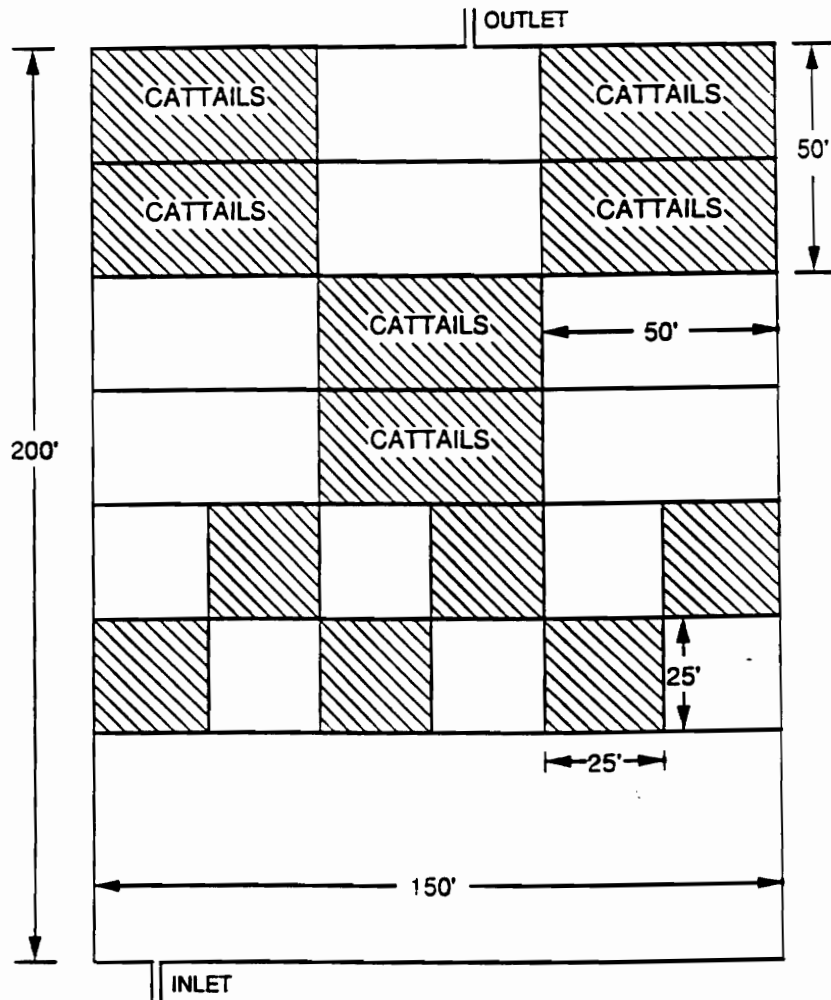


Figure 2. Diagram of cattail plantings. Shaded areas were planted with cattails. Large plots are 50' x 50', small plots are 25' x 25'.

water was analyzed for Fe, Mn and pH weekly by Environmental Monitoring Inc., Coeburn, Virginia. All other analyses were performed at Virginia Polytechnic Institute and State University. Additional analyses of total Fe, total Mn, and  $\text{SO}_4$  were done on wetland inlet, outlet, surface and subsurface (20 cm below mulch surface) water using atomic absorption spectrophotometry (Fe and Mn) and  $\text{BaCl}_2$  respectively (Methods for Chemical Analyses of Water and Wastes, 1985)

Substrate samples were collected to enumerate sulfate reducing bacteria (SRB). Three samples were taken from four or more areas in a random fashion (Petersen and Calvin, 1965) within the wetland using a pitchfork. They were placed in plastic bags and held on ice during transport to the laboratory. SRB enumeration was begun within 48 hours after sample collection using the Most Probable Number (MPN) method (Alexander, 1982) following incubation in Postgate's medium C (1979) at 30°C for 4 weeks. Comparisons between population size between areas with and without cattails were made.

### **Wetland Mesocosms**

Mesocosm construction. Wetland mesocosms were constructed of plexiglass sheets. Boxes measuring 6" x 6" x 24" were filled with 4 inches of limestone gravel under 17 inches of 10 year old weathered pine bark mulch (total volume of substrate and limestone = 12.7 L). Drains made of perforated PVC pipe were installed within the limestone bed and attached to outlet stand pipes to collect AMD after it flowed through the entire volume of the mesocosms. Water was maintained 2-4 inches

above the surface of the mulch substrate. Sampling ports fitted with serum stoppers were inserted down the front panel of each mesocosm at four inch intervals to allow for sampling of water at different levels within the mesocosm via syringe and needle.

Reservoirs containing 20 L of synthesized AMD were used to feed each of 3 mesocosms at an average rate of 0.1 L/h, equalling a 99% water replacement time of 18 days within the mesocosms.

Carbon experiments. In order to determine the effect of carbon availability on water quality improvement and SRB activity, inlet and outlet pH and concentrations of sulfate, total dissolved Fe and Mn, and dissolved sulfide were measured weekly for 27 weeks. Portal samples were collected weekly and analyzed for sulfate, pH, and total Fe and Mn. Hydrogen ion concentration was determined using a pH electrode and meter, dissolved sulfate and sulfide concentrations determined using  $\text{BaCl}_2$  and iodine respectively (Methods for Chemical Analyses of Water and Wastes, 1985), and total dissolved Fe and Mn concentrations determined using atomic absorption spectrophotometry. Three composite substrate samples (1 gram dry weight total) per box were collected at 4 inch depths every 3 weeks. SRB were enumerated by the MPN method (Alexander, 1982) following incubation in Postgate's medium C (1979) at 30°C for 4 weeks.

Simulated acid mine drainage was prepared containing a range of sulfate concentrations as sodium sulfate (1500-375 mg/L) and constant Fe (as ferrous chloride) and Mn (as manganous chloride) concentrations of 20 mg/L. Phosphorus

as potassium phosphate monobasic and ammonia-nitrogen as ammonium nitrate were added to AMD in concentrations of 115 and 100 mg/L of P and N respectively and assumed to be non-limiting.

Mulch was the only available carbon source for the first 14 weeks. After 14 weeks, 100 mg/L carbon as lactate was added to AMD, and then increased again to 200 mg/L at 18 weeks. At 20 weeks, experimentation was suspended due to the build up of hydrogen sulfide within the incubator. After the installation of an air filtering system, experimentation was restarted with a carbon concentration of 200 mg/L and increased to 300 mg/L after 4 weeks.

pH experiments. To determine the effect of pH on the reduction of sulfate and the precipitation of Fe and Mn, AMD pH was adjusted to 3.0, 4.5 and 6.0. Sulfate concentrations in AMD were maintained at 750 mg/L and Fe and Mn maintained at 20 mg/L. Based upon previous experimentation, carbon added at a concentration of 300 mg/L as lactate was non-limiting. Potassium phosphate monobasic and ammonium nitrate were added in concentrations of 115 and 100 mg/L of P and N respectively and assumed to be non-limiting. Inlet, outlet and portal samples were collected weekly for 7 weeks and analyzed for pH using a pH electrode and meter. Sulfate and dissolved sulfide were determined using BaCl and iodine respectively (Methods for Chemical Analyses of Water and Wastes, 1985), and total dissolved Fe and Mn by atomic absorption spectrophotometry. Three composite substrate samples per box were collected at a depth of 4 inches at 0, 3 and 7 weeks



and SRB enumerated using the MPN method (Alexander, 1982) following incubation in Postgate's medium C (1979) at 30°C for 4 weeks.

Fe experiments. In order to determine the effect of high concentrations of Fe on the reduction of sulfate and the precipitation of Fe and Mn, AMD Fe concentrations were adjusted to 75, 150 and 300 mg/L respectively as ferrous chloride. Initial AMD pH was 3.0 to insure Fe stayed in solution. Initial sulfate concentrations were 750 mg/L and Mn (as manganous chloride) 20 mg/L. Carbon added at a concentration of 300 mg/L was non-limiting. Potassium phosphate monobasic and ammonium nitrate were added in concentrations of 115 and 100 mg/L P and N respectively and assumed to be non-limiting. Inlet, outlet and portal samples were collected weekly for 7 weeks and analyzed for pH using an electrode and pH meter. Concentrations of sulfate and dissolved sulfide were determined using BaCl and iodine respectively (Methods for Chemical Analysis of Water and Wastes, 1985), and total dissolved Fe and Mn measured using atomic absorption spectrophotometry (Methods for Chemical Analysis of Water and Wastes, 1985). Three composite substrate samples (1 gram dry weight total) were collected per box at a depth of 4 inches at 0, 3 and 7 weeks and SRB enumerated using the MPN method (Alexander, 1982) following incubation in Postgates medium C (1979) at 30°C for 4 weeks.

## RESULTS

### Field Studies

Water chemistry. The effectiveness of a wetland constructed to treat AMD from an inactive coal refuse pile was determined over a period of 18 months. The wetland was constructed in the fall of 1990 at a cost of \$25,800. Prior to wetland treatment, annual costs for chemical treatment averaged \$7,200, including labor. After construction, annual costs for weekly water quality testing averaged only \$1,000.

Throughout the course of the 18 month study, the inlet AMD contained no acidity and the inlet and outlet pH was near neutral (Fig. 3A). For the first two months following construction, the wetland underwent an establishment period in which successful treatment of iron and manganese was not evident. Following establishment, however, effluent Fe concentrations were consistently reduced to levels below instream compliance standards set by the EPA (3 mg/L), and no additional chemical treatment was necessary (Fig. 3B). Manganese precipitation was not as consistent. During the first 2 months of the study, Mn concentrations in the effluent were higher than those in the influent (Fig. 3C). Throughout the following months the effluent met instream compliance standards set by the EPA (2 mg/L) with an average effluent Mn concentration of 2 mg/L. Sulfate concentration was monitored monthly from May through October of 1991 (Fig. 4). The reduction of  $\text{SO}_4$  in AMD was consistent over the sampling period, with an average decrease in dissolved sulfate concentration of 360 mg/L.

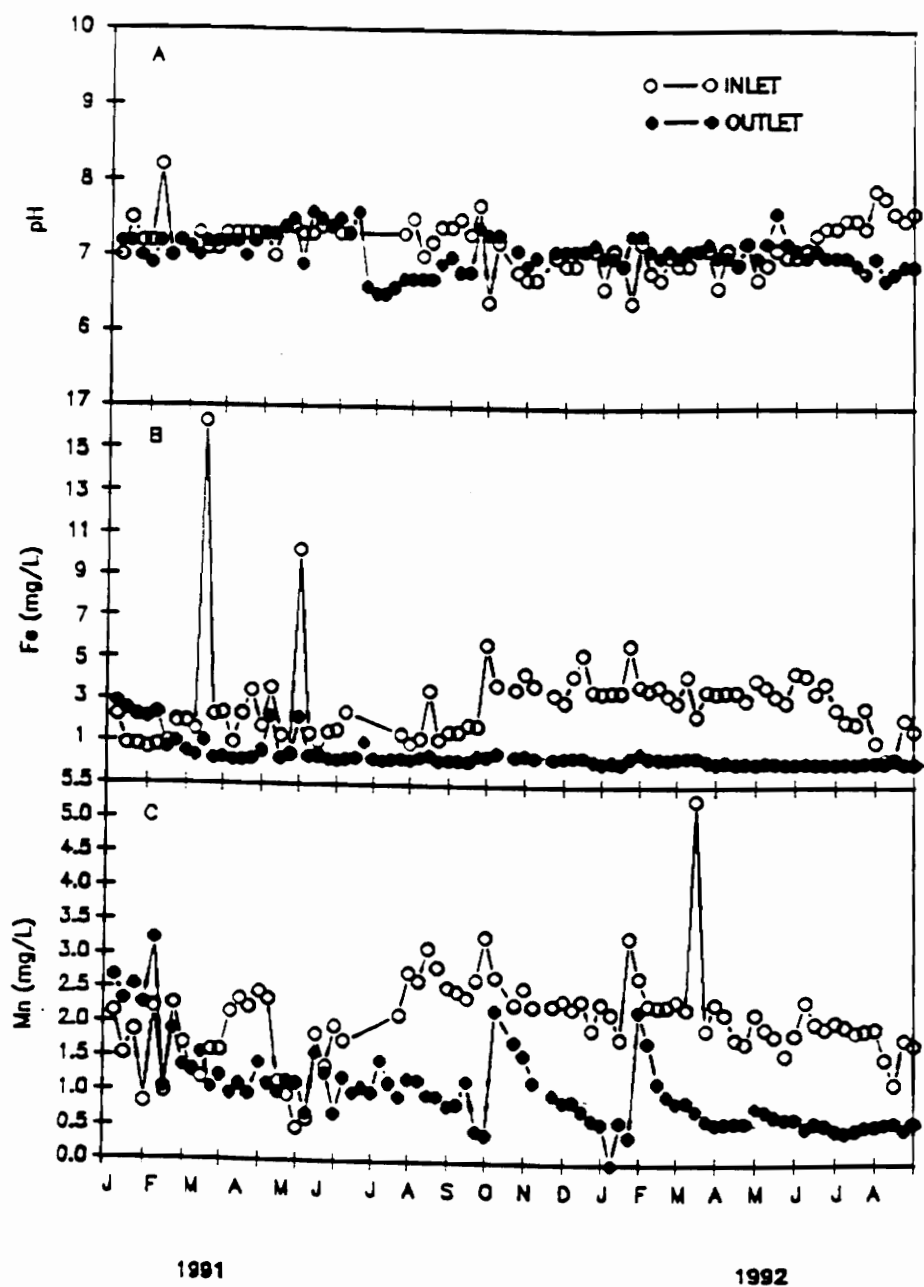


Figure 3. pH (A), and Fe (B) and Mn (C) concentration (mg/L) in influent and effluent AMD at Pine Branch wetland, Norton, Va.

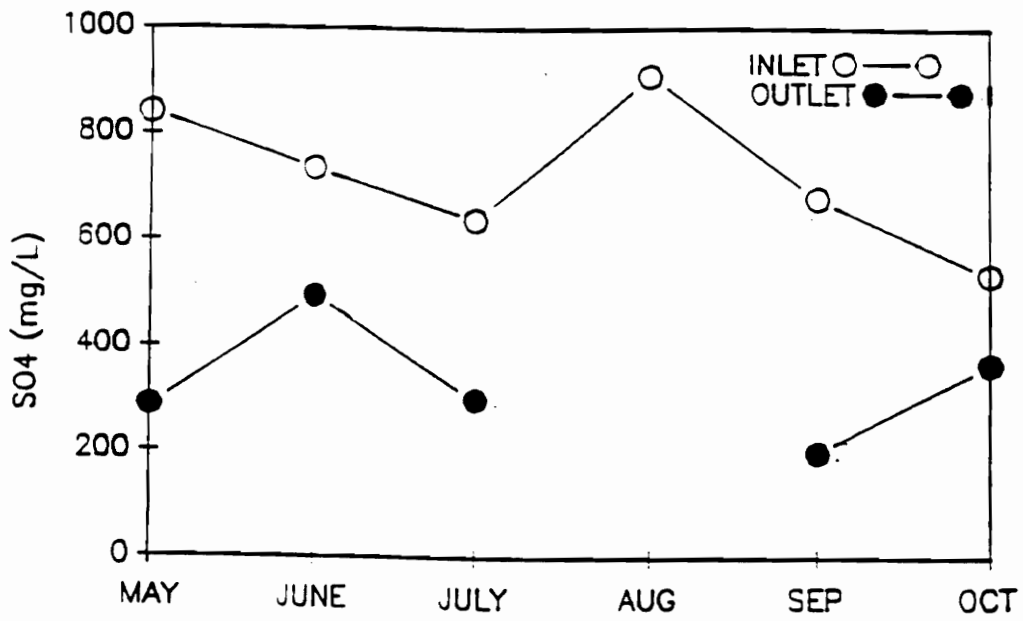


Figure 4. Influent and effluent sulfate concentration (mg/L) in AMD at Pine Branch wetland, Norton, Va.

Sulfate reducing bacteria enumeration. SRB were enumerated from February to September, 1991. The population averaged  $8.7 \times 10^4$  organisms/g dry mulch through the entire course of the study. March and September showed lower than average SRB populations, averaging  $4.9 \times 10^3$  and  $7.0 \times 10^3$  respectively. An increase to  $3.7 \times 10^5$  occurred in August (Table 1). Comparisons of SRB population size were made between areas with and without cattails. No difference in density was found, with numbers of SRB in areas with and without cattails, averaging  $3.4 \times 10^4$  and  $5.8 \times 10^4$  organisms/g dry mulch respectively. A change in population size of 2 or more  $\log_{10}$  was considered a significant change.

TABLE 1: MPN of Sulfate Reducing Bacteria in Pine Branch Wetland Mulch, Norton, Virginia.

Sample Date	MPN of Sulfate Reducers/gram dry mulch ( $\times 10^4$ ) <sup>*</sup>
Feb. 12, 1991	1.17 +/- 2.29 <sup>a</sup>
March 21, 1991	0.49 +/- 0.29
May 30, 1991	5.44 +/- 5.24
July 10, 1991	7.60 +/- 2.57
August 21, 1991	37.40 +/- 37.94
Sep. 30, 1991	0.72 +/- 0.55

\* average of 3 or more replicates

<sup>a</sup> standard deviation

## **Wetland Mesocosms**

Carbon experiments. The effect of carbon on microbial sulfate reduction and the subsequent precipitation of Fe was determined over a range of sulfate concentrations. The effect of carbon on effluent sulfate concentration at all sulfate levels tested are shown in figures 5, 6, and 7. During the first 98 days, utilizing mulch as the only available carbon source, a decrease in total sulfate concentration of 20% was found after AMD passed through the mesocosms, regardless of AMD influent sulfate concentration. An increase in sulfide concentration from 40 to 55 mg/L, 25 to 56 mg/L and 32 to 53 mg/L was detected in treatments with sulfate concentrations of 1554, 661 and 359 mg/L respectively (Figures 8, 9 and 10). The "uniform" increase in dissolved sulfide regardless of inflow concentration may have been due to a low rate of sulfate reduction that was not increased with the increased sulfate concentration. This indicated possible limitation of some nutrient other than sulfate. After 98 days, 100 mg/L carbon as sodium lactate was added to AMD, resulting in a decrease in sulfate concentration of 15% and no detectable increase in sulfide concentration. At 126 days the lactate concentration was increased to 200 mg/L and then at 144 days the experiment was suspended for a period of 2 months. The release of toxic levels of hydrogen sulfide required that an air filtering system be installed in order that experimentation continue. Experimentation was resumed with 200 mg/L carbon added as lactate to the AMD. An increase in effluent dissolved sulfide from 31 to 78 mg/L, 32 to 77 mg/L and 26 to 87 mg/L

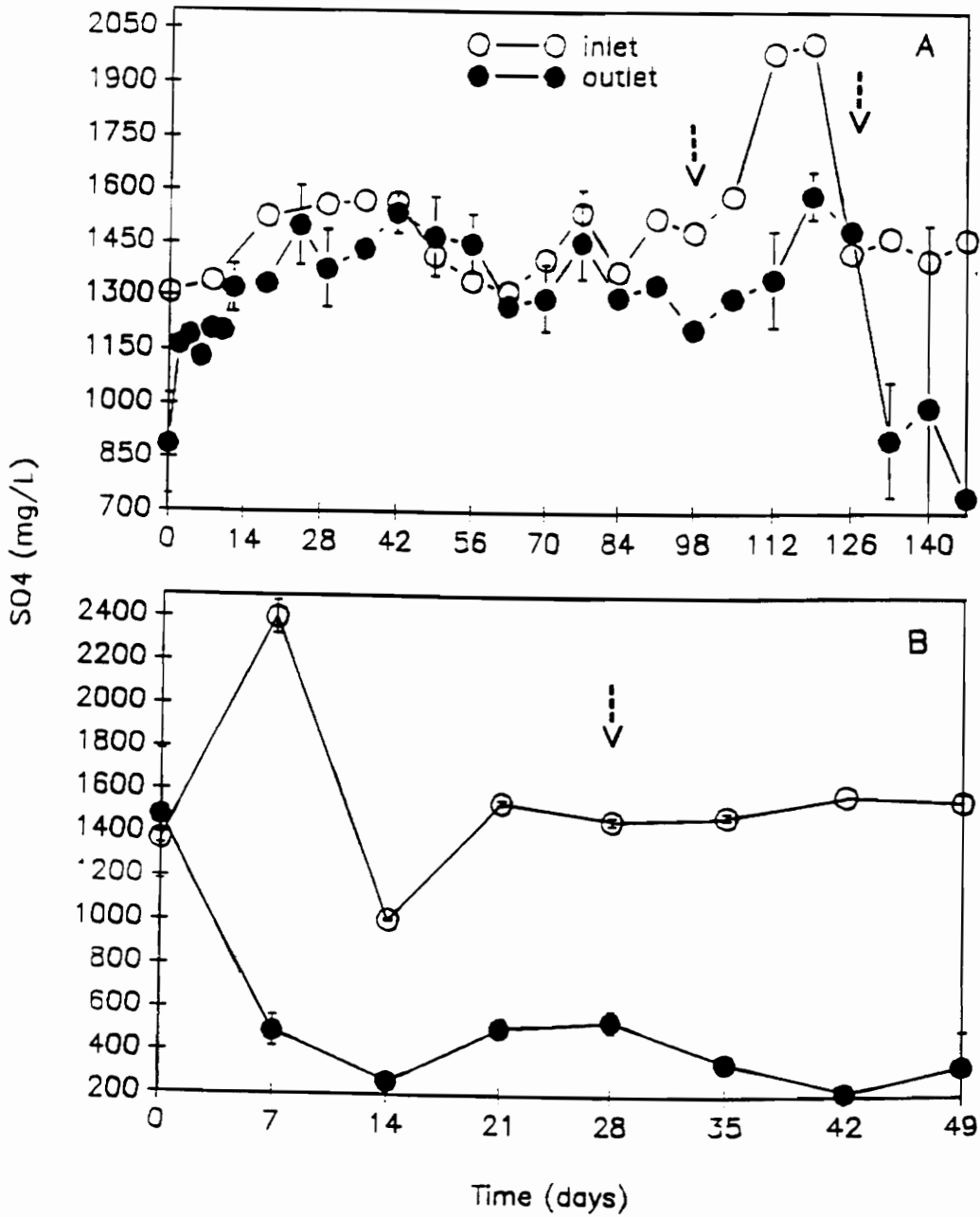


Figure 5. The effect of carbon on effluent sulfate concentration (mg/L) in AMD. Influent sulfate concentration averaged 1554 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.5. Vertical bars represent +/- SD.

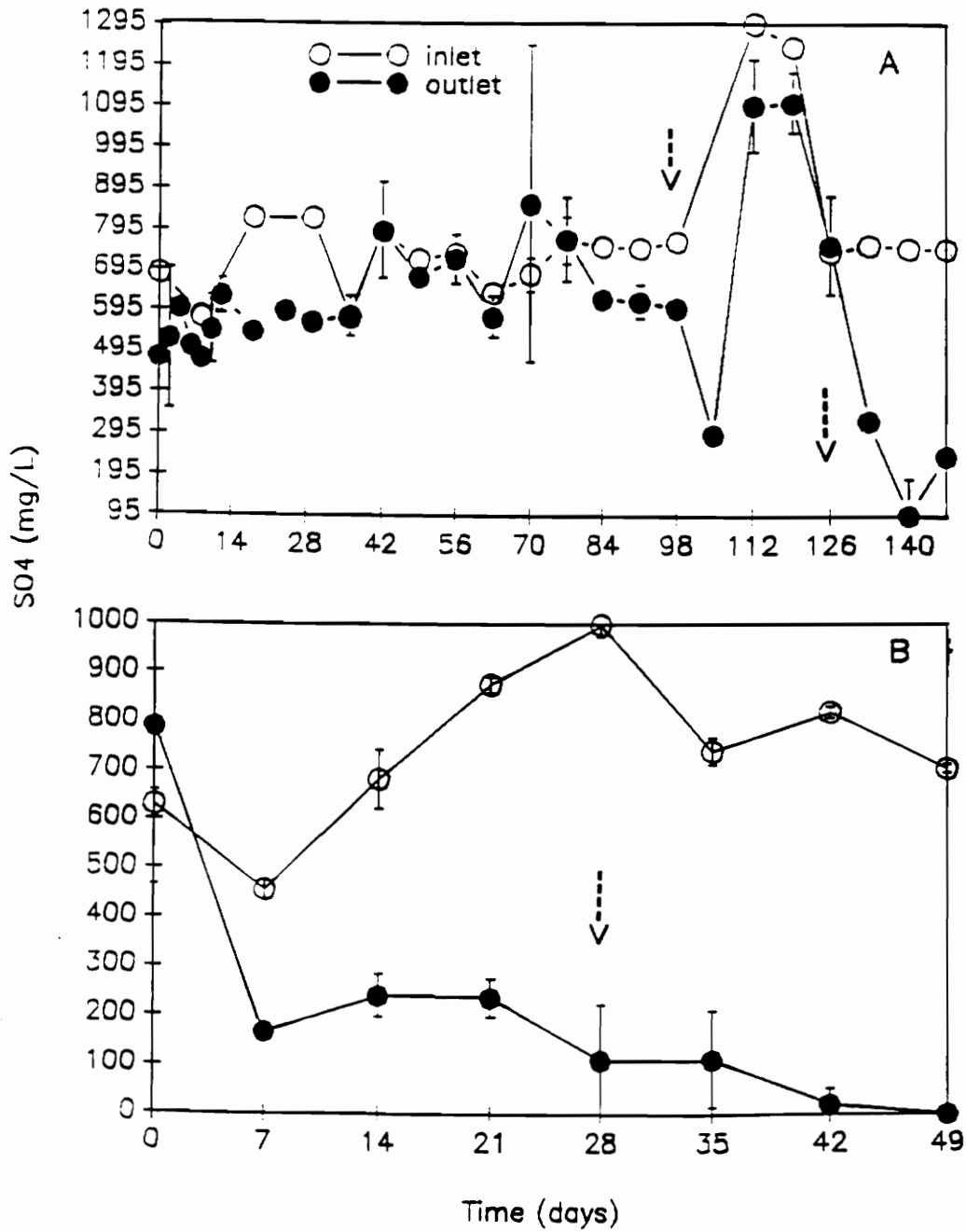


Figure 6. The effect of carbon on effluent sulfate concentration (mg/L) in AMD. Influent sulfate concentration averaged 661 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.4. Vertical bars represent  $\pm$  SD.



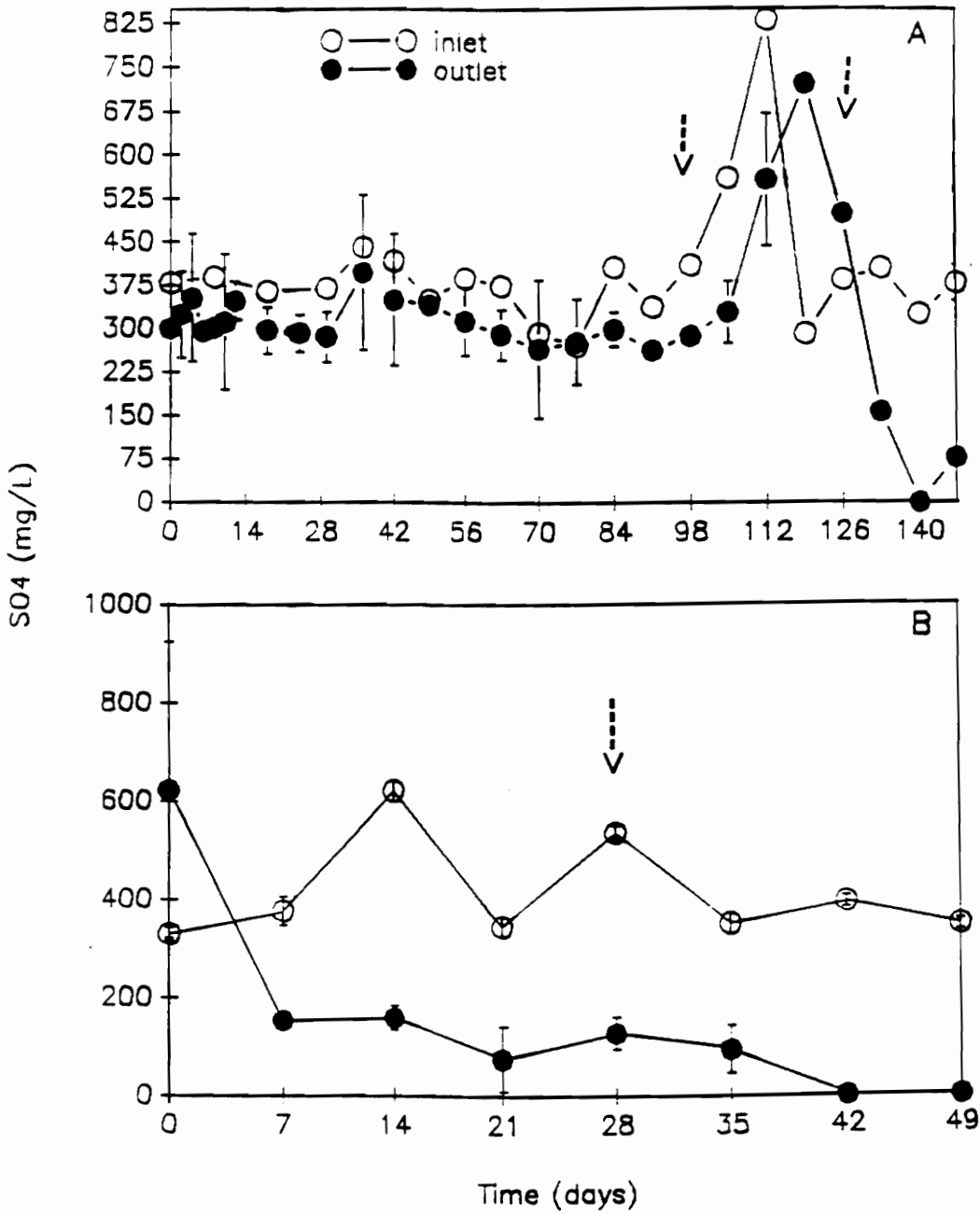


Figure 7. The effect of carbon on effluent sulfate concentration (mg/L) in AMD. Influent sulfate concentration averaged 359 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.5. Vertical bars represent  $\pm$  SD.

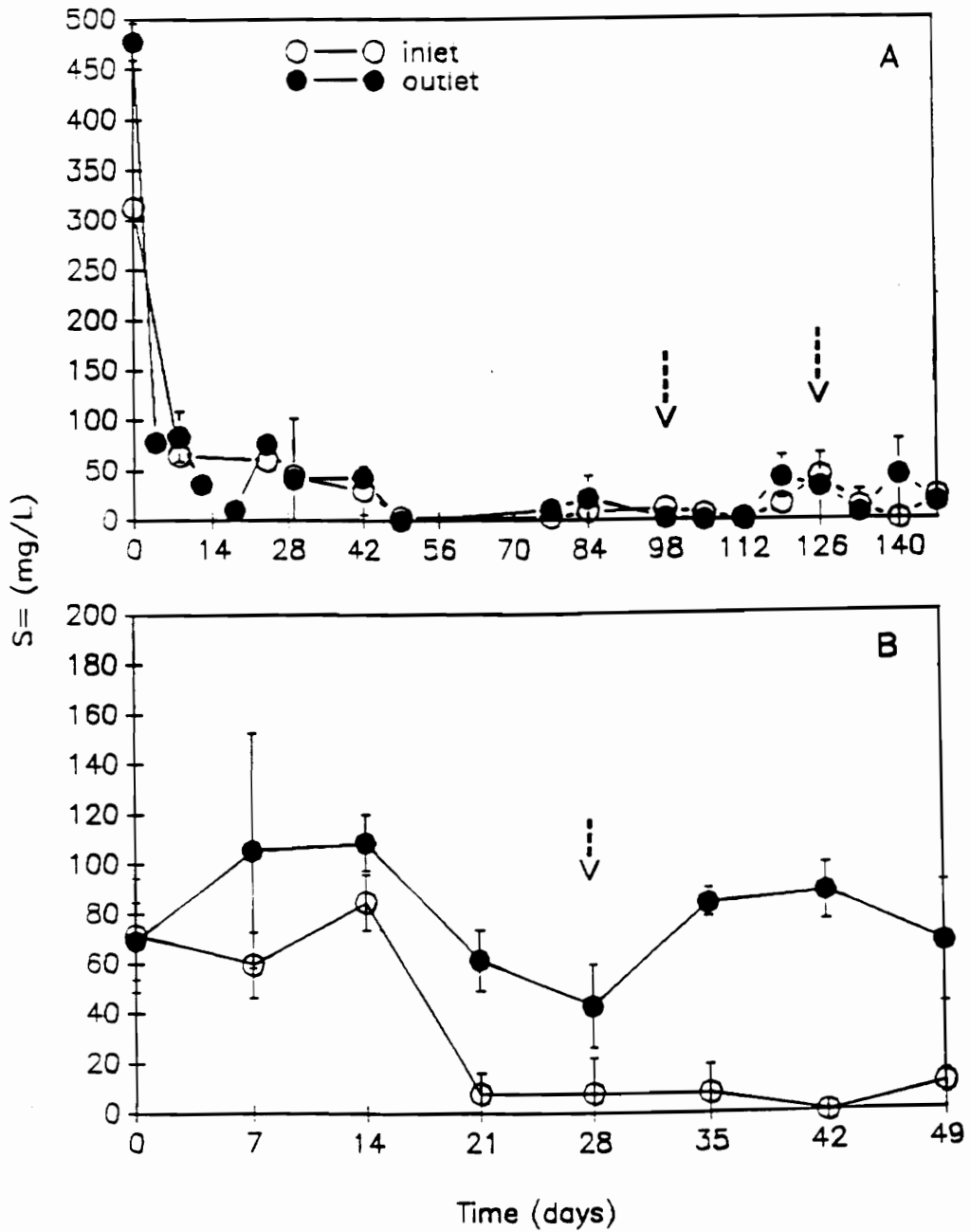


Figure 8. The effect of carbon on effluent sulfide concentration (mg/L) in AMD. Influent sulfate concentration averaged 1554 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.5. Vertical bars represent  $\pm$  SD.

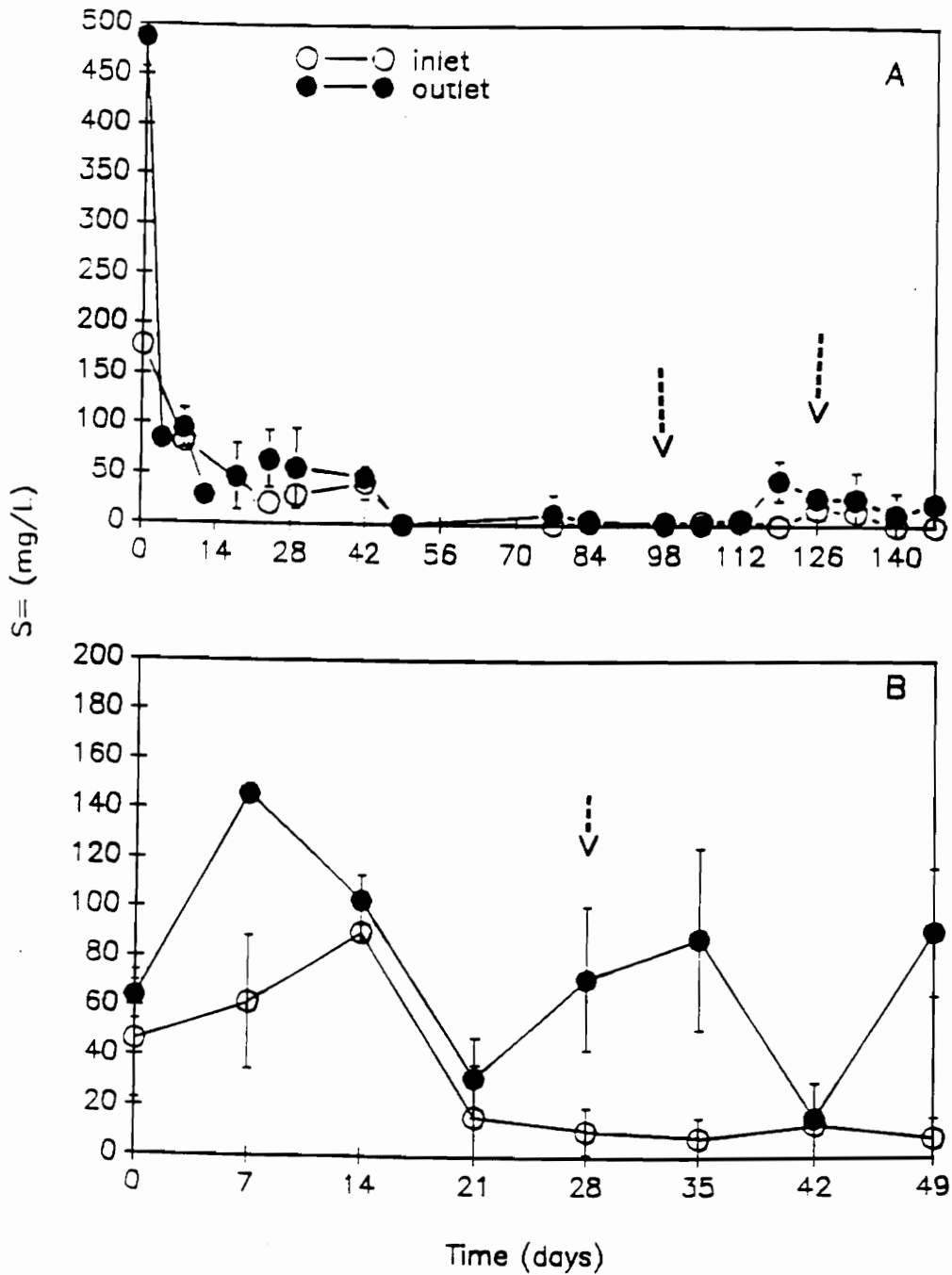


Figure 9. The effect of carbon on effluent sulfide concentration (mg/L) in AMD. Influent sulfate concentration averaged 661 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.4. Vertical bars represent  $\pm$  SD.

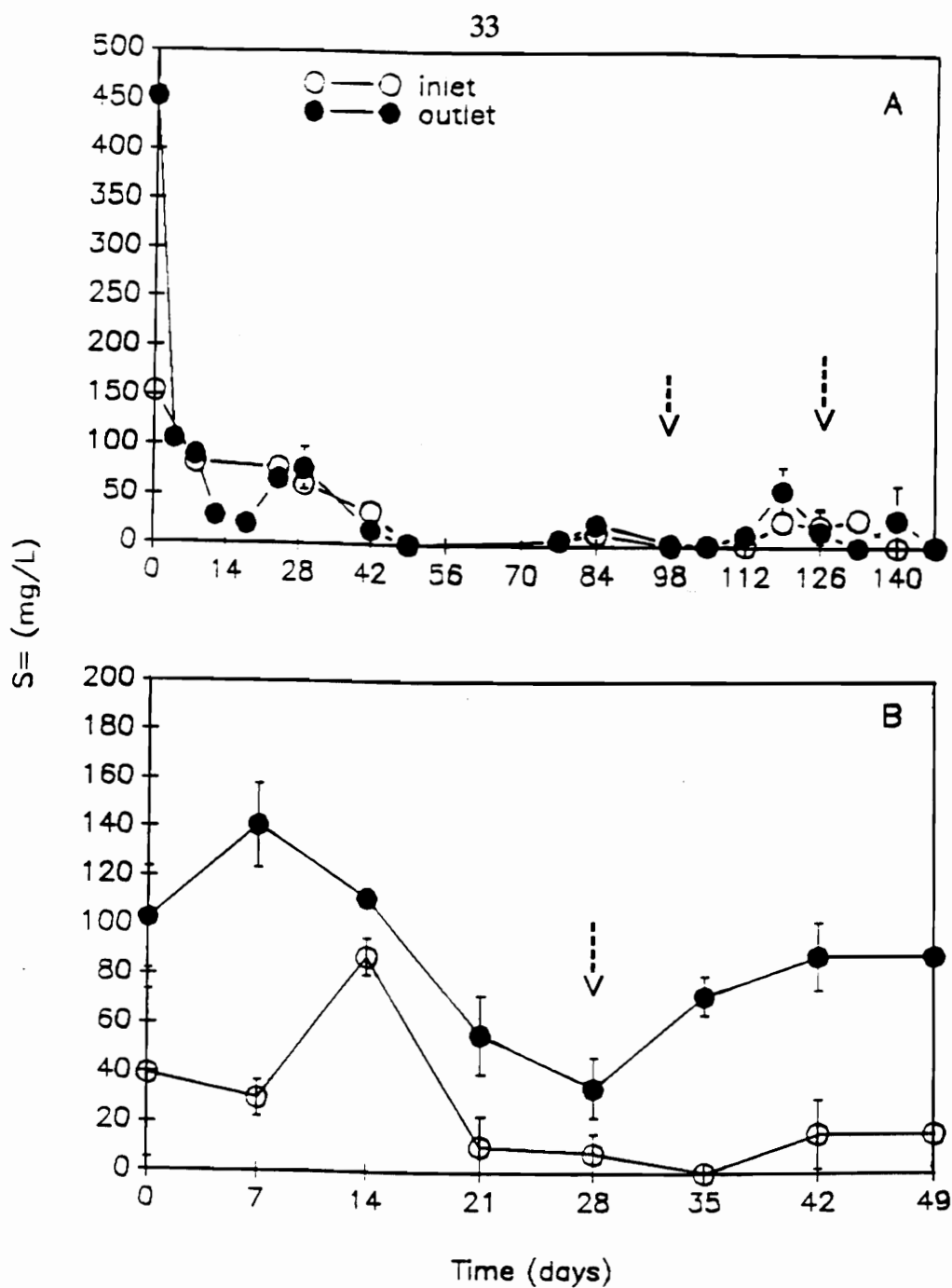


Figure 10. The effect of carbon on effluent sulfide concentration (mg/L) in AMD. Influent sulfate concentration averaged 359 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.5. Vertical bars represent  $\pm$  SD.

was found after the addition of both 200 and 300 mg/L carbon as lactate in treatments containing 1154 mg/L, 661 mg/L and 359 mg/L sulfate respectively (Figures 8, 9 and 10). The addition of 200 mg/L carbon to AMD resulted in significant decreases ( $p < 0.05$ ) in effluent sulfate concentrations of 57, 58 and 48% when inlet AMD sulfate concentrations were 1554, 661 and 359 mg/L respectively (Figs. 5, 6 and 7). After 4 weeks carbon was increased to 300 mg/L, resulting in a decrease in sulfate concentrations from 1544 to 305 mg/L (80%), from 778 to 44.9 mg/L (94%) and from 364 to 32 mg/L (91%).

Figures 11, 12 and 13 show the effect of carbon addition of the reduction in Fe concentration. Prior to carbon addition to AMD, total Fe concentrations decreased 88%, 90% and 92% from inflow concentrations in the three treatments respectively. After the addition of 200 and 300 mg/L carbon as lactate, Fe concentrations decreased 97%, 96% and 96% in the AMD containing inflow sulfate concentrations of 1554, 661 and 359 mg/L respectively. Manganese concentration was measured in inlet and outlet after resuming the experiment with 200 mg/L carbon. Manganese was reduced by 91%, 84% and 82% when inlet sulfate concentrations were 1500 mg/L, 750 mg/L and 375 mg/L respectively (Fig 14).

With mulch as the only available carbon source, SRB were present at an average density of  $1.4 \times 10^6$  organisms/g dry mulch ( $n=27$ ). At time 0 days, SRB averaged  $1.0 \times 10^6$ /g dry mulch in the treatment containing 1500 mg/L inlet sulfate (Table 2). SRB densities at time 0 averaged  $1.6 \times 10^5$  and  $5.0 \times 10^5$  organisms/g dry

mulch in AMD containing 750 and 375 mg/L sulfate respectively. A one  $\log_{10}$  increase was detected in treatments with 750 and 375 mg/L sulfate after 12 days. After 30 days, treatments with 1500 and 750 mg/L sulfate decreased one  $\log_{10}$  while the treatment containing 375 mg/L sulfate maintained at  $3.1 \times 10^6$  organisms/g dry mulch (Table 2). Due to the large error term associated with the MPN technique, a change in population size of 2 or more  $\log_{10}$  was considered significant. After the addition of 200 mg/L, SRB densities were not significantly increased over that of the addition of 0 or 100 mg/L carbon, averaging  $8.4 \times 10^6$  in all treatments. After the addition of 300 mg/L carbon, densities increased significantly, averaging  $1.5 \times 10^8$  organisms/g dry mulch (Table 2).

Table 2: Effect of Carbon on the Density of SRB in Wetland Mesocosms

DAY	SRB/g dry substrate (x 10 <sup>5</sup> )*			
Treatment (SO <sub>4</sub> mg/L)	1500	750	375	
0	19.0 +/- 15.0 <sup>a</sup>	5.5 +/- 6.7	5.0 +/- 6.0	
12	18.0 +/- 21.0	32.0 +/- 36.0	13.0 +/- 12.0	
30	8.1 +/- 3.6	5.6 +/- 3.3	7.2 +/- 6.2	
Addition of 100 mg/L carbon as lactate				
126	6.0 +/- 5.9	0.9 +/- 0.9	13.0 +/- 12.0	
Addition of 200 mg/L carbon as lactate				
144	45.0 +/- 9.9	50.0 +/- 17.0	37.0 +/- 1.4	
169	92.0 +/- 31.0	131.0 +/- 67.0	147.0 +/- 75.0	
Addition of 300 mg/L carbon as lactate				
193	1860.0 +/- 1440.0	1440.0 +/- 1280.0	1080.0 +/- 635.0	

\* mean of 3 replicates

<sup>a</sup> standard deviation

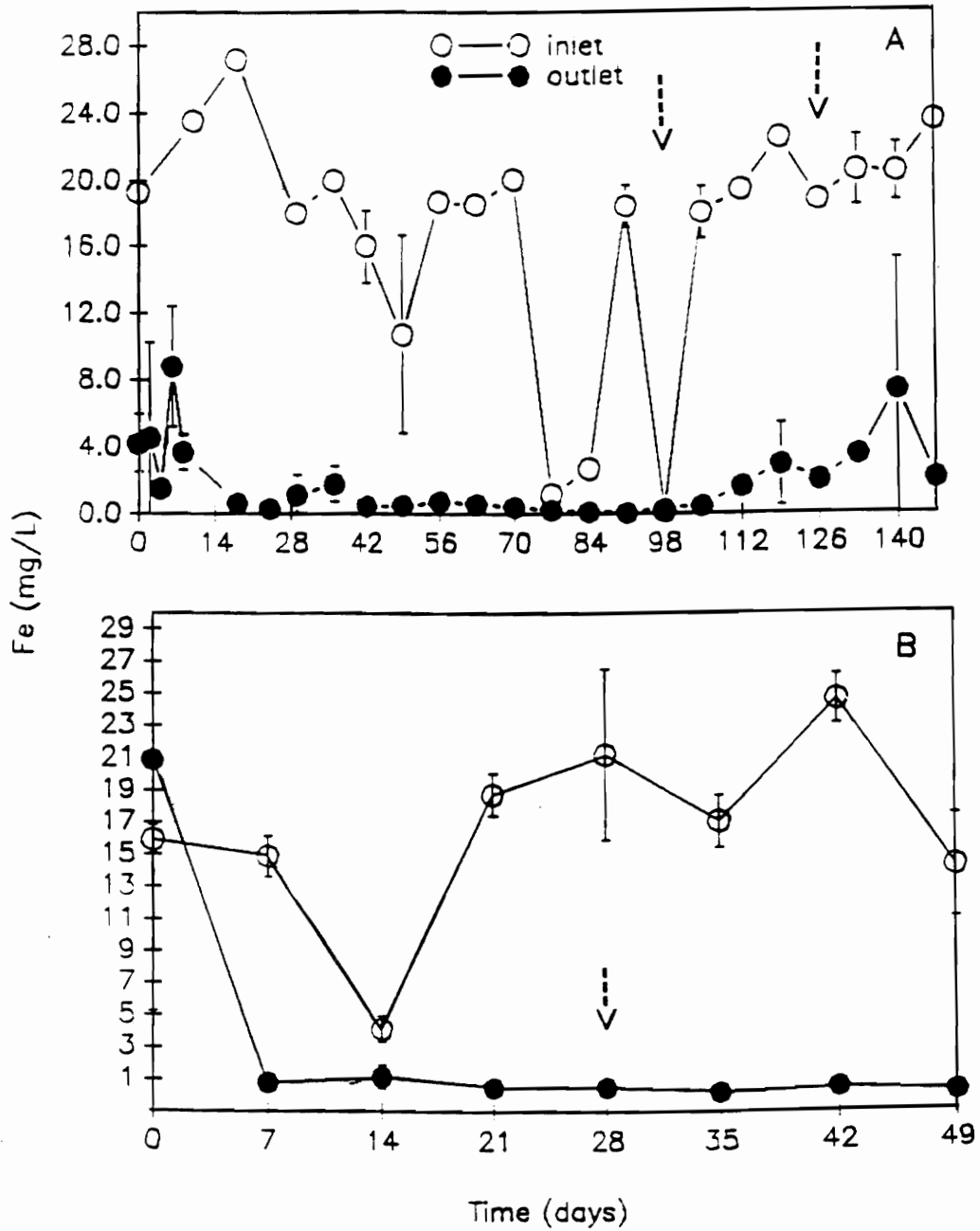


Figure 11. The effect of carbon on effluent Fe concentration (mg/L) in AMD. Influent sulfate concentration averaged 1554 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.5. Vertical bars represent  $\pm$  SD.

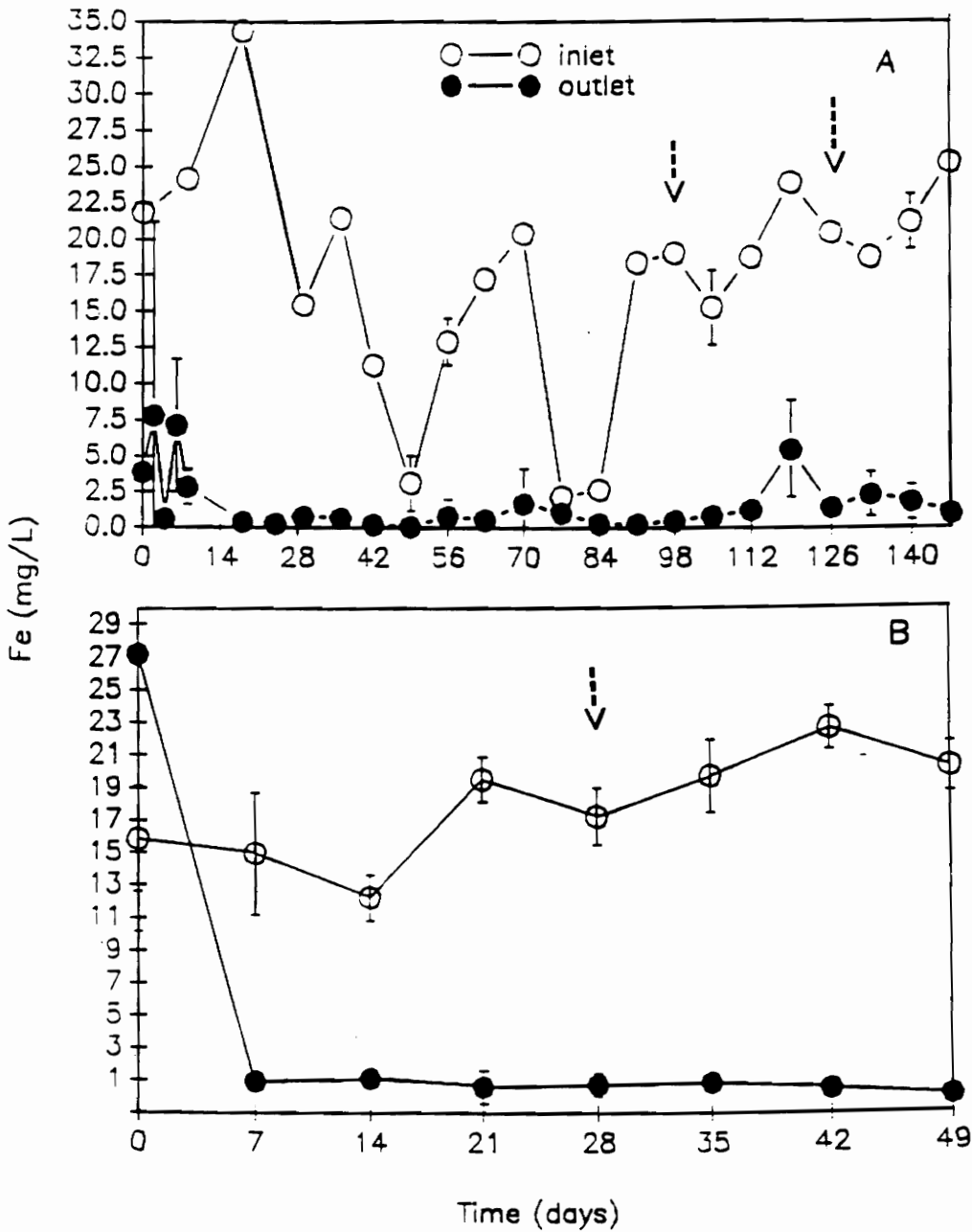


Figure 12. The effect of carbon on effluent Fe concentration (mg/L) in AMD. Influent sulfate concentration averaged 661 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.4. Vertical bars represent  $\pm$  SD.



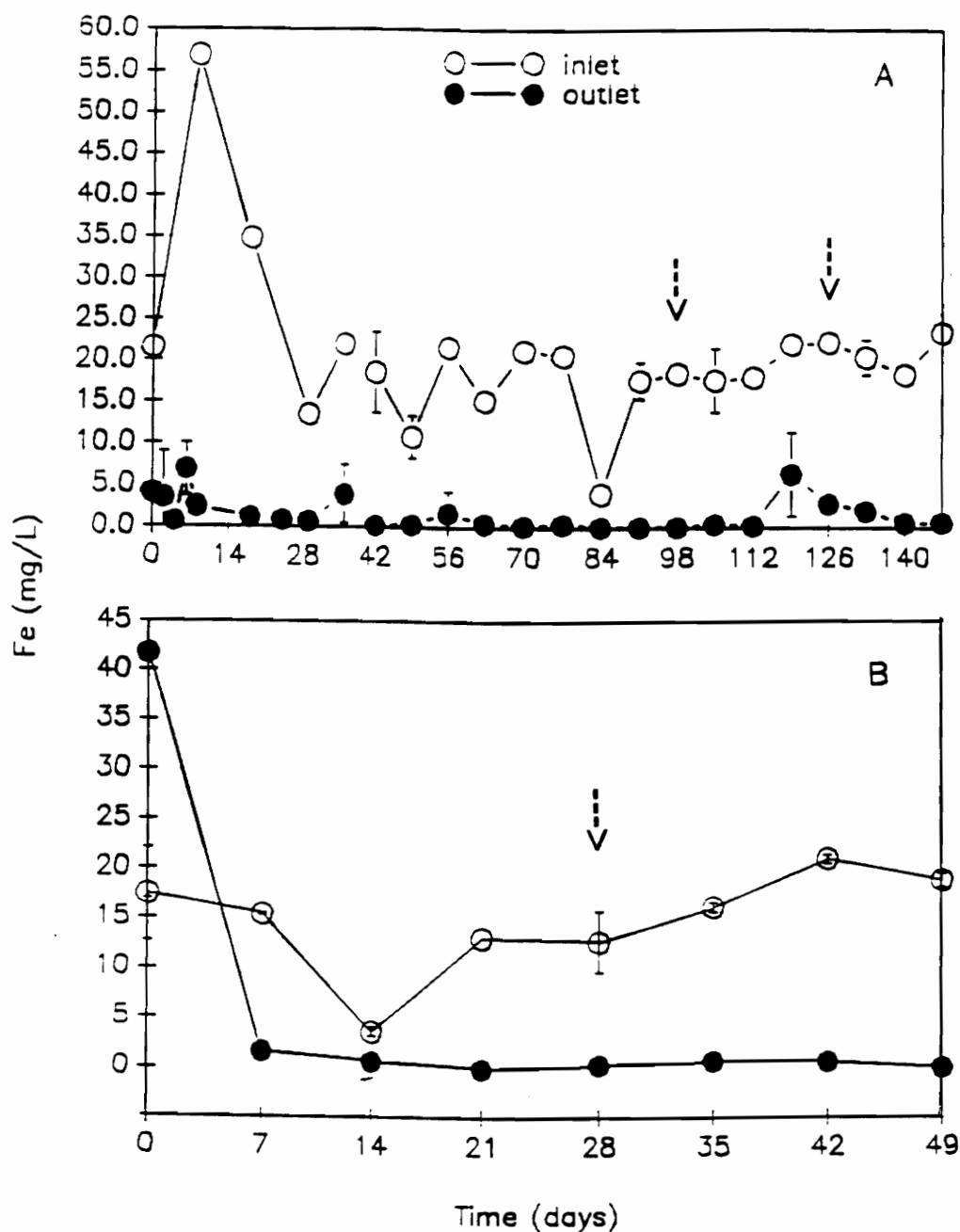


Figure 13. The effect of carbon on effluent Fe concentration (mg/L) in AMD. Influent sulfate concentration averaged 359 mg/L. Arrows represent the addition of 100, 200 and 300 mg/L carbon as lactate at 98, 126 and 28 days respectively. Average inflow pH was 5.5. Vertical bars represent  $\pm$  SD.

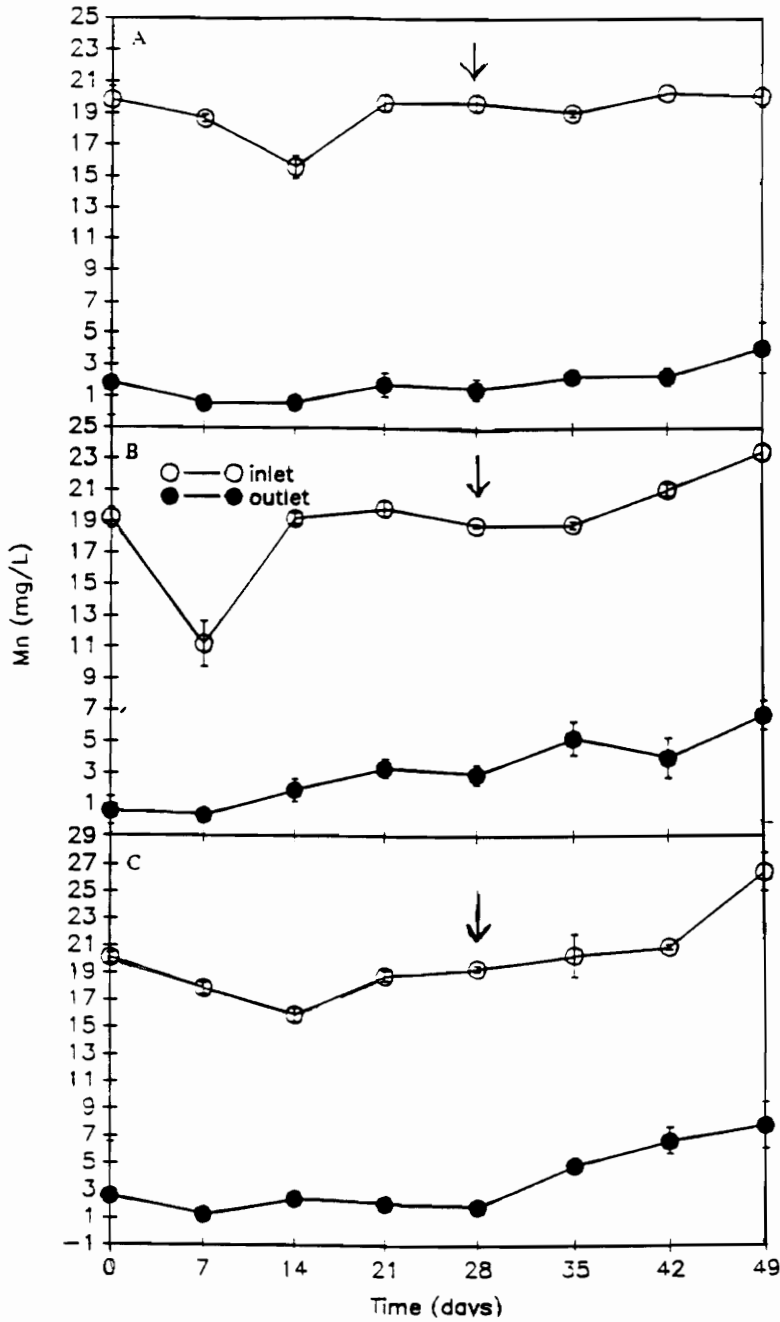


Figure 14. The effect of carbon on effluent Mn concentration (mg/L) in AMD. Influent sulfate concentrations averaged (A) 1554 mg/L, (B) 661 mg/L, and (C) 359 mg/L. Arrows represent the increase from 200 to 300 mg/L carbon as lactate at 28 days. Average inflow pH was 5.5, 5.4 and 5.5 respectively. Vertical bars represent  $\pm$  SD.

pH experiments. The effect of pH on microbial sulfate reduction and the subsequent precipitation of Fe and Mn was determined. Influent AMD maintained at pH 6.0 and 4.5 increased to an average pH of 7.4 and 7.2 respectively after passage through the mesocosms (Figs. 15a and 15b). AMD influent maintained at pH 3.5 increased to an average of 6.2 (Fig. 15c).

Figure 16 shows the effect of pH on total Fe concentration at pH 6.0, 4.5 and 3.5. At an influent AMD pH of 6.0 and 4.5, no significant difference was seen between effluent Fe concentrations ( $p > 0.05$ ). Effluent Fe concentrations were significantly lower at a pH of 6.0 and 4.5 than at a pH of 3.5 ( $p < 0.05$ ). Total Fe concentrations decreased 92% and 91% at pH 6.0 and 4.5 respectively, while effluent Fe concentration decreased 70% at an AMD pH of 3.5. At an influent AMD pH of 4.5, AMD effluent Fe concentration decreased from an average of 16.7 mg/L to less than 1 mg/L for the first 28 days of experimentation. During the last three weeks of experimentation, effluent Fe concentrations averaged 2 mg/L. At an AMD pH of 3.5, total Fe concentrations decreased from an average of 21.8 to 0 mg/L during the initial 3 weeks of experimentation. After 21 days, effluent Fe averaged 10.5 mg/L, with a high of 17 mg/L on day 35. Manganese concentration in AMD decreased 72%, 68% and 37% in treatments maintained at pH 6.0, 4.5 and 3.5 respectively (Fig 17). A significant difference in effluent Mn concentrations was found between AMD with a pH of 6.0 and 4.5 ( $p < 0.05$ ) and between AMD with a

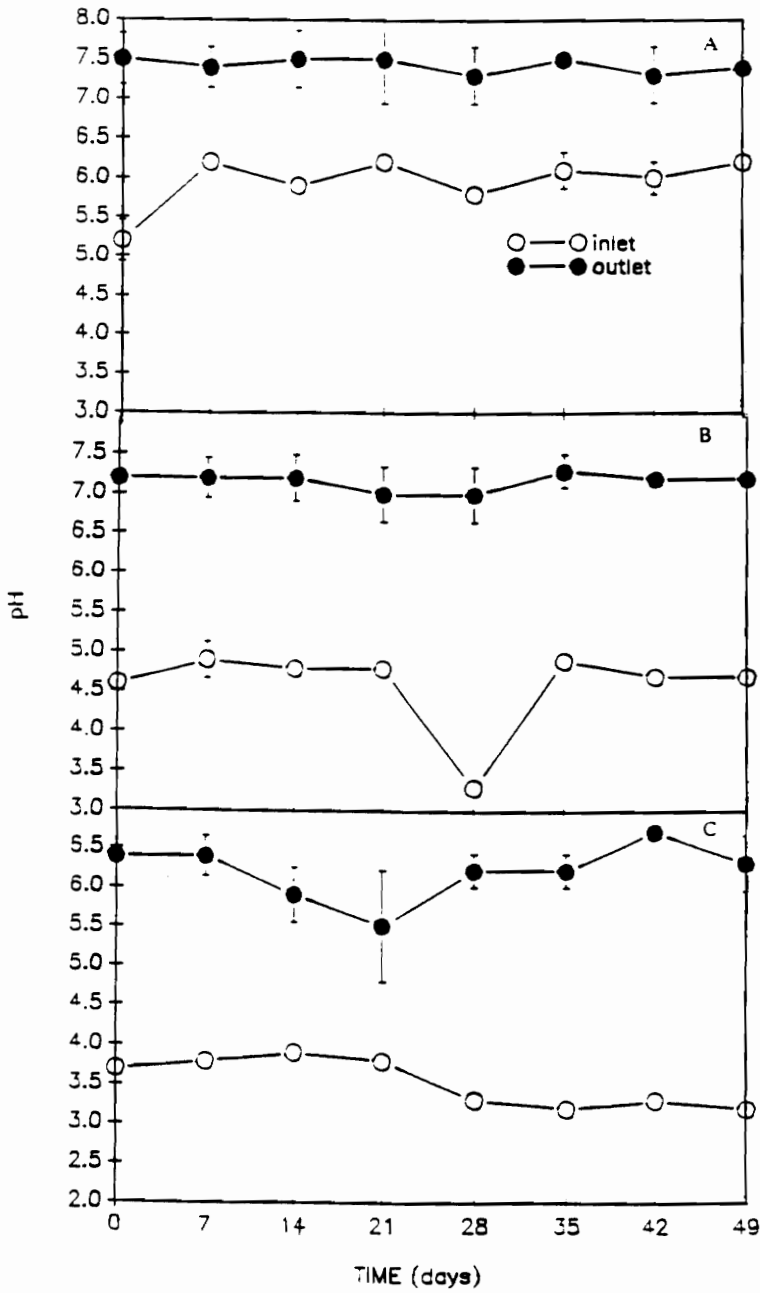


Figure 15. The effect of inflow pH on effluent pH in AMD. Influent pH averaged (A) 6.0, (B) 4.6 and (C) 3.5. Vertical bars represent  $\pm$  SD.

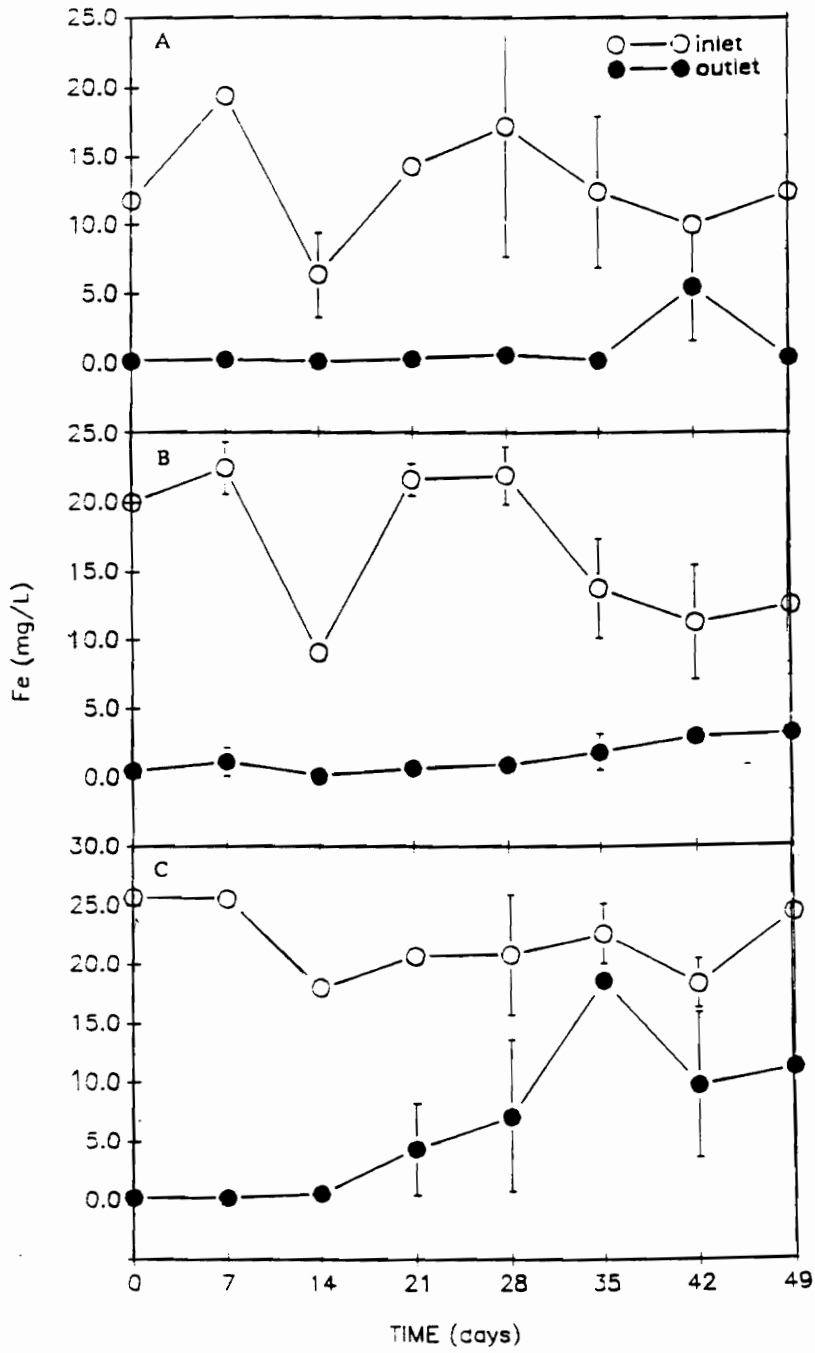


Figure 16. The effect of pH on effluent Fe concentration (mg/L) in AMD. Influent AMD pH averaged (A) 6.0, (B) 4.6 and (C) 3.5. Vertical bars represent  $\pm$  SD.

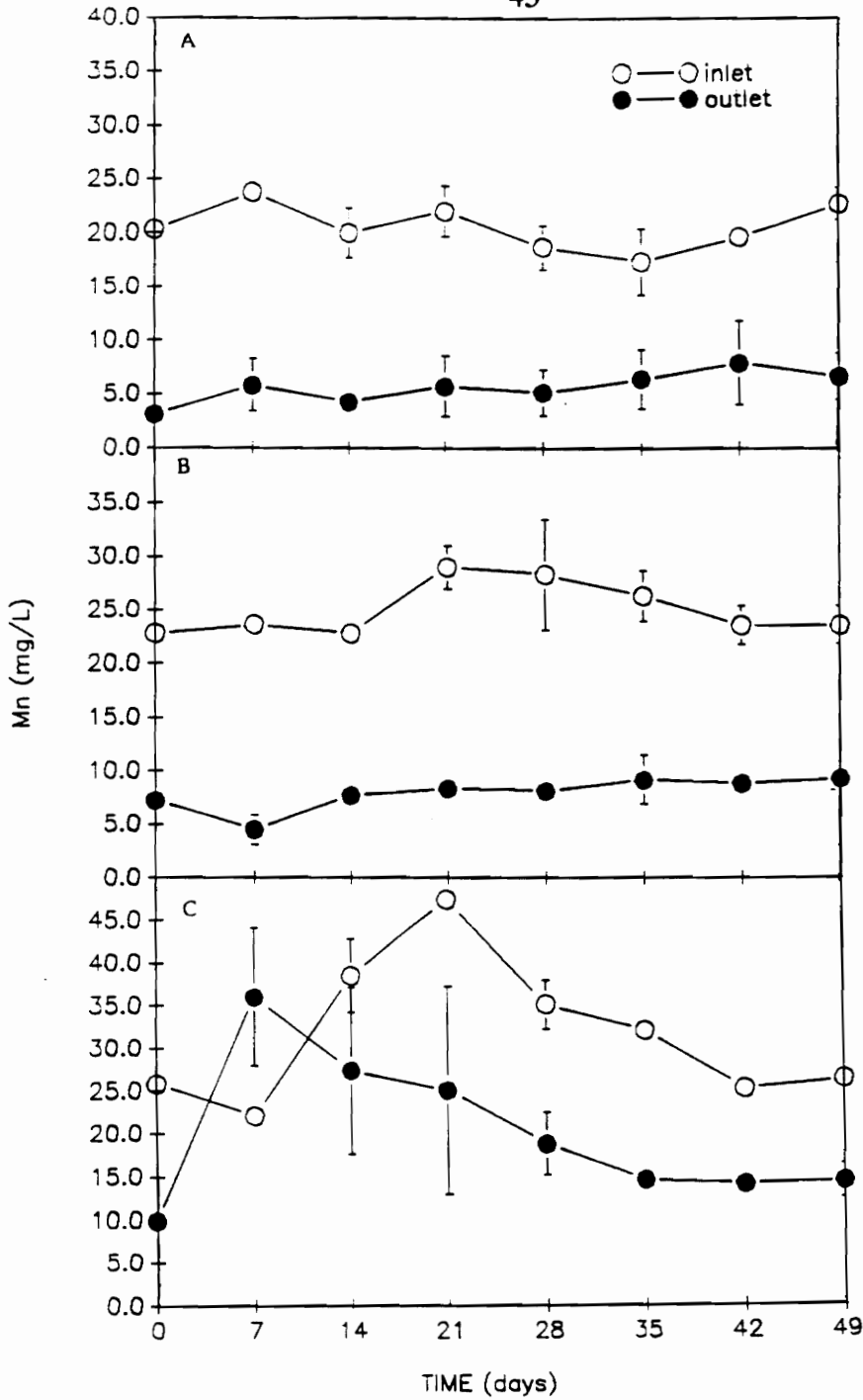


Figure 17. The effect of pH on effluent Mn concentration (mg/L) in AMD. Influent AMD pH averaged (A) 6.0, (B) 4.6 and (C) 3.5. Vertical bars represent +/- SD.

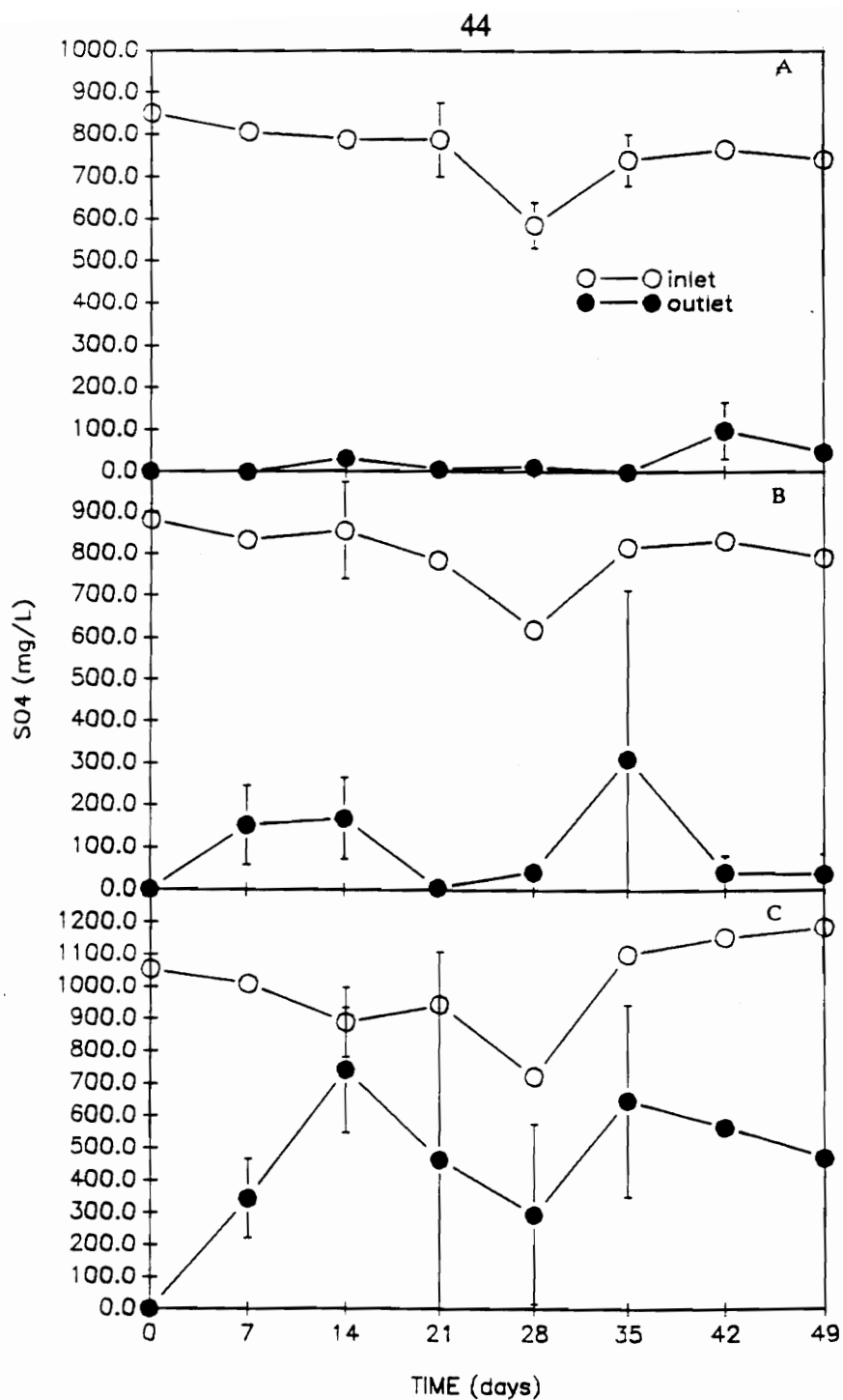


Figure 18. The effect of pH on effluent sulfate concentration (mg/L) in AMD. Influent AMD pH average (A) 6.0, (B) 4.6 and (C) 3.5. Vertical bars represent  $\pm$  SD.

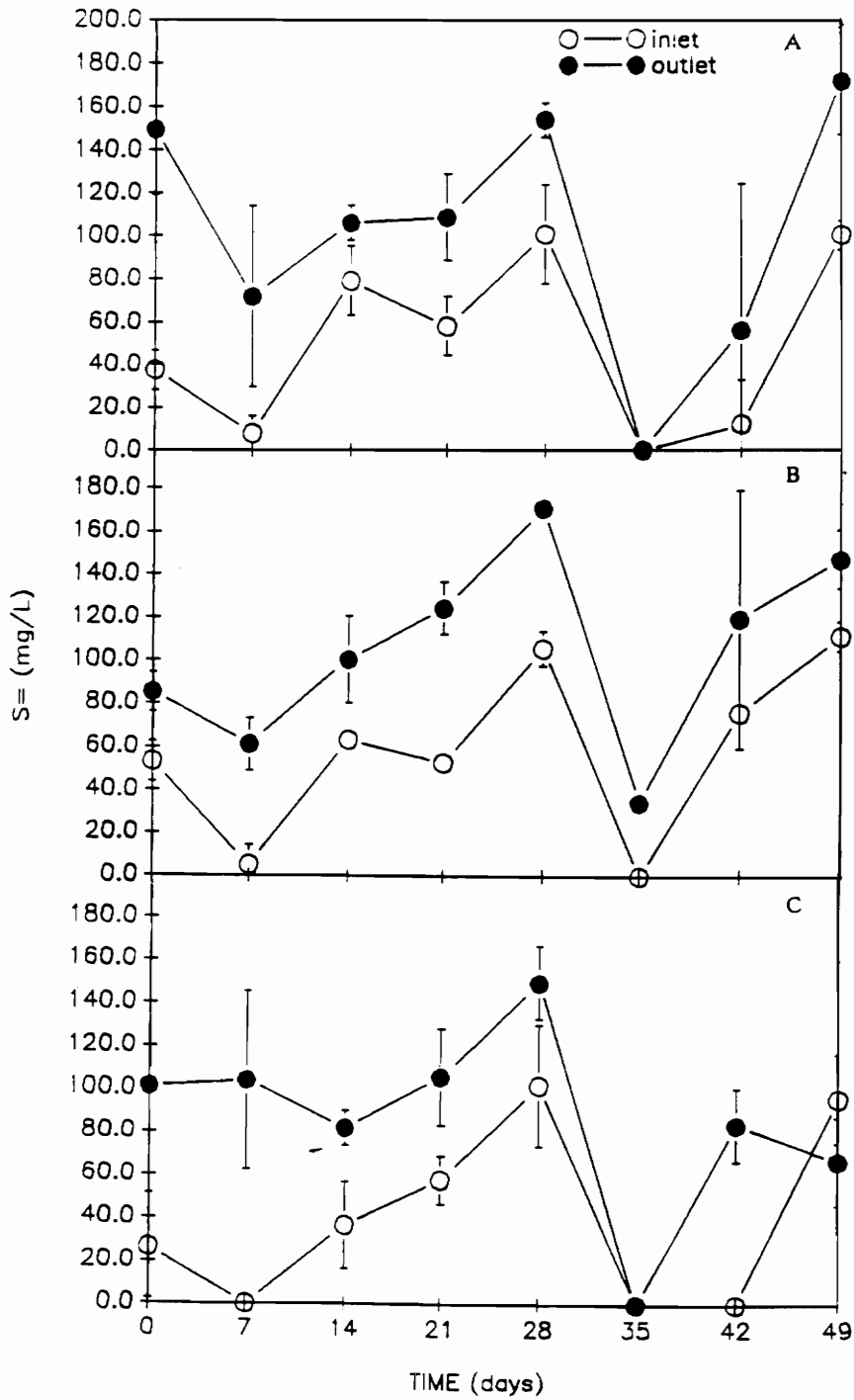


Figure 19. The effect of pH on effluent sulfide concentration (mg/L) in AMD. Influent AMD pH averaged (A) 6.0, (B) 4.6 and (C) 3.5. Vertical bars represent  $\pm$  SD.



pH of 6.0 and 3.5. There was also a significant difference in effluent Mn concentration between AMD with a pH of 4.5 and 3.5 ( $p < 0.05$ ).

Figure 18 shows the effect of AMD pH on dissolved sulfate concentration. A significant difference in effluent sulfate concentrations was seen between AMD maintained at a pH of 6.0 versus a pH of 3.5, and between AMD with a pH of 4.5 versus a pH of 3.5 ( $p < 0.05$ ). At an AMD pH of 6.0, sulfate concentration decreased from an average of 759 mg/L to 25 mg/L. At an AMD pH of 4.5 and 3.5, sulfate decreased from 799 mg/L to 96.2 mg/L and 1006 mg/L to 441.6 mg/L respectively. Dissolved sulfide increased from an average of 50 to 102 mg/L and 59 to 106 mg/L at an inlet pH of 6.0 and 4.5 respectively. At inlet pH 3.5, dissolved sulfide increased from 40 to 87 mg/L (Fig. 19). No significant difference in effluent dissolved sulfide concentrations was found between any pH tested ( $p > 0.05$ ). However, on days when outlet sulfate concentrations peaked, outlet dissolved sulfide concentrations were low (Figs. 18 and 19). Inlet sulfide concentrations averaged above 40 mg/L, yet no sulfide was added to the AMD mixture. This could be due to an error in the detection method, or the presence of a biofilm within the inlet tube which contained bacteria that produced detectable levels of sulfide which then dissolved into the AMD.

At an AMD pH of 3.5, SRB densities showed a significant decrease in population size over the 49 day period. At day 0 SRB averaged  $2.2 \times 10^8$ /g dry mulch, and after 21 days, SRB averaged  $1.4 \times 10^7$ . After 49 days the population

decreased 4  $\log_{10}$ , averaging  $2.4 \times 10^4$  (Table 3). SRB densities averaged  $2.4 \times 10^8$  and  $3.2 \times 10^8$ /g dry mulch on day 0 when inlet AMD pH was 4.5 and 6.0 respectively. At pH 4.5, densities decreased one  $\log_{10}$  after 49 days of experimentation. At a pH of 6.0, densities decreased from to  $3.2 \times 10^8$  to  $6.5 \times 10^7$ /g dry mulch after 21 days, but increased to  $1.1 \times 10^8$ /g dry mulch at 49 days (Table 3).

Table 3. MPN of SRB in Wetland Mesocosms at AMD pH 6.0, 4.5 and 3.5

Day	SRB/g dry mulch* (x 10 <sup>6</sup> )			
	pH	6.0	4.5	3.5
0		320.0 +/- 190.0 <sup>a</sup>	240.0 +/- 310.0	220.0 +/- 240.0
21		65.0 +/- 62.0	120.0 +/- 110.0	14.0 +/- 14.0
49		110.0 +/- 93.0	99.0 +/- 38.0	0.02 +/- 0.024

\* mean of 3 replicates -

<sup>a</sup> standard deviation

Fe experiments. The effect of Fe concentration on microbial sulfate reduction and the subsequent precipitation of Fe and Mn was determined. Although Fe was added as ferrous chloride, all concentration measurements were of total Fe, with no differentiation made between the ferrous and ferric form. The reservoirs were not made anaerobic, therefore, depending on the conditions present, the ferrous iron may have oxidized to the ferric form within the reservoir. However, the pH was maintained below 3.5 in all reservoirs to ensure that either form of Fe remained dissolved in solution. At all Fe concentrations examined, pH increased from an average of 3.3 to an average of 5.7 (Fig. 20). Figure 21 shows the effect of Fe concentration on total outlet Fe as AMD passed through the mesocosms. At inlet Fe concentrations averaging 85 mg/L, an 88% decrease in total Fe concentration occurred (Fig. 21a). Total Fe decreased 92% and 93% from inlet concentrations of 155 mg/L and 301 mg/L respectively (Figs 21b-21c).

The effect of Fe concentration on the precipitation of Mn is presented in Figure 22. At AMD inflow Fe concentrations averaging 85, 155 and 301 mg/L, total dissolved Mn decreased 12, 21 and 43% respectively. Dissolved sulfide was extremely variable regardless of the Fe concentration, with concentrations decreasing on days 0, 7 and 35 at all Fe levels tested (Fig. 23). No significant difference was detected between effluent sulfide concentrations at any Fe concentration examined ( $p > 0.05$ ). Figure 24 shows the effect of Fe concentration on the decrease in sulfate concentration. Sulfate concentrations decreased 37, 44 and 55% in treatments

containing 85 mg/L, 155 mg/L and 301 mg/L Fe respectively.

On day 0 of the experiment, sulfate reducing bacteria averaged  $5.3 \times 10^7$  SRB/g dry mulch and  $2.7 \times 10^8$  SRB/g dry mulch at inlet Fe concentrations of 85 mg/L and 155 mg/L respectively. At an AMD Fe concentration of 301 mg/L, SRB averaged  $1.2 \times 10^3$  /g dry mulch (Table 4). Over the 49 day period, SRB populations at inlet Fe concentrations 85 mg/L and 155 mg/L decreased significantly by 3  $\log_{10}$ . At an AMD Fe concentration of 301 mg/L, SRB population changed significantly, increasing 2  $\log_{10}$  over the 49 day study period (Table 4).

Table 4: MPN of SRB in Wetland Mesocosms at AMD Fe Concentrations of 85 mg/L, 155 mg/L and 301 mg/L.

Day	SRB/g dry mulch* (x 10 <sup>5</sup> )			
	Fe (mg/L)	85	155	301
0		530.0 +/- 680.0 <sup>a</sup>	270.0 +/- 440.0	0.01 +/- 0.007
21		11.0 +/- 9.7	52.0 +/- 85.0	3.8 +/- 0.630
49		0.6 +/- 0.63	1.1 +/- 1.8	4.7 +/- 8.100

\* mean of 3 replicates

<sup>a</sup> standard deviation

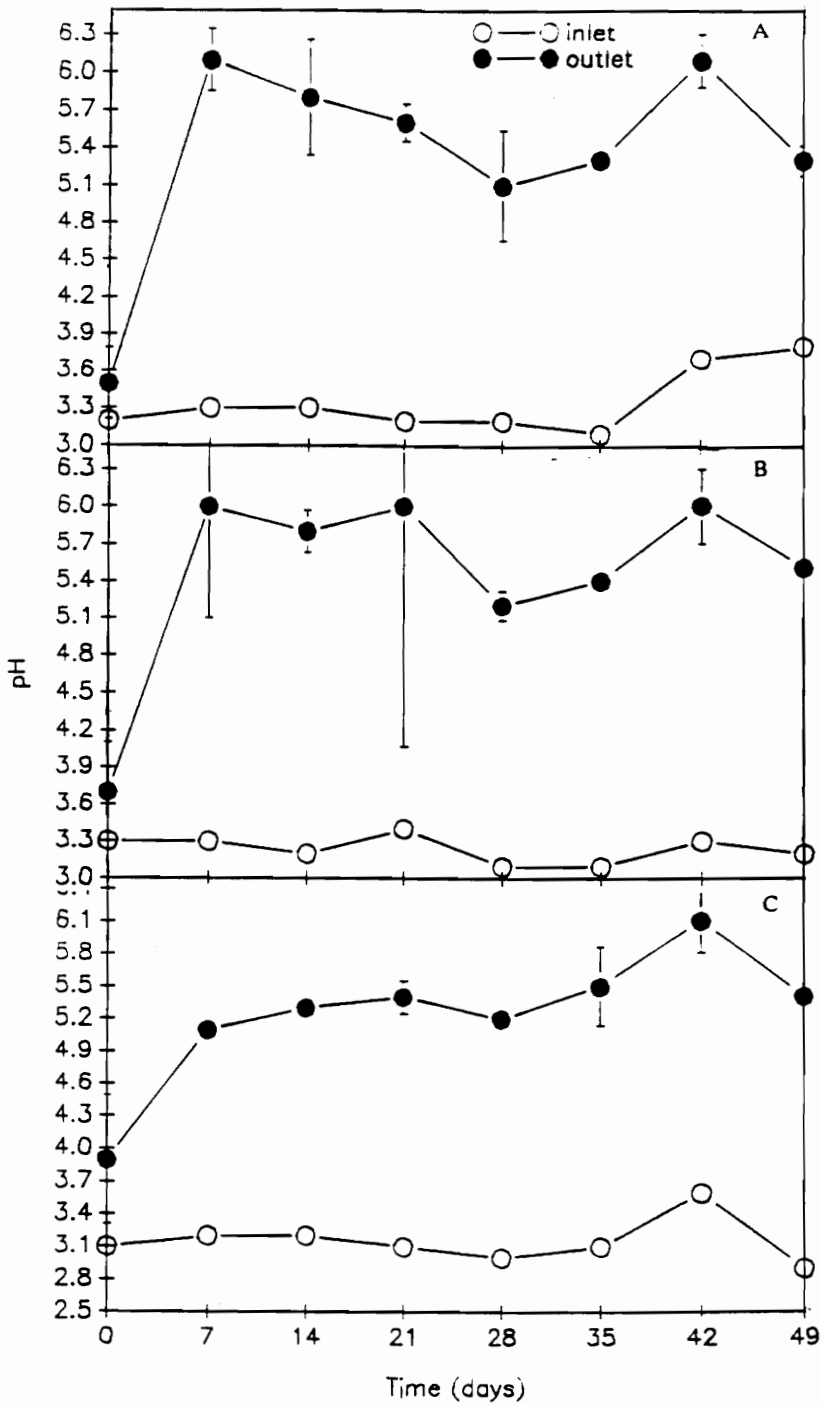


Figure 20. The effect of Fe concentration (mg/L) on effluent pH in AMD. Influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Vertical bars represent +/- SD.

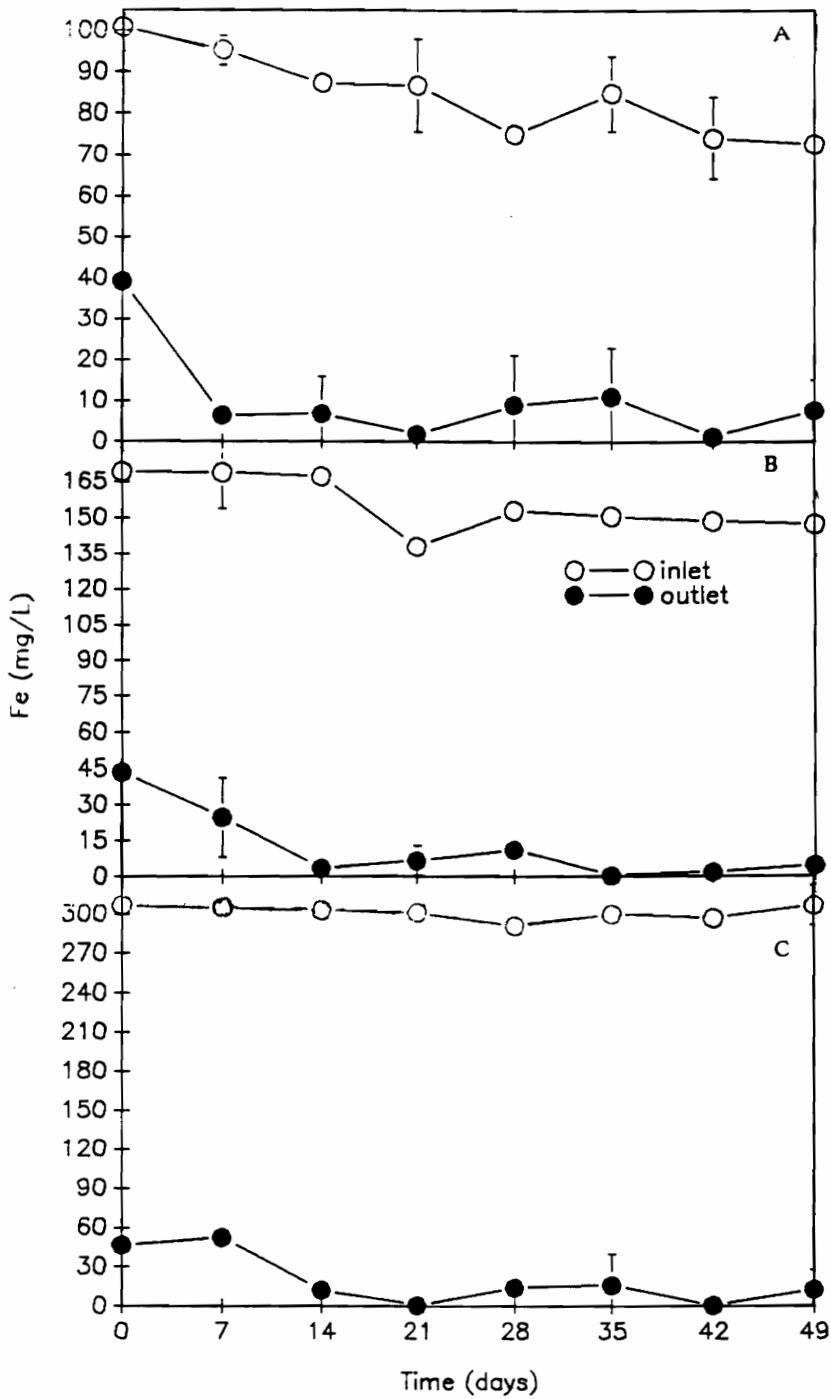


Figure 21. The effect of inflow Fe concentration (mg/L) on effluent Fe (mg/L) in AMD. Influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Vertical bars represent  $\pm$  SD.

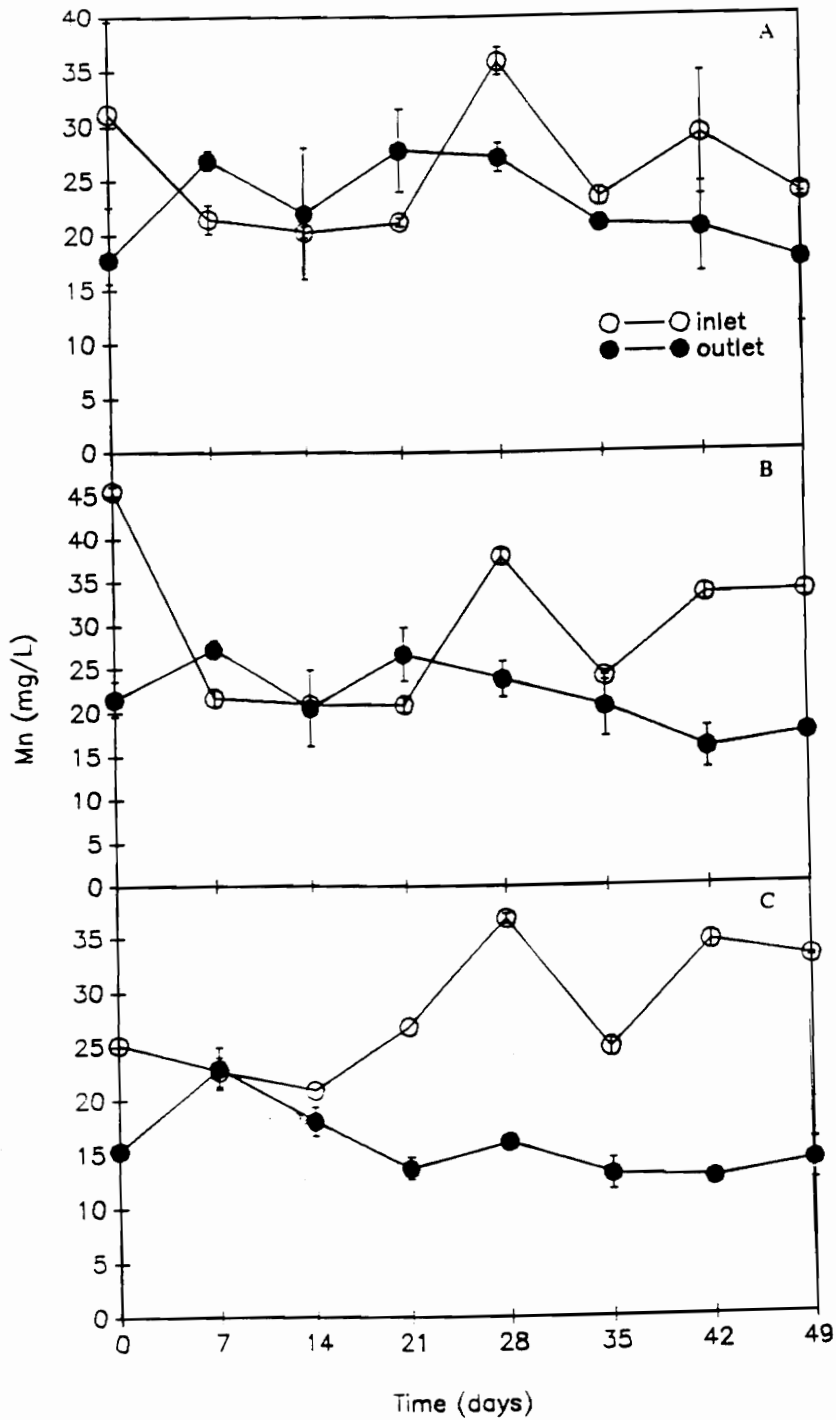


Figure 22. The effect of Fe concentration (mg/L) on effluent Mn (mg/L) in AMD. Influent Fe concentrations averaged (A) 85 mg/l, (B) 255 mg/L and (C) 301 mg/L. Vertical bars represent +/- SD.

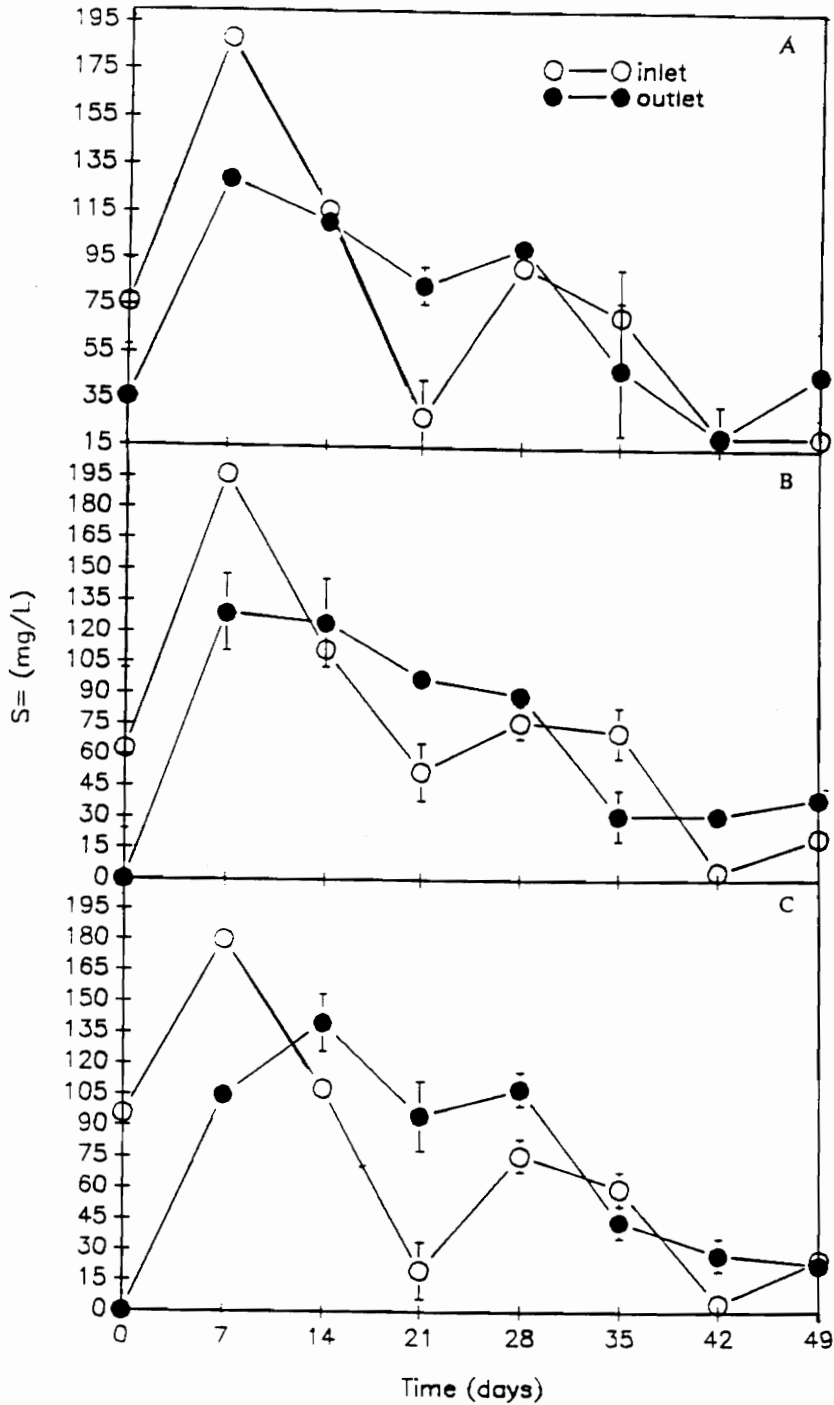


Figure 23. The effect of Fe concentration (mg/L) on effluent sulfide concentration (mg/L) in AMD. Inlet Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Vertical bars represent +/- SD.



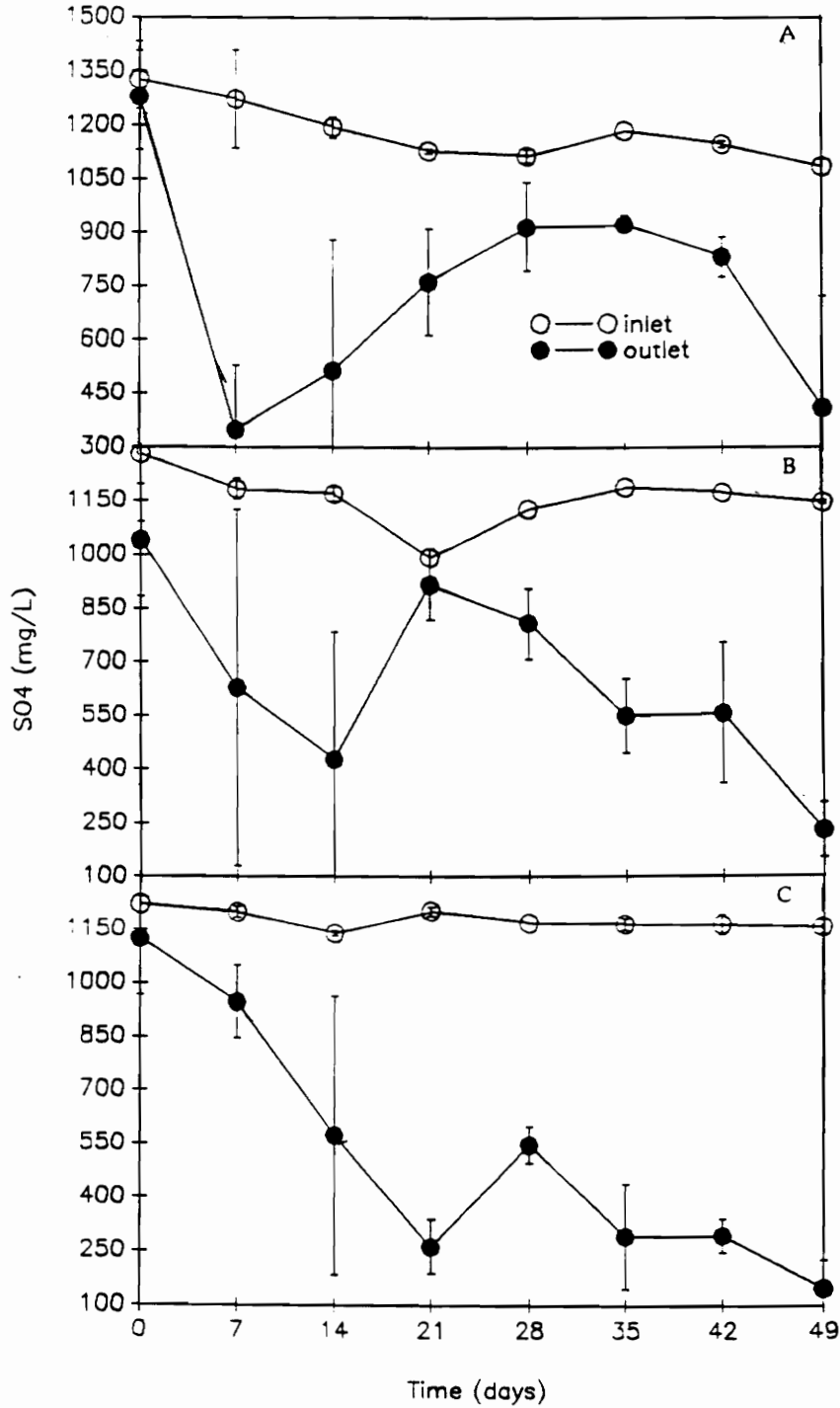


Figure 24. The effect of Fe concentration (mg/L) on effluent sulfate concentration (mg/L) in AMD. Influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Vertical bars represent +/- SD.

### Mesocosm Layer Analysis

Water from layers within the mesocosms was collected from portals stoppered with serum stoppers at depths of 4, 8, 12, 16, and 20 inches (from the top of the mesocosm) using a syringe and needle. Water was analyzed for pH, total dissolved Fe and Mn, and dissolved sulfate. Portal 1 (4 inch depth) was within the surface water layer, portals 2-4 within the mulch layer and portal 5 (20 inches) within the limestone layer. For the sake of brevity throughout the thesis, these will be referred to as portal analysis or portal data.

pH experiments. At an AMD inlet pH maintained at 6.0, all portals had a pH near neutral for the first 28 days, and averaging 8.0 for the last 3 weeks (Fig. 25A). Portal 5, within the limestone layer, had a pH of 8.8 on day 35, and 7.3 and 7.5 on days 42 and 49 respectively. Over the 49 day study period, total dissolved Fe concentrations within the surface water averaged 10 mg/L, fluctuating from as low as 2.0 to as high as 23 mg/L (Fig. 26A). For the first 14 days, portals 2-5 averaged less than 2.5 mg/L Fe. After 14 days, total Fe concentrations at all depths increased gradually. Portal 2 (8 inch depth, within mulch layer) consistently showed a higher Fe concentration than all other portals except on day 49. Total Mn concentrations throughout the depth of the mesocosms fluctuated over time when inlet pH averaged 6.0 (Fig. 27A). Dissolved sulfate averaged 400 mg/L within the surface water (Fig. 28A), decreasing from an average inlet concentration of 759 mg/L. Concentrations dropped to an average of 150 mg/L at 8 inches, and to less than 50 mg/L sulfate

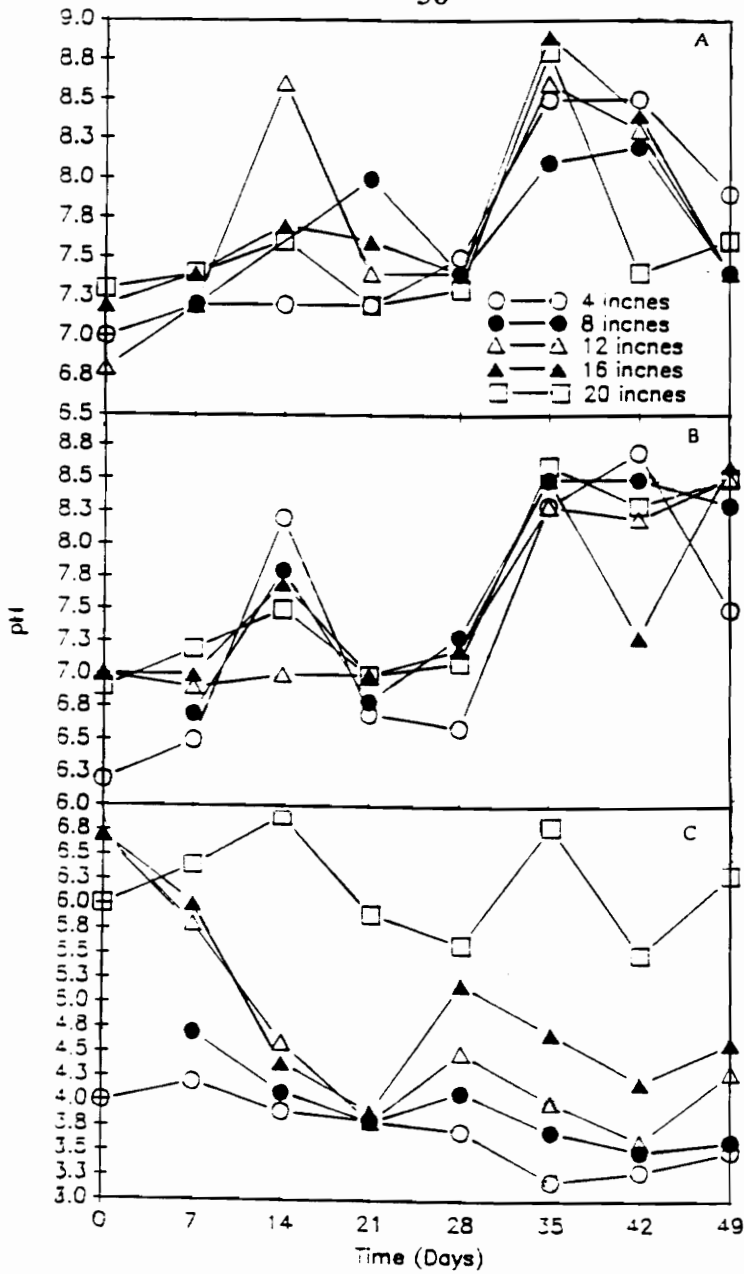


Figure 25. The pH at successive depths within the mesocosms at influent pH (A) 6.0, (B) 4.5 and (C) 3.5. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

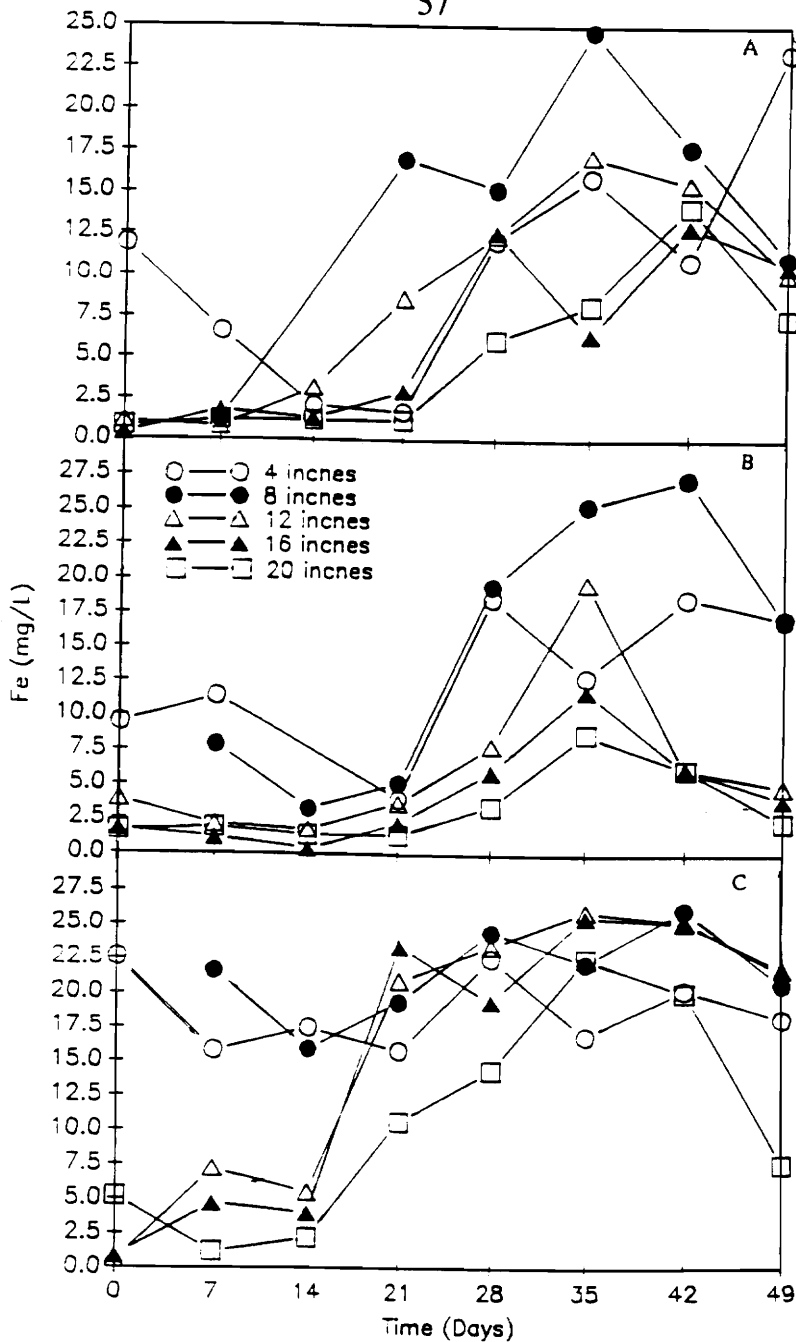


Figure 26. The concentration of Fe (mg/L) at successive depths within the mesocosms at influent pH (A) 6.0, (B) 4.5 and (C) 3.5. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

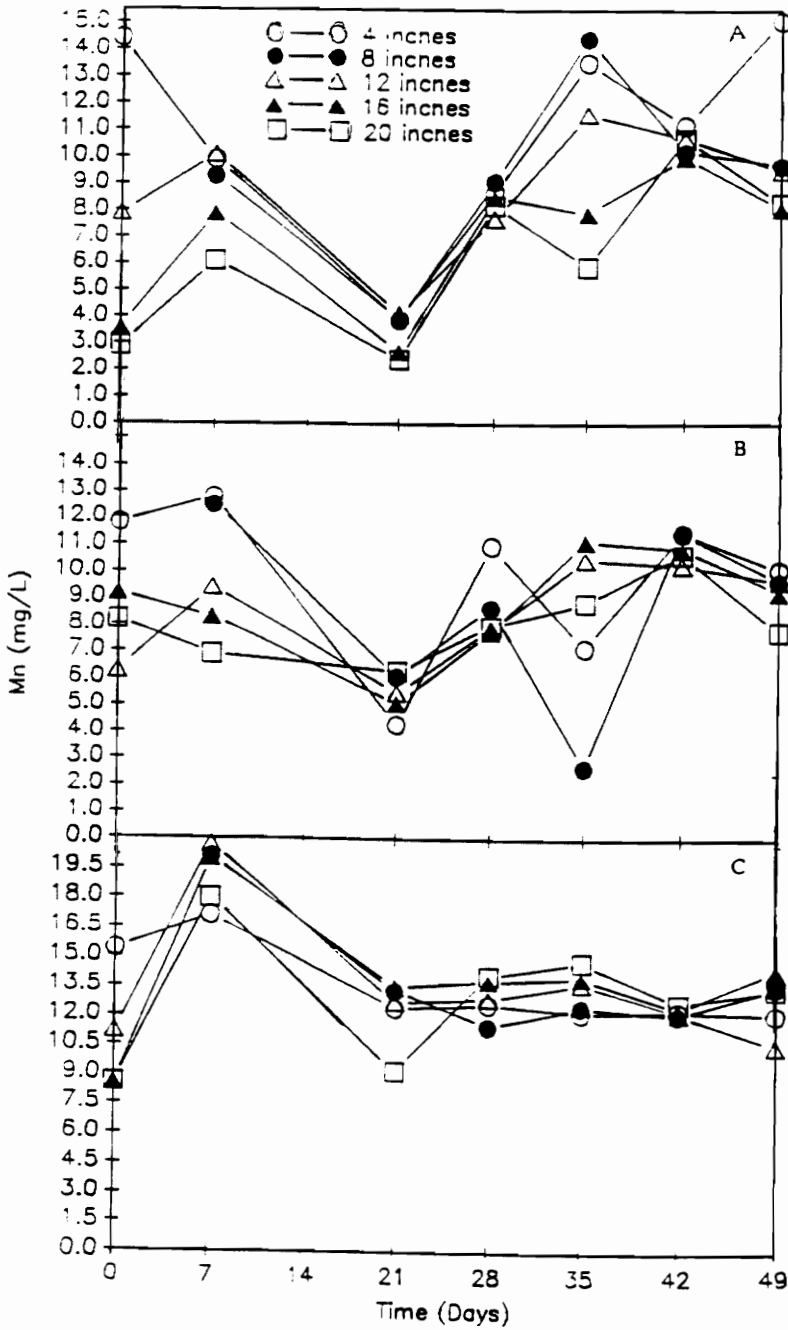


Figure 27. The concentration of Mn (mg/L) at successive depths within the mesocosms at influent pH (A) 6.0, (B) 4.5 and (C) 3.5. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

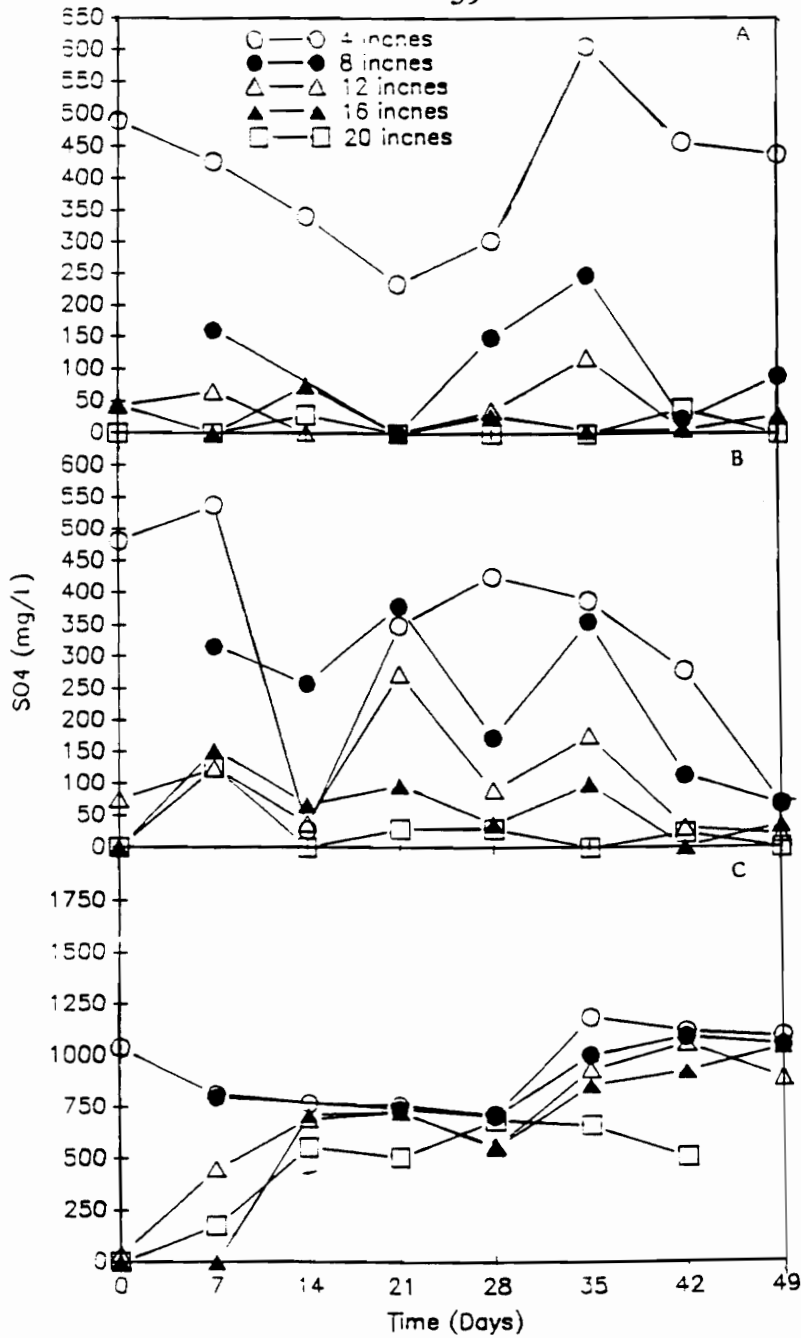


Figure 28. The concentration of sulfate (mg/L) at successive depths within the mesocosms at influent pH (A) 6.0, (B) 4.5 and (C) 3.5. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

below 8 inches (Fig. 28A).

At an inlet AMD pH of 4.5, pH averaged 6.8 within the surface water for the first 28 days and 8.3 for the last 3 weeks (Fig. 25B). At portals 2-5 (below 4 inch depth) pH averaged near neutral for the first 28 days and 8.2 for the last 3 weeks. Surface water total dissolved Fe concentrations averaged 13 mg/L, fluctuating as high as 18 mg/L on days 28 and 35 (Fig. 26B). Portals 2-5 had concentrations below 7.5 for the first 21 days of the experiment. After 21 days, Fe concentrations at 8 inch depths rose to as high as 27 mg/L, while all other depths averaged 12.5 mg/L. Total Mn concentrations averaged 10 mg/L at all depths within the mesocosm through the length of the experiment (Fig. 27B). Surface water Mn concentrations were consistently below 13 mg/L when AMD pH was 4.5. At an AMD pH of 4.5, surface water sulfate concentrations averaged 321 mg/L, decreased from an average inlet concentration of 799 mg/L (Fig. 28B). Portal 2 (8 inches, within mulch substrate) averaged 250 mg/L, while at a depth of 12 inches, sulfate averaged 100 mg/L. Depths of 16 and 20 inches averaged less than 50 mg/L (Fig. 28B).

When AMD pH was maintained at 3.5, surface water pH averaged 3.8 (Fig. 25C). At a depth of 8 inches, pH averaged 4.0. Hydrogen ion concentrations averaged 4.5, 4.2 and 6.5 at depths of 12, 16 and 20 inches respectively. Surface water and 8 inch depth total dissolved Fe concentrations averaged 18 mg/L at an AMD pH of 3.5 (Fig. 26C). For the first 14 days of the experiment, portals 3-5 averaged less than 7.5 mg/L Fe, increasing after 14 days to an average of 20 mg/L

for portals 3 and 4, and an average of 15 at a depth of 20 inches (Fig. 26B). Total Mn concentrations averaged 14 mg/L at all depths through the course of the study (Fig. 27C). Surface water sulfate concentrations averaged 1000 mg/L, decreasing from an inflow average of 1006 mg/L (Fig. 28C). At a depth of 8 inches, sulfate concentration averaged 850 mg/L. At depths of 12 and 16 inches, sulfate averaged 750 mg/L, while at a depth of 20 inches sulfate averaged 500 mg/L.

Fe experiments. Inflow Fe concentrations averaging 85 mg/L, 155 mg/L and 301 mg/L were examined for their effect on microbial sulfate reduction. Portal analysis shows that for those three concentrations studied, the pH at portal 1 (within surface water layer) measured 3.7, 3.6 and 3.2 respectively (Fig. 29). Where inflow concentrations (total Fe) were 85 and 155 mg/L, AMD pH was above 4.0 when measured at portal 2 (Fig. 29A and 29B). At an Fe inlet concentration of 301 mg/L, pH did not measure above 3.9 until portal 4 (Fig. 29C). Within the limestone layer (portal 5), the pH was 5.5, 5.4 and 5.2 at inflow Fe concentrations of 85, 155 and 301 mg/L respectively (Fig. 29). Figure 30A shows the portal analysis when inflow Fe concentrations averaged 85 mg/L. Surface AMD had a total Fe concentration of 33.2 mg/L. The concentration increased to 48.6 and 42.6 mg/L at portals 2 and 3 respectively, and to 36.2 and 36.4 in portals 4 and 5 respectively (Fig. 30A). When inflow Fe concentrations averaged 155 mg/L, surface water Fe concentrations averaged 47.7 (Fig 30B). Concentrations decreased to 47.6 at portal 2, 39.1 in portal 3 and 31.2 at portal 4. At portal 5, within the limestone layer, Fe



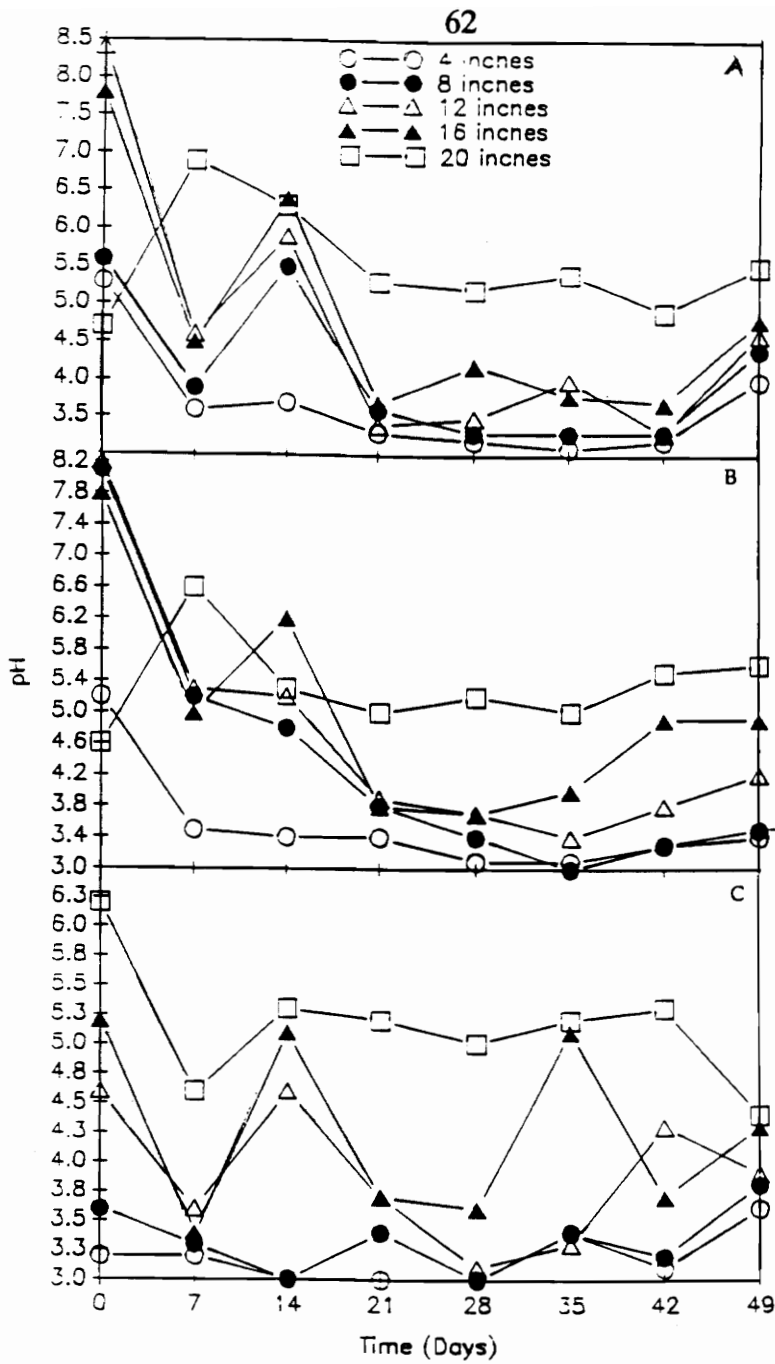


Figure 29. The pH at successive depths within the mesocosms when influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

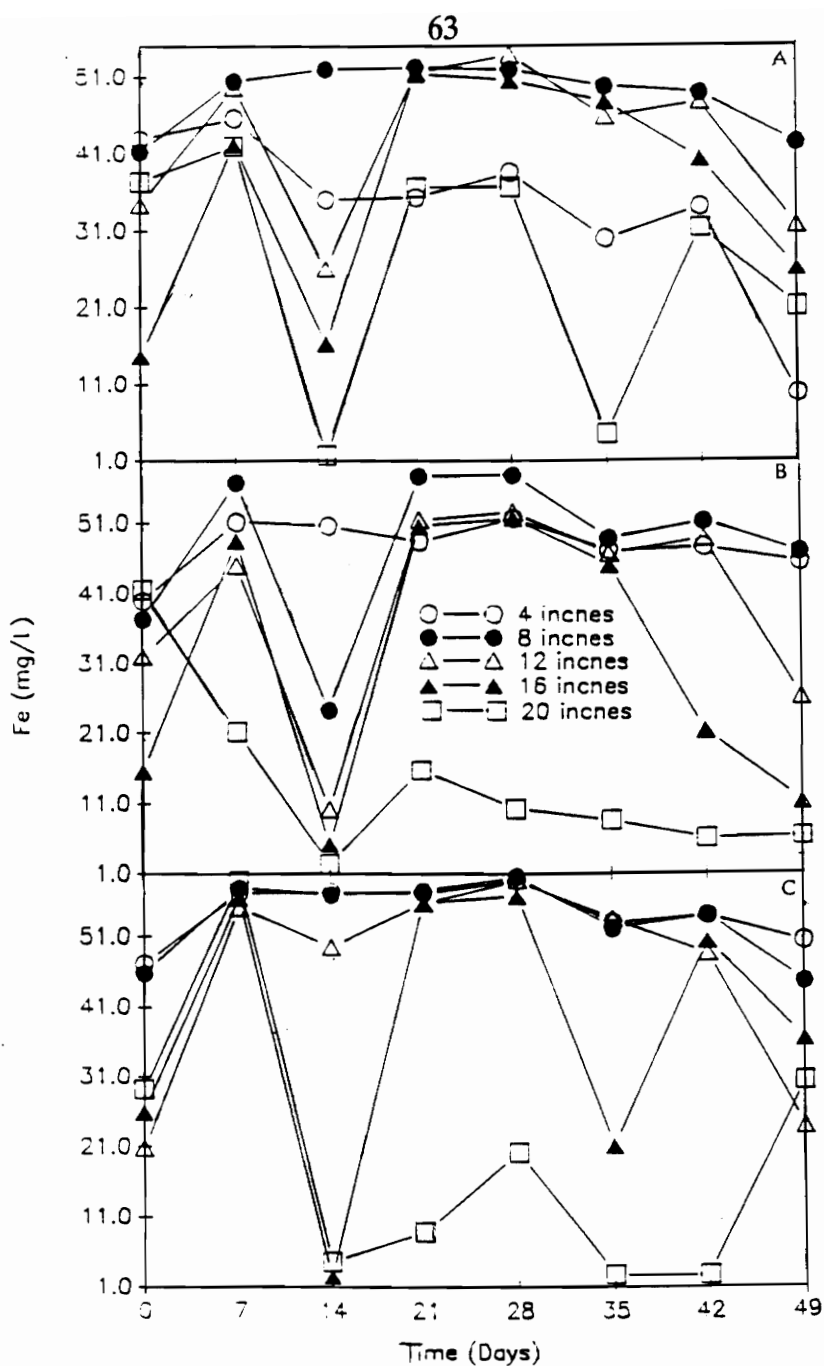


Figure 30. Iron concentration (mg/L) at successive depths within the mesocosms when influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

concentrations averaged 14 mg/L over the 49 day study period (Fig. 30B). When total inflow AMD Fe concentrations averaged 301 mg/L, surface water Fe concentrations averaged 54.4 mg/L (Fig. 30C). Concentrations averaged 53.6, 45.8 and 38.2 mg/L at portals 2, 3 and 4 respectively. Portal 5, within the limestone layer, averaged 19.7 mg/L Fe (Fig. 30C).

When total inflow Fe concentrations averaged 85 mg/L, and inflow total Mn concentrations averaged around 20 mg/L, the AMD at the surface of the mesocosm contained an average of 16.1 mg/L Mn over the course of the study (Fig. 31A). Concentrations of total Mn increased to 17.6, 16.4, 18.0 and 18.9 mg/L at portals 2, 3, 4 and 5 respectively. A similar trend was seen when inflow Fe concentrations averaged 155 and 301 mg/L. Surface AMD averaged 16.8 and 15.9 mg/L respectively (Fig 31B and 31C). Concentrations increased throughout the mesocosm, with concentrations averaging 20.3 and 17.6 at portal 5 when inflow Fe concentrations were 155 and 301 mg/L respectively (Fig. 31B and 31C).

At all Fe concentrations tested, inflow sulfate concentrations averaged above 1100 mg/L. When inflow Fe concentrations averaged 85 mg/L, surface AMD sulfate concentrations averaged 1003 through the course of the study (Fig. 32A). Sulfate concentrations increased to 1039 mg/L at portal 2, and decreased to 879 and 848 mg/L at portals 3 and 4 respectively. Portal 5, within the limestone layer, had an average sulfate concentration of 685 mg/L over the 49 day study period (Fig 32A). When inflow Fe concentrations averaged 155 mg/L, sulfate concentrations averaged

949 and 745 mg/L at portals 1 (surface water) and 2 (within mulch substrate) (Fig. 32B). Concentrations average 868 and 716 mg/L at portals 3 and 4 respectively, and averaged 594 mg/L within the limestone layer. At inflow Fe concentrations of 301 mg/L, surface sulfate concentration averaged 1168 mg/L (Fig. 32C). At portal 2 and 3, sulfate concentrations averaged 1124 and 1025 mg/L respectively. At portal 4, concentrations averaged 789 mg/L, and within the limestone layer, sulfate concentrations averaged 519 mg/L.

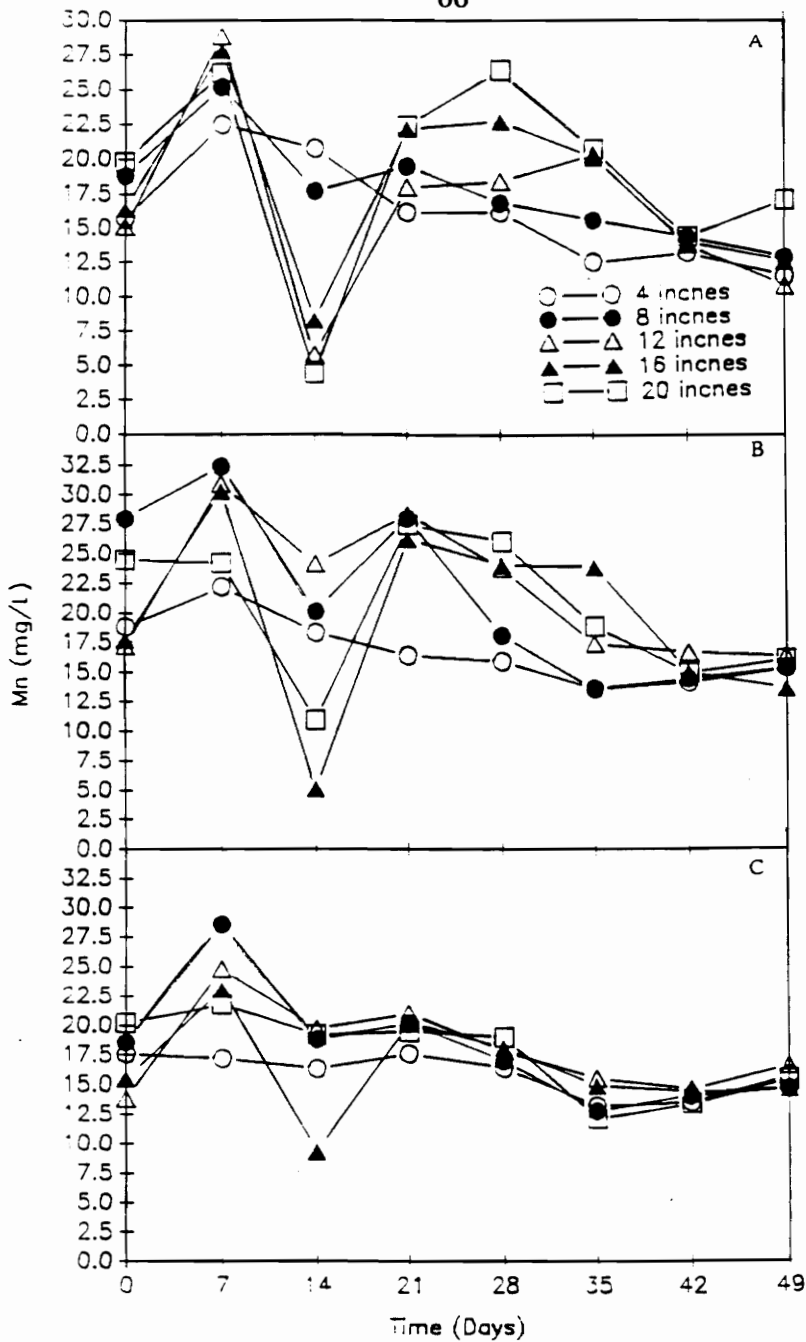


Figure 31. Manganese concentration (mg/L) at successive depths within the mesocosms when influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

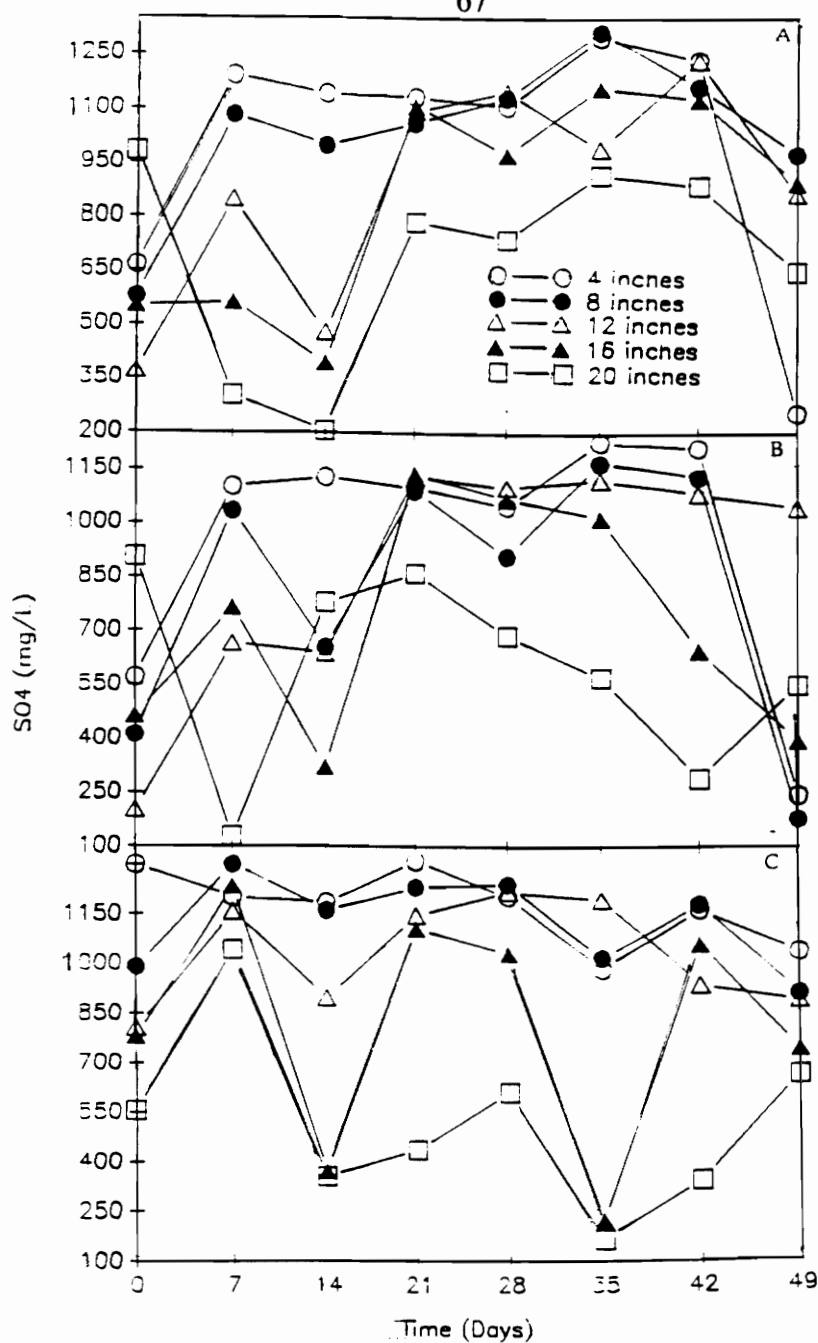


Figure 32. Sulfate concentration (mg/L) at successive depths within the mesocosms when influent Fe concentrations averaged (A) 85 mg/L, (B) 155 mg/L and (C) 301 mg/L. Depth is in inches from the top of the mesocosm. Four inch depth sampled within the AMD above the surface of the mulch substrate. Depths of 8, 12 and 16 inches were within the mulch substrate and 20 inch depth sampled within the limestone layer.

## DISCUSSION

### Field Studies

Water Chemistry. The purpose of this study was to evaluate the success of a wetland constructed to treat AMD from an inactive coal refuse pile. Figures 3 and 4 reveal that water quality was improved over the course of the study based on a rise in pH and a decrease in Fe and Mn concentrations to within instream compliance standards set by the EPA. Influent pH was already at a level within EPA standards (6-8), and Fe and Mn inlet concentrations were considerably low. Lundgren et al. (1972) classified AMD into 4 classes based on the characteristics of the discharge. Class I includes acidic discharges; class II, partially oxidized and neutralized, discharges; class III, oxidized and neutralized or alkaline; and class IV, neutralized and not oxidized. The AMD treated by the Pine Branch wetland falls between class III and class IV, as it is neutralized, with a pH between 6.5 and 8.5, it contains sulfate concentrations between 500 and 10,000 mg/L and has an Fe (total) concentration falling between 0 and 1,000 mg/L. Wieder (1989) found that 66% of 142 wetlands he surveyed east of the Mississippi were constructed to treat Fe concentrations less than 50 mg/L. About 37 treated AMD with Fe concentrations of 10 mg/L and below (Wieder, 1989). Fifty percent of the wetlands Wieder surveyed had treatment efficiencies for Fe of 81%. Wieder (1989) also found that treatment efficiencies for the removal of Mn averaged only 34%. The average inflow of Mn in these wetlands was 37.7 mg/L (+/- 3.5 mg/L).

Iron concentrations flowing into the Pine Branch wetland averaged about 4

mg/L over the course of the study with 2 exceptions, when inflow Fe concentrations were 16 and 10 mg/L respectively (Fig. 3B). Concentrations of Fe averaged less than 1 mg/L after the AMD passed through the wetland. Manganese concentrations entering the wetland from the Pine Branch refuse pile averaged 3 mg/L (Fig. 3C). Manganese concentrations in the wetland effluent averaged 1 mg/L, meeting the EPA water quality standard of 2 mg/L Mn instream. On the occasion that effluent was above 2 mg/L, EPA standards were met due to dilution upon entering Pine Branch, a tributary to the Powell River.

The ferric ion is soluble under strongly acidic and oxidizing conditions (Bohn et al., 1985). Under neutral to alkaline conditions however, the ferric ion precipitates as ferric hydroxide (Atlas and Bartha, 1993). This reaction occurs spontaneously as acidic AMD is aerated and flows into streams with a neutral pH. Studies conducted at the Pine Branch wetland site indicate that 80% of the total inflow Fe concentration was removed as the AMD entered the wetland (Hendricks and Duddleston, 1992). This was probably due to the formation of an iron hydroxide as the AMD flowed into the wetland. The presence of a thick orange-yellow precipitate, the typical color of ferric hydroxide, around the wetland inlet pipe further supports this conclusion. Other studies indicate that 40 to 70% of total iron removed from AMD in wetlands is in the form of hydroxides (Henrot and Wieder, 1990,; Calabrese et al., 1991,; Wieder, 1992).

Under anaerobic conditions, ferric iron is reduced to ferrous iron, which in the



presence of hydrogen sulfide gas, may precipitate as ferrous sulfide (Atlas and Bartha, 1993). An additional 19% of total inflow Fe was removed in the Pine Branch wetland before the AMD exited the wetland. The presence of a black precipitate below the surface of the wetland substrate suggests that the additional amount of total Fe (19%) was precipitated in the form of a metal monosulfide (Paul and Clark, 1989). Up to 30% of total Fe has been shown to be found in the form of iron sulfides, in particular mono and disulfides, within wetland substrates presumably from microbial sulfate reduction (McIntire and Edenborn, 1990;; Calabrese et al., 1991; Wieder, 1992). Under conditions of neutral pH, ferrous iron may also be removed by chelation with organic materials (Brock, 1991).

Data obtained from portal samples of wetland mesocosms supports experimental results obtained from field studies. Concentrations of Fe in the water above the surface of the mulch substrate were 60% lower than that in the influent AMD (20 mg/L) (Fig. 26). An orange precipitate, the typical color of ferric hydroxide, was present at the surface of the mulch substrate. A black precipitate was present within the substrate of the mesocosms, typical of an iron sulfide precipitate, and possibly a manganese oxide precipitate (Paul and Clark, 1989).

The manganous ion ( $\text{Mn}^{2+}$ ) is stable below pH 5.5 under aerobic conditions (Atlas and Bartha, 1993), as well as under anaerobic conditions between a pH of 5.5 and 7.0 (Atlas and Bartha, 1993). Manganese solubility increases however, as pH decreases and reducing conditions increase (Bohn et al., 1985). At a pH of 8 and

above, the manganous ion in the presence of oxygen will spontaneously oxidize to the manganic ion ( $\text{Mn}^{4+}$ ) and precipitate as manganic dioxide (Atlas and Bartha, 1993). The oxidation of manganese below pH 8 must be microbially mediated (Paul and Clark, 1989). Several soil bacteria and fungi have been shown to oxidize manganese, and one in particular, *Leptothrix*, accumulates manganic dioxide in its filaments and sheaths (Paul and Clark, 1989). Similarly, under aerobic conditions, rhizosphere bacteria oxidize the manganous ion to manganic ion and deposit manganic dioxide, a black precipitate, on the root (Paul and Clark, 1989). Influent Mn concentrations in the Pine Branch wetland averaged 3 mg/L (Fig 3C), and effluent concentrations averaged 1 mg/L over the course of the study. Concentration fluctuations ranged from 3 to less than 1 mg/L. A study at the Pine Branch wetland site showed that 63% of the Mn was precipitated prior to the AMD flowing through the volume of the wetland (Hendricks and Duddleston, 1992). The pH of the AMD was neutral in both the influent and effluent. This appears to be a transition zone for manganese transformation depending on the environmental conditions present. The formation of ferric hydroxide on the surface of the wetland is an acidity generating and oxygen consuming reaction. Measurement of pH in the surface water of the wetland was not done. If the water was rendered anaerobic as a result of ferric hydroxide formation, and the pH was lowered due to the generation of acidity, the manganese ion would be more soluble in the water (Bohn et al., 1985) and a 63% decrease in concentration would not be expected. However, if the water was not rendered

anaerobic, as is a likely scenario considering the low concentrations of Fe entering the wetland, the manganese could form a manganic dioxide precipitate at very low rates (Fig. 33, from Bohn et al., 1985). Another possibility is that the by diffusion of carbon dioxide and hydrogen sulfide from the anaerobic zone within the wetland, the manganese precipitated as a manganese carbonate or manganese sulfide (Fig 33, from Bohn et al., 1985). A positive Eh value would favor the formation of a carbonate, and a slightly negative Eh value favors the formation of a sulfide (Fig. 33, from Bohn et al., 1985).

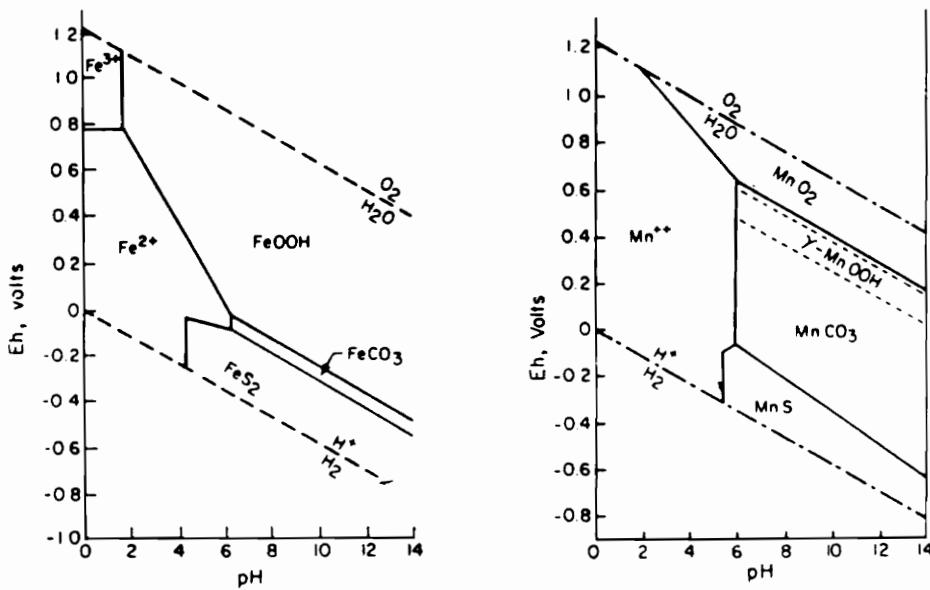


Figure 33. The Eh diagram of (A) iron and (B) manganese compounds. From Bohn et al., 1985.

An additional 15% of the dissolved manganese was shown to be removed after passage of the AMD through the wetland. The low amount is probably due to the continued formation of carbonate or sulfide precipitates. The presence of organotrophic manganese reducing bacteria, which reduce  $\text{Mn}^{4+}$  to  $\text{Mn}^{2+}$  (Brock, 1991) may also be present within constructed wetlands. After the reduction of manganic manganese to manganous manganese,  $\text{Mn}^{2+}$  is available for precipitation as manganous carbonate or manganous sulfide, or subsequent oxidation to manganic dioxide.

Sulfate Reducing Bacteria. The substrate used in the Pine Branch wetland was a 10-15 year old pine bark mulch. Studies with wetland mesocosms show that this weathered mulch supports a population of about  $10^5$  SRB (Table 2) and a 15% reduction in dissolved sulfate (Figs. 5A, 6A and 7A). Similarly, the Pine Branch wetland supports a population averaging  $8.7 \times 10^4$  SRB/g dry mulch (Table 1). However, sulfate concentration was reduced 58% as AMD passed through the wetland (Fig. 4). The wetland consists also of cattails, which contribute to the carbon available to SRB through the microbial degradation of their leaves and the release of plant root exudates. Available carbon is also constantly added to the wetland in the inflow of AMD in the form of end products resulting from the degradation of dead plant and animal material. The anaerobic degradation of the mulch by cellulose degraders would also contribute to the carbon available for SRB, as the end products of cellulose fermentation are ethanol, formic acid, acetic acid, lactic acid,

hydrogen and carbon dioxide (Rheinheimer, 1992). Ethanol, formate and lactate have been shown to be metabolized by SRB. Postgate (1979) states that formate acts as an "incomplete" substrate, as does isobutanol, where sulfate reduction, but not growth, is supported. These incomplete substrates are said to be acting as sources of inorganic hydrogen, and the reactions yield energy which can be used to assimilate carbon. Even though a low population of SRB ( $8.7 \times 10^4$ ) was found in the wetland during the study period, the metabolic activity of these organisms was sufficient to reduce the concentration of sulfate by an 58%. The metabolism of incomplete substrates supporting metabolic sulfate reduction may account for the low population of sulfate reducing bacteria but apparent high metabolic activity.

### **Wetland Mesocosms**

Carbon experiments. The purpose of these experiments was originally to determine if the concentration of dissolved sulfate had an effect on the removal of Fe from AMD. After the first 98 days when sulfate reducing activity was low (only 20% reduction) at all sulfate treatments, it was postulated that carbon was a limiting factor. This seemed logical considering the age of the mulch (10-15 years). Weathered mulch of this age would likely have been degraded to the point that only lignin and cellulose would constitute the remaining carbon. Both of these are forms of carbon which SRB apparently cannot use as a carbon source. Cellulose is degraded under both aerobic and anaerobic conditions by cellulytic microorganisms (Atlas and Bartha, 1987). In the mesocosms therefore, cellulose degraders should

be present. Considering the addition of both ammonia and nitrate nitrogen, phosphorus, and sulfate, it seems unlikely that these inorganic nutrients would limit either sulfate reduction or cellulose degradation. It is possible however, that the rate of cellulose degradation was not sufficient to release enough simple organics to support sulfate reduction by SRB.

Lignin degradation does not occur under anaerobic conditions (Atlas and Bartha, 1987). Intact wood is attacked by brown rot and white rot fungi. Brown rot fungi bypass the lignin directly, degrading first the associated cellulose and hemicellulose, leaving behind only lignin. White rot fungi on the other hand degrade the lignin, leaving behind the cellulose (Atlas and Bartha, 1987). Lignin is also degraded by aerobic bacteria of the genera *Arthrobacter*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas* (Atlas and Bartha, 1987). Periodic samples taken from portals inserted in the front panels of the mesocosms and analyzed for dissolved oxygen indicate that no more than 5.0 mg dissolved oxygen was present in the water above the surface of the substrate, and no detectable dissolved oxygen was found below the surface when mulch was the only available source of carbon in the mesocosm (Table 9, Appendix C). Most likely, carbonaceous material in the mulch was predominantly lignin, and in the absence of dissolved oxygen, degradation of lignin would be limited. A form of carbon easily utilizable by SRB would therefore not be present.

After the addition of 100 mg/L of carbon in the form of lactate to the AMD, only a 15% reduction in sulfate concentration occurred within the mesocosm (Figs.

5A, 6A and 7A). After the addition of 300 mg/L carbon as lactate, sulfate was decreased significantly. Similarly, no detectable increase in dissolved sulfide was detected until the addition of 300 mg/L carbon. The data suggests that carbon was a limiting factor affecting microbial sulfate reduction by SRB.

Sulfate reducing bacteria were enumerated throughout the course of the study. At time 0 days, SRB were present at an average of  $5.5 \times 10^5$ /g dry mulch. After 30 days with no additional carbon added, numbers increased to  $1.5 \times 10^6$ . However, at 126 days the average population of SRB again decreased to  $6.3 \times 10^5$ . After the addition of 200 mg/L carbon as lactate, numbers of SRB increased to  $4.4 \times 10^6$ , and after the addition of 300 mg/L carbon, increased to  $1.5 \times 10^8$  SRB/g dry mulch. The increase in population size by a factor of  $3 \log_{10}$ , in conjunction with the decrease in dissolved sulfate and increase in dissolved sulfide, supports the conclusion that carbon was limiting both growth and activity of SRB within the mesocosms. The data would suggest that carbon may also be a limiting factor within wetlands constructed to treat AMD. The population of SRB within the Pine Branch wetland averaged  $10^5$  SRB/g dry mulch over the course of the study. This population corresponds to that which was present in the mesocosms prior to lactate addition, yet apparent metabolic activity was greater in the wetland based on the percent removal of sulfate in the wetlands as compared to the mesocosms. It is possible that the inflow of carbon from plant root exudates and the end products of the microbial degradation of plant and animal matter may contribute to the apparent increase in

metabolic sulfate reduction over that found in the mesocosms. The study suggests that when AMD contains higher acidity and greater concentrations of metals than that which flows from the Pine Branch coal refuse pile, it may be necessary to consider carbon availability when choosing a substrate type in order to support a higher and more active population of SRB. This would in turn support a greater metal removal, especially at higher influent sulfate concentrations.

Hammack and Edenborn (1991) found that when adding 3500 mg/L lactate as sodium lactate to mine waters containing sulfate and nickel, nickel removal and sulfate reduction rates increased seven-fold. Sulfate concentrations in the effluent dropped dramatically, from an average of 2000 mg/L to a range of 700 to 1400 mg/L depending on the nickel concentration (Hammack and Edenborn, 1991). Interestingly, the amount of sulfate removed increased with increase in nickel concentration. They explained that increased nickel resulted in more of the sulfide produced being precipitated as a nickel sulfide, so sulfide was not inhibiting sulfate reduction as much as if sulfide were remaining dissolved in the water. High levels of sulfide are known to be inhibitory to sulfate reducing bacteria (Postgate, 1979; Widdel, 1988). Hammack and Edenborn (1991) also found that the increase in pH and alkalinity, and the sustained removal of nickel without the presence of added lactate, suggested that microbial sulfate reduction was taking place and nickel was precipitated as a sulfide. Hammack and Edenborn (1991) did not detect a reduction in sulfate during the periods of no lactate addition. The substrate they used was



mushroom compost, and they explained this occurrence as the result of sulfate already present in the compost, not as a lack of sulfate reduction (Hammack and Edenborn, 1991).

Mulch filled mesocosms used in this study showed a similar pattern. Even though no decrease in sulfate or increase in sulfide was detected during the first 98 days of the experiment, between 88 and 92% of Fe was precipitated as the AMD passed through the mesocosms. With a 15 to 20% removal of sulfate, and an influent Fe concentration of only 20 mg/L, it is feasible that all the Fe could have been precipitated as a metal monosulfide. Patches of black mulch present throughout the substrate indicate that at least some precipitation as a monosulfide was taking place. However, portal analysis show that a significant amount of Fe was removed from the AMD at the surface of the substrate prior to the addition of lactate. This suggests the formation of an iron hydroxide. The formation of an hydroxide may also explain why little or no dissolved oxygen was present in the surface water, as the formation of hydroxides is an oxygen consuming reaction. After the addition of 200 and 300 mg/L carbon as lactate, the entire volume of the mesocosm turned black. Data collected from portal samples shows that total Fe concentrations within the water above the surface of the substrate were still significantly lower (70%) than that in the influent AMD. This may be the result of the formation of ferric hydroxide, or the formation of a ferrous sulfide precipitate with sulfide seeping up from the substrate below. The latter explanation seems more feasible as the water above the surface

of the substrate had turned completely black.

After the addition of 200 and 300 mg/L carbon as lactate, the percent removal of total Fe increased from 88% to 97%, 90% to 96% and 92% to 96% at inflow sulfate concentrations of 1554, 650 and 360 mg/L respectively. This also suggests that more iron was precipitated as metal monosulfides than prior to carbon addition, indicating a stimulation of microbial sulfate reducing activity, and a lesser inhibition of sulfate reduction by sulfide (Hammack and Edenborn, 1991).

Manganese was not added as a component of AMD until after the carbon experiments were ended and restarted with 200 mg/L carbon as lactate. As was the case with Fe, a higher percentage of Mn was removed when AMD influent sulfate concentrations were higher. This suggests that Mn was possibly being removed as a monosulfide. Mn was also being removed as the AMD flows into the mesocosm, as indicated by portal analysis for dissolved Mn. Manganese was 57.5 % lower in the surface water than in the influent AMD. The pH was near neutral at the surface, supporting the probability that the manganese was in the form of a sulfide or carbonate precipitate, depending on the Eh value (Bohn et al., 1985).

pH Experiments. Experiments were conducted in order to determine the effect of low pH on microbial sulfate reduction, the increase in pH, and the precipitation of Fe and Mn. Figure 15 shows that at inflow averages of pH 6.0 and 4.6, the effluent pH averaged 7.4 and 7.2 respectively. At an average inlet pH of 3.5, pH was increased to an average of 6.2. The effluent pH when influent was

maintained at 3.5 was within Virginia's instream compliance standards, and was not significantly lower than the effluent from mesocosms treating AMD with a pH of 4.5 and 6.0. When influent AMD pH averaged 6.0, portal samples indicate that the pH at all depths within the mesocosm was near neutral, and sometimes as high as 8.8. Postgate (1979) states that because  $H_2S$  is volatile, large quantities of hydrogen sulfide will create alkaline conditions within the environment. The alkalinity produced by the volatilization of hydrogen sulfide is neutralized by carbon dioxide, and carbonate and bicarbonate accumulate. When sulfate reduction is active, and no acid forming metabolic reactions, or precipitation of hydrogen sulfide with metals, is taking place, the environment will become alkaline (Postgate, 1979). The fact that the pH was near 7.0 at all depths, and not just within the limestone base suggests that microbial sulfate reduction was contributing to the increase in pH. The total concentration of Fe was 20 mg/L, which probably did not result in the precipitation of enough hydrogen sulfide as  $FeS$  to prevent the occurrence of alkaline conditions. A similar trend was seen in mesocosms treating AMD with an influent pH of 4.5. All depths within the mesocosm showed a pH around neutral, and sometimes above 8.0. This indicates that at pH 4.5, adequate alkalinity is being produced by microbial sulfate reduction to raise the pH. In pure culture, sulfate generally occurs at very low rates at pH 5.0 and below (Postgate, 1979). In the mesocosms treating water with a pH of 4.5 it is likely that microenvironments with a buffered pH were present and that the SRB were able to establish their own optimal conditions within the

mesocosms through the production of hydrogen sulfide and bicarbonate.

The reduction in sulfate concentration at both a pH of 4.5 and 6.0, and the maintenance of SRB populations at an average of  $10^8$  SRB/g dry mulch, also supports the conclusion that sulfate reduction is responsible for the increase in pH. It is possible that the activity of other bacteria are contributing to the improvement of water quality, but considering the toxic effects that hydrogen sulfide have on other bacteria, both aerobic and anaerobic (Postgate, 1979), and considering the constant influx of high concentrations of sulfate and lactate, it is likely that sulfate reduction is the dominant microbial activity occurring within the mesocosms.

When treating AMD with a pH of 3.5, the effluent pH averaged 6.4 (Fig. 15). Portal analysis shows that at a depth of 4 inches, within the surface water layer the pH of the water was only 3.8. The pH increased from 3.8 to 4.2 after passing through 12 inches of mulch substrate within the mesocosm. It was not until the AMD passed through the limestone layer at the base of the mesocosm that the pH increased to 6.0. This suggests that at pH 3.5, SR activity is limited and the increase in pH is dependent on the limestone component of the mesocosm design. The decrease in population size (Table 3) as well as the decrease in sulfate removal from AMD at pH 3.5 also support the conclusion that sulfate reduction is inhibited at pH 3.5. The population of SRB decreased from an average of  $2.2 \times 10^8$  to  $2.4 \times 10^4$ /g dry mulch over the 49 day study period. Also, sulfate was reduced from 1000 mg/L to 500 mg/L at AMD pH 3.5 as opposed to less than 50 mg/L at AMD pH 4.5 and

6.0.

AMD pH 3.5 also had a negative effect on the removal of Fe. At a pH of 6.0 and 4.5, Fe was decreased by 92 and 91% respectively, but decreased 70% at AMD pH 3.5. Data from portal samples shows that at pH 6.0 and 4.5, about 38% of the Fe is removed as the AMD flows into the mesocosm. It is probable that Fe was being precipitated as an iron sulfide, as the entire volume of the mesocosm was black, including the water above the surface of the substrate. This is indicative of a reduced environment, under which the formation of an iron sulfide is favored.

At an AMD pH of 3.5, total Fe concentrations within the surface water were only 17% less than influent concentrations. In this case it appears that very little Fe was being precipitated even as an hydroxide on the surface of the substrate. The formation of ferric hydroxide is a reaction that requires oxygen and neutral to alkaline conditions in order to take place (Atlas and Bartha, 1993; Bohn et al., 1985). The formation of an iron sulfide would not be expected when little sulfate reducing activity was occurring. Although some aeration of the AMD occurred as it dripped into the mesocosm, it flowed at a rate of 0.1 L/h and it is probable that not enough dissolved oxygen was present to support the formation of a large amount of ferric hydroxide. Also, since the pH of the water above the surface of the substrate was below 4, and the iron was added in the form of ferrous chloride, it probably remained dissolved in the AMD.

The effluent Fe concentrations at pH 6.0 and 4.5 averaged less than 1 and 1.5

mg/L respectively. At an AMD pH of 3.5, effluent Fe concentrations averaged 0 mg/L for the first 21 days but then increased to 10 mg/L. This can be explained by the fact that mesocosms were not taken apart and refilled between experiments. Therefore, when the pH experiment was first started, residual buffer from the previous carbon experiment was still present within the mesocosm. A flow rate of 0.1 L/h resulted in a 99% water replacement time of 16 days within the mesocosms. It is therefore not unreasonable that the effects of the pH treatment were not seen until 21 days into the experiment.

The effect of pH on the removal of Mn was parallel to that of Fe. At pH 6.0 and 4.5, Mn decreased 72 and 68% respectively, whereas at pH 3.5 Mn decreased only 37%. This is not surprising however, as Mn is a more difficult metal to remove from AMD than Fe (Wieder, 1989; Skousen and Faulkner, 1992;), and is frequently not precipitated until after the addition of pH raising chemicals. Atlas and Bartha (1987) state that the Mn ion is stable under aerobic conditions below pH 5.5, but at pH's above 8.8 it is spontaneously oxidized. At AMD pH 3.5, the manganese remained dissolved in the reduced manganous form ( $\text{Mn}^{2+}$ )

Two possibilities may explain why Mn precipitation is inhibited at a pH of 3.5. Microorganisms which oxidize Mn, such as *Leptothrix*, *Bacillus*, *Pseudomonas* and *Arthrobacter* (Atlas and Bartha, 1987) are inhibited by low pH (Brock and Madigan, 1991), or are not present at all due to the lack of oxygen even within surface water (Table 9, Appendix C).  $\text{Mn}^{4+}$  reducing bacteria may also be inhibited by low pH.

However, the reduction of  $\text{Mn}^{4+}$  in anaerobic environments usually occurs as a chemical reduction with a product of microbial metabolism, such as hydrogen sulfide, which leads to the second possibility (Atlas and Bartha, 1987). The lack of sulfate reducing activity and thus lack of dissolved sulfide would in turn result in less Mn being precipitated as a manganous sulfide within the mesocosm substrate. Also,  $\text{Mn}^{4+}$  reduced to  $\text{Mn}^{2+}$ , and not precipitated as a sulfide, becomes more soluble in water with high acidity (Atlas and Bartha, 1987).

The effect of pH on sulfate reduction is consequently not easily overcome. The fact that a mesocosm with an established population of  $10^8$  organisms/g dry mulch, high rates of sulfate reduction based on the decrease in sulfate, increase in sulfide, and increase in pH, and a non-limiting supply of carbon and inorganic nutrients, cannot maintain an adequate number of actively reducing microenvironments suggests that treating AMD with a pH below 4.5 using microbial sulfate reduction may be very difficult. It may be necessary, especially in cases with high concentrations of metals, to incorporate other forms of treatment along with the wetland, such as a limestone drain, or post wetland chemical addition, into the overall treatment design in order to raise the pH and precipitate metals. The cost of treatment would be higher than with a wetland alone, but still less than treatment plants or sole chemical addition. Another solution may be a multiple pond design, where the pH is raised in the first 1 or 2 ponds, allowing for the establishment of active populations of SRB in subsequent ponds. In the following ponds, metals may

then precipitate as metal monosulfides. This design was used by Allied Signal in a wetland constructed to treat AMD flowing from an inactive pyrite mine. The design consists of 8 consecutive ponds. Analysis of populations of SRB showed an average  $2 \log_{10}$  increase in the last pond over the first (Neal and Hendricks, 1990). Analysis of Fe concentrations showed that when inflow Fe concentrations equalled 326 mg/L, outflow from the first pond contained 302 mg/L Fe and outlet from the last pond had a Fe concentration of 23 mg/L (Neal and Hendricks, 1990). The results generated from the mesocosm pH experiment also suggest that there may be a "critical" pH between 4.5 and 3.5 below which sulfate reduction is inhibited under the given experimental conditions. An experiment testing a closer range of pH may reveal what that pH is.

Fe experiments. The purpose of these experiments was to determine the effect of high levels of Fe on microbial sulfate reduction and the precipitation of Fe and Mn. In order to keep the  $\text{Fe}^{2+}$  in solution within the synthesized AMD, the pH of the AMD had to be maintained below 3.5. The pH of the AMD increased from an average of 3.3 to an average of 5.7 in all Fe levels tested (Fig. 20). Data collected from portal samples shows that an increase in pH above 5.0 did not occur until after the AMD passed through the limestone layer at the base of the mesocosm (Fig. 29) at all Fe levels tested. This indicates, based on the data generated from the pH experiment, that the low pH necessary to keep Fe in solution is having a negative effect on the pH increase within the mesocosms, probably due to a negative effect



on microbial sulfate reduction, and consequently the formation of carbonate within the mesocosm mulch. Fe decreased 88%, 92% and 93% at inlet Fe concentrations of 88, 155 and 301 mg/L respectively. It is apparent that the increase in Fe concentration up to 300 mg/L does not effect the ability of the mesocosms to remove Fe. In fact, higher inflow Fe concentrations showed greater Fe removal. The pH and carbon experiments showed greater than 90% Fe removal with inflow Fe concentrations of only 20 mg/L. However, low pH did have a negative effect on the removal of Fe, with only a 70% reduction as AMD passed through the mesocosms. Considering the pH of the AMD in the Fe experiments, and the fact that the pH remains low throughout the volume of the substrate, it appears that higher levels of Fe positively effect the removal of Fe from AMD up to inflow concentrations of 300 mg/L. It may be that iron is chelating with the organic material present within the substrate (Brock and Madigan, 1991) Data collected from portal samples (Fig. 31) show that at inlet Fe concentrations of 85, 155 and 301 mg/L, Fe was decreased by 60, 70 and 81% respectively as the AMD flowed into the surface of the mesocosm. The higher the inflow Fe concentration, the higher the percent total Fe precipitated as the AMD entered the mesocosm. Considering the pH of the AMD, and that it was added as ferrous chloride to the AMD mixture, it is probable that the Fe was not precipitated as an iron hydroxide (Bohn et al., 1985). The surface water above the substrate had no dissolved oxygen present (Table 9, Appendix C). The ferrous iron in the AMD would be quickly precipitated as a sulfide upon contact under reducing

conditions. Any ferrous iron in the reservoir that may have oxidized to the ferric form could also be reduced to the ferrous form and then precipitated as an iron sulfide (Bohn et al., 1985). Also, under acidic oxygenated conditions,  $\text{Fe}^{2+}$  is stable and may be oxidized by *Thiobacillus* sp. or other acidophiles to  $\text{Fe}^{3+}$  and water (Atlas and Bartha, 1993). This is an oxygen consuming reaction as well, and may be partly responsible for the lack of oxygen in the surface water. The oxygen could be used as an electron acceptor and reduced to water as fast as it enters the mesocosm. This is only speculation, as no enumeration of  $\text{Fe}^{2+}$  oxidizers or measurement of oxygen in the AMD reservoir was done.

Lovley and Phillips (1986) report that iron reducing bacteria are able to mineralize organic material within anaerobic sediments while utilizing  $\text{Fe}^{3+}$  as an electron acceptor and reducing it to  $\text{Fe}^{2+}$ . The reduction of iron by iron reducing microorganism GS-15 was determined to be optimal at pH 6.7-7.0, and did not occur in pure culture at a pH of 5.0 (Lovley and Phillips, 1988). Under the conditions present in the wetland mesocosm, the reduction of  $\text{Fe}^{3+}$  by iron reducing bacteria does not seem likely. However, microenvironments with a buffered pH may have some activity of these organisms. Lovley and Phillips (1986) also report that microbially mediated iron reduction can inhibit sulfate reduction and methanogenesis in anaerobic sediments. In situations where wetlands built to treat AMD using microbial sulfate reduction fail, the presence of ferric iron reducers may be a contributing factor.

At Fe inflow concentrations of 85, 155 and 301 mg/L, Mn decreased 12, 21 and 43% respectively as AMD passed through the mesocosms. The low levels of manganese removal, when considering the data generated by the pH experiment suggest that the pH needed to keep the Fe dissolved in the AMD was probably inhibiting the precipitation of Mn as a sulfide, carbonate or dioxide (Bohn et al., 1985). The percent removal of Mn was greater at greater inflow Fe concentrations. Brodie (1990) suggests the lack of Fe-Mn coprecipitation as a reason for the failure of a wetland to remove metals in a situation with a higher Mn than Fe concentration. Stolp (1988) discusses the formation of deep-sea nodules of ferromanganese, containing between 20-40% manganese and up to 50% Fe. Stolp (1988) makes no mention of ferromanganese nodules forming in freshwater environments, but Atlas and Bartha (1993) do make mention of freshwater nodules forming in aerobic sediments without discussing their composition. The coprecipitation of Mn and Fe would easily explain why the highest Fe inflow concentration resulted in the highest Mn percent decrease.

Figure 22A, which presents the Fe treatment with 75 mg/L influent Fe shows that sulfate is removed from 1200 mg/L to less than 500 for the first 14 days of the experiment and then gradually increases. The replacement time within the mesocosms at a flow rate of 0.1L/h is 16 days. The first 2 weeks data also reflect the residual effects of the pH experiment conducted previously. By day 21 the population of SRB had already decreased from  $5.3 \times 10^7$  to  $1.1 \times 10^6$  (Table 4).

At an Fe treatment of 150 mg/L, a similar the sulfate removed was as high as 400 mg/L for the first 14 days, and then effluent sulfate concentrations increased. However, after 28 days, effluent sulfate concentrations again dropped to levels equal to that of the first 14 days. The increase in effluent sulfate concentration on days 21 and 28 in the treatment with 155 mg/L Fe is difficult to explain. It may be that after the residual affects of the previous experiment wore off, the mesocosm took a week or two to establish "equilibrium". Also, after 21 days the population of SRB had decreased from  $2.7 \times 10^7$  to  $5.2 \times 10^6$ .

Figure 22C shows that when influent Fe concentration averaged 301 mg/L, sulfate concentrations decreased 55%, a much greater removal than the 37 and 44% seen at Fe concentrations 85 and 155 mg/L. Brock and Madigan (1991) state that under anaerobic conditions, phototrophic sulfur bacteria can oxidize  $\text{HS}_2^-$  to sulfate. However, this normally occurs where sufficient light can penetrate the anaerobic zone. This process is unlikely within the mesocosms as they were kept in the dark to prevent the growth of algae on the surface of the water and substrate. Although the decrease in sulfate is greater in the treatment with 301 mg/L Fe, it is still not near the levels seen in the pH experiment (treatment pH 6.0) or in the carbon experiments. It seems likely that the decreased removal of sulfate is due to the effect of low pH on microbial sulfate reduction. But that the treatment with highest level of Fe resulted in a greater decrease in sulfate suggests that more sulfide is being precipitated as an iron sulfide, and thus having less of an impact on what sulfate

reducing activity is occurring. This is supported by the findings of Hammack and Edenborn (1991)

Viewing the results of these experiments in terms of an overall picture of AMD treatment using constructed wetlands may lead to some insights as far as treatment capabilities and wetland design. That the addition of an available carbon source to AMD increases sulfate reducing activity and metal precipitation is not new. The fact that this finding is not novel allows one to speculate as to how one might enhance the activity of SRB in the field. It again leads to the question of what is the perfect substrate? Is it mushroom compost, mulch, peat, hay, or a combination of several? Although lab studies have indicated that substrate choice may make a difference, data from wetlands constructed to treat AMD do not support these results. No one substrate choice seems to give an overall better treatment efficiency than another (Wieder, 1988). This is logical considering the differences in the design of the wetland, AMD characteristics, and numerous other environmental factors. Certainly adding an additional source of carbon such as lactate is not a viable alternative. The incorporation of plants is extremely important as far as a source of carbon for SRB, as the degradation of plant material by aerobic and anaerobic microorganisms results in the subsequent release of more simple forms of carbon that SRB can utilize as an electron source. Also, the release of organic acids and sugars from plant roots directly into the substrate would constitute another source of carbon for SRB.

That low pH negatively affects microbial sulfate reduction is also not new. However in terms of wetland design, it is a parameter that would not be considered lightly, as pH, acidity and alkalinity are major determining factors to whether a wetland constructed to treat AMD through the enhancement of microbial sulfate reduction succeeds or fails. A multiple pond wetland design is a viable alternative to including chemical addition, or some other source of secondary treatment, along with a single cell constructed wetland. In a multiple pond design, the first pond would exist mainly to raise the pH to a level acceptable for sulfate reducing activity. This could be done by incorporating a limestone bed within the pond and utilizing subsurface flow. The studies presented here indicate that the rise in pH may not need to be as high as 6.0, as the mesocosm treating AMD with a pH of 4.5 had a large ( $10^7$ ) and active population of SRB that generated enough alkalinity to raise the pH to above 8.0. The subsequent ponds could then contain active populations of SRB that would generate alkalinity to raise the pH, and hydrogen sulfide to precipitate metals as metal monosulfides.

The results generated from the pH and Fe studies give some promising news as far as the precipitation of manganese is concerned. Low pH is known to have a negative effect on the precipitation of Mn as an oxide. Trying to enhance its precipitation as a sulfide or an oxide could also be accomplished with a multiple pond design. If sulfate reduction can generate enough alkalinity in subsequent ponds to create alkaline conditions, than the precipitation of Mn would be favored. Finally,

that an increased concentration of Fe resulted in the precipitation of a higher percentage of Mn suggests that trying to remove it as something other than an oxide may be a better road to follow. Unfortunately it was not determined whether the Mn was precipitated as a coprecipitate with Fe, or as a sulfide.

The results generated from the experimentation presented verified and supported the general conclusions of others that constructed wetlands built to treat AMD through microbial sulfate reduction are a viable alternative to traditional chemical treatment. However, there are a number of environmental factors that contribute to the success or failure of wetlands constructed to treat AMD, and each individual situation should be considered as an entity in itself. The design that succeeds for treating AMD at one particular site may not be adequate for treating AMD at another site, even if the AMD characteristics are exactly alike.

## LITERATURE CITED

- Alexander, M. 1982. Most Probable Number Method for Microbial Populations. pp. 813-820. In: Page, A. L., Miller, R. H., and D. R. Keney (eds.). Methods of Soil Analyses, Pt. 2. Am. Soc. of Agr., Inc. and Soil Sci. of Amer., Inc. Madison, Wisconsin.
- Atlas, R. M. and R. Bartha. 1987. Microbial Ecology: Fundamentals and Applications. Second Addition. Stephen M. Mautner (ed). The Benjamin/Cummings Publishing Co., Inc. California. pp. 321-322; 569-579.
- Atlas, R. M. and R. Bartha. 1993. Microbial Ecology: Fundamentals and Applications. Third Addition. Edith B. Brady (ed). The Benjamin/Cummings Publishing Co., Inc. California. pp. 353-355; 404-405.
- Badziong, W. and R. K. Thauer. 1980. Vectorial Electron Transport in *Desulfovibrio vulgaris* (Marburg) Growing on Hydrogen plus Sulfate as the Sole Energy Sources. Arch. Microbiol. 125:167-174.
- Bohn, H. L., McNeal, B. L., and G. A. O'Connor. 1985. Soil Chemistry. 4th Edition. John Wiley and Sons, Inc. New York. pp. 283-285.
- Brock, T. D. and M. T. Madigan. 1988. Biology of Microorganisms. 5th Edition. Prentice Hall, Inc. New Jersey. pp. 577; 633-637.
- Brock, T. D. and M. T. Madigan. 1991. Biology of Microorganisms. 6th Edition. Prentice Hall, Inc. New Jersey. pp. 591-592; 631-632; 752-753.
- Brodie, G. A. 1991. Constructed Wetlands for Treating Acid Drainage at Tennessee Valley Authority Coal Facilities. Tennessee Valley Authority, Environmental Affairs, Chattanooga, TN.
- Brodie, G. A. 1991. Achieving Compliance with Staged, Aerobic, Constructed Wetlands to Treat Acid Drainage. pp. 151-174. In: Proceedings of the 8<sup>th</sup> Annual Meeting of The American Society of Surface Mining and Reclamation, Durango, CO.
- Brodie, G. A., Britt, C. R., Taylor, H., Tomaszewski, and D. Turner. 1990. Passive Anoxic Limestone Drains to Increase Effectiveness of Wetlands Acidic Drainage Treatment Systems. In: Proceedings, 12<sup>th</sup> National Association of Abandoned Mined Land Conference, Breckenridge, CO.



- Butlin, K. R. and M. E. Adams. 1947. Autotrophic Growth of Sulphate-reducing Bacteria. *Nature (Lond.)* 160:154-155.
- Calabrese, J. P., Sexstone, a. J., Bhumbra, D. K., Bissonnette, G. K. and J. C. Sencindiver. 1991. Application of Constructed Cattail Wetlands for the Removal of Iron from Acid Mine Drainage. pp. 559-575. In: *Proceedings of the Second International Conference on the Abatement of Acidic Drainage, Tome 3.* Montreal Canada.
- Desrochers, R. and Fredette, V. 1960. Etude D'une Population De Bacteries Reductrices Du Soufre'. *Can. J. Microbiol.*, 6:349-54.
- Duddleston, K. N., Fritz, E., Hendricks, A. C. and K. N. Roddenberry. 1992. Anoxic Cattail Wetland for Treatment of Water Associated with Coal Mining Activities. pp. 249-254. In: *Proceedings of the 9<sup>th</sup> Annual Meeting of The American Society of Surface Mining and Reclamation, Duluth, MN.*
- Dvorak, D. H., Hedin, R. S., McIntire, S. P., and H. M. Edenborn, 1991. Treatment of Metal-contaminated Water Using Bacterial Sulphate Reduction- Results from Pilot-scale Reactors. pp. 301-314. In: *Proceedings of The Second International Conference on the Abatement of Acidic Drainage, Tome 2.* Montreal, Canada.
- Gussmann, H. W., Everett, D. J. and J. du Plessis. 1991. The Treatment of Underground Mine Waters for the Removal of Calcium and Sulphates by a Gyp-Cix Process. pp. 377-398. In: *Proceedings of The Second International Conference on the Abatement of Acidic Drainage, Tome 2.* Montreal, Canada.
- Hammack, R. W. and H. M. Edenborn. 1991. The Removal of Nickel from Mine Waters Using Bacterial Sulfate Reduction. pp. 97-107. In: *Proceedings of the the 8th Annual Meeting of The American Society of Surface Mining and Reclamation, Durango, CO.*
- Hammer, D. A. and R. K. Bastian. 1989. Wetlands as Ecosystems: Natural Water Purifiers? pp. 5-20 In: *Constructed Wetlands for Waste Water Treatment.* D. A. Hammer (ed.). Lewis Publishers, Chelsea, MI.

- Hart, W. M. and A. Stiller. 1991. Application of Phosphate Refuse to Coal Refuse for Amelioration of Acid Mine Drainage. pp. 173-190. In: Proceedings of the Second International Conference on the Abatement of Acidic Drainage, Tome 2. Montreal, Canada.
- Hedin, R. S., Hyman, D. M. and R. W. Hammack. 1988. Implications of Sulfate Reduction and Pyrite Formation Processes for Water Quality in a Constructed Wetland; Preliminary Observations. pp. 382-388. In: Mine Drainage and Surface Mine Reclamation, Vol. 1. Information Circular No. 9183. U.S. Bureau of Mines. Pittsburgh, PA.
- Hedin, R. S. and R. W. Nairn. 1992. Designing and Sizing Passive Mine Drainage Treatment Systems. In: Proceedings, 13<sup>th</sup> West Virginia Surface Mine Drainage Task Force Symposium, Morgantown, WV.
- Hendricks, A. C. 1991. The use of an Artificial Wetland to Treat Acid Mine Drainage. pp. 549-558. In: Proceedings of the Second International Conference on the Abatement of Acidic Drainage, Tome 3. Montreal, Canada.
- Hendricks, A. C. and K. N. Duddleston. 1992. Anoxic Cattail Wetland Development for the Treatment of Acid Mine Drainage at Pine Branch, Norton, Virginia. pp. 28-36. In: 1992 Powell River Project Symposium and Progress Report. Wise, VA.
- Henrot, J. and R. K. Wieder. 1990. Processes of Fe and Mn Retention in Laboratory Peat Microcosms Subjected to Acid Mine Drainage. *Journal of Environmental Quality*. 19:312-320.
- Herlihy, A. T., Mills, A. L., Hornberger, G. M., and A. E. Bruckner, 1987. The Importance of Sediment Sulfate Reduction to the Sulfate Budget of an Impoundment Receiving Acid Mine Drainage. *Water Resources Research*, 23:287-292.
- Hutchins, S. R., Davidson, M. S., Brierly, J. A., and C. L. Brierly. 1986. Microorganisms in Reclamation of Metals. *Ann. Rev. Microbiol.* 40:311-336.
- Ishimoto, M., Koyama, J., and Y. Nagai. 1954. pp. 86-96. In: Postgate, 1979. *The Sulphate-reducing Bacteria*. Cambridge University Press, Cambridge.

- Kimata, M., Kadota, H. and Y. Hata. 1955. pp. 86-96. In: Postgate, 1979. *The Sulphate-reducing Bacteria*. Cambridge University Press, Cambridge.
- Kleinmann, R. L. P. 1985. Treatment of Acid Mine Water by Wetlands. pp. 48-52. In: *Control of Acid Mine Drainage*. Information Circular No. 9027. U. S. Bureau of Mines, Pittsburgh, PA.
- Kleinmann, R. L. P., Brooks, R. P., Huntsman, B. E. and B. G. Pesavento. 1986. Constructing Wetlands for the Treatment of Mine Water. Short Course Notes. Presented at the National Symposium on Surface Mining, Hydrology, Sedimentology, and Reclamation. Lexington, KY. 53 pp.
- Lovett, R. J., Ziemkiewicz, P. F., and T. E. Rymer. 1991. Trapzene for Treatment of Acid Mine Drainage. pp. 35-46. In: *Proceedings of the Second International Conference on the Abatement of Acidic Drainage*, Tome 2. Montreal, Canada.
- Lovley, D. R. and E. J. P. Phillips. 1986. Organic Matter Mineralization with Reduction of Ferric Iron in Anaerobic Sediments. *Appl. Environ. Microbiol.* 51:683-689.
- Lovley, D. R. and E. J. P. Phillips. 1987. Competitive Mechanisms for Inhibition of Sulfate Reduction and Methane Production in the Zone of Ferric Iron Reduction in Sediments. *Appl. Environ. Microbiol.* 53:2636-2641.
- Lovley, D. R. and E. J. P. Phillips. 1988. Novel Mode of Microbial Energy Metabolism: Organic Carbon Oxidation Coupled to Dissimilatory Reduction of Iron or Manganese. *Appl. Environ. Microbiol.* 54:1472-1480.
- Lovley, D. R., Phillips, E. J. P., Gorby, Y. A. and E. R. Landa. 1991. Microbial Reduction of Uranium. *Nature* 350:413-416.
- Lundgren, D. G., Vestal, J. R. and F. R. Tabita. 1972. The Microbiology of Mine Drainage Pollution. pp. 69-88. In: *Water Pollution Microbiology*. Ralph Mitchell (ed.). Wiley-Interscience, N.Y.
- McIntire, P. E. and H. M. Edenborn. 1990. The Use of Bacterial Sulfate Reduction in the Treatment of Drainage from Coal Mines. pp. 409-415. In: *Proceedings of the 1990 Mining and Reclamation Conference and Exhibition*. Vol. II.

- Methods for Chemical Analyses of Water and Wastes. 1985. U. S. EPA Environmental Monitoring and Support Lab. Cincinnati, OH. pp. 151-155; 467-468; 476-477.
- Neal, J. L. and A. C. Hendricks. 1990. Sulfate Reducing Microbes and Ferrous Sulfide Formation in Huey Pit Wetlands. Allied-signal Research Report.
- Paul, E. A. and F. E. Clark. 1989. Soil Microbiology and Biochemistry. Academic Press, Inc. San Diego, CA. p. 257
- Postgate, J. R. 1979. The Sulphate-reducing Bacteria. Cambridge University Press, Cambridge. pp. 1-40; 86-96.
- Renton, J. J., Stiller, A. H., and T. E. Rymer. 1988. The Use of Phosphate Materials as Ameliorants for Acid Mine Drainage. pp. 67-75. In: Mine Drainage and Surface Mine Reclamation, Vol. 1. Information Circular No. 9183. U.S. Bureau of Mines. Pittsburgh, PA.
- Rheinheimer, G. 1991. Aquatic Microbiology. John Wiley and Sons, Inc. New York. pp. 201-205; 286.
- Silverman, M. P. and D. G. Lundgren. 1959. Studies on the Chemoorganotrophic Iron Bacteria *Ferrobacillus ferrooxidans*. Journal of Bacteriology. 77:642.
- Singer, P. C. and W. Stumm. 1970. Acidic Mine Drainage: The Rate Determining Step. Science 167: 1121-1123.
- Skousen, J. G. and B. B. Faulkner. 1992. Preliminary Results of Anoxic Limestone Drains in West Virginia. In: Proceedings of the 13<sup>th</sup> West Virginia Surface Mine Drainage Task Force Symposium, Morgantown, WV.
- Stark, L. R., Kolbash, R. L., Webster, H. J., Stevens, S. E., Dionis, K. A. Jr., and E. R. Murphy. 1988. The Simco #4 Wetland: Biological Patterns and Performance of a Wetland Receiving Mine Drainage. pp. 332-344. In: Mine Drainage and Surface Mine Reclamation, Vol. 1. Information Circular No. 9183. U.S. Bureau of Mines, Pittsburgh, PA.
- Starkey, R. L. 1966. Oxidation and Reduction of Sulfur Compounds in Soils. Soil Science. 101:297-306.

- Stille, W. and H. G. Truper. 1984. Adenylylsulfate Reductase in Some New Sulfate-reducing Bacteria. *Arch. Microbiol.* 137:145-150.
- Stillings, L. L., Gryta, J. J. and T. A. Ronning. 1988. Iron and Manganese Removal in a *Typha*-dominated Wetland During Ten Months Following its Construction. pp. 317-324. In: *Mine Drainage and Surface Mine Reclamation, Vol. 1. Information Circular No, 9183.* U.S. Bureau of Mines, Pittsburgh, PA.
- Stolp, H. 1988. *Microbial Ecology: Organisms, Habitats, Activities.* Cambridge University Press. Cambridge. 308 pp.
- Stumm, W. and J. J. Morgan. 1970. *Aquatic Chemistry.* Wiley-Interscience.
- Trudinger, P. A., and N. Williams. 1982. Stratified Sulfide Deposition in Modern and Ancient Environments. pp. 177-198. In: *Mineral Deposits and the Evolution of the Biosphere.* Holland and Schidlowski (eds.). Springer-Verlag, Heidelberg.
- Truper, H. G., and N. Pfennig. 1966. Sulphur Metabolism in Thiorhodaceae. III. Storage and Turnover of Thiosulphate Sulphur in *Thiocapsa floridana* and *Chromatium* species. *Antonie Leeuwenhoek J. Microbiol. Serol.* 32:261-276.
- Tuttle, J. H., Dugan, P. R., MacMillan, C. B., and C. I. Randles. 1968. Microbial Dissimilatory Sulfur Cycle in Acid Mine Water. *J. Bact.* 97: 594-602.
- Walsh, F. and R. Mitchell. 1972. A pH-Dependent Succession of Iron Bacteria. *Environmental Science and Technology, Vol. 6, No. 9.* pp. 809-812.
- Widdel, Friedrich. 1988. Microbiology and Ecology of Sulfate- and Sulfur-reducing Bacteria. pp. 469-585. In: *Biology of Anaerobic Microorganisms.* A. J. B. Zehnder (ed). John Wiley and Sons. New York.
- Wieder, R. K. 1988. Determining the Capacity for Metal Retention in Man-made Wetlands Constructed for Treatment of Coal Mine Drainage. pp. 375-381. In: *Mine Drainage and Surface Mine Reclamation, Vol. 1. Information Circular No. 9183.* U.S. Bureau of Mines, Pittsburgh, PA.

## APPENDIX A

Two Growth curves were performed on pure cultures of sulfate reducing bacteria isolated from mulch samples from Pine Branch Wetland, Norton VA. Mother cultures were prepared by injecting 1 ml of pure culture into side arm flasks for use in a Klett Somerson photoelectric colorimeter containing 300 ml of Postgate's (1979) medium C. Flasks were prepared by autoclaving media and capping with a stopper immediately upon removal from the autoclave to prevent excess oxygen from dissolving in the media. Mother cultures were allowed to grow to between 18-22 Klett units, and 2 ml of mother culture were then inoculated into each of 5 flasks using a syringe and needle. Klett readings were taken at 0, 5, 10 and 18 hours and then every 2 hours up to 30 hours. An uninoculated media blank was used as a negative control. Figure 34 shows the combined results of the 2 growth curves prepared. Experimentation was suspended following the second growth curves when it was determined that cultures were contaminated. Attempts to reisolate a pure culture were unsuccessful.

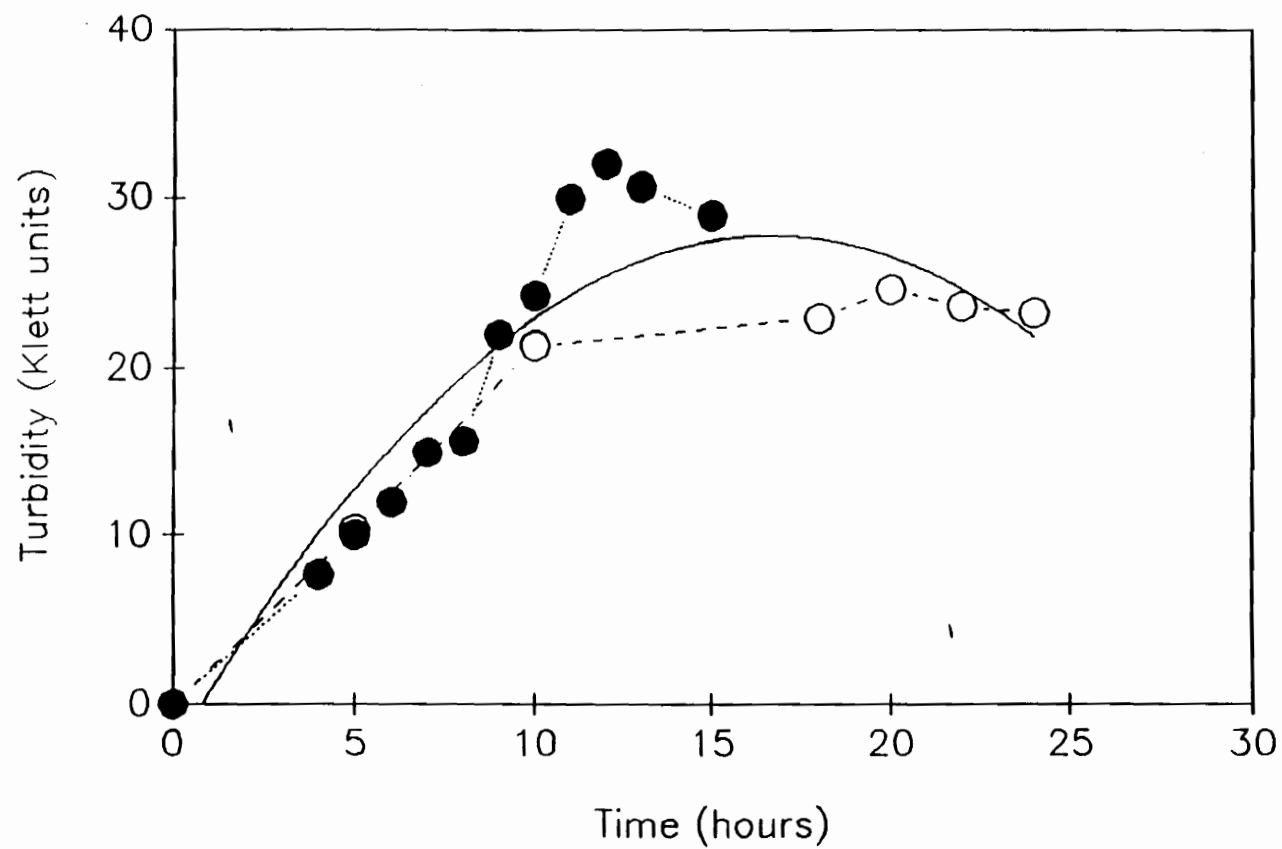


Figure 34. Growth curve of a pure culture of SRB.

## APPENDIX B

In order to determine the ratio of sulfate to iron necessary to optimize sulfate reduction and the removal of sulfate and iron, static soil cultures were prepared by placing 100 grams pine bark mulch or mushroom compost into 8 quart mason jars with the addition of a known volume of AMD synthesized as stated previously without the addition of lactate. AMD treatments contained Fe concentrations of 20 mg/L and sulfate concentrations in mg/L of (A) 200, (B) 150, (C) 100, (D) 50, and (E) 0. Treatments F and G contained 0 Fe and 100 and 0 mg/L sulfate respectively. Sodium Molybdate was added to negative controls as an inhibitor of sulfate reduction. Five hundred ml media was removed to provide 500 cc headspace. Serum stoppers were inserted into the jar lids to allow for collection of headspace gas samples for analysis of hydrogen sulfide gas using gas chromatography. In order to maintain a constant headspace of 500 cc while removing gas samples, a carbon dioxide impermeable membrane was suspended from the lid through a serum stopper with a piece of glass and rubber tubing inside. The tubing could be opened to the atmosphere and allowed to fill with air when samples were collected so that no change in pressure occurred. Analysis of dissolved sulfide, sulfate and Fe and enumerations of SRB were performed. Table 5 shows the water chemical analysis at 0 and 21 days following incubation at 30°C. No hydrogen sulfide gas was detected throughout the course of the experiments using gas chromatography, although the odor of sulfide gas was present.



TABLE 5: Concentration (mg/L) of  $\text{SO}_4$ ,  $\text{S}^{=}$  and Fe at time 0 and 21 days, static culture Run #3.

mg/L*	Time (days)	Treatment						
		A	B	C	D	E	F	G
$\text{SO}_4$	0	236.2	143.6	112.3	51.1	0	0	112.8
	21	0	0	0	0	0	0	0
	21M <sup>^</sup>	262.2	225.2	188.27	142.1	0	-	-
$\text{S}^{=}$	0	2.1	0.9	1.4	0.5	0.7	1.1	2.4
	21	17.8	58.7	48.5	42.1	44.3	43.2	46.4
	21M	33.6	17.6	38.4	38.4	6.4	-	-
Fe	0	27.8	45.5	24.4	21.0	34.4	0.2	0.1
	21	19.0	24.7	27.0	29.7	29.0	12.0	14.0
	21M	20.0	24.0	24.0	22.0	30.0	-	-

\* n=3 except time 0, where n=2, and with molybdate and treatments F and G, where n=1

<sup>^</sup> 21M = samples with the addition of molybdate after 21 days. Values for time 0 with the addition of molybdate are the same as those without molybdate

In order to determine if a minimum concentration of sulfate was necessary to precipitate 100% of a given Fe concentration, static soil cultures were prepared with mulch substrate. Cultures were prepared as described above with AMD containing 100 mg/L Fe and sulfate concentrations of (A) 200, (B) 100 and (C) 0 mg/L. Cultures were incubated for 60 days at 30°C. Tables 6 and 7 present the Fe and sulfate, and sulfide analysis at 0 and 60 and 0, 35 and 60 days respectively. Table 8 presents the enumeration of SRB at 0 and 60 days.

TABLE 8. Enumeration of SRB at Time 0 and 60 Days. Standard Deviations Represent 99% Confidence, n=3.

Treatment	Day			
	SRB/g dry substrate (x 10 <sup>4</sup> )			
	0		60	
A	0.00056 ± 0.00016		124.80 ± 195.00	
AM* <sup>a</sup>	0.0022		0.0011	
AL* <sup>b</sup>	0.0004		0.67	
B	0.00156 ± 0.00056		18.73 ± 16.33	
BM*	0.0059		1.30	
BL*	0.000096		29.00	
C	0.00296 ± 0.0036		20.33 ± 7.57	
CM*	0.00044		0.077	
CL*	0.00021		45.00	

\*n=1

<sup>a</sup> M= Molybdate Added

<sup>b</sup> L= Lactate Added

TABLE 6. Iron and Sulfate Concentrations at Time 0 and 60 Days. Standard Deviations represent 99% Confidence. n=3

Treatment	mg/L			
	Fe		SO <sub>4</sub>	
	Day		Day	
	0	60	0	60
A	63.77 ± 6.11	12.70 ± 6.35	237.00 ± 12.72	82.33 ± 71.44
AM* <sup>a</sup>	45.90	14.80	255.00	136.00
AL* <sup>b</sup>	50.70	13.90	246.00	0.00
B	65.80 ± 7.96	11.2 ± 5.86	152.00 ± 5.20	90.67 ± 80.34
BM*	48.3	17.10	155.00	136.00
BL*	67.50	16.21	146.00	0.00
C	45.17 ± 12.22	12.07 ± 1.17	0.00 ± 0.00	0.00 ± 0.00
CM*	53.50	16.30	0.00	0.00
CL*	62.30	18.01	0.00	0.00

\*n=1

<sup>a</sup> M= Molybdate Added<sup>b</sup> L= Lactate Added

TABLE 7. Dissolved Sulfide Concentrations at Time 0, 35, and 60 Days. Standard Deviations Represent 99% Confidence, n=3

Treatment	S <sup>2-</sup> (mg/L)		
	Day		
	0	35	60
A	0.00 ± 0.00	46.67 ± 6.11	1.02 ± 0.50
AM* <sup>a</sup>	13.00	28.00	0.40
AL* <sup>b</sup>	21.00	48.00	2.13
B	11.33 ± 10.60	41.00 ± 2.65	1.20 ± 0.00
BM*	0.00	47.00	0.93
BL*	0.00	40.00	0.93
C	22.00 ± 22.52	44.33 ± 4.62	0.22 ± 0.20
CM*	0.00	43.00	0.80
CL*	21.00	40.00	0.80

\*n=1

<sup>a</sup> M= Molybdate Added<sup>b</sup> L= Lactate Added

## APPENDIX C

Table 9 shows the oxygen concentration in mg/L, determined using the micro-Winkler method (Methods for Chemical Analysis of Water and Wastes, 1985). Oxygen was measured at depths of 4, 12 and 20 within wetland mesocosms when AMD was syntesized with lactate, and at 0 and 3 weeks after lactate was removed from AMD.

TABLE: 9. Oxygen concentration (mg/L) in AMD with and without the addition of lactate.

Sample	Oxygen (mg/L)			
	depth (inches)	4	12	20
AMD + Lactate <sup>b</sup>		0	0	0
AMD - Lactate <sup>a</sup>				
(day 0)		0	0	0
AMD - Lactate <sup>a</sup>				
(day 21)		1.29	0	0

<sup>a</sup> mean of 6 replicates

<sup>b</sup> 1 replicate

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Duddleston, K. N., J. L. Neal, and A. C. Hendricks. 1992. The Effect of Carbon on the Reduction of Sulfate in Simulated Wetland Mesocosms Built to Treat Acid Mine Drainage and the Subsequent Precipitation of Fe and Mn. Annual meeting of the Virginia Branch of The American Society for Microbiology. Williamsburg, VA.

**Presentations with Published Abstracts**

Duddleston, K., A. C. Hendricks and J. L. Neal. 1992. "An Evaluation of a Wetland Constructed to Treat Mine Seepage in Southwestern Virginia. In: Abstracts of the 1992 Virginia Water Resources Conference. Richmond, VA.

Duddleston, K. N., J. L. Neal and A. C. Hendricks. 1992. An Assessment of the Role of Sulfate Reducing Bacteria in Anoxic Wetlands Constructed to Treat Acid Mine Drainage. In: Abstracts of the 92nd Annual General Meeting of The American Society for Microbiology. New Orleans, LA.

Duddleston, K. N., A. C. Hendricks and J. L. Neal. 1993. The Effect of Carbon and pH on the Reduction of Sulfate and the Precipitation of Fe and Mn in Simulated Wetland Mesocosms Built to Enhance Microbial Sulfate Reduction. In: Abstracts of the 1993 Virginia Water Resources Conference. Richmond, VA.

Duddleston, K. N., J. L. Neal and A. C. Hendricks. 1993. The Effect of pH and Carbon of the Reduction of Sulfate by Sulfate Reducing Bacteria and the Subsequent Precipitation of Fe and Mn. In: Abstracts of the 93rd Annual Meeting of The American Society for Microbiology. Atlanta, GA.

Duddleston, K. N., A. C. Hendricks and J. L. Neal. 1993. The Effect of Carbon and pH on the Reduction of Sulfate by Sulfate Reducing bacteria and the Subsequent Precipitation of Fe and Mn in Simulated Wetland Mesocosms. In: Abstracts of the 10th Annual Meeting of The American Society of Surface Mining and Reclamation. Spokane, WA.

#### **Presentations with Published Proceedings**

Duddleston, K. N., E. Fritz, A. C. Hendricks and K. Roddenberry. 1992. Anoxic Cattail Wetland for Treatment of Water Associated with Coal Mining Activities. In: Proceedings of the 9th Annual Meeting of The American Society of Surface Mining and Reclamation. Duluth, Mn.

Hendricks, A. and K. Duddleston. 1991. Wetland Development for the Treatment of Water Associated with Coal Mining Activities. In: Proceedings of the Powell River Project Symposium and Progress Reports. Wise, VA.

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