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Development of Innovative Wastewater Treatment Technologies for Acid Mine Drainage and Municipal Wastewater Management in Energy Producing Regions

Dongyang Deng

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**Development of Innovative Wastewater Treatment Technologies for Acid Mine
Drainage and Municipal Wastewater Management in Energy Producing Regions**

Dongyang Deng

**Dissertation submitted
to the College of Engineering and Mineral Resources
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in
Civil and Environmental Engineering**

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**Keywords: Acid Mine Drainage, Municipal Wastewater, Sulfidogenic Treatment,
Kinetics, Microbial Ecology, Sludge Recycling, Biogeochemical Transformation
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ABSTRACT

Development of Innovative Wastewater Treatment Technologies for Acid Mine Drainage and Municipal Wastewater Management in Energy Producing Regions

By Dongyang Deng

Acid mine drainage (AMD) and municipal wastewater (MWW) are two major pollution sources in headwaters of Appalachia and energy producing regions worldwide. Incorporating the prevalent chemistry of the two wastes in designing treatment technologies for concurrent management of the wastes can provide multi-faceted benefits. First, alkalinity in MWW can raise the pH of AMD upon mixing, and promote chemical precipitation of metal hydroxides and carbonates. Second, low solubility of phosphate with multivalent metals (e.g., Fe and Al) can be an effective mechanism for recovering phosphate from MWW. Third, sulfate ions from AMD can serve as an electron acceptor for oxidation of organics from MWW. This can potentially eliminate the need of aeration for the biological treatment of MWW (e.g., activated sludge processes), which is the most energy-intensive operation at wastewater treatment plants. Additional benefits included significantly reduced greenhouse gas emission from the wastewater treatment and biological sludge production.

This research focuses on developing an innovative wastewater treatment method using iron as a green agent to render the abovementioned benefits. The research consists of two parts that reflect the development of the treatment concept: co-treatment of AMD and MWW, and iron-dosed wastewater treatment. The co-treatment method involves a two-staged treatment of field-collected AMD and MWW samples, which includes aerobic mixing of the two wastes followed by a sulfidogenic treatment of the mixture solution in batch-fed experiments. This part of the research focuses on examining treatment efficiency of a wide range of pollutants originating from the two wastes. The iron-dosed wastewater treatment involves anaerobic bioreactors for continuous treatment of MWW with an option of sludge recycle. Overall, the research activities are divided into four phases: 1) evaluation of technical feasibility of co-treatment of AMD and MWW using field-collected samples, 2) investigation of relevant factors on sulfidogenic wastewater treatment kinetics and its relationship with microbial ecology, 3) developing a anaerobic technology for continuous MWW treatment with iron dosing and 4) elucidating reaction biotic and abiotic reaction mechanisms at different stages of the continuous treatment process. Corroborated by phylogenetic tree, kinetic modeling, scanning electron microscope, X-ray photoelectron spectroscopy and X-ray diffraction analysis, these bio-chemical mechanisms were studied and used to optimize the treatment process.

Results indicate that AMD and MWW passive co-treatment is a viable cost-effective approach to improve water quality and can achieve multiple treatment objectives concurrently with promising treatment efficiency. Potential toxicity of iron and other metals can be avoided and favorable sulfidogenic treatment conditions can be achieved by proper mixing of the two wastes. Sulfidogenic treatment kinetics is closely related to microbial ecology in the bioreactors and can be optimized by chemical oxygen demand (COD)/sulfate ratio of the influent to the bioreactors.

Long-term operation of continuous treatment of MWW with iron dosing and sludge recycling under a range of COD/sulfate and Fe/S ratios was successfully demonstrated. Biogeochemical transformations of the two main elements, Fe and S, in the treatment process were examined using spectroscopic and phylogenetic analyses. The analyses included 1) mass balances of Fe, S in the treatment process, 2) qualitative characterization of the chemical and biological sludge materials, 3) estimations of mass fluxes of chemical and biological materials, and 4) identification of microbial species responsible for biological transformations of Fe and S at different stages of the treatment process. This innovative treatment process was found to exhibit long-term operation stability and consistent treatment performance with COD/sulfate and Fe/S as the primary two factors affecting the overall treatment performance.

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LIST OF SYMBOLS / NOMENCLATURE

1. **AMD** – Acid Mine Drainage
2. **MWW** – Municipal Wastewater
3. **COD** – Chemical Oxygen Demand
4. **TSS** – Total Suspended Solids
5. **TDS** – Total Dissolved Solids
6. **VSS** – Volatile Suspended Solids
7. **NVSS** – Non-Volatile Suspended Solids
8. **SRB** – Sulfate-Reducing Bacteria
9. **IRB** – Iron-Reducing Bacteria
10. **BOD** – Biochemical Oxygen Demand
11. **HRT** – Hydraulic Retention Time

CHAPTER 1: INTRODUCTION AND RESEARCH OBJECTIVES

1.1 INTRODUCTION/BACKGROUND INFORMATION

1.1.1 Acid mine drainage

Acid mine drainage (AMD) from active and abandoned mines represents a prevalent environmental pollution source and significant environmental liability in mining regions worldwide. AMD originating from oxidation of iron sulfide (e.g., pyrite) in coal and mine tailings can generate water containing high concentrations of metals (iron, aluminum and manganese and other toxic metals), metalloids, sulfate, and acidity. There are a number of major environmental and ecological problems caused by acid mine drainage: it disrupts growth and reproduction of aquatic plants and animals; diminishes valued recreational fish species; degrades outdoor recreation and tourism; contaminates surface and groundwater drinking supplies; and causes acid corrosion of infrastructure like wastewater pipes (Gray 1997). The northern Appalachian coal fields (bituminous or soft coal) extend from northwestern Pennsylvania, south of the New York state line and west of the Susquehanna River, through western Pennsylvania and southeastern Ohio, and through most of West Virginia and into western Maryland and southwestern Virginia, eastern Kentucky, and northeastern Tennessee. About 20,000 km of streams and rivers in USA are contaminated by AMD and over 90% of AMD affected streams originate from abandoned mines (Pierzynski et al. 2005). Over 95% of the acid problem is located in western Pennsylvania, almost all of West Virginia (WV), southwestern Virginia, and far western Maryland (EPA 2008). Runoff water, polluted by acid, iron, sulfur and aluminum, has often drained away from the mines and discharged into streams.

Various control measures may be performed at different stages in the mine water generation process to mitigate AMD impacts (Sengupta 1993). Active treatment utilizing acid neutralizing agents (e.g., hydrated lime, caustic soda, soda ash) to raise pH and remove metals through chemical precipitation is one of the most widely used treatment methods (D. B. Johnson and Hallberg 2005). Although active chemical treatment can effectively remediate AMD, high operation costs and disposal of large amounts of the produced sludge remain as a challenge (Chang et al. 2000). Given the sulfate prevalence in AMD, a common feature of the passive treatment is exploitation of sulfate-reducing bacteria (SRB) to facilitate sulfate reduction to (bi)sulfide, alkalinity generation, and subsequent metal sulfide precipitation (Lewis 2010; Waybrant et al. 1998). Passive treatment of AMD typically involves microbiological activities in systems such as lagoons, wetlands, and bioreactors to achieve treatment objectives (Neculita et al. 2007). A wide variety of organic sources have been examined for the biotic sulfate reduction (Benner et al. 2002; Dvorak et al. 1992; K. L. Johnson and Younger 2006; Jong and Parry 2003; W. Strosnider et al. 2011a; Tuttle et al. 1969). Waybrant et al. (1998) used single organic sources (e.g., sheep manure, sawdust, leaf mulch, and wood chips) for sulfate removal and found higher reduction rates with addition of sewage sludge than without the sludge. (W. Strosnider et al. 2011a; W. H. Strosnider et al. 2011) suggested that the diverse electron donors in the municipal wastewater (MWW) were suitable for supporting the growth of microbial communities and sulfate reduction.

1.1.2 Municipal wastewater

Municipal wastewater (MWW) originates from a combination of domestic, industrial, commercial or agricultural activities, surface runoff or stormwater, and from sewer inflow or infiltration (Hammer 1989). There are numerous processes that can be used to treat wastewaters depending on the type and extent of contamination (Rice et al. 2012). Treated wastewater is discharged into receiving water via an effluent pipe. Wastewaters generated in areas without access to centralized sewer systems rely on on-site wastewater systems. These typically comprise a septic tank, drain field, and optionally additional on-site treatment units.

MWW treatment is required to protect receiving water bodies from eutrophication and subsequent environmental degradation. Conventional MWW treatment generally consumes considerable economic, energy and material resources. Mechanical aeration, sludge scraping, clarifier skimming, sludge and effluent pumping, ultraviolet disinfection and other conventional MWW treatment practices require substantial energy, often supplied by non-renewable resources (Mannino et al. 2008; Metcalf et al. 2013; Muga and Mihelcic 2008).

Generally, MWW treatment needs to reduce the suspended solids, phosphorus, nitrogen and biochemical oxygen demand concentrations to certain limits. Suspended solids can be removed by biodegradation, flocculation, settling or filtration (Metcalf et al. 2013). Excess phosphorous and suspended solids are commonly removed from MWW by dosing of alum or ferric iron salt (Omoike and Vanloon 1999). However, aluminum or ferric iron salt dosing can be relatively expensive and coagulant/flocculant demand has increased over recent decades (Jarvis 2000; Ouellette 1996).

1.1.3 Co-treatment of acid mine drainage and municipal wastewater

Conventional active MWW and AMD treatment methods are commonly energy-intensive with higher operational and maintenance costs when compared to passive treatment approaches (Mannino et al. 2008; Younger et al. 2002) while passive methods generally require larger land areas and higher construction costs. Introducing AMD in MWW treatment can generate advantages over the traditional MWW treatment methods. First, alkalinity in MWW can raise the pH of AMD upon mixing, which promotes chemical precipitation, such as metal hydroxides and carbonates, and associated adsorption/co-precipitation mechanisms. Second, low solubility of phosphate with multivalent metals provides an effective mechanism for recovering phosphate from MWW. Specifically, dissolved Al and Fe in AMD can react with phosphate and form chemical precipitates that are readily removed by gravity (Bamforth et al. 2006; Fletcher and Beckett 1987; Omoike and Vanloon 1999). Alternatively, pre-existing AMD floc containing iron and aluminum can be an effective alternative reagent for removing soluble phosphorus to the conventional coagulant/flocculant sources (Menezes et al. 2010). Third, sulfate ions from AMD can serve as an electron acceptor for organics oxidation and removal from MWW. Sulfate reducing bacteria (SRB) can utilize the organic content of MWW in sulfate reduction to (bi)sulfide. The reaction generates alkalinity and promotes precipitation of metal sulfides. Studies have documented the effectiveness of sewage sludge as an SRB medium (Harris and Ragusa 2000; Waybrant et al. 1998). AMD containing a mixture of iron and aluminum hydroxide precipitates have

been reported to be a suitable medium for the adsorption of dissolved orthophosphate from solution. And it was demonstrated that Fe in AMD could promote phosphorous removal (Wei et al. 2008). Sulfidogenic treatment under an anaerobic condition can potentially eliminate the need for aeration, an energy-intensive operation required for the biological treatment of MWW such as activated sludge processes. These potential benefits represent an incentive and opportunities for developing innovative, energy-efficient treatment technologies for the two waste streams in mining regions.

1.2 LITERATURE REVIEW

1.2.1 Acid mine drainage formation and treatment technologies

AMD forms when sulfide minerals (mainly iron pyrite FeS_2 and other sulfidic minerals like CuS , ZnS , and PbS) are exposed to oxidizing conditions in coal and metal mining processes, highway construction and other large-scale excavation workings. Releases of AMD have low pH, high specific conductivity, high concentrations of iron, aluminum, and manganese, and relatively low concentrations of toxic heavy metals for coal mines in United States (Blowes et al. 2003; Skousen et al. 2000). Acidity in AMD is composed of metals acidity (Fe, Mn, Al, etc.) and proton (H^+) acidity. The oxidation of iron sulfides and conversion to acidity occur through several reactions and can be represented by a combination of reactions presented in Equation 1 (Akcil and Koldas 2006).

AMD may form in underground water of deep mines, when the mining operation is closed and abandoned. This could lead to discharge of contaminated groundwater. Acidic sulfur rich water may also form in mine tailings, where the mine drainage formed would be more concentrated and thus the contamination would be more severe (R. L. P. Kleinmann et al. 1981).

AMD causes environmental pollution worldwide, poses significant hazardous risks to aquatic life in streams and rivers and is a long-term pollution source. Mitigation techniques have been developed over the past 40 years (Skousen et al. 2000). They have been generally categorized into source control and migration control (Evangelou 1995; D. B. Johnson and Hallberg 2005; M. G. Li et al. 1997; Mehling et al. 1997; Swanson et al. 1997). Source control is a technique focused on prevention other than treatment. However, given the practical considerations, treatment techniques of AMD are much more widely utilized. Figure 1 presents various approaches that prevent and minimize the generation of AMD and treatment techniques. The remediation processes have been divided into active and passive techniques (Coulton et al. 2003; D. B. Johnson and Hallberg 2005; R. Kleinmann et al. 1998). Active methods usually refer to those requiring continuous inputs of resources to sustain the process, while passive methods indicate relatively little resource input into the operation.

Figure 1 Source control and migration control techniques for preventing and remediating AMD

1.2.2 Active methods of acid mine drainage treatment

The most widely-adopted technique of active treatment is aeration and addition of neutralizing chemicals (Coulton et al. 2003). Various neutralizing reagents have been used, which include lime (calcium oxide), calcium carbonate, slaked lime (calcium hydroxide), sodium carbonate, sodium hydroxide, and magnesium oxide and hydroxide. Addition of an alkaline material to AMD will raise pH, increase the rate of chemical oxidation of ferrous iron and lead to precipitation and settling of metal hydroxides and carbonates.

Off-line sulfidogenic bioreactors refer to the biogenic production of hydrogen sulfide to generate alkalinity and to remove metals as metal sulfides (e.g., compost bioreactors and permeable reactive barriers). However, off-line sulfidogenic bioreactors are constructed and operated to optimize production of hydrogen sulfide. Since the SRB used in these reactors are sensitive to even moderate acidity, the systems have to be engineered to protect the microorganisms from direct exposure to the inflowing AMD. Off-line sulfidogenic bioreactors systems have three potential advantages over passive biological remediation (Boonstra et al. 1999; D. Johnson 2000): 1) their performance is more predictable and readily controlled; 2) they allow heavy metals, such as iron, copper and zinc, present in AMD to be selectively recovered and reused; and 3) concentrations of sulfate may be significantly lowered. Overall, active chemical treatment can provide effective and rapid remediation of AMD, but it has the disadvantages of higher operating costs and problems with sludge disposal.

1.2.3 Passive methods of acid mine drainage treatment

Passive chemical method

Anoxic limestone drains: Passive methods generally refer to low cost, low maintenance techniques. The anoxic limestone drains (ALD) are an alternative approach for addition of alkalinity to AMD. Within the drain, the partial pressure of carbon dioxide is increased, accelerating the rate of limestone dissolution and consequently increasing the alkalinity concentration, which may reach up to 275 mg/L compared to an open system which produced only 50–60 mg alkalinity/L (D. B. Johnson and Hallberg 2005). However, this kind of technology is not suitable for AMD containing large amounts of iron which would form and accumulate hydroxide precipitates on the surface of limestone and cause failure (Evangelou 1998).

Passive biological methods

Passive biological methods for AMD treatment include aerobic wetlands, compost wetlands/bioreactors, and permeable reactive barriers. The main treatment processes involved include organics degradation, sulfate reduction, iron reduction and potentially methanogenesis. These processes usually would lead to pH increase, alkalinity generation, and reduction of sulfate to sulfide for removing heavy metals (Fe, Mn, Cu, Ni, Zn, and Pb). The reactions involved can be summarized in the following reactions (Gazea et al. 1996):

Specific features of the biological treatment methods are briefly described in the following:

Aerobic wetlands: Aerobic wetlands are assumed to treat mainly alkaline water. The main reaction occurs is shown in reaction (4) (Machemer and Wildeman 1992).

These wetland systems are generally constructed to allow enough surface area in contact with oxygen. The mechanism occurs in aerobic wetlands at near-neutral pH, and oxidation of iron proceeds rapidly in both chemical and biological ways (Ziemkiewicz et al. 2003; Mays and Edwards 2001).

Anaerobic wetlands/compost bioreactors: The major mechanism involved in the anaerobic treatment system is microbiological process that consumes organics and generates alkalinity and sulfide. In addition, the treated AMD is improved through adsorption and precipitation of suspended solids and metals onto the organic matter. These systems can treat acidic, metal-rich, high organics water. The choices of organics used vary according to local availability and the effectiveness. Generally, the composts are made of cow/horse manure, mushroom compost, sawdust, peat and straw. Waybrant et al. (1998) used single organic sources (e.g., sheep manure, sawdust, leaf mulch, and wood chips) for sulfate removal and found higher reduction rates with addition of sewage sludge than without the sludge. Iron- and sulfate-reducing bacteria are considered to have the major roles in AMD bioremediation in anaerobic wetlands/bioreactors.

Permeable reactive barriers (PRBs): Construction of PRBs involves digging of a trench in the flow path of contaminated groundwater, filling the void with reactive materials (a mixture of organic solids and possibly limestones) that are sufficiently permeable to allow flow of the groundwater. Reductive microbiological processes within a PRB generate alkalinity (which is further enhanced by dissolution of limestone and other basic minerals) and remove metals as sulfides, hydroxides, and carbonates (D. B. Johnson and Hallberg 2005; Waybrant et al. 1998).

Iron-oxidation bioreactors: In iron-oxidation bioreactors, oxidation of ferrous iron to ferric in acidic ($\text{pH} < 4$) mine waters is greatly accelerated by iron-oxidizing prokaryotes (bacteria and archaea). Among the well-studied bacteria include *Acidithiobacillus ferrooxidans*, an obligate acidophile that oxidizes a variety of reduced inorganic sulfur compounds. The rate-limiting factors in biological iron oxidation are often the numbers of iron oxidizing bacteria present, and the concentrations of iron and organics.

Technology choice for treating AMD is determined by many economic and environmental factors. Generally, active methods can deal with large volume of mine waters more rapidly and are more flexible and resistant to fluctuations. The requirement of land surface area may rule out passive systems in some situations. However, the mining industries are becoming increasingly attracted to passive biological systems, to avoid the high costs of lime addition and sludge disposal. The major advantages of passive systems are relatively low maintenance costs, and largely reduced amount of sludge. The disadvantage of the passive systems are: they are much more expensive to install and require large land area (wetlands), the performance is less predictable and stable than chemical treatment systems, and the long-term treatment efficacy remain uncertain (D. B. Johnson and Hallberg 2005; Skousen et al. 2000).

However, the problem of the large land area requirement can be resolved by using packed bed reactors for removing organics and heavy metals and generating alkalinity. Sustainability of AMD remediation system also becomes increasingly focused. Recently

an iron oxide sludge recovered from a drainage channel at an abandoned coal mine in Pennsylvania has been used to manufacture burnt sienna pigment in a commercially successful venture (Hedin 2003). Metals recovered by active biological treatment of AMD from metal mines provide some financial return on the investment and running costs of sulfidogenic bioreactors.

1.2.4 Municipal wastewater treatment technologies

In wastewater treatment facilities, unit operations are grouped together to provide various levels of treatment: preliminary, primary, advanced primary, secondary (without or with nutrient removal), and tertiary treatment (Metcalf et al. 2013). In the preliminary treatment, gross solids such as large objects, rags, and grit that may damage equipment are removed. In the primary treatment, a physical operation, usually sedimentation is used to remove the floating and settleable materials found in wastewater. For advanced primary treatment, chemicals are added to enhance the removal of suspended solids and, to a lesser extent, dissolved solids. Secondary treatment consists of biological and chemical processes to remove most of the organic matter. In tertiary treatment, additional combinations of unit operations are used to remove residual suspended solids and other constituents that are not reduced significantly by secondary treatment.

In traditional technology, bar screening, grit chamber and primary clarifier are designed to remove organic and inorganic solids by the physical processes of sedimentation and flotation. Primary treatment can reduce COD by 20% to 30% and suspended solids by up to 60% (Metcalf et al. 2013). In secondary treatment, the goal is to further achieve a certain degree of effluent quality with physical phase separation to remove settleable solids and a biological process to remove dissolved and suspended organic compounds. Secondary treatment can remove up to 85% of COD and total suspended solids (Metcalf et al. 2013).

Some wastewater treatment processes include tertiary treatment, which is any process that will further remove contaminants or specific pollutants. Tertiary treatment is typically used to remove pathogens. Treatment plant operators add chlorine as a disinfectant before discharging the water. Tertiary treatment can remove up to 99 percent of all impurities from sewage, but it is a very expensive process (Metcalf et al. 2013). However, pathogens can be removed by exposure to other unsuitable growth circumstances, such as elevated concentrations of dissolved metals and extreme pH (Hackney and Bissonnette 1978; Wortman and Bissonnette 1985). AMD can serve as economical disinfectant when mixed with MWW.

The amount of energy used for traditional wastewater treatment varies between treatment processes and facilities. Among the various treatment processes, aeration of activated sludge is the most energy consuming process, typically accounting for 45% of total energy consumption (Figure 2) (Martin M. 2011), and energy usage is around 0.28-0.71 kWh/m³ (Cooper et al. 2007).

Figure 2 Typical energy consumption of a conventional wastewater treatment plant (reprinted from from (Martin M. 2011))

1.2.5 Sulfate reducing bacteria (SRB)

Sulfate-reducing bacteria refer to bacteria and archaea that can obtain energy by oxidizing organic compounds or molecular hydrogen (H_2) while reducing sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S). There are SRB that can reduce other oxidized forms of inorganic sulfur (sulfite, thiosulfate, elemental sulfur), nitrate and nitrite, ferric iron and dimethyl sulfoxide (Barton and Tomei 1995; Hao et al. 1996).

SRB are, in general, heterotrophic bacteria and require organic matter as carbon and energy sources. However, hydrogen may substitute as an electron donor for sulfate reduction (Equation (5)).

The use of hydrogen is advantageous because it is more economical to use for high sulfate loadings and results in lesser production of bacterial biomass. Hydrogen may conveniently be formed by cracking methanol or from natural gas. In both cases, carbon dioxide is also produced, and some SRB are able to fix this as their source of carbon (Boonstra et al. 1999).

The largest group of sulfate-reducing bacteria (around 23 genera) lies in the Deltaproteobacteria, which include: Desulfobacterales, Desulfovibrionales and Syntrophobacterales (Muyzer and Stams 2008). Firmicutes contain the second largest group of sulfate-reducing bacteria including the genera *Desulfotomaculum*, *Desulfosporomusa*, and *Desulfosporosinus*. *Thermodesulfobivrio* species which belong to the Nitrospirae division also have the function of sulfate reduction. There are also three

genera of Archaea known to be capable of sulfate reduction: *Archaeoglobus*, *Thermocladium* and *Caldivirga* which are usually found in hydrothermal vents, oil deposits, and hot springs (Castro et al. 2000). In anaerobic digester sludge, when sulfate is present, *Desulfovibrio desulfuricans* is the dominating sulfate-reducing bacteria (Gerardi 2006).

Sulfidogenic treatment of wastes has been evaluated in various applications. Tuttle et al. (1969) suggested the use SRB for AMD treatment by adding organic waste which served as the carbon and electron donor source. Lab and pilot-scale tests using bioreactors and wetlands showed that sulfate reduction is effective in raising pH and removing organics, sulfate and metals from mine waters and municipal wastewaters (Chang et al. 2000; Jong and Parry 2003; Neculita et al. 2007; W. Strosnider et al. 2011a; Tuttle et al. 1969).

1.2.6 Co-treatment methods and applications

Co-managing AMD and MWW could be cost-effective and alleviate some of the infrastructure challenges of building separate treatment systems in areas where these two waste streams are prevalent and material and financial resources are limited. The concept of co-treatment of AMD and municipal wastewater (MWW) has long been explored. Roetman (1932) first proposed mixing of MWW and AMD to reduce pathogens by low pHs and elevated metal concentrations in AMD, but paid little attention to the co-treatment efficiency and its potentials. Joseph and Shay (1952) found that populations of *Escherichia coli* were rapidly decreased when exposed to AMD. Rogers and Wilson (1966) manipulated pH of water samples from the Monongahela River in West Virginia containing domestic sewage-related microorganisms, finding a marked decrease in microbial concentrations in low pH samples.

K. L. Johnson and Younger (2006) used a field-scale aerobic constructed wetland system to treat a low-strength secondary sewage effluent (~14 mg/L BOD₅) and mine water (net alkaline with ~3 mg/L Fe). The results showed that the treatment was successful in producing effluent meeting their effluent design standards for Fe, ammonia and BOD. Co-treatment of a high-strength AMD and secondary MWW effluent in an evaporation pond used by McCullough et al. (2008) also showed significant water quality improvement and bacterial sulfate reduction. W. H. Strosnider et al. (2013) tested the co-treatment of AMD and MWW under aerobic condition with limestone addition and concluded that the approach was a promising treatment method for removing metals and producing alkalinity. A four-stage passive system (clarifiers, Kaldnes, limestone, and wetlands) used by (W. Strosnider et al. 2011b, 2011a; W. H. Strosnider et al. 2011) for co-treatment of a high-strength synthetic AMD (pH 2.6, acidity 1,870 mg/L as CaCO₃) and MWW showed promising results for removing BOD₅, nutrient, and metals, but only achieved 5–12% sulfate reduction. (R. Li et al. (2011); Wei et al. 2008) both suggested utilization of AMD for phosphate removal from secondary effluents of wastewater to control eutrophication in receiving waters.

1.2.7 Iron applications in wastewater treatment

Incorporation of iron from sources such as AMD in sulfidogenic treatment of MWW can offer multiple environmental and energy benefits over conventional wastewater treatment methods such as activated sludge (Deng and Lin 2013; K. L.

Johnson and Younger 2006; Rao et al. 1992; R. Li et al. 2011; W. Strosnider and Nairn 2010; Wei et al. 2008; Winfrey et al. 2010; W. Strosnider et al. 2011a; W. H. Strosnider et al. 2011). Dosing iron, a redox active element, can facilitate multiple (bio)chemical functions that can be exploited to help remove a suite of contaminants in MWW. Chemical precipitation of iron phosphate can be used as an effective mechanism for retaining phosphorous from MWW and help alleviate eutrophication in receiving waters. Iron can form iron sulfide in the sulfidogenic bioreactors, which limits sulfide toxicity on SRB and lower sulfide levels in the treated effluents and the iron sulfide sludge material can be re-oxidized into ferric iron and sulfate to supplement iron and sulfate for continuous treatment.

1.2.7.1 Iron and phosphorous removal

Excessive discharge of phosphorus is a chief environmental concern associated with many water systems such as the Chesapeake Bay, Lake Erie, Gulf of Mexico, among many others (Glibert et al. 2001; Michalak et al. 2013; Tomlinson et al. 2004). MWW typically contains 4-11 mg/L phosphorus for low to high strength untreated wastewater (Table 3-18, (Metcalf et al. 2013). Although phosphorus is essential to the growth of aquatic plants, presence in excessive amount is detrimental because of noxious algal blooms.

Phosphorous removal can be accomplished either biologically or chemically. Biological phosphorus removal from wastewater is commonly achieved by the activity of phosphorous-accumulating organisms (PAOs) and removal of the microbial biomass from the water. Chemical removal is typically achieved through the use of common products such as alum ($\text{Al}_2(\text{SO}_4)_3 \cdot 14\text{H}_2\text{O}$), ferric iron salts ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous iron salts (e.g. FeCl_2 , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) or lime (Fytianos et al. 1998; Huang and Chiswell 2000; Reali et al. 2001). After chemical addition and mixing, phosphorous compounds are removed by either flocculation or sedimentation. Since AMD sludge typically contains high amounts of iron, it has a great potential for adsorbing phosphorus, which would create a cheap and effective use for an otherwise waste material.

1.2.7.2 Iron and organics and sulfide removal

Iron from AMD sludge can be an important source of ferric iron for the biological oxidation of carbon compounds and COD removal. Theoretically, the addition of ferric compounds can increase the metabolic activity and abundance of ferric iron-reducing bacteria (IRB), which would then facilitate carbon oxidation achieving organics removal (Lovley 1987; Lovley and Phillips 1986). In sulfidogenic systems, addition of iron from AMD sludge has the potential of further promoting organics oxidation together with SRB.

In sulfidogenic systems, IRB would transform ferric iron into ferrous iron which forms precipitates with sulfide as iron sulfide compounds and settle out of the aqueous solution (Davison and Heaney 1978; Morse et al. 1987). This mechanism can limit sulfide toxicity to SRB and other functional microorganisms (Kaksonen et al. 2004).

1.3 RESEARCH OBJECTIVES

In order to capitalize on the abovementioned benefits of the co-treatment method, the following two-phased approach with specific research objectives are proposed:

Phase I: Conduct bench-scale experiments to evaluate technical feasibility of co-treatment of AMD and MWW. This phase of research includes two-staged treatment of field-collected AMD and MWW in batch-fed experiments to achieve the following two research objectives:

- 1) Determine optimal operating conditions according to removal efficiency of multiple pollutants (COD, TSS, TDS, nutrients, and metals) and pH and acidity changes. The results have been summarized and reported in a peer-reviewed journal paper: “Deng, D., & Lin, L.-S. (2013). Two-stage combined treatment of acid mine drainage and municipal wastewater. *Water Science & Technology*, 67(5), 1000-1007.”
- 2) Investigate iron toxicity and microbial ecology for their effects on sulfidogenic treatment kinetics. The results have been summarized and reported in a peer-reviewed journal paper: “Deng, D., J.L. Weidhaas, Lin, L.-S. (2016). Kinetics and Microbial Ecology of Batch Sulfidogenic Bioreactors for Co-treatment of Municipal Wastewater and Acid Mine Drainage. *Journal of Hazardous Materials*, 305, 200-208.”

Phase II: Develop a continuous flow of co-treatment process for MWW treatment with iron dosing and iron sulfide sludge recycling. This phase of research includes bench-scale experiments to achieve two research objectives:

- 3) Investigate the relevant factors and optimize the operating conditions for the continuous MWW treatment. The factors to be evaluated include iron dosing, COD/sulfate/iron ratio, flow rate, and sludge recycling for their effects on sulfidogenic treatment kinetics and effluent quality. “Deng, D., & Lin, L.-S. (2017). Continuous sulfidogenic wastewater treatment with iron sulfide sludge oxidation and recycle. *Water Research*, 114, 210-217.”
- 4) Investigate biogeochemical transformations of Fe and S in the continuous MWW treatment process at different stages of the treatment process and associated microbial ecology. In addition, mass balance of key chemical elements were conducted.

Research methodology, experimental design, results, and implications are described in the following chapters of this dissertation with each chapter describing the scope of each research objective, methodology, results, discussions and conclusion when applicable.

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CHAPTER 2: PHASE I (STAGE 1): LAB-SCALE OF TWO-STEP TREATMENT PROCESS

Research Objective: Determine optimal operating conditions according to removal efficiency of multiple pollutants (COD, TSS, TDS, nutrients, and metals) and pH and acidity changes.

2.1 INTRODUCTION

Acid mine drainage (AMD) from active and abandoned mines is a prevalent environmental pollution source and significant environmental liability in mining regions worldwide. AMD originating from oxidation of iron sulfide (e.g., pyrite) in coal and mine tailings can generate water containing high concentrations of metals, sulfate, and acidity. To mitigate AMD impacts, various control measures may be performed at different stages in the mine water generation process (Sengupta 1993). Of those, chemical treatment utilizes acid neutralizing agents (e.g., hydrated lime, caustic soda, soda ash) to raise pH and remove metals through chemical precipitation (D. B. Johnson and Hallberg 2005). Although active chemical treatment can effectively remediate AMD, high operation costs and disposal of large amounts of the produced sludge remain a challenge (Chang et al. 2000). Biological treatment of AMD typically involves microbiological activities in systems such as lagoons, wetlands, and bioreactors to achieve treatment objectives (Neculita et al. 2007). Given the sulfate prevalence in AMD, a common feature of the passive treatment is exploitation of sulfate reducing bacteria (SRB) to render sulfate reduction to sulfide, alkalinity generation, and subsequent metal sulfide precipitation (Lewis 2010; Waybrant et al. 1998).

A wide variety of organic sources has been examined for biotic sulfate reduction (Benner et al. 2002; Dvorak et al. 1992; D. B. Johnson and Hallberg 2005; Jong and Parry 2003; Strosnider et al. 2011a; Tuttle et al. 1969). Waybrant et al. (1998) used single organic sources (e.g., sheep manure, sawdust, leaf mulch, and wood chips) for sulfate removal and found higher reduction rates with addition of sewage sludge than those without the sludge. (Strosnider et al. (2011a), 2011b)) suggested that the diverse electron donors in the MWW were suitable for supporting the growth of microbial communities and sulfate reduction.

The concept of combined treatment of AMD and municipal wastewater (MWW) has long been explored. However, there were only a few studies of such treatment approach reported in the literature. Roetman (1932) first proposed mixing of MWW and AMD to reduce pathogens by low pHs and elevated metal concentrations in AMD, but paid little attention to the co-treatment efficiency and its potentials. K. L. Johnson and Younger (2006) used a field-scale aerobic constructed wetland system to treat a low-strength secondary sewage effluent (~14 mg/L BOD₅) and mine water (net alkaline with ~3 mg/L Fe). The results showed that the treatment was successful in producing effluent meeting their effluent design standards for Fe, ammonia, and BOD. Co-treatment of a high-strength AMD and secondary MWW effluent in an evaporation pond used by McCullough et al. (2008) also showed significant water quality improvement and bacterial sulfate reduction. Strosnider and Nairn (2010) tested the co-treatment of AMD

and MWW under aerobic condition with limestone addition and concluded that the approach was a promising treatment method for removing metals and producing alkalinity. A four-stage passive system (clarifiers, Kaldnes, limestone, and wetlands) used by (Strosnider et al. (2011a), 2011b)) for co-treatment of a high-strength synthetic AMD (pH 2.6, acidity 1,870 mg/L as CaCO_3) and MWW showed promising results for removing BOD_5 , nutrient, and metals, but only achieved 5% - 12% sulfate reduction. (Li et al. (2011); Wei et al. (2008)) both suggested utilization of AMD for phosphate removal from secondary effluents of wastewater to control eutrophication in receiving waters.

Conceptually, combined treatment of AMD and MWW takes advantage of the prevalent chemistry of the waste streams and can potentially offer multiple environmental and energy benefits. First, alkalinity in MWW can raise the pH of AMD upon mixing, which promotes chemical precipitation such as metal hydroxides and carbonates, and associated adsorption/co-precipitation mechanisms. Second, low solubility of phosphate with multivalent metals (e.g., Fe and Al) provides an effective mechanism for recovering phosphate from MWW. Third, sulfate ions from AMD can serve as an electron acceptor for organics oxidation and removal from MWW. This can potentially eliminate the need of aeration, an energy-intensive operation required for the biological treatment of MWW such as activated sludge processes. These potential benefits represent an incentive and opportunities for developing innovative, energy efficient treatment technologies for the two waste streams in mining regions.

This study examined the feasibility of combined treatment of AMD and MWW using a two-stage treatment method by systematically evaluating its efficiency for a range of relevant chemical constituents. The two-stage treatment consisted of mixing of field-collected AMD and MWW samples, and anaerobic biological treatment of the mixtures. The mixing treatment was designed for phosphate removal, and conditioning pH and COD/sulfate ratio of the mixtures for the subsequent biological treatment. The biological treatment had a main function of COD and sulfate removal, and additional metal removal. Evaluation of the treatment approach also included acidity/alkalinity, TDS, TSS, nutrients, and selected metals.

2.2 MATERIALS AND METHODS

2.2.1 *Field sampling*

A total of five sampling trips were taken to collect AMD and MWW samples in the study. AMD samples were collected at six sites along Dunkard Creek downstream of Taylortown, Pennsylvania (PA), USA. Primary wastewater samples were obtained from the Bobtown wastewater treatment plant in PA (two trips), and the Star City wastewater treatment plant in West Virginia (WV, three trips), USA. In situ measurements of turbidity, electrical conductivity, and pH were taken during the trips. All the AMD and MWW samples were contained in acid-washed bottles and transported under refrigeration to laboratories where they were stored at 4 °C until laboratory analyses. The AMD samples collected from the six locations during the same sampling trip were first mixed in equal volumes to make an AMD composite solution for use in the experiments with the MWW sample collected on the same day.

2.2.2 Two-stage treatment

Two-stage batch experiments were conducted to evaluate the feasibility of combined treatment of collected MWW and AMD samples.

Stage 1: Aerobic mixing

The first stage involved mixing of the AMD composite solutions and MWW samples to promote chemical precipitation. A series of mixing reactors (1 L glass beakers) were used to test a range of volume ratios of the two wastes. Suspended solids including the formed chemical precipitates were then allowed to settle for 24 h before the aliquots and chemical sludge were taken for analyses. The aliquots were analyzed for pH, TSS, and alkalinity/acidity, and filtered with a membrane filter (0.45 μm) before measurements of other dissolved chemicals. The mixing experiments were labelled as M1, M2, M3, M4 and M5 corresponding to the five sampling trips. A meat extract material (Oxoid Lab-Lemco power, Thermo Scientific) was used to increase COD concentration in M4 treatment for comparisons with the other sets of experiments.

Stage 2: Biological treatment

In the second stage, the aliquots and sludge from the mixing step were treated in five series of biological reactors (1 L glass media bottles) under anaerobic conditions with mixing. Each bioreactor was packed with plastic media (Kaldnes K1, Evolution Aqua Ltd, UK, medium loading: $\sim 800 \text{ cm}^2/\text{L}$ reactor volume) for biofilm development. The bioreactors were first inoculated with 200 mL of anaerobic digester sludge collected from the Star City wastewater treatment plant, WV, and biomass enrichment was allowed to occur. The bioreactors were then used to treat the AMD/MWW mixtures from the five mixing experiments for 14 days. During the treatment, pH and redox potential were monitored and duplicate samples were taken from the reactors for analyses of alkalinity/acidity, COD, sulfate, sulfide, nutrients, and selected metals. At the end of the treatment period, sludge samples were taken and prepared for chemical element analysis. The experiments were labeled as B1–B5 corresponding to M1–M5.

2.2.3 Analytical methods

Conductivity and pH were measured using pre-calibrated pH/conductivity probes and meter (YSI 63) in the field during the sampling trips. Autotitrators were used for measuring alkalinity (Thermo Scientific Orion 950) and acidity (Mettler Toledo DL50) measurements. All samples were filtered with a 0.45 μm filter membrane prior to analyses for sulfate, sulfide, COD, chloride, metals, and nutrients following the Standard Methods (APHA 2005). Sulfate and nutrient concentrations were quantified by a UV-Vis spectrophotometer (Thermo Scientific Genesys 10S). Dissolved samples were preserved by acid digestion with a concentrated nitric acid ($\sim 70\%$, trace metal grade), and analyzed for metals using atomic adsorption spectroscopy (Perkin Elmer 3100). COD and sulfide concentrations were determined using a spectrophotometer (Hach DR 2800). Duplicates of sulfate and COD concentrations were measured.

2.2.4 Sludge characterization

The sludge samples were first dried at 103°C to remove the moisture content (Karamalidis et al. 2008), and then allowed to cool at room temperature until the weight

was constant. Sample pellets of 13 mm diameter were prepared for chemical element analyses using scanning electron microscopy (SEM, Hitachi S-4700) equipped with energy-dispersive spectroscopy (EDS, EDAX Genesis). The pellets were prepared using evacuable pellet dies (Specac Ltd, Rhode Island, USA) and the procedure is briefly described here. Using a paper chute, the well-ground and mixed powder of the sludge sample was poured into the bore of the cylinder body and compacted. The powder was evenly distributed across the face of the polished pellet by lightly tapping the side of the die. A stainless steel pellet was then used to push this polished face into the bore of the cylinder body, followed by insertion of a plunger into the cylinder body. The die assembly was then placed into a hydraulic press with a load of 7 tons for 15 seconds to make a compacted sludge pellet for SEM analysis. Elemental information of the pellet samples was obtained under an accelerating voltage of 10 kV, with on-line ZAF correction. Duplicate analyses were conducted on each sample for consistency, and the average composition was calculated.

2.3 RESULTS AND DISCUSSION

2.3.1 AMD and MWW characteristics

Chemical characteristics of the AMD samples (Table 1) were generally consistent with the mean chemical parameters of 156 coal mine drainages reported by (Watzlaf et al. 2004). The AMD samples contained high levels of Fe (112 ± 118 mg/L), acidity (327 ± 128 mg/L as CaCO_3), and SO_4^{2-} ($1,846 \pm 594$ mg/L), and may be classified as high strength (K. L. Johnson and Younger 2006). It is noted that the acidity/alkalinity of the AMD samples varied considerably among the five sampling trips. The MWW samples were net alkaline water and contained averaged COD values of 293 ± 262 mg/L and noticeable levels of sulfate. Ammonia was the predominant inorganic nitrogen in the MWW samples (20–33 mg/L) and phosphate concentration was around 2 mg/L.

Table 1 Mean concentrations and standard deviations (when n>3) of major chemical parameters for the AMD and MWW samples

Parameters	AMD	MWW (Bobtown)	MWW (Star City)
	(n = 5)	(n = 2)	(n = 3)
pH	4.2±0.9	6.9	6.9±0.4
TSS (mg/L)	20±25	97.5	208±138
TDS (mg/L)	2,423±396	132	243±175
Conductivity (S/m)	2,198±487	807	688±279
Turbidity (NTU)	9.4±9.9	113	148±72
Acidity (mg/L as CaCO ₃)	327±128	54	58±23
Alkalinity (mg/L as CaCO ₃)	13.4±18	163	205±49
Cl ⁻ (mg/L)	12.6±3.7	120.8	104±48
SO ₄ ²⁻ (mg/L)	1,846±594	68.4	69±57
COD (mg/L)	41±49	234	333±297
Metals			
Fe (mg/L)	112±118	12.7	0.4±0.3
Ca (mg/L)	292±129	67	76±30
Mg (mg/L)	86±43	10.7	7.2±4.4
Mn (mg/L)	6±2.4	0.1	0.03±0.05
Al (mg/L)	2±2.2	0.4	3.6±6.1
Na (mg/L)	199±51	86.2	63±10
Nutrients			
NO ₂ ⁻ (µg/L)	2.1±1.8	21	6.1
NO ₃ ⁻ (mg/L)	0.6±1.0	0.9	0.03
NH ₄ ⁺ (mg/L)	6.5±0.2	33	20
PO ₄ ³⁻ (mg/L)	0.6±0.005	2.2	2.1

2.3.2 Stage 1: Mixings

2.3.2.1 pH

The stage 1 mixings of the AMD and MWW samples caused significant increases of pH to the range of 6.2–7.9 compared with those of the AMD samples (Figure 3). This promoted formation of metal hydroxides and carbonate precipitation, and resulted in suitable pHs for the microbial activities (Crites and Technobanoglous 1998).

Figure 3 Values of pH of the AMD (5-trip average), MWW (5-trip average), and AMD/MWW mixtures with different volume ratios.

2.3.2.2 COD and sulfate

The mixings did not lead to significant changes in COD and sulfate concentrations except for the dilution effect (Figure 4). The high COD concentrations for M4 were caused by the meat extract addition. The mixture solutions had COD/sulfate concentration ratios in the range of 0.05–5.4. For this combined treatment approach, the mixing step played an important role in conditioning the mixture pH, and sulfate and COD concentrations, and in optimizing the performance of the biological treatment.

Figure 4 COD and sulfate concentrations and the resultant COD/sulfate concentration ratios of the AMD/MWW mixtures with different volume ratios.

2.3.2.3 Phosphate

Phosphate in the MWW was significantly reduced from the mixings by 9 to ~100% depending on the mixing ratio (Figure 5). The phosphate concentration of the mixture solutions was inversely proportional to the iron concentration (data not shown), and its removal was mostly due to formation of iron phosphate and its complexation with other chemical precipitates. It was noted that this mixing treatment was more effective for phosphate removal than co-treatment of sewage and mine waters in wetlands (10–50%) reported by K. L. Johnson and Younger (2006).

Figure 5 Phosphate concentrations of the AMD, MWW and AMD/MWW mixtures.

2.3.3 Biological treatment

2.3.3.1 COD and sulfate

The reducing environments in the bioreactors were confirmed by the redox potential measurements which ranged from -71 to -545 mV with its value depending on the residual COD level during the 14-day treatment. The biological treatment resulted in significant reductions of COD and sulfate, and the removal efficiency varied with the COD/ sulfate concentration ratio of the AMD/MWW mixtures (Figure 6). For COD/sulfate concentration ratios within 0.6–5.4, sulfate and COD removal was above 80%. When COD/sulfate ratio was below 0.2, sulfate removal decreased significantly due to insufficient COD (i.e., reducing power) for sulfate reduction. The biological treatment led to sulfide concentrations of 0.1–3 mg/L at the end of treatment. High bisulfide conditions occurred during the M2–B2 experiments. The second sampling trip took place during a heavy rain event and sample analyses indicated low metal levels in the AMDs for metal sulfide formation.

Figure 6 COD and sulfate removal efficiency of the biological treatment as a function of COD/sulfate concentration ratio.

It has been well established that sulfate reduction depends on COD/sulfate ratios, and the ratios for optimal COD and sulfate removal varied considerably with the types of organics (Al-Ani 1994; Damianovic and Foresti 2007; De Smul et al. 1999; Oude Elferink 1998; Velasco et al. 2008; Watzlaf et al. 2004; Waybrant et al. 1998). In general, higher ratios were preferred when complex organic carbon sources were used because not all the carbon in the organics could be used by SRB (Prasad et al. 1999). In the current study, the COD/sulfate ratios in the range of 0.6–5.4 consistently resulted in COD and sulfate removal above 80%. This suggested that, after an active biomass was established, the bioreactors could treat AMD/MWW mixtures with a fairly wide range of the concentration ratio. This is an important and beneficial feature for applications of this treatment method given the fluctuating chemical quality of AMD and MWW.

2.3.4 Additional chemical parameters

Additional chemical parameters were analyzed for a few treatment experiments and the results are used to illustrate the effects of the treatment method.

2.3.4.1 Acidity and alkalinity

The mixing treatment resulted in net alkaline conditions for the mixtures and additional alkalinity was produced in the biological treatment (Figure S 1 of Supplementary information). The primary mechanism for alkalinity production was biotic reduction of sulfate to hydrogen bisulfide and concurrent production of bicarbonate ions (D. B. Johnson and Hallberg 2005). This was corroborated by the net alkalinity results of experiments 4 (with 789, 786 and 799 mg/L of sulfate) and 5 (with 657, 199, and 119

mg/L of sulfate). The biological treatment exhibited significantly higher alkalinity production in B4 than in B5.

2.3.4.2 TSS and TDS

The MWW samples contained much higher TSS than the AMD samples, which were 367 and 4 mg/L, respectively for M5. The mixings increased the overall TSS concentration due to the formation of chemical precipitates in the solutions (Figure S 2 of Supplementary information). The biological treatment, B5, was found to greatly reduce the TSS concentrations of the AMD/MWW mixtures to 20–40 mg/L. The AMD samples contained much higher TDS than the MWW water, which were 2,050 and 375 mg/L, respectively for M5. The mixings did not significantly change the TDS concentration at high AMD/MWW ratios, but lowered TDS from the AMD level due to dilution and additional chemical precipitation formation (Figure S 2 of Supplementary information). The biological treatment slightly reduced the TDS levels of the AMD/MWW mixtures for the two higher mixing ratios (0.07 and 0.67) in M5.

2.3.4.3 Nitrogen nutrients

Inorganic nitrogen in the mixtures mostly originated from the MWW and existed in the ammonia form (Figure S 3 of Supplementary information). Nitrate and nitrite were present in relatively low concentrations: ≤ 0.2 mg/L and ≤ 12 μ g/L, respectively. The mixings caused the concentrations of these nitrogen chemicals to vary according to dilution of the mixture. The biological treatment, B5, resulted in reduction of 12–48% for ammonia compared to initial MWW concentration.

2.3.4.4 Metals

The mixing of the two wastes was effective for removing Fe and Al (Figure S 4 of Supplementary information). Concentrations of the remaining metals varied with the AMD/MWW mixing ratios. The biological treatment further reduced the metal concentrations. Compared to the AMD samples, the two-stage treatment overall resulted in excellent reductions of Fe (~100%), Al (~100%), and Mn (75 to ~100%). Calcium, magnesium and sodium were reduced significantly by 52–81%, 13–76%, and 56–76%, respectively.

The reduction of Fe and Al as a result of the mixings was attributed to formation of metals with phosphate (e.g., Fe and Al) and hydroxides (e.g., Fe, Al, and Mn). Combination with organic ligands also probably reduced their concentrations (Younger et al. 2002). Sulfide and bicarbonate generation in the biological treatment could promote precipitation of metal sulfides (e.g., Fe and Mn) and carbonate salts (e.g., Mn, Ca, and Mg) due to their relatively low solubilities (Stumm and Morgan 2012). In addition, bio-sorption of metals due to the binding ligands on cell walls and metabolism-related mechanisms may have contributed to the metal removal (Chen et al. 2000).

2.3.5 Sludge characterization

2.3.5.1 Sludge from the mixing

The SEM/EDS analysis of the sludge samples revealed the presence of metals (e.g., Fe and Al) and phosphorus, suggesting formation of iron- and aluminum-phosphate precipitates (data not shown). Strong signals for carbon and oxygen, along with

detectable calcium and magnesium, suggested presence of carbonate salts of the metals. Silicon probably originated from wastewater and AMD in the form of silica or silicates.

2.3.5.2 Sludge from the biological treatment

A SEM photomicrograph of the sludge obtained from the biological treatment is illustrated in Figure 7. The EDS qualitative analysis indicated the presence of metals including Fe, Al, Ca, Mg, and relatively smaller amount of Na. A strong signal for sulfur was also identified, suggesting iron and aluminum sulfides formation from the biological treatment. A weak peak for phosphorus was observed and presumably resulted from the chemical sludge in the mixing treatment and the phosphorus content of the biomass.

Figure 7 SEM photomicrograph and chemical element spectrum of a sludge pellet from the biological treatment.

2.4 CONCLUSION

This study denoted the feasibility of the two-stage treatment method for combined management of AMD and MWW. The treatment produced water with an average pH of 7.9 and net alkalinity of 290 mg/L as CaCO_3 . The treated water with the increased alkalinity has the potential to be partly recycled to neutralize the AMD in the mixing stage. The three-stream mixing would provide a flexible mechanism for conditioning the AMD/MWW mixture for the biological treatment. The mixings in this study consistently resulted in effective removal of phosphate, which is an important feature of the proposed method for removing one of the leading nutrients that cause eutrophication in receiving waters. The biological treatment consistently exhibited COD and sulfate removal above 80% for COD/sulfate ratios of 0.6–5.4. This indicated that proper conditioning of the AMD/MWW mixture can lead to sufficient removal of the organic matters and sulfate, and the biological treatment was robust to fluctuation of COD/sulfate ratio once an active biomass was established. The treatment also showed effective removal of multi-valent metals Fe, Al, and Mn, and to significant degrees Ca, Mg, and Na. The removed metal elements were mostly in the form of the produced sludge from both the mixing and biological treatment. Overall, the study showed promising results for combined

management of the two waste streams and denoted the potential of developing innovative energy-efficient engineering technologies for wastewater management.

2.5 SUPPLEMENTARY INFORMATION

Figure S 1 Net alkalinity values of AMD, MWW, and AMD/MWW mixtures from the M4/B4, M5/B5 treatment

Figure S 2 TSS and TDS concentrations of the AMD, MWW, and AMD/MWW mixtures from the M5 and B5 treatment

Figure S 3 Nutrient concentrations of the AMD, MWW and AMD/MWW mixtures from the M5 and B5 treatment.

Figure S 4 Metals concentrations of the AMD, MWW, and AMD/MWW mixtures from the M5 and B5 treatment.

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CHAPTER 3: PHASE I (STAGE 2): STUDY KINETICS AND MICROBIAL ECOLOGY OF SULFATE REDUCING CO-TREATMENT REACTORS.

Research Objective: Investigate iron toxicity and microbial ecology for their effects on sulfidogenic treatment kinetics.

3.1 INTRODUCTION

Co-treatment study of municipal wastewater (MWW) and acid mine drainage (AMD) can be traced back to 1900's when Roetman (1932) first proposed mixing the two to reduce pathogens in sewage. In more recent years, technical feasibility of the co-treatment was investigated by several research groups (Johnson and Younger 2006; Li et al. 2011; McCullough et al. 2008; Paul L. Younger 2014; Winfrey et al. 2010; Wei et al. 2008; Strosnider and Nairn 2010; W. Strosnider et al. 2011a, 2011b; W. Strosnider et al. 2013; W. H. Strosnider et al. 2013; W. H. Strosnider et al. 2011). Overall, these studies showed significant water quality improvements through removal of metals, nutrients, and organics along with increases in pH and alkalinity.

From a wastewater treatment perspective, incorporation of AMD in MWW treatment can provide significant environmental benefits over the widely adopted activated sludge processes, which were made possible by the complementary water chemistry of the two wastes. For examples, metals in AMD (e.g., iron and aluminum) can form chemical precipitation of low-solubility salts (i.e., iron phosphate) and help remove both dissolved and particulate materials. High levels of sulfate can be used as an electron acceptor by sulfate reducing bacteria (SRB) for oxidation of organic compounds under anaerobic conditions. This eliminates the need for aeration, which is the most energy-intensive operation in wastewater treatment facilities (Burton 1996; Droste 1997). The SRB-facilitated sulfate reduction to (bi)sulfide produces alkalinity and promotes metal sulfide precipitation. An additional benefit with the anaerobic treatment is the significant reduction in biological sludge production (Speece 1983).

A range of factors are critical for co-treatment system, including COD/sulfate ratios, mixed water chemistry, microbiological diversity, and reactor configuration (Neculita et al. 2007). Although deemed to play an important role in the treatment efficacy, there is scarce information about microbial ecology and its relationships with the co-treatment process kinetics. Schmidtova and Baldwin (2011) studied a bioreactor used to treat a landfill leachate and found a positive correlation between sulfate reduction rate and SRB abundance. Dann et al. (2009) investigated microbial profiles in a passive compost-based system used for remediating acidic, high iron and sulfate industrial wastewater, and concluded that compost/straw decomposition and associated sulfate and iron reductions were facilitated by a complex mix of aerobic and anaerobic bacteria. Sánchez-Andrea et al. (2014) recently reviewed and discussed important factors for utilizing SRB in sulfidogenic reactors used to treat AMD, as well as microbial communities in the bioreactors.

Metal toxicity needs to be taken into consideration in order to maintain active and diverse sulfate reducing microbial communities. Iron (Fe), one of the most prevalent

metals in AMD, was reported to inhibit SRB and lower sulfate reduction by 39-100% in two ways: deposit of FeS causing the inhibition of the cells activity (Utgikar et al. 2001; Utgikar et al. 2002; Zhang et al. 2009), and the competition of Fe³⁺-reducing bacteria for electron donors (Lovley and Phillips 1986b, 1986a; Van Bodegom et al. 2004). Metals such as Zn, Cd, Cu, Ni, Pb and Mn often remain at significant concentrations in acid mine drainage even after the pretreatment process such as alkaline chemical additions (Caraballo et al. 2009). Sulfate reduction by SRB was found to be completely inhibited at 2–50 mg Cu/L, 13–40 mg Zn/L, 75–125 mg Pb/L, 4–54 mg Cd/L, and 10–20 mg Ni/L (Utgikar et al. 2002). However, Castillo et al. (2012) evaluated the tolerance of SRB to Zn up to 260 mg/L and found SRB activities reduced Zn concentration almost completely by forming ZnS precipitation. These metal inhibitive effects are expected to vary depending on the reactor configuration, SRB species, metals concentration, pH, and *Eh* conditions (Hao et al. 1996; Hao et al. 1994; Jong and Parry 2003).

A previously reported two-stage process for co-treatment of field-collected AMD and MWW (i.e., mixing of the two wastes followed by sulfidogenic treatment of the mixture) has demonstrated effective removal of metals, COD, sulfate and acidity (Deng and Lin 2013). This study focuses on the kinetics, iron inhibitive effects, and microbial ecology in the sulfidogenic bioreactors. Specifically, COD degradation kinetics and inhibition by Fe were modeled to characterize the biological treatment. Bacterial 16S rRNA gene clone libraries were analyzed to describe microbial ecology and its relationship with the treatment kinetics. In addition, quantitative polymerase chain reaction (qPCR) was used to quantify the *dsrA* gene copies that encodes the dissimilatory (bi) sulfite reductase involved in biological sulfate reduction.

3.2 MATERIALS AND METHODS

3.2.1 Field Sampling

AMD samples were collected along Dunkard Creek downstream of Taylortown, Pennsylvania (PA) on five occasions. Primary wastewater samples were obtained from the wastewater treatment plant in Bobtown, PA and Star City in West Virginia (WV) on five occasions. Anaerobic digester sludge was sampled from the wastewater treatment plant in Star City, WV and used to inoculate the bioreactors. Organic compounds in the wastewater samples were the only carbon and energy sources for the sulfidogenic treatment. Major chemical parameters of the collected samples were described previously (Deng and Lin 2013).

3.2.2 Two-stage Treatment

Two-stage batch experiments were conducted. The first stage was aerobic mixing of AMD and MWW, which was conducted on 21 different COD/sulfate ratios ranged from 0.05 - 5.4 in the mixture and each ratio was tested in replicate treatments. Initial and final SO₄²⁻, COD and Fe concentrations and related parameters are listed in Supplementary information Table S1. The second stage was sulfidogenic treatment of the mixture solution using attached growth media (Kaldnes K1, Evolution Aqua Ltd., UK, plastic media with a surface area of 800 cm²/L of reactor volume) to retain the biomass. The study was performed in 1L glass Boston round bottles at room temperature (22±1 °C) and maintained in an anaerobic condition. COD and sulfate concentrations were monitored by periodic sampling (every 3 days) over a 15-day period. Initial rate method

was used to determine COD oxidation and sulfate reduction rates based on Michaelis-Menten model assumption (Chapra 1997). Details of the co-treatment design and sampling scheme can be found in a previous publication (Deng and Lin 2013).

3.2.3 Analytical Methods

COD analysis was performed using a dichromate reflux method (ASTM D1252-06 2006) with a spectrophotometer (HACH, model DR2800). Measurements of pH and redox potential were taken using HACH electrode (Ag/AgCl). Sulfate was analyzed following the Standard Methods (APHA 2005) with a spectrophotometer (HACH, model DR2800). Metal concentrations were determined using an atomic absorption spectrometer (PerkinElmer, model 3100, Shelton, CT, USA) after sample acidification with a concentrated HNO₃ solution (70% v/v, Fisher Scientific, Pittsburgh, PA).

3.2.4 Control Experiments

Control experiments were conducted to quantify the contribution of abiotic processes to COD and sulfate removal. Details of experimental setup are provided in Supplementary information.

3.2.5 COD Oxidation Kinetics Modeling

COD oxidation kinetics was conducted based on the 21 COD/sulfate ratios tested (0.05–5.4, Supplementary information Table S1). The Michaelis–Menten constant (K_m , mg/L) and maximum reaction rate (V_{max} , mg/L·min) were estimated by the Lineweaver–Burk transformation (Equation (6)) (A. Kaksonen et al. 2006).

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{S} \quad (6)$$

where V is the reaction rate (mg/L min), and S is the substrate concentration, COD (mg/L). The measured Fe concentrations (Supplementary information Table S1) were used to estimate the inhibition constants (K_i) using the following non-competitive inhibition model (Equation (7)) (Macbeth et al. 2004).

$$V = \frac{V_{max} \times S}{(K_m + S) \times (1 + \frac{I}{K_i})} \quad (7)$$

where I = inhibitor concentration (mg/L), and K_i = inhibition constant (mg/L). A non-linear least squares optimization subroutine, PROC NLIN of SAS (v. 9.2, SAS Institute Inc., Cary, NC, USA), was used to fit the model and estimate K_m , V_{max} and K_i .

3.2.6 Stoichiometry and mass balance on COD, iron and sulfur

Stoichiometric analyses were conducted on oxidation of COD (assumed to be C₁₀H₁₉O₃N (McCarty 1975)) coupled with reduction of SO₄²⁻ or Fe³⁺ (Supplementary information) to compare with the experimental results. Specifically, the theoretical value of grams sulfate reduced per gram of COD, and grams of iron reduced per gram of COD were compared to the observed values in the bioreactors to determine the predominant oxidation processes for COD. Mass balance on iron and sulfur was conducted based on their initial and final concentrations and those in the chemical sludge (Supplementary

information Table S1). The mass balance calculations were conducted for all 21 experimental runs.

3.2.7 Sludge characterization

Morphology and chemical composition of the filtered samples of AMD, MWW, AMD/MWW mixtures, and biological sludge were analyzed using a scanning electron microscope (SEM, Hitachi S-4700) coupled with energy dispersive X-ray spectroscopy (EDS, EDAX Genesis). Chemical elemental information of the samples was obtained under an accelerating voltage of 10 kV, with on-line ZAF correction.

3.2.8 Nucleic acid extraction, purification and 16S rRNA gene amplification

Bioreactors with initial COD/sulfate ratios of 0.2, 1, and 2 (labeled as B1, B2, and B3, respectively) were sampled for microbial community characterization. Microbial DNA was extracted from 50 ml of mixture sample from each bioreactor using the FastDNA SPIN Kit for Soil (MP Biomedicals, OH). The extracted DNA was purified using an ethanol precipitation method (Macbeth et al. 2004), followed by DNA quantification using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, DE). The purified microbial DNA was amplified by polymerase chain reaction (PCR, Eppendorf AG Mastercycler epgredient, Hamburg, Germany). Details of the PCR analysis and products can be found in Supplementary information and Figure S6.

3.2.9 Cloning and sequencing

The PCR amplicons of the 16S rRNA gene were cloned using the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Vectors were transformed into chemically competent *Escherichia coli* cells following the manufacturer's instructions. In total, 20 clones were selected from each of the bioreactors. The 16S rRNA gene fragments on the plasmids were amplified by the primer sets of M13F and M13R (Manual of TOPO TA cloning kit). The PCR products were visualized by agarose gel electrophoresis to verify the size and the existence of the inserts. The 16S rRNA genes of PCR amplicons were sequenced in the West Virginia University Genomics Core Facility using either 8F and 907R (clones B1-1- B1-12, B2-1 - B2-7, B3-1 - B3-3 and B3-8-B3-9) or 8F and 1492R (for clones B2-8- B2-9 and B3-4 - B3-7 and B3-10 - B3-12). Twelve clones from B1, nine clones from B2 and twelve clones from B3 were successfully sequenced.

3.2.10 Phylogenetic analysis and diversity calculations

The obtained 16S rRNA gene sequences were reassembled using Bioedit (version 7.1.3.0, Ibis Biosciences, Carlsbad, CA) to generate contigs. The contig sequences were checked for chimeras using Bellerophon tool and Decipher (Wright et al. 2012; Huber et al. 2004) and then aligned using MEGA 6 (Tamura et al. 2013). The sequences were classified into taxonomic groups using the ribosomal database project classifier (Wang et al. 2007). Evolutionary analyses were conducted in MEGA 6 and bootstrap resampling analyses were performed on 1000 replicates (Tamura et al. 2013). The sequences were submitted to NCBI Genbank and accession numbers are provided in Supplementary information

Table 5 and grouped into functional groups, as defined by putative function in Table 2. Details of diversity calculation can be found in the Supplementary information, Table S3 and Figure S7.

Table 2 Clone library results of bioreactors B1, B2 and B3.

Clone	Closet Species in GenBank [Accession no.]	Putative Function	Identity (%)	Abundance (%)	Phyla
B1-1	<i>Desulfovibrio idahonensis</i> [NR 114908.1]	Sulfate reduction	97%	33.3	Deltaproteobacteria
B1-3	<i>Desulfovirga adipica</i> [NR 036764.1]	Sulfate reduction	94%		Deltaproteobacteria
B1-5	<i>Desulfohalobium elongatus</i> [NR 029305.1]	Sulfate reduction	93%		Deltaproteobacteria
B1-11	<i>Desulfatibacillum alkenivorans</i> [NR 025795.1]	Sulfate reduction	92%		Deltaproteobacteria
B1-6	<i>Desulfomonile limimaris</i> [NR 025079.1]	Dehalogenation	94%	8.3	Deltaproteobacteria
B1-4	<i>Mucilaginibacter polysacchareus</i> [KM 019772.1]	Hydrolysis	86%	25.0	Bacteroidetes
B1-7	<i>Actinomyces bacterium</i> [DQ994722.1]	Hydrolysis	87%		Acidobacteria
B1-8	<i>Mucilaginibacter polysacchareus</i> [KM 019772.1]	Hydrolysis	86%		Bacteroidetes
B1-2	<i>Clostridium</i> sp. CYP5 [DQ 479415.1]	Fermentation	99%	33.3	Firmicutes
B1-9	<i>Acidaminobacter hydrogenoformans</i> [NR 028683.1]	Fermentation	98%		Firmicutes
B1-10	<i>Prolixibacter bellariivorans</i> [NR 043273.1]	Fermentation	86%		Bacteroidetes
B1-12	<i>Marinilabilia salmonicolor</i> [NR 104682.1]	Fermentation	86%		Bacteroidetes
B2-1	<i>Desulfomicrobium escambiense</i> [042018.1]	Sulfate reduction	99%	22.2	Deltaproteobacteria
B2-9	<i>Desulfococcus</i> sp. Hobo [EF 442977.1]	Sulfate reduction	85%	11.1	Deltaproteobacteria
B2-7	<i>Clostridium</i> sp. [AB596885.1]	Dehalogenation	96%		Bacteroidetes
B2-2	<i>Cloacibacillus porcorum</i> [NR 109636.1]	Fermentation	90%		Synergistetes
B2-3	<i>Leptolinea tardivitalis</i> [NR 040971.1]	Fermentation	89%		Chloroflexi
B2-4	<i>Gracilibacter thermotolerans</i> [NR 115693.1]	Fermentation	85%	66.7	Firmicutes
B2-5	<i>Gracilibacter thermotolerans</i> [NR 115693.1]	Fermentation	86%		Firmicutes
B2-6	<i>Gracilibacter thermotolerans</i> [NR 115693.1]	Fermentation	86%		Firmicutes
B2-8	<i>Ruminococcaceae bacterium</i> [LK 391549.1]	Fermentation	91%		Firmicutes
B3-2	<i>Desulfohalobium elongatus</i> [NR 029305.1]	Sulfate reduction	96%	16.7	Deltaproteobacteria

B3-7	<i>Desulfobulbus elongatus</i> [NR 029305.1]	Sulfate reduction	97%		Deltaproteobacteria
B3-3	<i>Bellilinea caldifistulae</i> [NR 041354.1]	Methanogenesis	90%		Chloroflexi
B3-5	<i>Syntrophus sp.</i> [AJ133796.1]	Methanogenesis	95%	33.3	Deltaproteobacteria
B3-6	<i>Cloacimonetes bacterium</i> [KJ535434.1]	Methanogenesis	93%		Cloacimonetes
B3-11	<i>Longilinea arvoryzae</i> [NR 041355.1]	Methanogenesis	90%		Chloroflexi
B3-8	<i>Thermophilic bacterium</i> [AJ242834.1]	Fermentation	84%	16.7	Firmicutes
B3-9	<i>Sedimentibacter sp.</i> [AY766466.1]	Fermentation, Dehalogenation	96%		Firmicutes
B3-1	<i>Clostridium sp.6-44</i> [AB596885.1]	Dehalogenation	94%	8.3	Bacteroidetes
B3-10	<i>Prolixibacter bellariivorans</i> [LC015091.1]	Nitrate-reducing	87%	8.3	Bacteroidetes
B3-4	<i>Smithella propionica</i> [NR 024989.1]	Acetogenesis	96%	8.3	Deltaproteobacteria
B3-12	<i>Mycobacterium llatzerense</i> [AJ 746071.2]	Hydrogen-oxidizing	99%	8.3	Actinobacteria

3.2.11 Quantification of *dsrA* gene

Quantification of *dsrA* gene (α -subunit of dissimilatory sulphite reductase) copies associated with sulfate reduction were determined by quantitative polymerase chain reaction (qPCR) (Kondo et al. 2006). The sequences of the primers used were: DSR1F+, (5'-ACSCACTGGAAGCACGGCGG-3') and DSR-R, (5' GTGGMRCCGTGCAKRTTGG-3') (Kondo et al. 2006). Details of the qPCR reaction can be found in Supplementary information and a representative standard curve is shown in Figure S 8.

3.3 RESULTS AND DISCUSSION

3.3.1 Co-treatment performance

The co-treatment showed promising results with respect to increases in pH and alkalinity, and concomitant reductions of COD, sulfate, suspended and dissolved solids, nutrients and metals (Deng and Lin 2013). Briefly, the first stage mixing of various volumetric ratios of AMD and MWW resulted in mixtures with pH (6.2 – 7.9), which was optimal for SRB (Samimi 2013), and varying degrees of phosphate removal (9-100 %). The mixed waste streams also allowed flexibility in adjusting COD/sulfate ratios (0.05 – 5.4), an important factor for the subsequent sulfidogenic treatment. The second stage biological treatment achieved >70% COD removal and sulfate reduction under COD/sulfate ratios 0.9 – 3.1 (Supplementary information Table S1). Alkalinity was produced during the biological treatment, which promoted metal removal from the solutions. Overall, the two-stage treatment achieved significant metal removal (Fe: >97%, Al: ~100%, Mn: 75% - ~100%, Ca: 52 – 81%, Mg: 13% - 76%, and Na: 56% - 76%).

3.3.2 Control experiments

No appreciable COD and sulfate removal was observed in the control experiments (biotic reaction inhibited) during the 15-day period. In contrast, the biological treatment

exhibited approximately 17% and 99% sulfate reduction, and 66% and 90% COD removal in the bioreactors with COD/sulfate ratios of 0.2:1 and 2:1, respectively (Deng and Lin 2013). These results indicated that COD and sulfate removal was predominantly due to biotic processes.

3.3.3 COD oxidation kinetics and models

The maximum COD oxidation rate (V_{max}) and Michaelis–Menten constant (K_m) were estimated as 0.33 mg/L min and 6220 mg/L, respectively (Figure 8 a). The K_m value was much higher than studies using mining granular sludge as a source of SRB (A. H. Kaksonen et al. 2003) and other studies using pure SRB cultures (Oude Elferink 1998; Schönheit et al. 1982; Widdel 1988) (Table 3). The much higher value of K_m obtained in this study suggested that V_{max} cannot be easily achieved in the co-treatment process given the typical COD values in MWW and anticipated mixing with AMD.

Figure 8 (a) Michaelis-Menten and non-competitive inhibition models for sulfidogenic COD oxidation rate (V), and (b) observed and predicted COD oxidation rates by the developed non-competitive inhibition model.

Table 3 Kinetic parameter estimates for COD oxidation by sulfate-reducing cultures.

Culture	K_m	Electron	T	Reference
	(mg l ⁻¹)	donor source	(°C)	
Mixed AMD and MWW culture	6220	AMD and MWW	20	Current Study
Mining granular sludge	4.3-7.1	Ethanol	35	A. H. Kaksonen et al. (2003)
Mining granular sludge	2.7-3.5	Acetate	35	A. H. Kaksonen et al. (2003)
<i>Desulfobacter postgatei</i>	13.6	Acetate	30	Schönheit et al. (1982)
<i>Desulfobacter postgatei</i>	3.8–4.5	Lactate	30	Ingvorsen et al. (1984)
<i>Desulfobacter postgatei</i>		n-Hexadecane	30	Widdel (1988)
<i>Desulforhabdus amnigenus</i>	35	Acetate	37	Oude Elferink (1998)
<i>Desulfobacca acetoxidans</i>	35	Acetate	37	Oude Elferink (1998)
Enrichment culture of SRB	5.9	Acetic acid	31	Middleton and Lawrence (1977)
Mixed culture of SRB and methanogens	9.5	Acetate,Propionate,Butyrate	30	Omil et al. (1998)

Inhibitive effects of iron on COD oxidation of SRB were apparent (Figure 8 b). The relationship between iron concentration and COD oxidation rate was nonlinear. The COD substrate utilization was almost completely inhibited with iron concentrations greater than 60 mg/L. Including the iron inhibitive effects in the kinetics (i.e., Equation 4) produced a closer model fit to the observed COD oxidation (Figure 8, AIC = 47.8 compared to 144.1 of the Michaelis-Menten model). The inhibition constant (K_i) for the attached growth system was estimated to be 6.5 mg/L.

3.3.4 Factors Affecting the COD Oxidation Kinetics

3.3.4.1 pH and Eh

The AMD/MWW mixtures had pH in the range of 6.2 – 7.9, a range optimal for SRB. Redox potential is another crucial factor for microbial reactions and controlling the fate of critical chemical elements such as iron and sulfur. Iron is expected to be in the form of Fe⁺³ prior to entering the bioreactors given the well aerated conditions at the AMD sampling sites and during the first stage mixing. Positive *Eh* values measured in the first stage “mixing” reactor (data not shown) were the further evidences of oxic conditions. In the three bioreactors, non-detectable DO level (~0 mg/L) and ORP values

ranging from -71 to -545 mV were recorded. Under these conditions, iron mostly exists as Fe^{+2} and sulfur in the form of (bi)sulfide, resulting in formation of iron sulfide.

3.3.4.2 Metals

The iron inhibitive effect observed under high iron concentrations in the kinetic model may have been caused by (1) iron (Fe^{+3})-reducing bacteria competing against SRB (Chapelle and Lovley 1992; Lovley and Phillips 1987), and (2) FeS deposited on the surface of SRB resulting in cell inhibition (Zhang et al. 2009). Ram et al. (2000) reported a similar Fe^{+3} inhibitive effect on anaerobic bacterial activities for biogas production from a rabbit waste slurry. Chapelle and Lovley (1992) observed competitive exclusion of sulfate-reducing activities by iron (Fe^{+3})-reducing bacteria in high iron groundwater environments. In this study, total iron in the mixtures for B1, B2, and B3 were 19.0, 12.3 and 3.6 mg/L, respectively. However these iron concentrations did not completely inhibit SRB activities and the low iron concentration in B3 allowed a high COD oxidation rate (Figure 8b). The reactor B1 had the highest iron concentration (19.0 mg/L) and the least number of *dsrA* gene copies (13.3 log gene copies/ μL) compared to other bioreactors (Figure 9).

Figure 9 COD oxidation rate as a function of COD/sulfate ratio (triangles) and SRB *dsrA* log gene copies/ μL (open squares) in the three bioreactors (B1, B2, and B3).

Other potential toxic metals such as Zn, Cd and Cu were not detected in the AMD samples. Mn was detected at low concentrations in the AMD samples (6 ± 2.4 mg/L), but significantly removed in both stages of the treatment (Deng and Lin 2013). Therefore, these elements were not included in the inhibitive model. Similar to pH, iron concentration can be controlled to avoid its inhibitive effects on SRB through dilution and iron phosphate precipitation by maintaining a proper MWW and AMD mixing ratio in the first stage of aerobic mixing.

3.3.4.3 COD/sulfate ratio

COD oxidation rate, V (mg/L·d), was found to increase with COD/sulfate ratio (Figure 9). The results suggest that, within the COD/sulfate range tested, COD was the limiting factor for supporting an active biomass of SRB. Quantification of the *dsrA* gene concentrations in the bioreactors showed a strong positive correlation between the gene concentration and COD oxidation rate (Figure 9). In all three bioreactors, *dsrA* gene concentrations (13.3, 14.7, 15.0 log gene copies/ μ L) were found to be significantly higher than the levels in the feed waste streams of AMD (9.7 log gene copies/ μ L) and MWW (12.5 log gene copies/ μ L). Therefore, it is likely that the *dsrA* genes were enriched as a result of the biological treatment. It has been reported that there are 2 to 3.5 copies of *dsrA* gene per SRB cell (Dar et al. 2009), and thus number of active SRB microorganisms can be reasonably estimated from the gene concentrations.

The COD/sulfate ratio has long been known to have significant effects on microbial community and electron flows with low and high COD/sulfate ratios favoring sulfidogenesis and methanogenesis, respectively (Dar et al. 2008; McCartney and Oleszkiewicz 1993). For this reason, COD/sulfate ratio is a better parameter for predicting substrate utilization rate in sulfidogenic bioreactors than COD concentration alone. A two-parameter kinetic model can therefore be developed to evaluate the combined effects of both COD/sulfate and iron concentration on COD oxidation (Figure 10). It is noted that the projections of the 3-D kinetic model on the x-z (black squares) and y-z planes (blue triangles) are the relationships between COD oxidation rate and predicting parameters in Figure 8 (i.e., COD/sulfate and Fe concentration).

Figure 10 COD oxidation rate, (V), (red solid circles) of the sulfidogenic treatment as a function of COD/sulfate ratio and iron concentration in the AMD/MWW mixtures. COD removal and sulfate reduction percentages as a function of COD/sulfate ratio from 1 to 8 (Results were obtained with inner recirculation ratio=5 and without sludge recycle and all results obtained under each COD/sulfate ratio is calculated on the average of all iron/sulfur molar ratios of 1, 2 and 4).

3.3.5 Stoichiometry and mass balance on COD, iron and sulfur

Theoretical mass of sulfate or iron reduced per mass of COD oxidized were estimated to be 1.5 g SO_4^{2-} /g COD and 7 g Fe^{3+} /g COD (Supplementary information). The observed ratio in the 21 experiments ranged from 0.2 – 10 g sulfate/g COD (Supplementary information Table S1), which frequently exceeded the theoretical value of 1.5. The additional sulfate reduction beyond the theoretical value may have been a result of additional electron donors from endogenous decay of microorganisms for sulfate reduction. In contrast, observed iron/COD utilization ratio ranged from 0.01 to 1.4 g Fe/g COD and were well below the theoretical estimation (i.e., 7 g Fe/g COD). These evidences suggest that sulfate reduction was the predominant COD oxidation pathway with relatively minor contribution of iron reducers to COD oxidation. The reaction pathways and treatment performances are illustrated in Figure 11.

Figure 11 Predominant degradation or removal processes for COD, sulfate and iron in the anaerobic co-treatment bioreactor. Half reactions are detailed in the supplemental material.

Mass balance analyses estimated $47\% \pm 15\%$ of sulfur in the chemical sludge, $8\% \pm 3\%$ of sulfur as dissolved, and the remaining $45\% \pm 11\%$ unaccounted for (e.g., lost through volatilization, incorporated into cells or unaccounted chemical precipitation in the bioreactors) (Supplementary information Figure S 5). A much higher fraction of iron was found in the chemical sludge ($87\% \pm 18.3\%$) compared to its soluble forms ($2\% \pm 1.2\%$). A total of $11\% \pm 3.5\%$ of the iron was unaccounted for in the bioreactors.

The results suggest that biologically mediated iron precipitation is likely occurring and this is further supported by the low iron concentrations in the effluent from the reactors (supplementary information Table S1). Further there may not be enough iron to drive the precipitation of sulfide generated in the reactor.

3.3.6 Sludge characterization

The filtered samples of the AMD and MWW, and biological sludge showed apparent morphological differences (Supplementary information Figure S 9). In comparison, there was a significantly stronger presence of sulfur in the biological sludge than the AMD, MWW, and AMD/MWW mixture (supplementary Figure S 10). A ZAF standardless quantitative analysis of the biological sludge indicated stoichiometric ratio

for Fe:S based on that the atomic percentages was close to 1:1 (1:0.93, supplementary Table S). This suggested precipitation of ferrous sulfide as a main product of the biological treatment.

3.3.7 Phylogenetic diversity

In total, 33 clones were detected in reactors B1 (COD/sulfate=0.2, 12 clones), B2 (COD/sulfate=1, 9 clones) and B3 (COD/sulfate=2, 12 clones). Eight phyla were identified including *Delta-proteobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria*, *Bacteroidetes*, *Synergistetes*, *Actinobacteria*, and *Cloacimonetes* (Table 2). Rarefaction curves (Supplementary information Figure S 7) suggested that majority of the functional group diversity (sulfate reducers, fermenters, and nitrate reducers) in the bioreactors have been identified. The phylogenetic trees of the microbial community clones from bioreactors B1, B2 and B3, and their closely related species are shown in Figure 12. Of the 12 clones in bioreactor B1 (Figure 12a), five were *Deltaproteobacteria* and four were closely related (>92%) to sulfate reduction species (*Desulfovibrio* sp., *Desulfovibrio* sp., *Desulfovibrio* sp. and *Desulfatibacillum* sp.) (Rampinelli et al. 2008). Clones B1-10 and B1-12 were most closely related (with a similarity of 86%) to nitrate-reducing, sugar fermentation bacteria *Prolixibacter bellariivorans* (Holmes et al. 2007) and agar-degrading *Marinilabilia salmonicolor* (Suzuki et al. 1999). The identification of clones related to potential nitrate-reducing microorganisms corresponded to the observed ammonia reduction in the reactor (Deng and Lin 2013).

The clone B2-7 (Figure 12b) belongs to *Bacteroidetes* and is closely related (>96%) to dehalogenating and fermentative *Clostridium* sp.6-44 (Lin et al. 2013) which are able to convert trichloroethene (TCE) to ethane (Ise et al. 2011) and also able to ferment organics to sugars, ethanol, lactate in anaerobic digesters (Palatsi et al. 2010). The presence of this microorganism suggests that the co-treatment reactor has the potential to treat high salinity wastewater. The clone B2-3 is 89% similar to *Leptolinea tardivitalis* which belongs to *Chloroflexi* subphylum and was found in mesophilic and thermophilic methanogenic sludge granules (Yamada et al. 2005) suggesting the possible co-existence of methanogenic and sulfidogenic bacteria while sulfidogenic bacteria remain dominant.

Bioreactor B3 (Figure 12c) contained the most diverse and evenly distributed microbial community based on Simpson's index (1-D of 0.79), Shannon index (H = 1.68) and evenness (E = 0.89) and also had the highest COD removal efficiency. Clone B3-1 is closely related (94%) to dehalogenating and fermentative *Clostridium* sp.6-44 similarly to clone B2-7. Clone B3-10 is related (87%) to anaerobic sugar fermenting, psychrotolerant nitrate-reducing *Prolixibacter bellariivorans* which can grow at temperatures as low as 4°C (Holmes et al. 2007). Clone B3-9 is 96% similar to *Sedimentibacter* sp. which was found in hexachlorocyclohexane (HCH) polluted soil and associated with dechlorination and growth of *Dehalobacter* sp.(van Doesburg et al. 2005).

Clones B1-1, B1-5, B3-2, B3-7 were highly related to neutrophilic and acidophilic SRB, namely *Desulfovibrio* sp. and *Desulfomicrobium* spp. which are known to oxidize substrates incompletely to acetate (G. Macfarlane 1991). Clones (in B1 and B3) related to nitrogen reducing bacteria supported the previous finding of nitrogen reduction in the bioreactors (Deng and Lin 2013). All these clones suggest that combined treatment

reactors have the potential to treat acidic, high-salinity, nutrient and sulfate rich wastewater under low to normal temperature conditions.

Microbial diversity in clone library was found to increase with increasing COD/sulfate ratios. All of the reactors have a significant amount of sulfate reducing organisms in different genera and the findings were further supported by the quantification of sulfate reduction associated *dsrA* gene copies. The highest concentration of *dsrA* gene copies was detected in B3 (COD/sulfate ratio = 2), which also had the highest pH (7.9) and lowest redox potential (-545 mv) compared to other bioreactors. Studies (Hiibel et al. 2011; Koschorreck et al. 2010; Sánchez-Andrea et al. 2011) suggest that pH values and redox potential conditions, and COD/sulfate ratios affect the development of diverse communities and the increase of microbial diversity stabilized the biofilm function under fluctuating conditions.

The co-treatment process integrates carbon, sulfur, and nitrogen cycles into wastewater treatment in one system. Unlike methanogenesis, a wide range of substrates can be utilized by sulfidogenes at a wide range of temperatures (10 °C to 45 °C). Wastewaters with pH 4 – 9 can be treated by moderate psychrophilic, thermophilic, neutrophilic and acidophilic SRB (Bijmans et al. 2010; Braissant et al. 2007; Sánchez-Andrea et al. 2014). This would promote the applications of the anaerobic treatment of various sulfate-rich industrial and municipal wastewater treatments.

a)

Figure 12 Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences in batch reactors B1, B2 and B3. Bootstrap values (1,000 replicates) above 50% are represented at the nodes. The scale bar represents 0.2 changes per 100 nucleotides. In Fig. 12, a), b) and c) refers to sequences from B1, B2 and B3 reactor specifically.

3.4 CONCLUSION

This study provides critical information regarding the performance of sulfidogenic bioreactors treating AMD and MWW. This work demonstrates that an SRB attached-growth reactor can efficiently facilitate removal of COD from wastewater while reducing sulfate, raising pH, and lowering concentrations of metals (Deng and Lin 2013). Proper control of the mixing ratio of MWW and AMD is necessary to avoid Fe inhibitive effects on SRB and to obtain favorable COD/sulfate ratios of the mixture solution for the biological treatment.

The present research demonstrated that in the co-treatment system, the dominant species belong to the *Deltaproteobacteria* group. The bioreactor which achieved the highest COD and sulfate removal rates (i.e., B3) supported the most active SRB biomass, and had both higher percentage of *Deltaproteobacteria* and more balanced microbial diversity.

The microbial community provided insights into the key microbes and metabolic pathways and how chemical substances (e.g., COD/sulfate ratio, Fe) would affect biological treatment. The microbial DNA analyses and chemical profiling demonstrate the feasibility of the treatment approach and the results provide a base line for future studies to further develop the technology. Further evaluations over extended time periods are necessary to determine how the co-treatment system performs for continuous treatment of the two wastes.

3.5 SUPPLEMENTARY INFORMATION

Table S1 Initial and final COD, sulfur and total iron concentrations, initial COD/sulfate ratios, and calculated mass of sulfate reduced per gram of COD oxidized for 21 COD/sulfate ratios tested. Experiments were conducted in replicates and averages are listed in the table.

All units are in mg/L										
Treatment	COD _{in}	Sulfate _{in}	Fe _{in}	COD/sulfate ratio	COD _{out}	Sulfate _{out}	Sulfide _{out}	Fe _{out}	g sulfate/g COD	g Fe/g COD
1	55.7	606.9	41.2	0.09	20.4	575.1	4.1	0.5	0.90	1.15
2	57.7	656.9	29.1	0.09	21	543.2	6.5	0.4	3.10	0.78
3	116.2	168.8	28.1	0.69	22.3	58.7	2.4	0.2	1.17	0.30
4	126.2	198.8	25.1	0.63	20.4	75.2	2.7	0.8	1.17	0.23
5	125.3	108.9	12.3	1.15	12.1	4.6	0.9	0.1	0.92	0.11
6	135.3	118.9	16.3	1.14	12.6	1.3	1.2	0.3	0.96	0.13
7	258.4	1090.2	19	0.24	87	900	9.1	0	1.11	0.11
8	475.9	610.2	42.8	0.78	173.5	39.4	4.9	0.3	1.89	0.14
9	321.6	536.5	54	0.60	35.8	34.6	4.3	0.1	1.76	0.19
10	857	158.3	51.4	5.41	254.6	24	1.5	0.4	0.22	0.08
11	51.4	879	45.6	0.06	0	702	8.3	1	3.44	0.87
12	29.5	553.1	33.1	0.05	5.3	387.1	5.6	0.2	6.86	1.36
13	25.6	526.6	33.4	0.05	0	266.1	3.4	0.1	10.18	1.30
14	42	329.9	32.3	0.13	3.5	135.5	3.7	0.7	5.05	0.82
15	436	268	31.4	1.63	83	42	3.2	0.9	0.64	0.09
16	424	224	3.6	1.89	42	3	2.9	0	0.58	0.01
17	473	196	39.5	2.41	90	16	2.6	0.5	0.47	0.10
18	524	167	33.6	3.14	154	28	2.4	0.8	0.38	0.09
19	103.6	777.2	32.2	0.13	27.5	692	5.5	0.5	1.12	0.42
20	660.8	781.6	39.5	0.85	79.6	111.6	6.7	0.8	1.15	0.07
21	2150	822.3	33.6	2.61	423.6	0	6.9	0	0.48	0.02

Theoretical electron balance calculation

(Redox reactions used include those concerning sulfate/domestic wastewater and iron/domestic wastewater).

All half reaction equations were obtained from Table 7-6 in (Metcalf et al. 2013) which was adapted from (McCarty 1975) and (Sawyer et al. 2003).

SO₄²⁻ half reaction:

Domestic wastewater (COD) half reaction:

Combined reaction of the COD oxidation and sulfate reduction, i.e., (1) + (2) $\rightarrow$ (3)

$\rightarrow$ 6.25 mole SO_4^{2-} are reduced by 1 mole of $C_{10}H_{19}O_3N$

According to

Oxidation of one mole of $C_{10}H_{19}O_3N$ requires 12.5 moles of O_2 . Therefore 0.5 mole SO_4^{2-} is to be reduced by 1 mole of O_2 (reaction 3), which results in **1.5 g SO_4^{2-} consumed per g of COD.**

Fe^{3+} half reaction:

Combined reaction of the COD oxidation and iron reduction, i.e., (5) + (2) $\rightarrow$ (6)

$\rightarrow$ 7 g Fe^{3+} reduced per g of COD.

Control experiments setup Pre-determined volumes of the AMD, MWW and sludge samples were mixed in 500 ml glass media bottles to obtain COD/sulfate ratios of 0.2:1 and 2:1. The mixture solutions were sampled and sonicated (2 min) before transferring into reactors. Microbial activities were inhibited by adding sodium azide (2 g/L) to the mixture solution. The control reactors were maintained under equivalent conditions as the sulfidogenic bioreactors and pH, COD and sulfate were measured over time.

PCR for 16S rRNA. The 16S rRNA genes for clones B2-8 to B2-9, B3-4 to B3-7 and B3-10 to B3-12 were amplified by PCR in 25 μ l mixtures containing 0.3 μ m of each

primer (8F and 1492R), 1X PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂ (Applied Biosystems), 0.5 mg/ml BSA (New England Biolabs, Ipswich, Massachusetts, USA), 0.2 mM dNTPs (Applied Biosystems), 0.5 U of *Taq* polymerase (Fisher Scientific, Pittsburgh, PA) and 25 to 185 ng of template DNA. The sequences of the primers used were: 8F, (5'- AGAGTTTGATCCTGGCTCAG -3'); 1492R, (5'- GGTACCTTGTACGCTT -3')(Weisburg et al. 1991). The thermocycling conditions included 15 min initial denaturation at 95°C, 35 cycles of denaturation (95 °C for 1 min), annealing (53.5 °C for 1 min), extension (72 °C for 1 min), and final extension for 5 min at 72 °C. The 16S rRNA genes for clones B1-1 to B1-12, B2-1 to B2-7, B3-1 to B3-3 and B3-8-B3-9 were amplified by PCR in 25 µl mixtures containing 1X PCR master mix (Applied Biosystems), 0.2 µm of each primer (8F and 1492R) and 25 to 185 ng of template DNA. The thermocycling conditions were the same as above. Each PCR run included negative controls (e.g., DNA-free water instead of template). The PCR products were purified with a QIAquick PCR Purification kit (Qiagen) and visualized by agarose gel electrophoresis using ethidium bromide stained (0.2 mg/L) 1% agarose gels (Supplementary information Figure S 6).

Diversity calculation details Shannon index and the Simpson's index of Diversity (Dunbar et al. 2000) were chosen to characterize the microbial diversity of bioreactor samples (supplementary material Table S) and rarefaction curves were calculated (supplementary material Figure S 7). Simpson's index of diversity, Shannon index, evenness and rarefaction curves were calculated using PAST (Hammer et al. 2001). In order to calculate the diversity indices, Shannon's H and evenness, the 16S rRNA clones were partially sequenced and clones were grouped into species; for rarefaction curve calculation, the clones were grouped into functional groups, as defined by putative function in Table 2.

qPCR reaction details for *dsrA*. The extracted and purified DNA from the bioreactors was used as template DNA. Amplification was performed using the real-time PCR system (7300 real-time PCR, Applied Biosystems, Foster City, CA). The qPCR mixture (25 µl) contained 1X of SYBR master mix (Applied Biosystems, Carlsbad, CA, USA), 0.5 mg/ml BSA, 0.4 µm of DSR1F+ and DSR-R primer and 5 to 10 ng of template DNA. The qPCR reactions were run under the following conditions: 15 min initial denaturation at 94°C, 40 cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec), extension (72°C for 1 min), and hold at 72°C for 7 min, followed by a dissociation curve analyses. A serial dilution of plasmid DNA containing inserted *dsrA* genes was used to generate a qPCR standard curve (supplementary information Figure S 8). Triplicate measurements of each standard concentration were made. The qPCR detection of the *dsrA* gene sequence remained linear from minimum 2.4×10^5 up to the maximum concentration of 1.8×10^9 copies per microliter of DNA extraction, and the linear regression R² value was 0.99, with an efficiency of 93.4%. Positive controls in each qPCR run consisted of plasmids containing the *dsrA* gene, while negative controls consisted of DNA-free water.

Table S2 Clones and corresponding accession number

Clone	Accession number	Clone	Accession number
B1-1	KP962316	B2-6	KP731372
B1-2	KP962317	B2-7	KP731373
B1-3	KP962318	B2-8	KP731374
B1-4	KP962319	B2-9	KP731375
B1-5	KP731362	B3-1	KP962296
B1-6	KP731363	B3-2	KP962297
B1-7	KP731364	B3-3	KP962298
B1-8	KP731365	B3-4	KP962299
B1-9	KP731366	B3-5	KP962300
B1-10	KP731367	B3-6	KP962301
B1-11	KP731368	B3-7	KP962302
B1-12	KP731369	B3-8	KP962303
B2-1	KP962320	B3-9	KP962304
B2-2	KP962321	B3-10	KP962305
B2-3	KP962322	B3-11	KP962306
B2-4	KP731370	B3-12	KP962307
B2-5	KP731371		

Table S3 Comparison of diversity indices, Shannon's H and evenness values for the B1, B2 and B3 bacterial communities, derived from three different methods

	B1	B2	B3
Simpson index of diversity (1-D)	0.68	0.72	0.79
Shannon (H)	1.24	1.43	1.68
Evenness	0.86	0.83	0.89

Figure S 5 Iron and sulfur mass balance in the batch reactor treatment. Initial sulfur is counted as total sulfur, final sulfur as the soluble, sulfur in the sludge as the precipitated, and the rest labelled as unaccounted. Error bars represent 21 times of reactor runs data ± 1 standard deviation.

Figure S 6 Electrophoresis gel run of PCR products after PCR amplification of extracted DNA from bioreactors B1, B2 and B3 (in duplicates) and positive control

Figure S 7 Rarefaction curves showing diversity of B1, B2 and B3 clone libraries vs. number of functional groups.

Figure S 8 A representative standard curve of CT values vs. Log gene concentration in sulfate reducers. The mean and standard deviation of triplicate samples is graphed vs. sulfate reducers 16S rRNA gene copies per μL .

Table S4 Chemical elements in the biological sludge from sulfidogenic treatment of a 1:1 AMW/MWW mixture using ZAF method standardless quantitative analysis

Element	Weight %	Atomic %	K-Ratio	Z	A	F
C	17.51	32.83	0.0698	1.0981	0.3629	1.0003
N	2.86	4.61	0.0106	1.085	0.3406	1.0006
O	24.8	34.92	0.1241	1.0729	0.466	1.0005
Fe	22.51	9.08	0.1202	0.8833	0.6046	1.0002
Na	0.36	0.35	0.0023	1.0004	0.6501	1.0008
Mg	0.79	0.73	0.0062	1.0237	0.7664	1.0015
Al	1	0.84	0.0084	0.9884	0.8473	1.0029
Si	1.58	1.27	0.0145	1.013	0.9011	1.005
P	0.87	0.64	0.008	0.9763	0.9348	1.0088
S	11.95	8.4	0.115	0.9987	0.9585	1.005
Ag	7.13	1.49	0.0549	0.76	1.0061	1.0071
Ca	8.62	4.84	0.0825	0.972	0.9814	1.0038
Total	100	100

Figure S 9 SEM micrographs of particulate matters in the a) AMD, b) MWW, c) 1:1 AMD/MWW mixture, d) 1:1 AMD/MWW mixture after the sulfidogenic treatment

Figure S 10 EDS spectra of the particulate matters in the AMD, MWW, 1:1 AMD/MWW mixture, and 1:1 AMD/MWW mixture after the sulfidogenic treatment

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CHAPTER 4: PHASE II (STAGE 3) CONTINUOUS FLOW OF SULFIDOGENIC WASTEWATER TREATMENT WITH FES SLUDGE RECYCLING

Research Objective: Investigate the relevant factors and optimize the operating conditions for the continuous MWW treatment. treatment performance based on COD/sulfate inflow/outflow concentrations, comparison with traditional treatment technology efficiency, redox potential, pH, alkalinity, iron, sulfide, TSS, VSS and TDS concentrations, and mass balance of iron and sulfur in combined treatment. The treatment system with FeS sludge recirculation would be compared with no FeS recirculation to determine if the recirculation is beneficial.

4.1 INTRODUCTION

Introducing acid mine drainage (AMD) in municipal wastewater (MWW) can offer multiple environmental and energy benefits over conventional methods such as activated sludge (Winfrey et al. 2010; W. Strosnider et al. 2011a, 2011b; McCullough et al. 2008; Deng and Lin 2013). In such co-treatment systems, sulfate reducing bacteria (SRB) are used to facilitate organic oxidation while sulfate is reduced to (bi)sulfide under anaerobic conditions. Such treatment is energy efficient because it does not require aeration for microbial oxidation of organics in the wastewater. Additional benefits include reduced CO₂ production and emission, removal of selected heavy metals (e.g., Cd, Cu, Hg, Pb and Zn) due to their low solubility with sulfide (Barnes et al. 1991; Bhattacharyya et al. 1981; Jong and Parry 2003; W. H. Strosnider et al. 2013), and low biological yield (Hoehler et al. 2001; Postgate 1979).

In sulfidogenic treatment, the degree of organics removal depends on the relative amount of sulfate, and the chemical oxygen demand (COD) to sulfate ratio was typically used to examine such effects (Damianovic and Foresti 2007; Friedl et al. 2009; Jeong et al. 2008; Lens et al. 1998; Vossoughi et al. 2003). Theoretically, enough sulfate is available to completely oxidize the organics when the COD/sulfate ratio is above 0.67 (Rinzema and Lettinga 1988). In reality, optimum COD/sulfate ratio is influenced by the composition and concentration of the organic matter. Active competition was observed between SRB and methane-producing bacteria (MPB) when the COD/sulfate ratio was 1.7-2.7, and SRB were dominant over MPB when the COD/sulfate ratio was greater than 1.7 (Choi and Rim 1991).

One potential issue with sulfidogenic treatment of MWW where AMD does not co-exist is an insufficient amount of sulfate for the oxidation of organics given the typical sulfate and organics concentrations of MWW (Metcalf et al. 2013). Incorporating iron in the sulfidogenic treatment can overcome this potential drawback and enable multiple (bio)chemical reactions in engineering designs that facilitate removal of a wide range of

contaminants from MWW. Specifically, low solubility of iron phosphate can be an effective mechanism for retaining phosphorus from wastewater and reducing nutrient loads to receiving waters. Precipitation of iron sulfide due to its low solubility (amorphous ferrous sulfide $K_{sp} \approx 10^{-3.05}$ (Emerson et al. 1983), can limit sulfide toxicity on SRB and control sulfide levels in the treated effluents. The formed iron sulfide sludge materials can be oxidized into ferric sulfate and recycled to the wastewater influent to supplement sulfate and iron for continuous treatment. With the abundance and widespread presence of iron, tremendous opportunities exist for incorporating iron in innovative MWW treatment technologies to realize the “green” benefits of this treatment approach.

The goal of this study was to evaluate the technical feasibility of an innovative iron-dosed sulfidogenic treatment process with iron sulfide sludge recycling to supplement sulfate and iron for continuous wastewater treatment. A bench-scale treatment process containing two packed-bed bioreactors was constructed to treat synthetic wastewater under a range of the key chemical factors (i.e., COD/sulfate ratio and Fe/S ratio) to optimize treatment performance. The treatment performance of the process and its potential were evaluated by COD removal efficiency and effluent quality. Physicochemical properties of the sludge materials were characterized and the effects of recycling were assessed by contrasting the treatment performance parameters between treatment periods with and without sludge recycling. Chemical states and mass balance of key elements, Fe and S, at different stages of the treatment process were examined to characterize the treatment process and illustrate the treatment mechanisms. Solid content and composition were monitored over long-term operation of the bioreactors.

4.2 MATERIAL AND METHODS

4.2.1 Bench-scale sulfidogenic treatment process

A bench-scale treatment process consisting of a wastewater reservoir, ferrous chloride reservoir, two parallel sulfidogenic bioreactors, and an oxidizing basin was constructed and used in this study (Figure 13).

Figure 13 Fe(II)-dosed sulfidogenic treatment process with sludge oxidation and recycle.

4.2.1.1 Wastewaters reservoir

A 57-L tank was used as a wastewater reservoir to supply wastewater for continuous sulfidogenic treatment. A synthetic wastewater containing 2.26 mM ethanol (C_2H_6O), 0.45 mM lactose ($C_{12}H_{22}O_{11} \cdot H_2O$) and 1.61 mM sodium acetate ($C_2H_3O_2Na \cdot 3H_2O$), 1.68 mM sodium bicarbonate ($NaHCO_3$), and trace elements (Supplementary information Table S5, 5 ml/L influent) (Diekert et al. 1992) was used in this study. In addition, different amounts of sodium sulfate (0.56-4.44 mM Na_2SO_4) were mixed with the synthetic wastewater to allow testing of the effects of COD/sulfate ratio on the sulfidogenic treatment.

4.2.1.2 Ferrous chloride reservoir

A 4-L tank containing a ferrous chloride solution ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, pH= 3.2-3.4) was used to evaluate the effects of iron dose on the sulfidogenic treatment of the wastewater. A range of ferrous chloride concentrations (0.56-17.76 mM) resulting in a range of Fe/S molar ratios (1-4) was used to investigate the effects of ferrous dosing

4.2.1.3 Sulfidogenic bioreactors

Duplicate sulfidogenic bioreactors (BR1 and BR2, 2.5 L each) were constructed and used to treat the synthetic wastewater. The bioreactors were made of an acrylic cylinder with inlets on the top for the synthetic wastewater and ferrous dosing. Each bioreactor was packed with plastic media (Kaldnes K1, specific surface area= $500 \text{ m}^2/\text{m}^3$, Evolution Aqua Ltd, UK) for attached growth of microorganisms. A perforated acrylic plate was used to support the packing media and a coned-shaped bottom was used to allow sludge settling and collection. There were ports on the side of the reactors for internal recirculation to promote hydraulic mixing and treatment efficiency. The attached growth design allowed development of microbial communities more resistant to potential toxic effects of metals (Parkin and Speece 1983) and convenient separation of iron sulfide precipitates from the attached biomass by their settling to the cone-shaped bottom of the bioreactors. The bioreactors each provided a working volume of approximately 1.5L with the packing media and sludge biomass.

Anaerobic digester sludge from a municipal wastewater treatment plant (Star City, West Virginia) and AMD sludge from a mine portal along Dunkard Creek (near Bobtown, Pennsylvania) were collected, mixed at 1:1 vol ratio and used to inoculate the bioreactors. No additional growth media was used during the inoculation. The bioreactors were operated at room temperature ($21 \pm 1^\circ\text{C}$) and under anaerobic condition. The air tight bioreactors were sparged with syringe filtered ($0.45 \mu\text{m}$, Fisherbrand, Ireland) N_2 gas prior to operation.

4.2.1.4 Oxidizing basin

The oxidizing basin was a 4L wide-mouth conical flask with 30 pieces of plastic media (Kaldnes K1, Evolution Aqua Ltd, UK) for attached growth of microorganisms. During sludge oxidation operation (6 days each time), sludge samples from the bioreactors were collected and added to the oxidizing basin daily. A magnetic stirrer was used to mix the sludge under aerobic conditions (i.e., open flask with mixing) to transform iron sulfide minerals into ferric and sulfate ions. At the end of the oxidation period, the oxidized sludge solution was then mixed with the wastewater influent (1:1 vol ratio) to evaluate the effects of sludge recycling. Samples of the sludge were taken for chemical analyses before and after mixing with the wastewater.

4.2.2 Experimental design

The bench-scale experiments were conducted in two phases. In phase I (450-day study period), the synthetic wastewater was continuously treated in the bioreactors to determine optimal conditions of COD/sulfate ratio and ferrous loading rate. In this phase, the experiments were conducted without sludge oxidation or recycling, and both BR1 and BR2 were operated under the same conditions as duplicates. The bioreactors were tested for their performance under a range of the COD/sulfate mass ratios (1, 1.3, 2, 4, and 8) and Fe/S molar ratios (1, 2 and 4) with each ratio combination lasting for approximately 2 months (1-month acclimation followed by a 1-month sampling period). In these experiments, influent flow rate (wastewater plus $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 3.5 L/d) and an internal recirculation (18.4 L/d, flow ratio = 5.3) were used. During each experiment, influent and effluent samples (20 each) were collected to quantify COD oxidation and sulfate reduction in the bioreactors. Iron retention, defined as loss of iron mass to chemical precipitation in the sludge or retained in the bioreactors, was calculated by the difference of the iron load between the influent and the effluent normalized by the influent load (%). The phase I results were analyzed to select the optimal chemical loading of COD/sulfate and ferrous iron for phase II experiments.

In phase II, the bioreactors were operated with periodic iron sulfide sludge collections and recycling over a 62-day period. Both BR1 and BR2 were operated under the same chemical loads. In each event, sludge materials (100 mL wet sludge from each bioreactor) were extracted from the bottom of the bioreactors daily and added to the oxidizing basin daily for six days. On day 6, the oxidized sludge solution was recycled to the bioreactors at the same flow rate as the synthetic wastewater. The averaged retention time for sludge oxidation was 3 days and the recycling of the oxidized solution lasted approximately 9 h each time. During the 62-day operation, 7 occasions of sludge recycling were performed. Regular samplings were done to allow comparisons of the treatment performance with and without the sludge recycling.

4.2.3 Control Experiments

Control experiments were also conducted to quantify the contribution of abiotic processes to COD removal and sulfate reduction. The mixed wastewater and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ solutions were sonicated (10 min) and then transferred into an anaerobic reactor which was maintained under same conditions as the sulfidogenic bioreactors. Microbial activities were inhibited by adding a 2% sodium azide solution. The control experiments lasted for 30 days, and samples were taken every 2 days. The samples from the control experiments were analyzed using the same methods as the sulfidogenic treatment samples.

4.2.4 Chemical analyses

An YSI meter with pre-calibrated probes (YSI 63) was used to measure pH and conductivity. Autotitrators were used for alkalinity (Thermo Scientific Orion 950,

Standard Method 2320 B) and acidity (Mettler Toledo DL50, Standard Method 2310 B) analyses. Samples for iron analyses were preserved with concentrated trace metal-grade nitric acid and stored at 4°C until nitric acid-perchloric acid digestion followed by determination using an atomic adsorption spectroscopy (AAS, Perkin Elmer 3100) following Standard Method 3030H. The digested samples were filtered through 0.45-µm nylon filters before the analysis for total iron concentration. Ferrous iron was determined using the 1, 10 Phenanthroline method (Standard Method 3500 B) and ferric iron was determined by the differences between total and ferrous iron concentrations (Standard Method 3500 B).

Total solids (TS), total suspended solids (TSS), total dissolved solids (TDS), and volatile suspended solids (VSS) were determined following the standard methods (APHA 2005). Sulfate concentrations were measured using a turbidimetric method (USEPA method 375.4) with a UV-Vis spectrophotometer (Thermo Scientific Genesys 10S). COD concentrations were determined using a closed reflux, colorimetric method with a spectrophotometer (ASTM D1252-06 2006), and sulfide concentrations measured by a methylene blue method (USEPA method 376.2). Duplicates of sulfate and COD concentrations were measured for each sample. Redox potential (ORP) was measured using a polished platinum probe with an Ag/AgCl electrode as a reference (EW-27018-40, Cole Parmer, Vernon Hills, IL, USA).

4.2.5 Sludge characterization

The sludge materials extracted from the bioreactors were analyzed for solid contents including TSS, TDS, VSS, and NVSS. They were also analyzed by X-ray photoelectron spectroscopy (XPS, Physical Electronics PHI 5000 VersaProbe) to determine the chemical states of iron and sulfur. The sludge samples were first dried in a closed chamber filled with calcium sulfate and flushed with nitrogen to remove the moisture content and prevent sludge oxidation (Karamalidis et al. 2008) until the weight was constant. The sludge samples were then powdered and the sample powder was mounted in the standard sample holder with a zero reflective quartz plate (MTI Corporation, CA) placed underneath. XPS spectra were obtained using XPS equipped with a monochromatized Al K α X-ray source (1487 eV). The base pressure in the analytical chamber was 10⁻⁷ Pa. The instrument work function was set to give a value of 84 eV for the Au line of metallic gold. Narrow region photoelectron spectra were acquired to obtain chemical state information for iron and sulfur.

Surface morphology and elemental composition were analyzed using a scanning electron microscope (SEM, Hitachi S-4700F) coupled with energy dispersive X-ray spectroscopy (EDX, EDAX Genesis). The sludge samples were prepared using the same method as XPS (dried in the closed chamber) and analyzed under an accelerating potential of 5-10 kV. Qualitative elemental analysis of the sludge samples was conducted

by EDX spectrometry operated under an accelerating potential of 20 kV. All samples were mounted on aluminum sample stubs and gold-coated to minimize surface charging.

4.3 RESULTS AND DISCUSSION

4.3.1 Phase I: sulfidogenic treatment without sludge recycle

4.3.1.1 Control vs. sulfidogenic treatment

In the control experiments, 10-25% COD removal was observed. This was substantially less than that observed in the sulfidogenic bioreactors. Sulfate reduction was negligible (Figure 14 a and b). This indicates that COD removal in the sulfidogenic bioreactors was predominantly biotic due to SRB.

Figure 14 Phase I a) COD removal, b) sulfate reduction, c) iron retention and d) pH and as a function of COD/sulfate mass ratio (1, 1.33, 2, 4, 8) and Fe/S molar ratio (1, 2, 4). Results were obtained with internal recirculation ratio=5.3 and without sludge recycle.

4.3.1.2. Steady state evaluation

A study of transient conditions during the 1-month acclimation period showed that the bioreactors reached a steady state by day 20 (Supplementary information Figure S 11). This suggested that the 1-month period followed by a 1-month sampling period was sufficient for studying the effects of different COD/sulfate and Fe/S ratios.

4.3.1.3. Effects of COD/sulfate and Fe/S ratios

Over the range of COD/sulfate ratios tested (i.e., 1-8), COD removal (%) decreased as the ratio increased (Figure 14a) reflecting the decreasing availability of sulfate as the electron acceptor for COD removal. Sulfate reduction (%) exhibited the opposite trend (Figure 14b). Under each COD/sulfate ratio, the bioreactors were progressively less efficient at COD oxidation and sulfate reduction as Fe/S molar ratio increased from 1 to 4. A stoichiometric ratio of 1:1 (Fe/S) yielded the most favorable treatment performance for both COD oxidation and sulfate reduction. The decreased treatment performance was mainly due to the effect of low pH on SRB from the increased loading of acidic ferrous chloride solution (Figure 14d). The optimal pH for SRB is 5.8-8.0 (Widdel 1988; Al Zuhair et al. 2008; Reis et al. 1992; Vogels et al. 1988). In addition to the pH effects, the excessive loads of ferrous ion over sulfate resulted in substantially higher levels of dissolved iron in the bioreactor effluent (Figure 14c).

Of the different combinations of the chemical ratios, a COD/sulfate of 2 (mass ratio) and Fe/S of 1 (molar ratio) yielded the best treatment performance with $84 \pm 9\%$ COD removal, $94 \pm 6\%$ sulfate reduction, and good iron retention ($99 \pm 1\%$) under favorable pH conditions (6.2-7.0). This optimal COD/sulfate ratio was consistent with previous sulfidogenic studies (Choi and Rim 1991; Damianovic and Foresti 2007; El Bayoumy et al. 1999; Hirasawa et al. 2008; Velasco et al. 2008).

4.3.1.4. COD oxidation rate

The COD oxidation rate was calculated based on the mass balance equation under steady-state conditions (Supplementary information). The average reaction rate ranged from 338 to 865 mg/L·d under different chemical load combinations (Figure 15a). These rates were comparable or better than the maximum reaction rates ($V_{\max}$) reported in other sulfidogenic studies using mixed cultures and in general lower than those using pure SRB cultures (Table 4). Sulfate reduction rates were also calculated and the average rates ranged from 105 to 726 mg/(L·d) under different chemical load combinations (Figure 15b). These reactions rates compared favorably to a cotreatment system used to treat a high-strength AMD and municipal wastewater (54 mg/(L·d), W. Strosnider et al. (2011a)) and a passive SRB bioreactor under optimum field conditions (29 mg/(L·d), URS (2003)). The sulfate reduction rates in the current study showed a wider range than those observed in a laboratory study using ethanol, lactic acid and glycerol as electron donors (250-300 mg/(L·d), Kolmert and Johnson (2001)).

Figure 15 Phase I (a) COD oxidation and (b) sulfate reduction rates estimated by steady-state concentrations of COD and sulfate under a range of COD/sulfate mass ratios (1, 1.33, 2, 4, 8) and Fe/S molar ratios (1, 2, 4) with hydraulic retention time 0.43 days.

Table 4 Michaelis-Menten kinetic parameters for COD oxidation by sulfate reducing cultures

Culture	v_{max} mg/(L·d)	K_m mg/L	Reference
Enriched culture from anaerobic digester sludge	475.2	4.3	Deng et al. (2016)
Enriched culture from mining areas	316.8	--	Sahinkaya et al. (2007)
Mixed culture of SRB and methanogens	47.5-50.4	2.7-3.5	Kaksonen et al. (2003)
Mixed culture of SRB and methanogens	936	9.5	Yoda et al. (1987)
<i>Desulfobacter postgatei</i>	576-1728	13.6	Schönheit et al. (1982)
<i>Desulfobacter postgatei</i>	4320-4464	3.8–	Ingvorsen et al. (1984)
<i>Desulfobacter postgatei</i>	10224-13536	4.5	
<i>Desulfobacter postgatei</i>	13536	NR	Widdel (1988)
<i>Desulforhabdus amnigenus</i>	2448	35	Oude Elferink (1998)
<i>Desulfobacca acetoxidans</i>	3600	35	Oude Elferink (1998)

4.3.1.5 Sludge morphology and chemical composition

SEM-EDX analysis indicated significant presence of iron and sulfur in the biogenic sludge (Supplementary information, Figure S 12). XPS analysis on the biogenic sludge material showed the presence of sulfide ion (161.4 and 162.9 eV) and significantly

strong presence of ferrous iron (708.6 eV) (Supplementary information, Figure S 13). The analysis revealed atomic percentages of C:O:Fe:S as 41:30:12:17, which corresponds to weight percentages of 22:22:31:25. These results suggest the co-existence of ferrous sulfide (FeS) and pyrite (FeS₂) as a result of the sulfidogenic treatment.

4.3.2 Phase II: sulfidogenic treatment with iron sulfide sludge oxidation and recycle

Based on the Phase 1 results, a COD/sulfate mass ratio of 2 and a Fe/S molar ratio of 1 (i.e., COD = 1384 mg/day, SO₄²⁻ = 692 mg/day, and Fe²⁺ = 404 mg/day) were selected to evaluate the technical feasibility of the sulfidogenic treatment with sludge recycling. Effects of the sludge oxidation and recycling are described and discussed in the following sections.

4.3.2.1 Sludge oxidation

In the oxidizing basin, the mixed influent pH ranged from 5.9 to 6.2 after the oxidized sludge solution (pH 2.3 ± 0.02) was mixed with wastewater influent. The relatively lower pH in the oxidizing basin compared to the synthetic wastewater and those in the bioreactors (6.2 ± 0.4), was a result of FeS/FeS₂ oxidization, which generated acidity (Schippers and Jørgensen 2002).

4.3.2.2 COD removal, sulfate reduction, and pH

Recycling of the oxidized sludge materials caused obvious effects on all relevant water quality parameters (Figure 16). Compared to baseline operation, sludge recycling caused significant increases in influent COD (653 ± 87 mg/L vs. 395 ± 22 mg/L) and sulfate-S (94 ± 12 vs. 71 ± 11 mg/L, respectively) concentrations (Figure 16 a and b). The changes in chemical loads due to sludge recycling resulted in enhanced COD removal (90 ± 6% vs. 75 ± 7%), but similar sulfate reduction efficiency (92 ± 4% vs. 93 ± 7%) as evident in the overall lower effluent COD (65 ± 33 mg/L vs. 100 ± 27 mg/L) and sulfate-S concentrations (7.0 ± 3.4 mg/L vs. 5.0 ± 5.1 mg/L) (Figure 16 a and b). The sludge recycling also caused slight decreases in influent pH (7.3 ± 0.2 vs. 7.7 ± 0.3, Figure 16 c). This did not negatively impact SRB, and resulted in an elevated pH in the effluent (6.8 ± 0.1 vs. 6.5 ± 0.2) due to additional alkalinity generation from increased sulfate reduction. Overall, sludge recycling yielded enhanced COD removal even with the additional COD loads from the recycling, and resulted in better effluent quality in terms of COD and pH.

Figure 16 Phase II a) COD, b) sulfate as S, c) pH, d) TSS, e) TDS, f) VSS, g) iron, and h) sulfide as S concentrations during the 62-day operation of the bioreactors. The shaded areas mark the sludge recycling occurrences. The box plots show the statistics of influent (Inf) and effluent quality during the baseline operation (BR) and time periods with sludge recycling (BR/R).

4.3.2.3 TDS, TSS and VSS

Sludge recycling resulted in elevated concentrations of influent TSS (884 ± 96 vs. 16 ± 5 mg/L) and VSS concentrations (493 ± 41 vs. 11 ± 5 mg/L), and lower TDS concentrations (756 ± 100 vs. 907 ± 60 mg/L) (Figure 16 d, e, f). The bioreactors were found to absorb the changes in these material loads efficiently judging from the slightly elevated TSS (34 ± 33 vs. 23 ± 17 mg/L), VSS (24 ± 20 vs. 14 ± 12 mg/L) and slightly higher TDS (917 ± 190 vs. 766 ± 95 mg/L) in the effluents.

4.3.2.4 Iron and sulfur

The sludge recycling also supplemented iron to the synthetic wastewater and resulted in increased total iron concentrations in the influent to the bioreactors (161.4 ± 8.8 vs. 118.0 ± 5.2 mg/L, Figure 16 g). The additional iron loads subsequently lowered the sulfideS levels in the effluents (0.3 ± 0.1 vs. 0.4 ± 0.1 mg/L, Figure 16 h). The lowered effluent iron concentrations (0.7 ± 0.5 vs. 1.9 ± 1.7 mg/L) concurrent with the

lowered sulfide concentrations suggested more efficient formation of iron sulfide precipitates as a result of the sludge recycling.

Figure 17 presents the means and standard deviations of iron and sulfur mass loads. In the influent ($n = 32$), both sulfur and iron were in dissolved forms with daily loads of 231 mg/day and 404 mg/day, respectively. During baseline operation ($n = 25$), average dissolved sulfur (sulfate and sulfide) in the effluents from the two bioreactors accounted for 7.3% of the influent sulfur load. Effluent particulate sulfur (estimated as sulfate after the sludge samples were acidified and oxidized) accounted for 50.5% of the influent sulfur load to the bioreactors. Similarly, average dissolved iron (total iron in the effluent) in the effluents accounted for only a small fraction of the influent iron load (5.7%) while particulate iron (total iron in the sludge materials) accounted for a much greater fraction (53%). During the sludge recycling operation ($n = 7$), smaller amounts of both dissolved sulfur and iron (6.4% and 1.5% of the influent load, respectively) were observed compared to the baseline operation. Conversely, both the effluent particulate sulfur and iron showed significant increases (62% and 68%, respectively). In the oxidizing basin ($n = 7$), dissolved sulfur (estimated by sulfate in the filtrate) accounted for a significantly higher percentage of the influent sulfur load (42%) compared to the bioreactor effluents. There was still a substantial but much lower particulate sulfur fraction (30%) compared to the bioreactors. Iron exhibited similar trends in both dissolved and particulate forms (45% and 18%, respectively). These results showed that the oxidation treatment transferred the majority of particulate S and Fe in the sludge materials to oxidized dissolved forms in the oxidizing basin. The unaccounted fractions of the chemical masses (i.e., loss) are attributable to several possible mechanisms including ferrous sulfide precipitation retained in void spaces or associated with the biomass in the bioreactors, evaporative loss of sulfide, and inaccuracy in chemical analyses.

Further analyses of the sludge extracted from the bioreactors (anaerobic sludge) and the oxidized sludge showed similar quantities of total iron. However, the distribution of chemical forms was different. In the anaerobic sludge, the iron was particulate with small percentages of dissolved Fe^{2+} and Fe^{3+} . In the oxidizing basin iron precipitates were partially converted to dissolved forms including Fe^{2+} and Fe^{3+} (Figure 18). Similarly, the sulfide precipitates were converted to dissolved sulfate, and partially oxidized forms including sulfite and thiosulfate.

Figure 17 Phase II iron and sulfur mass balance at the different stages of the treatment process, including the influent (Inf), bioreactors without sludge recycle (BR), bioreactors with sludge recycle (BR w/R). And the oxidizing basin (OxB). Legend: S(dis): soluble sulfur, S(par): particulate sulfur, S(loss): total sulfur in influent-S(dis)-S(par), and the same definitions apply for Fe(dis), Fe(par) and Fe(loss).

Figure 18 (a) Phase II iron and (b) sulfur content of the anaerobic and oxidized sludge in particulate (par) and dissolved (dis) forms.

4.3.3 Sludge solids evolution over long-term operation

During the 450-day study period (phase I), both VSS and TSS of the sludge materials from the bioreactors varied slightly with the different COD/sulfate and Fe/S ratios (Figure 19 a). VSS and TSS concentrations were 761 ± 39 mg/L, and 2477 ± 194 mg/L, respectively, resulting in a VSS/TSS ratio of 0.31 ± 0.02 . This suggested fairly uniform solid content and composition of the sludge materials even with variations in chemical loading during long-term operation. In phase II, sludge recycling occurrences were found to cause elevations in both VSS and TSS (Figure 19 b). The sludge recycling also caused increases in VSS/TSS ratio, indicating relatively higher quantities of VSS than NVSS in the oxidized sludge.

Figure 19 Solids content and VSS/TSS ratios of the (a) sludge materials from the bioreactors during Phase I (i.e., without sludge recycling), and (b) sludge materials from the bioreactors during Phase II on sludge recycling days compared to baseline days (Phase II).

4.4 CONCLUSIONS

This study represents an innovative treatment method with its novelty residing in the use of iron for sulfur recycling.

- The mass balance analysis and chemical analyses of Fe and S at different stages of the treatment process demonstrated that the designed biochemical reactions were successfully carried out and produced satisfactory results.

- With a COD/sulfate mass ratio of 2, and a Fe/S molar ratio of 1, this process operated under a condition conducive to sulfidogenic treatment of the wastewater and yielded the best treatment performance among the different chemical loads.
- Sludge oxidation and recycling significantly enhanced treatment performance. The oxidation in the oxidizing basin was found to only partially convert inorganic precipitates to soluble iron and sulfur and can be improved.
- The process exhibited treatment stability with reasonable variations under a range of COD/sulfate and Fe/S ratios. The sludge content was found to be fairly consistent over the long periods of operation without sludge recycling, and the bioreactors were found to efficiently absorb the changes in these material loads caused by the sludge recycling.

Additional studies are required to further optimize the treatment process and elucidate the treatment reactions. In particular, guidelines on C: Fe: S load ratios can be developed for optimized treatment performance. Studies investigating specific biochemical reactions in the sulfidogenic bioreactors and oxidizing basin, and their microbial communities are expected to generate useful results to further develop this treatment method. Further characterization of the sludge materials would also provide insights into the fate of two key chemical elements.

4.5 SUPPLEMENTARY INFORMATION

Table S5 Trace elements used in the synthetic wastewater

Trace element	mg/L
MnSO ₄ · H ₂ O	2.5
FeSO ₄ · 7H ₂ O	0.5
Co(NO ₃) ₂ · 6H ₂ O	0.5
ZnCl ₂	0.5
NiCl ₂ · 6H ₂ O	0.25
H ₂ SeO ₄	0.25
CuSO ₄ · 5H ₂ O	0.05
AlK(SO ₄) ₂ · 12H ₂ O	0.05
H ₃ BO ₃	0.05
Na ₂ MoO ₄ · 2H ₂ O	0.05
Na ₂ WO ₄ · 2H ₂ O	0.05

Steady state evaluation

Figure S 11 Averaged COD concentrations of the bioreactors during a 1-month acclimation period under COD/sulfate mass ratio 2 and Fe/S molar ratio 1.

COD reaction rate estimation:

Given the recirculation flow, we assumed a well-mixed condition in the bioreactors. At a steady state, the COD oxidation rate, ν (mg/L·d), can be estimated by

$$\nu = \frac{1}{\theta} (C_{in} - C_{ss})$$

where $\theta = \frac{1.5L}{3.5L/d} = 0.43d$, C_{in} : influent concentration; and C_{ss} : the steady-state concentration.

The equation was applied to estimate the COD oxidation and sulfate reduction rates under different COD/sulfate and Fe/S ratio combinations. The results are presented in Figure 15.

Figure S 12 SEM micrograph and EDX results for sludge materials from the sulfidogenic bioreactors.

Figure S 13 (a) S and (b) Fe XPS spectra for the biogenic sludge materials from the bioreactors.

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CHAPTER 5: PHASE II (STAGE 4) ELUCIDATION OF FE AND S BIOGEOCHEMICAL TRANSFORMATIONS AND KINETICS IN AN INNOVATIVE FE(II)-DOSED ANAEROBIC WASTEWATER TREATMENT PROCESS USING X-RAY SPECTROSCOPIC AND MICROBIAL PHYLOGENIC ANALYSES

Research Objective: Investigate biogeochemical transformations of Fe and S in the continuous MWW treatment process at different stages of the treatment process and associated microbial ecology. In addition, determine mass balance of key chemical elements.

5.1 INTRODUCTION

Anaerobic biological treatment has benefits of energy efficiency and potential recovery of useful products over aerobic treatment methods (van Lier et al. 2008; Chan et al. 2009). Building upon on previous findings on a co-treatment method for acid mine drainage and municipal wastewater (Deng et al. 2016; Deng and Lin 2013), an innovative Fe(II)-dosed process designed for anaerobic treatment of municipal wastewater was recently developed and shown to have treatment stability over long periods of operation under a range of COD/sulfate and Fe/S ratios (Deng and Lin 2017; Deng et al. 2016). This treatment process consisted of duplicate anaerobic bioreactors that employed primarily sulfate reducing bacteria (SRB) to facilitate oxidation of organic matters with sulfate as the primary electron acceptor. In the bioreactors, the produced hydrogen sulfide formed chemical precipitation with the dosed ferrous ion due to the low solubility of amorphous ferrous sulfide ($K_{sp} \approx 10^{-3.05}$, (Emerson et al. 1983). This mechanism not only limited sulfide toxicity on SRB but also safeguarded sulfide levels in the treated effluent. The treatment process also offered an option of sludge recycling for which the anaerobic sludge materials were periodically collected from the anaerobic bioreactors and oxidized mechanically in an oxidizing basin before recycled to mix with the wastewater influent. The periodic recycling of the sludge materials supplemented fully and partially oxidized sulfur (e.g., sulfate and thiosulfate) and ferric compounds to the influent for continuous wastewater treatment. Overall, the sludge recycling was found to enhance the biological treatment efficacy compared to the baseline operation without sludge recycling (Deng and Lin 2017).

The overall treatment performance of this iron-dosed process is closely tied to the biogeochemical transformations of Fe and S in the anaerobic bioreactors and the oxidizing basin. Under anaerobic conditions, formation of iron sulfides commonly occurs when sulfides are produced as a result of sulfate reduction (Doner and Lynn 1989; Ferris et al. 1987). Using sulfate as an external electron acceptor, SRB obtain energy and nutrients through oxidation of low molecular weight organics such as lactate and acetate, which also produces bicarbonate alkalinity (Yoda et al. 1987):

Aqueous hydrogen sulfide and ferrous iron subsequently precipitate as amorphous iron sulfide (Dvorak et al. 1992):

Potential forms of iron sulfides in the anaerobic bioreactors of the iron-dosed treatment process include ferrous monosulfide (FeS), greigite (Fe₃S₄), and ferrous disulfide (FeS₂) (D. Rickard 1969; Qiwei Wang and Morse 1996). Despite its metastable nature, FeS may persist for long periods of time in reducing environments before it transforms to more stable phases such as greigite and FeS₂ (Berner 1981; Benning et al. 2000; D. Rickard and Luther 2007). Recent studies have demonstrated that disordered FeS is a precursor phase to FeS₂ formation, with the FeS surface providing an initial nucleation site for FeS₂ growth (Schoonen and Barnes 1991; Wilkin and Barnes 1996; D. Rickard 1997; D. Rickard and Luther 1997). Kinetic studies further supported the formation of FeS₂ via reactions involving either intermediate sulfur species (e.g. polysulfides, S_n²⁻) (Schoonen and Barnes 1991; Wilkin and Barnes 1996) or dissolved hydrogen sulfide (D. Rickard and Luther 1997). FeS acting as a precursor phase to FeS₂ is important because, although FeS₂ is less soluble and thermodynamically more stable, the precursor phase may control the aqueous concentrations of sulfide and ferrous ions (Herbert et al. 1998; Berner 1967; D. Rickard 1969; D. T. Rickard 1975). The dominant formation path of FeS₂ has often been assumed to be reactions between a precursor monosulfide and zero-valent sulfur (reaction (10)) (Benning et al. 2000; Wilkin and Barnes 1996); however evidences also suggested that its formation may proceed via loss of ferrous iron from iron sulfide rather than via addition of zero-valent sulfur (reaction (11), (Wilkin and Barnes 1996)).

Presence of ferric iron in the anaerobic bioreactors due to the sludge recycling adds to their biogeochemical complexity because of potential ferric reduction to ferrous by dissimilatory iron reducing bacteria (IRB). IRB gain energy by coupling the oxidation of organic compounds or hydrogen to reduction of ferric oxides has long been studied but their biogeochemical importance was recognized only two decades ago (Thamdrup 2000; Lovley 1997). When in the presence of large quantities of reactive ferric source, microbial iron reduction could effectively compete against SRB and even inhibit sulfate reduction (King 1990). IRB have versatile metabolic pathways and can utilize short- and long-fatty acids, amino acids, sugars, H₂ and aromatic compounds as electron donors in ferric reduction (Erbs and Spain 2002).

In oxidizing environments such as the oxidizing basin of the treatment process in this study, iron sulfides could be oxidized by O_2 or Fe(III) abiotically to sulfate and ferric ion, and some intermediate products such as elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), and sulfite (SO_3^{2-}) (Pyzik and Sommer 1981; Burdige and Nealson 1986; Zhang and Millero 1993; Schippers and Jørgensen 2002). They could also be oxidized biotically by O_2 or Fe(III) to form sulfate and $Fe(OH)_3$. These biotic transformations can be mediated by sulfur oxidizing bacteria such as *Desulfobacter postgatei*, *Desulfobulbus propionicus*, *Desulfovibrio desulfuricans* and *Thiobacter subterraneus* (Dannenberg et al. 1992; Schippers and Jørgensen 2002; Hirayama et al. 2005) and iron oxidizing bacteria such as *Rubrivivax gelatinosus* (Watzlaf and Hammack 1989; Schoepp et al. 1995). The distribution of intermediate sulfur products (SO_3^{2-} , $S_2O_3^{2-}$ and S^0) during the pyrite oxidation process by oxygen and Fe(III) has been reported (Moses et al. 1987; Moses and Herman 1991). The iron species after abiotic and biotic oxidation were reported to be in the forms of Fe^{+2} , Fe^{+3} or $Fe(OH)_3$ depending on the pH (Schippers and Jørgensen 2002).

Microbial composition in the bioreactors is another critical factor that governs the biogeochemical transformations of Fe and S and the treatment performance of the bioreactors. The substrate, hydraulic retention time, temperature, type of support or carrier material, and source of inoculum among others are the main conditions that influence biofilm formation (Geesey and Bryers 2000). Characterization of microbial consortia in the bioreactors and examining their relationships with the environmental conditions in the anaerobic bioreactors and the oxidizing basin can help elucidate the designed biogeochemical reactions, and achieve reliable operation and eventual scale-up of the iron-dosed treatment process.

Using spectroscopic and microbial analytical tools, this study aims to elucidate the biogeochemical mechanisms that render the biogeochemical transformations of Fe and S in the Fe(II)-dosed anaerobic treatment process. Specifically, various forms of Fe and S were monitored at different stages of the process with and without sludge recycle to characterize and compare the treatment performance under the two sludge operating conditions. The anaerobic and oxidized sludge materials were characterized by their surface morphology, chemical composition, states, and structures as well as microbial functions and diversity. In addition, mass fluxes of Fe and S were estimated to help characterize degree of their biogeochemical transformations in the anaerobic bioreactors and oxidizing basin.

5.2 MATERIALS AND METHODS

5.2.1 Bench-scale treatment process

A bench-scale treatment process consisting of a wastewater reservoir, iron reservoir, two parallel anaerobic bioreactors, and an oxidizing basin were constructed and used in this study. Based on our previous findings, COD/sulfate mass ratio 2 and Fe/S

molar ratio 1 were chosen for the anaerobic treatment of wastewater to study the biogeochemical transformations of Fe and S. The wastewater reservoir (57-L tank) was used to supply a synthetic wastewater containing 2.26 mM ethanol ($\text{C}_2\text{H}_6\text{O}$), 0.45 mM lactose ($\text{C}_{12}\text{H}_{22}\text{O}_{11} \cdot \text{H}_2\text{O}$) and 1.61 mM sodium acetate ($\text{C}_2\text{H}_3\text{O}_2\text{Na} \cdot 3\text{H}_2\text{O}$), 1.68 mM sodium bicarbonate (NaHCO_3), and trace elements (5 ml/L influent) (Tindall 1992). A sodium sulfate solution (2.08 mM Na_2SO_4) was mixed with the synthetic wastewater to obtain COD/sulfate mass ratio 2. The iron reservoir (4-L tank) containing a ferrous chloride solution ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 2.08 mM, pH = 3.2–3.4) was used as a source for iron dosing.

The anaerobic bioreactors were made of acrylic cylinders (BR1 and BR2, 2.5 L each) and packed with plastic media (Kaldnes K1, specific surface area = $500 \text{ m}^2/\text{m}^3$, Evolution Aqua Ltd, UK) for attached growth of microorganisms. In each bioreactor, a perforated acrylic plate was used to support the packing media and a coned-shaped bottom was used to facilitate sludge settling and collection. The bioreactors were inoculated with anaerobic digester sludge from a municipal wastewater treatment plant (Star City, West Virginia) and AMD sludge from a mine portal along Dunkard Creek (near Bobtown, Pennsylvania) at 1:1 volume ratio. After biomass enrichment, the bioreactors were operated for 15 months under a range of COD/sulfate and Fe/S ratios without sludge recycle. The synthetic wastewater and ferrous solution were fed through inlets on the top of the bioreactors. The bioreactors were operated at room temperature ($21 \pm 1 \text{ }^\circ\text{C}$) under anaerobic conditions. Each bioreactor provided a working volume of approximately 1.5 L with the packing media and sludge biomass.

In the following 510-day period, the anaerobic bioreactors were operated with periodic sludge recycling. During that period, 10 sludge recycling events were conducted to examine their effects on treatment performance of the anaerobic bioreactors. A 4-L wide-mouth conical flask with 30 pieces of plastic media (Kaldnes K1, Evolution Aqua Ltd, UK) for attached growth of microorganisms was used as the oxidizing basin. During each event, sludge samples (100 mL) from the bioreactors were collected and added to the oxidizing basin daily for 6 days. A magnetic stirrer was used to mix the sludge under aerobic conditions to transform ferrous sulfides to their oxidized forms. At the end of the oxidation period, the oxidized sludge material was mixed with wastewater influent at 1:1 volume ratio to study the effects of sludge recycling. More details of the treatment process and operations can be found elsewhere (Deng and Lin 2017).

5.2.2 Sample preparation and spectroscopic analyses

5.2.2.1 Scanning Electron Microscopy (SEM)

Surface morphology and elemental composition of the anaerobic and oxidized sludge were analyzed using a scanning electron microscope (SEM, Hitachi S-4700F) coupled with energy dispersive X-ray spectroscopy (EDS, EDAX Genesis). The sludge

samples on the plastic media were first immersed in growth medium (synthetic wastewater) plus 1% glutaraldehyde and 1% formaldehyde. The samples were then dried in a closed chamber filled with calcium sulfate and flushed with nitrogen to remove the moisture content and prevent sludge oxidation (Karamalidis et al. 2008) until the weights were constant. To preserve the integrity of bacterial cell walls, sludge samples were processed through biological fixation. In this method, 1 ml of each sludge sample was washed with 2 ml 2.5% glutaraldehyde for 1 hour, and rinsed three times using phosphate-buffered saline (PBS) before being dehydrated by a graded ethanol series (30%, 50%, 70%, 90% and 100 % for 15 minutes each stage with very gentle periodic agitation). The sludge sample was then dried with hexamethyldisilazane (HMDS). With this technique, the liquid CO₂ changes to vapor without a change in density as the temperature of the sample is raised above the critical temperature for CO₂, therefore eliminating surface tension effects which would distort morphology and surface structure (Nordstrum 1986).

The SEM was operated under an accelerating potential of 5–20 kV. Qualitative elemental analysis of the sludge samples was conducted by EDS spectrometry operated under an accelerating potential of 20 kV. All samples were mounted on aluminum sample stubs and gold-coated to minimize surface charging. Five randomly selected areas were scanned and the combined spectra were used to determine the mean relative percentage of the most predominant chemical elements.

5.2.2.2 X-ray diffractometry (XRD) analysis

For XRD analyses, sludge was separated from the solution by high-speed centrifugation (5000 *xg*) for 10 min. The residue was washed several times with deionized water to remove solutes and recentrifuged to remove the supernatant liquid. The centrifuged sludge was then dried at room temperature in an anaerobic desiccator filled with calcium carbonate overnight.

Powder XRD analysis of samples was performed on the X-ray Diffractometer (PANalytical X'Pert Pro) with a Cu K α X-ray source operated at 45 KV and 40 mA. All samples were step-scanned from 10 to 90° (2 θ) using a step of 0.05 sec and counting time of 2.25 sec per step.

5.2.2.3 X-Ray photoelectron spectroscopic (XPS) analysis

The sludge materials were first separated from the aqueous portion of the sludge samples following the same method as that for the XRD analysis. XPS spectra were obtained using the X-Ray Photoelectron Spectroscope (Physical Electronics PHI 5000 VersaProbe) with a monochromatized Al K α X-ray source (1487 eV) operated at 15 kV and 25 W. The base pressure in the analytical chamber was on the order of 10⁻⁷ Pa. The instrument work function was set at 2.45 eV based on calibration with a Ag standard. Survey and narrow region XPS spectra were acquired with analyzer pass energy of 117.4

and 23.5 eV, respectively. An X-ray spot size of 100 μm was used for both scan types. Narrow region photoelectron spectra were acquired to obtain chemical state information for iron and sulfur. Spectra were fit with the PHI MultiPak software using a Shirley background (Shirley 1972) and an 80% Gaussian 20% Lorentzian peak model. The background hydrocarbon C(1s) peak at 284.8 eV was adopted as the reference for surface charging correction. Excessive charging of the sample surface was reduced by using argon ion gun neutralizer and electron flood gun neutralizer.

5.2.3 Nucleic acid extraction, purification and 16S rRNA gene amplification

Sludge samples (mixtures of the bottom sludge and packing media biofilms) from the anaerobic bioreactors and the oxidation basin were also collected and analyzed to characterize the microbial communities. Microbial DNA was extracted from 50 ml of mixture sample from each reactor using the FastDNA SPIN Kit for Soil (MP Biomedicals, OH). The extracted DNA was purified using an ethanol precipitation method (Macbeth et al. 2004), followed by DNA quantification using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, DE). The purified microbial DNA was amplified by polymerase chain reaction (PCR, Eppendorf AG Mastercycler eppgradient, Hamburg, Germany). The PCR mixture (25 μl) contained 0.25 μM of each primer (8F and 1492R), 1x PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl_2 (Applied Biosystems, Foster City, CA), 0.5 mg/ml BSA (New England Biolabs, Ipswich, Massachusetts, USA), 0.2 mM dNTPs (Applied Biosystems, Foster City, CA), 0.5 U of Taq polymerase (Fisher Scientific, Pittsburgh, PA) and 25~185 ng of template DNA. The sequences of the primers used were: 8F, (5'- AGAGTTTGATCCTGGCTCAG -3'); 1492R, (5'-GGTTACCTTGTTACGCTT -3') (Weisburg et al. 1991). The thermocycling conditions included 15 min of initial denaturation at 95°C, 35 cycles of denaturation (95 °C for 1 min), annealing (53.5 °C for 1 min), extension (72 °C for 1 min), and final extension for 5 min at 72 °C. Control samples for each PCR run included negative controls (e.g., DNA-free water instead of template).

5.2.4 Cloning and sequencing

The PCR amplicons of the 16S rRNA gene were cloned using the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Vectors were transformed into chemically competent *Escherichia coli* cells following the manufacturer's instructions. In total, 20 clones were selected for each of the sludge sample from the anaerobic bioreactor and the oxidizing basin. The 16S rRNA gene fragments on the plasmids were amplified by the primer sets of M13F and M13R (Manual of TOPO TA cloning kit). The PCR products were visualized by agarose gel electrophoresis to verify the size and the existence of the inserts. The 16S rRNA genes of PCR amplicons were sequenced in the West Virginia University Genomics Core Facility using 8F and 907R. Eight (8) clones from the anaerobic sludge and 7 clones from oxidized sludge were successfully sequenced.

5.2.5 Phylogenetic analysis

The obtained 16S rRNA gene sequences were reassembled using Bioedit (version 7.1.3.0, Ibis Biosciences, Carlsbad, CA) to generate contigs. The contig sequences were checked for chimeras using the Bellerophon tool and Decipher (Huber et al. 2004); (Wright et al. 2012) and then aligned using MEGA 6 (Tamura et al. 2013). The sequences were classified into taxonomic groups using the ribosomal database project classifier (Qiong Wang et al. 2007). Evolutionary analyses were conducted in MEGA 6 and bootstrap resampling analyses were performed on 1000 replicates. The sequences were submitted to NCBI Genbank and accession numbers are provided in Supplementary Table S6.

5.2.6 Chemical analyses

An YSI meter with pre-calibrated probes (YSI 63) was used to measure pH. Autotitrators were used for the analyses of alkalinity (Thermo Scientific Orion 950) and acidity (Mettler Toledo DL50) following the Standard Methods ((APHA 2005), 2320B and 2310B). Sludge samples for total iron analysis were digested and extracted following the Standard Methods. The sludge samples were filtered through 0.45- μ m membrane filters before the analysis for total dissolved iron concentration. Ferrous concentration was determined using the 1,10 Phenanthroline method ((APHA 2005), 3500B) and ferric concentration was determined by the differences between total dissolved and ferrous iron concentrations.

Total solids (TS), total suspended solids (TSS), total dissolved solids (TDS), and volatile suspended solids (VSS) were determined following the Standard Methods. Sulfur concentrations (sulfate, sulfite, and thiosulfate) were measured using an ion chromatography (Dionex ICS-1100). COD concentrations were determined using a closed reflux, colorimetric method with a spectrophotometer (Hach DR2800, (ASTM D1252-06 2006), and sulfide concentrations measured by a methylene blue method ((APHA 2005), 4500D). Duplicates of each sample were measured for sulfur, iron and COD concentrations.

5.3 RESULTS AND DISCUSSION

5.3.1 Treatment effects of sludge recycle

The recycling operations produced better effluent quality during the recycling time periods compared to the baseline operation (Table 5). Specifically, with the influent COD (412 ± 39 mg/L), sulfate (182 ± 7 mg/L), and total iron (167 ± 14 mg/L), and the anaerobic treatment resulted in better effluent quality (COD 44 ± 23 mg/L, sulfate 4.1 ± 2.7 mg/L, total iron 11 ± 5 mg/L) than the baseline condition (COD = 143 ± 32 mg/L, sulfate = 17 ± 9 mg/L, and total iron = 40 ± 16 mg/L). The anaerobic treatment reduced most of the

sulfate (91-98%) into solid sulfur and sulfide forms with significantly lower but detectable levels of the intermediates (e.g., sulfite and thiosulfate) than sulfate. Total iron was also more efficiently reduced into solid forms (iron or iron sulfides) during the sludge recycling periods.

The effluent pH was 6.2 ± 0.2 during the baseline operation and increased to 6.5 ± 0.2 with sludge recycling, corresponding to alkalinity of 173 ± 113 and 268 ± 49 mg/L as CaCO_3 , respectively. Effluent TSS and VSS were slightly elevated with sludge recycling (TSS 53 ± 21 mg/L, VSS 36 ± 15 mg/L) compared to the baseline condition (TSS 39 ± 32 mg/L and VSS 15 ± 11 mg/L). Overall, the sludge recycling operation improved effluent quality in terms of all measured water quality parameters except for slightly higher solid content.

Table 5 Treatment effects of system without and with recirculation. Effluent refer to baseline operation (without recirculation), Effluent (Re) refer to with recirculation condition.

Parameter		Influent	Effluent	Effluent (Re)
SO_4^{2-}		182 ± 6.9	17 ± 9.1	4.1 ± 2.7
SO_3^{2-}		5.3 ± 3.9	6.2 ± 3.8	1.5 ± 1.2
$\text{S}_2\text{O}_3^{2-}$		0	0.8 ± 0.1	0.3 ± 0.2
S^{2-}		0.006 ± 0.005	0.3 ± 0.2	0.12 ± 0.1
Fe(T)		166.8 ± 14	40.4 ± 15.6	10.5 ± 5
Fe(TD)	mg/L	123.1 ± 7.5	19.5 ± 9.7	4.3 ± 2
Fe^{+2}		80.3 ± 5	12.7 ± 7.3	3.0 ± 1.3
Fe^{+3}		42.8 ± 4.8	9.7 ± 5.9	1.3 ± 1.3
COD		412 ± 39.2	142.7 ± 32.1	43.5 ± 22.6
TSS		6.8 ± 2.9	39 ± 31.7	52.7 ± 21.0
TDS		1034.8 ± 104.8	940.3 ± 226.1	919.1 ± 107.9
VSS		5.5 ± 3.8	14.7 ± 11.3	35.6 ± 14.7
pH		7.9 ± 0.2	6.2 ± 0.2	6.5 ± 0.2
Acidity	mg/L as	4.1 ± 1.7	113.3 ± 45.9	66.3 ± 19.8
Alkalinity	CaCO_3	168.1 ± 2.9	173.3 ± 39.6	268 ± 48.8

5.3.2 Sludge morphology and mineralogy

5.3.2.1 SEM-EDS

The SEM analysis of the sludge samples revealed the presence of numerous bacterial cells in both the anaerobic (Figure 20a) and oxidized sludge materials (Figure 20b). The microorganisms were generally rod shaped with a size of 0.5-3.0 μm . The bacteria resembled SRB of genera *Desulfobulbus* or *Desulfomonas*, based on size and morphology (Holt et al. 1994). It is also probable that the cells were fermentative

bacteria, which often exist synergistically with SRB populations (Chapelle 2001). The density of bacterial cells was much higher in anaerobic sludge compared to the oxidized sludge in the areas under analysis. The anaerobic sludge also showed strands of exopolymeric substances responsible for bacterial adhesion. In addition, the cells intermixed in an iron sulfide matrix, and consequently the EDS analyses of the bacteria indicated the presence of Fe and S (Figure 20a). In the oxidized sludge, the EDS analyses also showed the presence of Fe and S (Figure 20b). The lack of sulfur in the presence of iron (Figure 20c) in anaerobic sludge suggests that iron may have been complexed by extracellular polymeric substances to the organic membranes of the microbial community (Sand and Gehrke 2006). Several peaks from the oxidized sludge correlated to sulfur particles (Figure 20d) suggesting the formation of elemental sulfur possibly through H_2S oxidation (Coelho et al. 2008).

Figure 20 SEM micrographs and energy-dispersive X-ray spectra of the (a) anaerobic sludge, (b) oxidized sludge, (c) iron particles in the anaerobic sludge and (d) sulfur particles in the oxidized sludge. The presence of gold is from the surface coating of the samples. Vertical scale in arbitrary units

Figure 21 XPS narrow region spectra for Fe (2p_{3/2}) and S (2p) of the anaerobic sludge (a and b) and the oxidized sludge (c and d), and XRD spectra of the (e) anaerobic and (f) oxidized sludge.

5.3.2.2 XPS and XRD

The narrow region Fe2p spectrum obtained for the anaerobic sludge exhibited a prominent peak at 711.1 eV (Figure 21a), which is within the reported binding energy range for high-spin Fe^{2+} dissociated from ferrous chloride. The additional peak on the low binding energy limb is corrected to 708.6 eV and representative of the presence of FeS_2 . The S2p spectrum (Figure 21b) was fit with two distinct peaks at 161.4 and 162.9 eV which are representative of typical binding energies associated with FeS and FeS_2 , respectively. Additional peaks within the 163-168 eV range are indicative of organosulfur compounds such as proteins.

In contrast, the Fe2p spectrum for the oxidized sludge consisted of a singular peak (Figure 21c) coinciding with the binding energy of FeO (Chastain et al. 1995). The slight elevation in binding energy can be attributed to the presence of another oxidized form, Fe_2O_3 , suggesting a mixture of both iron oxides as binding energies of pure FeO and pure Fe_2O_3 are lower and higher than the measured binding energy, respectively. The S2p spectrum (Figure 21d) of the oxidized sludge was fit with a prominent peak associated with iron sulfate and an additional peak corresponding to FeS_2 . The distinct lack of FeS compounds suggests that amorphous FeS was more readily oxidized to FeSO_4 than FeS_2 .

The XRD spectrum generated from the anaerobic sludge (Figure 21e) exhibited several large peaks representing crystalline iron sulfide phases (FeS and FeS_2). Based on quantity and intensity of the peaks, the precipitates isolated from the anaerobic sludge are best characterized as a mixture of amorphous and crystalline iron sulfides ranging from FeS to FeS_2 . Biogenic iron sulfides typically aggregate into amorphous, spherical clusters of Fe and S. Therefore, the large quantities of crystalline iron sulfide suggest the long-term conversion of amorphous FeS to more stable crystalline FeS or FeS_2 structures (Herbert et al. 1998).

XRD spectrum of the oxidized sludge did not show any reflections indicative of crystalline iron sulfide phases (Figure 21f) suggesting the breakdown of FeS and FeS_2 when exposed to the atmospheric oxygen. Additionally, major peaks corresponding to goethite (Fe_4O_8) have much lower intensity than the crystalline phases seen in the XRD analysis of the anaerobic sample (D. Rickard 1995). Based on these results and the narrow spectrum of the oxidized sludge, the precipitate may be described as largely amorphous phases of Fe(II) and Fe(III) oxides (e.g., wustite, hematite) along with oxidized iron sulfate.

5.3.3 Phylogenetic tree

5.3.3.1 Microbial composition

Of the twenty clones selected from each of the sludge samples, fifteen clones were detected in the anaerobic sludge (RB) and the oxidized sludge (OB) with quantifiable results (Figure 22 a, b). Six identified phyla were Alphaproteobacteria, Deltaproteobacteria, Betaproteobacteria, Chloroflexi, Saccharibacteria, and Bacterioidetes

(Table 6). Eight clones were identified in RB, three as Bacterioidetes, one as Alphaproteobacteria, one as Betaproteobacteria, one as Deltaproteobacteria, one as Saccharibacteria, and one as Chloroflexi. All clones in OB were identified as Alphaproteobacteria or Betaproteobacteria.

The phylogenetic analysis shows that microbial community in the anaerobic sludge was more diverse than that of the oxidized sludge (Figures 22 a, b). Clone RB-5 was most closely related (96% similarity) to dehalogenating, sulfur reducing *Desulfomonile tiedjei* sp. which reduces sulfate, sulfite, and thiosulfate (DeWeerd et al. 1990). RB-10 was mostly related (89% similarity) to *Candidatus Sacchaimonas*, commonly found in anaerobic sludge (Hugenholtz et al. 2001), and the clone also indicates a strong relationship (82% similarity) with *Alkaliphilus metalliredigens*, a species known for Fe(III)-reducing (Roh et al. 2007). Three species (RB-2, RB-4 and RB-8) belonging to the Bacterioidetes were identified in the anaerobic bioreactors. Of the three species, both RB-2 and RB-8 were most closely related (84% similarity) to polycyclic aromatic hydrocarbon-degrading function related species *Parapedobacter pyrenivorans* (Zhao et al. 2013) and RB-4 (95% similarity) was highly related to propionate-producing *Paludibacter propionigenes* (Ueki et al. 2006). Clone RB-7 was most closely related (99% similarity) to methyl degrading, *Acidovorax delafieldii* sp. known for its ability to breakdown biodegradable plastics and related compounds (Uchida et al. 2000). With a similarity of 94%, RB-17 was most closely related to *Pleomorphomonas diazotrophica*, a nitrogen fixing species (Madhaiyan et al. 2013). This species has the ability to complex nitrogen ion to be stored in an anabolic process and uses it as an energy source. RB-18 was found to be similar (81%) to *Dehalococcoides mccartyi*, an organohalide-respiring anaerobic bacteria relevant to halogen cycling (Löffler et al. 2013). The presence of clones closely related to fermentative species, as well as sulfur reducing, iron reducing, and nitrogen fixing bacteria species suggests that the bioreactors have the potential to treat acidic, nutrient and sulfate rich wastewater sources under anaerobic conditions.

The clone OB-13 was most similar (91%) to *Thiobacter subterraneus* sp., a thermophilic, sulfur oxidizing bacterium (Hirayama et al. 2005), which is consistent with the XPS analysis of the oxidized sludge. The clone OB-19 was most closely related (>98% similarity) to iron oxidizing species *Rubrivivax gelatinosus* (Schoepp et al. 1995) which was the sole iron oxidizing species identified in the oxidized sludge. OB-2 (99%) was closely related to methyl degradation *Piscinibacter aquaticus* (Stackebrandt et al. 2009). The clone OB-9 was most closely related (88% similarity) to species associated with nitrogen fixation and catabolism *Rhizobium* sp. LS-099 (Dreyfus et al. 1988). OB-14 was highly related to *Xanthobacter autotrophicus* which utilize halogenated short-chain hydrocarbons and halogenated carboxylic acids as sole carbon source for growth (Janssen et al. 1985). OB-16 was similar (96%) to aromatic degradation function related species *Sphingomonas* sp. KAR7 (Phillips et al. 2008). OB-17 was 95% similar to nitrogen fixing and iron chelating *Rhizobacter* Sp. NR 2-01 (Zakry and Rahim 2012). In re-oxidized sludge, all the successfully sequenced clones either belongs to Alphaproteobacteria or

Betaproteobacteria and related to organic degradation, dehalogenation, sulfur and iron oxidizing function.

Table 6 Microbial communities in the anaerobic bioreactors and oxidizing basin

Clone	Sequence Length	Closest Species in GenBank [Accession no.]	Putative function	Identity (%)	Phyla
RB-2	764	<i>Parapedobacter pyrenivorans</i> [NR109750.1]	Fermentation	84	Bacteroidetes
RB-4	1291	<i>Paludibacter propionicigenes</i> [AB910740.1]	Fermentation	95	Bacteroidetes
RB-5	1241	<i>Desulfomonile tiedjei</i> [NR074118.1]	Sulfur Reducing	96	Deltaproteobacteria
RB-7	1035	<i>Acidovorax delafieldii</i> [GQ284437.1]	Methyl Degrading	99	Betaproteobacteria
RB-8	1009	<i>Parapedobacter pyrenivorans</i> [NR109750.1]	Fermentation	84	Bacteroidetes
RB-10	696	<i>Candidatus Sacchaimonas</i> [KX028761.1]	Fermentation	89	Saccharibacteria
RB-17	1002	<i>Pleomorphomonas diazotrophica</i> [NR109585.1]	Nitrogen Fixing	94	Alphaproteobacteria
RB-18	1269	<i>Dehalococcoides mccartyi</i> [NR102515.1]	Dehalogenation	81	Chloroflexi
OB-2	1146	<i>Piscinibacter aquaticus</i> [KF253106.1]	Methyl Degrading	99	Betaproteobacteria
OB-9	1327	<i>Rhizobium</i> sp. LS-099 [KJ584032.1]	Nitrogen Fixing	88	Alphaproteobacteria
OB-13	1307	<i>Thiobacter subterraneus</i> [NR024834.1]	Sulfur Oxidizing	91	Betaproteobacteria
OB-14	1304	<i>Xanthobacter autotrophicus</i> [NR114104.1]	Dehalogenation	100	Alphaproteobacteria
OB-16	1180	<i>Sphingomonas</i> sp. KAR7 [EF451637.1]	Aromatic Degradation	96	Alphaproteobacteria
OB-17	811	<i>Rhizobacter</i> Sp. NR 2-01 [KM253106.1]	Iron Chelating	95	Betaproteobacteria
OB-19	830	<i>Rubrivivax gelatinosus</i> [FM886868.1]	Iron Oxidizing	98	Betaproteobacteria

Figure 22 Phylogenetic tree of microbial community in the anaerobic bioreactors (a) and in the oxidizing basin (b)

5.3.4 Biochemical mechanisms

5.3.4.1 Iron sulfide formation

Spectroscopic evidences indicate that the anaerobic sludge contained a mixture of amorphous and crystalline FeS and FeS₂. The formation of the iron sulfides was initiated by precipitation of biogenic hydrogen sulfide with ferrous iron as evident by the presence of sulfur- and iron-reducing bacteria identified in the phylogenetic analysis. The reduction of S and Fe was coupled to organics oxidation and the primary mechanisms contributing to organic waste removal. Crystallization of iron sulfides were likely to result from long-term conversion of amorphous FeS.

5.3.4.2 Sludge oxidation

FeS₂ oxidation

The FeS₂ oxidation in current study is similar to pyrite oxidation in uncovered mine tailings and the subsequent hydrolysis of ferric iron, which results in acidic metal-rich leachate. In general, FeS₂ oxidation occurs via two possible pathways. First, FeS₂ is chemically oxidized to sulfate in aqueous solutions when exposed to DO (reaction (12)) or ferric iron (reaction (13)), followed by ferrous oxidation to ferric ion (reaction (14)).

Second, FeS₂ can be oxidized biologically, in which the presence of iron-oxidizing bacteria can accelerate drastically accelerate ferrous oxidation in acid mine water by many orders of magnitude compared to abiotic conditions (Singer and Stumm (1970). Acidophilic sulfur and iron-oxidizing bacteria of the species such as *Thiobacillus ferrooxidans* (Keller and Murr 1982) are known to play an important role in catalyzing acid-producing reactions in metal sulfides environments within an optimum pH range of 2.0–3.5, utilizing energy from the oxidation of ferrous and sulfur compounds (elemental or reduced) using DO as the electron acceptor (Colmer and Hinkle 1947; Keller and Murr 1982; Southam and Beveridge 1992). *Thiobacter subterraneus* (OB-13) is a thermophilic, obligately chemolithoautotrophic, sulfur/thiosulfate-oxidizing bacterium with optimum pH 6.5-7.0 and it utilizes thiosulfate and elemental sulfur as energy source with oxygen as the only electron acceptor (Hirayama et al. 2005). *Rubrivivax gelatinosus* (OB-19) is able to oxidize iron and deposit the produced Fe(OH)₃ and Fe₂O₃ (Willems et al. 1991). Bacterial modes of FeS₂ oxidation may occur through the direct physical contact between bacteria and FeS₂ particles and the indirect contact in which bacterial oxidation of ferrous to ferric ion occurs, regenerating the ferric ion required for chemical oxidation of pyrite (Silverman and Ehrlich (1964).

FeS oxidation

FeS oxidation is a pH-dependent process and could occur before and after FeS dissolution (D. Rickard 2006). At acidic pHs (3.2-4.3), proton-promoted dissolution via reaction (15) prevails:

Both FeS solid and dissolved species (e.g., Fe^{2+} , FeS (aq), and $H_2S(aq)$) can be oxidized through surface-mediated oxidation and solution-phase oxidation, respectively. The relative contribution of either oxidation process varies significantly with pH. At acidic pHs (3.2-4.9), most of the Fe^{2+} in FeS is released into the solution before being oxidized (Jeong et al. 2010). The released $H_2S(aq)$ can be subsequently oxidized via reactions (16)-(19) or volatilized via reaction (20):

The solution pH in sludge oxidation was found to exhibit a lag with slight increases until day 2 (Supplementary materials Figure S14), suggesting slow iron sulfide dissolution kinetics (reaction (15)) at early stage of the sludge oxidation under circumneutral pH condition. The subsequent solution-phase oxidations of $H_2S(aq)$ via reactions (17)-(19) are proton-generating, corresponding to the pH drop during days 3-7. As evident by sulfide odor, the volatilization of $H_2S(aq)$ via reaction (20) also occurred. Therefore, the dissolved hydrogen sulfide resulting from FeS dissolution was partly oxidized and partly volatilized.

In parallel to the proton-promoted, nonoxidative dissolution, FeS may be dissolved through sulfide oxidation (Thomas et al. 1998; Thomas et al. 2001). However, these reactions are known to be slower by over three order magnitudes than the non-oxidative dissolution (Thomas et al. 2003). The dissolved Fe^{2+} is known to be subsequently oxidized into rust-like precipitates (Fe_2O_3) and goethite (α -FeOOH) at acidic pHs (Jeong et al. 2010).

Overall, FeS oxidation may occur through solution-phase oxidation (reactions (16)-(19)) and surface-mediated oxidation of sulfide via reactions (21)-(22) at acidic pHs. Besides FeS₂ and FeS, a variety of sulfur compounds (SO₄²⁻, S₂O₃²⁻, S⁰, and S²⁻) may be oxidized or reduced by bacteria such as sulfur-disproportionating bacteria (Fossing and Jørgensen 1990; Bharathi 2010).

5.3.4.3 Mass fluxes of Fe and S of the treatment process

Overall, a total iron load of 584±49 mg/d into the system resulted in 139±57 and 37±17 mg/d of total iron in the effluent without and with sludge recycling, respectively (Figure 23). This indicates that, even with the additional iron loads from the sludge recycling, iron was better retained in the anaerobic bioreactors (94%) compared favorably to the baseline operation (76%). Dissolved iron (Fe²⁺ + Fe³⁺) represented 41 – 55% of total iron in the effluent (i.e., 59 – 45% particulate iron). Specifically, dissolved Fe²⁺ (44±26 mg/d) and Fe³⁺ (32±22 mg/d) constituted 55% of the total iron (i.e., 45% particulate iron) without recycling, while dissolved Fe²⁺ (10.4±4.6 mg/d) and Fe³⁺ (4.6±4.7 mg/d) constituted 41% of total iron in the effluent (i.e., 59% particulate iron) with recycling.

The iron extracted from the anaerobic sludge was analyzed and yielded total iron concentrations of 382±170 mg/d, with only 7.2 ±0.3 mg/d in dissolved forms (3.8±0.3 mg/d Fe²⁺, 3.4±0.4 mg/d Fe³⁺). After the 6-day sludge oxidation with daily addition, the total iron concentration was measured comparably at 410±145 mg/d with dissolved iron concentration increasing to 86.0±0.7 mg/d (53.5±3.1 mg/d Fe²⁺ and 32.5±4.0 mg/d Fe³⁺).

For comparison purposes, all sulfur (SO₄²⁻, S₂O₃²⁻, and SO₃²⁻) forms are expressed on a sulfur basis (Figure 24). Overall, 212±8 mg/d of SO₄²⁻-S, 7.4±5.5 mg/d of SO₃²⁻-S, and 0.02 mg/d of S²⁻ (almost negligible) in the influent resulted in 19.7±10.7 mg/d of SO₄²⁻-S, 8.7±5.3 mg/d of SO₃²⁻-S, 1.5±0.3 mg/d of S₂O₃²⁻-S and 1.1±1.4 mg/d of S²⁻ in the effluent under the baseline condition, and 14.2±9.3 mg/d of SO₄²⁻-S, 5.3±4.4 mg/d of SO₃²⁻-S, 1±0.9 mg/d of S₂O₃²⁻-S and 0.42±0.35 mg/d of S²⁻ in the effluent with sludge recycling. These results indicate that 86% of S was retained in the anaerobic bioreactors without recycling and 91% with recycling. This is consistent with better iron retention in the bioreactors with sludge recycling, suggesting higher degree of iron sulfides formation with the sludge recycling.

For both the anaerobic sludge and oxidized sludge, only the dissolved forms of sulfur species in liquid phase were measured: 4.9±2.3 mg/d of SO₄²⁻-S, 2.9±1.4 mg/d of SO₃²⁻-S, 0.5±0.3 mg/d of S₂O₃²⁻-S and 0 mg/d of S²⁻ were present in the liquid phase of the anaerobic sludge. After oxidation, the sludge contained 144±3 mg/d of SO₄²⁻-S, 3.8±2.9 mg/d of SO₃²⁻-S, 0.9±0.6 mg/d of S₂O₃²⁻-S and non-detectable of S²⁻. Consistent

with the iron results, the 6-day oxidation with daily sludge addition did not result in complete oxidation of the sludge materials.

Figure 23 Iron concentrations of the (a) influent, (b) effluent, (c) effluent with sludge recycling, (d) anaerobic sludge, and (e) oxidized sludge under the baseline operation and the sludge recycling condition. Fe(T): total iron, Fe(TD): total dissolved iron, Fe²⁺: dissolved ferrous iron, and Fe³⁺: dissolved ferric iron.

Figure 24 Sulfur concentrations of the (a) influent, (b) effluent, (c) effluent with sludge recycling, (d) anaerobic sludge, and (e) oxidized sludge under the baseline operation and sludge recycling condition. All sulfur forms were measured in dissolved phase.

5.4 CONCLUSIONS

This iron-dosed wastewater treatment process relies on the designed biogeochemical transformations of Fe and S for continuous carbon oxidation. In the anaerobic bioreactors, formation of ferrous sulfides was initiated by sulfate reduction by SRB (*Desulfomonile tiedjei*) and subsequent ferrous sulfide precipitation. Sulfate reduction coupled organics oxidation was the primary mechanisms of removal of organic waste in this treatment process. Presence of IRB (*Alkaliphilus metalliredigens*) provided the evidence of their contributions to organics oxidation. In addition to the biomass, primary chemical sludge materials include both amorphous and crystalline FeS and FeS₂.

In the oxidizing basin, evidences indicate that iron sulfide oxidation was of both chemical (e.g., dissolution, Fe²⁺ and H₂S(aq) oxidation) and biological nature (*Rubrivivax gelatinosus* and *Thiobacter subterraneus*). The oxidized sludge contained a mixture of amorphous compounds (Fe₂O₃/FeO, FeSO₄, and FeS₂), and crystalline Fe₄O₈. Chemical forms of sulfur include the fully oxidized (SO₄²⁻), partially oxidized (i.e., SO₃²⁻, S₂O₃²⁻), and elemental sulfur (S⁰) indicating incomplete oxidation of sulfur. Future work on

enhancing sludge oxidation efficiency such as continuous sludge recycling and identifying favorable chemical and biological oxidizing conditions is needed.

The enhanced treatment performance with sludge recycling can be partly attributed to IRB that mediate additional organics oxidation coupled to ferric reduction because 79% of ferric mass load were reduced and co-precipitated with sulfide ions, estimated by the ferric fluxes in the oxidized and anaerobic sludge materials.

For practical applications of the iron-dosed treatment technology, the degree and quantity of sludge oxidation and recycling would depend on the influent wastewater characteristics such as the amount of iron, sulfate and organics. Optimal iron dosing rate would depend on the COD/sulfate and Fe/S ratios. Potential emission of hydrogen sulfide from the oxidizing basin will need to be prevented. Further reduction of sulfides and partially oxidized S, and ferrous ions in the effluent is also critical as these compounds cause biological instability in the receiving water and need to be taken into account for practical applications of the technology. Chlorine oxidation as a polishing treatment downstream of the anaerobic bioreactor can be a feasible option to oxidize these compounds as it is a commonly used chemical disinfectant.

5.5 SUPPLEMENTARY INFORMATION

Table S6 Clones and corresponding accession number

Clone	Accession number	Clone	Accession number
RB-2	KP962308	OB-2	KP731376
RB-4	KP962309	OB-9	KP731377
RB-5	KP962310	OB-13	KP731378
RB-7	KP962311	OB-14	KP731379
RB-8	KP962312	OB-16	KP962324
RB-10	KP962313	OB-17	KP731380
RB-17	KP962314	OB-19	KP731381
RB-18	KP962315

Figure S 14 pH change over 7 days of sludge oxidation

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CHAPTER 6: CONCLUSION

An innovative strategy for co-managing two prevalent pollution sources, municipal wastewater and acid mine drainage, was proposed in this dissertation. Two main treatment methods were developed and examined in two phases. In phase I, the bench scale study results of acid mine drainage and municipal wastewater using batch reactors were conducted to examine technical feasibility of the approach and the results were summarized as follows:

- The treatment produced water with an average pH of 7.9 and net alkalinity of 290 mg/L as CaCO_3 . The treated water with the increased alkalinity has the potential to be partly recycled to neutralize the AMD in the mixing stage.
- The three-stream mixing would provide a flexible mechanism for conditioning the AMD/MWW mixture for the biological treatment. The mixings in this study consistently resulted in effective removal of phosphate, which is an important feature of the proposed method for removing one of the leading nutrients that cause eutrophication in receiving waters.
- The biological treatment consistently exhibited COD and sulfate removal above 80% for COD/sulfate ratios of 0.6–5.4. This indicated that proper conditioning of the AMD/MWW mixture can lead to sufficient removal of the organic matters and sulfate, and the biological treatment was robust to fluctuation of COD/sulfate ratio once an active biomass was established.
- The treatment also showed effective removal of multi-valent metals Fe, Al, and Mn, and to significant degrees Ca, Mg, and Na. The removed metal elements were mostly in the form of the produced sludge from both the mixing and biological treatment.
- This work demonstrates that an SRB attached-growth reactor can efficiently facilitate removal of COD from wastewater while reducing sulfate, raising pH, and lowering concentrations of metals.
- Proper control of the mixing ratio of MWW and AMD is necessary to avoid Fe inhibitive effects on SRB and to obtain favorable COD/sulfate ratios of the mixture solution for the biological treatment.
- The present research demonstrated that in the co-treatment system, the dominant species belong to the Deltaproteobacteria group. The bioreactor which achieved the highest COD and sulfate removal rates (i.e., B3) supported the most active SRB biomass, and had both higher percentage of *Deltaproteobacteria* and more balanced microbial diversity.
- The microbial population provided insights into the key microbes and metabolic pathways and how chemical substances (e.g., COD/sulfate ratio, Fe) would affect biological treatment.

Overall, this study provides critical information regarding the performance of sulfidogenic bioreactors treating AMD and MWW. The study showed promising results for combined management of the two waste streams and denoted the potential of developing innovative energy-efficient engineering technologies for wastewater management. The microbial DNA analyses and chemical profiling demonstrate the feasibility of the treatment approach and the results provide a base line for future studies to further develop the technology. Further evaluations over extended time periods are necessary to determine how the co-treatment system performs for continuous treatment of the two wastes.

In phase II, the co-treatment treatment concept was extended to areas where the municipal wastewater need to be treated but AMD does not co-exist by doing iron in the anaerobic wastewater treatment process. Through incorporating iron in the anaerobic treatment, it can overcome the potential drawback and enable multiple biogeochemical reactions in engineering designs that facilitate removal of a wide range of contaminants from MWW. This study represents an innovative treatment method with its novelty residing in the use of iron as a green agent to facilitate the designed biogeochemical transformations.

- The mass balance analysis and chemical analyses of Fe and S at different stages of the treatment process demonstrated that the designed biochemical reactions were successfully carried out and produced satisfactory results.
- With a COD/sulfate mass ratio of 2, and a Fe/S molar ratio of 1, this process operated under a condition conducive to anaerobic treatment of the wastewater and yielded the best treatment performance among the different chemical loads.
- Sludge oxidation and recycling significantly enhanced treatment performance. The oxidation in the oxidizing basin was found to only partially convert inorganic precipitates to oxidized iron and sulfur and can be improved.
- The process exhibited treatment stability with reasonable variations under a range of COD/sulfate and Fe/S ratios. The sludge content was found to be fairly consistent over the long periods of operation without sludge recycling, and the bioreactors were found to efficiently absorb the changes in these material loads caused by the sludge recycling.
- The sludge isolated from the anaerobic bioreactors resulted in the selection of a more diverse and dense bacterial community than that observed in the oxidized sludge.
- Phylogenetic analysis of the anaerobic sludge revealed the presence of sequences closely related to *Desulfomonile tiedjei* (sulfur reducing) and *Alkaliphilus metalliredigens* (iron reducing) while in re-oxidized sludge, sequences related to *Thiobacter subterraneus* (sulfur oxidizing) and *Rubrivivax gelatinosus* (iron oxidizing) have been identified.

- In addition to biomass, the chemical sludge of the anaerobic sludge materials contained primarily amorphous and crystallized iron sulfides (i.e., FeS and FeS).

Future studies on investigating specific biogeochemical reactions in the anaerobic bioreactors and oxidizing basin, and their microbial communities would generate useful results to further develop this treatment method and identify optimal treatment conditions. Long-term microbial and chemical characterizations of the sludge materials would further provide insights into the roles of Fe and S, and microbial functions that responsible for the biogeochemical mechanisms of their transformations. Additional studies are also needed to further optimize the treatment process. In particular, guidelines on C: Fe: S load ratios would need to be developed for designing and operating the treatment process.