NOVEL ELECTRON DONORS FOR ANAEROBIC REMEDIATION OF ACID ROCK DRAINAGE

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Pedro Ayala-Parra entitled "*Novel Electron Donors for Anaerobic Remediation of Acid Rock Drainage"*, and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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DEDICATION

A ti amor (Gaby), la persona que más admiro; eres increíble.

A ustedes hijos (Carlos y Luis), por ser mis dos grandes motivos.

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ACRONYMS

AD	Anaerobic digestion
AMD	Acid mine drainage
ARD	Acid rock drainage
BMP	Biochemical methane potential
COD	Chemical oxygen demand
E-donor	Electron donor
Eh-pH	Redox potential-pH
Ein	Specific input energy
HM	Heavy metal
HRT	Hydraulic retention time
ICP-OES	inductively coupled plasma-optical emission spectrometry
LEA	Lipid extracted algae
LHV	Lower heating value
LS	limestone ($CaCO_3$)
MeS	Metal sulfide
MPN	Most probable number
PRB	Permeable reactive barrier
SA	Sonicated algae
SEM	Scanning electron microscopy
SEM-EDS	Scanning electron microscopy-energy-dispersive X-ray spectroscopy
SRB	Sulfate reducing bacteria
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WCA	Whole cell algae
XRD	X-ray diffractometer
ZVI	Zero valent iron (Fe^0)
ZVI-LS	Zero valent iron- limestone

ABSTRACT

We initially studied the treatment of acid rock drainage using a sulfate-reducing bioreactor with zero-valent iron as the electron donor. The results demonstrate that this electron donor can serve as the sole exogenous slow-release electron donor to drive sulfate reduction over 400 operational days at short HRTs (1–3 days). The synthetic acid rock drainage contained high heavy metal concentrations (up to 50 mg/L of copper) and pH values ranging from 3.0 to 7.0. Treatment of this acid rock drainage efficiently removed Cu, Cd and Pb (>99.7%) and increased pH to circumneutral values (7.3–7.7). Elemental analysis indicated that formation of insoluble metal sulfides was responsible for the effective metal removal in the zero valent iron columns.

In the second study, three inoculated columns containing anaerobic granular sludge were fed a synthetic medium containing H_2SO_4 and Cu^{2+} during the experimental period of 4 months. Algae biomass promoted 80% of sulfate removal (12.7 mg SO_4^{2-} d^{-1}), enabling near complete Cu removal (>99.5 %), and alkalinity generation, raising the effluent pH to 6.5. In the algae amended columns Cu^{2+} was precipitated with biogenic H_2S produced by sulfate reduction. Whole cell algae and lipid extracted algae biomasses were both shown to be effective e-donors in driving sulfate reduction of ARD, thus enabling the precipitation and removal of Cu^{2+}. The precipitate retained in the columns was composed mostly of insoluble copper sulfide formed from the biogenic sulfide, as shown by sequential extraction and X-ray diffraction.

In the third study, several pretreatments, i.e., thermal, chemical, sonication and combinations thereof that enhance anaerobic biodegradability of *Chlorella protothecoides* biomass were evaluated. The results demonstrate that anaerobic digestion of pretreated *Chlorella*

protothecoides biomass generates energy-rich methane and recovers nitrogen nutrients. Sonication of algal biomass under optimized conditions provided a significant increase in the methane yield (327 mL STP CH_4 g^{-1} VS) compared to untreated algae (146 mL STP CH_4 g^{-1} VS), as demonstrated in anaerobic digestion experiments incubated for 41 days. In contrast, thermal pretreatment provided only a moderate increase of the methane yield and alkaline treatment led to a decrease of the methane yield compared to the untreated algal biomass. Additionally, sonication treatment provided a 4.1-fold increase in the release of ammonia nitrogen during anaerobic digestion of the algal biomass.

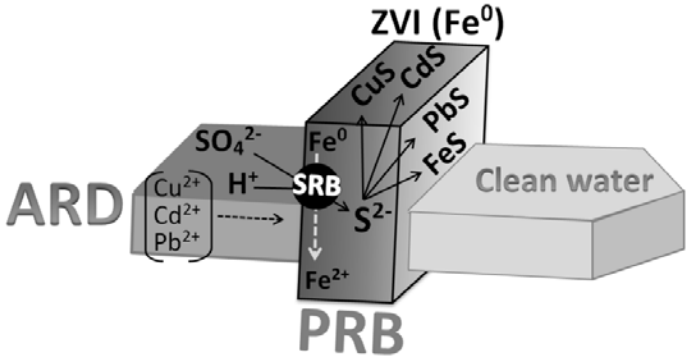
In the fourth study, the nutrient recovery and biogas generation from the anaerobic digestion of waste biomass from algal biofuel production was investigated. Anaerobic digestion of whole cell and lipid extracted *Chlorella sorokiniana*-1412 released 48.1 and 61.5% of the total algal nitrogen as NH_4^+-N, and 87.7 and 93.6% of the total algal P as soluble P, respectively. The biochemical methane potential, quantified through the methane yield of whole cell algae and lipid extracted algae, was 0.298 and 0.253 L methane/g algal volatile solids, respectively. The conversion of lipid extracted algae and whole cell algae biomasses to methane was very similar (38 and 41% on a COD basis, respectively), indicating that the energy yield was not significantly lowered by extraction of the lipid fraction (which accounted for 9% of algal dry weight). Sonication improved the access of hydrolytic enzymes to algal biopolymers, compensating in part for the energy lost due to lipid extraction. The above results demonstrate that algal waste from the biodiesel industry has the potential to be recycled through anaerobic digestion into valuable nutrients and energy.

These studies indicate that zero valent iron and algae biomass are promising reactive materials for the treatment of acid rock drainage in sulfate-reducing permeable reactive barrier

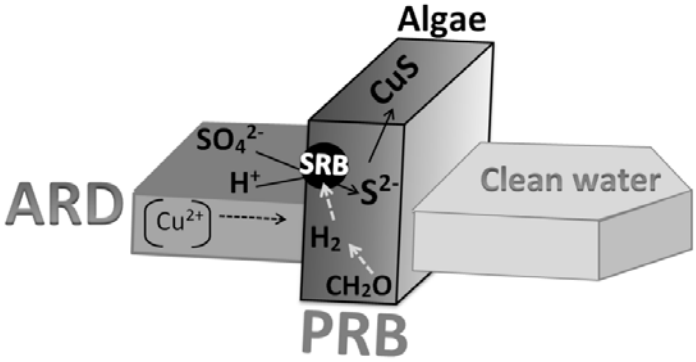
systems. Additionally, to promote algae cultivation for the biodiesel industry, the anaerobic digestion of algae residues can generate nutrients and energy, making algae cultivation more fiscally attractive.

OBJECTIVES AND GRAPHICAL ABSTRACTS

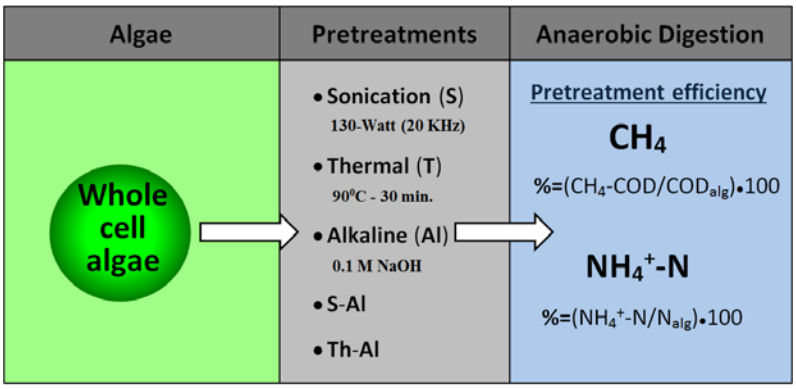
1. Investigate the effectiveness of zero-valent iron as a slow release electron donor for the bioremediation of a metal rich acid rock drainage effluent containing copper (Cu), lead (Pb) and cadmium (Cd) in a flow through sulfate-reducing reactor.



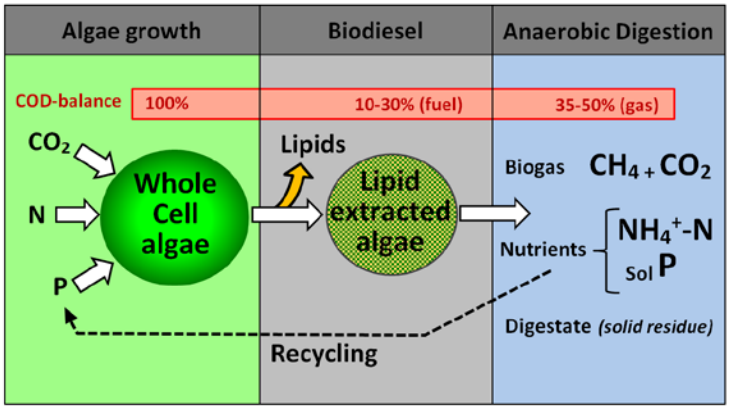
2. Investigate the effectiveness of *Chlorella sorokiniana*-1412 biomass as a slow release electron donor for the remediation of a metal rich acid rock drainage effluent containing Cu in a flow through sulfate-reducing bioreactor system.



3. Evaluate several pretreatment methods for their effectiveness in improving the anaerobic biodegradability of *Chlorella protothecoides* biomass.



4. Evaluate the anaerobic digestibility of the microalgae *Chlorella sorokiniana*-1412 before and after lipid extraction, and quantify the methane yield and the percentage of nutrients recovered through anaerobic digestion.



CHAPTER 1: INTRODUCTION

1.1 Global environmental issues

Current technological development has brought advances that lead to excessive generation of pollutants that affect the environment and disturb ecological balance [1]. Concerns about widespread pollution of water systems caused by acid rock drainage (ARD) have led to investigation of heavy metal (HM) remediation. The term “heavy metal” refers to any metallic element high in density and toxic even at low concentrations [2]. ARD occurs naturally but is more intense when rocks are crushed by anthropogenic activities.

Metal contamination can lead to various toxicological and ecological effects, depending on the particular element. Acidic effluent containing Zn and Cu has potentially adverse ecological impacts on aquatic ecosystems [3]. Soil containing a high concentration of HM increases the risk of cancer [4] in humans exposed to polluted drinking water. The best known toxic heavy metals are Pb, Hg and Cd. These HM are carcinogenic and toxic, affecting, among others, the central nervous system, kidneys or liver.

1.2 Acid rock drainage

The hydrological characteristic of sites where pyrite (FeS_2) is naturally abundant is the main framework for mobilization of ions to reach high metal concentrations in water systems [5]. ARD is the term used for effluent generated by the natural oxidation of sulfide minerals which produce sulfuric acid in the presence of water and dissolved oxygen. Iron sulfide minerals, especially pyrite and chalcopyrite ($CuFeS_2$), are the primary minerals that contribute to ARD

formation [6]. ARD is the most serious environmental concern associated with the mining industry due to its content of toxic HMs. The pollution of HM effluents varies greatly in composition from site to site [7]. Acid mine drainage (AMD) is the term commonly used when ARD is associated with coal mining activity [8]. ARD is also a term used in the mining industry. INAP for example, an International Network for Acid Prevention, collaborates and shares research projects for eliminating ARD [9]. Heavy metals, such as Zn, Cu, Cd, As, Ni, and Pb, are released from minerals exposed to high acidity, as well as SO_4^{2-} up to 5000 mg/L [10].

Several reactions are involved in ARD formation. The first reaction is mainly performed by acidophilic bacteria, such as *Acidithiobacillus ferrooxidans* [11, 12]. In the first step pyrite is partially oxidized to ferrous iron (Fe^{2+}), illustrated in Equation (1). In the second, Fe^{2+} is oxidized to ferric iron, Fe^{3+}, by lithotrophic prokaryotes as shown in Equation (2).

$$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe2+ + 2\ SO_4^{2-} + 2\ H^+ \quad (1)$$

$$Fe^{2+} + 1/4\ O_2 + H^+ \rightarrow Fe^{3+} + 1/2\ H_2O \quad (2)$$

Equation (3) illustrates that more sulfide minerals will be oxidized spontaneously where iron acts as an oxidant.

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+ \quad (3)$$

When comparing oxidants, Fe^{3+} promotes greater rates of pyrite oxidation than oxygen [13]. Thus, this process favors more ARD generation [11]. The complete balance of the reactions that generate ARD is illustrated in Equation (4), [14]. Once biological oxidation of pyrite has started, it is difficult to stop this process.

$$4FeS_2 + 15\ O_2 + 14\ H_2O \rightarrow 4Fe(OH)_3 + 8\ SO_4^{2-} + 16\ H^+ \quad (4)$$

The acid effluent, illustrated in Equation 4, leaches minerals out, increasing HM concentrations. Thus, high acidity increases HM dissolution of elements such as Fe, As, Cu, Zn, Cd, and Pb, in ground and surface waters and streams [15].

1.3 ARD remediation technologies

ARD must be treated to remediate HM and acidity prior to being discharged into streams in order to avoid severe environmental consequences. Several technologies are available to accomplish HM remediation [16, 17]. They are classified as active or passive, depending on whether they require energy or chemical inputs or frequent maintenance [18]. Active approaches for ARD remediation mostly rely on metal precipitation using alkaline or sulfidogenic bioreactors [19]. Traditionally, limestone is the chemical used to neutralize acidity and precipitate HM such as metal carbonates [20]. Other methods for HM remediation are: chemical oxidation and electrochemical treatment [17]. Advanced technologies, such as electrodialysis and ion exchange, are less attractive due to their high costs [21]. The most common passive methods use limestone channels, wetlands, and bioreactors [22].

1.4 Permeable reactive barriers

A permeable reactive barrier (PRB) is a groundwater cleanup technology that consists of a wall of reactive material installed in the path of flowing contaminated groundwater as it penetrates through the wall [18]. The composition of the reactive material used in the PRB is crucial to its efficiency [18]. PRBs remove pollutants, such as HM, by chemical and microbial

degradation processes. Sulfate reduction is a promising passive remediation technology coupled to a PRB. Figure (1.1) illustrates this technology.

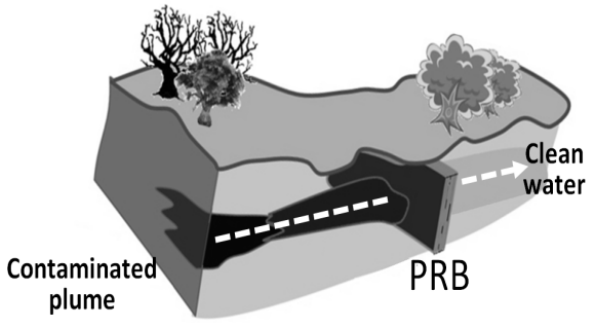


Fig. 1.1. Permeable reactive barrier, a passive HM-remediation technology.

PRBs coupled to sulfate reducing bacteria (SRB) require a source of carbon and an electron donor to facilitate microbial growth and sulfate reduction [23, 24]. The PRB's efficiency is evaluated by monitoring the pH and redox potential (Eh). The Eh of water entering the PRB is moderately oxidized at approximately 200 mV and becomes moderately reduced at approximately - 2 mV. The decrease in Eh promotes sulfate reduction [25]. Under anaerobic conditions, sulfate is reduced to sulfide with the dissolved HMs in situ.

SRB, a diverse anaerobic microbial community, [26], grow in anaerobic conditions of high sulfate (SO_4^{2-}) and H^+ concentrations. SRB use either organic compounds or molecular H_2 as an electron donor (e-donor) to reduce sulfate to sulfide, a process known as dissimilatory sulfate reduction. *Desulfovibrio* genus is a common member of this group of microorganisms. The electron acceptor of sulfate reduction is SO_4^{2-}, while the electron donor can be obtained from a variety of sources: simple or complex organic molecules, organic matter mixtures and inorganic elements [27]. The most common materials used as the electron donors for SRB include ZVI,

sewage sludge, animal manure, compost and other food processing wastes [10, 28]. A PRB primarily remediates ARD through SRB; the SRB transform the mobile inorganic contaminants into immobile forms [29].

When SRB are applied in a PRB, the electron donor duration is one of the most important factors to evaluate. The longer the electron donor lasts, the less PRB maintenance is required. Therefore, slow release electron donors, such as cellulose, lignocellulosic material or iron, are more attractive than readily soluble and easily biodegradable organic molecules, such as ethanol.

1.5 Electron donors

Materials such as cellulose, ethanol, molasses, manure and compost, are organic electron donors for SRB while ZVI is an inorganic electron donor [24]. In both cases molecular hydrogen generated either by iron corrosion or by anaerobic digestion is the preferable electron donor. Hydrogenotrophic SRB outcompete hydrogenotrophic methanogens and acetogens at limited H_2 concentrations. The maximum specific growth rate, the substrate affinity and substrate threshold explain the order of competition [24]. Figure (1.2) shows that sulfide, acetate and methane are the main products in environments with H_2.

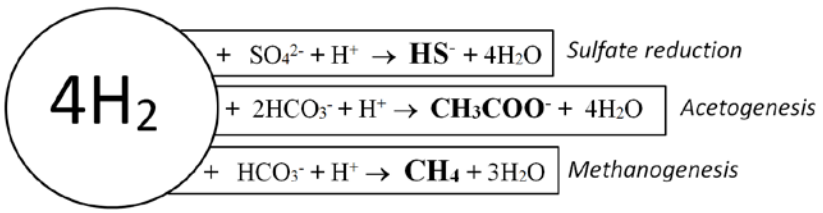


Fig 1.2 Anaerobic reactions where H_2 acts as electron donor [24].

1.5.1 Inorganic electron donor

ZVI, the elemental iron, is able to react with water under anaerobic conditions, producing H_2, by iron corrosion. Hydrogen-consuming microorganisms generally promote iron corrosion [30]. Figure (1.3) illustrates the redox hemi-reactions where ZVI is oxidized and H^+, the electron acceptor from an acidic environment, is reduced to molecular hydrogen.

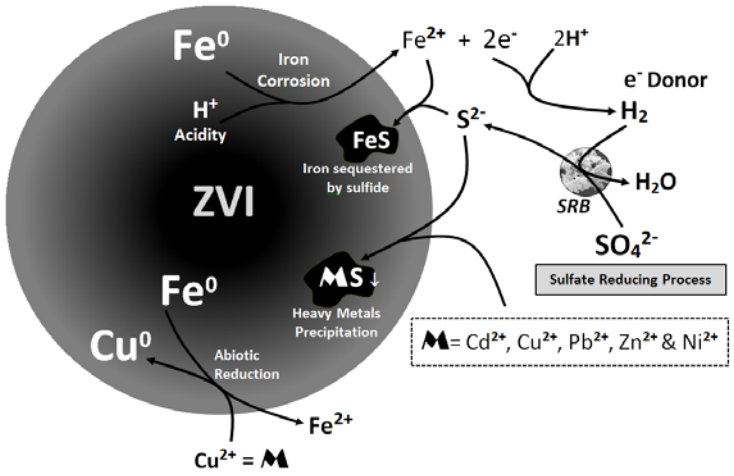


Fig 1.3 Mechanism of ZVI as an electron donor for sulfate reduction

Under sulfate reduction conditions, the sulfide released is combined with ferrous iron in dissolution produced by corrosion [31]. Equation (5) illustrates how ZVI as the electron donor and sulfate as the electron acceptor are catalyzed by SRB, with a molar ratio of 4 moles of iron per 1 mole of sulfate. The product of this reaction is biogenic sulfide which results in an increase in alkalinity and pH.

$$4\,Fe^{o} + SO_4^{2-} + 9\,H^{+} \rightarrow HS^{-} + 4\,Fe^{+2} + 4\,H_2O \qquad (5)$$

Due to the rate of ZVI corrosion and hydrogen utilization, this electron donor is also known as a slow release electron donor. All the processes previously mentioned are illustrated in figure (1).

1.5.2 Organic electron donors

In Equation (6) an organic electron donor is shown, where CH_2O represents dissolved organic carbon. Thus, only organic molecules with the same empirical formula, such as acetate, will have this particular acidity consumption based in molar ratio.

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

The anaerobic digestion is the central process that promotes the acetate and H_2 formation through acidogenesis and acetogenesis. The molecular H_2 is the main electron donor generated under this degradative process.

The organic electron donors are usually produced by anaerobic digestion (AD). AD is the key process in methanogenic and sulfate-reducing environments [32]. The AD of organic matter generates several intermediates, such as acetate and hydrogen. Both compounds serve as electron donors for SRB. It is noteworthy that methanogenesis occurs in the same anaerobic environment, thus methanogens will also compete for the same substrates. As is illustrated in Figure 1.4, the algae cell wall hydrolysis is the initial step of AD to break the cell wall.

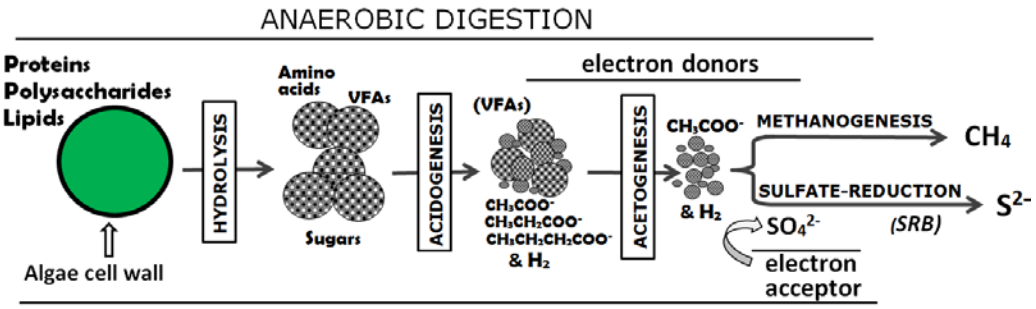


Fig. 1.4. Methanogenesis and sulfate reduction using algae as substrate.

Although lactate and pyruvate are good electron donors for SRB, hydrogen is the best electron donor. Equation (7) and (8) show the free Gibbs energy (ΔG) value for methanogenesis and sulfate reduction. Considering ΔG in both reactions, using hydrogen as the electron donor, the lower ΔG for SRB explains why H_2 is dominantly used by SRB [24].

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \quad \text{(Methanogenesis)} \qquad \Delta G = -32.7\ \text{KJ/mol} \qquad (7)$$

$$4\,H_2 + H^+ + SO_4^{2-} \rightarrow HS^- + 4\,H_2O \quad \text{(SRB)} \qquad \Delta G = -38.1\ \text{KJ/mol} \qquad (8)$$

In the presence of high concentrations of hydrogen sulfide (H_2S) produced by sulfate reduction, sulfide will inhibit methanogenic growth [33]. Thus, SRB will dominate methanogens. Contrarily, at low sulfate concentrations, methanogens can dominate SRB.

1.6 Mechanisms of heavy metal removal

Once sulfate reduction takes place, the main specie of sulfide will depend on the pH of the aqueous system. The sulfide dominant specie above pH 7 is HS^-. H_2S acidity constants are: pKa1

H_2S= 6.98 and pKa2= 19±2; thus, the HS^- concentration depends on chemical equilibrium [34]. In the following reaction, Equation (5), the HM precipitation is represented. Sulfide is able to generate stable precipitates with low solubility (MeS). Me^{2+} stands for divalent HM.

$$Me^{2+} + S^{2-} \rightarrow MeS \downarrow \quad (5)$$

The Pourbaix diagram (Eh-pH diagram), illustrated in Figure 1.5, panel 1.5A, shows an Eh-pH diagram for S and panel 1.5B shows an Eh-pH for the Cu-O-S system. The main specie in groundwater at acidic pH and under reductive conditions is H_2S. In the presence of Cu^{2+} and H_2S and/or HS^-, Cu will be precipitated as CuS. If the pH is increased and the reductive condition is lost, Cu will usually precipitate as cupric oxide (CuO) or copper hydroxide $Cu(OH)_2$, which are less stable forms of this HM.

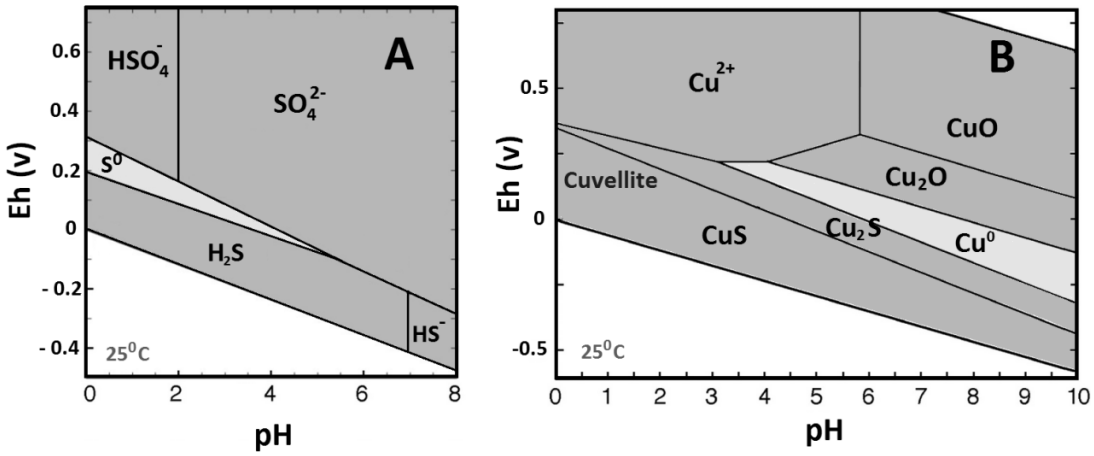


Fig. 1.5. Eh-pH diagram of S (4A) [35] and Cu-O-S (4B) [36] systems at 25°C.

In the presence of HM, the solubility product constant (Ksp), as well as the concentration of metal, define which sulfide metals will be precipitated first. Based on a Ksp comparison, metal sulfides are less soluble than metal hydroxides and metal carbonates. The table of Ksp is shown

in annex section (Table A1). In the corrosion process where SRB activity occurs, HM are first precipitated with sulfide and the remaining sulfur reacts with the ferrous iron. Both precipitates are deposited on the surface of ZVI and subsequently attached to the matrix. Figure 1.2 illustrates the whole mechanism where iron acts as the electron donor.

The solubility of HM sulfides and hydroxides is shown in Figure (1.6). As illustrated, sulfide precipitates are more insoluble than hydroxide metals. This also implies that HMs in their sulfide form are more stable and will be precipitated primarily in low pH.

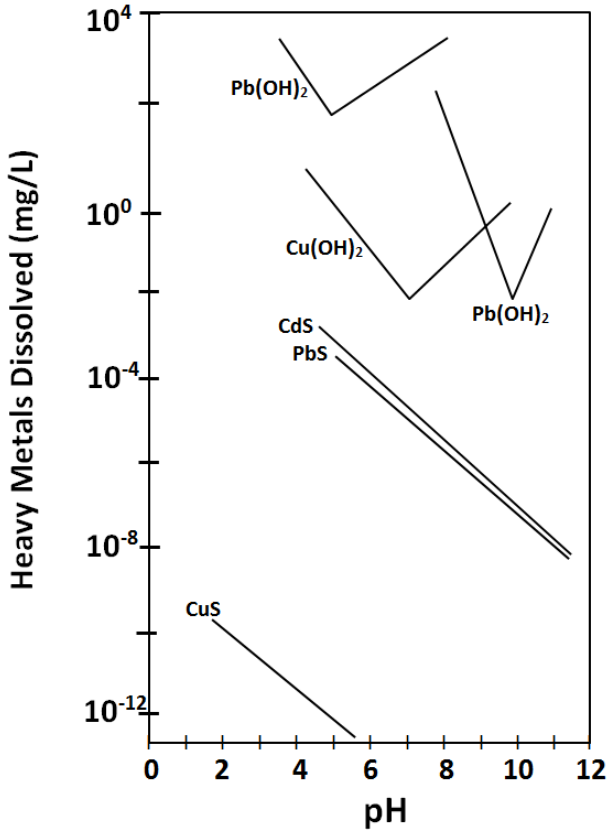


Fig. 1.6 Solubility of metal hydroxides and sulfides as a function of pH [37].

1.7 Algae as a promising source of biodiesel

Algae are characterized by their greater lipid production compared to other oil producing crops. Algae-lipids can be transformed into biodiesel through transesterification reactions. The lipidic fraction of algae is usually as high as 50% on a dry weight basis [38]. Further, lipid extracted algae (LEA) are still rich in proteins and polysaccharides after biodiesel production. Thus, 50% of the algae biomass might be considered as a residual biomass. Projections indicate that algae waste would exceed those uses and become an ecological risk. The excess biomass might, however, become a resource in other areas.

By integrating lipid extraction and anaerobic digestion, the economic feasibility of converting lipids into biodiesel would be increased. Algae biofuel production may become more attractive and environmentally sustainable if other processes, such as nutrient recovery after lipid extraction, CH_4 generation or electron donor formation (H_2 + VFAs) are coupled, as shown in Figure 1.7.

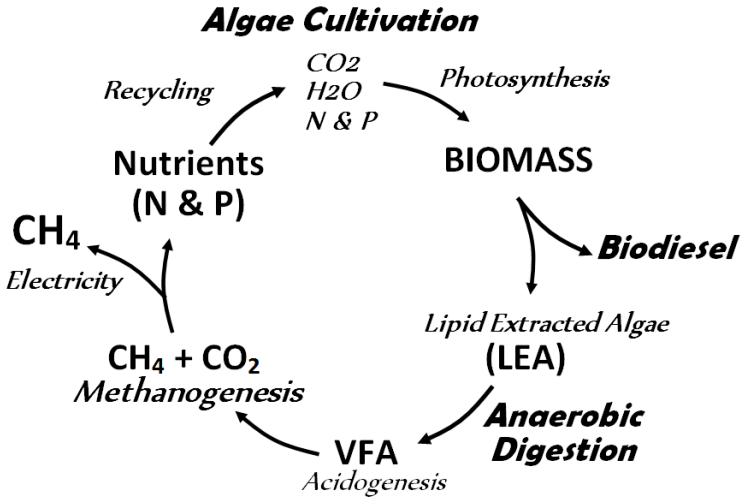


Fig. 1.7 Algae-biodiesel process coupled to anaerobic digestion.

Anaerobic digestion of organic substrates is a common technique to obtain renewable energy, such as CH_4, from waste products. This process is also a common pathway to obtain small VFAs and H_2, the main electron donors [39]. Due to resistance of the cell wall to degradation [40], algae are considered slow release electron donors.

The theoretical methane yield of different algae strains varies due to marked differences in chemical constituents: 6-52% proteins, 7-30% lipids and 5-30% carbohydrates were observed in various strains of algae. Sialve [41] estimated the theoretical biochemical methane potential (BMP) for unprocessed whole algae to be 0.47-0.80 L CH_4/g volatile solids (VS). In lab-scale studies, however, the specific methane yield from whole algae digestion usually ranges from 0.09–0.65 L CH_4/g VS. Some algae studies show that pretreatments that disrupt cell walls facilitate anaerobic degradation [42, 43]. The most common pretreatments are: thermal, chemical, enzymatic, sonication and liquefaction. Sonication is one of the more energy consuming procedures but also one of the most effective.

1.8. References

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CHAPTER 2

TREATMENT OF ACID ROCK DRAINAGE USING A SULFATE-REDUCING BIOREACTOR WITH ZERO-VALENT IRON

This study assessed the bioremediation of acid rock drainage (ARD) in flow-through columns testing zero-valent iron (ZVI) for the first time as the sole exogenous electron donor to drive sulfate-reducing bacteria in permeable reactive barriers. Columns containing ZVI, limestone or a mixture of both materials were inoculated with an anaerobic mixed culture and fed a synthetic ARD containing sulfuric acid and heavy metals (initially copper, and later also cadmium and lead). ZVI significantly enhanced sulfate reduction and the heavy metals were extensively removed (>99.7%). Solid-phase analyses showed that heavy metals were precipitated with biogenic sulfide in the columns packed with ZVI. Excess sulfide was sequestered by iron, preventing the discharge of dissolved sulfide. In the absence of ZVI, heavy metals were also significantly removed (>99.8%) due to precipitation with hydroxide and carbonate ions released from the limestone. Vertical-profiles of heavy metals in the columns packing, at the end of the experiment, demonstrated that the ZVI columns still had excess capacity to remove heavy metals, while the capacity of the limestone control column was approaching saturation. The ZVI provided conditions that enhanced sulfate reduction and generated alkalinity. Collectively, the results demonstrate an innovative passive ARD remediation process using ZVI as sole electron-donor.

***Keywords**:* Heavy metal, Copper, Permeable reactive barrier, Anaerobic, Bioremediation.

2.1. Introduction

Acid rock drainage (ARD) is the effluent generated from rock residues by oxidation of metal sulfides such as pyrite (FeS_2). ARD is often characterized by low pH values (2–6), and high sulfate and heavy metals content [1, 2]. The high acidity and heavy metal concentrations typically found in ARD pose serious ecological risks, particularly for aquatic ecosystems [3, 4]. High metal levels in drinking water resources or crops impacted by ARD can also have negative impacts on human health [5-7].

Remedial approaches for ARD tend to use low cost, low maintenance passive treatments, commonly limestone channels and constructed wetlands [2, 8, 9]. Another passive treatment option is to use permeable reactive barriers (PRB). PRB technology has been developed over the last two decades in order to provide passive, in-situ, treatment of groundwater. PRB is a subsurface emplacement of reactive materials through which a dissolved contaminant plume must move as it flows, typically under natural gradient [10-12]. Contaminants can be removed in PRBs by physicochemical mechanisms (e.g., adsorption, precipitation) or microbial mechanisms. In permeable reactive biobarriers, biological activity is enhanced so that biotic processes can mediate the treatment of contaminants. PRB relying on sulfate-reducing bacteria (SRB) have been shown to be effective for the immobilization of heavy metals in ARD [13, 14].

SRB are anaerobic bacteria that utilize sulfate (SO_4^{2-}) as an electron acceptor [15]. They reduce SO_4^{2-} to sulfide (S^{2-}) utilizing H_2 and organic molecules such as lactate, pyruvate, and ethanol as electron donors (e-donor), resulting in an increase in alkalinity and pH. The biogenic sulfide produced is an excellent ligand for precipitating heavy metals. Eq. (1) illustrates how divalent metals can easily be removed in the presence of sulfide; Me stands for metal.

$$Me^{+2}(aq) + HS^- \rightarrow MeS\downarrow(s) + H^+ \quad (1)$$

Sulfide minerals (MeS) are highly insoluble and can be dissolved only at highly acidic and/or strongly oxidizing conditions because their solubility constants (Ksp) are extremely low (Table A1). Under acidic conditions, metal sulfide precipitates are more stable than metal hydroxide and metal carbonate precipitates as indicated by their considerably higher Ksp constants.

Sulfate-reducing PRBs often utilize insoluble, slow release organic substrates such as sewage, manure, compost, lignocellulosic residues, and food waste as source of carbon and an e-donor [17-19]. This work determines for the first time whether zero-valent iron (ZVI or Fe^0) can be used as a sole e-donor to generate sulfide and stimulate heavy metal removal in a flow-through bioreactor simulating a PRB system. ZVI has been shown to serve as e-donor for SRB in batch bioassays [20, 21]. However, the application of ZVI as the sole e-donating substrate in sulfate-reducing systems for heavy metal remediation has not been described to date. ZVI is readily oxidized in water under anaerobic conditions to produce H_2. As iron is oxidized, Fe^0 produces $Fe^{2+} + 2e^-$ and H^+ is reduced to H_2. Concomitant with the formation of H_2 is the release of OH^- (Eq. (2)). SRB use the electrons via hydrogen formation to reduce sulfate (Eq. (3)).

$$Fe^o + 2H_2O \rightarrow Fe^{2+} + H_2 + 2OH^- \quad (2)$$

$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O \quad (3)$$

Sulfate reduction driven by abiotically formed H_2 has been demonstrated in a special culture flasks system where the abiotic corrosion of mild steel was separated from an H_2-consuming sulfate-reducing culture except for a shared headspace [22]. However there is also evidence for direct e-transfer from ZVI to microorganisms based on SRB accelerating the rate of ZVI corrosion beyond the abiotic rate [20, 23], evidence for involvement of c-type cytochromes in the electron transfer [24, 25], and evidence that enzymes in the cell-free-extract of a

methanogen could catalyze the formation of H_2 and formate from ZVI [26]. Eq. (4), which is obtained combining Eqs. (2) and (3), shows that a molar ratio of 4 moles of iron is required to reduce 1 mol of sulfate. Fe^{2+} and biogenic sulfide are produced and H^+ is consumed, leading to an increasing the pH.

$$4Fe^o + SO_4^{2-} + 9H^+ \rightarrow HS^- + 4Fe^{+2} + 4H_2O \quad (4)$$

The utilization of ZVI as substrate in sulfate-reducing PRBs designed to treat ARD has the potential to provide important benefits compared to conventional organic substrates. First, ZVI is a promising strong reductant and widely available material that can drive sulfate reduction and promote the precipitation of highly stable metal sulfides. Secondly, corrosion of ZVI leads to formation of Fe^{2+} ions that can sequester excess sulfide, minimizing discharge of this toxic and malodorous contaminant into the PRB effluent. Thirdly, corrosion of ZVI consumes acidity, thereby contributing to neutralize the high acidic levels often found in ARD. This study assessed the bioremediation of ARD in a laboratory-scale sulfate-reducing reactor using ZVI as the sole exogenous electron donor. Continuous-flow bioreactors packed with either limestone or limestone and ZVI were run in parallel to investigate the benefits of supplying limestone. The nature of the minerals deposited in the packing material of the reactors was elucidated to gain insights on the mechanisms of metal immobilization.

2.2 Materials and methods

2.2.1. Microorganisms

Anaerobic granular sludge was obtained from an upward-flow sludge bed reactor treating brewery effluent (Mahou, Guadalajara, Spain). The sulfate-reducing activity of this sludge was

demonstrated in batch experiments [27]. The sludge contained 7.13% volatile suspended solids (VSS) in wet–weight.

2.2.2. Chemicals

ZVI (325 mesh; 97%) was obtained from Sigma (St., Louis, MO),limestone ($CaCO_3$, 2–4 mm) from Oglebay Norton Industrial Sands (Buchanan, VA) and silica (2 mm) from Premier Silica (Colorado Springs, CO).

2.2.3. Basal medium

The basal mineral medium used to prepare the synthetic ARD contained (in mg L^{-1}): NH_4Cl (80); $NaHCO_3$ (50); K_2HPO_4(171);$CaCl_2.2H_2O$ (20), $MgCl_2{\cdot}6H_2O$ (29), yeast extract (20), and 1 mL L^{-1} of trace element solution [20]. Copper (added as $CuSO4{\cdot}5H_2O$) was gradually increased from 10 to 50 mg L^{-1}. Additional sulfate (250 mg SO_4^{2-} L^{-1}) was added as H_2SO_4. The pH was adjusted to the desired value (see Section 2.5) by addition of NaOH or HCl.

2.2.4. Continuous-flow bioreactors

Three up-flow packed-bed columns (0.385 L) were inoculated with anaerobic sludge (10 g VSS L^{-1}) and operated in parallel to evaluate the removal of heavy metals using ZVI as the only exogenous source of e-donor in the presence and absence of limestone (Fig. 2.1).

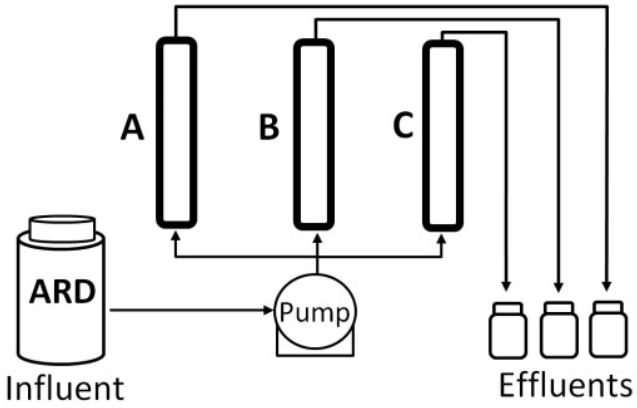

Figure 2.1. Diagram of the3 packed-bed columns used to test the ZVI as an e-donor for a sulfate reducing permeable reactive barrier. A) LS column, B) ZVI-LS column and C) ZVI column.

Limestone served as a pH buffer and as supplemental source of inorganic carbon for lithoautotrophic SRB that can grow using CO^2 as a sole carbon source [28]. The bioreactors were packed with sand and either ZVI (ZVI column), limestone (LS column), or limestone and ZVI (ZVI-LS column). Table 2.1 lists the amounts of limestone, sand and/or ZVI and anaerobic sludge supplied to each column. The columns were operated at 30 ± 2◦C in parallel for 400 days.

Table 2.1. Composition of the packing of the 3 continuous-flow columns utilized in this study.

		Columns[a]								
		LS			**ZVI-LS**			**ZVI**		
Component[b]	DBD[c]	Mass[d] (g)	Mass (%)	V/V[e] (%)	Mass (g)	Mass (%)	V/V (%)	Mass (g)	Mass (%)	V/V (%)
Limestone	1.50	183.6	36.5	36.9	183.6	31.5	36.8	0	0	0
Sand	1.53	320	63.5	63.1	215.0	36.9	42.2	400.0	68.6	78.9
ZVI	2.62	0	0	0	183.4	31.5	21.0	183.4	31.4	21.1

[a] Packing volume (0.335 L). [b] The composition shown in Table 2.1 does not include the addition of sludge inoculum (54 g wet sludge or 4.16 g dwt sludge). [c] DBD = dry weight bulk density (g cm^{-3}). [d] Mass is provided in dwt. [e] V = volume.

The feed consisted of synthetic ARD containing 250 mg $SO_4^{2-}L^{-1}$ and variable metal concentrations. The Cu^{2+} concentration varied from 0 to 50 mg L^{-1} depending on the period of operation (Fig. 2.2). Additional heavy metals (10 mg Cd^{2+} L^{-1} and 2.4 mg Pb^{2+} L^{-1}) were added during the final 60 days. During the initial 64 days (phase I), the influent pH was 7.2. During the next 65 days (phase II), the pH was gradually reduced from 7.2 to 3.0. Thereafter, the influent pH remained at 3.0 (phase III) (Fig. 2.2). The initial neutral pH conditions enabled rapid SRB enrichment. Fig. 2.2 shows the hydraulic retention times (HRT, calculated based on the empty-bed volume of the reactor).

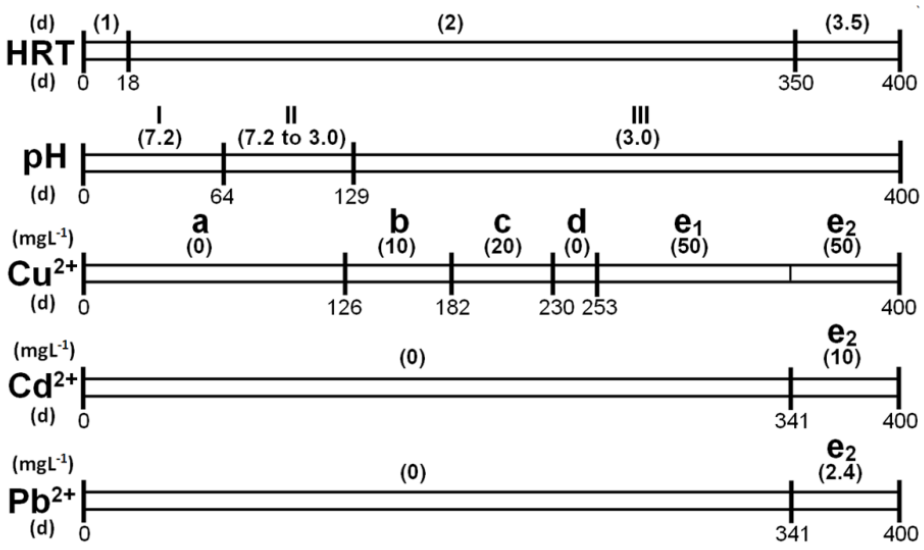

Figure 2.2. Diagram illustrating the conditions in each phase and period of column operation. For each parameter the units of the condition are on top and the days of reactor operation are on the bottom. The phases of the experiment (I, II, and III) are defined by the pH regimen. The periods of the experiment (a, b, c, d, e1 and e2) are defined by the heavy metal additions. HRT refers to the empty bed hydraulic retention time.

During the first 18 days, the reactors were operated at a HRT of approximately 1 day. From day 18 to 350, the HRT was 2 days. During the last 50 days, the HRT of the ZVI and ZVI-LS columns was3.5 days. Samples of the influent and effluent of the various reactors were collected regularly to determine pH, sulfide (S^{2-}), SO_4^{2-}, Cu^{2+}, Cd^{2+} and Pb^{2+}. Prior to the determination of SO_4^{2-} and soluble metals, samples were membrane filtered (0.45 μm).

2.2.5. Column packing characterization

2.2.5.1. Sequential extraction

As the experiments concluded, the packing of each column (334.5 mL) was divided into three vertical sections, each with the same volume. Sectional packing was weighed, crushed, homogenized and sampled in an anaerobic chamber to: 1 determine moisture, 2 enumerate SRB, and 3 characterize precipitates using scanning electron microscopy and energy-dispersive X-ray spectroscopy. Copper from all sections was sequentially extracted with water,1 M HCl, and a mixture of 16 M HNO_3–12 M HCl (3:1, v/v) following a procedure adapted from Cooper and Morse [29]. A wet sample of each section (3 g) was added to 10 mL of water and vortexed for 10 min (three repetitions). Test tubes were then centrifuged (4000 rpm, 7 min) to collect the supernatant. The solids remaining were extracted with 10 mL of 1 M HCl as previously described. Finally, the remaining solid was extracted with HNO_3–HCl (3:1) in the microwave at 120◦C for 35 min. The supernatant was centrifuged and collected for copper analysis.

2.2.5.2. Most probable number (MPN) determination

Counts of SRB were accomplished using the MPN technique [30]. Packing material (7 g wet weight) was blended in phosphate buffer (pH 7.4) and then diluted with basal medium to

attain 10 mL of solution in each tube. Consecutive dilutions from 10^2 to 10^{10} fold were performed. These dilutions were incubated in a solution containing ethanol (0.01 mL/10 mL), sulfate (250 mg L^{-1}), and an iron nail. The headspace of the tubes was flushed with N_2–CO_2 (80–20%) for 5 min to ensure anaerobic conditions. After three weeks of incubation at room temperature, sulfate reduction was indicated by the formation of black precipitates (iron sulfide) on the iron nail.

2.2.5.3. Scanning electron microscopy and energy-dispersive X-ray spectroscopy

SEM–EDS analyses were performed in a Hitachi S-4800 N Type II with a cold field emission electron gun and an accelerant voltage of 20 kV. The SEM was combined with a ThermoNORAN microanalyzer for energy dispersive spectroscopy (EDS). The samples were vacuum dried and crushed to a powder material. Then, the samples were adhered to a metallic base and coated with platinum.

2.2.6. Analyses

Sulfide was analyzed colorimetrically by the methylene blue method [31]. Sulfate was measured by ion chromatography with suppressed conductivity using an AS11-HC4 column (Dionex, Sunnydale, CA) and a conductivity detector. Copper was determined by inductively coupled plasma-optical emission spectrometry (2100 Optima ICP-OES, PerkinElmer, Waltham, MA). Wavelengths used for Cu^{2+}, Pb^{2+} and Cd^{2+} determinations were: 327.3, 220.3 and 228.8 nm, respectively. VSS and pH were determined according to standard methods [32].

2.3. Results and discussion

2.3.1. pH evolution

During phase I, the effluent pH increased to 9 and 10 in the two ZVI reactors, whereas the effluent pH remained neutral in the case of the LS column (Fig. 2.3). In phases II and III, as the influent pH decreased from 7.2 to 3.0, the effluent of the three columns was neutral to mildly alkaline. From day 250 and beyond, all effluents had similar pH values, between 7.2 and 7.7. The effluent of the LS column usually had a lower pH compared to the effluent of the ZVI-containing columns. The three columns were able to handle very low pH values, which are common in ARD, and increased the pH of the effluent to the circumneutral values typically required by SRB [33].

The increase in the pH of the effluent of the LS column was primarily due to the release of bicarbonate alkalinity from the limestone packing which is beneficial for acid neutralization and pH buffering. In acidic waters (pH less than 6.4), limestone reacts according to the following reaction [34]:

$$CaCO_3 + 2\ H^+ \rightarrow Ca^{2+} + H_2CO_3 \quad (5)$$

H_2CO_3 continues to react with limestone according to the following reaction:

$$CaCO_3 + H_2CO_3 \rightarrow Ca^{2+} + 2HCO_3^- \quad (6)$$

In ZVI-amended columns, alkalinity can be generated by anoxic corrosion of ZVI (Eq. (2)) and sulfate reduction (Eq. (3)), as both processes involve the consumption of H^+.

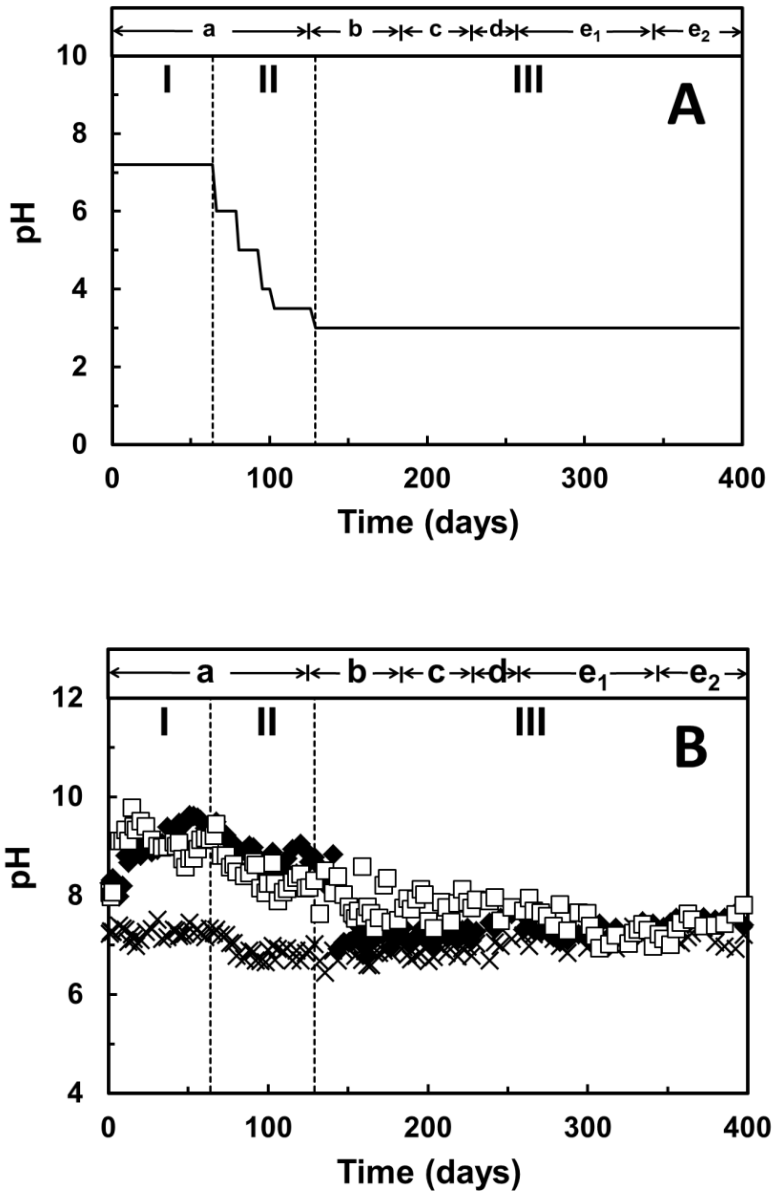

Figure 2.3. Panel A; influent pH. Panel B; effluent pH during three separate periods of feeding with influent's pH of: 7.2 (I), from 7.2 to 3.0 (II) and 3.0 (III). ZVI-LS column (♦), ZVI column (□) and LS column (×).

2.3.2. Sulfate reduction

Previous reports indicate that ZVI corrosion can provide electrons via H_2 to stimulate microbial sulfate reduction [20, 21]. Based on (Eq. (4)), 4 mol of ZVI are required to reduce 1 mol of SO_4^{2-}. The ZVI reactors were supplied with 183.4 g ZVI, which is 5.5-fold higher than the theoretical amount needed to reduce the cumulative amount of SO_4^{2-} supplied (14.3 g). These calculations are based on an HRT of 2 d (0.19 L d^{-1}) and 250 mg SO_4^{2-} L^{-1} over one year of operation. Fig. 2.4 shows sulfate removal efficiency differences between the ZVI and ZVI-LS reactors. During phase I, the ZVI reactors achieved sulfate removal efficiencies ranging from 40 to 50%; the best performance was achieved by the ZVI-LS column. In phase II, both columns removed sulfate as in phase I. In most of phase II and through period (e1) of phase III, there was a slightly improved performance by ZVI-LS compared to the ZVI column. The ZVI-LS column was approximately 10% more efficient than the ZVI reactor over phases I and II. During phase III, the ZVI-LS reactor was around 30% more efficient during periods (b) and (c), and 65% more efficient over periods (d) and (e1). In phase III period (e2), the sulfate reduction efficiency of the ZVI column was the same as the ZVI-LS. The better performance of ZVI-LS compared to ZVI column could be due to the improved inorganic carbon supply in the former. Some SRB are chemolithoautrophic utilizing CO_2 as carbon source [28].

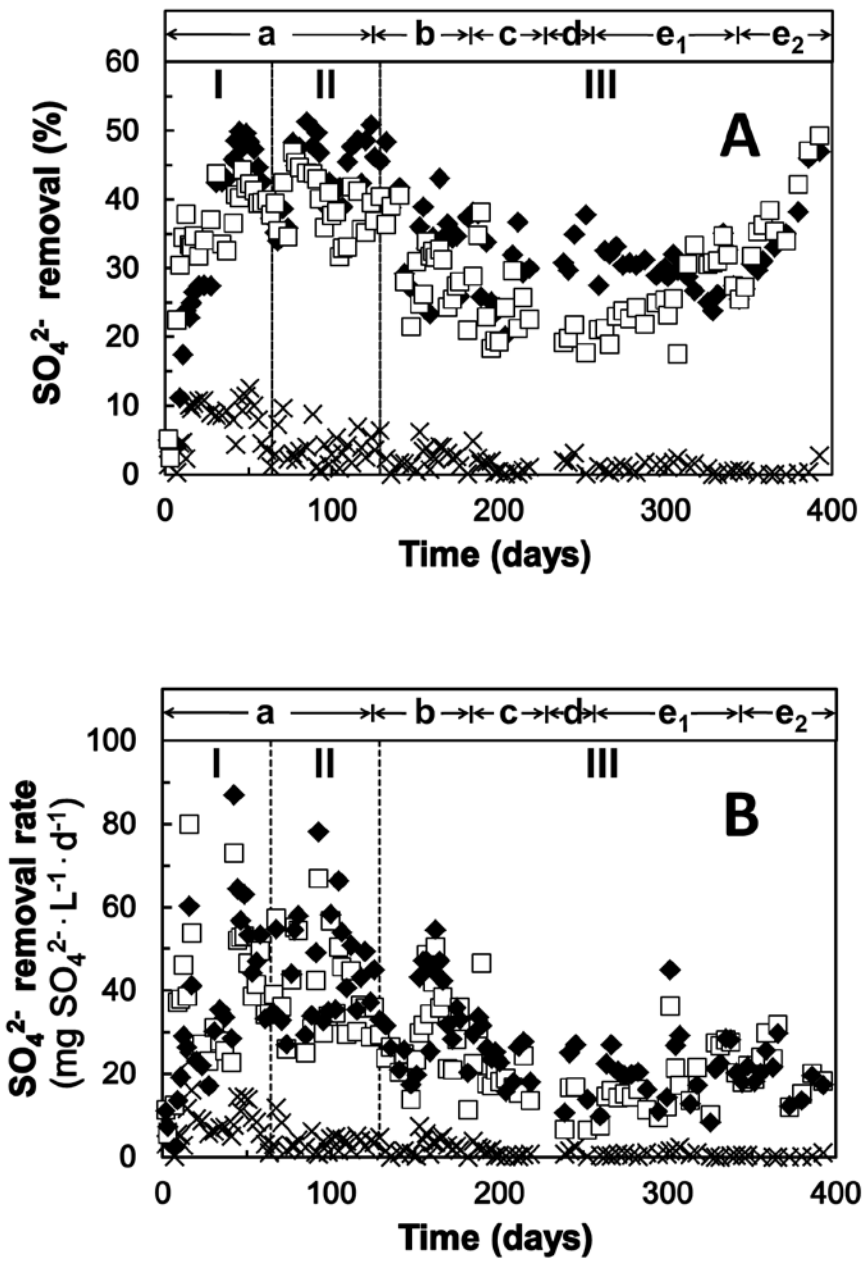

Figure 2.4. Percentage sulfate removal during the operation of the columns. ZVI-LS column (♦), ZVI column (□) and LS column (×).

At the beginning of phase III, sulfate removal decreased to 25% and 35% in the ZVI and ZVI-LS columns, respectively, due to the drop in influent pH from 7.2 to 3.0. The subsequent recovery of sulfate reduction was possibly due to an increase in the SRB population. Later, in phase III period (e2), when the HRT of the ZVI columns was increased from 2.0 to 3.5 days, the sulfate removal efficiency reached 50%. The high sulfate reduction rates maintained after 400 days of operation indicate that the e-donor capacity of ZVI was not exhausted. During phase I, the LS column achieved a sulfate removal of only 10%. The removal efficiency decreased to 5% in phase II and ceased at the beginning of phase III. Sulfate reduction in the LS column was driven by endogenous substrate derived from the decay of the inoculum, and lasted until the biomass decomposition was exhausted. A previous study showed that the inoculum used in this study had measurable endogenous e-donor contribution [35].

Soluble copper is known to inhibit SRB but the reported 50%inhibitory concentrations range drastically from only 0.84 mg L^{-1} to greater than 763 mg L^{-1} [36-39]. In this study, copper was sup-plied at concentrations between 10 and 50 mg L^{-1}. Fig. 2.4 shows that the sulfate removal efficiency of the ZVI columns was similar in periods (c), (d), (e_1) and (e_2), regardless of the copper concentration in the influent. The observation that the presence of Cu^{2+} in the influent had no measurable impact on inhibiting sulfate reduction is consistent with the sharp decrease in the Cu^{2+} concentration attained by the ZVI columns, as will be discussed later in Section 3.4. Previous studies have shown attenuation of copper toxicity by biogenic sulfide [27]. The apparent increase in the sulfate removal efficiency in the last period of operation (e_2, in Fig. 2.4), when the HRT was increased from 2.0 to 3.5 days, does not imply recovery from toxicity since the volumetric removal rate of sulfate remained the same as in the previous period (Fig. 2.4B). SRB were enumerated in the packing of the various columns at the end of the experiment. The SRB count in the methanogenic inoculum was very low (101 cells g^{-1}dwt). Continuous operation of

the various reactors for 400 days with a sulfate-containing feed led to a marked increase in the SRB counts. Whereas the lowest SRB count was in the in the bottom section of the LS column (4.5×10^5 cells g^{-1} dry packing), the SRB counts were highest in the bottom sections of the ZVI-LS and ZVI columns, 3.8×10^7 and 2.7×10^7 cells g^{-1} dry packing, respectively. The 60–85-fold higher SRB counts in the ZVI-amended reactors indicate that ZVI served as e-donor of the growth of the SRB population. Isotopic evidence from a previous study also demonstrated that ZVI enhanced sulfate reduction in biologically active columns [40]. The slightly improved performance in the ZVI-LS column versus the ZVI column may be due to its somewhat higher SRB population.

2.3.3. Sequestration of biogenic sulfide

Biogenic sulfide may be captured in the bioreactors due to the formation of metal sulfides. Fig. 2.5 shows the concentration of sulfide in the effluent of the bioreactors as a function of time. Sulfide was detected in the effluent of the LS column in phases I, II and the first part of phase III, indicating microbial sulfate reduction (Fig. 2.5).

Since the LS column did not receive an exogenous e-donor, microbial sulfate reduction was sustained by the endogenous substrate in the inoculum biomass. However, after 90 days of operation a sharp decrease in the concentration of sulfide released by the LS column was observed, most likely due to gradual depletion of the endogenous substrate in the inoculum. On the other hand, the ZVI and ZVI-LS columns discharged very little or no sulfide, despite these columns having much greater levels of sulfate reduction compared to the LS column (Fig. 2.5). Thus, the sulfide generated in the ZVI columns was for the most part sequestered in the column due to reaction with Fe^{2+} (periods a, d), or Fe^{2+} plus Cu^{2+} (during periods b, c and e).

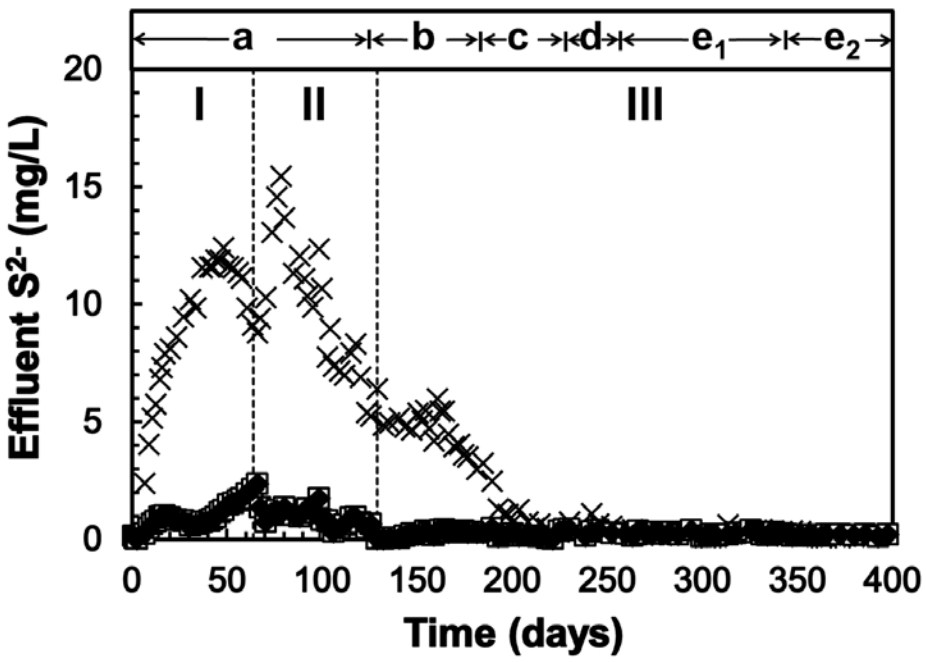

Figure 2.5. Effluent sulfide concentration during the operation of the columns. ZVI-LS column (♦), ZVI column (□) and LS column (×).

The effectiveness of ZVI to decrease the concentration of sulfide discharged in the effluent is illustrated in Fig. 2.6, which shows the S^{2-}-S sequestered, i.e., the difference between the concentration of SO_4^{2-}-S removed and S^{2-}-S discharged by the various columns, during two different periods of operation, with no copper addition (Fig. 2.6A) and with addition of 10 mg Cu^{+2} L^{-1} (Fig. 2.6B). The sulfide levels in the effluent of the columns amended with ZVI was very low indicating extensive sequestration of the biogenic sulfide in the columns.

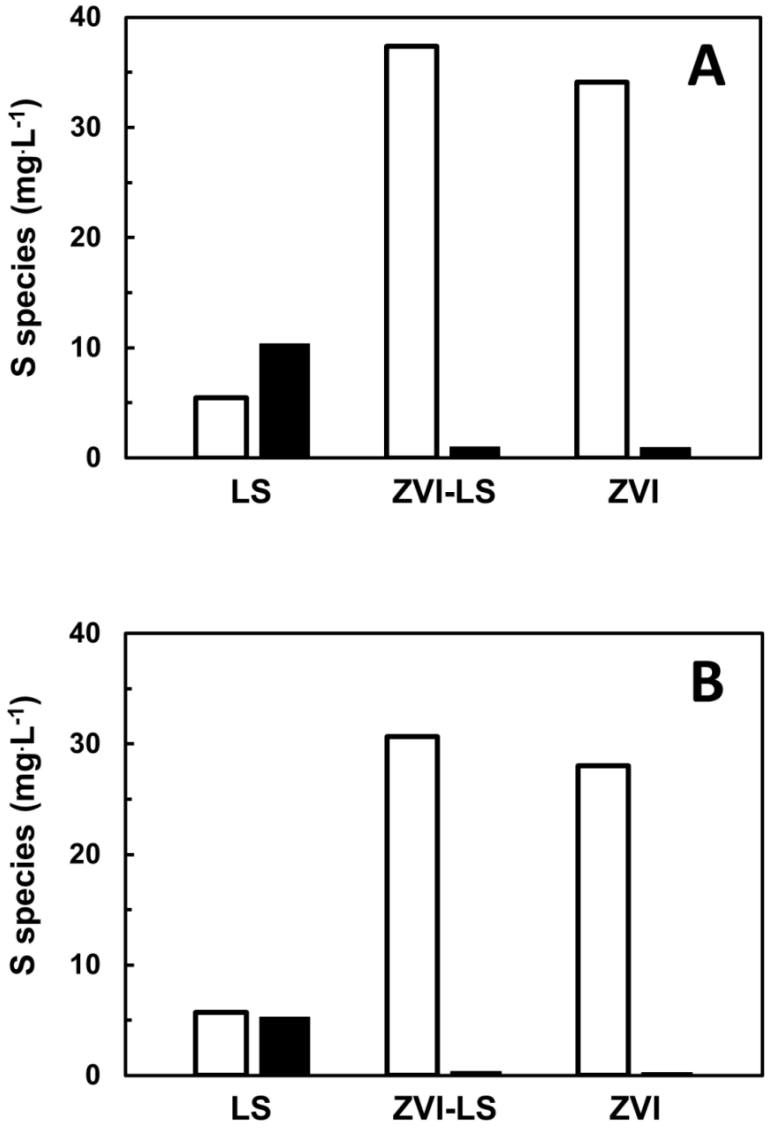

Figure 2.6. Sulfide sequestering in two different periods. Panel A represents the period from day 70 to day 110 with no copper addition and panel B represents the period from day 140 to day 170 with copper addition of 0.15 mM. Sulfate removed (open bars) and sulfide discharged with effluent (filled bars).

2.3.4. Removal of Cu, Cd and Pb

The concentration of soluble copper in the effluent of the three columns was always lower than 70 $\mu g L^{-1}$ (Fig. 2.7). All reactors demonstrated very high copper removal efficiencies ranging from 99.8 to 99.9%, even during the period when the feed contained cop-per concentrations as high as 50 mg L^{-1}. Bai and coworkers [41] also treated ARD in a high-rate SRB bioreactor using ZVI and lactate as e-donors and attained Cu^{2+} removal efficiencies up to 99%. How-ever, in contrast with our study, Bai used an organic substrate as the main e-donor source. Furthermore, the excellent treatment effectiveness obtained in the limestone columns is in agreement with several studies that demonstrate effective removal of heavy metals by using limestone channels via adsorption or precipitation [42, 43].

Metal–sulfide precipitation is the main mechanism expected to contribute to the removal of copper in the ZVI-amended columns. In the presence of biogenic sulfide, copper sulfide is formed due to the considerably lower Ksp value of CuS compared to $Cu(OH)_2$ and $CuCO_3$ (Table 1). In this sense, it is interesting to note that Sierra-Alvarez and coworkers [44] reported that the predominant copper sulfide mineral formed in a sequential sulfate-reducing bioreactor-crystallization reactor was covellite. The concentration of biogenic sulfide in the ZVI columns exceeded the stoichiometry requirement for the quantitative precipitation of the copper added. Considering the stoichiometry of CuS, 25.2 mg L−1of sulfide is needed to precipitate the highest concentration of copper added (50 mg L−1). When the columns were fed 50 mg L^{-1} copper, the average sulfate removal in both ZVI columns was 79.4 mg SO_4^{2-} L^{-1}, which is equivalent to 26.4 mg L^{-1} of S^{2-} produced. Thus, there was sufficient sulfide to assure that all the added Cu^{2+} could be sequestered in the columns as CuS. It should be noted that although sulfate reduction is the main process generating sulfide, FeS could also potentially immobilize heavy metals. Fe^{+2} in FeS

can be displaced by divalent metals with more affinity for S^{2-}. For example, copper and cadmium will form metal sulfides with FeS while Fe^{2+} is released in the aqueous phase [45].

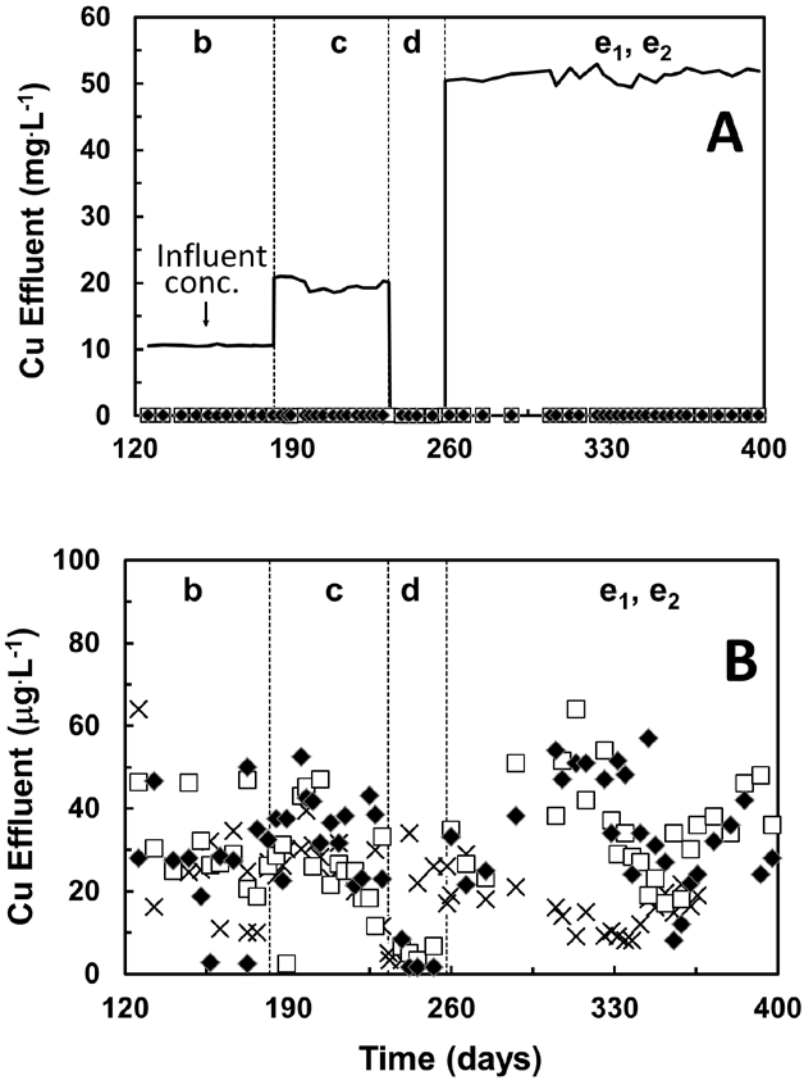

Figure 2.7. Copper effluent concentrations during four periods based on influent copper concentrations (μgL^{-1}): 10,000 (b), 20,000 (c), 0 (d) and 50,000 (e) into ZVI-LS column (♦), ZVI column (□) and LS column (×). Plot A shows complete results. Plot B zooms to 100 $\mu g\ L^{-1}$ to emphasize copper discharged from ZVI columns.

Biogenic sulfide also contributed to the removal of copper during the early stages of operation of the LS column. However, from period (c) onwards, the LS column was no longer reducing sulfate (Fig. 2.5), thus copper immobilization was likely mediated by precipitation with hydroxide and carbonate ions released from limestone ($CaCO_3$) dissolution. Solid phase analysis of Cu reacting with lime-stone ($CaCO_3$) in previous studies has provided evidence for $CuCO_3$ formation and its adsorption on calcite surfaces [46, 47]. The main advantages of using limestone are low costs and high efficiency of metal removal.

The disadvantage is that rock surfaces are saturated after a short time period [48, 49]. In period (e2), soluble Pb^{2+} (10.0 mg L^{-1}) and Cd^{2+} (2.4 mg L^{-1}) were added to the influent, in addition to Cu^{2+}. The effluent concentrations of Cd and Pb in all the columns were very low, averaging 17 and 11 μg L^{-1}, respectively (Fig. 2.8). Sulfate reduction in the LS column was negligible during this period (Fig. 2.4) suggesting that metal immobilization was due to carbonate and hydroxide precipitation. The solubility of Cd^{2+} and Pb^{2+} sulfides is extremely low (Table 2.1) and, therefore, Cd and Pb were most likely retained as metal sulfides in the sulfate-reducing bioreactors. The amount of sulfide required to precipitate 50 mg Cu^{2+} L^{-1}, 10 mg Cd^{2+} L^{-1} and 2.4 mg Pb^{2+} L^{-1} would be 25.2, 2.84 and 0.37 mg L^{-1}, respectively; requiring a total sulfide of 28.4 mg L^{-1}.

The average sulfate removal in the ZVI-amended columns was 40%, corresponding to reduction of 100 mg SO_4^{2-} and the concomitant generation of 33.3 mg L^{-1} of sulfide (Fig. 2.4), thus assuring a 17% excess of sulfide. Other studies have also reported that the main mechanism of Cu^{2+}, Cd^{2+} and Pb^{2+} removal in sulfate-reducing bioreactors is precipitation in the form of metal sulfides [44, 50-52].

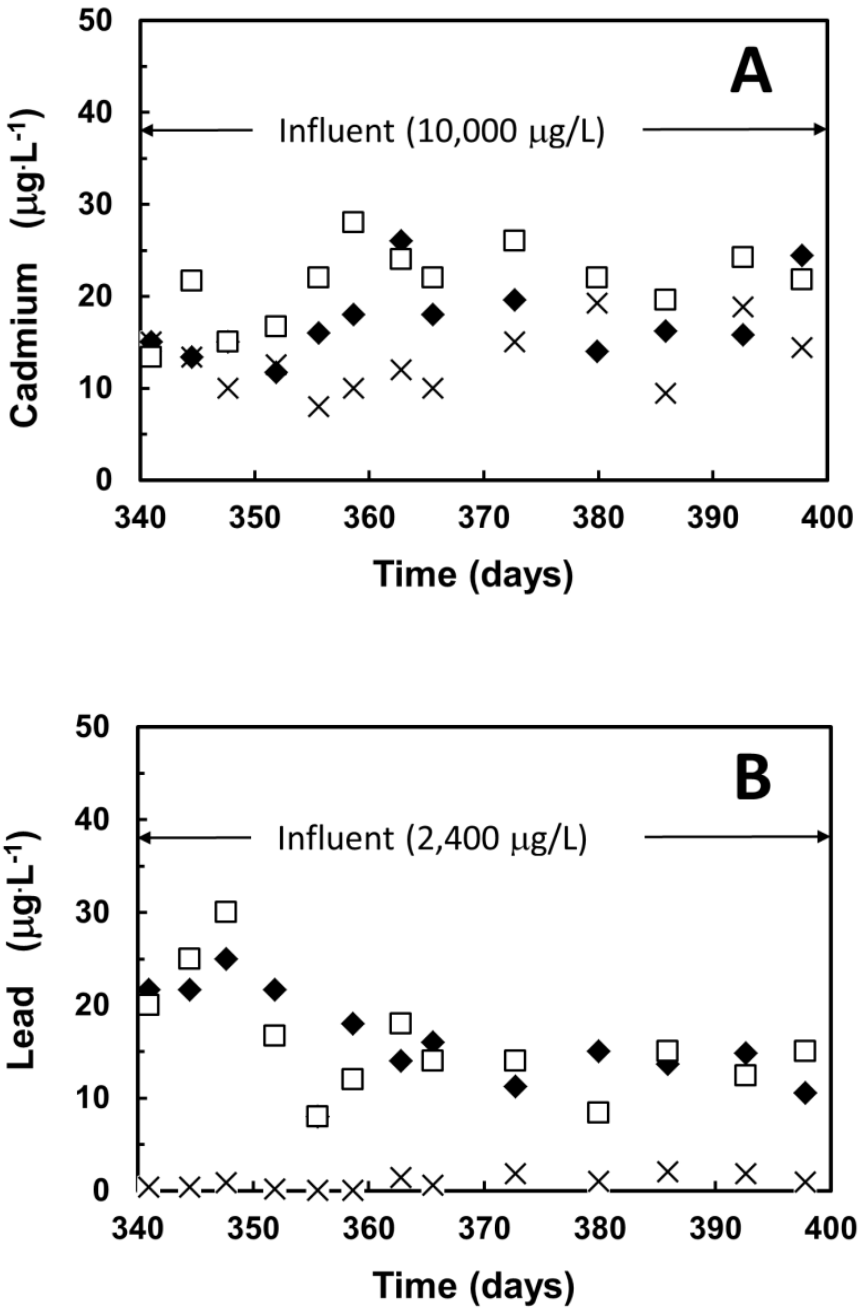

Figure 2.8. Cadmium and lead effluent concentrations during the last period, e_2. Panel A influent containing 10,000 μg L^{-1} Cd and panel B influent containing 2,400 μg L^{-1} of Pb, aside from 50,000 μg L^{-1} of Cu in each panel. ZVI-LS column (♦), ZVI column (□) and LS column (×).

2.3.5. Packing material characterization and mechanisms of metal removal

Packing material from each column was collected and extracted sequentially to assess the amount and nature of the copper retained in the columns. The total amount of copper recovered from the column packing was quite high, ranging from 84 to 93% of the cumulative copper removed from the effluent (Table 2.2.).

Table 2.2 Copper recovered by sequential extraction of the packing in different section of the up-flow columns: top (T), medium (M) and bottom (B).

Columns	Sections	Cu Extracted (%)			Cu Recovered (mg)		Removed (mg)[c]	Recovery (%) [d]
		H_2O	HCl[a]	HNO_3/HCl[b]	Section	Column		
	T	0.28	98.7	0	207			
LS	M	0.10	99.8	0.13	523	1383	1483	93
	B	0.07	99.8	0.09	653			
	T	0	0.0	100	1.6			
ZVI-LS	M	0	0.0	100	29.4	1254	1423	88
	B	0	0.05	99.9	1223			
	T	0	0.0	100	2.1			
ZVI	M	0	0.0	100	13.1	1087	1292	84
	B	0	0.27	99.7	1071.8			

[a] 1 M HCl.
[b] 16 M HNO_3-12 M HCl, (3:1 v/v).
[c] Cumulative copper removal calculated from difference between flux in and flux out
[d] % Recovery = 100 × (Cu extracted from packing material/cumulative Cu removed).

Most of the copper in the LS column was extracted with 1 M HCl, which is consistent with the high solubility constant of $Cu(OH)_2$ and $CuCO_3$. In contrast, 1 M HCl was ineffective in

extracting copper from the ZVI-containing columns. However, concentrated HNO_3–HCl extracted a large fraction of the copper from the ZVI columns, indicating that the precipitated copper was more stable and distinct from copper carbonates or hydroxides expected in the LS column. These results suggest that the copper minerals in the ZVI columns consisted of copper sulfide. This hypothesis is supported by laboratory experiments performed by Cooper and coworkers [28] confirming that sulfide minerals of copper such as covellite (CuS) and chalcocite (Cu_2S) are predominantly extracted by HNO_3 and not HCl. The formation of copper sulfide under sulfate reducing conditions is also consistent with the considerably lower Ksp of CuS compared to $Cu(OH)_2$ and $CuCO_3$ (Table A1).

Numerous sulfate-reducing studies have demonstrated that biogenic sulfide is an excellent ligand of different heavy metals, including Cu^{2+}, with a high tendency to form poorly soluble metal sulfides [44, 53, 54]. It is possible that corrosion products on the ZVI surface could have contributed to adsorb or co-precipitate some Cu^{2+}, but based on the sequential extraction data (Table 3) this does not seem to be the dominant removal mechanism.

As shown on Table 2.2, only a small fraction of the copper removed in the reactors amended with ZVI could be extracted with HCl. The bottom and mid-sections of the LS column contained relatively similar concentrations of copper, and the top section had about half the copper concentration as the lower two sections (Table 2.2). These results suggest that, by the end of the experiment, the capacity of the LS column to sequester copper was partially depleted. In contrast, in the ZVI and ZVI-LS columns most of the copper was immobilized in the bottom section of the reactors indicating that both columns still had significant capacity to sequester copper even after 400 days of continuous operation at HRTs ranging from 1 to 3.3 days. Much longer service times would be expected in practice since typical HRT values in operating PRB are considerably longer. For example, Barlett and Morrison [55] determined that the residence time

in two different locations of a full-scale PRB averaged 16.4 and 22.3 days. Field-scale application of iron-based technologies for the remediation of contaminated groundwater has shown promising results over relatively long treatment periods (e.g., up to ten years, [56]).

XRD and SEM–EDS measurements were performed to reveal the predominant elements in the columns. Unfortunately, XRD analysis did not provide conclusive evidence about the nature of the copper minerals due to interferences by the high concentrations of sand and calcite (limestone) and/or iron minerals with greater crystallinity in the various columns. SEM–EDS analyses on the other hand revealed that copper was an abundant element, along with calcium, in the packing of the LS column (Fig. 2.9).

In contrast, Fe was the most abundant element in the ZVI columns, but copper was also present. The elemental composition of the ZVI packing material was measured along a trajectory transecting a precipitate aggregate (Fig. 2.10). The background in the transect line analysis is mainly iron (dark gray area in the SEM). As the transect moves into the center of the precipitate microstructure (white area), the relative abundance of sulfur and copper increases as iron decreases. The molar ratio of Cu:S determined (1:1.04) suggests that CuS constituted the main mineral in the precipitate. These results, combined with the copper and sulfur balances (Table 3, Fig. 2.6), convincingly show that SRB activity was responsible for the effective removal of copper in the ZVI containing columns.

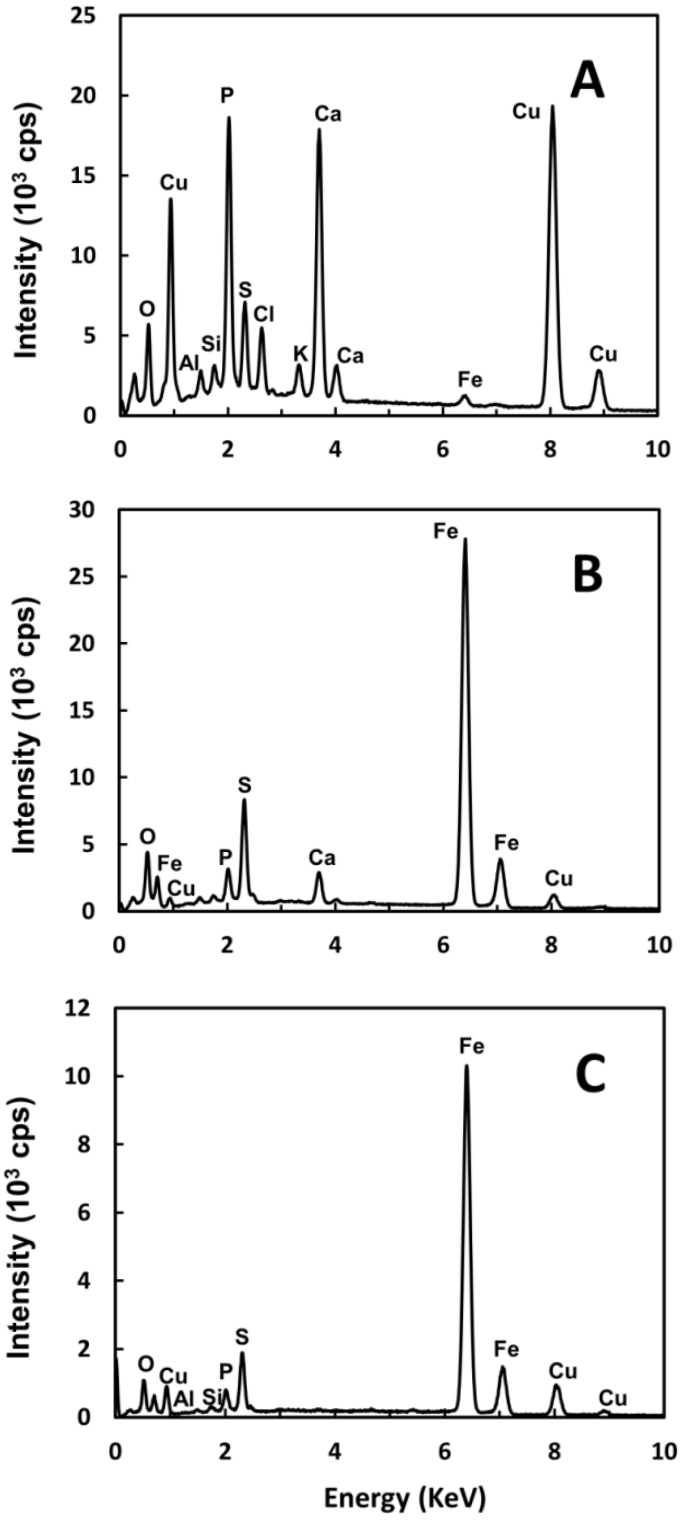

Figure 2.9. SEM-EDS spectra of: LS column (A), ZVI-LS column (B) and ZVI column (C).

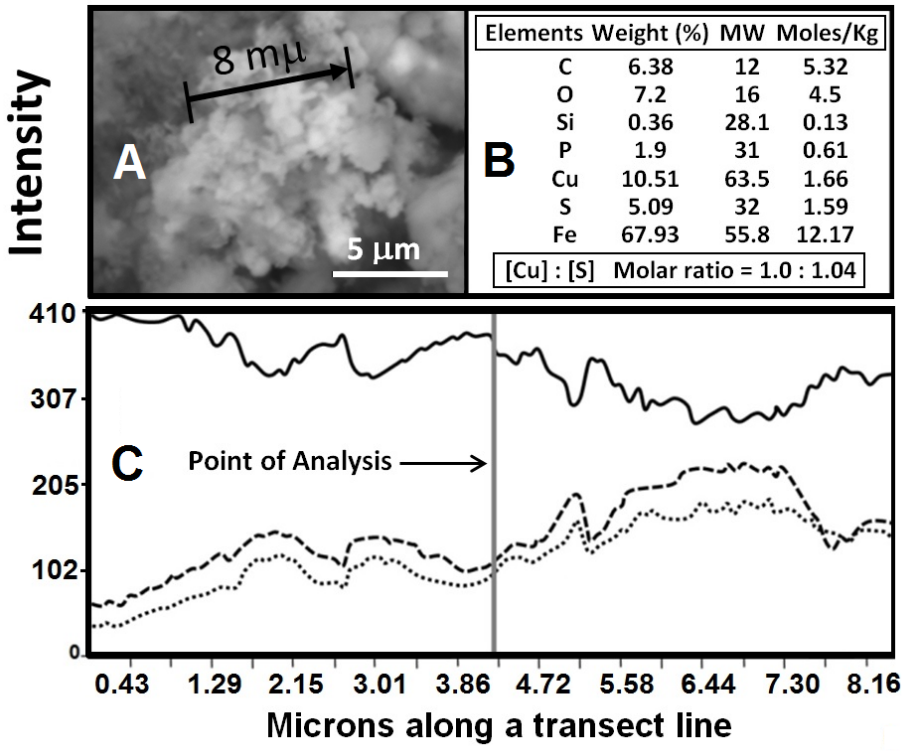

Figure 2.10. Panel A: SEM-EDS micrographs of the aggregate of Cu-sulfide from ZVI-LS column (30±2^{o}C, 400 d), Scale bars 5 μm. Panel B: Elemental composition of the aggregate, [Cu]:[S] ratio 1:1.04, and Panel C: Intensities' correlation among Fe, Cu and S for the aggregate along the transect line. Fe (─), S (- - -) and Cu (····).

2.4. Conclusions

The results obtained demonstrate that ZVI can serve as the sole exogenous slow-release electron donor to drive sulfate reduction over an extended time period in continuous-flow laboratory-scale columns treating a synthetic ARD containing high heavy metal concentrations (up to 50 mg/L of copper) and pH values ranging from 3.0 to 7.0. Treatment of this synthetic ARD was feasible and pro-vided very high removal efficiencies of copper, cadmium and lead

(>99.7%) and pH increase to circumneutral values (7.3–7.7) for over 400 days of operation at short HRTs (1–3 days). Moreover, the use of ZVI resulted in very low concentrations of toxic, malodourous hydrogen sulfide in the effluent due to its effective precipitation as metal sulfides, including sulfides of Fe^{2+} released from anoxic corrosion of ZVI. Element microanalysis and thermodynamic calculations using solubility products constants indicated that formation of insoluble metal sulfides was responsible for the effective metal removal in the ZVI columns. Continuous treatment of the synthetic ARD in a column packed with limestone also provided effective metal removal and acidity consumption. However, limestone did not contribute to sulfate reduction or to sequester biogenic sulfide and its treatment capacity had a lower longevity compared to ZVI. These results indicate that ZVI is a promising reactive material for the treatment of ARD in sulfate-reducing PRB systems.

2.5 Acknowledgements

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CHAPTER 3

ALGAE AS AN ELECTRON DONOR PROMOTING SULFATE REDUCTION FOR THE BIOREMEDIATION OF ACID ROCK DRAINAGE

This study assessed bioremediation of acid rock drainage in simulated permeable reactive barriers (PRB) using algae, *Chlorella sorokiniana*, as the sole electron donor for sulfate-reducing bacteria. Lipid extracted algae (LEA), the residues of biodiesel production, were compared with whole cell algae (WCA) as an electron donor to promote sulfate-reducing activity. Inoculated columns containing anaerobic granular sludge were fed a synthetic medium containing H_2SO_4 and Cu^{2+}. Sulfate, sulfide, Cu^{2+} and pH were monitored throughout the experiment of 123 d. Cu recovered in the column packing at the end of the experiment was evaluated using sequential extraction. Both WCA and LEA promoted 80% of sulfate removal (12.7 mg SO_4^{2-} d^{-1}) enabling near complete Cu removal (>99.5 %) and alkalinity generation raising the effluent pH to 6.5. No noteworthy sulfate reduction, alkalinity formation and Cu^{2+} removal were observed in the endogenous control. In algae amended-columns, Cu^{2+} was precipitated with biogenic H_2S produced by sulfate reduction. Formation of CuS was evidenced by sequential extraction and X-ray diffraction. LEA and WCA provided similar levels of electron donor based on the COD balance. The results demonstrate an innovative passive remediation system using residual algae biomass from the biodiesel industry.

Keywords: heavy metal, acid drainage, algae waste, biodiesel, permeable reactive barrier.

3.1 Introduction

Acid rock drainage (ARD) is produced by the contact of sulfide mineral residues of hard rock mining with moisture and air. ARD is characterized by low pH and dissolved heavy metals (HM). Low pH, ranging from 2 to 6, is due to oxidation of sulfides and iron generation of protons and sulfuric acid [1]. ARD samples from different mining locations are shown in Table A2 Acidity extracts and dissolves HM such as Cd, Pb and Cu. HM can reach surface waters and may accumulate to toxic levels causing severe impact on aquatic organisms [2].

Acidity and HM impact human health and the environment. In humans, exposure to high doses of Cu can cause headaches, dizziness, nausea, and diarrhea [3]. Cu is well known for its toxicity to aquatic life; lethal Cu concentrations (LC_{50}) ranging from 5 to 50 mg L^{-1} have been observed in several fish species [4]. Cu is toxic to green algae grown in fresh water at pH of 5.7 to 6.5; cell division is affected at concentrations as low as 1 μg L^{-1}, and Cu is 20-fold more toxic than uranium at the same concentrations [5]. Certain HM, e.g., Pb and Cd, are toxic to humans. Pb is known to cause neurotoxicity and affect the cardiovascular system and kidneys [6]. Cd is toxic to livers, lungs and kidneys and is a known carcinogen [7].

The main approaches to the remediation of ARD utilize alkali to precipitate HM and neutralize acidity or to use the activity of sulfate-reducing bacteria (SRB) to form biogenic sulfide and precipitate HM. Typically, either NaOH or limestone is used to promote the chemical precipitation of HM. Metal hydroxides precipitate metals due to an increase in pH, and metal carbonates precipitate using the soluble carbonate (CO_3^{2-}) from limestone [8]. HM precipitation with biogenic sulfide from SRB could be an economically attractive passive method for the treatment of ARD.

SRB are a group of anaerobic organisms that use organic compounds or molecular H_2 as electron donor (e-donor) to reduce sulfate (an external electron acceptor) to sulfide, a process known as dissimilatory sulfate reduction [9-11] (Eq. (1)). SRB reduce sulfate to sulfide, while organic matter is oxidized to CO_2 and acidity is simultaneously consumed [12].

$$SO_4^{2-} + 2\ CH_2O + 2\ H^+ \rightarrow\ H_2S + 2\ CO_2 + 2\ H_2O \qquad (1)$$

Formation of HM-sulfides (MeS) results from a chemical reaction between hydrogen sulfide produced by SRB and the soluble HM cation (Me^{2+}), Eq. (2).

$$Me^{2+} + S^{2-} \rightarrow\ MeS\downarrow \qquad (2)$$

A solubility constants (Ksp) comparison of hydroxide, carbonate and sulfide metals is shown in Table A1 of the SM. As shown, the Ksp values indicate the lower solubility of MeS compared to metal hydroxides and carbonates [13]. Biogenic sulfide (H_2S) generated during sulfate reduction is an excellent ligand of HM and is extensively used for remediation of HMs such as Cu, Pb, Zn and Cd in acidic effluents.

E-donating compounds enable sulfate reduction to convert sulfate to sulfide. For the remediation of ARD, slow release e-donors such as lignocellulosic materials and polysaccharide complex materials are generally preferred to enable passive treatment low maintenance systems. Cellulosic wastes, cow manure, municipal compost and wood chips have been tested as e-donor for SRB [12, 14-16]. The lower the lignin content of these materials, the higher their biodegradability and capacity for developing bacterial activity [17]. Lignocellulosic materials are slow release e-donors, which are desirable in a long-lasting system, such as a permeable reactive barrier (PRB).

A PRB is capable of removing contaminants in a passive treatment below-ground where a wall is installed to intercept an ARD plume, producing a clean effluent. PRBs are filled with pea-gravel and sand, mixed with reactive media. The sustainability of PRBs is greatly impacted by the selection of reactive media. The goal in biological PRBs is to use an organic carbon source with a life span exceeding 5 years. In HM remediation, the reactive materials vary from zeolite, limestone, zero-valent iron (ZVI) and local compost materials [18-20].

Residual algae from the biofuels industry may provide large quantities of biomass in the future. Microalgae can play the dual role of treating wastewater and generation of biomass for biodiesel production [21]. With current technology there is an excellent economic outlook for algae based biodiesel production [22]. Two microalgal strains have emerged as production candidates from recent U.S. Department of Energy research: *Chlorella sorokiniana*. (DOE 1412) and *Nannochloropsis gaditana* (CCMP-1775) [23]. *Chlorella sorokiniana*-1412, a genetically modified microalga has been widely tested for its biodiesel production potential. Lipid extracted algae (LEA) is the residual material left after lipid extraction from whole cell algae (WCA). The composition of algae cells lends itself as a slow release e-donor. LEA is expected to be a massive waste product [24-26] that could be used as an e-donor for bioremediation applications such as the treatment of HM in ARD by sulfate reduction. The effectiveness of algal biomass as both carbon source and energy to drive the process of sulfide generation by SRB has not been studied except in two cases evaluating extracellular polymeric substances (EPS) from algae, mainly composed by exopolysaccharides, as an e-donor for sulfate reduction [27, 28]. Due to the high content of complex cell wall polysaccharides in algae, LEA is expected to degrade slowly and last a long time in PRB as a slow-release e-donor, which is conducive to low cost and low maintenance. In this study, *Chlorella sorokiniana*-1412 is utilized as the e-donor. The objective

of this study is to compare WCA with LEA and determine if algae biomass residues could serve as an e-donor to support SRB in ARD remediation.

3.2 Materials and Methods

This study uses WCA and LEA as an e-donor for SRB to remediate ARD. Columns were packed with glass beads and inoculated with sludge having sulfate-reducing activity to mimic PRBs. The experiment compares an endogenous column (with no e-donor added) with columns containing WCA or LEA.

3.2.1 Algae growth conditions

Chlorella sorokiniana-1412 was cultivated on a BG-11 medium in a photo-bioreactor [29]. The chemical composition of WCA and LEA is illustrated in Table 3.1.

Table 3.1 Experimental set-up utilized in the anaerobic digestion studies.

Digestor	Mass of algae (g dwt)[a]	Initial total COD (g $COD_{alg\text{-}t0}$)
Endogenous control	0	0
Whole cell algae (WCA)	9.0	16.0
Sonicated algae (SA)	9.0	16.0
Lipid extracted algae (LEA)	8.5	14.2

[a] mass per 3 L of medium.

3.2.2 Anaerobic inoculum

Sulfate-reducing anaerobic granular sludge was obtained from a containerboard mill wastewater treatment plant (RockTenn, Syracuse, NY). It had a volatile suspended solids (VSS) content of 0.094 g VSS g^{-1} (wet weight of pellet after centrifugation). When unused, the sludge was stored anaerobically at 4˚C.

3.2.3 Chemicals

The main chemicals used were $CuSO_4 \cdot 5H_2O$, 98+%, CAS 7758-998 (Sigma-Aldrich, St. Louis, MO) to add a heavy metal to the synthetic ARD medium. H_2SO_4, 98% V/V;18M , CAS 7664-93-9 from Fisher Scientific (Fair Lawn, NJ) combined with cupric sulfate to prepare 250 mg SO_4^{2-} L^{-1} and lower the pH to 4. NaOH, ≥97% , CAS 1310-73-2, from Fisher Scientific (Fair Lawn, NJ) was used to adjust the ARD medium to pH 4.

3.2.4 Chemical Oxygen Demand content

The chemical oxygen demand (COD) content of *Chlorella sorokiniana*-1412 was quantified using standard methods [30], with H_2SO_4-$K_2Cr_2O_7$ at 150^oC for 2 h and measured at 600 nm. The COD (mg COD mg^{-1} dwt algae) was 1.43 for WCA and 1.32 for LEA. Table 3.2. shows the algae composition.

Table 3.2 Chemical composition of *Chlorella sorokiniana* biomass before and after lipid extraction.

Composition	WCA	LEA
Total solids (g TS/g wet wt)	0.20	1.0[a]
Volatile solids (g VS/g dwt)	0.96	0.94
Total nitrogen (% dwt)	5.5	5.6
Total phosphorous (% dwt)	0.57	0.53
Total carbon (% dwt)	51.0	48.4
Total lipids (% dwt)	9.4	ND

[a] LEA is dry powder.

3.2.5 Lipid extraction from microalgae biomass

Lipids were extracted using 7.5-g dry algae biomass with 1.43 g COD g^{-1} dwt algae in a mixture of methanol and chloroform (3:1, v/v) and microwaved at 80°C [31]. The remaining solids were washed with water 5 times and dried in a 160 mL bottle with nitrogen at 3 psi over 24 h. The total lipids extracted amounted to 9.4 %. The algal residue remaining is described as LEA, COD value of 1.32 g COD g^{-1} dwt algae.

3.2.6 Media for bioreactors

The basal mineral medium used for sulfate reducing experimentation contained (in mg L^{-1}): NH_4Cl (30); K_2HPO_4 (200); KH_2PO_4 (300); $CaCl_2 \cdot 2\ H_2O$ (20), $MgCl_2 \cdot 6\ H_2O$ (80), and 1mL per L of trace element solution [32]. Cu was added as $CuSO_4 \cdot 5\ H_2O$ (50), gradually increased from 10 to 50 mg L^{-1}, and additional sulfate was added as H_2SO_4 to reach 250 mg L^{-1}. The pH was adjusted by addition of NaOH.

3.2.7 PRB columns

This study was designed for a SRB process using 250 mg L^{-1} of SO_4^{2-} as the electron acceptor and algae as the e-donor, with no other exogenous source of e-donor. Fig. 3.1 illustrates three glass columns (0.07 L).

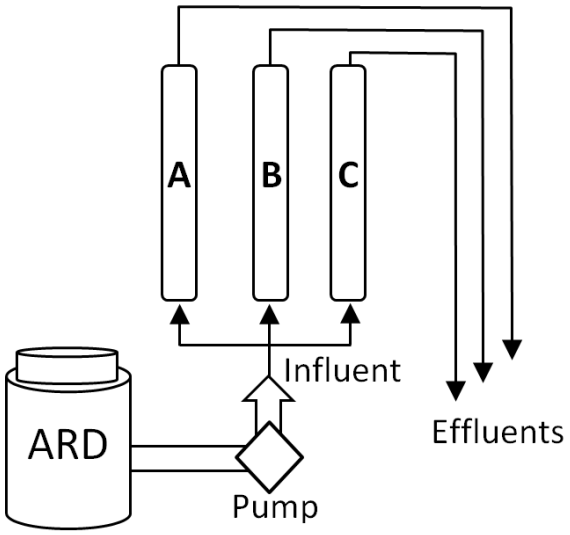

Figure 3.1 A descriptive diagram of the three up-flow packed bed columns used to test the algae biomass as electron donor for the SRB process. A) Endogenous column, B) WCA column, and C) LEA column.

The same amounts of granular sludge, equivalent to 12 g VSS $L^{-1}_{reactor}$, and 2-mm diameter glass beads were used to pack the columns and increase permeability. The endogenous column did not contain any algae. The WCA column was filled with intact *Chlorella* and the LEA column was filled with *Chlorella* after lipid extraction. The mass composition of the packing and the algae COD content are described in Table 3.3.

Table 3.3 Composition of the packed-bed columns.

		PRB Columns[a]		
	DBD[b]	Endogenous	WCA[c]	LEA[d]
Component	g/cm^3	---------- g dwt/ column ----------		
Glass beads (2 mm)	1.5	32	17.0	17.0
Sludge[e]	1.0	0.94	0.94	0.94
Whole Cell Algae (WCA)	0.42	0	4.9	0
Lipid Extracted Algae (LEA)[†]	0.41	0	0	5.3

[a] Packing volume (0.07 L).

[b] DBD = Dry weight bulk density.

[c] WCA (1.43 mg COD/mg dwt algae);

[d] LEA (1.32 mg COD mg^{-1} dwt algae)

[e] Sludge inoculum: 9 g wet sludge (0.094 g VSS g^{-1} wet sludge).

All columns were operated in continuous parallel mode at 24 ± 1^{o}C with the same source of influent (synthetic ARD). The influent was kept at 24±1^{o}C and then pumped upward using a peristaltic pump. The feed medium was pumped at a flow rate of 0.064 L·day^{-1} or an empty bed hydraulic retention time (HRT) of 1.1 day, lasting 123 days. The sulfate and acidity of the synthetic ARD medium (basal medium) were constant, while the Cu content was gradually increased at different periods (Table 3.4). The influent sulfate concentration was always 250 mg L^{-1} (supplied as sulfuric acid). The pH of the medium was kept at 4.0 by partially neutralizing the sulfuric acid with NaOH. Cu^{2+} was added after the first 26 days, at three intervals. As illustrated in Table 3.3, Cu^{2+} was not added during the first period (a). In the subsequent periods (b-d) the medium received 10, 30 and 50 mg L^{-1} Cu^{2+}. Cu was not added during the last period (e) to test the sulfate-reducing activity and the absence of toxicity due to Cu.

Table 3.4. Periods of column operation as defined by influent Cu^{2+} concentrations.

	Period				
	a	b	c	d	e
Time (d)	0 - 26	27 - 59	60 - 74	75 - 100	101 - 123
Influent Cu^{2+} (mg L^{-1})	0	10	30	50	0

3.2.8 Analysis

Influent and effluent were analyzed to determine pH, S^{2-}, SO_4^{2-} and Cu^{2+}. Sulfide was analyzed colorimetrically by the methylene blue method [33]. Sulfate was measured by ion chromatography with suppressed conductivity using a Dionex AS11-HC4 column (Dionex, Sunnyvale, CA) and a conductivity detector. To avoid sulfate formation from sulfide oxidation in sample handling and storage, the liquid samples were acidified, followed by stripping H_2S with N_2-CO_2, as recommended by Hughes and coworkers [34]. Cu was determined by inductively coupled plasma-optical emission spectrometry (2100 Optima ICP-OES, Perkin Elmer, Waltham, MA). Wavelength used for Cu^{2+} determination was 327.3 nm and the detection limit was 1.0 μg L^{-1}.

At the end of the experiment, the packing of each column was extracted, glass beads were removed and the remainder was homogenized. To compare the amount of Cu retained in the columns against the Cu recovered from the columns, sequential extractions were performed using water, 1M HCl and 3:1 HNO_3-HCl (15.7 and 12 M, respectively) sequentially to extract the Cu retained in the packing. A single sample was collected from each column and the precipitates were characterized by X-ray diffractometer (XRD), from (PANalytical X'Pert Pro, Netherlands). To estimate the washout of algal biomass, a daily sample was analyzed by the previously described COD method. The cumulative volume and the dilution factor were used to calculate the

total COD lost by algae washout. The COD was quantified using the whole solution of the algae amended columns and corrected by the endogenous column.

The total sulfide (TS) associated with the liquid and gas was calculated by Eq. (3). Effluent of 0.5 mL was analyzed for dissolved sulfide (DS), the sum of $H_2S + HS^- + S^{2-}$, by the methylene blue method. The concentration of undissociated sulfide [H_2S] was calculated based on the DS, accounting for pH and using the first dissociation constant (pKa1 = 6.98).

$$TS = DS \cdot (1 + \alpha_0 \cdot H \cdot F) \quad (3)$$

Where:

TS = total sulfide in system (gas & liquid) per liter liquid

DS = dissolved sulfide (measured with methylene blue method)

α_0 = fraction H_2S of DS

H = dimensionless H constant Cg/Caq (0.4)

F = headspace volume/liquid volume (stripping factor)

$\alpha_0 = 1/(10^{(pH-pKa)} + 1)$

pKa = dissociation constant of H_2S (6.98 at 25^{o}C)

Sulfide precipitated in the mineral fraction was estimated by the decrease in Cu. This calculation used a molar ratio of Cu/S = 1.0 with the formation of CuS as the only precipitate. Methane (CH_4) production was measured by volume, using a 1.0-L empty gas sampling bag Tedlar-SCV (Sigma-Aldrich, MO) connected to the upper part of each column. The amount of methane was significant only during the first three weeks; after that period the gas production declined to zero. In calculating methane produced, it was assumed the gas was composed 70% of methane [35].

The total initial algae COD (COD_{t0}) was 7 g. At the end of this study, the accounting of COD considered four fractions making up the total COD: cumulative H_2S-COD (COD in all forms of sulfide), algae washout-COD, CH_4-COD, and the COD remaining in reactor, which was calculated as follows:

$$COD_{remaining} = COD_{t0} - (H_2S\text{-}COD + washout\text{-}COD + CH_4\text{-}COD).$$

The sulfur balance was calculated considering: 1) soluble H_2S-S (measured + stripped), 2) SO_4^{2-}-S in the effluent, and 3) CuS-S due to the sulfur precipitated and adsorbed in the column in the periods when Cu was added.

3.3 Results

3.3.1 Sulfur

Both WCA and LEA contributed to sulfide formation in the effluent of the laboratory PRBs (Figure 3.2A). In the case of WCA, the highest effluent sulfide concentration, 50 mg L^{-1}, was observed in the latter half of period (b). In the case of LEA, the maximum sulfide concentration of 55 mg L^{-1} occurred at the end of period (e). Consistently, throughout reactor operation, the algae amended columns produced orders of magnitude higher effluent sulfide concentrations compared to the endogenous column. In fact, the endogenous column produced only very low levels of sulfide (maximally 5 mg L^{-1}) from d 5-40. Thereafter, there was no longer detectable sulfide in the effluent of the endogenous column.

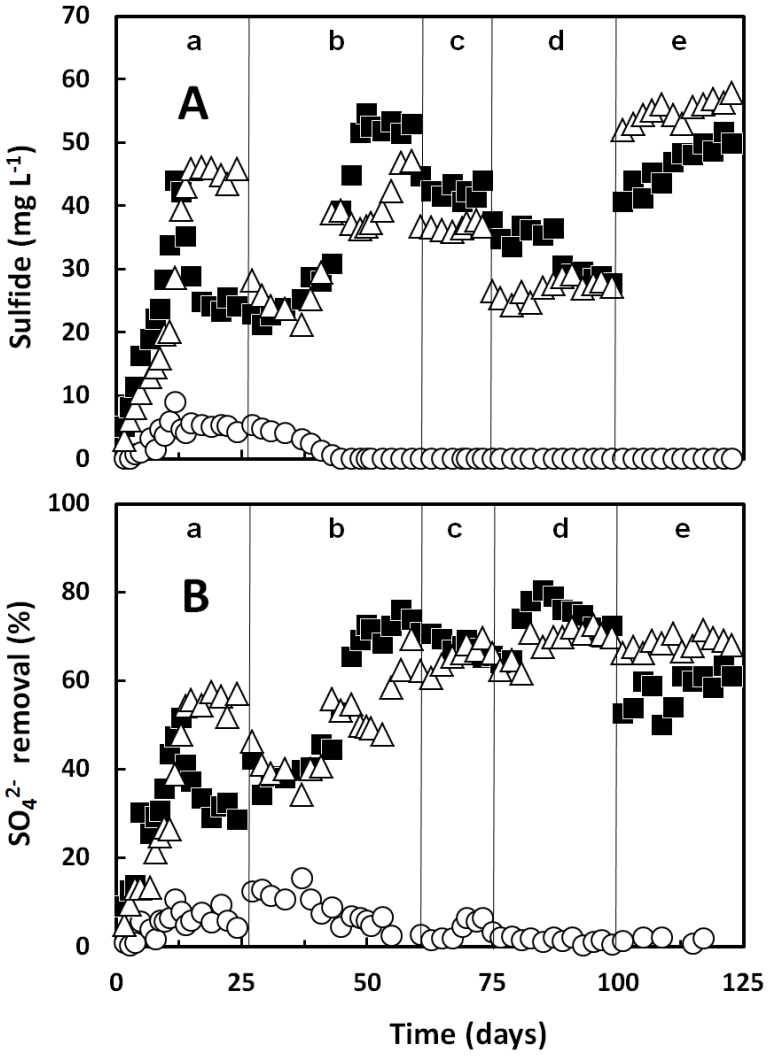

Figure 3.2. Panel A: Total sulfide in the effluent (sulfide measured + stripped): Endogenous column (○), WCA column (■) and LEA column (△). The concentration of Cu^{2+} in the influent during periods a, b c, d and e was 0, 10, 30, 50 and 0 mg L^{-1}, respectively. The maximum H_2S concentration stripped (at pH 6.5) was 0.23 mg L^{-1}-effluent during week 3. This corresponds to 5.1% of the total H_2S. Sulfide stripped was only corrected during the first 40 days of active gas production. Panel B: Sulfate removal (%).

Further evidence of sulfate reduction is supported by the removal of sulfate in the algae amended columns (Figure 2B). The highest sulfate removal occurred from the latter part of period

(b) to the end of period (d) in the WCA column or from period (c) through (e) in the LEA column, corresponding to 60-80% sulfate removal. There was little to no sulfate removal in the endogenous column.

Cu did not appear to impact sulfate reduction. Period (d), which had the highest Cu concentration, also had the highest sulfate removal (Figure 3.2B) and thus Cu inhibition was not witnessed. Sulfide levels dropped in periods (c) and (d) when Cu concentrations increased (Figure 3.2A), but the drop was due to copper sulfide precipitates (Figure 3) and not to toxicity.

In the beginning of period (e), a noteworthy difference in behavior was observed between the WCA column and the LEA column. Both the sulfate removal and the sulfide production increased at faster pace in the LEA column. This indicates a greater release of e-donor from LEA as compared to WCA in the final period as the experiment was ending. Alternatively, the sudden removal of Cu at the start of period (e) caused sulfide inhibition of SRB, and the LEA column acclimated to the toxicity faster. These observations taken together indicate that both algae e-donor sources were quite suitable as e-donor for sulfate reduction and there was no evidence of Cu inhibition.

Sulfur balances during the system operation are shown for the algae containing column in Figures 3.3A and 3.3B. The figures clearly show that sulfate was converted to aqueous sulfide (with a minor fraction of stripped S) and copper sulfide minerals. The balances are excellent on any given day; the sum of sulfur species was very similar to the inlet S quantity. The balance in the endogenous column primarily demonstrated that the effluent was sulfate-S equal to the input sulfate-S (Figure A1), what one would expect if no reaction had taken place.

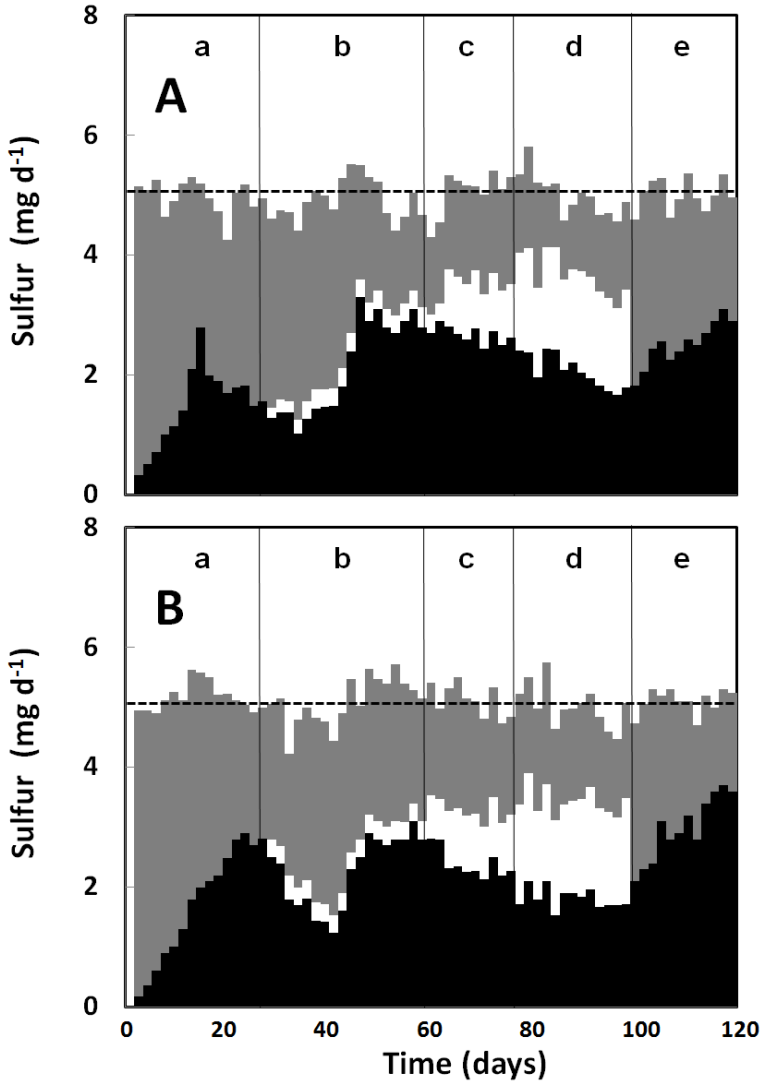

Figure 3.3. Sulfur balance in WCA (panel A) and LEA (panel B) columns: sulfide aqueous + stripped (■), sulfur in CuS (□), and sulfate effluent (■). The dash shows the sulfate concentration in the influent (5.1 mg SO_4^{2-}-S d^{-1}).

3.3.2 pH

Figure 3.4 shows the pH evolution of the effluent of the reactors. The algae amended columns had effluent pH values near neutral, ranging from 6.0 to 6.5. This contrasts starkly with the pH values in the endogenous columns, *i.e.*, approximately 4.0, from period (b) onwards. The

pH values were correlated with sulfate reduction activity. This activity was consistently high in algae-amended columns. The endogenous column, however, had low activity from the start of reactor operation until day 40, at which time activity decreased to zero and concomitantly as the pH dropped to 4.0.

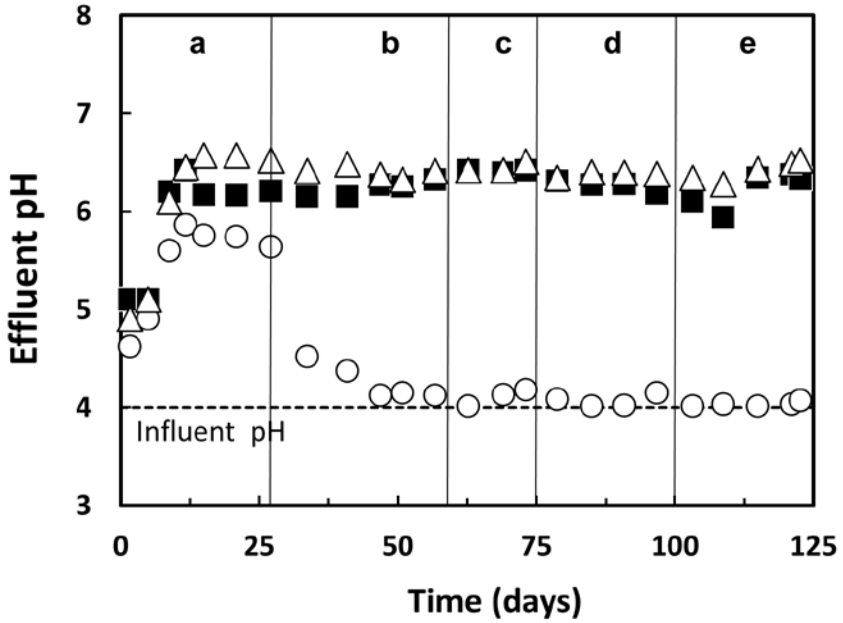

Figure 3.4. Effluent pH: Endogenous column (○), WCA column (■), and LEA column (△). The horizontal line represents the influent pH.

3.3.3 Copper

The influent and effluent Cu concentrations are shown in Figure 3.5. Figure 3.5A is zoomed out to show the influent and effluent concentrations ranging from 0 to 50 mg L^{-1}. Figure 3.5B is zoomed in to the concentration range from 0 to 0.5 mg L^{-1} in order to appreciate differences at very low effluent concentrations. The endogenous column reached Cu breakthrough towards the end of period (b). Thereafter, only partial or no Cu retention was

observed. At the end of period (d), 50 mg L^{-1} of Cu were discharged with the effluent and this was equal to the influent. This behavior is consistent with lack of sulfate reduction. Conversely, Cu was effectively retained in the algae-amended columns.

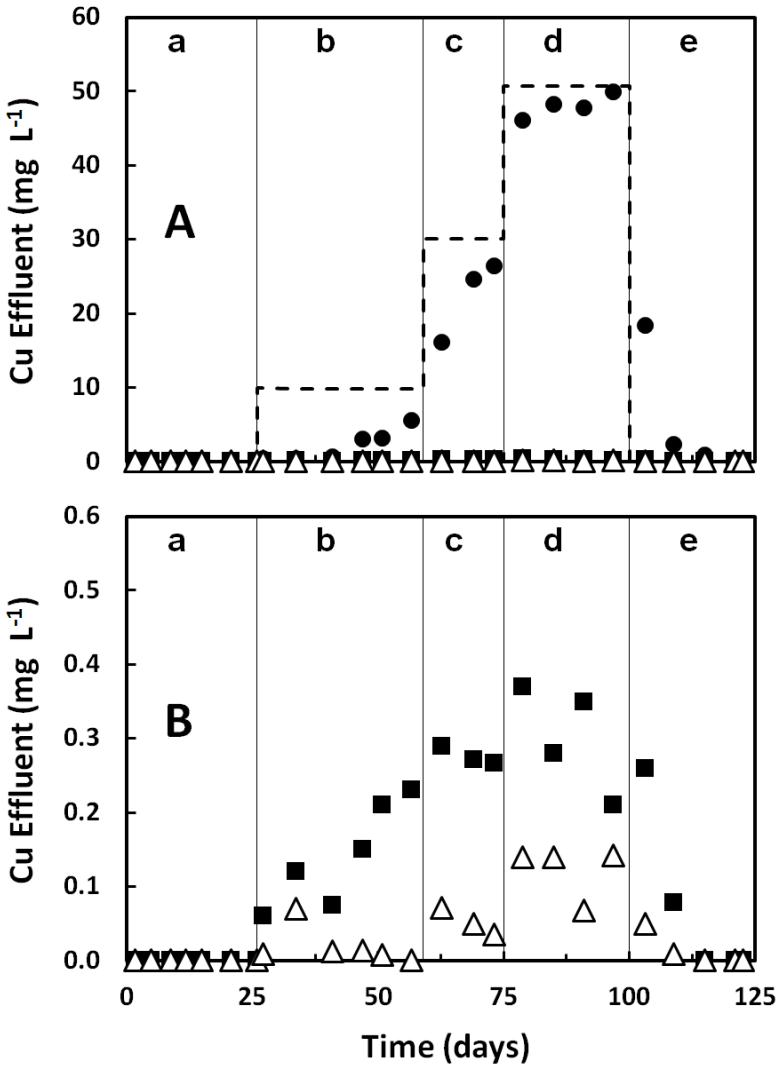

Figure 3.5. Effluent and influent copper concentration: Panel A: View of data in the range from 0 to 60 mg L^{-1}. Panel B: Close up view of effluent data in the range of 0 to 0.6 mg L^{-1}. Endogenous column (○), WCA column (■) and LEA column (△). The concentration of Cu^{2+} in

the influent during period a, b c, d and e was 0, 10, 30, 50 and 0 mg L^{-1}, respectively. The dash line represents the influent Cu^{2+} concentration in each of the periods.

Cu was retained in the reactor throughout the experiment as shown in Figure 3.5A. The Cu removal percentages were higher than 99.5 and 99.7% for the WCA- and LEA columns, respectively. The effluent Cu concentrations from these columns are shown in Figure 3.5B. The effluent Cu concentrations ranged from 50 to 400 μg L^{-1} in the WCA column and from non-detect to 450 μg L^{-1} in the LEA column.

Cu was precipitated as CuS as evidenced by XRD and sequential extraction measurements. XRD analyses showed clear evidence of CuS crystals that was only observable in the solids from the two algae-amended columns (Figure 3.6). No such evidence was found in the endogenous column.

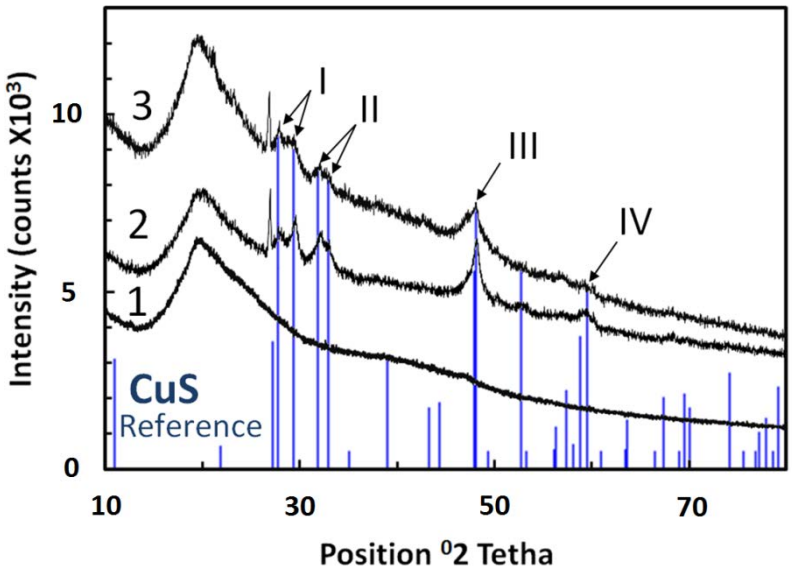

Figure 3.6. XRD spectrum of the column packing at the end of the experiment. Endogenous column (1), WCA column (2) and LEA column (3). Copper sulfide (covellite) used as the reference.

The cumulative Cu balance is presented in Table 4. Firstly, the table shows that there was approximately 4× more Cu retention in the algae-amended columns compared to the endogenous column. Secondly, the retained Cu speciation was mostly in the form of sulfides because most of the Cu could only be extracted by a strong oxidative acid (HNO_3-HCl, 3:1). In the endogenous column, HNO_3-HCl extraction did not extract much copper because 99% of the Cu was extracted with water and 1M HCl. Thirdly, the table shows a good balance of Cu extracted versus cumulative Cu that was retained in all columns (as estimated from inlet – outlet Cu concentrations). In the algae columns, the total recovery of Cu during the extractions ranged from 95 – 98% of that retained.

Table 3.5 Sequential extraction of total copper from the homogenized column packing at the end of the experiment by means of: water, 1M HCl and 1:3 ratio (v/v) of concentrated HNO_3:HCl.

	Sequential extraction of Cu (mg)					
Column	**Water**	**1 M HCl**	**HNO_3:HCl [a]**	**Total extracted**	**Cumulative[b] retained (mg)**	**% Cu Recovered[c]**
Endogenous	0.3	33.1	0.2	33.6	36.0	93.2
WCA	0.0	28.6	92.3	120.9	127.6	94.8
LEA	1.5	24.1	98.0	123.6	126.0	98.1

[a] HNO_3-HCl, 3**:**1 (v/v); 15.7 M and 12 M, respectively.

[b] Cumulative copper removal calculated from difference between flux in and flux out.

[c] % Recovery = (Cu extracted from packing material/cumulative Cu retained)*100.

3.3.4 Chemical oxygen demand

A cumulative COD balance is provided for the columns containing algae, WCA and LEA, in Figure 3.7. During the initial 26 d operational (period (a)), biogas production provoked washout of the biomass, accounting for the loss of 40.7 and 26.7% of the algae COD. The COD metabolized during the study corresponded to only 9.5 and 9.2% of the added algae-COD, respectively, to produce sulfide. Only 3.5% and 1.3% of the WCA- and LEA-COD was converted into methane-COD.

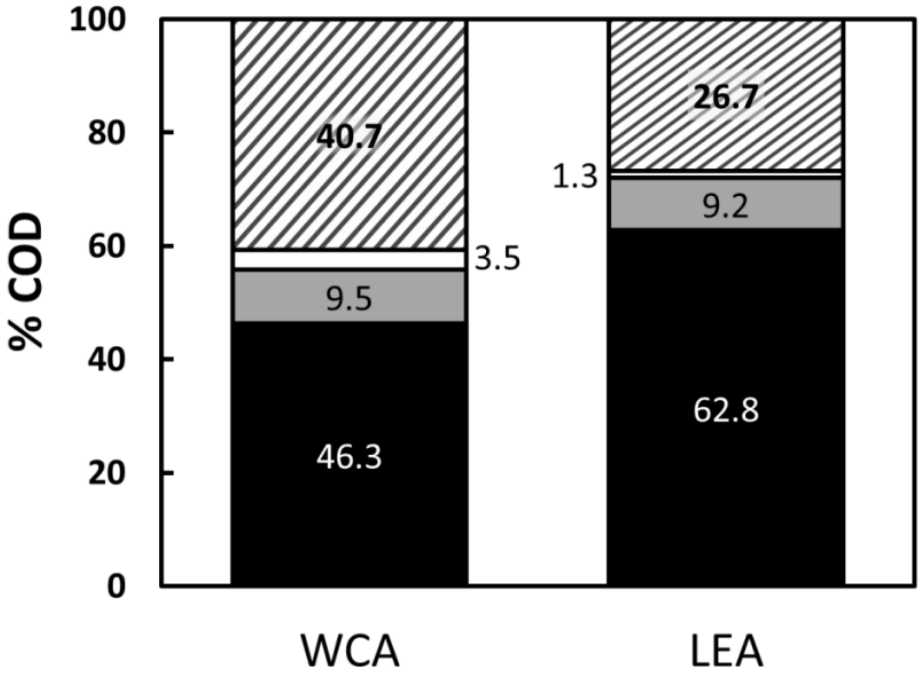

Figure 3.7. Algae-COD balance in WCA and LEA columns: Remaining algae (■), S^{2-}-COD (□), CH_4-COD (▩), and washed out algae (▨).

3.4 Discussion

3.4.1 Main Findings

Both WCA and LEA algae biomass served as e-donating substrates for sulfate reduction. The biogenic sulfide formed f precipitated Cu^{2+} in the form of copper sulfide as evidenced by the results of XRD and sequential extractions. Sulfate reduction was active in the algae-amended columns even as the experiment ended on day 123. By comparison, the endogenous reactor exhibited low sulfate-reducing activity during the first 40 days of the experiment and then activity ceased. Thus, algae could serve as a long-term e-donating substrate. Active sulfate reduction was linked to highly efficient Cu removal and an increase in the pH of the treated ARD.

3.4.2 Algae as Electron Donor in Anaerobic Conditions

This study provides direct evidence that WCA and LEA can serve as e-donors to drive sulfate reduction. Previously, microbially-driven sulfate reduction in a constructed wetland was hypothesized to be linked to the degradation of algal biomass from two genera of naturally occurring algae in that wetland, *Scenedesmus* and *Carteria* [27]. Additionally, extracellular polysaccharides produced by a mixed culture of algae in a high-rate pond were considered to be responsible for sulfate removal [28]. Studies of a wastewater ponding system containing HM [36] demonstrated that a combination of algae growing in the pond and co-disposed organic wastes served as a carbon source for sulfate reduction, enabling Cu removal with an efficiency of 80% (from 500 mg L^{-1}). This study provides for the first time direct evidence that WCA and LEA can be utilized as e-donor for sulfate reduction. In addition to algae, the use of phototrophic cyanobacterial biomass for sulfate reduction has also been reported. Sulfate reduction by a mixed culture utilized 31% COD from *Spirulina sp*. biomass as a sole carbon source [37]. The cell wall

of *Spirulina*, similar to that of other cyanobacteria, has a high glucan content [38]. The cell walls of *Chlorella* are rigid and more resistant to biodegradation because they consist of a matrix containing glucosamine, the dominant cell wall polymer, combined with hemicellulose with glucose and mannose [39, 40].

Utilization of cellulosic and hemicellulosic materials as e-donor by SRB has been demonstrated in several studies. A significant sulfate reduction rate of 12.3 mg L^{-1} d^{-1} was observed in a laboratory-scale study using rice straw as e-donor for the bioremediation of ARD (1.5 mg L^{-1}) Cu at pH 2.0 [41]. The corresponding Cu removal efficiency was 98%. In another study, grass cellulose was used as the carbon and energy source for microorganisms in rumen fluid, enabling sulfate removal efficiency of 86% [42].

While little is known about algae as a e-donor by sulfate-reducing consortia, it is well established that algal biomass can serve as an e-donor for methane production. In large-scale algae cultures, anaerobic digestion is a necessary step to make microalgal biodiesel sustainable [43]. The COD conversion of algal biomass from *Chlorella sorokiniana* and *Chlorella vulgaris* to methane by anaerobic digestion ranged from 40 - 73 % [44]. In a different study, around 50% of the biomass of *C. vulgaris* was converted to methane [45]. The methane yield of *C. vulgaris* biomass ranged from 189 to 450 mL CH4 g^{-1} VS, Anaerobic digestion can also release nutrients (nitrogen and phosphorus) from LEA [46], which are essential to make biodiesel production sustainable.

Sonication and thermal treatment enhance the methane yields that can be obtained during anaerobic digestion of algal biomass. Solvent extraction step applied to remove the lipid extraction is by itself a pretreatment that can increase methane production from LEA [47]. Thermal hydrolysis of *Nannochloropsis* biomass at 170^{o}C enhanced the methane yield by 40% for WCA and 15% for LEA [48]. Another study investigating the anaerobic digestion of

Scenedesmus biomass demonstrated a 2-fold and 1.6-fold increase in the methane yield, compared with untreated biomass, when the biomass was pretreated by sonication at 128.9 MJ/kg and thermal hydrolysis at 80^{o}C, respectively. of *Scenedesmus* biomass reported an increase if the methane yield [49]. The increase was attributed to cell wall disruption and COD solubilization.

3.4.3 Copper and Sulfide Toxicity to SRB and Methanogens

In this study, copper and sulfide had no apparent inhibitory effect on sulfate-reducing activity. The 50% Cu inhibiting concentration (IC_{50}) to acetoclastic and hydrogenotrophic sulfate reducers was 32.3 mg L^{-1} and over 200 mg L^{-1} respectively. In the same study the IC_{50} values of Cu^{2+} for acetoclastic and hydrogenotrophic methanogens were reported as 20.7 and 8.9 mg L^{-1}, respectively [50]. Greater IC_{50} values for SRB and methanogens have been reported for Cu^{2+}, 1136 and 130 mg L^{-1}, in a different study [51]. The reported Cu^{2+} inhibitory values on SRB were higher than the concentration used in this study of 50 mg L^{-1}, thus our findings are consistent with the lack of toxicity expected.

Undissociated sulfide (H_2S) is the main toxic form of sulfide, as only the neutral form permeates the cell membrane [52]. A previous study considering the toxicity of Cu^{+2} towards methanogens and SRB have shown that the average IC_{50} values of undissociated H_2S at pH of 6.8 towards acetoclastic and hydrogenotrophic SRB were 272 and 299 mg L^{-1}, respectively, while the IC_{50} values determined for acetoclastic and hydrogenotrophic methanogens were 97 and 136 mg L^{-1}, respectively [53]. In a similar study, the IC_{50} values determined for sulfide at pH of 6.8 in assays with acetoclastic and hydrogenotrophic SRB were 270 and 380 mg L^{-1} undissociated H_2S, respectively, while the IC_{50} values reported for acetoclastic and hydrogenotrophic methanogens were 160 and 220 mg L^{-1} of undissociated H_2S, respectively [54]. In our study, the pH of the effluent typically ranged from 6.4-6.7. In the worst case scenario, this corresponds to 62.5 mg L^{-1}

of undissociated H_2S (at pH= 6.5), which is much lower than the IC_{50} value reported in the literature for hydrogenotrophic SRB.

3.4.4 Implications of Algae Use as e-donor for AMD Remediation in PRB

The utilization of LEA biomass may have remarkable advantages as a slow release e-donor to remediate ARD in PRBs. Additionally, algae can sustain PRBs for years producing benefits to the environment by removing HM and acidity from ARD. LEA utilization could also add profitability to the biodiesel industry. The main challenge in using algae as a reactive material in PRBs, however, is how to reduce the washout of the suspended algae that we suspect was exacerbated by biogas production in the initial period. Eventually SRB will outcompete methanogens under prolonged sulfate reduction and low pH conditions in ARD plumes.

3.5 Acknowledgements

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CHAPTER 4

PRETREATMENTS TO ENHANCE THE ANAEROBIC BIODEGRADABILITY OF *CHLORELLA PROTOTHECOIDES* ALGAL BIOMASS

Anaerobic digestion (AD) of microalgae biomass is a promising approach for the production of energy and nutrients but the process is often limited by the resistance of the thick algal cell wall to biodegradation. This study investigated the effectiveness of sonication, thermal, and alkaline pretreatments to improve methane production and nitrogen release during AD of *Chlorella protothecoides* biomass. *C. protothecoides* can store high lipid levels and is a promising feedstock for biofuel production. Sonication experiments at 20 KHz showed that increasing the power level applied enhanced organic matter solubilization as well as the biochemical methane potential and nitrogen released during AD of the pretreated biomass. Sonication under optimized conditions provided a marked increase in the methane yield compared to the untreated algae following digestion for 41 days (327 and 146 mL_{STP} CH_4/g volatile solids, respectively). Sonication also led to 4.1-fold increase in ammonia nitrogen release. In contrast, thermal and alkaline pretreatment showed limited potential to enhance improve methane production and nitrogen release. The results indicate that AD of sonicated *C. protothecoides* biomass is a promising approach to generate methane gas, a valuable fuel material, and release nutrients that can be recycled to meet the high fertilizer demand of algal cultivation systems.

***Keywords*:** Biomass; Methane; Nutrient recovery, Nitrogen, Algal biofuels

4.1. Introduction

There is a growing interest in the application of photosynthetic microalgae to produce biodiesel [1-3] and remove nutrients from wastewater and other contaminants from wastewater [4-7]. An important byproduct from these processes is residual algal biomass. Although microalgae strains considered for biodiesel production contain high levels of lipids (20–50% dry weight) [1], more than half of the algal biomass remains as a residue after lipid extraction. Thus, large-scale cultivation of microalgae for biodiesel generation has the potential to generate large amounts of residual biomass. Lipid-extracted algae and excess biomass from algal-based processes for wastewater treatment are promising feedstocks for the production of energy and recovery of nutrients.

Anaerobic digestion of algal biomass can generate biogas containing high concentrations of methane (CH_4) gas, a valuable fuel material that can increase the economic feasibility of algal cultivation systems. Biochemical methane potentials (BMP) for algal biomass ranging from 0.09 to 0.45 L CH_4 under standard pressure and temperature (STP) conditions per gram of volatile solids (VS) of algal cells have been reported [8-11]. Assuming a chemical oxygen demand (COD) factor of 1.42 g COD per gram VS, these BMP values correspond to 18.1 to 90.5% conversion of the total chemical oxygen demand (COD) in the dry algae. The significant variability in the methane yield is likely related to variation in the applied digester operating conditions along with changes in microalgae composition due to strain selection and cultivation conditions. Obviously, when lipids are extracted from the algal biomass before digestion, the potential methane yield is lower compared to fresh algae since energy bearing lipids are removed [8, 11]. The methane produced can be used to generate electricity for algae biodiesel operations such as cultivation, harvesting, drying and lipid extraction. Extraction of lipids from algae requires as much as 70% of the total energy consumption in the biodiesel production process [12].

Anaerobic degradation of biomolecules containing organic nitrogen (N) and phosphorous (P) in algal biomass contributes to the release of soluble ammonia (NH_4^+) and phosphate (PO_4^{3-}). The nitrogen content in microalgae is high, typically ranging from 2 to 11% on a dry weight basis [11, 13]. These high nitrogen levels are related to the high protein content found in microalgae which can vary between 30 and 60% of the total dry weight [14]. The phosphorus concentration in microalgae cells varies with supply concentration [15]. On average, algal cells contain 0.1% phosphorus on a dry weight basis [16]. Nutrients released during the anaerobic digestion of algal biomass can be recycled to meet the high N and P demands involved in algal cultivation systems and reduce fertilizer costs. Nutrient recovery is critical for the sustainability of algal biofuels because algal growth requires high level of nitrogen and other nutrients and, if not already available in the water source, the addition of commercial fertilizers can significantly increase production costs, making the price of algae derived fuel cost prohibitive [4, 17].

Anaerobic digestion of biomass from *Chlorella* spp. and numerous other microalgae species is hindered by the resistance of the thick algae cell wall to biodegradation [8, 11]. The cell walls of most microalgae consist of tri-layered structures that include polysaccharides such as cellulose, proteins, and others, or tri-laminar layers of algaenan [18, 19], which confer the cell wall a high resistance to bacterial attack. Moreover, the cell wall of some *Chlorella protothecoides* species has been shown to contain up to 40% of a chitin–protein complex, resistant to enzymatic digestion (cellulase, chitinase, and glusulase) and acid hydrolysis [20]. Disruption of algae cells can facilitate the release of intracellular soluble organic matter and contribute to reduce particle size, enhancing the hydrolysis of insoluble polymers and the efficiency of anaerobic digestion. Pretreatments such as alkaline hydrolysis, thermal treatment, sonication, and enzyme dosing have been investigated widely to improve the digestibility of lignocellulosic biomass [21, 22] and waste activated sludge [23, 24], and some pretreatments

have reached full-scale application [24, 25]. However, information regarding the effect of pretreatments to improve the digestibility of algae biomass is still limited [8, 26].

The goal of this study was to improve methane production and nutrient release during the anaerobic digestion of *Chlorella protothecoides* biomass. *C. protothecoides* (also known as *Auxenochlorella protothecoides*) is a green microalga considered very promising species for biofuel production because it can produce very high levels of lipids (up to 55% dwt) when grown under optimal conditions [1, 27]. Ultrasound pretreatment at different energy levels was investigated and optimized in terms of organic matter solubilization, biochemical methane potential, and specific energy consumption. In addition, ultrasound pretreatment under optimized conditions was compared with thermal and alkaline pretreatments to identify the most effective process to improve the anaerobic biodegradability of *C. protothecoides* biomass.

4.2. Materials and Methods

4.2.1. Algae cultivation

Chlorella protothecoides UTEX 25 biomass was a kind gift of Dr. K. L. Ogden (University of Arizona). The microalgae was grown at 25°C in a synthetic medium containing 0.25 g L^{-1} of proteose peptones, 6 mg L^{-1} of $MgSO_4 \cdot 7\ H_2O$, and 0.45 g L^{-1} of $NaNO_3$ at an initial pH of 6.8 as described elsewhere [28].

4.2.2. Preliminary sonication pretreatments

Ultrasonic treatment of algal biomass was performed using a 130-Watt (20 KHz) ultrasonic processor (GEX 130, Cole-Parmer Instruments, Vernon Hills, IL, USA) using different power levels and times, including treatment at 70% intensity for 5, 10 and 15 min, and treatment at 40% and 100% intensity for 5 min. Sonication optimization tests were performed using centrifuge

tubes containing 10 mL of a suspension containing 8.7 g dry algal biomass L^{-1}. The tubes were kept in a bath containing water with ice to prevent increase of the sample temperature during sonication. Pretreated algal samples were tested for soluble chemical oxygen demand (COD) content and, subsequently, digested anaerobically to determine their biochemical methane potential (BMP).

The specific input energy (E_{in}) supplied in the ultrasonic pretreatment was calculated:

$$E_{in}\ (\text{kJ/g VS}) = P_d \cdot t \cdot I / (100 \cdot Cs \cdot V_d) \quad [1]$$

where P_d, t, I, Cs and V_d refer to device power energy (P_d, kJ/s), pretreatment duration time (t), device power level (%); microalgae concentration (C_s, g VS/L) and effective volume (V_d, L).

4.2.3. Algal biomass pretreatments

Improved anaerobic digestion of *C. protothecoides* was achieved via cell wall disruption by several pretreatments. All tests were performed in duplicate using 10 mL of a microalgae suspension. Experiments comparing sonication (5 min at 100% power level), alkaline, thermal pretreatments (or combinations of them) utilized suspensions containing 4.7 g dry algal biomass L^{-1}. Thermal pretreatment was conducted in a closed glass container at 90°C for 30 min in a water bath (2-L water bath, PolyScience WB02A11B model, 120V/60 Hz, Niles, IL, USA). In the alkaline pretreatment, the algal biomass was suspended in 0.1 M NaOH, vortexed, and allowed to incubate a room temperature for 10 min. Subsequently the solution was neutralized with HCl. Two combinations of these treatments were also examined: a thermal-alkaline pretreatment and an ultrasonic-alkaline pretreatment.

Liquid samples were collected at the end of the tests and analyzed for soluble COD to assess the degree of cell disruption. Samples were centrifuged at 10,000 rpm for 10 min and

stored at 4°C till further analysis. In addition, pretreated biomass samples were digested anaerobically to determine the impact of pretreatment on the methane productivity and the release of soluble N. All pretreatments were evaluated in duplicate.

4.2.4. Anaerobic biodegradability

4.2.4.1. Anaerobic basal medium

The basal mineral medium (pH 7.2) used in this anaerobic digestion experiment contained (in mg L^{-1}): NH_4Cl (40); $NaHCO_3$ (3000); K_2HPO_4 (171); $CaCl_2 \cdot 2H_2O$ (20), $MgCl_2 \cdot 6H_2O$ (29), yeast extract (20), and 1 mL of a trace element solution. The trace element solution contained (in mg L^{-1}): H_3BO_3 (50), $FeCl_2 \cdot 4\ H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2 \cdot 4\ H_2O$ (500), $(NH_4)6Mo_7O_2 \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H2O$ (92), $CuCl_2 \cdot 2\ H_2O$ (30), $NaSeO_3 \cdot 5H_2O$ (164), EDTA (1,000), and resazurin (200). Sodium bicarbonate was used to attain buffering capacity in the pH range of 7.0-8.0.

4.2.4.2. Source of inoculum

The anaerobic granular sludge was obtained from a full-scale upward-flow anaerobic sludge blanket reactor treating beer brewery wastewater (Mahou, Guadalajara, Spain) and contained 8.1% volatile suspended solids (VSS). The sludge was store anaerobically at 4°C under N_2 gas.

4.2.4.3 Biochemical methane potential (BMP) test

Glass serum flasks (160 mL) containing basal medium (100 mL) were supplied with algal biomass (0.66 g COD L^{-1}) and inoculated with anaerobic sludge (1.2 g VSS L^{-1}). Endogenous

controls lacking algal biomass were run in parallel to correct for any methane production caused by endogenous decay of the inoculum. All flasks were flushed with a N_2/CO_2 mixture (80:20, v/v) for 5 min to remove oxygen from the headspace and ensure anaerobic conditions. Subsequently, they were incubated at a temperature of 32±1°C in an orbital shaker operating at 120 rpm. The flasks were incubated in darkness to prevent photosynthetic reactions.

Headspace samples were withdrawn periodically and analyzed for methane. The net methane production due to anaerobic digestion of algal biomass was obtained by subtracting the endogenous methane production from the total methane production determined in the treatments supplied with algal biomass. Liquid samples were collected at the end of the tests for analysis of soluble COD, NH_4^+ and volatile fatty acids (VFA). Samples were centrifuged at 10,000 rpm for 10 min and stored at 4°C till further analysis. All bioassays were run in duplicate.

4.2.5. Analytical methods

The elemental C and N composition of the dry algal biomass was tested using a carbon and sulfur combustion analyzer (CS744 Series, LECO Corporation, St. Joseph, MI, USA). The lipid content was quantified by the Folch method using chloroform:methanol (2:1, v/v) [29]. The samples were extracted four times and water was added to remove residual minerals and water soluble impurities. The final extract was dried in a nitrogen evaporator (Model: N-EVAP 112; Organomation Associates, Inc., Berlin, MA). Total lipid content was determined by weight.

Ammonium (NH_4^+) was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH_4^+ electrode (Mettler Toledo, Columbus, OH, USA). Volatile fatty acids (VFAs), namely, acetic, propionic, butyric, isobutyric, valeric, and isovaleric, were quantified by gas chromatography (7890A GC System, Agilent Technologies, Santa Clara, CA) using a fused silica Stabilwax®-DA column (30 m × 530 μm × 0.25 μm, Restek, State College,

PA, USA) and a flame ionization detector. The temperature of the injector port and the detector was 280°C. The initial temperature of the column was 100°C and it was increased to 150°C at a rate of 8°C/min to achieve proper VFA separation. Helium was used as the carrier gas (5.2 mL/min), and air and hydrogen as the flame source. The injection volume was 1 μL. CH_4 in batch assays was measured by gas chromatography using the same apparatus, column, and detector. The temperature of the column, injector port, and detector was 140, 200, and 275°C, respectively. The injection volume was 100 μL.

Methods for the determination of the chemical oxygen demand (COD), total solids (TS), VS, VSS, and pH were adopted from standard methods [30]. The COD was determined using the colorimetric micro-method at a wavelength of 600 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The soluble COD was measured in the supernatant after centrifugation of the liquid phase at 10,000 rpm for 10 min. The moisture content of the algal pellets was determined by drying at 105°C for 24 h.

4.3. Results and Discussion

4.3.1. Algae composition

The volatile solid (VS) content and the lipid content of the *Chlorella protothecoides* biomass were 95.0% and 12.3% of the total dry weight (dwt). The elemental carbon and nitrogen content of the dry biomass was 45.5 and 8.0% of the total dwt, respectively. The total COD content of the algal biomass was 1.39 g COD g^{-1} dwt. Only 2.0-3.5% of the total algal COD was soluble in water at 30°C.

4.3.2. Impact of sonication on the susceptibility of algal biomass to anaerobic digestion

Preliminary experiments were performed to study the impact of sonication intensity and time on the biochemical methane potential (BMP) of *C. protothecoides* algal biomass. Ultrasonic treatment of fresh algal biomass (0.66 g COD L^{-1}) was performed at an acoustic frequency of 20 KHz using a 130-Watt ultrasonic processor operated at power levels of 40, 70 and 100% for 5 min. The impact of sonication time was evaluated in experiments where the biomass was sonicated at a power level of 70% for 1, 5 and 15 min. Sonicated samples were tested for soluble COD and, subsequently, digested anaerobically for 8 days to determine their methane yield.

4.3.2.1. Effect of sonication on COD solubilization

Sonication has previously been shown to promote cell disintegration, destruction of the cell wall integrity and reduction in particle size as confirmed by visual observations using light and electron microscopy [31]. In the present study, acoustic treatment disrupted algal cells leading to a marked increase in the fraction of organic matter that was soluble in water (Fig. 4.1). Sonication for 5 min at 40, 70 and 100% power level led to an increase in the soluble COD from just 3.5% of the total COD in the fresh biomass to 7.0, 13.4 and 40.9%, respectively. The release of soluble COD was also enhanced substantially by increasing the sonication time. As an example, the soluble fraction increased from 4.9% to as much as 40.2% of the total COD when the sonication time increased from 1 to 15 min in tests performed at 70% power level. Ultrasonic pretreatment generates bubble cavitation that causes intense heating and formation of free radicals and hydrogen peroxide [32], facilitating the disruption of algal cells and the solubilization of organic polymers [33].

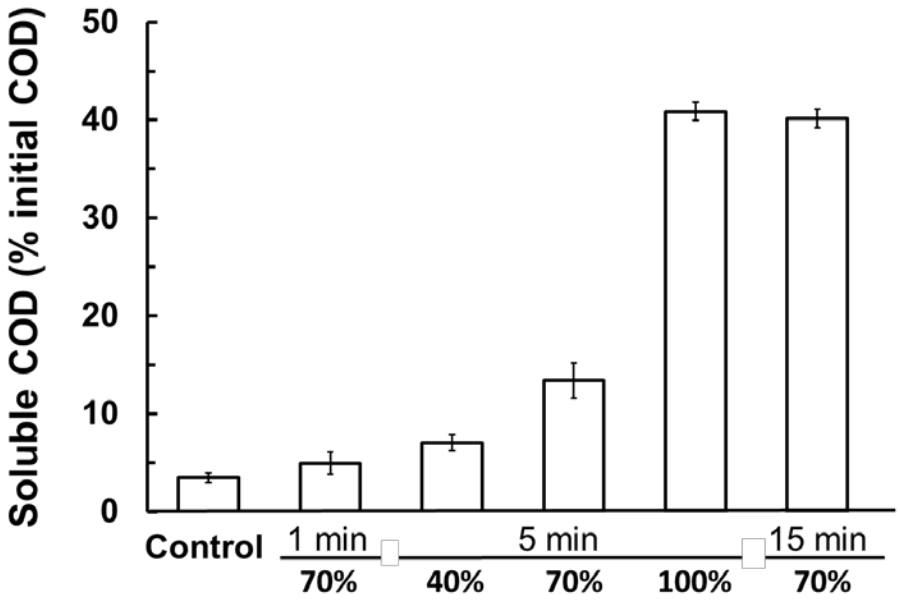

Figure 4.1. Soluble COD released (as % of the total initial COD) following sonication of *C. protothecoides* algal biomass at different sonication intensities.

In agreement with these results, enhanced organic matter solubilization with sonication intensity was also reported in recent studies with algal biomass [33-35] and with other materials such as waste activated sludge [24, 36]. The organic matter solubilization observed in the present study using the most aggressive sonication treatment (40.9%) is high compared to the results reported in some literature studies. For example, sonication of *Scenedesmus* biomass under stringent conditions (*i.e.,* 750W/20 KHz at 90% power amplitude for 15 min) was reported to result in solubilization of around 8% of the organic matter [34]. In a different study, ultrasonic treatment of *Chlorella vulgaris* biomass (20 kHz, constant power input of 150W for 2 min) provided 1.8-fold organic matter solubilization in comparison with untreated biomass [35]. Algal cells seem to be more resistant to disruption by ultrasonic treatment compared to activated sludge.

Experiments performed at the same sonication intensity (95 KJ L^{-1}) showed that the COD fraction in activated sludge and algal biomass (*Scenedesmus*) increased to 61 and 8% of the total initial COD, respectively [34, 37].

4.3.2.2. Effect of sonication on CH4 production

Sonication had a considerable impact on the total amount of methane produced during anaerobic digestion of *C. protothecoides* biomass for 8 days (Fig. 4.2). Pretreatment for 5 min at sonication intensities of 40, 70 and 100% increased the BMP of the algal biomass from just 10.9% (based on the total initial COD) to 23.6, 37.4 and 52.4% of the total COD, respectively. As expected, increasing sonication time enhanced the anaerobic degradability of the algal biomass. Sonication at 70% intensity for 1, 5, and 15 min increased the fraction of the total fresh algae COD transformed into CH_4 from 10.9% in the untreated control to 17.9, 37.4 and 57.7%, respectively. The reported BMP values are corrected for the methane production determined in bioassays lacking algal biomass, where the observed endogenous methane production was equivalent to 7.52% of the initial algal COD. In agreement with these results, enhanced methane generation with sonication intensity was also reported in recent studies with algal biomass and waste activated sludge.

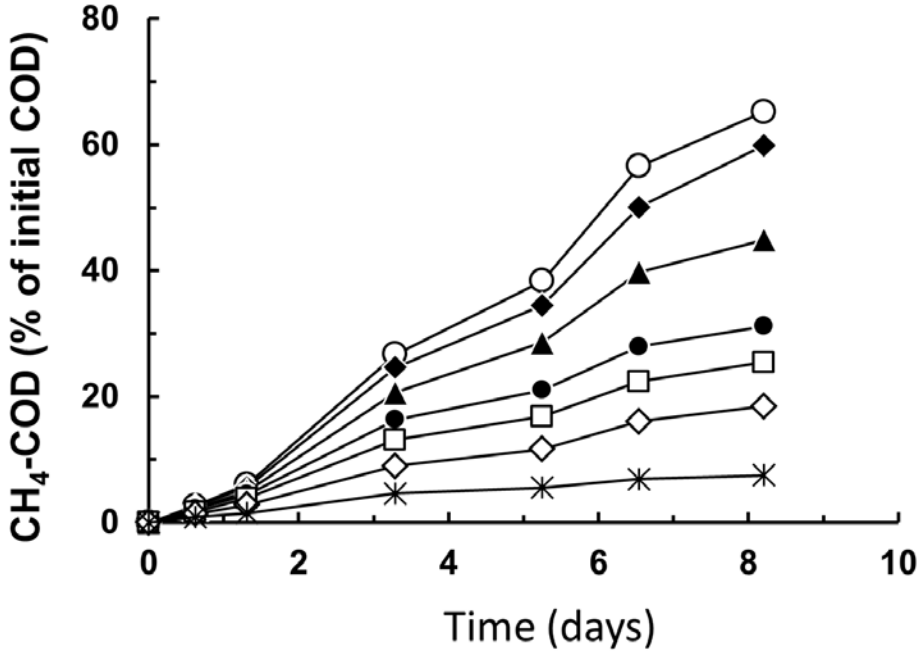

Figure 4.2 Biochemical methane potential of *Chlorella protothecoides* biomass pretreated by sonication at different power levels (PL) and times and without endogenous correction. Endogenous control (✳), untreated algae (◇),70% PL for 1 min (□), algae sonicated at 40% PL for 5 min (●), 70 PL for 5 min (▲), 100% PL for 5 min (◆), and 70% PL for 15 min (○). Note: the values shown are not corrected for the production of CH_4 in the endogenous control.

Methane increase by sonication of algal biomass vary widely, from only 2% to 96% or higher, depending on the type of algae used and the conditions of sonication [33-35, 38]. In some cases, the methane yields reported overestimate the BMP of algal biomass as some studies do not correct for the methane generated from the endogenous decomposition of the anaerobic sludge. Biogas enhancement by sonication of excess sewage sludge has been reported to range from 24% to 140% in batch systems and from 10% to 45% in continuous or semi-continuous systems [24]. Ultrasound technology has been implemented at the industrial scale as pretreatment for anaerobic digestion [39, 40].

4.3.2.3. Effect of energy consumption during sonication on methane production

Although the BMP of the algal biomass was enhanced with increasing sonication intensity and time, a disadvantage of applying high sonication intensities or long times of sonication is a sharp increase in the energy consumption. Fig. 4.3 shows the increase in the initial soluble COD fraction and the enhancement in the BMP of the treated algal biomass, respectively, as a function of the energy applied during sonication. The results demonstrate a sharp increase in the fraction of soluble COD when the energy supplied exceeded 329 kJ g^{-1} VS (Fig. 4.3A). The maximum soluble COD fraction, 40.9%, was reached at 470 kJ $^{g-1}$ VS and a further increase in energy supply did not provide additional benefits. In contrast with these results, the BMP determined after 8 days of incubation increased linearly with the amount of energy supplied during sonication up to 470 kJ g^{-1} VS (Fig. 4.3B). Applying higher energy levels during sonication only resulted in a small increase in the BMP. Previous sonication experiments with microalga biomass and waste activated sludge has shown that a threshold in the energy intensity applied must be exceeded before cell lysis occurs [24, 41]. For algal biomass, disruption of *Scenedesmus* cells by sonication was reported at specific energy levels of 76.5 kJ g^{-1} TS or greater [41].

The results obtained in this study indicate that sonication at 100% power level for 5 min was the most efficient pretreatment in terms of energy consumption to maximize cell disruption and enhance BMP. Therefore, this sonication treatment was selected for further research. It is important to note that due to the small scale of the experiments performed in this study (10 mL reaction volume), the calculated energy consumption is not an accurate measure of the required energy demand in large scale treatment systems.

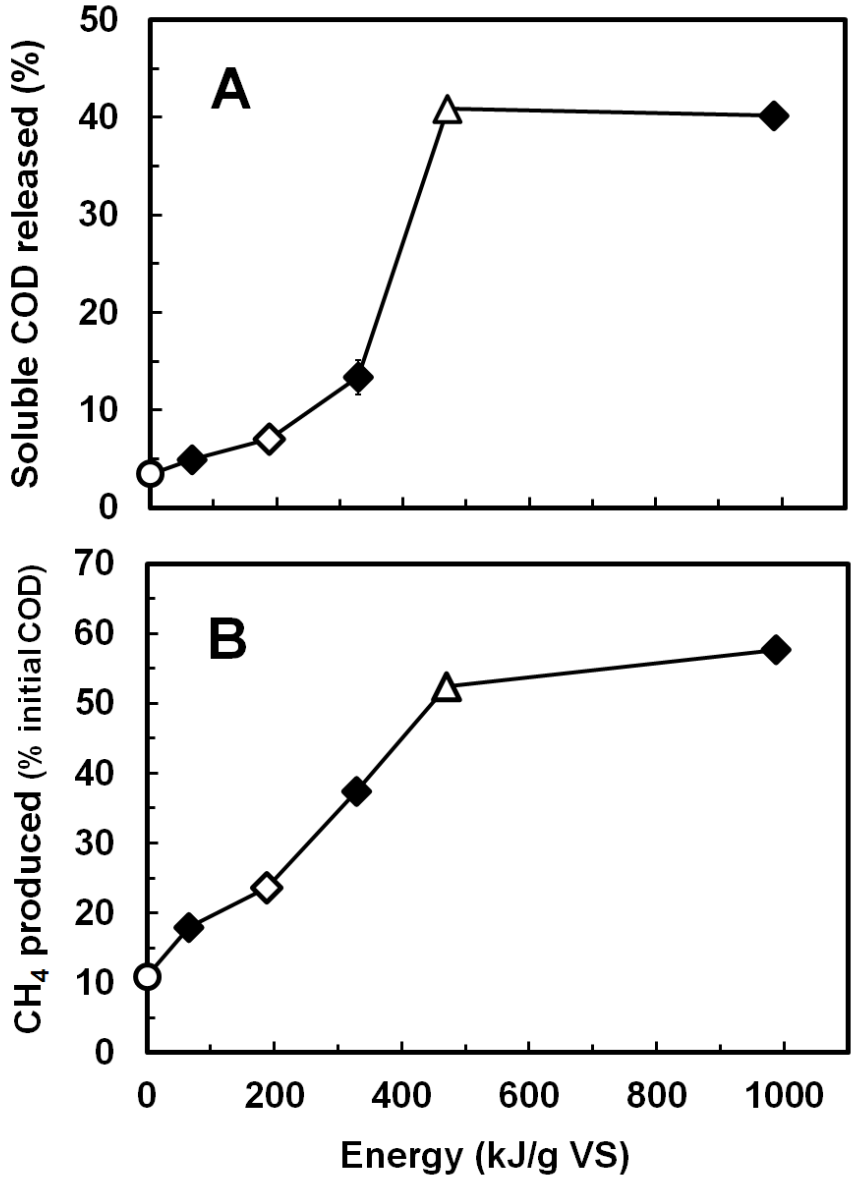

Figure 4.3. Soluble COD released (as % of the total initial COD) following sonication of *C. protothecoides* algal biomass (panel A), and methane produced after 8 days of anaerobic degradation (panel B). Untreated algae (○), 70% PL for 1 min, 5 min and 15 min, reading from left to right (◆), algae sonicated at 40% PL for 5 min (◇), and 100% PL for 5 min (△). Note: the values shown are corrected for the production of CH_4 in the endogenous control.

4.3.3. Evaluation of pretreatments to enhance the biochemical methane potential and organic nitrogen mineralization during the anaerobic digestion of algal biomass

The effectiveness of sonication pretreatment under optimized condition (130 Watt and 20 KHz for 5 min) to enhance the generation of methane and the release of ammonia N during anaerobic digestion of algal biomass was compared with that of thermal and mild alkaline treatments. Combinations of these methods were also assessed, including thermal/alkaline- and sonication/alkaline pretreatment.

4.3.3.1. Impact of pretreatment on initial algal solubilization

The water soluble fraction in untreated algal biomass at 30°C was low, accounting for only 2% of the total algal COD. All the pretreatments investigated disrupted the algal cells and increased the aqueous solubility of the cell organic matter. Sonication was the most effective method and led to solubilization of 33.7% of the algal COD, followed by thermal (15.8%) and alkaline treatment (10.6%) (Fig. 4.4). A combined alkaline-sonication pretreatment did not provide any advantage compared to the alkaline treatment only. On the contrary, sonication under mild alkaline conditions led to a 34.7% reduction in the soluble COD compared to sonication alone. Increased organic matter solubilization was also reported in recent studies where microalgae biomass was pretreated using sonication or thermal treatments [34, 35, 42]. The actual fraction of COD released in the latter studies varied depending on the treatment intensity and the composition of the algal biomass utilized. Thermal treatment has also been shown to provide a marked increase in the fraction of soluble organic matter of excess biological sludge in numerous studies [24]. As an example, [43] reported a 2.9- and 25.6-fold increase in soluble organic matter after treating excess sludge at temperatures of 70 and 90°C for an hour, respectively. Some

thermal processes are currently commercialized and applied at the full-scale level to increase sludge biodegradability during anaerobic digestion [24, 25].

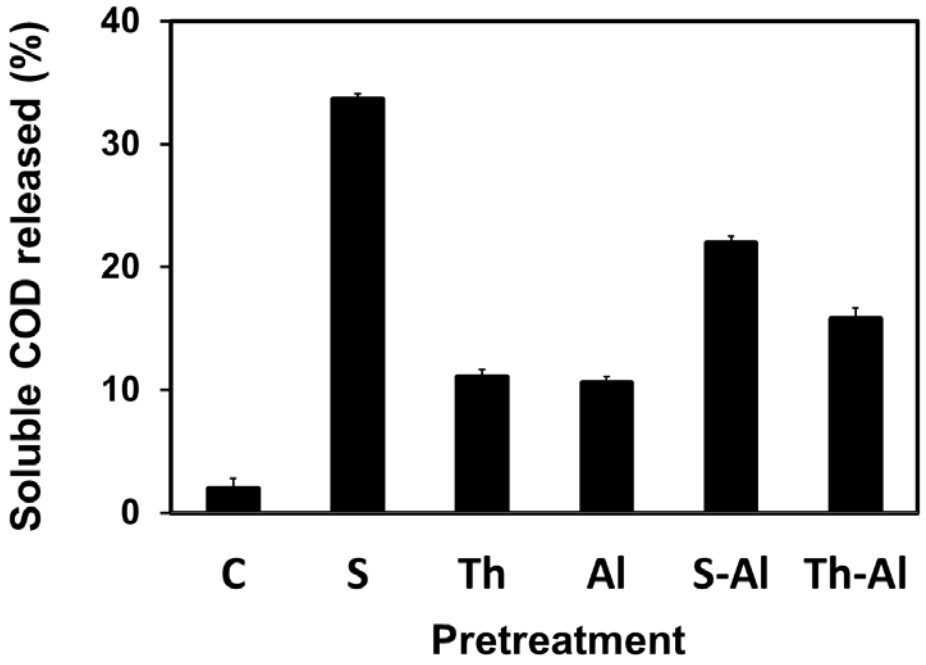

Figure 4.4. Soluble COD released (as % of the total initial COD) after pretreatment of *C. protothecoides* algal biomass using sonication (100% power level for 5 min); thermal treatment, alkaline treatment and combinations of these methods. Control (C); Sonication (S); Thermal (Th); Alkaline (Al); Sonication-alkaline (S-Al); and Thermal-alkaline (Th-Al).

4.3.3.2. Methane production

A rapid increase in the methane production was observed in all the treatments during the initial 10 days, which was followed by a considerable decrease in the rate of methane production during the rest of the incubation (Fig. 5). The slow increase in the total methane production with time after day 10 is mainly due to endogenous decay of the anaerobic inoculum as evidenced by similar slopes of the treatments with endogenous.

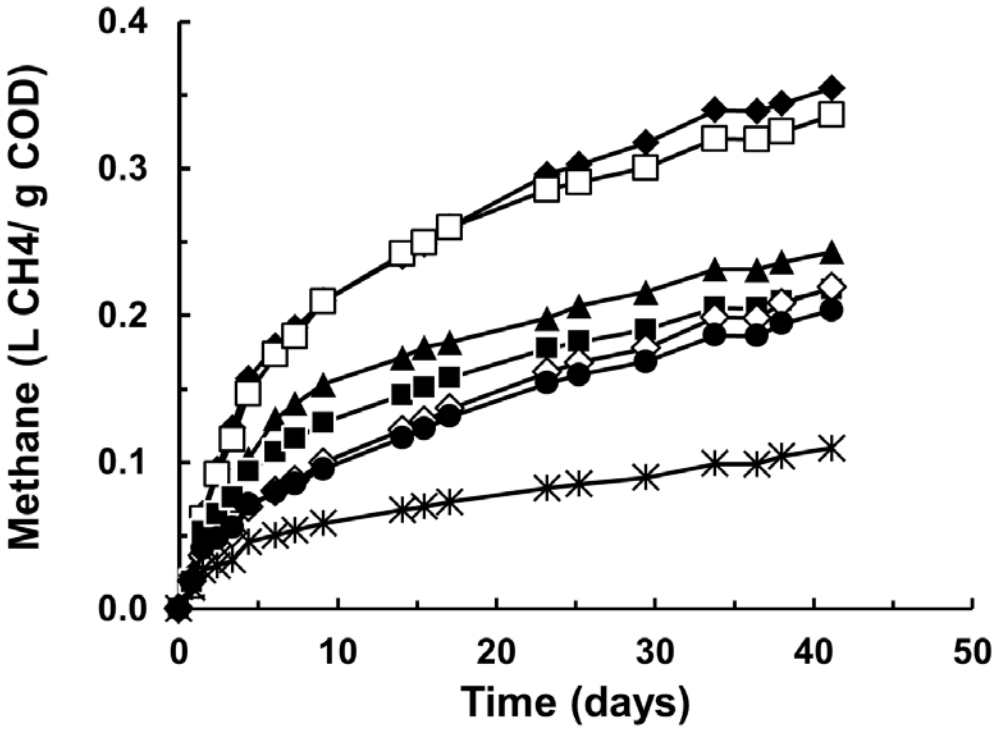

Figure 4.5. Methane production during anaerobic digestion of *C. protothecoides* biomass subjected to different pretreatments after 41 days of anaerobic digestion. Treatments: endogenous control (✳); untreated control (◇), alkaline (●); thermal-alkaline (■); thermal (▲); ultrasonic-alkaline (□); and ultrasonic (◆). Sonicated samples were treated at 100% power level for 5 min.

Detectable accumulation of volatile fatty acids (VFAs) was observed during the initial 4 days of incubation (Fig. 4.6). The main VFAs detected were acetate, propionate and butyrate. VFAs are key intermediate products of the microbial decomposition of organic matter in anaerobic environments. The maximum concentrations of VFA were correlated with the concentration of soluble COD in the various treatments. The highest VFA concentration was recorded in the assay with sonicated algae and accounted for only 4.8% of the initial COD. After day 4-5, the VFA concentrations detected in all the assays were negligible, indicating that the rate of methanogenesis exceeded the rate of VFA formation.

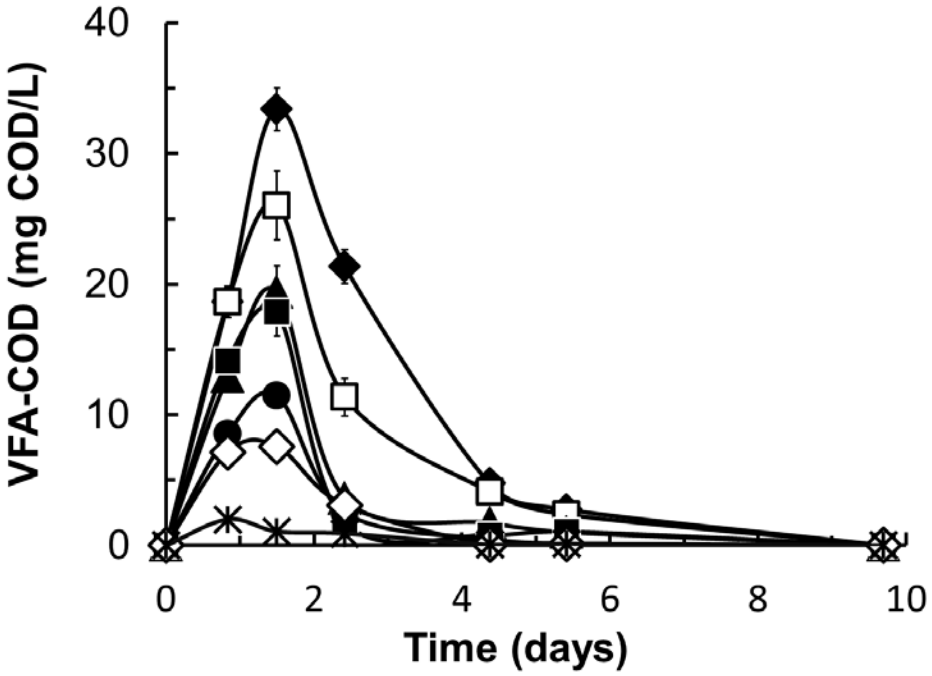

Figure 4.6. Production of volatile fatty acids (VFA) during the first week of anaerobic digestion of *C. protothecoides* biomass subjected to different pretreatments: endogenous control (✳); untreated algal biomass (◇), alkaline (●); thermal (▲); thermal-alkaline (■) ultrasonic-alkaline (□); and ultrasonic (◆).

The methane productivity in the assay with untreated algal biomass was 28.2% of the initial COD (146 mL_{STP} CH_4 g^{-1} algae VS). [44] reported a comparable methane yield of 136 mL_{STP} CH_4 g^{-1} VS in anaerobic digestion studies with *Chlorella* and *Scenedesmus* spp. biomass. However, higher methane yields (180-340 mL_{STP} CH_4 g^{-1} VS) have also been attained with *Chlorella* spp. biomass in some studies [26, 45-47]. The observed differences in the BMP could be due to differences in the experimental protocols utilized and/or differences in the composition of the algal biomass used in the various studies and, in particular, their lipid content. Lipids are high in energy content and have higher specific methane yields (1014 mL_{STP} CH_4 g^{-1} VS) compared to proteins (496 mL_{STP} CH_4 g^{-1} VS) and carbohydrates (415 mL_{STP} CH_4 g^{-1} VS) [48]. In agreement with these theoretical specific methane yields, a very high BMP (532 mL_{STP} CH4 g^{-1} VS) was reported in a recent study that utilized lipid-rich *A. protothecoides* biomass (57% of the dry cell mass) [47]. On the other hand, the high CH_4 productivity reported in some of the studies cited (e.g., [45-47] is also partly due to the fact that the data are not corrected for the CH_4 produced by endogenous decay of the anaerobic inoculum. Depending on the amount and nature of the inoculum used, the correction could be significant.

Sonication provided the highest improvements in methane generation potential (2.24-fold compared to the untreated algae) (Fig. 4.7). Thermal treatment of the fresh biomass achieved a moderate increase in BMP (22%), however, when combined with sonication it actually led to a small but measurable decrease in BMP. Sonication and thermal pretreatment have also been reported to enhance the methane yield of algal biomass in several studies [34, 35, 42, 47, 49]. However, [50] reported no benefit when a mixture of *Chlorella*, *Pseudokirchneriella* and *Chlamydomonas* microalgae biomass was treated at 80°C for 2.5 h. Finally, alkaline treatment was detrimental and resulted in a moderate decrease of the methane generation potential of the

treated biomass whether applied alone or in combination with alkaline treatment. Alkaline pretreatment was also observed to decrease methane productivity from algae biomass in several other studies [42, 46, 47, 51]. The positive correlation between the soluble COD values and the methane production determined in the current study, except for the alkali-pretreated samples, indicates that the hydrolysis of algal cells is the rate-limiting step in the anaerobic digestion of microalgal biomass.

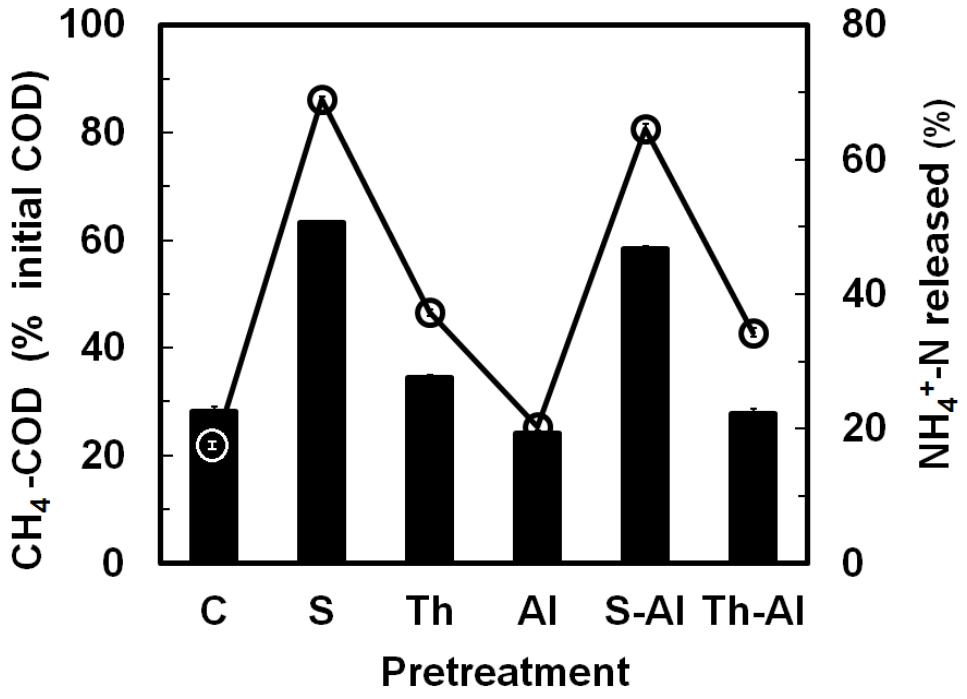

Figure 4.7. Methane production (■) and NH_4^+-N release (O) from algal biomass subjected to different pretreatments after 41 days of anaerobic digestion. Control (C); Sonication (S); Thermal (Th); Alkaline (Al); Sonication-alkaline (S-Al); and Thermal-alkaline (Th-Al). Sonicated samples were treated at 100% power level for 5 min. Methane and NH_4^+-N values were corrected for the corresponding values determined in algae-free endogenous controls.

4.3.3.3. Mineralization of organic nitrogen in algal biomass biomineralization

Anaerobic digestion released 17.1% of the total nitrogen in the untreated algal biomass (Fig.7). All the pretreatments enhanced the mineralization of organic nitrogen to some extent, but sonication was the most effective pretreatment providing a 4.1-fold increase in the release of nitrogen from the algal biomass at the end of the incubation (to 69.9% of the total N). Considerably lower levels of ammonia (20.5-37.7%) were detected when only thermal- or alkaline treatment was applied. As shown in Fig. 7, the enhancement in nitrogen mineralization was strongly correlated with the increase in the BMP of the algal biomass, as these are both governed by the biological degradation of the algae. High nitrogen solubilization accompanied by enhanced methane production has also been reported in studies where waste activated sludge [36, 52, 53] was subjected to ultrasonic pretreatment prior to anaerobic digestion, but comparable studies with algal cells seem to be lacking. In contrast, several reports have confirmed that thermal treatment of algal biomass can provide a significant increase in the release of nitrogen during anaerobic digestion. Keymer and coworkers [54] observed that the soluble nitrogen content in *Scenedesmus* algal biomass increased from only 2.5% in the raw algae to as much as 43% in the anaerobically digested biomass. When the algal cells were treated thermally, the fraction of nitrogen solubilized following anaerobic digestion increased to approximately 56% of the total nitrogen content. Similarly, Bohutskyi and coworkers also demonstrated that anaerobic digestion of lipid-extracted *Nannochloropsis salina* and *Auxenochlorella protothecoides* biomass provided significant release of soluble nitrogen content, approximately 28% and 40% of the total nitrogen, respectively [55, 56]. Lipid extraction in the later study involved thermochemical pretreatment (extraction with acetone at 65°C followed by extraction with hexane). These results confirm the potential of anaerobic digestion for nitrogen recovery and recycling.

4.4 Conclusions

The results of this study demonstrate that anaerobic digestion of pretreated *Chlorella protothecoides* biomass is a promising approach to generate energy-rich methane and recover nitrogen nutrients. Sonication of algal biomass under optimized conditions provided a significant increase in the methane yield (327 mL_{STP} CH_4 g-1 VS) compared to the untreated algae (146 mL STP CH_4 g^{-1} VS), as demonstrated in anaerobic digestion experiments incubated for 41 days. In contrast, thermal pretreatment only provided a moderate increase in the methane yield and alkaline treatment lead to decrease of the methane yield compared to the untreated algal biomass. Sonication treatment also provided a 4.1-fold increase in the release of ammonia nitrogen during anaerobic digestion of the algal biomass.

4.5 Acknowledgements

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CHAPTER 5

NUTRIENT RECOVERY AND BIOGAS GENERATION FROM THE ANAEROBIC DIGESTION OF WASTE BIOMASS FROM ALGAL BIOFUEL PRODUCTION

Microalgae are gaining popularity as a source of biodiesel. Recycling fertilizer nutrients is critical to sustain large-scale biodiesel production because the global supply of surplus fertilizer is limited. This study demonstrates that anaerobic digestion of residual algal biomass from biodiesel production can provide additional nutrients and energy. Anaerobic digestion of *Chlorella sorokiniana*-1412 whole cell algae (WCA), sonicated algae (SA), and SA subjected to lipid extraction (LEA) in bench-scale batch reactors operated at 30 ± 2^{0}C for 42 days released a considerable amount of the nitrogen and phosphorus in the algal cells. Digestion of WCA, SA, LEA released 48.1, 77.4, and 61.5% of the total algal nitrogen as NH_4^+-N, and 87.7, 99.4, and 93.6% of the total algal P as soluble P, respectively. The energy recovery from algae biomass was quantified through the methane yield. The biochemical methane potential of WCA, SA and LEA was 0.298, 0.388 and 0.253 L methane/g algal volatile solids, respectively. The conversion of LEA and WCA biomass to methane was very similar (38 and 41% on a COD basis, respectively), indicating that the energy yield was not significantly lowered by extraction of the lipid fraction (which accounted for 9% of algal dry weight). Sonication improved the access of hydrolytic enzymes to algal biopolymers (compensating in part for the energy lost due to lipid extraction). The results taken as a whole indicate that anaerobic digestion of LEA can provide considerable yields of methane and soluble nutrients.

Keywords**:** Methane, nutrient recovery, nitrogen, phosphorous, algal biofuels.

5.1. Introduction

Microalgae are being considered as a promising new approach to producing renewable biofuels. Biodiesel synthesized from lipids obtained from microalgal cultures has been studied intensively in the last decade as a potential partial replacement of the diesel fuel used in transportation [1]. The International Energy Agency expects that biofuels will contribute 6% of total fuel used by 2030 [2]. Photosynthetic microorganisms can produce lipids with yields per unit of land 100 times more than plants [3]. Additionally, microalgae-based fuels are anticipated to be an environmentally friendly way to obtain energy and while decreasing the net release of CO_2 into the atmosphere [4, 5]. Once the lipids are extracted from the whole cell algae (WCA), the remaining biomass in the lipid extracted algae (LEA), is still a valuable raw material with biopolymers and high levels of N and P.

The main costs drivers in algal aquaculture are carbon, water and nutrient inputs [2]. The Energy Independence and Security Act, enacted in 2007 by the U.S. Congress, called for meeting the renewable fuels standard of achieving 36 billion gallons of renewable fuel by 2022 [6], but production of microalgae-based biofuels is projected to be only 5 billion gallons per year (BGY) [7]. Approximately 10% of algal dry weight biomass is composed of N- and P- nutrients assuming the theoretical molecular formula $C_{106}H_{263}O_{110}N_{16}P$ [8]. Thus, under ideal nutrient uptake efficiency, 88 kg N and 12 kg P would produce one metric ton of dry weight algal biomass. Considering the 2022 goal of the Energy Independence and Security Act of 5 BGY, 32 to 49% of N and P from the current world surplus values would be used for two algal species, *Chlorella* and *Nannochloropsis* [9]. By integrating the anaerobic digestion of LEA, nutrients can be released and recycled and additional energy as methane could be recovered improving the economic viability and sustainability of biodiesel production [10].

Thus anaerobic digestion offers an attractive alternative to obtain additional energy as methane (CH_4) and nutrients recovery from LEA. Many species of algae are rich in lipids, starch and proteins and are well suited to anaerobic digestion. They lack lignin which makes them more readily biodegradable under anaerobic conditions compared to lignocellulosic biomass. Studies show pretreatments, like those characterized by energy-intensive lipid extraction, cause cell wall and cell membrane disruption which facilitates anaerobic degradation by releasing the intracellular content [11, 12] . The CH_4 generated under anaerobic digestion can be converted into renewable transportation fuels or combusted to generate electricity. The liquid digestate that contains water and fertilizers, such as ammonium (NH_4^+) and phosphate (PO_4^{3-}), can be recycled for the next algae generation and the digested solids can be composted.

The biochemical methane potential (BMP) is an index of anaerobic digestion, commonly expressed as L $CH_{4produced}$/g $VS_{substrate\text{-}fed}$. As the maximum amount of CH_4 expected under anaerobic conditions, is estimated by the chemical oxygen demand (COD) content of algae, BMP can be also expressed as % $COD_{CH4}/COD_{alg\text{-}t0}$, which indicates the degree of biodigestibility.

Stoichiometrically, 1 g of CH_4 is equal to 4 g COD, that under standard temperature and pressure (STP, 1 atm and 0 ^oC) is equivalent to 0.35 L CH_4/g COD. At STP, 0.515 and 0.648 L CH_4 are produced theoretically per g volatile solids (VS) assuming COD factors of 1.47 and 1.84 g COD/g VS for materials with 10% and 30% lipids, respectively [13]. In practical studies, the specific methane yield from whole algae digestion usually ranges from 0.09-0.44 L CH_4/gVS [14]. In studies of anaerobic performed in *Chlorella sorokiniana* and *Chlorella vulgaris,* the COD conversion in biogas ranged from 40 to 73% of algae-COD, where the maximum total methane yield was obtained for dry and milled algae [15].

Chlorella sorokiniana-1412 has been shown in multiple studies to be a highly productive and resilient algal strain for biodiesel production. This study focuses on nutrient recovery and

methane (CH_4) production through anaerobic digestion of *C. sorokiniana* biomass. The specific methane yield of *C. sorokiniana*, both with and without ultrasonic pretreatment, and its LEA counterpart were studied. The main objectives of the project were to evaluate the digestibility of the microalga *C. sorokiniana*-1412 before and after lipid extraction to quantify nutrients recovered through anaerobic digestion and the methane yield achieved.

5.2 Materials and methods

5.2.1 Algae growth conditions

C. sorokiniana-1412 was cultivated under standard growth conditions in a raceway pond, with sunlight. The synthetic medium contained (mg/L): urea (100), $MgSO_4 \cdot 7H_2O$ (12), $NH_4H_2PO_4$ (25), K_2CO_3 (75), $FeCl_3$ (3.15), Na_2CO_3 (20), EDTA (4.36), and 1 mL of Allen's nutrient solution. Allen's solution contained (in mg/L): H_3BO_3 (2860), $MnCl_2 \cdot 4H_2O$ (1810), $ZnSO_4 \cdot 7H_2O$ (220), $Na_2MoO_4 \cdot 2H_2O$ (390), $CuSO_4 \cdot 5H_2O$ (79), and Co $(NO_3)_2 \cdot 6H_2O$ (49.4). Carbon dioxide was bubbled from the bottom of the raceway pond to mix the culture of the pond and provide a complementary carbon source. Algae cells were harvested by centrifugation (Type 10 Evodos centrifuge, Evodos B.V., Breda, Netherlands) at 5000 rpm for 10 min.

5.2.2 Chemical composition of C. sorokiniana

5.2.2.1 Elemental composition

The elemental C, N and P composition of the dry algal biomass was analyzed using a carbon and sulfur Combustion Analyzer (CS744 Series, LECO Corporation, St. Joseph, MI, USA).

5.2.2.2 Lipid content

The algal biomass was dried for 10 h at 70^{o}C to facilitate lipid extraction prior to ultrasonic pretreatment. In order to facilitate lipid extraction, algae biomass was sonicated using a 130-Watt ultrasonic processor (GEX 130, Cole-Parmer Instruments, Vernon Hills, IL, USA) at 70% amplitude for 10 min. SA without lipid extraction was used in this study as control for LEA and also to understand how cell wall disruption in SA facilitate anaerobic digestion when compared against WCA. The lipid content of the algal cells was determined by Soxhlet extraction with hexane at 70^{o}C for 10 h. Extracted algal material was dried at room temperature for 24 h to remove excess hexane.

5.2.2.3 Chemical oxygen demand (COD)

The COD is expressed as g COD/g $VS_{alg\text{-}t0}$. The COD was determined using the colorimetric micro-method at a wavelength of 600 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Methods for the determination of the COD, total solids (TS), volatile solids (VS), volatile suspended solids (VSS) and pH were adopted from standard methods [16].

5.2.3 Anaerobic digestion experiments

5.2.3.1 Anaerobic inoculum

Digested anaerobic sludge was obtained from the Roger Road municipal wastewater treatment plant (Tucson, Arizona, USA). This sludge had a volatile suspended solids (VSS) content of 72 g VSS/kg wet weight (after centrifuge). When unused, the sludge was stored anaerobically at 4˚C.

5.2.3.2 Anaerobic basal medium

The basal medium used in the anaerobic digestion experiments contained (in mg/L): K_2HPO_4 (62.5), $CaCl_2$•$2H_2O$ (10), $MgCl_2$•$6H_2O$ (10), NH_4Cl (70), $NaHCO_3$ (3000), yeast extract (20), and trace element solution (1 mL). The trace elements solution contained (in mg/L): H_3BO_3 (50), $FeCl_2$•4 H_2O (2,000), $ZnCl_2$ (50), $MnCl_2$•$4H_2O$ (500), $(NH_4)_6Mo_7O_{24}$•$4H_2O$ (50), $AlCl_3$•$6H_2O$ (90), $CoCl_2$•$6H_2O$ (2,000), $NiCl_2$•$6H_2O$ (92), $CuCl_2$•$2H_2O$ (30), $NaSeO_3$•$5H_2O$ (164), EDTA (1,000), and resazurin (200). The pH of the medium solution was adjusted to 7.2 using diluted hydrochloric acid. Both solutions were prepared using deionized water.

5.2.3.3 Batch methanogenic activity assays

The acetoclastic and hydrogenotrophic methanogenic activities of the sludge were evaluated using batch assays carried out in serum bottles (160 mL) containing 60 mL of culture medium and 1.72 g VSS/L of anaerobic sludge. The headspace was flushed with a gas mixture of N_2/CO_2 (80:20, v/v) to ensure anaerobic conditions and provide buffering capacity (pH= 7.2-7.4). Acetate as a substrate (1.0 g/L as CH_3COONa) or H_2 (0.5 atm, supplied as H_2/CO_2 (80:20, v/v)) was added. The bottles were incubated in the dark at 30±2°C in an orbital shaker at 115 rpm. Head space samples (100 µL) were obtained periodically and analyzed for CH_4 content. The maximum specific methanogenic activities (mg CH_4-COD/g VSS•d) were calculated from the graph of the slope of the CH_4 content versus time as the mean value of triplicate assays. The maximum specific activity of hydrogenotrophic and acetate-utilizing methanogens were 96 and 102 mg CH_4-COD/g VSS•d, respectively.

5.2.3.4 Set-up of algae anaerobic digestion (bioreactor operational parameters)

Fig. 5.1 illustrates the bench-scale experimental set-up utilized to investigate the anaerobic digestion of whole cell algae (WCA), lipid-extracted algae (LEA), and sonicated algae (SA). The digestion vessels (4-L Erlenmeyer flasks) were supplied with basal medium (3 L), anaerobic inoculum (1.03 g VS/L), and algal biomass (3 g dwt/L, based on the original dwt of the WCA and SA and 2.83 g dwt/L of LEA). Thus, for the final designs of the reactors, the algal biomass concentration for WCA and SA (expressed in equivalent VS value) was 2.85 g $VS_{alg\text{-}t0}$/L; the LEA group had a substrate VS concentration of 2.69 g $VS_{alg\text{-}t0}$/L. An endogenous control lacking algal substrate supplementation was run in parallel. Table 5.1 shows details of the experimental protocol and settings.

Table 5.1. Experimental set-up utilized in the anaerobic digestion studies.

Digestor	Mass of algae (g dwt)[a]	Initial total COD (g $COD_{alg\text{-}t0}$)
Endogenous control	0	0
Whole cell algae (WCA)	9.0	16.0
Sonicated algae (SA)	9.0	16.0
Lipid extracted algae (LEA)	8.5	14.2

[a] mass per 3 L of medium.

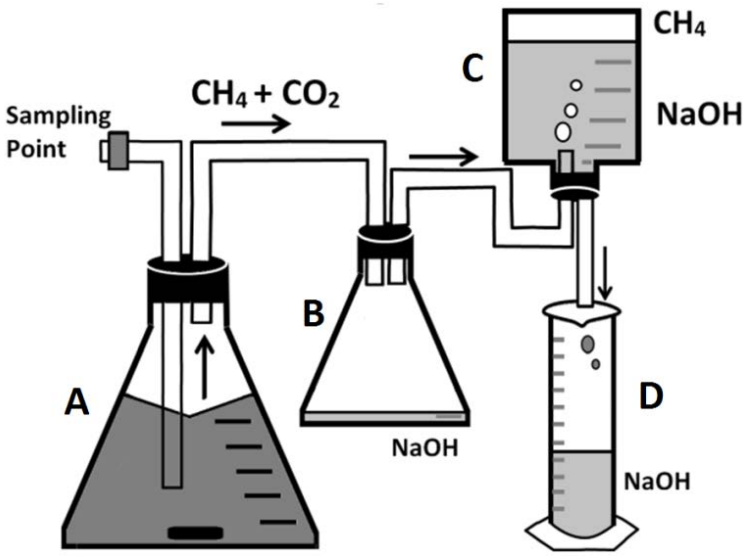

Figure 5.1. Schematic representation of the anaerobic digestion system utilized in this study. Digester (**A**), trap (**B**), Mariotte flask to quantify the volumetric production of methane (**C**), and NaOH collector (**D**).

Flasks were flushed with N_2/CO_2 (80/20) for 10 min to create anaerobic conditions and then sealed with a perforated rubber stopper which was connected to an inverted Mariotte bottle (1 L). The Mariotte bottle, which was filled with 2% NaOH solution to adsorb CO_2 from the biogas, was utilized to measure the methane produced by solution displacement. The NaOH solution was refilled as needed. The experiments were conducted in an incubator (Percival Scientific Inc., Perry, IA, USA) set at a constant temperature of 30±2˚C. The medium in all digesters was maintained under constant agitation at 200 rpm using magnetic stirrers. Digesters were kept in the dark to avoid algal photosynthesis.

5.2.4 Evolution of anaerobic digestion

The samples were centrifuged immediately at 10,000 rpm for 10 min (Spectrafuge 24D, Labnet Int'l Inc., Edison, NJ, USA). The supernatants were refrigerated at 4°C for further analysis. In the following sections, the parameters quantified are described.

5.2.4.1 Methane

The methane production was monitored periodically during the 42-day incubation period. Mariotte bottles, which were filled with 2% NaOH solution to adsorb CO_2 from the biogas, were utilized to measure the methane produced by solution displacement.

5.2.4.2 Phosphorous and nitrogen

Soluble PO_4^{3-} (or other soluble forms) was measured as P by inductively coupled plasma optical emission spectrometry (ICP-OES) (Model 2500DV, Perkin Elmer, Shelton, CT, USA) at 213.61 nm wavelength, with a detection limit of 10 µg/L. Ammonium (NH_4^+) was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH_4^+ electrode, a detection limit of 1 mg/L (Mettler Toledo, Columbus, OH, USA).

5.2.4.3 Volatile fatty acids

Volatile fatty acids (VFAs), namely, acetic, propionic, butyric, isobutyric, valeric, and isovaleric, were quantified by gas chromatography (7890A GC System, Agilent Technologies, Santa Clara, CA) using a fused silica Stabilwax®-DA column 30 m × 530 µm × 0.25 µm, (Restek, State College, PA, USA) and a flame ionization detector. The temperature of the injector port and the detector was 280°C. The initial temperature of the column was 100°C, and it was increased to 150°C at a rate of 8°C/min to achieve proper VFA separation. Helium was used as

the carrier gas (5.2 mL/min) and air and hydrogen as the flame source. The injection volume was 1 µL. CH_4 in batch assays was measured by gas chromatography using the same apparatus, column, and detector. The temperatures of the column, injector port, and detector were 140, 200, and 275°C, respectively. The injection volume was 100 µL.

5.3 Results

5.3.1. Algae composition

Table 5.2 describes the composition of *C. sorokiniana*-1412 before and after lipid extraction. Lipid extraction slightly increased NH_4^+-N content but slightly decreased the soluble P concentration per unit dwt. The COD per dwt decreased due to loss of energy-rich lipids after the lipid extraction.

Table 5.2 Chemical composition of biomass from *Chlorella sorokiniana* strain 1412 before and after lipid extraction.

Composition	Whole Cell Algae Biomass	Lipid-Extracted Biomass
Total solids (g TS/g wet wt)	0.20	1.00[b]
Volatile solids (g VS/g dwt)	0.95	0.95
Total nitrogen (% dwt)[a]	5.47	5.61
Total phosphorous (% dwt)	0.57	0.53
Total carbon (% dwt)	51.05	48.40
Total lipids (% dwt)	9.0	0
COD factor (g COD/g dwt)	1.78	1.65

[a] Expressed as percentage of the total dry weight (110°C, 24 h).

[b] Material received as dried mass, with no detectable water content.

5.3.2 N and P mineralization

The time course of the nutrient-nitrogen release as soluble NH_4^+-N is shown in Fig. 5.2 The concentration of NH_4^+-N on day 0 was 18.3 mg/L, which can mostly be accounted for by the NH_4Cl of the basal medium. Sonication treatment and lipid extraction did not significantly change the initial NH_4^+-N concentration. During the subsequent incubation, WCA slowly released NH_4^+-N over the course of 42 days. The release slowed down after 19 days. In contrast, SA and LEA rapidly released NH_4^+-N over the first 8 days of digestion, reaching maximum NH_4^+-N concentrations at day 8. The rates of N release were 23 mg NH_4^+-N/L•d for both SA and LEA; whereas this rate was only 3.9 mg NH_4^+-N/L•d for WCA. N release was greatly accelerated by 6-fold for NH_4^+-N, with treatments that disrupt cells such LEA and SA .By the end of the experiment, and corrected for endogenous N, a release of 48.1, 61.5 and 77.4 % of total algal N as NH_4^+-N from WCA, LEA and SA was observed, respectively. The release (in %) of NH_4^+-N is shown in Fig. 5.3.

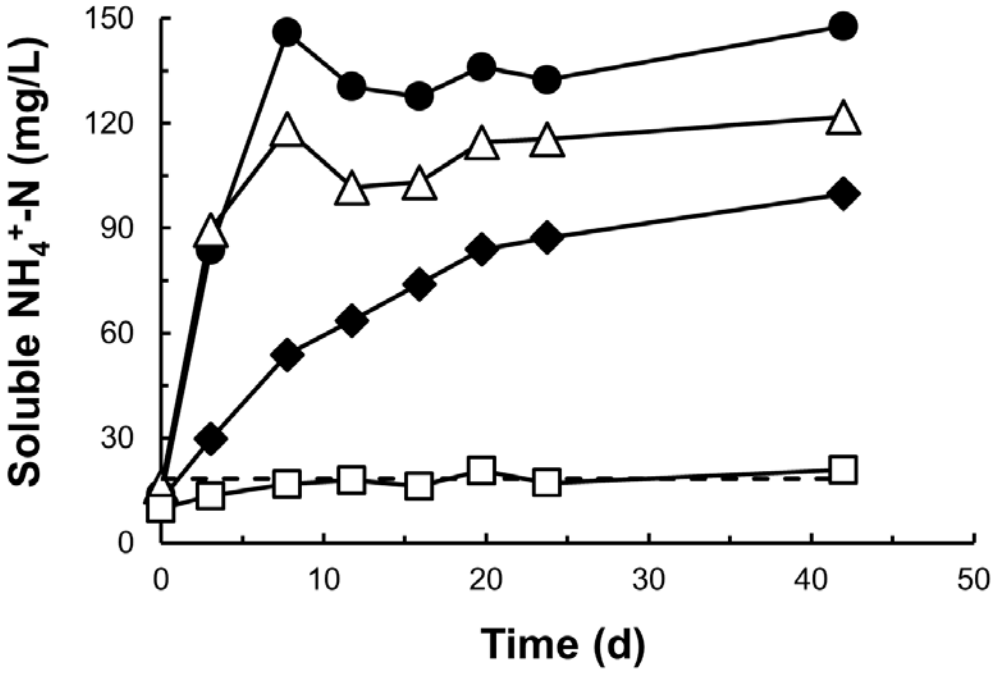

Figure 5.2. Release of soluble nitrogen (as mg/L NH_4^+-N) during the anaerobic digestion of *C. sorokiniana*-1432 as a function of time. Legends: whole cell algae (◆), sonicated algae (●), lipid-extracted algae (△), and endogenous, algae-free control (□). Dash line is N added in the basal medium.

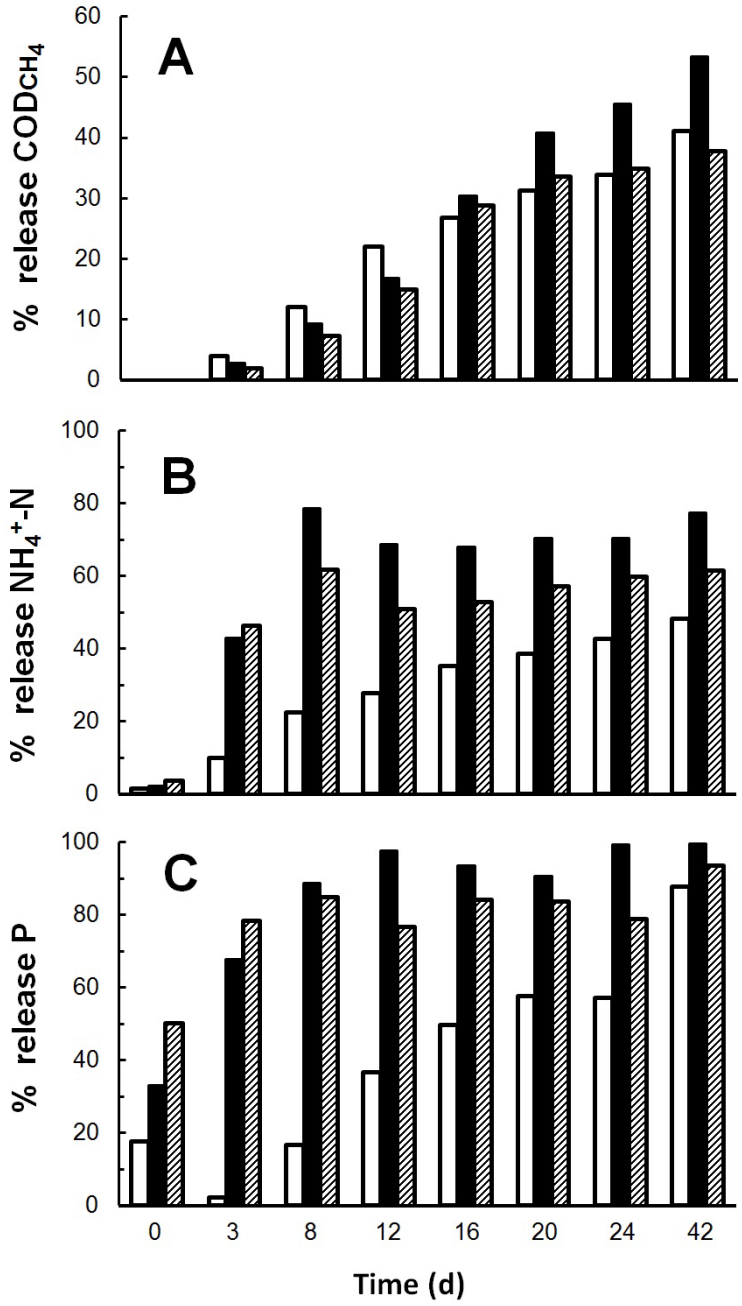

Figure 5.3. Methane production (panel A), NH_4^+-N released (panel B) and dissolved P released (panel C) from whole cell algal biomass (open bars), lipid-extracted algal biomass (stripped bars) and sonicated algal biomass (black bars) after 42 days of anaerobic digestion. All values were corrected for the corresponding values released in algae-free endogenous controls. 0.107 g CH_4-COD L^{-1}, 20.8 mg NH_4^+-N $L^{-1}$and 19.1 mg P L^{-1}.

Soluble phosphorous released during anaerobic digestion is shown in Fig. 5.4 The level of dissolved P on day 0 was approximately 10 mg/L, which could be mostly accounted for by the addition of K_2HPO_4 to the basal medium. In the endogenous digester, the P concentration is equal to the level of nutrients added. SA and LEA caused the initial P concentration to increase by up to 2-fold, suggesting release due to sonication or lipid extraction. Immediately upon starting the digestion, soluble P increased in all the digesters, including the endogenous control. During this period, the increase was greatest for LEA and SA, which released high concentrations of P with the maximum releases being achieved in 3 days.

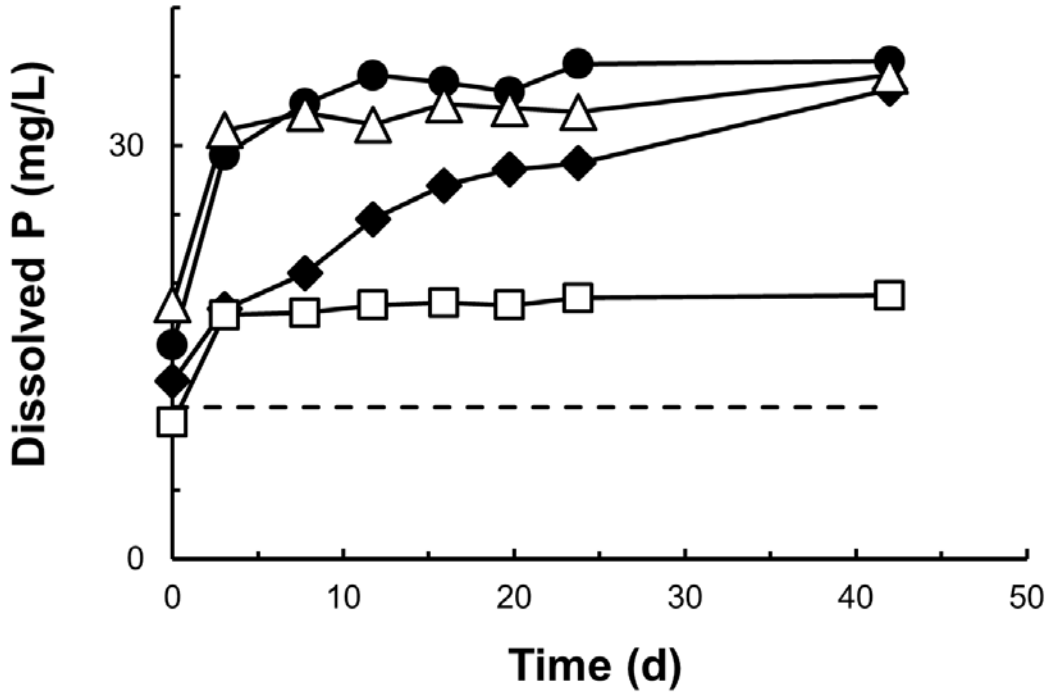

Figure 5.4. Release of soluble phosphorous during the anaerobic digestion of *C. sorokiniana* 1432 as a function of time. Legends: whole cell algae (◆), sonicated algae (●), lipid-extracted algae (△), and endogenous, algae-free control (□).Dash line is P added in the basal medium.

From day 3 onwards, there were no more increases of soluble P in the endogenous control. However, the P release continued slowly in WCA until the end of the experiment. The release (in %) of soluble P is shown in Fig. 5.3 The rates of P release were 4.5 mg P/L•d for both SA and LEA; whereas only 0.93 mg P/L•d was observed for the WCA. P was greatly accelerated by 5-fold for soluble P, with treatments that disrupt cells such LEA and SA. At day 42, the P released was, as a percentage of algal P and corrected for endogenous P, 87.7, 93.6 and 99.4 % for WCA, LEA and SA, respectively.

5.3.3 Conversion of algal-COD to methane

The cumulative methane yield for each of the digesters over 42 days of digestion is shown in Fig. 5.5.

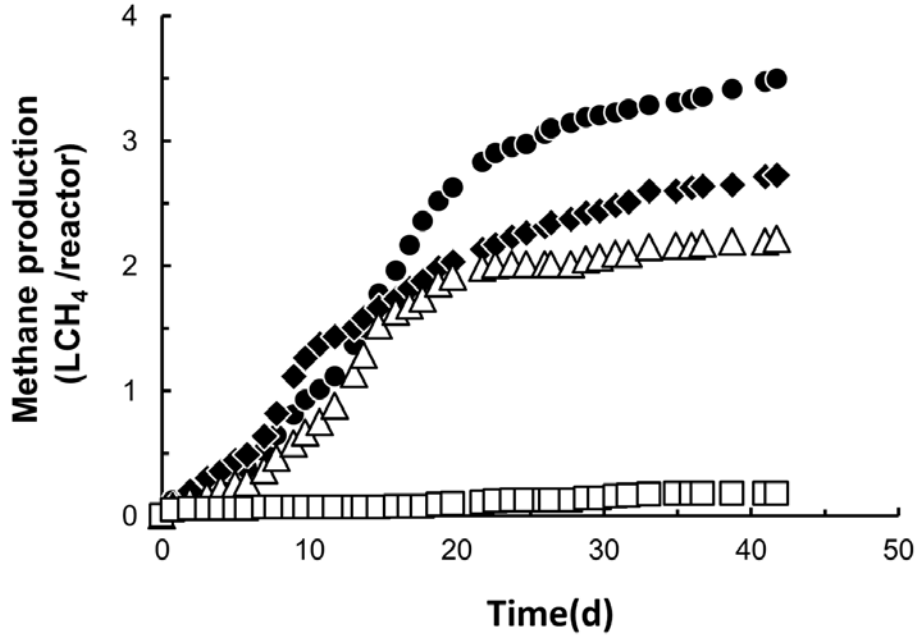

Figure 5.5. Time course of methane production during the anaerobic digestion of *C. sorokiniana* strain 1432 biomass. Legends: WCA (◆), SA (●), and LEA (Δ); and endogenous, algae-free control (□).

Methane production in the endogenous control was very low. All algae amended treatments produced methane to a much greater extent than the endogenous control. Over the first 14 d, the methane production was fastest in WCA. Thereafter, however, methane production was fastest and more extensive in the SA. LEA treatment started somewhat lower in methane production but by day 14 it caught up with WCA. Methane yield and their equivalent energy value are shown in Table 3. The percentage yield of $COD_{CH4}/COD_{alg\text{-}t0}$ after 42 days, corrected for endogenous COD_{CH4}, was 41.0, 37.9 and 53.4% for WCA, LEA and SA, respectively. The release of COD_{CH4} (in %) is shown in Fig. 5.3.

Table 5.3 Methane produced and energy generated during the digestion of WCA biomass, SA biomass, and LEA biomass (CH_4 at 30°C, after 42 days of digestion).

Reactor	**(L CH_4/ reactor)** measured	**BMP[a]**		**LHV[b]**
		(L CH_4/g $VS_{alg\text{-}t0}$)	(% $COD_{CH4}/COD_{alg\text{-}t0}$)	(MJ/kg VS)
Whole cell	2.726	0.298	41.0	10.6
Sonicated	3.496	0.388	53.4	13.8
LEA	2.215	0.253	37.9	9.0
Endogenous	0.175	N/A	N/A	N/A

[a] BMP = Biochemical methane potential corrected by endogenous (CH_4 total - CH_4 endogenous).

[b] LHV = Lower heating value at 30°C.

5.3.4 VFA production as an intermediate step of anaerobic digestion of algae

VFAs generated during anaerobic digestion of the algal biomass consisted mainly of acetate (C2), propionate (C3) and butyrate (C4), as shown in Fig. 5.6. Total VFA was 0 mg COD/L at the start of the experiment. At the peak of the VFA concentration, the VFA accounted for 3.0, 10.1 and 11.7% of the $COD_{alg\text{-}t0}$ for WCA, LEA and SA, respectively. VFA are produced by fermentation but also consumed by methanogenesis so their concentration is transient.

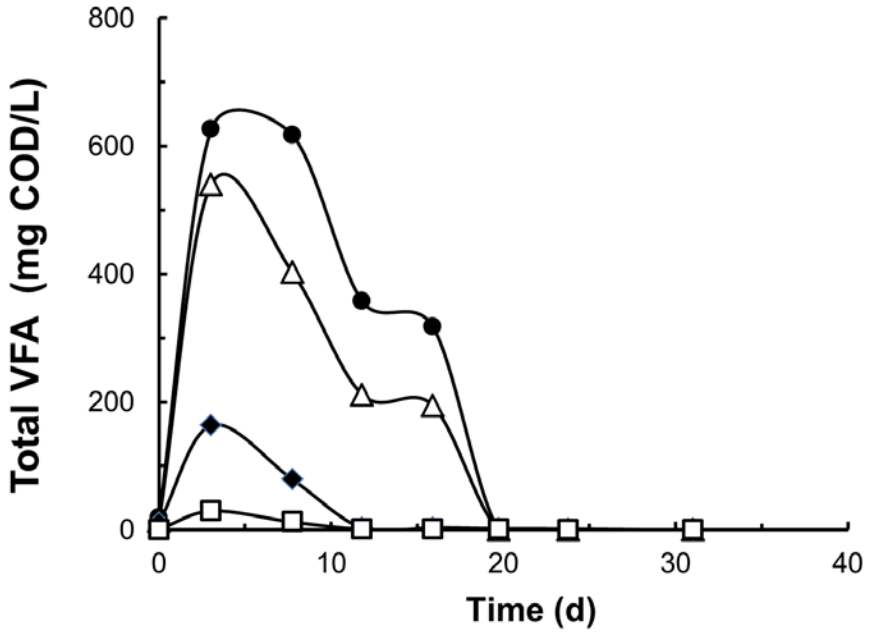

Figure 5.6. Total concentration of volatile fatty acids (VFA) produced during the anaerobic digestion of *C. sorokiniana* strain 1432 as a function of time. Legends: whole cell algae (◆), sonicated algae (●), lipid-extracted algae (△), and endogenous, algae-free control (□).

The peak concentrations were achieved somewhere between day 3 and 8. The highest concentration observed for SA and the lowest for WCA. These results suggest a more rapid fermentation of algal material in SA and LEA compared to WCA, suggesting that sonication and lipid extraction liberated bioavailable substrates from algae that were rapidly fermented. The total VFA composition had concentrations of individual VFA decreasing in this order: $C_2 > C_3 > C_4$. By day 20, the VFA concentrations decreased to below detection in all treatments, suggesting that rate of methanogenesis was dominating over the rate of VFA formation by fermentation in the latter period.

5.4 Discussion

5.4.1. Nutrient recovery

In our 42-day study of anaerobic digestion, organic N in WCA and LEA of *C. sorokiniana* was mineralized by 48% and 62% to NH_4^+-N, respectively, after correcting for endogenous and background NH_4^+-N production. Only one other study evaluated the release of NH_4^+-N during anaerobic digestion of the same phototrophically grown species (*C. sorokiniana*). That study lasted 74 d and reported a release of 59% N as NH_4^+-N in WCA with a recovery factor of 109% of total N [17]. The higher NH_4^+-N release may be due to N release from the inoculum in the endogenous control which was not corrected in the cited study. Our study also demonstrated a soluble P release of 88% and 94% from WCA and LEA, respectively. A similar release of P, 89%, was observed during anaerobic digestion of the WCA of the same algae species [17].

When considering the available literature, N and P recovery was highest following anaerobic digestion of *C. sorokiniana*. Studies conducted with WCA and LEA of *Scenedesmus* [12] and *Nannochloropsis salina* [18] at variable organic loading rates released much lower fractions of nutrients from the nutrients in algal biomass. *Scenedesmus* yielded 43% and 56% N

as total soluble N after anaerobic digestion from WCA and LEA, respectively. *N. salina* yielded 22-30% N as total soluble N after anaerobic digestion from LEA. It should be stated that no correction was made in the *Nannochloropsis* studies for endogenously produced NH_4^+-N. *Scenedesmus* yielded only 25% and 33% soluble P from WCA and LEA, respectively.

In our study sonication for 10 min at 130-watt was performed to enhance release of energy and nutrients during anaerobic digestion. Sonication enhanced the release of NH_4^+-N and soluble P from WCA by 61 and 13% respectively. It also enhanced COD_{CH4} yield by 30%. In the *Scenedesmus* study [12], also a high pressure thermal hydrolysis (HPTH) pretreatment, 17^0C at 800 kPa over 30 min was used on both WCA and LEA biomass. This pretreatment enhanced recovery of soluble N by 30% and 18%, respectively. After the HPTH pretreatment, 56% and 66% of soluble N was released from WCA and LEA, respectively. The soluble N quantified in the LEA-HPTH study is similar to the NH_4^+-N released from LEA in our study. LEA-HPTH pretreatment increased soluble P release by only 6% when compared to LEA alone [12]. Bohutskyi, et al [18] tested thermal and enzymatic pretreatments on *N. salina* biomass. Enzymatic pretreatments were performed for 4 h at 37 and 50°C using accellerase enzyme complex. Thermal pretreatments were performed at 120-190°C. From all their overall pretreatment results, 30-50% of soluble N and 60-70% of soluble P was released from LEA.

The unique contribution of our study is that we measure the rate of nutrient release. In WCA this rate was 3.9 and 0.93 mg nutrient/L•d for NH_4^+-N and soluble P; respectively. A surprising finding was that the nutrient was greatly accelerated by 6 and 5-fold for NH_4^+-N and soluble P, respectively, with treatments that disrupt cells such LEA and SA.

5.4.2. Biochemical methane potential

BMP has been studied in WCA of several species. The BMP from different species of the genus *Chlorella* ranged from 0.26 to 0.36 L CH_4/g VS after 50 days of anaerobic digestion [19]. That efficiency was similar to the results of our study with a BMP of 0.30 L CH_4/g VS of WCA after 42 days of anaerobic digestion. The anaerobic biodegradability of *C. sorokiniana* is similar to the average productivity obtained in the aforementioned study. This corresponded to a COD-normalized energy recovery of our WCA of 41 % $COD_{CH4}/COD_{alg\text{-}t0}$ based on the maximum methane production at 30°C. In Table 5.4, the methane yield of different photosynthetic microorganisms are shown.

In our study, LEA yielded 38% $COD_{CH4}/COD_{alg\text{-}t0}$, with a BMP of 0.25 L CH_4/g VS. In a similar study, using *Chlorella vulgaris* UTEX-395, lipids were extracted with chloroform and methanol [20]. The BMP of the LEA in that study was 0.314 L CH_4/g VS, which indicates a higher methane yield compared to that observed in the present study, which may be due to absence of a correction for endogenous methane production in the cited study. Bohutskyi et al [18] evaluated thermal and enzymatic pretreatments to increase methane yield from *N. salina* biomass. The enzymatic treatment enhanced the methane production by 7-17% compared to LEA and was not effective on WCA. Thermal treatment enhanced the methane production by 40-60% compared to WCA.

Table 5.4. Methane yields obtained during the anaerobic digestion of biomass from different photosynthetic microorganisms in batch reactors.

Microalgae species	**Temp** (˚C)	**HRT** (d)	**Methane yield** (L CH_4/g $VS_{alg\text{-}t0}$)	**References**
Chlorella sorokiniana	30	42	0.22-0.28[a]	This study
Spirulina dunaliella	35	20	0.12-0.14	[32]
Spirulina maxima	15-52	5~40	0.25-0.34	[33]
Chlorella vulgaris	35	28	0.24	[34]
Scenedesmus obliquus	38	32	0.18	[35]

[a] CH_4 volume at STP (0^oC and 1 atm).

5.4.3. Mechanisms

A global overview of literature on BMP results (excluding outliers) including the results of our study, indicates a range of 0.18 to 0.36 L CH_4/g VS for WCA and 0.14 to 0.31 L CH_4/g VS for LEA [12, 18-20]. If intensive pretreatments were performed (thermal, pressure, sonication), an increase of methane yield was observed. BMP for pretreated algae ranges from 0.30 to 0.45 L CH_4/g VS [12, 19, 21, 22].

The results taken as a whole indicate that LEA has a similar methane yield per unit of VS to WCA, even though energy rich lipids have been removed. A possible interpretation of this finding is that even though there is a loss in energy rich lipids for biodiesel, there is nonetheless a gain in enzymatic access to hydrolyzable substrates accomplished by the disruption of cells through treatments (e.g., sonication) that extract lipids. That increase comes from cell lysis. Cell

lysis enables improved access of hydrolyzable enzymes to substrates that are otherwise compartmentalized inside cellular or organelle membrane structures. LEA cell lysis greatly enhances the release of nutrients (to a much greater extent than the increase in methane yield). In addition to enhancing cell lysis, these pretreatments potentially disrupt the complex cell wall structure, thus improving their hydrolysis.

The accelerated nutrient release witnessed in this study due to sonication may be due to rapid hydrolysis of readily hydrolyzable substrates such as proteins. Anaerobic digestion includes fermentation, dephosphorylation, deamination and methanogenesis. These reactions promote biogas formation and N and P mineralization to NH_4^+ and PO_4^{3-}. Biodegradability under anaerobic digestion is enhanced according to the intensity and/or duration of sonication and thermal pretreatments [23]. The more intensive the pretreatment, the more soluble the COD becomes and the greater the rate of methane production [22]. Intense pretreatments tend to greatly increase the methane yield per unit of VS. Ultrasonic pretreatment for example is able to produce micro-cracks, micro-voids and ruptures in cell walls [24]. Ultrasonic pretreatment is used to maximize lipid extraction from microalgae cells and to increase methane production when compared to untreated biomass [25, 26]. Furthermore, hydrolysis of biopolymers will release more COD. Previous studies have shown that high cell wall resistance to bacterial attack might be a factor in reduced microalgal methane yields [27]. In our experiments and in most studies in the literature, there is never 100% conversion of algae biomass COD, indicating that a slowly biodegradable recalcitrant fraction is most likely due to cell wall polymers.

An important limiting factor of anaerobic digestion of algae is the efficiency of the cell wall disruption by the action of extracellular enzymes. Large solubilization of non-bacterial material increases the soluble COD and NH_4^+ from hydrolysis of organic matter [28]. Thus, the hydrolysis of organic polymers from microalgae cell wall is done by the action of extracellular enzymes

produced by hydrolytic microorganisms in the anaerobic sludge during the anaerobic digestion. Methane production of enzyme pretreated algae biomass was increased 20% compared untreated WCA by using xylanase and cellulose. This observation affirms that the recalcitrant fraction of algal cells is due to cell walls [29].

5.4.4. Evaluation of energy yield

In Table 3, BMP and lower heating value (LHV) are shown. The energy value of fuel can be derived from the LHV. LHV for algal lipids are 38.3 MJ/kg, while the LHV for methane is 35.6 MJ/m^3, under standard conditions [10, 30].Thus, the methane yield for WCA, SA and LEA are equivalent to 10.6, 13.8 and 9.0 MJ/kg VS_{alg}. For LEA, 9% of lipids obtained from the extraction process could yield a total energy value of 3.5 MJ/kg VS, while the subsequent methane production could yield an energy value of 9.0 MJ/kg VS, totaling 12.5 MJ/kg VS, which is only 9.4% less than solely conducting anaerobic digestion on SA. WCA generated 3-fold the energy obtained from the lipids extracted. Thus, there is more energy value CH_4 produced under anaerobic digestion than the energy obtained only from the lipid fraction. The caloric value of *C. protothecoides* was measured with a calorimeter [31]. These algae, with 38% proteins and 11% of lipids, yielded caloric values ranging from 22 to 26 MJ/Kg VS. Thus, if we assume 26 MJ/Kg VS as a typical energy value for *Chlorella*, the 13.8 MJ/Kg VS obtained from SA is equal to 53% of the total energy when compared with the cited study, which is also equivalent to the BMP, as % $COD_{CH4}/COD_{alg\text{-}to}$ obtained in our experiment.

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CHAPTER 6

CONCLUSIONS

In the first study we observed that ZVI significantly enhanced sulfate reduction, raising the ARD pH, and removed HM and sulfate. Cu, Cd and Pb were > 99.7% removed. Cu removal was mainly due to sulfide precipitation. The first evidence found, using SEM-EDS analysis, is the (1:1.04) molar ratio of Cu:S, the second evidence observed is the 60–85-fold higher SRB count in the ZVI-amended columns and the third evidence is that CuS was mainly extracted by concentrated HNO_3-HCl mixture. All these findings convincingly show that iron served as e-donor and that SRB activity was responsible for the effective removal of copper from the ZVI containing columns.

Furthermore, the excellent Cu removal effectiveness obtained in the LS column was likely mediated by precipitation with hydroxide and carbonate ions released from limestone ($CaCO_3$) dissolution. The increase in the pH of the effluent from the LS column was primarily due to the release of bicarbonate alkalinity from the limestone packing, which is beneficial for acid neutralization and pH buffering. The disadvantage is that LS-rock surfaces are saturated after a short time. The LS column was saturated after 400 d of continuous operation, while both zero valent amended columns still had significant capacity to sequester copper. We conclude that zero valent iron is a slow release electron donor able to promote heavy metal removal from acid rock drainage, using sulfide precipitation as the main mechanism.

The second study provides direct evidence for the first time that WCA and LEA of *Chlorella sorokiniana*-1412 can serve as e-donors to drive sulfate reduction. Active sulfate reduction was linked to highly efficient Cu removal and an increase in the pH of the treated ARD. The biogenic sulfide formed was used to precipitate Cu^{2+} in the form of CuS, as evidenced by the

results of XRD and sequential extractions using a strong oxidative acid (HNO_3-HCl, 3:1). Cu removal percentages were higher than 99.5 and 99.7% for the WCA- and LEA columns, respectively. Sulfate reduction was active in the algae-amended columns even as the experiment ended on day 123. By comparison, the endogenous reactor exhibited low sulfate-reducing activity during the first 40 days of the experiment and then activity ceased. The main challenge in using algae as a reactive material in PRBs, however, is how to reduce the washout of the suspended algae. We suspect the washout was exacerbated by biogas production in the initial period. Eventually SRB outcompeted methanogens under strong sulfate reduction and low pH conditions in ARD plumes. Based on all the findings, we conclude that algae can sustain PRBs for years, producing benefits to the environment by removing HM and acidity from ARD. Additionally, LEA utilization could also add profitability to the biodiesel industry.

The results in the third study demonstrate that anaerobic digestion of pretreated *Chlorella protothecoides* biomass by disrupting algal cells is a promising approach to generate energy-rich methane and recover nitrogen nutrients. The effectiveness of sonication pretreatment under optimized condition (130 Watt and 20 KHz for 5 min) to enhance the generation of methane and the release of ammonia N during anaerobic digestion of algal biomass was compared with that of thermal and mild alkaline treatments. All the pretreatments investigated disrupted the algal cells and increased the aqueous solubility of the cell organic matter and enhanced the mineralization of organic nitrogen to some extent, but sonication was the most effective pretreatment providing a 4.1-fold increase in the release of nitrogen from the algal biomass at the end of the incubation (to 69.9% of the total N).

Sonication was also the most effective method and led to solubilization of 33.7% of the algal COD, followed by thermal (15.8%) and alkaline treatment (10.6%). A combined alkaline-sonication pretreatment did not provide any advantage compared to the alkaline treatment only.

On the contrary, sonication under mild alkaline conditions led to a 34.7% reduction in the soluble COD compared to sonication alone.

The positive correlation between the soluble COD values and the methane production were determined in this study. Sonication provided the highest improvements in methane generation potential with methane yield of 327 mL_{STP} CH_4 g^{-1} VS (2.24-fold compared to the untreated algae). In contrast, thermal pretreatment only provided a moderate increase in the methane yield and alkaline treatment lead to decrease of the methane yield compared to the untreated algal biomass.

In the four study, we demonstrate that anaerobic digestion of residual algal biomass (*Chlorella sorokiniana*-1412) from biodiesel production can provide additional nutrients and energy. Under the pretreatment conditions used, 130-watt by 10 min., sonication enhanced the release of NH_4^+-N and soluble P from WCA by 61 and 13%, respectively. Organic N in WCA and LEA was mineralized by 48% and 62% to NH_4^+-N, respectively. The unique contribution of our study is that we measure the rate of nutrient release. In WCA this rate was 3.9 and 0.93 mg nutrient/L•d for NH_4^+-N and soluble P; respectively. A surprising finding was that the nutrient was greatly accelerated by 6 and 5-fold for NH_4^+-N and soluble P, respectively, with treatments that disrupt cells such LEA and SA.

AD of WCA yielded 41 % $COD_{CH4}/COD_{alg\text{-}t0}$, while LEA yielded 38% $COD_{CH4}/COD_{alg\text{-}t0}$. The results taken as a whole indicate that LEA has a similar methane yield per unit of VS to WCA, even though energy rich lipids have been removed. This similarity could be attributed to the gain in enzymatic access to hydrolyzable substrates accomplished by the disruption of cells through treatments. In the theoretically energy balance, based on lipids and methane yield, WCA generated 3-fold the energy obtained from the lipids extracted.

ANNEXES

Table A1. Solubility products (Ksp) at 25 ◦C of sulfides, carbonates and hydroxides of Cu^{2+}, Cd^{2+}, Pb^{2+} and Fe^{2+} [1].

Metal ion	Metal sulfide	Metal hydroxide	Metal carbonate
Cu^{2+}	6.3×10^{-36}	2.5×10^{-19}	3.1×10^{-12}
Pb^{2+}	1.0×10^{-27}	7.9×10^{-17}	7.9×10^{-14}
Cd^{2+}	7.9×10^{-27}	2.5×10^{-14}	1.0×10^{-12}
Fe^{2+}	6.3×10^{-18}	7.9×10^{-15}	3.5×10^{-11}

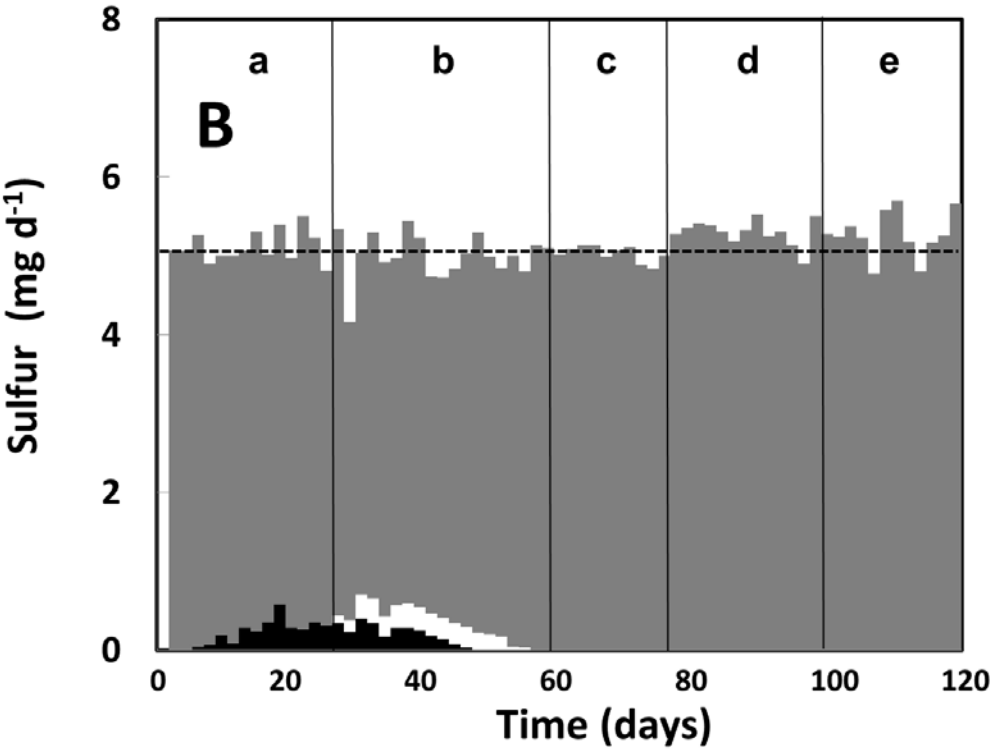
Table A2: 5 Case studies of typical acid mine drainage (AMD) through the influence of mining activity (contaminant concentrations in mg L^{-1}).

Parameter	Case 1	Case 2	Case 3	Case 4	Case 5
pH	1.9	1.8	2.47	2.5	3.2
SO_4^{2-}	3250	7540	3086	5760	2969
Fe	248	1440	315	324	631
Al	124	438	0.15	277	95.5
Cu	NR[a]	5.32	9.0	1060	0.08
Zn	NR[a]	1.39	20.1	2.86	22.3
Cd	NR[a]	0.282	0.11	0.007	0.11
AMDI[b]	5-28	12	23	18	23
Year	1995	1982	2012	2011	2006
Location	South Africa	CA & NV	SW Spain	Kerman, Iran	Sothern Brazil
Reference»	[2]	[3]	[4]	[5]	[6]

[a] NR : None Reported

[b] AMDI (Acid Mine Drainage Index) from 0 to 100; index based on pH, SO_4^{2-}, Fe, Zn, Cu, Cd and Al content [7]. The lower the AMDI, the higher the risk.

Figure A1: Sulfur balance in Endogenous column: sulfide aqueous + stripped (■), sulfur in CuS (□), sulfate effluent (■).The dash shows the sulfate concentration in the influent (5.1 mg SO_4^{2-}-S d^{-1}).



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