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Algae as an electron donor promoting sulfate reduction for the bioremediation of acid rock drainage

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HIGHLIGHTS

- Algal biomass can serve as an electron donor to drive reduction of sulfate to sulfide.
- Biogenic sulfide precipitates Cu^{2+} as stable sulfide mineral.
- Cu^{+2} removal in sulfidogenic bioreactors amended with algal biomass exceeded 99.5%.
- Acidity in synthetic acid rock drainage was consumed by sulfate reduction.

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ABSTRACT

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.

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 S1 of the Supplementary Data (SD). Acidity extracts and dissolves HM such as Cd, Pb and Cu. HM can reach surface waters and may accumulate to toxic levels causing severe impacts 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 5.7–6.5; 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 use the activity of sulfate-

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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 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. A previous study has shown effective removal of Zn, Cd, Co and Ni from contaminated groundwater using sulfate reduction [9].

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 [10–12] (Eq. (1)). SRB reduce sulfate to sulfide, while organic matter is oxidized to CO_2 and acidity is simultaneously consumed [13].

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

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).

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

The solubility constants (Ksp) of hydroxide, carbonate and sulfide metals is in Table S2 of the SD. The Ksp values indicate the lower solubility of MeS compared to metal hydroxides and carbonates [14]. Biogenic sulfide (H_2S) generated during sulfate reduction is an excellent ligand of HM and is extensively used for remediation of HMs in acidic effluents.

E-donating compounds enable sulfate 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 [13,15–17]. The lower the lignin content of these materials, the higher their biodegradability and capacity for developing bacterial activity [18]. 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 [19–21].

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 generating biomass for biodiesel production [22]. With current technology there is an excellent economic outlook for algae-based biodiesel production [23]. 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) [24]. *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 [25–27] 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 [28,29]. 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.

Table 1
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.

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.

2.1. Algal growth conditions

Chlorella sorokiniana-1412 was cultivated on a BG-11 medium in a photo-bioreactor [30]. The chemical composition of WCA and LEA is illustrated in Table 1.

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.

2.3. Chemicals

The main chemicals used were $CuSO_4{\cdot}5H_2O$, 98 +%, CAS 7758-998 (Sigma-Aldrich, St. Louis, MO) to add as heavy metal to the synthetic ARD medium. 18 M H_2SO_4, 98% v/v; 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.

2.4. Chemical oxygen demand content

The chemical oxygen demand (COD) content of *Chlorella sorokiniana*-1412 was quantified using standard methods [31], with H_2SO_4-$K_2Cr_2O_7$ at 150 °C 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 1 shows the algae composition.

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,

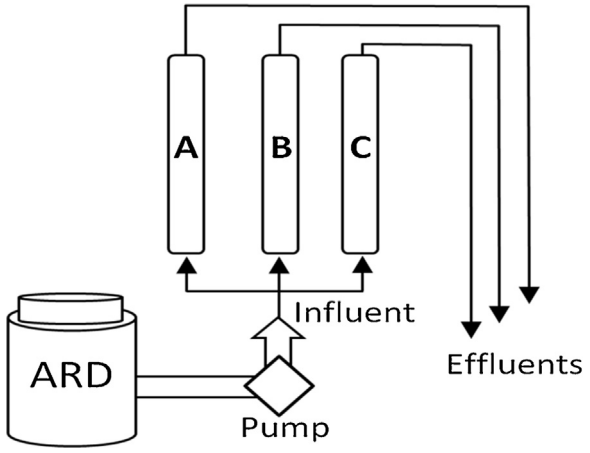

Fig. 1. A descriptive diagram of the three up-flow packed bed columns used to test the algae biomass as electron donor for the SRB process. A) Endogenous column, B) WCA column, and C) LEA column.

Table 2
Composition of the packed-bed columns.

Component		PRB Columns[a]		
	DBD[b] g/cm^3	Endogenous g dwt/column	WCA[c]	LEA[d]
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)[a]	0.42	0	4.9	0
Lipid Extracted Algae (LEA)[d]	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^{-1} 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).

v/v) and microwaved at 80 °C [32]. 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.

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 1 mL per L of trace element solution [33]. 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.

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. 1 illustrates three glass columns (0.07 L). 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* biomass and the LEA column was filled with *Chlorella* biomass after lipid extraction. The mass composition of the packing and the algae COD content are described in Table 2.

All columns were operated in continuous parallel mode at 24 ± 1 °C with the same source of influent (synthetic ARD). The influent was kept at 24 ± 1 °C and 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). 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, Cu^{2+} was not added during the first period (a). In 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
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

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 [34]. 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 [35]. Cu was determined by inductively coupled plasma-optical emission spectrometry (2100 Optima ICP-OES, Perkin Elmer, Waltham, MA). The wavelength used for Cu 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, 1 M 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). An effluent sample (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).

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

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 °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 [36].

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 the reactor, which was calculated as follows:

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

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 during periods when Cu was added.

3. Results

3.1. Sulfur

Both WCA and LEA contributed to sulfide formation in the effluent of the laboratory PRBs (Fig. 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, 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 day 5–40. Thereafter, there was no longer detectable sulfide in the effluent of the endogenous column.

Further evidence of sulfate reduction is supported by the removal of sulfate in the algae-amended columns (Fig. 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 (Fig. 2B) and thus Cu inhibition was not witnessed. Sulfide levels dropped in periods (c) and (d) when Cu concentrations increased (Fig. 2A), but the drop was due to copper sulfide precipitates (Fig. 3) and not to toxicity.

In the beginning of period (e), a noteworthy difference in behavior was observed between the WCA and the LEA columns. Both sulfate removal and sulfide production increased at a 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 Fig. 3A and B. 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 (Fig. S3 of SD), what one would expect if no reaction had taken place.

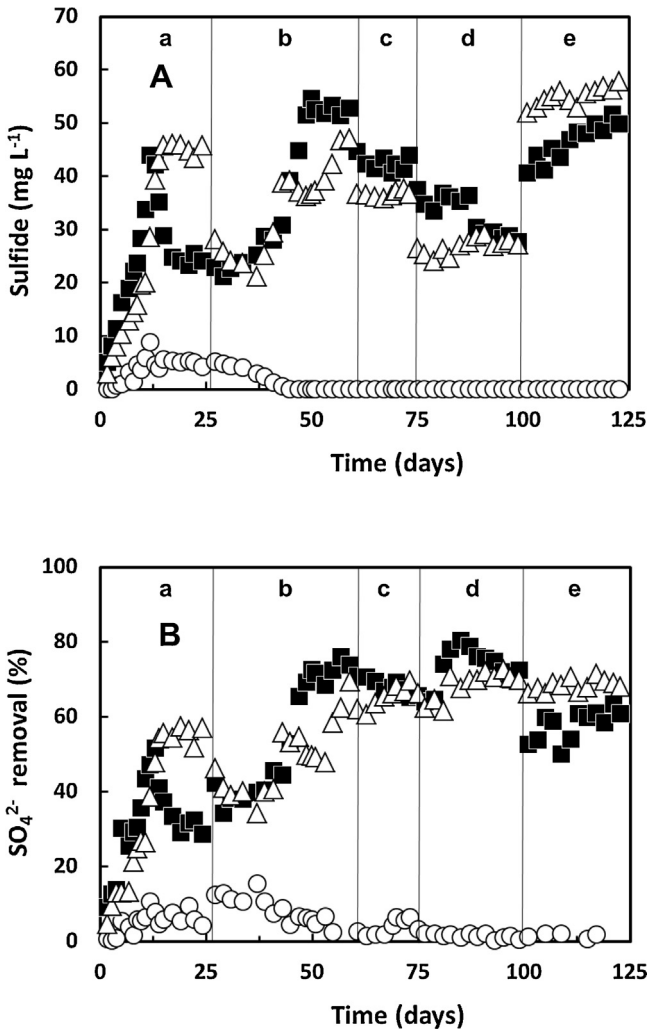

Fig. 2. Panel A: Total sulfide in the effluent (sulfide measured + stripped): Endogenous column (○), WCA column (■), and LEA column (△). The concentration of Cu^{2+} in the influent during periods (a–e) was 0, 10, 30, 50 and 0 mg L^{-1}, respectively. The maximum H_2S concentration stripped (at pH 6.5) 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 (%).

3.2. pH

Fig. 4 shows the pH evolution in 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.

3.3. Copper

The influent and effluent Cu concentrations are shown in Fig. 5. Fig. 5A is zoomed out to show the influent and effluent concen-

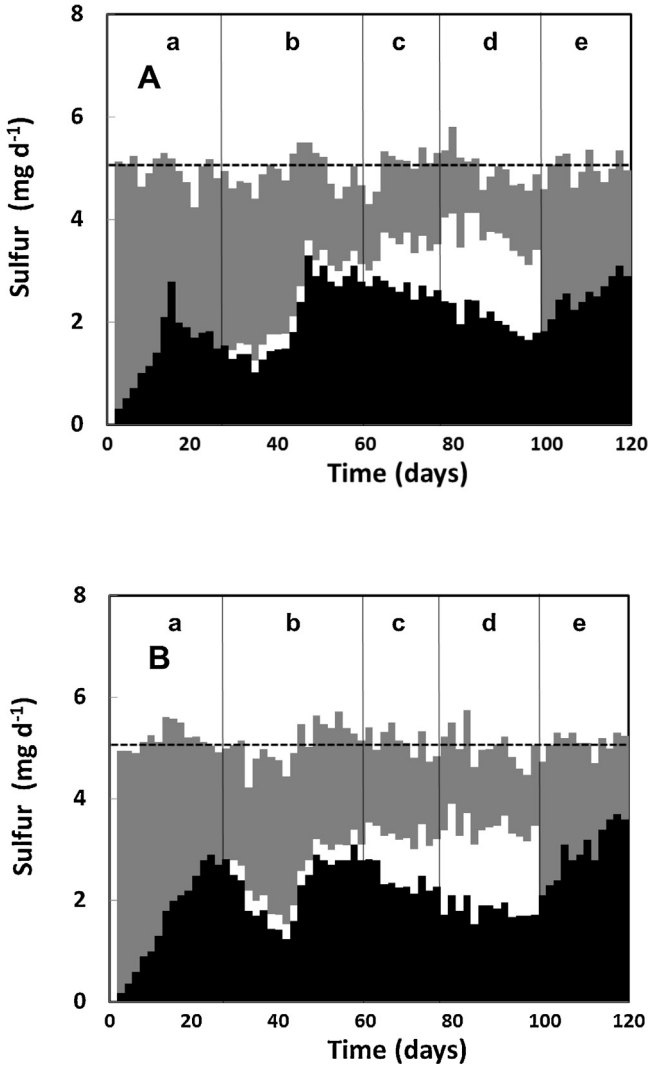

Fig. 3. Sulfur balance in WCA (panel A) and LEA (panel B) columns: sulfide aqueous + stripped (■), sulfur in CuS (□), and sulfate effluent (▩). The dash shows the sulfate concentration in the influent (5.1 mg SO_4^{2-}-S d^{-1}).

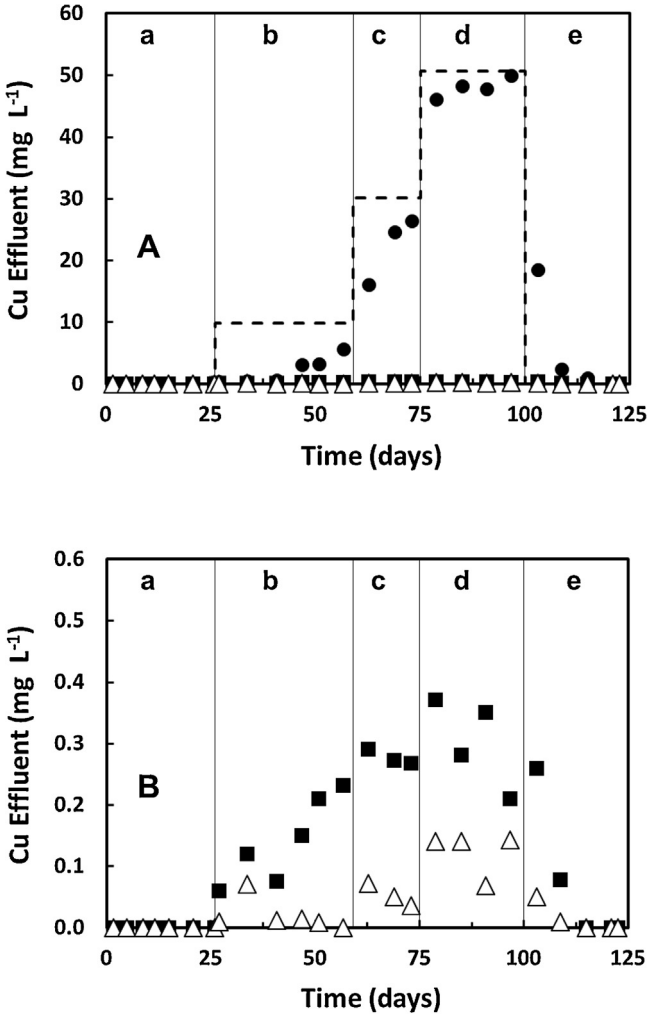

Fig. 5. Effluent and influent copper concentration: Panel A: View of data in the range from 0 to 60 mg L^{-1}. Panel B: Close up view of effluent data in the range of 0–0.6 mg L^{-1}. Endogenous column (○), WCA column (■) and LEA column (△). The concentration of Cu^{2+} in the influent during periods (a, b, c, d and e) was 0, 10, 30, 50 and 0 mg L^{-1}, respectively. The dash line represents the influent Cu^{2+} concentration in each of the periods.

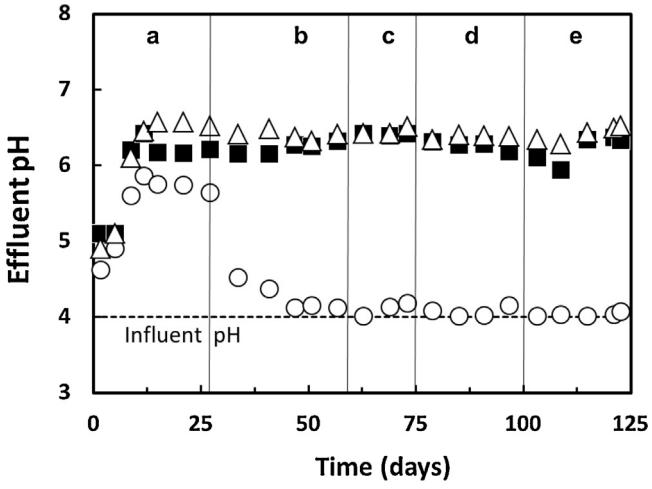

Fig. 4. Effluent pH: Endogenous column (○), WCA column (■), and LEA column (△). The horizontal line represents the influent pH.

trations ranging from 0 to 50 mg L^{-1}. Fig. 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. Cu was retained in the reactor throughout the experiment as shown in Fig. 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 Fig. 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 (Fig. 6). No such evidence was found in the endogenous column.

The cumulative Cu balance is presented in Table 4. Firstly, the table shows that there was approximately 4× more Cu retention

Table 4
Sequential extraction of total copper from the homogenized column packing at the end of the experiment by means of: water, 1 M 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.

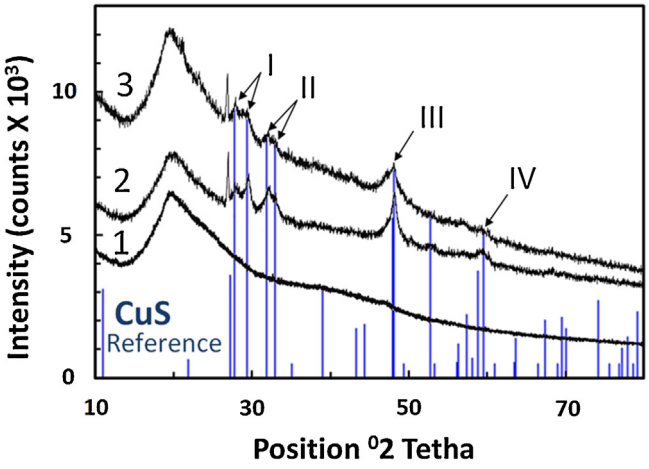

Fig. 6. XRD spectrum of the column packing at the end of the column experiment. Endogenous column (1), WCA column (2) and LEA column (3). Copper sulfide (covellite) used as the reference.

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 1 M 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 to 98% of that retained.

3.4. Chemical oxygen demand

A cumulative COD balance is provided for the columns containing algae, WCA and LEA, in Fig. 7. During the initial 26 d of operation (period (a)), biogas production provoked washout of the biomass, accounting for the loss of 40.7 and 26.7% of the algae COD, respectively. The COD metabolized to produce sulfide during the study corresponded to only 9.5 and 9.2% of the added algae-COD, respectively. Only 3.5% and 1.3% of the WCA- and LEA-COD was converted into methane-COD.

4. Discussion

4.1. Main findings

Both WCA and LEA algae biomass served as e-donating substrates for sulfate reduction. The biogenic sulfide formed 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.

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* [28]. Additionally, extracellular polysaccharides produced by a mixed culture of algae in a high-rate pond were considered to be responsible for sulfate removal [29]. Studies of a wastewater ponding system containing HM [37] 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 [38]. The cell wall of *Spirulina,* similar to that of other cyanobacteria, has a high glucan content [39]. 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 [40,41].

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 Cu L^{-1}) at pH 2.0 [42]. 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% [43].

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 [44]. The COD conversion of algal biomass from *Chlorella sorokiniana* and *Chlorella vulgaris* to methane by anaerobic digestion ranged from 40–73% [45]. In a different study, around 50% of the biomass of *C. vulgaris* was converted to methane [46]. 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 [47], 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. The solvent extraction step applied to remove the lipid extraction

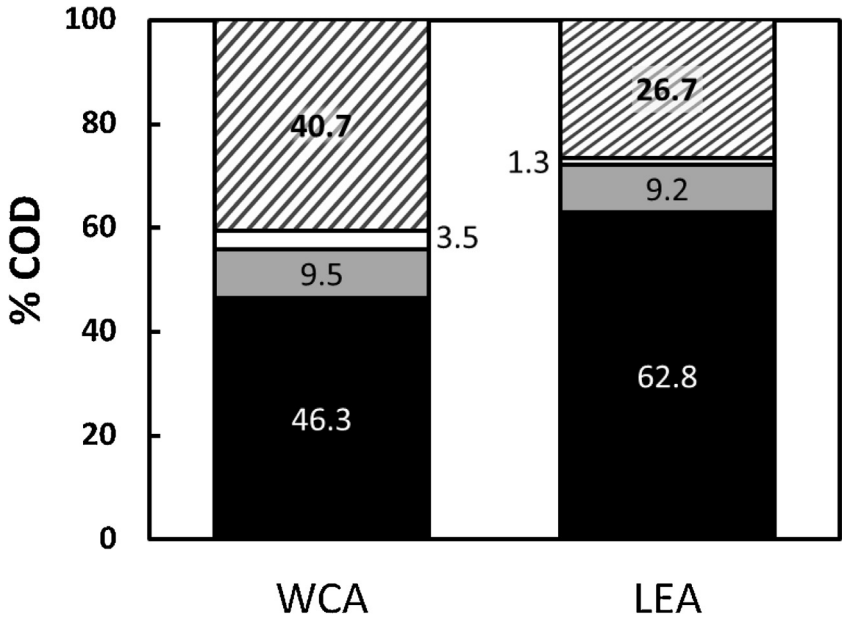

Fig. 7. Algae-COD balance in WCA and LEA columns: Remaining algae (■), S^{2-}-COD (□), CH_4-COD (▒), and washed out algae (▨).

is by itself a pretreatment that can increase methane production from LEA [48]. Thermal hydrolysis of *Nannochloropsis* biomass at 170 °C enhanced the methane yield by 40% for WCA and 15% for LEA [49]. 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^{-1} and thermal hydrolysis at 80 °C, respectively [50]. The increase was attributed to cell wall disruption and COD solubilization.

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 [51]. Greater IC_{50} values for SRB and methanogens have been reported for Cu^{2+}, 1136 and 130 mg L^{-1}, in a different study [52]. 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 [53]. 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 [54]. 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 [55]. 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.

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.

5. Conclusions

- WCA and LEA biomass were both shown to be effective e-donors to drive sulfate reduction of ARD, enabling the precipitation and removal of Cu^{2+} in laboratory-scale PRBs.
- Efficient sulfate reduction was maintained during the experimental period of 4 months, using a low pH, synthetic ARD influent. Sufficient algae-COD remained to sustain the operation for another 20 months.
- The Cu^{+2} removal efficiency was greater than 99.5% in the WCA- and LEA-amended columns.
- Due to metal sulfide precipitation, the SRB community was not inhibited at 50 mg Cu L^{-1} in the influent.
- The precipitate retained in the columns was composed mostly of insoluble copper sulfide formed from the biogenic sulfide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.06.011.

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