



Treatment of acidic sulfate-containing wastewater using revolving algae biofilm reactors: Sulfur removal performance and microbial community characterization



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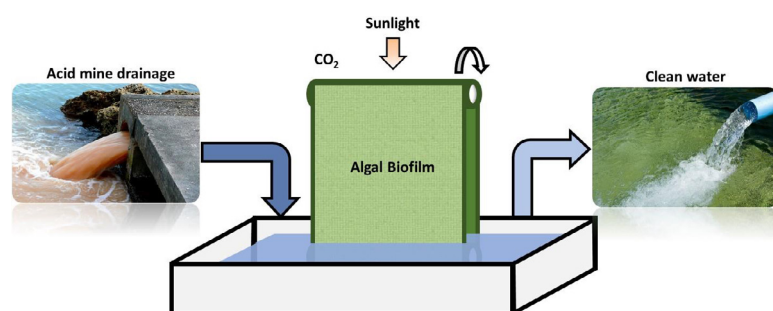
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GRAPHICAL ABSTRACT



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ABSTRACT

Industries such as mining operations are facing challenges of treating sulfur-containing wastewater such as acid mine drainage (AMD) generated in their plant. The aim of this work is to evaluate the use of a revolving algal biofilm (RAB) reactor to treat AMD with low pH (3.5–4) and high sulfate content (1–4 g/L). The RAB reactors resulted in sulfate removal efficiency up to 46% and removal rate up to 0.56 g/L-day, much higher than those obtained in suspension algal culture. The high-throughput sequencing revealed that the RAB reactor contained diverse cyanobacteria, green algae, diatoms, and acid reducing bacteria that contribute the sulfate removal through various mechanisms. The RAB reactors also showed a superior performance of COD, ammonia and phosphorus removal. Collectively, the study demonstrated that RAB-based process is an effective method to remove sulfate in wastewater with small footprint and can be potentially installed in municipal or industrial wastewater treatment facilities.

1. Introduction

Sulfur is a contaminant commonly found in municipal and industrial effluents generated from various operations such as medication,

tanning, mining, petrochemical, fermentation and food processing (Liu et al., 2012). The high sulfur-containing wastewater leads to severe environmental issues such as impoverishing aquatic flora and fauna, emissions of sulfur gases, subsidence and corrosion of foundations

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(Jarvis and Younger, 2000). Sulfate as the sulfur oxidation product is the most common sulfur compound in wastewater and is usually harmless to the environment. Under anaerobic environment, however, sulfate can be converted into sulfide by sulfate reducing bacteria (SRB). Compared to sulfate, sulfide is more toxic, corrosive and odorous, and more harmful to human health. The emission of sulfide-containing off-gases (e.g. H_2S) can lead to sulfur enrichment in a waterbody causing ecological and health hazards (Li et al., 2015). Because of the significant physiological and toxicological impacts on the environment, it is important to develop effective processes to remove sulfur contaminants from wastewater.

Sulfur in wastewater can be removed through physical, chemical and biological methods. The physical methods such as electro dialysis, ion exchange and membrane filtration require high energy input. Chemical methods, such as metal precipitation, need to use excessive chemicals and replace poisoned catalysts, and thus, cause liquid contamination and reactor corrosion (Lens et al., 1998). In the bacteria-based biological sulfur removal process, sulfate is reduced into sulfide and oxidized to elemental sulfur (Xu et al., 2014). This process can emit H_2S to the atmosphere as a result of sulfate reduction. It also requires strict anaerobic conditions which can be difficult to maintain. Considering these challenges, it is essential to develop a low-cost, simple and eco-friendly methods for sulfur removal.

In recent years, microalgae-based wastewater treatment is gaining increased attention due to its environmental friendliness and potential economic benefit compared to conventional wastewater treatment processes (Gross et al., 2015). Microalgae are capable of removing various pollutants such as oxygen consumption pollutants, nitrogen, and phosphorous and metals from wastewater, the biomass produced during the treatment process can be used as feedstock for fuels, feeds, and chemicals (Kesaano and Sims, 2014). When sulfur was the targeted pollutant, an algae-based sulfur removal process is also possible because algae need to absorb sulfur in the synthesis of amino acids cysteine and methionine (Mera et al., 2016).

Compared to the other nutrients such as nitrogen and phosphorous, however, sulfur removal from wastewater has been less studied. Among limited reports of algae-based sulfur removal, researchers have studied the municipal wastewater in which the sulfur concentrations were relatively low (~ 300 mg/L) and pH was neutral (Lv et al., 2017, Mera et al., 2016). Contrary to the municipal wastewater, effluents from industries such as medication, tanning, mining, and petrochemical operations contain a high sulfur level (> 2 g/L) (Galiana-Aleixandre et al., 2005). Among those industrial effluents, acid mine drainage (AMD) is a particular concern. In addition to high sulfur content, AMD also contains a diverse of metals (Orandi and Lewis, 2013) with an acidic pH ranging from 3.6–4.7 to 1.5 (Abinandan et al., 2018). Researches on AMD treatment have been mainly focusing on metal removal. For example, Orandi et al. (2012) demonstrated that an algal-microbial consortium in a rotating biological contactor was capable of removing heavy metals from AMD. Abinandan et al. (2018) reviewed the effect of microalgae and bacteria interaction on the metal removal and concluded that the algae-bacteria consortium can remediate AMD. Das et al. (2009) reviewed the role of algae and fungi in the metal removal during the AMD treatment and its effects on sulfate reduction bacteria. In another study of sulfur-removal from AMD by algae-bacteria system, Sheoran and Bhandari (2005) reported that the main role of algae is to adsorb metals and nitrogen, leading to a rise of alkalinity and serving as the carbon source for sulfur reducing bacteria, which is the ultimate sulfur remover.

Our research laboratory has recently developed a revolving algal biofilm (RAB) reactor as an effective way growing microalgae (Gross et al., 2015; Gross and Wen, 2018). The RAB reactor relies on a vertically oriented materials for attached algal growth. The material travels through the water absorbing nutrients, then rotates out of the water to facilitate light exposure and CO_2/O_2 exchange. Compared to the conventional suspended growth systems, the RAB reactor allows for greater

surface area exposure to sunlight in a much smaller footprint. The biomass productivity in the RAB reactor was 5–10 times higher than that of the open pond. Also the biomass can be harvested through scraping from the attachment material, which greatly reduced the cost compared to the centrifugation harvested processes (Gross et al., 2016). Recently, the RAB system has been successfully implemented in Metropolitan Water Reclamation District (MWRD) of Greater Chicago to remove nitrogen, phosphorus and metals from sludge thickening supernatant in MWRD facility (Kunetz et al., 2016, Zhao et al., 2018).

With prospective the success implementation of RAB reactor in municipal wastewater treatment, the aim of this study is to explore the utility of the RAB-based culture system for sulfur removal from AMD. Different from previous research on AMD treatment where metal removal was the focus (Orandi et al., 2012), this work focuses on a thorough evaluation of sulfur removal performance. In addition, a holistic view of the sulfur removal mechanisms was studied through identification and quantification of microbial consortium based on a high-throughput gene sequencing method.

2. Materials and methods

2.1. Microalgae culture

The microalgal seed culture was taken from a raceway pond (1000 L working volume) at the Algal Production Facility at Iowa State University in Boone, IA, USA. The pond was initially inoculated with *Chlorella vulgaris* (UTEX #265) and has been operated for four years. The pond culture has been maintained using Bold's Basal Medium (BBM) with half of the pond liquid being exchanged with fresh medium every 7 days. Over the years, a stable algal community containing various green algae and cyanobacteria species has been established. The abundance of the mixed algal culture, particularly the original strain *C. vulgaris*, was determined based on illumina high-throughput sequencing as described in Section 2.6. This algal polyculture was used as inoculum for the bubble column and RAB reactors.

2.2. Synthesis wastewater composition

Synthetic wastewater mimicking acid mine drainage commonly found in the mining industry was used in this work. The basic recipe of the synthetic wastewater composed of (per L) 200 mg NH_4Cl , 50 mg KH_2PO_4 , 66 mg $MgSO_4 \cdot 7H_2O$, 6 mg $CaCl_2$, 0.55 mg $FeSO_4 \cdot 7H_2O$, 2.86 mg H_3BO_3 , 1.84 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.39 mg $Na_2MoO_4 \cdot 2H_2O$, 0.08 mg $CuSO_4 \cdot 5H_2O$, 0.05 mg $Co(NO_3)_2 \cdot 6H_2O$ and 375 mg glucose. This receipt was adapted from the synthetic municipal wastewater reported previously (Lv et al., 2017). The addition of glucose was used to provide COD of the acid mining drainage. To mimic the high sulfur concentration in the acid mine drainage, sodium sulfate (Na_2SO_4) was added to the receipt at a concentration of 1 g/L, 2 g/L and 4 g/L sulfate, respectively. The pH of the wastewater was 3.5–4.0 adjusted by hydrochloric acid.

2.3. Bubble column cultures

Sulfur removal by microalgae was evaluated in suspension-based bubble column reactors in a batch culture mode. The bubble columns contained 1-L synthetic wastewater with different sulfate concentrations. To inoculate the bubble columns, the microalgae seed culture (with an inoculum ratio of 1:10, v/v) was first settled for 1–2 h, the settled slurry was then washed with DI water before being inoculated into the reactors. The bubble columns were placed at room temperature ($25^\circ C$) and aerated at a flow rate of 0.5 L/min throughout the culture. Fluorescent lights were used to provide 24-hr lighting at an intensity of $130 \mu mol cm^{-2} s^{-1}$. During the culture, cell density was determined based on optical density at 680 nm (OD_{680}).

2.4. RAB cultures

Lab-scale RAB reactors were used to treat sulfate-containing wastewater in a continuous operation mode (HRT = 3-day) under 24-hr lighting ($130 \mu\text{mol cm}^{-2} \text{s}^{-1}$). The details of the RAB reactor design and operation have been reported previously (Gross et al., 2015). In brief, the RAB reactor contained a liquid reservoir (1.5 L working volume) and a rotating belt with a surface area of 0.13 m^2 for algal attached growth. The belt rotated at 1.2 rpm with a linear velocity of 4 cm/s. The RAB reactors were started by inoculating the algal seed culture into the liquid reservoir and rotating the RAB belt. The RAB was run for three weeks during which suspended algae gradually attached to the belt surface to establish a stable algae biofilm. During this stage, the reservoir was supplemented with BBM as necessary to compensate for water evaporative loss.

After the three-week incubation period, the RAB reactor was operated for one additional week in a continuous mode by feeding 500-mL BBM and discharging spend medium daily. Then, the RAB reactors were switched to being fed with sulfate-containing wastewater. On a daily basis, the liquid reservoir was fed with 500 mL synthetic wastewater containing 1, 2 and 4 g/L sulfate, respectively; with the same amount liquid being discharged. This daily feeding and discharging operation were maintained for 21 days, during which the RAB reactors were regarded as a continuous operation with a hydraulic retention time (HRT) of 3-days. To keep the biomass healthy, the biofilm in the RAB belt was harvested every 7 days through scraping with a plastic blade. The harvested biomass is a paste-like material with a moisture content of 85–95% (w/w). After harvesting, the residual colonies remaining on the belts served as inoculum for the next cycle of growth. The RAB reactors were evaluated by its removal of various nutrient parameters (sulfate, phosphorus, ammonia and COD) as follows,

Nutrient removal rate (R , mg/L/day) representing mass of the nutrients removed per unit of reactor volume per day, i.e.,

$$R = F(C_{in} - C_{out})/V = (C_{in} - C_{out})/HRT \quad (1)$$

where F is the volumetric flow rate (500 mL/day), V is the RAB liquid reservoir working volume (1.5 L), C_{in} and C_{out} are the nutrient concentrations in the RAB reservoir influent and effluent, respectively, HRT is the hydraulic retention time of the liquid reservoir.

Nutrient removal efficiency (E , %) representing percentage of nutrients in the influent being removed, i.e.,

$$E = ((C_{in} - C_{out})/C_{in}) \times 100\% \quad (2)$$

Nutrient removal capacity based on belt surface (C_s , mg/m² belt surface/day) representing mass of nutrients removed per unit of belt surface per day, i.e.,

$$C_s = F(C_{in} - C_{out})/S \quad (3)$$

where S is the belt surface of the RAB reactor (0.13 m^2).

2.5. Chemical analyses

Sulfate concentration was measured based on Ion Chromatograph fusing APHA method 4500 (Arnold and Lenore, 1992). To measure total phosphorus (TP), water samples were digested with sulfuric acid and measured using the modified ascorbic acid method (Murphy and Riley, 1986). COD concentration was analyzed based on APHA method 5220D (Arnold and Lenore, 1992). Ammonium concentration was analyzed using the salicylate method, Hach Method 10023. To determine sulfur content of biomass of the RAB reactors, the samples were digested with nitric acid using Multiwave Go microwave (Anton Paar, Austria), and measured using the ICP-MS (Thermo Scientific, iCAP 7000 Series, USA) (Heilmann et al., 2004).

2.6. Microbial diversity determination

2.6.1. DNA extraction, PCR and high-throughput sequencing

Illumina high-throughput sequencing was used to characterize microbial community in the RAB reactors. The total genomic DNA of the freeze-dried biomass samples was extracted based on the protocols described previously (Sambrook and Russel, 2001). The concentration and purity of the extracted DNA was determined based on electrophoresis using 1% agarose gels. The V4 and V5 regions of 16S rRNA gene for bacterial diversity were amplified with the primer 515F (5'-GTGCCAGCMGCCGCGTAA-3') and 907R (5'-CCGTCGAATTCCTTTGAGTTT-3'), respectively. The V4 region of 18S rRNA gene for Eukaryota was amplified with primer 528F (5'-GCGGTAATTCAGCTCCAA-3') and 706R (5'-AATCCRAGAATTCACCTCT-3'). All the tags were barcoded at both ends to distinguish samples. PCR reactions were conducted in triplicate 30 μL mixture composed of 15 μL 2 \times Phusion Master Mix, 3 μL of each primer (6 μM), 10 μL template DNA (1 ng/ μL) and 2 μL H₂O. The PCR products were then purified with GeneJET™ Gel Extraction kit (Thermo Scientific, USA) and visualized on 1% agarose gel. Sequencing libraries were sequenced on an Ion S5TM XL platform and single-end reads of 450 bp for 16S rRNA and 250 bp for 18S rRNA, and constructed using Ion Plus Fragment Library kit 48 rxns (Thermo Scientific, USA) and quantified using the Qubit@ 2.0 Fluorometer (Thermo Scientific, USA).

2.6.2. Analysis of microbial community diversity and richness

Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence using Cutadapt software (Martin, 2011). The chimera sequences were detected based on the GOLD database (Reddy et al., 2014) using UCHIME algorithm (Edgar et al., 2011) and removed to obtain the Effective Tags for subsequent analysis (Haas et al., 2011). Sequences analysis were performed by UPARSE software (Uparse v7.0.1001) (Edgar, 2013) and the same Operational Taxonomic Units (OTUs) were defined at a sequence similarity level of 97%. For each OTU, the RDP classifier (Version 2.2) algorithm was used against the Silva Database (Quast et al., 2012) to annotate taxonomic information. OTUs abundance were normalized based on a standard sequence number relative to the sample with the least sequences. The output normalized data was used to perform Alpha diversity analysis. The ACE and Chao indices were used to characterize the community richness. Shannon index was used to determine the community diversity. All this indices were calculated with QIIME (Version 1.7.0) (Caporaso et al., 2010) and displayed with R software (Version 2.15.3).

3. Results and discussion

3.1. Sulfate removal in bubble column reactors

The sulfur removal performance by the batch culture of microalgae in the bubble column reactors is illustrated in Fig. 1. Here, the wastewater contained 1 g/L, 2 g/L and 4 g/L sulfate, respectively. The algae culture maintained in BBM (containing 36 mg/L sulfate) was used as control. As shown in Fig. 1A, the cell growth decreased when additional sulfate was added, indicating a sulfur inhibition to algal growth. Fig. 1B shows that the sulfate concentration reduced initially and then levelled off through the rest of culture, indicating the initial consumption of the sulfate is sufficient for the cell growth of the entire culture period. The sulfate removal efficiency was 20–25% among the three cultures (Fig. 1C), while the sulfate removal rate increased from 0.04 to 0.15 g/L-day with the initial sulfate concentration from 1 to 4 g/L (Fig. 1D). Collectively, results in bubble column batch culture experiment demonstrated an inhibitory effect on cell growth by high sulfate concentration. In addition, the elevated ionic strength and acidic pH (3.5–4.0) caused by high sulfate concentration may also contribute to the growth inhibition because algal growth commonly prefers neutral or

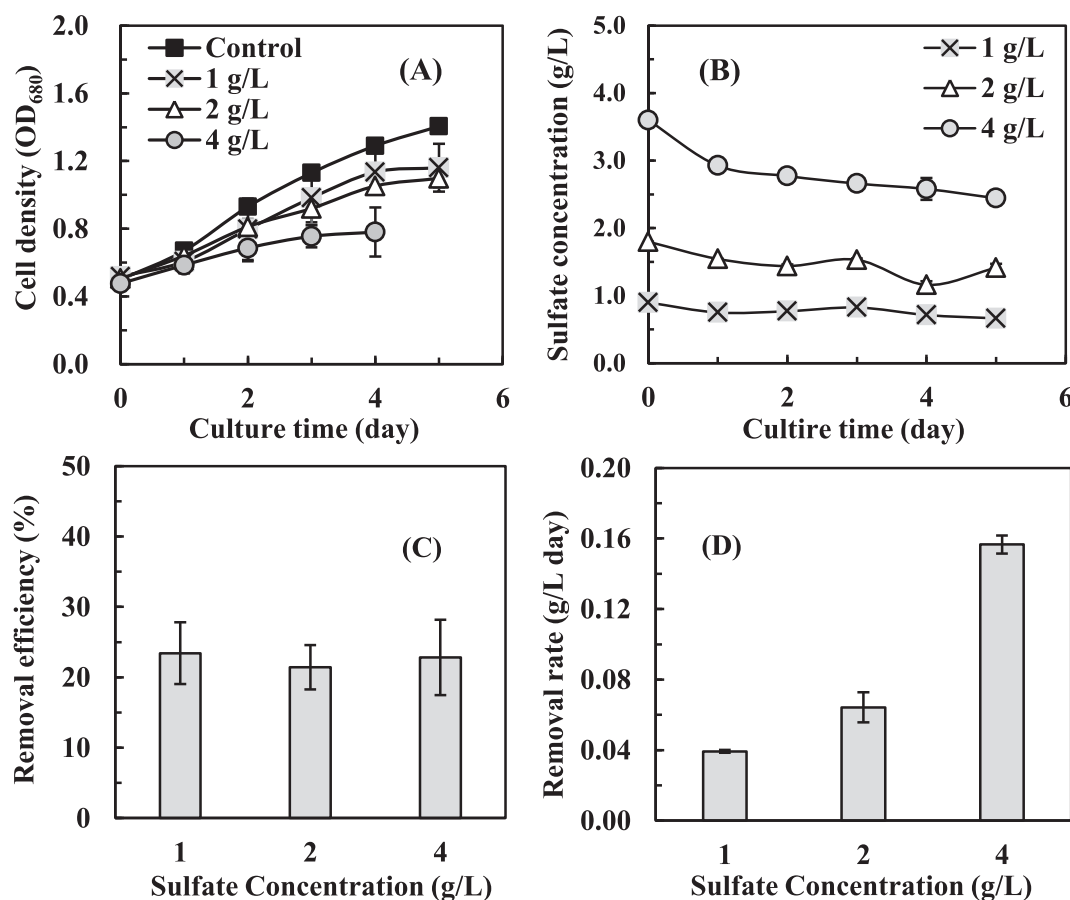


Fig. 1. Effects of sulfate concentration on the cell density (OD₆₈₀) (A); effluent sulfate concentration (B); sulfate removal efficiency (C); and sulfate removal rate (D) of algal polyculture in bubble column reactors in batch operation. Artificial wastewater with sulfate concentration of 1 g/L, 2 g/L, and 4 g/L were used. The control was performed in BBM medium with initial sulfate concentration of 0.036 g/L, with residual sulfate concentration not being presented in Fig. 2B. Removal rate was calculated by dividing the sulfate concentration reduction by the culture time.

weakly alkaline pH environment (Baldev et al., 2018).

3.2. Sulfate removal in RAB reactors

The performance of sulfate removal in RAB reactors was evaluated by feeding the reactors with wastewater containing 1 g/L, 2 g/L and 4 g/L sulfate, respectively. As shown in Fig. 2A, the effluent sulfate concentrations of the RAB reactors remained low values for the first 7 days when BBM (containing 0.036 g/L sulfate) was used as influent and a stable biofilm was established. At day 7 when BBM was switched to acidic high sulfate-containing wastewater, the effluent sulfate concentrations abruptly increased. The effluent sulfur concentration fluctuated thereafter and became stable after being operated for two weeks. The steady state was reached at this stage, during which the effluent sulfate concentration was used to evaluate the sulfate removal performance. Fig. 2B shows the pH change during the 28 days operation of the RAB reactors. The medium pH was maintained around 7.0 during the initial BBM-feeding stage. Starting from day 7 when acidic high-sulfate influents were fed to the RAB reactors, the pH gradually decreased to 4.4–4.6 at day 20, and then leveled off at this acidic pH level for the rest of culture, another indication of the steady state being reached.

The sulfur content in the biomass harvested at different stages of the continuous operation of the RAB reactors is illustrated in Fig. 2C. Here, we define the harvesting of biomass immediately before the sulfate-containing influent was fed to the RAB as the initial-harvest (Fig. 2A). Fig. 2C shows that the initial-harvested biomass had lower sulfur content than the rest of the biomass due to the low sulfate concentration in the feeding medium (BBM) during this stage. When the feeding

influent were switched to high sulfate-containing wastewater, the biomass sulfur content increased significantly. The increase of the biomass sulfur content was probably (partially) due to the physical adoption by the EPS in the algal biofilm (Kesaano and Sims, 2014). Fig. 2C also shows that higher sulfate concentrations resulted in more sulfate accumulation in the biomass. The biomass sulfur contents at 1 g/L and 2 g/L sulfate feeding were relatively stable among three consecutive harvests. At 4 g/L sulfate feeding, however, the sulfur content decreased with the harvesting passages, which was probably due to the inhibition of the algal growth at this high sulfate level leading to reduced biomass growth and EPS.

The sulfur removal performance of the RAB reactors were further evaluated through sulfate removal efficiency, removal rate and removal capacity (Eqs. (1)–(3)). As shown in Fig. 3A, the sulfate removal efficiency under three influent sulfate concentrations were relatively stable, ranging from 35 to 45%, which were higher than those in bubble column cultures (Fig. 1C). Both the sulfate removal rate (Fig. 3B) and removal capacity (Fig. 3C) increased with increasing sulfate concentration. Compared to the bubble column reactors, the RAB reactor resulted in a higher sulfate removal rate under each sulfate influent concentration level (Fig. 1C).

It has been reported that sulfate with over 300 mg/L was toxic to *Chlamydomonas moewusii* in BBM (Mera et al., 2016). While the growth of *Chlorococcum* sp. was not negatively affected when the alga was growing in synthetic wastewater with sulfate concentration ranging from 18 to 271 mg/L (Lv et al., 2017). In this work, the sulfate concentration used was much higher than those reported in previous studies. At such high sulfate level, the suspended algae in bubble columns

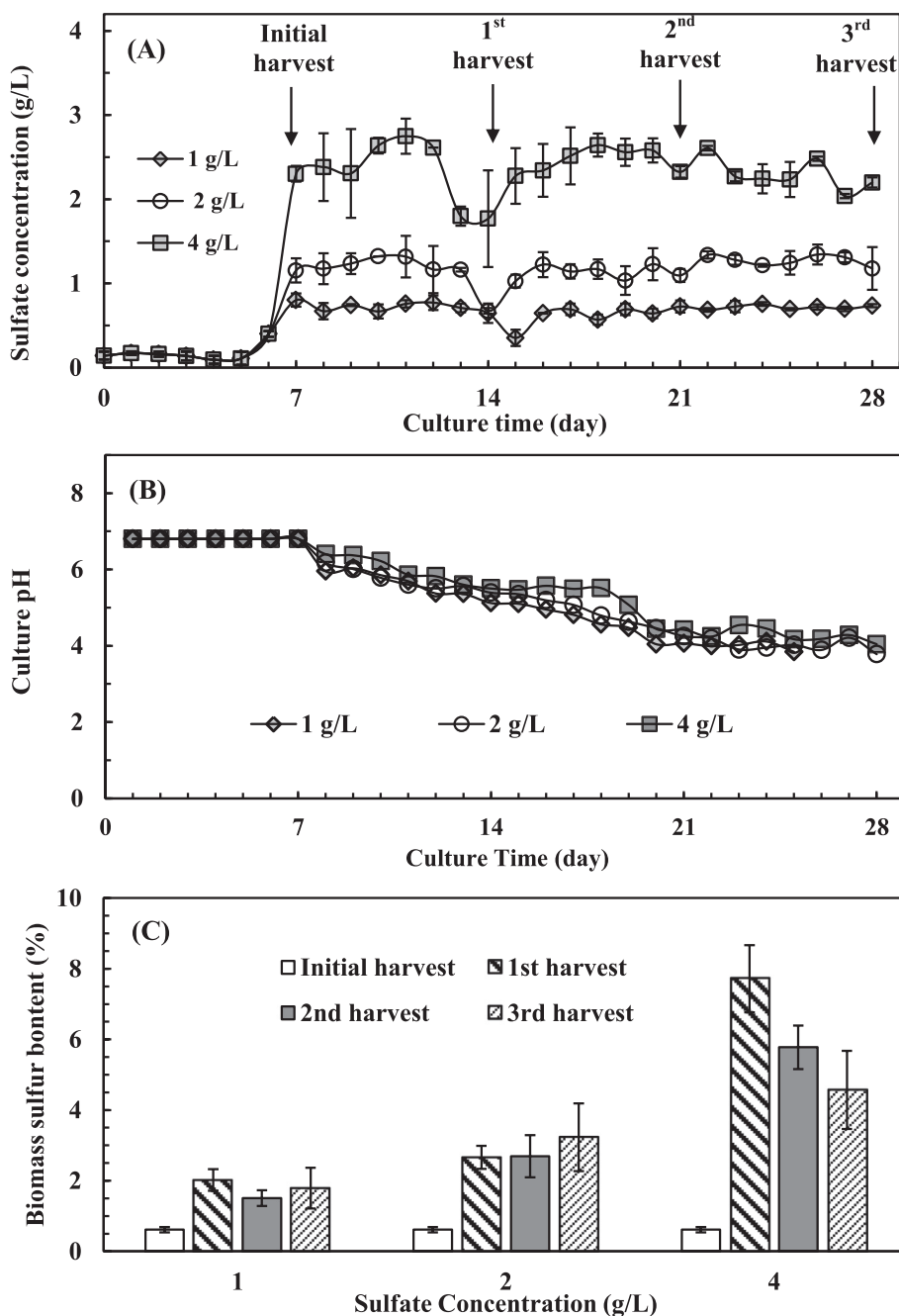


Fig. 2. Sulfate concentration in effluents (A), culture pH (B), and sulfur content of harvested biomass (C) of the RAB reactors operated at continuous mode (HRT = 3 day). The RAB reactors were fed with BBM for the first 7-days and then switched to wastewater influents containing 1 g/L, 2 g/L and 4 g/L sulfate, respectively. The harvest sequence in Fig. 3C refers to Fig. 2A.

were inhibited (Fig. 1A), while the attached algae in RAB reactors demonstrated a high performance for treating acidic high sulfate-containing wastewater (Fig. 3). This may be due to the contribution of the existing algal biofilm which contains a matrix of extracellular polymeric substances (EPS) to adsorb large amount of sulfate from the liquid (Kesaano and Sims, 2014). Indeed, EPS is commonly accumulated in the biofilm system (Gross et al., 2016). In this work, the harsh environment in the RAB reactors (high sulfate with low pH in influent) may lead the cells to produce more EPS to resist such an adverse environment so the metabolism can be maintained (Kesaano and Sims, 2014). Additionally, the attached biofilm in the RAB reactors enable the reactor to be operated at a shorter HRT without washing-out of algal cells, which eventually increased the sulfate removal rate. In the bubble column culture, it was found that applying the same HRT to the bubble

column resulted in cells washing-out (data not shown).

3.3. Diversity and richness of bacterial and eukaryotic community in RAB reactors

The microbial community of the RAB reactors treating sulfur-containing wastewater was characterized. After removing low quality sequences and chimeras, at least 82,396 raw reads and 80,070 clean reads were obtained for each sample with a length of 372 or 373 bp. The sequence depth was adequate to measure bacterial and eukaryotic diversity since all the coverage of each sample was higher than 99% (data not shown). As shown in Table 1, OTUs of the bacterial community decreased with the culture time; while at the same culture time (day 7 or day 28), low sulfate concentration resulted in low bacterial OTUs.

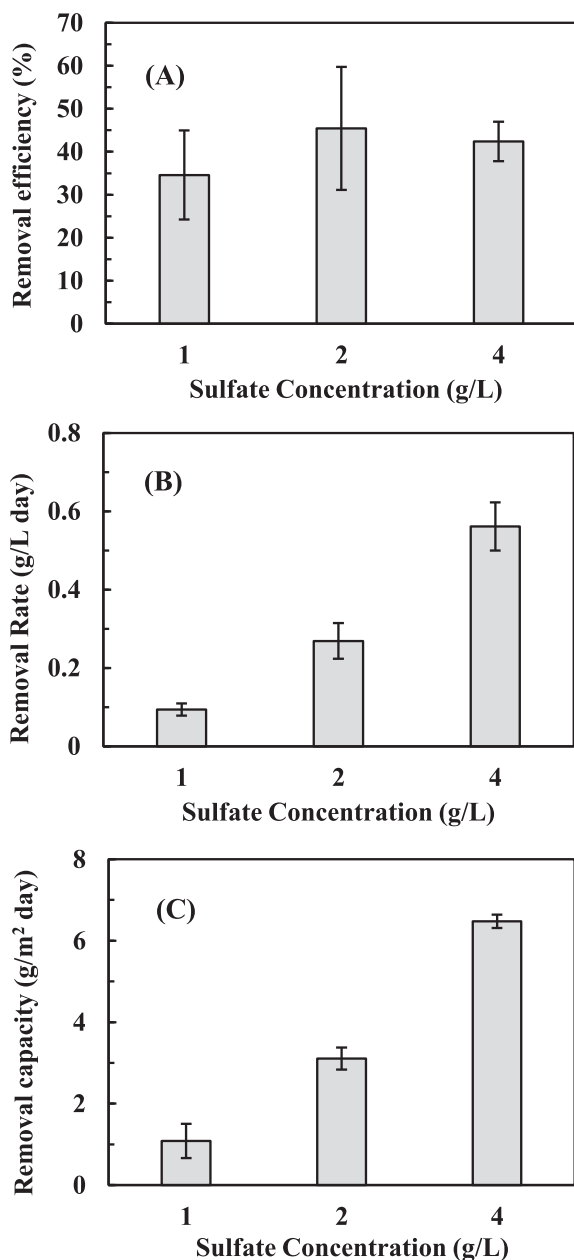


Fig. 3. Sulfate removal efficiency (A); sulfate removal rate (B); and sulfate removal capacity (C) of the RAB reactors operated at continuous mode (HRT = 3 day) with influent sulfate concentration of 1 g/L, 2 g/L and 4 g/L, respectively.

Table 1

Bacterial community richness and diversity indices of the biomass harvested at different culture times and sulfate feeding concentrations of the RAB reactors.

Biomass samples		Reads	OTUs	ACE	Chao	Shannon
Culture time	Sulfate concentration (g/L)					
Day 1	N/A	52,069	601	630	616	5.34
Day 7	1	56,383	474	505	497	4.28
	2	52,372	550	586	584	5.08
	4	59,890	611	650	643	5.37
Day 28	1	69,873	182	230	207	3.01
	2	68,207	279	339	338	2.61
	4	68,622	269	340	321	3.20

Table 2

Eukaryotic community richness and diversity indices of the biomass harvested at different culture times and sulfate feeding concentrations of the RAB reactors.

Biomass samples		Reads	OTUs	ACE	Chao	Shannon
Culture time	Sulfate concentration (g/L)					
Day 1	N/A	55,282	210	216	217	5.37
Day 7	1	65,194	232	237	235	5.14
	2	58,682	218	226	231	5.10
	4	59,708	219	228	229	5.16
Day 28	1	62,557	174	183	181	4.69
	2	58,704	179	191	185	4.98
	4	48,047	179	183	180	5.07

The ACE, Chao and Shannon estimators showed a similar trend, indicating reduced richness and diversity of the bacterial community with culture progressing (Table 1). Table 2 shows that richness and diversity indices of eukaryotic community. The OTUs decreased from the range of 218–232 at day 7 to the range of 174–179 at day 28. The ACE, Chao and Shannon values decreased from day 7 to day 28, indicating a reduced richness and diversity of the eukaryotic diversity with culture progressing.

The specific and common bacteria and eukaryotic OTUs were further evaluated at species level. Fig. 4A shows that the bacteria community had a total of 263 OTUs in common for the cultures in days 1 and 7 (with three sulfate concentrations), representing 28% of total sequences (940 OTUs) during this culture period. However, the common OTUs reduced to 96 (13% of total sequences) when culture extended to day 28 (Fig. 4B). The Venn diagrams for the eukaryotic communities (Fig. 4C and D) show that the common OTUs reduced from 137 (48% of total sequences) to 102 (37% of total sequences) when the culture was extended day 7 to day 28.

Overall, the above results indicated that the diversity the RAB biomass contained more diverse bacterial species than eukaryotic species. With the culture progressing, the community of both bacterial and eukaryotic communities reduced.

3.4. Compositions of bacterial and eukaryotic community in RAB reactors

Taxonomic classification revealed that bacteria were predominant of all classifiable 16S rRNA sequences. Fig. 5A shows that at the initial sulfur feeding stage (day 1), Proteobacteria and Cyanobacteria were dominant. Proteobacteria is a bacterial phylum some of which can facilitate the bacteria adherence to form biofilm (Mhedbi-Hajri et al., 2011); Cyanobacteria was predominant in our seed algal pond. After RAB reactors were fed sulfur-containing influent for 7 days, Proteobacteria and Cyanobacteria still dominated the community, while Bacteroidetes population increased. The abundance of Bacteroidetes has been detected in other sulfate reduction environments such as heavy-metal contaminated soil (Sitte et al., 2010) or metal-rich landfill leachate (Schmidtova and Baldwin, 2011). At day 28, Proteobacteria was still dominant in the community. Meanwhile, the acidophilic Acidobacteria increased to a range of 7.45%–15.53%, suggesting the acid environment provided a favorable condition for this bacteria to thrive (Lladó et al., 2016). Acidobacteria has been reported to withstand high metal content and acidic pH in other studies (Barns et al., 2007). The Cyanobacteria population still accounted as major population at day 28, but its relative abundance reduced compared to days 1 and 7. Bacteroidetes abundance also decreased particularly at 2 g/L and 4 g/L sulfate.

Regarding the composition of the present genus, Fig. 5B shows that *Leptolyngbya*, a very thin (< 3 μm) filamentous cyanobacteria (Albertano et al., 2000), was most abundant at day 1 but declined

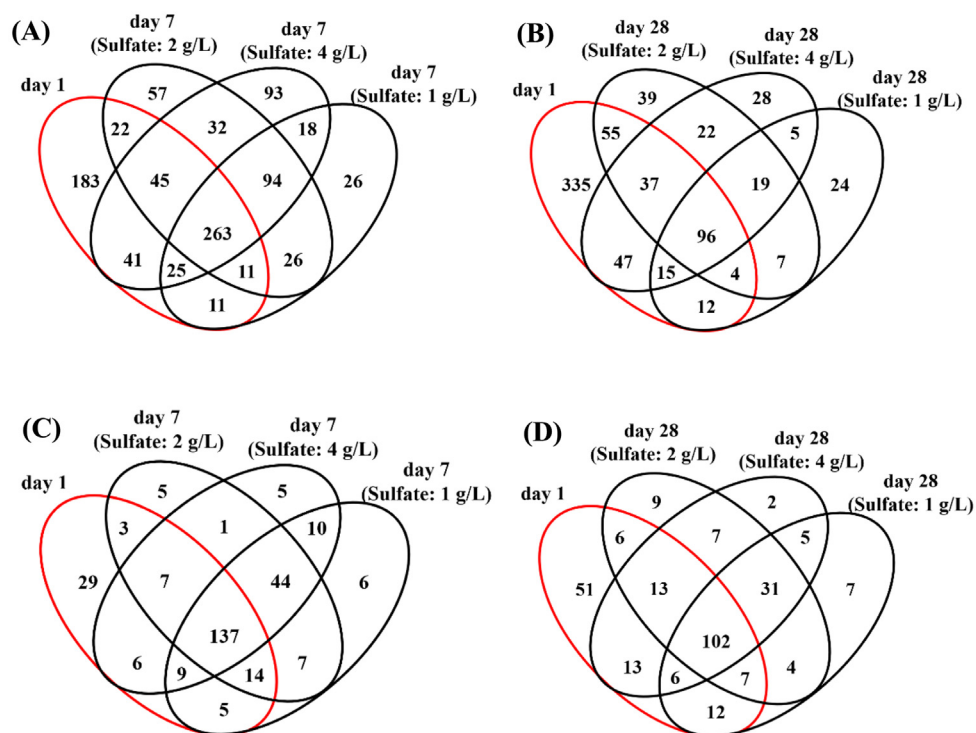


Fig. 4. Venn analysis of the bacterial and eukaryotic communities of the RAB reactors fed with influent containing different sulfate concentrations (based on OTUs at 3% distance). (A) Bacteria species progressing from day 1 to day 7; (B) Bacteria species progressing from day 1 to day 28; (C) Eukaryotic species progressing from day 1 to day 7; (D) Eukaryotic species progressing from day 1 to day 28.

sharply at days 7 and 28. An unidentified genus (Chloroplast class) and *Pseudanabaena* (both belonging to Cyanobacteria phylum) were abundant at day 7. While at day 28, *Terriglobus* and *Dyella*, respectively belonging to Acidobacteria and Proteobacteria phylum, became predominant. Previous researches have detected *Terriglobus* in the oil-contaminated soil (Abed et al., 2015) and sulfate reducing environment (Green-Saxena et al., 2014).

The abundance of eukaryotic community was also presented. As shown in Fig. 6A, Apicomplexa, an unidentified phylum (Eukaryota), Eustigmatophyceae, Chytridiomycota and Diatomea were dominant at day 1. At days 7 and 28, Ascomycota was more abundant; while Eustigmatophyceae and Chytridiomycota population decreased and the unidentified phylum (Eukaryota) maintained stable. The population of green alga Chlorophyta increased at day 7 but decreased at day 28. Previous studies have reported the existence of Ascomycota phylum during litter decay (Vofšková and Baldrian, 2013), and the existence of the green alga Chlorophyta phylum in a variety of environments (Paul and Fenical, 1987). At day 28, Basidiomycota population increased significantly attributing to the acid environment (Baker et al., 2009).

At genus level, Fig. 6B shows that *Nannochloropsis*, a diatom alga, was the most abundant member at day 1, but declined at days 7 and 28. However, *Acutodesmus* (belongs to Chlorophyta phylum), a microalga with resistant cell wall (Gruber-Brunhumer et al., 2015), increased its abundance with the culture progressing from day 1 to day 7 but decreased again to a relative low level at day 28. It should be noted that the alga *Chlorella vulgaris*, the original species inoculated for the open pond culture, was not detected in the RAB biofilm at day 1. The results indicate a drastic community change of the algal pond culture and RAB cultures.

Collectively, the above results show a dynamic change of the bacteria, cyanobacteria, eukaryotic algae and fungi during the treatment of acidic high sulfate-containing wastewater by the RAB reactors. The culture time and influent sulfate concentration significantly affected the bacterial and eukaryotic community constituents. With time evolving, various acidophilic bacteria, cyanobacteria, eukaryotic algae co-existed in the biofilm contributing to the sulfate removal. For example, in the bacterial community (Fig. 5B), *Hydrogenophaga* is a sulfate-reducing

bacteria (SRB) (Wei et al., 2010) while *Rhodobacter* was capable of assimilating sulfate (Cooper and Trüper, 1985). *Aquimonas*, *Curto-bacterium*, *Arenimonas*, and *Terriglobus* have also been found in various acidic sulfur-containing environments (Abed et al., 2015, Liu et al., 2015). Microalgae in the RAB reactors included cyanobacteria (*Cyanobacterium* and *Leptolyngbya*) (Fig. 5B), green algae (*Scenedesmus*, *Acutodesmus* and *Poterioochromonas*) and diatom (*Nannochloropsis* and *Nitzschia*) (Fig. 6B). It is believed that these microalgae removed sulfur from wastewater through assimilation as a nutrient (Lv et al., 2017, Mera et al., 2016). Such a diverse of species indicates that the sulfate removal by the RAB reactor may be based on a mixed mechanism such as bacteria-based reduction and microalgae-based assimilation. The bacterial and algae consortium can adapt to the wastewater environment and form a stable symbiosis system, realizing a robust pollutant removal. Further study is needed to elucidate the synergism of algae-bacteria interaction, and its implication in sulfur removal.

3.5. Nutrients (ammonia and phosphorus) and COD removal by the RAB reactors

The characteristics of the microbial community reported above also revealed that the RAB reactors have certain capability of removing nutrients (ammonia, phosphorus) and COD from the influent. For example, Proteobacteria, which was dominant in the bacterial community (Fig. 5A), contain some ammonia-oxidizing bacteria (Kowalchuk et al., 1997). Several algae (*Scenedesmus*, *Acutodesmus* and *Nannochloropsis*) were capable of removing phosphorus (Kim et al., 2016). The bacteria Acidobacteria (such as *Terriglobus*), and algae such as Eustigmatophyceae (*Nannochloropsis*) and Chlorophyta (*Acutodesmus*) can also consume organic carbon and thus benefit for COD removal (Kim et al., 2016, Velu et al., 2015). A mixed algae-bacteria based biofilm was capable of removing sulfate as well as other nutrients from secondary stage municipal wastewater (Shayan et al., 2016).

In this work, therefore, the RAB reactors were further evaluated for its capability of removing COD, ammonia and phosphorus. As shown in Table 3, the RAB reactors had an excellent performance for TP removal (~100%) under different influent sulfate concentrations. For ammonia

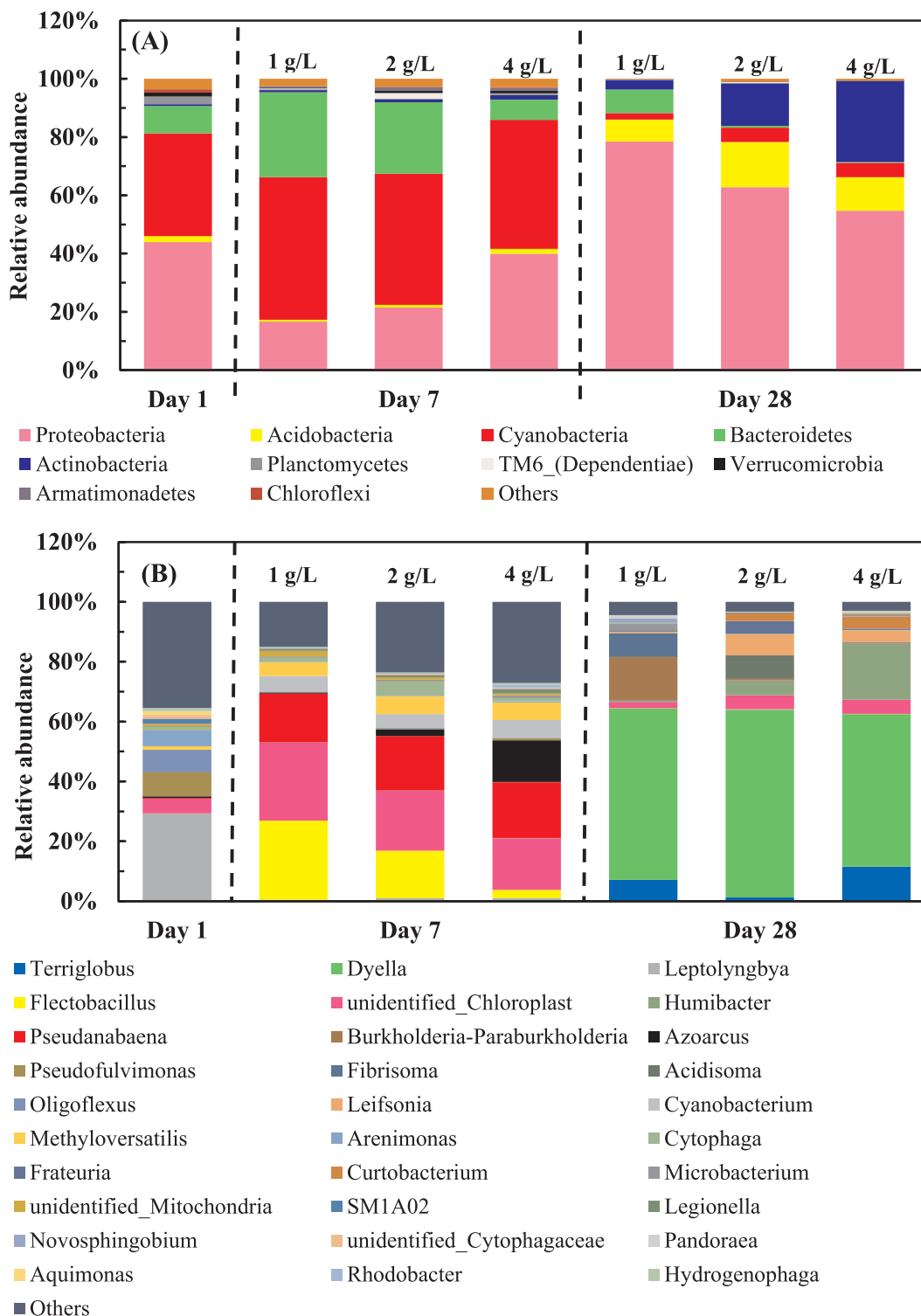


Fig. 5. Bacterial community abundance of the biomass harvested at days 1, 7 and 28. RAB reactors were fed with influent containing sulfate at 1 g/L, 2 g/L and 4 g/L. (A): phylum level, (B): genus level.

removal, the RAB reactors removed 69% (1 g/L sulfate), 68% (2 g/L sulfate) and 52% (4 g/L sulfate) ammonia from the influent, indicating high sulfate concentration may inhibit the ammonia removal from the influent. Comparison of TP and ammonia removal efficiency indicates that the RAB culture may be limited by phosphorus, while ammonia was supplied in excess. Table 3 also shows that the three RAB reactors had an excellent COD removal performance. For example, the influent fed to the reactors contained 400 mg/L COD; the effluent COD

concentration was lower than 50 mg/L, resulting in over 90% COD being removed. The high sulfate content in the influent did not exhibit any negative effect on the COD removal. Collectively, Table 3 indicate that influent sulfate concentration played an important role for the nutrients and COD removal efficiency, and 2 g/L was the optimal sulfate concentration for a maximum TP, ammonia and the COD removal. The effects of sulfur concentration on the nutrients and COD removal was also reported by other researchers. For example, Lv et al. (2017)

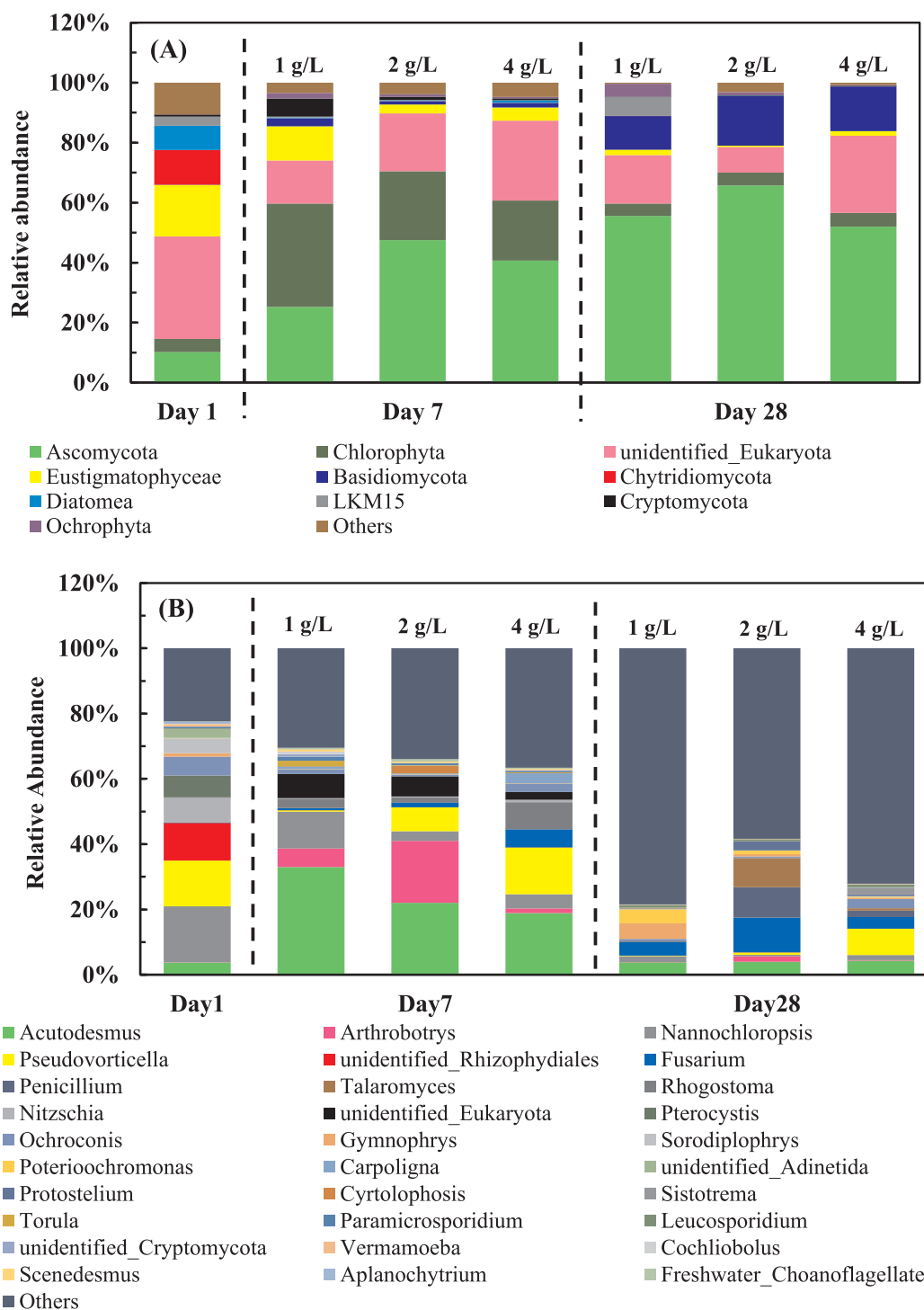


Fig. 6. Eukaryotic community abundance of the biomass harvested at days 1, 7 and 28. RAB reactors were fed with influent containing sulfate at 1 g/L, 2 g/L and 4 g/L. (A): phylum level, (B): genus level.

found that the removal efficiency of COD, ammonia and phosphorous decreased at sulfate deficient condition, while remained relatively stable when the sulfate concentration increased from 18 mg/L to 271 mg/L. Our results further indicated that excessive sulfate may result inhibition on nutrient removal.

4. Conclusion

RAB reactor is an effective system for treating acidic sulfate containing wastewater. The sulfate removal efficiency was within the range

of 35–46%, two times higher than the bubble column reactors. The sulfate removal rate and removal capacity reached up to 0.56 g/L-day and 6.47 g/m²-day, respectively. RAB reactors contained a diverse bacterial and algae community, which provided a superior sulfur removal performance through various mechanisms such as reduction and assimilation. In addition to the sulfate removal, the RAB reactors also achieved excellent ammonia, phosphorus and COD removal performance. Further efforts are needed to optimize the RAB operational conditions.

Table 3

Nutrients (ammonia and total phosphorus, TP) and COD removal performance by the RAB reactors fed with influents containing different sulfate concentrations.

		Sulfate concentration in the influent (g/L)		
		1	2	4
TP	Removal efficiency (%)	97.62 ± 4.01	98.56 ± 0.65	98.96 ± 0.69
	Removal rate (mg/L-day)	3.58 ± 0.15	3.62 ± 0.03	3.62 ± 0.05
	Removal capacity (mg/m ² -day)	41.30 ± 1.70	41.76 ± 0.31	41.74 ± 0.55
Ammonia	Removal efficiency (%)	69.52 ± 13.12	68.33 ± 10.59	52.11 ± 7.31
	Removal rate (mg/L-day)	15.53 ± 2.93	15.26 ± 2.37	11.64 ± 1.63
	Removal capacity (mg/m ² -day)	179.1 ± 33.81	176.1 ± 27.30	134.3 ± 18.83
COD	Removal efficiency (%)	93.98 ± 4.37	92.16 ± 4.23	95.53 ± 3.37
	Removal rate (mg/L-day)	125.3 ± 5.83	123.2 ± 5.64	127.4 ± 4.50
	Removal capacity (mg/m ² -day)	1445 ± 67.27	1422 ± 65.11	1469 ± 51.86

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