Sulfate removal and sulfur transformation in constructed wetlands:

The roles of filling material and plant biomass

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Abstract

Sulfate in effluent is a challenging issue for wastewater reuse around the world. In this study, sulfur (S) removal and transformation in five batch constructed wetlands (CWs) treating secondary effluent were investigated. The results showed that the presence of the plant cattail (*Typha latifolia*) had little effect on sulfate removal, while the carbon-rich litter it generated greatly improved sulfate removal, but with limited sulfide accumulation in the pore-water. After sulfate removal, most of the S was deposited with the valence states S (-II) and S (0) on the iron-rich gravel surface, and acid volatile sulfide was the main S sink in the litter-added CWs. High-throughput pyrosequencing revealed that sulfate-reducing bacteria (i.e. *Desulfobacter*) and sulfide-oxidizing bacteria (i.e. *Thiobacillus*) were dominant in the litter-added CWs, which led to a sustainable S cycle between sulfate and sulfide. Overall, this study suggests that recycling plant litter and iron-rich filling material in CWs gives an opportunity to utilize the S in the wastewater as both an electron acceptor for sulfate reduction and as an electron donor for nitrate reduction coupled with sulfide oxidation. This leads to the simultaneous removal of sulfate, nitrate, and organics without discharging toxic sulfide into the receiving water body.

Keywords

Constructed wetlands; Bacterial sulfate reduction; Sulfur oxidation; Denitrification; Plant litter

1 Introduction

Sulfate is a common contaminant of wastewater, and is not usually considered a health concern, but it can, under some circumstances, cause diarrhea. However, sulfate reduction may produce hydrogen sulfide (H_2S) and organic sulfur (S) compounds, which normally cause aesthetic problems (taste, color and/or odor) in the wastewater and the effluent-dominated river. Moreover, H_2S can cause serious corrosion to water pipes during the transportation of reused water and/or phytotoxicity to plants during irrigation (EPA, 2004). Therefore, the removal of sulfate in the effluent from wastewater treatment plants (WWTP) with minimum H_2S accumulation is of great importance to wastewater reuse around the world.

Constructed wetlands (CWs) are widely used as a tertiary treatment to polish the WWTP effluent for wastewater reuse due to their low implementation costs, simple operation, and efficient removal of effluent contaminants (Greenway, 2004; Jasper et al., 2014). CWs act as an eco-buffer zone between the WWTP and receiving waters, and could become promising artificial ecosystems for odor control in effluent-dominated rivers if the majority of the S could be immobilized or dissipated in CWs beds. Sulfur transformation in CWs has become increasingly important in recent years due to the high S reduction and oxidation activities shown in wetlands (Baldwin and Mitchell, 2012; Wu et al., 2013). In subsurface flow constructed wetlands (SSF CWs), the relatively low redox condition provides a high thermodynamic potential for sulfate reduction. However, the amount of internal carbon from the rhizosphere and external

carbon from secondary effluent are not enough to drive significant sulfate reductions in CWs (Stein et al., 2007). Plant litter is one of the most abundant carbon sinks in wetlands ($500\text{--}2000\text{ g C m}^{-2}\text{yr}^{-1}$) (Hume et al., 2002). While, the structure of SSF CWs prevents aboveground plant litter from reaching the subsurface water and inhibits the carbon release from plant litter. Therefore, recycling the carbon in plant litter could be a low cost and sustainable way to enhance sulfate reduction in CWs. Chen et al. (2014) showed that plant litter greatly stimulated sulfate reduction in CWs through the on-site production of carbon sources such as carbohydrate and volatile fatty acids. However, as far as can be ascertained, there have not been any studies on the effect of plant litter on S transformation in CWs treating secondary effluent.

Sulfide is considered to be the main product of sulfate reduction, and can severely inhibit ammonium/carbon removal and plant photosynthesis, which decreases the treatment efficiency of CWs. Sulfide detoxification can be achieved when CWs are supplied/filled with metal-enriched substrates, because sulfide can precipitate along with heavy metals (i.e. iron, zinc) (Stein et al., 2007; Wu et al., 2012). Wiessner et al. (2010) calculated that nearly half the sulfate-S was immobilized inside CWs. However, the amount and speciation of the immobilized S (solid-phase) is often unknown. Acid volatile sulfide (AVS) is considered to be the main component in the solid-phase S, and it is a complex and variable component that includes diverse reduced S forms (e.g. FeS , Fe_3S_4 , and FeS_2) (Rickard and Morse, 2005). At present, AVS detection relies on the

application of acid-based extraction methods, which are relatively efficient, but do not detect 100% of the AVS since not all of the Fe_xS_y can be fully extracted. X-ray photoelectron spectroscopy (XPS) has emerged as an element-sensitive technique for describing the speciation and distribution of S at the microscale in recent years (Sun et al., 2009). Despite the wide use of acid-based AVS extraction and S speciation identification by XPS (Baldwin and Mitchell, 2012; Johnston et al., 2014), very few studies have investigated the solid-phase S in the filling material of CWs. Therefore, there has not been complete elucidation of the S species distribution and related S transformations in CWs.

Apart from the precipitation of sulfide with metals, oxidation is another effective method of avoiding sulfide accumulation. Oxygen released from plant roots and the atmosphere oxidizes harmful sulfide to harmless forms (e.g. elemental S and sulfate) in CWs (Faulwetter et al., 2009). Previous studies have suggested that 41%–90% of the reduced S was re-oxidized by root-mediated oxygen in planted wetlands (Wiessner et al., 2010; Wu et al., 2011). Wu et al. (2011) further demonstrated the multiple S transformations (i.e. sulfide re-oxidation) in CWs using the ^{34}S isotope approach. Apart from oxygen, nitrate in the influent can also easily drive sulfide and elemental S oxidation to sulfate in the organic-rich wetlands (Krishnakumar and Manilal, 1999; Londry and Suflita, 1999). Chemical and microbial oxidation are the main sulfide oxidation processes in CWs (Wu et al., 2013). Bacterial sulfur oxidation is mainly

driven by S oxidizing bacteria (SOB), and sulfide is oxidized to sulfur (or sulfate) using oxygen or nitrate as electron acceptors (Faulwetter et al., 2009). At present, information on the SOB community in CWs is incomplete due to the inefficient detection of species that are present at low levels (Hallberg and Johnson, 2005; Nicomrat et al., 2006). Therefore, a sensitive and comprehensive detection method for S-related bacteria based on next-generation sequencing is urgently needed to improve understanding of the mechanism underlying microbial S oxidation in CWs.

In this study, S transformation was characterized in five iron-rich media containing CWs with or without cattail (*Typha latifolia*) and externally added carbon sources. The objectives were to (1) study the effects of plants and plant litter as carbon sources on sulfate removal, sulfide accumulation, and intermediate-S formation. (2) quantify the solid AVS and the multi-valence distribution of S in the iron-rich gravel; (3) quantify S species distribution and elucidate S transformation; and (4) characterize the structures of SRB/SOB communities in CWs.

2 Materials and Methods

2.1 Design and operation of the SSF CW

Five sequencing batch SSF CW microcosms, each with a bulk volume of 0.045 m³ (length: 0.3 m, width: 0.3 m, height: 0.5 m) and a pore volume of 12 L, were set up in this study. Five systems: an unplanted control (W0), two litter-added microcosms (W1: 100g; W2: 200g), a planted microcosm (W3: 22 plants m⁻²) and a planted plus litter

added microcosm (W4: 100g litter, 22 plants m⁻²), were established. All the microcosms were filled with iron-rich gravel (ϕ 8–13 mm, porosity = 0.4, iron content 4.7%, w/w) up to a height of 40 cm. The water level was adjusted to be 5 cm below the gravel bed surface. Two (W3 and W4) CWs were planted with cattail (*Typha latifolia*). The wetland microcosms have been located in an air-conditioned greenhouse at a temperature of 25 $\pm$ 1 °C since 2005. Prior to the start of the experiment, the five microcosms were fed, in batches, with a modified secondary effluent for 6 months pre-incubation in order to establish the plant shoots and microorganisms. Then, cattail litter (1~1.5 cm lengths) was added to the W1, W2, and W4 microcosms as the carbon source to drive sulfate reduction. The cattail litter was homogeneously mixed with gravel, and the mixed media were compacted with a tamping rod at 5 cm increments during loading and filled the microcosms to a height of 40 cm.

2.2 Batch experiment

The batch experiment began after a 6 month pre-incubation. The wetland microcosms were fed with the secondary effluent from a neighboring WWTP, and the characteristics of the wetland influent were seen in Table S1. Influent was introduced into the microcosm from the top and gravity drained from the bottom. The microcosm was operated in batch mode with five days for each batch (HRT = 5 d). Feeding, reaction and draining was designed as illustrated in Figure 1. Briefly, each batch started with a feeding stage (1 h), followed by a reaction stage (118 h), and terminated with a

draining stage (1 h). All the treatments (W0–W4) were triplicated and the duration of the batch experiment was 100 d, which included 20 batches. Water samples were taken from each microcosm and each batch. A 100 mL syringe was used to collect water samples at 5, 20, and 30 cm depths from the central sampling pipe. Only water samples taken from 20 cm depth were reported because no vertical gradients in the water chemistry were observed in the preliminary experiment and in previous experiments with the same microcosms (Wen et al., 2010).

2.3 S-based autotrophic denitrification kinetic tests

The autotrophic denitrification kinetic tests were carried out according to Chen et al. (2014b). Briefly, 1000 g of gravel was taken from W0–W4 before batch 20 and respectively transferred to 1 L serum bottles (S0–S4). After a 10 d pre-incubation period (removal of the original nitrate, sulfate, and endogenous organic matters inside the cell), nitrate ($10 \text{ mg L}^{-1} \text{ NO}_3^- \text{-N}$) was added to the serum bottles, which were then incubated in an anaerobic environment (25 °C) for five days. Nitrate and sulfate concentrations were measured every 12 h. There was no organic carbon in the feeding water, so nitrate loss in the serum bottles could be mainly attributed to autotrophic denitrification.

2.4 Aqueous-phase methods

Five 50 mL water samples, withdrawn at the appropriate time intervals, were membrane-filtered (0.22 μm) and immediately analyzed for dissolved sulfide. Zinc chloride solution was then added (prior to further analysis for other chemical

constituents) to the filtered samples in order to eliminate any soluble sulfide by precipitating it as zinc sulfide. Sulfate, sulfite, thiosulfate, and nitrate were detected using a DX ICS-3000 ion chromatography unit (Dionex Corporation, CA, USA) equipped with a conductivity detector and a self-regenerating suppressor ASRS-ULTRA II 4-mm (129 mA). Elemental sulfur was detected by HPLC (Agilent 1200, Agilent Technologies, CA, USA) using a Li-Chrospher 100, RP 18 column equipped with an UV detector at 263 nm. Dissolved sulfide and chemical oxygen demand (COD) were analyzed using standard methods (APHA, 1998). The Eh was measured using a portable mV/ pH /temperature meter (HACH, sensION1, USA) fitted with an Ag/AgCl Eh electrode. The details of the S compound analyses were reported in a previous study (Chen et al., 2014a).

2.5 Solid-phase methods

For the AVS-S analysis, 64 gravel samples were collected from four different layers (10, 20, 30, and 40 cm) after the experiment. Then, 500 g of composited gravel was extracted with 1 M HCl, and the produced H₂S was trapped in 0.05 M Zn acetate and quantified by iodometric titration. XPS experiments were carried out on an RBD upgraded PHI-5000C ESCA system (Perkin–Elmer) with Al K α radiation ($h\nu = 1486.6$ eV). Curve fitting of the carbon C1s peaks were achieved by fitting them to Gaussian curves using RBD AugerScan 3.21 software. A scanning electron microscope (Philips, XL30) and an energy dispersive spectrometer (Link 300) were used to observe the

surface morphology and analyze the elemental distribution on the surface of the gravel.

2.6 DNA extraction

After batch 20 (100d), approximately 200g of gravel and litter were collected from the top (5 cm), middle (20 cm), and bottom (40 cm) sections of the wetland microcosms. The three samples were combined for DNA extraction. Before DNA extraction, the gravel/litter samples were vigorously shaken at 225 rpm for 3 h in sterile glass bottles in order to suspend any attached biofilm in the liquid solution. The precipitate was collected in bottles for further analysis after they had been centrifuged twice at 5000g for 20 min. Total genomic DNA was extracted from the gravels and litters using an E.Z.N.A. ® Soil DNA Kit (OMEGA bio-tek). The quantity and quality of the extracted DNA were checked by measuring its absorbance at 260 and 280 nm using a UNICO-2100 UV/VIS spectrophotometer.

2.7 High-throughput 16S rRNA gene sequencing and analysis

High-throughput 454 GS-FLX pyrosequencing of the 16S rRNA gene was conducted according to standard protocols (Margulies et al., 2005). A BLAST search for taxonomic classification down to the phylum, class and genus levels was then undertaken using MOTHUR and the SILVA 106 database with a set confidence threshold of 80%. The pyrosequencing and analysis details were reported in a previous study (Chen et al., 2015).

2.8 Mass balance calculations

In this study, total sulfur was calculated by summing up all the determined sulfur compounds (SO_4^{2-} , S^0 , S^{2-} , $\text{S}_2\text{O}_3^{2-}$ and AVS) and undetermined sulfur compounds (other S). Assuming that the mass of total sulfur was constant after CW treatment, the following equation can be obtained:

$$\Delta m_{\text{total S}} = \Delta m_{\text{SO}_4^{2-}} + \Delta m_{\text{S}^0} + \Delta m_{\text{S}^{2-}} + \Delta m_{\text{S}_2\text{O}_3^{2-}} + \Delta m_{\text{AVS}} + \Delta m_{\text{other S}} = 0 \quad (1)$$

$$\Delta m_{\text{other S}} = -\Delta m_{\text{SO}_4^{2-}} - \Delta m_{\text{S}^0} - \Delta m_{\text{S}^{2-}} - \Delta m_{\text{S}_2\text{O}_3^{2-}} - \Delta m_{\text{AVS}} \quad (2)$$

The mass removal of the determined sulfur compounds is obtained from the following equation:

$$\Delta m_{\text{SO}_4^{2-}} = \sum_{i=1}^n (C_{i(\text{in})\text{SO}_4^{2-}} \times V_{i(\text{in})} - C_{i(\text{out})\text{SO}_4^{2-}} \times V_{i(\text{out})}) \quad (3)$$

$$\Delta m_{\text{S}^0} = \sum_{i=1}^n (C_{i(\text{in})\text{S}^0} \times V_{i(\text{in})} - C_{i(\text{out})\text{S}^0} \times V_{i(\text{out})}) \quad (4)$$

$$\Delta m_{\text{S}^{2-}} = \sum_{i=1}^n (C_{i(\text{in})\text{S}^{2-}} \times V_{i(\text{in})} - C_{i(\text{out})\text{S}^{2-}} \times V_{i(\text{out})}) \quad (5)$$

$$\Delta m_{\text{S}_2\text{O}_3^{2-}} = \sum_{i=1}^n (C_{i(\text{in})\text{S}_2\text{O}_3^{2-}} \times V_{i(\text{in})} - C_{i(\text{out})\text{S}_2\text{O}_3^{2-}} \times V_{i(\text{out})}) \quad (6)$$

$$\Delta m_{\text{AVS}} = m_{\text{AVS}-\text{ini}} - m_{\text{AVS}-\text{end}} \quad (7)$$

where $\Delta m_{\text{SO}_4^{2-}}$, Δm_{S^0} , $\Delta m_{\text{S}^{2-}}$, $\Delta m_{\text{S}_2\text{O}_3^{2-}}$ are respectively the mass removal of sulfate, elemental sulfur, sulfide and thiosulfate after CW treatment (mg); Δm_{AVS} is the mass change of acid volatile sulfide in CWs after the batch experiment (mg); $\Delta m_{\text{AVS}-\text{ini}}$ and

$\Delta m_{AVS-end}$ are the mass of acid volatile sulfide in gravel before and after the batch experiment, respectively (mg); Δm_{otherS} is the mass removal of undetermined sulfur (mg); i is the number of batch in sequence ($i=1,2,3...20$); n is the total number of batches; $C_{i(in)}$ and $C_{i(out)}$ are the concentrations of determined sulfur compounds in the influent and effluent of batch i , respectively ($\text{mg}\cdot\text{L}^{-1}$); $V_{i(in)}$ and $V_{i(out)}$ are the volume of pore water in the influent and effluent of batch i , respectively (L).

3. Results

The experiment was divided into three stages based on the sulfate removal characteristics: an initial stage (days 1–30, batches 1–6), a middle stage (days 31–70, batches 7–14), and a terminal stage (days 71–100, batches 15–20). The sulfate removal kinetics followed a similar pattern at each stage. Therefore, batches 4 (B4), 12 (B12), and 20 (B 20) were chosen as typical batches that represented the three stages, respectively.

3.1 Sulfur compound dynamics in CW pore water

3.1.1 Sulfate

Figure 2 shows that there were no significant sulfate decreases in the control (W0) and planted only systems (W3). This suggested that both the influent organic matter and plant root exudates have little effect on sulfate removal. In contrast, significant sulfate removals were observed in the litter-added microcosms, which indicated that cattail litter could act as carbon sources for sulfate reduction. Complete sulfate removal was

only achieved in the W2 microcosm during the initial stage, and sulfate removal rates gradually decreased over time due to the reduction in organic carbon provided by cattail litter. A previous study showed that the different sulfate removal behaviors were mainly due to the litter decomposition rates and carbon supply in the CWs (Chen et al., 2014a). Figure 2 also shows that sulfate concentrations increased in the litter-added CWs during initial period of each batch, but then decreased in the later stages. This indicated that sulfur oxidation of the deposited S compounds in the bed may also occur in litter-added CWs.

3.1.2 Sulfide

Sulfide is the final product during sulfate reduction, and its production is encouraged in order to precipitate metals such as Cu, Pb, Cd and Zn (Stein et al., 2007). In this study, the sulfide concentrations were small in the microcosms without litter, which was probably due to the lack of carbon sources to drive sulfate reduction. In contrast, sulfide was detected in all the litter-added microcosms, and its concentration decreased between the initial and the terminal stages. In this study, the sulfide concentrations were always below 1 mg L⁻¹ in the litter-added microcosms, which was much lower than the theoretical production through sulfate reduction. The efficient sulfate removal with little sulfide accumulation in the litter-added microcosms was probably due to hydrogen sulfide emissions, metal sulfide precipitation, and sulfide re-oxidation (Wu et al., 2013).

3.1.3 Intermediate S compounds

Elemental S, thiosulfate and sulfite are the common intermediate compounds in the wetland S cycle (Wiessner et al., 2010). These intermediate S compounds are not stable in wetlands and can be oxidized or reduced via different pathways (Wu et al., 2013). In this study, the elemental S was only detected in the pore water of the litter-added microcosms, and the maximum concentration increased from 0.9 mg L⁻¹ at the initial stage to 4.4 mg L⁻¹ at the terminal stage. The increased elemental S over time was probably caused by the gradual accumulation of reduced S in the microcosms, and it can be oxidized to elemental S once oxygen or nitrate is available. Figure 3 shows that thiosulfate was only detected in the litter-added microcosms during the initial and middle stages, and the highest concentration was observed in the W2 microcosm. This suggested that carbon sources played an important role in the production of thiosulfate. In this study, sulfite was not detected in all the microcosms, which indicated that the sulfite reduction rate outpaced its production rate.

3.2 Solid reduced S in the gravel

3.2.1 Acid-volatile sulfides (AVS)

AVS are sedimentary S pools that can generate gaseous H₂S following the addition of acid. Both S in the soluble phase (HS⁻, H₂S and FeHS⁺) and S in the solid phase (FeS, FeS₂ and Fe₃S₄) could be considered as sources of AVS (Rickard and Morse, 2005). In this study, solid reduced S was the main component of AVS due to the extraction of S in

the gravel. As shown in Figure S1, the highest AVS accumulation (1806 mg) was observed in the W2 microcosm gravel, which indicated that added carbon sources from plant litters increased AVS accumulation in wetlands. Furthermore, there was no significant difference in AVS accumulation between the W1 (824 mg) and W4 (859 mg) microcosms, suggesting that adding plants may not significantly affect AVS accumulation in wetlands. According to the mass calculation, 23.5, 66.3 and 15.8 mg soluble sulfide were detected in the W1, W2 and W4 microcosms, respectively, which were much lower than their individual AVS accumulations. This suggested that solid AVS sources other than soluble sulfide were the main sink for the reduced S in wetlands with added litter. The S valence was further analyzed using XPS in order to determine the diverse reduced and oxidized S forms in the wetland gravel.

3.2.2 Sulfur valences

One broad characteristic peak was observed on the gravel XPS S2p spectra (Figure 4). The broad peak located between 158 and 168 eV was divided into one doublet and one singlet (Sun et al., 2009): the S2p_{3/2-1/2} doublet at 161.5 and 162.6 eV were attributed to S (-II), and the S2p_{3/2} singlet at 163.6 eV was assigned to S (0). Figure 4 also shows that both the S (-II) and S (0) characteristic peaks were observed in the litter-added microcosms, which indicated that S reduction and oxidation coexisted in wetlands. Furthermore, the relative peak area ratios for S (0):S (-II) were 0.21:1.00, 0.36:1.00, and 0.22:1.00 in the W1, W2, and W4 microcosms, respectively. The larger

relative peak areas for S (-II) compared to of S (0) indicated that S (-II) was the main S valence in the gravel and that there was limited S transformation from a lower valence to a higher one. The electron binding energies (161.5 and 162.6 eV) suggested that FeS and FeS₂ could be the main components of the solid AVS in this study (Crist, 1999). The energy-dispersive X-ray spectroscopy (EDS) analysis also showed that Fe was present at high levels (4.66%), and there was no Cu, Pb, Cd, Zn, Ni, or Mn in the initial gravel (Figure S3), which indicated that Fe-S precipitation had a dominant role in the production of solid AVS.

3.3 Sulfur species distribution

After the experiment, the S species distribution was calculated based on the S mass balance (Section 2.8). There was very little sulfate removal in the control (W0) and planted microcosms (W3). Therefore, S species distribution data were only available for the litter-added microcosms (W1, W2, and W4). Figure 5 shows that the highest total sulfate removal was observed in W2 (2345.6 mg S), followed by W4 (1312.9 mg S) and W1 (1148.8 mg S). Furthermore, 65.4%–77.0% of the sulfate-S was transformed into AVS and immobilized in the gravel. As an intermediate product of sulfide oxidation, S⁰ accounted for 4.5%–7.5% of the sulfate removal in the litter-added microcosms. In addition to solid AVS, sulfide, elemental S and thiosulfate, there were some unidentified S compounds and they contributed 10.2%–25.9% to the total amount of sulfate-S that was transformed. These unaccounted S may mainly originate from the intermediate

products during the S cycling (i.e. S_x^{2-} , $S_4O_6^{2-}$) or the organic sulfides rather than gaseous sulfide (H_2S) (lower than detection limit in this study) (Isamu et al., 1999).

3.4 Sulfur-driven autotrophic denitrification

In this study, the accumulation of AVS/S^0 in the litter-added CWs and the production of nitrate in the secondary effluent provided an opportunity to connect sulfur oxidation to nitrate reduction (Eq. 1). In the batch experiment, the production of sulfate ($t < 20$ h, Figure 2) along with the removal of nitrate (Figure S2) indicated that sulfur-driven autotrophic denitrification may occur in CWs. In order to validate this hypothesis, autotrophic denitrification kinetic tests were carried out. Table 1 shows that without organic matter, the autotrophic denitrification rates ranged from $34.7\text{--}50.7\text{ mg N m}^{-2}\text{ d}^{-1}$ in litter-added CWs, and this accounted for 7%–16% of the total nitrate removal. The remaining proportion was removed by heterotrophic denitrification and plant uptake. Furthermore, the simultaneous formation of sulfate was observed in the litter-added CWs, and the observed sulfide oxidation rates (r_{SO-O}) were very close to the expected theoretical sulfide oxidation rates (r_{SO-T}), according to Eq. (1) (Table 1). This suggested that S-driven autotrophic denitrification occurred in litter-added CWs and this is represented by Eq. (1) below.

3.5 Microbial communities related to S cycling

Microbial community analyses were carried out for the gravel and litter in the

wetland microcosms at the end of the experiment. A total of 30884 high-quality sequence tags were obtained from the biofilms in the unplanted (W_0), litter-added (W_1), and planted (W_3) microcosms. Figure S4 shows that both SRB and SOB were found in the W_0 and W_1 microcosms. However, both types of bacteria were below the detection limit of the Roche 454 high-throughput pyrosequencer in the W_3 microcosm. *Desulfobacter*, *Desulfovibrio*, *Desulfobulbus*, *Desulfococcus* and *Desulfocapsa* were the dominant SRB genera (1.1%-9.3%), whereas *Sulfuricurvum* and *Thiobacillus* were the dominant SOB genera (1.5%-6.1%). Furthermore, uncultured species dominated SRB diversity in the microcosms. Interestingly, the relative abundances of SRB and SOB were higher in litter than in gravel. This indicated that the litter could act as a good biofilm carrier for the S-related microbes, and suggested that on-site S transformation may occur in the plant litter.

4. Discussion

4.1 Sulfate transformation in CWs

Sulfate is an electron acceptor commonly found in water and wastewater. In assimilatory sulfate reduction (ASR), sulfate can be integrated into organic S via uptake by plants and/or microorganisms. In dissimilatory sulfate reduction (DSR), sulfate is reduced to sulfide after the transfer of eight electrons. A previous study have shown that ASR contributed less than 0.3% to sulfate removal in wetlands, which means that DSR is probably the dominant pathway for sulfate removal (Wu et al., 2013). In this study,

the presence of cattail (*Typha latifolia*) made no significant contribution to sulfate reduction (Figure 2), which was consistent with the results from a previous study (Chen et al., 2014a). The low sulfate removal in the planted microcosms was probably due to root-mediated oxygen transfer, which would increase the redox potential and decrease the SRB activity (Stein et al., 2007). In contrast to the plants, adding cattail litter greatly stimulated the sulfate reduction due to the continuous input of labile organic carbon. However, the sulfate removal rates gradually decreased in the litter-added microcosms. The high sulfate removal rates during the initial stage were probably due to the rapid leaching of carbon sources (e.g. sugars) in the litter; and the low sulfate removal rates during the terminal stage were probably caused by the slow decomposition of recalcitrant materials (e.g. lignin) (Chimney and Pietro, 2006).

Despite the various sulfate removal rates observed in the microcosms, the sulfide concentrations were always low, and were much smaller than the theoretical sulfide production through sulfate reduction. In this study, the accumulation of solid AVS in the gravel indicated that the produced sulfide could have escaped from the water via metal sulfide precipitation. Additionally, S^0 formation (Figure 3) and increased sulfate concentration ($t < 20$ h, Figure 2) in the microcosms suggested that sulfide re-oxidation may have occurred. Previous studies found that 41%–90% of the reduced S was re-oxidized by root-mediated oxygen in planted wetlands (Wiessner et al., 2010; Wu et al., 2011). In this study, the nitrate in the influent was another electron acceptor that

could drive sulfide oxidation to sulfate in CWs (Section 3.4).

In wetlands, S^0 can be produced via either chemical or microbial oxidation of sulfide, or be eliminated through oxidation to sulfate or by bacterial disproportionation (Wu et al., 2011). Thus, S^0 is an important intermediate product in the wetland S cycle. Figure 3 shows that the S^0 concentrations were not stable during the initial and middle stages and this was probably caused by further oxidation to sulfate when sulfide is limited. The S^0 concentrations reached a steady state during the terminal stage, and there was a balance between S^0 formation and consumption. In DSR, the sulfate is firstly activated to form adenosine phosphosulfate (APS) and then APS is reduced to form sulfite. After this, sulfite can be directly reduced to sulfide or indirectly reduced to sulfide with the formation of thiosulfate (Ren et al., 2009). In this study, thiosulfate was only observed in the litter-added microcosms and its concentration rose as the carbon sources levels increased. Wiessner et al. (2010) also found that thiosulfate can accumulate in wetlands when there was a high carbon loading. The absence of thiosulfate under the carbon limited condition was probably due to the preferential utilization of a reduction pathway that allows SRB to obtain a higher energy yield when the electron donor is limited (Ren et al., 2009). In addition, some factors (e.g. temperature and pH) have also been reported to influence thiosulfate generation and accumulation (Qian et al., 2015).

Solid AVS was the most abundant S compound in the litter-added microcosms, and

accounted for 65.4%–77.0% of total sulfate removal (Figure 5). This suggested that a major proportion of the sulfide was precipitated along with metals and accumulated on the gravel after sulfate reduction. The AVS method (cold 1 N HCl) used in this study cannot quantitatively extract mackinawite, greigite and pyrite S (Rickard and Morse, 2005). This means that the AVS contents were probably underestimated. However, the disadvantages of this method do not affect the conclusions of this study. A previous study also found that most of the sulfate was converted to AVS in a wetland sediment with an S loading similar to this study (Baldwin and Mitchell, 2012). In this study, Fe oxides/oxyhydroxides are the dominant ferric mineral in the gravels. The reducing conditions in CWs should cause the reductive dissolution of Fe-oxys and thereby release ferric ion, indicating an important ferrous source for the formation of AVS. The intense S (-II) peak (Figure 4) and abundant Fe (4.7%, Figure S3) on the gravel surface further demonstrated the existence of Fe-S precipitation (FeS_2) in the litter-added microcosms. Pyrite (FeS_2) is widely considered to be a primary source of AVS in riverine, lake, and wetland sediments, and it can be oxidized via chemical and microbial pathways (Burton et al., 2009; Johnston et al., 2014; Zeng et al., 2013). In this study, the limited oxygen concentration in the influent and the long-term flooding operation created low redox conditions in the microcosms ($-200 \sim 50$ mV), which may have decreased the pyrite oxidation rates (Johnston et al., 2014). Furthermore, the organic carbon released by the litter could also compete with the reduced S for oxygen, thereby

slowing pyrite oxidation (Rigby et al., 2006). In addition to pyrite, some metastable iron sulfides (e.g. mackinawite and greigite) may also be present in the gravel surface layer and play a role in S cycling. Further studies need to produce quantitative descriptions of reactive Fe speciation.

4.2 Sulfur cycling pathways and the roles of microorganisms

Cattail is an aquatic plant that is widely used in CWs. During the decomposition of plant litters, lignocelluloses are first hydrolyzed by extracellular hydrolytic enzymes and then fermented into the liable carbon sources that drive sulfate reduction (Zhao et al., 2009). Bacterial sulfate reduction carried out by SRB is widely considered to be the dominant process involved in sulfate removal from CWs due to the significant enrichment of the heavier ^{34}S isotope (Wu et al., 2011). A previous study reported that SRB was usually divided into non-acetate oxidizers and acetate oxidizers (Hansen, 1993). The non-acetate oxidizers (*Desulfobulbus*, *Desulfovibrio* and *Desulfobacterium*) could utilize the liable fermentation products (hydrogen, lactate and pyruvate) as electron donors, and thus reduce sulfate to sulfide. The acetate oxidizers (*Desulfobacter*, *Desulfococcus* and *Desulfosarcina*) could oxidize acetate to CO_2 via TCA or acetyl-CoA pathways (Ren et al., 2009). Previous studies have also suggested that *Desulfobacter*, *Desulfovibrio*, *Desulfobulbus*, *Desulfococcus* and *Desulfobacterium* are the representative SRBs in wetlands (King et al., 2002; Lloyd et al., 2004; Russell et al., 2003). In this study, numerous different SRBs were found in the litter-added

microcosms. Among them, *Desulfobacter* and *Desulfovibrio* were very abundant in the litter-added microcosms (Figure S4), which indicated that the SRB could reduce sulfate using either acetate or other fermentation products as electron donors. The reduction in carbon sources produced during litter decomposition suggested that the SRB would compete with other microorganisms (e.g. methanogens) for available organic carbon (e.g. acetate), which could strongly influence the sulfate reduction rates during long operation of wetlands (Chen et al., 2014a).

After sulfate reduction, reduced S was produced and most of the sulfide was precipitated with Fe to form AVS on the gravel (Figure 5). Previous studies showed that the metal sulfide may be permanently immobilized in the sediments of wetlands if there is an anaerobic environment in the beds (Johnston et al., 2014; Wu et al., 2013). However, in this study, both oxygen and nitrate were present in the influent, which suggests that reduced S would become oxidized by chemical or microbial pathways. A previous study revealed that S microbial oxidation was much faster than chemical oxidation, and both aerobic oxidation (using oxygen as electron acceptor) and anoxic oxidation (using nitrate as electron acceptor) could contribute to the microbial oxidation of sulfide (Plas et al., 1992). If oxygen is unlimited, then a majority of the S would be oxidized by aerobic oxidation. However, the influent oxygen concentration was limited ($< 1 \text{ mg L}^{-1}$), and oxygen transport rate from air to water was very low ($k_{La} = 0.1 \text{ d}^{-1}$) in this study. The theoretical calculation indicated that the oxygen from the influent flow

and air transfer could only oxidize 1–1.5 mg L⁻¹ S to sulfate, much lower than the sulfate production in the litter-added microcosms (Figure 2). This suggested that anoxic microbial oxidation of sulfide to sulfate could be another pathway in the wetland S cycle. The mediation of sulfide oxidation by SOB has been reported by several groups (*Acidithiobacillus*, *Chromatium* and *Beggiatoa*) (Holmer and Storkholm, 2001). In this study, the SOBs, *Thiobacillus thioparus* and *Sulfuricurvum kujiense*, were abundant in the litter-added microcosms. They can use nitrate to oxidize sulfide, and couple the denitrification to S oxidation (Read-Daily et al., 2011). In this study, an increase in sulfate ($t < 20$ h, Figure 2) was concurrent with a decrease in nitrate (Figure S2). This indicated that they have roles in both S oxidation and nitrate reduction. The matched autotrophic denitrification rates (r_{AD}) and the sulfide oxidation rates (r_{SO-O}) further support this idea (Table 1). Additionally, the presence of *Desulfobulbus/Desulfocapsa* suggested that disproportionate S⁰ to sulfide/sulfate was possible to occur in CWs when sulfide concentrations were low (Finster et al., 1998; Lovley and Phillips, 1994).

4.3 Implications for tertiary wastewater treatment

In this study, the plant litter was reused as a self-supplying carbon source for sulfate reduction, and iron-rich gravel was used to efficiently immobilize the produced sulfide. This cooperation optimizes the carbon flow in wetlands and buffers sulfide toxicity in the receiving water body. Most significantly, simultaneous sulfur-driven autotrophic (S⁰ or AVS as the electron donor) and heterotrophic denitrification (litter carbon as the electron donor) can be achieved in one CW. When the influent nitrate

loading rates are low and carbon sources are sufficient for complete heterotrophic denitrification, the residual carbon sources will drive sulfate reduction and transfer the electron to sulfur (S^0 or AVS). Furthermore, S-driven autotrophic denitrification can occur during periods of high nitrate loading when heterotrophic denitrification alone is not sufficient to remove nitrate. Therefore, this study provides a promising, low-cost technology for tertiary or decentralized wastewater treatment when nitrogen loading rates are highly variable. Furthermore, S-based mixotrophic denitrification would greatly reduce the demand for organic carbon and wetland area compared to full heterotrophic denitrification. Although a litter-added CW with iron-rich filling material is an efficient ecosystem for sulfur and nitrogen removal, the benefits of iron-rich filling material must also be weighed against potential drawbacks. It has been reported that phosphate and sulfide removal efficiency declined in CWs with iron-rich gravel due to competition and iron exhaustion (Wu et al., 2012). Hence, further research is needed to investigate the dynamics of S, phosphate, and ferrous interactions during the long-term operation of CWs.

5. Conclusions

In this study, S transformations were investigated in CWs with and/or without plant. The results showed that the presence of *Typha latifolia* had a marginal effect on sulfate removal, but its carbon-rich litter greatly promoted sulfate removal. After sulfate reduction, most of the produced sulfide was immobilized on the iron-rich gravel surface

with only minor amounts lost through oxidation. Acid volatile sulfide and two valence states, S (-II) and S (0), were identified in the precipitate attached to the gravel surface. Elemental S and thiosulfate were detected as the intermediates in the pore water. Sulfur species quantification further showed that AVS was the main sink for the transformed sulfate-S (65%–77%), and elemental S and other unknown S compounds accounted for 5%–8% and 10%–26%, respectively. Most significantly, results showed that S-driven mixotrophic denitrification in CWs could effectively remove nitrate along with sulfide oxidation, which may lead to the simultaneous removal of organics, nitrate, and sulfate without excess toxic sulfide output during tertiary wastewater treatment.

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Appendix A. Supplementary material

Supplementary data associated with this article are available in the online version.

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Table caption

Table 1. Reaction rates for nitrate removal (r_{NR}), autotrophic denitrification (r_{AD}) and sulfide oxidation (r_{SO}) in five wetland microcosms.

Figure captions

Figure 1. The schematic diagram of experimental design concept of this study.

Figure 2. Sulfate and sulfide concentrations in the different wetland microcosms during the initial stage (a, batch 4), middle stage (b, batch 12) and terminal stage (c, batch 20).

Figure 3. Elemental S and thiosulfate S concentrations in the W0–W4 microcosms during the initial stage (a) batch 4, middle stage (b) batch 12, and terminal stage (c) batch 20.

Figure 4. Evolution of XPS $S2p$ spectra for the substances on gravel surface in the W1, W2 and W4 microcosms.

Figure 5. Distribution of S species in litter-added wetland microcosms based on the S mass balance calculation.

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	r_{NR}^{a} mg-N m ⁻² d ⁻¹	r_{AD}^{b} mg-N m ⁻² d ⁻¹	$r_{\text{SO-O}}^{\text{c}}$ mg-S m ⁻² d ⁻¹	$r_{\text{SO-T}}^{\text{d}}$ mg-S m ⁻² d ⁻¹
W0	44.4	8.0	nd.	20.1
W1	269.6	42.7	104.0	107.3
W2	611.0	50.7	114.7	127.4
W3	208.1	10.7	nd.	26.8
W4	491.8	34.7	93.3	87.2

a r_{NR} , nitrate removal rates, were obtained from the nitrate removal kinetic in batch 20.

b r_{AD} , autotrophic denitrification rates, were obtained from the variations of nitrate concentrations in the autotrophic denitrification kinetic tests (without organic matter).

c $r_{\text{SO-O}}$, observed sulfide oxidation rates, were obtained from the variations of sulfate concentrations in the autotrophic denitrification kinetic tests.

d $r_{\text{SO-T}}$, theoretical sulfide oxidation rates, were obtained from the theoretical calculations of sulfate formation for a complete sulfur-based autotrophic denitrification.

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