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Microbial sulfur transformations in novel laboratory-scale constructed wetlands treating artificial wastewater

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Microbial sulfur transformations in novel laboratory-scale constructed wetlands treating artificial wastewater

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SUMMARY

Constructed wetlands (CWs) are near-natural wastewater treatment systems. There, pollutant transformations are either direct components of or interlinked with the redox cycles of major chemical elements, such as sulfur. CWs have been applied in treatment of sulfate-rich wastewaters such as mining, tannery, textile wastewaters. However, hydrogen sulfide produced from sulfate reduction can cause environmental problems such as toxicity to plants and aquatic organisms. Hence, treatment of sulfur-rich wastewaters without sulfide accumulation is highly desirable. To date most prior work have focused on dynamics of sulfur compounds in CWs based on physicochemical evaluations, and little is known on the presence and the role of sulfur and sulfide oxidizing bacteria (SOB), specially in sulfide detoxification, in CWs.

The aim of the present study was to generate an enhanced view of microbial sulfur transformations and identify key microbial drivers for sulfur transformations with special focus on SOB in CWs. To this end, two newly designed laboratory-scale horizontal subsurface-flow constructed wetland models (CW1, CW2) were built. The wetlands's design favored a plug flow with the aim of limiting the physicochemical heterogeneity rectangular to the flow direction. Each wetland model had six separate compartments filled with gravel and was fed with artificial wastewater containing 300 mg/L of sulfate. In CW1, all six compartments were planted with soft rush, *Juncus effusus*, whereas only two middle compartments of CW2 were planted in order to observe microbial community shifts and effects of plants on sulfur transformations. Samples for physicochemical measurements and molecular analysis were collected from the individual compartments at the middle depth along the flow path. Next-generation 454 pyrosequencing was employed to dissect complex microbial communities in the wetland models, using the 16S rRNA gene as phylogenetic marker.

The results confirm previous findings that sulfate reduction and sulfur reoxidation occurred simultaneously in CWs. Sulfate reduction was predominant near the inlet zones, followed by the dominance of sulfur reoxidation. The role of *J. effusus* via oxygen release from the roots in enhancing sulfur reoxidation was observed. The abundances of SOB and sulfate reducing bacteria (SRB) were correlated to sulfur oxidation and sulfate reduction, respectively. Key

players in oxidation of inorganic sulfur compounds were found to be *Thiobacillus, Thiomonas,* and *Thiothrix* (at the roots). In addition, the identification of many other colorless and colored SOB in the systems reflects diverse SOB communities and their potential activities in sulfur oxidation. Dominant SRB were *Desulforhabdus, Desulfobacter, Desulfocapsa*, *Desulfovibrio*, and *Desulfobacula.* It appears that the roots of *J. effusus* were beneficial for the inhabiting and growth of SOB. In contrast, oxic environments at the roots' surfaces could inhibit growth of SRB. The results also demonstrate that plants significantly affected microbial community compositions in CWs. Furthermore, the findings add additional evidence for the interconnections between sulfur transformation processes and nitrogen and organic carbon removal in CWs. Nitrification and denitrification were likely to be inhibited by sulfide toxicity. Organic carbon removal was assumed to be mostly attributed to sulfate reduction. Detrimental effects of sulfide on growth of *J. effusus* were observed.

In conclusion, the results from this study enhance our understanding of microbial sulfur transformations in CWs by revealing key microbes involved in the sulfur cycle, underlining their role in sulfur transformations occurring in the wetlands and suggesting their interactions with plants. Such information could be of great help in optimizing design and operational conditions in CWs to achieve better performance in wastewater treatment.

TABLE OF CONTENTS

List of Abbreviations

ADP	Adenosine diphosphate
APAT	Adenylylsulfate phosphate adenylyltransferase
APS	Adenosine Phosphosulfate
Apr	Adenosine phosphosulfate reductase /APS reductase/Adenylyl sulfate reductase
ANAMMOX	Anaerobic Ammonium Oxidation
ATP	Adenosine triphosphate
BOD_5	Five-day Biological Oxygen Demand
COD	Chemical Oxygen Demand
CWs	Constructed Wetlands
DGGE	Denaturing Gradient Gel Electrophoresis
DNRA	Dissimilatory Nitrate Reduction to Ammonium
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
Dsr	Dissimilatory sulfite reductase
E_h	Redox potential
emPCR	Emulsion-based Polymerase Chain Reaction
Fcc	Flavocytochrome *c*
GSB	Green sulfur bacteria
HPLC	High Performance Liquid Chromatography
HSSF	Horizontal Subsurface-Flow
MID	Multiplex Identifiers

OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PFR	Planted Fixed-Bed Reactor
PP_i	Pyrophosphate
PNSB	Purple non-sulfur bacteria
PSB	Purple sulfur bacteria
PSO	*Paracoccus* sulfur oxidation
qPCR	Quantitative Polymerase Chain Reaction
Sat	Sulfate adenylyltransferase/ATP sulfurylase
SD	Standard deviations
SOB	Sulfur oxidizing bacteria
Sor	Sulfite acceptor oxidoreductase
Sox	Sulfur oxidizing system
Sqr	Sulfide quinone oxidoreductase
SRB	Sulfate reducing bacteria
TN	Total Nitrogen
TOC	Total Organic Carbon
TS	Total Sulfur

List of Tables

List of Figures

1. INTRODUCTION

1.1 Inorganic sulfur compounds in the environment

Inorganic sulfur exists in the environment in various compounds and multiple oxidation states, with -2 (sulfide), 0 (elemental sulfur) and +6 (sulfate) being the most significant in nature (Tang et al., 2009). Sulfur-containing minerals (e.g. pyrite, chalcopyrite) are abundant in rocks, and seawater is the largest sink of sulfate (Bitton, 2005). With the discovery that sulfur is widely distributed as sulfate minerals on the surface of Mars (Gendrin et al., 2005), the sulfur cycle has been argued to be an important geochemical cycle on the most-Earth-like planet in the solar system (King and McLennan, 2010).

Geological sulfur sources include volcanoes, hydrothermal vents and hot sulfur springs (Kuenen and Robertson, 1992). Human activities such as the burning of fossil fuels, mining industry, petroleum refining, tannery, and the pulp and paper industry also discharge large quantities of inorganic sulfur compounds into the environment (Mandeville, 2010). Considerable concentrations of sulfate have been found in various wastewaters such as textile and mining industries (Pokorna and Zabranska, 2015). Large discharge of inorganic sulfur compounds can cause disturbance of biological sulfur balance and detrimental impacts on all living organisms. Among inorganic sulfur compounds, hydrogen sulfide (H_2S) is highly toxic. With the typical smell of rotten eggs, hydrogen sulfide causes unpleasant condition and health effects such as headache, dizziness and respiratory problems. Serious eye damage can occur due to exposure to a concentration of 50 ppm of hydrogen sulfide (WHO, 2000) and at above 500 ppm, hydrogen sulfide can cause loss of consciousness (ATSDR, 2014). Furthermore, the problem of concrete corrosion caused by sulfide and oxidized sulfur species in sewer systems and digestors has been known for a long time (Olmstead and Hamlin, 1900). Sulfide can cause problems for anaerobic digestors by corrosion, inhibition of methanogens and subsequently affect biogas production efficiency (Bitton, 2005).

The conventional way for hydrogen sulfide removal, the use of strong oxidizing agents such as hydrogen peroxide (H_2O_2), chlorine gas (Cl_2), and hypochlorite (NaClO), is risky due to potential hazards associated with handling of these chemicals (Sorokin, 1994). Moreover, treatment technologies for sulfide removal based on physicochemical processes often require high demands of energy and operational cost (Oprime et al., 2001; Sorokin, 1994). At present, however, biological methods have been shown to be efficient and less expensive for sulfide removal (Pokorna and Zabranska, 2015). Microbial oxidation of inorganic sulfur compounds by sulfur oxidizing bacteria (SOB), one of the major processes involved in the global sulfur cycle (Friedrich et al., 2001) can be applied for sulfide removal. More than 100 years ago, the ability of oxidizing sulfide was described for the large filamentous SOB named *Beggiatoa* by Winogradsky (Larkin and Strohl, 1983; Winogradsky, 1887). Joshi and Hollis (1977) reported that *Beggiatoa* play a significant role in sulfide detoxification in the rice rhizophere. The ecological importance of SOB like *Beggiatoa* in wetland ecosystems like rice paddies suggest alternative biotechnologies, where wetlands can be employed in treatment of sulfur compounds. A comprehensive understanding on sulfur transformations in wetlands is therefore important. A brief overview on various sulfur transformation processes in wetland ecosystems is provided in the following section.

1.2 Sulfur transformations in constructed wetlands

Constructed wetlands are engineered systems designed to employ natural processes in wastewater treatment (Vymazal and Kröpfelová, 2008b). By utilizing ecological processes found in wetland ecosystems such as sedimentation, vegetation uptake and microbial interactions for pollution elimination, CWs play an important role in improving water quality in an environmentally friendly manner. In comparison with natural wetlands, there is a much greater degree of control of key parameters such as vegetation types, flow patterns, and overall dimensions in CWs (Vymazal and Kröpfelová, 2008b). Since the first experiments on the ability of wetland plants for treating wastewater in Germany in the early 1950s (Seidel, 1953; Vymazal, 2009), CWs have been widely applied for treating many different types of polluted water and wastewater of diverse origins such as acid mine drainage (Nyquist and Greger, 2009), landfill leachate (Bulc, 2006), municipal and agricultural wastewaters (Abou-Elela et al., 2013; Knight et al., 2000; Morari and Giardini, 2009).

The typical forms of inorganic sulfur in wetlands are sulfate in the oxidized zones and various sulfides in the reduced zones, though other sulfur intermediates (e.g. thiosulfate, elemental sulfur, sulfite) can also present (Sturman et al., 2008). Due to dynamic redox conditions in the rhizopheres of wetland plants, various sulfur transformation processes can occur simultaneously (Wiessner et al., 2010) (Figure 1.2-1). This section will discuss on the reduction of sulfate and oxidation of sulfide, physicochemical processes involving sulfur (e.g. mineral precipitation), sulfur disproportionation, the complex interconnections of sulfur transformations with wetland plants and other important redox processes, and the applications of sulfur transformations in wastewater treatment by CWs.

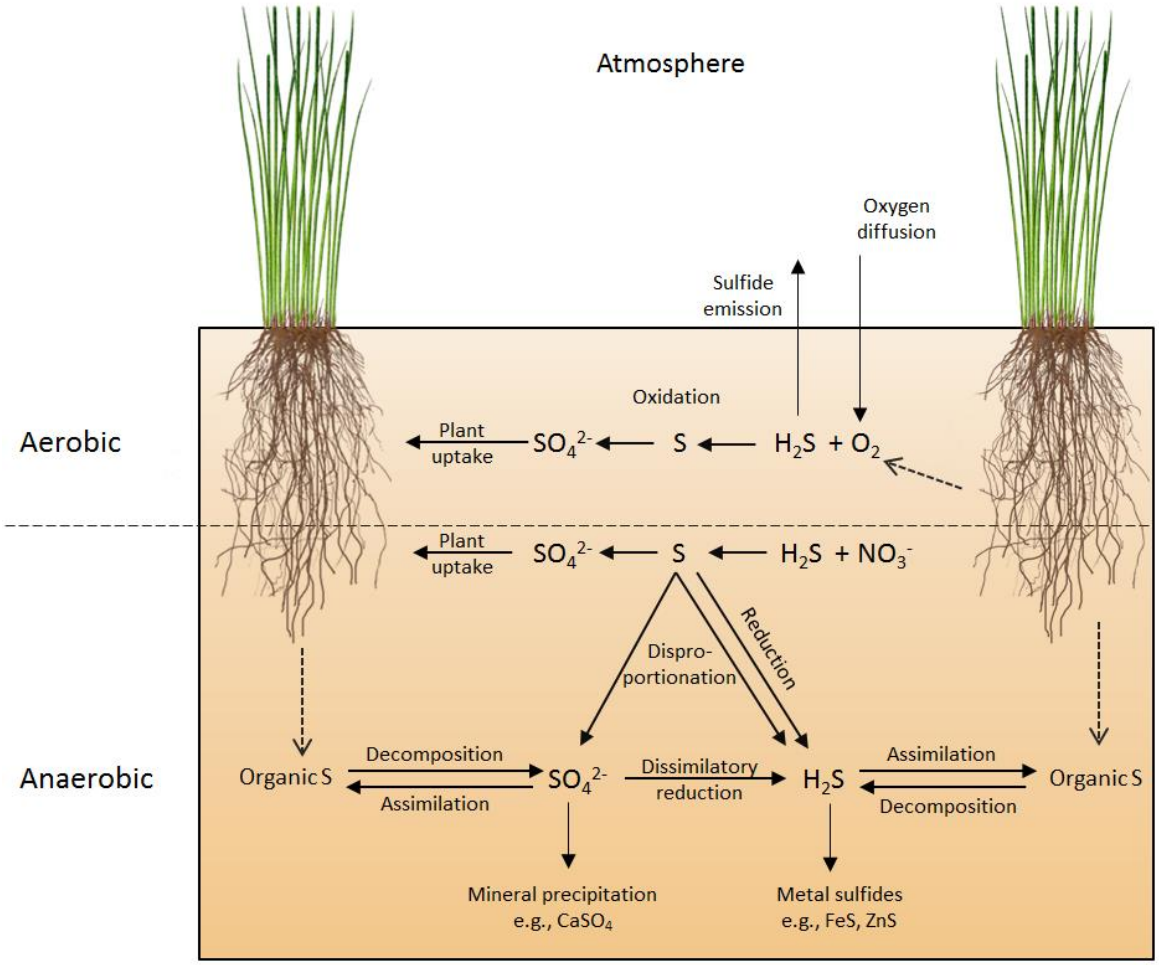


Figure 1.2-1. Main sulfur transformation processes in CWs (modified after Vymazal and Kröpfelová, 2008, and Wu et al., 2013)

1.2.1 Dissimilatory sulfate reduction

Dissimilatory sulfate reduction is the reduction of sulfate coupled with the oxidation of organic compounds or molecular hydrogen to generate sulfide under anaerobic conditions:

$$2\,[CH_2O] + SO_4^{2-} + H^+ \longrightarrow 2\,CO_2 + HS^- + 2\,H_2O$$

In CWs the process can be either enhanced by organic carbon from root exudates or inhibited by oxygen released from the roots of wetland plants (Stein et al., 2007; Wiessner et al., 2010). The consumption of protons increases the pH in the environment. Sulfate reduction has been reported at the pH range of 4 to 10 with an optimum at 6.8 (Vymazal and Kröpfelová, 2008b), and the redox value E_h at which sulfate reduction proceeds is typically below -100 mV (Reddy and D'angelo, 1994).

Dissimilatory sulfate reduction is catalyzed by sulfate-reducing bacteria (SRB) which are mainly strict anaerobes, although some species can tolerate oxygen (Rabus et al., 2006). SRB are classified into two groups: one group oxidizes organic compounds incompletely to acetate and the other one carries out complete oxidation of organic substrates into carbon dioxide (Table 1.2-1). The names of SRB usually begin with "*Desulfo*-" (Paul and Clark, 1996). Sulfur reducing bacteria (e.g. *Desulfuromonas, Desulfurella*) are unable to reduce sulfate but can utilize elemental sulfur as electron acceptor in anaerobic sulfur respiration to produce hydrogen sulfide (Bergey and Holt, 1994).

Most SRB can tolerate sulfide concentration of more than 10 mM, although at 4 – 7 mM, growth of some species (e.g. *Desulfotomaculum*) can be inhibited (Rabus et al., 2006). Due to the formation of sulfide, the presence and activity of SRB can be easily recognized in natural habitats by the smell of rotten eggs of hydrogen sulfide or black precipitation of ferrous sulfide (Rabus et al., 2006).

Table 1.2-1. Some sulfate- and sulfur-reducing bacterial genera

Genus	Oxidation of organic substrates	References
Desulfovibrio	Incomplete	Odom et al. (2013)
Desulfomicrobium	Incomplete	Odom et al. (2013)
Desulfobulbus	Incomplete	Odom et al. (2013)
Desulfobotulus	Incomplete	Odom et al. (2013)
Desulfofustis	Incomplete	Madigan et al. (2014)
Desulfobacula	Incomplete	Madigan et al. (2014)
Desulforhopalus	Incomplete	Madigan et al. (2014)
Thermodesulfobacterium	Incomplete	Odom et al. (2013)
Desulfomonas	Incomplete	Bergey and Holt (1994)
Desulforegula	Incomplete	Rees and Patel (2001)
*Sulfurospirillum**	Incomplete	Garrity et al. (2006)
Desulfosporosinus	Incomplete	Vos et al. (2011)
Desulfotomaculum	Both	Odom et al. (2013)
Desulfococcus	Complete	Odom et al. (2013)
Desulfobacterium	Complete	Odom et al. (2013)
Desulfobacter	Complete	Odom et al. (2013)
Desulfonema	Complete	Odom et al. (2013)
Desulfosarcina	Complete	Odom et al. (2013)
Desulfarculus	Complete	Odom et al. (2013)
Desulfomonile	Complete	Odom et al. (2013)
Desulfacinum	Complete	Madigan et al. (2014)
Desulforhabdus	Complete	Madigan et al. (2014)
Thermodesulforhabdus	Complete	Madigan et al. (2014)
*Desulfuromonas**	Complete	Bergey and Holt (1994)
*Desulfurella**	Complete	Bergey and Holt (1994)
Desulfovirga	Complete	Garrity et al. (2006)
Desulfobacca	Complete	Garrity et al. (2006)

Note: Incomplete: organic substrates oxidized to acetate; complete: organic substrates oxidized to CO_2
*: sulfur reducer

1.2.2 Assimilatory sulfate reduction

Assimilatory sulfate reduction results in the formation of organic sulfur. Sulfate is converted to sulfide intracellularly and incorporated into sulfur-containing amino acids, cysteine and

methionine, as well as some other cellular constituents such as coenzyme A (Brosnan and Brosnan, 2006; Vymazal and Kröpfelová, 2008b). Sulfide is released during the decomposition of biomass, but that amount is typically orders of magnitude lower than the amount of sulfide produced from dissimilatory sulfate reduction (Rabus et al., 2006). At high sulfate concentration, less than 0.3% sulfur removal from wastewater by mean of sulfur assimilation into plants was determined (Wu et al., 2013).

1.2.3 Mineral precipitation of sulfate

Abiotic mineral precipitation (e.g. $CaSO_4$, $BaSO_4$) can tribute to the decrease in sulfate concentrations in CWs. The application of gypsum (calcium sulfate dihydrate) crystallization in treating mine water has been well demonstrated (Geldenhuys et al., 2003). The precipitation of sulfate with calcium and barium has been shown to be effective in sulfate removal from laboratory wastewaters (Benatti et al., 2009). The solubility of minerals being precipitated should be taken into consideration as higher solubility requires larger concentration of cations to lower sulfate to the desired concentration (Tait et al., 2009).

1.2.4 Precipitation of metal sulfides

As a final product of dissimilatory sulfate reduction and biomass degradation, sulfide can react with cations of metals such as iron, zinc, copper to form metal sulfides. The precipitation of metal sulfides contributes to sulfide detoxification (Wu et al., 2013) and has the potential application in treatment of heavy metal-contaminated waters in CWs (Kosolapov et al., 2004). The following equation shows the precipitation of sulfide with Me^{2+} as metal cation:

$$Me^{2+} + H_2S \longrightarrow MeS\downarrow + 2H^+$$

Metal sulfides produced are highly stable in anoxic sediments in CWs (Kosolapov et al., 2004). In CWs receiving sulfate-rich wastewaters but containing low concentrations of heavy metals like domestic wastewater, precipitation of metal sulfides can cause detrimental effects for plants and microorganisms due to the lack of bioavailable trace elements (Wu et al., 2013).

1.2.5 Emission of hydrogen sulfide

Depending on pH and water temperature, hydrogen sulfide exists in the pore water of CWs as unionized form (H_2S), singly (HS^-) or doubly ionized (S^{2-}). In aquatic ecosystems like wetlands, the unionized form is dominant at low pH, whereas at high pH bisulfide (HS^-) is predominant (Kadlec and Wallace, 2008).

$$H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S^{2-} + 2H^+$$

In acidic waters such as mining water, the volatilization of H_2S causes unpleasant odor and toxicity to aquatic organisms. The emission of high amounts of H_2S to the atmosphere can lead to an increase in pH in the aquatic environment (Wu et al., 2013).

1.2.6 Oxidation of sulfide

In the presence of suitable electron acceptors such as oxygen and nitrate, sulfide produced from dissimilatory sulfate reduction can be reoxidized to sulfate and other sulfur compounds such as thiosulfate, sulfite and elemental sulfur as intermediates. Jørgensen (1990b) reported that thiosulfate is an important intermediate of the sedimentary sulfur cycle, accounting for 60 - 85% sulfide oxidation products in marine sediments in Denmark. Significant amounts of elemental sulfur were also found in laboratory-scale CWs fed with artificial sewage, and the presence of elemental sulfur provided evidence for the reoxidation of reduced sulfur compounds in CWs (Wiessner et al., 2010).

The oxidation of sulfide can occur via chemical reactions and biological pathways. Sturman et al. (2008) suggested that in the oxidized zones of CWs with high availability of oxygen, abiotic sulfide oxidation could be an important process. Thiosulfate and sulfate have been reported to be stable products of chemical sulfide oxidation (Zopfi et al., 2004). In hypoxic zones where oxygen is limited, rates of bacterial sulfide oxidation are highest (Sturman et al., 2008) and can outcompete chemical oxidation (Zopfi et al., 2001). While sulfide is a waste product from activities of SRB, SOB utilize sulfide as an energy source for growth. Chemolithotrophic SOB (or colorless SOB) are often found at the oxic-anoxic interface environments where they gain energy from the oxidation of sulfide to elemental sulfur and

sulfate (Zopfi et al., 2004). Phototrophic SOB such as purple and green sulfur bacteria often thrive in anoxic natural sediments where they can couple sulfide oxidation with carbon dioxide fixation in the presence of light (Sturman et al., 2008). Elemental sulfur produced from sulfide oxidation is accumulated intracellularly or extracellularly depending on the SOB species. Further oxidation of elemental sulfur to sulfate occurs under sulfide-limited conditions (Berg et al., 2014; Madigan et al., 2014). Additional details of SOB and microbial sulfur oxidation pathways are discussed in section 1.3.

1.2.7 Sulfur disproportionation

The term "sulfur disproportionation" has been used since the 1980s to describe a chemolithotrophic process involving the transformation of inorganic sulfur intermediates (Finster, 2008). Disproportionation of elemental sulfur, thiosulfate and sulfite are microbial mediated processes of the sulfur cycle and may occur in wetland ecosystems (Finster, 2008; Wu et al., 2013).

$4\ S^0 + 4\ H_2O \longrightarrow SO_4^{2-} + 3\ HS^- + 5\ H^+$ (Finster et al., 1998)

$S_2O_3^{2-} + H_2O \longrightarrow SO_4^{2-} + HS^- + H^+$ (Bak and Cypionka, 1987)

$4\ SO_3^{2-} + H^+ \longrightarrow 3\ SO_4^{2-} + HS^-$ (Finster, 2008)

Thus, intermediate products of sulfide oxidation, namely elemental sulfur, thiosulfate and sulfite are disproportionated into sulfate and hydrogen sulfide. Microbial sulfur disproportionation is carried out by bacteria also capable of dissimilatory sulfate reduction and which belong to the delta subclass of *Proteobacteria*, such as members of the genera *Desulfobacter*, *Desulfovibrio*, and *Desulfocapsa* (Finster, 2008) (Table 1.2-2).

Table 1.2-2. Some sulfur-disproportionating bacteria

Genus	Thiosulfate	Sulfite	Elemental sulfur
Desulfobacter curvatus	+*	-	n.d.
Desulfobacter hydrogenophilus	+*	-	n.d.
Desulfococcus multivorans	+*	-	n.d.
Desulfotomaculum nigrificans	+*	-	-
Desulfotomaculum thermobenzoicum	+	n.d.	n.d.
Desulfovibrio desulfuricans CSN	+	+*	n.d.
Desulfovibrio desulfodismutans	+	+	-
Desulfovibrio mexicanus	+*	+*	n.d.
Desulfovibrio aminophilus	+*	+*	n.d.
Desulfovibrio brasiliensis	+	n.d.	n.d.
Desulfovibrio oxyclinae	+	+	n.d.
Desulfomonile tiedje	+	n.d.	n.d.
Desulfobulbus propionicus	+	-	+
Desulfofustis glycolicus	n.d.	n.d.	+
Desulfocapsa thiozymogenes	+	+	+
Desulfocapsa sulfoexigens	+	+	+
Desulfocapsa Cad626	+	+	+

+: disproportionation with growth; + *: disproportionation without growth; -: unable to disproportionate; n.d.: not determined
Reference: Finster (2008)

While thiosulfate and sulfite disproportionation are commonly observed, sulfur disproportionation seems to be restricted to a few SRB. Furthermore, not all strains of SRB tested were able to couple that metabolism to growth (Finster, 2008). Although so far knowledge on the disproportionation of inorganic sulfur intermediates is not well advanced and the question why some SRB disproportionate sulfur compounds but do not grow remains unresolved (Finster, 2008), several studies have pointed out the important role of sulfur disproportionation in the sulfur cycle in marine and freshwater sediments. Thiosulfate disproportionation has been reported to be a key process in the transformation of sulfur intermediates in marine and freshwater environments (Jørgensen, 1990a; 1990b). It has been also revealed that several SRB (e.g. *Desulfobulbus propionicus*) are capable of oxidizing hydrogen sulfide to elemental sulfur using oxygen as electron acceptor as a mechanism to detoxify oxygen and subsequently undergo elemental sulfur disproportionation to obtain

energy for growth (Fuseler et al., 1996). This fascinating survival strategy may allow SRB to compete with SOB for substrates in (hyp)oxic environments (Finster, 2008).

1.2.8 Sulfur transformations in the interconnections with wetland plants and other important redox processes

Interactions with wetland plants

Sulfur is an important macronutrient for growth of plants. It is needed for the formation of enzymes and a constituents of some vitamins such as thiamine and biotin (Ceccotti, 1996). The sulfur content in wetland plants has been found to be in the range of 0.1 – 0.6% of the dry mass (0.35% on average) (Kadlec and Wallace, 2008). The important effect of hydrogen sulfide on several plant physiological processes, including photosynthesis and defense response to various abiotic stresses has been documented (Jin and Pei, 2015). However, high concentrations of sulfide produced from microbial sulfate reduction can reduce plant growth (van der Welle et al., 2006), affect photosynthesis (Tretiach and Baruffo, 2001), lead to root decay (Armstrong et al., 1996), and even plant death (Lamers et al., 1998). Values of sulfide concentration toxicity on wetland plant species are summarized in Table 1.2-3.

Table 1.2-3. Sulfide toxicity effects on some freshwater wetland plants

Wetland species	Concentration (µmol/L)
Calla palustris	150
Caltha palustris	170
Carex disticha	10 - 20
Carex nigra	10 - 20
Juncus alpinoarticulatus	30 - 50
Juncus effusus	500
Panicum hemitomon	1000
Phragmites australis	1400
Oryza sativa	170
Thelypteris palustris	150
Typha domingensis	920

Reference: Lamers et al. (2013)

In general, large freshwater wetland plants (e.g. *Phragmites australis*, *Typha domingensis*) showed higher sulfide tolerance (Lamers et al., 2013). The release of oxygen from the rhizosphere has been reported to be a special adaptation of wetland plants to survive under high sulfide concentrations (Armstrong et al., 1996). Oxygen released from the roots can inhibit sulfate reduction and favor the reoxidation of sulfide (Stein et al., 2007; Wiessner et al., 2010). SOB like *Beggiatoa* growing in the hypoxic layers of the root zones can detoxify sulfide by utilizing oxygen provided from the roots for sulfide oxidation (Joshi and Hollis, 1977). Plants on the other hand can enhance dissimilatory sulfate reduction by providing organic carbon in form of root exudates. Hsieh and Yang (1997) reported that sulfate reduction rates were significantly affected by the distribution of roots of *Juncus roemerianus*. It has been found that sulfate removal rates were three times higher in floating hydroponic root mats planted with *Phragmites australis* than with *Juncus effusus* (Saad et al., 2016). In addition, plant evapotranspiration can result in an increase in dissolved sulfate loads in the rhizosphere (El-Shatnawi and Makhadmeh, 2001).

Interactions with nitrogen transformation processes

Sulfur transformation processes are interconnected with nitrogen transformations in CWs (Wu et al., 2013). As a final product of dissimilatory sulfate reduction, sulfide can inhibit nitrogen uptake by plants (Chambers et al., 1998) and cause negative effects on nitrification capacity (Aesoy et al., 1998). At sulfide concentrations greater than 0.5 mg/L, microbial nitrification can be inhibited significantly (Aesoy et al., 1998). This inhibition could be due to sulfide toxicity to nitrifiers and the competition for oxygen between nitrifiers and sulfide oxidation reactions (Wu et al., 2013). It has been shown that high efficiency of dissimilatory sulfate reduction can inhibit ammonium removal (Wiessner et al., 2008; Wu et al., 2012). Furthermore, excessive sulfide concentrations can also inhibit activities of denitrifiers (Pokorna and Zabranska, 2015) and lower denitrification rates (Bowles et al., 2012; Pokorna et al., 2013).

The process called sulfur-driven autotrophic denitrification can be prevalent in anoxic sediments of CWs (Wu et al., 2013). Several chemolithotrophic SOB (e.g. *Thiobacillus denitrificans*) can couple sulfide oxidation with reduction of nitrate (Krishnakumar and

Manilal, 1999; Sorokin, 1994). Biological sulfide oxidation using nitrite as electron acceptor has been also reported (Cai et al., 2008). Simultaneous removal of nitrate and sulfate from wastewater containing high nitrate and sulfate loads by CWs has been described (Gruyer et al., 2013).

Interactions with organic carbon removal processes

The correlation of sulfate reduction and carbon removal in CWs has been well recognized (Garcia et al., 2010). Wiessner et al. (2005b) pointed out that carbon loads affected considerably sulfate reduction intensity. Doubling carbon load resulted in an immediate sulfate reduction of nearly 100% (Wiessner et al., 2005b), whereas carbon limited conditions caused an increase in sulfate concentrations along the flow path (Wiessner et al., 2010). In CWs, the release of organic carbon from root exudates could drive dissimilatory sulfate reduction, however studies on qualitative and quantitative on determinations of organic compounds from plants are limited (Wu et al., 2013). A study by Saad et al. (2016) suggested that difference in sulfate removal efficiencies in CWs planted with *P. australis* and *J. effusus* could be due to different flux of root exudates between those two helophyles.

On the other hand, concentrations of sulfate in the influents of CWs can cause significant effects on carbon removal efficiency (Wu et al., 2013). Higher carbon removal efficiency was achieved when sulfate was not present in the inflow (Caselles-Osorio and Garcia, 2007). In experiments with HSSF CWs receiving artificial sewage, Wu et al. (2012) also showed that better removal efficiency of dissolved organic carbon (DOC) was obtained at low sulfate concentrations in the inflow: DOC removal efficiency was 94% and 68% when sulfate concentration in the influent was 10 and 30 mg/L, respectively. A possible explanation for this might be that in systems with strong sulfate reduction and thus high generation rates of sulfide, sulfide can substitute organic carbon as electron donor for aerobic respiration and sulfur-driven denitrification process, therefore leading to the decrease in carbon consumption (Wu et al., 2013).

1.2.9 Sulfur transformations in constructed wetlands for sulfate-rich wastewater treatment applications

Sulfate is a common contaminant of wastewaters from industries such as mining, textile dyeing, tannery, and paper production (Kuschk et al., 2012). High sulfate concentrations of 20 – 500 mg/L can even be found in domestic wastewater, while in industrial wastewaters sulfate concentration can reach up to several thousand mg/L (Lens et al., 1998; Wiessner et al., 2008).

CWs have been widely applied for treatment of acid mine drainage (Nyquist and Greger, 2009; Riefler et al., 2008; Vymazal, 2009). Extremely acidic (pH = 1 – 3), high concentration of sulfate and dissolved metals are typical characteristics of mining wastewaters (Sturman et al., 2008). In CWs, sulfate can be reduced by microbial sulfate reduction, which leads to an increase in pH. As sulfide is produced from dissimilatory sulfate reduction, metals can be subsequently removed based on the precipitation of metal sulfides. Organic carbon is available in form of root exudates and dead plants (Kuschk et al., 2012). In technological applications, dissolved organic carbon is often added to promote microbial sulfate reduction when wetland-derived organic substrates are limitated (Lloyd et al., 2004). The applications of CWs in treating other industrial wastewaters such as wastewaters from textile, tannery industries, and pulp and paper production have been reported (Vymazal, 2009).

However, in general, treatment of sulfate by CWs is not as efficient as removal of other contaminants (Kadlec and Wallace, 2008; Wieder, 1989). Mean sulfate reduction efficiency in 32 different CWs treating various types of wastewaters (e.g. mining, textile, agricultural runoff, and municipal wastewaters) was only 14% (Kadlec and Wallace, 2008). Wiessner et al. (2005b) reported that mean sulfate removal from artificial domestic wastewaters was only 28%, whereas the removal of organic carbon (BOD_5) and ammonium were 83 and 44%, respectively. Similarly, mean sulfate reduction efficiency in 12 CWs treating domestic wastewaters in Czech Republic was approximately 26%, while nearly 77% BOD_5 and 32% ammonium were removed, correspondingly (Sturman et al., 2008). Low sulfate treatment efficiencies were probably due to the reoxidation of sulfide to sulfate by SOB activities in the wetland systems (Sturman et al., 2008). Evidence of sulfide reoxidation and SOB activity can also be observed by the formation of elemental sulfur as an intermediate of sulfide oxidation

(Sturman et al., 2008). The appearance of white blooms of *Thiobacillus* coincided with the high amount of sulfur deposits in wetland systems treating domestic wastewater (Winter and Kickuth, 1989). Therefore, knowledge regarding SOB and how their activities involved in the sulfur cycle in CWs will help to obtain better treatment performance. An insight into SOB (classification, physiology, common microbial oxidation pathways and current potential applications in environmental biotechnology) is provided in the following section.

1.3 Sulfur oxidizing bacteria

SOB are classified into two groups: colorless and colored SOB, which are capable of oxidizing reduced inorganic sulfur compounds, e.g. sulfide, thiosulfate, elemental sulfur for growth (Camacho, 2010; Robertson and Kuenen, 2006).

1.3.1 Colorless sulfur oxidizing bacteria

Colorless SOB lack photopigments and are thus differentiated them the colored (pigmented) phototrophic SOB (Lengeler et al., 1999; Robertson and Kuenen, 2006).

The colorless SOB are Gram-negative and comprise diverse genera falling into the phylum *Proteobacteria* (Table 1.3-1). Colorless SOB are ubiquitous in environments where reduced sulfur compounds are present, such as hydrothermal vents, sulfur springs, marine and freshwater sediments (Teske and Nelson, 2006). Most colorless SOB are mesophilic and neutrophilic (Robertson and Kuenen, 2006). Some SOB species are thermophilic (e.g. *Thermothrix*) and some are able to grow under extremely acidic conditions like acid mine drainage (e.g. *Thiobacillus ferrooxidans*) (Hazeu et al., 1988). Some archaea such as *Sulfolobus* and *Acidianus* can oxidize inorganic sulfur compounds, however since they are thermophilic and acidophilic (Lengeler et al., 1999), it is unlikely that they play an important role in sulfur oxidation in CWs.

Table 1.3-1. Genera of colorless sulfur bacteria

Genus	pH range		Anaerobic growth		References
	Neutro -philic	Acido- philic	NO_3^- as electron acceptor	S^0 as electron acceptor	
Traditional colorless SOB					
Thiobacillus (β)	+	+	+	v	Robertson and Kuenen (2006)
Thiomicrospira (γ)	+	-	+	-	Robertson and Kuenen (2006)
Thiosphaera (α)	+	-	+	-	Robertson and Kuenen (2006)
Thermothrix (β)	+	-	+	-	Robertson and Kuenen (2006)
Thiovulum (ε)	+	-	-	-	Robertson and Kuenen (2006)
Beggiatoa (γ)	+	-	+	+	Robertson and Kuenen (2006)
Thiothrix (γ)	+	-	+	-	Robertson and Kuenen (2006) Trubitsyn et al. (2013)
Thioploca (γ)	+	-	+	-	Robertson and Kuenen (2006)
Thiobacterium (γ)	+	-	-	+	Robertson and Kuenen (2006)
Thiomonas (β)	+	-	-	-	Garrity et al. (2006)
Thiospira (γ)	+	-	-	-	Robertson and Kuenen (2006)
Macromonas (β)	+	-	-	+	Robertson and Kuenen (2006)
Achromatium (γ)	+	-	-	+	Robertson and Kuenen (2006)
Sulfuritalea (β)	+	-	+	-	Kojima and Fukui (2011)
Sulfuricurvum (ε)	+	-	+	-	Kodama and Watanabe (2004)
Sulfurovum (ε)	+	-	+	+	Inagaki et al. (2004)
Sulfurimonas (ε)	+	-	+	+	Han and Perner (2015)
Other colorless bacteria capable of growth on reduced sulfur compounds					
Paracoccus (α)	+	-	+	-	Robertson and Kuenen (2006)
Hyphomicrobium (α)	+	-	-	-	Robertson and Kuenen (2006)
Alcaligenes (β)	+	-	+	-	Robertson and Kuenen (2006)
Pseudomonas (γ)	+	-	+	-	Robertson and Kuenen (2006)
Hydrogenobacter (A)	+	-	-	-	Robertson and Kuenen (2006)

+: example known to exist; - : example unknown; v: variable
α: *Alpha-proteobacteria*; β: *Beta-proteobacteria*; γ: *Gamma-proteobacteria*; ε: *Epsilon-proteobacteria*
A: *Aquificales*

Hydrogen sulfide produced from activities of SRB is an important substrate for colorless SOB (Robertson and Kuenen, 2006). Thiosulfate and elemental sulfur are other common inorganic sulfur substrates for colorless SOB. Oxygen is used as electron acceptor although several

species can utilize nitrate as electron acceptor under anaerobic conditions (Robertson and Kuenen, 2006). Some SOB (e.g. *Beggiatoa*) are able to use elemental sulfur as electron acceptor and molecular hydrogen or organic compounds (possibly acetate or stored carbohydrates) as electron donors to sustain anaerobic growth (Kreutzmann and Schulz-Vogt, 2016; Nelson and Castenholz, 1981; Schmidt et al., 1987; Schwedt et al., 2012). A few species can carry out complete denitrification to nitrogen (e.g. *Thiobacillus denitrificans*, *Thiomicrospira denitrificans)* while some are capable of reducing nitrate to nitrite only (e.g. *Thiobacillus thioparus, Thiothrix elkelboomii*) (Tang et al., 2009; Trubitsyn et al., 2013). Examples of biological oxidation reactions of inorganic sulfur compounds carried out by colorless SOB are summarized in Table 1.3-2.

Most colorless SOB can oxidize reduced sulfur compounds to sulfate as final oxidation product. Thiosulfate, elemental sulfur and tetrathionate can be also formed as intermediates (Lengeler et al., 1999). Elemental sulfur formed from sulfide oxidation can be accumulated intracellularly or extracellularly, creating white patches of microbial mats which can be recognized in natural environments. Filamentous colorless SOB such as *Beggiatoa, Thioploca* are living at the oxic-anoxic interfaces where they can compete successfully with chemical sulfide oxidation (Teske and Nelson, 2006). The growth of filamentous SOB like *Beggiatoa* and *Thiothrix* can cause problem of bulking in wastewater treatment plants (Tang et al., 2009).

Based on the carbon and energy source, colorless SOB are classified into three different groups: obligate chemolithotrophs, facultative chemolithotrophs, and chemolithoheterotrophs (Table 1.3-3).

Obligate chemolithotrophs (e.g. *Thiobacillus neopolitanus*, all of the known *Thiomicrospira* species) are strictly dependent on reduced inorganic sulfur compounds to grow and only use carbon dioxide as the carbon source. Facultative chemolithotrophs (e.g. *Thiosphaera pantotropha*, *Paracoccus denitrificans* and certain marine *Beggiatoa* species) have a versatile metabolism that allows them to grow either autotrophically, heterotrophically or mixotrophically. Mixotrophic growth can occur in the presence of both autotrophic and heterotrophic substrates, e.g. thiosulfate and acetate (Robertson and Kuenen, 2006).

Table 1.3-2. Examples of biological oxidation reactions of inorganic sulfur compounds by colorless SOB

Biological reactions			ΔG^o (kJ/reaction)
$H_2S + 0.5\ O_2$	$\rightarrow$	$S^o + H_2O$	-209.4
$S + 1.5\ O_2 + H_2O$	$\rightarrow$	$SO_4^{2-} + 2\ H^+$	-587.1
$H_2S + 2\ O_2$	$\rightarrow$	$SO_4^{2-} + 2\ H^+$	-798.2
$S_2O_3^{2-} + 2\ O_2 + H_2O$	$\rightarrow$	$2\ SO_4^{2-} + 2\ H^+$	-813.3
$S^{2-} + 1.6\ NO_3^- + 1.6\ H^+$	$\rightarrow$	$SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O$	-743.9
$S^{2-} + 0.4\ NO_3^- + 2.4\ H^+$	$\rightarrow$	$S^o + 0.2\ N_2 + 1.2\ H_2O$	-191.0
$S^{2-} + 4\ NO_3^-$	$\rightarrow$	$SO_4^{2-} + 4\ NO_2^-$	-501.4
$S^{2-} + NO_3^- + 2\ H^+$	$\rightarrow$	$S^o + NO_2^- + H_2O$	-130.4
$S^o + 1.2\ NO_3^- + 0.4H_2O$	$\rightarrow$	$SO_4^{2-} + 0.6\ N_2 + 0.8\ H^+$	-547.6
$S_2O_3^{2-} + 1.6\ NO_3^- + 0.2H_2O$	$\rightarrow$	$2\ SO_4^{2-} + 0.8\ N_2 + 0.4\ H^+$	-765.7

Reference: Tang et al. (2009)

Table 1.3-3. Colorless SOB classification based on physiological characteristics

Physiological types	Carbon source		Energy source	
	Inorganic	Organic	Inorganic	Organic
Obligate chemolithotroph	+	-	+	-
Facultative chemolithotroph	+	+	+	+
Chemolithoheterotroph	-	+	+	+

+: used by the group; -: not used (Adapted from Robertson and Kuenen, 2006)

Chemolithoheterotrophs (e.g. freshwater strains *Beggiatoa alba* B18LD, *Beggiatoa* sp. OH 75-2a) obtain energy from the oxidation of inorganic sulfur compounds but are unable to grow autotrophically (Nelson and Castenholz, 1981; Strohl et al., 1981).

Some chemoorganoheterotrophs (e.g. *Macromonas bipunctata*) can oxidize reduced sulfur compounds but are not capable of deriving energy from sulfur oxidation. An explanation could be that chemoorganoheterotrophs benefit from the oxidation of sulfide with hydrogen peroxide as a detoxification mechanism ($H_2O_2 + H_2S \rightarrow S + H_2O$) (Larkin and Strohl, 1983; Robertson and Kuenen, 2006).

1.3.2 Colored sulfur oxidizing bacteria

Colored (phototrophic) SOB utilize various reduced sulfur compounds (commonly sulfide) as electron donors for carbon dioxide fixation in the presence of light as energy source (Frigaard and Dahl, 2009). The photosynthetic growth of colored SOB is anoxygenic because instead of water (which is used as electron donor in oxygenic photosynthesis and oxygen is generated), reduced sulfur compounds are used electron donors which leads to the production of elemental sulfur or sulfate (Camacho, 2010):

$$2\ H_2S + CO_2 \xrightarrow{\text{light}} CH_2O + 2\ S^o + H_2O$$

$$H_2S + 2\ CO_2 + 2\ H_2O \xrightarrow{\text{light}} 2\ CH_2O + H_2SO_4$$

Colored SOB are divided into two main groups: purple sulfur bacteria and green sulfur bacteria (Table 1.3-4).

Table 1.3-4. Genera of colored sulfur bacteria and sulfur-metabolizing capabilities

Phototrophic SOB	**Sulfur substrates used**				**Sulfur deposits**	**Chemo-autotrophic growth**
	Sulfide	*Thiosulfate*	*Elemental sulfur*	*Sulfite*		
Purple sulfur bacteria						
Chromatiaceae						
Allochromatium	+	+	+	+	IC	+
Chromatium	+	-	+	-	IC	-
Halochromatium	+	+	+	+	IC	+
Isochromatium	+	-	+	-	IC	-
Lamprobacter	+	+	+	-	IC	+
Lamprocystis	+	+	+	-	IC	+
Marichromatium	+	+	+	+	IC	+
Rhabdochromatium	+	+	+	-	IC	-
Thermochromatium	+	-	+	-	IC	-

Thioalkalicoccus	+	-	+	-	IC	-
Thiobaca	+	-	-	-	IC	-
Thiocapsa	+	+	+	+	IC	+
Thiococcus	+	-	+	-	IC	-
Thiocystis	+	+	+	+	IC	+
Thiodictyon	+	-	+	-	IC	-
Thioflaviococcus	+	-	+	-	IC	-
Thiohalocapsa	+	+	+	+	IC	+
Thiolamprovum	+	+	+	-	IC	+
Thiopedia	+	-	+	-	IC	-
Thiorhodococcus	+	+	+	-	IC	+
Thiorhodovibrio	+	-	+	-	IC	+
Thiospirillum	+	-	+	-	IC	-
Ectothiorhodospiraceae						
Ectothiorhodosinus	+	+	-	-	EC	-
Ectothiorhodospira	+	+	+	+	EC	+
Halorhodospira	+	+	+	-	EC	-
Thiorhodospira	+	-	+	-	EC & IC	-
Green sulfur bacteria						
Chlorobiaceae						
Ancalochloris	+	-	-	-	EC	-
Chlorobaculum	+	+	+	-	EC	-
Chlorobium	+	+	+	-	EC	-
Chloroherpeton	+	-	-	-	EC	-
Prosthecochloris	+	-	+	-	EC	-

+: example known to exist; - : example unknown; IC: intracellularly; EC: extracellularly

Reference: Frigaard and Dahl (2009)

Purple sulfur bacteria (PSB)

The PSB are *Gammaproteobacteria* and are classified into two families: *Chromatiaceae* and *Ectothiorhodospiraceae.* The feature discriminating the two families is that the former deposit sulfur inside the cells, whereas the later accumulates sulfur outside the cells (except *Thiorhodospira sibirica*). The main photopigments are bacteriochlorophyll *a* and *b* and CO_2 is fixed via the Calvin-Benson-Bassham cycle (Frigaard and Dahl, 2009).

Some members of the family *Chromatiaceae*, e.g. *Chromatium okenii*, *Allochromatium warmingii*, are obligately phototrophic, strictly anaerobes and use only sulfide as electron donor, while other members, e.g. *Allochromatium vinosum, Thiorhodococcus*, *Thiocapsa* are able to grow chemolithotrophically on several different reduced sulfur compounds (Frigaard and Dahl, 2009). The ability to accumulate sulfur intracellularly brings advantages for *Chromatiaceae*: deposited elemental sulfur can be used as electron donor for photosynthesis when other electron donors (e.g. sulfide, thiosulfate) are unavailable and can be served as electron acceptor for the fermentation of stored carbohydrates under dark anoxic conditions (Camacho, 2010). Habitats of *Chromatiaceae* are freshwater, marine or saline inland water environments (Camacho, 2010).

In the family *Ectothiorhodospiraceae*, *Ectothiorhodospira* is the only known genus able to utilize sulfide, thiosulfate, sulfur and sulfite as electron donors, and chemolithotrophic growth has been recorded for *Ectothiorhodospira haloalkaliphila*, *Ectothiorhodospira shaposhnikovii* (Frigaard and Dahl, 2009). Some species can even grow under microaerobic or aerobic environments in the dark (Camacho, 2010). Members of *Ectothiorhodospiraceae* are mostly found in saline and hypersaline environments like soda lakes (Camacho, 2010).

The group called purple non-sulfur bacteria (PNSB) contains a number of bacteria able to use reduced sulfur compounds for photoautotrophic growth, however with much lesser extent compared to PSB (Frigaard and Dahl, 2009). Representatives of PNSB fall into three different taxonomic orders in the class *Alphaproteobacteria*: *Rhodospirillales*, *Rhizobiales* and *Rhodobacterales* (Frigaard and Dahl, 2009). Sulfur is the end product of sulfide oxidation in some species (e.g. *Rhodobacter capsulatus*), while other species (e.g. *Rhodopseudomonas palustris*) oxidize sulfide completely to sulfate. The ability to use thiosulfate was reported (e.g. *Rhodopila*, *Rhodovulum*) while sulfite utilization has not been observed (Frigaard and Dahl, 2009).

Green sulfur bacteria (GSB)

The GSB belong to the family *Chlorobiaceae*, phylum *Chlorobi*. Unlike some species of PSB where chemoautotrophic growth was observed, GSB are obligate photoautotrophs and grow under strictly anaerobic conditions. All GSB can utilize sulfide, and in an environment with

various reduced sulfur compounds present, sulfide is preferentially used (Frigaard and Dahl, 2009). *Chlorobium* and *Chlorobaculum* can utilize thiosulfate, sulfide and elemental sulfur as electron donors for sulfur oxidation. The ability to use sulfite has not yet been recorded in GSB (Frigaard and Dahl, 2009). The affinity for sulfide of GSB is much higher than that of PSB, and GSB are frequently found in the environments with low sulfide concentrations (Camacho, 2010).

Bacteriochlorophyll *c, d* and *e* are the main photopigments located in large organelles called chlorosomes, which allow light energy to be captured highly efficiently. Therefore, GSB can grow comparably well at low light intensities (Frigaard and Dahl, 2009). CO_2 fixation is carried out via reductive tricarboxylic acid cycle, which a lower ATP requirement per CO_2 molecule fixed compared to CO_2 fixation via the Calvin-Benson-Bassham cycle active in PSB. This explains the dominance of GSB over PSB under light-limiting conditions (Camacho, 2010; Frigaard and Dahl, 2009). The main habitats of GSB are anoxic freshwater and estuarine environments where light reaches sulfur-containing-water layers (Garrity et al., 2001).

1.3.3 Microbial sulfur oxidation pathways with gene systems involved

The sulfur oxidation systems in SOB are complex, with different enzyme systems involved such as sulfide quinone oxidoreductase (Sqr), flavocytochrome *c*/sulfide dehydrogenase (FccAB), dissimilatory sulfite reductase (Dsr), sulfate reductases (APS reductases or AprAB), sulfate adenylyltransferase (ATP sulfurylase or Sat), sulfite acceptor oxidoreductase (Sor) and sulfur oxidizing system (Sox). A brief overview of those complex systems is presented in the following sections.

1.3.1.1 Oxidation of sulfide to elemental sulfur: Sqr/Fcc system

Two enzyme systems are known to be able to catalyze the initial oxidation of hydrogen sulfide to elemental sulfur: (1) sulfide quinone oxidoreductase (Sqr) and (2) flavocytochrome *c*/sulfide dehydrogenases (FccAB) (Friedrich et al., 2005).

Sqr has been reported to be widely distributed among prokaryotes and is obligatory for growth on sulfide in photo- and chemolithoautotrophic bacteria (Griesbeck et al., 2000). Flavocytochrome *c* (Fcc) consists of a flavoprotein subunit, FccB and a *c*-type cytochrome subunit, FccA (Yamamoto and Takai, 2011). Different from Sqr, FccAB is not present in many SOB and this enzyme is not obligatory for sulfide oxidation (Griesbeck et al., 2000). Both Sqr and Fcc have been found to be expressed in the purple sulfur bacterium *A. vinosum*, however no effect on sulfide oxidizing ability after mutational inactivation of *fcc* was observed (Griesbeck et al., 2000). Activities of Fcc in sulfide oxidation of SOB is so far not fully understood and awaits further investigation.

1.3.1.2 Oxidation of elemental sulfur to sulfite: Dsr system

The Dsr system is essential for the oxidation of stored sulfur in many photo- and chemolithoautotrophic SOB which are able to form sulfur globules during the oxidation of reduced sulfur compounds (Grimm et al., 2010). The dependence of Dsr system for sulfur globule oxidation has been well demonstrated for *A. vinosum* (Dahl et al., 2005) and *Chlorobaculum tepidum* (Holkenbrink et al., 2011). The inability of growth on elemental sulfur in *Chlorobium ferrooxidans* is in accordance with the absence of *dsr* genes in this organism (Frigaard and Dahl, 2009).

The Dsr system has been extensively studied in *A. vinosum* (Dahl et al., 2005; Grimm et al., 2010; Sander et al., 2006). The system consists of DsrABEFHCMKLJOPNRS, in which DsrMKJOP is a transmembrane redox complex, which probably transfer electrons released from the sulfur oxidation to the photosynthetic electron transport (Sander et al., 2006). Is has been reported that each single protein of the DsrMKJOP complex is obligatory for sulfur oxidation in *A. vinosum* (Sander et al., 2006). DsrEFH and DsrC are responsible for translocate sulfur across the membrane into the cytoplasm to DsrAB (Grimm et al., 2010). Homologues of *dsr*EFH and *dsr*C are present in many *Gammaproteobacteria* lacking *dsr*AB, including *E. coli* and many other *Gammaproteobacteria* (Dahl et al., 2008).

1.3.1.3 Oxidation of sulfite to sulfate: SOR:sulfite acceptor oxidoreductase, APS reductase, ATP sulfurylase

Two pathways are known for the oxidation of sulfite to sulfate in SOB: (i) the direct pathway by sulfite:acceptor oxidoreductase (Sor) and (ii) the indirect pathway via adenylyl-sulfate (APS) as an intermediate (Kappler and Dahl, 2001).

Direct pathway by Sor

Direct oxidation of sulfite to sulfate is a widespread mechanism among SOB (Kappler, 2011). Sulfite is directly oxidized to sulfate by Sor and two electrons released are transferred to the catabolic electron transport system, ultimately generating ATP (Kappler and Dahl, 2001):

$$SO_3^{2-} + H_2O \xrightarrow{Sor} SO_4^{2-} + 2e^- + 2H^+$$

Indirect pathway via the intermediate APS

The enzymes involved in the indirect oxidation of sulfite to sulfate are APS reductase (AprAB) and ATP sulfurylase (Sat). First, APS reductase catalyzes the oxidation of sulfite to APS. In the next step, APS is converted to sulfate and either ATP or ADP is generated by the activities of ATP sulfurylase or APAT (adenylylsulfate phosphate adenylyltransferase), respectively.

$$SO_3^{2-} + AMP \xrightarrow{AprAB} APS + 2e^-$$

$$APS + PP_i \xrightarrow{Sat} SO_4^{2-} + ATP$$

$$APS + P_i \xrightarrow{APAT} SO_4^{2-} + ADP$$

The indirect pathway seems to be restricted to some GSB and the PSB *Chromatiaceae* (Frigaard and Dahl, 2009).

The ability to oxidize sulfite to sulfate via either one or both pathways vary among SOB, even in different strains of the same genus (Kappler and Dahl, 2001). Both pathways have been observed in *T. denitrificans*, *T. thioparus*, *A. vinosum* (Friedrich, 1997).

1.3.1.4 Direct oxidation of inorganic sulfur compounds to sulfate: Sox system

The oxidation of reduced sulfur compounds such as thiosulfate, hydrogen sulfide, elemental sulfur and sulfite by the Sox system has been reported for many SOB (Friedrich et al., 2001; Ghosh and Dam, 2009). The common current model of this enzyme system comprises four periplasmic complexes SoxXA, SoxYZ, SoxB and $Sox(CD)_2$ (Meyer et al., 2007), of which SoxB is widely distributed, highly conserved and has been frequently used as a genetic marker for phylogenetic analyses of SOB (Petri et al., 2001). SoxB has been detected in all investigated chemo- and phototrophic SOB that form sulfur granules, e.g. *Beggiatoa, Thiothrix, Thiobacillus, Chromatiaceae, Ectothiorhodospiraceae* (Meyer et al., 2007). However most sulfur-storing organism do not have SoxCD in their Sox systems (Frigaard and Dahl, 2009).

Two common sulfur oxidation pathways via the Sox system are known: (i) the *Paracoccus* sulfur oxidation (PSO) pathway, where thiosulfate is oxidized directly to sulfate without the formation of any sulfur intermediates by using SoxCD and (ii) the branched oxidation pathway, where thiosulfate is oxidized to other sulfur intermediates and sulfate without SoxCD (Ghosh and Dam, 2009). The PSO pathway is proposed to be a common mechanism in non-sulfur-storing organisms such as *Paracoccus pantotrophus* (Quentmeier et al., 1999), whereas the branched oxidation pathway appears to operate in sulfur-storing organism intra- or extracellularly such as *A. vinosum* and some species of *Beggiatoa*, *Thiothrix* and *Thiobacillus* (Friedrich et al., 2005; Ghosh and Dam, 2009; Hensen et al., 2006).

1.3.4 Sulfur oxidizing bacteria in wastewater treatment

Potential applications of SOB in treatment of sulfide-rich gaseous and liquid streams and various wastewaters have been documented (Tang et al., 2009). Removal of inorganic sulfur compounds based on activities of SRB and SOB in a biofilm of a fluidized bed reactor has

been reported (Celis-García et al., 2008). Treatment of textile wastewater by sulfate-reducing and sulfide-oxidizing membrane bioreactors has been recently published (Yurtsever et al., 2016).

Among SOB, the genus *Thiobacillus* has been most extensively studied for wastewater treatment applications. Sublette et al. (1998) showed that sulfide was removed by 99% from wastewater originating from an oil-field by *Thiobacillus denitrificans*. Application of *T. denitrificans* in sulfide removal coupled with nitrate reduction to treat industrial wastewaters has achieved promising results (Can-Dogan et al., 2010; Kleerebezem and Mendez, 2002). The potential use of *Thiobacillus* for sulfide removal from latex rubber wastewater has been also demonstrated (Kantachote and Innuwat, 2004). Lestari et al. (2016) reported that the efficiency of sulfide removal from biogas was 97.2% by using biofilm of *Thiobacillus* isolated from sludge of wastewater of a biogas plant.

The use of SRB in sulfate and organic matter removal and *Beggiatoa* species in sulfide removal by a microaerophilic upflow sludge bed reactor for domestic wastewater treatment has been reported (Basu et al., 1995). The green sulfur bacterium *Chlorobium limicola* has been successfully used for sulfide removal from liquid and gaseous effluents (Henshaw et al., 1998). Lee and Kobayashi (1992) also highlighted the potential application of the PNSB *Rhodobacter capsulatus* in elimination of unpleasant odor from animal feeding plants.

Despite successful applications of SOB in wastewater treatment, some challenges in sulfide-removing bioreactors using SOB remain. The use of photosynthetic SOB for sulfide removal requires strictly anaerobic conditions and constant sources of light energy. Furthermore, unwanted sulfate can be produced as a final oxidation product and the presence of organic compounds from phototrophic sulfide oxidation again stimulates growth of SRB and sulfide will be regenerated. Therefore, development of efficient light energy and anaerobic conditions for photosynthetic SOB and biological sulfide oxidation at the level of elemental sulfur in biotreatment systems are further investigated (Tang et al., 2009).

Several non-traditional SOB (e.g. *Alcaligenes*, *Pseudomonas*) have been isolated from an artificial wetland receiving tannery wastewater and the sharp decrease in sulfide concentrations was suggested to be attributed to SOB activities (Aguilar et al., 2008). The

colorless SOB *Thiobacillus* and *Thiomonas* have been also identified in CWs (Chen et al., 2016; Hallberg and Johnson, 2005; Li et al., 2016). In general, however studies on SOB in CWs for wastewater treatment applications are sparse.

1.4 Research gaps and objective of this study

Most earlier work was focused on the dynamics of sulfur species in CWs and the interactions of sulfur cycle with carbon and nitrogen removal based on chemical measurements (Wiessner et al., 2008; 2010; Wu et al., 2012). Microbial distribution of SRB and SOB in CWs with and without consideration of oxygen released from plant roots has been previously illustrated by HYDRUS-CWM1 model (Langergraber and Šimůnek, 2012). Distribution patterns of SRB and SOB in CWs have been also predicted by modelling results (Samsó and García, 2013). Recently, a study on nitrogen and sulfur transformation processes by two different helophytes has been published (Saad et al., 2016), however microbial analysis in the rhizospheres of those wetlands was not performed. There have been a few studies on microbial communities involved in the sulfur cycle in CWs that employed both cultivation-methods and molecular techniques (Chen et al., 2016; Hallberg and Johnson, 2005; Lloyd et al., 2004; Nicomrat et al., 2006). The present study aimed at generating an enhanced view of microbial sulfur transformations in CWs and to identify key microbes that are drivers for sulfur transformations with special focus on sulfur oxidation path. In order to provide an insight into the "black-box" CW systems, measurements of sulfur compounds as well as other physico-parameters related to sulfur transformations were combined with molecular microbial analysis in the rhizospheres of wetland plants. Two newly-designed HSSF CWs at laboratory-scale were employed in this study to achieve uniform flow path through the wetlands.

The hypotheses of the study are as following:

- Dynamic sulfur transformations with dissimilatory sulfate reduction and reoxidation of reduced sulfur compounds can be observed in CWs;
- Microbial communities involved in the sulfur cycle (SOB and SRB) are present and key players in sulfur transformations can be identified;
- Plants can enhance reoxidation of reduced sulfur compounds and affect distributions of microbial communities;
- Nitrification-denitrification and plant growth could be affected by sulfide toxicity.

The expected outputs of the study are anticipated to enhance wastewater treatment efficiency in CWs.

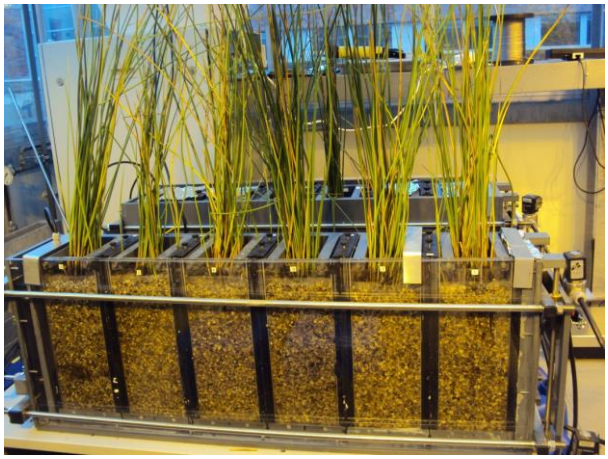
2. MATERIALS AND METHODS

2.1 Description of laboratory-scale constructed wetland models

Two HSSF CWs at laboratory-scale (CW1, CW2) were established in containers made from acrylonitrile-butadiene-styrene, glass, and metal (length 92 cm, width 15 cm, height 35 cm) (Figure 2.1-1). Each CW had six separated compartments (dimensions of each compartment: length 15 cm, width 15 cm, height 35 cm) and rectangular perforated plastic sieves were placed between each compartment. The design principles of the CWs were in order to achieve a plug flow through the wetlands aiming at limiting the physicochemical heterogeneity rectangular to the flow direction.

The compartments of both CW were filled with 48 kg gravel (2 – 6 mm in diameter) each up to a height of 30 cm. In CW1, all six compartments were planted with *Juncus effusus* (common or soft rush), whereas only the two middle compartments of CW2 were planted to observe effects of plants and associated microbial dynamics on sulfur transformation processes. *J. effusus* was chosen in the present study because of its wide application in treatment wetlands and its frequent use in wetland studies (Saad et al., 2016; Wiessner et al., 2010; Wu et al., 2012).

The two wetlands were placed in the Phytotechnicum at the UFZ. The temperature was set to 22^{o}C during the day (from 6 a.m. to 9 p.m.) and 16^{o}C during the night.



CW1 (water flow from left to right)

CW2 (at the front, planted in compartments 3 & 4)

Figure 2.1-1. Photographs of the employed CWs

2.2 Experimental set-up

Before being fed with artificial wastewater, the CWs were continuously fed with tap water for two months to allow plant roots to develop in the gravel beds. Subsequently, the CWs were fed with artificial wastewater from 6 March 2014 until the end of October 2015 for a total operation time of 605 days. A modified artificial wastewater according to a standard artificial domestic wastewater (DIN-38412-T24, 1981) was used (Wiessner et al., 2010). The ingredients (dissolved in tap water) of the influent are shown in Table 2.2-1.

Table 2.2-1. Compositions of artificial wastewater

Compound	Amounts added (mg/L)
CH_3COONa	204.9
C_6H_5COONa	107.1
K_2HPO_4 x $3H_2O$	36.7
NaCl	7
NH_4Cl	118
$MgCl_2$ x $6H_2O$	3.4
$CaCl_2$ x $2H_2O$	4
Na_2SO_4	222
Trace mineral solution	1 ml/L

Table 2.2-2. Compositions of trace mineral solution

Compound	Amounts added (g/L)
EDTA-Na	0.1
$FeSO_4$ x $7H_2O$	0.1
$MnCl_2$ x $4H_2O$	0.1
$CoCl_2$ x $5H_2O$	0.17
$CaCl_2$ x $6H_2O$	0.1
$ZnCl_2$	0.1
$CuCl_2$ x $5H_2O$	0.02
$NiCl_2$ x $6H_2O$	0.03
H_3BO_3	0.01
Na_2MoO_4 x $2H_2O$	0.01
H_2SeO_3	0.001
HCl	3 ml/L

The resulting concentrations (in mg/L) were as follows: theoretical total organic carbon (TOC): 122.4, NH_4^+-N: 30.9, PO_4^{3-} – P: 5 and SO_4^{2-} – S: 100. Final concentration of SO_4^{2-}- S in the inflow was 100 mg/L because it was the sum of 50 SO_4^{2-} - S in 222 Na_2SO_4 and 50 SO_4^{2-}- S (mg/L) as the mean concentration in tap water used.

The solution containing CH_3COONa, C_6H_5COONa, K_2HPO_4 and NaCl were injected into the systems by using a syringe pump. Other compounds, namely NH_4Cl, $MgCl_2$, $CaCl_2$, Na_2SO_4 and trace mineral solution, were dissolved in tap water in inflow tanks and introduced to the systems by a peristaltic pump. The experimental set-up with influents and effluents are shown in Figure 2.2-1.

The water level was adjusted to 4 cm below the gravel bed surface. The pore water volume of CW1 and CW2 was 11.03 and 11.31 L, respectively. The mean inflow and outflow rates and the hydraulic retention time (HRT) in each wetland during the whole operating period are shown in Table 2.2-3.

Table 2.2-3. Inflow, outflow rates and hydraulic retention time in laboratory-scale CWs

Wetland	Inflow (L/d)	Outflow (L/d)	HRT (d)
CW1	3.43 ± 0.07	1.51 ± 0.09	4.51 ± 0.09
CW2	3.24 ± 0.27	2.86 ± 0.09	3.72 ± 0.19

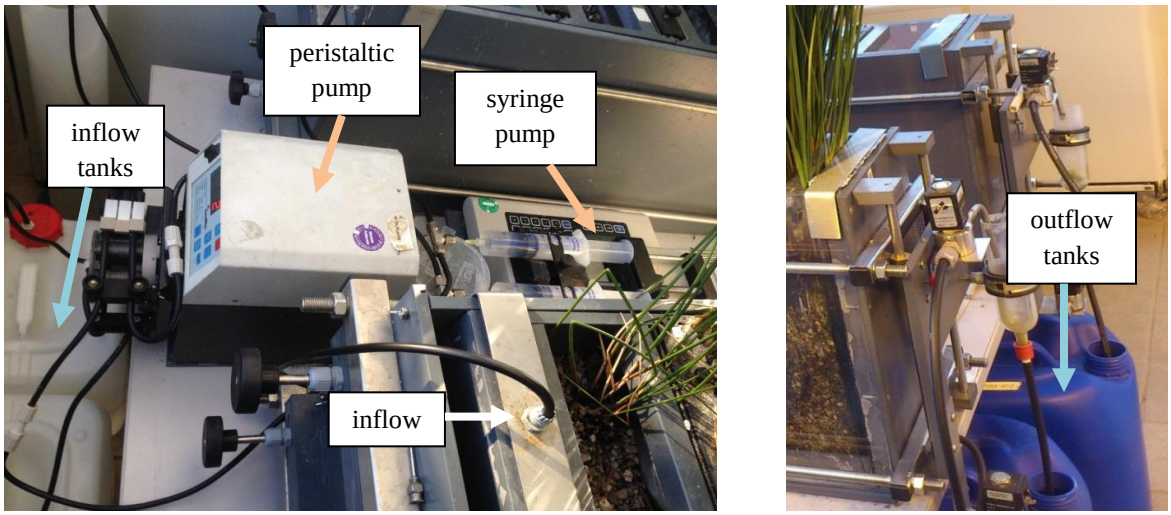


Figure 2.2-1. Experimental set-up with inflow- and outflow-tanks

System maintenance was done by cleaning water pipes once per week to avoid blockages caused by biomass accumulated in the pipes.

Water balance was done twice per week (every Monday and Thursday) to measure the inflow and outflow volumes, which were used for the calculations of water loss (due to evapotranspiration) and contaminant loads (details in calculation formulas are shown in section 2.4.6). The numbers of healthy plant shoots were counted twice per month in order to record plant growths and observe effect of sulfide toxicity on plants. Brown shoots which were easily to remove were discarded.

2.3 Conceptual framework

In order to achieve the research objectives "*obtain an enhanced view of microbial sulfur transformations and identify key microbial drivers for sulfur transformations in CWs (with special focus on SOB)*", the research design was divided into 4 phases as following:

Phase 1:

- The CWs were initially tested to detect any technical problems before experimental procedures started. Minor technical problems occurred frequently since the wetland systems had been newly designed and built.

- During this initial study phase, in parallel with testing the systems, a bioinformatic study on the sequenced genome of a model sulfur oxidizing bacterium, *Beggiatoa alba* B18LD was performed. This microbe is the type strain of the environmentally important genus *Beggiatoa* (Mezzino et al., 1984). Together with members of the closely related genus *Thioploca*, *Beggiatoa* is an important player in the sulfur cycle of many (semi)aquatic environments including wetlands (Teske and Nelson, 2006). The genomic analysis of the model sulfur oxidizer *B. alba* B18LD therefore helped to obtain more detailed mechanistic understanding of microbial sulfur oxidation in CWs. A manuscript titled "The genome of *Beggiatoa alba* B18LD – a large sulfur oxidizing *Gammaproteobacterium"* was prepared to submit to Journal *Standards in Genomic Sciences* (Appendix).

Phase 2:

- After technical start-up problems of the wetland models had been fixed, three sampling campaigns were conducted (April, July and August 2014) in order to establish experimental procedures. During each sampling campaign, pore water samples for chemical and microbiological analyses were collected from the inflow, outflow, and at the distance from 8 to 83 cm along the flow path at three different depths (8, 16 and 23 cm below the gravel surface) from all compartments. During this phase the wetland systems showed high variability of chemical parameters probably due to substantial differences of plant densities over time (see Figures 3.3-5 and 3.3-6).

- PCR targeting the 16S rRNA gene and functional gene markers (*sqr*, *apr*A and *sox*B) of total 84 extracted DNA samples was carried out. The 16S rRNA gene is highly conserved among different bacterial species and typically used for phylogenetic studies (Janda and Abbott, 2007). *sqr*, *apr*A and *sox*B are important functional genes involved in the microbial sulfur oxidation pathways (Kojima et al., 2014). The primer pair 27F/1492R was used for 16S rRNA gene-targeting PCR. PCR amplifications of functional gene markers were performed

by using primer pairs: Sqr 475F/964R (Pham et al., 2008), AprA-1-FW/AprA-5-RV (Meyer and Kuever, 2007) and SoxB 704F/1199R (Kojima et al., 2014).

- The PCR products of functional gene markers amplification were further analyzed by denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). The DGGE method was chosen for molecular analysis based on literature survey that DGGE has been widely applied for identification of bacteria of the sulfur cycle from environmental samples (Geets et al., 2006; Meyer and Kuever, 2007; Yamashita et al., 2011). However, due to apparent high diversity of sulfur-oxidizing communities in both wetland systems and the thus high demand for designing and testing appropriate PCR primers, analysis of functional gene markers via DGGE was discontinued in this study. Exemplary pictures of DGGE profiles are shown in Figure A1 (Appendix).

Phase 3:

- A total of 11 sampling campaigns were carried out for chemical measurements over a period from April to September 2015 in Phase 3. Samples were collected from the inflow, outflow, and at the distance from 0 to 83 cm along the flow path and at the middle depth of 16 cm below the gravel surface in all compartments (sampling points are indicated in Figure 2.3-2). The middle depth was chosen because there were no significant differences among depths observed in Phase 2.

- Results of chemical measurements showed that the CW systems were in stable operational modus in terms of sulfur- and nitrogen transformations (Chapter 3). To analyse microbial communities involved in the sulfur cycle (SOB and SRB) in the CW models, 16S rRNA gene amplicon sequencing via 454 pyrosequencing (454 Life Sciences, Branford, USA) was performed. The next-generation 454 Pyrosequencing technology has been proven to be a robust and highly reliable method to characterise microbial communities in natural samples (Pilloni et al., 2012). Here, DNA was isolated from pore water (1 – 15 ml), gravel (1 g) and root (0.25 g). The respective sample volume and weight of sample material for DNA extraction were chosen in order to be approximately representative of the wetland systems. Since 454 sequencing is a costly technique, limited numbers of samples were sequenced. Table 2.3-1 presents the samples selected for sequencing.

Table 2.3-1. DNA samples selected for 454 Pyrosequencing

Wetland	**Distance from inflow (cm)**	**DNA samples**			
		In Aug 2015		**In Oct 2015**	
		1st sampling campaign (13 Aug)	**2nd sampling campaign (19 Aug)**	**3rd sampling campaign* (14 Oct)**	
		Pore water	Pore water	Pore water	Root
CW1	8	x	x	x	x
	23	x	x	x	x
	38	x	x	x	x
	53	x	x	x	x
	68	x	x	x	x
	83	x	x	x	x
CW2	8	x	x	x	
	23	x	x	x	
	38	x	x	x	x
	53	x	x	x	x
	68	x	x	x	
	83	x	x	x	

* DNAs from mixture of pore water and roots were sequenced

No root samples were taken in August 2015 because roots were densely interwoven in each compartment (Figure 2.3-1a), and difficulty in retrieving samples without disturbance of the systems was expected. Thus, root samples were taken in October 2015 at the end of the operational period.

a) Plant roots distributed in the wetland system

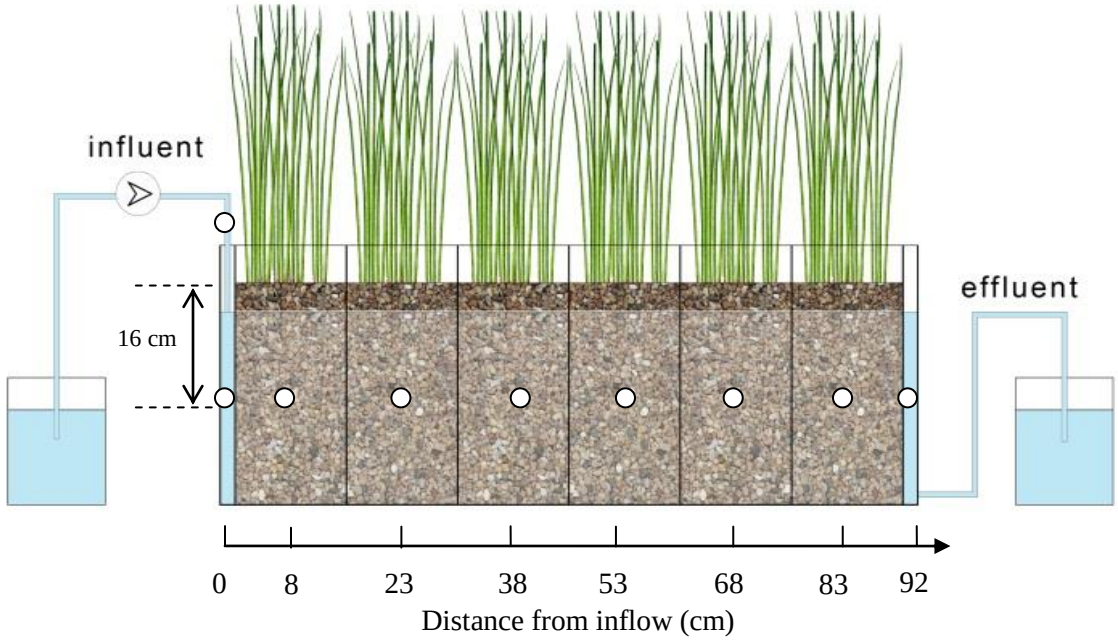
b) Plant roots taken out of the system

Figure 2.3-1. Plant roots in CW1

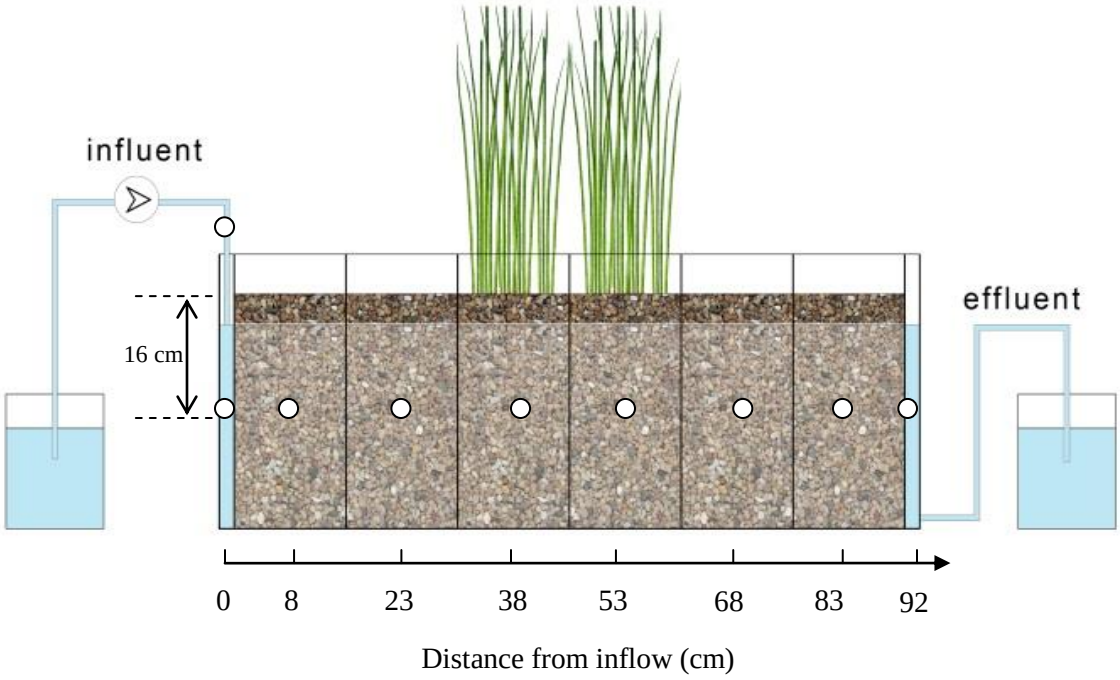
Phase 4:

In the final Phase of the thesis, data interpretation and analysis were performed. Data was visualized by using graphing software package SigmaPlot version 13.0 and the R statistical computing environment (R-Core-Team, 2013).

A manuscript with the working-title “Microbial sulfur transformations in novel laboratory-scale constructed wetlands” is being prepared for submission to the Journal *Environmental Microbiology*.



CW1



CW2

Figure 2.3-2. Schematic diagrams of the studied CWs indicating sampling points

2.4 Chemical analysis and calculations

2.4.1 Sulfur compounds

Sulfide and sulfate

Measurements of sulfide and sulfate were carried out by spectrophotometer CADAS 100, LPG 210 (Dr. Bruno Lange GmbH, Germany). Sulfide was analyzed by using the Test kit LCW 053 at 665 nm with the measuring range of 0.1 – 2 mg/L. The principle of the method is the reaction of dimethyl-*p*-phenylenediamine with sulfide to form an intermediate which merges into leuco methylene blue. The leuco methylene blue is further oxidized to methylene blue by iron (III) ion.
Sulfate was measured based on the turbidity of barium sulfate ($BaSO_4$) at 880 nm after the precipitation of sulfate ions and barium chloride in acidic gelatin solution.

Elemental sulfur, sulfite and thiosulfate

Elemental sulfur, sulfite and thiosulfate were analyzed according to Rethmeier et al. (1997). Elemental sulfur was measured by extracting pore water samples with chloroform and was subsequent detected by HPLC (Beckman, USA) using a Li-Chrospher 100, RP 18 column (5 µm, Merck, Germany) with a UV-detector at 263 nm. After derivatisation with monobromobimane, sulfite and thiosulfate were determined by HPLC (Beckman, USA) using fluorescence detector RF 551 (Shimadzu, Japan) and columns RP Li-Chrospher 60, RP Select B (250-4).

2.4.2 TOC, acetate and benzoate

After the filtration of pore water samples by using syringe filters with pore size of 5 µm, concentrations of TOC, acetate and benzoate were analyzed. TOC was analyzed by the multi N/C 2100S TOC analyzer (Analytik Jena). Acetate and benzoate were determined by Ion Chromatography ICS-5000.

2.4.3 Nitrogen species

Concentrations of nitrogen compounds (ammonium, nitrite, nitrate) were measured via a photometric method using the Photometer NOVA 60 A Spectroquant® (Merck KgaA, Germany). Test kits used for measurements of nitrogen species are shown in Table 2.4-1.

Table 2.4-1. Test kits for measurements of nitrogen species

Compound	Test kit	Standard	Wavelength (nm)	Detection range (mg/l)
NH_4^+ - N	Merck No. 1.00683.0001	DIN 38406 E5	690	2.0 – 75
NO_3^- - N	Merck No. 1.09713.0001	DIN 38405 D9	340	1.0 – 25
NO_2^- - N	Merck No. 1.14776.0001	DIN EN 26 777	525	0.02 – 1

2.4.4 Redox potential, temperature and pH

The redox potential of pore water samples was measured by $Pt/Ag^+/AgCl/Cl^-$ electrodes (Sentix ORP, WTW). Temperature was determined by PT-100 temperature sensor, Checktemp®1 Thermometer, Hanna Instruments. pH was determined by using pH meter, Sentix41 electrode (WTW).

2.4.5. Dissolved oxygen

Dissolved oxygen (DO) concentrations in pore waters were measured by trace oxygen meter (oxygen sensor PSt3, instrument FIBOX-4, PreSens GmbH, Regensburg, Germany).

2.4.6 Calculations

Calculations of water loss and contaminant loads were made according to the formulas described by Wu et al. (2012).

The water loss due to evapotranspiration (combination of evaporation and plant transpiration) was calculated by the measurement of influent and effluent volumes during a specific time

period (mostly 3 – 4 days in this study). The calculation of water loss was done using the equation:

$$\Delta V = V_{in} - V_{out}$$

where

ΔV: water loss (L/d)

V_{in}: influent volume (L/d)

V_{out}: effluent volume (L/d)

Water loss is an important parameter in CWs to calculate contaminant loads with the assumption of a linear increase in evapotranspiration along the flow paths and an ideal plug flow through the wetlands (Wu et al., 2012). The following equation shows the calculation of contaminant load (in mg/d) at a defined sampling point:

$$L = [V_{in} - V_{in} \times \Delta V \times f] \times C$$

where

L: contaminant load at a specific distance from the inflow (mg/d)

V_{in}: influent volume (L/d)

ΔV: water loss (L/d)

f: length fraction of the flow path

C: contaminant concentration at a specific distance from the inflow (mg/L)

The length fraction of the flow path (f) was calculated as following:

$$f = a/t$$

where

a: distance from inflow (cm)

t: total length of the system (cm)

Contaminant load can be present in mmol/d by dividing the load L to the molar mass of the specific substance:

$$L_i = L/M_i$$

where

L: load of a specific substance i (mg/d)

M_i: molar mass of the specific substance i (mg/mmol)

L_i: load of a specific substance i (mmol/d)

2.5 Molecular analysis

2.5.1 DNA isolation and quantification

DNA isolation from pore water (1 – 15 ml), gravel (1 g) and root (0.25 g) was performed using the DNeasy Blood & Tissue Kit (Qiagen, USA) according to instruction manual, following a modified protocol which is displayed in Table 2.5-1. Cell disruption was performed using FastPrep®-24 Instrument (MP Biomedicals, Illkirch, France), all centrifuge steps were carried out in a conventional table top centrifuge (Eppendorf AG, Hamburg, Germany). The extracted genomic DNAs were stored at - 20°C until further molecular analysis.

The quantity of extracted DNAs was determined by the NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH) at a wavelength of 260 nm.

Table 2.5-1. DNA extraction protocol

Step	Procedure
1.	Samples were centrifuged for 15 min, 13.000 rpm
2.	Discharge of supernatant and resuspension of pellet in 180 µl lysis buffer (2 mM EDTA, 20 mM TrisCl, 1.2 mM Triton x 100) and 20 mg/ml lysozyme (Sigma-Aldrich, Steinheim, Germany) appropriate 1x small spatula, vortex short (2 – 3 seconds)

3. Incubation at 37 °C for 45 min, 300 rpm rotation
4. Addition of 25 µl Proteinase K (Merck KGaA, Darmstadt, Germany) and 200 µl Buffer AL and subsequently mixing with vortex mixer
5. Addition of two times one small spatula (~100 mg) beads with different sizes (0.1 mm and 1.0 mm Zirconia/ Silica beads Biospec products)
6. Cell disruption at speed 4.0 for 30 seconds with FastPrep®-24 Instrument
7. Incubation at 65 °C for 45 min
8. Centrifugation at 8,000 rpm for 2 min, removal and continuation with 350µl supernatant
9. Addition of 200 µl ethanol (96 – 100 %), mixing by invertation
10. Transfer of the mixture including precipitate into an DNeasy column
11. Centrifugation at 13,000 rpm for 1 min and discharge of flow-through
12. Addition of 500 µl AW1 buffer, centrifugation at 13,000 rpm for 1 min and discharge of flow-through
13. Addition of 500 µl AW2 buffer, centrifugation at 13,000 rpm for 1 min and discharge of flow-through
14. Additional centrifugation at 13,000 rpm for 1 min in a new collection tube
15. Transfer of the column to a 1.5 ml tube, addition of 50 µl AE buffer and incubation at room temperature for 2 min. Repeat this step with the 50µl eluate
16. Centrifugation at 8,000 rpm for 1 min

Profiling of microbial communities via RNA (respectively cDNA) sequencing was attempted but eventually discontinued as isolation of high-quality RNA proved difficult although it was previously carried out successfully in our group on reactors planted with *J. effusus* (Lünsmann et al., 2016).

2.5.2 PCR amplification of the 16S rRNA gene

The 16S rRNA gene-based PCR was conducted using the primer pair 27F/1492R and HotStarTaq master mix kit (Qiagen). 49.5 µl of PCR mixture contained 1.5 µl genomic DNA, 3 µl of each primer, 17 µl ddH_2O and 25 µl HotStarTaq master mix. The PCR program for

amplification of the 16S rRNA gene fragment using the primer set 27F/1492R is shown in Table 2.5-2.

Table 2.5-2. 16S rRNA gene-based PCR program using the primer pair 27F/1492R

Step	Temperature	Time	Cycles
Initial denaturation	95°C	15 min	1
Denaturation	95°C	30 sec	
Annealing	55°C	30 sec	30
Extension	72°C	1.5 min	
Final extension	72°C	10 min	

2.5.3 Agarose gel electrophoresis

The PCR products were quality-checked via agarose gel electrophoresis [1.5% agarose in Tris–acetate–EDTA (TAE) buffer]. The electrophoresis was run for 45 minutes at 100 V and 103 mA. The gels were then stained with ethidium bromide and viewed under UV light. A FastRuler Middle Range DNA Ladder (# SM1113, ThermoScientific) was used to check the size of the bands of PCR products.

2.5.4 454 Pyrosequencing

454 Pyrosequencing includes four major steps: DNA library preparation, emulsion-based clonal amplification (emPCR amplification), sequencing run on the GS Junior Instrument and data processing.

Step 1 - DNA library prepararation

PCR products of pore water (from two sampling campaigns in Aug 2015) and from mixture of pore water and roots (from sampling campaign in Oct 2015) were amplified targeting the 16S rRNA gene by using the primer set U519F-MID/U909R and standard MyTaq protocol (Bioline, USA) for 454 Pyrosequencing (Table 2.5-3). The MID (multiplex identifier) has a

length of 10 nucleotides, which helps to identify the source of the read. Sequences of MIDs and primers (Roche) are shown in Table 2.5-4.

Table 2.5-3. 16S rRNA amplification program for 454 sequencing (MyTaq protocol, Bioline)

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95°C	1 min	1
Denaturation	95°C	15 sec	
Annealing	55°C	15 sec	30
Extension	72°C	10 sec	

26.5 µl of PCR mixture contained 2.5 µl PCR products, 0.7 µl of each primer, 17.3 µl dd H_2O, 0.3 µl MyTaq DNA Polymerase and 5 µl 5x MyTaq reaction buffer.

Table 2.5-4. Sequences of MIDs and primers for 454 sequencing

MID	Sequence
MID1	ACGAGTGCGT
MID2	ACGCTCGACA
MID3	AGACGCACTC
MID4	AGCACTGTAG
MID5	ATCAGACACG
MID6	ATATCGCGAG
MID7	CGTGTCTCTA
MID8	CTCGCGTGTC
MID10	TCTCTATGCG
MID11	TGATACGTCT
MID13	CATAGTAGTG
MID14	CGAGAGATAC
Forward primer (Primer A, Lib-L)	5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-ACGAGTGCGTCAGCM-template-specific sequence-3'
Reverse primer (Primer B, Lib-L)	5'- CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-CCGYGAATTCMTTTR-template-specific sequence-3'

Library quantitation was carried out by using the Quan-iT Picogreen dsDNA Assay Kit, (Roche). The DNA standard provided with Picogreen reagent was diluted according to amplicon library preparation method manual (Roche). Fluorescence was measured by QuantiFluor-ST Fluorometer (Promega) (Figure 2.5-1).

Figure 2.5-1. QuantiFluor-ST Fluorometer

Concentrations of amplicons in molecules/µl and volumes of TE buffer (µl) for the dilution of amplicons in order to obtain an concentration of 1 x 10^9 molecules/µl in each amplicon were calculated according to amplicon library preparation method manual (Roche).

Amplicon pools were prepared by transferring 5 µl of each amplicon library (concentration 1 x 10^9 molecules/µl) in an 1.5 ml-Eppendorf tube. From the 1.5 ml-Eppendorf tube, 2 µl of the pool was taken and 198 µl of molecular biology grade water was added to obtain a final concentration of 1 x 10^7 molecules/µl.

Step 2 - emPCR Amplification

In this step, the DNA library (from step 1) was loaded onto micron-sized beads. The reagents and emulsion oil were prepared by using the emPCR reagents kit (Roche). The DNA library fragments, capture beads, PCR reagents and emulsion oil were placed in a Turrax stirring tube (Figure 2.5-2). After mixture, droplets around the beads were formed ("water-in-oil" emulsion) (Figure 2.5-3). The emPCR amplification was carried out in the thermal cycler (C1000 Touch, Bio-Rad) according to emPCR amplification method manual – Lib L (Roche).

The emPCR allows DNA fragment in each droplet to be amplified into millions of copies of DNA (Roche). The emPCR program is described in Table 2.5-5.

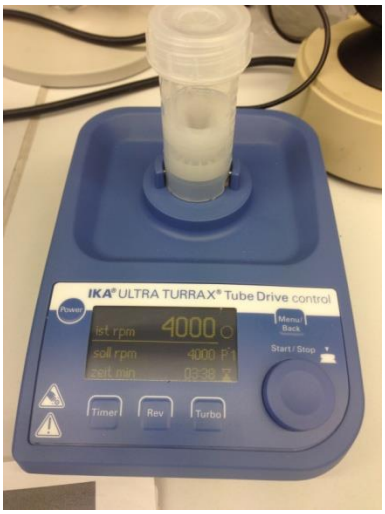


Figure 2.5-2. Ultra Turrax Tube Drive

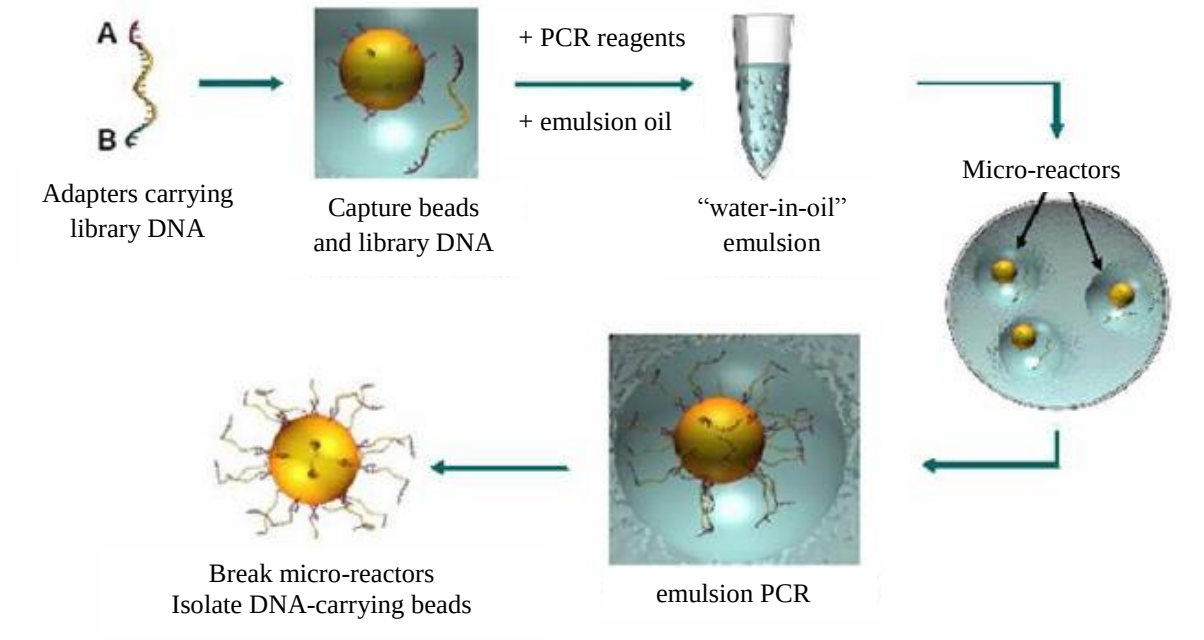


Figure 2.5-3. Loading DNA library onto beads and emPCR

(adapted from http://www.454.com/downloads/news-events/how-genome-sequencing-is-done_FINAL.pdf)

Table 2.5-5. emPCR program

Step	Temperature	Time	Cycles
Initial denaturation	94°C	4 min	1
Denaturation	94°C	30 sec	
Annealing	58°C	4.5 min	50
Extension	68°C	30 sec	
End	10°C	on hold	

After the PCR amplification, the bead recovery was carried out using the GS Junior Titanium emPCR Oil and Breaking Kit under a ventilated hood. Enriched DNA-carrying beads were counted by GS Junior Bead Counter. An input of 500,000 enriched beads is recommended for a GS Junior sequencing run (Roche).

Step 3 - Sequencing run on the GS Junior Instrument

Before the sequencing run, the 454 sequencing system instrument (GS Junior) was pre-washed with Sequencing Kit Buffers (Roche) (Figure 2.5-4).

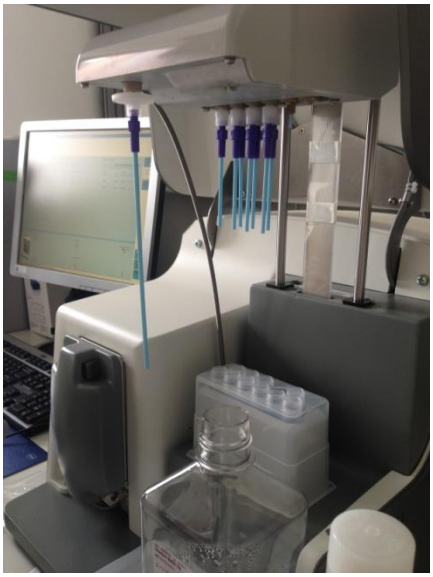


Figure 2.5-4. GS Junior Instrument pre-washed

DNA-capture beads were loaded in PicoTiterPlate device (Figure 2.5-5). One PicoTiterPlate contains 1.6 million wells. Each well has 75 picoliters in volume and 44 µm in diameter. The well was designed to capture one single DNA-carrying bead (Roche). After the PicoTiterPlate was filled with DNA-capture beads and enzyme beads (Figure 2.5-6), the plate was placed into the PicoTiterPlate cartrige of the GS Junior instrument.

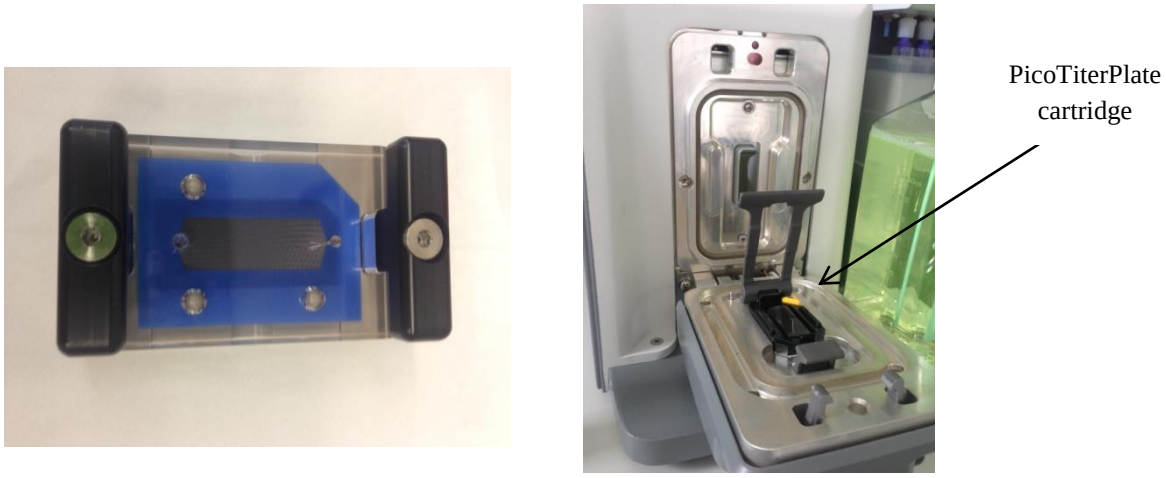


Figure 2.5-5. PicoTiterPlate device and PicoTiterPlate cartridge

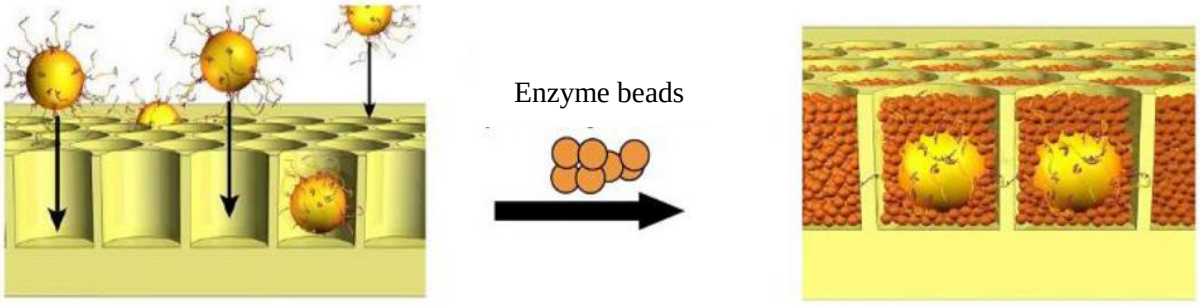


Figure 2.5-6. Loading DNA-carrying beads and enzyme beads into PicoTiterPlate

(adapted from http://www.454.com/downloads/news-events/how-genome-sequencing-is-done_FINAL.pdf)

The sequencing run was performed in GS Junior instrument according to the sequencing method manual (Figure 2.5-7).

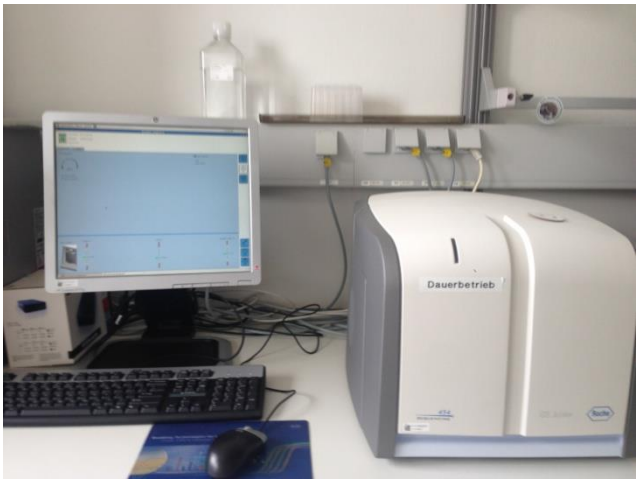


Figure 2.5-7. Sequencing run in GS Junior Instrument

The reagents containing the 4 deoxynucleotide triphosphates (dNTP) were sequentially flowed over the PicoTiterPlate. DNA polymerase catalyzed the incorporation of dNTP into DNA strands based on the complementary principle. Pyrophosphate released (PP_i) after each corporation was combined with adenosine 5'-phosphosulfate (APS) to ATP by ATP-sulfurylase. A light signal generated after the reaction of ATP with luciferin by the enzyme luciferase was detected by a charge-coupled device (CCD) camera. The light signal strength was proportional to the number of nucleotide incorporated (Ronaghi et al., 1996). Major reactions occurred in the sequencing run are described in the following equations:

$$(DNA)_n + dNTP \xrightarrow{\text{DNA polymerase}} (DNA)_{n+1} + PP_i$$

$$APS + PP_i \xrightarrow{\text{ATP-sulfurylase}} ATP$$

$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{oxyluciferin} + \text{light}$$

Step 4 – Data processing

Data processing after sequencing was conducted according to standard operating procedure by Schloss et al. (2011). The software package Mothur (version 1.37.0, released in April 2016) was used to extract and trim the sequences, followed by chimera removal by using

the chimera.uchime algorithm. Sequences derived from mitochondria and chloroplast were removed and alignment against the reference arb-silva (release 123) (Yilmaz et al., 2014) were performed. The Operational Taxonomic Unit (OTU) cutoff definition was 0.03, i.e., all reads sharing 97% similarity in sequences were clustered into one OTU. From the OTU table and based on literature survey on bacteria of the sulfur cycle (Camacho, 2010; Frigaard and Dahl, 2009; Robertson and Kuenen, 2006), sequences derived from SOB and SRB were selected and their relative abundances of total bacterial communities in each wetland sample was calculated. A taxonomic group was defined as abundant or rare group if it made up more than 1% or below 0.01% within a sample, respectively (Galand et al., 2009). Heatmaps showing relative abundances of SOB and SRB in the wetlands were generated by using R software (R-Core-Team, 2013).

2.5.5 Quantitative PCR

The quantitative PCR using the primer pair U519F/U909R and KAPA SYBR Fast qPCR master mix kit (Kapa Biosystems, USA) were applied to quantify copy numbers of 16S rRNA genes in pore water, gravel and root wetland samples. 11.5 µl of PCR mixture contained 1 µl genomic DNA, 0.25 µl of each primer, 4.75 µl ddH_2O and 6.25 µl KAPA SYBR Fast qPCR master mix. Table 2.5-6 shows the qPCR program (Kapa Biosystems) used in the study.

Table 2.5-6. qPCR program targeting 16S rRNA genes

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	3 sec	
Annealing	56°C	20 sec	40
Extension	72°C	20 sec	

3. RESULTS AND DISCUSSION

3.1 Dynamics of sulfur compounds in wetland models

Figure 3.1-1 illustrates overall dynamics of inorganic sulfur in CWs during Phase 3 (year 2015, Materials & Methods). Detailed data on loads of sulfur compounds (mean values) and standard deviations (SD) in CW1 and CW2 are presented in Table 3.1-1 and 3.1-2, respectively. As shown in Figure 3.1-1, co-occurrence of sulfate reduction and sulfur reoxidation with sequential dominance was observed in both CWs. After a drastic decrease from the inflow to 8 cm, sulfate-S was enriched towards the outflow. The decreases in sulfate-S were consistent with the formation of sulfide-S, the final product of dissimilatory sulfate reduction. The production of elemental sulfur in both CWs provides evidence for sulfur reoxidation. Amounts of elemental sulfur were about three times higher than that of thiosulfate-S. Amounts of sulfite-S were low.

In wetlands, reduced inorganic sulfur compounds contribute significantly to total sulfur when the latter is present at high concentrations (Giblin and Wieder, 1992). In this study, total sulfur of inorganic sulfur compounds was calculated as the sum of all five sulfur compounds determined. The decrease in total sulfur load from the inflow to the outflow in CW1 was 37.9%, whereas total sulfur load in CW2 remained quite stable. Sulfur depositions (e.g. metal sulfide precipitations, elemental sulfur deposits) and the emission of hydrogen sulfide can result in total sulfur loss (Kadlec and Wallace, 2008). It has been documented that CWs could harbor an important inorganic and organic sulfur pool (Spratt and Morgan, 1990; Stottmeister et al., 2003; Wiessner et al., 2010). Wiessner et al. (2007) found that 33.8% of total sulfur input was stored in a planted fixed-bed reactor (PFR). It has been suggested that sulfur was immobilized in wetlands mostly by organic substances such as dead plant matters, organo sulfate esters or microbial biomass (Giblin and Wieder, 1992; Wiessner et al., 2007).

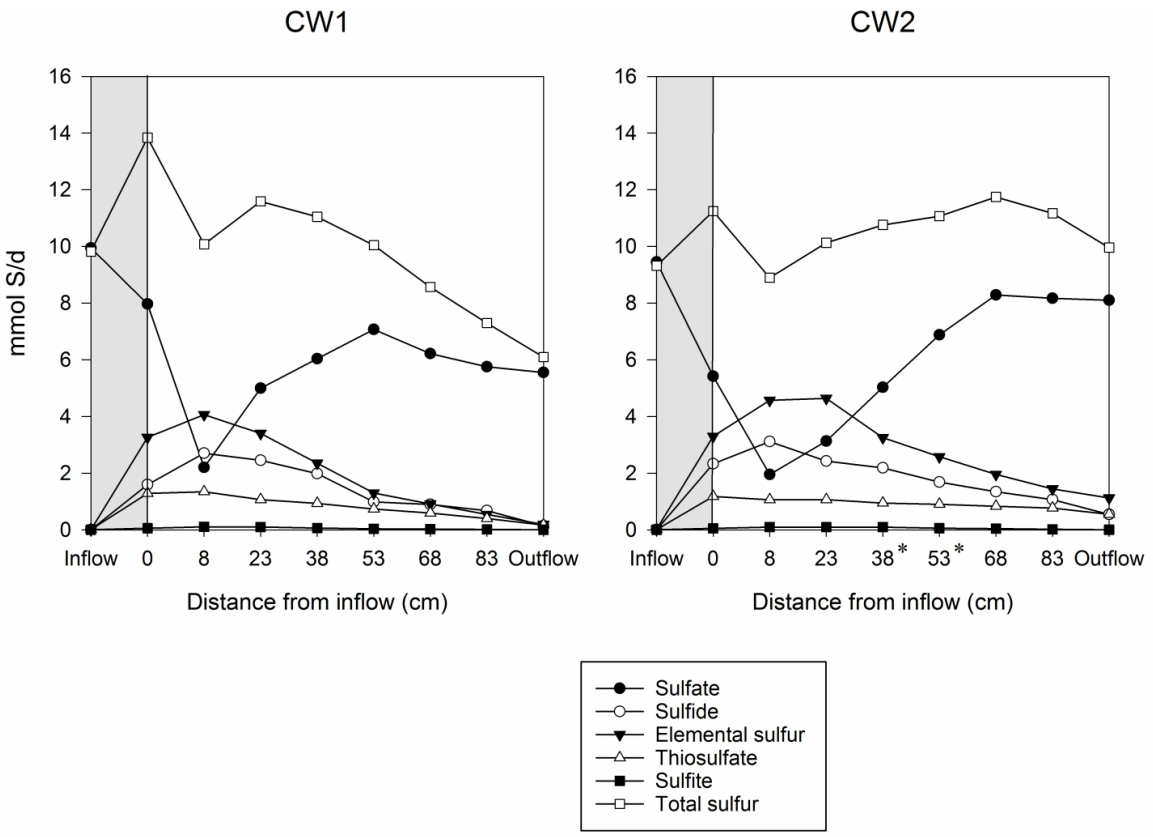


Figure 3.1-1. Overall behaviors of sulfur compounds in CWs

(SD are shown in Table 3.1-1 and 3.1-2 and not included in graphs to avoid the overlapping)

Table 3.1-1. Loads of sulfur compounds in CW1 (mmol/d)

Parameter	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	**0**	**8**	**23**	**38**	**53**	**68**	**83**	**Outflow**
SO_4^{2-} - S	n = 11	9.947	7.992	2.201	4.995	6.036	7.071	6.216	5.754	5.549
	SD	1.012	1.519	0.955	1.135	1.822	0.592	0.826	0.964	0.893
S^{2-}- S	n = 10	0.000	1.595	2.700	2.448	1.981	0.982	0.892	0.676	0.139
	SD	0.000	0.499	0.531	0.510	0.875	0.439	0.500	0.715	0.159
S^{o}- S	n = 9	0.005	3.270	4.063	3.400	2.352	1.301	0.907	0.541	0.189
	SD	0.014	0.727	0.864	0.344	0.751	0.557	0.368	0.415	0.302
$S_2O_3^{2-}$- S	n = 11	0.009	1.285	1.341	1.065	0.929	0.731	0.588	0.395	0.169
	SD	0.011	0.391	0.338	0.215	0.244	0.151	0.157	0.256	0.205
SO_3^{2-}- S	n = 11	0.002	0.056	0.104	0.100	0.060	0.033	0.025	0.015	0.001
	SD	0.005	0.087	0.080	0.077	0.057	0.038	0.022	0.024	0.002
TS	n = 8	9.819	13.880	10.078	11.594	11.047	10.047	8.567	7.294	6.095
	SD	1.107	1.128	1.676	1.829	1.068	1.049	0.798	1.505	1.238

Table 3.1-2. Loads of sulfur compounds in CW2 (mmol/d)

Parameter	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	**0**	**8**	**23**	**38***	**53***	**68**	**83**	**Outflow**
SO_4^{2-}- S	n = 11	9.452	5.418	1.949	3.129	5.028	6.879	8.289	8.174	8.104
	SD	0.763	1.952	0.960	1.481	1.571	1.592	0.939	1.095	1.405
S^{2-}- S	n = 10	0.002	2.332	3.113	2.425	2.185	1.680	1.343	1.062	0.539
	SD	0.006	0.787	0.894	0.636	0.715	0.623	0.442	0.561	0.335
S^{o}- S	n = 9	0.019	3.299	4.568	4.636	3.251	2.576	1.955	1.441	1.123
	SD	0.043	0.672	1.129	1.091	0.820	0.541	0.530	0.500	0.382
$S_2O_3^{2-}$- S	n = 11	0.008	1.178	1.060	1.062	0.942	0.900	0.831	0.764	0.549
	SD	0.008	0.388	0.346	0.383	0.249	0.154	0.223	0.160	0.130
SO_3^{2-}- S	n = 11	0.005	0.048	0.093	0.095	0.092	0.055	0.039	0.017	0.005
	SD	0.008	0.043	0.056	0.056	0.047	0.057	0.041	0.024	0.008
TS	n = 8	9.320	11.255	8.896	10.133	10.762	11.067	11.745	11.172	9.961
	SD	0.730	2.224	2.418	2.454	2.098	2.193	1.615	1.484	1.640

*: planted

Figure 3.1-2 distinguishes behavior of sulfur species between CW1 and CW2. Behavior of each sulfur compound is discussed in detail in the following.

Behavior of sulfate

In a short zone from the inflow to 8 cm, about 77.9% and 79.4% of sulfate-S was removed from CW1 and CW2, respectively. But despite the initial efficient sulfate-S reductions, overall sulfate-S removal efficiencies were 44.2 and 14.3% (equivalent to 31.87 mmol/m^2/d and 9.77 mmol/m^2/d as specific removal rates) in CW1 and CW2, respectively. The values are similar to previous findings on sulfate treatment efficiencies (14 – 26%) (Kadlec and Wallace, 2008; Sturman et al., 2008) and within the range of sulfate removal rates reported for CWs (11.56 – 54.7 mmol/m^2/d) (Saad et al., 2016; Wu et al., 2011). From 8 cm onwards, sulfate load elevated in both CWs. The enrichment of sulfate-S correlated well with the decreases in reduced sulfur compounds (sulfite-S, thiosulfate-S and elemental sulfur-S), which indicates sulfate production owing to reoxidation of reduced sulfur species. From 53 cm onwards, higher sulfate-S loads in CW2 compared to CW1 was the result of higher availability of reduced sulfur compounds in CW2.

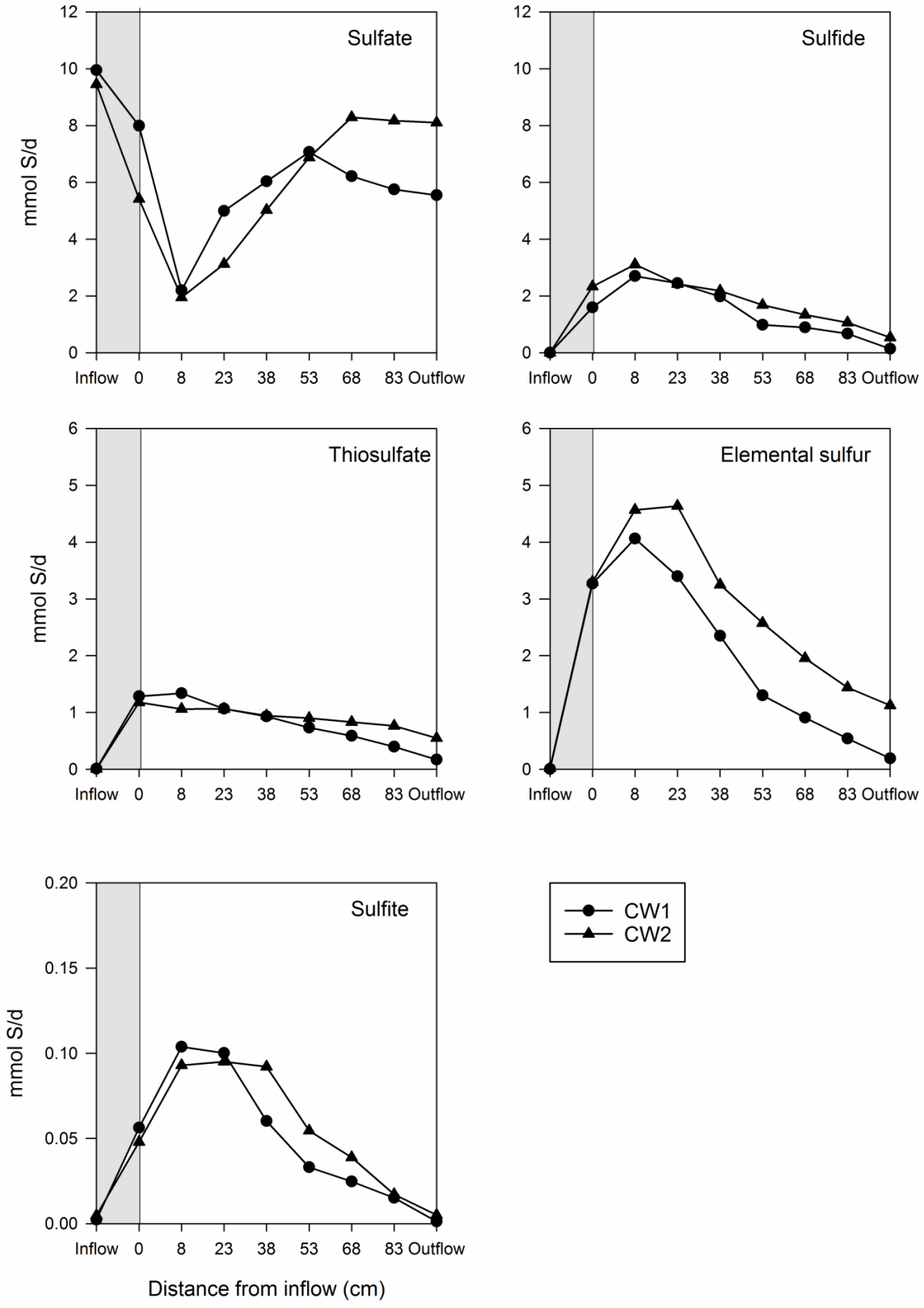


Figure 3.1- 2. Comparisons in sulfur transformations between CW1 and CW2

(SD are shown in Table 3.1-1 and 3.1-2)

The efficient reductions of sulfate-S in the first 8 cm in both CWs was attributed to dissimilatory sulfate reduction:

$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$
acetate

$C_6H_5COO^- + 3.75\ SO_4^{2-} + 4\ H_2O \rightarrow 3.75\ HS^- + 7\ HCO_3^- + 2.25\ H^+$ (Detmers et al., 2001)
benzoate

In the equations above, decimal fractions of some substrates and products are given to make comparisons between processes easier. Acetate and benzoate can be utilized by SRB as electron donors (Odom et al., 2013) and dissimilatory sulfate reduction by SRB has been shown to contribute considerably to the removal of organic carbons in CWs (Garcia et al., 2010). Root exudates from plants can also drive dissimilatory sulfate reduction. Although sulfate can be incorporated into sulfur-containing amino acids via assimilatory pathway in wetlands (Vymazal and Kröpfelová, 2008b), assimilatory sulfate reduction is considered to be of relatively minor importance compared to dissimilatory pathway (Wieder and Lang, 1988). Sulfur removal by assimilation into plants was reported to be less than 0.3% under high sulfate concentrations (Wu et al., 2013). Recently Saad et al. (2016) also found that total sulfur uptake of *J. effusus* was only 0.015 ± 0.002 g/m^2/d.

Behavior of sulfide

As the final product of dissimilatory sulfate reduction, sulfide was produced in both CWs. From the inflow, sulfide-S load increased at 8 cm to 2.7 ± 0.53 mmol/d in CW1 and 3.11 ± 0.89 mmol/d in CW2, followed by a decrease along the flow paths. From 8 cm onwards to the outflow, removal rates of sulfide were 20.33 and 20.43 mmol/m^2/d (equivalent to 94.9% and 82.7% relative concentration decrease) in CW1 and CW2, respectively.

Sulfide generated from sulfate reduction can precipitate with metals to form metal sulfides, volatilizes (hydrogen sulfide emission), form organic sulfur compounds and undergo reoxidation in wetland sediments (Vymazal and Kröpfelová, 2008b). It has been documented that sulfide was immobilized in reducing lake sediments only at a small fraction, whereas up to 90% of sulfide was reoxidized (Holmer and Storkholm, 2001). Since trace amounts of

metals (e.g. Fe^{2+}, Mn^{2+}) were present in the artificial wastewater feeding the systems, metal sulfides could be formed in the wetland models. The appearance of black precipitates showed that such precipitations occurred indeed (Figure 3.1-3).

Sulfate is the final oxidation product of sulfide reoxidation: $HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$ (van den Ende and Gemerden, 1993; Zopfi et al., 2001). Both abiotic and biotic processes can be involved in sulfide reoxidation (Holmer and Storkholm, 2001). It has been reported that sulfide oxidation occurs mainly via biologically mediated processes under oxygen-limiting conditions (Sturman et al., 2008), and 88% of total sulfide oxidation may result from biological pathways (Zopfi et al., 2001). SOB have special strategies which allow them to compete successfully with chemical sulfide oxidation at oxic-anoxic interface environments (Teske and Nelson, 2006; Zopfi et al., 2004). By having enzymes with high affinities for oxygen and sulfide together with the ability to move towards the oxic-anoxic interfaces, microaerophilic SOB such as *Beggiatoa*, *Thiothrix* can out-compete abiotic sulfide oxidation (Larkin and Strohl, 1983; Zopfi et al., 2004). In this study, it is likely that both abiotic and biotic sulfide oxidation occurred, however oxygen limitations in HSSF CWs (Stottmeister et al., 2003) could favor activities of microaerophilic SOB, therefore biotic sulfide oxidation could be important. In anoxic sediments, it is possible that sulfide oxidation can be coupled with nitrate reduction. SOB like *Thiobacillus denitrificans* can utilize nitrate as electron acceptor in sulfur-driven autotrophic denitrification process (Wu et al., 2013).

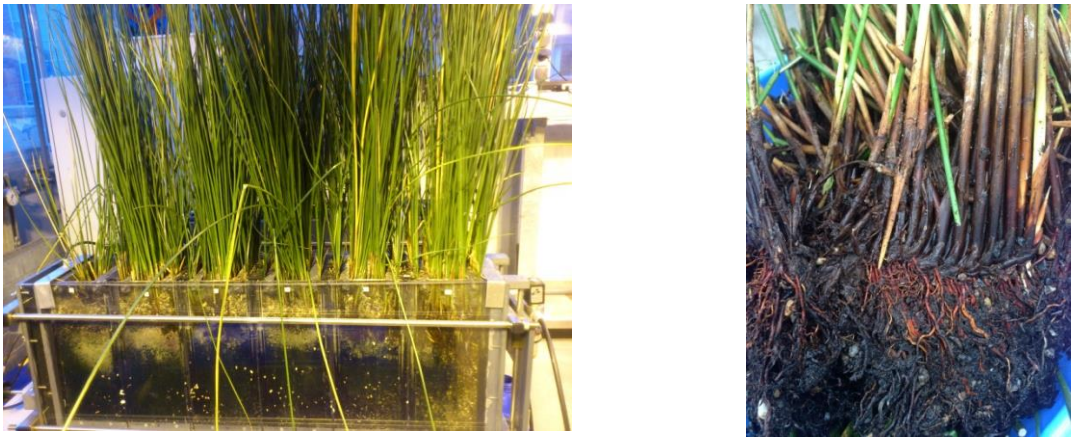


Figure 3.1-3. Black precipitates in CW1

Behavior of elemental sulfur

In CWs elemental sulfur can be formed via both chemical and biological sulfide oxidation ($2HS^- + O_2 \rightarrow 2S + 2OH^-$) (Chen et al., 2016; Wu et al., 2011). Elemental sulfur formed from bacterial sulfide oxidation can be deposited intracellularly or extracellularly. In many circumstances, white precipitates from elemental sulfur deposits have been observed in the outflows of treatment wetlands (Kadlec and Wallace, 2008).

In this study, elemental sulfur load increased steeply within the first centimeters and then declined along the flow path. From 8 cm to the outflow, higher elemental sulfur removal rates were recorded in CW1 compared to CW2: 30.74 against 27.33 mmol/m^2/d (equivalent to 95.3% and 75.4% relative concentration decrease) in CW1 and CW2, respectively. It is interesting to note that while elemental sulfur-S dropped by approximately 16.3% in the zone from 8 to 23 cm of CW1, no significant change in elemental sulfur-S load was observed in the corresponding yet unplanted compartment of CW2. Only from 23 cm onwards in CW2, elemental sulfur decreased significantly. These findings highlight the effects of plants in elemental sulfur removal. Oxygen released from plant roots has been reported to play an important role in oxidative processes in wetlands (Stottmeister et al., 2003; Wiessner et al., 2010). Oxidation of elemental sulfur can result in the elevation of sulfate in both CWs:
$S + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$.

In the wetlands, bacterial sulfur disproportionation could also contribute to the depletion of elemental sulfur, leading to the formation of sulfate and sulfide: $4S^0 + 4H_2O \rightarrow SO_4^{2-} + 3HS^- + 5H^+$ (Wu et al., 2013).

Overall, higher amounts of elemental sulfur in CW2 compared to CW1 were in good agreement with prior studies which showed that under more oxygen limiting conditions, more elemental sulfur was produced during sulfide oxidation (Celis-García et al., 2008; van den Ende et al., 1997).

Behavior of sulfite

Despite its modest amount in the systems (almost always below 0.1 mmol/d), sulfite-S loads experienced rapid turnovers. After fast enrichments from the inflow to 8 cm, sulfite-S was removed along the flow path: removal rates of sulfite-S were 0.81 and 0.70 mmol/m^2/d (equivalent to 98.8% and 94.5% relative concentration decrease) in CW1 and CW2, correspondingly). Interestingly, significant differences in sulfite-S behavior between two wetlands were observed for the zone from 23 to 38 cm: while almost no significant decrease in sulfite-S load occurred in CW2, a drastic decline in sulfite-S load was observed in CW1 (sulfite-S was decreased by 39.8%). In CW2, significant decrease in sulfite-S load (40.8%) was observed only from 38 to 53 cm flow path, where plants were present.

In general, the results demonstrate considerable effects of plants in sulfite-S removal, apparently due to more oxygen available in the rhizosphere (Stottmeister et al., 2003). Insignificant amounts of sulfite-S in this study corresponded well with the fact that sulfite is typically not detected at high concentrations in the environment because of its highly reactive nature (Zopfi et al., 2004). In the presence of oxygen, sulfite can be rapidly oxidized to sulfate: $SO_3^{2-} + 0.5O_2 \rightarrow SO_4^{2-}$ or can react with either sulfide or elemental sulfur to form thiosulfate: $SO_3^{2-} + HS^- + 0.5O_2 \rightarrow S_2O_3^{2-} + OH^-$; $SO_3^{2-} + S \rightarrow S_2O_3^{2-}$ (Zopfi et al., 2004). Sulfite can be utilized as electron acceptor by some SRB (Kappler, 2011; Simon and Kroneck, 2013) or as electron donor by some SOB (Frigaard and Dahl, 2009), but there is no evidence whether or not such activities occurred and competed successfully with abiotic reactions in the model CWs.

Behavior of thiosulfate

Thiosulfate-S loads increased substantially at the inflow of both wetlands. Thiosulfate has been reported to be an important product of bacterial sulfide oxidation (Holmer and Storkholm, 2001). Jørgensen (1990a) found that thiosulfate was a key intermediate of the sulfur cycle in freshwater sediments. Under oxygen limitations, chemical oxidation of sulfide with oxygen ($2HS^- + 2O_2 \rightarrow S_2O_3^{2-} + H_2O$) can also contribute to thiosulfate formation (Janssen et al., 1995). From 8 cm onwards to the outflow, thiosulfate-S removal rate in CW1 was approximately double that of CW2: 9.30 as opposed to 4.06 mmol/m^2/d (equivalent to

about 87.4% and 48.2% relative concentration decrease) in CW1 and CW2, respectively. As shown in Figure 3.1-4, there was a wide disparity in thiosulfate removal rates (in comparison with removal rates of sulfide, elemental sulfur and sulfite) between CW1 and CW2. Unlike sulfite, thiosulfate is less chemically reactive (Zopfi et al., 2004). At neutral pH thiosulfate was shown to be stable in the absence of microorganisms (Millero, 1991). Therefore, the decrease in thiosulfate along the flow paths of CWs was attributed to microbial activities.

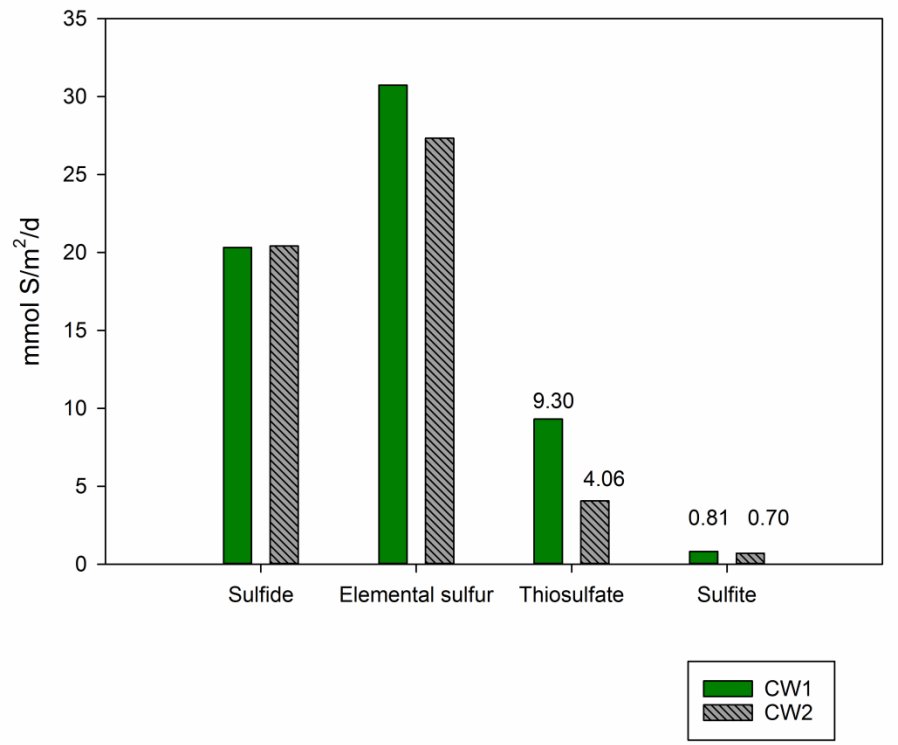


Figure 3.1-4. Removal rate of reduced sulfur species in CWs

(from 8 cm to the outflow)

Thiosulfate is commonly utilized by SOB in the presence of appropriate electron acceptors, e.g. oxygen or nitrate, according to the equations: $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$ and $S_2O_3^{2-} + 1.6NO_3^- + 0.2H_2O \rightarrow 2\ SO_4^{2-} + 0.8\ N_2 + 0.4\ H^+$ (Tang et al., 2009). Thiosulfate can also be reduced back to sulfide by activities of SRB in the presence of organic substrates: $S_2O_3^{2-} + CH_3COO^- + H^+ \rightarrow 2HS^- + 2CO_2 + H_2O$ (Jørgensen, 1990a). In the absence of both organic substrates and electron acceptors, thiosulfate disproportionation to sulfate and sulfide can be involved in thiosulfate removal: $S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$ (Bak and Cypionka, 1987; Jørgensen, 1990a). Thiosulfate-disproportionating bacteria are mostly SRB, e.g. members of the genera *Desulfovibrio* and *Desulfocapsa* (Finster, 2008). Concurrent oxidation,

reduction and disproportionation of thiosulfate could occur in CWs as a previous study revealed that all of these processes proceeded simultaneously in all sediment layers (Jørgensen, 1990b). In reduced sediments, thiosulfate disproportionation could be significant, accounting for 39% (Fossing and Jørgensen, 1990) and reoxidation can be enhanced in the presence of SOB and rooted macrophytes (Holmer and Storkholm, 2001).

In summary, rapid turnover of sulfur compounds with efficient sulfate reduction and subsequent reoxidation were observed in both CWs, which are consistent with prior studies that highlighted the importance of inorganic sulfur cycle in wetlands (Spratt and Morgan, 1990; Wieder and Lang, 1988; Wiessner et al., 2010). A closer look at dynamics of different sulfur species between the fully planted CW1 and partially planted CW2 revealed the significant effects of plants in sulfur transformations. Particularly, reoxidation was enhanced by oxygen released from the roots of *J. effusus*.

3.2 Sulfur transformations in the interconnections with other important redox processes

It is known that sulfur transformations have various interconnections with the nitrogen and carbon cycle in treatment wetlands (Garcia et al., 2010; Wu et al., 2013). Therefore, together with behavior of sulfur compounds, behavior of nitrogen species and carbon sources were also taken into account in this study. Results on behavior of nitrogen species and carbon sources as well as the interlink between sulfur transformation processes and nitrogen and organic carbon removal will be discussed in this section.

3.2.1 Nitrogen transformations

Behavior of nitrogen species in CWs are shown in Figure 3.2-1. Detailed data on loads of nitrogen species (means and SD) in CW1 and CW2 are presented in Table 3.2-1 and 3.2-2, respectively.

Overall, total nitrogen removal efficiency in CW1 was almost double that of CW2 (72% versus 39.4%, respectively). Ammonium-N loads decreased initially slowly, followed by

better removal towards the outflow. Better ammonium-N removal efficiency was achieved in CW1 compared to CW2 (72.6% as opposed to 40.2%, respectively). Nitrite-N and nitrate-N loads gradually decreased along the flow path. Nitrate-N removal efficiency was doubled that of CW2 (61.7% versus 29.5%, respectively). Nitrite-N loads were below 0.03 mmol/d.

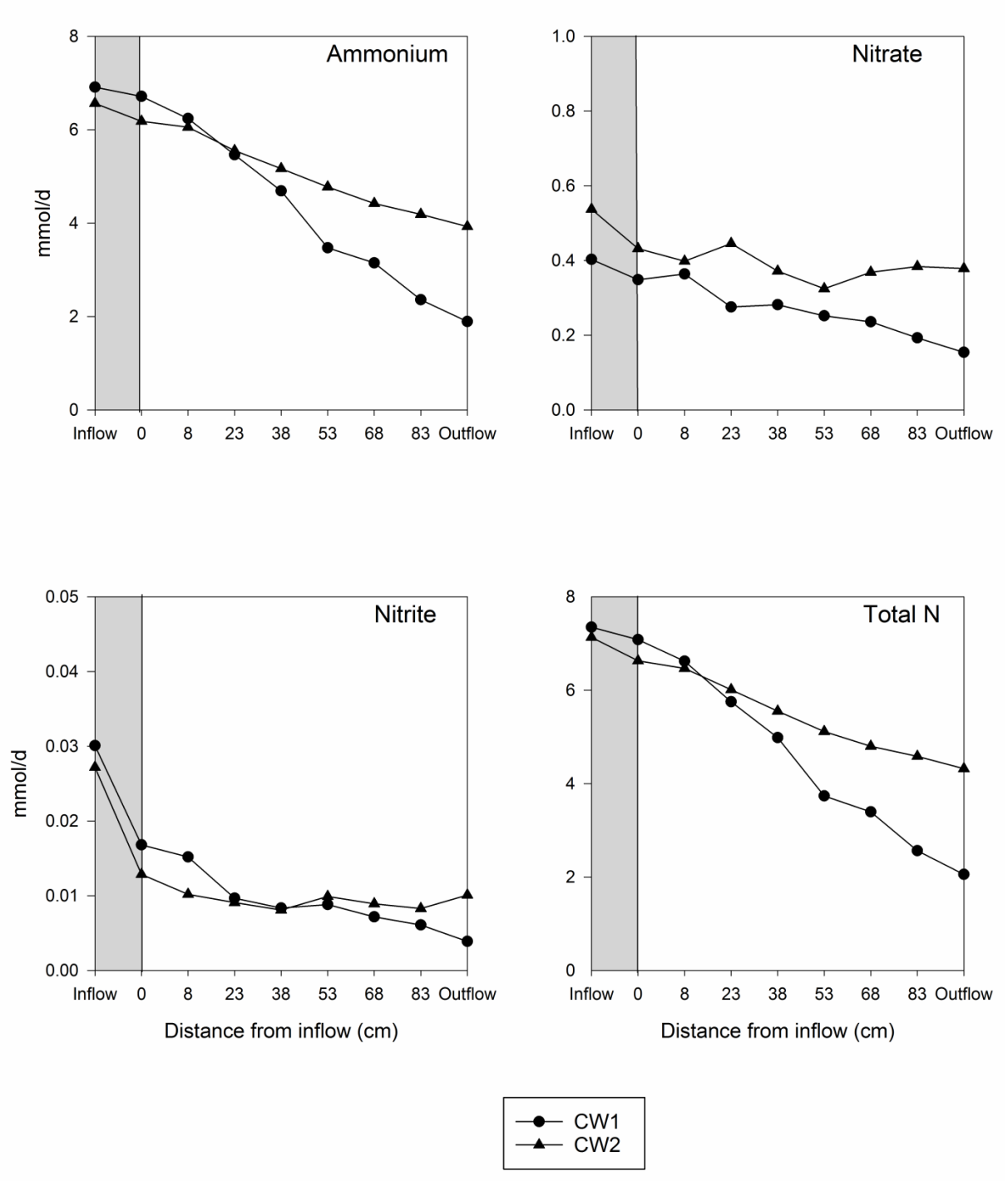


Figure 3.2-1. Behavior of nitrogen species in CWs

(SD are shown in Table 3.2-1 and 3.2-2)

Table 3.2-1. Loads of nitrogen species in CW1 (mmol/d)

Parameter	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	**0**	**8**	**23**	**38**	**53**	**68**	**83**	**Outflow**
NH_4^+ - N	n = 10	6.915	6.717	6.242	5.466	4.693	3.474	3.150	2.362	1.896
	SD	0.714	0.648	0.442	0.363	0.557	0.584	0.710	1.089	1.012
NO_3^- - N	n = 10	0.403	0.349	0.364	0.276	0.282	0.252	0.236	0.193	0.154
	SD	0.138	0.096	0.124	0.098	0.062	0.064	0.066	0.050	0.049
NO_2^- - N	n = 10	0.030	0.017	0.015	0.010	0.008	0.009	0.007	0.006	0.004
	SD	0.006	0.004	0.005	0.005	0.006	0.006	0.004	0.004	0.003
TN	n = 10	7.348	7.083	6.621	5.752	4.983	3.735	3.394	2.561	2.054
	SD	0.714	0.634	0.485	0.344	0.555	0.588	0.762	1.135	1.056

Table 3.2-2. Loads of nitrogen species in CW2 (mmol/d)

Parameter	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	**0**	**8**	**23**	**38***	**53***	**68**	**83**	**Outflow**
NH_4^+ - N	n = 10	6.568	6.183	6.058	5.555	5.170	4.779	4.421	4.191	3.931
	SD	1.041	0.776	0.540	0.619	0.527	0.569	0.518	0.555	0.611
NO_3^- - N	n = 10	0.537	0.432	0.399	0.446	0.372	0.325	0.369	0.384	0.379
	SD	0.180	0.100	0.161	0.143	0.117	0.095	0.129	0.208	0.153
NO_2^- - N	n = 10	0.027	0.013	0.010	0.009	0.008	0.010	0.009	0.008	0.010
	SD	0.010	0.007	0.006	0.007	0.006	0.006	0.006	0.005	0.006
TN	n = 10	7.132	6.628	6.466	6.010	5.550	5.113	4.799	4.583	4.319
	SD	1.193	0.770	0.633	0.704	0.614	0.583	0.631	0.583	0.618

*: planted

Several processes have been reported to be involved in nitrogen removal in CWs such as volatilization, adsorption, plant uptake, and microbial nitrogen transformations (Saeed and Sun, 2012). In HSSF CWs volatilization and adsorption are of minor importance in nitrogen removal (Vymazal and Kröpfelová, 2008b), while the role of plant uptake in nitrogen removal has been an interesting subject of former investigators (Chen et al., 2014; Scholz and Hedmark, 2010; Wiessner et al., 2013). Data from prior studies on plant contributions (by nitrogen uptake) in total nitrogen removal varied from 0.5 to 40% (Saeed and Sun, 2012). Microbial nitrification – denitrification processes are generally accepted to be the main mechanism in nitrogen removal in CWs, accounting for 60 – 96% total nitrogen removal (Chang et al., 2014; Chen et al., 2014; Lin et al., 2002; Stottmeister et al., 2003).

In the present study, the decreases in ammonium-N loads as well as the presence and decreasing amounts of nitrate-N and nitrite-N indicate that nitrification – denitrification processes occurred in CWs. However, both nitrification and denitrification in the two experimental CWs were likely to be inhibited by sulfide. Initial low efficiencies of ammonium-N removal in both CWs coincided with high sulfide loads: about 2.7 and 3.1 mmol/d sulfide-S (equivalent to 26 – 32 mg/L) were detected at 8 cm downstream the inflow in CW1 and CW2, respectively. Detrimental effects of sulfide on microbial nitrification have been reported in previous studies (Aesoy et al., 1998; Joye and Hollibaugh, 1995; Wiessner et al., 2007). Aesoy et al. (1998) found that at a concentration of only 0.5 mg/L, sulfide caused negative effects on nitrification capacity. Wiessner et al. (2007) documented that at sulfide concentration of above 25 mg/L, nitrification in CWs planted with *J. effusus* was completely inhibited. Furthermore, sulfide can also influence denitrification capacity. It has been shown that activities of denitrifiers were inhibited by excessive sulfide (Pokorna and Zabranska, 2015) and the presence of sulfide could considerably lower denitrification rates (Bowles et al., 2012; Pokorna et al., 2013).

In addition to denitrification where nitrate is reduced to dinitrogen via several intermediates ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Faulwetter et al., 2009), other possible pathways could also be involved in nitrate removal in CWs. Under highly reducing environments in HSSF CWs and with the presence of reduced inorganic sulfur compounds (thiosulfate, sulfide and elemental sulfur), sulfur-driven autotrophic denitrification possibly occurred (Dolejs et al., 2015; Tang et al., 2009; Wu et al., 2013):

$$HS^- + 1.6\ NO_3^- + 0.6\ H^+ \rightarrow SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O$$

$$HS^- + 0.4\ NO_3^- + 1.4\ H^+ \rightarrow S^0 + 0.2\ N_2 + 1.2\ H_2O$$

$$HS^- + 0.25\ NO_3^- + 1.5\ H^+ \rightarrow S^0 + 0.25\ NH_4^+ + 0.75\ H_2O$$

$$S^0 + 1.2\ NO_3^- + 0.4\ H_2O \rightarrow SO_4^{2-} + 0.6\ N_2 + 0.8\ H^+$$

$$S_2O_3^{2-} + 1.6\ NO_3^- + 0.2\ H_2O \rightarrow 2\ SO_4^{2-} + 0.8\ N_2 + 0.4\ H^+$$

From 8 cm towards the outflow, the concurrent decrease in thiosulfate-S, sulfide-S, elemental sulfur-S loads and nitrate-N loads and the increase in sulfate-S load suggest that sulfur-driven

autotrophic denitrification, an alternative route to carbon-driven heterotrophic denitrification (Kadlec and Wallace, 2008), took place.

Dissimilatory nitrate reduction to ammonium (DNRA) has been reported to occur in HSSF CWs (Vymazal and Kröpfelová, 2011) under carbon-rich and nitrate-limited environments (Kadlec and Wallace, 2008). To date information on the contribution of DNRA to nitrogen removal in CWs and freshwater ecosystems is limited (Burgin and Hamilton, 2007). In this study, DNRA was probably not an important process since organic carbon sources were consumed fast near the inlet zones (see section 3.2.2). Under carbon limited conditions, anaerobic ammonium oxidation (ANAMMOX) where ammonium is directly converted to dinitrogen gas could be another alternative pathway involved in nitrogen removal in anaerobic layers of HSSF CWs (Saeed and Sun, 2012). The importance of ANAMMOX in nitrogen removal in CWs may not be significant since ANAMMOX has been considered to be less important compared to conventional nitrification – denitrification in CWs (Coban et al., 2015a).

Better total nitrogen removal in the fully planted CW1 was consistent with earlier studies which showed that plants had positive effects on nitrification – denitrification (Faulwetter et al., 2009; Lin et al., 2002; Wiessner et al., 2010). As nitrification is an oxygen-requiring process ($NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O$; $NO_2^- + O_2 \rightarrow NO_3^-$), oxygen release from plant roots could facilitate nitrification (Coban et al., 2015b). Plants furthermore stimulate heterotrophic denitrification by organic carbon supply via rhizodeposition such as root exudates or dead plant matters (Hang et al., 2016; Zhai et al., 2013). Noteworthy, total nitrogen removal efficiency in CW1 (72%) was comparably high as the mean total nitrogen removal efficiency in CWs was found to be only in the range from 37 to 50% (Land et al., 2016; Verhoeven and Meuleman, 1999).

3.2.2 Behavior of organic carbon sources: acetate, benzoate, TOC

Behavior of carbon sources, including acetate, benzoate and TOC are shown in Figure 3.2-2. Detailed data on loads of organic carbons (means and SD) in CW1 and CW2 are presented in Table 3.2-3 and 3.2-4, respectively.

As can be seen from Figure 3.2-2, acetate, benzoate and TOC (as the sum of acetate and benzoate in the inflow) decreased drastically from the inflow up to 8 cm. From 8 cm towards the outflow, acetate, benzoate were completely removed, and little TOC remained in the systems. No significant difference in behavior of carbon sources between CW1 and CW2 was observed.

The rapid consumptions of acetate, benzoate and TOC matched with the effective sulfate reduction zones in CWs. This finding was expected since these organic substrates can be used as electron donors driving sulfate removal. It has been documented that in general more than 50% of the TOC is removed in the first quarter of treatment wetlands (Garcia et al., 2010). In the present study, in the first quarter of the CWs (23 cm from the inflow), about 88.9% and 93.2% of TOC was removed in CW1 and CW2, respectively, which shows highly effective organic carbon removal. From 23 cm, the remained TOC in the wetlands was the result of organic carbon derived from root exudates which could drive dissimilatory sulfate reduction.

In addition to sulfate reduction, acetate, benzoate and TOC can also be eliminated by other processes such as aerobic degradation, heterotrophic denitrification, and syntrophic fermentation (Garcia et al., 2004; Saeed and Sun, 2012). Heterotrophic microorganisms can use oxygen (from air diffusion or plant roots) as a terminal electron acceptor (aerobic respiration) or utilize nitrate in anoxic environments to degrade organic matter (denitrification) in CWs (Garcia et al., 2004). In HSSF CWs, anaerobic heterotrophic bacteria can carry out fermentation, producing organic acids, alcohols and molecular hydrogen which can be subsequently used by SRB for sulfate reduction (Saeed and Sun, 2012). From 8 cm onwards, the limitation of carbon sources was in accordance with the enrichment of sulfate along the flow path in both CWs. The results were similar to that of Wiessner et al. (2010), which found that sulfate was elevated along the flow path in HSSF CWs under carbon limited conditions.

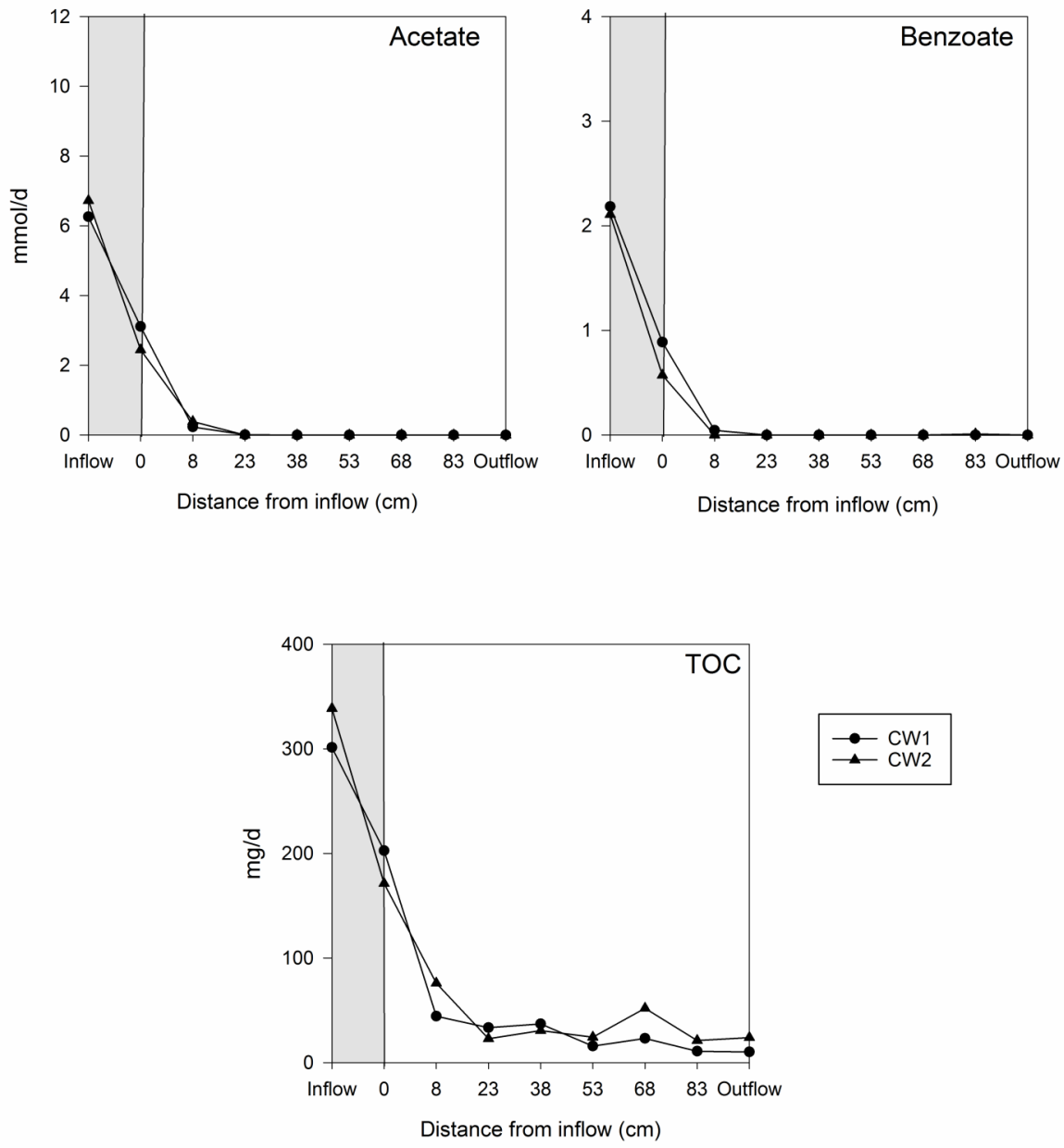


Figure 3.2-2. Behavior of carbon sources in CWs

(SD are shown in Table 3.1-3 and 3.1-4)

Table 3.2-3. Loads of organic carbons in CW1

Parameter	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	0	8	23	38	53	68	83	Out-flow
Acetate (mmol/d)	n = 9	6.263	3.116	0.235	0.012	0.000	0.000	0.000	0.000	0.000
	SD	2.782	0.891	0.404	0.036	0.000	0.000	0.000	0.000	0.000
Benzoate (mmol/d)	n = 8	2.185	0.888	0.047	0.000	0.000	0.000	0.000	0.000	0.000
	SD	0.705	0.132	0.132	0.000	0.000	0.000	0.000	0.000	0.000
TOC (mg/d)	n = 5	301.277	202.637	44.331	33.450	36.979	15.852	23.155	10.842	10.158
	SD	46.728	128.671	19.408	14.326	30.734	2.613	22.526	1.899	1.486

Table 3.2-4. Loads of organic carbons in CW2

Parameter	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	0	8	23	38	53	68	83	Out-flow
Acetate (mmol/d)	n = 10	6.725	2.447	0.390	0.000	0.000	0.000	0.006	0.000	0.000
	SD	1.996	1.850	0.405	0.000	0.000	0.000	0.020	0.000	0.000
Benzoate (mmol/d)	n = 8	2.110	0.573	0.000	0.000	0.000	0.000	0.000	0.011	0.000
	SD	0.455	0.341	0.000	0.000	0.000	0.000	0.000	0.031	0.000
TOC (mg/d)	n = 5	338.541	171.251	75.953	22.889	30.539	24.362	51.965	21.197	23.985
	SD	97.590	22.519	78.756	13.856	28.068	8.249	46.245	14.159	21.692

3.3 Other physicochemical parameters correlating with sulfur transformations

3.3.1 pH

pH is an important environmental factor influencing microbial processes and is associated with nutrient removal in CWs (Reddy and D'angelo, 1994). The pH behavior in the experimental wetland models is shown in Figure 3.3-1. From the inflow to 0 cm, pH increased immediately, followed by steady decreases towards the outflow (except a slight increase from 68 cm in CW2). In general, lower pH was observed in CW1 compared to CW2.

The rise in pH from the inflow to 0 cm could be explained by the production of conjugate base bicarbonate (HCO_3^-) from sulfate reduction [$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$ and $C_6H_5COO^- + 3.75SO_4^{2-} + 4H_2O \rightarrow 3.75HS^- + 7HCO_3^- + 2.25H^+$ (Detmers et al., 2001)] and the production of hydroxide from the formation of elemental sulfur via sulfide oxidation in

the wetlands ($2HS^- + O_2 \rightarrow 2S + 2OH^-$). Due to various chemical reactions occurred simultaneously, estimated concentrations of proton were calculated based on the oxidation of acetate and benzoate in dissimilatory sulfate reduction and sulfide oxidation with oxygen (Table A1, Appendix).

On the other hand, sulfate production from complete oxidation of reduced sulfur compounds resulting in the release of protons ($HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$; $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$; $S + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$) could explain the decrease in pH along the flow path in CWs. The lower pH in CW1 was correlated with the higher intensity of reoxidation occurring in this fully planted wetland. The neutral pH range in both CWs could favor various microbially mediated processes.

According to the pH values, it is conceivable that bisulfide (HS^-) was the most predominant form of sulfide in the CWs (Figure 3.3-1 and 3.3-2). As can be seen from Figure 3.3-2, at pH 7.5, over 70% sulfide is present in the form of bisulfide, and hydrogen sulfide makes up just under 30%. During the experimental period, the conspicuous smell of hydrogen sulfide was not noticed, which suggested that hydrogen sulfide was not emitted in significant amounts from the wetlands.

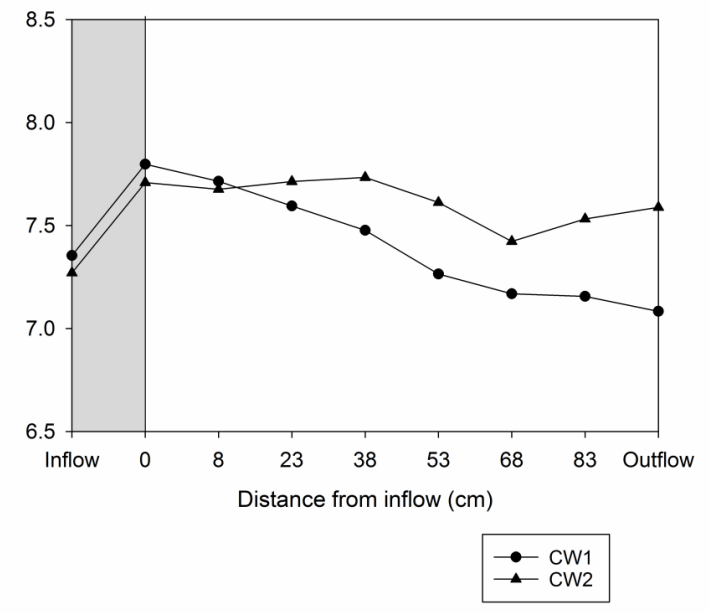


Figure 3.3-1. pH in CWs

(SD are shown in Table 3.3-1)

Table 3.3-1. pH in CWs

Wetland	No. of replicates/ SD	Distance from inflow (cm)								
		Inflow	**0**	**8**	**23**	**38**	**53**	**68**	**83**	**Outflow**
CW1	n = 10	7.35	7.80	7.72	7.60	7.48	7.26	7.17	7.16	7.08
	SD	0.09	0.21	0.19	0.15	0.15	0.10	0.10	0.14	0.09
CW2	n = 10	7.27	7.71	7.68	7.71	7.73	7.61	7.42	7.53	7.59
	SD	0.09	0.15	0.15	0.19	0.17	0.22	0.12	0.20	0.17

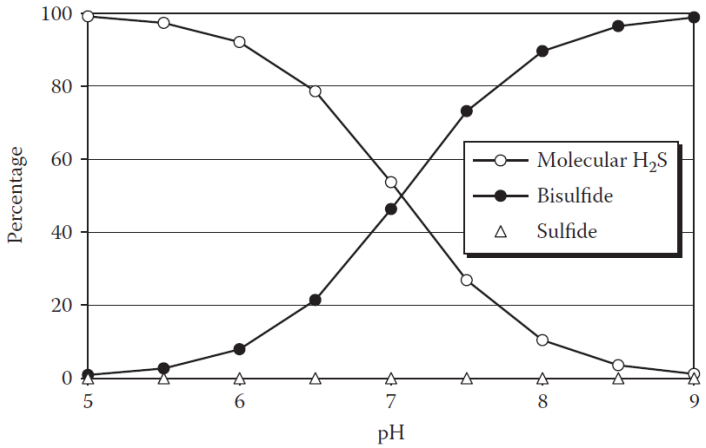


Figure 3.3-2. The forms of sulfide depending on pH values (Kadlec and Wallace, 2008)

3.3.2 Redox potential

The redox potential (E_h) shows the oxidation-reduction conditions and is related significantly to pollutant removal processes in CWs (Vymazal and Kröpfelová, 2008b; Wiessner et al., 2005a). Figure 3.3-3 shows the behavior of redox potential along the flow path in CWs.

Overall, redox potentials gradually increased towards the outflow after a decline in the first 8 cm of the wetlands. Similar behavior in E_h (reducing conditions near the inlet, followed by an increase towards the outlet) along the flow path of HSSF CWs were observed in previous studies (Garcia et al., 2003; Headley et al., 2005).

The decrease in E_h in the first 8 cm coincided with the net sulfate reduction zones of both wetland systems. The increase in E_h was associated with reoxidation of reduced sulfur compounds. In general, low E_h stimulates anaerobic processes such as sulfate reduction,

whereas high E_h promotes aerobic processes (Faulwetter et al., 2009). Redox values in the model CWs were found in the range of dissimilatory sulfate reduction (E_h < -100 mV) (Reddy and D'angelo, 1994).

Higher E_h in the fully planted CW1 in comparison with the partially planted CW2 was expected due to the effects of plants via oxygen release. A comparative study between a planted HSSF CW and an unplanted system also illustrated that higher E_h was recorded in the planted system (Tanner, 2001).

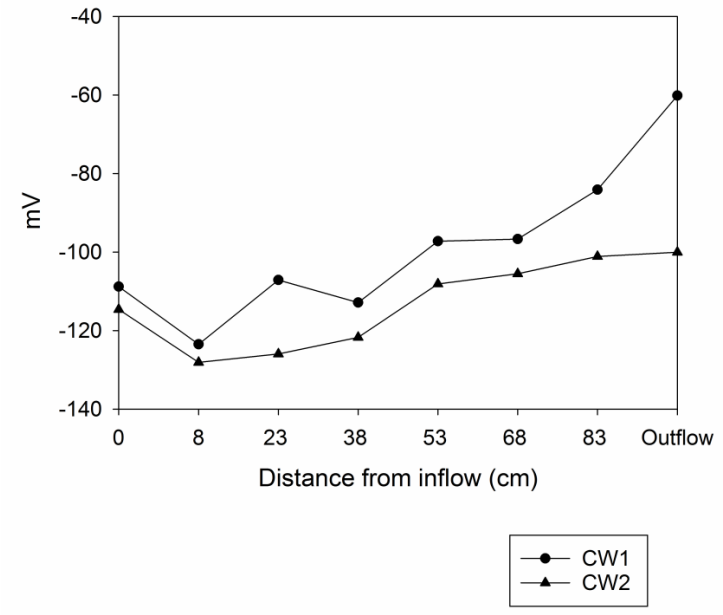


Figure 3.3-3. Redox potentials in CWs

(SD are shown in Table 3.3-2)

Table 3.3-2. Redox potential in CWs (mV)

Wetland	**No. of replicates/ SD**	**Distance from inflow (cm)**							
		0	**8**	**23**	**38**	**53**	**68**	**83**	**Outflow**
CW1	n = 8	-108.8	-123.5	-107.1	-112.9	-97.2	-96.7	-84.1	-60.1
	SD	22.96	21.24	30.51	18.28	18.68	20.49	15.81	25.71
CW2	n = 9	-114.6	-128.1	-126.0	-121.7	-108.1	-105.5	-101.1	-100.0
	SD	22.11	20.18	20.13	20.87	22.45	16.35	19.45	27.21

3.3.3 Dissolved oxygen

Dissolved oxygen (DO) is obviously needed for aerobic processes and activities of obligate aerobic microbes in CWs (Vymazal and Kröpfelová, 2008b). Behavior of DO in the model CWs is shown in Figure 3.3-4. As shown in the figure, a general decrease in DO was observed in CW1, while a peak of 0.05 mmol/d at 53 cm from the inflow was observed in CW2.

The decrease in DO in CW1 indicates consumptions of oxygen during aerobic processes such as nitrification and reoxidation of sulfur compounds. Higher DO at 53 cm of CW2 compared to that of other compartments could be related to the growth of plants in this zone, which provided more oxygen via release from the roots. The capacity of releasing oxygen of wetland plants has been well documented (Stottmeister et al., 2003; Vymazal and Kröpfelová, 2008b; Wiessner et al., 2002), however current available literature on oxygen release rates vary greatly. According to Garcia et al. (2010), oxygen release rate of cattail and bulrush in CWs with gravel beds ranged between 0.001 and 7.2 g $O_2/m^2/d$. The rate of oxygen release from plants also correlates with the redox states of the rhizosphere (Wiessner et al., 2002). Oxygen release rate was found to be highest under reduced conditions: -250 mV $< E_h <$ -150 mV (Wiessner et al., 2002). The leakage of oxygen from the roots can create an oxidized layer with an $E_h \approx 500$ mV on the root surface and $E_h \approx$ -250 mV at a distance of about 1 – 20 mm from the root surface (Faulwetter et al., 2009). The maximum oxygen release rate (0.5 mg/h/plant) of *J. effusus* was observed at E_h = - 210 mV (Wiessner et al., 2002).

In the study, values of DO in all sampling points were below 0.05 mmol/d (equivalent to 0.5 mg/L). The results were corroborated with traditional observation that HSSF CWs are mostly anaerobic systems where DO concentrations are very low or undetectable (Garcia et al., 2010; Vymazal and Kröpfelová, 2008a). However when considering high concentration of sulfide in CWs, values of DO were still high to some extent. This could be explained due to the high capacity to release oxygen of *J. effusus* or effects of sulfide on the electrodes used for measurement.

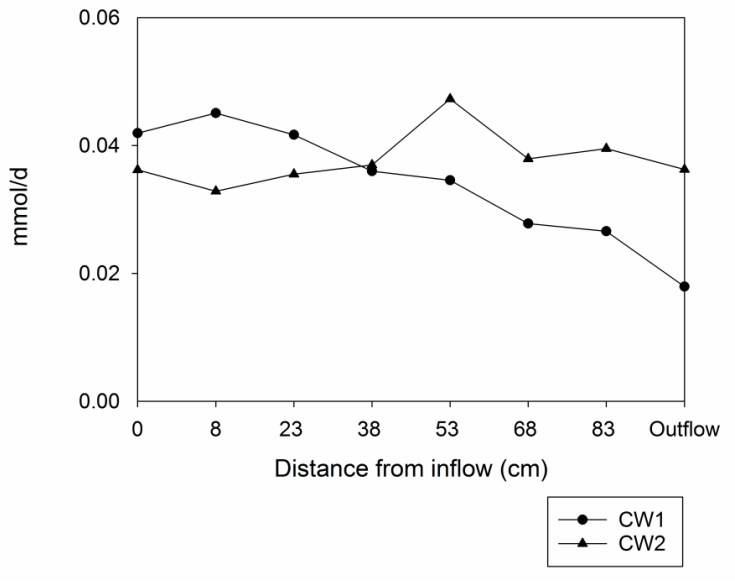


Figure 3.3-4. Dissolved oxygen in CWs

(SD are shown in Table 3.3-3)

Table 3.3-3. Loads of dissolved oxygen in CWs (mmol/d)

Wetland	**No. of replicates/ SD**	**Distance from inflow (cm)**							
		0	**8**	**23**	**38**	**53**	**68**	**83**	**Outflow**
CW1	n = 10	0.042	0.045	0.042	0.036	0.035	0.028	0.027	0.018
	SD	0.028	0.031	0.022	0.024	0.019	0.017	0.025	0.013
CW2	n = 8	0.036	0.033	0.036	0.037	0.047	0.038	0.040	0.036
	SD	0.028	0.021	0.021	0.022	0.042	0.026	0.026	0.029

3.3.4 Effects of sulfide on growth of wetland plants

The numbers of healthy shoots per specific area in CW1 and CW2 are shown in Figure 3.3-5 and 3.3-6, respectively (data recorded at the end of each month). The initial numbers of healthy shoots were about 1200 shoots/m^2 in each compartment of CWs. During the initial period when wetlands were fed with tap water (Jan – Feb 2014) to allow the plants develop their roots in the gravel beds, the numbers of healthy shoots in each compartment gradually increased. However, one month after the systems were being fed with artificial wastewaters, marked difference in plant growth in CWs were observed.

With respects to CW1, the most striking feature was the poor growth of plants in the first compartment (8 cm from in the inflow), whereas plants continued to grow well in the other compartments with increasingly healthy shoot numbers (Figure 3.3-5). Regarding CW2, death of plants was recorded in April 2014. Due to this death, replanting was carried out with an initial number of healthy shoots double than that initially. One month after replanting, the number of healthy shoots at the third compartment (38 cm from the inflow) of CW2 declined, while the number of healthy shoots increased in the fourth compartment (53 cm from the inflow) (Figure 3.3-6). The poor growth of plants in the first compartment of CW1 and the third compartment of CW2 was possibly due to sulfide toxicity. Concentrations of sulfide was 0.83 and 0.74 mmol/L in the first compartment of CW1 and the third compartment of CW2, respectively, while according to literature, growth of *J. effusus* is already inhibited at 0.5 mmol/L sulfide (Lamers et al., 2013).

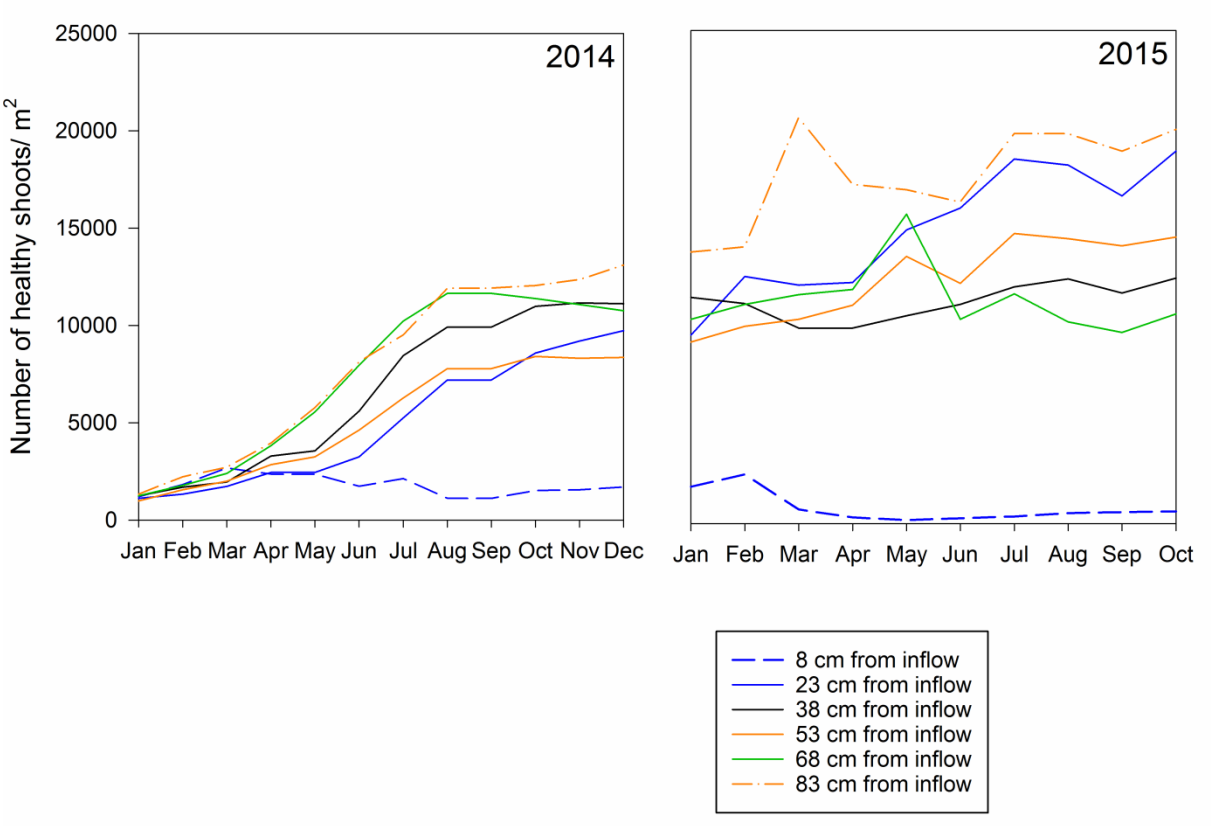


Figure 3.3-5. Plant growth in CW1

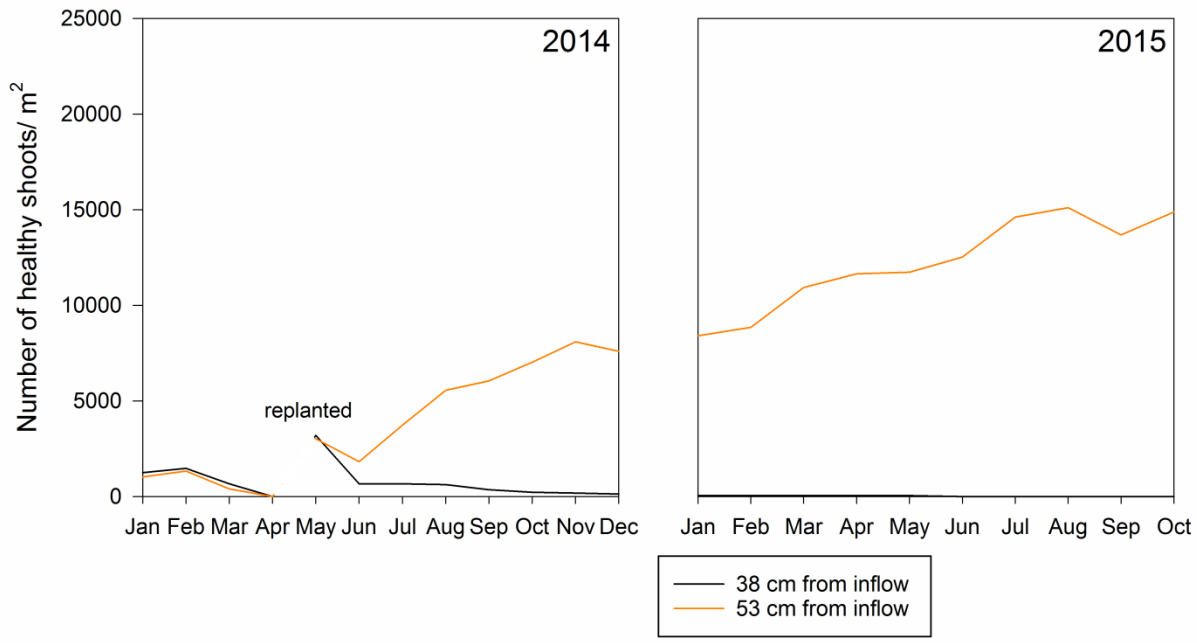


Figure 3.3-6. Plant growth in CW2

a) CW1 b) CW2 (at the front)

Figure 3.3-7. Photographs of CWs with plant growth (August 2014)

(water flow from left to right)

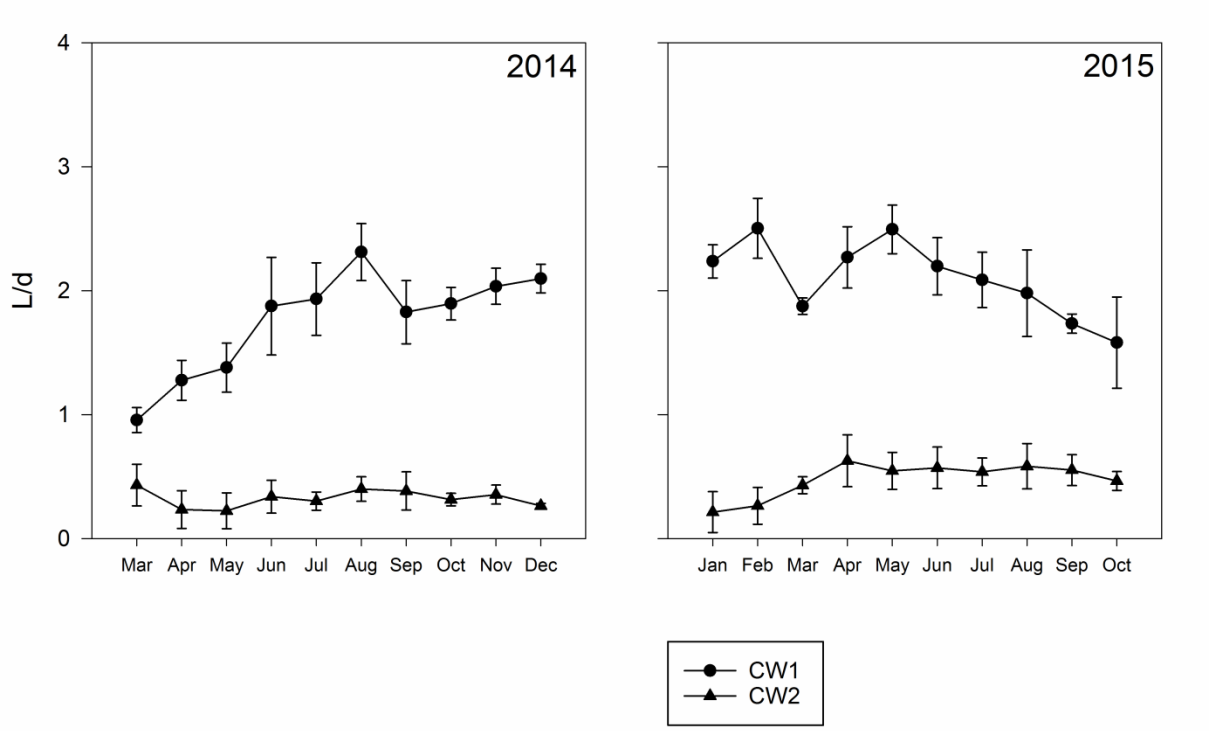


Figure 3.3-8. Water loss in CWs

Water loss due to evapotranspiration (combination of evaporation from water surfaces and plant transpiration) is one important parameter needed to be considered in wastewater treatment in CWs (Kadlec and Wallace, 2008; Stottmeister et al., 2003). Water loss in the studied CWs during the experimental period with artificial wastewater is shown in Figure 3.3-8. As illustrated in Figure 3.3-8, water loss in CW1 was almost four times greater than that of CW2: 1.93 ± 0.09 L/d versus 0.40 ± 0.05 L/d, equivalent to about 56% and 12.4% of the inflow in CW1 and CW2, respectively. The values are similar to the average water loss reported in CWs in central Europe: 20 – 50% of the inflow (Stottmeister et al., 2003).

3.4 Microbial community analysis in wetland models

Together with chemical measurements of sulfur compounds and other physicochemical parameters related to sulfur transformations, molecular microbial analysis of SOB and SRB was carried out in order to obtain a detailed view on microbial sulfur transformation in CWs at laboratory-scale.

3.4.1 SOB and SRB abundance and the correlations with behavior of sulfur compounds

3.4.1.1 SOB abundance and correlation with sulfur oxidation in CWs

Figure 3.4-1 shows the relative abundances of SOB during three sampling campaigns as based on 454 Pyrosequencing of 16S rRNA gene amplicons [once in October 2015 (DNA samples from pore water and roots) and twice in August 2015 (DNA samples from pore water)]. Total relative abundance of SOB in all compartments are given in Table A2-A3 (Appendix).

As illustrated in Figure 3.4-1, *Thiobacillus* was the most abundant SOB in all sampling campaigns (but not in all compartments). Results from sequencing of DNA samples of both pore water and roots (Figure 3.4-1a) showed that *Thiobacillus* accounted for 7% of total bacterial communities in the fourth compartment (53 cm from the inflow) of CW1 and about 18% and 15% of total bacterial communities in the last two compartments (68 and 83 cm from the inflow, correspondingly) of CW2. Relative abundances of *Thiobacillus* were much lower in pore water samples (Figure 3.4-1b and 3.4-1c), accounting for 1.3% of total bacterial communities in the third compartment of CW1 and 1.4 - 2.3% of total bacterial communities in the last two compartments of CW2.

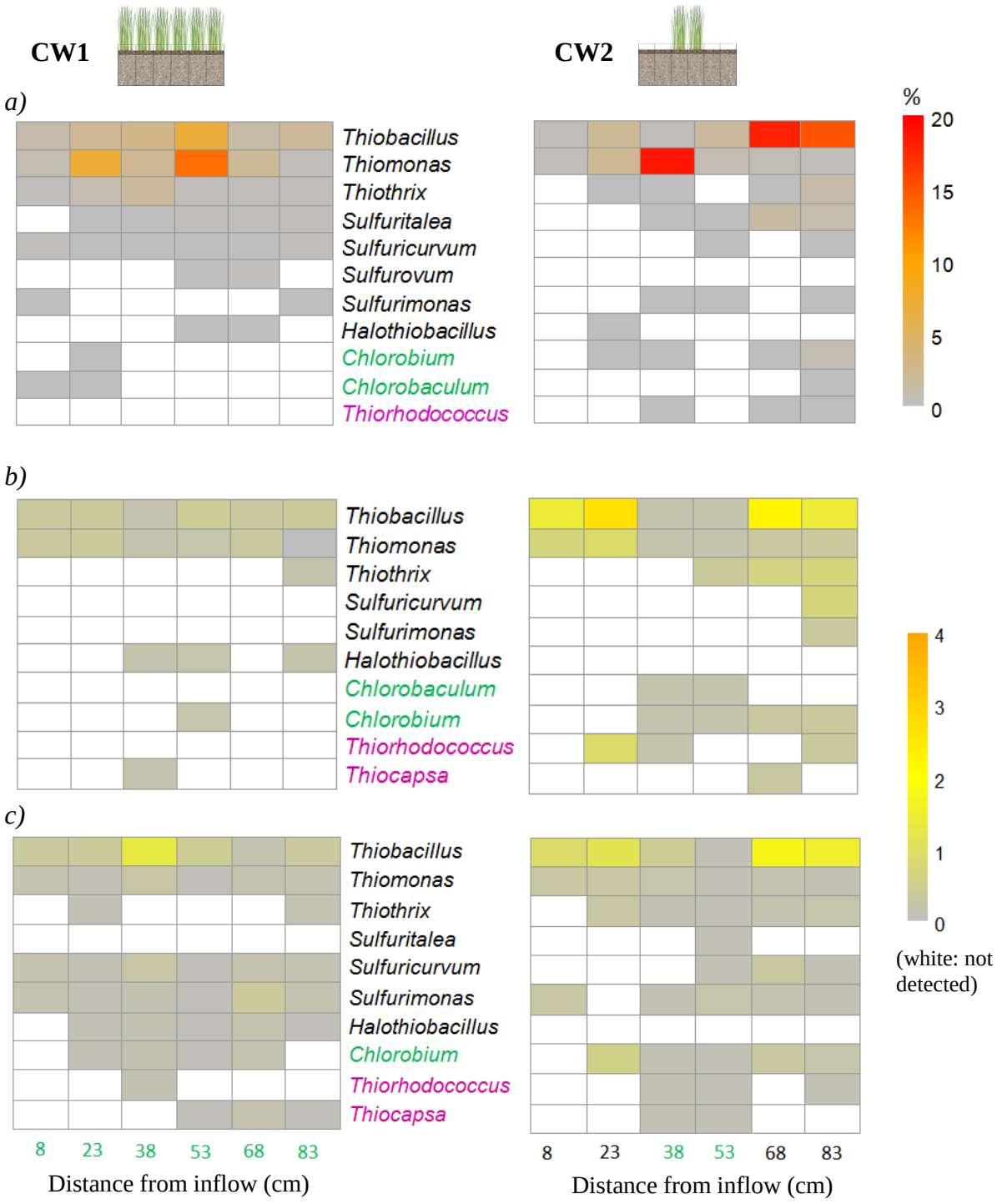


Figure 3.4-1. SOB abundances in CWs

(a: combined samples of pore water and roots in Oct, b: pore water samples on 13 Aug, c: pore water samples on 19 Aug)

Thiomonas was the second most abundant SOB with a maximum proportion of about 13% and 19% of total bacterial communities in the fourth compartment of CW1 and the third compartment of CW2, respectively (combined samples of pore water and roots). In pore waters, *Thiomonas* accounted for only 0.1 – 0.9% of total bacterial communities.

The third second most abundant SOB was *Thiothrix*, accounting for 1.9% and 1.2% of total bacterial communities in the third compartment of CW1 and the last compartment of CW2, respectively (combined samples of pore water and roots, Figure 3.4-1a). In pore water samples, *Thiothrix* was much less abundant (maximum abundance of 0.7% in the last compartment of CW2, samples on 13 Aug) and not present in several compartments (Figure 3.4-1b and 3.4-1c).

In addition, other colorless SOB, namely *Sulfuritalea*, *Sulfuricurvum*, *Sulfurovum*, *Sulfurimonas*, *Halothiobacillus* and colored SOB, namely green sulfur bacteria (*Chlorobium*, *Chlorobaculum*) and purple sulfur bacteria (*Thiorhodococcus*, *Thiocapsa*) were also present in the wetlands.

The results show that *Thiobacillus*, *Thiomonas* and *Thiothrix* (at the roots) could be key players in sulfur oxidation in CWs. SOB abundance and sulfur oxidation in CWs correlated. The high abundance of *Thiobacillus* and *Thiomonas* in the near-outlet compartments were in accordance with the low amounts of sulfide, elemental sulfur, thiosulfate-S and the enrichment of sulfate-S. *Thiobacillus* and *Thiomonas* were previously found in CWs by either cultivation methods (Hallberg and Johnson, 2005; Winter and Kickuth, 1989) or molecular-based approaches (Chen et al., 2016; Li et al., 2016; Wu et al., 2016). Bacteria of the genus *Thiobacillus* have been extensively studied and are widely applied in treatment of sulfur-containing wastewaters (Pokorna and Zabranska, 2015; Sorokin, 1994). Oxygen is a common electron acceptor for aerobic sulfide oxidation by colorless SOB like *Thiobacillus* (Tang et al., 2009; van den Ende and Gemerden, 1993). Furthermore, the ability of *T. denitrificans* in coupling nitrate reduction with oxidation of sulfur compounds under anaerobic conditions has been well reported (Krishnakumar and Manilal, 1999). The finding of genes responsible for sulfur oxidation (*sqr*, *dsr*, *apr*AB, *sat*, *sox*XAYZB) and all necessary genes for denitrification (nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase) in complete genome sequence of *T. denitrificans* further confirmed the ability to

denitrify using inorganic sulfur compounds as electron donors of this organism (Beller et al., 2006). Since *Thiobacillus* was the most abundant SOB in CWs and their dominant presence was consistent with the low concentrations of reduced sulfur compounds, oxygen and nitrate, it is possible that *Thiobacillus* could not only drive aerobic sulfide oxidation by using oxygen as electron acceptor but also played a role in sulfur-based autotrophic denitrification by using nitrate as an oxygen-substitute electron acceptor in anoxic wetland sediments, whenever nitrate was available.

In contrast to *Thiobacillus*, little attention has been paid to *Thiomonas* regarding sulfur oxidation in the literature. *Thiomonas* are obligate aerobes, facultative chemolithoautotrophs, capable of oxidizing reduced sulfur compounds (sulfide, thiosulfate, elemental sulfur) for growth but are unable to denitrify (Garrity et al., 2006). Although the ability to grow on reduced sulfur compounds has long been known (Garrity et al., 2006) and the presence of sulfur oxidation genes (*sqr*, *fcc*, *dsr*, *sor*, *sox*XAYZBCD) in genomes of several *Thiomonas* species has been documented (Arsene-Ploetze et al., 2010; Watanabe et al., 2014), most prior studies on *Thiomonas* have examined the capacity of this microbe for arsenite oxidation (Bruneel et al., 2003; Hovasse et al., 2016). There have been only a few reports on the potential application of *Thiomonas* in removal of hydrogen sulfide (Asano et al., 2012; Chen et al., 2004). In this study, high abundance of *Thiomonas* in the efficient sulfur oxidation zones in CW1 and CW2 together with previous findings on the dominance of this microbe in CWs underline the role of *Thiomonas* in sulfur oxidation in CWs. The potential application of *Thiomonas* in treating sulfur-containing wastewaters in CWs could therefore be an interesting topic for future research.

Bacteria belonging to the genus *Thiothrix* are filamentous SOB capable of utilizing reduced sulfur compounds and depositing sulfur granules intracellularly (Garrity et al., 2007). *Thiothrix* have often been found at oxic-anoxic interface environments where they can compete successfully with chemical sulfide oxidation (Teske and Nelson, 2006). *Thiothrix* is also commonly present in activated sludge in wastewater treatment plants and can cause bulking problems (Rossetti et al., 2003; Williams and Unz, 1985). Members of *Thiothrix* are aerobic or microaerophilic, facultative autotrophic, chemoorganotrophic or mixotrophic (small amount of organic compounds and reduced sulfur source are required for mixotrophic growth) (Garrity et al., 2007). The abundance of *Thiothrix* specially at the roots of CWs was

consistent with the observation that root surface environments can facilitate growth of SOB via oxygen leakage (Thomas et al., 2014). SOB living on the roots like *Thiothrix* can be also considered as rhizospheric protectors which prevent the wetland plants from sulfide toxicity. It has been reported that *Thiothrix* are able to respire nitrate to nitrite (Garrity et al., 2007; Trubitsyn et al., 2013). A recent study by Trubitsyn et al. (2014) revealed the versatile metabolism of *Thiothrix* by the finding that this microbe can reduce nitrite to gaseous products (NO and N_2O), and some species can carry out complete denitrification to molecular nitrogen under anaerobic conditions. The ability to respire nitrate to nitrite, nitric oxide and nitrous oxide is supported by the presence of respective genes in all *Thiothrix* genomes investigated thus far. The *nos*Z gene however, responsible for reduction of nitrous oxide to dinitrogen, has been not found in any *Thiothrix* genomes (Trubitsyn et al., 2014). Nevertheless, the metabolic diversity of *Thiothrix* could help the filaments to dominate in the wetlands, certainly at the oxic-anoxic interfaces or probably also in anoxic regions where reduced sulfur compounds and nitrate are present. According to current information on sulfur oxidation genes in *Thiothrix* genomes, the presence of *sqr*, *fcc*, *sox*XAYZB, *dsr*, *apr*AB, and *sat* confirm the capacity of oxidizing thiosulfate/sulfide to elemental sulfur and sulfate (Lapidus et al., 2011; Loy et al., 2009; Trubitsyn et al., 2013).

The finding of other colorless SOB in the wetlands, namely, *Sulfuritalea* (β-Proteobacteria), and *Sulfurimonas, Sulfuricurvum, Sulfurovum* (ε-Proteobacteria) is interesting since bacteria belonging to these four genera are able to carry out aerobic oxidation of reduced sulfur compounds and have the capacity to use nitrate as electron acceptor under anoxic conditions (Han and Perner, 2015; Inagaki et al., 2004; Kodama and Watanabe, 2004; Kojima and Fukui, 2011). While the ε-Proteobacteria *Sulfurimonas, Sulfuricurvum* and *Sulfurovum* can use sulfide, thiosulfate, and elemental sulfur as electron donors (Han and Perner, 2015; Kodama and Watanabe, 2004), *Sulfuritalea* grows only on thiosulfate and elemental sulfur (Kojima and Fukui, 2011; Watanabe et al., 2014). The apparent inability of *Sulfuritalea hydrogenivorans* sk43H to use sulfide contradicts with genome data, as both of the two gene systems known for sulfide oxidation, *sqr* and *fcc*, were found in the microbe's genome (Kojima and Fukui, 2011; Watanabe et al., 2014). A comparative genomic study suggested that the sulfur oxidation pathway of *S. hydrogenivorans* sk43H is similar to that of *Thiobacillus denitrificans* and different from *Thiomonas* sp. 3As, with the presence of *sqr, dsr*, *apr*AB, *sat, sox*XAYZB and the lack of *sox*CD (Watanabe et al., 2014) - but both *T.*

denitrificans and *Thiomonas* sp. 3As are able to use sulfide (Arsene-Ploetze et al., 2010; Beller et al., 2006). In the sulfur oxidizing ε-Proteobacteria group, *dsr* genes have not been found and sulfur oxidation is proposed to rely on the Sox system (Thomas et al., 2014). Key genes involved in sulfur oxidation (*sqr*, *sox*XAYZB) and all required genes for denitrification were found in *Sulfuritalea* (Watanabe et al., 2014), *Sulfurimonas* (Han and Perner, 2015; Sievert et al., 2008), *Sulfuricurvum* (Hamilton et al., 2015; Han et al., 2012; Tan and Foght, 2014) and *Sulfurovum* (Nakagawa et al., 2007). While *Sulfuritalea*, *Sulfurimonas* and *Sulfurovum* are able to oxidize sulfur compounds coupled with complete nitrate reduction to dinitrogen (Han and Perner, 2015; Inagaki et al., 2004; Kojima and Fukui, 2011), it has been reported that *Sulfuricurvum kujiense* converted nitrate to nitrite only (Kodama and Watanabe, 2004). So far all known *Sulfurimonas* species were isolated from marine sediments and deep seawater (Cai et al., 2014), *Sulfuritalea* are freshwater sulfur oxidizers (Kojima and Fukui, 2011), *Sulfuricurvum* has been found in an oil field, hydrothermal vents, and ground water environments (Han et al., 2012), and *Sulfurovum* is widely distributed in hydrothermal vents and marine sediments (Nakagawa et al., 2007). Among these organisms, *Sulfuricurvum* has been detected in CWs by molecular methods (Ansola et al., 2014; Chen et al., 2016). Due to their ability to couple the oxidation of reduced sulfur compounds with nitrate respiration, members of *Sulfuritalea*, *Sulfurimonas*, *Sulfuricurvum* and *Sulfurovum* are of ecological interest and their presence in the CWs (in the zones of low concentrations of reduced sulfur concentrations and elevated sulfate) suggest that they could play a role in both aerobic sulfur oxidation and sulfur-driven autotrophic denitrification.

Together with *Sulfuritalea*, *Sulfurimonas*, *Sulfuricurvum* and *Sulfurovum*, the presence of *Halothiobacillus*, which has been frequently found in hypersaline habitats (Sorokin et al., 2013), reveals a high diversity of the SOB communities in the wetlands. Some members of *Halothiobacillus*, formerly assigned to the genus *Thiobacillus* (Kelly and Wood, 2000), are obligate chemolithoautotrophic and capable of oxidizing reduced inorganic sulfur compounds (Kelly and Wood, 2000; Pokorna and Zabranska, 2015). The potential application of *H. neapolitanus* in hydrogen sulfide removal from biogas has been reported (Vikromvarasiri and Pisutpaisal, 2014). By a cultivation-based method, *Halothiobacillus* was also found in acid mine drainage (Hallberg and Johnson, 2005), however in general the role of *Halothiobacillus* in CWs is poorly understood. As aerobic environments favor the growth of *Halothiobacillus*

(Sorokin et al., 2013), this genus was mostly found in CW1, where oxygen is expected to be more available via release from plant roots.

In addition to diverse groups of colorless SOB, the presence of green sulfur bacteria (*Chlorobium, Chlorobaculum*) and purple sulfur bacteria (*Thiorhodococcus, Thiocapsa*) reveal a fascinating colorful world of sulfur oxidizers within the wetlands. In the presence of light, all members of green and purple sulfur bacteria detected in CWs have been known to oxidize sulfide, thiosulfate and elemental sulfur during anoxygenic photosynthesis (Frigaard and Dahl, 2009). The ability to utilize sulfite has been observed in the purple SOB *Thiorhodococcus* and *Thiocapsa* but not yet been recorded in any green SOB (Frigaard and Dahl, 2009). Chemotrophic growth under micro-oxic conditions in the dark was possible for some members of the purple sulfur bacteria *Thiorhodococcus* and *Thiocapsa* (Caumette et al., 2004; Frigaard and Dahl, 2009; Guyoneaud et al., 1997), while green sulfur bacteria are obligate phototrophs (Camacho, 2010). When sulfide is abundant, the green bacteria *Chlorobium, Chlorobaculum* can accumulate elemental sulfur extracellularly, whereas the purple bacteria *Thiorhodococcus, Thiocapsa* deposit elemental sulfur intracellularly (Frigaard and Dahl, 2009). Under sulfide limitation, both green and purple bacteria can further oxidize elemental sulfur to sulfate (Sturman et al., 2008). Therefore, in the wetland zones where sulfate was enriched and sulfide was limited, phototrophic SOB could play a role in complete sulfur oxidation to sulfate. Genes involved in sulfur oxidation: *sqr, fcc, sox*XAYZB, *dsr, apr*AB, *sat* were all found in *Chlorobium, Chlorobaculum* (Frigaard and Dahl, 2009), *Thiorhodococcus* (https://www.ncbi.nlm.nih.gov/genome/?term=thiorhodococcus) and *Thiocapsa* (https://www.ncbi.nlm.nih.gov/genome/?term=thiocapsa), which confirm their ability to grow on reduced sulfur compounds.

In this study, *Thiocapsa* was only detected in pore water samples (Figure 3.4-1). The concurrent dominant presence of *Thiobacillus* and the absence of *Thiocapsa* in samples of pore water and roots accord with earlier observation, which showed that *Thiobacillus* outcompeted *Thiocapsa* in the competition for sulfide as electron donor when oxygen was available, and their coexistence was observed only under oxygen limitation (van Gemerden, 1993). In general, low abundance of colored SOB suggests that their importance in sulfur oxidation may not be as high as colorless SOB like *Thiobacillus* in the wetlands.

In addition to traditional colorless SOB (e.g. *Thiobacillus, Thiomonas, Thiothrix*), green and purple SOB listed above, non-traditional colorless SOB (*Paracoccus, Pseudomonas, Hyphomicrobium*) and a variety of purple non-sulfur bacteria (e.g. *Rhodobacter, Rhodoplanes, Rhodococcus, Rhodomicrobium*) were also detected in CWs albeit with much lower abundance (Table A4-A9, Appendix).

In subsurface flow CWs, aerobic processes such as nitrification and sulfide oxidation occur mostly at the root surface, whereas anaerobic processes such as denitrification and sulfate reduction prevail in the rhizosphere (Stottmeister et al., 2003). Because of the oxic conditions created by oxygen leakage, root surface can be considered as important microbial niches for aerobic SOB. Due to the likely occurrence of nitrification mostly on the roots, nitrate produced from nitrification can also serve as electron acceptor for SOB. In this study, the presence of many SOB groups being known to be capable of denitrifying suggest their potential contribution in sulfur-driven autotrophic denitrification. Due to the capacity to release oxygen (Wiessner et al., 2002), the root surface can funtion as "microaerated zone" providing consistent oxygen flux for aerobic SOB, therefore sulfur-dependent denitrification may not as important as aerobic sulfur oxidation on the roots. In contrast, maximum nitrate flux has been reported to be in the range of 20 – 25 mm from the root apex of rice, maize, and wheat (Colmer and Bloom, 1998; Zhong et al., 2014) and since the environments become more anoxic with distance from the roots (Colmer and Pedersen, 2008), sulfur oxidation coupled with nitrate reduction may be important in the rhizosphere where oxygen is limited.

To summarize, the concurrent presence of different metabolic groups of SOB in CW1 and CW2 in the zones of efficient sulfur oxidation indicates diverse SOB communities and their potential activities in oxidation of reduced sulfur compounds in the wetlands. Root surface of *J. effusus* could foster growth and activities of SOB by oxygen release and by providing microbial attachment sites. The dominance of SOB near the outlet zones of the wetlands were in accordance with previous findings on distribution patterns of SOB in CWs based on cultivation methods (Aguilar et al., 2008) and modelling results (Langergraber and Šimůnek, 2012; Samsó and García, 2013). The presence of many SOB groups known to be capable of denitrifying under anoxic conditions suggests their potential contribution in sulfur-driven autotrophic denitrification in the wetlands.

3.4.1.1 SRB abundance and correlation with sulfate reduction in CWs

In order to obtain a comprehensive view of microbial sulfur transformations in CWs, SRB communities were also taken into account.

Figure 3.4-2 shows the abundance of SRB in three different sampling campaigns as revealed by 454 Pyrosequencing [once in October 2015 (DNA samples from pore water and roots) and twice in August 2015 (DNA samples from pore water)]. Total relative abundance of SRB in all compartments are given in Table A10-A11 (Appendix).

Overall, high abundance of SRB found in major sulfate reduction zones in CWs indicates a positive correlation between potential activities of SRB and sulfate reduction. The most abundant groups of SRB were *Desulforhabdus*, *Desulfobacter* and *Desulfocapsa*. In samples of pore water and roots, *Desulforhabdus* made up about 10% of total bacterial communities in the first compartment of CW1, whereas in CW2, the most abundant SRB were found in the first two compartments (unplanted zones): *Desulforhabdus* accounted for 7% the total bacterial communities (in the second compartment), *Desulfobacter* accounted for 10% and 16% total bacterial communities (in the first and the second compartment, respectively) (Figure 3.4-2a). In pore water, *Desulfocapsa* made up about 4% of total bacterial communities in each of the first two compartments of CW2 (Figure 3.4-2b). In addition, *Desulfovibrio*, *Desulfobacula* and the sulfur reducer *Desulfuromonas* were also dominant in the first two compartments of the wetlands. Similar to earlier studies, *Desulfobacter*, *Desulfovibrio*, and *Desulfocapsa* were determined as representative SRB in CWs (Chen et al., 2016; King et al., 2002; Lloyd et al., 2004; Russell et al., 2003). The dominant presence of SRB in the first two compartments fully corroborated with modelling results which suggested that SRB were over-represented near the inlet zones in CWs (Langergraber and Šimůnek, 2012; Samsó and García, 2013).

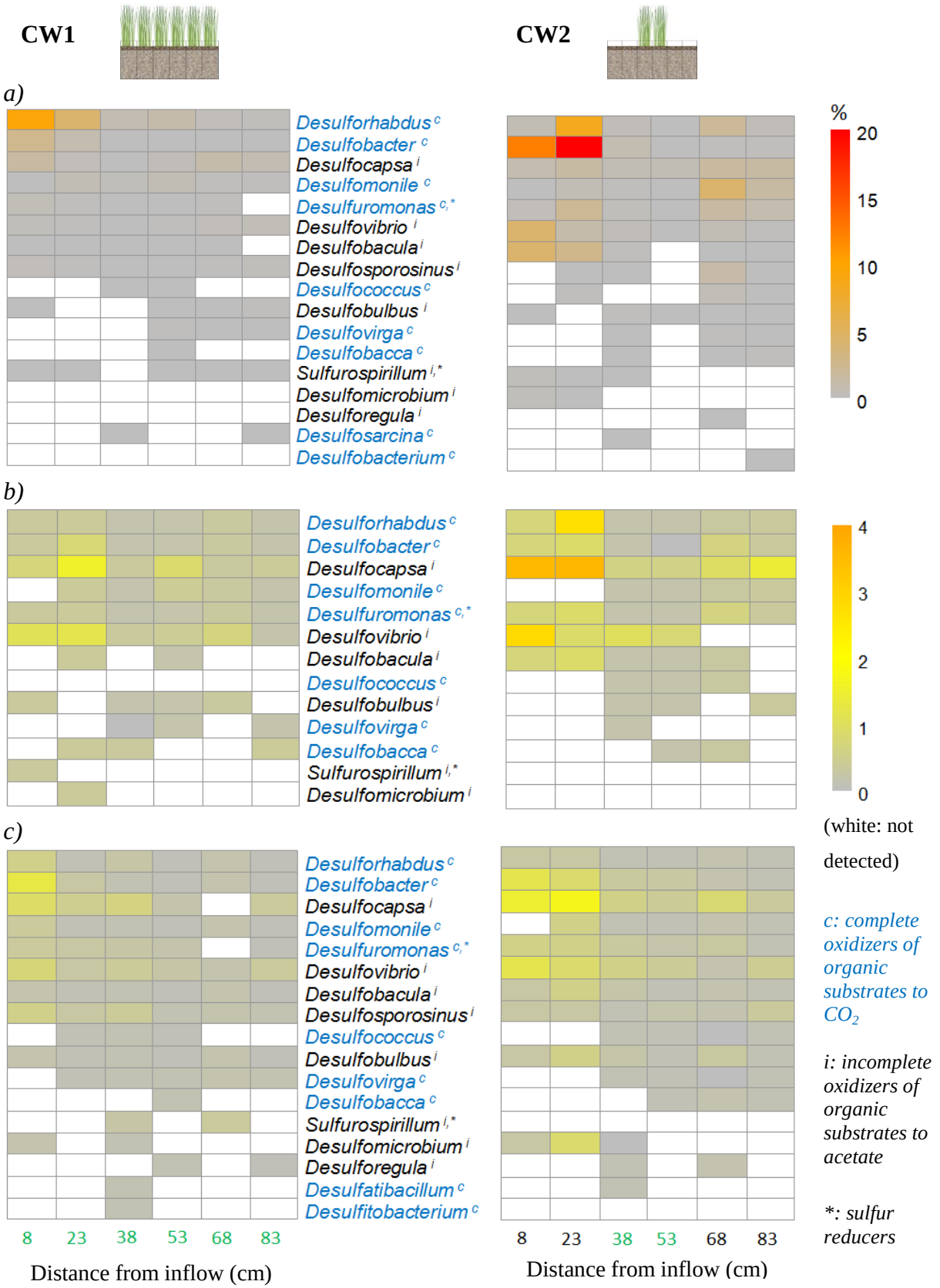


Figure 3.4-2. SRB abundances in CWs

(a: combined samples of pore water and roots in Oct, b: pore water samples on 13 Aug, c: pore water samples on 19 Aug)

In anoxic wetland sediments, SRB can utilize sulfate as terminal electron acceptor and generate acetate or inorganic carbon and sulfide (H_2S, HS^- or S^{2-}, depending on pH) (Kadlec and Wallace, 2008). A variety of carbon sources from simple organic compounds (e.g. acetate), aromatic compounds (e.g. benzoate), alcohols (e.g. ethanol, butanol) or inorganic compounds (e.g. hydrogen, carbon monoxide) can be used as electron donors by SRB to drive dissimilatory sulfate reduction (Odom et al., 2013). According to their metabolic capacity, SRB are classified into two groups: incomplete oxidizers (non-acetate oxidizers) (e.g. *Desulfovibrio*) and complete oxidizers (or acetate oxidizers) (e.g. *Desulfobacter*) (Sturman et al., 2008). The former carry out incomplete oxidation of organic substrates into acetate, while the latter are able to oxidize organic substrates completely to CO_2 (Odom et al., 2013). In this study, the dominance of *Desulforhabdus* and *Desulfobacter* (complete oxidizers) and the presence of various completely oxidizing SRB coincided with the concomitant fast depletion of acetate and benzoate as well as the rapid decrease in TOC (about 90%) in the first two compartments of both wetlands. The findings indicate the important role of SRB in degradation of organic matters, which has been previously highlighted by former investigators (Baptista et al., 2003; Jørgensen, 1982; Muyzer and Stams, 2008). In addition, after the high abundance in the first two compartments of CWs, the continuous presence of numerous SRB from the third compartment towards the outflow suggest that sulfate reduction was still occurring significantly in the systems [even dominant presence were still observed: *Desulfomonile* accounted for more than 3% of total bacterial communities in the fifth compartment of CW2 (Figure 3.4-2a), *Desulfocapsa* made up over 1% of total bacterial communities in the last compartment of CW2 (Figure 3.4-2b)]. SRB could use residual sulfate from the inflowing wastewater and sulfate produced from sulfur reoxidation by SOB activities. Furthermore, members of *Desulfobacter*, *Desulfocapsa* and *Desulfovibrio* have been reported to be capable of disproportionating sulfur compounds (Finster, 2008). Thus, the presence of those genera in both CWs points towards the possibility that sulfur disproportionation could have occurred. Sulfur disproportionation has been considered as a significant process in reduced sediments (Fossing and Jørgensen, 1990) and an important sulfur transformation process in anoxic wetland sediments and pore water (Wu et al., 2013).

Syntrophic growth of SRB with *Syntrophus* has been well documented (Elshahed et al., 2003; Morris et al., 2013). Interestingly, in this study, *Syntrophus* was present dominantly in the first two unplanted compartments of CW2 where relative abundance of SRB were also very

high. *Syntrophus* made up about 27% and 15% of total bacterial communities in the first and the second compartment of CW2, respectively, whereas in the fully planted CW1, their proportion was always below 1% (Table A12-A13, Appendix). Members of the genus *Syntrophus* are strict anaerobes and some can degrade benzoate to acetate, hydrogen and CO_2 (Garrity et al., 2006). Thus it is possible that acetate produced from benzoate degradation by *Syntrophus* could fuel acetate-oxidizing SRB like *Desulforhabdus* and *Desulfobacter* in the wetlands. The non-acetate oxidizer *Desulfovibrio* may also have profited from growth of *Syntrophus* due to the capacity to utilize hydrogen as electron donor for sulfate reduction (Auburger and Winter, 1996).

Another interesting finding to emerge from the data is the possible occurrence of dissimilatory sulfur reduction in CWs. Dissimilatory sulfur reduction is a process driven by microorganisms which are unable to reduce sulfate but able to reduce elemental sulfur to hydrogen sulfide (Madigan et al., 2014). In this study, key genera of sulfur reducers were detected in both CWs: *Desulfuromonas* and *Sulfurospirillum*. Both of those sulfur reducers can grow on elemental sulfur as electron acceptor, however only *Desulfuromonas* can use acetate as electron donor for sulfur reduction, whereas acetate oxidation has never been observed in *Sulfurospirillum* (Garrity et al., 2006). There may have been interrelationships among sulfur reducers, colorless and colored SOB in CWs. Sulfur reducers can utilize elemental sulfur produced from sulfide oxidation by SOB. Syntrophic growth of *Desulfuromonas* and phototrophic *Chlorobium* during acetate oxidation has been described by Warthmann et al. (1992). Elemental sulfur produced from sulfide oxidation by *Chlorobium* can be subsequently used as electron acceptor in sulfur reduction by *Desulfuromonas*, leading to the production of sulfide (Warthmann et al., 1992).

SRB have long been described as strictly anaerobic microorganisms that are ubiquitous in anoxic environments (Muyzer and Stams, 2008). However, the capacity of SRB to survive under oxic conditions has been revealed (Cypionka, 2000; van den Ende et al., 1997), and it is now acknowledged that SRB possess versatile metabolic capacities, much more than previously being thought (Brune et al., 2000; Zhou et al., 2011). An active coculture of an SRB, *Desulfovibrio*, and an aerobic SOB, *Thiobacillus*, has been reported (van den Ende et al., 1997). Apart from pure-culture study, the evidence of dissimilatory sulfate reduction taken place in oxic zones of natural environments has been published (Cypionka, 2000). Based on

genome studies, it has been shown that *Desulfovibrio* has fascinating defense mechanisms in response to reactive oxygen species, which could help the organisms being tolerant to oxygen exposure (Zhou et al., 2011). The metabolic flexibility of SRB therefore could explain their coexistence with SOB in the planted zones of CWs. Interestingly, both *Thiobacillus* and *Desulfovibrio*, two dominant genera detected in the two wetlands, have been listed as representative plant surface microbes (together with other plant-associated microbial groups such as *Azospirillum*, *Rhizobium*) (Andrews and Harris, 2000). On the other hand, although special mechanisms for oxygen adaptation (Zhou et al., 2011) and benefits from utilizing organic carbon from root exudates in wetland plants for sulfate reduction of SRB have been documented (Stein et al., 2007; Stottmeister et al., 2003), in general, it seems that oxygen is a critical factor negatively affecting the abundance of SRB in the wetlands in this study. This hypothesis is proposed from the observation that higher abundance of SRB was detected in the first two unplanted compartments of CW2 and the widespread distribution of SRB in samples of pore water (Figure 3.4-2b and 2c) compared to samples of pore water and roots in the wetlands (Figure 3.4-2a). The results were consistent with those of prior studies on interactions between SRB and plants (Stein et al., 2007; Wind et al., 1999) and fully match with the predicted higher distribution of SRB in CWs when oxygen released from plant roots was not taken into account by a stimulation study by Langergraber and Šimůnek (2012). The study on SRB in rice field soil and on rice roots by Wind et al. (1999) demonstrated that sulfate reducers on the roots were inhibited by oxygen and highest number of SRB was found in unplanted bulk soil. Inhibitory effects of plants on SRB activity by oxygen transfer from plant roots to the rhizosphere was also emphasized by Stein et al. (2007). Since the capacity of releasing oxygen from the roots of *J. effusus* has been well reported (Wiessner et al., 2002), it can thus be assumed that oxygen could inhibit growth of SRB on the roots in CWs. Nevertheless, in reviewing the literature, current knowledge regarding the complex SRB-plants interactions remains poorly understood, therefore further studies on this topic are recommended.

3.4.2 Quantitative qPCR targeting 16S rRNA genes

Quantitative PCR was carried out to determine copy numbers of 16S rRNA genes in wetland samples. The overall picture of copy numbers of 16S rRNA genes in wetland samples is shown in Figure 3.4-3. Samples in October (one sampling campaign) were from pore water,

gravel and roots, whereas samples in August (two sampling campaigns) were from pore water only.

As shown in Figure 3.4-3, in CW1 copy numbers of 16S rRNA genes were found highest in root samples in the first compartment (8 cm from the inflow) and decreased by about 7 times in the last compartment (83 cm from the inflow): 1.82×10^{12} against 2.68×10^{11} copy numbers/g root. In CW2, highest copy numbers of 16S rRNA genes were found in root sample in the fourth compartment (53 cm from the inflow): 1.56×10^{12} copy numbers/g root, about seven-fold greater than that of sample in the earlier planted compartment (38 cm from inflow): 2.05×10^{11} copy numbers/g root.

Regarding samples of pore water from three sampling campaigns along the flow path, in CW1 the highest copy numbers of 16S rRNA genes were found in the first two compartments: between 3.91×10^{10} and 1.55×10^{11} copy numbers/100 ml, whereas in CW2 the highest values were recorded in the first compartment (8 cm from the inflow): in the range from 8.02×10^{11} to 2.84×10^{12} copy numbers/100 ml.

As emerging from the data, low copy numbers of 16S rRNA genes were determined in gravel samples in the relative comparison with that of pore water and roots. As illustrated in Figure 3.4-3, 16S rRNA gene copy numbers in gravels were only at a similar level as in root-derived samples in the sample from the first compartment of CW2 (8 cm from inflow). This was also the highest value of 16S rRNA gene copy numbers recorded: 4.59×10^{10} copy numbers/g gravel, being over two orders of magnitude greater than that of the gravel sample in the first planted compartment of CW1 (3.17×10^{8} copy numbers/g gravel).

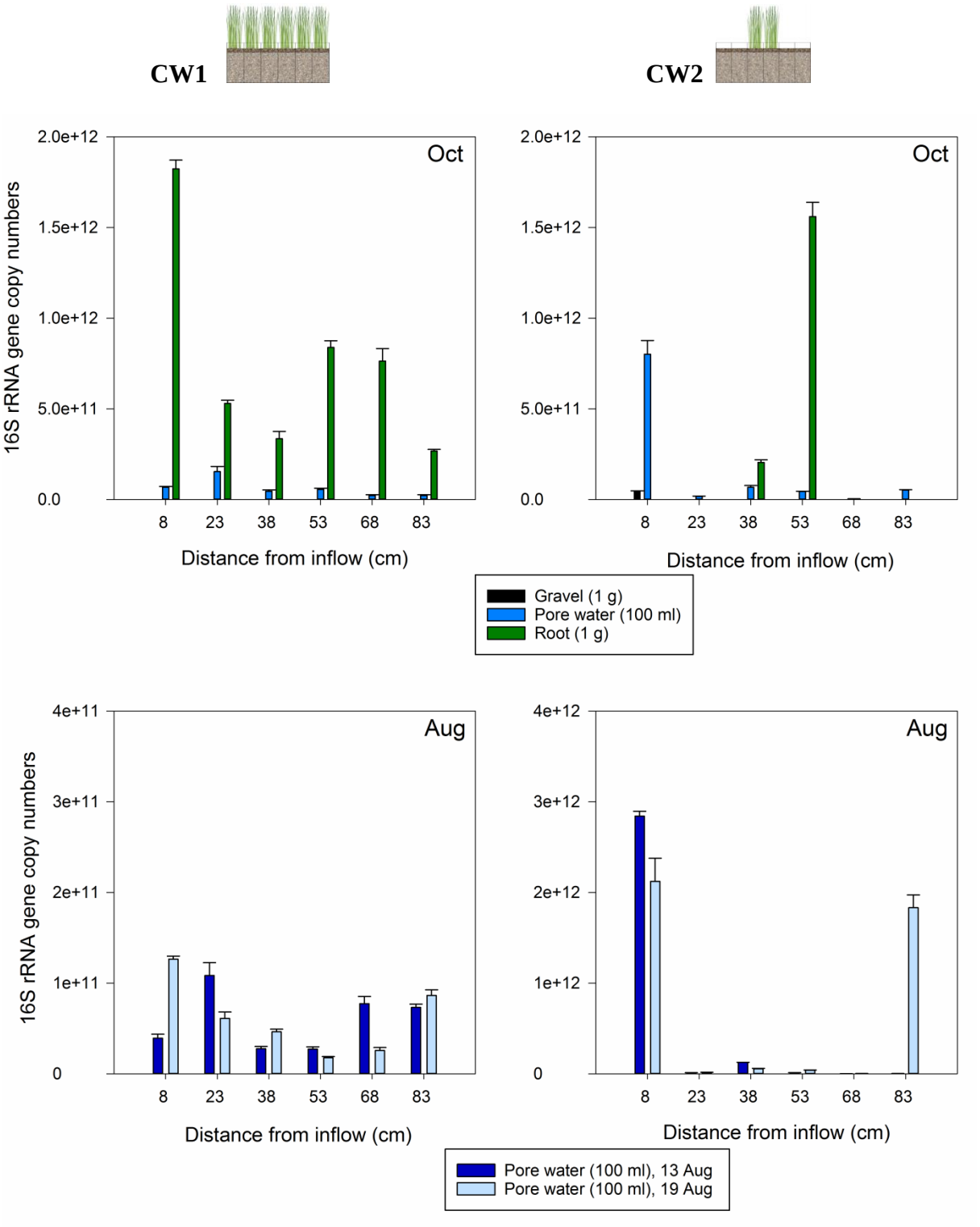


Figure 3.4-3. 16S rRNA gene copy numbers in CWs

(detailed data of 16S rRNA gene copy numbers and SD is shown in Table 3.4-1 and 3.4-2)

Table 3.4-1. 16S rRNA gene copy numbers in CW1

Samples	16S rRNA gene copy numbers	Distance from inflow (cm)					
		8	**23**	**38**	**53**	**68**	**83**
Roots (Oct)	Copy numbers	**1.82e+12**	**5.30e+11**	**3.35e+11**	**8.39e+11**	**7.64e+11**	**2.68e+11**
	SD	4.78e+10	1.78e+10	4.02e+10	3.73e+10	6.88e+10	9.51e+09
Gravel (Oct)	Copy numbers	**3.17e+08**	**2.96e+07**	**1.74e+06**	**1.83e+08**	**2.65e+07**	**4.08e+08**
	SD	4.91e+07	4.83e+06	7.32e+05	2.80e+07	3.12e+06	7.00e+07
Pore water (Oct)	Copy numbers	**6.76e+10**	**1.55e+11**	**4.66e+10**	**5.63e+10**	**2.25e+10**	**2.21e+10**
	SD	4.85e+09	2.88e+10	7.05e+09	6.59e+09	4.44e+09	4.60e+09
Pore water (13 Aug)	Copy numbers	**3.91e+10**	**1.08e+11**	**2.75e+10**	**2.71e+10**	**7.73e+10**	**7.32e+10**
	SD	4.36e+09	1.42e+10	2.65e+09	2.63e+09	7.98e+09	3.52e+09
Pore water (19 Aug)	Copy numbers	**1.26e+11**	**6.10e+10**	**4.63e+10**	**1.75e+10**	**2.55e+10**	**8.63e+10**
	SD	3.39e+09	7.05e+09	2.86e+09	1.36e+09	3.35e+09	6.24e+09

Root: 1 g, gravel: 1 g, pore water: 100 ml

Table 3.4-2. 16S rRNA gene copy numbers in CW2

Samples	16S rRNA gene copy numbers	Distance from inflow (cm)					
		8	**23**	**38***	**53***	**68**	**83**
Roots (Oct)	Copy numbers	**-**	**-**	**2.05e+11**	**1.56e+12**	**-**	**-**
	SD			1.44e+10	7.89e+10		
Gravel (Oct)	Copy numbers	**4.59e+10**	**1.39e+07**	**1.14e+08**	**3.66e+06**	**1.53e+08**	**1.69e+05**
	SD	2.13e+09	7.76e+05	6.23e+06	1.33e+06	8.04e+06	9.68e+04
Pore water (Oct)	Copy numbers	**8.02e+11**	**1.79e+10**	**6.79e+10**	**4.45e+10**	**3.98e+09**	**5.26e+10**
	SD	7.62e+10	1.88e+09	9.57e+09	2.05e+09	4.57e+08	2.67e+09
Pore water (13 Aug)	Copy numbers	**2.84e+12**	**1.01e+10**	**1.24e+11**	**1.12e+10**	**4.63e+08**	**2.11e+09**
	SD	5.20e+10	8.17e+08	1.26e+09	5.11e+08	2.76e+07	3.68e+08
Pore water (19 Aug)	Copy numbers	**2.12e+12**	**1.51e+10**	**5.45e+10**	**3.80e+10**	**1.89e+09**	**1.83e+12**
	SD	2.56e+11	7.89e+08	4.36e+09	3.11e+09	5.9e+07	1.40e+11

** planted; Root: 1 g, gravel: 1 g, pore water: 100 ml*

The finding of significantly high copy numbers of 16S rRNA genes in the root samples accords with earlier observations, which showed that the main favorable habitats for microorganisms in CWs are plant roots (Brix, 1997; Gersberg et al., 1986; Stottmeister et al., 2003). Plants provide beneficial surface area for root-attached microbes (Brix, 1997; Gagnon et al., 2007) and micro-oxic environments via oxygen leakage from roots, leading to stimulated growth of aerobic microorganisms on the rhizoplane (root surface) and in the rhizosphere (root zone) (Stottmeister et al., 2003) and accordingly enhancing aerobic processes such as microbial sulfur oxidation and nitrification (Brune et al., 2000; Faulwetter et al., 2009). It has been shown that density and activity of aerobic and facultative bacteria was greatly enhanced in planted microcosm (Gagnon et al., 2007). In addition to oxygen, organic compounds excreted from plant roots such as sugars, organic acids and vitamins (e.g. thiamine, riboflavin) can also facilitate growth of rhizopheric microbes (Stottmeister et al., 2003). Organic carbons from root exudates can be used as substrates for aerobes as well as anaerobes such as denitrifiers and sulfate reducers to drive denitrification and sulfate reduction (Brix, 1997; Stottmeister et al., 2003; Zhai et al., 2013). Therefore it is not surprising that high copy numbers of 16S rRNA genes were found in root samples in this study. Lower copy numbers of 16S rRNA genes of the root sample in the third compartment of CW2 compared to that in the next compartment were associated with deficient plant growth in the third compartment, which expectedly caused by sulfide toxicity (as mentioned earlier in section 3.3.4).

Among different sampling points along the flow path, the observed high copy numbers of 16S rRNA genes in pore water and gravel in the first compartment of each wetland can be explained by the availability of a wide range of substrates for microbial growth (e.g. sulfate, acetate, benzoate) in the inflowing wastewater. Higher copy numbers of 16S rRNA genes in the gravel sample from the unplanted compartment of CW2 compared to that in the first planted compartment of CW1 indicate that gravel surface could be important attachment sites for microorganisms in CW2, whereas major habitats for microbes in CW1 were assumed to be the rhizoplane rather than gravel. Other researchers have pointed out that solid surfaces provide area for bacterial colonization (Kurzbaum et al., 2010; van Loosdrecht et al., 1990), specially root surface is preferable for many functional bacterial groups (Hernandez et al., 2015; Varma et al., 2007). As shown earlier in section 3.4.1, relative abundance of SOB in the combined samples of pore water and roots were much higher than that of the pore water

alone. Here, high copy numbers of 16S rRNA genes found in root samples provides quantitative evidence for higher absolute abundance of SOB in the samples of pore water and roots compared to that of pore water only (in the range of 30 – 300 times). Hence it is plausible to suggest that growth of SOB was stimulated on the roots of *J. effusus*.

3.4.3 Microbial community compositions

The critical role of microorganisms in pollutant elimination in CWs has been well established (Faulwetter et al., 2009; Stottmeister et al., 2003), therefore information about the presence and compositions of microbial communities in CWs helps to obtain a better understanding on pollutant removal processes in treatment wetlands (Ansola et al., 2014). With the capacity to release organic carbons via root exudates (Stottmeister et al., 2003), effects of plants on microbial community structures in CWs has been demonstrated (Chen et al., 2015). An insight into dynamics of microbial community compositions in the CWs in this study will be presented in this section. Microbial community compositions of pore water and root samples in CWs (sampling campaign in Oct 2015) are illustrated in Figure 3.4-4, whereas that of pore water samples (sampling campaigns on 13 and 19 Aug 2015) are shown in Figure 3.4-5.

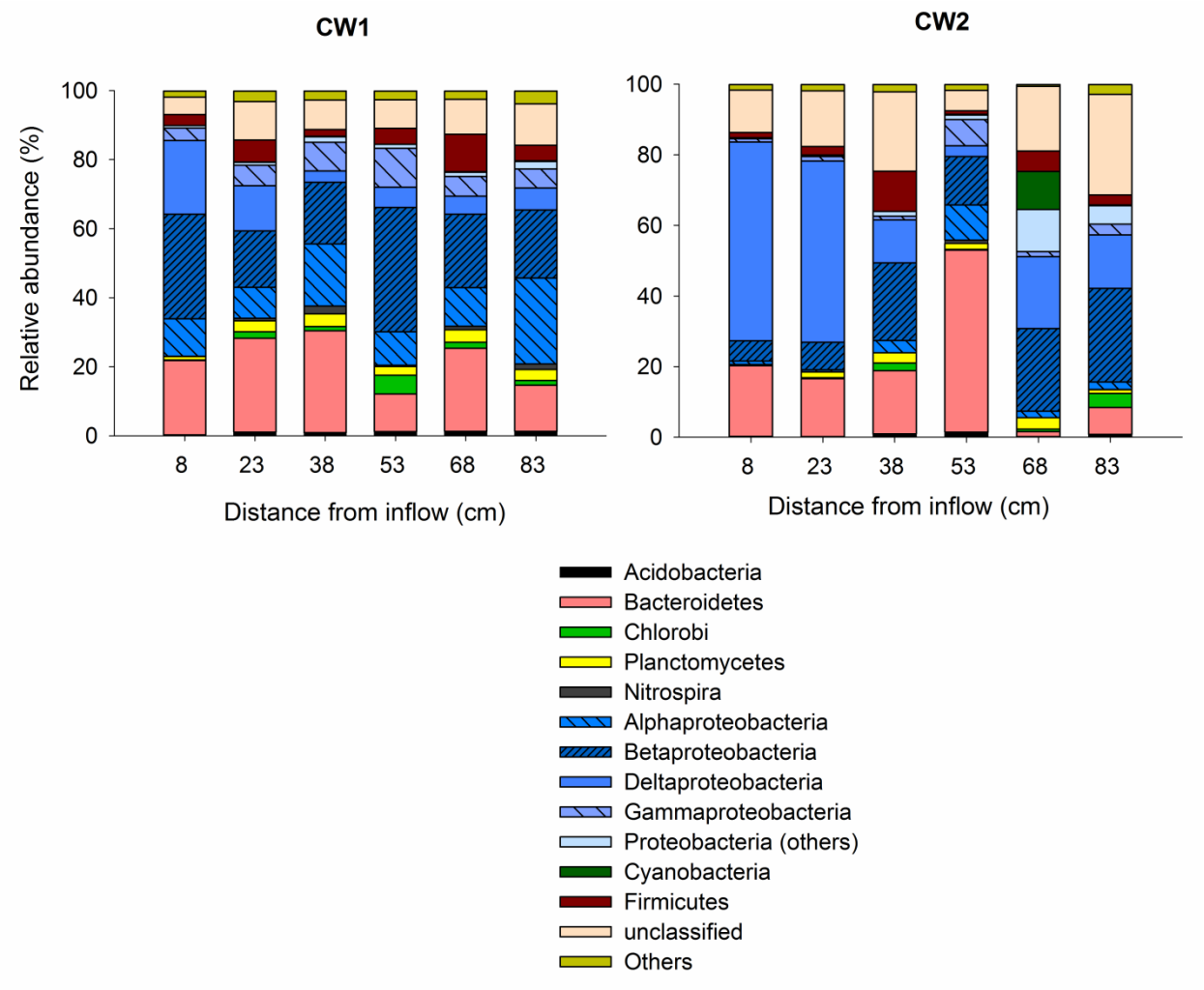


Figure 3.4-4. Microbial community compositions of pore water and root samples in CWs (samples in Oct 2015)

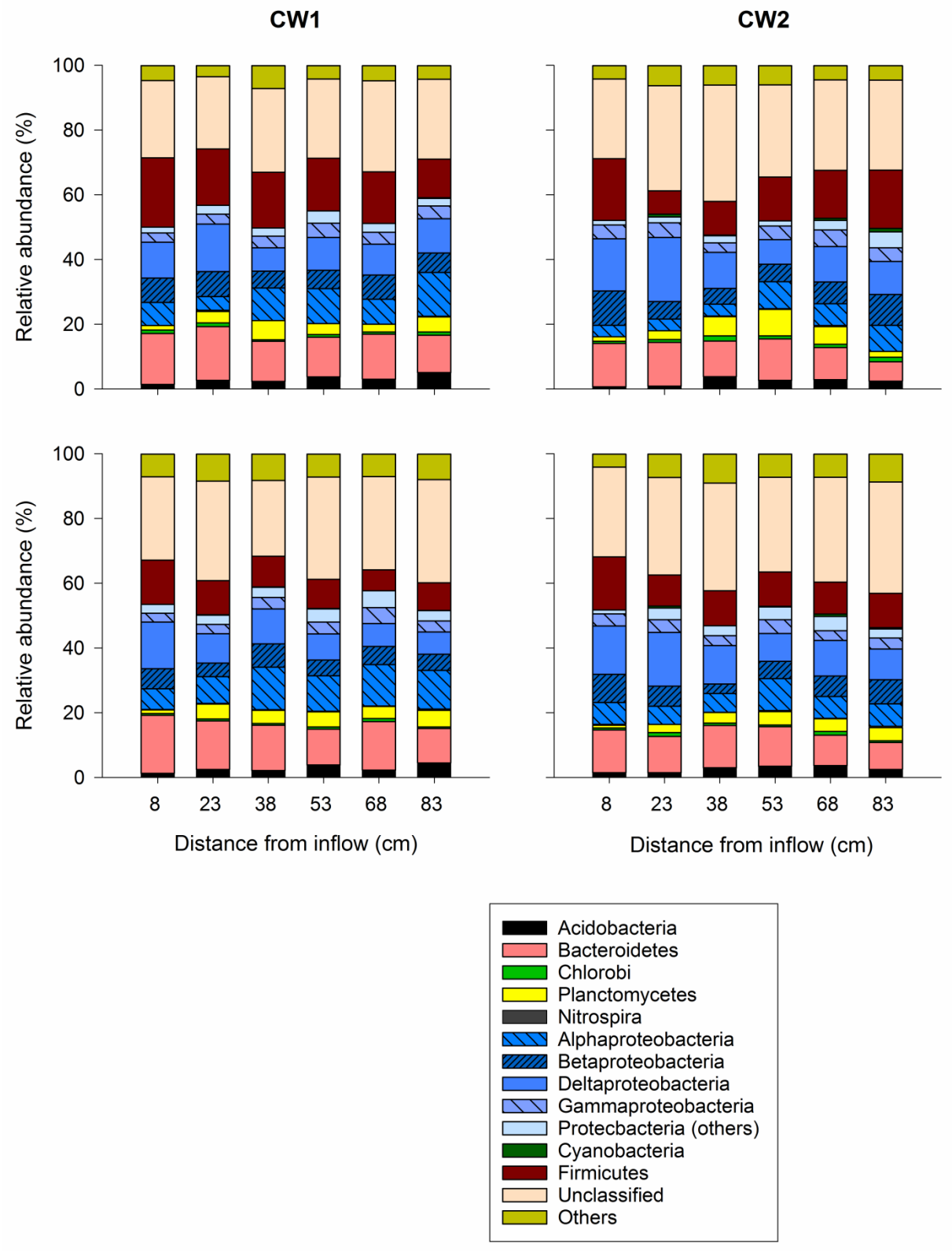


Figure 3.4-5. Microbial community compositions of pore water in CWs

(top: samples on 13 Aug 2015; bottom: samples on 19 Aug 2015)

The results showed that Proteobacteria was the most dominant phylum across all samples (except the mixed pore water and root samples in the fourth compartment of CW2, where Bacteroidetes was the most abundant phylum). In samples of pore water and roots, the relative abundances of Proteobacteria were in the range between 44.7 and 66.8% of bacterial communities in CW1. In CW2, Proteobacteria made up 35.4 - 64.2% of total bacterial communities. In pore water samples, the relative abundance of Proteobacteria remained around 30% in each wetland compartment. Major classes within the most abundant phylum Proteobacteria were Delta-, Beta-, Alpha-, and Gammaproteobacteria. The Deltaproteobacteria were present most predominantly in the first and second compartment of both CW1 (30.3% and 16.4%) and CW2 (56.3% and 51.3%, correspondingly) (Figure 3.4-4). The proportions of Epsilonproteobacteria were lower than those of other identified Proteobacteria classes (highest relative abundance at only 0.32% in the last compartment of CW1 (samples of pore water and roots) and 1.41% in the last compartment CW2 (samples of pore water)). Epsilonproteobacteria and unclassified Proteobacteria were classified together into the "other Proteobacteria group" category shown in Figure 3.4-4 and 3.4-5. Detailed data on relative abundance of single phylum and single class of Proteobacteria in CWs is provided in Table A14-A19 (Appendix). The dominant presence of Proteobacteria was in good agreement with other studies which found that Proteobacteria was the most abundant phylum in total microbial communities in CWs (Ansola et al., 2014; Chen et al., 2015; He et al., 2014; Wu et al., 2016). The widespread distribution of Deltaproteobacteria in the first two compartments of CWs was due to the high abundance of *Syntrophus* and SRB found in the wetlands. This result is reasonable since *Syntrophus* and numerous SRB are classified into the class Deltaproteobacteria (Garrity et al., 2006).

Ranking close behind Proteobacteria were the phyla Bacteroidetes and Firmicutes. In samples of pore water and roots, Bacteroidetes accounted for 13.4 – 29.5% in CW1. The most striking feature in the distribution of Bacteroidetes was noted in CW2. The proportion of Bacteroidetes exhibited a rapid increase from 17.9% in the third compartment to 51.5% in the fourth compartment (to be the most abundant phylum in this planted zone), followed by an immediate decrease to only 1.44% in the fifth compartment (unplanted) (Figure 3.4-4). This notable shift in Bacteroidetes distribution was not observed in pore water samples. Members of Bacteroidetes are commonly known as important degraders of complex organic matters (e.g. proteins and polysaccharides) in soils, freshwater and marine ecosystems (Parte et al.,

2011; Thomas et al., 2011). The observed sudden increase in the distribution of Bacteroidetes in the fourth compartment of CW2 could therefore be attributed to the availability of organic carbons derived from plants (Stottmeister et al., 2003), which could favor the growth of Bacteroidetes.

In samples of pore water and roots, the fractions of Firmicutes in CW1 varied from 1.9% to 10.9%. In CW2, modest distribution of the Firmicutes was recorded in the fourth compartment, accounting for only 0.98% of total bacterial communities. Much higher proportions of Firmicutes were found in pore water samples in both wetlands, ranging between 6.5% and 21.4% in CW1, and varying from 7.2% to 19% in CW2. In contrast to Bacteroidetes, the presence of plants appears to affect the distribution of the Firmicutes. Within the Firmicutes, Bacilli and Clostridia have usually been reported to be major classes (Sharmin et al., 2013; Sun et al., 2015). Members of the class Bacilli are aerobic, microaerophilic or facultative anaerobes, while Clostridia bacteria are obligate anaerobes (Vos et al., 2011). In this study, the dominant class within the Firmicutes was the strictly anaerobic Clostridia (Table A14-A19, Appendix). Therefore inhibitory effects of oxygen released from the roots of *J. effusus* could play as an explanation for the extremely low distribution of Firmicutes in the fourth compartment of CW2 and for the lower distribution of Firmicutes in the samples of pore water and roots (Figure 3.4-4) compared to that in samples of pore water (Figure 3.4-5).

Another interesting finding of the study was the substantial presence of the phylum Nitrospira in samples of pore water and roots. In CW1, Nitrospira accounted for 2.29% of the total bacterial community in the third compartment, whereas in the first two compartments, Nitrospira made up only 0.13 – 0.7%. In CW2, the highest relative abundance of Nitrospira was 0.98% in the fourth compartment (in contrast to the first two compartments, Nitrospira contributed only 0.01 – 0.05% of total bacterial communities). Nitrospira bacteria oxidize nitrite to nitrate (Faulwetter et al., 2009). The abundance of Nitrospira in the third compartment of CW1 and the fourth compartment of CW2 was in accordance with relatively low sulfide concentrations in this zones, which supports the hypothesis that nitrification was inhibited by sulfide toxicity elsewhere in the wetlands.

Within the wetlands, the "others" phyla group covered a wide range of different taxa such as Actinobacteria, Chloroflexi, Verrucomicrobia (Table A14-A19, Appendix). Large portions of unclassified bacteria in the samples of pore water indicate high diversity of bacterial communities in CWs.

Taken together, the findings indicate dynamics of microbial community compositions and the significant effects of plants on distributions of microbial communities in CWs. The most explicit disparity in microbial community structures was observed in the unplanted and planted zones of CW2 (samples of pore water and roots). No remarkable difference in microbial community compositions between two sampling campaigns of pore water (Figure 3.4-5) indicate the stability of microbial communities. The findings were supported by the fact that the results presented here were from the sampling campaigns after over one year of operation and stabilization of bacterial communities in CWs has been suggested to be achieved between 400 and 700 days after starting operation (Samsó and García, 2013). The presence of stable bacterial communities has been well recognized as an important factor for better performance of CWs in pollutant removal (Ramond et al., 2012; Samsó and García, 2013). The findings highlight the crucial role of bacterial communities in pollutant removal in CWs.

4. CONCLUDING REMARKS

The present study was designed to obtain a detailed view on microbial sulfur transformations and identify key microbes driving sulfur transformation processes in CWs. Earlier investigations have focused on sulfur transformation processes in CWs based on measurements of physicochemical parameters involved in the sulfur cycle (Wiessner et al., 2010; Wu et al., 2011). However thus far there was a lack of evidence to elucidate microbial sulfur transformation processes in CWs despite the essential role of microorganisms in pollutant removal has been well established (Faulwetter et al., 2009; Stottmeister et al., 2003). In this study, the combination of classical physicochemical measurements and comprehensive microbiological techniques (next-generation pyrosequencing, quantitative PCR) was employed to identify microbial communities involved in the sulfur cycle and describe the interplay between certain sulfur-metabolizing microbes and plants within the two newly designed CWs.

Returning to the hypotheses posed at the beginning of this study, it is now possible to state that

(i) Sulfate reduction and sulfur reoxidation co-occurred but with sequential dominance;
(ii) *Juncus effusus* enhanced oxidation of sulfur compounds by channelling oxygen into the rhizosphere and shaped microbial community compositions;
(iii) Reoxidation of sulfur was likely carried out mostly by *Thiobacillus*, *Thiomonas* and *Thiothrix* at the roots;
(iv) Key microbes driving sulfate reduction were found to be *Desulfohabdus*, *Desulfobacter*, *Desulfocapsa*, *Desulfovibrio*, and *Desulfobacula*;
(v) Sulfide inhibited nitrification – denitrification and plant growth.

The findings add to our understanding of the "black-box" wetland systems by revealing key microbes involved in the sulfur cycle and their potential metabolic activities in sulfur transformation processes occuring within the systems.

From this study, the following future investigations are invited:

vi) Assessment of microbial sulfur oxidation in CWs receiving different wastewater types such as acid mine drainage could be of interest. In this study, the role of *Thiomonas*, known as important arsenite oxidizer, in sulfur oxidation in CWs was shown. Therefore, further examination on the function of this microbe in terms of sulfur oxidation and potential application in treatment of sulfate and heavy metal-contaminated waters is recommended;

vii) Simultaneous removal of sulfate and nitrate in wastewaters by CWs could be investigated in more details with respect to denitrifying SOB. The use of hydrogen sulfide produced from sulfate reduction instead of organic carbons as electron donors will not only minimize sulfide accumulation but also reduce nitrate in autotrophic denitrification;

viii) Proteomic and metagenomic studies of microbial communities of the sulfur cycle could provide an extended picture of microbial sulfur transformations in CWs. Although 16S rRNA gene-based molecular analysis in this study has given a snapshot on SOB and SRB communities in the wetlands, their potential metabolic functions have only been proposed. Furthermore, not all functional bacterial groups involved in sulfur transformations may have been detected due to some limitations associated with universal primers and current public Genbank database;

ix) Experimental investigations regarding hydraulic behaviors of the newly designed wetlands in this study would be interesting for engineers to optimize design of wetland systems and therefore to achieve better treatment efficiency.

The decline in water quality and freshwater supply has increasingly raised environmental concerns, specially in developing countries. It has been estimated that at least 34 countries

will face water stress by 2025 (Stikker, 1998). Although CWs have been used in treatment of various wastewaters due to advantages such as low cost, easy operation and maintenance for many years (Kivaisi, 2001; Vymazal, 2009), insufficient knowledge on microbial processes driving pollutant removal limit their performance and widespread application in proper ways. The study therefore would be of importance to enhance performance of wetland technologies in wastewater treatment in an environmentally-friendly manner.

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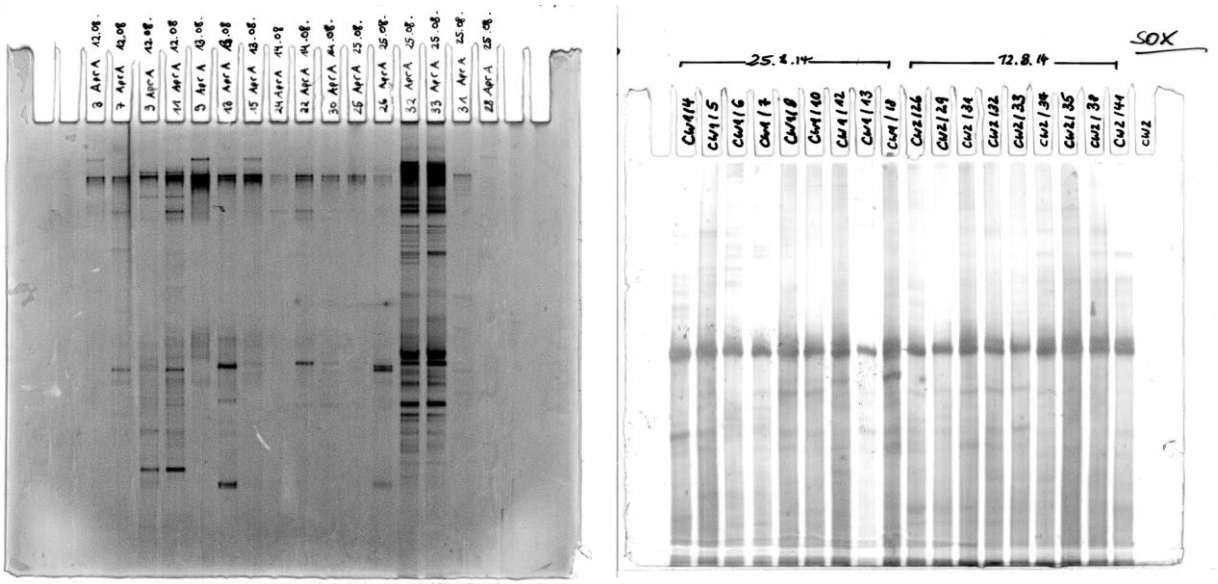
Appendix

Figures



*apr*A *sox*B

Figure A1. Exemplary pictures of DGGE profiles from wetland samples

Tables

Table A1. Estimated proton concentrations (mmol/L) in CWs (from inflow to 0 cm)

Wetlands	$[H^+]$ released from dissimilatory sulfate reduction[a]	Total $[H^+]$consumed[b]
CW1	0.820	1.426
CW2	1.114	2.097

[a] Calculated from oxidation of acetate and benzoate in dissimilatory sulfate reduction

[b] Calculation based on $[H^+]$ released in dissimilatory sulfate reduction and hydroxide produced from sulfide oxidation

Table A2. Total relative abundance of SOB in CW1 (%)

Samples	Distance from inflow (cm)					
	8	**23**	**38**	**53**	**68**	**83**
Pore water and roots (Oct)	2.16	10.24	7.18	20.89	3.92	2.87
Pore water (13 Aug)	0.71	0.77	0.72	1.06	0.68	0.78
Pore water (19 Aug)	0.91	0.98	2.36	0.82	1.48	1.10

Table A3. Total relative abundance of SOB in CW2 (%)

Samples	Distance from inflow (cm)					
	8	**23**	**38***	**53***	**68**	**83**
Pore water and roots (Oct)	0.58	4.63	19.26	2.43	20.12	18.26
Pore water (13 Aug)	2.11	4.51	1.02	1.16	3.86	4.22
Pore water (19 Aug)	1.46	2.25	1.24	1.04	2.83	2.45

*: planted

Table A4. Relative abundance of non traditional colorless SOB and PNSB in CW1 (%) (combined samples of pore water and roots in Oct)

Bacterial groups	**Distance from inflow (cm)**					
	8	**23**	**38**	**53**	**68**	**83**
Non traditional colorless SOB						
Paracoccus	0.009	0.047	0.272	0.146	0.108	0.136
Pseudomonas	0	0.070	0.033	0.008	0	1.009
Hyphomicrobium	0.009	0.163	0.380	0.195	0.341	0.746
PNSB						
Rhodobacter	0.099	0.062	0.256	0.073	0	0.300
Rhodoplanes	0	0.086	0.058	0.073	0	0.155
Rhodococcus	0	0	0	0	0	0
Rhodomicrobium	0	0	0	0.089	0	0
Roseomonas	0.018	0.163	0.025	0.065	0.232	0.236
Rhodoblastus	0	0	0.008	0	0	0
Rhodocista	0	0	0.008	0	0	0
Rhodopirellula	0.009	0	0.066	0	0	0

Table A5. Relative abundance of non traditional colorless SOB and PNSB in CW2 (%) (combined samples of pore water and roots in Oct)

Bacterial groups	**Distance from inflow (cm)**					
	8	**23**	**38***	**53***	**68**	**83**
Non traditional colorless SOB						
Paracoccus	0	0.054	0.044	0.093	0.152	0.150
Pseudomonas	0	0	0	0.025	0	0.007
Hyphomicrobium	0	0	0.027	0.261	0.040	0.021
PNSB						
Rhodobacter	0	0.022	0.018	0	0	0.041
Rhodoplanes	0	0	0	0.093	0.072	0.021
Rhodococcus	0	0	0	0	0.032	0
Rhodomicrobium	0	0	0	0	0	0
Roseomonas	0	0.076	0.053	0.017	0.575	0.267
Rhodopirellula	0	0	0	0	0	0.027

*: planted

Table A6. Relative abundance of non traditional colorless SOB and PNSB in CW1 (%) (pore water samples on 13 Aug)

Bacterial groups	**Distance from inflow (cm)**					
	8	**23**	**38**	**53**	**68**	**83**
Non traditional colorless SOB						
Paracoccus	0	0	0.180	0	0	0.196
Hyphomicrobium	0	0.386	0.180	0.422	0.339	0.587
PNSB						
Rhodobacter	0	0	0.180	0	0	0.196
Rhodoplanes	0	0	0.180	0	0	0.196
Roseomonas	0	0	0.180	0.211	0.339	0.196
Rhodoblastus	0.357	0	0.180	0	0	0
Blastochloris	0.000	0	0.180	0	0	0

Table A7. Relative abundance of non traditional colorless SOB and PNSB in CW2 (%) (pore water samples on 13 Aug)

Bacterial groups	**Distance from inflow (cm)**					
	8	**23**	**38***	**53***	**68**	**83**
Non traditional colorless SOB						
Paracoccus	0	0.901	0	0.194	0.322	0.352
Hyphomicrobium	0	0	0	0.194	0.322	1.056
PNSB						
Rhodobacter	0	0	0.203	0	0	0.352
Roseomonas	0	0	0.203	0.194	0.322	0.352

*: planted

Table A8. Relative abundance of non traditional colorless SOB and PNSB in CW1 (%) (pore water samples on 19 Aug)

Bacterial groups	**Distance from inflow (cm)**					
	8	**23**	**38**	**53**	**68**	**83**
Non traditional colorless SOB						
Paracoccus	0.182	0.098	0.131	0.063	0.186	0.073
Pseudomonas	0	0	0	0.063	0	0.147
Hyphomicrobium	0.365	0.197	0.393	0.253	0.371	0.220
PNSB						
Rhodobacter	0	0.098	0.131	0.063	0	0.073
Rhodoplanes	0	0	0.131	0	0	0.073
Rhodococcus	0	0	0	0	0	0.000
Roseomonas	0.182	0.098	0.131	0.127	0	0.073
Rhodoblastus	0	0.098	0.131	0	0	0
Rhodocista	0	0	0.131	0.063	0	0

Table A9. Relative abundance of non traditional colorless SOB and PNSB in CW2 (%) (pore water samples on 19 Aug)

Bacterial groups	**Distance from inflow (cm)**					
	8	**23**	**38***	**53***	**68**	**83**
Non traditional colorless SOB						
Paracoccus	0	0.282	0.113	0.104	0.157	0.117
Pseudomonas	0.292	0.282	0.113	0.104	0	0
Hyphomicrobium	0	0	0.113	0.207	0.157	0.350
PNSB						
Rhodobacter	0	0.282	0.113	0.104	0.157	0.117
Rhodoplanes	0	0	0	0	0	0
Rhodococcus	0	0	0	0.104	0	0
Roseomonas	0	0.282	0.226	0.104	0.157	0.233
Rhodoblastus	0	0	0	0.104	0.157	0

*: planted

Table A10. Total relative abundance of SRB in CW1 (%)

Samples	Distance from inflow (cm)					
	8	**23**	**38**	**53**	**68**	**83**
Pore water and roots (Oct)	14.75	6.32	1.11	2.45	2.07	1.84
Pore water (13 Aug)	3.57	5.79	1.98	2.95	2.71	1.96
Pore water (19 Aug)	5.29	2.26	3.40	1.52	1.86	1.54

Table A11. Total relative abundance of SRB in CW2 (%)

Samples	Distance from inflow (cm)					
	8	**23**	**38***	**53***	**68**	**83**
Pore water and roots (Oct)	18.24	29.07	2.25	0.77	9.65	3.82
Pore water (13 Aug)	9.16	9.91	3.25	2.71	3.86	3.17
Pore water (19 Aug)	5.85	7.04	3.05	2.28	2.67	2.21

*: planted

Table A12. Relative abundance of the genus *Syntrophus* in CW1 (%)

Samples	Distance from inflow (cm)					
	8	**23**	**38**	**53**	**68**	**83**
Pore water and roots (Oct)	0.26	0.11	0.01	0.11	0.03	0.04
Pore water (13 Aug)	0.36	0.39	0.18	0.21	0.34	0.00
Pore water (19 Aug)	0.91	0.10	0.26	0.06	0.19	0.07

Table A13. Relative abundance of the genus *Syntrophus* in CW2 (%)

Samples	Distance from inflow (cm)					
	8	**23**	**38***	**53***	**68**	**83**
Pore water and roots (Oct)	27.39	14.72	1.62	0.28	1.31	0.61
Pore water (13 Aug)	1.41	0.90	0.20	0.19	0.32	0.35
Pore water (19 Aug)	2.34	0.56	0.57	0.21	0.16	0.23

*: planted

Table A14. Relative abundance of microbial communities in CW1 (%)

(combined samples of pore water and roots in Oct)

Taxonomy	**Distance from inflow (cm)**					
	8	**23**	**38**	**53**	**68**	**83**
Acidobacteria	0.26	1.11	0.99	1.24	1.33	1.27
Actinobacteria	0.67	1.10	1.52	0.84	1.15	2.54
Armatimonadetes	0.02	0.09	0.03	0.05	0.09	0.03
Bacteroidetes	21.57	27.16	29.45	10.92	24.05	13.41
Chlorobi	0.17	1.84	1.21	5.42	1.73	1.35
Chloroflexi	0.30	0.75	0.38	0.68	0.65	0.51
Deferribacteres	0.04	0.00	0.00	0.00	0.02	0.00
Fusobacteria	0.00	0.00	0.00	0.00	0.00	0.07
Gemmatimonadetes	0.01	0.04	0.21	0.01	0.09	0.03
Lentisphaerae	0.00	0.01	0.00	0.00	0.00	0.00
Nitrospira	0.13	0.70	2.29	0.41	0.99	1.57
Planctomycetes	1.00	3.20	3.67	2.54	3.64	3.23
Proteobacteria	66.75	45.26	49.01	63.93	44.65	58.61
Alphaproteobacteria	10.78	8.98	18.02	9.66	11.24	24.90
Betaproteobacteria	30.34	16.36	17.82	35.96	21.19	19.71
Deltaproteobacteria	21.32	13.05	3.24	5.91	5.26	6.32
Epsilonproteobacteria	0.25	0.04	0.02	0.05	0.20	0.32
Gammaproteobacteria	3.57	5.97	8.32	11.22	5.73	5.57
Unclassified	0.49	0.85	1.59	1.14	1.04	1.79
Spirochaetes	0.00	0.11	0.00	0.06	0.02	0.00
Synergistetes	0.00	0.00	0.00	0.00	0.00	0.00
Verrucomicrobia	0.02	0.12	0.32	0.09	0.12	0.55
BRC1	0.03	0.08	0.02	0.11	0.06	0.02
Cyanobacteria	0.01	0.03	0.12	0.05	0.11	0.28
Firmicutes	3.19	6.37	1.94	4.60	10.87	4.47
Bacilli	0.07	0.27	0.28	0.58	0.56	0.90
Clostridia	2.28	4.83	1.53	3.52	9.84	3.19
Negativicutes	0.46	0.05	0.03	0.22	0.14	0.22
Unclassified Firmicutes	0.38	1.21	0.10	0.28	0.33	0.16
OP11	0.02	0.02	0.02	0.03	0.02	0.00
TM7	0.00	0.00	0.01	0.01	0.00	0.00
WS3	0.74	0.81	0.19	0.80	0.34	0.07
unclassified	5.10	11.19	8.59	8.23	10.08	11.98

Table A15. Relative abundance of microbial communities in CW2 (%)

(combined samples of pore water and roots in Oct)

Taxonomy	**Distance from inflow (cm)**					
	8	**23**	**38***	**53***	**68**	**83**
Acidobacteria	0.24	0.12	0.97	1.48	0.15	0.85
Actinobacteria	0.07	0.13	0.19	0.91	0.26	0.42
Armatimonadetes	0.00	0.00	0.17	0.00	0.00	0.03
Bacteroidetes	20.03	16.45	17.86	51.50	1.44	7.59
Chlorobi	0.04	0.31	2.18	0.20	0.65	3.96
Chloroflexi	0.18	0.63	0.61	0.17	0.18	1.40
Deferribacteres	0.53	0.06	0.06	0.00	0.00	0.01
Deinococcus-Thermus	0.02	0.00	0.00	0.00	0.00	0.00
Fusobacteria	0.00	0.02	0.00	0.00	0.00	0.00
Gemmatimonadetes	0.00	0.00	0.00	0.24	0.00	0.00
Lentisphaerae	0.00	0.25	0.00	0.01	0.04	0.01
Nitrospira	0.05	0.01	0.00	0.98	0.00	0.03
Planctomycetes	0.22	1.65	2.95	1.69	3.27	1.09
Proteobacteria	64.24	61.42	39.99	35.38	59.00	52.05
Alphaproteobacteria	1.00	0.62	3.43	9.97	1.89	2.15
Betaproteobacteria	5.77	7.76	22.02	13.66	23.35	26.51
Deltaproteobacteria	56.30	51.29	12.12	3.07	20.36	15.18
Epsilonproteobacteria	0.15	0.04	0.03	0.03	0.02	0.98
Gammaproteobacteria	0.89	1.28	1.12	7.40	1.41	3.01
unclassified	0.13	0.43	1.27	1.25	11.96	4.23
Spirochaetes	0.05	0.00	0.27	0.13	0.02	0.01
Synergistetes	0.00	0.00	0.04	0.00	0.00	0.00
Verrucomicrobia	0.00	0.01	0.38	0.23	0.00	0.03
BRC1	0.05	0.06	0.06	0.00	0.05	0.03
Cyanobacteria	0.00	0.00	0.00	0.24	10.72	0.21
Firmicutes	1.44	2.41	11.40	0.98	5.82	2.86
Bacilli	0.00	0.21	0.00	0.40	0.88	0.41
Clostridia	1.37	2.17	5.79	0.44	4.57	2.20
Negativicutes	0.07	0.02	0.00	0.01	0.15	0.05
Unclassified Firmicutes	0.00	0.01	5.61	0.14	0.22	0.21
OP11	0.00	0.00	0.04	0.00	0.00	0.01
TM7	0.00	0.00	0.00	0.03	0.00	0.00
WS3	0.75	0.68	0.31	0.03	0.06	0.91
unclassified	12.07	15.78	22.52	5.81	18.34	28.52

*: planted

Table A16. Relative abundance of microbial communities in CW1 (%)

(pore water samples on 13 Aug)

Taxonomy	**Distance from inflow (cm)**					
	8	**23**	**38**	**53**	**68**	**83**
Acidobacteria	1.43	2.70	2.33	3.80	3.05	5.09
Actinobacteria	0.00	1.54	3.41	1.05	1.36	1.96
Armatimonadetes	0.36	0.00	0.72	0.63	0.34	0.39
Bacteroidetes	15.71	16.60	12.39	12.24	13.90	11.55
Chlorobi	1.07	1.16	0.54	0.84	0.68	0.98
Chloroflexi	1.43	0.39	1.80	1.69	1.36	1.17
Deferribacteres	0.36	0.39	0.00	0.00	0.00	0.00
Fusobacteria	0.36	0.00	0.00	0.00	0.00	0.00
Gemmatimonadetes	0.00	0.00	0.18	0.00	0.00	0.20
Lentisphaerae	0.36	0.39	0.18	0.21	0.00	0.20
Nitrospira	0.00	0.39	0.00	0.00	0.00	0.20
Planctomycetes	1.43	3.47	5.92	3.38	2.37	4.70
Proteobacteria	30.36	32.43	28.55	34.81	31.19	36.40
Alphaproteobacteria	7.14	4.25	10.05	10.76	7.80	13.50
Betaproteobacteria	7.50	7.72	5.21	5.70	7.46	6.07
Deltaproteobacteria	11.07	14.67	7.18	10.13	9.49	10.57
Epsilonproteobacteria	0.36	0.00	0.00	0.00	0.00	0.00
Gammaproteobacteria	2.86	3.09	3.59	4.43	3.73	3.91
unclassified	1.43	2.70	2.51	3.80	2.71	2.35
Spirochaetes	0.71	0.00	0.36	0.00	0.34	0.00
Verrucomicrobia	0.36	0.39	0.18	0.00	0.68	0.20
BRC1	0.36	0.00	0.18	0.42	0.34	0.00
Cyanobacteria	0.00	0.00	0.00	0.00	0.00	0.20
Firmicutes	21.43	17.37	17.24	16.24	15.93	11.94
Bacilli	1.43	1.16	1.62	2.53	1.36	2.54
Clostridia	16.43	14.29	12.57	12.03	13.22	8.02
Negativicutes	2.14	0.77	1.26	0.42	0.34	0.20
Unclassified Firmicutes	1.43	1.16	1.80	1.27	1.02	1.17
WS3	0.36	0.39	0.18	0.21	0.34	0.20
unclassified	23.93	22.39	25.85	24.47	28.14	24.66

Table A17. Relative abundance of microbial communities in CW2 (%)

(pore water samples on 13 Aug)

Taxonomy	**Distance from inflow (cm)**					
	8	**23**	**38***	**53***	**68**	**83**
Acidobacteria	0.70	0.90	3.86	2.71	2.89	2.46
Actinobacteria	0.70	0.00	0.20	1.36	0.32	1.76
Armatimonadetes	0.00	0.00	0.41	0.19	0.00	0.35
Bacteroidetes	13.38	13.51	10.98	12.79	9.97	5.99
Chlorobi	0.70	0.90	1.63	0.97	0.96	1.41
Chloroflexi	0.70	4.50	4.07	3.10	2.89	1.76
Deferribacteres	0.70	0.90	0.00	0.19	0.00	0.00
Fusobacteria	0.70	0.00	0.00	0.00	0.00	0.00
Gemmatimonadetes	0.00	0.00	0.00	0.00	0.00	0.00
Lentisphaerae	0.70	0.00	0.20	0.00	0.00	0.00
Nitrospira	0.00	0.00	0.20	0.19	0.32	0.00
Planctomycetes	1.41	2.70	5.89	8.14	5.47	1.76
Proteobacteria	35.92	35.14	24.80	27.13	32.48	36.97
Alphaproteobacteria	3.52	3.60	3.66	8.33	6.75	8.10
Betaproteobacteria	10.56	5.41	4.88	5.43	6.75	9.51
Deltaproteobacteria	16.20	19.82	11.18	7.56	10.93	10.21
Epsilonproteobacteria	0.00	0.00	0.00	0.00	0.00	1.41
Gammaproteobacteria	4.23	4.50	2.85	4.26	5.14	4.23
unclassified	1.41	1.80	2.24	1.55	2.89	3.52
Spirochaetes	0.00	0.00	0.20	0.39	0.00	0.00
Verrucomicrobia	0.00	0.00	0.20	0.19	0.00	0.35
BRC1	0.00	0.00	0.20	0.19	0.64	0.00
Cyanobacteria	0.00	0.90	0.20	0.00	0.64	1.06
Firmicutes	19.01	7.21	10.37	13.57	14.79	17.96
Bacilli	0.70	1.80	0.61	1.16	1.29	3.17
Clostridia	16.90	4.50	8.54	9.11	11.25	12.68
Negativicutes	0.70	0.90	0.20	0.58	1.29	0.70
Unclassified Firmicutes	0.70	0.00	1.02	2.71	0.96	1.41
WS3	0.70	0.90	0.61	0.39	0.64	0.35
unclassified	24.65	32.43	35.98	28.49	27.97	27.82

*: planted

Table A18. Relative abundance of microbial communities in CW1 (%)
(pore water samples on 19 Aug)

Taxonomy	**Distance from inflow (cm)**					
	8	**23**	**38**	**53**	**68**	**83**
Acidobacteria	1.28	2.46	2.09	3.80	2.23	4.47
Actinobacteria	1.82	1.87	2.75	2.47	4.45	3.08
Armatimonadetes	0.91	0.69	0.39	0.57	0.19	0.81
Bacteroidetes	17.88	14.96	14.01	11.09	15.03	10.63
Chlorobi	0.55	0.59	0.52	0.70	0.93	0.44
Chloroflexi	2.01	3.64	2.62	2.53	1.11	2.27
Deferribacteres	0.18	0.10	0.00	0.00	0.00	0.00
Fusobacteria	0.36	0.10	0.00	0.00	0.00	0.00
Gemmatimonadetes	0.00	0.00	0.26	0.13	0.19	0.22
Lentisphaerae	0.36	0.20	0.13	0.06	0.19	0.15
Nitrospira	0.18	0.20	0.26	0.19	0.19	0.37
Planctomycetes	1.09	4.63	4.06	4.63	3.71	5.21
Proteobacteria	32.48	27.36	37.83	31.69	35.62	30.43
Alphaproteobacteria	6.39	8.27	13.09	10.96	12.80	11.95
Betaproteobacteria	6.20	4.23	7.20	4.88	5.57	4.99
Deltaproteobacteria	14.42	9.06	10.86	8.05	7.05	6.89
Epsilonproteobacteria	0.55	0.20	0.79	0.19	1.11	0.29
Gammaproteobacteria	2.74	2.85	3.53	3.68	5.01	3.37
unclassified	2.19	2.76	2.36	3.93	4.08	2.93
Spirochaetes	0.36	0.00	0.00	0.06	0.00	0.07
Verrucomicrobia	0.18	0.89	1.44	0.63	0.56	0.51
BRC1	0.55	0.49	0.26	0.51	0.19	0.59
Cyanobacteria	0.00	0.10	0.13	0.13	0.00	0.07
Firmicutes	13.69	10.53	9.42	9.00	6.49	8.58
Bacilli	0.91	0.59	1.31	1.14	0.93	1.10
Clostridia	10.77	8.17	6.94	6.08	5.01	6.01
Negativicutes	0.36	0.20	0.26	0.38	0.19	0.37
Unclassified Firmicutes	1.64	1.57	0.92	1.39	0.37	1.10
OP11	0.00	0.00	0.13	0.13	0.00	0.07
WS3	0.36	0.49	0.26	0.06	0.19	0.22
unclassified	25.73	30.71	23.43	31.62	28.76	31.82

Table A19. Relative abundance of microbial communities in CW2 (%)

(pore water samples on 19 Aug)

Taxonomy	**Distance from inflow (cm)**					
	8	**23**	**38***	**53***	**68**	**83**
Acidobacteria	1.46	1.41	2.94	3.42	3.62	2.45
Actinobacteria	1.17	1.97	2.60	2.69	2.52	2.10
Armatimonadetes	0.00	0.28	0.57	0.62	0.31	0.23
Bacteroidetes	13.16	11.27	13.12	12.22	9.43	8.28
Chlorobi	0.58	1.13	0.68	0.52	1.10	0.58
Chloroflexi	0.58	3.38	4.19	2.28	3.14	4.31
Deferribacteres	0.29	0.28	0.11	0.10	0.00	0.12
Deinococcus-Thermus	0.29	0.00	0.00	0.00	0.00	0.00
Fusobacteria	0.00	0.28	0.11	0.00	0.00	0.00
Gemmatimonadetes	0.00	0.00	0.00	0.41	0.00	0.00
Lentisphaerae	0.29	0.28	0.11	0.31	0.00	0.00
Nitrospira	0.29	0.00	0.11	0.31	0.16	0.35
Planctomycetes	0.88	2.54	3.28	4.14	3.93	4.08
Proteobacteria	35.38	36.06	26.70	32.09	31.45	30.07
Alphaproteobacteria	6.73	5.63	5.77	9.94	6.76	6.99
Betaproteobacteria	8.77	6.20	2.94	5.28	6.29	7.46
Deltaproteobacteria	14.91	16.62	11.88	8.59	11.01	9.44
Epsilonproteobacteria	0.29	0.28	0.23	0.31	0.63	0.35
Gammaproteobacteria	3.80	3.94	3.05	4.35	2.99	3.38
unclassified	0.88	3.38	2.83	3.62	3.77	2.45
Spirochaetes	0.29	0.00	0.11	0.00	0.00	0.00
Synergistetes	0.00	0.00	0.11	0.00	0.00	0.00
Verrucomicrobia	0.29	0.28	0.34	0.41	0.31	0.70
BRC1	0.58	0.28	0.34	0.21	0.31	0.70
Cyanobacteria	0.00	0.56	0.00	0.21	0.79	0.47
Firmicutes	16.37	9.58	10.86	10.56	9.91	10.61
Bacilli	0.58	0.56	0.45	0.93	1.10	1.28
Clostridia	12.87	7.04	8.82	7.87	6.45	7.69
Negativicutes	0.58	0.56	0.23	0.31	0.16	0.35
Unclassified Firmicutes	2.34	1.41	1.36	1.45	2.20	1.28
OP11	0.00	0.00	0.11	0.00	0.00	0.00
WS3	0.29	0.28	0.34	0.21	0.63	0.58
unclassified	27.78	30.14	33.26	29.30	32.39	34.38

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The genome of *Beggiatoa alba* B18LD^T – a large sulfur oxidizing Gammaproteobacterium

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Abstract

Members of the genus *Beggiatoa* are conspicuous Gammaproteobacteria which thrive at redox interfaces of freshwater or marine environments. There they may couple the oxidation of sulfide to sulfur or sulfate with oxygen or nitrate respiration. The capability to fix molecular nitrogen is widespread among this genus, and at least marine *Beggiatoa* are apparently autotrophs. In contrast to their biogeochemical importance and interesting biology, there is thus far only fragmented information available on the genomic basis for their manifold features. Here we present the complete genome sequence of a *Beggiatoa* species, *Beggiatoa alba* B18LD^T. The genome consists of one chromosome of 4.24 Mb and two small plasmids of 11.4 kb and 9 kb, respectively. In total there are 3569 annotated genes. About 18% of the genes originated from cyanobacteria, anoxygenic phototrophic bacteria, and sulfate-reducing bacteria. Strain B18LD^T has complete gene sets for oxidizing sulfide and thiosulfate to elemental sulfur but only an incomplete system for oxidizing sulfur further to sulfate. Distinct from marine *Beggiatoa*, key genes involved in autotrophic carbon fixation

pathways are absent and dissimilatory nitrate reduction may not be possible. Under anoxic conditions, sulfur can be respired coupled to the oxidation of hydrogen and maybe formate. We found genes involved in motility, redox and light sensing, as well as a complete system responsible for polyphosphate synthesis and release. The identified genomic features of *B. alba* B18LD^T highlight the microbe's niche specialization to dynamic redox interfaces and provide the basis for postgenomic investigations of this environmentally important genus.

Keywords:

Beggiatoa alba B18LD^T, *Thiotrichaceae*, sulfur-oxidizing bacterium, incomplete sulfur oxidation, microaerophilic, chemolithotroph

Abbreviations:

DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; PHB, poly-β-hydroxybutyric acid; PHA, poly-hydroxyalkanoates; TA, toxin - antitoxin

Introduction

With the studies on *Beggiatoa* of Winogradsky, the knowledge of chemolithotrophy, an important microbiological conception was revealed (Teske and Nelson, 2006). By the ability of oxidizing reduced inorganic sulfur compounds for growth, the filamentous sulfur oxidizing bacteria *Beggiatoa* play an important role in the sulfur cycle, one of the major biochemical cycles on Earth. *Beggiatoa* species forming mats at the oxic-anoxic interfaces by their gliding motility to gradients of sulfide and oxygen, there sulfide is oxidized to elemental sulfur or sulfate (Jørgensen and Revsbech, 1983). The ability of *Beggiatoa* in detoxification of sulfide is of ecological importance as organisms in oxic habitats are protected from toxic sulfide. The significant role of *Beggiatoa* in sulfide detoxification in the rice rhizophere has long been well documented (Joshi and Hollis, 1977). However, despite their environmental importance, genomic data of *Beggiatoa* species and knowledge regarding their metabolic pathways remains to be further investigated. Sequencing genome of the type strain sulfur oxidizing gamma-proteobacterium *B. alba* B18LD will help us to gain a better understanding of the role of this microbe and related strains in the cycling of sulfur, as well as carbon and nitrogen, in sulfide-rich habitats such as wetlands, rice paddy fields, methane seeps, and wastewater

treatment plants. Moreover, comprehensive knowledge of molecular components of behavioral responses to sulfide and oxygen and of gliding motility will be also able to obtain.

The present study provides an insight into the completely sequenced genome of *B. alba* B18LD with special focus on the genes involved in the sulfur cycle. Genomic data on oxygen respiration, nitrogen and carbon metabolism, phosphate accumulation, motility, redox and light sensing of *B. alba* B18LD were also analyzed. Based on genomic data analysis of *B. alba* B18LD, patterns of global gene expression analysis and identification of regulatory networks such as those involved in sulfide oxidation and carbon metabolism can be further examined, which will greatly enhance our understanding of the metabolic performance of this ubiquitous microbe under various environmental conditions.

Organism information

Classification and features

Beggiatoa are filamentous bacteria and widely distributed in freshwater environments such as sulfur springs, wetlands, rice paddies, lake sediments (Teske and Nelson, 2006), in marine habitats such as coastal sediments, submarine caves, hydrothermal vents (Jørgensen, 1977; MacGregor et al., 2013b; Mattison et al., 1996) and in activated sludge of wastewater treatment plants (Williams and Unz, 1985).

B. alba B18LD is a freshwater strain, isolated from sediments in a rice field ditch, Baton Rouge, Louisiana, USA and is determined as the type strain (ATCC33555) (Markowitz et al., 2012; Mezzino et al., 1984). The organism is Gram negative, motile by gliding by excreted slime, produces necridia and hormogonia and forms circuitan colonies on the agar surface (Mezzino et al., 1984). The filaments have rounded-ends and sheaths are absent. The filament length ranges from 60 to 120 µm and the width is 3.5 µm (Mezzino et al., 1984). The cells are colorless and 3 – 9 µm long (Garrity et al., 2007; Mezzino et al., 1984). Three types of inclusions have been observed in the cells: poly-β-hydroxybutyric acid (PHB) (0.5 to above 1 µm in diameter) (Lawry et al., 1981), polyphosphate (up to 1.2 µm in diameter) (Havemeyer, 2013), and sulfur (0.05 – 0.3 µm in diameter) (Lawry et al., 1981). Sulfur inclusions are located in the periplasm and external to the cytoplasmic membrane within the cells (Lawry et al., 1981). Raman spectroscopic observation revealed that cyclo-octasulfur (S_8) and inorganic

polysulfides (S_n^{2-}) are the forms of sulfur granules deposited in the filaments (Berg et al., 2014). Micrographs of *B. alba* B18LD is shown in Figure 1.

The strain is mesophilic, microaerophilic, catalase negative, cytochrome oxidase positive (Mezzino et al., 1984). *B. alba* B18LD has the ability to oxidize sulfide and thiosulfate to form sulfur globules during sulfur oxidation (Garrity et al., 2007; Mezzino et al., 1984; Schmidt et al., 1987). However, the organism lacks the capacity of further oxidizing sulfur to sulfate (Schmidt et al., 1987). Growth can be maintained under short period of anoxic conditions due to reduction of elemental sulfur to sulfide (Schmidt et al., 1987). *B. alba* B18LD is mixotrophic (Güde et al., 1981) and non-autotrophic (Strohl et al., 1981). The organism has the ablity to use a wide range of organic substrates (acetate, fumarate, lactate, malate, pyruvate, succinate, ethanol, and methanol) (Jewell et al., 2008; Mezzino et al., 1984). Sulfide oxidation could suppress acetate oxidation to some extent due to oxygen competition (Schmidt et al., 1987). The strain can utilize nitrate, nitrite, ammonia, casamino acids (Mezzino et al., 1984), urea, aspartate, asparagine, alanine and thiouerea (Vargas and Strohl, 1985a) as sole nitrogen sources.

B. alba B18LD belongs to the genus *Beggiatoa*, family *Thiotrichaceae*, order *Thiotrichales* (Table 1). The genera *Thiothrix* and *Thioploca* are classified in the same family with *Beggiatoa* and they share striking morphological and physiological similarities such as internal sulfur depositing ability, gliding motility (Garrity et al., 2007). Comparison in characteristics of those three genera have been well documented (Garrity et al., 2007; Larkin and Strohl, 1983; Teske and Nelson, 2006). Based on 16S rRNA sequence, two freshwater strains *Beggiatoa alba* B15LD and *Beggiatoa* sp. OH-75-2a are most closely related to *B. alba* B18LD (Ahmad et al., 2006; Teske et al., 1995). The phylogenetic tree based on the 16S rRNA sequences is shown in Figure 2.

Genome sequencing information

Genome project history

Growth conditions and genomic DNA preparation

Genome sequencing and assembly

Genome annotation

Sequences analysis was performed by using BLASTP searches from NCBI database, IMG/ER (Markowitz et al., 2012), Artemis (Rutherford et al., 2000), KEGG (Kanehisa and Goto, 2000), Mauve (Darling et al., 2004), and Geneious (Kearse et al., 2012) platforms. PHAST (Zhou et al., 2011) was used for identification of prophage sequences.

Genome Properties

The genome of *B. alba* B18LD consists of one circular chromosome of 4.24 Mb and two small plasmids of about 11.4 kb and 9 kb, respectively. Total genome size of *B. alba* B18LD is larger than that calculated previously (3.03 Mb) (Genthner et al., 1985). The number of annotated genes is 3569, of which there are 3516 protein-encoding genes (98.51%). There are 53 RNA genes, in which total number of tRNA is 46. There are 66 identified pseudogenes (1.85%). Genome statistics of *B. alba* B18LD are summarized in Table 3. Distribution of genes with COG functional categories is shown in Table 4.

We found two prophage regions in the chromosome of *B. alba* B18LD. The location of the first phage region is from 3636121 to 3649340 (13220 bp), and spans 12 coding sequences. The second prophage is located from 3751314 to 3760810 (9497 bp), covering 7 coding sequences. In plasmid pJKA1 (11.4 kb), there are 11 genes encoding hypothetical proteins. In plasmid pJKA2 (9 kb) there are 7 genes encoding hypothetical proteins, and the other genes encoding cytotoxic translational repressor of toxin-antitoxin (TA) stability system (WP_002692448), PhD/YefM antitoxin protein, type II TA system (WP_002692446) and ATPase (WP_002692433). The gene encoding YoeB toxin protein, which is known to interact with YefM to form TA complex (Polom et al., 2013) is absent. Since there are several proteins remained unknown funtions encoded in plasmid, whether any of those can serve as a substitution for the toxin protein could be further investigated. Nevertheless, the existence of genes encoding proteins involved in TA system in *B. alba* B18LD is predictable as the TA systems are distributed widespread in free-living prokaryotes (Pandey and Gerdes, 2005). TA systems encoded in plasmids are known to function as a maintenance mechanism of plasmid stability in bacterial lifestyle (Chan et al., 2016; Hayes, 2003). The two plasmids of *B. alba* B18LD share 9 regions with high similarities in nucleotide sequences (total length is 5779 bp).

Comparison in genome features of the completed sequence of *B. alba* B18LD^T with the first completed genome sequence of *Beggiatoa* species: *Beggiatoa leptomitiformis* D-402^T (Fomenkov et al., 2015), and other uncompleted sequence *Beggiatoa* strains is presented in table 5.

As shown in Table 5, it apparents that genomic statistics of the two completed genome sequence of *B. alba* B18LD and *B. leptomitiformis* D-402^T are quite similar. On the other hand, total genome size of *B. alba* B18LD is nearly a half of uncompleted genome of *Beggiatoa* PS, about 3.3 times larger than uncompleted genome of *Beggiatoa* SS and slightly smaller than that of uncompleted genome of *Beggiatoa* Orange Guaymas. *B. alba* B18LD has the same number of tRNA genes as *Beggiatoa* Orange Guaymas (46), whereas *Beggiatoa* PS and *B. leptomitiformis* D-402^T has 45 and 47 tRNA genes, respectively and only 5 tRNA genes were identified in *Beggiatoa* SS. Comparative genomic data suggests the genome sequence of *Beggiatoa* Orange Guaymas is close to be completed, followed by is the sequence of *Beggiatoa* PS, and the genome sequence of *Beggiatoa* SS is far from completion. It should be noted that the genome sizes of *Beggiatoa* PS and SS were estimated by optical mapping and the former was pyrosequenced with high coverage depth, the later was Sanger sequenced with low coverage depth (Mussmann et al., 2007), therefore the difference in genome sequencing states in *Beggiatoa* could probably be lied on the different methods have been used for sequencing methods and analysis.

Insights from the genome sequence

Sulfur Oxidation

The current model mechanism of sulfur oxidation in prokaryotes has been documented for the Sox enzyme system, which includes four periplasmic complexes SoxXA, SoxB, SoxYZ, and Sox$(CD)_2$ (Meyer et al., 2007). Based on the genomic data, *B. alba* B18LD has an incomplete Sox system. Genes encoding SoxXABYZ were found in the genome while genes encoding SoxCD are missing. So far many sulfur oxidizers that have the SoxXABYZ but not SoxCD have often been reported (Yamamoto and Takai, 2011). In marine *Beggiatoa* strains, the genes encoding Dsr (dissimilatory sulfite reductase) system responsible for the oxidation of sulfur globules consists of *dsr*ABEFHCMKJOPNRS (Mussmann et al., 2007). In the genome of *B. alba* B18LD, we found only six *dsr* genes, namely *dsr*EFHCKJ (Table 6). So far DsrC is found in all sulfur oxidizing bacteria and sulfate-reducing bacteria (Sander et al., 2006) and

shows high sequence similarities to TusE, a protein involved in the sulfur relay mechanism leading to thiouridine biosynthesis process (Numata et al., 2006). DsrEFH is highly similar to TusBCD, the sulfur donor of TusE and located upstream of *dsrC* (Grimm et al., 2010; Numata et al., 2006). It is noteworthy that *dsr*EFH and *dsr*C homologues are present in many *Gammaproteobacteria* which lack *dsr*AB (Cort et al., 2008), thus the presence of *dsr*EFHC and the absence of *dsr*AB in *B. alba* B18LD are not surprising. DsrEFH and DsrC are responsible for transfering sulfur into the cytoplasm to DsrAB (Grimm et al., 2010). DsrMKJOP is a transmembrane redox complex (Grimm et al., 2010) and it has been proven that each single protein of the DsrMKJOP complex is obligatory for sulfur oxidation in *Allochromatium vinosum*, a phototrophic purple sulfur bacterium (Sander et al., 2006). The Dsr system is thought to be essential for the oxidation of stored sulfur in many photo- and chemolithoautotrophic bacteria which are able to form sulfur globules as obligatory intermediates during the oxidation of reduced sulfur compounds (Grimm et al., 2010). Therefore, the absence of *dsr*AB and the incomplete *dsr*MKJOP gene system in *B. alba* B18LD may result in the inability of oxidizing stored sulfur in this strain. So far complete sulfur oxidation to sulfate has been recorded in most *Beggiatoa* spp. (Berg et al., 2014). Under limited-sulfide conditions, sulfate is produced, whereas under high-sulfide availability sulfur is accumulated in *Beggiatoa* filaments (Berg et al., 2014; Larkin and Strohl, 1983). As cannot be further oxidized, internal sulfur in *B. alba* B18LD can be respired to sulfide and the filaments can sustain growth in the short period of anoxia (Schmidt et al., 1987). In fact complete sulfur oxidizers, namely the freshwater *Beggiatoa* sp. OH75-2a (Nelson and Castenholz, 1981) and the marine strain *Beggiatoa* sp. 35Flor (Kreutzmann and Schulz-Vogt, 2016; Schwedt et al., 2012) undergo sulfur respiration as well. Therefore, it seems that strain B18LD possesses metabolic flexibilty with a lesser extent in comparison with other *Beggiatoa* species.

Regarding the oxidation of sulfite to sulfate, we found the genes encoding ATP sulfurylase (*sat*) and sulfite oxidase (*so*) but not adenosine phosphosulfate reductase (*aprAB*) in the genome of *B. alba* B18LD. As a result, due to the incomplete genes system responsible for oxidation pathway of elemental sulfur and the lack of *sox*CD for the direct oxidation of sulfide/thiosulfate to sulfate via Sox system (*Paracoccus* oxidation pathway) (Friedrich et al., 2001; Rother et al., 2001), it appears that *B. alba* B18LD has an incomplete sulfur oxidation

and the genomic picture matches with the fact that sulfate has never been observed as product of sulfide oxidation in *B. alba* B18LD (Schmidt et al., 1987).

There are two alternative enzymes postulated to catalyze the oxidation of hydrogen sulfide and/or thiosulfate to elemental sulfur: (1) sulfide quinone oxidoreductase (Sqr) and (2) flavocytochrome c/sulfide dehydrogenase (FccAB) (Friedrich et al., 2001; Mussmann et al., 2007). We found the genes encoding Sqr and FccAB in the genome of *B. alba* B18LD. Sqr has been reported to be widely distributed among prokaryotes and this enzyme is obligatory for growth on sulfide in photo- and chemolithoautotrophic bacteria (Griesbeck et al., 2000). Flavocytochrome c (Fcc) consists of a flavoprotein subunit, FccB and a *c*-type cytochrome subunit, FccA (Yamamoto and Takai, 2011) and Fcc do not occur in a variety of sulfide-oxidizing bacteria (Griesbeck et al., 2000). The neighbouring genes of *sqr* (WP_002685114) of *B. alba* B18LD are the genes encoding UDP-N-acetylmuramoyalanine-D-glutamate ligase and hypothetical protein. The neighbouring genes of *sqr* (WP_002690795) are the genes encoding uncharacterized conserved protein and GTPase subunit of restriction endonuclease (an enzyme involved in spliting phosphodiester bond within a polynucleotide chain), which suggests that the *sqr* genes of *B. alba* B18LD may come from other organisms. The fact that *sqr* is distributed among a number of different phylogenetically distant bacteria and considered to be an evolutionary old enzyme (Griesbeck et al., 2000) supports our hypothesis that *sqr* genes of *B. alba* B18LD may originated from other bacteria.

Respiration using sulfur and other electron acceptors

Sulfur respiration using molecular hydrogen or organic subtrates as electron donors are widely operated in bacteria and archaea (Hedderich et al., 1999; Schauder and Kröger, 1993). While anaerobic sulfur reduction to sulfide using hydrogen or formate as electron donors have been well documented in a number of sulfur reducing bacteria (Finster et al., 1997; Hedderich et al., 1999; L'Haridon et al., 1998; Laanbroek et al., 1978; Macy et al., 1986), thermophilic archaea (Schauder and Kröger, 1993) and methanogens (Stetter and Gaag, 1983) with the involvement of hydrogenase and formate dehydrogenase (Ma et al., 2000; Schröder et al., 1988), little is known about the capacity and mechanisms of sulfur reduction in sulfur oxidizing bacteria. The ability of using molecular hydrogen or organic compounds, possibly acetate or poly-hydroxyalkanoates (PHA) as electron donors in sulfur respiration has been observed in *Beggiatoa* (Kreutzmann and Schulz-Vogt, 2016; Nelson and Castenholz, 1981;

Schmidt et al., 1987; Schwedt et al., 2012) and other sulfur oxidizers such as *Chromatium* (Schmidt et al., 1987; van Gemerden, 1968), *Sulfurimonas* (Nakagawa et al., 2005).

We found hydrogenase genes (*hya*, *hyn*, *frh*) in the genome of *B. alba* B18LD, which is in agreement with the previous study demonstrated that *B. alba* B18LD has the capacity to produce sulfide under short-term anoxic conditions in the presence of hydrogen by using intracellular sulfur as an electron acceptor (Schmidt et al., 1987). The rate of sulfide production by *B. alba* B18LD was found to be higher than that determined in *Chromatium vinosum* as the positive control (6.7 versus 5 nmol/min per mg of protein) (Schmidt et al., 1987). The presence of three different hydrogenase systems in *B. alba* B18LD suggests that hydrogen is an important electron donor in anaerobic sulfide production and help the filaments adapt effectively to anoxic conditions. Sulfur respiration by using hydrogen (Kreutzmann and Schulz-Vogt, 2016) or stored PHA (Schwedt et al., 2012) as electron donors has been recently shown in the marine strain *Beggiatoa* sp. 35Flor (in the coculture with a *Pseudovibrio* sp.), whereas acetate was thought to be electron donor in sulfur respiration in *Beggiatoa* sp. OH75-2a (Nelson and Castenholz, 1981). By the capacity to respire stored sulfur under high-sulfide concentrations conditions, *Beggiatoa* filaments can not only gain energy for growth maintenance but also prevent the cells from being bursted by excessive sulfur loaded (Kreutzmann and Schulz-Vogt, 2016; Schwedt et al., 2012).

Genes encoding formate dehydrogenase are present in the genome of *B. alba* B18LD, which suggests that formate can probably act as an electron donor in sulfur reduction. The use of formate as electron donor in sulfur reduction to sulfide has been recorded in the sulfur reducer *Wolinella succinogenes* with the rapid doubling time under growth on formate and sulfur (1.2 h) (Macy et al., 1986). Polysulfide is possibly an intermediate of sulfur respiration (Hedderich et al., 1999; Schauder and Müller, 1993) and the actual electron acceptor in sulfur respiration in *W. succinogenes* is polysulfide (Klimmek et al., 1991; Schauder and Kröger, 1993). Due to the low solubility in water, elemental sulfur is transformed to polysulfide under presence of aqueous sulfide ($n/8\ S_8 + HS^- \rightarrow S_{n+1}^{2-} + H^+$) (Schauder and Müller, 1993). Polysulfide is subsequently reduced by either hydrogen or formate ($H_2 + S_n^{2-} \rightarrow HS^- + S_{n-1}^{2-} + H^+$; $HCO_2^- + S_n^{2-} + H_2O \rightarrow HCO_3^- + HS^- + S_{n-1}^{2-} + H^+$) (Hedderich et al., 1999). The formation of soluable polysulfide from elemental sulfur could play as an explanation for the presence of polysulfide observed in the filaments of *B. alba* B18LD (Berg et al., 2014). The mechanism of sulfur

respiration with the electron transfer from hydrogenase or formate dehydrogenase to polysulfide reductase (Psr) has been so far most extensively described in *W. succinogenes* (Gross et al., 1998; Hedderich et al., 1999; Klimmek et al., 1991; Krafft et al., 1995; Schauder and Kröger, 1993; Schröder et al., 1988). Genes encoding Psr are not found in the genome of *B. alba* B18LD while these genes are present in the completed genome sequence of *W. succinogenes* (Baar et al., 2003) and all published genome sequences of the sulfur oxidizers *Sulfurimonas* experienced sulfur respiration in their metabolism (Cai et al., 2014; Grote et al., 2012; Han and Perner, 2015; Sievert et al., 2008; Sikorski et al., 2010). Nevertheless, it seems that *psr* genes are not obligately required for sulfur respiration since in the absence of sulfide (the conditions that polysulfide was not produced), growth with elemental sulfur as terminal electron acceptor has been well confirmed in *W. succinogenes*, though less ATP was gained compared to growth on polysulfide respiration (Ringel et al., 1996).

Another pathway of anaerobic sulfur respiration with the involvement of thiosulfate reductase (*phs)* has been reported (Hinsley and Berks, 2002). The reduction of elemental sulfur via Phs has been well studied in the Gamma-proteobacterium *Salmonella enterica* (Hinsley and Berks, 2002). Phs catalyzes the transformation of thiosulfate to sulfide and sulfite ($S_2O_3^{2-} + H^+ + 2e^- \rightarrow HS^- + SO_3^{2-}$), followed by the reaction of sulfite with elemental sulfur to generate thiosulfate ($S + SO_3^{2-} \rightarrow S_2O_3^{2-}$), accordingly the reduction of elemental sulfur to sulfide was performed by the combination of those two reactions (Hinsley and Berks, 2002). The role of Phs in catalyzing the initial step of thiosulfate disproportionation to sulfide and sulfite has been also well demonstrated in *Desulfocapsa sulfoexigens*, a thiosulfate-disproportionating bacterium (Frederiksen and Finster, 2003). As *phs* genes were found in the uncompleted sequence genomes of *Beggiatoa* PS and SS, it has been stated that marine *Beggiatoa* could play a part in the disproportionation of thiosulfate (Mussmann et al., 2007), an important intermediate of the sulfur cycle in anoxic marine sediments (Jørgensen, 1990). Genes encoding Phs are absent from the genome of *B. alba* B18LD, which indicates that the organism might be unable to disproportionate thiosulfate and sulfur respiration via thiosulfate pathway seems to be not possible.

Regarding the ability of using dimethyl sulfoxide (DMSO), an organic sulfur compound often found in marine and freshwater environments (Madigan et al., 2011) as electron acceptors, DMSO reductase genes (*dms*ABC) were absent in the genome, which is in agreement with previous experimental observation indicated that *B. alba* B18LD does not have the ability to

grow on DMSO (Jewell et al., 2008). The reduction of DMSO to dimethyl sulfide (DMS) is commonly known in phototrophic purple bacteria and chemoorganotrophic bacteria such as *Campylobacter*, *Salmonella* (Lengeler et al., 1999; Madigan et al., 2011).

Oxygen Respiration

Cytochrome *c* bb_3-oxidase (cyt *c* bb_3 I-III) and quinol oxidase (*cyd*AB), which are responsible for oxygen respiration, were found in the genome of *B. alba* B18LD. Our finding of cytochrome *c* bb_3-oxidase confirms results from previous study which suggested the presence of a cytochrome *c* type in *B. alba* B18LD (Cannon et al., 1979). Both cyt *c* bb_3 I-III and *cyd*AB are known to be high-affinity for oxygen and express in microaerobic conditions (Govantes et al., 2000; Pitcher and Watmough, 2004). The presence of cytochrome *c* bb_3-oxidase and quinol oxidase and the lack of cytochrome *c* aa_3-oxidase in *B. alba* B18LD therefore correspond with the fact that *Beggiatoa* prefer to grow in microoxic environments (Moller et al., 1985; Teske and Nelson, 2006).

Nitrogen Metabolism

Our results show that *B. alba* B18LD has an assimilatory rather than dissimilatory nitrate reductase. Nitrate reductase (*nap*A) is present (WP_002690211, 70% similarity with that of *E.coli*) but without signal peptide, it seems unable for Nap of *B. alba* B18LD to receive electrons from the quinone pool to generate proton gradient, and ATP is accordingly not generated for growth on nitrate. As a result, we hypothesize that *nap* of *B. alba* B18LD encode for assimilatory nitrate reductase system and stay in cytoplasm. Our hypothesis fully corroborates with prior experimental study indicated that nitrate reductase of *B. alba* B18LD is likely assimilatory nitrate reductase type and nitrate reduction could not be coupled with sulfide oxidation in *B. alba* B18LD in anaerobic conditions (Teske and Nelson, 2006; Vargas and Strohl, 1985b). The absence of the genes *nar*GHIJ, *nir*S, *nor*AB, *nos*Z is in accordance with experimental observation that nitrogen and nitrous oxide have never been produced by *B. alba* B18LD (Vargas and Strohl, 1985b). Although study from Sweerts et al. (1990) suggested that *B. alba* B18LD is capable of using nitrate as terminal electron acceptor in anaerobic sulfide oxidation (Sweerts et al., 1990), other authors have doubts on this result by questions on the purity of the culture, to be specific, *Beggiatoa* filaments in Sweerts' experiments were thought to be possibly contaminated with denitrifiers (Jørgensen and Gallardo, 1999; Kamp et al., 2006; McHatton et al., 1996). So far only strain *Beggiatoa* sp. LPN was postulated to be

the first freshwater *Beggiatoa* having the ability to oxidize sulfide with nitrate under anaerobic conditions (Kamp et al., 2006).

We found glutamine synthetase (GS) and glutamate synthase (GOGAT), which are considered as the major pathway for ammonia assimilation in *B. alba* B18LD (Vargas and Strohl, 1985a). At high ammonia concentrations, glutamate dehydrogenase (GDH) is utilized as a secondary route for ammonia assimilation in many organisms (Tyler, 1978) and alanine dehydrogenase (ADH) can be used as an alternative enzyme in some organisms lacking GDH (Herbert et al., 1978). We found both GDH and ADH in *B. alba* B18LD. This result is partially consistent with the previous study on ammonia assimilation in *B. alba* B18LD, which demonstrated the activity of ADH under high ammonia concentrations but failed to detect GDH activity (Vargas and Strohl, 1985a); therefore the expression of GDH in *B. alba* B18LD needed to be further examined.

The ability to fix nitrogen of *B. alba* B18LD in microoxic conditions has been reported (Nelson et al., 1982), and genes encoding nitrogen fixation protein (*nif*) are present in *B. alba* B18LD. Nitrogenase in *B. alba* B18LD is apparently of the molybdenum type since the organism has genes encoding nitrogenase molybdenum-iron protein (MoFe protein) and nitrogenase iron protein (Fe protein). The activity of nitrogenase in *B. alba* B18LD was highest at limited oxygen concentrations (Polman and Larkin, 1988) and this is plausible since nitrogen fixation process is occurred under strictly anaerobic conditions. The presence of nitrate and nitrite inhibits nitrogenase activity (Nelson et al., 1982) perhaps due to nitrate or nitrite and nitrogen are competing for gaining electrons and nitrogen fixation is a high energy cost process so as to break the powerful triple bond of dinitrogen. The genes encoding regulator proteins (*nif*A, *ntr*C and *nif*L) were also found in the genome. Thus, *B. alba* B18LD has a complete gene system for nitrogen fixation.

Carbon Metabolism

The genome data shows that *B. alba* B18LD does not have the ability to fix carbon dioxide via autotrophic pathways. Two genes encoding key enzymes in the Calvin cycle, ribulose-bisphosphate carboxylase oxygenase (RubisCO) and ribulose-5-phosphate kinase are present in the genome, however glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$), which is involved in the reduction phase of the Calvin cycle is missing. Sedoheptulose-1,7-

bisphosphatase is also absent. Regarding the reductive citric acid cycle, the genes encoding fumarate reductase, 2-oxoglutarate synthase and ATP-citrate lyase were not found in the genome. The reductive acetyl-CoA pathway may also not operate in *B. alba* B18LD due to the lack of acetyl-CoA synthase/CO dehydrogenase and pyruvate synthase. For the 3-hydroxypropionate cycle, acetyl-CoA carboxylase is present, however propionyl-CoA carboxylase is absent. As a result, *B. alba* B18LD may not be able to fix carbon dioxide via autotrophic pathways due to the lack of key genes involved in both four autotrophic pathways known for carbon dioxide fixation. This finding correlated well with previous experimental observation, which proved that there was no autotrophic carbon dioxide fixation in *B. alba* B18LD and carbon dioxide is incorporated solely by heterotrophic pathways (Larkin and Strohl, 1983; Strohl et al., 1981).

We found genes encoding glycolate oxidase (*glc*DEF) in the genome of *B. alba* B18LD, which points to the possibility that the organism can utilize glycolate as carbon and energy sources. The genes encoding PHA/PHB synthase, including acetyl-CoA acetyltransferase and acetoacetyl-CoA reductase are present, which is in accordance with the observation of PHB inclusions in *B. alba* B18LD (Lawry et al., 1981). The presence of glycogen synthase suggests the possibility to synthesize glycogen as a storage carbon compound as similar to another large sulfur oxidizer *Thiomargarita namibiensis* (Schulz and Schulz, 2005) in *B. alba* B18LD. However to date glycogen synthesis has not been observed in *Beggiatoa*. The capacity to synthesize both PHB and glycogen as carbon storage compounds has been reported in several organisms, e.g., *Bacillus megaterium* (Wilkinson, 1963), *Sprirulina maxima* (De Philippis et al., 1992), *Rhizobium leguminosarum* (Lodwig et al., 2005), *Sinorhizobium meliloti* (Wang et al., 2007), which provides them survival strategies under the conditions of imbalanced nutrients in different environments.

Interestingly, similar to the well-known methylotroph *Methylococcus capsulatus* Bath (Ward et al., 2004), methanol dehydrogenase was present in the genome of *B. alba* B18LD, which provides genetic evidence for the ability to grow on methanol as a sole carbon and energy source of *B. alba* B18LD in prior experimental study (Jewell et al., 2008). Growth on methanol as carbon and energy sources has been also observed in the close relative strain *Beggiatoa* sp. OH75-2a (Jewell et al., 2008).

Phosphate Accumulation

The ability to accumulate phosphate has been observed in *Beggiatoa* spp. Marine *Beggiatoa* have polyphosphate inclusions with the diameter can reach up to 3 µm (Brock et al., 2012), whereas polyphosphate inclusions in freshwater *B. alba* can be up to 1.2 µm but are generally below 1 µm in diameter (Havemeyer, 2013). In the genome of *B. alba* B18LD, we found genes encoding high-affinity phoBRU-regulated ABC phosphate transporters, and those genes probably play an important role in polyphosphate uptake as similar to marine *Beggiatoa* strains (Mussmann et al., 2007). The gene encoding 3-phytase is also present. Phytase is a phytate-degrading enzyme widely distributed in plants and microorganisms (Konietzny and Greiner, 2002). Phytate is an important storage form of phosphorus and inorganic phosphate released from phytate by activity of phytase can be easily to absorb (Jain et al., 2016; Konietzny and Greiner, 2002), the presence of phytase therefore would be helpful for *Beggiatoa* to access inorganic phosphate (Mussmann et al., 2007). We also found the gene encoding polyphosphate kinase, which catalyzes the synthesis of polyphosphate granules after the uptake of polyphosphate in *B. alba* B18LD and the genes encoding polyphosphate degrading enzymes (polyphosphate:AMP phosphotransferase, adenylate kinase, and exopolyphosphatase), therefore *B. alba* B18LD has a complete gene system for polyphosphate synthesis and release. It has been reported that marine *Beggiatoa* sp. 35Flor stored polyphosphate under sulfide limitation by energy derived from sulfide oxidation while polyphosphate is released under high sulfide concentration conditions (Brock and Schulz-Vogt, 2011). In contrast, no effects of sulfide concentrations has been recorded on polyphosphate storage in the freshwater *B. alba* B15LD (Havemeyer, 2013). The mechanism of phosphate accumulation in *B. alba* B18LD so far remains unknown and still an interesting topic for future investigation.

Sensing

We found genes encoding proteins involved in light and redox sensing in the genome of *B. alba* B18LD, namely two genes encoding GAF domain and 45 genes encoding PAS domain. The number of genes encoding PAS domain proteins in *B. alba* B18LD is higher than that often found in other prokaryotes (18.3 in average) (Henry and Crosson, 2011). The gene coding for bPAC (photoactivated adenylyl cyclase), a domain protein for blue-light sensing using flavin is absent from the genome of *B. alba* B18LD and this characteristic is different from the soil *Beggiatoa* strain with the presence of bPAC (Stierl et al., 2011). Genes encoding

proteins involved in Light, Oxygen, or Voltage (LOV) sensing and Blue-Light-Utilizing flavin adenine dinucleotide (FAD) (BLUF) domains were also not found, which may indicate that *B. alba* B18LD does not have the ability to respond to blue light (320 – 500 nm). This probable incapacity of *B. alba* B18LD can cause detrimental impacts on the cells under circumstances of blue light exposure. Our result is consistent with prior observation that *B. leptomitiformis* was unable to respond to blue light due to the lack of blue-light photoreceptors and full sunlight inhibited *Beggiatoa* growth in spite of densely formed filaments before the exposure (Nelson and Castenholz, 1982).

The genome of *B. alba* B18LD also contains genes encoding chemotaxis proteins CheC, CheD, CheY, CheR, CheW and 8 genes encoding methyl-accepting chemotaxis proteins (MCPs). As a result, those findings suggest that *B. alba* B18LD can be flexible to respond to various environmental conditions.

Motility

Gliding motility of *Beggiatoa* has been reported with the speed of gliding of about 8µm s^{-1} (Larkin and Strohl, 1983). Unlike some nonflagelated-cyanobacteria with the ability to swim at rapid rates (Spormann, 1999), *Beggiatoa* cannot swim (Dunker et al., 2011) but may glide by excreted slime trails (Larkin and Strohl, 1983). Gliding by slime excretion has been widely observed in cytophaga-flavobacteria group (McBride, 2004). Slime formation has been reported to be often associated with gliding activities in filamentous cyanobacteria and *Beggiatoa* filaments (Larkin and Strohl, 1983; Spormann, 1999). In the genome of *B. alba* B18LD, homologs of *pil* genes (*pil*ABDEFMNOPQUZTVW) were also found. Since twitching motility has so far not been observed in *B. alba* B18LD, it is questionable whether those *pil* genes are expressed in the organism. In *Escherichia coli* K-12, the activities of type IV *pil* genes were not detected under laboratory conditions (Sauvonnet et al., 2000). The genes *cgl*, *agl* and *fla* are absent in the genome, which corresponds well with the fact that *B. alba* B18LD does not have flagella (Mezzino et al., 1984).

Comparative analysis

Based on genomic data, proposed metabolic characteristics of *B. alba* B18LD in comparison with other *Beggiatoa* and sulfur oxidizers are summarized in Table 7. *Thiothrix nivea* $JP2^T$ and *Thioploca ingrica* were chosen as other sulfur-oxidizing bacteria for comparison due to

their available completed genomic data. The genome sequence of *T. nivea* JP2^T is the first completed genome sequence of the family *Thiotrichaceae* (Lapidus et al., 2011) and that of *T. ingrica* is the first completed genome sequence of the group *Thioploca* (Kojima et al., 2015).

B. alba B18LD is the only strain which is not capable of oxidizing elemental sulfur to sulfate due to the incomplete gene system involved in this oxidation, while all the other strains possess complete sulfur oxidation. DMSO respiration could not be operated in *B. alba* B18LD and *B. leptomitiformis* D-402^T due to the absence of *dms* genes in their genomes, on the other hand, those genes are found in all the other strains. To the best of our knowledge, the reduction of sulfur to sulfide under anaerobic conditions has been reported only in the strain B18LD^T (Schmidt et al., 1987) among organisms chosen for the comparison. *Beggiatoa* sp. 35Flor and *Beggiatoa* sp. OH75-2a were the only two other *Beggiatoa* species that sulfur respiration to sulfide up to now has been tested with positive results (Nelson and Castenholz, 1981; Schwedt et al., 2012). Nitrate respiration is not operated in *B. alba* B18LD (Vargas and Strohl, 1985a) and *T. nivea* JP2 (Garrity et al., 2007; Larkin and Shinabarger, 1983), whereas marine *Beggiatoa* and *T. ingrica* appear to have this capacity (Kojima et al., 2015; MacGregor et al., 2013a; Mussmann et al., 2007). It is possible that sulfur reduction in freshwater strains to some extent considered to play a similar role as nitrate respiration in marine *Beggiatoa* and *Thioploca* in growth maintenance under short term anoxic conditions (Jørgensen and Gallardo, 1999). The ability to grow on methanol of *B. alba* B18LD is well confirmed with the presence of methanol dehydrogenase (Jewell et al., 2008). The gene encoding methanol dehydrogenase is also found in the genome of *B. leptomitiformis* D-402^T but not detected in the uncompleted genome sequences of marine *Beggiatoa*. While the ability to fix CO_2 autotrophically has been reported in *B. leptomitiformis* D-402^T, marine *Beggiatoa*, *T. ingrica* and so far remains unclear in *T. nivea* JP2, *B. alba* B18LD is undoubtful non-autotrophic. Genomic data showed that all the strains are capable of phosphate accumulation.

Conclusions

Our findings present an insight into the genome of *B. alba* B18LD^T. Genomic evidence for incomplete sulfur oxidation pathway, sulfur reduction to sulfide, inability in nitrate reduction coupled with sulfide oxidation, non-autotrophy and methylotrophy strongly confirms results from previous experimental studies (Jewell et al., 2008; Schmidt et al., 1987; Strohl et al., 1981; Vargas and Strohl, 1985a). The analysis of the completed genome sequence of *B. alba*

B18LD^T provides genomic-based-confirmation for differences in metabolic characteristics of strain B18LD with other *Beggiatoa* species and the far-completed genome sequences of *Beggiatoa* SS and nearly-completed genome sequences of *Beggiatoa* Orange Guaymas are suggested. The findings of this study can serve as a base for further proteomic studies to deepen our understanding in this ecologically important genus in future.

Authors' contributions

Phuong MN and Jochen AM carried out the data analysis and drafted the manuscript.

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Table 1. Classification and general features of *Beggiatoa alba* B18LD^T (Field et al., 2008)

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain *Bacteria*	TAS (Garrity et al., 2007)
		Phylum *Proteobacteria*	TAS (Garrity et al., 2007)
		Class *Gammaproteobacteria*	TAS (Garrity et al., 2007)
		Order *Thiotrichales*	TAS (Garrity et al., 2007)
		Family *Thiotrichaceae*	TAS (Garrity et al., 2007)
		Genus *Beggiatoa*	TAS (Garrity et al., 2007)
		Species *Beggiatoa alba*	TAS (Garrity et al., 2007)
		Type strain: *B18LD^T (ATCC33555)*	TAS (Garrity et al., 2007)
	Gram stain	Negative	TAS (Mezzino et al., 1984)
	Cell shape	Filaments	TAS (Mezzino et al., 1984)
	Motility	Motile	TAS (Mezzino et al., 1984)
	Sporulation	Not reported	
	Temperature range	0 - 38°C	TAS (Mezzino et al., 1984)
	Optimum temperature	*Beggiatoa* 28°C	TAS (Scotten and Stokes, 1962)
	pH range; Optimum	*Beggiatoa 5 – 9;* 7.2 - 8.2	TAS (Fjerdingstad et al., 1979; Scotten and Stokes, 1962)
	Carbon source	Acetate, fumarate, lactate, malate, pyruvate, succinate, ethanol, methanol	TAS (Jewell et al., 2008; Mezzino et al., 1984)
MIGS-6	Habitat	Freshwater	TAS (Mezzino et al., 1984)
MIGS-6.3	Salinity	0.5 – 1.0 % NaCl (w/v)	TAS (Mezzino et al., 1984)
MIGS-22	Oxygen requirement	Microaerophilic	TAS (Mezzino et al., 1984)
MIGS-15	Biotic relationship	Free-living	TAS (Markowitz et al., 2012)
MIGS-14	Pathogenicity	Non-pathogen	NAS
MIGS-4	Geographic location	Baton Rouge, Louisiana, USA	TAS (Mezzino et al., 1984)
MIGS-5	Sample collection	1976	TAS (Nelson et al., 1988)
MIGS-4.1	Latitude	Not reported	
MIGS-4.2	Longitude	Not reported	
MIGS-4.4	Altitude	Not reported	

[a] Evidence codes - TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project (Ashburner et al., 2000)

Table 2. Project information

MIGS ID	Property	Term
MIGS 31	Finishing quality	
MIGS-28	Libraries used	
MIGS 29	Sequencing platforms	
MIGS 31.2	Fold coverage	
MIGS 30	Assemblers	
MIGS 32	Gene calling method	
	Locus Tag	
	Genbank ID	
	GenBank Date of Release	
	GOLD ID	
	BIOPROJECT	
MIGS 13	Source Material Identifier	
	Project relevance	

Table 3. Genome statistics

Attribute	Value	% of total
Genome size (bp)	4,265,146	100.00
DNA coding (bp)	3,723,359	87.30
DNA G+C (bp)	1,736,797	40.72
DNA scaffolds	3	100.00
Total genes	3569	100.00
Protein coding genes	3516	98.51
RNA genes	53	1.49
Pseudo genes	66	1.85
Genes in internal clusters	440	12.33
Genes with function prediction	2720	76.21
Genes assigned to COGs	2271	63.63
Genes with Pfam domains	2861	80.16
Genes with signal peptides	331	9.27
Genes with transmembrane helices	864	24.21
CRISPR repeats	6	

Table 4. Number of genes associated with general COG functional categories

Code	Value	%age	Description
J	62	9.69	Translation, ribosomal structure and biogenesis
A	2	0.08	RNA processing and modification
K	111	4.38	Transcription
L	102	4.02	Replication, recombination and repair
B	2	0.08	Chromatin structure and dynamics
D	31	1.22	Cell cycle control, cell division, chromosome partitioning
V	69	2.72	Defense mechanisms
T	234	9.22	Signal transduction mechanisms
M	179	7.06	Cell wall/membrane biogenesis
N	62	2.44	Cell motility
U	64	2.52	Intracellular trafficking and secretion
O	143	5.64	Posttranslational modification, protein turnover, chaperones
C	198	7.8	Energy production and conversion
G	93	3.67	Carbohydrate transport and metabolism
E	185	7.29	Amino acid transport and metabolism
F	57	2.25	Nucleotide transport and metabolism
H	146	5.75	Coenzyme transport and metabolism
I	90	3.55	Lipid transport and metabolism
P	141	5.56	Inorganic ion transport and metabolism
Q	51	2.01	Secondary metabolites biosynthesis, transport and catabolism
R	201	7.92	General function prediction only
S	143	5.64	Function unknown
-	1298	36.37	Not in COGs

The total is based on the total number of protein coding genes in the genome.

Table 5. Summarized genome features of *B. alba* B18LD^T in comparison to other *Beggiatoa* genomes

Feature	***B. alba* B18LD^T**	***Beggiatoa leptomitiformis* D-402^T** (Fomenkov et al., 2015)	***Beggiatoa* PS** (Mussmann et al., 2007)	***Beggiatoa* SS** (Mussmann et al., 2007)	***Beggiatoa* Orange Guaymas** (MacGregor et al., 2013b)
Total genome size	4.3 Mb	4.3 Mb	> 7.6 Mb	>> 1.3 Mb	> 4.7 Mb
Total no. of genes	3569	3681	> 6686	>> 1441	> 5330
No. of tRNA genes	46	47	45	5	46
No. of replicons/contigs	3	2	6769	1091	822

Table 6. Sulfur oxidation and related genes in *B. alba* B18LD

Gene encoding protein	No. of amino acids	% Identity to *Beggiatoa* PS	Putative function	Accession no.
SoxXA	412	58 (413)	Sulfur oxidation protein	WP_002691438
SoxB	583	69 (589)	Sulfur oxidation protein	WP_002691637
SoxY	155	51 (57)	Sulfur oxidation protein	WP_002690118
SoxZ	104	65 (102)	Sulfur oxidation protein	WP_002690119
SoxYZ-like carrier	252	No close homolog	quinoprotein dehydrogenase-associated SoxYZ-like carrier	WP_002690522
FccA	100	36 (221)	Sulfide dehydrogenase	WP_002683362
FccB	433	52 (431)	Twin-arginine translocation pathway signal Uncharacterized NAD (FAD)-dependent dehydrogenase	WP_002683366
Sqr	423	70 (422)	Sulfide quinone reductase NADH dehydrogenase, FAD-containing subunit	WP_002690795
Sqr	377	No close homolog	Sulfide quinone reductase NADH dehydrogenase, FAD-containing subunit	WP_002685114
Sulfite oxidase-like oxidoreductase	322	No close homolog	Sulfite oxidation protein	WP_002685091
Sulfite oxidase	162	No close homolog	Sulfite oxidation protein	WP_002682695
DsrC-like protein	110	77 (110)	Dissimilatory sulfite/sulfate reductase	WP_002685049
DsrE	130	81 (79)	Dissimilatory sulfite/sulfate reductase	WP_002685059
DsrF	128	64 (89)	Dissimilatory sulfite/sulfate reductase	WP_002685058
DsrH	101	63 (101)	Dissimilatory sulfite/sulfate reductase	WP_002685051
DsrK	492	76 (475)	Dissimilatory sulfite/sulfate reductase	WP_002682853

DsrJ	129	49 (141)	Dissimilatory sulfite/sulfate reductase	WP_002682855
ATP sulfurylase, small subunit	329	No close homolog	Sulfate adenylyltransferase	WP_002684342
ATP sulfurylase, large subunit	426	No close homolog	Sulfate adenylyltransferase	WP_002684343
AprAB	-	-	Adenosine phosphosulfate reductases	Not found

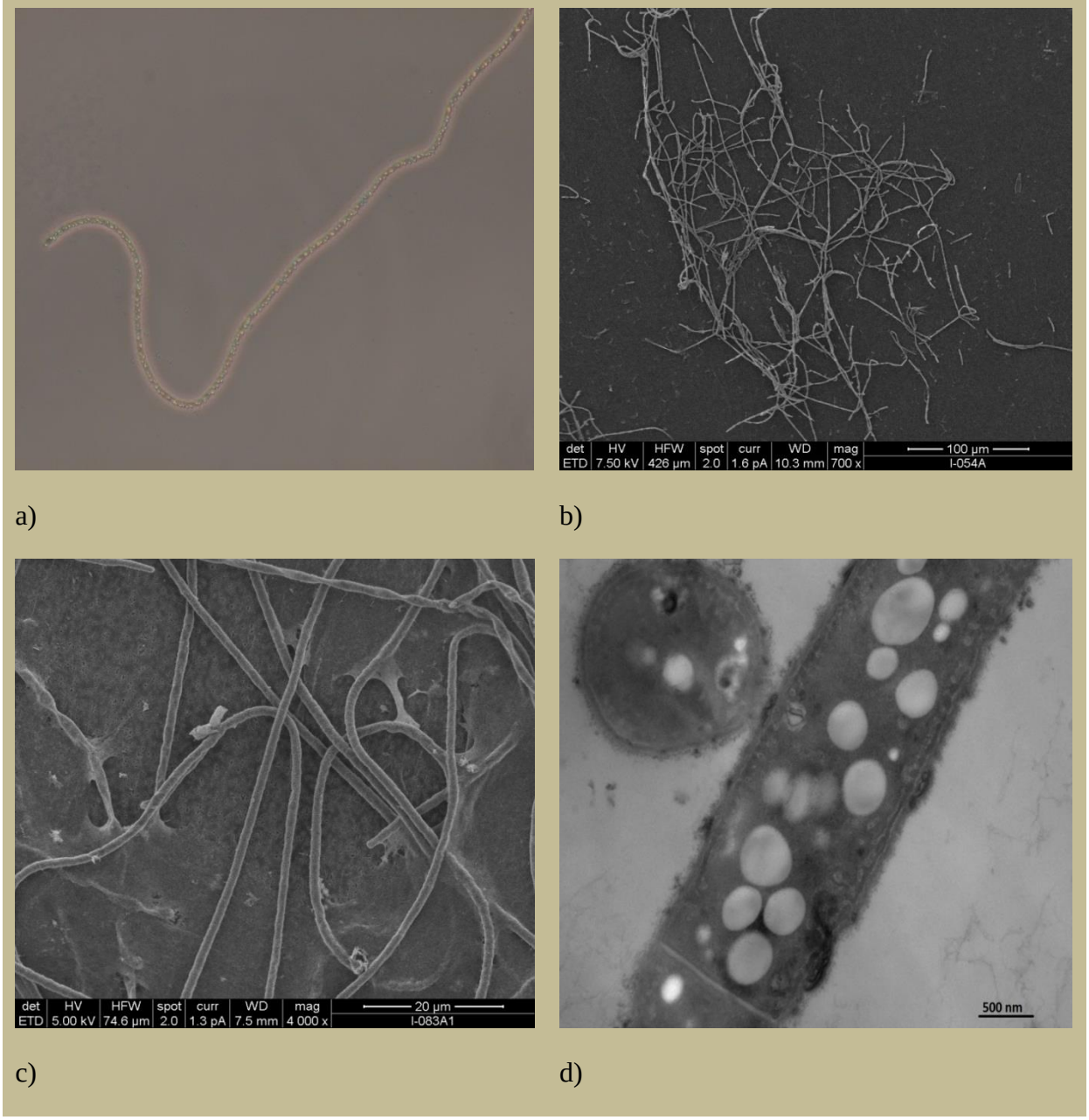
Data in the parentheses indicate the numbers of amino acids

Table 7. Metabolic characteristics of *B. alba* B18LD in comparison to other *Beggiatoa* and sulfur oxidizers proposed by genomic analysis

Metabolism	***B. alba* B18LD^{T}**	***Beggiatoa leptomiti-formis* D-402$^{T, a}$**	***Beggiatoa* PS, SS ^{b}**	***Beggiatoa* Orange Guaymas^{c}**	***Thiothrix nivea* JP2$^{T, d}$**	***Thioploca ingrica*^{e}**
Sulfur oxidation	Incomplete	Complete	Complete	Complete	Complete	Complete
Sulfur reduction to sulfide	+	nd	nd	nd	nd	nd
DMSO respiration	-	-	+	+	+	+
Nitrate respiration	-	nd	+	+	-	+
Methylotrophy	+	+	-	-	-	-
Autotrophy	-	+	+	+	nd	+
Phosphate accumulation	+	+	+	+	+	+

+ : positive, - : negative, nd: not determined

References: a: Fomenkov et al., 2015; Muntian et al., 2005; Patritskaia et al., 2001, and Blast search from ncbi; b: Mussmann et al., 2007; c: MacGregor et al., 2013a; d: Lapidus et al., 2011 and Blast search from ncbi; e: Kojima et al., 2015



a) b)

c) d)

Figure 1. Micrographs of *B. alba* B18LD. a) Phase-contrast micrograph of the filaments at 40x magnification. b and c) Electron micrographs showing densely formed filaments at 700x and 4000x magnification, respectively. d) Electron micrograph showing filamentous cells with different types and sizes of internal inclusions.

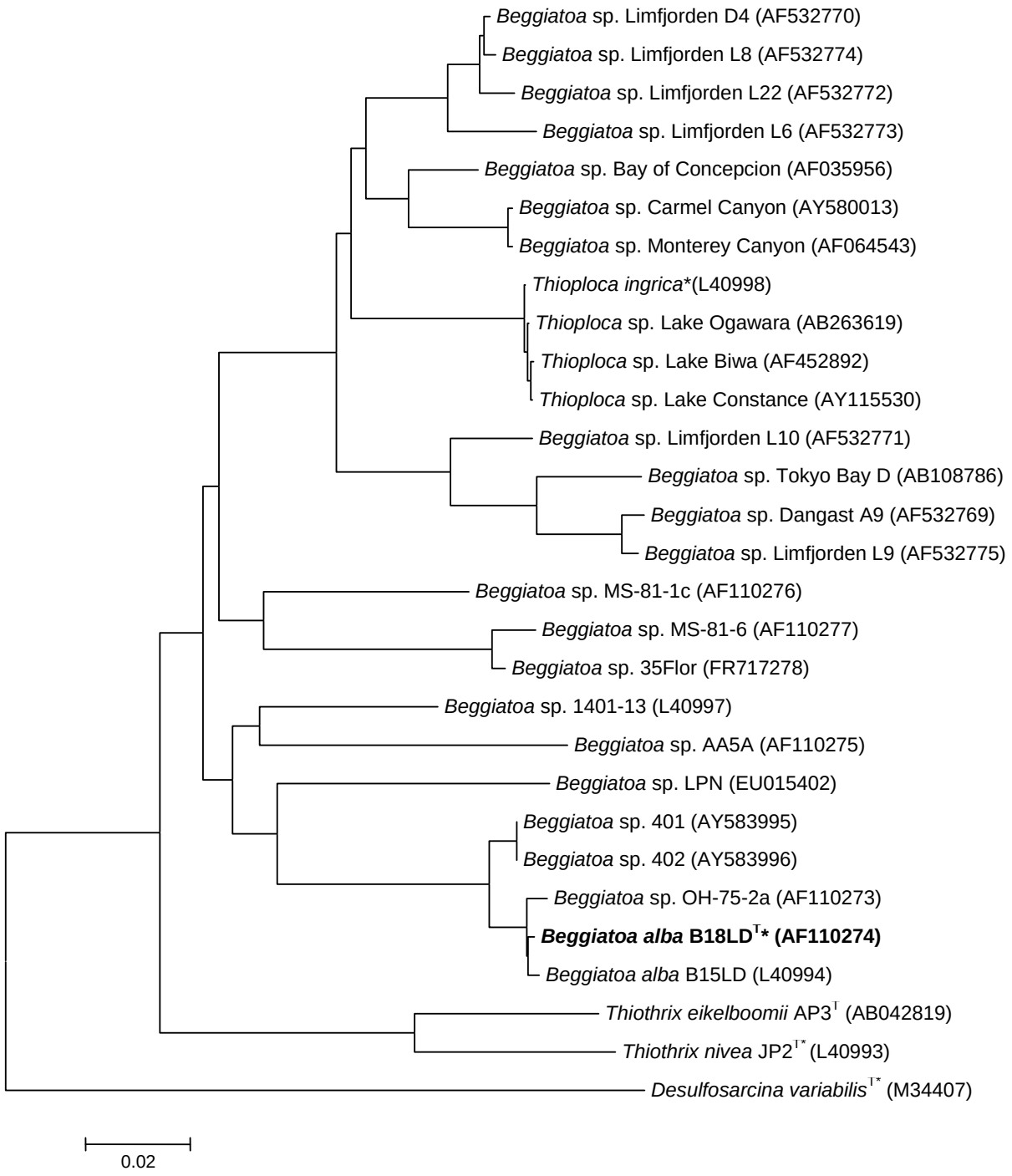


Figure 2. Phylogenetic tree based on 16s RNA gene sequences highlights current placement of *B. alba* B18LD. Other elements of the tree includes several species from the genus *Beggiatoa*, *Thiothrix*, *Thioploca*, and the delta-proteobacterium *Desulfosarcina variabilis* as the out-group. The scale bar corresponds to 2% estimated sequence divergence. GenBank accession numbers are shown in parentheses. The asterisks (*) indicates the availability of a genome. The tree was constructed by neighbor-joining clustering method and generated in MEGA6 (Tamura et al., 2013).

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremdem Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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