



# Royal Netherlands Academy of Arts and Sciences (KNAW) KONINKLIJKE NEDERLANDSE AKADEMIE VAN WETENSCHAPPEN

## Effect of the aerenchymatous helophyte *Glyceria maxima* on the sulfate-reducing communities in two contrasting riparian grassland soils

Miletto, M.; Bodelier, P.L.E.; Ferdelman, F.G.; Jørgensen, B.B.; Laanbroek, H.J.

### **published in**

Plant and Soil  
2013

### **DOI (link to publisher)**

[10.1007/s11104-013-1608-1](https://doi.org/10.1007/s11104-013-1608-1)

### **document version**

Peer reviewed version

[Link to publication in KNAW Research Portal](#)

### **citation for published version (APA)**

Miletto, M., Bodelier, P. L. E., Ferdelman, F. G., Jørgensen, B. B., & Laanbroek, H. J. (2013). Effect of the aerenchymatous helophyte *Glyceria maxima* on the sulfate-reducing communities in two contrasting riparian grassland soils. *Plant and Soil*, 370(1-2), 73-87. <https://doi.org/10.1007/s11104-013-1608-1>

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the KNAW public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the KNAW public portal.

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

### **E-mail address:**

[pure@knav.nl](mailto:pure@knav.nl)

**Effect of the aerenchymatous helophyte *Glyceria maxima* on the sulfate-reducing communities in two contrasting riparian grassland soils**

M. Miletto,<sup>1,†</sup> P.L.E. Bodelier,<sup>1</sup> T.G. Ferdelman,<sup>2</sup> B.B. Jørgensen,<sup>2,3</sup> H.J. Laanbroek<sup>1,4</sup>

<sup>1</sup> *Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands*

<sup>2</sup> *Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, Bremen, Germany*

<sup>3</sup> *Center for Geomicrobiology, Department of Bioscience, Aarhus University, Denmark*

<sup>4</sup> *Institute of Environmental Biology, Utrecht University, the Netherlands*

<sup>†</sup> Present address: Department of Plant and Microbial Biology, University of California, Berkeley, USA

\* Corresponding author

Email: [r.laanbroek@nioo.knaw.nl](mailto:r.laanbroek@nioo.knaw.nl)

Phone: +31 (0)317 473480

Fax: +31 (0)317 473675

**ABSTRACT**

28 *Aims:* The research aimed at studying the effect of flooding with sulfate-rich water on the activity,  
29 abundance and diversity of sulfate-reducing micro-organisms present in the root zone of an  
30 oxygen-releasing plant growing on two riparian grassland soils with contrasting amounts of iron.

31 *Methods:* A series of microcosms was used to investigate the effects. Plants were grown under  
32 controlled conditions in microcosms containing a rhizosphere and bulk soil compartment for a  
33 period of 12 weeks in the presence of sulfate-rich flood water. Molybdate-treated systems served  
34 as non-sulfate-reducing controls.

35 *Results:* At harvest, activity and numbers of sulfate-reducing micro-organisms were higher in the  
36 absence of molybdate, but a rhizosphere effect and an impact of the presence of high levels of  
37 iron were not observed on activity and numbers. Both soils had in common a diverse community  
38 of sulfate-reducing micro-organisms covering all major cultured bacterial taxa. The appearance of  
39 members of the *Desulfovibrionaceae* exclusively in the rhizosphere of *G. maxima* was the only  
40 unambiguous indication of a plant effect.

41 *Conclusion:* The presence of sulfate-rich flood water stimulated the activity and growth of a part  
42 of the sulfate-reducing community leading to a change in community composition. The  
43 proximity of aerenchymatous plant roots and the abundance of iron in the soil had a negligible  
44 effect on the sulfate-reducing community.

45

46 *Keywords:* rhizosphere, wetland, *Glyceria maxima*, microcosm, sulfate-reducing microorganisms  
47 (SRM)

48

49 **INTRODUCTION**

50

51 Aerenchymatous plants that leak oxygen and labile organic molecules into otherwise reduced  
52 wetland soils influence the biogeochemistry of elements in such water-saturated systems  
53 (Laanbroek 2010). The oxidation of organic carbon in natural environments is performed by  
54 different functional groups of aerobic and anaerobic microorganisms. Under conditions of limited  
55 access to degradable organic carbon these microbial groups compete for electron donors. The  
56 outcome of this competition determines the nature and amounts of oxidation products that are  
57 formed by the different reductive pathways (Laanbroek 1990). For this reason it is to be expected  
58 that the presence of oxygen and oxidized iron, which are energetically more favorable electron  
59 acceptors than sulfate, will lower the reduction of sulfate in the rhizosphere of aerenchymatous  
60 wetland plants. (Roden and Wetzel 1996) observed low rates of sulfate reduction in the root zone  
61 of the freshwater rush *Juncus effusus* in the presence of active iron-cycling. In spite of a possible  
62 repression of sulfate reduction in the oxidized rhizosphere, sulfate-reducing microorganisms (SRM)  
63 have been shown to colonize the rhizosphere of wetland plants (Bahr et al. 2005; Cifuentes et al.  
64 2003; Kusel et al. 1999; Stubner 2004). These SRM may be present as inactive cells and spores  
65 or they may function as hydrogen-producing, fermentative microorganisms living in syntrophy  
66 with hydrogen-consuming microbes, making them independent of sulfate for the generation of  
67 energy (Muyzer and Stams 2008). However, the presence of SRM in the rhizosphere of wetland  
68 plants may also be based on recycling of sulfide to sulfate in an oxygen-limited rhizosphere.  
69 Oxidation of sulfide has been shown in the rhizosphere of aerenchymatous plants (Choi et al.  
70 2006; Wind and Conrad 1995; 1997). A cryptic sulfur cycle driven by iron has also been  
71 demonstrated in the apparently sulfate-free, methane zone of marine sediment (Aarhus Bay,  
72 Denmark; (Holmkvist et al. 2011). Finally, an anoxic sulfur cycle may exist in wetland soils based  
73 on sulfate reduction combined with sulfide re-oxidation in the presence of redox-active organic  
74 matter (Heitmann and Blodau 2006; Heitmann et al. 2007). All these interactive sulfur-converting

75 processes may lead to low, hardly measurable concentrations of sulfate in vegetated freshwater  
76 wetlands.

77 In a survey on the presence of SRM in floodplains of three Dutch rivers, we observed a high  
78 diversity of these microorganisms (Miletto et al. 2008). The communities of SRM in the floodplain  
79 soils displayed a salinity- and plant-nutrient-dependent distribution. In a follow-up experiment we  
80 studied the effect of flooding and tide on the activity and diversity of SRM present in a former  
81 salt marsh soil (Miletto et al. 2010). The salt marsh had been disconnected from the sea since  
82 1971 and since then the tidal and salt water influence had been largely restricted. After a period  
83 of 4 months of flooding with brackish water containing 2.7 mM sulfate, anoxic sediment slurries  
84 from sods of the former salt marsh started to consume sulfate immediately upon addition of  
85 sulfate to the slurries. However, the same sulfate consumption rates were observed in slurries of  
86 sods that had received only freshwater without sulfate. Hence, both sod types contained an  
87 active community of SRM. During the 4 months of treatment, the community composition of the  
88 SRM in the former salt marsh soil changed only slightly in the presence of sulfate-containing  
89 brackish water by the specific appearance of *Desulfomonile*-related *dsrB* sequences.

90

91 In the present experiment we studied the effect of sulfate-containing flood water on the activity  
92 and diversity of the SRM community in the rhizosphere of the helophyte *Glyceria maxima* or Reed  
93 Sweetgrass, which is a common plant species in Dutch nutrient-rich wetlands. *G. maxima* is a  
94 highly aerenchymatous plant species (Smirnov and Crawford 1983) and the oxygen flux to the  
95 roots results in oxygen leakage into the rhizosphere (Brändle and Crawford 1987; Rees et al.  
96 1987) To separate the effects of flooding with sulfate-rich water on the rhizosphere of *G. maxima*  
97 from its effects on the bulk soil, the study was performed in microcosms, in which the  
98 rhizosphere compartment had been physically separated from the bulk soil by nylon gauze  
99 impervious for plant roots (Bodelier et al. 1998). We used additionally molybdate in the flood  
100 water of control microcosms to repress sulfate reduction. Molybdate functions as an intracellular  
101 competitor to sulfate (Oremland and Capone 1988). For the study we used two floodplains soils

102 that differ in their iron content. We hypothesize that the plant affects the activity, abundance and  
103 diversity of the community of SRM in its rhizosphere in the presence of sulfate-rich flood water  
104 and that the effects are most distinct in microcosms filled with the iron-poor soil, where the  
105 interference with iron cycling should be more limited.

106

107

## 108 **MATERIALS AND METHODS**

109

### 110 **Plant**

111 *Glyceria maxima* (Hartm.) Holmb. has been used as a model wetland plant in several earlier  
112 studies (e.g. (Bodelier et al. 1998; Nijburg et al. 1997)). We collected seeds from a *G. maxima*  
113 stand in a ditch near the institute during the growing season. All seeds were obtained from the  
114 same plant and germinated by incubation in a plant growth cabinet on a water-soaked cotton  
115 wool-filled Petri dish at day/night cycles of 10 h 25°C/14 h 15°C and a photosynthetic photon flux  
116 density of  $\pm 10 \mu\text{E m}^{-2} \text{s}^{-1}$  at the level of the seeds. After 7 days 80% of the seeds had  
117 germinated. 20 days-old seedlings (height:  $\pm 3.5$  cm) were transplanted into the microcosms.

118

### 119 **Soil**

120 The top 15 cm soil used for the microcosms was collected from two riparian zones at Ewijk  
121 ("EWK", the Netherlands, 51°53' N, 5°44' E) along the river Rhine and at Huis den Doorn ("HDD",  
122 the Netherlands, 52° 33' N, 6° 7' E) along the river Overijsselse Vecht. Soil was stored after air-  
123 drying. The HDD soil is rich in iron (total and amorphous iron of 693 and 459  $\mu\text{mol g}^{-1}$  dry weight,  
124 respectively), which originates from historical discharge of groundwater (Loeb et al. 2008). EWK  
125 soil that is not affected by groundwater seepage contains 103  $\mu\text{mol total iron g}^{-1}$  dry weight (LL  
126 soil in Conrad et al. (2008)). A summary of the chemical characteristics of the two soils is shown  
127 in supplementary Table S1.

128 The more silty EWK soil (40% clay, 60% sand) had the highest pore water pH and the highest  
129 contents of pore water and KCl-extractable nutrients. The more sandy HDD soil (21% clay, 79%  
130 sand) had slightly higher organic matter content and showed deposits of red iron oxides. Hence,  
131 although the amounts of pore water  $\text{Fe}^{3+}$  were quite similar between both soils, HDD soil had a  
132 larger storage of iron. Prior to use, clods were crushed, plants and roots removed and the  
133 remaining soil sieved (mesh size: 1 mm).

134

### 135 **Microcosms**

136 The microcosms used have been described before (Bodelier et al. 1998). Their most important  
137 feature is the separation between rhizosphere and bulk soils by nylon gauze (mesh size 30  $\mu\text{m}$ )  
138 supported by a metal cylinder. Pore water samples were collected with Rhizon soil solution  
139 samplers (Eijkelkamp, Giesbeek, the Netherlands) that were vertically installed in both  
140 rhizosphere and bulk soil compartment to a depth of 2 cm below the soil surface.

141 In total, 24 microcosms were filled with 780 g of dry soil each and flooded with a sterile oxygen-  
142 free aqueous solution containing 2 mM  $\text{Na}_2\text{SO}_4$  or 2 mM  $\text{Na}_2\text{SO}_4$  plus 2.5 mM  $\text{Na}_2\text{MoO}_4$ . Molybdate  
143 is a specific metabolic inhibitor of sulfate reduction (Oremland and Capone 1988). Per soil type, 6  
144 replicate microcosms were randomly selected for each treatment. Once the flooding solution  
145 completely penetrated the soil, randomly chosen *G. maxima* seedlings were planted in the  
146 rhizosphere compartment of all systems. The flood water level was then raised and kept constant  
147 at 2.5 cm above the soil surface by replenishing with sterile water throughout the experiment.

148 The systems were incubated in a plant growth cabinet (day/night cycles 16 h, 22°C/8 h, 15°C;  
149 relative humidity 65%; photosynthetic photon flux density at plant level  $\pm 300 \mu\text{E m}^{-2} \text{s}^{-1}$ ).

150 Microcosms were positioned randomly in the cabinet and were rearranged regularly.

151 After 12 weeks the systems were harvested. All microcosms were processed the same day. Plant  
152 dry matter was determined by weighing after drying at 70°C for 2 days. Plants were subdivided  
153 into aboveground biomass (leaf blades) and belowground biomass (rhizomes and roots). Relative  
154 growth rates (RGR) per week were calculated using initial ( $W_{\text{in}}$ ) and final ( $W_{\text{fin}}$ ) dry weights at

155 harvest according to the following equation  $RGR = (\ln W_{fin} - \ln W_{in}) / (t_{fin} - t_{in})$ , where  $t_{in} = 0$  and  $t_{fin} =$   
156 12 weeks. Soil moisture and organic matter content of the thoroughly mixed sediment were  
157 determined as describe above.

158

### 159 **Pore water analyses**

160 Pore water samples from the rooted and non-rooted compartments were collected weekly  
161 throughout the duration of the experiment. To minimize sampling biases (volume in the sampler:  
162 0.5 ml), the first ml of sample was discarded. Pore water pH was immediately measured and the  
163 sample stored at  $-20^{\circ}\text{C}$  until further analyses. A sub-sample of pore water (0.5 ml ) was saved to  
164 monitor sulfate and molybdate concentration in both compartments; sulfate was determined  
165 spectrophotometrically with the barium-gelatin method of (Tabatabai 1974), while molybdate was  
166 measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Spectro, Kleve,  
167 Germany). If necessary, a sterile anoxic  $\text{SO}_4^{2-}$  or  $\text{SO}_4^{2-}/\text{MoO}_4^{2-}$  aqueous solution was added at an  
168 appropriate volume/concentration, to maintain the pore water sulfate and molybdate content at  
169 the prefixed experimental value. The amendment was done by a syringe connected to a 15 cm  
170 long needle; to minimize disturbance, injections of maximum 5 ml were distributed randomly in  
171 the bulk soil and rhizosphere compartment. Pore water  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$  concentrations were  
172 analyzed on a continuous-flow analyzer. Volatile fatty acids and alcohols concentrations after 1  
173 and 12 weeks of incubation were determined after centrifugation (5 min. 13200 rpm) and two-  
174 fold dilution of the samples with 0.2 N  $\text{H}_2\text{SO}_4$  (volatile fatty acids) or 1 N formic acid (alcohols).  
175 Fatty acids were determined on a LKB-HPLC system (Pharmacia /LKB, Woerden, the Netherlands)  
176 equipped with a high-performance pump and a differential refractometer detector. The samples  
177 (20  $\mu\text{l}$ ) were applied to a Polyspher OA HY column (E. Merck), which was equilibrated with 0.01 N  
178  $\text{H}_2\text{SO}_4$  (flow rate 2 ml/min). Alcohols were determined on a Chrompack CP9001 gas  
179 chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a mol sieve 13X  
180 packed column (2 m  $\times$  4.3 mm i.d., 60-80 mesh) and a thermal conductivity detector. Internal  
181 and external standards were used for quantification.



182

183 **MPN counts**

184 Soil was resuspended in phosphate-buffered saline (PBS, per liter of milli-Q water: 8 g NaCl, 0.2  
185 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub>; pH 7.4) in a soil to buffer ratio of 1:6. Slurries were  
186 shaken for 2 h. Homogenates were immediately used for inoculation of MPN dilution series in  
187 microtiter plates (BRAND, 8 × 12 wells of 250 µl).

188 The bicarbonate-buffered basal mineral medium used had the following composition: 3 mM  
189 KH<sub>2</sub>PO<sub>4</sub>, 3 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM NaCl, 1 mM Na<sub>2</sub>S, 0.5 mM MgCl<sub>2</sub>, 0.75 mM CaCl<sub>2</sub>, 50 mM NaHCO<sub>3</sub>,  
190 5.6 mM NH<sub>4</sub>Cl, 2 µM resazurin. The medium was supplemented with 1 ml of trace element  
191 solution (final concentrations: 1 µM H<sub>3</sub>BO<sub>3</sub>, 0.5 µM MnCl<sub>2</sub>, 7.5 µM FeCl<sub>2</sub>, 0.5 µM CoCl<sub>2</sub>, 0.5 µM  
192 ZnCl<sub>2</sub>, 0.1 µM CuCl<sub>2</sub>, 0.5 µM NiCl<sub>2</sub>, 0.1 µM Na<sub>2</sub>SeO<sub>3</sub>, 0.1 µM Na<sub>2</sub>WO<sub>4</sub>, 0.1 µM Na<sub>2</sub>MoO<sub>4</sub>), 1 ml  
193 vitamins solution (final concentrations: 0.02 µM biotin, 0.2 µM nicotinic acid, 0.5 µM pyridoxine,  
194 0.1 µM riboflavin, 0.2 µM thiamin, 0.1 µM cyanocobalamin, 0.1 µM p-aminobenzoic acid, 0.1 µM  
195 pantothenic acid, 0.1 µM lipoic acid, 0.1 µM folic acid), 1 g of yeast extract. As electron acceptor,  
196 20 mM Na<sub>2</sub>SO<sub>4</sub> was used. A mix of acetate, propionate and lactate (15 mM each) served as  
197 electron donors. The reducing agent in the MPN medium was sodium thioglycolate (0.5 mM).  
198 FeSO<sub>4</sub> (0.2 mM) served as an indicator of sulfate reduction; the formation of a black FeS  
199 precipitate was indicative for sulfide formation by active SRM; black wells were counted as  
200 positive.

201 The microtiter plates were sealed with an adhesive foil (SecurSeal<sup>®</sup>, Simport, Beloeil, Canada)  
202 and put in anaerobic incubation bags (Anaerocult<sup>®</sup> A mini, Merck, Darmstadt, Germany). In the  
203 bags, a citric acid-based catalyst was used to create an oxygen-free N<sub>2</sub>/CO<sub>2</sub> atmosphere. The  
204 atmosphere became anoxic within 1 hour after sealing the bags, as shown by an indicator strip  
205 (Anaerotest<sup>®</sup>, Merck, Darmstadt, Germany). Cultures were incubated at 25°C for 3 months. After  
206 counting the number of positive wells per dilution, the MPN and confidence limits were calculated  
207 using standard MPN tables (Rowe et al. 1977). MPN counts were performed in triplicate samples  
208 per soil/microcosm type.

209

### 210 **Sulfate reduction rates**

211 First, replicate cores were collected from the bulk soil compartment using polymethyl  
212 methacrylate (PMMA) cylinders and immediately sealed at both ends with butyl rubber stoppers.  
213 Due to root growth, replicate samples of the rhizosphere compartment had to be obtained by  
214 longitudinal sectioning only after labeling. Hence, the whole inner cylinder together with the  
215 rhizosphere soil was removed from the microcosm and carefully sealed with saran wrap.  
216 Approximately 5  $\mu\text{l}$  (bulk soil cores) or 10  $\mu\text{l}$  (rhizosphere cylinder) of carrier-free  $^{35}\text{SO}_4^{2-}$  tracer  
217 ( $\sim 70$  kBq, Amersham) were injected at 1 cm intervals along the cores/cylinder. After 4 h of  
218 incubation at 25°C, transferring 2-cm slices of the cores or cylinders into 10 ml of cold 20% w/v  
219 zinc acetate stopped bacterial activity. The first and the last cm of soil were discarded. Blanks  
220 consisted of soil fixed in zinc acetate solution to which  $^{35}\text{SO}_4^{2-}$  was added. Samples were stored  
221 at  $-20^\circ\text{C}$  until analysis.

222  $^{35}\text{S}$  incorporated into total reducible inorganic sulfur (TRIS) was determined using the cold  
223 Chromium-II method (Kallmeyer et al. 2004). A Packard 2500 TR liquid scintillation counter  
224 (scintillation cocktail Lumasafe Plus®; Lamac LSC BV, the Netherlands) was used to quantify the  
225  $^{35}\text{SO}_4^{2-}$  and  $^{35}\text{S}_{\text{TRIS}}$  activities. Sulfate reduction rates were calculated according to (Jorgensen  
226 1978). Pore water sulfate concentrations were measured by anion exchange chromatography  
227 (Waters 510 HPLC Pump; Waters IC-Pak 50  $\times$  4.6 mm anion exchange column; Waters 430  
228 Conductivity detector) with an isophthalic acid eluent (1 mM, pH 4.7, in methanol, 10% v/v).

229

### 230 **DNA extraction**

231 DNA was extracted from 0.3 g [wet weight] of soil using the UltraClean Soil DNA kit (MoBio,  
232 Solana Beach, CA, USA). Quantification of the electrophoresed and ethidium bromide-stained  
233 DNA extracts was performed by comparison to two dilutions of the SmartLadder quantification  
234 standard (Eurogentec, Seraing, Belgium); digital images analysis was carried out using the  
235 software package Phoretics 1D Advanced (Nonlinear Dynamics, Newcastle upon Tyne, UK).

236

### 237 **Nested *dsrAB-dsrB* amplification and DGGE**

238 *dsrAB* (approximately 1.9-kb) and *dsrB* (approximately 350-bp) gene fragments were amplified as  
239 described by (Miletto et al. 2007). Primer mixtures DSR1Fmix and DSR4Rmix (Loy et al. 2004)  
240 were implemented with primers DSR1Fb, DSR1Fc and DSR4Rd, DSR4Re respectively, as  
241 suggested by (Zverlov et al. 2005). *dsrB* sequences were separated by DGGE, bands of interest  
242 isolated from the gel and purified as described previously (Miletto et al. 2007). The software  
243 package Phoretics 1D Advanced was used to analyze gel images; band detection and matching  
244 (by assignment of R<sub>f</sub> values using a suitable marker mix of 10 *dsrB* fragments run together with  
245 the samples to account for gradient heterogeneities) were performed automatically to avoid  
246 biases associated with manual band processing. Bands showing intensity under a certain value  
247 (15% of the largest peak within a lane) were omitted from further analyses. R<sub>f</sub> values were  
248 assigned to bands using as reference a suitable marker mix of 10 *dsrB* fragments run together  
249 with the samples to account for gradient heterogeneities. Bands having similar R<sub>f</sub> values were  
250 considered as corresponding and grouped into a match (the maximum acceptable displacement  
251 to call a match between bands was set to R<sub>f</sub> ± 0.001). At least one band per match was excised,  
252 sequenced and analyzed phylogenetically.

253

### 254 **Sequencing and phylogenetic analysis**

255 Sequencing was performed with an ABI PRISM 3730XL capillary sequencer (Applied Biosystems,  
256 Foster City, CA, USA) at the BMR Servizio Sequenziamento (CRIBI - Università di Padova, Italy,  
257 [<http://bmr.cribi.unipd.it>]). The ARB package (Ludwig et al. 2004); [<http://www.arb-home.de>])  
258 was used for the phylogenetic analyses. Partial *dsrB* sequences were added to an ARB alignment  
259 of 97 complete *dsrAB* sequences (Zverlov et al. 2005). The alignment of the corresponding amino  
260 acid sequences was carried out manually using the editor GDE 2.2 (Smith et al. 1994)  
261 implemented in ARB. Partial *dsrB* sequences were inserted one by one into a *dsrAB* core tree  
262 without distorting the overall tree topology. Phylogenetic inference was performed with a total of

263 95 amino acid residues (*Desulfovibrio vulgaris dsrB* amino acid sequence positions 172-267)  
264 corresponding to the length of the shortest sequence, and regions of insertion and deletions were  
265 excluded from the dataset (indel filter). Deduced partial *dsrB* sequences were compared to  
266 GenBank database (Benson et al. 2005) using the algorithm blastp (protein-protein BLAST,  
267 [<http://www.ncbi.nlm.nih.gov/BLAST>]). After checking for consistent clustering of bands  
268 belonging to the same match, one band per match was considered for further analyses. All *dsrB*  
269 sequences with DSRAB amino acid sequence identities equal to or greater than 97% were  
270 grouped into an operational taxonomic unit (OTU). One EWK/HDD sequence representative for  
271 each OTU, and sequences of the DGGE bands EWK1-6, and HDD1-2 were submitted to the EMBL  
272 database (accession numbers: AM901635-AM901654, AM901655, AM901657, AM901659,  
273 AM901661, AM901663, AM901665, AM901666, and AM901667).

274

#### 275 **Statistical analyses**

276 One-way ANOVA tests were performed using STATISTICA 7.1 (StatSoft, Inc., Tulsa, OK, USA) to  
277 determine the effects of different factors ('soil type' = HDD or EWK; 'microcosm type' =  
278 treatment or control; 'compartment' = bulk soil or rhizosphere; 'depth'). If the ANOVA revealed  
279 significant effects, a Tukey's HSD (Honestly Significant Differences) post hoc test was performed  
280 to group homogeneous means.

281 For DGGE bands presence-absence data, binary similarities for every pair of samples  
282 (hybridization/DGGE profiles) were inferred using the Jaccard coefficient calculated as

283

$$284 J = M11 / (M01+M10+M11)$$

285

286 where M11 represents the number of bands both lanes have in common, M01 the number of  
287 bands in one of the lanes en M10 the number of bands in the other lane.

288 Analysis of similarity (ANOSIM) between DGGE profiles was performed with the software PRIMER  
289 v5.2.9 (PRIMER-E Ltd, Plymouth, UK) on the similarity matrix, to test the effect of factors

290 'microcosm type' and 'compartment' on the SRM communities composition. Results with  $p > 0.05$   
291 were considered not significant.

292

## 293 **RESULTS**

294

### 295 **Plants**

296 In microcosms filled with EWK soil the presence of molybdate had a retarding effect on the  
297 growth of the plants. In the absence of molybdate, growth started approximately two weeks  
298 earlier. In microcosms with HDD soil, growth profiles overlapped throughout the experiment for  
299 sulfate and molybdate plus sulfate treatments, but growth was also delayed for two weeks similar  
300 to the plants on the EWK soil in the presence of molybdate. *G. maxima* shoot and root  
301 morphology differed between soils. At harvest after a growth period of 12 weeks, plant shoots on  
302 EWK soil displayed a light green color and wide leaves, whereas plants grown in HDD soil had  
303 narrow and dark green leaves. *G. maxima* grown in microcosms with EWK soil had filled the  
304 rhizosphere compartment completely with healthy looking roots at harvest time. Root density was  
305 higher in the absence of molybdate, in particular at the sides and bottom of the inner stainless  
306 steel cylinder. In contrast, the roots of *G. maxima* grown in microcosms with HDD soil grew more  
307 poorly both in the presence and absence of molybdate. The roots of these plants had a black  
308 coating especially in the absence of molybdate, and showed stunted growth. In all microcosms  
309 roots did not penetrate the nylon gauze. Hence, the outer zone of the microcosms remained free  
310 of plant roots throughout the growth period.

311 Plant height and biomass, shoot/root biomass ratio, and relative growth rate related to height  
312 after 12 weeks of growth are summarized in supplementary Table S2. Tukey's HSD test  
313 performed on data collected upon harvesting revealed that the shoot/root ratio did not  
314 significantly differ between soils and treatments. In contrast, addition of molybdate did interfere  
315 significantly with the plant height, total biomass and relative growth rate, but only with plants  
316 grown on EWK soil. After 12 weeks, *G. maxima* grown on EWK soils in the presence of molybdate

317 developed a smaller number of shoots, *i.e.* produced less biomass, but plants were slightly higher  
318 and had also a higher relative growth rate than in the absence of molybdate.

319

## 320 **Soil**

321 At the beginning of the experiment pore water pH was 7.2 and 5.9 and in EWK and HDD soils,  
322 respectively (Supplementary Table S1). After 12 weeks of plant growth pore water pH remained  
323 almost unchanged ( $7.2 \pm 0.3$ ) in the EWK microcosms, while pore water pH in the HDD  
324 microcosms had increased to  $7.4 \pm 0.3$ . The rhizosphere compartments in the microcosms  
325 displayed a pH slightly lower than the bulk soil compartments (EWK soil:  $7.1 \pm 0.0$  *vs*  $7.3 \pm 0.0$ ;  
326 HDD soil:  $7.3 \pm 0.0$  *vs*  $7.6 \pm 0.0$ ). Soil organic matter content slightly decreased both in EWK  
327 (10.5% to 9.9%) and in HDD microcosms (from 13.6% to 10.5%) after 12 weeks of incubation.  
328 EWK soil showed in general lower pore water ammonium concentrations during the course of the  
329 experiment compared to HDD soil. An increase in ammonium was registered during the first 4  
330 weeks in all systems, reaching a maximum value of 1.8 mM (EWK soil) and 2.8 mM (HDD soil),  
331 respectively. In the second part of the period of exponential shoot growth, ammonium  
332 concentrations started to vary considerably between microcosm and compartments of both soils,  
333 *i.e.* decreasing first in the absence of molybdate and in the rhizosphere (data not shown). Pore  
334 water nitrate concentrations rapidly decreased during the first 5 weeks with nitrate  
335 concentrations always lower in the presence of molybdate compared to its absence. After 5  
336 weeks nitrate remained constant in the range of  $0.1\text{-}0.5 \mu\text{g ml}^{-1}$  in both soils under all incubations  
337 (data not shown). Phosphate concentrations showed similar trends for EWK and HDD microcosms,  
338 *i.e.* decreasing over time, in particular during the first 5 weeks (data not shown). The decrease  
339 was most pronounced in the EWK soil irrespective of the presence or absence of molybdate. Pore  
340 water phosphate concentrations were always lower in the bulk soil compared to the rhizosphere  
341 soil.

342 Pore water methanol, ethanol, acetate and propionate concentrations at the end of the first week  
343 of incubation are shown in Figure 1. In general, EWK soil contained less of these small organic

344 compounds, except for ethanol in the presence of molybdate. Ethanol was never detected in the  
345 absence of molybdate. Surprisingly, methanol was observed in HDD soils in the presence of  
346 molybdate. No measurements of concentrations of alcohols and volatile fatty acids were done  
347 between the first and last week of the experiment. At the end they were all below the level of  
348 detection.

349

### 350 **SRM abundances and sulfate reduction rates**

351 The abundances of culturable SRM in the different compartments of the microcosms, as analyzed  
352 by most probable number (MPN) enumerations in microtiter plates, are presented in Table 1. In  
353 both soils, SRM densities at the end of the 12 weeks of incubation were significantly ( $p < 0.05$ )  
354 different between the absence and presence of molybdate (Tukey's HSD test) with several orders  
355 of magnitude lower numbers in its presence. In the absence of molybdate, numbers of culturable  
356 SRM were significant larger in the HDD soil. No significant differences were observed between  
357 rhizosphere and corresponding bulk soils.

358 Sulfate reduction occurred in EWK and HDD soils, both in the rhizosphere and bulk soil with  
359 values ranging from 0.4 to 1.4  $\mu\text{mol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$  (Figure 2). A statistically significant difference  
360 in sulfate reduction rates was only observed between the presence and absence of molybdate in  
361 both soils at all depths. Sulfate reduction rates were not significantly different between depths  
362 and between soils/compartments at all depths, except for the rhizosphere and the bulk soil of the  
363 upper layer of the EWK soil in the absence of molybdate, where the rates were significantly lower  
364 compared to the other layers of the EWK soil under the same conditions (Tukey's HSD test). Both  
365 abundance estimation and sulfate reduction activity data confirmed that the sulfate-reducing  
366 community was efficiently impaired by molybdate.

367

### 368 **SRM diversity assessment in EWK and HDD microcosms**

369 The SRM community composition in the two soils/compartments was investigated using  
370 denaturing gradient gel electrophoresis of *dsrB* amplicons. A total of 27 and 24 distinct

371 matches/sequences were recognized in gels of EWK and HDD, respectively, and considered for  
372 further phylogenetic and statistical analyses. All *dsrB* sequences with amino acid sequence  
373 identities equal to or greater than 97% were grouped into an operational taxonomic unit (OTU).  
374 This grouping produced a total of 10 OTUs (Table 2); 6 (OTU01-06) were present in both soils,  
375 while the remaining OTUs contained *dsrB* sequences that were found exclusively at HDD (OTU07)  
376 or EWK (OTU08-10).

377 The phylogenetic affiliations of deduced *dsrAB* sequences are shown in Figure 3 and in Table 2.  
378 The largest operational taxonomic unit, OTU01 comprising 12 sequences, formed a separate  
379 branch within the orthologues *Firmicutes*. OTU02 consisting of 9 sequences formed a  
380 monophyletic group with cultured representatives of the family *Synthrophobacteraceae*. OTU03,  
381 OTU05 and OTU07 with 8, 7 and 1 sequence in HDD, respectively, displayed high sequence  
382 identity with cultured and uncultured *Desulfotomaculum* species in the xenologues *Firmicutes*.  
383 The 7 sequences in OTU04 clustered with members of the *Desulfobulbacea*. OTU06 with 3  
384 sequences showed similarity with cultured representatives of the *Desulfovibrionaceae*, whereas  
385 OTU08 with 2 sequences only in EWK grouped within the *Desulfobacteraceae*. OTU09 and OTU10  
386 contained each 1 sequence derived from EWK microcosms. These latter OTUs formed an  
387 independent cluster deeply branching in the *Deltaproteobacteria* and different from any cultured  
388 SRM lineage (Figure 3).

389 In EWK microcosms, OTU02 and OTU05 were present irrespective of the absence or presence of  
390 molybdate, whereas OTU01, OTU03, OTU09 (only bulk soil) and OTU10 (only rhizosphere soil)  
391 were only present in presence of this inhibitor of sulfate reduction. OTU04 and OTU06 (only  
392 rhizosphere soil) were only present in the absence of molybdate. In HDD microcosms, the  
393 presence of OTU01, OTU02, OTU03 and OTU05 was independent of the absence or presence of  
394 molybdate. OTU07 was only present in the rhizosphere of HDD soil in the presence of molybdate,  
395 while OTU04 and OTU06 (only rhizosphere soil) were only detected in the absence of molybdate.  
396 Hence, OTU04 and OTU06 were favored in particular under conditions of active sulfate reduction,



397 *i.e.* in the absence of molybdate. In the case of OTU06 this stimulation in the absence of  
398 molybdate was only observed in the rhizosphere soils.  
399 DGGE profiles for EWK and HDD microcosms are depicted in Figures 4 and 5, respectively.  
400 Analysis of similarity (ANOSIM) between DGGE profiles of one soil showed that profiles did not  
401 significantly differ between compartments (data not shown). Instead a highly significant ( $p =$   
402 0.002) difference was highlighted between the absence and presence of molybdate (EWK,  $R =$   
403 0.993; HDD,  $R = 0.857$ ). However, OTUs occurrence was not uniform (Table 2).  
404 Whereas most OTUs were observed both in the rhizosphere and the bulk soil, the OTUs with  
405 smaller numbers of sequences were observed only in the rhizosphere soil, *i.e.* OTU06, OTU07  
406 (only in HDD microcosms) and OTU10 (only in EWK microcosms), or in the bulk soil, *i.e.* OTU09.  
407 Indicated in Figures 4 and 5, and also shown in Table 3, are the bands only observed in the  
408 absence of molybdate. They contain sequences mostly related to *Desulfobulbus rhabdoformis*  
409 (bands EWK01 and HDD01; OTU04), *Desulfovibrio magneticus* (band EWK02; OTU02),  
410 *Desulfacinum infernum* (band EWK03; OTU02), *Desulfonema limicola* (band EWK04; OTU08),  
411 and *Desulfovibrio longus* (bands EWK05, EWK06 and HDD02; OTU06).

412

## 413 **DISCUSSION**

414

### 415 **Plant effect on the SRM community**

416 We hypothesized that the root-driven processes such as the release of oxygen and labile organic  
417 carbon compounds would affect the community of SRM in the rhizosphere of the aerenchymatous  
418 helophyte *G. maxima*. In the absence of molybdate, a known inhibitor of microbial sulfate  
419 reduction, MPN numbers of sulfate reducing microorganisms and sulfate reduction rates were not  
420 significantly different between the rhizosphere and the bulk soil (Table 2 and Fig. 3, respectively).  
421 Numbers and rates in the absence of molybdate were on average 360 and 26 times, respectively,  
422 higher than in its presence. The presence of sulfate-rich floodwater without molybdate had a  
423 significant effect on the size and activity of the community of SRM in our microcosms, but the

424 influence of the plant itself as inferred from differences between rhizosphere and bulk soil was  
425 rather limited. It must be kept in mind that a part of the outer compartment of the microcosms  
426 defined as bulk soil is in direct contact with the roots occupying the inner compartments, only  
427 separated by impervious nylon gauze. Therefore the bulk compartment must be considered a  
428 mixture between the rhizosphere and the real bulk soil not influenced by plant roots. In addition,  
429 solutes may flow easily between the rhizosphere and bulk soil compartments in the flooded  
430 microcosms.

431 The SRM community was analyzed by means of DGGE profiling of the *dsrB* gene. Statistical  
432 analyses of DGGE profiles of soil DNA extracts also did not show significant differences between  
433 rhizosphere and bulk soil in both EWK and HDD microcosms in the absence of molybdate. In part,  
434 the absence of significant differences might be due to the fact that the ANOSIM analyses were  
435 carried out on DGGE profiles of soil DNA extracts that provide a screenshot of the total SRM  
436 community but do not allow a distinction between metabolically active and inactive populations.  
437 Furthermore, we may have missed a number of SRM that do not match the primers used  
438 (Kjeldsen et al. 2009). Nevertheless, by comparing the DGGE profiles of rhizosphere and bulk soil  
439 in the absence or presence of molybdate, we found that 26% of the EWK- and 25% of HDD-  
440 derived sequences occurred only in the absence of molybdate indicating a selective enrichment of  
441 the corresponding SRM under the conditions of sulfate reduction. In some cases the changes  
442 were only found in the rhizosphere compartments hinting to a direct plant effect. The emergence  
443 of new sequences encompass the *Desulfobulbus rhabdoformis*-related bands EWK01 (Figure 4)  
444 and HDD01 (Figure 5) both part of OTU04, the *Desulfovibrio longus*-related and rhizosphere-  
445 specific bands EWK05, EWK06 and HDD02 belonging to OTU06, and the *Desulfonema limicola*-  
446 related and rhizosphere-specific band EWK04 of OTU08. The appearance of *D. longus*- and *D.*  
447 *limicola*-related sequences in the rhizosphere compartments in the absence of molybdate seems  
448 to be the only sign of a plant effect on the community of SRM. The occurrence of members of the  
449 *Desulfobulbaceae*, *Desulfovibrionaceae* and *Desulfobacteraceae* has been described in paddy soils  
450 (Stubner 2004) and in *Spartina alterniflora*-dominated salt marshes (Hines et al. 1999). The

451 *Desulfobulbaceae* and the *Desulfovibrionaceae* are incomplete oxidizers that mineralize a wide  
452 variety of carbon substrates to acetate (Rabus et al. 2006) and *Desulfobulbus propionicus* is able  
453 to gain energy from fermentation in the absence of sulfate (Laanbroek et al. 1982). *D. longus* is  
454 closely related to *Desulfovibrio carbinolicus*, the only sulfate-reducing bacterium able to oxidize  
455 methanol (Joulian et al. 2001).

456 Also members of the family *Desulfobacteraceae* to which *D. limicola* belong, are metabolically  
457 versatile, but in addition they can oxidize acetate completely to CO<sub>2</sub> (Rabus et al. 2006). *D.*  
458 *limicola* is able to grow on larger fatty acids (Widdel 1980).

459

#### 460 **Soil effect on the SRM community**

461 By the potential interference between iron cycling and sulfate reduction, we hypothesized  
462 differences in SRM community characteristics between the iron-poor EWK soil and the iron-rich  
463 HDD soil. Whereas the pore water pH differed remarkably before flooding, with pH 7.2 for EWK  
464 and pH 5.9 for HDD soil, the pH values of both soils were almost similar, with pH 7.2 and 7.4 for  
465 the EWK and HDD soils, respectively, after 12 weeks of flooding. An increase of pH is common  
466 for acid soils upon flooding unless they are poor in active iron (Ponnamperuma 1972). Hence, the  
467 increase in pH in flooded HDD soil may indicate active iron reduction. However, the sulfate  
468 reduction rates observed were not significantly different between both soils (Fig. 3). Similar  
469 sulfate reduction rates in the presence of different iron reduction rates might be explained by the  
470 presence of different amounts of suitable electron donors in both soils among which the redox-  
471 active organic matter that may be present in wetland soils (Heitmann and Blodau 2006;  
472 Heitmann et al. 2007). Soil organic matter content decreased slightly more in EWK (10.5% to  
473 9.9%) than in HDD microcosms (from 13.6% to 10.5%) after 12 weeks of incubation. The  
474 measured sulfate reduction rates of 0.35 – 1.40 μmol cm<sup>-3</sup> d<sup>-1</sup> were at the higher end of the  
475 range of sulfate reduction rates observed in freshwater ecosystems summarized by (Pester et al.  
476 2012) and comparable with rates measured in 2 Appalachian peatland bogs (Wieder et al. 1990)  
477 and in littoral sediment of Lake Constance (Bak and Pfennig 1991).

478 MPN numbers of SRM observed after 12 weeks of flooding in the presence of molybdate are of  
479 the same size for both soils (Table 1) and of the same order of magnitude as determined in dry  
480 paddy soils by (Wind and Conrad 1995). However, the increased MPN numbers we found after 12  
481 weeks of flooding in the absence of molybdate were at the same higher level as found by these  
482 authors in planted and dynamically sulfur-recycling paddy soils that had been flooded with tap  
483 water for 13 weeks. Assuming that numbers observed in the dry soils of (Wind and Conrad 1995)  
484 are also indicative for the size of the SRM community in our soils (as numbers in the presence of  
485 molybdate remained at the same level), it can be concluded that flooding with sulfate-rich water  
486 stimulated the growth of SRM in our experiment in the absence of molybdate.

487 With respect to the diversity of the communities of SRM, large differences were also not observed  
488 between the incubated EWK and HDD soils; 74% of EWK- and 75% of HDD-derived sequences  
489 were ubiquitous among both soils or were present only in the presence of molybdate.

490 Interestingly, these include all bands related to the orthologues/xenologues *Firmicutes* (OTU01,  
491 OTU03, OTU05, OTU07, OTU09, and OTU10) and the *Syntrophobacteraceae*-related OTU02  
492 (Figure 3 and Table 3). The relatively high number and widespread distribution of *Firmicutes*-  
493 related *dsrB* found in microcosms filled with EWK and HDD soils might be explained by the  
494 capability of members of this group to produce spores in order to survive situations of  
495 environmental stress (Widdel 2006) such as the alternating oxic and anoxic conditions that  
496 depend on the water table fluctuations in the floodplains. The occurrence of the spore-forming  
497 *Desulfotomaculum* has been reported in similar variable environments such as rice paddies  
498 (Stubner and Meuser 2000). *Syntrophobacter* species have also been described as one of the  
499 predominant SRM groups in paddy soil studies (Scheid and Stubner 2001; Stubner 2004). These  
500 organisms do not form spores, but have extremely diverse nutritional capabilities being able to  
501 use a wide variety of electron donors and carbon sources, syntrophically or in pure cultures and  
502 in the presence or absence of sulfate as electron acceptor (Harmsen et al. 1998). This metabolic  
503 peculiarity confers these microorganisms adaptability to changing environmental conditions, and  
504 could explain the ubiquitous presence of *Syntrophobacter*-related *dsrB* genes in EWK and HDD

505 floodplains and microcosms, whether sulfate reduction is inhibited or not. In relation to this,  
506 drying of the floodplain soils before the start of the experiment may also have changed the SRM  
507 community composition.

508

### 509 **Fermentation intermediates**

510 In the presence of molybdate, alcohols and volatile fatty acids were observed in the microcosms  
511 filled with soil after one week of flooding with sulfate-rich water, although to a lower extent in  
512 EWK soil where also no methanol was found. (Parkes et al. 1989) observed also the accumulation  
513 of specifically acetate when they treated estuarine and marine sediments with molybdate. We do  
514 not know the origin of the methanol in our samples, but the other compounds are most likely  
515 fermentation products indicating the presence of actively fermenting microorganisms in the  
516 flooded soils. In the absence of molybdate, we did not observe the presence of alcohols, but the  
517 amounts of acetate were higher compared to the presence of the inhibitor. This was most evident  
518 in the microcosms filled with HDD soil. The change in fermentation products suggests ethanol  
519 oxidation by SRM in the absence of molybdate with acetate as the main product. Acetate  
520 accumulation from ethanol has been demonstrated before in mixed cultures of SRM capable of  
521 complete or incomplete oxidation of this alcohol (Laanbroek et al. 1984), but this was under  
522 sulfate-limited conditions. A number of sulfate-reducing microorganisms are able to consume  
523 hydrogen under sulfate-reducing conditions and may facilitate in this way the divergence of  
524 fermentative pathways to more oxidized products such as acetate (Plugge et al. 2011). The  
525 detection of fermentation products in the first week of the experiment only may have been  
526 caused by a slower start of the complete carbon-oxidizing SRM compared to incomplete oxidizing  
527 sulfate reducers leading to the temporary accumulation of these compounds as has been  
528 observed in arctic marine sediments (Finke et al. 2007).

529 The presence of alcohols and volatile fatty acids, especially in the microcosms filled with HDD soil,  
530 may also have been the reason for poorer plant growth. In reed (*Phragmites australis*) higher  
531 concentrations of acetic acid, but also of dissolved sulfide in the soil may lead to stunted

532 adventitious roots and laterals, callus blockages of the gas-pathways, and vascular blockages  
533 (Armstrong et al. 1996). Ethanol was also present in the microcosms filled with EWK soil in the  
534 presence of molybdate, but to a lesser extent than in the HDD microcosms (Fig. 2). The  
535 increased amounts of alcohols and volatile fatty acids might have been responsible for the  
536 retarded start of plant growth in the microcosms filled with HDD soil and in the EWK microcosms  
537 in the presence of molybdate. At the end of the incubation period all fermentation products were  
538 below the level of detection and likely less toxic to the plants.

539

#### 540 **Plant performance**

541 Like sulfate and phosphate, molybdate belongs to the group VI oxyanions and may therefore  
542 interfere with the sulfate and phosphate metabolism of plants. (McGrath et al. 2010) determined  
543 the threshold values of molybdenum toxicity for plant species in an experiment comprising 10  
544 different soils and 4 different plant species. Among the plant species tested, toxicity thresholds  
545 based on soil-added molybdenum solutions were higher for Rye grass (*Lolium perenne*) than for  
546 the tested dicotyledonous species on all the soils, indicating that shoot growth of Rye grass was  
547 less sensitive to molybdenum toxicity than the growth of the other plant species. The authors  
548 showed also that soil properties strongly affect the expression of molybdenum toxicity in the  
549 plant species. Toxicity threshold values were generally higher in acid (pH < 6.5) than in neutral  
550 and basic soils (pH > 6.5), indicating that molybdenum is less toxic to higher plants grown on  
551 acid soils. Selecting the two soils from the list of (McGrath et al. 2010) that are most comparable  
552 to our soils and looking to Rye grass, a concentration of 2.5 mM molybdenum as applied by us  
553 could have caused a 50% reduction in shoot yield after a growing period of three weeks. The  
554 plants in the analysis of McGrath and colleagues were more affected by molybdenum in  
555 calcareous than in acidic soils, which possibly agrees with our observation of a larger effect of  
556 molybdate addition in the more calcareous EWK soil. With *G. maxima*, we observed 29%  
557 reduction in biomass, but an increase of 26% in average height in EWK soils in the presence of  
558 molybdate. In HDD soils, these percentages were 8 and 0%, respectively. Since, no effect of

559 molybdate addition was observed the analyzed elements in the plant tissues, we may conclude  
560 that the presence of 2.5 mM of this anion on plant performance was minimal.

561

## 562 **Conclusions**

563 Although the original EWK and HDD soils were dry and oxidized at the time of sampling, they had  
564 the potential for active sulfate reduction when the conditions became favorable for SRM, *i.e.*  
565 flooding and non-limiting amounts of organic matter and sulfate. However, an effect of the  
566 presence of the aerenchymatous helophyte *G. maxima* was barely noticed as the phenomena  
567 related to active sulfate reduction such as increased reduction rates and numbers were hardly  
568 different between the rhizosphere and bulk soil compartments of the microcosms in which the  
569 plant were grown. Active sulfate reduction as inferred from increased rates and numbers was  
570 coupled to changes in SRM community composition, but these changes were rather limited. The  
571 emergence of *Desulfovibrionaceae*-related sequences exclusively in the rhizosphere of *G. maxima*  
572 grown on both soils suggests that this group is best adapted to the conditions created by the  
573 plants. Large differences in relation to the activity and composition of the SRM community  
574 between the iron-rich HDD soil en the iron-poor EWK soil were never observed, implying that iron  
575 biogeochemistry did not interfere with sulfate reduction to a large extent.

576

## 577 **ACKNOWLEDGEMENTS**

578 The authors are grateful to Maaïke Blauw, Fulvia Bracco and Miranda Kamst-van Agterveld for  
579 assistance during the microcosm experiment. Thanks also to Wim van Doesburg, Paul van der  
580 Ven and Thilo Behrends for their help with pore water analyses. Martijn Antheunisse, Roos Loeb,  
581 Leon Lamers and Caroline Plugge are acknowledged for stimulating discussions. We also want to  
582 acknowledge the valuable contribution of two unknown reviewers to the content of the paper.  
583 The Netherlands Organization for Scientific Research (NWO) funded this research. It was part of  
584 the TRIAS project 'Biogeochemical constraints for sustainable development of floodplains in

585 riverine regions' (835.80.010, research area 'Ecology and Soil Quality', theme 'Space for the  
586 River'). This is the publication no. xxxx of the Netherlands Institute of Ecology (NIOO-KNAW).  
587



588 TABLE 1: MPN counts of sulfate-reducing microorganisms (number of viable cells per gram of  
 589 dry soil) in the rhizosphere and bulk soil compartments of Ewijk (EWK) and Huis den Doorn (HDD)  
 590 microcosms. Values are means  $\pm$  SD of 3 replicate microcosms. Data followed by different  
 591 characters are significantly different from each other according to Tukey's test ( $p < 0.05$ ).  
 592

Soil	Presence of molybdate	Rhizosphere soil	Bulk soil
EWK	-	$6.3 \times 10^6 \pm 1.1 \times 10^4$ b	$5.9 \times 10^6 \pm 4.4 \times 10^3$ b
EWK	+	$2.0 \times 10^4 \pm 3.9 \times 10^3$ a	$2.1 \times 10^4 \pm 5.9 \times 10^3$ a
HDD	-	$8.5 \times 10^6 \pm 2.6 \times 10^4$ c	$8.4 \times 10^6 \pm 1.1 \times 10^4$ c
HDD	+	$2.0 \times 10^4 \pm 4.0 \times 10^3$ a	$2.1 \times 10^4 \pm 4.0 \times 10^3$ a

593 Table 2: OTUs of sulfate-reducing microorganisms based on comparative sequence analyses  
 594 of *dsrB* retrieved from Huis den Doorn (HDD) and Ewijk (EWK) microcosms  
 595

OTU <sup>a</sup>	Number of DGGE bands <sup>b</sup>		Occurrence <sup>c</sup>		Inferred phylogeny <sup>d</sup>
	EWK	HDD	EWK	HDD	
<b>01</b>	8	4	+Mo	I	orthologues <i>Firmicutes</i>
<b>02</b>	5	4	I	I	<i>Syntrophobacteraceae</i>
<b>03</b>	2	6	+Mo	I	xenologues Firmicutes
<b>04</b>	3	4	-Mo	-Mo	<i>Desulfobulbaceae</i>
<b>05</b>	3	4	I	I	xenologues Firmicutes
<b>06</b>	2	1	-Mo (R)	-Mo (R)	<i>Desulfovibrionaceae</i>
<b>07</b>	0	1	-	+Mo (R)	xenologues Firmicutes
<b>08</b>	2	0	-Mo	-	<i>Desulfobacteriaceae</i>
<b>09</b>	1	0	+Mo (B)	-	Deltaproteobacteria
<b>10</b>	1	0	+Mo (R)	-	Deltaproteobacteria

596

597 <sup>a</sup> *dsrB* sequences with deduced DsrAB sequence identity equal to or greater than 97% were  
 598 grouped in an OTU. OTUs were sequentially numbered according to the total number of  
 599 bands

600 <sup>b</sup> Based on one sequence per match, as determined by Phoretics analyses of DGGE gels

601 <sup>c</sup> Occurrence based on detection in the absence (-Mo) or presence (+Mo) of molybdate; I =  
 602 independent of +Mo or -Mo, (R) = only rhizosphere soil, (B) = only bulk soil

603 <sup>d</sup> Phylogeny of *dsrB* sequences as inferred from Fig. 4 and based on 95 amino acid sequence  
 604 positions

605

606 Table 3: Affiliation of the bands that appeared in the DGGE gels in the absence of molybdate.

607

DGGE band <sup>a</sup>	Accession number	OTU	Most similar <i>dsrB</i> sequence in GenBank		
			Species (Accession number)	Max. similarity	Reference
EWK01	AM901659	04	<i>Desulfobulbus rhabdoformis</i> (AY015603)	92%	(Chang et al. 2001)
EWK02	AM901655	02	<i>Desulfovibrio magneticus</i> (AP010904)	76%	(Nakazawa et al. 2009)
EWK03	AM901657	02	<i>Desulfacinum infernum</i> (AF482454)	100%	(Zverlov et al. 2005)
EWK04	AM901667	08	<i>Desulfonema limicola</i> (AY753100)	90%	(Madrid et al. 2006)
EWK05	AM901663	06	<i>Desulfovibrio longus</i> (GQ324675)	94%	(Joulian et al. 2001)
EWK06	AM901665	06	<i>Desulfovibrio longus</i> (GQ324675)	94%	(Joulian et al. 2001)
HDD01	AM901661	04	<i>Desulfobulbus rhabdoformis</i> (AY015603)	92%	(Chang et al. 2001)
HDD02	AM901666	06	<i>Desulfovibrio longus</i> (GQ324675)	94%	(Joulian et al. 2001)

608 <sup>a</sup> EWK and HDD bands indicated in Figures 5 and 6, respectively

609

610

611

612 **Legend to the figures**

613

614

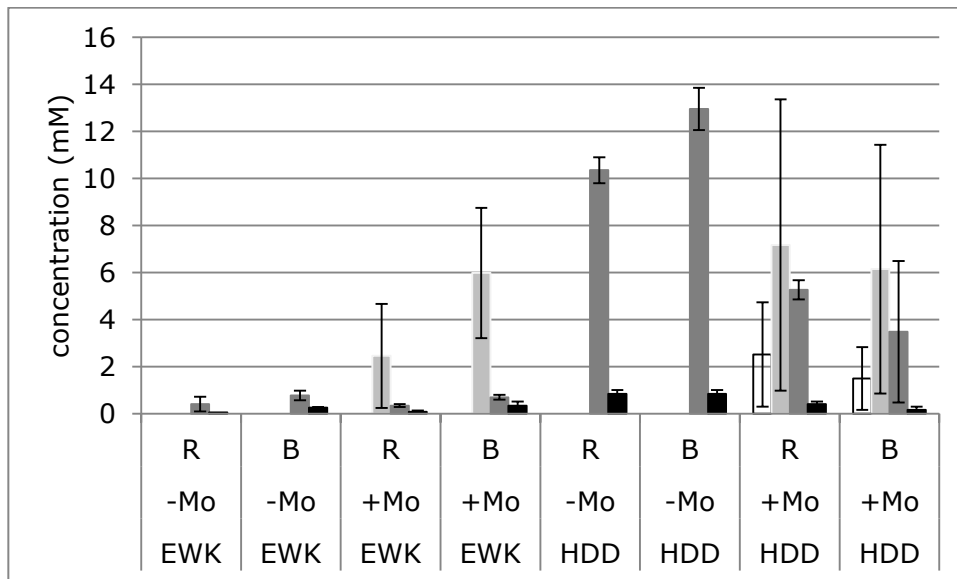
615 Figure 1: Concentrations of pore water methanol (white bars), ethanol (light grey bars),

616 acetate (dark grey bars) and propionate (black bars) at the end of the first week of the

617 incubation period. R = rhizosphere soil, B = bulk soil, -Mo = absence of molybdate, +Mo =

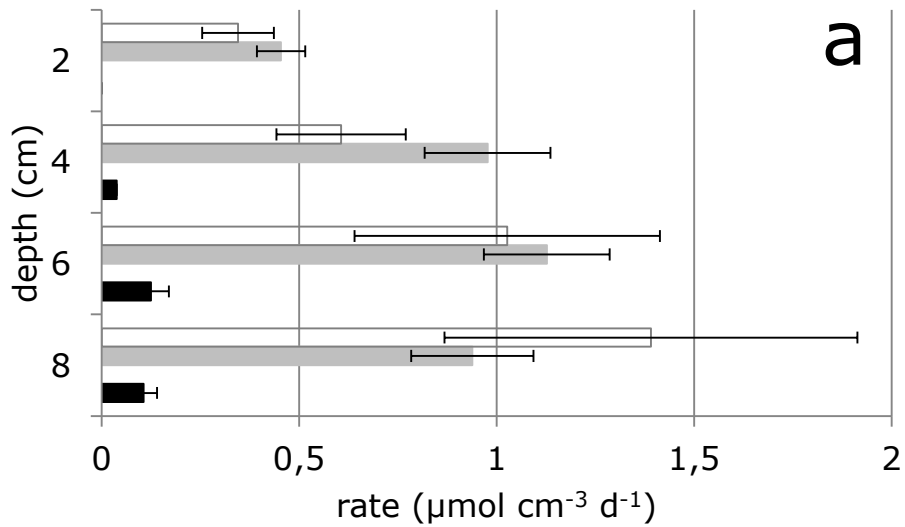
618 presence of molybdate, EWK = Ewijk soil, HDD = Huis den Doorn soil.

619

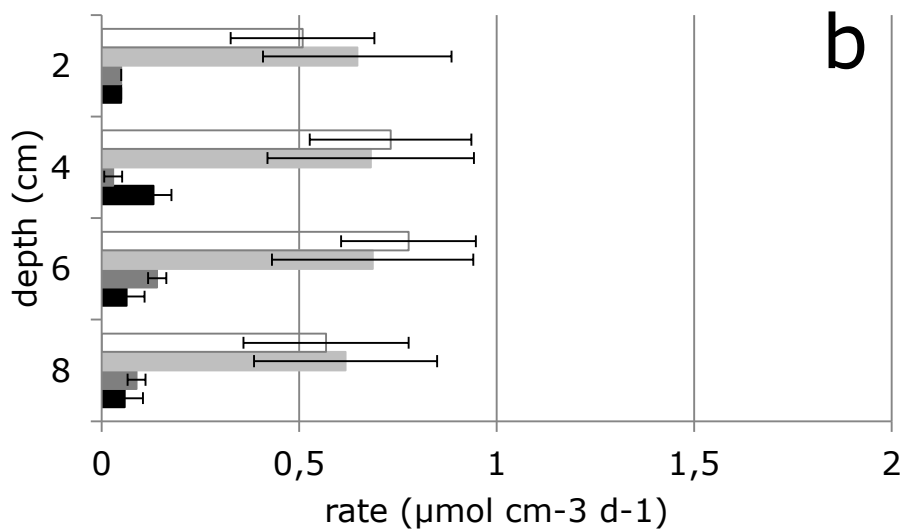


620

621 Figure 2: Depth profiles of sulfate reduction rates in (a) Ewijk (EWK) and (b) Huis den Doorn  
 622 (HDD) microcosms in the rhizosphere in the absence of molybdate (white bars), in the bulk  
 623 soil in the absence of molybdate (light grey bars), in the rhizosphere in the presence of  
 624 molybdate (dark grey bars), and in the bulk soil in the presence of molybdate (black bars).  
 625 Values are means  $\pm$  SD of 4 (rhizosphere) or 6 (bulk soil) replicate samples.  
 626



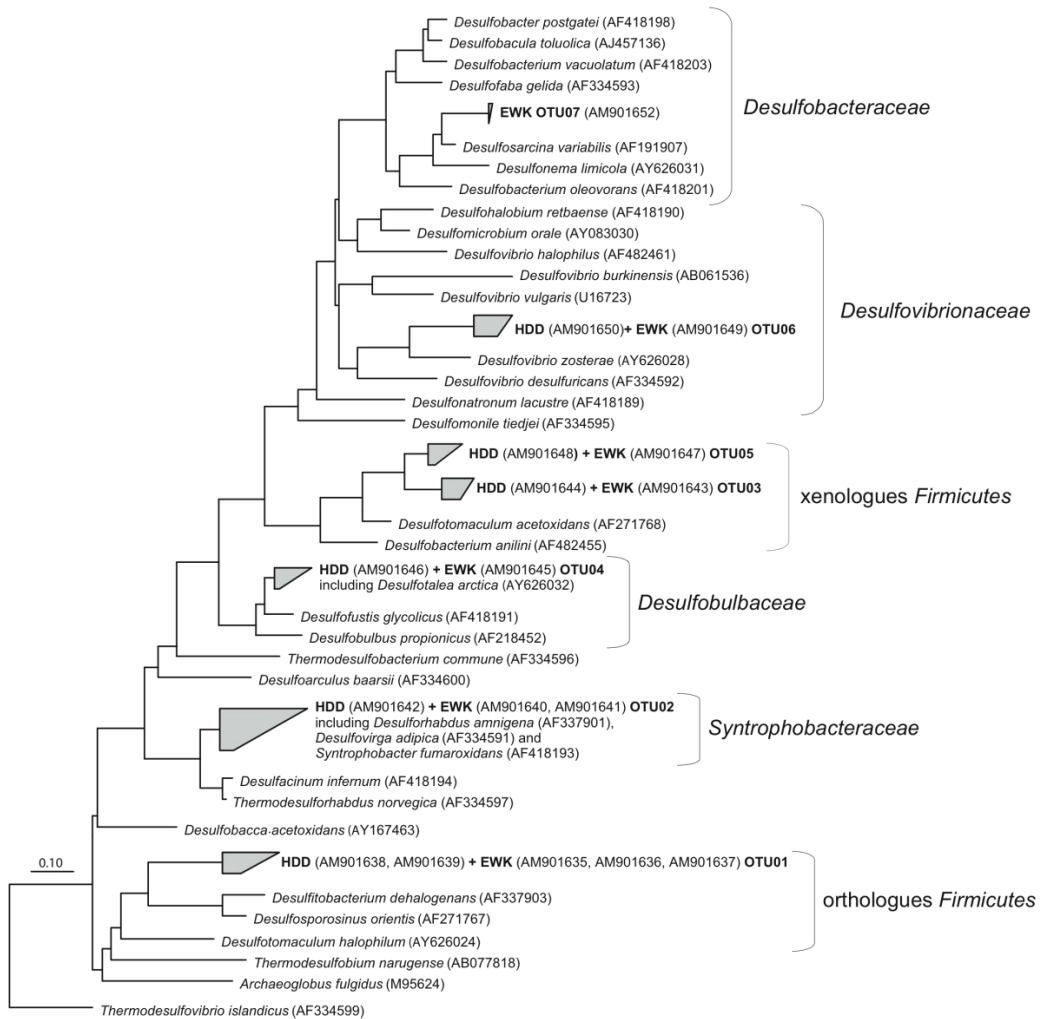
627



628

629 Figure 3: DSR phylogenetic tree showing the affiliation of Ewijk (EWK) and Huis den Doorn  
 630 (HDD) OTUs with known sulfate-reducing microorganisms. Partial *dsrB* sequences were  
 631 inserted one by one into a DSR core tree using the ARB parsimony tool (see text). Nucleotide  
 632 sequence accession numbers are given in parentheses. OTU02 includes bands EWK02  
 633 (AM901655) and EWK03 (AM901657); OTU04 includes bands EWK01 (AM901659) and HDD01  
 634 (AM901661); OTU06 includes bands EWK05 (AM901663), EWK06 (AM901665) and HDD2  
 635 (AM901666); OTU08 includes band EWK4 (AM901667). Bar indicates 10% sequence  
 636 divergence. Large ●, rhizosphere in the absence of molybdate; large ○, bulk soil in the  
 637 absence of molybdate; small ●, rhizosphere in the presence of molybdate; small ○, bulk soil  
 638 in the presence of molybdate.

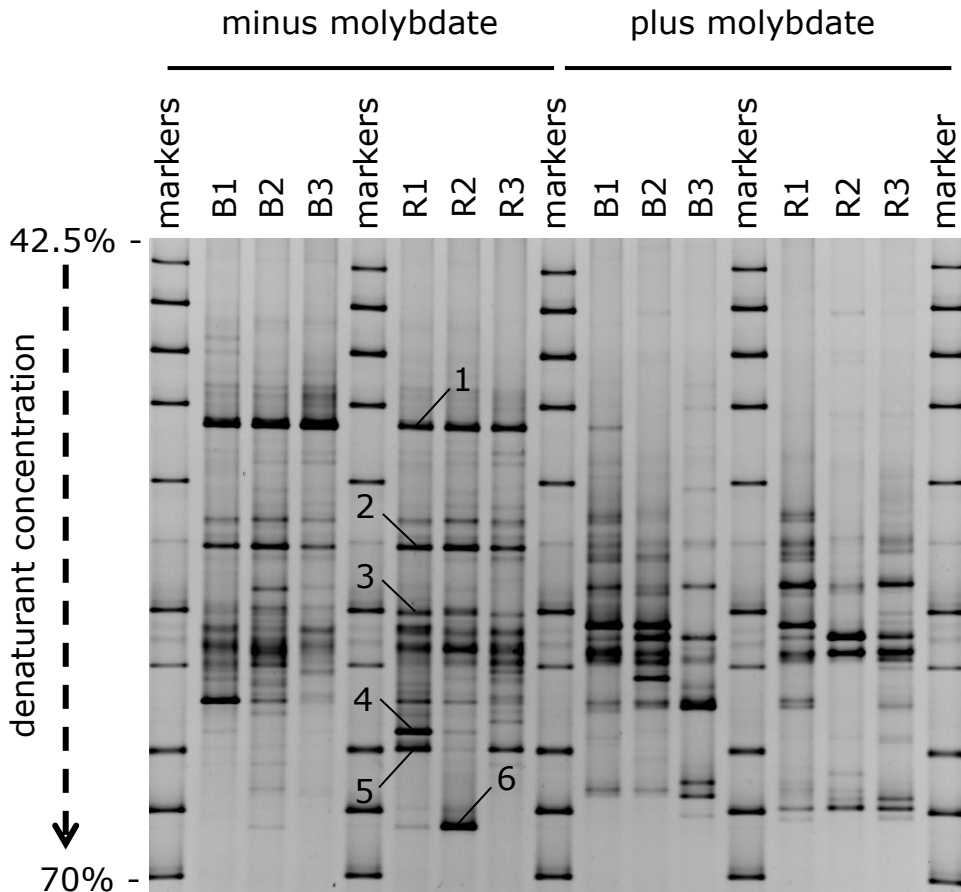
639



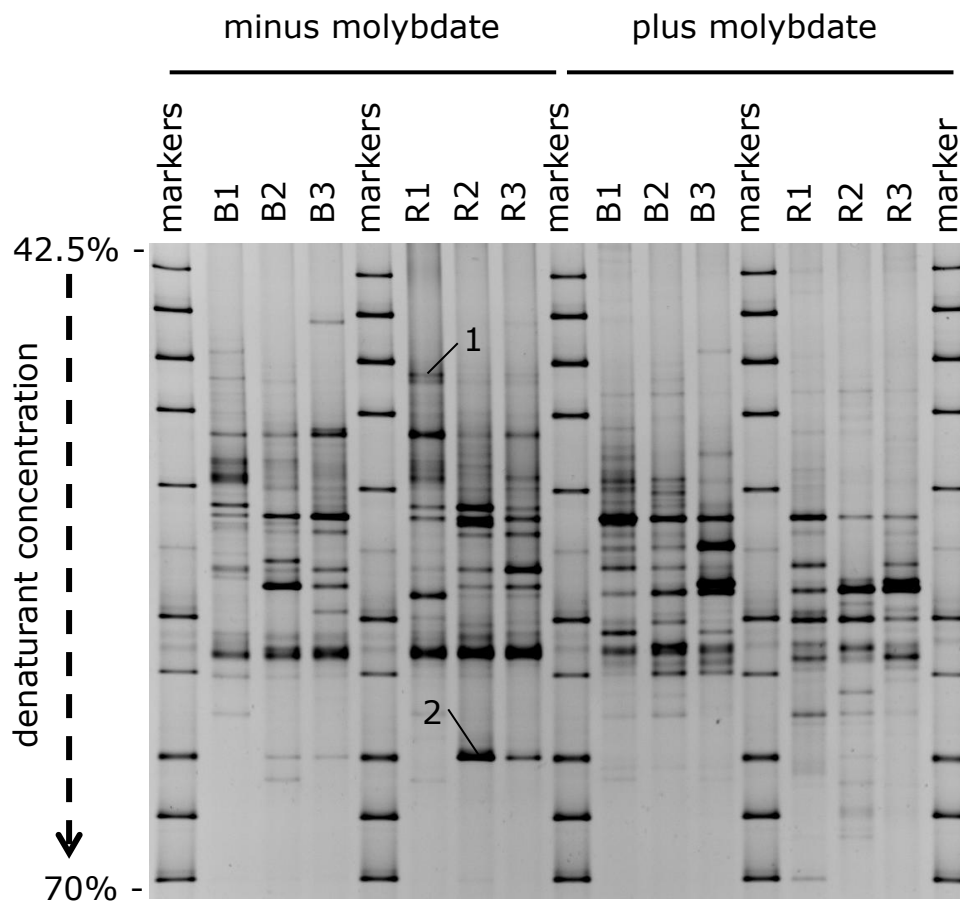
640

641 Figure 4: Denaturing gradient gel electrophoresis (DGGE) of *dsrB* PCR products amplified  
642 from EWK microcosms. Lanes coded B1-3 and R1-3 indicate samples collected from replicate  
643 bulk soil and rhizosphere samples, respectively. Bands 1-6 occurring only in the absence of  
644 molybdate are highlighted. *dsrB* DGGE marker lanes are indicated.

645  
646  
647



648 Figure 5: Denaturing gradient gel electrophoresis (DGGE) of *dsrB* PCR products amplified  
649 from Huis den Doorn microcosms. Lanes coded B1-3 and R1-3 indicate samples collected  
650 from replicate bulk soil and rhizosphere samples, respectively. Bands 1-2 occurring only in the  
651 rhizosphere of the treatment microcosms are highlighted. *dsrB* DGGE marker lanes are  
652 indicated.  
653





654 **REFERENCES**

- 655  
656 Armstrong J, AfreenZobayed F and Armstrong W 1996 Phragmites die-back: Sulphide- and acetic acid-  
657 induced bud and root death, lignifications, and blockages within aeration and vascular systems.  
658 *New Phytol.* 134, 601-614.
- 659 Bahr M, Crump B C, Klepac-Ceraj V, Teske A, Sogin M L and Hobbie J E 2005 Molecular characterization  
660 of sulfate-reducing bacteria in a New England salt marsh. *Environ. Microbiol.* 7, 1175-1185.
- 661 Bak F and Pfennig N 1991 Sulfate-reducing bacteria in littoral sediment of Lake Constance. *Fems*  
662 *Microbiology Ecology* 85, 43-52.
- 663 Benson D A, Karsch-Mizrachi I, Lipman D J, Ostell J and Wheeler D L 2005 GenBank. *Nucleic Acids*  
664 *Research* 33, D34-D38.
- 665 Bodelier P L E, Duyts H, Blom C W P M and Laanbroek H J 1998 Interactions between nitrifying and  
666 denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *glyceria*  
667 *maxima*. *FEMS Microbiol. Ecol.* 25, 63-78.
- 668 Brändle R and Crawford R M M 1987 Rhizome anoxia tolerance and habitat specialization in wetland  
669 plants. In *Plant life in aquatic and amphibious habitats*. Ed. R M M Crawford. pp 397-410.  
670 Blackwell Scientific, Oxford.
- 671 Chang Y J, Peacock A D, Long P E, Stephen J R, McKinley J P, Macnaughton S J, Hussain A, Saxton A M  
672 and White D C 2001 Diversity and characterization of sulfate-reducing bacteria in groundwater  
673 at a uranium mill tailings site. *Applied and Environmental Microbiology* 67, 3149-3160.
- 674 Choi J H, Park S S and Jaffe P R 2006 The effect of emergent macrophytes on the dynamics of sulfur  
675 species and trace metals in wetland sediments. *Environ. Pollut.* 140, 286-293.
- 676 Cifuentes A, Anton J, de Wit R and Rodriguez-Valera F 2003 Diversity of Bacteria and Archaea in  
677 sulphate-reducing enrichment cultures inoculated from serial dilution of *Zostera noltii*  
678 rhizosphere samples. *Environ. Microbiol.* 5, 754-764.
- 679 Conrad R, Klose M, Noll M, Kemnitz D and Bodelier P L E 2008 Soil type links microbial colonization of  
680 rice roots to methane emission. *Glob. Change Biol.* 14, 657-669.
- 681 Finke N, Vandieken V and Jorgensen B B 2007 Acetate, lactate, propionate, and isobutyrate as electron  
682 donors for iron and sulfate reduction in Arctic marine sediments, Svalbard. *FEMS Microbiol.*  
683 *Ecol.* 59, 10-22.
- 684 Harmsen H J M, Van Kwijk B L M, Plugge C M, Akkermans A D L, De Vos W M and Stams A J M 1998  
685 *Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing  
686 bacterium. *Int. J. Syst. Bacteriol.* 48, 1383-1387.
- 687 Heitmann T and Blodau C 2006 Oxidation and incorporation of hydrogen sulfide by dissolved organic  
688 matter. *Chemical Geology* 235, 12-20.
- 689 Heitmann T, Goldhammer T, Beer J and Blodau C 2007 Electron transfer of dissolved organic matter and  
690 its potential significance for anaerobic respiration in a northern bog. *Global Change Biology* 13,  
691 1771-1785.
- 692 Hines M E, Evans R S, Genthner B R S, Willis S G, Friedman S, Rooney-Varga J N and Devereux R 1999  
693 Molecular phylogenetic and biogeochemical studies of sulfate-reducing bacteria in the  
694 rhizosphere of *Spartina alterniflora*. *Applied and Environmental Microbiology* 65, 2209-2216.
- 695 Holmkvist L, Ferdelman T G and Jorgensen B B 2011 A cryptic sulfur cycle driven by iron in the methane  
696 zone of marine sediment (Aarhus Bay, Denmark). *Geochim. Cosmochim. Acta* 75, 3581-3599.
- 697 Jorgensen B B 1978 Comparison of methods for the quantification of bacterial sulfate reduction in  
698 coastal marine-sediments .1. Measurement with radiotracer techniques. *Geomicrobiology*  
699 *Journal* 1, 11-27.
- 700 Joulain C, Ramsing N B and Ingvorsen K 2001 Congruent phylogenies of most common small-subunit  
701 rRNA and dissimilatory sulfite reductase gene sequences retrieved from estuarine sediments.  
702 *Applied and Environmental Microbiology* 67, 3314-3318.
- 703 Kallmeyer J, Ferdelman T G, Weber A, Fossing H and Jorgensen B B 2004 A cold chromium distillation  
704 procedure for radiolabeled sulfide applied to sulfate reduction measurements. *Limnology and*  
705 *Oceanography-Methods* 2, 171-180.
- 706 Kjeldsen K U, Tang L, Jorgensen M G and Ingvorsen K 2009 Enumeration and identification of dominant  
707 types of sulfate-reducing bacteria in pulp from a paper-recycling plant: a multiphasic approach.  
708 *FEMS Microbiol. Ecol.* 69, 481-493.
- 709 Kusel K, Pinkart H C, Drake H L and Devereux R 1999 Acetogenic and sulfate-reducing bacteria  
710 inhabiting the rhizoplane and deep cortex cells of the sea grass *Halodule wrightii*. *Applied and*  
711 *Environmental Microbiology* 65, 5117-5123.
- 712 Laanbroek H J 1990 Bacterial cycling of minerals that affect plant-growth in waterlogged soils - a review.  
713 *Aquat. Bot.* 38, 109-125.
- 714 Laanbroek H J 2010 Methane emission from natural wetlands: interplay between emergent macrophytes  
715 and soil microbial processes. A mini-review. *Ann. Bot.* 105, 141-153.

- 716 Laanbroek H J, Abee T and Voogd I L 1982 Alcohol conversions by *Desulfobulbus propionicus* Lindhorst  
717 in the presence and absence of sulfate and hydrogen. Arch. Microbiol. 133, 178-184.
- 718 Laanbroek H J, Geerligs H J, Sijtsma L and Veldkamp H 1984 Competition for sulfate and ethanol among  
719 *Desulfobacter*, *Desulfobulbus*, and *Desulfovibrio* species isolated from intertidal sediments.  
720 Appl. Environ. Microbiol. 47, 329-334.
- 721 Loeb R, Lamers L P M and Roelofs J G M 2008 Effects of winter versus summer flooding and subsequent  
722 desiccation on soil chemistry in a riverine hay meadow. Geoderma 145, 84-90.
- 723 Loy A, Kusel K, Lehner A, Drake H L and Wagner M 2004 Microarray and functional gene analyses of  
724 sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal cooccurrence of recognized  
725 genera and novel lineages. Applied And Environmental Microbiology 70, 6998-7009.
- 726 Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G,  
727 Forster W, Brettske I, Gerber S, Ginhart A W, Gross O, Grumann S, Hermann S, Jost R, Konig A,  
728 Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N,  
729 Vilbig A, Lenke M, Ludwig T, Bode A and Schleifer K H 2004 ARB: a software environment for  
730 sequence data. Nucleic Acids Research 32, 1363-1371.
- 731 Madrid V M, Aller R C, Aller J Y and Chistoserdov A Y 2006 Evidence of the activity of dissimilatory  
732 sulfate-reducing prokaryotes in nonsulfidogenic tropical mobile muds. FEMS Microbiol. Ecol. 57,  
733 169-181.
- 734 McGrath S P, Mico C, Curdy R and Zhao F J 2010 Predicting molybdenum toxicity to higher plants:  
735 Influence of soil properties. Environ. Pollut. 158, 3095-3102.
- 736 Miletto M, Bodelier P L E and Laanbroek H J 2007 Improved PCR-DGGE for high resolution diversity  
737 screening of complex sulfate-reducing prokaryotic communities in soils and sediments. J.  
738 Microbiol. Methods 70, 103-111.
- 739 Miletto M, Loeb R, Antheunisse A M, Bodelier P L E and Laanbroek H J 2010 Response of the sulfate-  
740 reducing community to the re-establishment of estuarine conditions in two contrasting soils: a  
741 mesocosm approach. Microb. Ecol. 59, 109-120.
- 742 Miletto M, Loy A, Antheunisse A M, Loeb R, Bodelier P L E and Laanbroek H J 2008 Biogeography of  
743 sulfate-reducing prokaryotes in river floodplains. FEMS Microbiol. Ecol. 64, 395-406.
- 744 Muyzer G and Stams A J M 2008 The ecology and biotechnology of sulphate-reducing bacteria. Nature  
745 Reviews Microbiology 6, 441-454.
- 746 Nakazawa H, Arakaki A, Narita-Yamada S, Yashiro I, Jinno K, Aoki N, Tsuruyama A, Okamura Y,  
747 Tanikawa S, Fujita N, Takeyama H and Matsunaga T 2009 Whole genome sequence of  
748 *Desulfovibrio magneticus* strain RS-1 revealed common gene clusters in magnetotactic bacteria.  
749 Genome Research 19, 1801-1808.
- 750 Nijburg J W, Coolen M J L, Gerards S, Gunnewiek P J A K and Laanbroek H J 1997 Effects of nitrate  
751 availability and the presence of glyceria maxima the composition and activity of the  
752 dissimilatory nitrate-reducing bacterial community. Appl. Environ. Microbiol. 63, 931-937.
- 753 Oremland R S and Capone D G 1988 Use of specific inhibitors in biogeochemistry and microbial ecology.  
754 Advances in Microbial Ecology 10, 285-383.
- 755 Parkes R J, Gibson G R, Muellerharvey I, Buckingham W J and Herbert R A 1989 Determination of the  
756 substrates for sulfate-reducing bacteria within marine and estuarine sediments with different  
757 rates of sulfate reduction. J. Gen. Microbiol. 135, 175-187.
- 758 Pester M, Knorr K-H, M.W. F, Wagner M and Loy A 2012 Sulfate-reducing microorganisms in wetlands -  
759 fameless actors in carbon cycling and climate change. In Frontiers in Microbiology.
- 760 Plugge C M, Zhang W, Scholten J C M and Stams A J M 2011 Metabolic flexibility of sulfate-reducing  
761 bacteria. Frontiers in Microbiology 2, Article 81.
- 762 Ponnamperuma F N 1972 The chemistry of submerged soils. In Advances in Agronomy. pp 29-96.
- 763 Rabus R, Hansen T and Widdel F 2006 Dissimilatory sulfate- and sulfur-reducing prokaryotes. In The  
764 prokaryotes. Eds. M Dworkin, S Falkow, E Rosenberg, K H Schleifer and E Stackebrandt. pp  
765 659-768. Springer, New York, USA.
- 766 Rees A P T, Jenkins L E T, Smith A M and Wilson P M 1987 The metabolism of flood-tolerant plants. In  
767 Plant life in aquatic and amphibious habitats. Ed. R M M Crawford. pp 227-238. Blackwell  
768 Scientific Oxford.
- 769 Roden E E and Wetzel R G 1996 Organic carbon oxidation and suppression of methane production by  
770 microbial Fe(III) oxide reduction in vegetated and unvegetated freshwater wetland sediments.  
771 Limnology And Oceanography 41, 1733-1748.
- 772 Rowe R, Todd R and Waide J 1977 Microtechnique for most-probable-number analysis. Applied and  
773 Environmental Microbiology 33, 675-680.
- 774 Scheid D and Stubner S 2001 Structure and diversity of Gram-negative sulfate-reducing bacteria on rice  
775 roots. FEMS Microbiol. Ecol. 36, 175-183.
- 776 Smirnov N and Crawford R M M 1983 Variation in the structure and response to flooding of root  
777 aerenchyma in some wetland plants. Ann. Bot. 51, 237-249.

778 Smith S W, Overbeek R, Woese C R, Gilbert W and Gillevet P M 1994 The genetic data environment an  
779 expandable guide for multiple sequence-analysis. *Computer Applications in the Biosciences* 10,  
780 671-675.

781 Stubner S 2004 Quantification of Gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA  
782 gene-targeted real-time PCR. *J. Microbiol. Methods* 57, 219-230.

783 Stubner S and Meuser K 2000 Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S  
784 ribosomal nucleic acid analyses. *FEMS Microbiol. Ecol.* 34, 73-80.

785 Tabatabai M A 1974 Rapid method for determination of sulfate in water samples. *Environmental Letters*  
786 7, 237-243.

787 Widdel F 1980 Anaerober Abbau von Fettsäuren und Benzoessäure durch neu isolierte Arten sulfat-  
788 reduzierender Bakterien. p. 443. Georg-August-Universität Göttingen.

789 Widdel F 2006 The genus *Desulfotomaculum*. In *The prokaryotes*. Eds. M Dworkin, S Falkow, E  
790 Rosenberg, K H Schleifer and E Stackebrandt. pp 787-794. Springer New York, USA.

791 Wieder R K, Yavitt J B and Lang G E 1990 Methane production and sulfate reduction in 2 Appalachian  
792 peatlands. *Biogeochemistry* 10, 81-104.

793 Wind T and Conrad R 1995 Sulfur compounds, potential turnover of sulfate and thiosulfate, and  
794 numbers of sulfate-reducing bacteria in planted and unplanted paddy soil. *FEMS Microbiol. Ecol.*  
795 18, 257-266.

796 Wind T and Conrad R 1997 Localization of sulfate reduction in planted and unplanted rice field soil.  
797 *Biogeochemistry* 37, 253-278.

798 Zverlov V, Klein M, Lucker S, Friedrich M W, Kellermann J, Stahl D A, Loy A and Wagner M 2005 Lateral  
799 gene transfer of dissimilatory (bi)sulfite reductase revisited. *J. Bacteriol.* 187, 2203-2208.

800  
801  
802

803 TABLE S1: Initial chemical characteristics of Ewijk (EWK) and Huis den Doorn (HDD) soils

804

Soil	pH	OM (%)	Demineralized water extractable nutrients ( $\mu\text{g/g}$ )						KCl extractable nutrients ( $\mu\text{g/g}$ )			Total elements ( $\text{mg/g}$ )		
			$\text{Al}^{3+}$	$\text{Ca}^{2+}$	$\text{Fe}^{3+}$	$\text{K}^{+}$	$\text{Mg}^{2+}$	$\text{SO}_4^{2-}$	$\text{NO}_3^{-}$	$\text{NH}_4^{+}$	$\text{PO}_4^{3-}$	N	P	K
EW	7.	10.	0.2	254.	2.1	8.	9.9	63.9	168.	16.8	55.7	3.	1.	6.
K	5	5		8		1			5			3	0	8
HD	5.	13.	0.1	71.5	1.7	0.	1.4	58.5	107.	7.1	4.8	5.	1.	3.
D	9	6				5			1			1	4	0

805

806 Soil pH was determined in demineralized water suspensions using a standard pH meter (QIS  
807 Quality in Sensing, Oosterhout, The Netherlands). Organic matter content was determined by  
808 loss on ignition of dry ground soil at 550°C for 2.5 h.

809 Soil demineralized water extraction was performed as follows: 5 grams of dry soil were  
810 shaken for 1 hour in 100 ml of demineralized water, centrifuged for 4 minutes at 4000 rpm  
811 and filtered over a Whatman GF/C filter.  $\text{NH}_4^{+}$ ,  $\text{NO}_3^{-}$  and  $\text{PO}_4^{3-}$  concentrations in the filtrates  
812 were determined colorimetrically on a continuous flow analyzer (Skalar SA-40, Skalar, Breda,  
813 the Netherlands). Concentrations of  $\text{Al}^{3+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{K}^{+}$ ,  $\text{Mg}^{2+}$ , and  $\text{SO}_4^{2-}$  in the filtrate were  
814 analyzed by ICP-MS (X series, Thermo Electron Corporation, Marietta, Ohio) and ICP-OES  
815 (Spectroflame, VML2 and IRIS Interprid II XDL, Thermo Electron Corporation).

816 Concentrations of extractable ammonium and nitrate were determined by 0.2 M KCl  
817 extraction (Houba et al. 1989). Ten grams of dry soil was weighed, 100 ml of extraction  
818 solution was added and the sample was shaken for 1 h at 100 rpm. After centrifugation (4  
819 min 4000 rpm), the supernatant was filtered and stored at -20°C until analyses; the  
820 concentrations of  $\text{NH}_4^{+}$  and  $\text{NO}_3^{-}$  were determined colorimetrically on the continuous flow  
821 analyzer.

822 Total soil nitrogen, phosphorus and potassium concentrations were determined with a  
 823 salicylic acid thiosulphate modification of the Kjeldahl digestion method (Bremner and  
 824 Mulvaney 1982). N and P concentrations were determined colorimetrically on the continuous  
 825 flow analyzer and K concentrations by flame emission photometry (IL 543, Instrumentation  
 826 Laboratory, Lexington, MA, U.S.A.).

827 Available  $\alpha$ -phosphate was determined using the Bray P-2 method (Bray and Kurtz 1945).  
 828 Five grams of dry soil was extracted with a solution 0.03 M  $\text{NH}_4\text{F}$  and 0.1 M HCl, by shaking  
 829 the suspension for 20 minutes at 120 rpm. Phosphate concentrations were determined  
 830 colorimetrically on a spectrophotometer (Shimadzu UV-8-120-01, Kyoto, Japan) after the  
 831 formation of a phosphomolybdenum-blue complex by adding ammonium molybdate and  
 832 potassium antimonyltartrate (Houba et al. 1989).

833

834 TABLE S2: Plant height, plant biomass (total dry weight), shoot/root biomass ratio and  
 835 relative growth rate related to height of *Glyceria maxima* grown for 12 weeks in soils from  
 836 Ewijk (EWK) or Huis den Doorn (HDD) and in the absence or presence of molybdate. Values  
 837 are means  $\pm$  SD of 4 replicate microcosms. Data followed by different characters are  
 838 significantly different from each other according to Tukey's test ( $p < 0.05$ ).

839

Soil	Presence of molybdate	Plant height (cm)	Total plant biomass (g)	Shoot/root ratio	Relative growth rate ( $\text{week}^{-1}$ )
EWK	-	66.0 $\pm$ 9.4 a	12.02 $\pm$ 2.22 c	1.25 $\pm$ 0.26 b	0.35 $\pm$ 0.01 a
EWK	+	83.2 $\pm$ 5.6 b	8.51 $\pm$ 1.29 b	1.33 $\pm$ 0.12 ab	0.37 $\pm$ 0.01 b
HDD	-	56.1 $\pm$ 11.7 a	5.98 $\pm$ 0.59 a	1.17 $\pm$ 0.10 a	0.34 $\pm$ 0.01 a
HDD	+	56.1 $\pm$ 11.7 a	5.52 $\pm$ 0.29 a	1.11 $\pm$ 0.07 ab	0.33 $\pm$ 0.01 a

840

841 References

842 Bray R H and Kurtz L T 1945 Determination of total, organic, and available forms of phosphorus in soils.  
843 Soil Sci. 59, 39-45.  
844 Bremner J M and Mulvaney C S 1982 Salicylic acid-thiosulphate modification of Kjeldahl method to  
845 include nitrate and nitrite. In Methods of Soil Chemical Analysis, Part 2: Chemical and  
846 Microbiological Properties. Eds. A C Page, R H Miller and D R Keeney. pp 621-622. Soil Science  
847 Society of America, Madison WI.  
848 Houba V J G, Van der Lee J J, Novozamsky I and Wallinga I 1989 Soil and plant analysis. Part 5. In  
849 Soil analysis procedures. pp 154 -158. Agricultural University, Wageningen.  
850  
851