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

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ABSTRACT

Aims: The research aimed at studying the effect of flooding with sulfate-rich water on the activity, abundance and diversity of sulfate-reducing micro-organisms present in the root zone of an oxygen-releasing plant growing on two riparian grassland soils with contrasting amounts of iron.
Methods: A series of microcosms was used to investigate the effects. Plants were grown under controlled conditions in microcosms containing a rhizosphere and bulk soil compartment for a period of 12 weeks in the presence of sulfate-rich flood water. Molybdate-treated systems served as non-sulfate-reducing controls.
Results: At harvest, activity and numbers of sulfate-reducing micro-organisms were higher in the absence of molybdate, but a rhizosphere effect and an impact of the presence of high levels of iron were not observed on activity and numbers. Both soils had in common a diverse community of sulfate-reducing micro-organisms covering all major cultured bacterial taxa. The appearance of members of the *Desulfovibrionaceae* exclusively in the rhizosphere of *G. maxima* was the only unambiguous indication of a plant effect.
Conclusion: The presence of sulfate-rich flood water stimulated the activity and growth of a part of the sulfate-reducing community leading to a change in community composition. The proximity of aerenchymatous plant roots and the abundance of iron in the soil had a negligible effect on the sulfate-reducing community.

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Plant

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

Soil

The top 15 cm soil used for the microcosms was collected from two riparian zones at Ewijk ("EWK", the Netherlands, 51°53' N, 5°44' E) along the river Rhine and at Huis den Doorn ("HDD", the Netherlands, 52° 33' N, 6° 7' E) along the river Overijsselse Vecht. Soil was stored after air-drying. The HDD soil is rich in iron (total and amorphous iron of 693 and 459 µmol g^{-1} dry weight, respectively), which originates from historical discharge of groundwater (Loeb et al. 2008). EWK soil that is not affected by groundwater seepage contains 103 µmol total iron g^{-1} dry weight (LL soil in Conrad et al. (2008). A summary of the chemical characteristics of the two soils is shown 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 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 µm)
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 Na_2SO_4 or 2 mM Na_2SO_4 plus 2.5 mM Na_2MoO_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 ± 300 µE m^{-2} 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_{in}) and final (W_{fin}) dry weights at

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

Pore water analyses

Pore water samples from the rooted and non-rooted compartments were collected weekly throughout the duration of the experiment. To minimize sampling biases (volume in the sampler: 0.5 ml), the first ml of sample was discarded. Pore water pH was immediately measured and the sample stored at –20°C until further analyses. A sub-sample of pore water (0.5 ml) was saved to monitor sulfate and molybdate concentration in both compartments; sulfate was determined spectrophotometrically with the barium-gelatin method of (Tabatabai 1974), while molybdate was measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Spectro, Kleve, Germany). If necessary, a sterile anoxic SO_4^{2-} or SO_4^{2-}/MoO_4^{2-} aqueous solution was added at an appropriate volume/concentration, to maintain the pore water sulfate and molybdate content at the prefixed experimental value. The amendment was done by a syringe connected to a 15 cm long needle; to minimize disturbance, injections of maximum 5 ml were distributed randomly in the bulk soil and rhizosphere compartment. Pore water NH_4^+, NO_3^-, PO_4^{3-} concentrations were analyzed on a continuous-flow analyzer. Volatile fatty acids and alcohols concentrations after 1 and 12 weeks of incubation were determined after centrifugation (5 min. 13200 rpm) and two-fold dilution of the samples with 0.2 N H_2SO_4 (volatile fatty acids) or 1 N formic acid (alcohols). Fatty acids were determined on a LKB-HPLC system (Pharmacia /LKB, Woerden, the Netherlands) equipped with a high-performance pump and a differential refractometer detector. The samples (20 µl) were applied to a Polyspher OA HY column (E. Merck), which was equilibrated with 0.01 N H_2SO_4 (flow rate 2 ml/min). Alcohols were determined on a Chrompack CP9001 gas chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a mol sieve 13X packed column (2 m × 4.3 mm i.d., 60-80 mesh) and a thermal conductivity detector. Internal and external standards were used for quantification.

MPN counts

Soil was resuspended in phosphate-buffered saline (PBS, per liter of milli-Q water: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH 7.4) in a soil to buffer ratio of 1:6. Slurries were shaken for 2 h. Homogenates were immediately used for inoculation of MPN dilution series in microtiter plates (BRAND, 8 × 12 wells of 250 µl).

The bicarbonate-buffered basal mineral medium used had the following composition: 3 mM KH_2PO_4, 3 mM Na_2HPO_4, 5 mM NaCl, 1 mM Na_2S, 0.5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 50 mM $NaHCO_3$, 5.6 mM NH_4Cl, 2 μM resazurin. The medium was supplemented with 1 ml of trace element solution (final concentrations: 1 μM H_3BO_3, 0.5 μM $MnCl_2$, 7.5 μM $FeCl_2$, 0.5 μM $CoCl_2$, 0.5 μM $ZnCl_2$, 0.1 μM $CuCl_2$, 0.5 μM $NiCl_2$, 0.1 μM Na_2SeO_3, 0.1 μM Na_2WO_4, 0.1 μM Na_2MoO_4), 1 ml vitamins solution (final concentrations: 0.02 μM biotin, 0.2 μM nicotinic acid, 0.5 μM pyridoxine, 0.1 μM riboflavin, 0.2 μM thiamin, 0.1 μM cyanocobalamin, 0.1 μM p-aminobenzoic acid, 0.1 μM panthotenic acid, 0.1 μM lipoic acid, 0.1 μM folic acid), 1 g of yeast extract. As electron acceptor, 20 mM Na_2SO_4 was used. A mix of acetate, propionate and lactate (15 mM each) served as electron donors. The reducing agent in the MPN medium was sodium thioglycolate (0.5 mM). $FeSO_4$ (0.2 mM) served as an indicator of sulfate reduction; the formation of a black FeS precipitate was indicative for sulfide formation by active SRM; black wells were counted as positive.

The microtiter plates were sealed with an adhesive foil (SecurSeal®, Simport, Beloeil, Canada) and put in anaerobic incubation bags (Anaerocult® A mini, Merck, Darmstadt, Germany). In the bags, a citric acid-based catalyst was used to create an oxygen-free N_2/CO_2 atmosphere. The atmosphere became anoxic within 1 hour after sealing the bags, as shown by an indicator strip (Anaerotest®, Merck, Darmstadt, Germany). Cultures were incubated at 25°C for 3 months. After counting the number of positive wells per dilution, the MPN and confidence limits were calculated using standard MPN tables (Rowe et al. 1977). MPN counts were performed in triplicate samples 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 µl (bulk soil cores) or 10 µl (rhizosphere cylinder) of carrier-free $^{35}SO_4^{2-}$ tracer
217 (~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 $^{35}SO_4^{2-}$ was added. Samples were stored
221 at –20°C until analysis.
222 ^{35}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®; Lumac LSC BV, the Netherlands) was used to quantify the
225 $^{35}SO_4^{2-}$ and $^{35}S_{TRIS}$ 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 × 4.6 mm anion exchange column; Waters 430
228 Conductivity detector) with an isophtalic 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 DSRlFb, 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 Rf 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_f 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_f 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 $R_f \pm 0.001$). 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

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

Statistical analyses

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

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

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

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

Analysis of similarity (ANOSIM) between DGGE profiles was performed with the software PRIMER 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 0.1-0.5 µg 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

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

SRM abundances and sulfate reduction rates

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

Sulfate reduction occurred in EWK and HDD soils, both in the rhizosphere and bulk soil with values ranging from 0.4 to 1.4 µmol SO_4^{2-} cm^{-3} d^{-1} (Figure 2). A statistically significant difference in sulfate reduction rates was only observed between the presence and absence of molybdate in both soils at all depths. Sulfate reduction rates were not significantly different between depths and between soils/compartments at all depths, except for the rhizosphere and the bulk soil of the upper layer of the EWK soil in the absence of molybdate, where the rates were significantly lower compared to the other layers of the EWK soil under the same conditions (Tukey's HDS test). Both abundance estimation and sulfate reduction activity data confirmed that the sulfate-reducing community was efficiently impaired by molybdate.

SRM diversity assessment in EWK and HDD microcosms

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

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

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

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

i.e. in the absence of molybdate. In the case of OTU06 this stimulation in the absence of molybdate was only observed in the rhizosphere soils.

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

DISCUSSION

Plant effect on the SRM community

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

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

Also members of the family *Desulfobacteraceae* to which *D. limicola belong,* are metabolically versatile, but in addition they can oxidize acetate completely to CO_2 (Rabus et al. 2006). *D. limicola* is able to grow on larger fatty acids (Widdel 1980).

Soil effect on the SRM community

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

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

With respect to the diversity of the communities of SRM, large differences were also not observed between the incubated EWK and HDD soils; 74% of EWK- and 75% of HDD-derived sequences were ubiquitous among both soils or were present only in the presence of molybdate. Interestingly, these include all bands related to the orthologues/xenologues *Firmicutes* (OTU01, OTU03, OTU05, OTU07, OTU09, and OTU10) and the *Syntrophobacteraceae*-related OTU02 (Figure 3 and Table 3). The relatively high number and widespread distribution of *Firmicutes*-related *dsrB* found in microcosms filled with EWK and HDD soils might be explained by the capability of members of this group to produce spores in order to survive situations of environmental stress (Widdel 2006) such as the alternating oxic and anoxic conditions that depend on the water table fluctuations in the floodplains. The occurrence of the spore-forming *Desulfotomaculum* has been reported in similar variable environments such as rice paddies (Stubner and Meuser 2000). *Syntrophobacter* species have also been desribed as one of the predominant SRM groups in paddy soil studies (Scheid and Stubner 2001; Stubner 2004). These organisms do not form spores, but have extremely diverse nutritional capabilities being able to use a wide variety of electron donors and carbon sources, syntrophically or in pure cultures and in the presence or absence of sulfate as electron acceptor (Harmsen et al. 1998). This metabolic peculiarity confers these microorganisms adaptability to changing environmental conditions, and could explain the ubiquitous presence of *Syntrophobacter*-related *dsrB* genes in EWK and HDD

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

Fermentation intermediates

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

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

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

Plant performance

Like sulfate and phosphate, molybdate belongs to the group VI oxyanions and may therefore interfere with the sulfate and phosphate metabolism of plants. (McGrath et al. 2010) determined the threshold values of molybdenum toxicity for plant species in an experiment comprising 10 different soils and 4 different plant species. Among the plant species tested, toxicity thresholds based on soil-added molybdenum solutions were higher for Rye grass (*Lolium perenne*) than for the tested dicotyledonous species on all the soils, indicating that shoot growth of Rye grass was less sensitive to molybdenum toxicity than the growth of the other plant species. The authors showed also that soil properties strongly affect the expression of molybdenum toxicity in the plant species. Toxicity threshold values were generally higher in acid (pH < 6.5) than in neutral and basic soils (pH > 6.5), indicating that molybdenum is less toxic to higher plants grown on acid soils. Selecting the two soils from the list of (McGrath et al. 2010) that are most comparable to our soils and looking to Rye grass, a concentration of 2.5 mM molybdenum as applied by us could have caused a 50% reduction in shoot yield after a growing period of three weeks. The plants in the analysis of McGrath and colleagues were more affected by molybdenum in calcareous than in acidic soils, which possibly agrees with our observation of a larger effect of molybdate addition in the more calcareous EWK soil. With *G. maxima*, we observed 29% reduction in biomass, but an increase of 26% in average height in EWK soils in the presence of 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
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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 ± 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

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

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

[a] *dsrB* sequences with deduced DsrAB sequence identity equal to or greater than 97% were grouped in an OTU. OTUs were sequentially numbered according to the total number of bands

[b] Based on one sequence per match, as determined by Phoretics analyses of DGGE gels

[c] Occurrence based on detection in the absence (-Mo) or presence (+Mo) of molybdate; I = independent of +Mo or –Mo, (R) = only rhizosphere soil, (B) = only bulk soil

[d] Phylogeny of *dsrB* sequences as inferred from Fig. 4 and based on 95 amino acid sequence positions

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

607

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

608 [a] EWK and HDD bands indicated in Figures 5 and 6, respectively

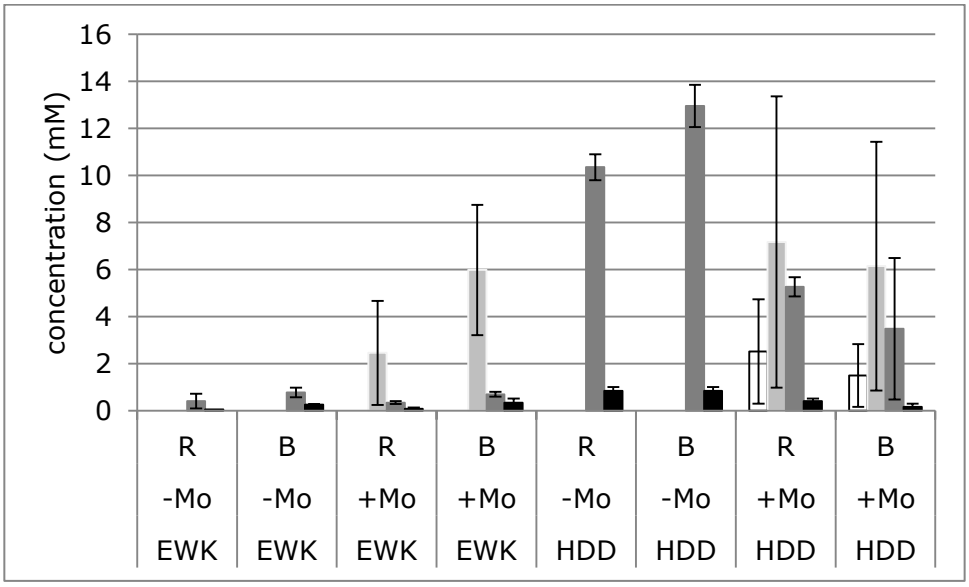
609

610

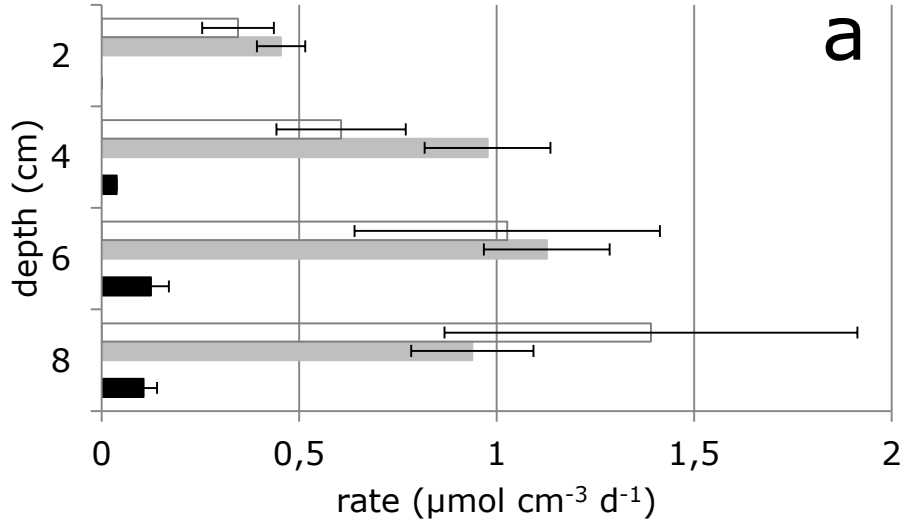
611

Legend to the figures

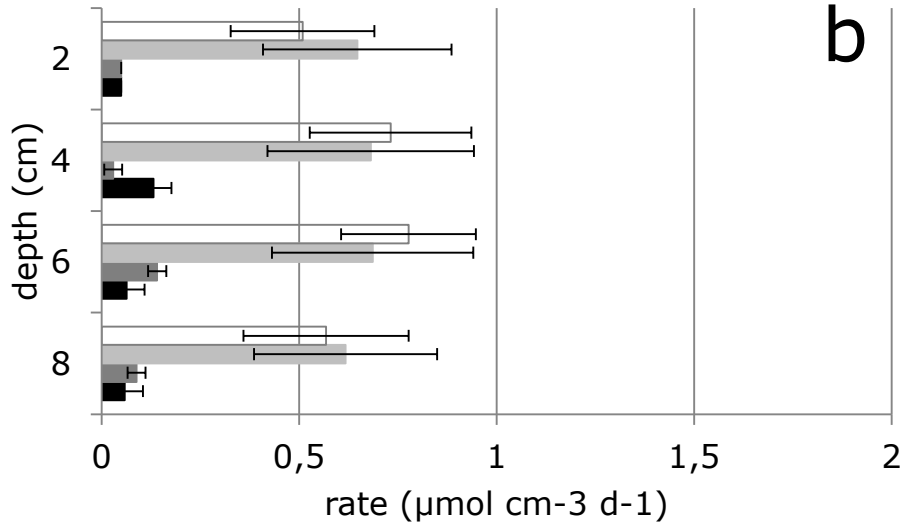
Figure 1: Concentrations of pore water methanol (white bars), ethanol (light grey bars), acetate (dark grey bars) and propionate (black bars) at the end of the first week of the incubation period. R = rhizosphere soil, B = bulk soil, -Mo = absence of molybdate, +Mo = presence of molybdate, EWK = Ewijk soil, HDD = Huis den Doorn soil.



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 ± SD of 4 (rhizosphere) or 6 (bulk soil) replicate samples.
626

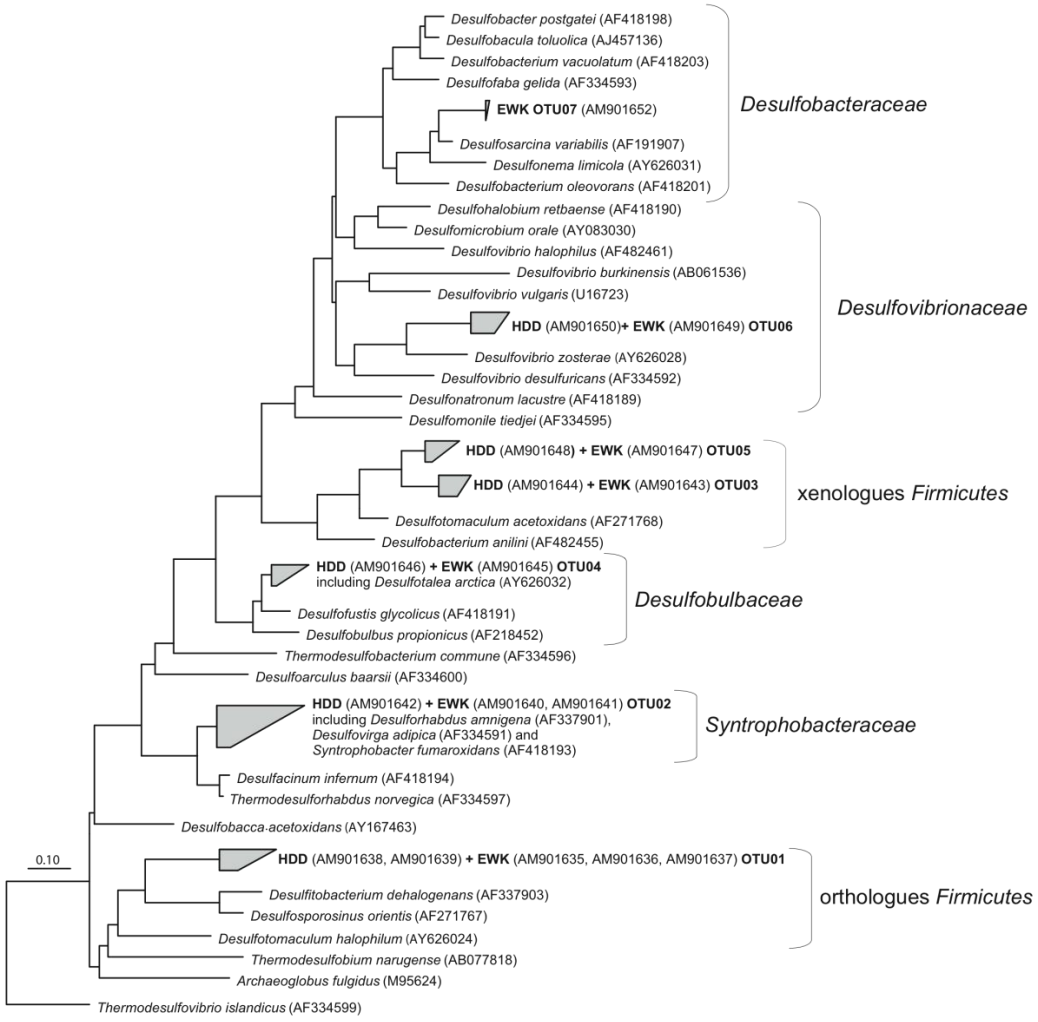


627



628

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



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

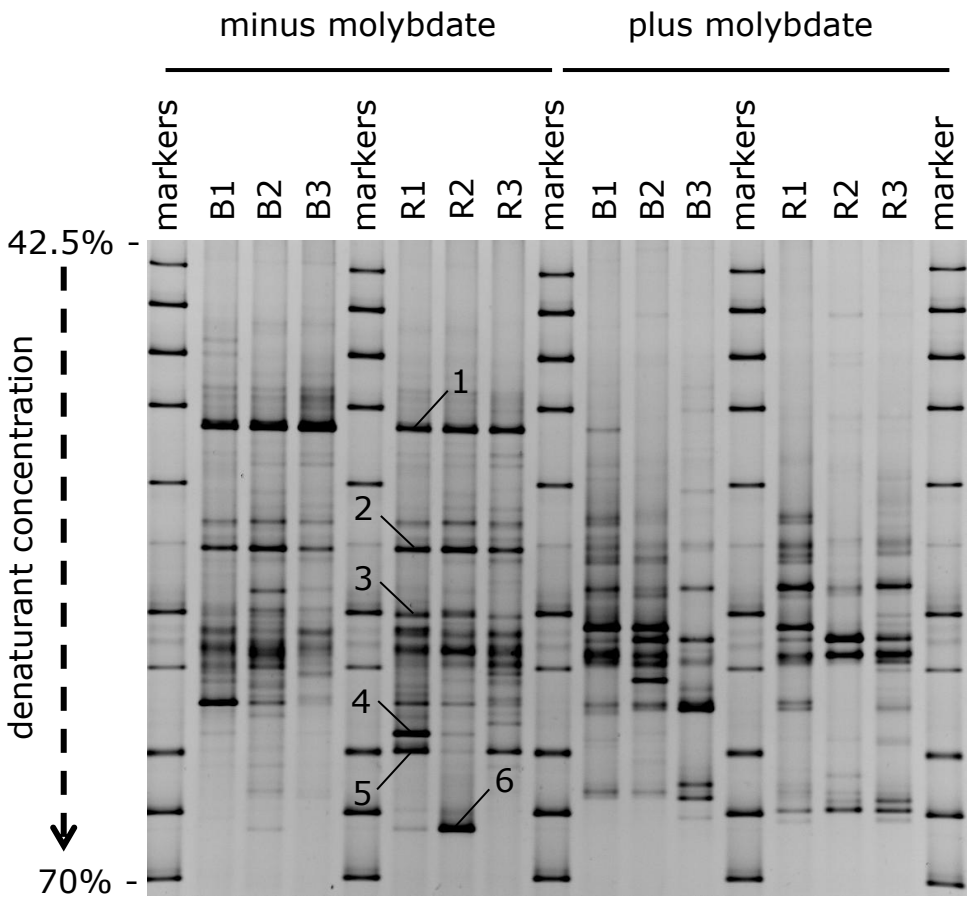
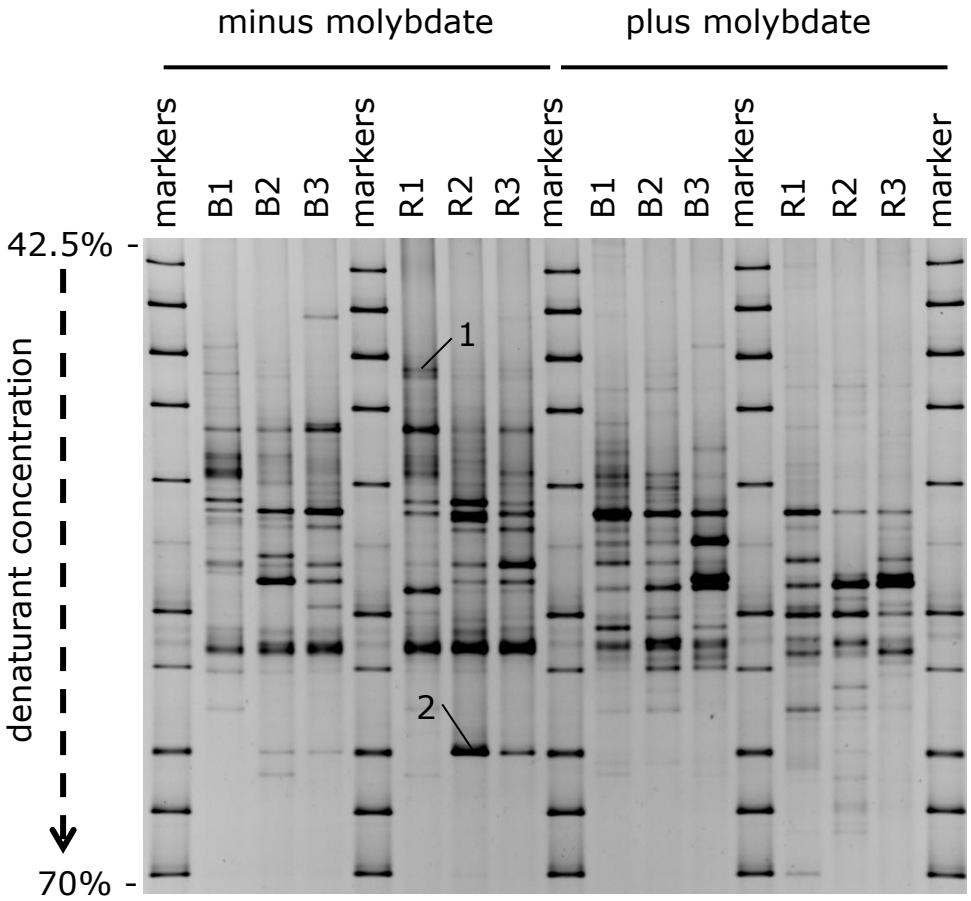


Figure 5: Denaturing gradient gel electrophoresis (DGGE) of *dsrB* PCR products amplified from Huis den Doorn microcosms. Lanes coded B1-3 and R1-3 indicate samples collected from replicate bulk soil and rhizosphere samples, respectively. Bands 1-2 occurring only in the rhizosphere of the treatment microcosms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



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

804

Soil	pH	OM (%)	Demineralized water extractable nutrients (µg/g)						KCl extractable nutrients (µg/g)			Total elements (mg/g)		
			Al^{3+}	Ca^{2+}	Fe^{3+}	K^{+}	Mg^{2+}	SO_4^{2-}	NO_3^{-}	NH_4^{+}	PO_4^{3-}	N	P	K
EWK	7.5	10.5	0.2	254.8	2.1	8.1	9.9	63.9	168.5	16.8	55.7	3.3	1.0	6.8
HDD	5.9	13.6	0.1	71.5	1.7	0.5	1.4	58.5	107.1	7.1	4.8	5.1	1.4	3.0

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. NH_4^+, NO_3^- and 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 Al^{3+}, Ca^{2+}, Fe^{3+}, K^+, Mg^{2+}, and 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 NH_4^+ and 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 *o*-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 NH_4F 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 ± 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 ($week^{-1}$)
EWK	-	66.0 ± 9.4 a	12.02 ± 2.22 c	1.25 ± 0.26 b	0.35 ± 0.01 a
EWK	+	83.2 ± 5.6 b	8.51 ± 1.29 b	1.33 ± 0.12 ab	0.37 ± 0.01 b
HDD	-	56.1 ± 11.7 a	5.98 ± 0.59 a	1.17 ± 0.10 a	0.34 ± 0.01 a
HDD	+	56.1 ± 11.7 a	5.52 ± 0.29 a	1.11 ± 0.07 ab	0.33 ± 0.01 a

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