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

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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)

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 $\pm 10 \mu\text{E m}^{-2} \text{s}^{-1}$ 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 $\mu\text{mol g}^{-1}$ 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.

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

Microcosms

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

In total, 24 microcosms were filled with 780 g of dry soil each and flooded with a sterile oxygen-free aqueous solution containing 2 mM Na_2SO_4 or 2 mM Na_2SO_4 plus 2.5 mM Na_2MoO_4 . Molybdate is a specific metabolic inhibitor of sulfate reduction (Oremland and Capone 1988). Per soil type, 6 replicate microcosms were randomly selected for each treatment. Once the flooding solution completely penetrated the soil, randomly chosen *G. maxima* seedlings were planted in the rhizosphere compartment of all systems. The flood water level was then raised and kept constant at 2.5 cm above the soil surface by replenishing with sterile water throughout the experiment. The systems were incubated in a plant growth cabinet (day/night cycles 16 h, 22°C/8 h, 15°C; relative humidity 65%; photosynthetic photon flux density at plant level $\pm 300 \mu\text{E m}^{-2} \text{s}^{-1}$).

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

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

harvest according to the following equation $RGR = (\ln W_{fin} - \ln W_{in}) / (t_{fin} - t_{in})$, 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 $\text{SO}_4^{2-}/\text{MoO}_4^{2-}$ 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 $\times$ 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 Na₂HPO₄, 0.24 g KH₂PO₄; 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 KH₂PO₄, 3 mM Na₂HPO₄, 5 mM NaCl, 1 mM Na₂S, 0.5 mM MgCl₂, 0.75 mM CaCl₂, 50 mM NaHCO₃, 5.6 mM NH₄Cl, 2 µM resazurin. The medium was supplemented with 1 ml of trace element solution (final concentrations: 1 µM H₃BO₃, 0.5 µM MnCl₂, 7.5 µM FeCl₂, 0.5 µM CoCl₂, 0.5 µM ZnCl₂, 0.1 µM CuCl₂, 0.5 µM NiCl₂, 0.1 µM Na₂SeO₃, 0.1 µM Na₂WO₄, 0.1 µM Na₂MoO₄), 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 pantothenic acid, 0.1 µM lipoic acid, 0.1 µM folic acid), 1 g of yeast extract. As electron acceptor, 20 mM Na₂SO₄ 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). FeSO₄ (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 N₂/CO₂ 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 $^{35}\text{SO}_4^{2-}$ 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 $^{35}\text{SO}_4^{2-}$ 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®; Lumatic 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 × 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).

Nested *dsrAB*-*dsrB* amplification and DGGE

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

Sequencing and phylogenetic analysis

Sequencing was performed with an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA) at the BMR Servizio Sequenziamento (CRIBI - Università di Padova, Italy, [<http://bmr.cribi.unipd.it>]). The ARB package (Ludwig et al. 2004); [<http://www.arb-home.de>]) was used for the phylogenetic analyses. Partial *dsrB* sequences were added to an ARB alignment of 97 complete *dsrAB* sequences (Zverlov et al. 2005). The alignment of the corresponding amino acid sequences was carried out manually using the editor GDE 2.2 (Smith et al. 1994) implemented in ARB. Partial *dsrB* sequences were inserted one by one into a *dsrAB* core tree 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.nlm.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

$$J = M_{11} / (M_{01} + M_{10} + M_{11})$$

where M_{11} represents the number of bands both lanes have in common, M_{01} the number of bands in one of the lanes and M_{10} 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

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

RESULTS

Plants

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

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

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

Soil

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

Pore water methanol, ethanol, acetate and propionate concentrations at the end of the first week 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 $\mu\text{mol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ (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 HSD 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

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,

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 CO₂ (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 $\mu\text{mol cm}^{-3} \text{ d}^{-1}$ 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 described 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 for 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

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

Conclusions

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

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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 $\pm$ 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

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 <i>Firmicutes</i>
02	5	4	I	I	<i>Syntrophobacteraceae</i>
03	2	6	+Mo	I	xenologues <i>Firmicutes</i>
04	3	4	-Mo	-Mo	<i>Desulfobulbaceae</i>
05	3	4	I	I	xenologues <i>Firmicutes</i>
06	2	1	-Mo (R)	-Mo (R)	<i>Desulfovibrionaceae</i>
07	0	1	-	+Mo (R)	xenologues <i>Firmicutes</i>
08	2	0	-Mo	-	<i>Desulfobacteriaceae</i>
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 <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%	(Joulain et al. 2001)
EWK06	AM901665	06	<i>Desulfovibrio longus</i> (GQ324675)	94%	(Joulain 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%	(Joulain et al. 2001)

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

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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.

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

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.

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

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

Soil	pH	OM (%)	Demineralized water extractable nutrients ($\mu\text{g/g}$)						KCl extractable nutrients ($\mu\text{g/g}$)			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
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

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

Soil demineralized water extraction was performed as follows: 5 grams of dry soil were shaken for 1 hour in 100 ml of demineralized water, centrifuged for 4 minutes at 4000 rpm and filtered over a Whatman GF/C filter. NH_4^{+} , NO_3^{-} and PO_4^{3-} concentrations in the filtrates were determined colorimetrically on a continuous flow analyzer (Skalar SA-40, Skalar, Breda, the Netherlands). Concentrations of Al^{3+} , Ca^{2+} , Fe^{3+} , K^{+} , Mg^{2+} , and SO_4^{2-} in the filtrate were analyzed by ICP-MS (X series, Thermo Electron Corporation, Marietta, Ohio) and ICP-OES (Spectroflame, VML2 and IRIS Interprid II XDL, Thermo Electron Corporation).

Concentrations of extractable ammonium and nitrate were determined by 0.2 M KCl extraction (Houba et al. 1989). Ten grams of dry soil was weighed, 100 ml of extraction solution was added and the sample was shaken for 1 h at 100 rpm. After centrifugation (4 min 4000 rpm), the supernatant was filtered and stored at -20°C until analyses; the concentrations of NH_4^{+} and NO_3^{-} were determined colorimetrically on the continuous flow analyzer.

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

Available α -phosphate was determined using the Bray P-2 method (Bray and Kurtz 1945). Five grams of dry soil was extracted with a solution 0.03 M NH_4F and 0.1 M HCl, by shaking the suspension for 20 minutes at 120 rpm. Phosphate concentrations were determined colorimetrically on a spectrophotometer (Shimadzu UV-8-120-01, Kyoto, Japan) after the formation of a phosphomolybdenum-blue complex by adding ammonium molybdate and potassium antimonyltartrate (Houba et al. 1989).

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

Soil	Presence of molybdate	Plant height (cm)	Total plant biomass (g)	Shoot/root ratio	Relative growth rate (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

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