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Response of the Sulfate-Reducing Community to the Re-establishment of Estuarine Conditions in Two Contrasting Soils: a Mesocosm Approach

Marzia Miletto · Roos Loeb · A. Martjin Antheunisse · Paul L. E. Bodelier · Hendrikus J. Laanbroek

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Abstract We studied the response of the sulfate-reducing prokaryote (SRP) communities to the experimental variation of salinity and tide in an outdoor mesocosm setup. Intact soil monoliths were collected at two areas of the Haringvliet lagoon (The Netherlands): one sampling location consisted of agricultural grassland, drained and fertilized for at least the last century; the other of a freshwater marshland with more recent sea influence. Two factors, i.e., "salinity" (freshwater/oligohaline) and "tide" (nontidal/tidal), were tested in a full-factorial design. Soil samples were collected after 5 months (June–October). Dissimilatory (bi)sulfite reductase β subunit-based denaturing gradient gel electrophoresis (*dsrB*-DGGE) analysis revealed that the SRP community composition in the agricultural grassland and in the freshwater marshland was represented mainly by microorganisms related to the *Desulfobulbaceae* and the *Desulfobacteraceae*, respectively. *Desulfovibrio*-related *dsrB* were detected only in the tidal treatments; *Desulfomonile*-related *dsrB* occurrence was related to the presence of oligohaline conditions. Treatments did have an effect on the overall SRP community composition of both soils, but not on the sulfate depletion rates in sulfate-amended anoxic slurry incubations. However, initiation of sulfate reduction upon sulfate addition was clearly different between the two soils.

M. Miletto · P. L. E. Bodelier · H. J. Laanbroek
Department of Microbial Ecology,
Netherlands Institute of Ecology (NIOO-KNAW),
Rijksstraatweg 6,
3631 AC Nieuwesluis, The Netherlands

R. Loeb
Department of Aquatic Ecology and Environmental Biology,
Institute for Water and Wetland Research,
Radboud University Nijmegen,
Toernooiveld 1,
6525 ED Nijmegen, The Netherlands

A. M. Antheunisse · H. J. Laanbroek
Department of Landscape Ecology, Institute of Environmental Biology, Utrecht University,
P.O. Box 800.84, 3508 TB Utrecht, The Netherlands

Present Address:
M. Miletto (✉)
Department of Microbiology, Morrill Science Center IV North,
University of Massachusetts,
Amherst, MA 01003, USA
e-mail: mmiletto@microbio.umass.edu

Introduction

In the past, the rivers Rhine and Meuse entered the North Sea in a combined delta in the southwestern part of the Netherlands. Within the framework of a larger plan aiming at safeguarding this densely populated area after a major storm surge in 1953, the Haringvliet estuary was cut off from the North Sea in 1970 by the construction of dams with sluices [1]. Besides safety reasons, this measure also ensured freshwater supply for the agricultural activities in the area. However, the closure of the estuary caused severe ecological damage. The original salinity gradient towards the sea was altered, in fact substituted by a large freshwater basin, and the tidal amplitude decreased drastically from 2 m before the closure to a maximum of 20 cm thereafter, resulting from some limited hydraulic connections with the sea. These physical–chemical changes had large repercussions on the entire area, altering the sedimentation–erosion equilibrium

and causing the disappearance of the characteristic oligohaline tidal marshland, which was substituted by freshwater vegetation typical of eutrophic conditions [2, 3]. Appreciating the ecological value of the former Haringvliet estuary compared to the current situation [4], the Haringvliet sluices will be gradually reopened in the near future in order to restore the natural transition zone between the sea and the rivers Rhine and Meuse [5]. The reintroduction of a salinity gradient and a tidal influence in the Haringvliet lagoon are expected to restore the former sedimentation–erosion equilibrium, the water quality, and the related vegetation and biogeochemistry of the soil [6].

An outdoor mesocosm experiment was set up to investigate the effects of the reintroduction of salinity and tide on nutrient cycling and vegetation development in two types of soil of the present-day Haringvliet lagoon: an agricultural polder, drained and fertilized for at least the last century, and a freshwater marshland with more recent sea influence. In order to be able to predict the effects of the estuarine ecosystem rehabilitation, the oligohaline and tidal treatments chosen mimicked the target conditions of the restoration process, while the freshwater and nontidal treatment served as control resembling the actual conditions in the Haringvliet lagoon. Within this framework, this study aimed at elucidating the effects of the restored estuarine conditions on the sulfate-reducing microbial community. Enhancement of sulfate reduction may have a strong impact on the overall biogeochemistry of the soil, as sulfate-reducing prokaryote (SRP) communities' principal metabolic product, i.e., sulfide, is a highly reactive compound known to promote eutrophication via phosphate mobilization [7]. In addition, sulfide is phytotoxic, especially for plants adapted to freshwater environments where sulfate reduction is generally low. SRPs constitute a phylogenetically and metabolically diverse group of anaerobic microorganisms united by the ability to couple the dissimilative reduction of sulfate to the oxidation of a wide variety of substrates [8–11]. It has been shown that the community composition of this group of microorganisms is greatly affected by salinity indicators [12].

We hypothesized that due to the introduction of oligohaline conditions and tide, changes in the diversity of the SRP community will occur. With respect to SRP activity, we expected an enhancement of sulfate reduction in the tidal treatment for both types of soil, as a constant flooding twice a day creates the anoxic environment suitable for the growth of these anaerobic microorganisms.

Materials and Methods

Sites

Two study sites were chosen in the Haringvliet lagoon, which used to be a part of the Rhine–Meuse estuarine system until its closing from the sea by the Haringvliet dam in 1970 (Fig. 1). The first site, named Beninger Slikken (BS, 51°47′ N, 4°13′ E), used to be a salt marsh, but is now a nature reserve where the original oligohaline marshland and halophyte vegetation has been partially replaced by a more freshwater marshland vegetation with many dicotyledonous species [13]. The second site, named Hitsertse Kade (HK, 51°44′ N, 4°21′ E), is covered by low-diversity vegetation dominated by a few forage grasses. It is a polder reclaimed from the sea already in the sixteenth century, extensively fertilized, and used as pasture for at least a hundred years. Due to the presence of a levee, it can be flooded only during the winter months when the water table rises due to higher river discharge. Soil characteristics differ significantly between the two locations [13]. Briefly, at the moment of sampling, the marshland soil contained a higher amount of extractable ammonium (3.8±0.6 vs. 0.3±0.3 mg kg^{-1} soil), whereas

Figure 1 The Haringvliet lagoon in the southwestern part of The Netherlands. Agricultural grassland monoliths have been collected at Hitsertse Kade, freshwater marshland monoliths at Beninger Slikken

the amount of extractable nitrate was higher in the agricultural grassland soil (13.1±1.5 vs. 2.4±0.5 mg kg^{-1} soil). Extractable amounts of sulfate (1,574±611 vs. 28±2 mg kg^{-1} soil) and sodium (101±10 vs. 8±2 mg kg^{-1} soil) were higher in the marshland soil than in the agricultural soil, showing the more recent influence of the sea in the freshwater marshland. The agricultural grassland soil is slightly poorer in organic matter (10.4±0.3% vs. 14.7±0.6% [*w*/*w*]) and had lower soil moisture content (0.26±0.01% vs. 0.70±0.03% [*w*/*w*]). No difference was observed between the pH values of the two soil types; both amounted to 7.6.

Mesocosm

Sixteen basins (∅=180 cm, *V*=2,500 L; Fig. 2) partially dug into the ground to reduce temperature fluctuations and lined with an impermeable cloth were used to set up the mesocosms. Each basin contained two soil–vegetation units encompassing the entire rooting depth (monoliths; *w*=60 cm, *l*=40 cm, *h*=20 cm), one of each sampling site, placed in a mulching cloth-lined open plastic crate. In the experiment, two factors, i.e., "salinity" (freshwater/oligohaline) and "tide" (nontidal/tidal), were tested in a full-factorial design: FN, freshwater-nontidal; FT, freshwater-tidal; ON, oligohaline-nontidal; OT, oligohaline-tidal. Every treatment had four replicates. The two artificial water types were prepared by dissolving an appropriate amount of a sea salt mixture (Meersalz Professional, Wiegandt GmbH, Krefeld, Germany) in tap water to reach a salinity of 0.1‰ (freshwater) and 3.0‰ (oligohaline), respectively. Salinity was determined with a salinity electrode (Multi 340i, WTW, Weilheim, Germany). NO_3^-, PO_4^{3-}, SO_4^{2-}, Cl^-, Na^+, and K^+ concentrations in the two water types were measured and reported before by Antheunisse et al. [13]. Briefly, the oligohaline treatments contained 4.4 times more sulfate than the freshwater treatments (260 vs. 59 mg L^{-1}). The nontidal water basins were filled to 5 cm below soil surface, and a pump ensured continuous water circulation. In the tidal basins, water level varied between 10 cm above and 20 cm below the soil surface by pumping the necessary amount of water to and from temporary reservoirs over a period of 6 h. This led to two ebb–flood cycles a day. The chosen levels were based either on the present situation for the former tidal flats in the Haringvliet estuary (nontidal treatment) or on the condition mimicking possible post-restoration levels, when these areas will be once more subjected to a tidal regime (tidal treatment). During the course of the experiment, salinity and water level were continuously monitored and, if necessary, adjusted to compensate for rain/drought periods.

The experiment started in June 2004. Soil samples for activity measurements, DNA, and lipid analyses were taken in October 2004 at the end of the growing season of the plants. Sterilized stainless steel cylinders (∅=3 cm, *h*=20 cm) were used to collect soil samples from all monoliths. Cores were transported under anoxic conditions to the laboratory at 4°C and, immediately upon arrival, roots were removed from the first 10 cm and the root-free soil was homogenized by passing through a sterilized 1-mm mesh sieve. All samples were processed within 4 h. A subsample of the homogenized soil was stored at −20°C until further DNA and polar lipid-derived fatty acids (PLFA) analyses. The remaining soil was immediately used to set up slurry incubations.

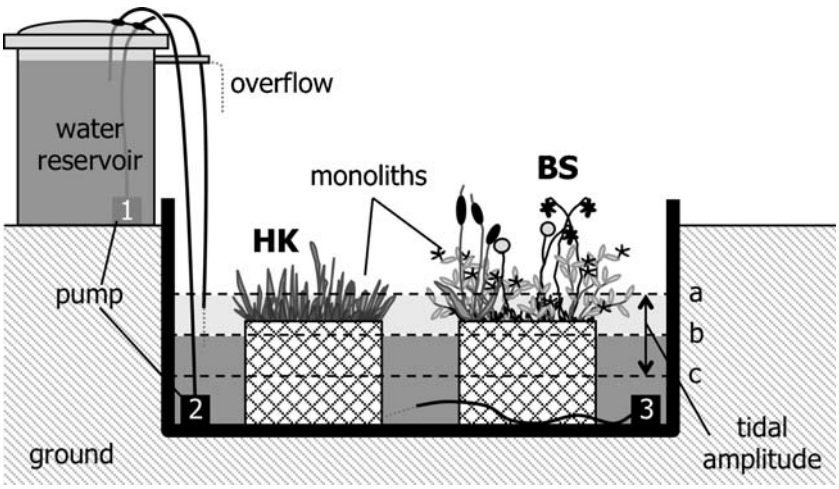

Figure 2 Mesocosm setup. Every basin housed one soil monolith from the grassland (*HK*) and one monolith from the marshland (*BS*). In the nontidal treatments, the water remained permanently at level *a* (−5 cm), and circulation in the basin was maintained with the constant action of pump *3*. In the tidal treatments, the tidal movement between levels *a* (−20 cm) and *b* (+10 cm) was ensured by the combined work of pump *1* (active during tide) and pump *2* (active during ebb)

Anoxic Slurry Incubations

Anoxic slurry incubations were set up as follows: 20 g fresh weight of soil were placed into 100 ml crimp-cap serum bottles and kept in an oxygen-free atmosphere by sealing the bottles with butyl-rubber stoppers and changing the gas phase to N_2. Activity measurements were started with the addition of 40 ml of a sterile, anoxic 2 mM Na_2SO_4 solution (ratio 1:2 [*w*/*v*]). To establish the biological nature of sulfate reduction, the specific inhibitor sodium molybdate was added to control slurry incubations from a sterile, anoxic stock solution (1 M) to a final concentration of 2.5 mM. Slurries were incubated at 25°C on a shaker (130 rpm) in the dark. One-milliliter samples were regularly collected with sterile, N_2-flushed syringes and sulfide was immediately fixed into 50 µl of 1 M zinc acetate. After centrifugation (5 min, 14,000 rpm, 4°C) and collection of the supernatant, sulfate was determined spectrophotometrically (Lambda 800 UV/Vis, PerkinElmer, Wellesley, MA, USA) with the barium–gelatin method of Tabatabai [14]. Sulfate depletion rates were calculated from the linear part of the depletion curves by linear regression.

PLFAs Analysis

PLFAs were extracted from 3 g of freeze-dried soil with a modified Bligh–Dyer extraction [15, 16]. Fractionation of total lipid extract into different polarity classes was performed on silicic acid by sequential elution with chloroform, acetone, and methanol. Fatty acids were extracted from the polar lipids of the methanol fraction and derivatized to fatty acid methyl esters (FAMEs) using mild alkaline methanolysis. $C_{12:0}$ and $C_{19:0}$ FAMEs were used as internal standards. FAMEs identification was performed by comparison of retention time data with known standards. FAMEs concentration was determined using a Thermo Finnigan TRACE GC-FID system equipped with a polar capillary column (SGE, BPX-70; 50 m×0.32 mm×0.25 µm). Oven conditions were the following: 80°C for 1.5 min, increase to 120°C at 20°C min^{-1}, increase to 240°C at 3°C min^{-1}. Thirty-three different PLFAs were measured. PLFAs nomenclature used was as described previously [17]. PLFA concentrations were expressed in nanomoles per gram of dry soil.

DNA Extraction

DNA was extracted from 0.3 g wet weight of soil using the UltraClean Soil DNA Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. Quantification of the electrophoresed and ethidium bromide-stained DNA extracts was performed by comparison to two dilutions of the SmartLadder quantification standard (Eurogentec, Seraing, Belgium). Digital image analysis was carried out using the 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) fragments were amplified as described in Miletto et al. [18]. Primer mixtures DSR1Fmix and DSR4Rmix [19] were implemented with primers DSR1Fb, DSR1Fc and DSR4Rd, DSR4Re, respectively [20]. *dsrB* were separated by denaturing gradient gel electrophoresis (DGGE), and bands of interest were isolated from the gel and purified as described previously [18]. The software package Phoretics 1D Advanced was used to analyze gel images; bands detection and matching were performed automatically to avoid biases associated with manual band processing. Bands showing intensity under a certain value (15% of the highest peak within a lane) were omitted from further analyses. Retardation factor (Rf) values were assigned to bands using as reference a suitable marker mix of ten *dsrB* fragments run together with the samples to account for gradient heterogeneities. Bands having similar Rf values were considered as corresponding and grouped into a match (the maximum acceptable displacement to call a match between bands was set to Rf±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 (http://www.arb-home.de [21]) was used for the phylogenetic analyses. Partial *dsrB* sequences were added to an ARB alignment of 97 complete *dsrAB* sequences [20]. The alignment of the corresponding amino acid sequences was carried out manually using the editor GDE 2.2 [22] implemented in ARB. Deduced partial DSR sequences were inserted one by one into a DSR core tree by using the parsimony tool implemented in ARB; this avoided distortions of the overall tree topology. Phylogenetic inference was performed with a total of 95 amino acid residues (*Desulfovibrio vulgaris* DSR β subunit 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). Partial *dsrB* sequences were compared to the GenBank database [23] using the algorithm BLASTX (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 DSR amino acid sequence identities ≥97% were grouped into an operational taxonomic unit (OTU). One marshland and/or grassland *dsrB* nucleotide sequence representative for each OTU was submitted to the EMBL database (accession numbers AM901621 to AM901634).

Statistical Analysis of DGGE and PLFAs Patterns

The software STATISTICA v7.1 (StatSoft, Inc., Tulsa, OK, USA) was used for cluster analysis using the unweighted pair group average algorithm of DGGE profiles. The initial data matrix consisted of DGGE bands as variables and the corresponding scores (band presence–absence data) as the values within each variable. Binary similarities for every pair of samples (DGGE profiles) were inferred using the Jaccard coefficient (C_J) calculated as:

$$C_J = \frac{c}{(a+b-c)} \times 100$$

where a is the number of bands in the first sample, b is the number of bands in the second sample, and c is the number of corresponding bands positive in both samples.

Analysis of similarity (ANOSIM) among DGGE profiles were performed with the software PRIMER v5.2.9 (PRIMER-E Ltd., Plymouth, UK) on the similarity matrix to test the effect of the different treatments on the DGGE profiles.

One-way analysis of variance (ANOVA) tests were performed using STATISTICA v7.1 to test the effect of the different treatments. If the ANOVA revealed significant effects, a Tukey's honestly significant differences post hoc test was performed to group homogeneous means. Results with $p>0.05$ were considered not significant.

Results

Anoxic Slurry Incubations

Sulfate depletion profiles in slurry incubations of marshland and grassland mesocosm soils are depicted in Fig. 3. In slurries made from marshland soils, sulfate consumption was registered immediately at the start of the incubation, but accelerated after 2 days and declined again after 6 days when the concentrations of sulfate became apparently limiting. Due to the high standard deviations (SD) of the means of the different treatments, no conclusion could be drawn on the effects of treatments on the sulfate depletion rates. In slurries made from grassland soils, sulfate concentration remained stable for the first 4 days, but decreased rapidly thereafter. The calculated rates of maximum depletion in slurries with grassland soils were on average 82% higher than the rates measured in slurries with marshland soils. In control incubations, 2.5 mM molybdate efficiently prevented sulfate reduction and sulfate concentrations remained constant over time at the initial value, proving that sulfate depletion was caused by microbial sulfate reduction (Fig. 3).

PLFAs

ANOVA tests revealed that the total concentration of PLFA was higher in the grassland mesocosm (146.7±26.7 nmol g^{-1}) compared to the marshland soil (74.6±20.1 nmol g^{-1}). Comparing treatments within the same soil, all PLFAs in grassland soil showed significantly higher concentrations in the freshwater/tidal mesocosms compared to the other treatments; in contrast, no significant difference was observed between the various treatments of the marshland mesocosms. These results are depicted in Fig. 4 for seven

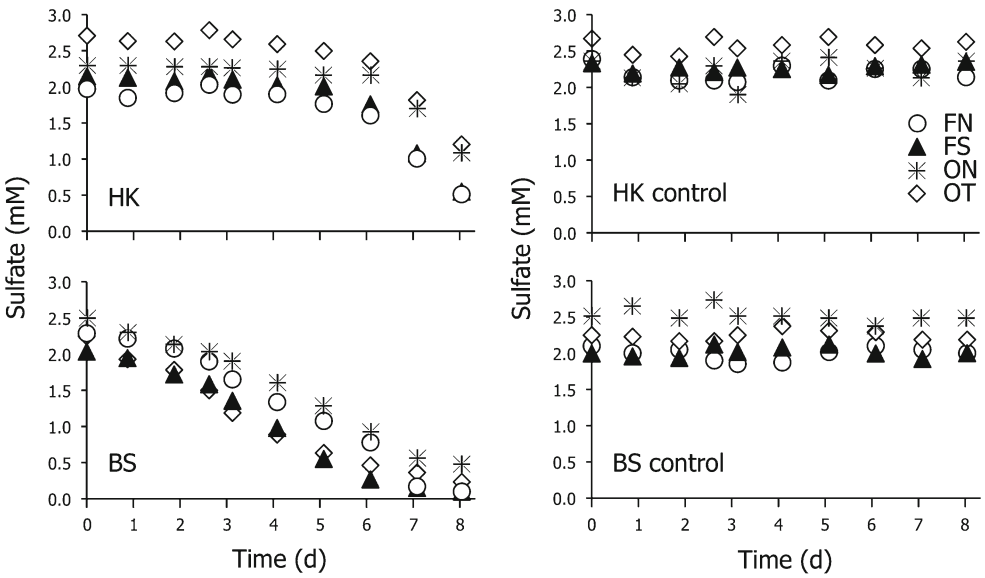

Figure 3 Sulfate depletion dynamics in slurry incubations of grassland (*HK*) and marshland (*BS*) mesocosm soils. Sulfate reduction was inhibited with molybdate in control incubations. Codes indicate the different treatments: *open circles* FN, *filled triangles* FT, *asterisks* ON, *open diamonds* OT. Values are means of four replicate microcosms, SD were omitted for graph readability

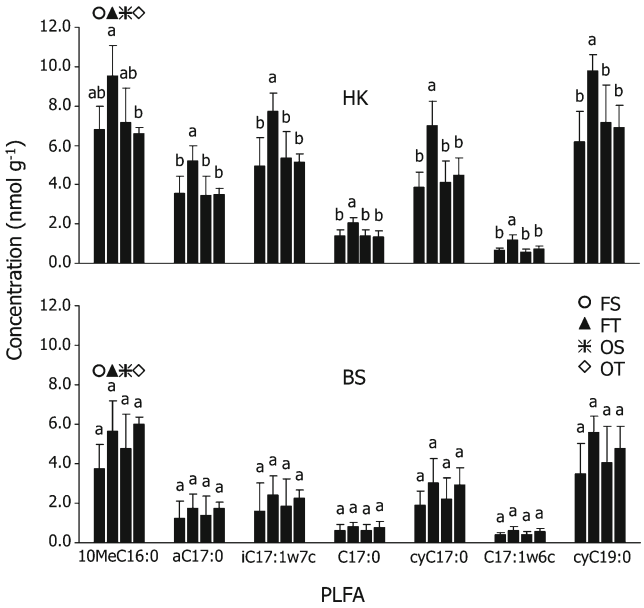

Figure 4 PLFA concentrations in grassland (*HK*) and marshland (*BS*) mesocosm soils. Only PLFAs commonly found in SRPs (see text) are shown. Codes indicate the different treatments: *open circles* FN, *filled triangles* FT, *asterisks* ON, *open diamonds* OT. Values are the means ± SD of four replicate microcosms. Per soil/PLFA, *letters* indicate grouping of significantly different means. Treatment codes sequence (identical for the different PLFAs) is indicated for the first PLFA only

PLFAs commonly found in SRPs [24], i.e., 10MeC16:0, aC17:0, iC17:1w7c, C17:0, cyC17:0, C17:1w6c, and cyC19:0; similar statistical results were obtained with all other PLFAs measured.

DGGE

The SRP community composition in grassland and marshland mesocosm soils was also investigated using SRPs-specific amplification of soil-borne DNA in combination with DGGE analyses. A total of 31 and 33 unique matches/sequences were obtained from grassland and marshland mesocosm soils, respectively, and considered for further phylogenetic and statistical analyses. All *dsrB* sequences with deduced amino acid sequence identities ≥97% were grouped into an OTU. This grouping produced a total of 12 OTUs (Table 1), two of which were represented by sequences retrieved from both soils (OTU01 and OTU02). All other OTUs were present in either grassland (OTU05, OTU06, OTU07, and OTU11) or marshland (OTU03, OTU04, OTU08, OTU09, OTU10, and OTU12) mesocosm soils. Figure 5 shows the phylogenetic affiliation of the deduced partial DSR amino acid sequences.

The largest OTU (OTU01; 28 sequences in total, 19 from grassland and nine from marshland) grouped with SRPs of the *Desulfotalea–Desulforhopalus–Desulfobulbus* line of descent (*Desulfobulbaceae*). In accordance, OTU01's most similar DSR sequences, as determined by

Table 1 OTUs of SRPs based on comparative sequence analyses of *dsrB* retrieved from grassland (HK) and marshland (BS) mesocosm soils

OTU[a]	Number of bands[b]		Inferred phylogeny[c]	Most similar DSR sequence as determined by BLAST search (accession no./% amino acid identity)	Reference
	HK	BS			
01	19	9	*Desulfotalea*, *Desulforhopalus*, *Desulfobulbus* (*Desulfobulbaceae*)	*Desulfobulbus*-related SRP (AAK15421/96–97)	[37]
02	5	10	Nonaffiliated with known SRPs (*Desulfobacteriaceae*)	*Desulfosarcina*-related SRP (AAK71956/81)	[38]
03		4	*Desulfatibacillum* (*Desulfobacteriaceae*)	*Desulfosarcina*-related SRP (AAX21261/96)	[26]
04		3	*Desulfosarcina* (*Desulfobacteriaceae*)	*Desulfosarcina*-related SRP (AAV68657/93)	[25]
05	2		*Desulfovibrio* (*Desulfovibrionaceae*)	*Desulfovibrio* sp. (AAK71942/89)	[38]
06	2		*Desulfomonile* (*Syntrophaceae*)	*Desulfomonile*-related SRP (AAV68678/92)	[25]
07	2		Nonaffiliated with known SRPs (orthologs *Firmicutes*)	*Thermodesulfobium*-related SRP (AAX21310/74)	[26]
08		2	*Desulfobacterium* (*Desulfobacteriaceae*)	*Desulfobacterium anilini* (AAQ05940/94)	[20]
09		2	*Desulfobacca* (*Syntrophaceae*)	*Desulfobacca acetoxidans* (AAQ05936/92)	[20]
10		2	*Desulfotomaculum* (orthologs *Firmicutes*)	*Desulfitobacterium*-related SRP (BAD06992/88)	[39]
11	1		*Desulfomicrobium* (*Desulfomicrobiaceae*)	*Desulfomicrobium*-related SRP (AAM03446/100)	[40]
12		1	*Desulfomonile* (*Syntrophaceae*)	*Desulfomonile tiedjei* (AAK83206/94)	[41]

[a] *dsrB* sequences with deduced DSR sequence identity ≥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] Phylogeny of *dsrB* sequences as inferred from Fig. 5 and based on 95 amino acid sequence positions

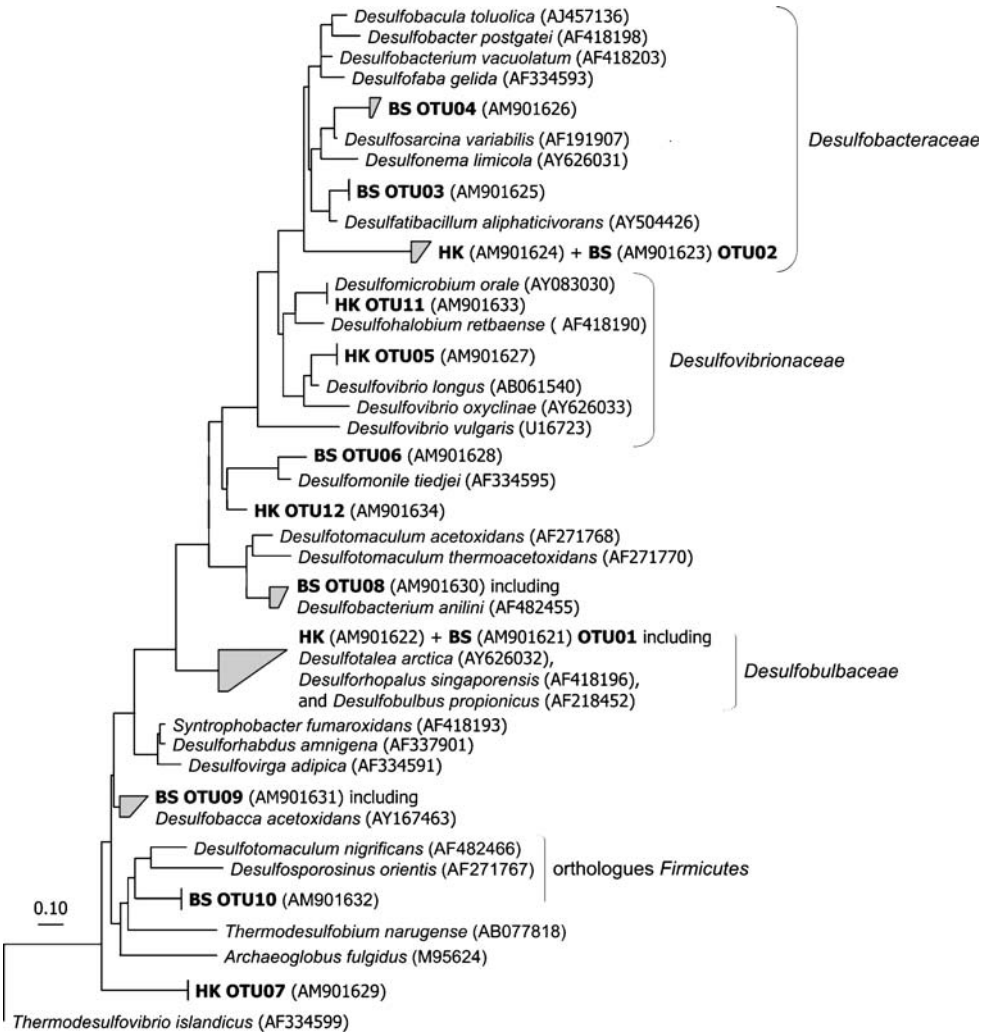

Figure 5 DSR phylogenetic tree showing the affiliation of grassland soil (*HK*) and marshland soil (*BS*) OTUs with known SRPs. Partial *dsrB* sequences were inserted one by one into a DSR core tree using the ARB parsimony tool (see text). Nucleotide sequence accession numbers are given in *parentheses*. *Bar* indicates 10% sequence divergence

BLAST search, belonged to a *Desulfobulbus*-related DSR clone obtained from groundwater at a uranium mill tailings site [25]. The other OTU shared by grassland and marshland mesocosms was OTU02 (grassland, five sequences; marshland, ten sequences). This OTU formed an independent branch within the deltaproteobacterial SRPs, different from any cultured SRP lineage (Fig. 5), and showed most sequence similarity with a *Desulfosarcina*-related DSR clone from estuarine sediments [25], although the amino acid identity was only 81% (Table 1).

The presence of *Desulfomonile*-related SRPs in both grassland and marshland soils was indicated by phylogenetic analysis of the two sequences of OTU06 (grassland) and the single sequence of OTU12 (marshland). The family *Desulfovibrionaceae* was exclusively present in grassland mesocosm soil. In particular, phylogenetic inference of *dsrB* belonging to OTU05 (two sequences) as depicted in Fig. 5 and confirmed in the GenBank revealed its affiliation with *Desulfovibrio*. In contrast, OTU11 (one sequence) showed 100% amino acid identity with SRPs belonging to the genus *Desulfomicrobium* (Table 1). Two other sequences also exclusively present in grassland and constituting OTU07 formed a deep branch in the DSR phylogenetic tree (Fig. 5) and were most similar to the DSR amino acid sequence of a *Thermodesulfobium*-related clone isolated from tropical mud [26].

The OTUs 03, 04, and 08, exclusively present in marshland mesocosms and including a total of nine *dsrB* sequences (Table 1), clustered together with *Desulfatibacillum aliphaticivorans*, *Desulfosarcina variabilis*, and *Desulfobacterium anilini*, respectively, in the DSR tree of Fig. 5 and were most similar to sulfate reducers belonging to the family *Desulfobacteraceae* present in GenBank [25, 26]. Finally, the last two OTUs comprising two *dsrB* each were exclusively retrieved in marshland mesocosms (Table 1). The first, OTU09, was related to *Desulfobacca acetoxidans*; the second, OTU10, clustered together with the representatives of the orthologs *Firmicutes.*

The overall relative abundance of different SRP groups varied between sites, as revealed by the analysis of the phylogenetic affiliation of the various *dsrB* sequences.

Representatives of the *Desulfobulbaceae* and the *Desulfobacteraceae* were the most numerous at both sites. However, the first family covered 61% of a total of 31 *dsrB* sequences from grassland soil, while the corresponding value for marshland was only 27% on a total of 33 sequences. In contrast, in marshland soil, the most frequently occurring group was the *Desulfobacteraceae* with 19 sequences out of 33 (58%), while in grassland soil, this family covered only 16% of all *dsrB* sequences. All other SRP groups present in grassland and/or marshland mesocosm soils did not exceed 10% of the total number of *dsrB* sequences.

The occurrence of OTUs according to treatments of the monoliths is summarized in Table 2. The *Desulfobulbaceae*-related OTU01 and the *Desulfobacteraceae*-affiliated OTU02, OTU03, OTU04, and OTU08 appeared generally ubiquitous between treatments. Other ubiquitous OTUs were the grassland-specific OTU07 and the marshland-specific OTU09 and OTU10, affiliated with SRPs of the lower part of the DSR phylogenetic tree, and the grassland-specific OTU11 related to the genus *Desulfomicrobium* (see Fig. 5). Interestingly, the *Desulfovibrio*-related OTU05 was observed only in the tidal treatments of grassland mesocosms. Noteworthy, OTU06 (grassland) and OTU12 (marshland), both comprising *Desulfomonile*-related *dsrB*, occurred only in the oligohaline treatments (Table 2).

Figure 6 shows the clustering of grassland (a) and marshland (b) mesocosm soils based on the 31 and 33 unique bands/matches identified in the respective DGGE profiles. The marshland samples showed more stable fingerprints when compared to the grassland samples. However, in both soils, replicate mesocosms for the different treatments tended to cluster. Accordingly, ANOSIM highlighted a significant effect of the treatment on the *dsrB*-based fingerprint of the SRP community, especially in grassland (grassland: global $R=0.7$, $p=0.01$; marshland: global $R=0.5$, $p=0.03$).

Discussion

Sulfate Depletion Rates

Sulfate depletion started immediately at the beginning of incubation with marshland soil slurries, while in grassland soil slurries, sulfate depletion started only after a lag phase of several days, regardless the particular treatment (Fig. 3). As both soils were generally rich in organic matter (14.7% in the marshland and 10.4% in the grassland; [13]) and this value even slightly increased during the growing season of June–October (Antheunisse, unpublished results), the availability of electron donors was likely sufficient to allow sulfate reduction in the slurries from both soils. Instead of organic carbon, sulfate availability might have been the factor influencing sulfate reduction in the two soil types. The marshland soil had higher concentration of sulfate compared to the grassland soil; at the start of the mesocosm experiment, sulfate concentrations in the pore water of the grassland and marshland soils were 0.5 and 3.8 mM, respectively [27], whereas the total quantities of sulfate amounted to 28 kg^{-1} soil for the grassland and 1,574 mg kg^{-1} soil for the marshland [13]. Although a direct link between in situ concentrations of sulfate and activities of SRP is not an established fact [28], lack of sulfate reduction at the

Table 2 Presence (+) and absence (−) of specific OTUs in monoliths from grassland (HK) and marshland (BS) soils treated differently

OTU[a]	HK				BS			
	FN	FT	ON	OT	FN	FT	ON	OT
01	+	+	+	+	+	+	+	+
02	+	−	+	+	+	+	+	+
03	−	−	−	−	+	+	+	+
04	−	−	−	−	+	+	+	+
05	−	+	−	+	−	−	−	−
06	−	−	−	+	−	−	−	−
07	+	+	+	+	−	−	−	−
08	−	−	−	−	+	+	+	+
09	−	−	−	−	+	+	+	+
10	−	−	−	−	+	+	+	+
11	+	+	+	+	−	−	−	−
12	−	−	−	−	−	−	+	+

FN freshwater-nontidal, *FT* freshwater-tidal, *ON* oligohaline-nontidal, *OT* oligohaline-tidal

[a] Numbering according to Table 1

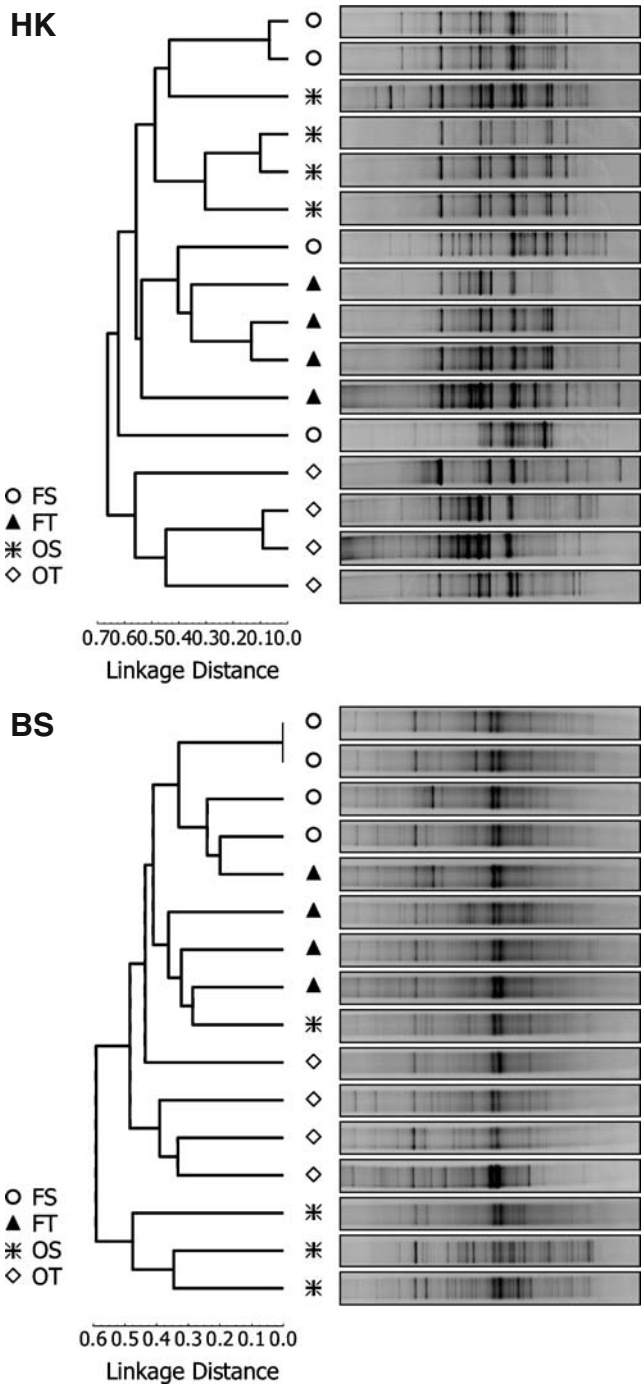

Figure 6 Clustering of DGGE profiles of *dsrB* PCR products amplified from grassland (HK) and marshland (BS) mesocosm soils. *Open circles* FN, *filled triangles* FT, *asterisks* ON, *open diamonds* OT

beginning of the sulfate-amended slurry incubation experiments might indicate the absence of actively sulfate-reducing microorganisms in the grassland soil monoliths. However, the presence of active SRPs with a syntrophic lifestyle should not be excluded [29]. When sulfate is supplied at the slurry incubation conditions, they may change their metabolism towards sulfate reduction within some days. Also, active denitrification could explain the initial absence of sulfate reduction in the slurry incubation of the grassland soil. In fact, the higher abundance of nitrate in grassland soil compared to marshland soil [13] likely promoted denitrification processes over sulfate reduction in the grassland monoliths. Higher denitrification rates were indeed measured in the grassland mesocosms compared to the marshland, irrespective of the treatment [13].

We postulated that the tidal treatment would enhance sulfate reduction in both types of soil, as flooding twice a day would create the anoxic environment suitable for the growth of these anaerobic microorganisms. However, we did not observe an effect of tide on the sulfate depletion rates. Other observations related to the marshland and grassland monoliths also contradict our hypothesis of more reduced conditions under a tidal regime. Loeb [27] observed the accumulation of reduced iron in the monoliths, but only under a nontidal regime. Denitrification was also relatively low in the presence of tide [13]. Both observations point to less reduced conditions in the tidal treatments.

A treatment effect with respect to fatty acids content was observed only in grassland soil; here, the concentration of all PLFAs measured was significantly higher in the FT mesocosms compared to the other treatments. This agrees with the relatively higher sulfate depletion rates we observed in this treatment compared to the other regimes. However, PLFAs concentrations are to be considered a measure of the total microbial biomass present in the soils and not specifically of the SRP community. Hence, differences in PLFAs concentrations between mesocosm soils are not conclusive with respect to sulfate depletion. Nevertheless, the significantly higher amounts of PLFAs under a tidal, freshwater regime in the grassland monoliths indicate that the total microbial community in these soils grows better due to tidal treatment only when the flooding water is fresh, but does not do so with oligohaline water. This implies that, in this soil, the microbial community is salt-sensitive, in contrast to the community in marshland soil. Consequently, the anticipated management scenarios will not affect all soils in the area to the same degree, with possible biogeochemical implications.

SRP Community Composition

The SRP community in grassland and marshland mesocosms was analyzed by means of DGGE profiling. Comparative sequence analysis revealed that most of the *dsrB* sequences retrieved from grassland and marshland soils were affiliated to representatives of the families *Desulfobulbaceae* and *Desulfobacteraceae*. Besides being the most represented, these groups were also ubiquitous among treatments, suggesting that they are likely the

dominant SRPs in these two soils. However, the two communities were different in the sense that the relative abundance of the two phylogenetic groups, based on *dsrB* occurrence, differed considerably between soil types. In grassland, the SRP community was dominated by the *Desulfobulbaceae* (61% of the total), while in marshland soil, the largest part of the retrieved *dsrB* (58%) were affiliated with the *Desulfobacteraceae*. The *Desulfobulbaceae* carry out an incomplete oxidation of organic substrates as they lack a mechanism for the terminal oxidation of acetyl-CoA [9]. Depending on the species, they oxidize a variety of carbon substrates to acetate or are able to perform complete fermentations. Also, members of the family *Desulfobacteraceae* are metabolically versatile, but in contrast to the *Desulfobulbaceae*, they can oxidize acetate completely to CO_2 [9]. In a recent study, the SRP communities of two mudflats characterized by contrasting salinity were compared [30]. The *Desulfobacteraceae* appeared to be the most abundant SRP phylotypes in the brackish mixing zone. In another recent study, the diversity of SRPs in sediments was investigated along a salinity gradient by means of *dsrAB* clone libraries [31]. The *Desulfobacteraceae*-related part of the clone libraries increased with salinity. Hence, the higher relative abundance of *dsrB* sequences related to the *Desulfobacteraceae* in the more recently sea-influenced and oligohaline marshland soil suggests that these organisms are probably better adapted to saline, sulfate-rich environments. In a recent biogeography study, the occurrence of *Desulfosarcina* (belonging to the family *Desulfobacteraceae*) based on microarray and DGGE analyses could be positively correlated with salinity [12]. In competition experiments between *Desulfobacter* and *Desulfobulbus* species isolated from the Ems-Dollard estuary in The Netherlands, it has been shown that the *Desulfobulbus* species are the better competitor for limiting amounts of sulfate and, therefore, the dominant species in sulfate-limited environments such as the freshwater part of an estuary [32, 33].

With respect to the occurrence of the other, less represented *dsrB* sequences found in the mesocosm soils, *Desulfovibrio*-related *dsrB* sequences were observed exclusively in replicate monoliths of the agricultural grassland soil treated with a tidal regime. The occurrence of SRPs related to this genus exclusively in the originally drier grassland soil and, in particular, in the tidal treatment is not surprising, given its selective capability to survive near oxic–anoxic interfaces [34, 35]. Noteworthy, *Desulfomonile*-related *dsrB* were found exclusively in most of the monoliths in the oligohaline basins, both in grassland and marshland soils. Also, in the biogeography study mentioned above, the distribution of uncultured *Desulfomonile*-related SRPs based on microarray and DGGE analyses could be explained by the difference in salinity of the soil [12].

Finally, the *dsrB* sequences deeply branching in the DSR tree found in grassland soil and those related to *Desulfobacca* and *Firmicutes* found in marshland soil were ubiquitous among treatments, probably representing the inactive part of the pool of SRPs in grassland and marshland soils. This idea is supported by the fact that the occurrence of microorganisms related to the *Firmicutes* and to *Desulfobacca* has generally been described in environments more fresh and with lower sulfate concentrations than marshland soil [21, 23]. With respect to the *Firmicutes*, similar results were obtained with DGGE analyses of SRP communities of freshwater riverine soils subjected to flooding with sulfate-rich water in microcosms (Miletto, unpublished results); *dsrB* sequences related to this group of gram-positive and spore-forming microorganisms [36] were abundant and ubiquitous, also in microcosms where sulfate reduction was inhibited with molybdate (Miletto, unpublished results).

Apparently, the different treatments had an effect on the composition of SRP communities, as DGGE profiles were more similar among replicates of the same treatment than between different treatments (ANOSIM). However, the relatively low global *R* value suggests that the difference between profiles might have been due to the presence or absence of a few bands corresponding to a single strain selectively enriched by the treatments and detected by the high resolution of the method used. The experimental time period (June–October) may have been not long enough to cause a substantial change in the overall SRP community composition. Supporting this observation, the variation in the overall SRP diversity was not significant enough to trigger differences between treatments in the sulfate depletion profiles.

Conclusions

Treatments did have an effect on the SRP community composition of both soils, but not on the sulfate depletion rates. The SRP community inhabiting the agricultural grassland soil was represented mainly by microorganisms related to the *Desulfobulbaceae*, while the *Desulfobacteraceae* were dominant in the freshwater marshland soil. The different composition of the two communities might have resulted from the different historical management practices at the two locations.

Our findings suggest that, if the reintroduction of salinity and tide in the Haringvliet lagoon will take place as planned, then a strong impact on the SRP community activity will not happen, at least not in the short term. However, a different response of the SRP community activity is expected in the different areas of the Haringvliet lagoon, as historical management practices and, consequently, the soil physical–chemical characteristics differ.

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