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Localization of sulfate reduction in planted and unplanted rice field soil

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Abstract. Rates of *in situ* sulfate reduction (SRR) in planted and unplanted rice field soil were measured by the $^{35}SO_4^{2-}$-radiotracer method using soil microcosms. The concentration of $^{35}SO_4^{2-}$ decreased exponentially with time. However, time course experiments indicated that incubation times of 10–30 min were appropriate for measurements of SRR using a single time point in routine assays. Unplanted microcosms showed high SRR of 177 nmol cm^{-3} d^{-1} in the uppermost centimeter where average sulfate concentrations were <33 μM. Fine scaled measurements (1 mm resolution) localized highest SRR (<100 nmol cm^{-3} d^{-1}) at the oxic/anoxic interface at 2–5 mm depth. In planted rice field soil, SRR of <310 nmol cm^{-3} d^{-1} were observed at 0–2 cm depth. Sulfate reduction rates were determined at a millimeter-scale with distance to a two dimensional root compartment. The SRR was highest at 0–1.5 mm distance to the root layer with rates up to 500 nmol cm^{-3} d^{-1}, indicating a high stimulation potential of the rice roots. SRR seemed to be mainly dependent on the *in situ* sulfate porewater concentrations. At the soil surface of unplanted microcosms sulfate concentration decreased from <150 μM to <10 μM within the first 8 mm of depth. In planted microcosms sulfate concentration varied from 87–99 μM sulfate at the 0–3 mm distance to the root layer to 48–62 μM sulfate at a root distance >4 mm from the roots.

The depth distribution of inorganic sulfur compounds was determined for planted and unplanted rice field soil. Sulfate, acid volatile sulfide (AVS) and chromium reducible sulfide (CRS) were up to 20 fold higher in planted than in unplanted microcosms. CRS was the major insoluble sulfur fraction with concentrations >1.7 μmol cm^{-3}. Organic sulfur accounted for 25–46% of the total sulfur present (269 μg/g dw) in an unplanted microcosm. The biogeochemical role of sulfate reduction for short-term accumulation of inorganic sulfur compounds (FeS, FeS_2 and S°) in rice soil was determined in a time course experiment with incubation periods of 5, 10, 20, 30 and 60 min. The relative distribution of CRS and AVS formation showed little depth dependence, whereas the formation of $^{35}S^\circ$ seemed to be the highest in the more oxidized upper soil layers and near the root surface. $AV^{35}S$ was the first major product of sulfate reduction after 20–30 min, whereas $CR^{35}S$ was formed, as $AV^{35}S$ and $^{35}S^\circ$ decreased, at longer incubation periods of >30 min.

Introduction

It is generally believed that sulfate reduction in freshwater environments is relatively unimportant for carbon mineralization because of relatively low sulfate concentrations. In marine habitats, on the other hand, sulfate reduction contributes up to 70% of the total organic carbon mineralized (Jorgensen 1982; Howarth 1984; Skyring 1987). Whereas sulfate reduction in marine habitats has been intensively studied (Jorgensen 1977a; Howarth & Giblin 1983; Thode-Andersen & Jorgensen 1989; Canfield 1989a; Thamdrup et al. 1994), the role of sulfate reduction in freshwater environments remains unclear. Several studies of freshwater environments indicate that sulfate reduction rates can be as high as in marine habitats (e.g. Ingvorsen et al. 1981; Bak & Pfennig 1991; Sinke et al. 1992; Urban 1994; Hadas & Pinkas 1995). However, there has been not much effort in the comparative characterization of sulfate reduction in vegetated and unvegetated freshwater sediments (Fukui & Takii 1990b).

Wetland rice is cultivated essentially under submerged conditions during its growth cycle. Many of the characteristics of rice wetlands, including the biological, physical, and chemical processes which occur in them, are similar to those of natural wetlands and freshwater sediments. Flooding of soil results in sequential reduction of available electron acceptors until the bulk of the soil is dominated by methanogenesis (e.g. Ponnamperuma 1978; Yamane 1978; Tian-ren 1985; Lefroy et al. 1993). With respect to O_2 supply the soil of unplanted flooded rice fields behaves similarly as freshwater sediments exhibiting an O_2 penetration to 3–4 mm depth (Frenzel et al. 1992), and an anoxic reduced zone below. Since the gas transport through flooded soil is very slow, the rice roots are supplied with O_2 via the aerenchyma system of the plant (Barber et al. 1962; Lee et al. 1981; Ando et al. 1983). Some of this internally transported O_2 is lost by diffusion to the surrounding soil, which results in oxic conditions close to the root surface (Armstrong 1971; Trolldenier 1988; Frenzel et al. 1992; Gilbert & Frenzel 1995). Therefore, the extension of the rice root system affects the chemistry of flooded rice soils (Begg et al. 1994; Kirk & Bajita 1995).

For rice field soil an intense sulfur cycling has been proposed (Freney et al. 1982; Lefroy et al. 1992). Sulfur can be supplied by fertilizer, by rain fall or it can be released from plant residues (Lefroy et al. 1992; Lefroy et al. 1994). However, the level of total sulfur and sulfate-sulfur in wetland rice soils varies widely (Lefroy et al. 1992), and the dynamics of the sulfur cycling of rice field soil have not been studied in detail. There has been some interest in understanding the cycling of sulfur in the rice field soil (e.g. Freney et al. 1982). Thus, sulfur is important in rice nutrition, e.g. for the synthesis of amino acids and proteins, which account for approximately

90% of organic S in the plant (Lefroy et al. 1992). In addition, high sulfide concentrations can be toxic for the rice plant (Prade et al. 1989; Jacq et al.1991), and thus can drastically decrease the yield of the crop. Sulfur is cycled between sulfide, as the most reduced sulfur compound, and sulfate, as the most oxidized one. The reduction of sulfate depends on the availability of organic substrates or H_2 that serve as electron donors. Organic substrates are supplied by degradation of soil organic matter and exudation by rice roots (Jacq 1975; Patnaik 1978). The degradation of organic matter is enhanced by the generally high soil temperatures in wetland rice fields. The oxidation of reduced sulfur compounds depends on the availability of oxidants that serve as electron acceptors such as O_2 or other oxidized compounds like nitrate, manganese(IV) or ferric iron (e.g. Canfield 1989b; Canfield et al. 1992; Elsgaard & Jorgensen 1992; Thamdrup et al. 1993). In flooded soil, oxidants are only available at the soil surface and in the rhizosphere, where O_2 leaks from the rice roots. In fact, it has been shown that porewater concentrations of sulfate and thiosulfate in planted microcosms were 20 to 40 times higher than in unplanted microcosms, indicating oxidation of reduced sulfur compounds to sulfate in the vicinity of rice roots (Wind & Conrad 1995). In drained soil, on the other hand, oxidants are much better available than in flooded soil, and reduced sulfur compounds are almost quantitatively oxidized to sulfate which then serves as electron acceptor during the subsequent flooding period.

Only a few publications have actually discussed sulfate reduction in flooded rice soils (i.e. Jacq 1975; Freney et al. 1982; Jacq et al. 1991; Wind & Conrad 1995). The sulfate and thiosulfate reduction potentials in planted microcosms were significantly higher than in unplanted microcosms (Wind & Conrad 1995). Although the site of sulfate reduction remained unclear, sulfate reduction was likely to occur at the oxic/anoxic interface, or even within oxic layers, where sulfate concentrations were highest and where high numbers of sulfate-reducing bacteria (SRB) have been observed (Wind & Conrad 1995). The survival and the activity of SRB in oxic zones have been reported for several freshwater and marine environments (Wakao & Furusaka 1976; Fukui & Takii 1990a; Furusaka et al. 1991; Canfield & Marais 1991; Jorgensen & Bak 1991; Fründ & Cohen 1992; Norsker et al. 1995). In fact, sulfate reduction is today no longer described as an entirely anaerobic process, since several sulfate reducers have been shown to oxidize reduced sulfur compounds with O_2 or nitrate as electron acceptor (Dilling & Cypionka 1990; Dannenberg et al. 1992; Krekeler & Cypionka 1995). Therefore, high sulfate reduction activity can also be assumed to occur in oxic zones in the rice field soil.

The objective of the present study was to clarify whether the rhizoplane is indeed a zone of enhanced sulfate reduction. Therefore, we measured sulfate reduction rates in planted and unplanted rice field soil at high resolutions of

1–2 mm. It was checked, whether the techniques usually applied for measurement of sulfate reduction rates were also appropriate for submerged rice field soil. In addition, we measured gradients of several inorganic sulfur compounds in planted and unplanted rice field soil (H_2S + FeS (AVS); FeS_2 + S° (CRS); S° (elemental sulfur); Fossing & Jorgensen 1989), and their dynamics as short term products of sulfate reduction.

Materials and methods

Soil microcosms and soil sampling. Soil samples were taken in the wetland rice fields of the Italian Rice Research Institute in Vercelli (northern Italy). The soil was sampled in April 1993 from yet unflooded fields and stored in dry state at room temperature in polyethylene containers. The sampling site, soil characteristics and management were described earlier (Schütz et al. 1989). The dry soil was sieved ($<$2 mm) and fertilized with nitrogen, phosphate and potassium, as described elsewhere (Wind & Conrad 1995). We used three different types of soil microcosms: (1) unplanted microcosms consisting of plastic tubes (3$\times$15 cm) were prepared in 36 parallels. The tubes were sealed with rubber stoppers at the bottom and each was filled with about 200 g dry weight (dw) of rice field soil. (2) Planted microcosms were prepared from 10 kg (dw) of rice field soil filled into a plastic container (40$\times$40$\times$20 cm). Rice seeds (*Oryza sativa*, var. Roma, type japonica) were germinated on water-soaked cotton wool and 36 seedlings were placed in approximately 5 cm distance to each other when the shoots had reached a length of 30–50 mm. (3) Microcosms with a defined root compartment were prepared in four parallels consisting of a polystyrole container (20$\times$10$\times$13 cm) that had four holes ($\emptyset$ = 3 cm) at 4 and 8 cm depth on two opposite sides (Figure 1). The root compartment was a bag (size 80$\times$80 mm, 3 mm thick) made of nylon cloth (25 μM mesh) placed in the middle of the polystyrole container (Gilbert & Frenzel 1995). The holes were sealed with rubber stoppers. Each container was constructed of two matching counterparts that were hold together with 4 clamps and sealed with silicone rubber. The nylon mesh was freely permeable to water and nutrients, but impermeable to the roots, so that the horizontal distance from the root compartment could be defined as the average distance to the root surface. The polystyrole container was then filled with 3.5 kg dw soil and then flooded. After one week of preincubation, six rice seedlings (30–50 mm length) were planted into the bag. The rice plants in the different microcosms were grown for 13 weeks at 25 °C and at an average light intensity of 40 W per m^2 using a 12/12 h light/dark cycle. Likewise, the incubation period for all unplanted microcosms was also 13

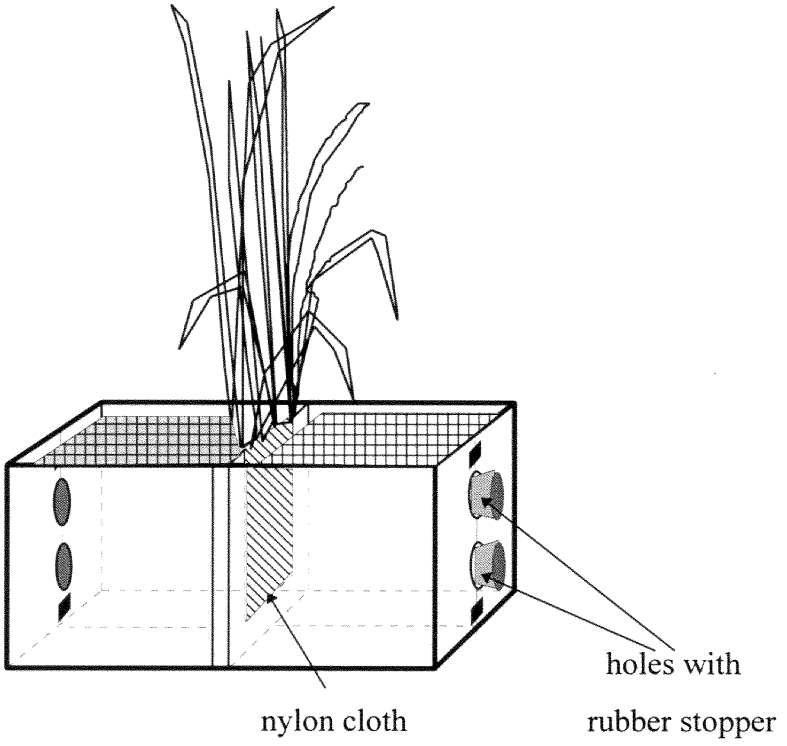

Figure 1. Polystyrole container (20×10×13 cm; w, d, h) constructed for horizontal core sampling.

weeks at 25 °C. All microcosms were initially flooded with tab water until they were waterlogged. Further watering was done with deionized water.

For chemical analysis and measurement of sulfate reduction rates unplanted microcosms were cut into horizontal soil sections of 1 cm thickness as described previously (Wind & Conrad 1995). For a resolution at the millimeter scale the microcosms were cut with a 0.2 mm stainless steel wire after stepwise pushing the soil out of the plastic tube with a microtome. Planted microcosms were sampled by cutting the leaves of one rice plant and taking a vertical soil core (⌀ = 2.6 cm; i.d.) of approximately 12 cm depth with the plant in its center. This core was then processed by sectioning as described above. Planted microcosms with a root compartment were sampled by taking four horizontal cores (⌀ = 2.6 cm; i.d.) through the drillings of the polystyrole container at the same time. Two thin copper plates (5×10 cm) were then pushed down along the root compartment. The cores were fixed by pressing the opposite cores against each other. The two parts of the container were taken apart and all the bulk soil detached to the outside of the core was

removed. The cores were then cut into 1.5 mm sections as described above. Millimeter sectioning was done in a glove box under a nitrogen atmosphere, except the experiments where $^{35}SO_4^{2-}$ was added. For these studies the time necessary for preparing and sectioning of the microcosms was too long using the glove box. Instead, the sectioning of the cores was done under a flow of nitrogen to prevent oxidation of the soil surface.

Concentration profiles. Concentration profiles of dissolved sulfur anions (SO_4^{2-}, $S_2O_3^{2-}$), acid volatile sulfide (AVS), chromium reducible sulfide (CRS), elemental sulfur (S°), porosity and density of the soil were measured in the uppermost 8 cm of planted and unplanted microcosms in triplicate experiments. The analysis of AVS, CRS, SO_4^{2-} and $S_2O_3^{2-}$ was carried out as described previously (Wind & Conrad 1995). Elemental sulfur was extracted from an aliquot of the soil sample by methanol (Blair et al. 1993; Ferdelmann pers. comm.). Therefore, the soil sample was suspended in 20% Zn-acetate (1:4 wt/wt) for preservation and centrifuged at 4000 rpm. Pure methanol (MeOH) was added to the soil pellet (1:4 vol./vol.) and horizontally shaken over night at 120 rpm and 25 °C. The suspension was centrifuged for 10 min at 4000 rpm and the sulfur-containing supernatant was filtered through a 0.45 μM PTFE filter (Acrodisc, Gelman Sciences, USA). Sulfur in the filtrate was analyzed by reversed-phase HPLC (Sykam, Germany), using a Zorbax ODS (Knaur, Berlin, Germany; 120×4 mm) separation column (Moeckel 1984; Ferdelmann in prep.), at 25 °C and 1 ml/min with MeOH/H_2O (90/10, vol%) as eluent. Total sulfur was analysed from 13-week-old unplanted rice field microcosms at 5 cm depth by soil ignition at 550 °C using the method of Tabatabai et al. (1988).

The density and the porosity of the rice field soil were determined by measuring the weight loss of core segments of known weights and volumes after drying for 24 h at 105 °C.

Sulfate reduction rates. To measure sulfate reduction rates (SRR) in planted and unplanted rice microcosms we injected trace amounts of $^{35}SO_4^{2-}$ into the soil cores and incubated at 25 °C for 10–30 min (Jorgensen 1978a; Howarth & Jorgensen 1984; Fossing & Jorgensen 1989). Since a better reproducibility is achieved when small amounts of "cold" $^{32}SO_4^{2-}$ were added as a carrier (Bak 1988; Bak & Pfennig 1991), the stock solutions of $^{35}SO_4^{2-}$ were prepared by dilution of $Na_2{}^{35}SO_4$ (37 kBq μl^{-1}) with pore water that was obtained from unplanted and planted soil microcosms and sterilized by autoclaving, i.e. ST1 containing approximately 30 μM and ST2 containing approximately 70 μM "cold" SO_4^{2-}, both diluted to 3.7 kBq μl^{-1}. The sulfate concentrations of these stock solutions were typical for the average sulfate concentration in the

pore water of unplanted and planted soil, respectively. Thus, it was avoided as best as possible to affect the SRR by a change of the sulfate concentration at the point of injection.

For measuring SRR in unplanted microcosms at 1 cm resolution, 3 μl of ST1 (12 kBq $^{35}SO_4^{2-}$) were injected horizontally through silicone stoppered ports at 1 cm intervals starting at 0.5 cm depth. For a higher resolution of SRR measurements in unplanted microcosms, three vertical injections each of 10 μl (ST1, 111 kBq) were made as continuos lines through the upper 15 mm. Similarly, in planted cores 3×15 μl of ST2 (167 kBq) were injected vertically at three positions as a line from the top of each of three cores down to 8 cm depth. For microcosms with a root compartment horizontal cores were pushed at opposite sides towards the root compartment, and 3×10 μl of ST2 (111 kBq) were injected into the 15 mm layers adjacent to the root plane. After incubation for 10 min, the cores were removed by detaching the two parts of the polystyrole container. The cores were then immediately sectioned into 1.5 mm sections as described above. The sections of two opposite cores were pooled together. In order to stop sulfate reduction and to fix the formed sulfides, each section was rapidly transferred into a centrifuge tube with 20% Zn-acetate (1:4 wt/wt). The incubation time was defined as the period from injection until the transfer into the Zn-acetate solution. To account for the exchange of radiolabel between different ^{35}S compounds, time zero samples were included (Fossing et al. 1992). For these samples, 1 cm-sections of different depths of unplanted and planted rice field soil were first transferred into Zn- acetate before 3 μl of ST1 (12 kBq) were added to each sample. All samples were immediately frozen until separation of ^{35}S-labeled compounds: SO_4^{2-}, AVS, CRS and S°.

The thawed soil samples were centrifuged at 4000 rpm for 5 min. The supernatant (100 μl subsample) was analyzed for radioactive $^{35}SO_4^{2-}$ by liquid scintillation counting (Hewlett Packard, U.S.A.). The soil pellet (1–2 g aliquots) was used for separation of AVS and CRS by following the destillation procedure described elsewhere (Howarth & Jorgensen 1984; Fossing & Jorgensen 1989; Fossing & Jorgensen 1990a). Tiny amounts of pulverized FeS_2 and 1–2 ml of a 10 mm ZnS solution were added before destillation to improve the recovery of radioactivity in CRS and AVS, respectively (Fossing & Jorgensen 1989). Another subsample of the soil pellet was used for the extraction of S° with MeOH as described above. After centrifugation (4000 rpm, 10 min) an aliquot of the supernatant was transferred to a vessel containing 10 ml of cyclohexane and 20 ml water. For extraction of S° the vessel was vigorously shaken for 5 min. The water phase containing $^{35}SO_4^{2-}$ was discarded. The extract was washed with water two times by repeating the procedure. The cyclohexane extract was transferred into a scintillation vial,

weighted and evaporated over night. The sulfur residue was dissolved in 5 ml of 100% MeOH, mixed with 5 ml of scintillation fluid (Quicksafe A, Zinsser Analytic, Germany) and counted in the scintillation counter. Quench correction was done by external standardization.

Sulfate reduction rates were calculated by two different models. The first one assumes that the rate is a linear function of time and was described by Jorgensen (1978a):

$$SRR = \frac{[\mathrm{SO_4^{2-}}] \cdot Ar \cdot \alpha}{At \cdot t} \qquad [\text{nmol } \mathrm{SO_4^{2-}} \text{ cm}^{-3} \text{ day}^{-1}] \tag{1}$$

where: $[\mathrm{SO_4^{2-}}]$ = the actual sulfate concentration [nmol $\mathrm{cm^{-3}}$]; A_r, = the radioactivity of the reduced ^{35}S-compounds (AV^{35}S, CR^{35}S, and ^{35}S°) per unit volume [dpm $\mathrm{cm^{-3}}$]; A_t = the total radioactivity of ^{35}S injected per unit volume [dpm $\mathrm{cm^{-3}}$]; α = the isotope fractionation factor (α = 1,03 − 1,06) (Jorgensen 1978b), and t = the incubation time [d].

The second model assumes that the rate is a logarithmic function of time and was described by Urban et al. (1994):

$$SRR = -k \cdot [\mathrm{SO_4^{2-}}] \cdot \alpha \qquad [\text{nmol } \mathrm{SO_4^{2-}} \text{ cm}^{-3} \text{ day}^{-1}] \tag{2}$$

where: k = first order rate constant [$\mathrm{d^{-1}}$]. The rate constants were obtained as the slopes of plots of $\ln(^{35}\mathrm{SO_4^{2-}}_{\text{ recovered}}/^{35}\mathrm{SO_4^{2-}}_{\text{ injected}})$ versus time, during the phase of logarithmic $^{35}\mathrm{SO_4^{2-}}$-depletion. Time course experiments in triplicates were done for 5, 10, 20, 30 and 60 min as described elsewhere (Bak 1988; Thode-Andersen & Jorgensen 1989).

Results

Profiles of porosity and bulk density were determined in unplanted and planted microcosms. In both microcosms porosity was highest in the upper soil layer where bulk densities were relatively low. A profile at a millimeter scale in unplanted microcosms showed at the surface (0–2 mm depth) an average porosity of 60% and an average density of 1.2 g $\mathrm{cm^{-3}}$ that changed to a porosity of 48% and a density of 1.5 g $\mathrm{cm^{-3}}$ at $>$15 mm depth, respectively (data not shown). Because of the formation of a root mat at the surface of the microcosms, bulk density and porosity of planted microcosms showed a relatively high porosity of $<$66% and a relatively low bulk density of $>$1.18 g $\mathrm{cm^{-3}}$ in the surface layer (0–1 cm). At a depth of 4–8 cm the porosity decreased to 53% and the density increased to 1.5 g $\mathrm{cm^{-3}}$ and did not change significantly with increasing distance to the root layer.

The sulfate concentrations in the floodwater were in the range of 50–165 μM. Averaged sulfate concentrations in the porewater of the uppermost 0–1 cm layer of unplanted and planted microcosms were 33 μM and 61 μM, respectively, and decreased gradually with depth reaching at 8 cm depth concentrations of 4 and 8 μM, respectively (Figure 2). Fine resolution at a millimeter scale in the surface layer (0–15 mm) of unplanted microcosms showed a steep sulfate concentration gradient. Sulfate concentrations were 165 μM at the surface and decreased gradually to <5 μM at a depth of 15 mm (Figure 3). Unlike previous studies, where thiosulfate porewater concentrations of planted microcosms were up to 150 μM (Wind & Conrad 1995), no thiosulfate was observed in either planted or unplanted bulk soil.

A vertical profile of insoluble sulfur fractions, AVS, CRS and S° for the uppermost 10 cm is shown in Figure 2. CRS concentrations in both planted and unplanted systems were 10 times higher than AVS concentrations and exceeded 1.7 μmol cm^{-3}. In the upper 3 cm, AVS concentrations were significantly higher in planted than in unplanted microcosms. In this layer $>$70% of the root dry weight was observed (determinations see Bosse 1995). Concentrations of S° were in the range of 50–200 nmol cm^{-3}, and thus were similar to concentrations of AVS. Elemental sulfur showed no significant depth distribution. However, S° concentrations of unplanted microcosms were up to 10 times higher than those of planted microcosms. The total sulfur content was 269 ($\pm$19.3) μg per g of dry soil. The thus resulting fraction of organic sulfur was in the range of 24.7–46.4% in the depth of 5 cm of an unplanted microcosm.

Time course experiments of sulfate reduction were done in unplanted microcosms to check the validity of equation (1) and (2) used for the calculation of SRR (Figures 4 and 5). Because of low sulfate concentrations and thus high specific radioactivities, relatively large fractions ($>$25%) of the $^{35}SO_4^{2-}$ were reduced during the relatively brief incubation periods of 5–60 min (Figure 4). Equation (1) assumes a linear decrease of $^{35}SO_4^{2-}$ with time. However, the decrease of $^{35}SO_4^{2-}$ was not linear with time for most of the incubation period. Only during a period of approximately 5 to 30 min after injection of the radiolabel, $^{35}SO_4^{2-}$ decreased linearly with time (Figure 4). If we nevertheless assume a time-linear sulfate reduction and calculate SRR according to equation (1), we obtain different rates for increasing periods of incubation (Table 1). Initially, SRR were low, increased to a maximum after 10–30 min at different depths and then decreased again.

Equation (2) assumes a logarithmic decrease of $^{35}SO_4^{2-}$ with time. Indeed, plotting the data on a semi-logarithmic scale resulted in linearity for incubation between 5 and 30 min (Figure 5). The determination of the rate constant from the linear part of the plots allowed the calculation of

Table 1. Sulfate reduction rates (SRR) determined in a time course experiment with samples from three different depths of an unplanted microcosm. The SRR were calculated by two different models and are given in nmol cm^{-3} d^{-1}. $^{(1)}$SRR calculated from equation (1). $^{(2)}$SRR calculated from equation (2); (± standard deviation of the mean, $n = 3$).

Depth [cm]	$SRR^{(1)}$ 0–5 min	$SRR^{(1)}$ 0–10 min	$SRR^{(1)}$ 0–20 min	$SRR^{(1)}$ 0–30 min	$SRR^{(1)}$ 0–60 min	$SRR^{(2)}$
0–1	19.84 (±13.50)	124.40 (±87.65)	84.15 (±16.52)	177.33 (±28.17)	89.82 (±16.49)	135.43 (±36.29)
1–2	4.32 (±3.60)	22.95 (±4.08)	63.82 (±16.75)	72.51 (±37.71)	32.93 (±3.82)	106.38 (±0.57)
3–5	0.81 (±0.12)	15.82 (±5.00)	21.36 (±1.34)	20.02 (±5.78)	11.95 (±1.49)	26.85 (±1.72)

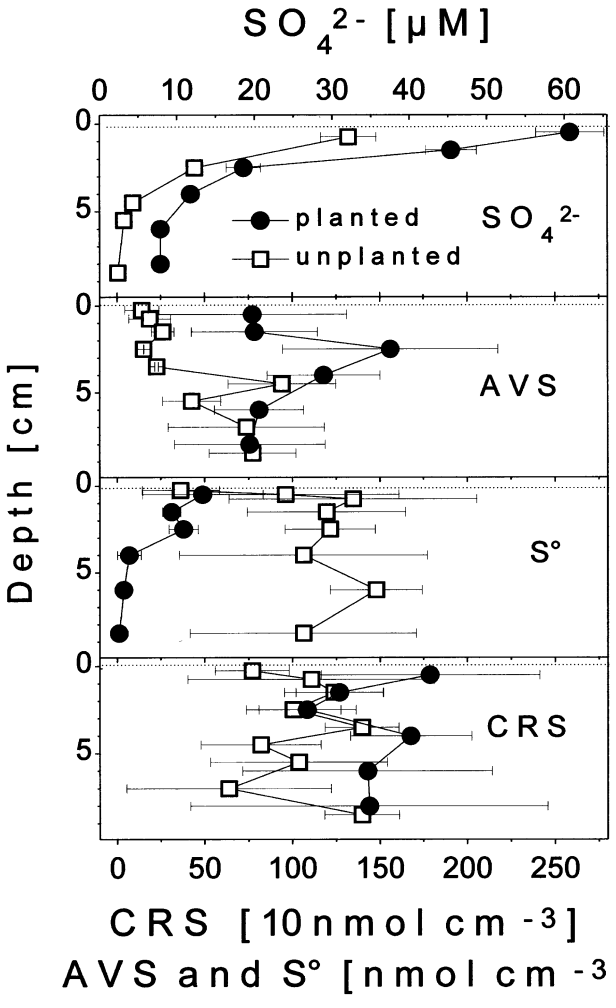

Figure 2. Depth distribution of acid volatile sulfide (AVS), elemental sulfur (S°), and chromium reducible sulfides (CRS) in 13 weeks-old unplanted and planted microcosms and (error bars = standard error, $n = 3$).

SRR for the different depths of the soil as the product of the slopes of $\ln(^{35}SO_4^{2-}{}_{\text{recovered}}/^{35}SO_4^{2-}{}_{\text{injected}})$ vs. time (Table 1). Therefore, the slopes were fitted with the maximum coefficient of regression in this time period. The SRR obtained with equation (2) were similar to those obtained with equation (1) if the latter was applied to incubation periods of 10–30 min. However, the data from the oxidized surface layer (0–1 cm depth) showed a relatively large scatter between the triplicate cores (coefficient of variation ±20–70%) and thus were relatively unreliable.

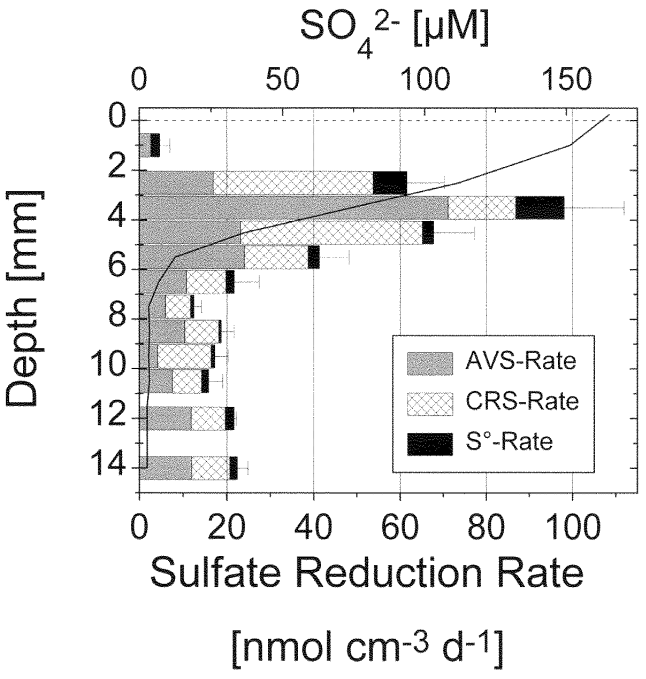

Figure 3. Distribution of radiolabel from $^{35}SO_4^{2-}$ reduction (SRR = sulfate reduction rates) in the pools of AVS, CRS and S° of 13-weeks-old unplanted microcosms at milimeter resolution. Incubation time 10–30 min. Each column represents the mean of triplicate experiments (error bars = standard deviation).

Short-term products of $^{35}SO_4^{2-}$ reduction were measured in unplanted microcosms at three different depths and for 5, 10, 20, 30 and 60 min of incubation, i.e. AV^{35}S, CR^{35}S and ^{35}S° (Figure 4). AV^{35}S was the more important product of sulfate reduction during the first 20 min, but then the proportion of CR^{35}S increased. After 60 min of incubation a significant fraction of the reduced ^{35}S (up to 70%) was recovered as CR^{35}S at all depths. In the reduced zone below 1 cm depth, CR^{35}S was recovered in similar proportions as AV^{35}S. Elemental sulfur (^{35}S°) was only important as short term product of sulfate reduction during the first 10 min when it represented up to 61% of the products. However, its fraction decreased to 10–26% at 60 min, probably due to further reduction and generation of $Fe^{35}S_2$. Only in the oxidized surface layer (0–1 cm depth) the fraction of S° was always >26%, which might be due to reoxidation of $H_2^{35}S$ to ^{35}S°.

A coarse vertical profile (>1 cm sections) of *in situ* SRR was determined in planted and unplanted microcosms (Figure 6). Sulfate reduction rates were determined after incubation for 10–30 min from a single time point using equation (1). The results show that SRR were highest at the surface layer (0–2

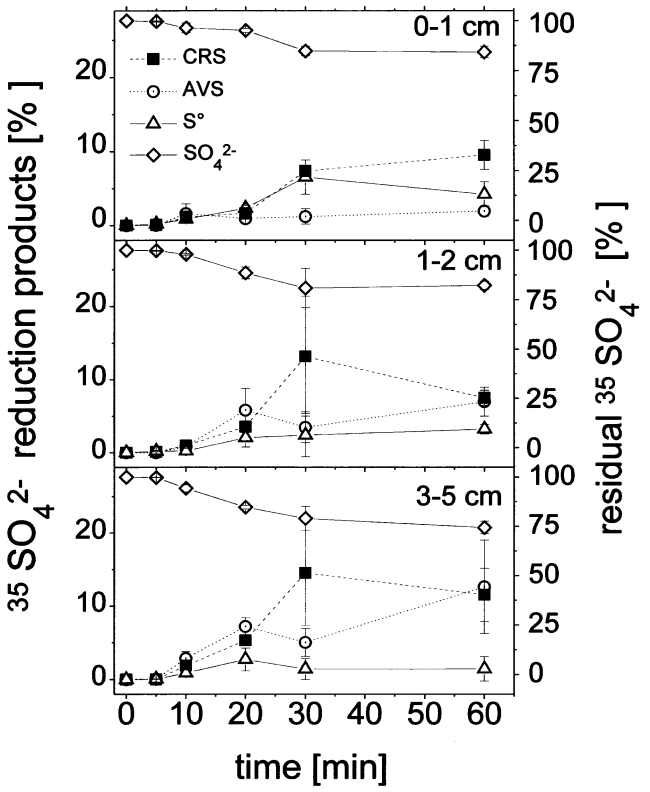

Figure 4. Time course experiment of $^{35}SO_4^{2-}$ reduction and generation of radiolabeled reduced sulfur species in 13 weeks-old unplanted microcosms at three different depths. Each point represents the mean of triplicate experiments (error bars = standard deviation).

cm) and decreased with depth. Furthermore, SRR were higher in planted than in unplanted microcosms. In unplanted microcosms maximum rates ($<$177 nmol cm^{-3} d^{-1}) were found in the uppermost 0–1 cm layer. Cores of planted microcosms showed highest sulfate reduction rates ($<$310 nmol cm^{-3} d^{-1}) in the uppermost 0–2 cm layer. Similarly as in the time course experiments with unplanted microcosms (Figure 4), a large fraction of $^{35}SO_4^{2-}$ was reduced to CR^{35}S and ^{35}S° in planted microcosms.

Since sulfate reduction rates were highest in the uppermost layer of unplanted microcosms and in the presence of rice roots, it was interesting to have a higher resolution of the distribution of SRR in the surface layer and at the rice root. Therefore, fine-scaled (1–2 mm resolution) sulfate reduction rates were measured. The upper 0–15 mm of unplanted microcosms showed a distinct maximum of SRR at 3–5 mm depth (Figure 3). At the 0–2 mm surface layer SRR were close to zero and increased to a rate of $>$98 nmol cm^{-3} d^{-1}

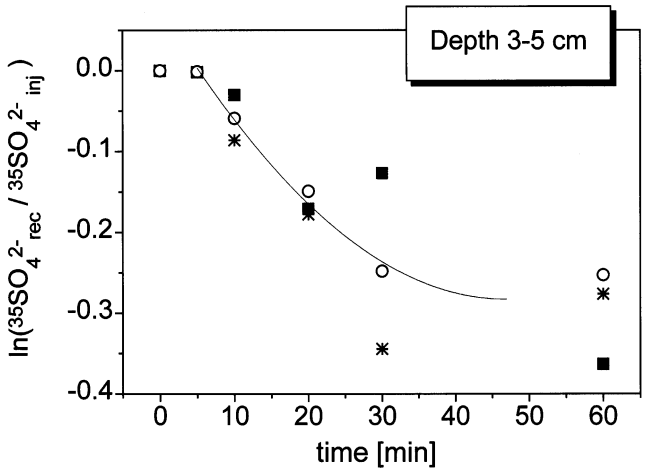

Figure 5. Time course of sulfate reduction at 3–5 cm depth in three replicates.The line is based on nonlinear regression through the mean of the data for the incubation period between 5 and 30 min.

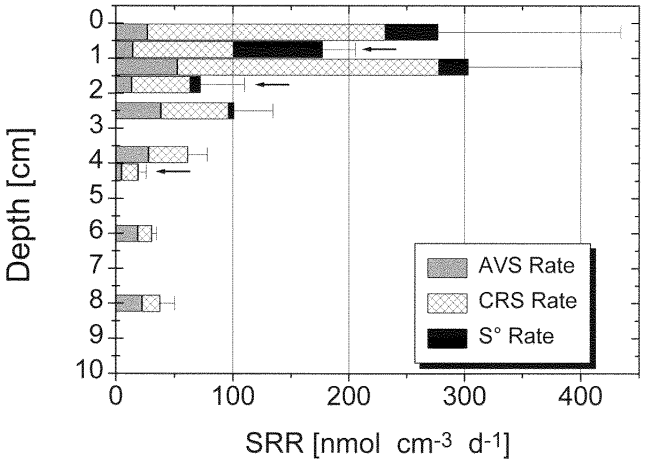

Figure 6. Distribution of radiolabeled products from $^{35}SO_4^{2-}$ reduction in the pools of AVS, CRS and S° in 13 weeks-old unplanted and planted microcosms. Arrows indicate the unplanted microcosms. Incubation time was 10–30 min. Each column represents the mean of triplicate experiments (error bars = standard deviation, SRR = sulfate reduction rates).

at 3–4 mm depth. Interestingly, $^{35}S°$ made up only a minor fraction (<13%) of the products of $^{35}SO_4^{2-}$ reduction. Instead $AV^{35}S$ and $CR^{35}S$ represented the largest fractions. This result is in contrast to the experiments shown in Figures 4 and 6, where $^{35}S°$ and $CR^{35}S$ made up one the largest fractions. Fine-scale SRR were also measured in the microcosms with a root compart-

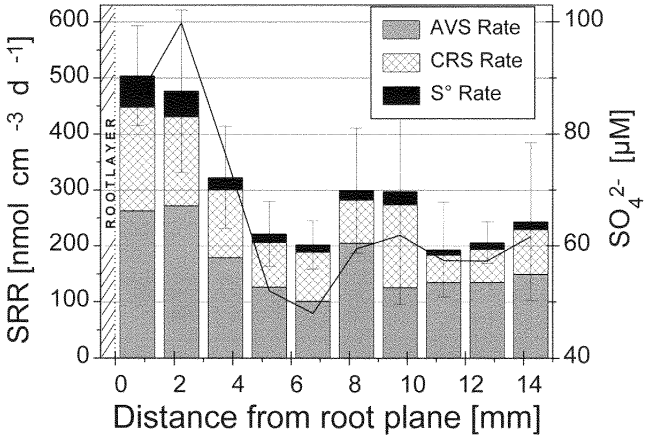

Figure 7. Horizontal distribution of radiolabeled products from $^{35}SO_4^{2-}$ reduction in the pools of acid volatile sulfide (AVS), chromium reducible sulfide (CRS, mainly FeS_2), and elemental sulfur (S°) in 13 weeks-old planted rice microcosms. Incubation time was 10–30 min. Each column represents the mean of four replicate experiments (error bars = standard deviation). Sulfate concentration (—) is the mean of four replicates at 4 and 8 cm depth. The coefficients of variation was in the order of ±4–16% for each depth (SRR = sulfate reduction rates).

ment horizontally from the root plane at 4 and 8 cm depth. The SRR observed in the two different depths showed a similar profile and thus were averaged (Figure 7). Figure 7 also shows the distribution of radioactivity in the AVS, CRS and S° fractions after 10–30 min of incubation and the average sulfate concentrations (n = 4). Sulfate concentrations at the root plane (0–3 mm) were 90–100 μM and thus significantly higher than in the bulk soil at $>$5 mm distance (u-test, α = 0.05). Sulfate concentrations at 4 cm depth were 20–40 μM higher than those at 8 cm depth. The root dry mass inside the flat root compartment was 2.8 ($\pm$0.2) mg cm^{-2} at 4 cm depth and 2.4 ($\pm$0.5) mg cm^{-2} at 8 cm depth, respectively. Correlated to the sulfate concentrations, SRR at the root surface were highest (500 nmol cm^{-3} d^{-1}), and decreased in the bulk soil to 200–300 nmol cm^{-3} d^{-1}. In general, AV^{35}S was the main product of $^{35}SO_4^{2-}$ reduction (50–70%), while CR^{35}S (20–40%) and $^{35}S^\circ$ (5–10%) were only minor products. However, the portion of $^{35}S^\circ$ among the products of $^{35}SO_4^{2-}$ reduction consistently increased to the root plane, indicating a higher reoxidation of H_2S to S° at the root surface. By contrast, total S° showed no significant distribution in neither the horizontal resolution at the root plane or the vertical depth resolution of planted and unplanted microcosms (data not shown).

Discussion

The measurement of SRR with $^{35}SO_4^{2-}$ in different natural habitats has been done for decades and the techniques used were improved by several investigators (Sorokin 1962; Jorgensen 1978a; Howarth & Teal 1979; Howarth & Jorgensen 1984; Hordijk et al. 1985; Thode-Andersen & Jorgensen 1989; Fossing & Jorgensen 1989). One procedure assumes time-linear kinetics of $^{35}SO_4^{2-}$ depletion. This model (equation (1)) is usually applied in marine sediments where sulfate concentrations are high and thus likely to be not rate-limiting. Prerequisites of the application of equation (1) are that during incubation sulfate concentration decreases negligibly, specific radioactivity remains constant (changes $<1\%$) and no reoxidation occurs (Howarth & Merkel 1984; Moeslund et al. 1994; Fossing 1995). In our experiments, reduction of $^{35}SO_4^{2-}$ was better described by exponential than by linear kinetics (equation (2)), such as proposed for freshwater environments where sulfate concentrations can be rate-limiting (Hordijk et al. 1985; Marnette et al. 1992; Urban et al. 1994). The use of equation (2) is based on the assumption of steady state conditions (i.e. sulfate concentrations stay constant) and that reoxidation of H_2S does not affect the specific radioactivity of sulfate. However, the measurement of extended time courses that are necessary for the application of equation (2) are very cumbersome for routine studies. Measurement of SRR based on single time points is easier. The comparison of the two methods (equation (1) and (2)) showed that equation (1) gave reliable results when the sulfate reduction was stopped after incubation periods of 10–30 min. Since sulfate depletion showed a lag phase during the first 5 min and nonlinearity after 30 min of incubation, the rates calculated from equation (1) for these incubation times were underestimated.

As discussed in other studies, the nonlinearity of $^{35}SO_4^{2-}$ depletion after a longer incubation (here >30 min) could be the result of reoxidation of reduced ^{35}S, e.g. by ferric iron (see below), to sulfate (Roden & Tuttle 1993; Moeslund et al. 1994; Thamdrup et al. 1994). Therefore, incubation times for the applied radiotracer had to be as short as possible. However, an incubation time of 5 min was too short due to the lag-phase. The lag phase may be caused by the localized injection of $^{35}SO_4^{2-}$ into the center of the soil core and the thus resulting diffusional limitations (Jorgensen 1978a). Alternatively, the lag phase may be caused by sulfate transport into the cells of sulfate-reducing bacteria. Freshwater strains of sulfate-reducing bacteria have the ability to maintain an up to 10^4times higher sulfate concentration in the cytoplasm than in the environment (Warthmann & Cypionka 1990). The exchange of $^{35}SO_4^{2-}$ with an intracellular sulfate pool could result in a lag phase of $^{35}SO_4^{2-}$ decrease until the intracellular $^{35}SO_4^{2-}$ pool is saturated.

In the present experiments, SRR in the surface layer (0–1 cm) showed a high variability. This may be the result of reoxidation and/or diffusional loss of $H_2^{35}S$ due to the heterogeneity with respect to oxic/anoxic microniches and bioturbutation, found in soil surface layers (Jorgensen 1977b; Bak 1988). Therefore, SRR measurements in the surface may be underestimated and should be interpreted with care. On the other hand, area-integrated SRR (in nmol cm^{-2} d^{-1}) calculated for low resolutions were higher than those calculated for high resolutions. For freshwater environments, where sulfate concentrations can show steep gradients (e.g. Figure 3), SRR may be overestimated, if an unrealistically high sulfate concentration is applied in equation (1) or (2). Therefore, the best possible resolution of sulfate concentrations is essential to determine accurate SRR.

Another error of the applied radiotracer technique by which SRR could be underestimated is the negligence of incorporation of $^{35}SO_4^{2-}$ into organic sulfur which is not covered by the chromium reduction technique (Howarth & Jorgensen 1984). Although, organic sulfur is generally believed to be irrelevant as a short-term product of sulfate reduction in marine habitats (Jorgensen 1977a; Fossing & Jorgensen 1989), organic sulfur apparently contributes to the major long term products of sulfate reduction in some lake sediments (Rudd et al. 1986). Although the fraction of organic sulfur in the present rice field soil was 25–46%, it can be $>$90% in other freshwater habitats (Freney et al. 1982; Lefroy et al. 1992; Urban & Brezonik 1993). We can presently not exclude that organic sulfur may also be formed during short term incubations of $^{35}SO_4^{2-}$ in rice field soil. Further work has to be done to clarify this problem. On the other hand, SRR may be overestimated when summing up the ^{35}S recovered in the AVS, CRS plus S° fractions, since part of the S° may be counted twice both in the CRS and in the S° fraction (Fossing 1989).

SRR in the top 10 cm of planted and unplanted rice field soil microcosms were in the same range as reported for other natural freshwater systems (Smith & Klug 1981; Ingvorsen et al. 1981; Hordijk et al. 1985; Bak & Pfennig 1991; Urban et al. 1994). In unplanted microcosms, maximum SRR were observed in the upper soil layer (0–1 cm). In planted rice microcosms, maximum SRR were found in the layer adjacent to the root surface (0–3 mm). In $>$1 cm depth, SRR were up to $>$300% (*u*-test, $\alpha = 0.05$, Lord 1950) higher in planted compared to unplanted soil. In the layers with high SRR sulfate porewater concentrations were also high suggesting that SRR may have been limited by the availability of sulfate. Indeed, SRR measured *in situ* were much lower than the potential sulfate reduction rates that were measured in a previous study after addition of sulfate to slurries of rice field soil (Wind & Conrad 1995). Similar observations were made for the freshwater littoral sediment

of “Lake Constance” (Bak et al. 1991). However, SRR may also be limited by the concentration of electron donors, which presumably are higher in the presence than in the absence of rice roots (see below).

Sulfate porewater concentrations of the 0–1 cm layer in this study varied between 32 μM in unplanted microcosms and 61 μM in planted rice field soil. Despite low sulfate concentrations, sulfate reduction rates were relatively high. The most freshwater sulfate-reducing bacteria (SRB) are well adapted to low sulfate concentrations and show low half saturation constants for sulfate of 5–77 μM (Smith & Klug 1981; Ingvorsen & Jorgensen 1984; Fukui & Takii 1994). SRB may even outcompete methanogenic bacteria at low (60–105 μM) freshwater sulfate concentrations (Lovley & Klug 1983), and indeed, Lovley & Klug (1986) suggested that a severe limitation of sulfate reduction occurs only at sulfate concentrations <30 μM. However, studies in other natural freshwater habitats showed that the activity of SRB was limited by sulfate porewater concentrations <100 μM (Ingvorsen et al. 1981; Bak & Pfennig 1991). Thus, it is possible that SRR in the rice field soil was limited by the availability of sulfate.

The supply of sulfate into the rice field soil could be due to either diffusion of sulfate from the flooding water or oxidation of reduced sulfur species at oxic/anoxic interfaces (Good & Patrick 1987). Measurements of concentration gradients in unplanted rice field soil, showed that O_2 diffuses downward to 4 mm depth (Frenzel et al. 1992; Gilbert & Frenzel 1995), and free sulfide diffuses upward to a depth of 3–4 mm (unpublished data). Since there is very little evidence for significant losses of sulfur as H_2S from rice wetlands (Freney et al. 1982), complete reoxidation of the sulfides in the top 5 mm layer can be assumed. Therefore, in unplanted rice field soil, a zone with rapid cycling of sulfur compounds apparently exists in the uppermost 0–10 mm with SRR being highest at a depth of 3–5 mm. For example, in the 2–3 mm depth zone a SRR of approximately 100 nmol cm^{-3} d^{-1} was observed. With a H_2S concentration of approximately 1 μM (unpublished data), the turnover time must therefore be in the order of <15 min. Much of the variability of sulfate reduction in planted systems and in the uppermost layers of unplanted microcosms could thus be due to fluctuation in concentrations of sulfate by reoxidation of reduced sulfur species mediated by the rice roots. The oxidizing power of rice roots was described previously (Armstrong 1971; Ando et al. 1983). Oxygen loss by rice roots has also been observed using microelectrode measurements (Frenzel et al. 1992; Gilbert & Frenzel 1995; Gilbert, pers. communication), and thus a direct oxidation of reduced sulfur species with O_2 might be concluded. The importance of the cycling between reduced and oxidized state of sulfur was also indicated in other aquatic environments. Urban et al. (1994) showed that the diffusive

supply of sulfate from the water into the sediments of Little Rock Lake, Wisconsin, was not sufficient to account for the high sulfate reduction rates. Therefore, they concluded that sulfate was regenerated by a rapid turnover of reduced sulfur species. Oxidation of reduced sulfur species has even been demonstrated in anoxic marine sediments (Aller & Rude 1988; Fossing & Jorgensen 1990a; Jorgensen & Bak 1991). This anoxic oxidation is controlled by oxidants such as nitrate, manganese(IV) and iron oxides (Howarth 1984; King 1990; Canfield 1989b; Canfield et al. 1992; Elsgaard & Jorgensen 1992; Thamdrup et al. 1993). These oxidants are also generated at the rice root surface and may contribute to intensive sulfur cycling in planted rice field soil (Begg et al. 1994; Kirk & Bajita 1995).

Beside an increased reoxidation potential of H_2S to sulfate due to the release of O_2 by the rice root or the generation of other oxidants, the stimulatory effect of rice roots on sulfate reduction could also be explained by the release of exudates from the roots. In this case, SRR should be limited by the availability of electron donors rather than electron acceptors. The effect of roots stimulating the growth of bacteria has been known for a long time (rhizosphere effect) and has also been described for SRB in a rice paddy (Jacq 1975; Loyer et al. 1982; Freney et al. 1982; Ouattara & Jacq 1992). Exudates released from rice-roots seem to differ widely and depend on the growth stage of the plants (Jacq 1975; Boureau 1977). Sulfate reduction can also be stimulated by fermentation products (e.g. H_2) of the exudates. Previous attempts to stimulate sulfate reduction in rice soil slurries using different electron donors have failed with the exception of H_2 (Wind & Conrad 1995). Hydrogen appears to be the predominant electron donor for sulfate reduction in Italian rice field soil (Achtnich et al. 1995a; Achtnich et al. 1995b). Although our present observations are satisfactorily explained by assuming stimulation of SRR by the regeneration of sulfate at the rice root surface, we cannot exclude that root exudates acting as electron donors contributed to the observed stimulation.

The in situ SRR were found to be especially high in the soil surface layer and in the rhizosphere. Both are most oxidized sites in the rice field soil. Sulfate reduction has long been believed to be an entirely anoxic process. Activity of SRB in oxic environments was thought to be restricted to anoxic microniches such as iron sulfide aggregates that are formed around hot spots of organic matter (Jorgensen 1977b; Fukui & Takii 1990a; Furusaka et al. 1991). However, the survival of SRB in the presence of O_2 has now repeatedly been observed (Battersby et al. 1985; Canfield & Marais 1991; Jorgensen & Bak 1991; Fründ & Cohen 1992; Norsker et al. 1995). It has been shown that SRB build up catalase and superoxide dismutase in the presence of O_2 (Hatchikian et al. 1977; Abdollahi & Wimpenny 1990) and that they are able

to respire O_2 at low O_2-tensions (Dilling & Cypionka 1990; Dannenberg et al. 1992; Marschall et al. 1993). In a recent study *Desulfovibrio desulfuricans* used O_2 preferentially as electron acceptor before reducing the other electron acceptors present (Krekeler & Cypionka 1995). The reduction of O_2 may be a strategy of SRB to remove inhibiting O_2 and thus sustain activity.

The radiotracer studies with ^{35}S-labeled SO_4^{2-} showed that the H_2S that has been formed as the product of bacterial sulfate reduction exhibited rapid diagenic transformations in unplanted rice field soil. The primary sulfate reduction product H_2S can spontaneously be transformed to FeS (Pyzik & Sommer 1981; Lovley 1991; Coleman et al. 1993). Indeed, AV^{35}S (mainly consisting of $H_2{}^{35}S$ + Fe^{35}S) was observed as an important product of sulfate reduction for the first 10 to 20 min. During extended incubations, the fraction of AV^{35}S decreased with time probably since Fe^{35}S was transferred to Fe$^{35}S_2$ (Berner 1970). After incubation periods of 30–60 min, CR^{35}S (mainly consisting of Fe$^{35}S_2$) accounted for the major product ($>$50%) of sulfate reduction. Turnover of CRS was probably marginal since FeS_2 is known as a compound that is difficult to mobilize by plants and bacteria. This conclusion is consistent with the vertical profiles of sulfur species which showed much higher concentrations of CRS than AVS in all depths. During the first 30 min of incubation elemental sulfur accounted for 23–61% of all the products of $^{35}SO_4^{2-}$ reduction in the soil surface layer (0–1 cm). Vertical profiles of S$^\circ$ showed higher concentrations in unplanted compared to planted microcosms. In marine sediments elemental sulfur exhibits a very dynamic turnover with a high initial rate of formation (Troelsen & Jorgensen 1982). In the upper oxic layer it can be oxidized to sulfate or thiosulfate and then serve again as an electron acceptor for organic matter oxidation. In the reduced zone, on the other hand, it can be further reduced to soluble sulfides (Howarth & Jorgensen 1984; Fossing & Jorgensen 1990a). However, fast isotopic exchange reactions occur between ^{35}S$^\circ$, $^{35}S_n$, $H_2{}^{35}$S, and Fe^{35}S, (but not FeS_2). Hence, the formation of ^{35}S$^\circ$ that was observed during the measurement of SRR in rice microcosms could partially be due to this exchange reactions (Fossing & Jorgensen 1990b; Fossing et al. 1992).

The present study showed that the average concentration of sulfur compounds (SO_4^{2-}, AVS and CRS), with the exception of S$^\circ$, was up to 20-fold higher in planted than in unplanted rice field soil. The concentration profiles of sulfur compounds in unplanted microcosms were in agreement with earlier observations (Wind & Conrad 1995), but those in planted microcosms were slightly different. In particular, sulfate concentrations were now about a third of those previously measured, AVS concentrations were only a tenth, and thiosulfate could not be detected at all. This may be due to the different types of planted microcosms used for the two studies. Whereas in

the previous study (Wind & Conrad 1995) the planted microcosms consisted of small tubes (⌀ = 3.6 mm), in this study they consisted of large containers. In the large microcosms, the roots formed an approximately 1–2 cm thick horizontal root mat starting from the soil surface. In small microcosms, on the other hand, roots were not restricted to the surface layer. They also grew at the edge of the microcosm in vertical direction down to deeper layers and thus resulted in relatively high root densities below 2 cm depth (Wind & Conrad 1995). The higher root density in small microcosms may result in a decrease of pH within the rhizosphere also in deeper layers of the microcosm, since rice roots are known to acidify their environment (Begg et al. 1994; Kirk et al. 1995). The lower pH may in turn result in an increased capacity for adsorption of sulfate to soil particles (Samosir et al. 1988; Lefroy et al. 1993), so that steady state pool concentrations of sulfate become higher. Since FeS is an initial product of sulfate reduction and is subsequently converted to either FeS_2 or reoxidized, lower sulfate concentrations should then also result in lower H_2S + FeS concentrations, such as observed in the present study. Unlike previous studies, no thiosulfate was observed in planted microcosms. The previously measured $S_2O_3^{2-}$ could have been a result of sulfide oxidation mediated by O_2 leaking from the roots during the sectioning procedure. Since not so many roots had to be cut by taking a core from the large microcosms compared to the small tubelike microcosms, less O_2 leakage could be assumed. However, experiments with intact rice roots incubated in a sulfide-reduced medium showed that $S_2O_3^{2-}$ was transiently formed from H_2S indicating that H_2S can be oxidized to $S_2O_3^{2-}$ in the presence of roots (unpublished data). Although we were unable to detect $S_2O_3^{2-}$ in the present study, we presently cannot exclude that $S_2O_3^{2-}$ plays some role in the sulfur cycling of flooded rice field soil, as it has been shown for other aquatic habitats (Jorgensen 1990a; Jorgensen 1990b; Elsgaard & Jorgensen 1992). More research will be necessary to evaluate the role of $S_2O_3^{2-}$ in rice fields.

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