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Temporal and spatial variation of sulfide invasion in eelgrass (*Zostera marina*) as reflected by its sulfur isotopic composition

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Abstract

Temporal and spatial variation of $\delta^{34}S$, total sulfur (TS) concentration, and elemental sulfur concentration (S^0) in leaves, roots, and rhizomes of *Zostera marina* was followed between June 2002 and May 2003 at four locations in Roskilde Fjord and Øresund, Denmark. These were related to temporal changes in sediment sulfide concentrations, sulfur pool size, and sulfur pool $\delta^{34}S$. The $\delta^{34}S$ of *Z. marina* was most negative in the roots, followed by rhizomes and leaves, indicating that roots were mostly affected by sulfide. A significant relationship between decreasing $\delta^{34}S$ and increasing TS in the plant tissues indicated that sulfide accumulated in the plant and, furthermore, a positive relation between TS and S^0 in the plant suggests that part of the sulfide is reoxidized to S^0. There were marked temporal changes in all variables at all sites, but the pattern of change varied between sites. The temporal and spatial heterogeneity in plant $\delta^{34}S$, TS, and S^0 depended on a variety of factors, such as sediment sulfide concentrations and the below : aboveground biomass ratio of the plants. This suggests that mechanisms of sulfide invasion are complex, and several factors (plant morphology, environmental variables) acting in concert or against each other need to be considered to successfully predict sulfide invasion in seagrasses.

Hydrogen sulfide (H_2S) is highly toxic to plants in concentrations as low as 10^{-3} to 10^{-5} mol L^{-1} (Howarth and Teal 1979; Raven and Scrimgeour 1997), and sulfide invasion may therefore pose a serious problem to the growth and survival of plants rooted in anaerobic and sulfide-rich sediments. Invasion of sulfide has been directly measured in seagrasses under unfavorable environmental conditions with low water column oxygen concentrations characteristic of eutrophic or special weather conditions (Pedersen et al. 2004; Borum et al. 2005). The isotopic composition of sulfur in seagrasses and wetland plants has been reported to reflect uptake or invasion of sulfide into plant tissues (Carlson and Forrest 1982; Fry et al. 1982). Accordingly, growth under different environmental conditions may be reflected in the plant's stable sulfur isotope composition ($\delta^{34}S$). The overall goal of the present study was to examine differences in $\delta^{34}S$ of eelgrass (*Zostera marina*) tissues from different seasons and locations to determine whether environmental conditions were reflected by differences in $\delta^{34}S$.

Submerged rooted macrophytes growing in marine environments may be supplied with sulfur from three different sources with different ratios of the stable isotopes, ^{34}S and ^{32}S. Leaves can take up sulfate directly from the water column, which has a constant $\delta^{34}S$ of around +21‰ (Rees et al. 1978). In the sediment, sulfur will be present as sulfate and as sulfide formed by sulfate reducing bacteria. Owing to bacterial fractionation, the produced sulfide is isotopically lighter (lower $\delta^{34}S$) than seawater sulfate (Kaplan et al. 1963). Consequently the remaining, non-reduced sulfate is heavier than seawater sulfate (Kaplan et al. 1963), but reoxidation processes and exchange of sulfate across the sediment–water interface mask this effect in natural systems to various degrees (Böttcher et al. 2004). Sediment sulfide may have $\delta^{34}S$ as low as −27‰ in the forms of free and acid volatile sulfide and as low as −42‰ in the form of pyrite (Kaplan et al. 1963). Owing to this large range in $\delta^{34}S$ among the different sulfur sources, it should be possible to identify invasion and movement of sulfide in the plant tissues by analyzing the sulfur isotopic composition of the different tissues. Mesocosm experiments have shown elemental sulfur (S^0) to accumulate in seagrasses exposed to high sulfide concentrations in the sediment, suggesting that oxidation of sulfide takes place in the plants (Holmer et al. 2005). Hence, sulfide invasion, in addition to changes in $\delta^{34}S$, may also be reflected in increased concentrations of compounds derived from the oxidation of sulfide.

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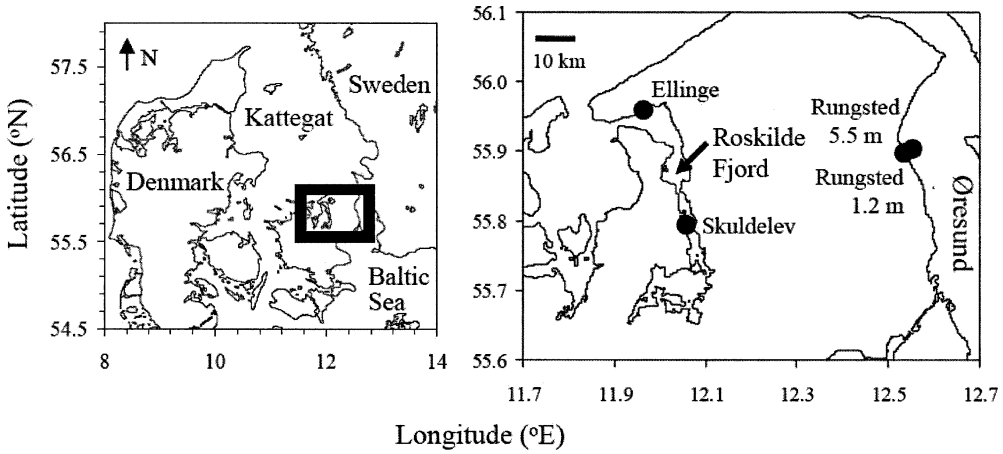

Fig. 1. Study area in Sjælland, Denmark, indicated by a square in left panel and magnified in right panel. Filled circles in right panel indicate the sites where the *Zostera marina* meadows were located.

Since sulfide is considered to enter plant tissues by gas phase diffusion from the sediment (Raven and Scrimgeour 1997), it is possible that sulfide invasion in eelgrass is a simple function of sediment sulfide concentrations. The concentration of sulfide in sediments is determined by the rate of sulfate reduction, which in turn depends on the amount of organic matter and the temperature (Moeslund et al. 1994). However, oxygen, oxidized iron, and manganese directly interfere with the sulfur cycle by reoxidizing H_2S to S^0 and by precipitation of FeS_2 and therefore play an important role in controlling the pool of H_2S in the sediment (Thamdrup et al. 1994). Sulfide is also rapidly reoxidized by bacteria in the transition zones between oxic and anoxic sediment around infaunal burrows and the water–sediment interface (Jørgensen and Revsbech 1983). In addition to the dynamics of sulfide within the sediment, the ability of seagrasses to maintain a continuous oxygen supply to rhizomes and roots via the air-filled lacunae and to subsequently leak oxygen to the sediment (Pedersen et al. 2004; Borum et al. 2005; Frederiksen and Glud 2006) may complicate the predictability of sulfide invasion into seagrasses. We therefore expect the relationship between sediment sulfide and the $\delta^{34}S$ of eelgrass tissues to be rather complex, being controlled both by the sediment sulfide concentrations and the oxygen status of the plants, which in turn is determined by plant photosynthesis and water column oxygen concentrations (Greve et al. 2003; Borum et al. 2005).

In spite of the expected complex relationship between sediment sulfide and $\delta^{34}S$ in eelgrass tissues, we hypothesize several general trends of sulfide invasion in eelgrass as reflected by its $\delta^{34}S$ values. As found earlier for seagrasses and wetland plants (Fry et al. 1982; Carlson and Forrest 1982), we expect that the $\delta^{34}S$ is low in roots and rhizomes since they are the primary sites of sulfide entry. Second, we expect $\delta^{34}S$ to be lowest in late summer with high temperature, higher organic matter input to the sediment, and potentially lower water column oxygen concentrations (Conley et al. 2000). Finally, we hypothesize that sulfide invasion should occur more frequently in areas with higher organic matter contents and sulfide concentrations within the sediment. To test these hypotheses, the composition of stable sulfur isotopes in *Z. marina* was investigated in four Danish eelgrass beds at different seasons from June 2002 to May 2003.

Methods

Study sites—Zostera marina plants, sediment, and water samples were collected at four Danish eelgrass meadows during June, July, August, October 2002 and in February and May 2003. The meadows were located in the central (Skuldelev) and outer part (Ellinge) of the Roskilde Fjord and in the Strait of Øresund (Fig. 1). At Rungsted in Øresund, two stations at different depths (1.2 and 5.5 m) were sampled. The depth limit of eelgrass in this part of Øresund is ~7 m (http://mads-en.dmu.dk), and plants at the chosen sampling depths therefore experienced significantly different light climates. Within the fjord the stations at Skuldelev and Ellinge were both sampled in shallow (1.2 m) water depths. Roskilde Fjord is a 50 km long, narrow estuary that receives large inputs of nitrogen and phosphorus from the rivers that discharge into the fjord and from treated municipal waste and atmospheric deposition (Middelboe and Sand-Jensen 2000, and references therein).

General sediment variables—The sediment organic content was measured on total sediment from 1 to 10 cm depth by loss of ignition (520°C for 6 h) of predried sediment (12 h at 105°C). The silt fraction (<63 μm) was obtained by sieving the dried sediment through a 63-μm sieve. Total nitrogen (TN) of total sediment was analyzed according to Kristensen and Andersen (1987) by elemental analysis using a Carlo Erba elemental analyzer (EA 1108). Total phosphorus was determined after acid digestion of combusted sediment followed by spectrophotometric determination of molybdate reactive phosphate (Koroleff 1983). The amount of dithionite extractable iron was determined by extracting 500 mg of sediment with a dithionite solution (50 g L^{-1}) for 1 h at room temperature (Lord 1980), and the Fe concentration was measured spectrophotometrically

(Stookey 1970). This method predominantly extracts free Fe oxides and some $FeCO_3$ and FeS, but not FeS_2. Rates of sulfate reduction were measured in June 2002 by the core injection technique (Jørgensen 1978) where radioactive ^{35}S-SO_4^{2-} was injected into a sediment core at 1-cm intervals down to 10 cm. After 2–4 h of incubation, the sediment was sliced in 1-cm intervals, fixed in zinc acetate, and kept frozen until analysis. The sediment was distilled according to the two-step procedure of Fossing and Jørgensen (1989), where the first step extracts the acid volatile sulfide (AVS) consisting of FeS and porewater sulfides and the second step extracts chromium reducible sulfur (CRS) consisting of FeS_2 and S^0. Radioactivity was determined on a scintillation counter, and the total sulfate reduction rate (SRR) was calculated by adding the contribution of the AVS and CRS fractions.

Seasonal sediment variables—Sediment porewater samples for measurement of the concentration of free sulfide were collected in situ using sippers ($n = 4$ per site) made of closed steel syringes with 0.4-mm holes drilled near the end as described in Berg and McGlathery (2001). The sippers were mounted on a sampling device similar in design to that of Burdige and Zimmerman (2002), capable of taking six simultaneous samples of 15 mL at 6 cm depth. The choice of depth was based on a study of root distribution, which showed that the highest root abundance occurred from 0 to 4 cm, gradually declining to nearly zero at depths between 6 and 10 cm. Taking out 15 mL of porewater from 6 cm depth integrates the sulfide distribution over a depth range that covered ~5.0 cm, assuming that porewater was drained from a sphere and that sediment porosity was 0.23 (range 0.19–0.26). Filtered samples were preserved in zinc acetate and analyzed spectrophotometrically by the method of Cline (1969). We also attempted to measure $\delta^{34}S$ of sulfide in the porewater, but it was not possible to obtain enough sample for isotopic analysis. Triplicate cores of sediment (diameter 5 cm) were sampled at each site to determine the depth of the sulfide front, the size of sediment sulfur pools, and $\delta^{34}S$ of sediment sulfides. The depth of the sulfide front was determined by inserting 10-cm-long silver sticks into the sediment (porewater sulfide reacts with the silver to form a black Ag_2S precipitate). From each core, sediment from the root zone (~1–5 cm) was preserved in zinc acetate and distilled according to the two-step procedure of Fossing and Jørgensen (1989) with the modification that the distillate was precipitated as Ag_2S instead of ZnS. The size of the AVS and CRS sulfur pools was determined by weighing the precipitates. For the sulfur isotope analysis of sediment sulfides, 0.5 mg of Ag_2S was loaded in tin boats together with ~3 mg vanadium pentoxide and measurements made by the National Isotope Geosciences Facility (Nottingham, UK) using Thermo Finnigan elemental analyzer+ Delta XL continuous flow mass spectrometer system. The sulfur isotope composition of a sample is expressed in the standard δ notation given by

$$\delta^{34}S = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000$$

where $R = {}^{34}S/{}^{32}S$. Values are expressed on a per mil (‰) basis and were calibrated to Canyon Diablo troilite (CDT). Precision was better than 0.4‰ based on internal standards. The $\delta^{34}S$ of seawater sulfate was assumed to be almost constant (Rees et al. 1978) and therefore only measured twice (July and February). In these sandy sediments variation of $\delta^{34}S$ in the porewater sulfate was also expected to be small (Böttcher et al. 2004) and was therefore measured once at each site in August 2002 ($n = 3$). This was the period where water temperatures and, consequently, the sulfate reduction rates were highest (Moeslund et al. 1994), which should correspond with the highest possibility of limitations in sulfate supply from the water column and, hence, the highest influence on sulfate $\delta^{34}S$. Samples were prepared by centrifuging the water sample or the sediment (10 min, 1500 *g*), boiling the supernatant under acidic conditions, and precipitating the sulfate with BaCl as $BaSO_4$. The $\delta^{34}S$ isotopic analysis of $BaSO_4$ was as described for Ag_2S.

Seagrass variables—Eelgrass shoot density was measured in summer (August 2002) and winter (February 2003) in 0.35-m^2 quadrats ($n = 7$) together with above and belowground biomass of actively growing tissues sampled by a 0.02-m^2 metal corer ($n = 3$). The depth distribution of roots in the sediment was measured once during the study by sectioning triplicate cores (i.d. 8 cm) at 2-cm intervals down to 10 cm depth.

For the temporal study, shoots of *Z. marina* were collected by digging up blocks of eelgrass that were washed free of sediment and divided into new leaves (youngest leaf), rhizomes (four youngest internodes), and roots (those attached to four youngest internodes). The plants were rinsed carefully in deionized water to remove excess salts and precipitates from the tissue surface and then freeze dried. Homogenized samples were analyzed for stable sulfur isotope ratio ($\delta^{34}S$), total sulfur content (TS), and elemental sulfur (S^0). For sulfur isotope analysis samples of plant material (8–10 mg) were weighed and analyzed the same way as described for sediment $\delta^{34}S$. TS was obtained during the analysis of $\delta^{34}S$. Plant content of S^0 was analyzed by high performance liquid chromatography (HPLC) according to the method of Zopfi et al. (2001) after extraction of dried plant tissue (5–20 mg) in 5 mL HPLC-grade methanol for 24 h.

Various S compounds such as iron sulfides may precipitate on the root surface and could potentially affect the plant results. Three different agents with increasing ability to dissolve the sulfide precipitates (reagent 1, distilled H_2O; reagent 2, 6 mol L^{-1} HCl to remove FeS; reagent 3, 1 mol L^{-1} Cr^{2+} to remove FeS_2 and S^0) were applied to roots and rhizomes and subsequently analyzed for the sulfur content. The results showed no significant difference between the three treatments, suggesting that precipitation was low and that S precipitates were removed by washing with distilled water, which was therefore used.

Statistical analyses—Data were tested for normality and homogeneity of variance. Basic sediment variables (organic material [OM], silt <63 μm, TN, total phosphorus [TP], total Fe, SRR) were evaluated using one-way analysis of

Table 1. Sediment content of organic material (OM), silt <63 μm, TN, TP (Aug 2001), total Fe (average Jul–Oct 2002), and sulfate reduction rates (SRR, Jun 2002). SE (n = 3) is shown in parentheses.

Site	OM (% dry wt)	Silt <63 μm (% dry wt)	TN (μmol [g dry wt]$^{-1}$)	TP (μmol [g dry wt]$^{-1}$)	Total Fe (μmol [g wet wt]$^{-1}$)	SRR (mmol m^{-2} d^{-1})
Skuldelev	1.91 (0.69)	5.68 (2.32)	37.8 (14.32)	12.7 (1.50)	5.81 (1.23)	40.4 (0.2)
Ellinge	0.27 (0.01)	0.11 (0.04)	2.8 (1.44)	3.5 (1.21)	0.96 (0.15)	15.5 (3.5)
Rungsted 1.2 m	0.57 (0.11)	2.52 (0.43)	6.2 (1.56)	7.6 (0.46)	2.69 (0.21)	22.9 (2.5)
Rungsted 5.5 m	0.31 (0.01)	0.47 (0.01)	2.6 (1.27)	2.9 (0.06)	3.01 (0.21)	19.3 (3.4)

variance (ANOVA) (factor: site). Sediment data on sulfide concentration, AVS and CRS pools, AVS and CRS $\delta^{34}S$, and basic plant variables (shoot density, biomass, and below : aboveground biomass ratio) were evaluated using two-way ANOVA (factors: month and site). Measurements from plant tissues ($\delta^{34}S_{eelgrass}$, TS, and S^0) were evaluated using three-way ANOVA (factors: month, site, and plant tissue). ANOVAs were followed by a Bonferroni adjusted Fisher's least significant difference (LSD) test using SAS version 8.02 (SAS Institute). For the three-way ANOVA it was a priori decided only to do multiple pairwise comparisons for main effects and double interactions. Because no data were available for Ellinge in June 2002, June was excluded from the two-way and three-way ANOVA tests of seasonal trends in sediment and plant variables, and analyzed separately by one-way ANOVA (factor: site) and two-way ANOVA (factors: site and plant tissue), respectively. Correlation analysis was likewise done in SAS 8.02 either on nontransformed or log-transformed data.

Results

Sediment variables—Sediment conditions varied considerably among the four sites (Table 1). Skuldelev had the highest organic content, nutrient concentrations, rates of sulfate reduction, and Fe content of all sites ($p < 0.05$ for TP and Fe). The lowest values were consistently at Ellinge, however ($p > 0.05$), whereas Rungsted 1.2 m and Rungsted 5.5 m had values in between these two extremes.

The concentration of free sulfide in the sediment pore water followed a seasonal pattern of high concentrations (maximum 283–580 μmol L^{-1}) in summer 2002 (July to September) and low concentrations (11–66 μmol L^{-1}) in winter (February 2003) at all sites (Fig. 2). There was generally no significant difference in sulfide concentrations among sites, except in July where sulfide concentrations at Skuldelev and Ellinge were significantly higher than at Rungsted 1.2 m ($p < 0.05$, no data available for Rungsted 5.5.m). The depth of the sulfide free zone was minimal during the summer where sulfide was present almost to the sediment surface at all sites (Fig. 2). The zone gradually increased during the autumn and winter to a maximum depth in February (range ~25–65 mm).

The sediment bound sulfides (AVS and CRS) in contrast to porewater sulfide showed no seasonal pattern at the four sites (Table 2). The AVS and CRS pools were always lowest at Ellinge ($p < 0.05$), whereas the other sites generally had similar concentrations except for the CRS pool at Rungsted 5.5 m, which was often several times (on average eight times) larger than at the three shallow sites. The $\delta^{34}S_{AVS}$ and $\delta^{34}S_{CRS}$ likewise showed little seasonal variation at the three shallow sites, but at Rungsted 5.5 m $\delta^{34}S_{CRS}$ and $\delta^{34}S_{AVS}$ were lower during summer/autumn (July–November) compared to winter/spring (February to May) ($p < 0.05$ for $\delta^{34}S_{CRS}$, Table 3).

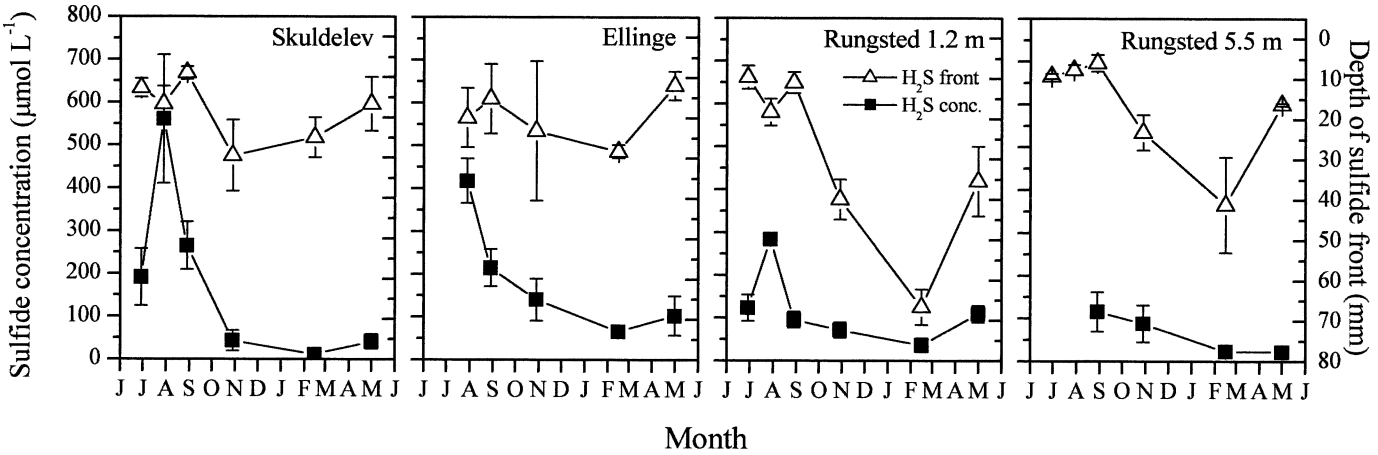

Fig. 2. Temporal variation in porewater sulfide concentrations (±SE, n = 4) and depth of the sulfide front (±SE, n = 3) at the four sites.

Table 2. Temporal variation in sediment sulfur pools (AVS and CRS) from ~1–5 cm depth at the four study sites ± standard error (SE, n = 2–3). At Ellinge the AVS pool was below the detection limit (<0.1 μmol S [g wet wt]$^{-1}$, b.d.) in July, February, and May. Ellinge was not sampled in June.

	AVS (μmol S [g wet wt]$^{-1}$ ± SE)				CRS (μmol S [g wet wt]$^{-1}$ ± SE)			
Month	Skuldelev	Ellinge	Rungsted 1.2 m	Rungsted 5.5 m	Skuldelev	Ellinge	Rungsted 1.2 m	Rungsted 5.5 m
Jun	0.9±0.2	—	1.0±0.8	1.8±1.0	5.0±1.0	—	4.6±1.8	40.3±20.3
Jul	0.1±0.0	b.d.	0.3±0.2	0.7	6.3±0.8	0.8±0.6	6.7±1.1	24.6±9.5
Aug	0.6±0.3	0.2	0.4±0.1	0.4±0.1	7.6±2.6	0.9±0.2	5.5±0.9	7.8±1.2
Oct	0.4±0.1	0.1	2.6±2.5	0.9±0.6	4.8±0.2	3.2±2.0	3.8±0.5	23.0±2.0
Feb	0.7±0.3	b.d.	0.1±0.1	0.4±0.2	7.9±1.2	0.9±0.2	4.9±0.4	23.4±11.1
May	0.1±0.1	b.d.	0.3±0.2	0.4±0.3	6.3±0.4	0.9±0.1	5.9±0.1	7.8±1.3

The measurements of $\delta^{34}S$ of seawater sulfate, $\delta^{34}S_{\text{seawater sulfate}}$, were similar at all sites (range +19.7‰ to +20.2‰) and similar to $\delta^{34}S$ of sulfate in the sediment porewater $\delta^{34}S_{\text{porewater sulfate}}$ (mean +22.2‰ ± 2.3, n = 12, all sites).

Seagrass variables—Eelgrass shoot density varied by almost an order of magnitude among sites, with the highest density at Skuldelev and the lowest density at Rungsted 5.5 m ($p < 0.05$, Table 4). Both shoot density and shoot biomass per m^2 declined in winter, except at Ellinge where shoot density remained constant during the investigation period. The below:aboveground biomass ratio showed a significant effect of season ($p < 0.05$) with higher values in winter, indicating that the relative amount of leaf biomass was reduced in the winter period.

The $\delta^{34}S$ of eelgrass varied between the plant tissues, with isotopic values in the roots being significantly more negative than in both rhizomes and leaves ($p < 0.05$, Fig. 3). All the plant tissues exhibited clear temporal trends that varied among locations. In Skuldelev, Ellinge, and Rungsted 5.5 m $\delta^{34}S$ was low or gradually decreasing during summer/autumn in all plant tissues and increased in winter and spring. Plants at Rungsted 1.2 m, in direct contrast with the other sites, had markedly less negative $\delta^{34}S$ in summer/autumn compared to winter/spring in all tissues. The same pattern of seasonal heterogeneity was apparent in the plant's TS and S^0 concentration. At Skuldelev, Ellinge, and Rungsted 5.5 m, eelgrass generally had the highest concentrations in summer/autumn, whereas at Rungsted 1.2 m maximum concentrations were found in winter/spring. (Fig. 4 and Table 5). Site specific comparisons of the four sites showed significant differences in $\delta^{34}S$ and TS ($p < 0.05$) but not in S^0 ($p > 0.05$). The lowest $\delta^{34}S$ values and highest TS concentrations were in Ellinge in summer/autumn and in Rungsted 1.2 m in winter/spring. The changes in TS and S^0 concentration mainly took place in the roots, whereas the concentration in leaves and rhizomes was more constant and significantly lower than in roots ($p < 0.05$). Elemental sulfur (S^0) concentration in leaves was below the detection limit (<0.1 μmol [g dry wt]$^{-1}$, data not shown). We found a significant positive relation between S^0 and TS ($p < 0.05$, $r^2 = 0.59$, Fig. 5), but S^0 constituted <4% of the TS pool.

The relationship between $\delta^{34}S$ and TS was tested by correlation analysis of log-transformed data (Fig. 6), which showed a significant decline of $\delta^{34}S$ with increasing amounts of TS in leaves ($r^2 = 0.79$, $p < 0.05$), rhizomes ($r^2 = 0.76$, $p < 0.05$), and roots ($r^2 = 0.70$, $p < 0.05$). In the roots the $\delta^{34}S$ tended to an asymptotic value of around −15‰ when the TS concentration was about 300 μmol (g dry wt)$^{-1}$.

In order to determine the relative contribution of sediment sulfide to the TS in the leaves, rhizomes, and roots, the fraction of the total sulfur pool derived from sulfide (F_{sulfide}) was estimated from the following equation:

$$F_{\text{sulfide}} = \frac{\delta^{34}S_{\text{tissue}} - \delta^{34}S_{\text{sulfate}}}{\delta^{34}S_{\text{sulfide}} - \delta^{34}S_{\text{sulfate}}}$$

where $\delta^{34}S_{\text{tissue}}$ is the value measured in the leaf, rhizome, or root; $\delta^{34}S_{\text{sulfate}}$ is the value measured in the seawater (+19.7‰ to + 20.3‰); and $\delta^{34}S_{\text{sulfide}}$ is the value measured in the CRS pools over the season (−22.4‰ to −32.1‰).

Table 3. Temporal variation in $\delta^{34}S$ of sediment AVS and CRS pools ± standard error (SE, n = 2–3) at the four study sites. Owing to very low amounts of AVS sulfur at Ellinge it was only possible to obtain enough material for $\delta^{34}S$ analysis in October.

	$\delta^{34}S_{AVS}$ (‰ ± SE)				$\delta^{34}S_{CRS}$ (‰ ± SE)			
Month	Skuldelev	Ellinge	Rungsted 1.2 m	Rungsted 5.5 m	Skuldelev	Ellinge	Rungsted 1.2 m	Rungsted 5.5 m
Jun	−21.0±1.0	—	−23.6±0.5	−24.7±1.6	−26.1±0.7	—	−23.0±0.8	−28.0±0.7
Jul	−25.1±1.5	—	−25.5±2.0	−17.2	−26.8±0.5	−24.4±0.5	−24.3±0.7	−26.6±1.4
Aug	−28.0±0.3	—	−24.7±1.3	−23.3±1.6	−26.4±0.5	−23.3±1.0	−23.3±0.3	−22.4±0.8
Oct	−25.9±0.1	−27.3	−24.8±1.2	−21.2±1.9	−27.7±0.1	−26.1±0.2	−23.3±0.4	−23.8±3.5
Feb	−26.1±2.1	—	−27.5±4.5	−30.0±1.5	−26.8±0.6	−25.3±1.4	−24.0±0.3	−32.1±0.9
May	−31.1	—	−27.4±1.3	−25.0±0.5	−27.7±1.2	−27.1±0.6	−24.2±0.8	−31.1±0.7

Table 4. General plant characteristics of *Zostera marina* at the four study sites. Standard error (SE) is shown in parentheses ($n = 3$ except for shoot density where $n = 7$).

	Shoot density (shoots $m^{-2} \pm$ SE)		Plant biomass (g dry wt $m^{-2} \pm$ SE)		Below : aboveground ratio (±SE)	
	August	February	August	February	August	February
Skuldelev	1,201 (99)	741 (80)	320.9 (35.2)	169.5 (25.8)	0.7 (0.03)	0.7 (0.11)
Ellinge	540 (32)	512 (33)	246.3 (38.9)	106.6 (7.3)	1.1 (0.29)	1.5 (0.24)
Rungsted 1.2 m	750 (67)	288 (38)	312.8 (5.4)	242.7 (56.2)	0.6 (0.05)	2.0 (0.67)
Rungsted 5.5 m	144 (17)	70 (16)	248.0 (76.3)	33.2 (3.8)	0.5 (0.07)	0.8 (0.05)

The calculations showed that roots derived a significantly higher proportion (57–77%) of their sulfur from sediment sulfides compared to rhizomes (35–59%) and leaves (31–50%, $p < 0.05$, Table 6).

Possible relationships between sulfide concentrations in the sediment and $\delta^{34}S$, TS, and S^0 concentrations in leaves, roots, and rhizomes were tested by correlation analysis and showed no significant relationships in leaves and rhizomes ($p > 0.05$, $r^2 < 0.01$). In the roots, correlations of log-transformed data, however, were significant for all three variables ($p < 0.05$), but r^2 values were relatively low ($r^2 = 0.21$, 0.43, and 0.28 for $\delta^{34}S$, TS, and S^0, respectively, Fig. 7). We tested whether plant variables such as shoot density, plant biomass, and below : aboveground ratio affected the plant $\delta^{34}S$, TS, and S^0 and found a significant linear relationship between the below : aboveground ratio and $\delta^{34}S$ and TS but not with S^0 (Figs. 8 and 9). The $\delta^{34}S$ decreased ($p < 0.05$, $r^2 = 0.65$ and 0.64 for leaves and rhizomes, respectively, $p > 0.05$, $r^2 = 0.20$ for roots, Fig. 8) and the TS concentration increased ($p < 0.05$, $r^2 = 0.70$ and 0.75 for leaves and rhizomes, respectively, $p > 0.05$, $r^2 = 0.32$ for roots, Fig. 9) with increasing below:aboveground biomass ratio, suggesting a greater likelihood of sulfide invasion in eelgrass plants with proportionally higher belowground biomass.

Discussion

The extensive range in $\delta^{34}S$ values of eelgrass, 21.4‰ in leaves (−11.5‰ to +9.9‰), 20.2‰ in rhizomes (−11.1‰ to +9.1‰), and 21.7‰ in roots (−16.‰ to +5.6‰), is well beyond what has previously been reported for *Zostera* species. Most of the previously published data was based on leaves with values ranging from +10‰ to +17.6‰ (e.g., Kharlamenko et al. 2001; Oakes and Connolly 2004). More depleted $\delta^{34}S$ values ranging from −0.4‰ to +10‰ have also been found (Mekhtiyeva et al. 1976; Peterson 1999), but it is not clear whether these values were from leaves only or included other tissues. The previously reported values for leaves are generally much more enriched than those observed during the present study, where leaf values down to −11.5‰ have been measured. Very few data exist for roots and rhizomes (Raven and Scrimgeour 1997; Kharlamenko et al. 2001), and they are within the range found in this study. Although not to the same extent as for *Z. marina*, considerable spatial and seasonal variation in $\delta^{34}S$ has also been found in seagrass species such as *Thalassia testudinum* (Chambers et al. 2001) and in salt marsh plants (Stribling et al. 1998, and references therein) suggesting this to be a general phenomenon of rooted macrophytes in marine environments.

A prerequisite for detecting sulfide invasion in seagrasses is that the isotopic signals of the sulfide sources are different from the $\delta^{34}S$ of the sulfate sources. The $\delta^{34}S$ of seawater sulfate ($\delta^{34}S_{\text{seawater sulfate}}$) in Roskilde Fjord and Øresund (around +20‰) was close to both the +21‰ found in other studies (Rees et al. 1978) and the $\delta^{34}S$ of sulfate (+22.2‰ ± 2.3‰) measured in the sediment porewater ($\delta^{34}S_{\text{porewater sulfate}}$) during August when sulfate reduction rates were at their highest. The $\delta^{34}S_{\text{sulfide}}$ was measured in the AVS and the CRS pool of the sediment, whereas it is the free/gaseous H_2S in the porewater that enters the

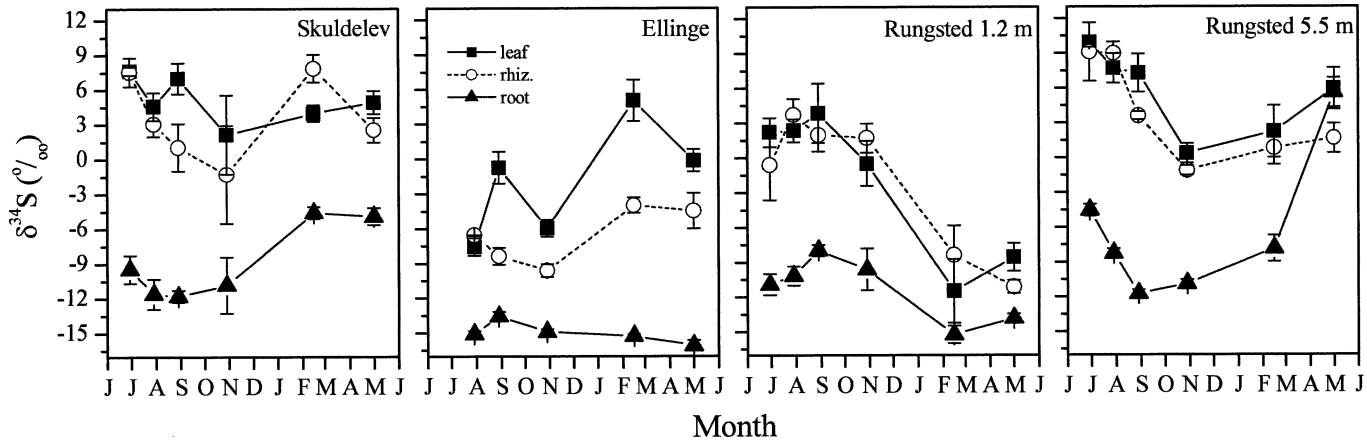

Fig. 3. Temporal variation in $\delta^{34}S$ of leaves, rhizomes, and roots at the four study sites. Error bars indicate SE ($n = 3$).

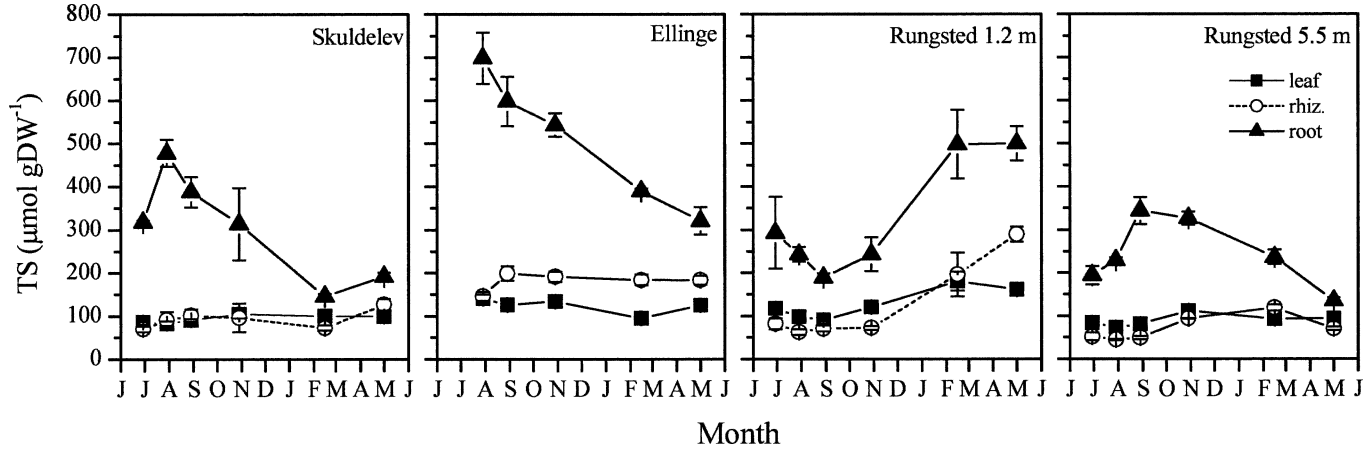

Fig. 4. Temporal variation in total sulfur content (TS) of leaves, rhizomes, and roots at the four study sites. Error bars indicate SE ($n = 3$).

seagrass (Pedersen et al. 2004). Despite relatively high concentrations of free sulfide (up to 580 μmol L^{-1}), it was not possible to obtain enough sample for isotopic analysis, but the AVS pool contains both the porewater sulfide and FeS and is considered more reactive to seasonal changes in SRR than the CRS pool (Schippers and Jørgensen 2002). Hence, the two pools together reflect the $\delta^{34}S$ value of sulfides both over shorter and longer time periods and provided the best possible estimate of the $\delta^{34}S$ of the free sulfide that may have entered the plants. The $\delta^{34}S$ of sulfide was always much more negative than $\delta^{34}S$ of sulfate, and this separation of source signals provides a basis for using stable sulfur isotopes to detect sulfide invasion in eelgrass.

The $\delta^{34}S$ of the eelgrass tissues varied systematically, with values in the roots being significantly more negative than in leaves and rhizomes. Isotopically depleted $\delta^{34}S$ values from eelgrass roots indicated that a larger part of the total sulfur in the roots was derived from sulfide compared to the rhizomes or leaves, as suggested by Fry et al. (1982). Mass balance computations based on $\delta^{34}S$ of plants, seawater sulfate, and sediment CRS showed that up to 77% of the S in roots was derived from sediment sulfides. The fact that $\delta^{34}S$ of eelgrass leaves and rhizomes was significantly depleted with respect to $\delta^{34}S$ of seawater/porewater sulfate furthermore suggests that sulfide or derivatives from sulfide moved from the belowground tissues to the leaves probably through gas phase lacunar diffusion as previously demonstrated by Pedersen et al. (2004). Low $\delta^{34}S$ relative to seawater sulfate has also been reported in leaves from other seagrass species, such as *T. testudinum, Halodule wrightii, Syringodium filiforme, Halophila engelmanni*, and *Ruppia maritima* (Fry et al. 1982; Pulich 1989; Chambers et al. 2001), while leaves of *Posidonia oceanica* had $\delta^{34}S$ almost identical to seawater sulfate at four locations across the Mediterranean (Frederiksen 2005). Thus the internal transport capacity of sulfide seems to vary between seagrass species. Sulfide may undergo various transformations when inside the plant, and the significant relation found between TS and $\delta^{34}S$ in roots, rhizomes, and leaves clearly shows that TS increased when $\delta^{34}S$ decreased, indicating that compounds derived from sulfide accumulated in the plants. In the lacunae the sulfide is exposed to oxygen and reoxidation will occur, and, if not excreted, the accumulation of reoxidation products could explain the higher concentrations of TS in plants with depleted $\delta^{34}S$ values. One possible reoxidation product is elemental sulfur, which showed a significant positive linear relationship with TS in the roots of eelgrass. Furthermore, the $\delta^{34}S$ of S^0 extracted from roots and rhizomes of *Z. marina* is very negative and close to the $\delta^{34}S$ of sediment sulfide (Frederiksen 2005), which strongly suggests that the S^0 was derived from sediment sulfide. The S^0 constituted only a small fraction of the total S pool ($< 4\%$), and the remaining S must be other oxidized sulfur

Table 5. Temporal variation in the content of elemental sulfur (S^0) ± standard error (SE, $n = 3$) in rhizomes and roots at the four study sites. S^0 concentration in leaves (data not shown) was below the detection limit (<0.1 μmol [g dry wt]$^{-1}$, b.d.).

	S^0 concentration in rhizomes (μmol [g dry wt]$^{-1}$ ± SE)				S^0 concentration in roots (μmol [g dry wt]$^{-1}$ ± SE)			
Month	Skuldelev	Ellinge	Rungsted 1.2 m	Rungsted 5.5 m	Skuldelev	Ellinge	Rungsted 1.2 m	Rungsted 5.5 m
Jun	0.1±0.1	—	0.4±0.3	b.d.	6.9±1.8	—	4.9±1.5	3.2±0.8
Jul	0.2±0.1	0.3±0.2	0.1±0.0	b.d.	10.8±3.2.	14.2±2.9	5.6±2.3	1.2±0.2
Aug	0.1±0.1	1.1±0.6	0.7±0.3	b.d.	7.7±2.0	12.8±3.6	2.7±0.4	2.9±0.8
Oct	0.2±0.2	0.2±0.1	0.1±0.0	0.1±0.0	9.7±6.0	5.9±1.1	2.0±0.6	2.7±0.3
Feb	0.2±0.1	0.6±0.2	1.7±1.1	0.1±0.0	2.5±0.8	5.4±1.6	18.9±5.4	1.4±0.2
May	b.d.	0.1±0.0	0.3±0.2	0.1±0.0	6.3±0.5	2.2±0.9	8.6±1.2	0.5±0.1

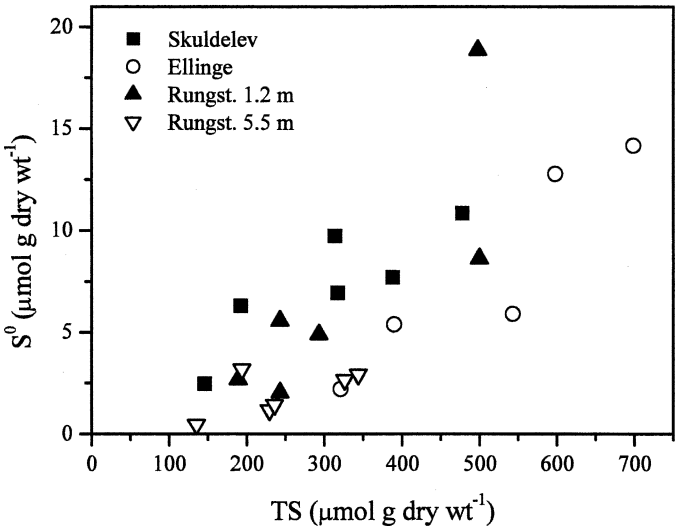

Fig. 5. Relationships between S^0 and TS in eelgrass roots.

species such as sulfate and thiosulfate, which are also likely end products of reoxidation (Fossing and Jørgensen 1990) as well as organic sulfur. Holmer et al. (2005) found an increased accumulation of S^0 in eelgrass plants experimentally exposed to high sulfide concentrations. In their study S^0 constituted up to 68% of the TS pool in the roots and up to 30% in rhizomes, suggesting that S^0 accumulation can be of greater importance than observed in this study.

It is possible that the changes in $\delta^{34}S$, TS, and S^0 resulted from precipitation of metal sulfides and oxidized sulfur compounds on the outer surface of the plant and not from the accumulation of sulfides inside the plant. Accordingly, Fry et al. (1982) found higher sulfur content and a lower $\delta^{34}S$ in the bark of mangrove roots compared to the interior, suggesting that this process may be important. However, the washing experiment showed that (1) there had been very little precipitation on the root surface and (2) that any sulfide associated with the exterior of the plant was removed during washing. Furthermore, isotopic changes also occurred in the leaves whose outer surfaces were not exposed to sulfide. Thus it is unlikely that external contamination by sulfides can explain the trend of decreasing $\delta^{34}S$ with increasing TS. It is also possible that low $\delta^{34}S$ in seagrass tissues could result from the uptake of sulfate with depleted $\delta^{34}S$ values derived from the oxidation of sulfides in the rhizosphere (Trust and Fry 1992). This mechanism has not yet been documented but would occur in close proximity to the root hairs. In the present study $\delta^{34}S$ of bulk pore water sulfate was measured (>+20‰), which would remain largely unaffected if the oxidation only occurred on such short spatial scales. However, although plants generally assimilate their S from sulfate (Rennenberg 1984), the depleted $\delta^{34}S$ values in the plant are unlikely to be due to an increasing proportion of sulfate derived from sulfide oxidized alongside the roots, since this processes would occur without any concomitant increase in plant TS. In fact the depletion in $\delta^{34}S$ was linked to an increase in TS when there was no obvious physiological reason why the eelgrass should accumulate S over and above the normal growth requirement. This and the fact that sulfide has been directly measured inside the eelgrass (Pedersen et al. 2004) both lend support to the contention that the changes in TS and $\delta^{34}S$ were not related to physiological processes and that the main part of the accumulating sulfur must have resulted from an uncontrolled invasion and accumulation of sediment sulfide.

Temporal and spatial variation in sulfide invasion—The study presented significant temporal and spatial variation in $\delta^{34}S$, TS, and S^0 of eelgrass, but whether the observed variation truly reflects seasonality or only relates to the conditions prevalent during 2003–2003 cannot be determined because of the lack of seasonal replication. The temporal and spatial variation in plant sulfur variables were expected to be closely related to seasonal and spatial changes in sediment sulfide concentrations, but relations between sediment sulfide concentrations and plant $\delta^{34}S$, TS, and S^0 were relatively weak. The porewater sulfide concentrations generally peaked in late July, and it was not until late August, late October, or even in February that the indications of sulfide invasion were strongest. The correspondence was even less with the sediment AVS and CRS sulfur pools. The spatial variation in sediment sulfide concentrations was remarkably small considering the relatively high variation in other sediment variables. The two sites in Roskilde Fjord represented the extremes in sediment OM, TN, TP, and SRR, with significantly higher values at Skuldelev in the central part of the fjord compared to Ellinge in the outer part. Sulfide concentra-

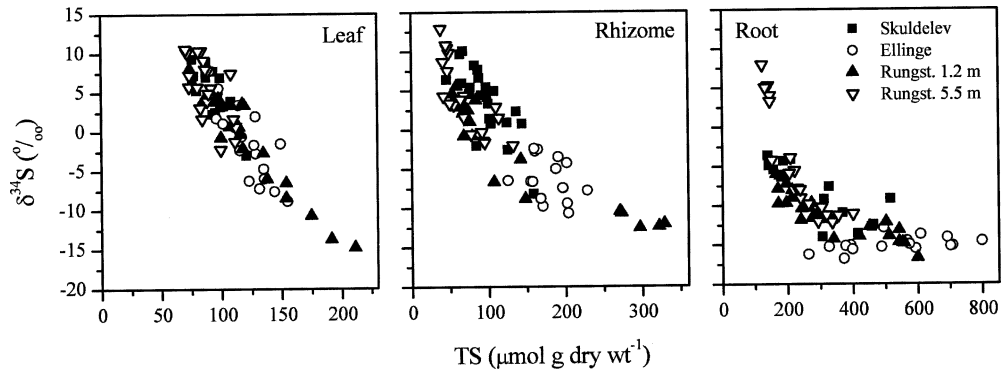

Fig. 6. Relationships between $\delta^{34}S$ and TS in eelgrass tissues.

Table 6. Annual means (±SE, n = 5–6) of the fraction of total plant sulfur ($F_{sulfide}$) that originated from sediment sulfides in each plant compartment (leaves, rhizomes, roots). See text for calculations.

	Leaf mean % (SD)	Rhizome mean % (SD)	Root mean % (SD)
Skuldelev	31.6 (4.0)	34.8 (7.4)	61.2 (7.3)
Ellinge	48.6 (11.4)	59.0 (6.3)	77.4 (1.2)
Rungsted 1.2 m	50.4 (14.3)	50.7 (13.6)	71.6 (5.8)
Rungsted 5.5 m	31.0 (8.1)	34.7 (9.7)	56.7 (16.6)

tions, however, were similar at the two sites probably because the higher sulfate reduction rates at Skuldelev were counterbalanced by the higher concentrations of Fe, which "buffers" the concentrations of sulfide through oxidation or precipitation of FeS and FeS_2 (Chambers et al. 2001). This is supported by the larger pools of AVS and CRS at Skuldelev. Despite similar levels of sulfide exposure, eelgrass at Ellinge showed stronger indications of sulfide invasion by having significantly more depleted $\delta^{34}S$ and higher TS and S^0 concentrations ($p < 0.05$ for $\delta^{34}S$ and TS) in the roots and rhizomes. The mechanism behind sulfide invasion is therefore not straightforward and is not controlled only by the free sulfide concentration in the sediment.

The oxygen concentration in the water column may strongly influence sulfide invasion in seagrasses by controlling the amount of oxygen diffusing into the leaves, down to the roots, and out into the sediment (Borum et al. 2005; Frederiksen and Glud 2006). We did not measure water column O_2 concentrations at the four sites, but measurements at two stations from the Danish National Monitoring Programme (NOVA, measuring depth 1 m, sampling interval 4–14 d) near our study sites in Roskilde Fjord (<9 km) showed reduced O_2 concentrations in August and September (~5 mg L^{-1}) compared to winter/spring (~9 mg L^{-1}, http://mads-en.dmu.dk). No data were available for the sites in Øresund. The study sites in Roskilde Fjord therefore may have experienced reduced water column O_2 concentrations in this period, and this may have contributed to the trend of low $\delta^{34}S$ and high TS concentrations plants in summer/autumn. However, direct O_2 measurements at the sites are required to verify this hypothesis.

The significant relations found between below : aboveground ratios and both $\delta^{34}S$ and TS in leaves and rhizomes suggest that plant morphology was an important controlling factor affecting sulfide invasion in the eelgrass. A high below : aboveground ratio results in a relatively higher root surface area over which sulfide can diffuse into the plant, and, furthermore, the respiration of belowground tissues is proportionally higher, reducing the oxygen available to prevent sulfide invasion. Relations, however, were only significant for leaves and rhizomes, since these organs have accumulated ca. 31–50% and 35–59%, respectively, of their TS from sulfide invasion. The mixing of these proportions of TS from sulfate and sulfide results in large and significant change in the $\delta^{34}S$ of the leaves and rhizomes. The roots, however, have a much higher proportion of their TS derived from sulfide invasion, and accumulation of further TS results in little or no significant change in $\delta^{34}S$. The general increase in below:aboveground ratio from August to February together with reduced light availability therefore could have affected plant oxygen status significantly and may explain the trend toward sulfide invasion in autumn compared to summer at most sites, even though sulfide concentrations were decreasing. Likewise the unexpected but very consistent indications of increased sulfide invasion in winter at Rungsted 1.2 m (low $\delta^{34}S$ and high concentrations of TS and S^0) when sulfide concentrations were low may also be related to the very large increase in below:aboveground ratio (from 0.6 to 2.0).

There was no effect of water depth, and hence light availability, on sulfide invasion in eelgrass at the two sites in Øresund. Basic plant characteristics clearly changed with depth as expected from other studies (e.g., Middelboe et al. 2003), e.g., with a shoot density at Rungsted 5.5 m constituting <25% of Rungsted 1.2 m and considerably larger shoots at deeper water. It could be expected that the eelgrass growing in deep water should be more susceptible to sulfide invasion because of lower light availability for photosynthetic oxygen production, but the plants at Rungsted 1.2 m generally had more depleted $\delta^{34}S$ values and higher concentrations of TS and S^0 than at the deep

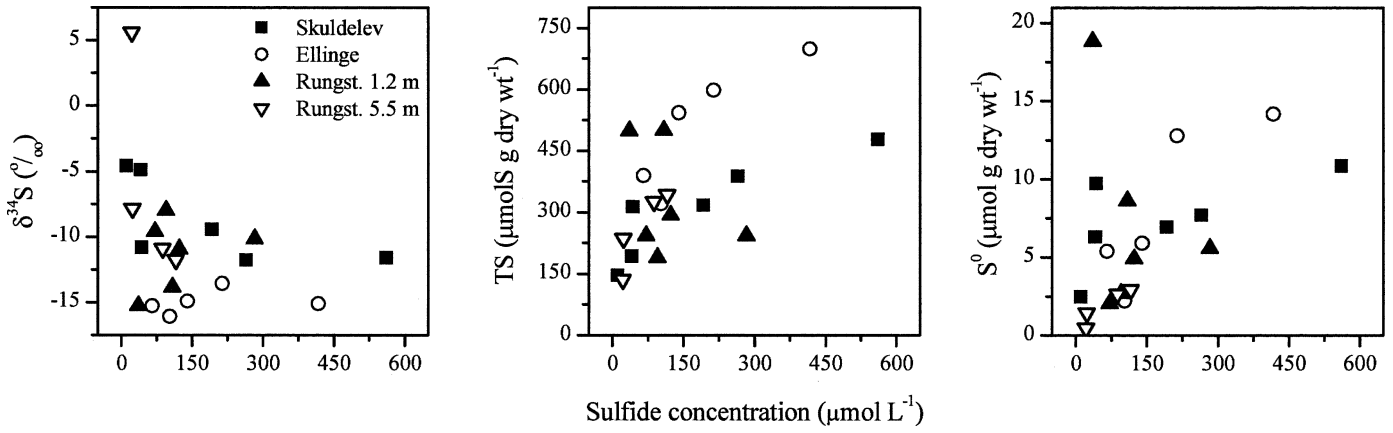

Fig. 7. Relationships between sulfide concentrations in the sediment and $\delta^{34}S$, TS, and S^0 concentrations in roots.

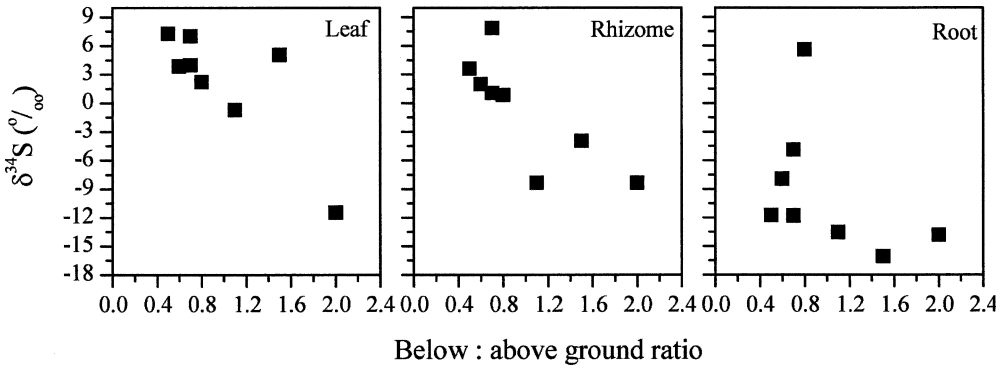

Fig. 8. Relationships between the below : aboveground ratio of *Z. marina* and $\delta^{34}S$ in leaves, roots, and rhizomes. Data are from August 2002 and February 2003.

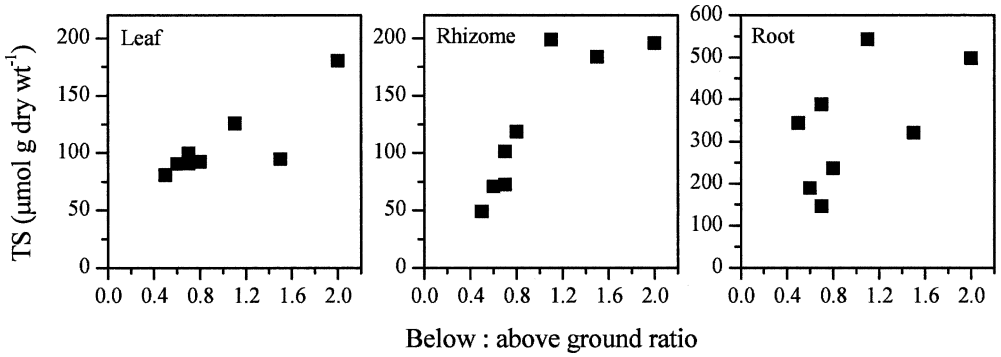

Fig. 9. Relationships between the below : aboveground ratio of *Z. marina* and TS concentration in leaves, roots, and rhizomes. Data are from August 2002 and February 2003.

site, indicating that sulfide invasion was greater at the shallow site. The difference in light climate therefore seemed to be overruled by other factors. Sediment conditions such as sulfide concentrations, SRR, and Fe content were not significantly different at these sites, and therefore differences in plant morphology, or as yet unquantified factors that affect the plant oxygen status, must have contributed to the differences between the two sites. This study only compared two sites, and general conclusions about the importance of water depth on sulfide invasion in seagrasses therefore cannot be made. Water depth is likely to be an important controlling factor in other places, since deeper meadows generally have higher contents of sediment organic matter due to reduced water movements with increasing depth (Holmer et al. 2003), but in the present study OM content was highest at the shallow site. More studies are therefore needed to evaluate the importance of depth for sulfide invasion in seagrass.

In conclusion, the present study shows that the mechanisms for sulfide invasion are complex and not only controlled by free sulfide concentrations in the sediment. Other factors influence invasion such as plant morphology and the internal oxygen status of the plant, which in turn is controlled by various environmental factors (light, temperature, etc.) that need to be considered when predicting the potential for sulfide invasion in seagrasses. The $\delta^{34}S$ isotopic signal in eelgrass tissues reflects sulfide invasion, and thus the isotopic signal can be used to indicate environmental stress despite the contradictions between expected and observed trends of depth and locality. Alternatively, sulfide invasion was also reflected by the TS and S^0 content, which in contrast to $\delta^{34}S$ does not require additional knowledge about the sulfur sources. Direct experimental work is required to obtain more robust knowledge on the relationship between sediment sulfide concentrations, plant oxygen status, and sulfide invasion.

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