Causes of sulfur isotope variability in the seagrass, 
*Zostera capricorni*

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Abstract

Sulfur has been proposed as a useful element to employ in addition to carbon and nitrogen in stable isotope studies of marine food webs, but variability in $\delta^{34}$S of primary producers may prevent food web resolution. $\delta^{34}$S values in green leaves of the seagrass, *Zostera capricorni*, showed considerable variability (12.7–17.6\%) in a survey in Moreton Bay, Australia. We demonstrated that $\delta^{34}$S values were correlated with sediment organic matter (OM) content and height of seagrass on the tidal gradient, but these relationships were opposite to those expected from work elsewhere. In our survey, $\delta^{34}$S values were relatively depleted at sites higher on the shore and with lower OM content. We did find the expected relationship of depleted $\delta^{34}$S values where sediment porewater sulfide concentrations were higher. Any influence of OM content on $\delta^{34}$S values would have been confounded in the survey by the relationship between height on shore and OM content itself. We separated the effects of height and OM content by creating the following treatments at one height on the shore: (1) OM added, (2) procedural control, and (3) untouched control. $\delta^{34}$S values of seagrass in OM added plots were significantly depleted (5.6\%) relative to procedural (10.1\%) and untouched (11.0\%) controls 8 weeks after the manipulation. This demonstrated that OM content on its own does have the expected effect on $\delta^{34}$S values of seagrass, so in the initial survey another factor, probably related to height on shore, must have overridden the influence of OM content. Seagrass roots are able to exude excess oxygen produced during photosynthesis, reoxygenising sulfides in surrounding porewater. We demonstrated that the above and below-ground biomass of seagrass

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was higher low on the shore, and contend that higher seagrass productivity low on the shore results in greater reoxidation of sulfides and leads to more enriched $\delta^{34}$S values of seagrass.

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### 1. Introduction

Stable isotopes are being used with increasing frequency in studies of the importance of estuarine habitats to secondary productivity (Peterson, 1999). Stable isotope analysis uses the ratio of a heavy, rare isotope, to a light, common isotope (e.g., $S^{34}/S^{32}$) of an element compared to that of an international standard. This ratio is referred to as the isotopic signature. The signature of a heterotrophic consumer reflects that of its food (Peterson et al., 1985; Currin et al., 1995). Where autotrophic producers in a system have unique isotopic signatures, comparison of these signatures to those of consumers may thus reveal the ultimate source of organic matter (OM) even after multiple trophic transfers (Peterson et al., 1986).

The elements most commonly used in stable isotope analysis of food webs are carbon and nitrogen. However, there is increasing interest in the use of sulfur to distinguish between producer signatures where a combination of carbon and nitrogen is unable to do so (e.g., Harrigan et al., 1989; Peterson, 1999; Connolly et al., in press). Improved automation has also enhanced the feasibility of sulfur analysis (e.g., Fry et al., 2002).

Sulfur is a component of a number of biochemical compounds and its importance in plant nutrition is well documented (Trust and Fry, 1992). The source of sulfur, and fractionation occurring during its uptake and assimilation, determines the sulfur isotopic signature of producers (Deegan et al., 1990). In estuaries, major sources of available sulfur are seawater sulfate, with a relatively constant signature of 20.3‰ (Jørgensen, 1979) and, in anoxic sediments, porewater sulfide which is depleted in $S^{34}$ relative to seawater sulfate (e.g., Fry et al., 1982 report $\delta^{34}$S for sulfide of $-23.4$‰ and $-24.5$‰).

For sulfur stable isotopes to be useful in food web studies, the sulfur isotopic signatures of producers must be unique and relatively constant (Peterson, 1999). Variability in $\delta^{34}$S of producer taxa may diminish the capacity to distinguish between producers and resolve food web issues. Reported values of $\delta^{34}$S within producer taxa have varied considerably, from $-9.6$‰ (Currin et al., 1995) to 24.5‰ (Weinstein et al., 2000) for the saltmarsh grass *Spartina alterniflora*, and from 9.1‰ (Kharlamenko et al., 2001) to 16.2‰ (Machás and Santos, 1999) for species of *Zostera* seagrass. At least part of the $\delta^{34}$S variability in rooted producers has been attributed to variation in sulfide concentrations. Where greater concentrations of $S^{34}$-depleted sulfides are available to producers, sulfur signatures are depleted (e.g., Currin et al., 1995; Stribling et al., 1998).

Sulfide is produced in anoxic sediments through bacterial reduction of sulfate to dihydrogen sulfide, which may enter macrophyte roots (Carlson and Forrest, 1982; Eldridge and Morse, 2000). Rates of production may be influenced by differences in OM content and factors associated with height on the tidal gradient, such as waterlogging.
Sediments low on the tidal gradient should have less opportunity to drain and thus be more waterlogged. Slow diffusion of oxygen through water filling the interstitial spaces in such sediments impedes replenishment of oxygen used in respiration (Hogarth, 1999). This leads to anoxic conditions in which sulfate-reducing bacteria populations develop (Adam, 1990), resulting in greater sulfide concentration, and depleted $\delta^{34}S$ values of rooted producers (e.g., Stribling et al., 1998).

During respiration, sulfate-reducing bacteria consume OM (Peterson and Howarth, 1987). Thus, where large volumes of OM occur, bacterial activity is expected to be greater, resulting in increased sulfide production and depleted producer $\delta^{34}S$ values (Carlson and Forrest, 1982).

Although sulfide is a potential phytotoxin (Koch and Erskine, 2001), in seagrasses oxygen produced during photosynthesis is transported to below-ground tissues via a well-developed lacunal system (Koch and Erskine, 2001). This allows respiration and prevents intrusion of sulfide (Lee and Dunton, 2000; Koch and Erskine, 2001). Where the oxygen produced exceeds that required, sediments surrounding seagrass roots and rhizomes may also be aerated (Pedersen et al., 1998), oxidising sedimentary sulfides in the vicinity (Terrados et al., 1999; Lee and Dunton, 2000). The sulfide concentration of sediments within seagrass beds is often low, and depending upon seagrass biomass, can be below that of adjacent unvegetated areas (Terrados et al., 1999; Eldridge and Morse, 2000).

The aims of this study were to, firstly, measure the extent of variability in $\delta^{34}S$ of seagrass leaves in Redland Bay, southeast Queensland, Australia. Secondly, we tested the relationship between $\delta^{34}S$ and the environmental variables of relative height of seagrass on the tidal gradient, OM content of sediment, and porewater sulfide concentration of sediment. We found a consistent relationship between OM and $\delta^{34}S$, but this relationship was confounded with height on the tidal gradient. We therefore did a manipulative experiment to separate the effect on $\delta^{34}S$ of OM content from that of height. Finally, because the relationship between OM and $\delta^{34}S$ determined through this manipulation contrasted with results of the field survey, we compared seagrass biomass high and low on the shore as a potential explanation for the $\delta^{34}S$ patterns observed.

2. Materials and methods

2.1. Field survey sample collection

The study was done in Redland Bay, southeast Queensland, Australia ($27^\circ32'\ S, 153^\circ19'\ E$) during Autumn 2002 in a Zostera capricorni seagrass meadow that extended from the mid-intertidal to shallow subtidal region. For both the field survey and the manipulative experiment described later, all samples were collected at a similar stage during the tidal cycle, shortly after spring tides, to minimise potential effects of tidal height variation.

A 10 m (width) × 50 m (length) transect traversing the tidal gradient from the highest position at which seagrass occurred (approximately mean sea level) to below mean low water mark was divided into 20 even blocks across the gradient, within each of which one site was haphazardly positioned. The relative height of each site was determined using
surveying equipment. The highest position at which seagrass occurred was designated a height of 0 cm. From each site, three sediment subsamples were collected by hand and combined for OM determination. It has been shown that in Moreton Bay, within which Redland Bay is located, most sulfate reduction occurs in the top 50 mm of sediments in seagrass beds (Moriarty et al., 1985). In the present study, all sediment was therefore collected to a depth of 50 mm. At alternating sites \( (n = 10) \), a number of \( Z. \) capricorni leaves were obtained for sulfur isotope analysis. All samples of \( Z. \) capricorni consisted only of green leaves to prevent confounding with any leaf age effects. All seagrass and sediment samples were frozen until processed.

Sediment porewater sulfide levels were determined at 13 of the sites using diffusive gradients in thin films (DGT). In this method, dissolved sulfide diffuses through a polyacrylamide hydrogel and binds to a further gel impregnated with AgI\(_{\text{(s)}}\) through reaction to form Ag\(_2\)S\(_{\text{(s)}}\) (Teasdale et al., 1999). The AgI\(_{\text{(s)}}\) in the binding gel on DGT probes is pale yellow in colour, and the Ag\(_2\)S\(_{\text{(s)}}\) formed by reaction with sulfide is black. The optical density and extent of black colouration on accumulating gels of DGT probes compared to known standards is an indication of sulfide concentration (Teasdale et al., 1999). As DGT is a time-integrated, kinetic method which accumulates sulfide using similar mechanisms to those operating within plant roots, the sulfide concentration thus determined is believed to be indicative of that which is available for uptake by plants (Zhang et al., 2001; Ernstberger et al., 2002). DGT probes inserted into the sediment remained in situ for 24 h to allow sulfide accumulation.

Results of the survey (above) and manipulative experiment (below) led us to consider the importance of seagrass biomass in explaining \( \delta^{34}\)S signatures. Differences in seagrass above and below-ground biomass at sites high and low on the tidal gradient were therefore determined through the collection of seagrass blades and root material, respectively. Above-ground biomass was collected by harvesting all seagrass material above the sediment surface in each of the five randomly placed \( 25 \times 25 \text{ cm} \) quadrats high and low on the shore. Below-ground biomass was collected in a core of diameter 75 mm and depth 50 mm in the centre of each of the quadrats from which above-ground biomass had been collected. Cores were washed through a 1-mm sieve to remove sediment, and live seagrass material was separated manually from dead and foreign material.

2.2. Manipulative experiment sample collection

The relationship established between OM content of sediments and seagrass leaf \( \delta^{34}\)S in the field survey was in contrast to that described in the literature. However, this relationship was confounded by the relationship between OM content and height on the shore. To remove the influence of height, an experiment was done in which enough OM was added to double the OM content of sites high on the shore (based on preliminary trials) to determine the effect upon \( \delta^{34}\)S of seagrass. An array of 18 plots \( (25 \times 25 \text{ cm}) \) separated by >1 m was set up. Six plots were randomly allocated to each of three treatments: organic matter added (OM added), procedural control (PC), and untouched control (UC). Treatments were established at low tide. A liter of OM was incorporated into the upper 50 mm of sediment in each OM added plot and a knife was used to cut around the perimeter of plots to a depth of 250 mm to limit assimilation of sulfur from outside the
manipulated area by rhizomes extending beyond plot boundaries. PC plots received identical actions as OM added plots, except that no OM was added. UC plots were left entirely undisturbed. OM was also added to sites low on the shore, aiming for a factor orthogonal with height, but OM was washed away soon after these plots were established, and the plots were therefore abandoned.

From each plot, sediment samples for OM content determination and seagrass leaves for sulfur analysis were collected 2, 4, and 8 weeks following manipulation. Measurement of sediment porewater sulfide concentrations using DGT in this sized plot potentially affects the oxidation state of porewater sulfur in a way that would alter the experimental outcome. We therefore decided to sacrifice a single plot from each treatment, and measure sulfide concentration using DGT immediately after the week 4 collection.

2.3. Sample processing

All frozen samples were thawed, and dried at 60 °C (unless otherwise specified). Seagrass leaves for sulfur analysis were gently scraped free of epiphytes using a scalpel and were then dried and ground. Sulfur stable isotope analysis was done by Iso-Analytical in the United Kingdom. Stable isotope signatures were reported in standard delta notation (units per mil, ‰), calculated as follows:

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

where $R$ is $^{34}S/^{32}S$.

Organic matter content of sediment was determined by loss on ignition (LOI) methodology. Although LOI may slightly overestimate the total organic matter content of sediments (Leong and Tanner, 1999), this overestimation is reduced by acid-treating sediments to remove calcium carbonate prior to burning; in any case, the technique was sufficient for the purposes of this study as only a relative measure of OM was required. A subsample of sediment from each site was dried. Approximately 2 g of dried sediment was placed into crucibles and acid-digested for 6 h in 1 M HCl before being dried at 100 °C to constant weight. Crucibles were then placed in a muffle furnace at 500 °C for 3 h.

2.4. Data analysis

2.4.1. Field survey

Relationships between seagrass leaf $\delta^{34}S$ and selected environmental variables, and among the environmental variables themselves, were tested using linear regressions. Comparisons of seagrass biomass low and high on the shore were made using one-way ANOVAs separately for above and below-ground biomass.

2.4.2. Manipulative experiment

Organic matter content and sulfur signature values were log transformed to make variances homogeneous. Comparison of OM content and sulfur signatures amongst the three treatments in the manipulative experiment required repeated measures models because the same plots were sampled at multiple times. For both variables, Mauchly's
test of sphericity was not significant, and univariate repeated measures analyses were thus used. Tukey’s tests ($\alpha = 0.05$) were used to examine differences between pairs of means where appropriate.

3. Results

3.1. Field survey

Seagrass leaf $\delta^{34}$S values ranged from 12.7‰ to 17.6‰. Values were related to the height at which seagrass was growing on the tidal gradient with more enriched $\delta^{34}$S values low on the shore (regression: $F_{1,8} = 5.8$, $p = 0.042$; Fig. 1A). Organic matter content of sediments was also related to height (regression: $F_{1,18} = 15.5$, $p < 0.001$), with less OM

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**Fig. 1.** Relationships between (A) sulfur signature and height, (B) organic matter content and height, (C) sulfur signature and organic matter content, (D) porewater sulfide concentration and height, and (E) sulfur signature and porewater sulfide concentration.
high on the shore, and generally higher, but more variable, OM content low on the shore (Fig. 1B). Seagrass $\delta^{34}\text{S}$ values were also therefore positively related to OM content (regression: $F_{1,8} = 12.3, p = 0.008$; Fig. 1C). This corresponded to an increase in porewater sulfide concentration with increased height on the shore (regression: $F_{1,11} = 1.1, p = 0.325$; Fig. 1D). Sulfide concentration was negatively correlated with $\delta^{34}\text{S}$ (regression: $F_{1,11} = 17.6, p = 0.002$, Fig. 1E).

Above-ground seagrass biomass low on the shore (82 ± 16 dry weight g m$^{-2}$ (mean ± S.E.)) was significantly greater than that high on the shore (22 ± 2 g m$^{-2}$; ANOVA: $F_{1,8} = 12.7, p = 0.007$). A similar pattern was apparent for below-ground material, with biomass low on the shore (361 ± 68 g m$^{-2}$) being greater than that high on the shore (226 ± 23 g m$^{-2}$; ANOVA: $F_{1,8} = 5.7, p = 0.044$).

![Graphs showing organic matter content and sulfur signatures](image)

**Fig. 2.** Effect of OM addition (OM) compared with procedural control (PC) and untouched control (UC) on organic matter content of sediment (LHS) and sulfur signatures of seagrass (RHS), 2, 4, and 8 weeks after manipulation (mean ± S.E.). Means with the same letters are not significantly different.
3.2. Manipulative experiment

The univariate repeated measures test for OM content among treatments showed an interaction with time (Huynh–Feldt: $F_{2,11} = 2.9$, $p = 0.045$). The effect of treatment was therefore investigated using separate Tukey’s tests at different times. At week 2, OM content was significantly higher in OM added plots, and there was no difference between PC and UC plots (Fig. 2). In week 4, the mean OM content of PC was not significantly different to that of either UC or OM added plots, however OM added plots had significantly greater OM content than UC (Fig. 2). By week 8, no difference was evident among any of the treatments (Fig. 2).

For seagrass leaf sulfur signatures, the univariate repeated measures test showed a significant effect of treatment ($F_{2,15} = 12.5$, $p = 0.001$) and a significant effect of time ($F_{2,4} = 13.1$, $p = 0.000$). Overall, the OM added treatment had values more depleted than PC and UC, which were not different from each other. However, the interaction term was close to significant (Huynh–Feldt: $F_{4, 24} = 2.7$, $p = 0.057$), and the most careful interpretation is to, again, compare means using separate Tukey’s tests at different times. These showed that in week 2, there were no significant differences in mean sulfur signature among treatments (Fig. 2). By week 4, OM added plots had sulfur signatures significantly more depleted than UC, with PC values intermediate and not significantly different from OM added or UC. By week 8, sulfur signatures in the OM added treatment (5.6 %) were significantly depleted compared to both PC (10.1 %) and UC (11.0 %), which were not significantly different from each other (Fig. 2). Tukey’s tests comparing how seagrass sulfur signatures changed within each treatment type over time showed that in OM added plots, sulfur signatures were significantly more depleted in week 8 than in week 2, with values at week 4 being intermediate and not significantly different to values in either week 2 or week 8 (Fig. 2). For both PC and UC plots, sulfur signatures did not differ significantly among weeks.

The sulfide concentration was only measured in week 4 and was much greater in the OM added plot (6.1 μmol l$^{-1}$) than in the UC (1.5 μmol l$^{-1}$) and PC plot (0.6 μmol l$^{-1}$).

4. Discussion

The naturally occurring values of $\delta^{34}$S for seagrass leaves in Redland Bay extend the range of values for Zostera species of seagrass, with some values more enriched than the previous maximum value of 16.2 % reported by Machás and Santos (1999). The negative relationship observed between seagrass $\delta^{34}$S and sediment sulfide concentration is as expected (e.g., Carlson and Forrest, 1982; Fry et al., 1982), as plants rooted in anoxic sediments should assimilate sulfides depleted in S$^{34}$ (Fry et al., 1982; Trust and Fry, 1992). The variation in seagrass $\delta^{34}$S may thus be explained by differences in porewater sulfide concentration, which may be influenced by OM content of sediments, height on the tidal gradient, or factors associated with these.

The relationships established between $\delta^{34}$S and height, $\delta^{34}$S and OM, and height and sulfide concentration are in contrast to those expected based on previous studies (Table 1). Whereas it has been reported that $\delta^{34}$S values of plants in anoxic sediments are more enriched where OM content is lowest and at greater heights on the shore (e.g., Stribling et al., 1998), the opposite was found in the field survey component of the present study. In
addition, although increased drainage of sediments high on the shore should result in aerobic sediment conditions in which sulfide concentration is low (e.g., Stribling et al., 1998), higher sulfide concentrations were found higher on the shore in the present study. This indicates that there is spatial and/or temporal variation in these mechanisms or that such relationships have not previously been adequately established.

The lower OM content of sediments higher on the shore is consistent with predictions based on literature (Lee and Dunton, 2000). Low OM high on the tidal gradient may occur for a number of reasons, including low OM input, removal of OM by water movement, rapid consumption of OM within the sediment, or a combination of these processes (Lee and Dunton, 2000). However, the association of depleted $\delta^{34}$S values with low OM high on the shore was unexpected (e.g., Stribling et al., 1998), and we suspected that the relationship between OM and $\delta^{34}$S was confounded by the height factor. The manipulative addition of OM was used to separate these factors.

The results of the OM addition experiment were consistent with results from previous surveys (e.g., Currin et al., 1995; Stribling et al., 1998) and clearly demonstrate that increased OM in sediments can cause depleted $\delta^{34}$S of seagrass. In studies such as that by Stribling et al. (1998), OM is proposed to result in depleted $\delta^{34}$S by increasing the activity of sulfate-reducing bacteria, and hence concentrations of S$^{34}$-depleted sulfides in sediment (Carlson and Forrest, 1982; Trust and Fry, 1992).

Sulfur signatures of seagrass leaves were most enriched in UC plots and most depleted in OM added plots. $\delta^{34}$S values in PC plots were never statistically different from those in

### Table 1

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Summary of relationships between $\delta^{34}$S values of rooted estuarine plants and environmental variables, as expected from literature (as cited) and as observed in the current study.
UC plots, so there was little if any effect of the procedures used to add the OM. In weeks 2 and 4, $\delta^{34}\text{S}$ in OM added plots was the most depleted of the three treatments. This trend became most evident by 8 weeks after manipulation, despite OM content of the sediment having returned to levels of untouched control plots. This may indicate some lag effect in sulfide production, with sulfide concentration increasing over time, or the steady accumulation of sulfide within the plant.

The manipulative experiment demonstrated that, in the absence of other environmental variables, enriched $\delta^{34}\text{S}$ values should occur where OM content is lowest, high on the shore. Given that the opposite relationship was observed in the survey, evidently some other factor, probably related to height of seagrass on the tidal gradient, overrides the relationship which would otherwise be expected to be produced by natural differences in OM content. Differences in the density of seagrass at different heights on the tidal gradient may provide a functional explanation for the observations.

The greater above- and below-ground biomass of $Z.\ capricorni$ found lower on the shore in the present study is consistent with previous reports from Moreton Bay that this species grows more densely where it is submerged for longer periods (Young and Kirkman, 1975). Greater above-ground biomass would enable a greater photosynthetic rate and potentially the production of more oxygen than is required by the plant. This excess oxygen may then be transported through the lacunal system to below-ground tissues and into surrounding sediments (Terrados et al., 1999; Koch and Erskine, 2001). A greater root mass in a given volume of sediment would increase the area of sediment able to be aerated by oxygen released from below-ground tissues (Pedersen et al., 1998; Eldridge and Morse, 2000). It would thus be expected that low on the tidal gradient, where more seagrass occurred, there would be greater potential for the reoxidation of sulfides compared to sites high on the tidal gradient, where a smaller above and below-ground biomass would reduce this capacity. Seagrass beds have the potential to re-oxidise up to 50% of reduced sulfides through this process, reducing porewater sulfide concentrations (Terrados et al., 1999). Our theory is that, since there is less seagrass high on the shore, any sulfides produced there are less likely to be reoxidised, resulting in elevated sulfide concentrations high on the shore compared to low on the shore.

This possible importance of plant biomass in driving $\delta^{34}\text{S}$ values might explain the apparent difference in trends in $\delta^{34}\text{S}$ with height between previous work in saltmarsh communities and the current study on seagrass. Whereas $Z.\ capricorni$ is distributed from the intertidal to subtidal zone, with greater biomass low on the tidal gradient, $Spartina$ communities have an intertidal to supratidal distribution and may have greater biomass with increased elevation. If greater plant biomass depresses sulfide concentration leading to enriched $\delta^{34}\text{S}$ values, as we propose, then this would explain the inverse relationships between $\delta^{34}\text{S}$ and height observed in these two communities.

5. Conclusion

The manipulative experiment confirmed that elevated OM content of sediments is associated with depleted $\delta^{34}\text{S}$ values of a rooted primary producer, as reported in the literature (e.g., Currin et al., 1995; Stribling et al., 1998). However, this relationship
between $\delta^{34}\text{S}$ and OM content of sediment was not observed in the survey across different shore heights, presumably because it was overridden by another factor related to shore height. A likely explanation for the survey results is that greater seagrass productivity low on the shore increases reoxidation of sulfides, lowering sulfide concentrations and resulting in enriched seagrass leaf $\delta^{34}\text{S}$ values. Further manipulative experiments are required to demonstrate, for example, the effects of seagrass density on porewater oxygen content, and consequently on sulfide concentration and $\delta^{34}\text{S}$ values of seagrass.

The use of stable isotopes in food web analysis requires that variation within primary producer species is small relative to differences among mean values for different producers. At the least, spatial and temporal variability in isotope signatures of producers must be fully accounted for. Whereas variability in carbon and nitrogen isotopes of producers is well described (e.g., Boon and Bunn, 1994), less work has been done on sulfur isotope variability. The data that do exist are mostly on Spartina saltmarsh grass (e.g., Stribling et al., 1998), so the present study is a step towards increasing awareness and understanding of variability in signatures for other rooted estuarine primary producers. Among other things, this study showed that $\delta^{34}\text{S}$ values of seagrass altered probably within 4 weeks, and certainly within 8 weeks of OM manipulation, and it may now be assumed that relatively small-scale temporal fluctuations in sulfide concentrations are reflected in the sulfur signatures of seagrass.

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