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Drivers of sulfide intrusion in *Zostera muelleri* in a moderately impacted estuary in south-eastern Australia

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Abstract

The seagrass *Zostera muelleri* is abundant in estuaries in Australia and is under pressure from coastal developments. We studied sulfide intrusion in *Z. muelleri* along a gradient of anthropogenic impact at five stations in the Wallis Lake estuary, Australia. Results showed differences in sediment biogeochemical conditions, seagrass metrics as well as nutrient content and sulfide intrusion along the gradient from the lower estuary (impacted) to the lagoon (unimpacted). Sulfide intrusion was driven by complex interactions and related to changes in seagrass morphology and sediment biogeochemistry and modified by the exposure to wind and wave actions. The sediments in the lower estuary had high contributions from phytoplanktonic detritus, whereas the organic pools in the lagoon were dominated by seagrass detritus. Despite high concentrations of organic matter, sulfide intrusion was lower at stations dominated by seagrass detritus probably due to lower sulfide pressure from the less labile nature of organic matter. Porewater Diffusive Gradients in Thin-films (DGT) sulfide samplers showed efficient sulfide reoxidation in the rhizosphere, with high sulfur incorporation in the plants from sedimentary sulfides likely due to sulfate uptake from reoxidised sulfide. This is a unique adaptation of *Z. muelleri*, which allows high productivity in estuarine sediments.

Keywords

Seagrass morphology, sediment biogeochemistry, nutrient enrichment, sulfide intrusion
Seagrasses are abundant in estuaries, where they benefit from good light conditions, nutrient inputs from land, shelter and rapid water circulation (Duarte 2002; Boström et al. 2014). These estuarine seagrass meadows provide many essential ecosystem services (e.g. biodiversity, shelter and food for juvenile fish and shellfish, coastal protection, nutrient retention), however, seagrasses in estuaries are also some of the most vulnerable ecosystems due to increasing anthropogenic pressure, with major losses having been observed worldwide (Waycott et al. 2009). Seagrass loss in estuaries can be attributed to natural and anthropogenic disturbances, and is often linked to eutrophication and coastal development (Baden et al. 2003; Waycott et al. 2009; Boström et al. 2014). High nutrient concentrations in the water column may enhance the growth of filamentous macroalgae and epiphytes, which can shade the seagrass and induce water-column anoxia, leading to seagrass decline. High organic matter loading of the sediments as a result of eutrophication increases the mineralization rates in the sediments and eventually the sulfide pressure on the plants, which may be detrimental to seagrasses and lead to loss of seagrass meadows (Frederiksen et al. 2006; Mascaro et al. 2009).

Seagrasses grow in reduced sediments in estuaries, where the oxygen penetration is often low due to high loading of labile organic matter (Glud 2008). Mineralization of organic matter occurs primarily by sulfate reducing bacteria and high pools of sulfides can be expected in the rhizosphere sediments (Hansen et al. 2000). Mineralization rates and in particular sulfate reduction rates are driven by the lability of the organic matter, where rates are stimulated by increasing inputs of phytoplanktonic detritus (Holmer et al. 2004). Sulfur stable isotope signals in seagrasses have been used to investigate sulfide intrusion into different plant tissues (leaves, rhizomes and roots) (Oakes and Connolly 2004; Frederiksen et al. 2006). Due to bacterial fractionation of sulfate during sulfate reduction, sedimentary sulfides are isotopically lighter than seawater sulfate, and intrusion of sulfides is measured as lower $\delta^{34}S$ signals in the plant tissues. Intrusion of sulfides into seagrasses is commonly observed (Holmer and Hasler-Sheetal 2014), and previous studies of Z. muelleri (formerly Z. capricorni) have shown lower $\delta^{34}S$ values in leaves compared to seawater sulfate, indicating sulfide intrusion (Oakes and Connolly 2004). Many seagrass species are capable of transporting oxygen to
the rhizosphere, which can influence the chemical composition of the rhizosphere sediments. Brodersen et al. (2015) showed that *Z. muelleri* releases oxygen from the entire root surface and from the rhizomes, whereas other seagrasses such as *Z. marina* only release oxygen from the root tip (Jensen et al. 2005). Hasler-Sheetal and Holmer (2015) found several sulfide detoxification mechanisms in *Z. marina*, including the reoxidation of sulfide to sulfate in the rhizosphere. Rhizosphere sulfate may be taken up by the plant showing an isotopic signal similar to the sedimentary sulfides, as there is no fractionation during the reoxidation process (Hasler-Sheetal and Holmer 2015). If the reoxidation capacity of the seagrasses is exceeded, due to anoxia in the water column or high sulfide pressure from the sediments for example, sulfide intrusion may reduce growth and increase mortality in seagrasses (Mascaro et al. 2009). Sulfide intrusion in seagrasses is complex and controlled by a range of different factors, including plant morphology and sediment biogeochemistry (Holmer and Kendrick 2013; Holmer and Hasler-Sheetal 2014). The biogeochemistry of iron, including the iron content of the sediments, plays an important role in mitigating sulfide intrusion, as dissolved ferrous iron can precipitate sulfides as iron monosulfides or pyrite in the sediments, thereby reducing the sulfide exposure from the porewaters on the seagrasses (Marbà et al. 2007). Furthermore, the oxidation of the sediments by oxygen released from seagrass roots, particularly during the day, can oxidize dissolved Fe$^{2+}$ to Fe(III) precipitates, which can then chemically oxidize sulfides to elemental sulfur or other sulfur compounds. Declining Fe$^{2+}$ concentrations during the day have been found in the rhizosphere of *Z. capricorni* (now *Z. muelleri*) by the use of Diffusive Gradients in Thin-films (DGT) techniques (Pagès et al. 2012). Recent research has shown that iron content can vary significantly along the west coast of Australia and effect sulfide intrusion in five different seagrasses (Holmer and Kendrick 2013). Hence sulfide intrusion in seagrasses is driven by a range of biogeochemical factors, which are all potentially linked to changes in anthropogenic pressures in estuaries.

Early detection of eutrophication is critical for management of coastal waters to avoid exceeding thresholds of no return (Duarte et al. 2008). As Wallis Lake is under pressure from run-off from land and coastal development it is important to monitor the seagrass meadows as they are key components of the Wallis Lake estuary. Traditional monitoring of water quality has been questioned (Pérez et al. 2008), whereas seagrass
bioindicators are now used for monitoring in several countries, and are for instance part of the Water Framework Directive in the European Union (Romero et al. 2007; Borja et al. 2013). Seagrass depth limits are widely used, but in shallow coastal waters such as Wallis Lake, light limitation may not be critical for seagrass distribution and other indicators are needed. Cover of epiphytes, blooms of filamentous algae and increase in tissue nitrogen content and change in $\delta^{15}N$ signal in leaves have been observed in areas with high nutrient inputs and are used as indicators of nutrient enrichment (Herbeck et al. 2014; Roca et al. 2015). In addition, the $\delta^{13}C$ of sediments may be used as an indicator of eutrophication, as seagrass meadows accumulate organic matter such as phytoplanktonic detritus and macroalgal debris due to increased sedimentation compared to unvegetated sediments, a likely situation in Wallis Lake, where increased phytoplankton production may increase inputs of organic matter to the sediments particularly at sheltered sites. Determining the $\delta^{13}C$ signal of the potential sources of sedimentation, therefore, makes it possible to identify the sources of organic matter and their origin in the sediments (Kennedy et al. 2010; Fourqurean et al. 2012). Higher sulfate reduction rates due to increased inputs of organic matter to the sediments may increase the sulfide pressure on the seagrasses in Wallis Lake. Intrusion of sulfides, indicated by low $\delta^{34}S$ or high $F_{sulfide}$ (percentage of plant sulfur derived from sedimentary sulfides), has been suggested as an early warning indicators of sulfide pressure, but $\delta^{34}S$ and $F_{sulfide}$ have not yet been fully developed as bioindicators (Holmer and Hasler-Sheetal 2015).

The main objective of this study was to examine potential sulfide intrusion in *Zostera muelleri* in a lightly to moderately impacted estuary on the south-eastern coast of Australia. *Z. muelleri* is confined to the south-eastern region of the Australian mainland and the waters surrounding Tasmania, New Zealand and Papua New Guinea and is a significant primary producer in this region (Kerr and Strother 1990; Udy and Dennison 1997; Matheson and Schwarz 2007; Kohlmeier et al. 2014). This species has similar morphometrics and growth requirements to the temperate *Z. marina*, and thus similarities in sulfide intrusion can be expected (Ferguson et al. 2016). Wallis Lake is, like many other estuaries, under pressure from an increasing population and increasing exploitation of the catchment, which increases the nutrient inputs to the estuary (Fiebig 2010). We explicitly tested two hypotheses on sulfide intrusion in *Z. muelleri*; that sulfide intrusion...
is driven by a) seagrass morphology and/or b) sediment biogeochemistry. The seagrass meadows were
examined along a gradient of anthropogenic impact, from the lower estuary (impacted) to the lagoon
(unimpacted) and we focused on seagrass morphology (shoot density, above- and below-ground biomass,
shoot size), plant nutrient content (as an indicator of eutrophication, Burkholder et al. 2007) and stable
isotopic composition, sulfide intrusion, and sediment biogeochemistry. The gradient is considered to be
driven by nutrient loading from land the populated northern lower estuary, stimulating phytoplankton
production at the expense of benthic primary production and increasing turbidity in the water column (Fiebig
2010).

**Materials and methods**

Wallis Lake is a large wave-dominated barrier estuary (Roy et al. 2001) located on the mid-north coast of
New South Wales, approximately 300 km north of Sydney (Fig. 1). The estuary has a catchment area of 1197
km², which is drained by the Wallamba River, Coolongolook River, Wang Wauk River and Pipers Creek.
The estuary has a surface area of 99 km² and a mean depth of 2.3 m, with a salinity range between 31-33 in
the central basin. Water column nutrient concentrations are generally low (0.2-0.3 µM NO₃, 0.4-0.9 µM
NH₄⁺ and 0.02-0.37 µM PO₄³⁻; (0.2-0.3 µM NO₃, 0.4-0.9 µM NH₄⁺ and 0.02-0.37 µM PO₄³⁻; Smith and
Heggie 2003) and there is a gradient of anthropogenic impact from the lower estuary to the lagoon (Fig. 1).
Bed shear stress is low within the estuary, driven primarily by small tidal currents (<0.1 m s⁻¹). In contrast,
bed shear stress in the lake basin is driven by wind wave exposure and tends to be high in shallow areas with
significant northeasterly and southeasterly fetches. *Zostera muelleri* is the dominant seagrass in the estuary
with small to large patches of *Ruppia megacarpa*, *Posidonia australis* and *Halophila ovalis*. The estimated
cover of seagrasses is 31.9 km², covering approximately one third of the estuary (Fiebig 2010). There are
significant beds of charophytes in the lagoon, and Wallis Lake is surrounded by mangroves and saltmarshes,
which contribute terrestrial organic matter to the estuary. The total N load to Wallis Lake is estimated to be
953 kg km⁻² yr⁻¹ (68 mmol m⁻² yr⁻¹, Fiebig 2010) and is low compared to other sites (Port Phillip Bay: 200
mmol m⁻² yr⁻¹ (Harris 1996); Basin d’Arcachon: 460 mmol m⁻² yr⁻¹ (de Wit et al. 2001), Limfjorden: 2700
mmol m⁻² yr⁻¹ (Møhlenberg 1999)).
Sampling

Five study stations with extensive cover of *Z. muelleri* were chosen along a nutrient enrichment gradient from the lower estuarine reach of a freshwater input (Pipers Creek) to the marine dominated lagoon (Fig. 1). Bed shear stress across this gradient was not measured, however, given the fetches of prevailing storms and water depth at each station, it is likely that the shear stress gradient was lowest at Sta. 1 followed by Sta. 5, Sta. 4 and Sta. 2 and highest at Sta. 3. The water depth varied between 1.2 to 2.1 m (Sta. 1: 1.5 m; Sta. 2- Sta. 3: 1.2 m; Sta. 4: 1.6 m; Sta. 5: 2.1 m). At each station five cores (i.d. 14.6 cm) were collected to a depth of 30 cm for measurements of shoot density and above- and below-ground biomass. An additional eight small cores (cut-off 50 ml syringes) were collected to measure sediment iron and sulfur content and 4 sediment cores (i.d. 8 cm and length 30 cm) were collected for sediment characteristics (density, porosity, C and N pools and stable C, N, and S isotope signals).

Four to five replicate Diffusive Gradients in Thin-films (DGT) porewater samplers containing a silver iodide binding layer, as described by (Robertson et al. 2008), were deployed in the seagrass meadows at each sampling station for two days to collect porewater sulfate and sulfide. Potential carbon sources (e.g. epiphytes, drifting macroalgae) to the seagrass meadow were collected by hand in the area during the sampling.

Processing of samples

Cores for seagrass biomass were sieved and seagrasses were separated into leaves, rhizomes and roots. The length and the width of the second oldest leaf were measured on five shoots and the number of flowering shoots was counted as described by Ferguson et al. (2016). The two youngest leaves, rhizomes and roots from ten shoots were collected for analysis of nutrient content and stable isotopes (C,N,S). The plant materials were dried at 60 °C for 1-2 days. Potential carbon sources were dried as above and analysed for stable isotopes (CNS) and nutrient content (CN).
The sediments for iron and sulfide analysis were adjusted to 5 cm and briefly homogenized before preservation in 0.5 M HCl (1 cm³ sediment and 9 mL HCl) and 1 M zinc acetate (20 cm³ sediment and 20 mL zinc acetate), respectively. The samples were stored refrigerated or frozen. Iron was measured on the supernatant after dilution according to (Stookey 1970) and Sørensen (1982). The samples for sediment characteristics were sliced at 2 cm intervals down to 10 cm and at 5 cm intervals down to 15-20 cm, depending on the length of the core. Sediments were processed for density, porosity and dry weight. A subsample of dried sediment was analysed for stable C and N isotopes. The sediments preserved in zinc acetate were distilled twice according to the two-step distillation procedure by Fossing and Jørgensen (1989) using either AgNO₃ or zinc acetate as a sulfide trap, and separated into AVS and CRS. The precipitated Ag₂S was packed into tin capsules together with vanadium pentoxide and analyzed using EA-IRMS (Delta V Advantage Isotope Ratio MS with Thermo Scientific EA) to obtain δ³⁴S in the AVS and CRS fraction. Standards analyzed were IRMS certified reference material EMA-P1, and an Ag₂S internal standard. The precipitated ZnS was analysed according to Cline (1969) to determine the sulfide pools.

Sulfide DGT samplers

DGT samplers for measuring dissolved porewater sulfide and sulfate were prepared as described previously (Robertson et al. 2008) in sediment probe housings purchased from DGT Research Ltd. The samplers are composed of a binding layer, which is a polyacrylamide hydrogel containing precipitated silver iodide, overlain with a diffusive layer, consisting of a polyacrylamide hydrogel of 0.8 mm thickness, to control diffusion of solutes into the sampler. Dissolved sulfide diffuses into the sampler from the sediment porewater and irreversibly binds as a black silver sulfide precipitate. The intensity of the black color is proportional to the concentration of dissolved sulfide in the sediment porewaters, and provides the unique capability of examining the two-dimensional distribution of dissolved sulfide in the seagrass rhizosphere. The sulfide distributions were obtained by scanning the retrieved gels, as described previously (Robertson et al. 2008). Porewater sulfate does not interact with the DGT binding layer, but simply reaches equilibrium. Prior to scanning the binding gels, they were back-equilibrated in a known volume of deionized water to obtain porewater sulfate for isotopic analysis.
Nutrient content and stable isotopes of C and N were obtained on ground plant and sediment material, and analyzed using EA-IRMS (Delta V Advantage Isotope Ratio MS with Thermo Scientific EA) with acetonilide and urea used as standards (Working standards, IVA- Analysentechnic). The contribution of different primary producers to sediment organic carbon was calculated by using the mass-balance model IsoSource 1.3 with increments of 1% and tolerance of 0.1, based on the primary producer’s δ\(^{13}\)C signatures.

Plant total sulfur (TS) and δ\(^{34}\)S was analyzed as described above by EA-IRMS by weighing ground plant material into tin capsules together with vanadium pentoxide. The water samples collected were kept frozen until analysis for δ\(^{34}\)S\(_{\text{sulfate}}\). Samples were prepared by boiling under acidic conditions followed by precipitation of sulfate with BaCl\(_2\) as BaSO\(_4\), and analysed as described for δ\(^{34}\)S in sediments and plants. The sulfate eluted from the DGT samplers was precipitated and measured as described for the water samples to obtain δ\(^{34}\)S for porewater sulfate. It was not possible to measure the collected sulfide on the samplers for δ\(^{34}\)S due to instrumental limitations, and they were only used to illustrate sulfide distributions in the rhizosphere sediment.

The percentage of sulfur in the plants derived from the sediment (F\(_{\text{sulfide}}\)) was calculated according to Frederiksen et al. (2006):

\[
F_{\text{sulfide}}(\%) = \frac{\delta^{34}S_{\text{tissue}} - \delta^{34}S_{\text{sulfate}}}{\delta^{34}S_{\text{sulfide}} - \delta^{34}S_{\text{sulfate}}} \times 100
\]

where δ\(^{34}\)S\(_{\text{tissue}}\) is the value measured in the leaf, rhizome or root, and δ\(^{34}\)S\(_{\text{sulfide}}\) and δ\(^{34}\)S\(_{\text{sulfate}}\) are the values measured in sediment and porewater samples, respectively.

Statistics

All figures and statistical analysis were conducted in GraphPad Prism version 6 for Mac (GraphPad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) was used as the test assumptions were
fulfilled (visual inspection and Brown-Forsythe test). For results of ANOVA that showed significant effects 
(p<0.05), the Tukey’s honestly significant difference post-hoc procedure was used to determine significantly 
different means. Linear regressions were conducted when test assumptions were fulfilled and reported with 
goodness of fit (R²) and p-values (F-tests to test significant deviations from zero). Data were adjusted for 
outliers using the Grubbs’ test. If not stated differently all mean values are presented with standard error of 
mean (mean ± SEM).

Results

The water column concentrations of TN decreased by a factor of 3 from Sta. 1 (~350 µg l⁻¹) to the marine 
lagoon sites (Sta. 4 and Sta. 5 ~100 µg l⁻¹). The sediments had the lowest density (1.2-1.3 gWW cm⁻³) and 
highest porosity (0.79-0.97) at Sta. 1 and Sta. 4 and 5, indicating less wind and wave exposed stations 
compared to Sta. 2 and Sta. 3, which had highest density (1.8-1.9 gWW cm⁻³) and lowest porosity (0.62-
0.66). The sediment organic matter pools represented a combination of expected high organic matter loading 
and exposure, where Sta. 1 had POC (3.4%DW) and PON pools (0.36%DW) in between the most sheltered 
(Sta. 4 and Sta. 5) and most exposed sites (Sta. 2 and 3, Fig. 2A and 2B, ANOVA p<0.05). This resulted in a 
large difference in C:N ratio among stations, with the lowest ratio (C:N = 11-15) at stations 1, 4 and 5 and 
the highest at Sta. 3 (C:N = 67, Fig. 2C, ANOVA p<0.05). Sediment Fe content was similar among stations 
(59-74 mmol Fe cm⁻³; Table 1) and did not show any response to eutrophication or exposure gradient. 
Similarly the AVS pools showed no response and were quite similar at all stations (0.5-2.7 µmol cm⁻³) and 
low compared to CRS. In contrast, the CRS pools varied along the gradient with low pools at Sta. 3 
(27.2±9.5 µmol cm⁻³) and the maximum at Sta. 1 (133.0±11.4 µmol cm⁻³, Fig. 2D, ANOVA p<0.05). The 
δ³⁴S_{AVS} ranged between -19.8‰ and -23.3‰ and δ³⁴S_{CRS} between -23.5‰ and -28.1‰ with no distinct 
pattern between stations (Table 1). Sulfate was collected by equilibration within the DGT samplers, and the 
porewater concentration in the rhizosphere sediment varied between 19.2-31.6 mM and the δ³⁴S_{sulfate} was 
20.84‰-21.19‰ (Table 1) with no major differences between stations. Unfortunately it was not possible to 
collect DGT profiles of porewater sulfides consistently at the 5 stations due to harsh weather conditions. 
Scanned images of selected DGT samplers from Z. muelleri rhizosphere sediments at Sta. 1 and Sta. 4
showed what appeared to be an absence of dissolved sulfide around two roots that were probably in contact with the DGT samplers during deployment (Fig. 3).

Seagrass morphological parameters varied among stations reflecting both the eutrophication gradient and degree of exposure and did thus not show a clear linear relationship with eutrophication or exposure. Shoot density of *Z. muelleri* varied by a factor of 4 between the five stations with lowest density of 836±335 shoots m⁻² at Sta. 2 and highest with 3285±1241 shoots m⁻² at Sta. 4 (Fig. 4, ANOVA p<0.05). This was reflected in above-ground biomass, which showed the same pattern, and the difference between Sta. 2 and Sta. 4 was 4.8 fold (98±49 gDW m⁻² and 487±287 gDW m⁻², respectively, ANOVA p<0.05). The ratio of above- to below-ground biomass (A:B) ranged from 1.3 to 1.9 with the highest ratio at Sta. 1 and 5 (Table 2, ANOVA p<0.05). Shoot morphology varied among stations with the longest and heaviest shoots at Sta. 5 (580±130 mm; 276±78 mgDW shoot⁻¹, ANOVA p<0.05), whereas there were no significant differences in the parameters at the other 4 stations (299-365 mm, 113-153 mgDW shoot⁻¹; Table 2, ANOVA p<0.05).

Intensity of flowering was highly variable among and within stations with flowering at Sta. 1-4 (36-137 flowers m⁻²), but no flowering at Sta. 5 (Table 2).

The N content in the plant leaves reflected the eutrophication gradient and showed a significant decrease from Sta. 1 to Sta. 5 of 1.54 to 1.29 %DW (ANOVA p<0.05, Table 3). The N content in the rhizomes (0.39-0.75 %DW) and roots (0.66-0.85 %DW) was approximately half of that in the leaves, but did not show the same decreasing trend along the gradient (Table 3). The TS content was highest in the roots at Sta. 1 (1.03±0.19 %DW) and lowest at Sta. 3 (0.62±0.12 %DW) (Table 3, ANOVA p<0.05) and showed no clear trend along the eutrophication gradient. The TS content was the same in leaves and rhizomes for Sta. 1 and Sta. 2, whereas TS was highest in the leaves at Sta. 3-5. F_{sulfide} was highest in the roots (57-80%), decreasing in the rhizomes (38-57%) and lowest in the leaves (18-48%, Table 3). F_{sulfide} was significantly lower at Sta. 3 compared to end points of the gradient (Sta. 1 and Sta. 5), whereas there were few significant differences among the other stations (ANOVA p<0.05). There were positive linear correlations between TS and F_{sulfide} for each type of tissue (Fig. 5, R²= 0.50-0.72).
The $\delta^{13}$C varied between -9.5‰ to -11.4‰ in the leaves, -9.9‰ to -11.5‰ in the rhizomes and -9.7‰ to -11.4‰ in the roots without any clear pattern among stations (Table 4). The $\delta^{15}$N was highest at Sta. 3 in all tissues (4.3‰-4.9‰) and lowest at Sta. 5 (0.95‰ in leaves increasing to 2.54‰ in roots; Table 2, ANOVA p<0.05). Seagrass leaves had very few epiphytes, though at several stations (Sta. 1, Sta. 4-5) the leaves were covered by particulates; probably resuspended particulates due to high winds during the sampling period. We collected *Halophila* sp. and some drifting macroalgae at the stations (Table 3) and $\delta^{13}$C and $\delta^{15}$N varied between -14.0‰ to -18.1‰ and 0.1‰-2.7‰, respectively, for these plant tissues. Results from the IsoSource model showed an increasing average contribution of seagrass detritus to the sediment organic carbon pool from 7.5% at Sta. 1 to 71-75% at Sta. 4 and 5 (Table 4).

There were significant correlations between $F_{\text{sulfide}}$ and seagrass morphology, seagrass nutrient content and sediment biogeochemistry (Table 5). The strongest correlations were with sediment biogeochemistry: a) contribution of seagrass to the organic matter pool (negative correlation $R^2=0.62$; p<0.0001), followed by b) sediment POC (negative correlation, $R^2=0.48$; p=0.0002) and c) sediment PON (negative correlation, $R^2=0.41$; p=0.0007). Seagrass nutrient enrichment, measured as increasing N content in the leaves ($R^2=0.32$; p=0.0037) and increasing $^{15}$N signal in the leaves ($R^2=0.49$; p=0.0001), also correlated with $F_{\text{sulfide}}$, whereas the correlations with seagrass morphology were more variable (negative correlation with leaf width $R^2=0.47$; p=0.0002, negative correlation with above-ground biomass $R^2=0.38$; p=0.0014, negative correlation with below-ground biomass $R^2=0.20$; p=0.027, negative correlation with shoot density $R^2=0.21$; p=0.024).

**Discussion**

The $F_{\text{sulfide}}$ in *Z. muelleri* was high in Wallis Lake (up to 80% in the roots) compared to other seagrass species in Australia (Holmer and Kendrick 2013) and similar to *Zostera marina*, where intrusion of sulfides can be high with up to 80-90% of the sulfur in the plants derived from sedimentary sulfides (Holmer and Hasler-Sheetal 2014). Our two hypotheses on possible links with a) seagrass morphology and b) sediment biogeochemistry on sulfide intrusion were confirmed, but as both seagrass morphology and sediment
biogeochemistry changed along the gradient, there were several potential effects of sulfide intrusion on Z. *muelleri* and several possible drivers of sulfide intrusion into Z. *muelleri*. The negative correlations between $F_{\text{sulfide}}$ and seagrass above-ground and below-ground biomass and shoot density suggest that sulfide intrusion negatively affected the above- and below-ground biomass and shoot density. This is consistent with findings by Mascaro et al. (2009), where sulfide intrusion stimulated by organic matter additions to the sediments and by hypoxia in the water column during day and night time, resulted in reduced leaf growth rate, increased mortality and lower above- and below-ground biomass in Z. *marina*. Sulfide toxicity damaging the seagrass tissues, in particular the meristems, were considered the main driver of lower seagrass performance. It is possible that the negative correlations in Wallis Lake could also be unrelated to sulfide toxicity, and instead a result of lower intrusion of sulfides in dense Z. *muelleri* meadows with high above- and below-ground biomass driven by oxidation of the sediments from oxygen released by the below-ground biomass. Brodersen *et al.* (2015) and Koren *et al.* (2015) found high leakage of oxygen from Z. *muelleri* compared to Z. *marina* as oxygen was released along the entire root and to some extent from the rhizomes. The radial oxygen loss (ROL) is much higher in Z. *muelleri* compared to Z. *marina*, where oxygen is only leaked to the sediments through the root tips (Jensen *et al.* 2005), suggesting that Z. *muelleri* has a larger capacity to oxidize the rhizosphere sediments compared to other seagrass species and thereby reduce sulfide intrusion in particular in dense meadows. This hypothesis is supported by previous findings, where major shifts in porewater Fe$^{2+}$ and sulfide concentrations between light and dark conditions in Z. *capricorni* (now Z. *muelleri*) rhizosphere sediments demonstrating a significant capacity of Z. *muelleri* to buffer sulfide accumulation in rhizosphere sediments (Pagès *et al.* 2012). In our study, two DGT samplers were by coincidence placed close to Z. *muelleri* roots and they showed that porewater sulfide was absent along the length of the roots (Fig. 5) confirming a high reoxidation of sulfides by leaked oxygen. Furthermore AVS pools were very low and the sedimentary sulfide pool was dominated by the CRS fraction (>96% of TRS). High pools of CRS are generally found in more oxidized sediments due to formation of elemental sulfur and pyrite (Holmer 2009). As $F_{\text{sulfide}}$ in Z. *muelleri* is high compared to other seagrasses despite the oxidized rhizosphere, it is most likely that a major fraction of the sulfides are reoxidized to sulfate immediately before entering the plant in the rhizosphere around individual roots. Reoxidation of sulfides to sulfate is considered an important
detoxification mechanism in *Z. marina* accounting for up to 12% of the sulfide intrusion (Holmer and Hasler-Sheetal 2014), and this mechanism is possibly even higher in *Z. muelleri* due to the extended oxidation of the rhizosphere. This may represent an important adaptation by *Z. muelleri* to increase survival in reduced estuarine sediments. The negative correlation between leaf width and sulfide intrusion further suggests higher release of oxygen from the larger seagrasses with wider leaves, a relationship which should be explored further.

The correlations between $F_{\text{sulfide}}$ and sediment biogeochemistry varied along the gradient. Most surprising was the negative correlations between $F_{\text{sulfide}}$ and pools of sediment organic carbon and nitrogen, showing that the sulfide intrusion decreased as the organic matter pools increased. This relationship was driven by high pools of organic matter in the lagoon sediments, where the organic matter probably accumulated as these sites were the most sheltered along the gradient. The organic matter pools in the lagoon sediments were high (average 6.5% POC and 0.5% PON) compared to global averages (1.5% POC and 0.15% PON) in seagrass sediments (Kennedy *et al.* 2010), which could be due to increased trapping efficiency in the dense meadows and lower physical energy at these stations. The $\delta^{13}$C sediment analysis showed that the buried organic matter primarily originated from seagrass detritus, as $>70\%$ of the sediment organic carbon pool could be attributed to seagrass detritus at these two stations, which is high compared to global average of about 50% in seagrass sediments (Kennedy *et al.* 2010). As seagrass detritus is more recalcitrant compared to phytoplanktonic detritus the rates of sulfate reduction and thus sulfide production is most likely lower in the lagoon sediments compared to the other stations. In the lower estuary, the contribution of seagrass detritus to the sediment organic matter was much lower and only 7.5% at Sta. 1, and the high contribution of phytoplanktonic detritus was also reflected in lower sediment C:N ratio at Sta. 1 and Sta. 2. We measured higher sediment oxygen uptake at these two sites compared to Sta. 3 (SOU was only measured at Sta. 1-3; Sta. 1: 48.0 mmol m$^{-2}$d$^{-1}$, Sta. 2: 32.8 mmol m$^{-2}$d$^{-1}$, Sta. 3: 30.0 mmol m$^{-2}$d$^{-1}$ (average, n=3) unpublished data) indicative of higher rates of mineralization and supporting higher sulfate reduction rates at Sta. 1 and Sta. 2. The correlations between $F_{\text{sulfide}}$ and sediment sulfide pools were not significant, probably due to interactions between seagrass oxidation capacity (e.g. below-ground biomass) and changes in sediment
biogeochemistry (e.g. sulfate reduction rates) along the gradient. The range of sediment organic matter content and other sediment characteristics found in Wallis Lake is similar to other studies showing that Z. muelleri has large plasticity to grow in variable environments (Matheson and Schwarz 2007; Kohlmeier et al. 2014).

Nutrient enrichment of Z. muelleri in Wallis Lake was indicated by higher N content in the leaves in the lower estuary, decreasing towards the lagoon. The N enrichment in the leaves at Sta. 1 and Sta. 2 may, in addition to inputs of N from terrestrial land run-off, derive from remineralized phytoplanktonic detritus in the rhizosphere sediments which was higher at Sta. 1 and Sta. 2 as discussed above. Wallis Lake appears to be a productive estuary, and above-ground and below-ground biomass was high compared to other studies of Z. muelleri in the distribution area (Kerr and Strother 1990 (South-Australia and Tasmania); Mellors et al. 2005 (Great Barrier Reef); Matheson and Schwarz 2007 (New Zealand); Kohlmeier et al. 2014 (New Zealand)) and given the high coverage of seagrasses in Wallis Lake, this suggests that seagrass meadows are an important component of the Wallis Lake ecosystem. The seagrass morphology varied significantly among stations, but there was no clear pattern from the lower estuary to the lagoon. The plants in the lagoon had the heaviest, longest and widest leaves. Shoot density at Sta. 5 was only one third of Sta. 4 suggesting light limitation due to self-shading, as the leaves were also longest at this station. Sta. 5 was 0.5 m deeper than Sta. 4 and with fine grained sediment, which probably negatively affected the light climate during resuspension events as experienced during sampling. Typical responses of Z. muelleri to decreased light conditions are broadening and elongation of leaves (Abal et al. 1994; Peralta et al. 2000). In the lower estuary the shoots were smaller and the meadow less dense and the total biomass was lower, which could be a result of several factors including light climate, physical energy and sulfide toxicity. There was no distinct pattern in the above:below ground (A:B) ratio along the gradient, probably due to this mix of factors acting on the ratio (Ferguson et al. 2016). The ratios were similar to other studies, but the range was lower, suggesting less variation in the environmental conditions compared to other study sites (Matheson and Schwarz 2007; Kohlmeier et al. 2014). There were no ecosystem signs of eutrophication in the seagrass meadows as the plants had few epiphytes and no blooms of filamentous algae were observed at the sampling
stations. The N content of the leaves thus appears to be a more sensitive indicator of nutrient enrichment.

The positive correlations between $F_{\text{sulfide}}$ and N content in the leaves and between $F_{\text{sulfide}}$ and $\delta^{15}N$ signal in the leaves suggests increasing intrusion of sulfides towards the source of nutrients and thereby the first signs of the potential negative impact of nutrient enrichment on seagrasses. Monitoring of N content and $\delta^{15}N$ signal of leaves along with $F_{\text{sulfide}}$ in the seagrasses are potential candidates as early indicators of nutrient enrichment and sulfide pressure on the seagrasses, which is consistent with findings in $Z. \text{marina}$ in the Black Sea (Holmer et al. 2016). It should be further explored if these early indicators can supplement the traditional monitoring of seagrass meadows, which typically consists of monitoring of water quality, sediment parameters and seagrass distribution. Seagrass distributions show large seasonal and inter-annual variations making the interpretation of monitoring data difficult. In contrast, early warning indicators of seagrass stress, such as N content, $\delta^{15}N$ signal of leaves, and $F_{\text{sulfide}}$, could provide a more reliable approach to seagrass monitoring.

In conclusion, $Z. \text{muelleri}$ in Wallis Lake grows in dense meadows from the lower estuary to the lagoon despite nutrient enrichment in the lower estuary. There were signs of nutrient enrichment, as the N content of the leaves was enriched in the lower estuary and at the same time the sediments had higher contribution of organic matter derived from phytoplanktonic detritus. The sulfide intrusion was generally high in $Z. \text{muelleri}$ and linked to N enrichment. As higher sulfide intrusion was concurrent with lower shoot density and biomass, the sulfide intrusion may be an early sign of the negative effects of nutrient enrichment. The deployment of DGT samplers, however, provided evidence of high oxidation of sulfides in $Z. \text{muelleri}$ rhizosphere sediments, which suggests that most of the sulfur entering the plants was in reoxidized and non-toxic forms. High oxygen release from $Z. \text{muelleri}$ provides high tolerance for sulfide intrusion and is a unique adaptation, which allows high abundance and productivity of $Z. \text{muelleri}$ in estuarine sediments.

**Acknowledgement**

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Figures legends

Fig. 1 Map of sampling stations in Wallis Lake. Modelled water column concentrations of TN (µg l$^{-1}$) are shown for the study area (Fiebig 2010).

Fig. 2 Sediment characteristics in Wallis Lake. (A) sediment organic carbon content (POC, %DW), (B) sediment organic nitrogen content (PON, %DW), sediment C:N ratio (molar) and sediment sulfides (TRS; µmol cm$^{-3}$). Letters indicate significant differences (ANOVA and Tukey’s test p>0.05; n=5, ±SEM).

Fig. 3 Two DGT samplers from Sta. 1 and Sta. 4 showing precipitation of porewater sulfides in greyscale. Areas with no sulfide precipitation (white) are found around roots of $Z$. muelleri.

Fig. 4 Seagrass shoot density (A, shoots m$^{-2}$) and above- and below-ground biomass (B, g DW m$^{-2}$) from lower estuary (Sta. 1) to lagoon (Sta. 4 and 5) in Wallis Lake. Letters indicate significant differences (ANOVA and Tukey’s test p>0.05; n=5, ±SEM).

Fig. 5 Relationship between total sulfur content (TS, %DW) in the plant tissue and $F_{\text{sulfide}}$ (%) in $Z$. muelleri in Wallis Lake. Best linear fit and correlation coefficients are given.
increasing sediment depth
The diagram shows the relationship between F$_{\text{sulfide}}$ (%) and TS (%) for leaves, rhizomes, and roots. The correlation coefficients ($R^2$) are:

- Leaves: $R^2 = 0.58$
- Rhizomes: $R^2 = 0.72$
- Roots: $R^2 = 0.51$

The data points for each category are represented by different symbols: diamonds for leaves, circles for rhizomes, and squares for roots.
Table 1: Sediment Fe content, porewater sulfate (SO$_4^{2-}$) from DGT gels and $\delta^{34}$S signals in porewater sulfate and sediment AVS and CRS in Lake Wallis (mean±SD; n=4-5). The contribute of seagrass detritus to sediment POC pool (\%seagrass) is calculated as average by use of IsoSource (see text for explanations).

<table>
<thead>
<tr>
<th></th>
<th>Fe (mmol cm$^{-3}$)</th>
<th>SO$_4^{2-}$ (mM)</th>
<th>$\delta^{34}$S Sulfate (‰)</th>
<th>$\delta^{34}$S AVS (‰)</th>
<th>$\delta^{34}$S CRS (‰)</th>
<th>%seagrass</th>
</tr>
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<tr>
<td>Sta. 1</td>
<td>67.26±4.71</td>
<td>21.2±5.2</td>
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<td>-22.53±1.65</td>
<td>-28.09±0.20</td>
<td>7.5</td>
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<tr>
<td>Sta. 2</td>
<td>58.97±9.76</td>
<td>21.9±9.7</td>
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<td>-22.30±2.37</td>
<td>-27.81±0.57</td>
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</tr>
<tr>
<td>Sta. 3</td>
<td>73.95±7.59</td>
<td>31.6±1.1</td>
<td>20.99±0.12</td>
<td>-20.01±1.03</td>
<td>-27.09±0.26</td>
<td>29</td>
</tr>
<tr>
<td>Sta. 4</td>
<td>59.56±14.86</td>
<td>19.9±3.4</td>
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<td>-23.33±1.16</td>
<td>-25.02±0.54</td>
<td>75</td>
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<tr>
<td>Sta. 5</td>
<td>69.77±14.30</td>
<td>19.2±4.2</td>
<td>21.11±0.14</td>
<td>-19.82±1.96</td>
<td>-23.49±1.19</td>
<td>71</td>
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Table 2: Seagrass morphological parameters in Lake Wallis Letters indicate significant differences (ANOVA and Tukey’s test p < 0.05); (n = 5, ±SD)

<table>
<thead>
<tr>
<th>A:B ratio</th>
<th>Shoot weight (mg DW⁻¹)</th>
<th>Leaf length (mm)</th>
<th>Leaf width (mm)</th>
<th>Flowering shoots</th>
</tr>
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<tbody>
<tr>
<td>Sta. 1</td>
<td>1.80±0.47</td>
<td>134±51 b</td>
<td>365±76 b</td>
<td>3.0±0.8 ac</td>
</tr>
<tr>
<td>Sta. 2</td>
<td>1.31±0.61</td>
<td>114±15 b</td>
<td>299±67 c</td>
<td>3.2±0.4 bc</td>
</tr>
<tr>
<td>Sta. 3</td>
<td>1.40±0.62</td>
<td>113±50 b</td>
<td>305±88 c</td>
<td>3.0±0.4 c</td>
</tr>
<tr>
<td>Sta. 4</td>
<td>1.34±0.45</td>
<td>153±75 b</td>
<td>358±71 b</td>
<td>3.5±0.4 a</td>
</tr>
<tr>
<td>Sta. 5</td>
<td>1.86±0.40</td>
<td>276±78 a</td>
<td>580±130 a</td>
<td>3.4±0.4 ab</td>
</tr>
</tbody>
</table>
Table 3: Average carbon (%C), nitrogen (%N), stable isotopic signal (δ¹³C, δ¹⁵N ‰), F₉ulfide, C:N ratio (molar) and sulfur (TS) content (%DW) sediment of *Z. muelleri* leaves, rhizomes and roots in Lake Wallis, respectively total reducible sulfide *(TRS)* in Lake Wallis sediments (*n=*5, mean±SD). Lower case letters indicate significant differences between stations. ANOVA and post hoc Tukey’s test; *p* < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>%C (%DW)</th>
<th>δ¹³C (‰)</th>
<th>%N (%DW)</th>
<th>δ¹⁵N (‰)</th>
<th>C:N (molar)</th>
<th>F₉ulfide</th>
<th>TS (%DW)/TRS (µmol cm⁻³)</th>
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<tr>
<td><strong>Leaves</strong></td>
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<td>1.54±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.71±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.0±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.4±1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.62±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>2.95±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.59±0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>-10.32±0.44&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>28.2±3.2&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sta. 1</td>
<td>3.48±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-18.05±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.35±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.64±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.6±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>*133.5±5.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sta. 2</td>
<td>0.97±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-16.04±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05±0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.9±1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd</td>
<td>*51.2±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sta. 3</td>
<td>0.76±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-16.34±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.0±17.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>*28.2±4.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sta. 4</td>
<td>6.82±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-14.63±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.25±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.6±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>*78.8±6.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sta. 5</td>
<td>6.71±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-14.02±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.39±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.2±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>*94.8±4.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 4: Average C (%C) and N (%N) content (%DW), stable isotopic signal ($\delta^{13}$C, $\delta^{15}$N ‰) and C:N ratio (molar) of potential carbon sources in sediments of Lake Wallis. n= number of replicates.

<table>
<thead>
<tr>
<th>Origin</th>
<th>n</th>
<th>%C (%DW)</th>
<th>$\delta^{13}$C</th>
<th>%N (%)</th>
<th>$\delta^{15}$N</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halophila sp.</td>
<td>2</td>
<td>30.46</td>
<td>-14.03</td>
<td>1.90</td>
<td>0.09</td>
<td>18.6</td>
</tr>
<tr>
<td>Seston</td>
<td>2</td>
<td>11.55</td>
<td>-18.10</td>
<td>0.90</td>
<td>4.39</td>
<td>14.9</td>
</tr>
<tr>
<td>Macroalgae</td>
<td>1</td>
<td>23.73</td>
<td>-18.10</td>
<td>1.32</td>
<td>2.74</td>
<td>21.0</td>
</tr>
<tr>
<td>Seagrass detritus</td>
<td>5</td>
<td>32.60</td>
<td>-11.79</td>
<td>0.78</td>
<td>2.32</td>
<td>51.1</td>
</tr>
</tbody>
</table>
Table 5: Linear regression of $F_{\text{sulfide}}$ in leaves versus seagrass morphology and sediment biogeochemistry

<table>
<thead>
<tr>
<th></th>
<th>Equation</th>
<th>$R^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot density</td>
<td>$Y = -0.005510X + 45.94$</td>
<td>0.21</td>
<td><strong>0.0243</strong></td>
</tr>
<tr>
<td>Above-ground biomass</td>
<td>$Y = -0.04267X + 47.51$</td>
<td>0.38</td>
<td><strong>0.0014</strong></td>
</tr>
<tr>
<td>Below-ground biomass</td>
<td>$Y = -0.04252X + 43.90$</td>
<td>0.20</td>
<td><strong>0.0273</strong></td>
</tr>
<tr>
<td>A:B</td>
<td>$Y = -1.964X + 39.29$</td>
<td>0.006</td>
<td>0.7091</td>
</tr>
<tr>
<td>Leaf width</td>
<td>$Y = -34.46X + 147.9$</td>
<td>0.48</td>
<td><strong>0.0002</strong></td>
</tr>
<tr>
<td>Leaf length</td>
<td>$Y = -0.02984X + 47.62$</td>
<td>0.23</td>
<td>0.2272</td>
</tr>
<tr>
<td>%N leaves</td>
<td>$Y = 49.14X - 32.84$</td>
<td>0.32</td>
<td><strong>0.0037</strong></td>
</tr>
<tr>
<td>$\delta^{15}$N</td>
<td>$Y = 5.693X + 21.78$</td>
<td>0.49</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td>%C seagrass</td>
<td>$Y = -0.3658X + 51.78$</td>
<td>0.62</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>sedPOC</td>
<td>$Y = -3.323X + 48.98$</td>
<td>0.48</td>
<td><strong>0.0002</strong></td>
</tr>
<tr>
<td>sedPON</td>
<td>$Y = -34.91X + 47.22$</td>
<td>0.41</td>
<td><strong>0.0007</strong></td>
</tr>
<tr>
<td>AVS</td>
<td>$Y = -6.718X + 46.09$</td>
<td>0.40</td>
<td><strong>0.0009</strong></td>
</tr>
<tr>
<td>CRS</td>
<td>$Y = -0.006269X + 36.69$</td>
<td>&lt;0.001</td>
<td>0.9309</td>
</tr>
</tbody>
</table>