Seagrass rhizosphere microenvironment alters plant-associated microbial community composition

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Summary

The seagrass rhizosphere harbors dynamic micro-environments, where plant-driven gradients of O2 and dissolved organic carbon form microhabitats that select for distinct microbial communities. To examine how seagrass-mediated alterations of rhizosphere geochemistry affect microbial communities at the microscale level, we applied 16S rRNA amplicon sequencing of artificial sediments surrounding the meristematic tissues of the seagrass Zostera muelleri together with microsensor measurements of the chemical conditions at the basal leaf meristem (BLM). Radial O2 loss (ROL) from the BLM led to ~300 μm thick oxic microzones, wherein pronounced decreases in H2S and pH occurred. Significantly higher relative abundances of sulphate-reducing bacteria were observed around the meristematic tissues compared to the bulk sediment, especially around the root apical meristems (RAM; ~57% of sequences). Within oxic microzones, elevated abundances of sulphide-oxidizing bacteria were observed compared to the bulk sediment and around the RAM. However, sulphide oxidisers within the oxic microzone did not enhance sediment detoxification, as rates of H2S re-oxidation here were similar to those observed in a pre-sterilized root/rhizome environment. Our results provide novel insights into how chemical and microbiological processes in the seagrass rhizosphere modulate plant-microbe interactions potentially affecting seagrass health.

Introduction

Seagrass meadows are high-value ecosystems (Costanza et al., 1997) that provide numerous ecosystem services to marine environments, such as facilitating nursery areas for many juvenile fish and crustaceans (Harborne et al., 2006; Larkum et al., 2006), improving water quality through increased sedimentation (Ward et al., 1984; Madsen et al., 2001), providing a main food source for iconic marine animals such as sea turtles and dugongs, and by having a high ability to sequester carbon in the sediment (Duarte et al., 2005; Fourqurean et al., 2012). Seagrass ecosystems harbour a unique microbiome including populations of microbes attached to seagrass leaves and within the rhizosphere, with microorganisms involved in the sulphur cycle known to play particularly important functional roles (e.g., Devereux, 2005; Jensen et al., 2007; Cucio et al., 2016; Fahimipour et al., 2017). However, a quantitative understanding of the importance of this microbiome on the fitness of seagrass plants is lacking (York et al., 2016).

Jensen and colleagues (2007) showed that sulphate-reducing bacteria (SRB) and sulphide-oxidizing bacteria (SOB) are located in different microniches within the seagrass rhizosphere, with their distributions largely defined by rhizospheric O2 availability, and the authors suggested that SOB may be beneficial to seagrasses by removing toxic H2S from the rhizosphere. However, this hypothesis was derived from bulk sample analysis and did not include measurements of H2S oxidation within oxic microzones harbouring SOB. To truly tease apart the relationships between seagrasses and communities of SRB and SOB, there is thus a need for combined microbiological and biogeochemical characterization of the seagrass rhizosphere microenvironment.

Seagrasses grow in largely anoxic, reduced marine sediments (Borum et al., 2005) that are often enriched in the potent phytotoxin H2S (Holmer et al., 2006; van der Heide et al., 2012; Lamers et al., 2013). These anoxic and sulphidic conditions are a consequence of the deposition of large amounts of organic matter in seagrass meadows driving microbial remineralization processes, which are dominated by SRB (Jorgensen, 1982; Blaabjerg and Finster, 1998; Blaabjerg et al., 1998; Blaabjerg et al., 1998; Nielsen et al., 2001). To accommodate growth in such hostile environments, seagrasses release O2 into their rhizosphere, that is, the small
volume of sediment directly influenced by root/rhizome secretions and associated microbes, from their basal leaf meristem and root apical meristems. This so-called radial O$_2$ loss (ROL), leads to the formation of localised oxic microniches around the seagrass roots/rhizome (Pedersen et al., 1998; Jensen et al., 2005; Brodersen et al., 2015a; 2016; Koren et al., 2015). Such plant-driven oxic microzones support local H$_2$S re-oxidation and thereby protect the most vulnerable parts of the plant against the H$_2$S produced by SRB in the surrounding anoxic sediments (Brodersen et al., 2015a). While oxic microniches mainly form around growing root tissues, mature regions of the roots possess physical barriers to ROL, which are typically composed of Casparian-band like structures that may function as an analogous barrier to H$_2$S intrusion (Barnabas, 1996; Colmer, 2003). Seagrass rhizomes and roots also release significant amounts of labile dissolved organic carbon (DOC), especially around the root-cap (Moriarty et al., 1986; Pollard and Moriarty, 1991), which can stimulate microbial activity including sulphate reduction in the rhizosphere (Isaksen and Finster, 1991; Blaabjerg et al., 1998; Hansen et al., 2000; Nielsen et al., 2001). Seagrasses thus substantially alter the biogeochemical conditions within their rhizosphere, resulting in a dynamic mosaic of chemical microgradients that influence both the distribution and activity of the plant-associated microbial community (Devereux, 2005; Jensen et al., 2007; Brodersen et al., 2015a; 2016; Cúcio et al., 2016).

Seagrass-driven modulation of the rhizosphere microhabitat has been suggested to initiate mutualistic relationships between seagrasses and diazotrophic SRB based on reciprocal nutrient exchange, whereby the seagrass host provides a carbon source to the diazotrophs that in return fix dinitrogen (Hansen et al., 2000; Welsh et al., 2000; Nielsen et al., 2001). Sulphide-oxidizing bacteria within oxic microenvironments in the rhizosphere have also been proposed to play an important role in sediment detoxification (Jensen et al., 2007; Cúcio et al., 2016; Fahimipour et al., 2017). Bacterial-mediated H$_2$S oxidation is $10^4$–$10^5$ times faster than spontaneous chemical oxidation (Jørgensen and Revsbech, 1983; Nelson et al., 1986) and given ample O$_2$ supply, SOB may thus efficiently remove toxic H$_2$S, facilitating seagrass colonization of highly sulfidic sediments. However, technical constraints in examining the distribution and activity of bacteria at the appropriate microscale resolution and at specific plant-driven chemical micro-hotspots have prevented confirmation of a beneficial role of SOB within the seagrass rhizosphere.

Here we present a detailed description of the microbial diversity surrounding the meristematic tissues of *Zostera muelleri* in combination with measurements of plant-modulated chemical micro-habitats in the seagrass rhizosphere, with the aim of elucidating the potential importance of SOB in detoxifying sediments for seagrasses, relative to plant-derived spontaneous chemical re-oxidation with O$_2$.

**Results and discussion**

The seagrass rhizosphere is dominated by bacteria involved in the sediment sulphur cycle (Jensen et al., 2007; Cúcio et al., 2016), but little is known about whether they alleviate or aggravate the exposure of below-ground seagrass biomass to phytotoxic H$_2$S. This lack of knowledge is particularly significant for the vital meristematic regions of the plants that lack barriers to ROL and are thereby most likely to experience H$_2$S intrusion (Colmer, 2003). However, this would only occur when ROL is insufficient to re-oxidise sulphide diffusing towards the below-ground tissue surface, as seen during severe events of water-column hypoxia in darkness (Brodersen et al., 2015a). Overall, the ROL-driven oxic microshields are understood as providing better protection of the below-ground tissues as they prevent phytotoxic sulphide from reaching the tissue surface (Koren et al., 2015; Brodersen et al., 2015a; 2017). Instead, the relatively impermeable mature parts of the below-ground tissues likely improve long-distance, internal transport of O$_2$ from leaves to distal root-tips (Colmer, 2003; Pedersen et al., 2004; Borum et al., 2006). Here, we combined detailed microscale measurements of the geochemical conditions and dynamics in the seagrass rhizosphere with analysis of the microbial community composition to determine, whether seagrasses benefit from rhizospheric bacterial-mediated sulphide oxidation.

We focused on the small volume of sediment surrounding the meristematic regions of the plant, as this part of the seagrass rhizosphere is most significantly affected by the seagrass host and exhibits strong chemical microgradients of O$_2$ and DOC availability (Moriarty et al., 1986; Pedersen et al., 1998; Jensen et al., 2007; Brodersen et al., 2015a). We were able to detect changes in the microbial community composition as a response to the activity of the seagrass host, without disturbing the below-ground biogeochemical micro-gradients and habitats during microsensor measurements, and determined that SOB are playing an important role in H$_2$S oxidation in the seagrass rhizosphere.

**Dynamics of the below-ground chemical microenvironment**

Radial O$_2$ loss (ROL) from the basal leaf meristem led to a $\sim$ 300 $\mu$m thick oxic microzone, protecting the seagrass plant from intrusion of H$_2$S through chemical oxidation at the oxic/anoxic interface (Fig. 1, Supporting Information Figs S1 and S2). In light, the O$_2$ efflux amounted to
A microsensor measurement in an artificial sediment matrix with added pore water microbes. C and D. Microsensor measurements in a presterilized environment, that is, sterilized artificial sediment matrix and below-ground tissue surface. A and C. Measurements in darkness. B and D. Measurements in light (photon irradiance of ~150 μmol photons m⁻² s⁻¹). Black line and symbols show the O₂ concentration; red line and symbols show the H₂S concentration; and blue line and symbols show pH. The dotted lines indicate the thickness of the plant-derived oxic microzone, and X = 0 indicates the surface of the basal leaf meristem. Symbols with error bars represent means ± SD (n = 3–4 technical replicates; biological replication of the below-ground chemical microenvironment dynamics is shown in Supporting Information Figs S1 and S2).

At the plant-driven rhizospheric oxic/anoxic interface (i.e., the dotted line in Fig. 1 ~ 300 μm from the tissue surface), we found a rapid decrease in sediment H₂S concentrations from ~100 μmol L⁻¹ at ~1 mm away from the below-ground tissue surface to 0 μmol L⁻¹ at the oxic/anoxic interface or slightly within the oxic microzone (Fig. 1 and Supporting Information Figs S1 and S2); thus showing complete detoxification of sedimentary H₂S before reaching the tissue surface. The ratio between the O₂ and total sulphide fluxes, that is, the amount of O₂ released from the below-ground tissue compared to the total amount of sulphide (H₂S, HS⁻ and S²⁻) oxidized at the basal leaf meristem in the artificial sediment was 0.6 in darkness and 1.7 in light for both treatments (Table 1). This is indicative of incomplete H₂S oxidation to elemental sulphur (S⁰) in the dark (the stoichiometry of sulphide oxidation to S⁰ is: 0.5O₂ → S⁰; Nielsen et al., 2006) and complete H₂S re-oxidation to sulphuric acid (H₂SO₄) in the light (stoichiometry of 2:1). Alternatively, the higher O₂ flux in the light could indicate higher microbial O₂ consumption rates and/or chemical oxidation of other reduced compounds, such as Fe²⁺, within the rhizosphere. Some of the rhizosphere sulphide (Sₗ₀₂⁻) could have precipitated as FeS during darkness, which would also explain the lower degree of co-existence between O₂ and H₂S in the seagrass rhizosphere in darkness as compared to in light (Fig. 1). This is in accordance with a recent study (Brodersen et al., 2017) that demonstrated Fe³⁺ reduction, and thus Fe²⁺...
mobilisation, in the seagrass rhizosphere predominantly during night-time (Brodersen et al., 2017). Moreover, at the seagrass-driven rhizospheric oxic/anoxic interface, we observed a rapid decrease in rhizosphere pH by ~ 2 pH units (Fig. 1 and Supporting Information Figs S1 and S2), most likely as a result of protons generated from the chemical reaction between \( \text{O}_2 \) and \( \text{H}_2\text{S} \) (Nielsen et al., 2006; Brodersen et al., 2015a; 2016). This resulted from acidification of the rhizospheric oxic microniches reaching pH 4–5 (Fig. 1).

**Microbial diversity in the seagrass rhizosphere**

The microbial community composition in the artificial sediment was similar to that reported from other seagrass-vegetated natural sediments (e.g., Jensen et al., 2007; Cúcio et al., 2016); with many members of the rhizospheric microbial community affiliated with the sulphur cycle (Cúcio et al., 2016; Ettinger et al., 2017; Fahimipour et al., 2017). Compared to the bulk sediment, we observed higher mean relative abundance of SRB taxa including OTUs matching *Desulfovibrio* sp., especially around the root apical meristems, where members of the SRB class *Clostridia* (Devereux, 2005; Sallam and Steinbüchel, 2009) dominated, with ~ 57% of sequences affiliated with this bacterial class (\( \text{t}(5)_{\text{RAM-BS}} = 4.015, p = 0.01; \text{t}(4)_{\text{RAM-NC}} = 16.944, p < 0.001 \); Fig. 2). This is notable given that many members of this class are also known to be diazotrophs, that is, N\(_2\) fixing SRB. It has previously been argued that diazotrophic bacteria (e.g., *Lachnospiracea* and *Desulfovibrio* sp.) live in a mutualistic relationship around the below-ground biomass of seagrasses (e.g., Welsh, 2000), where the plant provides DOC and diazotrophic SRB reciprocally provide fixed nitrogen to the seagrass. Nielsen and colleagues (2001), for example, showed higher rates of N\(_2\)-fixation and sulphate reduction around and on below-ground tissues of seagrasses as compared to rates in the sediment, and this study also showed that root/rhizome-associated SRB fixed more dinitrogen than needed for their own growth.

In addition, the occurrence of SRB within the rhizosphere may lead to increased phosphorus solubilisation owing to reduction of insoluble Fe\(^{3+}\) oxyhydroxides that can promote the release of previously adsorbed phosphate to the pore-water (Pagès et al., 2011; 2012; Brodersen et al., 2017).

Within the plant-derived oxic microzone in the microbe-enriched treatment, sulphide-oxidizing *Epsilonproteobacteria*, including *Arcobacter* sp. and *Sulfurimonas* sp., were detected (Fig. 2). *Epsilonproteobacteria* constituted ~ 34% of the community at the basal leaf meristem compared to other designated bacterial classes (including *Deltaproteobacteria*, *Clostridia* and *Bacteroidia*), with a relative increase of ~ 22% as compared to around the root apical meristems (Fig. 2). However, the higher abundance of SOB within plant-derived oxic microniches did not seem to enhance sulphide detoxification, as we observed similar \( \text{H}_2\text{S} \) re-oxidation rates within the pre-sterilized and microbe-enriched treatments (Fig. 1 and Supporting Information Figs S1 and S2 and Table 1); which was also shown in calculated rates of total sulphide oxidation (Supporting Information Figs S3 and S4 and Table 1). Spontaneous chemical \( \text{H}_2\text{S} \) re-oxidation via ROL thus seemed of similar magnitude as biological \( \text{H}_2\text{S} \) re-oxidation (Fig. 1 and Table 1). However, the mere presence of high populations of SOB indicates that they are playing an important role in \( \text{H}_2\text{S} \) oxidation in the seagrass rhizosphere.

Within the seagrass-derived rhizospheric low-pH microniches (corresponding to the oxic microzones; Fig. 1), we observed a slightly lower mean relative abundance of *Bacteroidia* (~ 13%) as compared to the root apical meristems and the bulk sediment (> 23%) albeit not significant for the porewater enriched sediment (\( \text{t}(4)_{\text{BLM-BS}} = -1.141, p > 0.05; \text{t}(5)_{\text{BLM-NC}} = -0.461, p > 0.05; \text{t}(4)_{\text{BLM-NC}} = -7.894, p = 0.001 \); Fig. 2). The growth rate of *Bacteroidetes* is pH dependent (Thomas et al., 2011), and the potentially impeded growth of these potential plant pathogens (Fig. 2) as a result of rhizosphere acidification driven by ROL from the below-ground tissues (Fig. 1) deserves further attention.

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**Table 1.** Radial O\(_2\) loss (ROL), plant-derived H\(_2\text{S}\) re-oxidation/sediment detoxification and \( \Delta\text{pH} \) in the immediate rhizosphere of *Z. muelleri*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ROL (nmol O(_2) cm(^{-2}) h(^{-1}))</th>
<th>( \text{H}_2\text{S} ) re-oxidation (nmol ( \text{H}_2\text{S} ) cm(^{-2}) h(^{-1}))</th>
<th>( \text{S}^{2-}<em>{\text{tot}} ) oxidation (nmol ( \text{S}^{2-}</em>{\text{tot}} ) cm(^{-2}) h(^{-1}))</th>
<th>( \Delta\text{pH} ) (pH units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>( -322 \pm 39 )</td>
<td>334 ± 1</td>
<td>555 ± 164(^a)</td>
<td>2.1 ± 0.4(^a)</td>
</tr>
<tr>
<td>Light</td>
<td>( -745 \pm 118 )</td>
<td>418 ± 4</td>
<td>440 ± 6</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Presterilized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>( -378 \pm 3 )</td>
<td>636 ± 136(^a)</td>
<td>651 ± 146(^a)</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Light</td>
<td>( -891 \pm 52 )</td>
<td>508 ± 33</td>
<td>533 ± 22</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

\( n = 2–3 \), biological replication. Values are mean ± SEM. Pore water indicates artificial sediment matrix with added native pore water microbes. Presterilized indicates a sterilized below-ground environment, that is, sterilized sediment and below-ground biomass. \( \text{S}^{2-}_{\text{tot}} \) = total sulphide.

\(^a\) Note relative high standard error of the mean (SEM).
Sulphate reduction thus seemed to be the microbial metabolism in the seagrass rhizosphere that was most strongly affected by the activity of the seagrass host, where locally enhanced DOC, especially at the root-tips of seagrasses (Moriarty et al., 1986; Pollard and Moriarty, 1991), could be responsible for the microbial community composition shift (Fig. 2 and Supporting Information Fig. S5). The higher relative abundance of the SRB class Clostridia at the root apical meristems is supportive of a potential mutualistic relationship between seagrasses and

![Microbial diversity in the rhizosphere of the seagrass Zostera muelleri determined via 16S rRNA amplicon sequencing.](image)

**Fig. 2.** Microbial diversity in the rhizosphere of the seagrass *Zostera muelleri* determined via 16S rRNA amplicon sequencing. The phylogenetic tree denotes the spatial separation of the microbial consortia as determined via beta diversity analysis by Jackknife comparison of the weighted sequences data. The heat-map shows the abundance of the respective bacterial class/genus within the selected regions of interest, where (o) and (f) denote order and family classification, respectively. The heat-map includes taxonomic groups within each sample that represent > 1% of the total sequences, which cumulatively represents > 85% of the total sequenced data. Diagrams (in %) show the mean relative abundance of designated bacterial classes present within the selected regions of interest of the artificial sediment matrix. All data originate from reduced, artificial sediment with added native pore water microbes (except from data given for the negative control, which originates from a presterilized environment as described in the Experimental procedures section); n = 2–3.
heterotrophic, diazotrophic bacteria based on reciprocal nutrient exchange.

Methodology

Our novel experimental approach allowed the combined use of molecular and microsensor techniques, without disturbing the below-ground biogeochemical microenvironment during detailed O₂, H₂S and pH microsensor measurements. Detailed information about the chemical microgradients around the below-ground tissues provided by the microsensor measurements enabled us to sample sediment corresponding to the plant-affected regions of the seagrass rhizosphere. While many modern ‘omics’ studies are often carried out ‘blindfolded’ by relying on bulk sample analysis, the combination of sampling with microenvironmental analyses, such as described in this study, may alleviate such limitations and guide more hypothesis-driven approaches.

Some of the limitations of using an artificial sediment matrix mainly relate to it being a simplification of complex natural sediments, where other elements and especially solid-phase species (such as Fe³⁺ oxyhydroxides) also play important roles for reactions affecting distributions of O₂, sulphide and pH in the seagrass rhizosphere (Brodersen et al., 2017). Insoluble Fe³⁺ oxyhydroxides are, for example, likely to accumulate in the seagrass rhizosphere during day-time as a result of chemical and biological oxidation of dissolved Fe²⁺ and precipitated FeS (Brodersen et al., 2017). At night time, in contrast, precipitated Fe³⁺ oxyhydroxides are reduced via sulphide, which re-generates dissolved Fe²⁺ leading to consumption of sulphide through precipitation of FeS (Brodersen et al., 2017). Hence, sulphide and O₂ consumption processes are more complex in natural sediments as compared to in artificial sediment. Different processes can also be temporally separated, where H₂S consumption at night is in part a result of ROL-driven Fe³⁺ formation during the day, and O₂ consumption in the day is partially due to re-oxidation of FeS that precipitated in the seagrass rhizosphere during darkness, to regenerate insoluble Fe³⁺ oxyhydroxides (Brodersen et al., 2017).

Conclusions

The novel combination of measuring microscale patterns in microbial diversity and sediment chemical characteristics within an artificial sediment matrix showed that seagrass-mediated alterations of rhizosphere geochemistry result in pronounced shifts of the rhizosphere microbial community composition. ROL from the basal leaf meristem resulted in a marked decrease in sediment pH and H₂S concentrations at the plant-driven oxic/anoxic interface, and within the plant-derived oxic microzones, H₂S was completely re-oxidized, protecting the most vulnerable part of the plant against phytoxic H₂S intrusion. We observed significantly elevated abundances of SRB in the seagrass rhizosphere and presence of SOB within oxic microniches. Our high-resolution characterization of rhizosphere chemistry and microbial communities indicate that SOB can play an important role for H₂S oxidation in the seagrass rhizosphere, especially within seagrass-generated oxic microniches.

Experimental procedures

Seagrass specimens and sediment sampling

Specimens of Z. muelleri subsp. capricorni (Asch.) S.W.L. Jacobs and marine sediment were collected from shallow waters (< 2 m depth) in a dense seagrass meadow in Narra-been Lagoon, NSW, Australia (~33.72°S, 151.29°E). Seagrass sediment was collected with open-barrel, push cores (PVC pipe, 25 cm length, 7 cm internal diameter) as described previously (e.g., Wesley, 2009). After sampling, the sediment and seagrass samples were transported to a greenhouse facility at University of Technology Sydney, where they were kept under natural sunlight in large, aerated and temperature-controlled seawater reservoirs (temperature of ~ 22°C; salinity of ~ 34) before further treatments. Prior to experiments, selected seagrass specimens were gently uprooted and washed free of any adhering sediment particles.

Experimental setup

To enable the examination of microscale gradients in chemical and microbiological properties near to and at the seagrass below-ground tissue surface, we employed a novel artificial sediment system consisting of a custom-made split flow-chamber, wherein the investigated seagrass specimens were grown in a transparent, artificial sediment matrix (Brodersen et al., 2014). Selected seagrass specimens (one plant at a time) were maintained in the flow-chamber with the leaf canopy positioned in the aerated, free-flowing seawater compartment and the below-ground biomass embedded in a reduced, deoxygenated agar matrix in the adjoining ‘sediment’ compartment. A detailed description of the casting procedure and chemical characteristics of the artificial sediment is given below.

A water pump submerged into an aerated and temperature-controlled seawater bath (temperature ~ 22°C; salinity ~ 34) provided a constant flow (~ 0.5 cm s⁻¹) of aerated seawater to the water compartment of the flow chamber. Within the sediment compartment, a ~ 3 cm-thick anoxic and HEPES buffered (10 mM) water layer, residing above the artificial sediment matrix, functioned as a liquid-phase diffusional barrier to O₂ invasion into the artificial sediment (Brodersen et al., 2014). The anoxic water layer was constantly flushed with humidified N₂ throughout the seagrass cultivation period. Below the artificial sediment matrix, pieces of gauze, pre-soaked in an acidic (pH 4) and anoxic 1 mM Na₂S solution, were deployed to ensure a continuous supply of H₂S to the overlying artificial sediment matrix. Finally, the sediment compartment was covered with aluminum foil to avoid
incoming stray light, retain N₂ and thus limit O₂ intrusion into the anoxic water layer.

Light was provided as a 12 h:12 h light/dark cycle with a fiber-optic tungsten halogen lamp (KL-2500; Schott GmbH, Mainz, Germany) connected to a timer and equipped with a collimating lens to restrict the illumination to the leaf canopy only. The incident photon scalar irradiance (PAR, 400–700 nm) at the leaf canopy during cultivation was ~150 μmol photons m⁻² s⁻¹. Scalar photon irradiance was measured with a spherical photon irradiance sensor (Walz GmbH, Effeltrich, Germany) connected to a calibrated photon irradiance meter (LI-250A; LiCor, Lincoln, NE, USA).

Seagrass cultivation period

Seagrass specimens were generally allowed 2 weeks to recover from sampling and to acclimatize to experimental conditions (temperature and salinity) before cultured in the reduced, transparent artificial sediment. Each experiment ran for a total of 8 days, where (i) pulse amplitude modulated (PAM) measurements were performed on the 4th and the second last day (7th) of the experiment, (ii) microsensor measurements on the 5th to the 7th day of the experiment and (iii) sediment samples for 16S rRNA amplicon sequencing were taken on the last day (8th) of the experiment, just after the microsensor and PAM measurements. To avoid effects of seasonal changes (such as temperature and nutrient availability) on the sediment microbial community composition, three plant replicates were chosen per treatment (giving a total of 48 experimental days). This is a minor limitation of the methodology, that is, when combining microsensor profiling with molecular techniques, as determining the chemical microenvironment around below-ground tissues in the casted artificial sediment in high spatio-temporal resolution is a time-consuming process.

Artificial sediment matrix in the sediment compartment

To enable identification of potential mutual beneficial relationships between seagrasses and SOB, two treatments were applied, whereby the artificial sediment matrix was either (i) sterilized, including the below-ground biomass surface (negative control), or (ii) enriched with native pore-water microbes. Each procedure is explained further in the following paragraphs.

Artificial sediment with pore water microbes. The transparent, reduced artificial sediment consisted of a ~0.7% (wt/vol) deoxygenated agar/seawater solution, buffered with an anoxic solution of HEPES buffer (final concentration of 10 mM; pH ~7) and amended with Na₂S (final H₂S concentration of 500 μM; at pH 7) and pore-water microbes (~50% pore-water in the final 0.7%, wt/vol, solution). During casting of the artificial sediment with pore-water microbes, the pore-water was homogenously incorporated into the pre-heated agar/seawater solution (~1.4%, wt/vol) shortly before the artificial sediment matrix was poured into the sediment compartment of the split flow chamber at a matrix temperature of ~38°C. Thereafter, the artificial sediment with microbes was rapidly cooled down to room temperature in the sediment compartment embedding the below-ground tissue of the investigated seagrass specimen (covering the rhizome with artificial sediment to a total depth of ~0.5 cm). The applied pore-water was extracted from sediment from the sampling site by means of (i) mild ultrasonication (30 s) in a 50 mL Falcon tube to dissociate microorganisms from the sediment grain surfaces and sediment aggregates (Ramsay, 1984; Lindahl and Bakken, 1995), (ii) centrifugation (2×3500 × g for 5 min at 20°C) and (iii) filtration of supernatants (continuously flushed with N₂ to avoid oxygenation; Polycarbonate membrane filters, 10 μm; Millipore®, USA) to exclude the remaining fine sediment particles.

Presterilized below-ground environment (negative control). The below-ground biomass of the investigated seagrasses was surface-sterilized by submerging sediment-free roots and rhizomes in a saline, anoxic ~1.05% (wt/vol) hydrochlorite solution for 30 s (Blaabjerg and Finster, 1998) followed by 3 × 1 min rinses in anoxic, filter-sterilized (0.2 μm) seawater. Prior to casting the presterilized artificial sediment, all added solutions and seawater were filter-sterilized (0.2 μm) and the agar solution was heated to 120°C in an oven for 30 min. The presterilized artificial sediment matrix consisted of a ~0.7% (wt/vol) deoxygenated agar/seawater solution, buffered with sterilized, anoxic HEPES (final concentration of 10 mM; pH 7) and amended with Na₂S to a final H₂S concentration of 500 μM (at pH 7); resulting in similar chemical properties to the artificial sediment with added pore-water microbes as described above. The transparent, artificial sediment matrix applied here permitted the precise and combined application of microsensor measurements and molecular characterisation of microbial communities within specific microzones of interest, that is, around the basal leaf meristem, root apical meristems and within the bulk sediment.

Specimen characteristics and performance

Seagrass specimens with a similar above- to below-ground biomass ratio were selected for this study, to ensure comparable below-ground tissue oxidation capabilities of the investigated specimens (e.g., Frederiksen et al., 2006; Frederiksen and Glud, 2006) (Supporting Information Table S1). The photosynthetic performance of the investigated seagrasses during cultivation was determined as the maximum PSII quantum yield in dark-adapted samples and the effective PSII quantum yield in illuminated samples by means of pulse amplitude modulated (PAM) variable chlorophyll fluorometry (Beer et al., 1998; PocketPAM, equipped with an optical fiber; Gademann Messtechnik GmbH, Germany) (Supporting Information Table S1) to confirm that the seagrasses were generally healthy and photosynthetically active under the experimental conditions (n = 3–7). Following the experiments, the Dry Weight (DW) biomass ratio of the above- to below-ground tissues was obtained after drying each seagrass specimen in an oven at 60°C until a constant weight was reached.

Microsensor measurements and flux calculations

We used microsensors to determine the chemical conditions and dynamics at the plant/sediment interface. Clark-type O₂ microsensors (OX-50; Unisense A/S, Aarhus, Denmark;
Revsbech, 1989) were used to measure the radial O$_2$ loss (ROL) from the below-ground tissue of *Z. muelleri*. The O$_2$ microsensors were linearly calibrated from signal readings in 100% air saturated seawater and anoxic seawater (obtained by flushing with N$_2$ and adding the O$_2$ scavenger sodium sulphite) at experimental temperature and salinity. To avoid drifting calibrations during measurements, the O$_2$ microsensors were precontaminated with H$_2$S before calibrations (Brodersen et al., 2015b). Clark-type H$_2$S microsensors (H$_2$S-50; Unisense A/S; Jeroschewski et al., 1996; KühI et al., 1998) were used to measure the H$_2$S concentration at and around the below-ground tissue of *Z. muelleri*. The H$_2$S microsensors were linearly calibrated in acidic (pH 4), anoxic Na$_2$S solutions of defined H$_2$S concentrations (0, 50 and 100 μM) at experimental temperature and salinity. pH measurements were performed by means of pH glass microelectrodes (pH-50, Unisense A/S; Revsbech, 2001) that were used in combination with a reference electrode (REF-RM; Unisense A/S) submerged in the split flow chamber to allow the pH microelectrode to develop an electric potential relative to the reference electrode. The pH microelectrodes were linearly calibrated from signal readings in pH buffers (pH 5, 8 and 9) at experimental temperature and salinity.

Microsensors were mounted on a motorized micromanipulator (MM33-2 and MC-232; Unisense A/S) and connected to a microsensor multimeter (Unisense A/S) that was interfaced with a PC running dedicated microsensor positioning and data acquisition software (SensorTrace PRO; Unisense A/S). The microsensors were carefully positioned at the surface of the basal leaf meristem (defined as 0 mm distance from the below-ground tissue on the figures) by manually operating the micromanipulator, while observing the tip of the microsensor relative to the surface of the below-ground tissue through a submerged hand-held lens (described in Brodersen et al., 2014) with a stereo microscope mounted on an articulating arm (SM-6TZ; Amscope, Irvine, CA, USA). All microprofiles were measured in distance increments of 50 μm. Plants were allowed to acclimatize to the experimental conditions for ~72 h before microsensor measurements commenced to ensure steady state geochemical conditions. During microsensor profiling, an additional source of N$_2$ was immersed into the anoxic seawater layer of the sediment compartment (described above) to avoid O$_2$ intrusion into the layer and loss of H$_2$S from the artificial sediment due to oxidation when removing the covering aluminium foil (Brodersen et al., 2014; 2015a; 2015b). Three-to-five microsensor measurements were performed in the artificial sediment at the basal leaf meristem and averaged to produce one replicate profile for each of the 2–3 investigated seagrass specimens (i.e., n = 3–5, technical replicates; n = 2–3, biological replicates; which gives a total of 6–15 microsensor profile replicates) in each treatment. Note, that one plant replicate was excluded from the sterilized environment as we could not convincingly determine the below-ground tissue surface during measurements (further described in Supporting Information Fig. S2).

**Flux calculations.** The radial O$_2$ loss (ROL) from the below-ground tissue (nmol O$_2$ cm$^{-2}$ h$^{-1}$) was calculated via a cylindrical version of Fick’s first law of diffusion (Steen-Knudsen, 2002) assuming a homogenous, cylinder-shaped O$_2$ flux from the surface:

$$J(r)_{BLM} = \varphi D_0 (C_1 - C_2)/r \ln \left(\frac{r_1}{r_2}\right)$$

where $\varphi$ is the porosity of the artificial sediment (here assumed to be similar to seawater); $D_0$ is the molecular diffusion coefficient of O$_2$ in seawater at experimental temperature and salinity; r is the radius of the basal leaf meristem and $C_1$ and $C_2$ are the O$_2$ concentrations measured at the radial distances $r_1$ and $r_2$ from the tissue surface respectively. The H$_2$S oxidation rates in the immediate rhizosphere (nmol H$_2$S cm$^{-2}$ h$^{-1}$) were calculated in a similar manner by correcting $D_0$ to the molecular diffusion coefficient for H$_2$S at experimental temperature and salinity (factor 0.7573; tabulated values are accessible on www.unisense.com).

The following equations where used to calculate the total sulphide concentration microprofiles and fluxes from the measured H$_2$S concentrations and pH microprofiles (equations are available at www.unisense.com):

$$pK_1 = -98.08 + \frac{5765.4}{T} + 15.0455 \times \ln(T) + (-0.157 \times (S^{0.5})) + 0.0135 \times S$$

where S is the salinity and T is the temperature in Kelvin.

$$\text{total sulphide } [S_{\text{tot}}^{-2}] = [H_2S] \times \left(1 + \frac{K_1}{[H_3O^+]^{1/2}}\right)$$

where $[H_3O^+] = [H^+] = 10^{-pH}$ and $K_1 = 10^{-pK_1}$ for pH < 9 (Jeroschewski et al., 1996).

**Sediment sampling for DNA extractions**

Artificial sediment samples were obtained from selected regions of interest, that is, around the basal leaf meristem (BLM; at the root/shoot junction), around the root apical meristem (RAM; at the root-tip) and from the bulk sediment (BS) using a sterilized surgical knife and spatula (Supporting Information Fig. S6). Samples around the below-ground tissues were carefully collected at a radial distance of up to ~1 mm from the tissue surface (final volume ~100 μL). Only one sediment sample was acquired from each of the meristem areas (i.e., the BLM and the RAM) of the three investigated seagrass plants cultured in artificial sediment enriched with native porewater microbes, as we were only interested in the small volume of sediment affected by the plant ($n = 3$). Whereas several samples (or large sediment volumes) were obtained from the bulk artificial sediment area ($n = 3–4$); taken from both the porewater enriched and presterilized (here used as a microbial negative control) treatments. After sampling, the sediment samples were stored in 2 mL Eppendorf tubes in a −80°C freezer until further analysis. Prior to DNA extraction, four rounds of washing were performed in order to remove the agarose. The artificial sediment samples were first liquefied in a dry bath at ~50°C and were then subsequently diluted via centrifugation with 1 mL of 3X PBS (2X 7500 × g at room
DNA extraction and PCR sequencing

A modified phenol:chloroform DNA extraction protocol was employed to extract microbial DNA from the artificial sediment matrix. We added 600 µL lysis buffer (TE buffer pH 8, 0.5% SDS, 0.1 mg mL\(^{-1}\)) to the pellets prior to incubation at 37°C for 1 h. Then, 100 µL of 5 M NaCl and 80 µL of 10% CTAB were added and the mixture was incubated at 65°C for 10 min. Lysates were transferred to sterile tubes, and DNA was extracted following standard phenol:chloroform procedures (Zhou et al., 1996). The obtained DNA was air-dried, resuspended in 20 µL of dH\(_2\)O and stored at −20°C until further analysis. DNA quantity and purity was evaluated using a Nanodrop-1000 Spectrophotometer (NanoDrop 1000; Thermo Scientific, USA).

PCR amplification and sequencing. To track shifts in the overall composition of the bacterial community, 16S rRNA amplicon sequencing was performed. Amplicons of variable composition of the bacterial community, 16S rRNA gene sequences were analysed using the QIIME pipeline (Caporaso et al., 2010; Kuczynski et al., 2012). Briefly, paired-end DNA sequences were joined, de novo Operational Taxonomic Units (OTUs) were defined at 97% of sequences, and identity was assigned against the Greengenes database (version 13/8/2013) using BLAST (Altschul et al., 1990). Chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011) and filtered out from the dataset. Chloroplasts and mitochondrial reads were removed before downstream analysis. Sequences were then rarefied to the same depth (7265 sequences per sample) to remove the effect of sampling effort upon analysis. Raw data files in FASTQ format were deposited in the NCBI Sequence Read Archive (SRA) with the study accession number SRP073850 under Bioproject number PRJNA315465.

Statistical analysis

Data were tested for equal variance prior to statistical analysis. Student’s t-tests were used to compare relative microbial abundances between the different regions of interest (i.e., RAM, BLM and BS).

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References


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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Chemical microenvironment as measured with microsensors at the surface of the basal leaf meristem of *Zostera muelleri* maintained in (i) a presterilized environment and (ii) with added native pore-water microbes – plant 2.

Fig. S2. Chemical microenvironment as measured with microsensors at the surface of the basal leaf meristem of *Zostera muelleri* maintained in reduced, artificial sediment with added native pore-water microbes – plant 3.

Fig. S3. Total sulphide concentration microprofiles at the surface of the basal leaf meristem of *Zostera muelleri* maintained in (i) a presterilized environment and (ii) with added native pore-water microbes – plant 1.

Fig. S4. Total sulphide concentration microprofiles at the surface of the basal leaf meristem of *Zostera muelleri* maintained in (i) a pre sterilized environment and (ii) with added native pore-water microbes – plant 2.

Fig. S5. Principal component analysis plot of the rhizosphere microbial community composition, illustrating the separation of the microbial consortia within selected rhizospheric regions of interest.

Fig. S6. Conceptual diagram visualizing sampling areas (i.e., region of interests) within the reduced, artificial sediment.

**Table S1.** Photosynthetic parameters as measured by variable chlorophyll fluorescence and measures of the above-ground:below-ground biomass ratios.