Original Article

Nanoparticle-based measurements of pH and O2 dynamics in the rhizosphere of Zostera marina L.: effects of temperature elevation and light-dark transitions

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ABSTRACT

Seagrasses can modulate the geochemical conditions in their immediate rhizosphere through the release of chemical compounds from their below-ground tissue. This is a vital chemical defence mechanism, whereby the plants detoxify the surrounding sediment.

Using novel nanoparticle-based optical O2 and pH sensors incorporated in reduced and transparent artificial sediment, we investigated the spatio-temporal dynamics of pH and O2 within the entire rhizosphere of Zostera marina L. during experimental manipulations of light and temperature. We combined such measurements with O2 microsensor measurements of the photosynthetic productivity and respiration of seagrass leaves.

We found pronounced pH and O2 microheterogeneity within the immediate rhizosphere of Z. marina, with higher below-ground tissue oxidation capability and rhizoplane pH levels during both light exposure of the leaf canopy and elevated temperature, where the temperature-mediated stimuli of biogeochemical processes seemed to predominate. Low rhizosphere pH microenvironments appeared to correlate with plant-derived oxic microzones stimulating local sulphide oxidation and thus driving local proton generation, although the rhioplane pH levels generally where much higher than the bulk sediment pH.

Our data show that Z. marina can actively alter its rhizosphere pH microenvironment alleviating the local H2S toxicity and enhancing nutrient availability in the adjacent sediment via geochemical speciation shift.

Key-words: microbial metabolism; nanoparticles; O2; pH; plant-sediment interactions; seagrass; temperature elevation.

INTRODUCTION

To accommodate growth in often highly reduced, sulphidic sediment environments, seagrasses possess aerenchymal tissue composed of a system of interconnected gas channels facilitating rapid transport of O2 from the seagrass leaves to the below-ground tissue (Larkum et al. 1989; McComb et al. 1999). Aerenchymal O2 supply supports aerobic metabolism at the root apical meristems, and also facilitates radial O2 loss (ROL) to the immediate rhizosphere from the basal meristems with leaf sheath, rhizome and the root apical meristems (Pedersen et al. 1998, 1999; Jensen et al. 2005; Frederiksen & Glud 2006; Brodersen et al. 2014, 2015a; Koren et al. 2015). The below-ground ROL drives local chemical oxidation of the surrounding sediment in plant-derived oxic microniches, wherein new actively growing roots can form and reach maturity with protective barriers to ROL and sulphide intrusion (Barnabas 1996; Enstone et al. 2003; Brodersen et al. 2014, 2015a). Most of these barriers to ROL are induced by anoxic, sulphidic conditions (Armstrong & Armstrong 2001, 2005) and inhibit gas-exchange over most of the root surface area ensuring an efficient internal gas transport to the apical parts of growing roots (Colmer 2003).

Seagrasses can thus actively alter their rhizosphere microenvironment through the release of O2 from their below-ground tissue, thereby enhancing the redox potential of the immediate rhizosphere and stimulating re-oxidation of sediment-produced reduced phyto-toxins, such as H2S (Lamers et al. 2013; Brodersen et al. 2014, 2015a). The oxidation capacity of the below-ground tissue is determined by numerous O2 sources and sinks (Borum et al. 2006), where the most important regulating parameters include the O2 conditions in the water column during night-time as the plants are completely dependent on passive diffusion of O2 into their leaves when photosynthesis ceases (O2 source) (Greve et al. 2003; Pedersen et al. 2004; Borum et al. 2005; Frederiksen & Glud 2006; Brodersen et al. 2015a), the light availability and quality during day-time strongly regulating rates of shoot photosynthesis (O2 source) (Brodersen et al. 2015a, 2015b), the ambient water temperature affecting plant and sediment respiratory needs and reaction kinetics (mainly regulating the O2 sinks, but also affects rates of leaf photosynthesis) (Raun & Borum 2013), as
well as the thickness of the seagrass leaf diffusive boundary layer impeding gas and nutrient exchange with the surrounding water-column and thereby the water flow (thus negatively affecting the O₂ source) (Binzer et al. 2005; Brodersen et al. 2015b).

Recently, Brodersen et al. (2015a) showed that the seagrass Zostera muelleri subsp. capricorni can modulate the pH microenvironment in its immediate rhizosphere, further alleviating the risk of H₂S intrusion through local sediment pH enhancements. This chemical defence mechanism, whereby pH enhancement changes the sulphide speciation in the rhizosphere towards non-permeable HS⁻ ions, is still poorly understood and there is therefore a need to elucidate the sediment pH microheterogeneity on a whole rhizosphere-scale.

Possible mechanisms behind such pH changes in the immediate rhizosphere are plant-derived allelochemicals. Rhizome/root exudation of organic carbon to the rhizosphere, as a result of internal carbon translocation, leads to enhanced bacterial productivity and growth in the seagrass rhizosphere (Moriarty et al. 1986). Rates of sulphate reduction have been coupled to plant photosynthesis and below-ground biomass (Pollard & Moriarty 1991; Blaabjerg & Finster 1998; Blaabjerg et al. 1998; Hansen et al. 2000; Nielsen et al. 2001) and young seagrass roots have also been found to stimulate the growth of epsilon- and gamma-proteobacteria that can utilize O₂ and nitrate as electron acceptors to re-oxidize sulphide (Jensen et al. 2007). Interestingly, the younger plant structures often leak O₂ from around the root-cap, where the presence of sulphide oxidizers overlaps with the plant-derived oxygenated microniches (Jensen et al. 2005; Frederiksen & Glud 2006; Brodersen et al. 2014).

The root-shoot junctions (including the basal leaf meristem) and the root apical meristems (Moriarty et al. 1986) have been suggested as sites of exudation, with rhizome/root organic carbon exudation amounting up to 18% of the total carbon fixed by the seagrass host (Hansen et al. 2000). The highest sulphate reduction rates in the seagrass rhizosphere have correspondingly been observed at the seagrasses rhizomes and roots, where, for example, Pollard & Moriarty (1991) found 6 times higher sulphate reduction rates in seagrass-vegetated sediment as compared to non-vegetated areas. Sulphate reducing bacteria associated with the below-ground tissue of seagrasses show high O₂ tolerance (Blaabjerg & Finster 1998), and several studies have shown that increasing temperature and light exposure of the seagrass leaf canopy has a pronounced positive impact on the rhizosphere sulphate reduction rate (Isaksen & Jørgensen 1994; Isaksen & Finster 1996; Blaabjerg et al. 1998). Sulphate reduction can have a positive impact on the availability of phosphate in marine sediment owing to its reducing properties (Pollard & Moriarty 1991), adding to the growing evidence of a specific relationship between the seagrass host and sulphate reducing bacteria based on a reciprocal exchange of nutrients (Moriarty et al. 1986; Blaabjerg et al. 1998; Hansen et al. 2000; Nielsen et al. 2001).

The consumption or production of protons as a result of microbial metabolisms and/or plant-derived allelochemicals plays an important role in the determination of sediment pH (Srinivasan & Mahadevan 2010; Brodersen et al. 2015a). Such sediment pH alterations can influence the chemical speciation and availability of vital nutrients (e.g. ammonium and phosphate) at the plant/sediment interfaces (Pollard & Moriarty 1991; Pagès et al. 2011, 2012; Brodersen et al. 2015a). Yet the understanding of rhizosphere pH dynamics in seagrasses is underexplored and data on pH microheterogeneity at plant/sediment interfaces are lacking.

In present study, we used novel O₂ and pH sensitive optical nanosensors incorporated in artificial, transparent sediment to investigate the pH and O₂ microdynamics in the rhizosphere of Zostera marina L. during light/dark transitions and temperature elevations. Our results provide new insights into the pH microheterogeneity and O₂ distribution in the Zostera marina L. rhizosphere during changing environmental conditions. We discuss how such pH and O₂ microgradients may alter the geochemical speciation of vital chemical species at plant/sediment andoxic/anoxic interfaces.

**MATERIALS AND METHODS**

**Seagrass sampling**

Zostera marina L. specimens were collected in shallow waters (less than 2 m depth) near Rungsted Harbour, Denmark and were transported in seawater from the sampling site to the laboratory within 1 h of sampling. The collected seagrass specimens were transplanted into sieved sediment from the sampling site to exclude burrowing animals from the holding tank. Specimens were held in a 30 L aquarium continuously flushed with aerated seawater (5 L h⁻¹; salinity of 34%; temperature of ~12°C) under a 14:10 h light/dark cycle. Illumination with a photon irradiance (400-700 nm) of ~200 μmol photons m⁻² s⁻¹ was provided by a combination of fluorescent and halogen lamps. Prior to experiments, selected plants were gently washed free of any adhering sediment particles and rhizome ends were carefully sealed with petroleum jelly to avoid gas leakage from damaged older rhizome parts, before placement in the custom-made, narrow split flow chamber (described below; Fig. 1). Relative small Z. marina specimens were used owing to the chamber dimension restrictions.

**Experimental setup and artificial, transparent sediment**

The applied experimental chamber consisted of a custom-made narrow, transparent acrylic split flow chamber attached to the side of a 30 L aquarium (inner dimensions 1 × 13 × 12 cm; Fig. 1). The split flow chamber was divided into an upper and lower compartment by means of an acrylic wall with numerous holes (inner diameter of ~1 mm) and was equipped with a removable front window for ease of access when casting the sediment and positioning the seagrass. A seagrass specimen was positioned in the upper compartment with the above-ground tissue in the free-flowing seawater phase and the below-ground tissue embedded in reduced, artificial sediment (Fig. 1). The artificial, transparent sediment with embedded nanosensors was designed to mimic chemical settings in natural marine sediment (Brodersen et al. 2014),
while enabling direct visual assessment of the below-ground tissue during measurements (Fig. 1; further described in Koren et al. 2015). The transparent artificial sediment consisted of a deoxygenated ~0.5% (w/v) agar-seawater gel, buffered with HEPES (final concentration of 10 mM; pH ~7), amended with O₂ or pH sensitive nanoparticles (~3 and 7 % v/v, respectively) and Na₂S*9H₂O to a final H₂S concentration of 500 μM (at pH7). The agar powder was pre-washed in continuously stirred cold seawater to improve clarity. The lower compartment of the split flow chamber contained a highly sulphidic (final H₂S concentration of 2500 μM) deoxygenated ~0.5% (w/v) agar-seawater solution buffered with HEPES (10 mM), ensuring a continuous supply of H₂S to the above artificial sediment with nanosensors during experiments, thereby maintaining a constantly high O₂ demand in the sediment (Brodersen et al. 2014, 2015a). After positioning of the plant and casting the sediments, the chamber was sealed and placed in front of the imaging system (described below).

Illumination of the leaf canopy was provided by a fibre-optic tungsten halogen lamp (KL-2500; Schott GmbH, Mainz, Germany) equipped with a collimating lens. The incident photon irradiance (PAR, 400-700 nm) at the level of the seagrass leaf canopy was measured with a calibrated irradiance sensor (Walz GmbH, Effeltrich, Germany) connected to a quantum irradiance meter (LI-250; LiCor, Lincoln, NE, USA). A constant flow of seawater (salinity of 34%) was maintained in the water-column of the upper flow chamber compartment via a connected pump submerged in an aerated and temperature-controlled seawater tank. The below-ground pH and O₂ microenvironment within the Zostera marina L. rhizosphere was investigated during light/dark transitions (incident photon irradiance of 500 μmol photons m⁻² s⁻¹) and at two different experimental temperatures (~16 and 24 °C). Plants were acclimatized to the experimental conditions for a minimum of 4 h prior to start of measurements to ensure steady-state biogeochemical conditions in the rhizosphere (as confirmed from repetitive image recordings). Temperature changes were induced by slowly increasing the temperature of the seawater reservoir for ~3 h until the desired temperature was reached and the plants were then allowed to acclimatize to the experimental temperature and irradiance for another 4 h before image recordings commenced.

Optical nanoparticle-based sensors

The optical nanoparticle-based pH sensors were prepared based on a modified literature method (Wang et al. 2012; Xie et al. 2013). Briefly, 1 mg of perylene (Sigma-Aldrich), 1 mg of lipophilic indicator 1-hydroxypyrene-3,6,8-tris-bis(2-ethylhexyl)sulphonamide (lipo-HPTS) (generously provided by Dr. Sergey Borisov TU Graz; Borisov et al. 2009) and 100 mg of the triblock copolymer Pluronic® F-127 (Sigma-Aldrich) were dissolved in 15 mL of tetrahydrofuran (THF). The mixture was poured into 100 mL of continuously stirred distilled water, the THF was evaporated under an air stream, and the particle suspension was concentrated to a final concentration of 5 mg mL⁻¹ at 60 °C. The obtained pH sensor nanoparticles had an average size of <100 nm as shown in the literature (Xie et al. 2013). The pH sensor nanoparticles were added to the pre-heated and previously deoxygenated artificial sediment in the last stage of the
casting procedure, i.e., during cooling at ~38 °C to obtain a final concentration of ~7 % (v/v) in the agar matrix.

A detailed description of the optical nanoparticle-based pH sensors, including optical properties and calibration procedures is provided in the Supporting Information (Fig. S1–4 and S6; Notes S1).

Artificial sediment with optical O2 sensor nanoparticles was prepared according to Koren et al. (2015). Briefly, 3 mg of platinum(II) meso-(2,3,4,5,6-pentafluoro)phenyl porphyrin (PtTFPP; indicator dye), 3 mg of Macrolex fluorescence yellow 10GN (MY; reference dye) and 200 mg of the styrene maleic anhydride copolymer (PSMA with 8% MA) XIRAN were dissolved in 20 g of Tetrahydrofuran (THF). This mixture was then poured into 200 mL of continuously stirred distilled water. THF was evaporated under an air stream, and the particle suspension was concentrated to a final concentration of 5 mg mL−1 at 60 °C. The optical O2 sensor nanoparticles were added to the pre-heated and previously deoxygenated artificial sediment in the last stage of the casting procedure at an agar temperature of ~38 °C to obtain a final concentration of ~3% (v/v) in the agar matrix.

Calibration curves of the optical O2 sensor nano particles at the two different experimental temperatures are provided in the Supporting Information (Fig. S5).

**Imaging setup and data acquisition**

A RGB camera setup (Larsen et al. 2011) was used for ratiometric pH and O2 imaging (Fig. 1). The imaging system consisted of a SLR camera (EOS 1000D, Canon, Japan) mounted on a tripod and equipped with a macro objective lens (Macro 100 f2.8 D, Tokina, Japan) and a long pass filter (pH imaging, 455 nm; O2 imaging, 530 nm; Uqgoptics.com). Excitation of the luminescent sensor nano particles was achieved by means of a multichip LED (LedEngin Inc, RS Components Ltd, Corby, UK) combined with a bandpass filter (pH imaging, 405 nm; O2 imaging, 455 nm). The applied LEDs were powered by a USB-controlled LED driver unit designed for luminescence imaging applications (imaging.fish-n-chips.de).

Data acquisition and control of the SLR exposure and LED light were achieved with a PC running custom software “look@RGB” (imaging.fish-n-chips.de).

**Image calibration and analysis**

The obtained SLR images were first split into red, green and blue channels and were then analysed via the Java-based image processing software ImageJ (rsbweb.nih.gov/ij/). In order to achieve images of pH and O2 dependent ratios, raw images were divided using the ImageJ plugin Ratio Plus (rsb.info.nih.gov/ij/plugins/ratio-plus.html). For O2 imaging, this implied dividing the red channel (emission of the O2 sensitive dye) with the green channel (emission of the reference dye). For pH imaging, the red channel (indicator dye) was divided with the blue channel (reference dye). The obtained ratio images were fitted with previously obtained calibration curves (Fig. S4 and S5) using the Curve Fitting function in ImageJ, by means of linking the ratio images to the respective O2 concentrations or pH units (see further details in Larsen et al. 2011; Koren et al. 2015).

**Net photosynthesis and plant respiration rates**

A seagrass leaf was positioned in a custom-made sample holder consisting of two 2 mm plexiglass plates to ensure a steady sample during microsensor measurements. Profiles were made through a hole in the plates (ø = 3 mm) towards the seagrass leaf surface. The sample holder was positioned in a flow chamber (25 × 8 × 5 cm), which was connected to an aquarium pump ensuring a steady flow of ~3 cm s−1 of aerated seawater (salinity = 34) from a 25 L aquarium, wherein the temperature was kept constant at either ~16 or 24 °C by a thermostate (F25-HD, Julabo GmbH, Germany). Light was provided with a fiber-optic tungsten halogen lamp (KL-2500 LCD, Schott GmbH, Germany) positioned at a 45° angle above the sample. The experimental photon irradiance (PAR) was 500 μmol photons m−2 s−1, measured at the position of the sample, i.e., the leaf canopy, with a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany) connected to a submersible spherical micro-quantum-sensor (US-SQS/L, Walz GmbH, Germany).

Vertical profiles of O2 concentration were measured in 50 μm increments from 0.5 mm above the leaf towards the tissue surface, using a Clark-type O2 microsensor with a tip diameter of <25 μm (OX-25, Unisense, Denmark; Revsbech 1989), with a fast response time (t90 < 0.5 s) and a low stirring sensitivity (1-2%). The microsensor was mounted on a motorized micro-manipulator (MU-1, PyroScience GmbH, Germany) and connected to a pA-meter (OXY-meter, Unisense, Denmark) that was interfaced to a PC via an A/D converter (DCR-16, PyroScience GmbH, Germany). Microsensor positioning and data acquisition were controlled by dedicated software (Profix, PyroScience GmbH, Germany).

Net photosynthesis and dark respiration rates were calculated from Fick’s 1st law of diffusion:

\[
J_{O2} = -D_0 \frac{\partial C}{\partial z}
\]

where \(D_0\) is the salinity and temperature dependent diffusion coefficient of O2 in seawater (www.unisense.com) and \(dC/dz\) is the linear concentration gradient of O2 in the diffusive boundary layer.

**RESULTS**

**Rates of photosynthesis and respiration**

The net photosynthesis and respiration rates of Zostera marina L. at the two experimental temperatures were determined via O2 concentration microprofiles measured towards the leaf tissue surface (Fig. 2). Measurements revealed a 2.2 times higher net photosynthesis rate at 24 °C as compared to 16 °C, amounting to an increase in O2 efflux from 0.117 to 0.252 nmol O2 cm−2 s−1, and a 1.4 times higher respiration rate at 24 °C as compared to 16 °C, which amounted to an increase in O2 influx from −0.116 to −0.159 nmol O2 cm−2 s−1. The measured temperature-induced enhancement in the rate
of net photosynthesis and respiration corresponded to $Q_{10}$ temperature coefficients of 2.6 and 1.5, respectively.

O2 distribution and microdynamics

The two-dimensional O2 distribution in the $Z$. marina rhizosphere at 16 and 24 °C during light-dark transitions is shown in Fig. 3. The O2 images showed an O2 release, i.e., radial oxygen loss, especially from the root-shoot junctions (nodiums) and the rhizome, leading to several oxic microniches in the immediate rhizosphere of $Z$. marina L. The seagrass was able to maintain oxic conditions around the rhizome even without photosynthetic activity (Fig. 3).

The O2 concentration images revealed a distinct increase in the belowground tissue oxidation capacity at 24 °C as compared to 16 °C; this temperature effect slightly predominated over light stimulation of the plants photosystems (Fig. 3). The extent of oxygenated regions and the below-ground tissue surface O2 concentration did only increase slightly during light exposure of the leaf canopy at 16 °C (incident irradiance of 500 μmol photons m$^{-2}$ s$^{-1}$; Fig. 3), whereas the effect of light stimulation on ROL was more pronounced at 24 °C. Some of the prophyllums (single leaves originating from the rhizome at the nodiums), as well as the leaf sheath at the base of the shoot also released O2 to the rhizosphere. The maximal width of the oxic microniches around the rhizome was ~5.0 mm at nodium 7 during light exposure at a temperature of 24 °C, corresponding to an oxic microshield thickness of ~0.75 mm surrounding the respective root-shoot junction (data obtained by subtracting the diameter of the rhizome), which is similar to previous findings in natural sediment (e.g. Pedersen et al. 1998; Jensen et al. 2005). The O2 concentrations determined within selected regions of interest (ROIs) in the $Z$. marina rhizosphere confirmed these observations (Fig. 4; Table 1). Based on O2 concentration measurements in ROI 1-7, we calculated a mean of a 1.1-fold increase in the oxidation capability of the belowground tissue as a result of the dark/light transitions as compared to a 1.3-fold increase in response to the 8 °C temperature elevation. The highest rhizome surface O2 levels were found at the root-shoot junctions (nodium 4, 5 and 7) corresponding to O2 concentrations reaching up to 122 μmol L$^{-1}$ (ROI 3, 4 and 5 in Fig. 4; Table 1). The O2 imaging thus documented pronounced spatial microheterogeneity and high spatio-temporal microdynamics of the belowground oxic microzones around the rhizome of $Z$. marina that was modulated by changes in light and temperature.

pH heterogeneity and dynamics

We found a high degree of pH heterogeneity within the seagrass rhizosphere, with distinct microzones of very low pH (down
Comparison of O2 and pH images revealed that areas of low pH (ROI 1-3 and 5-7; Fig. 6; Table 2). A distinct hotspot of low pH was measured in the region of nodium 7, internode 7 and nodium 8 with an up to 5.2 mm wide zone of pH < 5. The lowest rhizosphere pH levels were measured within this distinct zone with pH levels reaching the lower detection limit (pH 4) of the pH indicator (Fig. 5 & 6). The region of the belowground tissue with the highest pH levels was also found adjacent to nodium 7, corresponding to ROI 7 in Fig. 6 (Table 2).

**pH microheterogeneity at interfaces**

Extraction of cross-tissue pH values along line profiles in the pH images revealed pronounced pH microheterogeneity at interfaces (Fig. 7). The pH increased relative to the ambient sediment across internode 3 with the surrounding prophyllum, reaching pH levels of up to 8.3 on the rhizome surface and correlating with rapidly increasing pH levels at the rhizome/sediment interface (Fig. 7b; CTS 1). Interestingly, the cross tissue pH profile across internode 4 with prophyllum close to nodium 4 showed increasing pH levels at the approximate position of the oxic/anoxic interface with pH levels reaching up to 8.0 during light exposure of the leaf canopy (Fig. 7c; CTS 2). This was contrary to the rhizome/sediment interface where decreasing pH levels down to 4.1 were observed on the rhizome surface (measured during light exposure at 16 ºC), thus indicative of proton consuming and producing biogeochemical processes altering the geochemical microenvironment at this specific belowground oxic microniche (Fig. 7c; CTS 2).

A line microprofile across a root from root-bundle 6 showed similar microheterogeneity as found at internode 3, with increasing pH levels at the root/sediment interface, and root surface pH levels of up to 7.6 (Fig. 7d; CTS 3). Cross tissue microprofile 4 across internode 7 with prophyllum showed a pronounced decrease in pH at the approximate position of the oxic/anoxic interface with pH levels within the low pH hotspot approaching the lower detection limit of the pH indicator (Fig. 7e; CTS 4). Across nodium 9 at the end of the rhizome with a degraded prophyllum, pH increased at the approximate position of the rhizome up to pH 8.7 (Fig. 7f; CTS 5). These observations were

| ROI 1 represents measurements at the non-illuminated part of the shoot; ROI 2 = at the root-shoot junction (nodium 2); ROI 3 = at the base of the prophyllum close to the root-shoot junction (nodium 4); ROI 4 = at the root-shoot junction (nodium 5); ROI 5 = at the root-shoot junction (nodium 7); ROI 6 = internode 7 with prophyllum; ROI 7 = at the rhizome-end. |
|---|---|---|---|---|---|---|
| [O$_2$] | Dark | Light | Dark | Light |
| | % air sat. | μmol L$^{-1}$ | % air sat. | μmol L$^{-1}$ | % air sat. | μmol L$^{-1}$ | % air sat. | μmol L$^{-1}$ |
| ROI 1 | 5.8 (14.6) | 6.2 (15.4) | 11.8 (25.5) | 14.2 (30.6) |
| ROI 2 | 8.9 (22.3) | 9.0 (22.5) | 10.7 (23.1) | 13.2 (28.6) |
| ROI 3 | 37.2 (93.2) | 37.5 (94.0) | 49.3 (106.6) | 54.9 (118.6) |
| ROI 4 | 32.3 (81.0) | 36.1 (90.6) | 50.3 (108.7) | 52.7 (113.9) |
| ROI 5 | 34.6 (86.7) | 35.1 (88.0) | 47.3 (102.3) | 56.3 (121.8) |
| ROI 6 | 12.2 (30.6) | 12.5 (31.3) | 18.2 (39.3) | 23.2 (50.3) |
| ROI 7 | 24.0 (60.2) | 25.6 (64.3) | 35.2 (76.0) | 42.3 (91.5) |

Table 1. O$_2$ concentrations at selected regions of interest (ROI) within the immediate rhizosphere of *Zostera marina* L. Boxes and numbers indicate the measured ROI. O$_2$ concentrations are given in both % air saturation and μmol L$^{-1}$ at ~16 and 24 ºC during light-dark transitions.
supported by vertical pH microprofiles measured from the seawater/sediment interface down to the bottom of the pH sensitive sediment (Fig. 8). A rapid decrease in pH was observed within the uppermost 5 mm as typically observed in natural marine sediments (Stahl et al. 2006; Zhu et al. 2006), with pH levels decreasing from about ~7 at the water/sediment interface down to pH ~6 at 5 mm depth where after it stabilised.

A vertical pH microprofile extracted from pH images (VM1; Fig. 8b) showed the pH microdynamics and microheterogeneity at the interfaces between the sediment and the first prophyllum, as well as between the sediment and the basal meristem with leaf sheath. An increase in pH was measured at the position of the basal meristem with leaf sheath, i.e., the meristematic region of the rhizome, and along roots of the first root bundle (Fig. 8b). This was in contrast to pH conditions at the prophyllum/sediment interface, where we observed a rapid increase in pH towards the leaf tissue surface followed by a rapid decrease across the prophyllum, possibly due to oxic conditions and/or biological re-oxidation of H2S (Fig. 8b; VM1). Another vertical pH microprofile (Fig. 8c; VM2) showed a rapid pH decrease at the interface between the sediment and the base of the fifth prophyllum/internode 7. At nodium 8 (root-shoot junction), a rapid increase in pH was seen at the approximate position of the oxic/anoxic interface with pH levels up to 8.4, followed by a strong decrease in pH across the rhizome tissue with pH levels decreasing to ~4.6 (Fig. 8d; VM3). A root from root-bundle 8 may have interfered with the interpretation of the pH microdynamics at nodium 8 (see Fig. 8d; VM3; ~26 mm depth). Nevertheless, our results clearly showed that plant-derived alterations of the belowground chemical microenvironment caused pH changes in the rhizosphere with a high degree of spatial microheterogeneity.

**DISCUSSION**

Our results showed a high spatio-temporal pH and O2 microheterogeneity in the rhizosphere of *Zostera marina* L., where the chemical conditions in the immediate rhizosphere were highly affected by the plant (Fig. 3 and 5). Radial O2 loss (ROL) from the belowground tissue of *Zostera marina* resulted in oxic microniches around the root-shoot junctions and the rhizome (Fig. 3 & 4). Such oxic microniches have recently been shown to facilitate chemical re-oxidation of sediment-produced H2S, and ROL is therefore an important chemical defence mechanism whereby the plants can actively detoxify phytotoxins in the surrounding sediment (Brodersen et al. 2014, 2015a).

**Oxidation capacity of the below-ground tissue**

The higher oxidation capacity of the below-ground tissue observed at 24 °C as compared to 16 °C (Fig. 4; Table 1) was...
Table 2. pH values in selected regions of interest (ROI) within the immediate rhizosphere of *Zostera marina* L. Values are given as a mean of the entire ROI ± S.E; and as the relative difference in pH between the experimentally changed environmental conditions (ΔpH), n = 5–18. The average pH of the bulk, artificial sediment at similar vertical depth as the below-ground biomass was ~ 5.7 ± 0.0 (includes all treatments).

<table>
<thead>
<tr>
<th>ROI</th>
<th>16 °C dark</th>
<th>16 °C light</th>
<th>24 °C dark</th>
<th>24 °C light</th>
<th>16/24 °C dark</th>
<th>16/24 °C light</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.8 ± 0.0</td>
<td>5.8 ± 0.0</td>
<td>6.4 ± 0.0</td>
<td>6.4 ± 0.0</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>ROI 2</td>
<td>5.6 ± 0.0</td>
<td>5.7 ± 0.1</td>
<td>6.3 ± 0.0</td>
<td>6.5 ± 0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>0.7</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>ROI 3</td>
<td>5.6 ± 0.0</td>
<td>5.7 ± 0.1</td>
<td>6.3 ± 0.0</td>
<td>6.4 ± 0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>ROI 4</td>
<td>6.7 ± 0.0</td>
<td>6.7 ± 0.1</td>
<td>6.6 ± 0.0</td>
<td>6.8 ± 0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>ROI 5</td>
<td>3.9 ± 0.0</td>
<td>4.2 ± 0.1</td>
<td>4.8 ± 0.0</td>
<td>4.9 ± 0.0</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.1</td>
<td>0.9</td>
<td>0.0</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>ROI 6</td>
<td>5.9 ± 0.0</td>
<td>6.0 ± 0.1</td>
<td>6.3 ± 0.0</td>
<td>6.6 ± 0.1</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>ROI 7</td>
<td>6.6 ± 0.1</td>
<td>6.9 ± 0.2</td>
<td>7.1 ± 0.0</td>
<td>7.4 ± 0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.3</td>
<td>0.8</td>
<td>0.1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

ROI 1 represents measurements at the basal leaf meristem (nodium 1); ROI 2 = the root-shoot junction (nodium 4); ROI 3 = at the base of the prophyllum close to the root-shoot junction (nodium 4); ROI 4 = root-bundle at nodium 6; ROI 5 = internode 7 with prophyllum; ROI 6 = at the rhizome-end; ROI 7 = root-shoot junction (nodium 7).

Figure 7. Cross tissue line sections (CTS) determining the pH microdynamics at the plant/rhizosphere interface and on the plant tissue surface. The steady-state cross tissue line sections were determined at the two experimental temperatures (i.e. ~16 and 24 °C) during light-dark transitions (under an incident photon irradiance (PAR) of 500 μmol photons m⁻² s⁻¹). (a) Structural image of the seagrass *Z. marina* L. embedded in the artificial, transparent sediment with pH sensitive nanoparticles (pH colour coded image), illustrating the positions of the respective cross tissue line sections (CTS1-5). (b) Line microprofile across internode 3 with attached prophyllum (CTS1). (c) Line microprofile across internode 4 with prophyllum close to nodium 4 (CTS2). (d) Line microprofile across root from root-bundle 6 (CTS3). (e) Line microprofile across internode 7 with prophyllum at the base of the prophyllum (CTS4). (f) Line microprofile across nodium 9 at the end of the rhizome with degraded prophyllum (CTS5). n = 3. Note that the white areas on leaves/prophyllums (marked with black arrows on the figure) should be interpreted with caution, as some of these high pH microniches (pH of ≥9) seemed to be caused by epiphyte-derived red background luminescence (Notes S1; Figure S6).
due to a relatively higher rate of shoot photosynthesis (Fig. 2). The light-independent reactions, i.e., the enzyme-controlled reactions in the photosystems, are highly temperature dependent and the rate of photosynthesis, therefore, increases in direct proportion to temperature until it reaches a temperature optimum for the given plant, where after it rapidly decreases e.g. due to enzyme denaturation (Staehr & Borum 2011). The optimum temperature for oxygenic photosynthesis in summer acclimated \textit{Z. marina} plants is ~24 °C (Staehr & Borum 2011). The higher ROL from the rhizome in darkness at 24 °C as compared to 16 °C (Fig. 3 & 4) may be explained by a significantly higher O₂ diffusion coefficient in the temperature elevated water. As a water column temperature elevation of 8 °C results in a ~25% increase in the rate of O₂ diffusion across the diffusive boundary layer (DBL) and into the above-ground tissue from the surrounding aerated water column (Ramsing & Gundersen 2015), thus allowing enhanced internal O₂ supply through the aerenchyma (low-resistance internal gas channels) to the below-ground tissue during darkness. This enhancement of the internal O₂ concentration gradient may be supported by a simultaneous temperature-induced increase in ROL owing to (i) the relatively increased lateral molecular O₂ diffusion rate across the epidermal layer of the belowground tissue at higher temperatures (although this might be counter-balanced by the higher tissue respiration), and (ii) the high leaf surface-to-volume ratio of the small \textit{Z. marina} specimens used in this study leading to a relatively high efflux of O₂ from the leaves into the water column in light and a relatively high influx of O₂ from the water column into leaves in darkness.

Most prophyllums seemed to release O₂ into the rhizosphere (Fig. 3), and where prophyllum 1-5 potentially could be fueled by O₂ from the water-column, the fully buried prophyllum 6 at nodium 9 must be supplied with O₂ from the rhizome. Only a minor O₂ leakage was detected from the roots of the 2nd root-bundle close to the basal leaf meristem during light exposure and a temperature of 24 °C (Fig. 3). Structural tissue barriers to ROL (e.g. suberin; Barnabas 1996) minimize cross tissue gas permeability of mature roots (e.g. Colmer 2003; Jensen et al. 2005; Frederiksen & Glud 2006; Brodersen et al. 2015a). Frederiksen & Glud (2006) found that the root oxygenated zones diminished with root age and suggested that O₂ leakage from \textit{Z. marina} roots eventually ceased. Our results further support such anatomical root adaptation of \textit{Z. marina} to a life in a hostile reduced sediment environment. Barriers

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**Figure 8.** Vertical pH microprofiles (VM) illustrating the pH heterogeneity and microdynamics in the rhizosphere of \textit{Z. marina} L. The vertical pH microprofiles were determined at steady-state conditions during light-dark transitions (photon irradiance (PAR) of 500 µmol photons m⁻² s⁻¹) at ~16 and 24 °C. (a) Structural image of the \textit{Z. marina} L. plant illustrating the spatial positions of the vertical pH microprofiles (colour coded image). (b) Vertical pH microprofile from the water/sediment interface across the first prophyllum and the basal meristem with leaf sheath to the bottom of the artificial sediment (VM1). (c) Vertical pH microprofile from the water/sediment interface across the base of the fifth prophyllum and the rhizome (internode 7) to the bottom of the artificial sediment (VM2). (d) Vertical pH microprofile from the water/sediment interface across the root-shoot junction at nodium 8 to the bottom of the artificial sediment (VM3). Y-axis = 0 indicate the artificial sediment surface. The approximate position of the below-ground tissue is indicated on the graphs by means of colour coded boxes (i.e. P = Prophyllum (blue), BM = Basal meristem with leaf sheath (green), R = Roots (brown); IN7P = Internode 7 at the base of the prophyllum (green); N = Nodium 8 (green)). n = 3. Note that the white areas on leaves/prophyllums (marked with black arrows on the figure) should be interpreted with caution, as some of these high pH microniches (pH of ≥9) seemed to be caused by epiphyte-derived red background luminescence (Notes S1; Figure S6).
to ROL protect the plants against exposure to sediment-derived reduced phytotoxins such as H$_2$S and increase the amount of internal O$_2$ transported to the apical root meristems ensuring aerobic metabolism in distal parts of the plants.

**pH microheterogeneity in the rhizosphere**

The novel pH sensitive nanosensors incorporated in the transparent sediment matrix enabled the first detailed mapping of the spatio-temporal pH microheterogeneity in the whole rhizosphere of *Z. marina* (Fig. 5). A similar pattern was recently observed in the rhizosphere of *Zostera muelleri* spp. *capricorni* by means of point measurements using electrochemical microsensors (Brodersen et al. 2015a). Regions in the immediate rhizosphere of *Z. marina* with very low pH levels (pH <5) seemed to correlate with the plant-derived oxic microniches. Such acidification could be due to proton formation as a byproduct of the spontaneous chemical reactions between plant-released O$_2$ and sediment H$_2$S within the oxic microzone (Fig. 5 & 6). We also measured slightly lower pH values in the immediate rhizosphere during darkness as compared to in light (Fig. 5 & 6), owing to plant and sediment respiration processes in addition to the aforementioned plant-derived spontaneous chemical re-oxidation of H$_2$S.

At the end of the rhizome around nodium 9, the pH imaging revealed high pH levels in the adjacent sediment (Fig. 7f). We speculate that such local pH enhancement may be due to high levels of accessible organic carbon in this specific region of the rhizoplane, as a result of tissue degradation and rhizome exudates, leading to proton consumption through microbial metabolisms such as sulphate reduction (Isaksen & Finster 1996; Blaabjerg et al. 1998; Hansen et al. 2000; Nielsen et al. 2001). These plant-microbial mediated local changes in the rhizosphere pH microenvironment are potentially very important for seagrasses as enhanced pH levels in the immediate rhizosphere lead to a shift in the sulphide speciation away from H$_2$S and towards non-permeable and thus non-phytotoxic HS$^-$ ions. Besides formation of oxic microniches due to ROL (see above), rhizosphere pH changes represent another chemical defense mechanism, whereby the plants further detoxify the surrounding sediment to accommodate their own growth in the often reduced, anoxic environments (Brodersen et al. 2015a).

**Biogeochemical processes**

The enhanced photosynthetic activity of *Z. marina* L. at its photosynthetic temperature optimum (~24 °C) (Fig. 2), positively affects the production of photosynthates and thereby lead to diurnal increases in the secretion of root/rhizome exudates and ROL (Moriarty et al. 1986; Blaabjerg et al. 1998; Nielsen et al. 2001) that may stimulate the microbial activity (such as sulphate reduction and sulphide oxidation, respectively) on the root/rhizome surface and in the immediate rhizosphere. The overall higher pH levels measured in the immediate rhizosphere at 24 °C as compared to 16 °C (Fig. 5 & 6), may thus be a result of a temperature-induced enhancement in the plants photosynthetic activity leading to increased rhizome/root exudation of organic carbon to the rhizosphere (Moriarty et al. 1986; Blaabjerg et al. 1998). Such exudation could either directly increase the pH levels in the immediate rhizosphere and on the below-ground tissue surface through secreted allelochemicals like amines (although this would be an expensive chemical defense mechanism for the plants) and other alkaline substances, and/or indirectly via stimulation of microbial processes such as sulphate reduction (as indicated at the plant-derived oxic/anoxic interfaces (Fig. 7c & 8d)), in combination with the generally temperature-mediated increase of the sulphate reduction rates owing to reaction kinetics (Isaksen & Finster 1996; Blaabjerg et al. 1998). Sulphate reduction rates associated with rinsed *Zostera muelleri* spp. *capricorni* roots/rhizomes have been found to be up to 11 times higher than in the bulk sediment (Hansen et al. 2000), and both rhizome and roots have been shown to be important habitats for sulphate-reducing and N$_2$-fixing bacteria (Blaabjerg & Finster 1998; Nielsen et al. 2001). Sulphate-reducing bacteria associated with rhizomes/roots possess a high N$_2$-fixing activity that can cover up to 65% of the nitrogen needed by the seagrass plants (Hansen et al. 2000; Nielsen et al. 2001).

Notably, high sulphate reduction rates in the seagrass rhizosphere, furthermore, leads to a sulphide-induced release of sediment-bound phosphorus, as the reduction of Fe(III) (oxyhydroxides) to Fe(II) results in phosphate release to the pore water, which then becomes available for plant growth (Pollard & Moriarty 1991; Pagès et al. 2011, 2012). A mutual beneficial relationship between the *Zostera marina* L. plant host and sulphate reducing bacteria in the rhizoplane seems therefore likely during non-stressed environmental conditions, where the sulphate reducing bacteria provides nutrients in the form of nitrogen and phosphate to the plant host as a response to plant-mediated rhizome/root exudates. However, we note that this hypothesis remains speculative and needs further experimental support. Our study did not aim to investigate the role of sulphate reducing bacteria in the *Z. marina* rhizosphere, and as we have used a sterile artificial sediment any sulphate reducing bacteria in the immediate rhizosphere must have originated from the non-sterile plant tissue. Future studies could e.g. involve artificial sediment based on extracted pore water or even cultures of sulphate reducing bacteria in combination with quantification of bacteria around the root biomass, e.g. using FISH with group-specific probes.

In other microniches associated with the formation of oxic microzones (Fig. 7c & 8d) biological and/or spontaneous chemical sulphide re-oxidation processes reduced the rhizoplane pH levels (Fig. 5). Such hotspots of low pH may well be due to a relatively higher abundance of sulphide oxidizing bacteria at that specific region, as microbes associated with the below-ground tissue of seagrass show a patchy distribution (Nielsen et al. 2001).

**Optical nanoparticle-based sensors incorporated into transparent artificial sediment**

The combined use of O$_2$ and pH sensitive nanoparticles with transparent artificial sediments enabled combined chemical
and structural imaging on the whole rhizosphere level. This novel application of optical nanoparticle-based sensors represents an important supplement to existing methods, such as planar optodes and microsensors, when elucidating the rhizosphere of aquatic macrophytes, as the former rarely allows close contact to the entire belowground tissue at once and the latter rely on precise point measurements, which makes mapping the entire rhizosphere extremely tedious if not impossible. In addition, the optical nanoparticle-based sensors enable close spatial alignment of pH and O_2 concentration mapping thus facilitating co-localization of these important chemical parameters relative to particular plant/sediment and oxic/anoxic interfaces within the rhizosphere. However, at the current state, the present nanoparticle methodology only allows for O_2 and pH imaging in artificial sediments.

The strengths of employing such reduced artificial sediment, as compared to natural sediment, encompass: (i) significantly improved visual assessment within the investigated rhizosphere, thus allowing for determination of the exact position of the entire below-ground tissue during imaging, which is a necessity when determining the effects of plant/sediment interactions on the rhizosphere biogeochemistry, and (ii) changes observed within the homogenous artificial sediment can be assigned to plant-mediated alterations, which can be difficult to conclude in highly heterogeneous natural sediment. Weaknesses of using an artificial sediment matrix, as compared to natural sediment, include: (i) a significantly reduced microbial abundance in the bulk sediment, and (ii) a potential lower sediment pH buffering capacity, which may lead to slightly overestimated responses. Moreover, a minor limitation of current ratiometric pH imaging is that high energy excitation light has to be used when exciting the pH sensitive indicator dyes, potentially causing artefacts in the pH images owing to, for example, chlorophyll-derived red background luminescence. Further information on how to avoid/limit such potential artefacts in the pH images is available in the supporting information (Notes S1; Fig. S6). Nevertheless, nanoparticle-based imaging provides detailed information about the geochemical conditions and dynamics in the rhizosphere of aquatic macrophytes at high spatio-temporal resolution without the potential smearing effects seen with planar optodes and allows the first investigations of pH and O_2 dynamics in the entire seagrass rhizosphere in real-time and at all below-ground tissue/sediment interfaces. Nanoparticle-based imaging thus has the potential to further resolve important plant-sediment interactions, such as, for example, plant-derived sediment detoxification processes, in addition to, simply directing precise microsensor measurements to biogeochemical hotspots within natural sediment.

In conclusion, novel optical nanoparticle-based imaging revealed a pronounced spatio-temporal pH and O_2 microheterogeneity in the immediate rhizosphere of _Z. marina_. Light stimulation of the leaf canopy and temperature elevation to the plants photosynthetically temperature optimum, i.e., from ~16 to 24 °C, lead to higher oxidation capacity of the belowground tissue and higher pH levels in the immediate rhizoplane, where the temperature-induced stimulation seemed to predominate. Low rhizosphere pH levels correlated with the plant-derived oxic microniches. Patchy distributions of high rhizosphere pH levels were found on the tissue surface, and cross tissue pH microprofiles revealed enhanced pH levels at selected oxic/anoxic interfaces. We speculate that the higher pH levels on the tissue surface and at the oxic/anoxic interface may be due to a plant-derived stimulation of proton consuming microbial metabolisms such as sulfate reduction and excretion of alkaline substances. Protons produced or consumed during microbial metabolisms, in addition to plant-mediated allelochemicals and chemical re-oxidation of H_2S, thus seemed responsible for the photosynthesis/temperature-driven alterations of the geochemical microenvironment determined in the _Zostera marina_ L. rhizosphere.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest and no competing financial interest.

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