Spatial heterogeneity and short-term oxygen dynamics in the rhizosphere of Vallisneria spiralis: implications for nutrient cycling

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SUMMARY

1. Aquatic macrophytes modify the sediment biogeochemistry via radial oxygen loss (ROL) from their roots. However, the variation in ROL and its implication for nutrient availability remains poorly explored.

2. Here we use planar O\(_2\) optodes to investigate the spatial heterogeneity of oxic niches within the rhizosphere of Vallisneria spiralis and their alteration following variable light and ambient O\(_2\) levels. The effect of ROL on NH\(_4^+\) and PO\(_4^{3-}\) distribution in the rhizosphere was evaluated by a combination of \(^{15}\)N isotopic techniques, 2D sampling, and electron microscopy.

3. A single specimen of V. spiralis could maintain an oxidized sediment volume of 41 – 47 cm\(^3\) and 10 – 27 cm\(^3\) in the rhizosphere at 100% and 38% dissolved oxygen saturation in the overlying water, respectively. Whatever the environmental conditions, the ROL was however very heterogeneous and dependent on root age and architecture of the root system.

4. ROL stimulated the coupling between denitrification and nitrification in the sediment both under dark (+25 µmol N-N\(_2\) m\(^{-2}\) h\(^{-1}\)) and light (+70 µmol N-N\(_2\) m\(^{-2}\) h\(^{-1}\)) conditions. This, in combination with plant uptake, contributed to intense removal of NH\(_4^+\) from the pore water. Similarly, PO\(_4^{3-}\) was highly depleted in the rhizosphere. The detection of Fe-P plaques on the roots surface indicated substantial entrapment of P as a consequence of ROL.

5. The extensive spatio-temporal heterogeneity of oxic and anoxic conditions ensured that aerobic and anaerobic processes co-occurred in the rhizosphere and this presumably reduced potential nutrient limitation while maximizing plant fitness in an otherwise hostile reduced environment.

INTRODUCTION

Submerged aquatic macrophytes can transport variable amount of O\(_2\) to the roots to allow their respiration in waterlogged soils (Armstrong, 1979). Such transport primarily occurs through the aerenchyma that consists of air canals connecting leaves, stems and roots (Colmer, 2003b; Smirnoff & Crawford, 1983). The development of an aerenchyma is presumably an adaptation of submerged macrophytes to live within sediments that are strictly anoxic a few millimeters from the surface (Colmer, 2003b; Longhi, Bartoli, Nizzoli, & Viaroli, 2013). Part of the transported O\(_2\) may leak out and create oxidized sediment layers around the roots (Laskov, Horn, & Hupfer, 2006). In seagrasses like Zoostera marina and Ruppia maritima radial oxygen loss (ROL) may occur only in the proximity of the root tips (e.g. Jensen, Kuhl, Glud, Jorgensen, & Prieme, 2005; Jovanovic, Pedersen, Larsen, Kristensen, & Glud, 2015). In other plants, like Potamogeton perfoliatus the
whole rhizosphere displays ROL, suggesting that the whole root length is permeable to O\textsubscript{2} (Caffrey & Kemp, 1991). Whereas the onset of diffusion barriers at the root basal region allows to maximize longitudinal O\textsubscript{2} transport to the actively growing root apex (Colmer, 2003a), maintaining high permeability throughout the rhizosphere appears to be a strategy for optimizing nutrient uptake and maintaining positive redox conditions (e.g. Mei, Yang, Tam, Wang, & Li, 2014).

While O\textsubscript{2} transport via the aerenchyma is necessary to sustain the aerobic metabolism of the roots living in an otherwise anoxic environment, the leakage of O\textsubscript{2} to the surrounding sediments has several secondary implications. Radial oxygen loss can effectively oxidize reduced toxic compounds (i.e. free sulphides and metal ions) around and within the roots, where they otherwise may induce physiological stress and damage to the plant (Geurts et al., 2009; Koch & Mendelssohn, 1989). In addition, ROL may promote aerobic microbial processes that mobilize nutrients and trace-elements from recalcitrant organic matter (Harvey, Tuttle, & Bell, 1995; Magri et al., 2018), or on the contrary, favour nutrient immobilization (e.g. phosphorous and iron) via adsorption or precipitation (e.g. Christensen, Jensen, Andersen, Wigand, & Holmer, 1998; Povidisa, Delefosse, & Holmer, 2009; St-Cyr, Fortin, & Campbell, 1993). Implications for nutrient mobility and turn-over is expected to vary largely among different submerged macrophytes depending on ROL intensity, distribution along the roots, and temporal dynamics. In freshwater bodies, the same macrophyte species can be found across gradients of nutrients availability, water flow regimes, and water oxic level, which may vary from normoxic to suboxic levels on a daily basis. Especially eutrophic environments may display pronounced short-term variation in ambient O\textsubscript{2} levels, but for many species the implications for the ROL is unknown. This is relevant as hypoxic events are a menace for macrophytes due to limited capacity of sediment to re-oxidize the products of anaerobic microbial metabolisms, leading to chemically reduced conditions and the build-up of phytotoxic compounds. The plasticity of macrophytes and their capacity to vary the O\textsubscript{2} transport via ROL may determine their resilience in dynamic settings as well as provide a competitive advantage for the colonization of sediments with different organic content.

A large body of work has been conducted to explore the implications of ROL from the widely distributed freshwater macrophyte Vallisneria spiralis. Oxygen transport to sediments by V. spiralis was firstly indirectly inferred from an imbalance in the benthic O\textsubscript{2} and total inorganic carbon fluxes measured in the light in muddy vegetated sediments (Pinardi et al., 2009; Ribaudo, Bartoli, Racchetti, Longhi, & Viaroli, 2011). Based on the same approach, Soana and Bartoli (2013) demonstrated how V. spiralis varies the O\textsubscript{2} delivered to the sediment seasonally, depending upon
the pore water redox conditions, with highest delivery of \( \text{O}_2 \) in the late summer coinciding with the more reduced chemical conditions. The lower concentration of \( \text{CH}_4 \) and metal ions (\( \text{Fe}^{2+} \) and \( \text{Mn}^{2+} \)) and the stimulated coupling between denitrification and nitrification in vegetated versus non vegetated sediments further supported the hypothesis of rhizosphere-driven sediment oxygenation (Racchetti et al., 2010; Racchetti, Longhi, Ribaudo, Soana, & Bartoli, 2017; Ribaudo et al., 2011; Soana et al., 2015). Whereas the above mentioned studies provide indirect evidence of \( \text{O}_2 \) leakage from roots, direct measurement of ROL in \textit{V. spiralis} was only recently shown via planar optode application (Han, Ren, Tang, Xu, & Xie, 2016; Han et al., 2018). These studies demonstrated \( \text{O}_2 \) leakage of the roots, analyzed the effect of irradiance on ROL, and showed a link between ROL and the immobilization of porewater P.

In this work, we explored the fine-scale \( \text{O}_2 \) dynamics in the rhizosphere of \textit{V. spiralis} by comparatively analysing the effects of the ambient \( \text{O}_2 \) concentration and photosynthesis on ROL. Moreover, we further investigated the link between \( \text{O}_2 \) dynamics for nitrogen and phosphorous cycling in the rhizosphere by analysing the effect of light and dark cycles on the coupled denitrification and nitrification activity, and the formation of Fe-P plaques on the surface of roots of different age. The overall effect of the rhizosphere on nitrogen and phosphorous concentration and distribution was assessed via 2D sampling techniques integrating a two-week period.

**METHODS**

*Sediment, plant sampling and pre-incubation*

In May 2015, shoots of \textit{V. spiralis}, sediment, and water (200 L) were collected at one meter depth in the Mincio River in Massimbona (northern Italy). Single specimens of \textit{V. spiralis} were extracted by hand from the sediment to minimize root damage. Sediment was sampled from unvegetated banks in proximity of the \textit{V. spiralis} meadow using acrylic liners (inner diam. x length: 8 x 40 cm). Within a few hours, samples were brought to the laboratory and stored in a temperature-controlled room at 20°C ± 2 to resemble \textit{in situ} temperature typical for the season (Pinardi et al., 2009). Sediment was homogenized and sieved (mesh size 0.5 mm) to exclude macroinvertebrates, stones, and other irregularities, before being packed into four acrylic rhizotrons (H x W x D : 41 x 20 x 3 cm.) to about half of the volume. A shoot of \textit{V. spiralis} was then transplanted into each rhizotron and the rest of the volume was filled with \textit{in situ} water. An optode foil pre-installed on the inner wall of each rhizotron allowed for later oxygen imaging. The rhizotrons were placed into a large
tank (100 L) containing *in situ* water and tilted of 45°deg (optode wall face-down) to facilitate root growth along the optode foil (see below). Water in the tank and in the rhizotrons was kept mixed by submersible pumps and flushed with air. Plants were illuminated on a 13:11 h light/dark cycle using LED lights positioned above the aquarium. Irradiance during light phases was set to 200 µmol photons m$^{-2}$ s$^{-1}$ to match the daily average *in situ* light levels at the sampling site and to ensure photosynthesis light-saturation (Bartoli unpublished results). Plants were pre-incubated for 14 days to assure plant acclimation to the experimental conditions before the experiments started.

*Effect of ambient water O$_2$ level on ROL*

After the acclimation phase, one rhizotron that showed root grown along the planar optode wall, was extracted from the reservoir and positioned in front of the camera and LED setup for planar optode imaging (Fig. S1). The experiment consisted of two phases: i) a growth phase, characterized by continued expansion of the oxic area of the rhizosphere; and ii) a steady-state phase when plant growth ceased. During the growth phase, we comparatively analyse the extent of the ROL induced-oxic area around new, growing roots and old, not growing roots during light and dark conditions. The steady-state phase was used to assess the effect of dissolved oxygen saturation (100, 38, and 70%) on the ROL intensity, O$_2$ distribution and dynamics in the rhizosphere, both under light and dark conditions. Extent of the oxic area around single roots and the overall oxic area of the rhizosphere were measured by processing planar optode images via the software ImageJ (http://imagej.nih.gov). ROL intensity was estimated as described below.

Oxygen levels in the water overlying the sediment, was regulated by mixing air and N$_2$ via mass flow controllers (5850 S, Brooks Instruments, USA) controlled by a digital control/readout unit (type 0154, Brooks Instrument, USA). The O$_2$ levels and the temperature of the water were monitored throughout the experiment via a fiber-optic O$_2$ sensor and a thermometer connected to an oxygen meter (FireStingO$_2$, PyroScience, Germany). Planar optode images were acquired every 20 minutes. Image acquisition proceeded throughout at least one light/dark cycle (i.e. 24 h), while the overlying water O$_2$ level was kept constant. At the end of each O$_2$ treatment, the overlying water was replaced with fresh *in situ* water to avoid nutrient depletion and accumulation of waste products from the plant metabolism and sediment processes. Overall, images were recorded over 24 hours for the growth phase and 130 hours for the steady-state phase.
Oxygen imaging principle and optode sensor fabrication

The basic optode set up resembles the original description (Glud, Ramsing, Gundersen, & Klimant, 1996) but here we applied the color ratiometric sensing approach (Larsen, Borisov, Grunwald, Klimant, & Glud, 2011). The procedure is based on the relative change in intensity of O$_2$ sensitive red emission light versus the O$_2$ insensitive green emission light (Larsen et al., 2011). The O$_2$ images were recorded using a digital single lens reflex camera (Canon EOS 1000D) equipped with a 530 nm long-pass filter (Edmund Optics). Excitation light was delivered from seven high-power LEDs ($\lambda$ peak = 447.5 nm; SR-02-R0500, Luxeon Star LEDs) equipped with a 470 nm short-pass filter (Edmund Optics). The O$_2$ planar optode sensor applied in this study was fabricated as in Larsen et al. (2011). The applied O$_2$ optodes had an O$_2$ sensitive layer of $< 2$ μm that was coated by a 15 μm semi-transparent silicone layer with 1% (wt/wt) carbon powder. The coating ensured that any structures behind the sensor foil remained visible during O$_2$ measurements, without affecting the ratiometric approach (Larsen et al., 2011). The size of the optode foils was 23 x 17 cm. The excitation light was triggered and synchronized with the camera via a control unit (LED trigger light, Fish 'n' chips, Germany). Excitation light and image acquisition settings were regulated using the software Look@RGB (available at http://www.fish-n-chips.de/Look@RGB/publish.htm).

Images calibration

For calibration of the O$_2$ sensor, we used the luminescent intensity ratio ($R$) of the green and red images recorded simultaneously by the camera, according to Larsen et al. (2011):

$$R = \frac{\text{Red} - \text{Green}}{\text{Green}}$$

where Red and Green are the pixel intensities of the red and green images, respectively.

A modified Stern-Volmer equation adequately describes the response of the sensor (Klimant, Meyer, & Kuhl, 1995):

$$\frac{R}{R_0} = \left[\alpha + (1 - \alpha)\left(\frac{1}{1 + K_{sv} + C}\right)\right]$$

where $\alpha$ is the non-quenchable fraction of the luminescence signal, $K_{sv}$ the Stern-Volmer quenching constant, $R$ the (red-green)/green luminescent intensity ratio, $R_0$ is the luminescent intensity ratio in the absence of O$_2$, and $C$ is the O$_2$ concentration. Values for $\alpha$ and $K_{sv}$ were determined by curve fitting the variation in $R/R_0$ as a function of O$_2$ concentration. Finally, O$_2$ concentration can be calculated for each image pixel by rearranging equation 2 as follows:

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with $R_0$ determined in the anoxic sediment.

Estimation of sediment respiration and ROL

The areal O$_2$ consumption at the sediment-water interface ($R_{SWI}$) was calculated from microscale O$_2$ depth-profiles at the sediment-water interface, extracted from planar optode images and modelled with the algorithm developed by Berg et al. (1998). Due to the higher concentration of reduced species at depth, the O$_2$ respiration in proximity of the roots ($R_S$) can be higher than at sediment-water interface ($R_{SWI}$). $R_S$ was calculated as described above for $R_{SWI}$, but from depth-profiles measured right after the sediment was mixed and packed into the rhizotrons. Volume specific $R_S$ was calculated from the areal consumption divided by the O$_2$ penetration depth. The oxygen diffusion coefficient ($D_s$) was calculated according to Ullman and Aller (1982) from the sediment porosity and diffusion coefficient in water. The diffusion coefficient in water was calculated as in Boudreau (1997) as a function of temperature and salinity, while the porosity was determined from sediment density and water content measured as described in (Dalsgaard et al., 2000).

The detection limit of the optode was quantified as three times the standard deviation of the measured concentration at anoxia for an area of 5x5 cm and amounted to 2 µmol L$^{-1}$ (about 1% air saturation). Thus oxic areas were defined as values with a measured O$_2$ concentration above 2 µ mol L$^{-1}$. The spatial resolution achieved by the optode system was 210 µm pixel$^{-1}$.

The ROL per area of root surface was estimated with the equation for radial diffusion proposed by Fenchel (1996):

$$ ROL = \phi \times R_S \times L \times \left( \frac{L}{2A} + 1 \right) $$

where $\phi$ is the sediment porosity, $R_S$ is the respiration of the sediment, $L$ (mm) is half the width of the oxygenated zone measured at the planar optode wall, and $A$ (mm) is half the width of the root diameter. Since planar optode measurements were performed along a wall not consuming O$_2$, the measured extent of the oxic area around a single root was larger compared to a root surrounded by sediment only (Glud, 2008; Meysman, Galaktionov, Glud, & Middelburg, 2010; Wenzhöfer & Glud, 2004). Assuming that the oxic area around a root in contact with the planar optode can be approximated as a half-cylinder, and that the oxic volume around the roots is independent of the presence of a wall, the “true” radial O$_2$ distribution around a root surrounded by sediment can be calculated as in (Frederiksen & Glud, 2006). The total O$_2$ transport into the rhizosphere by ROL at
steady-state was calculated from the oxic volume of the rhizosphere multiplied by the respiration of the sediment (Rs). The oxic volume of the whole rhizosphere was calculated from the oxic area on the planar optode wall assuming hemispherical geometry (Frederiksen & Glud, 2006).

Coupled denitrification and nitrification rates in vegetated and unvegetated sediment

To investigate the influence of ROL and its diel variation on the coupling between nitrification and denitrification in the sediment at the rhizosphere, an additional number of V. spiralis shoots and about 20 litres of sediment was collected along with the samples for ROL studies as described above. In laboratory, the sediment was homogenized and transferred into cylindrical acrylic microcosms (inner diam. x length: 7.5 x 10 cm, n = 12) and in the half of the microcosms two specimens of V. spiralis were added, simulating in situ plant density. Each microcosm was provided with four series of vertical holes, spaced at 1 cm and filled with silicon glue. Plants were let acclimatize for three weeks in a tank with in situ water (renewed every two days) and exposed to 13:11 light:dark cycle (irradiance of 200 μmol photons m⁻² s⁻¹). After the acclimation period, light and dark incubations were performed as described in Soana et al. (2015). Briefly, an anoxic \(^{15}\text{NH}_4^+\) solution (10 mM, 98 atom%) was injected into the sediment through the lateral silicon ports using a glass syringe (Hamilton 725RN 250 μL, ga 22S/51 mm/pst 2). The volume of \(^{15}\text{NH}_4^+\) solution added to each microcosm was adjusted to enrich the natural \(\text{NH}_4^+\) pool by 30%. During incubations plants were submerged in a well-mixed tank kept at in situ temperature. At the beginning of the incubation all the liners were closed with a bottom stopper and a top lid. After four hours of incubation, 2 mL of 7 M ZnCl\(_2\) were added to the water phase in all the core liners to stop biological activity and the sediment and water phase was gently slurred. A subsample of slurry was collected and transferred into 12 mL exetainers spiked with 200 μl of 7 M ZnCl\(_2\). \(^{14}\text{N}\) and \(^{15}\text{N}\) abundance in N\(_2\) was analyzed by Membrane Inlet Mass Spectrometry (MIMS, Bay Instrument, USA). The subsurface denitrification (which refers to the coupled process occurring within the rhizosphere, below the oxygen penetration depth), was calculated as the sum of D\(_{15}\) and D\(_{14}\), which are the rates of coupled denitrification and nitrification (denitrification of \(^{15}\text{NO}_3^-\) and \(^{14}\text{NO}_3^-\) produced within the sediments via \(^{15}\text{NH}_4^+\) and \(^{14}\text{NH}_4^+\) oxidation, respectively), according to Risgaard-Petersen and Jensen (1997) and Risgaard-Petersen et al. (1998) and the assumptions of Nielsen (1992).
Microplates for 2D ammonium and phosphate distribution in the rhizosphere

The pore water concentration of NH$_4^+$ and PO$_4^{3-}$ in the rhizosphere was investigated by a modified version of a two-dimensional sampler originally described by Lewandowski, Ruter, and Hupfer (2002). Two polystyrene microplates (Sarstedt, Nubrecht, Germany) were assembled on the opposite sides of an acrylic chamber leaving a narrow space (5 mm) hosting the sediment and the roots. Each microplate consisted of 96 wells with an opening diameter of 6.9 mm (volume 385 µL), arranged in 8 rows and 12 columns. The wells were initially filled with O$_2$-free distilled water and covered by a membrane made from Spectra/por 1 dialysis membrane (Spectrum™) consisting of regenerated cellulose with a molecular weight cut off of 6-8 kDa (Mura et al., 1996). The sediment and the plant were added to the chamber, which was placed in a tank containing aerated in situ water for 14 days. The microplates were then retrieved from the chamber, the membrane was removed and the water from each well sampled for chemical analyses. Soluble reactive phosphorus (i.e. PO$_4^{3-}$) and NH$_4^+$ were determined using standard colorimetric methods (Bower & HolmHansen, 1980; Valderrama, 1977) and analyzed spectrophotometrically (iMark™ Microplate Absorbance Reader).

Environmental Scanning Electron Microscopy imaging on old and new roots and elemental composition of plaques

Presence and elemental spectra of the precipitates on the roots surface was analysed on new and old roots of *V. spiralis* by Environmental Scanning Electron Microscope (ESEM) coupled with Energy Dispersive X-ray Spectrometer. Specimens of *V. spiralis* were gently extracted from the sediment and rinsed with in situ water to remove sediment. Sections of new, light colored, and old, red-dark colored roots (5 mm in length) were excised with a sterile scalpel at different depths. Root sections were mounted on aluminum stubs of 12 mm with double-sided adhesive carbon tape. The prepared samples were then directly analyzed at the ESEM (QuantaTM 250 FEG, FEI, Hillsboro, OR, USA) at 15.0 kV, operating in wet mode (room internal relative moisture 100%, temperature 3-5°C and pressure 600-700 Pa).

RESULTS

Radial oxygen loss at overlying water at air saturation

The sediment not influenced by the plant had an O$_2$ penetration of 5.2 ± 0.05 (s.e.m., n = 5) mm (Table 1) at 100% air-saturation. The derived O$_2$ consumption within the top oxic layer of the
sediment was 234 ± 33 (s.e.m., n = 5) μmol m⁻³ h⁻¹. In the presence of the rhizosphere, radial
oxygen loss (ROL) was observed along all visible root segments, with higher O₂ concentrations
measured in the top, root-dense part of rhizosphere (Fig. 1A). During the light phase, O₂ saturation
reached 75% at about 3 cm depth and 25% at about 7 cm depth, in proximity of the root tips. After
the onset of darkness, the O₂ availability decreased for a period of 2.5 h before a new steady-state
O₂ distribution was established (see also Video S1 in supporting information). Under these
conditions, the prevailing O₂ saturation in the top part of the rhizosphere were 20 to 40%, with a
maximum at 50%. At about 7 cm depth, maximum O₂ saturation decreased to 15% and some of the
oxic areas at the root tips turned anoxic (Fig. 1B).

The comparative analysis of the light and dark phase images highlighted areas of the rhizosphere where condition shifted from oxic to anoxic levels during one diel cycle (Fig. 1C). Ninety-two percent of the rhizosphere (defined as maximum extent of the oxic area) remained oxic at all time, whilst 8% fluctuated between oxia and anoxia (Table 2). This corresponded to 41.3 cm³ of sediment remaining oxic and 5.5 cm³ that oscillated between oxic and anoxic condition. Such fluctuating areas were preferentially located at the peripheral zones of the rhizosphere. Ninety-four percent of such areas fluctuated between anoxia and ≤ 20 μmol L⁻¹ O₂, with a mode variation value of 2 μmol L⁻¹ (Fig. S2). The maximum diel amplitude of oxic-anoxic oscillations (50 μmol L⁻¹ O₂) was found where roots overlaid (Fig. 1C). Under light conditions, ROL per root surface area estimated across six roots ranged between 58 and 658 μmol m⁻² h⁻¹, with an average value of 324 ± 107 μmol m⁻² h⁻¹.

Radial oxygen loss in old versus new roots
Oxygen transects across an old, not growing root (not increasing in diameter nor length throughout a light period) and one new, growing root during subsequent light, dark, and light phases are shown in figure 2. At steady state in light (16:26, hh:mm), ROL by the old root led to stable O₂ saturation up to 39%. Oxygen was measured around the root over an area of 2.7 mm in diameter. During the following dark phase, maximum O₂ saturation decreased to 28% and the diameter of the area of net O₂ accumulation contracted to 2.4 mm. Subsequent O₂ saturation remained constant. With the next light phase, O₂ saturation realigned with the values of the previous light phase. No O₂ was measured in the sediment before the appearance of the new growing root (new root, time: 10:46). Net O₂ accumulation (maximum 3.5% O₂ saturation) was measured at 16:36 over an area with a diameter of 1.2 mm. At the first dark measurement (22:06), the maximum O₂ saturation had increased to
7.7% and the oxic area spanned over 1.4 mm. At 4:06, maximum O\textsubscript{2} saturation reached 9.4% with no substantial increase of the oxic diameter. The following measurement with light showed an increase of the O\textsubscript{2} saturation (maximum 17%) and of the oxic area diameter (1.7 mm). Similar dynamics, i.e., retreat of the oxic area during dark period in old roots versus continuous expansion of the oxic areas in new roots, was observed in four additional roots (two old roots and two new roots) (Fig. S3).

**Effect of changing O\textsubscript{2} saturation in the overlying water**

Figure 3 shows the variation of the average O\textsubscript{2} saturation within the rhizosphere as a function of O\textsubscript{2} saturation in the overlying water during light/dark periods. During light phases, under 100% O\textsubscript{2} saturation in the water column, the average O\textsubscript{2} saturation in the rhizosphere remained at 34% ± 0.2 (Mean ± SD) (Fig. 3B). However, O\textsubscript{2} saturation rapidly declined after the onset of darkness. Within 2 h 20 min, the average O\textsubscript{2} saturation stabilized at 22% ± 0.2 AS. Eighty-five percent of this decline was reached within the first 20 min. Under 38% O\textsubscript{2} saturation in the water column, the average O\textsubscript{2} saturation in the rhizosphere dropped to 15% ± 0.2 during the light phase, and to 4.2% ± 0.1 during the dark phase. Re-increasing the ambient O\textsubscript{2} saturation to 70%, increased of the average O\textsubscript{2} saturation of the rhizosphere to 27% ± 0.1 during the light phase, and to 13% ± 0.1 during the dark phase. Overall, the average steady state O\textsubscript{2} saturation in the rhizosphere decreased linearly with the ambient O\textsubscript{2} level of the overlying water under both light and dark conditions (light phase:

\[ \text{rhizosphere O}_2 \text{saturation} = 0.29 \times \text{ambient water O}_2 \text{saturation} + 5.0, \quad r^2 = 0.98; \]

dark phase:

\[ \text{rhizosphere O}_2 \text{saturation} = 0.29 \times \text{ambient water O}_2 \text{saturation} - 6.9; \quad r^2 = 0.99). \]

Similarly to the average O\textsubscript{2} saturation in the rhizosphere, the extension of the oxic area on the planar optode wall responded to the changes in ambient O\textsubscript{2} saturation and light condition (Table 2). The extension of the oxic area spanned from a maximum 25 cm\textsuperscript{2} at 100% O\textsubscript{2} saturation in overlying water during light phase to a minimum of 8.6 cm\textsuperscript{2} with overlying water at 38% O\textsubscript{2} saturation during the dark phase. Estimated total O\textsubscript{2} transport via ROL during the light (13 h) and dark (11 h) phases was 53.5 and 39.0 µmol, respectively at 100% O\textsubscript{2} saturation, and 29.8 and 10.4 µmol, respectively with water at 38% O\textsubscript{2} saturation.

**Effect of the rhizosphere on sediment denitrification coupled to nitrification**

Rates of denitrification coupled to nitrification in vegetated sediment were about 6 and 2.5 folds higher compared to unvegetated sediment exposed to light and darkness, respectively (Fig. 4).
light exposed vegetated sediment the subsurface denitrification coupled to nitrification (85 ± 10 
µmol N-N₂ m⁻² h⁻¹, mean ± sem, n = 6) was almost twice the values in darkness (45 ± 3 µmol N-N₂ 
m⁻² h⁻¹, mean ± sem, n = 6). Rates of subsurface denitrification coupled to nitrification measured in 
the unvegetated sediment indicate that some \(^{15}\text{NH}_4^+\) reached the oxic portion of the sediment, but 
rates remained largely unaffected by light (i.e. 15 ± 2 and 20 ± 2 µmol N-N₂ m⁻² h⁻¹ under light and 
dark conditions, respectively).

Nutrient availability in the rhizosphere as resolved by the microplate approach.
The prevailing \(\text{NH}_4^+\) concentration within the rhizosphere was around 15 µmol L⁻¹ (Fig. 5) while 
concentration in root-free areas reached over 100 µmol L⁻¹. Similarly, the phosphate concentration 
in the basal root zone amounted to 7.6 µmol L⁻¹ while values increased to about 200 µmol L⁻¹ in the 
periphery of the image. Thus nutrient availability in the rhizosphere was almost one order of 
magnitude lower than in zones with no roots. Two additional peripheral areas with no apparent link 
with the rhizosphere (at the sediment surface and at 8 cm depth) also appeared highly \(\text{PO}_4^{3-}\) depleted 
(0 – 6 µmol L⁻¹).

Environmental Scanning Electron Microscopy (ESEM) imaging on old and new roots and elemental 
composition of plaques
New roots of \textit{V. spiralis} appeared light-coloured and were clearly distinguishable from old, red to 
dark-coloured roots (Fig. 6b). The red-dark colour was associated with the formation of dense metal 
plaques as shown by ESEM imaging (Fig. 6c). By contrast, new-light coloured roots appeared bare, 
with only limited metal plaque precipitation (Fig. 6a). Comparative analysis of the elemental 
composition of the surface of the bare roots and plaque covered roots by Energy Dispersive X-ray 
Spectrometer revealed a relative enrichment of Fe on the plaques surface (27.0 %) compared to the 
bare root (2.5 %) (Fig. 6d and e); similarly, the P content was higher in the plaques (7.5 %) than in 
the bare roots (1.2 %).

DISCUSSION
Importance of ROL from \textit{V. spiralis} for benthic \textit{O}_2 consumption
To our knowledge the work of Han et al. (2016) and the present study provided the first direct 
estimates of ROL in \textit{V. spiralis} under conditions close to those met \textit{in situ}. Rates of ROL from 
single roots in our study (58.3 - 658 µmol m⁻² h⁻¹, Min. – Max.) are comparable to rates measured in

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rice plants (*Oryza sativa*) i.e., 19.2 – 441 µmol m$^{-2}$ h$^{-1}$ (Larsen, Santner, Oburger, Wenzel, & Glud, 2015) which are among the highest rates reported in the literature (Han et al., 2016 and references therein). Rates of ROL from single roots of *V. spiralis* measured by Han et al. (2016) via planar optode imaging ranged between 32 and 109 µmol m$^{-2}$ h$^{-1}$. Despite the difference in ROL from single roots between the two available studies on *V. spiralis*, our total ROL rate normalized for the root biomass (calculated from total ROL / whole roots dry weight) i.e., 9.5 - 10.8 µmol O$_2$ g$_{DW}$ root$^{-1}$ h$^{-1}$ were similar to the one reported by Han et al. (2016) i.e., 5.2 – 7.7 µmol O$_2$ g$_{DW}$ root$^{-1}$ h$^{-1}$. This convergence could be linked to a larger fraction of roots segments that do not facilitate or have limited ROL in our study. In chemically reduced sediments, ROL facilitates the re-oxidation of Fe$^{2+}$ and its precipitation as iron plaques on the root surface (Povidisa et al., 2009 and references therein). Such plaques can decrease the gas permeability of the roots and thus the ROL (Kaj Sand-Jensen, Møller, & Raun, 2008). Extensive Fe coating was also observed in our study on old roots of *V. spiralis* (Fig. 6 and later discussion). It is thus plausible that precipitation of Fe oxides have limited ROL in a substantial fraction of the rhizosphere.

In *V. spiralis*, the O$_2$ release via ROL occurs all along the root length. This results in a conspicuous transport of O$_2$ into the rhizosphere as compared to other submerged macrophytes where ROL occurs in partial section of the roots or solely at the root tips e.g., *Zostera sp.* (*marina* and *muelleri*) and *Ruppia maritima* (Brodersen, Nielsen, Ralph, & Kuhl, 2015; Jensen et al., 2005; Jovanovic et al., 2015; Koren, Brodersen, Jakobsen, & Kuhl, 2015). Based on the obtained O$_2$ images we estimated that one plant of *V. spiralis* increases the total oxic volume of the sediment 447 and 394 times during the day and night, respectively. Considering the ROL of a single plant (as reported in Tab. 2) and a minimum shoot density of 600 plant m$^{-2}$ (Ribaudo et al., 2011), the colonization of *V. spiralis* can enhance the total O$_2$ transport into the sediment from to 234 µmol m$^{-2}$ h$^{-1}$ to 2500 and 2200 µmol m$^{-2}$ h$^{-1}$ during day and night, respectively. On a daily basis, the ROL by the meadow (56.1 mmol O$_2$ m$^{-2}$ d$^{-1}$) can thus increase the sediment respiration ($R_{SW1}$ 5.6 mmol O$_2$ m$^{-2}$ d$^{-1}$) by approx. 10 times. This is substantially higher to what previously reported from marine meadows of *Zostera marina*, where ROL (2.16 – 2.48 mmol O$_2$ m$^{-2}$ d$^{-1}$) was estimated to accounted only for the 2-14% of the sediment respiration (Frederiksen & Glud, 2006; Jensen et al., 2005), and almost comparable to rice plants with highly gas permeable rhizospheres and where ROL (9.9 – 24.8 mmol O$_2$ m$^{-2}$ d$^{-1}$) was 144% of the sediment respiration (Larsen et al., 2015).
The oxic conditions in the rhizosphere exhibited considerable spatio-temporal variations. At the light-dark shift, the basal root zone of the rhizosphere remained oxic, whereas peripheral areas changed from oxic to anoxic with more pronounced anoxic/oxic oscillations detected where roots intersected. At the single root level, the oxic halo around the non-growing roots expanded and contracted regularly in response to light-dark and dark-light shifts, respectively. In contrast, for growing roots, the oxic halo kept expanding even during darkness (although at slower rates as compared to light conditions). The marginal expansion of the halo between the two successive dark measurements could have been determined by the widening of the root diameter (the root was observed to elongate overnight), or by a possible reduced sediment respiration linked with a lower release of roots exudates in the sediment at darkness (Watt & Evans, 1999). Overall our data indicate that the rhizosphere hosts a complex mosaic of microenvironments (microbial niches) created by light shifts, root age, assemblage and geometry. This could have important implications for plant performance and the biogeochemical function of the sediment.

In addition, our data show the relative importance of the O$_2$ saturation of the ambient water versus photosynthesis (at light saturation) in controlling ROL in *V. spiralis*. As reported for other submerged macrophytes, photosynthesis increases the O$_2$ partial pressure in the aerenchyma which enhances the ROL (e.g. Pedersen, Borum, Duarte, & Fortes, 1998; K. Sand-Jensen, Prahl, & Stokholm, 1982). In contrast, during darkness, only the O$_2$ gradient between the overlying water and the sediment drives the ROL. Thus the ratio between dark and light values indicates the contribution of the O$_2$ gradient to the total ROL. With ambient water at 100% and 70% O$_2$ saturation, the ratios between dark and light values were 0.88 and 0.73, respectively, indicating that the O$_2$ gradient was the main driver for ROL. At 38% O$_2$ saturation, the ratio lowered to 0.25 indicating that photosynthesis became more important for driving ROL at severely depleted O$_2$ levels. With water at 100% O$_2$ saturation, the ratio calculated for *R. maritima* and *Z. marina*, was approximately 0.4 indicating a relatively lower contribution of the water-sediment gradient to ROL (Jovanovic et al., 2015). The same ratio calculated from ROL estimated from single roots of *Z. marina*, (Frederiksen & Glud, 2006; Jensen et al., 2005) and *Cymodocea rotundata* (Pedersen et al., 1998), generally ranged between 0.39 to 0.45. Although this comparison is based on a limited amount of data, the available studies suggest a more effective transport of O$_2$ by *V. spiralis* in the sediment in absence of photosynthesis. The maintenance of elevated ROL during darkness by *V.*
*spiralis* is presumably important for colonization and growth in O$_2$ depleted eutrophic freshwater characterised by chemically reduced sediment.

In our experiment, i) the linear correlation between oxic level of the overlying water and ROL intensity, ii) the fast establishment of new steady oxic level in the rhizosphere in response to changing O$_2$ in the overlying water, and iii) the reversibility of such response, suggests that the variation of ROL is a passive response to the alterations of the O$_2$ level in the ambient water. The linear relationship between oxic level of the water and ROL also indicates that no or only marginal parts of the rhizosphere will remain oxic in darkness below 30\% O$_2$ saturation in the overlying water (average rhizosphere O$_2$ saturation about 2\%). It remains to investigate if anatomic adaptation such as variation of root porosity (Lemoine, Mermilod-Blondin, Barrat-Segreitain, Masse, & Malet, 2012) or size of the aerenchyma (Colmer, 2003b; Cronk & Fennessy, 2016) could happen in plants exposed to lower O$_2$ saturations and during longer exposure times than applied in the present study.

**Spatio-temporal heterogeneity of ROL and implications for N and P cycling**

The two-dimensional analysis of NH$_4^+$ and PO$_4^{3-}$ document an overall nutrient depletion in the rhizosphere, most likely due to macrophyte uptake and ROL-dependent processes. For nitrogen, the presence of plants clearly enhanced the denitrification as ROL stimulated subsurface nitrification – particularly during the day time. The plants thereby facilitated microbial driven removal of bioavailable nitrogen. This observation is consistent with previous data reported by Soana et al. (2015) and Racchetti et al. (2017). In bio-irrigated sediments the mobilization of N species (enhanced release of NH$_4^+$ in anoxic phase, enhanced nitrification in the oxic phase, and overall simulation of denitrification activity) is known to be favoured under oscillating redox conditions (Gilbert, Hulth, Grossi, & Aller, 2016). Similar dynamics could occur in the rhizosphere of *V. spiralis* in response to day and night shifts. In particular, pronounced redox oscillation are expected to occur in the peripheral areas where conditions shift from oxic to anoxic on a daily basis. In these areas, the activity of nitrifying microorganisms can be also stimulated by a higher availability of NH$_4^+$ compared to the root-dense, NH$_4^+$-depleted core of the rhizosphere where nitrification may face more intense competition with plant uptake (see later discussion). From the linear regression between ambient water O$_2$ level and oxic area of the rhizosphere, it can be estimated that a variation of ambient water O$_2$ saturation of 15\% will result in the oscillation of the oxic area of the rhizosphere similar to the one observed at light/dark shifts with ambient water at 100 \% O$_2$ saturation (i.e. contraction of the oxic area of 2 cm$^2$). An effect on the rhizosphere N
dynamics similar to the one induced by day/night shift can thus also be expected from moderate variations in ambient water O\textsubscript{2} level. All in all, our data indicate that the rapid (hours to days) modulation of ROL in response to variation in light regime and possibly in ambient water O\textsubscript{2} concentration can directly influence microbial-driven N transformations and overall enhance the ability of the rhizosphere to work as a N sink in the riverbed.

The variation of NH\textsubscript{4}\textsuperscript{+} concentration in the rhizosphere is ultimately determined by the balance between consumption processes (i.e. plant uptake and bacterial nitrification), and supply via organic matter decomposition (ammonification). Theoretical N uptake by \textit{V. spiralis}, estimated for plants from the same site ranged between 380 and 680 µm m\textsuperscript{-2} h\textsuperscript{-1} in spring and between 6600 and 10000 µm m\textsuperscript{-2} h\textsuperscript{-1} in summer months (Racchetti et al., 2017). This values are one to three orders of magnitude higher compared to our data on subsurface denitrification coupled to nitrification activity (45 – 85 µm m\textsuperscript{-2} h\textsuperscript{-1}). Uptake, more than nitrification, seems thus to drive the consumption of NH\textsubscript{4}\textsuperscript{+} in the rhizosphere, unless NO\textsubscript{3}\textsuperscript{-} uptake from the water column is significant. A maximum rate of ammonification in the rhizosphere can be estimated from the O\textsubscript{2} consumption rate of the vegetated sediment (assuming a respiratory coefficient O\textsubscript{2}:C = 1) and the sediment C:N ratio (i.e., 23 from Soana et al., 2015). The so calculated rate of ammonification (i.e. 109 µmol m\textsuperscript{-2} h\textsuperscript{-1}) is substantially lower than the sum of the NH\textsubscript{4}\textsuperscript{+} consuming processes. The mismatch between consumption and supply can thus explain the NH\textsubscript{4}\textsuperscript{+} depletion in the rhizosphere observed in our study.

Phosphate was also highly depleted in the rhizosphere. Similarly to NH\textsubscript{4}\textsuperscript{+}, net PO\textsubscript{4}\textsuperscript{3-} depletion is likely linked with potential P production in the sediment much lower than P uptake. Our data show that in addition to plant uptake, P-enriched Fe-plaques on root of \textit{V. spiralis} can also act as PO\textsubscript{4}\textsuperscript{3-} sink. The formation of Fe plaques on the roots surface results from the precipitation of pore water-dissolved ferrous iron as insoluble iron(III) oxides-hydroxides at the higher redox potential induced by ROL (e.g. Bacha & Hossner, 1977). Conversely, under prevailing anoxic conditions lower redox potential may favour the dissolution of Fe(III) oxide-hydroxides with consequent liberation PO\textsubscript{4}\textsuperscript{3-} (Azzoni, Giordani, Bartoli, Welsh, & Viaroli, 2001; Racchetti et al., 2010). Sedimentary pools can thus be made available during oxic-anoxic shifts. However, a recent publication (Han et al., 2018) suggests that P daily variations are much lower than those of O\textsubscript{2}, leaving space for further studies targeting how plants exploit sedimentary nutrients. The immobilization of P in Fe-plaques as induced by ROL is in apparent contrast with the need of the plant to take up dissolved P (Christensen & Sand-Jensen, 1998). To overcome this possible limitation recent studies showed that aquatic plants can ‘re-gain’ P from plaques by promoting their
dissolution (via stimulating acid production or Fe III reduction) for assimilation purposes (Brodersen et al., 2017; Xing et al., 2018). Our O₂ and environmental scanning electron microscopy coupled with energy dispersive X-ray spectrometer data show that conditions favouring precipitation or dissolution of Fe-P plaques coexist in the rhizosphere and that such heterogeneity persists at both macro and micro-scales. Maintaining high spatial and temporal heterogeneity of chemical niches, could thus represent a mechanisms per se to both accumulate (in plaques) and mobilize (in transiently or permanently reduced areas of the rhizosphere) P which has low concentrations in the water column and is generally assimilated from the sediment.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES


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### TABLES

**Table 1.** Characteristic of the plant and sediment. Values reported as mean (± s.e.m.). Radial Oxygen Loss (ROL), Oxygen penetration depth, volumetric O2 respiration at the sediment surface ($R_{SWI}$) and at depth ($R_s$) refer to the treatment with overlying water at 100% air saturation (AS) under light conditions.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves</td>
<td>8</td>
</tr>
<tr>
<td>Leaves dry weight (g)</td>
<td>0.386</td>
</tr>
<tr>
<td>Roots dry weight (g)</td>
<td>0.096</td>
</tr>
<tr>
<td>Average root diameter (mm)</td>
<td>0.37 (± 0.02, n = 6)</td>
</tr>
<tr>
<td>ROL (µmol m$^{-2}$ h$^{-1}$)</td>
<td>324 (± 107, n = 6)</td>
</tr>
</tbody>
</table>

**Table 2.** Average O2 level (AS %) in the rhizosphere, extension of the oxic area, extension of the oxic volume, Radial Oxygen Loss (ROL) as O2 flux by the whole plant under different experimental conditions at steady-state, and integrated O2 transport via ROL during the whole light and dark phases.

<table>
<thead>
<tr>
<th>100% (AS)</th>
<th>70% (AS)</th>
<th>38% (AS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>Mean $O_2$ sat. (% AS)</td>
<td>22.2</td>
<td>33.5</td>
</tr>
<tr>
<td>Area (cm$^2$)</td>
<td>22.9</td>
<td>24.9</td>
</tr>
<tr>
<td>Volume (cm$^3$)</td>
<td>41.3</td>
<td>46.8</td>
</tr>
<tr>
<td>ROL (µmol h$^{-1}$ plant$^{-1}$)</td>
<td>3.7</td>
<td>4.2</td>
</tr>
<tr>
<td>$O_2$ transport (µmol plant$^{-1}$)</td>
<td>39.0</td>
<td>53.5</td>
</tr>
</tbody>
</table>

**FIGURE CAPTIONS**

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Figure 1: Oxygen distribution in the rhizosphere of *V. spiralis*, with ambient water at 100% air saturation (AS) during light (panel a) and dark (panel b) periods. Zero on the X-axes indicates the approximate sediment surface. Air saturation level is denoted by colour. White dotted line in panel A shows the transect analysed for comparing ROL in old vs. new roots in figure 2. Panel c shows the areas of the rhizosphere that experience transition between oxic and anoxic (< 2 µmol L\(^{-1}\) O\(_2\)) condition during a light-dark cycle with overlying water at 100% AS. Intensity of the fluctuation in O\(_2\) concentrations are indicated by color and expressed in µmol L\(^{-1}\). Black areas indicate zones that are above the 2 µmol L\(^{-1}\) threshold even during the dark phase.

Figure 2. Oxygen levels along a transect crossing an old, not growing and a new, growing root. Measurements were repeated over a 24-hour during a light-dark-light cycle. Empty and full symbols indicate measurements time (hh:mm) during light and dark conditions, respectively. The location of the transect is illustrated in figure 1, panel a.

Figure 3. Variation of the oxic level in the rhizosphere (defined as the maximum expansion of the oxic area at O\(_2\) level at 100% air saturation in light) under repeated light and dark cycles, at three ambient O\(_2\) levels (as % of air saturation) (panel a). Average O\(_2\) level (as % of air saturation) in the rhizosphere at quasi steady-state under the various conditions (panel b).

Figure 4. Light and dark fluxes of denitrification coupled to nitrification in the subsurface sediment (Dn-S) measured in microcosms with and without *V. spiralis* (mean ± sem, n = 6).

Figure 5. Two-dimensional NH\(_4^+\) (panel b) and PO\(_4^{3-}\) (panel c) isoconcentration diagrams of the rhizosphere sediment obtained by a microplate sampler. Black circles indicate the opening of each sampling well. Panel a shows the sediment portion that was analysed before the application of the microplates.

Figure 6. Environmental Scanning Electron Microscopy (ESEM) images of young (panel a) and old (panel c) roots of *V. spiralis* (panel b) showing light and heavy plaques formation, respectively. ESEM-coupled to Energy Dispersive X-ray Spectrometer (EDS) image of root surface partially coated by Fe-P plaques (panel d). EDS spectrum showing elemental composition measured on the
bare root (circle) and on the plaque (star) (panel e). Relative enrichment (weight %) of Fe and P are reported on the panel. Numbers within brackets indicate three-SD.

Figure S1. Experimental set-up. CPU: computer; Ca: reflex camera; Emf: long-pass (590 nm) emission filter; LED: high power LED unit; Exf: short-pass (470 nm) excitation filter; Trig: trigger box used to coordinate LED, camera and light functioning; Light: 200 μmol photons m$^{-2}$ s$^{-1}$, PO: O$_2$-sensitive planar optode foil; RH: rhizotron containing sediment, plant and water; Gas mixer: digitally controlled mas mixing unit dosing air and N$_2$.

Figure S2. Distribution of O$_2$ fluctuating areas (pixels) as a function of the fluctuation amplitude (difference in concentration between light and dark phases).

Figure S3. Oxygen levels along a transect crossing two old, not growing (panel a and b) and two new, growing root (panel c and d). Measurements were repeated over a 12-hour period during a light-dark cycle. Empty and full symbols indicate measurements time (hh:mm) during light and dark conditions, respectively.

Video S1. Oxygen (as % of the air saturation level) dynamics in the rhizosphere of *V. spiralis* over 24 hours during a light-dark-light period. Images interval 20 min. Frame rate 7 frame per second.
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