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Oxygen as dynamic parameter in internal aeration and the rhizosphere of wetland plants

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2. A. C. Faußer et al. (2013). “Internal oxygen dynamics in rhizomes of *Phragmites australis* and presence of methanotrophs in root biofilms in a constructed wetland for wastewater treatment”. In: *Desalination and Water Treatment* 51.13-15, pp. 3026–3031. DOI: 10.1080/19443994.2012.748452

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Abstract

In wetland ecosystems, plants enlarge the oxic-anoxic borderline by releasing oxygen to the substrate and thus enhance processes which interconnect anaerobic and aerobic pathways. Actively ventilating wetland plants strongly influence the oxidation of reduced substances from anaerobic soil layers. Oxygen plays a key role in the carbon cycle of our oxygenated world. This work exploits the idea that processes, which are associated with the oxygenated rhizosphere, can be estimated by the assessment of oxygen supply to the substrate and potential rates of oxygen-consuming carbon dioxide production.

The objective of this thesis was to connect *in-situ* plant-mediated oxygen transport to submerged organs with oxygen usage associated with the roots. In order to reach this goal, plant-internal oxygen and carbon dioxide concentrations were measured *in-situ* continuously and concomitantly over several days. A greenhouse experiment was performed to reveal the influence of water vapour pressure deficit (VPD) on aeration efficiency of submerged organs and the rhizosphere. The root-associated biofilms were analysed for aerobic methane-oxidizing bacteria (MOB), in order to characterize the dominance of methane as substrate in the rhizosphere.

The diurnal dynamics of internal oxygen concentrations (O2conc) assessed *in-situ* inside culms and rhizomes of *Phragmites australis* (Cav.) Trin. ex Steud. showed clear ventilation patterns during daytime. High temporal dynamics were detected at the beginning of the gas-flow in the morning hours. The delay in the increase of rhizome-O2conc compared to culm-O2conc demonstrated that air was actively pressurized to submerged organs. In addition, the nocturnal declines of O2conc indicated regulation mechanisms for aerobic metabolism during times with reduced oxygen supply from the plant. The negative correlation of O2conc to internal carbon dioxide emphasizes the strong inter-dependency of plant-mediated oxygen supply and oxygen-consuming metabolic processes in the rhizosphere. Plant-internal carbon dioxide concentrations were highest at the beginning of the active aeration process. The peak detected in carbon dioxide concentration resulted from accumulated respiratory gases which were initially flushed from the submerged gas-spaces. During full activity of the plants in photosynthesis and ventilation, the internal carbon dioxide content decreased as expected.

Abstract

Changing of VPD influenced the aeration patterns of rhizomes and rhizospheres of *Typha angustifolia* L. strongly. This effect is assumed to be induced by stomatal narrowing in response to increasing VPD in the surrounding atmosphere. Dense biofilms were detected on the roots of *P. australis* and *Typha latifolia* L.. The proportions of MOB were higher than one third of total root-associated biofilm bacteria. By supplying oxygen to submerged organs and the rhizosphere, wetland plants support the proliferation of root-associated MOB. This demonstrates that the rhizosphere is a habitat for microorganisms which is dominated by methane diffusing from anaerobic soil layers.

The present study confirms that the combined assessment of plant-internal oxygen and carbon dioxide concentrations can be used to estimate oxygen-consuming processes in water-saturated soil associated with wetland plants. The large number of root-associated MOB indicates the high potential of methane consumption mediated through plants in wetland ecosystems. Further studies should be conducted in order to resolve patterns of carbon turnover during full seasons and different levels of organic load. The presented method of optical oxygen assessment allows to perform long-term *in-situ* studies with least obstruction of the plant.

Abstract (German) - Zusammenfassung

In Feuchtgebieten geben Pflanzen Sauerstoff an den Boden ab, wodurch die anoxisch-oxische Grenzfläche vergrößert wird und Prozesse gefördert werden, die die anaerobe mit der aeroben Welt verbinden. Feuchtgebietspflanzen, die ihre Gewebe aktiv belüften, beeinflussen stark die Oxidation von reduzierten Verbindungen aus anaeroben Bodenschichten. Sauerstoff spielt eine Schlüsselrolle im weltweiten Kohlenstoffkreislauf unserer oxidierten Welt. Diese Arbeit nutzt die Idee, dass sich Prozesse, die sich im oxidierten Wurzelraum abspielen, durch die Aufzeichnung von in den Boden geleitetem Sauerstoff und die möglichen Entstehung von Kohlenstoffdioxid abschätzen lassen.

Das Ziel der Arbeit war, den *in-situ* Transport von Sauerstoff durch die Pflanzen zu ihren untergetauchten Organen mit dem Sauerstoffverbrauch im Wurzelraum zu verbinden. Um diese Aufgabe zu erreichen, wurden die Konzentrationen von Sauerstoff und Kohlenstoffdioxid im Pflanzeninnern über mehrere Tage hinweg kontinuierlich und zeitgleich *in-situ* bestimmt. Im Gewächshaus wurde ein Experiment durchgeführt, um den Einfluss des Wasserdampfdruck-Defizits (*vapour pressure deficit*, VPD) auf die Effektivität der Pflanzenbelüftung aufzudecken. Die mikrobiellen Biofilme auf den Wurzeln wurden auf Methan-oxidierende Bakterien (MOB) untersucht, um den Einfluss von Methan als Substrat im Wurzelraum zu bestimmen.

Über den Tag zeigten die Dynamiken der pflanzeninternen Sauerstoffkonzentrationen (*oxygen concentration*, O2conc), die *in-situ* in Stengeln und Rhizomen von Schilffrohr (*Phragmites australis* (Cav.) Trin. ex Steud.) aufgenommenen wurden, deutliche Belüftungsmuster. In den Morgenstunden zu Beginn der Belüftung wurden starke zeitliche Dynamiken registriert. Der verspätete Anstieg der O2conc in den Rhizomen, verglichen mit O2conc in den Stengeln, weist daraufhin, dass Luft aktiv in die untergetauchten Pflanzenorgane geleitet wird. Außerdem deuten die nächtlichen Abnahmen der O2conc an, dass während der Zeit von geminderter Versorgung durch die Pflanze, Mechanismen aktiv werden, die den aeroben Stoffumsatz in den untergetauchten Regionen regulieren. Die negative Korrelation zwischen den Konzentrationen von Sauerstoff und Kohlenstoffdioxid bekräftigt die starke Wechselbeziehung zwischen der pflanzenvermittelten Sauerstoffversorgung und den sauerstoffverbrauchen-

den Stoffwechselprozessen im Wurzelraum. Die Konzentrationen von Kohlenstoffdioxid innerhalb der Pflanzen waren zu Beginn der aktiven Belüftung am höchsten. Die beobachtete Spitze der Kohlenstoffdioxid Konzentrationen ergab sich durch das erste Ausströmen von Luft aus den untergetauchten Gaskanälen, die sich über Nacht mit Atemgasen angereichert hatten. Wie erwartet nahm der interne Kohlenstoffdioxid Gehalt während der vollen Photosynthese- und Belüftungsaktivität der Pflanzen ab. Die Veränderung des VPD beeinflusste das Muster der Belüftung von Rhizomen und des Wurzelraums von *Typha angustifolia* L. stark. In der Arbeit wird vermutet, dass dieser Effekt durch das Verringern der Stomata-Öffnung verursacht wird, welche sich bei steigendem VPD der umgebenden Luft schließen. Auf den Wurzeln von *P. australis* und *Typha latifolia* L. wurden dichte Biofilme entdeckt. Der Anteil an MOB, unter den an die Wurzeln assozierten Biofilmbakterien, lag bei über einem Drittel. Durch die Versorgung von untergetauchten Organen und des Wurzelraums mit Sauerstoff, fördern Feuchtgebietspflanzen das Wachstum von wurzelnahen MOB. Dies veranschaulicht, dass der Wurzelraum ein Lebensraum für Mikroorganismen ist, der von Methan, das aus anaeroben Bodenschichten diffundiert, dominiert wird.

Diese aktuelle Arbeit bestätigt, dass die gemeinsame Erfassung der Konzentrationen von Sauerstoff und Kohlenstoffdioxid innerhalb von Pflanzen genutzt werden kann, um die Sauerstoff-verbrauchenden Prozesse in wassergesättigten Böden, die in engem Kontakt mit Feuchtgebietspflanzen stehen, abzuschätzen. Die große Anzahl der MOB auf den Wurzeln weist auf das hohe Potential des Methanabbaus hin, das Pflanzen in Feuchtgebieten darstellen. Es sollten zukünftige Studien durchgeführt werden, die die vollständige Struktur des Kohlenstoffumsatzes während einer gesamten Vegetationsperiode und bei unterschiedlichem Gehalt an organischer Substanz im Boden aufklären. Die vorgestellte Methode der optischen Sauerstofferfassung ermöglicht es, Langzeituntersuchungen anzuwenden, bei der die Pflanzen möglichst wenig gestört werden.

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List of Abbreviations

CET central european time.

COD chemical oxygen demand.

CW constructed wetland.

ICM intracellular membranes.

MDH methanol dehydrogenase.

MOB methane-oxidizing bacteria.

O2conc plant-internal oxygen concentrations.

O2drop oxygen drops.

O2pp oxygen partial pressures.

PE person equivalents.

PFD photon flux density.

RH relative air humidity.

ROL radial oxygen loss.

SG storage granules.

TEM transmission electron microscopy.

VPD water vapour pressure deficit.

1 Introduction

1.1 The development of aerobic microorganisms and an oxic atmosphere

The early atmosphere in the Archean eon

During the formation of Earth, an early atmosphere developed which consisted of water (H_2O), methane (CH_4), nitrogen (N_2), ammonia (NH_3) and hydrogen sulfide (H_2S) (Petsch, 2003). Free oxygen (O_2), nitrogen (N_2) and sulfur (S) were produced through photolysis of H_2O, NH_3 and H_2S, respectively. The generated oxygen subsequently reacted with CH_4 and H_2S, which produced carbon dioxide (CO_2), carbon monoxide (CO) and sulfur dioxide (SO_2). Once the partial pressure of CO_2 was high enough, methanogenic Archaea evolved out of the first microorganisms (~ 3.8 billion years ago) which oxidized hydrogen (H_2) by reducing CO_2 to CH_4 (Battistuzzi et al., 2004). During the Archean eon, the atmospheric equilibrium contained negligible traces of oxygen and lasted several hundred million years (see Fig. 1.1).

The first Great Oxygen Transition and Snowball Earth

The first evidence of at least localized elevated amounts of molecular oxygen, and thus oxygen producing photosynthesis (see below), in early oceans dates back about 3.5 billion years (Lyons et al. (2014) and Petsch (2003), see Fig. 1.1). For a long time, free oxygen became trapped in reactions with reduced compounds, e.g. seen in ferrous oxide and ferric oxide deposits (Banded Iron Formations) and sulfur oxide deposits (Petsch, 2003). Oxygenic photosynthesis is assumed to have developed through divergence from early non-oxygenic phototrophs, when adopting to stressful terrestrial habitats, but its chronological origin is still controversial (Battistuzzi et al. (2004); Fig. 1.2).

Not until 2.4 billion years ago, when the reservoirs of reduced compounds in the oceans and on land were exhausted, molecular oxygen accumulated in the atmosphere. During the following Great Oxygen Transition, the production of oxygen by the biosphere transformed the Earth's atmosphere and surface irreversibly into aerobic conditions (Lyons

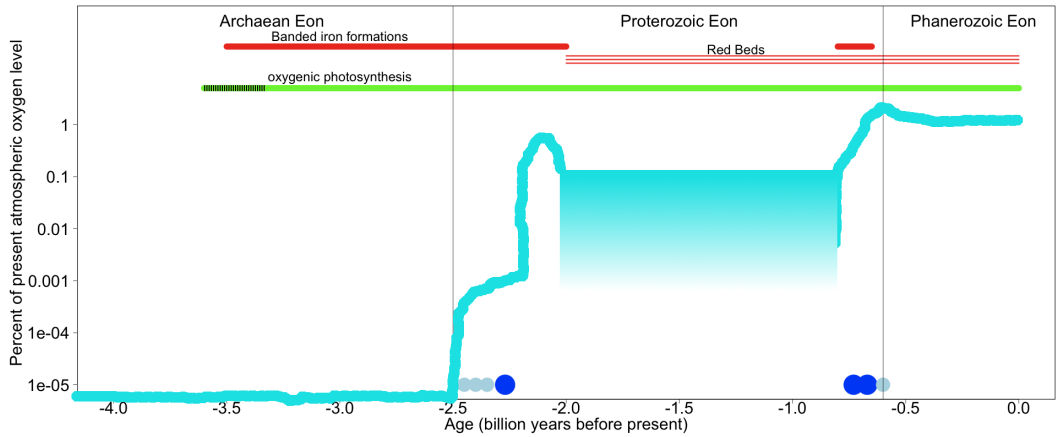

Figure 1.1: **The evolution of atmospheric oxygen.** Earth's early atmosphere contained traces of molecular oxygen. Through the evolution of oxygenic photosynthesis, the microorganisms produced enough oxygen to initiate a large increase of atmospheric oxygen (Archean eon). The second great oxygen transition is also believed to result from further innovations and prosperity of the biosphere on Earth. Blue circles: glaciation events. Based on Petsch (2003), Lyons et al. (2014), and Harada et al. (2015).

et al., 2014). A geological record for the elevated oxygenation state on Earth are the contemporaneously occurring Red Beds (ferric iron-rich sediments). At the interface between the still anoxic open ocean, first strains of methanotrophic eubacteria developed, which oxidized a great amount of the present methane (Battistuzzi et al. (2004); Fig. 1.2). During this period, the methanogenic Archaea in the oceans withdrew from the surface waters to the anaerobic deeper layers. With decreasing levels of methane in the atmosphere and the faint young sun, Earth experienced several glacial periods and a snowball glaciation from 2.4 to 2.1 billion years ago (Harada et al., 2015; Kopp et al., 2005).

Prosperity of oxygen-dependent life in the Proterozoic eon

During the ice-melt at 2.2 billion years ago, the primary productivity of oxygenic photosynthesis was highly stimulated (Harada et al., 2015). With the release of CO_2 after deglaciation, a geological shift occurred to climatic conditions which may have promoted the evolution of oxygen-dependent life on earth (Fig. 1.1). For about 1 billion years the oxygen concentration is suggested to vary between 0.001 and 0.1 of present atmospheric level, presumably as a result of low availability of nitrogen and molybdenum for the primary producers due to hydrogen sulfide-rich and oxygen-deficient oceans (Lyons et al., 2014; Petsch, 2003). In the stratosphere, the accumulating oxygen was photo-chemically transformed into ozone, which since then protects life from ultraviolet

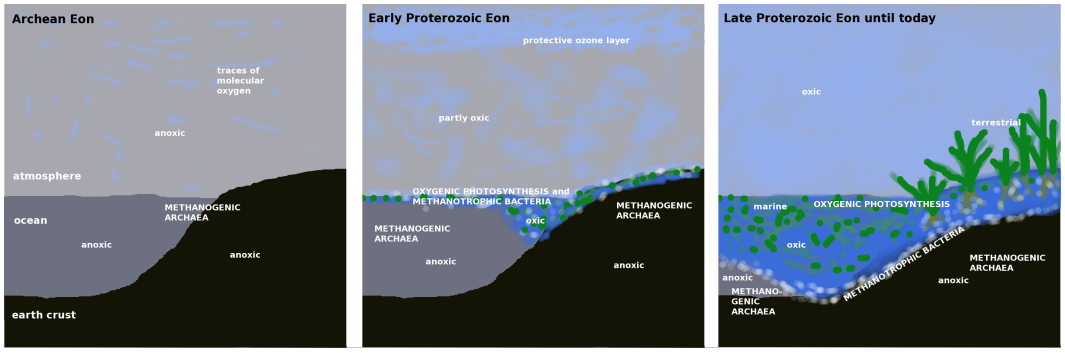

Figure 1.2: **Oxygenic photosynthesis transformed the Earth from anoxic to oxic.** With the development of oxygenic photosynthesis, the early anoxic Earth (left) became slowly oxygenated first in the atmosphere and surface waters (middle) and later additionally at all continental sites and deep waters (right). This figure focuses on oxygenic photosynthesis and methane-related carbon turnover. Green: organisms active in oxygenic photosynthesis; white: methane-oxidizing bacteria.

radiation from the sun. This allowed the first higher organized life-forms to settle on the continent.

During the Proterozoic eon, the oceans gradually became more and more oxygenated down to deeper layers. Several glaciation events followed between 700 to 600 million years ago and the atmospheric oxygen concentration again increased to modern levels at the beginning of the Phanerozoic eon (Harada et al. (2015); see Fig. 1.1 and Fig. 1.2). Until today ancient communities remain from the anoxic world before the two great oxygen transitions down in anaerobic layers of aquatic and semi-terrestrial sites and associated with volcano action. Ruminant animals use anaerobic archaean metabolism to pre-digest plant materials. In wetland ecosystems, anaerobic processes (e.g. methanogenesis) are in close contact with aerobic processes (e.g. methanotrophy) and relatively easy to reach, but still challenging to study (see Fig. 1.2 and Fig. 1.3).

Oxygenic organisms: source and sink for oxygen

Throughout Earth's history, the main source for molecular oxygen is the oxygenic photosynthesis which is at present performed by higher plants, algae, autotrophic unicellular eukaryotes, cyanobacteria and prochlorophytes (Petsch, 2003). In a first step, oxygenic organisms gather light energy to split $\mathrm{H_2O}$ in order to gain electrons and protons for chemical energy, while the produced oxygen is released. In a second step, the prior yielded energy is used to synthesize simple organic compounds by typically fixing carbon dioxide. Though this form of metabolism allows autotrophic life, in general, the de-

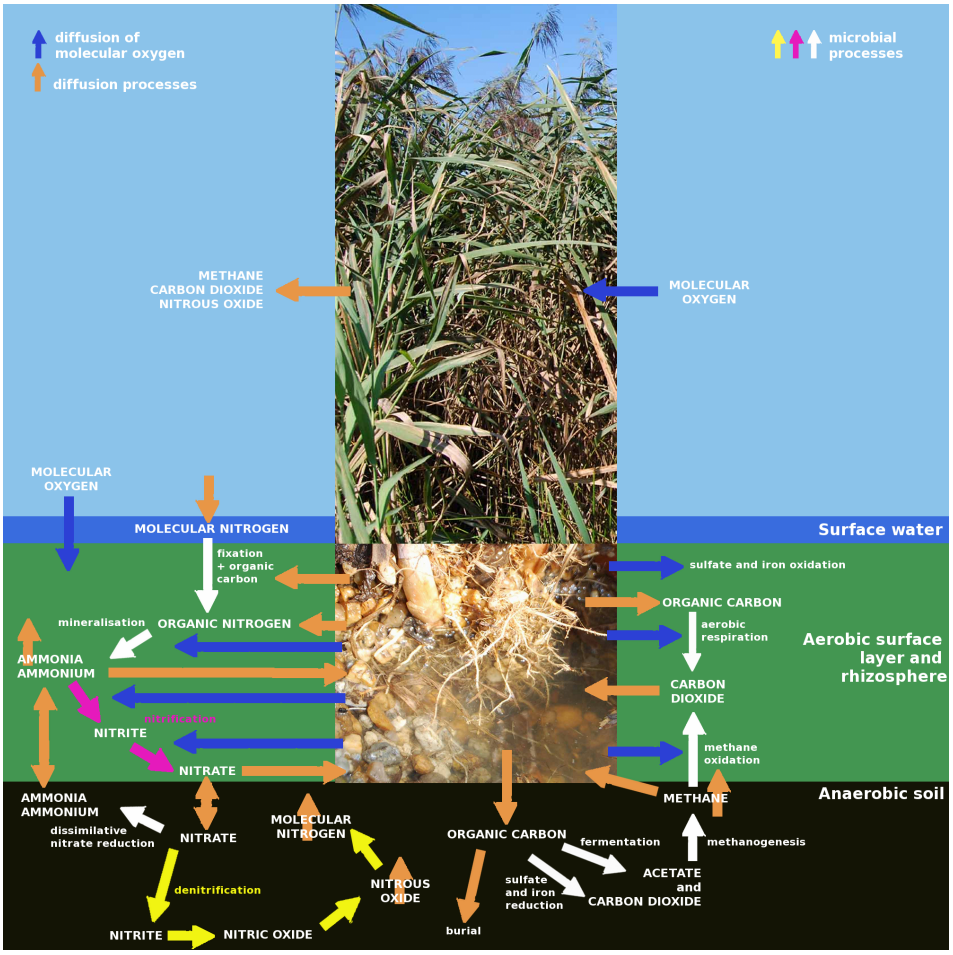

Figure 1.3: **The processes associated with submerged soils.** Oxygen-rich air is ventilated through the aerenchyma of the wetland plant and partly released into the rhizosphere. An interface between oxic and anoxic establishes from the root surface through the rhizosphere to the anaerobic soil layers. Soil-borne gases enter the roots and are transported along with the internal gas-flow to the atmosphere.

scribed eukaryotic oxygenic organisms depend on free oxygen for cellular respiration. Especially plants are supposed to originate from heterotrophic eukaryotic ancestors, which incorporated cyanobacteria (prochlorophytes) as their endosymbionts.

1.2 Challenges for plants on terrestrial and semi-terrestrial sites

Growing outside aquatic environments on land, gives plants the advantage to gather more light energy from the sun and maximum carbon dioxide compared to aquatic sites. On the other hand, the atmospheric situation implies the need for tissues which protect from water loss and irradiation, and requires organs which stabilize and supply

the whole organism. During their evolution, most land-growing plants have adopted to humid and arid sites, thus favouring well aerated soils. Under prolonged periods of water saturation, conditions in the substrate chemically turn to anaerobic and even phytotoxic (Ponnamperuma, 1972). In wetlands, plants live in an interface between the well known aerobic conditions and a totally different anaerobic world, which was there since time immemorial and still connects methanotrophic bacteria with methanogenic archaea (Fig. 1.3).

Internal ventilation

In wetland plants, large portions of the parenchymatic tissues are highly porous due to aerenchyma, i.e. air-filled channels between the cells which run from the stomata to the roots (Končalová, 1990). The internal ventilation through the aerenchymatic tissue is mainly based on diffusion and can be enhanced by pressurized gas-flow (W. Armstrong, 1980; W. Armstrong and J. Armstrong, 1991; Brix et al., 1992). In submerged plant organs, where pressurized gas-flow assists ventilation, the internal oxygen concentration can be maintained close to atmospheric levels (Brix et al., 1996; Konnerup et al., 2011). At least two types of ventilation mechanisms are identified which occur simultaneously, but with different dominance. Pressurizing wetland plants create internal elevated pressures by building gradients of humidity (humidity-induced convection) and/or temperature (thermal-transpiration) between the inner and outer atmosphere. During humidity-induced convection, the internal air is moisturized compared to the outer atmosphere and the contained gases (including oxygen) are diluted. Air is sucked inside the aerenchyma over a porous partition in the Knudsen regime ($<0.1\,\mu m$), which lies beneath the stomatal openings at the connection to the large gas-spaces, and flows downwards to the less pressurized tissues. During sunlight, the leaves, leaf-sheaths and the whole plant-body are warmed compared to the surrounding air. The elevated internal temperature creates pressure gradients across the above mentioned porous partition and thus induces thermal inward diffusion of air (thermal-transpiration). Thereby the pressurized gas-flow is enhanced during daily irradiance.

Plants with floating leaves like the lotus *Nelumbo nucifera* Gaertn. and the water lily *Nymphea alba* L. favour gas-flow by thermal-transpiration. Starting in the leaves on the water surface, air flows through channels from old to young leaves (Mevi-Schütz and Grosse, 1988a; Mevi-Schütz and Grosse, 1988b) or between channels within the same floating leaf (Dacey, 1980; Dacey, 1981; Schröder et al., 1986). In wetland plants with graminoid appearance, which is cylindrical shoots and linear leaves, ventilation is

mainly achieved by humidity-induced convection. In common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) a further through-flow mechanism is established, which arises from the large volume of the central pith cavities of shoots and rhizomes, which oppose less resistance to gas-flow. Winds blowing over the reed stand create pressure gradients (Venturi effects) between young and old or dead culms (J. Armstrong et al., 1992) and the resulting flow can more easily ventilate air through the central cavities.

The efficiency of the internal gas-flow also depends on additional factors tested for *P. australis* which include photosynthesis, stomatal aperture, aerenchymatic porosity, leaf sheath area and shoot height (W. Armstrong and J. Armstrong, 1991; Brix et al., 1992; Brix et al., 1996; Afreen et al., 2007). Several wetland macrophytes, including *Eleocharis spacelata* R.Br., *Juncus ingens* N.A.Wakef., *P. australis* and *Typha domingensis* Pers., were analysed for the influences of irradiance, temperature, plant-internal porosity and shoot height on convective flow (Brix et al., 1992). Also in *Typha angustifolia* L. and *Typha latifolia* L., the internal aeration is assumed to be influenced by leaf area, aerenchymatic proportion, stomatal movement and light (Bendix et al., 1994; Tornberg et al., 1994).

Aerobic rhizosphere

Many wetland plants cover their rhizome and root surfaces with an inducible and airtight barrier, to provide long-distance transport through the aerenchyma under waterlogged conditions (Colmer, 2002). In addition to the transport of air in order to supply submerged organs with oxygen for respiration, wetland plants release oxygen to the rhizosphere (Colmer (2003); see Fig. 1.3). The oxygen leaves the roots radially throughout the surface or, if a barrier is present, at the root tips and close to developing lateral roots.

Through the oxygenation of the rhizosphere, phytotoxic compounds become immobilized (e.g. ferric iron deposits on root surfaces; W. Armstrong (1980)) and nutrients are made available for uptake (nitrification vs. denitrification; see Fig. 1.3). The availability of free oxygen in combination with root exudates in the rhizosphere of wetland plants attracts various microorganisms (Bodelier, 2003). The microbes of diverse grades aggregate in biofilms of several cell-layers around the roots. Starting at the biofilms, an interface from aerobic conditions close to the roots to anaerobic conditions in the substrate establishes in the rhizosphere. The obligate methane-oxidizing bacteria grow in

the root-associated biofilms and use the oxygen released by the roots to oxidize the methane diffusing from anaerobic soil layers (see section 1.5.3).

Plant-mediated transport of soil-borne gases

The character of wetland plant aeration allows gases from the substrate to join the gas-flow stream. This means that while oxygen-saturated air flows into submerged plant organs, respiratory gases, originating from plant tissues and substrate-related respiration, vent to the atmosphere (Philippot et al. (2009); see Fig. 1.3). The soil-borne gases entering the aerenchymatic channels include CO_2 from aerobic substrate layers, and CO_2, CH_4, N_2O and H_2S diffusing from anaerobic layers. The soil-borne carbon dioxide can internally be used for photosynthesis prior to the release to the atmosphere (Raghoebarsing et al., 2005).

1.3 Why study wetlands?

We take the oxygenation of our Earth for granted which means that it is hard to imagine that there are still anaerobic organisms present from the very beginning of life on Earth (see section 1.1). Global cycles of nutrients, hydrogen and carbon all contain at some point essential anaerobic processes which recycle the substances to the aerobic world. Single biological and chemical conversions are hard to measure in soils, as all of them interact closely and depend on each other. Wetland ecosystems connect the ancient anoxic and methane-rich world with the modern oxic world and thus it is worth to analyse the wetland habitat more in detail. It is well accepted that wetland plants ventilate their submerged organs with fresh air by different mechanisms to supply themselves with oxygen for respiration (see section 1.2). Soil-borne gases enter the roots in return which are vented to the atmosphere. Some amount of the transported oxygen is released to the substrate which creates enlarged regions of partly oxic conditions close to the roots. An interface establishes between the anaerobic water-saturated soil and the rhizosphere. Specialized microorganisms are attracted which convert substances from anaerobic layers including greenhouse gases (methane, nitrous oxide) and compounds with potential phytotoxic effect (e.g. reduced iron/sulfate/nitrogen species, methanol, ethanol) to non-volatile agents (see Fig. 1.3). There is a need for studies concerning plant-internal oxygen transport and root-associated methane consumption. The reason is that wetland plants are the driving factor in wetland turnover, as they supply oxygen for the aerobic processes in the rhizosphere.

1.4 Aims of this thesis

The aim of this thesis is to connect the dynamics of plant-internal oxygen concentrations (O2conc) in wetland plants with internal carbon dioxide concentrations and methane-oxidizing communities in the rhizosphere. Plant-internal O2conc can serve as a direct indication for the ventilation status of the submerged organs and the rhizosphere. Plant-internal carbon dioxide concentrations in combination with O2conc can display the activities of below ground aerobic processes, i.e. aerobic respiration and methane oxidation. The presence of methane-oxidizing bacteria close to wetland plant roots implies that the methane consumption is located in the rhizosphere in wetland ecosystems. Thus, it is of essential importance to combine the *in-situ* assessment of O2conc with carbon dioxide concentrations inside ventilating wetland plants for accurate estimations of the global carbon budget in wetlands. The achieved results will clearly help understanding the role of plants in wetlands concerning the oxidation of the greenhouse gas methane. In order to reach this goal, subsequently described hypotheses have to be evaluated.

1.4.1 Hypothesis 1: Internal O2conc are related to diurnal dynamics of plant activity

Main ideas for this hypothesis: Photosynthesis and stomatal aperture are expected to influence the *in-situ* internal aeration in the morning hours and during the day. Additionally, it is supposed that *in-situ* plant-aeration is affected by irradiation and relative air humidity according to the above mentioned ventilation mechanisms (see section 1.2). **Predictions on positively tested hypothesis:** Oxygenation patterns will be different when comparing day and night-time oxygen runs. Characteristic transition periods will occur in early morning and evening hours when pressurization starts and ends, respectively. High temporal resolutions will be necessary in the measurement technique to clarify the involved processes more precisely.

In order to evaluate hypothesis 1, research was performed *in-situ* on *P. australis* (Cav.) Trin. ex Steud. (common reed) growing in a constructed wetland. Hypothesis 1 was tested by measuring *in-situ* daily courses of plant-internal O2conc in culms and rhizomes. The evaluation of both culm and rhizome O2conc assessments is available in section 2.1 (Faußer et al., 2016) and summarized in section 1.5.1. In this implementation, O2conc are presented in combination with the carbon dioxide results of the below addressed hypothesis 2. The work illustrated in Faußer et al., 2013 combines

rhizome O2conc together with biofilm bacteria of *P. australis* in sections 2.4 and 1.5.4 (summary).

1.4.2 Hypothesis 2: Carbon dioxide concentrations are correlated negatively to O2conc inside plant cavities

Main ideas for this hypothesis: Wetland plants are expected to supply oxygen for submerged organs and the rhizosphere. The detected plant-internal carbon dioxide is assumed to accumulate in the gas-spaces from below ground respiratory processes. **Predictions on positively tested hypothesis:** Concentrations of carbon dioxide will increase during the night, when the plant is inactive in ventilation and respiratory gases accumulate. Starting in the morning hours with the activity of the wetland plant, internal carbon dioxide concentrations will decline to minimal values while plant-internal oxygen concentrations are maximal.

Hypothesis 2 was investigated by determining the plant-internal carbon dioxide concentrations in the same *P. australis* plants which were analysed for O2conc (hypothesis 1, see above). The combined evaluations of plant-internal O2conc (hypothesis 1) and carbon dioxide concentrations (hypothesis 2) are presented in section 2.1 (Faußer et al., 2016). The summary of the results is available in section 1.5.1.

1.4.3 Hypothesis 3: There is a direct influence in the oxygenation efficiency of the rhizosphere when the water vapour pressure deficit (VPD) changes

Main ideas for this hypothesis: The mechanisms of humidity-induced convection implies that the best efficiency is achieved when the relative air humidity of the surrounding atmosphere is lower than the internal humidity (see section 1.2). During very dry periods of high VPD in the atmosphere, the stomata are expected to be narrowed to avoid increased water evaporation during photosynthesis. Thus, the active ventilation mechanisms are supposed to be influenced when the relative humidity decreases below a certain level. **Predictions on positively tested hypothesis:** The aeration efficiency will be reduced when VPD rises. Less air will be transported to the roots and less oxygen will be released into the substrate. On account of that, O2conc will decrease in rhizome and rhizosphere when VPD is increased.

Hypothesis 3 was tested by performing a greenhouse experiment with plants of *T. angustifolia* L. (narrowleafed cattail). The evaluation of the results is presented in section 2.2. The summary of the results is available in section 1.5.2.

1.4.4 Hypothesis 4: Methane-oxidizing bacteria (MOB) thrive in root-associated biofilms

Main ideas for this hypothesis: The rhizosphere is expected to serve as aerobic habitat for various communities of soil microbiota. The anaerobically produced methane is assumed to have a great impact on the composition of the microbial communities associated with wetland plant roots. It is known that MOB depend on both molecular oxygen and methane for their metabolism. **Predictions on positively tested hypothesis:** The specialized MOB will grow in the interface which is established by the oxygenation of anaerobic wetland soils through the release of molecular oxygen into the rhizosphere. Thus, wetland plants will increase the methane consumption in wetland ecosystems, in case of MOB in the rhizosphere.

Hypothesis 4 was tested by checking the root surfaces of *T. latifolia* L. (broadleafed cattail) and *P. australis* which were monitored during hypothesis 1 (see above) for bacterial communities containing MOB. The evaluation was done in the work Faußer et al. (2012), available in section 2.3 and summarized in section 1.5.3. The article Faußer et al. (2013) presents the results of the MOB survey on *P. australis* roots in terms of hypothesis 4 together with rhizome O2conc courses recorded during the testing of hypothesis 1 (see above). The combined work is illustrated in section 2.4 and summarized in section 1.5.4.

1.5 Summary of the results

1.5.1 *In-situ* diurnal dynamics of oxygen and carbon dioxide concentrations in shoots and rhizomes of *Phragmites australis* in a constructed wetland

Permanently water-saturated soils of wetlands possess stressful habitats for plants. A major characteristic of emergent wetland plants is the active ventilation of oxygen to submerged organs and the rhizosphere (see section 1.2). Although gas-flow studies were performed on excised culms and under laboratory conditions, the interest remained in the *in-situ* composition and dynamics of plant-internal gases under field conditions.

In this study, internal dynamics of oxygen were analysed *in-situ* inside common reed (*P. australis* (Cav.) Trin. ex Steud.), growing in a CW situated in Czech Republic, over several days. With the use of optical oxygen sensors (Fa. PreSens) implanted in central pith cavities of culms and vertical rhizomes, the internal gas composition was not altered during the acquisition of oxygen concentrations (O2conc). Concomitantly, internal carbon dioxide concentrations were measured via individual gas samples from the central cavities of another internode.

The recorded oxygen and carbon dioxide concentrations were highly dynamic but persistent comparing daily courses. In the morning hours, O2conc in both culms and rhizomes increased rapidly from minimal predawn values (6.5–13 %) to maximal concentrations of up to 22 % according to the start of ventilation. Though the overall similar diurnal runs, there was a time lag observed between the rise of culm and rhizome O2conc. This delay indicates pressurized gas-flow from shoots to rhizomes. Until the late afternoon, the O2conc remained high but declined exponentially with sunset towards sunrise, when conditions for pressurized ventilation ceased. The concentrations of culm-internal carbon dioxide showed an inverse pattern with lowest values during the day (0.04–0.94 %), when the plants actively ventilated. Highest CO_2 values were recorded before midday (1.55–17.5 %), when CO_2-enriched air is initially flushed from below-ground gas-channels.

The results indicate that the observed oxygen and carbon dioxide concentration correlate in a way which reflects the interdependency of oxygen supply via the wetland plant and oxygen consumption by plant respiration and rhizosphere-related biological and geochemical processes. From the nocturnal exponential decline of O2conc, a regulation mechanism is interpreted which controls the demand of oxygen in the submerged system beneath *P. australis*, in order to sustain sufficient oxic levels.

1.5.2 Relationship between oxygen saturation in rhizomes and rhizosphere oxygenation by *Typha angustifolia* L.

The ventilation efficiency of emergent wetland plants is enhanced by pressurized gas-flow through the aerenchymatic tissues (see section 1.2). The humidity condition of the surrounding air is supposed to have a major influence on the aeration mechanisms. The aim of this study was to investigate the effect of water vapour pressure deficit (VPD) on plant-mediated oxygen supply to the substrate.

Plants of *T. angustifolia* L. were analysed in a growth chamber experiment to determine the relationship between the below-ground oxygenation capacity and the VPD. Oxygen concentrations (O2conc) were measured with optical sensors inside buried horizontal rhizomes(% of air saturation), in the rhizosphere (in $\mathrm{mg\,l^{-1}}$) and the root-free soil (in $\mathrm{mg\,l^{-1}}$). The atmospheric VPD was adjusted during the measurement subsequently from maximal $0.8\,\mathrm{kPa}$ over $1.5\,\mathrm{kPa}$ to $1.9\,\mathrm{kPa}$. Conditions of maximal VPD were reached concomitantly with highest irradiation during midday.

Highest values of O2conc were found in parallel in the rhizomes and the rhizosphere. During low VPD ($0.8\,\mathrm{kPa}$), the values of O2conc were significantly highest in rhizomes (mean $42.4\,\%$ air saturation) compared with medium VPD ($1.5\,\mathrm{kPa}$; $30\,\%$) and high VPD ($1.9\,\mathrm{kPa}$; $21\,\%$). Diurnal dynamics of O2conc showed similarities between rhizomes and the rhizosphere. Values of O2conc seemed to be maximal in the morning and decrease slowly until the evening. During midday, occasional increases of O2conc were recorded. At the low VPD treatment, the rhizosphere was supplied by up to three times more oxygen (mean $1.3\,\mathrm{mg\,l^{-1}}$) compared to the medium and high VPD treatments. The root-free substrate showed O2conc of less than $0.5\,\mathrm{mg\,l^{-1}}$.

The decline of O2conc in rhizomes at rising VPD can be explained likely due to decreased ventilation efficiency as a result of reduced stomatal conductivity. The stomata are narrowed at rising VPD to avoid increased loss of water vapour through the stomatal openings. The concomitant patterns in the oxygenation of rhizomes and the rhizosphere indicate that oxygen is actively supplied below-ground by the wetland plant and released to the substrate. During the treatments, there were no significant effects of VPD on the O2conc in root-free soil. The study confirms that a decrease of internal aeration in rhizomes due to changes in VPD consequently leads to a decline of rhizosphere oxygenation.

1.5.3 Roots of the wetland plants *Typha latifolia* and *Phragmites australis* are inhabited by methanotrophic bacteria in biofilms

Water-saturated soils of wetland ecosystems are dominated by anaerobic microbial processes which produce mainly carbon dioxide and methane from the anaerobic degradation of organic matter. In the presence of wetland plants partly aerobic conditions are created in the rhizosphere as a result of oxygen penetrating from the roots (Fig. 1.3). Several aerobic processes have been identified to be associated with wetland plant roots in metabolic studies (e.g. Bodelier (2003)). The target of the present study

was to describe the root-associated biofilms with reference to the arrangement of rhizobacteria and the proportions of methane-oxidizing bacteria (MOB) in relation to root part and organic load.

Root samples from *T. latifolia* and *P. australis* were prepared for analysis of the root surfaces using light microscopy and and transmission electron microscopy. Total bacterial numbers were assessed in the rhizoplane biofilms. The included MOB were identified with an immunological probe generated from a subunit of a crucial enzyme involved in methane turnover (α-methanol dehydrogenase).

Divers bacterial communities were arranged around the roots of both plants in microbial biofilms with thicknesses of several cells. The total bacterial densities were higher around basal root sections ($\sim$53 bacteria per root exodermis cell) compared to section closer to the root tip ($\sim$36 bacteria per root cell) of *T. latifolia*. Around roots of *P. australis* similar total bacterial numbers were counted for both sampling sites (high organic load: $\sim$31; low organic load: $\sim$28).

Characteristic internal membrane systems of MOB were identified in many biofilm bacteria and was confirmed by immunological labelling. The proportions of MOB were highly variable among the different consideration types and showed highest divergence around basal root sections of *T. latifolia*. Among the root-associated microbial communities of both plant species, 34 to 43 % of the total bacteria were potentially active in methane turnover.

The high abundance of microorganisms on the roots of the two plants studied here, confirm the great impact of the oxygenation of the rhizosphere by wetland plants. Regarding the proportion of over 30 % of MOB, methane obviously is one of the main resources for bacterial growth in water-saturated soils of wetlands.

1.5.4 Internal oxygen dynamics in rhizomes of *Phragmites australis* and presence of methanotrophs in root biofilms in a constructed wetland for waste water treatment

It has been recognized that constructed wetlands (CW) planted with macrophytes efficiently clean domestic waste water in terms of biological degradation (Dušek et al., 2008). Additionally to aerobic microbial decomposition processes, methanogenic archaea produce methane in the anaerobic layers of the CW. The produced methane can be oxidized in great amounts by methanotrophic bacteria in the aerobic rhizosphere

in return. Thus methane-oxidizing bacteria (MOB) are assumed to represent a great amount of the biofilm microorganisms around the roots of *P. australis*.

In this implementation, the prior *in-situ* assessed oxygen proportions in the central pith cavity of *P. australis* rhizomes (see section 1.5.1) were combined with the analysis of the root-associated biofilms (see section 1.5.3). The total amount of microorganisms were counted on the root surface. The proportions of MOB were identified by immunological labelling in light microscopy and transmission electron microscopy.

Oxygen concentration in the rhizomes followed diurnal patterns which are attributed to the activity of internal ventilation of *P. australis*. Steep increases were recorded soon after sunrise close to ambient atmospheric levels of around $185\,\mathrm{hPa}$. This concentration was maintained during midday and decreased exponentially after sunset to lowest values overnight ($\sim 80\,\mathrm{hPa}$). Biofilms with numerous bacteria were detected on the root surfaces of which about 34 to $37\,\%$ were identified to be potential MOB.

In this context, the importance of the aerobic rhizosphere is emphasized which the wetland plant provide for degradation processes and methane oxidation. Diurnal and seasonal dynamics of plant-internal oxygen concentration may account for variation of biogeochemical processes in wetland habitats.

1.6 Conclusions

In wetland soils, organisms produce and utilize carbon compounds at all stages from the most reduced ($\mathrm{CH_4}$) to the most oxidized ($\mathrm{CO_2}$) carbon. In anaerobic layers, large organic carbon compounds are degraded to smaller substances by anaerobic bacteria using fermentation processes. Methanogenic archaea generate methane from the prior produced substrates carbon dioxide and hydrogen, methanol, formate, acetate and methylamines (see Fig. 1.3; Wrede (2011)). Special bacterial communities thrive in the interface between anaerobic and aerobic soil conditions. Mechanisms of anaerobic oxidation of methane are present, which are coupled to the reduction of sulfate or nitrate (Battistuzzi et al., 2004; Wrede, 2011). It is assumed that this methane consuming metabolism is performed by a consortium of a sulfate or nitrate-reducing bacterium and a methane-oxidizing archaeon (Raghoebarsing et al., 2006). Methanotrophic bacteria oxidize methane over several steps to carbon dioxide while methane serves as single source for carbon and energy. One step further in the carbon cycle are methylotrophic bacteria, which grow on less reduced single carbon compounds, e.g. methanol, by generating carbon dioxide through aerobic respiration.

Wetland plants enlarge the oxic-anoxic borderline by releasing oxygen to the substrate and thus enhance processes which interconnect anaerobic and aerobic pathways. Specifically, actively ventilating macrophytes strongly influence the oxidation of methane from wetlands. By supplying oxygen to submerged organs and the rhizosphere, they support the proliferation of root-associated methane-oxidizing bacteria (MOB). The analysis of metabolic processes in soils are often hindered as these habitats are dominated by microbial communities of different clades which are influenced by numerous parameters of soil composition. Oxygen plays a key role in the modern carbon cycle of our oxygenated world. This work exploits the fact that processes, which are associated with the oxygenated rhizosphere, can be assessed by the estimation of oxygen supply to the substrate and potential rates of oxygen-consuming carbon dioxide production.

The objective of this thesis was to connect *in-situ* plant-mediated oxygen transport to submerged organs with oxygen usage, namely plant-internal carbon dioxide concentrations and MOB associated with the roots. The diurnal dynamics of internal oxygen concentrations (O2conc) assessed *in-situ* inside culms and rhizomes of *P. australis* showed clear ventilation patterns during daytime (see sections 1.5.1 and 2.1). The combined article with rhizoplane MOB and rhizome-O2conc only (Faußer et al., 2013) is available in sections 1.5.4 and 2.4. High temporal dynamics at the beginning of the gas-flow in the morning hours were detected. The observation that O2conc in culms increased before O2conc in rhizomes indicated that air was actively pressurized to submerged organs. In addition, the nocturnal declines of O2conc reflected regulation mechanisms for aerobic metabolism during times with reduced oxygen supply from the plant. These results positively answer hypothesis 1 (see section 1.4.1). The correlation of O2conc to internal carbon dioxide emphasizes the strong interdependency of plant-mediated oxygen supply and oxygen-consuming metabolic processes in the rhizosphere (see sections 1.5.1 and 2.1). Plant-internal carbon dioxide concentrations were highest at the beginning of the active aeration process. The peak detected in carbon dioxide concentration resulted from accumulated respiratory gases which were initially flushed from the submerged gas-spaces. This positively answers hypothesis 2 (see section 1.4.2). Water vapour pressure deficit (VPD) showed strong influence on aeration patterns of rhizomes and rhizospheres of *T. angustifolia* (see section 1.5.2 and 2.2). This effect is assumed to be induced by stomatal narrowing in response to increasing VPD in the surrounding atmosphere. It was shown that plant-internal ventilation is also inefficient at low air humidity, which means high VPD, similarly than at high humidity (see humidity-induced convection in section1.2). From these results, hypothesis 3 can be answered positively

(see section 1.4.3). Dense biofilms were detected on the roots of *P. australis* and *T. latifolia* (Faußer et al., 2012, see sections 1.5.3 and 2.3 and Faußer et al., 2013, see sections 1.5.4 and 2.4). The proportions of MOB were higher than one third of total root-associated biofilm bacteria. This demonstrates that the rhizosphere is a habitat for microorganisms which is dominated by methane diffusing from anaerobic soil layers. Thus, hypothesis 4 can be answered positively (see section 1.4.4).

The present study confirms that the combined assessment of plant-internal oxygen and carbon dioxide concentrations can be used to estimate oxygen-consuming processes in water-saturated soil associated with wetland plants. The large number of root-associated MOB indicated the high potential of methane consumption mediated through plants in wetland ecosystems.

1.6.1 Further prospects

The *in-situ* examination of internal O2conc should be extended in regard of photosynthetic activity, stomatal conductance, seasonal changes, organic load and plant age. Several question arose which are worth regarding in future studies: It should be quantified how much of the oxygen produced during photosynthesis is ventilated belowground. It should also be analysed in what extent the transported soil-borne carbon dioxide is used in photosynthesis. The actual movements of the stomata should be assessed by measuring the stomatal conductivity during dry and humid air conditions in parallel to oxygen measurements. Another concern should be to estimate the amount of oxidized methane by e.g. calculating isotope balances. Methanogenesis favours *light*-carbon species and again the oxidation of methane should result in *light*-carbon dioxide ventilating through the wetland plants. An advantage of the optical oxygen sensors is, that longer time periods can be measured without obstructing the plant too much. Thus seasonal experiments can be conducted with the presented method. The proposed further studies can enhance the understanding of methane turnover in wetlands in consideration of increased methane levels since the industrial revolution.

2 Publications and Manuscripts

2.1 In-situ diurnal dynamics of oxygen and carbon dioxide concentrations in shoots and rhizomes of *Phragmites australis* in a constructed wetland

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- **State of submission:** Unsubmitted

After the thesis defence, an extended and revised version of this article was accepted and published in Faußer et al., 2016. This section, starting from summary and ending with conclusions and acknowledgement, is a pre-version of the article in Faußer et al., 2016. The contributions of each author can be found in the appendix, see chapter B.

2.1.1 Summary

- Wetland plants actively provide oxygen for aerobic processes in submerged tissues and the rhizosphere. The novel contribution of this article is the concomitant assessment of diurnal dynamics of oxygen and carbon dioxide concentrations under field conditions.
- Oxygen concentrations (O2conc) were monitored *in-situ* in central culm and rhizome pith cavities of common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) using optical oxygen sensors. The corresponding $\mathrm{CO_2}$ concentrations were assessed via gas samples from the culms.
- Highly dynamic diurnal courses of O2conc were recorded, which started at 6.5–$13\,\%$ in the morning, increased rapidly up to $22\,\%$ during midday and declined ex-

ponentially during the night. Internal CO_2 concentrations were high in the morning (1.55–17.5 %) and decreased (0.04–0.94 %) during the rapid increase of O2conc in the culms.

- The observed correlations between O_2 and CO_2 concentrations particularly describe the *in-situ* below ground relationship between plant-mediated oxygen supply and oxygen demand by respiration and biogeochemical processes in the rhizosphere. Furthermore, the nocturnal declining slopes of O2conc in culms and rhizomes indicated a downregulation of the demand for oxygen in the complete below ground plant-associated system.

Keywords: *aeration*, in-situ *field study*, *internal carbon dioxide*, *internal oxygen dynamics*, Phragmites australis, *wetland*

2.1.2 Introduction

In permanently water-saturated soils, as in wetland ecosystems, there are usually strongly anoxic conditions without free oxygen (Ponnamperuma, 1972). There, oxygen is a very rare resource with limited occurrence and, if present, it is immediately utilized by biological and chemical processes. Plants growing in waterlogged conditions frequently develop an enlarged gas-space continuum which runs from shoots through the extensive rhizome system to the roots (Končalová, 1990; Jackson and W. Armstrong, 1999). This aerenchyma system enables emergent wetland plants to supply submerged organs with oxygen. In return respiratory gases (e.g. CO_2) from aerobic processes as well as methane from the anaerobic sediments can ventilate to the atmosphere (W. Armstrong, 1980; W. Armstrong et al., 2000; Le Mer and Roger, 2001; Laanbroek, 2010; Maltais-Landry et al., 2009; Kayranli et al., 2010).

Wetland plants enhance their internal ventilation capacity by pressurized gas-flow through the aerenchymatic tissue (W. Armstrong and J. Armstrong, 1991; Brix et al., 1992). Pressurization is achieved by the generation of gradients of temperature (thermal-transpiration) and/or humidity (humidity-induced convection) over a porous partition. The following free flow of gas inside the aerenchymatic tissue is realized under Knudsen regime between the inner and outer atmosphere (W. Armstrong et al., 1996). Through stems and rhizomes, gas-flow in *Phragmites australis* (Cav.) Trin. ex Steud. can also occur as a result of wind blowing over the stand by which air pressure gradients (Venturi effects) are created between young and old or dead culms (J. Armstrong et al., 1992). In *P. australis* gas through-flow occurs mainly via the pith cavity, as the cortex cylinder has very low porosity (Afreen et al., 2007).

The described ventilation mechanisms can occur simultaneously, but prevailing pressurization types vary between species. Pressurization in emergent wetland macrophytes with graminoid growth type is mainly driven by humidity-induced convection (e.g. *Eleocharis spacelata* R.Br., *Typha* sp., *Juncus ingens* N.A.Wakef., *P. australis*). In parallel, pressurization efficiency is affected by other factors including light intensity, shoot height, leaf sheath area, photosynthesis and stomatal aperture (W. Armstrong and J. Armstrong, 1991; Brix et al., 1992; Brix et al., 1996; Bendix et al., 1994; Tornberg et al., 1994; Afreen et al., 2007). The combined effects of pressurization processes can result in internal overpressure between 4 and $11\,\mathrm{hPa}$ (J. Armstrong and W. Armstrong, 1990; Afreen et al., 2007), which sustains the internal oxygen concentration (O2conc) in submerged organs close to ambient levels (Brix et al., 1996; Konnerup et al., 2011).

Most roots of wetland plants develop barriers to prevent radial loss of oxygen to the soil by impeding radial diffusion in the epidermal and hypodermal cell layers (W. Armstrong et al., 2000; Soukup et al., 2002). Nonetheless, oxygen is released into the rhizosphere through porous regions at the root tips, lateral roots and through gas permeable 'windows' opposite of developing laterals (W. Armstrong et al., 2000). Thus, partly oxic conditions occur in the rhizosphere (Mainiero and Kazda, 2005) which allow oxidation of the phytotoxic compounds which characterize anaerobic soils. While aerobic soil microbes benefit from partly oxic conditions in the rhizosphere (Faußer et al., 2012), plants can utilize products of the mineralization processes by the rhizobacterial communities (Rudrappa et al., 2008). In such interactions, oxygen supplied below ground is readily consumed and soil borne respiratory gases may enter the internal gas-spaces (Colmer, 2003). Consequently, diurnal courses of oxygen and carbon dioxide can be observed in internal plant cavities in both above and below ground plant parts (Brix et al., 1996; Konnerup et al., 2011). It was reported that the internal partial pressure of $\mathrm{CO_2}$ in *Typha latifolia* leaves is up to ten times elevated compared to ambient air (Constable et al., 1992). Laboratory experiments on rice plants showed $\mathrm{CO_2}$ concentrations of above $9\,\%$ when growing in $\mathrm{CO_2}$ enriched media (Higuchi, 1982).

In this field study, diurnal dynamics of oxygen concentration (O2conc) inside rhizomes and culms were connected to plant-internal $\mathrm{CO_2}$ concentrations while testing the following hypothesis:

1. Plant-internal concentrations of $\mathrm{O_2}$ and $\mathrm{CO_2}$ are negatively correlated.
2. In the morning hours, O2conc increase rapidly and internal $\mathrm{CO_2}$ should decline concomitantly due to photosynthesis and aeration mechanisms of the culms.

3. The nocturnal values of O2conc behave dynamically, reflecting respiration of submerged organs and oxygen demand in the rhizosphere.

Mechanisms for internal ventilation and pressurization in *P. australis* and other wetland plants are well documented (Bendix et al., 1994; Brix et al., 1996; Sorrell and Brix, 2003; Colmer, 2003; Afreen et al., 2007; Konnerup et al., 2011). The contribution in this work is a detailed investigation of how the internal oxygen concentration and CO_2 concentrations in culms and rhizomes of *P. australis* vary *in-situ* under natural conditions of a CW. These measurements *in-situ* in a CW are of added value to previous experiments, as our study was conducted in the plant's natural condition and not under laboratory conditions or on excised plants.

2.1.3 Materials and Methods

Site description

This study was conducted in a subsurface horizontal flow constructed wetland (CW) located in Slavošovice in South Bohemia, Czech Republic in August 2009. Situated at 480 m above sea level, the annual average air temperature was $7.9\,^{\circ}C$ and the annual precipitation was 634 mm. Tab. 2.1 gives meteorological data at the study site for month August. The facility consisted of a pretreatment (screen, sand trap, sedimentation tank) and two parallel vegetated treatment beds each of 17 m length and 22 m width (total treatment area 374 m^2) planted with common reed (*P. australis* (Cav.) Trin. ex Steud.). The CW was created for municipal waste water treatment of 150 person equivalents and started operation in August 2001. The vegetated part of the treatment beds was filled with fine gravel (3–20 mm) while the margins at inlet and outlet (1.5 m each) consisted of coarse gravel (50–100 mm). Water and substrate properties are characterized in detail in earlier studies (Dušek et al., 2008; Picek et al., 2007). Analysis of the final effluent showed a high efficiency of the system in removing organic pollution (reduction of biological oxygen demand, BOD_5, by $82\,\%$ and chemical oxygen demand, COD, by $74\,\%$), total nitrogen ($63\,\%$), total phosphorus ($75\,\%$) and suspended solids ($52\,\%$) (Dušek et al., 2008). On average, the waste water inflow rate was regulated to $0.12 \pm 0.10\,l\,s^{-1}$ (mean $\pm$ SD), with a maximum of $1.0\,l\,s^{-1}$ during periods of extremely strong precipitation. During the vegetation season, the water level was controlled to 20–30 mm under the gravel surface.

Until this study in August 2009, the common reed successfully established a dense stand covering all available CW area. The stand was estimated as healthy according

Table 2.1: Meteorological conditions of Slavošovice constructed wetland (August 2009 and 7 years average values with s.d. are shown)

Average values		Period of measurements August 2009	7 year averages August 2002 - 2009
Air temperature [°C]		14.6 ± 5.2	14.9 ± 4.0
Water temperature [°C]	0.2 m depth	14.2 ± 1.8	14.9 ± 1.8
	0.5 m depth	14.1 ± 1.9	15.2 ± 1.9
Inflow rate [l s^{-1}]		0.18	0.30
Precipitation [mm]		69.2	45.3

to the shoot density, average shoot height and appearance (cf. Dickopp et al. (2011)). Above and below ground biomass of the common reed stand was evaluated earlier (Picek et al., 2007).

During the period of measurements, we recorded microclimatic factors inside the stand in a height of 1 m above the gravel surface. These parameters included photon flux density (PFD) over the waveband 400–700 nm, air temperature and relative air humidity (RH) (PFD sensors by LI-COR Biosciences, Lincoln NE, USA; temperature and RH sensors by Delta-T, Burwell, UK). Additionally, substrate water temperature of the reed bed was logged. PFD outside the stand was gathered in a height of 1.5 m. Stable weather conditions prevailed during diurnal oxygen recording (cf. Fig. 2.1a). In all data, time of day is presented as Central European Time (CET) without summer time adjustment.

Plant selection

Measurements in the internal gas-spaces of reed plants were performed in two regions of the southern vegetation bed of the CW: the first half closer to the inflow and the second half closer to the outflow. Two shoots of *P. australis* plants were selected for discontinuous measurements of internal oxygen concentration (O2conc) and carbon dioxide concentration each in the inflow region (In1, In2) and outflow region (Out1, Out2), respectively. The culms had similar appearance and counted 14 to 17 nodes (above the sensor implantation site, see below) in the inflow zone and 14 to 20 nodes in the outflow zone. The internodal diameters in the inflow region ranged at the bases from 11 to 11.5 mm and at the apices from 2 to 5 mm. In the outflow region the reed culms had basal diameters of 10 to 11.5 mm and apical diameters of 2.5 to 3 mm.

For intensive assessment of O2conc in culms and directly adjacent rhizomes, two additional plants (A1, A2) were assigned in the second half of the CW vegetation bed.

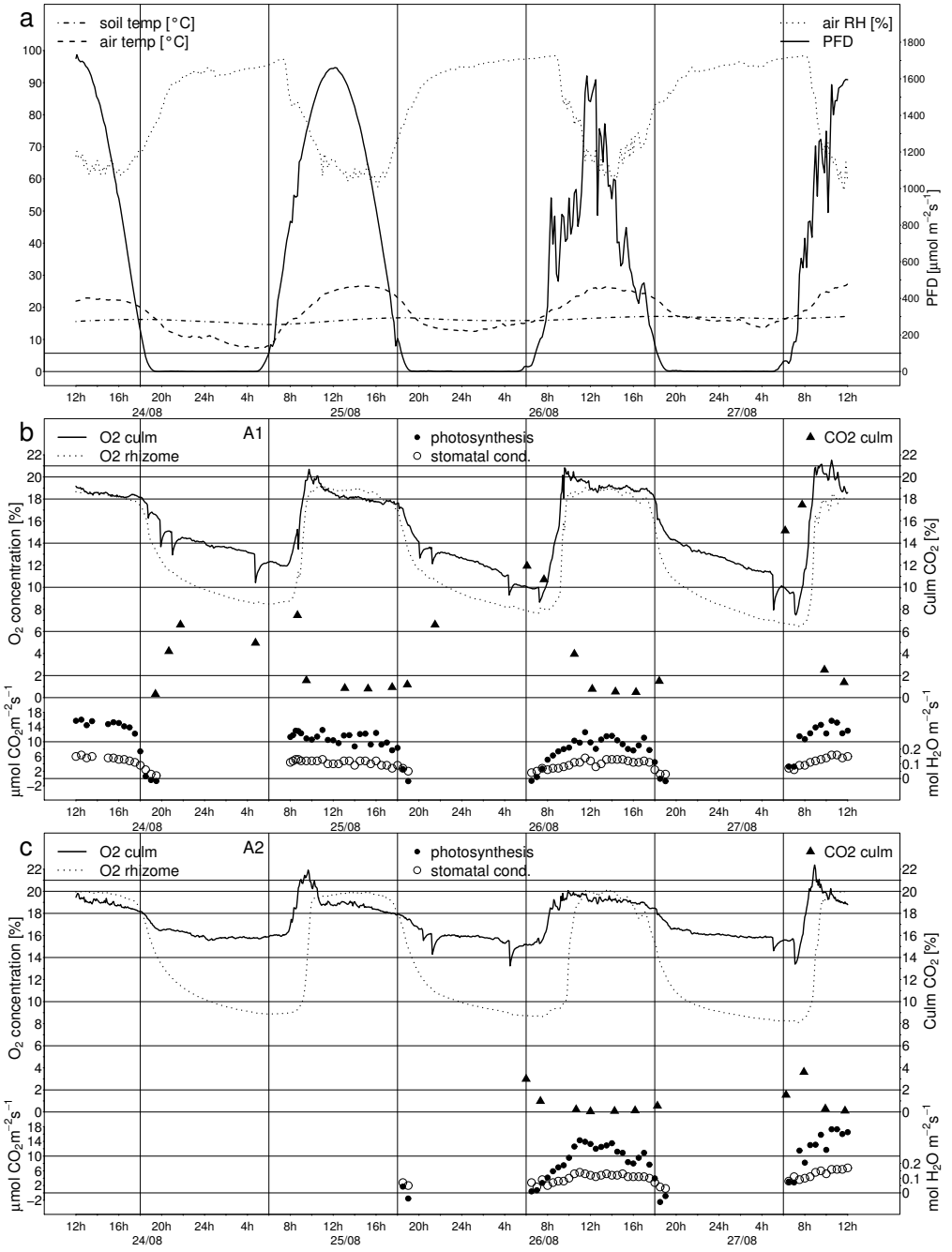

Figure 2.1: Diurnal oxygen courses in central pith cavities of culms and rhizomes of two *P. australis* plants and microclimatic conditions during measurements (a). Plants A1 (b) and A2 (c) analysed grew in a constructed wetland in Slavošovice, Czech Republic. Optodes for recording oxygen concentration were implanted about 30 cm above the substrate in shoots and in vertical rhizome ends elevating the water table. Values were recorded continuously every five minutes over 72 hours in August 2009. Time is given in CET without summer time adjustment. RH: relative air humidity; PFD: photosynthesic photon flux density.

In this region high amplitudes in internal oxygen were detected in rhizomes (Dickopp et al., 2011). The rhizome systems of the reed plants were gently excavated from leaf litter and gravel (each layer of about 50 mm in thickness). Two dead ending vertical rhizomes, with short stumps of shoots from earlier years (1–2 years old), were chosen in order to connect to directly adjacent this year's culms. The culms of A1 and A2 counted 15 nodes above the sensors and had heights of 2.1 and 2.0 m, respectively. The basal diameters of the culms were 8 and 11 mm and the apical diameters were 3.5 and 4 mm for A1 and A2, respectively. On those two plants also gas exchange measurements of top leaf blades and analysis of internal CO_2 concentration were performed.

Oxygen measurement

For the measurement of O2conc in the reed culms (A1, A2, In1, In2, Out1, Out2), optical oxygen sensors (diameter 4 mm, type PSt3, PreSens GmbH, Regensburg, Germany; Klimant, Meyer & Kühl 1995) were implanted into internodes (300 mm above ground) through drilled openings. On the plants A1 and A2, the ends of the corresponding rhizomes were cut open 25 to 55 mm above the water table (i.e. within the gravel layer) and equipped with the same type of oxygen sensor as in the culms. The generated openings in shoots and rhizomes were sealed with several layers of laboratory film (Parafilm, American National Can, Chicago, USA) subsequently after sensor implantation. Oxygen concentrations in In1, In2, Out1, Out2 were recorded by FIBOX 3 (PreSens GmbH, Regensburg, Germany) every hour on a daily course. The use of a four channel gauge (Oxy-4 mini, PreSens, Regensburg, Germany) connected to the culms and rhizomes of A1 and A2, enabled us to monitor changes in O2conc continuously every five minutes for three complete diurnal courses (August 24th to 27th 2009). During measurements, the excavated rhizome systems were covered with aluminium foil to protect them from any direct sunlight which might penetrate the reed stand. Data recorded from the sensors were recalculated to values of O2conc according to the temperatures of air (for values in culms) or in water (for values in rhizomes), which were assessed close to the implanted optodes.

Experimental limitation of oxygen supply

At midday (12:15) on the final day of O2conc measurement campaign (27/08/09), culms A1 and A2 were cut at the internodes above the oxygen sensor insertion position and

sealed with laboratory film. Oxygen measurements in culms and rhizomes continued for the next 20 hours.

Carbon dioxide measurement

Concentrations of CO_2 were measured with a mobile gas analyser based on laser absorption technology (Los Gatos Research, inc., USA, Model: 908-0007). The air samples (10 ml) from the reed culms were taken by a gas-tight syringe from the internodes above the oxygen sensor insertion position. The syringe needles were mechanically fixed and sealed with laboratory film to reduce possible leakage of air out of the reed culms. Any manipulation was limited to the sampling procedure only. The needles were closed by empty syringes or by rubber caps during each interval between two sampling times. Immediately after sampling, the air samples were analysed on the laser gas analyser. The CO_2 measurements were performed on average every two hours during daylight.

Photosynthetic gas exchange measurement

In order to observe the activity of the investigated reed plants A1 and A2, the rate of photosynthetic assimilation (A) and stomatal conductance (g_s) were assessed every half an hour on leaf blades (second from the top inflorescence) using a LI-6400 Portable Photosynthesis System (LI-COR Biosciences, Lincoln NE, USA) at ambient conditions of light and CO_2. It was observed that the leaf blades turned towards the sun during the day. Thus the measurement chamber was arranged carefully with the turning leaf blades in direction of the sunlight to gather maximum PFD on the leaves.

Evaluation and modelling of diurnal oxygen courses

Relationships between courses of O2conc and microclimatic parameters (PFD, temperature of air and water, RH), and O2conc and CO_2, were evaluated based on the Pearson's product moment correlation in R (function 'cor.test', R Development Core Team (2010)). Temporal courses of O2conc at nocturnal declines and after cutting of the culms were fitted using the exponential equation:

$$f(t) = a \cdot exp(b \cdot t) + c \tag{2.1}$$

Table 2.2: Correlations (Pearson's product moment) between O2conc in culms and rhizomes of A1 and A2 and microclimatic factors. Calculations for RH (relative air humidity), air temperature (Air T) and water temperature (Water T) included measurements 12–24 h on 24/08/09, 0–24 h on 25/08/09 and 26/08/09, and 0–12 h on 27/08/09. Correlations with photon flux density (PFD) were evaluated during sun hours: 5–19 h (i.e. 12–19 h on 24/08/09 and 5–12 h on 27/08/09.) According to t-tests all correlations had p-values of $p < 0.001$ or indicated as n.s.

r	culms ($n = 2$)				rhizomes ($n = 2$)			
	RH	Air T	Water T	PFD	RH	Air T	Water T	PFD
24/08/09	< -0.93	> 0.96	n.s.	> 0.75	< -0.98	> 0.97	n.s.	> 0.80
25/08/09	< -0.73	> 0.72	n.s.	> 0.69	< -0.96	> 0.95	> 0.50	> 0.57
26/08/09	< -0.80	> 0.89	> 0.46	> 0.66	< -0.95	> 0.93	> 0.52	> 0.68
27/08/09	< -0.70	> 0.78	> 0.55	> 0.73	< -0.97	> 0.94	> 0.80	> 0.96

where t is time in seconds and $a, b, c \in \mathbb{R}$ are the parameters to be fitted. Curve fitting was performed by minimising the least squares with Genetic Algorithms (R, package gafit, Telford (2009)). Intercept 'c' in this formula indicates the levels to which internal O2conc possibly converged. Gradients of the plots given by Eq. (2.1) are expressed by its derivative:

$$\frac{\partial f(t)}{\partial t} = a \cdot b \cdot exp(b \cdot t) \tag{2.2}$$

Slopes of O2conc are assumed to reflect the extent of oxygen consumption due to respiration in plant tissue and rhizosphere related oxygen demand or loss when main convective ventilation mechanisms are missing overnight or after culm cut-off.

2.1.4 Results

Diurnal oxygen dynamics

The internal oxygen concentration, measured *in-situ* in culms and rhizomes, showed distinct diurnal patterns as shown in detail for the intensively assessed plants A1 and A2 (Fig. 2.1). On a daily basis, culm-O2conc was correlated strongly with RH ($r \leq -0.70$; see Tab. 2.2) and air temperature ($r \geq 0.72$), and moderately with PFD ($r \geq 0.57$). Courses of rhizome-O2conc in A1 and A2 were similar to those observed in culms and were correlated strongly to RH ($r \leq -0.94$) and air temperature ($r \geq 0.93$), and moderate with surface water temperature ($r \geq 0.50$) and PFD ($r \geq 0.57$).

In the morning hours, minimum O2conc ranged in culms from 9.4 to $16.1\,\%$ and in rhizomes from 6.7 to $9.0\,\%$. Following sunrise, between 07:25 and 07:40, culm-O2conc increased steeply (Fig. 2.1, Tab. 2.3). This pattern was recurrent on each morning at PFD between 200 and 900 µmol m^{-2} s^{-1}. At the same time, RH was mainly high but started to decline and the air temperature had risen above $15\,°C$. Rhizome-O2conc

Table 2.3: Details of morning increase of oxygen air concentration (O2conc) in *P. australis* culm and rhizome pith cavities A1 and A2. The times given indicate the period between the first sharp increase in O2conc ('start time') until highest values were reached and slopes flattened ('end time'). Values of ΔO2conc show the extent of increase in O2conc. Time in [hh:mm] CET; O2conc in [%]; PFD: photon flux density in the open in [$\mu mol\,m^{-2}\,s^{-1}$]; RH: relative air humidity inside stand in [%].

date			start time	PFD	RH	O2conc	end time	PFD	RH	O2conc	ΔO2conc
24/08	A1	culm	08:10	935	81	7.9	10:45	1611	70	19.6	12
		rhizome	08:20	993	80	6.8	10:10	1503	70	18.3	12
25/08	A1	culm	07:40	716	93	11.9	09:45	1368	75	20.7	9
		rhizome	07:45	739	90	8.7	09:45	1368	75	18.4	10
	A2	culm	07:40	716	93	16.2	09:40	1347	76	21.9	6
		rhizome	08:35	956	84	9.2	09:55	1409	73	18.5	9
26/08	A1	culm	07:40	231	98	9.7	09:35	778	85	20.8	11
		rhizome	09:05	585	94	9.2	09:55	837	83	18.4	9
	A2	culm	07:40	231	98	15.9	09:55	837	83	20.1	4
		rhizome	09:35	779	84	9.8	11:25	1364	68	19.9	10
27/08	A1	culm	07:25	376	98	8.3	09:35	1203	70	21.2	13
		rhizome	08:10	569	98	7.0	08:55	1126	86	15.5	9
	A2	culm	07:25	376	98	14.3	08:55	1126	86	22.4	8
		rhizome	08:40	733	97	10.0	09:35	1203	71	19.0	9

followed this steep slope subsequently or with a delay of up to 100 minutes. Especially on 26/08/09, which was a more windy day with some clouds, the delay in rhizome-O2conc increase was more prolonged compared to the culm-O2conc increase.

Maximum O2conc values above 19.9 % were reached in the culms for periods from 70 to 120 minutes typically starting from 08:50 and at RH below 85 %. From 10:30 on during high PFD and minimum RH (around 60 %), culm-O2conc evened out at values around 18.8 %. Maximum O2conc in rhizomes were reached 45–120 minutes later than in culms. Rhizome-O2conc maintained at high levels between 18.8 and 19.9 % for up to six hours, thus oxygen concentrations were elevated in the rhizomes compared to the culms for some periods.

In the late afternoon, first rhizome-O2conc (17:00) and then culm-O2conc (18:00) began to decline rapidly. Meanwhile PFD dropped below 200 $\mu mol\,m^{-2}\,s^{-1}$ and RH increased while air temperature was still above 20 °C. The O2conc decline was more pronounced in the rhizomes than in the culms, and more distinct in culm A1 compared to culm A2 (Fig. 2.1). During the night-time, O2conc levelled to minimum values occurring before sunrise.

O2conc in the culms of the other investigated plants (In1, In2, Out1, Out2) followed similar diurnal courses as in plants A1 and A2. Minimum morning values occurred between 07:00 and 10:00 and ranged from 9.6 to 17.3 % in the inflow zone and from

to 6.1 to 19.6 % in the outflow zone. Maximum O2conc were recorded earlier in the inflow zone (09:00-12:00; 16.9 ± 4.8 % s.d. to 19.9 ± 1.9 %) than in the outflow zone (11:00-13:00; 18.6 ± 2.1 % to 20.1 ± 2.1 %).

Short-term drops in culm O2conc

During the late evening and early morning, several short-term oxygen drops (O2drop) were recorded in culms A1 and A2 (Fig. 2.1). The change in internal culm-O2conc during the O2drop ranged from -0.5 to -3.2 % (mean -1.8 ± 0.6 % s.d.). O2conc recovered to levels before the O2drop events within 10 to 85 minutes. In both culms, two to three succeeding O2drops were recorded from 19:00 to 21:00 and between 04:20 and 08:40 (25/08/09 culm A1 only). Shortly after a O2drop took place in culm A1 on 25th and 26/08/09, peaks of O2conc were detected in rhizome A1. Subsequently to an evening O2drop event, O2conc in rhizome A1 increased up to 0.1 %, and up to 0.5 % after a morning O2drop.

Gas-exchange of leaf blades

Both *Phragmites* plants A1 and A2 had positive rates of photosynthetic assimilation (A) in the leaf blades when PFD exceeded 100 µmol m^{-2} s^{-1} (typically between 07:00 and 18:00). The stomatal conductance (g_s) was constantly high during the measurement. The rise of A rates above 10 µmol (CO_2) m^{-2} s^{-1} coincided mainly with the time of culm-O2conc increase after sunrise (typically 07:30). During more cloudy and windy conditions on 26/08/09, A rates above 10 µmol (CO_2) m^{-2} s^{-1} were reached later (10:00) and for shorter periods. Rates of A above 15 µmol (CO_2) m^{-2} s^{-1} were recorded when PFD exceeded 1600 µmol m^{-2} s^{-1}. After 16:00, photosynthesis exhibited a decline parallel to O2conc in rhizomes.

Carbon dioxide in the culms

The ambient levels of CO_2 concentrations at the study site during stable atmospheric conditions reached maximum levels of 0.050 to 0.063 % around sunrise (05:00), which decreased to 0.038 % during the day (06:00 to 16:00) and subsequently increased over 0.04 %. The CO_2 concentrations in the culms showed high amplitudes that ranged from 0.32 to 17.5 % (A1; Fig. 2.1) and from 0.044 to 3.61 % (A2). Maximum internal CO_2 concentrations were recorded in the early morning hours and ranged from 11.1–12.2 %

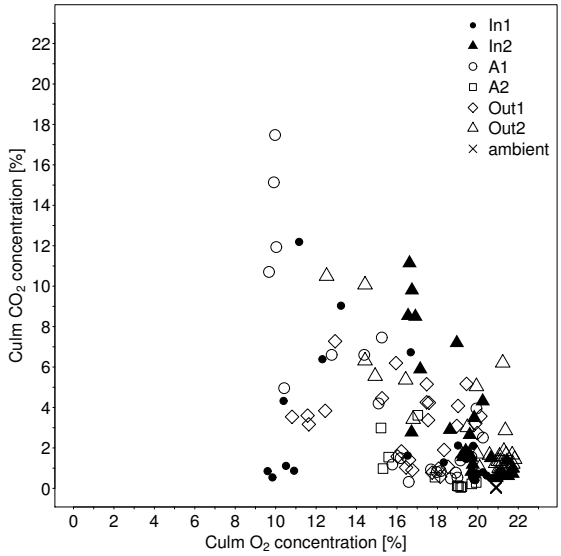

Figure 2.2: Scatterplot of culm internal oxygen concentration against internal carbon dioxide concentration in the six investigated *P. australis* plants.

(plants In1, In2), 7.28–10.5 % (Out1, Out2), 4.96–17.5 % (A1), and 1.55–3.61 % (A2), respectively. Starting with the time of O2conc increase (after 07:30), internal $\mathrm{CO_2}$ concentrations decreased to minimum values over midday of 0.32–0.94 % in culm A1 and 0.04–0.13 % in culm A2. In the inflow region and outflow region, the minimum values varied from 0.40 to 0.52 % (In1, In2) and from to 0.58 to 0.66 % (Out1, Out2), which was always higher than in A1 and A2 plants. After 18:00, internal $\mathrm{CO_2}$ concentrations increased in all culms up to the latest measurement (21:00).

Relationship between oxygen and carbon dioxide in the culms

On the whole data basis, $\mathrm{CO_2}$ concentrations were correlated negatively with O2conc in the culms (Fig. 2.2). We found overall strong correlations ($p < 0.01$) in four of the six investigated plants: A1 ($r = -0.90$), A2 ($r = -0.83$), In2 ($r = -0.80$) and Out2 ($r = -0.88$). Although the courses of $\mathrm{CO_2}$ varied according to plant and day, they were still mainly correlated negatively with O2conc on a daily basis. It should be mentioned, that on 25/08/09 $\mathrm{CO_2}$ concentrations in Out1 were constantly high (>3 %), while the observed O2conc remained below 17.8 %. On the contrary, on 27/08/09 the $\mathrm{CO_2}$ concentrations in Out1 were constantly below 2 % and the observed O2conc was constantly above 16.7 %.

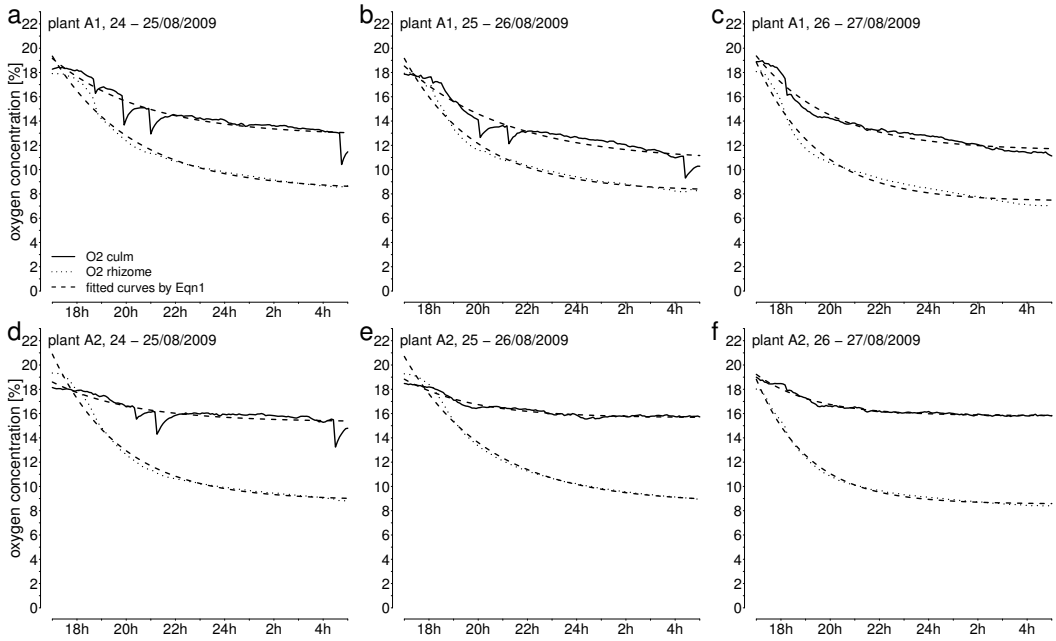

Figure 2.3: Oxygen concentration recorded overnight from 5pm (17 h) until 5am (5 h) in culms and rhizomes of *P. australis* plant A1 (a-c) and plant A2 (d-f). Exponential curves were fitted with time t in [s] against the oxygen courses with Eq. (2.1).

Modelling nocturnal oxygen courses

Nocturnal courses of O2conc in culms and rhizomes of A1 and A2 were fitted using an exponential equation Eq. (2.1). The coefficients of determination (r^2) were above 0.9 (Fig. 2.3) except culm A2 from 25 - 26/08/09 ($r^2 = 0.76$). The fitted intercept (c) was interpreted as the level of internal O2conc convergence due to the balance between oxygen demand by aerobic respiration of plant tissues, sediment organism activity, and radial oxygen loss, and oxygen supply through the aerenchyma under the non-pressurization condition. Thus O2conc approached a night-time steady-state oxygen level of about 10.7–$12.7\,\%$ in culm A1, and 15.5–$15.7\,\%$ in culm A2. In both plants, rhizome-O2conc converged to values around 6.7–$9.0\,\%$.

The initial declining slopes of O2conc varied between -1.7 and $-2.8\,\%\,\mathrm{h}^{-1}$ in culm A1 and between -1.1 and $-2.2\,\%\,\mathrm{h}^{-1}$ in culm A2. The internal rhizome-O2conc declined initially with -3.3 to $-4.6\,\%\,\mathrm{h}^{-1}$ in rhizome A1 and with -3.5 to $-4.9\,\%\,\mathrm{h}^{-1}$ in rhizome A2 twice as steep as in the corresponding culms. Rates of oxygen decrease were apparently reduced in the morning hours (05:00) to values between -0.02 and $-0.12\,\%\,\mathrm{h}^{-1}$ in culm A1, and between -0.01 to $-0.02\,\%\,\mathrm{h}^{-1}$ in culm A2. Rhizome-O2conc decrease

rates varied in the early morning between -0.04 and $-0.09\,\%\,\mathrm{h}^{-1}$ in rhizome A1 and between -0.02 and $-0.11\,\%\,\mathrm{h}^{-1}$ in rhizome A2.

Experimental limitation of oxygen supply

The analysed culms A1 and A2 were cut at the internodes above the oxygen sensor insertion on 27/08/09 at 12:15 and the stumps were given an airtight seal. O2conc in both culms and rhizomes showed a sudden and steep decrease after cutting (Fig. 2.4). Clearly, internal O2conc decreased despite high PFD and low RH. The initiated effect was similar to, but faster than, the evening decreases observed previously. Initial slopes were $-8.4\,\%\,\mathrm{h}^{-1}$ in culm A1 and $-1.5\,\%\,\mathrm{h}^{-1}$ in culm A2, and from -6.3 to $-8.4\,\%\,\mathrm{h}^{-1}$ in the rhizomes. In plant A2, O2conc dropped to values in the range of overnight levels $18\,\mathrm{h}$ after the culm was cut (culm stump: $\sim$16.7 %, rhizome: $\sim$8.4 %). About $12\,\mathrm{h}$ after the culm was cut, O2conc levels in culm stump and rhizome of plant A1 were also similar to overnight decline. However, during the following 6 hours, the internal O2conc decreased further to about $7.3\,\%$ and $5.2\,\%$ in culm stump and rhizome, respectively.

2.1.5 Discussion

Diurnal oxygen dynamics in culms and rhizomes

Dynamics of oxygen concentration inside wetland plant gas-spaces have been studied mainly in laboratory or greenhouse experiments or on excavated plants and culms (J. Armstrong and W. Armstrong, 1990; J. Armstrong et al., 1992). Studies on intact plant shoot-rhizome systems in the field are rare (Brix et al., 1996; Konnerup et al., 2011). The diurnal courses of O2conc recorded *in-situ* in this study in culms and rhizomes confirm the strong influence of RH on internal oxygen concentration, especially in rhizomes (Tab. 2.2), which aligns with previous works demonstrating that internal ventilation is mainly driven by humidity-induced convection (W. Armstrong and J. Armstrong, 1991; J. Armstrong et al., 1992; Brix et al., 1992; Brix et al., 1996; Bendix et al., 1994; Tornberg et al., 1994; Afreen et al., 2007; Dickopp et al., 2011). Close correlations with air temperature can be explained by considering that increasing air temperature resulted in decreasing RH and higher leaf temperature by which elevated internal water vapour pressures were built (J. Armstrong et al., 1992). Thus thermal transpiration supports pressurization efficiency of wetland macrophytes during hours of high PFD (Brix et al., 1996; Konnerup et al., 2011).

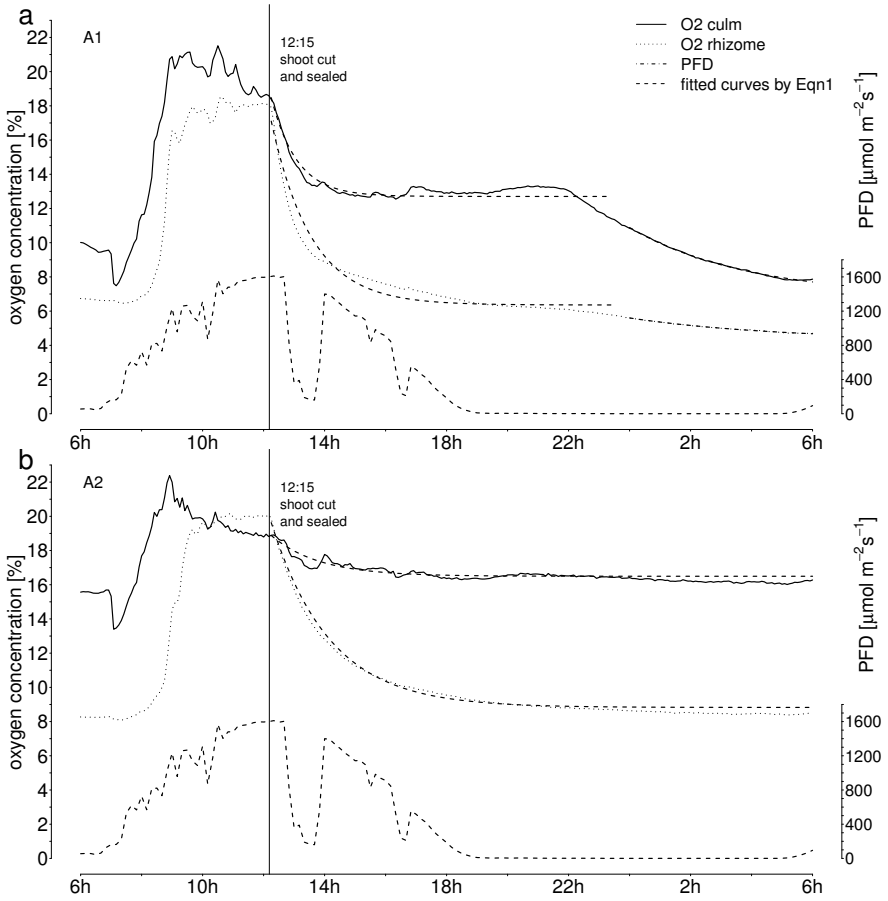

Figure 2.4: Concentrations of internal oxygen in *P. australis* culm stumps after experimental removal of the culms above the implanted oxygen sensor. The pith cavities of the remaining stumps were sealed airtight and measurements continued for 20 h overnight. Exponential curves were fitted with time (t in [s]) against the oxygen courses with Eq. (2.1); compare to Fig. 2.3.

In the work presented here, culm-O2conc obviously increased before rhizome-O2conc in the morning, at a similar time irrespective of RH or PFD. This shows that the pressurization in the culms started already before low RH conditions were reached and demonstrates the active role of the shoot tissues of *P. australis* for pressurized gas-flow (W. Armstrong and J. Armstrong, 2005; Afreen et al., 2007). The steep slopes of O2conc resulted from the widening of stomatal aperture in the early morning, and in the early evening hours from the narrowing of the stomata (cf. J. Armstrong et al. (1992) and Brix et al. (1996)).

The difference in levels of O2conc between rhizomes and culms during the middle of the day indicates that oxygen is actively pressurized from culm to rhizome gas-spaces during that period (Brix et al., 1996). In the evening rhizome-O2conc decreased as

soon as RH increased and PFD declined below $100\,\mu mol\,m^{-2}\,s^{-1}$ while culm-O2conc remained high for another hour (Fig. 2.1). Thus, throughout the measurements, there was a significant lag between the oxygen courses in culms and rhizomes, which reveals the importance of pressurized gas-flow for rhizome aeration.

During the night, micro-climatic parameters were unfavourable for pressurization mechanisms and, as a result, ventilation was dominated by diffusion or, if present, by Venturi effects (J. Armstrong et al., 1992; Afreen et al., 2007). As pressurization decreased in the evenings, internal O2conc declined rapidly. Aerobic respiration of submerged plant organs (Čížková and Bauer, 1998), radial oxygen loss (W. Armstrong et al., 2000) and biological and geochemical oxygen demand in the rhizosphere (Dušek et al., 2008) deplete oxygen reserves in the submerged organs. Brix et al. (1996) found that 5–20 % of the oxygen vented through below ground organs of *P. australis* is consumed by oxygen-dependent processes like respiration. The gradual reduction in rhizome-O2conc by as much as 50 % of the maximum values indicates very high oxygen demand in the rhizosphere of *Phragmites* plants at this study site.

The O2drops observed in the reed culms during the night can be interpreted as sudden, strong effects of convective gas-flow in the culm-rhizome-system. The oxygen optode placed at the end of the rhizome concomitantly registered a slight O2conc increase. O2drops might be also related to stomatal widening (J. Armstrong et al., 1992) and narrowing as they occurred in the early morning and evening hours (cf. Fig. 2.1). Another explanation may be that O2drops are the readings of local effects of a Venturi driven through-flow event (J. Armstrong et al., 1992). These sudden changes in internal gas concentrations indicate that there are other gas exchange patterns besides of diffusion and convective gas-flow.

Oxygen and carbon dioxide content

High diurnal variation in O2conc was accompanied by high changes in CO_2 concentration implying high negative correlations (cf. Fig. 2.1 and 2.4). Obviously, CO_2 accumulated in the pith cavities during the night when ventilation processes were inactive. The highest CO_2 values were detected at the beginning of the sharp increase in culm-O2conc, which resembles the flush of CO_2-rich air from the rhizome system when the through-flow started in the morning (Konnerup et al., 2011). Subsequently, CO_2 concentrations declined steeply, showing that the below ground organs were well supplied with fresh air. On the other hand, constantly low levels of O2conc resulted in less diurnal

variation of CO_2 (plant Out1: O_2 <17.8 %, CO_2 >3 %; 25/08/09). Conversely, constantly high O2conc resulted in lower CO_2 concentrations (plant Out1: O_2 >16.7 %, CO_2 <2 %; 27/08/09).

On average, a reduction of oxygen was replenished by an increase of CO_2. The high CO_2 levels reported here can only originate from a combination of all biological processes below ground including aerobic respiration of rhizomes, roots and rhizosphere microorganisms and anaerobic fermentation processes. Specifically in plant A1, the molar CO_2/O_2 ratio exceeded 1, which is typical for respiratory quotients of anaerobic systems (Dilly, 2003). In the plant In1, situated in the inflow zone of the CW, the CO_2 declined at low culm-O2conc, however. In this part of the CW with high organic load, Dickopp et al. (2011) observed low diurnal oxygen dynamics and argued that below ground plant organs might be less permeable in order to prevent diffusion of toxic fermentation products.

Oxygen consumption and oxygen supply

Steep slopes of O2conc decline in rhizomes began to decelerate when oxygen concentrations dropped below 14.6 %, and later oxygen levels remained above 6.7 % (cf. Fig. 2.1 and 2.2). In horizontal rhizomes of the same part of the studied CW the mean minimum rhizome-O2conc recorded was around 5.8 ± 1.5 % s.d. (Dickopp et al., 2011). Minimum values in the rhizomes may represent the balance between oxygen supply by diffusion and oxygen demand by submerged plant organs and rhizosphere-related aerobic processes. Also barriers against radial oxygen loss were presumably more effective at lower oxygen gradients between inner gas lacunae and the surrounding anoxic environment (W. Armstrong et al., 2000). Further, plant organs and rhizosphere microorganisms reduced their aerobic respiration at low oxygen availability. Our data indicates that substrate-related respiration will already slow down when air with around 14.6 % O2conc is supplied through the pith cavities. Hypoxic internal conditions in the submerged organs can be expected when O2conc converged to levels less than 6.3 % in culms and 4.2 % in rhizomes (W. Armstrong et al., 2009; Gupta et al., 2009). As oxygen is transported inside the roots by diffusion only, it can be assumed that root internal oxygen concentrations reach the range of the critical oxygen pressure (i.e. 45 hPa or <3.1 % O2conc) which causes a downregulation of respiration (Berry and Norris Jr., 1949; Čížková and Bauer, 1998; W. Armstrong et al., 2000; Gupta et al., 2009; Zabalza et al., 2009; W. Armstrong et al., 2009). The mechanism of oxygen conservation was modelled for respiring root segments by W. Armstrong and Beckett (2011), by which the

spread of an anoxic core through the root diameter is described when downregulation occurs.

The excision of the investigated culms at midday, when micro-climatic conditions were generally favourable for pressurization, showed the dependence of oxygen on pressurized gas-flow from upper plant parts (Fig. 2.4). Removing major parts of the above ground plant organs caused O2conc to drop sharply, although the remaining culm stumps had $30\,\mathrm{cm}$ of functional leaf sheath area left. Differences in below ground plant organization are presumably the main reason why different levels of internal O2conc were observed in the two individuals analysed for a $18\,\mathrm{h}$ period after the culms were cut. The reduction of oxygen levels to about $50\,\%$ of predawn values indicates that the rhizome system connected to plant A1 was highly dependent on the experimentally removed shoot. Also the high $\mathrm{CO_2}$ concentrations of up to $18\,\%$ pointed out that there is a large respiratory turnover in the submerged organs and rhizosphere of plant A1. $18\,\mathrm{h}$ after the culm removal of plant A2, the O2conc levels in culm stump and rhizome approached similar values to previous morning concentrations. This indicated that plant A2 was connected to an extended rhizome system which had good ventilation properties, and could compensate better for the loss of the current culm A2 (cf. Čížková and Lukavská (1999)).

2.1.6 Conclusions

Common reed (*P. australis*) has a high capacity to actively ventilate oxygen rich air to submerged organs and to the rhizosphere. Our continuous *in-situ* measurements of internal oxygen concentration revealed for the first time sudden changes in internal gas concentrations and indicate also other gas exchange patterns besides of diffusion and pressurized gas-flow. The relationship between internal oxygen and carbon dioxide concentrations measured *in-situ* reflects the way that respiration of submerged organs and biogeochemical processes in the rhizosphere feed on internal oxygen reserves, while $\mathrm{CO_2}$ accumulates in the internal gas-spaces. During late night, steady-state oxygen levels in culms and rhizomes indicate a balance between below ground oxygen demand and oxygen supply via the aerenchyma. Further, we conclude that the observed slopes of declining O2conc in culms and rhizomes during the night and in particular after culm-cut notably demonstrate the regulation of the oxygen consumption rate for the complete below ground plant-associated system.

2.1.7 Acknowledgement

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2.2 Relationship between oxygen saturation in rhizomes and rhizosphere oxygenation by *Typha angustifolia* L.

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- **State of submission:** Unsubmitted.

2.2.1 Abstract

A growth chamber experiment was conducted to determine the relationship between internal oxygen saturation of *Typha angustifolia* L. rhizomes and substrate oxygenation in the course of a day in comparison to root-free soil. During measurements days' maxima of atmospheric vapour pressure deficit (VPD) were altered in three steps from $0.8\,\mathrm{kPa}$ over $1.5\,\mathrm{kPa}$ to $1.9\,\mathrm{kPa}$. Our results indicate a positive correlation between oxygen saturation in rhizomes and oxygen concentration in substrate close to roots (rhizosphere). Significantly highest values of oxygen saturation in rhizomes to a mean of $42.4\,\%$ were detected at low VPD ($0.8\,\mathrm{kPa}$) around midday (highest PFD $900\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$). At medium VPD of $1.5\,\mathrm{kPa}$ a short-time increase of oxygen saturation ($30\,\%$) was obvious, whereas at high VPD ($1.9\,\mathrm{kPa}$) oxygen in rhizomes stayed around $21\,\%$ over time. Daily dynamical behaviour of oxygen concentration in the rhizosphere ran nearly parallel with rhizome oxygen saturation, bearing highest values in the early mornings. Oxygenation in the rhizosphere (mean of $1.3\,\mathrm{mg\,l^{-1}}$ at a VPD of $0.8\,\mathrm{kPa}$) increased two to three times compared to root-free soil (less than $0.5\,\mathrm{mg\,l^{-1}}$). When raising VPD we recorded a decrease in substrate oxygenation likely due to lower oxygen saturations in the plant rhizomes. The findings support a temporal pattern of rhizosphere oxygenation generated through radial oxygen loss (ROL), as there were close correlations between oxygenation of rhizomes and rhizospheres but no significant changes of oxygen concentrations in root-free soil. It can be assumed that atmospheric VPD causes changes in stomata aperture that overrules humidity induced pressurization. This may lead to a decrease of internal aeration in rhizomes and consequently to a decline of rhizosphere oxygenation.

Keywords: *Internal aeration*, *Radial oxygen loss* (ROL), *VPD*, Typha angustifolia

2.2.2 Introduction

Wetland ecosystems are for many plants unfavourable habitats due to oxygen deficiency and reductive soil conditions (J. Armstrong et al., 1996; Brix et al., 1996). With increasing water content the gases are replaced from soil pores and the remaining oxygen in the soil is consumed by respiration and chemical processes. Oxygen supply from the atmosphere is prevented due to lowered gas diffusion in waterlogged soils. Hypoxic or anoxic soil conditions arise within a few hours or days after water saturation (Drew and Lynch, 1980; Feng et al., 2002; Ponnamperuma, 1972).

One of the major adaptations allowing wetland plants to grow in waterlogged soils, is the development of aerenchyma. Aerenchyma facilitates oxygen transport to below ground organs (W. Armstrong, 1980) thereby maintaining aerobic root respiration. In addition to transporting oxygen to submersed organs, wetland plants are able to release oxygen into soil in a process known as 'radial oxygen loss' (ROL); (Sorrell, 1999). Oxygen emitted by ROL differs in respect to plant species in the amount and in the location of the release and occurs often at lateral roots and close to the root tips (Colmer, 2003; Conlin and Crowder, 1989). The appearance of molecular oxygen in waterlogged soil is a net balance of oxygen supply from the root surface and consumption due to chemical bonding and respiration by plant roots and rhizomes and soil biota. As oxygen diffusion rates in waterlogged soils are restricted, free molecular oxygen in the substrate only occurs when ROL is high enough to exceed oxygen consumption.

Several studies have shown that aeration of the roots and rhizomes is supported by pressurized ventilation. Pressurized ventilation has been found in species with floating leaves (Dacey, 1980) and in several emergent species including *Phragmites australis* (Cav.) Trin. ex Steud., *Typha angustifolia* L. and *Typha latifolia* L. (Brix et al., 1992; Brix et al., 1996; Bendix et al., 1994). The major mechanisms responsible for pressurized ventilation are thermal transpiration and humidity-induced diffusion. Thermal transpiration is driven by temperature gradients between ambient temperature and internal leaf temperature (Dacey, 1981; Große, 1996). Humidity induced pressurization is generated by a humidity gradient between ambient air and water vapour saturated air in the intercellular space (J. Armstrong and W. Armstrong, 1990). The wetland species *T. angustifolia* is known for pressurized ventilation (Bendix et al., 1994) where air enters the middle-aged leaves and is convected down to the rhizomes and roots, where it can

leak as ROL. Gases from the rooting zone can also be vented back to the atmosphere through old or damaged leaves or even other shoots via horizontal rhizomes (Tornberg et al., 1994).

Atmospheric factors including solar radiation, air temperature and relative humidity generate temporal changes of ROL in several wetland species (e.g. Christensen et al. (1994) and Sorrell (1999)). These studies have investigated ROL and its connection to climatic conditions including air temperature, relative humidity and radiation along with external oxygen demand in soil. However, the relationship between internal aeration of rhizomes and roots, ROL and enhanced oxygen concentration in the flooded soil remains to be examined and understood. The aims of this study were to examine the effects of vapour pressure deficit (VPD) and photon flux density (PFD) on the oxygen saturation in rhizomes of *T. angustifolia* and to determine the relationship between internal aeration, ROL and soil oxygen.

2.2.3 Materials and Methods

Experimental design

Plants of *T. angustifolia* were grown from rhizomes and cultivated under waterlogged condition in the Botanical Garden at the Ulm University. Eight horizontal rhizomes with lengths of 180–220 mm which terminated with one shoot at each end, thus forming U-shaped aeration systems, were selected for experiments. The shoots were between 1.6 and 2.4 m high and submerged organs (rhizomes and roots) held an average ratio of 0.6 of the plants' total biomasses. Injuries of selected rhizomes were avoided in order to prevent callus formation and thus a blockage of ventilation in rhizomes. The rhizomes were planted in glass vessels (25 cm $\times$ 25 cm $\times$ 30 cm) filled 10 cm deep with a mixture of sand and organic-rich soil. The water level was kept 10 cm above the substrate surface thereby facilitating waterlogged conditions throughout the study. The experiment was performed in a phytotrone (Vötsch Industrietechnik GmbH, Balingen, Germany) which allows to control temperature, relative humidity and photon flux density (PFD). One week prior to the start of the experiment, the plants were moved in the phytotrone to adapt to the chamber conditions. To simulate diurnal weather conditions a daily course was programmed with the temperature ranging between 15–25 °C (Fig. 2.5a). In total, three different diurnal treatments were applied, 11 h long on two following days each. The same light regime was used for each of the three treatments (Fig. 2.5). The light (metal halide lamps HPI-T400 W, Royal Philips Electronics, Netherlands) was switched

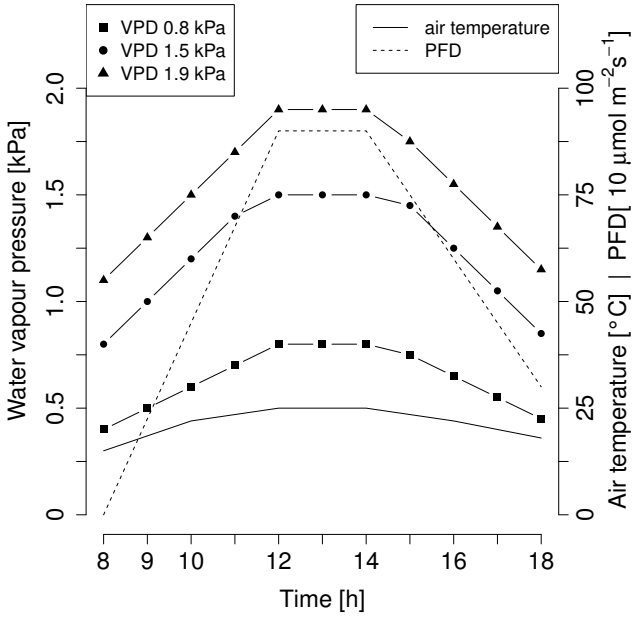

Figure 2.5: Course of time for temperature, PFD and VPD with maximum values for the three treatments respectively.

on at 8:00 and the maximum PFD of $900\,\mu\text{mol photons}\,m^{-2}\,s^{-1}$ was reached at 12:00. After two hours of maximum, the PFD decreased to $300\,\mu\text{mol photons}\,m^{-2}\,s^{-1}$ before the lamps were switched off at 19:00. The three treatments differed in the relative air humidity consisting of $75\,\%$, $55\,\%$ and $40\,\%$. Due to identical maximum air temperature a corresponding maximum VPD of $0.8\,\text{kPa}$ (treatment 1), $1.5\,\text{kPa}$ (treatment 2) and $1.9\,\text{kPa}$ (treatment 3) was reached in each treatment, respectively. Maximum VPD was reached for about two hours between 12:00 and 14:00 (Fig. 2.5). The treatments were referred to as VPD $0.8\,\text{kPa}$, VPD $1.5\,\text{kPa}$ and VPD $1.9\,\text{kPa}$, and were conducted consecutively with increasing VPDmax from June 25/26th, July 2/3th to July 6/7th 2005, respectively.

Oxygen measurements

Oxygen measurements were performed using optical sensors (PreSens, Regensburg, Germany). The system consists of a light source, an optical fiber as signal transducer (plastic or glass fiber), a photodetector and the optical sensor (indicator immobilized in a solid matrix). The sensors have neither cross sensitivity for carbon dioxide, hydrogen sulfide, ammonia, pH nor any ionic species like sulfide, sulfate or chloride (Klimant et

al., 1995). The sensors were finally calibrated and adjusted before the plants were put into the vessels. Two different types of optical sensors were used. Glass fiber-based microoptodes were implanted into the rhizomes taking advantage of the thin tips ($<50\,\mu m$) of microoptodes (Microx TX). The microoptodes were injected approximately $1–2\,mm$ into the rhizome and fixed. These fine optodes are susceptible to mechanical damage, which is then indicated directly on the optical service gauge. Such failure occurred in two plants and led to missing values for the measurement at run 2 of the VPD $0.8\,kPa$ treatment in plant S3 and at both runs of the $1.9\,kPa$ treatment in plant S8.

Soil oxygen concentration was assessed by plastic fiber optodes (Fibox with a diameter of $2\,mm$), where the sensor is additionally protected by a steel tube. These optodes were placed in between the fine basal lateral roots originating from the rhizome measured. Oxygen concentration in the root-free soil was obtained from sensors placed on distant parts of the growing containers, obviously without plant roots.

In the gaseous system of the rhizome lacunar oxygen values were expressed in percent air saturation. Oxygen in the substrate was assumed as completely dissolved and the values were given in $mg\,l^{-1}$. The oxygen measurements were temperature compensated with a Pt 100 temperature sensor connected directly to the oxygen measuring unit (PreSens, Regensburg, Germany) close to the implanted optodes. Oxygen measurements on all optodes were done approximately every 60 minutes for the three treatments during the light period in each of their two diurnal courses.

Statistical analysis

Statistical tests were performed using Statistica Rel. 6.1 (StatSoft Inc., Tulsa, Okla, USA) and graphics were built with R v. 2.8.1 (R Development Core Team, 2010). Differences in oxygen saturation in rhizomes and oxygen concentration in substrate between the diurnal courses were determined using repeated measures ANOVA. Prior to final evaluation, we tested wheter there are significant differences between the two runs of the same VPDmax. As for each treatment both runs were similar, data of the same VPDmax were pooled. To account for scourged distribution, the data were $log10(x)$ transformed, where x is the respective oxygen value. Linear regression was used to assess the relationship between internal oxygen saturation of the rhizomes and rhizosphere oxygen concentration, as well as between rhizosphere and root-free soil oxygen concentrations.

Table 2.4: Means of oxygen values during the three treatments in rhizome, rhizosphere and root-free soil. As both runs were similar, data of each treatment were pooled. Time intervals show means before, during and after high radiation period. Different letters after the means indicate significant differences at $p < 0.05$ level in TukeyHSD test.

Oxygen content	VPDmax [kPa]	Time interval 8-11 h	12-14 h	15-18 h	Daily mean
Oxygen saturation in rhizomes [%]	0.8	27.4	35 b	24.7	28.5
	1.5	22.9	24.1 a	20.5	23.7
	1.9	21.8	21.7 a	18.8	20.6
Oxygen saturation in rhizosphere [mg l^{-1}]	0.8	1.20 b	1.09 b	0.99 b	1.09
	1.5	0.57 a	0.44 a	0.24 a	0.42
	1.9	0.46 a	0.32 a	0.22 a	0.34
Oxygen saturation in root-free soil [mg l^{-1}]	0.8	0.30	0.36	0.30	0.32
	1.5	0.25 a	0.30	0.24	0.26
	1.9	0.28 a	0.23	0.23	0.25

2.2.4 Results

The measured oxygen saturations in rhizomes and concentrations in rhizospheres and root-free soil are illustrated in Tab. 2.4 and Fig. 2.6. In the rhizomes the mean oxygen saturation ranged between 20–30 % at the beginning of each treatment (8-11h), displaying slightly higher values at lower VPD. Figure 2a illustrates that at low VPDmax corresponding to 0.8 kPa, oxygen saturation increased gradually up to 42.4 ± 10.9 % ($\pm$ SE) at highest PFD over midday (14 h). In individual plants oxygen saturation values in the rhizome even exceeded 100 %. After this maximum the saturation decreased significantly within two hours to 18.1 ± 4.6 % and stayed at levels around 25 % thereafter. During the VPDmax 1.5 kPa treatment, only a short-time increase up to 30.8 ± 6.5 % was found at the beginning of maximum light intensity (12 h). Thereafter the oxygen saturation in rhizomes decreased to 21.9 ± 5.1 % within one hour and remained at approximately this level until the end of the day. Oxygen saturation in rhizomes at VPDmax 1.9 kPa didn't change significantly within the treatment's runs. However, a marginal increase of the oxygen saturation in the rhizomes from 20.5 ± 5.9 % up to 25.3 ± 6.9 % was recorded at 11 h. Oxygen values decreased to saturation levels around 20 % before the VPD reached the maximum of 1.9 kPa during high PFD period.

Similarly to oxygen saturation in rhizomes, the oxygen concentration in the rhizosphere showed highest values at lowest VPD of 0.8 kPa (Fig. 2.6b). Levels of oxygen concentrations at VPD 1.5 kPa and 1.9 kPa were less than half the values of the treatment with VPDmax 0.8 kPa (Tab. 2.4) and were highest in the early morning while declining gradually over daytime. During the run with VPDmax 0.8 kPa, there was a moderate decline of mean oxygen concentration in the rhizosphere from around 1.25 mg l^{-1} down

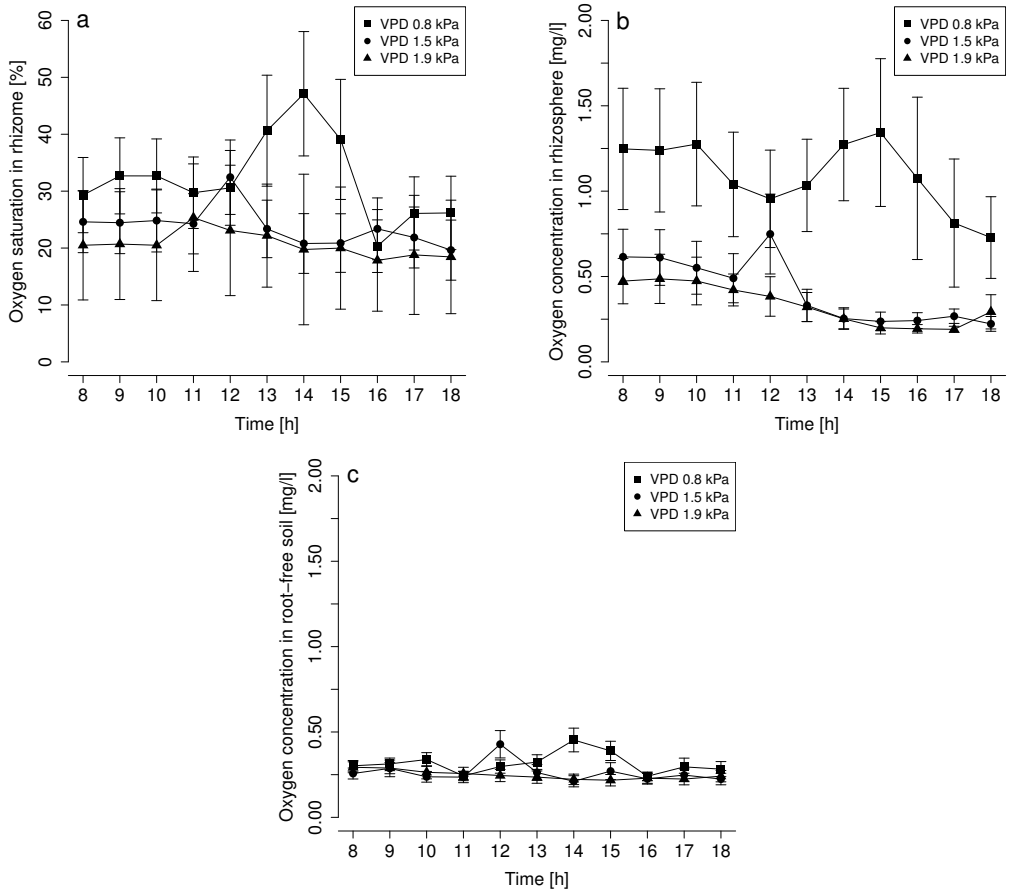

Figure 2.6: Oxygen saturation (mean $\pm$ SE) in rhizomes (a) and oxygen concentration (mean $\pm$ SE) in rhizosphere (b) and root-free soil (c) during the three treatments. Means were calculated over the eight replicates and two days of measurement ($n = 2 \times 8$). Note different y-axis scaling in (a) and (b), (c), respectively.

to $0.96 \pm 0.29\,\mathrm{mg\,l^{-1}}$ over the period of high radiation (11–13 h). Thereafter the oxygen concentrations recovered up to $1.34 \pm 0.43\,\mathrm{mg\,l^{-1}}$ (15 h) before they got almost halved in the later hours to $0.73 \pm 0.24\,\mathrm{mg\,l^{-1}}$ (18 h). Mean oxygen concentrations at the beginning of the treatment with VPDmax 1.5 kPa ranged around $0.57\,\mathrm{mg\,l^{-1}}$ and were followed by a short-time increase up to $0.75 \pm 0.23\,\mathrm{mg\,l^{-1}}$ at the onset of high radiation period (12 h). After this peak the mean rhizosphere oxygen concentration declined to about $0.25\,\mathrm{mg\,l^{-1}}$ and stayed around there until the end of the treatment. At the highest VPDmax – treatment (1.9 kPa) values of oxygen concentration in the rhizospheres declined continuously from $0.47 \pm 0.13\,\mathrm{mg\,l^{-1}}$ (8 h) to $0.19 \pm 0.02\,\mathrm{mg\,l^{-1}}$ (17 h). Oxygen concentrations in root-free soil averaged out to $0.28\,\mathrm{mg\,l^{-1}}$ (Fig. 2.6c). At VPDmax - treatments of 0.8 kPa and 1.5 kPa detected values for oxygen increased temporarily to

Table 2.5: Results of repeated measures ANOVA for oxygen saturation in rhizomes and oxygen concentration in substrate. Categorial 'position' refers to rhizosphere and root-free soil. N rhizome: 3 (VPD) $\times$ 8 (plants) $\times$ 22 (2 days with 11 measurements each) – 35 (missing values) $=$ 493 and N subtrate: 2 (rhizosphere and root-free soil) $\times 3 \times 8 \times 22 = 1056$. Effects were tested post-hoc with TukeyHSD.

Parameter	Effect	df	F-ratio	p-value
Oxygen saturation in rhizome	VPD	2	0.06	n.s.
	Time	10	7.92	< 0.001
	VPD x Time	20	2.33	< 0.01
Oxygen concentration in substrate	VPD	2	5.48	< 0.01
	Position	1	7.43	< 0.01
	Time	10	18.57	< 0.001
	VPD x Position	10	0.8	n.s.
	VPD x Time	20	7.17	< 0.001
	Position x Time	10	6.74	< 0.001
	VPD x Position x Time	20	1.1	n.s.

about $0.4\,\mathrm{mg\,l^{-1}}$ during the high PFD period of the daily courses. In treatment with VPDmax $1.9\,\mathrm{kPa}$ free oxygen in soil stayed constantly around $0.25\,\mathrm{mg\,l^{-1}}$.

Repeated measures ANOVA for oxygen saturation in the rhizomes revealed, that VPD treatments had no significant effect on rhizome oxygenation (Tab. 2.5, VPD). However, oxygen saturation in the rhizomes was most significantly influenced by time. Moreover, the significant VPD x Time interaction confirmed, that the VPD treatment had an effect on the daily course of oxygen in *T. latifolia*'s rhizomes.

Statistical analysis showed a significant influence of VPD treatments on oxygen concentration in the rhizosphere (TukeyHSD: $p < 0.05$) additionally to the effect of time (Tab. 2.4 and 2.5). In root-free soil the values of free oxygen were significantly lower compared with the rhizosphere (Position) and independently from the VPD treatments (interaction VPD x Position not significant), as it is obvious from Tab. 2.4 and Fig. 2.6c. Also oxygen concentration in the substrate showed significant differences within the daily courses (Time). Moreover, the influence of time during the measurements proved significant for rhizosphere oxygenation at different VPD treatments (VPD x Time) as well as when rhizosphere was compared with root-free soil (Position x Time).

Relationship between oxygen saturation in rhizomes and oxygen concentration in the rhizosphere during the different VPD treatment are displayed in Fig. 2.7. Both days of measurements are illustrated separately and show similar characteristics for each treatment. At all treatments there were highly significant correlations between rhizome and rhizosphere oxygenation, except for first run of VPDmax $1.9\,\mathrm{kPa}$ treatment (Fig 2.7c). Moreover, the negative intercept for all linear regressions proved that a threshold oxygen saturation in the rhizomes must be reached before free oxygen can be

detected in the rhizosphere and thus is higher than the oxygen consumption rate by microorganisms. Coefficients of determination differed between the VPD treatments and decreased for pooled data from the $0.8\,\mathrm{kPa}$ to the $1.9\,\mathrm{kPa}$ treatment. Close positive correlations ($r^2 > 0.59$) were found for six of the analysed plants in the VPDmax $0.8\,\mathrm{kPa}$ treatment (Fig. 2.7a, b: S1, S3, S4, S6, S7, S8). Four of those plants showed even closer correlations during treatment with VPDmax $1.5\,\mathrm{kPa}$ ($r^2 > 0.7$, Fig. 2.7c, d: S1, S3, S4, S6). Whereas during the VPDmax $1.9\,\mathrm{kPa}$ treatment oxygenation between rhizome and rhizosphere correlated only slightly in one of those ($r^2 = 0.2$, Fig. 2.7e, f: S4).

2.2.5 Discussion

Under low VPD and high PFD the internal aeration of *T. angustifolia* was very effective. The oxygen saturation in the aerenchyma system in the rhizomes under these conditions reached or even exceeded atmospheric oxygen saturation. Similarly high levels of atmospheric oxygen saturation in the intercellular lacunar system of stems were found for *Phragmites australis* Cav. Trin. Ex Steud. by J. Armstrong and W. Armstrong (1990) and W. Armstrong et al. (2000) and for *Eleocharis sphacelata* R. Br. by Sorrell and Tanner (2000). Oxygen saturations in rhizomes exceeding $100\,\%$ indicate that additional oxygen from photosynthesis was supplied in the leaf lacunar system and vented down to the below ground organs. Previous investigations on *P. australis* provided no evidence of photosynthetic oxygen aerating intercellular systems of the culms and rhizomes (Brix, 1988). In field experiments oxygen saturations of gases venting through syringes into living stems of *P. australis* were marginally greater than atmospheric oxygen saturation (W. Armstrong and J. Armstrong, 1991), whereas photosynthetic enhancement of radial oxygen loss has been recorded for submerged species (Sand-Jensen et al., 1982; Kemp and L. Murray, 1986). The results of the present study suggest an additional oxygen supply into the intercellular lacunae and aerenchyma of *T. angustifolia* through photosynthesis. This is visible by means of increasing oxygenation of both rhizome and rhizosphere during the period of high radiation in treatment 1 with VPDmax $0.8\,\mathrm{kPa}$. During all three of the 2-day VPD treatments, even under high atmospheric VPD ($1.9\,\mathrm{kPa}$), the oxygen saturation in rhizomes never declined below $20\,\%$ of air saturation. Therefore it can be assumed that *T. angustifolia* was capable of maintaining aerobic respiration in the below ground organs even over night-time.

Previous studies reported the influence of atmospheric condition on internal aeration (J. Armstrong and W. Armstrong, 1990; Brix, 1988). The static pressures in the lacunar

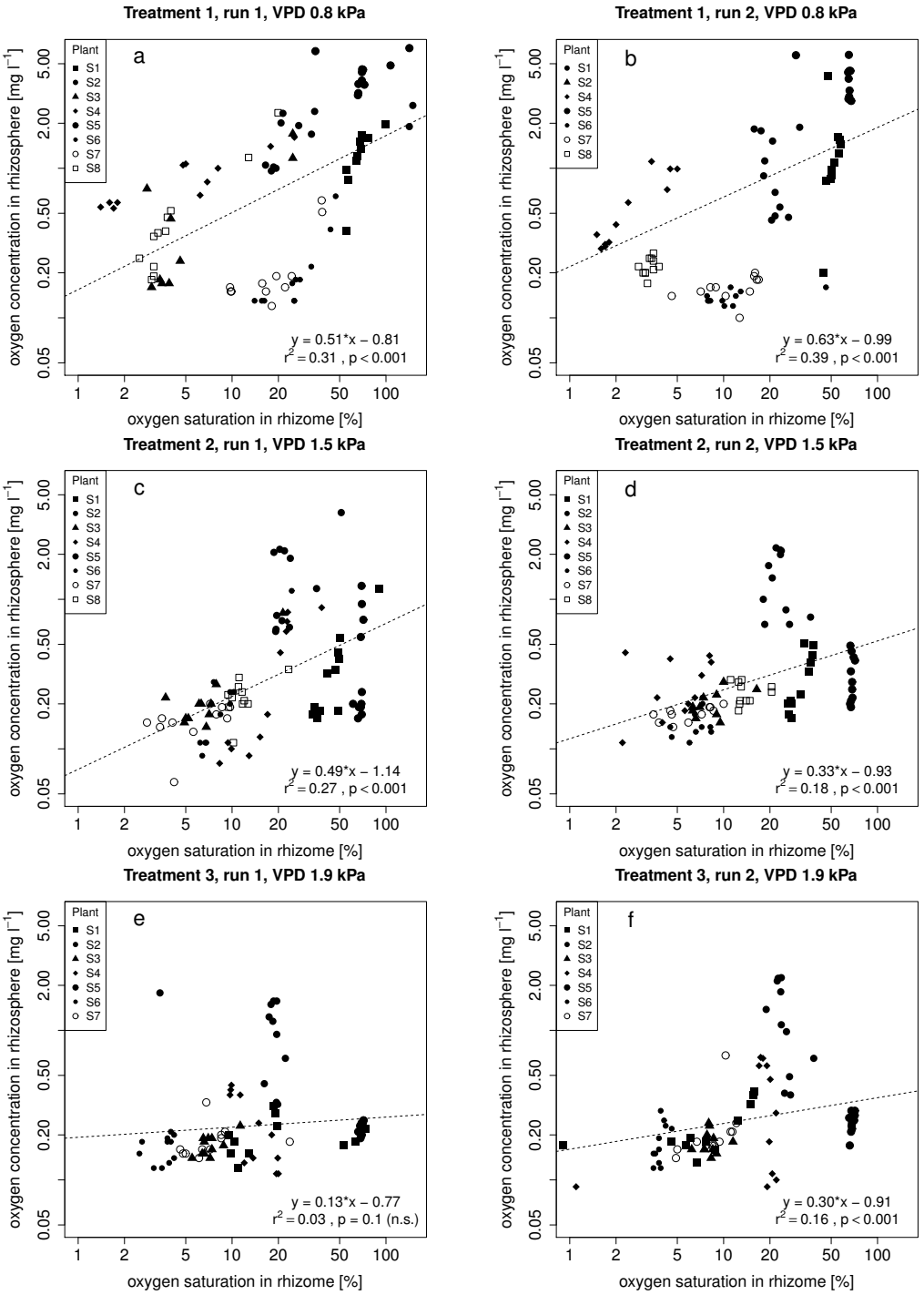

Figure 2.7: Relationship between oxygen saturation in rhizomes and oxygen concentration in the rhizosphere of single plants during the three different treatments on first day (a-c) and on second day (d-f). Because of sensor defect there is no data for plant S8 at VPD 1.9 kPa (treatment 3) and for plant S3 at VPD 0.8 kPa (treatment 1) at run 2.

system and convective flows in *P. australis* increased markedly in response to light and raised the oxygen partial pressure in rhizomes up to almost atmospheric pressure. The present experiment cannot reveal whether a through flow convection occurred as internal pressures and flows were not measured. According to previous investigations with *T. angustifolia* by Bendix et al. (1994) it can be assumed that convective flow took place. The authors accounted humidity-induced pressurization as major ventilation mechanisms for *T. angustifolia*. The highest oxygen saturations in rhizomes recorded during low VPD and high PFD in the present study indicate that thermal transpiration had an higher effect than previously reported. The mechanisms of thermal transpiration and humidity-induced pressurization are highly interdependent. Both pressurization mechanisms are responsible for internal ventilation in emergent wetland plants. Higher air temperatures together with higher VPD causes transpiration increases and thus provides the precondition for humidity-induced pressurization (J. Armstrong and W. Armstrong, 1990). However, the internal aeration in *T. angustifolia* was effective at low humidity gradients between ambient and lacunar air at treatment with VPD 0.8 kPa.

The pressurization efficiency is known to be positively correlated to stomatal conductance (White and Ganf, 2000). A lower oxygen transfer to the below ground organs under high VPD presumably resulted from changed stomatal aperture. In most terrestrial plants, the stomatal conductance decreases with an increase of atmospheric VPD in order to prevent water loss. In wetland species, the response of stomatal conductance is similar, but responses may not be so evident (Eisele, 2003; Jones and Muthuri, 1984; Jones, 1987). Gas exchange measurements on *T. angustifolia* at the Kuybyshev water reservoir in Russia showed a decrease of stomatal conductance even at fully waterlogged plants with increasing atmospheric VPD (unpublished data). Knapp and Yavitt (1995) recorded higher stomatal conductance in morning compared to the afternoon in *T. latifolia*. Furthermore, hysteresis behavior of stomatal conductance became evident in *Carex nigra* L., *Salix cinerea* L. and *T. latifolia* during measurement of daily courses with different VPD (Eisele, 2003). The hysteresis would explain the short-time and marginal increase of oxygen saturation in rhizomes in the courses with higher VPD maximum during increasing PFD and VPD, whereas under the same atmospheric conditions under declining PFD no increase of oxygen saturation was recorded. A similar reaction of *T. angustifolia* in the present investigation is indicated by the significant interaction of VPD x Time, especially during the treatment with VPDmax 1.5 kPa (Tab. 2.5). The degree of stomata opening is critical and achieves an optimum for diffuse inflow and Poisseuille outflow (J. Armstrong and W. Armstrong, 1990; W. Armstrong and J.

Armstrong, 1991). If stomata were mostly closed under increased VPD, the oxygen flow to the rhizomes and consequently ROL to the rhizosphere ceased.

The results of the present study and previous studies confirm that the ROL is a dynamic property influenced by the VPD and the intensity of radiation. Investigations already indicated relevance of PFD and photosynthesis for ROL. In light ROL of *Juncus effusus* L. was 1.75 times higher than in dark (Sorrell, 1999). Oxygenation of the rhizosphere reacted very sensitively to changes in rhizome saturation. Concomitant to higher photosynthesis, internal oxygen saturation in the plant increased and oxygen concentration in the rhizosphere behaved accordingly as was expressed by high positive correlations between these oxygen values. For the whole diurnal course oxygen saturations in rhizomes were not significantly affected by the three VPD treatments (Tab. 2.5), but it was obvious, that at high atmospheric humidity (VPD 0.8 kPa) free oxygen in the rhizosphere rose two-fold due to ROL compared with drier conditions (Fig. 2.6b). But still at more severe VPD oxygen saturation in rhizomes produced sufficient ROL high enough to elevate rhizosphere oxygen over oxygen concentrations in root-free soil.

Data illustrating the significant link between internal aeration of underground organs and ROL presented in this study shows the ability of effective internal aeration and soil substrate oxygenation mechanisms in *T. angustifolia*. Such adaptations allow the cattail to grow at static water depths up to 2 m (Sainty and Jacobs, 1981; Grace and Wetzel, 1982). Root aeration is enhanced by diel pulses of oxygen saturation in the below ground organs. ROL detoxifies reduced substances and maintains aerobic respiration in the below ground organs (W. Armstrong, 1980). Our data suggest that the emergent wetland plant *T. angustifolia* can maintain significant internal aeration even at low humidity gradients between ambient and lacunar air in the leaves and this may be beneficial for aeration particularly in wetland areas with high atmospheric humidity.

2.2.6 Acknowledgement

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2.3 Roots of the wetland plants *Typha latifolia* and *Phragmites australis* are inhabited by methanotrophic bacteria in biofilms

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This section, starting from the abstract and ending with discussion and acknowledgements, is a full excerpt of the article in Faußer et al., 2012. The contributions of each author can be found in the appendix, see chapter B.

2.3.1 Abstract

Wetland plants create partly aerobic conditions in the rhizosphere by releasing oxygen to the waterlogged substrate. The present study was conducted to characterize the arrangement of rhizobacteria, especially those active in methane oxidation, in root-associated biofilms of wetland plants. Root cross sections sampled from *Typha latifolia* L. (broadleafed cattail) and *Phragmites australis* (Cav.) Trin. ex Steud. (common reed) were scanned using light and electron microscopy. Methane-oxidizing bacteria were identified and quantified by immunological labelling of the α-subunit of the methanol dehydrogenase (α-MDH; encoded in mxaF.) On roots of both species there was a diverse subset of bacteria arranged in a microbial biofilm around the roots' exodermis. Similar bacterial densities in the root-associated biofilm were detected in more basal regions and closer to the root tip. Many microbes carried notable internal membrane systems that are characteristic of methanotrophic bacteria. This morphological characterization was confirmed by immunogold labelling with α-MDH antibodies. Quantification of labelled bacteria revealed that 34 to 43 % of the biofilm bacteria were potentially capable of methane turnover. These findings confirm that methane oxidation is a common and

important microbial process related to oxygen-releasing roots of wetland macrophytes such as *T. latifolia* and *P. australis*.

Keywords: *Methane-oxidizing bacteria*, *rhizoplane biofilm*, *immunological labelling*, *methanol dehydrogenase*, *rhizosphere oxygenation*, *wetlands*.

2.3.2 Introduction

Methane emissions from wetlands mirror the balance between methanogenesis and methanotrophy (Le Mer and Roger, 2001). The rates of both processes depend on activities of specialized soil microbial communities. Methane production by methanogenic archaea, which occurs in strictly anoxic soils and sediments, is related to substrate availability, temperature and flooding regime (Nahlik and Mitsch, 2011; Schimel and Gulledge, 1998; Sjögersten and Wookey, 2002). Methane oxidation as an emission-mitigating process in wetlands is dependent on the activity and abundance of methane-oxidizing bacteria (MOB) that are able to oxidize $\mathrm{CH_4}$ to $\mathrm{CO_2}$ under aerobic conditions (R. S. Hanson and T. E. Hanson, 1996). Low emissions can occur where methane is consumed by MOB in the uppermost aerobic layer or within the anearobic-aerobic interface in the rhizosphere of wetland plants before reaching the atmosphere (Bodelier, 2003; Whalen, 2005). Through methane oxidation the total methane produced in a rice paddy for instance thus can be reduced by up to $80\,\%$ before emission (see Conrad (1996) and Holzapfel-Pschorn et al. (1985).

Aerobic MOB are obligate methylotrophic bacteria that are capable of utilizing methane, in addition to other reduced carbon substrates with no C-C bonds, as their sole source for energy and carbon (Chistoserdova et al., 2009). Oxidation of methane to methanol is a unique feature that distinguishes MOB from other methylotrophic bacteria. According to the metabolism of methane after oxidation to methanol and formation of formaldehyde, at least two types of aerobic MOB can be distinguished (R. S. Hanson and T. E. Hanson, 1996; Lidstrom, 2006): Type I methanotrophs are γ-Proteobacteria that assimilate carbon via the ribulose monophosphate-pathway (RuMP) and type II MOB are α-Proteobacteria using the serine-pathway. Typically MOB of type I carry characteristic internal membrane systems aligned to vesicular stacks, while the intracellular mebranes (ICM) of type II MOB run parallel to the cytoplasmic membrane (Dalton, 2005; R. S. Hanson and T. E. Hanson, 1996). New findings of unusual methane mono oxygenases and alternative assimilation pathways in filamentous γ-Proteobacteria *Crenothrix polyspora* Cohn (backwash water from groundwater treatment, Stoecker et al. (2006))

and *Clonothrix fusca* Roze (artesian well, Vigliotta et al. (2007)) indicate, that methane oxidation is a more complex mechanism and more widespread feature than previously suggested. As reviewed by Trotsenko and Khmeleninao (2002) methanotrophic bacteria are ubiquitously distributed even in extreme environments. Recently a novel group of acidophilic and non-Proteobacteria, phylogenetically belonging to *Verrucomicrobia*, has been isolated from geothermal areas in New Zealand, Italy and Russia which members appear to be capable of methane oxidation (Op den Camp et al., 2009).

The activity of aerobic rhizobacteria (including methanotrophs) in wetlands relies on oxygen availability. Plants mainly of the order Poales have managed to combat anoxic conditions in wetland soils by evolving an aeration system that runs through all plant organs (Brix et al., 1992), including rhizomes interconnecting individual culms (Klimešová and Čížková, 1996). This aerenchyma system allows emerged wetland plants to supply oxygen for submerged tissue and, in addition, to provide partly oxic conditions in the rhizosphere (W. Armstrong, 1972; W. Armstrong et al., 2000; Colmer, 2002; Jespersen et al., 1998). Oxygenation of the rhizosphere occurs through radial oxygen loss (ROL) primarily at young roots and root tips, as older root parts develop a barrier against oxygen loss (Colmer et al., 2006; Matsui and Tsuchiya, 2006; Matsui and Tsuchiya, 2008). Oxygen released via ROL is rapidly consumed by chemical and microbiological processes in the soil (e.g. nitrification, metal oxidation, methane oxidation) by which the rhizosphere becomes detoxified and nutrients mobilized for plant uptake (Bodelier, 2003; Ponnamperuma, 1972).

Gilbert and Frenzel (1998), Holzapfel-Pschorn et al. (1985) and King (1994) have shown that the aerobic microbial conversion of methane in waterlogged soil is preferentially associated with roots of several wetland plants and paddy rice. Microbial isolates from leaf surfaces and roots of wetland plants as well as the rhizosphere showed high rates of methane consumption. Using oligonucleotide probes and other molecular techniques numerous MOB were detected in such samples (Bodelier, 2003; Bosse and Frenzel, 1997; Dedysh, 2009; Gilbert et al., 1998; King, 1994; McDonald et al., 2008).

With this study we intend to get closer insight into the ultrastructural organization of the root associated rhizobacterial communities of wetland plants in situ. The hypothesis is that oxygen release by wetland macrophytes provides habitat for methane-oxidizing bacteria and leads to formation of biofilms directly on root surfaces. The aim of this study is to identify the proportion of methylotrophic bacteria including MOB integrated within undisturbed natural structures of roots and associated microbial biofilms of

emerged macrophytes *Typha latifolia* L. (broadleafed cattail) and *Phragmites australis* (Cav.) Trin. ex Steud. (common reed).

2.3.3 Materials and Methods

Sampling sites and sample preparation

Root samples of *T. latifolia* L. were taken in mid-July 2009 from a natural pond in the Botanical Garden of the University of Ulm, Germany. *P. australis* (Cav.) Trin. ex Steud. plants, analysed by mid-August 2009, grew in a constructed wetland for biological waste water treatment in Slavošovice, Czech Republic. This facility was supervised by the Faculty of Science at the University of South Bohemia, Czech-Republic and is subject to previous publication (see Dušek et al. (2008)). Common reed plants in the constructed wetland were sampled (i) from the inflow zone with high organic load in flooding water and (ii) from the outflow zone with low organic load. The sampling regions were located on roots of both plants in the apical part at a distance of 2 to $3\,\mathrm{cm}$ from the tip. An additional sample was taken from the *T. latifolia* root closer to the basal region (6 to $7\,\mathrm{cm}$ from the tip). The collected root samples (*T. latifolia*: apical and basal; *P. australis*: low organic load and high organic load) were divided into sets of several subsamples (see below) prior to fixation.

Plant root samples were prepared for immunological analysis in transmission electron microscopy (TEM) and in light microscopy in order to detect and localize methylotrophic bacteria associated with the roots. To provide the original structure of roots and associated microorganisms, it was crucial to obtain fully undisturbed samples. The procedure for root and biofilm fixation was optimized in several test runs. The plants' root systems were excavated carefully. The sampled roots were first washed with water from the wetland and later rinsed with deionized water directly before fixation. Root samples of *T. latifolia* were prepared by high-pressure freezing as described by Buser and Walther (2008) with some modifications: The roots were immersed in hexadecen and cut into subsamples of $200\,\mu\mathrm{m}$ thickness and $2\,\mathrm{mm}$ width using razor blades, mounted on aluminum planchettes and high-pressure frozen with a Wohlwend Compact 01 high-pressure freezer (Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland). After freeze-substitution in a medium containing acetone with $0.1\,\%$ uranyl acetate and $5\,\%$ of water, the root samples were embedded in LR-Gold resin. Fixation of *P. australis* root samples (as subsamples in pieces of about $5\,\mathrm{mm}$ length) was performed chemically with $0.5\,\%$ glutaraldehyde in isoosmotic phosphate buffer at $\mathrm{pH}\ 7.0$. Half of

these subsamples were then infiltrated with LR-Gold according to the same protocol as above, apart from high-pressure freezing. The other half was embedded in Lowicryl K4M (Heller et al., 2008; Roth et al., 1981).

TEM, light microscopy and immunological labelling

Semithin and ultrathin sections were obtained according to established procedures (Wrede et al., 2008). Immunological labelling was performed as described by Takizawa and Robinson (2003) and Wrede et al. (2008). For detection of the highly conserved α-subunit (encoded in mxaF) of the methanol dehydrogenase (MDH) polyclonal antibodies were used. The α-MDH antibodies directed against the α-subunit of MDH in the model methylotroph Methylobacterium extorquens AM1 were generated and characterized as described by Wrede (2011). For immunofluorescence light microscopy a secondary antibody coupled to Alexa-Fluor® dye (Molecular Probes, Eugene, Oregon) was used. Microscopy was carried out with a Zeiss Axioscope fluorescence microscope using an AxioCam MRm (1388×1038 pixels) for image recording. Fluorescence excitation light energy was maintained constant during image recording to obtain comparable fluorescence intensities. For immunogold labelling secondary antibodies coupled to $10\,\mathrm{nm}$ colloidal gold were used (BBI, Cardiff, UK). Transmission electron microscopy was carried out with a Jeol 1011 instrument using a Gatan Orius SC200 CCD digital camera ($4\,\mathrm{MP}$) for image recording. Gold particles in images were digitally enhanced as described (Kämper et al., 2004).

In preparations from *P. australis* roots, cytoplasmatic membranes could not be detected inside attached bacterial cells using TEM. Due to technical facilities at investigations on the constructed wetland only chemical fixation of *P. australis* roots was possible. It is consistent with other studies showing that membranes are much better visible when high-pressure freezing and freeze substitution was applied, compared to chemical fixation (Walther and Ziegler, 2002).

Evaluation of biofilm bacteria and the proportions of MOB

Microorganisms in the root-associated biofilm were also quantified using light microscopy. Total numbers of attached microbes were obtained by counting bacterial cells, regardless of shape or clustering, outside on the root exodermis. In the root subsamples biofilm microbes were counted in six semithin cross sections ($1\,\mu\mathrm{m}$) taken at a distance of $5\,\mu\mathrm{m}$. This analysis was performed per plant species and sampling site

(apical: closer to root tip or basal root part and high or low organic load, respectively) on the following numbers of cross sections (subsample x section): *T. latifolia* apical: $4 \cdot 6 = 24$, basal: $3 \cdot 6 = 18$; *P. australis* high org. load: $2 \cdot 6 = 12$, low org. load: $2 \cdot 6 = 12$. The reason for unbalanced sample numbers was due to different preparation protocols and sample availability. As the roots analysed differed in perimeter, the total number of counted biofilm bacteria was set in relation to the number of exodermis cells of the particular root section analysed. For better visualization in light microscopy the sections were stained with toluidine blue before counting total bacterial cells.

Abundances of methylotrophic bacteria (i.e. MOB) in the biofilms were gained by counting the number of fluorescing cells on the root sections that were labelled with α-MDH in the immunofluorescence assays (two sections per species and sampling site). As immunological labelling had to occur on unstained sections, counts for methylotrophic bacteria could not be performed on the same sections as for total root-associated bacteria. Thus on the corresponding root subsample sections both parameters, i.e. total biofilm bacteria and shares of methylotrophs were expressed in counts per exodermis cell.

2.3.4 Results

Structure and bacterial densities in root-associated biofilms

Heterogeneous bacterial communities were detected on the exodermis of aerenchymatic roots of both *T. latifolia* and *P. australis* (Fig. 2.8). Abundances of rhizobacteria were not evenly distributed around the cross sections. Biofilms consisted of one to several bacterial cell layers with a thickness of 2–$12\,\mu\mathrm{m}$ and up to $20\,\mu\mathrm{m}$. In regions where two exodermis cells had contact, the bacteria accumulated in particular in the resulting groove between adjacent cells (Fig. 2.8 A, C). At places where remnants of rhizodermis cells were still present, the remaining cell structures were used by microbes as additional space for colonization (Fig. 2.8 B). The microbial community consisted of many different bacterial morphotypes (Fig. 2.8 C): Coccoid, rod-shaped, filamentous, streptobacilloid. As an additional feature, up to $46\,\%$ of exodermis cells in *P. australis* were densely packed with structures of bacterial shape (Fig. 2.8 D). But it has to be mentioned that the α-MDH immunological labelling did not respond to those intracellular microorganisms (Fig. 2.11). Colonization of reed exodermis cells seemed to be more developed at the site with higher organic load in the constructed wetland. In *T. latifolia*, bacterial structures within plant cells were found only scarcely.

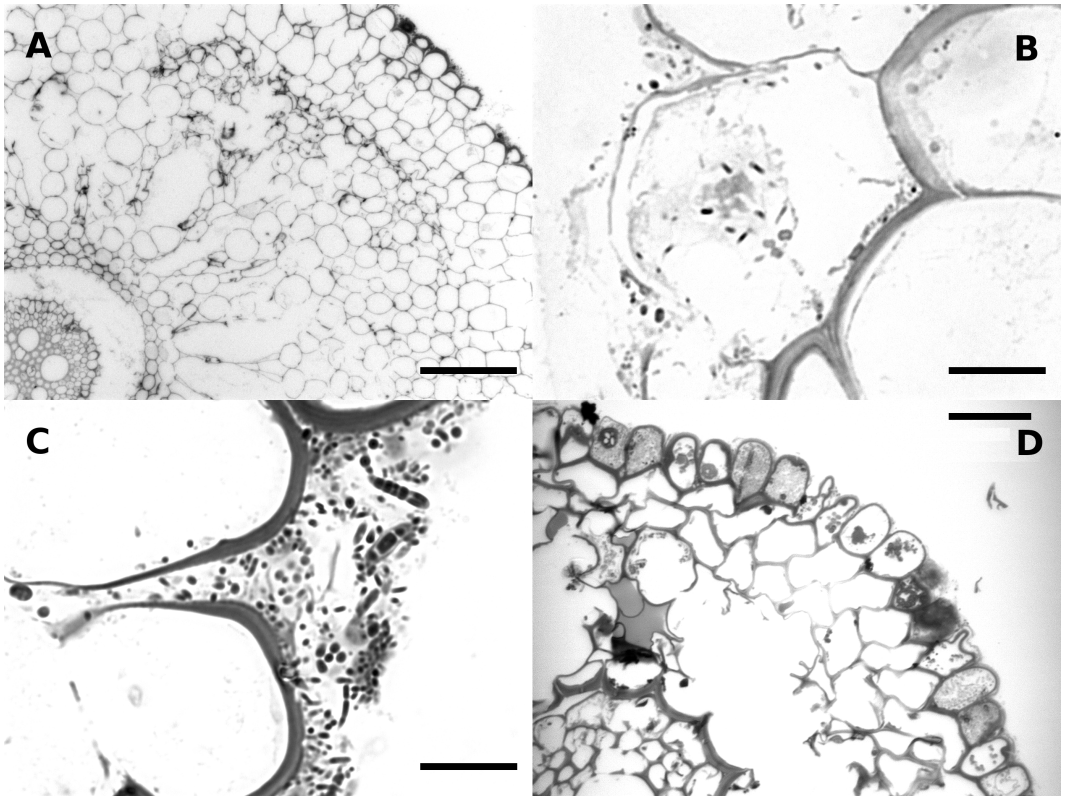

Figure 2.8: Sections of *T. latifolia* (A-C) and *P. australis* (D) roots observed via light microscopy showing a microbiological biofilm on the exodermis. (A) Organization of the root from central region to exodermis in early aerenchymatic state. Bar 200 µm. (B) Bacterial cells are visible outside of the exodermis and inside the remaining structures of decaying rhizodermis cells. Bar 10 µm. (C) A closer view reveals diverse morphotypes of microorganisms inside the biofilm. Bar 10 µm. (D) Fine root of *P. australis* exhibiting over one third of exodermis cells densely packed with bacterial structures. Bar 20 µm.

Most of the microorganisms were embedded in an amorphous extracellular matrix (Fig. 2.9 A, B, C). In some occasions bacteria were located inside engraved channels within the cell wall of exodermis cells (Fig. 2.9 A). Very often clusters of biofilm bacteria were enclosed by electron dense capsule like structures (Fig. 2.9 E). The majority of the single standing and filamentous forms among the biofilm bacteria carried well developed sheaths (Fig. 2.9 D, F). It was frequently observed in *T. latifolia* samples that the bacteria were arranged side by side perpendicularly with respect to their long axis to the plant root surface (Fig. 2.9 D, cf. Fig 2.8 C). The micrographs of such undisturbed cross sections also revealed that many bacteria had notable structures of intracellular membranes (ICM). The black granules visible in great numbers inside many microbial cells were accumulated storage materials (Fig. 2.9 A, B). Many bacteria found in the rhizoplane biofilm of *T. latifolia* showed ICM which were organized in loops and

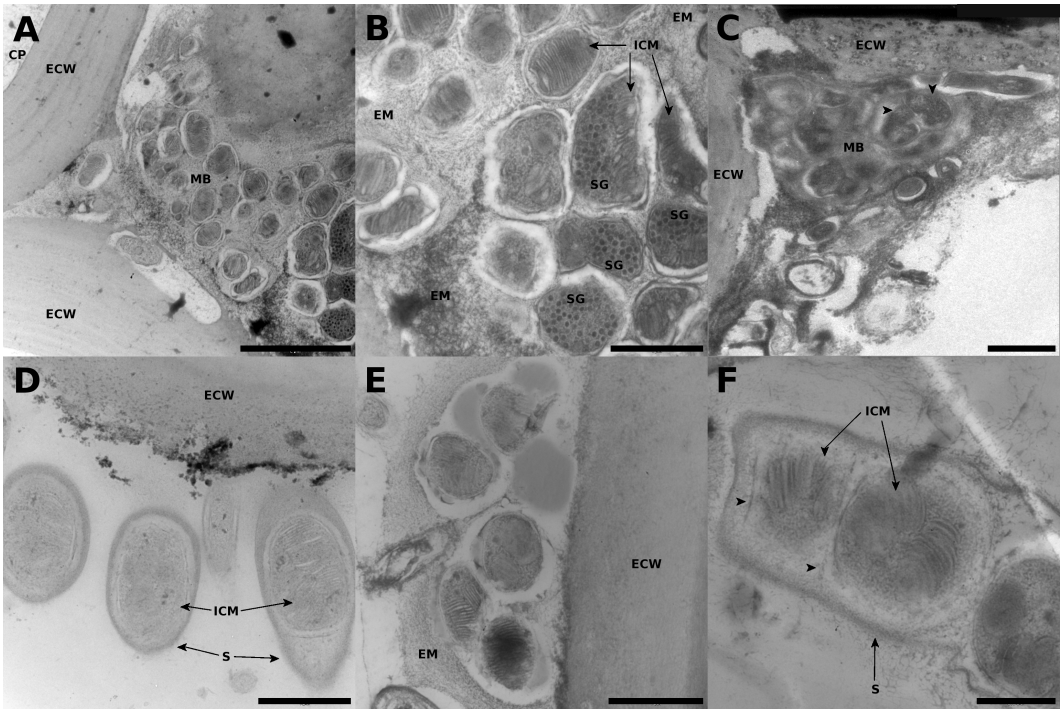

Figure 2.9: Ultrastructure of the microbial biofilm on the root surface of the wetland plants *T. latifolia* and *P. australis*. (A,B) *T. latifolia*: Overview and further enlarged biofilm bacteria and potential MOB with internal structures: storage granules (SG) and intracellular membranes (ICM). Note the bacterium in engraved channel in wall of exodermis cell; (C) *P. australis*: biofilm bacteria with less preserved ICM (arrow heads); (D-F) *T. latifolia*: (D) single standing bacteria with tight sheaths arranged perpendicularly to exodermis; (E) biofilm bacteria with ICM grouped together in electron dense outer layer; (F) filamentous bacterial morphotype with ICM and tight sheath; arrow heads point on possible growth division marks in the sheath. CP: Cytoplasma of plant root cell; ECW: Exodermis cell wall; EM: Extracellular matrix; MB: Microbial biofilm; S: Sheath. Bars (A): 2 µm; (B-F): 1 µm.

piled up in stacks, predominantly crossing the whole cell lumina perpendicular to the cytoplasmic membrane (Fig. 2.9 B, D, E, F). In this study the other type of membrane arrangement in MOB, in which the loops run parallel to the cell wall (a formerly assumed characteristic for type II MOB; Smith and Ribbons (1970)), was not observed among the bacteria in the root-associated biofilms of the studied macrophytes.

Quantification of bacterial cells on roots of *T. latifolia* revealed no difference in abundances of biofilm bacteria per exodermis cell close to the root tip (40.4 ± 10.4; mean $\pm$ standard deviation; 24 cross sections) in comparison to more basal regions (40.8 ± 13.2; 18 cross sections). On the exodermis of *P. australis*, numbers of associated biofilm bacteria were lower than on *T. latifolia* roots, regardless of organic load. At sites grown with *P. australis* and higher chemical oxygen demand (COD $50\,\mathrm{mg\,l^{-1}}$, Dušek et al. (2008)) the bacterial density varied between 5.7 ± 1.1 and 31.1 ± 6.8 (12 cross sections). At

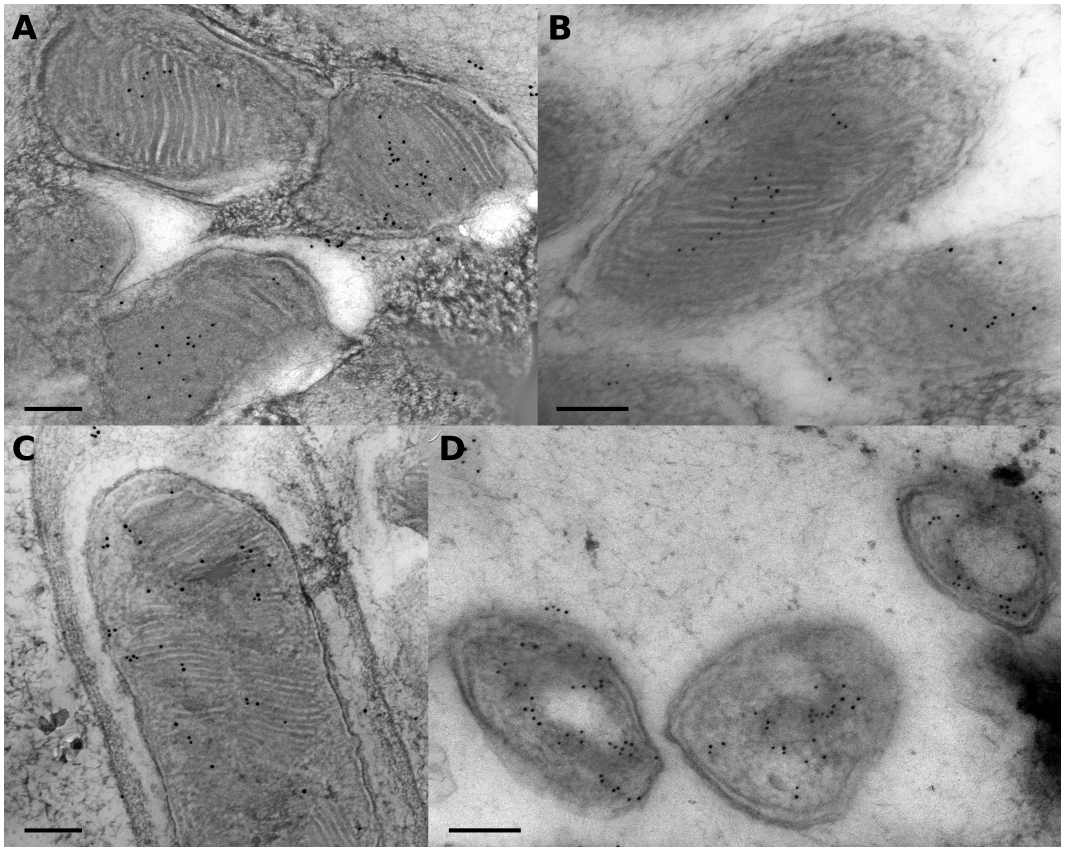

Figure 2.10: Immunogold labelling of methylotrophic bacteria in the root-associated biofilms of *T. latifolia*. Primary antibodies against the α-subunit of methanol dehydrogenase (α-MDH) were detected in TEM by 10 nm gold particles conjugated to secondary antibodies. Methylotrophs with intracellular membranes (ICM) are regarded as methane-oxidizing bacteria (MOB) and were observed in up to 95 % of the labelled bacteria. (A-B) Cluster of bacteria with labelled ICM; (C) Filamentous bacterium with labelled ICM; (D) α-MDH labelled methylotrophic bacteria without ICM. ECW: Exodermis cell wall. Bars: 200 nm.

lower COD ($30\,\mathrm{mg\,l^{-1}}$, Dušek et al. (2008)) in average 21.6 ± 6.8 (12 cross sections) bacteria per exodermis cell were recorded. Please note that the given numbers of biofilm bacteria were evaluated from subsamples of the same sampling sites and no further statistical evaluation was performed on this data set.

Root-associated methylotrophic bacteria

Immunofluorescence labelling with α-MDH antibodies provided consistent results for root samples of both plants regarding the expression of methanol dehydrogenase (MDH) in the bacteria of the analysed root biofilms (Fig. 2.10). Despite high variance of labelled bacteria counted per exodermis cell on corresponding subsamples, the

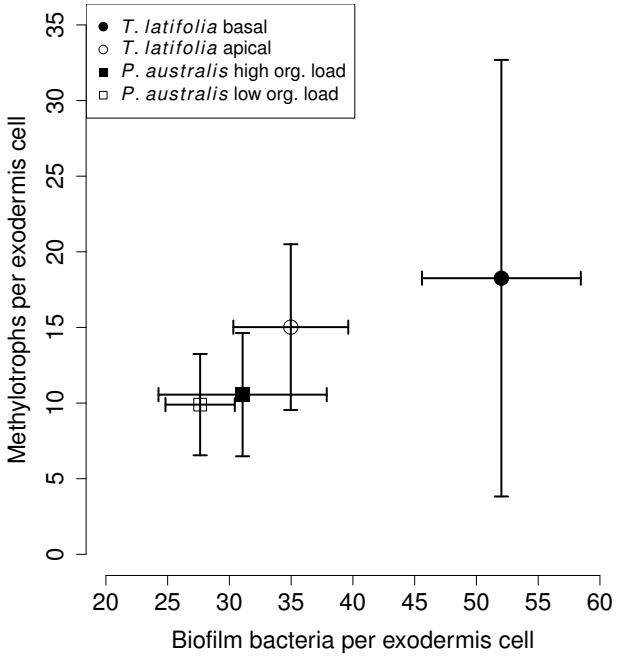

Figure 2.11: Abundance and range of immunologically labelled methylotrophs in relation to total biofilm bacteria counted on respective corresponding root samples. Numbers of bacteria are given per root exodermis cell each for *T. latifolia* (circles) and *P. australis* (squares) root samples. Values represent means with corresponding standard deviation in both dimensions. Numbers of total biofilm bacteria illustrate only the abundances of root-associated bacteria in the corresponding subsamples the immunofluoresence-assays were performed on.

immunofluorescence assays indicated that 34 to 43 % of biofilm bacteria on roots of both species contained MDH (Fig. 2.10, Fig. 2.11), i.e. were either methanotrophic or methylotrophic. Fractions of methylotrophs appeared to be higher at apical regions in *T. latifolia* (43 %) compared with basal root parts (35 %). For *P. australis* growing under different levels of organic load, the share of methylotrophs was almost the same (34 vs. 36 %). Please note that the differences in abundances were not proven statistically due to sample availability. Both the percentage and variation of labelled bacteria seemed to increase with the total amount of biofilm bacteria per exodermis cell (Fig. 2.11). Numbers of total biofilm bacteria illustrated in Fig. 2.11 represent only the abundances of root-associated bacteria in the corresponding subsamples the immunofluoresence assay was performed on.

By immunogold labelling it was observed for biofilms on *T. latifolia* roots in TEM that more than 90 % of the biofilm bacteria showing ICM contained also MDH (Fig. 2.12). On the other hand, less than 5 % of MDH-labelled biofilm bacteria carried no ICM and were thus regarded as methylotrophs. The common feature of both ICM and MDH

characterized those bacteria as methanotrophs among the methylotrophs in the root-associated biofilm. The connection between ICM and MDH was not possible for labelled bacteria in root-associated biofilms of *P. australis* as the resolution of cytoplasmatic structures in TEM was impaired due to the fixation method.

2.3.5 Discussion

Microbial biofilms are communities of metabolically linked microorganisms from different taxa (Beer and Stoodley, 2006), thus possessing many different bacterial morphotypes. This feature was also shown in the presented biofilms on roots of both wetland plants *T. latifolia* and *P. australis*. The cohesion of the MOB community was strong enough to withstand fixation and embedding procedures and thus can be regarded as integral part of oxygen-releasing root surfaces. The root-associated bacterial community is stabilized by plant exudates as well as by bacterial extracellular polymer matrix (Beer and Stoodley, 2006), which build the biofilm structure at the rhizoplane. Moreover, many of the identified MOBs formed clusters surrounded by fine sheaths (c.f. Fig. 2.9, 2.10), which assure internal coherence and gives some protection from external influences of e.g. toxic compounds under oxygen-deprived conditions. Such stable association explains why Bosse and Frenzel (1997) and King (1994) isolated MOB from washed and surface-sterilized roots of wetland plants and rice and thereupon postulated MOB to be closely attached or even inside the root tissue. Methane-oxidizing bacteria live in the interface, where oxygen released by plant roots and diffusive methane from anoxic sediments are mixing (Beckett et al., 2001). Perpendicular arrangement to the root surface can be regarded as optimization of gas exchange (cf. Fig. 2.9 D). Such close association of MOB to emergent macrophytes explains, why oxygen release via ROL was mostly detected under laboratory conditions (Bodelier, 2003) but only exceptionally in the field (Mainiero and Kazda, 2005).

High numbers of attached biofilm bacteria analysed on *T. latifolia* roots exhibited noticeable and distinct intracellular membrane systems (ICM). Different arrangements of these triple-layered membrane units in the micrographs result from cuttings of bacterial cells in diverse orientations present in the biofilm. A reason why there were only traces of internal membranes visible in TEM in bacteria on *P. australis* roots was presumably due to chemical instead of cryo-fixation of the reed samples. This holds true as the immunofluorescence assays revealed similar proportions of methylotrophic bacteria on both *T. latifolia* and *P. australis* roots. Taking into account that more than $95\,\%$ of labelled bacteria were capable of methane-oxidation (simultaneously indicated by MDH

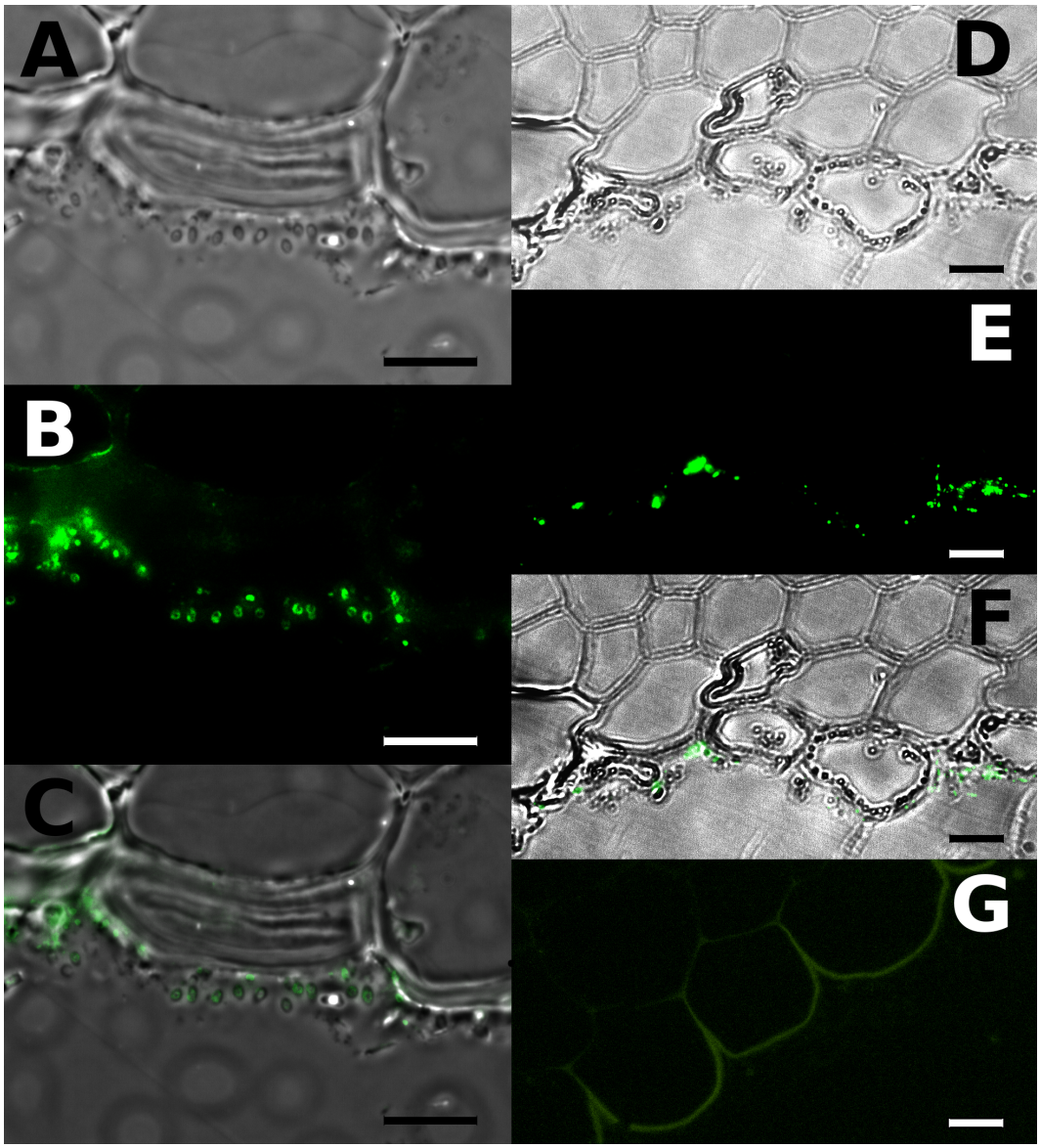

Figure 2.12: Immunofluorescence of methane-oxidizing bacteria (MOB) on the root surface of *T. latifolia* (A-C, G) and *P. australis* (D-F). Methylotrophic bacteria and MOB were labelled with antibodies directed against the conserved α-subunit of the methanol dehydrogenase (α-MDH). (A,D): Images in phase contrast / dark field; (B,E): Emitted fluorescence light; (C,F): Overlay. (G): Control labelling of a root section only with second antibody and without first antibody (α-MDH). Bars 10 µm.

and ICM) then methanotrophs made up more than one third of the total bacterial community associated with the roots of both wetland plant species investigated (cf. Fig. 2.11).

The ultrastructure of ICM reflects special metabolic features of the bacteria under consideration and thus ICM can serve as a characteristic in addition to e.g. immunological methods. Several studies have shown that autotrophic microorganisms gain energy mainly with extended membrane-bound electron transport chains and thus carry morphologically very similar ICM, ie. phototrophic and chemolithotrophic bacteria (purple bacteria: Cohen-Bazire and Kunisawa (1963); nitrifiers: R. G. E. Murray and Watson (1965); methanotrophic bacteria: Bowman (2006), R. S. Hanson and T. E. Hanson (1996), and Smith and Ribbons (1970)). It had been well accepted that MOB carry characteristic internal membrane systems, either running parallel to the cytoplasmic membrane (Type II) or aligned to vesicular stacks (Type I), where the methane oxidation occurs (Dalton, 2005; R. S. Hanson and T. E. Hanson, 1996; Lidstrom, 2006). But recently described genera like *Methylocella* isolated from peat bogs (Dedysh et al., 2000) suggest that those membranes involved in methane oxidation have not necessarily to be arranged in this manner. Raghoebarsing et al. (2005) described a novel symbiosis of an acidophilic MOB of type II (sequence similarity $93\,\%$ to both *Methylocella palustris* and *Methylocapsa acidiphila*, Dedysh et al. (2000) and Dedysh (2009)) living inside hyaline cells of peat bog (*Sphagnum cupsidatum* Ehrh. ex Hofin.). This bacterium does not possess membranes arranged in such a specific way. The detected ultrastructure of potential MOB is characteristic of, but not necessarily present in type I MOB and was described e.g. for a Methylomonas sp. (Boer and Hazeu, 1972). Especially the appearance of the filamentous forms in the biofilms associated with *T. latifolia* roots (Fig. 2.10 C) showed high similarity with the ultrastructure of *Clonothrix fusca* (Vigliotta et al., 2007), which was recently discovered to be an unusual freshwater methanotroph.

Former investigations regarding MOB in macrophytes' rhizospheres indicated that there are more MOB of type II than of type I (Bodelier, 2003; Bosse and Frenzel, 1997; Gilbert et al., 1998; King, 1994). The type of membrane arrangement in MOB (a formerly assumed characteristic for type II MOB; Smith and Ribbons (1970)), in which the loops run parallel to the cell wall, was not observed among the bacteria in the biofilms studied here. A reason for the reported majority of type II over I might be that most of the previous studies were based on cultivation-dependent investigations. Recently, Wu et al. (2009) detected higher abundances for MOB type I over type II on roots of rice compared with rhizosphere or bulk soil when applying a cultivation-independent method (ter-

minal restriction fragment length polymorphism (T-RFLP)). Nevertheless type II MOB do not necessarily carry such noticeable ICM, as described for *Methylocella* species (Dedysh et al., 2000). Such MOB species might be also detected by immunological labelling but eventually were not distinguished from other methylotrophic non-MOB bacteria in the biofilm in our ultrastructural investigations.

The α-subunit of MDH (encoded in mxaF) was used as a target for immunological assays as this enzyme subunit is highly conserved in both methylotrophs and methanotrophs and well approved in MOB detection. Nevertheless we are aware, that novel MOB possessing different MDH and methane assimilation pathways exist, that could not be recognized by antibodies against the usual MDH (Chistoserdova et al., 2009; Dedysh, 2009). Therefore the relative abundances of methylotrophic bacteria within the root-associated biofilms could be higher than 34 to $43\,\%$ as enumerated in this study. This view is supported by high numbers of bacteria with extensive ICM characteristic for MOB (Lidstrom, 2006) like it was observed and labelled in the microbial community on *T. latifolia* roots. Although non-MOB can possess internal membranes, too, those are arranged in a distinguishably different manner. Nitrifying bacteria for example were likely also present in the rhizoplane, which carry ICM (R. G. E. Murray and Watson, 1965) and oxidize methane at low rates ($5\,\%$ of the methanotroph rate; R. S. Hanson and T. E. Hanson (1996)). But nitrifiers lack a MDH (Hyman and Wood, 1983) and thus were not recognized by the α-MDH antibodies used here.

Bacterial communities attached to aerenchymatic, potentially oxygen-releasing roots of both plant species studied, as far as they are aerobic microorganisms, depend on molecular oxygen released by roots of the host wetland plants (Gilbert and Frenzel, 1998; Holzapfel-Pschorn et al., 1985; King, 1994; Kravchenko, 2002). W. Armstrong et al. (2000) and Colmer (2002) showed that oxygen release is highest at the root tip and declines towards the emerging point of the root. Therefore higher bacterial abundance was expected closer to the root tip compared to basal regions. But our results have not yet proven such an expectation. This may be due to the fact that although the bacteria have more favourable conditions for development close to the root tips, the time for their development there is shorter. Rudrappa et al. (2008) showed that in aerated soils root associated biofilms were fewer and less developed close to the root tips and central elongation regions, while highest microbial abundances were localized on mature root surfaces and at the emerging points of roots.

For *P. australis* no difference in abundance of biofilm bacteria on roots was found on sites with different organic load. Neither did proportions of methylotrophs within the

biofilms vary between the two sites. The internal bacterial colonization of exodermis cells of the fine roots of *P. australis* at the site with high orgnic load has to be evaluated further.

Aerobic methanotrophic bacteria have been detected and characterized in the rhizosphere of wetland plants and rice by several research groups (Bodelier, 2003; Bosse and Frenzel, 1997; Calhoun and King, 1998; Gilbert and Frenzel, 1998; Gilbert et al., 1998; King, 1994). Murase and Frenzel (2007) identified a methane driven metabolic food web in rice soils, revealing that methane concentrations and thus MOB essentially shape the composition of the microbial and microeukaryotic communities. According to their results, it can be suspected that MOB play an important role as primary oxidizers in the microbial ecology of wetland habitats with methane as prevailing carbon substrate. In this study MOB were identified first by their characteristic internal membrane structures (ICM) and second by immunological labelling with α-MDH. The immunogold assays revealed that also a small proportion of the labelled bacteria carry no ICM (less than $5\,\%$). In natural wetlands, the supply of methane from the anoxic sediments is the definitely prevailing substrate over solely methanol which is frequent on atmospheric plant surfaces. Neither pathway of methane production by methanogenic archaea using acetate or $\mathrm{H_2}$ and $\mathrm{CO_2}$ as substrates includes intermediate steps of methanol synthesis. Therefore the MDH labelling applied in this study pointed out individuals active in methane oxidation.

The present study provided evidence for a close and stable association between roots of two wetland macrophytes and MOB-containing microbial communities. The ecological impact of methanotrophs and methanogens has been extensively reviewed elsewhere (Conrad, 2007; Laanbroek, 2010; Philippot et al., 2009) and the presented study is the first to show the ultrastructure of methanotrophic rhizobacterial communities attached to the root surface of wetland plants in situ. High proportion of methanotrophic bacteria within these biofilms points out their importance for methane oxidation. Mitigation of methane emission from wetlands by these microbial associations is still highly dependent on the magnitude of oxygen transport to the rhizosphere and its release through root surfaces. Aerenchymateous plants in wetlands and littorals therefore not only vent methane from anoxic layers to the atmosphere (Joabsson et al., 1999; Juutinen et al., 2003) but they provide also habitats for methane-oxidizing bacteria. These findings strengthen the importance of wetland plants regarding carbon cycle and methane emissions and turnover.

2.3.6 Acknowledgements

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2.4 Internal oxygen dynamics in rhizomes of *Phragmites australis* and presence of methanotrophs in root biofilms in a constructed wetland for waste water treatment

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This section, starting from the abstract and ending with conclusions, is a full excerpt of the article in Faußer et al., 2013. The contributions of each author can be found in the appendix, see chapter B.

2.4.1 Abstract

Oxygen partial pressures (O2pp) inside rhizomes of *Phragmites australis* were measured to monitor diurnal dynamics. Root-associated biofilms were characterized regarding methane-oxidizing bacteria (MOB) by immuno labelling. Runs of O2pp showed distinct diurnal patterns repeating day-to-day. Soon after sunrise O2pp increased steeply to around 185 hPa, remained on this level over midday and decreased exponentially overnight to about 80 hPa. Root surfaces were densely packed with bacteria of which 34 to 37 % accounted for potential MOB. This emphasizes the importance of the oxic rhizosphere in degradation of organic matter and methane oxidation and diurnal fluctuation of O2pp may implicate for variation of biogeochemical processes involved in waste water treatment.

Keywords: *aerenchyma*, *macrophyte*, *constructed wetland*, *internal oxygen partial pressure*, *methane-oxidizing bacteria*

2.4.2 Introduction

Wetlands are a challenging environment for plant growth due to anoxic and often reducing conditions in soil (Ponnamperuma, 1972). Plants growing effectively in waterlogged conditions possess the major characteristic to develop an enlarged gas-space continuum running through all plant organs including shoots, roots and the extensive rhizome system interconnecting shoots (Končalová, 1990; Jackson and W. Armstrong, 1999). This aerenchyma system allows emerged wetland plants to supply oxygen for submerged tissue and to provide partly oxic conditions in the rhizosphere (W. Armstrong, 1980; W. Armstrong et al., 2000; Mainiero and Kazda, 2005). Wetland plants enhance their internal ventilation based on diffusion additionally by pressurized air flow through the aerenchymatic tissue in shoots and rhizomes (W. Armstrong and J. Armstrong, 1991; Brix et al., 1992; Colmer, 2003). Pressurized gas flow is achieved by gradients of temperature (thermal-transpiration) or humidity (humidity-induced convection) over a porous partition (diameter $<0.1\,\mu\mathrm{m}$) between the inner and outer atmosphere enabling Knudson-type diffusion (W. Armstrong, 1980; Colmer, 2003). In common reed (*Phragmites australis* (Cav.) Trin. ex. Steud.) additional through flow can occur as a result of wind blowing through the stand by which pressure gradients (venturi effects) are created between young and old or dead shoots and air is sucked through the pith cavities of shoots and rhizomes (J. Armstrong et al., 1992). The through flow of air raises the internal oxygen partial pressure which can reach levels close to ambient air (Colmer, 2003). Root aeration operates by diffusion only but profits from elevated oxygen levels in the rhizomes (W. Armstrong, 1980; W. Armstrong et al., 2000; Colmer, 2003).

Oxygen is depleted in waterlogged soils by chemical (e.g. oxidation of reduced metals) (Ponnamperuma, 1972) and biological processes that are involved in soil organic matter degradation. Thus the redox potential (Eh) can reach low values from -200 to $-400\,\mathrm{mV}$ (Dušek et al., 2008; Wießner et al., 2005). In anoxic layers of saturated soils organic matter is decomposed anaerobically by reduction processes including denitrification, fermentation and methanogenesis. Greenhouse gases ($\mathrm{CO_2}$, $\mathrm{CH_4}$, $\mathrm{N_2O}$) generated in submerged soils are emitted directly or through the gas-spaces of the wetland plants (Picek et al., 2007). The wetland macrohytes release oxygen to the rhizosphere through special porous regions at the root tips (W. Armstrong et al., 2000) to oxidize phytotoxic compounds characteristic for reduced anaerobic soils (i.e. reduced metals). Thus

the redox potentials are increased (Wießner et al., 2005; Flessa, 1994) and nutrients become available for uptake. Aerobic soil microbes profit from the partly oxic conditions in the rhizosphere and aggregate in biofilms around the roots in comparison to bulk soil (Wießner et al., 2005; Bodelier, 2003; Rudrappa et al., 2008). Metabolically linked microbial communities develop on the root surface which use the oxygen released to decompose organic matter (Wießner et al., 2005) and oxidize reduced substances like methane (Bodelier, 2003; Shrestha et al., 2008). Up to $80\,\%$ of soil borne methane can thus be quenched before reaching the atmosphere (Conrad, 1996) and methane-oxidizing bacteria in the rhizosphere can be considered as crucial controllers of methane emission.

As mechanisms for internal ventilation of *P. australis* and gas flows in the aerenchyma of several wetland plants are well documented (Colmer, 2003), we intended to gain insight in the daily dynamics of oxygen concentration in the central rhizome gas-spaces under natural *in-situ* conditions of a constructed wetland utilising *P. australis* for waste water treatment. The hypothesis was that oxygen is actively pressurized from shoot to rhizome pith cavities by the above described ventilation processes. During night, submerged plant tissues and rhizobacteria will consume the oxygen from the rhizomes' gas-spaces in a way that mirrors biological and chemical oxygen demand in the rhizosphere. Furthermore, as anaerobic processes in anoxic layers of the wetland soil produce methane, we hypothesize that parts of the bacterial community are involved in methane oxidation. As a result diurnal variation in oxygen supply to the rhizosphere can influence also biogeochemical processes used for waste water treatment in constructed wetlands.

2.4.3 Materials and methods

Site description and plant species analysed

This study was conducted in August 2009 in a subsurface horizontal flow constructed wetland (CW) located in Slavošovice in South Bohemia, Czech Republic (48N57'44.34", 14E39'39.997"; $480\,\mathrm{m}$ a.s.l., Fig. 2.13). The CW was created for municipal waste water treatment and started operating in August 2001. It consisted of a pretreatment (screen, sand trap, sedimentation tank) and two parallel vegetated beds (size of each $17\ \mathrm{m} \cdot 22\ \mathrm{m}$; total area $748\,\mathrm{m}^2$) planted with common reed (*P. australis* (Cav.) Trin. ex. Steud.). The CW had a purification capacity of 150 person equivalents (PE) with $5\,\mathrm{m}^2\,\mathrm{PE}^{-1}$ and about 100 persons were connected during the study period. Analysis

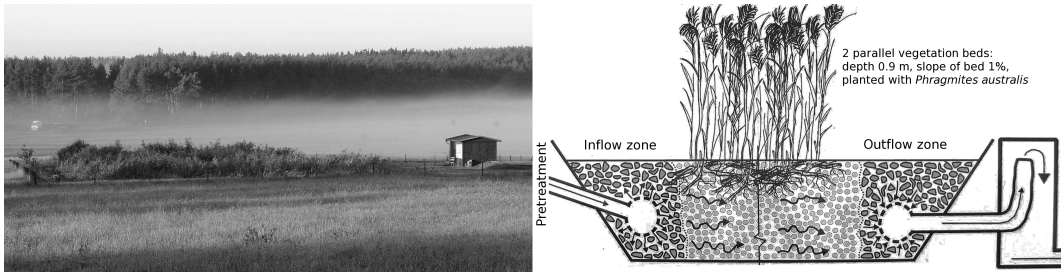

Figure 2.13: Diagram and photograph of the subsurface horizontal flow constructed wetland in Slavošovice, Czech Republic. Photo author.

of the water passed the CW showed a high efficiency in removing organic pollution (Biological Oxygen Demand (BOD_5) $82\,\%$ and Chemical Oxygen Demand $74.0\,\%$, total nitrogen ($62.6\,\%$), total phosphorus ($75.4\,\%$) and suspended solids ($52\,\%$) (Dušek et al., 2008). In average, the waste water inflow rate was regulated to $0.12 \pm 0.10\,\mathrm{l\,s^{-1}}$, with a maximum of $1.0\,\mathrm{l\,s^{-1}}$ during periods of extremely strong precipitation. Hydraulic retention time ranged from 8 to 16 days (Conrad, 1996). Inflow and outflow zones ($1.5\,\mathrm{m}$ each) of the treatment beds were filled with coarse gravel (5–$20\,\mathrm{cm}$), and the vegetated part with fine gravel (0.5–$1\,\mathrm{cm}$).

General climate and microclimate during the measurement

The annual average air temperature and sum of precipitation at the study site were $7.9\,^{\circ}\mathrm{C}$ and $634\,\mathrm{mm}$, respectively. Microclimatic factors (irradiation inside and outside the stand, air and surface water temperature, air humidity) were recorded during the period of measurement via appropriate sensors (all Delta-T, Burwell, UK). Weather conditions during oxygen recording were sunny summer days with more than $12\,\mathrm{h}$ sun light (05:45 until 18:15 PFD $>100\,\mathrm{\mu mol\,m^{-2}\,s^{-1}}$) and almost no clouds. Microclimatic parameters were consistent over the three days of measurement. Air temperatures ranged from $5.4\,^{\circ}\mathrm{C}$ at night to $29.8\,^{\circ}\mathrm{C}$ at day time. Temperatures of the sediment water laid between $14.6\,^{\circ}\mathrm{C}$ and $17.8\,^{\circ}\mathrm{C}$. Relative air humidity was recorded between $55\,\%$ at day up to $98\,\%$ at night.

Oxygen measurement

Partial pressures of oxygen were measured in the gaseous phase of pith cavities in rhizomes of two *P. australis* plants (A1, A2). Both plants grew in the outflow half of

the CW. After gentle excavation of the rhizome systems, those plants were chosen as they had this year's shoot directly adjacent to a dead-ended vertical rhizome. The rhizomes were one or two years old and emerged over the water table of the CW, but were buried inside the gravel and litter layer of the vegetation bed. Optical oxygen sensors (diameter 4 mm, Fibox-type: PreSens, Regensburg, Germany) were implanted into cut ends of the two rhizomes (diameter A1: 13 mm; A2: 16 mm) and sealed airtight. The excavated rhizome systems were covered with aluminium foil to protect them from sunlight. Oxygen partial pressures (O2pp) from the optodes were recorded every five minutes for several diurnal courses. The oxygen sensors are designed to show no cross sensitivity for carbon dioxide, hydrogen sulfide, ammonia, pH or any ionic species like sulfide, sulfate or chloride (Klimant et al., 1995). Values of oxygen measurements were compensated for water temperatures close to the implanted optodes.

Biofilm characterization

Adventitious roots (diameter 2–3 mm) were collected from the measured rhizomes from a depth of 7–10 cm below the water table of the CW. Root samples were prepared for transmission electron microscopy (TEM) and immunofluorescence analysis via light microscopy. Fixation was performed chemically with 0.5 % glutaraldehyde in isoosmotic phosphate buffer at pH 7.0 and the samples were infiltrated with LR-Gold or Lowicryl K4M (Roth et al., 1981). In the immunoassays polyclonal antibodies directed against methanol dehydrogenase were used to detect methylotrophic and methane-oxidizing bacteria (MOB) in the root-associated biofilms. By counting fluorescing cells and total abundances of bacteria, the share of MOB inside the biofilms was evaluated.

2.4.4 Results and discussion

Diurnal oxygen recording

Oxygen measurements in rhizomes of both plants showed distinct diurnal patterns repeating every day of measurement (Fig. 2.14). Before sunrise oxygen partial pressures (O2pp) were around 80 hPa. With little delay after sunrise and at the moment when relative air humidity (RH) began to fall, oxygen concentrations increased steeply. Over midday when RH was low, O2pp stayed at a plateau level of around 185 hPa until 18:00. As soon as radiation decreased and RH rose again, O2pp in rhizomes began to fall rapidly. The decline of O2pp was exponential overnight and levelled out low before sun-

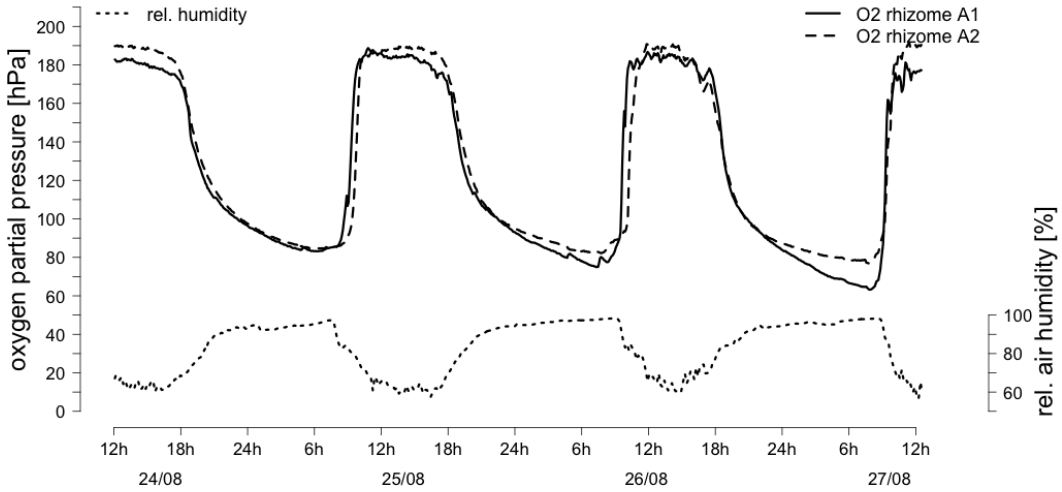

Figure 2.14: Diurnal courses of oxygen partial pressures in rhizomes A1 and A2, and of relative humidity of the ambient air.

rise. Diurnal O2pp courses in both rhizomes showed high negative correlations with relative air humidity (A1: $r \leq -0.94$; A2: $r \leq -0.95$; for all correlations $p < 0.001$).

Pressurization in emergent wetland plants with cylindrical shoots and linear leaves has been investigated to be mainly driven by humidity-induced convection (*Eleocharis spacelata* R.Br., *Typha domingensis* Pers., *Typha latifolia* L., *Typha angustifolia* L., *Juncus ingens* N.A.Wakef., *P. australis* (Cav.) Trin. ex Steud.; (W. Armstrong and J. Armstrong, 1991; Brix et al., 1992; Colmer, 2003; J. Armstrong et al., 1992; Brix et al., 1996; Bendix et al., 1994; Tornberg et al., 1994)). Until now little has been recorded about dynamics of internal oxygen partial pressures in the field. The diurnal O2pp courses recorded in this study clearly demonstrate the dependencies between internal oxygen concentration and relative air humidity (Afreen et al., 2007) as reflected in close negative correlations. This relationship is thus confirmed also for a constructed wetland with an organic load exceeding the natural wetland conditions.

Overnight when pressurization mechanisms were missing and photosynthesis ceased only restricted oxygen is available in the gas-space continuum of *P. australis* plants, especially in the rhizomes and roots. Submerged plant tissue and substrate microorganisms fed on the oxygen reserves generated over the day time. When oxygen partial pressures dropped below values of $120\,\mathrm{hPa}$, steep course O2pp slopes flattened out. One explanation is that below a certain partial pressure, oxygen can not cross the barriers for radial oxygen loss (W. Armstrong et al., 2000) to the substrate and thus remains inside the rhizome and root gas-spaces. Another possibility is that substrate related

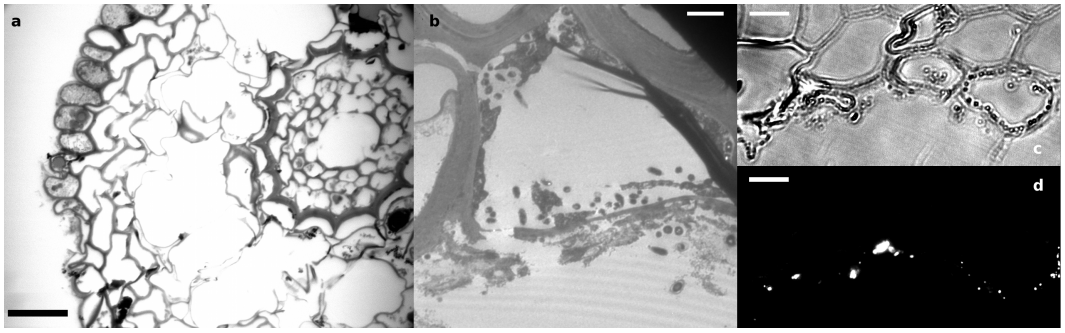

Figure 2.15: Root cross sections from *P. australis* showing (a) the organization of a lateral root, internal gas spaces and colonized exodermis cells. (b) Enlargement of root-associated biofilms in TEM. High abundances of bacteria active in methane turnover in biofilms: Bright-light image (c) and fluorescing bacteria labelled by antibodies against methanol-dehydrogenase (d). Bars: (a) 20 µm, (b) 2 µm, (c, d) 10 µm.

oxygen demand slowed down because plant tissue as well as root-associated microorganisms reduced their respiration below this level of oxygen availability. A recent study showed that root respiration in pea seedlings declined when cortical O2pp in the root tips decreased below 5 to 45 hPa (W. Armstrong et al., 2009). Here we have measured oxygen concentrations in rhizomes emerging from the water table which did not drop below 70 hPa. The oxygen concentrations in horizontal rhizomes of the same study site decreased below 40 % (∼45 hPa) during the night (Dickopp et al., 2011). Thus it can be assumed that O2pp in the roots dropped to values in the range of the proposed critical oxygen pressures of roots in anoxic sediments (W. Armstrong et al., 2009) which results in reduced activity and respiration. A third explanation is that around 70 hPa resembles the level of oxygen that is reached in this part of the rhizome by diffusion alone through the aerenchymatic tissues of shoots and rhizomes (W. Armstrong, 1980; Colmer, 2003). Also wind driven venturi effects can ventilate the rhizome system at night (Colmer, 2003; J. Armstrong et al., 1992).

Root-associated methanotrophic biofilms

Cross sections of *P. australis* roots revealed high proportions of gas-spaces in the tissue (Fig. 2.15a). Attached to the root surface numerous bacteria were detected in biofilms (Fig. 2.15b). Additionally, up to 45 % of root exodermis cells seemed to be colonized with bacteria (Fig. 2.15a). Evaluation of root-associated biofilms revealed 34–37 % of the bacteria to be potential methane-oxidizing bacteria (Fig. 2.15c, d).

By principle, the water purification capacity of constructed wetlands depends on the oxygen supply to the rhizosphere, where oxidative processes degrade organic pol-

lutants. Certain microbial communities develop in the rhizosphere of different plant species (Yuan et al., 2011). On such sites with high organic load and high oxygen demand many aerobic bacteria rely on spatially restricted oxygen release by roots of aerenchymatic wetland plants. Outside of these oxic zones, methanogenesis is the prevailing process of organic matter degradation. In this context, root-associated methanotrophs play a crucial role in the carbon cycle and in mitigation of methane emissions from the wetland to the atmosphere.

High shares of potential methanotrophic bacteria detected on roots of *P. australis* demonstrate that the latter microorganisms play an essential role in the carbon cycle in ecosystems with waterlogged soils (Bodelier, 2003; Conrad, 1996; Jespersen et al., 1998). Especially on sites with high organic load and high oxygen demand like constructed wetlands aerobic bacteria like methanotrophs rely on spatially restricted oxygen release by roots of aerenchymatic wetland plants.

Wetland plants like *P. australis* also provide easily degradable organic substances to the rhizosphere that result in an additional emission of carbon dioxide from the CW (Picek et al., 2007). Plant roots release oxygen in a passive process that is restricted to the root tips (W. Armstrong, 1980; Colmer, 2003) and was thus occasionally regarded as irrelevant for the purification capacity of CW. But root oxygen release is enhanced by convective throughflow that rises the oxygen levels in the rhizomes close to ambient air (W. Armstrong et al., 2000; Colmer, 2003). It could be shown that the oxygen released to the soil is effectively used in the planted CW to oxidize methane and phytotoxic compounds, and supports the waste water purification processes, especially denitrification (Wießner et al., 2005; Picek et al., 2007). Oxygen is also provided for bacterial processes in cold periods without active plant growth (Picek et al., 2007) by diffusion and venturi effects through the submerged rhizomes of *P. australis*.

2.4.5 Conclusions

Common reed (*P. australis*) showed high capacity to ventilate oxygen actively to submerged tissues. Diurnal oxygen dynamics in the rhizomes revealed very consistent courses of oxygen supply. Internal oxygen reserves created during the day were consumed rapidly over the night but not depleted as respiration can be assumed to slow down and diffusion processes sustain a steady-state but low oxygen level. This indicates the huge demand for oxygen in rhizomes, roots and the rhizosphere. A dense biofilm on the rhizoplane shows that the oxygen released to the rhizosphere is ade-

quate to supply bacterial communities active in purifying the waste water of the CW. Also methane-oxidation is common process around the roots, as about one third of the attached bacteria are potential methane-oxidizers. Through the continuous gas-spaces of *P. australis* oxygen can be provided to below ground even in periods outside the growing season, as can be seen in the high purification efficiency of the studied CW.

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Many, many thanks go to my family, Stefan and Ylva, for being there and helping me when I needed it most.

B Declaration of own work and cooperations

In-situ diurnal dynamics of oxygen and carbon dioxide concentrations in shoots and rhizomes of *Phragmites australis* in a constructed wetland

During the field trip to Slavošovice, Czech Republic, I, Anna Faußer, participated strongly in the design of the study and performed the measurements of oxygen concentrations, photosynthetic gas-exchange and micro-climate on the *Phragmites australis* plants in the constructed wetland. Jiří Dušek analysed the carbon dioxide content in the gas samples from the reed culms and provided the data for me. I evaluated the data for the preparation and writing of the manuscript. These included: oxygen concentrations recorded in the six culms and two rhizomes, rate of photosynthesis and stomatal conductance, micro-climate parameters. I compared and interpreted the oxygen and carbon dioxide concentrations and evaluated correlation with micro-climate and plant activity. Prof. Marian Kazda and Prof. Hana Čížková were the supervisors of the project.

Relationship between oxygen saturation in rhizomes and rhizosphere oxygenation by *Typha angustifolia* L.

The experiments of this greenhouse study were performed by Norbert Gäng under supervision of Marian Kazda. I, Anna Faußer, evaluated the data and prepared the manuscript to the current version. Because I have performed the above mentioned study, I was already experienced in the evaluation of plant-internal oxygen concentrations.

Roots of the wetland plants *Typha latifolia* and *Phragmites australis* are inhabited by methanotrophic bacteria in biofilms

I, Anna Faußer, collected and fixed the root samples chemically from the studied *Phragmites australis* plants (see section 2.1) in Slavošovice, Czech Republic. In the Central

Microscopy Unit around Paul Walther, I participated in the performance of high-pressure freezing fixation of the *Typha latifolia* roots samples. In his facility, the root samples were also embedded and most of them sectioned. Michael Hoppert and his group advised me in the chemical fixation method and provided the facility and antibodies for the immunological labelling of the methane-oxidizing bacteria (MOB). In his lab in Göttingen, I conducted many of the sections for the MOB-labelling and its evaluation via fluorescence microscopy and transmission electron microscopy. I evaluated the data and wrote the article. Prof. Marian Kazda was the supervisor of this study.

Internal oxygen dynamics in rhizomes of *Phragmites australis* and presence of methanotrophs in root biofilms in a constructed wetland for waste water treatment

This implementation is the combination of both the above studied plant-internal oxygen dynamics in rhizomes of *Phragmites australis* and the analysis of methane-oxidizing bacteria on the root-associated biofilms. I, Anna Faußer, evaluated the data and wrote the manuscript. The article was presented by me in a talk on the CEMEPE Conference in Skiathos, Greece, in 2011.

C Erklärung

Ich versichere hiermit, daß ich die Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

Ulm, den .....

Anna Charlott Faußer

D Academic curriculum vitae

Personal information

Anna Charlott Faußer, née Honold
Born in Göppingen, Germany

Education

since October 2008 **Doctoral degree 'Dr. rer. nat' in Biology (equivalent to Ph.D.)** from the University of Ulm, Ulm, Germany. This work was done at the Institute of Systematic Botany and Ecology, University of Ulm, Germany.

October 2002 - October 2008 **Diploma in Biology (equivalent to M.Sc.)** from the University of Ulm, Ulm, Germany. **Main subjects:** *Molekulare Botanik* (molecular botany), *Ökologie* (ecology), *Pharmakologie* (pharmacology). **Secondary subject:** *Informatik* (computer science). **Diploma thesis:** Diplomarbeit: *Analyse von Mutanten der chloroplastidären Transportwege in* Arabidopsis thaliana (Analysis of mutants of chloroplastic transport ways in *Arabidopsis thaliana*).

Work experience

June 2006, June 2008, since October 2008 **Teaching assistant** at the Institute of Systematic Botany and Ecology, University of Ulm, Germany. **Main activities and responsibilities:** Planning and holding practical courses and field trips in ecology: Undergraduate courses (basic ecology and soil ecology), graduate courses (tropical ecology, mediterranean ecology).

April 2007 - May 2007 **Study-related practical training** at the *Kompetenzzentrum Obstbau-Bodensee*, Ravensburg, Germany. **Main activities and**

responsibilities: Planning and conducting experiments in agriculture.

June 2005 - July 2005 **Study-related practical training** at the *Museum für Naturgeschichte* (museum of natural history), Mainz, Germany. **Main activities and responsibilities:** Excavation of miocene fossils in *Ur-Rhein*, close to Eppelsheim, Germany.

Research interests

Interpretation and analysis of the ecological importance of oxygen and carbon species in upland and wetland soils.

Thesis-related travelling

June 2015 (4 days) **Maastricht, The Netherlands:** Rhizosphere 4 Conference. Oral presentation.

February 2015, March 2015 (4 days each) **Montpellier, France:** Study at the ECOTRON.

May 2012 (4 days) **Northern Germany:** Excursion to wetlands of Northern Germany.

March 2012 (1 day) **Stuttgart, Germany:** 7th *Umweltbildungskongress*.

August 2009 (3 days), August 2011 (5 days) **Göttingen, Germany:** Immunological labelling of MOB at the Institute of Microbiology at the Georg-August University of Göttingen.

June 2011 **Island Skiathos, Greece:** CEMEPE Conference. Oral presentation.

May 2009, May 2011 (12 days each) **Island Cres, Croatia:** Excursion and student supervision.

April 2011 (3 days) **Rostock, Germany:** Workshop of DBG on wetlands.

May 2010 (8 days) **London, Great Britain:** Excursion to the Botanical Garden of Kew and the Seed Bank.

March 2010 (4 days) **Kiel, Germany:** Meeting AKOE. Poster presentation.

August 2009 (12 days) **Slavosovice, Czech-Republic:** Field study to a constructed wetland. Cooperation with Jiří Dušek and Prof. Hana Čížková.

April 2009 (4 days) **Bayreuth, Germany:** Workshop about isotopes in research.

Journal publications (peer-reviewed)

A. C. Faußer et al. (2016). "Diurnal dynamics of oxygen and carbon dioxide concentrations in shoots and rhizomes of a perennial in a constructed wetland indicate down-regulation of below ground oxygen consumption". In: *AoB Plants*. DOI: 10.1093/aobpla/plw025

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