Phosphate uptake by aquatic macrophytes at varying phosphorus availability

PhD Thesis

By

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Front page photo: Cross section of adventitious roots of rice. Photo by Nina Høj Christiansen
This PhD dissertation represents a part of the requirements for obtaining the degree of Doctor of Philosophy at the Department of Biology, Faculty of Science, at the University of Southern Denmark. Most of the work in this dissertation has been carried out at the Department of Biology, The University of Southern Denmark under the supervision of Dr. Henning S. Jensen and Dr. Frede Ø. Andersen. A part of the research has been carried out at School of Plant Biology, at the University of Western Australia, under the supervision of Dr. Timothy D. Colmer, Dr. Dennis Konnerup and Dr. Ole Pedersen. This dissertation was financed by the Danish Council for Independent Research, Natural Sciences grant No. 09-067485, and supported by the Centre for Lake Restoration, a Villum Kann Rasmussen Centre of Excellence.

The dissertation includes three manuscripts (Chapter 1-3) and all three are under preparation for submission. Fieldwork and laboratory work for Chapter 1 was conducted in Lake Hampen and at the Department of Biology, University of Southern Denmark. Fieldwork and laboratory work involved in Chapter 2 and 3 was conducted at School of Plant Biology, at The University of Western Australia, and partly at the Department of Biology, University of Southern Denmark.

The field of research in this dissertation focuses on aquatic macrophytes and their phosphate uptake characteristics at varying phosphate availability. This dissertation includes an introduction to the field of research, including a short outline of the three chapters and a conclusion where results are put into perspective.

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Enjoy!

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Phosphate uptake by aquatic macrophytes at varying phosphorus availability
Summary
In the freshwater ecosystems the macronutrient phosphorus is one of the major compounds limiting plant growth. As a consequence of the increased input of phosphorus to our surface waters during the last century, aquatic macrophyte community compositions have changed. In many oligotrophic lakes the macrophyte communities have changed in a direction where fast growing, large macrophyte species outcompetes small slow growing species. These community shifts leads to major changes in the ecosystems. Plants take up phosphorus in the dissolved inorganic form phosphate. To be able to manage and control macrophyte community compositions in the future, it is important to understand how the different species compete for phosphate.

To investigate this, phosphate uptake kinetic experiments were conducted using a $^{33}$P radioisotope technique to accomplish the phosphate uptake rates at a wide range of phosphate concentrations. The phosphate uptake kinetics were determined for six aquatic macrophyte species, two isoetid species (Littorella uniflora and Isoetes australis), three submerged elodeid species (Potamogeton perfoliatus, Myriophyllum alterniflorum and Elodea canadensis) and one emergent macrophyte species (Oryza sativa). For O. sativa it was additionally investigated whether the barrier to radial oxygen loss (ROL), being cell wall depositions that impede oxygen diffusion from the root aerenchyma to the rhizosphere, induced when grown under anaerobic conditions, would affect the phosphate uptake kinetics in the roots. For I. australis the short term phosphate translocation was examined in a $^{33}$P pulse-chase experiment.

Combined the studies showed that aquatic macrophytes possess species specific phosphate uptake kinetics. The isoetid species possess a low range phosphate concentration where they are competitively dominant. I. australis’ short term phosphate translocation was most evident in a root to shoot direction. The elodeid and emergent species show great plasticity and possess phosphorus uptake characteristics that make them strong competitors at a broad range of phosphate concentrations. O. sativa improved its phosphate affinity and uptake rate when grown anaerobic. Submerged macrophyte species showed phosphate uptake both in shoot and root tissues. However root uptake was most significant at ambient phosphate concentrations. When phosphate availability in the overlying water increases the elodeids can benefit from a large shoot uptake.

This study has shown that phosphate availability must be kept at a low level to meet the isoetid species where they are competitively superior. Especially increased phosphate concentrations in the overlying water may result in the isoetids disappearance. However other chemical and physical factors affect the macrophyte communities and should also be taken into account in the progress of preserving these unique species and to ensure high macrophyte diversity in our freshwater systems.
**Resume**

I ferskvandsøkosystemer er fosfat en af de centrale næringsstoffer der begrænser plantevæksten. Som en konsekvens af de øgede mængder fosfor der er tilført vores overfladevand igennem det sidste århundrede, har plantesamfundene med de akvatiske makrofyter ændret sig. I mange oligotrofe søer har de akvatiske makrofytsamfundene ændret sig i en retning, hvor store og hurtigt voksende arter udkonkurrere små langsamt voksende arter. Disse forandringer i makrofytsamfundene resulterer i omfattende ændringer i økosystemerne. Makrofyter optager fosfor i den opløste uorganiske form fosfat. For i fremtiden at være i stand til at kunne forvalte makrofytsamfund er det vigtigt at få en forståelse for, hvordan de enkelte arter konkurrerer om fosfaten.

For at undersøge fosfatoptagelseskinetikken for akvatiske makrofyter, blev der opstillet kinetik forsøg ved brug af en $^{33}\text{P}$ radioisotop teknik for at finde frem til raterne for fosfatoptaget ved varierende fosfakoncentrationer. Fosfatoptagelsesrater blev bestemt for seks arter af akvatiske makrofyter, to isoetider (Littorella uniflora og Isoetes australis) tre arter af neddykkede elodeider (Potamogeton perfoliatus, Myriophyllum alterniflorum og Elodea canadensis) og én emergent art (Oryza sativa). For O. sativa blev det derudover undersøgt, om barrieren for radial tab afilt (ROL), havde en effekt på rodens kinetiske fosfat-optagelse. Den kortvarige fosfat translokering for I. australis blev undersøgt ved hjælp af et $^{33}\text{P}$ "Pulse-Chase" forsøg.


Dette studie viser at fosfatconcentrationen skal holdes på et lavt niveau for at arterne af isoetider er konkurrencedygtige. Specielt kan forøgede fosfatconcentrationer i overfladevandet resulterer i at isoetiderne kan forsvinne. Dog påvirker andre fysiske og kemiske faktorer også makrofytsamfundene og disse faktorer bør inkluderes i processen som skal sikre disse unikke arter samt for at sikre en høj diversitet af makrofyter i vores ferskvandsøkosystemer.
General introduction

Background and purpose
Freshwater ecosystems provide a great variety of habitats for aquatic macrophytes and algae. Both lentic and lotic freshwater ecosystems host aquatic macrophytes and algae in various adapted forms. This thesis will focus on aquatic macrophyte species adapted to live in the shallow lentic ecosystems, even though many aquatic macrophyte species can inhabit both the lentic and lotic systems. The lentic system refers to the standing water and can be found in ecosystems such as lakes, ponds, swamps and wetlands (Wetzel 2001). Such standing waters not only consist as closed systems but also include its full drainage basin (Wetzel 2001). Aquatic macrophytes dominate in the land-water interface in the littoral zones of the lakes and in the wetlands. The work in this PhD study has been conducted on a variety of rooted aquatic macrophyte species adapted for growth under different lentic conditions.

As a consequence of the increased nutrient input to the freshwater systems during the last century and the resulting eutrophication of many freshwater environments, the plant communities have changed in a direction where epiphytes, filamentous algae and elodeids are spreading in the lakes and as a result are competing with isoetids for nutrients and light (Roelofs 1983; Sand-Jensen et al. 2000; Brouwer et al. 2002; Pedersen et al. 2006). Since the eutrophication in most freshwater ecosystems is driven by phosphorus (e.g. Christiansen et al. 1985; Wetzel 2001) the focus of this PhD thesis has been on the phosphate uptake characteristics of different macrophyte species. The primary aim of this PhD thesis has been to gain a better understanding of why these community shifts happen in relation to the macrophytes phosphate uptake. From this knowledge the competitive advantages and disadvantages of the different macrophyte species phosphate uptake characteristics at varying phosphate availability will be discussed. Six aquatic macrophyte species were included in the investigations conducted; two isoetid species (*Littorella uniflora* L. and *Isoetes australis* S. Williams), three submerged elodeid species (*Potamogeton perfoliatus* L., *Myriophyllum alterniflorum* DC. and *Elodea canadensis* L. C. Rich.) and one emergent macrophyte species (*Oryza sativa* L.). These different species were expected to cover both different lentic freshwater environments and to have different adaptations in phosphate uptake in relation to morphology and to phosphate availability. A wide range of phosphate concentrations were used in the experiments; from extremely low phosphate concentrations, including most ambient phosphate contraptions in oligotrophic environments, up to extremely high phosphate concentrations, which were expected sufficient to reach the macrophytes
maximum phosphate uptake rates. It is the hope that the gained knowledge in this thesis can contribute to the future administration and management of the freshwater environments to ensure preservation of habitats and to prevent species loss.

In the following, a short introduction to the aquatic macrophytes will be given in relation to their phosphate uptake and in relation to their phosphate availability. In addition, the importance of phosphorus in the freshwater ecosystems will be introduced. Finally an outline of thesis and a concluding perspective will be given.
Aquatic Macrophytes

The aquatic macrophytes belong to different taxonomic classes and families and have very diverse growth forms. Grime (1977) defined two categories of factors that influence plant growth forms; one category includes the stress factors that limit plant growth such as nutrient and light availability, the other category includes the disturbing factors defined as lost biomass from disturbances such as grazing or wave exposure. Different macrophyte species have different adaptations and traits that enable them to live in their respective environment and niches. Plants growing together will compete for the resources they share within their immediate environment, which can impact growth outcomes for one individual versus another. Competition can be defined as the interaction between individuals that share the same limiting resources (Grime 1977). This section will give a brief introduction to the rooted aquatic macrophytes.

Emergent macrophyte species growth in the land-water interface in waterlogged soils, which is generally defined to be 0.5 meter above the water table to about 2 m depth (Wetzel 2001), and is characterized by the shoot being present above the surface of the water. These emergent macrophytes are usually different perennial reeds (e.g. Phragmites and Typha) characterized by growing in large monocultures with shoot height up 4 m (Schierup 1986) and often having rhizomes or corms.

Floating-leaved macrophytes are angiosperms, rooted in submerged sediments from about 0.5 to 3 m depth with both submerged and floating leaves (e.g. Nuphar and Potamogeton natans) (Wetzel 2001). Both the emergent and the floating-leaved macrophytes are wind and wave exposed and are usually found growing in areas along the shorelines with low wave activity.

Submerged aquatic macrophytes are rooted, fully submerged plants (angiosperms and pteridophytes) with the exception of a few species which are also amphibian (Moeslund et al. 1990; Wetzel 2001). Given the sufficient light availability these species grow to a depth of about 10 m, with the mosses and charophytes capable of growing to greater depths (Wetzel 2001). The amphibian species are able to grow both fully submerged in water and in waterlogged soils with leaves exposed above the waters surface. The submerged aquatic macrophyte species covers both the isoetid and the elodeid species. In the littoral zone and in shallow areas, the vegetation is dominated by the amphibian isoetids, while the submerged elodeid species grow on deeper water.

The isoetids belong to both the angiosperms and the pteridophytes (the quillwort Isoetes) but are all characterized by having similar rosette growth form inhabiting nutrient poor softwater lakes with high visibility (Moeslund et al. 1990). These species are evergreen and slow-growing, and are characterized by being stress-
tolerant with short leaves and a high root to shoot ratio (Brouwer et al. 2002; Murphy 2002; Pedersen et al. 2006). They possess several adaptations to be able to cope with low carbon and nutrient availability. In addition, most isoetids develop symbiosis with mycorrhizae, which enhance the ability to extract phosphorus and nitrogen from a larger sediment volume (Wigand et al. 1998; Andersen and Andersen 2006). This gives these species a competitive advantage in nutrient poor environments, compared to, for example, the elodeids, which have not been found to create mycorrhizae (Beck-Nielsen and Madsen 2001). These species are amphibian and their morphology is adapted to cope with wind and wave exposure by having short, thick, stiff leaves and a large root biomass to anchor them.

The elodeids belongs to the angiosperms and inhibit both softwater lakes and more alkaline waters with higher nutrient content (Hutchinson 1970; Roelofs et al. 1984). Elodeids are characterized by having long erect shoots and a relatively high growth rates. Elodeids can form large canopies, and up to several meters long (Moeslund et al. 1990), towards the surface where the light intensity is highest. These characteristics convey a significant competitive advantage for light compared to the isoetids.

Aquatic macrophytes are important in several ways for the freshwater environments (Carpenter and Lodge 1986). They stabilize the sediment and thereby inhibit sediment resuspension and erosion induced by wave action from wind and benthic fish species, and promotes sediment accumulation (Carpenter 1981; Barko and James 1997). Resuspension and erosion enhance nutrient cycling, which reduces water clarity and leads to high phytoplankton biomass (Søndergaard et al. 1992; Barko and James 1997). Aquatic submerged macrophytes provide shelter and refugia for zooplankton, macroinvertebrates and fish and thereby contributing to a lower phytoplankton level in shallow lakes, and creating more clear water (Scheffer et al. 1993; Lauridsen et al. 1997). Aquatic macrophytes also interact with sediment nutrients, both directly and indirectly (Barko and James 1997). Direct interactions are characterized by nutrients moving directly from the sediment to tissues in the water, such as nutrient leaching and degradation of dead organic matter. Indirect nutrient interaction, for example, includes photosynthesis and respiration, which can create changes in pH, CO₂ and O₂ in the surrounding water (James and Barko 1991; Barko and James 1997). In phosphorus rich sediments changes in pH can result in significant phosphorus release from the sediment (Barko and James 1997). For example is the sorption capacity for phosphorus high in sediment with high Fe:P ratios (>15 by weight) as long as the microzone remains oxidized (Jensen et al. 1992). However, phosphorus release from littoral sediments can be enhanced at higher pH.
even under aerobic conditions. Increases in pH from about 8 to 9 can result in at least a doubling in the rate of phosphorus release from aerobic littoral sediments (Boers 1991).
Sources of phosphorus
The natural sources of phosphorus are related to the soil characteristics in the drainage basin (Wetzel 2001). Surface drainage is the major source of phosphorus to streams and lakes, but atmospheric precipitation from dust generated over land and from ground water also contributes to phosphorus levels (Wetzel 2001). The increased phosphorus input to the freshwater systems observed during the last century is due to anthropogenic activities (Wetzel 2001). Chemical inputs to the ecosystems are classified as ‘point’ or ‘nonpoint’ sources (Carpenter et al. 1998; Fig. 1). The major source of phosphorus to surface waters is nonpoint sources, which result primarily from agriculture and urban activity, including industry (Carpenter et al. 1998). Typical nonpoint sources result from runoff associated with agricultural land use involving phosphorus rich fertilizers, and urban runoff from unsewered areas, septic tank leachate, failed septic systems, construction sites, fish farms, altered land use and activities on land that generate contaminants (Carpenter et al. 1998; Wetzel 2001). Point sources are typically industrial wastewater, including wastewater from treatment plants, overflows from sanitary sewers, runoff and leachate from waste disposal sites and industrial and construction sites (Carpenter et al. 1998).
Phosphorus availability
All living organisms need a variety of essential ions for living. The availability of these ions affects the species composition of plant and animal communities. Some of these ions may be present in insufficient concentrations in relation to the needs of the organisms and thus are limiting for their growth. In the aquatic environment, ions such as phosphate, ammonium and nitrate are some of the main factors limiting the growth rate and biomass production of aquatic macrophytes and algae (Wetzel 2001). A large input of nutrients such as phosphorus and nitrogen into the aquatic systems can thereby result in an eutrophication of the system, which is defined by an aquatic ecosystems response to enrichment in inorganic nutrients (Wetzel 2001). The most vulnerable aquatic systems to an increase in phosphorus and nitrogen supply are the lakes and estuaries. An increase in the availability of these ions in the aquatic environments can result in major changes in the aquatic plant communities (Roelofs 1983; Sand-Jensen et al. 2000; Brouwer et al. 2002), where some species, such as the isoetids (Pedersen et al. 2006), are outcompeted by the

Figure 2 Box plots of data on submerged macrophyte coverage in percentage (number of lakes 30-60) and phytoplankton biovolume (number of lake years 495) along a TP gradient at low alkalinity (low TA) and high alkalinity (high TA) and in deep and shallow lakes. For each box 10% (bottom end of line), 25% (bottom edge of box), median (connected by lines), 75% (top edge of box) and 90% (top end of line) percentiles are shown. Modified from Søndergaard et al. (2005).
longer and faster growing elodeid species (Smolders et al. 2002). Søndergaard et al. (2005) found when relating chemical and biological data from 709 Danish lakes that both macrophyte coverage and their maximum depth decreased along a total phosphorus gradient (Fig. 2). Especially isoetid species showed in their proportional contribution to macrophyte cover a decrease from a median of 59% in lakes with total phosphorus concentrations < 25µg P L\(^{-1}\) to 0% in lakes with total phosphorus concentrations >100 µg P L\(^{-1}\) (Søndergaard et al. 2005). Pedersen et al. (2006) found similar evidence when comparing physical and chemical parameters in 472 lakes from where L. uniflora had disappeared from the 218. The disappearance of L. uniflora was primary explained by higher total phosphorus and total nitrogen in the water, as well as decreased transparency, increased chlorophyll in the water, catchment area and alkalinity (Pedersen et al. 2006). In the end the eutrophication will lead to a shift in the plant communities from a dominance of rooted macrophytes to a dominance of phytoplankton (Wetzel 2001), which is also evident from the investigations by Søndergaard et al. (2005; Fig. 2) and Pedersen et al. (2006). These phytoplankton blooms result in decreased light penetration and oxygen depletion at the bottom leading to a cascade of effects. In the 20\(^{th}\) century the input of especially phosphorus and nitrogen to the aquatic environments from urban wastewater and intensified agricultural production increased in the northern part of Europe and as a consequence up to 90% of the aquatic environments have changed to an eutrophic status (Sand-Jensen et al. 2000, Brouwer et al. 2002; Pedersen et al. 2006).

In the freshwater systems, especially lakes, phosphorus has been the most important limiting nutrient (Carpenter et al. 1998). For this reason, no other element in the fresh water systems has been studied so intensively as phosphorus (Wetzel 2001). Plants take up phosphorus in the dissolved inorganic form called orthophosphate or phosphate (PO\(_4^{3-}\)), but phosphate only constitutes a few percent of total phosphorus in the freshwater systems. More than 90% of the phosphorus in the freshwater systems is incorporated in organic matter or adsorbed to inorganic or organic particles and material (Wetzel 2001). Phosphate is immobilized by oxides of for example iron (Fe) and aluminium (Al). Most important in lakes is the binding to iron. Phosphate binds to the oxidized form of iron (ferric iron/Fe\([\text{III}]\)), and dissolves again when sediments get reduced. When this happens, the phosphate will be released to the water phase and be available for the primary producers. Oxygen concentrations, pH, and the presence of these ions therefore affect the concentrations of available phosphate for plant uptake (Jensen et al. 1992; Smolders et al. 2002; Geurts et al. 2008). For example is the solubility of aluminum phosphate (AlPO\(_4\)) and ferric phosphate (FePO\(_4\)) at a minimum at pH 6 and increases at both higher and lower pH
(Wetzel 2001). Also adsorption to positively charged surfaces, particularly clay, affects the inorganic soluble phosphorus concentrations, and here high phosphate adsorption is pH 5-6 (Wetzel 2001). Oxygen content in the sediment-water interface is influenced by the respiratory metabolism of bacteria, algae, fungi, invertebrates, plants and the degradation of dead organic matter. Diffusion of oxygen from the overlying water to the sediment is slow and will only penetrate a few mm into the sediment. The oxygen penetration into the sediment will thereby depend both on the oxygen supply to the sediment and on the oxygen demand of the sediment (Wetzel 2001). Sediments and waterlogged soils therefore become anoxic. Under these conditions, the macrophytes experience a higher availability of phosphate (Wium-Andersen and Andersen 1972; Wetzel 2001; Smolders et al. 2002). Some aquatic macrophytes (e.g. O. sativa, Chapter 3; Typha domingensis and Cladium mariscus; Brix et al. 2010) are able to benefit from this higher phosphate availability by increasing their phosphate uptake rate when grown in waterlogged anaerobic conditions. O. sativa showed in a study (Chapter 3) that roots grown in anaerobic conditions had a higher (up to about 2.5 times) uptake rate for phosphate compared to roots grown in aerated conditions (Fig. 3).

Figure 3 Phosphate uptake rates (µmol g⁻¹ DM h⁻¹) as a function phosphate concentrations (µmol L⁻¹) for adventitious root segments of O. sativa (E = 0-13.3 mm; F = 13.4-26.6; G = 26.7-39.9 mm; H = 47-60 mm behind root tip). Every graph illustrates root segments cultivated in aerated (clear symbols) and stagnant (filled symbols) conditions (mean ± SE, n = 3). Modified from chapter 3.
The isoetids oxidize the rhizosphere by transporting oxygen to the sediment via lacunae in leaves to roots with consequent radial oxygen loss (ROL) from the roots. When the sediment is oxidized, phosphate precipitates as FePO$_4$, consequently decreasing the phosphate concentrations in the sediment porewater. High densities of isoetids can thereby change the sediment characteristics and make it harder for other species to assimilate phosphate (Wium-Andersen and Andersen 1972; Sand-Jensen et al. 1982). Oxidized sediment also reduces the transport of phosphate into the surface water (Smolders et al. 2002; Geurts et al. 2008). On the contrary, many aquatic macrophytes develop a barrier in the basal zones of the roots to minimize radial O$_2$ loss (ROL) to the surrounding environment (Colmer et al. 1998). A barrier to ROL is, for example, known to develop in adventitious roots of rice (Oryza sativa L.), when grown in stagnant solution (Colmer et al. 1998), and it is considered to be a result of suberin and lignin depositions in the exodermis and in the sclerenchyma of the root (Kotula et al. 2009; Chapter 3). In this way the plants does not loose valuable oxygen to the surrounding reduced sediment and in the same time benefit from the higher phosphate availability due to the anoxic conditions.

**Figure 4** Phosphate uptake rates (µmol g$^{-1}$ DW h$^{-1}$) as a function of phosphate concentration (µmol L$^{-1}$) illustrated by Michaelis-Menten saturation kinetics for root and leaf tissue of *L. uniflora*, *P. perfoliatus* and *M. alterniflorum* in June (solid lines). Solid lines are best fit to the Michaelis-Menten model and dotted lines are 95 % confidence intervals. Notice that axes scales are not the same. Modified from chapter 1.
Macrophytes and their phosphate uptake

As mentioned above, plants take up phosphorus in the dissolved inorganic form called phosphate. For this reason phosphate is the most significant form in the freshwater systems (Wetzel 2001). Phosphate is incorporated in the plants in organic matter and is primarily used as building blocks in DNA, ATP and in phospholipids in the plants’ cell membranes (White and Hammond 2008). When temperature and light availability is high enough for primary production the phosphate in the water phase is used as soon as it becomes available and the phosphate concentrations here may therefore be extremely low. In the sediment porewater, however, the phosphate pools are high from the degradation of organic matter. Rooted emergent aquatic macrophytes rely on their roots to cover their phosphorus requirements (Wetzel 2001, Brix et al. 2010) whereas submerged macrophytes also benefit from shoot uptake. It has been widely discussed which organs are most important for the nutrient uptake by submerged macrophytes, shoots or roots. Some studies have argued that phosphate uptake by the shoot is more important than root uptake (Schults and Adams 1971), but it is now generally accepted that rooted macrophytes can take up phosphate from the sediment (Barko and Smart 1980; Carignan and Kalff 1980; Barko et al. 1991) and in this way satisfy their P requirement at 90-100% level (Carignan and Kalff 1980; Barko and Smart 1981). When phosphate concentrations in the above laying water increase (for example as a consequence of eutrophication) shoot tissue becomes more important in covering the plants phosphorus demand (Chapter 1). Carignan and Kalff (1980) and Christiansen et al. (Chapter 1) found, that next to the different phosphate uptake kinetic characteristics at different species, root and shoot tissue within the same species also showed different uptake characteristics (Fig. 4). Phosphate uptake is therefore primary via the root system, when concentrations in the surface water are low, but most submerged aquatic macrophytes can take up phosphate both by leaves and roots, and are therefore able to take up phosphate from the most available source (Fig. 4).

The phosphate uptake mechanism in aquatic macrophytes is not well known (Thiébaut 2008). Transporter proteins in the plasma membrane transport the phosphate ions over the membrane into the cell (Schachtman et al. 1998; Nussaume et al. 2011). Macrophytes can via these transporter proteins make an active absorption of phosphate against a concentration gradient. This absorption of phosphate is limited by the affinity for phosphate by the transporter proteins and/or number of transporter proteins per surface area and by the availability of phosphate in the surrounding environment. Next to the direct uptake over the cell membrane, aquatic macrophytes also have other mechanisms to facilitate the phosphorus pools in the sediment. Some aquatic macrophyte species
release organic acids from the roots to mobilize phosphorus bound in the particulate organic fraction and in the sediment mineral fraction. For example, formate and lactate have been found in the rhizosphere of the isoetid species, *Littorella uniflora* (Thomsen et al. 2005). Many aquatic macrophyte species also have symbiosis with mycorrhizae to facilitate the phosphorus bound in organic matter and in the sediment minerals (Wigand et al. 1998). In addition, adaptations in root and shoot morphology can improve nutrient uptake (e.g. *P. perfoliatus* and *M. alterniflorum*; Chapter 1) as well as adaptations in root to shoot ratio (*I. australis*; Chapter 3).
Outline of thesis
As mentioned above, this study has determined the phosphate uptake by six aquatic macrophyte species at a range of phosphate concentrations from low to high. From this work the potential effect of changes in phosphate availability on the aquatic macrophytes community composition will be discussed in a broader perspective. In the three manuscripts (Chapter 1-3) it was possible to examine the phosphate uptake kinetics at very low phosphate concentrations (down to 0.016 \( \mu \text{mol P L}^{-1} \)) for root and leaf tissue, by the use of a radioactive isotope \( ^{33}\text{P} \) tracer technique (Nielsen et al. 2006). Determination of the uptake kinetics at low phosphate concentrations is essential, since macrophytes may experience low phosphate concentrations in the lake and porewater with resultant phosphate limitation of macrophyte growth. Since proteins are responsible for the uptake of phosphate, we used the Michaelis-Menten model to describe the characteristics of the uptake kinetics.

Chapter 1 determines and compares the phosphate uptake kinetics in four submerged macrophyte species (\( L. \text{uniflora}, M. \text{alterniflorum}, P. \text{perfoliatus} \) and \( E. \text{canadensis} \)) living in the temperate softwater Lake Hampen (Denmark; Fig. 4). Phosphate is known to limit macrophyte growth in this lake, and this enabled us to compare the species’ competitive characteristics in their phosphate uptake. The isoetid \( L. \text{uniflora} \) showed highest affinity for phosphate in the root tissue. \( M. \text{alterniflorum} \) had the highest affinity for phosphate in the leaf tissue and the highest \( V_{\text{max}} \) and showed as the only species a higher \( V_{\text{max}} \) in leaves compared to roots. For all species root tissue was the dominant organ for phosphate uptake at the phosphate concentrations measured in lake water and porewater in Lake Hampen. However \( M. \text{alterniflorum} \) covered about 40% of its phosphate uptake by the leaf tissue even though phosphate concentrations in the water was about 2 times lower than in the porewater. The results also showed that \( M. \text{alterniflorum} \) had a morphological adaption to the phosphate uptake, where the large shoot surface area explained about half the leaf uptake. \( E. \text{canadensis} \) and \( P. \text{perfoliatus} \) were not as competitively strong at low phosphate concentrations, and especially \( P. \text{perfoliatus} \) performed better at higher concentrations. The result indicates that at low lake water phosphate concentrations \( L. \text{uniflora} \) will be able to survive on the phosphate pools in the sediment porewater. \( M. \text{alterniflorum} \) turned out to have an efficient uptake at both low and high phosphate concentrations by both roots and shoots, suggesting that \( M. \text{alterniflorum} \) will be a strong competitor to isoetids at increasing phosphate concentrations in the water.

Chapter 2 determines the uptake kinetics and the short-term translocation of phosphate in the isoetid species \( \text{Isoetes australis} \) growing in vernal rock pools situated in southwestern Australia in an environment where phosphate availability limits plant growth. \( I. \)
I. *australis* showed a large plasticity in its Michaelis-Menten kinetics across a broad range of phosphate concentrations, from as low as 0.016 µmol L⁻¹ up to several hundred fold higher than the ambient phosphate concentrations. *I. australis* also showed a morphological adaptation to the environment having about twice as much root tissue as leaf tissue, facilitating use of the higher phosphorus pools in the sediment compared to the shallow water column. As a result of the large root surface and better uptake kinetics, roots accounted for 87% of the plants phosphate. The white basal part of the leaves, constituting 23% of the leaf biomass, did not contribute to the phosphate uptake at ambient phosphate concentrations. A pulse-chase experiment revealed that the short-term translocation of phosphate internally in the plant primarily seemed to go from root tissues to the leaf tissue (Fig. 5).

Chapter 3 investigates whether morphological changes induced by growth in anaerobic conditions, in this case the barrier to radial oxygen loss (ROL) in the adventitious of the emergent macrophyte species rice (*O. sativa*), would result in changes in the phosphate uptake capacity. Adventitious roots of *O. sativa* showed a high plasticity in the phosphate uptake characteristics. *O. sativa* showed a high affinity to phosphate irrespective of the surrounding phosphate concentrations. Roots with a well developed barrier to ROL had a greater affinity for phosphate compared to roots with no barrier. The uptake rate was up to 60% greater where the barrier to ROL was present in the root (Fig. 3). Overall, *O. sativa* express both physiological and morphological plasticity, highly adapted to cope with the extreme environment it experiences.

Morphological modifications induced by growth in anaerobic conditions, such as the development of a barrier to ROL, shorter root length, more adventitious roots, reduction in the root to shoot ratios and increased phosphate uptake capacity likely contribute to this tolerance.

**Figure 5** Activity per dry mass (DPM g⁻¹ DM) as a function of time (minutes) in youngest leaves, second youngest leaves, third youngest leaves and roots of *Isoetes australis* (mean ± SD, n = 3). (a) Leaves exposed and (b) roots exposed to radioactive ³³P media. From chapter 2.
**Conclusion and perspectives**

**Community composition related to phosphate availability**

Aquatic macrophytes show characteristic traits in their phosphate uptake kinetics in root and leaf tissue in relation to phosphate availability. Different species show different adaptations to low and/or high phosphate concentrations. These uptake characteristics can help us explain why we see a macrophyte community shift in our lakes in relation to the increased phosphorus input to our freshwater systems towards dominance of elodeids and emergent macrophytes. Isoetids are competitively strong relative to other species in the competition for phosphate at low phosphate concentrations, with roots showing especially high affinity for phosphate at this phosphate range. On the contrary, when phosphate concentrations increase, the isoetids cannot benefit from this larger phosphate availability due to their low $V_{\text{max}}$. But the elodeids and the emergent macrophytes have the advantage when phosphate availability increases. Especially *M. alterniflorum* and *O. sativa* showed a great plasticity in their uptake kinetics, being able to benefit both from low and from extremely high phosphate concentrations. These species can thereby benefit from sudden rises in phosphate availability which can be an advantage in phosphate limited environments (Thiébaut 2008). Some emergent macrophyte species can even adjust their phosphate uptake kinetics in relation to plant demand (*Typha domingensis* and *Cladium mariscus*; Brix et al. 2010) and phosphate availability (*O. sativa*; Fageria 1974; Fig. 6). Our data also showed that both root and shoot tissue can function as a source for phosphate uptake in

![Phosphate uptake rate (µg g⁻¹ FW h⁻¹) by O. sativa as a function of phosphate concentration (µmol L⁻¹). Circles illustrate experimental data. Solid line illustrates theoretical curve calculated according to Michaelis-Menten kinetics. Dashed line is maximal uptake rate at low phosphate concentrations. From Fageria (1974).](image)

**Figure 6** Phosphate uptake rate (µg g⁻¹ FW h⁻¹) by *O. sativa* as a function of phosphate concentration (µmol L⁻¹). Circles illustrate experimental data. Solid line illustrates theoretical curve calculated according to Michaelis-Menten kinetics. Dashed line is maximal uptake rate at low phosphate concentrations. From Fageria (1974).
submerged macrophytes, but that different uptake kinetics appeared for species and organs. Elodeid species seemed to be adapted to take up phosphate from the most available source, whereas *L. uniflora* seemed to prefer roots as primary organ for phosphate uptake.

To be able to manage and control macrophyte growth and the composition of plant communities in the future, it is important to understand and determine which factors (e.g. nutrients, light, sediment characteristics, alkalinity) that contributes to the competition between the macrophytes. As phosphorus is the primary determining factor for numerous biological processes in lake ecology, total phosphorus is chosen as one of the chemical variables used for detecting the ecological status in relation the European Water Framework Directive, where standing waters are one of the four main surface water categories to be dealt with (Moss et al. 2003; Søndergaard et al. 2005). The main target of the European Water Framework Directive is to protect and improve the quality of all surface water resources and to achieve as a minimum ‘good ecological status’ in all waterbodies (Søndergaard et al. 2005). The productivity of most ‘non-polluted’ shallow lakes is dominated by macrophytes with associated epiphytic algae and therefore must lakes, to provide a high or good ecological status, be dominated by bottom-living macrophyte communities (Moss et al. 2003). The macrophyte communities should be of sufficient diversity (not dominated by one or a few species) to provide all the chemical and biological traits of the submerged macrophytes, such as refuges for animals that graze on epiphytes and phytoplankton (Moss et al. 2003). These macrophyte communities can persist if they are not influenced by external impacts, such as eutrophication, which can lead to greater growth of larger and more competitive species and increasing epiphyte biomass and/or phytoplankton (Moss et al. 2003).

In the next River Basin Management Plans (2016-2021) aquatic macrophytes will also be implemented as a biological variable for detecting the ecological status in relation the European Water Framework Directive. Today the target for the total phosphorus level in the surface water of Lake Hampen is 23 µg L$^{-1}$ according the River Basin Management Plans. When comparing this target level with the investigation by (Søndergaard et al. 2005) of the macrophyte coverage in relation to total phosphorus in the water, this target level is just below the critical total phosphorus concentration, given that the largest decline in macrophyte coverage in shallow lakes changed most markedly from the 25-50 to the 50-100 µg TP L$^{-1}$ category (Fig. 2). In low alkalinity lakes the same pattern was showed for the isoetid coverage Søndergaard et al. (2005; Fig. 2).

Of additional concern in flowing waters is nutrient enrichment, as the P dynamics in relation to the aquatic macrophytes have received little attention (Thiébaut 2008).
European Water Framework Directive, one of the only tools used to administrate and control the macrophyte community shifts towards aggressive growth of emergent macrophytes is weed cutting. However, some ‘problem’ species even seem to benefit from this type of expensive management (Christiansen, N.H. unpubl. data). The results from the studies in this PhD indicate that nutrient enrichment in the streams might be worth incorporating in the future River Basin Management Plans 2016-2021.
References


Chapter 1
Phosphate uptake kinetics for four species of submerged freshwater macrophytes measured by a \(^{33}\)P phosphate radioisotope technique

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Abstract

Phosphate (Pi) uptake kinetics were determined in shoot and root tissues for four freshwater macrophyte species, \textit{Littorella uniflora}, \textit{Potamogeton perfoliatus}, \textit{Myriophyllum alterniflorum} and \textit{Elodea canadensis}, using a radioactive \(^{33}\)P phosphate technique. Collection of plant material in the oligotrophic softwater lake, Lake Hampen, Denmark, where Pi limits macrophyte growth, enabled us to characterize and compare the Pi uptake kinetics and competitive characteristics of the four species in a low level Pi environment. The maximum Pi uptake rates (V\textsubscript{max}), the half saturation constants (K\textsubscript{m}) together with the affinity at low Pi concentrations (K\textsubscript{m}/V\textsubscript{max}) were determined by fitting data to the Michaelis-Menten kinetics.

\textit{L. uniflora} showed the highest K\textsubscript{m}/V\textsubscript{max} in the root tissue and the lowest K\textsubscript{m}. \textit{M. alterniflorum} showed the highest and \textit{E. canadensis} and \textit{P. perfoliatus} the lowest K\textsubscript{m}/V\textsubscript{max} in leaf tissue. \textit{M. alterniflorum} had the highest V\textsubscript{max} and, as the only species, a higher V\textsubscript{max} in leaves than in roots. Morphology explained about half of this high V\textsubscript{max} in \textit{M. alterniflorum} leaves. Roots were the dominant organ for Pi assimilation for all species at the Lake Hampen Pi concentrations.

The results indicate that at low lake water Pi concentrations \textit{L. uniflora} will be able to survive on the Pi pools in the sediment porewater. \textit{M. alterniflorum} turned out to have an efficient uptake at both low and high Pi concentrations by both roots and shoots, suggesting that \textit{M. alterniflorum} will be a strong competitor to isoetids at increasing Pi concentrations.

Introduction

Softwater lakes are characterized by low nutrient and inorganic carbon concentrations and low to neutral pH (Roelofs et al. 1984; Murphy 2002; Pedersen et al. 2006). The vegetation is typically dominated by isoetids such as \textit{Littorella uniflora} (L.) Aschers., \textit{Lobelia dortmana} L., and \textit{Isoetes lacustris} L. (Roelofs et al. 1984). These slow-growing, stress-tolerant, and short-leafed evergreen rosette species form dense stands with a high root to shoot ratio and are able to exploit the carbon dioxide pool in the sediment (Brouwer et al. 2002; Murphy 2002; Pedersen et al. 2006). The plant communities in northern European softwater lakes have changed during the last century and continue to change in a direction where epiphytes and elodeids are spreading in the lakes and are believed to compete with the isoetids for nutrients and light (Roelofs 1983; Sand-Jensen et al. 2000; Brouwer et al. 2002). In Denmark, eutrophication has led to a major decline in the \textit{L. uniflora} population; prior to 1990 it was found in 472 lakes, but
now it only occurs in 218 lakes (Pedersen et al. 2006). It is important to obtain an understanding of why these community shifts happen to ensure maintenance of the high species diversity in these habitats. Factors such as acidification and eutrophication are believed to be some of the main reasons why more than 90% of these habitats have disappeared in the 20th century (Arts et al. 1990; Brouwer et al. 2002; Murphy 2002).

Since eutrophication in softwater lakes is driven by phosphorus (P) (e.g. Christiansen et al. 1985; Wetzel 2001), a better understanding of the adaptation of different macrophyte species to low $P_i$ availability and identification of species benefitting from increasing $P_i$ concentrations are essential. Macrophytes are able to take up nutrients through both roots and shoots (Carignan and Kalff 1980; Brix and Lyngby 1985). In softwater lakes, where the $P_i$ concentration in the surface water is low, $P_i$ in the porewater is a more easily accessible P source for the rooted macrophytes (Carignan and Kalff 1980; Barko and Smart 1981; Roelofs et al. 1984). Therefore, macrophytes with a well developed root system and a high root to shoot ratio presumably have a competitive advantage in taking up $P_i$ in softwater lakes.

The relationship between eutrophication and the community shift to more fast-growing, short-lived species suggest that fast-growing species require higher nutrient supplies than slow-growing, long-living species, the latter being expected to be better adapted to growth at low $P_i$ concentrations (Pedersen and Borum 1996; 1997). We investigate these expectations by comparing the $P_i$ uptake kinetics for roots and shoots of four macrophyte species, a slow-growing, long-living isoetid, *L. uniflora*, and three faster-growing elodeids, *Potamogeton perfoliatus* L., *Myriophyllum alterniflorum* DC. and *Elodea canadensis* L. C. Rich., in relation to dry weight, but also in relation to surface area since a larger surface area expectedly results in a higher uptake rate. Phosphate is taken up via transport proteins in the plant cell membrane (Schachtman et al. 1998; Nussaume et al. 2011), so a larger surface and thereby a larger membrane might result in more transport proteins and, in consequence, a higher uptake rate. Since proteins are responsible for the uptake of $P_i$, we use the Michaelis-Menten model to describe the characteristics of uptake kinetics. The maximum $P_i$ uptake rates ($V_{max}$) and the half saturation constants ($K_m$) will be calculated from the Michaelis-Menten kinetics.

Determination of the uptake kinetics at low $P_i$ concentrations is essential, since macrophytes experience low $P_i$ concentrations in the lake and porewater with resultant $P_i$ limitation of macrophyte growth and macrophyte competition for $P_i$. The increase from these low concentrations represents the first step in the eutrophication process. To determine the short-term uptake kinetics at low $P_i$ concentrations we used a $^{33}P$ radioisotope technique (Nielsen et al. 2006), allowing
determination of Pi affinity at low Pi concentrations, equal to $V_{\text{max}}/K_m$ (Healey 1980; Pedersen and Borum 1997).

**Methods**

**Study site**

Lake Hampen is situated in central Jutland, Denmark (N 56°1.083', E 9°23.259'). The catchment area is mainly covered by nature areas dominated by forest; however, there is a small coverage of agricultural land along the eastern shoreline of the lake (Kidmose et al. 2011; Ommen et al. 2012). The soil in the catchment area and the sediment in the littoral zone of the lake mainly consist of sand, gravel, silt and peat (Kidmose et al. 2011). Lake Hampen is a nutrient poor groundwater and rainwater fed, softwater lake, which hosts different macrophyte populations including both isoetids and elodeids (Kidmose et al. 2011; Vestergaard and Sand-Jensen 2000). Since the lake accumulates nutrients over time, it is expected to be in the initial stage of eutrophication (Ommen et al. 2012).

**Sampling**

Sampling of the four macrophyte species (L. uniflora, P. perfoliatus, M. alterniflorum and E. canadensis) was conducted in June and July 2011 and May, June and August 2012 in the southern middle bay of Lake Hampen.

Elodeid material was collected by scuba diving and was gently dug up by hand to make sure that the tissue would be intact. L. uniflora was brought back as turfs. Plant material was taken to the laboratory in lake water and kept outside in open containers in water from Lake Hampen and in a room with a fixed temperature (15 °C). Lake water was transported back in containers and kept at 4 °C prior to use. All four species were sampled and analyzed in June. Additionally, E. canadensis was collected in May and August to allow determination of seasonal changes in this species.

The L. uniflora vegetation was found along the shore line in sandy sediments. The three elodeid species grew in deeper water mixed in between each other in softer more organic sediment. To analyze sediment characteristics ten centimeter deep turfs ($n = 3$) were sampled within the L. uniflora vegetation, and undisturbed sediment cores ($n = 5$) from the elodeid habitat were taken to the laboratory and kept at 4 °C. Samples were analyzed the next day for Pi concentrations in

<table>
<thead>
<tr>
<th>Species</th>
<th>L. uniflora</th>
<th>P. perfoliatus</th>
<th>M. alterniflorum</th>
<th>E. canadensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>May</td>
<td>0.91 ± 0.21</td>
<td>0.04 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>June</td>
<td>0.12</td>
<td>0.14 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>August</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*No data. 0.12 was estimated as an average between May and August ratios and this value was used in further calculations.
the porewater, easily (water) extractable Pi, loss on ignition (LOI) and total phosphorus (TP) content. Surface water samples were also analyzed for Pi concentration. L. uniflora grew in conditions characterized by a slightly lower ambient Pi concentration in the porewater and a lower pool of easily available adsorbed Pi than the elodeid species (Table 3).

**The experimental setup**

The experiment was conducted in a constant temperature room at 15 °C, which corresponds to a typical Danish lake summer temperature. The incubations were conducted in polycarbonate bottles (150 and 250 ml bottles) which have a very low adsorption of Pi onto the walls. The bottles were mounted on a rotating disc to optimize mixing in the bottles and thereby minimize the boundary layer. A light-dark incubation (n = 3, 90 min, 0.032 and 3.23 µmol P L\(^{-1}\), 1 µCi \(^{33}\)Pi) was conducted in light at 379 µmol photons m\(^{-2}\) s\(^{-1}\) and in darkness (aluminum foil covered bottles) to test if light had an effect on the Pi uptake. No significant differences in Pi uptake were observed in the 90 minute experimental period. Following this, we decided to run the Pi uptake kinetics incubations at the room background light (PAR = 2 µmol quanta m\(^{-2}\) s\(^{-1}\)).

After incubation the plant tissue was rinsed following a three step washing procedure: 1) in 2 mg P L\(^{-1}\) Na\(_2\)HPO\(_4\) to remove adsorbed \(^{33}\)Pi, 2) in 0.02 mol L\(^{-1}\) HCl, and 3) in distilled H\(_2\)O for approximately 30 seconds in each wash. After rinsing and drying (on paper tissues), the plant material was freeze dried and dry weight was present (mean ± SD, n = 10).

**Table 2** Tissue concentrations of total phosphorus (TP), total nitrogen (N) and total carbon (C) in percentage of dry weight for L. uniflora, P. perfoliatus, M. alterniflorum and E. canadensis in June and additionally for E. canadensis in May and August 2012. Additionally, the surface area to dry weight ratio is presented (mean ± SD, n = 10).

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Month</th>
<th>TP</th>
<th>N</th>
<th>C</th>
<th>Surface area to dry weight ratio cm(^{2}) mg DW(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. uniflora</td>
<td>Leaf</td>
<td>June</td>
<td>0.28</td>
<td>2.39</td>
<td>36.38</td>
<td>0.72 ± 0.16</td>
</tr>
<tr>
<td>L. uniflora</td>
<td>Root</td>
<td>June</td>
<td>0.18</td>
<td>3.13</td>
<td>40.89</td>
<td>0.88 ± 0.21</td>
</tr>
<tr>
<td>P. perfoliatus</td>
<td>Leaf</td>
<td>June</td>
<td>0.26</td>
<td>2.68</td>
<td>33.20</td>
<td>3.07 ± 0.80</td>
</tr>
<tr>
<td>P. perfoliatus</td>
<td>Root</td>
<td>June</td>
<td>0.31</td>
<td>3.82</td>
<td>39.94</td>
<td>2.18 ± 1.10</td>
</tr>
<tr>
<td>M. alterniflorum</td>
<td>Leaf</td>
<td>June</td>
<td>0.43</td>
<td>4.68</td>
<td>44.46</td>
<td>1.82 ± 0.42</td>
</tr>
<tr>
<td>M. alterniflorum</td>
<td>Root</td>
<td>June</td>
<td>0.15</td>
<td>1.37</td>
<td>38.68</td>
<td>1.28 ± 0.51</td>
</tr>
<tr>
<td>E. canadensis</td>
<td>Leaf</td>
<td>May</td>
<td>0.34</td>
<td>3.56</td>
<td>36.33</td>
<td>-</td>
</tr>
<tr>
<td>E. canadensis</td>
<td>Root</td>
<td>May</td>
<td>0.27</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. canadensis</td>
<td>Leaf</td>
<td>June</td>
<td>0.27</td>
<td>4.01</td>
<td>40.96</td>
<td>1.55 ± 0.23</td>
</tr>
<tr>
<td>E. canadensis</td>
<td>Root</td>
<td>June</td>
<td>0.25</td>
<td>2.57</td>
<td>36.17</td>
<td>0.99 ± 0.12</td>
</tr>
<tr>
<td>E. canadensis</td>
<td>Leaf</td>
<td>August</td>
<td>0.30</td>
<td>3.31</td>
<td>40.36</td>
<td>-</td>
</tr>
<tr>
<td>E. canadensis</td>
<td>Root</td>
<td>August</td>
<td>0.22</td>
<td>2.30</td>
<td>37.97</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3  Surface water and sediment characteristics (mean ± SD, n = 3 for isoetids, n = 5 for elodeids). Percent dry weight (DW), percent loss of ignition (LOI), total phosphorus (TP), dissolved inorganic phosphate in surface and porewater (P_i) and loosely adsorbed phosphate (H_2O IP).

<table>
<thead>
<tr>
<th></th>
<th>May</th>
<th>June</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isoetid</td>
<td>Elodeid</td>
<td>Isoetid</td>
</tr>
<tr>
<td>Surface water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P_i (µmol L(^{-1}))</td>
<td>0.07</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>Sediment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DW (%)</td>
<td>71.7 ± 5.20</td>
<td>17.3 ± 10.00</td>
<td>80.2 ± 3.30</td>
</tr>
<tr>
<td>LOI (%)</td>
<td>0.57 ± 0.12</td>
<td>19.8 ± 12.51</td>
<td>0.48 ± 0.13</td>
</tr>
<tr>
<td>TP (µmol cm(^{-3}))</td>
<td>1.76 ± 0.19</td>
<td>4.4 ± 0.99</td>
<td>1.65 ± 0.17</td>
</tr>
<tr>
<td>P_i (µmol L(^{-1}))</td>
<td>1.3 ± 0.31</td>
<td>1.5 ± 0.06</td>
<td>3.3 ± 0.34</td>
</tr>
<tr>
<td>H_2O IP (µmol dm(^{-3}))</td>
<td>14.3 ± 5.11</td>
<td>19.5 ± 13.08</td>
<td>18.2 ± 2.53</td>
</tr>
</tbody>
</table>

determined. Freeze dried tissue was bleached and destructed in NaClO after which scintillation fluor (HionicFluor) was added prior to counting. Activity was determined by counting the radioactivity of the tissue in a PerkinElmer Liquid Scintillation Analyzer Tri-Carb 2910 TR. Counts were corrected for background using blank samples and quenching was corrected using external standard.

Activity in the media was determined before and after each incubation. Samples were counted in the liquid scintillation analyzer (100 µL media and 5 mL HionicFluor). P_i analyses of the media were also conducted.

From the relationship between the uptake and the available \(^{31}\)P_i and \(^{33}\)P_i, the assimilated \(^{31}\)P_i was determined accordingly:

\[
(\text{P_i in tissue [DPM]} / \text{added } P_i \text{ activity [DPM]}) \times \text{added } P_i \text{ amount [µmol]}
\]

The incubation experiment

Time series incubations were conducted in June 2011 prior to the \(^{33}\)P_i uptake kinetics incubations in order to determine an adequate incubation time with a nearly constant \(^{33}\)P_i uptake rate. Root and leaf fragments of similar size and age were cut off, carefully rinsed and incubated in 100 or 200 ml media (91.7 CaCl_2, 69.0 MgSO_4, 58.4 NaHCO_3, 15.4 KHCO_3, 1.29 NH_4NO_3 mg L\(^{-1}\), Smart and Barko 1985) with two P_i concentrations (0.032 µmol L\(^{-1}\) and 3.23 µmol L\(^{-1}\)) added in form Na_2HPO_4 and 3 incubation times (30, 60 and 120 min) for *P. perfoliatus* leaf tissue (n = 3), 10 incubation times up to 60 minutes (n = 1) for *L. uniflora* root tissue, and 10 incubation times up to 30 minutes (n = 1) for *L. uniflora* leaf tissue and *M. alterniflorum* tissue; 1 µCi \(^{33}\)P_i being added to all incubations. If epiphytes were present, these were carefully removed by scraping. All time series for *P. perfoliatus* leaf tissue and *L. uniflora* root tissue showed a linear relationship at an incubation time of 50
Table 4 Maximum phosphate uptake rates \( (V_{\text{max}}) \) and half saturation constants \( (K_{\text{m}}) \), phosphate affinity at low phosphate concentrations \( (V_{\text{max}}/K_{\text{m}}) \) calculated from the Michaelis-Menten model for leaf and root tissue for *L. uniflora*, *P. perfoliatus*, *M. alterniflorum* and *E. canadensis* in June and additionally for *E. canadensis* in May and August 2012 (mean ± SE, \( n = 3 \)). Parameters are calculated per dry weight (g DW) and per surface area (cm\(^2\)).

<table>
<thead>
<tr>
<th></th>
<th>Uptake rates in respect to dry weight</th>
<th>Uptake rates in respect to surface area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} ) ( \mu \text{mol g DW}^{-1} \text{ h}^{-1} )</td>
<td>( K_{\text{m}} ) ( \mu \text{mol L}^{-1} )</td>
</tr>
<tr>
<td><strong>Leaf tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. uniflora</em></td>
<td>Jun</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td><em>P. perfoliatus</em></td>
<td>Jun</td>
<td>0.59 ± 0.16</td>
</tr>
<tr>
<td><em>M. alterniflorum</em></td>
<td>Jun</td>
<td>13.6 ± 3.75</td>
</tr>
<tr>
<td><em>E. canadensis</em></td>
<td>May</td>
<td>&gt;0.98( ^{1*} )</td>
</tr>
<tr>
<td><em>E. canadensis</em></td>
<td>Jun</td>
<td>0.20 ± 0.17</td>
</tr>
<tr>
<td><em>E. canadensis</em></td>
<td>Aug</td>
<td>0.83 ± 0.13</td>
</tr>
<tr>
<td><strong>Root tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. uniflora</em></td>
<td>Jun</td>
<td>1.5 ± 0.15</td>
</tr>
<tr>
<td><em>P. perfoliatus</em></td>
<td>Jun</td>
<td>&gt;2.51( ^{2*} )</td>
</tr>
<tr>
<td><em>M. alterniflorum</em></td>
<td>Jun</td>
<td>2.95 ± 0.49</td>
</tr>
<tr>
<td><em>E. canadensis</em></td>
<td>May</td>
<td>1.11 ± 0.24</td>
</tr>
<tr>
<td><em>E. canadensis</em></td>
<td>Jun</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td><em>E. canadensis</em></td>
<td>Aug</td>
<td>1.46 ± 0.18</td>
</tr>
</tbody>
</table>

\( ^{1*} \) when the model estimated unlikely high \( K_{\text{m}} \) values or when saturation was not reached, parameters were changed to a ">" value, estimated by choosing the substrate concentration at the measured \( V_{\text{max}} \) and dividing this by two. \( V_{\text{max}}/K_{\text{m}} \) is calculated from these estimated values.

\( ^{1,2,3...9*} \) The Michaelis-Menten estimated parameters: \( 5.92, 9.17, 31.4, 12.8, 65.0, 623.3, 67.9, 3.82, 4.21. \)
minutes, indicating that the Pi uptake by the tissue was not limited within this time frame, and an incubation time of 30 minutes was finally chosen for all species. *L. uniflora* leaf tissue and *M. alterniflorum* tissue time series showed a linear relationship in the 30 minute incubation period. Time series were not conducted on *E. canadensis* tissue and root tissue of *P. perfoliatus* on the assumption that these would not be limited in Pi within the 30 minute time frame and based on our previous experience with the other species.

Incubations for the Pi uptake kinetics were conducted in June and July 2011 and in May, June and August 2012. The experimental design for the Pi uptake kinetics corresponded to that of the time series experiment, except that the incubation time

### Table 5 Phosphate uptake at ambient Pi concentrations in Lake Hampen in June 2012 for leaf and root tissue of *L. uniflora*, *P. perfoliatus* and *M. alterniflorum* and in May, June and August for *E. canadensis*. (A) Measured uptake rates at Pi concentrations similar to the ambient Pi concentrations (mean ± SE, n = 3). (B) Michaelis-Menten model estimated uptake rates at ambient Pi concentrations. C) Percentage contribution by the root to the whole plant’s phosphate uptake. The calculation is based on the parameters Vmax and Km estimated from the Michaelis-Menten model, on the ambient Pi concentrations and on the average root and shoot biomass. (D) Estimated parameters from Carignan’s (1982) model using ambient Pi concentrations.

<table>
<thead>
<tr>
<th></th>
<th>A) Measured uptake rate</th>
<th>B) Michaelis-Menten model estimated uptake rates</th>
<th>C) Calculated for Lake Hampen</th>
<th>D) Carignan’s model</th>
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<tr>
<td></td>
<td>Lake water Pi</td>
<td>At 0.065 µmol L⁻¹</td>
<td>Pi uptake by roots</td>
<td>Pi uptake by roots</td>
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<td><strong>Leaf tissue</strong></td>
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<tr>
<td><em>L. uniflora</em></td>
<td>0.06 µmol L⁻¹</td>
<td>0.014 ± 0.006 µmol g DW⁻¹ h⁻¹</td>
<td>0.009</td>
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<tr>
<td><em>P. perfoliatus</em></td>
<td>0.06 µmol L⁻¹</td>
<td>0.007 ± 0.006 µmol g DW⁻¹ h⁻¹</td>
<td>0.001</td>
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<tr>
<td><em>M. alterniflorum</em></td>
<td>0.06 µmol L⁻¹</td>
<td>0.062 ± 0.023 µmol g DW⁻¹ h⁻¹</td>
<td>0.061</td>
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<tr>
<td><em>E. canadensis¹</em></td>
<td>0.07 µmol L⁻¹</td>
<td>0.006 ± 0.002 µmol g DW⁻¹ h⁻¹</td>
<td>0.006</td>
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<tr>
<td><em>E. canadensis²</em></td>
<td>0.06 µmol L⁻¹</td>
<td>0.004 ± 0.002 µmol g DW⁻¹ h⁻¹</td>
<td>0.001</td>
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<tr>
<td><em>E. canadensis³</em></td>
<td>0.12 µmol L⁻¹</td>
<td>0.069 ± 0.030 µmol g DW⁻¹ h⁻¹</td>
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<td><strong>Porewater Pi</strong></td>
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<td><strong>Root tissue</strong></td>
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<tr>
<td><em>L. uniflora</em></td>
<td>1.33 µmol L⁻¹</td>
<td>0.918 ± 0.300 µmol g DW⁻¹ h⁻¹</td>
<td>0.912</td>
<td>99</td>
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<tr>
<td><em>P. perfoliatus</em></td>
<td>1.75 µmol L⁻¹</td>
<td>0.269 ± 0.021 µmol g DW⁻¹ h⁻¹</td>
<td>0.231</td>
<td>89</td>
</tr>
<tr>
<td><em>M. alterniflorum</em></td>
<td>1.75 µmol L⁻¹</td>
<td>0.883 ± 0.431 µmol g DW⁻¹ h⁻¹</td>
<td>0.888</td>
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<tr>
<td><em>E. canadensis¹</em></td>
<td>1.54 µmol L⁻¹</td>
<td>0.107 ± 0.019 µmol g DW⁻¹ h⁻¹</td>
<td>0.165</td>
<td>73</td>
</tr>
<tr>
<td><em>E. canadensis²</em></td>
<td>1.75 µmol L⁻¹</td>
<td>0.056 ± 0.019 µmol g DW⁻¹ h⁻¹</td>
<td>0.090</td>
<td>96</td>
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<tr>
<td><em>E. canadensis³</em></td>
<td>1.76 µmol L⁻¹</td>
<td>0.499 ± 0.241 µmol g DW⁻¹ h⁻¹</td>
<td>0.535</td>
<td>42</td>
</tr>
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¹,²,³ *E. canadensis* in ¹ May, ² June and ³ August.
*Specified at 0.161 µmol L⁻¹, which is more similar to the Lake Hampen August concentration.
was constant (30 min) and the Pi concentrations varied. The Pi concentrations in the media were 0.016, 0.032, 0.065, 0.161, 0.323, 0.807, 1.61, 3.23, 6.46 and 12.9 µmol L⁻¹ (and for P. perfoliatus also 25.8 µmol L⁻¹). During all incubations the Pi concentrations in the media remained relatively constant and did not decline with more than 10%.

Additional analysis
Plant material was analyzed for total phosphorus content (TP), total nitrogen (N) and total carbon content (C) and root to shoot relationship (n = 5; Table 1). TP in root and shoot material was measured spectrophotometrically as dissolved inorganic P (Pi) with the molybdenum-blue method (Koroleff 1983) after extraction from boiling in 1 M HCl (120 °C, 0.5 h) after combustion (520 °C, 2 h). N and C content in dried root and shoot material was analyzed using a CarloErba CHN EA1108-Elemental Analyzer (Table 2).

WinRhizo was used to determine the plant surface area to dry weight ratio (n = 10; Table 2), and this ratio was used to convert uptake rates per dry weight to per surface area.

The upper ten centimeters of the turf and sediment cores were cleaned for plant material and were homogenized prior to analysis. Homogenized wet sediment samples were dried to determine dry weight (105 °C, 24 h). Dried sediment was used to determine loss on ignition (520 °C, 5 h). The sediment TP concentration was measured as Pi (described above) on extracts of the ignited sediment (boiled in 1 mol L⁻¹ HCl at 120 °C, 1 h). Loosely adsorbed Pi was extracted from 1 g wet sediment by shaking twice in 25 ml DI water for 1 h and finally measured as Pi after filtration. Porewater was retrieved by centrifugation and filtration and analyzed for Pi, and lake water was filtered and analyzed for Pi (Table 3).

Calculations and statistics
Uptake rates were calculated with the assumption that the uptake of Pi was unidirectional, with no loss of Pi from the tissue back into the media. Vₘₐₓ and Kₘ were determined by plotting the Pi uptake rate to the Pi concentration and fitting the data to the nonlinear Michaelis-Menten model. The Michaelis-Menten kinetics was fitted in GraphPad Prism 5 and the specified standard errors (SE) and 95 % confidence intervals were calculated by this program as well. The fitted values were considered different when there was no overlap between the 95% confidence intervals. Six outliers from a total of 246 numbers in the dataset were removed when fitting the data to the model. Several Kₘ values in Table 4 were changed to a “>value” when saturation was not reached at the highest measured uptake rate or when the model estimated an unlikely high Kₘ value. Kₘ was then
estimated by choosing the substrate concentration at the highest measured uptake rate and dividing this by two. When using the Michaelis-Menten model for calculating the total Pi uptake in a whole plant, the estimated parameters were used. As the model parameters are estimated from mainly low Pi concentrations, estimated Pi uptake corresponds well with the measured uptake rates at these low concentrations and is therefore proper for use in such calculations. \(K_m/V_{max}\) values were calculated on the basis of

Figure 1 (A) Phosphate uptake rates (\(\mu\text{mol g}^{-1}\ \text{DW h}^{-1}\)) as a function of phosphate concentration (\(\mu\text{mol L}^{-1}\); mean ± SE, \(n = 3\)) illustrated by Michaelis-Menten saturation kinetics for root and leaf tissue of \(L.\ uniflora\), \(P.\ perfoliatus\) and \(M.\ alterniflorum\) in June. Solid lines are best fit to the Michaelis-Menten model and dotted lines are 95 % confidence intervals. (B) Phosphate uptake rates at low phosphate concentrations (0.016, 0.032 and 0.065 \(\mu\text{mol L}^{-1}\)). Dotted lines indicate best fit to the Michaelis-Menten model and solid lines indicate linear regression models. Notice that axes scales are not the same.
the model values.

Carignan (1982) presented an empirical model for estimating the relative importance of roots in the phosphorus uptake by aquatic macrophytes and the relative phosphorus availability,

\[ P = \frac{9.8}{(1 + 2.66(s/w)^{0.83})} \]

where \( P \) represents the percentage contribution by the root to the whole plant phosphorus uptake, and \( s \) and \( w \) are the dissolved reactive phosphorus concentrations in the sediment porewater and the overlying water, respectively. We used this model with the measured \( P_i \) concentrations in Lake Hampen to compare Carignan’s \( P \) with our own calculations of percentage \( P_i \) uptake by root tissue using our own model with the estimated Michaelis-Menten parameters, porewater and lake water \( P_i \) concentrations, and root and shoot biomass. The designed experiment met the criteria applied by Carignan (1982) in his model; thus, 1) the measurements were conducted on plants recently collected from their natural sites, 2) \( P_i \) concentrations in the medium in contact with the tissue were specified, 3) uptake of \( ^{33}P \) per weight of the plant was transformed into \( ^{31}P \) uptake per total plant weight, and 4) epiphytic communities were removed, if present.

**Results**

In general, all species showed a positive non-linear relationship between the \( P_i \) uptake rate and the \( P_i \) concentration, except \( P. perfoliatus \) for root tissue and \( M. alterniflorum \) for leaf tissue that did not reach saturation and showed almost linear relationships with the applied \( P_i \) concentrations (Fig. 1 and 2). The maximum \( P_i \) uptake rates (\( V_{\text{max}} \)) and the half saturation constants (\( K_m \)) calculated from the Michaelis-Menten kinetics together with the affinity at low \( P_i \) concentrations (\( K_m/V_{\text{max}} \)) for leaf and root tissue are summarized for the four different species in Table 4.

For leaf tissue in June, \( V_{\text{max}} \) varied 68-fold among the four species with \( M. alterniflorum \) reaching the highest rate (13.6 ± 3.75 µmol g DW\(^{-1}\) h\(^{-1}\); Table 4), whereas the three other species showed much lower maximum uptake rates (0.20 to 0.59 µmol g DW\(^{-1}\) h\(^{-1}\); Table 4). \( M. alterniflorum \) also had the highest \( V_{\text{max}} \) in the root tissue together with \( P. perfoliatus \) (2.95 and 2.51 µmol g DW\(^{-1}\) h\(^{-1}\), respectively; Table 4), whereas \( L. uniflora \) demonstrated a \( V_{\text{max}} \) of about half this value, and \( E. canadensis \) had the lowest \( V_{\text{max}} \) (0.32 ± 0.07 µmol g DW\(^{-1}\) h\(^{-1}\); Table 4). \( M. alterniflorum \) showed, as the only species, a higher \( V_{\text{max}} \) in the leaf tissue than in the root tissue (Fig. 1). \( L. uniflora \) exhibited the lowest \( K_m \) values in both root and leaf tissue (2.46 ± 1.41 and 0.85 ± 0.31
The $V_{\text{max}}/K_m$ ratio for leaf tissue in June was highest for *M. alterniflorum* (1.06; Table 4), being up to 100 times lower for *P. perfoliatus* and *E. canadensis* (0.01 to 0.02, respectively; Table 4). In contrast, *L. uniflora* demonstrated the highest $V_{\text{max}}/K_m$ in the root tissue (1.76; Table 4); otherwise, this value varied up to 25-fold for the four species.

When using the surface area to dry weight ratio (Table 2) to calculate uptake rates per surface area (Table 4), the difference in $V_{\text{max}}$ between the four species was still evident, although the difference between, for instance, shoots of *M. alterniflorum* and *L. uniflora* decreased from a factor 38 to a factor 15, while the difference between *M.*

Figure 2 (A) Phosphate uptake rates ($\mu$mol g$^{-1}$ DW h$^{-1}$) as a function of phosphate concentration ($\mu$mol L$^{-1}$; mean $\pm$ SE, $n = 3$) illustrated by Michaelis-Menten saturation kinetics for root and leaf tissue of *E. canadensis* in May, June and August 2012. Solid lines are best fit to the Michaelis-Menten model and dotted lines are 95% confidence intervals. (B) Phosphate uptake rates at low phosphate concentrations (0.016, 0.032 and 0.065 $\mu$mol L$^{-1}$). Dotted lines indicate best fit to the Michaelis-Menten model and solid lines indicate linear regression models. Notice that axes scales are not the same.
alterniflorum and P. perfoliatus increased from a factor 23 to a factor 39. For root tissue the $V_{\text{max}}$ for P. perfoliatus became relatively smaller when calculated per surface area. Otherwise, the differences among species remained nearly unchanged. When comparing the $V_{\text{max}}/K_{\text{m}}$ ratios calculated per surface area, the difference between L. uniflora and M. alterniflorum was not as pronounced as before in the leaf tissue (0.22 to 0.54), whereas in the root tissue the difference in $V_{\text{max}}/K_{\text{m}}$ between the two species increased from a factor 2.4 to a factor 3.7 in favor of L. uniflora (Table 4).

E. canadensis exhibited a seasonal change from May to August (Fig. 2; Table 4), $V_{\text{max}}$ being lowest in June compared to May and August in both root and leaf tissue, whereas May and August had similar $V_{\text{max}}$ values (Table 4). $V_{\text{max}}$ did not show any pronounced difference between leaf and root tissue at the 3 dates. $K_{\text{m}}$ decreased over the season in both root and leaf tissue (8.82 to 3.05 and 6.46 to 0.87 µmol L$^{-1}$, respectively; Table 4). $V_{\text{max}}/K_{\text{m}}$ showed highest affinity in August in both leaf and root tissue, affinity of root tissue being a factor 2 higher than that of leaf tissue. May and June had similar affinity in leaf and root tissue.

The Michaelis-Menten model based uptake rates calculated from Lake Hampen Pi concentrations are very similar to the actually measured rates (Table 5), rendering the Michaelis-Menten estimates reliable for these low Pi concentrations. When calculating the total Pi uptake in the whole plant in Lake Hampen using the Michaelis-Menten parameters, the measured Pi concentrations in lake and porewater, and the total biomass of root and shoot tissue in the macrophytes, we were able to determine how much of the total Pi uptake the roots were responsible for (Table 5). In June L. uniflora showed the highest and M. alterniflorum the lowest percentage uptake by roots (99 vs. 62%; Table 5). When using Carignan’s 1980 model for calculating the quantity of total Pi uptake that the roots were responsible for, all four species showed similar values (83 - 86%; Table 5).

$K_{\text{m}}$ and $V_{\text{max}}/K_{\text{m}}$ in the elodeid species showed positive and negative correlations, respectively, with TP ($r^2=0.6123$ and $r^2=0.659$, respectively) and N ($r^2=0.7269$ and $r^2=0.659$, respectively) concentrations in the root tissue (Fig. 3), whereas no correlations emerged for leaf tissue. The isoetid L. uniflora with the very low $K_{\text{m}}$ stood out from the other species (Fig. 3) and was not included in the correlations.

**Discussion**

To examine Pi uptake kinetics at very low Pi concentrations in four freshwater macrophyte species from oligotrophic softwater Lake Hampen (L. uniflora, P. perfoliatus, M. alterniflorum and E. canadensis), we used the
radioactive isotope $^{33}$P tracer technique, previously used for the marine macrophyte Thalassia testudinum (Nielsen et al. 2006). The present study showed that the four different macrophyte species had different strategies for $Pi$ uptake relative to $Pi$ availability. At low $Pi$ concentrations $L. uniflora$ showed highest affinity for $Pi$ in the root tissue and the lowest Km, indicating that $L. uniflora$ is well adapted to live in habitats with low porewater $Pi$ concentrations and competitive to the three other species at low $Pi$ concentrations. Also at low lake water $Pi$ concentrations, leaf tissue from $L. uniflora$ had a high affinity, about ten times higher than $E. canadensis$ and $P. perfoliatus$. $M. alterniflorum$ had the highest affinity, however, it being more than six times higher than that of $L. uniflora$, indicating that both $L. uniflora$ and $M. alterniflorum$ are well adapted to low lake water $Pi$ concentrations, but that $M. alterniflorum$ becomes competitively dominant at increasing concentrations. For root tissue $M. alterniflorum$ had the second highest affinity of the four species, which suggests that it is the most severe competitor for $L. uniflora$ at intensifying lake eutrophication. High affinity for $Pi$ in lake water through the shoot tissue is an advantage in lakes where the main $P$ source is drain and surface water from the catchment. $P. perfoliatus$ and $E. canadensis$ both showed high $K_m$ values and low affinity for $Pi$ at low $Pi$ concentrations, suggesting that they both are less competitive for $Pi$ at low concentrations than the two other species. In our experiment $P. perfoliatus$ never reached saturation at root uptake, even when incubated at a twice as high $Pi$ concentration than the other three species, and it may, therefore, benefit from very high $Pi$ concentrations in the sediment. $M. alterniflorum$ demonstrated the highest maximum uptake rates ($V_{max}$) of $Pi$, and in contrast to the other three species it had a higher $V_{max}$ in leaf tissue than in root tissue. Morphology explained about half of the relative difference between $M. alterniflorum$ and the other species, indicating that $M. alterniflorum$ has changed its shoot morphology and developed a large surface area as an adaptation to optimize $Pi$ uptake. However, $M. alterniflorum$, even when taking the shoot surface area into account, still had a much higher $V_{max}$ in its shoot tissue than did the other species. This may possibly be explained by the mechanism by which it takes up $Pi$ over the membrane, either by having more transport proteins per surface area in the membrane or by having more efficient transport proteins in the membrane. $P. perfoliatus$ exhibited the highest surface area to dry weight ratio in both shoots and roots, and as for the root tissue the $V_{max}$ of $P. perfoliatus$ was clearly influenced by the surface area when compared with the other species. This indicates that $P. perfoliatus$ has
developed a large surface area to optimize Pi uptake. In general, our study showed that macrophytes have at least two possible methods to optimize their Pi uptake: 1) via number and/or species of transport proteins in the membrane (Nussaume et al. 2011) and 2) via morphology of roots and shoots, for example specific surface area. Other morphological adaptations may be the root to shoot ratio, where *L. uniflora* had a ratio close to ten times higher than that of *M. alterniflorum* and *E. canadensis* and almost twenty times higher than the ratio of *P. perfoliatus*. This clearly demonstrates the importance of root tissue for *L. uniflora* living in clear softwater lakes with good light penetration but low water nutrient and inorganic carbon concentrations (Roelofs 1983; Brouwer et al. 2002). Here, it is an advantage to develop a large root biomass to utilize the much richer nutrient and carbon sources in the sediment. In contrast, the other three species, with their long shoots and large shoot biomass, are well adapted to live in more eutrophic waters with poor light penetration and nutrient- and carbon-rich surface water (Murphy 2002).

It has been pointed out in the literature that isoetids have several advantages that can help them in the competition with elodeids in softwater oligotrophic lakes (Smolders et al. 2002; Spierenburg et al. 2010). These advantages combined with the findings in this study show *L. uniflora* to be highly competitive to elodeids in habitats with low Pi concentrations in lake water and porewater. In addition to the mentioned adaptations, *L. uniflora* is able to create a symbiosis with mycorrhizae which act as an expansion of the root surface area, thereby enhancing the ability to extract P and N from a larger sediment volume and giving *L. uniflora* a competitive advantage in nutrient poor environments (Wigand et al. 1998; Andersen and Andersen 2006). Unfortunately, incubations could not be conducted on *L. uniflora* roots with functional mycorrhizae but were made on the root tissue alone. However, *L. uniflora* would expectedly have had an even higher uptake rate and affinity for Pi if this symbiosis had been intact. According to Beck-Nielsen and Madsen (2001) mycorrhizae have not been found on the three other studied species.

*L. uniflora* oxidizes the rhizosphere by transporting oxygen to the sediment via lacunae in leaves to roots with consequent radial oxygen loss (ROL) from the roots. When the sediment is oxidized, Pi precipitates as Fe(III)PO$_4$, consequently decreasing the Pi concentrations in the sediment porewater. High densities of isoetids can thereby change the sediment characteristics and make it harder for the elodeids to assimilate Pi (Wium-Andersen and Andersen 1972; Sand-Jensen et al. 1982; Spierenburg et al. 2010). In
contrast to most elodeids, isoetids also have the advantage that they are able to exploit the CO$_2$ pools in the sediment via root uptake (Sand-Jensen and Prahl 1982; Frandsen et al. 2012). The findings in the present experiment of low $K_m$ and high affinity for $Pi$ for L. uniflora at low concentrations further add to the benefits of the above mentioned adaptations that make L. uniflora competitive to elodeids at low Pi concentrations in the porewater. However, our study also indicates that M. alterniflorum is able to compete with L. uniflora at low Pi concentrations in the porewater if the Pi concentration increases in the lake water and the sediment becomes more organic.

To be able to manage and control macrophyte growth and the composition of plant communities, it is important to understand and determine the nutrient sources for the plants. It has therefore been widely discussed which organs are most important for the nutrient uptake by macrophytes, shoots or roots. Some studies have argued that P uptake by the shoot is more important than root uptake (Schults and Adams 1971), but it is now generally accepted that rooted macrophytes can take up P from the sediment (Barko and Smart 1980; Carignan and Kalff 1980; Barko et al. 1991) and in this way satisfy their P requirement at 90-100% level (Carignan and Kalff 1980; Barko and Smart 1981). Our experiment showed that both root and shoot tissue can function as a source for Pi uptake, but different uptake kinetics appeared for species and organs. Elodeid species seemed to be adapted to take up Pi from the most available source, whereas L. uniflora seemed to prefer roots as primary organ for Pi uptake. For all four species, our experimental results for June reveal roots to be the dominant organ for Pi uptake (62-99%). For M. alterniflorum, however, leaf tissue was responsible for about 40% of the uptake despite the relatively low Pi concentration in the lake water. When applying Carignan’s (1982) model to the measured Pi concentrations in water and sediment in Lake Hampen, roots emerged as the dominant organ for Pi uptake (83-86%) for all four species. In comparison with the calculated percentage Pi uptake from the measured Michaelis-Menten uptake kinetics, our results support, to a certain extent, Carignan’s (1982) model. However, our results demonstrate that species have different adaptations to Pi uptake and that Carignan’s model is not specific for an individual species. For instance, at increasing lake water concentrations of Pi, our uptake kinetics results suggest that some species, especially M. alterniflorum, may easily shift to leaf tissue uptake to assimilate Pi. Carignan and Kalff (1980) found similar results for Myriophyllum sp., namely that Pi uptake by shoot tissue becomes more important with increasing Pi concentrations in the water, Myriophyllum sp. thus acting as an opportunistic...
species that take up P from the most available source (Nichols and Shaw 1986). In contrast, *L. uniflora* was not able to increase the Pi uptake rate in the leaf tissue to the same degree as *M. alterniflorum* and had a low $V_{\text{max}}$. These results show that *L. uniflora* relies on the roots for assimilation of Pi. At increasing Pi concentration in surface water and groundwater, the vegetation of softwater lakes might therefore experience a shift in species composition that will threaten the isoetid vegetation.

When discussing which organ is most important in the Pi uptake, the affinity ($V_{\text{max}}/K_{\text{m}}$) for Pi assimilation is an important parameter to include, especially at low Pi concentrations where competition for Pi limits growth (Christiansen et al. 1985). By limiting macrophyte growth low Pi concentrations can be a controlling factor for the macrophyte species composition. Species with high affinity at low Pi concentrations are therefore expected to dominate in habitats with limiting Pi concentrations. The present experiment showed that especially *L. uniflora* but also *M. alterniflorum* had high affinity for Pi assimilation in the root tissue at low Pi concentrations, whereas *M. alterniflorum* demonstrated the highest affinity for Pi assimilation in the leaf tissue. The root tissue of *L. uniflora* and root and shoot tissue of *M. alterniflorum* had about a tenfold higher uptake rate at low Pi concentrations (0.016, 0.032 and 0.065 µmol L$^{-1}$) than *P. perfoliatus* and *E. canadensis*. These results corresponds with Thiébaut et al. (2004) findings that also demonstrated that Pi can be a controlling factor for macrophyte species composition. Thiébaut et al. (2004) ranked different macrophyte species’ distribution to trophic level in streams, where some species were more abundant at low water Pi concentrations at an oligotrophic nutrient status (e.g. *L. uniflora*), and other species more abundant as water Pi concentration increased to a mesotrophic or eutrophic nutrient status (e.g. *E. canadensis*, *M. alterniflorum* and *P. perfoliatus*).

All four species exhibited a positive correlation with $K_{\text{m}}$ and a negative correlation with $V_{\text{max}}/K_{\text{m}}$ in relation to the TP concentration in the root tissue, indicating that Pi uptake might depend on P deficiencies in the tissue (Fig. 3; Table 2). As an average for several species Gerloff and Krombholz (1966) estimated the critical P value to be 0.13%, whereas Christiansen et al. (1985) for *L. uniflora* in Lake Hampen estimated the critical tissue content of P to be 0.28% or more. We found root tissue TP concentrations around 0.13% for *L. uniflora* and *M. alterniflorum* and around 0.28% for *P. perfoliatus* and *E. canadensis*. This suggests that the growth of all four species was partly P limited (Table 2). However, when looking at the $K_{\text{m}}$ values found in this experiment and comparing them to the actual Pi concentrations
Figure 3 Half saturation constants \( (K_m) \) and phosphate affinities \( (V_{\text{max}}/K_m) \) correlated to root tissue concentration of (A) total nitrogen (N %) and (B) total phosphorus (P %) for \textit{P. perfoliatus}, \textit{M. alterniflorum} and \textit{E. canadensis} in June and additionally for \textit{E. canadensis} in May and August 2012. Results for \textit{L. uniflora} were not included in the correlation.

recorded in the porewater in Lake Hampen (in June 1.33 µmol L\(^{-1}\)), \textit{L. uniflora} should be close to \( V_{\text{max}} \) in the root tissue \( (K_m=0.85 \ \text{µmol L}^{-1}) \), whereas the other three species must have been limited at this Pi concentration with a \( K_m \) at least 4 to 13 times higher. This implies that P remains a limiting factor for macrophyte growth in Lake Hampen. Yet, the results do not allow us to draw conclusions as to how low the porewater concentration in Lake Hampen should be to limit elodeid growth sufficiently enough for \textit{L. uniflora} to win in the competition for Pi.

Tissue P concentration varies according to plant species, type of tissue, time of year and trophic level of sediment and water (Gerloff and Krombholz 1966; Thiébaut and Muller 2003). Thiébaut (2005) demonstrated in soft water streams that plant tissue P concentrations were lowest in spring and highest in autumn for aquatic vascular plants. We cannot explain the seasonal change observed in tissue TP content and in \( V_{\text{max}} \) for \textit{E.}
canadensis with a lower TP content and a very low $V_{\text{max}}$ in late June. This midsummer minimum might indicate that $E. \textit{canadensis}$ was stressed, potentially limiting its physiological performance in late June. Similarly, Best (1977) found seasonal changes in organic and mineral components with a $V_{\text{max}}$ minimum in midsummer for $E. \textit{canadensis}$, and Pieczynska and Tarmanowska (1996) demonstrated that high biomasses of both living and decomposing filamentous algae have a negative effect on the growth of $E. \textit{canadensis}$. In our study, $E. \textit{canadensis}$ was collected in between much taller elodeids and at high densities of filamentous algae (personal observation). Our results indicate that seasonal changes in uptake kinetics are a highly interesting subject for further study.

Comparison of our results with the only other study conducted with a similar $\text{Pi}$ addition technique reveals somewhat similar uptake kinetics for macrophytes living in marine and freshwater environments low in $\text{Pi}$. Thus, Nielsen et al. (2006) found that $\textit{Thalassia testudinum}$ was adapted to live at extremely low $\text{Pi}$ concentrations (as low as ~0.010 $\mu$mol L$^{-1}$) and recorded similar $V_{\text{max}}$ (0.58-1.28 $\mu$mol g DW$^{-1}$ h$^{-1}$) and $K_m$ (2.18-7.89 $\mu$mol L$^{-1}$) values as in our experiment. Their results and ours imply that macrophyte species in general are able to utilize periods of increasing $\text{Pi}$ availability (Nielsen et al. 2006), except $L. \textit{uniflora}$ that actually, as described above, reached saturation in the roots at almost the ambient concentration of $\text{Pi}$ in the sediment porewater.

In conclusion, the results of our study of $\text{Pi}$ uptake kinetics for four macrophyte species in Lake Hampen, Denmark, $L. \textit{uniflora}$, $M. \textit{alterniflorum}$, $P. \textit{perfoliatus}$ and $E. \textit{canadensis}$, indicate that slow-growing stress tolerant isoetids can exploit $\text{Pi}$ resources through efficient uptake by the roots at low $\text{Pi}$ concentrations even more efficiently than faster-growing macrophyte species. Our findings point to that at low $\text{Pi}$ concentrations in the lake water $L. \textit{uniflora}$ will be able to survive on the $\text{Pi}$ pools in the sediment porewater. If $\text{Pi}$ concentrations increase in the lake water and porewater, elodeids will have an advantage and might outcompete $L. \textit{uniflora}$. $M. \textit{alterniflorum}$ had an efficient uptake at both low and high $\text{Pi}$ concentrations by both roots and shoots, suggesting that $M. \textit{alterniflorum}$ will be a strong competitor to isoetids at low $\text{Pi}$ concentrations. However, $E. \textit{canadensis}$ and $P. \textit{perfoliatus}$ are not as competitive at low $\text{Pi}$ concentrations, and especially $P. \textit{perfoliatus}$ performs better at higher concentrations.

For all species root tissue was the dominant organ for $\text{Pi}$ assimilation at the $\text{Pi}$ concentrations measured in lake water and porewater in Lake Hampen. The leaf tissue of $M. \textit{alterniflorum}$ may potentially be responsible for more than 40% of the $\text{Pi}$ uptake. Its large shoot
surface area is an advantage that explains about half the leaf uptake, indicating that *M. alterniflorum* is competitively strong also at high Pi concentrations in the lake water.

**Acknowledgement**

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Phosphate uptake by the aquatic macrophyte *Isoetes australis* growing in oligotrophic vernal rock pools in south-western Australia

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Abstract

The aquatic freshwater macrophyte species *Isoetes australis* S. Williams is growing in rock pools situated in south-western Australia in an environment where dissolved inorganic phosphorus (Pᵢ) availability limits plant growth. The Pᵢ uptake characteristics are described for *I. australis*, by using a radioactive $^{33}$Pᵢ technique to determine Pᵢ uptake kinetics. Root tissue showed better Pᵢ uptake by having a lower $V_{\text{max}}$ and $K_{\text{m}}$, and a higher Pᵢ affinity at low ambient Pᵢ concentrations compared to leaf tissue. *I. australis* also showed a morphological adaptation to the environment having about twice as much root tissue as leaf tissue, facilitating use of the higher P pools in the sediment compared to the shallow water column. As a result of the high root surface and better uptake kinetics, roots accounted for 87% of the plants Pᵢ uptake and the green part of the leaves for about 13%. The white basal part of the leaves, constituting 23% of the leaf dry mass, did not seem to contribute to the Pᵢ uptake at ambient Pᵢ concentrations. A pulse-chase experiment revealed that root tissues as well as the oldest green leaves also turned out to be the most important organs in taking up Pᵢ. Translocation of Pᵢ internally in the plant seemed to go from root and oldest leaves to youngest leaves.

Introduction

Phosphorus (P) is, among other elements, an essential nutrient required for plant growth. In the soils of south-western Australia, P and many other elements (e.g. N, K, S, Cu, Zn, Mn and Mo) are limiting plant growth and the content of organic matter is also in general low (Moore 2001, Housley and Walcott 2013). The infertile soils reflect that south-western Australia is one of the oldest stable surfaces on earth, so the landscape and soils are highly weathered (for 65 million years) and soil renewal is minimal (Moore 2001, Lambers et al. 2010; Housley and Walcott 2013). The soils also have a high P adsorption capacity (Moore 2001), which further reduces P availability to most plants (Lambers et al. 2012).
One of the plant species inhabiting a niche within this extremely nutrient poor environment with low P availability is the aquatic macrophyte *Isoetes australis* S. Williams, which is endemic to the south-western Australia (Brunton 2001). *I. australis* is found in broad shallow pits eroded out of the granite bedrock outcrops, which contain sediment deposits and become seasonally wet; such habitats have been referred to as ‘vernal pools’ (Keeley and Zedler 1998) (in the following ‘vernal pools’ will be referred to as ‘rock pools’). Here it grows a few months a year in the wet season, when the rock pools fill with rainwater. The remaining part of the year the pools dry out and the sediment is desiccated. The thin sediment layer mainly consists of wind borne weathered granite, sand, gravel and debris (Brunton 2001; Keeley and Zedler 1998). Besides from growing in a nutrient poor environment, *I. australis* also experiences dramatic diurnal changes in temperature, CO$_2$, O$_2$ and pH (Keeley and Zedler 1998). Combined, all these factors provide an extremely challenging habitat for plants inhabiting these rock pools.

The isoetid species *I. australis* is a small and distinctive species with 1-6 straight, bright green leaves (about 0.7-2 cm in length) arranged in ranks, unlike most other isoetid species that have leaves arranged in a rosette around the corm (Williams 1944, Brunton 2001). *Isoetes* species (quillworts) belongs to the class Lycopodiopsida and reproduce via spores. In *I. australis* the sporangia are imbedded in the basal adaxial side of fertile leaves (Brunton 2001). New leaves and roots emerge from the central leaf rank and old leaves shed from the two ends of the rank. The corm and the white leaf bases are buried in the sediment, and the upper part of each leaf is exposed to light and surface water (Pedersen et al. 2011b). The leaves contain four gas-filled lacunae and have well developed cuticle on the green parts and the roots also are of relatively high gas-filled volume (Pedersen et al. 2011b) which facilitates internal gas transport between roots and leaf tissues (CO$_2$ from roots to leaves and O$_2$ from leaves to roots) as shown for other isoetids (Wium-Andersen 1971). Isoetid species are characterized in having a high root to shoot ratios (ratios of 1 to > 1 are common; Sand-Jensen and Søndergaard 1979; Sand-Jensen et al. 1982). Pedersen et al. (2011b) found for *I. australis* a root to shoot ratios close to 1. This high root dry biomass could be an advantage for exploiting the CO$_2$ and nutrient pools in the sediment (Brouwer et al. 2002; Murphy 2002; Pedersen et al. 2006). In addition, *I. australis* has the ability to use crassulacean acid metabolism (CAM) photosynthesis (Pedersen et al. 2011c; Keeley 1983). Under the right conditions *I. australis* can live for several years and it is viable and germinates after having been completely dry for several months (Williams 1944). Thus, *I. australis* populations persist in the extreme environment of these seasonally wet, shallow, rock pools. However, knowledge is limited of how
these macrophytes, growing in the rock pools, cope with this nutrient poor environment.

No one has yet studied nutrient availability in rock pools of south-western Australia (other aspects have been studied, Pedersen et al. 2011a); so, the present study investigated the P-pools and turnover of a few pools and P acquisition characteristics of *I. australis*. Submerged macrophytes take up inorganic P (Pi) both through roots and shoots (Carignan and Kalff 1980; Brix and Lyngby 1985) and since Pi concentrations in surface waters usually are low in oligotrophic freshwater ecosystems, porewater Pi normally is the major source of P for rooted submerged macrophytes (Carignan and Kalff 1980; Barko and Smart 1981; Roelofs et al. 1984; Christiansen et al. paper in preparation). Macrophytes with a well-developed root system and high root to shoot ratio therefore have an advantage in exploiting the P source in the sediment. We are expecting to find similar conditions (i.e. water vs. sediment P-pools in the rock pools). In addition to the advantage to plants in having a high root to shoot ratio in environments low in P, we also expected that *I. australis* had a high affinity for Pi uptake in root and leaf tissues, evaluated by a $^{33}$P radioisotope technique (Nielsen et al. 2006). High affinity for Pi uptake has previously been shown for other submerged macrophyte species growing in low-P environments (Nielsen et al. 2006; Christiansen et al. paper in preparation).

We determined the Pi uptake rates and Pi affinities for roots, leaves including the white basal part and leaves not including the white basal part to assess whether the different tissues of *I. australis* have different functions in the Pi uptake. Pedersen et al. (2011b) found that the buried white basal part of the leaves represented about 30% of the total leaf length, that they had a reduced cuticle compared to the green part of the leaves, and suggested that this part of the leaves could function as an extension of the root system and thereby play a role in nutrient uptake.

In addition to Pi uptake, we designed a pulse-chase experiment in order to determine the internal Pi transport of *I. australis* including the short term translocation of Pi from roots to leaves and from leaves to roots. We also studied whether different tissues, divided into roots, youngest leaves, second youngest leaves and third youngest leaves, received different amounts of Pi via short term Pi translocation. Thus, we were able to determine which organs were most important in Pi uptake and which were receiving the largest amounts via internal redistribution.

**Methods**

**Study site**

Plant material was collected from granite rock pools near Mukinbudin, Western Australia (118.2896 °E, 30.7468 °S); for photographs see Pedersen et al. (2011b). The pools were inhabited
Table 1 Concentrations of total phosphorus (P), total nitrogen (N) and total carbon (C) in percentage of dry mass for the green part of the leaves (leaf G), whole leaves including both the green parts and white bases (leaf GW), newly produced leaves, old yellow leaves and roots of *Isoetes australis* collected from 1 rock pool near Mukinbudin, south-western Australia.

<table>
<thead>
<tr>
<th></th>
<th>P%</th>
<th>N%</th>
<th>C%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf G</td>
<td>0.256</td>
<td>3.25</td>
<td>39.1</td>
</tr>
<tr>
<td>Leaf GW</td>
<td>0.214</td>
<td>2.94</td>
<td>37.1</td>
</tr>
<tr>
<td>Leaf New</td>
<td>0.435</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leaf Yellow</td>
<td>0.145</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Root</td>
<td>0.192</td>
<td>1.65</td>
<td>27.7</td>
</tr>
</tbody>
</table>

by a few macrophyte species, where *I. australis* was dominating at the time of collection (September 2012). At the time of sampling the sediment in the pools was 1-3 cm deep in depressions on top of solid granite and the pools were shallow (about 1-6 cm water depth), but water depth is variable with time, relying on rain as the only source of water. The four pools sampled had a surface area of 3.89 ± 0.22 m².

**Sampling**

Sampling was conducted in September 2012. Four rock pools containing *I. australis* populations were evaluated for physical and chemical characteristics. The pools length and width were measured as well as water depth and sediment depth. Surface water samples were taken and kept at 4 °C until being analyzed the next day for Pi concentrations. To analyze sediment characteristics, sediment turfs (20x20 cm) were sampled and kept submerged in the natural water in buckets at 4 °C until analyses the next day. Sediment was analyzed for concentrations of Pi in the porewater, easily (water) extractable Pi, total phosphorus (TP) and total iron (Fe) contents and loss on ignition (LOI).

Plant material was collected from one of the four rock pools. Plants were brought back as turfs submerged with rock pool water in trays, so that they remained intact. The trays were kept in a phytotron (naturally lit, 20 °C/15 °C d/n) for up to 12 weeks. The temperature in these shallow, open trays could rise to 29 °C during the day. Deionized water was added daily or every second day to maintain 3-5 cm water depth above the leaves, so that leaves were always submerged.

**Pi uptake rates**

To be able to determine affinity in roots and leaves at low Pi concentrations we used a ³³P radioisotope technique (Nielsen et al. 2006; Christiansen et al. paper in preparation) to determine the short-term uptake rates at low Pi concentrations. Pi is taken up by transport proteins in the cell membrane (Schachtman et al. 1998; Nussaume et al. 2011), so next to determining the Pi uptake in relation to dry mass, we also determine the uptake rate in relation to root and leaf surface area, since it could be expected, that a higher surface area would result in a higher uptake rate.

Time series incubations were conducted as part of the ³³ Pi uptake kinetics incubation experiment in order to determine an adequate incubation time with a nearly constant
The experiment was conducted on roots and leaves. Only ‘white roots’ with no plaque were chosen for the incubations and between 6-8 leaves (n=3), with incubations in 100 ml of media (described below). The time-series incubations were carried out at two Pi concentrations (0.032 μmol L\(^{-1}\) and 3.229 μmol L\(^{-1}\)), 3 incubation times (30, 60 and 120 min) and with 1 μCi \(^{33}\)P added to all incubations. No epiphytes were present.

The experiment was conducted, using the same method as Christiansen et al. (paper in preparation). Incubation media was a dilute nutrient solution to represent ‘artificial rock-pool water’ made using the same recipe as Pedersen et al. (2011b): 100 HCO\(_3\)-, 150 Ca\(^{2+}\), 100 Mg\(^{2+}\), 100 K\(^+\), 300 Cl\(^-\) and 100 SO\(_4^{2-}\) μmol L\(^{-1}\) resulting in a final electrical conductivity of 85 μS cm\(^{-1}\). The incubations were conducted at 24 °C in polycarbonate bottles (150 mL). The bottles were mounted on a rotating incubator to provide mixing in the bottles. Incubations were conducted at the room background light (PAR = 10 μmol quanta m\(^{-2}\) s\(^{-1}\)). Christiansen et al. (paper in preparation) found that light intensity had no influence on Pi uptake at short term incubations.

After incubation the roots and leaves were rinsed following a three step washing procedure to remove \(^{33}\)Pi, adsorbed to the surface: (1) in 64.6 μmol L\(^{-1}\) Na\(_2\)HPO\(_4\), (2) in 0.02 mol L\(^{-1}\) HCl (pH = 1.7), and (3) in distilled H\(_2\)O, each washing step was for approximately 30 sec. After rinsing and drying (on paper tissues) leaves and root tissue fresh mass was determined prior to activity determination. Tissue was treated in NaClO after which scintillation cocktail (HionicFluor) was added. Activity of samples was determined using a LS 6500 Beckman scintillation counter. Counts were corrected for background using blanks and quenching was corrected by using internal standards. Activity in the media was determined before and after each incubation (100 μL media and 5 ml HionicFluor). Since the Pi uptake in the 4 time series experiments could not be explained with a clear linear model with an intercept on the x-axis through a value ≥0 and passing through 30 and 60 minute incubation times (Fig. 1), this probably is an indication that the linear uptake in the initial phase was taking place during the first 30 minutes. After 30 minutes, the tissues appear to be transporting some of the absorbed P to other plant parts or in this case efflux back to the media. Activity in the media decreased by less than 5% even at the 120 min incubation time, so supply did not influence the uptake pattern. In the pulse-chase experiment it seemed (see description of the pulse-chase experiment further down in the methods), that the internal transport of P to other tissues started after an incubation time of 60 minutes. For this reason, and since incubation times shorter than 30 minutes could introduce more uncertainty due to handling times, an incubation of 30 minutes was chosen for the Pi uptake kinetics experiment. Both leaf and root tissue uptakes reached saturation at a Pi concentration of 3.229 μmol L\(^{-1}\) after 120 min. At
Pi of 0.323 μmol L⁻¹ roots also reached saturation after 120 min incubation.

The experimental design for the Pi uptake kinetics corresponded to that of the time series experiment, except that the incubation time was constant (30 min) and the Pi concentrations varied. The experiment was conducted on roots, whole leaves including the green top and the white bases (leaf GW) and on leaves with only the green top (leaf G). The Pi concentrations in the media were 0.016, 0.032, 0.065, 0.323, 1.61, 3.23 and 12.9 μmol L⁻¹. During all incubations the Pi concentrations in the media remained relatively constant (less than 5% decline) owing to the large volume of media relative to tissue sample.

The pulse-chase experiment
Phosphate absorption by roots and transport to the leaves as well as the absorption by leaves and transport to the roots was examined using ³²³P as a tracer for analysis of Pi uptake and distribution. In a pulse-chase experiment leaves and roots, respectively, were exposed to radioactive labeled ³²³Pi, which over time was chased in unexposed tissue. Intact plants (n=3) were placed with only root or leaves exposed to a 24.5 ml nutrient solution containing sufficient Pi (13 μmol L⁻¹), for plants not to be limited by Pi within the incubation time, and 1 μCi ³²³Pi at 4 different time periods, 15, 30, 60 and 120 minutes. All plants used had three leaves. The exposures were conducted at room temperature (24 °C) in closed containers wrapped with water saturated tissue to ensure high humidity for air-exposed tissues.

After exposure, leaves and roots were excised and divided into 4 classes: roots, youngest leaves, second youngest leaves and third youngest leaves, only the green part of the leaf was analyzed. Root and leaf tissue was then rinsed following a three step washing procedure and activity in the tissue was determined as described above in the ‘Pi uptake rates’ experiment.

Leaf turnover and density
From the 11th of October to the 15th of December 2012, a period of 66 days, 30 individuals of I. australis were marked in the trays by placing a toothpick with a specific color code in the sediment next to the plant. Every second day all leaves were counted and dates of new leaf emergence recorded. Leaf color was visually assessed and also recorded. From these data leaf lifespan and leaf turnover rate were determined. Density of I. australis was determined by counting all individuals in 10X10 cm squares (n = 3).

Plant and sediment analysis
Plant tissue was sampled as roots, youngest new leaves, mature green leaves and old yellow leaves, respectively, for analysis of total phosphorus (TP), total nitrogen (N) and total carbon (C) concentrations (Table 1). The plant material was dried at 60 °C and combusted (520
°C, 2 h). The combusted tissue was then boiled in 1 M HCl (120 °C, 0.5 h), and TP in roots and leaves was measured spectrophotometrically as dissolved inorganic P (DIP) with the molybdenum-blue method (Koroleff 1983) on the extractions. N and C concentrations in dried plant samples were analyzed using a CarloErba CHN EA1108-Elemental Analyzer. Also root to shoot ratio (dry mass basis) was determined on dried plant samples (n = 12). Surface area to dry mass ratio of fresh leaves and roots (n = 10) was determined by 2 dimension picture analysis using WinRhizo (Regent Instruments Inc.). This ratio was used to convert uptake rates per unit dry mass to uptake rate per unit surface area.

Freshly collected pool water samples were filtered through 1.2 µm glass fiber filter (GF/C) prior to DIP analysis (described above). Porewater from freshly collected sediment samples was retrieved by centrifugation, filtered through 1.2 µm GF/C filter, and analyzed for DIP. Fresh sediment was homogenized prior to analysis. Easily extractable Pi (termed H₂O Pi) was extracted from 1 g homogenized wet sediment by shaking twice in 25 ml deionized water for 1 h, filtered through 1.2 µm GF/C and measured as DIP. Homogenized wet sediment samples were dried at 60 °C to determine dry mass. Dried sediment was used to determine loss on ignition (520 °C, 5 h). On extracts of the ignited sediment (boiled in 1 mol L⁻¹ HCl at 120 °C, 1 h) TP was subsequently measured as described for DIP. Total-Fe was measured on the same extracts by inductively coupled plasma optical emission spectrometry (ICP-OES).

Table 2 Phosphorus per m² in rock pools in sediment, including inorganic Pi in porewater (DIP) and loosely adsorbed inorganic Pi (H₂O Pi), in surface water, and in root and leaf tissue. Sediment and water samples were collected from 5 rock pools near Mukinbudin, south-western Australia.

<table>
<thead>
<tr>
<th></th>
<th>µmol P m⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>293920</td>
</tr>
<tr>
<td>- of which H₂O Pi</td>
<td>1232</td>
</tr>
<tr>
<td>- of which DIP porewater</td>
<td>1.43</td>
</tr>
<tr>
<td>Surface water</td>
<td>&lt; 3.28</td>
</tr>
<tr>
<td>Root tissue</td>
<td>1493</td>
</tr>
<tr>
<td>Leaf tissue</td>
<td>749</td>
</tr>
</tbody>
</table>

Calculations

V_{max} and K_m were determined by plotting the Pi uptake rate against the Pi concentration and fitting the data to the nonlinear Michaelis-Menten model in GraphPad Prism 5, with standard deviations also calculated by this program. Affinities were determined by plotting the Pi uptake as a function of the medium Pi concentrations at low concentrations (0.016, 0.032, 0.065 and 0.3229 µmol L⁻¹) where the uptake rates were linear, and by fitting the data to the linear regression model. The linear regression models determined from the data were used, together with other plant and environment data, to calculate the total Pi uptake per hour in a whole plant at measured ambient rock pool phosphate concentrations in pool water and porewater. Uptake rates were calculated with the assumption that the $^{33}$Pi transport was
unidirectional, with no loss from the tissues back into the external environment.

Disintegrations per unit time (DPM) were calculated from counts per minute (CPM) by use of internal standards in both the incubation media and in vials with tissue samples and NaClO. From the relationship between the $^{32}$Pi uptake and the concentration of $^{33}$P and $^{31}$Pi in the solution, the absorbed $^{31}$Pi was determined accordingly:

\[ \left( \frac{^{32}Pi \text{ in tissue [DPM]}}{\text{added } ^{33}Pi \text{ activity to media [DPM]}} \right) \times \text{added } ^{31}Pi \text{ amount [µmol]} \]

**Statistics**

Statistics were performed in GraphPad Prism 5. For the estimated Michaelis-Menten models 95% confidence intervals were determined, and fitted values were considered different when there was no overlap between these. Slopes and intercepts estimated from the 3 linear regression models were tested for significant differences using an Analysis of Covariance (ANCOVA) and 95% confidence intervals are shown. Two-way ANOVA was used to test if the factors ‘tissue type’ and ‘incubation time’ in the pulse-chase experiment had a significant effect, following a Bonferroni posthoc test to compare each treatment.

**Results**

**Plant and rock pool characteristics**

*I. australis* used in this study were characterized by having 2-3 leaves per individual with an average leaf turnover rate of 1 leaf month$^{-1}$, where 32.7% of the new leaves were produced between -1 to 2 days after an old leaf was senescent and decay, and 55.1% of the new leaves were produced within 6 days after an old leaf was senescent and decay (not shown). Tissue TP concentrations varied with the youngest new leaves containing more TP per unit dry mass (0.435%) compared to the other tissues, being 3 times as much as than the yellow senescent leaves (0.145%), which contained the lowest TP concentration (Table 1).

From the sediment characteristics for September 2012 DIP concentrations in surface water and porewater (< 0.1 and 0.23 ± 0.07 µmol

**Table 3** The maximum Pi uptake rates ($V_{max}$) and the half saturation constants ($K_m$) calculated from the Michaelis-Menten model estimated for the green part of the leaves (leaf G), whole leaves including the green part and white basis (leaf GW) and roots of *Isoetes australis* (mean ± SD, n = 3). Parameters are calculated per dry mass (g DM). Leaf G and leaf GW did not reach saturation and consequently $V_{max}$ and $K_m$ for these two tissues are probably underestimated. Additionally, the surface area to dry mass ratio is presented (mean ± SD, n = 10).
Table 4 Affinity ($\alpha$: L g$^{-1}$ DM h$^{-1}$) for $Pi$ at low concentrations (0.016, 0.032, 0.065 and 0.3229 µmol L$^{-1}$) determined via linear regression on the $Pi$ uptake rate as a function of $Pi$ concentration for the green part of the leaves (leaf G), whole leaves including the green part and white basis (leaf GW) and roots for *Isoetes australis* (mean ± SD, n = 3) (Fig. 3). The linear regression model is determined both on uptake rates per dry mass (g) as well as per surface area (cm$^2$). Letters indicate significant different affinities (ANCOVA, $p<0.001$).

<table>
<thead>
<tr>
<th>Affinity in respect to dry mass</th>
<th>Affinity in respect to surface area</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>$\gamma^2$</td>
</tr>
<tr>
<td>Leaf G</td>
<td>0.134 ± 0.017$^a$</td>
</tr>
<tr>
<td>Leaf GW</td>
<td>0.068 ± 0.015$^b$</td>
</tr>
<tr>
<td>Root</td>
<td>0.154 ± 0.008$^a$</td>
</tr>
</tbody>
</table>

L$^1$, respectively) and H$_2$O $Pi$ sediment content (< 0.1 µmol cm$^{-3}$) were low, whereas TP content in the sediment was ~200 fold higher (16.70 ± 1.50 µmol cm$^{-3}$). The high Fe content in the sediment (348 ± 43 µmol cm$^{-3}$) resulted in a Fe:TP ratio of 20.9 ± 1.7. The organic content in the sediment was 6.25 ± 0.95 % and the sediment dry weight was 64.74 ± 4.41 % of wet weight.

An estimate of the $P$ pools in sediment, water, and leaf and root tissues of *I. australis* per m$^2$ (Table 2) was made on the basis of the surface water DIP and the TP content in the sediment and the TP content in root and leaf dry mass of the *I. australis* population (density was 15667 ± 3055 individuals per m$^3$). There was a large $P$ pool bound in the sediment (99.2 % of the total $P$; Table 2). The root to shoot ratio (2.16 ± 1.44) explains why there was about twice as much $P$ in root tissue per m$^2$ than in the leaf tissue.

$Pi$ uptake rates

Both leaf and root tissue showed a positive relationship between the $Pi$ uptake rate and the $Pi$ concentration (Fig. 3). Leaf tissue (both leaf G and leaf GW) did not reach saturation across the range of $Pi$ concentrations used but showed a linear relationship between the $Pi$ uptake rate and the $Pi$ concentration in the media. Consequently, reliable maximum $Pi$ uptake rates ($V_{max}$) and half saturation constants ($K_m$) from the non-linear hyperbolic Michaelis-Menton model could not be determined for the leaf tissue. Root tissue on the other hand did show a positive non-linear relationship between the $Pi$ uptake rate and the $Pi$ concentration, and the Michaelis-Menton kinetics were used to determine $V_{max}$ and $K_m$ (0.82 ± 0.19 µmol g$^{-1}$ DM h$^{-1}$ and 10.54 ± 4.47 µmol L$^{-1}$ respectively; Table 3). Both leaf G and leaf GW had higher $V_{max}$ and $K_m$ than roots, but it was not possible from this dataset to conclude whether $V_{max}$ and $K_m$ differed between leaf G and leaf GW, since these tissues did not reach saturation.

In addition, linear regression was used to describe the $Pi$ uptake rates at low $Pi$ concentrations for leaf G, leaf GW and root tissues (Fig. 3). Roots and leaf G did not show significantly different affinities for $Pi$ (0.154 ± 0.008 and 0.134 ± 0.017 L g$^{-1}$ DM h$^{-1}$, respectively, ANCOVA, $p=0.301$) whereas leaf GW showed 50%
lower affinity \((0.068 \pm 0.015 \text{ L g}^{-1} \text{ DM h}^{-1})\), ANCOVA, \(p=0.0004\), Table 4). From the estimated linear regression models (based on low \(\Pi\) concentrations, on the ambient surface water and porewater \(\Pi\) concentrations \(I.\ australis\) experience in the rock pools, and on the root and leaf dry mass) it is possible to calculate how much \(\Pi\) roots and leaves, respectively, take up per time unit (Table 5). From these results it appears, that at ambient \(\Pi\) concentrations in surface water and porewater that the root and leaf G \(\Pi\) uptake rates differ by a factor 3 (0.036 and 0.012 \(\mu\text{mol g}^{-1} \text{ DM h}^{-1}\), respectively, Table 5). Because of this higher uptake rate at ambient \(\Pi\) concentrations and since the root to shoot ratio was around 2, the roots ended up being responsible for 87% of the \(\Pi\) uptake (Table 5). Additionally, the green part of the leaf (leaf G) was responsible for 100% of the leaf uptake at the ambient \(\Pi\) concentration (0.1 \(\mu\text{mol L}^{-1}\)) even though the white part of the leaves corresponded to about 23% of the shoot dry mass and experienced a higher \(\Pi\) concentration (0.23 versus 0.1 \(\mu\text{mol L}^{-1}\)). However, since the \(\Pi\) concentration in the surface water was below the detection limit (<0.1 \(\mu\text{mol L}^{-1}\)) it is not possible to tell whether the leaf tissue had an even lower \(\Pi\) uptake rate.

The \(\Pi\) uptake per surface area did not show any difference compared to “per dry mass based uptake rates” at low ambient \(\Pi\) concentrations (Table 5).

**The pulse-chase experiment**

When leaf tissue was exposed to the radioactive media in the pulse-chase experiment the two-way ANOVA showed, that only the type of tissue had a significant effect on the activity in the tissue (\(F=23.85, p<0.0001\)), whereas incubation time did not have an significant effect (Fig. 4a). When roots were exposed to the radioactive media (Fig. 4b) the two-way ANOVA showed that both incubation time and type of tissue had a significant effect on the tissue activity (\(F=9.96, p<0.0001\) and \(F=3.40, p=0.029\), respectively). The incubation time had the most important effect with a general increase in the activity in all tissue types over time, whereas the interaction was not significant (\(F=1.77, p=0.114\)). The Bonferroni

### Table 5 Calculated \(\Pi\) uptake at measured ambient \(\Pi\) concentrations in rock pools in September 2012 for leaf G, white leaf basis and root tissues of \(I.\ australis\) based on the linear regression model

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Ambient DIP (\mu\text{mol L}^{-1})</th>
<th>Dry mass per individual (mg)</th>
<th>Linear regression model estimated uptake rates (\mu\text{mol} \text{ Pi} \text{ uptake g}^{-1} \text{ DM h}^{-1})</th>
<th>(\Pi) uptake per individual (\mu\text{mol h}^{-1})</th>
<th>Linear regression model estimated uptake rates per surface area (\mu\text{mol} \text{ Pi} \text{ uptake cm}^{-2} \text{ h}^{-1})</th>
<th>(10^3)</th>
<th>Percentage of whole plant (\Pi) uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf G White basis</td>
<td>&lt;0.1</td>
<td>0.69</td>
<td>0.012</td>
<td>0.009</td>
<td>0.008</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Root</td>
<td>0.23</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>1.54</td>
<td>0.036</td>
<td>0.055</td>
<td>0.026</td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>
Phosphate uptake per dry mass (µmol g⁻¹ DM) as a function of incubation time (30, 60, and 120 min) by leaves and roots of *Isoetes australis* at two different Pi concentrations: (a) 0.0323 µmol L⁻¹ and (b) 3.2285 µmol L⁻¹ (mean ± SD, n = 3).

The Bonferroni posthoc test showed, that when roots were exposed, there was no significant difference between the activity level in the different tissue types at the first 3 incubation times, whereas after 120 minutes incubation, roots had a significantly higher activity than the youngest and second youngest leaves (p<0.05). When leaves were exposed, the Bonferroni posthoc test showed, that third youngest leaves had significantly higher activity than all other tissue types after 15 minutes incubation time (p<0.05), whereas after 30 and 120 minutes, the activity in the third youngest leaves were only significantly higher than the activity in the root tissue (p <0.05 and p<0.001, respectively), because the activity in the youngest and second youngest leaves increased after 60 and 120 minutes (Fig. 4b).

The time-series experiment (especially for the leaves) as well as the pulse-chase experiment indicated that the rate of ³²P uptake in the tissue might not have been linear with time even below 30 minutes incubation time. This means that we could have underestimated uptake rates slightly which means that affinity and V_max could be underestimated while K_m values could be lower than reported here.

Discussion

This experiment showed that root tissue of *I. australis* was responsible for 87 % of the Pi uptake at ambient Pi concentrations, emphasizing the importance of the root tissue in covering this species P demand. Root and leaf tissue showed different uptake kinetics. Root tissue for *I. australis* had a lower V_max (0.82 ± 0.19 µmol g⁻¹ DM h⁻¹) and K_m (10.55 ± 4.47 µmol L⁻¹) than the 2 leaf tissue types (leaf G and leaf GW), which did not reach saturation when incubated at the highest Pi concentration (14 µmol L⁻¹). Nielsen et al. (2006), found similar values of V_max (0.74 ± 0.22 µmol P g⁻¹ DM h⁻¹ and 1.28 ± 0.24 µmol P g⁻¹ DM h⁻¹) and K_m (3.34 ± 3.40 µmol P L⁻¹ and 7.89 ± 3.60 µmol P L⁻¹) values for root and leaf tissue for *T. testudinum*. Christiansen et al. (paper in...
Figure 2  Phosphate uptake rates (µmol g\(^{-1}\) DM h\(^{-1}\)) as a function of Pi concentrations (µmol L\(^{-1}\)) illustrated by Michaelis-Menten saturation kinetics for: (a) the green part of the leaves, (b) whole leaves including the green part and white base and (c) roots of *Isoetes australis* (*n* = 3). Solid lines are best fit to the Michaelis-Menten model and dotted lines are 95% confidence intervals.

These species indicate that the roots are more important than the leaves in meeting the P requirements by Pi uptake at low Pi concentrations.

The rock pools are characterized by having an extremely large water volume per unit soil surface due to a water depth of 1-10 cm and a surface area of about 4-6 m\(^2\). This might result in periodically similar Pi concentrations in porewater and surface water in contrast to what usually characterize oligotrophic lakes, where the ratio between the water volume and the water depth is much larger, and porewater Pi concentration usually exceed surface water Pi concentrations (Wetzel 2001). This potentially higher Pi concentration in the surface water in the rock pools in relation to the porewater Pi concentration, could explain the high *V*\(_{max}\) in leaf tissue found for *I. australis* as an adaptation to this environment. Nevertheless, this experiment clearly demonstrated, that the Pi uptake rate for *I. australis* was P limited in the rock pools, with a *K*\(_m\) for Roots of 10.55 ± 4.47 µmol P L\(^{-1}\), a Pi concentration which is about 50 times higher than the ambient porewater Pi concentration (0.23 µmol P L\(^{-1}\)), and a *K*\(_m\) for leaves more than 269 and 1155 times higher the ambient Pi concentration preparation) found for another isoetid species, *L. uniflora*, that root tissue reached a higher *V*\(_{max}\) (1.5 ± 0.15 µmol g\(^{-1}\) DM h\(^{-1}\)) and a lower *K*\(_m\) (0.85 ± 0.31µmol L\(^{-1}\)) than leaf tissue (0.38 ± 0.08 µmol g\(^{-1}\) DM h\(^{-1}\) and 2.45 ± 1.41 µmol L\(^{-1}\), respectively). The lower *K*\(_m\) values for root tissues found for
Figure 3 Phosphate uptake rates at low Pi concentrations (0.016, 0.032, 0.065 and 0.3229 µmol L\(^{-1}\)). Solid lines indicate the linear regression models for (a) the green part of the leaves (leaf G), (b) whole leaves including the green part and white base (leaf GW) and (c) roots for *Isoetes australis*. Dotted lines are 95% confidence intervals.

estimated the critical P level in aquatic macrophytes tissue, which is the minimum tissue content associated with maximum growth, to be 0.13% as an average for several species. Christiansen et al. (1985) and Christiansen et al. (paper in preparation) found higher critical P values up to around 0.28 %. TP content found for *I. australis* in this study was about 0.20 % in root and leaf tissue and about 0.40 % in new leaf tissue. This plasticity in the tissue being capable of taking up Pi at several hundred folds higher Pi concentrations could indicate, that *I. australis* has adapted to periods of increased Pi availability, which is similar to what Nielsen et al. (2006) found for *T. testudinum* and what Christiansen et al. (paper in preparation) found for *Myriophyllum alterniflorum* and leaf tissue of *L. uniflora*. The N to P ratio was 8.6 and 13.7 (weight) in root and leaf tissue, respectively, and did not show evidence of P limitation (Ventura et al. 2008).

In environments where Pi concentrations are low enough to limit growth, the affinity for Pi at low concentrations is an important parameter to include when determining which organ is most important for meeting the plants Pi requirements. Linear regression was used to compare leaf G, leaf GW and root Pi uptake rates and affinities at low Pi concentrations (0.016 to 0.3229 µmol L\(^{-1}\)). The more than 2 times higher Pi affinity (0.154 ± 0.008 versus 0.068 ± 0.015) and the lower \(K_m\) value found for Root tissue for *I. australis*, indicate that Root tissue could be more efficient concentration (for leaf G and leaf GW, respectively). Gerloff and Krombholz (1966)
in taking up $P_i$ at the low ambient $P_i$ concentrations compared to the leaf tissue. The affinity found for leaf G tissue ($0.134 \pm 0.017$) was similar to what was previously found for $T. testudinum$ (0.12) by Nielsen et al. (2006), but the $K_m$ values estimated for leaf tissue for $I. australis$ were several fold higher compared to what Nielsen et al. (2006) found for $T. testudinum$. When comparing the results with what Christiansen et al. (paper in preparation) found for Root tissue of $L. uniflora$, $K_m$ was about 7 fold higher and the affinity 15 times lower (2.00) for $L. uniflora$ than for $I. australis$. The uptake rates at ambient $P_i$ concentrations calculated for leaf G, white basis and roots of $I. australis$ (0.012, 0.00 and 0.036 μmol $P_i$ g$^{-1}$ DM h$^{-1}$, respectively), in combination with the high root to shoot dry mass ratio, indicate that the roots were responsible for about 87% of the plants $P_i$ uptake. It is generally accepted that rooted macrophytes can fulfill 90-100% of their $P$ requirements via uptake from the sediment (Carignan and Kalff 1980, Barko and Smart 1981). Christiansen et al. (paper in preparation) found similar results but also that both root and leaf tissue can function as a source for $P_i$ uptake and that different species and organs show different uptake kinetics. The results found in this experiment therefore correspond well with what is previously described in the literature. The high $P_i$ affinity found for just the green part of the leaves, leaf G, the only tissue exposed to the surface water, indicates that leaf tissue could be more important in covering the $P$ requirements for $I. australis$ if $P_i$ concentration should increase in the surface water.

Individuals had 2-3 leaves and produced 1 leaf per month as well as decayed 1 leaf per month in this experiment. Phosphorus concentration in tissue was about 3 times higher in newly produced leaves compared to defoliating leaves. Data and observations showed that individuals in general produced new leaves when another leaf defoliated, indicating that resorption from defoliating leaves to new leaves was taking place. Pedersen et al. (2011b) found for the same species sampled from the same population a leaf turnover rate of 1 leaf week$^{-1}$. However, it is not clear from Pedersen et al. (2011b) how this turnover rate was measured and neither if it was measured in situ or in lab, which could have an effect on the turnover rate (Reich 1998). In addition to the species specific genotype leaf life-span depends on several environmental factors such as temperature, light, water, nutrients and diseases, and in seasonally dry tropical environments some species have a plastic leaf life-time that typically extends as long as the plant remains well hydrated (Reich 1995, Reich 1998). For $I. australis$ the poor nutrient availability could have an effect. The nutrient demands of the plants can be estimated from leaf growth rates and plant $P$ content (Gras et al. 2003). Average leaf production for $I. australis$ was in this experiment estimated to 1 leaf month$^{-1}$ and from the average leaf dry mass ($0.18 \pm 0.031$ mg DM) this corresponds to $6.667 \times 10^{-3}$ g DM d$^{-1}$. 
Using the mean percent P in new leaves (0.435%) for *I. australis*, assuming no P resorption between plant parts, the average P demand in leaf production of *I. australis* in the rock pools would be 0.039 µmol P g⁻¹ DM h⁻¹. The calculated uptake rates from the linear regression models at ambient Pi concentrations for leaf G, leaf GW and roots were estimated to 0.012, 0.009 (not shown) and 0.036 µmol Pi g⁻¹ DM h⁻¹, respectively (Table 5). From this it seems, that the leaf Pi uptake only covers about 25% of the P demand for leaf production, and *I. australis* therefore also depends on contribution from the roots. However, since *I. australis* does have resorption of P from old leaf tissue (up to 42 % of the leaf P content in mature leaves in this study) and since *I. australis* also decayed 1 leaf month⁻¹, this calculated P demand for leaf production (0.039 µmol P g⁻¹ DM h⁻¹) is over estimated. When taking root, rhizome and spore production into account as well, which was not estimated in this study, it is clear, that *I. australis* must rely on roots to cover the P requirements for tissue production at ambient Pi concentrations. Gras et al. (2003) calculated the P demand for *T. testudinum* leaf tissue to be 0.0216 µmol P g⁻¹ DM h⁻¹ at ambient surface water Pi concentrations in Florida Bay. From this demand Nielsen et al. (2006) also concluded, that *T. testudinum*, Florida Bay, only could cover 13-30% of the P requirements from leaf uptake and therefore had to rely on the P pools in the sediment. If we assume that the roots of *I. australis* have the same turnover rate as the leaves and that resorption of P from old root tissue is comparable to what we estimated for leaf tissue (42 %), we can calculate a net requirement of P by *I. australis*, namely the 58% P the plant does not resorb from old tissue but loosen in senescent decayed tissue. These 58 % net requirement of P for *I. australis* corresponds to 16.6 µmol P m⁻² d⁻¹ in leaf and root tissue. If leaf tissue contributes with 13 % of the Pi uptake, the surface water has a Pi turnover of about 1.5 days, whereas the porewater Pi pool must be renewed about 10 times per day to satisfy the roots contribution of 87 % of the plant’s P requirement. In comparison, Jensen et al. (1998) found for *T. testudinum*, at two different sites in Bailey’s Bay, net leaf production rates on 1.18 and 0.666 g DM m⁻² d⁻¹ respectively, and P requirements to be 72 and 28 µmol P m⁻² d⁻¹. When Jensen et al. (1998) compared these requirements to the available DIP in porewater and overlying water, they revealed that these P pools would last 1 d and 3 d respectively, if they were not renewed.

The high TP content in the sediment is a potential source of P for *I. australis*, but the availability of this P pool to the surrounding porewater is regulated by sediment adsorption/desorption reactions (Nielsen et al. 2006). As mentioned in the introduction laterite soils have high concentrations of iron and aluminium near the surface, which have a high P adsorption capacity (More 2004). We measured a high iron content in the sediment (348 ± 43 µmol
cm$^3$), and in oxidized sediments $P_i$ can adsorb to iron (III) and consequently decrease the $P_i$ concentration in the loosely adsorbed inorganic $P_i$ pool and in porewater and thereby reduce $P_i$ availability to roots. The low water depth, which allow oxygen transport to the sediment surface, and *I. australis* characterized by oxidizing the rhizosphere due to radial oxygen loss from the roots (Pedersen et al. 2011b), could contribute to a high iron (III) pool. The high P adsorption capacity of the sediment could explain the low $P_i$ concentrations in the porewater and could be the reason for roots being adapted to low porewater $P_i$ concentrations. However, the relatively higher loosely adsorbed inorganic $P_i$ pool (1232 µmol P m$^{-2}$) compared to the porewater $P_i$ pool (1.43 µmol m$^{-2}$) found in the rock pools seems to be large enough to supply the high $P_i$ turnover rate in the porewater with $P_i$ for months.

The high TP content in the sediment was a quite surprising result in this extremely nutrient poor environment where many elements are deficient for plant growth and organic matter also is generally low (Moore 2001). It could be speculated that the source for the high P content is wind-borne fine soil with adsorbed P added as fertilizer in the surrounding wheat belt, where agriculture dominate the landscape (Grierson and Adams, 1999; Housley and Walcott, 2013).

The morphology in terms of the relatively high root to shoot dry mass ratio (2.16 ± 1.44) found for *I. australis* indicate, up to 2 times higher than what is usual for other isoetid species (Sand-Jensen and Søndergaard 1979; Sand-Jensen et al. 1982), that the root tissue of *I. australis* could be an adaptation to meeting its $P_i$ requirements, since a large root system could be an advantage in utilizing the nutrient sources in the sediment. *I. australis*, in contrast to most other isoetids (Wigand et al. 1998), lack mycorrhizae (O. Pedersen unpubl. data) that might have facilitated mobilization of P from the large P pool bound in sediment minerals and in particulate organic matter (solid-fraction P) in the rock pool sediment. Release of organic acids from the roots may be another possibility to mobilize the solid-fraction P, however, it is unknown whether *I. australis* is capable of this. Formate and lactate have been found in the rhizosphere of another isoetid species, *Littorella uniflora*, but in so low concentrations that, they were insufficient to contribute significantly to desorption of sediment P (Thomsen et al. 2005).

The white basal part of the leaves was also investigated to clarify if it would have any effect on the $P_i$ uptake. At ambient $P_i$ concentrations (0.23 µmol L$^{-1}$) the white basis had did not seem to have an contribute in the $P_i$ uptake where the white basal part was responsible for than 0% of the leaf $P_i$ uptake, even though it corresponded about 23% of the leaf dry mass and experience a more than 2 times higher $P_i$ concentration. This result is surprising and it seems that the buried part of the leaves actually turned out to be a trade off in respect to
Pi uptake even though the species lives in an P limited environment. The purpose of the white basal part is therefore still a question to be answered.

In the pulse-chase experiment we investigated the short term translocation of phosphorus from roots to leaves, where leaves were divided into youngest, second youngest and third youngest. Similarly short term translocation of phosphorus from leaves to roots was investigated as well as translocation between leaves. There was a clear Pi transport from roots to leaves after 60 and 120 min, whereas there was no clear Pi transport from leaves to roots in the 120 min exposure time. The third youngest leaves showed the highest Pi uptake and seemed to be the main source for Pi uptake when only leaves were exposed to the media, since the activity in the youngest and second youngest leaves mainly increased after 60 and 120 min. This result implies that translocation mainly happened after 30 min exposure time. We know from the time series experiment that tissue show saturation after 30-60 minutes incubation time. But translocation of phosphorus in the plant can still take place.

![Figure 4](image)

**Figure 4** Activity per dry mass (DPM g⁻¹ DM) as a function of time (minutes) in youngest leaves, second youngest leaves, third youngest leaves and roots of *Isoetes australis* (mean ± SD, n = 3). (a) Leaves exposed and (b) roots exposed to radioactive ³²P media.

In conclusion, this experiment revealed that *I. australis* lives in an environment with a low Pi availability but that *I. australis* exhibit a large plasticity in its Michaelis-Menten kinetics across a broad range of Pi
concentrations, from as low as 0.016 µmol L$^{-1}$ up to several hundred fold higher than the ambient $Pi$ concentrations. Our results suggest that $P$ may be a limiting nutrient for $I. australis$ at ambient $Pi$ concentrations, and that $I. australis$ needs root $Pi$ uptake to cover the $P$ requirements for tissue production. At ambient $Pi$ concentrations roots were the most important organ in taking up $Pi$, responsible for about 87% of the whole plant $Pi$ uptake. The white basal part of the leaves turned out not to be important in the $Pi$ contribution (0%) at ambient $Pi$ concentrations (0.23 µmol L$^{-1}$) even though it represented 23% of the leaf dry mass. $I. australis$ also showed a morphological adaptation to the nutrient poor environment by having about twice as much root tissue as leaf tissue, which is expected to be an advantage in utilizing the larger $P$ pools in the sediment. The pulse-chase experiment revealed that the short-term translocation of $Pi$ in the plant seemed to go primarily from roots and oldest leaves to the youngest leaves.

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Chapter 3
The barrier to radial oxygen loss does not inhibit phosphate uptake of rice (Oryza sativa L.) roots grown in stagnant solution

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Abstract

Waterlogged and consequently anaerobic conditions result in physiological and morphological adaptations in roots of rice (Oryza sativa L.). One of these adaptations involves development of a barrier to radial oxygen loss (ROL) in the adventitious roots – being cell wall depositions that impede oxygen diffusion from the root aerenchyma to the rhizosphere. Another adaptation involves an increased number of adventitious roots. The objective of this paper was to reveal whether these physiological and morphological modifications would result in changes in the inorganic phosphorus (Pᵢ) uptake capacity in the adventitious roots. Using a radioactive ^3³P-technique, it was possible to determine the Pᵢ uptake rates directly in adventitious roots cultivated in stagnant anaerobic and aerated conditions, respectively.

Adventitious roots of O. sativa showed a high affinity for Pᵢ at low Pᵢ concentrations. Roots grown in stagnant conditions had a higher Pᵢ uptake rate in the full range of Pᵢ concentrations up to 32.3 μmol L⁻¹ compared to roots grown in aerated conditions. The barrier to ROL did not inhibit the Pᵢ uptake rate, but on the contrary, the Pᵢ uptake rate was up to 60% greater by roots from the stagnant treatment even though a barrier to ROL was present. Overall, O. sativa roots express both physiological and morphological plasticity to root-zone oxygen supply, being highly adapted to cope with the extreme environment it experiences from low oxygen to aerobic.

Introduction

Most wetland plant species experience fluctuations in water levels, but are often exposed to completely water-saturated (i.e. waterlogged) soils. When soils are waterlogged, oxygen (O₂) becomes deficient and usually, due to O₂ consumption by respiration and due to the low diffusion rate of O₂ in water, conditions become anaerobic (Ponnamperuma, 1984; Armstrong and Drew, 2002). Anaerobic conditions lead to several environmental changes in the root zone, such as changes in pH and accumulation of different reduced compounds and microbial metabolites (e.g. CO₂, Mn²⁺, Fe²⁺, S²⁻, carboxylic acids and ethylene) (Ponnamperuma, 1984). Therefore, many wetland plant species have developed different adaptations to make it possible to cope with these environmental changes. Morphological adaptations and responses to growth in
anaerobic conditions are, as examples, increased number of adventitious roots per individual, reduced maximum root length and reduced root to shoot ratio (Colmer, 2003b; Colmer and Greenway, 2011). Furthermore, since the roots depend on \( \text{O}_2 \) for respiration, an anatomical adaptation to anaerobic conditions is aerenchyma formation in the root tissue, which makes internal gas transport between root and leaf tissue efficient (\( \text{O}_2 \) from leaves to roots and \( \text{CO}_2 \) from roots to leaves) (Wium-Andersen, 1971; Colmer, 2003b). In addition, many wetland species develop a barrier in the basal zones of the roots to minimize radial \( \text{O}_2 \) loss (ROL) to the surrounding environment (Colmer et al. 1998). A ‘tight’ barrier to ROL is, for example, known to develop in adventitious roots of rice (\( \text{Oryza sativa} \) L.), when grown in stagnant solution (Colmer et al. 1998; Colmer 2003b). How the barrier prevents ROL in roots and how the microstructure of the barrier is composed is not yet fully understood, but it is considered to be a result of suberin and lignin depositions in the exodermis and in the sclerenchyma of the root (Kotula et al. 2009; Shiono et al. 2011).

It could be speculated, that this deposition of suberin and lignin in the exodermis and in the sclerenchyma under stagnant conditions are trade-offs for the plants. Sorrel and Orr (1993) suggested in their study on 3 emergent wetland species (\( \text{Cyperus involucratus} \) Rottb., \( \text{Eleocharis spacelata} \) R. Br. and \( \text{Juncus ingens} \) N. A. Wakef.) that nutrient uptake was largely restricted to the small unlignified part of the root system. It has also been suggested, that the fine lateral roots are the most important source for nutrient uptake, and that the axial root with aerenchyma is inefficient in nutrient uptake (Sorrell and Orr, 1993; Kirk, 2003). Nevertheless, whether the barrier to ROL leads to a disadvantage in nutrient uptake is still not fully resolved and only a few studies on this topic have been done until now (Colmer, 2003a; Insalud et al. 2006; Colmer and Greenway 2011). Colmer and Bloom (1998) found in a study on net fluxes of \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) along adventitious roots of \( \text{O. sativa} \) and the primary seminal roots of \( \text{Zea mays} \) L., that net uptake declined in the more basal regions of \( \text{O. sativa} \) roots containing sclerenchymatic fibres, whereas net uptake in the basal regions of \( \text{Z. mays} \) did not decline, compared to the uptake near the apex. By contrast, Rubinigg et al. (2002) concluded in an experiment with \( \text{O. sativa} \) that the barrier to ROL had no effect on the capacity of adventitious roots to take up nitrate (\( \text{NO}_3^- \)) up to 22 d after plants were exposed to anaerobic conditions (deoxygenated stagnant agar nutrient solution). Another study by Insalud et al. (2006) on the P uptake in roots of \( \text{O. sativa} \) showed, that roots in the initial phase after exposure to anaerobic solution, developed a barrier to ROL within the first 2 d and that the roots relative P uptake also decreased when exposed to a sudden \( \text{O}_2 \) deficiency up to 8 d after exposure to anaerobic conditions. However, Insalud et al. (2006) did not
measure the direct uptake of inorganic phosphorus (Pi) in the root, but measured the relative P content per dry mass. Therefore, other factors than the Pi uptake in the cell membrane could explain the differences they found.

In this study, we were interested in looking more into a possible effect of the barrier to ROL on the phosphate uptake in adventitious roots of *O. sativa*. This study was designed to answer whether adventitious roots are efficient in taking up Pi when cultured in anaerobic condition and whether the barrier is a disadvantage in the Pi uptake. To investigate these questions we compared the Pi uptake rates for adventitious roots of *O. sativa* cultivated in aerated and stagnant solutions at four different distances behind the apex. The Pi uptake rates were determined in relation to dry mass, but also in relation to surface area, since a larger surface area could result in a higher uptake rate. Phosphate absorption in plants takes place in the cell membrane (Schachtman et al. 1998; Nussaume et al. 2011), and therefore a larger surface area and thereby a larger membrane could result in more transport proteins, and in perhaps a higher Pi uptake rate. To determine the short-term Pi uptake rates we used a ³³P radioisotope technique (Nielsen et al. 2006; Christiansen et al. paper in preparation), and thereby allowing us to minimize the potential source of error from internal Pi transport in the root. This method also allows determination of Pi affinity at low Pi concentrations which is equal to the slope at low Pi concentration where uptake is linear. Determination of the uptake rates were conducted at a range of Pi concentrations from low to high, to determine whether a possible effect on the Pi uptake rate caused by the barrier to ROL would depend on the Pi concentration. In addition, shoot and root morphology was described for plants grown in aerated and stagnant conditions.

**Methods**

**Initial preparations**

Seeds of *O. sativa* were prepared for germination following the procedure also used by Rubinigg et al. (2002). Day 1 seeds were imbibed for 3 h in aerated 0.5 CaSO₄ mmol L⁻¹ and then rinsed in deionized water prior to germination in a foil covered container on a mesh screen floating in a 2 L aerated nutrient solution (a quarter-strength of the composition given below). The germination was initiated in a 30 °C room with high humidity (70-80%). Day 3 seedlings were exposed to light 12 h per day (photon flux density 600 µmol m⁻² s⁻¹) under same conditions. Day 5 the seedlings were transferred to containers, 4 seedlings per container, with 4.5 L of aerated nutrient solution consisting of: 1.5 CaSO₄; 0.4 MgSO₄; 0.625 NH₄NO₃; 0.2 KH₂PO₄; 0.1 Na₂O₃Si; 0.05 Fe-EDTA; 3.75 KNO₃; 2.5 MES (buffer); 0.05 KCl; 0.025 H₃BO₃; 0.002 MnSO₄; 0.002 ZnSO₄; 0.0005 CuSO₄; 0.0005 Na₂MoO₄; 0.001 NiSO₄ mmol L⁻¹; pH adjusted to 6.5 using KOH; [P] adjusted to 200 µmol L⁻¹ using KH₂PO₄.
Day 17 the plants were transferred to the two treatments; aerated: plants continued growing in the aerated nutrient solution, and stagnant: the aerated nutrient solution was replaced with a N₂-flushed deoxygenated nutrient solution containing agar (0.1% w/v). Additionally 2.5 mmol NH₄NO₃ L⁻¹ was added to both treatments to prevent diffusive limitations to nitrogen uptake in the stagnant agar solution (Wiengweera et al. 1997). Plants were grown for 6 d in this under these conditions to make sure that the barrier for ROL would be well developed (Shiono et al. 2011).

**Test for ROL**

Before the Pi uptake rate experiment was initiated, it was important to test whether the adventitious roots of plants grown in the stagnant solution had developed a barrier to ROL. To test this, ROL measurements were conducted on 3 plants from each treatment 6 d after treatments were initiated. The ROL measurements were performed on adventitious roots (at positions 5, 10, 20, 30, 40, 50 and 56 mm behind root tip) of intact plants using root-sleeving O₂ electrodes with an inner diameter of 2.25 mm and a height of 5 mm (Armstrong 1967, Armstrong and Wright 1975). Measurements were conducted in a 30 °C constant temperature and light (photon flux density of 110 µmol m⁻² s⁻¹). Intact plants were fixed at the shoot base on the top of the chamber, with the shoot in the air and roots immersed in anoxic conditions in a stagnant (0.1% agar) nutrient solution consisting of 5.0 K⁺, 5.0 Cl⁻, 0.5 Ca²⁺, 0.5 SO₄²⁻ mmol L⁻¹ in chambers (50x50x250 mm) (Colmer 2003b). After 1 h acclimatization the measurements were initiated. Measurements were conducted on 1 adventitious root on each plant. The root

![Figure 1](attachment:figure1.png)

**Figure 1** Rates of ROL (nmol O₂ m⁻² s⁻¹) along adventitious roots of O. sativa 5, 10, 20, 30, 40 and 55 mm behind root tip for plants grown in aerated conditions, and 5, 10, 20, 30, 40 and 50 mm behind root tip for plants grown in stagnant deoxygenated conditions (mean ± SE, n = 3).
was led through the cylindrical electrode with guides to keep it central and ROL measurements were conducted.

**Pi uptake rates**

Time series incubations were conducted as part of the $^{33}$Pi uptake rate experiment in order to determine an adequate incubation time with a nearly constant $^{33}$Pi uptake rate. The experiment was conducted on excised adventitious root segments (from root tip to 60 ± 10 mm behind root tip) 6 d after treatments were initiated, only on roots cultured in aerated conditions. The time-series incubations were carried out with incubations in 100 ml of media (described below), at two Pi concentrations (0.032 and 3.229 µmol P L$^{-1}$), three incubation times (30, 60 and 120 min) and with 1 µCi $^{33}$Pi added to all incubations.

The experiment was conducted, using the same method as Nielsen et al. (2006) and Christiansen et al. (paper in preparation). Incubation media was the same nutrient solution as the plants had been cultured in (but always without any agar), except the Pi concentration, which instead corresponded to the 2 specific Pi concentrations mentioned above. The incubations were conducted at 24 °C in polycarbonate bottles (150 ml). The bottles were mounted on a rotating incubator to provide mixing in the bottles.

After incubation the roots were rinsed following a three step washing procedure to remove adsorbed $^{33}$Pi: (1) in 64.6 µmol L$^{-1}$ Na$_2$HPO$_4$, (2) in 0.02 mol L$^{-1}$ HCl (pH = 1.7)), and (3) in distilled H$_2$O, each washing step was for approximately 30 sec. After rinsing and drying (on paper tissues) root tissue fresh weight was determined prior to activity determination.

**Figure 1** Rates of ROL (nmol O$_2$ m$^{-2}$ s$^{-1}$) along adventitious roots of *O. sativa* 5, 10, 20, 30, 40 and 55 mm behind root tip for plants grown in aerated conditions, and 5, 10, 20, 30, 40 and 50 mm behind root tip for plants grown in stagnant deoxygenated conditions (mean ± SE, n = 3).

**Figure 2** Pi uptake per dry mass (µmol g$^{-1}$ DM) as a function of incubation time (30, 60, and 120 min) of roots of *O. sativa* at two different Pi concentrations: (A) 0.0323 µmol L$^{-1}$ and (B) 3.2285 µmol L$^{-1}$ (n = 3).
Tissue was treated in NaClO after which scintillation cocktail (HionicFluor) was added. Activity of samples was determined using a scintillation counter (LS 6500 Beckman Coulter Inc., Brea, CA, USA). Counts were corrected for background using blanks and quenching was corrected by using internal standards. Activity in the media was determined before and after each incubation (100 μL media and 5 ml HionicFluor).

To be able to decide an adequate incubation time for the Pi uptake rate experiment, the Pi uptake was plotted as a function of the incubation time to examine whether the roots showed a constant $^{33}\text{Pi}$ uptake with time (Fig. 2). At 3.229 μmol P L$^{-1}$ the Pi uptake showed a linear relationship ($r^2 = 0.9762$) at all three incubation times (Fig. 2a). This indicated that the Pi uptake by the tissue was not limited within the 3 time frames. The patterns observed was not an expression of that the tissue was Pi limited in the media at 3.229 μmol P L$^{-1}$ within the chosen incubations times, since the activity in the media decreased by less than 10% even at the 120 min incubation time. At 0.032 μmol P L$^{-1}$ the Pi uptake showed a weak linear model ($r^2 = 0.5396$) with an intercept on the x-axis through a value ≥0 and through 30 to 60 minutes incubations time (Fig. 2b). This could indicate that the linear uptake in the initial phase is taking place up to the 30 minutes incubation time. The activity in the media at this Pi concentration decreased by about 16-35% at the 60 and 120 min incubation time frames indicating that the tissue was Pi limited in the media within these incubations times at 0.032 μmol L$^{-1}$. For this reason, an incubation time of 20 minutes was finally chosen for the Pi uptake rate experiment.

The experimental design for the Pi uptake rates corresponded to that of the time series experiment, except that the incubation time was constant (20 min) and the Pi concentrations varied (0.032, 0.161, 0.807, 3.23, 12.9 and 32.3 μmol P L$^{-1}$). Adventitious root segments, with no lateral roots, were used. Segments were about 65 and 70 mm long, from root tip, from the aerated and stagnant solution respectively. After incubation the upper 10 mm (55-65 and 60-70 behind root tip) was cut off and discarded, to minimize any effect from diffusion of $^{33}\text{Pi}$ from the media into the tissue via the cut end. The remaining 55 and 60 mm of the incubated roots were divided into 3 smaller segments; 0-13.3, 13.4-26.6, 26.7-39.9 mm. This experiment was conducted 6 d after treatments were initiated. The ROL measurements showed that roots cultivated in stagnant conditions did develop a barrier for ROL after 6 d and that it was most apparent 50 mm behind root tip (Fig. 1). Therefore it was important to measure Pi uptake in root segments 50 mm behind root tip on roots grown in the stagnant conditions. Therefore a follow up experiment was conducted again 12 d after roots were introduced to the treatment. Here only segments 40-53.3 mm (roots from aerated solution) and 47-60 mm (roots from
General morphological characteristics of the plants cultured in the two treatments were registered at harvest, 7 d after treatments were individual, number of leaves per individual, maximum shoot and root length and number of adventitious roots (initial and adventitious) (n=3). Also shoot and root dry mass was determined and from this the root to shoot ratio was calculated (n=3).

Surface area of fresh adventitious roots from each treatment was determined (n=6) by using a 2 dimensional picture analyzing software WinRhizo (Regent Instruments Inc.) followed by dry mass determination. The surface area to dry mass ratio was later used to convert uptake rates per dry mass to uptake rate per surface area.

To investigate the modifications of apoplastic barriers, histochemical studies of cross sections on randomly selected adventitious roots from both treatments were made 10 and 60 mm behind the root tip (n = 10) and were stored in 70% ethanol. To visualize the suberin lamellae in the roots exodermis, cross sections were stained for 1 h with 0.1% Fluorol Yellow 088 in Figure 3 Identification of suberin lamellae in the exodermis of adventitious roots of *O. sativa*, cultivated in aerated (A, C) or stagnant (B, D) conditions, at 10 (A, B) and 60 mm (C, D) behind root tip (n = 10). Presence of suberin lamellae was visible by yellow fluorescence. Arrows show where to find the suberin lamellae in exodermis if present. Bars = 50 µm.
polyethylene glycol-glycerol (Brundrett et al. 1991) and viewed under UV light where it appeared as a yellow band in a microscope (Zeiss Axioskop 2, Carl Zeiss AG, Oberkochen, Germany). To visualize lignified sclerenchyma cells, cross sections were stained for several min with phloroglucinol-HCl and viewed under white light the lignin was visual as a red band on the Zeiss Axioskop 2.

Calculations
Since proteins are responsible for the uptake of Pi, we tried to describe the Pi uptake rates by fitting the data to the nonlinear Michaelis-Menten model. However, the roots did not reach saturation in this experiment and linear regression was used to describe data. Linear regression models were determined by plotting the Pi uptake rate as a function of the medium Pi concentrations and by fitting the data to the linear regression model in GraphPad Prism 5 (Graphpad Software Inc.), and the specified standard errors were calculated by this program as well. Affinities were determined by plotting the Pi uptake as a function of the medium Pi concentrations at low Pi concentrations (0.032, 0.161 and 0.807 μmol P L⁻¹), and fitting the data to the linear regression model. Uptake rates were
Table 1  Slopes from the linear regression all Pi concentrations (0.032, 0.161, 0.807, 3.23, 12.9 and 32.3 μmol L⁻¹) and slopes (affinity= α) for Pi at low Pi concentrations (0.032, 0.161 and 0.807 μmol L⁻¹) in adventitious root segments of O. sativa (mean ± SE, n = 3) determined via linear regression on the Pi uptake rate as a function of Pi concentration (Fig. 5, 6). Segments are given as distance (mm) behind root tip.

<table>
<thead>
<tr>
<th>Aerated</th>
<th>At all concentrations slope ± SE</th>
<th>r²</th>
<th>At low concentrations α ± SE</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-13.3 mm</td>
<td>0.808 ± 0.037</td>
<td>0.9915</td>
<td>1.54 ± 0.076</td>
<td>0.9976</td>
</tr>
<tr>
<td>13.4-26.6 mm</td>
<td>0.677 ± 0.031</td>
<td>0.9920</td>
<td>1.85 ± 0.131</td>
<td>0.9951</td>
</tr>
<tr>
<td>26.7-39.9 mm</td>
<td>0.666 ± 0.031</td>
<td>0.9912</td>
<td>2.07 ± 0.096</td>
<td>0.9979</td>
</tr>
<tr>
<td>40-53.3 mm</td>
<td>0.613 ± 0.050</td>
<td>0.9157</td>
<td>1.06 ± 0.049</td>
<td>0.9979</td>
</tr>
<tr>
<td>Stagnant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-13.3 mm</td>
<td>0.972 ± 0.016</td>
<td>0.9989</td>
<td>0.705 ± 0.042</td>
<td>0.9965</td>
</tr>
<tr>
<td>13.4-26.6 mm</td>
<td>0.935 ± 0.024</td>
<td>0.9973</td>
<td>0.783 ± 0.023</td>
<td>0.9991</td>
</tr>
<tr>
<td>26.7-39.9 mm</td>
<td>1.1 ± 0.013</td>
<td>0.9994</td>
<td>1.14 ± 0.025</td>
<td>0.9995</td>
</tr>
<tr>
<td>47-60 mm</td>
<td>1.56 ± 0.126</td>
<td>0.9050</td>
<td>1.60 ± 0.003</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

calculated with the assumption that the $^{33}$Pi transport was unidirectional, with no loss from the tissues $^{33}$Pi back into the external environment.

Disintegrations per unit time DPM were calculated from CPM by use of internal standards in both the incubation media and in vials with tissue samples and NaClO. From the relationship between the $^{33}$Pi uptake and the concentration of $^{33}$Pi and $^{31}$Pi in solution, the absorbed $^{31}$Pi was determined accordingly:

\[
\left(\frac{^{33}\text{Pi in tissue (DPM)}}{\text{added } ^{33}\text{Pi activity to media (DPM)}}\right) \times \text{added } ^{31}\text{Pi amount (μmol)}
\]

Statistics

Statistics were performed in GraphPad Prism 5. Slopes estimated from the linear regression models were tested for significant differences using an Analysis of Covariance (ANCOVA). 2 outliers were removed from a total of 144 data points in the dataset when fitting the data to the linear regression models. Morphological characteristics were tested if equal between the two treatments using Students t-test and a significant level of 5%.

**Results**

Adventitious roots of O. sativa cultured in stagnant conditions showed contrary patterns of ROL compared to roots of O. sativa cultured in aerated conditions from 5 to 55 mm behind root tip (Fig. 1). Roots grown in stagnant conditions showed a decrease in ROL in relation to the distance behind the root tip from about 520 nmol O₂ m⁻² s⁻¹ at 5 mm behind the root tip to about 40 nmol O₂ m⁻² s⁻¹ at 55 mm behind the root tip. In contrast, roots grown in aerated conditions showed an increase in ROL, as a function of the distance behind the root tip, starting at around 130 nmol O₂ m⁻² s⁻¹ at 5 mm behind the root tip to about 270 nmol O₂ m⁻² s⁻¹ at 55 mm behind the root tip. These results indicated that a barrier to ROL had developed when plants were cultured in stagnant conditions, and that the barrier was most pronounced from 50 mm behind the root tip, as ROL was about 6 times lower than for plants cultured in aerated conditions.
Table 2: Slopes from the linear regression models estimated on the P_i uptake rates per dry mass of adventitious root segments of O. sativa were tested (ANCOVA) if equal (Fig. 5). Tests were conducted between the two treatments (aerated and stagnant) at same distance behind root tip (n = 3).

<table>
<thead>
<tr>
<th>Distance (mm)</th>
<th>F</th>
<th>P</th>
<th>Df</th>
<th>F</th>
<th>P</th>
<th>Df</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-13.3 mm</td>
<td>9.24</td>
<td>0.0047</td>
<td>32</td>
<td>51.1</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>13.4-26.6 mm</td>
<td>11.7</td>
<td>0.0017</td>
<td>32</td>
<td>73.0</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>26.7-39.9 mm</td>
<td>9.17</td>
<td>0.0048</td>
<td>32</td>
<td>65.5</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>40-53.3 and 47-60 mm</td>
<td>40.4</td>
<td>&lt;0.0001</td>
<td>30</td>
<td>6.69</td>
<td>0.0215</td>
<td>14</td>
</tr>
</tbody>
</table>

The development of suberin lamellae in the exodermis in the adventitious roots also showed different results between the two treatments (Fig. 3). In both treatments, suberin was absent 10 mm behind root tip (Fig. 3A-B). At 60 mm behind the root tip suberin lamellae were evident as a bright yellow fluorescence in roots cultivated in stagnant conditions (Fig. 3D), whereas roots cultivated in aerated conditions did not show suberin lamellae at this distance behind the root tip (Fig. 3C).

The development of lignification in the sclerenchyma in the cell walls also showed different patterns in the 2 treatments. In roots of plants cultivated in aerated conditions, lignin was absent in the sclerenchyma 10 mm behind the root tip (Fig. 4A), whereas at 60 mm behind root tip, weak red stains were observed (Fig. 4C). In roots of plants cultivated in stagnant conditions sclerenchyma cells, already 10 mm behind root tip, showed a weak red stain (Fig. 4B), and at 60 mm behind the root tip the sclerenchyma cells showed intense lignifications, evident by clear red stains in the cell wall (Fig. 4D).

In the P_i uptake experiment all root segments from the two treatments showed a positive linear relationship between the P_i uptake rate and the P_i concentration (Fig. 5, 6), but did not reach saturation across the range of P_i concentrations used. Consequently, data were not fitted to the non-linear hyperbolic Michaelis-Menten model, since the estimated maximum P_i uptake rates (V_{max}) and half saturation constants (K_m) were not reliable or could not be

Table 3: Slopes from the linear regression models estimated on the P_i uptake rates per surface area of adventitious root segments of O. sativa were tested (ANCOVA) if equal. Tests were conducted between the two treatments (aerated and stagnant) at same distance behind root tip (n = 3).

<table>
<thead>
<tr>
<th>Distance (mm)</th>
<th>F</th>
<th>P</th>
<th>Df</th>
<th>F</th>
<th>P</th>
<th>Df</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-13.3 mm</td>
<td>30.2</td>
<td>&lt;0.0001</td>
<td>32</td>
<td>36.5</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>13.4-26.6 mm</td>
<td>22.5</td>
<td>&lt;0.0001</td>
<td>32</td>
<td>54.8</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>26.7-39.9 mm</td>
<td>14.1</td>
<td>0.0007</td>
<td>32</td>
<td>35.3</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>40-53.3 and 47-60 mm</td>
<td>15.9</td>
<td>0.0004</td>
<td>30</td>
<td>10.0</td>
<td>0.0068</td>
<td>14</td>
</tr>
</tbody>
</table>
determined. Instead linear regression was used to describe the $Pi$ uptake rates, both over the full range of $Pi$ concentrations (0.032, 0.161, 0.807, 3.23, 12.9 and 32.3 $\mu$mol L$^{-1}$) and at low $Pi$ concentrations (0.032, 0.161 and 0.807 $\mu$mol L$^{-1}$), from which the slope ($\alpha$) is a measure for the $Pi$ uptake affinity (Table 1, Fig. 5, 6).

Distance behind root tip had an influence on the uptake rate (Fig. 6). Root segments, from roots cultivated in aerated conditions, showed significantly different slopes both when compares for low and the full range of $Pi$ concentrations (ANCOVA, $p < 0.0001$ p = 0.03, respectively, Fig. 6A, B). At both $Pi$ concentrations 40-53.3 mm behind the root tip showed lowest $Pi$ uptake rate and the root tip (0-13.3 mm) showed a higher uptake rate than the three other distances at the full range of $Pi$ concentrations. On the contrary, when roots were cultivated under stagnant conditions, the tissue furthest from the root tip (47-60 mm behind root tip) showed the highest $Pi$ uptake rate (ANCOVA, $p < 0.0001$), both when tested at low and the full range of $Pi$ concentrations (Fig. 6C,D).

When comparing the slopes, estimated by the linear regression models (Table 1), between the two treatments (aerated versus stagnant conditions) at the full range of $Pi$ concentrations at the 4 different distances behind the root tip (0-13.3, 13.4-26.6, 26.7-39.9 mm, and 40-53.3 mm for roots from aerated solution) and (0-13.3, 13.4-26.6, 26.7-39.9 mm, and 47-60 mm for roots from stagnant solution), all 4 comparisons showed significantly different slopes (ANOVA, Table 2). In all cases roots cultivated in stagnant conditions reached a higher $Pi$ uptake rate at 32.3 $\mu$mol L$^{-1}$ than roots cultivated in aerated conditions (Fig. 5a, B, C, D). On the contrary, when the estimated slopes at low $Pi$ concentrations (Table 1) were compared for the two treatments, roots cultivated in aerated conditions showed significantly higher $Pi$ uptake rates (ANOVA, Table 2) than roots cultivated in stagnant conditions (Fig. 5E, F, G, H).

The surface area to dry mass ratio was significantly different between the two treatments (Students t-test, $p = 0.03$, $n = 6$), where roots cultivated in aerated conditions had a 14% higher ratio than roots cultivated in stagnant conditions ($3671 \pm 206$ and $3149 \pm 306$ cm$^2$ g$^{-1}$, respectively). When comparing the slopes, estimated from linear regressions on the $Pi$ uptake rates per surface area at the 4 different distances behind the root tip, all 4 comparisons showed significant differences between the two treatments, both when estimated on the full range on $Pi$ concentrations and at low $Pi$ concentrations (Table 3).

*O. sativa* showed different morphology when grown for 7 d in a stagnant solution compared to an aerated solution. Maximum root length was about 44% shorter (Students t-test, $p < 0.05$, $n = 3$; Table 4), total root dry mass was 60% smaller (Students t-test, $p < 0.05$, $n = 3$; Table 4) and total plant dry mass was about 43% larger (Students t-test, $p < 0.01$, $n$
= 3; Table 4) when grown in stagnant conditions. These were the only morphological features, besides surface area to dry mass ratio mentioned above, that were significantly different between the two treatments.

**Discussion**

Roots grown in stagnant conditions had a higher (up to about 2.5 times higher) uptake rate for $\text{Pi}$ in the full range of $\text{Pi}$ concentrations up to 32.3 $\mu\text{mol P L}^{-1}$ compared to roots grown in aerated conditions. This higher uptake rate for $\text{Pi}$ in roots, grown in stagnant conditions, was present both when determined per root mass and per root surface area. Rubio et al. (1997) found in a similar study on *Paspalum dilatatum* (a waterlogging-tolerant grass) comparable results. They found in a P uptake kinetics experiment, that plants cultivated in waterlogged and anaerobic conditions had about a 4 times higher affinity for P in roots than plants grown in aerated conditions. Rubio et al. (1997) concluded from this, that even though root dry mass decreased, the changed root morphology and the higher P affinity in waterlogged roots, compensated in terms of P nutrition for the reduction in root growth. Another study similarly found, for *O. sativa*, an increased Pi uptake rate (up to 18 % increase), when plants were cultivated under anaerobic conditions John et al. (1974). The same was observed in a study by Brix et al. (2010) for phosphate on two other wetland species, *Typha domingensis* and *Cladium mariscus* spp. *jamaicense* (up to about 3.5 and 2 times higher uptake rates, respectively, when grown at low oxygen accessibility).

The results in this present study demonstrated, that adventitious root of *O. sativa* increase their Pi uptake rate when exposed to anaerobic conditions. This may possibly be explained by the mechanism by which *O. sativa* takes up Pi over the membrane, either by having more transport proteins per surface area in the membrane or by having more efficient transport proteins in the membrane (Nussaume et al. 2011). Since the total root dry mass and total plant dry mass for *O. sativa*, when grown in stagnant conditions, increased, it could be assumed, that the total P demand for the plant would increase as well. This higher P demand could therefore be attributed to *O. sativa* in at least two possible mechanisms, 1) via the changed root morphology, and 2) via number and/or species of transport proteins in the membrane to optimize the Pi affinity, comparable to what Rubio et al. (1997) concluded in their study. These findings also clarify one of the questions this study aimed to reveal, namely whether adventitious roots of *O. sativa* were inefficient in Pi acquisition when grown in stagnant solution so that an ROL barrier was induced. The results disproved this hypothesis and showed that the adventitious roots of *O. sativa* were very efficient in taking up Pi at a very wide range of Pi concentration, and even at a
higher capacity in roots previously raised in anaerobic conditions.

This study aimed to clarify whether the barrier to ROL would inhibit the Pi uptake, which also has been suggested previously in the literature. When grown in stagnant conditions adventitious roots of *O. sativa* showed a reduction in ROL on 85% from the basal zones of the root (about 50 mm behind root tip), compared to adventitious roots grown in aerated conditions. On the contrary, ROL did not decline near the root tip, which indicates that the O₂ supply to the root apex was not limiting (Rubinigg et al. 2002). These results are consistent with what earlier studies found (Rubinigg et al. 2002; Insalud et al. 2006; Kotula et al. 2009), that *O. sativa* roots develop a physical barrier to ROL, when exposed to anaerobic conditions, as an adaptation to be able to survive in the stressful anaerobic environment. Kotula et al. (2009) compared the ROL for *O. sativa* grown in stagnant conditions with a histochemical and biochemical study of the development of the Casparian bands and suberin lamellae in the exodermis and lignified sclerenchyma cells in the roots. They concluded that the ROL could be effectively restricted by both suberin and lignin deposited. Our results showed that the barrier to ROL did not inhibit the Pi uptake rate. Surprisingly, the results showed that the Pi uptake rate actually peaked 47-60 mm behind the root tip in the adventitious roots grown in the stagnant condition (up to 66% higher), where the barrier to ROL was present. These findings contribute to the understanding of how *O. sativa* is capable of surviving and thriving in waterlogged anaerobic conditions. The results are also supported by the observed changes in root morphology, where the production of more adventitious roots in waterlogged anaerobic conditions and the barrier to ROL increases the Pi uptake capacity. These adaptations seems to be very beneficial and maybe not that surprising to find in a species that evolutionary has adapted to life in primarily waterlogged conditions. The question could be asked then, why adventitious roots cultivated in aerated conditions showed the highest Pi affinity at the lowest Pi concentrations. It could be speculated that this would be an advantage due to the different behavior of Pi and iron in aerobic and anaerobic conditions. Phosphate in soil (not waterlogged) is not available in the same high concentrations, mainly because Pi can adsorb to iron (III) in oxidized sediments and consequently decrease the Pi concentration in the loosely adsorbed inorganic Pi pool and in porewater and thereby reduce Pi availability to roots. This was also what Rubio et al. (1997) found in their experiment when comparing the available P from waterlogged soils with non-waterlogged soil, where the available P increased almost 3 times per soil volume. Nevertheless, this question needs further investigation.

In this study *O. sativa* did not reach saturation in the uptake rate experiment at the
highest $P_i$ concentration incubated (32.3 µmol L$^{-1}$). Fageria (1974) found 2 mechanisms in *O. sativa* when incubated over a range of $P_i$ concentrations from 0.16 to 161 µmol L$^{-1}$, where *O. sativa* reached to saturation at about 2.5 µmol L$^{-1}$ at the low range of $P_i$ concentrations whereas in the high range of $P_i$ concentrations saturation was reached at about 160 µmol L$^{-1}$. It was concluded from this study that *O. sativa* has two separate systems of different affinity. It was also concluded in the study of Fageria (1974) that the low concentration mechanism would be the most important in $P_i$ absorption by plants growing in soil, where the high concentration mechanism would be involved in other circumstances. In this present study we were not able to rediscover the
Figure 5 $P_i$ uptake rates (µmol g$^{-1}$ DM h$^{-1}$) as a function of low (A, B, C, D) $P_i$ concentrations (0.032, 0.161 and 0.807 µmol L$^{-1}$) and as a function of all (E, F, G, H) $P_i$ concentrations (0.032, 0.161, 0.807, 3.23, 12.9 and 32.3 µmol L$^{-1}$) for adventitious root segments of O. sativa (A, E = 0-13.3 mm; B, F = 13.4-26.6; C, G = 26.7-39.9 mm; D, H = 47-60 mm behind root tip). Every graph illustrates root segments cultivated in aerated (clear symbols) and stagnant (filled symbols) conditions. (Mean ± SE, n = 3).
Figure 6 Pi uptake rates (µmol g⁻¹ DM h⁻¹) as a function of low (A, B) Pi concentrations (0.032, 0.161 and 0.807 µmol L⁻¹) and as a function of all (E, F) Pi concentrations (0.032, 0.161, 0.807, 3.23, 12.9 and 32.3 µmol L⁻¹) for adventitious root segments (0-13.3, 13.4-26.6, 26.7-39.9, 47-60 mm behind root tip) of O. sativa. Root segments cultivated in aerated (A, B, clear symbols) and stagnant (B, D, filled symbols) conditions are illustrated (mean ± SE, n = 3).

low concentration mechanism in the Pi range from 0.0323 to 3.23 µmol L⁻¹ in the two treatments, however we did not conduct incubation in the Pi concentration range from 0.807 to 3.23 µmol L⁻¹ and maybe data would have fitted the Michaelis-Menten model better, if these data were obtained.

Brix et al. (2010) found for T. domingensis and C. marscus, that they adapted their Pi uptake capacity and showed different uptake kinetics depending on the Pi concentration they were acclimated to prior to the experiment. If they were acclimated to low Pi concentrations they had about twice as high Pi concentrations (comparable to what Brix et al. (2010) found for T. domingensis and C. marscus when they were acclimated to low Pi concentrations) and still did not reach to saturation. This could imply that O. sativa was Pi limited prior to the incubation experiment, even when cultivated at a Pi concentration on 200 µmol L⁻¹. These results also implies that O. sativa exhibits a huge plasticity and advantage in the competition for Pi in that it shows a high affinity and V_max for Pi at extremely low and high Pi concentrations, indicating that O. sativa can survive in environments with fluctuating Pi concentrations and benefits from sudden high pulses of Pi concentrations, both in waterlogged and aerated conditions, but also at both high and low Pi concentrations.

In conclusion, this study showed that anaerobic conditions had an effect on almost all measured parameters. Adventitious roots of O. sativa were capable of taking up Pi with a high affinity for Pi and reached to several fold higher V_max than if acclimated to high Pi concentrations. In this present study the O. sativa plants were acclimatized to a high Pi concentration prior to the incubations (200 µmol L⁻¹) but still had a high affinity a the lowest Pi concentrations (0-13.3, 13.4-26.6, 26.7-39.9, 47-60 mm behind root tip) of O. sativa.
affinity both in conditions with extremely low $P_i$ concentrations and in conditions with extremely high $P_i$ concentrations. Roots grown in stagnant conditions had a greater affinity for $P_i$ in the full range of $P_i$ concentrations up to 32.3 $\mu$mol L$^{-1}$ compared to roots grown in aerated conditions. In this study, the barrier to ROL did not inhibit the $P_i$ uptake rate, but on the contrary, the $P_i$ uptake rate was actually greater where the barrier to ROL was present in the root. The study also demonstrated that adventitious root of *O. sativa* increase their $P_i$ uptake rate per unit dry mass and per surface area when exposed to anaerobic conditions. Overall, *O. sativa* express both physiologically and morphologically plasticity, highly adapted to cope with the extreme environment it experiences. The development of a barrier to ROL, shorter root length, more adventitious roots, reduction in the root to shoot ratios and increased $P_i$ uptake capacity likely contribute to this tolerance.

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Table 4  Morphological characteristics of *O. sativa* at harvest cultivated in the two treatments for 7 d (aerated and stagnant) (mean ± SD, *n* = 3). Different letters indicate significant differences (Students t-test, *p*<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Shoots per individual</th>
<th>Leaves per shoot</th>
<th>Maximum shoot length cm</th>
<th>Maximum root length cm</th>
<th>Roots per individual</th>
<th>Dry mass shoot g DM</th>
<th>Dry mass root g DM</th>
<th>Dry mass total g DM</th>
<th>Root to Shoot ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerated</td>
<td>4.67 ± 1.16</td>
<td>3.00 ± 0.00</td>
<td>58.5 ± 4.3</td>
<td>57.33 ± 8.08</td>
<td>4.67 ± 1.15</td>
<td>0.63 ± 0.21</td>
<td>0.026 ± 0.038</td>
<td>0.65 ± 0.24</td>
<td>0.031 ± 0.043</td>
</tr>
<tr>
<td>Stagnant</td>
<td>5.33 ± 0.58</td>
<td>3.07 ± 0.06</td>
<td>65.3 ± 5.1</td>
<td>76.33 ± 9.61</td>
<td>5.67 ± 1.15</td>
<td>0.91 ± 0.29</td>
<td>0.067 ± 0.017</td>
<td>1.14 ± 0.14</td>
<td>0.062 ± 0.009</td>
</tr>
</tbody>
</table>
References


Nussaume, L., Maréchal, E., Thibaud, M.C. and Block, M.A. 2011. Plant plasma membrane and PO$_4^{3-}$ deprivation. In A.S. Murphy et al. [Eds.]. The


