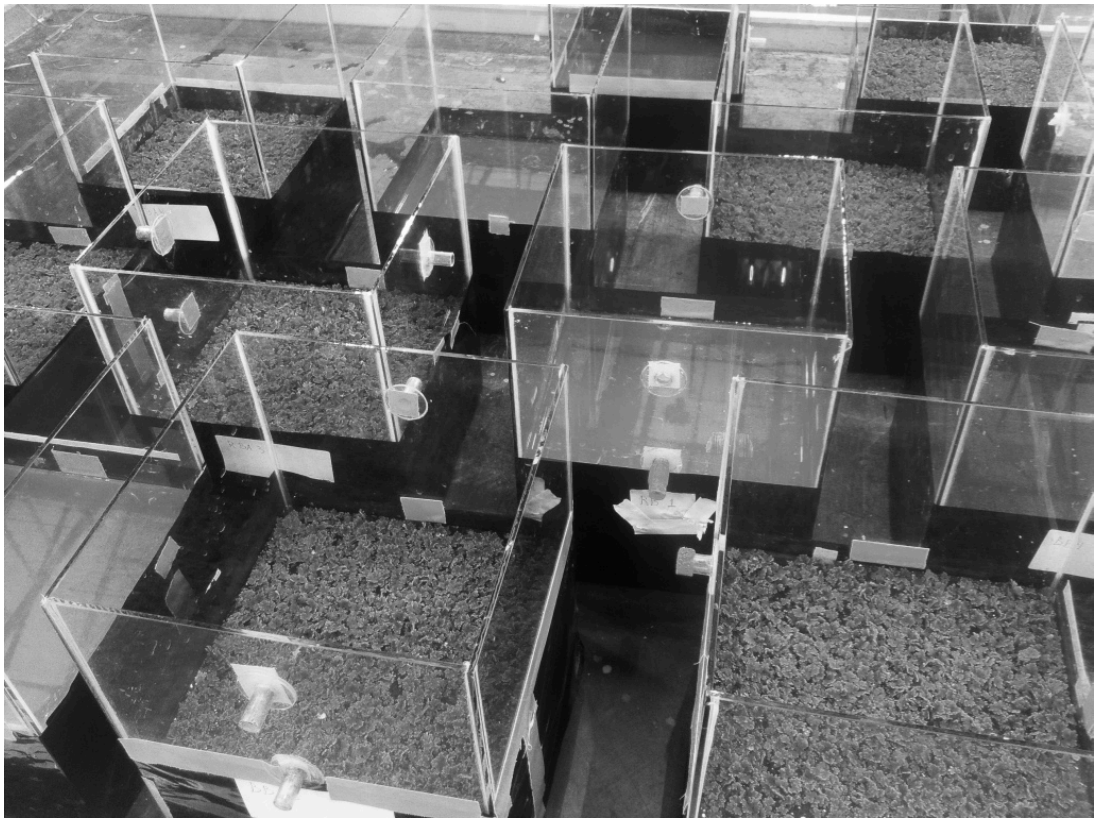
Phosphate mining from iron-rich sediments by means of *Azolla filiculoides* cultivation

Bachelor Thesis

Final Report



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August, 2016

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Abstract

Eutrophication because of heightened phosphorus and nitrogen input into freshwater ecosystems is an increasing problem, while at the same time phosphorus availability for agricultural purposes is decreasing on a high pace. B-Ware Research Centre and Radboud University Nijmegen developed a novel biocascade water purification system to contribute to the solution of both problems. In this system, most phosphorus is immobilized via sorption with oxidized iron, in contrast to conventional helophyte filters, which often utilize phosphorus immobilization via sorption with aluminium in clay-rich soil. This research investigated if immobilized phosphorus can be recovered by means of growing the floating fern *Azolla filiculoides* on both iron-rich sediment and clay-rich sediment, and if adding glucose could be a suitable management tool to enhance oxygen depletion by increasing the biological oxygen demand in the water layer.

Addition of sulphate and sufficient plant density proved to be key factors in facilitating phosphorus mobilization in the iron-rich treatment, resulting in increased phosphate concentrations in the water column after 40 days. Optimum biomass production rates of 0.465 g dw m^{-2} day^{-1} and a concomitant phosphorus yield of 1.85 kg ha^{-1} $year^{-1}$ were found.

No phosphorus mobilization in the clay-rich treatment was observed, resulting in decreasing biomass due to phosphorus deficiency. The glucose pilot experiment showed that addition of glucose to the water layer resulted in very low dissolved oxygen concentrations and increased phosphorus and iron mobilization. Glucose concentrations of 0.5 and 1 g/L resulted in extremely high iron concentrations in the pore- and surface water. 0.2 g/L resulted in the most stable anaerobic conditions and was considered the most suitable option in large scale application. The results of this study show that recovering phosphorus from iron-rich soil by using *A. filiculoides* has great potential to meet the need of sustainable solutions to eutrophication and phosphorus mining.

Acknowledgements

First of all, I would like to thank my supervisor/examiner Ms. Jenny van der Welle for her advice and help. Furthermore, I would like to thank my supervisor at B-Ware Research Centre Prof. Dr. Jan Roelofs for giving me the possibility to work on this research, and for his infectious enthusiasm and immense amount of knowledge and expert opinion. Very special thanks go to my second supervisor Tamara van Bergen from the Radboud University who offered great help in difficult times and provided me with so much input and advice regarding this report. I would also like to thank Ankie de Vries-Brock for her instructions and advice concerning laboratory work. I also would like to thank Sebastian Krosse and Germa Verheggen for performing the elemental analyses of my samples.

Last, but definitely not least, I would like to thank my girlfriend Wiebke for always being there for me.

Table of contents

List of figures

1 Introduction

Since the Green Revolution, phosphorus (P) use – mostly mined from non-renewable phosphorus rock – to increase plant productivity for agricultural purposes has increased significantly (Liu et al., 2008; Verhoeven, 2014). However, only a fraction of the phosphorus is taken up by plants and a substantial part ends up in aquatic ecosystems via surface runoff and seepage, causing eutrophication in aquatic ecosystems (Liu et al., 2008). Eutrophication by increased phosphorus or nitrogen (N) loads in aquatic ecosystems is often unwanted, because it may trigger a rapid shift to a system dominated by phytoplankton, resulting in poor water quality (Scheffer et al., 2001).

Although freshwater systems can be both P and N limited (Kosten et al., 2009), P is often the limiting nutrient for growth in freshwater systems (Hecky & Kilham, 1988; Smith et al., 1999). Therefore, P is one of the priorities in policy frameworks regarding water quality of freshwater systems such as the European Water Framework Directive in Europe. To meet the desired P concentration in surface waters, constructed wetlands are experiencing increased interest as a relatively inexpensive way to polish wastewater (Brix, 1997; Vymazal & Kröpfelová, 2008). These manufactured wetlands utilize some of the ecosystem services of natural wetlands related to water quality such as nutrient and contaminant retention (Vymazal et al., 1998; De Groot et al., 2002).

The most popular type of constructed wetland in Europe is the free water surface flow-helophyte filter (Brix, 1993). Helophytes are rooted emergent macrophytes with resting buds below the water surface (Hickey & King, 2000). *Phragmites australis*, or common reed, is a helophyte species that is often used in free water surface filters. These systems are very effective in filtering out suspended particles and nitrogen removal via nitrification and denitrification but P-removal rates are relatively low (Vymazal et al., 1998). The major processes involved in phosphorus removal are sorption with Fe, Al and Ca ions, but because of relatively little contact between the soil and the overlying water compared to sub-surface flow- or vertical flow systems, the sorption is limited (Vymazal et al., 1998).

Clay-rich soil – naturally containing high concentrations of aluminium – is often used in constructed wetlands (Sakadevan & Bavor, 1998). Aluminium has a very strong P-binding capacity (Rydin & Welch, 1998), and it has been suggested to be more effective in its phosphate-binding capacity than iron (Cooke et al., 2016). Aluminium is also redox-insensitive

and the formation of aluminium salt-phosphorus complexes are insoluble under a wide range of chemical conditions (Cooke et al., 2016). P sorption in the sediment is however a finite process as over time the sediment will become saturated, after which it is often replaced (e.g. Pant et al., 2001).

As part of the project "Rich Water World" in park Lingezegen, B-Ware Research Centre and Radboud University Nijmegen developed a novel constructed wetland utilizing a cascade of biological and chemical processes not only to retain and store nutrients such as phosphorus – like most constructed wetlands – but also to recover these valuable compounds in a sustainable way (Kwakernaak et al., 2015) (Figure 1).

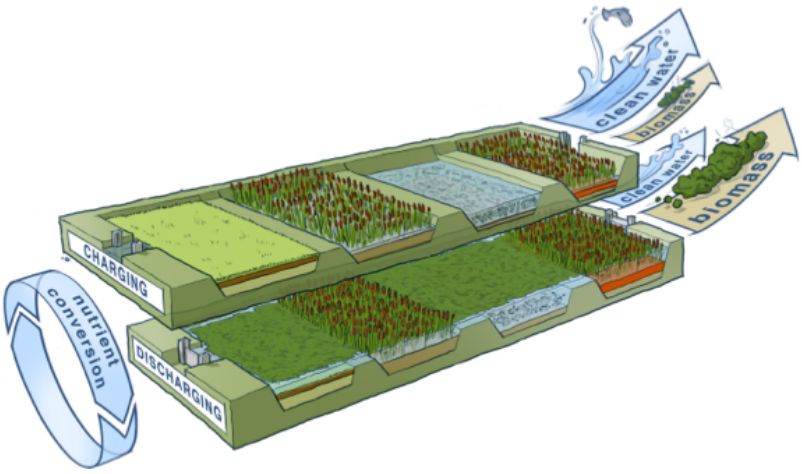

Figure 1: Artist impression of the biocascade water purification system, showing both the charging- (top) and discharging phase (Kwakernaak et al., 2015).

The major share of phosphate removal takes place in the first compartment of the biocascade water purification. Here, sorption of PO_4^{3-} with oxidized Fe(III) compounds takes place, forming iron phosphate, iron(hydr)oxide-phosphate and humic-iron-phosphate complexes (Patrick & Khalid, 1974; Richardson, 1985; Lamers et al., 1998; Smolders et al., 2006). To ensure that the Fe(III) compounds in the soil stay in the oxidized state – and PO_4^{3-} sorption is most effective – the first compartment consists out of a vertical infiltration bed, allowing O_2 exchange between the atmosphere and the soil and enlarging the contact between soil and water to increase P sorption.

Iron (Fe) is a redox-sensitive element, which means that in an anaerobic environment with a redox potential below +77 mV (Stumm & Morgan, 1981), oxidized Fe(III) may function as

an alternative electron acceptor. It is then reduced to Fe(II) by iron-reducing bacteria, resulting in the mobilization of Fe(II) and dissociation of the adsorbed phosphorus (Khalid et al., 1977; Boström et al., 1988). Mobilization of P from the sediment to the overlying water is often referred to as internal (P) loading. Besides oxygen fluctuations being one of the main stimulants in P mobilization from iron-rich sediments (Einsele & Vetter, 1938; Mortimer, 1941), the mobilization of P from iron-rich soils may increase substantially when the sulphate (SO_4^{2-}) concentration in the water layer heightens (Smolders et al., 1995). Under anaerobic conditions with a low redox potential, sulphate is reduced to sulphide (S^{2-}) and may form highly insoluble iron-sulphides (FeS_x) (Boström et al., 1988). Iron-sulphide has significant fewer sorption sites for P, resulting in an increased mobile P-fraction (Caraco et al., 1989; Roelofs, 1991; Smolders & Roelofs, 1993).

The biocascade water purification system aims to recover phosphorus by remobilization of the iron-bound P. In order to realise P mobilization, anaerobic conditions are required.
The concentration of oxygen in the water column depends on the balance of production and respiration of all organisms in the water column, and the exchange of oxygen with the atmosphere (Odum, 1956). The dissolved oxygen (DO) concentration may decrease when the respiration rate increases.
Floating macrophytes are able to influence the O_2 balance by limiting oxygen exchange with the atmosphere, while simultaneously limiting oxygen production in the water column due to decreased light availability (Lewis & Bender, 1961; Rai & Munshi, 1979; Pokorný & Rejmánková, 1983; Janes et al., 1996). In addition, floating macrophytes have the ability to take up phosphorus directly from the water layer and can be easily harvested and used in various applications such as green fertilizer or digestion for biogas production.

In the biocascade water purification system, *Azolla filiculoides* (Figure 2) will be used in the discharging-phase. *A. filiculoides* is a heterosporous floating fern of the *Azolla* genus that in turn belongs to the *Azollaceae* family. *Azollaceae* is a family of aquatic ferns in the division of Pteridophyta (ferns) (Kempen, 2015). *Azolla* spp. live in symbiosis with the nitrogen-fixing bacteria *Anabaena azollae* (Moore, 1969; Peters & Mayne, 1974), and therefore are unlikely nitrogen limited. This gives them the advantage of being able to grow in relatively

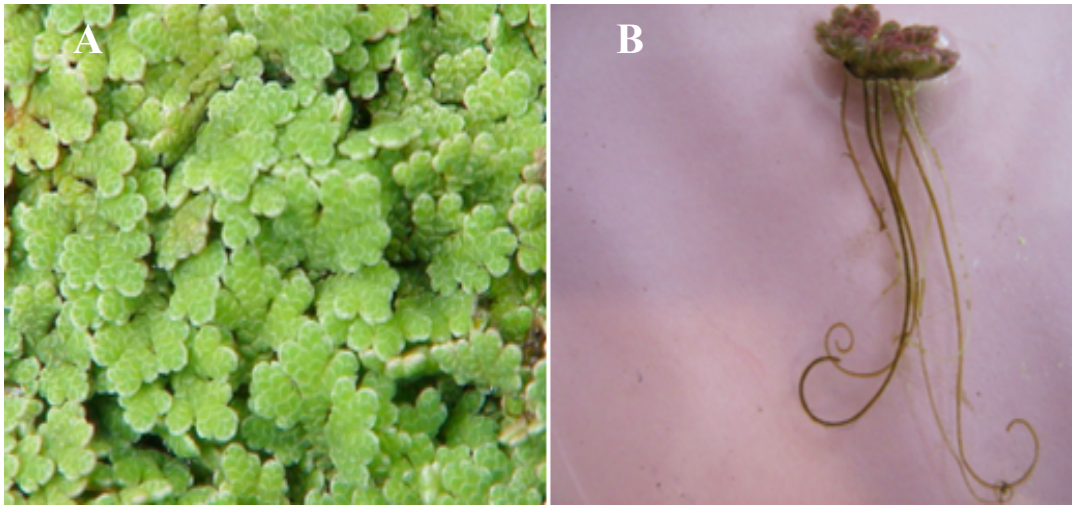

Figure 2: (A) Zoomed-in picture of dense A. filiculoides bed (Stüber, 2004), and (B) zoomed in picture of single A. filiculoides plant (Wikipedia, 2006)

high phosphorus concentrations and makes *Azolla* one of the fastest growing plants on earth (Kempen, 2015).

Because of its fast growth rate, ability to absorb phosphorus directly from the water column, and potential applications, *Azolla* spp. are under increased interest as a low-cost wastewater purification method (Scharpenseel & Knuth, 1985; Forni et al., 2001). By harvesting on a regular basis, nutrients in the form of biomass are taken out. Much research has been conducted on the application of *Azolla spp.* as a green fertilizer in rice paddies or biomass production on sewage sludge (e.g. Rains & Talley, 1979; Singh, 1979; Subudhi & Watanabe, 1981; Vermaat & Hanif, 1998; Forni et al., 2001; Costa et al., 2009). The potential of *Azolla spp.* as a means to control internal loading processes however remains largely unexplored.

The aim of this research was to investigate whether *A. filiculoides* is suitable for phosphate mining from both iron-rich- and clay-rich soil. It was hypothesized that *A. filiculoides* causes DO levels in the water column to decrease by limiting oxygen exchange with the atmosphere and light in the water below. In this way *A. filiculoides* is expected to mobilize P, due to the anaerobic reduction of Fe(III) to Fe(II) and the concomitant dissociation of the iron-bound phosphates. Thereafter, the mobilized P is expected to be taken up by the *A. filiculoides*.
It was also hypothesized that P-mobilization induced by *A. filiculoides* was expected to be less effective in clay-rich soil, due to the redox-insensitiveness of aluminium – to which most P is expected to be bound.

2 Materials and methods

Within this research, investigations were made on DO concentrations in the water column, biomass production of *A. filiculoides* and phosphorus concentrations in the plant biomass and the water column. Furthermore, various chemical parameters were monitored such as iron and sulphate concentrations. To test whether phosphate mining by using *A. filiculoides* could also work with clay-rich soil, clay-rich soil from the FWS-helophyte filter at the Radboud University Nijmegen (see Figure 3 B in the next chapter) was tested together with the iron-rich soil from the biocascade water purification system.

Furthermore, addition of glucose as a possible management measure to promote anaerobic conditions was tested in a small scale pilot experiment. It was hypothesized that the higher the added glucose concentration, the faster anaerobic conditions will occur due to an increase in microbial activity resulting in a higher biological oxygen demand.

2.1 Mesocosm experiment

2.1.1 Experimental setup

A mesocosm experiment was conducted in the greenhouse complex of the Radboud University, using square aquaria with a volume of 15.63 litre. Iron-rich- and clay-rich soil were collected from the constructed wetland 'test ditches' (Figure 3) located near the greenhouse complex of the Radboud University Nijmegen. The two soil types were incubated with and without *Azolla*, resulting in four different treatments with five replicates per treatment.

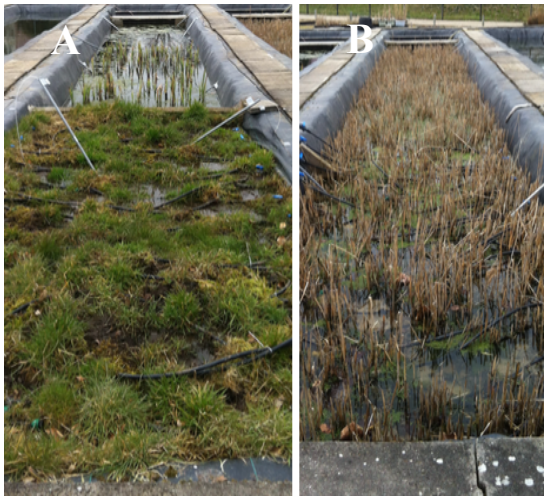

Figure 3: Biocascade (A) and helophyte filter (B) test ditches at the Radboud University

The iron-rich- and clay-rich soils were distributed equally among ten aquaria (25x25 cm) per soil-type, resulting in a soil layer of approximately 1.6 cm (~ 1 L) per aquaria.

Thereafter, 10 centimetres (6.25 L) of tap water was added to each aquarium. The aquaria were randomly placed on a table in a compartment of the greenhouse of the Radboud University with a day-temperature between 21 and 31 ˚C, a night-temperature between 17 and 21 ˚C. The compartment had a day-night regime of 16 and 8 hours respectively. Lights (Philips

GreenPower 400V/1000WE) automatically switch on when radiation from the sun is less than 155 W/m^2 and switch off at a radiation of more than 205 W/m^2.
The water level was maintained throughout the whole experiment with a fluctuation of ± 2 cm by adding demineralized water weekly. The aquaria were covered with lightproof black plastic on the sides (to prevent light from penetrating the water column from the side) and left for two days to let suspended particles settle (Figure 4). After two days, 40 grams (± 0.1) of cultivated *A. filiculoides* was introduced to five aquaria of each soil type. The plants were cultivated in a big basin containing a nutrient solution to support optimum growth.

In order to prevent potassium deficiency in *A. filiculoides*, potassium chloride (KCl) from a stock solution (186.5 g/L) was added on a regular basis with a target value of 400 µmol/L, which is a concentration found during field studies in the Netherlands where *A. filiculoides*

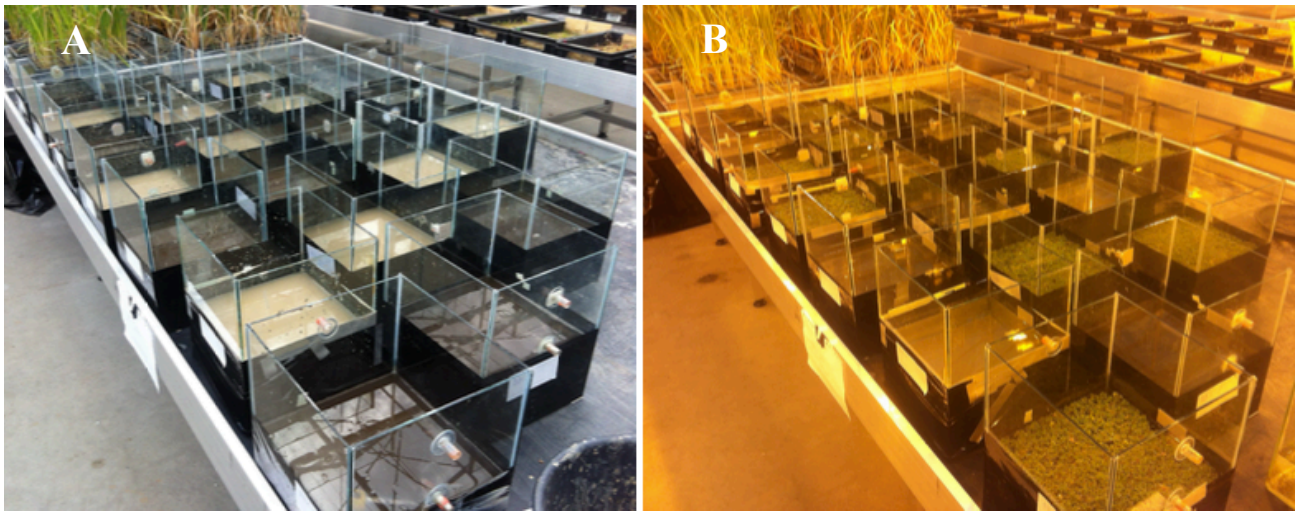

Figure 4: Experimental setup of the aquaria experiment. Picture (A) showing the setup before the introduction of A. filiculoides and picture (B) showing the setup after the addition of A. filiculoides

was abundantly present (De Lyon & Roelofs, 1986). The amount of stock solution required to meet the target value was calculated from the most recent available data of elemental analysis of the surface water. Furthermore, SO_4^{2-} was added in the form of $NaSO_4 \cdot 10\ H_2O$ from a stock solution (302.1 g/L), with a target concentration of 1500 umol/L (common for surface- and groundwater in the Netherlands, according to Lamers et al. (1998)). The amount of stock solution required to reach the target concentration was calculated from last available data from elemental analysis.

The total duration of the experiment was 112 days. In this time, the experiment was conducted twice, because a die-off of *A. filiculoides* occurred. Biomass decrease and phosphorus deficiency signs (purple colourization of the leaves) indicated there was no phosphorus being mobilized. To stimulate this and prevent a die-off of the newly introduced *A. filiculoides*, additional measures were taken to boost P mobilization: the density of *A. filiculoides* was increased to block the air-water O_2 exchange sufficiently and the aquaria were flushed with N_2 (g) at the start.

2.1.2 Water biochemistry

DO and water temperature were monitored by measuring between 12:00 and 16:00 using a multi-meter probe (Hach Company, Loveland, Colorado, USA). A dark measurement of the DO was performed once by covering the aquaria with black lightproof plastic during night time. The DO concentration was measured the morning after. DO concentrations at this point are the minimum concentration over a 24-hour period as it is the longest period without production. This gives an idea about effect of algal growth in the aquaria without the *A. filiculoides* cover on DO fluctuations, and thus the potential P-mobilization in these treatments.
Furthermore, surface water was sampled in the top 5 cm every 14 days and analysed on pH and total inorganic carbon (TIC). Subsamples were stored at 4 ˚C and -20 ˚C for elemental analysis. To prevent precipitation of dissolved metals, the samples stored at 4 ˚C were acidified with 0.1 mL HNO_3 (65%) prior to storage.
The concentration of PO_4^{3-} was measured colorimetrically and the concentration of K^+ was measured using a flame-photometer on an Auto Analyser system (Bran & Luebbe, Norderstedt, Germany). Elemental analysis of total phosphorus (TP), total potassium (K), total iron (TFe) and total sulphur (TS) were conducted using inductively coupled plasma - optical emission spectrometry (ICP-OES iCAP 6000; Thermo Fischer Scientific, Waltham, Massachusetts, USA).

2.1.3 Plant biomass, plant density and nutrient composition

To obtain a measure of (cumulative) biomass production and the quantitative amount of P-uptake, plant material of each aquaria was harvested three times in the first period- and three times in the second period of the experiment. Furthermore, to obtain a measure of biomass production rate (g m^{-2} d^{-1}), harvesting in the second period was done by collecting plant mate-

rial from a fixed surface area. The production rate was calculated by taking the plant density (g m^{-2}) from the last two harvesting points and determine the difference in biomass. In addition, the relative growth rate (RGR) was calculated according to the following formula:

$$RGR = (lnW_2 - lnW_1)/(t_2 - t_1) \qquad (eq.\ 1)$$

Where ln is the natural logarithm, t_1 is time one (in days), t_2 is time two (in days), W_1 is dry weight of plant material at time one (in grams) and W_2 is the dry weight of plant material at time two (in grams).

The harvested material was drained for ten seconds, after it was weighed (drained weight) and dried for 24 hours at 70 degrees and weighed again to obtain the dry weight and moisture loss. Dried plant material was ground using a mortar and pestle, after which it was stored at room temperature.

To determine the amount of P in the harvested *A.* filiculoides, subsamples of 200 (± 5) mg from the ground plant material were weighed for destruction and 4 mL HNO_3 (65%) and 1 mL H_2O_2 (35%) were added. Subsequently, the plant material was destructed using an Ethos D microwave (Milestone, Sorisole Lombardy, Italy) (Kingston & Haswell, 1997). Thereafter, the samples were stored at 4 ˚C until further elemental analysis on phosphorus (P) by means of ICP-OES.

2.2 Glucose pilot experiment

In order to start P-mobilization, necessary for *A. filiculoides* growth, the water in all aquaria of the mesocosm experiment were flushed with nitrogen gas to lower the DO concentration artificially. However, this would not be a suitable management measure on a large scale. Therefore, an additional pilot experiment was conducted to investigate whether biochemical measures can be taken to increase O_2 consumption in the water column and achieve a similar result as flushing with N_2 (g). The dissolved organic carbon (DOC) concentration in the water column was increased to stimulate microbial activity and therefore increasing the BOD.

2.2.1 Experimental setup

In this study, glucose was used as an organic carbon source and 5 different glucose concentrations were tested: 0.1, 0.2, 0.3, 0.5 and 1 g/L. Iron-rich soil was collected (as in chapter 2.1) and 3 cm (84.8 mL) was added to glass cylinders, with a diameter of 6 cm and a total

length of 20 cm. The soil was covered by 9 cm of demineralized water. This water level was maintained during the experiment. The experiment was conducted with two replicates per treatment, resulting in 12 cylinders, including two cylinders without added glucose, as a control treatment. The working solutions with the above described glucose concentrations were made from a 500 mL stock solution containing 200 g/L glucose. Glucose was added at the beginning of the experiment and monitored afterwards. The cylinders were placed in a water bath at a constant temperature of 20 ˚C in a dark room to prevent primary production in the water column (Figure 5).

Figure 5: Experimental setup of the sugar pilot. Treatments consist out of duplicates which were randomly placed in a water bath

2.2.2 Surface- and pore water analyses

DO was measured after 0, 4, 19 and 29 hours, after which it was measured every 24 hours until the experiment ended after 13 days. The measurements were conducted using a multi-meter probe (Hach Company, Loveland, Colorado, USA). Surface water samples for glucose concentration analysis were taken daily from day 1 until day 7 and at day 10 and 13. This was done by sampling 5 mL of unfiltered water from each cylinder with a pipette and measure for glucose concentration colorimetrically with a spectrophotometer (D-glucose assay kit; Megazyme, Bray, Ireland). The reactions involved are the enzyme-facilitated oxidase and peroxidase of glucose (Figure 6).

Glucose was measured by adding 0.1 mL of water sample to 3 mL of reagent into a glass test tube and incubate at 40 ˚C for 20 minutes. The reagent consisted out of enzymes (glucose

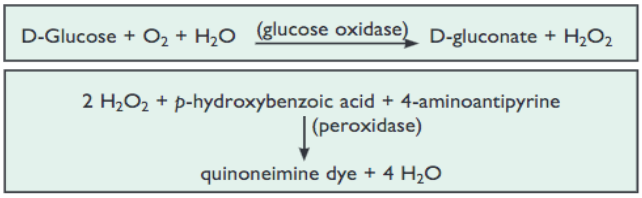

Figure 6: Chemical reactions involved with the colorimetric change due to the enzymatic oxidase and peroxi-

oxidase plus peroxidase and 4-aminoantipyrine) and a buffer (50 mL, pH 7.4 of 0.095% w/v p-hydroxybenzoic acid and sodium azide).

Thereafter, the samples were measured on a spectrophotometer at 510.0 nm and a light path of 1 cm. All samples were measured in duplicates to rule out technical errors. Thereafter, with the measured values, the glucose concentration in solution could be calculated according to the following formula:

$$\text{D-glucose}(\mu g/0.1\ \text{mL}) = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{D-glucose (100 µg)}}} \times 100 \qquad \textit{(Eq. 2)}$$

Where D-glucose is the amount of glucose in the sample, ΔA_{sample} is the absorbance measured in the sample, and $\Delta A_{\text{D-glucose (100 µg)}}$ is the absorbance measured in the standard solution (1.0 g/L D-glucose in in 0.2% w/v benzoic acid).

The surface water was analysed on elemental composition by means of ICP-OES. Surface water was sampled after 48 hours and at the end of the experiment, at a depth of 4 cm with a repetitive pipet. Furthermore, filtered pore water was collected once at the end of the experiment by connecting vacuum infusion flasks (30 mL) to a pore-water sampler (Rhizon SMS-

10 cm; Eijkelkamp Agrisearch Equipment). To prevent precipitation of dissolved metals in the sample, they were acidified with 0.1 mL HNO_3 (65%) and subsequently stored at 4 ˚C until further analysis on the ICP-OES. In this way, total dissolved phosphorus (TDP) and total dissolved iron (TDFe) could be measured.

The measured values in the surface water of glucose, TFe and TP were corrected for dilution by multiplying the measured value with a correction factor:

$$1 * \frac{5}{270} \qquad (Eq.\ 3)$$

Where 5 is the amount of sampled water in mL and 270 the total volume of the water column in mL.

2.3 Statistical analysis

For the mesocosm experiment, DO concentrations during night- and day-time were compared using one-way ANOVA followed by Tukey HSD post-hoc test ($p < 0.05$) to determine significant differences between the treatments. Furthermore, biomass production and P yield in biomass were compared using one-way ANOVA ($p < 0.05$). Data were log-transformed to achieve normal distribution and equality of variances where possible.

For the glucose pilot, correlations between (initial) sugar concentrations, TDP and TFe in the pore water and TP and TFe in the surface water were tested. All data was log-transformed to achieve normality and meet the assumptions necessary for the Pearson's product-moment correlation. When normality was not achieved after log-transforming the Spearman rank-order correlation method was used, assuming a non-normal distribution. All statistical analyses were carried out using SPSS 21.0 for OS X (SPSS Inc., Chicago, IL, U.S.A.). All mean values in the report are shown ± the standard error of the means (SEM).

3 Results

3.1 Mesocosm experiment

3.1.1 Dissolved oxygen

The DO in the water column was substantially lower in the treatments with *Azolla* at day four of the experiment. With Mean values of 13.97 ± 0.7 (SEM) mg/L in the iron-rich treatment without *Azolla*, 6.04 ± 0.04 mg/L in the iron-rich treatment with *Azolla*, 14.03 ± 0.26 mg/L in the clay-rich treatment without *Azolla* and 6.76 ± 0.12 mg/L in the clay-rich treatment with *Azolla*.

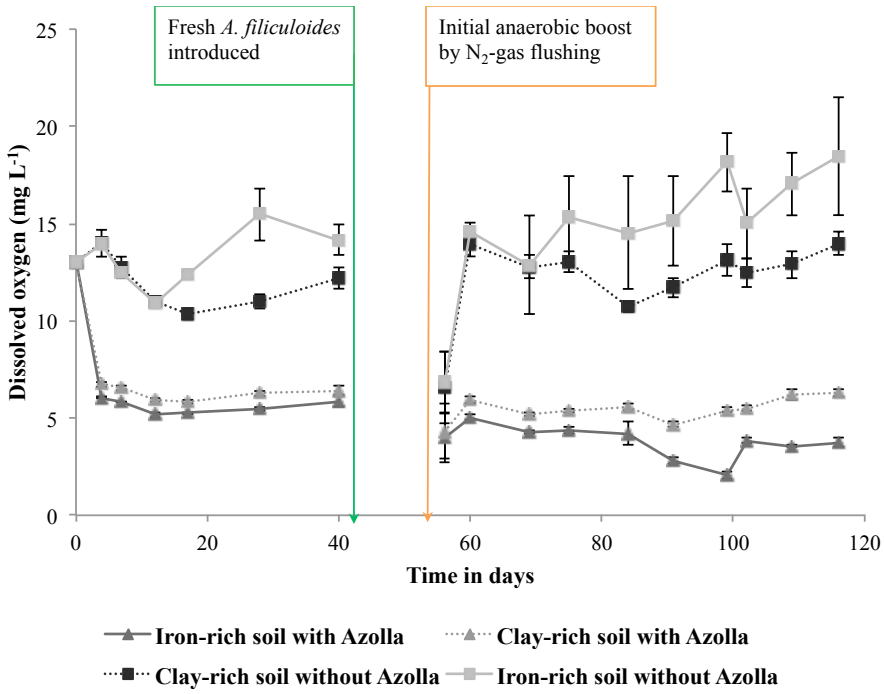

Figure 7: Mean DO (± SEM) in the four treatments over time (n=5). Standard error of the mean is indicated by the bars.

DO concentrations were relatively stable in both treatments with *Azolla*. In the second period – after flushing with N_2 (g) and increasing the *Azolla* density – the DO concentration in both *Azolla* treatments decreased compared to the DO levels in the first 40 days of the experiment. DO levels in the treatments without *Azolla* returned to concentrations similar to the concentrations in the first period within a few days. The iron-rich treatment with *Azolla* had a mean DO concentration of 5.6 ± 0.076 (SEM) mg/L in the first period and 3.65 ± 0.175 mg/L in the second period. The clay-rich treatment with *Azolla* had a mean DO concentration of 6.29 ± 0.082 mg/L in the first period and 5.23 ± 0.171 mg/L in the second period.

The treatments without *Azolla* showed more fluctuations: 13.21 ± 0.382 mg/L in the first period- and 13.55 ± 0.870 mg/L in the second period of the iron-rich treatment. The clay-rich treatment without *Azolla* had a mean DO concentration of 11.88 ± 0.276 mg/L in the first period and 11.31 ± 0.489 mg/L in the second period.
When looking at the iron-rich treatment with *Azolla* at day 100 there is an increase of DO, this event coincides with harvesting half of the total amount of plants, decreasing the density of the floating *A. filiculoides* bed.

At day 14, the iron-rich treatment without *Azolla* was experiencing increased algal growth in some of the replicates (see Appendix A, Figure 20 for a picture). To measure the effect of algal growth on DO fluctuations, a day- and night-measurement was conducted (Figure 8). The day-measurement is representative for the maximum DO concentration because of highest net production rates. The night-measurement is representative for the minimum DO concentration because of highest net respiration rates.
DO at the day-measurement (maximum DO) in the iron-rich treatment without *Azolla* was significantly higher ($p < 0.05$) than the other treatments, and also significantly lower in the night-measurement (minimum DO) compared to the other treatments.

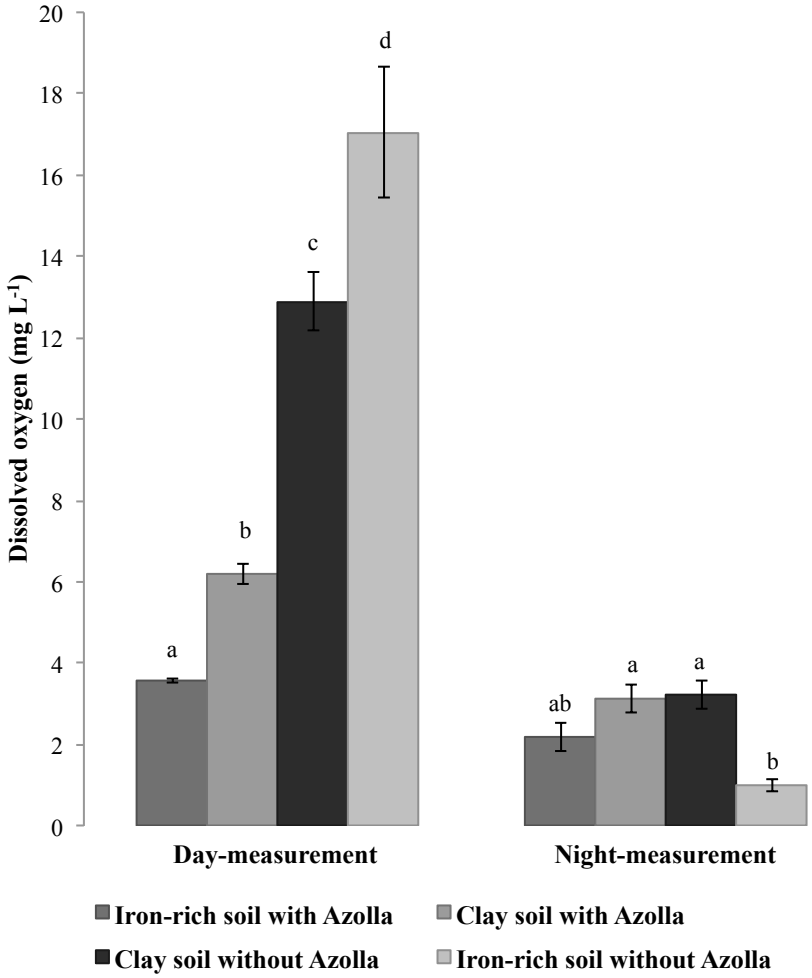

Figure 8: Mean DO (± SEM) in the water column during day-time and after a dark period (night) of 15 hours (n=5). Standard error of the mean is indicated by the bars. Different letters on the top indicate significant differences ($p < 0.05$)

Significant differences were also found between all treatments at the day-measurement ($p < 0.05$). No significant differences were found between the iron-rich treatment with *Azolla*, the clay-rich treatment with *Azolla* and the clay-rich treatment without *Azolla*.

3.1.2 Phosphorus

TP and PO_4^{3-} concentrations in the surface water were relatively low (< 1 µmol/L) in both clay-rich treatments from the start of the experiment to the end of the experiment (Figure 9 and Figure 10). Concentrations of TP and PO_4^{3-} were generally very high in the iron-rich treatments *Azolla,* with 43.87 µmol/L ± 4.49 (SEM) and 7.90 µmol/L ± 1.75, respectively.

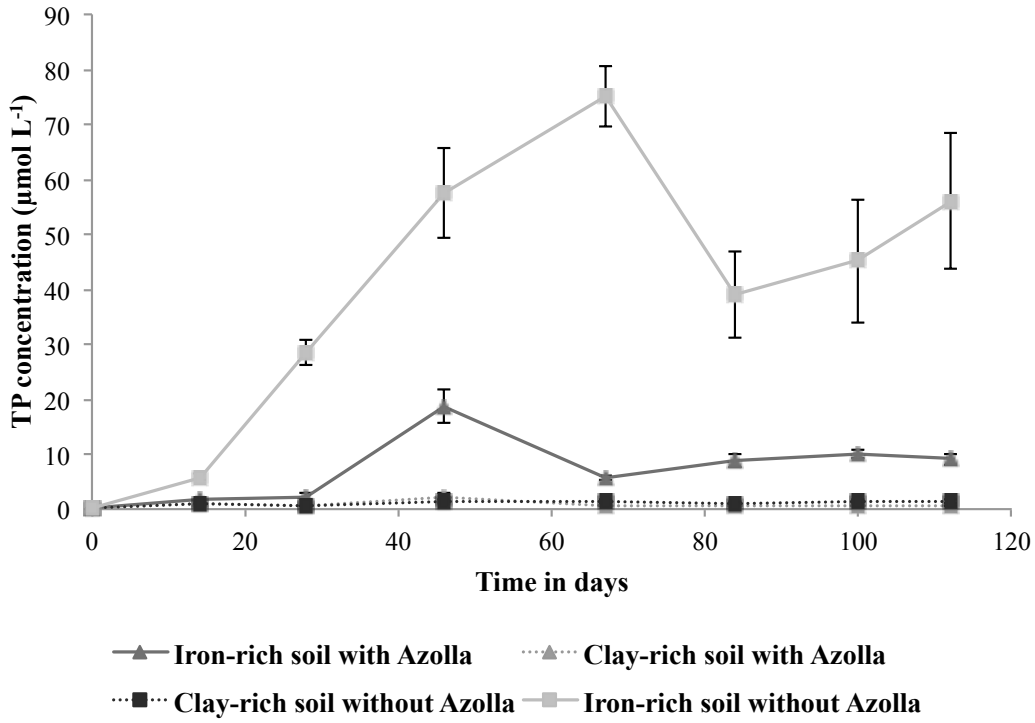

Figure 9: Mean total phosphorus concentrations (± SEM) in the water column over time (n=5). Standard error of the mean is indicated by the bars.

TP concentrations in the iron-rich soil with *Azolla*-treatment were relatively low (6.25 µmol/L ± 1.65 (SEM)) during the experiment, but showed an increase after flushing with N_2 (g) at day 47 (18.83 µmol/L ± 3.13). PO_4^{3-} concentrations in this treatment were relatively low throughout the whole experiment (period 1 + 2) (2.25 µmol/L ± 0.98) but showed a rising trend the last 30 days of the experiment, reaching concentrations of 5.26 µmol/L ± 0.63 at the end of the experiment.

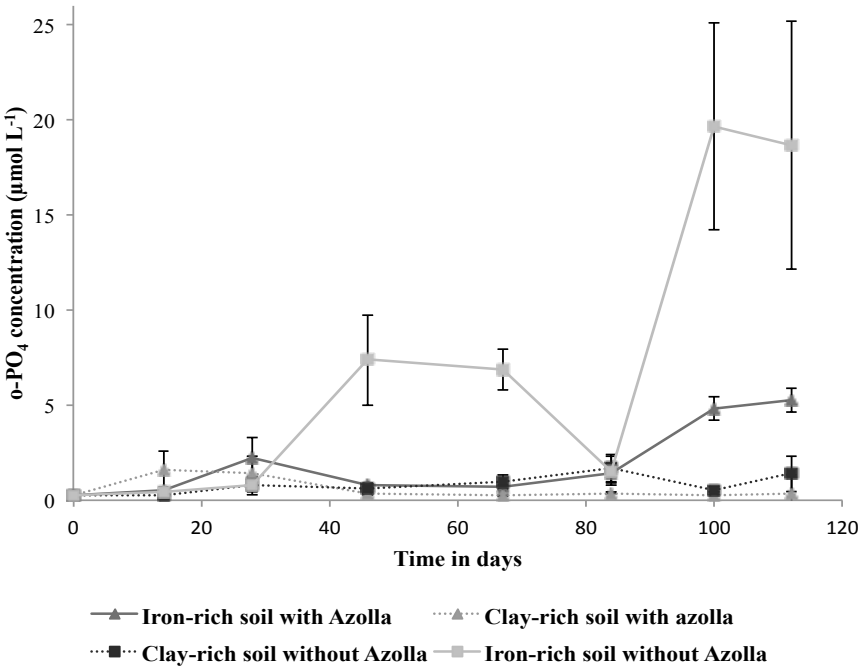

Figure 10: Mean phosphate concentrations (± SEM) in the water column over time (n=5). Standard error of the mean is indicated by the bars.

The increased PO_4^{3-} availability in the water column during the last 30 days of the experiment coincides with increased P concentrations in the plant tissue, while at low PO_4^{3-} concentrations in the first period of the experiment also low values of P in the plant tissue were found (Figure 11).

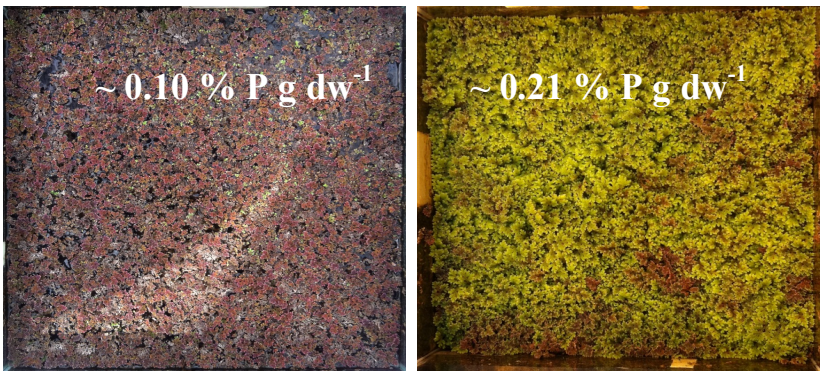

Figure 11: Percentage of P per gram dry weight in the plant tissue in replicate 1 of the iron-rich treatment with Azolla. The picture on the left was taken in period 1 on 14-04-2016 and the picture on the right was taken in period 2 on 21-06-2016

3.1.3 Iron

The TFe concentrations in the water column were relatively high in both the iron-rich treatment with- and without *Azolla* throughout the whole experiment (period 1 + 2), with 85.64 µmol/L ± 14.68 (SEM) and 197.30 µmol/L ± 22.76, respectively (Figure 12).

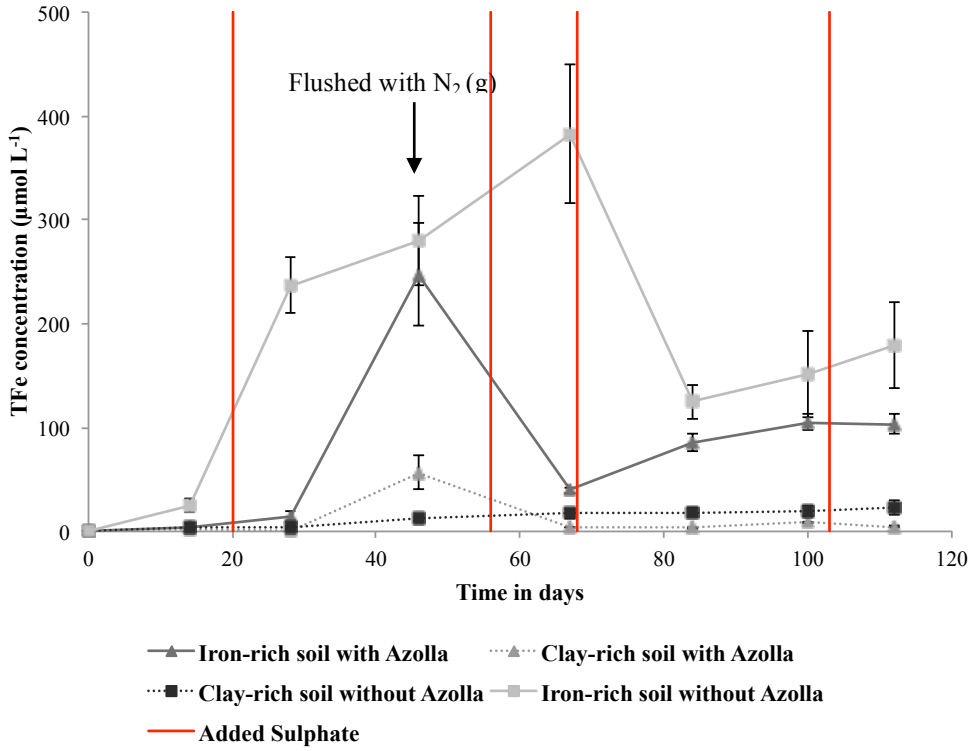

Figure 12: Mean total iron concentrations (± SEM) in the water column over time with sulphate addition moments. Standard error of the mean is indicated by the bars.

Flushing with N_2 (g) in all treatments at day 47 coincides with an increase in TFe in the water column in both iron-rich treatments. In the second period of the experiment, addition of sulphate coincided with decreasing TFe concentrations in the iron-rich treatment with *Azolla.* Addition of sulphate also coincided with decreasing TFe concentrations in the iron-rich treatment without *Azolla* at the last two sulphate addition-points. During the last 40 days of the experiment the TS concentration (as a proxy for the sulphate concentration) in the water column dropped relatively quick in both iron-rich treatments after each point where sulphate was replenished to the target concentration of 1500 µmol/L (see Appendix B, Figure 21).
There was little variation in TFe concentrations throughout the experiment (period 1 + 2) in both the clay-rich treatment with- (11. 47 µmol/L ± 3.82 SEM) and without *Azolla* (14.01 µmol/L ± 1.93). With the exception of a small peak (56.78 µmol/L ± 16.07) in TFe in the clay treatment with *Azolla* after flushing with N_2 at day 47. Furthermore, TS concentrations in the

clay treatments remained relatively stable (see Appendix B), resulting in less sulphate addition than in the iron-rich treatments.

3.1.4 Plant biomass, plant density and nutrient composition

During the first period (40 days) of the experiment the plants showed signs of phosphorus deficiency in both the iron-rich and clay treatments from day 20 (Appendix C, Figure 22), suggesting there was little phosphorus mobilization. The mean P content (% P g dw^{-1}) in the plant tissue during the first period of the experiment was 0.11% ± 0.0040 (SEM) in the iron-rich treatment with *Azolla* and 0.10% ± 0.0039 in the clay-rich treatment with *Azolla*.

After the introduction of fresh *A. filiculoides* plants (period 2), P concentrations in the plant tissue increased in the treatment with iron-rich soil (0.16 % ± 0.0137 (SEM)) (Figure

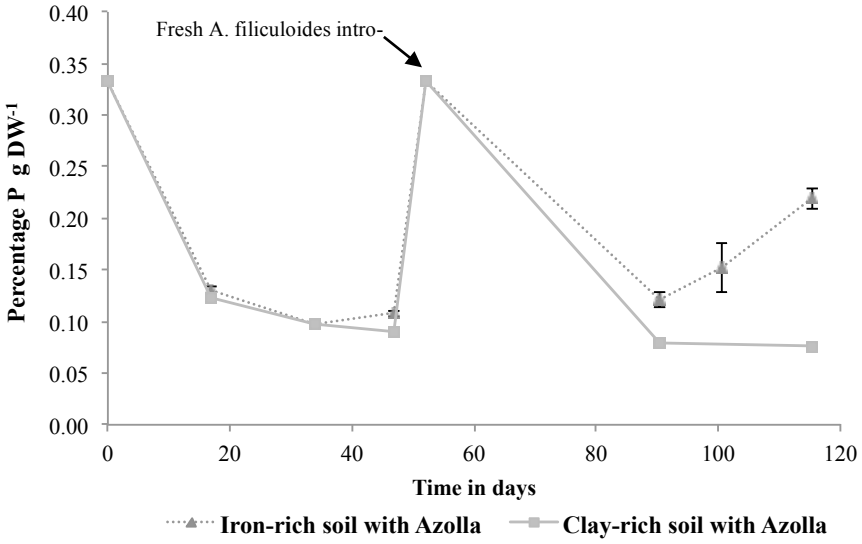

Figure 13: Mean percentage of phosphorus (± SEM) in A. filiculoides plant tissue over time (n=5). Standard error of the mean is indicated by

13).

The increased availability of P in the iron-rich treatment with *Azolla* in the second period can also be seen when looking at the PO_4^{3-}-availability in the last 30 days of the experiment (Figure 10).

The plants in the clay-rich soil started to show phosphate deficiency symptoms again after 17 days since the introduction of the fresh plants. In the clay-rich treatment with *Azolla* the per-

centage of P in the plant tissue is lower in the second period (0.08 % ± 0.0017 SEM), compared to the first period of the experiment (0.10% ± 0.0039).

Both treatments showed a negative total phosphorus (incorporated in plant tissue) yield from plant biomass in the first period of the experiment (Figure 14). The iron-rich treatment with *Azolla* had a mean P yield of -15.24 µmol ± 6.77 (SEM) and the clay-rich treatment with *Azolla* had a mean P yield of -20.04 µmol ± 3.96. No significant difference between treatments was found in the first period.

In the second period however, *Azolla* growth increased in the iron-rich treatment (as can be seen in the biomass production, Figure 15), resulting in a higher phosphorus yield (982 µmol ± 79.4). P yield in the clay-rich treatment in the second period showed a very high net loss compared to the first period (-367 µmol ± 13.5). It should be noted that the second period (56 days) of the experiment lasted longer than the first period (40 days).

The P yield in the iron-rich treatment was significantly higher ($p < 0.000$) than the P yield in the clay-rich treatment.

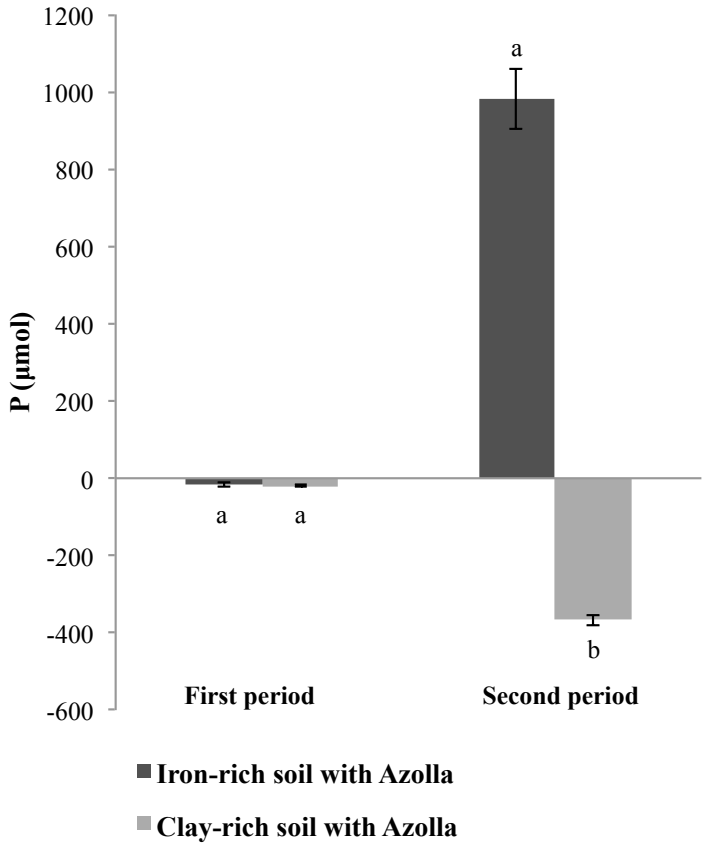

Figure 14: Mean Total phosphorus yield (± SEM) in both treatments in the first- and second (after introduction of new plants) period (n=5). Standard error of the mean is indicated by the bars. The letters on top indicate significant differences ($p < 0.05$)

No significant differences in biomass production were found in the first period (Figure 15). Biomass production in the iron-rich treatment in the second period was significantly higher ($p < 0.000$) than the biomass production in the clay-rich treatment in the second period. Biomass production in the second period increased considerably in the iron-rich treatment (20.46 g dw ± 0.38 (SEM)) compared to the first period (4.68 g dw ± 0.23), whereas biomass production in the clay-rich treatment decreased in the second period (2.34 g dw ± 0.57) compared to the first period (5.43 g dw ± 0.19).

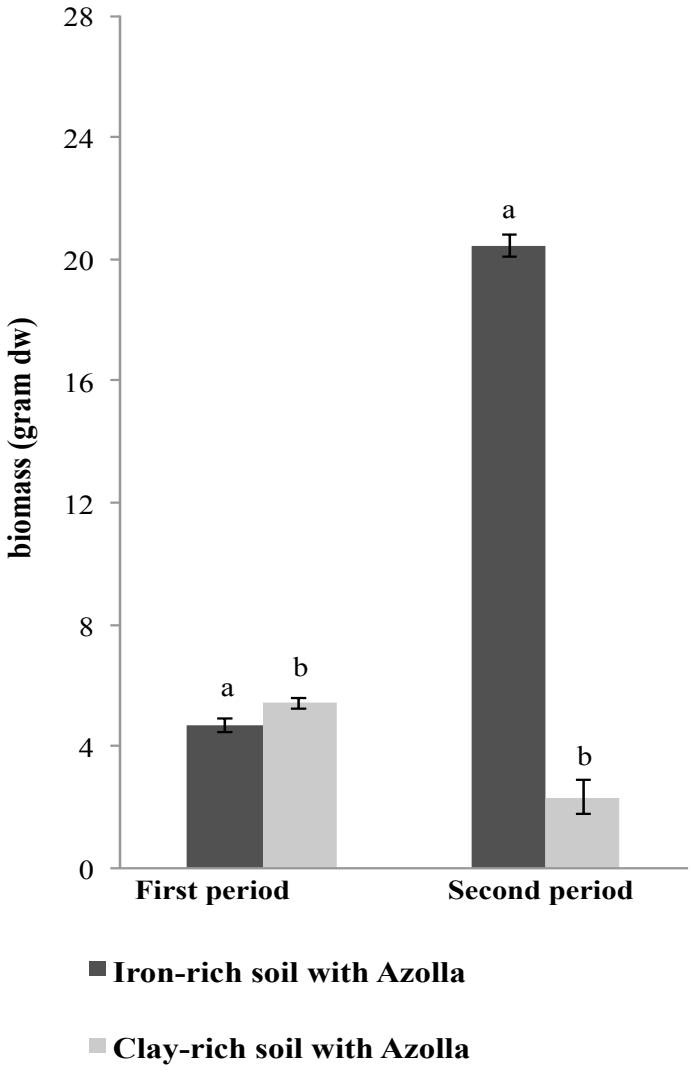

Figure 15: Mean biomass yield (± SEM) in both treatments in the first- and second (after introduction of new plants) period (n=5). Standard error of the mean is indicated by the bars. The letters on top indicate significant differences ($p < 0.05$)

3.2 Sugar pilot

Dissolved oxygen concentrations in the surface water declined fast within the first 30 hours of the experiment in all treatments except the control (Figure 16). The treatments with the two lowest initial glucose concentrations (0.1 and 0.2 g/L) showed anaerobic conditions (< 1 mg/L O_2) for 100 hours from experiment start and the 0.2 treatment even maintained anaerobic conditions for more than 200 hours. The treatments with higher sugar concentrations showed inhibited BOD between hour 20 and 50.

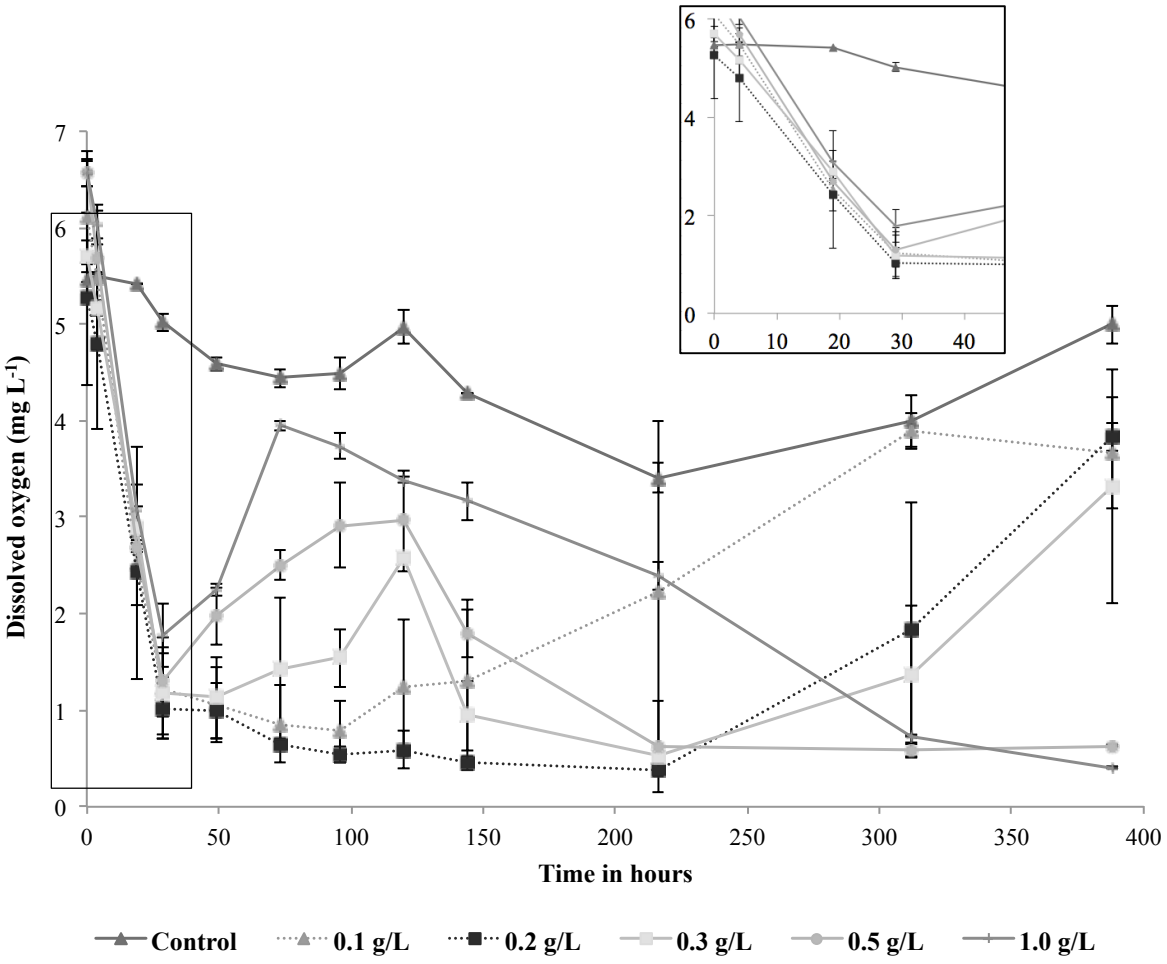

Figure 16: Mean DO (± SEM) over time in treatments with varying initial glucose concentrations (n=2). Standard error of the mean is indicated by the bars.

Glucose consumption rates were highest in the 1 g/L treatment with 0.076 g L^{-1} day^{-1} ± 0.00029 (SEM). Glucose consumption was lowest in the 0.1 g/L treatment with 0.020 g L^{-1} day^{-1} ± 0.00019 (Figure 17).

Glucose was completely consumed in treatment 0.1 and 0.2 g/L after 96 hours and after 120 and 216 hours in treatment 0.3 g/L and 0.5 g/L respectively (Figure 17). The treatment with 1 g/L was only depleted of glucose after 312 hours.

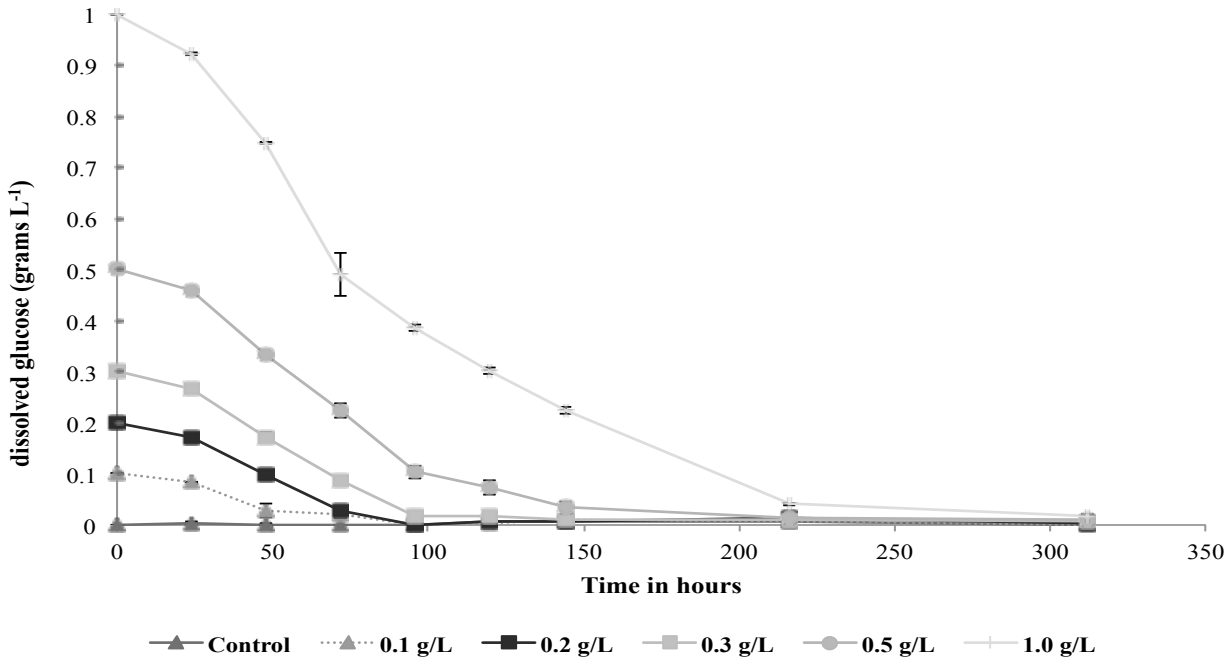

Figure 17: Mean glucose concentrations (± SEM) in the surface water plotted against time (n=5). Standard error of the means is indicated by the bars.

There was a strong positive correlation ($r = 0.903$, $n = 12$, $p < .0005$) between the initial glucose concentration and the measured TP in the surface water at the end of the experiment. Where higher initial glucose concentrations resulted in higher TP concentrations.

Mean TP concentrations (± SEM) in the surface water decreased between day 2 and day 13 in the control and the treatment with 0.1 g/L glucose (n=2), whereas in the other treatments TP increased (Appendix D, Figure 23).

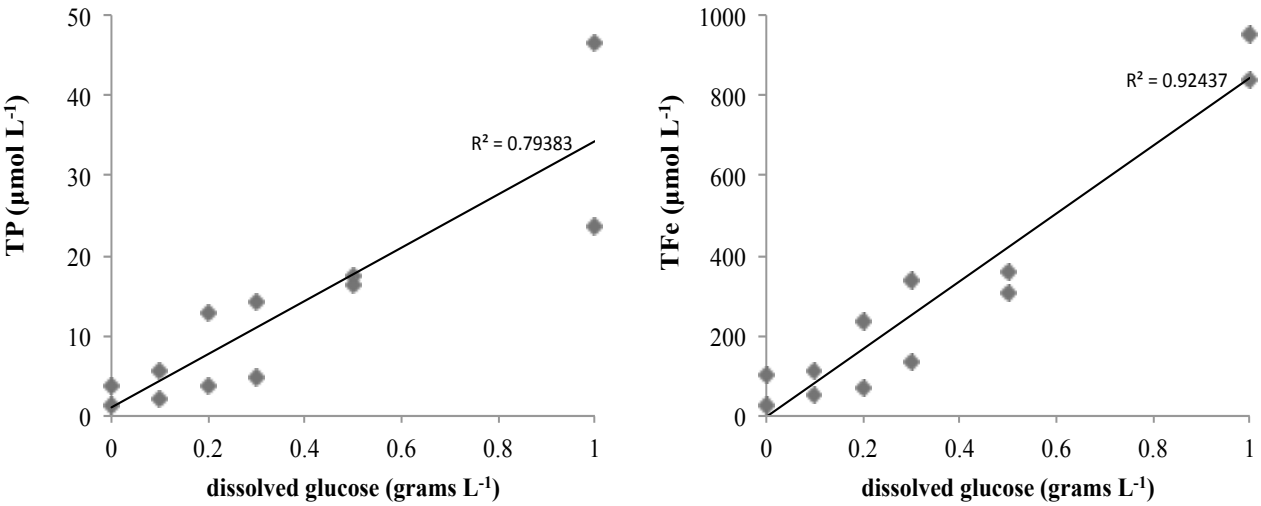

Figure 19:Correlation graphs between TP at the end of the experiment and the initial glucose concentration (left), and the TFe at the end of the experiment and initial glucose concentration (right)

Furthermore, a strong positive correlation ($r = 0.900$, $n = 12$, $p < .0005$) was found between the initial glucose concentration and the TFe concentration in the surface water at the end of the experiment. Higher initial glucose concentrations resulted in higher TFe concentrations (Figure 19).

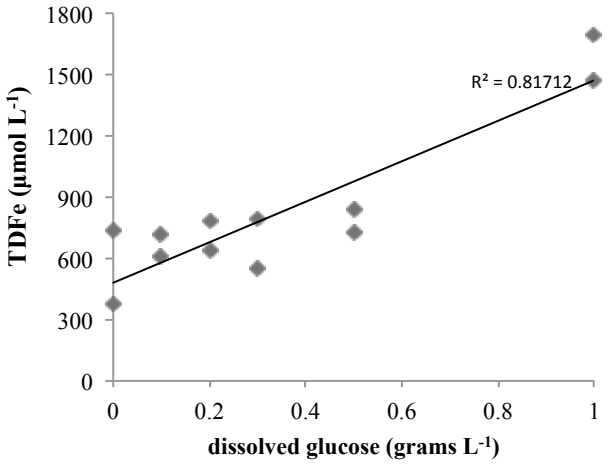

Figure 18: Correlation graph between TDFe and initial glucose concentration

A strong positive correlation ($r = 0.868$, $n = 12$, $p < 0.0005$) between dissolved Fe in the pore water and initial glucose concentration in the water column was found. Higher initial glucose

concentrations resulted in higher dissolved Fe in the pore water (Figure 18).

Dissolved Fe concentrations in the pore water were extremely high – even in the control group (553 µmol/L ± 178 SEM). However, the dissolved Fe concentration in the highest glucose treatment (1 g/L), was almost three times higher (mean = 1586 µmol/L ± 112 SEM). No correlation between initial glucose concentration and dissolved P in the pore water was found (Appendix E, Figure 24).

4 Discussion

4.1 Mesocosm experiment

During the first period of the experiment both treatments with *Azolla* showed purple colorization of the leaves, indicating that the plant suffered from phosphorus deficiency. This was probably due to the absence of P mobilization from the sediment, indicated by the low P concentrations in the plant tissue. Compared to the P percentage in the plants from the cultivation the P in the plant decreased during the experiment. According to Ali & Watanabe (1986), *Azolla* spp. show severe P defiency when the percentage of P per g dw^{-1} decreases to 0.1 % or lower. This is in full agreement with this study, where similar concentrations were found in the first period of the experiment (Figure 11).

When the DO concentration in the iron-rich treatment with *Azolla* was above 5 mg/L during daytime (as in period one), P mobilization was very limited, as judged from the P uptake of the plant and PO_4^{3-} concentration in the water layer. It is well documented that P mobilization from iron-rich sediment is directly linked to a low redox potential (Patrick et al., 1973; Roelofs, 1991), this suggest that redox potential in the first period was too high to facilitate iron reduction and concomitant P dissolution.

Increasing plant density and flushing with N_2 (g) at the start of period two had substantial effect on the DO in the water column in the iron-rich treatment with *Azolla*. Once the DO reached a concentration below 4 mg/L, P mobilization increased, as judged from the PO_4^{3-} concentration in the water, the increased plant growth and the decreasing TS concentration as a measure for anaerobic FeS_x production (Figure 7, Figure 10, Figure 15 and Appendix B, Figure 21).

The results show that sulphate addition had a positive effect on P mobilization in both iron-rich treatments – as demonstrated in many papers (e.g. Caraco et al., 1989; Smolders & Roelofs, 1993; Lamers et al., 1998). Sulphate addition – especially in the second period of the experiment – coincides with decreasing TFe concentrations in the surface water and an increase of PO_4^{3-} in the surface water in both iron-rich treatments. Because oxygen typically only penetrates waterlogged soil up to 2 cm (Wetzel, 2001), it is expected that sulphate reduction in the soil of the biocascade water purification system will occur more quickly than observed in this research.

Judging from the high peak of PO_4^{3-} and the large differences in the DO concentration during day- and night-time in the iron-rich treatment without *Azolla*, algae growth seemed to have caused increased P mobilization, which is well documented in various studies (Søndergaard et al., 1990; Welch & Cooke, 1995). Excessive algae growth may have a huge impact on net production in the water column by producing oxygen during day-time and consuming oxygen due to respiration at night-time. High respiration rates during the night can lead to temporary near anoxic conditions – as shown is this experiment – resulting in P mobilization (Khalid et al., 1977; Boström et al., 1988).

P mobilization in the clay-rich treatments was very low, resulting in a rapid decline and die-off of *A. filiculoides* in this treatment both in the first period and second period of the experiment. It is likely that most P was bound to aluminium, as reducing DO and SO_4^{2-} addition had no observed effect on the P mobilization. The redox insensitiveness of aluminium-phosphate (Cooke et al., 2016), is the most plausible explanation, it could, however, not be confirmed by sediment analysis.

The biomass production of *A. filiculoides* in the iron-rich treatment was the highest in the last two weeks of the experiment (day 101 – 116). Taking the biomass production from this period as the optimum biomass production rate, this results in a biomass production rate of 0.465 g dw m^{-2} day^{-1} and a relative growth rate (RGR) of 0.0024 g g^{-1} day^{-1}. This is much lower than relative growth rate of 0.097 g g^{-1} day^{-1} up to 0.242 g g^{-1} day^{-1} reported by Cary & Weerts (1992). However, higher RGR's may have been achieved if the duration of the experiment was prolonged, as P mobilization showed an increasing trend in the last two weeks.

4.2 Glucose pilot experiment

The relatively fast decrease in DO compared to the control group suggests that adding glucose may be a suitable measure to enhance oxygen depletion in the water column. It also shows the effect of anaerobic conditions on dissolved iron concentrations in the pore- and surface water when sulphate concentrations are very low. Surprisingly, the two treatments with the highest glucose concentration (0.5 and 1.0 g/L) appear to cause microbial growth inhibition – as judged from the increase in the DO concentration – and may be considered counterproductive. Competition between bacteria and fungi may partly explain the increase in DO (0, Figure 25). Some fungi have been reported to release antibiotic substances that may inhibit bacterial growth (Redhead & Wright, 1980; Cueto et al., 2001). Furthermore, research by Reischke et al. (2014) suggests that fungi are more tolerant to high glucose concentrations than bacteria, increasing their competitive advantage over bacteria in high glucose concentrations. Increased fungal growth could help to explain the observed decline in glucose concentration even though the DO concentration increases during this period. The DO concentration decreased again in the 1 g/L and 0.5 g/L treatment after 73 and 120 hours, respectively, indicating a tipping point at glucose concentrations of 0.5 g/L and 0.1 g/L, respectively. The observed tipping point may be caused by a combination of relatively lower glucose concentrations compared to the start of the experiment, and an increased tolerance of the bacterial community to glucose.

Glucose also had substantial impact on the mobilization of Fe and P. There was a strong positive correlation between increasing glucose and TP concentrations in the water column, with higher glucose concentrations resulting in more TP. The increase in TP under increased glucose concentrations indicates more P is being mobilized. The relatively small increase in TP in the treatments with 0.1, 0.2 and 0.3 g/L of glucose can be explained by the fast depletion of glucose and the subsequent increase of dissolved oxygen in the water column, resulting in immobilization by forming complexes with oxidized Fe(III) (Patrick & Khalid, 1974; Richardson, 1985; Lamers et al., 1998). P mobilization rates in these treatments may have been higher if the glucose concentrations were replenished once depleted.

TFe concentration in the water column shows a more than tenfold (~80 µmol/L to ~880 µmol/L) increase during the experiment in the treatment with 1 g/L glucose. In an anaerobic environment without sufficient supply of sulphate, such a high iron concentration is likely to have detrimental effects to plant tissue, as is extensively investigated in rice plants (e.g.

Tanaka et al., 1966; Sahrawat, 2005). However, iron toxicity in *A. filiculoides* is not well documented as of yet and may differ from the iron tolerance of rice plants. Unpublished work by Tang (2013) suggests that glucose concentrations of 1 g/L and 2 g/L have detrimental effects on *A. filiculoides* and causes shedding of the roots and brown colorization of the leaves. The brown colorization of the leaves may indicate iron toxicity. Because the experiment was carried out in a dark room, the oxygen balance did not include production in the water layer. The observed effect of the increased BOD on the DO concentration may be less when there is also production in the water column.

5 Conclusion

Aluminium-bound P in clay-rich soil proves to be very stable under changing oxygen concentrations and may be considered a great phosphorus sink, however, it is unfavourable over iron-rich soil in terms of P recovery. Triggering P mobilization in the iron-rich treatment with *Azolla* has proven to be a challenge. It is essential to overcome the vicious circle of decreasing plant density due to the absence of P and an absence of P due to too low plant density. Increasing the sulphate concentration to ~ 1500 µmol/L proved to be very effective in the immobilization of Fe and the stimulation of P mobilization in the second period of the experiment. Sulphate concentrations in surface waters in the Netherlands have been reported to be much higher (Lamers et al., 1998), especially near wastewater treatment plant (WWTP) outflows (STOWA, 2011). Constructed wetlands such as the biocascade water purification system are often used to polish effluent of WWTP, therefore it is expected that sufficient concentrations of sulphate will be available. Furthermore, increasing the density of the *A. filiculoides* layer had a positive effect on the DO and the concomitant P mobilization.

In natural ecosystems, floating water plants such as *A. filiculoides* limit light – and thus primary production in the water layer – and they block O_2 exchange of the water with the atmosphere. The glucose pilot experiment showed that adding 0.2 g/L of glucose results in the most stable low DO concentration over time. With this in mind, and considering the low costs, 0.2 g/L may be the most suitable concentration when applied on a large scale in order to trigger P-mobilization until the density of *A. filiculoides* is large enough to create anaerobic conditions.

The optimum biomass production rate of *A. filiculoides* found in this study (0.465 g dw m^2 day^1) – just after P-mobilization was triggered by means of sulphate addition and mechanically creating temporary anaerobic conditions through flushing with N_2 (g) – resulted in a phosphorus yield of 1.85 kg ha^{-1} $year^{-1}$. In conclusion, this research shows that phosphate mining from iron-rich sediments by cultivation of *A. filiculoides* in constructed wetlands may proof to be a sustainable strategy to create a regenerative water purification system while simultaneously recovering phosphorus.

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Appendices

Appendix A Algae growth in iron-rich soil without *Azolla*-treatment

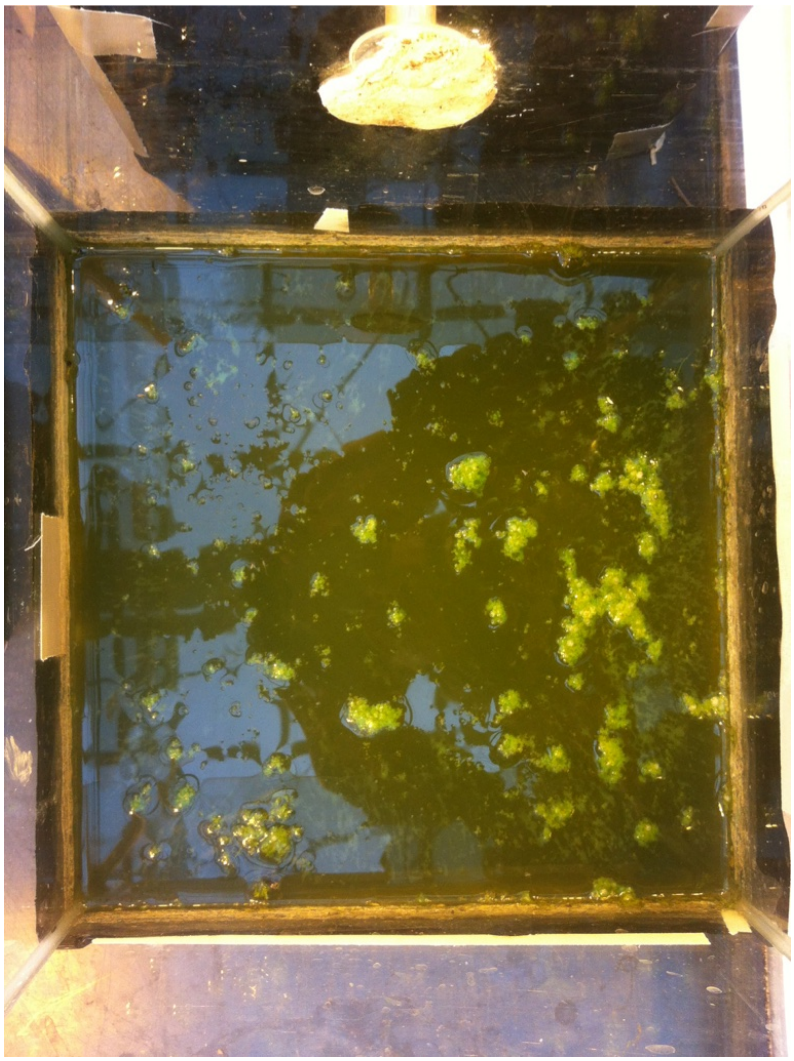

Figure 20: Algae growth in one of the replicates from the iron-rich soil without Azolla-treatment (29/03/16)

Appendix B Total sulphur concentrations in the water column

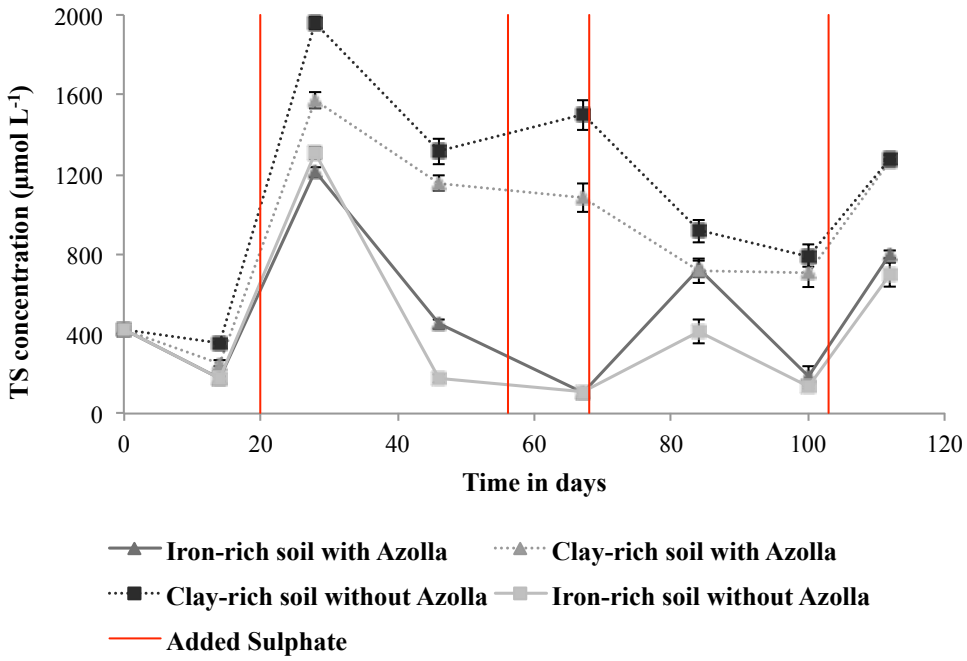

Figure 21: Mean total sulphur (± SEM) in the water column over time as a proxy for the sulphate concentration in the water column (n=5). The red lines indicate the sulphate addition moments. The standard error of the means is indicated by the bars.

Appendix C P deficiency vs sufficient P in plant tissue

Figure 22: Phosphorus deficiency after 20 days in both the iron-rich soil-treatment (A) and the clay-rich soil-treatment (B)

Appendix D Glucose pilot TP in the water column

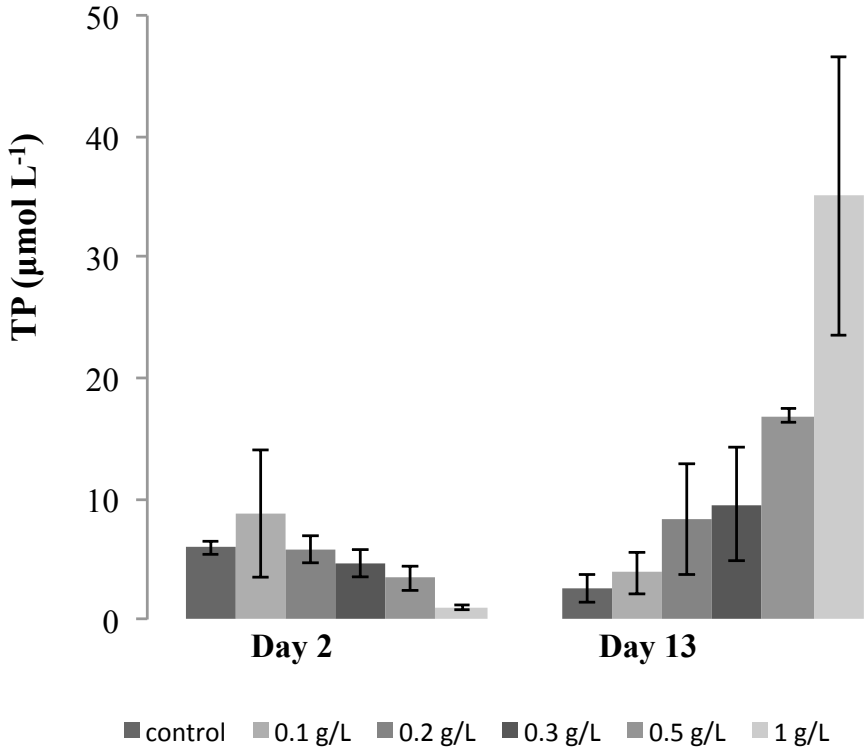

Figure 23: Mean TP (± SEM) in the surface water at day 2 and the end of the experiment (n=2). The standard error of the mean is indicated by the bars.

Appendix E Correlation graph TDP and glucose

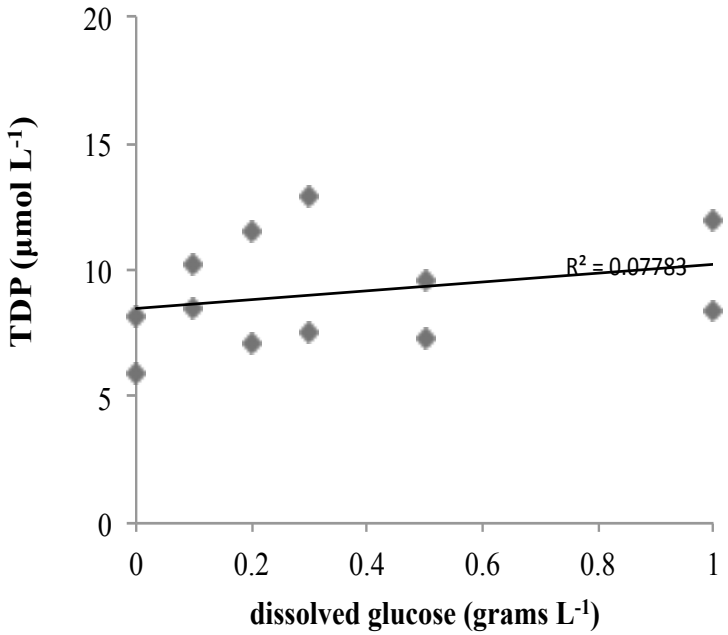

Figure 24: Correlation graph between TDP in the pore water at the end of the experiment and initial glucose concentration

Appendix F Fungal activity in highest glucose treatment

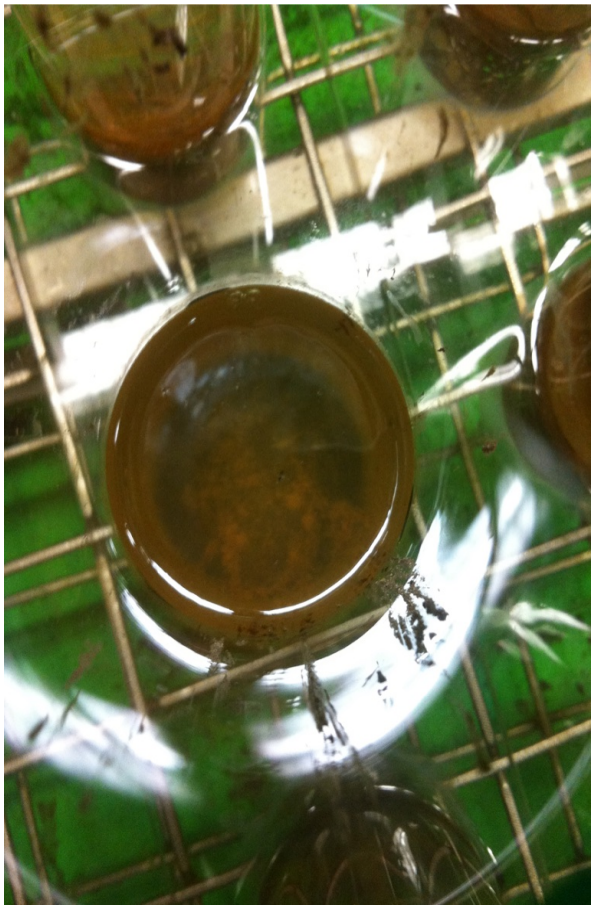

Figure 25: Fungi growth on the soil in one of the replicates with 1 g/L glucose, indicated by the whitish colour and the fuzzy appearance