Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/189958

Please be advised that this information was generated on 2018-04-11 and may be subject to change.

SCIENTIFIC REPORTS

Azolla along a phosphorus gradient: biphasic growth response linked to diazotroph traits and phosphorus-induced iron chlorosis

Ralph J. M. Temmink [1], Sarah F. Harpenslager[1,3], Alfons J. P. Smolders[2,1], Gijs van Dijk[2,1], Roy C. J. H. Peters[1], Leon P. M. Lamers[1,2] & Monique M. L. van Kempen[1]

Azolla spp., a water fern often used for phytoremediation, is a strong phosphorus (P) accumulator due to its high growth rate and N_2 fixing symbionts (diazotrophs). It is known that plant growth is stimulated by P, but the nature of the interactive response of both symbionts along a P gradient, and related changes in growth-limiting factors, are unclear. We determined growth, and N and P sequestration rates of *Azolla filiculoides* in N-free water at different P concentrations. The growth response appeared to be biphasic and highest at levels $\geq$10 P µmol l^{-1}. Diazotrophic N sequestration increased upon P addition, and rates were three times higher at high P than at low P. At 10 µmol P l^{-1}, N sequestration rates reached its maximum and *A. filiculoides* growth became saturated. Due to luxury consumption, P sequestration rates increased until 50 µmol P l^{-1}. At higher P concentrations ($\geq$50 µmol l^{-1}), however, chlorosis occurred that seems to be caused by iron- (Fe-), and not by N-deficiency. We demonstrate that traits of the complete symbiosis in relation to P and Fe availability determine plant performance, stressing the role of nutrient stoichiometry. The results are discussed regarding *Azolla*'s potential use in a bio-based economy.

The genus *Azolla* comprises a broad range of floating aquatic fern species growing in tropical, subtropical and temperate freshwater ecosystems[1,2]. *Azolla* spp. are able to reach high growth rates by asexual reproduction and are very strong phosphorus (P) and nitrogen (N) accumulators[3], which makes them very suitable for phytore-mediation, bio-gas production, animal food and crop fertilization[1,4,5]. Due to their symbiosis with atmospheric nitrogen (N_2) fixing microorganisms (diazotrophs), the primary production of the plants is hardly ever N-limited under natural conditions. The diazotrophs live inside *Azolla*'s leaf cavities (Supplementary Fig. S1) and include the cyanobacteria *Nostoc/Anabaena azollae*[6,7] that forms unbranched, multi-cellular chains that contain both photosynthetic, vegetative cells and N_2 fixing heterocysts[8]. Using nitrogenase enzymes, the diazotrophs reduce atmospheric N_2 to ammonium (NH_4^+), which is then excreted into the *Azolla* leaf cavity and taken up by the fern[9]. In response to N-limitation, when there is no exogenous N available to *Azolla*, the heterocyst fraction increases in diazotroph chains[10,11].

Given its high potential P uptake rates, luxury accumulation of P[12,13] and high growth rates, *Azolla* may well be used for phytoremediation with respect to P[4,12,14–17]. The cultivation of *Azolla* spp. in P-enriched water could therefore offer a sustainable way of P recovery and recycling from eutrophic water[12,18–22]. Sustainable practices of P recycling are important, as global reserves of phosphate rock are becoming increasingly depleted[23,24]. Next to P recycling, *Azolla* cultivation may yield annually up to 35 t dry N-rich biomass, independent of N-fertilizer[25]. Although nutrient supply can easily be managed in hydro-cultures, nutrient management is more challenging when growing *Azolla* in flooded fields, due to biogeochemical interactions in the flooded soil and surface water[26]. The supply of macronutrients such as P and iron (Fe) and micronutrients by inflowing water, or by mobilization from the sediment to the overlying water should be sufficient to support *Azolla*'s growth[26,27]. Natural waters may

[1]Aquatic Ecology and Environmental Biology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525 AJ, Nijmegen, The Netherlands. [2]B-WARE Research Centre, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands. [3]School of Biological and Chemical Sciences, Queen Mary University, E1 4NS, London, United Kingdom. Correspondence and requests for materials should be addressed to R.J.M.T. (email: r.temmink@science.ru.nl)
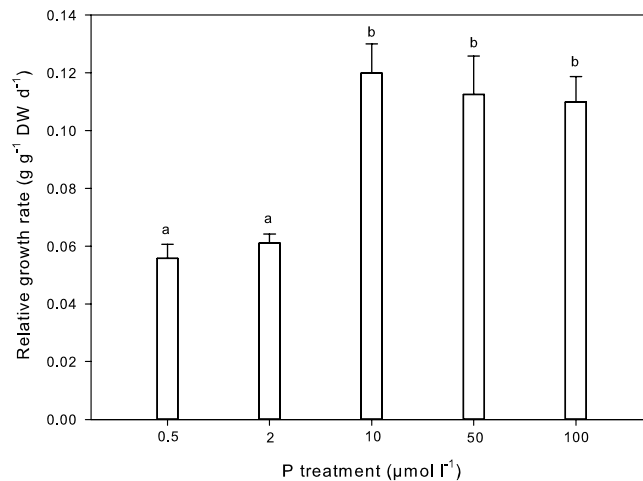
Figure 1. Relative growth rates (RGRs, g g^{-1} DW d^{-1}) $\pm$ SE of *A. filiculoides* after 21 days of growth at 0.5, 2, 10, 50 and 100 μmol P l^{-1} (n = 5). Significant differences are indicated by different letters (Tukey HSD post hoc test). Additional results of the statistical analysis are presented in Supplementary Table S2.

well show non-optimal nutrient stoichiometry resulting in nutrient deficiency, impeded *Azolla* growth or even chlorosis[1,28] due to P or Fe limitation[28,29].

High P concentrations in surface waters are well known to stimulate the growth of *Azolla* spp.[30,31] and to increase N$_2$ fixation rates of their symbionts[31–33]. For efficient and broad application of *A. filiculoides* in a biobased economy, however, a profound understanding of the interactive responses of this species and its diazotroph microbiome to different P loadings is required, which is currently lacking. To test the type of growth response to P and find an explanation for this, *A. filiculoides* was grown in a greenhouse experiment in N-free surface water at different P concentrations. We hypothesized that both N and P sequestration rates are positively related to exogenous P concentrations, but level off because endogenous N provided by the diazotrophs becomes limited. We further hypothesized that at high P levels, Fe deficiency may occur which may induce chlorosis and limit *Azolla* growth, as this element is not only essential for *Azolla* but also for the production of the nitrogenase enzyme[1,28].

Results

Growth response to P availability.
RGRs of *A. filiculoides* growing in the 0.5 and 2 μmol P l^{-1} treatments were significantly lower than those of the higher P treatments (Fig. 1). *Azolla's* growth response to increasing P showed a biphasic response (Michaelis-Menten fit: R^2 = 0.62, p < 0.001). During 21 days, *A. filiculoides* growing in 0.5 and 2 μmol P l^{-1} treatments increased their biomasses 2.2 times, whereas in 10, 50 and 100 μmol P l^{-1} treatments, biomasses increased 3.4 times. During the experiment *Azolla's* biomass was lower than the self-crowding threshold of 2 kg FW m^{-2} (data not shown).

Morphology of *A. filiculoides* also changed in response to different P treatments. Plants from the 0.5 and 2 μmol P l^{-1} treatments were smaller and more fragile than those from the 10, 50 and 100 μmol P l^{-1} treatments. Furthermore, plants from the lowest two concentrations turned reddish, while plants from the 10 μmol P l^{-1} treatment stayed green. Plants from the 50 and 100 μmol P l^{-1} treatments turned chlorotic (yellowish) after 21 days of growth.

Plant nutrient composition.
Plant P contents increased when P concentrations in the nutrient solution increased, but declined over time (Fig. 2a). P sequestration rates also increased, until the 50 μmol P l^{-1} treatment (Fig. 3a). Plant N content was stable over time, and highest at 50 μmol P l^{-1}. Lowest N content was found for the 0.5 and 2 μmol P l^{-1} treatments (Fig. 2b). P concentrations $\geq$ 10 μmol l^{-1} in the nutrient solution significantly increased N sequestration rates of *Azolla* and its symbionts (Fig. 3b). N sequestration rates were approximately 4.5 and 2.5 times higher in the higher P treatments ($\geq$10 μmol l^{-1}) compared to the 0.5 and 2 μmol P l^{-1} treatments, respectively. Plant N: P ratios showed an extensive range, and decreased with increasing P availability (Fig. 2c). At day 21, the highest value (55 $\pm$ 1.7 mol mol^{-1}) was reached in the 0.5 μmol P L^{-1} treatment, and the lowest in the 50 and 100 μmol P l^{-1} treatments (12.4 $\pm$ 0.3 and 10.9 $\pm$ 0.3 mol mol^{-1}, respectively). Plant K, P, N, Na and S contents were significantly higher in the >10 μmol P l^{-1} treatments, whereas Zn and Ca contents were significantly lower and Fe and Si were similar (Supplementary Table S3). Further elemental composition of the plants is shown in Supplementary Table S3.

Deficiency testing.
To test the cause of the observed chlorosis an additional, but similar, experiment was carried out, in which plants also turned chlorotic after 5 weeks of 100 μmol P l^{-1} treatment. They were subsequently treated with Fe or NH$_4^+$-NO$_3^-$. After two weeks, only the plants treated with Fe became green again and had a significantly higher chlorophyll a + b content (15.2 $\pm$ 2.3 mg g^{-1} DW) than the control (10.8 $\pm$ 2 mg g^{-1} DW) or the N treatment (9.5 $\pm$ 2.7 mg g^{-1} DW, Fig. 4a). In addition, highest N contents were found in plants treated with Fe (3000 $\pm$ 11 μmol N g^{-1} DW) and lowest in the control (2125 $\pm$ 20 μmol N g^{-1} DW; Fig. 4b). Plants treated with N showed an intermediate N content of 2529 $\pm$ 18 μmol N g^{-1} DW (Fig. 4b and Supplementary
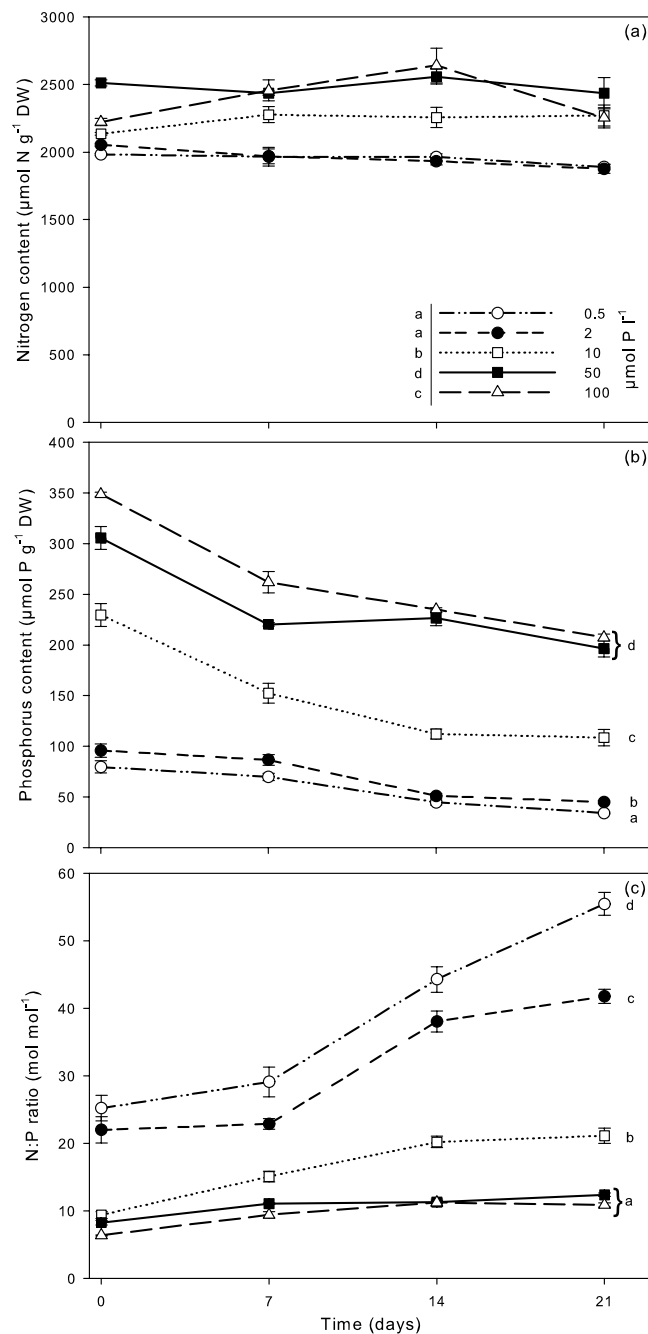
Figure 2. P content (µmol P g^{-1} DW; (**a**) N content (µmol N g^{-1} DW; (**b**) and N: P ratios (mol mol^{-1}; (**c**) ±SE of *A. filiculoides* during 21 days of growth at 0.5, 2, 10, 50 and 100 µmol P l^{-1} (n = 5). Different letters indicate significant differences (Bonferroni post hoc test). Additional results of the statistical analyses are presented in Supplementary Table S2.

Table S4). Compared to both the control and the N treatment, the Fe content (55.8 ± 8 µmol Fe g^{-1} DW) in the Fe treatment was 1.6 and 1.5 times as high (Supplementary Table S4). Additional elemental composition of the plants is shown in Supplementary Table S4.

Discussion

Both *Azolla filiculoides* growth and the diazotrophic activity of its microbiome (resulting in N sequestration) showed a biphasic response to P availability, when grown without external N supply. For high P levels, after maximum fixation rates by diazotrophic symbionts had been reached, the plants turned chlorotic. Additional external N supply did not increase plant health, but additional Fe supply did increase chlorophyll a + b and plant N contents. This not only proves that Fe was limiting at high P availability, but also means that traits of the complete symbiosis in relation to P and Fe availability determine *Azolla's* performance, stressing the role of nutrient stoichiometry.
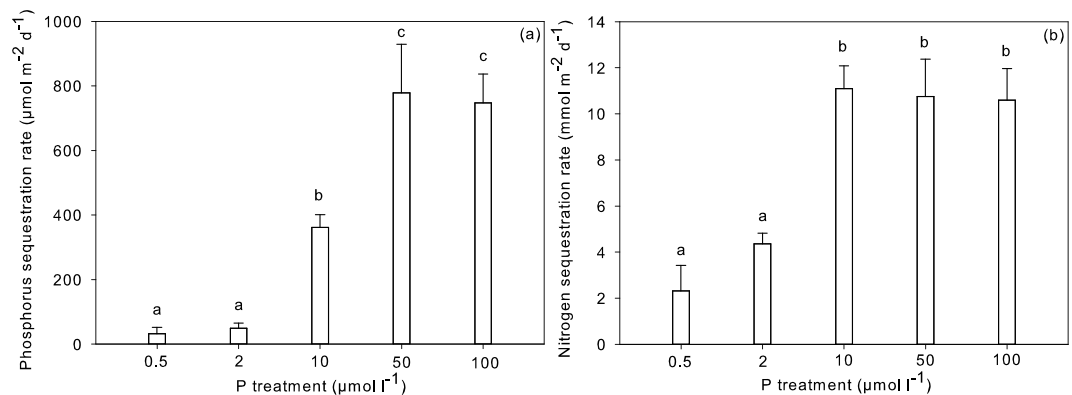
Figure 3. P (panel a) and N (panel b) sequestration rates (µmol m^{-2} d^{-1}; n = 5) ± SE by *A. filiculoides* for the 0.5, 2, 10, 50 and 100 µmol P l^{-1} treatments. Different letters indicate significant differences (ANOVA Tukey post hoc test). Additional results of the statistical analyses are presented in Supplementary Table S2.
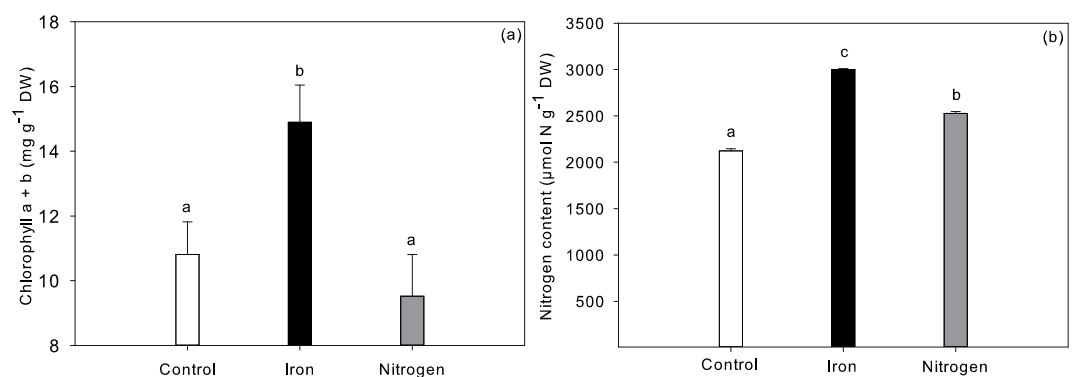

Figure 4. Chlorophyll a + b (mg g^{-1} DW, (**a**) and plant N content (µmol N g^{-1} DW, (**b**) of *A. filiculoides* treated with 100 µmol P l^{-1} (Control), after Fe (Iron) or nitrogen addition (limitation experiment, n = 4) ± SE. Significant differences are indicated by different letters (ANOVA Tukey HSD pairwise comparison). Additional statistical results are presented in Supplementary Table S2.

We found that *A. filiculoides* is able to double its biomass in one week in an N-free nutrient solution growing in a P-rich environment, entirely relying on the symbiosis with diazotrophs for its N supply. The growth rates measured are in the range reported in literature[32,34–37]. However, higher RGRs may be obtained when growing *Azolla* under optimal conditions using synthetic media in aquaculture[1,38]. Self-crowding may also result in lower RGRs of the fern, however, during the experiment the biomass was lower than the self-crowding threshold of 2 kg FW m^{-2} and was therefore not responsible for the biphasic growth response[39]. Here we show that the RGR of *A. filiculoides* shows a biphasic response to P availability, and becomes saturated at 10 µmol P l^{-1}. The lack of differences between RGRs of plants grown with 10, 50 and 100 µmol P l^{-1}, together with the substantially higher P content in plants grown at high P, either suggests that the maximum growth of this strain of *A. filiculoides* and its diazotrops were reached, or that a factor other than P became limiting above 10 µmol P l^{-1}.

In aquatic plants, N: P ratios >17 (mol mol^{-1}) generally indicate P deficiency, whereas a N: P ratios < 10 (mol mol^{-1}) indicate N deficiency[40–42]. Based on their N: P ratios, *A. filiculoides* grown at P levels ≤ 2 µmol l^{-1} were severely P-limited[43,44], which was also indicated by their red color[1]. Addition of P shifted the limitation through N and P co-limitation (in plants receiving 10 µmol P l^{-1}) towards N-limitation (in plants receiving 50 and 100 µmol P l^{-1}), based on N:P ratios and color.

A. filiculoides from the 50 and 100 µmol P l^{-1} treatment had a similar N: P ratio around 10 mol mol^{-1}, which may indicate that the plant's N-requirement and N-supply by the diazotrophs were in equilibrium to sustain *Azolla's* growth. Apparently, the diazotrophs were not able to fix additional N that could be utilized to increase biomass growth. Interestingly, plants supplied with additional Fe had significantly higher N content compared to plants that were only supplied with N and plants that did not receive additional Fe or N, indicating Fe limitation for both plant growth and N$_2$ fixation by the diazotrophs[1,25]. Luxury consumption of N did not seem to occur, probably because N$_2$ fixation rates reached their maximum due to Fe limitation. The ferns, however, continued to accumulate P up to average rates of 778 µmol m^{-2} d^{-1}, indicating luxury consumption (Fig. 5). N and P sequestration rates were indeed positively related to exogenous P concentrations. For N the maximum was reached at 10 µmol P l^{-1}, whereas for P this was at 50 µmol P l^{-1}. Our results clearly show that the diazotrophic community and *A. filiculoides* are able to respond dynamically to P availability in the aquatic environment, but become saturated or limited at a certain level.
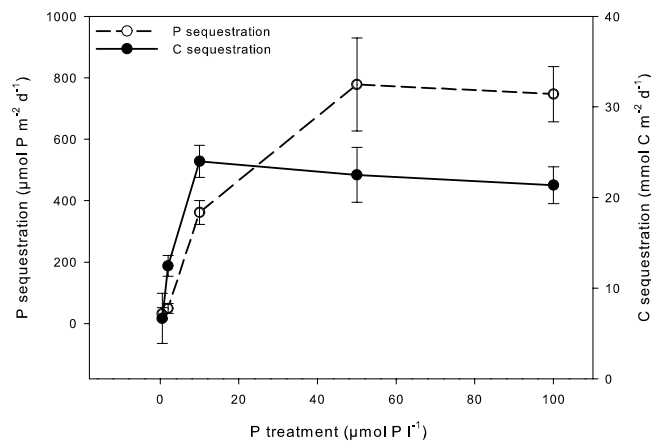
Figure 5. P (µmol P m^{-2} d^{-1}; left y-axis) and carbon (C; mmol C m^{-2} d^{-1}; right y-axis) sequestration rates in *A. filiculoides* under different P loadings (n = 15; ± SE).

In natural environments, eutrophication usually includes increased loading of both N and P. Presence of exogenous N influences both N_2 fixation and growth of *A. filiculoides*. Availability of N, has been shown to inhibit nitrogenase activity in *Azolla*[11,15,45,46], while not lowering plant growth[47]. In temperate regions, under natural conditions, surface water N concentrations rapidly decrease in spring as a result of high denitrification rates due to increased microbial activity, but surface water P availability remains high, especially if sediment P gets mobilized due to anoxia in the water layer[27,48]. Under these environmental conditions, *Azolla* can become highly competitive, using N produced by the diazotrophs combined with enhanced P availability.

At high P levels, plants may turn chlorotic, which has been attributed to absolute N-limitation in literature[1]. Here, we show that chlorosis and lower chlorophyll contents are caused by Fe deficiency at high P levels. Fe limitation can be expected to have resulted in lower growth and N sequestration rates in the high P treatments. The fact that *Azolla's* condition (chlorophyll a + b) did improve after supplying exogenous Fe is in agreement with the hypothesis that Fe was deficient for *Azolla* grown at high P levels. In addition, it has been shown that *Azolla* is capable of higher growth rates when grown at >100 µmol P l^{-1} provided Fe is sufficiently available[2,32]. Fe availability in the environment or total Fe content of the plant are not the critical processes leading to chlorosis; multiple mechanisms can be responsible for Fe induced chlorosis including Fe precipitation in the apoplast, which is physiologically unavailable[49–51]. The presence of P can also affect plant Fe availability[44,52,53]. Interaction of P and Fe leading to Fe chlorosis can be caused by an internal immobilization of Fe probably due to formation of Fe-PO$_4$[54–57].

Our results indicate that nutrients in the water should be balanced when growing Azolla to optimize plant health and maximize yields. The use of *A. filiculoides* (and its diazotrophic community) as a tool to recover and recycle P in a bio-based economy[12,25] is also dependent on Fe availability. When Azolla is grown on inundated agricultural lands (Azolla farming) for example, not only P re-mobilization from the sediment[27,48], but also Fe mobilization should be considered.

Materials and Methods

Experimental set-up. Before the start of the experiment, *A. filiculoides* was acclimatized to experimental conditions in 15 l tanks containing an N-free nutrient solution to which 0.5, 2, 10, 50 or 100 µmol P l^{-1} was added (as NaH$_2$PO$_4$ * 2H$_2$O). The general composition of the solution was similar to the quality of natural surface waters where *Azolla* is abundant (Supplementary Table S1). After sufficient *Azolla* biomass had been produced in the greenhouse for 30 days[37], the plants were pretreated for 10 days in larger basins without harvesting to minimize P history effects[13]. Nutrient solutions were replaced 3 times a week. After this period[13], 7.5 g (surface cover of 90%) of carefully dry-blotted *A. filiculoides* was transferred to a 1 l glass aquarium (10 cm × 10 cm × 12 cm; L × W × H; 750 g FW m^{-2}), which was continuously fed by the same nutrient solution (pH = 7.3) as during the acclimatization period, at a rate of 1 l d^{-1} using peristaltic pumps (Masterflex L/S; Cole-Palmer, Chicago, IL, USA). Nutrient concentrations were checked biweekly. Each P treatment had 5 replicates. The water level was kept constant by an overflow outlet. Aquaria were randomly placed in a temperature controlled water bath at 18 °C situated in the greenhouse facilities, at a mean day temperature of 21.2 °C (SE ± 0.007) and night temperature of 18.6 °C (SE ± 0.007) during the experimental period of 21 days. Light was mostly natural, but an artificial light regime of 16 h: 8 h (light: dark) was maintained by four 400 W high-pressure sodium lamps (Hortilux-Schréder, Monster, The Netherlands) to illuminate the experiment whenever light intensity fell below 250 W m^{-2} (300 µmol m^{-2} s^{-1} PAR; Quantum sensor, Skye Instruments LTD, Wales, England). Sides of the aquaria were covered with black plastic foil to avoid light penetration from the sides.

Growth measurement. Total plant biomass in each aquarium was determined at 0, 7, 14 and 21 days and subsamples were taken to determine fresh weight (FW) to dry weight (DW) ratios. Samples were rinsed, carefully blotted dry and weighed. After FW determination, 7.5 g (750 g FW m^{-2}) of *A. filiculoides* was returned into its original aquarium to prevent crowding effects. The rest of the biomass was dried for 48 h at 60 °C, ground and

homogenized using a ball mill (type Mixer Mill 301, Retsch GmbH, Haan, Germany) for further analyses. From the biomass increase, relative growth rates were calculated (RGR; g g^{-1} DW d^{-1}).

Plant nutrient analyses.   200 mg dried plant material was digested using 4 ml HNO$_3$ (65%) and 1 ml H$_2$O$_2$ (35%) in Teflon vessels using an Ethos D microwave (1200 MLS, Milestone, Sorisole, Italy), after which digestates were analyzed for P, Fe, aluminum (Al), manganese (Mn), sodium (Na), sulfur (S), silicon (Si), zinc (Zn) using an inductively coupled plasma emission spectrophotometer (ICP-OES; model IRIS Intrepid II XDL, Thermo Fisher Scientific, Franklin, USA). N contents of 3 mg homogenized dried plant material were determined by an elemental CNS analyzer (model NA 1500, Carlo Erba; Thermo Fisher Scientific, Franklin, USA). Plant N and P sequestration rates were calculated by combining plant biomass production and N and P contents for every harvest. As *A. filiculoides* was grown in an N-free medium, the increase in plant N could fully be attributed to N$_2$ fixation by the diazotrophs.

Iron versus nitrogen limitation experiment.   To determine which element caused the chlorotic appearance of *A. filiculoides* found at 100 µmol P l^{-1}, the original experiment was repeated for this treatment. *Azolla* may turn yellow/chlorotic due to the depletion of chlorophyll[1]. Also, Fe is often a limiting element for *Azolla* because it is an essential component of nitrogenase[1,28]. Chlorosis induced by iron deficiency, however, does not always implicate low total Fe contents in plant tissue[58–62], because Fe may be immobilized in the apoplast and not be physiologically available[63,64]. Iron deficiency can be identified optimally when spraying plants with Fe, which causes regreening and an increase of chlorophyll a + b content[57,59,61,65]. To test N limitation, increasing both NO$_3^-$ and NH$_4^+$ in the medium is the optimal approach[45]. When *A. filiculoides* became chlorotic after 5 weeks, plants were daily treated with iron (sprayed with Fe-EDTA [*Pro Analysis quality*]; 10 µmol l^{-1}) or nitrogen (NH$_4$-NO$_3$ [*Pro Analysis quality*]; 100 µmol l^{-1}) for 2 weeks. Both methods were used to ensure optimal uptake of either Fe or N. To ensure that there would not be a time effect on the chlorotic appearance of *A. filiculoides*, the original treatment was continued (control, 100 µmol P l^{-1}). Treatments were replicated 4 times. Nutrient content and chlorophyll a + b content[66] were determined in all treatments at the end of the experiment, after 2 weeks of Fe and N application.

Statistical analyses.   Homogeneity of variance and normality of the residuals were tested, and Analyses of Variances (ANOVAs) were used to analyze potential differences in RGR, N and P sequestration rates and plant chlorophyll a + b in response to different P concentrations or to element addition (response chlorophyll a + b). Differences between treatments were determined using Tukey HSD post hoc tests. Data of P sequestration were square root transformed to authorize the use of parametric analyses. Linear mixed models (LMMs) were used to analyze differences in plant P and N content and N: P ratio over time at different P concentrations. Time was nested in aquaria and the covariance type was selected based on the smallest Hurvich and Tsai's Criterion (AICC). P content or N: P ratio of the plants was set as a dependent variable while the P treatment was set as a fixed factor. Overall effects and P treatments were displayed in Estimated Marginal Means of Fitted Models and the main effects were compared using Bonferroni pairwise comparisons. Plant P content was log transformed and N: P ratios were square root transformed. All statistical analyses were carried out using SPSS 21.0 (SPSS Inc., Chicago, IL, U.S.A.).

Data availability.   The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Wagner, G. Azolla: A review of its biology and utilization. *The Botanical Review* **63**, 1–26 (1997).
2. Sadeghi, R., Zarkami, R., Sabetraftar, K. & Van Damme, P. A review of some ecological factors affecting the growth of Azolla spp. *Caspian Journal of Environmental Sciences* **11**, 65–76 (2013).
3. Lumpkin, T. & Plucknett, D. Azolla: Botany, physiology, and use as a green manure. *Economic Botany* **34**, 111–153 (1980).
4. Valderrama, A., Tapia, J., Peñailillo, P. & Carvajal, D. E. Water phytoremediation of cadmium and copper using Azolla filiculoides Lam. in a hydroponic system. *Water and Environment Journal* **27**, 293–300 (2013).
5. van Hove, C. & Lejeune, A. In *Cyanobacteria in Symbiosis* (*Eds* N. Rai, B. Bergman & U. Rasmussen) (Kluwer Academic Press, 2002).
6. Baker, J. A., Entsch, B. & McKay, D. B. The cyanobiont in an Azolla fern is neither Anabaena nor Nostoc. *FEMS Microbiology Letters* **229**, 43–47, https://doi.org/10.1016/s0378-1097(03)00784-5 (2003).
7. Ran, L. *et al*. Genome erosion in a nitrogen-fixing vertically transmitted endosymbiotic multicellular cyanobacterium. *PLoS ONE* **5**, e11486, https://doi.org/10.1371/journal.pone.0011486 (2010).
8. Moore, A. W. Azolla: Biology and agronomic significance. *The Botanical Review* **35**, 17–34 (1969).
9. Peters, G. A. & Meeks, J. C. The Azolla-anabaena symbiosis: basic biology. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 193–210, https://doi.org/10.1146/annurev.pp.40.060189.001205 (1989).
10. Singh, R. P. & Singh, P. K. Effect of nitrogen fertilizers on nitrogen fixation and heterocyst frequency of cyanobacterium Anabaena azollae in 7 species of Azolla. *Biochemie und Physiologie der Pflanzen* **185**, 429–433, https://doi.org/10.1016/S0015-3796(89)80068-X (1989).
11. Hechler, W. D. & Dawson, J. O. Factors affecting nitrogen fixation in Azolla caroliniana. *Transactions of the Illinois State Academy of Science* **88**, 97–107 (1995).
12. Shilton, A. N., Powell, N. & Guieysse, B. Plant based phosphorus recovery from wastewater via algae and macrophytes. *Curr Opin Biotechnol* **23**, 884–889, https://doi.org/10.1016/j.copbio.2012.07.002 (2012).
13. Peeters, E. T. H. M., Neefjes, R. E. M. & Zuidam, B. G. v. Competition between Free-Floating Plants Is Strongly Driven by Previously Experienced Phosphorus Concentrations in the Water Column. *PLOS ONE* **11**, e0162780, https://doi.org/10.1371/journal.pone.0162780 (2016).
14. Forni, C., Chen, J., Tancioni, L. & Grilli Caiola, M. Evaluation of the fern Azolla for growth, nitrogen and phosphorus removal from wastewater. *Water Research* **35**, 1592–1598 (2001).
15. Costa, M. L., Santos, M. C. R., Carrapio, F., and Pereira, A. L. Azolla-anabaena's behaviour in urban wastewater and artificial media influence of combined nitrogen. *Water Research* **43** (2009).

16. Song, U., Park, H. & Lee, E. Ecological responses and remediation ability of water fern (Azolla japonica) to water pollution. *J. Plant Biol.* **55**, 381–389, https://doi.org/10.1007/s12374-012-0010-5 (2012).

17. Tang, Y. *et al.* Aquatic macrophytes can be used for wastewater polishing but not for purification in constructed wetlands. *Biogeosciences* **14**, 755–766, https://doi.org/10.5194/bg-14-755-2017 (2017).

18. Peñuelas, J. *et al.* Human-induced nitrogen–phosphorus imbalances alter natural and managed ecosystems across the globe. *Nat Commun* **4**, https://doi.org/10.1038/ncomms3934 (2013).

19. Brouwer, P. *et al.* Azolla domestication towards a biobased economy? *New Phytologist* **202**, 1069–1082, https://doi.org/10.1111/nph.12708 (2014).

20. Choudhury, A. T. M. A. & Kennedy, I. R. Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. *Biology and Fertility of Soils* **39**, 219–227, https://doi.org/10.1007/s00374-003-0706-2 (2004).

21. de Macale, M. & Vlek, P. G. The role of Azolla cover in improving the nitrogen use efficiency of lowland rice. *Plant and Soil* **263**, 311–321, https://doi.org/10.1023/B:PLSO.0000047742.67467.50 (2004).

22. Bhuvaneshwari, K. & Singh, P. K. Response of nitrogen-fixing water fern Azolla biofertilization to rice crop. *Biotech* **5**, 523–529, https://doi.org/10.1007/s13205-014-0251-8 (2015).

23. Van Vuuren, D. P., Bouwman, A. F. & Beusen, A. H. W. Phosphorus demand for the 1970-2100 period: A scenario analysis of resource depletion. *Global Environmental Change-Human and Policy Dimensions* **20**, 428–439 (2010).

24. Carpenter, R. S. & Bennett, E. M. Reconsideration of the planetary boundary for phosphorus. *Environmental Research Letters* **6**, 014009–014009 (2011).

25. Brouwer, P. *et al.* Metabolic Adaptation, a Specialized Leaf Organ Structure and Vascular Responses to Diurnal N_2 Fixation by Nostoc azollae Sustain the Astonishing Productivity of Azolla Ferns without Nitrogen Fertilizer. *Frontiers in Plant Science* **8**, https://doi.org/10.3389/fpls.2017.00442 (2017).

26. van Diggelen, J. M. H. *et al.* New Insights into Phosphorus Mobilisation from Sulphur-Rich Sediments: Time-Dependent Effects of Salinisation. *PLoS ONE* **9**, e111106, https://doi.org/10.1371/journal.pone.0111106 (2014).

27. Zak, D., Wagner, C., Payer, B., Augustin, J. & Gelbrecht, J. Phosphorus mobilization in rewetted fens: the effect of altered peat properties and implications for their restoration. *Ecological Applications* **20**, 1336–1349 (2010).

28. Watanabe, I. In *Microbiology of Tropical Soils and Plant Productivity. Developments in Plant and Soil Sciences* Vol. 5 (eds Y. R. Dommergues & H. G. Diem) 169–185 (Springer Netherlands, 1982).

29. Rains, D. W. & Talley, S. N. In *Nitrogen and rice symposium proceedings.* (International Rice Research Institute, Los Baons, Philippines).

30. Cheng, W., Sakai, H., Matsushima, M., Yagi, K. & Hasegawa, T. Response of the floating aquatic fern Azolla filiculoides to elevated CO_2, temperature, and phosphorus levels. *Hydrobiologia* **656**, 5–14 (2010).

31. Singh, R. P. & Singh, P. K. Symbiotic algal nitrogenase activity and heterocyst frequency in seven Azolla species after phosphorus fertilization. *Hydrobiologia* **169**, 313–318 (1988).

32. Cary, P. R. & Weerts, P. G. J. Growth and nutrient composition of Azolla pinnata R. Brown and Azolla filiculoides Lamarck as affected by water temperature, nitrogen and phosphorus supply, light intensity and pH. *Aquatic Botany* **43**, 163–180 (1992).

33. Kushari, D. P. & Watanabe, I. Differential responses of azolla to phosphorus deficiency. *Soil Science and Plant Nutrition* **37**, 271–282 (1991).

34. Subudhi, B. P. R. & Watanabe, I. Differential phosphorus requirements of Azolla species and strains in phosphorus-limited continuous culture. *Soil Science and Plant Nutrition* **27**, 237–247 (1981).

35. Moretti, A. & Siniscalco Gigliano, G. Influence of light and pH on growth and nitrogenase activity on temperate-grown Azolla. *Biology and Fertility of Soils* **6**, 131–136, https://doi.org/10.1007/bf00257662 (1988).

36. van Kempen, M. M. L., Smolders, A. J. P., Bögemann, G. M., Lamers, L. P. M. & Roelofs, J. G. M. Interacting effects of atmospheric CO_2 enrichment and solar radiation on growth of the aquatic fern Azolla filiculoides. *Freshwater Biology* **61**, 596–606, https://doi.org/10.1111/fwb.12724 (2016).

37. van Kempen, M. M. L. *et al.* Responses of the Azolla filiculoides Stras.–Anabaena azollae Lam. association to elevated sodium chloride concentrations: Amino acids as indicators for salt stress and tipping point. *Aquatic Botany* **106**, 20–28 (2013).

38. Peters, G. A. *et al.* Characterization and comparison of five N_2-fixing Azolla-Anabaena associations, I. Optimization of growth conditions for biomass increase and N content in a controlled environment. *Plant Cell & Environment* **3**, 261–269 (1980).

39. van Hove, C. Azolla and its multiple uses with emphasis on africa. *Technical report, Food and Agricultural Organization of the United Nations (FAO), Rome, Italy.* (1989).

40. Atkinson, M. J. & Smith, S. V. C:N:P ratios of benthic marine plants. *Limnology and Oceanography* **28**, 568–574, https://doi.org/10.4319/lo.1983.28.3.0568 (1983).

41. Hellström, T. An empirical study of nitrogen dynamics in lakes. *Water Environment Research* **68**, 55–65 (1996).

42. Ekholm, P. N:P ratios in estimating nutrient limitation in aquatic systems. *Finnish Environment Institute* (2008).

43. Epstein, E. *Plant Biochemistry*. (Academic Press, 1965).

44. Marschner, H. *Mineral nutrition of higher plants*. (Academic, 2nd edition edition, 1995).

45. Ito, O. & Watanabe, I. The relationship between combined nitrogen uptakes and nitrogen fixation in Azolla-anabaena symbiosis. *New Phytologist* **95**, 647–654 (1983).

46. Singh, P. K., Singh, D. P. & Singh, R. P. Growth, acetylene reduction activity, nitrate uptake and nitrate reductase activity of Azolla caroliniana and Azolla pinnata at varying nitrate levels. *Biochemie und Physiologie der Pflanzen* **188**, 121–127, https://doi.org/10.1016/S0015-3796(11)80019-3 (1992).

47. Manna, A. B. & Singh, P. K. Effects of nitrogen fertilizer application methods on growth and acetylene reduction activity of Azolla pinnata and yield of rice. *Fertilizer research* **28**, 25–30, https://doi.org/10.1007/bf01048852 (1991).

48. Zak, D. & Gelbrecht, J. The mobilisation of phosphorus, organic carbon and ammonium in the initial stage of fen rewetting (a case study from NE Germany). *Biogeochemistry* **85**, 141–151 (2007).

49. Mengel, K. Iron availability in plant tissues-iron chlorosis on calcareous soils. *Plant and Soil* **165**, 275–283, https://doi.org/10.1007/BF00008070 (1994).

50. Kosegarten, H. & Koyro, H.-W. Apoplastic accumulation of iron in the epidermis of maize (Zea mays) roots grown in calcareous soil. *Physiologia Plantarum* **113**, 515–522, https://doi.org/10.1034/j.1399-3054.2001.1130410.x (2001).

51. Rediske, J. H. & Biddulph, O. The Absorption and Translocation of Iron. *Plant Physiology* **28**, 576 (1953).

52. Morrissey, J. & Guerinot, M. L. Iron uptake and transport in plants: The good, the bad, and the ionome. *Chemical reviews* **109**, 4553–4567, https://doi.org/10.1021/cr900112r (2009).

53. Kuo, S. & Mikkelsen, D. S. Effect of P and Mn on growth response and uptake of Fe, Mn and P by sorghum. *Plant and Soil* **62**, 15–22, https://doi.org/10.1007/bf02205021 (1981).

54. Römheld, V. The chlorosis paradox: Fe inactivation as a secondary event in chlorotic leaves of grapevine. *Journal of Plant Nutrition* **23**, 1629–1643, https://doi.org/10.1080/01904160009382129 (2000).

55. Fageria, V. D. Nutrient interactions in crop plants. *Journal of Plant Nutrition* **24**, 1269–1290, https://doi.org/10.1081/PLN-100106981 (2001).

56. Olsen, C. Iron absorption and chlorosis in green plants. *Compt. rend. trav. lab. Carlsberg, Sér. chim* **21**, 15–52 (1935).

57. Rajaie, M. & Tavakoly, A. R. Iron and/or acid foliar spray versus soil application of Fe-EDDHA for prevention of iron deficiency in Valencia orange grown on a calcareous soil. *Journal of Plant Nutrition*, 1–9, https://doi.org/10.1080/01904167.2017.1382523 (2017).

58. De Kock, P. C. & Wallace, A. Excess Phosphorus and Iron Chlorosis. *California Agriculture* **19**, 3–4 (1965).
59. Bienfait, H. F. Prevention of stress in iron metabolism of plants. *Acta Botanica Neerlandica* **38**, 105–129, https://doi.org/10.1111/j.1438-8677.1989.tb02035.x (1989).
60. Smolders, A. J. P. & Roelofs, J. G. M. The roles of internal iron hydroxide precipitation, sulphide toxicity and oxidizing ability in the survival of Stratiotes aloides roots at different iron concentrations in sediment pore water. *New Phytologist* **133**, 253–260, https://doi.org/10.1111/j.1469-8137.1996.tb01892.x (1996).
61. Smolders, A. J. P., Hendriks, R. J. J., Campschreur, H. M. & Roelofs, J. G. M. Nitrate induced iron deficiency chlorosis in Juncus acutiflorus. *Plant and Soil* **196**, 37–45, https://doi.org/10.1023/a:1004293012462 (1997).
62. Mengel, K., Bubl, W. & Scherer, H. W. Iron distribution in vine leaves with HCO_3^- induced chlorosis. *Journal of Plant Nutrition* **7**, 715–724, https://doi.org/10.1080/01904168409363236 (1984).
63. Mengel, K. & Geurtzen, G. Relationship between iron chlorosis and alkalinity in Zea mays. *Physiologia Plantarum* **72**, 460–465, https://doi.org/10.1111/j.1399-3054.1988.tb09151.x (1988).
64. Sattelmacher, B. The apoplast and its significance for plant mineral nutrition. *New Phytologist* **149**, 167–192, https://doi.org/10.1046/j.1469-8137.2001.00034.x (2001).
65. Fernández, V. & Ebert, G. Foliar Iron Fertilization: A Critical Review. *Journal of Plant Nutrition* **28**, 2113–2124, https://doi.org/10.1080/01904160500320954 (2005).
66. Harpenslager, S. F., Lamers, L. P. M., van der Heide, T., Roelofs, J. G. M. & Smolders, A. J. P. Harnessing facilitation: Why successful re-introduction of Stratiotes aloides requires high densities under high nitrogen loading. *Biological Conservation* **195**, 17–23, https://doi.org/10.1016/j.biocon.2015.12.031 (2016).

Acknowledgements

Author Contributions

R.J.M.T., A.J.P.S., L.P.M.L. and M.M.L.v.K. designed the experiments. R.J.M.T., S.F.H., R.C.J.H.P. and G.v.D. performed the experiments. R.J.M.T. analyzed the data. R.J.M.T., S.F.H., A.J.P.S., G.v.D., L.P.M.L. and M.M.L.v.K. interpreted the results and co-wrote the paper.

Additional Information