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

Ralph J. M. Temmink1, Sarah F. Harpenslager3,3, Alfons J. P. Smolders2,1, Gijs van Dijk2,1, Roy C. J. H. Peters1, Leon P. M. Lamers1,2 & Monique M. L. van Kempen1

Azolla spp., a water fern often used for phytoremediation, is a strong phosphorus (P) accumulator due to its high growth rate and N2 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 ≥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 (≥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 ecosystems1,2. Azolla spp. are able to reach high growth rates by asexual reproduction and are very strong phosphorus (P) and nitrogen (N) accumulators3, which makes them very suitable for phytoremediation, bio-gas production, animal food and crop fertilization1,4,5. Due to their symbiosis with atmospheric nitrogen (N2) 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 azollae6,7 that forms unbranched, multi-cellular chains that contain both photosynthetic, vegetative cells and N2 fixing heterocysts8. Using nitrogenase enzymes, the diazotrophs reduce atmospheric N2 to ammonium (NH4⁺), which is then excreted into the Azolla leaf cavity and taken up by the fern9. In response to N-limitation, when there is no exogenous N available to Azolla, the heterocyst fraction increases in diazotroph chains9,10,11.

Given its high potential P uptake rates, luxury accumulation of P12,13 and high growth rates, Azolla may well be used for phytoremediation with respect to P14,15-17. The cultivation of Azolla spp. in P-enriched water could therefore offer a sustainable way of P recovery and recycling from eutrophic water18,19-22. Sustainable practices of P recycling are important, as global reserves of phosphate rock are becoming increasingly depleted23,24. Next to P recycling, Azolla cultivation may yield annually up to 35 t dry N-rich biomass, independent of N-fertilizer25. 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 water26. 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 growth26,27. Natural waters may

1Aquatic Ecology and Environmental Biology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525 AJ, Nijmegen, The Netherlands. 2B-WARE Research Centre, Toeronoield 1, 6525 ED, Nijmegen, The Netherlands. 3School 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)
well show non-optimal nutrient stoichiometry resulting in nutrient deficiency, impeded *Azolla* growth or even chlorosis\(^1\) due to P or Fe limitation\(^2\)\(^9\)\(^,\)\(^\)\(^2\)\(^8\).

High P concentrations in surface waters are well known to stimulate the growth of *Azolla* spp.\(^3\)\(^0\)\(^,\)\(^3\)\(^1\) and to increase N\(_2\) fixation rates of their symbionts.\(^3\)\(^1\)\(^–\)\(^3\)\(^3\). 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\(^1\)\(^,\)\(^2\)\(^8\).

**Results**

**Growth response to P availability.** RGRs of *A. filiculoides* growing in the 0.5 and 2 \(\mu\)mol 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 \(\mu\)mol P l\(^{−1}\) treatments increased their biomasses 2.2 times, whereas in 10, 50 and 100 \(\mu\)mol 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 \(\mu\)mol P l\(^{−1}\) treatments were smaller and more fragile than those from the 10, 50 and 100 \(\mu\)mol P l\(^{−1}\) treatments. Furthermore, plants from the lowest two concentrations turned reddish, while plants from the 10 \(\mu\)mol P l\(^{−1}\) treatment stayed green. Plants from the 50 and 100 \(\mu\)mol 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 \(\mu\)mol P l\(^{−1}\) treatment (Fig. 3a). Plant N content was stable over time, and highest at 50 \(\mu\)mol P l\(^{−1}\). Lowest N content was found for the 0.5 and 2 \(\mu\)mol P l\(^{−1}\) treatments (Fig. 2b). P concentrations \(\geq 10 \mu\)mol 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 (\(>10 \mu\)mol l\(^{−1}\)) compared to the 0.5 and 2 \(\mu\)mol 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 \(\mu\)mol P l\(^{−1}\) treatment, and the lowest in the 50 and 100 \(\mu\)mol 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 \(\mu\)mol 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 \(\mu\)mol 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 \(\mu\)mol N g\(^{−1}\) DW) and lowest in the control (2125 \(\pm\) 20 \(\mu\)mol N g\(^{−1}\) DW; Fig. 4b). Plants treated with N showed an intermediate N content of 2529 \(\pm\) 18 \(\mu\)mol N g\(^{−1}\) DW (Fig. 4b and Supplementary Table S3).
Table S4). Compared to both the control and the N treatment, the Fe content \(55.8 \pm 8 \mu\text{mol Fe g}^{-1} \text{DW}\) 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.
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. However, higher RGRs may be obtained when growing *Azolla* under optimal conditions using synthetic media in aquaculture. 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.

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 diazotrophs 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. Based on their N: P ratios, *A. filiculoides* grown at P levels ≤ 2 µmol l\(^{-1}\) were severely P-limited, which was also indicated by their red color. 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. 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 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.
In natural environments, eutrophication usually includes increased loading of both N and P. Presence of exogenous N influences both N₂ 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 growth⁴₇. 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 layer²⁷,₄₈. 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 literature¹. 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 available²,₂³. 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 unavailable¹⁹–₂¹. The presence of P can also affect plant Fe availability⁴₄,⁵₂,⁵₃. Interaction of P and Fe leading to Fe chlorosis can be caused by an internal immobilization of Fe probably due to formation of Fe-PO₄³⁴–₅₇.

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 economy¹₂,²⁴ 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 sediment²⁷,₄₈, 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 NaH₂PO₄ *•* 2H₂O). 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 days²⁷, the plants were pretreated for 10 days in larger basins without harvesting to minimize P history effects¹². Nutrient solutions were replaced 3 times a week. After this period¹³, 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⁻³), which was continuously fed by the same nutrient solution (pH = 7.3) as during the acclimatization period, at a rate of 11 d⁻¹ using peristaltic pumps (Masterflex L/S; Cole-Parmer, 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. Also, Fe is often a limiting element for *Azolla* because it is an essential component of nitorgenase\(^{1,28}\). Chlorosis induced by iron deficiency, however, does not always implicate low total Fe contents in plant tissue\(^{29–62}\), because Fe may be immobilized in the apoplastic 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\(^{55,56,61,65}\). To test N limitation, increasing both NO\(_3\)\(^{-}\) and NH\(_4\)\(^{+}\) in the medium is the optimal approach\(^{66}\). 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 (ANOVA) 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 square root transformed to authorize the use of parametric analyses. Linear mixed models (LMMs) were used to analyze differences in RGR, N and P sequestration rates and plant nutrient analyses.

**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*. (Lewis, New York, 2004).
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., Enth, B. & McKay, D. B. The cyanobiont in an *Azolla* fern is neither *Anabaena* nor *Nostoc*. EMS Microbiology Letters 229, 43–47, https://doi.org/10.1016/j.emslre.2003.09.007 (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 fixation on nitrogen fixation and nitrocytosis frequency of cyanobacterium *Anabaena azollae* in 7 species of *Azolla*. Biochemie und Physiologie der Pflanzen 185, 429–433, https://doi.org/10.1007/s00015-0079(89)00068-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. Foroni, 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., Carrapiço, F., and Pereira, A. L. *Azolla-anabaena* 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/s12737-012-0010-5 (2012).
17. Tang, Y. et al. Aquatic macrophytes can be used for wastewater polishing but not for purification in constructed wetlands. *Biogesosciences* 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. Choudhary, A. T. M. & 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/10.1007/BF02874724 (2004).
22. Bhuvaneshwari, K. & Singh, P. K. Response of nitrogen-fixing water fern Azolla biofiltration to rice crop. *Biotrop* 5, 523–529, https://doi.org/10.13205/biotrop5.04-02518 (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, 428–443 (2010).
24. Carpenter, R. S. & Bennett, E. M. Reconsideration of the planetary boundary for phosphorus. *Environmental Research Letters* 6, 140009–140009 (2011).
25. 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).
26. Zak, D., Wagner, C., Payer, B., Augustin, J. & Gelbrecht, J. Phosphorus mobilization in rewetted fens: the effect of altered peat hydrology. *Hydrobiologia* 169, 313–318 (1988).
27. Cary, P. R. & Weerts, P. G. J. Growth and nutrient composition of Azolla pinnata R. Brown and Azolla filiculoides Lam. as affected by water temperature, nitrogen and phosphorus supply, light intensity and pH. *Aquatic Botany* 43, 163–180 (1992).
28. Koshgarian, H. & Koyro, H.-W. Apoplastic accumulation of iron in the epidermis of maize (Zea mays) roots grown in calcareous soil. *Plant Physiology* 113, 515–522, https://doi.org/10.1104/pp.113.3054.2011.1130410.x (2001).
29. Rediske, J. H. & Biddulph, O. The Absorption and Translocation of Iron. *Plant Physiology* 28, 576 (1953).
30. 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/cr090112r (2009).
31. Singh, R. P. & Singh, P. K. Symbiotic algal nitrogenase activity and heterocyst frequency in seven Azolla species after phosphorus fertilization. *Aquatic Botany* 51, 27–37 (1996).
32. Cary, P. R. & Weerts, P. G. J. Growth and nutrient composition of Azolla pinnata R. Brown and Azolla filiculoides Lam. as affected by water temperature, nitrogen and phosphorus supply, light intensity and pH. *Aquatic Botany* 43, 163–180 (1992).
33. Koshgarian, H. & Koyro, H.-W. Apoplastic accumulation of iron in the epidermis of maize (Zea mays) roots grown in calcareous soil. *Plant Physiology* 113, 515–522, https://doi.org/10.1104/pp.113.3054.2011.1130410.x (2001).
34. Rediske, J. H. & Biddulph, O. The Absorption and Translocation of Iron. *Plant Physiology* 28, 576 (1953).
35. 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/cr090112r (2009).
36. Koshgarian, H. & Koyro, H.-W. Apoplastic accumulation of iron in the epidermis of maize (Zea mays) roots grown in calcareous soil. *Plant Physiology* 113, 515–522, https://doi.org/10.1104/pp.113.3054.2011.1130410.x (2001).
37. Rediske, J. H. & Biddulph, O. The Absorption and Translocation of Iron. *Plant Physiology* 28, 576 (1953).
38. 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/cr090112r (2009).
39. Kim, H. & Jeong, B. J. Iron acquisition and utilisation in the rhizosphere. *Compt. rend. trav. lab. Carlsberg, Ser. chin* 21, 15–52 (1935).
40. Mengel, K. Iron availability in plant tissues-iron chlorosis on calcareous soils. *Plant Physiology* 113, 515–522, https://doi.org/10.1104/pp.113.3054.2011.1130410.x (2001).
41. Rediske, J. H. & Biddulph, O. The Absorption and Translocation of Iron. *Plant Physiology* 28, 576 (1953).
42. 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/cr090112r (2009).
43. 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).
44. 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/10.1080/0190416009382129 (2000).
45. Fageria, N. K. Nutrient interactions in crop plants. *Journal of Plant Nutrition* 24, 1269–1290, https://doi.org/10.1081/PLN-100106981 (2001).
46. Olsen, C. Iron absorption and chlorosis in green plants. *Compt. rend. trav. lab. Carlsberg, Ser. chin* 21, 15–52 (1935).
47. Rajase, M. & Tavakoly, A. R. Iron and/or acid foilar 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/10.1080/01904167.2013.1382523 (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
The authors would like to thank Jelle Eygensteyn, Paul van der Ven and Sebastian Krosse for their help with chemical analyses. Furthermore, we thank Gerard van der Weerden for the use of the experimental greenhouse facilities. RJMT, SFH and MMLvK were supported by the European Union GO ERDF 2007–2013 (Water-Rijk, Rich Water World). RJMT was also supported by TTW-Open Technology Program grant “Bridging Thresholds” (#14424).

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
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-22760-5.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018