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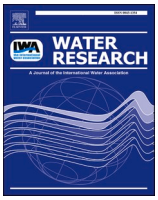

Azolla cultivation enables phosphate extraction from inundated former agricultural soils

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ABSTRACT

To combat the global loss of wetlands and their essential functions, the restoration and creation of wetlands is imperative. However, wetland development is challenging when soils have been in prolonged agricultural use, often resulting in a substantial nutrient legacy, especially of phosphorous (P). Inundating these soils typically leads to P mobilization, resulting in poor water quality and low biodiversity recovery. As a potential novel means to overcome this challenge, we tested whether cultivation of the floating fern *Azolla filiculoides* could simultaneously extract and recycle P, and provide a commercial product. *Azolla* has high growth rates due to the nitrogen fixing capacity of its microbiome and is capable of luxury consumption of P. *Azolla* cultivation may also accelerate soil P mobilization and subsequent extraction by causing surface water anoxia and the release of iron-bound P. To test this approach, we cultivated *Azolla* on 15 P-rich former agricultural soils in an indoor mesocosm experiment. Soils were inundated and either left unvegetated or inoculated with *A. filiculoides* during two 8-week cultivation periods. Biomass was harvested at different intervals (weekly/monthly/bimonthly) to investigate the effect of harvesting frequency on oxygen (O_2) and nutrient dynamics. We found that *Azolla* attained high growth rates only on soils with high mobilization of labile P, as plant cover did not reduce surface water O_2 concentrations in the first phase after inundation. This concurred with low porewater iron to P ratios (<10) and high porewater P concentrations. *A. filiculoides* cultivation substantially reduced surface water nutrient concentrations and extracted P at rates up to 122 kg ha^{-1} yr^{-1}. We conclude that rapid P extraction by *A. filiculoides* cultivation is possible on soils rich in labile P, offering new perspectives for wetland rehabilitation. Additional field trials are recommended to investigate long-term feasibility, seasonal variations, and the influence of potential grazers and pathogens.

1. Introduction

Human impacts have led to the loss of an estimated 21 % of global inland wetlands between 1700 and 2020, with extreme losses (>75 %) occurring in multiple European and Asian countries (Fluet-Chouinard et al., 2023). As wetlands and their biodiversity deteriorate or disappear entirely, so do essential wetland ecosystem functions, including carbon (C) sequestration, water retention, and water purification (Acreman and Holden, 2013; Albert et al., 2021; Zedler and Kercher, 2005). Therefore, the restoration and novel creation of wetlands is imperative to ensure water safety, C sequestration, and biodiversity conservation, especially in light of rapid global change (Mitsch et al., 2013; Moomaw et al., 2018; Trenberth, 2011). However, creating or restoring wetlands on soils that have been in prolonged agricultural use can be severely constrained by high soil nutrient contents, especially of phosphorous (P) (Kreyling et al., 2021; Pfadenhauer and Klötzli, 1996). Wetland development by rewetting or inundation generally leads to the mobilization of labile and iron-bound P to the surface water (Hooda et al., 2000; Pant and Reddy, 2003; Zak and Gelbrecht, 2007). The resulting high nutrient loads lead to algal and cyanobacterial blooms, poor water quality, and, generally, a low biodiversity, both locally and downstream of inundated areas (Surridge et al., 2012). This also leads to downstream losses of P, which is a valuable and finite resource (Edixhoven et al., 2014).

Nutrient removal prior to nature development on former agricultural soils is commonly achieved by top soil removal (Harpenslager et al., 2015; Quadra et al., 2023; Smolders et al., 2008; Zak et al., 2017).

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However, this is a highly invasive method which can have adverse effects on C storage and microbial activity, and can still lead to P mobilization at the site where the soil is deposited (Daun et al., 2023; Geissen et al., 2013). Depending on the depth and the fate of the removed soil, it can also be a costly procedure (Klimkowska et al., 2010). A novel, less invasive, and cost-efficient approach for rapid P removal and recovery from inundated former agricultural soils may be the cultivation of *Azolla filiculoides*. *Azolla* is a genus of floating ferns that is capable of fixing atmospheric nitrogen (N) through a symbiosis with the cyanobacterium *Nostoc/Anabaena Azollae* (Peters and Meeks, 1989). Its growth and P uptake is therefore not affected by N availability, which is often limiting after inundation due to high rates of denitrification and anaerobic ammonium oxidation (Geurts et al., 2020). As a result, *Azolla* spp. can grow extremely fast, with a doubling time of as few as two days (Wagner, 1997). *Azolla* spp. are efficient accumulators of both P and N (Peeters et al., 2016; Temmink et al., 2018), resulting in their widespread use as a biofertilizer in rice systems (Mandal et al., 1999) and in phytoremediation of eutrophic water (Forni et al., 2001; Rezania et al., 2021). *Azolla* biomass is protein rich and can also be used as animal feed or, potentially, human food, depending on its chemical content (e.g. heavy metal concentrations) (Brouwer et al., 2014; Sela et al., 1989).

Azolla growth is often limited by P availability, with optimal surface water concentrations of $\geq$10 μmol P L^{-1} and a plant N:P quotient below 20 mol mol^{-1} at which growth limitation occurs (Temmink et al., 2018). If P availability is high, *Azolla filiculoides* can rapidly cover the water surface with dense mats (Pieterse et al., 1977; Sheppard et al., 2006). These mats can hamper oxygen (O_2) diffusion and thereby cause O_2 depletion of the underlying surface water (Pinero-Rodríguez et al., 2021). Surface water O_2 depletion and concurrent anoxia at the sediment-water interface, can increase soil P mobilization through the release of Fe-bound P (Smolders et al., 2006), thereby potentially enhancing P availability and concomitant P extraction rates by *Azolla*.

Besides P, *Azolla* requires the (micro)nutrients iron (Fe), potassium (K), calcium (Ca), magnesium (Mg), molybdenum (Mo), cobalt (Co), sulphur (S), and zinc (Zn) (Peters et al., 1980; Sadeghi et al., 2013; Widiastuti and Davis, 2020). Furthermore, *Azolla* spp. grow optimally at a pH between 4.5 and 7.0, with a higher pH leading to reduced N fixation rates (Dawar and Singh, 2001; Nickell, 1958). Accordingly, P extraction using *Azolla* may not be possible on all P-rich soils. Additionally, nutrient deficiencies and other environmental stressors can lead to lower protein and higher condensed tannin contents in *Azolla* (Nham Tran et al., 2020), thereby impacting its protein availability and potential use as an animal feed.

In this study, we investigate the potential of *Azolla filiculoides* (hereafter, '*Azolla*') cultivation as novel, cost-efficient eco-tool to 1) extract and recycle P from P-saturated agricultural soils after inundation, 2) accelerate P extraction by causing surface water anoxia and the subsequent release of iron-bound P, and 3) provide a high-grade, economically interesting product (figure S1). To test this concept, we carried out a mesocosm experiment using 15 agricultural soils designated for wetland development. Specifically, we investigate 1) which soil indicators can be used to determine the suitability for P extraction using *Azolla*, 2) how different soils affect chemical composition and protein availability of *Azolla* (tannin:protein ratio), and 3) how different harvesting regimes can prevent overcrowding and affect nutrient and oxygen dynamics in the surface water. We hypothesize that *Azolla* growth and P sequestration is positively linked to P mobilization potential, which we defined by a low Fe:P ratio and high porewater P concentration (Geurts et al., 2010). We hypothesize that protein availability is positively linked to the growth rate (i.e., that non-stressed, fast growing *Azolla* has a lower tannin:protein ratio). We expect that a higher harvesting frequency leads to higher P extraction rates, by preventing *Azolla* overcrowding. Lastly, we expect that the presence of an *Azolla* mat will lead to surface water O_2 depletion and the concomitant release of Fe-bound P.

2. Materials & methods

2.1. Soil collection

Soils were collected from 15 sampling sites in the Netherlands. These sites were located within five main locations, in which two to four differentiating sites (different plots) were selected (represented by the letters a-d in the results): 'Allemanskamp' (52°03′27″N 5°33′50″E; 'AK', n = 4), 'Brouwketel' (51°55′14″N 5°58′25″E; 'BK'; n = 2), 'Leegveld' (51°24′31″N 5°51′23″E; 'LV'; n = 3), 'De Vlotter' (52°31′20″N 4°38′40″E; 'VL'; n = 2), and 'EVZ de Run' (51°22′39″N 5°23′37″E; 'EV'; n = 4) (table S1). In all locations excluding BK, a transition from agriculture to nature development, involving a rise in water table, was planned. BK was located in a floodplain, and was flooded at least once a year during high discharge events. At the moment of sampling, these plots were either in agricultural use or agricultural activity had ceased recently (<1 year before sampling). All soils had a high expected P mobilization potential based on high Olsen P (>2000 μmol L^{-1} FW), and low Fe (<100 mmol L^{-1} FW) and Ca (<50 mmol L^{-1} FW) content in the upper 20 cm (data collected in preceding sampling campaigns). After removing vegetation and the majority of roots (if present) we collected the topsoil (upper 20 cm) with a spade. Soil was stored in plastic bags at 4 °C until processing.

2.2. Experimental setup

Each soil was homogenized using a concrete mixer. Then, three mesocosms (30 L black HDPE containers, diameter 33 cm, height 42 cm) were filled with 16 L (20 cm) of soil, resulting in a total of 45 mesocosms. Rainwater was gently added to each mesocosm to a level of 18 cm above the soil surface. All mesocosms were placed in the Radboud University greenhouse facilities, with light conditions (irradiance) maintained at 186 Wm^{-2} or higher for 16 h per day, using grow lights if sunlight was not sufficient. After inundation, mesocosms were covered loosely with opaque plastic covers to prevent algae growth but still allow gas exchange. Rainwater was added weekly to compensate for evaporation, keeping the water level at +18 cm.

The experiment comprised two *Azolla* cultivation periods (Fig. 1). After a stabilization period of three weeks (experimental day 0), the first cultivation period started. Three different treatments were applied to each soil: two mesocosms per soil were inoculated with 30 g of *Azolla filiculoides*, cultivated in the same greenhouse, which was either harvested each week if 100 % coverage was reached ("weekly harvested" treatment) or harvested only at the end of the cultivation period (after 8 weeks; "bimonthly harvested" treatment). The third mesocosm was used as an unplanted control, with the plastic cover remaining to prevent algae growth ("control" treatment). After 8 weeks ("first cultivation"), all *Azolla* was harvested and plastic covers were placed back on all mesocosms to observe nutrient dynamics after *Azolla* removal ("inter-cultivation period").

After seven weeks (on day 104), the mesocosms were inoculated for the second cultivation period ("second cultivation"), similar to first cultivation period, with the exception that *Azolla* in the second treatment was harvested monthly instead of bimonthly ("monthly harvested" treatment), i.e. in week 4 and 8 of the second cultivation.

To prevent macro-ion limitation of *Azolla* due to the use of rainwater, we added a stock solution to increase mesocosm surface water concentrations with 500 μmol L^{-1} magnesium sulphate ($MgSO_4$), 1000 μmol L^{-1} calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), and 500 μmol L^{-1} potassium chloride (KCl). This was done on day 0 and repeated for KCl on day 29.

2.3. Soil sampling & analyses

Soil samples were collected at the start of the experiment, after homogenization. Prior to filling a mesocosm, approximately 50 g of soil was added to a plastic bag, resulting in three soil samples per soil.

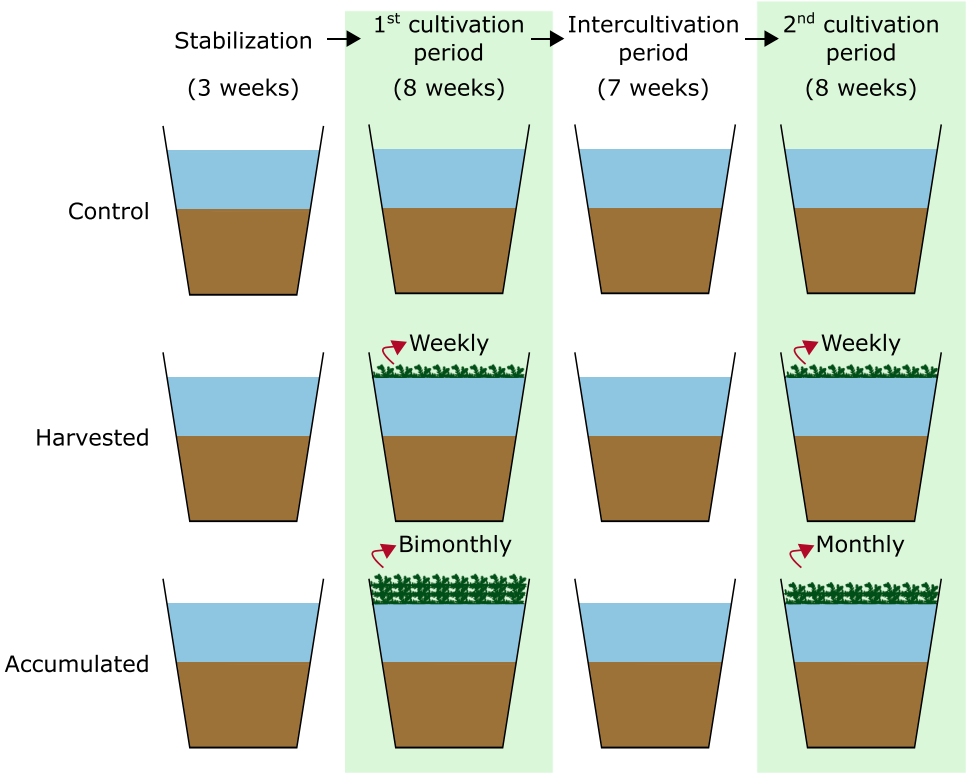

Fig. 1. Overview of the experimental setup with different treatments, periods, and harvesting frequencies. For each soil ($n = 15$), one of the three treatments was applied to one mesocosm ($n = 3$ mesocosms per soil, $n = 45$ mesocosms in total).

Samples were stored at 4 °C until further analyses.

To determine bio-available nutrient concentrations, extractions were carried out using 17.5 g of fresh soil, incubated with 50 mL of 0.2 M sodium chloride (NaCl, so called 'salt extractions'), after which pH of the supernatant was determined with an Ag/AgCl electrode (Orion Research, Beverly, MA, USA) (for details, see Tomassen et al., 2004). Bio-available P (Olsen-P) was measured by 30 min incubation of 3 g dried and ground soil in 60 mL 0.5 M sodium carbonate ($NaHCO_3$) (Henriksen, 1965). Amorphous Al and Fe and Fe/Al-bound P were determined after 2 h incubation with 50 mL of 0.11 M ammonium oxalate monohydrate, 0.9 M oxalic acid dehydrate, and fresh soil material equal to 5 g DW (Hooda et al., 2000). All incubations were carried out on a shaker (New Brunswick Scientific, Edison, US) at 105 RPM, followed by fluid extraction using Rhizon samplers (Rhizosphere Research Products B.V.) under vacuum. Extracts were stored at −20 °C (salt extracts) or 4 °C (all other extracts) until further analysis. Total phosphorous (TP) concentration was determined by digesting 200 mg soil in 4 mL HNO_3 (65 %) and 1 mL H_2O_2 (35 %) in Teflon vessels, heated in an Ethos I microwave for 1 h (Milestone, Sorisole Lombardy, Italy). After digestion, samples were samples were diluted to 100 ml with deionized water (ELGA LabWater, High Wycombe, UK) and stored at 4 °C until chemical analysis. Subsamples of fresh soil were dried at 70 °C for 48 h to determine dry weight and bulk density.

2.4. Water sampling & analyses

Surface water and pore water samples were taken approximately every other week (weekly in the beginning of each cultivation period). Surface water samples were taken from the upper 5 cm (*Azolla* root zone) using a syringe and were flushed through a coarse sieve (1 mm mesh) to remove *Azolla* roots. Pore water was extracted from the upper 10 cm of soil using Rhizon samplers attached to 60 mL syringes under vacuum. Water samples were divided in two fractions and stored at 4 °C (after adding 0.1 mL of 65 % HNO_3 to a 10 mL subsample) and at −20 °C until further analyses. Surface water O_2 concentrations and pH were determined every other week at 5 cm water depth using a Hach HQ40D portable multimeter (Hach, Loveland, CO, USA).

2.5. Biomass sampling and analyses

Azolla was harvested by hand and all biomass was gently placed in a mesh bag. This bag was closed with a string and was spun eight times to remove excess water without damaging the plants. The biomass was then weighed (fresh weight; FW) and, in the case of the harvested or monthly accumulated treatment, separated: 30 g was gently placed back in the mesocosm, and the remainder was dried at 70 °C for four days. After drying, dry weight (DW) was determined and samples were ground to a fine powder in a ball mill (Mixer Mill 301, Retsch GmbH, Haan, Germany). TP and total potassium (TK) contents were determined after microwave digestion as described above. Total nitrogen (TN) and total carbon (TC) were measured by an elemental CNS analyser (Vario Micro Cube, Elementar, Langenselbond, Germany) using 3 mg dried plant material. Relative growth rates (RGR) (g g^{-1} DW d^{-1}) and doubling times (DT) (d) were calculated from the biomass increases according to Kimani et al. (2020).

On day 35, biomass samples of approximately 1 g FW were frozen for colorimetric assays to estimate tannin:protein ratio, which was used as a proxy for digestibility, with a higher ratio indicating a lower digestibility (Brouwer et al., 2018). Prior to analysis, *Azolla* samples were dried overnight at 60 °C and subsequently homogenized with a mortar and pestle. Tannin content was determined using the acid butanol assay (Güngör et al., 2021): 1.2 ml of butanol-HCl solution (5 % v/v) and 10 µl of 2 % $NH_4Fe(SO_4)_2$ in 2 M HCl was added to about 0.5–2 mg of dried biomass, incubated for 15 min. at 95 °C, spun down, and placed on ice before the absorption was measured at 550 nm. Protein content was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions: briefly, 0.5–2 mg of dried biomass was incubated with reagent for 10 min. at 37

°C, centrifuged and placed on ice before the absorption was measured at 562 nm. Wine tannins and bovine serum albumin were used for calibration curves. The average of three technical replicates was expressed in %-equivalent and divided to evaluate the amount of tannins per protein (tannin:protein ratio).

2.6. Chemical analyses

NH_4^+, NO_3^-, and PO_4^{3-} concentrations were determined by colorimetric methods (Auto Analyser III, Seal Analytical GmbH, Norderstedt) in the water samples that were stored at −20 °C (Geurts et al. 2008). NH_4^+ was measured based on the Berthelot reaction (Searle, 1984), NO_3^- according to the cadmium reduction method (Green et al., 1982), and PO_4^{3-} was measured according to the molybdenum blue method (Murphy and Riley, 1962). Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to measure concentrations of Al, Fe, Mn, P, and Zn (iCAP 6000, Thermo Fischer Scientific, Carlsbad, USA) in the samples that were stored at 4 °C (water samples, extractions, and digestions).

The degree of phosphorous saturation (DPS; in %) of the soils was calculated according to Van der Zee et al. (1990):

$$DPS = \frac{P_{ox}}{0.5 * (Al_{ox} + Fe_{ox})} * 100\%$$

With P_{ox}, Al_{ox} and Fe_{ox} being the concentrations of P, Al, and Fe obtained from oxalate extractions (mmol kg DW^{-1}).

PO_4^{3-} mobilization rates were calculated from surface water PO_4^{3-} concentrations, using the slope of the linear increase in the first weeks of the experiment (in the stabilization period, prior to *Azolla* cultivation).

We calculated an indicative duration to reduce soil Olsen-P concentrations to 1000 μmol L^{-1} FW using *Azolla* cultivation (in optimal conditions) by dividing soil P surplus (kg ha^{-1}) by *Azolla* P sequestration rate (kg P ha^{-1} yr^{-1}) for each soil. Soil P surplus was calculated based on the ratio between soil total P and Olsen-P:

$$P_{Surplus} = \left(TP - P_{Olsen,\ t1} * \frac{TP}{P_{Olsen,\ t0}}\right) * V$$

Where TP is total soil P in kg L^{-1} FW, $P_{Olsen,t1}$ is the target Olsen-P concentration in kg L^{-1} FW (equal to 1000 μmol L^{-1}), $P_{Olsen,t0}$ is the start Olsen-P concentration in kg L^{-1} FW, and V is the volume of topsoil (0–20 cm depth) in L ha^{-1}.

2.7. Statistical analyses

Average *Azolla* growth rate, P sequestration rate, and P content were compared between treatments (weekly, monthly or bimonthly harvest) using linear mixed models with soil as a random intercept, as this significantly lowered AIC values. Differences in these variables between both cultivation periods within the weekly harvested treatment were compared using paired samples *t*-tests.

To unravel the most important soil predictors without collinearity issues, a partial least squares regression (PLS) (e.g. Bodmer et al., 2020; Carrascal et al., 2009; Sobek et al., 2003) was performed. Soil variables DPS, Olsen-P, P mobilization rate, Al_{salt}, Fe_{salt}, Mn_{salt}, P_{salt}, pH_{salt}, Zn_{salt}, Fe:P_{salt}, Al_{PW}, Fe_{PW}, Mn_{PW}, P_{PW}, pH_{PW}, Zn_{PW}, and Fe:P_{PW} were used as predictors (average of all three treatments after 3 weeks of stabilization, prior to *Azolla* introduction), with 'salt' representing concentrations from NaCl extractions, and PW representing pore water concentrations. *Azolla* growth rate, P sequestration and tannin:protein were added as response variables. We only used data from the weekly harvested treatment (both cultivation periods) for these response variables, to avoid the impact of space limitation on *Azolla* growth rate which likely occurred in the other treatments. Skewness and min:max ratio were calculated for all variables, and if >2 or <0.1, respectively, variables (in this case PO_4^{3-} mobilization rate, Fe:P_{salt}, Fe:P_{PW}, Mg_{salt}, Zn_{salt} and Al_{PW}) were log-transformed prior to further analyses. Data was scaled to unit variance and mean centered prior to analysis. A model with two components was selected based on highest R^2 value. Values for variance importance for projections (VIPs) were extracted as a measure for predictor importance. Predictors with a VIP>1 were labelled as 'High influence', with a VIP between 0.8 and 1 as 'Medium influence' and with VIP<0.8 as 'Low influence' (Sobek et al., 2003). The explanatory variables with the highest absolute standardized coefficients extracted from these models were tested individually using linear regression. Relations between P mobilization rate and Fe:P_{PW}, P_{PW}, and DPS were tested using linear regression.

Differences in water chemistry (surface water O_2, NO_3^-, NH_4^+, PO_4^{3-}, and pH, and pore water P and NH_4^+) over time were compared using linear (mixed) models, with treatment, time, and their interaction as explanatory variables. We added soil as a random intercept and/or an auto-regressive moving average (ARMA) variance structure to correct for temporal auto-correlation, if this significantly lowered AIC values (tested with analysis of variance (ANOVA)). ANOVAs followed by Tukey post hoc tests were performed on the chosen models to assess differences between individual treatments and days. Model assumptions were verified by visual examination of residual plots. Variables surface water PO_4^{3-}, NO_3^-, and NH_4^+ and pore water P and NH_4^+ were log transformed to obtain normal distribution of residuals. A significance threshold of $p < 0.05$ was used. In-text values are reported as mean ± standard error for all soils, unless specified otherwise. Reported R^2 values are adjusted R^2. All statistical analyses and visualizations were performed using R Studio (version 4.0.3) and packages ggplot2, lsmeans, multcomp, nlme, psych, plsdepot and viridis.

3. Results

3.1. Driving factors of Azolla growth and composition on different soils

Soils varied greatly in chemical composition and available nutrients (table S1). Although all soils had high Olsen-P concentrations (>2000 μmol L FW^{-1}), porewater P concentrations after inundation varied widely, from 32 to 1154 μmol L^{-1} (table 1).

Azolla growth rates varied strongly between soils, with absolute growth rates ranging from 1.45 to 5.03 g DW m^{-2} d^{-1} in weekly, 1.74 to 4.65 g DW m^{-2} d^{-1} in monthly, and 0.40 to 3.31 g DW m^{-2} d^{-1} in bimonthly harvested treatments (Fig. 2). During the first cultivation period, absolute growth rates were higher in the weekly harvested than in the bimonthly harvested treatments ($p < 0.001$). During the second cultivation, average absolute growth rates were higher in the monthly harvested treatment than the weekly harvested treatment ($p < 0.001$). Average *Azolla* RGR was almost thrice as low in the bimonthly harvested than the weekly harvested *Azolla* ($p < 0.001$). RGR did not differ between weekly harvested and monthly harvested *Azolla* ($p = 0.60$). The highest growth rates were observed in all AK and LV soils, and in EVd. RGR in the weekly harvested treatment was lower in the second than in the first cultivation period (on average 33 % lower, $p < 0.001$), but absolute growth rates did not differ between the two periods ($p = 0.678$).

Extrapolated P sequestration rates by *Azolla* strongly varied and ranged between 3 and 122 kg ha^{-1} yr^{-1} (table S2). During the first cultivation, P sequestration rates were almost twice as low in the bimonthly than the weekly harvested treatment ($p = 0.002$). P sequestration rates were similar in the monthly and weekly harvested treatments in the second cultivation ($p = 0.497$). Harvesting frequency had no effect on *Azolla* P content in the first ($p = 0.601$) or second ($p = 0.923$) cultivation period, with P content ranging between 0.06 and 0.71 % DW. There was no effect of harvesting frequency on tannin:protein ratio ($p = 0.385$). Indicative duration of P extraction to reduce P-Olsen concentrations to 1000 μmol L^{-1} ranged from 4 to 384 years, depending on the soil and harvest frequency.

Azolla tannin:protein ratio was excluded from the PLS analysis as it

Table 1
Azolla growth rates and P extraction characteristics at different harvesting regimes on 15 different soils (P seq = *P* sequestration rate, P extr time = indicative duration of P extraction to 1000 μmol L^{-1} FW P-Olsen). The weekly harvested treatment includes both cultivation periods. Capital letters indicate the main sampling locations, a-d indicate individual plots within these sites.

Harvest frequency				Weekly				Monthly				Bimonthly			
Soil	P-Olsen (μmol L^{-1})	P_{PW} (μmol L^{-1})	Fe:P_{PW}	Growth rate (g m^{-2} d^{-1})	%P	P seq (kg ha^{-1} yr^{-1})	P extr time (yr)	Growth rate (g m^{-2} d^{-1})	%P	P seq (kg ha^{-1} yr^{-1})	P extr time (yr)	Growth rate (g m^{-2} d^{-1})	%P	P seq (kg ha^{-1} yr^{-1})	P extr time (yr)
AK a	4486	628	1.5	4.11	0.32	46.6	44	3.47	0.21	24.9	82	1.82	0.54	36.1	57
AK b	4642	544	3.2	3.67	0.27	36.5	59	4.48	0.22	35.9	60	3.31	0.41	49.3	43
AK c	3655	335	8.7	4.01	0.39	52.8	34	4.24	0.13	20.1	90	2.95	0.21	22.1	82
AK d	3725	1051	1.2	3.95	0.50	74.9	15	3.90	0.71	101.1	11	2.10	0.55	42.5	26
BK a	4184	42	4.3	1.97	0.10	9.7	194	3.69	0.07	9.6	197	0.95	0.17	6.0	313
BK b	5346	56	2.3	2.10	0.14	12.9	187	4.07	0.10	13.8	174	0.81	0.21	6.2	384
EV a	2443	19	146.3	1.45	0.08	5.5	126	1.84	0.06	3.1	220	1.20	0.09	3.9	177
EV b	2783	32	40.6	1.64	0.09	6.7	115	1.74	0.06	3.3	235	2.06	0.10	7.3	105
EV c	2214	90	22.0	1.62	0.09	6.9	72	2.11	0.07	5.0	99	1.91	0.09	6.4	78
EV d	3778	1154	1.1	3.61	0.66	85.7	16	3.79	0.63	88.2	15	2.20	0.58	46.5	29
LV a	3270	249	0.2	5.03	0.47	87.2	10	4.58	0.53	90.4	10	2.88	0.42	44.3	20
LV b	2060	557	0.3	4.80	0.66	113.1	5	4.55	0.70	122.2	4	2.88	0.52	54.6	9
LV c	5183	53	2.4	2.91	0.14	18.8	56	4.65	0.10	17.3	60	3.27	0.19	22.4	47
VL a	3209	587	0.2	2.15	0.29	24.9	50	3.60	0.26	34.4	36	0.68	0.34	8.4	147
VL b	3417	241	0.2	2.06	0.24	17.8	66	3.26	0.17	20.0	59	0.40	0.33	4.8	248

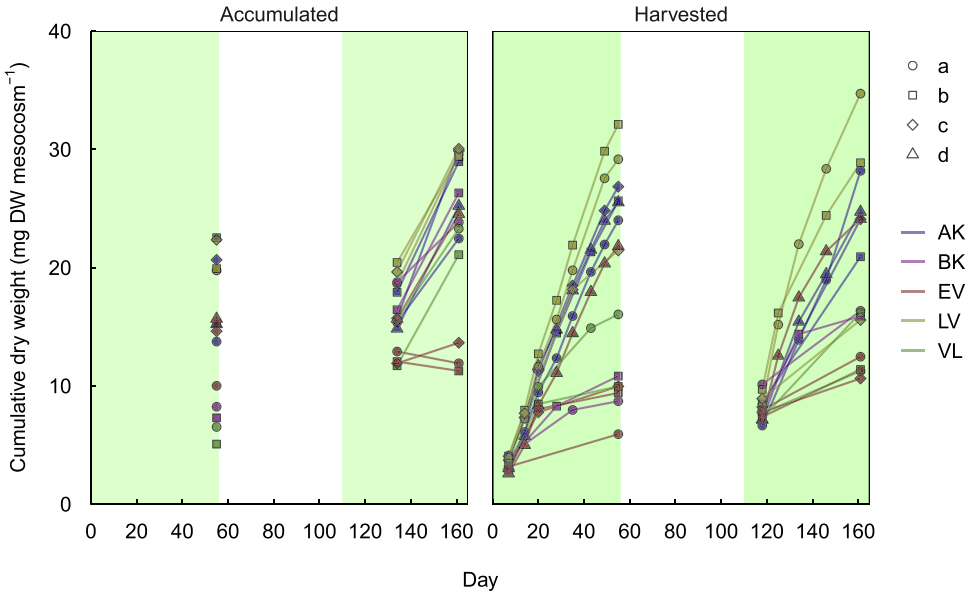

Fig. 2. Cumulative *Azolla* production of the accumulated (first cultivation harvested after 8 weeks, second cultivation harvested after 4 and 8 weeks) and the harvested (harvested weekly if 100 % cover in both periods) treatments. Green shaded areas indicate the period during which *Azolla* was present in the mesocosms: 30 g FW of *Azolla* was inoculated on days 0 and 104 and all *Azolla* was harvested on days 56 and 161. Capital letters indicate the main sampling locations, a-d indicate individual plots within these sites.

resulted in a negative predictive power (Q^2_{cum}) of the model, suggesting overfitting. The two components of the resulting PLS model, including *Azolla* absolute growth rate and P content, explained 78 % of the variance, with good predictive power (Q^2_{cum}= 0.519) (Fig. 3). Clustering of independent variables shows that soils with a high PO_4^{3-} mobilization rate had a high porewater P concentration and a low Fe:P_{PW} ratio – in

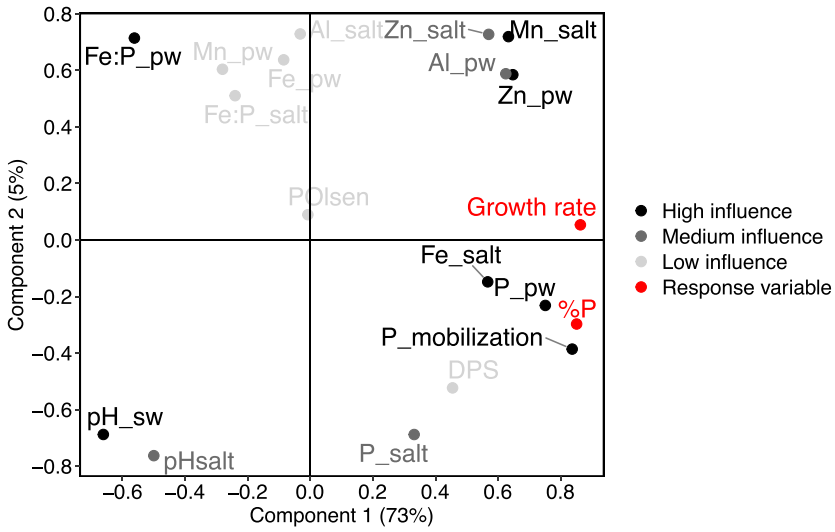

Fig. 3. PLS analysis of soil predictors for *Azolla* absolute growth rate and P content (%P) in the harvested treatment (both cultivation periods). Variables containing 'salt' were measured after salt extraction, whereas 'pw' indicates pore water, and 'sw' indicates surface water measurements. Predictors with a VIP>1 were labelled as 'High influence', with a VIP between 0.8 and 1 as 'Medium influence' and with VIP<0.8 as 'Low influence'.

line with our supposition that these variables are good indicators for P mobilization rates. The clustering also showed that soils with a high pH generally had low porewater and salt extractable Al, Mn, and Zn concentrations. *Azolla* growth rate and P content are positioned close to each other, suggesting they have similar drivers. Highly influential predictors (VIP>1) for these were Fe_{salt}, Mn_{salt}, P_{PW}, P mobilization rate, and Zn_{PW} (positive influence) and Fe:P_{PW}, and pH_{SW} (negative influence). Linear regression between response variables showed a positive relationship between *Azolla* growth rate and P content (R^2=0.63, p < 0.001) (Fig. 4A), and marginally significant negative relationships between tannin:protein ratio and growth rate (R^2=0.17, p = 0.071) and P content (R^2=0.19, p = 0.057). Linear regressions of the largest standardized coefficients (>0.14 or <−0.14) (table S3) showed a positive relationship between PO_4^{3-} mobilization rate and *Azolla* growth rate (R^2=0.58, p = 0.001), and P content (R^2=0.64, p < 0.001) (Fig. 4B, D). Furthermore, Fe:P_{PW} was negatively related to both growth rate (R^2=0.24, p = 0.038) and P content (R^2=0.32, p = 0.017) (Fig. 4C, E). P content was positively related to P_{PW} (R^2=0.61, p < 0.001) and Fe_{salt} (R^2=0.42, p = 0.006) (Fig. 4F, G).

3.2. Influence of Azolla cultivation on water chemistry

Surface water O_2 concentrations were relatively stable, fluctuating around 6.4 ± 0.4 mg L^{-1} in all treatments and soils (Fig. 5A). On day 42, O_2 concentrations in the accumulated treatment became significantly lower than the control (p < 0.001) and harvested treatment (p < 0.001). This trend continued, with concentrations in the accumulated treatment dropping to 2.7 ± 0.4 mg L^{-1} on day 54. After all *Azolla* was harvested, O_2 concentrations in the accumulated treatment increased, becoming similar to the other treatments on day 110. *Azolla* cultivation led to a slightly lower surface water pH than the control (average 6.8 ± 0.3) in both the harvested (6.5 ± 0.3, p < 0.001) and accumulated (6.3 ± 0.3, p < 0.001) treatments (figure S2A).

PO_4^{3-} mobilization to the surface water was substantial in most soils, with large variations between soils (Fig. 5B). On the day prior to *Azolla* inoculation, surface water PO_4^{3-} concentrations ranged from 0 to 99 μmol L^{-1} with an average of 44.2 ± 5.1 μmol L^{-1} on all soils. In the controls, six out of fifteen soils mobilized very high amounts of PO_4^{+} (>50 μmol L^{-1} in the surface water for at least one time point), whereas surface water concentrations of the remaining soils remained much lower throughout the experiment (<50 μmol L^{-1}). In three of the five EV soils, surface water PO_4^{3-} concentrations were relatively low (≤3 μmol L^{-1}) throughout the experiment in all treatments. In general, surface water PO_4^{3-} concentrations were very high compared to boundary values for good-moderate water quality used in the European Water Framework Directive (0.2 – 15.6 μmol L^{-1} TP; Poikane et al., 2019). After *Azolla* inoculation, surface water PO_4^{3-} concentrations dropped substantially becoming about three times lower than in control treatments. During the first cultivation, concentrations in the accumulated treatments became similar to the control on day 45 (p = 0.386). During the second cultivation, on day 145, concentrations decreased to below control values again (p < 0.001). In the harvested treatment, surface water PO_4^{3-} concentrations remained lower than the control during the first cultivation, rising to similar concentrations after harvest (p = 0.728). During the second cultivation, concentrations decreased significantly again after day 113 (p = 0.036), remaining low during the rest of the experiment. Porewater P concentrations varied per soil, with averages per soil ranging from 0 to 555 μmol L^{-1} (average 376 ± 59 μmol L^{-1}) at the start, but were not affected by *Azolla* cultivation throughout the experiment (p = 0.851) (figure S2B). PO_4^{3-} mobilization showed a positive relation with P_{PW} (R^2=0.54, p = 0.001) and a strong negative relation with Fe:P_{PW} (R^2=0.70, p < 0.001) (Fig. 6). A weaker, but significant positive correlation with DPS was also found (R^2=0.41, p = 0.006).

Surface water NO_3^- concentrations decreased significantly by *Azolla* cultivation. After *Azolla* inoculation, concentrations became lower in the controls than in the harvested (p = 0.005) and accumulated treatments (p = 0.012) (Fig. 5C). Concentrations in harvested treatments then remained low throughout the first cultivation and second cultivation, but increased to levels similar to the controls during the inter-cultivation period (day 72, p = 0.663). In accumulated treatments, concentrations during the first cultivation increased again to values similar to the control. During the second cultivation, concentrations dropped below control concentrations after day 113 (p = 0.002), remaining lower until the end of the experiment. Surface water NH_4^+ concentrations were similar in controls and harvested treatments (around 16 μmol L^{-1}), but were significantly lower in harvested treatments on day 6 (p = 0.013) and 16 (p = 0.002), and higher in the inter-cultivation period on day 72 (p = 0.003) (Fig. 5D). Concentrations rose in the accumulated treatment after day 56, becoming significantly higher than the harvested (p < 0.001) and control (p < 0.001) treatments. In the second cultivation period, concentrations did not differ significantly among the three treatments. *Azolla* cultivation did not affect pore water NH_4^+ concentrations (p = 0.589) (figure S2C).

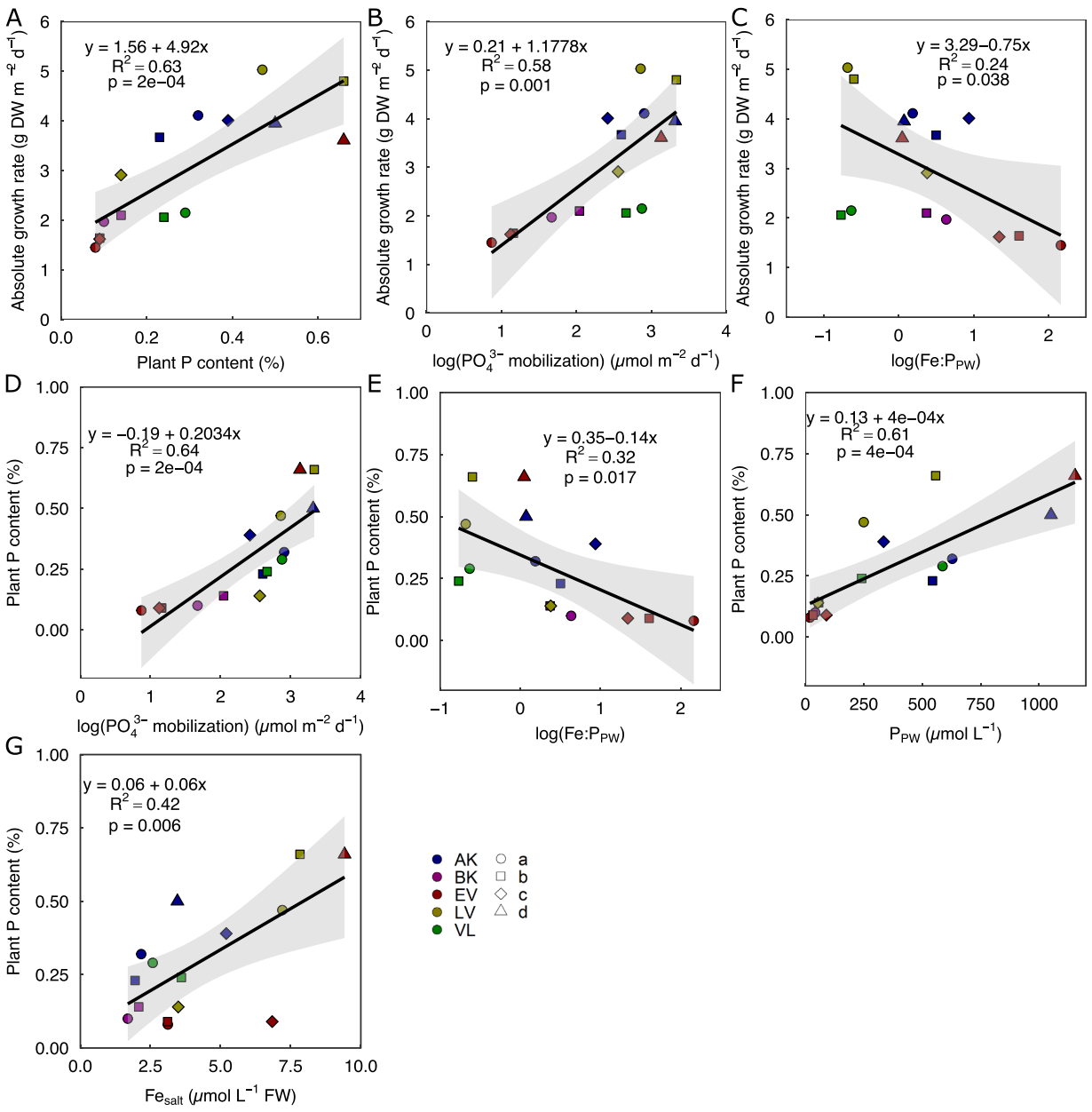

Fig. 4. Linear regressions between *Azolla* absolute growth rate (A-C) and P content (D-G) in the harvested treatment (both cultivation periods) and other response and explanatory variables. Black lines represent linear models; grey shaded areas represent 95 % confidence intervals. Capital letters indicate the main sampling locations, a-d indicate individual plots within these sites.

4. Discussion

P legacies in former agricultural soils hamper restoration and novel development of biodiverse wetlands. Topsoil removal, a commonly adopted method to avoid nutrient mobilization, can be invasive and often costly (Klimkowska et al., 2010). *Azolla* cultivation could be an alternative means to extract and recycle P from these soils while enabling biomass production. In this study using 15 agricultural soils, we show that *Azolla* can efficiently extract P and other nutrients when sufficient labile P is present in the soil. Contrary to our hypothesis, the presence of an *Azolla* mat did not lead to surface water O_2 depletion when *Azolla* was frequently harvested, implying that the release of iron-bound P was limited in the time span of this experiment. Optimal P extraction rates were attained with a weekly to monthly biomass harvest, avoiding *Azolla* P sequestration and overcrowding.

4.1. P mobilization rate in oxic conditions is a driving factor for Azolla growth

Azolla growth rates in weekly harvested treatments (RGR: 0.08–0.15 g^{-1} DW d^{-1}, doubling time 6.7 to 13.3 days) were similar to those observed in other laboratory studies using synthetic nutrient mixtures and similar temperatures (e.g. Cary and Weerts, 1992; Temmink et al., 2018; van Kempen et al., 2016). Contrary to our expectations, surface water O_2 depletion was observed only after six weeks of *Azolla* accumulation. This could be explained by a lower *Azolla* cover in harvested treatments, enabling O_2 diffusion into the water (Kosten et al., 2016). Additionally, space limitation in the bimonthly harvested treatment may have led to an increase in decaying plant material and accumulation of reactive organic matter on the sediment. Decaying plant material, consisting mostly of roots, was indeed observed on top of the sediment in all mesocosms. This decay could have led to a substantial increase in O_2

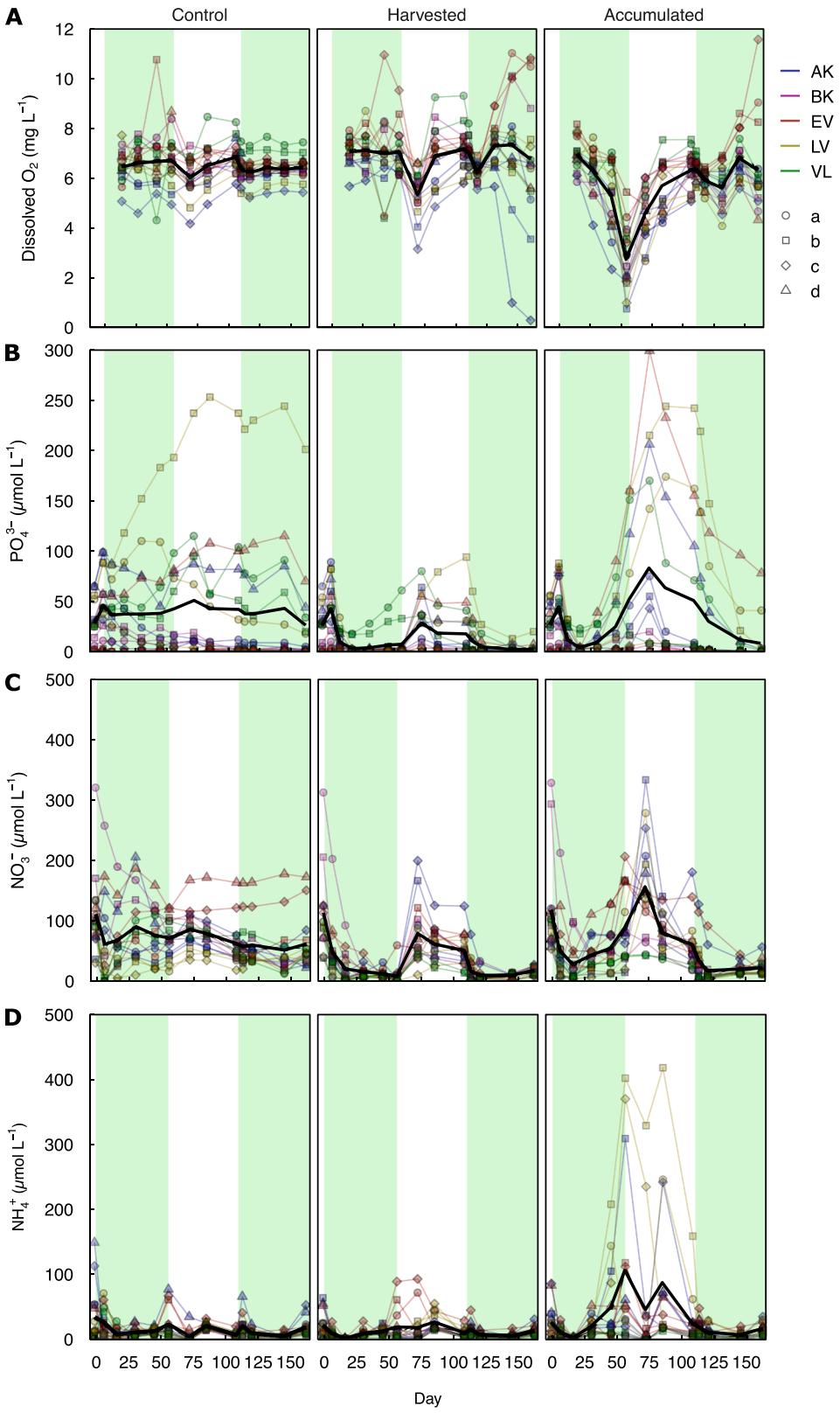

Fig. 5. Development over time of surface water dissolved O_2 (A), PO_4^{3-} (B), NO_3^- (C), and NH_4^+ (D) concentrations for 15 different soils. Black lines indicate average trends over all soils. Green shaded areas represent periods of *Azolla* cultivation in the harvested (weekly) and accumulated (monthly or bimonthly harvested) treatments (same area shown in the control panels without *Azolla* to facilitate comparison). Capital letters indicate the main sampling locations, a-d indicate individual plots within these sites.

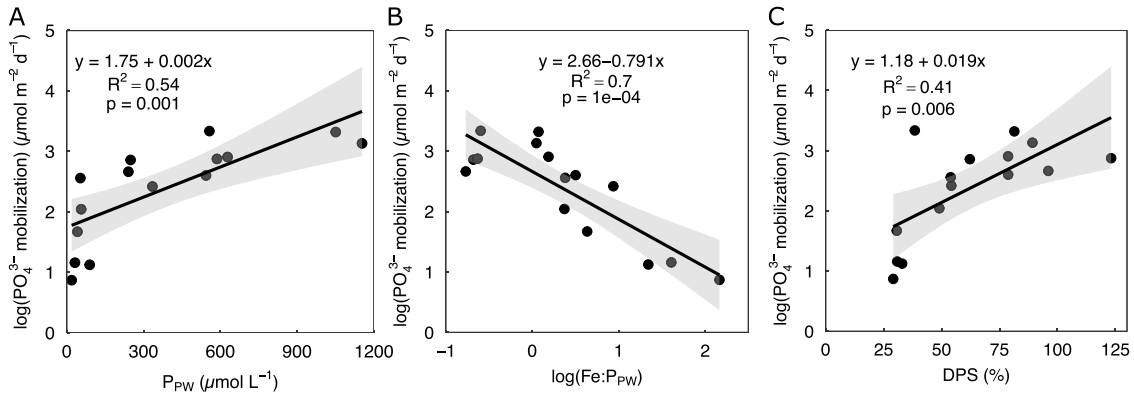

Fig. 6. Linear regressions of soil PO_4^{3-} mobilization and porewater P (A), porewater Fe:P (B), and DPS (C) in the harvested treatment (both cultivation periods). Black lines represent linear models; grey shaded areas represent 95 % confidence intervals.

consumption over time at the soil-water interface (as also observed in Aben et al., 2022), especially if soil respiration rates at the onset of inundation were low. The presence of O_2 at the sediment-water interface in the harvested treatments imply that the mobilization of Fe-bound P was limited, and thus not accelerated by *Azolla* cultivation.

As *Azolla* requires P for growth, it could grow successfully only on soils with high P mobilization rates – at least directly after inundation, when oxic conditions persist. This tends to happen only when porewater Fe:P ratios are low, and our threshold of Fe:$P < 10$ corroborates with earlier studies on soil P mobilization (Forsmann and Kjaergaard, 2014; Smolders et al., 2001; Zak et al., 2010). Furthermore, a sufficiently high porewater P concentration is necessary: in our study PO_4^{3-} mobilization was substantial at a porewater P above 100 μmol L^{-1}, with a linear increase in mobilization rates at higher concentrations. Therefore, *Azolla* growth rate and P content were negatively related to pore water Fe:P ratio, and positively to porewater P. Porewater P concentrations were extremely high in some soils (e.g. AKd and EVd), due to ongoing or only recent cessation of intensive agricultural activity and fertilization resulting in very high labile P contents. In soils with a high Fe:P ratio and/or low porewater P concentration, a typical red discoloration of *Azolla* was observed, also suggesting P limitation (Wagner, 1997). When soil P mobilization potential is low, P sequestration using rooted vegetation (e.g. *Typha latifolia* or *Phragmites australis*) may prove more efficient (Ren et al., 2019; Vroom et al., 2018). Alternatively, when P mobilization potential is high, a polyculture of Azolla with emergent vegetation might accelerate P extraction.

In our study, *Azolla* tannin:protein content, related to its digestibility, showed a near-significant negative link with growth rate and P content, which suggests that P-rich *Azolla* has a higher digestibility. This is in line with earlier findings showing a lower tannin:protein ratio in *Azolla* that did not suffer from nutrient stress (Nham Tran et al., 2020), and would suggest that fast-growing, P rich *Azolla* has a higher suitability as an animal feed.

4.2. *Azolla cultivation requires sufficient Fe*

We observed two soils ("VLa" and "VLb") in which PO_4^{3-} mobilization rates were high, but growth and P sequestration were low. These soils also had a low Fe_{PW} and the *Azolla* had the lowest biomass Fe content, and showed the typical yellow chlorosis indicating Fe deficiency (Temmink et al., 2018). Surface water Fe concentrations were below 0.9 μM, a threshold below which N fixation in *Azolla* can be limited, as Fe is an essential constituent of nitrogenase (Yatazawa et al., 1980). A significant, although not very strong, link between *Azolla* P content and Fe_{salt} further supports this mechanism. This shows that not only PO_4^{3-}, but also sufficient Fe mobilization is important for successful *Azolla* cultivation, as suggested by Temmink et al. (2018).

4.3. *Azolla cultivation successfully extracts nutrients from surface water when harvested*

The substantial decline in surface water NO_3^-, NH_4^+, and PO_4^{3-} when *Azolla* was harvested weekly or monthly corresponds with findings from *Azolla* grown on wastewater (Forni et al., 2001; Schuijt et al., 2021; Tang et al., 2017). However, when *Azolla* was not harvested, remobilization of nutrients from decaying *Azolla*, including additional N acquired by nitrogen fixation, took place. Furthermore, this decaying organic material probably caused the observed decline in dissolved O_2, in turn enhancing nutrient mobilization from the soil. Regular harvesting (in our case, at least every month) is therefore recommended to keep surface water nutrients at a minimum. Thus, to optimize surface water quality and prevent nutrient remobilization form decaying *Azolla*, harvesting frequency needs to be adapted to growth speed to prevent overcrowding.

In soils where nutrient conditions were suitable and *Azolla* growth rates were high (i.e. >4 g DW m^{-2} d^{-1}), extrapolated P sequestration rates between 17 and 122 kg ha^{-1} yr^{-1} were obtained, with an average of 54 kg ha^{-1} yr^{-1}. In these soils, indicative duration of P extraction down to 1000 μmol L^{-1} Olsen-P ranged from four years to several decades, of course depending on the initial Olsen-P concentration. Although in situ rates will also depend on light and temperature conditions year-round, our rates were comparable to potential P removal rates of *T. latifolia* (20–80 kg P ha^{-1}) and higher than those of *P. australis* (10–60 kg P ha^{-1}) stands (varying in age) on rewetted peat and mineral soils (Geurts et al., 2020). The removal rates for both species were limited by N availability, which would not be the case for *Azolla*. *Azolla* removal rates exceeded the rates of methods based on temporarily establishing potassium-fertilized grass-clover swards which are regularly mowed (34 kg P ha^{-1} yr^{-1}) on similar former agricultural mineral soils (Timmermans and van Eekeren, 2016). Rates are also comparable to P extraction rates based on agricultural cultivation and mowing of N-fertilized grass sward (28–50 kg ha^{-1} yr^{-1}) (van der Salm et al., 2009), with the considerable advantage that *Azolla* does not require additional N input.

4.4. *Implications and recommendations*

Our study shows that using *A. filiculoides* could be a feasible option for P extraction from soils in the process of wetland restoration or novel wetland creation. This approach could be scalable in space as well as time, and may serve as a transition-phase between agricultural use and the development of new nature on inundated fields. After sufficient P has been extracted by *Azolla* cultivation, we envision that natural wetland vegetation can develop in prevailing flooded conditions, or at a slightly lower water table (e.g. at soil surface level), depending on the target vegetation. To assess the suitability of a soil for P extraction by this method, simple analyses using wet soil incubations can be carried

out to determine P mobilization. Alternatively, DPS, calculated from oxalate-extractable P, Fe, and Al, also would be a reliable measure. However, additional field trials are strongly advised to unravel long-term and seasonal variations in *Azolla* yield, P extraction, and nutrient and O_2 dynamics, as well as the influence of potential grazers and pathogens (Sadeghi et al., 2013). Although we did not observe surface water O_2 depletion and subsequent enhanced P mobilization in regularly harvested *Azolla*, this may still occur in the longer term due to the accumulation of decaying organic matter (e.g. shed *Azolla roots*). Monitoring O_2 concentrations during a longer-term *Azolla* cultivation experiment is therefore advised. Additionally, we suggest comparing and especially combining *Azolla* with alternative vegetation types (e.g. emergent macrophytes) to elucidate optimal P extraction strategies on inundated former agricultural lands.

5. Conclusions

- Azolla cultivation is feasible on soils with a low porewater Fe:P ratio (<10), high porewater P concentrations (>100 μmol L^{-1}), and sufficient Fe mobilization.
- When frequently harvested, *Azolla* does not lower surface water O_2 concentrations in the first months after inundation, and therefore does not cause the release of Fe-bound P from the sediment-water interface .
- A weekly to monthly harvesting frequency is recommended for optimal P extraction rates and reduction of surface water N and P concentrations.
- *A. filiculoides* cultivation is a viable strategy for P extraction on inundated agricultural soils with potentially higher extraction rates than currently applied methods based on the cultivation of terrestrial vegetation.
- *Azolla* cultivation can therefore be used to create more suitable conditions for further wetland development on former agricultural lands.

CRediT authorship contribution statement

RJE Vroom: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **AJP Smolders:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **BP Van de Riet:** Supervision, Resources, Project administration, Conceptualization. **LPM Lamers:** Writing – review & editing, Funding acquisition, Conceptualization. **E Güngör:** Writing – review & editing, Investigation. **S Krosse:** Writing – review & editing, Investigation. **GM Verheggen-Kleinheerenbrink:** Investigation. **NR Van der Wal:** Investigation, Data curation. **S Kosten:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121411.

References

Aben, R.C.H., Velthuis, M., Kazanjian, G., Frenken, T., Peeters, E.T.H.M., Van de Waal, D. B., Hilt, S., de Senerpont Domis, L.N., Lamers, L.P.M., Kosten, S., 2022. Temperature response of aquatic greenhouse gas emissions differs between dominant plant types. Water Res 226, 119251. https://doi.org/10.1016/j.watres.2022.119251.

Acreman, M., Holden, J., 2013. How wetlands affect floods. Wetlands 33, 773–786. https://doi.org/10.1007/s13157-013-0473-2.

Albert, J.S., Destouni, G., Duke-Sylvester, S.M., Magurran, A.E., Oberdorff, T., Reis, R.E., Winemiller, K.O., Ripple, W.J., 2021. Scientists' warning to humanity on the freshwater biodiversity crisis. Ambio 50, 85–94. https://doi.org/10.1007/s13280-020-01318-8.

Bodmer, P., Wilkinson, J., Lorke, A., 2020. Sediment Properties Drive Spatial Variability of Potential Methane Production and Oxidation in Small Streams. J. Geophys. Res. Biogeosciences 125, 1–15. https://doi.org/10.1029/2019jg005213.

Brouwer, P., Bräutigam, A., Külahoglu, C., Tazelaar, A.O.E., Kurz, S., Nierop, K.G.J., van der Werf, A., Weber, A.P.M., Schluepmann, H., 2014. Azolla domestication towards a biobased economy? New Phytol 202, 1069–1082. https://doi.org/10.1111/nph.12708.

Brouwer, P., Schluepmann, H., Nierop, K.G.J., Elderson, J., Bijl, P.K., van der Meer, I., de Visser, W., Reichart, G.J., Smeekens, S., van der Werf, A., 2018. Growing Azolla to produce sustainable protein feed: the effect of differing species and CO2 concentrations on biomass productivity and chemical composition. J. Sci. Food Agric. 98, 4759–4768. https://doi.org/10.1002/JSFA.9016.

Carrascal, L.M., Galván, I., Gordo, O., 2009. Partial least squares regression as an alternative to current regression methods used in ecology. Oikos 118, 681–690. https://doi.org/10.1111/j.1600-0706.2008.16881.x.

Cary, P.R., Weerts, P.G.J., 1992. 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. Aquat. Bot. 43, 163–180. https://doi.org/10.1016/0304-3770(92)90041-G.

Daun, C., Huth, V., Gaudig, G., Günther, A., Krebs, M., Jurasinski, G., 2023. Full-cycle greenhouse gas balance of a Sphagnum paludiculture site on former bog grassland in Germany. Sci. Total Environ. 877, 162943 https://doi.org/10.1016/j.scitotenv.2023.162943.

Dawar, S., Singh, P., 2001. Growth, nitrogen fixation and occurrence of epiphytic algae at different pH in the cultures of two species of Azolla. Biol. Fertil. Soils 34, 210–214. https://doi.org/10.1007/s003740100374.

Edixhoven, J.D., Gupta, J., Savenije, H.H.G., 2014. Recent revisions of phosphate rock reserves and resources: a critique. Earth Syst. Dyn. 5, 491–507. https://doi.org/10.5194/esd-5-491-2014.

Fluet-Chouinard, E., Stocker, B.D., Zhang, Z., Malhotra, A., Melton, J.R., Poulter, B., Kaplan, J.O., Goldewijk, K.K., Siebert, S., Minayeva, T., Hugelius, G., Joosten, H., Barthelmes, A., Prigent, C., Aires, F., Hoyt, A.M., Davidson, N., Finlayson, C.M., Lehner, B., Jackson, R.B., McIntyre, P.B., 2023. Extensive global wetland loss over the past three centuries. Nature 614, 281–286. https://doi.org/10.1038/s41586-022-05572-6.

Forni, C., Chen, J., Tancionil, L., Grilli Caiola, M., 2001. Evaluation of the fern Azolla for growth, nitrogen and phosphorus removal from wastewater. Water Res 35, 1592–1598. https://doi.org/10.1016/S0043-1354(00)00396-1.

Forsmann, D.M., Kjaergaard, C., 2014. Phosphorus release from anaerobic peat soils during convective discharge - effect of soil Fe: p molar ratio and preferential flow. Geoderma 21–32. https://doi.org/10.1016/J.GEODERMA.2014.01.025, 223–225.

Geissen, V., Wang, S., Oostindie, K., Huerta, E., Zwart, K.B., Smit, A., Ritsema, C.J., Moore, D., 2013. Effects of topsoil removal as a nature management technique on soil functions. Catena 101, 50–55. https://doi.org/10.1016/j.catena.2012.10.002.

Geurts, J.J.M., Oehmke, C., Lambertini, C., Eller, F., Sorrell, B.K., Mandiola, S.R., Grootjans, A.P., Brix, H., Wichtmann, W., Lamers, L.P.M., Fritz, C., 2020. Nutrient removal potential and biomass production by Phragmites australis and Typha latifolia on European rewetted peat and mineral soils. Sci. Total Environ. 747, 141102 https://doi.org/10.1016/j.scitotenv.2020.141102.

Geurts, J.J.M., Smolders, A.J.P., Banach, A.M., van de Graaf, J.P.M., Roelofs, J.G.M., Lamers, L.P.M., 2010. The interaction between decomposition, net N and P mineralization and their mobilization to the surface water in fens. Water Res 44, 3487–3495. https://doi.org/10.1016/j.watres.2010.03.030.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126, 131–138. https://doi.org/10.1016/0003-2697(82)90118-X.

Güngör, E., Brouwer, P., Dijkhuizen, L.W., Shaffar, D.C., Nierop, K.G.J., de Vos, R.C.H., Sastre Toraño, J., van der Meer, I.M., Schluepmann, H., 2021. Azolla ferns testify: seed plants and ferns share a common ancestor for leucoanthocyanidin reductase enzymes. New Phytol 229, 1118–1132. https://doi.org/10.1111/nph.16896.

Harpenslager, S.F., van den Elzen, E., Kox, M.A.R., Smolders, A.J.P., Ettwig, K.F., Lamers, L.P.M., 2015. Rewetting former agricultural peatlands: topsoil removal as a

prerequisite to avoid strong nutrient and greenhouse gas emissions. Ecol. Eng. 84, 159–168. https://doi.org/10.1016/j.ecoleng.2015.08.002.
Henriksen, A., 1965. An automatic method for determining nitrate and nitrite in fresh and saline waters. Analyst 90, 83–88. https://doi.org/10.1039/AN9659000083.
Hooda, P.S., Rendell, A.R., Edwards, A.C., Withers, P.J.A., Aitken, M.N., Truesdale, V.W., 2000. Relating soil phosphorus indices to potential phosphorus release to water. J. Environ. Qual. 29, 1166–1171. https://doi.org/10.2134/jeq2000.00472425002900040018x.
Kimani, S.M., Kanno, T., Tawaraya, K., Cheng, W., 2020. Floating Azolla cover influences evapotranspiration from flooded water surfaces. Wetlands 40, 1425–1432. https://doi.org/10.1007/s13157-020-01282-9.
Klimkowska, A., Dzierza, P., Brzezińska, K., Kotowski, W., Medrzycki, P., 2010. Can we balance the high costs of nature restoration with the method of topsoil removal? Case study from Poland. J. Nat. Conserv. 18, 202–205. https://doi.org/10.1016/J.JNC.2009.09.003.
Kosten, S., Piñeiro, M., de Goede, E., de Klein, J., Lamers, L.P.M., Ettwig, K., 2016. Fate of methane in aquatic systems dominated by free-floating plants. Water Res 104, 200–207. https://doi.org/10.1016/j.watres.2016.07.054.
Kreyling, J., Tanneberger, F., Jansen, F., van der Linden, S., Aggenbach, C., Blüml, V., Couwenberg, J., Emsens, W.J., Joosten, H., Klimkowska, A., Kotowski, W., Kozub, L., Lennartz, B., Liczner, Y., Liu, H., Michaelis, D., Oehmke, C., Parakenings, K., Pleyl, E., Poyda, A., Raabe, S., Röhl, M., Rücker, K., Schneider, A., Schrautzer, J., Schröder, C., Schug, F., Seeber, E., Thiel, F., Thiele, S., Tiemeyer, B., Timmermann, T., Urich, T., van Diggelen, R., Vegelin, K., Verbruggen, E., Wilmking, M., Wrage-Mönnig, N., Wołejko, L., Zak, D., Jurasinski, G., 2021. Rewetting does not return drained fen peatlands to their old selves. Nat. Commun. 12, 1–8. https://doi.org/10.1038/s41467-021-25619-y.
Mandal, B., Vlek, P.L.G., Mandal, L.N., 1999. Beneficial effects of blue-green algae and Azolla, excluding supplying nitrogen, on wetland rice fields: a review. Biol. Fertil. Soils. https://doi.org/10.1007/s003740050501.
Mitsch, W.J., Bernal, B., Nahlik, A.M., Mander, Ü., Zhang, L., Anderson, C.J., Jørgensen, S.E., Brix, H., 2013. Wetlands, carbon, and climate change. Landsc. Ecol. 28, 583–597. https://doi.org/10.1007/s10980-012-9758-8.
Moomaw, W.R., Chmura, G.L., Davies, G.T., Finlayson, C.M., Middleton, B.A., Natali, S. M., Perry, J.E., Roulet, N., Sutton-Grier, A.E., 2018. Wetlands in a changing climate: science, policy and management. Wetlands 38, 183–205. https://doi.org/10.1007/s13157-018-1023-8.
Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta 27, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5.
Nham Tran, T.L., Miranda, A.F., Abeynayake, S.W., Mouradov, A., 2020. Differential production of phenolics, lipids, carbohydrates and proteins in stressed and unstressed aquatic plants, Azolla filiculoides and Azolla pinnata. Biology (Basel) 9, 342. https://doi.org/10.3390/biology9100342.
Nickell, L.G., 1958. Physiological studies with azolla under aseptic conditions. I. Isolation and preliminary growth studies. Am. Fern J. 48, 103. https://doi.org/10.2307/1545557.
Pant, H.K., Reddy, K.R., 2003. Potential internal loading of phosphorus in a wetland constructed in agricultural land. Water Res 37, 965–972. https://doi.org/10.1016/S0043-1354(02)00474-8.
Peeters, E.T.H.M., Neefjes, R.E.M., Van Zuidam, B.G., 2016. Competition between free-floating plants is strongly driven by previously experienced phosphorus concentrations in the water column. PLoS ONE 11, 1–18. https://doi.org/10.1371/journal.pone.0162780.
Peters, G., Meeks, J., 1989. The Azolla-anabaena symbiosis: basic biology. Annu. Rev. Plant Physiol. Plant Mol. BioI. 40, 193–210.
Peters, G., Toia, R., Evans, W., Crist, D., Mayne, B., Poole, R., 1980. Characterization and comparisons of five N2-fixing Azolla-Anabaena associations, I. Optimization of growth conditions for biomass increase and N content in a controlled environment. Plant. Cell Environ. 3, 261–269. https://doi.org/10.1111/1365-3040.ep11581825.
Pfadenhauer, J., Klötzli, F., 1996. Restoration experiments in middle European wet terrestrial ecosystems: an overview. Vegetatio 126, 101–115. https://doi.org/10.1007/bf00047765.
Pieterse, A.H., De Lange, L., Van Vliet, J., 1977. A comparative study of Azolla in the Netherlands. Acta Bot. Neerl. 26, 433–449.
Pinero-Rodríguez, M.J., Fernández-Zamudio, R., Arribas, R., Gomez-Mestre, I., Díaz-Paniagua, C., 2021. The invasive aquatic fern Azolla filiculoides negatively impacts water quality, aquatic vegetation and amphibian larvae in Mediterranean environments. Biol. Invasions 23, 755–769. https://doi.org/10.1007/s10530-020-02402-6.
Poikane, S., Kelly, M.G., Salas Herrero, F., Pitt, J.A., Jarvie, H.P., Claussen, U., Leujak, W., Lyche Solheim, A., Teixeira, H., Phillips, G., 2019. Nutrient criteria for surface waters under the European water framework directive: current state-of-the-art, challenges and future outlook. Sci. Total Environ. 695, 133888 https://doi.org/10.1016/J.SCITOTENV.2019.133888.
Quadra, G.R., Boonman, C.C.F., Vroom, R.J.E., Temmink, R.J.M., Smolders, A.J.P., Geurts, J.J.M., Aben, R.C.H., Weideveld, S.T.J., Fritz, C., 2023. Removing 10cm of degraded peat mitigates unwanted effects of peatland rewetting: a mesocosm study. Biogeochemistry 163, 65–84. https://doi.org/10.1007/s10533-022-01007-6.
Ren, L., Eller, F., Lambertini, C., Guo, W.Y., Brix, H., Sorrell, B.K., 2019. Assessing nutrient responses and biomass quality for selection of appropriate paludiculture crops. Sci. Total Environ. 664, 1150–1161. https://doi.org/10.1016/j.scitotenv.2019.01.419.
Rezania, S., Kamyab, H., Rupani, P.F., Park, J., Nawrot, N., Wojciechowska, E., Yadav, K. K., Lotfi Ghahroud, M., Mohammadi, A.A., Thirugnana, S.T., Chelliapan, S., Cabral-Pinto, M.M.S., 2021. Recent advances on the removal of phosphorus in aquatic plant-based systems. Environ. Technol. Innov. 24, 101933 https://doi.org/10.1016/j.eti.2021.101933.
Sadeghi, R., Zarkami, R., Sabetraftar, K., Van Damme, P., 2013. A review of some ecological factors affecting the growth of Azolla spp. CJES Casp. J. Environ. Sci. Casp. J. Env. Sci 11, 65–76.
Schuijt, L.M., van Bergen, T.J.H.M., Lamers, L.P.M., Smolders, A.J.P., Verdonschot, P.F. M., 2021. Aquatic worms (Tubificidae) facilitate productivity of macrophyte Azolla filiculoides in a wastewater biocascade system. Sci. Total Environ. 787, 147538 https://doi.org/10.1016/j.scitotenv.2021.147538.
Searle, P.L., 1984. The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. A review. Analyst 109, 549–568. https://doi.org/10.1039/AN9840900549.
Sela, M., Garty, J., Tel-Or, E., 1989. The accumulation and the effect of heavy metals on the water fern Azolla filiculoides. New Phytol 112, 7–12. https://doi.org/10.1111/j.1469-8137.1989.tb00302.x.
Sheppard, A.W., Shaw, R.H., Sforza, R., 2006. Top 20 environmental weeds for classical biological control in Europe: a review of opportunities, regulations and other barriers to adoption. Weed Res 46, 93–117. https://doi.org/10.1111/j.1365-3180.2006.00497.x.
Smolders, A.J.P., Lamers, L.P.M., Lucassen, E.C.H.E.T., Van Der Velde, G., Roelofs, J.G. M., 2006. Internal eutrophication: how it works and what to do about it - A review. Chem. Ecol. 22, 93–111. https://doi.org/10.1080/02757540600579730.
Smolders, A.J.P., Lamers, L.P.M., Moonen, M., Zwaga, K., Roelofs, J.G.M., 2001. Controlling phosphate release from phosphate-enriched sediments by adding various iron compounds. Biogeochemistry 54, 219–228. https://doi.org/10.1023/A:1010660401527/METRICS.
Smolders, A.J.P., Lucassen, E.C.H.E.T., van der Aalst, M., Lamers, L.P.M., Roelofs, J.G.M., 2008. Decreasing the abundance of juncus effusus on former agricultural lands with noncalcareous sandy soils: possible effects of liming and soil removal. Restor. Ecol. 16, 240–248. https://doi.org/10.1111/j.1526-100X.2007.00267.x.
Sobek, S., Algesten, G., Bergström, A.K., Jansson, M., Tranvik, L.J., 2003. The catchment and climate regulation of pCO2 in boreal lakes. Glob. Chang. Biol. 9, 630–641. https://doi.org/10.1046/j.1365-2486.2003.00619.x.
Surridge, B.W.J., Heathwaite, A.L., Baird, A.J., 2012. Phosphorus mobilisation and transport within a long-restored floodplain wetland. Ecol. Eng. 44, 348–359. https://doi.org/10.1016/j.ecoleng.2012.02.009.
Tang, Y., Harpenslager, S.F., Van Kempen, M.M.L., Verbaarschot, E.J.V., Loeffen, L.M.J. M., Roelofs, J.G.M., Smolders, A.J.P., Lamers, L.P.M., 2017. 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.
Temmink, R.J.M., Harpenslager, S.F., Smolders, A.J.P., Van Dijk, G., Peters, R.C.J.H., Lamers, L.P.M., Van Kempen, M.M.L., 2018. Azolla along a phosphorus gradient: biphasic growth response linked to diazotroph traits and phosphorus-induced iron chlorosis. Sci. Rep. 8, 3–10. https://doi.org/10.1038/s41598-018-22760-5.
Timmermans, B.G.H., van Eekeren, N., 2016. Phytoextraction of soil phosphorus by potassium-fertilized grass-clover swards. J. Environ. Qual. 45, 701–708. https://doi.org/10.2134/jeq2015.08.0422.
Tomassen, H.B.M., Smolders, A.J.P., Limpens, J., Lamers, L.P.M., Roelofs, J.G.M., 2004. Expansion of invasive species on ombrotrophic bogs: desiccation or high N deposition? J. Appl. Ecol. 41, 139–150. https://doi.org/10.1111/j.1365-2664.2004.00870.x.
Trenberth, K.E., 2011. Changes in precipitation with climate change. Clim. Res. 47, 123–138. https://doi.org/10.3354/cr00953.
van der Salm, C., Chardon, W.J., Koopmans, G.F., van Middelkoop, J.C., Ehlert, P.A.I., 2009. Phytoextraction of phosphorus-enriched grassland soils. J. Environ. Qual. 38, 751–761. https://doi.org/10.2134/jeq2008.0068.
van Kempen, M.M.L., Smolders, A.J.P., Bögemann, G.M., Lamers, L.P.M., Roelofs, J.G.M., 2016. Interacting effects of atmospheric CO2 enrichment and solar radiation on growth of the aquatic fern Azolla filiculoides. Freshw. Biol. 61, 596–606. https://doi.org/10.1111/fwb.12724.
Vroom, R.J.E., Xie, F., Geurts, J.J.M., Chojnowska, A., Smolders, A.J.P., Lamers, L.P.M., Fritz, C., 2018. Typha latifolia paludiculture effectively improves water quality and reduces greenhouse gas emissions in rewetted peatlands. Ecol. Eng. 124, 88–98. https://doi.org/10.1016/j.ecoleng.2018.09.008.
Wagner, G.M., 1997. Azolla: a Review of Its Biology and Utilization. Bot. Rev. https://doi.org/10.1007/BF02857915.
Widiastuti, D., Davis, J.G., 2020. Optimization of the nutrient growing solution and inoculation rate for Azolla mexicana production and use as fertilizer. J. Plant Nutr. 44, 1404–1419. https://doi.org/10.1080/01904167.2020.1849287.
Yatazawa, M., Tomomatsu, N., Hosoda, N., Nunome, K., 1980. Nitrogen fixation in azolla-anabaena symbiosis as affected by mineral nutrient status. Soil Sci. Plant Nutr. 26, 415–426. https://doi.org/10.1080/00380768.1980.10431227.
Zak, D., Gelbrecht, J., 2007. 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. https://doi.org/10.1007/s10533-007-9122-2.
Zak, D., Meyer, N., Cabezas, A., Gelbrecht, J., Mauersberger, R., Tiemeyer, B., Wagner, C., McInnes, R., 2017. Topsoil removal to minimize internal eutrophication in rewetted peatlands and to protect downstream systems against phosphorus pollution: a case study from NE Germany. Ecol. Eng. 103, 488–496. https://doi.org/10.1016/j.ecoleng.2015.12.030.
Zak, D., Wagner, C., Payer, B., Augustin, J., Gelbrecht, J., 2010. Phosphorus mobilization in rewetted fens: the effect of altered peat properties and implications for their restoration. Ecol. Appl. 20, 1336–1349. https://doi.org/10.1890/08-2053.1.
Zedler, J.B., Kercher, S., 2005. Wetland resources: status, trends, ecosystem services, and restorability. Annu. Rev. Environ. Resour. https://doi.org/10.1146/annurev.energy.30.050504.144248.