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Growing *Azolla* to produce sustainable protein feed: the effect of differing species and CO_2 concentrations on biomass productivity and chemical composition

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Abstract

BACKGROUND: Since available arable land is limited and nitrogen fertilizers pollute the environment, cropping systems ought to be developed that do not rely on them. Here we investigate the rapidly growing, N_2-fixing *Azolla*/*Nostoc* symbiosis for its potential productivity and chemical composition to determine its potential as protein feed.

RESULTS: In a small production system, cultures of *Azolla pinnata* and *Azolla filiculoides* were continuously harvested for over 100 days, yielding an average productivity of 90.0–97.2 kg dry weight (DW) ha^{-1} d^{-1}. Under ambient CO_2 levels, N_2 fixation by the fern's cyanobacterial symbionts accounted for all nitrogen in the biomass. Proteins made up 176–208 g kg^{-1} DW (4.9 × total nitrogen), depending on species and CO_2 treatment, and contained more essential amino acids than protein from soybean. Elevated atmospheric CO_2 concentrations (800 ppm) significantly boosted biomass production by 36–47%, without decreasing protein content. Choice of species and CO_2 concentrations further affected the biomass content of lipids (79–100 g kg^{-1} DW) and (poly)phenols (21–69 g kg^{-1} DW).

CONCLUSIONS: By continuous harvesting, high protein yields can be obtained from *Azolla* cultures, without the need for nitrogen fertilization. High levels of (poly)phenols likely contribute to limitations in the inclusion rate of *Azolla* in animal diets and need further investigation.

Keywords: *Azolla*; growth; feed; protein; lipids; polyphenols

INTRODUCTION

The human population is expected to grow to 9 billion by 2050.[1–3] Additionally, increasing wealth will generate a greater demand for meat and dairy products,[3,4] which require a 3.2- to 40-fold input of plant protein to produce, hence further increasing the pressure on global food supply.[5]

Here we consider floating freshwater ferns from the genus *Azolla* as an alternative crop for the production of protein feed. While species diversity was higher in episodes in the geologic past,[6] the genus *Azolla* today consists of seven species worldwide[7] that thrive in tropical to temperate regions of the world.[8–10] *Azolla* ferns exhibit high relative growth rates (RGRs) when grown individually on an open water surface, i.e. not competing for light. RGRs of over 0.5 d^{-1}, or biomass doubling times of less than 2 days have been reported – much higher than generally encountered in land plants.[11,12] A unique feature of *Azolla* ferns is that they host nitrogen (N_2)-fixing cyanobacteria, *Nostoc azollae*, in the cavities of their leaves.[13–15] These phototrophic symbionts fix N_2 during the day and likely release it as NH_4^+ in the leaf cavities to be taken up by the ferns.[16,17] Nitrogen fixation by *N. azollae* of 0.15–0.17 mg N h^{-1} per gram of dry biomass is remarkably high

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compared to 0.08 mg N h^{-1} per gram of dry biomass fixed by *Rhizobia* residing in the root nodules of soybean (*Glycine max*).[16,18] Effectively, the *N. azollae* symbiont is capable of supplying all the nitrogen (1100–1200 kg N ha^{-1} per year) required for the fern's rapid growth.[16,19]

As a result of their high growth and N_2 fixation rates, *Azolla* ferns have been used as a bio-fertilizer in rice paddies in south-east Asia for several centuries.[7] Using *Azolla* as bio-fertilizer increases the sustainability of rice cultivation by reducing fertilizer requirement as well as reducing nitrogen and greenhouse gas (GHG) emissions from rice paddies.[20–22] However, a direct use of *Azolla* biomass for feed production may provide more environmental benefits as well as economic value to farmers, but is not yet common on a large scale.

Azolla is of interest as a protein feed due to the high protein content of its biomass, which was reported to be between 200–400 g kg^{-1} of the dry weight (DW).[23–27] *Azolla* biomass was further reported to contain 79–160 g kg^{-1} lipids, which may contribute to the gross energy of whole *Azolla* feed, while also containing up to 50 g kg^{-1} (poly)phenolic compounds, of which condensed tannins in particular may decrease the digestibility of *Azolla* feed.[28–37] The large variation between the chemical compositions reported in these studies suggests that the strains and growth conditions used will not only determine the quantity but also the feed quality of *Azolla* biomass produced. Since the relevant output of a farming system is the product of quantity and quality, simultaneous analysis of biomass productivity and chemical composition is needed to determine the potential for growing *Azolla* for protein feed.

The growth of *Azolla* has generally been characterized as relative growth rates (RGRs). RGRs, however, cannot be extrapolated to the productivity per surface area of a farming system since RGRs represent exponential growth in a situation where surface area is not limiting and no inter- and intra-species competition for light and nutrients occurs. Additionally, most studies on *Azolla*'s chemical composition and/or feeding value have not used *Azolla* biomass obtained from productive cultures, or did not report biomass productivity data of the plants analyzed.

Hence the aim of this study was to analyze *Azolla*'s biomass productivity and associated chemical composition in a small production system to assess its potential as a protein feed for various animal species. We therefore investigate two *Azolla* species that differ in habitat and genetic makeup, i.e. *A. filiculoides* growing in temperate climates in Europe and *A. pinnata* growing in tropical climates in Asia and Australia. We first perform growth curve analyses for the two species to obtain key growth parameters, which are subsequently used to design a small production system in which cultures are continuously harvested over long time periods. This production system is then used to estimate yield potential for each species and to investigate the effect of elevating CO_2 (to 800 ppm) as a means to boost productivity while recycling CO_2 waste streams. Lastly, protein, amino acid, crude lipid and total (poly)phenol contents were determined for both species grown under ambient and elevated CO_2 and are discussed in relation to the application of *Azolla* biomass as feed.

METHODS

Growth conditions

Azolla filiculoides and *A. pinnata* were obtained from the International Rice Research Institute under accession numbers 1052 and 534, respectively. Plants were grown in a growth room with the following conditions: average photosynthetic flux density 300 µmol m^{-2} s^{-1} for a 16 h light period. Light was provided by fluorescent tubes and incandescent lamps. Unless mentioned otherwise, day temperature was set at 23 °C and night temperature at 20 °C, resulting in an average daytime water and air temperature (1 cm above the canopy) of 24.8 and 24.6 °C, respectively. Average night-time temperature of water and air were 23.3 and 21.1 °C, respectively.

For growth curve analysis, plants were grown in aerated 30 L containers with a surface area of 1505 cm^2 containing a non-limiting nutrient solution including macronutrients: 2 mmol L^{-1} KNO_3, 0.34 mmol L^{-1} $Ca(NO_3)_2.4H_2O$, 0.42 mmol L^{-1} KH_2PO_4 and 0.21 mmol L^{-1} $MgSO_4.7H_2O$; and trace elements: 3.6 µmol L^{-1} Fe-EDTA, 3.6 µmol L^{-1} $MnSO_4.H_2O$, 0.16 µmol L^{-1} $Na_2Mo_4.2H_2O$, 13.4 µmol L^{-1} H_3BO_3, 0.10 µmol L^{-1} $CuSO_4.5H_2O$ and 0.53 µmol L^{-1} $ZnSO_4.5H_2O$. The solution was replaced every 2 weeks; pH varied between 6 and 7, and when necessary pH was adjusted using H_2SO_4. The CO_2 concentration in the growth room was maintained at ambient concentrations (400–450 ppm). Each container was divided into 15 compartments using a Plexiglas construction. In each compartment two plants were inserted. Twice a week one randomly chosen compartment, per species and per container ($n = 6$) was harvested. Plants were oven dried for 72 h at 35 °C and subsequently their dry weight was determined. In the analysis two growth phases were distinguished: exponential growth and linear growth. Plant growth, as dry weight, was modelled by

$$\text{for } t < t_{e\to l} : y_t = y_0 e^{\mathrm{RGR}\cdot t} \quad (1)$$

$$\text{for } t_{e\to l} < t : y_t = \mathrm{AGR}\cdot t + b \quad (2)$$

Where Eqn (1) follows from the definition of RGR: t (dy/dt)·(1/y) = time (days), $t_{e\to l}$ = time point when the growth phase shifts from exponential to linear phase (days), y_t = standing crop at time t (kg ha^{-1}), y_0 = standing crop at the start of the experiment (kg ha^{-1}), RGR = relative growth rate (per day, d^{-1}), b = standing crop at the start of the linear growth phase (kg ha^{-1}) and AGR = absolute growth rate (kg ha^{-1} d^{-1}).

For the harvest experiment, *A. filiculoides* and *A. pinnata* were grown indoors using 30 L containers similar to the growth analysis experiment ($n = 5$ for each species). The net surface area of each container was 1495 cm^2. At the start of the experiment the surface was fully covered. Twice a week, 33% of the surface area was harvested. The growth medium was changed to better fit plant requirements and included macronutrients: 0.7 mmol L^{-1} KNO_3, 0.1 mmol L^{-1} $Ca(NO_3)_2.4H_2O$, 0.13 mmol L^{-1} KH_2PO_4 and 0.1 mmol L^{-1} $MgSO_4.7H_2O$; and trace elements: 4.7 µmol L^{-1} Fe-EDTA, 2.2 µmol L^{-1} $MnSO_4.H_2O$, 0.1 µmol L^{-1} $Na_2Mo_4.2H_2O$, 8.1 µmol L^{-1} H_3B_3, 0.06 µmol L^{-1} $CuSO_4.5H_2O$ and 3.1 µmol L^{-1} $ZnSO_4.5H_2O$. The complete 30 L solution was replaced every 2 weeks; in between these periods, medium concentrate was supplied to 1× concentration every 3.5 days. CO_2 concentration in the growth room were maintained either at ambient level (400–450 ppm) or elevated level (800 ppm). Harvested biomass was oven dried for 72 h at 35 °C, weighed and stored until further analyses.

Nitrogen and phosphorus concentrations

Nitrogen and phosphorus concentration in the growth medium were analyzed during the harvest experiments at ambient CO_2, using a DR 5000 analyzer (Hach Lange, Manchester, UK). Shortly

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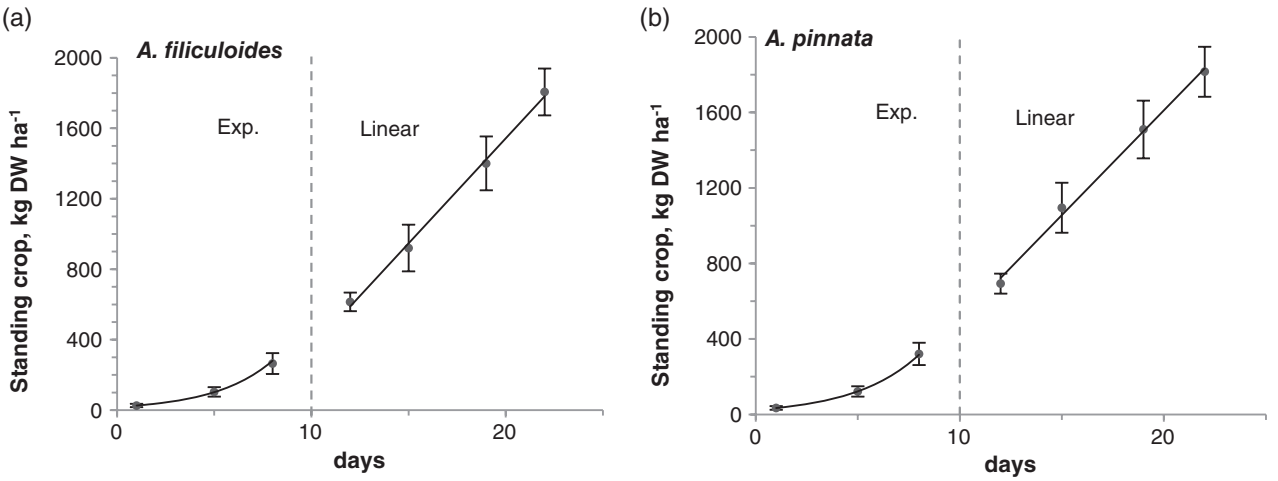

Figure 1. Growth curve analyses. Standing crop as dry weight (DW) over time is given for growth curve analysis of *A. filicuoides* (a) and *A. pinnata* (b) cultures. Data points are averages with standard deviations ($n = 6$).

after the solution was replaced, aliquots of the solution were taken from each container and analyzed for nitrate-N and *ortho*-P concentrations. The same procedure was followed 3 days later shortly before harvest of 33% of the surface area. After harvesting, water samples were taken and fresh nutrients were added as described above. This provided data for nutrient depletion over the course of three harvest intervals.

Biomass nitrogen and amino acid analysis

The oven-dried plant material was frozen in liquid nitrogen, ground and subsequently freeze dried. Total biomass nitrogen was analyzed in all growth replicates using the Dumas method (according to ISO 16634-1 analytical standard) and total amino acid analyses were performed according to the Ph. EUR 2.2.56 analytical standard (Silliker, Mérieux NutriSciences, Ede, Netherlands).[38,39] Briefly, amino acid analysis was performed in triplicate and using three different methods: (i) acid hydrolysis for most of the amino acids; (ii) oxidation followed by acid hydrolysis for cysteine and methionine; (iii) alkaline hydrolysis for tryptophan. Amino acids in acid hydrolysates were determined using ion-exchange liquid chromatography with post-column Ninhydrin derivatization and photometric detection (BiochromAAA) and Li-citrate and Na-citrate buffer system elution. Tryptophan was quantified by reversed-phase high-performance liquid chromatography. Amino acids were expressed as milligrams per gram of total amino acids (AA). Protein concentrations were estimated by correcting total AA for the gain of a water molecule during hydrolysis.

Crude lipid and total phenol analysis

The oven-dried plant material was ground using a Retsch ZM200 grinder equipped with a 1 mm sieve. One gram of material was used for dry weight determination after further oven drying at 105 °C for 24 h, followed by 1 h cooling in a desiccator with activated silica. The remainder of the material was Soxhlet extracted with a 7.5:1 dichloromethane–methanol solution for 24 h with a water bath temperature of 80 °C.[30] The collected extracts were dried using a rotary evaporator and weighed to determine the amount of 'crude lipids'.

After Soxhlet extraction plant residues were collected and dried. Total phenol was determined in triplicate after extraction of 100 mg biomass with 10 mL water–acetone (30:70, v/v) for 24 h at 20 °C using the Folin–Ciocalteu assay in two technical replicates.[40] Tannic acid was used as the calibration standard. Literature data, used for comparison, presented in gallic acid equivalents, were converted to tannic acid equivalents according to the relative reactivity with the Folin–Ciocalteu reagent.[41]

Statistical analysis

Statistical analyses were performed using the IBM SPSS 20 statistics software package. For comparing growth rates, lipid concentrations and total phenol concentrations between species or CO_2 treatments a standard homoscedastic, two-tailed *t*-test was used. For determining the effect of species and CO_2 treatment on protein and amino acid content, a two-way analysis of variance (ANOVA) was performed followed by Fisher's LSD post hoc test. Differences were deemed significant for a *P*-value below 0.05.

RESULTS AND DISCUSSION

Growth curve analyses

Growth was exponential for the first 8 days for both species ($R^2 = 0.999$ for *A. filiculoides* and $R^2 = 1.000$ for *A. pinnata*), then shifted to linear 8–12 days after the start of the experiment, when cultures reached a standing crop, i.e. the total dry weight biomass present on the surface area, between 300 and 600 kg ha^{-1} (Fig. 1). Growth remained linear at least until a standing crop of 1800 kg ha^{-1}.

Relative growth rates (RGR) calculated over the first three time points in Fig. 1 representing the exponential growth phase were 0.337 ± 0.078 d^{-1} for *A. filiculoides* and 0.317 ± 0.034 d^{-1} for *A. pinnata* (non-significant difference) and were within the range 0.12–0.5 d^{-1} previously reported.[11,24,42–46] RGRs obtained compared with those from the macrophytes *Salvinia natans* (0.28 d^{-1})[47] and *Eichhornia crassipes* (0.23 d^{-1}),[48] but were somewhat lower than those reported for duckweeds *Wolffiella hyaline* (0.52 d^{-1}) and *Lemna gibba* (0.50 d^{-1}).[49] The high RGRs reflect the ability of *Azolla* plants, and other floating macrophytes, to expand horizontally on the surface, avoiding competition as long as the surface is not fully covered, in contrast to the vertical growth of land plants, for which competition for light starts shortly after emergence.[50]

However, by definition, the highest growth per unit of surface area is not obtained in the exponential growth phase but in

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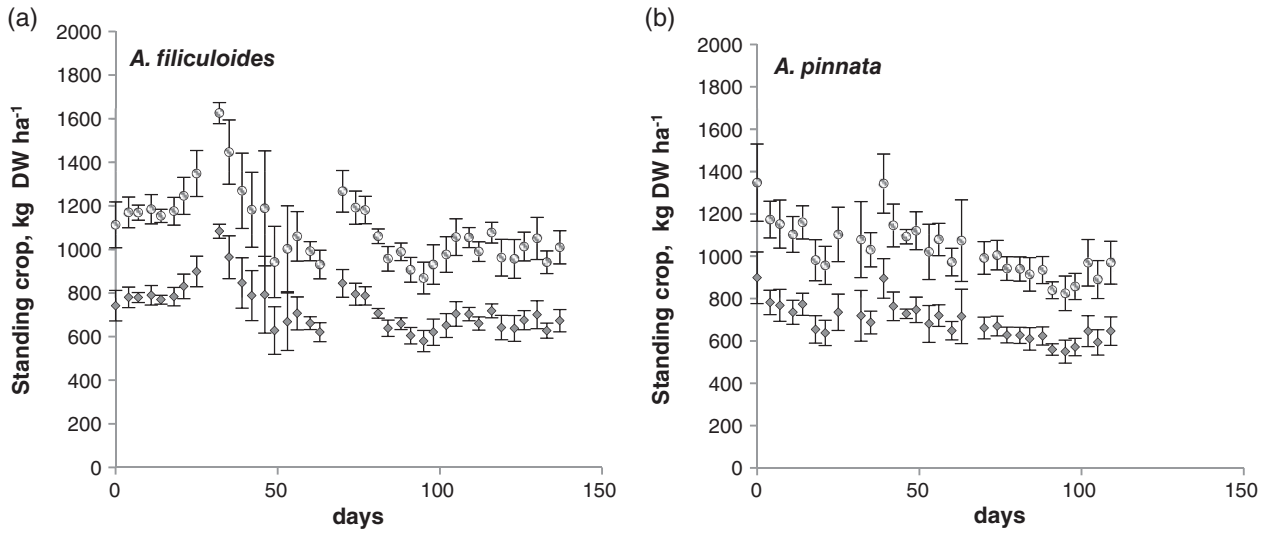

Figure 2. Continuous harvest experiments in controlled conditions. Standing crop DW is given before (circle) and after (diamond) harvest of *A. filicuoides* (a) and *A. pinnata* (b) cultures. Data points are averages with standard deviations ($n = 5$).

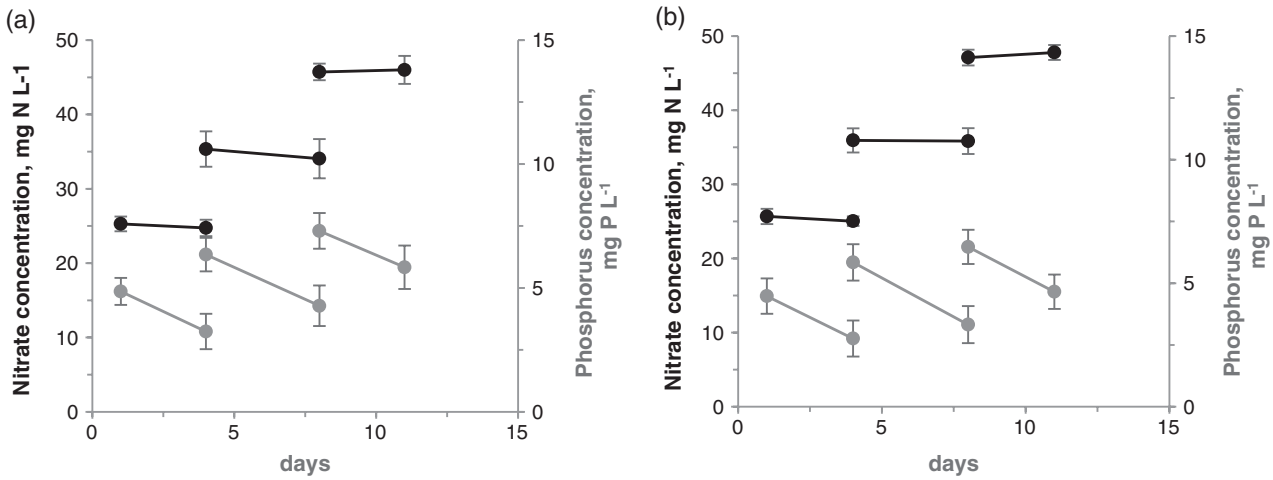

Figure 3. Nutrient use of *A. filiculoides* (a) and *A. pinnata* (b) under continuous harvest conditions. Nitrate (black lines) and phosphate (grey lines) concentrations in the medium over a monitoring period of 11 days during the ambient CO_2 experiment. Data points are averages with standard deviations ($n = 3$).

the linear growth phase, as the slope of standing crop *versus* time reaches is maximum (Fig. 1**)**. Hence, when considering *Azolla* species for their potential biomass yields, absolute growth rates (AGR) relative to production surface needed to be considered instead of RGRs. AGRs were at a maximum during the linear growth phase when increases of 119 ± 14 kg dry weight (DW) ha^{-1} d^{-1} and 111 ± 7 kg DW ha^{-1} d^{-1} were determined for *A. filiculoides* and *A. pinnata* respectively (Fig. 1). Growth rates of the two species did not differ significantly ($P < 0.05$) from each other.

Continuous harvest experiment

Based on the growth analyses a small production system was designed. By continuously harvesting a part of the cultivation area, plants were kept within the linear growth phase to maximize productivity. Theoretically, the harvesting period and harvest area can be chosen freely as long as standing crop remains in the linear growth phase before and after harvest, whereas each combination of harvest period and area will result in a different equilibrium standing crop, but identical yields.

This concept was tested by maintaining cultures of *A. filiculoides* and *A. pinnata* for over 100 days, while harvesting 33% of the surface area twice a week. *Azolla filiculoides* cultures equilibrated at around 1000 kg ha^{-1} before harvest and 670 kg ha^{-1} after harvest (Fig. 2a). *Azolla pinnata* cultures reached a lower equilibrium, i.e. 900 kg ha^{-1} before harvest and 600 kg ha^{-1} after harvest (Fig. 2b). For both, the equilibrium in standing crop was reached later than expected due to perturbations caused by delaying two harvests at days 27 and 67. However, at all time points, the standing crop remained within the linear phase defined in the growth analyses.

We therefore conclude that continuous harvesting can be used to maintain cultures in the linear growth phase and to obtain predictable biomass yields under controlled conditions.

Nutrient input during indoor harvest experiment

Monitoring nitrogen and phosphate nutrients in the medium during indoor growth experiments revealed that phosphate was, as expected, depleted during growth and replenished when adding nutrients. The nitrate in the medium, however, was not depleted

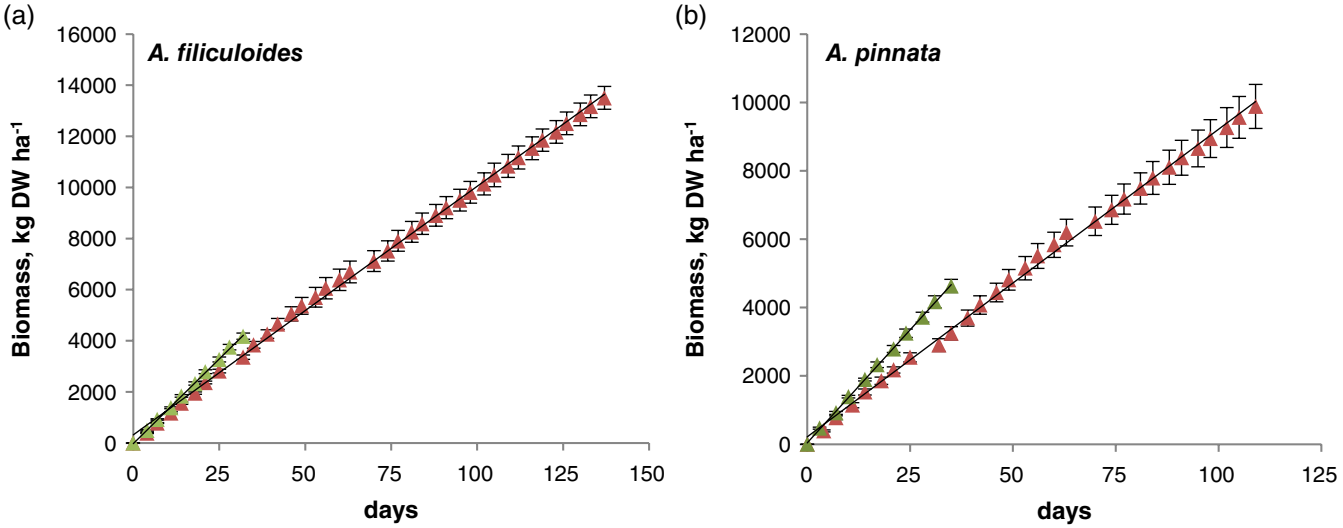

Figure 4. Biomass yields of *A. filiculoides* (a) and *A. pinnata* (b) under continuous harvest conditions. Cumulative harvest in dry weight (DW) for ambient (red) and elevated (800 ppm) CO_2 conditions (green). Data points are averages with standard deviations ($n = 5$).

Table 1. Average concentrations of nitrogen ($n = 6$), total amino acids (AA) and protein ($n = 3$) (as % of dry weight) and derived ratios for *A. filiculoides* and *A. pinnata* grown at ambient and elevated CO_2 (800 ppm)

	Concentration, g kg^{-1} dry weight			
	Ambient CO_2		Elevated CO_2	
	A. filiculoides	*A. pinnata*	*A. filiculoides*	*A. pinnata*
Total nitrogen	40 ± 3a	37 ± 3b	41 ± 2a	37 ± 1b
Total AA	227 ± 21a	205 ± 19b	243 ± 15a	212 ± 3b
AA nitrogen	35 ± 5a	31 ± 3b	36 ± 2a	32 ± 0b
Non-AA nitrogen	4 ± 1a	5 ± 1a	6 ± 1a	06 ± 01a
Protein estimate	195 ± 18a	176 ± 16b	208 ± 13a	182 ± 03b
Ratio of protein:AA nitrogen	5.6 ± 0.9a	5.7 ± 0.7a	5.8 ± 0.5a	5.7 ± 0.1a
Ratio of protein:total nitrogen	4.9 ± 0.1a	4.9 ± 0.1a	5.0 ± 0.1a	4.9 ± 0.1a

Letters after entries indicate significant differences ($P < 0.05$), based on a two-way ANOVA.

and as a consequence increased stepwise, reaching 3.4 mmol L^{-1} after 2 weeks (Fig. 3). The N_2-fixing symbiont *N. azollae* apparently provided all nitrogen incorporated in both species, and nitrate concentrations up to 3.4 mmol L^{-1} did not inhibit N_2 fixation. Previously, addition of 4 mmol L^{-1} nitrate resulted in increased nitrate reductase activity in *A. filiculoides* and a small increase in growth and biomass nitrogen content, but nitrate uptake was not quantified in this study.[16] In *A. pinnata* 80–97% of the N incorporated into the biomass was shown to derive from N_2 fixation at 1 mmol L^{-1} NO_3.[45] However, at concentrations of nitrate and, especially, ammonium above 5 mmol L^{-1}, N_2 fixation was inhibited in *Azolla* ferns.[51] Hence the lack of nitrate uptake observed here may be restricted to low (<4 mmol L^{-1}) nitrate concentrations. Results indicate that *Azolla* ferns have particular potential for production systems without N fertilizer, consistent with their long-established use as bio-fertilizers in rice paddy fields.[7,52]

Biomass yields in ambient and elevated CO_2

The cumulative harvested biomass from the indoor experiments is depicted in Fig. 4. Harvested biomass accumulated linearly over time, yielding 97.2 ± 4.0 kg DW ha^{-1} d^{-1} and 90.0 ± 6.2 kg DW ha^{-1} d^{-1} for *A. filiculoides* and *A. pinnata* respectively (no significant difference, *P*-value = 0.058). These yields were somewhat lower than expected from the growth analysis, which could be attributed to changes made to the growth media and/or increased stress due to frequent handling and introduction of competing algae and/or pathogens when cultures are maintained over a long time period.

The only previously reported continuous harvest pilot using *Azolla*, carried out over 154 days in outdoor ponds recycling chicken farm waste in Colombia, reached similar productivities (107 kg DW ha^{-1} d^{-1}).[53] Compared to crops grown on arable land, the biomass productivity of *Azolla* was not exceptionally high: maximum biomass increases for *Zea mays* and *Brassica napus* of over 200 kg DW ha^{-1} d^{-1} have been observed.[54,55] The high growth rates of these crops, however, are restricted to vegetative growth after seedling establishment. In contrast, *Azolla* ferns exhibit continuous, rapid vegetative growth as long as they are regularly harvested and given sufficient nutrients.

The possibility that productivity could be increased further by adding CO_2 was tested by elevating CO_2 concentrations in the growth chamber to 800 ppm, while keeping all other environmental factors constant. Again, harvested biomass accumulated linearly over the 40 days tested; the productivities, however, increased to 132.4 ± 4.0 kg DW ha^{-1} d^{-1} for *A. filiculoides* and

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Table 2. Amino acid concentrations in biomass of *A. filiculoides* and *A. pinnata* grown under ambient and elevated (800 ppm) CO_2 concentrations ($n = 3$)

	Amino acid concentration, g total amino acids kg^{-1} dry weight			
	Ambient CO_2		Elevated CO_2	
Amino acid	*A. filiculoides*	*A. pinnata*	*A. filiculoides*	*A. pinnata*
Arginine (Arg)	69.5 ± 5.2b	66.9 ± 4.7b	71.4 ± 7.3a	69.7 ± 1.4b
Histidine (His)	21.4 ± 1.9b	18.9 ± 1.1b	21.7 ± 1.7a	21.6 ± 0.3b
Isoleucine (Ile)	50 ± 4.2a	51.8 ± 3.4a	49.7 ± 5.6b	50.2 ± 0.9a
Leucine (Leu)	88.6 ± 8.1ab	88.1 ± 5.5b	90.3 ± 9.5a	92.2 ± 1.5c
Lysine (Lys)	57.6 ± 6.0b	54.2 ± 3.3a	55.2 ± 6.9a	53.6 ± 0.5a
Methionine (Met)	18.1 ± 0.6a	19.2 ± 1.5a	19.1 ± 1.2a	19.5 ± 0.5a
Phenylalanine (Phe)	59.2 ± 5.4b	54.2 ± 2.8b	59.4 ± 5.8a	58.9 ± 0.8b
Threonine (Thr)	50.8 ± 4.9b	53.2 ± 3.3a	49.1 ± 4.3c	50.7 ± 0.8b
Tryptophan (Trp)	15.5 ± 1.0a	18.3 ± 0.9bc	17.2 ± 1.0c	16.6 ± 0.5ab
Valine (Val)	60.3 ± 5.2a	60.4 ± 3.5a	60.3 ± 6.3a	60.3 ± 0.8a
Alanine (Ala)	63.7 ± 5.9a	74 ± 5.5b	69.3 ± 6.0d	71.6 ± 1.4c
Aspartic acid (Asp) + Asparagine (Asn)	104 ± 8.2b	104 ± 6.5a	99.6 ± 8.4b	101 ± 1.8a
Cysteine (Cys)	11.6 ± 0.3b	9.98 ± 0.5b	11.8 ± 0.6a	11.2 ± 0.1b
Glutamic acid (Glu) + Glutamine (Gln)	144 ± 18.2b	137 ± 7.4a	137 ± 9.8ab	131 ± 2.0a
Glycine (Gly)	54.9 ± 4.6a	55.2 ± 3.1a	55.2 ± 5.3a	55.5 ± 0.6b
Proline (Pro)	42.9 ± 4.2a	42.6 ± 2.4b	45.6 ± 5.3a	45 ± 0.3b
Serine (Ser)	49 ± 4.6a	51.6 ± 3.4b	50.3 ± 4.3c	51.8 ± 0.8c
Tyrosine (Tyr)	38.9 ± 3.9ab	40.8 ± 3.0a	38.3 ± 4.0c	39.7 ± 1.1bc

Letters after entries indicate significant differences ($P < 0.05$), based on two-way ANOVA and Fisher's LSD post hoc test.

132.3 ± 5.4 kg DW ha^{-1} d^{-1} for *A. pinnata* (Fig. 4), corresponding to a significant increase of 36% and 47% for *A. filiculoides* and *A. pinnata* respectively. This yield increase was lower than observed for ferns growing exponentially in batch experiments (50%), but still provides a case for adding CO_2 to production systems.[45]

Nitrogen and protein content in *Azolla* biomass

Total nitrogen and amino acid (AA) contents were measured in the biomass of *A. filiculoides* and *A. pinnata*, obtained from the indoor harvest experiments. Nitrogen concentrations in *Azolla* biomass varied between 37 and 41 g kg^{-1} DW (Table 1). The sum of amino acids (AA) ranged from 208 to 244 g kg^{-1} DW and the AAs contained 82–88% of the total nitrogen in the biomass. Protein concentration was estimated at 176–208 g kg^{-1} DW (Table 1). The ratio of protein concentration over nitrogen concentration in AAs ranged from 5.6 to 5.7. Since a considerable fraction of the nitrogen was not incorporated into amino acids, the overall ratio between protein and total nitrogen in the biomass was calculated at 4.9–5.0. The simplified estimation of crude protein by multiplying total nitrogen with 6.25 is therefore unsuitable for *Azolla* biomass, which has also been reported for leaves of Nigerian crops (3.24–5.17), duckweed (4.8), water hyacinth (4.6), and *Ulva lactuca* (4.6).[56–58] In *Azolla*, the additional nitrogen may be present as inorganic nitrogen, such as ammonium in the leaf pockets, or as nitrogen-containing organic compounds other than amino acids.[59]

Statistical analyses indicated that *A. filiculoides* contained significantly more nitrogen and protein ($P < 0.05$) than *A. pinnata*. This significant difference also held for the total AA and AA nitrogen, while the amount of non-AA nitrogen was similar across species and treatments. Increasing CO_2, although increasing growth rates, did not lead to a decrease in nitrogen, nor in protein content. Hence net nitrogen assimilation increases significantly under elevated CO_2 (800 ppm). Increased nitrogen fixation under elevated CO_2 has been reported in the N_2-fixing legumes *Galactia elliottii* and *Medicago sativa*, which was attributed to increased transport of photosynthates to the nodules.[60–62] Alternatively, increased CO_2 may have directly increased N_2 fixation by cyanobacteria in the fern leaf cavities. This is in line with previous studies wherein, firstly, nitrogenase activity increased 2.6-fold when the cyanobacterium *Anabaena fertilissima* was grown at 60 000 ppm CO_2 compared to ambient CO_2 and, secondly, 900 ppm CO_2 increased nitrogen fixation rates 1.5- to 3-fold compared to ambient CO_2 in the marine cyanobacterium *Trichodesmium*.[63,64] Still, experiments carried out here did not rule out enhanced nitrate uptake at elevated CO_2. Irrespective, the addition of waste CO_2 from industry, electricity or heat generation to an *Azolla* production system is thus expected to boost biomass productivity with no reduction of protein content.

Based on the productivities obtained in this study, annual yields of 32.8–35.5 t DW ha^{-1} per year and 48.3 t DW ha^{-1} per year can be expected from a fully controlled indoor production system using ambient CO_2 and 800 ppm CO_2 concentrations, respectively. Depending on the species, the annually harvested biomass contains 6.8–7.9 t protein ha^{-1} per year for a controlled system at ambient CO_2 and 10.3–11.8 t protein ha^{-1} per year at elevated CO_2.[65] The most established protein crop, soybean (*Glycine max*), can yield 2 t protein ha^{-1} per year in subtropical regions, but only fixes 50% of the organic nitrogen exported from the field as seed.[66] Legumes used for protein feed in Europe, while yielding 15–21 t DW ha^{-1} per year or 2.2 t N ha^{-1} per year, still require at least 150 kg N fertilizer ha^{-1} per year.[67,68] Hence the major advantage of *Azolla* over established crops derives from the combination of no N input, no requirement for arable land and high yields.

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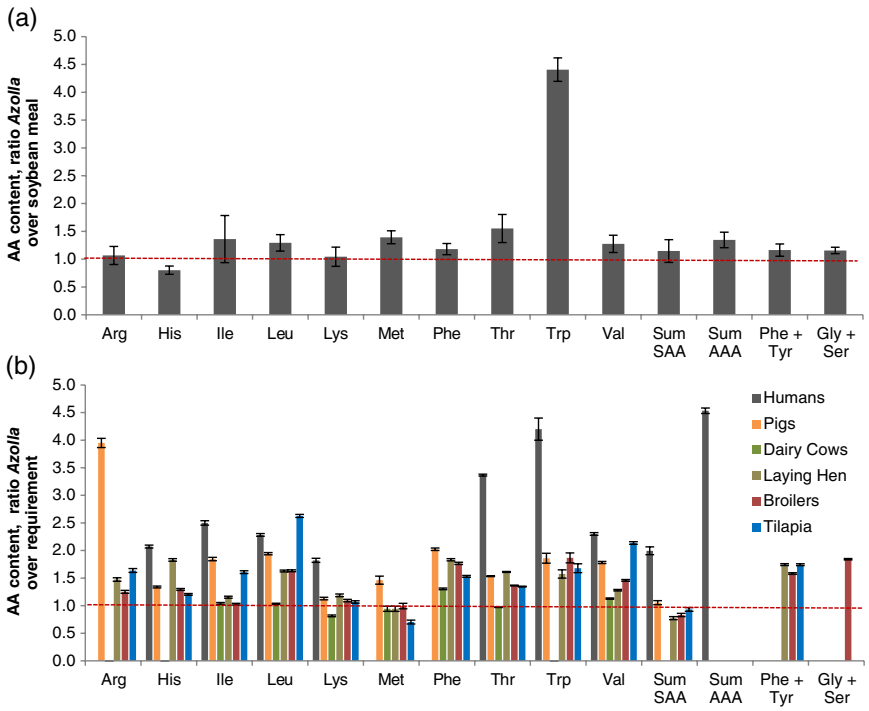

Figure 5. Essential amino acid (AA) concentrations in protein from *Azolla* compared to soybean (*Glycine max*) meal and their suitability for feed. (a) Ratios of the average AA concentration in *Azolla* species over the average of soybean meal, from three independent studies.[70–72] The sum of sulfur-containing amino acids (Sum SAA), the sum of the aromatic amino acids (Sum AAA) and the combinations of Phe+Tyr and Glyc+Ser are also compared. (b) Ratios of amino acid concentrations in *Azolla* over concentration minima required in food for humans and feed for pigs, dairy cows, laying hens, broilers and tilapia.[73–77] Standard deviations indicate variation between *Azolla* biomass of both species and treatments.

Azolla biomass amino acid profile suits feed applications

In feeding trials *Azolla* could be included in diets at rates of 10% for poultry, 15% for sows and 25% for tilapia fish, but higher inclusion rates negatively affected animal weight and overall digestibility, meaning that commercial soybean meal or fish feed could not be fully replaced by *Azolla*.[25,26,53,69] The amino acid composition of *Azolla* was previously suggested to limit the inclusion rate.[25,65] To determine whether the *Azolla* biomass obtained from the continuous harvest system described here was suitable as a source of feed, we analyzed individual amino acids for all species and treatments. Although concentrations of several individual amino acids differed between species and CO_2 treatment, differences were small (<10%) (Table 2). Noteworthy exceptions include concentrations of tryptophan and alanine, which increased by 18% and 17% when *A. filiculoides* was grown in elevated *versus* ambient CO_2 concentrations. Concentrations of cysteine and histidine in *A. filiculoides* decreased by 14% and 11%, respectively, in ferns grown at elevated *versus* ambient CO_2 (Table 2).

Compared to soybean meal, *Azolla* contained a higher proportion of all essential amino acids except for histidine (Fig. 5a). Histidine is, however, not the limiting amino acid in many feeds (Fig. 5b). For Nile tilapia and chicken the sulfur containing AA (SAA) were limiting (Fig. 5b). Levels of methionine were 68–72% of the level recommended for Nile tilapia and 91–97% of those for laying hens. Levels of combined methionine and cysteine equaled 92–95% of the recommended concentration for Nile tilapia, 77–79% of that for laying hens and 82–85% of that for broilers. For dairy cows, lysine (79–85%), methionine (91–97%) and threonine (93–102%) were slightly limiting (Fig. 5b). However, when compared to soybean meal, *Azolla* was superior because of its higher relative concentrations of methionine and threonine (Fig. 5a). As Becerra *et al.* reported, an improved composition of essential amino acids, including methionine and cysteine, was consistently seen in pig diets, of which 15–30% of soy protein was replaced by *Azolla*.[53] *Azolla* essential amino acid composition, furthermore, met all requirements for growing pigs (50–88 kg) and adult humans (Fig. 5b).[73,74] We conclude that slight deficiencies in specific essential amino acids with respect to recommended levels for chicken, tilapia and dairy cows cannot explain the limited inclusion rate of *Azolla* biomass in feeding trials. To domesticate *Azolla* as a protein crop, therefore, other components affecting biomass digestibility need to be characterized, then either bred or extracted out.

Selecting species and growth conditions on (poly)phenol- and lipid content to improve biomass value

Suggested causes for lowering digestibility of *Azolla* biomass, other than AA composition, included high lignin and low energy content.[25,26,53,69] Lignin is, however, not present in *Azolla*, which instead contains (poly)phenolic tannins.[35] These tannins likely decrease digestibility when binding to proteins upon digestion of the biomass.[36,37] Total soluble (poly)phenols and crude lipids were therefore measured in biomass harvested from *A. filiculoides* and *A. pinnata* grown at ambient and elevated CO_2 levels (Fig. 6). Soluble (poly)phenol levels, assayed as tannic acid equivalents per unit of dry weight, were very high (20.9–69.1 g kg^{-1}; Fig. 6a) compared to foodstuff, such as fruits (1.47–6.22 g kg^{-1}), beans (1.26–6.67 g kg^{-1}) and nuts (0.56–19.2 g kg^{-1}).[41,78–82] Soluble (poly)phenols were 3.3 times more abundant in *A. pinnata*, compared to *A. filiculoides,* reaching 69.1 g kg^{-1} DW (tannic acid equivalents) at ambient CO_2 (Fig. 6a). Crude lipid yields represented roughly 100 g kg^{-1} DW when ferns of either species were

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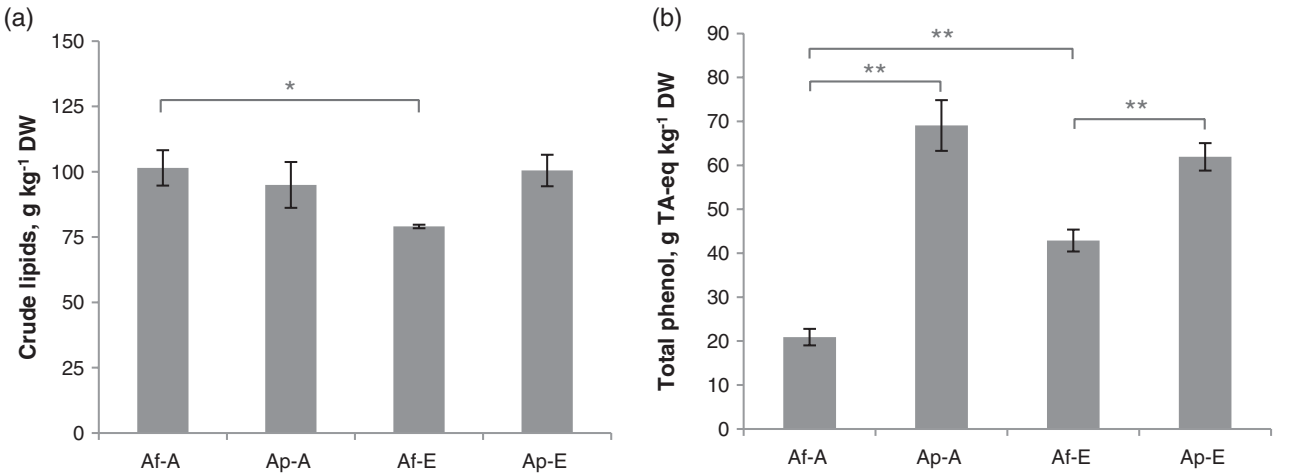

Figure 6. Total phenol and crude lipid content in different *Azolla* species grown at different CO_2 concentrations. Total phenol is expressed as milligrams of tannic acid (TA) equivalent (a) and lipids are measured by weight (b) in dry biomass of *A. filiculoides* (Af) and *A. pinnata* (Ap) grown under ambient (Ap-A, Af-A) and elevated (800 ppm) CO_2 (Ap-E, Af-E). Data points are averages with standard deviations ($n = 3$). Asterisks indicate significant differences: **P*-value < 0.05; ***P*-value < 0.01.

grown at ambient CO_2 (Fig. 6b). Hence, besides higher protein content, *A. filiculoides* contained fewer (poly)phenols than *A. pinnata* when grown at ambient CO_2, making it a more suitable protein feed.

In *A. pinnata*, elevated CO_2 altered neither lipids nor soluble (poly)phenols significantly. In *A. filiculoides*, however, elevated CO_2 significantly decreased (0.78-fold) crude lipid and increased (2.1-fold) total soluble (poly)phenols (Fig. 6). Although adding CO_2 increased protein yield in *A. filiculoides*, it reduced cysteine and lipid content and increased total (poly)phenol content, which may reduce gross energy and digestibility of the *Azolla* biomass. More research is needed to confirm the effects of polyphenol and lipid content on biomass digestibility.

CONCLUSIONS

Production systems using continuous harvesting allow us to obtain high biomass yields for *A. filiculoides* and *A. pinnata*, without use of nitrogen fertilizer. The high levels of protein and favorable amino acid content compared to soybean suit application of *Azolla* biomass as feed. The high content of (poly)phenols (up to 69 g kg^{-1} dry weight) is likely responsible for the reported limits in the inclusion rate of *Azolla* in the diet of animals. In terms of protein content and (poly)phenol content, *A. filiculoides* is a more suitable species for use as animal feed than *A. pinnata*. Our results show a clear case for combining *Azolla* cultivation with CO_2 waste streams, as elevating CO_2 concentrations to 800 ppm boosted productivity to 48.3 t DW ha^{-1} per year without loss in protein content. Increasing CO_2 concentrations did increase (poly)phenol content and decreased lipid content. The effects of (poly)phenols on applications of *Azolla* as protein feed need further investigation. Breeding and selection for more favorable *Azolla* strains as well as bio-refining to produce multiple value-adding products likely will further enhance the value of an *Azolla* production chain.

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