Growing *Azolla* to produce sustainable protein feed: the effect of differing species and CO₂ concentrations on biomass productivity and chemical composition

Paul Brouwer, a,b* Henriette Schluepmann, a Klaas GJ Nierop, b Janneke Elderson, c Peter K Bijl, b,d Ingrid van der Meer, e Willem de Visser, c Gert-Jan Reichart, b,f Sjef Smeekens a and Adrie van der Werf c

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, N₂-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 CO₂ levels, N₂ 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 CO₂ treatment, and contained more essential amino acids than protein from soybean. Elevated atmospheric CO₂ concentrations (800 ppm) significantly boosted biomass production by 36–47%, without decreasing protein content. Choice of species and CO₂ 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.

© 2018 The Authors. *Journal of the Science of Food and Agriculture* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

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 worldwide7 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 (N₉)–fixing cyanobacteria, *Nostoc azollae*, in the cavities of their leaves.13–15 These phototrophic symbionts fix N₂ during the day and likely release it as NH₄⁺ 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

© 2018 The Authors. *Journal of the Science of Food and Agriculture* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
compared to 0.08 mg N h\(^{-1}\) per gram of dry biomass fixed by \(\text{Rhizobia}\) residing in the root nodules of soybean (\(\text{Glycine max}\)).\(^{1,6,18}\)

Effectively, the \(\text{N. azollae}\) 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 \(\text{N}\)\(_2\) fixation rates, \(\text{Azolla}\) ferns have been used as a bio-fertilizer in rice paddies in south-east Asia for several centuries.\(^7\) Using \(\text{Azolla}\) 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 \(\text{Azolla}\) 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.

\(\text{Azolla}\) 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).\(^{23–27}\) \(\text{Azolla}\) biomass was further reported to contain 79–160 g kg\(^{-1}\) lipids, which may contribute to the gross energy of whole \(\text{Azolla}\) feed, while also containing up to 50 g kg\(^{-1}\) (poly)phenolic compounds, of which condensed tannins in particular may decrease the digestibility of \(\text{Azolla}\) 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 \(\text{Azolla}\) 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 \(\text{Azolla}\) for protein feed.

The growth of \(\text{Azolla}\) 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 \(\text{Azolla}\)’s chemical composition and/or feeding value have not used \(\text{Azolla}\) 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 \(\text{Azolla}\)’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 \(\text{Azolla}\) species that differ in habitat and genetic makeup, i.e. \(\text{A. filiculoides}\) growing in temperate climates in Europe and \(\text{A. pinnata}\) 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 \(\text{CO}_2\) (to 800 ppm) as a means to boost productivity while recycling \(\text{CO}_2\) waste streams. Lastly, protein, amino acid, crude lipid and total (poly)phenol contents were determined for both species grown under ambient and elevated \(\text{CO}_2\) and are discussed in relation to the application of \(\text{Azolla}\) biomass as feed.

**METHODS**

**Growth conditions**

\(\text{Azolla filiculoides}\) and \(\text{A. pinnata}\) 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 \(\text{µmol m}^{-2} \text{s}^{-1}\) 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 analyses, 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\(^{-1}\) \(\text{KNO}_3\), 0.34 mmol L\(^{-1}\) \(\text{Ca(NO}_3\)\(_2\))\(_2\), 0.42 mmol L\(^{-1}\) \(\text{KH}_2\text{PO}_4\) and 0.21 mmol L\(^{-1}\) \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\); and trace elements: 3.6 mmol L\(^{-1}\) \(\text{Fe-EDTA}\), 3.6 mmol L\(^{-1}\) \(\text{MnSO}_4\cdot\text{H}_2\text{O}\), 0.16 mmol L\(^{-1}\) \(\text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O}\), 13.4 mmol L\(^{-1}\) \(\text{H}_2\text{BO}_3\), 0.10 mmol L\(^{-1}\) \(\text{CuSO}_4\cdot5\text{H}_2\text{O}\) and 0.53 mmol L\(^{-1}\) \(\text{ZnSO}_4\cdot\text{H}_2\text{O}\). The solution was replaced every 2 weeks; pH varied between 6 and 7, and when necessary pH was adjusted using \(\text{H}_2\text{SO}_4\). The \(\text{CO}_2\) 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

\[
\text{AGR} = \lambda \cdot t + b
\]

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

For the harvest experiment, \(\text{A. filiculoides}\) and \(\text{A. pinnata}\) 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\(^{-1}\) \(\text{KNO}_3\), 0.1 mmol L\(^{-1}\) \(\text{Ca(NO}_3\)\(_2\))\(_2\), 0.13 mmol L\(^{-1}\) \(\text{KH}_2\text{PO}_4\) and 0.1 mmol L\(^{-1}\) \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\); and trace elements: 4.7 mmol L\(^{-1}\) \(\text{Fe-EDTA}\), 2.2 mmol L\(^{-1}\) \(\text{MnSO}_4\cdot\text{H}_2\text{O}\), 0.1 mmol L\(^{-1}\) \(\text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O}\), 8.1 mmol L\(^{-1}\) \(\text{H}_2\text{BO}_3\), 0.06 mmol L\(^{-1}\) \(\text{CuSO}_4\cdot5\text{H}_2\text{O}\) and 3.1 mmol L\(^{-1}\) \(\text{ZnSO}_4\cdot\text{H}_2\text{O}\). The complete 30 L solution was replaced every 2 weeks; in between these periods, medium concentrate was supplied to 1x concentration every 3.5 days. \(\text{CO}_2\) 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 \(\text{CO}_2\), using a DR 5000 analyzer (Hach Lange, Manchester, UK). Shortly
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). 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. 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. 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.

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 CO2 treatments a standard homoscedastic, two-tailed t-test was used. For determining the effect of species and CO2 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 (R² = 0.999 for A. filiculoides and R² = 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. 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.

However, by definition, the highest growth per unit of surface area is not obtained in the exponential growth phase but in
Figure 2. Continuous harvest experiments in controlled conditions. Standing crop DW is given before (circle) and after (diamond) harvest of *A. filiculoides* (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 CO₂ 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 $119 \pm 14$ kg dry weight (DW) ha$^{-1}$ d$^{-1}$ and $111 \pm 7$ 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...
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\(^{-1}\) \(N\)_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 \(A.\) filiculoides ferns.\(^{51}\) Hence the lack of nitrate uptake observed here may be restricted to low (\(<4\) mmol L\(^{-1}\)) nitrate concentrations. Results indicate that \(A.\) filiculoides 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 \(A.\) filiculoides, 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 productivities of \(A.\) filiculoides 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, \(A.\) filiculoides 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...
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 CO₂ 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 CO₂, although increasing growth rates, did not lead to a decrease in nitrogen, nor in protein content. Hence net nitrogen assimilation increases significantly under elevated CO₂ (800 ppm). Increased nitrogen fixation under elevated CO₂ has been reported in the N₂-fixing legumes Galactia elliottii and Medicago sativa, which was attributed to increased transport of photosynthates to the nodules.⁶⁰–⁶² Alternatively, increased CO₂ may have directly increased N₂ 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 Anaebaena fertilissima was grown at 60 000 ppm CO₂ compared to ambient CO₂ and, secondly, 900 ppm CO₂ increased nitrogen fixation rates 1.5 to 3-fold compared to ambient CO₂ in the marine cyanobacterium Trichodesmium.⁶³,⁶⁴ Still, experiments carried out here did not rule out enhanced nitrate uptake at elevated CO₂. Irrespective, the addition of waste CO₂ from industry, carried out here did not rule out enhanced nitrate uptake at elevated CO₂. Hence net nitrogen assimilation increases significantly under elevated CO₂ (800 ppm). Increased nitrogen fixation under elevated CO₂ can be expected from a fully controlled indoor production system at ambient CO₂ and 800 ppm CO₂ concentrations, respectively. Dependent on the species, the annually harvested biomass contains 6.8–7.9 t protein ha⁻¹ per year at ambient CO₂ and 10.3–11.8 t protein ha⁻¹ per year at elevated CO₂.¹ 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.

### Table 2. Amino acid concentrations in biomass of A. filiculoides and A. pinnata grown under ambient and elevated (800 ppm) CO₂ concentrations (n = 3)

| Amino acid | Ambient CO₂ | Elevated CO₂ |
|------------|-------------|--------------|
|            | A. filiculoides | A. pinnata | A. filiculoides | A. pinnata |
| Arginine (Arg) | 69.5 ± 5.2b | 66.9 ± 4.7b | 71.4 ± 7.3a | 69.7 ± 4.4b |
| Histidine (His) | 21.4 ± 1.9b | 18.9 ± 1.1b | 21.7 ± 1.7a | 21.6 ± 0.3b |
| Isoleucine (Ile) | 50 ± 4.2 | 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 ± 0.9b | 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 ± 5.5b | 69.3 ± 6.0d | 71.6 ± 1.4c |
| Aspartic acid (Asp) | 104 ± 4.2b | 104 ± 6.5a | 99.6 ± 8.4b | 101 ± 1.8a |
| Aspartagine (Asn) | 11.6 ± 0.3b | 9.98 ± 0.5b | 11.8 ± 0.6a | 11.2 ± 0.1b |
| Cysteine (Cys) | 144 ± 18.2b | 137 ± 7.4a | 137 ± 9.8ab | 131 ± 2.0a |
| Glutamic acid (Glu) | 54.9 ± 4.6a | 55.2 ± 3.1a | 55.2 ± 5.3a | 55.5 ± 0.6b |
| Glutamine (Gln) | 42.9 ± 4.2a | 42.6 ± 2.4b | 45.6 ± 5.3a | 45 ± 0.3b |
| Glycine (Gly) | 49 ± 4.6a | 51.6 ± 3.4b | 50.3 ± 4.3c | 51.8 ± 0.8c |
| Alanine (Ala) | 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.
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.\textsuperscript{25,26,53,69} The amino acid composition of Azolla was previously suggested to limit the inclusion rate.\textsuperscript{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\textsubscript{2} treatment, differences were small (<10%) (Table 2). Noteworthy exceptions include concentrations of tryptophan and alanine, which increased by 18% and 17% when \textit{A. filiculoides} was grown in elevated versus ambient CO\textsubscript{2} concentrations. Concentrations of cysteine and histidine in \textit{A. filiculoides} decreased by 14% and 11%, respectively, in ferns grown at elevated versus ambient CO\textsubscript{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 \textit{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.\textsuperscript{53} Azolla essential amino acid composition, furthermore, met all requirements for growing pigs (50–88 kg) and adult humans (Fig. 5b).\textsuperscript{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.\textsuperscript{25,26,53,69} Lignin is, however, not present in Azolla, which instead contains (poly)phenolic tannins.\textsuperscript{35} These tannins likely decrease digestibility when binding to proteins upon digestion of the biomass.\textsuperscript{36,37} Total soluble (poly)phenols and crude lipids were therefore measured in biomass harvested from \textit{A. filiculoides} and \textit{A. pinnata} grown at ambient and elevated CO\textsubscript{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\textsuperscript{-1}; Fig. 6a) compared to foodstuff, such as fruits (1.47–6.67 g kg\textsuperscript{-1}), beans (1.26–6.67 g kg\textsuperscript{-1}) and nuts (0.56–19.2 g kg\textsuperscript{-1}).\textsuperscript{41,78–82} Soluble (poly)phenols were 3.3 times more abundant in \textit{A. pinnata}, compared to \textit{A. filiculoides}, reaching 69.1 g kg\textsuperscript{-1} DW (tannic acid equivalents) at ambient CO\textsubscript{2} (Fig. 6a). Crude lipid yields represented roughly 100 g kg\textsuperscript{-1} DW when ferns of either species were...
grown at ambient CO₂ (Fig. 6b). Hence, besides higher protein content, A. filiculoides contained fewer (poly)phenols than A. pinnata when grown at ambient CO₂, making it a more suitable protein feed.

In A. pinnata, elevated CO₂ altered neither lipids nor soluble (poly)phenols significantly. In A. filiculoides, however, elevated CO₂ significantly decreased (0.78-fold) crude lipid and increased (2.1-fold) total soluble (poly)phenols (Fig. 6). Although adding CO₂ 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 CO₂ waste streams, as elevating CO₂ concentrations to 800 ppm boosted productivity to 48.3 t DW ha⁻¹ per year without loss in protein content. Increasing CO₂ 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.

ACKNOWLEDGEMENTS
We thank Niño Paul Maynard Banayo, Stephan Haefele and Inez Hortense Slamet-Loedin from the International Rice Research Institute (IRRI, Philippines) for providing IRRI Azolla accessions. We further acknowledge funding from Climate KIC for the Pathfinder project AzozFaSt and financial support by the LPP foundation.

REFERENCES
1 United Nations, World population prospects: the 2015 revision, key findings and advance tables. Working Paper No. ESA/P/WP.241. 2015.
2 Lukovenkov AV, Belokurov VV and Varfolomeev SD, The stability and limits of human population growth. Moscow Univ Chem Bull 66:318–321 (2011).
3 Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF et al., Food security: the challenge of feeding 9 billion people. Science 327:812–818 (2010).
4 Speedy AW, Global production and consumption of animal source foods. J Nutr 133:40485–40535 (2003).
5 Shepon A, Eshel G, Noor E and Milo R, Energy and protein feed-to-food conversion efficiencies in the US. Environ Res Lett 11:105002 (2016).
6 Collinson ME, Smith SY, van Konijnenburg-van Cittert JHA, Batten DJ, Refda C, Barke J et al., New observations and synthesis of paleogene heterosporous water ferns. Int J Plant Sci 174:350–363 (2013).
7 Wagner GM, Azolla: a review of its biology and utilization. Bot Rev 63:1–26 (1997).
8 Lumpkin TA and Plucknett DL, Azolla: botany, physiology, and use as a green manure. Econ Bot 34:111–153 (1980).
9 van Hove C, Azolla. Its Multiple Uses with Emphasis on Africa. Rome, FAO, (1989).
10 van Hove C and Lejeune A, The Azolla–Anabaena symbiosis. Biol Environ 102:23–26 (2002).
11 Maejima K, Kitoh S, Uheda E and Shiomi N, Response of 19 Azolla strains to a high concentration of ammonium ions. Plant and Soil 234:247–252 (2001).
12 van der Werf A, Geerts RHEM, Jacobs FHH, Korevaar H, Oomes MJM and De Visser W, The importance of relative growth rate and associated traits for competition between species during vegetation succession, in Inherent Variation in Plant Growth. Physiological Mechanisms and Ecological Consequences, ed. by Lambers H, Poorter H and Van Vuuren MMi. Backhuys, Leiden, pp. 489–502 (1998).
13 Ran L, Larsson J, Vigil-Stenman T, Nylander JAA, Ininbergs K, Zheng W et al., Genome erosion in a nitrogen-fixing vertically transmitted endosymbiotic multicellular cyanobacterium. PloS ONE 5:e11486 (2010).
14 Peters GA and Meeks JC, The Azolla–Anabaena symbiosis: basic biology. Annu Rev Plant Physiol Plant Mol Biol 40:193 – 210 (1989).
15 Dijkstraen L, Brouwer P, Bolhuis H, Reichart G-J, Koppers N, Huettel B et al., Is there fowl play in the leaf pocket? The metagenome of floating fern Azolla reveals endophytes that do not fix N₂ but may denitrify. New Phytol 202:1069–1082 (2017). https://doi.org/10.1111/nph.
16 Brouwer P, Bräutigam A, Buiks JA, Tazelaa AOE, van der Werf A, Schütter U et al., Metabolic adaptation, a specialized leaf organ structure and vascular responses to diurnal N2 fixation by Nostoc azollae sustain the astonishing productivity of Azolla ferns without nitrogen fertilizer. Front Plant Sci 8:442 (2017).

17 Meeks JC, Steinberg N, Joseph CM, Enderlin CS, Jorgensen PA and Parker GA, Assimilation of exogenous and dinitrogen-derived 13NH4+ by Anabaena azollae separated from Azolla caroliniana Willd. Arch Microbiol 142:229–233 (1985).

18 Hung NVP, Watanabe S, Ishikawa S, Ohtake N, Suyeishi K, Sato T et al., Quantitative analysis of the initial transport of fixed nitrogen in nodulated soybean plants using 15N as a tracer. Soil Sci Plant Nutr 59:888–895 (2013).

19 Hall DO, Markov SA, Watanabe Y and Krishna Rao K, The potential applications of cyanobacterial photosynthesis for clean technologies. Photosynth Res 61:159–167 (1995).

20 Watanabe I, Ventura W, Masarca G and Eskew DL, Fate of Azolla spp. and urea nitrogen applied to wetland trout (Oryza sativa L.). Biol Fertil Soils 8:102–110 (1989).

21 Xu H, Zhu B, Liu J, Li D, Yang Y, Zhang K et al., Azolla planting reduces methane emission and nitrogen fertilizer application in double rice cropping system in southern China. Agron Sustain Dev 37:29 (2017).

22 Liu J, Xu H, Jiang Y, Zhang K, Hu Y and Zeng Z, Methane emissions and microbial communities as influenced by dual cropping of Azolla along with early rice. Environ Exp Biol 27:215–221 (1987).

23 Buckingham KW, Ela SW, Morris JG and Goldman CR, Nutritive value of Azolla caroliniana biomass. J Agric Food Chem 26:1230–1234 (1978).

24 Cary PR and Weerts PGJ, Growth and nutrient composition of Caryophyllus lamarckii and Azolla filiculoides. Biochem System Ecol 5:102–110 (1989).

25 Alalade OA and Iyayi EA, Chemical composition and the feeding value of Azolla (Azolla pinnata) meal for egg-type chicks. Int J Poultry Sci 8:123–134 (2009).

26 Leterme P, Londoño AM, Ordoñez DC, Rosales A, Estrada F, Bindelle J et al., Nutritional value and intake of aquatic ferns (Azolla filiculoides Lam. and Salvinia molesta Mitchell) in sows. Anim Feed Sci Technol 155:55–64 (2010).

27 Datta SN, Culture of Azolla and its efficacy in diet of Labeorohita. Aquaculture 310:376–379 (2011).

28 Paolotti C, Bocci F, Lercker G, Capella P and Materassi R, Lipid composition of Azolla caroliniana biomass and its seasonal variation. Phytochemistry 26:1045–1047 (1987).

29 Abou Y, Fiogbé ED and Michá J-C, A preliminary assessment of growth and production of Nile tilapia, Oreochromis niloticus L., fed Azolla-based-diets in earthen ponds. J Appl Aquacult 19:55–69 (2007).

30 Borer P, van der Werf A, Schleppmann H, Reichart GJ and Nierop KJG, Lipid yield and composition of Azolla filiculoides and the implications for biodiesel production. Bioenergy Res 9:369–377 (2015).

31 Dewanjii A, Amino acid composition of leaf proteins extracted from some aquatic weeds. J Agric Food Chem 41:1232–1236 (1993).

32 Sánchez-Vizcaíros G, Ferrera-Cerrato R and Alarcón A, Short-term effects of arsenate-induced toxicity on growth, chlorophyll and carotenoid contents, and total content of phenolic compounds of Azolla filiculoides. Water Air Soil Pollut 217:455–462 (2011).

33 Forni C, Braglia R, Harren FJM and Cristescu SM, Stress responses of Oreochromis niloticus to anionic surfactants sodium-dodecyl-sulphate (SDS). Aquat Toxicol 80:117–133 (2007).

34 Deval CG, Mane AV, Joshi NP and Saratale GD, Phytoremediation of Arsenic contaminated water by Azolla–Anabaena association: historical perspective. J Appl Phycol 28:1–15 (2016).

35 Canini A, GrilliCaiola M and Mascini M, Ammonium content, nitrogenease activity and heterocyst frequency within the leaf cavities of Azolla filiculoides Lam. FEBS Microbiol Lett 71:205–210 (1990).

36 Deval CG, Mane AV, Joshi NP and Saratale GD, Phytoremediation of Arsenic contaminated water by Azolla–Anabaena association: historical perspective. J Appl Phycol 28:1–15 (2016).

37 Bravo L, Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 56:317–333 (1998).

38 International Organization for Standardization, ISO 16634-1. Determination of nitrogen in cereal and vegetable proteins. ISO 16634-1:2015 (2017).

39 Bravo L, Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 56:317–333 (1998).

40 European Pharmacopoeia Commission & Council of Europe, Ph. Eur. 2.2.56 Amino Acid Analysis. Council of Europe, Strasbourg (2016).

41 Cachelin EM, Bryant QM, Green AM, Abbey YA, Wangla GW and Walker RB, Thorough study of reactivity of various compound classes toward the Folin–Ciocalteu reagent. J Agric Food Chem 58:8139–8140 (2010).

42 Debusk WF and Reddy KR, Growth and nutrient uptake potential of Azolla caroliniana Willd. and Salvinia rotundifolia Willd. as a function of early-season temperature. Environ Exp Biol 27:215–221 (1987).

43 Deval CG, Mane AV, Joshi NP and Saratale GD, Phytoremediation of Arsenic contaminated water by Azolla–Anabaena association: historical perspective. J Appl Phycol 28:1–15 (2016).

44 Sah RN, Goyal SS and Rains DW, Interactive effects of exogenous combined nitrogen and phosphorus on growth and nitrogen fixation by azolla. Plant Soil 117:1–8 (1989).

45 Debush WF and Reddy KR, Growth and nutrient uptake potential of Azolla caroliniana Willd. and Salvinia rotundifolia Willd. as a function of early-season temperature. Environ Exp Biol 27:215–221 (1987).

46 Ziegler P, Adelmann K, Zimmer S, Schmidt C and Appenroth K, Relative growth rates of duckweeds (Lemnaceae): the most rapidly growing higher plants. Plant Biol 17:33–41 (2015).

47 Goudriaan J and Monteith JL, A mathematical function for crop growth based on light interception and leaf area expansion. Ann Bot 55:695–701 (1990).

48 Ito O and Watanabe I, The relationship between combined nitrogen uptake and nitrogen fixation in Azolla–Anabaena symbiosis. New Phyto 95:647–654 (1983).

49 Shi D and Hall DO, The Azolla–Anabaena association: historical perspective, symbiosis and energy metabolism. Bot Rev 54:333–386 (1988).

50 Beccera M, Murgueitio E, Reyes G and Preston TR, Azolla filiculoides as partial replacement for traditional protein supplements in diets for growing fattening pigs based on sugar cane juice. Livestock Res Rural Dev 2:15–22 (2010).

51 Rajcan I and Tollenaar M, Source:sink ratio and leaf senescence in maize. I. Dry matter accumulation and partitioning during grain filling. Field Crops Res 60:245–253 (1999).

52 Diepenbrock W, Yield analysis of winter oilseed rape (Brassica napus L): a review. Field Crops Res 67:35–49 (2000).

53 Magomya AM, Kubmarawa D, Ndahi JA and Yebpella GG, Determination of plant proteins via the Kjeldahl method and amino acid analysis: a comparative study. Int J Sci Technol Res 3:68–72 (2014).

54 Zhao Y, Fang J, Yin Y, Huang J, Bao S, Fu T et al., Potential of duckweed in the conversion of wastewater nutrients to valuable biomass: a pilot-scale comparison with water hyacinth. Bioresour Technol 163:82–91 (2014).

55 Blikker P, van Kriempen MM, van Wijkelaar MM, Houten Tan B, Scaccia N, Velial JW et al, Biofimery of the green seaweed Ulva lactuca to produce animal feed, chemicals and biofuels. J Appl Phycol 28:1–15 (2016).

56 Canini A, GrilliCaiola M and Mascini M, Ammonium content, nitrogenease activity and heterocyst frequency within the leaf cavities of Azolla filiculoides Lam. FEMS Microbiol Lett 71:205–210 (1990).

57 Houten Tan B, Djikstra P, Johnson DW, Hinkle CR and Drake BG, Elevated CO2 increases nitrogen fixation and decreases soil nitrogen mineralization in Florida scrub oak. Glob Chang Biol 5:781–789 (1999).

58 Luscher A, Hartwig UA, Suter D and Nosberger J, Direct evidence that symbiotic N2 fixation in fertile grassland is an important trait for a strong response of plants to elevated atmospheric CO2. Glob Chang Biol 6:655–662 (2000).

59 Southan JF and Hartwig UA, The effects of elevated CO2 on symbiotic N2 fixation: a link between the carbon and nitrogen cycles in grassland ecosystems. Plant and Soil 187:321–332 (1996).
Chinnasamy S, Ramakrishnan B, Bhatnagar A, Goyal SK and Das KC, Carbon and nitrogen fixation by Anabaena fertilissima under elevated CO2 and temperature. *J Freshwater Ecol* **24**:587–596 (2009).

Levitan O, Rosenberg G, Setlik I, Setlikova E, Grigel J, Klepetar J et al., Elevated CO2 enhances nitrogen fixation and growth in the marine cyanobacterium Trichodesmium. *Glob Chang Biol* **13**:531–538 (2007).

Sangina N and Van Hove C, Amino acid composition of Azolla as affected by strains and population density. *Plant Soil* **117**:263–267 (1989).

Salvagiotti F, Cassman KG, Specht JE, Walters DT, Weiss A and Dobermann A, Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. *Field Crops Res* **108**:1–13 (2008).

Anglade J, Billen G and Garnier J, Relationships for estimating N2 fixation in legumes: incidence for N balance of legume-based cropping systems in Europe. *Ecosphere* **6**:37 (2015).

Pugesgaard S, Schelde K, Larsen SU, Lærke PE and Jørgensen U, Comparing annual and perennial crops for bioenergy production: influence on nitrate leaching and energy balance. *GCB Bioenergy* **7**:1136–1149 (2015).

Abdel-Tawwab M, The preference of the omnivorous–macrophagous, *Tilapia zillii* (Gervais), to consume a natural free-floating Fern, *Azolla pinnata*. *J World Aquacult Soc* **39**:104 –112 (2008).

Ravindran V, Abdollahi MR and Bootwalla SM, Nutrient analysis, metabolizable energy, and digestible amino acids of soybean meals of different origins for broilers. *Poult Sci* **93**:2567–2577 (2014).

Eweedah N, Gundel J and Mátrai T, Protein degradability, amino acid composition, trypsin inhibitor and urease activity of raw and heat-treated fullfat soybean. *Arch Tierernahr* **50**:361–367 (1997).

Karr-Lilienthal LK, Grieshop CM, Spears JK and Fahey GC Jr, Amino acid, carbohydrate, and fat composition of soybean meals prepared at 55 commercial US soybean processing plants. *J Agric Food Chem* **53**:2146–2150 (2005).

FAO, *Dietary Protein Quality Evaluation in Human Nutrition*. FAO, Rome (2013).

NRC, *Nutrient Requirements of Swine*, 10th edn. National Academy Press, Washington, DC (1998).

NRC, *Nutrient Requirements of Dairy Cattle*, 7th edn. National Academy Press, Washington, DC (2001).

NRC, 1994: *Nutrient Requirements of Poultry*, 9th edn. National Academy Press, Washington, DC.

Santiago CB and Lovell RT, Amino acid requirements for growth of Nile tilapia. *J Nutr* **118**:1540 –1546 (1988).

Nicoletto C, Tosini F and Sambo P, Effect of grafting and ripening conditions on some qualitative traits of ‘Cuore di bue’ tomato fruits. *J Sci Food Agric* **93**:1397–1403 (2013).

Martínez R, Torres P, Meneses MA, Figueroa JP, Pérez-Álvarez JA and Viuda-Martos M, Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate. *Food Chem* **135**:1520–1526 (2012).

Wolosiak R, Druzynska B, Pieczyk M, Worobiej E, Majewska E and Lewicki PP, Influence of industrial sterilisation, freezing and steam cooking on antioxidant properties of green peas and string beans. *Int J Food Sci Technol* **46**:93–100 (2011).

Malencic D, Popovic M and Miladinovic J, Phenolic content and antioxidant properties of soybean (*Glycine max* (L.) Merr.) seeds. *Molecules* **12**:576–581 (2007).

Kornsteiner M, Wagner K and Elmadfa I, Tocopherols and total phenolics in 10 different nut types. *Food Chem* **98**:381 – 387 (2006).