Interacting effects of atmospheric CO$_2$ enrichment and solar radiation on growth of the aquatic fern *Azolla filiculoides*

MONIQUE M. L. VAN KEMPEN*, ALFONS J. P. SMOLDERS*, GERARD M. BÖGEMANN†, LEON P. M. LAMERS* AND JAN G. M. ROELOFS*

*Department of Aquatic Ecology and Environmental biology, Institute for Water and Wetland Research, Radboud University Nijmegen, Nijmegen, the Netherlands
†B-WARE Research Centre, Nijmegen, the Netherlands.
‡Department of Experimental Plant Ecology, Institute for Water and Wetland Research, Radboud University Nijmegen, Nijmegen, the Netherlands

SUMMARY

1. *Azolla* spp. rank among the fastest growing plants in the world. Increased atmospheric CO$_2$ concentrations can be expected to have a strong impact on the biomass production of this fast growing floating fern, especially at northern latitudes.

2. We investigated the growth of the northernmost occurring species, *A. filiculoides*, under past (Eocene), present and predicted future CO$_2$ levels. To study the interacting effects of temperature and solar radiation, we performed the atmospheric CO$_2$ fertilisation experiments in spring, summer and autumn.

3. *Azolla filiculoides* grown at 1600 ppm CO$_2$ produced twice as much biomass as when grown at 400 ppm CO$_2$ in spring and autumn. However, high summer temperature and solar radiation seemed to significantly lower its maximum growth potential, presumably as a result of a metabolic shift as indicated by water loss.

4. Nutrient availability became more important at higher atmospheric CO$_2$ concentration. In addition, high temperature and solar radiation affected the nitrogen concentrations in *A. filiculoides*, most likely by inducing photorespiration and photoinhibition.

5. Our results indicate that temperate *Azolla* may become more productive in temperate regions as a result of rising atmospheric carbon dioxide concentrations. In addition, our results can help to explain the reported massive *Azolla* event in the Eocene.

Keywords: *Azolla filiculoides*, climate change, CO$_2$ enrichment, photosynthetic active radiation, temperature

Introduction

Azollaceae represent a family of small, free floating freshwater ferns ranking among the fastest growing plants in the world. Although the genus is generally considered a noxious weed in natural ecosystems, it is valued as a green fertiliser in paddy fields as it is able to fix atmospheric dinitrogen (N$_2$) by the activity of its symbiotic cyanobacteria *Anabaena azollae* (Peters & Mayne, 1974a,b), and also is a strong accumulator of phosphorus (Lumpkin & Plucknett, 1982). Furthermore, it is being widely applied as a phytoremediation tool for contaminated surface waters and wastewaters, and for the production of biogas and animal food (Wagner, 1997; van Hove & Lejeune, 2002).

*Azolla filiculoides* is the northernmost occurring *Azolla* species (Lumpkin & Plucknett, 1980). It can be found in ditches, ponds and water reservoirs that show relatively high phosphorus (P) concentrations (Bloemendaal & Roelofs, 1988; Janes, 1998). Nitrogen (N) is often limiting biomass production in these waters, which therefore offer the competitive advantage to plants and cyanobacteria that, like the *Azolla-Anabaena* symbiosis, are able to fix N$_2$ from the atmosphere to meet their N demand.
Variation in temperature and solar radiation probably explains the seasonal abundance of the species and the strong variations observed between years (Peeters et al., 2013). Light saturation of Azolla photosynthesis generally occurs at a relatively low level of 375 W m⁻² (Lumpkin & Plucknett, 1980), and as a result, the growth of Azolla spp. is usually reduced in full sunlight (Wagner, 1997). The optimal temperature range for Azolla spp. is assumed to lie between 18 and 28 °C (Wagner, 1997), and A. filiculoides is often reported to be the species least tolerant of high temperatures, but most tolerant of low temperatures (Watanabe & Berja, 1983; Wagner, 1997; Janes, 1998).

As Azolla spp. fix atmospheric CO₂ via the C3 pathway (Ray et al., 1979), high atmospheric CO₂ concentrations can be expected to enhance their growth rates. In temperate regions, where biological process rates may still increase towards optima rather than in warmer regions, the relative and combined effects of CO₂ enrichment and other climate variables are probably stronger (Rosenzweig & Liverman, 1992). Present day atmospheric CO₂ concentrations are around 400 volume parts per million (ppm), but are estimated to reach values between 550 and 970 ppm by the end of this century (IPCC, ISAM model, 2007). In the past, atmospheric CO₂ concentrations during the Eocene are estimated to have varied between 200 and 300 times pre-industrial values (Pearson & Palmer, 2000; Royer et al., 2001; Yapp, 2004; Brinkhuis et al., 2006). The Azolla fossil record begins in the mid Cretaceous (Collinson, 2001, 2002), and high abundances of fossil spores of Azolla spp. have been reported from Eocene marine sediments of the central Arctic Ocean and adjacent Nordic (Brinkhuis et al., 2006; Collinson et al., 2009, 2010; van der Burgh et al., 2013). The mass occurrence of Azolla during the Eocene coincided with declining atmospheric CO₂ concentrations (Pearson & Palmer, 2000; Pagani et al., 2005) and it has been suggested that the massive growth of Azolla and its burial in the deep ocean water layers of the Eocene Arctic basin contributed to the climatic shift from greenhouse to icehouse by creating a significant biological sink for atmospheric CO₂ (Brinkhuis et al., 2006; Speelman et al., 2009).

We studied the growth responses of temperate A. filiculoides at mean diurnal atmospheric CO₂ concentrations of 400, 1000 and 1600 ppm during spring, summer and autumn. We expected temperate Azolla to grow faster at elevated carbon dioxide levels. However, as the growth of Azolla spp. is usually reduced in full sunlight and temperate A. filiculoides is intolerant of temperatures >28 °C, we expected the growth-promoting effect of high carbon dioxide levels to be reduced in summer. Our study provides information about Azolla biomass production rates under different climate conditions in temperate regions. The results can also help explain the reported massive Azolla event in the Eocene.

Methods

Species collection and cultivation

Azolla filiculoides was collected in the Netherlands (N51°82′29″, E5°87′16″) and cultivated for several months in the experimental greenhouse of the Radboud University Nijmegen, the Netherlands. Plant stocks were grown in an artificial nutrient solution based on water quality data for natural Azolla stands (de Lyon & Rollofs, 1986). The solution was refreshed twice a week and contained 1.75 mM NaHCO₃, 1.75 mM CaCl₂, 25 μM NaH₂PO₄, 1 mM K₂SO₄, 1 mM MgSO₄, 10 μM Fe-EDTA, 1 μM CuSO₄, 20 μM MnCl₂, 10 μM ZnSO₄, 3 μM Na₂MoO₄, 20 μM H₃BO₃ and 4 μM CoCl₂ (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). No nitrogen was added to the solution.

Experimental design

Azolla filiculoides was grown in glass aquaria with a water volume of 15.6 L (L × W × H: 25 cm × 25 cm × 25 cm) and a headspace of 3.4 L. Except for a gas overflow outlet, the aquaria were airtight. They were placed randomly in a water bath. As air temperature is more important for Azolla growth during winter, whereas water temperature is more important for Azolla growth during summer (Watanabe & Berja, 1983), and the effect of solar radiation on water temperature is strongly influenced by vegetation cover (van Hove, 1989), we decided to keep the water temperature constant at 15 °C, which reflects the mean annual temperature of inland surface waters in the Netherlands. The water temperature minimised the effect of microclimate differences and buffered the air temperature fluctuations in the greenhouse, so that these were comparable to field conditions. The underwater parts of the aquaria were kept dark by plastic foil.

Azolla filiculoides was grown at atmospheric CO₂ concentrations set to 400 (430 ± SE 16), 1000 (1005 ± SE 43) and 1600 (1665 ± SE 23) ppm CO₂ (n = 8 for each atmospheric CO₂ concentration). The different atmospheric CO₂ concentrations were attained by mixing compressed air with custom-made mixtures of various concentrations of CO₂ in synthetic air (Air Liquide, Eindhoven, the Netherlands) using mass flow controllers (Bron-
khorst Hi-Tec, Veenendaal, the Netherlands). Each gas mixture was uniformly distributed among the headspaces of the aquaria in order to refresh the total air volume of each aquarium every 5 min. CO₂ concentrations in the headspaces of the aquaria were monitored by taking air samples at the gas overflow outlets using syringes, and measuring them directly using an Infrared Gas Analyser (IRGA, type ABB Advance Optima, Etten-Leur, the Netherlands). These measurements showed that during the week the pre-set atmospheric CO₂ concentrations in the aquaria decreased to 385 ± 33, 886 ± 104 and 1465 ± 76 ppm respectively (n = 24; 3 weeks of measuring eight replicates per atmospheric CO₂ treatment) due to the increase in Azolla biomass during the week. The amount of Azolla biomass in each aquarium was reset weekly.

To study the interacting effect of atmospheric CO₂ concentrations and seasonal variation in solar radiation (J cm⁻²), radiation sum (J cm⁻²) and air temperature (air T), experiments were conducted in early spring (19 February to 16 March), summer (17 June to 13 July) and early autumn (28 August to 23 September) 2008. Radiation, radiation sum and air T were logged every hour using the Priva monitoring system of the greenhouse complex. The average daily radiation and radiation sum, and the average mean, highest and lowest air T were calculated (Table 1). The experiments lasted 27 days.

**Plant growth**

The experiments were started by introducing 10 g of fresh *A. filiculoides* into each aquarium, covering approximately a quarter of the water surface. Total fresh biomass in each aquarium was determined weekly by collecting the plants from the aquarium and carefully blotting them dry with tissue paper before weighing. Only 10 g of fresh Azolla was returned to each aquarium to prevent overcrowding, while the rest of the biomass was dried (48 h, 60 °C) to calculate moisture contents and dry weight (dw) production rates, and for the analyses of nutrient composition.

Cumulative biomass was calculated on a dw basis by subtracting the calculated dw at the beginning of the sampling period from the dw at the end of the sampling period, and adding this to the cumulative biomass of the previous sampling period. Relative growth rates (RGRs) were calculated on a dry weight basis: 

\[
RGR = \frac{(\ln W_2 - \ln W_1)}{(t_2 - t_1)}, \quad \text{where} \quad W_1 \text{ and } W_2 \text{ represented the dw at the beginning (}t_1\text{) and end (}t_2\text{) of the sampling period.}
\]

**Plant nutrient analyses**

Dried plant material was ground in liquid nitrogen after which 200 mg of the plant material was digested in 4 mL HNO₃ (65%) and 1 mL H₂O₂ (35%) (Kingston & Haswell, 1997), in Teflon vessels heated in an Ethos D microwave (Milestone, Sorisole Lombardy, Italy). The digestates were analysed for elemental P concentrations using an inductively coupled plasma emission spectrophotometer (ICP-OES; model IRIS Intrepid II XDL, Thermo Fisher Scientific, Franklin U.S.A.). For analyses of total C and total N concentrations, the ground plant material was further homogenised using a ball mill (type Mixer Mill 301, Retsch GmbH, Germany), and 2 mg of the plant material was weighed in pressed, ultra-lightweight tin capsules that were analysed by an elemental CNS analyser (model EA 1110, Carlo Erba; Thermo Fisher Scientific, Franklin, U.S.A.). In both the elemental composition analyses, and in the plant total C and N analyses, standard reference materials were included.

**Nutrient solution analyses**

Fresh nutrient solution was supplied at a rate of 0.2 L h⁻¹ from containers using peristaltic pumps. The water level was kept constant by an overflow outlet. Nutrient solutions of all aquaria were sampled weekly....

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**Table 1** The average solar radiation (J cm⁻²), radiation sum (J cm⁻²) and mean, maximum and minimum air temperature (air T mean, air, T max, air, min in °C) measured during 27 days in the different seasons ± SE. Significant differences between seasons, as determined with Bonferroni post hoc analyses, are indicated by different letters.

<table>
<thead>
<tr>
<th></th>
<th>Spring</th>
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<td>P</td>
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</table>

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for nutrient analyses using 30 mL acid-rinsed glass bottles. Total concentrations of Al, Ca, Fe, K, Mg, Mn, Na, P, S, Si and Zn were determined by ICP-OES (model IRIS Intrepid II XDL, Thermo Fisher Scientific, Franklin, U.S.A.) using standard reference materials. Fe, Mn and P concentrations in the aquaria showed small fluctuations during the experiments, and on average decreased by 38, 38 and 31%, respectively, whereas all other nutrient concentrations remained at their pre-set concentrations (data not shown).

pH was measured using an electrode with a double Ag/AgCl reference (model, HI 1043; Hanna Instruments, Ijsselstein, the Netherlands) and total inorganic carbon (TIC) as CO2 with an infrared carbon analyser (IRGA; model ABB Advance Optima, Zurich, Switzerland) after conversion of all TIC to CO2 in 2.1% phosphoric acid and stripping by N2. Neither pH nor TIC showed differences among the different atmospheric CO2 treatments during the different seasons (data not shown).

Statistical analysis

We used IBM SPSS Statistics 20 for Windows. Mixed Linear Models (MLM) were used for analyses of the plant growth rates, plant moisture contents and plant nutrient concentrations. Time was nested as a repeated co-variable of the aquaria and covariance type was selected based on the smallest Akaike's Information Criterion. Season (spring, summer, autumn) and atmospheric CO2 concentration (400 ppm, 1000 ppm, 1600 ppm) were entered as fixed factors. Pairwise comparisons were made based on estimated marginal means and these were adjusted for multiple comparisons using Bonferroni. When the MLM gave a significant interaction effect between season and atmospheric CO2, data were further analysed per season using ANOVA with atmospheric CO2 concentration as a fixed factor to determine the differences between the CO2 treatments.

Cumulative biomasses were analysed using ANOVA, considering the effects of season and atmospheric CO2 concentration and their interaction. Pairwise comparisons were made based on estimated marginal means and when a significant interaction was found, data were further analysed per season to determine the differences between the CO2 treatments using ANOVA.

Differences in climate variables (radiation, radiation sum and mean, high and low air T) were analysed using ANOVA with season as a fixed factor. For the analyses of air T, univariate contrasts in the ANOVA were set on repeated to correct for the fact that temperature on \( t = 2 \) depends on temperature on \( t = 1 \).

In ANOVAs where only season or only atmospheric CO2 was considered as a fixed factor, differences were determined using Bonferroni post hoc analyses. When Levene’s Test returned a significant result, Games How- ell post hoc analyses were used. In all statistical analyses, care was taken that the model assumptions were met. For this purpose, the following variables were transformed: Square root (SQRT) (Cumulative biomass), dry weight/fresh weight ratio (i.e. for moisture content), SQRT [SQRT (P concentration)], Log [SQRT (N concentration)], Log [SQRT (C concentration)], SQRT (Radiation sum), SQRT (Radiation), SQRT [SQRT (T mean)], 1/ SQRT (T max).

We performed linear regression analyses to identify how much of the variation in the relative growth rates of \( A. \) filiculoides could be explained by the different variables. First z-scores were calculated of the observed variables to a scale with a mean of 0 and standard deviation (SD) of 1, and entered in a factor analysis to fix collinearity problems. The factor analysis was performed (Extraction Method: Principal Component Analysis; Rotation Method: Varimax with Kaiser normalisation) to identify which of the following set of observed variables could explain the pattern of correlations the most: the atmospheric CO2 concentration, the radiation, and the maximum and minimum air T. The factor scores resulting from the factor analyses were entered in the linear regression analyses with RGR as the dependent variable. Similar analyses were performed per CO2 treatment to study possible differences in the effects of the climate variables on the RGRs of the plants.

Results

Plant growth

In spring \( A. \) filiculoides doubled its biomass in 1.5 to 3 days. Just before the weekly harvests, the plants generally covered the water surface completely. In all three CO2 treatments, the plant covers were multilayered and green, especially in the highest CO2 treatment where the leaves sometimes grew in a more upright position. In autumn, \( Azolla \) also doubled its biomass in 2.5 to 3 days, though in this experiment, the plants were more tightly stacked and some had turned darker green, especially at the higher atmospheric CO2 concentrations. In summer, \( A. \) filiculoides did not gain much biomass between sampling times, and cyanobacteria started to grow among the plants. At the end of the last sampling period, the plants mostly occurred in single-layered groups that were almost completely enclosed by a relatively thick
floating cyanobacterial bed. Many of the plants had turned from green to dark green and red. In a light microscopic examination of the floating cyanobacterial beds, *Anabaena* spp., *Planktothrix* spp., and *Nostoc* spp. were recognised.

In line with the general appearances of the plants in the different experiments, the cumulative biomasses of *A. filiculoides* (Fig. 1A) differed among seasons (Table 2). Highest cumulative biomasses were obtained in spring and lowest in summer. Higher CO₂ concentrations generally resulted in higher cumulative biomasses, but the dose–responses differed among seasons (Table 2). In spring [ANOVA: $F(2) = 74.78$, $P < 0.001$] and autumn [ANOVA: $F(2) = 47.26$, $P < 0.001$], the cumulative biomasses of *A. filiculoides* increased with increasing CO₂ concentrations. In spring, the cumulative biomass of the plants grown at the highest CO₂ treatments (1600 ppm) amounted to 7 g dry weight, which was 1.4 and 2.5 times higher than the cumulative biomass of the plants grown at 1000 ppm and 400 ppm atmospheric CO₂ respectively. The cumulative biomasses of *A. filiculoides* in autumn were somewhat lower, amounting to $5.56 \pm 0.2$, $4.58 \pm 0.19$ and $3.06 \pm 0.14$ (mean $\pm$ SE) g dry weight in the 1600, 1000 and 400 ppm CO₂ treatments respectively. In summer, a difference was found only between the 400 and 1000 ppm CO₂ treatments (ANOVA: $F(2) = 3.90$, $P < 0.05$).

Relative growth rates (RGRs) of *A. filiculoides* (Fig. 1B) differed among seasons (Table 2). Highest RGRs were measured in spring, followed by those in autumn and summer. Higher atmospheric CO₂ concentrations generally resulted in higher RGRs, but there were differences among seasons (Table 2). In spring RGRs increased with increasing concentrations, but only up to 1000 ppm CO₂ (ANOVA: $F(2) = 15.41$, $P < 0.001$). The same was true for the RGRs measured in autumn (ANOVA: $F(2) = 11.69$, $P < 0.001$). In summer, atmospheric CO₂ had no effect on RGR (ANOVA: $F(2) = 1.57$, ns).

Moisture contents of *A. filiculoides* (Fig. 1C) differed among seasons (Table 2), being higher in spring than in autumn and lowest in summer. Higher atmospheric CO₂ concentrations reduced the moisture contents of the plants, regardless of season (Table 2).

**Plant growth in relation to climate variables**

The linear regression model explained 62% ($R^2 = 0.6250$) of the variation in the data and showed that air T and radiation, and to a lesser extent atmospheric CO₂, had a significant effect on the relative growth rates of the

**Fig. 1** (a) Cumulative biomasses after 27 days (g DW), (b) relative growth rates (g g⁻¹ DW day⁻¹) and (c) moisture contents (% of fresh weight) of *A. filiculoides* grown at 400, 1000 and 1600 ppm atmospheric CO₂ during different seasons (means $\pm$ SE). For cumulative biomasses $n = 8$. For RGRs $n = 24$ for spring and $n = 32$ for summer and autumn. For moisture contents $n = 32$ for spring and $n = 40$ for summer and autumn. Differences between CO₂ treatments are indicated by different letters and are specified separately for each season. Note that the moisture content y-axis intercept is at 90% and that the statistics for this variable can be found in Table 2.

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Table 2 Results of the ANOVA considering the effects of season and atmospheric CO\textsubscript{2} and their interacting effects on the cumulative biomass of \textit{A. filiculoides} after 27 days, and the results of the MLMs, considering the effects of season and atmospheric CO\textsubscript{2} and their interacting effect on the RGRs and moisture contents of the plants. The results of the ANOVA per CO\textsubscript{2} treatment are given in the text and in Fig 1.

<table>
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<tr>
<th>Cumulative biomass (g)</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
<th>Bonferroni adjusted pairwise comparisons</th>
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<table>
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<th>Relative growth rate (g g\textsuperscript{-1} dry weight day\textsuperscript{-1})</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
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<tbody>
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<th>$P$</th>
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<td>1.756</td>
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Plants (ANOVA: $F(4) = 11.671$, $P < 0.001$ (Table S1). Minimum air T was most important in determining the growth rate of the plants, followed by radiation and maximum air T, and finally atmospheric CO\textsubscript{2} concentration (Table S1). Running the linear regression model on the individual atmospheric CO\textsubscript{2} treatments showed that none of the climatic variables could significantly explain the RGR variation of \textit{A. filiculoides} in the 400 ppm treatment. In the 1000 ppm atmospheric CO\textsubscript{2} treatment, radiation was the most important predictor variable, whereas in the 1600 ppm atmospheric CO\textsubscript{2} treatment minimum and maximum air T were additional predictor variables (Table S1).

Plant nutrient analyses

Phosphorus concentrations in \textit{A. filiculoides} were lower in autumn than in the other seasons (Table 3). Higher atmospheric CO\textsubscript{2} concentrations generally resulted in lower plant P concentrations, but not to the same extent for the different seasons (Table 3). In spring [ANOVA: $F(2) = 22.73$, $P < 0.001$], summer [ANOVA: $F(2) = 8.03$, $P < 0.001$] and autumn [ANOVA: $F(2) = 14.75$, $P < 0.001$] P concentrations decreased at and above 1000 ppm CO\textsubscript{2} (Table 3).

Plant N concentrations were significantly higher in spring and autumn than in summer (Table 4). Generally,
N concentrations decreased with increasing atmospheric CO$_2$, but not to the same extent for the different seasons (Table 4). In spring, plant N concentrations decreased at higher atmospheric CO$_2$ concentrations [ANOVA: $F(2) = 26.51$, $P < 0.001$], whereas in autumn N concentrations only decreased for the lowest two CO$_2$ treatments [ANOVA: $F(2) = 62.11$, $P < 0.001$]. For the summer [ANOVA: $F(2) = 0.29$, ns] experiment, no differences were found (Table 4).

Carbon concentrations in $A$. filiculoides were found to differ among seasons (Table 5), being highest in autumn, followed by spring and summer. Unlike for P and N, there was no effect of the atmospheric CO$_2$ concentration on the C concentrations of the plants. However, small differences were found among seasons (Table 5). In summer, plant C concentrations in the 1000 and 1600 ppm CO$_2$ treatments were higher than those in the 400 ppm treatment [ANOVA: $F(2) = 6.01$, $P < 0.01$]. In autumn, C concentrations in the 1000 ppm atmospheric CO$_2$ treatment were significantly higher than those in the 1600 ppm treatment [ANOVA: $F(2) = 3.17$, $P < 0.05$]. Spring [ANOVA: $F(2) = 0.11$, ns] did not show differences among CO$_2$ treatments (Table 5).

**Discussion**

Plant growth responses in relation to atmospheric CO$_2$ and climate variables

The study investigated the interacting effects of atmospheric CO$_2$ enrichment and solar radiation on the growth responses of temperate $A$. filiculoides. In line with
our hypothesis, the results show that its growth strongly increased with increasing atmospheric CO2 concentrations. A fourfold increase in atmospheric CO2 led to a twofold stimulation of growth. However, high air T and radiation significantly lowered the maximum growth potential of the plants, especially at high atmospheric CO2 concentrations. The high air T effect on Anabaena filiculoides is consistent with the results of van der Heide et al. (2006). In contrast, CO2 enrichment generally stimulates the photosynthesis of subtropical Azolla strains at radiation up to 1200 W m$^{-2}$ (Allen et al., 1988), while enhancing survival at mean daily temperatures of up to 34 °C (Idso et al., 1989). The different outcome in our research may have resulted from differences in summer day lengths, which are shorter at lower latitudes than at higher latitudes. In addition, they may be explained by phenotypical and ecotypical differences among Azolla species with respect to their temperature optima and sensitivity to high light intensities (Uheda, Kitoh & Shiomi, 1999; Lechno-Yossef & Nierzwicki-Bauer, 2002).

With respect to future climate change scenarios, our results suggest that temperate Anabaena filiculoides will increase its biomass production rates in response to elevated atmospheric CO2 concentrations in spring and autumn, but will only be able to profit in summer as long as very high radiation and high air temperatures do not occur.

### Growth limitation

Azolla filiculoides generally showed lower tissue N and P concentrations at higher CO2 levels than at ambient concentrations. This shows that nutrient availability becomes increasingly important at higher CO2 levels due to increasing RGRs. Nutrient limitation may cause starch accumulation, especially at higher CO2 concentrations, as it changes the source to sink ratio in plants (Idso, Kimball & Mauney, 1988; Geigenberger, 2011). Starch accumulation in turn may lower plant moisture contents and alter the fixed carbon sources within the plant tissue (Idso et al., 1988; Geigenberger, 2011), which may explain the differences in moisture contents and plant C concentrations among seasons and CO2 treatments, especially for the autumn and summer.

In autumn, some of the plants had turned darker green, which might indicate that P was becoming limiting. The concentrations we found in the 1000 and 1600 ppm CO2 treatments were at the low end of the range commonly found in Azolla; P concentrations in the plant tissue may reduce to ~0.2% (65 μmol g$^{-1}$) of the dry weight, at which point it becomes limiting for growth. On the other hand, when Azolla growth is limited by another environmental factor at relatively abundant P supply, the plants may accumulate P up to 1.6% of their dry weight (Lumpkin & Plucknett, 1982).

In summer, plant N concentrations became much lower than in the other seasons. As no N was added to the nutrient solutions, this suggests that in this experiment, the nitrogenase activity of Anabaena azollae, living inside the frond cavities of Anabaena filiculoides, was reduced. The fact that free-living Anabaena spp. were also thriving in the aquaria in the summer experiment, makes it unlikely that cyanobacterial growth was inhibited by high T, as was suggested earlier by Cheng et al. (2010) who found a decrease in nitrogenase activity of subtropical Anabaena filiculoides–A. azollae in response to higher temperatures.
Bar, Kulasooriya & Telor (1991) showed that the nitrogenase activity of A. azollae is directly regulated by light, increasing significantly at wavelengths between 690 and 710 nm. They suggested that nitrogenase activity in the Azolla-Anabaena association is inhibited by O2 produced by photosynthesis. The plants in the summer season were exposed to longer day lengths than those in spring and autumn. In addition, radiation sometimes exceeded the light saturation level of 375 W m−2 (Lumpkin & Plucknett, 1980). Both the relatively longer photoperiod and the high light intensities may have contributed to a relatively high O2 production in the leaves of A. filiculoides during the summer, inhibiting the nitrogenase activity of the cyanobiont and hence N availability for Azolla. High O2 concentrations may also have contributed to increased photorespiration in Azolla (Ray et al., 1979; Bar et al., 1991), especially at the high air T during the summer season (Ainsworth & Rogers, 2007). Furthermore, the red colour of A. filiculoides in the summer experiment, caused by anthocyanins, suggests that the plants were suffering from photoinhibition. Bright sunlight generally contains a relatively higher contribution of blue light to the total radiation sum, whereas these phenolic compounds are usually produced by plants to protect their photosynthetic apparatus, also in response to high T (Moore, 1969; Lumpkin & Plucknett, 1980; Wagner, 1997).

Thus, our results show that nutrient availability becomes more important at higher atmospheric CO2 concentrations due to its growth enhancing effect. In addition, our results suggest that high T and high radiation affect the N concentrations in temperate A. filiculoides, most likely by inducing photorespiration and photoinhibition, which both may limit growth either directly, or indirectly via A. azollae.

**Azolla growth potential under past and future climate conditions**

Changes in atmospheric CO2 concentrations are commonly regarded as a mechanism forcing global climate change on geological time scales because of the strong and predictable effect of CO2 on temperature (Pearson & Palmer, 2000). Here, we showed a strong effect of atmospheric CO2 enrichment on the biomass production of A. filiculoides. Speelman et al. (2009) computed that the massive growth and burial of Azolla during the Eocene Azolla interval may have resulted in a 0.9 1018-3.5 1018 g carbon storage giving rise to a potential 55 to 470 ppm drawdown of global atmospheric CO2. From that, it may be concluded that Azolla blooms, at magnitudes like those in the Eocene Arctic Ocean, may have had a significant effect on global climate, given that the carbon sequestered by the plants remained in storage. Our results can help to account for the probable development of massive Eocene Azolla occurrences, as ambient CO2 concentrations are thought to have exceeded 2000 ppm during the early Eocene, while maximum annual temperatures at the Arctic are assumed to have been around 25 °C (Basinger, Greenwood & Sweda, 1994; Greenwood, Basinger & Smith, 2010).

With respect to future climate change, our results suggest that temperate Azolla species may become more abundant in small eutrophic waterbodies as a result of increased CO2 concentrations. Higher CO2 concentrations will result in increased growth of Azolla during spring and increased coverage of surface waters, at least at the start of the summer period. This may give Azolla a competitive advantage over free-living cyanobacteria, which otherwise could become dominant earlier in the year as a result of climate change (Winder & Schindler, 2004).

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**References**


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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. $R^2$ values and ANOVA results of the overall linear regression analysis and the linear regression analyses per CO$_2$ treatment with RGR as the dependent variable and the factor scores of the z-scores of the weekly averages of the climatic variables as the predictor variables. For interpretation of the results, the rotated component matrixes of the factor analyses are included.

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