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The interactive effects of rising atmospheric CO_2 and nutrient supply on carbon and water relations of grassland ecosystems: An analysis of stable carbon isotopes and water-use efficiency in vegetation samples from the Park Grass Experiment

Iris Heike Köhler

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‚Das Gras wächst nicht schneller,
wenn man daran zieht.'
Afrikanisches Sprichwort

Summary

The present study analysed how water-use efficiency (WUE) of grassland ecosystems responded to the increase in atmospheric CO_2 during the last century. The aim was to improve our understanding of the long-term responses of WUE – and of the underlying carbon and water relations – in grassland ecosystems under rising atmospheric CO_2. Interactions with other environmental parameters like plant available soil water and nutrient availability were also considered. The results help to understand how WUE in grassland ecosystems responds to the increasing availability of CO_2 and how these responses may feed back on climate and climate change.

The stand-scale response of WUE in grassland was derived from stable carbon isotope analysis of archived hay and herbage samples from the Park Grass Experiment (PGE) in Rothamsted, England. This experiment was started in 1856 on an old grassland site, which was divided into experimental plots given different combinations and amounts of fertilizers and lime. Annual samples of hay and herbage have been stored in the Rothamsted Sample Archive since the beginning of the experiment. In the present study, time series of carbon isotope discrimination ($^{13}\Delta$) in samples from five differently fertilized treatments were analysed. $^{13}\Delta$ is a proxy for c_i/c_a, the ratio of intercellular to atmospheric CO_2 concentration. This ratio is controlled by the net carbon assimilation rate (A) and stomatal conductance (g_s). $^{13}\Delta$ can thus be used to estimate W_i, the *intrinsic* WUE (A / g_s).

First, two time-series of spring (first cut) and summer / autumn (second cut) $^{13}\Delta$ covering 150 years (1857 – 2007, first cut) resp. 132 years (1875 – 2007, second cut) were derived for the unlimed and unfertilized control treatment. Second, for comparison of nutrient effects, $^{13}\Delta$ chronologies from limed plots with different fertilizer treatments were analysed, covering 95 years (1915 – 2009). The shorter period was chosen to avoid confounding effects with changes in botanical composition induced by fertilization during the first decades of the experiment. Furthermore, for the analysis of nutrient effects only spring growth (first cut) was analysed as it profited most from fertilizer application. The selected limed fertilizer treatments received different amounts of

nitrogen (N; 0, 48 or 96 kg ha^{-1} yr^{-1}), phosphorus (P; 0 or 35 kg ha^{-1} yr^{-1}) and potassium (K; 0 or 225 kg ha^{-1} yr^{-1}) and lime as required to maintain soil pH near 7.

In the 1857 – 2007 period, W_i increased by +33% during spring growth and by +18% during summer / autumn growth on the unlimed *Control (U)* treatment. The inter-annual variation in $^{13}\Delta$ was mainly related to weather conditions. Plant available soil water explained 51% and 40% of the variation in spring growth $^{13}\Delta$ and summer / autumn growth $^{13}\Delta$, respectively. On the limed treatments, the increase of W_i was +28% on the high N treatment (*High N+PK*) and only +13% on the limed *Control (L)* treatment in the 1915 – 2009 period. The other limed and fertilized treatments showed intermediate changes. Plant available soil water explained between 39% and 55% of the inter-annual variation in $^{13}\Delta$ on the limed treatments. N status, assessed as N fertilizer supply *plus* an estimate of biologically-fixed N, was negatively related ($r^2 = 0.88$; $P < 0.02$) to the trend for $^{13}\Delta$ against CO_2 on the limed treatments. Other indices of N status exhibited similar relationships. But, the relationship did not hold when the unlimed *Control (U)* treatment was also included in the analysis. However, it was found that the CO_2 responsiveness of $^{13}\Delta$ was related to the grass content of the plant community. This may have been due to the reported greater CO_2 responsiveness of g_s in grasses relative to forbs. Thus, the stronger increase of W_i on grass-rich swards may be related to effects of nutrient supply and soil pH on botanical composition. As yields have not increased in the observation period, it was assumed that community-level A has not increased either. Therefore, the increased W_i probably resulted from a decreased g_s in the studied grassland vegetation.

These physiological adjustments were in contrast to results from short-term studies, but consistent with results from long-term studies in forest ecosystems, showing that short-term responses may not mirror long-term ecosystem responses. This has to be taken into account when results from short-term experiments are used for predictions about grassland ecosystem responses under rising atmospheric CO_2. The future analysis of stable oxygen isotopes (^{18}O) in the PGE samples will help to partition the response of the functional components of W_i (A and g_s) in this grassland ecosystem.

Zusammenfassung

Die vorliegende Arbeit untersucht die Reaktion der Wassernutzungseffizienz (WUE) in Graslandökosystemen auf den Anstieg der atmosphärischen CO_2-Konzentration während des letzten Jahrhunderts. Das Ziel dieser Arbeit war die Verbesserung unser Verständnisses der Langzeitreaktion der WUE – und des ihr zu Grunde liegenden Kohlenstoff- und Wasserhaushalts – in Graslandökosystemen unter steigender atmosphärischer CO_2-Konzentration. Interaktionen mit weiteren Umweltfaktoren wie z.B. pflanzenverfügbarem Bodenwasser und Nährstoffverfügbarkeit wurden ebenfalls untersucht. Die Ergebnisse können dazu beitragen die Reaktion der WUE in Graslandökosystemen auf die ansteigende Verfügbarkeit von CO_2 zu verstehen und abzuschätzen wie sich diese Reaktion auf das Klima und den Klimawandel auswirkt.

Die Reaktion der WUE auf Pflanzenbestandesebene im Grasland wurde mit Hilfe der Analyse stabiler Kohlenstoffisotope aus archivierten Heuproben des Park Grass Experiments (PGE) in Rothamsted, England abgeleitet. Das Experiment wurde 1856 auf einer alten, etablierten Grünlandfläche angelegt. Diese wurde in Versuchparzellen mit unterschiedlichen Düngermengen und -arten in Kombination mit Kalkung unterteilt. Seit Beginn des Experiments wurden jährlich Heuproben in das Rothamsted Sample Archive eingelagert. In der vorliegenden Studie wurden Zeitreihen der Kohlenstoff-Isotopendiskriminierung ($^{13}\Delta$) von unterschiedliche gedüngten Parzellen untersucht. $^{13}\Delta$ ist ein indirektes Maß für das Verhältnis der interzellulären zur atmosphärischen CO_2-Konzentration, c_i/c_a. Dieses Verhältnis wird durch die Nettokohlenstoffassimilationsrate (A) und die stomatäre Leitfähigkeit (g_s) bestimmt. $^{13}\Delta$ kann daher zur Bestimmung der W_i, der *intrinsischen* WUE (A / g_s) verwendet werden.

Zunächst wurden zwei $^{13}\Delta$ Zeitreihen für das Frühjahr (erster Schnitt) sowie den Sommer / Herbst (zweiter Schnitt) auf der ungekalkten und ungedüngten Kontrollparzelle über 150 Jahre (1857 – 2007, erster Schnitt) bzw. 132 Jahre (1875 – 2007, zweiter Schnitt) abgeleitet. Des Weiteren wurden für den Vergleich von Nährstoffeffekten $^{13}\Delta$ Zeitreihen von unterschiedlich gedüngten Parzellen untersucht. Diese deckten 95 Jahre ab (1915 – 2009). Der kürzere Zeitraum wurde gewählt um Störeinflüssen durch die starken Veränderungen in der botanischen Zusammensetzung,

welche durch die Düngung in den ersten Jahrzehnten des Experiments auftraten, zu vermeiden. Für die Untersuchung von Nährstoffeffekten wurde nur der Frühlingsaufwuchs (erster Schnitt) analysiert, da er am stärksten von der Düngergabe profitiert. Die ausgewählten gedüngten Parzellen erhielten unterschiedliche Mengen an Stickstoff (N; 0, 48 oder 96 kg ha^{-1} yr^{-1}), Phosphor (P; 0 oder 35 kg ha^{-1} yr^{-1}) und Kalium (K; 0 oder 225 kg ha^{-1} yr^{-1}) sowie Kalk um einen Boden pH-Wert von 7 zu halten.

Im Zeitraum von 1857 – 2007 stieg die W_i im Frühjahrsaufwuchs auf der ungekalkten Kontrolle *Control (U)* um +33% und von 1875 – 2007 im Sommer / Herbstaufwuchs um +18% an. Die interannuellen Schwankungen der $^{13}\Delta$ waren mit den Witterungsbedingungen korreliert. Das pflanzenverfügbare Bodenwasser erklärte jeweils 51% bzw. 40% der Streuung von $^{13}\Delta$ im Frühjahrs- bzw. Sommer- / Herbstaufwuchs. Auf den gekalkten Parzellen betrug der Anstieg der W_i +28% auf der Hochstickstoffvariante *High N+PK* und nur +13% auf der gekalkten Kontrollvariante *Control (L)* im Zeitraum von 1915 – 2009. Die anderen gekalkten und gedüngten Parzellen zeigten dazwischen liegende Reaktionen. Das pflanzenverfügbare Bodenwasser erklärte zwischen 39% und 55% der interannuellen Streuung der $^{13}\Delta$ auf den verschiedenen Parzellen. Der N-Status – berechnet aus der ausgebrachten Düngermenge und einem Schätzwert für den biologische fixierten N – war auf den gekalkten Parzellen negativ mit dem $^{13}\Delta$ Trend gegenüber CO_2 korreliert ($r^2 = 0.88$; $P < 0.02$). Weitere Kennzahlen der N-Versorgung wiesen ähnliche Beziehungen auf. Die Beziehungen hatten jedoch keinen Bestand wenn die ungekalkte Kontrolle *Control (U)* in die Betrachtung miteinbezogen wurde. Es zeigte sich jedoch, dass die CO_2 Reaktion von $^{13}\Delta$ mit dem Grasanteil im Pflanzenbestand zusammenhing. Dies könnte auf die in der Literatur berichtete größere CO_2-Reaktion von g_s in Gräsern im Verhältnis zu Kräutern zurückzuführen sein. Demzufolge wäre die stärkere Zunahme der W_i in grasreichen Beständen auf die Effekte der Nährstoffversorgung und des Boden pHs auf die botanische Zusammensetzung zurückzuführen. Da sich die Erträge im Beobachtungszeitraum nicht verändert haben ist anzunehmen, dass A auf Bestandesebene nicht zugenommen hat. Folglich ist der Anstieg der W_i in der untersuchten Graslandvegetation wahrscheinlich auf eine Abnahme von g_s zurückzuführen.

Diese physiologischen Anpassungen stehen im Gegensatz zu Ergebnissen aus Kurzzeituntersuchungen, stimmen aber mit Ergebnissen aus Langzeitstudien in Wäldern

überein. Dies zeigt, dass Reaktionen in Kurzzeitstudien nicht unbedingt die langfristige Reaktion von Ökosystemen widerspiegeln. Dies muss berücksichtigt werden wenn Ergebnisse aus Kurzzeitexperimenten für Voraussagen über die Reaktion von Graslandökosystemen unter steigendem CO_2 verwendet werden sollen. Weiterführende Analysen von stabilen Sauerstoffisotopen (^{18}O) in den PGE Proben werden dazu beitragen die Reaktionen der funktionalen Komponenten von W_i (A und g_s) in diesem Graslandökosystem detaillierter aufzuschlüsseln.

Contents

List of Figures

List of Tables

1. Introduction

Vegetation and the atmosphere on planet earth are strongly linked through the exchange of carbon (C) and water. In the C cycle, vegetation takes up CO_2 from the atmosphere in the process of photosynthesis and releases CO_2 in the process of respiration. C is stored in plants or sequestered in soils and may be released from these pools with time lags up to decades and centuries. In the water cycle, plants take up water from the soil and release water vapour in the process of transpiration from aboveground tissue into the atmosphere, contributing e.g. to cloud and rain formation.

Both biogeochemical cycles are intimately coupled as the C and water fluxes between plants and the atmosphere share the same path: the stomatal pores in the leaf epidermis. The ratio of C gain per unit water lost is termed water-use efficiency (WUE) and is a key parameter for understanding C and water relations at different organizational and temporal scales: from the leaf to the ecosystem, from minutes to centuries (Bacon, 2004; Jones, 2004). The study of WUE helps us to understand how ecosystem gas exchange responds to environmental changes. One of the biggest environmental changes that plants are currently exposed to is the rise in atmospheric CO_2 concentrations. In order to help predict future climate, it is crucial to understand how ecosystem WUE responds to the increased availability of C and how these responses feed back on climate and climate change.

The present work investigates long-term trends in WUE of grassland ecosystems under increasing atmospheric CO_2 in the Anthropocene. Amongst terrestrial ecosystems, grasslands play an important role in the global C and water cycle. They cover 41% of the world's land area (excluding Antarctica and Greenland), and store about one-third of the global stock of C in terrestrial ecosystems, predominantly in the soil (White *et al.*, 2000). Understanding how grassland ecosystems regulate their C and water relations under elevated atmospheric CO_2 concentrations and varying nutrient supply will help to better understand possible feedback effects on climate in a CO_2-rich world.

Rising atmospheric CO₂ – Effects on plant physiology and feedbacks on climate

From the end of the Industrial Revolution in the 19th century until today, atmospheric CO_2 concentration has increased by approximately +31% from 290 µmol mol^{-1} in the 1850's to over 380 µmol mol^{-1} in 2009 (Friedli *et al*., 1986; Francey *et al*., 1999; Keeling *et al*., 2009). Predictions suggest that atmospheric CO_2 concentrations will rise to between 530 and 970 µmol mol^{-1} until 2100 (Prentice *et al*., 2001).

During the last 24 million years, terrestrial plants have not faced such elevated atmospheric CO_2 concentrations as we observe today (Pearson & Palmer 2000). At a given CO_2 concentration, C uptake is controlled by the demand for CO_2 via the C assimilation rate (*A*) and by the supply of CO_2 through the stomatal pores, as regulated by the stomatal conductance (g_s). As *A* is not CO_2-saturated in C_3 plants under present conditions (Farquhar *et al*., 1980) increasing availability of CO_2 in the atmosphere may lead to increased *A*. At the same time, an increased gradient between the CO_2 concentration in the atmosphere and inside the leaf would facilitate CO_2 diffusion into the leaf. A lower g_s would then be sufficient to maintain C supply. In accordance with these theoretical considerations, C_3 plants showed higher light-saturated *A* (on average +31%) and decreased g_s (on average -22%) under elevated CO_2 concentration (567 µmol mol^{-1}, in comparison to CO_2 concentrations of 366 µmol mol^{-1}, average of all studies; Ainsworth & Rogers, 2007) in multi-year FACE (Free Air CO_2 Enrichment) experiments. Thus, by affecting *A* and g_s, elevated atmospheric CO_2 may modify the exchange of C and water between terrestrial plants and the atmosphere: Increased *A,* and thus enhanced C uptake, may mitigate the increase in atmospheric CO_2 as C-sequestration in terrestrial ecosystems could increase under rising CO_2 when surplus C is sequestered in biomass and soils (Broecker *et al*., 1979; Woodward *et al*., 2009; Jansson *et al*., 2010). At the same time, decreases in g_s may reduce ecosystem evapotranspiration (Bernacchi *et al*., 2007), which would affect hydrological cycles and air temperature near the surface. For example, Gedney *et al*. (2006) found that increased trends of continental runoff in the 20th century were consistent with reduced transpiration due to reduced g_s.

However, in complex natural ecosystems, interactions and feedback processes may occur on all spatio-temporal scales, and co-occurring processes might even cancel each other out. Thus, short-term results under controlled conditions may not accurately

reflect such long-term responses making extrapolation difficult (Luo & Reynolds, 1999; Körner, 2006; Leuzinger *et al.*, 2011). Only few FACE studies have been running for more than one decade (Ainsworth & Long, 2005). In particular, our knowledge about long-term interactions between C and nitrogen (N) under rising atmospheric CO_2 is very limited (Reich *et al.*, 2006*b*).

One way to overcome these limitations and to study the linked CO_2 response of *A* and g_s over long time scales, different geographic regions, biome types, plant functional groups, and environmental conditions is the retrospective analysis of WUE under the recent increase in atmospheric CO_2. Past values of WUE can be derived from the analysis of stable C isotopes in plant biomass. The only prerequisite for such studies is the availability of suitable plant biomass samples from the past, e.g. in the form of tree rings or herbarium species.

Water-use efficiency – a measure for the regulation of gas exchange

WUE can be defined in different ways, depending on the context (Jones, 2004). In the present work, I analyzed long-term trends of the *intrinsic water-use efficiency* (W_i) and the *instantaneous water-use efficiency* (W_t).

W_i is a physiological efficiency reflecting the leaf-level relationship between plant CO_2 uptake and transpiration at standard humidity. It represents the ratio of *A* to g_s, which determines the CO_2 concentration gradient between the atmosphere (c_a) and the leaf internal (intercellular) air space (c_i). The ratio can be derived from the analysis of C isotope discrimination ($^{13}\Delta$; see Material & Methods, Eqns. 2 to 7) in plant biomass (Farqhuar *et al.*, 1982). In addition to W_i, the actual leaf-level 'instantaneous' water use efficiency (W_t) can be estimated when data on atmospheric evaporative demand are available. W_t represents the ratio of assimilation rate (*A*) to transpiration (*E*), and unlike W_i, accounts for the effect of the leaf-to-air vapour pressure deficit (VPD) on *E* (see Material & Methods, Eqn. 8). W_t has also been termed 'transpiration efficiency' (Farqhuar *et al.*, 1989*a*).

The analysis of $^{13}\Delta$ in plant material from the past enables retrospective studies of W_i and, provided that VPD data are available, also of W_t. Indeed, time-series analyses of W_i of vegetation during the 20th century have provided direct and compelling evidence for changes in C uptake relative to potential water loss of plants under the recent increase in atmospheric CO_2: Numerous tree-ring studies have investigated the response of W_i to rising atmospheric CO_2 concentration and climate change, as the woody tissue and annual rings provide convenient archives of stable C isotopes. Due to the short-lived nature of biomass from herbaceous plants, such studies have been scarce for grassland ecosystems.

The effect of rising atmospheric CO_2 on W_i (or resp. the c_i/c_a ratio) depends on the plant's reponse, i.e. the adjustment of A and g_s. Three theoretical response scenarios of gas exchange under rising atmospheric CO_2 have been proposed by Saurer *et al.* (2004) and help to interpret the reaction of $^{13}\Delta$ and W_i:

- Scenario (1): c_i remains constant
- Scenario (2): the c_i/c_a ratio remains constant
- Scenario (3): the difference $c_a - c_i$ remains constant

For a response following scenario (1) or (2), A must increase and / or g_s must decrease, leading to increasing W_i. Scenario (1) leads to stronger increases in W_i and requires thus a stronger reaction of A or g_s than scenario (2) ("active response"). For scenario (3), every increase in c_a leads to an equal increase in c_i. For a response following scenario (3) plants do not need to actively respond in terms of A and g_s, leading to unchanged W_i ("passive response") (McCarroll *et al.*, 2009). Figure 1 exemplarily shows the theoretical responses for the 1915 – 2009 period, assuming a c_i/c_a ratio of 0.7 in 1915. The underlying equations are described in Material & Methods (Eqn. 3 – 7).
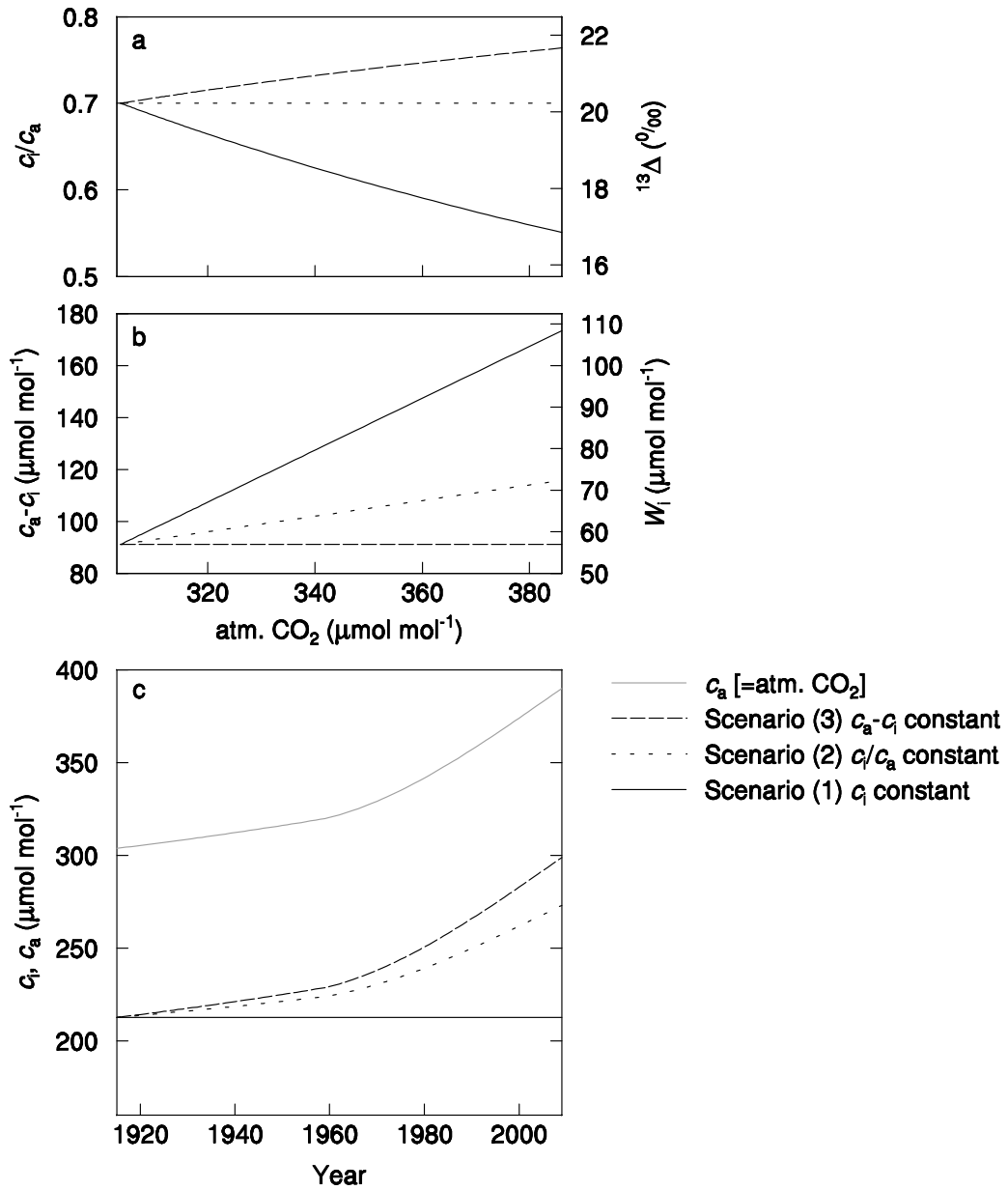
Figure 1: Theoretical response scenarios (after Saurer *et al.*, 2004), taking 1915 as starting year and assuming a c_i/c_a ratio of 0.7 in 1915. Calculated with Eqns. 3 – 7.

Studies of $^{13}\Delta$ in forest trees often found increases in W_i with rising CO_2 (e.g. Bert *et al*., 1997; Duquesnay *et al*., 1998; Feng, 1999; Arneth *et al*., 2002; Saurer *et al*., 2004; Andreu-Hayles *et al*., 2011) across a wide range of locations (e.g. the Iberian Peninsula, Western / Northern Europe, Siberia or North America) and tree species (e.g. from the *Pinus*, *Quercus*, *Picea* or *Fagus* genera). Decreasing W_i was an exception and was only observed in a few cases (Marshall & Monserud, 1996; Feng, 1999). Others observed that the increase in W_i levelled off in recent decades (Waterhouse *et al*., 2004; Gagen *et al*., 2011). Long-term changes in W_i also differed within the same tree species or genus and between study sites (Peñuelas *et al*., 2008: *Fagus sylvatica*; Andreu-Hayles *et al*., 2011: *Pinus sp*.).

In the majority of long-term tree-ring studies, trends in W_i were close to the 'constant c_i/c_a' response following scenario (2). For a summary of the responses see Table 1 and Peñuelas *et al*. (2011) for a meta-analysis of more tree-ring studies. By contrast, long-term $^{13}\Delta$ chronologies from herbaceous plants are rare and have been analysed in only a few studies (Peñuelas & Azcón-Bieto, 1992; Zhao *et al*., 2001; Pedicino *et al*., 2002; Barbosa *et al*., 2010). Peñuelas & Azcón-Bieto (1992) and Pedicino *et al*. (2002) used herbarium samples. They found that changes in W_i differed between species (Table 1): In most cases W_i increased, only in one case (*Papaver alpinum,* Peñuelas & Azcón-Bieto, 1992) a decrease was observed.

However, the use of herbarium samples complicates the interpretation of the results, as samples are based on a small number of individuals collected at different sites with mostly unknown environmental conditions during plant growth. Ecosystem- or stand-scale responses to rising CO_2 cannot be derived from such data. Zhao *et al*. (2001) analysed wheat grain and straw samples from the Broadbalk Continuous Wheat Experiment at Rothamsted, England, which offered the possibility of analysing archived stand-scale plant samples dating back until 1845. They observed increasing W_i until approximately 1965 and decreasing W_i afterwards. No such studies have been available for the grassland biome so far, except for one study by Barbosa *et al*. (2010), who analysed a time-series of *Capra ibex* horns and inferred W_i of alpine grassland between 1938 and 2006 from this animal tissue. They found that W_i has increased, but W_t remained unchanged due to increasing VPD. A summary of the results from long-term isotopes studies is given in Table 1.

Table 1: Summary of trends in W_i as reported for C_3 plants in literature and comparison with theoretical response scenarios (cf. Fig. 1 and Saurer *et al.*, 2004).Where the necessary data values were not directly given in the literature, they were derived from graphs or reported regression analyses. Thus, data may deviate slightly from reported data in the literature. Changes in W_i per 1 µmol mol^{-1} CO_2 increase are +0.63 for responses following scenario (1) ('constant c_i') and 0 for responses following scenario (3) ('constant c_a-c_i'). Changes in W_i per 1 µmol mol^{-1} CO_2 increase for responses following scenario (2) ('constant c_i/c_a') depend on the starting value of c_i/c_a, and were thus calculated separately for each data set.

Sample site and elevation in meters above sea level (where available) and sample type (for "Herbarium and other")	Period of time (Start year – end year)	Species	Intrinsic water-use efficiency (W_i)				Calculation and origin of data (Figure and table numbers refer to the respective reference)	Reference
			Absolute values in start and end year of observation period (µmol mol^{-1})	Total change in %	Change in µmol mol^{-1} per 1 µmol mol^{-1} CO_2 increase for			
					observed values	'constant c_i/c_a', scenario (2)		
Tree-ring studies								
Iberian Peninsula, 1600 – 2100	1901 – 2000	*Pinus nigra, P. uncinata, P. sylvestris*	93 (±7) – 116 (±8)	+26 (±9)	+0.32 (±0.10)	+0.31 (±0.02)	Mean (±SD) for five sites derived from reported regression analysis (Fig. S3)	Andreu-Hayles *et al.* (2011)
Montseny Mountains, Spain, 940 – 1665	1978 – 2003	*F. sylvatica* High forest limit Central forest area Lower forest limit	77 – 78 74 – 77 81 – 84	0 +4 +4	0 +0.07 +0.09	+0.23 +0.22 +0.24	Data derived from reported regression analysis (Fig. 3)	Peñuelas *et al.* (2008)
North-eastern France	1885 – 1990 1855 – 1980	*Fagus sylvatica* High forest Coppice-with-standards	72 – 104 77 – 91	+44 +18	+0.52 +0.27	+0.25 +0.27	Derived from reported $^{13}\Delta$ data	Duques-nay *et al.* (1998)
French Jura Mountains 550 to 1350	1860s – 1930s 1930s – 1980s	*Abies alba*	77 – 82 82 – 105	+6 +28	+0.27 +0.69	+0.27 +0.27	Derived from reported $^{13}\Delta$ data	Bert *et al.* (1997)

Table 1 continued

Central Siberia	1856 – 1991	*P. sylvestris*	71 (±3) – 96 (±4)	+34 (±0.5)	+0.36 (±0.01)	+0.25	Mean (±SD) of three (1856 - 1991) resp. two (1920 - 1991) sites; data derived from Fig. 2	Arneth *et al*. (2002)
	1920 – 1991		85 (±2) – 103 (±10)	+22 (±15)	+0.34 (±0.23)	+0.28		
Northern Eurasia	1861 – 1890 vs. 1961 – 1990 (mean values)	*Larix* sp.	63 – 74	+17	+0.26	+0.22	Data as reported in Table 3	Saurer *et al*. (2004)
		Pinus sp.	65 – 76	+17	+0.26	+0.22		
		Picea sp.	69 – 84	+23	+0.36	+0.24		
Norther Europe	1895 – 1994	*Quercus robur, F. sylvatica, P. sylvestris*	76 (±9) – 106 (±14)	+39 (±9)	+0.43 (±0.12)	+0.26	Mean (±SD) for 7 tree-ring chronologies; data derived from Fig. 3	Waterhouse *et al*. (2004)
Laanila, Finland	1840 – 2000	*P. sylvestris*	75 – 85	+13	+0.11	+0.26	Data derived from Fig. 4	Gagen *et al*. (2011)
Western North America 0 to 3500	1850 – 1985	*Pinus* sp., *Picea sitchensis, Quercus lobata* (without series SD-PC, MB-PE)	103 (±12) – 126 (±19)	+22 (±9)	+0.38 (±0.17)	+0.36 (±0.04)	Mean (±SD) for 17 sites derived from reported regression analysis (Table 2); series with negative trend reported separately	Feng (1999)
		Picea sitchensis (series KI-PS)	83 – 76	–8	–0.1	+0.29		
Rocky Mountains, USA	1910 – 1991	*Pinus ponderosa, P. monticola, Pseudotsuga menziesii*	79 – 78	–2	–0.03	+0.27	Data derived from reported regression analysis (Fig. 2a)	Marshall & Monserud (1996)
Herbarium and other								
Bernese Alps, Switzerland, Horn	1938 – 2006	*Capra ibex* (Alpine grassland)	64 – 75	+18	+0.16	+0.21	Data derived from reported regression analysis (Fig. 3c)	Barbosa *et al*. (2010)

Introduction

Table 1 continued

North of Sweden Needles	1970 – 2002	*Pinus sylvestris:* N0	72 – 73	0	0	+0.22	Data derived from reported regression analyses in Fig. 2	Betson *et al*. (2007)
		N1 (30 kg N ha^{-1} yr^{-1})	77	0	0.01	+0.24		
		N2 (60 kg N ha^{-1} yr^{-1})	76 – 75	−1.2	−0.02	+0.23		
		N3 (90 kg N ha^{-1} yr^{-1})	77 – 75	−3.4	−0.05	+0.24		
Catalonia, Spain Leaves	1850 – 1890 vs. 1982 – 1988 (mean values)	*Pinus uncinata*	72 – 85	+18	+0.23	+0.25	Data derived from reported $^{13}\Delta$ values for the respective periods (Table 1)	Peñuelas & Azcón-Bieto (1992)
		P. pinea	48 – 63	+32	+0.27	+0.18		
		Juniperus communis	80 – 82	+2	+0.03	+0.24		
		Alnus glutinosa	51	0	0	+0.15		
		Betula pendula	48 – 80	+67	+0.57	+0.23		
		Ceratonia siliqua	69 – 71	+3	+0.03	+0.21		
		Buxus sempervirens	57 – 103	+81	+0.82	+0.30		
		Pistacia lentiscus	51 – 63	+24	+0.22	+0.18		
		Rhododendron ferrugineum	56 – 84	+49	+0.49	+0.24		
		Helleborus foetidus	51 – 94	+84	+0.77	+0.27		
		Papaver alpinum	51 – 40	−22	−0.20	+0.12		
		Gentiana alpina	57 – 92	+62	+0.63	+0.27		
England Grain & Straw		*Triticum aestivum*					Data derived from reported regression analysis	Zhao *et al*. (2001)
	1845 – 1965	Grain	78 – 85	+10	+0.21	+0.27		
	1965 – 1997	Grain	85 – 87	+2	+0.04	+0.27		
	1845 – 1965	Straw	61 – 70	+14	+0.25	+0.21		
	1965 – 1997	Straw	70 – 68	−2	−0.04	+0.22		
South-western USA, Leaves	1894 – 1992	*Ephedra viridis*	81 – 91	+13	+0.17	+0.27	Derived from original data using 3rd order poly-nomials (cf. Fig. 5)	Pedicino *et al*. (2002)
	1874 – 1996	*Pinus edulis*	85 – 125	+46	+0.54	+0.29		
	1883 – 1996	*P. flexilis*	89 – 95	+7	+0.09	+0.30		
	1887 – 1996	*Juniperus scopulorum*	80 – 97	+20	+0.23	+0.27		
	1891 – 1994	*Quercus turbinella*	69 – 76	+9	+0.09	+0.24		

The role of nutrient supply for the response of gas exchange under rising CO_2

The great variability in published responses of W_i to rising atmospheric CO_2 suggests interactions with other environmental factors which could e.g. include different hydrological conditions, differences in climate and nutrient status. Such factors could affect W_i via changes of A or g_s or both and may thus enhance or counteract the response of plants under rising atmospheric CO_2. Nutrient supply, and in particular N availability, is thought to play a major role for the long-term CO_2 response of ecosystems under rising atmospheric CO_2: The down-regulation of photosynthesis under elevated CO_2 observed in long-term studies (Ainsworth & Long, 2005) could result from reduced or insufficient sink capacity (Leakey *et al*., 2009), which is influenced strongly by N availability (Isopp *et al*., 2000). Therefore, N availability constrains ecosystem C accumulation in response to rising CO_2 (Hungate *et al*., 2003), with progressive N limitation (Luo *et al*., 2004) decreasing N availability further. Nutrient supply could also indirectly affect the response of gas exchange at ecosystem level by leading to changes in species composition and / or functional groups, which can have different CO_2 responsiveness (Ainsworth & Rogers, 2007).

However, long-term experiments that have studied the interactions between nutrient supply and rising atmospheric CO_2 for several decades are scarce. According to Reich *et al*. (2006*b*), none of the FACE experiments with contrasting N treatments has been running for more than two decades (Swiss FACE (since 1993), Jasper Ridge Mini FACE (since 1997) and the BioCON FACE (since 1998). Often, a stimulation of photosynthesis and growth was found with higher nutrient supply: For perennial grassland species (including legumes, non-leguminous forbs, warm season C_4 grasses and cool season C_3 grasses), Reich *et al*. (2006*a*) found that addition of 40 kg N ha^{-1} yr^{-1} stimulated the response of biomass accumulation under elevated CO_2 (560 μmol mol^{-1}) in a 6 year study. In a more intensively managed monoculture of *Lolium perenne*, Daepp *et al*. (2000) found that yield response increased in the high N treatment (560 kg N ha^{-1} yr^{-1}) under elevated CO_2 (600 μmol mol^{-1}) from 7% in 1993 to 25% in 1998. In contrast, yield responses at low N (140 kg N ha^{-1} yr^{-1}) were low (5% in 1993 and 9% in 1998). Arp *et al*. (1998) also found increased growth under elevated CO_2 (560 μmol mol^{-1}) at high N supply (160 kg N ha^{-1} yr^{-1}), but not at low N supply (40 kg N ha^{-1} yr^{-1}) for six

perennial plant species (including the grassland species *Arrhenatherum elatius, Molinia caerulea* and *Rumex obtusifolius*). In a review of controlled-environment studies, Stitt & Krapp (1999) found that the acclimation of photosynthesis under elevated CO_2 was stronger in N limited plants and any stimulation of growth was diminished and finally ceased when N supply was decreased. Main reactions were a decrease of Rubisco, accumulation of carbohydrates and decrease of N concentration.

There are only two long-term studies (Betson *et al.,* 2007; Leonardi *et al.*, 2012) that analysed the responses of gas exchange under rising atmospheric CO_2 in relation to nutrient supply. W_i, derived from stable C isotope chronologies, was analysed in both studies. Betson *et al.* (2007) analyzed W_i in needles of *Pinus sylvestris* from a 32 years-long N loading experiment (1970 – 2002). They found no significant increase in W_i and the responses of the N treatments did not differ from the control, indicating that N supply did not modify the CO_2 response in this system. Leonardi *et al.* (2012) analyzed 89 tree-ring chronologies (1850 – 2000) from 53 different sites and covering 23 conifer and 13 angiosperm tree species. By contrast, they found that in conifers and angiosperms, the annual rate of N deposition was associated with W_i and $^{13}\Delta$, and cumulative N deposition was positively related with the increase in W_i in angiosperms. To my knowledge, there are so far no studies on the long-term CO_2 response of grassland ecosystems under differing nutrient supply.

The grassland response under rising CO₂: Aims and Objectives

The present study analysed stable C isotopes in archived hay and herbage samples from the Park Grass Experiment (PGE) in Rothamsted, England. The aim was to assess the long-term responses of C and water relations in grassland ecosystems under rising atmospheric CO_2 and the interactions with nutrient supply.

First, a 150-year-long record of W_i was derived from community level $^{13}\Delta$, using hay and herbage samples of the unfertilized, unlimed control treatment *Control (U)* of the PGE between 1857 and 2007. VPD data were available from 1915 on, thus allowing a comparison of W_i and W_t for the 1915-2007 period. Furthermore, seasonal differences between the responses in spring and summer / autumn were analysed (published as Köhler *et al.*, 2010).

Second, I analysed in how far other environmental parameters may affect $^{13}\Delta$ in addition to CO_2, and how the inter-annual variation of the $^{13}\Delta$ signal influenced the interpretation of long-term trends. The high-frequency variation of the $^{13}\Delta$ time-series used for the analysis of the nutrient effect was studied in detail. A multiple linear regression model was developed for the subsequent analysis of $^{13}\Delta$ long-term trends. It was assessed in how far the high-frequency variation of the $^{13}\Delta$ data affected model outputs of the estimated long-term trends (unpublished).

Third, the role of long-term nutrient status and especially N supply for the CO_2 response of W_i in temperate semi-natural C_3 grassland was studied. Long-term trends (1915–2009) of $^{13}\Delta$ and W_i were derived for five fertilizer treatments [*Control (L)*, *PK*, *Low N*, *Low N+PK*, *High N+PK*] at Park Grass, each with different annual N (0, 48 or 96 kg ha^{-1} yr^{-1}), phosphorus (P; 0 or 35 kg ha^{-1} yr^{-1}) and potassium (K; 0 or 225 kg ha^{-1} yr^{-1}) applications and with lime as required to maintain soil pH near 7. Differences in the CO_2 responses of $^{13}\Delta$ resp. W_i between the treatments were analysed with the multiple linear regression model, and the responses were explored with regard to different nutrient measures. The effect of botanical composition on the response was also considered (published as Köhler *et al*., 2012).

2.   Material & Methods

Stable carbon isotope theory and water-use efficiency

Long-term trends of W_i, i.e. the ratio between A and g_s, can be derived by retrospective analysis of stable C isotopes in plant biomass. This makes it possible to assess how rising atmospheric CO_2 concentrations affect the biological coupling of C and water cycles.

What are stable carbon isotopes?

Chemical elements are defined by the number of protons in their nucleus. The number of neutrons in the nucleus differs between the different "isotopes" of a particular element. Stable isotopes (i.e. isotopes that do not undergo radioactive decay) are used widely in plant (eco)physiological and ecological studies (Dawson *et al.*, 2002; Fry, 2008). Due to their slight difference in mass, the reaction rates of each isotope in chemical and physical processes differ slightly. This can lead to shifts (known as '*isotope fractionation*') in the ratios of the isotopes in the product relative to the substrate of a reaction. These fractionations can provide information about the underlying processes that cause these shifts.

In this study, the focus is on the analysis of stable C isotopes and their role for understanding gas exchange between plants and the atmosphere. C possesses two stable isotopes: ^{12}C, the "light" isotope with six protons and six neutrons in the nucleus and the "heavy" isotope ^{13}C with six protons and seven neutrons. The natural abundances are 98.9% for ^{12}C and 1.1% for ^{13}C (Dawson *et al.*, 2002). A comprehensive study on the variation of $^{13}C/^{12}C$ in nature was given by Craig (1953).

The isotopic composition of a substrate is expressed in the small delta notation (δ). δ values denote the difference in isotopic composition relative to an international standard. For C ($\delta^{13}C$):

$$\delta^{13}C = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1, \tag{1}$$

with R the $^{13}C/^{12}C$ ratio in the sample or standard (Pee Dee Belemnite, PDB). Multiplication with the factor 1000 yields per mill (‰). Negative $\delta^{13}C$ values indicate that the sample contains less ^{13}C in relation to ^{12}C than the standard (the sample is '*depleted*') while positive values indicate that the sample contains more ^{13}C in relation to ^{12}C than the standard (the sample is '*enriched*').

Carbon isotope discrimination and the linear Farquhar model

Fractionation in biological processes is also termed '*discrimination*' (Δ), and is calculated as the deviation of the isotope effect α ($\alpha = R_{\text{source}}/R_{\text{product}}$) from unity:

$$\Delta = \frac{R_{\text{source}}}{R_{\text{product}}} - 1. \tag{2}$$

For the effect during carbon assimilation by plants, R_{source} refers to the $^{13}C/^{12}C$ ratio in atmospheric CO_2 and R_{product} to $^{13}C/^{12}C$ ratio in plant biomass: A model explaining C isotope discrimination ($^{13}\Delta$) during C_3 photosynthesis has been developed by Farquhar *et al*. (1982). Fractionations occurring along the path from the atmosphere to the site of C fixation by Rubsico are considered additive and are weighed by the respective CO_2 partial pressure drawdown involved in each step. The simple linear form of this model considers two processes, diffusion through the stomatal pores and fixation by Rubisco. $^{13}\Delta$ is then linearly related to the c_i/c_a ratio:

$$^{13}\Delta = a \frac{c_a - c_i}{c_a} + b \frac{c_i}{c_a} = a + (b - a) \frac{c_i}{c_a}, \tag{3}$$

where a denotes the fractionation of $^{13}CO_2$ (relative to $^{12}CO_2$) that occurs during diffusion in air (4.4‰) and b is the net fractionation due to carboxylations (27‰) (Farquhar *et al*., 1989a). In addition to Eqn. 3 a more complex model, including also effects of diffusion through the boundary layer, mesophyll conductance, and respiration on $^{13}\Delta$ exists (Farquhar *et al*. 1982, 1989*b*). For the analyses done in this study, the simple model is considered as adequate. For a discussion see Köhler *et al*. (2010).

$^{13}\Delta$ can be obtained from the C isotope composition of plant material ($\delta^{13}C_p$) and that of the atmosphere ($\delta^{13}C_a$; Friedli *et al.*, 1986; Francey *et al.*, 1999; Keeling *et al.*, 2010), by combining Eqns. 1 and 2 into:

$$^{13}\Delta = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p}, \tag{4}$$

In contrast to $\delta^{13}C_p$, $^{13}\Delta$ is independent of the source isotopic composition, thus allowing for comparison of plants growing under different $\delta^{13}C_a$. By rearranging Eqn. 3 and substituting with Eqn. 4 we can derive c_i, the intercellular CO_2 concentration:

$$c_i = c_a \frac{^{13}\Delta - a}{b - a} = c_a \frac{\dfrac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p} - a}{b - a} \tag{5}$$

Linking carbon isotopes with water-use efficiency

W_i (µmol mol^{-1}) is defined as the ratio between A and g_s. In retrospective, these parameters cannot be measured directly via gas exchange measurements. However, A is determined by the CO_2 concentration gradient between the ambient air and the leaf intercellular air space and the stomatal resistance to diffusion of CO_2 (or its reciprocal stomatal conductance). Thus according to Fick's law, A (µmol m^{-2} s^{-1}) at the leaf level can be defined as

$$A = g_{CO_2}(c_a - c_i), \tag{6}$$

where g_{CO2} is the leaf conductance to CO_2 (µmol m^{-2} s^{-1}), c_a the CO_2 concentration (µmol mol^{-1}) in ambient air (assumed to equal atmospheric CO_2 concentration) and c_i the leaf intercellular concentration. As the ratio of the diffusivities for water vapour and CO_2 in air is 1.6 ($g_{H_2O} = 1.6 g_{CO_2}$) we get

$$W_i = \frac{A}{g_{H_2O}} = \frac{g_{CO_2}(c_a - c_i)}{1.6 g_{CO_2}} = \frac{c_a - c_i}{1.6} = \frac{c_a\left(1 - \dfrac{c_i}{c_a}\right)}{1.6}. \tag{7}$$

As data for c_a are available for the past centuries from measurements of CO_2 in gas bubbles from ice cores and in free air (Friedli *et al*., 1986; Francey *et al*., 1999; Keeling *et al*., 2009) the only unknown is c_i. But, as shown in Eqn. 5, c_i can be estimated from the known relationship between c_i/c_a and $^{13}\Delta$ in C_3 photosynthesis.

When data on the vapour pressure deficit of the atmosphere are available we can also derive W_t (mmol mol^{-1}), which is defined as:

$$W_t = \frac{A}{E} = \frac{g_{CO_2}(c_a - c_i)}{g_{H_2O}\nu} = \frac{c_a - c_i}{1.6\nu} = \frac{c_a\left(1 - \dfrac{c_i}{c_a}\right)}{1.6\nu}, \tag{8}$$

where E is transpiration rate (mmol m^{-2} s^{-1}), ν is the leaf-to-air difference in the mole fraction of water vapour, i.e. the vapour pressure deficit ($e_i - e_a$; the difference between water vapour pressures in the leaf and in the ambient air) divided by total atmospheric pressure. e_i is calculated from saturation vapour pressure assuming that air temperature equals leaf temperature. For the calculations see Allen *et al*. (1998) and Köhler *et al*. (2010).

The Park Grass Experiment – history, treatments and experimental site details

The PGE was started in 1856 at the Rothamsted agricultural research station, located approximately 40 km north of London in Hertfordshire, England (0° 21' West, 51° 49' North, 128 m height above sea level). About 2.8 ha of old grassland were divided into experimental plots which received different fertilizer, manure and lime treatments (Silvertown *et al*., 2006).

Fertilizer treatments

Figure 2 gives an overview of the experimental layout and the applied treatments. N fertilizer was applied as sodium nitrate at two levels (N*1: 48 kg ha^{-1} yr^{-1} N*2: 96 kg ha^{-1} yr^{-1}) or as ammonium sulphate at three different levels (N1: 48 kg ha^{-1}

yr^{-1}, N2: 96 kg ha^{-1} yr^{-1}, N3: 144 kg ha^{-1} yr^{-1}). Both N fertilizers were applied in different combinations with other minerals (phosphorus (P): 35 kg ha^{-1} yr^{-1}, potassium (K): 225 kg ha^{-1} yr^{-1}, sodium (Na): 15 kg ha^{-1} yr^{-1}, magnesium (Mg): 10 kg ha^{-1} yr^{-1}, silicium (Si): 135 kg ha^{-1} yr^{-1}). N fertilizers were applied in spring, while minerals were applied in winter. Some treatments received organic fertilizers in the form of farm yard manure or poultry manure or fishmeal. The treatments are labelled by numbers and are subdivided by letters (*U*, *L*, *a*, *b*, *c*, and *d*) according to their liming scheme (Rothamsted Research, 2006).

Liming treatments

The main liming scheme on Park Grass was introduced in 1903 when selected plots were split into limed *(L)* and unlimed *(U)* areas; the remaining plots were divided in 1920. Chalk (4 t ha^{-1}) was applied on the southern half *(L)* of the plots every fourth year from 1903 resp. 1920 on. In 1965 the plots were further divided into four sub-plots: The former '*L*' half was divided into '*a*' and '*b*', the former '*U*' half into '*c*' and '*d*', which received different amounts of chalk to maintain the soil (0-23 cm) at a pH (in water) of about 7, 6 and 5 on the '*a*', '*b*' and '*c*' sub-plots; the '*d*' sub-plots received no chalk so that the soil pH reflects inputs from the different fertiliser inputs and atmospheric deposition only (Rothamsted Research, 2012*a*, 2012*b*). The whole experiment received a small amount of lime in the 1880's (Rothamsted Research, 2006).

The Park Grass Experiment

	a	b	c	d
13/2		FYM/PM		
13/1		(FYM/Fishmeal)		
12		Nil		
11/2		N3 P K Na Mg Si		
11/1		N3 P K Na Mg		
10		N2 P Na Mg		
9/2		N2 P K Na Mg		
9/1		(N2) P K Na Mg		
8		P Na Mg		
7		P K Na Mg		
6		N1 P K Na Mg		
5		These areas used for microplot experiments.		
4/2		N2 P		
4/1		P		
3		Nil		
2/2		Nil		
2/1		K since 1996		
1		N1		
14/2		N*2 P K Na Mg		
14/1		(N*2) P K Na Mg		
15		P K Na Mg		
16		N*1 P K Na Mg		
17		N*1		

18d	
18c	
18/2	N2 K Na Mg
18b	
18a	
19/1	
19/2	FYM
19/3	
20/1	
20/2	FYM/ N°PK
20/3	

N

Plot layout and current treatments

Plot treatments
(per hectare per year unless indicated)

Nitrogen (applied in spring)

N1, N2, N3: ammonium sulphate supplying
48, 96, 144 kg N and 55, 110, 165 kg S

N*1, N*2: sodium nitrate supplying
48, 96 kg N and 78, 157 kg Na

(N2), (N*2): last applied 1989

Minerals (applied in winter)

P: triple superphosphate supplying 35 kg P

K: potassium sulphate supplying 225 kg K
    and 99 kg S

Na: sodium sulphate supplying 15 kg Na
     and 10 kg S

Mg: magnesium sulphate (Epsom salts)
     supplying 10 kg Mg and 13 kg S

Si: water soluble sodium silicate supplying
     135 kg Si and 63 kg Na

Plot 20: rates of fertilizer in years when FYM
is not applied; 30 kg N*, 15 kg P, 45 kg K

Organics (applied every 4th year)

FYM: 35 t farmyard manure supplying
      c. 240 kg N, 45 kg P, 350 kg K,
      25 kg Na, 25 kg Mg, 40 kg S, 135 kg Ca

PM: Pelleted poultry manure (replaced
     fishmeal in 2003) supplying c. 65 kg N

On plot 13/2 FYM and PM (previously fishmeal)
are applied in a 4-year cycle ie:
FYM in 2005, 2001, 1997, 1993 etc
PM in 2003, fishmeal in 1999, 1995, 1991 etc

(FYM/Fishmeal): FYM and fishmeal last applied
in 1993 and 1995 respectively.

Lime

Sub-plots a, b and c: differential amounts of
chalk applied, if needed, every three years to
maintain soil pH 7, 6 and 5, respectively

Sub-plot d receives no chalk

Plots 1-13 started in 1856, plots 14-17 in 1858,
plot 18 in 1865 and plots 19 and 20 in 1872.

Sub-plots range in size from 75 - 634 m^2

Figure 2: Schematic treatment layout and aerial view of the Park Grass Experiment (reproduced with permission from Rothamsted Research, 2006).

Harvesting

The herbage was cut and made into hay each year from the beginning of the experiment until 1960. The first cut was usually taken in mid-June and samples of hay were taken from the material dried *in situ*. The harvesting method was changed in 1960. Since then, strips have been cut with a forage harvester and vegetation samples were taken directly from the fresh material and dried afterwards. The herbage on the rest of the plot was made into hay as before. This change in method has led to higher recorded yields after 1960. For the first 19 years of the experiment the subsequent-regrowth after the first cut was often grazed by sheep penned on individual plots but, since 1875, a second cut has been taken, usually between late September and early November. The second cut has usually been cut and carted green. Samples from all plots and both cuts have been dried and stored in the Rothamsted Sample Archive since the beginning of the experiment (Silvertown *et al.,* 2006). Most plots have not been replicated nor randomized, however the meadow was reasonably uniform before the experiment began (Crawley *et al*., 2005) and the size of the plots (450-1910 m^2) compensated to some extent for the lack of replication.

Botanical composition

The original vegetation of the PGE has been classified by Dodd *et al*. (1994) as dicotyledon-rich *Cynosurus cristatus-Centaurea nigra* grassland, which is one of the mesotrophic grassland communities in the British National Vegetation Classification system. Vegetation analysis in years shortly after the beginning of the experiment counted approx. 60 species on the experimental area (eRA, 2011). Today species numbers vary from 3 to 44 per 200 m² (Crawley *et al*., 2005), showing that fertilization and liming led to strong changes in botanical composition (Silvertown *et al*., 1980, 2006; Crawley *et al*., 2005). According to Silvertown *et al*. (2006), by 1910 the botanical composition had reached a dynamic equilibrium at the functional group level and subsequent inter-annual changes were due to yearly differences in climate. For a summary of percentage contribution of functional groups see Fig 3.
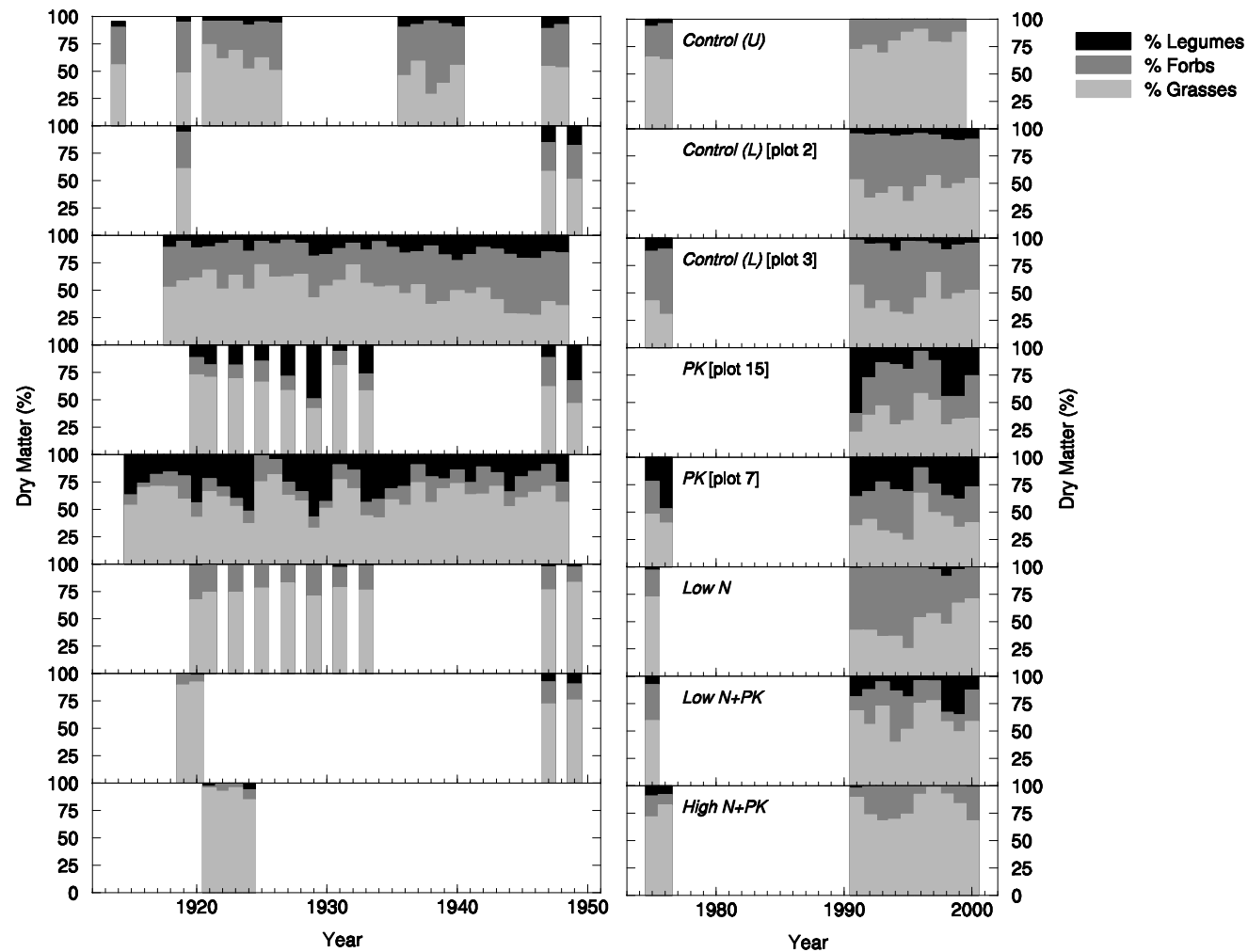
Figure 3: Percent contribution of functional groups (legumes, forbs, grasses) to total dry matter of the first cut on the studied treatments. Mean botanical composition at the functional group level was calculated from botanical separation data available for individual years in the 1915 – 1976 period and for each year in the 1991 – 2000 period with data from eRA (2011).

Material & Methods

Soil characteristics

The experiment is located on a moderately well-drained silty clay loam overlying clay-with-flints and chalk, the soil pH was slightly acid when the experiment began (5.4-5.6) and the nutrient status was poor (Silvertown *et al*., 2006). It is classified as stagnogleyic paleo-argillic brown earth by the Soil Survey of England and Wales or as Aquic Paleudalf according to USDA classification (Avery & Catt, 1995). The water holding capacity (or maximum plant available soil water, $PAW_{capacity}$) of the soil has not been measured directly, but was inferred from measurements on similar soils. It was 135 mm.

Climatic conditions and plant available soil water

Data on mean temperature, mean vapour pressure deficit (VPD), total rainfall and mean plant available soil water (PAW) at Park Grass are presented separately for the spring and summer/autumn growth in Fig. 4 (1915 – 2009 period). Mean temperature, VPD and total rainfall were calculated from April to June daily values for the spring growth and from July to September daily values for the summer / autumn growth. Data were available from eRA (2011). Mean daily temperature increased slightly by +0.8°C in spring and +1.3°C in summer / autumn during the 1915 – 2009 period. The strongest increase occurred after 1990. VPD remained constant in spring (0.4 ±0.05 kPa) but increased slightly by +0.1 kPa during summer in the 1915 – 2009 period ($P < 0.01$). The sum of rain averaged 166 (±46 mm) in spring and (189 ±64 mm) in autumn, no long-term change was observed. PAW was calculated from rainfall and potential evapotranspiration (PET) data (for the model see Schnyder *et al*., 2006). PET was estimated with the FAO Penman–Monteith equation for a standard grass reference crop (Allen *et al*., 1998; see also Köhler *et al*., 2010). The estimated PAW provides a standard measure for the overall evaporative conditions and water availability in individual years, but does not account for eventual treatment effects on PAW. The parameter was therefore termed PAW_{ref} (plant available soil water for the standard grass *reference* crop) in this study. Mean PAW_{ref} in June remained constant (64 ±30 mm), while mean PAW_{ref} in September decreased slightly by –28 mm, in the 1915 – 2009 period ($P < 0.05$). For more details on the calculations of weather parameters and PAW_{ref} see Köhler *et al*. (2010, 2012).
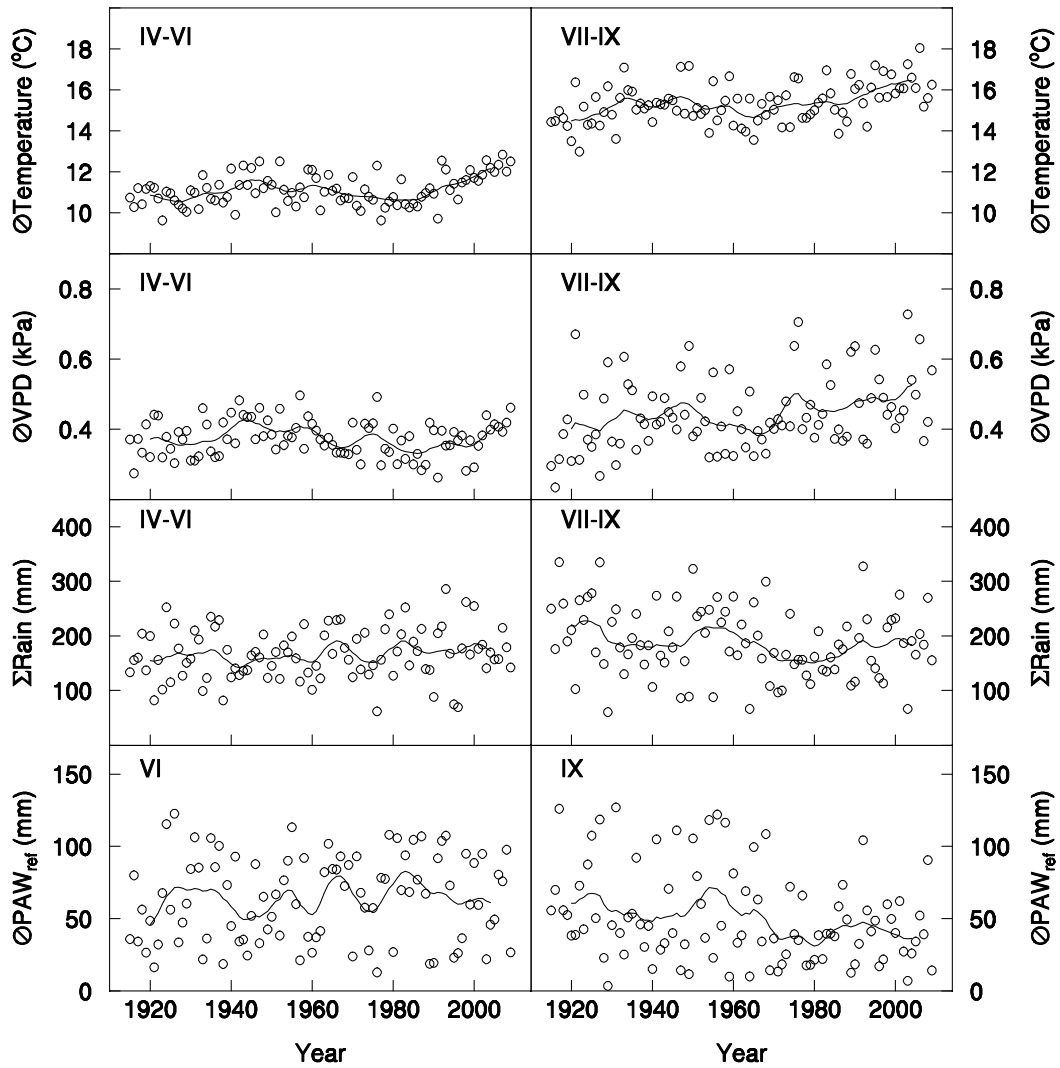
Figure 4: Climate data (temperature, VPD, rain) and plant available soil water (PAW_{ref}) during the growth season. Roman numerals indicate the months over which daily values were averaged (PAW_{ref}, VPD, temperature) or summed up (rain). Significant long-term trends (1915 – 2009, $P < 0.05$) were observed for temperature in both seasons (+0.8 resp. +1.3°C), and for VPD (+0.1 kPa) and PAW_{ref} (−28 mm) in the summer / autumn season. Data from eRA (2011). The black lines are the 11-year weighted average.

Selection of suitable treatments for carbon isotope analysis

Treatments suitable for the aims of this study were identified by considering nutrient supply, soil pH and botanical composition (for details on the chosen treatments and sampled plots see Table 2).

For the long-term record of the CO_2 response since the end of the Industrial Revolution the grassland sward on the unlimed control treatment, where no fertilizer or lime has been applied since the beginning of the experiment, was identified as most suitable. It is referred to as *"Control (U)"* in the following text. The botanical composition on this treatment resembles most the original vegetation on the site and no major changes in botanical composition have occurred. Dominant species on the unlimed and unfertilized plot 3*U* in 1858 were *Plantago lanceolata*, *Holcus lanatus* and *Lolium perenne* (> 10% contribution to hay weight; Williams *et al.*, 1978), in 1991 – 2000 the dominant species on the unlimed and unfertilized subplot 3 *d* were *Agrostis capillaris*, *Festuca rubra* and *Centaurea nigra* (eRA, 2011). The derived time series from the *Control (U)* covered the 1857 – 2007 period for the first cut and the 1875 – 2007 period for the second cut. Soil pH on the unlimed subplots 3*U* and *d* was 5.6. In order to study if the CO_2 response of $^{13}\Delta$ and W_i resp. W_t differed between seasons, samples from both cuts were analysed.

For the analysis of the effect of nutrient supply, plots with different levels and combinations of fertilizer supply were chosen. Chosen treatments included none, low (48 kg ha^{-1} yr^{-1}) and high (96 kg ha^{-1} yr^{-1}) N fertiliser inputs (applied as sodium nitrate) with or without additional P (35 kg ha^{-1} yr^{-1} as triple superphosphate) and K (225 kg ha^{-1} yr^{-1} as potassium sulphate). Five treatments were selected, they are referred to as *Control (L)*, *PK*, *Low N*, *Low N+PK* and *High N+PK* in the following. For details see Table 2. Treatments with N applied as sodium nitrate were preferred over treatments with N applied as ammonium sulphate because soil pH on the latter underwent strong changes. Subplots '*a*' with a target soil pH of 7 were chosen for the study, as soil pH was relatively stable over time on these subplots and was not negatively affecting plant growth. The control plot 3 *a* and its replicate 2 *a* were therefore sampled for comparisons within the same pH range. This limed control dataset is named *Control (L)* to distinguish it from the unlimed plot 3 *d Control (U)* data.

The soil pH of the limed (*L* and *a*) sub-plots used in this study varied between 6 and 7. As N fertilizers are applied in spring, the first cut profits most from the fertilizer application. Therefore only the first cut (spring growth) was used for the study of the effect of nutrient supply on the long-term $^{13}\Delta$ response. Strong changes in botanical composition occurred during the first decades of the experiment on the fertilized plots, therefore the time series was restricted to the 1915 – 2009 period for the analysis of nutrient effects. Botanical composition of the swards differed between treatments, but the functional groups within treatments were relatively constant (see Fig. 3).

Table 2: Details of treatments, sampled subplots, fertilizer and lime application, and dry matter production on selected treatments of the Park Grass Experiment.

Treatment	Sampled plots	Time period covered	Number of analysed samples	Annual nutrient applications of N, P and K (kg ha^{-1})	Total amount of lime applied since beginning of liming scheme in 1903 (CaCO$_3$ in t ha^{-1})	Mean annual dry matter production* (t ha^{-1}) for 1960 – 2009 of the first resp. second cut ±SD
Unlimed						
Control(U)	3U, 3d	1857 – 2007[†] 1875 – 2007[§]	144[†] 103[§]	nil	0[†] 0[§]	1.5 ±0.5[†] 1.4 ±1.0[§]
Limed[†]						
Control(L)	2L, 2a, 2-2a 3L, 3a	1915 – 2009	93 92	nil	85.9 87	2.1 ±0.6 2.1 ±0.6
Low N	17L, 17a	1915 – 2009	92	N: 48	69.5	2.7 ±0.6
PK	7L, 7a, 15L, 15ab, 15a	1915 – 2009	90 95	P: 35 K: 225	92.9 78.9	5.0 ±0.8 4.5 ±0.8
Low N+PK	16L, 16a	1915 – 2009	94	N: 48 P: 35 K: 225	84.7	5.0 ±0.6
High N+PK	14L, 14a, 14-2a	1915 – 2009	89	N: 96 P: 35 K: 225	66.7	5.0 ±1.0

[†]First cut; [§]Second cut; *from forage harvester yield

Sampling, sample preparation and stable isotope measurements

Sampling in the Rothamsted Sample Archive

Dried hay and herbage samples from the PGE have been stored in the Rothamsted Sample Archive since the beginning of the experiment. Older samples before 1919 were stored in glass bottles sealed with cork; newer samples were stored in tins. Subsamples of 2-3 g were taken from the original samples using a pair of long tweezers (Fig. 5). When necessary, original containers were cleaned with a paper cloth before opening in order to avoid contamination of the sample. Some samples from older cork-sealed bottles contained weevils, which were picked out of the subsample. If this was not possible, the samples were not taken. Some samples of the second cut were heavily contaminated with soil or tree leaves and were therefore not taken. In general, samples were dry and clean, suggesting no biological activity during the storage time. A typical example of a sample from 1920 is shown in Fig. 5. Subsamples were kept in glassine bags and stored in air tight plastic bags until further processing took place.

Figure 5: Taking of subsamples in the Rothamsted Sample Archive.

Sample preparation for isotope measurements

Plant samples were dried at 40°C for 48 h, ball milled to a homogenous fine powder and then dried again at 60°C for 3 h. Aliquots of 0.7 mg ±0.05 mg were weighed into tin cups (IVA Analysentechnik e.K., Meerbusch, Germany). The remaining sample was stored in capped microcentrifuge tubes ("Eppendorf tube").

Stable carbon isotope measurements

The sample aliquots were combusted in an elemental analyzer (NA 1110, Carlo Erba, Milan, Italy) interfaced (Conflo III, Finnigan MAT, Bremen, Germany) with an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT). Each sample was measured against a laboratory working standard CO_2 gas, which was previously calibrated against a secondary isotope standard (IAEA-CH6 for ^{13}C). After every tenth sample a solid internal laboratory standard (SILS) with similar C/N ratio as the respective sample material (fine ground wheat flour) was run as a control. The SILS were calibrated against an international standard (IAEA-CH6). The precision for sample repeats was better than 0.1‰ (SD of 57 SILS replications) for the analysis of the *Control (U)* samples. For the samples studied for the analysis of nutrient effects [*Control (L)*, *PK*, *Low N*, *Low N+PK*, *High N+PK*], the precision for sample repeats was better than 0.11‰ (SD of 190 SILS replications).

It was tested if the change of the harvesting method in 1960 had an effect on the $\delta^{13}C$ of the vegetation samples. This analysis was possible as samples for the first cut in 1992, 1993 and 1994 had been obtained with both harvesting methods. The comparison revealed that $\delta^{13}C$ of the samples taken from plot 3 *d* as hay were less negative by 0.18‰ (±0.06 SD). This phenomenon was probably related to larger losses of leaf material during hay making before 1960. To correct for this, 0.18‰ were subtracted from the $\delta^{13}C$ of the first cut samples collected before 1960. For the '*a*' subplots used for the analysis of the nutrient effect, only the *Control (L)* (plot 3), the *Low N+PK* and the *High N+PK* treatment had been harvested with both methods in the comparative sampling. Differences between $\delta^{13}C$ in forage and hay samples varied between years ($\delta^{13}C_{forage} -$ $\delta^{13}C_{hay}$: *Control (L)*: -0.3‰, -0.2‰, 0.02‰; *Low N+PK*: -0.2‰, -0.6‰, 0.1‰; *High*

N+PK: -0.5‰, -0.6‰, 0.03‰; in 1992, 1993, 1994 resp.). Thus a general correction factor could not be derived. As both negative and positive differences were observed, it was assumed that $\delta^{13}C$ values prior to 1960 were not systematically biased by sampling as hay and in this case I used the original data for the analyses.

Statistical Analysis

All statistical analysis was done with *R* (R Development Core Team, 2012). Long-term trends of $^{13}\Delta$ over time (and accordingly with increasing atmospheric CO_2 concentration) were analysed using simple and multiple linear regression. The *multcomp* package (Hothorn *et al*., 2008) in *R* was used for multiple comparisons of model parameters.

3. Results

Trends in carbon isotope discrimination ($^{13}\Delta$) and water-use efficiency of a semi-natural, unfertilized grassland since 1857

On the unlimed and unfertilized *Control (U)* treatment, $\delta^{13}C_p$ values have declined by approximately 2‰ during the last century. This reflects the decline in the source $\delta^{13}C$ of atmospheric CO_2 ($\delta^{13}C_a$) (see Figs. 2a and 2d in Köhler *et al*., 2010). $\delta^{13}C_a$ has decreased during the last century (Friedli *et al*., 1986) due to the burning of fossil fuels that are depleted in ^{13}C (this is also known as 'Suess effect').

$^{13}\Delta$ was derived from $\delta^{13}C_p$ and $\delta^{13}C_a$ using Eqn. 4. For the analysis of long-term trends over time on the *Control (U)* treatment the simple linear regression model

$$^{13}\Delta = \beta_0 + \beta_1 yr + \varepsilon \qquad\qquad (9)$$

was used, where yr is the year, β_0 and β_1 are fitting parameters to be estimated and ε is random error. It was tested if β_1 differed significantly from zero (i.e. did $^{13}\Delta$ change significantly with time?). The analysis was carried out for both cuts. During spring growth (first cut), $^{13}\Delta$ averaged 21.0‰ ($\pm$0.5‰ SD) and did not show a long-term trend ($P = 0.5$) since 1857. $^{13}\Delta$ of the summer/autumn growth (second cut) however, increased from 21.3‰ to 22.0‰ ($P < 0.001$) between 1875 and 2007 (see Figs. 2b and 2e in Köhler *et al*., 2010). Part of the variation in the $^{13}\Delta$ data could be explained by interannually changing weather conditions (see Table 1 in Köhler *et al*., 2010). The best relationship was found with PAW_{ref}, which explained 51% resp. 40% of the variation in $^{13}\Delta$ (first resp. second cut, June resp. July – September average of PAW_{ref} used for calculation; see Fig. 4 in Köhler *et al*., 2010). The relationship between $^{13}\Delta$ and PAW_{ref} could only be established for the 1947 – 2007 period for this dataset as wind speed data – which are needed for calculation of potential evapotranspiration (PET) with the Penman-Montheith model (Allen *et al*., 1998) – were not available for the period before 1947 at the time of the data analysis.

W_i was derived from $^{13}\Delta$ using Eqn. 7. For the spring growth (first cut) an increase of W_i by +33% since 1857 was estimated. W_i of summer/autumn growth (second cut) increased less, by only +18% since 1875 (see Figs. 2c and 2f in Köhler *et al*., 2010). VPD data were available since 1915. VPD during spring growth (March – June) has not changed since 1915, thus leading to an increase in instantaneous water-use efficiency (W_t) from 14.8 to 19.6 mmol mol^{-1} in spring. Conversely, VPD in the months between the first and second cut has increased by +0.07 kPa since 1915, offsetting the effect of increased W_i on W_t during summer and early autumn. Consequently, W_t remained constant at 13.9 mm mol^{-1} (see Figs. 3a and 3b in Köhler *et al*., 2010). For the detailed results see Köhler *et al*. (2010).

Interannual variation of $^{13}\Delta$ and its effect on the estimation of long-term trends

The long-term $^{13}\Delta$ trends on the *Control (U)* treatment during the 1857 resp. 1875 to 2007 periods revealed large interannual variation in the data, which could be partly explained by environmental parameters (see Table 1 in Köhler *et al*., 2010). The nature of the interannual $^{13}\Delta$ variation in the long-term data sets was studied in more detail for the extended first cut dataset from the *Control* (*U* and *L*), *PK*, *Low N*, *Low N+PK* and *High N+PK* treatments covering the 1915 – 2009 period.

$^{13}\Delta$ values, as calculated with Eqn. 4, showed large interannual variation in the range of 2.8‰ for the first cut on all treatments. Residuals ($^{13}\Delta_{Resid}$) of the simple linear regression of $^{13}\Delta$ values against CO_2 are shown in Fig. 6. Where replicate plots were available they are shown separately. A similar pattern was revealed on all plots and correspondingly, correlation coefficients between the plots were high and positive (between 0.6 and 0.9). This result strongly suggested that interannual variation of $^{13}\Delta$ resp. $^{13}\Delta_{Resid}$ was not random, but influenced by a factor that affected all treatments in the same way within the spring season.

Therefore, relationships between $^{13}\Delta_{Resid}$ of the first cut and the monthly averages (March to June) of several environmental factors (temperature, rainfall, VPD, PAW$_{ref}$) for the 1915 – 2009 period were tested for the six treatments. For this analysis the PAW$_{ref}$ dataset was extended back until 1915 as necessary wind speed data could be derived from wind force data (Beaufort scale) for the 1915 – 1947 period. Temperature

could not explain the observed variation, the sum of rainfall in May explained only between 8% and 22% of the variation and average VPD in May and June explained between 10% and 23% resp. 8% and 28% of the variation. Averages calculated for other months explained less variation. The percentage variance accounted for was highest for PAW_{ref} averaged for June (between 39 to 55%). I used a logarithmic transformation of PAW_{ref}, as the increase in $^{13}\Delta$ with increasing PAW followed closely a logarithmic relationship in the observed range. Details of the regression statistics for the different treatments, environmental factors and months are shown in Table 3. Only cases where the explained variance was > 8% are shown.
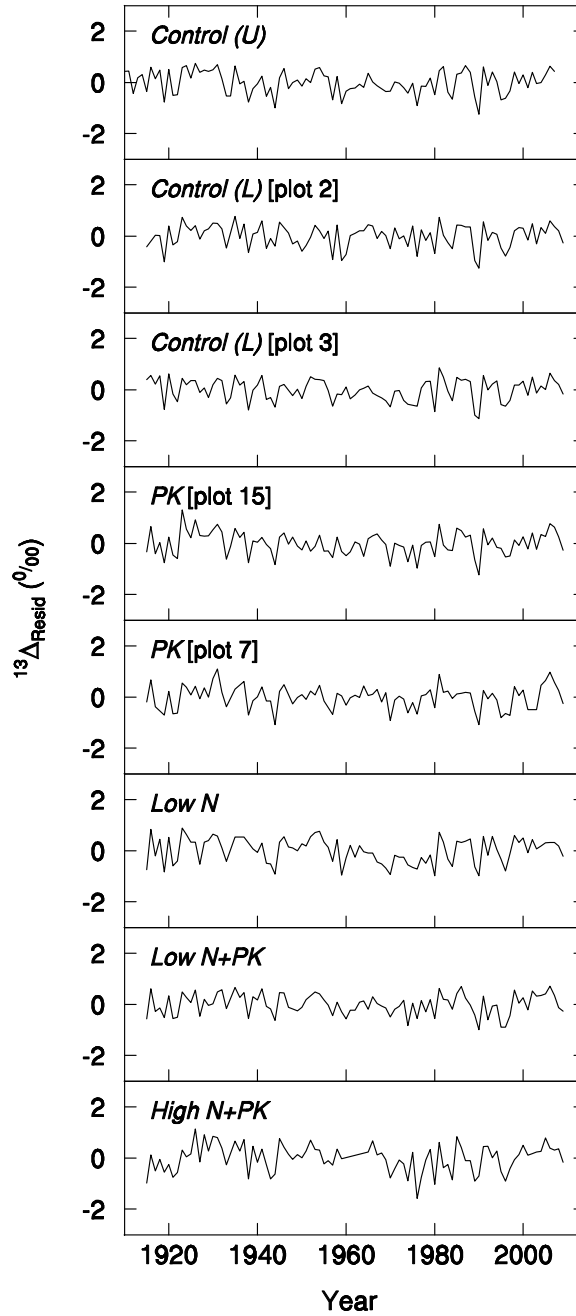
Figure 6: Residuals of the simple linear regression of $^{13}\Delta$ *vs*. CO_2 plotted against time show a similar high-frequency variation on all treatments. Replicate plots of the limed *Control (L)* (plot 2 and 3) and the *PK* (plot 7 and 15) treatments are shown separately.

Table 3: Summary of regression statistics for the simple linear regressions between $^{13}\Delta_{Resid}$ of the first cut and the average $\ln(PAW_{ref})$ in mm, average VPD in kPa and the rain sum in mm. Roman numerals indicate the months for which the daily data values of environmental parameters were averaged or summed up. The regressions were calculated for the 1915 – 2009[†] period.

	Control (U) df = 90			Control (L) df = 184			PK df = 183			Low N df = 90			Low N+PK df = 92			High N+PK df = 87		
	β_1	SE	r^2	β_1	SE	r^2	β_1	SE	r^2	β_1	SE	r^2	β_1	SE	r^2	β_1	SE	r^2
Ø $\ln(PAW_{ref})$																		
V	0.61***	0.13	0.20	0.70***	0.08	0.29	0.74***	0.08	0.29	0.55***	0.15	0.12	0.64***	0.11	0.27	0.82***	0.14	0.27
VI	0.58***	0.06	0.52	0.56***	0.04	0.52	0.59***	0.04	0.55	0.56***	0.07	0.39	0.51***	0.05	0.49	0.63***	0.08	0.44
Ø VPD																		
V	-2.61***	0.64	0.15	-2.61***	0.43	0.16	-3.35***	0.41	0.23	-2.35***	0.70	0.10	-2.64***	0.55	0.19	-2.67***	0.76	0.11
VI	-2.31***	0.38	0.28	-1.89***	0.27	0.20	-1.77***	0.28	0.17	-2.04***	0.44	0.19	-1.43***	0.37	0.13	-1.45**	0.51	0.08
∑ Rain																		
V	0.006***	0.00	0.13	0.006***	0.00	0.16	0.008***	0.00	0.22	0.005***	0.00	0.08	0.006***	0.00	0.15	0.007***	0.00	0.13

[†]1915 – 2007 for *Control (U)*

It was tested if the intercepts and slope parameters of the regressions between $^{13}\Delta_{Resid}$ and PAW_{ref} in June differed significantly between the treatments. As no significant difference was found data were pooled for a global analysis of the relationship (Fig. 7). PAW_{ref} in June explained 49% of the total variation in the $^{13}\Delta_{Resid}$ dataset.
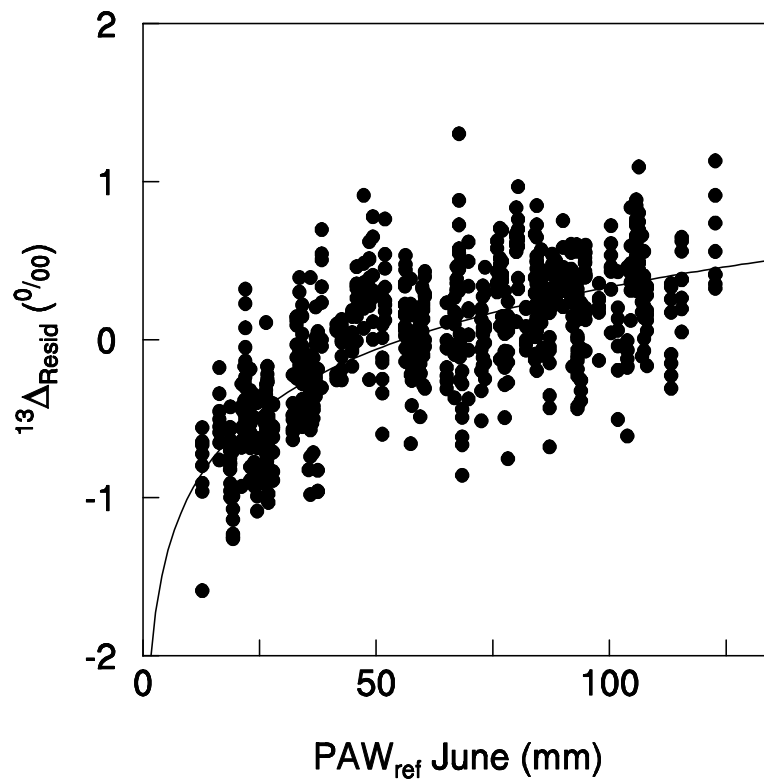
Figure 7: Relationship between $^{13}\Delta_{Resid}$ and PAW_{ref} in June for all plots of the first cut (pooled data from all treatments, 1915 – 2009 period). The black line is the fitted regression line ($^{13}\Delta_{Resid} = 0.57048 \ln(PAW_{refJUNE}) - 2.2937$, $P > 0.001$, adj. $r^2 = 0.49$, $df = 736$).

Based on these findings, a multiple linear regression model for the subsequent analysis of $^{13}\Delta$ long-term trends on the limed treatments with varying nutrient supply was developed. Atmospheric CO_2 concentration $[CO_2]$ and the natural logarithm of PAW_{ref} as explanatory variable were included in the model. Furthermore, treatment was included as a factor. The model

$$^{13}\Delta = \beta_0 + \beta_1 \text{Treatment}[CO_2] + \beta_2 \ln(PAW_{ref}) + \varepsilon \qquad (10)$$

was fitted to the $^{13}\Delta$ data of the limed treatments. β_0, β_1 and β_2 are fitting parameters to be estimated and ε is random error. The model can be used to assess if $^{13}\Delta$ changed significantly with increasing CO_2 by testing if β_1 differed significantly from zero. It was also tested if β_1 differed significantly between treatments.

High-frequency variation can mask long-term trends if it is not accounted for. It was checked if adjusting for PAW_{ref} affected the outcomes of the model parameter β_1 (i.e. the change of $^{13}\Delta$ with changing atmospheric CO_2) for the 1915 – 2009 period. For this test, the unlimed *Control (U)* was also included in the analysis. Omission of PAW_{ref} from the model did not change the slopes significantly (Table 4), however, without PAW_{ref} included it was not possible to detect the difference in the trends between the limed *Control (L)* and the *PK*, *Low N* and *Low N+PK* treatments (Table 5).

For the given time period (1915 – 2009) I estimated how big the maximum standard deviation of $^{13}\Delta$ could be, in order to still detect a certain long-term change in $^{13}\Delta$ as significantly different from zero at a given significance level. This was done by rearranging and solving the following test statistic (Kreyszig, 1979)

$$t_0 = s_x \sqrt{(n-1)(n-2)} \frac{b_1}{\sqrt{(n-1)\left(s_y^2 - b^2 s_x^2\right)}} \qquad (11)$$

of the test of $\beta_1 = 0$ against the alternative $\beta_1 > 0$ for s_y, where s_y is the standard deviation of the y variable ($^{13}\Delta$) and s_x the standard deviation of the x variable (atmospheric CO_2 concentration), n is the number of observations and b_1 is the slope estimate of β_1. t_0 was replaced by the critical t-value for a significance level of 5% and $(n-2)$ degrees of freedom (one-sided t-test).

The standard deviation of $^{13}\Delta$ in the 1915 – 2009 period averaged over all treatments at the PGE was 0.5‰. With Eqn. 11 it can be shown that s_y can be more than

twice as big (1.1‰) to still detect an increase of 0.009‰ $(\mu mol\ mol^{-1})^{-1}$ as observed on the *Control (L)* in the 1915 – 2009 period. The slope of the change in $^{13}\Delta$ with increasing atmospheric CO_2 (β_1) following the response scenario (3) ('constant c_a-c_i', cf. Fig. 1a) depends on the value of c_i/c_a in the beginning year of the observation. With the observed c_i/c_a ratio of 0.7 at the PGE in 1915, β_1 for response scenario (3) was calculated to be 0.017‰ $(\mu mol\ mol^{-1})^{-1}$. For comparison with results from studies with different c_i/c_a I calculated the maximum standard deviations (s_y) of $^{13}\Delta$ for decreasing slopes from 0.029‰ $(\mu mol\ mol^{-1})^{-1}$ (the resulting slope for response scenario 3 with a c_i/c_a ratio of 0.5) to 0.001‰ $(\mu mol\ mol^{-1})^{-1}$ [in steps of 0.001 ‰ $(\mu mol\ mol^{-1})^{-1}$] and for time periods consecutively shortened by increasing the starting year 1915 in one year steps. Table 6 summarizes the results for the PGE and shows the minimum starting years required to detect a specific slope as statistically significant from zero (for $s_y = 0.5$‰ and c_i/c_a in 1915 = 0.7). The full analysis is attached in Appendix I. Calculations were only done for positive β_1, as responses with negative slopes (cf. scenario (1) 'constant c_i', Fig. 1a) were hardly observed in long-term studies (Table 1). Furthermore, for equivalent values of negative slopes, the results reported in Appendix I would be the same.

Table 4: Summary of regression statistics for the 1915 – 2009[†] period for the relationship between $^{13}\Delta$ of the first cut and CO_2, with and without PAW_{ref} included in the linear regression model. P-values < 0.05 are in bold.

Model	$^{13}\Delta = \beta_0 + \beta_1$ Treatment [CO_2] $+ \beta_2 \ln(PAW_{ref}) + \varepsilon$			$^{13}\Delta = \beta_0 + \beta_1$ Treatment [CO_2] $+ \varepsilon$		
	adj. r^2=0.55, df=725			adj. r^2=0.12, df=726		
	β_1	SE	P	β_1	SE	P
Control (U)	0.001	0.001	n.s.	0.001	0.002	n.s.
Control (L)	0.009	0.001	**< 0.001**	0.009	0.001	**< 0.001**
PK	0.004	0.001	**< 0.001**	0.004	0.001	**< 0.01**
Low N	0.003	0.001	n.s.	0.003	0.002	n.s.
Low N+PK	0.003	0.001	n.s.	0.004	0.002	n.s.
High N+PK	0.000	0.001	n.s.	0.001	0.002	n.s.

[†]1915 – 2007 for *Control (U)*

Table 5: Adjusted P-values of the differences between the slopes of the multiple and simple linear regression models for the first cut data. Results of comparisons of the multiple linear regression ($^{13}\Delta = \beta_0 + \beta_1$ Treatment [CO_2] $+ \beta_2 \ln(PAW_{ref}) + \varepsilon$) are shown in the right upper-half, results for the simple linear regression ($^{13}\Delta = \beta_0 + \beta_1$ Treatment [CO_2] $+ \varepsilon$) are shown in the left lower-half of the table (divided by the diagonal black line), P-values < 0.05 are in bold.

	Control (U)	*Control (L)*	*PK*	*Low N*	*Low N+PK*	*High N+PK*
Control (U)		**< 0.001**	0.443	0.873	0.787	1
Control (L)	**0.014**		**0.005**	**0.006**	**0.012**	**< 0.001**
PK	0.839	0.094		0.993	0.999	0.274
Low N	0.987	0.097	0.997		1	0.758
Low N+PK	0.963	0.151	1	1		0.645
High N+PK	1	**0.003**	0.625	0.933	0.870	

Table 6: Overview of the minimum start years of the simple linear regression which would be necessary to detect a certain slope parameter (change of $^{13}\Delta$ with 1 μmol mol^{-1} CO_2 increase) as statistically significant from zero ($\alpha = 5\%$) under the assumption that the standard deviation of the $^{13}\Delta$ values is 0.5‰ as observed on the PGE. End year for all regressions was 2009, the minimum start year was 1915. 0.017 is the value of β_1 if the $^{13}\Delta$ response followed scenario (3) ('constant c_a-c_i').

β_1	0.017	0.016	0.015	0.014	0.013	0.012	0.011	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Minimum start year	1988	1987	1986	1984	1983	1981	1979	1977	1975	1971	1967	1960	1950	1929	na	na	na

Trends in $^{13}\Delta$ and intrinsic water-use efficiency (W_i) of grasslands with varying nutrient supply (1915 – 2009)

On the studied limed treatments (*Control (L)*, *PK*, *Low N*, *Low N+PK* and the *High N+PK*), $\delta^{13}C_p$ values have declined during the last century, reflecting also the decline in $\delta^{13}C_a$ (see Fig. 1 in Köhler *et al.*, 2012). The multiple linear regression model (Eqn. 10) with average PAW$_{ref}$ in June showed that $^{13}\Delta$ increased significantly ($P < 0.001$) on the *Control (L)* (0.9‰ per 100 μmol mol^{-1} CO_2 increase). This trend differed significantly ($P < 0.01$) from those observed on the fertilized treatments (*PK*: 0.4‰ per 100 μmol mol^{-1} CO_2 increase, $P < 0.001$; *Low N*, *Low N+PK* and *High N+PK*: no significant increase; see Fig. 2a in Köhler *et al.*, 2012). The $^{13}\Delta$ trends on fertilized treatments did not differ significantly from each other.

Mean W_i on the limed treatments in 1915 (derived from modelled $^{13}\Delta$) was 53.1 μmol mol^{-1} (±0.8 SD) and increased between +13% on the *Control (L)* to +28% on the *High N+PK* treatment. Other treatments lay in-between (*PK*: +22%, *Low N* and *Low N+PK*: +23%). The changes in W_i per 1 μmol mol^{-1} CO_2 increase ranged between +0.08 [*Control (L)*] and +0.17 (*High N+PK*). Responses were close to the 'constant c_i/c_a' scenario (2) except for the *Control (L)*, which showed a considerably lower increase in W_i (Fig. 8).

The effect of nutrient supply was first studied within treatments with similar soil pH [*Control (L)*, *PK*, *Low N*, *Low N+PK*, *High N+PK*]. N status, assessed as N fertilizer supply plus an estimate of biologically fixed N, was negatively related ($r^2 = 0.88$; $P < 0.02$) to the trend for $^{13}\Delta$ against CO_2 on these limed treatments. Other indices of N status were also tested: The nitrogen nutrition index (NNI) and the Ellenberg N indicator value both exhibited similar relationships, but were only significant on the 10% α-level (see Fig. 3 in Köhler *et al.*, 2012). The NNI is an ecophysiologically based indicator of canopy N status (Lemaire & Gastal, 1997) derived from actual plant N concentrations and an estimate of the critical plant N value. The Ellenberg N value is an ecological indicator value derived from observations of the occurrence of plant species along gradients of soil N availability or soil fertility (Ellenberg *et al.*, 1992; Hill *et al.*, 1999; Diekmann, 2003).
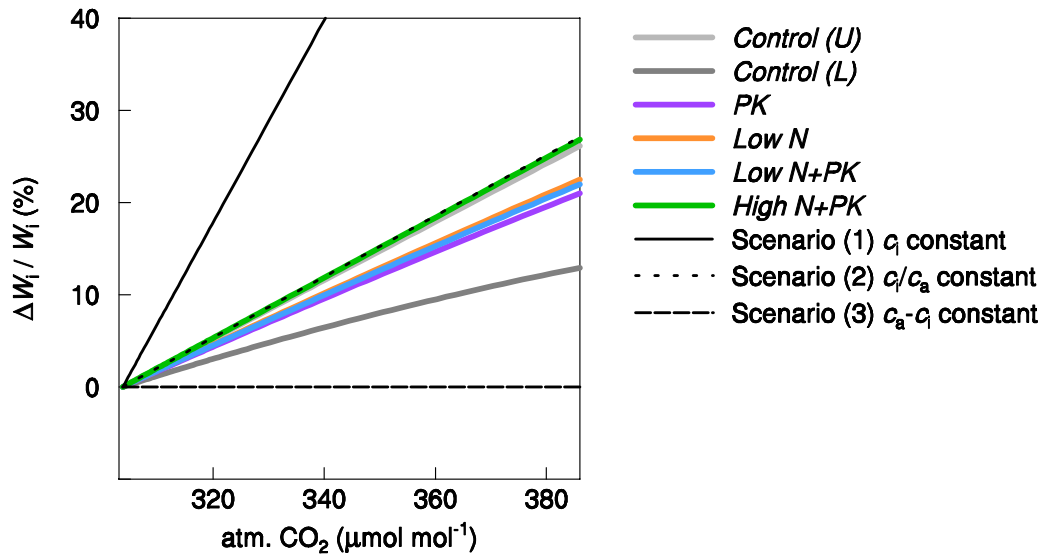
Figure 8: Change in W_i ($\Delta W_i/W_i$ in %) on the studied treatments during spring growth relative to W_i in 1915 (equivalent to 303 μmol mol^{-1} atmospheric CO_2) and comparison to the response scenarios by Saurer *et al.* (2004) with a c_i/c_a ratio of 0.7 in 1915.

The relationship with N status, NNI and the Ellenberg N value suggested an effect of nutrient supply on the $^{13}\Delta$ response. Accordingly, the increase in W_i on the *High N+PK* treatment was twice that of the *Control (L)* treatment (+28% resp. +13% relative to 1915; see Fig. 2b in Köhler *et al.*, 2012). For the detailed results see Köhler *et al.* (2012). However, the unlimed *Control (U)* treatment did not fit with the relationships between the $^{13}\Delta$ response and nutrient measures. Its $^{13}\Delta$ response matched the response of the *High N+PK* treatment and differed significantly from the limed *Control (L)*. As botanical composition differed between the treatments, it was tested if the $^{13}\Delta$ responses were related to the percentage of functional groups in the swards. A significant relationship between the $^{13}\Delta$ response and percentage contribution of grasses to total biomass of the swards was observed when all treatments where considered (see Fig. 4 in Köhler *et al.*, 2012).

4. Summarising Discussion

Increasing W_i caused by decreased stomatal conductance?

Trends in W_i of spring growth on the six studied treatments [*Control (U)*, *Control (L)*, *PK*, *Low N*, *Low N+PK*, *High N+PK*] revealed general increases in W_i (Köhler *et al*., 2010, 2012). $^{13}\Delta$ trends followed closely the 'constant c_i/c_a' response scenario (2) (Fig. 8) with the exception of the limed unfertilized *Control (L)*. For the observed increases of W_i under increasing atmospheric CO_2, c_i must increase less strongly than c_a (Fig. 1c). This requires an increase in A and / or a decrease in g_s (McCarroll *et al*., 2009). Possible combinations of the underlying percentage changes in A and g_s are illustrated in Fig. 9. Against the background of the general results from short-term studies (Ainsworth & Rogers, 2007), the situation where A decreases and g_s increases is not considered here. If g_s remained constant, A would have had to increase by between +13% to +28%. If A remained constant, then g_s would have had to decrease by between –22% and –12%.

Using yield as a proxy for stand-scale A, the observed increases in W_i then strongly suggest that grassland vegetation on the PGE has adjusted physiologically to elevated CO_2 mainly by decreasing g_s: An analysis by Jenkinson *et al*. (1994) indicates that yields (harvested aboveground biomass) have not increased at the PGE, at least not on the unlimed plots. Belowground biomass has not been measured, but data on soil organic C (SOC) at the PGE suggests that increased CO_2 has not led to increased C accumulation in the PGE soil through changes in the allocation of C to the roots: No increases of SOC on limed or unlimed subplots were observed (Hopkins *et al*., 2009; Fornara *et al*., 2011). This supports the assumption that aboveground yield can serve as a proxy for A at stand-scale on the PGE. Increases in A per unit leaf area during the study period would then have been minor or have not occurred at all, provided that leaf area index (LAI) stayed constant. According to Long *et al*. (2004), LAI under elevated CO_2 seems to remain unchanged in the long-term.
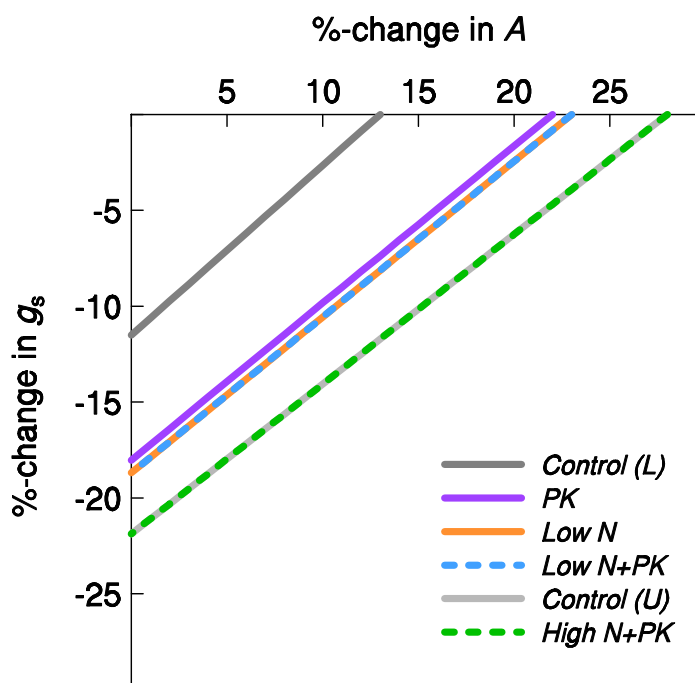
Figure 9: Possible combinations of the percentage changes in *A* and g_s for the observed changes in W_i (= *A* / g_s) between 1915 and 2009 on the studied treatments.

The indication of unchanged *A* at Park Grass contrasts with results for C_3 plants from *short-term* FACE studies (Ainsworth & Rogers, 2007), where a general increase in *A* (+31%) was observed alongside a decrease in g_s (–22%). In these experiments c_a was increased from 366 to 567 μmol mol^{-1} on average, but even in experiments where CO_2 concentration was varied between pre-industrial and today's levels, strong increases of W_i and the above-ground biomass per plant were found (Polley *et al*., 1993).

However, results from *long-term* studies in different (agro)ecosystems are consistent with the observations at Park Grass: For wheat, Zhao *et al*. (2001) observed increases in W_i for the 1845 – 1965 period, but not afterwards. No increases in yields were observed and they concluded that N limitation constrained the increase of C assimilation under rising CO_2. Peñuelas *et al*. (2011) found that increased W_i during the 20th century has not led to an overall increased tree growth. Independent of the biome type, half of the study sites showed enhanced growth, the other half negative or no trends. They suggested that environmental factors like drought or nutrient limitation may

have limited the growth response. At the same time, decreases in g_s were observed by Lammertsma *et al.* (2011), who analysed leaf material of nine C_3 species from 1880 to present and found a –34% (±12%) reduction in maximum g_s per 100 μmol mol^{-1} CO_2 increase. Using subfossil leaf measurements of stomatal density and geometry, Gagen *et al.* (2011) demonstrated that the maximum g_s in *Betula nana* declined by –15% between 1850 and 2000.

The results from the PGE thus indicate that the effect of rising atmospheric CO_2 on C uptake by plants may also be overestimated in grassland ecosystems, when predictions are based on short-term results. This means that both in forests and in grassland ecosystems, the proposed increases in C sequestration (Melillo *et al.*, 1993; Friedlingstein *et al.*, 2006) may not be realized. Instead, the reduction in g_s can affect hydrological cycles, as has already been shown for the 20th century (Betts *et al.*, 2007) and is predicted to continue throughout the 21st century (de Boer *et al.*, 2011).

Increases in W_i are not directly related to varying nitrogen supply

For the limed Park Grass treatments it was shown that the changes in $^{13}\Delta$ under increasing atmospheric CO_2 were negatively correlated with N input (Fig. 3a in Köhler *et al.*, 2012). W_i thus increased more strongly with higher N input. However, when the unlimed *Control (U)* was included in the analysis the relationship with N input was no longer apparent as the response of the unlimed *Control (U)* treatment was similar to the response of the *High N+PK* treatment. This is in contrast to results from short-term experiments with grassland species under elevated CO_2: Arp *et al.* (1998) reported that WUE (in g drymass per dm³ total water use) was highest under high N supply and elevated CO_2. They found stronger increases in biomass with higher N supply in comparison to low N supply. This was also observed by Reich *et al.* (2006*a*) and Daepp *et al.* (2000), however, they did not analyse water use. Still, the stronger increases in biomass indicate increases in *A* and thus probably also in WUE. On the contrary, the results from the unlimed *Control (U)* and the *High N+PK* treatment show that increases in W_i at Park Grass were not generally lower in N limited grassland swards. This indicated that other factors in addition to nutrient supply play a role for the long-term W_i response in (semi)natural grassland ecosystems.

As shown in Fig. 4 of Köhler *et al.* (2012), the CO_2 response of $^{13}\Delta$ correlated with the proportion of grasses in the swards. From Fig. 3 it becomes apparent that botanical composition on the unlimed *Control (U)* and *High N+PK* treatment is characterized by a high contribution of grasses, in contrast to the limed *Control (L)*. Under the assumption of unchanged *A*, the similar CO_2 response of $^{13}\Delta$ on the unlimed *Control (U)* and the limed *High N+PK* treatment indicates that g_s decreased strongly on both treatments while the g_s decrease on the limed *Control (L)* was lower (cf. Fig. 9). To my knowledge, direct effects of nutrient supply on g_s, have not been measured in short-term FACE studies. However, it has been shown that the responsiveness of g_s to elevated CO_2 differed between functional groups. Ainsworth & Rogers (2007) reported that the response of g_s was greater in C_3 grasses (approx. –36%) than in forbs and legumes (approx. –21%) when c_a was increased from 366 to 567 μmol mol^{-1}.

The observed effect of N supply on the CO_2 reponse (Fig. 3a, Köhler *et al.*, 2012) may thus have been due to indirect effects of N supply on botanical composition. Other factors that influence botanical composition, like e.g. soil pH, may also play a role, as was indicated by the different responses of the limed and unlimed *Control* treatments, *Control (U)* and *Control (L)*. While both the unlimed *Control (U)* and the limed *Control (L)* received no fertilization, they differed in the amount of applied chalk, soil pH and in botanical composition. For mixed C_3 / C_4 plant communities, it has been shown that N addition can lead to shifts in plant community composition, suppressing the CO_2 growth response (Langley & Megonigal, 2010). The results from the PGE show that this may also be true in pure C_3 grassland ecosystems, which can rapidly undergo changes in species composition following changes in nutrient availability. Effects of shifts in botanical composition will thus have to be considered in models for the estimation of N effects.

High-frequency variation of environmental parameters and its effects on the detection of long-term trends of W_i

The analysis of long-term trends of stable C isotopes has been widely used to assess gas exchange responses of plants to increasing atmospheric CO_2 concentration. However, the $^{13}\Delta$ signal is not only influenced by responses of *A* or g_s to atmospheric CO_2 concentrations, but also by effects of other environmental parameters (Farqhuar *et*

al., 1989*a*) on A and g_s. Long-term changes in these environmental parameters can interfere with the effect of CO_2 on the long-term response of W_i, but environmental parameters which affected the high-frequency variation of $^{13}\Delta$ of spring growth (Table 3) showed no significant long-term trends at the PGE experimental site (Fig. 4). The effect of the high frequency variation of environmental parameters on $^{13}\Delta$ variation, however, can impede the detection of long-term trends and thus hinder comparison of responses and classification according to the theoretical response scenarios.

A review of reported standard deviations of $^{13}\Delta$ in long-term studies (including the PGE data) showed that they varied between 0.3‰ and 1.0‰, with a mean of 0.6‰ (Saurer *et al*., 2004; Arneth *et al*., 2002; derived from SE: Peñuelas & Azcón-Bieto, 1992; calculated from own data: Barbosa *et al*., 2010; Köhler *et al*., 2010; Köhler *et al*., 2012). It was shown that with a standard deviation of 0.6‰ of the $^{13}\Delta$ values, it would be possible to detect trends as small as 0.005‰ per 1 μmol mol^{-1} CO_2 increase as significantly different from zero in the studied time period 1915 – 2009 (Appendix I), even without adjusting for high frequency variation. Nonetheless, adjusting for high frequency variation can facilitate the detection of long-term trends, in particular when time-periods are short. It is therefore recommended that, whenever possible, the high-frequency variation should be accounted for. The best variables to explain scatter caused by inter-annually changing weather conditions seem to be integrative values that account for a variety of environmental parameters, as shown for PAW_{ref} in grasslands (Table 3; Schnyder *et al*., 2006; Köhler *et al*., 2010). This parameter is derived from precipitation and PET, which integrates temperature, wind speed, vapour pressure deficit and solar radiation. For forests, Betson *et al*. (2007) tested a fire hazard index (FHI) which integrated temperature, wind speed, relative humidity and precipitation and also found that this integrative parameter could explain variation better than single climatic parameters.

The W_i response of grasslands and forests in comparison

The comparison of the W_i long-term response of grassland vegetation with results from tree-ring studies (Table 1) showed no systematic difference. This suggests that, irrespective of the structural and functional differences between short-lived herbaceous vegetation and long-lived woody plants, both have responded in a similar way during the

increase of atmospheric CO_2 concentrations in the last century. Some studies (Gagen *et al*., 2011; Waterhouse *et al*., 2004) reported a lower CO_2 responsiveness of trees and concurrently reduced increases of W_i since about 1970. When the time series from the PGE were analysed for the 1970 – 2009 period only, significant increases of $^{13}\Delta$ [ranging from 0.008 to 0.015 ‰ $(\mu mol\ mol^{-1})^{-1}$] were found on all treatments, reducing the rate of increase of W_i. Grassland ecosystems with short-lived species and related fast generation changes should be able to adapt quicker to environmental changes by genetic adaptations or changes in species compositions in addition to plastic responses. Rapid genetic adaptation under the different nutrient regimes at the PGE have been shown e.g. for *Anthoxanthum odoratum* (Freeland *et al*., 2010). Yet, the results of long-term responses at stand-scale (grassland ecosystem) show no general difference to results at single individual scale, i.e. trees, which are limited to plastic adaptations. As yet, the observed increase in atmospheric CO_2 has likely not lead to a selection of more CO_2 responsive species or genotypes at the PGE.

Conclusions and Outlook

The responses of A and g_s cannot be separated by analysis of stable C isotopes alone. Yet, for estimation of feedback effects on C and water cycles it is necessary to understand how A and g_s were regulated. In this study, yield data was used as a proxy for A. Another option is the analysis of stable oxygen isotopes (^{18}O), which can be used to disentangle the responses of A and g_s (Scheidegger *et al*., 2000). Future additional analysis of ^{18}O in plant biomass at the PGE will help to confirm the reaction of A and g_s and contribute to our understanding how g_s and transpiration have already been affected in grasslands under rising atmospheric CO_2.

The results of this study showed that the $^{13}\Delta$ and W_i long-term responses of (semi)natural grassland ecosystems under rising atmospheric CO_2 resembled that of trees. The varying responses of grassland swards that differ in nutrient supply and in botanical composition indicated that the shorter-term responses of single species under controlled environments cannot be directly upscaled to stand-scale responses under gradually increasing CO_2. For predictions of future atmospheric CO_2 concentrations it is therefore necessary to consider the effect of N (Hungate *et al*., 2003) and recent models are taking this into account (Sokolov *et al*., 2008; Thornton *et al*., 2009).

However, it has to be considered that effects of additional environmental parameters on other components of the system can lead to responses not directly connected to the initially changed parameter (e.g. the effect of nutrients on botanical composition). In addition, large amounts of N may be needed to support an increase in *A* under rising atmospheric CO_2. For *Lolium perenne* it was shown that an excess input of N (yearly average: +83 kg ha^{-1}) was needed for an increased yield response under elevated CO_2 (Daepp *et al*., 2000). In forests, Oren *et al*. (2001) observed no difference between a nutritionally poor and moderate site: biomass increased only after additional N fertilization (+112 kg ha^{-1} yr^{-1}). The apparent lack of an increase in *A* on the PGE may be due to a growth limiting N availability as indicated by moderate NNI (Köhler *et al*., 2012) on all treatments despite different levels of nutrient supply. Thus, the ability of (semi)natural ecosystems to sequester additional C under elevated atmospheric CO_2 may be strongly limited unless a deliberate eutrophication was accepted.

5. Acknowledgements

6.   References

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Appendix I: Maximum allowed standard deviations of $^{13}\Delta$ in order to detect a certain slope [in the range of 0.001 to 0.029‰ (μmol mol^{-1})$^{-1}$] as significantly different from zero within a certain period of time, calculated for periods prior to 2009 and extending back until 1915. The gray-shaded area marks the timeperiods where a detection of significant trends is not possible if the standard deviation is > 0.5‰.

Start year	c_a (μmol mol^{-1})	n	.029	.028	.027	.026	.025	.024	.023	.022	.021	.020	.019	.018	.017	.016	.015	.014	.013	.012	.011	.010	.009	.008	.007	.006	.005	.004	.003	.002	.001
1915	304	95	3.6	3.5	3.4	3.3	3.1	3.0	2.9	2.8	2.6	2.5	2.4	2.3	2.1	2.0	1.9	1.8	1.6	1.5	1.4	1.3	1.1	1.0	0.9	0.8	0.6	0.5	0.4	0.3	0.1
1916	304	94	3.6	3.5	3.4	3.2	3.1	3.0	2.9	2.7	2.6	2.5	2.4	2.2	2.1	2.0	1.9	1.7	1.6	1.5	1.4	1.2	1.1	1.0	0.9	0.7	0.6	0.5	0.4	0.2	0.1
1917	304	93	3.6	3.5	3.3	3.2	3.1	3.0	2.8	2.7	2.6	2.5	2.3	2.2	2.1	2.0	1.9	1.7	1.6	1.5	1.4	1.2	1.1	1.0	0.9	0.7	0.6	0.5	0.4	0.2	0.1
1918	305	92	3.6	3.4	3.3	3.2	3.1	2.9	2.8	2.7	2.6	2.5	2.3	2.2	2.1	2.0	1.8	1.7	1.6	1.5	1.3	1.2	1.1	1.0	0.9	0.7	0.6	0.5	0.4	0.2	0.1
1919	305	91	3.5	3.4	3.3	3.2	3.0	2.9	2.8	2.7	2.6	2.4	2.3	2.2	2.1	1.9	1.8	1.7	1.6	1.5	1.3	1.2	1.1	1.0	0.9	0.7	0.6	0.5	0.4	0.2	0.1
1920	305	90	3.5	3.4	3.3	3.1	3.0	2.9	2.8	2.7	2.5	2.4	2.3	2.2	2.1	1.9	1.8	1.7	1.6	1.5	1.3	1.2	1.1	1.0	0.8	0.7	0.6	0.5	0.4	0.2	0.1
1921	306	89	3.5	3.4	3.2	3.1	3.0	2.9	2.8	2.6	2.5	2.4	2.3	2.2	2.0	1.9	1.8	1.7	1.6	1.4	1.3	1.2	1.1	1.0	0.8	0.7	0.6	0.5	0.4	0.2	0.1
1922	306	88	3.5	3.3	3.2	3.1	3.0	2.9	2.7	2.6	2.5	2.4	2.3	2.1	2.0	1.9	1.8	1.7	1.5	1.4	1.3	1.2	1.1	1.0	0.8	0.7	0.6	0.5	0.4	0.2	0.1
1923	306	87	3.4	3.3	3.2	3.1	3.0	2.8	2.7	2.6	2.5	2.4	2.2	2.1	2.0	1.9	1.8	1.7	1.5	1.4	1.3	1.2	1.1	0.9	0.8	0.7	0.6	0.5	0.4	0.2	0.1
1924	307	86	3.4	3.3	3.2	3.1	2.9	2.8	2.7	2.6	2.5	2.3	2.2	2.1	2.0	1.9	1.8	1.6	1.5	1.4	1.3	1.2	1.1	0.9	0.8	0.7	0.6	0.5	0.4	0.2	0.1
1925	307	85	3.4	3.3	3.1	3.0	2.9	2.8	2.7	2.6	2.4	2.3	2.2	2.1	2.0	1.9	1.7	1.6	1.5	1.4	1.3	1.2	1.0	0.9	0.8	0.7	0.6	0.5	0.3	0.2	0.1
1926	307	84	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.5	2.4	2.3	2.2	2.1	2.0	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.0	0.9	0.8	0.7	0.6	0.5	0.3	0.2	0.1
1927	308	83	3.3	3.2	3.1	3.0	2.9	2.8	2.6	2.5	2.4	2.3	2.2	2.1	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.3	0.2	0.1
1928	308	82	3.3	3.2	3.1	3.0	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.3	0.2	0.1
1929	308	81	3.3	3.2	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.3	0.2	0.1
1930	309	80	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.4	0.3	0.2	0.1
1931	309	79	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.4	0.3	0.2	0.1
1932	309	78	3.2	3.1	3.0	2.9	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.5	0.4	0.3	0.2	0.1
1933	310	77	3.2	3.1	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.5	0.4	0.3	0.2	0.1
1934	310	76	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.6	0.5	0.4	0.3	0.2	0.1
1935	310	75	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1936	311	74	3.1	3.0	2.9	2.8	2.7	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1937	311	73	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1938	311	72	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1939	312	71	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1940	312	70	3.0	2.9	2.8	2.7	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1941	313	69	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1942	313	68	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1943	313	67	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1944	314	66	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	2.0	1.9	1.8	1.6	1.5	1.4	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1945	314	65	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1946	315	64	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1947	315	63	2.7	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1948	315	62	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.7	0.6	0.5	0.4	0.3	0.2	0.1
1949	316	61	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.6	0.5	0.4	0.3	0.2	0.1
1950	316	60	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.5	0.4	0.3	0.2	0.1
1951	316	59	2.6	2.5	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.5	0.4	0.4	0.3	0.1
1952	317	58	2.6	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.5	0.4	0.4	0.2	0.1
1953	317	57	2.5	2.4	2.3	2.3	2.2	2.1	2.0	1.9	1.8	1.7	1.6	1.6	1.5	1.4	1.3	1.2	1.1	1.0	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.3	0.2	0.1
1954	318	56	2.5	2.4	2.3	2.2	2.1	2.0	2.0	1.9	1.8	1.7	1.6	1.5	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.3	0.2	0.1
1955	318	55	2.4	2.4	2.3	2.2	2.1	2.0	1.9	1.8	1.8	1.7	1.6	1.5	1.4	1.3	1.3	1.2	1.1	1.0	0.9	0.8	0.8	0.7	0.6	0.5	0.4	0.3	0.3	0.2	0.1
1956	319	54	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.8	1.7	1.7	1.6	1.5	1.4	1.3	1.2	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.2	0.1	
1957	319	53	2.4	2.3	2.2	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.5	1.5	1.4	1.3	1.2	1.1	1.1	1.0	0.9	0.8	0.7	0.6	0.6	0.5	0.4	0.3	0.2	0.2	0.1
1958	319	52	2.3	2.2	2.2	2.1	2.0	1.9	1.8	1.8	1.7	1.6	1.5	1.4	1.4	1.3	1.2	1.1	1.0	1.0	0.9	0.8	0.7	0.6	0.6	0.5	0.4	0.3	0.2	0.2	0.1

Appendix I continued

Start year	c_a	n	.029	.028	.027	.026	.025	.024	.023	.022	.021	.020	.019	.018	.017	.016	.015	.014	.013	.012	.011	.010	.009	.008	.007	.006	.005	.004	.003	.002	.001
1959	320	51	2.3	2.2	2.1	2.0	2.0	1.9	1.8	1.7	1.6	1.6	1.5	1.4	1.3	1.3	1.2	1.1	1.0	0.9	0.9	0.8	0.7	0.6	0.5	0.5	0.4	0.3	0.2	0.2	0.1
1960	320	50	2.2	2.1	2.1	2.0	1.9	1.8	1.8	1.7	1.6	1.5	1.5	1.4	1.3	1.2	1.1	1.1	1.0	0.9	0.8	0.8	0.7	0.6	0.5	0.5	0.4	0.3	0.2	0.2	0.1
1961	321	49	2.2	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.6	1.5	1.4	1.3	1.3	1.2	1.1	1.0	1.0	0.9	0.8	0.7	0.7	0.6	0.5	0.4	0.4	0.3	0.2	0.1	0.1
1962	322	48	2.1	2.1	2.0	1.9	1.8	1.8	1.7	1.6	1.5	1.5	1.4	1.3	1.2	1.2	1.1	1.0	1.0	0.9	0.8	0.7	0.7	0.6	0.5	0.4	0.4	0.3	0.2	0.1	0.1
1963	323	47	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.6	1.5	1.4	1.4	1.3	1.2	1.1	1.1	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5	0.4	0.4	0.3	0.2	0.1	0.1
1964	323	46	2.0	2.0	1.9	1.8	1.7	1.7	1.6	1.5	1.5	1.4	1.3	1.3	1.2	1.1	1.0	1.0	0.9	0.8	0.8	0.7	0.6	0.6	0.5	0.4	0.3	0.3	0.2	0.1	0.1
1965	324	45	2.0	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.4	1.4	1.3	1.2	1.2	1.1	1.0	1.0	0.9	0.8	0.8	0.7	0.6	0.5	0.5	0.4	0.3	0.3	0.2	0.1	0.1
1966	325	44	1.9	1.9	1.8	1.7	1.7	1.6	1.5	1.5	1.4	1.3	1.3	1.2	1.1	1.1	1.0	0.9	0.9	0.8	0.7	0.7	0.6	0.5	0.5	0.4	0.3	0.3	0.2	0.1	0.1
1967	326	43	1.9	1.8	1.7	1.7	1.6	1.6	1.5	1.4	1.4	1.3	1.2	1.2	1.1	1.0	1.0	0.9	0.8	0.8	0.7	0.6	0.6	0.5	0.5	0.4	0.3	0.3	0.2	0.1	0.1
1968	327	42	1.8	1.8	1.7	1.6	1.6	1.5	1.4	1.4	1.3	1.3	1.2	1.1	1.1	1.0	0.9	0.9	0.8	0.8	0.7	0.6	0.6	0.5	0.4	0.4	0.3	0.3	0.2	0.1	0.1
1969	328	41	1.8	1.7	1.7	1.6	1.5	1.5	1.4	1.3	1.3	1.2	1.2	1.1	1.0	1.0	0.9	0.9	0.8	0.7	0.7	0.6	0.6	0.5	0.4	0.4	0.3	0.2	0.2	0.1	0.1
1970	329	40	1.7	1.7	1.6	1.5	1.5	1.4	1.4	1.3	1.2	1.2	1.1	1.1	1.0	0.9	0.9	0.8	0.8	0.7	0.7	0.6	0.5	0.5	0.4	0.4	0.3	0.2	0.2	0.1	0.1
1971	330	39	1.7	1.6	1.6	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.1	1.0	1.0	0.9	0.9	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.3	0.3	0.2	0.2	0.1	0.1
1972	331	38	1.6	1.6	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.1	1.1	1.0	0.9	0.9	0.8	0.8	0.7	0.7	0.6	0.6	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1
1973	333	37	1.6	1.5	1.5	1.4	1.3	1.3	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.8	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1
1974	334	36	1.5	1.5	1.4	1.4	1.3	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.9	0.8	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1
1975	335	35	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.9	0.8	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1
1976	336	34	1.4	1.4	1.3	1.3	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.9	0.8	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.0
1977	338	33	1.3	1.3	1.3	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.0
1978	339	32	1.3	1.2	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.9	0.8	0.8	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.0
1979	340	31	1.2	1.2	1.2	1.1	1.1	1.0	1.0	0.9	0.9	0.9	0.8	0.8	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.3	0.2	0.2	0.1	0.1	0.0
1980	342	30	1.2	1.1	1.1	1.1	1.0	1.0	0.9	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.0
1981	343	29	1.1	1.1	1.1	1.0	1.0	0.9	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.0
1982	345	28	1.1	1.0	1.0	1.0	0.9	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.2	0.2	0.1	0.1	0.1	0.0
1983	346	27	1.0	1.0	1.0	0.9	0.9	0.8	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.0
1984	348	26	1.0	0.9	0.9	0.9	0.8	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.5	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.0
1985	349	25	0.9	0.9	0.9	0.8	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.0
1986	351	24	0.9	0.8	0.8	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.0
1987	352	23	0.8	0.8	0.8	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0
1988	354	22	0.8	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0
1989	355	21	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0
1990	357	20	0.7	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0
1991	359	19	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0
1992	360	18	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.0
1993	362	17	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0
1994	364	16	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0
1995	365	15	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
1996	367	14	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
1997	369	13	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
1998	370	12	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
1999	372	11	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2000	374	10	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
2001	376	9	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2002	377	8	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2003	379	7	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2004	381	6	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2005	383	5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2006	385	4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2007	386	3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Summary of Publication "Köhler et al. (2010)"

Abstract

A 150 years-long record of intrinsic water-use efficiency (W_i) was derived from community-level carbon isotope discrimination ($^{13}\Delta$) in the herbage of the unfertilized, unlimed control treatment (plot 3) of the Park Grass Experiment at Rothamsted (England) between 1857 and 2007. $^{13}\Delta$ during spring growth (first cut harvested in June) averaged 21.0‰ (±0.5‰ SD) and has not shown a long-term trend ($P = 0.5$) since 1857. $^{13}\Delta$ of summer / autumn growth (second cut harvested between September and November) increased from 21.3‰ to 22.0‰ ($P < 0.001$) between 1875 and 2007. W_i during spring growth has therefore increased by 33% since the beginning of the experiment, and W_i of summer / autumn growth has increased by 18%. The variation in $^{13}\Delta$ was mainly related to weather conditions. Plant available soil water explained 51% and 40% of the variation in spring growth $^{13}\Delta$ and summer / autumn growth $^{13}\Delta$, respectively. In the 1857-2007 period yields have not increased, suggesting that community-level photosynthesis has not increased either. Therefore, the increased W_i probably resulted from a decreased stomatal conductance. Vapour pressure deficit (VPD) during spring growth (March to June) has not changed since 1915, meaning that instantaneous water-use efficiency (W_t) in spring time has increased and transpiration has probably decreased, provided that leaf temperature followed air temperature. Conversely, VPD in the months between the first and second cut has increased by 0.07 kPa since 1915, offsetting the effect of increased W_i on W_t during summer and early autumn. Our results suggest that vegetation has adjusted physiologically to elevated CO_2 by decreasing stomatal conductance in this nutrient limited grassland.

The candidate's contribution

The candidate conducted the sampling, sample preparation and measurements. She analysed the data, discussed the results, composed the graphs and tables, developed the story of the paper, wrote the first draft of the paper and revised the paper. The overall contribution of the candidate to the final paper was 85%.

Summary of Publication "Köhler et al. (2012)"

Abstract

Under the increase in atmospheric CO_2 during the last century, variable increases in the intrinsic water-use efficiency (W_i), i.e. the ratio between carbon assimilation rate (A) and stomatal conductance (g_s), of C_3 vegetation have been observed. Here we ask if long-term nutrient status and especially nitrogen supply have an effect on the CO_2 response of W_i in a temperate semi-natural C_3 grassland. This analysis draws on the long-term trends (1915-2009) in W_i, derived from carbon isotope analysis, of archived hay and herbage from the Park Grass Experiment at Rothamsted (South-East England). Plant samples came from five fertilizer treatments, each with different annual nitrogen (N; 0, 48 or 96 kg ha^{-1}), phosphorus (P; 35 kg ha^{-1}) and potassium (K; 225 kg ha^{-1}) applications, with lime as required to maintain soil pH near 7. Carbon isotope discrimination ($^{13}\Delta$) increased significantly ($P < 0.001$) on the *Control* (0.9‰ per 100 ppm CO_2 increase). This trend differed significantly ($P < 0.01$) from those observed on the fertilized treatments (*PK* only: 0.4‰ per 100 ppm CO_2 increase, $P < 0.001$; *Low N* only, *Low N+PK*, *High N+PK*: no significant difference). The $^{13}\Delta$ trends on fertilized treatments did not differ significantly from each other. However, N status, assessed as N fertilizer supply *plus* an estimate of biologically-fixed N, was negatively related ($r^2 = 0.88$; $P < 0.02$) to the trend for $^{13}\Delta$ against CO_2. Other indices of N status exhibited similar relationships. Accordingly, the increase of W_i at *High N+PK* was twice that of the *Control* (+28% resp. +13% relative to 1915). In addition, the CO_2 responsiveness of $^{13}\Delta$ was related to the grass content of the plant community. This may have been due to the greater CO_2 responsiveness of g_s in grasses relative to forbs. Thus, the greater CO_2 response of grass-rich fertilized swards may be related to effects of nutrient supply on botanical composition.

The candidate's contribution

The candidate developed the project plan and conducted the sampling. She developed the statistical models and conducted all statistical analyses. She analysed and interpreted the results. All graphs and tables were created by her. She discussed the results, developed the story of the paper, wrote the first draft of the paper and revised the paper. The overall contribution of the candidate to the final paper was 90%.