

**Impact of elevated CO<sub>2</sub> on biomass production,  
forage quality and population dynamics  
of an extensive managed permanent grassland ecosystem**

*Results from the long-term Giessen Free Air CO<sub>2</sub> Enrichment (GiFACE) experiment*



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*For Mom and Dad*  
*I miss you every day*



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This thesis is based on the following 4 papers:

- 1) Andresen, L.C., Yuan, N., Seibert, R., Moser, G., Kammann, C.I., Luterbacher, J., Erbs, M., Müller, C. (2018) Biomass responses in a temperate European grassland through 17 years of elevated CO<sub>2</sub>. *Global Change Biology* 24, 3875-3885.
- 2) Seibert, R., Donath, T.W., Moser, G., Laser, H., Günhage, L., Schmid, T., Müller, C. (2021) Effects of long-term CO<sub>2</sub> enrichment on forage quality of extensively managed temperate grassland. *Agriculture, Ecosystems and Environments* 312, 107347.
- 3) Seibert, R., Andresen, L.C., Jarosch, K.A., Moser, G., Kammann, C.I., Yuan, N., Luterbacher, J., Laughlin, R.L., Watson, C., Erbs, M., Schmid, T., Müller, C. (2021) Plant functional types differ in their long-term nutrient response to eCO<sub>2</sub> in an extensive grassland. *Ecosystems* (under review).
- 4) Seibert, R., Grünhage, L., Müller, C., Otte, A., Donath, T.W. (2019) Raised atmospheric CO<sub>2</sub> levels affect soil seed bank composition of temperate grasslands. *Journal of Vegetation Science* 30, 86-97.

In paper 1, I was one of the co-authors and carried out statistical analysis of the data and creation of graphics and I was involved to the interpretation and discussion of the results and manuscript writing. In paper 2 and 3, I was responsible for data analysis and writing, while the co-authors were involved in interpretation and discussion of the results and gave helpful comments. In paper 4, I had the main responsibility for the study design, field work, data analysis and writing. The co-authors contributed constructive suggestions and helpful comments.

# Table of contents

<b>Summary .....</b>	<b>1</b>
<b>Zusammenfassung .....</b>	<b>3</b>
<b>1 Synopsis.....</b>	<b>5</b>
1.1 Introduction .....	5
1.2 Grassland biomass production.....	6
1.3 Forage quality.....	8
1.4 Nutrients concentrations and yield.....	9
1.5 Population dynamics of grassland communities .....	10
1.6 General objectives and hypotheses .....	12
1.7 Study site .....	14
1.8 Grassland biomass response to eCO <sub>2</sub> .....	16
1.9 Effects of eCO <sub>2</sub> on forage quality.....	18
1.10 Effects of eCO <sub>2</sub> on nutrients concentrations and yield .....	20
1.11 Effects of eCO <sub>2</sub> on soil seed bank composition .....	23
1.12 Conclusion.....	24
1.13 Outlook.....	26
<b>2 Biomass responses in a temperate European grassland through 17 years of elevated CO<sub>2</sub>.....</b>	<b>28</b>
2.1 Introduction .....	29
2.2 Materials and Methods .....	32
2.2.1 Field site .....	32
2.2.2 Free air carbon dioxide enrichment (FACE) .....	33
2.2.3 Bi-annual harvests .....	35
2.2.4 Environmental conditions.....	35
2.2.5 Statistical analysis.....	36
2.2.5.1 Consistency within plots .....	36
2.2.5.2 Linear regression.....	36
2.2.5.3 Effect size.....	36
2.2.5.4 Repeated-measures ANOVA and linear mixed model .....	37
2.2.6 Break point analysis.....	37

<b>2.3 Results .....</b>	<b>38</b>
2.3.1 Aboveground biomass .....	38
2.3.2 Biomass and soil moisture .....	39
2.3.3 Break point analysis of biomass response .....	41
2.3.4 Grasses vs. forbs .....	41
2.3.5 Soil moisture and temperature .....	42
<b>2.4 Discussion .....</b>	<b>43</b>
<b>2.5 Acknowledgements.....</b>	<b>45</b>
<b>2.6. Appendix .....</b>	<b>46</b>
 <b>3 Effects of long-term CO<sub>2</sub> enrichment on forage quality of extensively managed temperate grassland.....</b>	 <b>66</b>
 <b>4 Plant functional types differ in their long-term nutrient response to eCO<sub>2</sub> in an extensive grassland .....</b>	 <b>67</b>
4.1 Introduction .....	69
4.2 Materials and Methods .....	72
4.2.1 Field site .....	72
4.2.2 Elevated CO <sub>2</sub> treatments.....	72
4.2.3 Local weather and climatic conditions .....	73
4.2.4 Bi-annual harvest .....	73
4.2.5 Plant leaf nutrient analysis.....	74
4.2.6 Data analysis .....	74
4.2.6.1 Nutrient uptake response.....	76
4.3 Results .....	76
4.3.1 Concentration of nutrients aboveground in plant functional types.....	76
4.3.2 Plant nutrient yield responses .....	79
4.4 Discussion.....	81
4.5 Conclusion.....	84
4.6 Acknowledgements.....	85
4.7 Appendix .....	85
 <b>5 Raised atmospheric CO<sub>2</sub> levels affect soil seed bank composition of temperate grasslands.....</b>	 <b>98</b>

<b>5.1 Introduction .....</b>	<b>99</b>
<b>5.2 Materials and Methods .....</b>	<b>102</b>
5.2.1 Experimental site .....	102
5.2.2 Study design of the seed bank analysis .....	103
5.2.3 Treatment design and FACE system .....	103
5.2.4 Data analysis .....	104
<b>5.3 Results .....</b>	<b>105</b>
5.3.1 Seed bank and vegetation .....	105
5.3.2 Seed density, species number and diversity indices .....	106
5.3.3 Differences in functional traits .....	108
5.3.3.1 Seed bank type .....	108
5.3.3.2 Reproduction type .....	110
<b>5.4 Discussion .....</b>	<b>112</b>
<b>5.5 Acknowledgements .....</b>	<b>114</b>
<b>5.6 Appendix .....</b>	<b>115</b>
 <b>6 References .....</b>	 <b>132</b>
<b>Danksagung.....</b>	<b>148</b>
<b>Eigenständigkeitserklärung.....</b>	<b>150</b>

## List of figures

**Figure 1-1:** Aerial view of the GiFACE study site, with the six FACE-rings assign to the CO<sub>2</sub> treatment elevated CO<sub>2</sub> (eCO<sub>2</sub>) and ambient CO<sub>2</sub> (aCO<sub>2</sub>). Picture taken from google maps (<https://www.google.de/maps>; last access: 09.02.2021).....15

**Figure 2-1:** (a) Schematic drawing of one plot (ring). Numbers 1 to 25 in the central ring indicate the subplots for harvest of biomass. A 0.9 m wide buffer-zone encircled the treatment area of 6.2 m in diameter. M (middle): CO<sub>2</sub> concentration measurement. W: wind speed and direction sensors. Full blue lines: cables and TDR probes (0-15 cm soil depth). Numbers 1 to 24 at the edge of the outer ring indicate CO<sub>2</sub> fumigation and CO<sub>2</sub> resorption tubes. (b) Aerial photograph of the field site, 2016 by © Thomas Wissner. Plots with elevated CO<sub>2</sub> (eCO<sub>2</sub>-1, eCO<sub>2</sub>-2 and eCO<sub>2</sub>-3, pink circles) and ambient CO<sub>2</sub> (aCO<sub>2</sub>-1, aCO<sub>2</sub>-2 and aCO<sub>2</sub>-3, blue circles) are indicated.....34

**Figure 2-2:** Total aboveground biomass (TAB) in g m<sup>-2</sup> at (a) harvest 1 (H1) and (b) harvest 2 (H2); and biomasses of grasses, forbs and legumes at (c) H1 and (d) H2; for ambient CO<sub>2</sub> (aCO<sub>2</sub>, filled symbols) and elevated CO<sub>2</sub> (eCO<sub>2</sub>, open symbols). Error bars indicate standard errors. Note that CO<sub>2</sub> enrichment started in 1998, pretreatment measurements are shown for 1997. The results from the mixed model analysis are indicated for time, moisture (moist), CO<sub>2</sub> treatment (treat). \* indicates  $p < .05$  and \*\*  $p < .01$ , \*\*\* indicates  $p < .001$ . For  $p$  values and the effect of block and interaction, see Table A. 2-5. Note the different scale at y axis between (a-d).....39

**Figure 2-3:** Response (in %) of aboveground biomasses to elevated CO<sub>2</sub> treatment, for grasses, forbs (incl. legumes) and TAB (total aboveground biomass) at (a) harvest 1 (H1) and (b) harvest 2 (H2), in the years 1998 to 2014. Effect size was calculated as described in Eq. (1). The BP year 2006 is indicated. Differences between grasses and forbs (type) response within the time sections are indicated as “Compare effect size grasses vs. forbs” with  $p < .05$  as \*, from rmANOVA; for details see Table A. 2-9.....40

**Figure 2-4:** Correlation of biomasses of forbs and grasses from both harvests for elevated CO<sub>2</sub> (eCO<sub>2</sub> open circles, full trend line) and ambient CO<sub>2</sub> (aCO<sub>2</sub> filled circle, grey trend line),

in years 1998 to 2014 ( $n = 102$ ). The slopes of  $eCO_2$  and  $aCO_2$  were significantly different ( $P_{com}$ , Table A. 2-8).....41

**Figure 2-5:** Soil moisture differences (volumetric soil water content %) between elevated  $CO_2$  ( $eCO_2$ ) and ambient  $CO_2$  ( $aCO_2$ ) by monthly mean differences. Significant effects of the soil moisture differences (moi) and the interaction of moisture and time (time  $\times$  moi) on total aboveground biomass (TAB) response at harvest 1 (H1) or 2 (H2) in the full time series or within the given time sections are indicated by \* when  $p < .05$  and \*\* when  $p < .01$ . For details on p values, and effects of grasses and forbs, see Table A. 2-9. The volumetric soil moisture monthly data are presented in Table A. 2-2.....42

**Figure 4-1:** Concentration of N (a; b; c), P (d; e; f), Ca (g; h; i), Mg (j; k; l) and K (m; n; o) in % of grasses, forbs and legumes, separated in harvest 1 (H1; circles) and harvest 2 (H2, triangles). Error bars indicate standard deviation. The results of the mixed model analysis are indicated for treatment ( $CO_2$ ), ring-pair (Bl.) time (Y) and soil moisture (SWC). The significance levels were reported as significant  $p \leq 0.05$  [\*], much significant  $p \leq 0.01$  [\*\*] and highly significant  $p \leq 0.001$  [\*\*\*]. Highly significant results in bold.....78

**Figure 4-2:** Response to  $CO_2$  for the macro nutrient elements K, N, Mg, P, Ca, S and the micro nutrient elements Cu, Mn, Fe and Zn, seen as the effect size of plant nutrient yield (% reduction or increase from  $eCO_2$ ) at  $Y_0$  which is the extrapolated point of zero biomass response to  $eCO_2$ , jointly for harvest 1 and 2. The intercept  $Y_0$  was found using standardized major axis test considering variance at both axis (SMATR) on the response function of the nutrient (i) yield response (Y) relative to the biomass response (x) (Eq. 3) for each of the three functional groups grasses (a), forbs (b) and legumes (c) and is presented in Table A. 4-6.  $Y_0$  is the intercept with Y-axis at  $X = 0$ , and is the percentage reduction of (or increase in) nutrient yield at zero biomass response. The bar length represent the 95 % confidence interval of the intercept  $Y_0$  and the line in the middle of each bar represents the mean value. Asterisks (\*) emphasize significance where all both upper and lower confidence interval excludes (or includes) point zero.....80

**Figure 5-1:** NMS Ordination diagram of seed bank (triangle upside down = 0 – 1 cm; chequer = 1 – 5 cm; triangle = 5 – 10 cm) and aboveground vegetation (circles). Seed bank samples

and aboveground vegetation were separated in elevated CO<sub>2</sub>-rings (black) and ambient CO<sub>2</sub>-rings (grey). Final stress of the NMS Ordination was 11.64.....106

**Figure 5-2:** Mean species number [a], mean seed density [b], Shannon-index [c] and evenness [d] of the seed bank samples, separated in total depth (0 – 10 cm) and seed bank layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm). Error bars indicate standard deviation. The significance levels were reported as significant,  $p \leq 0.05$  [\*], significant by tendency  $p \leq 0.1$  [(\*)] and not significant [n.s.].....107

**Figure 5-3:** Mean species number and mean seed density of species with long-term persistent seed bank type [a + b] and short-term persistent seed bank type [c + d], separated in total depth (0 – 10 cm) and seed bank layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm). Error bars indicate standard deviation. The significance levels were reported as significant  $p \leq 0.05$  [\*], much significant  $p \leq 0.01$  [\*\*], significant by tendency  $p \leq 0.1$  [(\*)] and not significant [n.s.].....109

**Figure 5-4:** Mean species number and mean seed density of species with generative reproduction type [a +b] and vegetative reproduction type [c + d], separated in total depth (0 – 10 cm) and seed bank layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm). Error bars indicate standard deviation. The significance levels were reported as significant,  $p \leq 0.05$  [\*], significant by tendency  $p \leq 0.1$  [(\*)] and not significant [n.s.].....111

## List of supplementary figures

**Figure A. 2-1:** Daily air temperature means (black dots and line), minimum (blue line) and maximum (red line) at day of year (DOY) for the years 1997 to 2014, in sections of DOY 1 to 180 (a and b) and 181 to 360 (c and d). The red arrows indicate spring start, as the first DOY with a daily mean air temperature  $\geq +5^{\circ}\text{C}$ , after the winter period. The green vertical line at winter, indicates the last hard frost event when the minimum temperature dropped below  $-10^{\circ}\text{C}$ . Harvest 1 and 2 indicated by vertical line in May-June and August-September. Temperature anomalies are indicated in Figure A. 2-3.....46

**Figure A. 2-2:** The ratio of grasses to forbs (incl. legumes) biomasses dry weight, at the two harvests (H1 and H2), at ambient  $\text{CO}_2$  ( $\text{aCO}_2$ ) and elevated  $\text{CO}_2$  ( $\text{eCO}_2$ ). 1997 was a pre-treatment year.....49

**Figure A. 2-3:** Anomalies of four meteorological variables: a) mean air temperature ( $^{\circ}\text{C}$ , 1995-2014), b) precipitation (mm, 1995-2014), c) air humidity (% , 1995-2014), and d) global radiation ( $\text{W m}^{-2}$ , 1996-2014). The anomalies are defined as differences between the observed values and their monthly average from the entire time series. The monthly data are presented in Table A. 2-3.....49

**Figure A. 4-1:** PCA ordination diagram of the plant nutrient concentrations of N, P, K, Ca, Mg, Zn, and Mn from each year, separated in grasses (circles), forbs (triangles) and legumes (squares), from the two harvests H1 and H2, where H1  $\text{eCO}_2$  is red, and  $\text{aCO}_2$  is blue, and H2  $\text{eCO}_2$  is dark red and  $\text{aCO}_2$  is dark blue. The axes explain 89 % of the total variance.....85

## List of tables

**Table 4-1:** Significant differences in nutrient concentrations between eCO<sub>2</sub> and aCO<sub>2</sub>, separated for the functional types of grasses, forbs and legumes and by harvest 1 (H1) and harvest 2 (H2). The significance levels for CO<sub>2</sub> effect were reported as significant  $p \leq 0.05$  [\*], much significant  $p \leq 0.01$  [\*\*] and highly significant  $p \leq 0.001$  [\*\*\*]. Downward arrow [↓] means negative CO<sub>2</sub> effect; upward arrow [↑] means positive CO<sub>2</sub> effect, analyzed by a linear mixed model. Grey colors indicate decreasing trends, black color indicate increasing trends.....77

**Table 5-1:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number, seed density, Shannon-index and evenness in the soil seed bank (total depth 0 – 10 cm). Soil moisture was included as a co-variable. Df = degrees of freedom; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italics.....107

**Table 5-2:** Percentage of seed bank species, separated in treatment and functional traits.....109

**Table 5-3:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) of species with long-term- and short-term persistent seed bank type. Soil moisture was included as a co-variable. Df = degrees of freedom; ssq = sum of squares; p = p-values. Significant values marked in bold. \* = Log10 transformed data.....109

**Table 5-4:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) of species with generative- and vegetative reproduction type. Soil moisture was included as a co-variable. Df = degrees of freedom; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data...111

## List of supplementary tables

**Table A. 2-1:** Fertilization management of the GiFACE areas through the years 1993 to 2014. All of the areas and the eCO<sub>2</sub> and aCO<sub>2</sub> plots received the same fertilizer treatment. The added amount of each element (Nitrogen, N; Phosphor, P; Potassium, K; Magnesium, Mg; Calcium, Ca and Sulphur, S) in g·m<sup>-2</sup>. The chemical fertilizer products ‘Thomaskali’ and ‘Kalkammonsalpeter’ were applied each year in April prior to the main growing season.....49

**Table A. 2-2:** Monthly mean soil moisture (%) from 1997 to 2013. Volumetric soil water content recorded on daily intervals by permanent TDR-sensors in 0 to 15 cm depth at each plot (aCO<sub>2</sub> 1 to 3 and eCO<sub>2</sub> 1 to 3).....50

**Table A. 2-3:** Monthly meteorological data in years 1995 to 2014. Mean air temperature (Temp; °C), relative humidity (RH; %) and global radiation (GR; W·m<sup>-2</sup>) were measured in 2 m above ground, the precipitation is presented as monthly sums (Precip; mm). nd. means no data available. Anomalies are displayed in Figure A. 2-3.....55

**Table A. 2-4:** Intra-class correlation (ICC) analysis correlation coefficients of biomasses in the 25 sub-plots (Fig. 2-1a), within each of the three ambient CO<sub>2</sub> (aCO<sub>2</sub>) plots and three elevated CO<sub>2</sub> (eCO<sub>2</sub>) plots, at harvest 1 and harvest 2 for grasses, forbs and total aboveground biomass (TAB) across the years 1998 to 2012. Upper and lower 95 % confidence interval (CI<sub>up</sub> and CI<sub>low</sub>) in brackets. ICC correlation coefficients higher than 0.8 indicates very good consistency (Shrout & Fleiss, 1979; McGraw & Wong, 1996).....60

**Table A. 2-5:** Mixed model analysis of effects on biomass. Significant p values (significant at p < 0.05 and by tendency when 0.05 < p < 0.1), and the F-values in brackets, for effects of the factors: CO<sub>2</sub> treatment (Treatment), time and block (Time, fixed terms), and soil moisture (as random term), on the biomass (dependent variable: total aboveground biomass: TAB, grasses and forbs). The effect was assessed for each harvest (1 and 2) by a mixed model analysis (restricted maximum likelihood) with first-order autoregressive process, no interactions of the main factors were significant. The model was tested both for the full time series and for the two half time sections indicated, as determined by break point analysis. See Figure 2-2.....61

**Table A. 2-6:** Plant biomass and soil moisture. Regression analysis of plant aboveground biomasses (total aboveground biomass TAB, grasses and forbs, in  $\text{g}\cdot\text{m}^{-2}$ ) from both harvests with mean soil moisture during the respective growing periods for elevated  $\text{CO}_2$  ( $\text{eCO}_2$ ) and ambient  $\text{CO}_2$  ( $\text{aCO}_2$ ) treated plots in the years 1998 to 2013 (biomass data were not corrected for pre-treatment differences). Regression slopes and Y-axis intercept for the  $\text{eCO}_2$  and  $\text{aCO}_2$  treatments with upper and lower 95 % confidence intervals (CI),  $R^2$  and  $P_{\text{reg}}$  for the regression are given. Test results comparing slopes for  $\text{eCO}_2$  and  $\text{aCO}_2$  are significant at  $P_{\text{com}} < 0.05$  (different slopes).....62

**Table A. 2-7:** Response of plant functional groups. Difference of grasses and forbs biomass effect size and time, for the entire time series (a) and the two time sections divided at year 2006 (b). Significant effects of type (functional group), time and the interaction of type and time ( $T \times T$ ) are indicated by p value  $< 0.05$ , and the F-values in brackets, obtained from repeated measures ANOVA. 'ns.' indicated non-significant effect at  $p > 0.1$ . The biomass effect size is for whole plots. Significant p values are indicated in Figure 3 by 'Compare effect size grasses vs. forbs' .....63

**Table A. 2-8:** Regression analysis of biomasses of forbs and grasses, from both harvests for elevated  $\text{CO}_2$  ( $\text{eCO}_2$ ) or ambient  $\text{CO}_2$  ( $\text{aCO}_2$ ) treated plots in years 1998 to 2014. Regression slopes and Y-axis intercept for  $\text{eCO}_2$  and  $\text{aCO}_2$  treatment with upper and lower 95 % confidence intervals (CI),  $R^2$  and  $P_{\text{reg}}$  for the regression are given. Test results comparing slopes for  $\text{eCO}_2$  and  $\text{aCO}_2$  are significant at  $P_{\text{com}} < 0.05$  (different slopes). See Figure 2-4....63

**Table A. 2-9:** Effects on plant response. Significant p values (significant at  $p < 0.05$  and by tendency when  $0.05 < p < 0.1$ ), and the F-values in brackets, for effects of the main factors (fixed terms) time and soil moisture difference ( $\text{eCO}_2 - \text{aCO}_2$ , from the relevant growing period) on the biomass effect size (dependent variable: TAB, grasses and forbs). Assessment made for each harvest by a mixed model analysis (restricted maximum likelihood) with first-order autoregressive process, using time as repeated measure term (random term). The model was tested both for the full time series and for the two half time sections as determined by break point analysis. See Figure 2-5.....64

**Table A. 4-1.** Fertilization management of the GiFACE areas through the years 1993 to 2014. All of the areas and the elevated  $\text{CO}_2$  and ambient  $\text{CO}_2$  treated plots received the same

fertilizer treatment. The added amount of each element (Nitrogen, N amended as  $\text{NH}_4\text{NO}_3$ ; Phosphorus, P amended as  $\text{P}_2\text{O}_5$ ; Potassium, K amended as  $\text{K}_2\text{O}$ ; Magnesium, Mg amended as  $\text{MgO}$ ; Calcium, Ca amended as  $\text{CaO}$  and Sulphur, S) in  $\text{g m}^{-2}$ . The chemical fertilizer products ‘Thomaskali’ and ‘Kalkammonsalpeter’ were applied each year in April prior to the main growing season (identical to the supplementary Table A. 2-1 in the Andresen *et al.*, 2018).....86

**Table A. 4-2:** Grasses linear mixed model analysis of the effect of  $\text{CO}_2$ , block, year, soil moisture, standard temperature index (STI) and standard precipitation index (SPI) for nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), Potassium (K) and Zinc (Zn), separately for harvest 1 (H1) and harvest 2 (H2). The model analysis was started with the full-factorial model and was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion. Df = degrees of freedom; F = F-value; p = p-values. Significant values in bold.....87

**Table A. 4-3:** Forbs linear mixed model analysis of the effect of  $\text{CO}_2$ , block, year, soil moisture, standard temperature index (STI) and standard precipitation index (SPI) for nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), Potassium (K) and Zinc (Zn), separately for harvest 1 (H1) and harvest 2 (H2). The model analysis was started with the full-factorial model and was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion. Df = degrees of freedom; F = F-value; p = p-values. Significant values in bold.....89

**Table A. 4-4:** Legumes linear mixed model analysis of the effect of  $\text{CO}_2$ , block, year, soil moisture, standard temperature index (STI) and standard precipitation index (SPI) for nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), Potassium (K) and Zinc (Zn), separately for harvest 1 (H1) and harvest 2 (H2). The model analysis was started with the full-factorial model and was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion. Df = degrees of freedom; F = F-value; p = p-values. Significant values in bold.....91

**Table A. 4-5:** PerMANOVA analysis for the coordinated effect of  $\text{eCO}_2$  on plant nutrients concentrations for grasses, forbs and legumes, separated into harvest 1 (H1) and harvest 2

(H2). Df = degrees of freedom; ss = sum of squares; F = F-value; p = p-value. Graphical presentation in Fig. A. 4-1.....93

**Table A. 4-6:** Correlation analysis of the CO<sub>2</sub> effect size for nutrient yield (Y-axis) versus the effect size for biomass (X-axis) standardized major axis test considering variance at both axis (SMATR), to compare the slope ('common') for grasses (G), forbs (F) and legumes (L), as combined for harvest 1 and 2. N is number of data (16 years with two annual harvests), R<sup>2</sup> and p express the significance of the linear regression (p < 0.05), with the presented slope and Y<sub>0</sub> the y-axis intercept (at X = 0 the Y<sub>0</sub> is the percental reduction of nutrient uptake) with upper and lower intercept confidence interval (CI). The 'p(test)' indicates if the two compared slopes are significantly common (p < 0.05; or by tendency p < 0.1). Graphical presentation of the Y<sub>0</sub> data in Fig. 4-2.....93

**Table A. 5-1:** Species list of the aboveground vegetation of the ambient CO<sub>2</sub>-rings, with mean coverage from 2004 till 2013 and the functional traits seed bank type, reproduction type and life span (Klotz *et al.*, 2002; Poschlod *et al.*, 2003). Species with highest coverage are marked in bold.....115

**Table A. 5-2:** Species list of the aboveground vegetation of the elevated CO<sub>2</sub>-rings, with mean coverage from 2004 till 2013 and the functional traits seed bank type, reproduction type and life span (Klotz *et al.*, 2002; Poschlod *et al.*, 2003). Species with highest coverage are marked in bold.....117

**Table A. 5-3:** Soil seed bank species of the single rings with seedling number per depth and the functional traits seed bank type, reproduction type and life span (Klotz *et al.*, 2002; Poschlod *et al.*, 2003). Species with highest seedling number are marked in bold. E = elevated CO<sub>2</sub>; A = ambient CO<sub>2</sub>.....119

**Table A. 5-4:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively). Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic.....124

**Table A. 5-5:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on Shannon-index and evenness in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively). Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic.....125

**Table A. 5-6:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with long-term persistent seed bank type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.....126

**Table A. 5-7:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with short-term persistent seed bank type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.....127

**Table A. 5-8:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with generative reproduction type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.....129

**Tabelle A. 5-9:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with vegetative reproduction type. Soil moisture was included as a co-variable. Df = degrees of

freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic.....130

## List of abbreviations

aCO <sub>2</sub>	ambient Carbon dioxide concentration
ADF <sub>OM</sub>	Organic acid detergent fibre
ANCOVA	Analysis of covariance
BP	Break point
C	Carbon
Ca	Calcium
CI <sub>low</sub>	Lower confidence interval
CI <sub>up</sub>	Upper confidence interval
CO <sub>2</sub>	Carbon dioxide
CPER	Central Plains Experimental Range
Cu	Copper
Df	Degree of freedom
DM	Dry matter
DOY	Day of the year
ECE	Extreme climate events
eCO <sub>2</sub>	elevated atmospheric Carbon dioxide concentration
Eq	Equation
ESOM	Enzyme-soluble organic matter
FACE	Free air carbon dioxide enrichment
Fe	Iron
GE	Metabolisable gross energy
GiFACE	Giessen Free air carbon dioxide enrichment
GR	Global radiation
H1	First harvest of the year
H2	Second harvest of the year
ICC	Intra-class correlation
IPCC	International Panel on Climate Change
K	Potassium
Log10	Common logarithm
ME	Metabolisable energy
Mg	Magnesium

MJ	Mega Joule
Mn	Manganese
moi	Soil moisture
N	Nitrogen
N <sub>2</sub> O	Nitrous oxide
NEL	Net energy for lactation
NfE	Nitrogen-free extractive matter
NH <sub>4</sub> <sup>+</sup>	Ammonium
NMS	Nonmetric multi-dimensional scaling
NO <sub>3</sub> <sup>-</sup>	Nitrate
NZ-FACE	New Zealand Free air carbon dioxide enrichment
P	Phosphorus
PCA	Principal Component analysis
PerMANOVA	Permutational multivariate analysis
pH	potentia Hydrogenii
PHACE	Prairie-Heating and CO <sub>2</sub> Elevation
ppm	Parts per million
PNL	Progressive nitrogen limitation
Precip	Precipitation
RH	Relative humidity
rmANOVA	repeated measurement analysis of variance
S	Sulphur
SD	Standard deviation
SMART	Standardized Major Axis Tests and Routines
soil moist	Soil moisture
SPI	Standard Precipitation Index
ssq	Sum of squares
STI	Standard Temperature Index
TAB	Total aboveground biomass
TasFACE	Tasmania Free air carbon dioxide enrichment
Temp	Temperature
TNC	Total non-structural carbohydrates
WUE	Water use efficiency
XA	Ash

XF	Crude fibre
XL	Crude fat
XP	Crude protein
Zn	Zinc

## Summary

Future increase in atmospheric CO<sub>2</sub> concentrations will potentially enhance grassland biomass production and shift the functional group composition as well as nutrient concentrations in plant tissues, with consequences for ecosystem functioning.

Despite several studies about climate change impact on grassland ecosystems, long-term measurements and observations over more than 10 years continuous monitoring are scarce. To understand effects of elevated atmospheric CO<sub>2</sub> concentration (eCO<sub>2</sub>) on biomass production, forage quality and nutrients concentration of the biomass, as well as effects on regeneration ability and longevity of the grassland ecosystem, four different studies were conducted.

The studies were carried out at the Giessen Free Air CO<sub>2</sub> Enrichment (GiFACE) experiment, which was set up in 1998. The main objectives were to assess the influence of eCO<sub>2</sub> (+ 20 % above ambient CO<sub>2</sub> concentrations) on yield production, forage quality, biomass nutrient concentration and population dynamics of the ecosystem. The biomass consists of C3 grasses and forbs, with a small proportion of legumes and was harvested twice per year (end of May, beginning of September).

The results of the long-term GiFACE experiment revealed effects of eCO<sub>2</sub> concentration on biomass production, forage quality and nutrients concentration, as well as population dynamics in the form of the soil seed bank in different ways. The functional groups grasses and forbs responded differently to eCO<sub>2</sub> through time. In general, total biomass increased under eCO<sub>2</sub> accompanied with a small increase in soil moisture. The forage quality declined due to eCO<sub>2</sub>, especially in forbs and most nutrient element concentrations were reduced under eCO<sub>2</sub>, especially in grasses. Forbs and legumes showed a positive eCO<sub>2</sub> effect in nutrient yield for single elements. Although the soil seed bank is a system where environmental changes trace only slowly into the seed bank, several significant changes in seed bank compositions and shifts in functional traits were indicated due to eCO<sub>2</sub>.

The CO<sub>2</sub> effect on biomass yield and forage quality is most pronounced under soil and climatic conditions that did not restrict plant growth under eCO<sub>2</sub>. As soon as other conditions and resource limitations limited the CO<sub>2</sub> fertilization effect, factors like water or nutrient availability and factor interactions dominated the biomass and forage quality results under eCO<sub>2</sub>. Thus, to obtain reliable evidence of climate change effects on biomass yield, forage quality and nutrients content, as well as the population dynamics, it is important to consider all relevant site conditions. Extreme climate events (i.e. drought, heat waves, heavy rainfall),

which are expected to increase in the future, may lead to a decrease of the CO<sub>2</sub> fertilization effect on grassland biomass and also to a negative impact on forage quality.

Furthermore, the observed changes in nutrient contents and yields of grassland biomass, accompanied with changes in the soil seed bank composition may have serious impact on ecosystem services, e.g. forage production and grassland management and adjustment of fertilization and harvest regimes might be needed.

## **Zusammenfassung**

Ein Anstieg der atmosphärischen CO<sub>2</sub> Konzentration, wird möglicherweise zu einer Steigerung der Biomasseproduktion im Grünland führen. Desweiteren sind Veränderungen in der Zusammensetzung der funktionellen Gruppen, sowie der Nährstoffkonzentrationen im Pflanzengewebe zu erwarten, was erhebliche Konsequenzen für das Grünlandökosystem und seine Funktion bedeutet.

Trotz zahlreicher Studien über die Auswirkungen des Klimawandels auf Grünlandökosysteme, sind Langzeitstudien mit einem Untersuchungszeitraum von über 10 Jahren selten. Um die Auswirkungen von erhöhter atmosphärischer CO<sub>2</sub> Konzentration (eCO<sub>2</sub>) auf die Biomasseproduktion, die Futterqualität und die Nährstoffkonzentration der Biomasse, sowie Auswirkungen auf die Regenerationsfähigkeit und Langlebigkeit des Grünlandökosystems zu verstehen, wurden vier verschiedene Untersuchungen durchgeführt.

Die Untersuchungen wurden im Giessener „Free Air CO<sub>2</sub> Enrichment“ (GiFACE) Experiment durchgeführt, welches 1998 startete. Die Hauptziele der Untersuchungen waren, den Einfluss von eCO<sub>2</sub> (+ 20 % höheres CO<sub>2</sub> gegenüber der aktuellen Umgebungskonzentration) auf die Ertragsproduktion, die Futterqualität, die Nährstoffkonzentration der Biomasse und die Populationsdynamik des Ökosystems zu untersuchen. Die Vegetation, bestehend aus C3 Gräsern und Kräutern, mit einem geringen Anteil an Leguminosen, wurde zweimal pro Jahr (jeweils Ende Mai und Anfang September) geerntet.

Die Ergebnisse aus dem GiFACE-Langzeitexperiment zeigten unterschiedliche Auswirkungen von CO<sub>2</sub>. Vor allem Gräser und Kräuter zeigten verschiedene Reaktionen bezüglich der erhöhten atmosphärischen CO<sub>2</sub> Konzentration. Im Allgemeinen nahmen die Gesamtbiomasse und auch die Bodenfeuchte unter eCO<sub>2</sub> zu. Die Futterqualität zeigte eine Abnahme auf Grund von eCO<sub>2</sub>, besonders bei den Kräutern. Die meisten Elemente der Nährstoffe zeigten eine Reduzierung der Konzentration unter eCO<sub>2</sub>, besonders bei den Gräsern. Kräuter und Leguminosen wiederum zeigten in den Nährstoffträgen bei einzelnen Elementen einen positiven eCO<sub>2</sub>-Effekt. Obwohl die Bodensamenbank ein System ist, in dem Umweltveränderungen nur langsam Auswirkungen zeigen, wurden mehrere signifikante Veränderungen in der Zusammensetzung der Samenbank und Verschiebungen in den funktionellen Eigenschaften auf Grund von eCO<sub>2</sub> gezeigt.

Der CO<sub>2</sub>-Effekt auf den Biomasseertrag und die Futterqualität ist unter Boden- und Klimabedingungen, welche das Pflanzenwachstum unter eCO<sub>2</sub> nicht einschränken, am stärksten ausgeprägt. Sobald allerdings der sogenannte CO<sub>2</sub>-Düngeeffekt durch andere

Bedingungen und begrenzte Ressourcen eingeschränkt wird, dominieren Faktoren wie Wasser- oder Nährstoffverfügbarkeit und Interaktionen der Faktoren die Ergebnisse zur Biomasse und Futterqualität unter eCO<sub>2</sub>. Dies bedeutet, um zuverlässige Aussagen über die Auswirkungen des Klimawandels auf den Biomasseertrag, die Futterqualität und den Nährstoffgehalt der Biomasse, sowie der Populationsdynamik zu erhalten, ist es wichtig, alle relevanten Standortbedingungen zu berücksichtigen. Extreme Klimaereignisse (z.B. Trockenheit, Hitzewellen, Starkregen), die in Zukunft voraussichtlich zunehmen werden, können zu einer Abnahme des CO<sub>2</sub>-Düngeeffekts auf die Grünlandbiomasse und auch zu einem negativen Einfluss auf die Futterqualität führen.

Darüber hinaus können die beobachteten Veränderungen in den Nährstoffgehalten und Nährstofferträgen der Biomasse, begleitet von Veränderungen in der Zusammensetzung der Bodensamenbank, schwerwiegende Auswirkungen auf die Ökosystemdienstleistungen, z. B. Futterproduktion und das Grünlandmanagement haben, was eine Anpassung von Dünge- und Ernteregimen erfordern würde.

# 1 Synopsis

## 1.1 Introduction

Temperate grasslands, which cover 26 % of the world's terrestrial area (Contant, 2010; FAO, 2008), consist of permanent plant communities dominated by forbs and grasses and are used for the production of the majority of the world's livestock (Augustine *et al.*, 2018; Becker *et al.*, 2014). Plant communities develop based on the interplay between natural site conditions and anthropogenic management (Becker *et al.*, 2014). Any changes in grassland productivity due to intensified management, e.g. higher cutting frequencies and increased fertilization, leads to a continuous decline in grassland diversity and quality (Becker *et al.*, 2014). Besides management factors, climate drivers like elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) also affects grassland vegetation (Bütof *et al.*, 2012; Jablonski *et al.*, 2002; Kammann *et al.*, 2005; Obermeier *et al.*, 2017).

The current atmospheric CO<sub>2</sub> concentration exceeds 400 ppm (Dlugokencky & Tans, 2016), and will rise to 550 ppm by the year 2050 (Leakey *et al.*, 2009; Yuan *et al.*, 2018). Elevated atmospheric CO<sub>2</sub> is an important influencing factor on grassland vegetation often driving a 'CO<sub>2</sub> fertilization effect' with increasing biomass production under eCO<sub>2</sub> (Cantarel *et al.*, 2013; Feng *et al.*, 2015; Kammann *et al.*, 2005; Leadley *et al.*, 1999; Morgan *et al.*, 2004a, b; Newton *et al.*, 2014).

Increased biomass production under eCO<sub>2</sub> leads to higher N requirement for plant growth (Augustine *et al.*, 2018; Hovenden *et al.*, 2017; Reich *et al.*, 2014). However, plant growth under eCO<sub>2</sub> often leads to an increase in C assimilation, accompanied by decreasing N content in plant biomass (Feng *et al.*, 2015; Lüscher *et al.*, 2004). This reduction in leaf nitrogen concentration in plants may lead to downward regulation of photosynthesis and respiration (Woodward, 2002). The resulting eCO<sub>2</sub> depressed photorespiration in C3 plants leads to higher rates of photosynthetic CO<sub>2</sub> assimilation and provides more carbohydrate for metabolism and export to sinks like leaf starch (Paul *et al.*, 2001). Photosynthetic sink regulation is dependent on the plant physiology, and Augustine *et al.* (2018) indicated physiological changes, such as increased stomatal closure, in C3 plants when exposed to eCO<sub>2</sub>. Hence, besides declining transpiration and leaf N content, Rubisco activity and its amount declines, while leaf carbohydrate content increases at the expense to N (Augustine *et al.*, 2018; Leakey *et al.*, 2009; Lüscher *et al.*, 2004; Paul *et al.*, 2001; Woodward, 2002).

Altered C allocation accompanied by reduced N-rich compounds and concomitant increase in C-rich structural compounds may lead to a C sink limitation by limited nutrient availability

(e.g. N) in plants (Ainsworth *et al.*, 2003). Elevated CO<sub>2</sub> induced reductions of stomatal conductance and evapotranspiration leading to improved plant water-use-efficiency (WUE). This leads to nutrient limitation because plant nutrient uptake by downregulating of the passive inflow of nutrients to the root with the transpiration stream is affected (Haworth *et al.*, 2016; Houshmandfar *et al.*, 2018; Shaw *et al.*, 2002; Tausz-Posch *et al.*, 2014).

The eCO<sub>2</sub> induced nutrient limitation and changed C/N ratio in plants lead to reductions in protein supply (Newton *et al.*, 2010) and reproductive tissues (i.e. seeds, seed mass, seed weight and germinability) (Wagner *et al.*, 2001). Changes in reproductive fitness due to rising atmospheric CO<sub>2</sub> affects grassland plant communities above- and belowground (Bloor *et al.*, 2010; Wagner *et al.*, 2001), phenology performances (Jablonski *et al.*, 2002), and therefore the quantity and quality of forage in grassland ecosystems (Augustine *et al.*, 2018; Dumont *et al.*, 2015; Soussana & Lüscher, 2007).

## 1.2 Grassland biomass production

To investigate the effects of rising atmospheric CO<sub>2</sub> on grassland ecosystem processes, Free-air carbon dioxide enrichment (FACE) experiments are the most appropriate technology (Müller *et al.*, 2019; Obermeier *et al.*, 2017). FACE systems simulate the effect of future atmospheric CO<sub>2</sub> concentrations on vegetation under natural conditions.

Mainly positive responses of plant biomass to eCO<sub>2</sub>, with increasing yield, were revealed by different studies (Feng *et al.*, 2015; Leadley *et al.*, 1999; Morgan *et al.*, 2004a, b; Newton *et al.*, 2014; Owensby *et al.*, 1999). However, no uniform response pattern can be found across worldwide FACE experiments in grassland. At a grassland prairie study in Wyoming USA (PHACE), the aboveground biomass increased in the first three years due to eCO<sub>2</sub>, but in the further process it became nonresponding, when root biomass was stronger affected than aboveground biomass (Carrillo *et al.*, 2014; Mueller *et al.*, 2016; Zelikova *et al.*, 2014). Another study at perennial grassland (BioCON, Minnesota USA) revealed increased biomass under eCO<sub>2</sub>, depended on precipitation (Reich *et al.*, 2006, 2014). At the Tasmanian TasFACE experiment, periodical positive biomass responses to eCO<sub>2</sub> depended on the seasonal rainfall balance and ranged from years with negative to years with positive growth stimulation (Hovenden *et al.*, 2014). Furthermore, the Jasper Ridge FACE in California USA showed a weak response of aboveground biomass, with the CO<sub>2</sub> response being independent on precipitation and temperature (Dukes *et al.*, 2005; Zhu *et al.*, 2016).

Generally, a positive biomass response trend was found in the various FACE grassland studies across different climatic conditions, CO<sub>2</sub> concentrations, nutrient fertilization intensities and management practices, like simulated grazing or mowing (Feng *et al.*, 2015).

Changes of aboveground biomass because of eCO<sub>2</sub> can also be related to a shift in plant community composition. Proportions of forbs and legumes initially increased in response to eCO<sub>2</sub> in the long-term NZ-FACE study (New Zealand), however, this treatment effect disappeared after six years (Newton *et al.*, 2014). In contrast, at the PHACE experiment, the biomass production of the dominant species decreased under eCO<sub>2</sub>, while subdominant species increased after eight years (Zelikova *et al.*, 2014).

Plant biomass response to eCO<sub>2</sub> is considered to affect soil moisture, because plants grown under eCO<sub>2</sub> have lower stomatal conductance and therefore increased water use efficiency (WUE) (Leakey *et al.*, 2009; Morgan *et al.*, 2004a). When WUE is increased on a leaf area basis, a larger grown canopy caused by eCO<sub>2</sub> might consume more water (Tricker *et al.*, 2009), therefore, eCO<sub>2</sub>-induced water savings per leaf area may have no effect on soil moisture at the ecosystem scale (Leuzinger *et al.*, 2011). However, eight years of eCO<sub>2</sub> treatment at a temperate perennial grassland at the BioCON experiment, the soil moisture increased on average 23 % under eCO<sub>2</sub> (Adair *et al.*, 2011), while soil moisture increased up to 16 % during the first four years in a shortgrass steppe study (CPER, Colorado USA; Nelson *et al.*, 2004).

In general, plant performance and production are controlled by climatic factors (Rapacz *et al.*, 2014). Episodes with anomalous weather or climatic conditions, i.e. extreme climatic events (ECE), can alter ecosystem reactions and lead to changes in the plant community and ecosystem functions (Smith, 2011). ECE drivers like heat waves, drought, heavy rainfall and frost episodes, potentially reduce yield for herbaceous plants and shrubs (Orsenigo *et al.*, 2014). The sensitivity of grasslands to subzero temperatures, particularly in spring, has a strong potential to reduce biomass yield (Höglind *et al.*, 2013). This effect is based on the loss or reduction of frost tolerance of the grassland species and earlier starts of the growing seasons due to warm temperatures in the winter (Höglind *et al.*, 2013; Rapacz *et al.*, 2014; Schuerings *et al.*, 2014). Therefore, the risk of winter and spring frost damage on grassland species is predicted to increase in several regions, i.e. boreal, oceanic, alpine and continental in NW Europe (Rapacz *et al.*, 2014). Hence, both high temperature during heat waves and low temperatures during late spring frost events may inhibit plant growth under eCO<sub>2</sub> (Obermeier *et al.*, 2017; Yuan *et al.*, 2018).

### 1.3 Forage quality

Since aboveground biomass yield increases under eCO<sub>2</sub>, the question arises whether forage quality is also influenced. Several studies revealed negative and positive impacts of eCO<sub>2</sub> on forage quality parameters like crude protein, C/N ratios, crude fibre, ash, total non-structural carbohydrates or lipids (Augustine *et al.*, 2018; Campbell *et al.*, 2000; Dumont *et al.*, 2015; McGranahan & Yurkonis, 2018; Picon-Cochard *et al.*, 2004; Schenk *et al.*, 1997).

In a two-year open-top chambers experiment, Schenk *et al.* (1997) showed an increase in crude protein and a decrease in crude fibre and ash by eCO<sub>2</sub>. Furthermore, McGranahan & Yurkonis (2018) revealed an increase in total crude protein content in C3 and C4 grass forage, due to eCO<sub>2</sub> in a growth chamber experiment. In contrast, Augustine *et al.* (2018) indicated an increase in fibre content and a reduction of N (and hence crude protein) in a seven years long FACE-experiment. Other studies of different length revealed also a decrease in forage N or crude protein (Campbell *et al.*, 2000; Dumont *et al.*, 2015; Milchunas *et al.*, 2005) and an increase in total non-structural carbohydrates under eCO<sub>2</sub> (Campbell *et al.*, 2000; Dumont *et al.*, 2015; Picon-Cochard *et al.*, 2004).

In general, concentrations of crude protein, fibre, as well as non-structural carbohydrates and minerals, i.e. ash (Izaurrealde *et al.*, 2011), are key quality parameters. Higher contents of nutrients, especially N and crude protein content, will have a positive impact on metabolisable energy [ME] and net energy of lactation [NEL] (Buchgraber *et al.*, 2008; Herzog *et al.*, 2009). Therefore, impacts of future climate change on these parameters may result in profound changes in livestock production (Augustine *et al.*, 2018; Craine *et al.*, 2010, 2017; DaMatta *et al.*, 2010; Dumont *et al.*, 2015).

Plant growth under eCO<sub>2</sub> often leads to an increase in C assimilation and a decrease of N content in plant biomass (Feng *et al.*, 2015; Lüscher *et al.*, 2004). Lower N concentrations may affect the nutritive value of the forage, which in turn affect digestibility, forage intake and productivity of ruminants (Lüscher *et al.*, 2004; Owensby *et al.*, 1996; Soussana & Lüscher, 2007). Newton *et al.* (2010) indicated reduced N in plant biomass under eCO<sub>2</sub> over a 10 year period in a FACE experiment, which may result in reduced protein supply in the forage. Reduction in the protein content correlates with high fibre content in the forage (Ball *et al.*, 2001; Buchgraber *et al.*, 2008). Elevated CO<sub>2</sub> induces the synthesis of secondary phenolic compounds, mainly the indigestible cell wall component lignin (Gifford *et al.*, 2000; Sanz-Sáez *et al.*, 2012). Thus, increased plant growth due to eCO<sub>2</sub> would increase fibre fractions (Milchunas *et al.*, 2005). Higher fibre content in the forage means higher cell wall

components, which can limit digestibility, palatability, intake of forages and thus, the forage quality (Sanz-Sáez *et al.*, 2012).

#### **1.4 Nutrients concentrations and yield**

Plant physiological reactions such as reduced stomatal conductance and elevated photosynthetic rates can directly induce by eCO<sub>2</sub> (Haworth *et al.*, 2016; Tausz-Posch *et al.*, 2014). These physiological reactions jointly affect the plant nutrient uptake by downregulating the passive inflow of nutrients to the root with the transpiration stream (Houshmandfar *et al.*, 2018) and possibly by driving a more active uptake of certain elements required in photosynthesis. Shifts of photosynthetic activity under eCO<sub>2</sub> can lead to limited electron transport, which can decrease plant nutrient requirements and uptake. However, micro nutrients like Fe and Mn, which are involved in electron transport, may increase in concentration under eCO<sub>2</sub> (Natali *et al.*, 2009). On the other hand, plant uptake of nutrients like Ca and Mg is controlled by mass flow, which is directly driven by evapo-transpiration (Nord & Lynch, 2009). Hence, decreased transpiration rates by eCO<sub>2</sub> and therefore decreased mass flow, may lead to decreased uptake of nutrients such as Ca and Mg in aboveground plant biomass (Houshmandfar *et al.*, 2018; Tausz-Posch *et al.*, 2014). Increased C sink strength in grassland plants induced by elevated photosynthesis under eCO<sub>2</sub> was revealed by Ainsworth *et al.* (2003). Furthermore, plants grown under eCO<sub>2</sub> increase carbohydrate content, which in some theories leads to dilution of nutrient concentration in plant tissues from increased C assimilation (Ainsworth & Long, 2021; Loladze, 2002).

Besides the direct effects on plant productivity, biodiversity and physiological responses, also soil nutrient cycles can directly and indirectly affect by eCO<sub>2</sub> (Hawkesford *et al.*, 2014). Availability of nutrients is also controlled by soil properties, such as pH, which affects nutrient ion diffusion and adsorption (Marschner, 2002). Because of increased root exudation and respiration, changed cation-anion exchange balance or organic anion release under eCO<sub>2</sub>, a slight decrease in soil pH can be expected (Hinsinger *et al.*, 2003; Natali *et al.*, 2009). Additionally, increasing inputs of carbonic acid from plant roots and microbial respiration, due to eCO<sub>2</sub>, may increase soil acidity (Natali *et al.*, 2009). For slightly acidic soils, such a decrease in the soil pH could directly affect the availability of soil nutrients. The consequences would be a decrease in the availability of macro nutrients such as N, P, K, Ca, S and Mg and an increase in the availability of micro nutrients such as Fe, Mn, Zn and Cu (Marschner, 2002), which might in turn affect the nutritional status of plants.

One of the most important plant nutrients is N and a controlling factor for soil N availability is the process of mineralization of soil organic matter. While net N mineralization varied strongly in response to eCO<sub>2</sub> (Hovenden *et al.*, 2017; Reich *et al.*, 2018), the gross N mineralization rates were often not responding to eCO<sub>2</sub> in grasslands (Müller *et al.*, 2009; Rütting & Andresen, 2015; Rütting & Hovenden, 2020). The presence of legumes in grassland ecosystems adds to the complexity as these are often associated with N<sub>2</sub> fixing microorganisms (Gamper *et al.*, 2004; Lüscher & Nösberger, 1997). Legumes are found to have even greater benefit to eCO<sub>2</sub> as the N<sub>2</sub> fixation increases the nodule size, number of nodules per plant and promotes a higher nitrogenase activity (Rogers *et al.*, 2009). Hereby, the addition of N to the ecosystem from symbiotic N fixation can, through years, sustain an otherwise decreasing N availability for all plant species (Liang *et al.*, 2016; Rütting, 2017).

Across many short-term FACE experiments, decreases in plant nutrient concentrations have been observed, usually with small but significant effects (Myers *et al.*, 2014). These changes in the nutrient concentrations suggest an adjustable plant nutrient stoichiometry, however, an increased C to N ratio under eCO<sub>2</sub> is often observed (Dijkstra *et al.*, 2012; Feng *et al.*, 2015; Sistla & Schimel, 2012). Also, in a meta-analysis of 1418 studies, Yuan and Chen (2015) found that N to P ratios decreased under eCO<sub>2</sub> in general, but observations suggest high variability in responses. Likewise, in USA, Australia and Japan, decreased concentrations of Zn and Fe in wheat, rice, beans and soybeans occurred due to eCO<sub>2</sub> treatment (Myers *et al.*, 2014; Smith & Myers, 2018). These results provide an indication of a poorer nutritive value in the future food quality, as the plant nutrient status directly links to human and livestock nutrition (Augustine *et al.*, 2018; Dumont *et al.*, 2015; Myers *et al.*, 2014; Seibert *et al.*, 2021; Smith & Myers, 2018; Yuan & Chen, 2015).

## **1.5 Population dynamics of grassland communities**

Today, grasslands with low management intensities, i.e. extensive grassland, are remnants of formerly widespread species-rich grassland communities (Becker *et al.*, 2014). Any changes in management, e.g., in cutting dates and frequencies, have a profound impact on the aboveground vegetation composition, and will also affect plant regeneration. In this context, soil seed banks play an important role in providing a buffer against environmental changes and ensure the long-term persistence of plant species and communities (Burmeier *et al.*, 2010; Thompson, 2000). Therefore, in species-rich grasslands a vital soil seed bank is crucial for diversity preservation (Fenner & Thompson, 2005; Wellstein *et al.*, 2007). While this holds for management or temporary environmental changes (Zechmeister *et al.*, 2003), long-term

climatic changes are considered to affect the soil seed bank composition (Akinola *et al.*, 1998).

Seed or fruit production, which is responsible for soil seed bank input, depends on different climatic factors. It may increase with warming, decrease with drought, or remain unchanged with warming and water addition (Walck *et al.*, 2011). Temperature effects could have a direct influence on seed persistence, because increasing temperature in the soil may reduce dormancy in a greater proportion of seeds (Ooi *et al.*, 2009). Furthermore, increasing soil temperatures due to global warming may approach thresholds for seed death in those ecosystems where high temperatures are already apparent. Ooi *et al.* (2009) indicated that viability of seeds and the rate of germination were also affected by increased temperature. Viability declined at some species after exposure to predicted high temperature for 70 days, while germination rate was increased. Thus, increased soil temperature can affect seed dormancy, viability and germination rate.

Several studies focused on the effects of eCO<sub>2</sub> (Edwards *et al.*, 2001; Kammann *et al.*, 2005; Newton *et al.*, 2014) and/or elevated air temperature (Bloor *et al.*, 2010; Mueller *et al.*, 2016; Obermeier *et al.*, 2017; Zelikova *et al.*, 2014) on aboveground vegetation in grassland and showed that in comparison to eCO<sub>2</sub> effects, warming has a greater influence on species- and community-level response (Bloor *et al.*, 2010; Engel *et al.*, 2009; Hovenden *et al.*, 2008b). Hovenden *et al.* (2008b) showed that for a temperate grassland, flowering time is sensitive to experimental warming but insensitive to eCO<sub>2</sub>, which highlights that reproductive traits are key characteristics for predicting the response of grassland communities and ecosystems to global change. In contrast to Hovenden *et al.* (2008b), Engel *et al.* (2009) and Bloor *et al.* (2010), a range of studies with growth chamber, greenhouse, field chamber and FACE rings showed that eCO<sub>2</sub> affected reproductive traits, i.e. more flowers, more fruits and more seeds (Jablonski *et al.*, 2002).

In general, life history, or longevity of plant species, will play a role in the response to global change (Hovenden *et al.*, 2008a). While annual species depend on flowering and seed production to ensure population survival, perennial species have several years to maintain their population. Furthermore, perennial species are under different selective pressures, which are reflected in their reproduction responses under global change (Hovenden *et al.*, 2008a). Trait-mediated differences in the responses of plant species to changing environmental conditions, e.g. climate, could change the way how species interact and this may affect plant community composition (Engel *et al.*, 2009). Plant communities can be regarded as the result

of an abiotic and biotic hierarchy that constrains which species and traits can prevail (Lavorel & Garnier, 2002).

## **1.6 General objectives and hypotheses**

Despite of several and diversified studies about impacts of climate change factors on grassland, long-term measurements and observations over more than 10 years continuous monitoring are rarely available. To understand eCO<sub>2</sub> effects on biomass production, forage quality and nutrients concentration of the biomass yield, as well as effects on regeneration ability and longevity of the grassland ecosystem, long-term investigations are important and necessary to obtain reliable evidence of climate change effects on grassland ecosystems.

Thus, four different studies were conducted at the long-term Giessen FACE experiment (GiFACE), which was set up in 1998 (Jäger *et al.*, 2003), for regarding influence of eCO<sub>2</sub> on yield production, forage quality and nutrient concentration of grassland biomass and population dynamics of the grassland ecosystem.

Based on the results from available grassland FACE studies which cover typically only a few years, one hypothesis for study 1 for the GiFACE is, that eCO<sub>2</sub> will have a positive effect on aboveground biomass in long-term (see chapter 2, study 1). We also hypothesized that the biomass response with eCO<sub>2</sub> will also be associated with changes in functional group proportions. However, the conditions in the different grassland FACE studies are different, and therefore, we expected an ecosystem-specific response regarding the CO<sub>2</sub> effect on functional groups, their direction and the temporal dynamics for the extensively managed moist grassland on the GiFACE site at study 1.

Because of eCO<sub>2</sub>-induced water savings per leaf area by plants and reduced plant water consumption, there will be a limited impact on the soil moisture at the grassland ecosystem. Thus, for study 1, we hypothesized changes in soil moisture due to eCO<sub>2</sub> would not be pronounced at GiFACE. Furthermore, because of alterations of ecosystem reactions on episodes with anomalous weather or climatic conditions, we hypothesized that frost episodes in the late winter to early spring may affect the vegetation response to eCO<sub>2</sub>.

Based on the hypothetical assumption of a positive eCO<sub>2</sub> effect on aboveground biomass, the question arises whether forage quality of the biomass is also influenced. Still, despite this obvious relevance for agriculture, long-term measurements of eCO<sub>2</sub> effects on forage quality are rare (Augustine *et al.*, 2018). Therefore, we assessed the effects of eCO<sub>2</sub> on different forage quality and energy parameters of the extensively managed GiFACE grassland biomass (see chapter 3, study 2). The main objectives was to assess the differences in the quality and

energy content of grassland forage, exposed to eCO<sub>2</sub> and ambient CO<sub>2</sub> (aCO<sub>2</sub>) concentrations, based on the following research questions and hypotheses for study 2:

- 1.) Is there an influence of eCO<sub>2</sub> on the content of crude protein, C/N ratio, crude fat, total non-structural carbohydrates, ash and crude fibre in the grassland biomass?
  - a) We expect a decrease of the crude protein content because of changing C/N ratios under eCO<sub>2</sub>.
  - b) We hypothesize an increase in the crude fibre content, due to eCO<sub>2</sub>.
- 2.) Are there differences in the energy content, i.e. metabolisable energy (ME) and net energy for lactation (NEL), of the biomass between elevated and ambient CO<sub>2</sub> treatment?
  - a) We hypothesize a decrease of the energy content of the forage, because of declining crude protein and rising crude fibre content under eCO<sub>2</sub>.

Beside eCO<sub>2</sub> effects on grassland biomass production and on its forage quality, changes of nutrient concentrations in plant tissues and of nutrient yield in plant biomass can be assumed. To reveal which of the macro- and micro nutrients were affected by eCO<sub>2</sub>, we addressed the following hypotheses for study 3 (see chapter 4):

1. We expected differences in N concentration and its CO<sub>2</sub> response among the functional groups because of the different CO<sub>2</sub> responses of plant N uptake in forbs and grasses and the N<sub>2</sub> fixation mechanism in symbionts with legumes.
2. We expected decreased leaf concentrations of Ca, K, N, S and Mg under eCO<sub>2</sub>, due to down regulated transpiration and therefore reduced passive inflow of nutrients, and to a slight soil acidification of the soil during eCO<sub>2</sub> making these nutrients less available.
3. We expected increased nutrient concentrations in response to eCO<sub>2</sub> for Fe and Mn due to stimulated active uptake to meet increased photosynthesis under eCO<sub>2</sub> and that a slight acidification under eCO<sub>2</sub> increases the availability of these elements.

Soil seed banks are important to provide plant species and communities against environmental changes and ensure their long-term persistence (Burmeier et al., 2010; Thompson, 2000).

Long-term climatic changes are assumed to influence the composition of the soil seed bank (Akinola et al., 1998). While some studies had a look at the effect of warming or precipitation on the soil seed bank in grassland ecosystems (Leishman *et al.*, 2000; Ooi *et al.*, 2009; Ooi, 2012), investigations of effects of eCO<sub>2</sub> on soil seed banks are rare (Hovenden *et al.*, 2008a). To reveal the long-term effect of eCO<sub>2</sub> on soil seed banks of a grassland ecosystem, we carried out study 4 on the GiFACE site (see chapter 5). Our main objective was to assess the differences in the soil seed bank between plots under eCO<sub>2</sub> and aCO<sub>2</sub> concentrations. Especially we addressed the following research questions for study 4:

- 1.) Are there general differences in the species composition between aboveground vegetation and seed bank and are these related to eCO<sub>2</sub>?
- 2.) What are the impacts of eCO<sub>2</sub> on seed density and species composition of the soil seed bank?
- 3.) Are there differences in the functional traits of the seed bank species between eCO<sub>2</sub> and aCO<sub>2</sub> treatments?

## 1.7 Study site

The study, which resulted in the following four papers (chapter 2 - 5), was conducted at the research field of the Environmental Monitoring and Climate Impact Research Station Linden (172 m a.s.l., 50°32'N 8°41'E). The study site is an extensively managed and species rich permanent grassland, with surface-reaching groundwater during winter and dry soil conditions during the summer. The dominant plant species are *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl., *Holcus lanatus* L., *Trisetum flavescens* (L.) P.Beauv., *Alopecurus pratensis* L. and *Poa pratensis* L. for grasses and *Galium album* Forssk., *Geranium pratense* L., *Sanguisorba officinalis* L. and *Plantago lanceolata* L. for forbs. The main legumes are *Lathyrus pratensis* L., *Trifolium repens* L. and *Trifolium pratense* L. (for more details see Jäger *et al.*, 2003).

The soil of this grassland is a Fluvic Gleysol with a texture of sandy clay loam over a clay layer at variable depths (Kammann *et al.*, 2005), with a pH of 5.7 and a C/N ratio of 9.6 (Guenet *et al.*, 2012). The mean soil organic matter content at a depth of 7.5 cm is 2952 ( $\pm$  255) C g m<sup>-2</sup> for the aCO<sub>2</sub> plots and 2924 ( $\pm$  146) C g m<sup>-2</sup> for the eCO<sub>2</sub> plots (Keidel *et al.*, 2018). The volumetric soil moisture was recorded five days a week with permanent TDR-

sensors (Imko, Germany, type P2G) in 0 – 15 cm depth at each FACE-ring. The precipitation sum was measured at the site every 30 minute and the mean annual precipitation was  $556 \pm 27$  mm (average  $\pm$  standard deviation (SD)). The precipitation data were used to calculate the 3-months standardized precipitation index (SPI), which is a drought index based only on precipitation (WMO, 2012). Two meter above ground temperature was recorded continuously since 1995 at two stations within the field site and the mean annual temperature was  $9.4 \pm 6.5^\circ \text{C}$ . The aboveground temperature data were used to calculate the 3-months standardized temperature index (STI), which is an index representing the probability of temperature value occurrences when compared with temperature values on a longer period (Fasel, 2015).

Besides vegetation sampling according to the Braun-Blanquet method (Dierschke, 1994) and two aboveground harvests of 25 sub-plots in each FACE-ring every year (end of May, H1 and beginning of September, H2), the extensive management included annual fertilization of 50 - 80 kg ha<sup>-1</sup> yr<sup>-1</sup> calcium ammonium nitrate fertilization until 1995 and 40 kg N ha<sup>-1</sup> yr<sup>-1</sup> since then. For an adequate supply of minerals, 60 kg P<sub>2</sub>O<sub>5</sub> + 90 kg K<sub>2</sub>O + 18 kg MgO; 198 kg CaO + MgO (PK fertilizer) were applied in April every year (Kammann *et al.*, 2005).



**Figure 1-1:** Aerial view of the GiFACE study site, with the six FACE-rings assign to the CO<sub>2</sub> treatment elevated CO<sub>2</sub> (eCO<sub>2</sub>) and ambient CO<sub>2</sub> (aCO<sub>2</sub>). Picture taken from google maps (<https://www.google.de/maps>; last access: 09.02.2021).

For characterization of the field site, a five years preliminary monitoring was done, before the start of the experiment in May 1998 (Jäger *et al.*, 2003). Three ring pairs with an inner diameter of 8 m and an inner circular buffer-zone of 0.9 m to the ring segments, cover a slight soil moisture gradient caused by differences in average ground water table depth and individual clay layer depths (Jäger *et al.*, 2003; Kammann *et al.*, 2005). The assignment of the CO<sub>2</sub> treatment to one ring of each pair was randomly allocated (Fig. 1-1).

Each ring was constructed of 24 segments with inner and outer air-flow channels. Before the release of CO<sub>2</sub> by pipes at the outer channels, it has been diluted with ambient air for enhance mixing with the air entering the monitoring-plots inside the rings. Pipes at the inner channels partly recycled downwind CO<sub>2</sub> enriched air. The upwind and amount CO<sub>2</sub> release was controlled by wind direction and wind speed. The CO<sub>2</sub> fumigation was started year-round during daylight hours to +20 % above ambient CO<sub>2</sub> conditions, i.e. CO<sub>2</sub> enrichment to 415 ppm, when aCO<sub>2</sub> was 364 ppm in 1998 and CO<sub>2</sub> enrichment to 524 ppm when aCO<sub>2</sub> was 397 ppm in 2014 (Jäger *et al.*, 2003); rings will be referred to as “elevated CO<sub>2</sub> rings” (eCO<sub>2</sub>) and “ambient CO<sub>2</sub> rings” (aCO<sub>2</sub>) (Fig. 1-1). Variations of the CO<sub>2</sub> enrichment among and within years were caused by variable wind conditions and technical failures due to material wear and subsequent repairs at the FACE rings in 2012, 2013 and 2014 (Obermeier *et al.*, 2017).

## 1.8 Grassland biomass response to eCO<sub>2</sub>

The CO<sub>2</sub> enrichment of +20 % at the GiFACE experiment and through the investigation period 1998-2014 had a variable effect on the biomass production. The response pattern of total aboveground biomass (TAB) remained positive in the long term, as hypothesized for study 1 (see chapter 1.6 & chapter 2). The increase in TAB of 15 % in the period 2006-2013 was comparable in magnitude to increase in other grassland FACE studies (Feng *et al.*, 2015). A long-term perspective could reveal a delayed but certain development in the yield response, as a response can change dramatically from short term to long term (Kimball *et al.*, 2007; Leuzinger *et al.*, 2011; Luo *et al.*, 2011; Andresen *et al.*, 2016; Drake, 2014).

A relative abundance of forbs increased during the course of the experiment in both aCO<sub>2</sub> and eCO<sub>2</sub> plots. We suggest that this is partly due to the management regime of the GiFACE experiment (two harvests per year). A functional plant type shift in grassland was also reported from the NZ-FACE, where the proportion of legumes declined through 10 years of eCO<sub>2</sub> treatment. It is assumed, that this was also due to the management (grazing) (Newton *et al.*, 2014).

Across all years, forbs were slightly suppressed by grasses in the eCO<sub>2</sub> treatment. The different response of grasses and forbs to eCO<sub>2</sub>, confirming our second hypothesis for study 1 (see chapter 1.6 & chapter 2), was evident in the first period (1998-2006, positive for grasses and negative for forbs) and equalized in the last period (2007-2014, only positive responses), and resulted in an overall positive response of TAB. Hence, TAB response to eCO<sub>2</sub> through time relied on the response of different functional groups, as reported by Reich *et al.* (2004). In fact, the forbs response was effectively stronger than the grasses response, as it climbed from negative to positive response.

The positive link between biomass and soil moisture was evident from correlation and covariation at the analyses (see chapter 2; study 1). A low frequent year-round positive soil moisture effect of eCO<sub>2</sub> at the last half of the experiment was associated with the positive biomass response of both functional groups forbs and grasses. Thus, biomass and soil moisture were significantly connected, however, there is no simple causality in the GiFACE experiment, as we did not manipulate the soil moisture (Volk *et al.*, 2000). Hence, it cannot be deduced whether the direct biomass response to eCO<sub>2</sub> caused the eventual small positive soil moisture effect, or whether the indirect soil moisture effect caused an indirect biomass response. A plant physiological study by Haworth *et al.* (2016) in the GiFACE revealed a reduction in stomatal conductance due to eCO<sub>2</sub>. This reduction in stomatal conductance is likely to be the regulating mechanism connecting biomass and soil moisture response, potentially leading to reduced water loss from plant and soil. However, the substantial increase in TAB of 15 % under eCO<sub>2</sub> coincided with only a water saving of c. 3 % which was lower compared to other ecosystems (23 %, Adair *et al.*, 2011 and 16 %, Nelson *et al.*, 2004). One possible reason is that the ground water table was rather close to the surface at our site, and thus, the plant-driven regulation of soil moisture had only limited impact. Another possible mechanism is that an increased biomass under eCO<sub>2</sub> produces a larger canopy, adding up to increased water use in spite of the water savings at leaf level (Leuzinger *et al.*, 2011).

An unusual late severe frost episode in 2001 most probably damaged the group of forbs during a critical phenological stage. This frost event was followed by a reduced abundance of forbs relative to grasses in eCO<sub>2</sub> plots for the years 2001 and 2002, and we suggest that this triggered the negative response of forbs to eCO<sub>2</sub>. The effect on the abundance of forbs gradually disappeared throughout the subsequent eight years, which was possibly accelerated by the heat wave in the year 2003 (García-Herrera *et al.*, 2010). An increased frost sensitivity of forbs under eCO<sub>2</sub> can explain this pattern (Obrist *et al.*, 2001; Martin *et al.*, 2010; Rapacz

*et al.*, 2014). Thus, forbs under eCO<sub>2</sub> responded more negative to the frost episode in 2001 than grasses, underlining our hypothesis that climatic episodes with frost may affect plant response to eCO<sub>2</sub> (see chapter 1.6 & chapter 2; study 1).

## 1.9 Effects of eCO<sub>2</sub> on forage quality

Study 1 (see chapter 1.8 & chapter 2) showed a long-term average +15 % biomass yield increase, with a high dependency on abiotic conditions like soil moisture and temperature during the relevant growth periods. The results of study 2 (see chapter 3) showed that the CO<sub>2</sub> effect on forage quality of the GiFACE grassland biomass is also highly dependent on soil moisture and seasonal climatic conditions. Forbs showed a significant CO<sub>2</sub> induced decline for most forage quality parameters at both harvests, while grasses revealed a significant negative CO<sub>2</sub> effect only for ash (XA) at the first harvest of the year (H1) (see table 3-1, chapter 3, study 2). This answered our first research question of study 2 regarding the influence of eCO<sub>2</sub> on the content of diverse forage quality parameters (see chapter 1.6 & chapter 3). Functional groups responded differently to eCO<sub>2</sub> and single forage quality parameters did not all decrease in crude protein (XP) and increase in crude fibre (XF) as hypothesized at study 2.

The crude protein (XP) content in forbs at both harvests only showed a tendency with respect to CO<sub>2</sub>, but a significant soil moisture effect at the second harvest of the year (H2) (see figure 3-1, chapter 3, study 2). In study 1, we revealed increased soil moisture (+ 3 %) under eCO<sub>2</sub>, which was associated with increasing biomass yield for grasses and forbs (see chapter 2). Higher biomass production increased the N requirement for plant growth, but different studies indicated a reduced soil N availability under eCO<sub>2</sub> (Augustine *et al.*, 2018; Hovenden *et al.*, 2017; Reich *et al.*, 2014). Besides eCO<sub>2</sub> induced N limitation, the C assimilation increased under eCO<sub>2</sub> (Augustine *et al.*, 2018; Newton *et al.*, 2010; Owensby *et al.*, 1996; Reich & Hobbie, 2013), which may cause a growth dilution of nutrients (e.g. N) in the biomass (Feng *et al.*, 2015; Gifford *et al.*, 2000; Lüscher *et al.*, 2004). Furthermore, C3 plants can exhibit physiological changes, when exposed to eCO<sub>2</sub>, such as increased stomatal closure, therefore, transpiration, leaf N content, Rubisco activity and its amount declined, while leaf carbohydrate content increased at the expense of N (Augustine *et al.*, 2018; Lüscher *et al.*, 2004). This corresponds to earlier observations at the GiFACE site (Haworth *et al.*, 2016), revealing a reduced stomatal conductance under eCO<sub>2</sub>, as well as an increased C/N ratio under eCO<sub>2</sub>. Nitrogen is essential for proteins and a decline of N concentration in aboveground

biomass (mainly with C3 species) is equivalent to a decrease of crude proteins in forbs in the forage (Picon-Cochard *et al.*, 2004).

Despite a tendency of decreasing crude protein contents in forbs under eCO<sub>2</sub>, C/N ratio showed no significant CO<sub>2</sub> effects (see chapter 3, study 2). These results did not confirm our hypothesis 1a for study 2 (see chapter 1.6 & chapter 3), because the induced decrease of crude protein content, especially in forbs, were not due to changing C/N ratios under eCO<sub>2</sub>. These changes were more likely induced by other side conditions like soil moisture or climatic conditions, which in turn were indirectly affected by eCO<sub>2</sub> (Obermeier *et al.*, 2017; Yuan *et al.*, 2018).

Impacts of side and climatic conditions, as well as eCO<sub>2</sub> altered the crude fat (XL) content in forbs, despite of unchanging carbon concentration in our investigated biomass. This suggests a species-specific reaction on C assimilation, especially for forbs. Berauer *et al.* (2020) indicated that the impact on crude fat depended on the origin of the tested community. Forage quality of species-rich communities is highly variable and influenced by species composition, nutrient availability and stage of maturity, furthermore, plant diversity affects quality-adjusted biomass yield (Berauer *et al.*, 2020; Haughey *et al.*, 2018; Hovenden *et al.*, 2008b; Schaub *et al.*, 2020).

Ash content decreased under eCO<sub>2</sub> in grasses and forbs, but a significant negative CO<sub>2</sub> effect was only revealed for H1 (see figure 3-3, chapter 3, study 2). But significant side and climatic conditions, as well as diverse significant interactions relativize the CO<sub>2</sub> effect. Elevated CO<sub>2</sub> induced effects on plant physiological mechanisms, i.e. reductions of stomatal conductance and evapo-transpiration, leading to improved plant water-use efficiency (WUE). However, despite a relatively higher WUE, a sharp increase in biomass production under eCO<sub>2</sub> can increase total water consumption and lead to nutrient limitation (Augustine *et al.*, 2018; Haworth *et al.*, 2016; Lüscher *et al.*, 2004; Toreti *et al.*, 2020). Therefore, a nutrient dilution via increased C assimilation might explain the decrease of the ash content. A more detailed investigation of the nutrients was performed in study 4 (see chapter 1.10 & chapter 4).

Crude fibre content was the only parameter, which showed a significant increase under eCO<sub>2</sub>, but only for forbs at H2 (see figure 3-4, chapter 3, study 2). This indicates an altered C allocation with reductions of N-rich compounds and concomitant increases in C-rich structural compounds. This increase in crude fibre in forbs might indicate a C sink limitation by limited nutrient and N availability. Other studies point out that elevated CO<sub>2</sub> concentrations seem to affect fibre composition of plant tissue by increasing fibre fractions (Akin *et al.*, 1995; Augustine *et al.*, 2018; Dumont *et al.*, 2015; Milchunas *et al.*, 2005;

Schädel *et al.*, 2010), which is partly supported by our results and confirms our hypothesis 1b for study 2 (see chapter 1.6 & chapter 3). However, our analysis also indicated an impact of side conditions like soil moisture. These side and climatic conditions in terms of the factors soil moisture and year were involved in all significant results of the tested factors (see chapter 3, study 2). That implies that there was not just an influence of eCO<sub>2</sub> on the tested forage quality parameters, which answers our first research question for study 2 (see chapter 1.6 & chapter 3), but side and climatic conditions should be taken into account.

Same patterns were revealed for the energy parameters metabolisable energy (ME) and net energy for lactation (NEL). Only forbs showed a tendency for negative CO<sub>2</sub> effects, with an energy decrease under eCO<sub>2</sub> at both harvests (NEL only at H1). Otherwise both parameters revealed significant year effects, as well as significant soil moisture effects for forbs at H1 (see figure 3-5 and A. 3-2, chapter 3, study 2). Lower concentrations of crude protein and crude fat will lead to a decline in ME and NEL (Just, 1982). This is what our results confirmed with a tendency of declining XP and XL in forbs due to eCO<sub>2</sub>. We also showed an increase in crude fibre content in forbs under eCO<sub>2</sub>. Forage intake by ruminants is limited, if the fibre content in the forage is high (Sanz-Sáez *et al.*, 2012). Limited forage intake may lead to a reduced utilization of energy in ruminants.

The decreased crude protein and crude fat content in our biomass, as well as increased crude fibre content may cause the decline of ME and NEL under eCO<sub>2</sub>. This answered our second research question and confirmed our hypothesis 2a for study 2 at least for forbs that eCO<sub>2</sub> decreased the energy content of the forage (see chapter 1.6 & chapter 3).

### **1.10 Effects of eCO<sub>2</sub> on nutrients concentrations and yield**

A closer look at the nutrient concentrations of the GiFACE biomass revealed reduced plant nutrient concentrations in grasses (N, P, Ca, Mg, K), and reducing effects only for Ca and Mg concentrations in forbs and legumes, but increasing effects on the concentrations of K in forbs and P in legumes, all due to eCO<sub>2</sub> (see Tab. 4-1; Fig. 4-1; chapter 4, study 3).

The nutrient yield response also pointed at reduced nutrient yields in grasses, while forbs and legumes had increased as well as reduced nutrient responses (see Fig. 4-2; Tab. A. 4-6; chapter 4, study 3). This partly confirmed our hypothesis 1 in study 3 of differences among the functional groups regarding N, and the found increases in concentration and yields are contradictory to hypothesis 2 of study 3 which suggested decreases (see chapter 1.6 & chapter 4). Compared to forbs and legumes, the concentration reductions in grasses were more intense at H2 than at H1. This reduction occurred especially at H2 at the end of the regrowth phases,

when the availability of spring applied fertilizer nutrients was low and dry soil conditions caused reduced transpiration rates. The GiFACE management protocol requires only one fertilization during the first growing period and requires the removal of the biomass upon harvest, therefore, plant nutrients were relatively more limited at H2 compared to H1. Accepting that the spring fertilization is part of the driver of the nutrient availability, which is equally available for all species, we also need to consider the physiological differences of the functional groups, as we suggest that this caused differences in the responses. As stated in hypothesis 3 for study 3, increased active uptake of Fe and Mn is expected as consequence of upregulated photosynthesis under eCO<sub>2</sub> (see chapter 1.6 & chapter 4). Contrastingly to this hypothesis, grasses had a reduced Fe and Mn yield, suggesting downregulated photosynthesis, however, forbs and legumes proved increased nutrient response supporting the upregulation hypothesis.

Three hypothetical physiological mechanisms may decrease plant nutrient concentrations under eCO<sub>2</sub>: i. dilution in the plant tissue of the acquired nutrient amount by an increased C assimilation (Ainsworth & Long, 2021; Loladze, 2002), ii. decreased water mass flow carrying fewer nutrients caused by higher water use efficiency through reduced stomatal conductance (Houshmandfar *et al.*, 2018; Tausz-Posch *et al.*, 2014), or iii. decreased water mass flow caused by downregulation of photosynthesis because of low C sink strength (Ainsworth *et al.*, 2003) and therefore reduced stomatal conductance.

The meta-analysis across FACE sites by Feng *et al.* (2015) suggested that the dilution (i. above) in plant tissue cannot fully explain reduced N yields, but rather suggests that a reduced uptake of N is realistic (ii. & iii. above), which was the case in our study 3, indicated by the negative Y-axis intercept  $Y_0$  at zero biomass response to eCO<sub>2</sub> during the analysis of the plant nutrient yield (see Fig. 4-2; Tab. A. 4-6; chapter 4, study 3). This negative  $Y_0$  implies that the nutrient yield was still smaller under eCO<sub>2</sub> compared to aCO<sub>2</sub>, when the biomass was non-responding to eCO<sub>2</sub>. Between elements and functional groups the  $Y_0$  indicated differences in the eCO<sub>2</sub> response in nutrient yield versus response in biomass in the GiFACE. For K, Mg, Ca, S, Cu, Mn, Fe in grasses, P, Ca, S in forbs and Mg in legumes the significantly negative  $Y_0$  indicated that eCO<sub>2</sub> had a negative effect on nutrient yield, independent of the effect on biomass. Hence, these plant nutrient yields are in its effect not responding proportionally to productivity stimulation with increased C assimilation, which indicates that nutrient dilution does not explain reduced nutrient concentrations.

The mechanisms behind the reduced nutrient yield under eCO<sub>2</sub> can be related to plant physiological constraints, such as root-cell absorption sites (Welch & Shuman, 1995), or a

high assimilation efficiency and therefore a reduced nutrient in-flow due to the documented reduced transpiration caused by reduced stomata aperture under eCO<sub>2</sub> (Haworth *et al.*, 2016). Furthermore, a downregulation of photosynthesis and therefore stomata aperture because of low C sink strength (Ainsworth *et al.*, 2003) can reduce nutrient yields. Soil properties like soil pH, buffer capacity, soil moisture and soil structure affect the solubility and sorption of most nutrient elements in the soil (Hawkesford *et al.*, 2014). Investigations by Brenzinger *et al.* (2017) at the GiFACE site indicated only marginally and non-significant differences in soil characteristics (i.e. pH, soil moisture, C-content, N-content) between eCO<sub>2</sub> and aCO<sub>2</sub>. However, reduced pH, due to eCO<sub>2</sub> was assumed in different other studies (Hinsinger *et al.*, 2003; Natali *et al.*, 2009). These changes could lead to an increasing (e.g. Fe, Mn, Zn and Cu) or decreasing (e.g. N, P, K, Ca, S and Mg) availability of nutrients for plants. Even though no pH shift in bulk soil was observed in the GiFACE (Brenzinger *et al.*, 2017), this pH controlled nutrient availability seems to be partly reflected in the rhizosphere by the observed forb (and legume) nutrient yield responses, e.g. more Fe, Mn and Zn yield and less N, P, Ca, Mg yield (see Fig. 4-2, chapter 4, study 3) and partly confirmed our second and third hypothesis for study 3 (see chapter 1.6 & chapter 4). However, the grass nutrient yield response does not support these hypotheses. In that case the limited C sink strength compared to the one of forbs and legumes might cause a downregulation of photosynthesis and therefore stomata aperture in grasses causing reduced transpiration and reduced passive nutrient uptake by reduced water mass flow. But an analysis of wheat under eCO<sub>2</sub> showed an increase in the ratio of nutrient uptake per unit of transpired water for Ca, Mg and Mn, supporting a compensatory mechanism, albeit insufficient to prevent some decline in nutrient contents (Houshmandfar *et al.*, 2018).

Like in study 1 (see chapter 1.8 & chapter 2) and in study 2 (see chapter 1.9 & chapter 3), also the CO<sub>2</sub> effect on nutrient concentrations was dependent on site and seasonal climatic conditions which control transpiration and photosynthetic assimilation, as was evident from significant interactions of the tested factors. The highest CO<sub>2</sub> fertilization effects occurred under warm and humid climatic conditions during the growth periods, which were similar to the long-term average conditions of the respective seasons. Climatic conditions below or above the long-term average conditions lead to a reduction and suppression of the CO<sub>2</sub> fertilization effect (Obermeier *et al.*, 2017; Yuan *et al.*, 2018). For example, during extreme climatic events like heat and drought waves plants under drought stress reduced transpiration via reduced stomata aperture and therefore the CO<sub>2</sub> assimilation and fertilization effect was

reduced. During cold and wet seasons plant transpiration and therefore nutrient uptake via mass flow was reduced by low vapor pressure deficit.

### **1.11 Effects of eCO<sub>2</sub> on soil seed bank composition**

Besides increasing biomass production (see study 1), accompanied by changes in forage quality (see study 2) and nutrients concentrations (see study 3) due to eCO<sub>2</sub>, different studies have shown that eCO<sub>2</sub> affects seed production and seed germination in grassland too (Bloor *et al.*, 2010; Edwards *et al.*, 2001; Jablonski *et al.*, 2002; Marty & BassiriRad, 2014).

On average 60 % of the seed bank species occurred in the above- and belowground vegetation at the GiFACE site, which leads to a relatively high similarity between the species composition of the seed bank and the aboveground vegetation. In general, grassland species show a low potential for seed dispersal (Donath *et al.*, 2003), supporting a high similarity between above- and belowground vegetation. Our analysis at study 4 (see chapter 5) showed no clear separation within the both compartments according to the CO<sub>2</sub> treatment, which is in line with other studies, where similarities between above- and belowground vegetation were lower than similarities within seed bank and within aboveground vegetation (Bekker *et al.*, 2000; Schmiede *et al.*, 2009). These results answers our first research question for study 4 regarding the seed bank (see chapter 1.6 & chapter 5). At the GiFACE site were no general differences in the species composition between aboveground vegetation and seed bank and no clear CO<sub>2</sub> effect. A reason for this may be a delayed response of the seed bank to environmental changes (Thompson, 2000).

To answer the second research question for study 4 (see chapter 1.6 & chapter 5), a closer look at the seed bank revealed several significant effects because of the CO<sub>2</sub> treatment, like higher seed densities under eCO<sub>2</sub>. Beside CO<sub>2</sub> effects, our results also corroborate the importance of soil moisture for seed bank development. Changes in the soil moisture content can have direct impacts on the longevity of seeds in the soil (Bekker *et al.*, 1998). A 3 % increase of the soil moisture accompanied by 15 % increase of total biomass due to eCO<sub>2</sub> was indicated at study 1 (see chapter 2). In general, higher biomass production under eCO<sub>2</sub> may result in more seeds because more resources can be allocated to reproduction (Jablonski *et al.*, 2002). This effect is enhanced by changes in resource acquisition due to eCO<sub>2</sub>, which also supports higher seed production (Marty & BassiriRad, 2014).

In contrast to seed densities, the species composition of the seed bank showed no significant differences of species diversity and Shannon diversity between the CO<sub>2</sub> treatments. However,

a small decrease of evenness under eCO<sub>2</sub> was seen and these small changes can be considered as the first signals of changes in species composition (Bossuyt & Honnay, 2008).

With regard to the functional traits of the species, proportion of long-term-persistent seeds and species should increase with the unpredictability of a system, since a long-term seed bank allows species to bridge periods that are unfavorable for growth (Baskin & Baskin, 2001).

When unpredictability in habitats induces variability in establishment success and reproductive output, a vital seed bank ensures persistence of species (Thompson, 2000). Changes in CO<sub>2</sub> concentration increase the species dynamics or species shift and therefore the unpredictability of the system (Hopfensperger, 2007). In line with this expectation, a significant increase by about one-third under eCO<sub>2</sub> of the seed density of long-term-persistent species was observed, while the proportion of short-term-persistent seeds almost halved (see figure 5-3, chapter 5, study 4). Since species that produce long-term-persistent seeds tend to produce more but smaller seeds (Eriksson & Eriksson, 1997), the shift in this functional trait shows up first differences in seed density and is less pronounced in number of species. The slower shift in species numbers belowground might also be linked to the low dispersal ability of grassland species, i.e., while site conditions change fast, better-adapted species move in slowly (Bakker *et al.*, 1996).

Species which produce long-term-persistent seeds not only produce more and smaller seeds, but also tend to be short-lived (Grime, 2001), thus relying on generative reproduction, seed densities of species of this reproduction type increase under eCO<sub>2</sub>. At the same time, species with vegetative reproduction decrease in number (see chapter 5). While vegetative growth is more frequent in little disturbed sites, generative growth prefers more heavily disturbed areas (Kontanen, 1996, Vitová *et al.*, 2017). Establishment by seedling is very sensitive to abiotic conditions and biotic interactions and thus very rare (Vitová *et al.*, 2017). However, there is a species-specific response to these abiotic and biotic interactions and optimal conditions for seedling emergence for species often differ from those for establishment (Vitová *et al.*, 2017). While differences between treatments seemed rather low at the species level, shifts in the species' spectra of functional traits were more apparent at GiFACE and answer our third research question for study 4 (see chapter 1.6 & chapter 5).

## **1.12 Conclusion**

The results of the studies 1 to 4 from the long-term GiFACE experiment revealed effects of eCO<sub>2</sub> on biomass production, forage quality and nutrients concentrations, as well as

population dynamics in the form of the soil seed bank in different ways, especially with regard to the functional groups grasses, forbs and legumes.

After 17 years of observation of the biomass response to eCO<sub>2</sub> (1998–2014) in study 1 (see chapter 2), the functional groups grasses and forbs responded differently to eCO<sub>2</sub> through time, at the extensively managed, permanent, species-rich grassland. The forbs were slightly suppressed by grasses in the eCO<sub>2</sub> treatment and resulted in positive eCO<sub>2</sub> effects for grasses and negative eCO<sub>2</sub> effects for forbs in the first study period (1998–2005). This was equalized in the last study period (2006–2014), where all responses were positive, which resulted in an overall positive total biomass response (TAB). Hence, TAB response to eCO<sub>2</sub> through time relied on the response of different functional groups. In fact, the forbs response was effectively stronger than the grasses response, as it climbed from negative to positive response. We suggest that the CO<sub>2</sub> response of forbs biomass was affected negatively in 2001, due to a frost damage which was boosted by eCO<sub>2</sub>. After this extreme weather event, an acclimation of forbs took place followed by the overall final positive CO<sub>2</sub> response of both grasses and forbs. Soil moisture responses were significantly connected to biomass response, but only a small increase in soil moisture of +3 % accompanied an increase in 15 % total biomass. The results in study 1 indicated that grassland FACE experiments should be performed over long-term time scales to obtain reliable responses of perennial ecosystems to eCO<sub>2</sub> and weather extremes.

Investigations of the forage quality of the GiFACE biomass (study 2; chapter 3) indicated a decline in forage quality, especially of forbs caused by reduced crude proteins and fat contents, as well as increased crude fibre contents after 18 years of CO<sub>2</sub> enrichment (1998–2015). The increasing proportion of forbs in the biomass (20 to 50 %), accompanied with declining proportion of grasses (80 to 50 %) during the period 1998 till 2015 (see figure A 3-1, chapter 3, study 2), may explain a general decrease of the forage quality at the GiFACE study site. However, site and climatic conditions should be considered showing the importance of long-term observations that differing climatic conditions. It appears that CO<sub>2</sub> effect on biomass yield (see study 1) and similarly on forage quality is most pronounced under soil and climatic conditions that do not restrict plant growth under eCO<sub>2</sub>. As soon as other conditions and resource limitations limit the CO<sub>2</sub> fertilization effect, other factors like water or nutrient availability and factor interactions dominate the biomass and forage quality results under eCO<sub>2</sub>.

A closer look at the nutrients in the GiFACE biomass (study 3; chapter 4) revealed differences in their concentrations and yield responses throughout 16 years of CO<sub>2</sub> treatment

(1998–2013). In particular, plant functional types of grasses, forbs and legumes differed in their nutrient specific responses to eCO<sub>2</sub>. Most element concentrations were reduced under eCO<sub>2</sub>, especially for grasses, while single element concentrations in forbs and legumes showed a positive eCO<sub>2</sub> effect of nutrient yields at zero biomass response. The results suggest that under eCO<sub>2</sub> the nutrient concentrations were not diluted by the CO<sub>2</sub> fertilization effect. Rather, altered plant nutrient acquisitions via changed physiological mechanisms prevail at increased C assimilation under eCO<sub>2</sub>. Furthermore, other factors like water or nutrient availability affected plant nutrient concentrations under eCO<sub>2</sub>. We conclude that the nutritive value of extensively managed temperate grassland may be reduced in response to eCO<sub>2</sub>. Especially Ca, Mg, N, P and K indicated a negative response to eCO<sub>2</sub> compared to the remaining investigated elements. Particularly grasses are more affected by this process than forbs and legumes because of different C sink strength. Nevertheless, like in study 1 and study 2, it appears that the CO<sub>2</sub> effect on nutrient concentrations is also most pronounced under average site and climatic conditions, which do not restrict the CO<sub>2</sub> fertilization effect on plant biomass, while it is reduced during extreme weather events.

Although the soil seed bank is a system where environmental changes trace only slowly into the seed bank, study 4 (see chapter 5) revealed several significant changes in seed bank composition and shifts in functional traits after 17 years of eCO<sub>2</sub> (1998–2014). The seed densities were higher under eCO<sub>2</sub> and the Evenness decreased significantly under eCO<sub>2</sub>. Seed densities of long-term-persistent species increased, while short-term-persistent species decreased. Seed densities of species with generative reproduction increased under eCO<sub>2</sub>, while numbers of species with vegetative reproduction decreased. The observed trait compositions of the seed bank under eCO<sub>2</sub> indicate that species relying on generative reproduction and production of long-term-persistent seeds have a competitive advantage under eCO<sub>2</sub>. Based on the study results, it can be assumed that in response to a higher frequency of unfavorable periods for growth caused by climate change, the proportion of short-lived species producing long-term-persistent seeds will increase.

### **1.13 Outlook**

The studies 1 to 3 showed the dependence of the CO<sub>2</sub> effect on soil and climatic conditions, which do not restrict plant growth under eCO<sub>2</sub>. Thus, to obtain reliable evidence of climate change effects on biomass yield, forage quantity and quality, as well as on nutrient concentrations and yield, it is important to consider all relevant site conditions (Izaurrealde *et al.*, 2011) in future climate impact research experiments. As mentioned above, FACE

experiments at grasslands and comparable perennial ecosystems should be performed over long-term time scales to uncover the effect of different environmental conditions and weather extremes. Because periods of heat, drought and heavy rainfall, which are expected to increase in the future, may lead to a decrease of the CO<sub>2</sub> fertilization effect on grassland biomass (Hatfield *et al.*, 2011; Obermeier *et al.*, 2017; Yuan *et al.*, 2018) and also to a negative impact on forage quality and nutrient contents (Berauer *et al.*, 2020; Craine *et al.*, 2010, 2017; Hatfield *et al.*, 2011; Izaurralde *et al.*, 2011).

Furthermore, the identified changes in the soil seed bank (study 4) suggest an ongoing change of the extensively managed grassland system towards a more ruderal state. These changes are still within the range of natural variability covered by resilience but they show signs of the development of new persistent habitats (Hobbs *et al.*, 2006). It remains to be seen how these changes translate into the most prominent ecosystem services of extensively managed grasslands, such as forage quality and quantity, as well as functioning as a harbor for species diversity. A decrease of individual ruminant performance because of declining forage quality can be expected, which would have to be compensated by increasing amounts of concentrate supplements in dairy cow diets. The observed changes in nutrient contents and yields of grassland biomass, accompanied with changes in the soil seed bank composition, may have serious impact on ecosystem services like forage production, thus, adjustments of the grassland management, e.g. fertilization regimes, might be needed.

## 2 Biomass responses in a temperate European grassland through 17 years of elevated CO<sub>2</sub>

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### Abstract

Future increase in atmospheric CO<sub>2</sub> concentrations will potentially enhance grassland biomass production and shift the functional group composition with consequences for ecosystem functioning. In the “GiFACE” experiment (Giessen Free Air Carbon dioxide Enrichment), fertilized grassland plots were fumigated with elevated CO<sub>2</sub> (eCO<sub>2</sub>) year-round during daylight hours since 1998, at a level of +20 % relative to ambient concentrations (in 1998, aCO<sub>2</sub> was 364 ppm and eCO<sub>2</sub> 399 ppm; in 2014, aCO<sub>2</sub> was 397 ppm and eCO<sub>2</sub> 518 ppm). Harvests were conducted twice annually through 23 years including 17 years with eCO<sub>2</sub> (1998 to 2014). Biomass consisted of C3 grasses and forbs, with a small proportion of legumes. The total aboveground biomass (TAB) was significantly increased under eCO<sub>2</sub> ( $p = .045$  and  $.025$ , at first and second harvest). The dominant plant functional group grasses responded positively at the start, but for forbs, the effect of eCO<sub>2</sub> started out as a negative

response. The increase in TAB in response to eCO<sub>2</sub> was approximately 15 % during the period from 2006 to 2014, suggesting that there was no attenuation of eCO<sub>2</sub> effects over time, tentatively a consequence of the fertilization management. Biomass and soil moisture responses were closely linked. The soil moisture surplus (c. 3%) in eCO<sub>2</sub> manifested in the latter years was associated with a positive biomass response of both functional groups. The direction of the biomass response of the functional group forbs changed over the experimental duration, intensified by extreme weather conditions, pointing to the need of long-term field studies for obtaining reliable responses of perennial ecosystems to eCO<sub>2</sub> and as a basis for model development.

**Keywords:** climate change, forbs, free air carbon dioxide enrichment, frost, Giessen free air carbon dioxide enrichment, grasses, long-term response, soil moisture

## 2.1 Introduction

Grassland worldwide covers approximately 3.5 billion ha, that is 26 % of the world land area (Contant, 2010; Freibauer *et al.*, 2004). The vast areas of native (prairies, pampas, steppes or savannah) or managed grassland spanning from cold-dry to warm-humid climates are essential components of the landscape and provide a range of ecosystem services including food for humans, livestock and wild animals. Any changes in productivity or product quality can have severe consequences for human livelihood and well-being and for the management options. Among other factors, for example excessive N supply or N deposition, the changes may be driven by climate change (Myers *et al.*, 2014).

The current atmospheric CO<sub>2</sub> concentration recently crossed 400 ppm (Dlugokenchy & Tans, 2016), a concentration that has never been reached for at least 800.000 years (IPCC, 2013). Many elevated CO<sub>2</sub> (eCO<sub>2</sub>) experiments started in the 1980s, when the ambient CO<sub>2</sub> concentrations reached 350-360 ppm (Kimball & Idso, 1983). Effects of rising atmospheric CO<sub>2</sub> concentrations have since then been studied across different types of grasslands with field scale manipulations such as open-top chambers and Free Air Carbon dioxide Enrichment (FACE) techniques. In these experiments, the response of plant biomass to eCO<sub>2</sub> was mainly positive (Feng *et al.*, 2015; Owensby *et al.*, 1999). Furthermore, crop yields were increased by eCO<sub>2</sub> by about 19% on average (grasses: wheat, ryegrass, rice, barley; forbs and tubers: clover, soya bean, rape, mustard, cassava, potato, sugar beet; and fruits, etc. from woody plants: grape, cotton and coffee) (Kimball, 2016). Across FACE studies in grasslands

worldwide, no uniform response pattern can be found. At a grassland prairie eCO<sub>2</sub> study (Wyoming USA, the Prairie-Heating and CO<sub>2</sub> Elevation: “PHACE”), aboveground biomass increased in the first three years, but later became nonresponding, when root biomass was stronger affected than aboveground biomass (Carillo *et al.*, 2014; Mueller *et al.*, 2016; Zelikova *et al.*, 2014). At another perennial grassland (Minnesota USA, “BioCON”), biomass increases (here a total of above- and belowground biomass) in response to eCO<sub>2</sub> through 13 years, depended on precipitation (Reich, Hobbie, & Lee, 2014; Reich *et al.*, 2006). Likewise, in a Southern Hemisphere grassland (Tasmania, “TasFACE”), periodical positive biomass responses to eCO<sub>2</sub> depended on the seasonal rainfall balance and ranged from years with negative to years with positive growth stimulation (Hovenden *et al.*, 2014). Furthermore, Californian grassland (Jasper Ridge FACE) showed a weak response of aboveground biomass, with the CO<sub>2</sub> response being independent on precipitation and temperature (Dukes *et al.*, 2005; Zhu *et al.*, 2016). Generally, a positive biomass response trend was found in the various FACE grassland studies across different climatic conditions, concentrations of eCO<sub>2</sub>, nutrient fertilization intensities and management practices such as simulated grazing or mowing (Feng *et al.*, 2015). Thus, based on the results from available grassland FACE studies which cover typically only a few years, we hypothesized for the GiFACE that eCO<sub>2</sub> will have a positive effect on above ground biomass in the long term.

Aboveground biomass responses to eCO<sub>2</sub> changes can also be related to a shift in plant community composition. In a long-term pasture study (New Zealand, “NZ-FACE”), the relative proportions of forbs and legumes initially increased in response to eCO<sub>2</sub>, but after six years, this treatment effect disappeared (Newton *et al.*, 2014). In contrast, at a grassland prairie ecosystem (PHACE), the aboveground biomass production of the dominant species decreased at eCO<sub>2</sub>, while subdominant species increased in growth after eight years (Zelikova *et al.*, 2014). Furthermore, in a brackish wetland, one dominant graminoid species increased and another dominant species decreased in response to eCO<sub>2</sub> during years with raised sea level (Drake, 2014). Based on these findings, we hypothesized for the GiFACE that the biomass response with eCO<sub>2</sub> will also be associated with changes in functional group proportions. However, the conditions in each grassland study are different, and therefore, we expected an ecosystem-specific response regarding the CO<sub>2</sub> effect on functional groups, their direction and the temporal dynamics, for the extensively managed moist grassland in our study.

The plant biomass response to eCO<sub>2</sub> is considered to affect soil moisture, because plants grown under eCO<sub>2</sub> have lower stomatal conductance and therefore increased water use

efficiency (WUE) (Leakey *et al.*, 2009; Morgan *et al.*, 2004a). However, a larger grown canopy caused by eCO<sub>2</sub> stimulation might consume more water, even when WUE is increased on a leaf area basis (Tricker *et al.*, 2009). Thus, the eCO<sub>2</sub>-induced water savings per leaf area may have no effect on soil moisture at the ecosystem scale (Leuzinger *et al.*, 2011). At a temperate perennial grassland (BioCON) with eight years of eCO<sub>2</sub> treatment, the soil moisture increased at eCO<sub>2</sub> on average by 23 %, but with a seasonal trend (Adair *et al.*, 2011). Likewise, in a shortgrass steppe (Colorado USA, Central Plains Experimental Range, “CPER”) up to 16 % increase in soil moisture was observed during the first four years (Nelson *et al.*, 2004). The high ground water level at the GiFACE site (volumetric soil moisture periodically approaching 60 %) does, however, suggest that water is normally not a limiting factor. Hence, any reduced plant water consumption should have only limited impact on the soil moisture. We hypothesized the changes in soil moisture due to eCO<sub>2</sub> would not be pronounced at GiFACE.

In general, climatic factors control plant performance and production (Rapacz *et al.*, 2014). Furthermore, the occurrence of extreme climatic events (ECE) defined as episodes with anomalous weather or climate conditions (drivers) can alter ecosystem reactions and lead to community changes or altered ecosystem functioning (Smith, 2011). For herbaceous plants and shrubs, ECE drivers such as heat waves, droughts, the irregular timing of snow melt, heavy rainfall and frost episodes, all potentially reduce yield, possibly with a delayed response occurring months after the ECE-trigger (Orsenigo *et al.*, 2014). When identifying climatic thresholds by experimentally intensifying such a parameter, one approach is to investigate tipping points (Kreyling *et al.*, 2014). Alternatively, during long-term field experiments, the natural climatic variation can also by chance provoke ECEs (Kreyling *et al.*, 2011). For instance, a strong dependency between rainfall and response to eCO<sub>2</sub> has been found in grassland ecosystems (Hovenden *et al.*, 2014; Morgan *et al.*, 2004a; Reich *et al.*, 2014).

The sensitivity of grasslands to subzero temperatures, particularly in spring, has a strong potential to reduce biomass yield (Höglind *et al.*, 2013). This effect is based on the loss or reduction of frost tolerance and an earlier start of the growing season due to warm winter temperatures (Höglind *et al.*, 2013; Rapacz *et al.*, 2014; Schuerings *et al.*, 2014). Several studies indicated that eCO<sub>2</sub> increased frost sensitivity and tissue damage (Rapacz *et al.*, 2014). This is mainly controlled by the following physical and biological factors: (i) change in leaf temperature due to reduced transpiration and evaporative cooling at eCO<sub>2</sub>, (ii) higher ice nucleation temperature at eCO<sub>2</sub> and (iii) down-regulation of genes responsible for cold

acclimation under eCO<sub>2</sub> (Rapacz *et al.*, 2014). Interestingly, for grassland species, the leaf freezing resistance decreased at eCO<sub>2</sub>, even though concentrations of sugars, starch and carbohydrates increased (Obrist *et al.*, 2001). In addition, in alpine vegetation, the freezing tolerance of leaves during spring was reduced at eCO<sub>2</sub> or indifferent (Martin *et al.*, 2010). Therefore, the risk of winter and spring frost damage on grassland species is predicted to increase in boreal, oceanic, alpine and continental regions in NW Europe (Rapacz *et al.*, 2014). Hence, we hypothesized that (daytime) frost episodes in the late winter to early spring may affect the vegetation response to eCO<sub>2</sub>. Hence, both high temperatures during heatwaves as well as low temperatures during late spring frost events may inhibit plant growth under eCO<sub>2</sub>. The effect of eCO<sub>2</sub> on these rather irregularly occurring episodes can only be realistically investigated with long-term data sets.

At a permanent, managed, NKP fertilized grassland in Germany, a field scale experiment (GiFACE) was set up to examine effects of eCO<sub>2</sub> on ecosystem processes. Before exploration of the 17 years of grassland data, we hypothesized to find:

- a positive effect of eCO<sub>2</sub> on total aboveground biomass;
- a different response of the main functional groups of grasses and forbs to eCO<sub>2</sub>;
- only limited or absent positive soil moisture response along a positive biomass response; and
- effects of climatic episodes with frost or heatwaves on the CO<sub>2</sub> response of sensitive forbs.

## 2.2 Materials and Methods

### 2.2.1 Field site

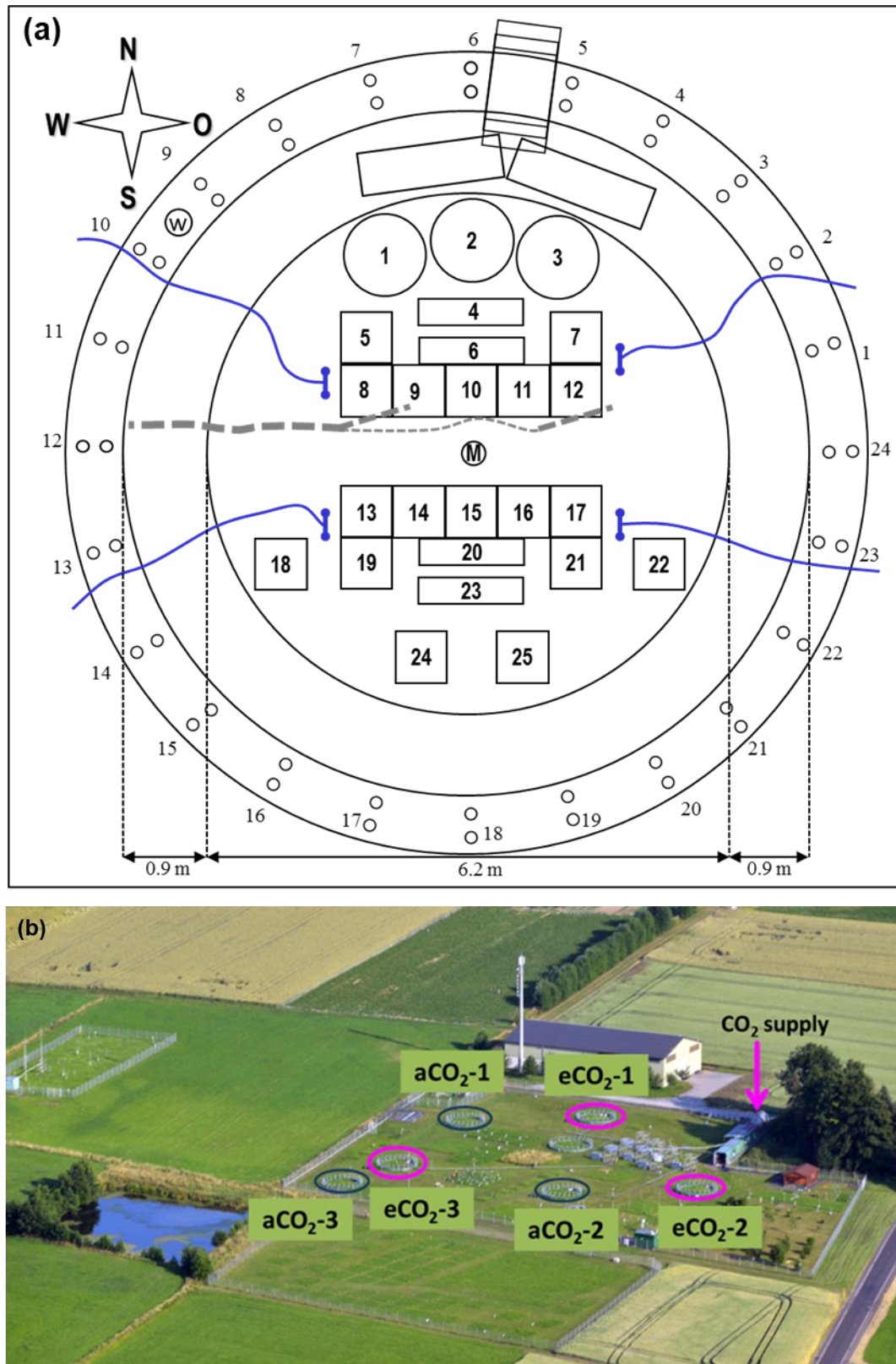
The experiment was carried out on a field site (Fig. 2-1a, b) in the German federal state of Hesse, near the town of Giessen (50°32'N 8°41'E) at 172 m a.s.l. The research area has been managed as a meadow mown twice a year and never ploughed for at least 100 years. The permanent, nongrazed grassland had been fertilized with 80 kg N ha<sup>-1</sup> yr<sup>-1</sup> up to 1995, and thereafter, the fertilizer rate was reduced to 40 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Tab. A. 2-1). The annual N deposition of NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup> and N containing dust amounted to 18.5 kg N ha<sup>-1</sup> yr<sup>-1</sup> (unpublished, Grünhage *et al.*). The vegetation was an “Arrhenatheretum elatioris–Filipendula ulmaria subcommunity” (Grüters *et al.*, 2006) according to the Braun-Blanquet classification. At the start of the study, the dominating grasses in terms of biomass were *Arrhenatherum elatius*, *Holcus lanatus*, *Trisetum flavescens*, *Poa pratensis*, *Anthoxanthum odoratum* and *Poa*

*trivialis*, and the dominant forbs were *Galium album*, *Geranium pratense*, *Plantago lanceolata* and *Sanguisorba officinalis*. The dominating legume species *Lathyrus pratensis* and *Medicago lupulina* were present in small proportions (<1 to 2 % by mass) when the study was initiated.

The field site was located in a floodplain and the soil type was a Fluvisol with a texture of sandy clay loam over a clay layer with the top 10 cm soil consisting of 28% sand with a 45–50 % porosity. The soil pH was 5.7. The carbon-to-nitrogen ratio of the soil was 9.6 (Guenet *et al.*, 2012).

### 2.2.2 Free air carbon dioxide enrichment (FACE)

The GiFACE experiment (Jäger *et al.*, 2003) was started in May 1998, after five years of preliminary data collection to characterize the site and to investigate the yield before the onset of CO<sub>2</sub> fumigation (Kammann *et al.*, 2005). The mean atmospheric CO<sub>2</sub> concentration enrichment of +20 % above ambient (i.e. enrichment to 399 ppm, when the ambient CO<sub>2</sub> concentration (aCO<sub>2</sub>) was 364 ppm in 1998; and to 518 ppm in 2014, when ambient was 397 ppm) was at the lower end of concentration increases used in comparable FACE experiments (475 ppm, Newton *et al.*, 2010; 510 ppm, Mikkelsen *et al.*, 2008; 550 ppm, Hovenden *et al.*, 2014; 560 ppm, Reich *et al.*, 2004; 600 ppm, Zelikova *et al.*, 2014; 720 ppm, Nelson *et al.*, 2004; and 550 to 700 Weigel *et al.*, 1994; Manderscheid *et al.*, 2014). Three circular plots were subjected to eCO<sub>2</sub>, while three additional circular plots surrounded by the same ring structures served as aCO<sub>2</sub> controls. They were arranged in a randomized block design (three blocks). The rings had an inner diameter of 8.0 m (Fig. 2-1a) with an inner circular buffer-zone to the ring structure of 0.9 m. Each ring construction consisted of 24 segments with an outer and inner air-flow channel. CO<sub>2</sub> was diluted with ambient air before it was released by the pipes at the outer channel to enhance mixing with the air entering the plots. This upwind CO<sub>2</sub> release was controlled by wind direction, and the amount of CO<sub>2</sub> release was controlled by wind speed. Downwind, the CO<sub>2</sub> enriched air was partly recycled by pipes installed at the inner ring channel. Since May 1998, the FACE rings were active year-round during daylight hours (Fig. 2-1b; Jäger *et al.*, 2003).



**Figure 2-1:** (a) Schematic drawing of one plot (ring). Numbers 1 to 25 in the central ring indicate the subplots for harvest of biomass. A 0.9 m wide buffer-zone encircled the treatment area of 6.2 m in diameter. M (middle):  $\text{CO}_2$  concentration measurement. W: wind speed and direction sensors. Full blue lines: cables and TDR probes (0-15 cm soil depth). Numbers 1 to 24 at the edge of the outer ring indicate  $\text{CO}_2$  fumigation and  $\text{CO}_2$  resorption tubes. (b) Aerial photograph of the field site, 2016 by © Thomas Wissner. Plots with elevated  $\text{CO}_2$  (e $\text{CO}_2$ -1, e $\text{CO}_2$ -2 and e $\text{CO}_2$ -3, pink circles) and ambient  $\text{CO}_2$  (a $\text{CO}_2$ -1, a $\text{CO}_2$ -2 and a $\text{CO}_2$ -3, blue circles) are indicated.

### 2.2.3 Bi-annual harvests

Since 1997, the aboveground biomass was harvested twice per year in each circular plot within 25 subplots of 0.36 m<sup>2</sup> each, subplot 1, 2 and 3 had a larger area of 0.69 m<sup>2</sup> each as these were also used for greenhouse gas flux measurements (Fig. 2-1a). Prior to 1997, 16 different 100 m<sup>2</sup> plots at the field site were monitored for selection of the later FACE ring locations by (total) vegetation surveys and harvests. From these 100 m<sup>2</sup> plots, three blocks were selected and the CO<sub>2</sub> treatment was randomly assigned to one of the paired plots (Jäger *et al.*, 2003; Kammann *et al.*, 2005). At the harvests from 1998 on (harvest 1, H1 and harvest 2, H2), the vegetation was cut manually with garden scissors to a height of 3–5 cm above the soil surface. After each harvest, the remaining aboveground biomass was cleared away by hand-operated mowing tools. Harvests were H1 between the end of May and the beginning of June and H2 at the beginning of September. The harvested total aboveground biomass (TAB) was stored at 4° C and sorted by hand into the three functional groups: grasses, forbs and legumes followed by oven drying at 105° C to weight constancy. As legumes contributed only a very small biomass portion (c. 3.5 %), they were included within the group of forbs in the following data analysis.

### 2.2.4 Environmental conditions

Volumetric soil water content was recorded on daily intervals since spring 1997 by four permanent TDR-sensors (Imko, Germany, type P2G) installed in 0–15 cm depth at each circular plot (Tab. A. 2-2). This measurement system worked until January 2014 and was replaced in 2015 by water content reflectometers (CS616, Campbell Scientific). Hence, data on soil water content were not available for 2014. Soil temperature was logged as 30-min. averages since March 1998 in 10 cm depth, with three probes in each circular plot (Pt-100 sensors, Imko, Germany). Rainfall was measured by up to three Hellmann samplers with tipping buckets (Thies, Gottingen, Germany). The mean annual precipitation at the site was 558 ± 92 mm (18-year average). Air temperature was recorded continuously since 1995 at two locations at 2 m height. The mean annual air temperature at the site was 9.4 ± 0.1° C (18-year average). Relative humidity (RH) and global radiation (GR) (Tab. A. 2-3) were obtained from meteorological stations on the field site operated by the Hessian Agency for Nature Conservation, Environment and Geology (HNLUG) and the Environmental Monitoring and Climate Impact Research Station Linden (UKL) and the German Meteorological Service.

The day of year (DOY) with the last hard frost below 10° C is indicated in Fig. A. 2-1. The number of days with frost during a year was calculated as the number of days with more than

five consecutive hours of frost, for the winter ending in March of the given year (starting in November of the previous year). Our observations of plant growth start coincided with Eddy covariance measurements, indicating the days when net ecosystem CO<sub>2</sub> sink-activity occurred (Grünhage, unpublished data). The spring growing period was defined to last from the first DOY when mean air temperature was  $\geq +5^{\circ}\text{C}$  until H1 (Fig. A. 2-1). For this spring growing period and the summer growing period between H1 and H2, we separately calculated the means, minima and maxima of soil moisture and soil temperature as well as the precipitation sum and air temperature for the eCO<sub>2</sub> and aCO<sub>2</sub> rings, to assess for relations with the respective biomass harvest yields.

### 2.2.5 Statistical analysis

#### 2.2.5.1 Consistency within plots

For the biomass obtained from 25 subplots (Fig. 2-1a), the within-plot consistency (variability) was assessed by intraclass correlation (ICC) (McGraw & Wong, 1996; Shrout & Fleiss, 1979). We evaluated the consistency among the 25 subplots within each plot by a two-way random, average consistency approximation. At an ICC larger than 0.8, there was very good consistency among different subplots (McGraw & Wong, 1996; Shrout & Fleiss, 1979). ICC was assessed for the years 1998 to 2012, but not for 2013 and 2014 as in these last two years, the subplots 24 and 25 were part of a <sup>13</sup>C and <sup>15</sup>N pulse-labelling study and could therefore not be harvested (Moser *et al.*, unpublished).

#### 2.2.5.2 Linear regression

SMATR (a freeware program for Standardized Major Axis Tests and Routines) was used for bivariate linear regression, using standardized major axis regression and testing for possible common slopes in the eCO<sub>2</sub> and aCO<sub>2</sub> data sets for biomasses (TAB, grasses and forbs) and soil moisture (Falster *et al.*, 2006; Warton *et al.*, 2006).

#### 2.2.5.3 Effect size

Effect size expresses the relative treatment effect (response) in percentage (Leuzinger *et al.*, 2011) and was calculated for aboveground biomasses of grasses, forbs and TAB (denoted as X, representing the average of three replicates per treatment) as:

$$Effect\ size = \frac{eCO_2(X) - aCO_2(X)}{aCO_2(X)} \times 100\ [%] \quad (1)$$

The 1997 biomass functional group data were not used as a correction factor in the effect size calculation, because one single year of functional group biomass data might not be representative. We rate it as not justified to define such short time duration as pretreatment conditions, particularly as this year was not representative of the average weather conditions during the experimental duration (Fig. A. 2-3).

#### *2.2.5.4 Repeated-measures ANOVA and linear mixed model*

Difference in treatment response (effect size) between the two functional groups (grasses and forbs, incl. legumes) was assessed by rmANOVA starting at year 1998, with the factors functional group (type), time and the interaction time  $\times$  type, by SPSS Statistics 20; the response was not significantly affected by the factors block and block  $\times$  time. The functional group and treatment rmANOVA model was used at the full time series and the two half time sections as determined by break point analysis.

Effects of the factors treatment, time, soil moisture and block (“ring-pair” 1, 2, 3; Fig. 2-1b) and their interactions were addressed by a mixed model analysis (restricted maximum likelihood), with first-order autoregressive process using soil moisture as covariate (random term), and treatment, time and block as fixed terms, by SPSS Statistics 23. The interactions were removed from the model when  $p > .1$  (Bandoly *et al.*, 2016; Engqvist, 2005; Safari *et al.*, 2016). Likewise, effects of the factors time and growing season soil moisture difference (between aCO<sub>2</sub> and eCO<sub>2</sub>; fixed terms) on the biomass effect size (dependent variable: TAB, grasses and forbs) were assessed for each harvest by a mixed model analysis (restricted maximum likelihood), using time as repeated-measure term (random term) by SPSS Statistics 23. The mixed model analyses were conducted both for the full time series and for the two half time series as determined by break point analysis (see below). Effects with  $p$  values  $< p < .10$  were considered as tendencies.

#### *2.2.6 Break point analysis*

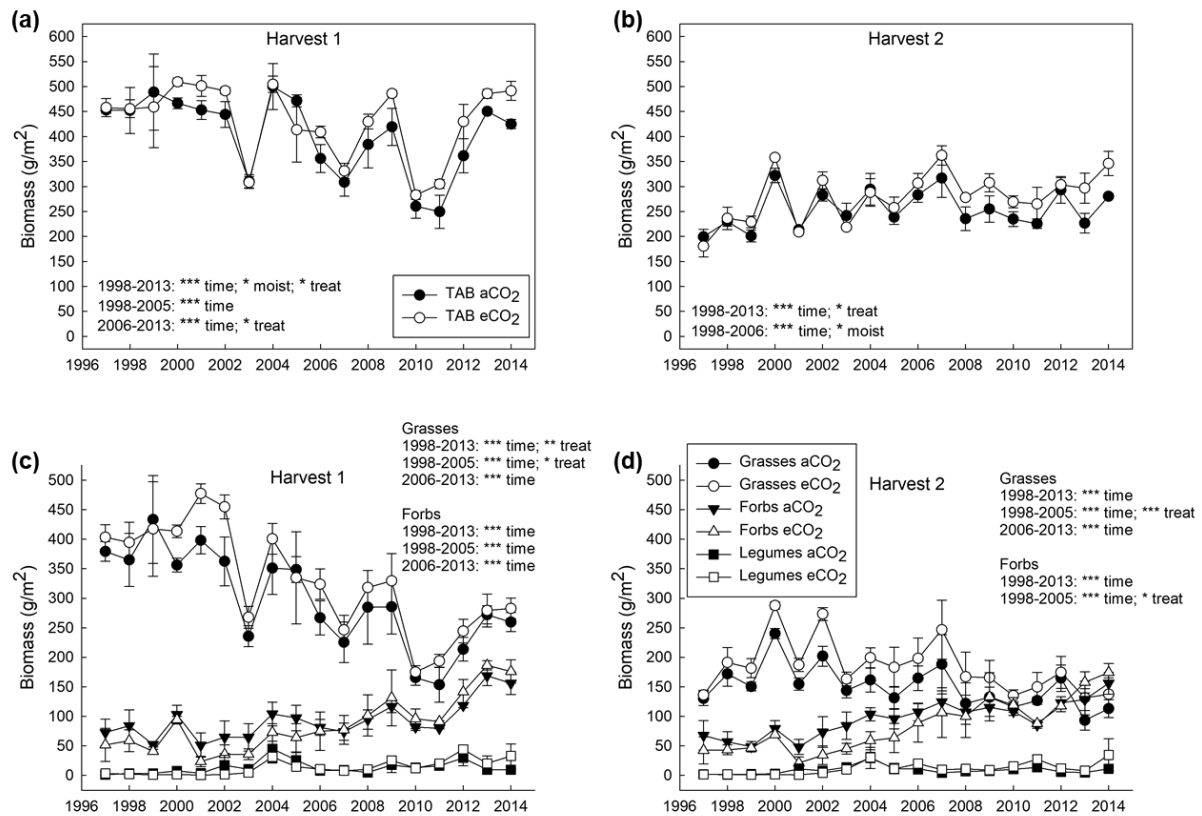
Determination of break points (BP; Vieth, 1989) for the treatment response of aboveground biomass of grasses, forbs and TAB in the period 1998–2014 was done separately for H1 and H2 by the “Change-point Analyzer” version 2.3, which used cumulative sum charts (CUSUM) and bootstrapping to detect changes (Taylor, 2000a, b). Thus, BP analysis was applied to (i) the temporal variation of the effect size of eCO<sub>2</sub> on biomass and (ii) the temporal variation of soil moisture differences between the eCO<sub>2</sub> and aCO<sub>2</sub> plots.

## 2.3 Results

### 2.3.1 Aboveground biomass

The total aboveground biomass (TAB) harvest throughout the 17- year study period (1998 to 2014) was  $658 \pm 47 \text{ g m}^{-2}$  (mean standard error;  $n = 3$ ) in the aCO<sub>2</sub> plots and  $714 \pm 38 \text{ g m}^{-2}$  in the eCO<sub>2</sub> plots (Fig. 2-2a, b). Through the five years prior to the start of the CO<sub>2</sub> enrichment, no statistically significant differences were found in TAB between plots (Kammann *et al.*, 2005). In the years 2003, 2007, 2010 and 2011, TAB at harvest 1 (H1), but not at H2, was rather small in both eCO<sub>2</sub> and aCO<sub>2</sub> plots (Fig. 2-2a), which was attributed predominantly to timing of harvest and weather conditions during spring (Tab. A. 2-2, A. 2-3; Fig. A. 2-1, A. 2-3). The relative proportion of the grass biomass increased in the years 2001 and 2002 and then decreased in the following years as forbs (including legumes) increased in proportion (Fig. A. 2-2). The abundance of legumes was on average 3.5 and 3.7 % across all years for aCO<sub>2</sub> and eCO<sub>2</sub> (Fig. 2-2a–d), but boosted in spring 2004 after the heat wave during summer 2003 (Fig. A. 2-3; see García-Herrera *et al.*, 2010 for a review), and again in 2011, 2012 and 2014 reaching up to 10 %. The response of individual species will be discussed elsewhere (Seibert *et al.*, unpublished results).

Among all aCO<sub>2</sub> and eCO<sub>2</sub> plots, grasses, forbs and TAB showed good consistency between subplots, as all the intraclass correlation (ICC) coefficients were higher than 0.9 (Tab. A. 2-4).



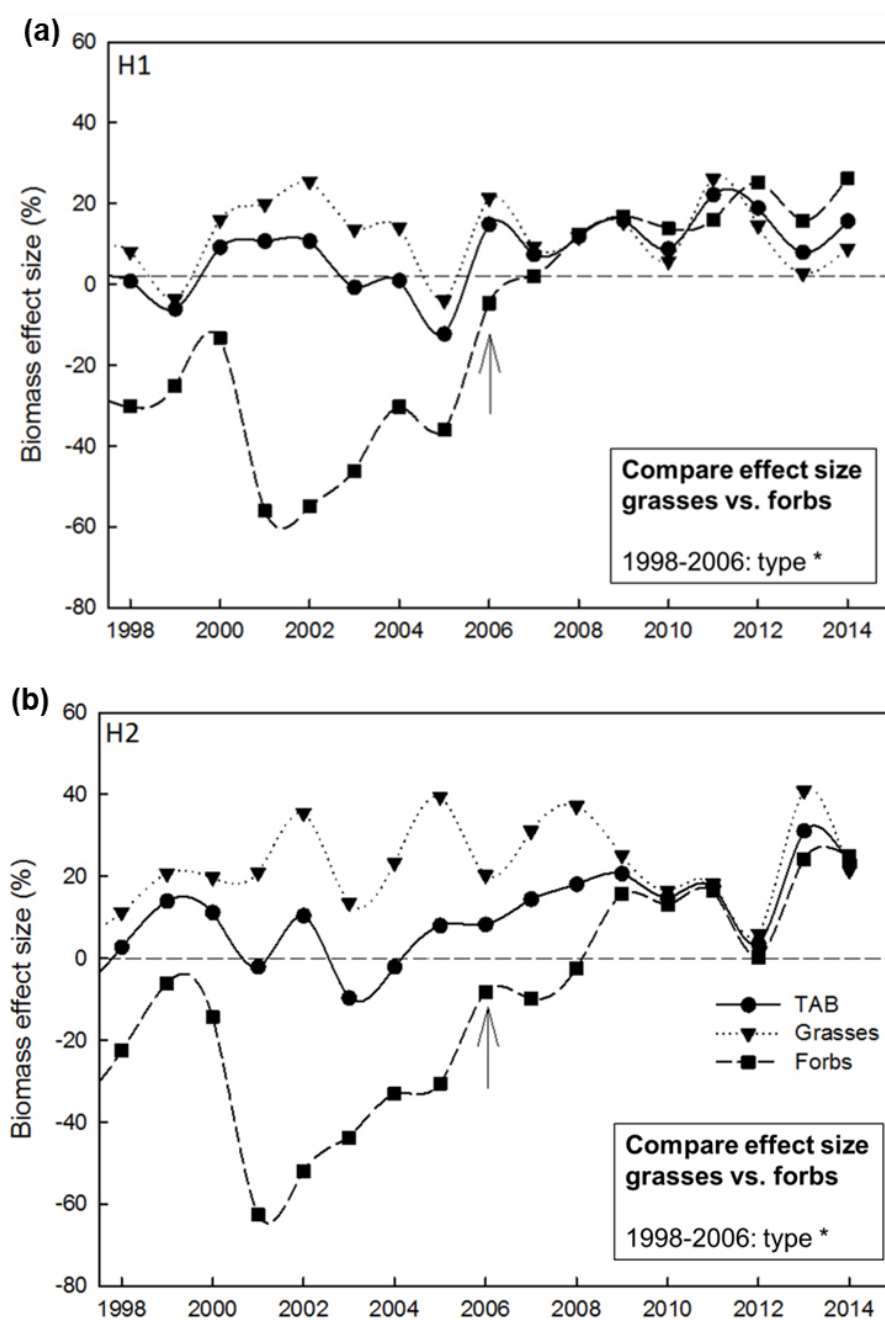
**Figure 2-2:** Total aboveground biomass (TAB) in g m<sup>-2</sup> at (a) harvest 1 (H1) and (b) harvest 2 (H2); and biomasses of grasses, forbs and legumes at (c) H1 and (d) H2; for ambient CO<sub>2</sub> (aCO<sub>2</sub>, filled symbols) and elevated CO<sub>2</sub> (eCO<sub>2</sub>, open symbols). Error bars indicate standard errors. Note that CO<sub>2</sub> enrichment started in 1998, pretreatment measurements are shown for 1997. The results from the mixed model analysis are indicated for time, moisture (moist), CO<sub>2</sub> treatment (treat). \* indicates  $p < .05$  and \*\*  $p < .01$ , \*\*\* indicates  $p < .001$ . For  $p$  values and the effect of block and interaction, see Table A. 2-5. Note the different scale at y axis between (a-d).

### 2.3.2 Biomass and soil moisture

For the full time series, when soil moisture data were available (years 1998 to 2013), the TAB was positively and significantly affected by the factors time ( $p < .001$ , both harvest 1 (H1) and harvest 2 (H2)) and treatment ( $p = .045$ , H1 and  $p = .025$ , H2; mixed model analysis, Fig.2-2a, b; see Tab. A. 2-5 for F-values). Furthermore, growing period soil moisture significantly affected TAB at harvest 1 ( $p = .019$ , H1), and in the first half section of the time series (see BP analysis below) significantly at harvest 2 ( $p = .012$ , H2). In the second half of the time series, the main factor CO<sub>2</sub> treatment positively affected TAB ( $p = .041$ , H1 and  $.069$ , H2). Treatment affected grass biomass at both harvests positively ( $p = .006$ , H1 and  $.062$ , H2; full time series), especially at the first half of the time series ( $p = .017$ , H1 and  $p = .001$ , H2; see BP analysis below). Treatment only affected forbs biomass significantly negative at harvest 2 in the first time section ( $p = .047$ , H2; Fig. 2-2c, d). Time was a dominant significant factor at

all levels ( $p \leq .003$ ), while soil moisture was not significantly important for grasses and forbs alone (mixed model analysis, see Tab. A. 2-5 for  $p$  and  $F$ -values; Fig. 2-2).

Taking both harvests together, TAB and grass biomass (but not forbs) correlated significantly positive with soil moisture in the relevant growing period ( $P_{\text{reg}} < .001$ , for both  $e\text{CO}_2$  and  $a\text{CO}_2$ ; Tab. A. 2-6) throughout the years 1998 to 2013. Only forbs showed a treatment effect of the moisture—biomass regression, as the slopes significantly differed by CO<sub>2</sub> level ( $P_{\text{com}} = .001$ ; Tab. A. 2-6).

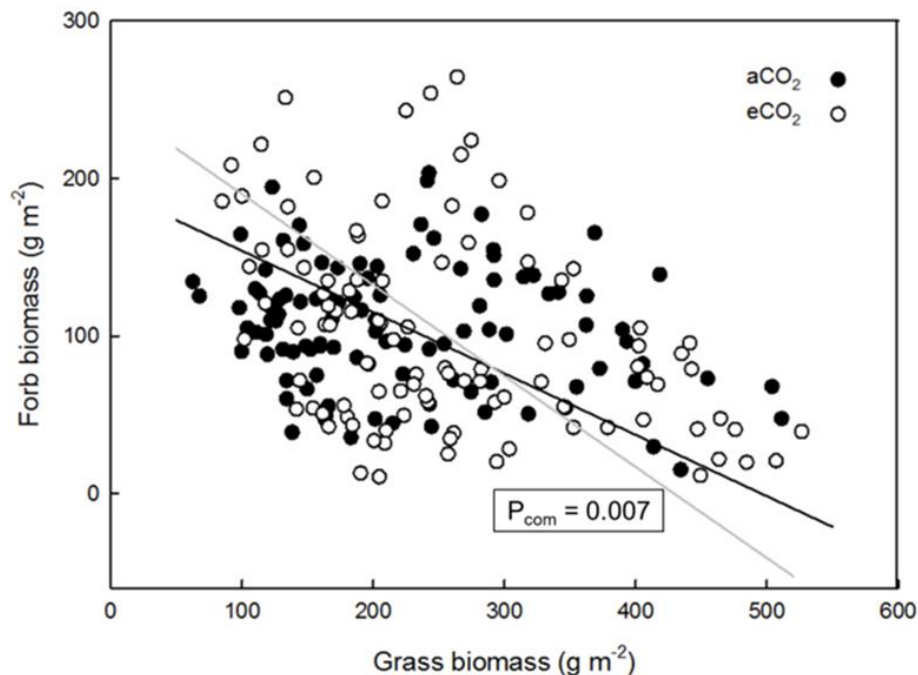


**Figure 2-3:** Response (in %) of aboveground biomasses to elevated CO<sub>2</sub> treatment, for grasses, forbs (incl. legumes) and TAB (total aboveground biomass) at (a) harvest 1 (H1) and (b) harvest 2 (H2), in the years 1998 to

2014. Effect size was calculated as described in Eq. (1). The BP year 2006 is indicated. Differences between grasses and forbs (type) response within the time sections are indicated as “Compare effect size grasses vs. forbs” with  $p < .05$  as \*, from rmANOVA; for details see Table A. 2-9.

### 2.3.3 Break point analysis of biomass response

By the break point (BP) analysis of effect sizes at each harvest, the forbs and TAB responses were apportioned into two periods: for forbs at the year 2006 (H1:  $p < .001$  and H2:  $p < .001$ ; indicated by arrows in Fig. 2-3a, b) and for TAB at the year 2006 for H1 ( $p < .04$ ) and by tendency in the year 2007 for H2 ( $p < .06$ ); grasses showed no BPs. The BP year marks the point in time where the forbs eCO<sub>2</sub> response shifted from negative to positive. The annual TAB (sums of H1 and H2) increased by 15 % in response to eCO<sub>2</sub> throughout the period 2006 to 2014.

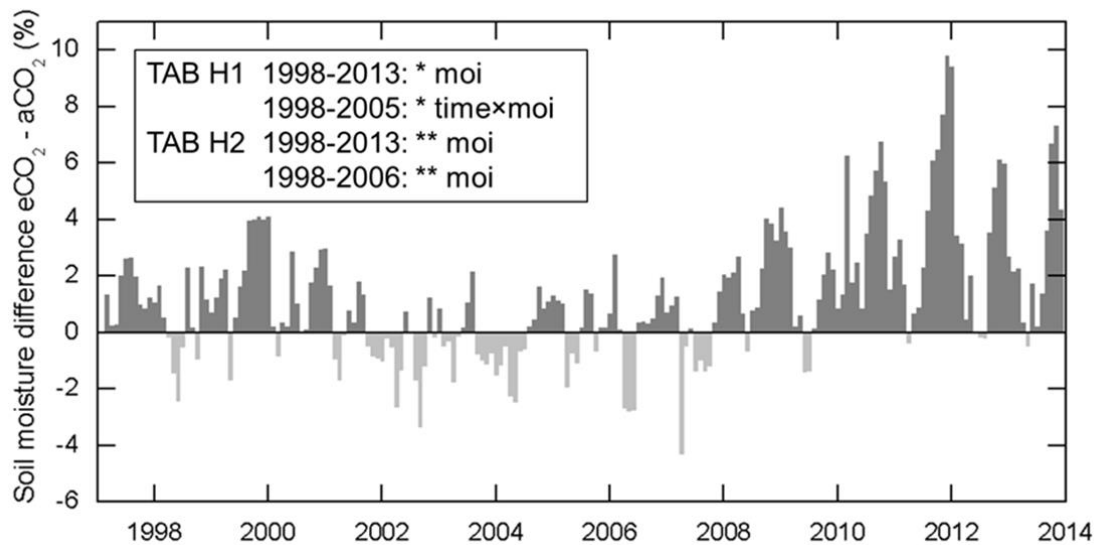


**Figure 2-4:** Correlation of biomasses of forbs and grasses from both harvests for elevated CO<sub>2</sub> (eCO<sub>2</sub> open circles, full trend line) and ambient CO<sub>2</sub> (aCO<sub>2</sub> filled circle, grey trend line), in years 1998 to 2014 ( $n = 102$ ). The slopes of eCO<sub>2</sub> and aCO<sub>2</sub> were significantly different ( $P_{\text{com}}$ , Table A. 2-8).

### 2.3.4 Grasses vs. forbs

Forbs and grasses had significantly different responses (functional group effect sizes, mixed model analysis) during the first time period (1998 to 2006; H1  $p = .006$  and H2  $p = .022$  see Tab. A. 2-7 for F-values), but not in the last (2007 to 2014; Fig. 2-4; Tab. A. 2-7). Furthermore, biomasses of forbs and grasses correlated negatively (significantly for both

treatments, see Tab. A. 2-9), with a significant treatment effect ( $P_{\text{com}} = .007$ ; Fig. 2-4; Tab. A. 2-8).



**Figure 2-5:** Soil moisture differences (volumetric soil water content %) between elevated CO<sub>2</sub> (eCO<sub>2</sub>) and ambient CO<sub>2</sub> (aCO<sub>2</sub>) by monthly mean differences. Significant effects of the soil moisture differences (moi) and the interaction of moisture and time (time  $\times$  moi) on total aboveground biomass (TAB) response at harvest 1 (H1) or 2 (H2) in the full time series or within the given time sections are indicated by \* when  $p < .05$  and \*\* when  $p < .01$ . For details on  $p$  values, and effects of grasses and forbs, see Table A. 2-9. The volumetric soil moisture monthly data are presented in Table A. 2-2.

### 2.3.5 Soil moisture and temperature

The soil moisture difference between eCO<sub>2</sub> and aCO<sub>2</sub> (Fig. 2-5) had a BP in year 2008 ( $p = .01$ ). During the last period, a positive effect of eCO<sub>2</sub> on soil moisture was observed on average with a 2.8 % increase from 2008 to 2013, contrasting to only 0.2 % increase (a difference at the detection limit of the soil moisture sensors) in the first period from 1998 to 2007.

For the full 17-year time period, the soil moisture difference between aCO<sub>2</sub> and eCO<sub>2</sub> (during the growth period) affected the response of TAB ( $p = .017$ , H1 and  $0.007$ , H2) and of forbs ( $p = .020$ , H2; time  $p = .038$ , H1; mixed model analysis, see Tab. A. 2-9) significantly and of grasses by tendency ( $p = .072$ , H1). During the first time section (set by BP for the biomass responses; see above), the soil moisture difference during the growth period likewise affected the TAB response ( $p = .052$ , H1 and  $p = .009$ , H2) and the forbs response significantly ( $p = .0036$ , H2) and the grasses response by tendency ( $p = .053$ , H1; mixed model analysis, Tab. A. 2-9). Furthermore, time (only for grasses  $p = .004$ , H1) as well as the soil moisture  $\times$  time interaction affected the biomass responses (TAB  $p = .017$ , H1; grasses  $p = .002$ , H1; Tab. A.

2-9). Only forbs significantly responded to soil moisture differences during the last time section ( $p = .040$ ; Tab. A. 2-9).

The cold temperatures (frost and hard frost) potentially interfered with effects on plants at the beginning of the year near to growing season start ( $\geq +5^{\circ}\text{C}$  DOY, Fig. A. 2-1). A late frost down to  $10^{\circ}\text{C}$  in 2001 came as late as 20 days after the first  $+5^{\circ}\text{C}$  day. Likewise, in the years 2005, 2010, 2011 and 2013, a late frost occurred after the first  $+5^{\circ}\text{C}$  DOY (Fig. A. 2-1). Anomalies of monthly mean air temperature, precipitation, air humidity and global radiation were calculated by subtracting the specific monthly average for all years (1995 to 2014) from the monthly average (Fig. A. 2-3). However, none of the anomaly patterns directly matched with the biomass response pattern.

## 2.4 Discussion

The moderate CO<sub>2</sub> enrichment of +20 % through 17 years had a variable effect on the biomass production. The response pattern of TAB occasionally dropped to zero or negative, but in the long term, it remained positive as hypothesized, which suggests a long-term steady biomass increase in this fertile grassland. The increase in TAB of 15 % in the last eight years was comparable in magnitude to increases in other grassland FACE studies (Feng *et al.*, 2015) and forest FACE studies (17–18 % in Hoosbeek *et al.*, 2011). A random or prefixed point in time selected for recording biomass response might not fully reflect the system response, but provide a snapshot. A long-term perspective could reveal a delayed but certain development in the yield response, as a response can change dramatically from short term to long term (Andresen *et al.*, 2016; Drake, 2014; Kimball *et al.*, 2007; Leuzinger *et al.*, 2011; Luo *et al.*, 2011).

In the current study, the relative abundance of forbs (which was initially nonsignificantly lower in the eCO<sub>2</sub> plots) increased during the course of the experiment in both aCO<sub>2</sub> and eCO<sub>2</sub> plots. We suggest that this is partly due to management (the harvest programme). A functional plant type shift in grassland was also reported from the NZ-FACE, where the proportion of legumes declined through 10 years of eCO<sub>2</sub> treatment. Newton *et al.* assumed that this response was also related to the management (grazing) (Newton *et al.*, 2014). Within the plots of the GiFACE, a homogeneous biomass structure at TAB and functional group level existed between the 25 subplots (ICC analyses). This strengthens the conclusions drawn from correlation and mixed model analysis made at the plot level, as “plot level” is an averaging of subplot data.

The forbs were slightly suppressed by grasses in the eCO<sub>2</sub> treatment (across all years). The different response of grasses and forbs to eCO<sub>2</sub>, confirming our second hypothesis, was evident in the first period (positive for grasses and negative for forbs) and equalized in the last period (only positive responses), and resulted in an overall positive response of TAB. Hence, TAB response to eCO<sub>2</sub> through time relied on the response of different functional groups, as reported by Reich *et al.* (2004). In fact, the forbs response was effectively stronger than the grasses response, as it climbed from negative to positive response. The positive link between biomass and soil moisture was evident from correlation and covariation. In addition, the CO<sub>2</sub> response in soil moisture significantly covaried with the plant response, especially in the first half of the experiment. Most probably, the covariation expresses concurrent changes in the modes (increase or decrease between two years) of the responses. Moreover, a low frequent year-round positive soil moisture effect of eCO<sub>2</sub> at the last half of the experiment was associated with the positive biomass response of both functional groups forbs and grasses. Thus, biomass and soil moisture were significantly connected; however, there is no simple causality in this experiment, as we did not manipulate the soil moisture (Volk *et al.*, 2000). Hence, it cannot be deduced whether the direct biomass response to eCO<sub>2</sub> caused the eventual small positive soil moisture effect, or whether the indirect soil moisture effect caused an indirect biomass response. In a plant physiological study by Haworth *et al.* (2016) in the GiFACE grassland, the stomata opening (aperture) was reduced between April and May 2012 in eCO<sub>2</sub>, around the sampling time for H1 (after 15 years of eCO<sub>2</sub>), while stomatal density remained constant in eCO<sub>2</sub> compared to aCO<sub>2</sub> treatments in the six investigated mass-dominant species. This reduction in stomatal conductance is likely to be the regulating mechanism connecting biomass and soil moisture response, potentially leading to reduced water loss from plant and soil. However, at the last half of the time series, the substantial increase in TAB of 15 % under eCO<sub>2</sub> coincided with only a modest water saving of c. 3 % which was lower compared to other ecosystems (23 %; Adair *et al.*, 2011; and 16 %; Nelson *et al.*, 2004). One possible reason is that the ground water table was rather close to the surface at our site, and thus, the plant-driven regulation of soil moisture had only limited impact. Another possible mechanism is that an increased biomass under eCO<sub>2</sub> produces a larger canopy, adding up to an increased water use in spite of the water savings at leaf level (Leuzinger *et al.*, 2011).

In 2001, the unusual late severe frost episode most probably damaged the group of forbs during a critical phenological stage. This frost event was followed by a reduced abundance of forbs relative to grasses in eCO<sub>2</sub> plots for two years (2001 and 2002), and we suggest that this

triggered the negative response of forbs to eCO<sub>2</sub>. The effect on the abundance of forbs gradually disappeared throughout the subsequent eight years, which was possibly accelerated by the heat wave in year 2003 (García-Herrera *et al.*, 2010). An increased frost sensitivity of forbs (but not the grasses) under eCO<sub>2</sub> can explain this pattern (Martin *et al.*, 2010; Obrist *et al.*, 2001; Rapacz *et al.*, 2014). Thus, forbs under eCO<sub>2</sub> responded more negative to the frost episode in 2001 than grasses, underlining our hypothesis that climatic episodes with frost may affect plant response to eCO<sub>2</sub>. However, no eCO<sub>2</sub> responses with declining proportion of forbs were observed since the 2001 incident. This possibly indicates that forbs slowly adapted to eCO<sub>2</sub>, thus explaining that through the final eight years the eCO<sub>2</sub> biomass response of forbs was identical to that of grasses and was no longer markedly hampered by occurrence of late frosts. However, the years 2010 and 2011 had abnormal small grass biomasses (and TAB) at H1, which we also attribute to the occurrence of late hard frosts. The climatic episodes in 2001 (late frost) and in 2003 (European heatwave) can tentatively be termed ECEs.

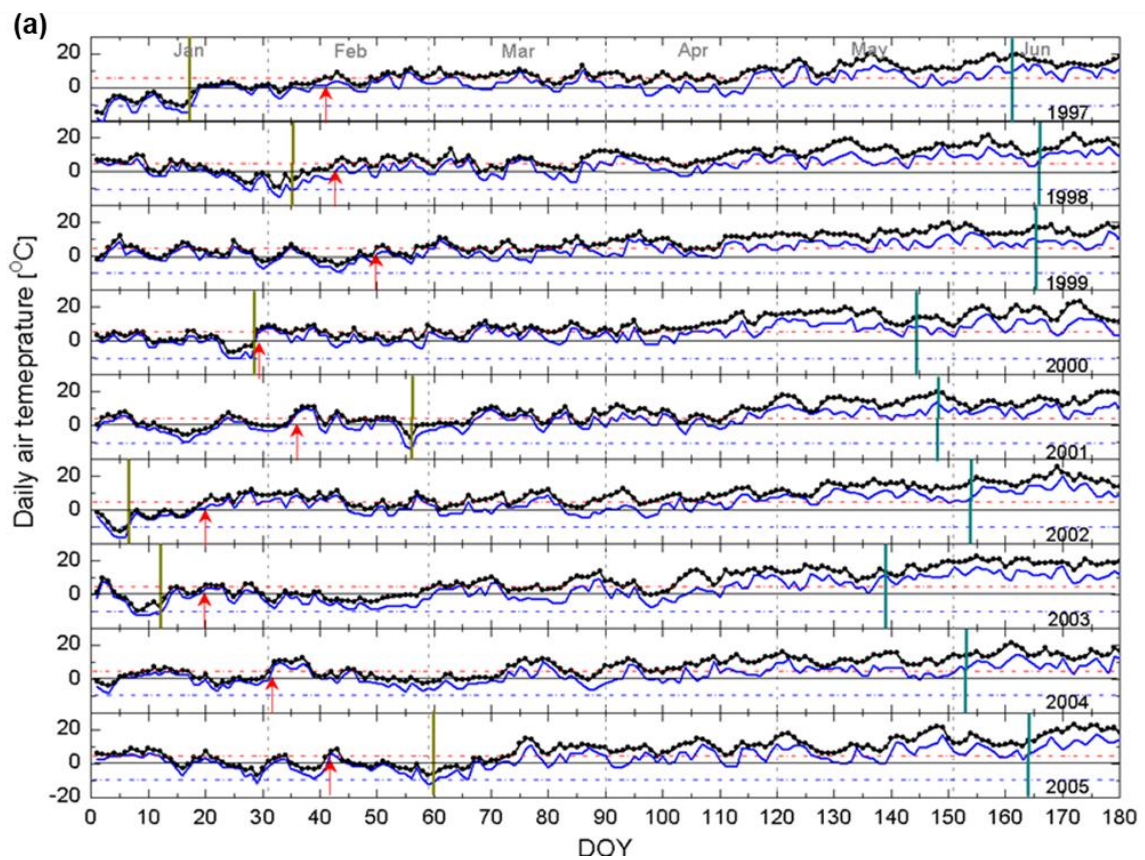
We conclude that at the moderately fertilized, permanent, species-rich GiFACE site, the functional groups grasses and forbs responded differently to eCO<sub>2</sub> through time, initially suggesting a suppression of the forbs by grasses at eCO<sub>2</sub>, and later converging to a positive CO<sub>2</sub> effect, from a certain BP on at 8 years after treatment start. We suggest that the CO<sub>2</sub> response of forbs biomass was affected negatively in 2001, due to a frost damage which was boosted by eCO<sub>2</sub>. After this extreme weather event, an acclimation of forbs took place followed by the overall final positive CO<sub>2</sub> response of both grasses and forbs. Soil moisture response was significantly connected to biomass response, but only a small increase in soil moisture of 3 % accompanied an increase in total biomass of 15 % in the last half period. We suggest that FACE experiments at grasslands and comparable perennial ecosystems should be performed over “long-term” time scales to uncover the effect of different environmental conditions and weather extremes (Obermeier *et al.*, 2017), for harvests both at spring and fall, and to confirm observed positive biomass responses beyond the extreme years.

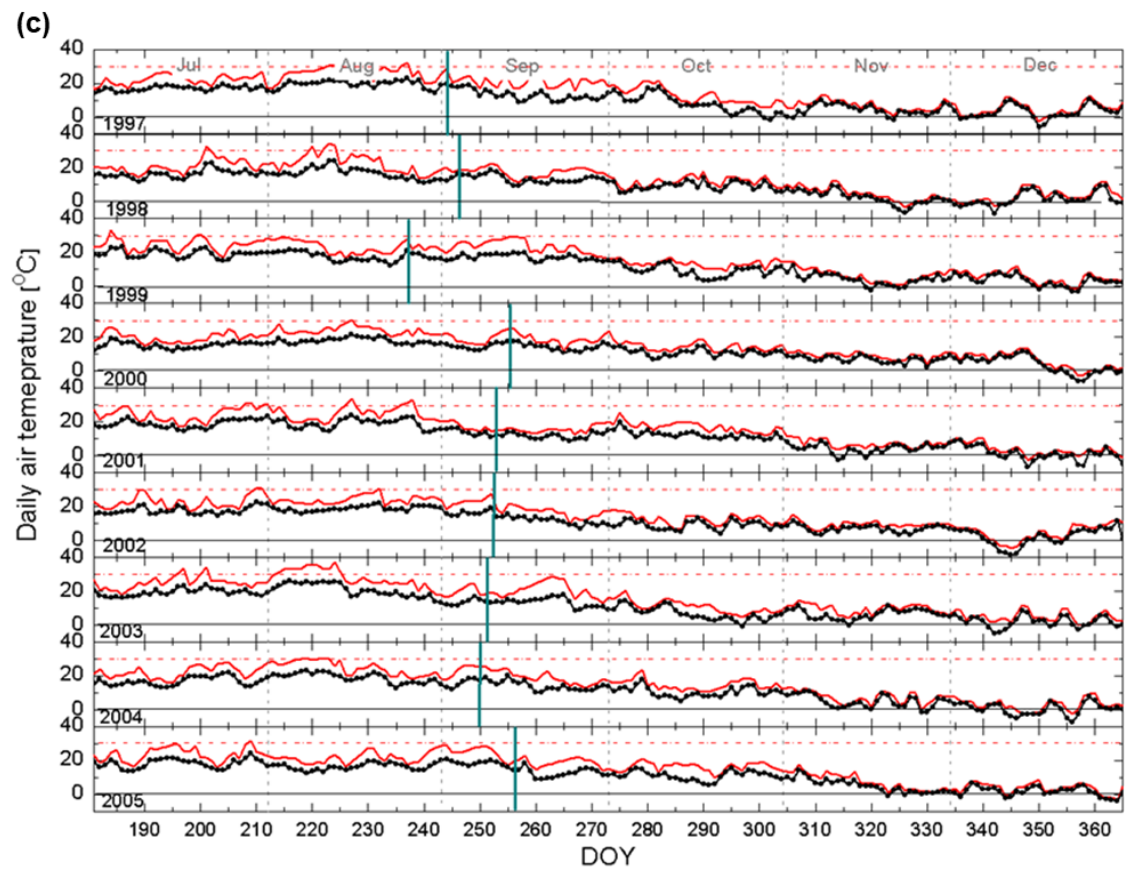
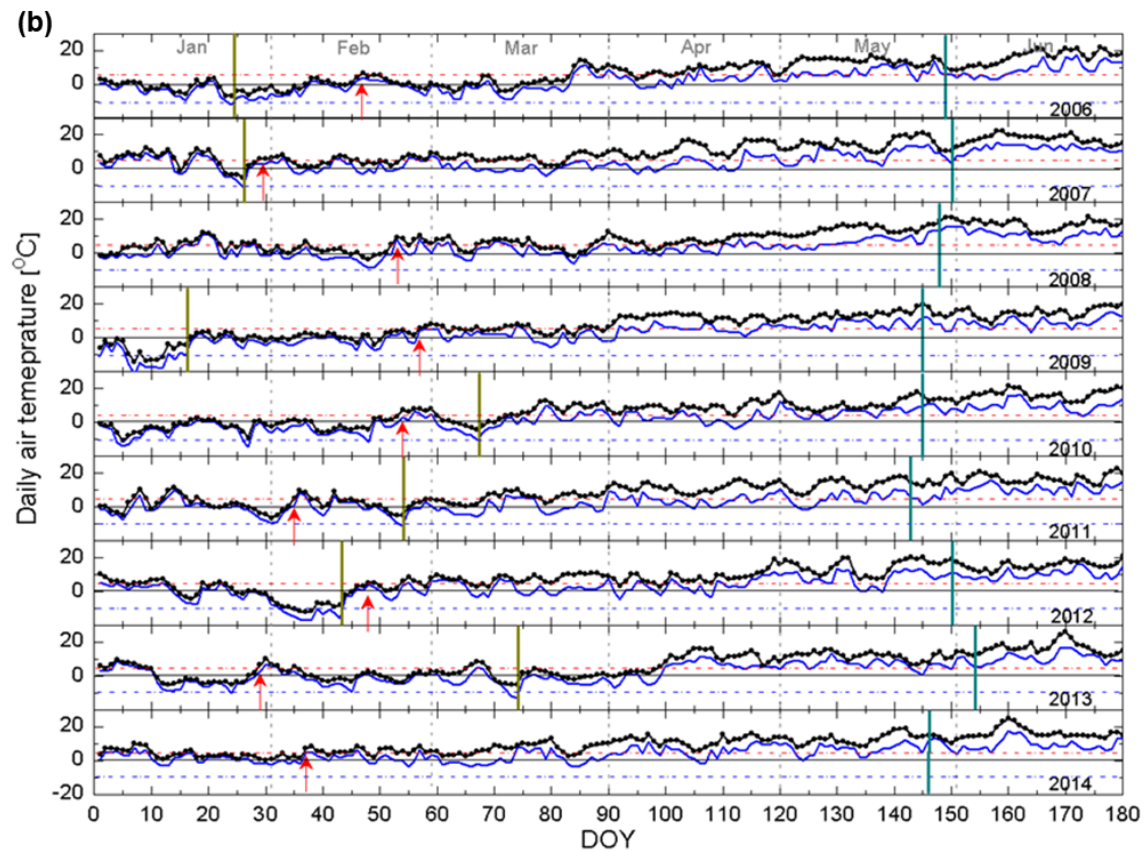
## 2.5 Acknowledgements

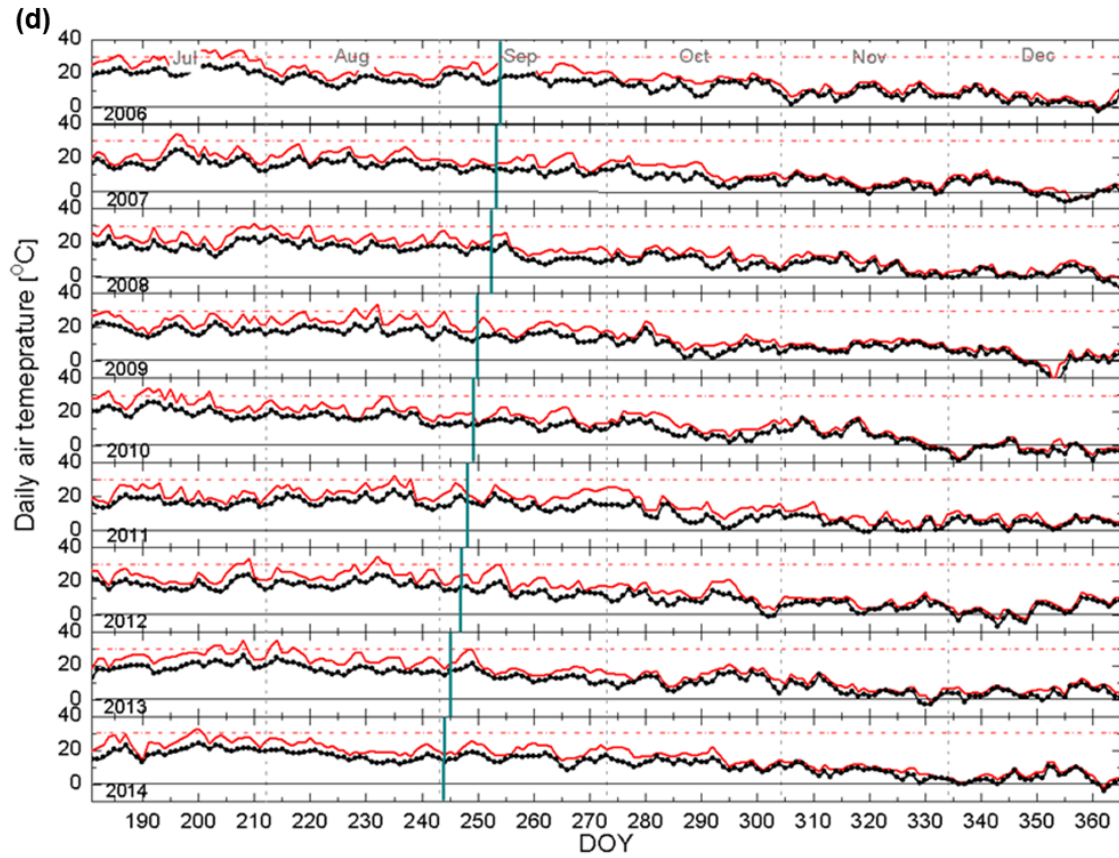
We thank Ludger Grünhage, Jürgen Franz, Gerlinde Lehr, Birte Lenz, Gerhard Mayer, Siegfried Schmidt, Jochen Senkbeil, Wolfgang Stein, Nicol Strasilla and Till Strobusch at JLU-Giessen for long-term assistance with the management of the FACE site, the harvests, the soil moisture measurements and the data recording. We thank Matthias Meine for the pH measurements, soil pore space and sand grain size analysis during his diploma thesis at

Phillips University Marburg. We thank Tobias Rütting and the anonymous reviewers for constructive comments. We are grateful for long-term financial support of the Hessian Agency for Nature Conservation, Environment and Geology (HLNUG), and we acknowledge the funding by the LOEWE excellence cluster FACE<sub>2</sub>FACE from the Hessian State Ministry of Higher Education, Research and the Arts. We keep a respectful memory of the deceased Professor Dr. h.c. Hans-Jürgen Jäger who initiated and sustained the GiFACE experiment.

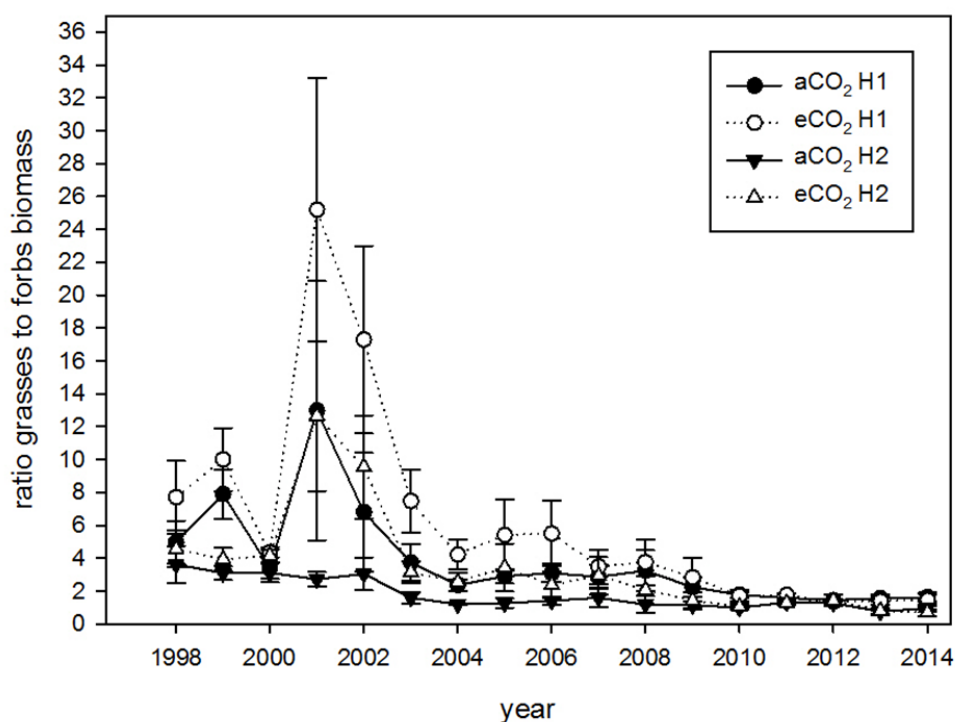
## 2.6. Appendix



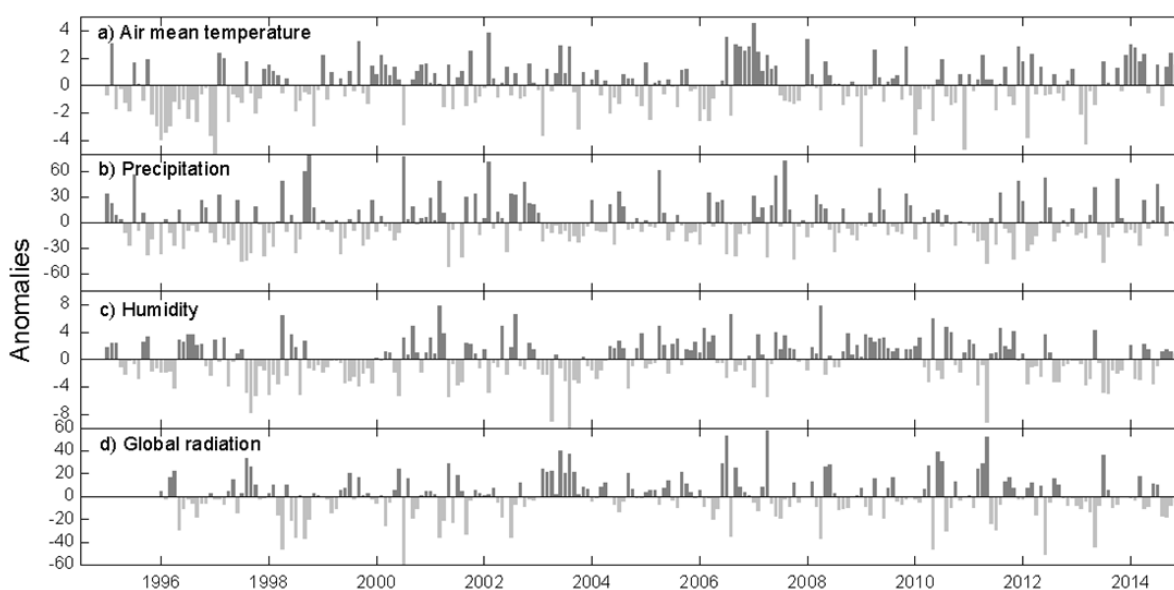




**Figure A. 2-1:** Daily air temperature means (black dots and line), minimum (blue line) and maximum (red line) at day of year (DOY) for the years 1997 to 2014, in sections of DOY 1 to 180 (a and b) and 181 to 360 (c and d). The red arrows indicate spring start, as the first DOY with a daily mean air temperature  $\geq +5^{\circ}\text{C}$ , after the winter period. The green vertical line at winter, indicates the last hard frost event when the minimum temperature dropped below  $-10^{\circ}\text{C}$ . Harvest 1 and 2 indicated by vertical line in May-June and August-September. Temperature anomalies are indicated in Figure A. 2-3.



**Figure A. 2-2:** The ratio of grasses to forbs (incl. legumes) biomasses dry weight, at the two harvests (H1 and H2), at ambient  $\text{CO}_2$  ( $\text{aCO}_2$ ) and elevated  $\text{CO}_2$  ( $\text{eCO}_2$ ). 1997 was a pre-treatment year.



**Figure A. 2-3:** Anomalies of four meteorological variables: a) mean air temperature ( $^{\circ}\text{C}$ , 1995-2014), b) precipitation (mm, 1995-2014), c) air humidity (% , 1995-2014), and d) global radiation ( $\text{W m}^{-2}$ , 1996-2014). The anomalies are defined as differences between the observed values and their monthly average from the entire time series. The monthly data are presented in Table A. 2-3.

**Table A. 2-1:** Fertilization management of the GiFACE areas through the years 1993 to 2014. All of the areas and the  $\text{eCO}_2$  and  $\text{aCO}_2$  plots received the same fertilizer treatment. The added amount of each element (Nitrogen, N; Phosphor, P; Potassium, K; Magnesium, Mg; Calcium, Ca and Sulphur, S) in  $\text{g m}^{-2}$ . The chemical

fertilizer products ‘Thomaskali’ and ‘Kalkammonsalpeter’ were applied each year in April prior to the main growing season.

<b>Year</b>	<b>N</b> (NH <sub>4</sub> NO <sub>3</sub> )	<b>P</b> (P <sub>2</sub> O <sub>5</sub> )	<b>K</b> (K <sub>2</sub> O)	<b>Mg</b> (MgO)	<b>Ca</b> (CaO)	<b>S</b> (S)
<b>1993</b>	8.0	0	0	0	0	0
<b>1994</b>	8.0	6.0	9.0	1.8	21.3	0
<b>1995</b>	8.0	6.0	9.0	1.8	21.3	0
<b>1996</b>	4.0	6.0	9.0	1.8	21.3	0
<b>1997</b>	4.0	6.0	9.0	1.8	21.3	0
<b>1998</b>	4.0	6.0	9.0	1.8	21.3	0
<b>1999</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2000</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2001</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2002</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2003</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2004</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2005</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2006</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2007</b>	4.0	6.0	9.0	1.8	15.9	1.2
<b>2008</b>	4.0	6.0	9.0	2.4	14.7	1.8
<b>2009</b>	4.0	6.0	9.0	3.0	14.7	2.4
<b>2010</b>	4.0	6.0	9.0	3.0	1.5	2.4
<b>2011</b>	4.0	4.8	9.0	3.6	1.5	0
<b>2012</b>	4.0	4.8	9.0	3.6	1.5	0
<b>2013</b>	4.0	4.8	9.0	3.6	1.5	0
<b>2014</b>	4.0	6.0	11.3	7.5	1.5	0

**Table A. 2-2:** Monthly mean soil moisture (%) from 1997 to 2013. Volumetric soil water content recorded on daily intervals by permanent TDR-sensors in 0 to 15 cm depth at each plot (aCO<sub>2</sub> 1 to 3 and eCO<sub>2</sub> 1 to 3).

<b>Year</b>	<b>Month</b>	<b>aCO<sub>2</sub>-1</b>	<b>aCO<sub>2</sub>-2</b>	<b>aCO<sub>2</sub>-3</b>	<b>eCO<sub>2</sub>-1</b>	<b>eCO<sub>2</sub>-2</b>	<b>eCO<sub>2</sub>-3</b>
1997	3	40.71	50.43	46.16	44.13	49.20	47.96
1997	4	31.10	42.58	35.30	34.48	36.98	38.26
1997	5	28.93	37.22	32.57	32.14	32.72	34.63
1997	6	21.91	31.55	26.92	27.91	29.41	29.02
1997	7	22.90	33.11	27.64	30.95	31.90	28.61
1997	8	12.08	19.23	14.96	17.37	20.08	16.76
1997	9	11.19	17.65	16.98	15.74	19.53	16.47
1997	10	28.62	34.49	34.61	34.53	34.79	31.32
1997	11	35.63	41.85	41.37	42.86	41.68	36.87
1997	12	38.57	46.85	46.36	46.87	47.51	41.08
1998	1	37.90	47.47	45.93	46.48	47.23	40.75
1998	2	31.53	40.06	38.52	39.62	38.97	36.49
1998	3	38.33	48.89	45.58	46.58	46.92	40.81

1998	4	38.68	51.67	47.26	44.97	48.56	43.64
1998	5	27.41	40.98	32.60	31.08	35.31	30.37
1998	6	35.04	45.74	40.86	39.75	39.83	34.83
1998	7	29.86	39.72	36.35	35.15	35.58	33.64
1998	8	23.04	28.63	26.65	28.09	29.84	27.28
1998	9	35.84	48.50	43.72	41.87	45.15	41.50
1998	10	41.03	59.80	62.98	51.11	60.60	49.30
1998	11	42.71	61.65	66.71	56.05	69.60	52.39
1998	12	42.45	59.95	60.46	53.30	61.92	51.08
1999	1	43.86	62.57	62.43	55.63	62.87	52.39
1999	2	43.32	59.11	59.37	54.68	59.76	51.06
1999	3	42.56	56.87	56.96	53.56	59.41	49.07
1999	4	42.27	57.66	57.78	54.48	61.09	48.84
1999	5	31.13	52.09	34.99	37.63	43.03	32.49
1999	6	23.75	39.45	25.85	29.33	32.05	29.24
1999	7	28.50	33.90	30.91	31.86	33.67	32.61
1999	8	23.57	24.55	21.74	24.44	28.09	23.91
1999	9	21.77	24.89	22.20	26.50	29.47	24.70
1999	10	33.72	36.54	34.73	39.82	41.24	35.88
1999	11	38.88	41.25	41.62	46.11	46.33	41.55
1999	12	40.41	44.36	44.71	48.05	49.42	43.97
2000	1	40.35	44.67	44.68	47.21	50.78	44.03
2000	2	43.72	58.39	50.24	48.23	55.25	49.47
2000	3	43.07	59.85	49.05	46.27	54.35	48.89
2000	4	38.32	53.66	40.62	41.95	48.59	43.06
2000	5	27.89	39.24	29.18	30.96	34.36	31.54
2000	6	24.13	32.98	27.31	30.22	32.26	30.52
2000	7	35.04	43.44	37.68	39.38	42.68	37.10
2000	8	36.24	44.24	37.30	38.73	42.01	37.03
2000	9	38.78	48.88	40.59	41.07	47.29	40.16
2000	10	45.76	58.60	51.15	50.44	59.26	51.08
2000	11	49.62	63.66	56.64	54.23	66.30	56.26
2000	12	50.51	65.53	59.03	55.31	69.84	58.65
2001	1	49.64	62.22	59.20	54.25	68.78	56.94
2001	2	51.52	60.80	57.66	54.04	68.00	52.93
2001	3	56.10	66.99	62.20	54.08	72.97	55.45
2001	4	53.15	60.63	55.44	50.44	62.75	50.96
2001	5	33.24	44.26	31.33	34.92	40.42	33.22
2001	6	25.81	36.04	28.89	29.15	32.77	31.11
2001	7	26.99	32.36	28.22	27.46	32.57	28.58
2001	8	20.47	24.42	20.42	21.12	26.15	23.37
2001	9	30.69	33.18	31.51	31.31	35.22	32.81
2001	10	42.77	47.27	44.93	43.73	46.06	43.71
2001	11	47.39	55.67	50.64	48.22	53.39	49.61
2001	12	46.87	57.58	50.97	48.03	55.74	48.94

2002	1	44.71	55.63	52.13	47.90	53.60	48.03
2002	2	48.01	63.58	60.95	55.67	63.92	52.42
2002	3	45.95	60.81	51.41	50.63	56.33	49.64
2002	4	37.70	55.28	40.12	39.51	45.48	40.20
2002	5	38.97	57.03	42.89	42.51	50.21	42.25
2002	6	26.99	39.53	27.76	29.87	35.04	31.55
2002	7	29.53	35.67	29.94	29.27	34.73	31.19
2002	8	39.57	49.93	39.20	38.90	44.32	40.44
2002	9	38.66	54.21	39.11	38.13	43.51	40.31
2002	10	46.99	63.80	48.56	47.95	58.06	49.83
2002	11	51.39	69.08	55.90	55.69	67.52	56.82
2002	12	46.86	64.61	53.68	50.88	59.85	54.00
2003	1	46.44	61.95	54.98	53.03	58.59	54.29
2003	2	37.28	51.00	44.80	41.02	45.61	45.06
2003	3	43.10	52.60	44.40	44.81	47.55	46.83
2003	4	36.60	53.84	35.33	38.45	42.81	39.29
2003	5	33.38	45.98	32.07	35.66	37.53	37.93
2003	6	28.43	36.71	29.88	30.64	32.22	32.69
2003	7	24.02	24.62	23.64	23.81	26.55	25.02
2003	8	18.53	15.18	18.17	17.86	20.07	20.42
2003	9	29.61	29.55	28.05	27.48	31.40	26.11
2003	10	30.77	32.49	29.56	29.72	32.32	27.93
2003	11	34.96	36.76	34.21	34.13	36.75	31.68
2003	12	40.47	44.76	41.87	42.12	44.03	38.77
2004	1	43.81	53.71	48.48	46.79	51.14	43.57
2004	2	43.27	53.40	46.66	46.53	50.38	43.02
2004	3	41.25	50.06	43.55	44.54	47.67	41.19
2004	4	34.91	43.60	33.63	36.05	36.20	33.14
2004	5	37.84	46.93	38.59	38.41	41.54	36.00
2004	6	27.00	37.84	27.55	29.88	30.04	30.48
2004	7	34.51	41.84	34.15	34.22	37.28	37.28
2004	8	35.19	38.58	34.78	35.03	35.87	38.22
2004	9	37.85	41.15	38.72	37.60	41.08	40.41
2004	10	40.10	45.61	42.63	42.44	46.99	43.73
2004	11	47.53	58.02	51.63	51.11	56.80	51.80
2004	12	47.90	54.86	51.38	49.36	56.72	51.36
2005	1	49.83	57.26	55.16	51.74	58.97	55.46
2005	2	48.40	56.35	53.29	49.94	57.93	53.56
2005	3	44.89	49.03	47.31	45.82	50.42	47.99
2005	4	47.64	59.47	53.58	51.06	56.24	47.59
2005	5	46.02	58.14	50.40	49.64	56.77	46.04
2005	6	29.32	41.20	30.10	30.72	34.67	31.96
2005	7	30.54	36.67	29.80	30.91	33.89	32.68
2005	8	29.40	34.08	30.82	31.29	33.89	33.68
2005	9	31.04	35.37	32.27	32.71	34.98	35.04

2005	10	37.97	44.33	39.49	38.43	40.91	40.55
2005	11	44.08	54.28	49.73	47.19	51.93	49.45
2005	12	46.47	59.17	54.32	51.60	55.63	53.19
2006	1	37.16	51.55	45.25	44.04	47.17	44.74
2006	2	35.52	44.28	43.53	43.07	43.81	44.70
2006	3	42.82	53.76	51.07	47.98	50.49	49.43
2006	4	43.35	62.31	50.64	47.79	53.57	46.96
2006	5	35.49	49.46	38.48	37.09	39.24	38.80
2006	6	38.33	46.53	38.76	36.67	40.71	38.07
2006	7	26.27	29.05	28.60	27.72	27.83	29.36
2006	8	29.02	26.25	29.14	28.66	28.41	28.51
2006	9	25.65	27.98	25.07	25.26	28.13	26.19
2006	10	33.34	36.55	30.00	32.78	36.50	32.12
2006	11	39.31	43.20	37.03	39.96	43.89	39.61
2006	12	44.39	51.09	44.28	46.88	50.81	47.84
2007	1	46.42	58.00	48.91	49.42	53.80	52.17
2007	2	46.52	58.97	49.52	50.00	53.67	54.18
2007	3	47.11	62.85	47.87	49.80	56.35	55.50
2007	4	31.34	48.13	31.21	31.13	34.11	32.53
2007	5	26.43	31.84	25.67	26.95	27.38	28.20
2007	6	37.86	43.68	39.21	39.94	42.11	39.05
2007	7	40.65	48.72	41.49	42.84	43.04	40.90
2007	8	43.36	54.53	46.17	45.05	53.11	42.94
2007	9	36.83	52.63	38.28	39.03	49.38	35.26
2007	10	39.54	55.83	42.10	40.75	54.07	39.07
2007	11	45.34	62.96	50.24	47.61	64.51	47.40
2007	12	47.01	67.14	53.16	50.81	70.20	50.63
2008	1	49.95	51.40	54.81	51.71	60.15	50.47
2008	2	49.04	50.65	53.87	51.30	58.45	49.63
2008	3	50.66	56.13	57.21	53.60	64.89	51.79
2008	4	50.29	61.71	56.29	54.20	65.62	56.47
2008	5	26.01	33.12	29.72	29.42	31.72	29.65
2008	6	35.02	46.69	41.90	37.46	44.50	39.72
2008	7	22.30	25.73	27.25	25.03	25.47	27.04
2008	8	26.33	27.52	28.26	28.10	28.90	27.71
2008	9	32.07	34.30	32.31	34.30	36.58	34.52
2008	10	42.97	49.78	44.74	48.43	52.70	48.46
2008	11	45.38	56.22	50.16	52.07	56.58	54.63
2008	12	46.41	61.92	55.14	53.84	58.06	61.26
2009	1	29.64	42.78	37.04	38.99	40.43	43.24
2009	2	41.52	54.73	47.73	49.85	50.48	54.33
2009	3	45.64	60.15	50.95	55.09	56.26	54.42
2009	4	37.09	47.07	38.10	40.66	43.98	38.28
2009	5	40.95	54.50	47.28	45.75	55.96	42.78
2009	6	40.25	51.44	44.13	42.27	48.15	41.25

2009	7	33.54	39.82	37.24	34.13	37.82	34.60
2009	8	32.53	36.95	31.01	32.34	36.29	32.29
2009	9	31.64	37.14	33.15	35.13	37.75	32.54
2009	10	38.37	45.22	42.57	41.88	48.80	41.62
2009	11	45.68	56.43	53.11	50.83	61.26	51.63
2009	12	47.57	61.37	54.85	52.13	64.45	53.81
2010	1	45.88	58.46	54.58	50.76	59.29	51.42
2010	2	46.66	57.79	53.85	50.70	59.15	52.41
2010	3	53.08	63.20	55.00	54.98	75.21	59.88
2010	4	40.52	52.34	40.71	41.48	54.27	43.13
2010	5	43.00	49.47	42.47	43.84	54.71	43.77
2010	6	36.43	47.32	38.89	38.69	46.66	39.82
2010	7	21.25	27.70	26.93	26.81	31.86	27.65
2010	8	30.70	32.35	32.08	32.76	41.63	35.30
2010	9	42.66	42.51	43.10	45.53	53.91	45.96
2010	10	44.45	46.86	45.60	48.71	59.78	48.66
2010	11	49.58	54.25	51.37	52.51	66.00	52.68
2010	12	51.38	61.76	57.66	56.06	64.97	54.26
2011	1	54.00	69.61	61.11	58.35	76.87	57.53
2011	2	52.79	58.04	54.45	53.42	66.81	54.86
2011	3	49.92	48.99	45.13	46.38	54.93	47.85
2011	4	37.40	40.75	34.19	36.11	38.31	36.88
2011	5	22.79	25.37	24.01	24.35	23.14	26.66
2011	6	28.59	33.36	26.25	29.23	29.62	31.93
2011	7	23.91	27.91	20.19	24.65	26.33	27.92
2011	8	34.78	37.09	30.20	37.53	39.25	38.16
2011	9	37.35	38.93	38.08	39.93	49.70	42.90
2011	10	38.46	40.71	41.78	42.86	52.96	44.53
2011	11	39.41	40.12	41.61	43.25	56.74	44.22
2011	12	44.61	51.36	50.55	53.61	68.59	53.64
2012	1	45.95	57.20	53.55	56.33	73.51	55.05
2012	2	27.90	28.15	31.67	32.20	31.82	33.97
2012	3	44.60	48.54	43.21	46.55	55.94	43.27
2012	4	36.12	45.52	34.83	39.23	42.94	35.68
2012	5	34.21	38.80	31.73	38.54	37.75	34.49
2012	6	45.92	43.68	41.57	44.03	47.69	39.46
2012	7	46.96	47.35	41.98	46.05	49.76	39.99
2012	8	29.37	32.72	29.72	30.60	32.55	28.09
2012	9	26.55	29.55	28.54	31.74	31.68	31.78
2012	10	38.10	42.94	41.38	43.46	47.77	46.53
2012	11	46.05	53.15	47.64	52.21	58.97	53.99
2012	12	49.63	58.34	50.82	59.23	64.50	52.98
2013	1	51.98	56.62	47.96	54.22	59.29	51.11
2013	2	56.67	60.48	50.96	58.22	62.45	53.82
2013	3	52.59	53.56	48.43	54.75	57.49	49.09

2013	4	46.43	48.34	42.49	45.61	51.08	41.65
2013	5	48.64	53.73	46.79	47.50	54.81	45.43
2013	6	43.19	51.16	39.64	42.94	54.45	41.81
2013	7	29.24	33.91	28.68	29.60	32.66	30.14
2013	8	25.98	27.89	24.20	26.09	27.92	28.20
2013	9	37.56	34.38	31.44	35.84	40.28	38.03
2013	10	47.26	43.15	42.05	49.29	56.23	47.00
2013	11	53.46	51.98	50.63	56.79	69.69	51.56
2013	12	56.61	56.66	54.79	58.78	71.56	50.71

**Table A. 2-3:** Monthly meteorological data in years 1995 to 2014. Mean air temperature (Temp; °C), relative humidity (RH; %) and global radiation (GR; W·m<sup>-2</sup>) were measured in 2 m above ground, the precipitation is presented as monthly sums (Precip; mm). nd. means no data available. Anomalies are displayed in Figure A. 2-3.

Year	Month	Temp	Precip	RH	GR
1995	1	0.36	69.35	88.59	nd.
1995	2	5.37	58.10	86.58	nd.
1995	3	3.47	41.20	81.52	nd.
1995	4	9.13	43.90	73.51	nd.
1995	5	12.06	48.70	74.18	nd.
1995	6	14.38	27.30	75.90	nd.
1995	7	19.81	130.60	75.54	nd.
1995	8	17.91	45.30	73.92	nd.
1995	9	12.72	56.30	84.82	nd.
1995	10	11.56	12.10	90.43	nd.
1995	11	3.16	24.90	88.15	nd.
1995	12	-1.19	38.30	87.53	nd.
1996	1	-2.95	0.90	84.96	34.72
1996	2	-1.11	39.40	82.39	57.47
1996	3	2.25	20.90	77.53	126.51
1996	4	8.20	13.40	70.38	194.19
1996	5	11.56	74.70	79.09	174.35
1996	6	15.27	23.70	78.55	223.42
1996	7	15.77	65.80	79.84	212.36
1996	8	16.78	52.30	80.26	184.12
1996	9	11.15	35.80	84.27	112.82
1996	10	9.08	75.80	89.27	69.28
1996	11	5.12	60.80	88.81	26.94
1996	12	-1.90	27.30	86.49	24.26
1997	1	-4.00	13.60	89.57	28.93
1997	2	4.65	68.25	83.82	58.13
1997	3	7.21	15.00	82.27	103.25
1997	4	6.73	14.80	70.66	176.53
1997	5	12.70	40.00	75.95	218.99
1997	6	15.40	79.20	76.85	219.79

1997	7	16.92	29.30	77.70	217.36
1997	8	19.53	11.50	71.88	223.45
1997	9	13.27	10.80	74.54	156.54
1997	10	7.66	68.00	81.84	84.71
1997	11	4.40	41.80	88.90	30.54
1997	12	2.92	0.00	87.17	21.15
1998	1	2.53	25.08	81.67	33.21
1998	2	3.29	7.87	82.05	69.80
1998	3	5.90	33.70	75.69	94.46
1998	4	8.78	88.80	81.08	126.03
1998	5	13.79	50.30	73.93	213.52
1998	6	16.24	62.20	79.59	213.09
1998	7	16.33	39.90	77.99	178.84
1998	8	16.71	35.90	71.58	191.12
1998	9	13.34	105.20	84.93	94.68
1998	10	9.07	130.80	85.79	54.97
1998	11	2.32	60.10	88.22	35.07
1998	12	1.47	30.60	87.99	22.37
1999	1	3.20	38.80	84.99	30.33
1999	2	1.29	28.59	83.10	57.59
1999	3	6.12	21.70	79.06	95.95
1999	4	9.37	42.33	74.40	161.09
1999	5	13.74	24.80	75.86	208.95
1999	6	15.48	35.60	72.64	241.12
1999	7	19.17	78.50	73.18	235.31
1999	8	17.38	45.60	74.21	187.84
1999	9	17.00	60.70	78.30	147.91
1999	10	9.14	23.20	85.14	75.77
1999	11	4.01	24.21	88.54	35.41
1999	12	3.21	64.40	85.27	19.58
2000	1	1.78	26.50	86.98	24.67
2000	2	4.52	42.40	84.07	60.96
2000	3	6.68	28.90	80.27	84.60
2000	4	10.06	30.70	75.54	166.76
2000	5	14.64	40.70	74.53	209.68
2000	6	16.67	42.40	70.74	257.38
2000	7	15.33	150.99	79.30	155.76
2000	8	17.78	58.31	77.22	205.55
2000	9	14.24	64.80	87.15	112.01
2000	10	10.71	48.32	88.09	64.56
2000	11	6.78	47.40	87.93	32.97
2000	12	3.32	45.30	89.69	26.87
2001	1	1.19	64.80	89.86	35.25
2001	2	3.20	37.49	84.97	61.11
2001	3	5.28	79.96	87.07	74.15

2001	4	7.81	50.80	78.39	150.70
2001	5	14.76	9.10	70.86	232.76
2001	6	14.57	46.30	75.43	210.67
2001	7	18.76	73.92	72.54	232.85
2001	8	18.83	14.76	73.41	195.11
2001	9	12.34	75.90	84.53	97.72
2001	10	12.21	49.70	89.36	72.07
2001	11	4.03	76.70	90.62	38.18
2001	12	0.95	25.00	87.54	24.46
2002	1	0.87	41.00	88.17	31.32
2002	2	6.11	106.47	79.30	61.77
2002	3	5.66	25.30	78.69	117.40
2002	4	8.47	52.60	73.49	166.69
2002	5	13.47	65.70	81.20	185.84
2002	6	17.57	19.40	73.81	233.70
2002	7	17.48	107.65	78.00	179.21
2002	8	18.62	87.45	83.13	183.19
2002	9	12.88	37.30	81.36	143.07
2002	10	8.89	96.85	85.70	66.73
2002	11	6.83	65.10	92.08	30.53
2002	12	1.90	60.10	90.10	19.25
2003	1	0.69	47.85	85.39	30.36
2003	2	-1.35	14.09	81.99	83.50
2003	3	6.39	25.10	77.08	131.38
2003	4	8.94	28.35	65.62	194.23
2003	5	14.18	58.10	76.96	205.59
2003	6	19.16	40.50	74.81	273.68
2003	7	19.04	65.90	73.07	235.23
2003	8	20.59	33.50	66.75	227.47
2003	9	13.34	30.40	79.29	152.76
2003	10	6.52	26.90	83.63	76.60
2003	11	6.28	28.40	90.14	40.95
2003	12	1.80	34.60	87.80	28.67
2004	1	1.40	62.15	85.26	26.82
2004	2	3.41	27.35	81.37	58.86
2004	3	4.51	22.05	77.68	118.58
2004	4	9.65	30.05	74.40	183.37
2004	5	11.30	81.70	78.13	203.92
2004	6	15.40	27.80	77.57	226.52
2004	7	16.89	110.15	78.88	201.37
2004	8	18.57	73.85	78.20	186.72
2004	9	14.27	37.90	78.00	151.16
2004	10	10.18	43.10	86.22	81.40
2004	11	4.55	47.80	91.43	31.84
2004	12	0.29	28.50	92.45	20.55

2005	1	2.69	39.25	85.63	34.28
2005	2	-0.20	32.16	83.60	64.95
2005	3	5.35	26.50	78.80	116.00
2005	4	9.65	100.10	79.52	164.88
2005	5	12.67	71.25	78.30	211.25
2005	6	16.62	33.65	74.02	247.85
2005	7	18.12	64.60	78.46	211.13
2005	8	16.25	62.85	79.62	181.08
2005	9	14.89	42.75	81.48	151.90
2005	10	10.91	29.00	88.48	86.08
2005	11	4.95	32.35	91.04	36.40
2005	12	1.54	28.20	91.33	21.38
2006	1	-1.57	12.15	87.72	36.30
2006	2	0.59	34.28	88.78	51.60
2006	3	2.62	67.30	81.67	109.17
2006	4	8.39	36.25	78.09	151.97
2006	5	13.26	84.00	75.81	193.76
2006	6	16.56	79.60	75.58	261.97
2006	7	21.67	37.45	73.65	267.55
2006	8	15.61	53.15	83.20	155.58
2006	9	16.75	7.00	80.63	155.82
2006	10	12.49	37.65	86.27	83.26
2006	11	7.76	40.80	88.26	35.74
2006	12	4.57	25.60	89.20	22.46
2007	1	5.54	67.10	82.70	25.63
2007	2	4.71	41.64	87.81	47.21
2007	3	6.24	49.77	79.88	118.65
2007	4	11.54	0.33	69.17	229.25
2007	5	14.48	80.54	75.64	198.53
2007	6	17.65	107.74	79.89	216.18
2007	7	17.48	70.39	77.69	195.99
2007	8	16.72	127.07	80.06	188.46
2007	9	12.68	60.69	83.78	122.19
2007	10	8.37	7.17	88.52	86.71
2007	11	4.24	38.72	89.48	27.89
2007	12	1.70	40.80	88.73	21.83
2008	1	4.39	20.62	85.25	30.34
2008	2	3.07	30.38	85.97	72.73
2008	3	5.07	64.62	79.98	101.23
2008	4	7.56	60.20	82.37	134.79
2008	5	15.01	76.09	74.11	230.11
2008	6	16.94	46.23	76.49	261.54
2008	7	18.26	40.26	75.12	217.17
2008	8	17.91	43.66	75.50	178.60
2008	9	12.41	62.05	83.34	120.95

2008	10	8.94	42.96	90.80	65.21
2008	11	5.52	29.85	90.42	32.42
2008	12	0.98	18.11	90.83	22.84
2009	1	-3.39	34.30	87.07	37.60
2009	2	1.60	32.35	87.71	51.40
2009	3	5.01	42.57	82.35	94.13
2009	4	11.91	35.82	77.07	187.07
2009	5	13.81	99.67	79.35	203.44
2009	6	15.11	67.51	79.16	214.83
2009	7	18.40	60.73	77.78	222.31
2009	8	18.23	51.20	77.69	207.18
2009	9	14.54	35.80	83.83	127.37
2009	10	8.66	37.63	87.12	68.09
2009	11	8.14	77.20	91.30	30.34
2009	12	1.07	58.11	90.13	21.58
2010	1	-2.55	17.60	88.62	28.35
2010	2	0.61	34.30	87.24	55.40
2010	3	4.97	37.95	78.19	116.64
2010	4	9.13	6.35	71.40	198.38
2010	5	10.75	71.51	82.29	157.61
2010	6	16.68	67.59	74.51	272.32
2010	7	20.05	70.80	73.46	244.79
2010	8	16.99	63.88	81.33	160.42
2010	9	12.42	43.91	86.19	121.88
2010	10	8.45	23.76	86.26	88.20
2010	11	6.05	44.39	87.90	29.29
2010	12	-2.91	38.68	89.69	21.63
2011	1	1.82	34.51	89.63	31.44
2011	2	1.90	24.87	86.32	50.26
2011	3	5.64	11.17	75.43	134.18
2011	4	11.55	19.75	73.92	200.63
2011	5	13.70	12.74	67.10	256.48
2011	6	16.65	58.42	76.87	210.15
2011	7	16.43	49.74	77.22	185.04
2011	8	17.88	89.17	81.22	183.67
2011	9	15.15	39.35	84.17	143.90
2011	10	8.91	38.83	88.46	91.72
2011	11	3.87	0.62	93.87	39.89
2011	12	4.61	86.44	88.63	20.19
2012	1	2.77	60.94	87.59	28.91
2012	2	-1.50	2.81	80.69	67.19
2012	3	7.51	7.05	78.15	121.61
2012	4	8.71	24.40	73.72	155.48
2012	5	14.65	61.75	73.88	213.31
2012	6	15.56	105.58	79.73	183.47

2012	7	17.56	91.32	77.23	209.94
2012	8	18.56	33.85	73.32	205.82
2012	9	13.31	34.28	79.02	141.24
2012	10	8.59	52.04	86.24	73.90
2012	11	5.58	38.80	89.20	25.24
2012	12	2.98	54.27	88.60	20.43
2013	1	1.07	23.22	86.64	22.55
2013	2	0.21	24.31	83.50	49.19
2013	3	0.95	14.24	75.55	105.93
2013	4	8.93	48.34	71.91	158.34
2013	5	11.85	101.83	80.49	160.04
2013	6	16.25	39.25	75.52	226.12
2013	7	19.88	28.84	71.44	250.45
2013	8	17.98	38.67	71.64	195.38
2013	9	13.80	40.36	80.66	121.77
2013	10	10.98	100.52	85.07	68.00
2013	11	4.89	47.72	88.28	32.38
2013	12	3.92	27.01	88.71	21.52
2014	1	3.96	29.42	88.77	29.70
2014	2	5.05	24.75	81.42	56.88
2014	3	6.92	6.12	76.32	127.35
2014	4	11.60	66.08	76.72	161.61
2014	5	12.76	54.33	77.73	195.17
2014	6	16.27	56.15	72.43	244.68
2014	7	19.68	119.66	75.23	224.28
2014	8	16.28	72.82	77.76	173.25
2014	9	15.13	30.20	83.62	113.04
2014	10	12.07	50.72	88.15	67.10
2014	11	6.81	33.80	90.66	28.12
2014	12	3.50	39.05	86.16	16.67

**Table A. 2-4:** Intra-class correlation (ICC) analysis correlation coefficients of biomasses in the 25 sub-plots (Fig. 2-1a), within each of the three ambient CO<sub>2</sub> (aCO<sub>2</sub>) plots and three elevated CO<sub>2</sub> (eCO<sub>2</sub>) plots, at harvest 1 and harvest 2 for grasses, forbs and total aboveground biomass (TAB) across the years 1998 to 2012. Upper and lower 95 % confidence interval (CI<sub>up</sub> and CI<sub>low</sub>) in brackets. ICC correlation coefficients higher than 0.8 indicates very good consistency (Shrout & Fleiss, 1979; McGraw & Wong, 1996).

	Grasses (ICC; CI <sub>up</sub> ; CI <sub>low</sub> )	Forbs (ICC; CI <sub>up</sub> ; CI <sub>low</sub> )	TAB (ICC; CI <sub>up</sub> ; CI <sub>low</sub> )
<i>Harvest 1</i>			
aCO <sub>2</sub> -1	0.968 (0.940; 0.987)	0.937 (0.880; 0.975)	0.964 (0.932; 0.986)
aCO <sub>2</sub> -2	0.972 (0.946; 0.989)	0.949 (0.902; 0.980)	0.966 (0.936; 0.987)
aCO <sub>2</sub> -3	0.987 (0.975; 0.995)	0.954 (0.912; 0.982)	0.985 (0.971; 0.994)
eCO <sub>2</sub> -1	0.980 (0.961; 0.992)	0.977 (0.956; 0.991)	0.968 (0.938; 0.987)
eCO <sub>2</sub> -2	0.979 (0.960; 0.992)	0.981 (0.964; 0.993)	0.971 (0.946; 0.989)

eCO <sub>2</sub> -3	0.977 (0.956; 0.991)	0.960 (0.923; 0.984)	0.965 (0.933; 0.986)
<i>Harvest 2</i>			
aCO <sub>2</sub> -1	0.953 (0.911; 0.981)	0.926 (0.860; 0.971)	0.947 (0.898; 0.979)
aCO <sub>2</sub> -2	0.953 (0.910; 0.981)	0.949 (0.904; 0.980)	0.944 (0.892; 0.978)
aCO <sub>2</sub> -3	0.974 (0.950; 0.989)	0.956 (0.915; 0.982)	0.967 (0.938; 0.987)
eCO <sub>2</sub> -1	0.981 (0.964; 0.992)	0.970 (0.942; 0.988)	0.962 (0.928; 0.985)
eCO <sub>2</sub> -2	0.946 (0.879; 0.978)	0.964 (0.931; 0.986)	0.943 (0.892; 0.977)
eCO <sub>2</sub> -3	0.969 (0.941; 0.988)	0.946 (0.897; 0.978)	0.949 (0.903; 0.980)

**Table A. 2-5:** Mixed model analysis of effects on biomass. Significant p values (significant at  $p < 0.05$  and by tendency when  $0.05 < p < 0.1$ ), and the F-values in brackets, for effects of the factors: CO<sub>2</sub> treatment (Treatment), time and block (Time, fixed terms), and soil moisture (as random term), on the biomass (dependent variable: total aboveground biomass: TAB, grasses and forbs). The effect was assessed for each harvest (1 and 2) by a mixed model analysis (restricted maximum likelihood) with first-order autoregressive process, no interactions of the main factors were significant. The model was tested both for the full time series and for the two half time sections indicated, as determined by break point analysis. See Figure 2-2.

<b>Full time series 1998 to 2013</b>	Treatment	Time	Soil moisture	Block
<i>Harvest 1</i>				
TAB	0.045 (4.54)	<0.001 (14.92)	0.019 (5.80)	0.031 (3.89)
Grasses	0.006 (9.44)	<0.001 (17.06)	ns.	<0.001 (17.50)
Forbs	ns.	<0.001 (14.66)	ns.	ns.
<i>Harvest 2</i>				
TAB	0.025 (6.31)	<0.001 (3.80)	ns.	ns.
Grasses	0.062 (5.09)	<0.001 (6.74)	ns.	ns.
Forbs	ns.	<0.001 (4.00)	ns.	ns.
<b>First section</b>	Treatment	Time	Soil moisture	Block
<i>Harvest 1</i>				
TAB	ns.	<0.001 (7.69)	ns.	0.032 (4.53)
Grasses	0.017 (7.99)	0.001 (14.64)	ns.	<0.001 (5.76)
Forbs	ns.	<0.001 (18.11)	ns.	ns.

*Harvest 2*

TAB	ns.	0.003 (4.25)	0.012 (7.00)	ns.
Grasses	0.001 (27.34)	<0.001 (7.47)	ns.	0.016 (6.25)
Forbs	0.047 (7.88)	0.003 (4.53)	ns.	ns.

Last section	Treatment	Time	Soil moisture	Block
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*Harvest 1*

TAB	0.041 (6.23)	<0.001 (32.68)	ns.	ns.
Grasses	ns.	<0.001 (16.22)	ns.	0.092 (3.37)
Forbs	ns.	<0.001 (11.48)	ns.	ns.

*Harvest 2*

TAB	0.069 (5.92)	ns.	ns.	ns.
Grasses	ns.	0.001 (4.99)	ns.	ns.
Forbs	ns.	ns.	ns.	ns.

**Table A. 2-6:** Plant biomass and soil moisture. Regression analysis of plant aboveground biomasses (total aboveground biomass TAB, grasses and forbs, in g·m<sup>-2</sup>) from both harvests with mean soil moisture during the respective growing periods for elevated CO<sub>2</sub> (eCO<sub>2</sub>) and ambient CO<sub>2</sub> (aCO<sub>2</sub>) treated plots in the years 1998 to 2013 (biomass data were not corrected for pre-treatment differences). Regression slopes and Y-axis intercept for the eCO<sub>2</sub> and aCO<sub>2</sub> treatments with upper and lower 95 % confidence intervals (CI), R<sup>2</sup> and P<sub>reg</sub> for the regression are given. Test results comparing slopes for eCO<sub>2</sub> and aCO<sub>2</sub> are significant at P<sub>com</sub> < 0.05 (different slopes).

		TAB	Grasses	Forbs
aCO <sub>2</sub>	P <sub>com</sub>	ns.	ns.	0.001
	N	96	96	96
	Slope	11.39	11.45	4.12
	CI <sub>up</sub>	13.08	13.3	5.03
	CI <sub>low</sub>	9.93	9.86	3.37
	R <sup>2</sup>	0.54	0.46	0.02
	P <sub>reg</sub>	< 0.001	< 0.001	ns.
	Intercept	-122	-227.2	-59.73
	CI <sub>up</sub>	-58.1	-157.4	-25.47
eCO <sub>2</sub>	CI <sub>low</sub>	-185.9	-297.1	-93.99
	N	96	96	96

Slope	12.1	12.98	6.79
CI <sub>up</sub>	14.05	15.32	8.31
CI <sub>low</sub>	10.41	11	5.54
R <sup>2</sup>	0.46	0.34	0.01
P <sub>reg</sub>	< 0.001	< 0.001	ns.
Intercept	-129.8	-258.6	-177.49
CI <sub>up</sub>	-55.4	-170.1	-120.2
CI <sub>low</sub>	-204.1	-347.2	-234.79

**Table A. 2-7:** Response of plant functional groups. Difference of grasses and forbs biomass effect size and time, for the entire time series (a) and the two time sections divided at year 2006 (b). Significant effects of type (functional group), time and the interaction of type and time ( $T \times T$ ) are indicated by p value < 0.05, and the F-values in brackets, obtained from repeated measures ANOVA. 'ns.' indicated non-significant effect at  $p > 0.1$ . The biomass effect size is for whole plots. Significant p values are indicated in Figure 3 by 'Compare effect size grasses vs. forbs'.

(a)

1998 – 2014	Type	Time	$T \times T$
<i>Harvest 1</i>	ns.	ns.	ns.
<i>Harvest 1</i>	ns.	ns.	ns.

(b)

Time sections	First section			Last section		
	Type	Time	$T \times T$	Type	Time	$T \times T$
<i>Harvest 1</i>	0.006 (20.27)	ns.	ns.	ns.	ns.	ns.
<i>Harvest 2</i>	0.022 (13.06)	ns.	ns.	ns.	ns.	ns.

**Table A. 2-8:** Regression analysis of biomasses of forbs and grasses, from both harvests for elevated CO<sub>2</sub> (eCO<sub>2</sub>) or ambient CO<sub>2</sub> (aCO<sub>2</sub>) treated plots in years 1998 to 2014. Regression slopes and Y-axis intercept for eCO<sub>2</sub> and aCO<sub>2</sub> treatment with upper and lower 95 % confidence intervals (CI), R<sup>2</sup> and P<sub>reg</sub> for the regression are given. Test results comparing slopes for eCO<sub>2</sub> and aCO<sub>2</sub> are significant at  $P_{com} < 0.05$  (different slopes). See Figure 2-4.

Grasses & Forbs		
aCO <sub>2</sub>	P <sub>com</sub>	0.007
	N	102
	Slope	-0.3895

	CI <sub>up</sub>	-0.3211
	CI <sub>low</sub>	-0.4725
	R <sup>2</sup>	0.04
	P <sub>reg</sub>	0.045
	Intercept	193
	CI <sub>up</sub>	212.5
	CI <sub>low</sub>	173.5
eCO <sub>2</sub>	N	102
	Slope	-0.5777
	CI <sub>up</sub>	-0.4817
	CI <sub>low</sub>	-0.6927
	R <sup>2</sup>	0.152
	P <sub>reg</sub>	< 0.000
	Intercept	247.9
	CI <sub>up</sub>	278.1
	CI <sub>low</sub>	217.7

**Table A. 2-9:** Effects on plant response. Significant p values (significant at  $p < 0.05$  and by tendency when  $0.05 < p < 0.1$ ), and the F-values in brackets, for effects of the main factors (fixed terms) time and soil moisture difference (eCO<sub>2</sub> - aCO<sub>2</sub>, from the relevant growing period) on the biomass effect size (dependent variable: TAB, grasses and forbs). Assessment made for each harvest by a mixed model analysis (restricted maximum likelihood) with first-order autoregressive process, using time as repeated measure term (random term). The model was tested both for the full time series and for the two half time sections as determined by break point analysis. See Figure 2-5.

<b>Full time series 1998 to 2013</b>	Time	Soil moisture	Time × Soil moisture
<i>Harvest 1</i>			
TAB	ns.	0.017 (6.35)	ns.
Grasses	ns.	0.072 (3.47)	ns.
Forbs	0.038 (2.90)	ns.	0.095 (2.20)
<i>Harvest 2</i>			
TAB	ns.	0.007 (8.23)	ns.
Grasses	ns.	ns.	ns.
Forbs	ns.	0.020 (6.76)	ns.
<b>First section</b>	Time	Soil moisture	Time × Soil moisture

*Harvest 1*

TAB	ns.	0.052 (5.50)	0.017 (9.20)
Grasses	0.004 (18.17)	0.053 (5.22)	0.002 (20.85)
Forbs	ns.	ns.	ns.

*Harvest 2*

TAB	ns.	0.009 (10.10)	ns.
Grasses	ns.	ns.	ns.
Forbs	ns.	0.036 (5.64)	ns.

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<b>Last section</b>	<b>Time</b>	<b>Soil moisture</b>	<b>Time × Soil moisture</b>
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*Harvest 1*

TAB	ns.	0.094 (3.34)	ns.
Grasses	ns.	ns.	ns.
Forbs	ns.	ns.	ns.

*Harvest 2*

TAB	ns.	ns.	ns.
Grasses	ns.	ns.	ns.
Forbs	ns.	0.040 (6.02)	ns.

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### **3 Effects of long-term CO<sub>2</sub> enrichment on forage quality of extensively managed temperate grassland**

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## 4 Plant functional types differ in their long-term nutrient response to eCO<sub>2</sub> in an extensive grassland

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## **Author contribution**

CM, CIK and LCA managed and scheduled the sample analysis. RJL and CJW conducted ICP-MS analysis at AFBI. RS and LCA carried out the statistical analysis of the data and created the graphics; CIK, GM, CM conducted the year-long field work and coordinated the eCO<sub>2</sub> experiment. All authors (RS, LCA, KAJ, GM, CIK, NY, JL, RJL, CJW, ME, TS and CM) took part in the planning of this synthesis paper, contributed to the interpretation and discussion of the results and writing of the final manuscript.

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## **Abstract**

Increasing atmospheric CO<sub>2</sub> enhances plant biomass production and may thereby change nutrient concentrations in plant tissues. The objective in this study was to reveal the differences in the nutrient concentration of grassland biomass through 16 years of CO<sub>2</sub> fumigation.

Grassland biomass grown at the extensively managed Giessen FACE experiment, fumigated with ambient and elevated CO<sub>2</sub> (aCO<sub>2</sub>; eCO<sub>2</sub>; + 20 %) was harvested twice annually. Concentrations of C, N, P, K, Ca, Mg, Mn, Fe, Cu and Zn were determined separately for grasses, forbs and legumes in 1998-2013.

Under eCO<sub>2</sub> the concentration of N was reduced in grasses, Ca was reduced in grasses and forbs, P was reduced in grasses but increased in legumes, Mg concentration was reduced in grasses, forbs and legumes and K was reduced in grasses but increased in forbs.

The nutrient yield (in g nutrient yield of an element per m<sup>-2</sup>) of most elements indicated negative yield responses at a zero biomass response to eCO<sub>2</sub> for grasses. K and Zn nutrient yields responded positively to eCO<sub>2</sub> in forbs and Mn and Fe responded positively in forbs and legumes.

The results suggest that under eCO<sub>2</sub> the nutrient concentrations were not diluted by the CO<sub>2</sub> fertilization effect. Rather, altered plant nutrient acquisitions via changed physiological mechanisms prevail for increased C assimilation under eCO<sub>2</sub>. Furthermore, other factors like water or nutrient availability affected plant nutrient concentrations under eCO<sub>2</sub>.

**Keywords:** FACE; macro nutrients; micro nutrients; elevated CO<sub>2</sub>; climate change; GiFACE

## Highlights

- Grasses revealed most reduced element concentrations due to eCO<sub>2</sub>.
- In grasses, most elements indicated a negative nutrient yield under eCO<sub>2</sub>.
- Site and climatic conditions affected CO<sub>2</sub> effect on nutrients concentrations.

## 4.1 Introduction

Grassland ecosystems play a critical role in providing food and forage to a large number of the world's human population and their livestock (White *et al.*, 2000). Elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) is an important influencing factor on grassland vegetation often driving increased biomass production under eCO<sub>2</sub> (Andresen *et al.*, 2018; Cantarel *et al.*, 2013; Feng *et al.*, 2015; Newton *et al.*, 2014).

Elevated CO<sub>2</sub> can induce direct plant physiological reactions such as reduced stomatal conductance and elevated photosynthetic rates (Haworth *et al.*, 2016; Tausz-Posch *et al.*, 2014). These physiological reactions jointly affect the plant nutrient uptake by downregulating the passive inflow of nutrients to the root with the transpiration stream (Houshmandfar *et al.*, 2018) and possibly by driving a more active uptake of certain elements required in photosynthesis. Shifts of photosynthetic activity under eCO<sub>2</sub> can lead to limited electron transport, which can decrease plant nutrient requirements and uptake. However, micro nutrients like Fe and Mn, which are involved in electron transport, may increase in concentration under eCO<sub>2</sub> (Natali *et al.*, 2009). On the other hand, plant uptake of nutrients like Ca and Mg is controlled by mass flow, which is directly driven by evapo-transpiration (Nord & Lynch, 2009). Hence, decreased transpiration rates by eCO<sub>2</sub> and therefore decreased mass flow, may lead to decreased uptake of nutrients such as Ca and Mg in aboveground plant biomass (Houshmandfar *et al.*, 2018; Tausz-Posch *et al.*, 2014).

Ainsworth *et al.* (2003) revealed increased C sink strength due to elevated photosynthesis in grassland plants grown under eCO<sub>2</sub>. Furthermore, plants grown under eCO<sub>2</sub> increased carbohydrate content, which in some theories leads to dilution of nutrient concentration in plant tissues from increased C assimilation (Ainsworth & Long, 2021; Loladze, 2002). However, in a meta-analysis, Feng *et al.* (2015) showed that the N nutrient yield was generally reduced under eCO<sub>2</sub> in grassland, cropland and forest ecosystems, likely due to reduced N acquisition under eCO<sub>2</sub> and not due to C dilution. Complementarily, Houshmandfar *et al.* (2018) found that nutrient uptake of several elements (Ca, K, N, S, Mg and Mn) in wheat correlated with plant transpiration.

Besides the direct effects on plant productivity, biodiversity and physiological responses, eCO<sub>2</sub> can also indirectly and directly affect soil nutrient cycles (Hawkesford *et al.*, 2014). Availability of nutrients is also controlled by soil properties, such as pH, which affects nutrient ion diffusion and adsorption (Marschner, 2002). A slight decrease in soil pH can be expected under eCO<sub>2</sub> conditions because of increased root exudation and respiration, changed cation-anion exchange balance or organic anion release (Hinsinger *et al.*, 2003; Natali *et al.*, 2009). Additionally, increasing inputs of carbonic acid from plant roots and microbial respiration, due to eCO<sub>2</sub>, may increase soil acidity (Natali *et al.*, 2009). For slightly acidic soils, such a decrease in the soil pH could directly affect the availability of soil nutrients. The effect would be a decrease in the availability of macro nutrients such as N, P, K, Ca, S and Mg and an increase in the availability of micro nutrients such as Fe, Mn, Zn and Cu (Marschner, 2002), which might in turn affect the nutritional status of plants.

One of the most important plant nutrients is N and a controlling factor for soil N availability is the process of mineralization of soil organic matter. Net N mineralization varied strongly in response to eCO<sub>2</sub> in grasslands (Hovenden *et al.*, 2017; Reich *et al.*, 2018), while gross N mineralization rates were often not responding to eCO<sub>2</sub> (Müller *et al.*, 2009; Rütting & Andresen, 2015; Rütting & Hovenden, 2020). Furthermore, the presence of legumes in ecosystems adds to the complexity as these are often associated with N<sub>2</sub> fixing microorganisms (Gamper *et al.*, 2004; Lüscher & Nösberger, 1997). Legumes are found to have even greater benefit to eCO<sub>2</sub> as the N<sub>2</sub> fixation increases because of increasing nodule size, number of nodules per plant and a higher nitrogenase activity due to eCO<sub>2</sub> (Rogers *et al.*, 2009). Hereby, the addition of N to the ecosystem from symbiotic N fixation can, through years, sustain an otherwise decreasing N availability for all plant species (Liang *et al.*, 2016; Rütting, 2017). In ecosystems without N<sub>2</sub> fixing organisms a situation with a progressive nitrogen limitation (PNL) has been predicted but rarely observed. For PNL, a decline in plant

available N can restrain the biomass from responding positively to eCO<sub>2</sub> (Newton *et al.*, 2010). Hence, changes in soil N availability for plant uptake are difficult to predict under eCO<sub>2</sub>.

Across many short-term FACE experiments, decreases in plant nutrient concentrations have been observed, usually with small but significant effects (Myers *et al.*, 2014). These changes in the nutrient concentrations suggest an adjustable plant nutrient stoichiometry, however, an increased C to N ratio under eCO<sub>2</sub> is often observed (Dijkstra *et al.*, 2012; Feng *et al.*, 2015; Sistla & Schimel, 2012). Also, in a meta-analysis of 1418 studies, Yuan and Chen (2015) found that N to P ratios decreased under eCO<sub>2</sub> in general. However, observations suggest high variability in responses. Likewise, in USA, Australia and Japan, decreased concentrations of Zn and Fe in wheat, rice, beans and soybeans occurred due to eCO<sub>2</sub> treatment (Myers *et al.*, 2014; Smith & Myers, 2018). These results provide an indication of a poorer nutritive value in the future food quality, as the plant nutrient status directly links to human and livestock nutrition (Augustine *et al.*, 2018; Dumont *et al.*, 2015; Myers *et al.*, 2014; Seibert *et al.*, 2021; Smith & Myers, 2018; Yuan & Chen, 2015).

Long-term observations of nutrient concentrations in eCO<sub>2</sub> experiments over more than 10 years continuous monitoring are rarely available. Investigations on the species rich, extensively managed, temperate grassland at the long-term Giessen Free-Air Carbon dioxide Enrichment experiment (GiFACE) in Germany (Jäger *et al.*, 2003; Müller *et al.*, 2019) revealed eCO<sub>2</sub> effects on aboveground biomass yield and its forage quality (Andresen *et al.*, 2018; Kammann *et al.*, 2005; Obermeier *et al.*, 2017; Seibert *et al.*, 2021; Yuan *et al.*, 2018). During the study period at the GiFACE experiment since 1998 (Jäger *et al.*, 2003), the biomass of grasses decreased, while forbs (including legumes) increased in proportion (Andresen *et al.*, 2018; Seibert *et al.*, 2021). Furthermore, the total biomass production increased (+15 %) under eCO<sub>2</sub> (Andresen *et al.*, 2018) and the forage quality declined, especially in forbs (Seibert *et al.*, 2021).

To reveal which of the macro- and micro nutrients were affected by eCO<sub>2</sub>, we addressed the following hypotheses:

1. We expected differences in N concentration and its CO<sub>2</sub> response among the functional groups because of the different CO<sub>2</sub> responses of plant N uptake in forbs and grasses and the N<sub>2</sub> fixation mechanism in symbionts with legumes.

2. We expected decreased leaf concentrations of Ca, K, N, S and Mg under eCO<sub>2</sub>, due to down regulated transpiration and therefore reduced passive inflow of nutrients, and to a slight soil acidification of the soil during eCO<sub>2</sub> making these nutrients less available.
3. We expected increased nutrient concentrations in response to eCO<sub>2</sub> for Fe and Mn due to stimulated active uptake to meet increased photosynthesis under eCO<sub>2</sub> and a slight acidification under eCO<sub>2</sub> increases the availability of these elements.

## 4.2 Materials and Methods

### 4.2.1 Field site

The GiFACE site is a 1.5 ha large research site in the German state Hessen, near Giessen (50°32'N 8°41'E) at 172 m.a.s.l. The soil is a Fluvic Gleysol in a floodplain. Since more than 100 years the area has been managed as permanent grassland (semi-natural). Until 1995 the site was fertilized with 50 to 80 kg N ha<sup>-1</sup> yr<sup>-1</sup>. Since the Justus- Liebig- University Giessen started renting the area in 1993, the commercial fertilizers 'Thomas-Kali' and 'Kalk-Ammonsalpeter' were applied once a year in April at the beginning of the growing season. Since 1996 a fertilization level of 40 kg N ha<sup>-1</sup> yr<sup>-1</sup> was applied, which is equivalent to the following nutrient amounts: nitrogen: 4 g N m<sup>-2</sup> yr<sup>-1</sup>, phosphorus: 6 g P m<sup>-2</sup> yr<sup>-1</sup>, potassium: 9 g K m<sup>-2</sup> yr<sup>-1</sup>, magnesium: 1.8 g Mg m<sup>-2</sup> yr<sup>-1</sup> and calcium: 21.3 g Ca m<sup>-2</sup> yr<sup>-1</sup> (Table A. 4-1, Andresen *et al.*, 2018). The mean content of the soil organic matter at a depth of 7.5 cm is 2952 (± 255) C g m<sup>-2</sup> for the aCO<sub>2</sub> plots and 2924 (± 146) C g m<sup>-2</sup> for the eCO<sub>2</sub> plots (Keidel *et al.*, 2018). The vegetation is an 'Arrhenatheretum elatioris - Filipendula ulmaria sub-community' (Grüters *et al.*, 2006) according to the Braun-Blanquet classification. At the start of the study, the dominating grasses in terms of biomass were: *Arrhenatherum elatius* (L) P.Beauv. ex J.Presl & C.Presl., *Holcus lanatus* L., *Trisetum flavescens* (L) P.Beauv., *Poa pratensis* L., *Anthoxanthum odoratum* L. and *Poa trivialis* L., the dominant forbs were *Galium album* Forssk., *Geranium pratense* L., *Plantago lanceolata* L., *Filipendula ulmaria* (L.) Maxim. and *Sanguisorba officinalis* L.. The legume species *Lathyrus pratensis* L., *Trifolium pratense* L. and *Trifolium repens* L. were present in small proportions (< 1 to 2 % by mass) when this study was initiated (Grüters *et al.*, 2006).

### 4.2.2 Elevated CO<sub>2</sub> treatments

Three circular plots (rings) were subjected to air with elevated CO<sub>2</sub> concentration (eCO<sub>2</sub>), while three rings were subjected to air with ambient CO<sub>2</sub> concentration, in a randomized

block design (3 blocks). The 6 rings had 8 meters inner diameter (Andresen *et al.*, 2018) and had an inner circular buffer-zone of 1 m width where no harvests or soil samplings were carried out. In 1998 the randomized block design was set up and since then CO<sub>2</sub> fumigation (+ 20 % above ambient conditions) is active all-year round, from 2 hours after astronomical sun rise until 2 hours before astronomical sun set (Andresen *et al.*, 2018; Jäger *et al.*, 2003).

#### 4.2.3 Local weather and climatic conditions

Volumetric soil water content was measured daily since spring 1997 by 4 permanent TDR-sensors (Imko, Germany, type P2G) in 0 to 15 cm depth in each ring. Soil temperature was recorded every 30 min. since March 1998 at 10 cm depth with 3 probes in each ring (Pt-100 sensors, Imko, Germany). The precipitation sum was measured at the site in 30 min. intervals; the mean annual precipitation was  $556 \pm 27$  mm (average  $\pm$  standard deviation (SD)); 18 year average from 1995 Dec to 2013 Nov) with the seasonal averages during winter (December to February) of  $108 \pm 33$  mm; spring (March to May)  $130 \pm 48$  mm; summer (June to August)  $172 \pm 49$  mm; and autumn (September to November):  $142 \pm 55$  mm. The precipitation data were used to calculate the 3 months standardized precipitation index (SPI), which is a drought index based only on precipitation (WMO, 2012). Two meter above ground temperature was recorded continuously since 1995 at two stations within the field site. The mean annual temperature was  $9.4 \pm 6.5$  °C with the seasonal averages: winter  $1.44 \pm 1.67$  °C; spring  $9.24 \pm 1.02$  °C; summer  $17.37 \pm 0.78$  °C; and autumn  $9.50 \pm 1.01$  °C. The aboveground temperature data were used to calculate the 3 months standardized temperature index (STI), which is an index representing the probability of temperature value occurrences when compared with temperature average of a longer period (Fasel, 2015). For details and further data presentations see Andresen *et al.*, (2018) and Obermeier *et al.*, (2017), Seibert *et al.*, (2021) and Yuan *et al.*, (2018).

#### 4.2.4 Bi-annual harvest

Since 1993, harvests were conducted within 25 sub-plots with an area of 0.36 m<sup>2</sup> per sub-plot per ring (Andresen *et al.*, 2018). All vegetation was cut with garden scissors at 3 to 5 cm above soil surface on two occasions each year: harvest 1 at the end of May and harvest 2 at the beginning of September. The fresh biomass was stored under cool conditions and sorted by hand into three functional groups: grasses, forbs and legumes and was then oven dried at 105 °C. The dried material was ground in a mill (SM 300, Retsch, Germany). From each sub-

plot a proportional sample (relative to the biomass yield in each sub-plot) was mixed to one composite sample from the ring for further chemical analysis.

#### 4.2.5 Plant leaf nutrient analysis

The element concentration of dried ground plant material was analyzed by standard methods. At the department of Plant Ecology, University of Giessen, the samples for the years 1993 to 2013 were analyzed for total N and C with a Vario Max CNS (Elementar). Other elements were analyzed in two large batches assembled through the years. Plant samples from the years 1993 to 2006 were analyzed at the Agri-Food and Biosciences Institute, Belfast, where they were digested by microwave using nitric acid (0.25 g plant material was added 5 ml concentrated nitric acid) in 45 ml deionized water to give a final volume of 50 ml. The elements were then determined using an ICP-MS (Inductively Coupled Plasma Mass Spectrometer; Varian Liberty Series II) for analysis of P, K, Ca, Mg, Mn, Fe, Cu, Zn and S. Plant samples from the years 2007 to 2013 were analyzed at Geisenheim University laboratories in the department of soil science and plant nutrition, where they were digested at 400 °C by a Kjeldahl mixture consisting of: hydrochloric acid, peroxide, lithium sulphate, selen (0.6 g plant material + 10 ml) for analysis of P, K, Ca, Mg, Mn, Fe, Cu and Zn with an ICP-OES (Arcos), and of N at a FIA (Foss). A small-scale comparison between labs was made with samples that were initially analyzed in Belfast and then re-analyzed in Geisenheim (7 years later) and confirmed that there was no baseline shift for all elements except for Fe and Cu. S was not analyzed in Geisenheim and thus determined only until 2006. However, since all samples from any harvest time were always consistently analyzed within the same lab, this did not have consequences for determining the CO<sub>2</sub> treatment effect. Legume biomasses were only analyzed when sufficient plant material was available.

#### 4.2.6 Data analysis

Plant leaf concentrations of the elements N, P, K, Ca, Mg, Fe, Zn, Mn, and Cu in each of the plant functional types grasses, forbs, and legumes, were used for data analysis separately for harvest 1 (H1) and harvest 2 (H2).

The plant nutrient yield of a nutrient element ( $N_i$ ) was calculated per functional group and harvest according to the following equation (Tausz-Posch *et al.*, 2014 in Hawkesford).

$$\text{Nutrient yield of } N_i [\text{g } N_i \text{ m}^{-2}] = N_i \text{ concentration } [\text{g } N_i \text{ g}^{-1}] \times \text{biomass } [\text{g m}^{-2}] \quad (1)$$

The annual nutrient yield for an element was the summed yield of  $N_i$  from the two harvests (H1 and H2) and all functional groups.

The eCO<sub>2</sub> treatment effect on plant growth and nutrient yield was quantified by determining the effect size respectively. Effect size expresses the relative treatment effect in percentage (Leuzinger *et al.*, 2011) and was calculated as follows for the biomass X (each functional group) or the nutrient yield of element  $N_i$  ( $N_i$  represents the average of the three treatment rings for each functional group):

$$\text{Effect size [\%]} = ((\text{eCO}_2(\text{X or } N_i) - \text{aCO}_2(\text{X or } N_i)) / \text{aCO}_2(\text{X or } N_i)) \times 100 \quad (2)$$

To compare the functional groups of all six FACE rings, separated in eCO<sub>2</sub> and aCO<sub>2</sub>, as well as in H1 and H2, a principal component analysis (PCA) biplot of nutrient concentrations was used. Cross-products matrix contains correlation coefficients among nutrient concentrations and 7 axes were interpreted (McCune & Grace, 2002). Before analysis, the data were subjected to square root transformation (van der Maarel, 1979). The PCA was performed using PC-Ord 6 (McCune & Mefford, 2011). To analyze the coordinated effect of eCO<sub>2</sub> on plant nutrients concentrations a one-way permutational multivariate analysis of variance (PerMANOVA) with Sorensen distance measure was done for each functional group and harvest (Anderson & Walsh, 2013; McCune & Grace, 2002).

To test the significance of the differences of all nutrient concentrations and yields between eCO<sub>2</sub> and aCO<sub>2</sub>, a linear mixed model analysis with repeated measures was used for the dataset 1998 – 2013. As covariance type, first-order autoregressive process [AR(1)] was used. All mixed model analyses were carried out in SPSS version 23.0 (IBM Corp., Armonk, NY, USA).

The model analysis was started with the full-factorial model with the factors CO<sub>2</sub>, block, year and the co-variables soil moisture, STI and SPI, including the interactions  $\text{CO}_2 \times \text{block}$ ,  $\text{CO}_2 \times \text{year}$ ,  $\text{CO}_2 \times \text{soil moisture}$ ,  $\text{CO}_2 \times \text{STI}$ ,  $\text{CO}_2 \times \text{SPI}$ ,  $\text{block} \times \text{soil moisture}$ ,  $\text{block} \times \text{STI}$ ,  $\text{block} \times \text{SPI}$ ,  $\text{year} \times \text{soil moisture}$ ,  $\text{year} \times \text{STI}$  and  $\text{year} \times \text{SPI}$ . Soil moisture, STI and SPI data were aggregated to averages for the respective three months preceding the harvest (March – May for H1 and June to August for H2). The model was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion (Andresen *et al.*, 2018; Bandoly *et al.*, 2016; Safari *et al.*, 2016). Effects with  $p$  values  $\leq 0.05$  were considered significant,  $\leq 0.01$  very significant and  $\leq 0.001$  highly significant.

#### 4.2.6.1 Nutrient uptake response

Linear regression was made for the CO<sub>2</sub> responses measured as effect size (Eq. 2) of nutrient yield ( $Y_i$ ) versus the effect size of biomass ( $x$ ):

$$Y_i = 'a' \times x_i + Y_0 \quad (3)$$

The intercept with the Y-axis ( $Y_0$ ) at  $x_i = 0$  is the percent reduction or increase in nutrient yield at zero biomass response. If this intercept is a negative number for the theoretical point of no response in biomass, it means the nutrient yield under eCO<sub>2</sub> is smaller than under aCO<sub>2</sub>. We judged significant negative (or positive)  $Y_0$  by its 95 % confidence interval ( $CI_{up}$  and  $CI_{low}$ ) including '0' or not. We compare the  $Y_0$  across element and functional types as expression of loss in nutrient yield under eCO<sub>2</sub>. SMATR (a freeware program for Standardised Major Axis Tests and Routines; Falster *et al.*, 2006; Warton *et al.*, 2006) was used for bivariate linear regression, using standardised major axis regression and testing for 'common slopes' ( $p(\text{test}) < 0.05$ ) of the regression lines being compared. For each element, we compared in Eq. 3 the plant types grasses, forbs, legumes pairwise. A difference of slopes indicates that one functional plant type has better nutrient uptake under eCO<sub>2</sub> than the other type.

### 4.3 Results

#### 4.3.1 Concentration of nutrients aboveground in plant functional types

Elevated CO<sub>2</sub> decreased the nitrogen (N) concentration in grasses only for H2 and not in forbs and legumes ( $p = 0.001$ ; Fig. 4-1a & b; Tab. 4-1; Tab. A. 4-2; linear mixed model).

Phosphorus (P) had both negative and positive eCO<sub>2</sub> effects seen as declined concentrations in grasses (H1,  $p = 0.011$ ; H2,  $p = 0.024$ ; Fig. 4-1d; Tab. 4-1; Tab. A. 4-2) and increased concentrations in legumes (H2,  $p = 0.045$ ; Fig. 4-1f; Tab. 4-1; Tab. A. 4-4). There was also a significant CO<sub>2</sub>  $\times$  soil moisture interaction for P in grasses H1 (Tab. A. 4-2; Tab. A. 4-3).

**Table 4-1:** Significant differences in nutrient concentrations between eCO<sub>2</sub> and aCO<sub>2</sub>, separated for the functional types of grasses, forbs and legumes and by harvest 1 (H1) and harvest 2 (H2). The significance levels for CO<sub>2</sub> effect were reported as significant  $p \leq 0.05$  [\*], much significant  $p \leq 0.01$  [\*\*] and highly significant  $p \leq 0.001$  [\*\*\*]. Downward arrow [↓] means negative CO<sub>2</sub> effect; upward arrow [↑] means positive CO<sub>2</sub> effect, analyzed by a linear mixed model. Grey colors indicate decreasing trends, black color indicate increasing trends.

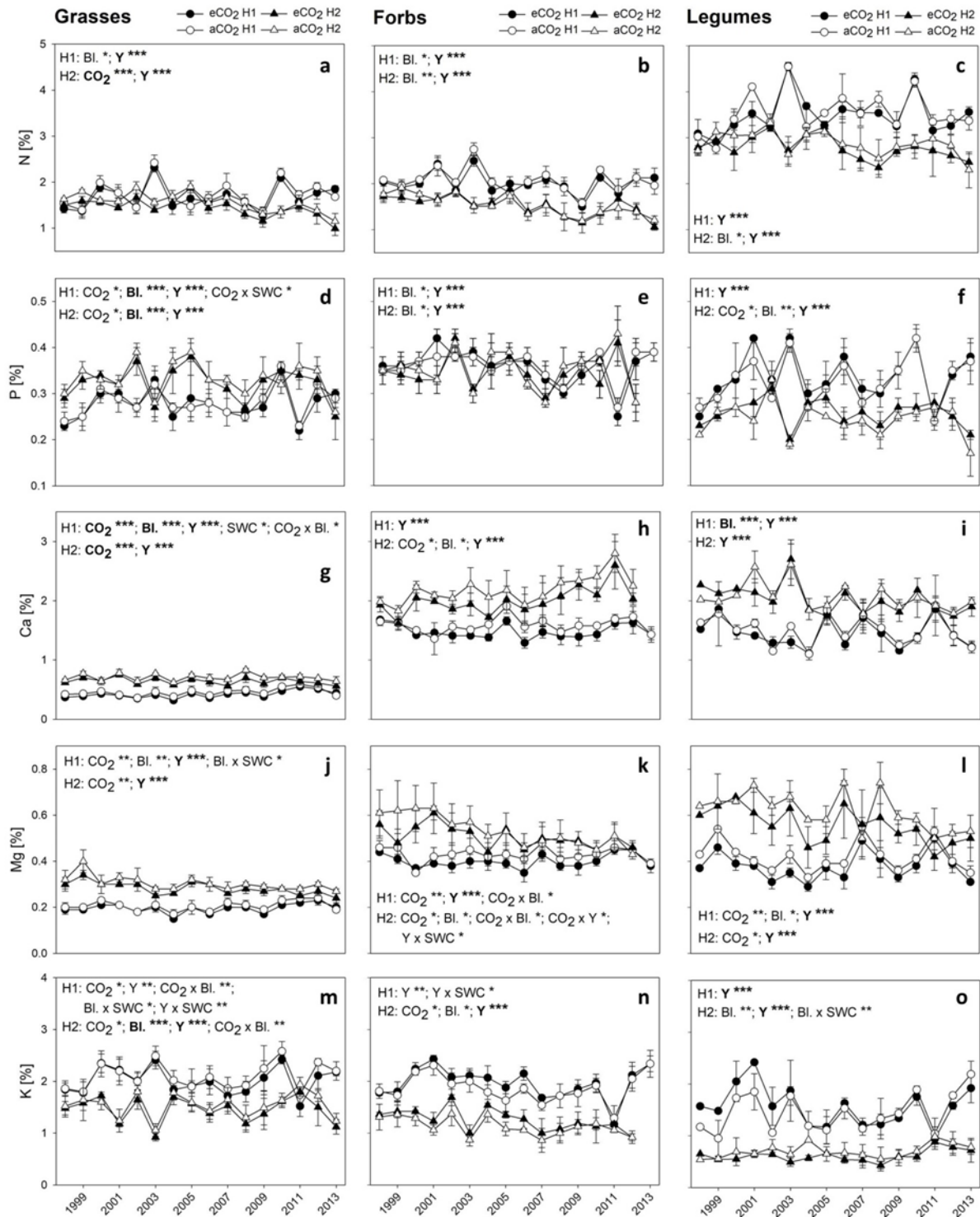
Nutrient	Grasses		Forbs		Legumes	
	H1	H2	H1	H2	H1	H2
Nitrogen	-	*** ↓	-	-	-	-
Phosphorus	* ↓	* ↓	-	-	-	* ↑
Calcium	*** ↓	*** ↓	-	* ↓	-	-
Magnesium	** ↓	** ↓	** ↓	* ↓	** ↓	* ↓
Potassium	* ↓	* ↓	-	* ↑	-	-
Zn, Fe	-	-	-	-	-	-
Mn, Cu	-	-	-	-	-	-

CO<sub>2</sub> had a negative effect for calcium (Ca) in grasses, with decreased concentrations under eCO<sub>2</sub> for both harvests, (both  $p \leq 0.001$ ; Tab. 4-1; Fig. 4-1g; Tab. A. 4-2). Forbs also showed lower Ca concentrations under eCO<sub>2</sub> ( $p = 0.030$ ; Tab. 4-1; Fig. 4-1h; Tab. A. 4-3).

The magnesium (Mg) concentrations decreased significantly in all functional groups and both harvests under eCO<sub>2</sub> (Tab. 4-1; Tab. A. 4-2, A. 4-3 and A. 4-4). Mg concentrations were smaller under eCO<sub>2</sub> for both harvests in grasses (Fig. 4-1j), except in 2013 for H1 (H1,  $p = 0.008$ ; H2,  $p = 0.004$ ; Fig. 4-1j; Tab. 4-1; Tab. A. 4-2). Forbs had a negative eCO<sub>2</sub> effect for both harvests (H1,  $p = 0.005$ ; H2,  $p = 0.043$ ; Tab. 4-1; Tab. A. 4-3), except in 2000 for H1 and in 2005, 2008, 2010 and 2013 for H2 (Fig. 4-1k). In legumes, a smaller Mg concentration under eCO<sub>2</sub> occurred for the whole time series for H1 ( $p = 0.009$ ; Fig 4-1l; Tab. 4-1; Tab. A. 4-4) and with only one exception in 2000 for H2 ( $p = 0.030$ ; Fig 4-1l; Tab. 4-1; Tab. A. 4-4).

Potassium (K) concentration in grasses showed negative eCO<sub>2</sub> effects for both harvests, with decreasing K concentrations, except in 2000 for H2 (H1,  $p = 0.041$ ; H2,  $p = 0.024$ ; Fig. 4-1m; Tab. 4-1; Tab. A. 4-2). Forbs had a positive CO<sub>2</sub> effect for H2, with higher K concentrations under eCO<sub>2</sub>, except in 2010 ( $p = 0.036$ ; Fig. 4-1n; Tab. 4-1; Tab. A. 4-3).

Concentrations of Zn, Fe, Cu and Mn indicated no significant CO<sub>2</sub> effects in any of the functional groups (Tab. 4-1). Significant effects of site conditions ('block'), year and interactions in the linear mixed model are presented in detail in the supplementary tables.



**Figure 4-1:** Concentration of N (a; b; c), P (d; e; f), Ca (g; h; i), Mg (j; k; l) and K (m; n; o) in % of grasses, forbs and legumes, separated in harvest 1 (H1; circles) and harvest 2 (H2, triangles). Error bars indicate standard deviation. The results of the mixed model analysis are indicated for treatment ( $\text{CO}_2$ ), ring-pair (Bl.) time (Y) and soil moisture (SWC). The significance levels were reported as significant  $p \leq 0.05$  [\*], much significant  $p \leq 0.01$  [\*\*] and highly significant  $p \leq 0.001$  [\*\*\*]. Highly significant results in bold.

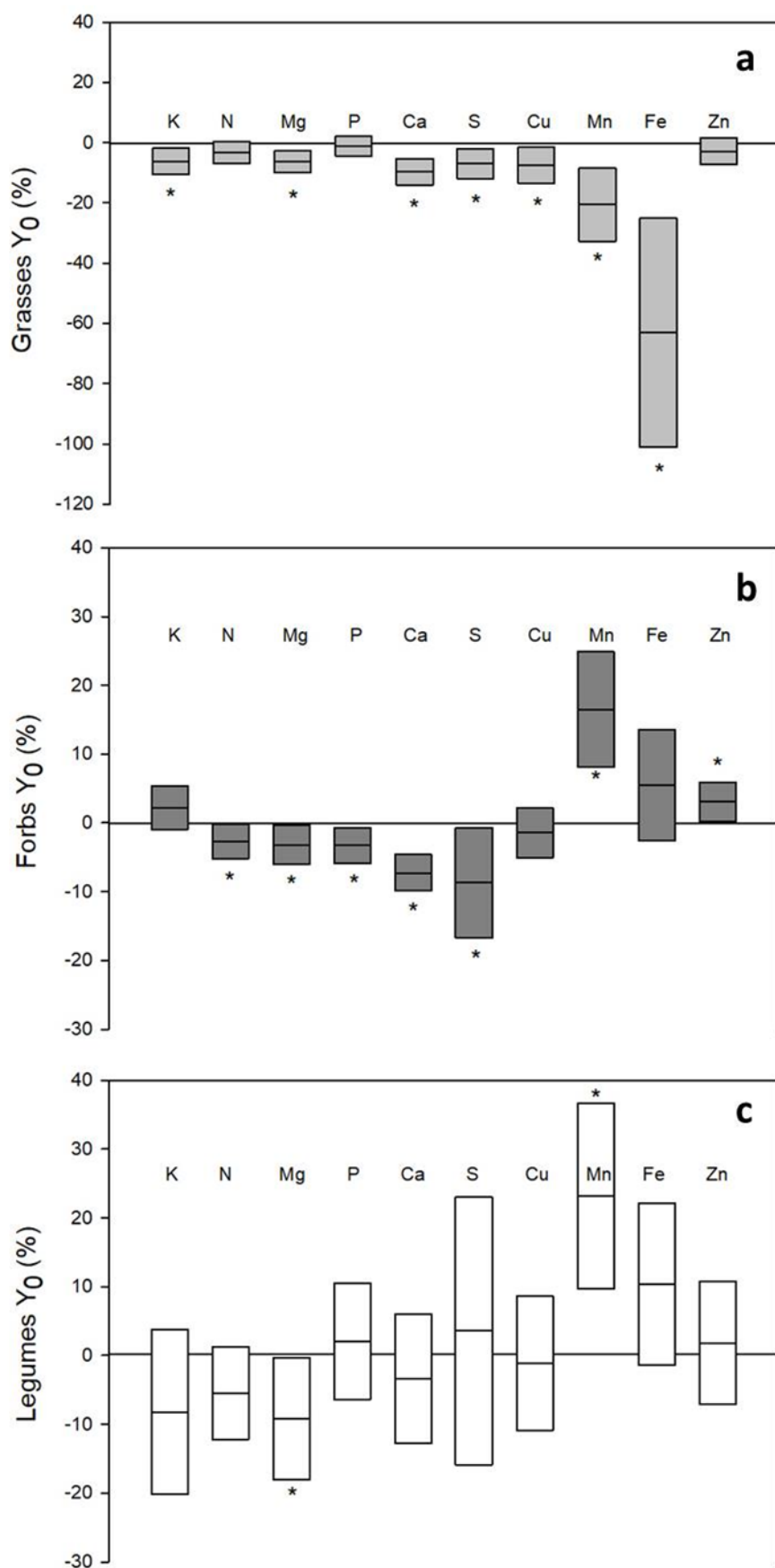
A joint analysis of the three functional groups (grasses, forbs and legumes) revealed a clear separation among all nutrient concentrations under ambient and elevated  $\text{CO}_2$  by the un-

rotated s-mode principal components analysis (PCA), which also revealed a clear separation between H1 (spring) and H2 (summer) (Fig. A. 4-1). However, a clear effect in the functional groups as response to CO<sub>2</sub> treatment was not found and this was confirmed by PerMANOVA analysis (Tab. A. 4-5).

#### 4.3.2 Plant nutrient yield responses

The response to eCO<sub>2</sub> measured as effect size (Eq. 3) of the nutrient yields varied as both positive and negative for all elements and functional groups across all years (Eq. 2, data not shown), and this inherently followed the temporal trends from the biomass response (Andresen *et al.*, 2018). The nutrient yield response relative to the biomass response correlated positively between all functional types (Tab. A. 4-6). For all elements these slopes were not different for grasses and forbs except for Fe, where grasses had the steepest slope. Furthermore, for all elements the slopes were not different for grasses and legumes, except for Fe and Mn, where again grasses had a steeper slope. Forbs and legumes had common slopes for all elements, except Zn, where the slope for legumes was steepest (Tab. A. 4-6).

A relative reduction of nutrient yield under eCO<sub>2</sub> compared to aCO<sub>2</sub> in zero biomass response was found for grasses, as a negative Y-axis intercept  $Y_0$  (Eq. 3) occurred for all elements, significantly for K, Ca, Mg, Fe, Mn, Cu and S (95 % confidence interval excluding the zero point; Fig. 4-2a; Tab. A. 4-6). Forbs, however, had mainly negative  $Y_0$  for N, P, Ca, Mg and S but had contrastingly significant positive  $Y_0$  for Mn and Zn (Fig. 4-2b). For legumes the  $Y_0$  was negative only for Mg and positive for Mn, (Fig. 4-2c). The largest reduction of Fe and Mn nutrient yields (−63 % and −20 %, respectively;  $Y_0$  in Tab. A. 4-6) was in grasses, while these had smaller yield reduction of other nutrients (between 1 and 10 %). Contrastingly, forbs and legumes showed the largest yield increase of Mn of 8 % and 10 % (Fig. 4-2b & c; Tab. A. 4-6). The mean N yield of grasses and forbs were ca. 3 % reduced, while the mean N yield of legumes was 5 % reduced (*e.g.*  $Y_0 = -3.29, -2.66$  and  $-5.44$  respectively; Tab. A. 4-6).



**Figure 4-2:** Response to  $CO_2$  for the macro nutrient elements K, N, Mg, P, Ca, S and the micro nutrient elements Cu, Mn, Fe and Zn, seen as the effect size of plant nutrient yield (% reduction or increase from  $eCO_2$ ) at  $Y_0$  which is the extrapolated point of zero biomass response to  $eCO_2$ , jointly for harvest 1 and 2. The intercept  $Y_0$

was found using standardized major axis test considering variance at both axis (SMATR) on the response function of the nutrient (i) yield response (Y) relative to the biomass response (x) (Eq. 3) for each of the three functional groups grasses (a), forbs (b) and legumes (c) and is presented in Table S6.  $Y_0$  is the intercept with Y-axis at  $X = 0$ , and is the percentage reduction of (or increase in) nutrient yield for zero biomass response. The bar length represent the 95 % confidence interval of the intercept  $Y_0$  and the line in the middle of each bar represents the mean value. Asterisks (\*) emphasize significance where all both upper and lower confidence interval excludes (or includes) point zero.

## 4.4 Discussion

In this study, we found that eCO<sub>2</sub> reduced plant nutrient concentrations in grasses (N, P, Ca, Mg, K; Tab. 4-1; Fig. 4-1), while eCO<sub>2</sub> had reducing effects only for Ca and Mg concentrations in forbs and legumes, but had increasing effects on the concentrations of K in forbs and P in legumes (Tab. 4-1; Fig. 4-1). The nutrient yield response  $Y_0$  (Fig. 4-2 and Tab. A. 4-6) also pointed towards reduced nutrient yields in grasses, while forbs and legumes had increased as well as reduced nutrient responses. This partly confirmed our hypothesis 1 of differences among the functional groups regarding N, and the found increases in concentration and yields are contradictory to hypothesis 2 which suggested decreases. Compared to forbs and legumes, the concentration reductions in grasses were more intense for H2 than for H1 (Tab. 4-1). This reduction occurred especially in H2 for the end of the regrowth phases, when the availability of spring applied fertilizer nutrients was low and dry soil conditions caused reduced transpiration rates. The GiFACE management protocol requires only one fertilization during the first growing period and requires the removal of the biomass upon harvest therefore, plant nutrients were relatively more limited for H2 compared to H1. Accepting that the spring fertilization is part of the driver of the nutrient availability, which is equally available for all species, we also need to consider the physiological differences of the functional groups, as we suggest that this caused differences in the responses. As stated in hypothesis 3, increased active uptake of Fe and Mn is expected as consequence of upregulated photosynthesis under eCO<sub>2</sub>. Contrastingly to this hypothesis, grasses had a reduced Fe and Mn yield, suggesting downregulated photosynthesis, however, forbs and legumes proved increased nutrient response supporting the upregulation hypothesis. Three hypothetical physiological mechanisms may decrease plant nutrient concentrations under eCO<sub>2</sub>: i. dilution in the plant tissue of the acquired nutrient amount by an increased C assimilation (Ainsworth & Long, 2021; Loladze, 2002), ii. decreased water mass flow carrying fewer nutrients caused by higher water use efficiency through reduced stomatal

conductance (Houshmandfar *et al.*, 2018; Tausz-Posch *et al.*, 2014), or iii. decreased water mass flow caused by downregulation of photosynthesis because of low C sink strength (Ainsworth *et al.*, 2003) and therefore reduced stomatal conductance.

The meta-analysis across FACE sites by Feng *et al.* (2015) suggested that the dilution (i. above) in plant tissue cannot fully explain reduced N yields, but rather suggests that a reduced uptake of N is realistic (ii. & iii. above), which was the case in our study, indicated by the negative Y-axis intercept  $Y_0$  for zero biomass response to eCO<sub>2</sub> (Fig. 4-2; Tab. A. 4-6). This negative  $Y_0$  implies that the nutrient yield was still smaller under eCO<sub>2</sub> compared to aCO<sub>2</sub>, when the biomass was non-responding to eCO<sub>2</sub>. Between elements and functional groups the  $Y_0$  indicated differences in the eCO<sub>2</sub> response in nutrient yield versus response in biomass in the GiFACE (Fig. 4-2). For K, Mg, Ca, S, Cu, Mn, Fe in grasses, P, Ca, S in forbs and Mg in legumes the significantly negative  $Y_0$  indicated that eCO<sub>2</sub> had a negative effect on nutrient yield, independent of the effect on biomass. Hence, these plant nutrient yields are in effect not responding proportionally to productivity stimulation with increased C assimilation, which indicates that nutrient dilution does not explain reduced nutrient concentrations.

The mechanisms behind the reduced nutrient yield under eCO<sub>2</sub> can be related to plant physiological constraints, such as root-cell absorption sites (Welch & Shuman, 1995), or a high assimilation efficiency and therefore a reduced nutrient in-flow due to the documented reduced transpiration caused by reduced stomata aperture under eCO<sub>2</sub> (Haworth *et al.*, 2016). Furthermore, a downregulation of photosynthesis and therefore stomata aperture because of low C sink strength (Ainsworth *et al.*, 2003) can reduce nutrient yields. Soil properties like soil pH, buffer capacity, soil moisture and soil structure affect the solubility and sorption of most nutrient elements in the soil (Hawkesford *et al.*, 2014). Investigations by Brenzinger *et al.* (2017) at the GiFACE site indicated only marginally and non-significant differences in soil characteristics (i.e. pH, soil moisture, C-content, N-content) between eCO<sub>2</sub> and aCO<sub>2</sub>. Results by Moser *et al.* (2018) in the GiFACE indicated increased N mineralization rates under eCO<sub>2</sub> that contributed to increased N<sub>2</sub>O emissions and caused also changed nutrient availability and competition between plants and microbes. Reduced pH, due to eCO<sub>2</sub> was also assumed in different studies (Hinsinger *et al.*, 2003; Natali *et al.*, 2009). These changes could lead to an increasing (e.g. Fe, Mn, Zn and Cu) or decreasing (e.g. N, P, K, Ca, S and Mg) availability of nutrients for plants. Even though no pH shift in bulk soil was observed in the GiFACE (Brenzinger *et al.*, 2017), this pH controlled nutrient availability seems to be partly reflected in the rhizosphere by the observed forb (and legume) nutrient yield responses, e.g. more Fe, Mn and Zn yield and less N, P, Ca, Mg yield (Fig. 4-2) and partly confirmed our second and

third hypothesis. However, the grass nutrient yield response does not support these hypotheses. In that case the limited C sink strength compared to the one of forbs and legumes might cause a downregulation of photosynthesis and therefore stomata aperture in grasses causing reduced transpiration and reduced passive nutrient uptake by reduced water mass flow. But an analysis of wheat under eCO<sub>2</sub> showed an increase in the ratio of nutrient uptake per unit of transpired water for Ca, Mg and Mn, supporting a compensatory mechanism, albeit insufficient to prevent some decline in nutrient contents (Houshmandfar *et al.*, 2018).

The CO<sub>2</sub> effect on nutrient concentrations was dependent on site and seasonal climatic conditions which control transpiration and photosynthetic assimilation, as was evident from significant interactions of the tested factors (Tab. A. 4-2; Tab. A. 4-3; Tab. A. 4-4). This was in line with previous studies at the GiFACE, where the CO<sub>2</sub> fertilization effect on biomass yield (Andresen *et al.*, 2018), as well as the CO<sub>2</sub> effect on forage quality (Seibert *et al.*, 2021) showed high dependency on abiotic conditions. The highest CO<sub>2</sub> fertilization effects occurred under warm and humid climatic conditions during the growth periods, which were similar to the long-term average conditions of the respective seasons. Climatic conditions below or above the long-term average conditions lead to a reduction and suppression of the CO<sub>2</sub> fertilization effect (Obermeier *et al.*, 2017; Yuan *et al.*, 2018). For example, during extreme climatic events like heat and drought waves plants under drought stress reduced transpiration via reduced stomata aperture and therefore the CO<sub>2</sub> assimilation and fertilization effect was reduced. During cold and wet seasons plant transpiration and therefore nutrient uptake via mass flow was reduced by low vapor pressure deficit.

In the current analysis, P and Ca in grasses H1 showed significant negative CO<sub>2</sub> effects, but the significant site conditions (denoted as block and soil moisture effect), interactions (i.e.  $CO_2 \times \text{soil moisture}$  in P and  $CO_2 \times \text{block}$  in Ca; Tab. A. 4-2) relativize the CO<sub>2</sub> effect. These interactions indicated the dependence of the CO<sub>2</sub> fertilization effect on soil moisture. The significant  $\text{block} \times \text{soil moisture}$  interaction in Mg and K for grasses H1 (Tab. A. 4-2), clearly reflects the moisture gradient at the GiFACE site, which led to the block partitioning of the FACE rings (Jäger *et al.*, 2003). Nevertheless, this interaction did not affect the negative eCO<sub>2</sub> effect for Mg concentration, seen by the CO<sub>2</sub> effect ( $p = 0.008$ ; Tab. A. 4-2; Fig. 4-1j). However, K concentrations indicated further significant interactions, like for grasses H2 (Tab. A. 4-2), which again relativized the CO<sub>2</sub> effect. The same pattern was seen for Mg (H1 and H2) and K (H1) in forbs and K (H1) in legumes. This dependence of the CO<sub>2</sub> fertilization effect on site and climatic conditions and the occurrence of extreme climatic events (denoted

as year) with significant factor interactions were also confirmed by the forage quality analysis of Seibert *et al.* (2021), seen in the quality parameter ash.

## 4.5 Conclusion

Our results revealed differences in plant nutrient concentrations and nutrient yield responses in an extensively managed grassland throughout 16 years of CO<sub>2</sub> treatment. In particular, plant functional types of grasses, forbs and legumes differed in their nutrient specific responses to eCO<sub>2</sub>. Most element concentrations were reduced under eCO<sub>2</sub>, especially for grasses, while single element concentrations in forbs and legumes showed a positive eCO<sub>2</sub> effect of nutrient yields for zero biomass response. This supports the hypothesis that under eCO<sub>2</sub> the plant nutrient concentration was not generally diluted by the CO<sub>2</sub> fertilization effect through increased C assimilation, but that it was caused by altered plant nutrient acquisition. For grasses, the general reduced plant nutrient yield was likely due to physiological mechanisms, such as reduced water uptake because of a downregulation of photosynthesis because of C sink limitation and therefore reduced photosynthesis and transpiration via reduced stomata aperture. We furthermore suggest that an additional soil acidification in the rhizosphere under eCO<sub>2</sub> might slightly decrease the availability of N, P, K, Ca, S and Mg and slightly increase the availability of Fe, Mn, Zn and Cu to plants.

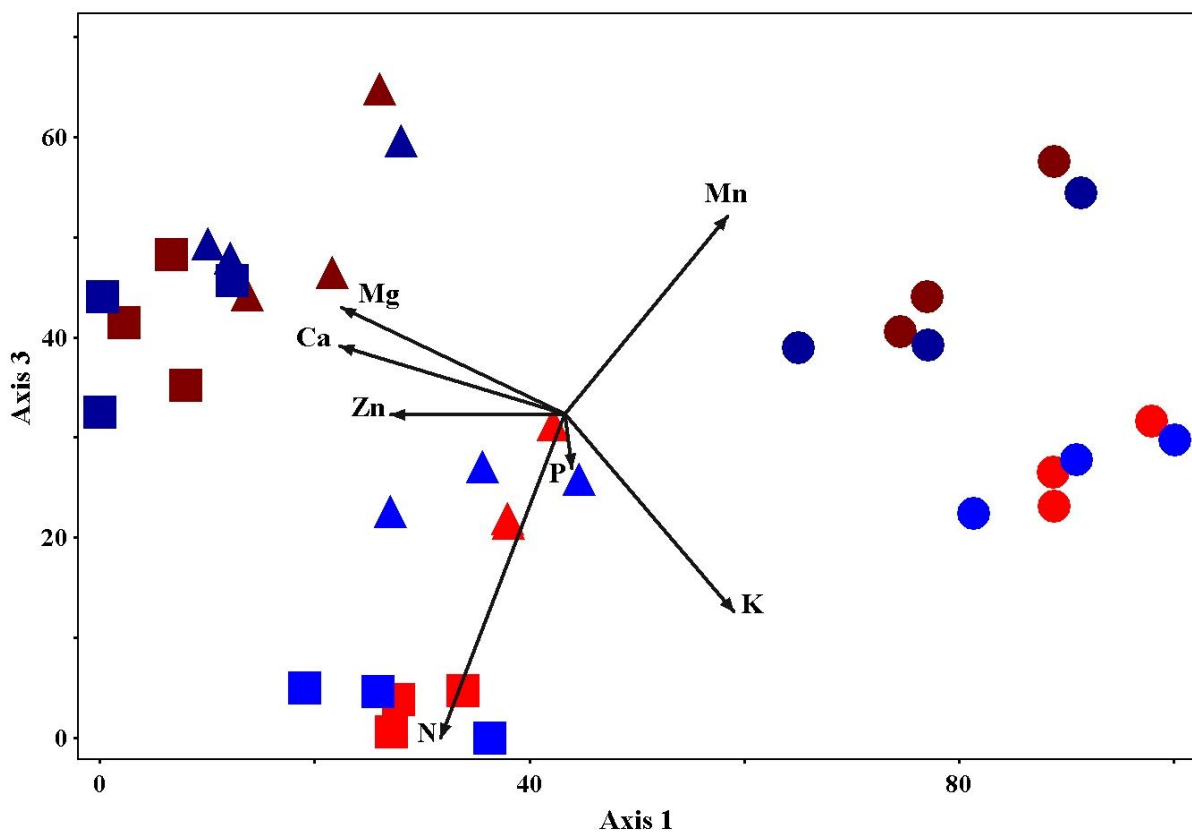
We conclude that the nutritive value of extensively managed temperate grassland may be reduced in response to eCO<sub>2</sub>. Especially Ca, Mg, N, P and K indicated a negative response to eCO<sub>2</sub> compared to the remaining investigated elements. Particularly grasses are more affected by this process than forbs and legumes because of different C sink strength. However, it appears that the CO<sub>2</sub> effect on nutrient concentrations is most pronounced under average site and climatic conditions, which do not restrict the CO<sub>2</sub> fertilization effect on plant biomass, while it is reduced during extreme weather events. As soon as the CO<sub>2</sub> fertilization effect is limited by other factors like water or nutrient availability, interactions dominate the nutrient concentrations under eCO<sub>2</sub>. These observed changes in nutrient contents and yields of grassland biomass may have serious impact on ecosystem services, e.g. forage production and grassland management, and adjustment of fertilization regimes might be needed.

## 4.6 Acknowledgements

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Conflict of Interest: The authors declare that they have no conflict of interest.

## 4.7 Appendix



**Figure A. 4-1:** PCA ordination diagram of the plant nutrient concentrations of N, P, K, Ca, Mg, Zn, and Mn from each year, separated in grasses (circles), forbs (triangles) and legumes (squares), from the two harvests H1 and H2, where H1 eCO<sub>2</sub> is red, and aCO<sub>2</sub> is blue, and H2 eCO<sub>2</sub> is dark red and aCO<sub>2</sub> is dark blue. The axes explain 89 % of the total variance.

**Table A. 4-1:** Fertilization management of the GiFACE areas through the years 1993 to 2014. All of the areas and the elevated CO<sub>2</sub> and ambient CO<sub>2</sub> treated plots received the same fertilizer treatment. The added amount of each element (Nitrogen, N amended as NH<sub>4</sub>NO<sub>3</sub>; Phosphorus, P amended as P<sub>2</sub>O<sub>5</sub>; Potassium, K amended as K<sub>2</sub>O; Magnesium, Mg amended as MgO; Calcium, Ca amended as CaO and Sulphur, S) in g m<sup>-2</sup>. The chemical fertilizer products ‘Thomaskali’ and ‘Kalkammonsalpeter’ were applied each year in April prior to the main growing season (identical to the supplementary Table A. 2-1 in the Andresen *et al.*, 2018).

	N	P	K	Mg	Ca	S
<b>1993</b>	8.0	0	0	0	0	0
<b>1994</b>	8.0	6.0	9.0	1.8	21.3	0
<b>1995</b>	8.0	6.0	9.0	1.8	21.3	0
<b>1996</b>	4.0	6.0	9.0	1.8	21.3	0
<b>1997</b>	4.0	6.0	9.0	1.8	21.3	0
<b>1998</b>	4.0	6.0	9.0	1.8	21.3	0
<b>1999</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2000</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2001</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2002</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2003</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2004</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2005</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2006</b>	4.0	6.0	9.0	1.8	21.3	0
<b>2007</b>	4.0	6.0	9.0	1.8	15.9	1.2
<b>2008</b>	4.0	6.0	9.0	2.4	14.7	1.8
<b>2009</b>	4.0	6.0	9.0	3.0	14.7	2.4
<b>2010</b>	4.0	6.0	9.0	3.0	1.5	2.4
<b>2011</b>	4.0	4.8	9.0	3.6	1.5	0
<b>2012</b>	4.0	4.8	9.0	3.6	1.5	0
<b>2013</b>	4.0	4.8	9.0	3.6	1.5	0

**Table A. 4-2:** Grasses linear mixed model analysis of the effect of CO<sub>2</sub>, block, year, soil moisture, standard temperature index (STI) and standard precipitation index (SPI) for nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K) and zinc (Zn), separately for harvest 1 (H1) and harvest 2 (H2). The model analysis was started with the full-factorial model and was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion. Df = degrees of freedom; F = F-value; p = p-values. Significant values in bold.

Source of variation	Nitrogen H1			Nitrogen H2			Phosphorus H1			Phosphorus H2			Calcium H1			Calcium H2		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
CO <sub>2</sub>	1	4.271	0.052	<b>1</b>	<b>18.675</b>	<b>0.001</b>	<b>1</b>	<b>6.796</b>	<b>0.011</b>	<b>1</b>	<b>5.900</b>	<b>0.024</b>	<b>1</b>	<b>19.907</b>	<b>0.001</b>	<b>1</b>	<b>25.359</b>	<b>&lt;0.001</b>
Block	<b>2</b>	<b>4.612</b>	<b>0.018</b>	2	1.366	0.274	<b>2</b>	<b>15.899</b>	<b>&lt;0.001</b>	<b>2</b>	<b>27.887</b>	<b>&lt;0.001</b>	<b>2</b>	<b>9.389</b>	<b>0.001</b>	2	0.959	0.397
Year	<b>13</b>	<b>57.112</b>	<b>&lt;0.001</b>	<b>13</b>	<b>17.758</b>	<b>&lt;0.001</b>	<b>13</b>	<b>40.347</b>	<b>&lt;0.001</b>	<b>13</b>	<b>18.121</b>	<b>&lt;0.001</b>	<b>13</b>	<b>23.400</b>	<b>&lt;0.001</b>	<b>13</b>	<b>7.077</b>	<b>&lt;0.001</b>
Soil moisture (Co-variable)	1	1.660	0.202	1	0.177	0.675	1	0.024	0.876	1	1.235	0.270	<b>1</b>	<b>4.106</b>	<b>0.046</b>	1	0.014	0.906
STI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
SPI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × Block	-	-	-	-	-	-	-	-	-	-	-	-	<b>2</b>	<b>5.054</b>	<b>0.021</b>	-	-	-
CO <sub>2</sub> × Year	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × Soil moisture	-	-	-	-	-	-	<b>1</b>	<b>6.552</b>	<b>0.013</b>	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block × STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block × SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year × STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year × SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

**Table A. 4-2: continued**

Source of variation	Magnesium H1			Magnesium H2			Potassium H1			Potassium H2			Zinc H1			Zinc H2		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
CO <sub>2</sub>	<b>1</b>	<b>9.011</b>	<b>0.008</b>	<b>1</b>	<b>11.752</b>	<b>0.004</b>	<b>1</b>	<b>5.913</b>	<b>0.041</b>	<b>1</b>	<b>6.135</b>	<b>0.024</b>	1	0.007	0.935	1	3.100	0.090
Block	<b>2</b>	<b>5.727</b>	<b>0.005</b>	2	2.645	0.094	2	3.006	0.058	<b>2</b>	<b>16.880</b>	<b>&lt;0.001</b>	2	2.964	0.065	<b>2</b>	<b>6.315</b>	<b>0.005</b>
Year	<b>13</b>	<b>13.279</b>	<b>&lt;0.001</b>	<b>13</b>	<b>9.837</b>	<b>&lt;0.001</b>	<b>13</b>	<b>2.977</b>	<b>0.003</b>	<b>13</b>	<b>14.103</b>	<b>&lt;0.001</b>	0	>1.000	>1.000	0	>1.000	>1.000
Soil moisture (Co-variable)	1	0.404	0.527	1	<0.001	0.983	1	2.934	0.092	1	1.107	0.296	1	1.565	0.219	1	1.214	0.278
STI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
SPI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × Block	-	-	-	-	-	-	<b>2</b>	<b>9.492</b>	<b>0.007</b>	<b>2</b>	<b>9.228</b>	<b>0.002</b>	2	0.560	0.585	<b>2</b>	<b>11.463</b>	<b>0.002</b>
CO <sub>2</sub> × Year	-	-	-	-	-	-	-	-	-	-	-	-	13	1.699	0.127	13	1.247	0.314
CO <sub>2</sub> × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	1	<0.001	0.991	1	3.083	0.091
CO <sub>2</sub> × STI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × SPI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
Block × Soil moisture	<b>2</b>	<b>4.581</b>	<b>0.014</b>	-	-	-	<b>2</b>	<b>3.212</b>	<b>0.049</b>	-	-	-	<b>2</b>	<b>4.244</b>	<b>0.023</b>	<b>2</b>	<b>5.543</b>	<b>0.009</b>
Block × STI	-	-	-	-	-	-	-	-	-	-	-	-	2	0.425	0.657	<b>2</b>	<b>4.622</b>	<b>0.021</b>
Block × SPI	-	-	-	-	-	-	-	-	-	-	-	-	2	0.286	0.753	<b>2</b>	<b>4.394</b>	<b>0.020</b>
Year × Soil moisture	-	-	-	-	-	-	<b>15</b>	<b>2.695</b>	<b>0.006</b>	-	-	-	15	1.768	0.091	15	1.141	0.365
Year × STI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
Year × SPI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000

**Table A. 4-3:** Forbs linear mixed model analysis of the effect of  $CO_2$ , block, year, soil moisture, standard temperature index (STI) and standard precipitation index (SPI) for nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K) and zinc (Zn), separately for harvest 1 (H1) and harvest 2 (H2). The model analysis was started with the full-factorial model and was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion. Df = degrees of freedom; F = F-value; p = p-values. Significant values in bold.

Source of variation	Nitrogen H1			Nitrogen H2			Phosphorus H1			Phosphorus H2			Calcium H1			Calcium H2		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
$CO_2$	1	2.497	0.131	1	<0.001	0.994	1	0.353	0.563	1	0.591	0.454	1	4.203	0.067	<b>1</b>	<b>6.436</b>	<b>0.030</b>
Block	<b>2</b>	<b>4.638</b>	<b>0.018</b>	<b>2</b>	<b>5.513</b>	<b>0.009</b>	<b>2</b>	<b>5.308</b>	<b>0.015</b>	<b>2</b>	<b>4.739</b>	<b>0.019</b>	2	0.485	0.626	<b>2</b>	<b>5.461</b>	<b>0.020</b>
Year	<b>13</b>	<b>31.133</b>	<b>&lt;0.001</b>	<b>13</b>	<b>11.399</b>	<b>&lt;0.001</b>	<b>13</b>	<b>19.185</b>	<b>&lt;0.001</b>	<b>12</b>	<b>14.544</b>	<b>&lt;0.001</b>	<b>13</b>	<b>7.955</b>	<b>&lt;0.001</b>	<b>12</b>	<b>14.518</b>	<b>&lt;0.001</b>
Soil moisture (Co-variable)	1	0.597	0.446	1	0.680	0.412	1	0.930	0.338	1	2.113	0.151	1	0.028	0.867	1	0.775	0.382
STI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
SPI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
$CO_2 \times$ Block	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$CO_2 \times$ Year	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$CO_2 \times$ Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$CO_2 \times$ STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$CO_2 \times$ SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block $\times$ Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block $\times$ STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block $\times$ SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year $\times$ Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year $\times$ STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year $\times$ SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

**Table A. 4-3: continued**

Source of variation	Magnesium H1			Magnesium H2			Potassium H1			Potassium H2			Zinc H1			Zinc H2		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
CO <sub>2</sub>	<b>1</b>	<b>13.140</b>	<b>0.005</b>	<b>1</b>	<b>6.513</b>	<b>0.043</b>	1	1.471	0.266	<b>1</b>	<b>5.636</b>	<b>0.036</b>	1	0.063	0.803	1	1.045	0.316
Block	2	1.814	0.195	<b>2</b>	<b>4.729</b>	<b>0.034</b>	2	1.751	0.224	<b>2</b>	<b>3.774</b>	<b>0.046</b>	<b>2</b>	<b>4.987</b>	<b>0.014</b>	2	0.393	0.678
Year	<b>13</b>	<b>3.889</b>	<b>&lt;0.001</b>	12	1.901	0.075	<b>13</b>	<b>2.741</b>	<b>0.006</b>	<b>12</b>	<b>8.751</b>	<b>&lt;0.001</b>	0	>1.000	>1.000	0	>1.000	>1.000
Soil moisture (Co-variable)	1	0.185	0.668	1	1.178	0.284	1	3.040	0.087	1	3.863	0.054	1	0.692	0.412	1	0.009	0.925
STI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
SPI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × Block	<b>2</b>	<b>5.130</b>	<b>0.029</b>	<b>2</b>	<b>9.640</b>	<b>0.011</b>	-	-	-	-	-	-	2	3.975	0.094	2	0.631	0.584
CO <sub>2</sub> × Year	-	-	-	<b>14</b>	<b>2.479</b>	<b>0.028</b>	-	-	-	-	-	-	13	1.523	0.185	12	0.642	0.787
CO <sub>2</sub> × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	1	0.003	0.959	1	0.662	0.424
CO <sub>2</sub> × STI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × SPI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
Block × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	<b>2</b>	<b>5.936</b>	<b>0.007</b>	2	0.688	0.511
Block × STI	-	-	-	-	-	-	-	-	-	-	-	-	<b>2</b>	<b>5.383</b>	<b>0.012</b>	2	0.060	0.941
Block × SPI	-	-	-	-	-	-	-	-	-	-	-	-	2	1.537	0.234	2	1.634	0.212
Year × Soil moisture	-	-	-	<b>14</b>	<b>2.110</b>	<b>0.043</b>	<b>15</b>	<b>2.415</b>	<b>0.013</b>	-	-	-	15	1.732	0.107	14	1.354	0.239
Year × STI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
Year × SPI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000

**Table A. 4-4:** Legumes linear mixed model analysis of the effect of CO<sub>2</sub>, block, year, soil moisture, standard temperature index (STI) and standard precipitation index (SPI) for nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K) and zinc (Zn), separately for harvest 1 (H1) and harvest 2 (H2). The model analysis was started with the full-factorial model and was reduced for non-significant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion. Df = degrees of freedom; F = F-value; p = p-values. Significant values in bold.

Source of variation	Nitrogen H1			Nitrogen H2			Phosphorus H1			Phosphorus H2			Calcium H1			Calcium H2		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
CO <sub>2</sub>	1	1.583	0.247	1	2.158	0.164	1	0.001	0.978	<b>1</b>	<b>5.018</b>	<b>0.045</b>	1	1.837	0.193	1	1.463	0.241
Block	2	0.776	0.481	<b>2</b>	<b>4.309</b>	<b>0.025</b>	2	0.963	0.413	<b>2</b>	<b>8.302</b>	<b>0.003</b>	<b>2</b>	<b>15.616</b>	<b>&lt;0.001</b>	2	2.921	0.070
Year	<b>13</b>	<b>28.305</b>	<b>&lt;0.001</b>	<b>13</b>	<b>4.449</b>	<b>&lt;0.001</b>	<b>13</b>	<b>24.561</b>	<b>&lt;0.001</b>	<b>13</b>	<b>9.565</b>	<b>&lt;0.001</b>	<b>13</b>	<b>7.793</b>	<b>&lt;0.001</b>	<b>13</b>	<b>5.537</b>	<b>&lt;0.001</b>
Soil moisture (Co-variable)	1	3.448	0.070	1	0.066	0.798	1	0.016	0.899	1	0.456	0.503	1	0.006	0.937	1	0.082	0.775
STI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
SPI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × Block	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × Year	13	2.052	0.053	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO <sub>2</sub> × SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block × STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Block × SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year × STI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year × SPI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

**Table A. 4-4: continued**

Source of variation	Magnesium H1			Magnesium H2			Potassium H1			Potassium H2			Zinc H1			Zinc H2		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
CO <sub>2</sub>	<b>1</b>	<b>9.558</b>	<b>0.009</b>	<b>1</b>	<b>7.093</b>	<b>0.030</b>	1	0.149	0.715	1	0.780	0.419	1	0.620	0.444	1	0.076	0.946
Block	<b>2</b>	<b>4.547</b>	<b>0.024</b>	2	2.744	0.105	2	1.862	0.223	<b>2</b>	<b>5.474</b>	<b>0.007</b>	2	1.173	0.337	2	0.105	>1.000
Year	<b>13</b>	<b>6.821</b>	<b>&lt;0.001</b>	<b>13</b>	<b>4.772</b>	<b>&lt;0.001</b>	<b>13</b>	<b>14.866</b>	<b>&lt;0.001</b>	<b>13</b>	<b>5.358</b>	<b>&lt;0.001</b>	0	>1.000	>1.000	0	>1.000	>1.000
Soil moisture (Co-variable)	1	1.100	0.299	1	2.927	0.094	1	<0.001	0.989	1	2.553	0.117	1	0.271	0.610	1	0.780	0.395
STI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
SPI (Co-variable)	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × Block	-	-	-	-	-	-	-	-	-	-	-	-	2	2.282	0.179	2	<0.001	>1.000
CO <sub>2</sub> × Year	-	-	-	-	-	-	-	-	-	-	-	-	13	1.111	0.427	12	1.046	0.470
CO <sub>2</sub> × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	1	0.798	0.386	1	3.377	0.091
CO <sub>2</sub> × STI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
CO <sub>2</sub> × SPI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
Block × Soil moisture	-	-	-	-	-	-	-	-	-	<b>2</b>	<b>6.875</b>	<b>0.003</b>	2	1.294	0.304	<b>2</b>	<b>9.282</b>	<b>0.004</b>
Block × STI	-	-	-	-	-	-	-	-	-	-	-	-	2	0.276	0.763	<b>2</b>	<b>5.853</b>	<b>0.017</b>
Block × SPI	-	-	-	-	-	-	-	-	-	-	-	-	2	0.166	0.849	<b>2</b>	<b>6.568</b>	<b>0.012</b>
Year × Soil moisture	-	-	-	-	-	-	-	-	-	-	-	-	15	0.723	0.726	<b>14</b>	<b>2.928</b>	<b>0.036</b>
Year × STI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000
Year × SPI	-	-	-	-	-	-	-	-	-	-	-	-	0	>1.000	>1.000	0	>1.000	>1.000

**Table A. 4-5:** PerMANOVA analysis for the coordinated effect of eCO<sub>2</sub> on plant nutrients concentrations for grasses, forbs and legumes, separated into harvest 1 (H1) and harvest 2 (H2). Df = degrees of freedom; ss = sum of squares; F = F-value; p = p-value. Graphical presentation in Fig. S1.

Source of variation	Grasses H1				Grasses H2		
	df	ss	F	p	ss	F	p
CO <sub>2</sub>	1	0.615	0.204	0.896	-0.109	-0.197	1.000
Residual	4	0.120			0.221		
Total	5	0.121			0.220		

Source of variation	Forbs H1				Forbs H2		
	df	ss	F	p	ss	F	p
CO <sub>2</sub>	1	0.210	0.216	0.683	0.448	0.233	0.498
Residual	4	0.390			0.768		
Total	5	0.411			0.813		

Source of variation	Legumes H1				Legumes H2		
	df	ss	F	p	ss	F	p
CO <sub>2</sub>	1	0.548	0.715	0.705	0.101	0.520	0.703
Residual	4	0.306			0.778		
Total	5	0.312			0.788		

**Table A. 4-6:** Correlation analysis of the CO<sub>2</sub> effect size for nutrient yield (Y-axis) versus the effect size for biomass (X-axis) standardized major axis test considering variance at both axis (SMATR), to compare the slope ('common') for grasses (G), forbs (F) and legumes (L), as combined for harvest 1 and 2. N is number of data (16 years with two annual harvests), R<sup>2</sup> and p express the significance of the linear regression (p < 0.05), with the presented slope and Y<sub>0</sub> the y-axis intercept (at X = 0 the Y<sub>0</sub> is the percental reduction of nutrient uptake) with upper and lower intercept confidence interval (CI). The 'p(test)' indicates if the two compared slopes are significantly common (p < 0.05; or by tendency p < 0.1). Graphical presentation of the Y<sub>0</sub> data in Fig. 2.**Nitrogen**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.646	0.000	-3.29	-7.00; 0.42	0.788	.	.
Forbs	32	0.935	0.000	-2.66	-5.19; -0.14	0.958	.	.
Legumes	32	0.916	0.000	-5.44	-12.20; 1.31	0.980	.	.

Common G & F	64	.	.	.	.	0.930	0.105	yes
Common G & L	64	.	.	.	.	0.941	0.085	(yes)
Common F & L	64	.	.	.	.	0.968	0.751	yes

**Phosphorus**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.796	0.000	-1.14	-4.38; 2.11	0.914	.	.
Forbs	31	0.935	0.000	-3.27	-5.86; -0.68	0.949	.	.
Legumes	32	0.877	0.000	2.07	-6.37; 10.51	1.010	.	.
Common G & F	63	.	.	.	.	0.940	0.682	yes
Common G & L	64	.	.	.	.	0.972	0.351	yes
Common F & L	63	.	.	.	.	0.970	0.410	yes

**Potassium**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.707	0.000	-6.26	-10.62; -1.90	1.021	.	.
Forbs	31	0.909	0.000	2.22	-0.97; 5.41	0.989	.	.
Legumes	32	0.693	0.000	-8.20	-20.17; 3.77	0.880	.	.
Common G & F	63	.	.	.	.	0.996	0.777	yes
Common G & L	64	.	.	.	.	0.950	0.305	yes
Common F & L	63	.	.	.	.	0.962	0.294	yes

**Calcium**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.637	0.000	-9.64	-13.99; -5.29	0.963	.	.
Forbs	31	0.935	0.000	-7.26	-9.90; -4.62	0.972	.	.
Legumes	32	0.841	0.000	-3.40	-12.76; 5.96	0.978	.	.

Common G & F	63	.	.	.	.	0.971	0.932	yes
Common G & L	64	.	.	.	.	0.973	0.905	yes
Common F & L	63	.	.	.	.	0.974	0.945	yes

**Magnesium**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.765	0.000	-6.37	-9.96; -2.77	0.944	.	.
Forbs	31	0.939	0.000	-3.18	-6.00; -0.36	1.067	.	.
Legumes	32	0.848	0.000	-9.20	-18.04; -0.36	0.946	.	.
Common G & F	63	.	.	.	.	1.040	0.239	yes
Common G & L	64	.	.	.	.	0.945	0.986	yes
Common F & L	63	.	.	.	.	1.031	0.157	yes

**Iron**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.081	0.115	-63.13	-101.21; -25.05	4.753	.	.
Forbs	31	0.719	0.000	5.51	-2.54; 13.55	1.391	.	.
Legumes	32	0.824	0.000	10.35	-1.43; 22.14	1.167	.	.
Common G & F	63	.	.	.	.	1.822	0.001	no
Common G & L	64	.	.	.	.	1.390	0.001	no
Common F & L	63	.	.	.	.	1.246	0.169	yes

**Zinc**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.664	0.000	-2.82	-7.30; 1.66	0.978	.	.
Forbs	31	0.919	0.000	3.06	0.20; 5.92	0.940	.	.
Legumes	32	0.894	0.000	1.84	-7.06; 10.74	1.148	.	.

Common G & F	63	.	.	.	.	0.948	0.744	yes
Common G & L	64	.	.	.	.	1.106	0.198	yes
Common F & L	63	.	.	.	.	1.026	0.012	no

**Manganese**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.314	0.001	-20.60	-32.71; -8.49	1.807	.	.
Forbs	31	0.654	0.000	16.49	8.12; 24.86	1.294	.	.
Legumes	32	0.785	0.000	23.19	9.69; 36.68	1.204	.	.
Common G & F	63	.	.	.	.	1.450	0.082	(yes)
Common G & L	64	.	.	.	.	1.322	0.021	no
Common F & L	63	.	.	.	.	1.237	0.629	yes

**Copper**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.599	0.000	-7.48	-13.45; -1.52	1.187	.	.
Forbs	31	0.898	0.000	-1.45	-5.04; 2.15	1.053	.	.
Legumes	32	0.856	0.000	-1.10	-10.84; 8.64	1.073	.	.
Common G & F	63	.	.	.	.	1.079	0.370	yes
Common G & L	64	.	.	.	.	1.102	0.446	yes
Common F & L	63	.	.	.	.	1.061	0.818	yes

**Sulfur**

	N	R <sup>2</sup>	p	Y <sub>0</sub>	CI <sub>low</sub> ; CI <sub>up</sub>	slope	p(test)	common
Grasses	32	0.778	0.000	-6.94	-11.88; -2.00	0.971	.	.
Forbs	31	0.804	0.000	-8.71	-16.74; -0.69	0.909	.	.
Legumes	32	0.603	0.000	3.57	-15.88; 23.01	1.004	.	.

Common G & F	63	.	.	.	.	0.938	0.670	yes
Common G & L	64	.	.	.	.	0.983	0.864	yes
Common F & L	63	.	.	.	.	0.940	0.605	yes

## 5 Raised atmospheric CO<sub>2</sub> levels affect soil seed bank composition of temperate grasslands

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### Abstract

**Questions:** Soil seed banks buffer plant populations against environmental variability. But environmental changes can have profound impact on it. Several studies addressed the effect of climate change on aboveground vegetation, but studies on changes in the seed bank are rare. Thus, we studied the seed bank of a temperate grassland at a long-term FACE (Free-Air Carbon dioxide Enrichment) site, with the following questions: (1) are there general differences in the species composition between aboveground vegetation and seed bank; (2) what are the impacts of elevated CO<sub>2</sub> (eCO<sub>2</sub>) on seed density and species composition of the seed bank; and (3) are there differences in the functional traits of the seed bank species under eCO<sub>2</sub> vs. ambient CO<sub>2</sub> (aCO<sub>2</sub>)?

**Location:** Temperate grassland, Gießen, Germany.

**Method:** Thirty soil cores were taken in six FACE-rings. Emerging seedlings were identified to species level after germination and aboveground vegetation was sampled. From the seed bank data we derived diversity measures and weighted means of species traits, e.g. seed longevity, regeneration type and compared eCO<sub>2</sub>- (+20 % CO<sub>2</sub> above ambient conditions) with aCO<sub>2</sub>-treatment.

**Results:** NMS-Ordination revealed a clear separation between seed bank and aboveground vegetation but no clear CO<sub>2</sub>-effect. Analyses revealed higher seed densities under eCO<sub>2</sub>. Species diversity and Shannon diversity were not significantly affected. Evenness decreased significantly under eCO<sub>2</sub>. There are shifts in functional traits of seed bank species. Seed density of long-term persistent species increased, while short-term persistent species decreased. Seed densities of species with generative reproduction increased under eCO<sub>2</sub>, while species numbers with vegetative reproduction decreased.

**Conclusion:** The observed trait compositions of the seed bank under eCO<sub>2</sub> indicate that species relying on generative reproduction and production of long-term persistent seeds have a competitive advantage under eCO<sub>2</sub>. These changes in the plant communities, may lead to profound changes in the supply of grassland ecosystem services.

**Keywords:** FACE; climate change; elevated CO<sub>2</sub>; seed bank; grassland; species composition; functional traits; GiFACE

## 5.1 Introduction

Temperate grasslands, which cover 26 % of the world's terrestrial area (Contant, 2010; FAO, 2008), consist of permanent plant communities dominated by forbs and grasses. Plant communities develop based on the interplay between natural site conditions with anthropogenic management (Becker *et al.*, 2014). Due to intensified management, e.g. higher cutting frequencies and increased fertilization, grassland diversity declined continuously during the last decades in Germany (Becker *et al.*, 2014). Today, grasslands with low management intensities, so called extensive grassland, are remnants of formerly wide-spread species-rich grassland communities (Becker *et al.*, 2014).

Any changes in management, e.g. in cutting dates and frequencies, have a profound impact on the aboveground vegetation composition, and will also affect plant regeneration. In this context, soil seed banks play an important role in providing a buffer against environmental changes and ensure the long-term persistence of plant species and communities (Burmeier *et*

*al.*, 2010; Thompson, 2000). Therefore, in species-rich grasslands a vital soil seed bank is crucial for diversity preservation (Fenner & Thompson, 2005; Wellstein *et al.*, 2007). While this holds for management or temporary environmental changes (Zechmeister *et al.*, 2003), long-term climatic changes are considered to affect the soil seed bank composition (Akinola *et al.*, 1998).

The effects of changes in site conditions and management on species-rich grasslands are well studied. Engel *et al.* (2009) revealed that soil moisture was the dominant factor affecting species richness, evenness and diversity. Moreover, several studies focused on the effects of elevated atmospheric CO<sub>2</sub> [eCO<sub>2</sub>] (Andresen *et al.*, 2018; Edwards *et al.*, 2001; Kammann *et al.*, 2005; Newton *et al.*, 2014) and/or elevated air temperatures (Bloor *et al.*, 2010; Mueller *et al.*, 2016; Obermeier *et al.*, 2017; Zelikova *et al.*, 2014) on aboveground vegetation in grasslands.

Several studies showed that in comparison to the effects of eCO<sub>2</sub>, warming is more influential on species- and community-level responses (Bloor *et al.*, 2010; Engel *et al.*, 2009; Hovenden *et al.*, 2008b). Hovenden *et al.* (2008b) showed that for a temperate grassland, that flowering time is sensitive to experimental warming but insensitive to eCO<sub>2</sub>, which highlights that reproductive traits are key characteristics for predicting the response of grassland-communities and -ecosystems to global change. In contrast to Hovenden *et al.* (2008b), Engel *et al.* (2009) and Bloor *et al.* (2010), a range of studies with growth chamber, greenhouse, field chamber and FACE rings showed that eCO<sub>2</sub> affected reproductive traits, i.e. more flowers, more fruits and more seeds (Jablonski *et al.*, 2002). At the site of the present study (GiFACE), aboveground biomass increased significantly under eCO<sub>2</sub> but was not accompanied by differences in species diversity and distribution between CO<sub>2</sub> treatments (Andresen *et al.*, 2018; Kammann *et al.*, 2005). Besides the impact of eCO<sub>2</sub> on aboveground biomass and species composition, other studies at the GiFACE site focused on soil microbial communities, N-cycling and soil respiration (Brenzinger *et al.*, 2017; Keidel *et al.*, 2015; Moser *et al.*, 2018). The long history of continuous experimental CO<sub>2</sub> elevation at the GiFACE site provided us with the opportunity to study the response of the soil seed bank to elevated CO<sub>2</sub> levels, which to our knowledge has not been studied in extensive grassland so far.

Seed or fruit production, which is responsible for soil seed bank input, depends on different climatic factors. It may increase with warming, decrease with drought, or remains unchanged with warming and water addition (Walck *et al.*, 2011). Temperature effects could have a direct influence on seed persistence, because increasing temperature in the soil may reduce

dormancy in a greater proportion of seeds (Ooi *et al.*, 2009). Temperature may also influence seed survival. Increasing soil temperatures due to global warming may approach thresholds for seed death in those ecosystems where high temperatures are already apparent. Ooi *et al.* (2009) indicated that viability of seeds and the rate of germination were also affected by increased temperature. Viability declined at some species after 70 days exposure to predicted high temperatures, while germination rate was increased. Thus, increased soil temperature can affect seed dormancy, viability and germination rate. However, plasticity of dormancy and germination traits is species-specific in response to raised temperature treatments (Ooi *et al.*, 2009).

In general, life history, or longevity of plant species, will play a role in the response to global change (Hovenden *et al.*, 2008a). While annual species are depending on flowering and seed production to ensure population survival, perennial species have several years to maintain their population. Furthermore, perennial species are under different selective pressures, which are reflected in their reproduction responses under global change (Hovenden *et al.*, 2008a).

The specific change under global warming depends on the prevailing strategies of growth, demography and productivity in plant communities (Engel *et al.*, 2009; Violle *et al.*, 2007). Trait mediated differences in the responses of plant species to changing environmental conditions, e.g. climate, could change the way how species interact and this may affect plant community composition (Engel *et al.*, 2009).

In general, morphological, physiological or phenological plant traits, are features that can be determined at the species level (Violle *et al.*, 2007) and they are results of processes, i.e. evolutionary and community assembly, in response to abiotic and biotic environmental requirements (Kattge *et al.*, 2011; Valladares *et al.*, 2007). Plant communities can be regarded as the result of an abiotic and biotic hierarchy that constrains which species and traits can prevail (Lavorel & Garnier, 2002).

While some studies had a look at the effect of warming or precipitation on the soil seed bank in grassland ecosystems (Leishman *et al.*, 2000; Ooi *et al.*, 2009; Ooi, 2012), investigations of effects of elevated CO<sub>2</sub> on soil seed banks are rare (Hovenden *et al.*, 2008a). To reveal the effect of eCO<sub>2</sub> on the soil seed bank of a grassland system, we carried out our study in an extensive managed temperate C<sub>3</sub> grassland at the long-term free-air carbon dioxide enrichment [GiFACE] site, which was set up in Germany in 1998 (Jäger *et al.*, 2003). Our main objective was to assess the differences in the soil seed bank between plots under elevated CO<sub>2</sub>- and ambient CO<sub>2</sub>-concentrations.

Specifically we addressed the following research questions:

- 1) Are there general differences in the species composition between aboveground vegetation and seed bank and are these related to eCO<sub>2</sub>?
- 2) What are the impacts of eCO<sub>2</sub> on seed density and species composition of the soil seed bank?
- 3) Are there differences in the functional traits of the seed bank species between elevated and ambient CO<sub>2</sub>-treatments?

## 5.2 Materials and Methods

### 5.2.1 Experimental site

The research area of the experimental site is permanent grassland, with groundwater levels, reaching the surface during winter. The vegetation is dominated by the grass species *Arrhenatherum elatius*, *Holcus lanatus*, *Alopecurus pratensis* and *Poa pratensis*. *Galium album*, *Sanguisorba officinalis* and *Plantago lanceolata*, are the dominant forbs. The main legumes are *Lathyrus pratensis*, *Trifolium repens* and *Trifolium pratense* (for more details see Jäger et al., 2003). The most dominant species of the aboveground vegetation during the last ten years were *Arrhenatherum elatius*, *Trisetum flavescens*, *Galium album* and *Geranium pratensis* at both treatments (Tab. A. 5-1; Tab. A. 5-2).

Before the onset of CO<sub>2</sub> enrichment, the experimental site (Environmental Monitoring and Climate Impact Research Station Linden, 50°32'N 8°41'E) had been managed as a meadow for at least 100 years and never been ploughed during this period (Kammann *et al.*, 2005). The extensive management included two cuts per year and fertilization, i.e. 50 - 80 kg ha<sup>-1</sup> a<sup>-1</sup> calcium ammonium nitrate until 1995 and 40 kg N ha<sup>-1</sup> a<sup>-1</sup> since then. In addition, 600 kg ha<sup>-1</sup> a<sup>-1</sup> of 10 % P<sub>2</sub>O<sub>5</sub> + 15 % K<sub>2</sub>O + 3 % MgO; 33 % CaO + MgO were applied in spring each year to ensure a sufficient supply of these minerals (Kammann *et al.*, 2005).

The soil is a Fluvic Gleysol with a texture of sandy clay loam over a clay layer at varying depths (FAO classification). The pH ranges between 5.8 and 6.0 in 0 - 40 cm depth, the organic carbon and nitrogen contents in 0 - 5 and 5 - 15 cm depth are 4.6 % and 3.6 % (C), and 0.44 % and 0.36 % (N), respectively (Kammann *et al.*, 2005).

Annual mean precipitation at the site is 639.7 ± 92.9 mm (1998 – 2013) and the mean annual temperature is 9.8 ± 0.5° C (1998 – 2013; Environmental Monitoring and Climate Impact

Research Station Linden). A detailed description of the experimental site and the Giessen FACE system is provided by Jäger *et al.* (2003).

### 5.2.2 Study design of the seed bank analysis

We analyzed soil seed densities and composition using the seedling emergence method (Roberts, 1981). The collection of the seed bank samples took place in July 2014. Thirty soil cores per ring were taken with an auger of 3 cm diameter down to a depth of 10 cm. The samples were divided into three depth layers (0 – 1 cm, 1 – 5 cm, 5 – 10 cm). Before transferring the soil samples into a greenhouse they were stratified in the dark at 4° C for five weeks.

Subsequently, the samples were spread thinly (0.5 – 1 cm) in “Multi-Plant-Pots” (soil layer of 0 – 1 cm) and 10 x 10 cm plant pots (soil layer of 1 – 5 cm and 5 – 10 cm) over a base of moistened, sterilized standard potting soil (Einheitserde, Classic, Typ 0). Plant pots filled only with sterilized standard potting soil acted as control pots for seed input from outside. During the experiment, no seed inputs from outside the experimental area were registered.

The pots were randomly placed in a greenhouse with automatic watering, controlled temperature (~ 20° C), air humidity (~ 70 %) and light ( $\geq 10$  klx) in a day-night-rhythm of 12/12 hours in Gießen, Germany.

Emerging seedlings were identified to species level as soon as possible after germination (monitored weekly), counted and removed. Those specimens that could not be identified at the seedling stage were transferred to pots and grown until identification was possible.

When germination ceased, the soil material was carefully stirred to stimulate germination of the remaining seeds. After 14 weeks, the pots were allowed to air dry for 5 weeks, whereupon the soil was carefully stirred and the pots were incubated for another 10 weeks in the greenhouse. During the drying process the pots were covered with a fine gaze to prevent seed input from outside.

### 5.2.3 Treatment design and FACE system

In 1997, six of 16 previously monitored 100 m<sup>2</sup> plots were selected for three ring pairs and the CO<sub>2</sub> treatment was randomly assigned, to one of the rings. The rings cover a slight soil moisture gradient caused by different depths of the clay layer as well as from average ground water table depth, which was accounted for in the data analysis as a covariable (Jäger *et al.*, 2003; Kammann *et al.*, 2005).

The rings had an inner diameter of 8.0 m with an inner circular buffer-zone to the ring structure of 0.9 m. Each ring construction consisted of 24 segments with an outer and inner air-flow channel. CO<sub>2</sub> was diluted with ambient air before it was released by the pipes at the outer channel to enhance mixing with the air entering the monitoring-plots inside the rings. This upwind CO<sub>2</sub> release was controlled by wind direction, and the amount of CO<sub>2</sub> release was controlled by wind speed. Downwind, the CO<sub>2</sub> enriched air was partly recycled by pipes installed at the inner ring channel (Andresen *et al.*, 2018; Jäger *et al.*, 2003). The CO<sub>2</sub> enrichment started in May 1998 and was performed year-round during daylight hours to +20 % above ambient conditions (Jäger *et al.*, 2003); from subsequently referred to as “elevated CO<sub>2</sub>-rings” (eCO<sub>2</sub>) and “ambient CO<sub>2</sub>-rings” (aCO<sub>2</sub>).

Since the start of the experiment vegetation sampling took place annually in May and at the end of August according to the Braun-Blanquet method (Dierschke, 1994). In addition, volumetric soil moisture was measured five days a week using four permanent TDR-sensors (Imko, Germany, type P2G) in the upper 15 cm depth.

#### 5.2.4 Data analysis

For the data analysis we used seed density in seeds cm<sup>-3</sup>, species number and cover percentage based on the aboveground vegetation of 2013.

A nonmetric multidimensional scaling (NMS) was used for detecting differences in species composition between the aboveground vegetation and the soil seed bank (McCune and Grace, 2002). For conducting the NMS, the Sørensen-distance measure, two dimensions and a starting configuration by random number were used. For the final solution we used 49 iterations. Before analysis the data were subjected to root transformation (van der Maarel, 1979). Prior to the combined analyses of aboveground and belowground vegetation, seed densities of the soil seed bank were converted to percentages.

To determine the species diversity of the seed bank, we used the Shannon- (Shannon & Weaver, 1949) and evenness-index (Pielou, 1966).

A two-factorial analysis of covariance (ANCOVA) was used to test for significant differences ( $p \leq 0.05$ ) of the seed density and species number present in the soil seed bank between the different treatments and the three different depths. Effects with  $p$  values  $\leq 0.05$  were regarded as significant and  $0.05 < p \leq 0.1$  were regarded as significant by tendencies. Basic requirements (e.g. normality and homoscedasticity) for conducting a parametric ANCOVA were visually checked using diagnostic plots. As a result, data of species number of long-term persistent seed bank type (Tab. 5-3, Tab. A. 5-6), seed density of short-term persistent seed

bank type (Tab. 5-3, Tab. A. 5-7) and seed density of generative reproduction type (Tab. 5-4, Tab. A. 5-8) were log<sub>10</sub> transformed prior to the statistical analyses.

The main factors induced into the analysis were CO<sub>2</sub> treatment (k = 2; ambient CO<sub>2</sub> vs. elevated CO<sub>2</sub>) and the soil seed bank layers (k = 3; 0 – 1 cm, 1 – 5 cm; 5 – 10 cm). To account for the potential influence of soil moisture on the soil seed bank (Bekker *et al.*, 1998), the annual soil moisture measurements of September 2011 – 2013 were used as co-variables.

The following traits of the species and seeds were tested:

- (1) *Seed bank type*: according to the database BIOPOP (Poschlod *et al.*, 2003). The species were classified as long-term persistent (>5 years), short-term persistent (1 - 5 years) or transient (<1 year, Thompson *et al.*, 1997). 63 % of the present species in the soil seed bank were covered by this three seed bank types (Tab. A. 5-3).
- (2) *Reproduction type*: three reproduction types generative, vegetative and generative + vegetative were calculated according to the database BiolFlor (Klotz *et al.*, 2002; see Tab. A. 5-3).

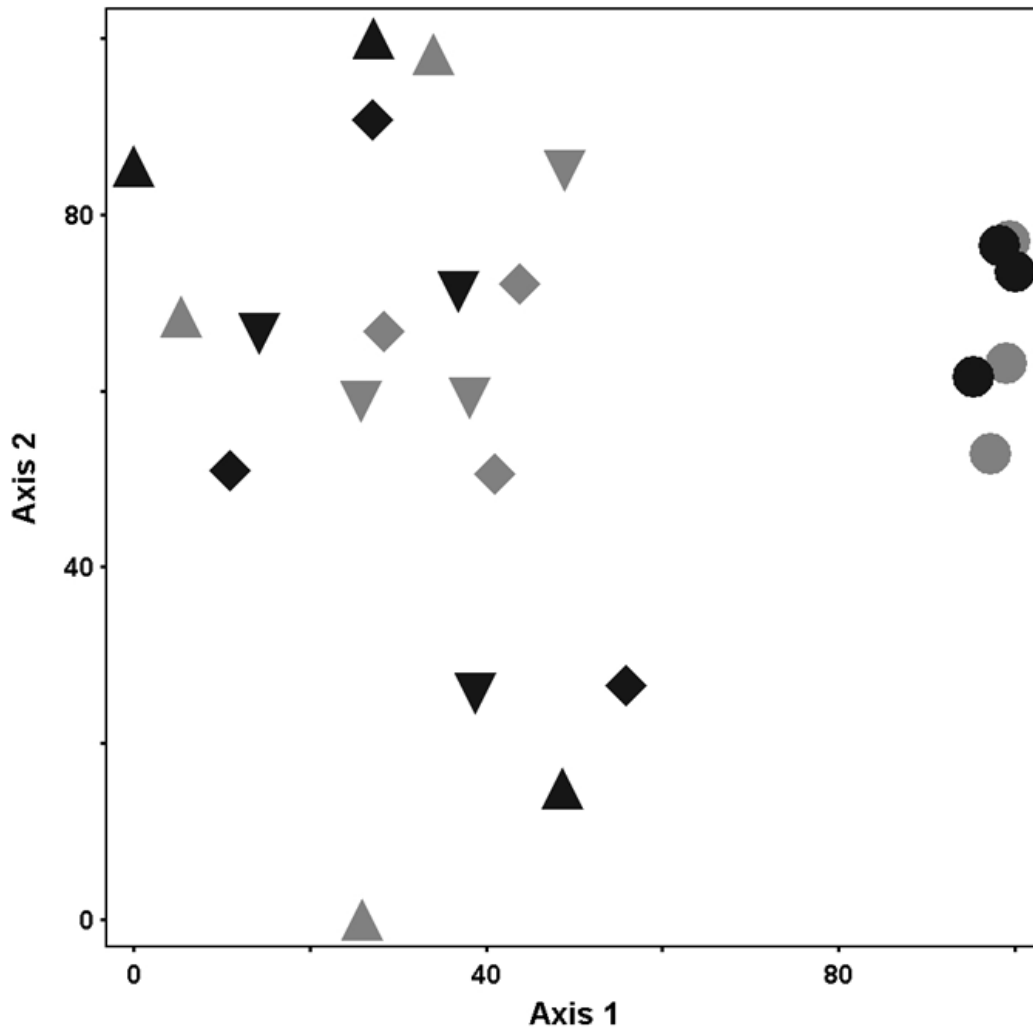
All statistical analyses were carried out in IBM SPSS Statistics (version 23.0). The NMS was performed using PC-Ord 6 (McCune and Mefford, 2011).

## 5.3 Results

### 5.3.1 Seed bank and vegetation

The soil samples comprised 2155 seedlings in total. Of these, 898 seedlings were found in the samples of the ambient CO<sub>2</sub>-rings and 1257 seedlings were found in the elevated CO<sub>2</sub>-rings. The seedlings of the ambient CO<sub>2</sub>-rings comprised 32 species in total, of these, 20 were found in the aboveground vegetation of 2013 and 12 species were found only belowground. The seedlings from the elevated CO<sub>2</sub>-rings comprised 30 species in total. Eleven of these were found only belowground and 19 were found in the aboveground vegetation of 2013. Species with highest seedling number under both treatments were *Cerastium holosteoides*, *Cardamine pratensis* and *Erophila verna* (Tab. A. 5-3).

The differences between aboveground vegetation and seed bank was confirmed by the NMS Ordination, which revealed a separation of the seed bank and aboveground vegetation, but no obvious CO<sub>2</sub> effect on both the seed bank and aboveground vegetation (Fig. 5-1).

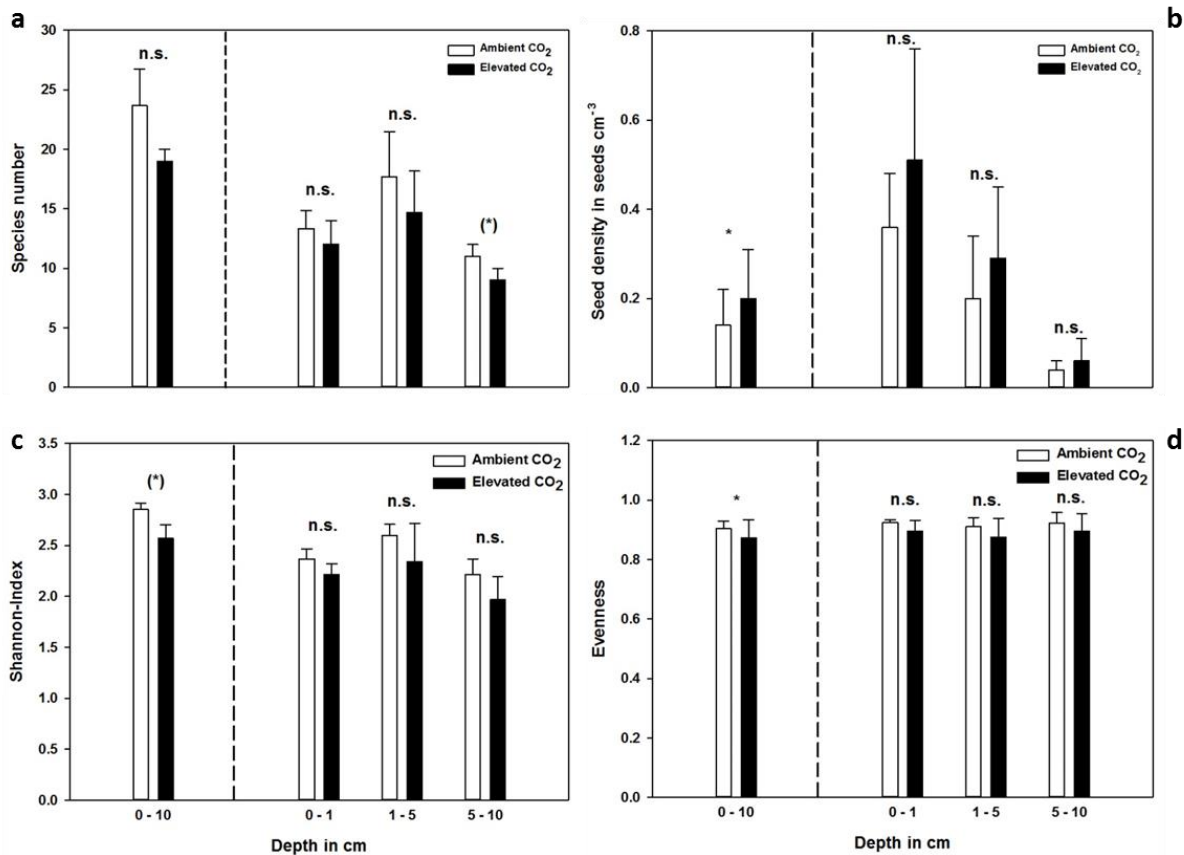


**Figure 5-1:** NMS Ordination diagram of seed bank (triangle upside down = 0 – 1 cm; chequer = 1 – 5 cm; triangle = 5 – 10 cm) and aboveground vegetation (circles). Seed bank samples and aboveground vegetation were separated in elevated CO<sub>2</sub>-rings (black) and ambient CO<sub>2</sub>-rings (grey). Final stress of the NMS Ordination was 11.64.

### 5.3.2 Seed density, species number and diversity indices

In all depths, a higher species number was detected in aCO<sub>2</sub> than eCO<sub>2</sub> (Fig. 5-2a + b). The seed density decreased significantly with increasing soil depth (Fig. 5-2b). Significant CO<sub>2</sub>- and soil moisture effects on seed density were observed when all three layers (0 – 10 cm) were taken together (Tab. 5-1, Tab. A. 5-4), with higher seed densities under eCO<sub>2</sub>. In case of species number, there was a significant decline of species number with depth in case of the combined data, but no general CO<sub>2</sub>-effect (Tab. 5-1). Only in the layer 5 – 10 cm higher species numbers under aCO<sub>2</sub> were significant by tendency ( $p = 0.088$ ; Fig. 5-2a, Tab. A. 5-4). The species diversity revealed a significant decline of Shannon diversity with depth (Tab. 5-1). Only when all soil layers were included the CO<sub>2</sub>-effect was significant by tendency ( $p = 0.066$ ; Fig. 5-2c, Tab. A. 5-5). All other responses patterns were not significant (Tab. 5-1, Tab.

A. 5-5). In case of evenness, the CO<sub>2</sub>-effect was also significant ( $p = 0.035$ ; Fig. 5-2d). While soil moisture seemed to be significant by tendency ( $p = 0.091$ ), all other responses patterns in case of evenness were not significant (Tab. 5-1, Tab. A. 5-5).



**Figure 5-2:** Mean species number [a], mean seed density [b], Shannon-index [c] and evenness [d] of the seed bank samples, separated in total depth (0 – 10 cm) and seed bank layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm). Error bars indicate standard deviation. The significance levels were reported as significant,  $p \leq 0.05$  [\*], significant by tendency  $p \leq 0.1$  [(\*)] and not significant [n.s.].

**Table 5-1:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number, seed density, Shannon-index and evenness in the soil seed bank (total depth 0 – 10 cm). Soil moisture was included as a co-variable. Df = degrees of freedom; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italics.

Source of variation	Species number			Seed density in seeds cm <sup>-3</sup>		
	0 - 10 cm			0 - 10 cm		
	df	ssq	p	df	ssq	p
CO <sub>2</sub>	<b>1</b>	6.561	0.321	<b>1</b>	<b>0.117</b>	<b>0.012</b>
Depth	<b>2</b>	<b>114.778</b>	<b>0.004</b>	<b>2</b>	<b>0.437</b>	<b>&lt;0.001</b>
CO <sub>2</sub> x Depth	2	2.111	0.843	2	0.011	0.650
Soil moisture (Co-variable)	<b>1</b>	3.235	0.481	<b>1</b>	<b>0.114</b>	<b>0.012</b>
Error	11	66.765		11	0.140	

Source of variation	Evenness 0 - 10 cm			Shannon-index 0 - 10 cm		
	df	ssq	p	df	ssq	p
CO <sub>2</sub>	<b>1</b>	<b>0.009</b>	<b>0.035</b>	<i>1</i>	<i>0.188</i>	<i>0.066</i>
Depth	2	0.001	0.681	<b>2</b>	<b>0.431</b>	<b>0.032</b>
CO <sub>2</sub> x Depth	2	6.14E-05	0.981	2	0.010	0.900
Soil moisture (Co-variable)	<i>1</i>	<i>0.005</i>	<i>0.091</i>	1	0.010	0.649
Error	11	0.017		11	0.494	

### 5.3.3 Differences in functional traits

#### 5.3.3.1 Seed bank type

Thirty-seven percent of the seed bank species in the eCO<sub>2</sub>-rings and 35 % in the aCO<sub>2</sub>-rings belonged to the long-term persistent seed bank type (Tab. 5-2, Tab. A. 5-3). Consequently, species of this seed bank type had a significantly higher seed density under eCO<sub>2</sub> across soil layers (Fig. 5-3b, Tab. 5-3). This trend was also true when layers were analyzed separately in case of the top layer and lowermost layer ( $p \leq 0.1$ ; Fig. 5-3b, Tab. 5-3, Tab. A. 5-6). Also a significant soil moisture effect ( $p = 0.006$ ) was observed when all soil layers were included.

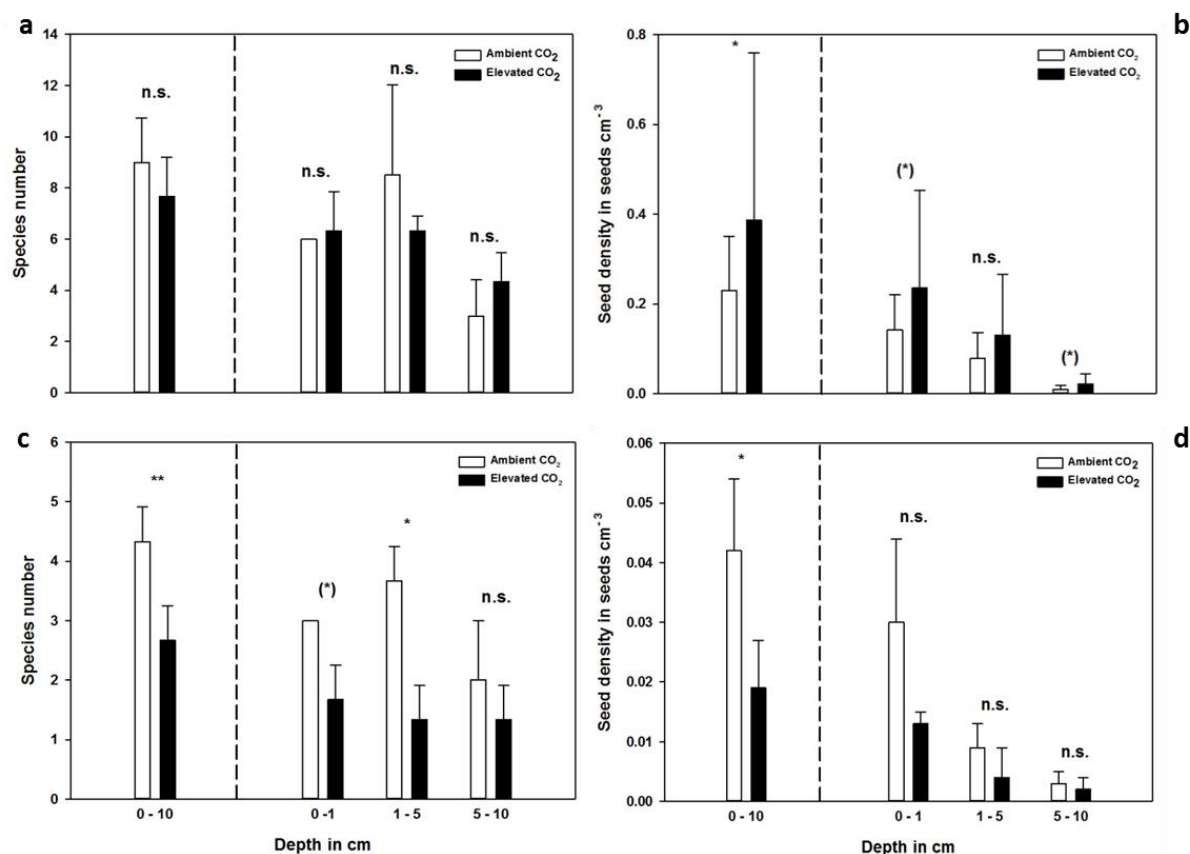
CO<sub>2</sub> and soil moisture had no effect on number of species producing long-term persistent seeds (Fig. 5-3a, Tab. 5-3). Only depth had a significant influence on this response variable.

Thirteen percent of the seed bank species under eCO<sub>2</sub> and 19 % under aCO<sub>2</sub> belonged to the short-term persistent seed bank type (Tab. 5-2, Tab. A. 5-3). Thus, in contrast to long-term persistent species, density of short-term persistent species was significantly higher under ambient compared to eCO<sub>2</sub> levels (Fig. 5-3c + d, Tab. 5-3); the same pattern was found for species number. Seed density but not species number of short-term persistent species decreased with depth (Fig. 5-3c + d). Only in case of species numbers, a general CO<sub>2</sub> effect was revealed when soil layers were analyzed separately (Tab. A. 5-7).

Seventeen percent of the seed bank species in the elevated CO<sub>2</sub>-rings and 13 % in the ambient CO<sub>2</sub>-rings belonged to the transient seed bank type (Tab. 5-2, Tab. A. 5-3). Although, seed density of transient species was higher under eCO<sub>2</sub> across all depth, this pattern was not significant (results not shown). The same pattern was found for number of transient species in the seed bank (results not shown).

**Table 5-2:** Percentage of seed bank species, separated in treatment and functional traits.

	Seed bank type			Reproduction type		
	Long-term	Short-term	Transient	Generative	Vegetative	Generative + Vegetative
eCO <sub>2</sub>	37	13	17	40	10	50
aCO <sub>2</sub>	35	19	13	44	9	47

**Figure 5-3:** Mean species number and mean seed density of species with long-term persistent seed bank type [a + b] and short-term persistent seed bank type [c + d], separated in total depth (0 – 10 cm) and seed bank layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm). Error bars indicate standard deviation. The significance levels were reported as significant  $p \leq 0.05$  [\*], much significant  $p \leq 0.01$  [\*\*], significant by tendency  $p \leq 0.1$  [(\*)] and not significant [n.s.].**Table 5-3:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) of species with long-term- and short-term persistent seed bank type. Soil moisture was included as a co-variable. Df = degrees of freedom; ssq = sum of squares; p = p-values. Significant values marked in bold. \* = Log10 transformed data.

Source of variation	Long-term persistent seed bank type					
	Species number			Seed density in seeds cm <sup>-3</sup>		
	0 - 10 cm*			0 - 10 cm		
	df	ssq	p	df	ssq	p

CO <sub>2</sub>	1	0.038	0.146	<b>1</b>	<b>0.065</b>	<b>0.010</b>
Depth	<b>2</b>	<b>0.266</b>	<b>0.006</b>	<b>2</b>	<b>0.090</b>	<b>0.012</b>
CO <sub>2</sub> x Depth	2	0.030	0.414	2	0.005	0.708
Soil moisture (Co-variable)	1	0.034	0.168	<b>1</b>	<b>0.079</b>	<b>0.006</b>
Error	11	0.170		11	0.073	

Source of variation	Short-term persistent seedbank type					
	Species number			Seed density in seeds cm <sup>-3</sup>		
	0 - 10 cm			0 - 10 cm*		
	df	ssq	p	df	ssq	p
CO <sub>2</sub>	<b>1</b>	<b>7.338</b>	<b>0.001</b>	<b>1</b>	<b>0.698</b>	<b>0.021</b>
Depth	2	2.333	0.101	<b>2</b>	<b>2.676</b>	<b>0.001</b>
CO <sub>2</sub> x Depth	2	2.111	0.121	2	0.182	0.418
Soil moisture (Co-variable)	1	0.155	0.551	1	0.172	0.208
Error	11	4.511		11	1.057	

### 5.3.3.2 Reproduction type

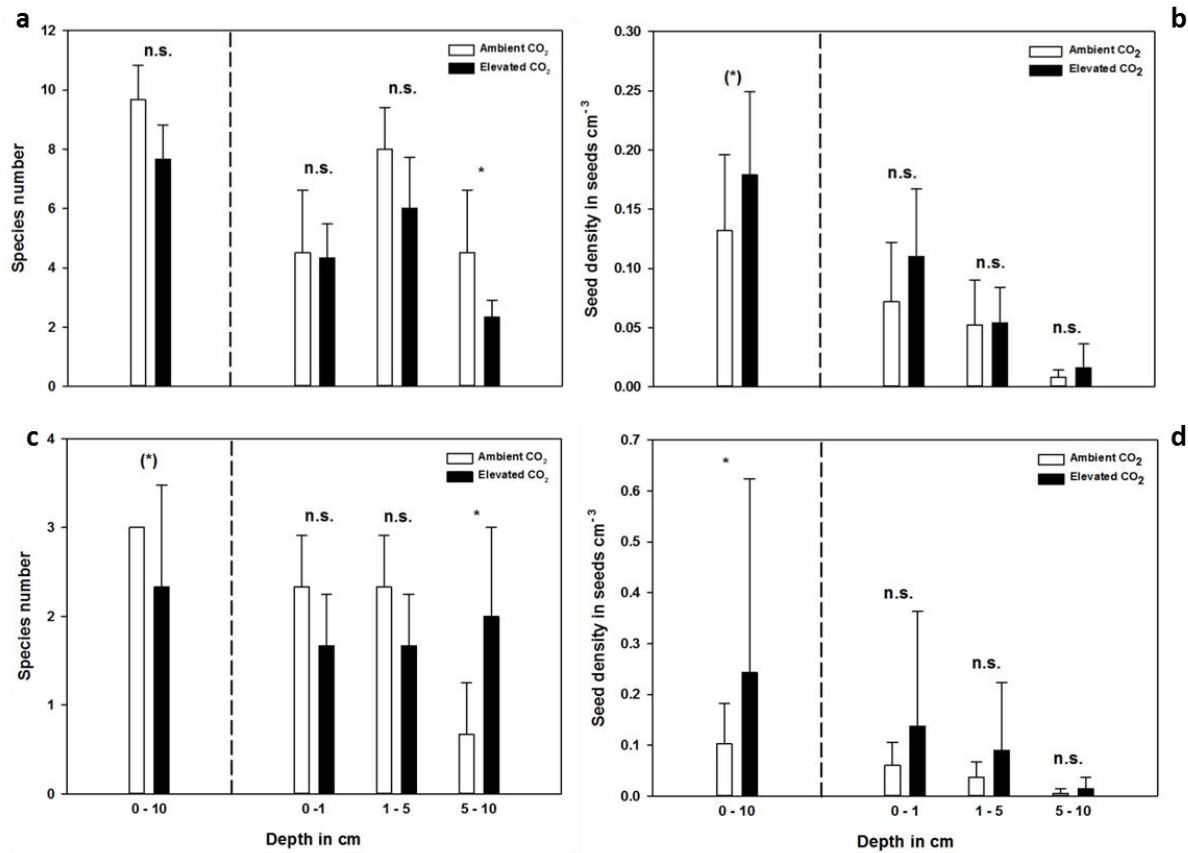
There was only a small difference in the percentage of species belonging to the generative reproduction type between aCO<sub>2</sub> and eCO<sub>2</sub> (40 % vs. 44 %; Tab. 5-2). Although these species were found in higher seed densities under eCO<sub>2</sub>, this difference was only significant by tendency ( $p = 0.078$ ) when all soil layers were included but not when layers were analyzed separately. There was only a significant soil moisture effect for the layer 1 – 5 cm ( $p = 0.05$ ). In addition, with increasing depth seed density of mainly generatively reproducing species decreased significantly (Fig. 5-4a + b, Tab. 5-4, Tab. A. 5-8).

With regard to the number of generative species, a significant CO<sub>2</sub>-effect was only detected for the layer 5 – 10 cm, which did not translate into a significant CO<sub>2</sub>-effect across soil layers. Species with a vegetative reproduction type accounted for 10 % of the seed density under eCO<sub>2</sub> and 9 % in aCO<sub>2</sub> (Tab. 5-2, Tab. A. 5-3). The CO<sub>2</sub>-treatment had a significant effect ( $p = 0.022$ ) on seed density of species with a vegetative reproduction strategy only when all three layers (0 – 10 cm) were taken together (Fig. 5-4d, Tab. 5-4).

In case of number of vegetative species, differences between the CO<sub>2</sub>-treatments were too small to reach significance across soil layers ( $p = 0.088$ ) but the difference was significant in 5 – 10 cm ( $p = 0.011$ ; Fig. 5-4c, Tab. A. 5-9). Soil moisture had a significant effect across soil layers ( $p = 0.030$ ).

Percentage of those species in the seed bank, which combine the two reproduction types, differed only slightly between treatments (50 % in eCO<sub>2</sub>-rings; 47 % in aCO<sub>2</sub>-rings; Tab. 5-2). Although, their seed density was higher under eCO<sub>2</sub> and the mean species number was higher

under aCO<sub>2</sub>, there were no significant CO<sub>2</sub>-effects (results not shown). Overall the seed density with depth was highly significant ( $p = <0.001$ ).



**Figure 5-4:** Mean species number and mean seed density of species with generative reproduction type [a + b] and vegetative reproduction type [c + d], separated in total depth (0 – 10 cm) and seed bank layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm). Error bars indicate standard deviation. The significance levels were reported as significant,  $p \leq 0.05$  [\*], significant by tendency  $p \leq 0.1$  [(\*)] and not significant [n.s.].

**Table 5-4:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) of species with generative- and vegetative reproduction type. Soil moisture was included as a co-variable. Df = degrees of freedom; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.

Source of variation	Generative reproduction type					
	Species number			Seed density in seeds cm <sup>-3</sup>		
		0 - 10 cm		0 - 10 cm*		
	df	ssq	p	df	ssq	p
CO <sub>2</sub>	1	3.130	0.249	1	0.398	0.078
Depth	2	<b>47.444</b>	<b>0.002</b>	2	<b>3.382</b>	<b>0.001</b>
CO <sub>2</sub> x Depth	2	4.778	0.358	2	0.023	0.897
Soil moisture (Co-variable)	1	0.054	0.876	1	0.420	0.072
Error	11	23.280		11	1.161	

Source of variation	Vegetative reproduction type					
	Species number			Seed density in seeds cm <sup>-3</sup>		
	0 - 10 cm			0 - 10 cm		
	df	ssq	p	df	ssq	p
CO <sub>2</sub>	1	0.885	0.088	<b>1</b>	<b>0.052</b>	<b>0.022</b>
Depth	2	3.517	0.066	2	0.012	0.238
CO <sub>2</sub> x Depth	<b>2</b>	<b>4.000</b>	<b>0.007</b>	<b>2</b>	<b>0.002</b>	<b>0.013</b>
Soil moisture (Co-variable)	<b>1</b>	<b>2.553</b>	<b>0.009</b>	1	0.064	0.784
Error	11	2.780		11	0.007	

## 5.4 Discussion

We observed a relatively high similarity between the species composition of seed bank and aboveground vegetation, i.e. on average 60 % of the seed bank species occurred in both compartments, which is in line with several other studies (e.g. Bossuyt & Honnay, 2008; Henderson *et al.*, 1988; Hopfensperger, 2007; Osem *et al.*, 2006). In general, grassland species show a low potential for seed dispersal (Donath *et al.*, 2003), supporting a high similarity between above- and belowground vegetation. Still, differences in abundances and species diversity, i.e. on average 47 % of the aboveground species, were high enough to induce a clear separation of samples of the seed bank and aboveground vegetation in the NMS ordination (Fig. 5-1). The missing separation within both compartments according to the CO<sub>2</sub>-treatment is in line with other studies, where similarities between above- and belowground vegetation were lower than similarities within seed bank and within aboveground vegetation (Bekker *et al.*, 2000; Schmiede *et al.*, 2009). A reason for this may be a delayed response of the seed bank on environmental changes (Thompson, 2000). Still, a closer look at the effects of the CO<sub>2</sub>-treatment revealed several significant effects, such as higher seed densities under eCO<sub>2</sub>. Different studies have shown that eCO<sub>2</sub> affects seed production and seed germination in grassland (Bloor *et al.*, 2010; Edwards *et al.*, 2001; Jablonski *et al.*, 2002; Marty & BassiriRad, 2014).

Our results also corroborate, at least partly, the importance of soil moisture for seed bank development. Changes in the soil moisture content can have direct impacts on longevity of seeds in the soil (Bekker *et al.*, 1998). Studies by Andresen *et al.* (2018) in the same system observed an increase in soil moisture under eCO<sub>2</sub>. Since the start of the experiment a 3 % increase of soil moisture was accompanied by 15 % increase of total biomass (Andresen *et al.*, 2018). In general, higher biomass production under CO<sub>2</sub> enrichment may result in more seeds because more resources can be allocated to reproduction (Jablonski *et al.*, 2002). This

effect is enhanced by changes in resource acquisition due to elevated CO<sub>2</sub>, which also supports higher seed production (Marty & BassiriRad, 2014).

In contrast to seed densities, species diversity and Shannon diversity were not significantly different between CO<sub>2</sub>-treatments. Although the decrease of evenness under eCO<sub>2</sub> seems small, this is evaluated against the background of a resilient seed bank. Therefore, these small changes can be considered as the first signals of changes in species composition (Bossuyt & Honnay, 2008).

While differences between treatments – albeit significant in some cases - seemed rather low at the species level, shifts in the species' spectra of functional traits were more apparent. From theory the proportion of long-term persistent seeds and species should increase with the unpredictability of a system, since a long-term seed bank allows species to bridge unfavorable periods for growth (Baskin & Baskin, 2001). When unpredictability in habitats induces variability in establishment success and reproductive output, a vital seed bank ensures persistence of species (Thompson, 2000). Changes in CO<sub>2</sub> concentrations increase the species dynamics or species shift and therefore the unpredictability of the system (Hopfensperger, 2007). Under these conditions the portion of species capable of producing long-term persistent seeds should increase (Hopfensperger, 2007). In line with this expectation a significant increase by about one-third under eCO<sub>2</sub> of the seed density of long-term persistent species was observed while the proportion of short-term persistent seeds almost halved (Fig. 5-3). Since species that produce long-term persistent seeds tend to produce more but smaller seeds (Eriksson & Eriksson, 1997), the shift in this functional trait shows up first in differences in seed density and less pronounced in number of species. The slower shift in species numbers belowground might also be linked to the low dispersal ability of grassland species, i.e. while site conditions change fast, better adapted species move in slowly (Bakker *et al.*, 1996).

Changes in proportion of the reproductive type seem in accordance to shifts observed in seed longevity (Fig. 5-4). Since species producing long-term persistent seeds not only produce more and smaller seeds but also tend to be short-lived (Grime, 2001), thus relying on generative reproduction, seed densities of species of this reproduction type (esp. *Juncus bufonius* and *Lolium perenne*) increase under eCO<sub>2</sub>. At the same time, species with vegetative reproduction decreased in number. Although this trend was only significant by tendency it also meets expectations that in line with a decrease of species producing short persistent seeds, number of perennial species, relying on vegetative reproduction increases. Vitová *et al.* (2017) showed that most grassland species are clonal, with a predominant vegetative spread.

Due to the beneficial support from the maternal plant, the clonal spread seems more successful (Vitová *et al.*, 2017). While vegetative growth is more frequent in low disturbed sites, generative growth prefers more heavily disturbed areas (Kontanen, 1996; Vitová *et al.*, 2017). Establishment by seedling (i.e. generative regeneration) is very sensitive to abiotic conditions and biotic interactions and thus very rare (Vitová *et al.*, 2017). However, there is a species-specific response to this abiotic and biotic interactions and species optimal conditions for seedling emergence often differ from those for establishment (Vitová *et al.*, 2017).

Our results highlight that CO<sub>2</sub> enrichment affects the soil seed bank of mesic moist temperate grassland. Although the soil seed bank is a system, where environmental changes trace only slowly into the seed bank, our study revealed several significant changes in seed bank composition and shifts in functional traits after 18 years of eCO<sub>2</sub>. Based on our results, it can be assumed that in response to a higher frequency of unfavorable periods for growth caused by climate change the proportion of short-lived species, producing long-term persistent seeds will increase. Thus, the observed changes suggest an ongoing change of the extensively managed grassland system towards a more ruderal state. These changes are still within the range of natural variability covered by resilience but they show signs of the development of new persistent habitats (Hobbs *et al.*, 2006). It remains to be seen how these changes translate into most prominent ecosystem services of extensively managed grasslands, such as fodder quality and quantity as well as functioning as a harbor for species diversity.

## 5.5 Acknowledgements

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## 5.6 Appendix

**Table A. 5-1:** Species list of the aboveground vegetation of the ambient CO<sub>2</sub>-rings, with mean coverage from 2004 till 2013 and the functional traits seed bank type, reproduction type and life span (Klotz *et al.*, 2002; Poschlod *et al.*, 2003). Species with highest coverage are marked in bold.

Ambient CO <sub>2</sub>	Mean coverage in %										Traits		
	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Seed bank type	Reproduction type	Life span
<i>Achillea millefolium</i>									0.5	0.5	Short-term	vegetative + generative	perennial
<i>Agrostis tenuis</i>								2.5			N/A	vegetative + generative	perennial
<i>Ajuga reptans</i>				1	0.5	0.4	0.75	0.3			N/A	vegetative + generative	perennial
<i>Alopecurus pratensis</i>	3.75	3	2.21	1.17	1.38	1.09	0.68	0.52	0.69	0.75	Long-term	vegetative + generative	perennial
<i>Anthoxanthum odoratum</i>	1		1.75	2	1.5	1.5	1.25	1.5	1.5	1.25	N/A	vegetative + generative	perennial
<b><i>Arrhenatherum elatius</i></b>	<b>42.09</b>	<b>32.92</b>	<b>21.05</b>	<b>22.71</b>	<b>20.83</b>	<b>18.75</b>	<b>18.54</b>	<b>12.67</b>	<b>15</b>	<b>14.17</b>	<b>Short-term</b>	<b>generative</b>	<b>perennial</b>
<i>Avenochloa pubescens</i>	1		1	1	1	1	0.55	1	1	1	N/A	vegetative + generative	perennial
<i>Bellis perennis</i>			0.5	1	1	0.4	1	1	1	1	Long-term	vegetative + generative	perennial
<i>Bromus hordeaceus</i>			0.78	0.55		0.1	2.25			1	Short-term	generative	annual
<i>Bromus racemosus</i>							0.75	1	1	1	N/A	generative	annual
<i>Cardamine pratensis</i>	0.5							0.5			Long-term	vegetative	perennial
<i>Cerastium holosteoides</i>	1		0.75	0.75	1.17		0.6	1.38	1.5	1.5	N/A	vegetative + generative	perennial
<i>Cirsium arvensis</i>								0.1	0.5	0.5	Transient	vegetative + generative	perennial
<i>Crepis biennis</i>	0.5										Transient	generative	biennial
<i>Cynosurus cristatus</i>			1	1							Transient	vegetative	perennial
<i>Dactylis glomerata</i>	5.88	4.29	4.42	6.42	6.21	6.04	4.08	1.42	1	0.88	N/A	generative	perennial
<i>Deschampsia cespitosa</i>								1		0.5	N/A	generative	perennial
<i>Erophila verna</i>								0.75	1	0.88	Long-term	generative	annual
<i>Festuca pratensis</i>	1				1	1.13	1	0.5	1	0.5	Transient	generative	perennial
<i>Festuca rubra</i>	2.71	3.21	2.88	2.75	2.13	1.88	1.75	2.29	1.25	1	Short-term	vegetative + generative	perennial

<i>Filipendula ulmaria</i>	0.77	1	2	0.89	1.88	1.32	0.59	1	0.69	0.5	Short-term	vegetative + generative	perennial
<b><i>Galium album</i></b>	<b>24.17</b>	<b>25.79</b>	<b>28.96</b>	<b>28.75</b>	<b>23.75</b>	<b>24.17</b>	<b>25.83</b>	<b>12.5</b>	<b>13.33</b>	<b>11.67</b>	<b>Short-term</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Galium verum</i>	0.53							0.1			Short-term	vegetative + generative	perennial
<b><i>Geranium pratense</i></b>	<b>12.46</b>	<b>20.94</b>	<b>25.79</b>	<b>18.79</b>	<b>24.75</b>	<b>22.25</b>	<b>32.5</b>	<b>18.75</b>	<b>21.67</b>	<b>23.13</b>	<b>Transient</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Glechoma hederacea</i>	2.5		2.13	1.29	0.71	1.21	0.78	1.88	0.96	0.79	Long-term	vegetative + generative	perennial
<i>Hieracium pilosella</i>					0.1	0.5	1	1	1		Short-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	2.96	3.13	6.46	8.13	5.83	7.29	4.5	4.5	2.75	1.5	Long-term	vegetative + generative	perennial
<i>Hypocoris radicata</i>					0.1	0.1		0.3			Short-term	generative	perennial
<i>Lathyrus pratensis</i>	16.57	13.88	9.51	3.57	3.01	3.08	2.54	2.38	1.25	0.88	Transient	generative	perennial
<i>Leucanthemum vulgare</i>	2.5	1	1								N/A	vegetative + generative	perennial
<i>Lolium perenne</i>	1.25	2.25	1.75	0.83	1	0.75	0.65		0.5	0.5	Long-term	generative	perennial
<i>Lysimachia nummularia</i>								2.5			N/A	vegetative	perennial
<i>Medicago lupulina</i>								7.5	3.75	1	Long-term	vegetative + generative	a, b, p*
<i>Plantago lanceolata</i>	3.3	3.08	2.64	5.13	5.29	2.79	1	2.04	0.96	0.92	Short-term	generative	perennial
<i>Poa pratensis</i>	2.5		2.38	1.88	1.46	1.71	1.38	2.75	5.67	2.92	Long-term	vegetative + generative	perennial
<i>Poa trivialis</i>	2.92		2.17	1.25	1.75	1.5	1.38	0.83	1	1	Long-term	vegetative + generative	perennial
<i>Prunella vulgaris</i>			0.5		0.55						Short-term	vegetative + generative	perennial
<i>Ranunculus acris</i>	0.47	1	0.5	0.43	0.38	0.48	0.41	0.53	0.88	0.88	Short-term	generative	perennial
<i>Rumex acetosa</i>	0.53	0.4	0.54	0.5	0.52	0.67	0.59	1.67	0.88	0.83	Long-term	vegetative + generative	perennial
<i>Sanguisorba officinalis</i>	2.29	1.92	2.92	2.63	6.04	3.88	3.29	3.29	1.8	1.09	Transient	vegetative + generative	perennial
<i>Saxifraga granulata</i>	0.45		0.75	0.53	0.93	0.75	1.17	0.92	0.92	0.83	Long-term	vegetative	perennial
<i>Stellaria graminea</i>						0.75					N/A	vegetative + generative	perennial
<i>Taraxacum officinale</i>	0.5		0.1		0.1	0.3	0.3	0.3	0.5	0.5	N/A	generative	perennial
<i>Trifolium pratense</i>				0.1		0.5		0.75	1	0.5	Short-term	generative	perennial
<i>Trifolium repens</i>				1			0.3				Long-term	vegetative + generative	perennial
<b><i>Trisetum flavescens</i></b>	<b>6.63</b>	<b>12.46</b>	<b>8.21</b>	<b>6.54</b>	<b>5.5</b>	<b>7.71</b>	<b>9.58</b>	<b>7.38</b>	<b>5</b>	<b>5.5</b>	<b>Transient</b>	<b>generative</b>	<b>perennial</b>
<i>Veronica serpyffolia</i>								0.5			Long-term	vegetative + generative	perennial

**Table A. 5-2:** Species list of the aboveground vegetation of the elevated CO<sub>2</sub>-rings, with mean coverage from 2004 till 2013 and the functional traits seed bank type, reproduction type and life span (Klotz *et al.*, 2002; Poschlod *et al.*, 2003). Species with highest coverage are marked in bold.

Elevated CO <sub>2</sub>	Mean coverage in %										Traits		
	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Seed bank type	Reproduction type	Life span
<i>Agrostis tenuis</i>								2.5			N/A	vegetative + generative	perennial
<i>Ajuga reptans</i>				0.5	0.5		0.75	0.5			N/A	vegetative + generative	perennial
<i>Alopecurus pratensis</i>	1.75	1.75	4	1.59	1.25	1.13	0.75	0.75	0.83	0.88	Long-term	vegetative + generative	perennial
<i>Anthoxanthum odoratum</i>	2.5		2	1.25	1	1	1.25	2.6	1.5	1.42	N/A	vegetative + generative	perennial
<b><i>Arrhenatherum elatius</i></b>	<b>47.5</b>	<b>42.5</b>	<b>35</b>	<b>31.88</b>	<b>21.67</b>	<b>23.54</b>	<b>19.58</b>	<b>18.75</b>	<b>23.13</b>	<b>23.13</b>	<b>Short-term</b>	<b>generative</b>	<b>perennial</b>
<i>Avenochloa pubescens</i>	1	0.5	1	1	0.75	0.75	1	1	2.5		N/A	vegetative + generative	perennial
<i>Bellis perennis</i>			0.5	1	0.65	0.5	0.75	0.63	0.5	0.5	Long-term	vegetative + generative	perennial
<i>Bromus hordeaceus</i>	0.1										Short-term	generative	annual
<i>Bromus racemosus</i>							0.1				N/A	generative	annual
<i>Cardamine pratensis</i>	0.5			0.1			0.3	0.37			Long-term	vegetative	perennial
<i>Cerastium holosteoides</i>	1	0.5			1.63		1.08	1	1.17	0.92	N/A	vegetative + generative	perennial
<i>Cirsium vulgare</i>							0.5				N/A	generative	perennial
<i>Colchicum autumnale</i>			0.1								Transient	vegetative + generative	perennial
<i>Dactylis glomerata</i>	5.71	2.59	3.17	4	2.55	3	2.96	1.63	1.13	0.92	N/A	generative	perennial
<i>Deschampsia cespitosa</i>	2.5	1						1			N/A	generative	perennial
<i>Erophila verna</i>								1.38	0.75	0.5	Long-term	generative	annual
<i>Festuca pratensis</i>	1	0.5			0.5	0.75		1	1	1	Transient	generative	perennial
<i>Festuca rubra</i>	2.13	3	2.5	1.75	1.75	1.57	1.19	1.92	1.09	1.21	Short-term	vegetative + generative	perennial
<b><i>Galium album</i></b>	<b>24.38</b>	<b>27.29</b>	<b>31.46</b>	<b>28.75</b>	<b>30.84</b>	<b>25.42</b>	<b>29.38</b>	<b>12.71</b>	<b>14.17</b>	<b>15</b>	<b>Short-term</b>	<b>vegetative + generative</b>	<b>perennial</b>
<b><i>Geranium pratense</i></b>	<b>3.5</b>	<b>7.25</b>	<b>9.88</b>	<b>14.02</b>	<b>17.38</b>	<b>22.67</b>	<b>22.71</b>	<b>16.46</b>	<b>18.75</b>	<b>18.75</b>	<b>Transient</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Glechoma hederacea</i>	2.84	1.75	2.17	2.13	1.71	2.21	1.7	1.71	1	0.79	Long-term	vegetative + generative	perennial
<i>Hieracium pilosella</i>					0.1						Short-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	2.13	7.09	7.17	3.92	5.38	4	2.13	2.88	2.13	2.13	Long-term	vegetative + generative	perennial

<i>Lathyrus pratensis</i>	10.04	4.13	7.3	4.38	5.46	5	4.54	6.71	1.46	1.17	Transient	generative	perennial
<i>Leucanthemum vulgare</i>	0.5		1								N/A	vegetative + generative	perennial
<i>Lolium perenne</i>	1	2.25	1.94	1.38	0.78	0.72	0.88	0.53	0.5	0.5	Long-term	generative	perennial
<i>Lotus corniculatus</i>	1				1	0.5	0.5	0.5	1	1	Long-term	generative	perennial
<i>Luzula campestris</i>								0.5	0.75	0.5	Long-term	vegetative + generative	a, b, p*
<i>Lysimachia nummularia</i>									1		N/A	vegetative	perennial
<i>Medicago lupulina</i>								1			Long-term	vegetative + generative	a, b, p*
<i>Plantago lanceolata</i>	1.04	0.75	1.54	1.42	1.09	0.71	1.58	1.63	0.88	0.88	Short-term	generative	perennial
<i>Poa pratensis</i>	1.75	1	2	2	1.63	1.63	1.5	1.5	2.75	3.63	Long-term	vegetative + generative	perennial
<i>Poa trivialis</i>	1.75	1	2.08	1.67		1.5		0.92	0.92	0.92	Long-term	vegetative + generative	perennial
<i>Prunella vulgaris</i>			0.5	1							Short-term	vegetative + generative	perennial
<i>Ranunculus acris</i>	0.5	0.5	0.23	0.23	0.3	0.54	0.58	0.4	0.58	0.63	Short-term	generative	perennial
<i>Rumex acetosa</i>	0.52	0.65	0.67	0.58	0.79	0.75	0.88	1.75	0.83	0.83	Long-term	vegetative + generative	perennial
<i>Sanguisorba officinalis</i>	1.42	1.46	3.25	1.96	3.04	3.79	2.29	3.68	1.21	2.63	Transient	vegetative + generative	perennial
<i>Saxifraga granulata</i>	1	0.5	0.37	0.5	0.92	0.75	1	0.92	0.96	1.08	Long-term	vegetative	perennial
<i>Senecio jacobaea</i>	0.5		1			1					Short-term	generative	perennial
<i>Stellaria graminea</i>						0.5					N/A	vegetative + generative	perennial
<i>Taraxacum officinale</i>	0.5	0.1	0.1	0.1	0.1	0.1	0.5				N/A	generative	perennial
<i>Trifolium pratense</i>								0.88	1	0.5	Short-term	generative	perennial
<i>Trifolium repens</i>							0.5	1			Long-term	vegetative + generative	perennial
<b><i>Trisetum flavescens</i></b>	<b>7.38</b>	<b>18.88</b>	<b>6.46</b>	<b>7.29</b>	<b>7.67</b>	<b>8.71</b>	<b>8.5</b>	<b>8.63</b>	<b>4.38</b>	<b>4.25</b>	<b>Transient</b>	<b>generative</b>	<b>perennial</b>
<i>Veronica serpyffolia</i>				1			0.75				Long-term	vegetative + generative	perennial

**Table A. 5-3:** Soil seed bank species of the single rings with seedling number per depth and the functional traits seed bank type, reproduction type and life span (Klotz *et al.*, 2002; Poschlod *et al.*, 2003). Species with highest seedling number are marked in bold. E = elevated CO<sub>2</sub>; A = ambient CO<sub>2</sub>.

Ring	Seedlings per depth			Traits		
E1	0 - 1 cm	1 - 5 cm	5 - 10 cm	Seed bank type	Reproduction type	Life span
<i>Arrhenatherum elatius</i>	-	-	1	Short-term	generative	perennial
<i>Bellis perennis</i>	2	-	-	Long-term	vegetative + generative	perennial
<i>Cardamine pratensis</i>	1	-	-	Long-term	vegetative	perennial
<b><i>Cerastium holosteoides</i></b>	<b>77</b>	<b>200</b>	<b>49</b>	<b>N/A</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Cirsium arvense</i>	-	1	-	N/A	vegetative + generative	perennial
<i>Cynosurus cristatus</i>	1	9	-	Transient	generative	perennial
<b><i>Erophila verna</i></b>	<b>25</b>	<b>20</b>	<b>5</b>	<b>Long-term</b>	<b>generative</b>	<b>annual</b>
<i>Festuca pratensis</i>	6	-	-	Transient	generative	perennial
<i>Festuca rubra</i>	2	-	-	Short-term	vegetative + generative	perennial
<i>Galium album</i>	1	1	1	Short-term	vegetative + generative	perennial
<i>Lolium perenne</i>	1	7	-	Long-term	generative	perennial
<i>Lotus corniculatus</i>	-	1	-	Long-term	generative	perennial
<i>Lysimachia numularia</i>	-	1	1	N/A	vegetative	perennial
<i>Poa pratensis</i>	3	6	-	Long-term	vegetative + generative	perennial
<i>Poa trivialis</i>	3	-	-	Long-term	vegetative + generative	perennial
<i>Rumex acetosa</i>	3	7	1	Long-term	vegetative + generative	perennial
<i>Saxifraga granulata</i>	1	10	2	Long-term	vegetative	perennial
<i>Trisetum flavescens</i>	4	-	-	Transient	generative	perennial
<i>Urtica dioica</i>	-	-	3	Long-term	vegetative + generative	perennial
E2	0 - 1 cm	1 - 5 cm	5 - 10 cm	Seed bank type	Reproduction type	Life span
<i>Arrhenatherum elatius</i>	1	-	-	Short-term	generative	perennial
<i>Bellis perennis</i>	2	-	2	Long-term	vegetative + generative	perennial

<i>Bromus hordaceus</i>	-	1	-	Short-term	generative	annual
<i>Cardamine pratensis</i>	<b>83</b>	<b>204</b>	<b>41</b>	<b>Long-term</b>	<b>vegetative</b>	<b>perennial</b>
<i>Cerastium holosteoides</i>	<b>35</b>	<b>84</b>	<b>24</b>	<b>N/A</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Cirsium arvense</i>	-	1	-	N/A	vegetative + generative	perennial
<i>Cynosurus cristatus</i>	-	4	-	Transient	generative	perennial
<i>Dactylis glomerata</i>	1	-	-	N/A	generative	perennial
<i>Erophila verna</i>	<b>10</b>	<b>28</b>	<b>6</b>	<b>Long-term</b>	<b>generative</b>	<b>annual</b>
<i>Festuca pratensis</i>	5	16	-	Transient	generative	perennial
<i>Galium album</i>	1	-	1	Short-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	4	3	1	Long-term	vegetative + generative	perennial
<i>Juncus bufonius</i>	<b>2</b>	<b>21</b>	<b>35</b>	<b>Long-term</b>	<b>generative</b>	<b>annual</b>
<i>Lolium perenne</i>	-	3	-	Long-term	generative	perennial
<i>Lysimachia numularia</i>	-	-	1	N/A	vegetative	perennial
<i>Poa pratensis</i>	-	1	-	Long-term	vegetative + generative	perennial
<i>Rumex acetosa</i>	-	1	-	Long-term	vegetative + generative	perennial
<i>Saxifraga granulata</i>	2	4	1	Long-term	vegetative	perennial
<i>Trisetum flavescens</i>	-	1	-	Transient	generative	perennial
<i>Urtica dioica</i>	1	3	-	Long-term	vegetative + generative	perennial
<b>E3</b>	<b>0 - 1 cm</b>	<b>1 - 5 cm</b>	<b>5 - 10 cm</b>	<b>Seed bank type</b>	<b>Reproduction type</b>	<b>Life span</b>
<i>Ajuga reptans</i>	-	1	-	N/A	vegetative + generative	perennial
<i>Alopecurus pratensis</i>	1	1	1	Long-term	vegetative + generative	perennial
<i>Arrhenatherum elatius</i>	-	3	-	Short-term	generative	perennial
<i>Cerastium holosteoides</i>	<b>20</b>	<b>31</b>	<b>5</b>	<b>N/A</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Cynosurus cristatus</i>	-	8	1	Transient	generative	perennial
<i>Festuca pratensis</i>	10	3	1	Transient	generative	perennial
<i>Festuca rubra</i>	3	4	4	Short-term	vegetative + generative	perennial
<i>Geranium pratense</i>	-	2	-	Transient	vegetative + generative	perennial

<i>Glechoma hederacea</i>	-	1	-	Long-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	2	2	1	Long-term	vegetative + generative	perennial
<i>Lolium perenne</i>	1	4	-	Long-term	generative	perennial
<i>Phleum pratense</i>	-	1	-	Transient	vegetative + generative	perennial
<i>Poa pratensis</i>	1	5	3	Long-term	vegetative + generative	perennial
<b><i>Rumex acetosa</i></b>	<b>4</b>	<b>13</b>	<b>5</b>	<b>Long-term</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Saxifraga granulata</i>	1	12	1	Long-term	vegetative	perennial
<i>Sonchus asper</i>	-	1	-	N/A	generative	annual
<i>Taraxacum officinale</i>	3	5	-	N/A	generative	perennial
<i>Trisetum flavescens</i>	-	1	1	Transient	generative	perennial
<b>A1</b>	<b>0 - 1 cm</b>	<b>1 - 5 cm</b>	<b>5 - 10 cm</b>	<b>Seed bank type</b>	<b>Reproduction type</b>	<b>Life span</b>
<i>Ajuga reptans</i>	1	-	2	N/A	vegetative + generative	perennial
<i>Bellis perennis</i>	2	-	-	Long-term	vegetative + generative	perennial
<i>Bromus hordaceus</i>	-	1	-	Short-term	generative	annual
<i>Cardamine pratensis</i>	-	1	-	Long-term	vegetative	perennial
<b><i>Cerastium holosteoides</i></b>	<b>24</b>	<b>39</b>	<b>25</b>	<b>N/A</b>	<b>vegetative + generative</b>	<b>perennial</b>
<b><i>Erophila verna</i></b>	<b>24</b>	<b>25</b>	<b>1</b>	<b>Long-term</b>	<b>generative</b>	<b>annual</b>
<i>Festuca pratensis</i>	-	2	-	Transient	generative	perennial
<i>Festuca rubra</i>	3	4	1	Short-term	vegetative + generative	perennial
<i>Galium album</i>	3	3	2	Short-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	4	3	-	Long-term	vegetative + generative	perennial
<i>Juncus bufonius</i>	-	-	1	Long-term	generative	annual
<i>Lathyrus pratensis</i>	-	1	-	Transient	generative	perennial
<i>Lolium perenne</i>	-	3	-	Long-term	generative	perennial
<i>Lysimachia numularia</i>	2	4	-	N/A	vegetative	perennial
<i>Plantago lanceolata</i>	1	2	-	Short-term	generative	perennial
<i>Poa pratensis</i>	1	2	1	Long-term	vegetative + generative	perennial

<i>Poa trivialis</i>	1	-	-	Long-term	vegetative + generative	perennial
<i>Saxifraga granulata</i>	<b>12</b>	<b>12</b>	<b>2</b>	<b>Long-term</b>	<b>vegetative</b>	<b>perennial</b>
<i>Trifolium pratense</i>	-	-	1	Short-term	generative	perennial
<i>Trisetum flavescens</i>	1	2	-	Transient	generative	perennial
<i>Urtica dioica</i>	-	11	1	Long-term	vegetative + generative	perennial
<b>A2</b>	<b>0 - 1 cm</b>	<b>1 - 5 cm</b>	<b>5 - 10 cm</b>	<b>Seed bank type</b>	<b>Reproduction type</b>	<b>Life span</b>
<i>Agrostis stolonifera</i>	-	-	1	Long-term	vegetative + generative	perennial
<i>Arrhenatherum elatius</i>	1	2	1	Short-term	generative	perennial
<i>Bellis perennis</i>	1	-	-	Long-term	vegetative + generative	perennial
<i>Cardamine pratensis</i>	2	-	-	Long-term	vegetative	perennial
<i>Cerastium holosteoides</i>	<b>34</b>	<b>47</b>	<b>10</b>	<b>N/A</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Cynosurus cristatus</i>	-	1	2	Transient	generative	perennial
<i>Erophila verna</i>	1	2	-	Long-term	generative	annual
<i>Epilobium parviflorum</i>	-	-	1	N/A	vegetative + generative	perennial
<i>Epilobium hirsutum</i>	-	1	-	N/A	vegetative + generative	perennial
<i>Festuca pratensis</i>	3	6	1	Transient	generative	perennial
<i>Festuca rubra</i>	1	4	-	Short-term	vegetative + generative	perennial
<i>Galium album</i>	1	1	-	Short-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	2	10	2	Long-term	vegetative + generative	perennial
<i>Juncus bufonius</i>	-	2	6	Long-term	generative	annual
<i>Lolium perenne</i>	-	3	-	Long-term	generative	perennial
<i>Lysimachia numularia</i>	-	1	-	N/A	vegetative	perennial
<i>Plantago lanceolata</i>	-	1	3	Short-term	generative	perennial
<i>Poa pratensis</i>	3	-	1	Long-term	vegetative + generative	perennial
<i>Rumex acetosa</i>	2	-	-	Long-term	vegetative + generative	perennial
<i>Saxifraga granulata</i>	1	15	-	Long-term	vegetative	perennial
<i>Taraxacum officinale</i>	-	-	1	N/A	generative	perennial

<i>Trisetum flavescens</i>	-	1	-	Transient	generative	perennial
<i>Urtica dioica</i>	-	-	1	Long-term	vegetative + generative	perennial
<b>A3</b>	<b>0 - 1 cm</b>	<b>1 - 5 cm</b>	<b>5 - 10 cm</b>	<b>Seed bank type</b>	<b>Reproduction type</b>	<b>Life span</b>
<i>Ajuga reptans</i>	-	-	1	N/A	vegetative + generative	perennial
<i>Arrhenatherum elatius</i>	-	1	-	Short-term	generative	perennial
<i>Bellis perennis</i>	4	2	1	Long-term	vegetative + generative	perennial
<i>Bromus hordaceus</i>	1	-	-	Short-term	generative	annual
<i>Cardamine pratensis</i>	<b>19</b>	<b>59</b>	<b>17</b>	<b>Long-term</b>	<b>vegetative</b>	<b>perennial</b>
<i>Cerastium holosteoides</i>	<b>46</b>	<b>139</b>	<b>36</b>	<b>N/A</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Cynosurus cristatus</i>	2	8	-	Transient	generative	perennial
<i>Erophila verna</i>	<b>3</b>	<b>49</b>	<b>3</b>	<b>Long-term</b>	<b>generative</b>	<b>annual</b>
<i>Epilobium hirsutum</i>	-	-	1	N/A	vegetative + generative	perennial
<i>Festuca pratensis</i>	<b>6</b>	<b>17</b>	<b>1</b>	<b>Transient</b>	<b>generative</b>	<b>perennial</b>
<i>Festuca rubra</i>	3	2	1	Short-term	vegetative + generative	perennial
<i>Galium album</i>	5	2	-	Short-term	vegetative + generative	perennial
<i>Glechoma hederacea</i>	-	4	-	Long-term	vegetative + generative	perennial
<i>Holcus lanatus</i>	-	2	-	Long-term	vegetative + generative	perennial
<i>Juncus bufonius</i>	-	1	-	Long-term	generative	annual
<i>Lolium perenne</i>	1	2	-	Long-term	generative	perennial
<i>Lotus corniculatus</i>	-	1	-	Long-term	generative	perennial
<i>Lysimachia numularia</i>	1	-	-	N/A	vegetative	perennial
<i>Poa pratensis</i>	6	3	-	Long-term	vegetative + generative	perennial
<i>Poa trivialis</i>	-	1	-	Long-term	vegetative + generative	perennial
<i>Rumex acetosa</i>	-	2	1	Long-term	vegetative + generative	perennial
<i>Saxifraga granulata</i>	2	2	-	Long-term	vegetative	perennial
<i>Sonchus oleaceus</i>	-	1	-	Long-term	generative	perennial
<i>Taraxacum officinale</i>	2	-	2	N/A	generative	perennial

<i>Trisetum flavescens</i>	-	1	-	Transient	generative	perennial
<i>Urtica dioica</i>	<b>2</b>	<b>12</b>	<b>9</b>	<b>Long-term</b>	<b>vegetative + generative</b>	<b>perennial</b>
<i>Veronica chamaedris</i>	-	1	-	N/A	vegetative + generative	perennial

**Table A. 5-4:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively). Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic.

Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 10 cm					0 - 10 cm				
Source of variation	df	F	ssq	p	Source of variation	df	F	ssq	p
CO <sub>2</sub>	1	1.081	6.561	0.321	CO <sub>2</sub>	<b>1</b>	<b>9.152</b>	<b>0.117</b>	<b>0.012</b>
Depth	<b>2</b>	<b>9.455</b>	<b>114.778</b>	<b>0.004</b>	Depth	<b>2</b>	<b>17.141</b>	<b>0.437</b>	<b>&lt;0.001</b>
CO <sub>2</sub> x Depth	2	0.174	2.111	0.843	CO <sub>2</sub> x Depth	2	0.448	0.011	0.650
Soil moisture (Co-variable)	1	0.533	3.235	0.481	Soil moisture (Co-variable)	<b>1</b>	<b>8.935</b>	<b>0.114</b>	<b>0.012</b>
Error	11		66.765		Error	11		0.140	
Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 1 cm					0 - 1 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.048	0.165	0.840	CO <sub>2</sub>	1	4.335	0.099	0.129
Soil moisture (Co-variable)	1	0.704	2.408	0.463	Soil moisture (Co-variable)	1	3.780	0.087	0.147
Error	3		10.259		Error	3		0.069	
Species number					Seed density in seeds cm <sup>-3</sup>				
1 - 5 cm					1 - 5 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.142	2.218	0.732	CO <sub>2</sub>	1	3.881	0.050	0.143

Soil moisture (Co-variable)	1	0.404	6.327	0.570	Soil moisture (Co-variable)	1	4.259	0.055	0.131
Error	3		47.006		Error	3		0.039	
Species number					Seed density in seeds cm <sup>-3</sup>				
5 - 10 cm					5 - 10 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	6.258	6.454	0.088	CO <sub>2</sub>	1	4.132	0.003	0.135
Soil moisture (Co-variable)	1	0.878	0.906	0.418	Soil moisture (Co-variable)	1	4.644	0.003	0.120
Error	3		3.094		Error	3		0.002	

**Table A. 5-5:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on Shannon-index and evenness in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively). Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic.

Shannon-Index					Evenness				
0 - 10 cm					0 - 10 cm				
Source of variation	df	F	ssq	p	Source of variation	df	F	ssq	p
CO <sub>2</sub>	1	4.183	0.188	0.066	CO <sub>2</sub>	<b>1</b>	<b>5.810</b>	<b>0.009</b>	<b>0.035</b>
Depth	<b>2</b>	<b>4.789</b>	<b>0.431</b>	<b>0.032</b>	Depth	2	0.397	0.001	0.681
CO <sub>2</sub> x Depth	2	0.106	0.01	0.900	CO <sub>2</sub> x Depth	2	0.020	6.14E-05	0.981
Soil moisture (Co-variable)	1	0.219	0.01	0.649	Soil moisture (Co-variable)	1	3.425	0.005	0.091
Error	11		0.494		Error	11		0.017	
Shannon-Index					Evenness				
0 - 1 cm					0 - 1 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	1.192	0.015	0.355	CO <sub>2</sub>	1	1.933	0.002	0.259
Soil moisture (Co-variable)	1	0.195	0.002	0.689	Soil moisture (Co-variable)	1	0.465	<0.001	0.544

Error	3		0.038		Error	3		0.002	
Shannon-Index					Evenness				
1 - 5 cm					1 - 5 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.603	0.063	0.494	CO <sub>2</sub>	1	1.362	0.004	0.327
Soil moisture (Co-variable)	1	<0.001	5.18E-05	0.984	Soil moisture (Co-variable)	1	0.715	0.002	0.460
Error	3		0.311		Error	3		0.008	
Shannon-Index					Evenness				
5 - 10 cm					5 - 10 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	4.280	0.143	0.130	CO <sub>2</sub>	1	2.264	0.004	0.229
Soil moisture (Co-variable)	1	1.573	0.052	0.299	Soil moisture (Co-variable)	1	2.161	0.004	0.238
Error	3		0.100		Error	3		0.006	

**Table A. 5-6:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with long-term persistent seed bank type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.

Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 10 cm*					0 - 10 cm				
Source of variation	df	F	ssq	p	Source of variation	df	F	ssq	p
CO <sub>2</sub>	1	2.445	0.038	0.146	CO <sub>2</sub>	<b>1</b>	<b>9.760</b>	<b>0.065</b>	<b>0.010</b>
Depth	<b>2</b>	<b>8.616</b>	<b>0.266</b>	<b>0.006</b>	Depth	<b>2</b>	<b>6.721</b>	<b>0.090</b>	<b>0.012</b>
CO <sub>2</sub> x Depth	2	0.956	0.030	0.414	CO <sub>2</sub> x Depth	2	0.357	0.005	0.708
Soil moisture (Co-variable)	1	2.176	0.034	0.168	Soil moisture (Co-variable)	<b>1</b>	<b>11.806</b>	<b>0.079</b>	<b>0.006</b>

Error	11		0.170		Error	11		0.073	
Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 1 cm					0 - 1 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.901	0.858	0.413	CO <sub>2</sub>	<i>1</i>	<i>7.426</i>	<i>0.067</i>	<i>0.072</i>
Soil moisture (Co-variable)	1	2.600	2.476	0.205	Soil moisture (Co-variable)	<i>1</i>	<i>8.907</i>	<i>0.080</i>	<i>0.058</i>
Error	3		2.857		Error	3		0.027	
Species number					Seed density in seeds cm <sup>-3</sup>				
1 - 5 cm					1 - 5 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.402	1.943	0.571	CO <sub>2</sub>	1	5.396	0.024	0.103
Soil moisture (Co-variable)	1	2.520	12.174	0.211	Soil moisture (Co-variable)	<i>1</i>	<i>6.959</i>	<i>0.031</i>	<i>0.078</i>
Error	3		14.493		Error	3		0.013	
Species number					Seed density in seeds cm <sup>-3</sup>				
5 - 10 cm					5 - 10 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	4.831	4.511	0.115	CO <sub>2</sub>	<i>1</i>	<i>6.147</i>	<i>0.001</i>	<i>0.089</i>
Soil moisture (Co-variable)	1	1.997	1.865	0.252	Soil moisture (Co-variable)	<i>1</i>	<i>5.576</i>	<i>0.001</i>	<i>0.099</i>
Error	3		2.802		Error	3		<0.001	

**Table A. 5-7:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with short-term persistent seed bank type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.

Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 10 cm					0 - 10 cm*				
Source of variation	df	F	ssq	p	Source of variation	df	F	ssq	p
CO <sub>2</sub>	<b>1</b>	<b>17.893</b>	<b>7.338</b>	<b>0.001</b>	CO <sub>2</sub>	<b>1</b>	<b>7.270</b>	<b>0.698</b>	<b>0.021</b>
Depth	2	2.845	2.333	0.101	Depth	<b>2</b>	<b>13.928</b>	<b>2.676</b>	<b>0.001</b>
CO <sub>2</sub> x Depth	2	2.574	2.111	0.121	CO <sub>2</sub> x Depth	2	0.946	0.182	0.418
Soil moisture (Co-variable)	1	0.379	0.155	0.551	Soil moisture (Co-variable)	1	1.790	0.172	0.208
Error	11		4.511		Error	11		1.057	
Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 1 cm					0 - 1 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	6.835	1.247	0.079	CO <sub>2</sub>	1	1.264	<0.001	0.343
Soil moisture (Co-variable)	1	0.654	0.119	0.478	Soil moisture (Co-variable)	1	1.537	<0.001	0.303
Error	3		0.547		Error	3		<0.001	
Species number					Seed density in seeds cm <sup>-3</sup>				
1 - 5 cm					1 - 5 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	<b>1</b>	<b>26.189</b>	<b>7.400</b>	<b>0.014</b>	CO <sub>2</sub>	1	2.650	4.89E-05	0.202
Soil moisture (Co-variable)	1	1.719	0.486	0.281	Soil moisture (Co-variable)	1	0.651	1.20E-05	0.479
Error	3		0.848		Error	3		5.53E-05	
Species number					Seed density in seeds cm <sup>-3</sup>				
5 - 10 cm					5 - 10 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.858	0.731	0.423	CO <sub>2</sub>	1	2.278	4.07E-06	0.228
Soil moisture (Co-variable)	1	0.129	0.110	0.744	Soil moisture (Co-variable)	1	2.975	5.31E-06	0.183

Error	3	2.557	Error	3	5.36E-06
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**Table A. 5-8:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with generative reproduction type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic. \* = Log10 transformed data.

Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 10 cm					0 - 10 cm*				
Source of variation	df	F	ssq	p	Source of variation	df	F	ssq	p
CO <sub>2</sub>	1	1.479	3.130	0.249	CO <sub>2</sub>	1	3.766	0.398	0.078
Depth	2	<b>11.209</b>	<b>47.444</b>	<b>0.002</b>	Depth	2	<b>16.019</b>	<b>3.382</b>	<b>0.001</b>
CO <sub>2</sub> x Depth	2	1.129	4.778	0.358	CO <sub>2</sub> x Depth	2	0.109	0.023	0.897
Soil moisture (Co-variable)	1	0.025	0.054	0.876	Soil moisture (Co-variable)	1	3.977	0.420	0.072
Error	11		23.280		Error	11		1.161	
Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 1 cm					0 - 1 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.983	1.843	0.394	CO <sub>2</sub>	1	0.834	0.003	0.428
Soil moisture (Co-variable)	1	1.626	3.046	0.292	Soil moisture (Co-variable)	1	0.242	0.001	0.657
Error	3		5.621		Error	3		0.011	
Species number					Seed density in seeds cm <sup>-3</sup>				
1 - 5 cm					1 - 5 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.950	2.351	0.662	CO <sub>2</sub>	1	3.906	0.001	0.143
Soil moisture (Co-variable)	1	0.233	0.576	0.402	Soil moisture (Co-variable)	1	<b>10.116</b>	<b>0.004</b>	<b>0.050</b>
Error	3		7.424		Error	3		0.001	

Species number					Seed density in seeds cm <sup>-3</sup>				
5 - 10 cm					5 - 10 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	<b>1</b>	<b>11.162</b>	<b>8.342</b>	<b>0.044</b>	CO <sub>2</sub>	1	1.238	<0.001	0.347
Soil moisture (Co-variable)	<i>1</i>	<i>5.920</i>	<i>4.424</i>	<i>0.093</i>	Soil moisture (Co-variable)	1	0.899	<0.001	0.413
Error	3		2.242		Error	3		0.001	

**Table A. 5-9:** Results of two-factorial ANCOVA about the effect of CO<sub>2</sub>, depth and their interaction on species number and seed density in the soil seed bank (total depth 0 – 10 cm) and separated into three layers (0 – 1 cm; 1 – 5 cm; 5 – 10 cm, respectively) of species with vegetative reproduction type. Soil moisture was included as a co-variable. Df = degrees of freedom; F = F-value; ssq = sum of squares; p = p-values. Significant values marked in bold and significant by tendency marked in italic.

Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 10 cm					0 - 10 cm				
Source of variation	df	F	ssq	p	Source of variation	df	F	ssq	p
CO <sub>2</sub>	1	3.500	0.885	0.088	CO <sub>2</sub>	1	7.117	0.052	0.022
Depth	2	1.778	3.517	0.066	Depth	2	1.640	0.012	0.238
CO <sub>2</sub> x Depth	2	7.913	4.000	0.007	CO <sub>2</sub> x Depth	2	0.249	0.002	0.013
Soil moisture (Co-variable)	1	10.100	2.553	0.009	Soil moisture (Co-variable)	1	8.694	0.064	0.784
Error	11		2.780		Error	11		0.007	

Species number					Seed density in seeds cm <sup>-3</sup>				
0 - 1 cm					0 - 1 cm				
	df	F	ssq	p		df	F	ssq	p
CO <sub>2</sub>	1	0.221	0.062	0.670	CO <sub>2</sub>	1	4.304	0.053	0.130
Soil moisture (Co-variable)	1	1.719	0.486	0.281	Soil moisture (Co-variable)	1	5.555	0.069	0.100
Error	3		0.848		Error	3		0.037	

Species number					Seed density in seeds cm <sup>-3</sup>				
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	1 - 5 cm			
	df	F	ssq	p
CO <sub>2</sub>	1	0.306	0.102	0.619
Soil moisture (Co-variable)	1	1.012	0.336	0.389
Error	3		0.997	
Species number				
	5 - 10 cm			
	df	F	ssq	p
CO <sub>2</sub>	<b>1</b>	<b>32.612</b>	<b>4.830</b>	<b>0.011</b>
Soil moisture (Co-variable)	<b>1</b>	<b>15.007</b>	<b>2.222</b>	<b>0.030</b>
Error	3		0.444	

	1 - 5 cm			
	df	F	ssq	p
CO <sub>2</sub>	1	3.796	0.020	0.147
Soil moisture (Co-variable)	1	4.214	0.022	0.132
Error	3		0.015	
Seed density in seeds cm <sup>-3</sup>				
	5 - 10 cm			
	df	F	ssq	p
CO <sub>2</sub>	1	5.088	0.001	0.109
Soil moisture (Co-variable)	1	6.269	0.001	0.087
Error	3		<0.001	

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\*) The reference list contains the references used from chapter 3.

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## **Eigenständigkeitserklärung**

Ich erkläre hiermit, dass ich die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt habe, die ich in der Dissertation angegeben habe.

Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Ruben Seibert

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