

# Plant Functional Types Differ in Their Long-term Nutrient Response to eCO<sub>2</sub> in an Extensive Grassland

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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 of this

study was to identify the effect of elevated atmospheric CO<sub>2</sub> concentrations on nutrient concentrations of grassland biomass that have been grown for 16 years (1998–2013). The 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. 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 posi-

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tively 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 such as water or nutrient availability affected plant nutrient concentrations under eCO<sub>2</sub>.

**Key words:** FACE; macronutrients; micronutrients; elevated CO<sub>2</sub>; climate change; GiFACE.

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## 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.

## 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 and others 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 and others 2018; Cantarel and others 2013; Feng and others 2015; Newton and others 2014).

Elevated CO<sub>2</sub> can induce direct plant physiological reactions such as reduced stomatal conductance and elevated photosynthetic rates (Haworth and others 2016; Tausz-Posch and others 2014). Together, these physiological reactions affect the plant nutrient uptake by downregulating the passive inflow of nutrients to the root with the transpiration stream (Houshmandfar and others 2018) and possibly stimulate 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, concentrations of micronutrients such as Fe and Mn, which are involved in electron transport, have been found to increase under eCO<sub>2</sub> (Natali and others 2009). On the other hand, plant uptake of nutrients like Ca and Mg is controlled by mass flow,

which associated with the transpiration stream (Nord and 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 and others 2018; Tausz-Posch and others 2014).

Ainsworth and others (2003) revealed increased C sink strengths due to elevated photosynthesis in grassland plants grown under eCO<sub>2</sub>. Moreover, plants grown under eCO<sub>2</sub> increased carbohydrate contents, which led to the explanation of dilution of nutrient concentrations in plant tissues by increased C assimilation (Ainsworth and Long 2021; Loladze 2002). However, in a meta-analysis, Feng and others (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. Similar evidence was reported by Houshmandfar and others (2018) for nutrient uptakes Ca, K, N, S, Mg and Mn in wheat that were 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 and others 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 and others 2003; Natali and others 2009). Additionally, increasing inputs of carbonic acid from plant roots and microbial respiration, due to eCO<sub>2</sub>, may increase soil acidity (Natali and others 2009). For slightly acidic soils, such a pH decrease could directly affect the availability of soil nutrients. The effect would be a decrease in the availability of macronutrients such as N, P, K, Ca, S and Mg and an increase in the availability of micronutrients such as Fe, Mn, Zn and Cu (Marschner 2002), which might in turn affect the nutritional status of plants (Chapin 1980).

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 and others 2017; Reich and others 2018), whereas gross N mineralization rates were often not responding to eCO<sub>2</sub> (Müller and others 2009; Rütting and Andresen 2015; Rütting and 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 and others 2004; Lüscher and Nösberger 1997). Legumes are found to benefit even more under eCO<sub>2</sub> as the N<sub>2</sub> fixation increases due to increasing nodule size, number of nodules per plant and a higher nitrogenase activity (Rogers and others 2009). The N supply via symbiotic N fixation can, through years, sustain an otherwise decreasing N availability for all plant species (Liang and others 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 and others 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 and others 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 and others 2012; Feng and others 2015; Sistla and Schimel 2012). Also, in a meta-analysis of 1418 studies, Yuan and Chen (2015) found that N-to-P ratios generally decreased under eCO<sub>2</sub>. However, observations suggest high variability in responses. Lower concentrations of Zn and Fe in response to eCO<sub>2</sub> were also observed in wheat, rice, beans and soybeans (Myers and others 2014; Smith and Myers 2018), indicating that the nutritive value of crops decline with negative impacts on human and livestock nutrition (Augustine and others 2018; Dumont and others 2015; Myers and others 2014; Seibert and others 2021; Smith and Myers 2018; Yuan and Chen 2015).

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

Furthermore, the total biomass production increased (+15%) under eCO<sub>2</sub> (Andresen and others 2018) and the forage quality declined, especially in forbs (Seibert and others 2021).

To reveal which of the macro- and micronutrients 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 lower availability of these nutrients due to a slight soil acidification.
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 because a slight acidification under eCO<sub>2</sub> increases the availability of these elements.

## MATERIALS AND METHODS

### 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 50–80 kg N ha<sup>-1</sup> y<sup>-1</sup>. Since 1993, the commercial fertilizers 'Thomas-Kali' and 'Kalk-Ammon-salpetere' 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> y<sup>-1</sup> was applied, which is equivalent to the following nutrient amounts: nitrogen: 4 g N m<sup>-2</sup> y<sup>-1</sup>, phosphorus: 6 g P m<sup>-2</sup> y<sup>-1</sup>, potassium: 9 g K m<sup>-2</sup> y<sup>-1</sup>, magnesium: 1.8 g Mg m<sup>-2</sup> y<sup>-1</sup> and calcium: 21.3 g Ca m<sup>-2</sup> y<sup>-1</sup> (Table S1, Andresen and others 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 and others 2018).

The vegetation is an 'Arrhenatheretum elatioris—Filipendula ulmaria sub-community' (Grüters and others 2006) according to the Braun-Blanquet classification. At the start of the study, the dominating grasses in terms of biomass were: *Ar-*

*rhenatherum 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 (< 1–2% by mass) when this study was initiated (Grüters and others 2006).

## Elevated CO<sub>2</sub> Treatments

Three circular plots (8 m inner diameter rings) were subjected to CO<sub>2</sub> enriched air (eCO<sub>2</sub>; + 20% above ambient conditions), while three rings were subjected to ambient CO<sub>2</sub> concentrations (aCO<sub>2</sub>), arranged in a randomized block design (3 blocks). CO<sub>2</sub> fumigation started in May 1998 and continued all-year round, from 2 h after astronomical sunrise until 2 h before astronomical sun set (Jäger and others 2003).

## 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 0–15 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 December to 2013 November) 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 aboveground 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), 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 and others (2018) and Obermeier and others (2017), Seibert and others (2021) and Yuan and others (2018).

## Biannual Harvest

Since 1993, harvests were conducted within 25 subplots with an area of 0.36 m<sup>2</sup> per subplot per ring (Andresen and others 2018). All vegetation was cut with garden scissors at 3–5 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 subplot a representative sample (relative to the biomass yield in each subplot) was mixed to one composite sample from the ring for further chemical analysis.

## Plant Leaf Nutrient Analysis

The element concentration of dried ground plant material was analyzed by standard methods. The samples for the years 1993–2013 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 sulfate, selenic (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 laboratories was made with samples that were initially analyzed in Belfast and then re-analyzed in Geisenheim (7 years later) which 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 al-

ways consistently analyzed within the same laboratory, 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.

## 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 and others 2014).

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

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 and others 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):

$$\text{Effect size } [\%] = ((eCO_2(X \text{ or } N_i) - aCO_2(X \text{ or } N_i)) / aCO_2(X \text{ or } N_i)) \times 100 \quad (2)$$

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 product matrix contains correlation coefficients among nutrient concentrations and 7 axes were interpreted (McCune and 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 and Mefford 2011). To analyze the coordinated effect of eCO<sub>2</sub> on plant nutrient concentrations, a one-way permutational multivariate analysis of variance (PerMANOVA) with Sorensen distance measure was carried out for each functional group and harvest (Anderson and Walsh 2013; McCune and 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 CO<sub>2</sub> × block, CO<sub>2</sub> × year, 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 and year × SPI. Soil moisture, STI and SPI data were aggregated to averages for the respective 3 months preceding the harvest (March–May for H1 and June to August for H2). The model was reduced for nonsignificant interactions ( $p \geq 0.1$ ) and evaluated for their goodness of fit based on the Akaike information criterion (Andresen and others 2018; Bandoly and others 2016; Safari and others 2016). Effects with  $p$  values  $\leq 0.05$  were considered significant,  $\leq 0.01$  very significant and  $\leq 0.001$  highly significant.

## 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 compared 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 standardized major axis tests and routines; Falster and others 2006; Warton and others 2006) was used for bivariate linear regression, using standardized 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-type grasses, forbs, legumes pairwise. A difference of slopes indicates that one functional plant type has a better nutrient uptake under eCO<sub>2</sub> than the other type.

## RESULTS

### 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 ( $p = 0.001$ ; Figure 1a and b; Table 1; Table S2; 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,  $p = 0.024$ ; Figure 1d; Table 1; Table S2) and increased concentrations in legumes (H2,  $p = 0.045$ ; Figure 1f; Table 1; Table S4). There was also a significant CO<sub>2</sub> × soil moisture interaction for P in grasses H1 (Table S2; Table S3).

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  $p \leq 0.001$ ; Table 1; Figure 1g; Table S2). Forbs also showed lower Ca concentrations under eCO<sub>2</sub> ( $p = 0.030$ ; Table 1; Figure 1h; Table S3).

The magnesium (Mg) concentrations decreased significantly in all functional groups and both harvests under eCO<sub>2</sub> (Table 1; Table S2, S3 and S4). Mg concentrations were smaller under eCO<sub>2</sub> for both harvests in grasses (Figure 1j), except in 2013 for H1 (H1,  $p = 0.008$ ; H2,  $p = 0.004$ ; Figure 1j; Table 1; Table S2). Forbs had a negative eCO<sub>2</sub> effect for both harvests (H1,  $p = 0.005$ ; H2,  $p = 0.043$ ; Table 1; Table S3), except in 2000 for H1 and in 2005, 2008, 2010 and 2013 for H2 (Figure 1k). In legumes, a smaller Mg concentration under eCO<sub>2</sub> occurred for the whole time series for H1 ( $p = 0.009$ ; Figure 1l; Table 1; Table S4) and with only one exception in 2000 for H2 ( $p = 0.030$ ; Figure 1l; Table 1; Table S4).

Potassium (K) concentrations 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,  $p = 0.024$ ; Figure 1m; Table 1; Table S2). Forbs had a positive CO<sub>2</sub> effect for H2, with higher K concentrations under eCO<sub>2</sub>, except in 2010 ( $p = 0.036$ ; Figure 1n; Table 1; Table S3).

Concentrations of Zn, Fe, Cu and Mn indicated no significant CO<sub>2</sub> effects in any of the functional groups (Table 1). Significant effects of site conditions ('block'), year and interactions in the linear mixed model are presented in detail in the supplementary tables (Table S2; Table S3; Table S4).

A joint analysis of the three functional groups (grasses, forbs and legumes) revealed a clear separation among all nutrient concentrations under ambient and elevated CO<sub>2</sub> by the un-rotated s-

mode principal components analysis (PCA), which also revealed a clear separation between H1 (spring) and H2 (summer) (Figure S1). However, a clear effect in the functional groups as a response to CO<sub>2</sub> treatment was not found and this was confirmed by the PerMANOVA analysis (Table S5).

### 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 and others 2018). The nutrient yield response relative to the biomass response correlated positively between all functional types (Table S6). 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 (Table S6).

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  $Y_0$ -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; Figure 2a; Table S6). 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 (Figure 2b). For legumes, the  $Y_0$  was negative only for Mg and positive for Mn, (Figure 2c). The largest reduction of Fe and Mn nutrient yields ( $-63\%$  and  $-20\%$ , respectively;  $Y_0$  in Table S6) was found in grasses, although they had smaller yield reductions of other nutrients (between 1 and 10%). Contrastingly, forbs and legumes showed the largest yield increase of Mn of 8% and 10% (Figure 2b and c; Table S6). The mean N yield of grasses and forbs were about 3% reduced, while the mean N yield of legumes was 5% reduced (for example,  $Y_0 = -3.29\%$ ,  $-2.66\%$  and  $-5.44\%$ , respectively; Table S6).

## DISCUSSION

In this study, we found that eCO<sub>2</sub> reduced plant nutrient concentrations in grasses (N, P, Ca, Mg, K; Table 1; Figure 1), although eCO<sub>2</sub> had reducing effects only for Ca and Mg concentrations in forbs and legumes, but showed increasing effects on K

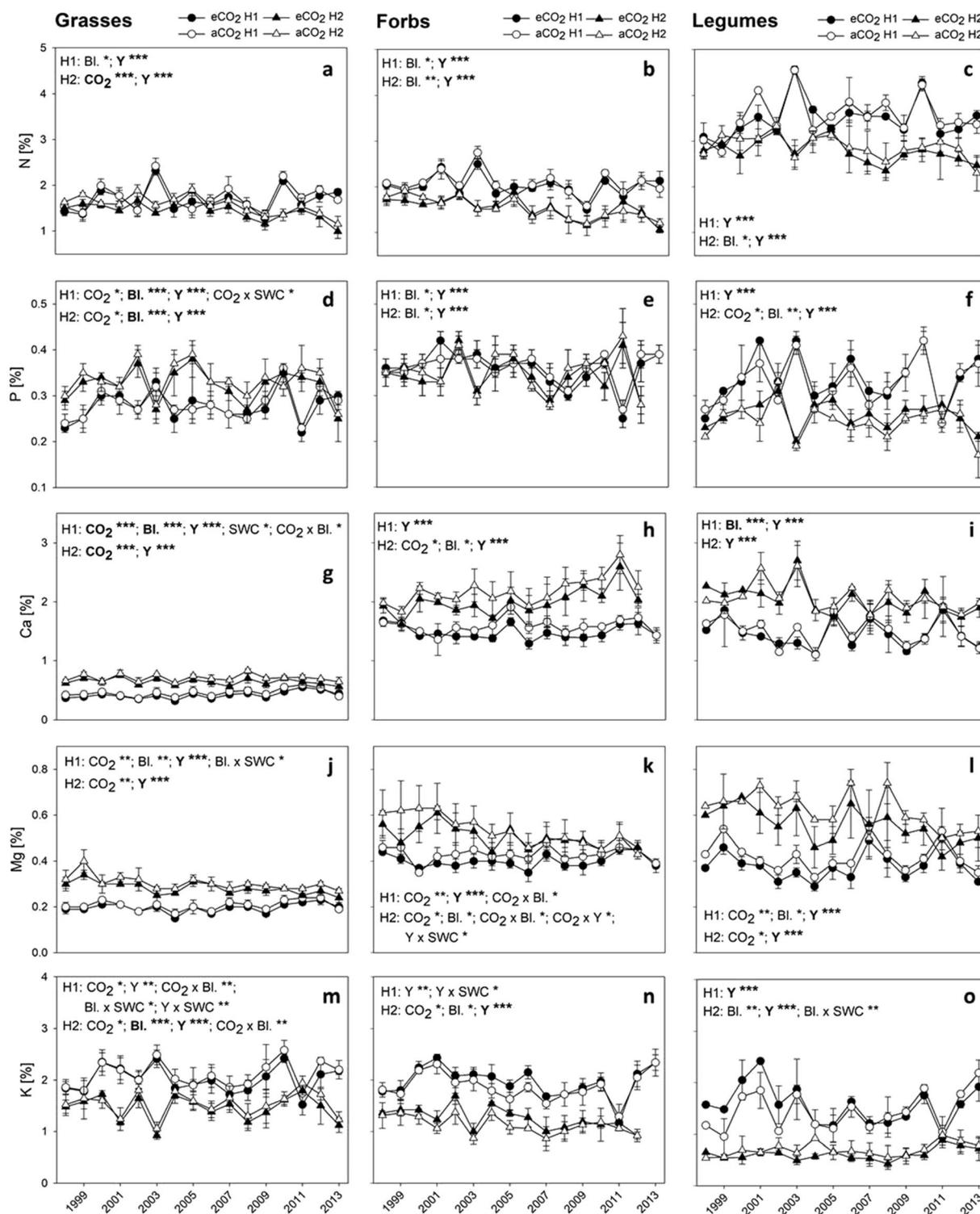


Figure 1. Concentrations of N (a, b, c), P (d, e, f), Ca (g, h, i), Mg (j, k, l) and K (m, n, o) in % of grasses, forbs and legumes, separated in harvest 1 (H1; circles) and harvest 2 (H2; triangles). Error bars indicate standard deviation. The results of the mixed model analysis are indicated for treatment (CO<sub>2</sub>), block (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

**Table 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)

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

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. Gray color indicates decreasing trends, and black color indicates increasing trends

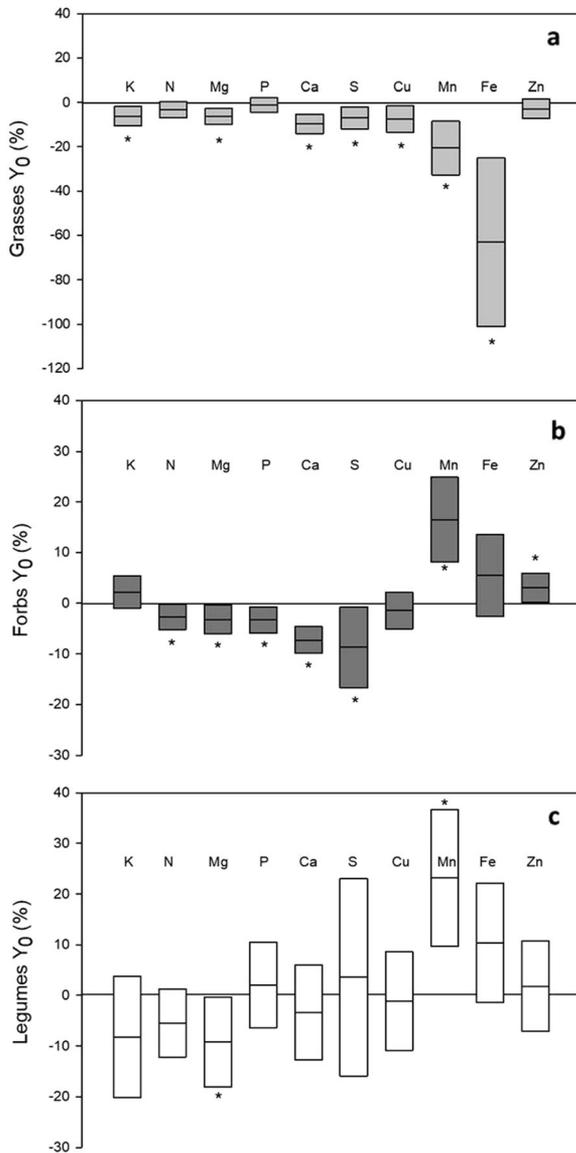
and P concentrations in forbs and in legumes, respectively (Table 1; Figure 1). The nutrient yield response  $Y_0$  (Figure 2 and Table S6) also pointed toward reduced nutrient yields in grasses, while in forbs and legumes they increased and reduced. This partly confirmed our hypothesis 1 of differences among the functional groups regarding N, but the increase in concentration and yields contradicted hypothesis 2 which suggested a decrease. Compared to forbs and legumes, the concentration reductions in grasses were more intense for H2 than for H1 (Table 1). This reduction occurred especially in 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 than at H1. Provided that the spring fertilization is enhancing nutrient availability for all species, the physiological differences of the functional groups for nutrient acquisition may become more important to explain the observed differences. 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 were in line with 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 and Long 2021; Loladze 2002), (ii)

decreased water inflow due to improved water use efficiency, carrying fewer nutrients (Houshmandfar and others 2018; Tausz-Posch and others 2014) or (iii) decreased water mass flow caused by downregulation of photosynthesis because of low C sink strength (Ainsworth and others 2003) and therefore reduced stomatal conductance.

The meta-analysis across FACE sites by Feng and others (2015) suggested that the dilution (i. above) in plant tissue cannot fully explain reduced N yields, rather a reduced uptake of N is realistic (ii. and iii. above), which was the case in our study, as indicated by the negative Y-axis intercept  $Y_0$  for zero biomass response to eCO<sub>2</sub> (Figure 2; Table S6). 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 (Figure 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 and Shuman 1995), or a high assimilation efficiency and therefore a reduced nutrient inflow via reduced transpiration under eCO<sub>2</sub> (Haworth and others 2016). Furthermore, a downregulation



**Figure 2.** Response to CO<sub>2</sub> for the macronutrient elements K, N, Mg, P, Ca, S and the micronutrient elements Cu, Mn, Fe and Zn, seen as the effect size of plant nutrient yield (% reduction or increase from eCO<sub>2</sub>) at Y<sub>0</sub> which is the extrapolated point of zero biomass response to eCO<sub>2</sub>, jointly for harvest 1 and 2. The intercept Y<sub>0</sub> was found using standardized major axis test considering variance at both axes (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<sub>0</sub> 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<sub>0</sub> 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

of photosynthesis and low C sink strength (Ainsworth and others 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 and others 2014). Investigations by Brenzinger and others (2017) at the GiFACE site indicated only marginally and nonsignificant differences in soil characteristics (that is, pH, soil moisture, C content, N content) between eCO<sub>2</sub> and aCO<sub>2</sub>. Results by Moser and others (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>, is a widespread effect in different pH studies (Hinsinger and others 2003; Natali and others 2009) leading to an increasing (for example, Fe, Mn, Zn and Cu) or decreasing (for example, 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 and others 2017), this pH controlled nutrient availability seems to be partly reflected in the rhizosphere by the observed forb (and legume) nutrient yield responses, for example, more Fe, Mn and Zn yield and less N, P, Ca, Mg yield (Figure 2) and therefore at least 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. 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 a decline in nutrient contents (Houshmandfar and others 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 (Table S2; Table S3; Table S4). This was in line with previous studies at the GiFACE, where the CO<sub>2</sub> fertilization effect on biomass yield (Andresen and others 2018), as well as the CO<sub>2</sub> effect on forage quality (Seibert and others 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 sim-

ilar 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 and others 2017; Yuan and others 2018). For example, during extreme climatic events like heat and drought, transpiration reduced via 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 were 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) and interactions (that is, CO<sub>2</sub> × soil moisture in P and CO<sub>2</sub> × block in Ca; Table S2) put the CO<sub>2</sub> effect into perspective. These interactions indicated the dependence of the CO<sub>2</sub> fertilization effect on soil moisture. The significant block × soil moisture interaction in Mg and K for grasses H1 (Table S2), reflects the moisture gradient at the GiFACE site, which led to the block partitioning of the FACE rings (Jäger and others 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 ( $p = 0.008$ ; Table S2; Figure 1j). However, K concentrations indicated further significant interactions, like for grasses H2 (Table S2), 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 and others (2021), seen in the quality parameter ash.

## 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> enrichment. 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, whereas single element concentrations in forbs and legumes showed a positive eCO<sub>2</sub> effect of nutrient yields. 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 due to C sink limitation and therefore reduced photosynthesis and transpiration via reduced stomata aperture. Furthermore, we 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>. In particular, 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, for example forage production and grassland management, and adjustment of fertilization regimes might be needed.

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## Declaration

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

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