



Rising CO₂ concentrations reduce nitrogen availability in alpine grasslands

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ABSTRACT

Alpine grasslands, local biodiversity hotspots with very high nature conservation and cultural value, belong to one of the most affected ecosystems by global change. Yet, the potential effects of others than global warming factors on alpine plant functioning are poorly understood. To address this gap, we made use of 359 herbarium specimens from nine vascular plant species collected in the Bavarian Alps, Germany, extending back 200 years (1807–2018) to reconstruct historical changes in foliar N content and stable isotope composition ($\delta^{15}\text{N}$), indicators of plant response to long-term N atmospheric deposition and rising atmospheric CO₂ concentrations ([CO₂]). These changes were interpreted in terms of three competing hypotheses (eutrophication, oligotrophication and photorespiration), representing alternative explanations for the response of plants to changes of N and CO₂ availability.

Foliar $\delta^{15}\text{N}$ decreased significantly over time but an explanation by an increased input of reactive N from long-distance transport ('eutrophication' hypothesis) was unlikely because foliar N contents decreased significantly as well. An increased carbon gain due to increasing [CO₂] ('oligotrophication') also was unlikely because instantaneous water use efficiency remained unchanged and indicated no increase in C gain. The detected patterns agreed well with the 'photorespiration' hypothesis that biochemically links N assimilation and C assimilation. Increasing concentration of ambient CO₂ that decreases photorespiration explained decreasing $\delta^{15}\text{N}$ values ($R^2 = 0.84$, $p < 0.001$) and decreasing N contents ($R^2 = 0.40$, $p < 0.036$).

Our results suggest that increasing [CO₂] by suppressing photorespiration reduces N availability to alpine plants. These findings contradict the generally accepted assumption of negative effects of eutrophication on alpine grasslands caused by air-borne N deposition. We conclude that increasing [CO₂] should be considered as an alternative driver of long-term changes in alpine ecosystems, as it affects directly the plant C:N stoichiometry, a key plant trait determining several important ecosystem processes.

1. Introduction

With the beginning of industrialization in the late 18th century, the Earth entered a period of anthropogenic environmental change that has continuously gained momentum (Lang et al., 2019). Over the past two centuries, human activities have drastically increased the input of greenhouse gases into the atmosphere, altered the global cycles of carbon, nitrogen (N) and phosphorous considerably and transformed vast pristine ecosystems into urban and agricultural land. This has resulted in global warming and the increased frequency and magnitude of extreme climate events, eutrophication of terrestrial and aquatic ecosystems and land degradation, respectively (Bobbink et al., 2010; IPCC, 2013). A vast

body of research has revealed numerous ecological fingerprints of environmental change across a wide range of biomes and organizational hierarchies. The evidence most often reported includes biodiversity loss, shifts in spatial and temporal distributional patterns, altered species interactions and habitat fragmentation (Walther et al., 2002; Parmesan and Yohe, 2003). Future predictions suggest that these negative effects on Earth's biosphere will be even greater over the next decades, due to the continuously rapid growth of the world's population, which inevitably leads to agricultural, industrial and urban expansion (Tilman et al., 2001; Araujo et al., 2004).

In Europe, a number of low-temperature and nutrient-limited ecosystems (e.g. tundra, boreal forests, peatlands) are particularly prone to

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global environmental change. Among them are nutrient-poor alpine grasslands, local biodiversity hotspots with very high nature conservation and cultural value (Theurillat and Guisan, 2001; Ellenberg and Leuschner, 2010). Specifically, alpine grasslands are experiencing some of the highest rates of temperature rise resulting in warming-induced shifts in species distribution patterns, altered species richness and homogenization of alpine vegetation (Lamprecht et al., 2018; Steinbauer et al., 2018). Furthermore, this ecosystem type has been suggested to be strongly affected by increasing N deposition as well (Bobbink et al., 2010). Due to its position in the centre of Europe and the intensive use of the valley floors, the Alps are directly exposed to N emissions from urban, agricultural and industrial areas, with the highest impacts recorded on the fringes of the Northern and Southern Alps, particularly in Southern Germany (Bavaria), Northern Switzerland and Northern Italy (Lombardy; Kirchner et al., 2014). Although there are no observational studies on N deposition effects in alpine ecosystems, a few fertilization experiments have indicated that increased N input from the atmosphere inevitably leads to biomass increase, shifts in community composition and species turnover, whereby the grassland communities on infertile soils are most affected (Bobbink et al., 2003; Hiltbrunner et al., 2005). Finally, yet importantly, rising atmospheric CO₂ concentrations [CO₂] might have large impacts on biodiversity (Dawes et al., 2011), community composition (Körner et al., 1997) and some other processes in this ecosystem including plant-herbivore interactions and nutrient cycling (Schappi and Körner, 1997). Yet, despite the complex nature of the environmental change in the alpine grasslands, the existing studies have primarily attributed the patterns of vegetation changes observed in this habitat to rising temperatures (Lamprecht et al., 2018; Steinbauer et al., 2018). At the same time, the potential effects of long-term increasing [CO₂] and N deposition on this ecosystem are largely unknown as related experimental studies in alpine environments are still rare (e.g. Xu et al., 2004; Fengzhan et al., 2019).

In this context, natural history collections are an underused treasure of temporally and geographically broad samples that we have just begun to dust off (Barbosa et al., 2010; Carine et al., 2018; Lang et al., 2019). Particularly, data obtained from herbaria have been already used to reveal the effects of global environmental change on plants. Due to their sessile nature and, thus, a particular sensitivity to environmental change, historical herbarium specimens have been shown to successfully capture information about ambient abiotic and biotic environment and plants' responses to them (Lang et al., 2019). For example, herbarium-based studies qualify changes in plant morphology (Leger, 2013), phenology (Willis et al., 2017), anatomy (Lammertsma et al., 2011) and chemistry (Peñuelas and Filella, 2001; Gritcan et al., 2016) as response to global warming, rising CO₂ concentrations and N deposition, respectively.

Among herbarium-inferred traits foliar N content and stable isotope composition ($\delta^{15}\text{N}$) have a large potential to be used as indicators of plant response to global change. The $\delta^{15}\text{N}$ values generally indicate the openness of the N cycle because losses are usually associated with large fractionations (ϵ). In particular, NH₃ volatilization (ϵ ; 29–60 ‰), N₂O and NO production during nitrification (ϵ ; 35–60 ‰) and N₂O and N₂ production during denitrification (ϵ ; 28–33 ‰; Högberg, 1997; Robison, 2001) can create large effects on the isotopic composition of the residual N but also during NH₃ deposition. Losses in agriculturally used systems, mainly by NH₃ volatilization, increase with increasing N surplus and cause $\delta^{15}\text{N}$ values to increase within the agricultural operations (Schwertl et al., 2005). The lost NH₃ is strongly depleted in ¹⁵N and causes especially low $\delta^{15}\text{N}$ values in gaseous atmospheric deposition (Weigel et al., 2000). Long-distance transport of NH₃ (Asman et al., 1998) depleted in ¹⁵N may contribute to the low $\delta^{15}\text{N}$ values and higher foliar N contents found in unfertilized systems (Krupa, 2003; Bragazza et al., 2004) and may be indicators of an 'eutrophication' hypothesis. Previous herbarium-based research, done mainly on historical specimen of bryophytes, confirms this inference; foliar N content tends to increase with increasing N deposition (e.g. Baddeley et al., 1994; Solga et al.,

2006), whereas $\delta^{15}\text{N}$ decreases with increasing NH₃ emissions (Pitcairn et al., 2006).

An alternative explanation for variability in foliar $\delta^{15}\text{N}$ values can be the 'oligotrophication' or 'progressive nitrogen limitation' hypothesis (Luo et al., 2004; McLauchlan et al., 2010; Craine et al., 2018). The simultaneous decreases in foliar N content and $\delta^{15}\text{N}$ values over time suggests a decline in N supply relative to plant demand, which is induced by the increasing carbon gain due to increasing atmospheric CO₂ concentrations. This hypothesis is based on the documented increase in intrinsic water use efficiency (WUEi) over time, suggesting that plants make better use of the available water and increase their carbon uptake (McLauchlan et al., 2010). Yet, this interpretation may fall short as WUEi does not consider the water demand of the atmosphere (vapor pressure deficit, VPD). Due to the increase in ambient temperatures, VPD may have also increased and increasing WUEi may indicate increasing stomatal closure instead of increasing carbon gain. For example, Barbosa et al. (2010) have shown for a 70-yr time series of ibex (*Capra ibex*) horn data from an alpine site in Switzerland that WUEi increased over time but this increase was fully compensated by the simultaneous increase in VPD. Consequently, instantaneous water use efficiency (WUEt), which takes into account CO₂ concentration and VPD, did not change over time.

A third explanation for variation in foliar N and $\delta^{15}\text{N}$ (the 'photorespiration' hypothesis) is based on the biochemical coupling of the C and N assimilation in plants that occurs during photorespiration, a physiological process causing loss of previously fixed CO₂ and energy (Keys et al., 1978). Under current atmospheric CO₂ levels, it dissipates about 25% of the carbon fixed during CO₂ assimilation (Sharkey, 1988; Rachmilevitch et al., 2004). Short-term exposure to elevated CO₂ levels (up to several hours) reduces photorespiration and increases CO₂ assimilation, whereas longer exposure time (days to weeks) slows it down (CO₂ acclimation; Rachmilevitch et al., 2004). This is caused by the twofold linkage between photorespiration and the N cycle in plants. Firstly, photorespiration converts two molecules of glycine to one molecule of serine releasing CO₂ and NH₃ in equal amounts (Keys et al., 1978; Keys, 2006). This flux of NH₃ through the photorespiratory N cycle is an order of magnitude larger than that resulting from primary assimilation and plants could lose rapidly all their organic N, if NH₃ would not be re-assimilated. Second, *de novo* assimilation of nitrogen in leaves of C3 plants can occur via the photorespiratory pathway and a decrease in photorespiration lowers nitrate assimilation (Busch et al., 2018). The additional reductant generated during photorespiration and used for nitrate reduction is considered to be the main explanation for the continued dominance of C3 plants during the past 23 million years despite the low atmospheric CO₂ concentrations (Bloom, 2015). Consequently, increasing CO₂ levels lower nitrate uptake. Synthesis of existing data showed a 14 % reduction of N content in plant shoots generated under elevated CO₂ regimes (Cotrufo et al., 1998), a change which is nearly double the effect of dilution by the additional biomass production that is the basis of the oligotrophication hypothesis. The reduced N content then slows down photosynthesis (Rachmilevitch et al., 2004).

Photorespiration is associated with an isotope effect and increases ¹⁵N abundance. Several processes contribute to this effect, such as the improved nitrate assimilation that decreases discrimination of the nitrate reductase against ¹⁵N-NO₃ (Bloom, 2015). Also, some of the NH₃ released during photorespiration may back diffuse to the atmosphere (Schjoerring et al., 2000; Tcherkez and Hodges, 2008). In a closed system, the atmospheric NH₃ equilibrates at an isotopic composition 8.1 ‰ lighter than N in leaf protein. In an open system below the NH₃ compensation point, the fractionation can be even larger (Johnson and Berry, 2013). The effect of photorespiration on ¹⁵N abundance thus may be large (Tcherkez and Hodges, 2008). With rising [CO₂] concentration, we hence expect that foliar N contents decrease due to the hampered nitrate uptake and the increase in CO₂ assimilation, while ¹⁵N abundance decreases due to the isotope effects (the 'photorespiration'

hypothesis). Similarly to the ‘oligotrophication’ hypothesis, it predicts a decrease of $\delta^{15}\text{N}$ values and of N contents over time and explains that with one consistent explanation, but not by combining two independent changes in the two properties as required for the oligotrophication hypothesis. The eutrophication hypothesis also predicts a decrease in $\delta^{15}\text{N}$, but this should be associated with an increase in N content or at least a constant N content if increased CO_2 assimilation is also taken into consideration.

Following previous studies in other ecosystem types (Peñuelas and Filella, 2001; Bragazza et al., 2005; Pitcairn et al., 2006; Huang et al., 2016), we make use of herbaria specimens to reconstruct historical changes (1807–2018) in foliar N and $\delta^{15}\text{N}$ of alpine plants, in order to infer their responses to the anthropogenic global change. The historical variation in $\delta^{15}\text{N}$ and foliar N content are interpreted in terms of three hypotheses – eutrophication, oligotrophication and photorespiration – representing different plant responses to the increased N atmospheric deposition, altered WUEi and the rising CO_2 concentrations in alpine ecosystems with high nature conservation values.

2. Materials and methods

2.1. Study region and focal species

The study region comprises alpine areas in the central part of the Northern Calcareous Alps at an elevation above 1000 m a.s.l. The majority of samples was collected in the Berchtesgaden Alps at the territory of Berchtesgaden National Park. A relatively small part of the samples (10–15%) were collected in the adjacent Lofer, Leogang and Tennen Mountains (Fig. 1).

For the purpose of this study, nine alpine herbaceous species with main distributions in montane (1000–1700 m a.s.l.) and alpine (1700–2600 m a.s.l.) vegetation belts in the Northern Calcareous Alps were chosen (Table 1). All chosen species occur only in calcareous grasslands and share similar habitat preferences with regard to light, water and soil pH (Ellenberg et al., 1991; Oberdorfer, 2001).

2.2. Stable isotopes and foliar nitrogen measurements

Leaf tissue from 359 specimens of focal species were collected from eleven natural history collections located in Germany (Berlin, Munich, Jena, Regensburg, Stuttgart) and Austria (Linz, Salzburg, private herbarium of Peter Pils, Vienna). Contemporary leaf samples were collected in 2009–2018 in the study area within projects involving leaf trait measurements (Rosbakh et al., 2014; Rosbakh et al., 2015).

To avoid variability resulting from leaf age, position and growth light environment of the collected herbarium specimen, we took whole leaves or their parts from the same terminal position of the herbarium

specimen in a natural history collection or a living plant in the field (Peñuelas and Filella, 2001). The contemporary leaf material was collected from several individuals growing close to each other in a sampling site. The fresh leaves without visible damage were collected from each of ten randomly selected individuals growing in species-specific habitats (Ellenberg et al., 1991; Oberdorfer, 2001), to avoid possible environmental effects on plant growth. When possible, leaves from several individuals from the herbarium specimen were collected and pooled. In all years, collections were made during the main flowering period of corresponding species. Plant specimens were dried immediately after collection, so foliar N content and nitrogen stable isotopes were preserved. To our knowledge, no nitrogen-containing compounds have been used in the herbaria for preservation.

Prior to measurements, all leaves were rinsed with deionized water, in order to remove any extraneous material, oven-dried at 60 °C for 72 h, frozen in liquid nitrogen and ground to a fine powder manually by pestle and mortar. An amount of 0.6 – 0.8 mg of the milled material was weighed into tin capsules and combusted in an elemental analyzer (NA 1110, Carlo Erba, Thermo Fisher Scientific, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT, MasCom GmbH, Bremen, Germany) via a Conflow interface (Conflo III, Finnigan MAT). Each sample was calibrated against the laboratory standard, a fine ground wheat flour of known C and N isotope composition, which was previously calibrated against IAEA secondary standards (IAEA-CH6 and IAEA-NO3). After every tenth sample, wheat flour was run as a blind control. The precision of repeats (SD) was better than 0.20 ‰ for flour. Isotope data are presented in δ notation as

$$\delta X = R_{\text{sample}}/R_{\text{standard}} - 1, \quad (1)$$

where X is either ^{13}C or ^{15}N and R is the $^{13}\text{C}:^{12}\text{C}$ or the $^{15}\text{N}:^{14}\text{N}$ abundance ratio. The subscript ‘standard’ refers to the International Atomic Energy Agency AIR standard for nitrogen and to V-PDB (Vienna-Pee-Dee Belemnite) for carbon.

2.3. Data analysis

Carbon isotope composition of plants depends on carbon isotope composition of ambient CO_2 and carbon isotope discrimination ($^{13}\Delta$, in ‰) during photosynthesis and by post-photosynthetic processes. Discrimination is defined as:

$$^{13}\Delta = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p/1000} \quad (2)$$

where $\delta^{13}C_a$ and $\delta^{13}C_p$ apply for atmospheric CO_2 (a) and plant carbon (p). Farquhar et al. (1989) set up a model that describes $^{13}\Delta$ depending on processes during photosynthesis. In its simplified version it relates

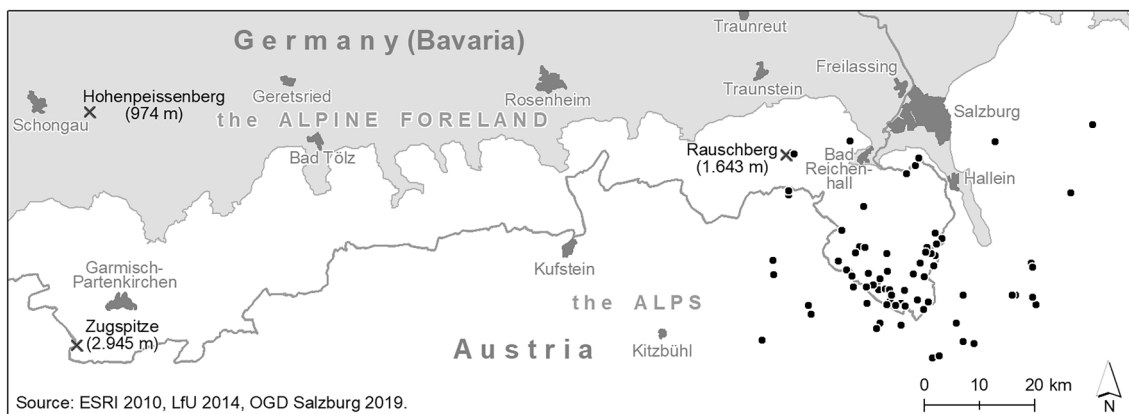


Fig. 1. Plant sampling sites (circles) and meteorological stations (crosses and names); the white background indicates the Northern Calcareous Alps while the grey area includes the Flysch mountains of upthrust molasse and the moraine foreland.

Table 1
Characteristics of the nine vascular plant species used in the present study.

Species	Number of specimen	Oldest specimen	Youngest specimen	Time span covered, years	Foliar $\delta^{15}\text{N}$ (‰)		Foliar N (%)	
					Mean	SE \pm	Mean	SE \pm
<i>Aconitum tauricum</i> Wulf.	37	1846	2018	172	-1.62	0.13	2.71	0.32
<i>Betonica alopecuroides</i> L.	28	1832	2018	186	-2.62	0.12	2.87	0.26
<i>Campanula alpina</i> Jacq.	54	1807	2018	211	-3.95	0.82	2.27	0.23
<i>Carex atrata</i> L.	56	1807	2018	211	-0.46	0.59	2.33	0.26
<i>Heracleum austriacum</i> L.	36	1850	2018	168	-1.82	0.77	3.25	0.5
<i>Horminum pyrenaicum</i> L.	36	1832	2018	186	-2.15	0.11	2.75	0.27
<i>Pimpinella major</i> (L.) Huds.	16	1902	2018	116	-1.45	0.18	2.85	0.68
<i>Salix retusa</i> L.	42	1827	2018	191	-2.29	0.21	3.08	0.42
<i>Senecio abrotanifolius</i> L.	54	1846	2018	172	-3.75	0.08	1.85	0.23

$^{13}\Delta$ with fractionation during diffusions of CO_2 in air (a), the net fractionation caused by carboxylation (b) and the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) as:

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

The relationship between $^{13}\Delta$ and c_i/c_a allows estimating water-use efficiency during photosynthesis. Instantaneous water-use efficiency WUEt (W_i in formulas; in $\mu\text{mol mol}^{-1}$), i.e. the ratio between assimilation and transpiration, depends on the ratio c_i/c_a and the water vapor concentration gradient v :

$$W_i = \frac{c_a \left(1 - \frac{c_i}{c_a}\right)}{1.6v} \quad (4)$$

where the factor 1.6 considers the difference in diffusivities of water vapor and CO_2 in air. The water vapor concentration gradient v is given by:

$$v = \frac{e_i - e_a}{P} \quad (5)$$

where vapor gradient driving the loss of water is the water vapor pressure deficit (VPD in hPa) between the vapor pressure in the intercellular space of a leaf (e_i) and that of the air (e_a), divided by atmospheric pressure (P in hPa). Intercellular vapor pressure is assumed to be equal to saturated vapor pressure at ambient temperature.

Under conditions where v does not vary, e.g. when comparing canopies in close neighborhood, v can be assumed to be identical and thus be ignored, which then gives the intrinsic water use efficiency WUEi (W_i in formulas; in $\mu\text{mol mol}^{-1}$) (Ehleringer et al., 1993):

$$W_i = \frac{c_a \left(1 - \frac{c_i}{c_a}\right)}{1.6} \quad (6)$$

For historic time series, v is often not available and thus WUEi is used instead of WUEt (Saurer et al., 2004; McLauchlan et al., 2010) although a change in VPD cannot be excluded.

Meteorological data at daily resolution (air temperature, air humidity, rainfall) for the main growing period (May to September) were taken from the globally oldest mountain meteorological station Hohenpeissenberg, which started operation in 1781 and thus covered the entire study period (www.opendata.dwd.de). Like the collection sites of the plant specimen, Hohenpeissenberg is also located at the northern fringe of the Alps. The station is at an elevation of 977 m a.s.l. (above sea level) and at a distance of about 180 km from the center of the plant sampling area (Fig. 1). The Hohenpeissenberg data were complemented by stations at other elevations, namely Rauschberg and Zugspitze. The station Rauschberg near Ruhpolding is at a distance to the main sampling area of about 40 km and at an elevation of 1640 m a.s.l. The station Zugspitze is at a distance of about 190 km to the main sampling area and at an elevation of 2964 m a.s.l., which is 400 m higher than the highest plant sampling location.

Saturation vapor pressure was estimated from air temperature according to Buck (1981). VPD was calculated as difference between saturation and ambient vapor pressure. Ambient vapor pressure was not available before 1851 and appeared unreliable before 1880 due to sudden shifts. As there was no trend in VPD between 1851 and 1930, the mean VPD during 1880 to 1930 was used before 1880. VPD strongly decreases with elevation, mainly because of the altitudinal change in temperature. Growing period VPD for the individual specimen sampled at different elevations was then estimated from

$$(e_i - e_a)_A = (e_i - e_a)_{977} \times (5.8 - 0.7 \times \ln(A)) \quad (7)$$

where $(e_i - e_a)_A$ is VPD in hPa at a certain elevation A and $(e_i - e_a)_{977}$ is VPD at Hohenpeissenberg at 977 m a.s.l. while $\ln(A)$ is the natural logarithm of altitude. This equation explained the variation in VPD at other high-elevation meteorological stations within the northern fringe of the Alps well. For Hohenpeissenberg, mean growing period VPD varied between 2.4 and 7.3 hPa for individual years between 1880 and 2018 and its elevation corresponds with the lowest elevation of our plant samples (1000 m). At Rauschberg (1640 m a.s.l.), data were available from 1963 to 1998 and ranged from 1.9 to 4.0 hPa. The prediction error (root mean squared error RMSE when comparing the prediction of Eq. (1) with the measured values) was 0.2 hPa. At Zugspitze (2964 m a.s.l.) data were available from 1901 to 2018 and ranged from 0.5 to 1.7 hPa. The RMSE was again 0.2 hPa. Eq. (7) in combination with the Hohenpeissenberg data should hence cover the entire range of sampling locations.

Annual mean isotopic composition of CO_2 ($\delta^{13}\text{C}_{\text{CO}_2}$) was taken from ice cores (Friedli et al., 1986; Francey et al., 1999) and from the Mauna Loa Observatory (<https://www.esrl.noaa.gov/gmd/dv/data/>). Annual mean CO_2 concentrations [CO_2] were taken from the same sources (Fig. 2A). This assumes that the atmosphere is a well-mixed pool regarding CO_2 . A seasonal variation of $\delta^{13}\text{C}_{\text{CO}_2}$ and [CO_2] was neglected although $\delta^{13}\text{C}_{\text{CO}_2}$ is slightly higher by 0.29 ‰ during the main growing season (April to September) compared to the annual value at station Hohenpeissenberg, and [CO_2] is lower by 6 ppm (data also taken from <https://www.esrl.noaa.gov/gmd/dv/data/>). Consideration of this seasonal variation would only cause constant shifts but would not influence the general relations.

We used ammonium (NH_3) concentration in precipitation as a proxy for air-borne N deposition, as it contributes considerably to the total amount of deposited N (Döscher et al., 1996). Due to the lack of a long time series (1820–2020) of N deposition to the Central Europe, we compiled a data set by combining published time series of deposition data to the Swiss Alps (1820–1966; Döscher et al., 1996) and the Baltic Sea (1966–2006; Ruoho-Airola et al., 2012), which agrees well with the shorter time series of high-elevation data for the Czech mountains (Kopáček et al., 2012). The temporal trends of Döscher et al. (1996) and Ruoho-Airola et al. (2012) agree with $R^2 = 0.96$ for the overlapping period 1845 to 1966 (Fig. 2A). The time series by Ruoho-Airola et al. (2012) ended 2006 and was extrapolated until 2018 assuming the same trend as observed between 1996 and 2006.

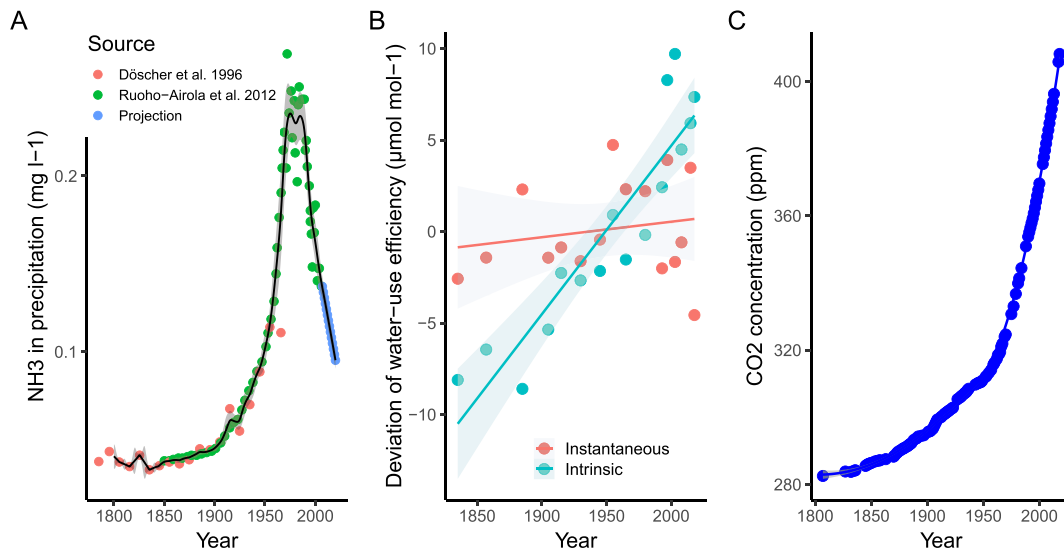


Fig. 2. Drivers of the three hypotheses ('eutrophication', 'oligotrophication' and 'photorespiration') during two centuries (a) NH_3 concentration in precipitation in Central Europe drives eutrophication; (b) instantaneous water use efficiency relates to carbon gain and drives oligotrophication (for comparison, intrinsic water use efficiency is shown as well data were normalized by species mean, to account for species-specific differences); (c) atmospheric CO_2 concentration drives photorespiration. The shaded areas denote the 95% confidence interval.

Due to high species-specific difference in the study parameters (see the Results section) and uneven sampling effort of herbarium material over the study period (e.g. a big sampling gap during the World War II), two data transformations were necessary prior to the analysis. First, the measured parameters were normalized by the species mean (i.e. the deviation of an individual measurement from the species mean covering the entire study period), to remove the scatter due to the species-effect. This also causes a parameter to be zero, if no temporal effect occurred. Second, to account for the fact that more herbarium specimens were collected in the last 5 decades and that individual samples are subject to a large scatter due uncontrolled site and year effects, each sample was assigned to one of 16 time periods that aimed at balancing a high temporal resolution and a sufficient number of specimens within each group (from 14 to 36; Table 2). For each time period we calculated mean-normalized values for the variable under focus and used them for further statistical calculations.

Data analysis was carried out in two steps. First, we performed linear weighted models using foliar N contents and $\delta^{15}\text{N}$ as dependent variable and mean year of a period, to detect any significant temporal changes of

the study parameters. Number of samples per period was included into the models as weights (i.e. periods with more samples had a stronger influence). In the same manner, we regressed the change in ^{13}C discrimination, WUE_i and the WUE_t against the collection year, to estimate the changes in gas exchange during photosynthesis over the same period of time. In the second step, to provide causal explanation for the detected temporal changes, we regressed period means for foliar stable N isotopes and total N against the potential drivers [CO_2], WUE_i and NH_3 in precipitation of the corresponding periods using linear weighted models with number of herbarium samples as weights.

Model requirements and assumptions were met in all the cases, as variances were homogeneous and residuals normally distributed. All statistical calculations were done with the help of the R software (R Core Development Team, 2020). The package 'np' (Hayfield and Racine, 2008) was used to draw the curve in the Fig. 2A. CI95 indicates the 95% interval of confidence.

3. Results

3.1. Changes in N deposition, WUE and atmospheric CO_2 concentration

The NH_3 concentration in rain exponentially increased from 0.04 mg l^{-1} in the 1820s to 0.24 mg l^{-1} in the 1980s suggesting high levels of airborne N deposition during the 20th century (Fig. 2A). From the late 1980s on these values started to decline continuously to ca. 0.11 mg l^{-1} in the 2010s, levels of N deposition similar to those before the WWII.

Discrimination of ^{13}C differed significantly among species. It was lowest in *Aconitum tauricum* (18.6 ‰ , CI95: 0.6) and highest in *Senecio abrotanifolius* (20.9 ‰ , CI95: 0.4). Discrimination slightly but significantly ($R^2 = 0.26$, $p = 0.03$) increased over time with a rate of 0.004 ‰ yr^{-1} (in total by 0.84 ‰ ; Table 3, Appendix 2). Average c_i/c_a was 0.67 with slight but significant increase over time. Together with the strong increase in ambient CO_2 concentration c_a from about 283 ppm in 1820 to about 408 in 2018 (Fig. 2C), this indicated a pronounced increase in intercellular CO_2 concentration. WUE_i increased very highly significantly ($R^2 = 0.82$, $p < 0.001$) from 54 µmol mol^{-1} in 1807 to 76 µmol mol^{-1} in 2018 (Table 3, Fig. 2B). However, VPD during the growing period had increased pronouncedly at Hohenpeissenberg from on average 3.5 hPa before 1900 to an average of 5.5 hPa after 2000 in

Table 2

Sampling periods and number of samples available per each period. All herbarium specimens were assigned to one of 16 time periods with a comparable number of samples, to account for uneven data coverage over the study period.

No	Time period	Year for regression	Number of samples
1	1807–1849	1835	16
2	1850–1863	1857	27
3	1870–1899	1885	28
4	1900–1909	1905	25
5	1910–1919	1915	20
6	1920–1939	1930	22
7	1940–1949	1945	17
8	1950–1959	1955	15
9	1960–1969	1965	35
10	1970–1989	1980	20
11	1990–1995	1993	25
12	1995–1999	1997	29
13	2000–2005	2003	15
14	2006–2009	2008	15
15	2010–2017	2015	14
16	2018	2018	36

Table 3

Effects of sampling year on foliar $\delta^{15}\text{N}$, foliar N content, change in ^{13}C discrimination ($\Delta^{13}\text{C}$), intrinsic water use efficiency (WUEi) and the instantaneous water use efficiency (WUEt) as based on linear weighted models. Data were normalized by mean, to account for species- and elevation-specific differences in the study parameters. Bold entries are statistically significant ($p < 0.05$).

Parameter	Intercept			Slope			R^2
	Estimate	SE \pm	p-value	Estimate	SE \pm	p-value	
Foliar $\delta^{15}\text{N}$	19.5	4.1	<0.001	-0.01	0.002	<0.001	0.59
Foliar N content	2.9	1.3	<0.001	-0.0015	0.0007	0.038	0.22
$\Delta^{13}\text{C}$	-7.3	2.9	0.026	0.004	0.002	0.026	0.26
WUEi	-183.6	21.7	<0.001	0.09	0.01	<0.001	0.82
WUEt	-5.9	25.1	0.82	0.003	0.01	0.82	0.003

parallel to the rising air temperature (12.4 °C during the May to September before 1900 and 14.3 °C after 2000). In consequence, WUEt was on average 24 $\mu\text{mol mol}^{-1}$ and did not change over time ($R^2 = 0.004$, $p = 0.82$; Table 3, Fig. 2B). Hence, the large change in WUEi just compensated the change in VPD.

3.2. Stable nitrogen isotopes and foliar nitrogen

Among studied samples, foliar $\delta^{15}\text{N}$ varied from -7.9 to 4.2 ‰ with an average of -2.4 ‰ (Appendix 2). The samples of *Campanula alpina* had the lowest mean $\delta^{15}\text{N}$ values (-4.19 ‰; CI95: 0.47 ‰), whereas *Carex aterrima* had the highest $\delta^{15}\text{N}$ (-0.46 ‰, CI95: 0.51 ‰). During the period from 1820 to 2018, normalized foliar $\delta^{15}\text{N}$ declined very highly significantly ($R^2 = 0.59$, $p < 0.001$) over time with the rate 0.01‰ yr^{-1} (in total by 2.2 ‰: Table 3, Fig. 3A).

Foliar N content among samples varied by over 4 % (from 0.7 to 4.7 %, on average 2.6 %) across the study species during the studied period. On average, foliar N content were the lowest in *Senecio abrotanifolius* (on average 1.8 %, CI95: 0.2 %) and the highest in *Heracleum austriacum* (3.3 %, CI95: 0.3 %). Normalized foliar N content displayed a moderate statistically significant decrease ($R^2 = 0.22$, $p = 0.038$) over time with the rate 0.0015% yr^{-1} (in total by 0.3 %: Table 3, Fig. 3B).

3.3. Drivers of foliar $\delta^{15}\text{N}$ and N changes

Linear weighted models revealed that $[\text{CO}_2]$ had a strong significant negative effect on both foliar $\delta^{15}\text{N}$ ($R^2 = 0.84$, $p < 0.001$: Fig. 4A) and N

values ($R^2 = 0.40$, $p = 0.036$: Fig. 4D). Furthermore, foliar $\delta^{15}\text{N}$ correlated significantly negatively with NH_3 concentrations in precipitation ($R^2 = 0.31$, $p = 0.04$: Fig. 4C) but N contents indicated no significant NH_3 uptake. Foliar N contents did not respond to increasing NH_3 concentration in precipitation ($R^2 = 0.01$, $p = 0.46$, Fig. 4F). WUEi did not have any significant effects on both N parameters ($R^2 = 0.01$, $p = 0.96$, Fig. 4B and $R^2 = 0.08$, $p = 0.41$, Fig. 4E, respectively).

4. Discussion

In this study, we employed eutrophication, oligotrophication and photorespiration hypotheses to infer alpine plant responses to airborne N eutrophication and rising $[\text{CO}_2]$ from the historical variation in foliar $\delta^{15}\text{N}$ and N content. To achieve that, we compiled a unique data set that is specifically well suited for this purpose. First, it covers a comparatively long period of 200 years that captures acclimated responses of plants to the anthropogenic global change (in contrast to rather short FACE and fertilization experiments, e.g. Kim et al., 2003; Körner et al., 1997, respectively). Second, the leaf tissue samples originate from an area, which is remote enough to exclude direct anthropogenic influence. Thirdly, we complemented the data set with meteorological data from the world's longest running mountain meteorological station to improve quantification of leaf gas exchange. Finally, and more importantly, we directly correlated the historical foliar $\delta^{15}\text{N}$ and N values with the data on NH_3 concentration in precipitation, WUEt and $[\text{CO}_2]$ instead of using time as a proxy for these changes. Such approach solves the well-known problem of analysing time-series (including herbarium-based research)

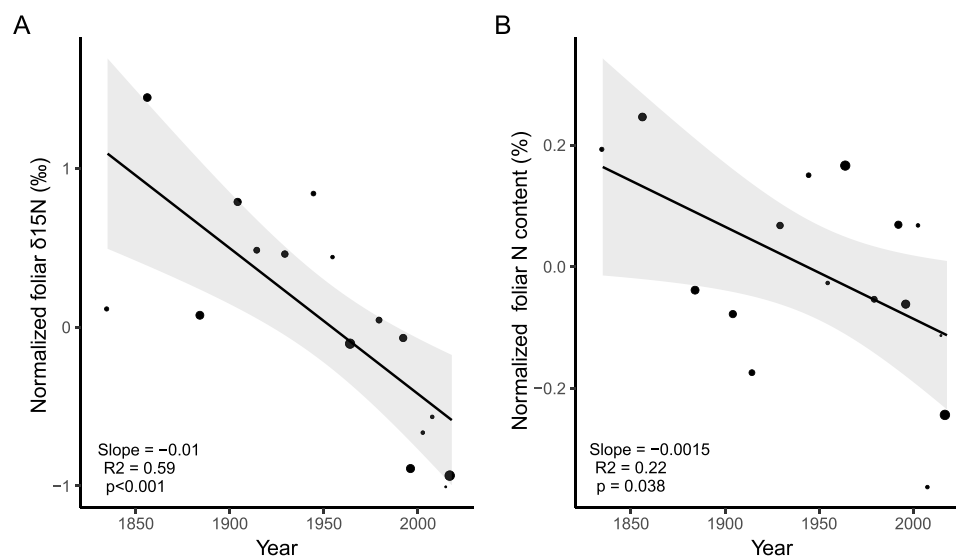


Fig. 3. Change in normalized $\delta^{15}\text{N}$ (a) and nitrogen content (b) in leaves of nine alpine species during two centuries deduced from linear weighted models averaged for 16 periods. Data were normalized by species mean, to account for species-specific differences in the study parameters. The shaded area denotes the 95% confidence interval. Marker size and weight increase with number of samples within a period (see Table 2).

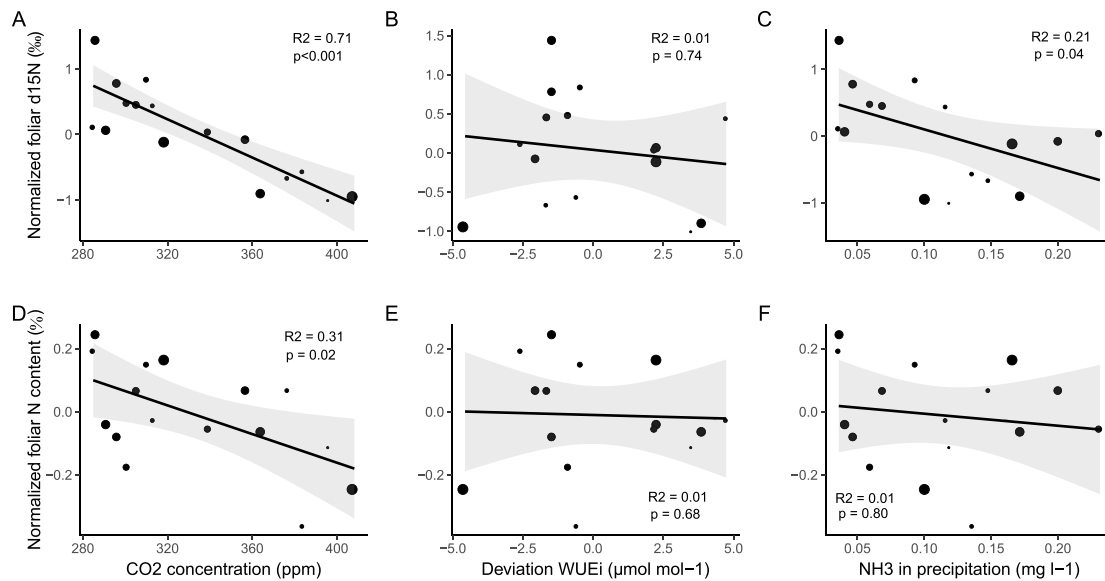


Fig. 4. Effects of air CO_2 concentration, intrinsic water use efficiency and NH_3 in precipitation on foliar $\delta^{15}\text{N}$ and nitrogen content in plant material of nine alpine species based on linear weighted models. Data were normalized by mean, to account for species- and elevation-specific differences in the study parameters. The shaded area denotes the 95% confidence interval. Marker size increases with number of samples (see Table 2 for details).

that temporal variability in one parameter of interest can be correlated to temporal variability in the another, even if there is no a direct interaction between them. Furthermore, the time course of NH_3 in precipitation and $[\text{CO}_2]$ exhibited divergent patterns thereby allowing us to estimate their effects on foliar N stoichiometry in alpine plants independently from each other.

The eutrophication hypothesis focuses solely on impacts of atmospheric N deposition on foliar N status and predicts that increasing N input into an ecosystem should result in an increased foliar N content, whereby foliar $\delta^{15}\text{N}$ might decrease, if the main source of deposited N comes from ^{15}N depleted sources (Krupa, 2003; Bragazza et al., 2004; Pitcairn et al., 2006). Despite the comparatively high NH_3 concentrations in precipitation in the second half of the 20th century, the foliar N content in the study alpine plants declined strongly ($R^2 = 0.59$) in continuous manner during the last two centuries. Moreover, we found no significant relationship between foliar N and NH_3 concentrations in precipitation, higher levels of which are supposed to result in higher foliar N contents (Baddeley et al., 1994; Solga et al., 2006). Collectively these findings contradict the assumptions of the eutrophication hypothesis and suggest that the alpine grasslands were not affected by the high levels of atmospheric N deposition in the past, at least not at the individual plant level.

There are two possible explanations for that. First, and the most parsimonious explanation, is that the studied alpine grasslands either received none or a very low amount of regional (e.g. agriculture, traffic Krupa, 2003; Kirchner et al., 2014) and/or remote (e.g. long-distance transport of reactive N derived from fossil fuel combustion as well as fertilizer production and application; Fagerli et al., 2018) N emissions, due to their remote position from the N emitting sources. Second, the comparatively high variability in foliar $\delta^{15}\text{N}$ and N content caused by the extreme heterogeneity of alpine environment (e.g. slope orientation and steepness, soil properties; Körner, 2007) combined with relative low temporal resolution of the herbarium samples (359 specimens collected in 200 years) and the species-specific responses to N fertilization (Körner et al., 1997) might mask the plant responses to the increased N inputs. Thus, future herbarium-based research should consider potential impacts of these factors.

In contrast to the peak-shaped time course of NH_3 in precipitation, $[\text{CO}_2]$ follows an exceptional and accelerating increase during the entire

research period. The oligotrophication hypothesis postulates that the rising $[\text{CO}_2]$ increases carbon assimilation rates, due to the CO_2 fertilization effects on plant growth resulting in decreasing foliar N contents and foliar $\delta^{15}\text{N}$ depletion ('dilution' effect; Luo et al., 2004; McLauchlan et al., 2010; Craine et al., 2018). The CO_2 fertilization effect, i.e. an increase in assimilation rate, is inferred from an increase in WUEi, which is the ratio of assimilation rate and stomatal conductance, by assuming that stomatal conductance did not change. At first glance, this seems to be supported by the sharp increase in WUEi. However, it is known that stomatal conductance is decreased by increasing $[\text{CO}_2]$, which turns the required assumption rather unlikely. Stomatal conductance is even more decreased by increasing VPD (Sionit et al., 1981; Tyree and Alexander, 1993; Ainsworth and Rogers, 2007; Baca Cabrera et al., 2020). WUEi, which considers VPD, showed no change over time, indicating that the plants reduced stomatal conductance in response to increasing VPD. Also, the decreasing N content indicated a decrease in photosynthetic capacity. All our findings thus point to a decrease in stomatal conductance but not to an increase in assimilation rate as suggested by the oligotrophication hypothesis. This agrees with findings in similar (Barbosa et al., 2010) or different systems (Köhler et al., 2012). Even more, the notion that stomatal conductance has changed but assimilation rate has not increased agrees with the finding that grassland yields have not changed over time even on N fertilized plots (Jenkinson et al., 1994; Köhler et al., 2012) and that N supply had no influence on the CO_2 response of discrimination by trees during recent climate change (Betson et al., 2007). Also Baca Cabrera et al. (2021) have shown by analyzing $\delta^{18}\text{O}$ in cellulose from the Park Grass experiment, that reductions of stomatal conductance correlated with yield trends, nitrogen acquisition, and the nitrogen nutrition index. The effects were larger for dicot rich communities than for grass dominated communities. They concluded that temperate grassland may have attained saturation of C sink activity more than one century ago, which is in contradiction to the oligotrophication hypothesis.

From this follows that the photorespiration hypothesis (e.g. Tcherkez and Hodges, 2008) is the most parsimonious explanation of our findings as it simultaneously explained the decline in the both foliar properties and exhibited the highest coefficient of determination. Similar to the oligotrophication, it considers the rising $[\text{CO}_2]$ as the main driver of reduction in the both foliar properties, but explains these changes by a

decreasing N assimilation that is increasingly suppressed by increasing CO₂ levels (Bloom et al., 2010; Bloom et al., 2014; Bloom, 2015). This hypothesis is superior to the oligotrophication, because we did not detect any increase in photosynthetic rates, a key assumption of the latter. Furthermore, the photorespiration provides a clear mechanistic explanation (e.g. modified nitrate assimilation; Bloom, 2015) for the decline in foliar $\delta^{15}\text{N}$, whereas the oligotrophication simply uses this parameter as a proxy for terrestrial N availability (McLauchlan et al., 2010) and assumes an increase in assimilation that overcompensate the increasing N availability resulting in the oligotrophication.

These results have several important implications. Firstly and most importantly, they demonstrate that, since the beginning of the Anthropocene, alpine ecosystems have been affected not only by global warming (Lamprecht et al., 2018; Steinbauer et al., 2018), but also increasing [CO₂]. In contrast to other study systems (e.g. McLauchlan et al., 2010; Craine et al., 2018), the effects of the latter were found to be negative in the alpine grasslands as exponentially rising [CO₂] had no positive fertilization effect on alpine plant growth and increased N limitation of the comparatively nutrient-poor alpine grasslands. Thus, [CO₂] should be considered as alternative driver of observed long-term changes in alpine grassland biodiversity and community composition in the study region (Rosbakh et al., 2014) and throughout of Europe (Steinbauer et al., 2018; Rumpf et al., 2019).

Secondly, the detected continuous decrease in the foliar N traits contradicts the assertions of negative effects of N eutrophication on alpine grasslands (Grytnes et al., 2014; Steinbauer et al., 2018); in reality, this ecosystem has been becoming even nutrient-poorer. Because plant N availability determines several aspects of (alpine) grasslands function, its decline will inevitably affect the plant C:N stoichiometry resulting in nutritional stress for herbivores, both in wildness and managed populations (e.g. grazers; Bloom et al., 2014; Craine et al., 2018). At the community level, the lower N availability might reduce alpha diversity (Yang et al., 2011) and change community composition (Boutin et al., 2017). At the ecosystem level, it will have some negative effects on net primary productivity in this ecosystem (Johnson et al., 2008) and recycling of nutrients through decomposition (Cotrufo et al., 1998).

Finally, herbaria have been recently used as unique data source on effects of long-term environmental change on plants, including global warming (Leger, 2013; Willis et al., 2017), rising CO₂ concentrations (Lammertsma et al., 2011), air pollution (Hallingbäck, 1992; Foan et al., 2010) and eutrophication in terrestrial (Peñuelas and Filella, 2001) and aquatic ecosystems (Gritcan et al., 2016). Along with other, very limited, historical records of archived leaf tissue (Peñuelas and Filella, 2001; McLauchlan et al., 2010), our study is a timely and important contribution to the ongoing discussion on global change effects on N availability in terrestrial ecosystems (Craine et al., 2018; Craine et al., 2019; Hiltbrunner et al., 2019). In this regard, it extends the applicability of natural history collections to capture information about historical changes in the environment (Barbosa et al., 2010; Carine et al., 2018; Lang et al., 2019).

5. Data availability

Data used for statistical analysis is available in the Appendix 1.

Authors contribution

SR, KA and PP conceived the paper, SR collected the data, SR, and KA performed the statistical analysis. SR and KA wrote the paper. All authors contributed substantially to the paper and gave approval for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2021.107990>.

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