Effects of combined ozone and nitrogen deposition on the in situ properties of eleven key plant species of a subalpine pasture

6 AUTHORS, INCLUDING:

Seraina Bassin
Agroscope
34 PUBLICATIONS **444** CITATIONS

SEE PROFILE

Roland A. Werner
ETH Zurich
85 PUBLICATIONS **3,203** CITATIONS

SEE PROFILE

Karin Sörgel
Leibniz Institute for Zoo and Wildlife Resea…
10 PUBLICATIONS **313** CITATIONS

SEE PROFILE

Matthias Volk
Agroscope
24 PUBLICATIONS **443** CITATIONS

SEE PROFILE

GLOBAL CHANGE ECOLOGY - ORIGINAL PAPER

Effects of combined ozone and nitrogen deposition on the in situ properties of eleven key plant species of a subalpine pasture

Seraina Bassin · Roland A. Werner · Karin Sörgel · Matthias Volk · Nina Buchmann · Jürg Fuhrer

Abstract Tropospheric O_3 and deposition of reactive N threaten the composition and function of natural and semi-natural vegetation even in remote regions. However, little is known about effects of these pollutants individually or in combination on plant species in alpine habitats. We analyzed 11 frequent plant species of a subalpine *Geo-Montani-Nardetum* pasture exposed at 2,000 m a.s.l. in the Swiss Alps during 3 years using a factorial free-air exposure system with three concentrations of O_3 and five rates of N application. The aim was to detect subtle effects on leaf chlorophyll and N concentrations, leaf weight, specific leaf area (SLA), and $\delta^{18}O$ and $\delta^{13}C$ as proxies for gas exchange. We expected that the species' responsiveness to O_3 and N would be related to their functional traits and that N-induced changes in these traits would modify the species' response to O_3 via increased growth and higher leaf conductance (g_s). Most species reacted to N supply with the accumulation of N and chlorophyll, but with no change in SLA, g_s, and growth, except *Carex sempervirens* which showed increased water use efficiency and leaf weight. Elevated O_3 reduced g_s in most species, but this was not related

to a reduction in leaf weight, which was recorded in half of the species. Contrary to our expectation, the magnitude of the response to both O_3 and N was not related to species-specific traits such as SLA or g_s. No pronounced $O_3 \times N$ interactions were observed. In conclusion, since for most species neither N nor gas exchange limited growth, their short-term response to O_3 and N and to their combination was small. $O_3 \times N$ interactive effects are expected to be more pronounced in habitats where species are more responsive to N due to favorable growth conditions in terms of nutrient availability and temperature.

Keywords Functional trait · Species composition · Stable isotope

Communicated by Andrea Polle.

S. Bassin (✉) · M. Volk · J. Fuhrer
Air Pollution and Climate Group,
Agroscope Research Station ART,
Reckenholzstrasse 191, 8046 Zurich, Switzerland
e-mail: Seraina.Bassin@art.admin.ch

R. A. Werner · K. Sörgel · N. Buchmann
Institute of Plant Sciences, ETH Zurich,
8092 Zurich, Switzerland

Introduction

Concentrations of tropospheric O_3 and reactive N compounds have risen on a global scale during the last decades as a result of increasing anthropogenic emissions (Matson et al. 2002; Ashmore 2005). Today, both O_3 concentrations and N inputs exceeding current critical levels and loads are widespread and threaten the structure and function of sensitive natural and semi-natural plant communities even in remote regions (Bobbink et al. 1998). Yet there is considerable uncertainty with respect to the tolerance of many plant species and communities to these pollutants, and about possible interactive effects in areas where both atmospheric contaminants occur together.

O_3 is the most important phytotoxic air pollutant at a regional scale in Europe (Ashmore 2005). In sensitive plant species, chronic O_3 exposure has been shown to reduce CO_2 assimilation by negatively affecting photosynthesis (A) through enhanced chlorophyll and protein degradation

(Grandjean and Fuhrer 1989) and impairment of stomatal function through effects on stomatal guard cells (Torsethaugen et al. 1999). Possible consequences include accelerated leaf senescence and thus alterations of the plants' C budget. On longer time scales, this can negatively affect growth, productivity and seed output (Ojanperä et al. 1998). But species respond differently to elevated O_3 depending on specific O_3 uptake, mesophyll exposure, and detoxification capacity (Fuhrer and Booker 2003). Thus, in plant communities, effects on plant–plant interactions can lead to shifts in species composition (Nussbaum et al. 2000).

Because of its key role in O_3 uptake and O_3 tolerance, particular attention needs to be paid to leaf gas exchange. This can be done either by direct measurements of A and g_s, or, when not possible, e.g., because of the small size of leaves in alpine plants, by analyzing stable isotopic signatures. The stable C isotopic signature of plant organic matter ($^{13}C/^{12}C$ ratio expressed as $\delta^{13}C$) has been widely used as an integral signal of leaf gas exchange (Farquhar et al. 1989). $\delta^{13}C$ is proportional to the ratio of the internal CO_2 concentration (c_i) to the atmospheric CO_2 concentration (c_a) (Farquhar et al. 1989). Foliar $\delta^{13}C$ values of tree and grassland species have been shown to be less negative with increasing O_3 (Saurer et al. 1995; Jäggi et al. 2005), but also with increased N availability, thus indicating improved water use efficiency (WUE) under both conditions. However, as both g_s and A influence c_i/c_a, the main driver of changes in $\delta^{13}C$ remained unknown. The isotopic composition of oxygen (the $^{18}O/^{16}O$ ratio expressed as $\delta^{18}O$) enables one to separate the effects of changes in g_s from those of changes in A on leaf gas exchange as it shares dependence on g_s with $\delta^{13}C$, but is independent of A (Scheidegger et al. 2000). $\delta^{18}O$ of bulk leaf material is mainly determined by the isotopic composition of soil water and evaporative and diffusion effects during transpiration. Assuming uniform $\delta^{18}O$ of soil water, the degree of enrichment in leaf water depends on the ratio of the vapor pressure in the atmosphere and the intercellular spaces within the leaf (Barbour 2007). A conceptual model developed by Scheidegger et al. (2000) to study the influence of micro-climatic conditions on plant physiology, was semi-quantitatively expanded by Grams et al. (2007) for investigating the effects of elevated CO_2 or O_3 concentrations on g_s and A.

As an essential nutrient, N has the potential to strongly affect plant performance either directly by stimulating growth or indirectly by affecting competition in communities (Bobbink et al. 1998). Generally, higher N availability enhances leaf N concentrations (%N) and, consequently, enzyme quantities and activities which, together with higher chlorophyll contents, stimulate A (Evans 1989). In turn, this improves instantaneous WUE when leaf conductance to water vapor (g_s) is maintained (Lee et al. 2001). Investment of C in leaf area expansion at the expense of leaf thickness results in higher specific leaf area (SLA), which enables individuals to efficiently occupy space aboveground when competition for nutrients shifts to competition for light (Schieving and Poorter 1999).

Co-occurring O_3 and N deposition might interact via plant functional traits. Species-specific O_3 sensitivity is assumed to be determined mainly by g_s controlling the major pathway of O_3 uptake (Grulke et al. 2007), and by leaf morphology, e.g., SLA, determining the mesophyll resistance to O_3 diffusion (Ferdinand et al. 2000). It was suggested that plant species characterized by high relative growth rates (RGR), and with related leaf traits such as high g_s and high SLA, would be more susceptible to O_3 than slow-growing species (Bassin et al. 2007a). Similarly, the growth response to increased N supply is stronger in species with a high initial SLA, a parameter that serves as a proxy for potential RGR (Grime et al. 1996). Consequently, as a result of phenotypic plasticity, O_3 tolerance could be modulated by N availability: plants might become less tolerant to O_3 stress when RGR is stimulated by higher N availability or, alternatively, more tolerant when detoxification is increased through N stimulation of A. In *Plantago major,* high nutrient supply protected individuals from O_3 damage (Whitfield et al. 1998). So far, at the level of intact plant communities, interactions between O_3 exposure and N input have not been tested.

Using a free air fumigation system established at 2,000 m a.s.l., we were able for the first time to expose intact monoliths of a species-rich subalpine *Geo-Montani-Nardetum* pasture in situ to different combinations of O_3 and N deposition (Bassin et al. 2007b). After 3 years of treatment, increasing N input but not elevated O_3 had a significant effect on the plant community composition and on aboveground productivity, with sedges benefiting over-proportionally. As an integrated measure for leaf area index and chlorophyll content, the change in the normalized difference vegetation index (NDVI) suggested increasing leaf chlorophyll concentrations after N addition, as well as increased aboveground necromass with elevated O_3, thus suggesting the presence of subtle effects that may be present in the absence of changes in growth. This possibility could be tested by investigating shifts in physiological and morphological characteristics of selected key species. Moreover, the analysis of the relationships between specific leaf traits and the tolerance to O_3, N, and to their combined deposition, could lead to more general conclusions about community responses to theses atmospheric pollutants.

Therefore, the aim of the present study was to test the following hypotheses:

1. Elevated N deposition has a positive effect on leaf gas exchange, as indicated by shifts in $\delta^{13}C$ and $\delta^{18}O$, total leaf chlorophyll ($a + b$) (Chl) and N concentration, and

stimulates leaf growth, with the extent of the response being positively related to the species' initial SLA and g_s.

2. Elevated O_3 negatively affects Chl and N concentrations and gas exchange, with the response being more pronounced in species with high initial SLA and g_s.

3. N-induced stimulation of growth and related changes in SLA and g_s increase the specific sensitivity to O_3.

Materials and methods

Study site

The experimental site is located at Alp Flix, Sur, Switzerland (2,000 m a.s.l., N 9° 39′/E 46° 32′), on a high plateau near the Julier Pass in the Central Alps. Climatic conditions are characterized by cold winters with a permanent snow cover and a growing season extending from April to October with average temperatures of 5.6, 7.2 and 7.0°C and total precipitation of 722.6, 488.7, and 368.7 mm in 2004, 2005, and 2006, respectively (Bassin et al. 2007b).

The vegetation type is a *Geo-Montani-Nardetum*, a typical alpine pasture community, covering large areas in the subalpine zone of the European Alps and Pyrenees. The most abundant species are the grasses *Festuca* spp. *(*mixture of *Festuca rubra* and *Festuca violacea)*, *Nardus stricta* and the sedge *Carex sempervirens*, which together account for approximately one-half of the cover (Table 1). The most frequent forbs and legume species each contribute approximately 2–4% to the total cover. More than 70 forbs and a few grasses and legumes account for the remaining 40%

Table 1 Key plant species of the *Geo-Montani-Nardetum* pasture used for the investigation of individual species' responses to the N and O_3 treatment, grouped by functional group affiliation

Scientific name	Functional group	Cover[a] (%)	Abbreviation
Festuca violacea Gaudin and *Festuca rubra* L.	Grasses	16.3	*Fesspp*
Nardus stricta L.	Grasses	11.2	*Narstr*
Helictotrichon versicolor (Vill.) Pilg	Grasses	2.4	*Helver*
Carex sempervirens Vill.	Sedges	12.3	*Carsem*
Ranunculus villarsii DC.	Forbs	3.5	*Ranvil*
Leontodon helveticus Mérat	Forbs	3.1	*Leohel*
Ligusticum mutellina (L.) Crantz	Forbs	2.9	*Ligmut*
Potentilla aurea L.	Forbs	2.7	*Potaur*
Arnica montana L.	Forbs	1.9	*Arnmon*
Gentiana acaulis L.	Forbs	1.3	*Genaca*
Trifolium alpinum L.	Legumes	3.2	*Trialp*

[a] Mean relative cover in control plots (%) (from Bassin et al. 2007b)

(Bassin et al. 2007b). In the experimental plots, management with one cut per year replaced previous management as a dairy cattle pasture with ca. 1.3 livestock units ha^{-1} grazing for 3–4 weeks every year but without additional manure application.

The soil is a slightly acidic Cambisol (pH 4.8–6) developed on serpentinite bedrock. Soil depth varies between 20 and 40 cm, but rooting depth is limited typically to a maximum of 20 cm.

Experimental design, O_3 and N treatment

Experimental plots consisted of 180 turf monoliths (30 × 40 cm, 20 cm depth) excavated in the fall 2003 from a nearby pasture, placed in drained plastic boxes, and assigned randomly to nine fumigation rings. Monoliths were placed in shallow pits flush with the surrounding surface.

A free air fumigation system consisting of nine rings of 7-m diameter (for details see Volk et al. 2003; Bassin et al. 2007b) supplied three O_3 treatment levels (ambient [control], 1.2 × ambient O_3 [O_3+] and 1.6 × ambient O_3 concentration [O_3++]), starting on 16 June 2004. With mean O_3 concentrations at these altitudes being relatively high (ca. 45–47 p.p.b. during the vegetation period) but only small diurnal fluctuations, we increased O_3 concentration during day and night between April and October. Cumulative O_3 exposures expressed as AOT40 during the three seasons (2004, 2005, 2006) amounted to 12.6, 11.1, 11.6 p.p.m.-h in the control treatment, 15.2, 27.4, 29.5 p.p.m.-h in the O_3+ treatment, and 28.4, 45.9, 64.9 p.p.m.-h in the O_3++ treatment, respectively (Bassin et al. 2007b). Starting in spring 2004, five N treatments equivalent to 0, 5, 10, 25, 50 kg N ha^{-1} year^{-1} were applied during the growing season as 12 bi-weekly applications of a 200 ml solution of NH_4NO_3 in well water. Each N level was replicated 4 times in each fumigation ring. Average combined annual background deposition (ca. 4 kg N ha^{-1} year^{-1}, Bassin et al. 2007b) and N applications amounted to 4 (N4), 9 (N9), 14 (N14), 29 (N29), and 54 kg N ha^{-1} year^{-1} (N54).

Leaf sampling

Leaves of the 11 most frequent species, accounting together for ca. 61% of the cover in the control plots (Table 1) were sampled during the last week of July 2005 and 2006. After being collected from different individuals, 20–25 fully developed leaves per species, from which petioles were removed, were pooled for the four *N* replications in each fumigation ring and then divided into two sub-samples. One sub-sample was immediately immersed in liquid nitrogen and stored in the dark at −80°C until Chl extraction. The other sub-sample was stored (maximum 30 min) between layers of moist tissue paper until leaf area measurements.

Leaf area measurement

Leaf number and leaf area were determined by using flat-bed scanners (EPSON perfection 4990; EPSON, Switzerland) and image processing software (Winfolia and Winseedle; Régent Instruments, Quebec). Sub-samples were dried to constant weight at 60°C and weighed. Mean leaf weight is the sub-sample weight divided by leaf number. SLA was obtained by dividing mean leaf area by mean leaf weight for each sub-sample (except for *Festuca* spp. owing to technical reasons).

Stable isotope analysis

Dried leaves were ground to a fine powder with a ball mill (MM301 mixer mill; Retsch, Germany). $\delta^{13}C$ was measured using a Flash EA 1112 series elemental analyzer (Thermo Italy, Rhodano, Italy) coupled to a Finnigan MAT Delta^{plus}XP isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) via a six-port valve and a ConFlo III (Werner et al. 1999). Bulk leaf $\delta^{18}O$ was determined using a high temperature oxygen analyzer (Hekatech, Wegberg, Germany) coupled to a Finnigan MAT Delta^{plus}XP isotope ratio mass spectrometer via a four-port valve (Vici, Schenkon, Switzerland) and a ConFlo III.

Samples of 5 mg ground leaves were transferred into tin capsules (Säntis Analytical, Teufen, Switzerland) for $^{13}C/^{12}C$ analysis and for measurement of leaf N concentration (%), and 0.5 mg plant material was placed in silver capsules for $^{18}O/^{16}O$ analysis. Analysis of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ($\delta^{15}N$ data not presented here) were done using the combustion gases of the same sample after chromatographic separation of the peaks (Brooks et al. 2003). Oxygen in plant samples was pyrolized to CO for the determination of $^{18}O/^{16}O$. All values are expressed in per-mill relative to the standards Vienna Pee Dee belemnite for C and Vienna-standard mean ocean water for $\delta^{18}O$. The long-term precision (1 year) for analysis of the internal quality control standard was 0.04‰ for $\delta^{13}C$. For $\delta^{18}O$ analysis, a long-term quality control is not available yet, but the routine standard deviation of a sample triplet is <0.1‰. Owing to time restrictions, only three out of the five N treatment levels were analyzed for $\delta^{13}C$, $\delta^{18}O$, and %N (N4, N14, N54).

$\delta^{18}O$ in soil water extracted at 5–10 cm depth was determined at three occasions. The mean value was −10.5‰ (±0.41, SE).

Chlorophyll concentration and chlorophyll content

Frozen fresh leaves (−80°C) were lyophilized for 96 h and ground to a fine powder. Chl was extracted by incubating 10–15 leaves for 3 h at 60°C in CaCO$_3$-saturated dimethyl sulfoxide (Fluka, Buchs, Switzerland). After centrifugation, absorption of the diluted supernatant was determined at 664.9 and 648.2 nm with a UV/VIS spectrophotometer (UVIKON 923; Kontron Instruments, Schlieren, Switzerland). To prevent chlorophyll degradation, all steps were carried out under dimmed green light. Chl concentration (Chl$_{conc}$; mg g^{-1}}, was calculated as described by Barnes et al. (1992). Chl content (Chl$_{leaf}$; mg leaf^{-1}), was calculated from Chl$_{conc}$ and leaf weight.

Statistical analyses

Treatment effects on leaf weight, SLA, Chl$_{conc}$ and Chl$_{leaf}$, N concentration, $\delta^{13}C$ and $\delta^{18}O$ ratios of bulk leaf material were tested in a split-plot ANOVA with O$_3$ at the main-plot level and N at the sub-plot level. Block and O$_3$ entered the model as class variables, while N was defined as a continuous variable for the parameters measured at five N levels (SLA, leaf weight, Chl$_{conc}$, Chl$_{leaf}$). Inclusion of higher order interactions and quadratic effects of N in the model were assessed with Akaike's information criterion with correction for small sample sizes (Burnham and Anderson 2002). For the parameters measured only at three N levels (N concentration, $\delta^{13}C$, and $\delta^{18}O$) N entered the model as class variable, and the O$_3 \times$ N interaction was always included. Correlations between parameters were calculated using Pearson's correlation coefficient. All analyses were carried out with the statistical software SAS (SAS Institute, N.C.), applying the procedures proc mixed. To meet the assumptions of ANOVA, data were transformed when necessary. For figures and tables, data were pooled from all O$_3$ treatments to show N effects and from all N treatments to show O$_3$ effects.

Results

SLA and N concentration

In the control treatment (N4), the average SLA for the 11 species ranged from 67 to 208 cm^2 g^{-1} in 2005 and from 70 to 207 cm^2 g^{-1} in 2006. In this treatment, SLA was positively correlated to N concentration, with the latter ranging between 1.40 and 2.85% in 2005 and between 1.46 and 2.78% in 2006 [Pearson's correlation $R = 0.71$, $P = 0.014$ (2005) and $R = 0.58$, $P = 0.075$ (2006)] (Fig. 1). Species characterized by the lowest SLA and N concentration were the grasses *N. stricta* and *Festuca* spp. (N concentration, <1.8%; SLA, <84 cm^2 g^{-1}), while the legume *Trifolium alpinum* together with the forbs *Ranunculus villarsii*, *Potentilla aurea*, and *Leontodon helveticus* (N concentration, >2.2%; SLA, >150 cm^2 g^{-1}) had the highest values of both parameters.

In agreement with our first hypothesis, addition of N in the N54 treatment significantly increased N concentration
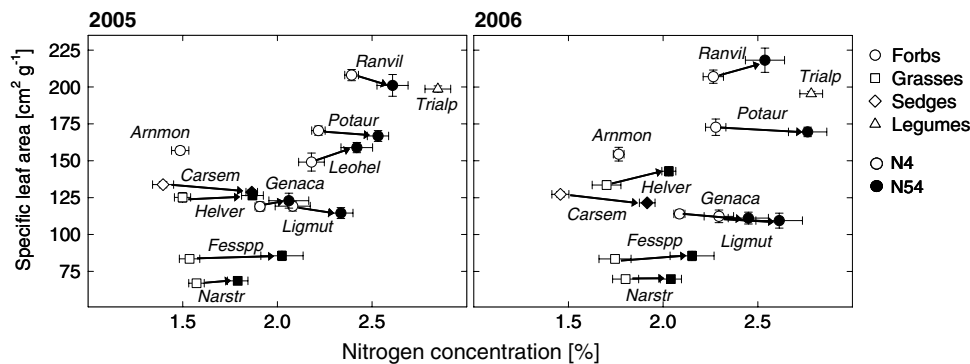
Fig. 1 Effect of N treatment [*N4* (control), *N54*] on leaf N concentration (%) and specific leaf area (cm² g⁻¹) of 11 key plant species of a subalpine pasture in 2005 and 2006. Means ± SE are shown {where *arrows* are missing there were non-significant effects on both parameters according to ANOVA [Tables S8 and S9 in the Electronic supplementary material (ESM)]}. For abbreviations, see Table 1

in all species, except *Arnica montana* and *T. alpinum* (Table S1; Fig. 1). The strongest relative N increase was seen in species characterized by low initial SLA and N concentration, i.e., *C. sempervirens* (+34% in 2005, +31% in 2006; Table S1), *Festuca* spp. (+32% in 2005, +23% in 2006), and in *Helictotrichon versicolor* (+25% in 2005, +19% in 2006). Effects of N addition on N concentration and SLA were mostly linear (Tables S8–S9). SLA was generally not affected by N addition (Table S2), except for *C. sempervirens* with reductions of −4% in 2005 and −5% in 2006, and significant increases of 7% and 6% in 2006 for *H. versicolor* and *L. helveticus*, respectively.

In contrast to N, elevated O_3 had only marginal and inconsistent effects on both SLA and N concentration of all species investigated (Tables S1, S2).

Chl_{conc}, Chl_{leaf} and leaf weight

Vector analysis of shifts in Chl_{conc} versus Chl_{leaf} in relation to leaf weight can be used to analyze plant responses to changing nutrient availability (Timmer and Morrow 1984) (Fig. 2). As expected, after 3 years of N addition in N54, Chl_{conc} had increased significantly relative to N4: by 45–54% in *C. sempervirens*, by 20–30% in *H. versicolor*, *L. helveticus*, *R. villarsii*, *Festuca* spp. and *Ligusticum mutellina*, and by 10–20% in *P. aurea*, *Gentiana acaulis* and *N. stricta* (Table S4). Contrary to our expectation, leaf weight was only slightly stimulated (<10%) by N addition in most species, as indicated by the positive shifts along leaf weight isolines (Fig. 2a, b). Leaf weight significantly increased in the N54 treatment in both years in two species only: in *C. sempervirens* by +59% (2005) and +52% (2006), in *P. aurea* by +20% (2005) and +23% (2006) and in *N. stricta* by +22% in 2006 (Table S5).

Of the 11 species, only two showed an expected O_3-induced reduction in Chl_{conc}, namely *R. villarsii* [−7% (O_3+) and −19% (O_3++) in 2005] and *A. montana* [−3%

(O_3+) and −20% (O_3++) in 2006] (Table S4). In contrast, Chl_{conc} of *C. sempervirens* increased by +8% (O_3+) and by +14% (O_3++) in 2006. Thus, the general trend towards reduced Chl_{leaf} in the O_3++ treatment compared to the control (Table S3), reflected in Fig. 2c, d as shifts towards the lower left corner, resulted from reduced leaf weight. The O_3++ treatment tended to decrease leaf weight (>10%) in five species in 2005 and in three species in 2006 (Table S5), but this effect was significant only for *L. helveticus* (−20% in 2005, −7% in 2006) and for *P. aurea* (−25% in 2006).

Only a small number of species showed significant $O_3 \times N$ interactive effects (Chl_{conc} in 2005, one species; Chl_{conc} in 2006, three species; leaf weight 2006, two species) (Tables S4–S5). In three of these six cases, the stimulating effect of N was strongest in the intermediate O_3+ treatment: for Chl_{conc} of *L. mutellina* (2006) and for leaf weight of *H. versicolor* and *R. villarsii* (2006).

## $\delta^{18}O$ and $\delta^{13}C$

Contrary to our first hypothesis, N addition affected bulk leaf isotopic signature only marginally. However, in leaves of *C. sempervirens* with the strongest N-induced increase in leaf weight (see above), less negative foliar $\delta^{13}C$ in the N54 treatment relative to the N4 treatment was consistent and significant (Fig. 3 a, b; Table S6), while $\delta^{18}O$ remained unchanged (Table S7). In contrast, $\delta^{13}C$ of *L. helveticus* was significantly more negative (−0.3‰) in the N54 treatment in 2005 with constant $\delta^{18}O$. Yet another pattern was found in *Festuca* spp., showing a decrease in $\delta^{18}O$ (−0.4‰) in response to N addition in 2006, while $\delta^{13}C$ values remained unaffected. *A. montana* showed a non-linear reaction in both years.

Leaf isotopic signature was affected by O_3 only in a few species (Fig. 3c, d; Tables S6, S7), in contrast to our second hypothesis. In 2005, in *L. mutellina*, foliar $\delta^{13}C$ was unaffected while $\delta^{18}O$ increased by +0.6‰ in the O_3++

Fig. 2 Effect of N treatment [N4 (control), N54] (**a**, **b**) and O₃ treatment [*O₃ control*, *O₃+*, *O₃++*] (**c**, **d**) on leaf weight (mg), total chlorophyll content (mg leaf⁻¹), and total chlorophyll concentration (mg g⁻¹) of 11 key plant species of a subalpine pasture in 2005 and 2006 [only significant responses according to ANOVA (Tables S10, S11 and S12 in ESM) are displayed]. Means ± SE are shown. *Isolines* represent constant leaf weight (mg leaf⁻¹). For abbreviations, see Table 1 and Methods
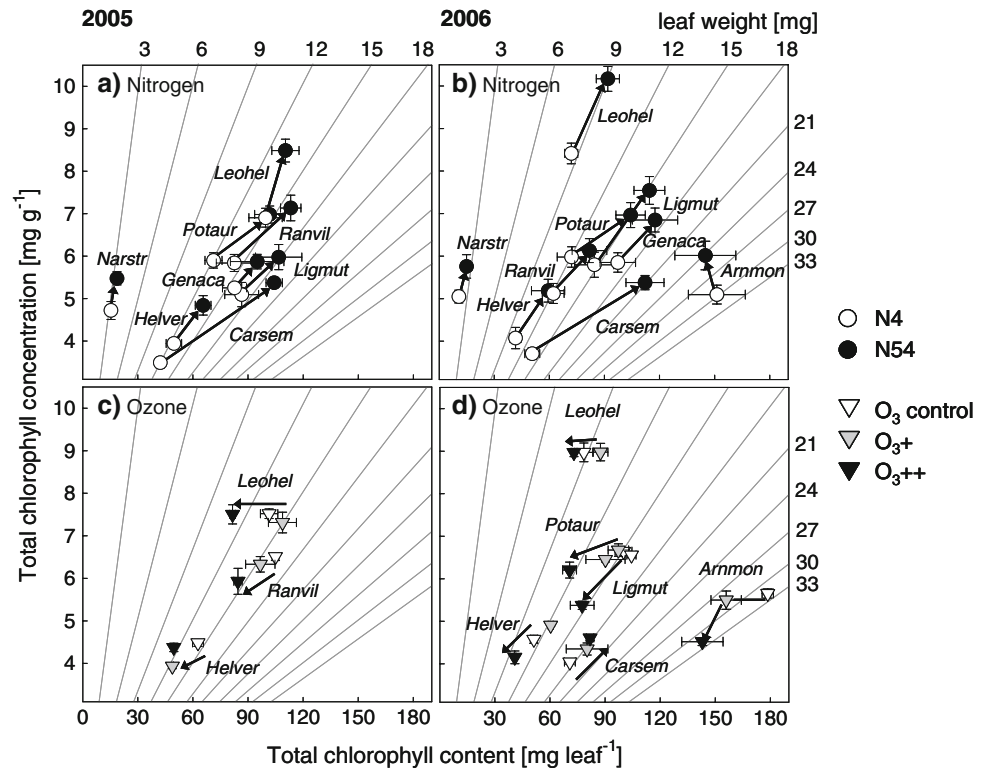
Fig. 3 Effect of N treatment [N4 (control), *N14*, N54] (**a**, **b**) and O₃ treatment [O₃ control, O₃+, O₃++] (**c**, **d**) on δ¹³C [‰] and δ¹⁸O [‰] values of bulk leaf organic material of 11 key plant species of a subalpine pasture in 2005 and 2006 [only significant responses according to ANOVA (Tables S13, S14 in ESM) are displayed]. Means ± SE are shown. For abbreviations, see Table 1 and Methods
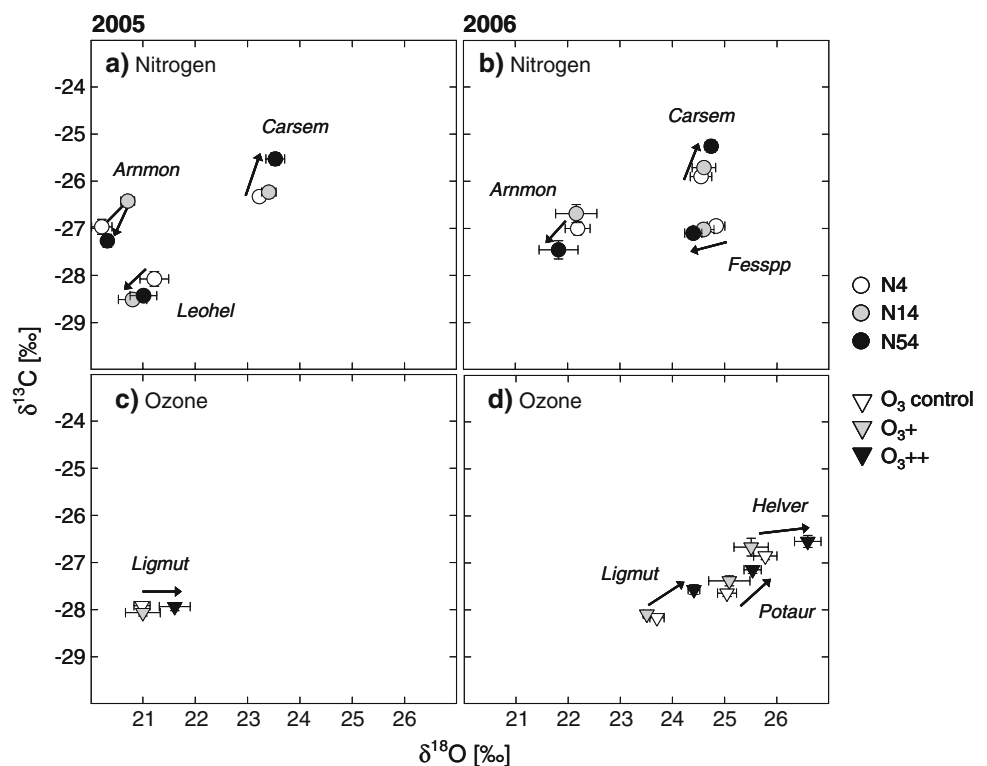
treatment. In 2006, the situation was similar in *H. versicolor*, with unchanged δ¹³C and increased δ¹⁸O (+0.8‰). In the same year, *L. mutellina* had less negative δ¹³C (+0.6‰) together with higher δ¹⁸O (+0.7‰) in the O₃++ treatment. In *P. aurea*, δ¹³C shifted towards less negative values (+0.5‰) at constant δ¹⁸O.

O₃ effects on δ¹⁸O (2005, three species; 2006, seven species) were more prominent than on δ¹³C (2005, one species;

2006, four species), and effects of N on $\delta^{18}O$ (2005, no species; 2006, one species) or $\delta^{13}C$ (2005, one species; 2006, two species) were less frequent than effects of O_3, but these observations of trends include considerable but non-significant differences (e.g., changes of $\geq 0.5‰$).

Finally, our third hypothesis, suggesting $O_3 \times N$ interactive effects through N-induced changes in g_s, was only confirmed by significant interactive effects on $\delta^{18}O$ in *H. versicolor* (both years), in *T. alpinum* (2005) and in *N. stricta* (2006), but the pattern was not consistent. For $\delta^{13}C$, the only interaction was observed in *L. mutellina* in 2005.

Relationship between treatment effect and initial SLA and $\delta^{18}O$ signature

To test whether or not measured shifts in the selected parameters with increased N (N54) or elevated O_3 (O_3++) were related to initial plant traits in the control treatment (N4 at O_3 control, 2-year averages), Pearson's correlation analysis was performed across all species. Correlations were found to be either weak or absent (Table 2). A slight positive correlation was observed in both years between initial $\delta^{18}O$ and N-induced change in N concentration (Δ%N) ($P = 0.02$ in 2005, $P = 0.05$ in 2006), and a negative correlation between Chl_{conc} and Δ%N. The effect of O_3 was more pronounced in the second year when initial $\delta^{18}O$ was negatively and leaf weight was positively related to ΔSLA. No significant relationships were found in either

N or O_3 treatments between initial SLA and any other parameter.

Discussion

N effect

The N addition affected the species investigated after 2 and 3 years rather differently. Earlier, we reported that N addition exceeding 10 kg ha^{-1} year^{-1} strongly altered species composition in favor of sedges which increased their fractional biomass from 10% in the control to 30% in the N54 treatment (Bassin et al. 2007b). In support of that, two proxies for growth (SLA and leaf weight) indicated an effect of N on aboveground productivity only in two out of the 11 species, i.e., leaf weight increased in N54 in the sedge *C. sempervirens* and in *P. aurea*. Hence, the rapid change in community composition was likely favored by the absence of a growth response in most species, allowing N-responsive species such as *C. sempervirens* to quickly gain in abundance.

In contrast to its effect on growth, N addition increased Chl_{conc} and N concentration in the majority of species. This effect of increased N supply is common, especially in cool climates (Michelsen et al. 1996), and it is often observed when growth is restricted by other environmental factors (Timmer and Morrow 1984). It supports an earlier notion

Table 2 Relationship between initial leaf traits (control treatment, averaged over 2 years) of 11 key plant species and the relative changes in these traits (Δ) caused by the 54 kg N ha^{-1} year^{-1} treatment (*N54*) and the 1.6 $\times$ ambient O_3 concentration treatment (O_3++), respectively, compared to the control

	$\Delta\delta^{13}C$		$\Delta\delta^{18}O$		ΔChl_{conc}		Δ leaf weight[a]		Δ%N		ΔSLA	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Change caused by N54 relative to control												
Initial $\delta^{13}C$	−0.24	−0.09	0.45	−0.06	0.08	0.03	0.39	0.24	0.04	0.09	−0.08	−0.29
Initial $\delta^{18}O$	−0.54	−0.36	−0.30	−0.08	0.47	0.26	0.29	0.58	**0.69***	**0.63***	0.12	0.21
Initial Chl_{conc}	**0.63***	0.33	−0.18	0.47	−0.52	−0.54	−0.51	−0.52	**−0.72***	**−0.68***	0.26	0.24
Initial leaf weight	0.23	0.30	0.45	−0.52	−0.14	0.13	−0.22	−0.50	−0.07	−0.19	−0.27	−0.13
Initial %N	0.28	0.27	0.06	0.31	−0.60	−0.53	−0.18	−0.42	**−0.83***	−0.63	−0.15	0.14
Initial SLA	−0.02	−0.02	0.22	0.13	−0.21	−0.22	0.11	−0.29	−0.54	−0.39	−0.36	0.28
Change caused by O_3++ relative to control												
Initial $\delta^{13}C$	0.06	0.46	−0.37	0.03	0.42	−0.51	0.26	0.16	0.25	0.33	−0.18	−0.10
Initial $\delta^{18}O$	0.18	0.28	−0.34	0.35	0.48	−0.33	−0.49	**0.79***	0.06	−0.22	0.14	**−0.73***
Initial Chl_{conc}	0.11	−0.26	0.56	−0.15	−0.09	0.28	0.14	−0.37	0.23	−0.04	0.23	0.15
Initial leaf weight	−0.55	−0.57	0.03	−0.25	**−0.65***	0.42	−0.07	−0.53	−0.31	−0.12	−0.08	**0.90***
Initial %N	−0.01	−0.30	**0.70***	−0.13	−0.19	0.09	0.47	−0.18	−0.01	0.17	−0.12	−0.05
Initial SLA	−0.27	−0.43	0.41	−0.02	−0.16	−0.11	0.01	−0.46	0.23	0.19	0.23	0.32

Pearson's correlation coefficients are shown. Chl_{conc} Leaf chlorophyll ($a + b$) concentration (mg g^{-1}), *%N* % leaf N concentration, *SLA* specific leaf area (cm^2 g^{-1})

* $P < 0.05$

[a] Milligrams

(Bassin et al. 2007b) that accumulation of Chl was the major cause for the observed N-induced increase in NDVI in the plots receiving extra N. This accumulation of Chl and N in leaves should lead to a stimulation of A and, consequently, increased g_s (Evans 1989). Analysis of stable isotope ratios by means of a semi-quantitative model relating $\delta^{18}O$ to g_s was used to test if co-occurring changes in $\delta^{13}C$ were related to changes in either A or g_s (Grams et al. 2007). According to this model, a simultaneous increase in $\delta^{13}C$ and $\delta^{18}O$ indicates a decline in c_i/c_a (indicated by less negative $\delta^{13}C$) as a result of reduced g_s (indicated by increased $\delta^{18}O$). The negative relationship between $\delta^{18}O$ and g_s is empirically well supported (Barbour et al. 2000). The prerequisites for a relationship between $\delta^{18}O$ and g_s such as equal $\delta^{18}O$ in soil water and air humidity in all treatments were given in this study since monoliths were isolated from the surrounding soil by drained boxes and exposed to homogeneous atmospheric conditions. Potential differences in $\delta^{18}O$ due to dilution of leaf water by depleted transpiration water were considered to be negligible. The analysis of leaf isotopic composition revealed only a weak relationship of gas exchange to the observed increase in Chl and N. The only consistent effect of N in both years was detected in *C. sempervirens* as a shift towards less negative $\delta^{13}C$ at constant $\delta^{18}O$. This reaction suggests increased A at constant g_s, thus reflecting enhanced WUE with increased N, similar to findings for prairie grassland species (Lee et al. 2001). It corresponds well to the pronounced growth stimulation observed specifically in *C. sempervirens*. The simultaneous stimulation of A and g_s by N addition observed in *Festuca* spp. has also been observed elsewhere (Gordon et al. 1999), but here it was not associated with increased growth.

The pronounced interspecific differences in the response to increasing N are difficult to explain. The strong increase in leaf N concentration observed in most species suggests that they are related to differences in N investment rather than in differential N uptake efficiency or preference of certain N forms (NO_3^-, NH_4^+, amino acids). Theory suggests that species characterized by a competitive growth strategy (e.g., high initial SLA, high potential RGR) should react most strongly to extra N (Grime et al. 1996). However, under the conditions of this experiment, *C. sempervirens*, a stress-tolerant species (Caccianiga et al. 2006), was most responsive to N addition, despite the presence of species with a more competitive growth strategy (e.g., *L. mutellina*, *Agrostis capillaris*). A relationship between RGR-related leaf traits such as SLA and g_s and the response to N in terms of any of the measured parameters could not be observed. Interspecific differences could be related to growth restrictions caused by specific uptake and storage of P or K. Güsewell (2004) assumed that the often observed dominance of clonal graminoids after N deposition results

from the adaptation of these species to low P availability through their high efficiency of P resorption from senescing leaves. Analysis of nutrient ratios in leaf tissue following nutrient addition could indicate shifts in nutrient balances (Güsewell 2004), although such investigations may also not be conclusive, since growth could equally be limited by other environmental factors such as low temperature or a short growing season (Körner 2003; see below). Hence, in view of the lack of data for foliar nutrient levels, and because of the difficulty in interpreting such data, the reasons behind the interspecific difference in the short-term response to increasing N observed in this experiment remain open.

O$_3$ effect

Elevated O_3 exposure had no significant effect on productivity and community composition (Bassin et al. 2007b). Given the low number of replicates ($n = 3$), the magnitude of effects of O_3 to be statistically significant must be much larger than those observed after only 3 years of treatment. However, it was expected that elevated O_3 could have subtle negative effects on Chl_{conc}, N concentration and gas exchange. In fact, in a few species elevated O_3 caused reduced Chl_{conc} associated with enhanced leaf yellowing, a typical symptom of O_3 stress (Grandjean and Fuhrer 1989), thus contributing to the decrease in NDVI observed at elevated O_3 at the community level (Bassin et al. 2007b). The decrease in leaf longevity should be associated with changing A and g_s, as found in many earlier studies (cf. Fuhrer and Booker 2003). In species from temperate grassland, a reduction in g_s at constant A was revealed by analysis of $\delta^{13}C$ and $\delta^{18}O$ in O_3-exposed leaves (Jäggi and Fuhrer 2007). Here, only *L. mutellina* showed this response pattern in 2006, while the increase in $\delta^{18}O$ at constant $\delta^{13}C$ suggested reduced A and g_s in *L. mutellina* and *H. versicolor*. A weak but consistent increase in $\delta^{18}O$ observed in the O_3++ treatment in seven species, especially in 2006, indicated a general trend towards O_3-induced stomatal closure, in support of our hypothesis. But it remains unclear whether O_3 affects g_s directly, or whether stomatal closure is the downstream consequence of an increase in c_i as a result of reduced A (Paoletti and Grulke 2005).

Stomatal closure and reduced A are often considered major causes for reductions in aboveground biomass, for instance in *P. major* (Zheng et al. 2002) and birch (Oksanen and Saleem 1999). For half of the species studied here, elevated O_3 caused small reductions in leaf weight (Table S5). Remarkably, the reduction in leaf weight was not strongest in the species showing the most pronounced decline in A, g_s, Chl, and N concentration (*L. mutellina* in 2005 and *L. mutellina*, *P. aurea,* and *H. versicolor* in 2006), but rather in *L. helveticus*. The latter species showed

consistent reductions in both leaf weight and leaf thickness with elevated O_3 but remained unaffected by O_3 in terms of Chl and N concentration, and in isotopic composition. In this species, enhanced resource demand for biochemical defense could have reduced the C investment in leaf construction, as was suggested for O_3-sensitive clones of birch (Oksanen and Saleem 1999).

In contrast to our hypothesis, the data reveal no relationship between RGR-related leaf traits (SLA and g_s) and the specific O_3 sensitivity (Table 2). This agrees with data for a range of species presented by Hayes et al. (2007), but contrasts suggestions by Bassin et al. (2007a), Pleijel and Danielsson (1997) and Bungener et al. (1999), who found relationships between tolerance to O_3 and specific initial RGR. The strongest O_3 response was detected in species with intermediate SLA and ^{18}O enrichment. Also, contrary to the commonly high O_3 sensitivity of legumes relative to grasses and forbs (e.g., Hayes et al. 2007), and despite a high SLA, the only legume under investigation (*T. alpinum*) remained unaffected by elevated O_3.

Overall, the modest O_3 responses of individual species are in line with the absence of O_3 effects on aboveground productivity and functional group composition observed at community level (Bassin et al. 2007b). This contrasts with findings from a meta-analysis of data from fumigation experiments with pot-grown plants, which suggested alpine plant communities to be highly O_3 sensitive (Mills et al. 2007). Thus the present study gives additional evidence that plants react less to elevated O_3 when grown in their natural environment (Bassin et al. 2007a), and that late successional plant communities are rather resilient (Körner 2003). The lack of a relationship between reductions in growth and A and/or g_s suggests that in most of these species, A is not limiting growth. At high elevations, growth is probably rather limited by low temperatures slowing down cell construction and maturation, thus limiting A through limited C sink capacity as observed in a CO_2 enrichment experiment with alpine grassland (Körner 2003). Nevertheless, observed subtle effects of elevated O_3 on leaf longevity in some species could in the longer run lead to altered community composition, as observed by Volk et al. (2006) in montane grassland.

$O_3 \times N$ interaction

N-induced specific changes in SLA and g_s were expected to be related to increasing O_3 sensitivity. However, these traits were only marginally modified by N addition and most of the observed significant $O_3 \times N$ interactions were not statistically related to changes in SLA and $\delta^{18}O$. Due to its exceptional response to N addition, *C. sempervirens* was the only species in which pronounced $O_3 \times N$ interactions could be expected. However, this sedge remained largely unaffected by O_3 in any of the N treatments, which corresponds to the observed absence of an $O_3 \times N$ interaction in terms of aboveground productivity (Bassin et al. 2007b). The probability for observing $O_3 \times N$ interactive effects may be similarly low in other ecosystems characterized by singular species' dominance after N supply (e.g., heaths, bogs and tundra, see Bobbink et al. 1998), which could be the result of co-occurring growth limitations. In contrast, $O_3 \times N$ interactions are more likely in habitats where more species are stimulated by elevated N input due to more favorable growth conditions.

Conclusion

The absence of N-induced stimulation of growth despite increased N accumulation, and of any relationship between reductions in A and aboveground productivity with elevated O_3 suggest that in most of the species studied, growth was not limited by N supply and C assimilation. This indicates that most subalpine species are comparatively unresponsive to both O_3 and N in the short term. However, the exceptional ability of *C. sempervirens* to invest extra N into enhanced A, improved WUE, and growth is likely responsible for the over-proportional increase in biomass in response to N addition in this sedge. Thus it cannot be excluded that in the longer run, differences in the extent and quality of the responses to either O_3 or N among species could have an important effect on community composition.

The extent of the responses to both N and O_3 was not related to specific initial SLA or g_s (representing potential RGR). The species most responsive to O_3 and N were characterized by intermediate SLA and g_s. This suggests that commonly used principles of functional growth analysis do not directly hold under the specific conditions of this experiment, which limits the application of functional principles to predict the sensitivity to environmental changes of such old, subalpine plant communities.

References

Ashmore MR (2005) Assessing the future global impacts of ozone on vegetation. Plant Cell Environ 28:949–964

Barbour MM (2007) Stable oxygen isotope composition of plant tissue: a review. Funct Plant Biol 34:83–94

Barbour MM, Fischer RA, Sayre KD, Farquhar GD (2000) Oxygen isotope ratio of leaf and grain material correlates with stomatal

conductance and grain yield in irrigated wheat. Aust J Plant Physiol 27:625–637

Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW (1992) A reappraisal of the use of DMSO for the extraction and determination of chlorophyll-*a* and chlorophyll-*b* in lichens and higher-plants. Environ Exp Bot 32:85–100

Bassin S, Volk M, Fuhrer J (2007a) Factors affecting the ozone sensitivity of temperate European grasslands: an overview. Environ Pollut 146:678–691

Bassin S, Volk M, Suter M, Buchmann N, Fuhrer J (2007b) Nitrogen but not ozone affects productivity and species composition of subalpine grassland after 3 year of treatment. New Phytol 175:523–534

Bobbink R, Hornung M, Roelofs JGM (1998) The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation. J Ecol 86:717–738

Brooks PD, Geilmann H, Werner RA, Brand WA (2003) Improved precision of coupled $\delta^{13}C$ and $\delta^{15}N$ measurements from single samples using an elemental analyser/isotope ratio mass spectrometer combination with a post-column 6-port valve and selective CO_2 trapping; improved halide robustness of the combustion reactor using CO_2. Rapid Commun Mass Spectrom 17:1924–1926

Bungener P, Nussbaum S, Grub A, Fuhrer J (1999) Growth response of grassland species to ozone in relation to soil moisture condition and plant strategy. New Phytol 142:283–293

Burnham KP, Anderson DR (2002) Model selection and multimodel inference: a practical–theoretic approach. Springer, Berlin

Caccianiga M, Luzzaro A, Pierce S, Ceriani RM, Cerabolini B (2006) The functional basis of a primary succession resolved by CSR classification. Oikos 112:10–20

Evans JR (1989) Photosynthesis and nitrogen relationships in leaves of C-3 plants. Oecologia 78:9–19

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 40:503–537

Ferdinand JA, Fredericksen TS, Kouterick KB, Skelly JM (2000) Leaf morphology and ozone sensitivity of two open pollinated genotypes of black cherry (*Prunus serotina*) seedlings. Environ Pollut 108:297–302

Fuhrer J, Booker F (2003) Ecological issues related to ozone: agricultural issues. Environ Int 29:141–154

Gordon C, Woodin SJ, Alexander IJ, Mullins CE (1999) Effects of increased temperature, drought and nitrogen supply on two upland perennials of contrasting functional type: *Calluna vulgaris* and *Pteridium aquilinum*. New Phytol 142:243–258

Grams TE, Kozovits AR, Häberle KH, Matyssek R, Dawson TE (2007) Combining $\delta^{13}C$ and $\delta^{18}O$ analyses to unravel competition, CO_2 and O_3 effects on the physiological performance of different-aged trees. Plant Cell Environ 30:1023–1034

Grandjean A, Fuhrer J (1989) Growth and leaf senescence in spring wheat (*Triticum aestivum*) grown at different ozone concentrations in open-top field chambers. Physiol Plant 77:389–394

Grime JP, Hodgson JG, Hunt R (1996) Comparative ecology. A functional approach to common British species. Chapman & Hall, London

Grulke NE, Neufeld HS, Davison AW, Roberts M, Chappelka AH (2007) Stomatal behavior of ozone-sensitive and -insensitive coneflowers (*Rudbeckia laciniata* var. *digitata*) in Great Smoky Mountains National Park. New Phytol 173:100–109

Güsewell S (2004) N:P ratios in terrestrial plants: variation and functional significance. New Phytol 164:243–266

Hayes F, Jones MLM, Mills G, Ashmore MR (2007) Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. Environ Pollut 146:754–762

Jäggi M, Saurer M, Volk M, Fuhrer J (2005) Effects of elevated ozone on leaf $\delta^{13}C$ and leaf conductance of plant species grown in semi-natural grassland with or without irrigation. Environ Pollut 134:209–216

Jäggi M, Fuhrer J (2007) Oxygen and carbon isotopic signatures reveal a long-term effect of free-air ozone enrichment on leaf conductance in semi-natural grassland. Atmos Environ 41:8811–8817

Körner C (2003) Alpine plant life. Springer, Heidelberg

Lee TD, Tjoelker MG, Ellsworth DS, Reich PB (2001) Leaf gas exchange responses of 13 prairie grassland species to elevated CO_2 and increased nitrogen supply. New Phytol 150:405–418

Matson P, Lohse KA, Hall SJ (2002) The globalization of nitrogen deposition: consequences for terrestrial ecosystems. Ambio 31:113–119

Michelsen A, Jonasson S, Sleep D, Havstrom M, Callaghan TV (1996) Shoot biomass, $\delta^{13}C$, nitrogen and chlorophyll responses of two arctic dwarf shrubs to in situ shading, nutrient application and warming simulating climatic change. Oecologia 105:1–12

Mills G, Hayes F, Jones MLM, Cinderby S (2007) Identifying ozone-sensitive communities of (semi-)natural vegetation suitable for mapping exceedance of critical levels. Environ Pollut 146:736–743

Nussbaum S, Bungener P, Geissmann M, Fuhrer J (2000) Plant–plant interactions and soil moisture may be important in determining ozone impacts on grasslands. New Phytol 147:327–335

Ojanperä K, Patsikka E, Ylaranta T (1998) Effects of low ozone exposure of spring wheat on net CO_2 uptake, Rubisco, leaf senescence and grain filling. New Phytol 138:451–460

Oksanen E, Saleem A (1999) Ozone exposure results in various carry-over effects and prolonged reduction in biomass in birch (*Betula pendula* Roth). Plant Cell Environ 22:1401–1411

Paoletti E, Grulke NE (2005) Does living in elevated CO_2 ameliorate tree response to ozone? A review on stomatal responses. Environ Pollut 137:483–493

Pleijel H, Danielsson H (1997) Growth of 27 herbs and grasses in relation to ozone exposure and plant strategy. New Phytol 135:361–367

Saurer M, Maurer S, Matyssek R, Landolt W, Günthardt-Goerg MS, Siegenthaler U (1995) The influence of ozone and nutrition on $\delta^{13}C$ in *Betula pendula*. Oecologia 103:397–406

Scheidegger Y, Saurer M, Bahn M, Siegwolf R (2000) Linking stable oxygen and carbon isotopes with stomatal conductance and photosynthetic capacity: a conceptual model. Oecologia 125:350–357

Schieving F, Poorter H (1999) Carbon gain in a multispecies canopy: the role of specific leaf area and photosynthetic nitrogen-use efficiency in the tragedy of the commons. New Phytol 143:201–211

Timmer VR, Morrow LD (1984) Predicting fertilizer growth response and nutrient status of jack pine by foliar diagnosis. In: Stone EL (ed) Forest soils and treatment impacts. The University of Tennesse, Knoxville, pp 335–351

Torsethaugen G, Pell EJ, Assmann SM (1999) Ozone inhibits guard cell K^+ channels implicated in stomatal opening. Proc Natl Acad Sci USA 96:13577–13582

Volk M, Geissmann M, Blatter A, Contat F, Fuhrer J (2003) Design and performance of a free-air exposure system to study long-term effects of ozone on grasslands. Atmos Environ 37:1341–1350

Volk M, Bungener P, Contat F, Montani M, Fuhrer J (2006) Grassland yield declined by a quarter in 5 years of free-air ozone fumigation. Global Change Biol 12:74–83

Werner RA, Bruch BA, Brand WA (1999) Conflo III—an interface for high precision $\delta^{13}C$ and $\delta^{15}N$ analysis with an extended dynamic range. Rapid Commun Mass Spectrom 13:1237–1241

Whitfield CP, Davison AW, Ashenden TW (1998) The effects of nutrient limitation on the response of *Plantago major* to ozone. New Phytol 140:219–230

Zheng Y, Shimizu H, Barnes JD (2002) Limitations to CO_2 assimilation in ozone-exposed leaves of *Plantago major*. New Phytol 155:67–78