DISS. ETH NO. 17373

Effects of combined ozone and nitrogen deposition on a species-rich subalpine pasture

A dissertation submitted to
ETH ZURICH

for the degree of
DOCTOR OF SCIENCES

presented by
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2007

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Summary

Continuously increasing concentrations of tropospheric ozone (O_3) and reactive nitrogen compounds (N) threaten the composition and function of plant communities. To protect ecosystems from excess pollutant deposition, effect-based abatement strategies were developed under the UNECE Convention on Long-Range Transboundary Air Pollution on the basis of critical loads and levels, which rely on experimental sensitivity assessment. Uncertainty exists with regard to critical levels/loads for herbaceous semi-natural vegetation, and hardly anything is known about interactive effects between elevated O_3 and N exposure.

The aims of this thesis were a) to review the literature in order to develop an approach for classifying the O_3 sensitivity of different grassland types, and to formulate a hypothesis related to the interactive effects of O_3 and N and b) to test this approach, the hypothesis, and the underlying assumptions in a field experiment. For this purpose a factorial free-air fumigation experiment was installed at Alp Flix, Sur, Switzerland at 2000 m asl on a *Geo-Montani-Nardetum* pasture. 180 monoliths taken from this pasture were exposed under realistic climatic conditions to a combination of three levels of O_3 (ambient concentration, 1.2 x ambient concentration, 1.6 x ambient concentration) and five levels of N addition (ambient, +5, +10, +25, +50 kg N ha^{-1} y^{-1}). During three years, treatment effects were studied on ecosystem productivity, functional group composition and vegetation development, as well as on leaf morphology and physiology of the 11 most frequent plant species.

Most of the literature available for the review reported results from O_3 fumigation experiments exposing isolated plants in pots in open-top chambers (OTC). Three plant traits were identified as determinants for O_3 sensitivity: stomatal conductance (g_s), specific leaf area (SLA), and defence capacity. We hypothesized that the basis for interactive effects of O_3 and N is given by the fact that due to phenotypic plasticity, SLA and g_s and consequently O_3 sensitivity may be modulated by altered growth rates through external factors such as nutrient availability and micro-climate. While studies testing freshly sown species-poor plant mixtures consistently revealed O_3 to cause quick and strong compositional changes, responses developed slowly or were absent in the few experiments on established grassland. Since these findings were in line with general ecological principles, we proposed that an ecosystems' ability to tolerate O_3 stress would be higher in low-productivity plant communities dominated by species of the stress-tolerant growth strategy than in productive communities.

In the free-air fumigation experiment, productivity and community composition of the subalpine pasture was not affected after three years of elevated O_3 exposure. Nevertheless, several of the 11 plant species studied in detail showed symptoms of O_3 stress: in most of the species, g_s was reduced and less frequently, chlorophyll content. Reductions in photosynthesis and g_s were not related to reductions in leaf weight, which was observed in one half of the species. In contrast to our hypothesis, no relationship was found between the species' SLA and g_s, respectively, and the extent of their O_3 response. The experiment also revealed that N input exceeding 10 kg ha^{-1} y^{-1} had altered the community composition of the monoliths in favour of sedges. Those alone accounted for the 30%-increase in ecosystem productivity in the highest N treatment. In fact, despite strong N-induced leaf N and chlorophyll accumulation, no increase of photosynthetic activity (*A*), g_s - as revealed by the analysis of the stable isotopes ($\delta^{18}O$, $\delta^{13}C$) - and growth was observed for most of the species, except for the sedge *Carex sempervirens.* Together, these findings suggest that neither gas exchange nor N availability are growth limiting factors in this ecosystem, making plants comparatively unresponsive to elevated O_3 and N exposure in the short term. However, subtle species-specific differences in susceptibility to these pollutants might lead to changes in community composition in the longer term, as already indicated by the behavior of sedges.

In contrast to our hypothesis, no O_3 x N interactive effects on productivity and species composition were detected. In fact, leaf analysis revealed that, probably as a result of the absent growth stimulation, neither SLA nor g_s was affected by N addition in most of the species, making this ecosystem an inappropriate model for investigating the mechanisms of interacting effects of O_3 and N over only three years. Measurements of canopy reflectance assessed by normalized difference vegetation index (NDVI), however, revealed positive effects of N addition on canopy greenness being counteracted by accelerated leaf senescence in the highest O_3 treatment. Prolongation of the experiment will show whether or not reduced leaf life span will affect productivity in the long-term through continuous reductions in carbon assimilation.

This is the first study investigating the interactive effects of elevated O_3 and N deposition on established grassland. Although it is too early to draw final conclusions for the establishment of critical levels/loads after three years of treatment, our results suggest a comparatively low risk for O_3, but a danger by increasing background concentrations of reactive N compounds for the species composition of subalpine pastures, a hot-spot of biodiversity.

Zusammenfassung

Stetig steigende Konzentrationen von Ozon (O_3) und reaktiven Stickstoff-Verbindungen (N) in der Troposphäre stellen eine Bedrohung für die Zusammensetzung und Funktion von natürlichen und halb-natürlichen Pflanzengemeinschaften dar. Um diese vor übermässigem Luftschadstoffeinfluss zu schützen, wurden im Rahmen der UN/ECE Konvention für grenzüberschreitende Luftverschmutzung wirkungsbezogene Reduktionsstrategien basierend auf einem Critical level/load Konzept entwickelt. Nach wie vor gibt es grosse Wissenslücken in Bezug auf halbnatürliche Vegetation, während über die interaktive Wirkung von O_3 und N praktisch nichts bekannt ist. Gross angelegte Langzeitversuche, welche der Komplexität von alten artenreichen Pflanzengesellschaften gerecht werden, sind sehr selten.

Diese Studie verfolgte zwei Hauptziele: Erstens sollte im Rahmen einer Literaturstudie ein Ansatz für die Abschätzung der O_3-Sensitivität für verschiedene Graslandtypen entwickelt, sowie eine Hypothese über die interaktive Wirkung von O_3 und N hergeleitet werden. Zweitens sollte dieser Ansatz, die Hypothese, sowie die zugrunde liegenden Annahmen in einem Feldexperiment getestet werden. Dafür wurde ein Freiluftbegasungs-experiment in einer *Geo-Montani-Nardetum* Weide auf der Alp Flix, Sur, Schweiz auf 2000 m ü.M. installiert. 180 Monolithen wurden aus dieser Weide ausgegraben und unter realistischen Bedingungen einer Kombination von drei O_3-Belastungsstufen (Umgebungs-konzentration (UK), 1.2 x UK, 1.6 x UK) und fünf N-Stufen (Hintergrundbelastung, +5, +10, +25, +50 kg N ha^{-1} y^{-1}) ausgesetzt. Währen dreier Jahren wurden die Auswirkungen auf die Produktivität, der Anteil verschiedener funktioneller Gruppen und die Vegetations-entwicklung des Ökosystems, sowie die Blattmorphologie und -physiologie der elf häufigsten Pflanzenarten untersucht.

In der Mehrzahl der für die Literaturstudie verfügbaren Experimente wurden die Auswirkungen von O_3 auf Einzelpflanzen in Töpfen unter kontrollierten Bedingungen in Open-Top Kammern untersucht. Aus solchen Experimenten konnten drei Eigenschaften abgeleitet werden, welche vermutlich auf die O_3-Sensitivität von Pflanzen bestimmen: stomatäre Leitfähigkeit (g_s), spezifische Blattfläche (SLA) und Abwehrkapazität. Wir vermuteten, dass eine mögliche O_3 x N Wirkungsinteraktion darauf basiert, dass sich g_s und SLA und in der Folge auch die O_3-Sensitivität von Pflanzen ändert, wenn diese ihre Wachstumsraten sich ändernden Umweltbedingungen wie Ressourcenverfügbarkeit,

Mikroklima oder Phänologie anpassen. Die relativ zahlreichen Studien mit angesäten artenarmen Pflanzenmischungen zeigten rasch starke O_3-induzierte Änderungen in der Artenzusammensetzung, während in den vier bisher durchgeführten Experimenten mit etablierten Wiesen die O_3-Reaktion spät oder gar nicht auftrat. Weil dies mit allgemeingültigen ökologischen Prinzipien übereinstimmt, schlossen wir, dass artenreiche, schwach-produktive Pflanzenbestände, welche von stresstoleranten Arten dominiert werden, erhöhte O_3-Belastung besser tolerieren als produktive, artenarme Bestände.

Im Freiluftbegasungsexperiment hatte die erhöhte O_3-Belastung nach drei Jahren keinen Einfluss auf die Produktivität bzw. die Artenzusammensetzung der Pflanzengesellschaft. Diese O_3-Toleranz kann entweder durch spezifische Ökosystemeigenschaften, durch die Anpassung alpiner Pflanzen an oxidativen Stress oder durch das Fehlen von besonders schädigenden O_3-Belastungsspitzen erklärt werden. Trotzdem zeigten eine Reihe der elf untersuchten Pflanzenarten Symptome von O_3-Stress. Erhöhte O_3-Belastung verminderte in der Mehrzahl der untersuchten Arten den Gaswechsel und sporadisch trat ein Chlorophyll-Verlust auf. Beide Effekte waren jedoch nicht mit einer durch O_3 verursachten Reduktion des Blattgewichts korreliert, welche bei der Hälfte der untersuchten Arten auftrat. Unerklärlicherweise fanden wir im Gegensatz zu früheren Studien keine Beziehung zwischen artspezifischem SLA bzw. g_s und dem Ausmass der O_3-Schädigung. Das Experiment zeigte auch, dass ein N-Eintrag von mehr als 10 kg N ha^{-1} y^{-1} die Artenzusammensetzung der Monolithen zugunsten von Seggen verändert. Diese waren allein für den Ökosystem-Produktivitätszuwachs von 30% in der höchsten N-Behandlung verantwortlich. Tatsächlich erhöhten die meisten der elf Arten trotz starker N und Chlorophyll-Anreicherung weder ihre Photosynthese, noch g_s, noch ihr Wachstum. Einzig die Segge *Carex sempervirens* steigerte ihr Wachstum beträchtlich. Zusammengefasst lassen diese Resultate vermuten, dass in diesem Ökosystem weder Gaswechsel noch N-Verfügbarkeit das Wachstum der Pflanzen limitieren, was die meisten Arten gegenüber O_3 und N relativ unempfindlich macht. Trotzdem können kleine Unterschiede in der Reaktion gegenüber diesen Substanzen längerfristig zu Änderungen in der Artenzusammensetzung führen.

Im Widerspruch zu unserer Hypothese wurde keine interaktive Wirkung von O_3 und N auf Produktivität und Artenzusammensetzung des Bestandes beobachtet. Blattanalysen zeigten jedoch, dass bei den meisten Arten die N Zugabe, vermutlich wegen der ausbleibenden Wachstumsstimulation, weder SLA noch g_s veränderte, was dieses Ökosystem zu einem ungeeigneten Modell für die Untersuchung von Mechanismen einer Wirkungsinteraktion macht und auch die fehlenden Effekte erklärt. Hingegen wiesen Messungen der Grünfärbung des Bestandes (Normalized Difference Vegetation Index, NDVI) auf eine O_3 x N Interaktion hin: die positive Wirkung von N wurde durch beschleunigte Blattalterung in der höchsten O_3-Belastungsstufe aufgehoben. Längerfristig könnte sich die mit einer verkürzten Blattlebensdauer einhergehende Reduktion der Kohlenstoffassimilation auch auf die Produktivität auswirken.

Dies ist die erste Studie, welche die Wirkungsinteraktion von erhöhter O_3- und N-Exposition in einem Feldexperiment untersucht. Durch die Verwendung einer grossen Zahl von Monolithen in einer Freiluftbegasungsanlage konnten realistische klimatische und edaphische Bedingungen geschaffen werden, welche eine Extrapolation der Ergebnisse auf reale Pflanzenbestände erlauben. Nach drei Jahren können noch keine definitiven Schlüsse in Bezug auf Critical levels/loads gezogen werden. Die Resultate deuten jedoch darauf hin, dass subalpine Weiden gegenüber erhöhter O_3-Belastung relativ tolerant sind, während weiterhin steigende N-Hintergrundkonzentrationen eine grosse Gefahr darstellen für die Artenzusammensetzung dieser Pflanzengesellschaften, welche zu letzten Biodiversität-Hotspots in Europa zählen.

Chapter 1

General introduction

1 Air pollution

Concentrations of tropospheric ozone (O_3) and reactive nitrogen (N) have risen several-fold during the last century (Galloway & Cowling, 2002; Vingarzan, 2004). Initially, this trend was limited to North America and Europe, but meanwhile, background concentrations are increasing globally as a consequence of industrial development in Asia and Africa (Matson *et al.*, 2002; Ashmore, 2005). Today, O_3 and reactive N compounds are among the most harmful air pollutants threatening human health as well as structure and functioning of natural and semi-natural ecosystems (Bobbink, 1998).

1.1 Ozone

Tropospheric O_3 is a secondary pollutant formed when nitrogen dioxide (NO_2) is photolyzed by UV radiation (< 420 nm) to NO and a free oxygen radical, which in turn reacts with oxygen (O_2) to form O_3 (Fig. 1, for more details see Atkinson, 2000).

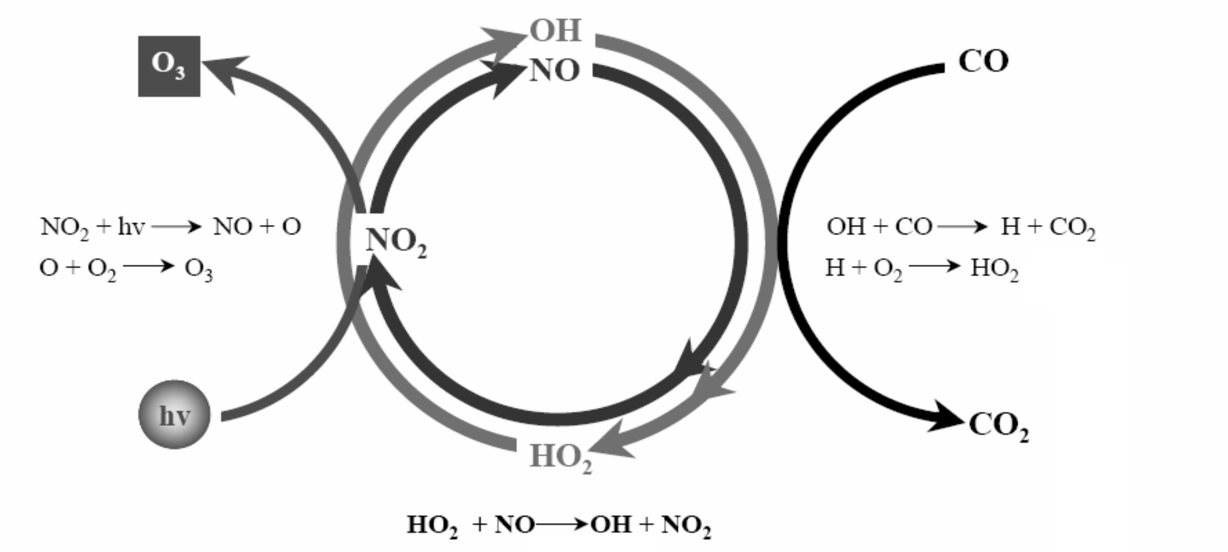

Fig. 1. Scheme of O_3 production with CO as surrogate for VOCs. CO is consumed, NO_x and HO_x are recycled. Modified after Spirig (2003).

In an atmosphere consisting of an even balance of oxidising and reducing agents, net O_3 formation is prevented by the O_3-consuming oxidation of NO back to NO_2 and O_2. In the troposphere, the presence of hydrocarbons allows the oxidation of NO to NO_2 without any consumption of O_3 by reaction products formed in the breakdown of VOCs to CO_2 and water. The latter kind of NO_2 regeneration is the basis for further O_3 formation, resulting in enhancement of O_3 concentrations. Fossil-fuel combustion by traffic and industry is considered the major source of precursor substances such as NO_x and VOC. In urbanized and industrialized areas, O_3 concentrations may remain relatively low because perpetually emitted NO_x quickly consumes O_3 in a continuous production/reduction cycle. In contrast, in pollutant air masses carried downwind from the emission source into rural or mountain areas, O_3 concentrations are considerably higher, due to the reduced levels of NO_x, which creates a more favourable ratio between NO_x and VOCs for increased O_3 formation (Spirig, 2003).

Over Europe, total O_3 background concentrations account for 10-40 ppb at the surface. In Switzerland, yearly averaged 1h means (1990 to 2004) range between 20 to 45 ppb in city centres to 47 to 52 bbp in rural areas (NABEL, 2006). 1h maxima consistently decreased over this period due to successful NO_x and VOC emission control at regional scale, while background concentrations, depending on global emissions, increased. Average O_3 concentrations generally increase with altitude, first due to the comparatively cleaner air preventing O_3 degradation, and secondly because inversions and associated depletion of ground-level air layers through deposition occur less frequently, resulting in maintenance of high O_3 concentrations over the entire diurnal period (Sandroni *et al.*, 1994).

Due to its reactive characteristics, O_3 not only affects human health and material surfaces, but also plant tissues. It is considered the major phytotoxic pollutant in Europe (Ashmore, 2005). O_3 enters the leaves by diffusion through the stomata. In the sub-stomatal cavities, O_3 decays to O_2 and a free oxygen radical that oxidises membranes as well as cell compounds (Fig. 2b). Acute symptoms of O_3 damage occur as stipples and necrotic flecks at the upper leaf sides (Fig. 2c). Long-term effects on plant performance include reduced stomatal conductance by impaired K+ channels (Torsethaugen *et al.*, 1999), chloroplast degradation (Oksanen *et al.*, 2004), and lowered enzyme quantity and activity (Reid *et al.*, 1998). Together, this reduces CO_2 assimilation and eventually leads to accelerated leaf senescence (Ojanperä *et al.*, 1998).

These effects have the potential to affect plants' productivity, root-shoot ratio and seed production (Davison & Barnes, 1998). Considerable variation in O_3 sensitivity exists within and among species (Bungener *et al.*, 1999; Bassin *et al.*, 2004). By influencing productivity and reproductive fitness of species unequally, O_3 has the potential to alter the composition of plant communities. Reduced carbon allocation and stomatal conductance of plants may affect both carbon and water budget of entire ecosystems (Fuhrer & Booker, 2003).

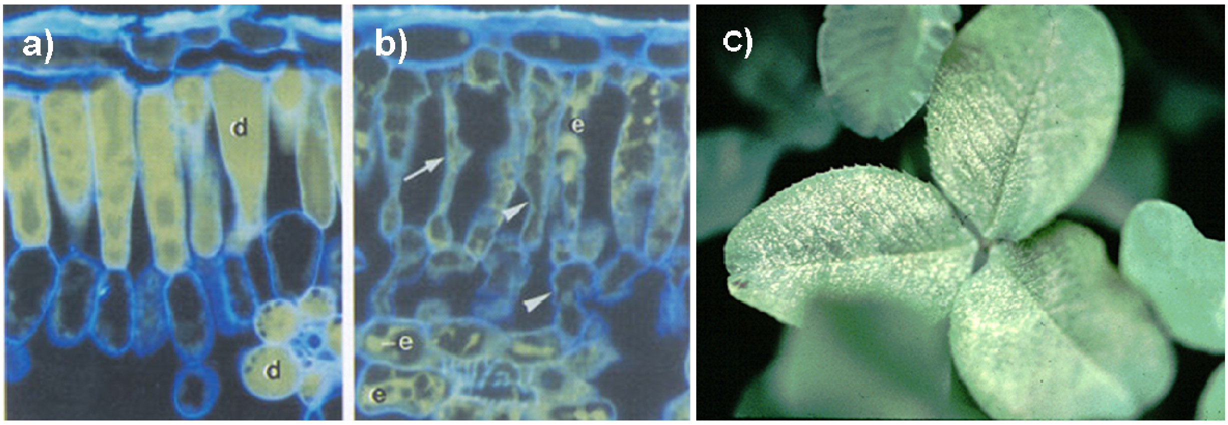

Fig. 2. Palisade and upper epidermal cells of a Prunus serotina leaf a) exposed to 50% of ambient O_3 concentration b) exposed to 50% ambient + 30 ppb O_3 concentration (reproduced after Günthardt-Goerg et al., 2000). c) Visible O_3 injury on white clover, Trifolium repens.

1.2 Nitrogen

Nitrogen oxides (NO_x) are emitted to the atmosphere by the combustion of fossil fuel in electricity generating power stations, industrial plants, road transport, shipping and aircrafts, while ammonia (NH_3) stems from the hydrolysis of urea and/or uric acid present in large quantities in animal wastes, i.e., slurries and farm yard manures (Ferm, 1998). In Switzerland, a total amount of around 77.2 kt of oxidized and reduced N compounds are emitted annually, 60% by agriculture, 27% by traffic, and 11% by industries (NABEL, 2006). While 54.3 kt is exported annually by long-range transport to neighboring countries, 41.4 kt are imported. Gaseous NH_3 is typically deposited close to the sources, whereas a minor fraction reacts with atmospheric acids (e.g., H_2SO_4, HNO_3) to form aerosols such as $[NH_4]_2SO_2$ and NH_4NO_3. Such aerosols have a smaller deposition velocity, and hence may remain in the air for several days and therefore be dispersed and carried over long distances by winds. Depending on precipitation patterns, the particles are removed from the atmosphere by either dry, wet (rain/snow) or occult (fog) deposition. Of the annual sum of N deposited in Switzerland, 54% is imported by transboundary long-range transport, namely 80% of oxidized and 38% of reduced N compounds deposited (EKL, 2005). Current annual N deposition rates reach values below 10 kg N ha^{-1} y^{-1} in remote areas (e.g., alpine zones), but amount to 50 kg N ha^{-1} y^{-1} in regions with a high density of livestock farming (Fig. 3b) (Rihm & Kurz, 2001).

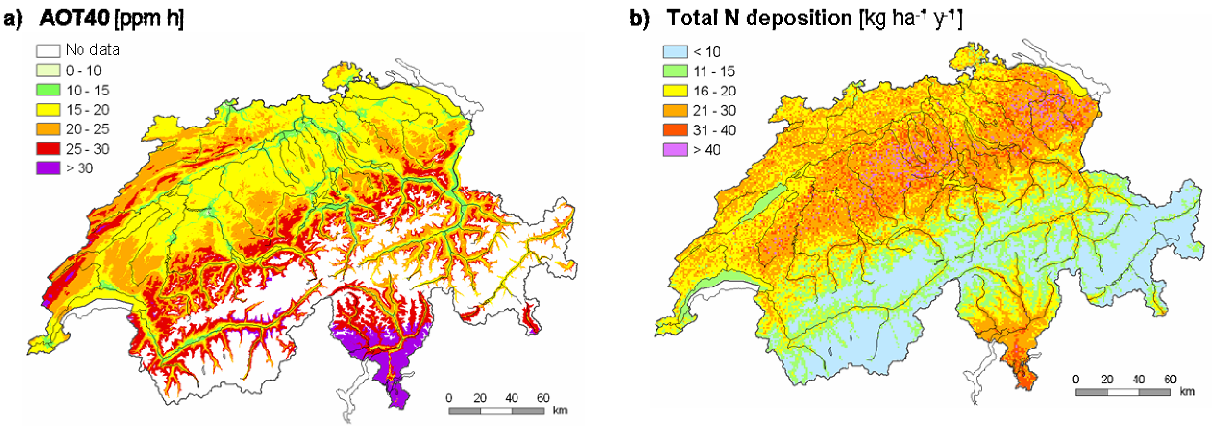

Fig. 3. Maps of Switzerland, showing a) cumulative ozone exposure expressed as six-month AOT40 values [ppm h] for forests averaged for the years 1998–2002 (http://www.bafu.admin.ch/luft/) and b) total nitrogen deposition [kg N ha^{-1} y^{-1}] for the year 2000 (EKL, 2005).

As an essential nutrient, N has the potential to affect plant performance either directly by growth stimulation or indirectly through altered competition. Generally, N supply stimulates photosynthesis by increasing leaf chlorophyll and N concentrations, which leads to enhanced enzyme quantities and activity. By investing carbon in leaf area expansion at the expense of leaf thickness, plants efficiently occupy aboveground space when competition for nutrients shifts to aboveground competition for light (Schieving & Poorter, 1999). Anthropogenic N input may affect ecosystem properties such as productivity, C and N cycling (Vitousek *et al.*, 1997), and biodiversity. In natural habitats, species adapted to nutrient-poor conditions may be displaced by a few competitive species as shown by N addition experiments (Bobbink *et al.*, 1998). Thus, the loss of rare specialist plant species observed over the last 30 years in nutrient-poor Swiss grasslands, unexplained by changes of management practices, was probably caused by increased atmospheric N deposition (Peter, 2007).

2 Air pollution abatement strategies

2.1 Critical loads/levels

In order to protect human beings, plants, ecosystems, or materials from excess pollutant input, effect-based abatement strategies were developed under the Convention on Long-Range Transboundary Air Pollution (CLRTAP) of the United Nations Economic Commission for Europe (UNECE, 2002). The Gothenburg Protocol to abate acidification, eutrophication and ground-level O_3 was ratified by 23 European countries as well as the United States and entered into force in 2005. It sets country-specific emission ceilings for 2010 for four pollutants (sulphur, NO_x, VOCs and NH_3), which have to be implemented by the countries' governments. These ceilings were negotiated on the basis of scientific assessments of pollution effects based on a critical level/load concept. Once the Protocol is fully implemented, Europe's sulphur emissions should be reduced at least by 63%, its NO_x emissions by 41%, its VOC emissions by 40%, and its NH_3 emissions by 17% compared to 1990 (UNECE, 2002).

The critical level for O_3 stands for the concentrations of O_3 in the atmosphere above which direct adverse effects on receptors, such as vegetation, may occur according to present knowledge. Presently, the Critical level for O_3 is expressed as Accumulated Ozone exposure over a Threshold concentration of 40 ppb during daylight hours (AOT40) over a defined period (Fig. 3a) (CLRTAP, 2004). Critical levels are set for three broad vegetation types (forests, crops and semi-natural vegetation) but they rely on sensitivity studies using individual plant species or species mixtures, with the aim to protect the most sensitive species or the most sensitive plant community from O_3 impact. While current critical levels for O_3 for forests and crops are well supported by experimental data, considerable gaps of knowledge were identified for semi-natural vegetation (Fuhrer & Booker, 2003). This vegetation type includes a broad range of herbaceous ecosystems, from annual Mediterranean pastures over wetlands, temperate grasslands, alpine hay meadows to northern heath. Nevertheless, a critical level of 5 ppm h over a six-month period was set for semi-natural vegetation (CLRTAP, 2004).

The critical load for N deposition stands for the annual sum of N deposition (NH_y and NO_x [kg N ha^{-1} y^{-1}]) an ecosystem can tolerate without being affected in structure and functioning. Two independent methods are simultaneously used to estimate critical loads: the "steady state mass balance" method based on the computation of N budgets, and the "empirical" method based on experiments and observations in the field. The latter relies on indicators for N-induced alterations in community characteristics and functioning, such as species composition changes, N leaching, or susceptibility to pathogens (Achermann & Bobbink, 2003). Empirical critical loads for N are set specifically for different vegetation types according to the "European Nature Information System" (EUNIS) (http://eunis.eea.europa.eu/habitats.jsp). They are well supported by empirical data from N-addition experiments, e.g., for forests, whereas substantial gaps of experimental data were identified for wet heaths, dune grassland, hay meadows as well as arctic, alpine and subalpine habitats. Therefore, the current critical load for alpine and subalpine grasslands set at 10-15 kg N ha^{-1} $y\text{-}^{1}$ is based on expert judgement only (Achermann & Bobbink, 2003).

Although the Gothenburg Protocol is the first protocol including the interrelationship of multiple pollutants, interactive effects of O_3 and N have not been considered in the critical level/load concept so far. Theoretically, the stimulating effect of N and the detrimental effect of O_3 may act interactively on plant performance, demanding an adaptation of existing critical levels in relation to N deposition. There is evidence for O_3 x N interactive effects from single-plant studies. For instance, *Plantago major* was protected against O_3 damage by high nutrient supply (Whitfield *et al.*, 1998). Data from *in situ* experiments, however, including multiple factor combinations to test the interaction between N and O_3 are still lacking globally.

2.2 Sensitivity assessment

To provide reliable results, experimental conditions in sensitivity assessment studies should be representative for the original habitat of the species under investigation, especially in terms of climate, resource availability, plant age, and competition. Extrapolation beyond climatic and edaphic conditions created in an experiment can be far misleading (Bassin *et al.*, 2007a).

Sensitivity assessment experiments for N deposition are typically performed by irrigation of intact ecosystems with an aqueous solution of ammonium nitrate (e.g., Lee & Caporn, 1998; Shaver *et al.*, 2001; Bowman *et al.*, 2006; Phoenix *et al.*, 2006). Studies investigating the differential effects of NH_y and NO_x (de Graaf *et al.*, 1998; Nordin *et al.*, 2006) or including dry and wet deposition scenarios (Leith *et al.*, 2002) are rare. Moreover, to short-cut N accumulation over time, in many of the previous experiments, treatment doses exceeded the annual N deposition rate at the respective sites (Theodose & Bowman, 1997; Carroll *et al.*, 2003; Soudzilovskaia & Onipchenko, 2005), ignoring the boosting effect of a sharp increase of N availability on organisms. According to Achermann & Bobbink (2003), several N addition levels, ranging around the background N deposition at the site, should be added by irrigation in small doses at numerous dates over the whole vegetation period for more than three years. These requirements are mostly fulfilled by recently established experiments (Britton & Fisher, 2007; Madan *et al.*, 2007).

O_3 sensitivity assessment studies are commonly performed by means of fumigation systems providing different O_3 concentrations as charcoal filtered and/or O_3-enriched ambient air (Heagle *et al.*, 1979; Volk *et al.*, 2003). The cost-intensive fumigation technique restricts experiments in plot size, treatment levels, replication number, and duration. Thus, most studies exposed individual plants grown from seeds in comparatively small open-top chambers (OTCs) for 1-3 months during 1-2 years. Compared to ambient conditions, the air inside OTCs is highly turbulent, enhancing the O_3 flux to leaves and stomata. Moreover, the chamber microclimate characterized by higher temperatures and lower water pressures deficit (VPD) relative to ambient conditions enhances stomatal conductance, which in turn stimulates O_3 uptake by 20-50% (Nussbaum & Fuhrer, 2000). To avoid artifacts introduced by chamber effects, free-air fumigation systems are most useful (Volk *et al.*, 2003).

Experimental limitations are the main reason for missing data on the sensitivity to O_3 of the different vegetation types representing “semi-natural vegetation” (see above). Differences in the ability of plant communities to tolerate elevated O_3 exposure might be substantial considering the large vegetation-type specific differences in stress tolerance observed in calcareous grasslands (Grime *et al.*, 2000). It is uncertain whether the vegetation types most at risk for O_3 have been identified yet, and if they are protected properly by the current critical level for O_3. Thus, it was proposed that grassland communities most at risk should be identified by extrapolation from the available literature instead (Mills *et al.*, 2007). The same authors suggested that the sensitivity of plant communities is determined by the O_3 sensitivity of the species they are composed of. However, the community response is presumably more than the sum of responses of individuals predicted from experiments with isolated plants, since canopy structure and complex interactions among species might modulate the effects of O_3 (Bassin *et al.*, 2007a). A holistic approach would be more appropriate, i.e., considering general community characteristics such as species richness, functional composition, and successional status to classify O_3 sensitivity. This issue is treated in Chapter 2 of this thesis.

3 O_3 and N sensitivity assessment for subalpine pastures

3.1 Subalpine pastures

Subalpine pastures cover large areas of the European Alps and Pyrenees but they have not been studied in terms of air pollution impact yet. Swiss subalpine grasslands are typically managed as cattle pastures during three months of the year (June to September). The combination of traditional low-intensity grazing, the large climatic, edaphic and topographic variability, and the low anthropogenic N input results in habitats that are very rich in plant and arthropod species (Väre *et al.*, 2003) and thus are of high conservation value. Climatic conditions of the subalpine zone are characterized by cold winters with permanent snow cover and a growing season from April to October. Average annual temperatures vary between 0.5 and 3°C depending on altitude and exposition (Körner, 2003). As a result, subalpine soils show low turnover rates and thus high contents of soil organic matter and low N mineralization rates. Moreover, strong N immobilization was observed in alpine soils (Körner, 2003). Overall, the soil solution is dominated by ammonium N, while nitrate N is rare. Therefore, a considerable number of (sub-)alpine plant species is able to take up, or even prefers, ammonium or small amino-acids as main N source (Miller & Bowman, 2003).

Due to their intermediate location between the alpine and colline zone, subalpine pastures are composed by both colline (*Briza media*, *Carex caryophyllea*, *Carex ornithopoda*) and alpine species (*Poa alpina*, *Nardus stricta*, *Carex sempervirens*, *Trifolium alpinum*). Irrespective of their original distribution area, species of subalpine grasslands are characterized by low statures and small or needle-like leaves indicating phenotypic adaptation to the climatic conditions (Körner, 2003). They also share physiological characteristics with species of the alpine zone, such as low growth rates and low leaf turnover rates, commonly assigned to the stress-tolerant growth strategy (Grime *et al.,* 1996).

With O_3 and N exposures at these elevations being mainly controlled by background concentrations (Staehelin *et al.*, 1994), subalpine pastures must have experienced very low levels of N deposition but comparatively high O_3 concentrations in the past (see above). In future, they might be subjected to globally increasing background O_3 and N concentrations. However, no information is available on their response to these air pollutants. Overall, both ecosystem and physiological characteristics of the subalpine pasture differ strongly from systems tested before in comparable experiments.

3.2 Experimental set-up

To investigate the effects of combined elevated O_3 x N deposition on a subalpine *Geo-Montani-Nardetum* pasture, a factorial fumigation experiment (Fig. 4) was installed at Alp Flix, Sur, Switzerland, 2000 m asl, a high plateau near Julier Pass in the Central Alps (Bassin *et al.*, 2007b). During three years, a combination of three levels of O_3 (ambient concentration, 1.2 x ambient concentration, 1.6 x ambient concentration) and five levels of additional N (ambient, +5, +10, +25, +50 kg N ha^{-1} y^{-1}) was applied (Table 1). Mean ambient O_3 concentrations at the site amount to 45-47 ppb, average annual background N deposition is 3-4 kg N ha^{-1} y^{-1}.

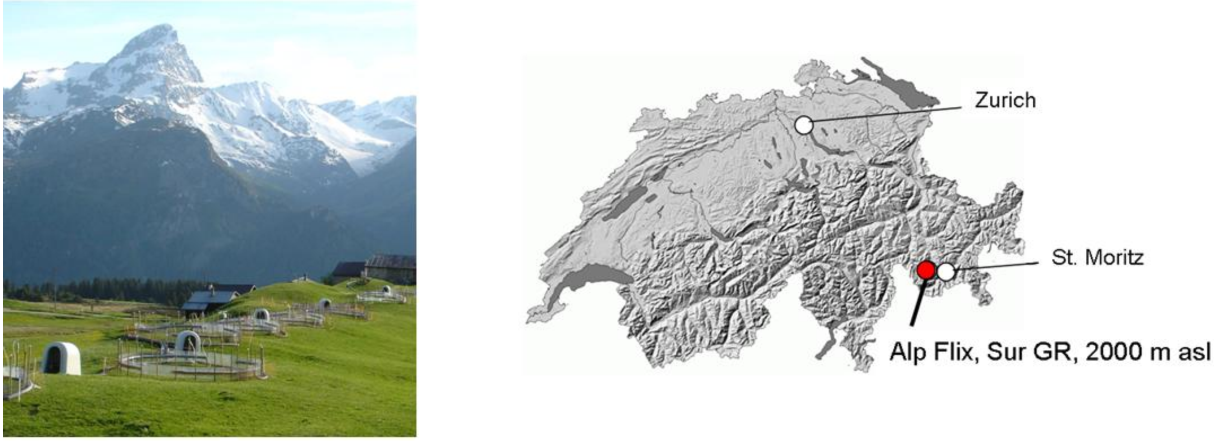

Fig. 4. Free-air fumigation system used for the investigation of interactive effects of elevated O_3 and N deposition on subalpine grassland, installed at Alp Flix, 2000 m asl, and map of Switzerland displaying the research site.

The free air fumigation system consisted of nine rings of 7 m diameter (for details see Volk *et al.*, 2003), which were arranged in three linear blocks on a ridge of 150 m length, orthogonal to the two main wind directions. The three O_3 treatments were randomly assigned to one of the three rings in each block. O_3 concentrations were increased during day and night. Experimental plots consisted of monoliths (40 x 30 cm, 20 cm depth), which were cut from a nearby pasture, placed in drained plastic boxes and randomly assigned to the rings. Boxes were placed in shallow pits flush with the surrounding surface. Annual N amounts were supplied as twelve bi-weekly applications of a 200 ml solution of ammonium nitrate in well water. Each N level was replicated four times in each fumigation ring according to a split-plot design. Treatment effects were measured at ecosystem (Chapter 3) and individual species level (Chapter 4).

Table 1. Treatments applied, background exposure, cumulative addition, and total exposure to O_3 and N in the factorial fumigation experiment at Alp Flix.

	Treatment	Background exposure	Addition	Total exposure
Ozone		AOT40* [ppm h]	Cumulative O_3 addition, AOT40* [ppm h]	AOT40* [ppm h]
		12		
	O_3 control		+ 0	12
	O_3+		+ 12	24
	O_3++		+ 36	48
Nitrogen		Annual N deposition [kg N ha^{-1} y^{-1}]	N addition [kg N ha^{-1} y^{-1}]	Annual N deposition [kg N ha^{-1} y^{-1}]
		< 4		
	N4		+ 0	4
	N9		+ 5	9
	N14		+ 10	14
	N29		+ 25	29
	N54		+ 50	54

*AOT40 = Accumulated ozone exposure over a threshold of 40 ppb for a period of six months (April-October, averaged over the 3 experimental years).

4 Outline of the thesis

This thesis had two aims. The first is to develop an approach for estimating the O_3 sensitivity of different grassland types and to formulate a hypothesis on the interactive effects of O_3 and N. The second aim is to test this approach, the hypothesis and the underlying assumptions under appropriate experimental conditions in a case study, namely in a subalpine pasture exposed to different levels and combinations of elevated O_3 and N concentrations. In an applied context, the study is intended to improve the basis for establishment of a critical level for O_3 exposure and a critical load for N deposition for subalpine grassland under consideration of the combined effects of both pollutants.

In **Chapter 2,** we review the currently available literature for O_3 fumigation experiments with herbaceous plant species in order to describe the biotic and abiotic factors modifying the effects of O_3, but also to identify physiological and ecological principles associated with O_3 sensitivity of temperate European grassland species and communities (Bassin *et al.*, 2007a). This would allow for the development of an approach to estimate the O_3 sensitivity of different grassland types and to derive a hypothesis on O_3 x N interactive effects.

In **Chapter 3,** using the free-air fumigation experiment described above, we test the hypotheses developed in Chapter 2: a) late-successional grasslands dominated by stress-tolerant species will be more O_3 tolerant in terms of above-ground productivity, functional group composition and vegetation development than plant communities of productive habitats, and b) the susceptibility to O_3 will be modified through increased N availability (Bassin *et al.*, 2007b).

In **Chapter 4**, we investigate the individual responses of 11 key species to the combined O_3 and N treatments in terms of proxies for gas exchange and growth (leaf chlorophyll and N content, mean leaf weight, SLA, carbon and oxygen isotopic compositions). According to the hypotheses stated in Chapter 2, we expect that a relationship exists between the species' initial leaf traits and the extent of the response to both N and O_3 and that N-induced changes in these traits will modify the species' reaction to O_3 (Bassin *et al.,* in preparation).

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Chapter 2

Factors affecting the ozone sensitivity of temperate European grasslands: an overview

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Environmental Pollution 2007, 146, 678-691

Abstract

This overview of experimentally induced effects of ozone (O_3) aims to identify physiological and ecological principles, which can be used to classify the sensitivity to O_3 of temperate grassland communities in Europe. The analysis of data from experiments with single plants, binary mixtures and multi-species communities illustrates the difficulties to relate individual responses to communities, and thus to identify grassland communities most at risk. Although there is increasing evidence that communities can be separated into broad classes of O_3 sensitivity, the database from experiments under realistic conditions with representative systems is too small to draw firm conclusions. But it appears that risk assessments, based on results from individuals or immature mixtures exposed in chambers, are only applicable to intensively managed, productive grasslands, and that the risk of O_3 damage for most of perennial grasslands with lower productivity tends to be less than previously expected.

1 Introduction

Air pollution by ozone (O_3) occurs over wide areas and represents a major threat to vegetation across Europe and elsewhere (Ashmore, 2005). Adverse effects of elevated O_3 on agricultural crops and forest trees have been documented and results from experiments and field observations were summarized in numerous articles (e.g., Fuhrer *et al.*, 1997). Much less is known about effects on herbaceous vegetation (Davison & Barnes, 1998), although evidence exists for widespread impacts on sensitive species (Benton *et al.*, 2000). Lack of data for O_3 effects has made it difficult to establish guidelines or critical levels for the protection of semi-natural vegetation, including grasslands (Fuhrer *et al.*, 2003; Fuhrer *et al.*, 2005). This is unfortunate because grasslands and wetlands cover a major fraction of European landscapes. They are important for conservation of biodiversity and - in many cases - agricultural use. Many of these systems such as temperate perennial grasslands are exposed to O_3 over long growing seasons and for many years. Therefore, effects of O_3 may not become apparent in the short-term, but may instead develop over time. This is in contrast to other systems, including Mediterranean therophytic grasslands, where short-term effects on reproductive traits of annuals may be most important (Gimeno *et al.*, 2004a).

Mechanisms of O_3 effects on plants have been reviewed recently by Fuhrer & Booker (2003), Matyssek & Sandermann (2003), and others. Briefly, short-term O_3-induced damage on leaves can be observed as stipples, flecks or bronzing (Skelly *et al.*, 1999). They result from a light-dependent hypersensitive-like response and subsequent cell death (Langebartels *et al.*, 2002). In many species, elevated O_3 exposure reduces CO_2 assimilation in various ways (Fiscus *et al.*, 2005): (i) O_3 decreases stomatal conductance by affecting stomatal guard cells (Torsethaugen *et al.*, 1999); (ii) O_3 reduces photosynthetic activity by reducing the levels and activity of Rubisco (Pell *et al.*, 1997); (iii) O_3 leads to chloroplast degradation and accelerated leaf senescence (Grimm & Fuhrer, 1992). O_3 was shown to increase the concentrations of soluble and insoluble carbohydrates in leaves, which can be explained by O_3-induced reduction of phloem loading (Andersen, 2003). As a result of the altered carbon source-sink balance, O_3-exposed plants that have not flowered or set fruits often show a decreased root/shoot ratio (Cooley & Manning, 1987). Most likely, the different effects do not reflect a climax of damage, but rather different response types. This would explain why some species show severe visible injury, but their productivity remains unaffected (Pleijel & Danielsson, 1997), whereas others suffer reduced productivity, despite a lack of visible injury (Reiling & Davison, 1992b).

Spatially explicit assessments of potential impacts of O_3 on the floristic composition and productivity of grasslands, and identification of communities at risk, in the context of the UNECE Convention on Long-Range Transboundary Air Pollution, rely on knowledge of the sensitivity to O_3 of different grassland types. The difficulty rests in their complexity and biological heterogeneity, possible interactions with environmental and edaphic factors, and the effect of time, but also in the lack of suitable experimental data. The aim of this overview is to summarize possibilities and constraints to predict the sensitivity of different grassland types in terms of growth and productivity, based on physiological and ecological principles. The initial assumption was that community sensitivity is determined by inherent properties of the component species, environmental conditions, and community-level processes. The overview uses a hierarchical approach by first looking at factors controlling O_3 sensitivity of individual plants and then at species mixtures and intact communities. It ends with a discussion of experimental limitations and recommendations for future experiments.

2 Individual plant level

Numerous studies have been carried out to assess the O_3 response of selected species in terms of visible injury, aboveground/belowground biomass, relative growth rate RGR, etc. (Table 1). Both O_3 injury and biomass reductions were found, although in some vegetation types visible injury is more prominent than biomass reduction. Interspecific differences are mainly due to differences in the specific 'O_3 sensitivity-relevant traits'. The bulk of results from experiments with single plants suggest that three traits can be identified as important controls of specific O_3 sensitivity: stomatal control, specific leaf area (SLA, i.e., unit leaf area per unit weight), and biochemical defense capacity. These traits are not independent of each other; but are linked through their relationship to the plant's relative growth rate, RGR.

2.1 Stomatal uptake of O_3

The cumulative dose of O_3 taken up by leaves, which is determined by both stomatal conductance (g_s) and O_3 concentration at leaf level, is assumed to be a key factor influencing O_3 damage to plants (Pleijel *et al.*, 2004). Consequently, differences in O_3 sensitivity between species and cultivars have often been positively related to differences in g_s measured under identical, usually non-limiting environmental conditions (Bungener *et al.*, 1999a; Power & Ashmore, 2002). However, since environmental factors modify g_s (for details see section 3.1), O_3 sensitivity in the field presumably depends on the species-specific ability to control stomata under varying environmental conditions (*cf.* Fuhrer & Booker, 2003, Fuhrer *et al.*, 2003).

2.2 Detoxification, and physical defense

Detoxification processes are based on a generic response to oxidative stress, induced by increased levels of free radicals (Sharma & Davis, 1997). This can include increased activity of peroxidase (Ranieri *et al.*, 2002) and higher contents of antioxidants such as ascorbate (Turcsanyi *et al.*, 2000). Scavenging of free oxygen radicals, derived from the decay of O_3 (Sharma & Davis, 1997), prevents peroxidation of a variety of mesophyllic compounds, including enzymes, cell wall lipids and proteins. The leaf area-based antioxidant content has been found to increase with age (Wieser *et al.*, 2003). In addition, physical defense in the form of cell wall thickening has been observed in a variety of taxa (Bass *et al.*, 2006; Bussotti *et al.*, 2005; Paakkonen *et al.*, 1998; Rinnan & Holopainen, 2004). Both biochemical and physical responses require energy for regeneration and transport of antioxidants; thus, O_3 tolerance could depend on the amount of carbohydrates available and on the energy supplied from photosynthesis (*cf.* Fuhrer & Booker, 2003).

2.3 Leaf morphology

High specific O_3 sensitivity was related to thinner palisade mesophyll layers and a high ratio of spongy to palisade mesophyll cells (Bennett *et al.*, 1992; Ferdinand *et al.*, 2000). Such leaf traits decrease mesophyll resistance to O_3 diffusion and, in turn, increase the cumulative dose per cell. As cell density and probably also mesophyll resistance are negatively correlated with SLA (Garnier & Laurent, 1994), O_3 sensitivity may increase with increasing SLA. However, leaf morphology shows high phenotypic plasticity, such that individuals of the same species may produce completely different leaves when grown under climatically different conditions (Körner, 2003).

Table 1. Overview of studies showing inter-specific differences in O_3 sensitivity in herbaceous species originating from European semi-natural habitats. Sensitivity assessment was carried out on individual plants grown from seed, cultivated in pots with unlimited water and nutrient supply, and exposed to O_3 in open-top chambers (OTC) or growth chambers.

Vegetation type/origin	**Species number**	**% of species showing visible injury**	**% of species showing reduced above-ground productivity**	**% of species showing reduced below-ground biomass**	**% of species showing changes (+ or-) in root:shoot ratio**	**Reference**
UK native	32	19	44	Not tested	28 – 19 +	Reiling & Davison, 1992
Swiss mesic grassland	33	70	Not tested	Not tested	Not tested	Nebel & Fuhrer, 1994
German native	12	42	8	Not tested	Not tested	Bergmann *et al.*, 1995
Calcareous grassland	5	40	20	Not tested	20 - 0 +	Warwick & Taylor, 1995
Swedish native	27	11	67	Not tested	Not tested	Pleijel *et al.*, 1997
Danish grassland	8	87	75	Not tested	Not tested	Mortensen 1997
Swiss native	24	96	21	Not tested	Not tested	Bungener *et al.*, 1999a Bungener *et al.*, 1999b
German native	25	32	Not tested	Not tested	Not tested	Bergmann *et al.*, 1999
Wetland	10	10	30	40	10 - 10 +	Franzaring *et al.*, 2000
Fen-meadow	12	75	25	17	40 – 0 +	Power & Ashmore, 2002
Mediterranean annual grassland	22	72	42	32	16 - 16+	Bermejo *et al.*, 2002 Gimeno *et al.*, 2004a
UK upland vegetation	33	24	15	Not tested	Not tested	Hayes *et al.*, 2007

2.4 RGR and SLA

In several studies, O_3-induced growth reduction in herbaceous species was positively related to inherent RGR (Bungener *et al.*, 1999b; Danielsson *et al.*, 1999; Reiling & Davison, 1992b), which in turn is positively related to both g_s and SLA (Hunt & Cornelissen, 1997; Poorter & DeJong, 1999). Presumably, high growth rates can only be achieved at high rates of carbon assimilation and with low investments in structural elements. Poorter & Garnier (1999) presented evidence that interspecific differences in maximum RGR are largely due to inherent differences in SLA, and they suggested that selection in the field under adverse conditions might act on parameters related to SLA. An inherently low SLA diminishes losses of nutrients and biomass due to stress and, therefore, may be advantageous for plants exposed to elevated O_3.

In support of the notion that SLA may be a key determinant for species-specific O_3 sensitivity, Batty & Ashmore (1997) reported that woodland and wetland species with high SLA were most sensitive to O_3, and similarly, Franzaring *et al.* (2000) found that the most tolerant wetland species in their study had lowest SLA and RGR. Finally, a survey of 72 European species by Franzaring (2000) generally confirmed higher O_3 tolerance in species with a lower SLA.

Among plant families, Fabaceae appeared consistently to be most sensitive to O_3, despite high interspecific variability (Hayes *et al.*, 2007). This high sensitivity cannot be related to SLA. Inherent SLA of legumes such as *Trifolium repens*, a highly O_3-sensitive species (Becker *et al.*, 1989), is low (Poorter & De Jong, 1999). But legumes are characterized by high RGR and a specific chemical response to oxidative stress (Ranieri *et al.*, 2002).

3 Factors modifying O_3 sensitivity

3.1 Climatic factors

Some of the 'O_3 sensitivity-relevant traits' discussed above are dynamic, i.e., they vary in time with environmental conditions and plant development. It was suggested that herbaceous vegetation would generally be most at risk under environmental conditions favoring high g_s (Davison & Barnes 1998). The same conditions also maximize RGR. Most importantly, g_s is regulated by radiation (PAR), soil water content (SWC) and air vapor pressure deficit (VPD), with the dependence of g_s on these factors being species-specific. Hence, in addition to inherent stomatal properties of a species, the response of g_s to variations in the above factors is important. Bungener *et al.* (1999a and 1999b) observed that protection from O_3 under increased VPD or low SWC occurs in some, but not all, of 24 tested grassland species, depending on the specific response of g_s to changes in these parameters. As a result of reduced irrigation, low SWC decreased the detrimental O_3 effect on productivity, as well as on the competitive ability of grassland species grown in model communities (Nussbaum *et al.*, 2000). However, these findings may only apply to taxa from mesic sites, as opposed to those from drier or wetter habitats. Unfortunately, the vast majority of European studies used taxa from mesic sites (Franzaring, 2000).

In a field experiment, Jäggi *et al.* (2005) analyzed the C stable isotope composition ($\delta^{13}C$) as an integrated measure of gas exchange in several species in intact, old grassland and found that lack of moisture in the topsoil layer did not always protect from O_3 uptake. They concluded that in species such as *T. pratense* individuals could maintain high g_s during rain-free periods at levels similar to those in well-watered control plants, possibly because the roots of these plants reached deeper soil layers with abundant water. Such testing of the influence of variable SWC on O_3 impacts is more valuable when carried out in the original habitat of the respective species, rather than in pot-grown plants. This is important because optimal conditions for growth, which are associated with the highest risk of O_3 damage, are rarely found in nature. Instead, in temperate regions O_3 episodes are often associated with hot, dry weather. Therefore, results from experiments under ‘optimized’ conditions likely overestimate O_3 impacts.

3.2 Phenological development and plant age

During their life cycle, plants pass through different phases characterized by specific patterns of growth and allocation. In perennials, which typically dominate temperate grasslands, three phases can be separated: establishment phase, vegetative growth phase, and recurring generative growth phases. The initial establishment phase is characterized by expansion with high RGR, large carbon investments in roots and shoots, and high g_s. Based on the above considerations, these characteristics are related to high O_3 sensitivity. During the vegetative phase, growth rates are reduced as compared to the establishment phase and carbon is mainly invested in growth of storage organs, but also in structures and biochemical defense. Thus, O_3 sensitivity can be expected to be low. In the generative phase, carbon allocation is primarily to stem, flowers and shoots, while root and leaf allocation decreases. In the generative phase, both grasses and herbs had a higher SLA (Reekie & Reekie, 1991; Sylvester *et al.*, 2001), and detoxification capacity was found to decrease during flowering (Ye *et al.*, 2000). Therefore, during this phase plants may be more sensitive to O_3 than during the vegetative phase. Moreover, strong shoot growth results in taller plants with their leaves exposed in the upper part of the canopy (see 4.5).

Experimental support for differences in O_3 sensitivity during individual developmental stages in herbaceous species is weak. *Plantago major* was found to be more sensitive in the seedling stage (Lyons & Barnes, 1998; Reiling & Davison, 1992a). *Centaurea jacea* showed enhanced sensitivity during the reproductive phase (Bassin *et al.*, 2004), but it was not possible to distinguish whether this was due to physiological changes, or due to the fact that the leaves were more exposed to O_3. More direct evidence of increased sensitivity during the reproductive stage comes from studies with wheat (Pleijel *et al.*, 1998).

3.3 Nutrient availability

Nutrient availability was considered to be an important factor modifying O_3 response of semi-natural vegetation (Davison & Barnes, 1998). Increasing nutrient supply could have opposing effects: increasing sensitivity due to stimulated growth and higher SLA, or alternatively, increasing tolerance due to enhanced detoxification capacity through stimulated photosynthesis. Several studies have been carried out in open-top chambers (OTC) using a cross factorial design with two or more O_3 treatments and at least two nutrient levels (either complete fertilizer or nitrogen only). Whitfield *et al.* (1998) claimed that high nutrient supply protected *P. major* plants against O_3 damage. This was not confirmed for *T. subterraneum*, where fertilization (only N) reduced early senescence, but not negative effects of elevated O_3 on biomass (Sanz *et al.*, 2005). No significant interaction of O_3 and nutrient supply (complete fertilizer) was found in the biomass response of *Arrhenatherum elatius*, *Bromopsis erectus*, and *Lathyrus pratensis* (Thwaites, 1997), nor in mixtures of *L. perenne* and *T. repens* (Bass *et al.*, 2006), or in complex mixtures (Samuelsson *et al.*, 2006). In contrast, preliminary results from a recently established experiment with alpine pasture using three O_3 and five nitrogen levels indicate that O_3 sensitivity may be enhanced by elevated N deposition (Bassin *et al.*, 2005). The contrasting results may be related to different experimental protocols, differences in the nutrient status in the control, and because in most cases plants have not been tested in their natural habitat (see 5.1).

Little is known about the role of mycorrhiza in the relation between nutrient availability and O_3 sensitivity in perennial grasslands. Miller *et al.* (1997) found significant interactions between O_3 and symbiont type and showed that the presence of an arbuscular mycorrhizal fungus suppressed growth of the host *T. subterraneum* even at low O_3 concentrations. Presumably, this was due to the extra carbon demands by the fungus, but the question deserves more attention.

4 O_3 sensitivity of communities

To assign a measure of O_3 sensitivity to a particular system, the simplest assumption would be that the sensitivity of the component species determines the sensitivity of the community. Consequently, in communities dominated by species with traits associated with high sensitivity, a shift in species cover or frequency, or loss of productivity would be most likely. It was proposed to use this principle to map sensitive communities across Europe (Mills *et al.*, 2007). However, the overall community response is likely to be more than the sum of responses of individuals predicted from experiments with isolated plants because the cohabitation of individuals is characterized by multiple mutualistic and competitive interactions, and specific microclimatic conditions, which may all influence the individual O_3 response. Grime *et al.* (2000) proposed a set of community characteristics, which determine the sensitivity of a community to climate change. The same principles could be applied to O_3 stress.

4.1 Species diversity

In agreement with the insurance theory (Yachi & Loreau, 1999), it is commonly assumed that higher species richness enhances both resistance (capacity to tolerate perturbation) and resilience (capacity to recover after perturbation) of plant communities. Unfortunately, this notion has never been tested for O_3 stress.

Table 2. Overview of phytometer experiments carried out in the framework of the EU project BIOSTRESS. Sensitivity assessment were carried out on plants grown from seed, cultivated in pots with unlimited water and nutrient supply, and exposed to O_3 in open-top chambers (OTC).

Vegetation type	**No. of individuals**	**Phytometer species**	**Target plant species**	**Response parameter**	**O_3 effect on target species**	**O_3 x competition interaction on target species**	**Duration**	**Reference**
Mediterranean annual pasture	4	*Briza maxima*	*Trifolium cherleri*	flower production	Yes	yes	1 season	Gimeno *et al*., 2004b
			Trifolium subterraneum		Yes	No		
			Trifolium striatum		Yes	No		
Wet temperate grasslands	4	*Agrostis capillaris*	*Holcus lanatus*	biomass	No	No	3 seasons	Toneijck *et al*., 2004
			Lychnis flos-cuculi		No	No		
			Molinia caerulea		No	No		
			Plantago lanceolata		No	No		
Temperate grassland	4	*Poa pratensis*	*Anthoxanthum odoratum* *Achillea millefolium* *Rumex acetosa* *Veronica chamaedrys*	biomass	No No No No	No No No Yes, 1st season	3 seasons	Bender *et al*., 2002 Bender *et al*., 2006

Table 3. Overview on studies using intact plant communities for the assessment of O_3 impact.

Vegetation type	**Species number**	**Scale**	**Age of the community**	**Reduction of total aboveground biomass**	**Changes in species composition**	**Duration**	**Reference**
Temperate grassland	7 (dominant)	Field	Established	Yes	Increase of forb and legume fraction	2 seasons	Evans & Ashmore, 1992
Calcareous grassland	38	Monoliths	Established	Not tested	Reduction of *Carex flacca*, loss of *Campanula rotundifolia*	3 seasons	Thwaites *et al*., 2006
Forest understorey	40	Field	Early succession	No	Increase of *Rubus cuneifolius*	2 seasons	Barbo *et al*., 1998
Pre-alpine grassland	53	Field	Established	Yes	Reduction of legume fraction	5 seasons	Volk *et al*., 2006

4.2 Functional types

The use of functional types, rather than species, makes it possible to link communities that are separated in time and space into a conceptual framework, and effects of O_3 on grassland communities could be compared and mapped across Europe. Attempts have been made to develop predictors of O_3 sensitivity based on functional types according to Grime *et al.* (1996). The competitor (C) *vs.* stress-tolerant (S) *vs.* ruderal-discrimination (R) (C-S-R system) is based on characteristics such as reproduction, phenology, potential RGR etc. of each individual species. Importantly, these parameters are not unrelated to the 'O_3 sensitivity-relevant traits' discussed above (see 2). According to Poorter & Garnier (1999), fast-growing species are found in habitats favorable for plant growth, either in the short-term (annuals, R-types) or in the longer-term (C-types), whereas species found in stressed environments (S-types) generally have a lower potential RGR. S-types are also known to have small, leathery, or needle-like leaves (Grime *et al.*, 1996). In their analysis of plants collected from 15 habitats, Poorter & De Jong (1999) confirmed that species from highly productive habitats had higher SLA than those from sites of low productivity. In agreement, community C-S-R type composition was found to change along gradients of productivity in Nordic grassland (Vandvik *et al.*, 2002; Ejrnaes *et al.*, 2000). Therefore, S-type dominated communities at low-productivity sites could be less affected by O_3 than with highly productive communities in typical C or R-type habitats.

However, according to most screening experiments, the predictive value of the C-S-R system for O_3 sensitivity seems limited (Davison & Barnes, 1998; Timonen *et al.*, 2004). So far, only one study confirmed the relationship between C-S-R types and O_3 sensitivity (Bungener *et al.*, 1999b), while several other studies found no evidence to support it (Gimeno *et al.*, 2004a; Pleijel & Danielsson, 1997; Warwick & Taylor, 1995). In a recent meta-analysis of O_3 sensitivity among European herbaceous species, including more than 80 plant species from ecologically different habitats, Hayes *et al.* (2007) again found no significant correlation between O_3 sensitivity and C-S-R types. Instead, Ellenberg indicator values turned out to be more valuable predictors of O_3 sensitivity. These values represent a scoring system reflecting the plants natural habitat preferences. Scores are provided for several site factors: light, temperature, continentality, soil moisture, soil reaction (acidity) and soil nitrogen. Among Ellenberg values, light, nitrogen, and salinity number were positively correlated with O_3 sensitivity, whereas moisture number was negatively correlated. In the same study, Raunkiaer classes of life form were found to be associated to some extent with O_3 sensitivity. While therophytes responded with a strong biomass reduction, helophytes showed increased productivity at elevated O_3 concentrations.

Overall, while ecological theory suggests that species functional type composition might determine O_3 sensitivity of communities, experimental results do not unequivocally support this. This is surprising considering that plant traits, on which the C-S-R type classification is based (e.g., SLA and RGR), have turned out to be well-related to O_3 sensitivity (see section 2.4). However, the correlation between SLA or RGR, respectively, and O_3 sensitivity is based on measurements of SLA/RGR made on the individuals used for sensitivity assessment (Bungener *et al.*, 1999b; Danielsson *et al.*, 1999; Franzaring *et al.*, 2000). In contrast, the C-S-R type classification is typically taken from the literature (Reiling & Davison, 1992b; Pleijel *et al.*, 1997; Hayes *et al.*, 2007), thus ignoring the effective growth rates under the experimental conditions. Most importantly, growth rate and plant shape of species from resource-limited habitats, namely S-types, are strongly affected by modifications introduced by the experimental growing conditions. Pleijel *et al.* (1997) showed that the effect of OTC enclosure on above-ground biomass was stronger for S-strategists than for C and R-strategists. It is likely that under these non-representative conditions S-strategists are more sensitive to O_3 than they would be under more natural conditions. Perhaps for the same reason, the regression analysis of Hayes *et al.* (2007) suggested that species from dry habitats were more O_3 sensitive than those from wet habitats. The authors assumed that drought-adapted species showed stronger O_3-responses because they reacted more readily with stomatal opening to the unlimited water availability in the pot experiments than under typical habitat conditions.

4.3 Genotypes

Species composition in semi-natural and natural grasslands is the result of natural selection of species and genotypes best adapted to local conditions. When O_3 levels start to increase, a system is exposed to an additional stressor, giving the selective pressure a new direction. Thus, previous exposure to O_3 stress may change the genotypic composition of a population through selection of O_3-resistant genotypes. Several studies showed that genotypes of the same species differ in O_3 sensitivity. This was true for genotypes from populations of different geographic origin, e.g., *P. major* (Lyons *et al.*, 1997) and *C. jacea* (Bassin *et al.*, 2004), and from within a population, e.g., *C. jacea* (Bungener *et al.*, 2003) and *T. repens*

(Nebel & Fuhrer, 1994). In studies with *P. major*, the distribution of O_3 resistance was positively related to O_3 exposure during previous years (Lyons *et al.*, 1997), thus indicating that genetic selection for O_3 resistance may have taken place. But in this type of survey it is not possible to separate O_3 effects from the influence of drought and high light intensities, factors typically co-occurring with high O_3 concentration episodes. However, Heagle *et al.* (1991) and Whitfield *et al.* (1997) confirmed experimentally that the selective pressure of previous exposure to elevated O_3 concentrations increased the tolerance of the community to O_3 (*cf.* Barnes *et al.*, 1999). Hence, at the community level specific O_3 responses could be buffered through genetic variability. On longer time scales, this selection could result in the loss of genetic diversity.

4.4 Competition

In non-managed systems, competitive mechanisms are among the determining factors of shifts in the floristic composition in response to changing environmental conditions (Bishop & Cook, 1981). Neighboring individuals compete for resources both below- and aboveground. Consequently, changes in resource availability, resulting from effects of O_3 on one of the competing species, lead to adaptations of the other competing species. Rooting pattern is strongly influenced by the presence of neighbors (Callaway *et al.*, 2003). Likewise, in dense stands competition for light causes changes in shoot growth and architecture (Turkington *et al.*, 1994). For instance, rosette plants raise their leaves when shaded (Vanhinsberg & Vantienderen, 1997). Thus, it could be hypothesized that the sensitivity to O_3 depends on whether the plant is grown in isolation, in a monoculture, or in intraspecific competition. In a competitive environment, growth could be enhanced or reduced depending on the relative responses to O_3 of the target species and its neighbors.

O_3 impacts on these interactions are important, but little is known about interspecific interactions under O_3 stress (*cf.* Fuhrer & Booker, 2003). Blackberry (*Rubus cuneifolius*) under high O_3 became dominant in an early successional community previously dominated by sumac (*Rhus copallina*) despite the great sensitivity of blackberry to O_3 (Barbo *et al.*, 1998). However, these results of studies with natural communities cannot be analyzed for competition effects, because competition is not a controlled factor in the design (Ashmore & Ainsworth, 1995; Barbo *et al.*, 1998; Evans & Ashmore, 1992; Volk *et al.*, 2006). Under more controlled conditions, Nussbaum *et al.* (2000) using an experiment with binary mixtures showed that competition changed the species reaction to O_3. *Trisetum flavescens*, planted with either *T. repens* or *C. jacea* in different mixing ratios, exhibited a significant O_3 x mixing ratio interaction with *T. repens*, but not with the other species. In mesocosm studies, species from different habitats across Europe (annual Mediterranean Dehesa, wet grassland, mesic grassland, see Table 2) were exposed to O_3 early in the growing season. It was assumed that competition is highest during phases of high productivity (Dunnett & Grime, 1999). Species were grown in monocultures and binary mixtures consisting of a target plant surrounded by three phytometer plants. In three clover species from Dehesa Mediterranean therophytic grasslands, tested in competition with *Briza maxima*, both O_3 and competition reduced the target plant growth and flower production. No O_3 x competition interaction was found, except for one of six harvests where the flower output in filtered air was 61% higher in monocultures than in mixtures (Gimeno *et al.*, 2004b). In a study of four wetland species grown in competition with *Agrostis capillaris*, O_3 had no impact on species productivity, but competition substantially influenced the biomass of the target species (Tonneijck *et al.*, 2004). Out of six perennials from temperate grasslands in binary mixtures with *Poa pratensis*, only *Veronica chamaedris* showed a significant O_3 x competition interaction. *V. chamaedris* depressed the phytometer plant growth in filtered air, but its competitive advantage disappeared under increasing O_3 stress (Bender *et al.*, 2002). Bass *et al.* (2006) using established mesocosms with high numbers of individuals found that the O_3-induced growth reduction of white clover *T. repens* in binary mixtures with *L. perenne* was similar to the reduction in monocultures. Interestingly, the only experiment showing a strong O_3 x competition interaction was using two different irrigation treatments (Nussbaum *et al.*, 2000). This suggests that O_3 effects on competitive interactions must be viewed in the context of variable resource availability.

4.5 Canopy structure/plant position in the canopy

In studies with isolated potted plants, all leaves are equally exposed to O_3, thus maximizing exposure. In real communities, O_3 exposure depends on the plants' position in the canopy. As the air passes through the canopy, O_3 is depleted due to uptake via stomata, destruction at surfaces, or gas-phase reactions. It is replaced by air mixing in from above the canopy, which is controlled mainly by turbulence intensity above the canopy (Jäggi *et al.*, 2006). Both O_3 influx and degradation of O_3 along the in-canopy pathway are related to the physical structure of the canopy (Figure 1).

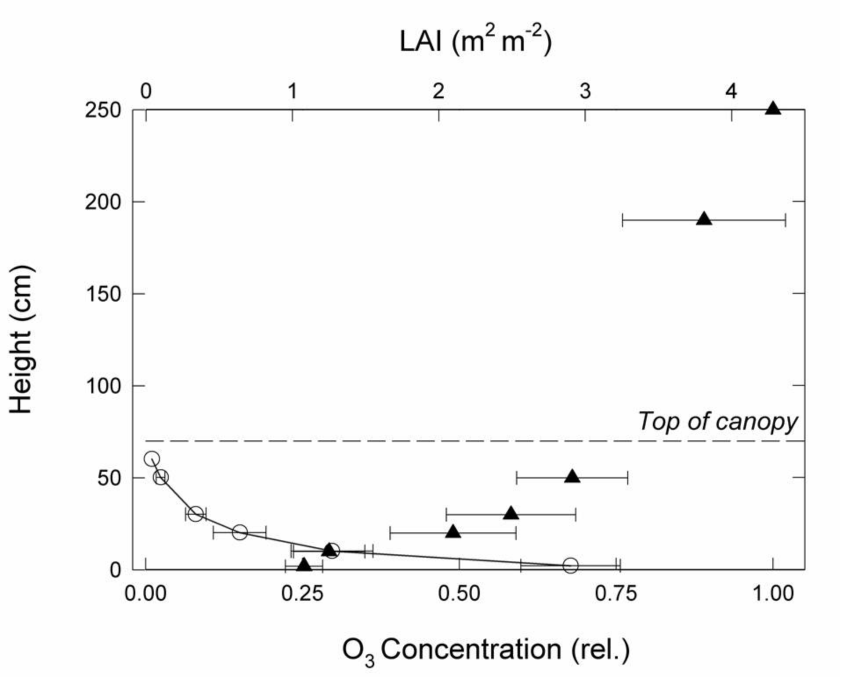

Figure 1. *Average daytime vertical profiles of O_3 concentration (relative to reference at 2.5 m) (triangles) and cumulative leaf area index (LAI, open circles) in a mature canopy of semi-natural grassland (Le Mouret, Switzerland, 12 May-12 June 2003). Bars indicate standard deviation.*

In addition, O_3 uptake is modified by changes in g_s due to in-canopy gradients of microclimatic factors such as VPD and PAR. According to studies with trees, leaf morphology and defense capacity substantially differed between sun-leaves and shade leaves, with corresponding differences in O_3 sensitivity (Wieser *et al.*, 2003). However, only Davison *et al.* (2003) and Jäggi *et al.* (2006) actually measured O_3 profiles in herbaceous stands. Davison *et al.* (2003) found a much greater reduction in PAR than in O_3 along gradients in a canopy of *Rudbeckia laciniata*, a species that regularly shows visible injury in the Great Smoky Mountains National Park (USA). At 50 cm above ground, which included the four lowest leaves, the O_3 concentration varied from only 15% to 90% of ambient levels, whereas PAR was consistently below 10%. It was concluded that O_3 uptake of low leaves was limited by PAR rather than by the level of O_3. In contrast, Jäggi *et al.* (2006) found a moderate PAR reduction throughout the canopy in a two-layered grass canopy with less than 20% of the total leaf area index in the upper half. In this stand g_s in leaves of *T. repens* at 25 cm above ground was similar to g_s in *Alopecurus pratensis* leaves at 50 cm. During daytime, leaves of *A. pratensis* were exposed to 92%, while leaves of *T. repens* were exposed to only 64% of the reference O_3 concentration above the canopy. Consequently, species occupying the lower part of this type of canopy are less exposed to O_3 than taller species.

4.6 Management

Cutting or grazing is often used to maintain temperate grasslands. Consequently, the phases of intensive growth and leaf development are distributed over much of the growing season. This increases the probability of co-occurring sensitive developmental phases and peak O_3 episodes. In one of the few studies available, Wilbourn *et al.* (1995) found that effects of O_3 effects on yield and species composition of *T. repens – L. perenne* mixtures differed between harvests. As a result of cutting, canopy structure and related exposure along gradients of O_3 are removed and species occupying lower canopy layers become exposed to higher O_3 levels and more light during the initial phase of re-growth. Thus, frequent cutting could contribute to the relatively higher sensitivity of productive systems.

In ambient air, periods of elevated O_3 concentration occur at irregular intervals so that some growth periods experiences substantially lower O_3 concentrations than others. Thus, O_3 tolerance includes the capacity to recover between O_3 episodes or between cuts. For example, *T. repens* replaced damaged leaves rather quickly, albeit at the expense of the stolons (Wilbourn *et al.*, 1995). The same species recovered from damage in previous periods during growth periods with lower O_3 concentrations during the same year (Nussbaum *et al.*, 1995) or in the course of two consecutive years (Fuhrer *et al.*, 1994). Also, Bass *et al.* (2006) found no season-to-season carry over effects of damage in white clover. Theses results underline the important influence of the regime of management for community sensitivity.

4.7 Responses of sown model communities

It could be expected that favorable growth conditions and the establishment-phase character of most experimental communities would shift plant traits towards higher O_3 sensitivity. Accordingly, in experiments with sown 2-5-species mixtures, representing intensively managed, productive meadows, high O_3 sensitivity was found (Table 4). Generally, grass-legume communities (e.g., *L. perenne – T. repens*) have been found to shift towards pure grass stands during O_3 fumigation (*cf.* Fuhrer *et al.*, 1997). Fuhrer *et al.* (1994) and Nussbaum *et al.* (1995) reported no O_3 effect on the total yield, but a decrease of the clover fraction. The study of Pleijel *et al.* (1996) revealed a linear decrease in forage yield with increasing O_3, but no effect on the clover fraction, while Bass *et al.* (2006) and Heagle *et al.* (1989) found reductions in both yield and clover fraction. The data suggest that O_3 often accelerates the clover decline in highly productive grass/clover mixtures, but that the magnitude of the effect depends on the species combination, cultivars and genotypes.

Table 4. Overview of open-top chamber studies (after 1985) using sown mixtures as model plant communities for the assessment of O_3 impacts.

Vegetation type	**Taxa**	**Species tested**	**Scale**	**Biomass reduction**	**Changes in species composition**	**Additional factors tested**	**O_3 x factor interaction**	**Duration**	**References**
Productive meadow	*Phleum pratense* *Trifolium pratense*	2	Field	Yes	Reduction of clover fraction	Sulphur-dioxide	No	2 seasons	Kohut *et al*., 1988
Productive meadow	*Festuca arundinacea* *Trifolium repens*	2	Pot	Yes	Reduction of clover fraction	Water availability	Yes (1season)	2 seasons	Rebbeck *et al*., 1988 Heagle *et al*., 1989
Productive pasture	Grasses Legumes Weeds	> 4	Field	Small	Reduction of legume fraction	No	-	2 seasons	Fuhrer *et al*., 1994
Productive meadow	*Lolium perenne* *Trifolium repens*	2	Pot	No	Reduction of clover fraction	Episodic O_3 exposure	Yes	1 season	Nussbaum *et al*., 1995
Productive pasture	*Phleum pratense* *Festuca pratensis* *Trifolium pratense*	3	Field	Yes	No	No	-	2 seasons	Pleijel *et al*., 1996
Productive meadow	*Lolium perenne* *Trifolium repens*	2	Pot	Yes, small	Reduction of clover fraction	Nutrient availability Competition	No No	2 seasons	Bass *et al*., 2006
Natural grassland	Grasses Clover Forbs	4	Pot	No	Reduction of clover fraction	Cutting	Yes (on forb fraction)	1 season	Ashmore & Ainsworth, 1995
Calcareous grassland	Grasses Forbs	10	Pot	No	Reduction of forb fraction (mainly *Leontodon hispidus*)	No	-	1 season	Ashmore *et al*., 1995
Natural grassland	*Trisetum flavescens* *Trifolium pratense* *Centaurea jacea*	3	Pot	Yes	Reduction of clover fraction	Water availability	Yes	2 seasons	Nussbaum *et al*., 2000
Natural grassland	Grasses Forbs Legumes	10	Pot	Yes, small	Reduction of *Briza media, Phleum bertonii,* increase of *Alopecurus pratensis*	Nutrient availability Hemi-parasitism	No Yes	2 seasons	Samuelsson *et al*., 2006
Nordic dry grassland	Grasses Forbs Legumes	7	Field	Yes	Reduction of forb fraction; reduction of *Campanula rotundifolia* and *Vicia cracca*	Elevated CO_2 concentrations	No	3 seasons	Rämö *et al*., 2006

Three out of the five studies with model communities of semi-natural grasslands found only small or no effects of O_3 on aboveground biomass, but strong changes in species composition (Table 4). Ashmore & Ainsworth (1995) used mixtures of two grasses, a forb and a legume and found little effect on total biomass, but a decline in the legume component (*T. repens*) with increasing O_3 exposure. In a ten-species mixture of taxa from calcareous grassland total biomass remained unaffected by O_3, while the grass fraction increased mainly at the expense of *Leontodon hispidus* in the forb fraction (Ashmore *et al.*, 1995). The relatively high O_3 tolerance of grasses was recently confirmed under low and high fertility conditions in a mesocosm study using species from temperate natural grasslands (*L. perenne*, *A. pratensis*, *Ranunculus acris/repens*, *Briza media*, *Geranium silvaticum*) (Samuelsson *et al.*, 2006). The 2-year fumigation (30 and 50 ppb) had no effect on aboveground biomass, but the more tolerant grasses were favored over the less tolerant forbs. Interestingly, at species level, only the grasses experienced significant changes. While *Alopecurus pratensis* increased its fraction, *B. media* and *Phleum bertolonii* were strongly reduced by O_3. The single legume species *Lotus corniculatus* remained unaffected.

There are two studies showing O_3-induced biomass-reduction in addition to the changes in species ratios. Binary mixtures of *T. flavescens* with either *C. jacea* or *T. repens* showed a change in species composition as well as a strong reduction of total biomass in the clover-mixtures, but not in mixture with *C. jacea* (Nussbaum *et al.*, 2000). In a mesocosm experiment, using species from a northern European lowland hay meadow, aboveground biomass was reduced up to 40% in elevated O_3 concentrations (Rämö *et al.*, 2006). Since the most strongly affected species (*Campanula rotundifolia*, *Fragaria vesca* and *Vicia cracca*) contributed only marginally to total biomass, it is possible that decreased nitrogen fixation caused the decline in total productivity.

These results support the idea that, in general, sown model communities show a quick and strong O_3 response in terms of species composition, similar to individuals, with legumes often, but not always, being most strongly affected and grasses generally less. However, additional experiments with different vegetation types under representative climatic and edaphic conditions are necessary to test the hypothesis that O_3 sensitivity is positively related to productivity (as hypothesized in the context of C-S-R strategy, see 4.2).

4.8 Response of mature communities

Four studies were carried out dealing with the response of older or mature communities (Table 3). Considering that species number, plant age, number of mutualistic interactions and genetic variability are higher in mature compared to young communities, the O_3 response of species composition and community productivity should be less and develop slower than in sown systems. In a 3-year study, Thwaites *et al.* (2006) exposed large turf monoliths from calcareous grassland to elevated O_3 concentrations. Changes in community composition started to occur during the second season. But, likely because of year-to-year variations in climate, the change was not consistent over time, and the final difference between treatments was small. Evans & Ashmore (1992) compared plots of semi-natural grassland under charcoal-filtered air with plots in ambient air. Effects on aboveground biomass and species composition were observed in one of the two seasons, when ambient O_3 concentration was high. In contrast, Barbo *et al.* (1998) was able to show consistent O_3 effects on early successional understory vegetation in terms of species performance, canopy structure, and species richness. However, in the latter study plants were exposed to O_3 while establishing from the seed bank of disturbed soil, and thus the results cannot be compared directly with those of mature grasslands.

When studying intact, established communities it is important to consider long-term effects to account for year-to-year variations in climate and O_3, as well as for recovery and carry-over effects. In a 5-year exposure experiment, ambient and elevated O_3 concentrations were applied to a 60-year-old, extensively managed pasture using a free-air fumigation system. Productivity declined progressively resulting in a 23% annual yield reduction by the end of the last season (Volk *et al.*, 2006). The legume fraction decreased from 12 to 4%, whereas the forb fraction increased from 25 to 41%. However, significant O_3 effects appeared only after several years of exposure, in contrast to sown productive systems (see 5). This supports the idea proposed by Grime *et al.* (2000) that mature systems exhibit a greater inertia to stress and disturbance than newly established communities. Higher resilience could result from long-term community-level processes, leading to a balanced mixture of mostly intermediate C-S-R-strategists, as was the case in the experimental system used by Volk *et al.* (2006) (data not shown). Also the large root system of a mature community could help to buffer short-term impacts of O_3 on belowground allocation of carbon.

5 Limitations to predictions

5.1 Extrapolation across scales

Regarding the vast amount of data from studies with individual plants (Figure 2) and mesocosms (Table 4), the question arises as to whether or not it is possible to extrapolate results across scales. Single pots and mesocosms are used in experiments as simplified models of a real community, but they can differ in a variety of parameters such as climate, resource availability, species number, plant age, competition. The more closely these parameters represent real ecosystem conditions, the more useful are the results of model-experiments for extrapolation to the real world. For productive grasslands, model and real systems are best comparable in most of the characteristics (species number, plant age, soil nutrient content, water availability, genotypic variation; for comparison see references in Table 4 and Whitehead, 1995), and thus extrapolation seems feasible. Also, 'O_3 sensitivity-relevant traits' are similar in both situations. Conversely, in low-productivity grasslands, community characteristics, including the establishing-phase character and resource availability, differ in most cases between model and natural communities (for comparison see references in Table 4 and Grime *et al.*, 2000; Niklaus & Körner, 2004). In agreement, Thwaites *et al.* (2006) observed that contrary to expectations from pot experiments biomass of some species in the turf, especially *L. corniculatus,* increased under elevated O_3 fumigation. Also Barbo *et al.* (1998) showed that blackberry (*Rubus* spp.), which was expected to be O_3-sensitive, dominated field communities in non-filtered, but not in the filtered air treatment, possibly because of the reduction of interspecific competition for light. A similar argument was used to explain the unexpected reduction of forbs (including legumes) in filtered vs. unfiltered air in the experiment by Evans & Ashmore (1992).

According to these findings, the predictability of community responses to O_3 based on results for single plants or model communities seems low, except perhaps for productive grassland. This is in agreement with conclusions drawn from results of CO_2-enrichment experiments, e.g., by Poorter & Navas (2003). Thus, extrapolation approaches like those of Mills *et al.* (2007) and Jones *et al.* (2007) to estimate community sensitivity based on individual plant responses are most useful in cases, in which experimental conditions correspond best to the typical habitat conditions of the species tested, namely productive and mesic grasslands. In fact, using the statistical approach it was possible to predict the reduction in above-ground biomass in a field fumigation experiment with mesic grassland (Jones *et al.*, 2007). However, similar tests of this approach for other vegetation types are lacking.

5.2 Experimental constraints

Extrapolation beyond the climatic and edaphic conditions created in experimental systems can be misleading. Since phenotypic plasticity enables plants to adapt growth rates and plant shape to resource availability, the experimental climatic and edaphic conditions should be representative for the original habitat in terms of climate, resource availability, plant age, and competition (see above). This is particularly the case for plants from dry, nutrient-limited, or alpine habitats. Also, experiments should focus on the dominant species of relevant communities, or artificial communities with representative ratios of species and functional types, and plant densities.

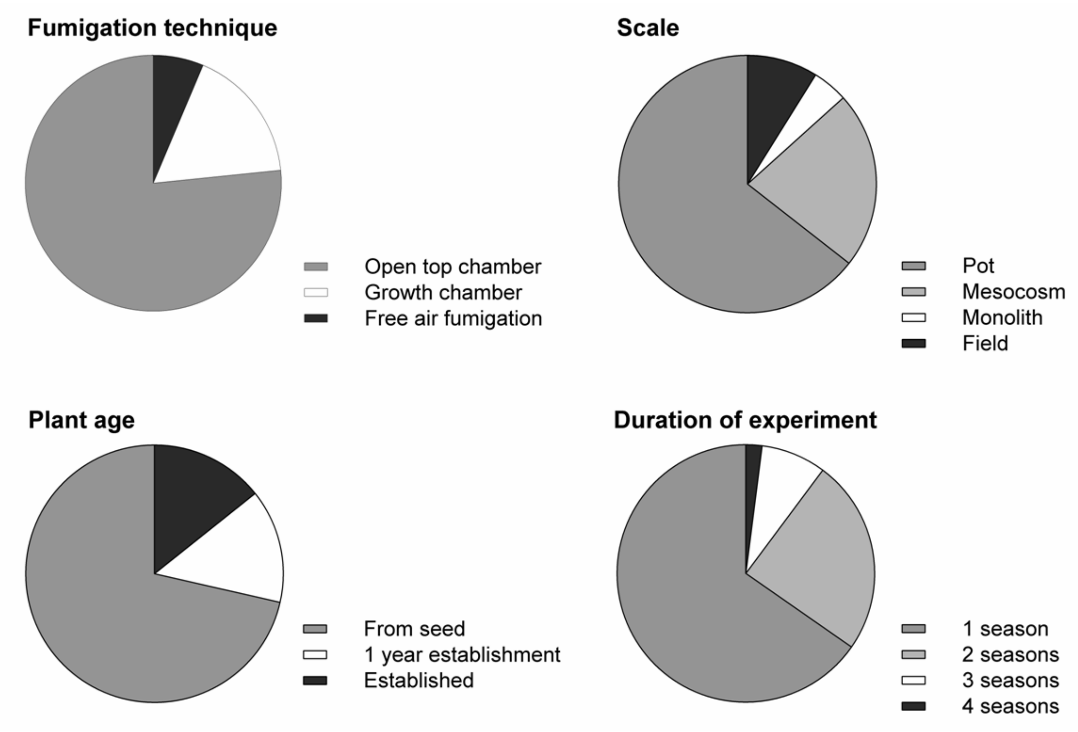

Figure 2. *Pie charts classifying the 47 fumigation experiments used in this article according to the criteria "Fumigation technique", "Scale", "Plant age", and "Duration of experiment".*

Most studies with herbaceous species used open-top chambers (OTCs) with selected individuals grown from seeds in pots and exposed to different levels of O_3 during 1-3 months within 1-2 years (Figure 2). Typically, unlimited nutrient and water supply in pots is provided to prevent co-limitations, but space for root expansion is limited. These artificial conditions for root growth may affect the response of the whole plant to O_3: maximum RGR during the establishment phase exacerbate O_3 sensitivity, followed by limited growth during later stages associated with declining sensitivity, as observed in several studies (Bungener *et al.*, 1999b; Bender *et al.*, 2006; Tonneijck *et al.*, 2004).

Compared to ambient conditions, the air inside OTCs is highly turbulent, reducing both aerodynamic and boundary layer resistances to near zero (Nussbaum & Fuhrer, 2000). Moreover, the chamber microclimate enhances g_s due to higher temperatures and lower VPD relative to ambient conditions, which stimulates O_3 uptake by a factor of 1.2-1.5 (Nussbaum & Fuhrer, 2000). Not surprisingly, Novak *et al.* (2005) showed that several plant species respond differently to O_3 when grown in OTCs compared to ambient conditions. Moreover, enclosure of mature communities in OTCs for fumigation purposes may cause large microclimatic effects on community growth and composition (Fuhrer, 1994). The above constraints can be overcome by measures such as (i) controlling water (precipitation) and nutrient supply at levels typical of natural habitats, (ii) growing mixtures in natural soils with no restriction to root growth, and (iii) minimizing microclimatic modifications due to forced turbulence and changes in the energy balance. To avoid artifacts introduced by altered microclimatic conditions, free-air fumigation systems are most useful (Volk *et al.*, 2003). They provide close-to-natural conditions and allow for sufficient plot size and space to study the interactions with additional factors such as water and nutrition.

6 Implications for critical levels to protect semi-natural vegetation

To define critical levels for semi-natural vegetation has been difficult for reasons discussed by Fuhrer *et al.* (2003). The most recently proposed critical level for semi-natural vegetation is based on five experiments carried out either with plants grown in mesocosms (Mills *et al.* 2005, Rämö *et al.*, 2006), monoliths (Samuelsson *et al.*, 2006; Thwaites *et al.*, 2006) or in the field (Volk *et al.*, 2005). In these experiments, the AOT40 necessary to induce biomass reductions or changes in species composition ranged from 8 (mesocosm study from Finland) to 34 ppm.h (field experiment), reflecting the heterogeneity of this receptor. However, the critical level should protect the most sensitive plants, and thus it is based on the results from the study showing the most sensitive O_3 response, although in reality this value may overestimate the sensitivity of a majority of semi-natural vegetation.

7 Summary and conclusions

This overview reveals difficulties in relating responses to O_3 of individuals to those of communities, and to identify communities of temperate grassland most at risk. At the level of individual plants, results are more consistent and suggest that three traits are main determinants for O_3 sensitivity: g_s, SLA, and defense capacity. Phenology and external factors (microclimate, nutrients, management) can modify the specific sensitivity via effects on g_s and/or RGR. Consistently, legumes are more sensitive than other forbs and grasses.

At the level of communities, data are less conclusive and important ecological theories such as the assumption that increasing species richness may increase the tolerance of a community to O_3 have not yet been tested. Other results tentatively suggest that the sensitivity to O_3 of communities is positively related to productivity, and therefore, that species grown under favorable growth conditions or in productive habitats are most sensitive (e.g., EUNIS mesic grassland classes E 2.6. “Agriculturally improved grassland”, and E 2.1. “Permanent mesotrophic pastures”), in contrast to less productive habitats (e.g., EUNIS classes E4 “alpine grassland”, and E1 “dry grassland”). These are characterized by the presence of S-types with low RGR and low SLA, which are less sensitive than fast growing C and R types, but evidence is limited. Also, among Raunkiaers life forms, therophytes with high RGR are relatively more O_3 sensitive than other life forms.

Sown communities, representing leys or intensively managed grasslands, respond quickly to elevated O_3 with shifts in species composition and sometimes with reductions in aboveground productivity. In contrast, in older communities the reaction develops more slowly and remains small after typical experimental periods of a few years. While in sown communities, the change in species composition largely reflects the species sensitivity observed in single plant studies, this is not always the case in older communities. Co-factors like plant-plant interactions, canopy structure, and microclimate can influence the response of individuals and, consequently, of the respective community. This would be typical of older perennial grasslands covering large areas of the non-forested landscape in Europe, which are characterized by balanced mixtures of different functional types and adapted genotypes. Moreover, in these systems O_3 effects co-occur with seasonal and inter-annual climate weather variability, and spatial heterogeneities in biological and edaphic conditions make it difficult to identify O_3 effects with statistical significance.

Results from short-term OTC experiments using young individuals in pots under unlimited resource availability cannot easily be extrapolated to semi-natural and natural communities. The experiments required to make O_3 risk assessments at community level should be carried out under realistic conditions in terms of soil substrate, nutrient and water availability, plant productivity, climatic conditions, community structure and composition (species, genotypes), and micrometeorology.

In conclusion, there is emerging evidence that on the basis of physiological or ecological principles communities may be separated into broad classes of O_3 sensitivity, but at present the database from experiments under realistic conditions with representative systems is too small to make firm recommendations. However, it seems quite evident that risk assessments based on knowledge gained with individuals or immature mixtures in OTCs are only applicable to a minor fraction of grasslands, and that O_3 risks for most of the perennial grasslands tend to be lower than previously expected.

Acknowledgments

This work was supported by the Swiss Federal Office of Environment in the framework of the International Cooperative Programme ‘Vegetation’ (ICP Vegetation) of the UNECE Convention on Long-Range Transboundary Air Pollution. We thank J. Barnes for providing unpublished data.

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Chapter 3

Nitrogen deposition but not ozone affects productivity and community composition of subalpine grassland after three years of treatment

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New Phytologist 2007, 175, 523-534

Abstract

A field experiment was established at 2000 m a.s.l. in the central Swiss Alps with the aim to investigate the effects of elevated ozone (O_3) and nitrogen deposition (N), and of their combination, on aboveground productivity and species composition of subalpine grassland. 180 monoliths were extracted from a species-rich *Geo-Montani-Nardetum* pasture and exposed in a free-air O_3-fumigation system to one of three concentrations of O_3 (ambient, 1.2 x ambient, 1.6 x ambient) and five concentrations of additional N (0-50 kg N ha^{-1} y^{-1}). Aboveground biomass, proportion of functional groups and Normalized Difference Vegetation Index (NDVI) were measured annually. After three years of treatment, the vegetation responded to the N addition with an increase in aboveground productivity and altered species composition, but without changes due to elevated O_3. N input > 10 kg N ha^{-1} y^{-1} was sufficient to affect the composition of functional groups, with sedges benefiting over-proportionally. No interaction of O_3 x N was observed, except for NDVI; positive effects of N addition on canopy greenness were counteracted by accelerated leaf senescence in the highest O_3 treatment. The results suggest that effects of elevated O_3 on the productivity and floristic composition of subalpine grassland may develop slowly, regardless of the sensitive response to increasing N.

1 Introduction

During the last century, concentrations in the troposphere of ozone (O_3) and reactive nitrogen compounds (N) have risen substantially on a global scale due to increasing anthropogenic emissions (Matson *et al.*, 2002; Ashmore, 2005). Today many regions are exposed to O_3 concentrations and N deposition rates that exceed several times pre-industrial levels (Volz & Kley, 1988; Preunkert *et al.*, 2003). In Switzerland, current annual rates of N deposition in remote mountain areas are around 5 kg N ha^{-1} y^{-1}, but reach up to 60 kg N ha^{-1} y^{-1} in regions with intensive livestock farming (Rihm & Kurz, 2001). Both N input and O_3 have been found separately to affect plant communities of natural and semi-natural habitats in ways that threaten the structure and function of these systems (Ashmore, 2005; Phoenix *et al.*, 2006). To protect sensitive communities from excess pollutant input, effect-based abatement strategies applying critical levels for O_3 and critical loads for N have been developed under the UNECE Convention on Long-range Transboundary Air Pollution (CLRTAP). However, due to the lack of suitable experimental data possible interactive effects of O_3 and N have not been considered.

As an essential nutrient, atmospheric N can alter ecosystem properties such as productivity, cycling of carbon (C) and N (Vitousek *et al.*, 1997) or biodiversity (Stevens *et al.*, 2004). In particular, in natural and semi-natural ecosystems species characteristic of nutrient-poor conditions may be displaced by a few fast growing and highly competitive species (Bobbink *et al.*, 1998). When due to increasing nutrient availability belowground competition for resources shifts to aboveground competition for light, species that effectively invest available N into high relative growth rates are favoured (Schieving & Poorter, 1999), as observed in several N addition experiments in dune vegetation (van den Berg *et al.*, 2005), and calcareous grasslands (Bobbink, 1991). Herbaceous systems in climatically extreme environments such as mountain regions may be most susceptible to even low rates of N addition, as suggested by Bowman & Steltzer (1998). These systems are currently marginally exposed to air pollutants, and in the absence of high levels of N input they are typically nutrient-poor, species-rich, and thus of high conservation value. In the alpine zones, N addition experiments concentrated on unmanaged alpine heaths (Soudzilovskaia & Onipchenko, 2005) and tundra (Theodose & Bowman, 1997; Bowman *et al.*, 2006), but little is known about effects of increasing N inputs on subalpine grasslands which play an important role for both conservation and agriculture.

O_3 is the major phytotoxic air pollutant on regional scales. There is ample evidence that current ambient levels are sufficiently high to cause widespread visible O_3 injury in plants in Europe and elsewhere (Benton *et al.*, 2000). Elevated O_3 impairs photosynthesis, leaf life span, reproduction, and growth (Ojanperä *et al.*, 1998). In species-poor productive grass/legume swards, experimental exposure to elevated O_3 consistently caused an increase in the dominance of grasses at the expense of legumes (cf. Fuhrer *et al.*, 1994). In contrast, in natural multi-species communities, few available studies revealed less consistent responses with either declining (Volk *et al.*, 2006) or rising proportions of legumes (Evans & Ashmore, 1992), and unaffected (Barbo *et al.*, 1998) or reduced total yield (Evans & Ashmore, 1992; Volk *et al.*, 2006). These conflicting results suggest that in complex mature communities multiple plant-plant and plant-environment interactions might strongly modulate the effect of O_3.

In grasslands, large variability in responsiveness to O_3 stress exists among and within species (Bungener *et al.*, 1999; Bassin *et al.*, 2004b). Recently, the responses to O_3 of different species and community types have been compared (Bassin *et al.*, 2006; Jones *et al.*, 2006; Mills *et al.*, 2006). Bassin *et al.* (2006) discussed the importance of physiological and ecological traits in determining O_3 sensitivity. They suggested that a high relative growth rate with related leaf traits such as high stomatal conductance and high specific leaf area (SLA) promote the specific sensitivity to O_3. Accordingly, low productivity communities such as alpine and subalpine grasslands could be considered less responsive to O_3 due to their high proportion of slow-growing, stress tolerant species. In contrast, Mills *et al.* (2006) classified this type of grasslands among the most sensitive vegetation types based on individual species responses to O_3 observed under controlled conditions.

Theoretically, an increase in N availability and in O_3 exposure could have opposing effects. Due to phenotypic plasticity, plants might become more susceptible to O_3 when growth rates are stimulated by extra available N, or alternatively enhanced detoxification capacity from N-stimulated photosynthesis could reduce O_3 sensitivity. For instance, high nutrient supply protected *Plantago major* against O_3 damage (Whitfield *et al.*, 1998) and *Trifolium repens* from accelerated leaf senescence (Sanz *et al.*, 2005). However, data from in situ experiments with intact communities including multiple treatment levels and factor combinations to test the interaction between N and O_3 are lacking.

This study was designed to investigate the long-term impact of elevated O_3 and N input, and of their combination, in extensively managed grassland in the Swiss Alps. The aim was to determine i) total aboveground productivity, ii) productivity and proportion of functional groups (grasses, forbs, sedges, and legumes), iii) individual species' abundance and diversity, and iv) the development of the vegetation greenness as an indicator of leaf senescence, in relation to individual and combined levels of O_3 and N deposition. Here we report results from the initial three experimental years.

2 Materials and Methods

2.1 Study area

The experimental site is located at Alp Flix, Sur, Switzerland (2000 m a.s.l., 9° 39' N / 46° 32' E), a high plateau near Julier Pass in the Central Alps. The plateau, located about 100 m below the climatic tree line, is dominated by a raised bog surrounded by extensively managed pastures and hay meadows. Climatic conditions are characterized by cold winters with permanent snow cover and a growing season from April to October. Precipitation peaks in summer (long-term average 120-140 mm monthly from May to September) with an annual sum of 1200 mm. Long-term mean annual temperature is 2.8°C, and monthly average temperatures are highest in July and August (9-10°C).

2.2 Vegetation and soil

The vegetation is a *Geo-Montani-Nardetum,* which is a typical pasture community covering large areas in the subalpine zone of the European Alps and Pyrenees (EUNIS classification 35.1, http://eunis.eea.europa.eu/). The most frequent species are *Festuca violacea*, *Nardus stricta* and *Carex sempervirens,* which together account for approximately one half of the cover. The remaining half is composed of more than 70 forb and a few legume species. The low-intensity management, as a cattle pasture with ca. 1.3 livestock units ha^{-1} grazing during 3-4 weeks per year, was replaced in the present study by a cutting regime. It is uncertain to which extent above-ground productivity and species composition might be affected in the long term due to the release from grazing selection and due to potential modifications of the nutrient turnover.

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

2.3 Experimental design, fumigation and N treatment

A free air fumigation system was installed in spring 2004 consisting of nine rings of 7 m diameter (for details of the system and of its performance see Volk *et al.* (2003)). Rings were arranged in three linear blocks on a small ridge of 150 m length. The ridge is east-westerly orientated and orthogonal to the two main wind directions. Three fumigation treatments (ambient [control], 1.2 x ambient [O_3+], 1.6 x O_3 ambient concentration [O_3++]) were randomly assigned to the three rings in each block. With O_3 concentrations at these altitudes being mainly controlled by the global background concentration (Staehelin *et al.*, 1994), we aimed at increasing O_3 concentration during the day as well as during night. Exposure to elevated O_3 levels lasted from 16 June to 25 October in 2004, from 15 April to 24 October in 2005, and from 21 April to 23 October 2006. In 2004, late snow-melt delayed the installation of the fumigation system. Meteorological conditions allowed O_3 release in 74, 62 and 78% of the time during the fumigation periods in the years 2004, 2005, and 2006, respectively. O_3 concentrations were measured at canopy height in control and fumigation treatments and at reference height (2 m) in ambient air.

Experimental plots consisted of monoliths (L x W x D = 40 x 30 x 20 cm). These were excavated in fall 2003 from a nearby pasture, placed in drained plastic boxes and randomly assigned to the rings. Boxes were placed in shallow pits flush with the surrounding surface. They were rearranged every year between the rings within each O_3 treatment to minimize confounding effects of microclimatic differences between rings.

Five levels of N application were established to simulate atmospheric N deposition during the growing season. Extra N input in these treatments was equivalent to 0, 5, 10, 25, 50 kg N ha^{-1} y^{-1}. These annual N amounts were separated into bi-weekly additions of 200 ml of a solution of ammonium nitrate in well water. Each N level was replicated four times in each fumigation ring. The amount of added water was equivalent to only 2% of the annual rainfall amount, and N addition from well water was less than 0.05 kg N ha^{-1} y^{-1}. Considering the annual background deposition measured at the study site (about 4 kg N ha^{-1} y^{-1}, see Results), total N loads in the different treatments were 4, 9, 14, 29, 54 kg N ha^{-1} y^{-1}. In the following, treatments are referred to as N4, N9, N14, N29, N54, respectively.

2.4 Micrometeorological measurements

Meteorological parameters were recorded at 2 m reference height outside the fumigation rings: global radiation [W m^{-2}], wind speed [m s^{-1}], air temperature [°C], relative humidity [%], and precipitation [mm h^{-1}]. For details concerning measurement equipment see Volk (2003).

2.5 Background N deposition measurements

Background N deposition was calculated from concentrations measured in air, rainwater, and snow. During the growing season, bi-weekly average ammonia (NH_3) and nitrogen dioxide (NO_2) concentrations in air were determined by means of passive samplers (NH_3, Radiello ® Fondazione Salvatore Maugeri, Pavia, Italy; NO_2, constructed after Palmes (1976)). Fluxes of NH_3-N and NO_3-N were estimated as a function of the serial resistances R_a (aerodynamic resistance), R_b (boundary layer resistance), R_s (stomatal resistance), and in the case of NH_3, R_w (leaf surface resistance), following the parameterization proposed by Bassin *et al.* (2004a) and Smith *et al.* (2000). Total gaseous N deposition per growing season (1 May – 31 Oct) was calculated as the sum of hourly fluxes. Monthly rates of wet deposition of ammonium (NH_4^+) and nitrate (NO_3^-) were obtained from concentrations measured in rainwater collected in a wet-only sampler (Aerochem Metrics Inc., Florida, USA). During winter, NH_4^+ and NO_3^- loads of the snowpack were measured shortly before snow-melt, assuming that at this time the snow pack contained the accumulated wet and dry deposition.

Chemical analysis of NO_2 in passive samplers (as nitrite), and NH_4^+ in rainwater and melted snow were measured by spectrophotometry (Lambda 25, Perkin Elmer). NH_4^+ from NH_3 in passive samplers and NO_3^- in water were analysed by ionchromatography (DX1-03, DIONEX). In 2006, the methods for the determination of NH_4^+ were replaced by flow injection analysis (Fiastar, FOSS).

2.6 Vegetation measurements

Plants were cut at 2 cm above the surface once each year in August. Plant material was stored at 4°C until separation into functional groups (grasses, forbs, sedges, and legumes). Samples were dried at 60 °C to constant weight.

The frequency of individual plant species was recorded each year during the first two weeks of July using the point-quadrat (PQ) method (Stampfli, 1991) with 18 sampling points within a 6 cm grid. Based on these values, canopy density (= total number of PQ-hits per monolith) as well as diversity index were derived (Shannon-Wiener index, H') according to

$$H' = \sum_{i=1}^{s} p_i \ln p_i$$

where *s* is the species number and p_i is the relative abundance of the ith species.

Vegetation development was monitored by measuring the Normalized Difference Vegetation Index (NDVI) at 2-week intervals during June and July. In sparse canopies, NDVI provides an integrative measure of leaf area index (LAI) and chlorophyll content of the vegetation (Gamon *et al.*, 1995). A multi-spectral radiometer sensor (MSRSYS16R system, Cropscan Inc., Rochester, USA) to measure reflectance at 810 and 680 nm was placed 90 cm above the centre of a monolith. During the measurement the surrounding monoliths were covered. Measurements were taken only on cloudless days during hours with a sun angle >60° (from 10 a.m. to 5 p.m.). NDVI was calculated following Tucker (1979):

$$\mathrm{NDVI} = \frac{\mathrm{NIR} - \mathrm{RED}}{\mathrm{NIR} + \mathrm{RED}}$$

where RED and NIR are the measured spectral reflectance in the red and near-infrared regions, respectively.

2.7 Data analysis

Effects of treatments on aboveground dry matter weight, proportions of functional groups, species number per monolith, diversity index H', and NDVI were tested in a repeated measures split-plot analysis of variance (ANOVA) with O_3 at the main-plot level and N at the sub-plot level. All parameters except NDVI were repeated by year; NDVI was analysed separately for the two years 2005 and 2006 and repeated by day of the year. Block, O_3, and year entered the model as class variables, while N and day (NDVI) were defined as continuous variables. Inclusion of higher order interactions and quadratic effects into the model were assessed with the AICc criterion (Burnham & Anderson, 2002) with one exception: the interaction of N x N was always included since it refers to the curvature of N as a design variable. To evaluate differences between dry matter weight of functional groups at different N levels, values were grouped by N level separately for 2005 and 2006, and a one-way ANOVA followed by a Tukey post-hoc test was performed. Treatment-induced changes in the abundance of the 11 most frequent species were analysed qualitatively. All analyses were carried out with the statistical software SAS (SAS Institute Inc., NC, USA), applying the procedures “proc mixed” and “proc anova”. To meet the assumptions of ANOVA (normal distribution and homogeneity of residual variance), data were transformed when necessary. To appropriately account for the multidimensional data structure, estimated means and standard errors (SE) resulting from back-transformation are displayed in figures and text.

3 Results

3.1 Climatic conditions, O_3 exposure, background N deposition

Weather conditions varied considerably over the three years (Tab. 1) with the summer in 2004 being the wettest and in 2006 being the warmest and driest. Overall, the region experienced warmer and drier years in 2004-2006 compared to the long-term annual mean (data from Swiss Meteorological Service, http://www.meteosuisse.ch/web/en/weather.html).

Ambient O_3 concentrations (24-h means) during the fumigation period were about 46 ppb (Tab. 1) showing only a small diurnal variation. Accumulated exposures above a threshold of 40 ppb (AOT40) averaged 12 ppm.h in the control plots, 24 ppm.h in the O_3+ treatment and 48 ppm.h in the O_3++ treatment. Year-to-year variations in AOT40 in the elevated O_3 treatments were associated with variations in meteorological conditions and variable length of fumigation duration.

Estimated total annual N deposition was between 3.2 and 3.8 kg N ha^{-1} y^{-1}, with 1.33 to 2.7 kg N ha^{-1} y^{-1} deposited with rain and about 0.5 kg N ha^{-1} y^{-1} as dry deposition (Tab. 1). Winter N input to the vegetation was low (0.14 – 0.58 kg N ha^{-1} y^{-1}) due to small amount of snow accumulation.

Table 1. Mean temperatures [°C], sum of precipitation [mm], wind velocity [m s^{-1}] and AOT40 [ppm.h], mean O_3 concentrations [ppb], and mean daily O_3 concentration maxima [ppb] measured in the O_3 fumigation experiment at Alp Flix during the growing seasons (April-October) 2004-2006 and contribution of reduced (NH_3, NH_4^+) and oxidised (NO_2, NO_3^-) N to the annual total N deposition, separated by deposition form (winter: snow-pack, summer: wet and dry deposition).

	2004			**2005**			**2006**		
	Temperature [°C]	Precipitation [mm]	Wind speed [m s^{-1}]	Temperature [°C]	Precipitation [mm]	Wind speed [m s^{-1}]	Temperature [°C]	Precipitation [mm]	Wind speed [m s^{-1}]
	5.6	722.6	3.3	7.2	488.7	3.2	7.0	368.7	3.6
	AOT40 [ppm.h]	Mean conc. [ppb]	Daily max [ppb]	AOT40 [ppm.h]	Mean conc. [ppb]	Daily max [ppb]	AOT40 [ppm.h]	Mean conc. [ppb]	Daily max [ppb]
Ambient	13.8*	46.9*	55.9*	15.4	45.7	52.8	14.9	44.9	53.1
Control	12.6**	46.0**	54.7**	11.1	44.0	50.8	11.6	42.1	50.3
O_3+	15.2	51.2	61.0	27.4	51.6	64.5	29.5	54.9	70.0
O_3++	28.4	64.2	81.5	49.9	65.0	84.7	64.9	74.2	101.7
	Snow pack [kg N ha^{-1} $wi.^{-1}$]	Wet deposition [kg N ha^{-1} $su.^{-1}$]	Dry deposition [kg N ha^{-1} $su.^{-1}$]	Snow pack [kg N ha^{-1} $wi.^{-1}$]	Wet deposition [kg N ha^{-1} $su.^{-1}$]	Dry deposition [kg N ha^{-1} $su.^{-1}$]	Snow pack [kg N ha^{-1} $wi.^{-1}$]	Wet deposition [kg N ha^{-1} $su.^{-1}$]	Dry deposition [kg N ha^{-1} $su.^{-1}$]
NH_4^+-N	n.a.	0.75	0.53	0.23	1.67	0.45	0.05	1.53	0.64
NO_3^--N	n.a.	0.58	0.05	0.35	1.05	0.06	0.09	0.82	0.06

* reconstructed for the entire growth period with values from the O_3-monitoring station at Davos (1590 m a.s.l).

** reconstructed for the period of 16 August to 20 October with values from the O_3-monitoring station at Davos (1590 m a.s.l.).

n.a. = not available

wi. = winter

su. = summer

Table 2. Effects of O_3, N, and year on total harvested aboveground biomass [g m^{-2}], the proportion of grasses [%], forbs [%], and sedges [%], and on the total number of point-quadrat hits (PQ-hits), species number, and Shannon diversity index H' in monoliths of a free-air fumigation experiment at Alp Flix in 2004-2006. Numerator (N df) and denumerator degrees of freedom (D df) and F-values are displayed. Terms with "-" were not fitted in the particular model. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	Total dry matter			Grasses proportion			Forbs proportion			Sedges proportion			No. of PQ hits			Species No.			Shannon Index H'		
	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
Block	2	4	5.83	2	4	1.30	2	4	4.41	2	4	0.85	2	4	4.60	2	4	6.62	2	4	0.25
O_3	2	4	0.56	2	4	0.52	2	4	0.67	2	4	1.25	2	4	1.17	2	4	0.73	2	4	0.92
N	1	169	28.99***	1	169	9.20**	1	169	1.11	1	169	19.50***	1	169	23.78***	1	169	3.12	1	169	0.28
N x N	1	169	1.46	1	169	0.29	1	169	0.84	1	169	0.26	1	169	1.37	1	169	0.09	1	169	0.10
Year	2	350	65.47***	2	356	62.05***	2	352	3.35*	2	352	190.96***	2	350	110.87***	2	350	13.22***	2	358	2.11
Year x Block	4	350	6.26***	-	-	-	4	352	4.33**	-	-	-	4	350	6.39***	4	350	5.15***	-	-	-
Year x O_3	4	350	3.44**	-	-	-	-	-	-	4	352	4.24**	4	350	6.22***	4	350	3.05*	-	-	-
Year x N	-	-	-	2	356	8.03***	2	352	12.19***	2	352	38.39***	-	-	-	-	-	-	-	-	-

3.2 Total aboveground biomass

Aboveground productivity generally did not respond to elevated O_3 during the three years (Fig. 1, Tab. 2). The O_3 effect pattern was not consistent over time, as indicated by the significant O_3 x year interaction: in year 2004, there was a reduction of total dry matter with O_3+ (Fig. 1). N addition persistently stimulated biomass, but no O_3 x N interaction was observed (Fig. 1a, Tab. 2). Growth stimulation in the N29 treatment relative to control plots was +24% ($P < 0.05$, Tukey test for multiple comparisons) in 2005 and +25% ($P < 0.05$) in 2006 (supplementary Fig. S1), while in the N54 treatment, the growth increase amounted to +34% in both years ($P < 0.05$ in both years). In the N9 treatment, biomass was also slightly, but non-significantly stimulated by 8 and 6% in the two years, respectively, and in the N14 treatment by 13 and 10%, respectively.

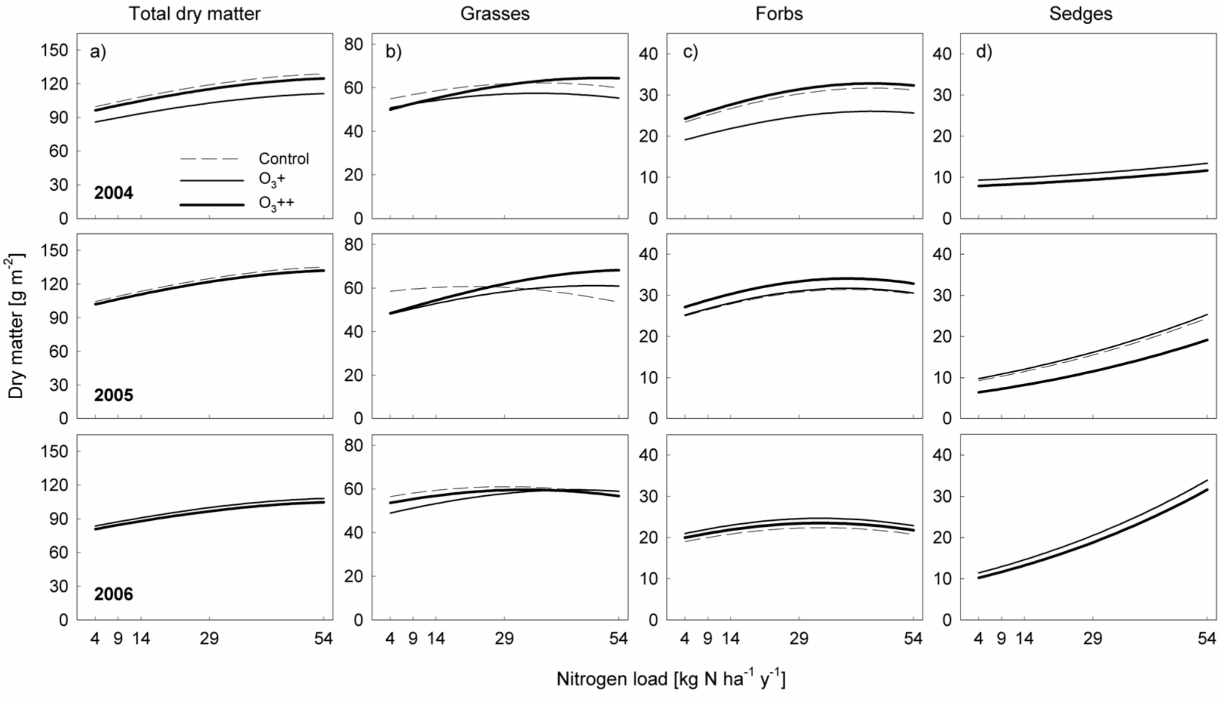

Fig. 1. Total aboveground total dry matter [g m-2] (a) and dry matter of the functional groups of grasses (b), forbs (c), and sedges (d) as a function of O3, N, and year. To allow a clear interpretation, only fitted lines based on the ANOVA model (supplementary Tab. S2) are displayed.

Harvested aboveground biomass differed significantly among the years (Tab. 2). In control plots, it dropped from about 100 g m^{-2} in 2004 and 2005 to 83 g m^{-2} in 2006 (Fig. 1a). So far it is unclear whether this drop was caused by the dry weather conditions in 2006 (see Tab. 1) or whether it is an early indication of long-term effects of the management change.

3.3 Dry matter and proportion of functional groups

N addition but not O_3 modified the functional group composition with no significant O_3 x N interaction. The group of sedges benefited most from the additional N input and the N-induced increase in biomass became more pronounced over the years (Fig. 1d). In 2006, even the N14 level (+55%) was significantly different from the N4 level ($P < 0.05$, supplementary Fig. S1b). Biomass of sedges was simulated by 124 and 257% in the N29 and N54 treatments, respectively ($P < 0.05$). In terms of proportions, the fraction of sedges increased from 11% in the control plots to 14% in the N54 treatment in 2004, and more than doubled from 14% (N4) to 31% (N54) in 2006 (Tab. 2: year x N interaction); thus sedges became the second most abundant functional group in this treatment (Fig. 2).

Grasses as the dominating functional group with an average dry matter yield of around 60 g m^{-2} did not benefit from the extra N input (supplementary Tab. S2). As a result, their fraction declined in the N54 treatment progressively from 2004 to 2006 (Fig. 2, Tab. 2: year x N interaction).

In contrast, forbs with approximately 30 g m^{-2} were significantly promoted by N addition, but the stimulating effect declined during the three years (Fig. 1c), and in 2005 and 2006 differences between N treatments disappeared (supplementary Fig. S1).The fraction of forbs declined from 27% (2005 and 2006) in the N4 treatment to 24% (2005) and to 22% (2006) in the N54 treatment (Fig. 2, Tab. 2: year x N interaction).

Dry matter of legumes ranged between 2 and 3 g m^{-2} and accounted for only 2 to 5% of the total biomass (Fig. 2). Statistical analyses were not feasible due to the heterogeneous distribution among plots, but N addition tended to slightly reduce the fraction of legumes, while neither O_3 nor the O_3 x N interaction caused a detectable response.

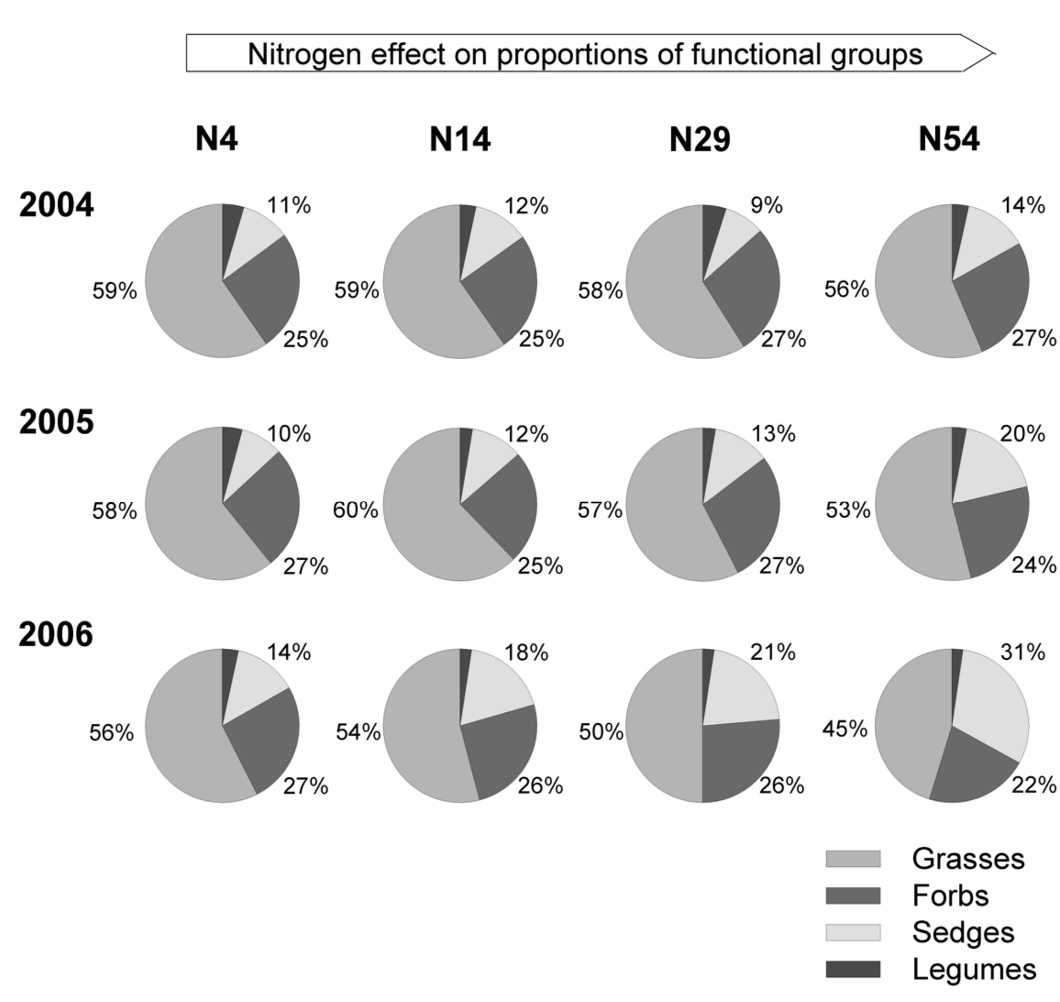

Figure. 2. Relative contribution [%] to the total harvested aboveground biomass of the four functional groups (grasses, forbs, sedges, and legumes) in relation to the N treatments and the three experimental years (2004-2006).

3.4 Species composition and frequency

The total number of vascular species was 91 (supplementary Tab. S3) with 10% grasses, 7% sedges, 4% legumes, and a majority (79%) of forbs. The dominating species with an average of more than five point-quadrat hits (PQ-hits) were the grasses *F. violacea* and *N. stricta,* and the sedge *C. sempervirens* (Tab. 3). The six most frequent forb species (*Ranunculus villarsii*, *Leontondon helveticus*, *Ligusticum mutellina*, *Potentilla aurea*, *Arnica montana* and *Gentiana acaulis*), and one legume species (*Trifolium alpinum*) averaged between 0.5 and 1.7 PQ-hits while the majority (80 species) touched the probe on the average less than 0.5 times per monolith (0.12 m^2). Neither the number of species per monolith nor the Shannon diversity index was influenced by O_3 or N treatments, but the total species number differed significantly among years (Tab. 2).

Table 3. The 11 most abundant species in the O_3 fumigation experiment at Alp Flix, their functional group, the three-year average frequency per monolith in the control plots [No. of point-quadrat hits ± SE], and the extent or direction of their response to the O_3 and N treatments, respectively.

	Functional group	Frequency [No. PQ hits]	Effect of O_3	Effect of N
Festuca violacea	Grasses	7.7 ± 1.1	no	no
Carex sempervirens	Segdes	5.9 ± 0.5	no	++
Nardus stricta	Grasses	5.0 ± 0.7	no	-
Ranunculus villarsii	Forbs	1.7 ± 0.3	no	no
Trifolium alpinum	Legumes	1.5 ± 0.2	no	-
Leontodon helveticus	Forbs	1.4 ± 0.1	no	no
Ligusticum mutellina	Forbs	1.4 ± 0.1	no	no
Potentilla aurea	Forbs	1.3 ± 0.3	no	+
Helictotrichon versicolor	Grasses	1.2 ± 0.3	no	no
Arnica montana	Forbs	0.8 ± 0.1	no	-
Gentiana acaulis	Forbs	0.6 ± 0.1	no	no

Corresponding to measured dry matter, N but not O_3 affected the abundance of the most frequent 11 species (Tab. 3). In some cases, individual species reflected the performance of the functional group to which they belong. For instance, *C. sempervirens* as the most abundant sedge showed an N response similar to the respective functional group. Among the grasses, only the fraction of *N. stricta* decreased in response to N addition (Tab. 3), while *F. violacea* and *Helictotrichon versicolor* remained unaffected. In the group of forbs, N affected *P. aurea* positively and *A. montana* negatively, while all other forbs did not respond to any of the treatments. The legume *T. alpinum* declined with increasing N addition.

3.5 NDVI

NDVI clearly reflected differences in phenological development. NDVI peaked on 10 July 2005 at values between 0.82 and 0.85 followed by a slight decline due to the senescing vegetation (Fig. 3a). In 2006, NDVI only reached values between 0.72 and 0.75 without a detectable maximum, thus indicating that the vegetation developed only marginally after the start of the measurements (Fig. 3b). This pattern corresponded to the comparatively low dry matter yield in 2006 (Fig. 1a). In both years, NDVI increased with higher N levels (Fig. 3, Tab. 4). In the O_3++ treatment, this effect was less pronounced (non-significant trend in 2005) or marginal (2006, Tab. 4: N x O_3 interaction). In both years, NDVI curves levelled off only in the O_3++ treatment, which resulted in a significant day x O_3 interaction (Tab. 4).

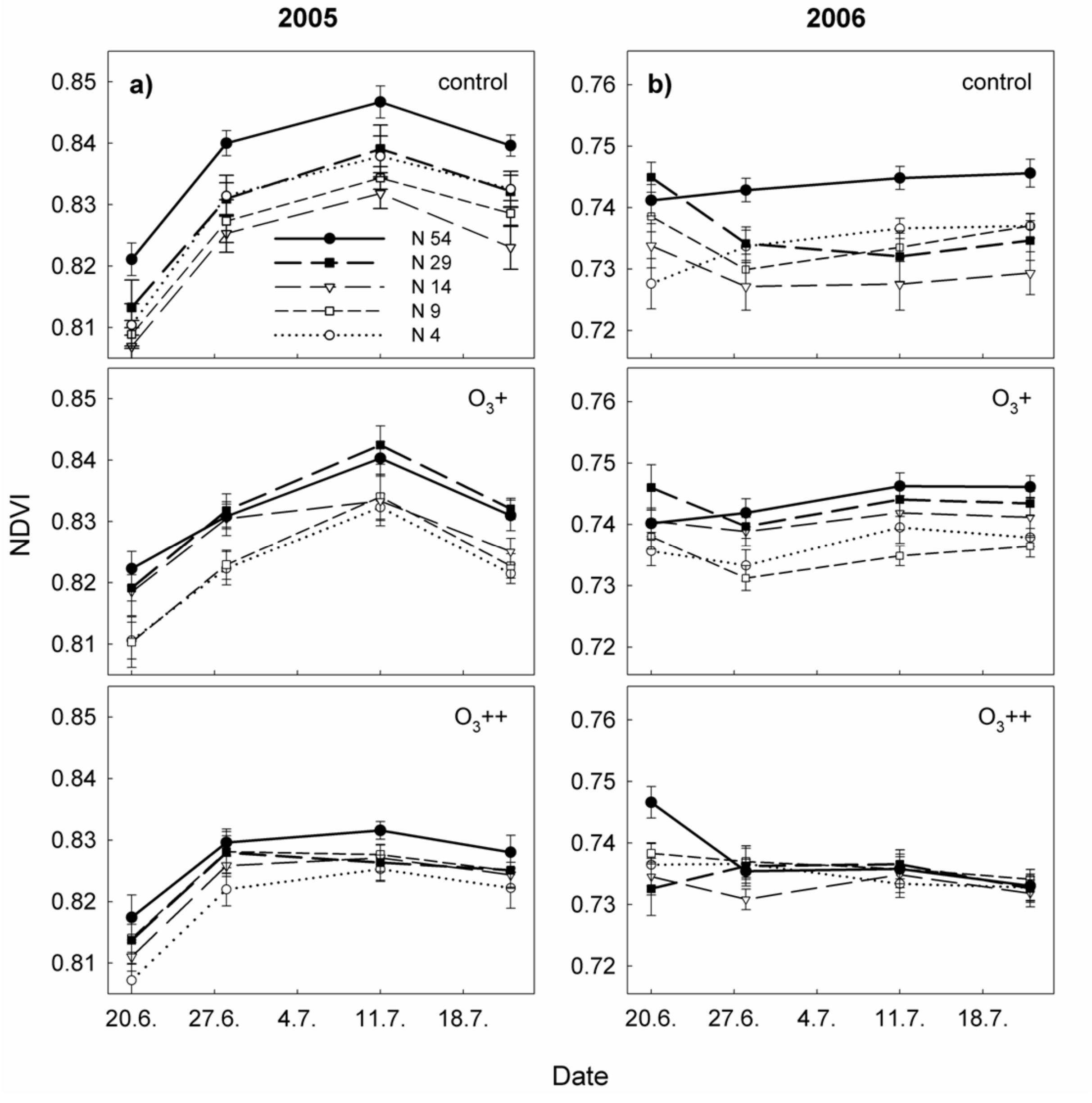

Fig. 3. Normalized Difference Vegetation Index (NDVI, means ± SE) during June and July of 2005 (a) and 2006 (b) in plots exposed to different levels and combinations of N and O_3.

Tab. 4. Effects of O_3, N, and day on Normalized Difference Vegetation Index (NDVI) in vegetation monoliths of a free-air fumigation experiment at Alp Flix in 2005 and 2006. Numerator (N df) and denumerator degrees of freedom (D df) and F-values are displayed. Terms with "-" were not fitted in the particular model. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	NDVI 2005			NDVI 2006		
	N df	D df	F	N df	D df	F
Block	2	4	0.42	2	4	4.19*
O_3	2	4	4.18	2	4	8.72**
N	1	170	31.24***	1	168	25.87***
N x O_3	-	-	-	2	168	3.46*
Day	1	551	382.97***	1	503	0.53
Day x Day	1	551	509.54***	-	-	-
Day x O_3	2	551	13.02***	2	503	6.25**
Day x Day x O_3	2	551	10.10***	-	-	-

4 Discussion

4.1 Elevated N deposition

The measured background N deposition of 3-4 kg N ha^{-1} y^{-1} corresponds to deposition rates measured at comparable alpine sites in the central Swiss Alps (Hiltbrunner *et al.*, 2005). Such alpine and sub-alpine ecosystems together with moorland and heathland habitats in northern Europe (Smith *et al.*, 2000; Fremstad *et al.*, 2005) represent relic sites with little influence from anthropogenic N input in the past. In most of the published studies on herbaceous plant communities, the simulated N deposition rates by far exceeded the range of anthropogenic deposition for the respective study site (Theodose & Bowman, 1997; Soudzilovskaia & Onipchenko, 2005). However, such efforts to shortcut long-term accumulation of low N deposition rates disregard the boosting effects of an abrupt rise in N availability on soil microorganisms and plants. Therefore, long-term experiments including high resolution treatments between 5 and 50 kg ha^{-1} y^{-1} in regions with low background N deposition are required to elucidate the effects of chronic low-level N deposition on nutrient poor vegetation (Achermann & Bobbink, 2003).

Increased N deposition caused quick and strong changes in dry matter production and species composition. Due to the lack of suitable experiments in the sub-alpine zone, these results can only be compared to those of studies in the alpine zone. However, alpine and subalpine grasslands have similar edaphic and climatic conditions and share various component species and characteristics, such as sparse canopies, prevailing slow-growing plants of low stature, or small and often needle-like leaves. In different studies, alpine vegetation did not respond in a consistent way to N addition, with stimulated productivity in some cases (Theodose & Bowman, 1997; Soudzilovskaia & Onipchenko, 2005) and no change in others (Bowman *et al.*, 2006). The discrepancies might be related to differences in N-binding capacity of alpine soils (Körner, 2003), or to a co-limitation by other macro-nutrients such as phosphorus (P) (e.g., in Theodose & Bowman (1997) and Soudzilovskaia & Onipchenko (2005)).

The N-induced change in vegetation composition was determined largely by an increase in sedges. Neither productivity of the other functional groups, nor species number per monolith, nor evenness were negatively affected by the success of sedges, in contrast to decreasing species richness generally found in low-land communities after N addition (Bobbink *et al.*, 1998). A similar increase in the cover of a few responsive species without a concomitant decrease in productivity of other species was observed in alpine plant communities (Soudzilovskaia & Onipchenko, 2005; Bowman *et al.*, 2006). It was suggested that limited competition for light in sparse alpine canopies (Körner, 2003) allows unresponsive species to maintain their productivity.

Alpine studies consistently found sedges to benefit more from experimental N addition than grasses and forbs (Theodose & Bowman, 1997; Soudzilovskaia & Onipchenko, 2005). This contradicts common theory (e.g. C-S-R (Grime *et al.* 1996)) that species with a competitive growth strategy should benefit most. But according to Aerts (1999), in nutrient limited habitats typical sedge traits such as low nutrient loss through low tissue concentration, slow turnover rates and high resorption efficiency (Shane *et al.*, 2006) are more important for competitive success than investing into high growth rates.

4.2 Elevated O_3

After three years of fumigation, elevated O_3 concentrations had no detectable effects on harvested above-ground dry matter and species composition of the subalpine grassland. The most likely explanation for the absence of a response to O_3 might be specific characteristics of this old, multi-species community. More pronounced O_3 effects were observed in past studies using individual plants, monocultures or binary mixtures of newly sown plants (e.g., Fuhrer *et* *al.*, 1994). Due to their high growth rates, high stomatal conductance, high specific leaf area, and in the absence of a large rooting system, plants in the establishing phase are assumed to be most sensitive to O_3, whereas established communities generally show weaker O_3 responses (Bassin *et al.*, 2006). Transient or absent O_3 effects on dry matter production were also observed in open-top chamber experiments with established vegetation (Evans & Ashmore, 1992; Thwaites *et al.*, 2006) and were explained by the large heterogeneity of the community composition due to edaphic and weather conditions. In a previous free-air fumigation experiment, productivity of old semi-natural grassland was reduced only after four and five years of exposure to elevated O_3 (Volk *et al.*, 2006). The old communities' ability to tolerate elevated O_3 concentrations has been related to functional redundancy, inter-specific interactions, a large rooting system, and high genetic diversity (Bassin *et al.*, 2006).

Alternatively, the absence of a strong O_3 effect might be due to the species composition of the monoliths. Low-productivity vegetation such as subalpine grassland is mainly composed of species with a stress tolerant growth strategy, which have been considered unresponsive to O_3 (Bassin *et al.*, 2006). Adaptation to tolerate low nutrient availability and climatic conditions at the respective sites might protect (sub-)alpine plant species from O_3 damage. In particular, the capacity to cope with O_3-induced oxidative stress could be pronounced in species from elevated sites due to their high levels of anti-oxidants as an adaptation to natural oxidative stress by UV-radiation (Wildi & Lutz, 1996) and historically higher average O_3 levels (Staehelin *et al.*, 1994). Moreover, the monoliths contained only small proportions of legumes as their abundance decreases with elevation, and their altitudinal distribution is limited to below 2600 m a.s.l. (Jacot *et al.*, 2000). In a large number of studies, legumes consistently reacted highly sensitive to elevated O_3 concentrations (Hayes *et al.*, 2006) presumably due to their high relative growth rates and their specific chemical response to oxidative stress (Ranieri *et al.*, 2002). Accordingly, in grass/clover mixtures as well as in multi-species communities, observed changes in species composition resulted predominantly from a reduction in the legume fraction (Fuhrer *et al.*, 1994; Nussbaum *et al.*, 2000). In the present situation, an O_3-induced reduction in legumes would not lead to strong compositional changes due to their low abundance. Legume-poor communities could also be less sensitive to O_3 because they depend less on N supplied by N-fixation, which often impaired by O_3 stress (e.g., see Rämö *et al.*, 2006).

Lastly, the absence of O_3 effects could be related to the fumigation regime and/or the experimental design. The constant enrichment of background air during the entire growing season led to a seasonal O_3 exposure (AOT40) that was substantially higher than in previous studies (for comparison see (Rämö *et al.*, 2006; Thwaites *et al.*, 2006)). Mean O_3 concentrations in ambient air persistently exceeded 40 ppb and AOT40 of control plots exceeded by far the critical level for protecting semi-natural vegetation of 5 ppm.h over a 6-month period (CLRTAP, 2004). However, peak O_3 concentrations above 120 ppb that have been found to affect plants most effectively (Nussbaum *et al.*, 1995) were absent. Finally, the low number of replication at main plot level (O_3) limits the power of the experiment to detect significant O_3 effects.

4.3 Interactive effects of elevated O_3 and N deposition

After three years, no interactive effect of O_3 and N deposition on productivity and species composition was detected, thus suggesting that possible modifications in leaf morphology and stomatal conductance associated with increased growth due to N addition did not affect the specific sensitivity to O_3. The alternative hypothesis that increased N availability would reduce O_3 sensitivity could not be tested because of the absence of an O_3 effect in the monoliths receiving no additional N.

An interactive effect of O_3 and N appeared for NDVI. N increased NDVI, but the effect was reduced in the O_3++. The N-induced increase in NDVI can be explained by a higher canopy density (PQ-hits) and by increased chlorophyll content, as shown in Bell *et al.* (2004). Since there is no O_3 x N interaction in any measure of productivity, we can assume that the observed O_3 and O_3 x N effects on NDVI was rather the result of declining chlorophyll contents. O_3-induced reductions in NDVI in crops have been related to visible and non-visible O_3 damage (Kraft *et al.*, 1996). The leaves of some of the species in the monoliths showed visible O_3 symptoms exclusively in the O_3++ treatment (e.g., *A. montana*) but more pronounced early leaf senescence was observed in the form of yellowing leaf tips (e.g., in *C. sempervirens, N. stricta*). Early leaf senescence is a common symptom of O_3 stress leading to reduced leaf longevity and C assimilation (Grandjean & Fuhrer, 1989). It could thus be expected that on longer time scales a continuous reduction of assimilation might affect the plants' C budget and, consequently, lead to long-term effects on productivity.

5 Conclusions

After three years of treatment under realistic environmental conditions it can be concluded that N inputs in the range of the current critical load for alpine- and subalpine grassland in Europe of 10-15 kg N ha^{-1} y^{-1} stimulate the productivity of a *Geo-Montani-Nardetum* pasture at 2000 m and alter community composition in favour of a few species, mainly sedges. When considering the possibility of N accumulation in the system, it seems likely that under conditions of low N export and non-limiting P availability in soils even lower levels of N deposition could induce vegetation changes in the long-term. Such long-term effects merit further studies.

Productivity and community composition do not respond to three years of increased O_3 exposure above current ambient levels. The lack of an O_3 response in spite of high cumulative exposures (AOT40) suggests considerable O_3 tolerance of the system. Inherent community characteristics or the absence of effective peak concentrations could be reasons for the absence of a detectable biomass response.

Elevated O_3 reduced the positive effects of N on greenness of the canopy (NDVI) through accelerated leaf senescence. This effect on leaf life span may be manifested as growth reductions in the longer term due to continuous reductions in C assimilation.

Prolonged treatment of this subalpine grassland will be necessary before drawing firm conclusions related to possible interactive effects of increased N input and elevated O_3.

Acknowledgements

This work was supported by the Swiss Federal Office of Environment in the framework of the International Cooperative Programme ‘‘Vegetation’’ (ICP Vegetation) of the UNECE Convention on Long-Range Transboundary Air Pollution. The help of V. Spinas, M. Montani, R. Giger, F. Contat and the ART field team in setting up the experiment and performing the fieldwork and vegetation analysis is greatly acknowledged. All chemical analyses were performed at the FUB-Research Group for Environmental Monitoring (Rapperswil, Switzerland).

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Supplementary material

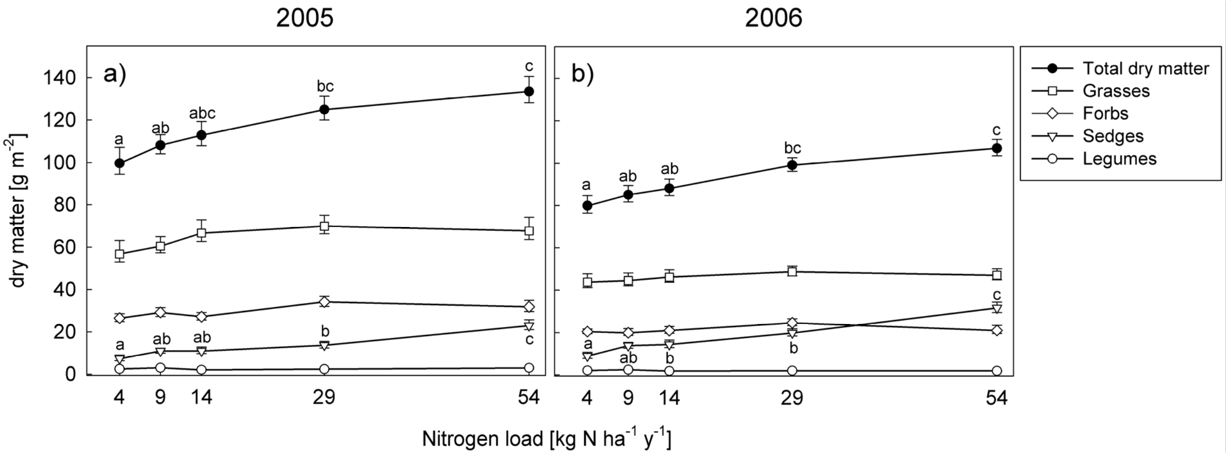

Figure S1. Means (± SE) of total aboveground dry matter [g m^{-2}] and dry matter of the functional groups (grasses, forbs, and sedges) in the years 2005 (a) and 2006 (b). Back transformed values from ANOVA are shown. Letters indicate significant differences at $P < 0.05$ (Tukey test for multiple comparisons) among the N treatments within each functional group.

Table S2. Effects of O_3, N, and year on harvested aboveground biomass [g m^{-2}] of grasses, forbs, and sedges in monoliths of a free-air fumigation experiment at Alp Flix in 2004-2006. Numerator (N df) and denumerator degrees of freedom (D df) and F-values are displayed. Terms with "-" were not fitted in the particular model. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	Grasses dry matter			Forbs dry matter			Sedges dry matter		
	N df	D df	F	N df	D df	F	N df	D df	F
Block	2	4	2.58	2	4	6.63	2	4	1.91
O_3	2	4	0.78	2	4	0.89	2	4	1.18
N	1	167	2.83	1	169	5.77*	1	169	39.9***
N x N	1	167	1.30	1	169	2.58	1	169	0.02
N x O_3	2	167	0.85	-	-	-	-	-	-
Year	2	344	112.28***	2	348	51.38***	2	352	101.04***
Year x Block	4	344	3.39**	4	348	9.32***	4	352	3.66**
Year x O_3	4	344	1.37	4	348	3.98**	-	-	-
Year x N	2	344	0.83	2	348	3.22*	2	352	40.46***
Year x Nx O_3	4	344	2.35	-	-	-	-	-	-

Table S3. List of species recorded by point-quadrat method in the *Geo-Montani-Nardetum* monoliths exposed in the fumigation experiment on Alp Flix, 2000 m a.s.l.

Species name
Achillea millefollium L.
Agrostis capillaris L.
Agrostis rupestris All.
Alchemilla fissa aggr.
Alchemilla glabra aggr.
Alchemilla hybrida L.
Alchemilla xanthochlora aggr.
Androsace chamaejasme Wulfen
Anemone narcissifolia L.
Antennaria dioica (L.) Gaertn.
Anthoxanthum alpinum Á. & D. Löve
Anthyllis vulneraria L.
Arnica montana L.
Aster bellidiastrum (L.) Scop.
Bartsia alpina L.
Biscutella laevigata L.
Briza media L.
Botrychium lunaria (L.) Sw.
Campanula barbata L.
Campanula scheuchzeri Vill.
Cerastium fontanum Baumg.
Carex sempervirens Vill.
Carex caryophyllea Latourr.
Carex ericetorum Pollich
Carex montana L.
Carex ornithopoda Willd.
Carlina acaulis L.
Centaurea nervosa Willd.
Cerastium alpinum L.
Coeloglossum viride (L.) Hartm.
Crepis aurea (L.) Cass.
Crepis pontana (L.) Dalla Torre
Crocus albiflorus Kit.
Daphne striata Tratt.
Elyna myosuroides (Vill.) Fritsch
Euphrasia minima Schleich.
Lloydia serotina (L.) Rchb.
Festuca violacea Gaudin
Galium anisophyllon Vill.
Gentiana acaulis L.
Gentiana campestris L.
Gentiana verna L.
Geum montanum L.
Gymnadenia conopsea (L.) R. Br.
Helictotrichon versicolor (Vill.) Pilg.
Helianthemum nummularium (L.) Mill.
Hieracium hoppeanum Schult.
Homogyne alpina (L.) Cass.
Hypochaeris uniflora Vill.
Leontodon helveticus Mérat
Leontodon hispidus L.
Leucanthemum vulgare Lam.
Ligusticum mutellina (L.) Crantz
Loiseleuria procumbens (L.) Desv.
Lotus alpinus (DC.) Rämönd
Luzula multiflora (Retz.) Lej.
Myosotis alpestris F. W. Schmidt
Nardus stricta L.
Nigritella nigra auct.
Pedicularis tuberosa L.
Phleum rhaeticum (Humphries) Rauschert
Phyteuma orbiculare L.
Plantago alpina L.
Plantago atrata Hoppe
Poa alpina L.
Polygonum viviparum L.
Potentilla aurea L.
Potentilla erecta (L.) Raeusch.
Primula farinosa L.
Primula integrifolia L.
Pulmonaria australis (Murr) W. Sauer
Pulsatilla alpina (L.) Delarbre subsp. *apiifolia* Nyman
Pulsatilla vernalis (L.) Mill.
Ranunculus villarsii DC.
Salix herbacea L.
Scabiosa columbaria L.
Selaginella spec.
Silene nutans L.
Soldanella alpina L.
Solidago virgaurea L. subsp. *minuta* (L.) Arcang.
Thesium alpinum L.
Thymus serpyllum aggr.
Tofieldia spec.
Trifolium alpinum L.
Trifolium pratense L. subsp. *nivale* (W. D. J. Koch) Arcang.
Trollius europaeus L.
Vaccinium myrtillus L.
Vaccinium uliginosum L.
Vaccinium vitis-idaea L.
Viola calcarata L.

Chapter 4

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

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Manuscript submitted to Oecologia

Tropospheric ozone (O_3) and deposition of reactive nitrogen (N) threaten the composition and function of natural and semi-natural vegetation even in remote regions. However, almost nothing is known about possible interactive effects of these pollutants on plant species and their communities. We analyzed plants of the 11 most frequent species of a subalpine *Geo-Montani-Nardetum* pasture exposed at 2000 m asl in the Swiss Alps during three years using a factorial free air exposure system to three concentrations of O_3 and five concentrations of N. Individual species performance was assessed on the basis of leaf chlorophyll and N concentrations, $\delta^{18}O$, $\delta^{13}C$, specific leaf area (SLA) and leaf weight. 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 exposure via increased growth and higher leaf conductance (g_s).

Most species reacted to increased N supply with strong N and chlorophyll accumulation, but no change in SLA, g_s, and growth, except *Carex sempervirens*. Elevated O_3 reduced g_s in most of the species. Leaf chlorophyll concentration was less affected. These effects were not related to O_3-induced leaf weight reduction, 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 initial SLA or g_s. No pronounced O_3 x N interactions were observed.

In conclusion: Since for most species neither N availability nor gas exchange limited growth, and thus their short-term response to O_3 and N was small, this subalpine could not be used as a model for testing interactive effects of O_3 and N. However, as a result of large interspecific differences in the responsiveness to these pollutants, community composition might change in the long-term.

1 Introduction

Concentrations of tropospheric ozone (O_3) and reactive nitrogen compounds (N) 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 considerable uncertainties exist with respect to the tolerance of plant species and plant 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 the regional scale in Europe (Ashmore, 2005). In sensitive species, chronic O_3 exposure has been shown to reduce CO_2 assimilation by negatively affecting photosynthesis rates (A) by enhancing chloroplast degradation (Grandjean & Fuhrer, 1989) and impairing stomatal functioning through effects on stomatal guard cells (Torsethaugen *et al.*, 1999). Possible consequences include accelerated leaf senescence and thus alterations of the plants' carbon (C) budget. On longer time scales, this can affect growth, productivity and seed output (Ojanperä *et al.*, 1998). In plant communities, effects on plant-plant interactions can lead to shifts in species composition (Volk *et al.*, 2006).

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, 1998). In turn, this improves instantaneous water use efficiency (WUE) when leaf conductance to water vapour (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 & Poorter, 1999).

Effects of O_3 and N might interact via plant functional traits. Species-specific O_3 sensitivity is assumed to be mainly determined 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 for O_3 (Ferdinand *et al.*, 2000). It is assumed that plant species characterized by high relative growth rates (RGR) and with related leaf traits such as high g_s and high SLA are 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 and N input have not been tested.

Using a free air fumigation system at 2000 m asl, 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 three 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 (LAI) and chlorophyll content, the change in normalized difference vegetation index (NDVI) suggested increasing leaf chlorophyll concentrations after N addition as well as the presence of increasing leaf necromass with elevated O_3 (Bassin *et al.*, 2007b). To evaluate possible subtle effects at the level of individual plant species, it is thus necessary to investigate shifts in physiological and morphological characteristics of selected key species and to test the relationship between leaf traits and the specific tolerance to O_3, N, and their combined deposition.

Because of its key role in O_3 uptake and O_3 tolerance, particular attention needs to be paid to leaf gas exchange, 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 using 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 crop and grassland species have been shown to be less negative with increasing O_3 (Saurer *et al.*, 1991; Jäggi *et al.*, 2005), but also with increased N availability, thus indicating improved WUE under both conditions (Saurer *et al.*, 1995). However, as both g_s and *A* modify c_i/c_a, the main driver of changes in $\delta^{13}C$ remained unknown. The oxygen (O) isotopic composition (the $^{18}O/^{16}O$ ratio expressed as $\delta^{18}O$) provides additional information as it shares the 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 diffusional effects during transpiration. Assuming uniform $\delta^{18}O$ of soil water, the degree of enrichment in leaf water depends on the ratio of the vapour pressure in the atmosphere (e_a) and the intercellular spaces within the leaf (e_i) (Barbour, 2007). Consequently, g_s and $\delta^{18}O$ are negatively correlated (Barbour *et al.*, 2000). Thus, it is possible to separate the effects of changes in g_s from those of changes in A on leaf gas exchange. The correlation of $\delta^{13}C$ and $\delta^{18}O$ values of leaf bulk material revealed decreasing g_s at constant A in grassland species and trees exposed to elevated O_3 concentrations (Jäggi & Fuhrer 2007; Grams *et al.,* 2007).

The aim for the current study was to test the following hypotheses: a) 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 it stimulates leaf growth, with the extent of the response being positively related to the species' initial SLA and g_s; b) 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; c) N-induced stimulation of growth and related changes in SLA and g_s increase the sensitivity to O_3.

2 Materials and Methods

2.1 *Study site*

The experimental site is located at Alp Flix, Sur, Switzerland (2000 m asl, N 9° 39' / E 46° 32'), on a high plateau near 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 6.6°C (for details see Bassin *et al.,* 2007b).

The vegetation type studied is a *Geo-Montani-Nardetum*, a typical alpine pasture community, covering large areas in the subalpine zone of the European Alps and Pyrenees. Most abundant species are the grasses *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 contribute approximately 2-4% to the total cover each. More than 70 forbs and a few grasses and legumes account for the remaining 40% (Bassin *et al.*, 2007b).

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 to a maximum of 20 cm.

2.2 Experimental design, O_3 and N treatment

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

A free air fumigation system (for details see Bassin *et al.*, 2007b and Volk *et al.*, 2003) supplied three O_3 treatment levels (ambient [control], 1.2 x ambient O_3 [O_3+] and 1.6 x ambient O_3 concentration [O_3++]), starting on 16 June, 2004. With O_3 concentrations at these altitudes showing only small diurnal fluctuations, we increased O_3 concentration during day and night between April and October. AOT40 values for the two seasons considered for this study amounted to 27.4 and 49.9 ppm h in 2005 in the O_3+ and O_3++ treatments respectively, but reached 29.5 and 64.9 ppm h in 2006 in the two treatments, respectively (Bassin *et al.*, 2007b). Five N treatments equivalent to 0, 5, 10, 25, 50 kg N ha^{-1} y^{-1} were applied during the growing season as twelve bi-weekly applications of a 200 ml solution of ammonium nitrate in well water starting in spring 2004. Each N level was replicated four times in each fumigation ring. Average combined annual background deposition (*c.* 4 kg N ha^{-1} y^{-1} Bassin *et al.*, 2007b) and N applications amounted to 4, 9, 14, 29, and 54 kg N ha^{-1} y^{-1}. The respective treatments are thus referred to as N4, N9, N14, N29, and N54.

2.3 Leaf sampling

Leaves of the 11 most frequent species, accounting together for *c.* 61% of the cover in the control plots (Table 1) were sampled during the last week of July in 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 (max. 30 min) between layers of moist tissue paper until leaf area measurements.

Table 1. 11 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. Mean relative cover in control plots [%] (from Bassin *et al.*, 2007b), and abbreviations used in the Figures are displayed.

Scientific name	**Functional group**	**Cover [%]**	**Abbreviation**
Festuca violacea Gaudin	grasses	16.3	*Fesvio*
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*

2.4 Leaf area measurements

Leaf number and leaf area were determined by using flatbed scanners (EPSON perfection 4990, EPSON, Switzerland) and image processing software (Winfolia® and Winseedle®, Régent Instruments Inc., Quebec, Canada). Sub-samples were dried to constant weight at 60°C and weighed. SLA was obtained by dividing mean leaf area by mean leaf weight for each sub-sample (except for *F. violacea* owing to technical reasons).

2.5 Stable isotope analysis

Dried leaves were ground to a fine powder with a ball mill (Mixer Mill, Retsch MM301, 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 6-port valve and a ConFlo III (Werner *et al.*, 1999). Bulk leaf $\delta^{18}O$ was determined using a high temperature oxygen analyser (Hekatech GmbH, Wegberg, Germany) coupled to a Finnigan MAT Delta^{plus}XP isotope ratio mass spectrometer via a 4-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 %N measurements, 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 V-PDB for C and V-SMOW for oxygen isotope ratios. 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 solution extracted at 5-10 cm depth was determined at three occasions. Mean value was -10.5‰ (± 0.41, SE). Soil water $\delta^{18}O$ signature was assumed to be uniform in the rooting zone since root extension was limited by the depth of the boxes.

2.6 Chlorophyll concentration and chlorophyll content

Frozen fresh leaves (-80°C) were lyophilized during 96 h and ground to a fine powder. Chl was extracted by incubating 10-15 leaves during 3 h at 60°C in $CaCO_3$-saturated DMSO (Fluka, Buchs, Switzerland). After centrifugation, absorption of the diluted supernatant was determined at 664.9 und 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. Total chlorophyll (a + b) concentration, Chl_{conc} [mg g^{-1}], was calculated as described by Barnes (1992). Chl content, Chl_{leaf} [mg $leaf^{-1}$], was calculated from Chl_{conc} and leaf weight.

2.7 Statistical analyses

Treatment effects on leaf weight, SLA, Chl_{conc} and Chl_{leaf}, %N, $\delta^{13}C$ and $\delta^{18}O$ ratios of bulk leaf material were tested in a split-plot analysis of variance (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 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 into the model were assessed with the AICc criterion (Burnham & Anderson, 2002). For the parameters measured only at three N levels (%N, $\delta^{13}C$, and $\delta^{18}O$) N entered the model as class variable, and the O_3 x N interaction was always included. Correlations between parameters were calculated using Pearson's correlation coefficient. All analyses were carried with the statistical software SAS (SAS Institute Inc., NC, USA), 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.

3 Results

3.1 SLA and %N

In the control treatment (N4), SLA ranged from 67 to 208 $cm^2\ g^{-1}$ in 2005 and from 70 to 207 $cm^2\ g^{-1}$ in 2006. It was positively correlated to %N, which ranged 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 lowest SLA and %N were the grasses *N. stricta* and *F. violacea* (%N: <1.8%, SLA: < 84 $cm^2\ g^{-1}$), while the legume *T. alpinum* as well as the forbs *R. villarsii*, *P. aurea* and *L. helveticus* (%N: >2.2%, SLA: > 150 $cm^2\ g^{-1}$) showed the highest values in both parameters.

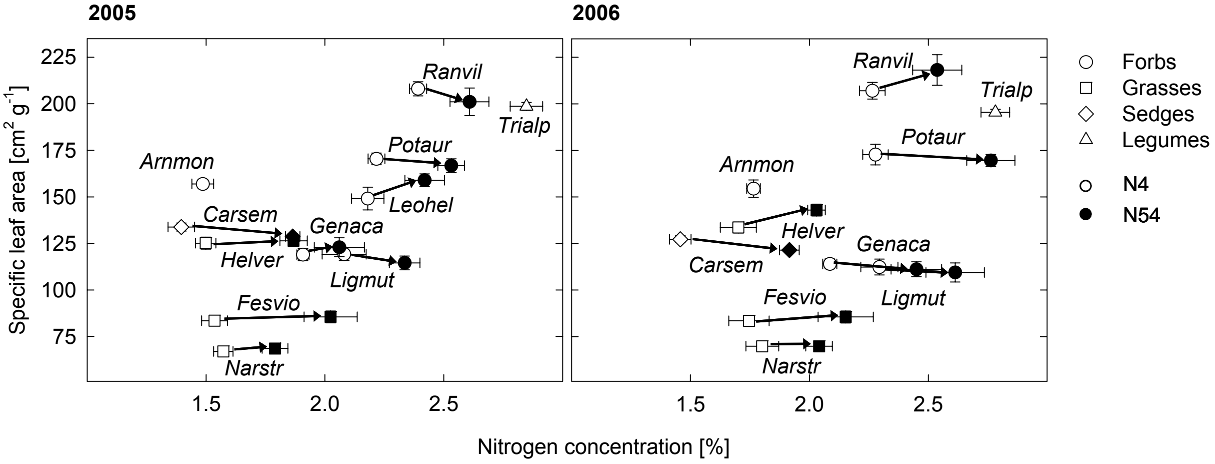

Fig. 1. Effect of nitrogen (N) treatment on leaf N concentration [%] and specific leaf area [$cm^2\ g^{-1}$] of 11 key plant species of a subalpine pasture in 2005 and 2006. Means ± SE are shown (only significant responses and changes which could not be tested due to inhomogeneous distribution are displayed, according to ANOVA Tables S8 and S9 in Supplementary material).

In agreement with our first hypothesis, e.g., suggesting stimulating effects of N on plant performance, addition of N in the N54 treatment significantly increased %N in all species, except *A. montana* and *T. alpinum* (Table S1, Fig. 1). The strongest relative N increase was seen in species characterized by low initial SLA and %N, i.e., *C. sempervirens* (+34 in 2005, +31% in 2006, Table S1), *F. violacea* (+32% in 2005 and +23% in 2006), and *H. versicolor* (+25 in 2005 and +19% in 2006). Effects of N on %N 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. On the other hand, elevated O_3 had only sporadically significant and inconsistent effects on either SLA or %N for the 11 key species investigated (Table S1, S2).

3.2 Chl concentration, Chl content 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 & Morrow, 1984) (Fig. 2). As expected, after three years N addition (N54) significantly increased Chl_{conc} in *C. sempervirens* by 45-54% relative to N4, by 20-30% in *H. versicolor*, *L. helveticus*, *R. villarsii*, *F. violacea*, and *L. mutellina*, and by 10-20% in *P. aurea*, *G. acaulis* and *N. stricta* (Table S4). In agreement with our second hypothesis, assuming negative effects of O_3 on plant performance, O_3 generally decreased $Chl_{conc,}$ although not consistently for all plant species, compared to the ambient controls (Fig. 2 c & d), with significant Chl_{conc} reductions of -7% (O_3+) and -19% (O_3++) for *R. villarsii* in 2005 (Table S4), and of -3% (O_3+) and of -20% (O_3++) in 2006 for *A. montana*. In contrast, Chl_{conc} for *C. sempervirens* increased by +8% (O_3+) and by +14% (O_3++) in 2006. Furthermore the effect of O_3 became more pronounced over the years (Tab S4).

Contrary to our expectations, leaf weight remained unaffected by N addition, as indicated by the positive shifts along leaf weight isolines (Fig. 2 a & b). Only in two species, leaf weight significantly increased in the N54 treatment in both years: 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). According to our hypothesis, O_3 tended to decrease leaf weight of six species in 2005 and of five species in 2006 (Table S5), but this effect was significant only for *L. helveticus* (O_3+: +9% (2005), +11% (2006), O_3++: -20% (2005), -7% (2006)) and for *P. aurea* (O_3+: -3%, O_3++: -25% (2006)).

In contrast to our assumptions, less than three of the 11 key species showed significant O_3 x N interactive effects (Chl_{conc} in 2005: 1, Chl_{conc} in 2006: 3, leaf weight 2006: 2) (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).

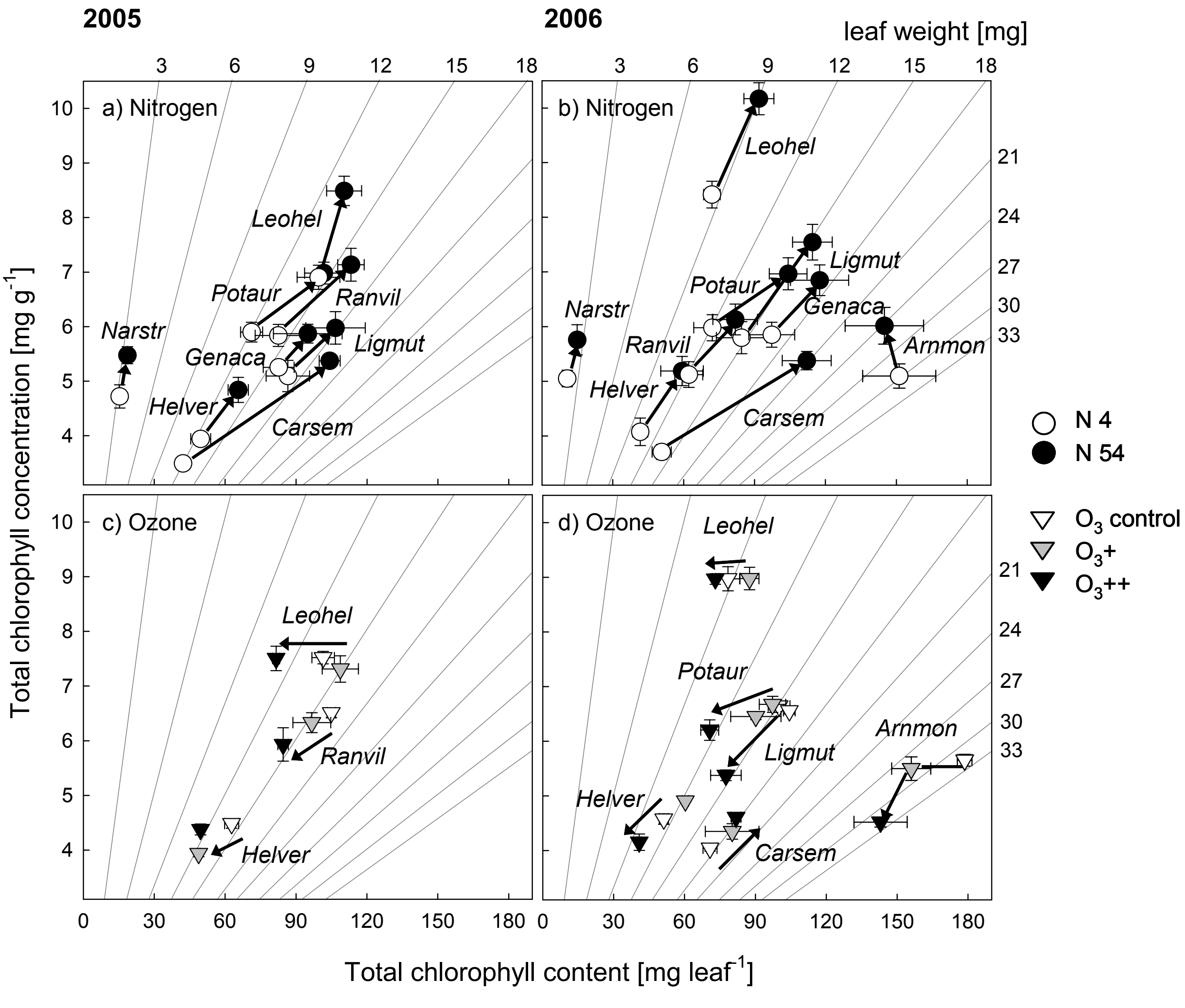

Fig. 2. Effect of N (a, b) and O_3 (c, d) treatment on leaf weight [mg], total Chl content [mg leaf^{1}], and total Chl concentration [mg g^{-1}] of 11 key plant species of a subalpine pasture in 2005 and 2006 (only significant responses are displayed, according to ANOVA Tables S10, S11 and S12 in Supplementary material). Means ± SE are shown. Isolines represent constant leaf weight [mg leaf^{1}].

3.3 $\delta^{18}O$ and $\delta^{13}C$

Contrary to our expectation, N addition affected bulk leaf isotopic signature only in a few species. However, leaves of *C. sempervirens*, i.e., the species with the strongest N-induced increase in leaf weight (see above), were significantly enriched in ^{13}C in the N54 treatment by +0.8‰ (2005) and +0.6‰ (2006), relative to the N4 treatment (Fig. 3 a & b, Table S6). $\delta^{13}C$ in leaves of *A. montana* shifted by +0.6‰ (2005) and +0.3‰ (2006) only in the N14 treatment. In contrast, $\delta^{13}C$ values of *L. helveticus* were significantly more negative (-0.3‰) in the N54 treatment in 2005. In contrast to our hypothesis, O_3 affected bulk leaf isotopic signature only in 2 species (Fig. 3 c & d, Table S6): In 2006, foliar $\delta^{13}C$ of *L. mutellina* and *P. aurea* shifted towards less negative values (+0.6 and +0.5‰, respectively) in the O_3++ treatment compared to the control.

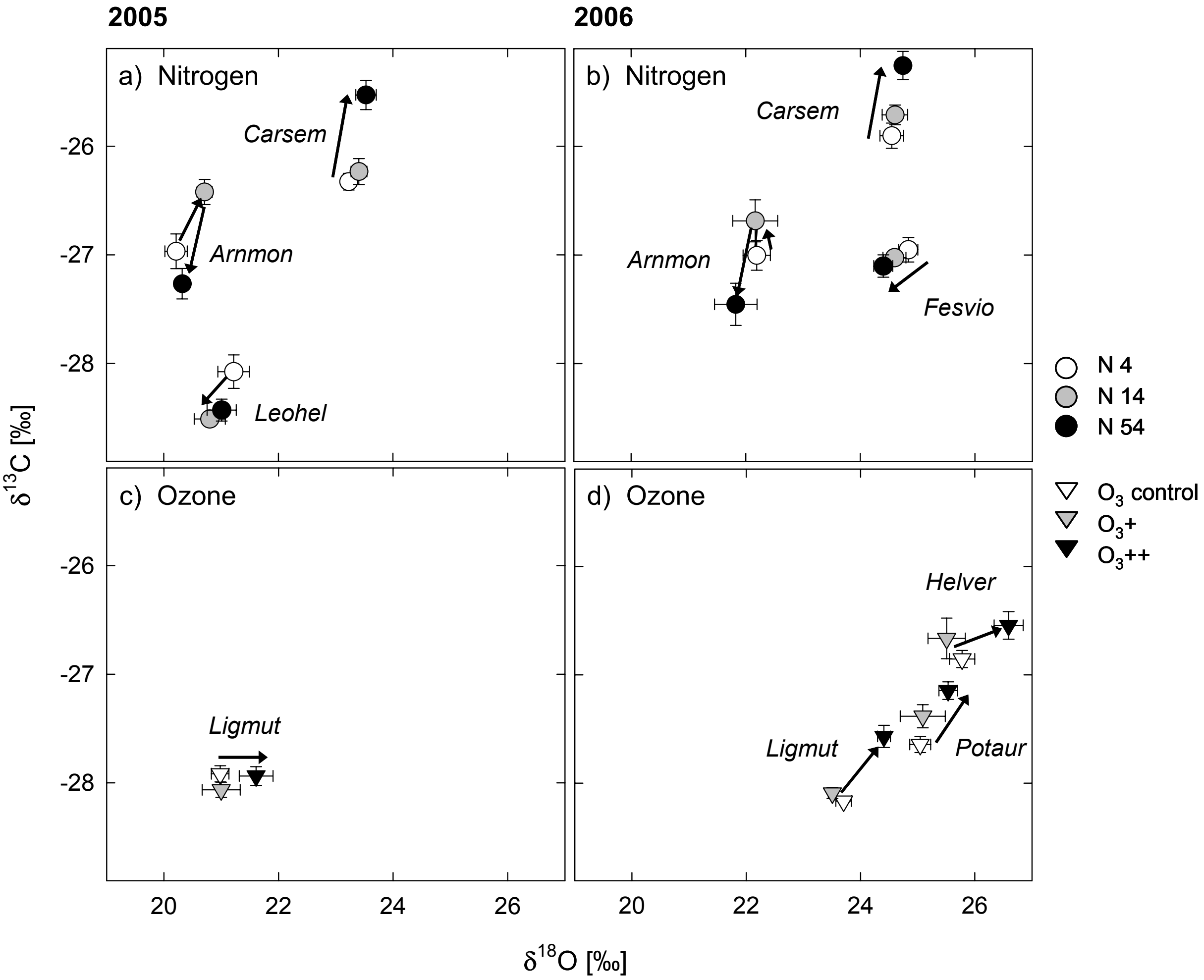

Fig. 3. Effect of N (a, b) and O_3 (c, d) treatment on $\delta^{13}C$ [‰] and $\delta^{18}O$ [‰] values of bulk leaf organic material of 11 key plant species of a subalpine pasture in 2005 and 2006 (only significant responses are displayed, according to ANOVA Tables S13 and S14 in Supplementary material). Means ± SE are shown.

Foliar $\delta^{18}O$ values were enriched in most species by 1-2‰ in 2006 compared to 2005 (Table S7). However, contrary to our expectations, $\delta^{18}O$ remained unaffected by the N addition in most species, with two exceptions: In 2005, leaves of *A. montana* were enriched by +0.5‰ in the N14 treatment in 2005, while *F. violacea* reacted with a decrease in $\delta^{18}O$ (-0.4‰) in response to the N addition in 2006 (Fig. 3 a & b). In contrast, $\delta^{18}O$ consistently (but not always significantly) shifted towards more positive values in the O_3++ treatment, relative to ambient air, especially in 2006 (Table S7). A significant enrichment in ^{18}O by was observed for *L. mutellina* (+0.6‰) in 2005 and for *H. versicolor* (+0.8‰) and *L. mutellina* (+0.7‰) in 2006 in the O_3++ treatment (Fig. 3 c & d).

In contrast to our third hypothesis, suggesting O_3 x N interactive effects through N-induced changes in g_s, the rare significant interactions on $\delta^{18}O$ showed an inconsistent pattern. They were only found in *H. versicolor* (both years) and in *T. alpinum* (2005) and in *N. stricta* (2006). For $\delta^{13}C$, the only interaction observed was in *L. mutellina* in 2005.

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

To test whether or not responses of the measured parameters to increased N (N54) or elevated O_3 (O_3++) were related to initial plant traits measured in the control treatment (N4 at O_3 control, two-year averages), Pearsons' correlation analysis was performed across all species, but correlations were either absent or weak (Table 2). A slight positive correlation was observed in both years between the species' initial $\delta^{18}O$ and the N-induced change in %N (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, thus more significant correlations were found: e.g., initial $\delta^{18}O$ was negatively and leaf weight positively related to ΔSLA. No significant relationships were found in both N or O_3 treatments between initial SLA and any other parameter.

Table 2. Relationship between initial leaf traits (control treatment, averaged over two years) of 11 key plant species and the relative changes in these traits (Δ) caused by the N54 treatment (top panel) and the O_3++ treatment (bottom panel), respectively, compared to the control. Pearson's correlation coefficients are displayed; bold figures indicate significant correlations (*P* < 0.05). Parameters measured: δ^{18}O [‰], δ^{13}C [‰], total Chl$_{conc}$ [mg g^{-1}], leaf weight [mg], %N [%], and SLA [cm^2 g^{-1}].

Change caused by N relative to control	**Δ δ^{13}C**		**Δ δ^{18}O**		**Δ Chl$_{conc}$**		**Δ leaf weight**		**Δ %N**		**Δ SLA**	
	2005	**2006**	**2005**	**2006**	**2005**	**2006**	**2005**	**2006**	**2005**	**2006**	**2005**	**2006**
initial δ^{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 δ^{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 δ^{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 δ^{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

4 Discussion

4.1 Nitrogen effect

In this species-rich *Geo-Montani-Nardetum* pasture, an N addition exceeding 10 kg ha^{-1} y^{-1} strongly altered species composition in favour of the sedges, which increased their fraction from 10% in the control to 30% in the N54 treamtent, and eventually accounted alone for the observed N-induced increase in aboveground productivity. Grasses, forbs, and legumes remained unaffected by the additional N supply (Bassin *et al.*, 2007b). In agreement, the proxies for growth measured here (SLA, leaf weight) indicated no N effect on aboveground productivity in 9 out of the 11 species studied. Leaf weight increased in the N54 treatment only in the sedge *C. sempervirens* and the forb *P. aurea* with associated positive effects on abundance (Bassin *et al.*, 2007b). Based on the results presented here, the rapid change in community composition was likely favoured by the absence of a growth response in most other species, allowing N-responsive species such as *C. sempervirens* to quickly gain in proportion.

Chl_{conc} and %N increased after the N addition in 9 out of 11 key plant species studied in more detail. This is a common reaction of plants, especially in cool climates (Michelsen *et al.*, 1996), and it is often associated with luxury consumption (Timmer & Morrow, 1984). It supports the earlier notion by Bassin *et al.* (2007b) that accumulation of Chl was the major cause for the observed N-induced increase in NDVI. Following Evans (1989), Chl and N accumulation, resulting in higher enzyme quantity and activity, should stimulate A and, consequently, increase g_s. However, the only consistent N effect on gas exchange assessed by stable isotopic signature of bulk leaf material was detected in *C. sempervirens* and *A. montana* (in the N14 treatment) as a shift towards less negative $\delta^{13}C$ at constant $\delta^{18}O$ in both years. This reaction strongly suggests increased A at constant g_s, thus reflecting enhanced WUE with increased N supply, which is similar to findings for prairie grassland species (Lee *et al.*, 2001).

These pronounced interspecific differences are difficult to interpret. 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 characterized by 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 the measured parameters (Chl_{conc}, leaf weight, %N, SLA, $\delta^{13}C$, $\delta^{18}O$) could not be observed. Differences in the responsiveness to N could be related to differences in phosphorous or potassium uptake (Theodose & Bowman, 1997), which would depend on root morphology and/or potential symbiotic interactions with micro-organisms, but this possibility needs to be investigated in more detail.

4.2 Ozone effect

After three years, elevated O_3 exposure had no effect on productivity and communtiy composition of the subalpine pasture (Bassin *et al.,* 2007b). However, from the literature we expected that elevated O_3 would have a negative effect on Chl_{conc}, %N and gas exchange of individual species. In fact, in a few species, elevated O_3 caused reduced Chl_{conc} associated with enhanced leaf yellowing. Chloroplast degradation is a typical symptom for O_3 stress, often being associated with accelerated leaf senescence (Grandjean & Fuhrer, 1989) and reduced leaf longevity, which was suggested to be the cause for the decrease in NDVI at elevated O_3 (Bassin *et al.*, 2007b).

In earlier experiments with species from temperate grassland, consistently less negative $\delta^{13}C$ values were found in O_3-exposed leaves (Saurer *et al.*, 1991; Jäggi *et al.*, 2005), which was attributed to the stronger reduction in g_s relative to the reduction in carboxylation. Here, only *L. mutellina* and *P. aurea* showed this response pattern, while in most other species $\delta^{13}C$ remained unaffected by elevated O_3 (Table S7). However, based on $\delta^{13}C$ it is not possible to exclude that reductions in g_s were compensated by a decline in carboxylation. More insights can be obtained from the measurement of $\delta^{18}O$. Increasing ^{18}O enrichment reflects increasing stomatal restriction to water vapour exchange as a result of either climatic conditions (Barbour, 2007) or the effect of stress on g_s. $\delta^{18}O$ was significantly higher in *L. mutellina* and *H. versicolor* at elevated O_3 than in ambient air, whereas the difference between treatments was small in another six out of the 11 species studied in 2006, but still indicating a general trend towards O_3-induced stomatal closure, supporting our hypothesis as well as findings from previous studies (Jäggi & Fuhrer 2007; Grams *et al.,* 2007). Reduced g_s is a common response to O_3 often coinciding with Chl and N reductions (Ojanperä *et al.*, 1998), and thus probably being the downstream consequence of an increase in c_i as a result of reduced A (Paoletti & Grulke, 2005).

Often, stomatal closure and reduced A are considered the major cause for reductions in aboveground biomass such as in *P. major* (Zheng *et al.*, 2002) and birch (Oksanen & Saleem, 1999). For half of our 11 subalpine species studied here, elevated O_3 caused modest reductions in leaf weight (Table S5), similar to effects observed in other experiments (e.g., Pearson *et al.*, 1996), and in agreement with our hypothesis. Remarkably, the reduction in leaf weight was not strongest in the species showing the most pronounced decline in Chl and N concentration (*L. mutellina* in 2005 and in *L. mutellina*, *P. aurea,* and *H. versicolor* in 2006), but *L. helveticus*. Moreover, the only species (*L. helveticus*) showing consistent reductions in both leaf weight and SLA with elevated O_3 remained unaffected by O_3 in terms of Chl and N concentration, and in isotopic composition. Enhanced resource demand for biochemical defence could have reduced the C investment in leaf construction in this species, as it was observed in O_3-sensitive clones of birch (Oksanen & 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 tolerance (Table 2). This agrees with data presented by Hayes *et al.* (2007) for a range of species, but contrasts suggestions by Bassin *et al.,* (2007a) and data presented by Pleijel *et al.*, (1997) and Bungener *et al.* (1999) who found relationships between tolerance to O_3 and specific initial RGR. The strongest O_3 responses were detected in species of intermediate SLA and ^{18}O enrichment. Also, contrary to the low O_3 tolerance in legumes relative to grasses and forbs found in previous experiments (e.g., Hayes *et al.*, 2007), and despite its high SLA, *T. alpinum*, the only legume species under investigation, remained unaffected by elevated O_3. Differences between the results obtained here and those of other studies may occur, firstly, because of the different response parameters used, e.g., visible injury, leaf weight, or aboveground biomass; and secondly, because the correlation strongly depends on the number of species and the range of selected traits considered; despite the considerable variation in SLA among the 11 species, only a limited range of growth strategies is covered.

In conclusion, the results presented here, showing no correlation between growth and reduced A and/or g_s, suggest that in most of these subalpine species, CO_2 assimilation is not a limiting factor for growth. At high elevations, growth is rather limited by low temperatures slowing down cell construction and maturation, thus limiting A through limited C sink capacity (Körner, 2003). C saturation at ecosystem level was proposed as the cause of the inertia of an alpine *Caricetum curvulae* community to CO_2 enrichment (Körner *et al.*, 1997). The same authors also claimed that as a result of their old age, late successional plant communities are characterized by a high resilience to any environmental influence. Nevertheless, our study provides evidence for considerable O_3 stress in several plant species, thus in the longer term, small differences in species-specific tolerance to O_3 might lead to altered community composition or even loss of the most sensitive species.

4.3 O_3 x N interaction

N-induced specific changes in SLA and g_s were expected to be related to decreasing O_3 tolerance. However, these traits were only marginally modified by N addition and most of the observed significant O_3 x N interaction patterns were not statistically related to changes in SLA and $\delta^{18}O$. Hence, the results of this analysis support the absence of O_3 x N interaction in terms of aboveground productivity after three years of experimentation (Bassin *et al.*, 2007b).

4.4 Conclusions

Lack of N-induced stimulation of growth despite increased N accumulation, and absence of effects of O_3-induced reductions in leaf gas exchange on aboveground productivity indicate that in most species studied, growth was not limited by N and gas exchange; in the short term, this makes most subalpine species comparatively unresponsive to both O_3 and N. However, the exceptional ability of *C. sempervirens* to invest the extra N taken up into enhanced *A*, improved WUE, and growth is probably responsible for the sedges' over-proportional increase in biomass in response to N addition. This shows that in the long run, differences in the extent and quality of the responses to either O_3 or N among species could alter 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 most responsive species to O_3 (*L. mutellina*, *H. versicolor*, *L. helveticus*, *P. aurea*) and to N (*C. sempervirens* and *P. aurea*) 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 subalpine plant communities.

Due to the absence of N-induced growth responses and changes in SLA and g_s in most species, this subalpine plant community turned out to be an inappropriate model system for testing the mechanisms of interacting N and O_3 over three years, which explains the lack of O_3 x N interactive effects on aboveground productivity. As suggested by the results for more productive montane grassland (Volk *et al.*, 2006), longer O_3 exposures, and additional field experiments carried out in habitats of different responsiveness to N are needed to elucidate the biological consequences of co-occurring elevated O_3 and N deposition.

Acknowledgements

This work was supported by the Swiss Federal Office of Environment in the framework of the International Cooperative Programme ‘Vegetation’ (ICP Vegetation) under the UNECE CLRTAP. The help of V. Spinas, M. Montani, R. Giger, P. Scheuber, F. Contat and the ART field team in setting up the experiment and performing fieldwork and vegetation analysis is greatly acknowledged.

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Supplementary material

Tables S1-S7. Means ± SE for the three O_3 levels (n = 3) and five N levels (n = 9) for 11 key plant species from *Geo-Montani-Nardetum* monoliths exposed 3 years to elevated O_3 and N concentrations at the site ‘Alp Flix’, 2000 m asl. Significance levels from split-plot ANOVA are indicated as (*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For leaf N concentration, $\delta^{13}C$, $\delta^{18}O$, only three N treatment levels were analyzed.

S1: leaf N concentration [%]
S2: specific leaf area [cm^2 g^{-1}]
S3: total Chl content [mg $leaf^{-1}$]
S4: total Chl concentration [mg g^{-1}]
S5: leaf weight [mg],
S6: $\delta^{13}C$ [‰]
S7: $\delta^{18}O$ [‰]

Tables S8-S14. Effects of ozone (O_3), nitrogen (N), and their interactions on leaf traits of 11 key species in monoliths of a free-air fumigation experiment at the site ‘Alp Flix’ in 2005 and 2006. Numerator (N df) and denominator degrees of freedom (D df) and F-values are displayed. Terms with "-" were not fitted in the particular model. (*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

S8: leaf N concentration [%]
S9: specific leaf area [cm^2 g^{-1}]
S10: total Chl content [mg $leaf^{-1}$]
S11: Chl concentration [mg g^{-1}]
S12: leaf weight [mg]
S13: $\delta^{13}C$ [‰]
S14: $\delta^{18}O$ [‰]

Table S1.

Nitrogen concentration [%]	**Ozone (O_3) effect**				**Nitrogen (N) effect**				**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N14	N54	sign.	sign.
A. montana	1.57±0.08	1.58±0.02	1.54±0.07		1.49±0.05	1.57±0.08	1.62±0.04		
C. sempervirens	1.58±0.08	1.53±0.04	1.61±0.03		1.40±0.06	1.47±0.04	1.86±0.03	***	
F. violacea	1.72±0.11	1.69±0.07	1.71±0.04		1.54±0.05	1.58±0.03	2.02±0.11	***	
G. acaulis	1.88±0.10	1.91±0.09	1.96±0.06		1.91±0.03	1.79±0.06	2.06±0.11	(*)	
H. versicolor	1.71±0.02	1.66±0.02	1.66±0.03		1.50±0.04	1.64±0.05	1.87±0.06	**	
L. helveticus	2.24±0.05	2.17±0.10	2.42±0.04		2.18±0.07	2.23±0.07	2.42±0.08	(*)	
L. mutellina	2.30±0.04	2.26±0.04	1.96±0.10	(*)	2.08±0.09	2.10±0.06	2.34±0.06	*	
N. stricta	1.64±0.04	1.71±0.02	1.68±0.01		1.57±0.04	1.67±0.03	1.79±0.05	*	
P. aurea	2.35±0.06	2.44±0.04	2.41±0.06		2.22±0.04	2.45±0.06	2.53±0.06	***	
R. villarsii	2.42±0.03	2.51±0.07	2.45±0.11		2.39±0.04	2.38±0.06	2.61±0.08	*	
T. alpinum	2.82±0.07	2.62±0.07	2.81±0.12		2.85±0.07	2.77±0.05	2.66±0.10		
2006									
A. montana	1.87±0.02	1.85±0.02	1.71±0.04		1.77±0.03	1.79±0.05	1.87±0.05		
C. sempervirens	1.58±0.01	1.66±0.01	1.71±0.02		1.46±0.04	1.57±0.04	1.92±0.04	***	
F. violacea	1.87±0.00	1.89±0.04	1.82±0.11		1.75±0.08	1.68±0.03	2.15±0.12	***	*
G. acaulis	2.15±0.01	2.28±0.07	2.25±0.07		2.09±0.03	2.14±0.06	2.45±0.11	*	
H. versicolor	1.82±0.01	1.88±0.03	1.74±0.03	nnd	1.70±0.06	1.71±0.05	2.03±0.04	nnd	
L. helveticus				na				na	
L. mutellina	2.29±0.04	2.60±0.14	2.40±0.12		2.29±0.08	2.39±0.06	2.61±0.12	*	
N. stricta	1.81±0.15	1.87±0.02	1.85±0.09	nnd	1.80±0.06	1.76±0.04	2.04±0.06	nnd	
P. aurea	2.51±0.01	2.53±0.03	2.36±0.05		2.28±0.05	2.36±0.08	2.76±0.10	**	
R. villarsii	2.23±0.09	2.31±0.20	2.51±0.02		2.26±0.05	2.24±0.08	2.54±0.10	***	
T. alpinum	2.86±0.06	2.69±0.04	2.84±0.04		2.78±0.06	2.77±0.07	2.82±0.08		

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
na, not available
nnd, not normally distributed even after transformation

Table S2.

Specific leaf area [cm^2 g^{-1}]	**Ozone (O_3) effect**				**Nitrogen (N) effect**						**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N9	N14	N29	N54	sign.	sign.
A. montana	153.7±6.6	151.8±2.5	149.0±1.2		156.9±2.6	151.6±4.6	147.4±5.1	151.7±2.1	155.9±3.2		*
C. sempervirens	124.5±10	122.8±2.7	124.5±1.3		133.8±2.2	138.0±2.6	136.6±1.7	131.2±2.0	128.7±1.8	**	
F. violacea	84.7±1.3	82.7±1.3	84.9±2.4		83.2±2.8	83.6±2.8	82.6±1.9	80.0±1.5	87.6±2.7		
G. acaulis	113.9±2.6	110.0±4.4	114.5±3.1		118.9±3.2	117.6±2.1	119.1±3.4	117.4±1.8	122.9±5.1		
H. versicolor	134.0±1.4	134.0±2.6	136.5±1.4		125.1±3.3	128.2±1.6	127.9±2.2	122.7±3.3	126.4±1.9		
L. helveticus	172.9±2.3	165.6±3.1	177.1±1.9	*	149.1±6.1	163.1±4.4	156.6±2.4	159.5±5.5	158.9±3.5		
L. mutellina	106.5±3.1	114.1±5.2	108.4±5.9		119.2±3.4	113.8±2.3	118.0±3.9	111.5±1.6	114.5±3.7		
N. stricta	67.2±1.1	70.9±2.6	71.5±3.5		66.9±1.3	68.4±1.6	68.2±1.1	67.8±0.9	68.6±1.2		
P. aurea	163.2±1.3	167.7±4.1	176.9±4.9		170.4±3.1	166.2±2.7	164.1±1.5	163.6±2.6	166.8±3.7		*
R. villarsii	218.0±3.8	211.4±7.3	215.1±7.2		208.1±3.8	201.7±5.3	200.2±4.8	198.4±5.0	201.1±7.4		
T. alpinum	195.1±1.6	193.6±0.7	199.5±1.7	(*)	198.6±2.1	193.6±3.9	201.0±3.9	199.1±3.3	199.6±3.1		
2006											
A. montana	153.1±4.7	152.7±5.5	153.0±3.0		154.5±4.6	149.4±5.8	151.1±4.5	147.6±3.1	155.0±4.2		
C. sempervirens	132.7±0.4	133.5±2.7	134.8±2.2		127.3±1.9	124.1±1.5	124.2±1.3	122.6±0.7	121.5±1.2	**	
F. violacea	85.4±1.8	83.5±1.6	83.5±1.9		83.5±1.9	87.2±1.5	80.7±1.3	83.6±1.8	85.5±3.2		
G. acaulis	118.9±2.4	117.8±1.5	120.8±5.0		114.0±2.7	111.5±1.4	113.6±2.8	113.8±2.2	111.2±4.0		
H. versicolor	122.9±1.8	126.7±0.4	128.5±1.2		133.6±2.9	132.1±2.7	131.8±3.3	133.7±2.1	143.0±3.2	**	
L. helveticus	155.0±3.3	150.5±3.1	166.9±2.2		170.4±5.2	170.6±6.3	166.0±3.0	172.4±3.7	179.9±4.0	*	
L. mutellina	116.1±3.8	116.8±3.8	114.2±1.8		112.3±4.3	107.3±1.9	109.1±5.1	110.2±2.7	109.5±5.1		
N. stricta	68.0±0.4	67.8±1.2	68.1±0.5		69.8±1.8	71.3±2.9	69.7±2.2	68.6±1.4	69.8±1.0		
P. aurea	165.9±3.5	168.3±3.6	164.3±3.8	*	172.8±5.6	170.4±3.6	166.6±3.1	167.2±3.7	169.6±3.2		
R. villarsii	199.5±2.0	195.9±3.0	210.3±4.4		207.0±4.5	217.8±2.5	216.3±6.3	214.9±3.3	218.2±8.2		*
T. alpinum	202.0±1.1	191.6±2.0	201.5±2.8		195.4±5.1	192.1±7.4	196.1±4.6	196.9±7.3	200.2±5.4	*	

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S3.

Chlorophyll content [mg leaf^{-1}]	**Ozone (O_3) effect**				**Nitrogen (N) effect**						**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N9	N14	N29	N54	sign.	sign.
A. montana	175.1±26.9	147.4±7.7	139.6±10.3		124.0±10.2	163.0±21.0	196.8±19.1	168.1±17.2	118.3±12		
C. sempervirens	68.9±3.5	66.3±3.7	58.7±2.9		42.2±2.0	46.0±2.8	54.2±5.2	76.5±4.8	104.2±4.4	***	
F. violacea				na						na	
G. acaulis	89.4±9.3	98.8±14.6	83.7±5.4		82.7±6.5	102.9±12.6	86.0±10.9	86.7±8.0	94.9±14.6		
H. versicolor	62.7±2.4	48.8±2.4	49.5±1.7	*	49.7±4.2	46.4±5.2	53.7±3.8	53.3±3.2	65.5±4.3	**	
L. helveticus	101.4±4.0	108.7±4.9	81.6±3.0	(*)	99.5±9.0	77.3±5.6	89.6±7.1	109.5±7.9	110.3±7.4	***	
L. mutellina	115.1±9.9	87.1±14.0	88.6±9.8		86.5±9.2	88.7±11.4	98.9±13.2	103.9±22.5	106.7±12.6		
N. stricta	15.6±0.8	17.7±0.4	16.0±0.1		15.4±0.8	13.1±1.0	15.6±1.4	18.9±1.1	18.7±0.8	***	
P. aurea	95.9±4.3	86.5±2.4	81.4±2.1		71.2±4.6	78.0±4.2	85.4±6.5	103.3±7.2	101.7±8.1	***	
R. villarsii	104.9±3.3	96.7±5.9	84.5±5.6		82.5±9.8	87.1±3.5	94.7±6.4	99.2±5.7	113.2±5.6	*	*
T. alpinum	57.5±2.7	59.1±2.1	55.2±4.5		59.8±2.6	56.2±3.8	55.2±5.2	55.4±3.3	60.6±4.8		
2006											
A. montana	178.5±4.1	155.9±16.5	143.0±10.8		151.2±15.5	165.6±17.2	155.4±10.6	167.4±15.8	144.9±16.6		
C. sempervirens	70.8±1.6	80.2±5.6	81.8±2.2		50.7±4.1	66.4±5.5	68.5±3.6	90.2±6.6	112.1±10.4	***	
F. violacea				na						na	
G. acaulis	97.1±6.3	114.2±9.0	112.0±3.8		97.2±9.7	115.4±9.7	103.6±3.3	104.9±7.8	117.5±12.3		
H. versicolor	51.2±1.3	60.3±1.1	40.8±5.0	*	41.6±3.2	49.4±5.5	45.8±4.4	56.6±4.0	59.3±9.0	**	*
L. helveticus	78.4±0.8	87.5±3.7	73.0±3.0	(*)	71.9±3.5	76.7±3.9	75.4±2.3	82.3±3.5	91.8±6.3	***	
L. mutellina	104.3±8.7	90.2±3.3	77.5±9.4	nnd	84.5±10.7	74.6±8.8	93.0±9.7	86.9±10.5	114.5±8.4	nnd	
N. stricta	13.1±0.3	11.8±0.5	12.7±1.3		10.7±1.2	11.9±1.1	11.2±0.5	14.0±0.8	14.9±1.0	***	
P. aurea	100.0±3.8	97.2±4.9	70.6±5.9	*	72.1±7.8	82.1±4.9	95.9±6.5	92.1±7.6	104.2±8.0	***	
R. villarsii	66.0±9.3	70.7±14	63.4±0.8		62.1±5.9	63.8±4.6	63.2±7.0	64.0±11.6	81.8±9.4	*	*
T. alpinum	58.3±1.8	68.2±2.2	57.9±2.1		60.0±4.3	67.0±4.9	57.6±3.4	60.7±5.2	62.0±6.5		*

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
na, not available
nnd, not normally distributed even after transformation

Table S4.

Chlorophyll concentration [mg g^{-1}]	**Ozone (O_3) effect**				**Nitrogen (N) effect**						**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N9	N14	N29	N54	sign.	sign.
A. montana	4.6±0.2	4.2±0.2	4.0±0.1		4.1±0.2	4.1±0.1	4.3±0.2	4.5±0.1	4.4±0.3		
C. sempervirens	4.2±0.1	4.2±0.1	4.1±0.0		3.5±0.1	3.5±0.1	3.8±0.1	4.6±0.1	5.4±0.1	***	
F. violacea	4.8±0.2	4.8±0.2	4.6±0.1		4.5±0.2	4.4±0.2	4.4±0.1	4.7±0.2	5.6±0.2	***	
G. acaulis	5.2±0.2	5.2±0.1	5.2±0.1		5.3±0.1	5.0±0.2	4.8±0.1	5.1±0.2	5.9±0.2	***	
H. versicolor	4.5±0.0	3.9±0.1	4.4±0.1		3.9±0.1	4.0±0.2	4.2±0.2	4.3±0.2	4.8±0.2	***	
L. helveticus	7.5±0.2	7.3±0.1	7.5±0.2		6.9±0.2	6.7±0.3	7.1±0.2	8.0±0.2	8.5±0.3	***	
L. mutellina	5.6±0.3	5.7±0.4	4.4±0.3		5.1±0.3	5.0±0.3	4.7±0.4	5.4±0.4	6.0±0.3	**	
N. stricta	4.9±0.3	5.0±0.1	5.1±0.1		4.7±0.2	4.4±0.2	4.8±0.2	5.5±0.2	5.5±0.2	***	*
P. aurea	6.6±0.2	6.4±0.1	6.4±0.2		5.9±0.2	6.3±0.1	6.5±0.3	6.7±0.2	7.0±0.2	***	
R. villarsii	6.5±0.2	6.3±0.3	5.9±0.2	(*)	5.8±0.2	6.0±0.2	6.0±0.2	6.3±0.2	7.1±0.3	***	
T. alpinum	6.2±0.2	6.0±0.3	5.9±0.2		6.1±0.2	5.8±0.2	6.1±0.3	6.3±0.4	6.0±0.4		
2006											
A. montana	5.7±0.2	5.5±0.0	4.5±0.1	**	5.1±0.2	5.0±0.2	4.9±0.2	5.1±0.2	6.0±0.3	***	(*)
C. sempervirens	4.0±0.2	4.3±0.1	4.6±0.0	(*)	3.7±0.1	4.0±0.1	3.9±0.1	4.8±0.2	5.4±0.2	***	
F. violacea	4.5±0.3	4.5±0.1	4.4±0.1		4.1±0.1	4.4±0.2	4.2±0.2	4.7±0.2	5.1±0.1	***	
G. acaulis	5.8±0.1	5.9±0.1	6.4±0.1		5.9±0.2	5.7±0.2	5.8±0.1	6.2±0.2	6.8±0.3	***	
H. versicolor	4.6±0.0	4.9±0.2	4.2±0.4		4.1±0.3	4.5±0.4	4.2±0.3	4.8±0.1	5.2±0.3	***	
L. helveticus	9.0±0.1	9.0±0.2	9.0±0.2		8.4±0.2	8.8±0.2	8.1±0.2	9.3±0.2	10.2±0.3	***	
L. mutellina	6.6±0.2	6.5±0.2	5.4±0.3	(*)	5.8±0.3	5.5±0.3	5.9±0.3	5.9±0.3	7.5±0.3	***	*
N. stricta	5.3±0.1	5.1±0.3	5.3±0.2		5.1±0.2	5.0±0.2	4.9±0.2	5.5±0.1	5.8±0.3	**	
P. aurea	6.6±0.1	6.7±0.1	6.2±0.2		6.0±0.2	6.4±0.1	6.6±0.2	6.6±0.2	7.0±0.3	**	
R. villarsii	5.2±0.3	5.2±0.5	5.5±0.2		5.1±0.2	5.3±0.2	5.2±0.4	4.9±0.4	6.1±0.3	*	
T. alpinum	5.8±0.1	6.0±0.1	5.9±0.1		5.6±0.2	6.0±0.2	5.7±0.2	6.1±0.3	6.2±0.4		(*)

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S5.

Leaf weight [mg]	**Ozone (O_3) effect**				**Nitrogen (N) effect**						**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N9	N14	N29	N54	sign.	sign.
A. montana	39.3±4.7	34.8±11.1	35.5±10.6		31.1±3.1	40.6±5.8	45.9±4.1	36.6±3.0	28.5±4.4		
C. sempervirens	16.2±0.6	15±4.2	14.1±3.5		12.2±0.7	13.1±0.6	14.3±1.0	16.5±0.8	19.4±0.8	***	
F. violacea				na						na	
G. acaulis	17.2±2.3	18.7±7.3	16.2±4.7		15.8±1.4	20.2±2.1	17.9±2.2	16.9±1.4	16.0±2.2		
H. versicolor	14.0±0.7	12.2±3.4	11.3±3.1		12.6±1.0	11.5±1.1	12.7±0.9	12.5±0.6	13.6±0.6		
L. helveticus	13.6±0.6	14.8±3.5	10.8±2.4	*	14.5±1.3	11.6±0.7	12.5±0.6	13.7±1.0	13.1±0.9		
L. mutellina	19.8±1.0	16.3±5.5	20.1±5.1		16.8±1.4	17.8±1.9	20.7±2.4	21.2±1.7	17.8±1.7		
N. stricta	3.2±0.1	3.5±0.9	3.2±0.8		3.3±0.2	3.1±0.2	3.3±0.2	3.4±0.2	3.5±0.2		
P. aurea	14.3±0.4	14.3±2.6	12.6±3.1		12.0±0.5	12.4±0.6	14.3±1.2	15.4±1.0	14.4±0.8	**	
R. villarsii	16.2±0.1	15.1±2.7	14.3±3.5		13.9±1.5	14.5±0.4	15.7±0.7	15.8±0.7	16.0±0.8		
T. alpinum	9.3±0.5	10.0±1.5	9.3±2.9		9.9±0.5	9.6±0.4	9.0±0.4	8.9±0.5	10.3±1.0		
2006											
A. montana	32.3±2.1	29.4±2.6	31.3±1.9		29.6±2.6	33.3±3.3	32.2±2.4	33.1±2.8	24.4±2.4		
C. sempervirens	17.3±0.6	17.9±1.0	17.5±0.4		13.6±0.9	16.6±1.0	17.9±0.9	19.0±1.2	20.7±1.4	***	
F. violacea				na						na	
G. acaulis	16.6±0.8	19.0±1.1	17.5±0.8		16.5±1.4	19.9±1.3	18.0±0.9	17±1.0	17.0±1.4		
H. versicolor	11.1±0.2	11.8±0.9	9.9±0.5		10.3±0.7	10.4±0.7	10.9±0.8	11.8±0.8	11.1±1.3		*
L. helveticus	8.8±0.1	9.8±0.5	8.2±0.4	*	8.6±0.5	8.7±0.5	9.3±0.4	8.8±0.3	9.0±0.6		
L. mutellina	16.0±1.1	13.9±0.7	14.1±1.8		14.3±1.4	13.4±1.2	15.7±1.4	14.5±1.3	15.4±1.2		
N. stricta	2.5±0.0	2.3±0.1	2.4±0.2		2.1±0.2	2.3±0.1	2.3±0.1	2.6±0.1	2.6±0.2	*	
P. aurea	15.1±0.4	14.6±1.0	11.4±0.9	(*)	12.0±1.3	12.8±0.8	14.4±0.7	14.5±1.3	14.8±0.8	*	
R. villarsii	12.6±1.3	13.1±1.6	11.4±0.3		12.1±1.1	12.1±0.6	11.8±1.0	12.6±1.3	13.2±1.2		(*)
T. alpinum	10.1±0.5	11.3±0.4	9.7±0.4		10.7±0.7	11.1±0.6	10.1±0.5	10.0±0.6	10.0±0.8		

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
na, not available

Table S6.

$\delta^{13}C$ **[‰]**	**Ozone (O_3) effect**				**Nitrogen (N) effect**				**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N14	N54	sign.	sign.
A. montana	-27.1±0.2	-26.9±0.2	-26.6±0.1		-27.0±0.2	-26.4±0.1	-27.3±0.1	***	
C. sempervirens	-26.0±0.1	-26.1±0.1	-26.0±0.2		-26.3±0.1	-26.2±0.1	-25.5±0.1	***	
F. violacea	-27.4±0.1	-27.2±0.1	-27.1±0.1		-27.2±0.1	-27.1±0.1	-27.3±0.1		
G. acaulis	-25.5±0.2	-25.2±0.2	-25.6±0.2		-25.4±0.3	-25.2±0.2	-25.7±0.2		
H. versicolor	-26.8±0.1	-26.6±0.2	-26.7±0.0		-26.9±0.2	-26.7±0.1	-26.5±0.2		
L. helveticus	-28.3±0.2	-28.3±0.1	-28.4±0.1		-28.1±0.2	-28.5±0.1	-28.4±0.1	*	
L. mutellina	-27.9±0.1	-28.1±0.1	-27.9±0.1		-28.0±0.1	-28.0±0.1	-27.9±0.1		*
N. stricta	-26.5±0.1	-26.4±0.1	-26.4±0.0		-26.4±0.1	-26.5±0.1	-26.4±0.1		
P. aurea	-27.5±0.1	-27.6±0.1	-27.3±0.1		-27.6±0.2	-27.5±0.1	-27.4±0.1		
R. villarsii	-26.8±0.1	-26.8±0.1	-26.6±0.1		-26.8±0.1	-26.7±0.1	-26.7±0.1		
T. alpinum	-26.2±0.0	-26.2±0.1	-26.0±0.2		-26.1±0.1	-26.1±0.1	-26.2±0.1		
2006									
A. montana	-27.4±0.1	-27.0±0.2	-26.7±0.2		-27.0±0.1	-26.7±0.2	-27.5±0.2	*	
C. sempervirens	-25.7±0.0	-25.5±0.2	-25.6±0.1		-25.9±0.1	-25.7±0.1	-25.3±0.1	***	
F. violacea	-27.1±0.0	-27.2±0.1	-26.8±0.1		-27.0±0.1	-27.0±0.0	-27.1±0.1		
G. acaulis	-25.9±0.1	-25.3±0.1	-25.8±0.2		-25.6±0.2	-25.7±0.2	-25.8±0.2		
H. versicolor	-26.9±0.1	-26.7±0.2	-26.5±0.1		-26.7±0.1	-26.7±0.1	-26.7±0.2		
L. helveticus	-28.5±0.0	-28.3±0.1	-28.4±0.1		-28.5±0.1	-28.3±0.1	-28.4±0.1		
L. mutellina	-28.2±0.0	-28.1±0.1	-27.6±0.1	*	-27.9±0.1	-28.0±0.2	-27.9±0.2		
N. stricta	-26.4±0.1	-26.4±0.0	-26.4±0.1		-26.3±0.1	-26.4±0.1	-26.5±0.1		
P. aurea	-27.6±0.1	-27.4±0.1	-27.1±0.1	(*)	-27.4±0.2	-27.4±0.0	-27.4±0.1		
R. villarsii	-27.0±0.3	-27.0±0.2	-26.3±0.1		-26.8±0.2	-26.7±0.2	-26.7±0.2		
T. alpinum	-27.0±0.1	-27.2±0.1	-26.9±0.2		-26.9±0.1	-26.9±0.1	-27.2±0.1		

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S7.

$\delta^{18}O$ **[‰]**	**Ozone (O_3) effect**				**Nitrogen (N) effect**				**N x O_3**
2005	O_3 control	O_3+	O_3++	sign.	N4	N14	N54	sign.	sign.
A. montana	20.6±0.1	20.4±0.1	20.3±0.2		20.2±0.2	20.7±0.1	20.3±0.1	*	
C. sempervirens	23.4±0.2	23.2±0.2	23.5±0.1		23.2±0.1	23.4±0.1	23.5±0.2		
F. violacea	23.4±0.3	23.1±0.1	23.2±0.3		23.4±0.2	23.3±0.2	23.0±0.2		
G. acaulis	22.0±0.3	22.6±0.5	22.3±0.5		21.9±0.3	22.7±0.4	22.2±0.3		
H. versicolor	24.7±0.1	24.3±0.2	24.6±0.2		24.6±0.1	24.4±0.3	24.6±0.2		*
L. helveticus	21.0±0.3	20.8±0.0	21.3±0.5		21.2±0.3	20.8±0.3	21.0±0.3		
L. mutellina	21.0±0.2	21.0±0.3	21.6±0.3	*	21.1±0.2	21.1±0.2	21.2±0.1		
N. stricta	24.5±0.4	24.0±0.2	24.2±0.4		24.4±0.3	24.1±0.2	24.2±0.2		
P. aurea	22.3±0.7	22.5±0.4	23.1±0.3		22.5±0.5	22.6±0.2	22.9±0.3		
R. villarsii	21.1±0.1	21.7±0.2	21.7±0.4		21.4±0.2	21.6±0.2	21.5±0.2		
T. alpinum	21.0±0.1	20.9±0.2	21.4±0.3		21.0±0.1	21.2±0.2	21.0±0.1		(*)
2006									
A. montana	22.0±0.2	22.0±0.5	22.1±0.7		22.2±0.2	22.2±0.4	21.8±0.4		
C. sempervirens	24.3±0.1	24.7±0.3	25.0±0.3		24.5±0.2	24.6±0.2	24.7±0.1		
F. violacea	24.5±0.01	24.7±0.3	24.7±0.3		24.8±0.2	24.6±0.2	24.4±0.2	(*)	
G. acaulis	23.3±0.2	23.9±0.2	23.8±0.6		23.6±0.2	23.5±0.2	23.8±0.4		
H. versicolor	25.8±0.2	25.5±0.3	26.6±0.3	*	25.9±0.2	25.9±0.3	26.0±0.3		*
L. helveticus	24.4±0.2	24.6±0.4	24.8±0.0		24.4±0.2	24.6±0.3	24.9±0.1		
L. mutellina	23.7±0.1	23.5±0.1	24.4±0.1	*	23.7±0.3	24.1±0.2	23.8±0.1		
N. stricta	24.0±0.2	24.3±0.2	24.5±0.1		24.1±0.2	24.5±0.2	24.2±0.1		(*)
P. aurea	25.0±0.2	25.1±0.4	25.5±0.2		25.2±0.3	25.2±0.1	25.2±0.3		
R. villarsii	23.9±0.3	24.2±0.2	24.8±0.6		24.3±0.4	24.4±0.2	24.2±0.3		
T. alpinum	22.9±0.2	22.8±0.3	23.1±0.4		22.8±0.2	22.9±0.1	23.0±0.2		

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S8.

Nitrogen concentration [%]	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	2.41	2	4	0.12	2	12	1.31	4	12	0.57
C. sempervirens	2	4	4.76	2	4	1.34	2	12	46.45***	4	12	0.66
F. violacea	2	4	0.68	2	4	0.13	2	10	14.46***	4	10	1.75
G. acaulis	2	4	1.91	2	4	0.36	2	12	3.46(*)	4	12	0.37
H. versicolor	2	4	0.10	2	4	0.69	2	11	12.21**	4	11	1.20
L. helveticus	2	4	0.52	2	4	3.09	2	12	3.24(*)	4	12	0.47
L. mutellina	2	4	0.32	2	4	5.99(*)	2	12	8.28*	4	12	0.42
N. stricta	2	4	0.15	2	4	0.64	2	12	5.46*	4	12	0.51
P. aurea	2	4	0.23	2	4	0.60	2	12	13.61***	4	12	1.80
R. villarsii	2	4	4.28	2	4	0.64	2	12	4.97*	4	12	0.60
T. alpinum	2	4	0.29	2	4	1.94	2	11	2.28	4	11	1.89
2006												
A. montana	2	4	0.23	2	4	4.40	2	12	1.73	4	12	0.42
C. sempervirens	2	4	0.00	2	4	2.08	2	12	27.36***	4	12	0.39
F. violacea	2	4	0.37	2	4	0.23	2	12	13.21***	4	12	4.23*
G. acaulis	2	4	0.45	2	4	0.85	2	12	6.68*	4	12	0.66
H. versicolor	nnd											
L. helveticus	na											
L. mutellina	2	4	1.92	2	4	2.69	2	12	4.68*	4	12	0.36
N. stricta	nnd											
P. aurea	2	4	0.07	2	4	1.59	2	12	11.96**	4	12	2.13
R. villarsii	2	4	1.60	2	4	1.60	2	12	13.28***	4	12	0.92
T. alpinum	2	4	0.04	2	4	1.49	2	11	0.09	4	11	0.98

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
na, not available
nnd, not normally distributed even after transformation

Table S9.

Specific leaf area [cm^2 g^{-1}]	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**			**N x N**			**O_3 x N x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	0.74	2	4	0.00	1	34	0.28	2	32	3.00(*)	-	-	-	-	-	-
C. sempervirens	2	4	3.06	2	4	0.45	1	35	9.98**	-	-	-	-	-	-	-	-	-
F. violacea	nnd																	
G. acaulis	2	4	0.70	2	4	0.19	1	35	0.95	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	0.77	2	4	1.99	1	34	0.16	-	-	-	-	-	-	-	-	-
L. helveticus	2	4	1.17	2	4	7.19*	1	35	0.77	-	-	-	-	-	-	-	-	-
L. mutellina	2	4	0.86	2	4	0.16	1	34	1.17	-	-	-	-	-	-	-	-	-
N. stricta	2	4	0.50	2	4	0.04	1	29	0.34	-	-	-	-	-	-	-	-	-
P. aurea	2	4	7.86*	2	4	1.07	1	30	0.64	2	31	3.68*	1	21	5.87*	-	-	-
R. villarsii	2	4	0.03	2	4	3.52	1	35	0.49	-	-	-	-	-	-	-	-	-
T. alpinum	2	4	0.37	2	4	6.32(*)	1	34	0.44	-	-	-	-	-	-	-	-	-
2006																		
A. montana	2	4	0.34	2	4	0.25	1	32	0.09	-	-	-	-	-	-	-	-	-
C. sempervirens	2	4	4.21	2	4	0.61	1	35	11.02**	-	-	-	-	-	-	-	-	-
F. violacea	2	4	0.04	2	4	0.31	1		0.23	-	-	-	-	-	-	-	-	-
G. acaulis	2	4	10.65*	2	4	2.44	1	35	0.75	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	0.62	2	4	0.55	1	30	7.98**	-	-	-	-	-	-	-	-	-
L. helveticus	2	4	0.46	2	4	3.47	1	35	4.88*	2	33	2.77	-	-	-	-	-	-
L. mutellina	2	4	1.56	2	4	0.97	1	35	0.11	-	-	-	-	-	-	-	-	-
N. stricta	2	4	11.17*	2	4	3.81	1	35	0.08	-	-	-	-	-	-	-	-	-
P. aurea	2	4	4.00	2	4	7.13*	1	34	0.19	-	-	-	-	-	-	-	-	-
R. villarsii	2	4	9.65*	2	4	1.02	1	33	1.15	2	33	4.47*	-	-	-	-	-	-
T. alpinum	2	4	1.50	2	4	4.53	1	34	6.48*	-	-	-	-	-	-	-	-	-

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
nnd, not normally distributed even after transformation

Table S10.

Chlorophyll content [mg leaf^{-1}]	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**			**N x N**			**O_3 x N x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	1.14	2	4	1.23	1	34	1.253	-	-	-	1	34	10.89**	-	-	-
C. sempervirens	2	4	3.65	2	4	3.81	1	35	201.63***	-	-	-	-	-	-	-	-	-
F. violacea	na																	
G. acaulis	2	4	4.62	2	4	0.85	1	35	0.02	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	0.00	2	4	7.40*	1	29	15.41***	2	29	0.14	1	29	0.37	2	29	3.93*
L. helveticus	2	4	1.27	2	4	6.87(*)	1	35	7.85**	-	-	-	-	-	-	-	-	-
L. mutellina	2	4	0.40	2	4	1.52	1	35	1.37	-	-	-	-	-	-	-	-	-
N. stricta	2	4	0.76	2	4	1.32	1	29	9.92**	-	-	-	-	-	-	-	-	-
P. aurea	2	4	0.30	2	4	2.63	1	29	19.14***	2	29	1.36	1	29	6.80*	2	29	3.30(*)
R. villarsii	2	4	4.64	2	4	7.68*	1	30	23.87***	2	30	5.10*	1	30	0.26	2	30	3.17(*)
T. alpinum	2	4	1.38	2	4	0.41	1	34	0.09	-	-	-	-	-	-	-	-	-
2006																		
A. montana	2	4	0.08	2	4	1.56	1	32	0.07	-	-	-	-	-	-	-	-	-
C. sempervirens	2	4	1.09	2	4	1.72	1	34	60.28***	-	-	-	1	34	4.70*	-	-	-
F. violacea	na																	
G. acaulis	2	4	0.59	2	4	1.70	1	35	1.27	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	1.19	2	4	8.84*	1	29	9.29**	2	29	3.85*	1	29	0.95	2	29	5.12*
L. helveticus	2	4	0.94	2	4	6.68(*)	1	35	19.40***	-	-	-	-	-	-	-	-	-
L. mutellina	nnd																	
N. stricta	2	4	2.08	2	4	0.92	1	35	13.99***	-	-	-	-	-	-	-	-	-
P. aurea	2	4	0.34	2	4	8.12*	1	34	15.29***	-	-	-	-	-	-	-	-	-
R. villarsii	2	4	3.33	2	4	0.26	1	32	5.61*	2	32	3.50*	-	-	-	-	-	-
T. alpinum	2	4	0.81	2	4	3.13	1	32	0.01	2	32	5.07*	-	-	-	-	-	-

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
na, not available
nnd, not normally distributed even after transformation

Table S11.

Chlorophyll concentration **[μg mg^{-1}]**	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**			**N x N**			**O_3 x N x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	0.86	2	4	2.80	1	35	2.40	-	-	-	-	-	-	-	-	-
C. sempervirens	2	4	0.21	2	4	1.14	1	35	229.11***	-	-	-	-	-	-	-	-	-
F. violacea	2	4	1.73	2	4	0.23	1	35	21.56***	-	-	-	-	-	-	-	-	-
G. acaulis	2	4	0.77	2	4	0.01	1	30	26.66***	2	30	2.41	1	30	12.88**	2	30	10.79***
H. versicolor	2	4	0.25	2	4	0.06	1	29	21.58***	2	29	1.78	1	29	0.71	2	29	3.09
L. helveticus	2	4	1.16	2	4	0.87	1	30	41.27***	2	30	2.31	1	30	0.48	2	30	2.42
L. mutellina	2	4	0.47	2	4	4.80	1	35	11.53**	-	-	-	-	-	-	-	-	-
N. stricta	2	4	2.92	2	4	0.72	1	33	20.72***	2	33	3.46*	-	-	-	-	-	-
P. aurea	2	4	2.63	2	4	0.56	1	35	13.32***	-	-	-	-	-	-	-	-	-
R. villarsii	2	4	9.72*	2	4	5.63(*)	1	30	30.98***	2	30	1.56	1	30	0.64	2	30	3.37*
T. alpinum	2	4	2.63	2	4	0.36	1	34	0.03	-	-	-	-	-	-	-	-	-
2006																		
A. montana	2	4	1.96	2	4	20.10**	1	32	24.82***	2	32	3.25(*)	1	32	6.13*	-	-	-
C. sempervirens	2	4	1.22	2	4	6.24(*)	1	35	96.36***	-	-	-	-	-	-	-	-	-
F. violacea	2	4	5.15	2	4	0.61	1	33	23.34***	-	-	-	-	-	-	-	-	-
G. acaulis	2	4	0.71	2	4	4.92	1	35	22.35***	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	0.79	2	4	1.64	1	34	14.38***	-	-	-	-	-	-	-	-	-
L. helveticus	2	4	0.33	2	4	0.00	1	35	37.30***	-	-	-	-	-	-	-	-	-
L. mutellina	2	4	0.55	2	4	5.82(*)	1	30	49.26***	2	30	3.48*	1	30	8.35**	2	30	3.36*
N. stricta	2	4	2.29	2	4	0.23	1	35	12.02**	-	-	-	-	-	-	-	-	-
P. aurea	2	4	1.19	2	4	2.40	1	35	7.75**	-	-	-	-	-	-	-	-	-
R. villarsii	2	4	0.96	2	4	0.36	1	33	6.21*	-	-	-	1	33	4.34*	-	-	-
T. alpinum	2	4	0.08	2	4	0.31	1	32	2.33	2	32	3.04(*)	-	-	-	-	-	-

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.00$

Table S12.

Leaf weight [mg]	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**			**N x N**			**O_3 x N x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	0.29	2	4	0.29	1	34	2.81	-	-	-	1	34	3.73(*)	-	-	-
C. sempervirens	2	4	3.43	2	4	4.36	1	35	69.34***	-	-	-	-	-	-	-	-	-
F. violacea	na																	
G. acaulis	2	4	4.28	2	4	0.94	1	35	0.98	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	0.04	2	4	4.85	1	34	2.21	-	-	-	-	-	-	-	-	-
L. helveticus	2	4	1.48	2	4	11.77*	1	35	0.08	-	-	-	-	-	-	-	-	-
L. mutellina	2	4	0.25	2	4	2.18	1	33	0.04	-	-	-	1	33	3.56(*)	-	-	-
N. stricta	2	4	0.48	2	4	0.90	1	29	1.30	-	-	-	-	-	-	-	-	-
P. aurea	2	4	0.02	2	4	2.32	1	30	7.32*	2	30	1.13	1	30	6.30*	2	30	3.13(*)
R. villarsii	2	4	0.68	2	4	1.97	1	35	2.66	-	-	-	-	-	-	-	-	-
T. alpinum	2	4	0.25	2	4	0.24	1	34	0.06	-	-	-	-	-	-	-	-	-
2006																		
A. montana	2	4	0.06	2	4	0.36	1	32	2.36	-	-	-	-	-	-	-	-	-
C. sempervirens	2	4	1.71	2	4	0.12	1	35	17.46***	-	-	-	-	-	-	-	-	-
F. violacea	na																	
G. acaulis	2	4	0.33	2	4	1.32	1	35	0.51	-	-	-	-	-	-	-	-	-
H. versicolor	2	4	1.77	2	4	2.87	1	30	1.13	2	30	4.03*	1	30	1.59	2	30	3.87*
L. helveticus	2	4	3.60	2	4	7.36*	1	35	0.21	-	-	-	-	-	-	-	-	-
L. mutellina	2	4	0.05	2	4	0.53	1	34	0.84	-	-	-	-	-	-	-	-	-
N. stricta	2	4	1.28	2	4	0.53	1	35	4.76*	-	-	-	-	-	-	-	-	-
P. aurea	2	4	0.97	2	4	6.31(*)	1	34	5.52*	-	-	-	-	-	-	-	-	-
R. villarsii	2	4	6.13(*)	2	4	1.47	1	33	1.21	2	33	3.25(*)	-	-	-	-	-	-
T. alpinum	2	4	1.75	2	4	3.82	1	34	1.79	-	-	-	-	-	-	-	-	-

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
na, not available
nnd, not normally distributed even after transformation

Table S13.

$\delta^{13}C$ [‰]	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	1.44	2	4	2.84	2	12	19.58***	4	12	0.53
C. sempervirens	2	4	2.54	2	4	0.34	2	12	18.94***	4	12	2.16
F. violacea	2	4	0.46	2	4	1.70	2	10	1.59	4	10	0.23
G. acaulis	2	4	0.07	2	4	0.46	2	12	0.92	4	12	1.39
H. versicolor	2	4	0.24	2	4	0.33	2	11	1.17	4	11	0.19
L. helveticus	2	4	3.70	2	4	0.23	2	12	4.97*	4	12	1.14
L. mutellina	2	4	0.90	2	4	1.03	2	12	1.47	4	12	4.73*
N. stricta	2	4	0.80	2	4	1.12	2	12	0.37	4	12	2.28
P. aurea	2	4	2.60	2	4	2.06	2	11	0.86	4	11	1.56
R. villarsii	2	4	0.17	2	4	1.00	2	12	0.77	4	12	2.40
T. alpinum	2	4	0.02	2	4	0.68	2	11	0.54	4	11	0.40
2006												
A. montana	2	4	3.56	2	4	4.37	2	12	6.61*	4	12	1.34
C. sempervirens	2	4	1.67	2	4	0.75	2	12	9.52**	4	12	0.12
F. violacea	2	4	0.15	2	4	4.05	2	12	0.84	4	12	1.51
G. acaulis	2	4	0.57	2	4	3.15	2	12	0.31	4	12	0.41
H. versicolor	2	4	0.48	2	4	1.06	2	12	0.01	4	12	0.81
L. helveticus	2	4	0.35	2	4	1.61	2	12	0.67	4	12	0.41
L. mutellina	2	4	0.33	2	4	10.27*	2	12	0.10	4	12	2.03
N. stricta	2	4	0.09	2	4	0.14	2	12	0.30	4	12	0.08
P. aurea	2	4	0.18	2	4	5.90(*)	2	11	0.26	4	11	2.11
R. villarsii	2	4	0.73	2	4	3.09	2	12	0.20	4	12	1.95
T. alpinum	2	4	0.21	2	4	0.92	2	12	2.03	4	12	1.31

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S14.

$\delta^{18}O$ [‰]	**Block**			**Ozone (O_3)**			**Nitrogen (N)**			**O_3 x N**		
2005	N df	D df	F	N df	D df	F	N df	D df	F	N df	D df	F
A. montana	2	4	3.56	2	4	2.36	2	12	5.13*	4	12	1.20
C. sempervirens	2	4	6.73(*)	2	4	1.60	2	12	1.86	4	12	2.05
F. violacea	2	4	4.75	2	4	0.96	2	9	1.59	4	9	0.61
G. acaulis	2	4	0.59	2	4	0.43	2	12	1.33	4	12	0.69
H. versicolor	2	4	2.63	2	4	2.16	2	11	0.55	4	11	4.89*
L. helveticus	2	4	2.78	2	4	0.44	2	9	1.65	4	9	2.06
L. mutellina	2	4	10.43*	2	4	7.35*	2	8	0.71	4	8	0.74
N. stricta	2	4	6.23(*)	2	4	1.23	2	11	0.45	4	11	0.28
P. aurea	2	4	13.09*	2	4	4.01	2	12	1.30	4	12	2.32
R. villarsii	2	4	2.88	2	4	2.25	2	12	0.51	4	12	0.04
T. alpinum	2	4	1.02	2	4	0.79	2	10	1.17	4	10	3.00(*)
2006												
A. montana	2	4	3.56	2	4	0.02	2	12	0.53	4	12	0.89
C. sempervirens	2	4	3.98	2	4	3.99	2	10	2.67	4	10	1.00
F. violacea	2	4	0.93	2	4	0.06	2	9	3.87(*)	4	9	1.11
G. acaulis	2	4	4.63	2	4	2.18	2	12	0.51	4	12	0.56
H. versicolor	2	4	8.93*	2	4	13.28*	2	12	0.09	4	12	3.56*
L. helveticus	2	4	0.72	2	4	0.34	2	11	2.56	4	11	1.03
L. mutellina	2	4	0.58	2	4	8.97*	2	12	1.28	4	12	0.60
N. stricta	2	4	1.78	2	4	3.55	2	11	2.01	4	11	2.96(*)
P. aurea	2	4	3.25	2	4	1.79	2	12	0.01	4	12	1.03
R. villarsii	2	4	1.72	2	4	1.70	2	12	0.12	4	12	1.35
T. alpinum	2	4	2.97	2	4	0.36	2	10	0.65	4	10	1.06

(*) $P < 0.075$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Chapter 5

Synthesis

1 Aim of the study

The overall aim of this study was to investigate the effects of elevated ozone (O_3) exposure and their interaction with environmental factors, in particular nitrogen (N) deposition, on subalpine grassland vegetation. The first goal was to develop an approach for classifying the O_3 sensitivity of temperate grasslands and to formulate a hypothesis concerning the interactive effect of O_3 and N (Chapter 2). This objective was pursued by means of a literature review, searching for biotic and abiotic factors modifying the sensitivity of herbaceous plants to O_3 at different scales, i.e., from the individual plant level to artificial mixtures and to intact multi-species plant communities. The second and main goal was to test the approach, the hypothesis, and the underlying assumptions in a case study in the field using a factorial experimental design. A subalpine pasture was exposed in a free-air fumigation system to combinations of different levels of O_3 and N. During three years, treatment effects were studied at ecosystem (Chapter 3) and individual species level (Chapter 4).

2 Factors influencing the O_3 sensitivity of grassland vegetation

Most of the literature available for the review reported results from O_3 fumigation experiments exposing isolated plants grown from seed under controlled conditions in open-top chambers (OTC). At the individual plant level, three traits were identified as being the main determinants for O_3 sensitivity: a) stomatal conductance (g_s) controlling O_3 uptake, b) specific leaf area (SLA) determining the mesophyll resistance for O_3, and c) defence capacity as related to resource investments into chemical and physical defence (Bassin *et al.*, 2007a). It was hypothesized that the basis for interactive effects of O_3 and environmental factors is provided by the fact that due to phenotypic plasticity, SLA, g_s and, consequently, O_3 sensitivity may be modulated through altered growth rates by external factors such as nutrient availability (especially N), phenology, and micro-climate.

At the plant community level, the available literature was rare and less conclusive. Artificial, sown communities commonly reacted quickly to elevated O_3 exposure with shifts in relative species composition and/or reduced productivity, while in intact perennial grasslands the responses developed either slowly or were not detectable. By relating these findings to general ecological principles it was assumed that O_3 sensitivity of plant communities is determined by their species-richness, functional type composition, genetic variability, and age.

3 O_3 x N interaction experiment

The results of the literature study underlined the need for experiments in which species-rich, nutrient-poor plant communities would be exposed under realistic edaphic and climatic conditions to elevated O_3 concentrations. Also, the hypothesis suggesting high O_3 tolerance for low-productivity grassland and interactive effects of O_3 and N due to N-induced changes in leaf morphology and physiology was yet untested due to the lack of appropriate field experiments.

A factorial free-air fumigation experiment was established in 2004 at Alp Flix, Sur, Switzerland, at 2000 m asl. 180 monoliths of a *Geo-Montani-Nardetum* pasture were exposed to three levels of O_3 (ambient concentration, 1.2 x ambient concentration, 1.6 x ambient concentration) and five levels of N (ambient with 4, +5, +10, +25, +50 kg N ha^{-1} y^{-1}). At the ecosystem level, aboveground productivity, contribution of functional groups, and development of vegetation greenness assessed by normalized difference vegetation index (NDVI) were measured. Individual species *in situ* performance in terms of parameters related to growth (total chlorophyll (a + b) and N concentrations, specific leaf area (SLA), leaf weight) and the stable istopic composition ($\delta^{18}O$, $\delta^{13}C$) related to gas exchange (photosynthesis, A, and stomatal conductance, $g_{s)}$, were recorded in 2005 and 2006 for the eleven most dominant species of the community (grasses: *Festuca violacea*, *Nardus stricta*, *Helictotrichon versicolor*, sedges: *Carex sempervirens*, forbs: *Ranunculus villarsii*, *Potentilla aurea*, *Leontodon helveticus*, *Ligusticum mutellina*, *Arnica montana*, *Gentiana acaulis*, legumes: *Trifolium alpinum*).

3.1 O_3 effects

After three years, elevated O_3 exposure had no effect on productivity and community composition of the monoliths, suggesting high O_3 tolerance of the subalpine pasture (Bassin *et al.*, 2007b). This result supports the above hypothesis and is probably related to ecosystem characteristics such as the high proportion of stress-tolerant species, low proportion of legumes, high biodiversity, and old age of the community (Bassin *et al.*, 2007a). The absence of O_3 effects might also be due to the ability of alpine species to tolerate oxidative stress (Wildi & Lutz, 1996) or due to the specific O_3 fumigation pattern used here simulating high background concentrations during day and night, but without harmful peak concentrations (Nussbaum *et al.*, 1995). However, analysis of vegetation greenness (NDVI) and individual species' leaf analysis revealed physiological symptoms of O_3 stress, such as accelerated leaf senescence (Ojanperä *et al.*, 1998). Also, elevated O_3 exposure reduced g_s in most of the eleven investigated species, but only in three of them significantly (Bassin *et al.*, in prep.). In contrast to earlier studies suggesting reduced A and g_s to be the major cause for O_3-induced growth reductions (Renaud *et al.*, 1998; Zheng *et al.*, 2002), here, these effects do not correlate with reductions in leaf weight, which was observed in half of the species, neither with growth. It seems that, in agreement with findings from an experiment with elevated CO_2 within a high-altitude *Caricetum-curvulae* community (Körner *et al.*, 1997), in this subalpine ecosystem gas exchange was not a primary limiting factor for growth, thus making the community comparatively unresponsive to O_3 stress in the short-term. Limitation by CO_2 assimilation might be an additional ecosystem trait related to the higher O_3 sensitivity in more productive habitats.

After three years, it seems too early to conclude that effects of O_3 on productivity and species composition will not appear in the long-term, since firstly, old alpine plant communities in general respond extremely slowly to changing environmental conditions (Körner, 2003), and secondly, effects of an altered carbon budget might accumulate over time: For instance, in a previous experiment in a montane perennial grassland the O_3 effect was significant only in the forth year, but in the fifth year, the O_3-induced reduction in productivity amounted already to -25% (Volk *et al.*, 2006). Thirdly, even subtle species-specific differences in O_3 sensitivity may lead to altered community composition in the long-term. Finally, also indirect effects such as altered ecosystem water budgets as a consequence of reduced g_s, or altered soil microbial communities or mycorrhizal infection rates as a consequence of reduced carbon allocation, might affect ecosystem productivity and composition in the longer term.

3.2 N effects

N addition exceeding 10 kg N ha^{-1} y^{-1} caused quick and strong changes in dry matter production and species composition of the monoliths, in favour of the sedges, which accounted alone for the 30% ecosystem productivity increase in the highest N treatment (Bassin *et al.*, 2007b). The productivity of grasses, forbs and legumes remained unaffected by additional N supply. This was confirmed by findings at individual species level, showing that despite substantial N and chlorophyll accumulation, most of the eleven species studied were not stimulated in photosynthetic activity (A), g_s or growth by extra N, except for the sedge *Carex sempervirens* and partly for the forb *Potentilla aurea* (Bassin *et al.*, in prep.). Remarkably, in agreement with findings from alpine studies (Soudzilovskaia & Onipchenko, 2005; Bowman *et al.*, 2006), the shift in community composition was not accompanied by a reduction in species number per monolith, which is in contrast to decreasing species richness generally found in lowland communities (Bobbink *et al.*, 1998). We suggested that limited competition for light in sparse alpine canopies (Körner, 2003) allows unresponsive species to maintain their productivity. Prolongation of the experiment will show whether or not the observed shift in species composition was only a transient phenomenon, as observed by Bowman *et al.* (2006). However, according to Körner *et al.* (1997) it will take several decades to reach a new steady state in such late-successional alpine communities.

3.3 O_3 x N interactive effects

This is the first time interactions of effects of elevated O_3 and N exposure have been tested in a field experiment. In contrast to the initial hypothesis, no O_3 x N interaction was observed in terms of species composition or productivity (Bassin *et al.*, 2007b). In fact, leaf analysis revealed that, possibly as a result of the absent growth stimulation, neither SLA nor g_s were affected by N addition in most of the investigated species; this makes the monoliths at this site an inappropriate model system for investigating the mechanisms of interacting effects of O_3 and N (Bassin *et al.*, in prep.). Further field experiments conducted in plant communities differing in responsiveness to N are needed to gain more insight into this interaction.

However, O_3 x N interactive effects were found on NDVI, a measure integrating leaf area index and vegetation chlorophyll content: elevated O_3 reduced the positive effects of N on canopy greenness. In agreement, individual species analysis revealed a stimulation of leaf chlorophyll content with increasing N addition and a reduction at elevated O_3 due to accelerated leaf senescence. No interactive effects were observed here. This might be due to the fact that dead leaves, which are recorded by NDVI sensors, were not included in the chlorophyll analysis. Overall, NDVI measurements turned out to be a powerful tool to detect non-destructively early physiological changes at ecosystem level.

4 Leaf traits

It is commonly assumed that the responsiveness of plant species to environmental change is related to growth strategy, which in turn is reflected by specific leaf traits (Grime *et al.*, 1996). For instance, the capacity to react with stimulated growth to additional N supply is higher in plant species characterized by high SLA, a trait commonly associated with high potential relative growth rates (Poorter & Garnier, 1999). By investing into thinner leaves with a large area, fast growing individuals successfully outcompete others by efficiently occupying aboveground space when - due to increased N availability - competition for resources shifts to competition for light (Schieving & Poorter, 1999). In agreement, a shift towards higher average SLA was observed in subalpine grassland as a result of fertilization-induced changes in community composition (Quetier *et al.*, 2007). Based on findings from the literature study, it was also suggested that susceptibility to O_3 should be higher in fast-growing species characterized by high SLA and high g_s (see above). Such correlations can be used to predicting community responses to environmental change (e.g., Gross *et al.,* 2007).

Our experimental results were in strong contrast to these assumptions: No relationship was found between the species' initial leaf traits and their response to either O_3 or N. The species most responsive to O_3 (*L. mutellina*, *H. versicolor* and *L. helveticus*) were characterized by intermediate SLA and g_s (Bassin *et al.,* in prep.). This inconsistency may have occurred, firstly, because of the different response parameters used to quantify O_3 effects in earlier studies (Bassin *et al.*, 2007a) and secondly, because the correlation strongly depends on the number and characteristics of the species included. Despite the considerable variation in SLA across the eleven species, they represented only a limited range of growth strategies and plant statures.

The sedge *C. sempervirens*, a stress-tolerant species (Caccianiga *et al.*, 2006), characterized by intermediate SLA and g_s, showed an exceptional growth response to increased N supply. Although several studies from alpine ecosystems found sedges to benefit most from increased N addition (Soudzilovskaia & Onipchenko, 2005; Bowman *et al.*, 2006), the reason for their relative success remains uncertain. The absence of a growth response to N addition in most of the other species studied (see above), suggests an influence of additional growth-limiting factors such as soil phosphorus (P) availability or temperature. We can assume that under these conditions, nutrient storage and investments in nutrient uptake and in structural components prolonging leaf life span are most important for competitive success (Aerts, 1999).

5 Critical levels/loads

For the establishment of critical levels for O_3 and critical loads for N deposition, it is too early to draw firm conclusions after only three years of experimentation. Although the lack of an O_3 response in spite of high cumulative exposures (AOT40) suggests considerable O_3 tolerance of the system, possible alterations in carbon allocation and small species-specific differences in susceptibility to O_3 potentially accumulate over time. The literature review revealed that owing to the lack of free-air fumigation field experiments under realistic conditions, the O_3 sensitivity of intact ecosystems may be overestimated on the one hand, but on the other hand, the risk of O_3 might be underestimated due to the lack of long-term experiments.

Considering the rapid changes in species composition caused by the N addition already at an additional N input of 10, but not at 5 kg ha^{-1} y^{-1}, the current critical load for N deposition set on the basis of expert knowledge at 10-15 kg ha^{-1} y^{-1} for alpine and subalpine vegetation seems to be reasonable. Effects of a potential long-term N accumulation in the system can not be estimated yet (Providoli *et al.*, 2006). Therefore, our experiment, providing one N supply level below the current critical load, will be continued for another four years. Also, considering that the effectiveness of extra N and thus the critical load might depend on P availability (Gordon *et al.*, 2001), a possible P co-limitation will be investigated in a separate small experiment. Finally, it would be necessary to improve the representativeness of the results by numerous smaller N addition experiments using the same vegetation type in a variety of locations differing in edaphic and climatic conditions.

In the rationale it was argued that due to interactive effects between atmospheric O_3 and N deposition an adaptation of the critical levels for O_3 in relation to N deposition would be required. With regard to the observed physiological changes and possible long-term consequences on productivity, prolongation of the experiment and studying aspects of carbon allocation such as root:shoot ratio, defense against oxidative stress, and flowering is necessary. Even if an O_3 x N interaction on productivity and species composition would be observed in the future, the very weak growth response in most species to N addition would limit the possibility to extrapolate the results to more productive ecosystems.

6 Overall conclusions

The work presented here contributes to the overall knowledge of morphological and physiological effects of O_3 and N air pollution on herbaceous plant species. The unique long-term experiment has proven a valuable set-up for testing the interactive effects of O_3 and N under realistic environmental conditions, thus allowing us to extrapolate the results to real plant communities. Considering the results after the first three years, one tends to assume that species composition of this subalpine species-rich pasture is threatened by elevated N deposition rather than from increased O_3 exposure. To protect these habitats from further loss of diversity, measures that have already been successful in reducing NO_x, NH_3 (but also VOC) emissions need to be continued or even reinforced on a national, but more urgently on a global scale. Otherwise, world-wide industrialization and intensification of agriculture would pose a nearly unavoidable threat to subalpine pastures, which are among the last hot-spots of biodiversity in Europe.

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Acknowledgements

Most of the work for this thesis was performed in the free-air fumigation experiment at Alp Flix. This work would not have been possible without the large number of people involved in the installation and support of the fumigation system. Besides many thanks to them, I would like to express my gratitude to:

Dr. Matthias Volk, my project partner, for the creative contribution to the genesis of this project, his enduring support at the research site, his willingness to help anytime, and the inspiring time we spent together on long car trips and at the Alp.

Prof. Jürg Fuhrer, for providing me the exceptional opportunity to write my PhD thesis in my position as permanent employee, for his confidence and for his motivating support.

Prof. Nina Buchmann, for her valuable scientific input and the helpful editing work.

Prof. Alex Widmer, for accepting to act as Co-Examiner.

Dr. Matthias Suter for patiently supporting the statistical analyses.

Karin Soergel and Dr. Roland Werner, the Isolab team at ETH, for their loyalty (despite my status as external PhD student).

The Air Pollution/Climate Group of Agroscope Research Station Reckenholz-Tänikon, for the friendly, joyful and stimulating working environment.

Victoria Spinas and Alfons Cotti, for their assistance and for welcoming us so friendly any time at Alp Flix.

The “Schatzinsel Alp Flix” research foundation and its board member Jürg Paul Müller for providing us access to infrastructure, public relations, and valuable research networks.

My parents, for giving me the curiosity about anything growing and flowering to take along with me and for their semi-voluntary work in the field experiment.

And finally Tobias Schmid, for his endless love and patience.

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