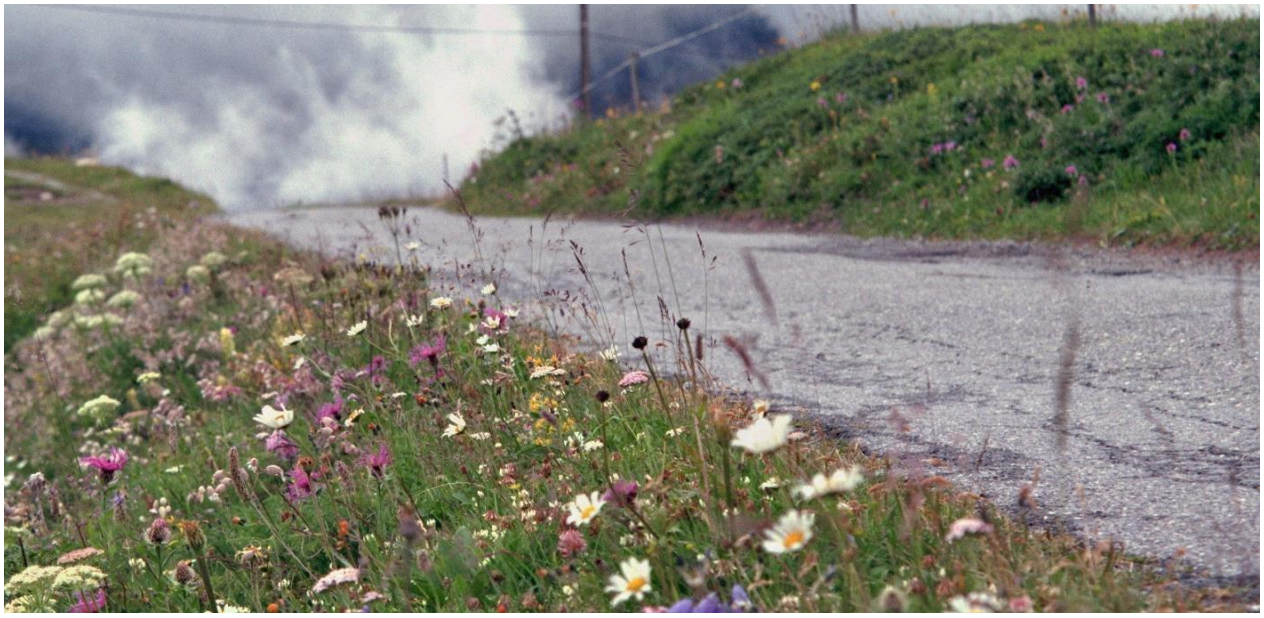

A Study of Changes in Biomass Production and Mycorrhizal Symbiosis in a Subalpine *Geo-Montani-Nardetum* due to Simultaneous Long-Term N Fertilisation and O_3 Fumigation

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Impacts on above and belowground Biomass Production and Mycorrhizal Symbiosis in a Subalpine *Geo-Montani-Nardetum* due to Simultaneous Long-Term N Fertilisation and O_3 Fumigation

Diploma Thesis

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Zusammenfassung

Die weltweit zunehmenden Luftschadstoffbelastungen anthropogenen Ursprungs durch Stickstoff und Ozon wirken sich auch auf die entlegenen Regionen der Alpen aus. Die möglichen Auswirkungen die diese Einträge und ihrer Wechselwirkungen auf die alpine Vegetation haben könnten, wurden bisher kaum im Feld untersucht. Diese Diplomarbeit widmet sich der Frage, wie sich erhöhte Stickstoff- und Ozoneinträge auf die oberirdische und unterirdische Biomasseproduktion eines nährstoffarmen subalpinen Borstgrasrasens sowie auf die Besiedelung der Wurzeln mit Arbuskulären Mycorrhizapilzen auswirken. Dazu wurde zwischen April und August 2010 dreimal Proben in einem Langzeit-Experiment auf der bündnerischen Alp Flix genommen. Sieben Jahre lang wurden dort erhöhte Einträge durch Stickstoffdüngung und Ozonbegasung simuliert.

Für die vorliegende Studie wurde oberirdische und unterirdische Biomasse geerntet und die Besiedelung durch die Mykorrhizapilzstrukturen Mycel, Arbuskel und Vesikel unter dem Mikroskop untersucht. Die Ergebnisse zeigen, dass Stickstoffzugabe zu einer Zunahme sowohl der oberirdischen als auch der unterirdischen der Biomassse führte, während Ozon keinen Einfluss auf die Biomasseproduktion aufwies. Stickstoff und Ozon alleine hatten keine Auswirkungen auf die Wurzelbesiedelung durch Mycel und Arbuskel. Es gab jedoch eine Wechselwirkung von Ozon und Zeiteffekt, die durch eine starke Zunahme der Mykorrhizierung in unbehandelten Proben und eine Abnahme in mit Ozon begasten Proben für den letzten Probezeitpunkt zum Ausdruck kam. Beim Verhältnis von Arbuskel zu Mycel, das auch als Indikator für die Art der Pilz-Pflanzen-Beziehung gesehen werden kann, kam es zu einer Ozon-Stickstoff-Interaktion: Ozonbegasung wirkte sich mit gleichzeitiger Düngung negative auf das Arbuskel:Mycel-Verhältnis aus. Die Besiedelung durch Vesikel nahm sowohl mit Stickstoffdünung als auch mit Ozonbegasung zu.

Hinsichtlich der oberirdischen Biomasseproduktivität konnten vorherige Ergebnisse mit dieser Studie bestätigt werden. Eine Destabilisierung des Bodengefüges aufgrund von reduziertem Wurzelwachstum oder geringerer Mykorrhizierung wurde nicht beobachtet und die Speicherung von Kohlenstoff im Boden beibehalten. Unter gleichzeitiger erhöhter Einwirkung von Stickstoff- und Ozon nahmen die Kosten gegenüber dem Nutzen der Symbiose für die Pflanze zu. Obwohl sich die Auswirkungen von Ozon und Stickstoff als nicht so gravierend darstellten wie ursprünglich angenommen, waren dennoch Tendenzen zu erkennen, die Stoff für weitere Untersuchungen geben.

Keywords

Alpine, Arbuscular Mycorrhizal Fungi, *Geo-Montani-Nardetum*, Nitrogen, Ozone, Root colonisation, Shoot:Root Ratio

Abstract

The possible reaction of plant communities to a simultaneous rise of tropospheric deposition of nitrogen (N) compounds and ozone (O_3) due to human emission has hardly been studied in the field. Aim of this diploma thesis is to investigate the effects of elevated N and O_3 deposition and the impacts of their interaction on the above and belowground biomass productivity and root colonisation by arbuscular mycorrhizal fungi in nutrient poor subalpine grassland. The fieldwork was conducted during three sampling dates from April to August 2010, after seven years of O_3 fumigation and N fertilisation in a long-term field experiment at Alp Flix, Grisons, Switzerland. Above and belowground biomass was harvested and mycorrhizal root colonisation by mycelium, arbuscules and vesicles determined under the microscope. The results showed that N addition led to an increase of both, above and belowground biomass production while O_3 did not have any significant effect on biomass productivity. N and O_3 did not have a main effect on root colonisation by mycelium and arbuscules, but there was a variance in reaction to O_3 treatment through time: percentage of root length colonised differed in August. The indicator of the plant fungus relationship, the ratio of arbuscules to mycelium (A/M) was affected by both treatments interactively: O_3 affected A/M negatively. Abundance of vesicles increased significantly in response to N and O_3 separately. Concerning aboveground biomass productivity previous findings could be confirmed. Concerns that the response to additional N and O_3 would lead to destabilisation of soil through reduced belowground productivity and decreased abundance of mycorrhiza were not approved. As response to both treatments the cost of a symbiosis for plants increased. Even though the results of this study were not as momentous as expected the observed tendencies give subject to further studies.

List of Abbreviations

A/M	Ratio of arbuscules to mycelium
AMF	Arbuscular mycorrhizal fungi
ART	Agroscope Reckenholz Tänikon
a.s.l.	Above sea level
C	Carbon
DW	Dry weights (after 48h at 60°C)
e.g.	Exempli gratia, for example
et al.	Et alii, and other
FW	Fresh weights
LME	Linear mixed effect model
N	Nitrogen
N0	Ambient ammonium nitrate deposition
N50	Ambient ammonium nitrate deposition + 50 $kg*ha^{-1}*y^{-1}$
O_3	Ozone
O_3 Control	Ambient O_3 treatment
O_3++	Treatment with 1.6 x ambient O_3
P	Phosphorous
%RLC	Percentage of root length colonised
%RLCA	Percentage of root length colonised by arbuscules
%RLCM	Percentage of root length colonised by mycelium
%RLCV	Percentage of root length colonised by vesicles
ROOT	Root (Variable)
SHOOT	Shoot (Variable)
S/R	Shoot:Root ratio

Introduction

Due to rising anthropogenic emission, concentrations of tropospheric reactive nitrogen (N) compounds have increased throughout the last century and influenced natural ecosystems in different ways (Matson et al. 2002, Scheffer & Schachtschabel 2002), even in commonly remote regions like the Swiss Alps (Körner 2003). Concomitantly and in further consequence to photochemical oxidation processes an increase of tropospheric ozone (O_3) can be observed, especially in elevated mountain regions (Matson et al. 2002, Scheffer & Schachtschabel 2002, Vingarzan 2004). Assumptions arise, that impacts of this altered tropospherical chemistry could lead to a decline of the plant community's robustness and result in a change of nutrient poor alpine ecosystems (Körner 2003, Wookey et al. 2009).

As a limiting element in many natural ecosystems, N is taking a key role concerning plant metabolism, diversity of species composition, dynamics and function of numerous ecosystems (Vitousek et al. 1997, Matson et al. 2002, Körner 2003). Long-term N fertilisation experiments on nutrient poor alpine pasture in the mid 20th century for example, still revealed considerable effects forty years after treatment (Ellenberg 1996). N fertilisation can alter circumstances of competition by shifting ecosystems from nutrient limited to light limited conditions. Nitrophilic plants profiting from this change reduce their allocation of carbon (C) compounds belowground in favour of augmented aboveground allocation. This can result in an increased shoot:root-ratio (S/R) (Craine 2005, Johnson et al. 2008).
Being an essential phytotoxic air pollutant in the Northern Hemisphere (Vingarzan 2004), the impact of O_3 on plants reveals itself in restricted plant growth and damaged cell structures, lower photosynthetic activity and less resistance to cold (Ellenberg 1996).

Hitherto, few is known about the response of alpine plant communities to simultaneous elevated N and O_3 deposition and their possible interactions are hardly studied (Bassin et al. 2009). Considering the different responses of plants to additional N and O_3, a simultaneous rise of both pollutants could reveal contrasting effects: It is possible that N fertilisation intensifies O_3 uptake through augmented stomatal conductance and Specific Leaf Area, and thus increases plant damage by O_3. Apart from that, additional N could as well improve the plant's capacity to withstand O_3 damage through enhanced photosynthesis (Bassin et al. 2007, Jones et al. 2010).

To improve their access to limiting soil nutrients, mainly phosphorous (P) but also N compounds, more than 80% of all terrestrial plant species form mutualistic symbioses with fungi, predominantly with arbuscular mycorrhizal fungi (AMF) from the fungal phylum Glomeromycota (Smith & Read 1997, Wang & Qui 2006). Besides being beneficial for plants by means of increased nutrient uptake, AMF improve also water uptake of plants and can protect them against pathogens. With their influence on individual plant level, mycorrhiza can manipulate plant community structure and species composition (Hartnett et al. 1999). They also play an important role in soil cycles and soil stability, as well as for C fixation belowground (Brundrett 1991, Scheffer & Schachtschabel 2002, Bardgett et al. 2005,Rillig & Mummey 2006).

While AMF are obligate symbionts, the plant's dependency on the trade of self-generated photosynthates for fungal-acquired soil nutrients can range from non-mycorrhizal to facultative or obligate (Brundrett et al. 1996, Klironomos 2003, Bidartondo 2005). The host-symbiont relationship is generally mutualistic, however sometimes can be expressed in a parasitic way too (Klironomos 2003).
Within and between plant root cells penetrating fungal mycelium takes up C compounds from the plant (Douds et al. 2000) signifying the plant's cost of the symbiosis (Fitter 1991, Dekkers & van der Werff 2001). Responsible for the plant's benefit of nutrient uptake by AMF, are

tree-shaped fungal structures inside plant cells, so-called arbuscules (Dekkers & van der Werff 2001, Strack et al. 2003). Therefore, the ratio of arbuscules to mycelium (A/M) may indicate a benefit to cost ratio for the plant and may be seen as indicator for the manner of the plant-fungus relationship (Dekkers & van der Werff 2001). Further morphological fungal structures formed by some AMF species are intracellular vesicles, which are lipid-rich storage organs (Strack et al. 2003). A plate of all described AMF structures is found in Appendix 1.

Despite the important role of AMF for nutrient supply (Smith & Read 1997), little is known yet about their role in ecosystem change due to elevated N (van der Heijden et al. 2008). N fertilisation may provide plants a sufficient access to N and therefore reduce their need for a mycorrhizal symbiosis: It may result in a reduction of belowground C allocation and thereby in decreased mycorrhizal colonisation (Blanke et al. 2005, Johnson et al. 2008).
O_3 fumigation might not induce direct effects on AMF, but indirect secondary effects via the plant response (McCool & Menge 1984). As it is assumed that O_3 reduces below-ground C allocation it can also have negative effects on mycorrhizal symbiosis (Yoshida et al. 2001, Jones et al. 2010). However, there is still a lack in understanding effects of O_3 on soil processes and AMF (Yoshida et al. 2001), especially in alpine biomes where general research about AMF has sparsely been carried out (Cripps & Eddington 2005).

While plant nutrient uptake remains more or less stable in the case of reduced mycorrhizal colonisation due to fertilisation, a reduction caused by O_3 may impair the plant's access to nutrients (Andersen 2003). Assuming that plants reduce C allocation to the roots in consequence of fertilisation induced O_3 damage, a concomitant rise of N and O_3 may induce an even stronger decrease of colonisation rate than the rise of one pollutant alone would do. On the other hand, if N would mediate O_3 damage, enhanced photosynthesis may lead to a lesser decrease of mycorrhizal colonisation rate.

The aim of this study is to determine the effects upon aboveground biomass production and belowground biomass production, upon shoot:root ratio (S/R) as well as upon root colonisation by AMF, caused by simultaneous long-term deposition of elevated N and O_3 on an alpine pasture in the Swiss Alps.

It was expected that
(I) N fertilisation would a) increase S/R as it would lead to an increase in aboveground biomass production but not in belowground biomass production and furthermore b) reduce colonisation by AMF.
(II) Elevated O_3 conditions would a) reduce aboveground and belowground biomass production and b) result in a reduction of AMF colonisation.
(III) Simultaneous N and O_3 elevation would lead to interactive effects a) on biomass production and b) mycorrhizal colonisation.

This study is part of a research project, established in autumn 2003 by scientists from the Air Pollution and Climate Group of the ART Research Institute of the Swiss Federal Office for Agriculture in Zurich, to investigate the effects of elevated N and O_3 deposition and the impacts of their interaction on subalpine grassland (Bassin et al. 2007).

Materials and Methods

The **study site** was situated at a plateau called Alp Flix, 2000 m a. s. l. near Sur, Grisons, Switzerland (9°39′N / 46°32′E). The area is characterized by an alpine climate with a mean annual temperature of 2.8°C, a permanent snow cover during winter and vegetation period from April to October. Highest mean temperatures of 9 - 10°C are recorded during the summer months July and August. The annual sum of rainfall is about 1200 mm, which also peaks in summer (120-140 mm / month). Alp Flix is used extensively as pastures and hay meadows (Bassin et al. 2007). The

dominating vegetation, a *Geo-Montani-Nardetum*, is typical for this altitude above sea level, for the use as pasture and for the slightly acidic soil (pH 4.8 - 6) (Ellenberg 1996). Grasses like *Festuca violacea* SCHLEICH. ex GAUD. and *Nardus stricta* L. and sedges like *Carex sempervirens* VILL. (Schmeil & Fitschen 2006) are the most abundant species and make up about 50% of the vegetation cover, while more than 70 forbs species and some legumes account for the rest of the cover. Rooting depth reaches down to about 10 cm. Bedrock to the 20 - 40 cm deep Cambisol soil is Serpentinite (Bassin et al. 2007).

The experiment consisted of nine rings (Ø 7 m) of a free-air-O_3-fumigation system with three different levels: O_3 Control = ambient air (ca. 47 ppb); O_3+ = 1.2 × ambient; O_3++ = 1.6 × ambient. Rings were set up in a row on a small ridge and arranged at random in three blocks. Every treatment was found in each block (for details see Appendix 2). Each ring contained 20 monoliths (40 cm long × 30 cm wide × 20 cm deep) of a *Geo-Montani-Nardetum* from a nearby pasture. To simulate tropospheric N deposition, 200 ml of aqueous solutions of ammonium nitrate (NH_4NO_3) in five different concentrations were applied every two weeks to the different monoliths during the vegetation period. Always four monoliths of the same concentration, N0 = ambient (= ~ 4), N5 = +5, N10 = +10, N25 = +25 and N50 = +50 kg*ha^{-1}*yr^{-1}, were allocated randomly within each ring.
Once a year in the beginning of August, shoot biomass was harvested from all monoliths 2 cm above the surface. For more detailed information about the free-air fumigation system see Volk et al. 2003, about the experiment and set-up at Alp Flix see Bassin et al. 2007.

The **field work** presented in this study was accomplished in 2010, the seventh and last year of treatment. At May 15th, July 12th and the 30th of August, soil cores with a height of 10 cm and a diameter of 6 cm were taken with an adequate sampler from monoliths with N0 and N50 treatment, situated in the rings with O_3 Control and O_3++ fumigation. Two cores at a time were extracted from each of the 36 sampled monoliths (see Appendix 3).
The 72 cores were kept cool in boxes and taken to the laboratory in Zurich on the same day, where they were stored at 4 °C and processed as soon as possible. First, vegetation cover was recorded as estimated percentages of the following divisions: sedges, grasses, forbs, bare soil, mosses, lichens and rock. The last four divisions contribute to the core surface that is not covered by vascular plants and are referred to together as "not covered".
Samples were processed following the block order from the ridge at Alp Flix. Therefore a possible block effect describes not only effects caused by different ring positions in space but also variation in processing and effects caused by different processing time.

Aboveground biomass was collected from each core (Appendix 3), separated from the plant litter and dried to constant weight at 60°C. In July aboveground biomass was harvested in two portions because data was needed for a parallel study: First, shoot biomass above 2 cm was collected, then below 2 cm.

For methodological reasons (different parallel projects, representativeness) the two cores of each monolith were cut into halves. Two halves, one half of each core, were merged into only one sample per monolith. One sixth of each remaining half was kept and dried at 60°C to serve as calculation reference. Prior to further processing of the samples, soil was gained for a parallel project by sieving on a sieve with 2 mm mesh size. Roots were separated with tweezers and finer roots that passed the sieve were recaptured during further processing of the soil.

Belowground biomass of the merged sample was acquired by washing and rinsing the roots on a sieve with 0.063 mm mesh size (Appendix 3). The parts of leaves and stems which were embedded in the topsoil layer, e.g. from sedges or tussock grasses, were obtained after washing process, separated from the roots and amalgamated with aboveground biomass.

A subsample of about 2 g of the homogenised root biomass was fixed in a mixture of 70 ml of 25% Glutaraldehyde and 430 ml of Sørensen's phosphate buffer solution (0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 (1:9)) to examine the root colonisation by AMF by further investigation under the microscope. By recording fresh weights of total root mass, root mass without subsample (rest) and constant dry weights at 60 °C of the rest, total root biomass could be calculated for each sample using the rule of three.
Similarly, it was possible to calculate the average root mass per core using fresh weights of the complete soil cores, of the halves before merging, of the reference sixths and of the dry weights of the latter (see Appendix 4 for calculation details). All weights were finally converted into kg / m^2.

For **microscopical examination** fixed roots were bleached for 15 min in 10% potassium hydroxide at 90°C, rinsed with tap water twice, acidified for 10 min in 3.7% hydrochloric acid and then stained for 5 min in a 5% ink-vinegar solution (5 ml Pelikan royal blue in 95 ml 5% acetic acid (household vinegar)) at 90°C. Decolourization of the plant cell structures in pure vinegar for 30 min made mycorrhizal structures visible.
Only root segments with a diameter smaller than 1 mm were examined under the microscope. 500 fields of view of each sample were checked for the presence or absence of mycorrhizal structures at 100fold magnification with a Leika Laborlux S light microscope using the line-intersect method (McGonigle et al. 1990) in a modified way. Percentage of root-length colonised (%RLC) by entire mycelium, arbuscules and vesicles could be calculated.

Data were digitalized with MS Office Excel 2007. For **statistical analysis** R 2.12.0 was the software of choice. As always three repeated measures of each of the 36 sampled monoliths were examined, altogether 108 samples entered statistical analysis. A linear mixed effect model (LME) was a suitable statistical approach for analysing if there were any significant effects of elevated N and O_3 concentrations on the dependent variables: shoot (SHOOT) and root (ROOT) biomass, S/R, %RLC by entire mycelium (%RLCM), %RLC by arbuscules (%RLCA), %RLC by vesicles (%RLCV) and A/M. Fixed factors were Block, O_3, N and Time, while Ring and Subunit (= monolith number) entered the statistics as a random variable. In appropriate cases, cover fractions of the different vegetation divisions were used as co-variables. Predominant presence of grasses was assumed to be the usual state. Co-variables to some analyses were also soil pH-values, which were measured in solution of $CaCl_2$ in July 2010.
To fulfil needed assumptions of LMEs, data were checked for normal distribution (Shapiro-Wilk, Shapiro-Francia, Cramer van Mises, Lilliefors (Kolmogorov-Smirnov), Anderson-Darling and Pearson chi^2 normality test) and homoscedasticity (descriptive method). For the variables SHOOT, ROOT, S/R and %RLCA a logarithmic transformation was used to reach normal distribution. %RLCV data was square root transformed and data of A/M by using arcsine function. Only %RLCM data did not need transformation.
To test for significances of factor effects, of their interactions and of co-variables, model comparisons were carried out by testing one LME against another using likelihood ratio tests. Results with a p-value less than 0.07 were considered as trend, lower than 0.05 were considered as significant, inferior to 0.01 as very significant and lower than 0.001 as highly significant. Figures and graphics were created using MS Excel 2007 and MS Word 2007.

Results

The factor Time produced significant p-values for all three variables concerning biomass production: SHOOT, ROOT and S/R. Furthermore there were significant N effects on overall biomass production. (Tab. 1, Fig. 1). The time effect on **SHOOT** resulted in highest biomass in July for both N treatments, while May and

Tab. 1: Effects of the factors Block, O_3, N and Time and of the co-variables (Forbs, Not Covered, pH) on **biomass production** are displayed and levels of significance indicated with asterisks as follows: $^{(*)}$p<0.07, * p < 0.05, ** p < 0.01, *** p < 0.001. Data (log transformed) were analysed with linear mixed effect models; models were compared by performing likelihood ratio tests in R: **a)** Effects on shoot biomass (SHOOT) **b)** Effects on root biomass (ROOT) **c)** Effects on shoot:root Ratio (S/R).

		a) SHOOT (log)			b) ROOT (log)			c) S/R (log)		
Variable	Df effect	Likelihood ratio	p-value	Estimate (log)	Likelihood ratio	p-value	Estimate (log)	Likelihood ratio	p-value	Estimate (log)
Block	2	5.2871	0.0711		2.4096	0.2997		8.1098	0.0173 *	
O_3	1	0.0027	0.9582		2.2845	0.1307		0.9375	0.3329	
N	1	14.2173	0.0002 ***		8.8968	0.0029 *		6.1707	0.013 *	
Time	2	25.3767	<0.0001 ***		8.3889	0.0151 *		30.4742	<0.0001 ***	
O_3 x N	1	0.1964	0.6576		0.7655	0.3816		0.8425	0.3587	
O_3 x Time	2	0.9250	0.6297		1.4880	0.4752		2.1667	0.3385	
N x Time	2	0.2708	0.8734		7.1523	0.028 *		4.3574	0.1132	
O_3 x N x Time	2	0.6914	0.7077		2.8908	0.2357		3.5327	0.171	
Forbs	1	18.9173	<0.0001 ***	-0.0036	0.7942	0.3728	-0.0005	13.1736	0.0003 ***	-0.0034
Not covered	1	5.5190	0.0188 *	-0.0036	Not tested	Not tested	Not tested	1.8977	0.1683	-0.0024
pH	1	Not tested	Not tested	Not tested	1.3788	0.2403	0.0302	0.0104	0.9187	-0.0047

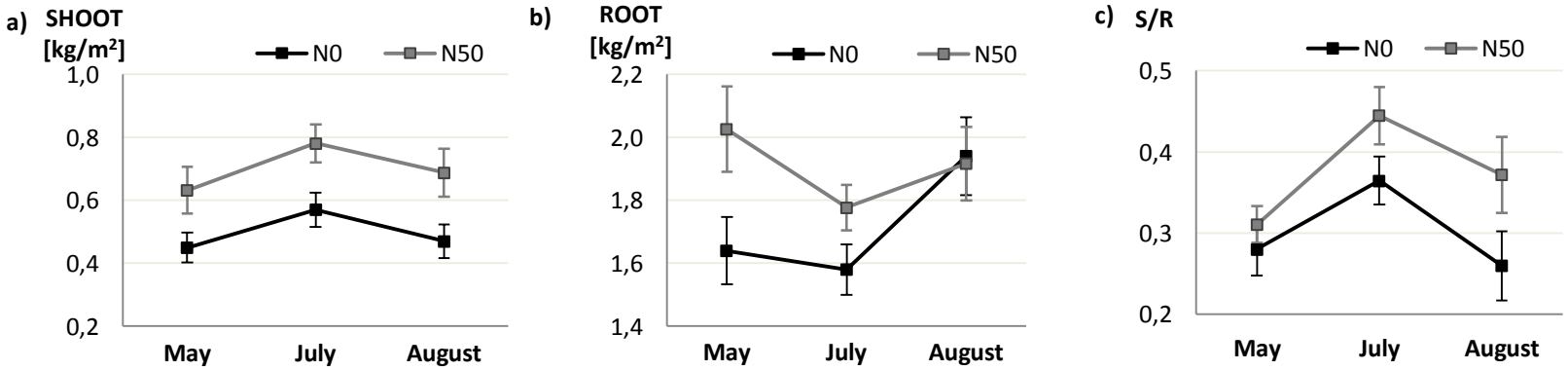

Fig. 1: Time effects and impacts of N treatment averaged over O_3 treatment: **a)** on shoot biomass (SHOOT) **b)** on root biomass (ROOT) **c)** on shoot:root-ratio (S/R). Mean values ± standard errors are displayed. N0= ambient N deposition, N50=fertilisation with 50kg*ha^{-1}*yr^{-1}.

August displayed resembling values. Comparison of the two portions of SHOOT in July shows that a major part (78.6%) of SHOOT is situated between soil surface and 2 cm above soil surface (Fig. 2). N fertilisation led to SHOOT biomass allocation that was about 50% higher than unfertilised SHOOT (Fig. 1a)). Tested co-variables influenced SHOOT significantly. It declined with rising forb cover fraction and percentage of core surface not covered (Tab. 1 a)).

Time and N had a significant effect on **ROOT** and their interaction produced a significant p-value too (Tab. 1b)). Values averaged over time increased only about 10%, from 1.720 kg / m^2 in N0 monoliths to 1.906 kg / m^2 in N50 monoliths. However, giving a closer look to the change of ROOT with time, a huge difference between N0 and N50 reveals itself in May. This difference diminished with time until in August samples of both N treatments were almost equal, N50 = 1.916 kg / m^2 and N0 = 1.940 kg / m^2. Lowest ROOT for both N treatments was found in July (Fig. 1b)).

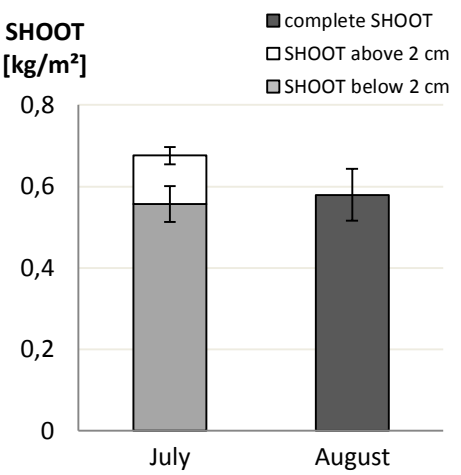

Fig. 2: Difference of aboveground biomass production (SHOOT) between July and August at Alp Flix. Displayed are mean values averaged over all treatments ± standard errors.

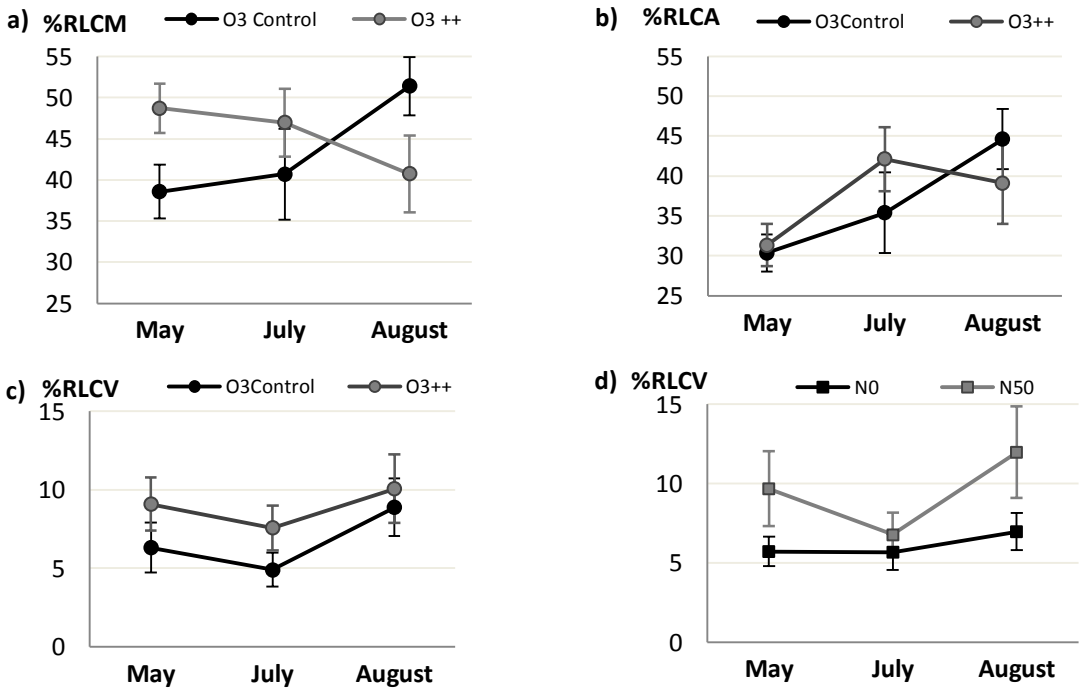

Fig. 3: Time effects and impact of O_3 fumigation averaged over N treatment on **a)** Root-Length colonised by mycelium (%RLCM) **b)** Root-Length colonised by arbuscules (%RCLA) **c)** Root-Length- colonised by vesicles (%RLCV). **d)** Time effect and impact of N fertilisation averaged over O_3 on %RLCV. **a)-d)** Mean values ± standard errors are displayed. O3 Control= ambient O_3 deposition, O3++= 1.6 x ambient; N0= ambient N deposition, N50= ambient + 50kg*ha^{-1}*yr^{-1}.

O_3 treatment caused neither a significant effect on SHOOT nor on ROOT and there were also no significant N × O_3 interactions on biomass production (Tab. 1 a) and b)).

All examined variables with regard to the **mycorrhizal root colonisation** except %RLCV, resulted neither in a main effect due to N fertilisation nor due to fumigation with O_3. But the factor Time produced highly significant p-values for all examined mycorrhizal structures. Moreover, all variables relating to mycorrhizal colonisation apart from A/M produced a significant Block effect. (Tab. 2 a-d)). On average, Block 2 had lower %RLC for all mycorrhizal structures.

The main Time effect on **%RLCM** was expressed in a slight increase from May to August. There was also a significant response to O_3 fumigation through time: In May and July %RLCM in O_3++ was higher than in O_3 Control. This was different in August, where O_3++ was lower than O_3 Control. O_3++ led to a steady decrease of %RLCM from May to August, while O_3 Control induced an increase on %RLCM with time (Fig. 3 a)). Only on the margin to significance (p=0.063) but nevertheless visible (Tab. 2, Fig. 3 a)) is this O_3 × Time interaction on %RLCM. Co-variables pH value and percentage of surface covered by forbs significantly affected %RLCM (Tab. 2 a)). With increasing forb cover and rising pH values %RLCM declined.

Averaged over all treatments %RLCA increased steadily from May to August. In addition it was significantly influenced by an O_3 × Time interaction (Tab. 2 b), Fig. 3 b)): O_3++ was highest in July, while May and August were lower. O_3 Control rose continuously from May to August (Fig. 3 b)). Equivalent to %RLCM, pH and cover of forbs affected %RLCA.
The factor Time affected **A/M** significantly (Tab. 2 c)). An average of all sampled treatments had lowest A/M in May and a peak in July (Fig. 4 a)). There was also a significant N x O_3 interaction on A/M (Tab. 2 c)): In unfertilised monoliths A/M was resembling for both O_3 treatments when averaged over time. In fertilised monoliths however, A/M differed between the two O_3 treatments, as O_3++ led to a decrease of A/M (Fig. 4 b)). Corresponding to %RLCM and %RLCA, A/M decreased significantly with declining pH.

Analysis of **%RLCV** displayed no interactive but only main effects. Block, Time, N and O_3 had significant influence (Tab. 2 d)). In July %RLCV was lowest. Furthermore both, N50 and O_3++ treatment led to higher %RLCV (Fig. 3 c) and d)).

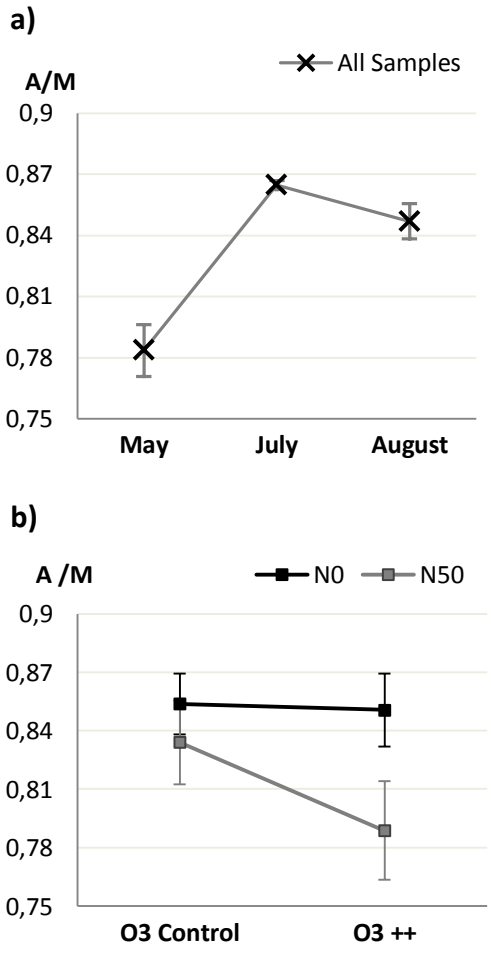

Fig. 4: a) Variation of the ratio of arbuscules to mycelium (A/M) with time is shown. Displayed are means of all samples ± standard errors. **b)** N x O_3 Interaction plot displaying A/M in colonized roots. Displayed are means averaged over time ± standard errors. O3 Control = ambient O_3 deposition, O3++ = 1.6 x ambient deposition; N0= ambient N deposition, N50= fertilisation with 50kg*ha^{-1}*yr^{-1}.

Tab. 2: Effects of the factors Block, O_3, N and Time and of the covariables (Forbs, pH, Sedges) on **mycorrhizal colonisation** are displayed and levels of significance indicated with asterisks as follows: $^{(*)}$ p < 0.07, * p < 0.05, ** p < 0.01, *** p < 0.001. Data were analysed with linear mixed effect models; models were compared by performing likelihood ratio tests in R: **a)** Root-Length Colonised by Mycelium (%RLCM) **b)** Root-Length Colonised by Arbuscules (%RLCA, log transformed) **c)** Ratio Arbuscules to Mycelium (A/M, arcsine transformed) **d)** Root-Length Colonised by Vesicles (%RLCV, sqrt transformed)

		a) Root-Length Colonised by Mycelium			**b) Root-Length Colonised by Arbuscules (log)**		
Variable	Df effect	Likelihood ratio	p-value	Estimate	Likelihood ratio	p-value	Estimate (log)
Block	2	12.8381	0.0016 **		9.0994	0.0106 *	
O_3	1	2.6848	0.1013		1.1258	0.2887	
N	1	0.1916	0.6616		2.0102	0.1562	
Time	2	15.8522	0.0004 ***		20.5867	<0.0001 ***	
O_3 x N	1	0.4467	0.5039		2.5900	0.1075	
O_3 x Time	2	5.5289	0.063 $^{(*)}$		8.9619	0.0113 *	
N x Time	2	0.8663	0.6485		1.9857	0.3705	
O_3 x N x Time	2	2.2588	0.3232		1.2941	0.5236	
Forbs	1	5.6656	0.0173 *	-0.1851	4.9786	0.0257 *	-0.0019
pH	1	11.8561	0.0006 ***	-13.6993	15.5157	0.0001***	-0.1922
Segdes	1	0.0232	0.879	-0.0092	0.1536	0.6951	-0.0003

		c) Ratio of Arbuscules to Mycelium (arcsine)			**d) Root-Length Colonised by Vesicles (sqrt)**		
Variable	Df effect	Likelihood ratio	p-value	Estimate (arcsine)	Likelihood ratio	p-value	Estimate (sqrt)
Block	2	3.3515	0.1872		9.5382	0.0085 **	
O_3	1	0.9925	0.3191		5.3221	0.0211 *	
N	1	1.8639	0.1722		3.8797	0.0489 *	
Time	2	12.5280	0.0019 **		8.8718	0.0118 *	
O_3 x N	1	5.1920	0.0227 *		1.6831	0.1945	
O_3 x Time	2	0.9909	0.6093		1.9412	0.3788	
N x Time	2	0.7268	0.6953		2.1118	0.3479	
O_3 x N x Time	2	2.7879	0.2481		1.0930	0.579	
Forbs	1	0.0521	0.8194	-0.0003	1.8393	0.175	-0.0079
pH	1	7.4907	0.0062 **	-0.1970	0.4505	0.5021	0.2447
Segdes	1	0.8664	0.352	-0.0009	0.0591	0.8079	0.0012

Discussion

The observed growth stimulation of **aboveground biomass** in response to N fertilisation is a common feature (e.g. Bowman et al. 1995, Ellenberg 1996, Soudzilovskaia et al. 2005, Bassin et al. 2007), but occurs not necessarily (Cunha et al. 2002, Bowman et al. 2006). The increase in shoot at Alp Flix caused by N fertilisation indicates that N is a limiting factor at the site. With additional N plants were able to overcome nutrient limitation and invest more biomass aboveground to meet the needs of the now occurring light limitation (Johnson et al. 2008). This corresponds to the findings for alpine environments of Soudzilovskaia et al. 2005 and Bassin et al. 2007.
Since declining fraction of forb cover coincided with rising shoot biomass, the conclusion could be drawn, that graminoides, which made up the dominant fraction of vegetation (Bassin et al. 2007) also constituted the major part of aboveground biomass. Other studies in alpine environments reported analogous, especially for sedges (e.g. Bowman et al. 1993, Soudzilovskaia et al. 2005).

Besides the response to N fertilisation, a seasonal behaviour of aboveground productivity was observed: low shoot biomass in May and August and a peak in July. This corresponds to the common phenology for plants in alpine environments, where annual shoot biomass production begins with birth of new leaves at the start of vegetation period, then is maintained stable and finally ends with dieback of older leaves (Körner 2003). However, the total harvest of aboveground biomass for a parallel study in the beginning of August also contributed to reduce shoot biomass in August. Even when taking in mind that plants were cut only above 2 cm and that the major part of biomass still remained on the monoliths, the difference in shoot biomass from July to August arises obviously from the harvest in July.

That there was no significant effect of O_3 on aboveground productivity after seven years of fumigation treatment designates the examined plant community to be very tolerant towards O_3. The missing response may have its origin in the well-adapted stress-tolerance of alpine plants in general (Körner 2003). But it could also be the result of a moderate constant simulation of depositions (about 60-70 ppb in O_3++ monoliths) at Alp Flix (Bassin et al. 2007) without plant harming peak concentrations (e.g. up to 105 ppb in Jones et al. 2010). However, Volk et al. 2006 suggested responses of temperate grassland to moderate long-term O_3 exposition to occur cumulative after several years. Probably exposition to O_3 in this experiment was not long enough and cumulative effects might have occurred later.

Processes affecting root growth in alpine vegetation are hardly studied and poorly understood (Jackson et al. 1996, Körner 2003). That O_3 did not affect **belowground biomass** could be due to similar reasons like for shoot.

The general finding that N fertilisation leads to an increase in shoot biomass production but not in root biomass production (e.g. Zerihun 1998, Andrews et al. 2001) could not be approved for this study at Alp Flix. On the contrary, consistent with the findings for arctic tundra of van Wijk et al. 2003, belowground production increased significantly. This may be explained by a strategy of exaggerated nutrient consumption, developed by otherwise slow growing nutrient limited alpine species. As nutrient supply in alpine ecosystems is not continuous but cumulative in time and/or space, plants might have developed such strategies to be able to profit from ephemeral high nutrient supply e.g. during snowmelt (Körner 2003, van Wijk et al. 2003). Fertilisation with the limiting nutrient, in this case N, could trigger this strategy and may have led to the observed increase in root biomass.
Another possible process could be that N fertilisation initiates limitation of another nutrient, e.g. P. To improve their access to the newly limited nutrient, plants might

use photosynthates acquired due to fertilisation to invest belowground.

At peak growing season in July lowest root biomass was obtained. This reflects possibly a seasonal course (Körner 2003): Roots that died during winter were still present in May but could have been decomposed by soil organisms in July, when biomass allocation preferably might have been aboveground and not belowground due to peak growing season. Hence, if re-growth of roots was not as fast as decomposition, lower root biomass in July could be explained. In the end of August allocation of biomass to belowground structures for hibernation could be reflected by the again higher root biomass (Bardgett et al. 2005)

An explanation of why belowground productivity differed widely between N treatments in May but not in August might be, that N50 treatment led to belowground investment in persistent root structures that can survive dormancy during winter and live up to 20 years (Körner 2003), whereas N0 might have formed nutrient capturing young side- or adventitious roots with fast turnover. As root composition was not examined, this can only be speculated about.

Plants in alpine environments have considerable parts of their biomass below ground because they invest more in roots and less in stems (Jackson et al. 1996, Fisk et al. 1998). This was also true for the vegetation examined in this study. Plants obviously favoured investment in belowground and not in aboveground structures, what may have enhanced their ability to survive climatic stress, increased their tolerance to strong grazing pressure (Körner 2003) and may also indicate nutrient limitation (Frey & Lösch 2004) at Alp Flix.
Other experiments in alpine environment (Körner 2003) gave reason to expect **shoot:root ratio** values to be higher than 0.5. But that was not the case, not even in fertilised monoliths. An increase in shoot:root ratio due to fertilisation leads to the suggestion that shoots benefited more from the extra nutrient supply than roots. This conforms with the idea that nutrient addition shifts competition from belowground competition for nutrients to aboveground competition for light respectively photosynthetic C compounds (Craine 2005, Johnson et al. 2008). Shoots might benefit from being closer to the source of photosynthates through stimulated growth (Zerihun et al. 1998, Andrews et al. 2001).

The peak of shoot:root ratio in July is a consequence to the seasonal course of shoot and root. A decline of shoot:root ratio with rising forb cover most likely reflects the higher level of performance of graminoides also observed in other studies (Soudzilovskaia et al. 2005, Bassin et al. 2007).

In the present study, about twice as much root biomass was measured than was found in another study using samples from the same pasture at Alp Flix where the monoliths for the present study originated from (J. Leifeld personal communication). The pasture was still in use for cattle grazing from June to September, while the monoliths were cut only once a year since seven years. Several studies in different ecosystems about grazing impact on root biomass productivity however, gave no evidence that grazing reduced belowground productivity (McNaughton et al. 1993, Milchunas & Lauenroth 1993, Pucheta et al. 2004).
If the possibility of a cattle grazing effect might be ruled out, other possible influences have to be considered. In contrast to vegetation at the original pasture, monoliths were embedded belowground in plastic boxes and protected on the surface with wire mesh during winter. This might have influenced vegetation as disturbance of voles (Gervais et al. 2010) or other soil perturbing animals could be excluded from the monoliths. Imaginable is also that the boxes affected soil water content or soil composition.

About the reasons of root biomass difference between original pasture and monoliths can only be speculated. It is an example to show that advanced research

on belowground processes and dynamics is necessary as they are understood least. This is also true for mycorrhizal symbiosis in alpine environments (Jackson 1996, Wookey et al. 2009) even though it is known, that most alpine plants form mutualisms with AMF (Cripps & Eddington 2005)

Highest **%RLC values for all mycorrhizal** structures were obtained in August. For the present study C movements inside the plants were not examined directly but the results suggested that increased C allocation belowground in root and storage structures as preparation for hibernation (Körner 2003) stimulated mycorrhizal colonisation (Bardgett et al. 2005).
A steady increase of %RLC by mycelium and arbuscules with time in O_3 Control treatment reflects natural AMF behaviour during vegetation period when plants might invest actively in the symbiosis to improve nutrient uptake for enhanced growth.
According to Schadt et al. 2003 fungi are most abundant during winter in alpine environments. Even when Schadt et al. 2003 found out that AMF contributed only 10% to wintery fungal community it may be an explanation why %RLC by mycorrhizal fungi was highest in August at Alp Flix.

Bassin et al. 2007 stated a significant increase in sedges at Alp Flix caused by N fertilisation. Species composition of roots was not examined for the present study, but it might reflect the composition of shoots (van Wijk et al. 2003). Commonly Cyperaceae are said to be non- or less-mycorrhizal (Wang & Qui 2006) and consequently V. Blanke (personal communication) found no evidence of mycorrhizal symbioses in sedges from Alp Flix. A rising proportion of non-mycorrhizal sedge roots in fertilised monoliths might give reason to expect a noticeable reduction of **%RLC by mycelium**. However, this was not the case; there was no significant N effect.

The picture that could be drawn from the fact that there was no visible change in %RLC by mycelium due to fertilisation might be as follows: Even if the proportion of root that forms symbiosis with AMF was reduced, this proportion might nevertheless be more colonized and therefore compensate the lesser proportion of symbiotic host root. It is on the one hand possible, that not only plants but also AMF themselves were limited by N and with additional N supply were able to overcome limitation and build up more biomass (Treseder & Allen 2002). On the other hand it is likely that due to N addition, limitation of a different nutrient, e.g. P was intensified and mycorrhizal colonisation stimulated to enhance nutrient uptake.

Contrary to expectations, O_3 fumigation alone did not have a significant influence on the percentage of mycorrhizal colonisation. Since AMF may respond to O_3 via vegetation response (McCool & Menge 1984) this might be explained by the ecosystem's tolerance towards O_3, that was already recognised with regard to biomass production.

In contrast to the findings of Yoshida et al. 2001, **%RLC by mycelium and arbuscules** was higher in O_3++ than in O_3 Control, probably because plants might have invested even more in the symbiosis to be able to repair O_3 damage. After the harvest of aboveground biomass in the beginning of August, plants in O_3 control intensified symbiosis, most likely to support rebuilding of aboveground plant components. In O_3++ treatments however, the cut back may have imposed additional stress on plants as exposure to O_3 of the remaining rose severely (Volk et al. 2006). Plants might in response not have been able to afford the investment of photosynthates in AMF anymore, what led to a decline of %RLC by both, mycelium and arbuscules. This could explain the O_3 × Time interactive effect on %RLC by arbuscules and the O_3 × Time interactive trend on %RLC by mycelium.

Since arbuscules are the fungal structures from which the plants benefit most (Dekkers & van der Werff 2001, Strack et al. 2003), it is likely that rising **%RLC by**

arbuscules improved nutrient uptake during vegetation period and probably supported formation of new buds for next season (Körner 2003) by stimulating photosynthesis.
A closer look on the ratio of cost to benefit, **arbuscules:mycelium ratio**, reveals that plants benefited least in May and most in July. The course over time of arbuscules:mycelium ratio is similar to the course of shoot biomass productivity and shoot:root ratio. This is a likely support for the idea that plant biomass productivity, especially at peak growing season is linked to the benefit from AMF colonisation.

A significant O_3 × N interaction on arbuscules:mycelium ratio led to the assumption that under fertilised conditions the mycorrhizal symbiosis became less mutualistic when exposed to O_3. Benefit for plants might have declined if N fertilisation reduced their need for symbiosis, whereas fungi still depended on and demanded for plant C deliveries. O_3 fumigation may have contributed to lower photosynthetic activity, and thus lowered C availability in the plants whilst fungi still demanded for C. Thus, the addition of both, N and O_3, may have resulted in even lesser benefit for plants, expressed in a decreased arbuscules:mycelium ratio.

With a least %RLC in July, the course of **vesicles** resembled the course of root biomass. This acts in concert with the idea that allocation of C compounds belowground was least in July during peak growing season. The lesser C delivery may have negatively influenced the formation of vesicles, fungal storage organs. Furthermore it matches with arbuscules:mycelium ratio being least in July, meaning that AMF benefited least and possibly invested less in the storage organs.

A possible explanation why fumigation led to an increase of %RLC by vesicles might be, that AMF derived some of the increased plant C delivery that was originally meant to improve nutrient uptake for the reparation of O_3 damage, to stimulate vesicle formation. Fertilisation might have stimulated vesicle formation as with increased root biomass due to fertilisation, C compounds were available whilst there was not need of formation of nutrient delivery to plants by arbuscules.

Alternatively, since not all AMF species form vesicles, a shift to vesicle forming AMF species could have occurred due to the treatments. It may be hypothesised that AMF species which form vesicles reacted more tolerant towards N fertilisation and O_3 fumigation than other species and thus increased. Nevertheless, about this can only be speculated because an identification of AMF on species level was not accomplished in this study.

As %RCL by all mycorrhizal structures became lower with increasing fraction of forbs, graminoides seem to be the main host for AMF at the study site. Since sedges at Alp Flix were not found to be mycorrhizal (V. Blanke personal communication) it can be assumed that Poaceae, "true grasses" were the main hosts for mycorrhizae at Alp Flix.
Mycorrhization is said to be more intense on alpine calcareous soils (Körner 2003). There is a highly significant influence of pH on overall %RLC by mycorrhiza in this study, but it states that %RLC increases with lower pH. This may occur due to other soil processes dependent on pH.

Ecosystem response to fertilisation may also be dependent on other soil and topographic parameters (Bowman et al. 1993, Fisk et al. 1998). These parameters have not been examined in this study but their effects may be hidden in the factor Block. During previous data analysis for other studies at Alp Flix the factor Block never gave reason to believe that it originated from the experimental set up (S. Bassin personal communication). Block in the present study produced significant p-values for some variables related to mycorrhizal colonisation and for the variable S/R.

Conclusions

After seven years of treatment a significant influence of N fertilisation on biomass production could be stated. There was an overall increase in biomass production, above ground as well as below ground, albeit an increase in shoot:root ratio might indicate a higher benefit of shoot.
The response of the examined *Geo-Montani-Nardetum* to N fertilisation led to the suggestion that the site at Alp Flix is limited by N. However, some findings with regard to belowground biomass' reaction to fertilisation might indicate that N is not the only limiting factor at the site.

Zero reaction of biomass production to O_3 fumigation attested a considerable O_3 tolerance to the plants also belowground and could confirm the results of Bassin et al. 2007.
%RLC by mycorrhizal structures was increased in O_3++. In addition to another impact on the plants, e.g. biomass loss through harvest however, mycorrhizal colonisation declined.
Assumptions arise that ecosystem response to O_3 may occur more slowly and that seven years of treatment might have been sufficient to indicate the direction of change but not the impact on a bigger scale.

If an increase of the fraction of sedges (Bassin et al. 2007) prevails with continuing treatment, this could have a negative impact on mycorrhizal colonisation.
A decreased benefit for plants forming mycorrhizal symbiosis may weaken their ability to compete in the plant community and thus result in a change of community composition.

Concerns, that the response to additional N and O_3 would be destabilisation of soil and lesser belowground C fixation could so far not be approved, as there was no evidence that root biomass or abundance of mycorrhizal fungi would decline with the treatments.

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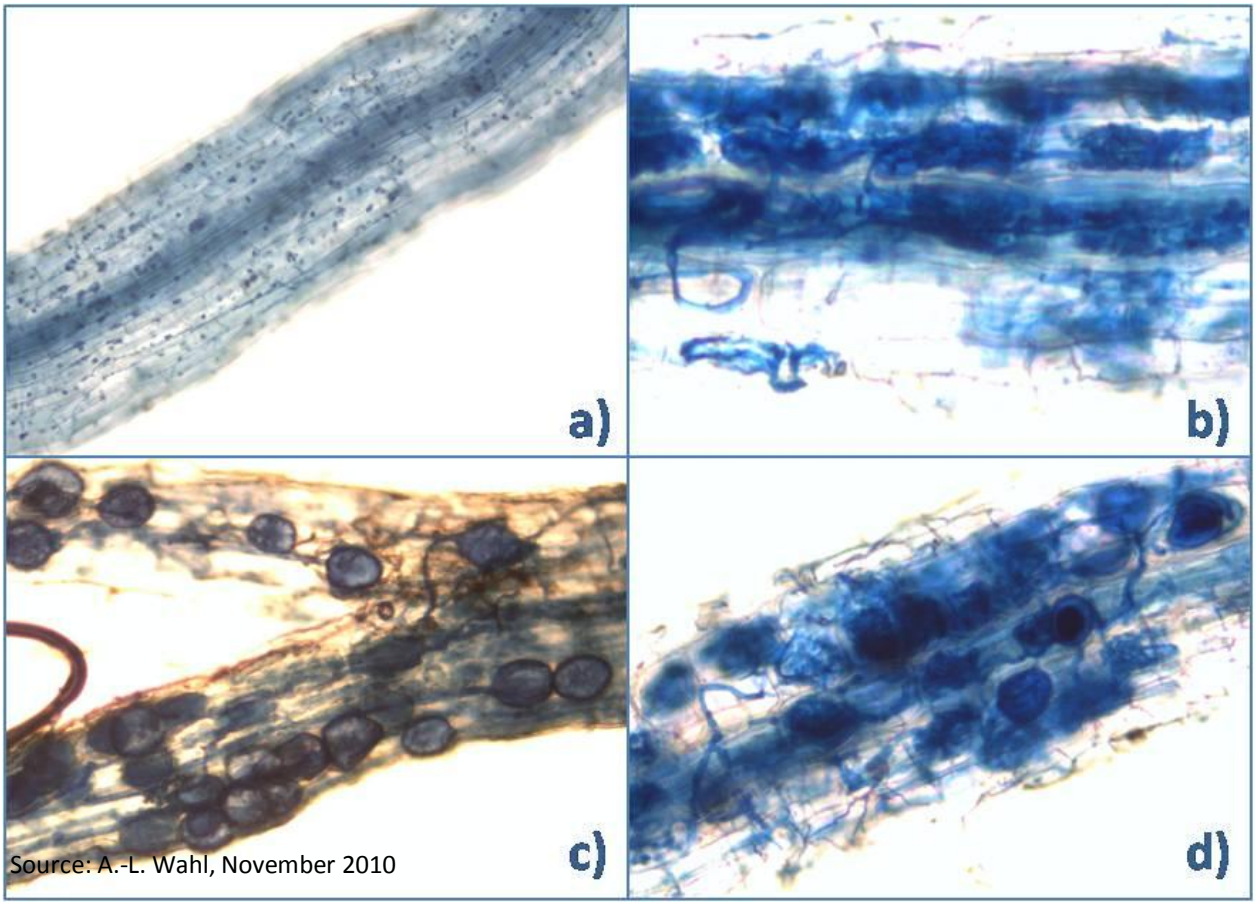
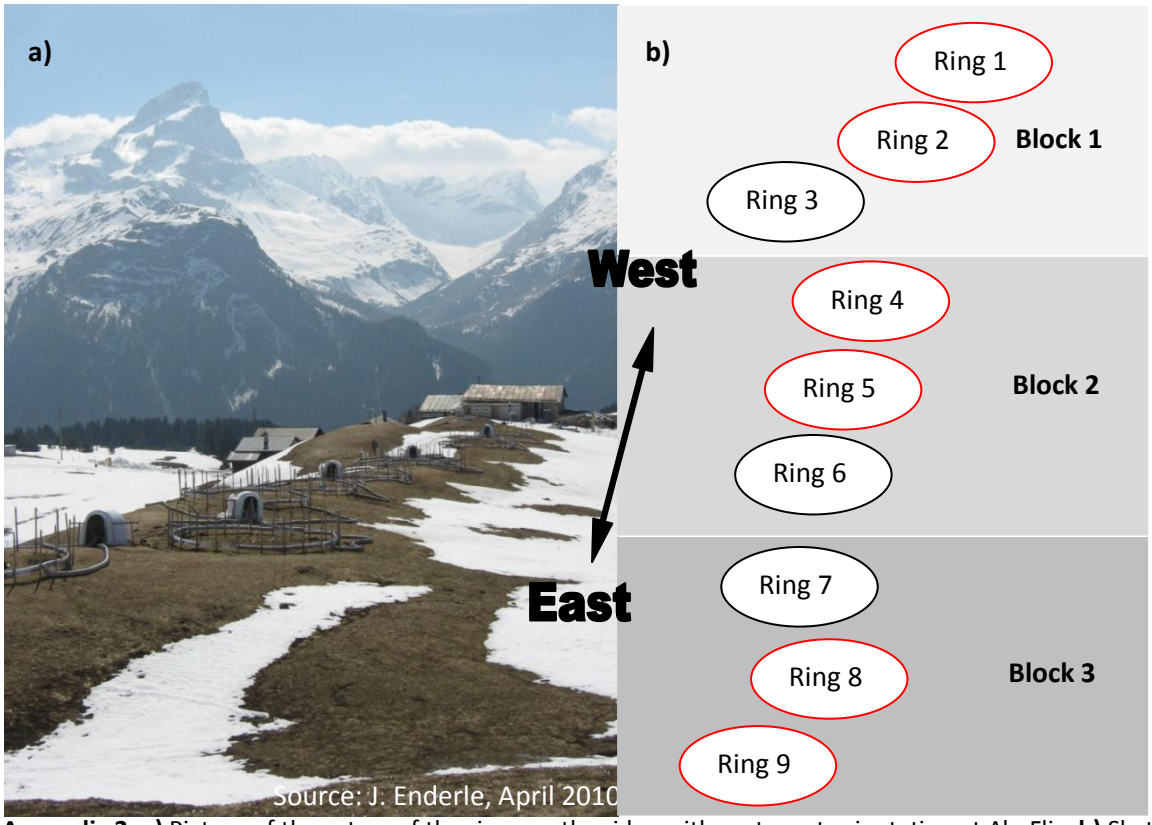
Appendix

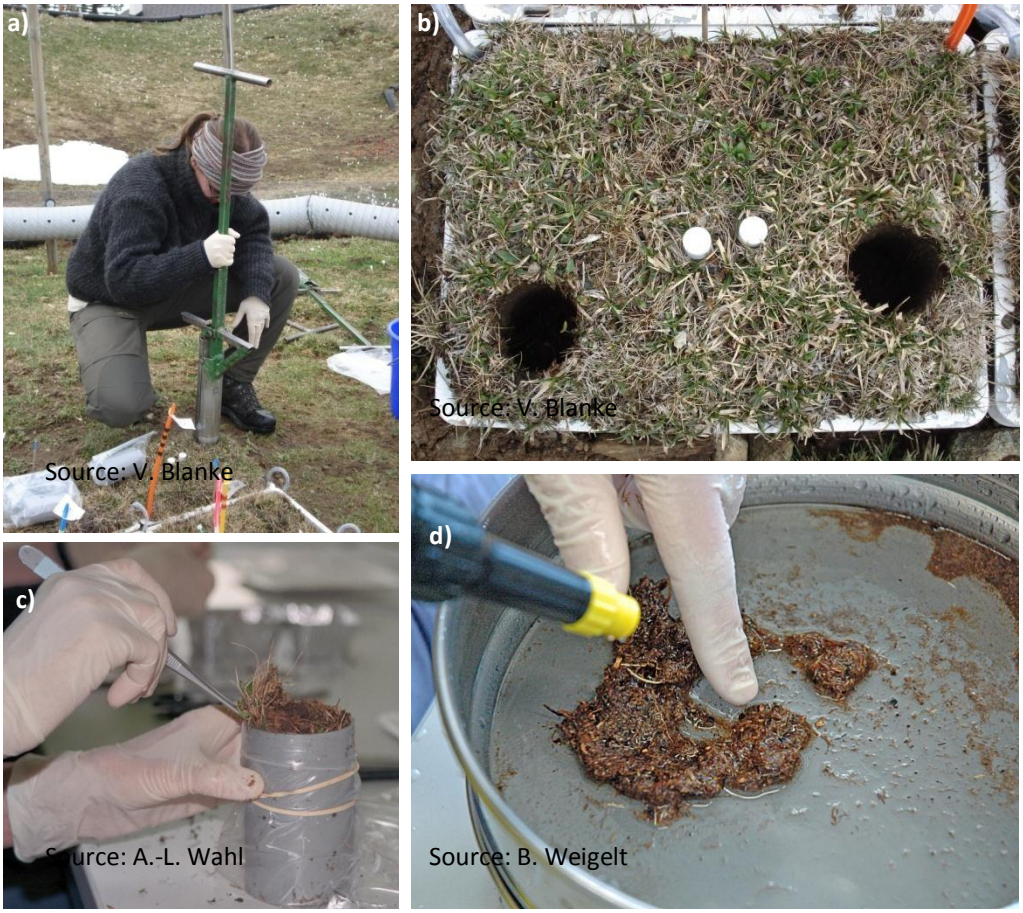
Appendix 1: Pictures of mycorrhizal structures inside roots sampled at Alp Flix. **a)** Root fraction with no clearly visible mycorrhizal structure but a well visible vascular cylinder. **b)** Tree-shaped arbuscules inside root cells linked by mycelium (blue lines). **c)** Round vesicles with a distinct border. **d)** Fraction of root colonized by arbuscules (e.g. left side centerd) and vesicles (e.g. right upper corner). Internal mycelium is well visible.





Appendix 2: a) Picture of the set up of the rings on the ridge with east-west orientation at Alp Flix. **b)** Sketch of the set up of the rings at Alp Flix from the same angle of vision as the picture in Appendix 2 a). Ring one, five and nine were not fumigated (ambient = O_3 Control), ring two, four and eight were fumigated with highest O_3 concentrations (1.6 × ambient = O_3 ++) and ring three, six and seven were fumigated with intermediate O_3 concentrations (1.2 × ambient = O_3 +). Only monoliths from rings with O_3 Control and O_3++ were sampled. The three blocks are marked in different shades of grey. Sampled rings are marked with a red outline. **c)** Position of the experiment at Alp Flix, marked with a red outline.

Appendix 3: Pictures, illustrating the soil core processing. **a)** Taking samples at Alp Flix. **b)** N50 monolith after the sampling in May. **c)** Harvest of aboveground biomass. **d)** Washing and rinsing of root biomass on a sieve to gain belowground biomass.



Appendix 4: Table to explain calculation details for dry weights of total root mass.

Item	FW/DW No.	FW (Fresh weight [g])	DW (Dry weight at 60 °C [g])
Core A	1	measured	----------
Core B	2	measured	----------
Core AB	3	= FW 1 + FW 2	= (DW 6) / (FW 6) * (FW 3)
Core $^{1}/_{6}$ A	4	measured	measured
Core $^{1}/_{6}$ B	5	measured	measured
Core $^{1}/_{6}$ AB	6	= FW 4 + FW 5	= DW 4 + DW 5
Core $^{1}/_{2}$ A	7	measured	----------
Core $^{1}/_{2}$ B	8	measured	----------
Core $^{1}/_{2}$ AB	9	= FW 7 + FW 8	= (DW 6) / (FW 6) * (FW 9)
ROOT $^{1}/_{2}$ AB	10	measured	= (DW 11) / (FW 11) * (FW 10)
ROOT $^{1}/_{2}$ AB – Root Subsample	11	measured	measured
ROOT A + B	12	----------	= (DW 10) / (DW 9) * (DW 3)

Declaration

Herewith I confirm that I produced this thesis all by myself, including all inserted figures, pictures and tables. My results were accomplished by no other means but those specified. References are given for all segments originating from other sources like books, papers or analogous.

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