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Spatial heterogeneity confounded ozone-exposure experiment in semi-natural grassland

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Abstract Interpretation of observations from manipulative experiments is often complicated by a multitude of uncontrolled processes operating at various spatial and temporal scales. As such processes may differ among experimental plots there is a risk that effects of experimental treatments are confounded. Here we report on a free-air ozone-exposure experiment in permanent semi-natural grassland that suggested strong ozone effects on community productivity after 5 years. We tested ozone effects and investigated the potential of confounding due to changes in nutrient management. Repeated-measure ANOVA revealed mainly negative temporal trends for frequency of abundant productive plant species. Constrained ordination additionally showed converging trajectories of species compositions for ozone and control treatments with time. Yields sampled prior to the start of the experiment and soil nitrogen concentrations revealed that spatial heterogeneity in the soil nutrient status was not accounted for by the random allocation of treatments to plots with a bias towards less productive patches in the elevated-ozone plots. Re-analysis of yield data using

repeated-measure ANOVA with a covariable to account for productivity prior to the start of fumigation revealed effects on the temporal changes in total yield and yield of legumes that cannot be separated between ozone and pre-treatment nutrient status. Changes in species composition favour an ecological interpretation with spatial heterogeneity as the major cause of different yield declines. Although elevated ozone may cause subtle physiological changes with longer term implications, our new results suggest that species-rich mature grassland such as the one studied at Le Mouret may be less sensitive to elevated ozone than previously assumed. In this experiment a confounded design was hidden at the start by transitory effects of a prior change in nutrient treatments.

Keywords Air pollution · Experimental design · Permanent grassland · Productivity · Vegetation dynamics

Introduction

Air pollution by ozone has long been considered an important large-scale threat to vegetation. Data from field observations and manipulative exposure experiments suggested that in regions with elevated concentrations ozone may reduce crop productivity (Ashmore 2005), or cause changes in grassland biodiversity because of inter-specific differences in ozone sensitivity (Bassin et al. 2007a). Models for future atmospheric conditions project that ozone risks may even increase with climate change (Royal Society 2008; Fuhrer 2009). However, experimental results often suffer from artefacts caused by altered microclimatic conditions in field exposure chambers, or results obtained with artificial plant communities are difficult to extrapolate to natural situations (Bassin et al. 2007a). To overcome

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these limitations, recent ozone-exposure studies have used a free-air fumigation technique in mature grassland systems (Volk et al. 2003; Bassin et al. 2007b). But, as in any manipulative experiment with such systems, processes operating at various spatial and temporal scales can complicate the interpretation of observations.

The experiment at Le Mouret (Switzerland) has investigated ozone impacts at the community level over the longest period so far in intact semi-natural, mature grassland. Due to the high technical costs of running the free-air fumigation system over several years, a minimal number of six circular plots were used in a design in which the single factor ozone had two levels, i.e. ozone enrichment and ambient-air control, and three replicates without block structure (Volk et al. 2006). Such a design bears a high risk that treatment effects are confounded with a spatial pattern, and observed responses may have an unrecognised cause (Huston 1997). The six 7-m-diameter plots were established in permanent grassland that was managed as hay pasture. Based on yield samples representing 7.5% of the entire plot area, Volk et al. (2006) suggested that ozone effects progressively built up over the period of fumigation from 1999 to 2003, resulting in a 23% loss of dry matter yields at the community level and in a decline of the legume fraction during the fifth year. Data of annual surveys that aimed at tracking species abundance responses to ozone in the entire plot area at Le Mouret remained to be analysed.

We investigate the potential problem that cumulative effects of ozone might be confounded. A difference between progressive yield declines in both treatments could have resulted from a change in the nutrient treatment if plots differed in productivity or nutrient status. Under the assumption that experimental treatments are not confounded, we tested whether or not ozone had an effect on the entire species composition, plant functional groups and single species, and on the temporal changes of these variables. We hypothesized that controversial interpretations based on the analysis of frequency and yield data resulted from a confounded design of the experiment, and we asked whether the apparent contradiction can be reconciled by considering differences in productivity and soil nitrogen concentrations between plots at the onset of the ozone treatment.

Materials and methods

Before the start of the experiment, the permanent grassland at Le Mouret (754 m a.s.l., 46°45'N, 7°10'E) had been used for haymaking in summer and subsequent sheep grazing. Applied for over 30 years, this regime was maintained during the fumigation experiment between the

experimental plots but was replaced inside the plots by mowing 3 times per year (mid-June, early August, and late October). In 1998, prior to setting up the plots, four cuts were carried out (early May, mid-June, early August, and early November) to even out the impact of irregular grazing. A small amount of ammonium nitrate fertilizer (equivalent to 20 kg nitrogen ha⁻¹) was added in early 1998 as a one-time pulse in order to bring annual nitrogen inputs to the plots and their surrounding areas to a similar level after the substitution of sheep grazing by mowing. The plots were established in 1998, and ozone treatment was started in spring 1999 and was maintained during the growing seasons until autumn 2003. In the elevated-ozone treatment, ozone concentrations in the air were increased by approximately a factor of 1.5 on average (Volk et al. 2006). The seasonal cumulative ozone exposure (AOT40) at canopy level fluctuated between years in the range of 1.0–20.7 p.p.m. h in the control and 13.3–59.5 p.p.m. h in the ozone-treated plots, with ozone exposure minima in 1999 and maxima in 2003 in both treatments. For further details on the experimental plots and a description of the ozone-exposure system, its characteristics and the meteorological measurements, see Volk et al. (2003, 2006).

During the period of ozone exposure the bayonet technique (Poissonet and Poissonet 1969) was used each year before harvesting in mid-June to sample species frequency data at 120 points per plot, with a grid distance of 0.4 m, representing the central 5-m-diameter circle, i.e. an area of ca. 20 m² in each plot. Such data accurately represent year-to-year fluctuations in abundant grassland species (Stampfli 1991, 1995). No such data were recorded in 1998, the year before ozone exposure started. Species frequency defined as the number out of 120 points at which species *x* was intercepted. In tests for ozone and time effects, repeated-measure ANOVA with Greenhouse-Geisser adjusted probability was applied to the frequency sum over all species, the frequency sums of grasses, forbs, and legumes, and to the arcsine-transformed frequencies of the 11 most abundant species that were present in all plots. These species contributed 71% to the frequency sum over all species. Subsequent linear contrasts with 2 *df* in the numerator and *n* – 2 *df* in the denominator (*n* = number of plots) tested the trend across 5 years (see Stampfli and Zeiter 2004 for a similar analysis). A multivariate approach used data for all species and plots to determine the effects of ozone and time on species composition in constrained ordinations [redundancy analysis (RDA) in CANOCO, version 4.5, Centre for Biometry, Wageningen, the Netherlands]. In the search for a linear temporal trend, a main effect of ozone, and an effect of ozone on temporal changes in species composition, we calculated three separate RDAs with one constrained axis and specific

covariables (Lepš and Šmilauer 2003), i.e. we used the ordinal variable year, the nominal variable ozone, or the year \times ozone interaction, respectively, as a single explanatory variable (see below). The significance of the constrained axis was tested in Monte Carlo permutation tests that account for the split-plot structure of the repeated measures across time. Trajectories of annual centroids of the species compositions were calculated in a RDA with ozone and time as explanatory variables to visualise temporal changes in ozone-exposed and control plots.

Total nitrogen concentration was measured in pooled samples of five 5-cm-diameter soil cores (0–20 cm) per plot on 3 November 1999 and 27 October 2003 using the standard Kjeldahl procedure.

Aboveground plant matter was separately harvested in six permanent 0.25-m² sub-plots in each plot for all cuts from 1998 to 2003. Harvests were completely sorted by fractions of grasses, forbs, and legumes, and then dried, and weighed (Volk et al. 2006). Total dry matter yield and fractions of species groups were calculated as averages of sub-plots and sums of cuts in each year. An alternative approach, compared here to Volk et al. (2006), used yield in 1998 as a covariable to account for between-plot differences in productivity prior to ozone fumigation. We employed GENSTAT 6.0 (Payne 2000) and used the log-transformed covariable in repeated-measure ANOVAs of log-transformed annual yields across the fumigation period (1999–2003).

Results

Point frequency surveys found a total of 47 species, 3.4 species per point on average in 1999 and 2.0 species in 2003. The frequency sum over all species showed a negative trend across 5 years but no effects of ozone (Table 1). Grasses and legumes contributed to this negative trend while forbs did not. Ozone showed an effect on the temporal change of grasses. Six out of 11 abundant species showed a negative and one species a positive 5-year trend, but none appeared to be affected by ozone (Table 1). The group of declining species included principal constituents of highly productive grasslands (Fig. 1): *Poa pratensis* and the three most abundant species among the grasses at the onset of the experiment, *Alopecurus pratensis*, *Poa trivialis*, and *Lolium perenne*; declining species also included two forbs of short stature that often occur in pastures, *Cerastium caespitosum* and *Trifolium repens*, which were the most abundant species at the onset. *Plantago lanceolata*, the only increasing species, is a characteristic forb of low productive meadows, while characteristic grasses of such habitats, *Anthoxanthum odoratum* and *Holcus lanatus*, remained in steady state and hence advanced to the top in the abundance ranking of grasses. *Ranunculus friesianus* and *Taraxacum officinale*, two forbs that often occur in nutrient-rich meadows, increased from year 1999 to 2000 and advanced in abundance rank by remaining constant over 5 years. In 2003, *R. friesianus* was ahead of all species.

Table 1 Effects of ozone and time on frequency sums of all species, grasses, forbs, and legumes and on arcsine-transformed frequency of abundant species: repeated-measure ANOVA of data 1999–2003. *F*-statistic with *df*, Greenhouse-Geisser ϵ (ϵ), direction (*Trend* +/–) and *P* of 5-year trend

Frequency	Ozone <i>F</i> _{1,4}	Time <i>F</i> _{4,16}	Time \times Ozone <i>F</i> _{4,16}	ϵ	Trend	<i>P</i>
All species	0.5	45.6***	1.7	0.34	–	<0.01
Grasses (19 species)	2.7	176.1***	6.0*	0.63	–	<0.01
<i>Alopecurus pratensis</i>	1.4	7.7*	2.0	0.50	–	0.04
<i>Anthoxanthum odoratum</i>	0.2	0.4	0.1	0.27		0.90
<i>Holcus lanatus</i>	3.0	0.7	1.2	0.48		0.64
<i>Lolium perenne</i>	2.0	12.8**	0.2	0.64	–	<0.01
<i>Poa pratensis</i>	0.0	16.7**	1.5	0.42	–	<0.01
<i>Poa trivialis</i>	1.4	17.1**	0.5	0.48	–	0.01
Forbs (23 species)	2.5	3.0	0.8	0.39		0.89
<i>Cerastium caespitosum</i>	5.6	10.5*	0.8	0.41	–	0.02
<i>Plantago lanceolata</i>	0.6	7.0*	1.0	0.57	+	0.04
<i>Ranunculus friesianus</i>	0.1	10.5*	1.2	0.39		0.28
<i>Taraxacum officinale</i>	0.9	4.0	0.5	0.59		0.11
Legumes (5 species)	0.5	14.2**	1.5	0.58	–	0.01
<i>Trifolium repens</i>	1.4	14.2**	0.6	0.54	–	0.01

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (ϵ -adjusted *P*-levels)

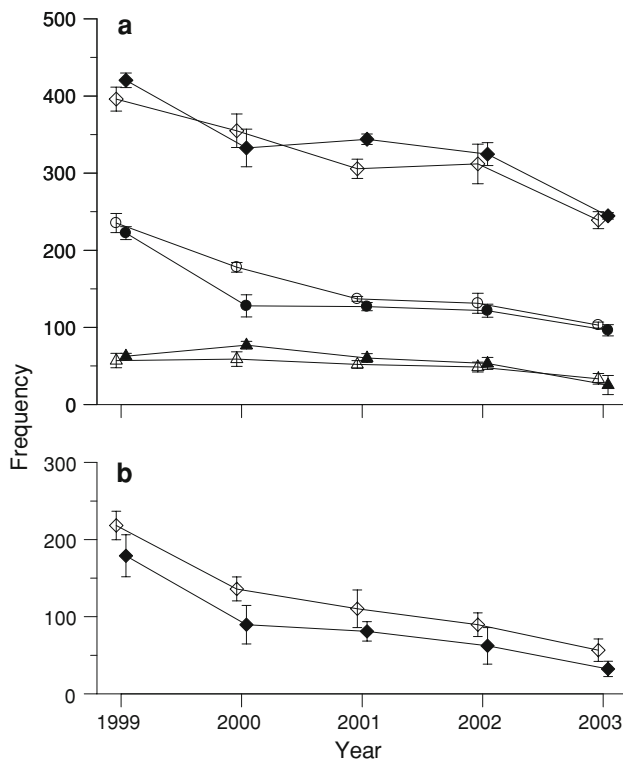


Fig. 1 Temporal change in absolute frequency sums (plot mean \pm SE, $n = 3$) of **a** all species (diamonds), grasses (circles), legumes (triangles), and **b** a group of abundant, declining species (*Alopecurus pratensis*, *Lolium perenne*, *Poa pratensis*, *Poa trivialis*, *Cerastium caespitosum*, *Trifolium repens*) across a period of experimental fumigation in control plots (open symbols) and ozone plots (filled symbols). A difference between treatments in temporal change appeared for grasses

Table 2 Effect of year, ozone, and year \times ozone in three constrained ordinations (redundancy analysis; RDA) with different explanatory variables, year (ordinal), ozone (nominal), and covariables, plot (nominal), year (nominal; Yr), applied to entire set of species frequency data. Percentage of variability explained by constrained axis and correlation (r) of species and explanatory variable on constrained axis, F -ratio statistics for test of trace and P -value obtained by test using 499 random permutations

RDA	Explanatory variable	Covariables	First axis			
			Variability %	r	F	P
I	Year	Plot	15.9	0.94	11.9	0.016
II	Ozone		12.8	0.62	4.1	1.000
III	Year \times Ozone	Yr, Plot	1.7	0.63	1.8	0.054

The constrained ordination found a significant temporal trend (Table 2). The six species that fit this trend best (variance explained by 1st axis of RDA $> 20\%$), *P. pratensis* (50%), *L. perenne* (42%), *C. caespitosum* (41%), *P. trivialis* (36%), *T. repens* (32%), *A. pratensis* (22%), all declined with time. A marginal effect of year \times ozone was

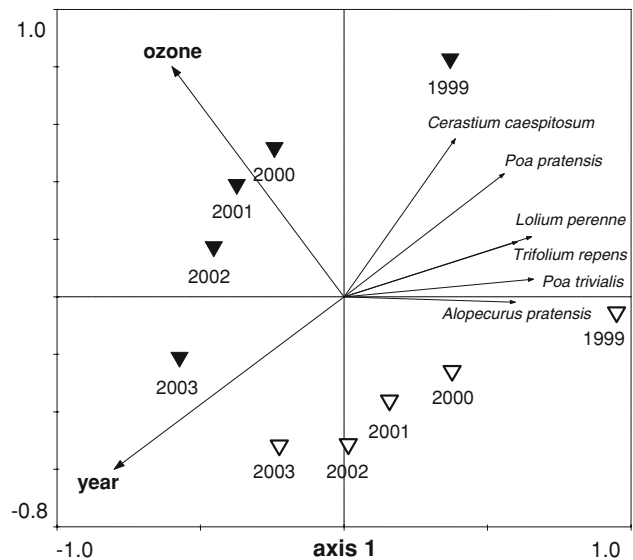


Fig. 2 Trajectories of centroids of species compositions in ozone-exposed (filled triangles) and control (open triangles) plots in redundancy analysis (RDA) biplot with explanatory variables (year, ozone) and six species that fit the temporal trend best (Table 2, RDA I), $\lambda_{\text{axis1}} = 0.198$, $\lambda_{\text{axis2}} = 0.089$

apparent but only explained a very small fraction of variation (Table 2) and the constrained axis was best fit by rare species with insignificant representation in both treatments (data not shown). Trajectories of species composition show almost parallel shifts in both treatments during the period 1999–2000 and a tendency to converge towards the end of the experiment (Fig. 2).

Variation in total dry matter harvested prior to the start of fumigation showed a spatially confounded pattern with the allocation of the ozone treatment, i.e. the three least productive plots in 1998 were those that were exposed to ozone (Fig. 3a). Yield differed to a similar degree between treatments in the fifth year of ozone exposure but no difference was apparent at the start of the experiment in 1999 (Fig. 3b). Plant dry matter in 1998 was positively correlated ($r = 0.91$, $P < 0.01$) with soil nitrogen concentration (means of measurements in 1999 and 2003, soil nitrogen concentrations in 1999 and 2003 were positively correlated, $r = 0.93$, $P < 0.01$) and, similarly, yield differences between treatments in 2003 were mirrored by differences in soil nitrogen concentration in 2003.

Plant dry matter showed no significant main effects of ozone but did show effects of time (Table 3). Total yield strongly increased in the interval 1998–1999 (Fig. 4a) and the inter-annual difference was negatively correlated with total yield in 1998 at the sub-plot scale (Fig. 5). During the fumigation experiment total yield steadily declined to before experimental levels (Fig. 4a). A tendency to return to the former productivity pattern is also illustrated at the sub-plot scale as total yields in 2003 reflected those in 1998

Fig. 3 Pattern of productivity-related variables measured in control plots (*open symbol*) and fumigated plots (*filled symbols*). **a** Relationship between soil nitrogen concentration in 1999 and total yield before experiment began in 1998 (*diamonds*). **b** Relationship between soil nitrogen concentration and yield in 1999 (*triangles*) and 2003 (*circles*)

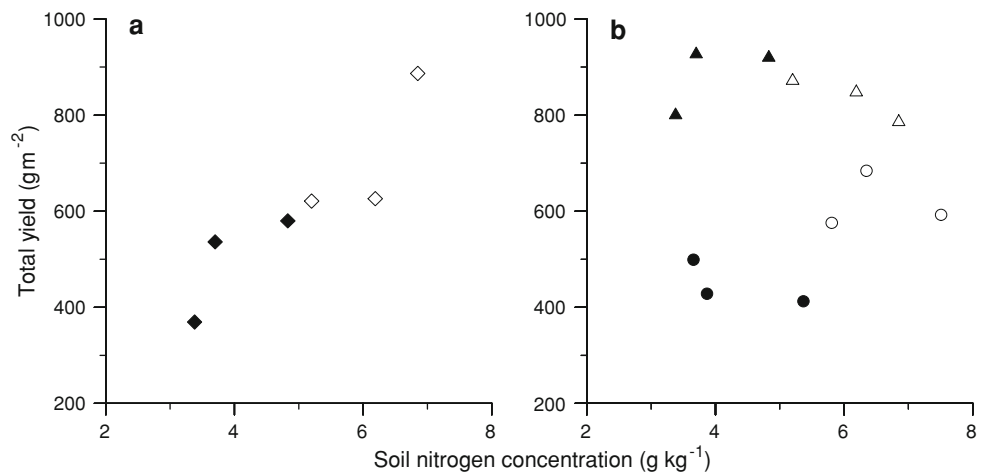


Table 3 Effects of ozone and time on plant matter: repeated-measure ANOVA of log-transformed yield totals and fractions of grasses, forbs, and legumes. *F*-statistic with *df* and ϵ

Yield	Covariable <i>F</i> _{1,3}	Ozone <i>F</i> _{1,3}	Time <i>F</i> _{4,16}	Time × Ozone <i>F</i> _{4,16}	ϵ
Total	0.1	0.8	47.2***	7.5*	0.45
Grasses	1.6	0.5	28.6***	1.6	0.48
Forbs	0.0	0.2	0.6	3.5	0.45
Legumes	0.1	0.0	24.4***	5.0*	0.51

* $P < 0.05$, *** $P < 0.001$ (ϵ -adjusted probability level)

($r = 0.49$, $P < 0.01$) but not those in 1999 ($r = 0.12$, $P > 0.1$). Time × ozone interactions indicate that the decline in total yield and legumes differed between treatments (Table 3; Fig. 4b).

Discussion

Species frequency data representing the entire experimental area instead of small sub-plots clearly indicate a succession over 5 years towards low-productivity grassland. This is supported by a continuous decline in the frequency sum of species, the frequency changes in a majority of abundant species, a decrease of species indicating highly productive grasslands consistently identified by ANOVA and constrained ordination (Figs. 1, 2), and the increase or persistence of species indicative of low productivity. The succession resembles a process caused by nutrient impoverishment (Oomes and Mooi 1985; Bakker 1989; Bischof 1992). Compared to this development, exposure to ozone-enriched air appears to have had a much weaker influence on community development (Table 1). An effect of ozone on the temporal change in the legume fraction, as

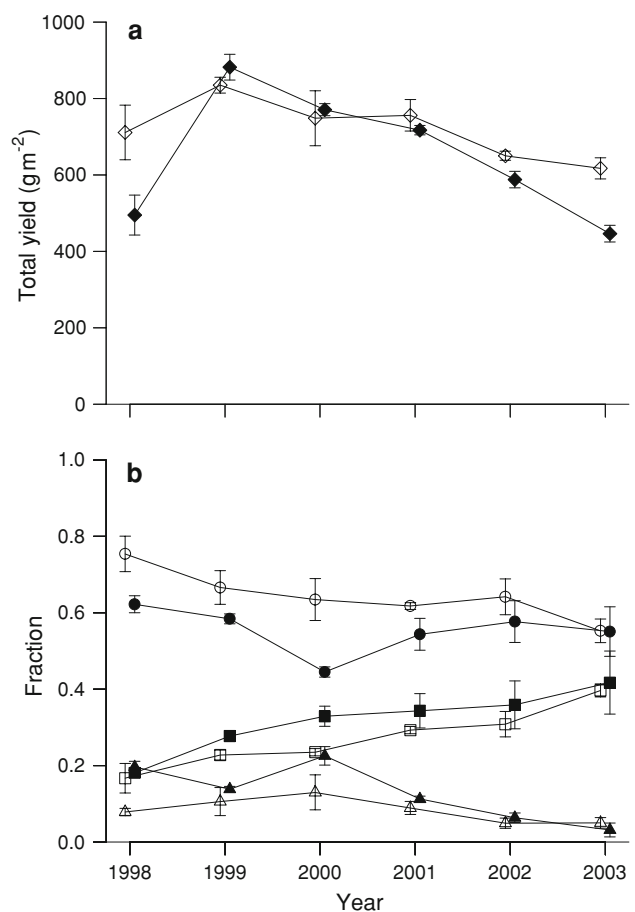


Fig. 4 Plant matter yields of experimental plots in previous year (1998) and during period of continuous ozone fumigation (1999–2003). In spring 1998, nitrogen fertilizer was added as a one-time application and sheep were excluded from then on. Total yield (**a**) and fraction of three species groups (**b**), grasses (*circles*), forbs (*squares*), legumes (*triangles*) in fumigated plots (*filled symbols*) and control (*open symbols*), mean ± SE, $n = 3$ (extended after Volk et al. 2006: Fig. 3a, 5a)

suggested by yield trends observed in sub-plots (Table 3), is not confirmed by the frequency sum of legumes (Table 1). The patchy distribution of legumes can explain this difference. On the other hand, a supposed effect of ozone on the temporal change in the frequency sum of grasses (Table 1) is not confirmed by grass yields (Table 3) although both variables showed a consistent negative deviation under the ozone treatment in the second year of the experiment (Figs. 1, 4). Yield fractions provide evidence for continuous trends in the composition of grasses, forbs, and legumes since 1998, the year before fumigation started (Fig. 4b).

Change in species composition over 5 years of ozone fumigation (1999–2003), as presented in the constrained ordination, and plant matter yield, as analysed by Volk et al. (2006), support different ecological interpretations. Species composition (Fig. 2) reveals convergence of control and ozone-exposed plots with time. In contrast, yield difference between treatments increases with time and suggests a gradually diverging development (Volk et al. 2006).

Random allocation of treatments coincided with grassland patches of different productivity as was consistently indicated by yield measurements in 1998 and 2003 and soil nitrogen measurements in 1999 and 2003 (Fig. 3). At the onset of ozone exposure in 1999, such differences escaped notice because they were hidden by the effect of factors that had stimulated the production of plant matter in the less productive patches (Fig. 5). The switch from hay pasture management to four hay cuts per year and a

nitrogen pulse in the preceding year could have caused transient effects on productivity of this kind. Probably, sheep had visited and grazed some areas more intensively, e.g. those areas closer to a shelter situated at one edge of the pasture, while other areas remained less grazed (Hopkins 2000). Minor differences in topography may have further contributed to the formation of patches due to an accumulation of nitrogen from dung and urine at slightly lower locations, and differences in soil characteristics affecting the soil water regime. Cutting in spring 1998 removed patches of litter and dead plant matter due to irregular grazing. More frequent cutting increases tiller density by the stimulation of branching (Hopkins 2000). Presumably tillering was increased more in the less productive and formerly less frequently grazed patches that had lower tiller density. Nitrogen fertilization and nutrient storage in plants under lower grazing intensity may have supported this site-filling process in 1998. Yield reached a maximum in 1999 as warm temperatures in May and June (4–7 K above the means of later years; Volk et al. 2006) favoured plant growth at a time when the sward had improved its use of space and relatively high nutrient reserves were still available in plant tissues. Seasonal harvests (Volk et al. 2006) show that this stock was continuously depleted by the removal of plant matter in the following seasons and years. Hence, ozone-exposed plots with a lower nitrogen status showed exceptional growth under the particular conditions caused by the change in management with spring yields exceeding those of the normally more productive plots, which influenced the starting conditions of the analyses presented by Volk et al. (2006). Productive hay pastures such as the Le Mouret grassland are expected to have high nutrient demands (Hopkins 2000). Therefore, nutrient limitations build up quickly when the import of dung and urine is lost and one or two additional hay cuts per year come into effect. After 5 years of plant matter removal, the pattern of annual yield reflects that of soil nutrient concentration.

Frequency data representing the entire experimental plots did not show a faster decline in ozone-treated than in control plots. The negative deviation of grasses in ozone-treated plots in the second year is not credibly caused by ozone as it was not maintained in later years despite higher ozone concentrations (see Volk et al. 2006). A depletion of plant storage tissue following exceptional growth in these less productive plots can explain the negative deviation that contributed to a significant year \times ozone interaction. The conclusion of Volk et al. (2006) that difference in total yield trends between ozone and control treatment are caused by ozone during the 5-year fumigation period may have been biased to a large extent by pre-experimental spatial heterogeneity while sample size may have added a further bias in the case of legume yields.

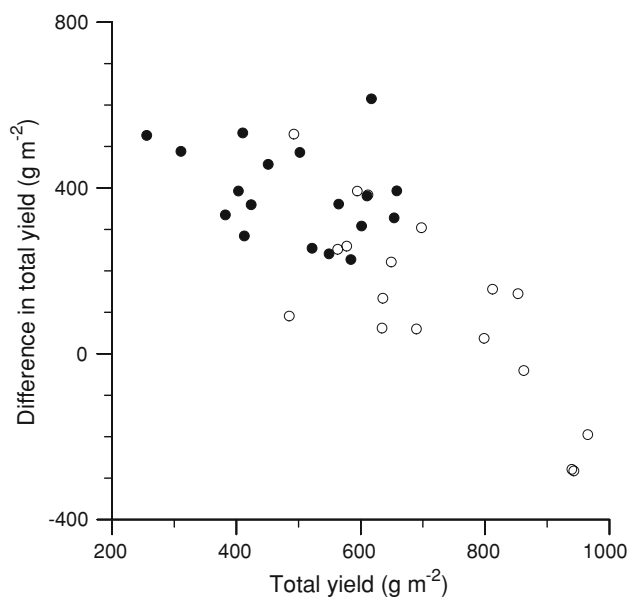


Fig. 5 Inter-annual difference in total yield (1999–1998) on total yield in 1998 for 36 sub-plots ($r = -0.78$, $P < 0.001$). Sub-plots of control plots (open symbols) and fumigated plots (filled symbols)

As pre-treatment nutrient status and ozone are confounded it is not possible to partition the observed effects of ozone from those of nutrient status in this study. Biased situations are probably not uncommon in manipulative ecological experiments as multiple processes operating in parallel but at different rates may render interpretation difficult. Space filling is a fast process happening within a season, and the depletion of plant storage tissue probably lasts for a few years, while the depletion of soil nutrients clearly extends the time scale of this study. During this experiment vegetation dynamics appear to have been driven by several processes connected to a change in management which involved the enhanced removal of plant matter from a patchy hay pasture site. Hence, a difference in annual yield between patches of different productivity, temporarily hidden by the effect of management change, set the circumstances in which an interpretation of the strong role of ozone as an important driver of vegetation dynamics in semi-natural grassland (see Volk et al. 2006) likely received too much emphasis. Similarly, only small effects of elevated ozone on community composition were observed in calcareous grassland turfs studied by Thwaites et al. (2006). Studies exposing transplanted grassland turfs to different ozone environments bear a high risk of confounding treatment effects with those of transplantation.

These results indicate that the mature semi-natural grassland studied here may be less sensitive to long-term cumulative ozone exposure than expected on the basis of short-term observations in species screening experiments (Mills et al. 2007), in which a relatively high sensitivity of legumes such as *T. repens* was observed (Hayes et al. 2007). It also contrasts the model prediction based on species Ellenberg values (Jones et al. 2007), which suggested effects of ozone of the same order of magnitude as reported for the Le Mouret experiment by Volk et al. (2006). However, the present result is in line with suggestions based on a literature analysis by Bassin et al. (2007a), which attributed moderate ozone sensitivity to mature low-productivity grasslands, and with more recent results from a similar free-air ozone experiment in subalpine grassland showing rapid community responses to increasing nitrogen addition but no effect of elevated ozone (Bassin et al. 2007b). However, results from physiological investigations point to the possibility of subtle ozone effects, which over much longer time scales may lead to changes at the community level. The observed changes include changes in carbon and oxygen stable isotopic signatures that indicate effects of ozone on the water economy of some species (Jäggi and Fuhrer 2007; Bassin et al. 2009), or accelerated leaf senescence (Bassin et al. 2007b).

In conclusion, the more detailed analysis of species and soil data from a 5-year ozone-exposure experiment

indicates that the difference between ozone and control treatment reported earlier were likely overestimated because overlapping effects of species dynamics caused by heterogeneous initial conditions and a change in management were not considered. The new results thus support the view that mature, permanent grassland such as the one studied in this experiment may be less sensitive to increasing ozone levels than previously assumed.

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