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Short-term responses of greenhouse gas emissions and ecosystem carbon fluxes to elevated ozone and N fertilization in a temperate grassland

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Abstract

Growing evidence suggests that tropospheric ozone has widespread effects on vegetation, which can contribute to alter ecosystem carbon (C) dynamics and belowground processes. In this study, we used intact soil mesocosms from a semi-improved grassland and investigated the effects of elevated ozone, alone and in combination with nitrogen (N) fertilization on soil-borne greenhouse gas emissions and ecosystem C fluxes. Ozone exposure under fully open-air field conditions was occurred during the growing season. Across a one-year period, soil methane (CH_4) and nitrous oxide (N_2O) emissions did not differ between treatments, but elevated ozone significantly depressed soil CH_4 uptake by 14% during the growing season irrespective of N fertilization. Elevated ozone resulted in a 15% reduction of net ecosystem exchange of carbon dioxide, while N fertilization significantly increased ecosystem respiration during the growing season. Aboveground biomass was unaffected by elevated ozone during the growing season but significantly decreased by 17% during the non-growing season. At the end of the experiment, soil mineral N content, net N mineralization and extracellular enzyme activities (i.e., cellobiohydrolase and leucine aminopeptidase) were higher under elevated ozone than ambient ozone. The short-term effect of single application of N fertilizer was primarily responsbible for the lack of the interaction between elevated ozone and N fertilization. Therefore, results of our short-term study suggest that ozone exposure may have negative impacts on soil CH_4 uptake and C sequestration and contribute to accelerated rates of soil N-cycling.

Keywords: Air pollutant; Fertilizer management; Ground level O_3; Plant-soil feedbacks; Soil nutrient cycling

1. Introduction

Owing to the global increase in ozone precursor emissions (i.e., nitrogen oxides, carbon monoxide and volatile organic compounds), further increases of background ozone concentrations in the Northern Hemisphere may occur over this century unless precursor emissions are effectively controlled (Fowler et al., 2008; Meehl et al., 2007). Tropospheric ozone is not only the third-most-important contributor to the human-induced greenhouse gas (GHG) effect after carbon dioxide (CO_2) and methane (CH_4) (IPCC, 2007), but also the important gaseous air pollutant in terms of its effects on net primary production, soil carbon (C) sequestration, as well as other ecosystem services (Ainsworth et al., 2012; Sicard et al., 2017).

Ozone may have important indirect effects on global change through its effects on CH_4 and nitrous oxide (N_2O) emissions. In the past decade, the effects of elevated ozone on CH_4 and N_2O emissions, especially from agroecosystems, have been increasingly studied. For example, studies in rice fields conducted under either free-air open conditions or the open-top chambers (OTC) have consistently shown that CH_4 emissions were substantially less under elevated ozone than under ambient or charcoal-filtered air conditions (Bhatia et al., 2011; Tang et al., 2015; Zheng et al., 2011). The impacts of elevated ozone on N_2O emissions have been examined in a range of agricultural systems, including rice fields (Bhatia et al., 2011; Kou et al., 2015; Tang et al., 2015), a soybean field (Decock et al., 2012), an annual grassland (Sánchez-Martín et al., 2017) and a meadow ecosystem (Kanerva et al., 2007). However, the results from these experiments are conflicting, showing either positive (Sánchez-Martín et al.,

2017), negative (Bhatia et al., 2011; Kanerva et al., 2007) or no significant (Decock et al., 2012; Kou et al., 2015; Tang et al., 2015) responses of N_2O emissions to ozone exposure. Such inconsistencies may stem from the complex impacts of other factors (e.g. exposure methodology, exposure duration and plant type etc.) on the responses of N_2O emissions to elevated ozone (Tang et al., 2015).

On the other hand, ozone may also have important indirect effects on global change through its effect on terrestrial C sequestration. At the global scale, Sitch et al. (2007) used a global land C cycle model by including the ozone deposition effect and demonstrated that the negative impact of elevated ozone on plant productivity may result in a significant suppression of the global land-C sink. Such modeling studies (Sitch et al., 2007), however, are based on the effects of ozone on photosynthetic rates and aboveground growth, and do not consider its effects on soil C fluxes. Furthermore, studies now support the view that ozone may also have a profound impact on belowground processes because of altered C allocation to the roots and associated rhizosphere (Andersen, 2003; Fuhrer et al., 2016; Grantz et al., 2006; Wang et al., 2019). To better understand how increasing tropospheric ozone will affect global C fluxes, studies addressing ozone effects on soil C fluxes are therefore required.

Net ecosystem exchange (NEE) of CO_2 represents the balance between gross ecosystem productivity (GEP) and ecosystem respiration (R_{eco}) (Niu et al., 2010). It has been suggested that directly measuring NEE can be used to evaluate the response of ecosystem C sequestration to climatic factors (Luyssaert et al., 2007). To date, divergent responses of ecosystem C fluxes

to elevated ozone have been reported in an annual grassland (Calvete-Sogo et al., 2014), peatlands (Haapala et al., 2011; Toet et al., 2011), and a subalpine grassland (Volk et al., 2011). These contrasting results of studies from different ecosystem types under distinct ozone exposure conditions signify a limited understanding of ozone effects on ecosystem C fluxes.

Despite considerable awareness of the potential interaction between elevated ozone and nitrogen (N) fertilization, very little attention has been paid to investigate their combined effects on ecosystem production and function (Mills et al., 2016). In a seven-year study from a subalpine grassland, plant biomass was positively affected by N addition but neither ozone nor its interaction with N addition (Volk et al., 2014). In contrast, results of a recent meta-analysis show that elevated ozone generally tends to exacerbate the negative impact of limiting N on plant growth (Yendrek et al., 2013). Similarly, in an annual grassland ozone-induced reductions in N content and C/N ratio of plant biomass can be counterbalanced by N addition (Sánchez-Martín et al., 2017), despite the absence of their interaction on GHG emissions and ecosystem C fluxes. Altogether, these conflicting results suggest that the direction and magnitude of the combined effect of elevated ozone and N fertilization on ecosystem processes remains unpredictable.

As the dominant form of agriculture by land area, grasslands represent over two thirds of utilized agricultural land area in the UK (Defra, 2016). Semi-natural vegetation is well known to be adversely affected by elevated ozone concentrations across Europe (Hayes et al., 2007; Mills et al., 2011). The aforementioned studies have mainly focused on croplands and annual

grasslands, but less is known about how elevated ozone and its interaction with N fertilization affect ecosystem production and function (e.g. fodder production, trace gas emissions and soil properties) in temperate grasslands (Wang et al., 2019). Our objectives for this study were therefore (i) to determine the effects of elevated ozone on CH_4 and N_2O fluxes from a temperate grassland in the UK throughout a one-year period; (ii) to investigate how elevated ozone would affect ecosystem C fluxes during the growing season (i.e., ozone exposure period); and (iii) to test whether the responses of these fluxes to elevated ozone would be interacted with N fertilization. To this end, we carried out a field experiment with intact soil mesocosms from a temperate semi-improved grassland and exposed them to elevated ozone for one growing season using a ozone free-air controlled enrichment (O_3-FACE) platform.

2. Materials and methods

2.1 Experimental design

The experiment was conducted in 2017 in a O_3-FACE system at CEH Bangor Air Pollution Facility, Abergwyngregyn, North Wales, UK (13 m asl, 53°15'N, 4°01'W). The climate at the site is classed as temperate-oceanic with a mean annual soil temperature of 11°C at 10 cm depth and a mean annual rainfall of 1250 mm. Experimental plots consisted of 16 intact soil mesocosms (31 cm diameter × 25 cm deep), which were excavated in the early spring of 2017 from a semi-improved upland grassland located at the Henfaes Research Station, Abergwyngregyn, North Wales, UK (53°13'N, 4°0'W). The upland site is located at approximately 270 m altitude with a mean growing season temperature of 10 °C and a mean

annual precipitation of 1200 mm. This semi-improved grassland site had not received any fertilizer applications and is grazed at low stocking densities (1-2 ewes ha^{-1}). The vegetation was classified as *Cynosurus cristatus-Centaurea nigra* grassland (NVC MG5; Rodwell, 1992). The soil is classified as an Orthic Podzol (FAO, 1981). The soil of this site had a slightly acidic pH of 5.39±0.04, total soil C of 8.45±0.09%, and total N of 0.81±0.01%.

Four treatments were established to determine the effects of elevated ozone, N fertilization and their potential interaction. The low and high ozone treatments from the O_3-FACE system as described below (hereafter called the ambient and elevated ozone treatments, respectively) were used in this study. The exposure period of ozone was from May 26 to October 9, 2017. Each quarter of these mesocosms was randomly assigned to one of the four combinations of ozone and N fertilizer treatments. Nitrogen was added as NH_4NO_3 at a rate of 100 kg N ha^{-1} dissolved in 200 mL of deionized water per mesocosm on May 26, 2017. For the control treatment, an equal amount of deionized water was added. To ensure all other macro-nutrients were not limiting pasture growth, and that any nutrient response was the result of the N addition, calcium superphosphate for P and potassium chloride for K were also used and applied to all treatments at a rate of 10 and 50 kg ha^{-1}, respectively. The fertilizer addition rates were based on national guidelines.

The O_3-FACE system was established in the spring of 2014, consisting of nine rings of 4 m diameter as described previously (Wang et al., 2019). Breifly, the rings were arranged in a replicated 3 × 3 Latin square with 10 m between the centers of each ring. Low (ambient air)

and high ozone (ambient air + 20 ppb) treatments were used in this study. Ozone was generated from oxygen concentrated from air (Integra 10, SeQual) using a G11 ozone generator (Pacific Ozone). Small fans (200 mm, Explair) were used to push the ozone through the delivery pipe (65 mm, with 3 mm holes every 10 cm). Ozone delivery was achieved via computer controlled (LabView version 2012) solenoid valves operating using pulse width modulation. Wind speed was monitored continuously (WindSonic, Gill Instruments Ltd, UK) and was used to instantaneously adjust solenoid operation and thus ozone delivery. Ozone release was reduced at wind speeds below 16 m s^{-1} and did not occur when wind speeds fell below 2 m s^{-1}. At very high wind speeds the ozone concentrations may not be well controlled and thus did not reach the target maximum concentrations. Despite this, we still got elevated ozone with the 'high' ozone treatment compared to the low ozone treatment as the solenoid valves were <1 m from the O_3-FACE rings, the response time of ozone delivery to track windspeed was fast. Ozone was sampled adjacent to the plants in each ring at a height of 30 cm for approximately 3.5 min in every 30-min using an ozone analyzer (ThermoScientific, Model 49i, Reading, UK).

2.2 Soil GHG emissions and ecosystem C fluxes

Soil GHG fluxes were measured throughout the experimental year. Gas samples were collected by placing a handmade opaque chamber over the intact soil mesocosms and sealing with a wide rubber band to ensure that the headspace inside the chamber was air-tight. Three gas samples were taken within a 40-min enclosure time and immediately transferred into pre-evacuated 20-mL screw-cap vials (QUMA Elektronik & Analytik GmbH, Germany). Gas samples were

160 stored under positive pressure and analyzed using a Perkin Elmer Clarus 580 gas
161 chromatograph equipped with a Turbo Matrix 110 autosampler (PerkinElmer Inc., Shelton, CT,
162 USA). CH_4 and CO_2 were detected with a flame ionization detector connected to a methanizer
163 and N_2O with a ^{63}Ni electron-capture detector. Three standard gases were used for calibration
164 with concentrations of 1.42, 2.92, and 10.4 ppm CH_4; 258, 496 and 1276 ppm CO_2; and 308,
165 641, and 1500 ppb N_2O (BOC Gases Ltd, Guildford, UK). Gas fluxes were calculated from the
166 concentration change in the chamber versus time and were adjusted for air temperature and
167 atmospheric pressure at the time of sampling. The annual fluxes of these gases were
168 approximated by applying the trapezoid rule assuming constant flux rates per day.

169 Ecosystem C fluxes were measured during the growing season using a static-chamber
170 method, which has been used and validated in previous studies (Niu et al., 2010; Volk et al.,
171 2011). The measurements were made from June to September in 2017 and was taken between
172 8:00 and 11:00 am on sunny days. If it is rained or cloudy on a scheduled sampling date, we
173 postponed the measurements and selected the next sunny day. In total, complete sets of
174 measurements were made seven times during the growing season. A portable infrared gas
175 analyzer (EGM-4 Environmental Gas Monitor for CO_2; PP Systems Ltd, Hitchin, UK), which
176 was attached to a handmade transparent plastic chamber, was employed to sample and measure
177 CO_2 concentration ([CO_2]) *in situ* as described elsewhere (Williamson et al., 2016). A small fan
178 created moderate turbulence inside the chamber to facilitate air mixing. A temperature probe
179 was also installed inside to determine the chamber air temperature. During the measurement,
180 the chamber was tightly sealed to the rim of the mesocosm using a cell foam band. The chamber

181 [CO_2] were consecutively recorded at 20 s intervals during a 2-min measurement period per
182 mesocosm. The chamber [CO_2] did not drop below 340 ppm or rise above 500 ppm. After the
183 net ecosystem exchange (NEE) of CO_2 measurement, the chambers were opened to allow the
184 chamber [CO_2] to return to the ambient atmospheric concentration. Subsequently, the chamber
185 was again placed over the mesocosm and covered with a shade cloth, and the [CO_2]
186 measurement was repeated over a 2-min period (i.e., dark respiration). Due to the light removal
187 inside the chamber, the data of the chamber [CO_2] were considered to represent ecosystem
188 respiration (R_{eco}). To eliminate the disturbance effect during measurement, the first 20 s of data
189 was omitted in subsequent analysis to allow for initial adjustment of the chamber [CO_2]. The
190 quality of the measurement was considered acceptable if a linear regression of [CO_2] versus
191 time during the following 100 s yielded $R^2 \geq 0.9$, indicating strictly linear changes in the
192 chamber [CO_2]. Gross ecosystem productivity (GEP) was computed by the sum of absolute
193 values of NEE and R_{eco}. By convention, negative NEE values refer net C uptake by the
194 ecosystem, while positive NEE values represent net C loss from the ecosystem.

195 *2.3 Soil abiotic and biotic properties and plant analysis*

196 Air temperature and rainfall were recorded hourly on site with an automated weather station.
197 Parallel to gas sampling and ecosystem C flux measurements, soil temperature and volumetric
198 water content (*v*/*v*, %) at a depth of 10 cm in each mesocosm were measured by a handheld
199 thermometer (HI98509 Checktemp®1; Hanna Instruments Ltd, Leighton Buzzard, UK) and
200 moisture sensor (ML3 ThetaProbe; Delta-T, Cambridge, UK), respectively. Water-filled pore

space (WFPS) was calculated by dividing volumetric water content by total porosity (Linn and Doran, 1984). Total porosity was calculated as [1-(bulk density/particle density)] × 100% and using a particle density of 2.65 g cm^{-3}.

In May 2018, two soil cores (10-cm deep; 5-cm diameter) were collected from each mesocosm. Fresh soils were passed through a 2-mm sieve to remove visible plant material and small stones, and then placed at 4 °C to await analysis. Soil water content was determined gravimetrically by drying soil in an oven (24 h, 105 °C). Available soil C and N pools were quantified by extracting soil subsamples with 0.5 M K_2SO_4 (1:5 *w*/*v*). The concentrations of ammonium (NH_4^+) and nitrate (NO_3^-) in the extracts were determined via the colorimetric salicylate procedure of Mulvaney (1996) and the vanadate method of Miranda et al. (2001), respectively. For soil microbial biomass, additional subsamples were fumigated for 24 h with chloroform and similarly extracted with 0.5 M K_2SO_4 (1:5 *w*/*v*) (Vance et al., 1987). Dissolved organic C (DOC) and total dissolved N (TDN) in the 0.5 M K_2SO_4 extracts (fumigation and non-fumigation samples) were quantified using a Multi N/C 2100 TOC analyzer (AnalytikJena, Jena, Germany). Dissolved organic N (DON) was calculated as the difference between TDN and soil mineral N. Microbial biomass C and N concentrations were corrected using correction factors of 0.45 for C and 0.54 for N (Brookes et al., 1985; Wu et al., 1990). Total C (TC) and N (TN) contents of ground soil and plant samples were determined with a TruSpec® elemental analyzer (Leco Corp., St Joseph, MI). Net N mineralization and nitrification rates were determined by the aerobic incubation of soil samples for 14 days at 10 °C in the dark (Hart et al., 1994), followed by extraction with 0.5 M K_2SO_4 and analyzing for soil mineral N as

described above. To assess the effects of elevated ozone and N fertilization on hydrolytic extracellular enzyme activities, the potential activities of four extracellular enzymes: β-glucosidase, cellobiohydrolase, N-acetyl-glucosaminidase and leucine aminopeptidase were measured according to the fluorometric protocol (DeForest, 2009; Saiya-Cork et al., 2002).

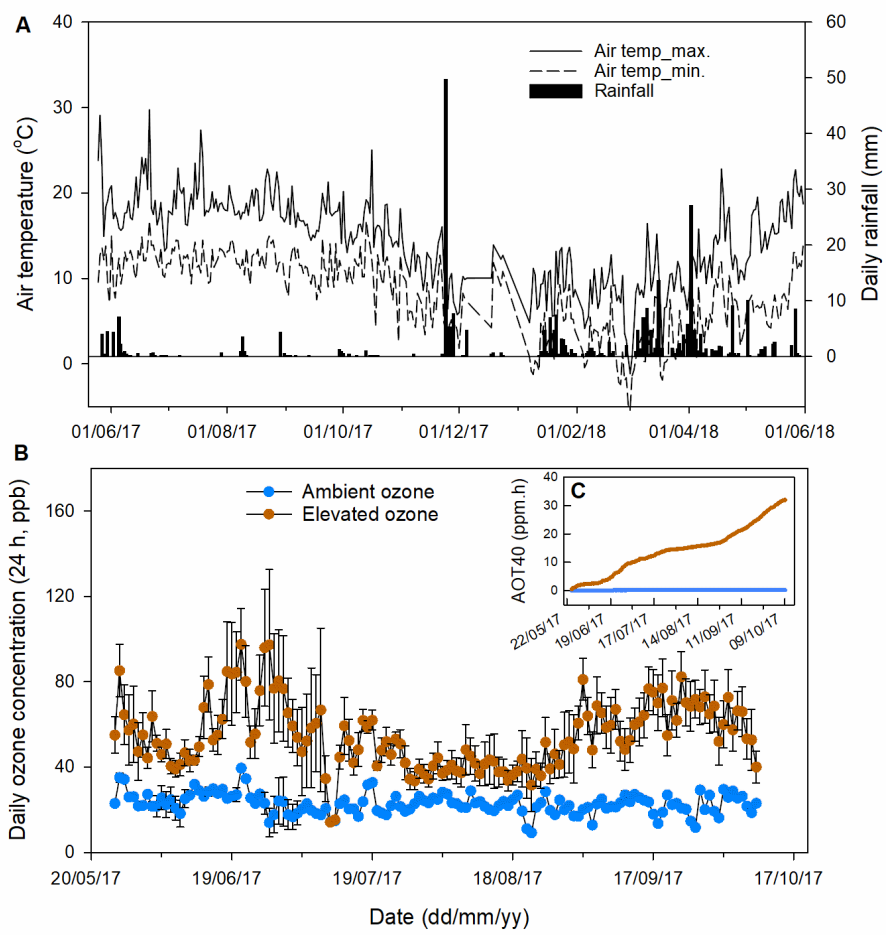
Plant biomass was measured twice by cutting all plants at 2 cm above the ground at the end of growing season (early November of 2017) and in May 2018, respectively. For root biomass measurement, two additional soil cores (10-cm deep; 5-cm diameter) was sampled from each mesocosm. The harvested soil cores were rinsed thoroughly and passed through a 0.5-mm aperture sieve and the root fragments remaining in the sieve recovered. All plant samples were oven-dried at 65 °C and weighed to determine their dry biomass.

2.4 Statistical analysis

Each parameter was tested for normal distribution (using Shapiro-Wilk's test) and equality of variance (using Levene's test), and parameters with non-normal distributions or unequal variances were either logarithmically or square-root transformed when necessary. For analysis of time-series data (i.e., repeated measurements of soil gas fluxes and soil factors), we used linear mixed effects models (LME, *package* LME4; Bates et al. 2014) to test for the fixed effects of ozone, N fertilization and their possible interaction. The spatial replication and time (sampling days) were included as random effects. If the Akaike's information criterion (AIC) showed an improvement in the LME models, we included a first-order temporal autoregressive function to account for the decreasing correlation of the measurements with increasing time

242 and/or a variance function (varIdent) to account for heteroscedasticity in the fixed-factor
243 variances (Crawley, 2012). We used linear mixed effects models to test the fixed effects of
244 ozone, N fertilization and their interaction on soil properties, extracellular enzyme activities
245 and plant biomass. Multiple comparisons were made using the Tukey HSD test.

246 To assess the relationships between soil GHG emissions or ecosystem C fluxes and soil
247 factors (temperature and WFPS), we used the mean values of the four replicate mesocosms on
248 each sampling day, and conducted Pearson correlation tests over the entire sampling period. In
249 all statistical tests, differences among treatments were considered significant at $P \leq 0.05$ and
250 marginally significant at $P \leq 0.09$. Statistical analyses were performed in R version 3.2.2 (R
251 Development Core Team, 2015).



252

253 **Figure 1.** Daily maximum and minimum air temperature and daily rainfall (A) over the period

254 of May 25, 2017 to May 31, 2018, daily ozone concentration (B) and AOT40 during daylight

255 hours (C) from ambient and elevated ozone treatments in the 2017 growing season. Values

256 represent mean±SEM (n = 3).

257 **3. Results**

258 *3.1 Climatic conditions and ozone exposure*

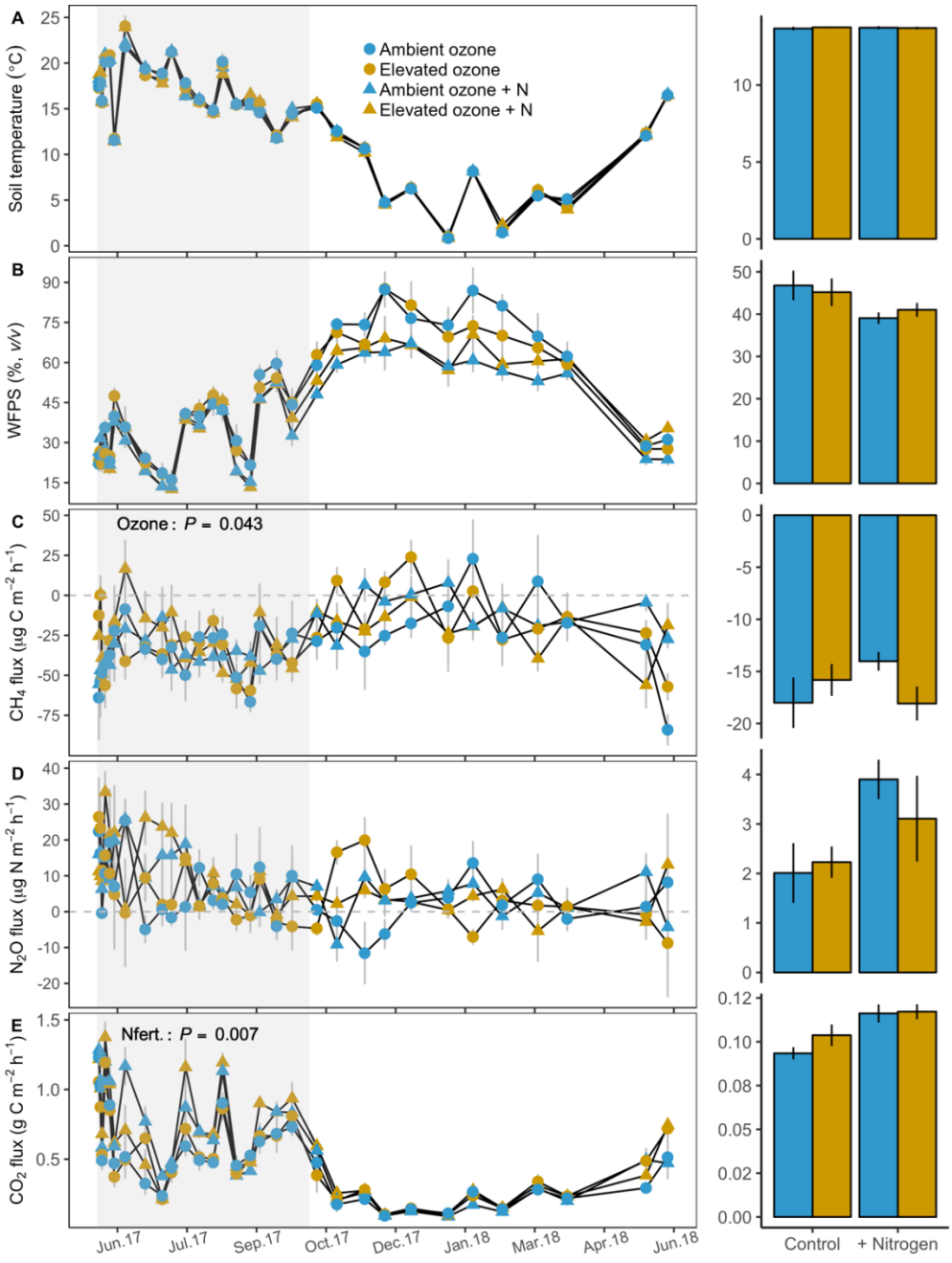
259 Over the experimental period, air temperature ranged from –5 °C to 30 °C, with an annual

260 average of 11 °C (Fig. 1A). The total rainfall was 324 mm, of which only 13% occurred during

the growing season (May–September 2017). Intact soil mesocosms were exposed to ozone in the field from May 25 to October 9, 2017, with a total of 136 days effective exposure (Fig. 1B). During the exposure period, the 24-h mean ozone concentrations were 22.9±0.6 ppb and 54.9±6.1 ppb in the ambient and elevated ozone treatments, respectively. As shown in Fig. 1C, AOT40 (accumulated ozone exposure over a threshold of 40 ppb) in the elevated ozone treatment was 31.9±10.1 ppm.h being markedly higher than that (< 0.5 ppm.h) in the ambient treatment.

3.2 Seasonal variability of soil microclimate and GHG emissions

Soil temperature followed a clear seasonal pattern with an annual mean of 13.7 °C across all treatments (Fig. 2A). Soil temperatures were highest in July 2017 and lowest during January 2018. Soil WFPS varied seasonally in response to rainfall and ranged from 12.6% to 87.6% (Fig. 2B). Soil WFPS periodically increased following summer rainfall. Soil water contents steadily increased during the winter season when a large proportion of the annual rainfall occurred. Neither soil temperature nor WFPS differed between treatments (all $P > 0.1$).



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276 **Figure 2.** Soil temperature (A), water-filled pore space (WFPS) (B) at a depth of 10 cm, and

277 year-round fluxes of CH_4 (C), N_2O (D) and night-time CO_2 (E) from a temperate semi-improved

278 grassland exposed to different levels of ozone and N. The averages of soil microclimate

279 parameters and gas fluxes over the experimental year are shown in the right panel. Values

280 represent mean±SEM (n = 4). The horizontal dashed line marks the zero flux. Grey shading

281 marks the ozone exposure period during the growing season. Results from the linear mixed
282 effects model at $P \leq 0.05$ are shown.

283 This semi-improved grassland was a small sink of atmospheric CH_4. Across all treatments,
284 CH_4 fluxes ranged from –84.1 to 23.9 μg C m^{-2} h^{-1} (Fig. 2C). During the phase of ozone
285 exposure, CH_4 uptake rates were significantly affected by elevated ozone with a 14% reduction
286 (P = 0.043) regardless of N fertilizer. A marginally significant interaction between elevated
287 ozone and N fertilizer was detected during the winter season (P = 0.077). In parallel with high
288 soil WFPS, CH_4 fluxes tended to be high and occasionally positive during the winter season.
289 Overall, CH_4 fluxes were negatively correlated with soil temperature and positively correlated
290 with soil WFPS (r = –0.41 and 0.51, both $P < 0.001$; Table 1), indicative of an increase in CH_4
291 uptake rates with an increase in soil temperature but a decrease of that with an increase in soil
292 water content. Because of the large spatial and temporal variations, neither elevated ozone nor
293 N fertilization had a significant effect on CH_4 emissions throughout the experimental period.

294 As with CH_4, N_2O emissions were temporally and spatially variable (Fig. 2D). Across all
295 treatments, N_2O fluxes ranged from –11.6 to 33.3 μg N m^{-2} h^{-1}. On a few occasions, fluxes
296 were negative indicating the occurrence of N_2O consumption by the soil. In contrast to CH_4,
297 N_2O fluxes correlated positively with soil temperature and negatively with soil WFPS (r = 0.36
298 and –0.33, both $P < 0.001$; Table 1). For both seasons, neither elevated ozone nor N fertilizer
299 affected N_2O emissions.

Table 1 Pearson correlation coefficients between greenhouse gas (CH_4, N_2O and night-time CO_2) fluxes or ecosystem C fluxes and soil environmental factors (soil temperature and water-filled pore space (WFPS) at a depth of 10 cm). NEE, net ecosystem exchange of CO_2; R_{eco}, ecosystem respiration; GEP, gross ecosystem productivity.

Parameter	*n*	Soil temperature	WFPS
Greenhouse gas fluxes			
CH_4	123	-0.41***	0.51***
N_2O	123	0.36***	-0.33***
Night-time CO_2	123	0.69***	-0.53***
Ecosystem C fluxes			
NEE	28	0.04	-0.67***
R_{eco}	28	0.45*	0.44*
GEP	28	0.26	0.67***

*, **, *** Values are significantly different from zero at $P < 0.05$, 0.01, and 0.001, respectively

The variation in seasonal night-time CO_2 fluxes was more pronounced as compared with CH_4 and N_2O emissions (Fig. 2E). Greatest fluxes occurred during the growing season despite a few occasions when small fluctuations occurred. Lowest emissions occurred after the autumn harvest of aboveground biomass in 2017 and remained relatively stable throughout the winter season. In addition, night-time CO_2 fluxes were best correlated with soil temperature and WFPS ($r = 0.69$ and –0.53, both $P < 0.001$; Table 1). Overall, night-time CO_2 fluxes were negatively and positively correlated with CH_4 and N_2O emissions, respectively ($r = –0.41$ and 0.46, both $P < 0.001$).

3.3 Seasonal variability of ecosystem C fluxes

During the growing season, ecosystem C fluxes were measured seven times in total (Fig. 3). Marked fluctuations in ecosystem C fluxes occurred in July 2017, which may have resulted from substantial variations in soil WFPS (Fig. 3). Ecosystem C fluxes were strongly correlated with soil WFPS (r = 0.44 to 0.67, P < 0.05–0.001; Table 1), while only the seasonal pattern of R_{eco} had a positive relationship with soil temperature (r = 0.45, P < 0.05).

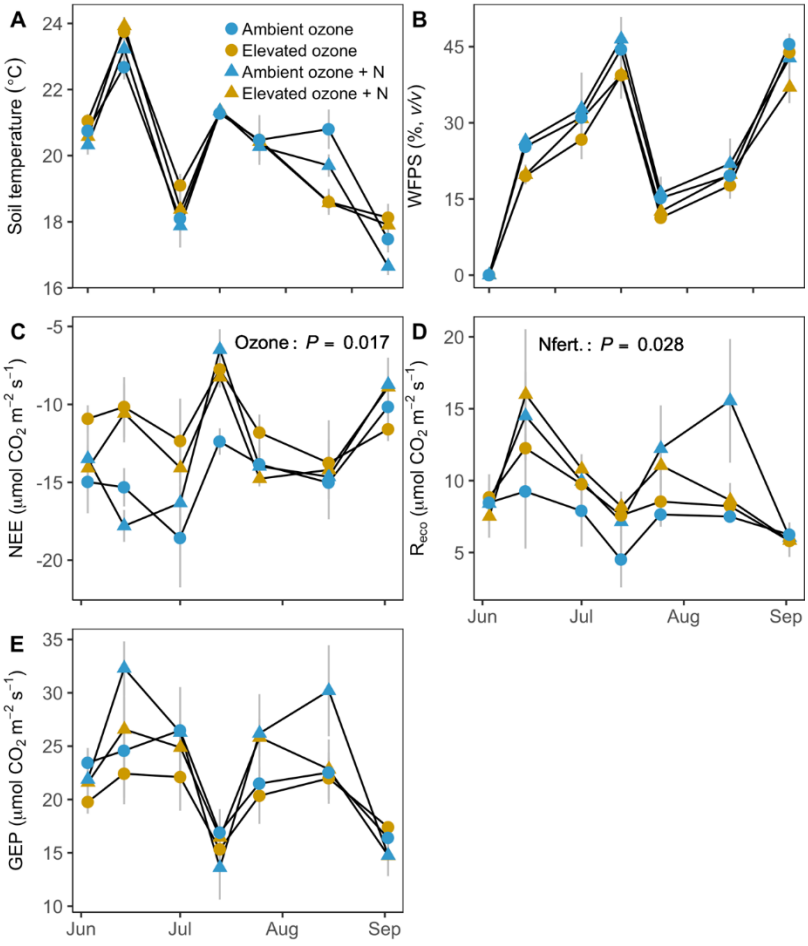

Figure 3 Soil temperature (A), water-filled pore space (WFPS) (B) at a depth of 10 cm, net ecosystem carbon exchange of CO_2 (NEE, C), ecosystem respiration (R_{eco}, D) and gross ecosystem productivity (GEP, E) during the growing season from a temperate semi-improved

grassland exposed to different levels of ozone and N. Values represent mean±SEM (n = 4). Results from the linear mixed effects model at $P \leq 0.05$ are shown.

Across the growing season, elevated ozone significantly decreased NEE (P = 0.017), whereas no effects of N fertilization (P = 0.823) or its interaction with elevated ozone (P = 0.161) were detected (Fig. 3C). Elevated ozone resulted in a 15% reduction of seasonal mean NEE. By contrast, R_{eco} was significantly increased by N fertilization (P = 0.028) but not by elevated ozone or their interaction (P = 0.315 and 0.155; Fig. 3D). Irrespective of ozone exposure, N fertilization significantly increased mean R_{eco} by 11%. Unlike NEE and R_{eco}, none of elevated ozone, N fertilization, or their interaction affected GEP (Fig. 3E).

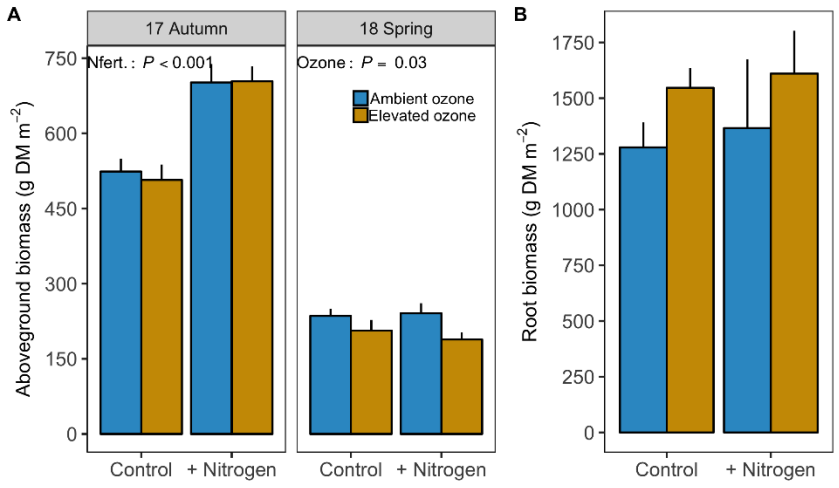

Figure 4 Aboveground biomass (A) and root biomass (B) in a temperate semi-improved grassland exposed to different levels of ozone and N. Values represent mean±SEM (n = 4). Results from the linear mixed effects model at $P \leq 0.05$ are shown.

3.4 Aboveground and root biomass

Harvested aboveground biomass differed significantly between cut events ($P < 0.001$; Fig. 4A). In the 2017 autumn harvest, aboveground biomass from the N fertilized treatment (mean: 703±23 g DM m^{-2}) was higher than from the control treatment (mean: 515±20 g DM m^{-2}, $P < 0.001$), but there was no difference between N treatments at the 2018 spring harvest ($P = 0.736$). Aboveground biomass was affected by elevated ozone in the 2018 spring harvest but not in the 2017 autumn harvest. In the 2018 spring harvest, aboveground biomass decreased from 238±12 g DM m^{-2} in the ambient ozone treatment to 197±13 g DM m^{-2} in the elevated ozone treatment ($P = 0.03$). The average root biomass was 1450 g DM m^{-2} and did not differ between either elevated ozone or N fertilization treatments (Fig. 4B).

3.5 Soil properties, net N-cycling rates and enzyme activities

Towards the end of the experiment, neither elevated ozone or N fertilization nor their interaction affected NH_4^+ concentrations, DON or microbial biomass C (Table 2). Nitrate concentrations and microbial biomass N were higher in the elevated ozone plots than in the ambient ozone plots ($P = 0.003$ and 0.012, respectively), but there was no difference for both parameters between control and N-fertilized plots. A marginally significant interaction between ozone and N fertilizer was detected for DOC ($P = 0.081$). Microbial biomass C:N ratio was lower in the elevated ozone plots than in the ambient ozone plots ($P = 0.018$). Net N mineralization, which is often used as an index of plant available mineral N, was higher in the elevated ozone treatment than in the ambient ozone treatment ($P = 0.056$) but did not differ between the control

and N fertilized treatments. Further, net nitrification was higher in the elevated ozone than the ambient ozone treatment (P = 0.011).

Potential activities in two of studied soil extracellular enzymes were not affected by elevated ozone, N fertilization or their interaction (Table 2). Elevated ozone and N fertilization significantly increased cellobiohydrolase activity by 26% and 73%, respectively (P = 0.04 and 0.003). Elevated ozone also marginally stimulated leucine aminopeptidase activity by 15% as compared to under ambient ozone (P = 0.069).

4. Discussion

In the past decade, many conclusions about the impacts of elvated ozone have been drawn from croplands (Bhatia et al., 2011; Decock et al., 2012; Kou et al., 2015; Tang et al., 2015; Zheng et al., 2011), peatlands (Toet et al., 2017, 2011) and subalpine grasslands (Volk et al., 2011). The effects of elevated ozone and N fertilization alone or in combination on GHG emissions and ecosystem C fluxes have been rarely reported, especially in temperate grasslands (Sánchez-Martín et al., 2017; Wang et al., 2019). This is to our knowledge the first study to report how GHG emissions, coupled with ecosystem C fluxes, in response to elevated ozone and N fertilization alone or in combination from a temperate semi-improved grassland under fully open-air field conditions.

373 **Table 2** Soil properties, net N-cycling rates and potential enzyme activities measured in May

374 2018 from a temperate semi-improved grassland exposed to different levels of ozone and N.

375 Values represent mean±SEM (n = 4).

	Ambient ozone		Elevated ozone	
	Control	+ Nitrogen	Control	+ Nitrogen
NH_4^+ (mg N kg^{-1})	4.48±0.54	6.25±0.54	5.30±0.27	5.12±0.90
NO_3^- (mg N kg^{-1})	0.43±0.03^{B*}	0.46±0.21^{B}	1.28±0.26^{A}	1.01±0.50^{A}
DOC (mg C kg^{-1})	336±13†	295±14	314±11	318±8
DON (mg N kg^{-1})	59.7±1.8	54.6±3.1	55.6±2.4	57.1±1.0
Microbial biomass C (mg C kg^{-1})	2445±67	2371±103	2548±172	2461±73
Microbial biomass N (mg N kg^{-1})	227±3^{B}	229±15^{B}	259±24^{A}	253±9^{A}
Microbial biomass C:N	10.8±0.3^{A}	10.4±0.3^{A}	9.9±0.3^{B}	9.7±0.1^{B}
Net N mineralization (mg N kg^{-1} day^{-1})	–0.03±0.11‡	–0.16±0.09	0.22±0.15	0.13±0.15
Net nitrification (mg N kg^{-1} day^{-1})	0.04±0.04^{B}	0.06±0.09^{B}	0.33±0.06^{A}	0.19±0.08^{A}
ß-glucosidase (nmol g^{-1} h^{-1})	126±24	121±15	113±2	132±16
Cellobiohydrolase (nmol g^{-1} h^{-1})	16.7±2.8^{Bb§}	27.1±5.7^{Ba}	19.7±3.0^{Ab}	36.0±3.5^{Aa}
N-acetyl-glucosaminidase (nmol g^{-1} h^{-1})	83.6±6.2	94.3±7.5	83.5±7.8	84.3±9.9
Leucine aminopeptidase (nmol g^{-1} h^{-1})	42.7±3.4‡	37.8±1.7	45.1±2.3	47.1±3.8

376 * Values with different uppercase letters indicate significant differences between ambient and

377 elevated ozone treatments (linear mixed effects model with the Tukey HSD test at $P \leq 0.05$)

378 † Marginally significant interaction between ozone and N fertilized treatments (linear mixed

379 effects model at $P \leq 0.09$)

380 ‡ Marginally significant effect of elevated ozone (linear mixed effects model at $P \leq 0.09$)

381 § Values with different lowercase letters indicate significant differences between control and
382 N-fertilized treatments (linear mixed effects model with the Tukey HSD test at $P \leq 0.05$)

383 *4.1 Response of CH_4 fluxes to elevated ozone and N fertilization*

384 The fluxes of CH_4 from our studied grasslands were generally negative throughout the
385 experimental period, which agrees with the argument that temperate grassland soils generally
386 act as an atmospheric CH_4 sink (Hörtnagl et al., 2018; Smith et al., 2000). We found that
387 elevated ozone tended to decrease the magnitude of CH_4 uptake (a slightly significant effect)
388 compared to the ambient plots during the growing season but not the winter period, suggesting
389 a negative impact on the CH_4 uptake capacity. This is unlikely to be related to a direct effect of
390 ozone on soil methanogens and methanotrophs since ozone does not penetrate far into the soil
391 (Blum and Tingey, 1977; Toet et al., 2009). The reduced CH_4 uptake during the growing season
392 could be possibly due to altered methanotroph activity at elevated ozone. Across the one-year
393 period CH_4 emissions were not affected by elevated ozone, which agrees with findings of
394 several studies under OTC conditions (Kanerva et al., 2007; Sánchez-Martín et al., 2017). For
395 example, studies in an annual Mediterranean grassland reveal that ozone exposure for a short-
396 term period (49 days) did not affect CH_4 emissions (Sánchez-Martín et al., 2017). Similarly,
397 the three-year field study of Kanerva et al. (2007) reported no overall effect of ozone exposure
398 on CH_4 fluxes from ground-planted meadow mesocosms. By contrast, studies in rice fields
399 under either OTC or O_3-FACE conditions show that elevated ozone can decrease CH_4 emissions
400 during the rice growing season (Bhatia et al., 2011; Tang et al., 2015; Zhang et al., 2016). Either

401 a negative or transient effect of elevated ozone on CH_4 emissions from peatlands is also reported
402 (Mörsky et al., 2008; Toet et al., 2011).

403 Although it has been suggested that N addition decreases CH_4 uptake in upland ecosystems
404 (Liu and Greaver, 2009), our results show that neither N fertilization nor its interaction with
405 elevated ozone affected CH_4 uptake. This is supported by the findings of a meta-analysis where
406 they found that soil CH_4 uptake in natural or with short-term N fertilization sites showed a non-
407 significant change when N is added (Aronson and Helliker, 2010). Thus, the lack of CH_4 uptake
408 in response to N addition could be attributed to a one-time application of N fertilizer into this
409 semi-improved grassland. On the other hand, our result contradicts with their finding that N
410 addition effects on soil CH_4 uptake may be switched from stimulation to inhibition when N
411 addition exceeds a threshold value of 100 kg N ha^{-1} yr^{-1} (Aronson and Helliker, 2010). This
412 implies that a shift from stimulation to inhibition of soil CH_4 uptake might be related to not only
413 the amount of N added but also the inherent soil N status of the studied system. We are aware
414 of only one previous field study where they examined the interaction effect between elevated
415 ozone and N fertilization on GHG emissions from a simplified annual grassland (Sánchez-
416 Martín et al., 2017). Consistent with our findings, the response of CH_4 emission to ozone
417 exposure from their experiment site was not N fertilization dependent. While we found either
418 elevated ozone or N fertilization may have a negligible effect on soil CH_4 uptake in this
419 temperate grassland, future multiple-year studies are needed to reveal the inter-annual
420 variability and its underlying mechanisms.

4.2 Response of N_2O fluxes to elevated ozone and N fertilization

This semi-improved grassland acted as a source of N_2O, although negative N_2O fluxes were recorded occasionally, especially during the winter season (Fig. 2D). Our results showed that neither elevated ozone or N fertilization nor their interaction had an appreciable effect on N_2O emission over the experimental year. The lack of responsiveness of N_2O emissions to N addition suggests that a one-time application of 100 kg N ha^{-1} (the first fertilizer N application to this grassland for >25 years) at the beginning of the experiment would not have been sufficient to produce significant N_2O emissions in this semi-improved grassland. Annual N_2O emissions from this study were much higher (0.18–0.34 kg N ha^{-1} yr^{-1}) than that (ca. 0.06 kg N ha^{-1} yr^{-1}) from the field study of Marsden et al. (2018) in the same grassland. This is likely due to the differences in microclimate between the two study sites. In their study, the large amount of N applied in the form of sheep urine (ca. 2000 kg N ha^{-1} yr^{-1}) had no marked effect on N_2O fluxes (Marsden et al., 2018), which partly supports our finding. Furthermore, N fertilization significantly increased aboveground biomass during the growing season, but had minor resultant effects on plant yield and soil characteristics at the end of the experimental year (Fig. 4A; Table 2). Therefore, these findings collectively point to the consumption of applied N fertilizer tightly coupled to plant uptake and microbial immobilization in this semi-improved grassland.

To date, the divergent responses of N_2O fluxes to elevated ozone have been reported. For example, some studies showed a decrease in N_2O emissions under elevated ozone (Bhatia et al.,

2011; Kou et al., 2015), other reported stimulated emissions (Sánchez-Martín et al., 2017) or no marked effects (Decock et al., 2012; Kanerva et al., 2007) compared to the ambient ozone concentrations. As soil nitrification and denitrification processes are major pathways of N_2O emissions, changes in the substrate availability due to elevated ozone are expected to alter N_2O emissions. We found that elevated ozone resulted in a significant decline of aboveground biomass at the 2018 spring harvest, which may, in turn, contribute to accelerating soil N-cycling (i.e., a build-up of soil nitrate, higher rates of net N mineralization and nitrification; Table 2). The unchanged N_2O emissions but stimulated N-cycling processes could be explained by the low intensity of soil N_2O emission and/or other pathways being altered which might be primarily responsible for N loss from this system. Indeed, in a soybean agroecosystem under fully open-air conditions, Decock et al. (2012) also demonstrated that elevated ozone stimulated soil N-cycling but had no effect on soil N_2O emissions. In contrast, in an annual Mediterranean grassland, ozone exposure reduced the fertilization effect of N on plant growth and thereby resulted in a significant reduction of soil N_2O emissions (Sánchez-Martín et al., 2017). Clearly, this was not the case for our study because of the absence of an interaction between elevated ozone and N fertilization on plant yield, especially during the growing season (Fig. 4). Nevertheless, it should be noted that the single N application in our study might counterbalance the negative impact of ozone exposure on plant growth during the growing season.

4.3 Effects of elevated ozone and N fertilization on ecosystem C fluxes

A growing number of studies have documented the detrimental effects of elevated ozone on grassland plant species, ranging from visible injury of leaves to substantially decreased productivity (Hayes et al., 2007; Volk et al., 2006). In our study, elevated ozone resulted in a small reduction (3.2% for the control plots) but a significant reduction of 17.1% in aboveground biomass during the growing and non-growing seasons, respectively (Fig. 4A). During the growing season, elevated ozone caused a significant reduction of NEE (14.9%), which is consistent with other studies in an annual temperate grassland (Calvete-Sogo et al., 2014; Sánchez-Martín et al., 2017) and peatland microcosms (Haapala et al., 2011). Because of the unresponsiveness of total aboveground biomass, the reduced NEE by elevated ozone at the canopy level is likely due to the changed photosynthetic and/or respiration rates (Andersen, 2003). We noted that these semi-improved grassland mesocosms had a higher growth rate, in terms of stimulated fluxes of trace gas and night-time CO_2 (Fig. 2C-E), under the O_3-FACE field condition than *in situ* on the upland site (Marsden et al., 2018). Thus, our results support the view that plant communities with fast-growing rates (i.e., annual and newly established systems) would be more responsive to elevated ozone than these perennial and well established systems that are characterized by slower growth rates (Grantz et al., 2006; Grime, 2000). In contrast, we are aware of a transient effect of elevated ozone on NEE in peatlands (Haapala et al., 2011; Niemi et al., 2002; Rinnan et al., 2003). On a subalpine grassland, Volk et al. (2011) found no effect of elevated ozone on NEE measured during the third growing season. Taken together, our results and those of previous findings suggest that further long-term investigations are warranted to evaluate the inter-annual response of NEE to elevated ozone in grasslands.

Ecosystem respiration as the sum of the respiration from plants and heterotrophs was not affected by elevated ozone during the growing season (Figs. 2E and 3D). This is in agreement with findings from other studies in subalpine and annual grasslands (Calvete-Sogo et al., 2014; Volk et al., 2011). Similarly, studies from peatlands suggested no effect of elevated ozone on ecosystem respiration, especially during the first experimental year (Haapala et al., 2011; Kanerva et al., 2007; Toet et al., 2011). In our study, the unchanged ecosystem respiration might be due to the response of heterotrophic respiration to elevated ozone which was unresponsive or masked by the larger proportion of plant respiration (Volk et al., 2011). As expected, stimulation of plant productivity provides more substrate for plant and soil respiration, leading to increases in ecosystem respiration following N fertilization (Figs. 2E and 3D). The positive response of ecosystem respiration to N addition is consistent with that in a subalpine grassland (Volk et al., 2011). In contrast, the lack of N effects on ecosystem respiration in an annual grassland was attributed to the fact that the low N doses were only enough to meet plant N demand but had no effect on the yield or gas exchange rates (Calvete-Sogo et al., 2014). Since the assimilation processes was primarily N-limited, we expected to see the positive response of GEP to N fertilization in this semi-improved grassland. However, the differential responses of NEE and ecosystem respiration to elevated ozone and N fertilization resulted in non-significant changes in GEP, suggesting N fertilization counterbalanced the effect of elevated ozone on GEP in our studied grassland.

4.4 Short-term effects of elevated ozone and N fertilization on belowground processes

While the intact grassland mesocosms were exposed to ozone only for one growing season, our results indeed showed that soil N dynamics were altered at the end of the experimental year. Increased soil mineral N availability under elevated ozone may be due to the significant reduction of aboveground biomass during the non-growing season, which may have contributed to reduced plant N uptake. On the other hand, we found that the activities of cellobiohydrolase and leucine aminopeptidase were higher in the elevated ozone plots than in the ambient plots, suggesting that elevated ozone stimulates microbes in the soil to produce enzymes that degrade cellulose and peptide-containing moieties. Parallel to the marginal increase of net N mineralization, our results suggest an increased soil N mineralization by elevated ozone in this grassland soil. Under elevated ozone, the unchanged soil NH_4^+ is likely related to increased microbial N immobilization, whereas stimulated soil net nitrification may have resulted in an accumulation of soil NO_3^-. Consistent with our findings, other studies in the O_3-FACE systems have reported the increased soil mineral N availability under elevated ozone in both soybean and wheat fields (He et al., 2014; Wu et al., 2016). Note that our soil sampling was carried out in early spring of 2018, which probably contributes to masking the possible N fertilization effect or its interaction with ozone, especially during the growing season. As soil mineral N availability is mainly regulated by plant uptake and microbial-mediated N transformation processes (Schimel and Bennett, 2004), our results collectively point to a positive feedback of soil N transformation in this semi-improved grassland to short-term of ozone exposure.

5. Conclusions

To our knowledge, this study is the first to report the effects of elevated ozone on GHG and ecosystem C fluxes in temperate grasslands under fully open-air field conditions. Our results demonstrate that elevated ozone may reduce atmospheric CH_4 uptake and net C uptake during the growing season in this semi-improved grassland. Given that the reported responses of CH_4 emission and NEE to elevated ozone are still inconsistent and controversial yet, we speculate that the depressed CH_4 uptake under elevated ozone could be transient, which warrants further investigation. The lack of any interaction between elevated ozone and N fertilization is likely due to the one-time N fertilization at the beginning of experiment, which probably was not synchronized with the occurrence of ozone injury of the plants. Further, our results suggest that short-term of ozone treatment may have contributed to accelerated soil N cycling in this grassland. Further investigations are warranted to examine the long-term impact of elevated ozone on ecosystem production and function.

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