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Four years of experimental climate change modifies the microbial drivers of N₂O fluxes in an upland grassland ecosystem

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Abstract

Emissions of the trace gas nitrous oxide (N₂O) play an important role for the greenhouse effect and stratospheric ozone depletion, but the impacts of climate change on N₂O fluxes and the underlying microbial drivers remain unclear. The aim of this study was to determine the effects of sustained climate change on field N₂O fluxes and associated microbial enzymatic activities, microbial population abundance and community diversity in an extensively managed, upland grassland. We recorded N₂O fluxes, nitrification and denitrification, microbial population size involved in these processes and community structure of nitrite reducers (*nirK*) in a grassland exposed for 4 years to elevated atmospheric CO₂ (+200 ppm), elevated temperature (+3.5 °C) and reduction of summer precipitations (–20%) as part of a long-term, multifactor climate change experiment. Our results showed that both warming and simultaneous application of warming, summer drought and elevated CO₂ had a positive effect on N₂O fluxes, nitrification, N₂O release by denitrification and the population size of N₂O reducers and NH₄ oxidizers. *In situ* N₂O fluxes showed a stronger correlation with microbial population size under warmed conditions compared with the control site. Specific lineages of *nirK* denitrifier communities responded significantly to temperature. In addition, *nirK* community composition showed significant changes in response to drought. Path analysis explained more than 85% of *in situ* N₂O fluxes variance by soil temperature, denitrification activity and specific denitrifying lineages. Overall, our study underlines that climate-induced changes in grassland N₂O emissions reflect climate-induced changes in microbial community structure, which in turn modify microbial processes.

Keywords: AOB, climate change, denitrification, diversity, grasslands, N₂O, *nirK*, nitrification, *nosZ*

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Introduction

In recent decades, changes in land use and human activities have had significant impacts on gaseous nitrogen (N) losses and the global cycle of N (Galloway *et al.*, 2004), contributing to regional and global changes in the atmosphere (IPCC, 2007). Emissions of nitrous oxide (N₂O) are of particular interest because this trace gas has a strong global warming potential (ca. 310 times greater than that of carbon dioxide) and is the single most important ozone-depleting emission (Ravishankara *et al.*, 2009). The magnitude of N₂O emissions depends on both microbial activities (nitrifiers and/or denitrifiers, Bremner, 1997; Wrage *et al.*, 2004) and abiotic factors, including soil temperature, oxygenation, mineral nitrogen, pH, carbon availability and water

content (Simek *et al.*, 2002; Smith *et al.*, 2003; Jones *et al.*, 2005). Consequently, understanding the interplay between microbial and environmental variables is critical for the estimation of potential N₂O fluxes from soils under climate change.

Despite a large number of studies documenting gaseous N₂O emissions from grassland ecosystems, few have focused on impacts of climate change drivers on N₂O fluxes and associated microbial processes (Clayton *et al.*, 1997; Flechard *et al.*, 2007; but see Avrahami & Bohannon, 2009). In theory, warming is expected to have positive effects on nitrification and denitrification rates (Gödde & Conrad, 1999), with cascading effects on N₂O emissions. However, warming responses of both nitrification and denitrification appear to be highly variable across sites (Emmett *et al.*, 2004; Horz *et al.*, 2004; Malchair *et al.*, 2010; Szukics *et al.*, 2010), which may partly reflect variable soil water content status during experiments (Barnard & Leadley, 2005). Impacts

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of reduced soil moisture status on microbial processes are well established (Barnard *et al.*, 2004; Barnard & Leadley, 2005; Bateman & Baggs, 2005), typically promoting denitrification at the expense of nitrification *via* changes in soil aeration and O₂ content (Smith *et al.*, 2003). In addition, elevated CO₂ may indirectly alter microbial processes by both increasing soil moisture (Smith & Tiedje, 1979) and carbon substrate availability (Luo & Mooney, 1999). Previous study suggests that elevated CO₂ may have greater positive effects on denitrification than nitrification (Baggs *et al.*, 2003; Barnard *et al.*, 2004), but considerable variation is observed across studies.

Although information on N₂O emissions and microbial activities subjected to individual climate change drivers is becoming increasingly available (Bateman & Baggs, 2005; Kammann *et al.*, 2008; Malchair *et al.*, 2010), data on N₂O flux responses to multiple and simultaneous environmental changes remain scarce. In a recent study, examining the impact of co-occurring climatic changes on N₂O fluxes in an upland grassland, Cantarel *et al.* (2011) found that N₂O fluxes responded equally strongly to both warming alone and the combination of summer drought or elevated CO₂ and warmed conditions. Results from laboratory incubations suggest that interactions between soil moisture and temperature can generate complex patterns of N₂O emissions under controlled conditions (Avrahami & Bohannon, 2009), but the importance of multiple climate changes for field N₂O emissions remains unclear.

In addition to direct climate-induced changes in microbial activities, climate change drivers can impact N transformations and N₂O emissions via indirect effects on the abundance of different microbial populations, and microbial community structure. Variation in soil N₂O emissions may reflect differences in terms of abundances and/or composition of AOB (ammonium oxidizing archaea seem to be not involved in N₂O emission; Di *et al.*, 2010) and denitrifying microorganisms (Avrahami & Bohannon, 2009; Philippot *et al.*, 2010; Brown *et al.*, 2011). To date, only AOB community structure has been studied for grasslands subjected to complex, multiple climate change treatments (Horz *et al.*, 2004). Horz *et al.* found that abundance of AOB decreased in response to combined elevated CO₂ and increased precipitation, but these effects appeared to be buffered under elevated temperature conditions. To our knowledge, no study has yet focused on changes in denitrifiers community structure under climate change. Hence, the potential impact of multiple climatic variables on the microbial community structure, and the respective contributions of AOB and denitrifying microorganisms to N₂O fluxes on terrestrial ecosystems remain poorly understood.

In the present study, we investigated the relationship between field N₂O fluxes and soil microbial parameters under three key climate change drivers at the Clermont Climate Change Experiment facility (Bloor *et al.*, 2010). This long-term grassland climate change facility manipulates air temperature (+3.5 °C), atmospheric CO₂ (+200 ppm) and summer drought (−20% summer rainfall) in an additive experimental design. The aims of our study were to determine impacts of sustained single and combined climate change treatments on N₂O fluxes, nitrification, denitrification, abundance of microorganisms (AOB and nitrite reducers), denitrifiers community structure and to estimate the existing relationships between N₂O fluxes, abiotic parameters and microbial parameters. Specifically, we asked: (1) How do nitrification, denitrification, abundances and composition of microbial nitrifiers/denitrifiers respond to multiple and simultaneous climate changes? (2) Are variations in field N₂O fluxes mirrored by changes in microbial activities, abundance or community structure of specific microbial functional groups?

Materials and methods

Experimental design and climate treatments

The studied ecosystem was an upland permanent grassland in the French Massif Central region (45°43'N, 03°01'E, 850 m a.s.l.), characterized by a Cambisol soil (59.5% sand, 19.7% silt, 20.8% clay, pH 6.2), and a grass-dominated plant community (*Festuca arundinaceae*, *Elytrigia repens*, *Poa pratensis*; described in Bloor *et al.*, 2010). The study area has a mean annual temperature of 8.7 °C and a mean annual rainfall of 780 mm.

The Clermont Climate Change Experiment was established in 2005, manipulating air temperature, summer rainfall and atmospheric CO₂ in line with IPCC projections for the study area in 2080 (ACACIA A2 scenario, IPCC, 2007; see Bloor *et al.*, 2010 for full details). In brief, the experimental design consisted of 80 grassland monoliths (0.5 × 0.5 × 0.4 m in size), excavated from the study grassland site and allocated at random to one of four climate treatments; C (control), T (+3.5 °C), TD (+3.5 °C, 20% reduction in summer rainfall) and TD_{CO₂} (+3.5 °C, 20% reduction in summer rainfall, CO₂ levels of 600 ppm). Each experimental treatment comprised of five experimental units (or repetitions), formed by grouping four monoliths together in specially prepared cavities in the ground. Elevated temperatures were achieved by transporting monoliths to a nearby lower-altitude site (Clermont-Ferrand, 350 m a.s.l.). Summer drought was established by the use of rain screens and reduced watering regimes during June, July and August. Enrichment of atmospheric CO₂ was obtained by Mini-FACE (Free Air Carbon dioxide Enrichment) technology; the target CO₂ concentration was only operational during daylight hours.

Meteorological measurements were achieved using a Campbell Scientific automatic weather station and logged to a

CRX-10 data logger (Campbell scientific Inc., Utah, USA) at 30 min intervals for both the upland and lowland sites. Volumetric soil moisture (0–20 cm) was recorded hourly using ECH₂O-20 probes (Dielectric Aquameter; Decagon Devices, Inc., Pullman, WA, USA). To stimulate the management prior to monolith extraction (i.e., low-intensity sheep grazing and no fertilization), vegetation in all experimental units was cut to a height of 5 cm at 6 month intervals (April and October). Monoliths were not fertilized throughout the study, in keeping with extensive management practices.

N₂O flux measurements and soil sampling

N₂O fluxes were determined on four dates between May and November 2009, using medium-size, closed and non-vented manual chambers on one monolith per experimental unit (following Cantarel *et al.*, 2011). During each N₂O measurement campaign, chambers were fixed onto a permanent base for each target monolith and gas samples were taken at 520 min intervals using a quick release pneumatic connector (TST Tansam Inc, Kocaeli, Turkey) and a PTFE-Teflon tube connected to an INNOVA 1412 photoacoustic multi-gas analyzer (INNOVA AIR Tech Instruments, Ballerup, Denmark). The INNOVA gas analyzer was encased in an air-conditioned box maintained at 20–25 °C to avoid confounding effects of temperature on analyzer measurements. N₂O fluxes were calculated by linear regression of N₂O in the chamber against time; flux data were rejected if the statistic *P*-value was above 0.05 and *r*² < 0.95 (Cantarel *et al.*, 2011). Soil temperature in the topsoil layer (2–5 cm) was recorded by thermocouples (TC S.A., Dardilly, France) during N₂O measurement campaigns. Immediately following *in situ* N₂O measurements, three soil cores (diameter 1.5 cm) were taken from the top layer (0–10 cm) of each target monolith, pooled together and sieved at 4 mm. Soils were stored for less than 5 days at 4 °C before carrying out assays for nitrification and denitrification enzyme activity (NEA, DEA respectively). A subsample of ca. 2 g fresh soil was frozen at –18 °C for subsequent molecular analyses.

Denitrifying and nitrifying enzyme activities

Denitrification enzyme activity (DEA) was measured in fresh soils from each monolith following the protocol described in Patra *et al.* (2006). Two sub-samples (10 g equivalent dry soil) from each soil sample were placed into 150 ml plasma flasks, and 7 ml of solution containing KNO₃ (50 µg NO₃[–] N g^{–1} dry soil), glucose (0.5 mg C g^{–1} dry soil) and glutamic acid (0.5 mg C g^{–1} dry soil) were added. Additional distilled water was provided to achieve 100% water holding capacity and optimal conditions for denitrification. The atmosphere was replaced by helium to provide anaerobic conditions and for one flask of each pair, 10% C₂H₂ was added to inhibit N₂O reductase activity. During incubation at 28 °C, gas samples were taken at 2, 3h30, 5, 6h30 and immediately analyzed for N₂O quantitation using a gas chromatograph (R3000µGC; SRA instrument, Marcy l'Etoile, France). For the first series of samples without C₂H₂, we measured N₂O accumulation, i.e.,

potential N₂O emission rates of our soil (N₂O_{DEA}). The second series of samples with C₂H₂ allowed the determination of maximal N₂O production (N₂O_{TOT}). We estimated potential fluxes of N₂ (N₂DEA) by subtracting N₂O_{DEA} from N₂O_{TOT}.

Nitrification enzyme activity (NEA) was determined following the protocol described in Dassonville *et al.* (2011). Briefly, subsamples of fresh soil (3 g equivalent dry soil) were incubated with 6 ml of a solution of N-NH₄ (50 µg N-(NH₄)₂SO₄ g^{–1} dry soil). Distilled water was adjusted in each sample to achieve 24 ml of total liquid volume in flasks. The flasks were sealed with Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA) and incubated at 28 °C with constant agitation (180 rpm). During incubation, 1.5 ml of soil slurry was sampled at 1, 2h30, 4, 5h30 and 7h, filtered (0.2 µm pore size). Samples were stored at –20 °C until analysis of NO₂[–]/NO₃[–] concentrations on an ionic chromatograph (DX120 Dionex, Salt Lake City, USA). A linear rate of NO₂[–] + NO₃[–] production with time was always observed, and the rates of NEA were determined from the slope of this linear regression. The intercept was used to estimate pools of soil nitrate (NO₃[–]).

Soil DNA extraction and quantitation of AOB, nirK and nosZ abundances

DNA was extracted for each frozen soil subsample (0.5 g equivalent dry soil) using the 96 Well Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) and manufacturer protocols. The quantity of the DNA extraction was checked using the Quant-iT™ PicoGreen® method (Quant-iT™ PicoGreen® dsDNA Assay kit; Molecular Probes Inc., Eugene, OR, USA).

All gene quantitations were obtained by qPCR, using a Lightcycler 480 (Roche Diagnostics, Meylan, France). The abundance of β-proteobacterial AOB, that represented known AOB in soil, and which are potentially implied in N₂O emissions (Wrage *et al.*, 2004) was measured by qPCR targeting 16S rRNA gene sequences specific to this group (Hermansson & Lindgren, 2001). The final qPCR reaction volume was 20 µl, with 0.5 µM of a 2 : 1 ratio of primer CTO189fA/B (GGAGR AAAGCAGGGGATCG) and CTO189fC (GGAGGAAAGT AGGGGATGC; Kowalchuk *et al.*, 1997), 0.5 µM of RT1r primer (CGTCCTCTCAGACCARCTACTG; Hermansson & Lindgren, 2001), 0.5 µM of TPM1 probe (CAACTAGCTAATCAGR CATCRGCCGCTC), 0.4 mg ml^{–1} bovine serum albumin (BSA), 10 ng of sample DNA or standard DNA with known number of copies. The samples were run as follow: 10 min at 95 °C; 45 cycles at 95 °C for 10 s, 58 °C for 20 s, and 1 s at 72 °C; and 30 s at 40 °C.

The abundance of *nirK* genes was determined using SYBR Green as the detection system in a reaction mixture of 20 µl, with 10 µl of SYBR Green PCR master mix, including HotStar Taq™ DNA polymerase, QuantiTect SYBR Green PCR buffer, dNTP mix with dUTP SYBR Green I, ROX and 5 mM MgCl₂ (QuantiTect™ SYBR® Green PCR Kit; Qiagen, Courtaboeuf, France), 1 µM of *nirK876* primer (ATYGGCGGVAYGGCGA), 1 µM of *nirK1040* primer (GCCTCGATCAGRTTRTGGTT), according to Henry *et al.*, 2006, 0.4 µg of T4 gene protein 32 (QBiogene, France), 5 ng of soil DNA and Rnase-free water to complete the 20 µl volume. The conditions for *nirK* qPCR were 15 min at 95 °C for

denaturation; 45 cycles at 95 °C for 15 s, 63 °C for 30 s and 72 °C for 30 s for amplification; 1 s at 95 °C and 20 s at 68 °C for acquisition step and 10 s at 40 °C to finish analysis.

For *nosZ* gene quantitation, the primers *nosZ2F* (5'-CGCRACGGCAASAAGGTSMSSGT-3') and *nosZ2R* (5'-CAK-RTGCAKSGCRTGGCAGAA-3'), according to Henry *et al.* (2006) were used. The final volume 25 µl PCR mix contained: QuantifTect SybrGreen PCR Master Mix 1X (Qiagen), 0.1 µg of T4 gene protein 32 (QBiogene), 1 µM of each primer, and 5 ng of soil DNA extract or 5 µl of ten-fold standard serial dilution ranging from 10⁷ to 10² *nosZ* copies of genomic DNA from *Pseudomonas aeruginosa* PA14. Thermal cycling was carried out by an initial enzyme activation step at 95 °C for 10 min followed by 55 cycles of denaturation at 95 °C for 15 s, annealing at 68 °C for 30 s with a touchdown of -1 °C by cycle until reach 63 °C and elongation at 72 °C for 30 s.

Characterization of *nirK* community by cloning-sequencing

Characterization of *nirK* community was achieved on DNA extracted from samples taken at the beginning and at the end of flux measurements, i.e., May and November 2009 with/without field N₂O fluxes respectively. We used the conditions described by Wertz *et al.* (2006) to amplify partial *nirK* gene sequences prior to cloning procedures. Briefly, PCR was performed using the primers Copper 583F (5'-TCATGGT GCTGCCGCGKACGG-3') and Copper 909R (5'-GAAC TTGCCGGTPGCCAGAC-3') according to Liu *et al.* (2003) and 30 ng of extracted DNA. The final reagent concentrations for PCR were 1 µM primers, 200 µM of each dNTP, 1.75 U of Taq (QBiogene, Carlsbad, USA), and 0.5 µg of T4 protein in 50 µl of 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, pH 9. Thermal cycling was carried out by an initial step at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 72 °C for 1 min with a touchdown of -1 °C by cycle until reach 67 °C and elongation at 72 °C for 1 min and a final elongation cycle at 72 °C for 7 min. PCR products were purified using the NucleoSpin[®] Extract II kit (Macherey-Nagel, Düren, Germany) and were cloned using the pGEM T-Easy vector system (Promega Ltd., Southampton, UK) and DH10B electrocompetent *Escherichia coli* cells (Fisher Scientific-Invitrogen, Illkirch, France). For each treatment, three clone libraries were constructed from three (out of the four) randomly selected replicates. From each clone library, at least 28 clones were randomly picked and their vector sent for purification and sequencing (LGC Genomics, Berlin, Germany). Nucleotide sequences have been deposited in GenBank under the following accession numbers: JQ770451–JQ771049.

DNA sequence process, phylogenetic assessment and comparison of microbial communities

Vector and primer sequences were trimmed from the raw sequence dataset. Chimera formations were detected using ChimeraCheck (Cole *et al.*, 2005) and sequences shorter than 358 bp were removed from the original dataset. From a total

of 631 remaining 'clean' sequences random normalization of sample sizes was carried out using the Daisy Chopper tool (Gilbert *et al.*, 2009), based on the smallest sample i.e., 25 sequences. This subsampled dataset was then aligned together, included the outgroup sequence of the *nirS* gene of *Dechloromonas aromatica* using MUSCLE (Edgar, 2004) and the resulting alignment was manually checked using Seaview (Galtier *et al.*, 1996). From the resulting optimized alignment, a maximum likelihood phylogenetic tree was inferred using RAxML (Stamatakis, 2006) under a GTR + Gamma + Invariable model of sequence evolution (Appendix 1). The resulting tree was then imported into the UNIFRAC on-line tool (Lozupone *et al.*, 2006) for comparison of community composition and detection of lineages specific to the various treatments. The RAMI tool was used to measure accurate branch lengths and distances between nodes containing (Pommier *et al.*, 2009). Proportional abundances of selected nodes were depicted using the KRONA tool (figure 2, Ondov *et al.*, 2011).

Statistical analyses

Effects of climate treatment on N₂O, NEA, NO₃⁻, N₂O_{DEA}, N₂DEA, and abundance of gene copies (16S rRNA of AOB, *nirK*, *nosZ*) were analyzed using mixed model repeated measures analysis of variance (ANOVA) with both treatment and date as fixed factors (Zar 1998). Effects of individual climate change drivers (temperature, drought, and CO₂) were analyzed using orthogonal contrasts (Gilligan, 1986). Effects of warming were determined by comparing the C and T treatment; effects of summer drought under elevated temperature by comparing T and TD; effects of elevated (CO₂) under elevated temperature and drought by comparing TD and TDCO₂; effects of simultaneous application of warming, summer drought, and CO₂ enrichment (2080 climate scenario) were investigated by the C vs. TDCO₂ comparison. All data used were checked for normality and non-normal data were log transformed to conform with assumptions of normality and homogeneity of variances. Relationships between field N₂O fluxes, potential activities and gene abundances were examined using Spearman correlation coefficients. All analyses were carried out using Statgraphics Plus 4.1[®] (Statistical Graphics Corp., Rockville, Maryland, USA).

We performed a restricted maximum likelihood method (REML) with the software JMP8[®] (SAS Institute Inc., SAS Campus Drive, NC, USA) considering monoliths as a random factor to determine, which variables (among soil temperature, WFPS, NO₃⁻ and NH₄⁺ contents, abundances, activities and composition of denitrifiers) were significantly related to *in situ* N₂O fluxes in May and November (i.e., dates with diversity analyses). To compare field measures of N₂O fluxes to denitrification activities measured in the laboratory, which differed in experimental temperature, we linearly transformed the denitrification values (N₂O_{DEA} corr) as suggested by their known linear correlation between 4 and 25 °C (Braker *et al.*, 2010).

Structural equation modeling (SEM) was performed using Amos18[®] (Amos Development Corporation, Crawfordville, FL, USA) with the data from May and November to explore the causal links between denitrification, microbial community

structure, abiotic factors and the *in situ* N₂O fluxes, using the following parameters: soil temperature, WFPS, pool of NO₃⁻, N₂O_{DEA}, N₂O_{DEA} corr, percentage of sequences in *nirK* lineages A and B (Appendix 2). In a SEM, a χ^2 test is used to determine whether the covariance structures implied by the model adequately fit the actual covariance structures of the data. A non-significant chi-squared test ($P > 0.05$) indicates adequate model fit. The coefficients of each path as the calculated standardized coefficients were determined using the analysis of correlation matrices. Paths in this model were considered significant with a P -value < 0.1 . These coefficients indicate by how many standard deviations the effect variable would change if the causal variable was changed by one standard deviation.

Results

Characteristics of climate treatments

During the study period (May–November 2009), the difference in mean monthly temperature between control and elevated temperature treatments was 3.4 ± 0.03 °C (Appendix 3). In summer (June, July, and August), the drought treatments (TD, TDCO₂) were subjected to a 21% reduction in rainfall compared with the no-drought treatments (C, T). Mean daily CO₂ differences between the TDCO₂ treatment and the ambient CO₂ treatments (C, T, and TD) were 193.3 ± 13.1 ppm (data not shown). Meteorological variables (i.e., soil moisture and soil temperature) recorded on days of N₂O measurement indicated higher soil temperature in the warmed treatments (T, TD and TDCO₂) compared with the control (C). No significant differences in soil moisture between the C, T and TDCO₂ treatments were found for the four sampling dates (T -test, Table 1). However, the TD treatment showed lower soil moisture values than the T treatment in July and September. We found no significant difference between soil moisture

Table 1 Mean soil moisture (WFPS, %) and soil temperature recorded during each N₂O measurement date for experimental climate treatments. Means and SEs are presented ($n = 5$)

	29th May	27th July	23rd September	28th November
WFPS (%)				
C	31.9 ± 0.0	35.9 ± 0.0	50.1 ± 0.0	52.9 ± 0.3
T	29.3 ± 1.1	32.7 ± 1.6	45.2 ± 2.9	52.6 ± 2.3
TD	32.4 ± 0.8	29.4 ± 0.3	30.6 ± 0.3	48.8 ± 0.2
TDCO ₂	29.5 ± 0.8	37.3 ± 1.8	47.1 ± 1.7	51.8 ± 1.5
Soil temperature (°C)				
C	17.3 ± 0.4	17.3 ± 0.4	14.3 ± 0.2	4.2 ± 0.3
T	22.7 ± 1.5	23.8 ± 0.6	17.1 ± 0.5	6.3 ± 0.6
TD	23.5 ± 1.2	24.9 ± 1.1	17.3 ± 0.7	5.7 ± 0.7
TDCO ₂	22.6 ± 1.2	22.9 ± 0.9	17.4 ± 0.8	6.7 ± 0.6

and air temperature measured on the days of sampling and the averages recorded on the five previous days (all dates and treatments). This consistence between measurements allows considering measurements of each sampling date as representative of the preceding week.

Effects of climate change drivers on *in situ* N₂O fluxes

During the four measurement dates, N₂O fluxes ranged from -5 to 369 $\mu\text{g N}_2\text{O-N.m}^{-2}.\text{hr}^{-1}$ across treatments. N₂O fluxes showed significant climate treatment effects for measurement dates during the growing season, but no response to climate treatments in November (significant treatment \times date interaction; $F_{1,9} = 2.16$, $P < 0.05$; Fig. 1). This significant interaction was driven by very low N₂O fluxes in November across all climate treatments. With the exception of the November sampling date, warming had a positive effect on N₂O emissions (C vs. T comparison; $F_{1,16} = 23.1$, $P < 0.001$; $F_{1,16} = 6.6$, $P < 0.05$ and $F_{1,16} = 14.6$, $P < 0.01$ respectively for May, July and September). This pattern of response was also found for the combined climate change drivers (C vs. TDCO₂) in May and July. Unlike warming and combined climate change, summer drought (T vs. TD) and elevated CO₂ (TD vs. TDCO₂) had little impact on N₂O fluxes. However, drought was associated with a significant negative effect on N₂O fluxes in September ($F_{1,16} = 15.5$, $P < 0.01$; Fig. 1).

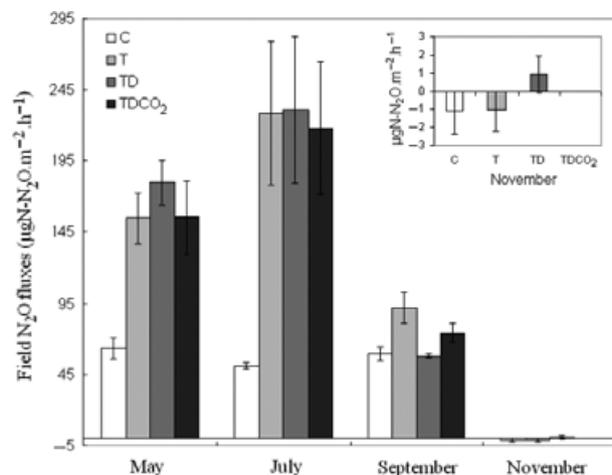


Fig. 1 Effects of climate manipulations on N₂O fluxes for measurement dates in spring, summer and autumn 2009. Treatments are given by: C, control; T, elevated temperature (+3.5 °C); TD, elevated temperature and summer drought (+3.5 °C, -20% rainfall); TDCO₂, elevated temperature, summer drought and CO₂ enrichment (+3.5 °C, -20% rainfall, +200 ppm CO₂). Means and standard errors are presented per treatment and measurement date ($n = 5$).

Changes in nitrifying and denitrifying enzyme activities

Over the course of the study, climate treatments had a significant effect on NEA, N₂O_{DEA} and soil nitrate pools (Table 2). Warming and combined climate treatments had a positive impact on nitrification (NEA; $F_{1,38} = 5.7$, $P < 0.05$ and $F_{1,16} = 6.9$, $P < 0.05$ respectively) and on NO₃⁻, which is the product of the nitrification ($F_{1,38} = 10.67$, $P < 0.01$ and $F_{1,38} = 10.85$, $P < 0.01$ for C vs. T and C vs. TDCO₂ respectively). In addition, combined warming, drought and elevated CO₂ had a positive effect on N₂O_{DEA} across all measurement dates (C vs. TDCO₂, $F_{1,38} = 5.4$, $P < 0.05$). In contrast, the potential fluxes of N₂ (N_{2DEA}) and denitrification product ratio (N₂O_{DEA}/[N₂O_{DEA} + N_{2DEA}]) showed no response to climate treatments. Neither summer drought under warmed conditions (T vs. TD) nor elevated CO₂ in combination with warming and summer

drought (TD vs. TDCO₂) had any significant effect on nitrifying and denitrifying enzyme activities. Across treatments, NO₃⁻, N₂O_{DEA}, N_{2DEA}, and denitrification product ratio showed a significant effect of measurement date (Table 2). NO₃⁻ and N_{2DEA} showed a continuous increase over time ($r^2 = 37.7$, $P < 0.001$ and $r^2 = 23.8$, $P < 0.001$ respectively), whereas N₂O_{DEA} and denitrification product ratio showed a progressive decrease overtime ($r^2 = 18.3$, $P < 0.001$ and $r^2 = 49.6$, $P < 0.001$ respectively).

Changes in the abundances of AOB, nirK and nosZ

Responses of gene abundances to climate change treatments varied depending on the gene considered (Table 3). Both warming and combined climate change had a positive effect on abundance of N₂O reducers (*nosZ*; $F_{1,16} = 6.1$, $P < 0.05$ and $F_{1,16} = 6.4$,

Table 2 Effects of climate change treatment on (a) nitrifying enzyme activities (NEA), (b) nitrates (NO₃⁻), (c) potential N₂O fluxes (N₂O_{DEA}), (d) potential N₂ fluxes (N_{2DEA}) and (e) denitrification product ratio (means and standard errors; $n = 5$). Results from repeated measures ANOVA testing the effects of climate change treatments, measurement dates and their interaction are presented (significant P values are shown in bold)

	Climate treatments				Repeated measures ANOVA			
	C	T	TD	TDCO ₂	P	Treatments	Dates	Treatments × dates
(a) NEA ($\mu\text{g N}(\text{NO}_2 + \text{NO}_3) \text{g}^{-1}\text{h}^{-1}$)								
May	0.51 ± 0.09	0.82 ± 0.09	0.87 ± 0.16	0.89 ± 0.16	*	0.003	0.238	0.807
July	0.53 ± 0.06	0.70 ± 0.16	0.89 ± 0.14	0.91 ± 0.08				
September	0.72 ± 0.10	0.94 ± 0.13	0.82 ± 0.09	0.92 ± 0.12				
November	0.81 ± 0.10	0.91 ± 0.13	0.82 ± 0.08	1.06 ± 0.18				
(b) NO ₃ ⁻ ($\mu\text{g N-NO}_3\text{-g}^{-1}$)								
May	3.1 ± 0.5	2.8 ± 0.6	2.5 ± 0.6	3.2 ± 0.6	***	0.002	< 0.001	<i>0.080</i>
July	4.3 ± 0.4	5.5 ± 0.9	6.2 ± 1.9	7.2 ± 0.8				
September	6.8 ± 1.1	14.7 ± 3.9	15.5 ± 3.2	10.3 ± 2.3				
November	5.2 ± 0.8	13.3 ± 2.8	14.3 ± 3.6	14.9 ± 2.9				
(c) N ₂ O _{DEA} ($\mu\text{g N-N}_2\text{O g}^{-1}\text{h}^{-1}$)								
May	1.17 ± 0.04	1.39 ± 0.13	1.51 ± 0.22	1.39 ± 0.01	***	0.022	< 0.001	0.712
July	1.05 ± 0.07	1.15 ± 0.09	1.15 ± 0.01	1.30 ± 0.09				
September	1.19 ± 0.06	1.15 ± 0.06	1.21 ± 0.13	1.28 ± 0.10				
November	0.98 ± 0.04	1.03 ± 0.07	1.03 ± 0.06	1.19 ± 0.1				
(d) N _{2DEA} ($\mu\text{gN-N}_2 \text{g}^{-1}\text{h}^{-1}$)								
May	0.07 ± 0.04	0.04 ± 0.01	0.06 ± 0.01	0.17 ± 0.09	**	0.555	< 0.001	0.557
July	0.08 ± 0.04	0.19 ± 0.05	0.20 ± 0.06	0.09 ± 0.03				
September	0.08 ± 0.03	0.27 ± 0.11	0.25 ± 0.15	0.19 ± 0.04				
November	0.29 ± 0.04	0.29 ± 0.05	0.38 ± 0.09	0.37 ± 0.03				
(e) Denitrification product ratio (% N ₂ O _{DEA} /[N ₂ O _{DEA} + N _{2DEA}])								
May	93.1 ± 2.2	97.7 ± 2.2	96.8 ± 2.1	95.7 ± 1.9	***	0.129	< 0.001	0.197
July	92.0 ± 3.6	85.8 ± 3.8	85.5 ± 3.6	93.1 ± 2.4				
September	93.3 ± 2.2	81.6 ± 10	80.1 ± 5.8	86.7 ± 3.6				
November	77.2 ± 2.6	77.8 ± 4.2	73.8 ± 8.9	75.7 ± 2.1				

*** indicates significant differences at $P < 0.05$; 0.01 and 0.001, respectively and italic indicates marginal differences ($0.05 < P < 0.1$).

Table 3 Effects of climate change treatments on the (a) AOB, (b) *nirK* and (c) *nosZ* gene abundances (means and standard errors are shown; $n = 5$). Results from repeated measures ANOVA testing the effects of climate change treatments, measurement dates and their interaction are presented (significant P -values are shown in bold)

	Climate treatments				Repeated measures ANOVA			
	C	T	TD	TDCO ₂	P	Treatments	Dates	Treatments × dates
(a) Mean copy numbers of ammonia-oxidizing bacteria (10 ⁶ copy per g of dry soil)								
May	3.97 ± 0.15	5.58 ± 0.67	4.34 ± 0.80	4.13 ± 0.15	***	0.083	<0.001	0.021
July	4.84 ± 0.70	4.95 ± 0.46	4.77 ± 0.69	5.32 ± 0.76				
September	5.14 ± 0.39	4.73 ± 0.50	4.40 ± 0.66	5.01 ± 0.32				
November	5.12 ± 0.43	6.40 ± 0.59	7.23 ± 0.54	8.36 ± 0.86				
(b) Mean copy numbers of Cu nitrite reductors <i>nirK</i> (10 ⁷ copy per g of dry soil)								
May	1.70 ± 0.23	1.93 ± 0.12	1.48 ± 0.14	1.64 ± 0.18	ns	0.371	0.051	0.740
July	2.10 ± 0.38	2.06 ± 0.35	2.31 ± 0.37	2.22 ± 0.33				
September	1.62 ± 0.25	2.20 ± 0.33	1.82 ± 0.31	1.94 ± 0.19				
November	1.36 ± 0.23	1.76 ± 0.26	1.98 ± 0.24	2.03 ± 0.84				
(c) Mean copy numbers of nitrous oxide reductors <i>nosZ</i> (10 ⁷ copy per g of dry soil)								
May	2.24 ± 0.39	2.46 ± 0.38	2.54 ± 0.90	2.57 ± 0.85	*	0.017	0.048	0.942
July	1.49 ± 0.26	2.76 ± 0.28	3.76 ± 0.91	2.98 ± 0.84				
September	1.64 ± 0.13	2.78 ± 0.38	3.22 ± 0.91	3.56 ± 0.94				
November	2.33 ± 0.12	3.93 ± 0.45	4.13 ± 0.66	4.71 ± 0.97				

C, control treatment; T, elevated temperature treatment; TD, temperature and drought treatment; TDCO₂, temperature, drought, and elevated CO₂.

*,*** indicates significant differences at $P < 0.05$ and 0.01 , respectively and italic indicates marginal differences ($0.05 < P < 0.1$).

$P < 0.01$ respectively) at all measurement dates (no significant Treatment × Date interaction). In addition, *nosZ* gene abundance was found to increase over time (Table 3, $r^2 = 12.5$, $P < 0.05$). Climate treatment also had significant effects on the abundance of AOB sequences, but treatment effects varied overtime (significant treatment × date interaction, Table 3). In general, numbers of AOB copies were significantly higher in November compared with those in May and July. Warming had a positive effect on the abundance of AOB in May and November ($F_{1,16} = 6.3$, $P < 0.05$ and $F_{1,16} = 9.1$, $P < 0.01$ respectively), whereas combined climate and elevated CO₂ alone was only associated with an increase in AOB in November

($F_{1,16} = 8.5$, $P < 0.05$ for combined climate and $F_{1,16} = 16.5$, $P < 0.001$ for elevated CO₂). Unlike *nosZ* and AOB, abundance of *nirK* genes showed no significant response to climate treatments over the four measurement dates.

Relationship between microbial activities, microbial population abundances and abiotic factors

Across treatments, field N₂O fluxes showed a positive correlation with denitrification product ratio, which is consistent with its positive correlation with the denitrification enzyme activity producing N₂O (N₂O_{DEA}) and a negative correlation with the reduction of N₂O to N₂

Table 4 Correlation coefficients (Spearman) between field N₂O fluxes, microbial activities and gene abundances pooled across experimental climate treatments and dates ($n = 80$), and for each climate treatment pooled across dates ($n = 20$). Significant P values ($P < 0.05$) are shown in bold and marginal P values ($0.1 > P > 0.05$) are in italic

N ₂ O fluxes	Microbial activities				Gene abundances			
	NEA	N ₂ O _{DEA}	N ₂ DEA	Denitrification product ratio	AOB	<i>nirK</i>	<i>nosZ</i>	<i>nosZ/nirK</i>
Pooled treatments	0.016	0.335	-0.291	0.411	-0.179	0.117	-0.166	-0.216
C	-0.462	0.477	-0.734	0.768	-0.205	0.281	0.017	-0.211
T	-0.428	0.221	-0.321	0.398	0.006	<i>0.359</i>	-0.177	-0.426
TD	0.206	0.275	-0.356	0.475	-0.314	-0.252	-0.447	-0.327
TDCO ₂	-0.101	0.305	-0.393	0.599	-0.346	0.120	-0.264	-0.394

(N₂DEA) during the study period (Table 4). This pattern was mirrored by N₂O fluxes in the control treatment. In addition, N₂O fluxes in the C treatment showed a significant negative correlation with NEA (Table 4). Variation in gene abundances played a relatively more important role for N₂O fluxes under warmed conditions compared with the control. In the T treatment, N₂O fluxes showed a significant negative correlation with both NEA and the *nosZ/nirK* ratio (Table 4). In the TD treatment, N₂O fluxes were positively correlated with the denitrification product ratio, but negatively correlated with *nosZ* abundance (Table 4). Finally in the TDCO₂ treatment, N₂O fluxes showed a positive correlation with the denitrification product ratio, but a negative correlation with N₂DEA and the *nosZ/nirK* ratio. No significant relationships were observed between N₂O fluxes and gene abundances across treatments (Table 4).

Changes in *nirK* community and diversity structure

On the basis of the complete maximum likelihood tree (Appendix 1), both the Unifrac significance and the *P*-test significance indicated that the *nirK* community sampled in May was significantly different in its structure from the community sampled in November ($P < 0.03$). In addition, the sequences from the November samples had a significant number of unique branches compared to the rest of the tree ($P \leq 0.01$). When clus-

tering the environments according to the full tree topology (Appendix 4) the *nirK* communities from the treatments T and TDCO₂ shared the most sequences, and were closer to the control *nirK* community than to the TD community. All pairwise comparisons of the treatments showed significant differences (Jackknife analysis, $P < 0.05$). At a branch threshold of 0.05, two lineages showed significant biases toward specific treatments (Fig. 2). Lineage A showed significant dominance in the TDCO₂ treatment (dominance 18 observed while 8.5 expected) and the C treatment (recession 2 observed instead of 8.5 expected). Lineage B showed significant dominance in all elevated temperature treatments compared with control ($C = 3$; $T = 22$; $TD = 20$; $TDCO_2 = 19$; expected = 16). Compared to the rest of the tree, the sequences belonging to both lineages showed significant differences in high-GC% sequences (mean GC% = 62.92% for lineages A and B; GC% = 62.03% for all other sequences; Kruskal–Wallis, $X^2 = 32.5$, $P < 0.001$).

Microbial drivers of N₂O fluxes, a structural equation modeling

Structural equation modeling (SEM) identified potential causal relationships between variables significantly correlated with *in situ* N₂O fluxes ($\chi^2 = 5.204$, $P = 0.391$; Fig. 3). Non-standardized path coefficients and tests of path significance are available in Appendix 5. Almost all of the N₂O flux variance (87%) was

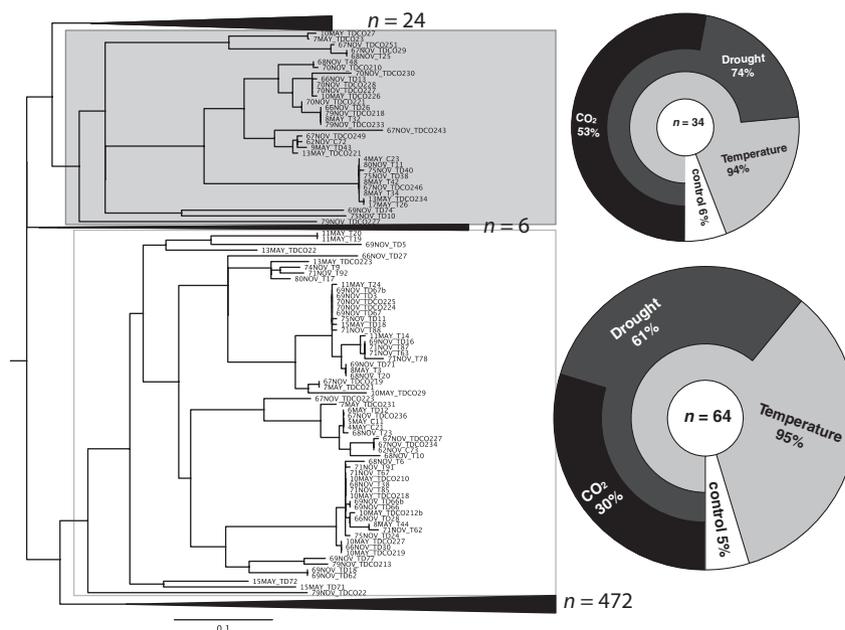


Fig. 2 Maximum likelihood tree based on GTR–GAMMA model of substitution. All but two nodes were collapsed to illustrate significant biases toward climate (lineage A in dark grey and lineage B in light grey). Bar legend indicates 0.1 substitutions/nucleotide.

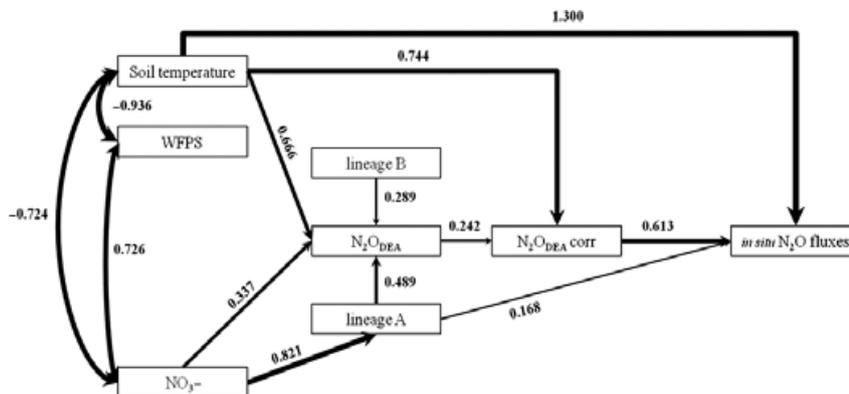


Fig. 3 Structural equation model results for effects of *nirK* denitrifiers on *in situ* N₂O fluxes. Path coefficients (values indicated next to the arrows) correspond to the standardized coefficients calculated based on the analysis of correlation matrices. Tests of path significance are given in Appendix 5.

explained by denitrifier processes (N₂O_{DEA}, N₂O_{DEA} corr), relative abundance of specific *nirK* lineages (lineage A, B) and by abiotic factors (soil temperature). Abundances of *nirK* and *nosZ* *per se* or their ratio had no effect on denitrification activity and *in situ* N₂O fluxes in the SEM (data not shown). NO₃⁻ availability impacted *in situ* N₂O fluxes indirectly *via* impacts on potential denitrification and the *nirK* community structure (lineage A). The *nirK* community structure influenced N₂O fluxes either directly with lineage A or indirectly *via* impacts on potential N₂O emissions (N₂O_{DEA} with lineage A and B; Fig. 3). Soil temperature was identified as the driver of denitrification and N₂O fluxes. The path coefficients indicated that changes in soil temperature were the major driver of altered *in situ* N₂O fluxes. However, neither soil temperature nor WFPS measured on the day of sampling were related to changes in *nirK* community structure (Fig. 3, Appendix 5). SEM performed with nitrification (i.e., NEA and AOB gene abundances) were not significant ($P < 0.05$; data not shown) implying the weak effect of nitrifiers-related parameters on field N₂O fluxes. Similarly, SEM performed with gene abundances of denitrifiers (*nirK* and *nosZ*) were not significant ($P < 0.05$; data not shown), implying uncoupled responses of gene abundances and the denitrification process.

Discussion

Global changes are known to enhance soil N₂O fluxes (Cantarel *et al.*, 2011; Carter *et al.*, 2011; Niboyet *et al.*, 2011). In the present study, we aimed to improve the mechanistic understanding of soil microbial functioning and the processes contributing to the emissions of N₂O for grasslands subjected to sustained climate change.

Warming and all combined climate change drivers induced strong modifications in field N₂O fluxes and microbial functioning

Throughout our study, we found that N₂O fluxes and microbial activities responded more strongly to warming and combined climate changes (simultaneous application of warming, summer drought and elevated CO₂) than to summer drought or elevated CO₂ under warmed conditions. Flux data from the present study confirms the importance of warming as a key driver of climate-induced changes for N₂O-N losses in grassland ecosystems (Cantarel *et al.*, 2011). Accordingly, we found positive effects of warming on N₂O fluxes recorded during the growing season, but no significant warming effects for the winter sampling date (Cantarel *et al.*, 2011) due to an insufficient warming to compensate for the winter temperature. Such seasonal variation may reflect interactions between soil temperature and soil moisture on microbial processes (Flechard *et al.*, 2007), as well as variation in root exudation and soil nutrient availability. Moreover, both nitrification enzyme activity (NEA) and the *in situ* nitrate pool increased in response to elevated temperature, in agreement with previous results observed in well-aerated soils (Barnard & Leadley, 2005). Warming was found to have a positive impact on AOB abundance in May, whereas combined warming, drought and elevated CO₂ had a positive impact on AOB abundance in November. Given the relatively limited changes in gene abundances observed, and their transient nature, it is likely that the increase of AOB abundances was probably a result of indirect effects, most likely mediated by the plant community (Horz *et al.*, 2004). AOB are believed to be inferior competitors for nutrient resources (Belsler, 1979), and temporal changes in AOB community size may reflect a shifting competitive

hierarchy for nutrient resources (mainly NH₄⁺) between AOB, heterotrophic microbes and plants.

Irrespective of measurement date, combined climate change (TDCO₂) was found to increase N₂O_{DEA} and N₂O + N₂ emissions in the laboratory measurements (N₂O_{tot}). Surprisingly, these two variables did not show a significant response to warming alone. This lack of response did not result from the confounding effects of soil moisture during the measurement campaigns, since similar soil moisture conditions were observed in the C and T treatments. Barnard & Leadley (2005) recently reported that denitrification enzyme activity (DEA) was generally less responsive to temperature in field experiments compared with laboratory studies, a phenomenon attributed to acclimation of DEA to ambient environmental conditions over time (French *et al.*, 2009). Denitrifying bacteria harboring *nosZ* genes also carry *nirK* or *nirS* genes, though denitrifying bacteria may solely harbor *nirK* and/or *nirS* genes (Jones *et al.*, 2008). Therefore, shifts in *nosZ* community may not always reflect *nirK* and/or *nirS* community changes. In our study, the abundances of *nosZ* denitrifiers increased more than those of *nirK* denitrifiers in response to warming and combined climate changes, suggesting a shift in *nirK* and/or *nirS* community structure. Between *nirK* and *nirS* communities, the former have been shown to respond to environmental changes (Hallin *et al.*, 2009; Szukics *et al.*, 2010). Herein, changes in *nirK* community structure were found under warming and combined climate change treatments. Indeed, we found two deeply branching lineages with significant biases for warmed treatments. This result suggests a selective process under warmed treatments; it is noteworthy that the sequences included in these two lineages harbored a higher GC-content than the other sequences on the tree, consistent with bacteria adapted to higher temperatures (Madigan & Martinko, 2006).

Low variation in soil water status modifies microbial community structure but does not affect N-related microbial activities and abundance

Combined summer drought and warming had no significant effect on microbial parameters (enzymatic activities and gene abundances) compared with warming alone. Previous study indicates that decreases in soil moisture are often associated with a decrease in DEA and an increase in NEA products (Barnard & Leadley, 2005; Bateman & Baggs, 2005). In our study, the variation in soil water status across T and TD treatments on measurement dates was weak, despite a 20% reduction of summer precipitation. Consequently, the limited effects of drought treatment on soil moisture conditions may have diminished the impact of experimental drought on

soil processes. Another explanation could be that changes in nitrite community structure under warmed and drought treatment mitigated drought responses in enzymatic activities and gene abundances. Irrespective of the sampling dates (May or November), phylogenetic analysis of *nirK* sequences indicated a strong divergence between the nitrate reducer community in the TD treatment and those communities found in the other climate change treatments. This suggests a key selective process linked to drought under warmed conditions, which could explain why no difference was found in denitrification activities in the various treatments.

Elevated CO₂ was expected to increase soil water status due to reduced plant stomatal aperture and transpiration rates (Schulze, 1986), which can have indirect consequences on denitrification by releasing the soil O₂ partial pressure (Smith *et al.*, 2003). Although we measured significantly higher soil moisture conditions in July and September in the TDCO₂ treatment compared with the TD treatment (37% vs. 29% and 47% vs. 31% for July and September respectively), we found no impact of elevated CO₂ on enzymatic activities and N₂O fluxes. The lack of response to drought and elevated CO₂ observed here mirrors the patterns of N₂O fluxes recorded in 2007–08 at the same site (Cantarel *et al.*, 2011) and suggests that N₂O-related microbial processes may also be insensitive to minor variations in soil water content. Moreover, drought and elevated CO₂ did not highly modify the relationship between field N₂O fluxes and microbial activities and gene abundances. The maintenance of soil functioning in combined warming, drought and elevated CO₂ conditions despite substantial modifications in the bacterial community structure, agrees with other studies which show high-functional redundancy of microbial communities (Wertz *et al.*, 2007; Cabrol *et al.*, 2011).

Relationships among microbial parameters and field N₂O emissions

N₂O flux variations were better explained by the denitrification product ratio (N₂O_{DEA}/[N₂O_{DEA} + N₂DEA]) both across treatments and under cool, wet conditions (control site). Changes in DEA products over time (i.e., decrease of N₂O_{DEA} and increase of N₂DEA) were correlated with a decrease in field N₂O fluxes. This may result from continuous N losses *via* N₂ fluxes in our grassland ecosystem even when no N₂O fluxes were detected. The relative importance of microbial activities and microbial population size was modified under warmed conditions, with stronger correlations between field N₂O fluxes and gene abundances in the T, TD and TDCO₂ treatments. Nevertheless, field N₂O fluxes showed a stronger correlation with enzymatic activities

than with community abundance across climate treatments. Links between N₂O fluxes and microbial abundances are known to be elusive, and may depend on soil properties or ecosystem type (Ma *et al.*, 2008). Furthermore, the activity of a given enzyme may be uncoupled from the size of the corresponding functional gene pool due to subsequent enzyme regulation. Additional study is needed to examine the relative importance of other denitrifying and nitrifying genes on patterns of microbial activities and associated field N₂O fluxes under future climate conditions.

A challenge in this study was to link *in situ* N₂O fluxes and functioning microbial ecosystem, and particularly the *nirK* denitrifiers community. The SEM supported the importance of changes in abiotic conditions (i.e., soil temperature) toward *in situ* N₂O fluxes. However, additional significant path coefficients suggested that other factors e.g., changes in denitrification activities and community structure were important in determining field N₂O fluxes. Moreover, the availability of NO₃⁻ pool influenced *in situ* N₂O fluxes indirectly by providing substrate for denitrification and impacting the *nirK* community structure (lineage A). The observed direct and indirect influences of *nirK* diversity suggest that the mechanisms driving field N₂O fluxes are subtler than simple warming effects on denitrifying enzymatic activities. Taken together, our results strongly suggest that the combined effects of soil temperature, denitrifier community structure and activity, provide a much better predictor of N₂O fluxes than nitrifier-related parameters. Further study coupling automated N₂O measurements with more frequent soil sampling over the course of the year is required to confirm these findings, and improve our understanding of climate change impacts on annual N₂O fluxes and N-related microbial functioning.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Maximum likelihood tree based on GTR-GAMMA model of substitution. Bar legend indicates 0.1 substitutions/nucleotide.

Appendix S2. The initial structural equation models.

Appendix S3. Daily air temperature (a), rainfall (b) and soil moisture (c) recorded during the study period (April–November 2009). The control, upland site is given by gray line, whereas the warmer, lowland site is presented by black line (T, full line; TD, dashed line; TDCO₂, dotted line). Arrows represented day of N₂O measurement and soil sampling.

Appendix S4. Clustering environments according to the full tree topology.

Appendix S5. Full SEM results for each path in the model. UnStd Est, unstandardized path coefficient estimates; SE, standard error of the unstandardized path estimate; CR, critical ratio for regression weight (UnStd Est/SE); *P*-value, test of significance of path estimate; Std., standardized path coefficient estimates.

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