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SPECIAL TOPIC: IN HONOR OF CHRISTIAN KÖRNER

Effects of drought and N-fertilization on N cycling in two grassland soils

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Abstract Changes in frequency and intensity of drought events are anticipated in many areas of the world. In pasture, drought effects on soil nitrogen (N) cycling are spatially and temporally heterogeneous due to N redistribution by grazers. We studied soil N cycling responses to simulated summer drought and N deposition by grazers in a 3-year field experiment replicated in two grasslands differing in climate and management. Cattle urine and NH_4NO_3 application increased soil NH_4^+ and NO_3^- concentrations, and more so under drought due to reduced plant uptake and reduced nitrification and denitrification. Drought effects were, however, reflected to a minor extent only in potential nitrification, denitrifying enzyme activity (DEA), and the abundance of functional genes characteristic of nitrifying (bacterial and archaeal *amoA*) and denitrifying (*narG*, *nirS*, *nirK*, *nosZ*) micro-organisms. N_2O emissions, however, were much reduced under drought, suggesting that this effect was driven by environmental limitations rather than by changes in the activity potential or the size of the respective microbial communities. Cattle urine stimulated nitrification and, to a lesser extent, also DEA, but more so in the absence of drought. In contrast, NH_4NO_3 reduced the activity of nitrifiers and denitrifiers due to top-soil acidification. In summary, our data demonstrate that complex interactions between drought, mineral N availability, soil acidification, and plant nutrient uptake control soil N cycling and associated N_2O emissions. These interactive effects differed between processes of the soil N cycle, suggesting that the spatial heterogeneity in pastures needs to be taken into account when predicting changes in N cycling and associated N_2O emissions in a changing climate.

Keywords Climate change · Enzyme activities · Functional genes · Quantitative PCR · Nitrification and denitrification · N_2O fluxes

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Introduction

Summer heat waves over western Europe have doubled in length since 1880 (Della-Marta et al. 2007), and climate modeling predicts a further increase in summer drought frequency in central Europe, associated with reduced precipitation and decreased soil moisture (Fischer and Schär 2010; Schär et al. 2004). Drought directly or indirectly affects most organisms and processes in ecosystems, including soil nitrogen (N) cycling and nitrous oxide (N_2O) emissions.

Atmospheric N_2O is a powerful greenhouse gas that also contributes to stratospheric ozone depletion (Ravishankara et al. 2009). Globally, soils are the single largest source of

N_2O, with ca. 6 Tg year^{-1} emitted from natural soils and 4 Tg year^{-1} from agricultural soils (IPCC 2007; Stehfest and Bouwman 2006). N_2O essentially originates from nitrification and denitrification (Conrad 1996; Prosser 1989; Zumft 1997). During nitrification, ammonium (NH_4^+) is oxidised to nitrite (NO_2^-) and nitrate (NO_3^-), releasing N_2O as a by-product. During denitrification, NO_3^- is sequentially reduced to N_2O and N_2. At a proximal level, nitrification is controlled by the availability of O_2 and NH_4^+; denitrification depends on O_2 and the availability of NO_3^- and organic substrates. At a more distal level, nitrification and denitrification also depend on the processes controlling mineral N availability, i.e., N mineralization and immobilization rates and plant N uptake, as well as factors affecting diffusion rates in the soil such as structure, temperature, aggregation, and cation exchange capacity. Ultimately, nitrification and denitrification may be controlled by climate, soil type, and plant communities (Robertson 1989). As a consequence of the many direct and indirect factors affecting N_2O production, the effects of management and environmental change remain difficult to predict despite our fairly good process understanding.

In pastures, N cycling is strongly modulated by grazing. Grazing animals incorporate only a small fraction of ingested plant N, and redistribute the rest as excreta over the pasture. Although excreta typically cover only a small fraction of a pasture, they constitute hotspots of N transformations and N_2O emissions that can dominate ecosystem-level fluxes (Yamulki et al. 1998). A mechanistic understanding of grazing effects on N_2O fluxes in pastures therefore requires that the patch-level processes, in particular in hotspots in urine patches, are sufficiently well understood.

In a companion study, we have examined effects of severe summer drought on soil–atmosphere exchange of N_2O and CH_4 in two pastures contrasting in climate and management intensity (Hartmann et al. 2011; Stiehl-Braun et al. 2011). The respective experiments simulated drought in factorial combination with a N fertilization treatment simulating deposition rates as they occur under urine patches. This approach allowed application of N as a randomized, replicated treatment, with a defined treated area and defined N deposition rates, thus circumventing some of the difficulties encountered when studying natural excreta patches. Measurements of soil N_2O exchange over two growing seasons indicated a one to two orders of magnitude increase in N_2O emissions when N fertilizers were applied; under drought, the stimulation of emissions was only in the order of a factor of two (summary given in Table 1; for details, see Hartmann and Niklaus 2012).

Here, we present a detailed analysis of the effects of drought and N application on the underlying soil processes. We combine measurements of soil mineral N concentrations with measurements of mineralization rates as well as potential nitrification and denitrification activities. We further estimated the size of the respective microbial functional groups by quantitative (real-time) PCR of selected functional and 16S rRNA genes. Specifically, our objectives were (1) to test for effects of the applied treatments on the investigated processes, in particular nitrification and denitrification; (2) to develop a mechanistic understanding of the interactions between plants, environmental conditions, and soil microbial communities that led to the observed effects on N_2O fluxes; and (3) to test whether and which of the measured soil parameters (e.g., potential denitrification or functional gene abundances) can be used as proxies for the observed N_2O fluxes. Identifying such integrated indicators is of great practical relevance, since N_2O emissions are very difficult to quantify in the field due to their very dynamic nature and spatial heterogeneity.

Materials and methods

Field site and experimental design

A field experiment was set up in September 2006 to study effects of drought and N inputs by mineral fertilizer and grazing animals on soil N cycling and N_2O emissions. Effects on CH_4 fluxes were also studied but are published elsewhere (Hartmann et al. 2011). The experiment was replicated at two research farms that represent typical Swiss grassland farming systems.

The first site, Früebüel, is located on a montane plateau in central Switzerland (47.1135°N, 8.5415°E, 1,000 m a.s.l.). Vegetation is dominated by *Alopecurus pratensis*, *Dactylis glomerata*, *Trifolium repens*, and *Ranunculus bulbosus*. Other species accounting for at least a few percent cover are *Heracleum sphondylium*, *Lolium perenne*, *Poa pratensis*, *Poa trivialis*, *Rumex acetosa*, *Taraxacum officinale*, and *Veronica filiformis*. Before the experiment started, the site was managed at intermediate intensity, predominantly grazed by non-dairy cattle or mown for hay three to four times a year. Fertilizer inputs consisted of excreta when cattle were on the pastures and of manure when they were not. Since most of this N ultimately originates from plant N growing on the site, net fertilizer inputs are difficult to quantify. The growing period starts in early April and ends in late October. The topsoil is a silt loam containing 37 % sand, 56 % silt, and 7 % clay, and with a pH of ~4.7.

The second site, Alp Weissenstein, is located in subalpine grassland situated in the eastern Swiss Alps (46.5833°N, 9.7859°E, 1,975 m a.s.l.). *Alchemilla xanthochlora* and

Table 1 Estimated growing season average of soil N_2O emissions

Site	Years	Drought	N fertilizer treatment N_2O emissions (μmol N_2O m^{-2} day^{-1})		
			NIL	NH_4NO_3	Urine
Früebüel	2007	Control	10 ± 1	83 ± 13	100 ± 20
		Drought	12 ± 3	20 ± 3	17 ± 4
	2008	Control	10 ± 2	370 ± 160	940 ± 260
		Drought	5.5 ± 1.3	9.1 ± 2.1	9.2 ± 1.9
Alp Weissenstein	2007	Control	7.0 ± 1.7	15 ± 4	21 ± 11
		Drought	2.5 ± 0.2	4.2 ± 0.9	3.3 ± 0.3

The data presented are estimates based on static chamber measurements conducted on 41 dates for Früebüel (2007 and 2008) and 20 dates for Alp Weissenstein (2007). While the seasonal average is probably a relatively crude estimate due to the very dynamic nature of N_2O fluxes, relative treatment effects should be reflected accurately. N_2O fluxes are rounded to two significant digits; the associated standard error is rounded to the same precision. Fluxes were not measured at sufficient temporal resolution to reliably estimate average fluxes at Alp Weissenstein in 2008. See Hartmann and Niklaus (2012) for details

Festuca rubra were the most abundant species. Further species exceeding several percent cover were *Trifolium pratense*, *Trifolium repens*, *Plantago alpina*, *Crocus albiflorus*, *Potentilla aurea*, *Leontodon hispidus*, *Crepis aurea*, *Agrostis capillaris*, *Nardus stricta*, *Phleum rhaeticum*, and *Poa alpina*. Due to the short growing period (mid-May to mid-September), the site is only grazed during the summer. The pasture is managed extensively and no fertilizer is applied except for excreta of non-dairy cattle and horse grazing two to three times a year. The topsoil is a silt loam containing 35 % sand, 59 % silt, and 6 % clay, and with a pH of ~5.0.

The experimental sites were fenced in October 2006 and vegetation clipped at 4 cm height when the surrounding pastures were grazed by livestock or mown. At each site, five blocks consisting of two 3.5 × 3 m plots were established. The 2 × 2.2 m core area of each plot was further subdivided into four subplots, which were separated by PVC sheets inserted 15 cm deep into the soil.

Drought was simulated by excluding precipitation from one plot per block with rain exclusion roofs consisting of a metal frame covered with a 200-μm-thick plastic foil (polyethylene co-extruded with ethyl-vinyl-acetate; Gewächshausfolie UV 5; Folitec Agrarfolien-Vertriebs, Westerburg, Germany; Hartmann et al. 2011). In 2007, the rain exclusion roofs were installed on the drought-treated plots from August 3 to September 27 and from July 31 to September 25 at Früebüel and Alp Weissenstein, respectively. In 2008, roofs were installed at each site from June 26 to August 13 and from July 14 to September 26, respectively.

The fertilizer treatment consisted of the application of ammonium nitrate (NH_4NO_3), non-dairy cattle urine, or no fertilizer (NIL treatment) to one of three subplots each per plot. The fourth subplot remained unused. The cattle urine applied in the field was collected from non-dairy cattle kept in stables. In 2007, a small amount of fertilizer was added on August 9 at Früebüel and on August 10 at Alp Weissenstein (5 g urine-N and 10 g NH_4NO_3-N m^{-2}). A larger application (15 g urine-N and 30 g NH_4NO_3-N m^{-2}) was added on September 19 at Früebüel and on September 24 at Alp Weissenstein. In 2008, a single large application (15 g urine-N and 30 g NH_4NO_3-N m^{-2}) was added on July 9 at Früebüel and on July 29 at Alp Weissenstein. The differences in N application rates among treatments resulted from the fact that the N content of urine was only known after it had been applied to field plots. Since most of urine-N is in the form of urea, which quickly hydrolyses to NH_4^+ (Haynes and Williams, 1992), approximately equivalent amounts of NH_4^+ and urea-N were added to the sub-plots. All fertilizers were applied as aqueous formulation at a rate of 4.9 L m^{-2}, and equivalent amounts of water were applied to the control subplots (NIL treatment). All applications took place during periods when rain exclusion roofs were installed (Früebüel: August 9 and September 19, 2007, and July 9, 2008; Alp Weissenstein: August 10 and September 24, 2007, and July 29, 2008).

Meteorological conditions at both sites were recorded by an automatic on-site weather station (Table 2). Ten-minute averages of soil temperature and soil moisture were recorded using automatic data loggers (CR1000; Campbell Scientific, Logan, UT, USA). Probes were installed in two blocks per site at depths of 8 and 25 cm at Früebüel, and, because of shallower soils, at 8 and 20 cm depths at Alp Weissenstein. Additional soil moisture readings were recorded in all subplots using a portable soil moisture sensor (ThetaProbe ML2x; Delta-T Devices, Burwell, Cambridge, UK). For details, see Hartmann et al. (2011).

Soil sampling

Soils were sampled twice in 2007 and twice in 2008. In 2007, the first sampling took place after application of the small but before application of the large amount of

Table 2 Precipitation, soil temperature and soil moisture of control and drought-treated soils at the two study sites Früebüel and Alp Weissenstein

Site and month	Precipitation (mm)			Soil temperature (°C)		
	2007	2008	2009	2007	2008	2009
Früebüel						
January	102	52	33	3.4	1.9	1.4
February	55	43	110	2.8	1.8	1.6
March	149	104	115	3.6	3.2	1.4
April	13	202	56	9.6	5.7	7.1
May	209	46	184	11.8	12.1	11.6
June	238	196	294	15.9	16.0	
July	317	290	120	17.2	17.8	
August	336	293		16.9	17.2	
September	131	187		14.5	13.1	
October	64	152		10.9	10.6	
November	37	46		5.1	5.1	
December	114	44		2.9	2.8	
Sum/Mean	1,765	1,656		9.5	8.9	
Alp Weissenstein						
January	82[a]	66[a]	46[a]		0.9	1.4
February	48[a]	11[a]	81[a]		0.8	1.6[b]
March	54[a]	52[a]	44[a]		0.9	1.7[b]
April	18	84	87		3.0[b]	3.3[b]
May	112	101	43		8.2	9.3
June	166	140	120	17.2	11.0	10.0
July	123	217	61	14.2	12.6	11.9[b]
August	172	117		13.6	13.6	
September	48	148		9.6	10.1	
October	19	75		6.5	7.0[b]	
November	105[a]	107[a]		2.4[b]	2.4	
December	25[a]	73[a]		0.2[b]	1.7	
Sum/Mean	970	1,189			6.2	

Soil moisture (m^3 H_2O m^{-3})

2007		2008		2009	
Control	Drought	Control	Drought	Control	Drought
0.37	0.37	0.41	0.35	0.39	0.37
0.37	0.37	0.39	0.34	0.41	0.40
0.38	0.38	0.42	0.36	0.43	0.40
0.33	0.33	0.43	0.38	0.39	0.38
0.34	0.34	0.29	0.26	0.41	0.41
0.35	0.35	0.33	0.30		
0.41	0.40	0.37	0.18		
0.42	0.35	0.39	0.13		
0.41	0.24	0.39	0.29		
0.40	0.25	0.40	0.34		
0.41	0.33	0.42	0.38		
0.42	0.36	0.41	0.38		
0.38	0.34	0.39	0.31		

Table 2 continued

Soil moisture (m^3 H_2O m^{-3})

2007		2008		2009	
Control	Drought	Control	Drought	Control	Drought
		0.47	0.42	0.49	0.50
		0.47	0.43	0.50[c]	0.50[c]
		0.51	0.46	0.50[c]	0.50[c]
		0.50[c]	0.50[c]	0.50[c]	0.50[c]
		0.50	0.48	0.47	0.44
0.56	0.55	0.48	0.47	0.42	0.44
0.50[c]	0.50[c]	0.47	0.49	0.39	0.34
0.55	0.19	0.50	0.33		
0.48	0.31	0.50	0.10[d]		
0.48	0.43	0.40[c]	0.10[d]		
0.50[c]	0.50[c]	0.53	0.50		
0.50[c]	0.50[c]	0.52	0.51		
		0.49	0.40		

The N fertilization treatment did not affect these parameters. Summer precipitation was measured by rain-gauges installed at the two farms (for details, see Zeeman et al. 2010)

[a] Data interpolated based on measurements from a nearby weather station with heated rain-gauge

[b] Data interpolated based on soil temperature readings at the next weather station of the Swiss federal office of meteorology and climatology

[c] Soil moisture estimated by visual interpolation due to incomplete logger data

[d] Very dry soils; soil moisture readings outside calibrated range

fertilizer. In 2008, the first sampling took place before application of any fertilizer. The second sampling in each year took place several weeks after the last application of fertilizer. Therefore, soil analyses reflect short-term effects of the small (first sample) and the small plus the large (second sample) fertilizer application in 2007. In 2008, soil analyses reflect longer-term effects of the previous years' fertilizer application (first sample) and the combined short- plus long-term effects of all fertilizer applications (second sample). Each sample consisted of four to five soil cores per subplot (2 cm diameter × 10 cm depth) that were pooled and sieved (2 mm mesh size). Not all analyses were conducted on all sampling dates—we refer to the respective figures for details.

Soil mineral N concentrations

To determine soil NH_4^+ and NO_3^- concentrations, soil subsamples were extracted with 2 M KCl on a table shaker (30 min of vigorous shaking). Suspensions were centrifuged, filtered, and extracts analyzed for NH_4^+ and NO_3^- (Autoanalyzer; Bran and Luebbe, Norderstedt, Germany).

N mineralization and nitrification

Anaerobic N mineralization rates were determined by incubating soil subsamples equivalent to 10 g dry weight under waterlogged conditions (40 °C, 7 days). The incubated samples were analyzed for NH_4^+ concentrations as described above, and anaerobic N mineralization rates calculated as increase in extractable NH_4^+ during the incubation.

Potential nitrification rates of soil subsamples were calculated from NO_3^- produced during a short-term incubation in a buffer containing excess NH_4^+, using a method described in Niklaus et al. (2006). Briefly, fresh soil equivalent to 10 g dry weight was added to 100 mL buffer (0.5 mM KH_2PO_4/K_2HPO_4, adjusted to a pH of 7 by adding K_2CO_3). 1 mL of 0.05 M $(NH_4)_2SO_4$ was added and the suspension incubated on a table shaker (25 °C, 50 rpm). After 1, 4, 7, and 23 h, aliquots of the suspension were analyzed for NO_3^- concentrations. Potential nitrification rates were calculated by linear regression of NO_3^- concentration against time.

Denitrifying enzyme activity

Denitrifying enzyme activity (DEA) was assessed over a short time by making all the factors affecting denitrification rate non-limiting (Smith and Tiedje 1979; Tiedje et al. 1989). For each subsample, 5 g equivalent dry soil were placed in a 150-ml plasma flask. The atmosphere of the flask was replaced by a 90:10 He–C_2H_2 mixture providing anaerobic conditions and inhibition of N_2O-reductase activity. A solution containing 1 mg glucose C g^{-1} dry soil, 1 mg glutamic acid C g^{-1} dry soil, and 0.1 mg NO_3^--N g^{-1} dry soil was added, the volume of which was adjusted to bring the soil to 100 % water-holding capacity. N_2O production rate was calculated from the N_2O concentration measured in the flask after 60, 90, and 120 min of incubation at 26 °C. Preliminary assays showed that no de novo synthesis of denitrifying enzyme occurred within a 2-h incubation. N_2O concentrations were analyzed on a gas chromatograph (Agilent 6890 equipped with an electron capture detector; Agilent Technologies, Santa Clara, CA, USA).

Nitrifier and denitrifier functional genes

These analyses were conducted at Früebüel only. DNA was extracted from soil with the MoBIO PowerSoil DNA Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the protocol of the manufacturer and redissolved in 100 µl H_2O. Prior to extraction, samples were homogenized briefly using a Fast Prep FP 120 (Qbiogene, Illkirch, France). Extracted DNA was quantified (Nanodrop ND1000; NanoDrop Technologies, Wilmington, NC, USA) and each sample diluted to 5 ng DNA μl^{-1} with DEPC water and stored at −24 °C. The abundance of ammonia oxidation (nitrification) and denitrification pathway genes was assessed by real-time quantitative PCR (qPCR) using primers targeting *amoA* of archaea (AOA) and bacteria (AOB) and *narG*, *nirK*, *nirS* and *nosZ* gene fragments, respectively (Keil et al. 2011; Regan et al. 2011). The qPCR results were used as a proxy for the abundance of the nitrifier and denitrifier community. The 16S rRNA gene abundance was quantified and used a measure of the size of total bacterial community (Henry et al. 2006). Functional gene fragments were amplified on an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Tests for the potential presence of PCR inhibitors in DNA extracted from soil were performed and soil DNA extracts diluted until inhibition was not longer detectable. Total reaction volume was 15 µl, containing 7.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 2 µM of each primer, 100 ng of T4gp32 (MP Biomedicals Europe, Illkirch, France) and 15 ng (AOA and AOB) or 7.5 ng (denitrifiers) of template DNA. For each functional gene, serial dilutions of standard soil DNA extract (AOA and AOB) and cloned, linearized plasmids harboring the respective catalytic subunits (denitrifiers) were used to generate standard curves.

Soil acidity

When the experiment was destructively harvested in 2009, soil blocks of 20 × 20 cm surface area were excavated, divided into 0–5, 5–10, and 10–15 cm depth layers and sieved (4 mm mesh). Five grams of air-dried soil were mixed with 25 ml 0.1 M KCl solution and soil pH measured with a glass electrode (780 pH Meter; Metrohm, Zofingen, Switzerland).

Data analysis

To analyze the data, mixed-effects models fitted by maximum likelihood were used (*lme* function of the *nlme*-package of R 2.8.1, http://www.r-project.org). The models included the nested random effects site, block, plot and subplot, and the fixed effects site, drought and fertilization. For soil pH, the terms depth (fixed effect) and soil layer (random effects) were also fitted. Note that the main effect of site was not tested, since there is no well-defined unit of replication (this would require replicated experiments per site located at some distance of each other; interactions with site, however, were tested). When data were assessed on several occasions, effects of time plus the respective interactions with drought and fertilization were included in the model. We tested for systematic covariance of residuals between these repeated measures, but residuals from

repeated measures were essentially uncorrelated so that there was no need to include this term in the final model. Differences were considered significant when $P < 0.05$. All error estimates in text and figures are standard errors of treatment means.

Results

Soil temperature and moisture

Soil moisture exhibited large intra-annual variation, with pronounced natural and treatment-induced drying cycles. Soil moisture dynamics are discussed in detail in Hartmann et al. (2011); a summary is presented in Table 2. The installation of rain exclusion roofs reduced precipitation water inputs in 2007 and 2008 by 450 and 410 mm, respectively, at Früebüel, and by 210 and 315 mm, respectively, at Alp Weissenstein. This amounted to a reduction of annual precipitation of 25–30 % (i.e., a 30–40 % reduction of growing season precipitation). Neither simulated drought nor N fertilization affected soil temperature.

Soil NH_4^+ and NO_3^- concentrations

The amount of extractable NH_4^+ strongly depended on both experimental treatments (Fig. 1a; Table 3). Drought increased soil NH_4^+ concentrations by 275 % ($P < 0.01$). However, this drought effect was mainly due to fertilized subplots, since unfertilized subplots (NIL) showed no significant increase in soil NH_4^+ when exposed to severe drought. Both N fertilizers increased soil NH_4^+ ($P < 0.001$), but NH_4NO_3 application caused a higher increase in soil concentrations (+330 %) than cattle urine application (+70 %). The effect of both fertilizers depended on the drought treatment (drought × N: $P < 0.01$), resulting in higher concentrations of NH_4^+ when fertilizers were applied under rain exclusion roofs.

Soil NO_3^- concentrations (Fig. 1b; Table 3) showed similar patterns as NH_4^+ concentrations. Drought treatment increased soil NO_3^- by a factor of four (+280 %, $P < 0.05$), mainly due to high soil NO_3^- concentrations in subplots fertilized with NH_4NO_3. Soil NO_3^- concentrations were also strongly affected by the applied fertilizer type ($P < 0.001$). NH_4NO_3 application caused higher soil

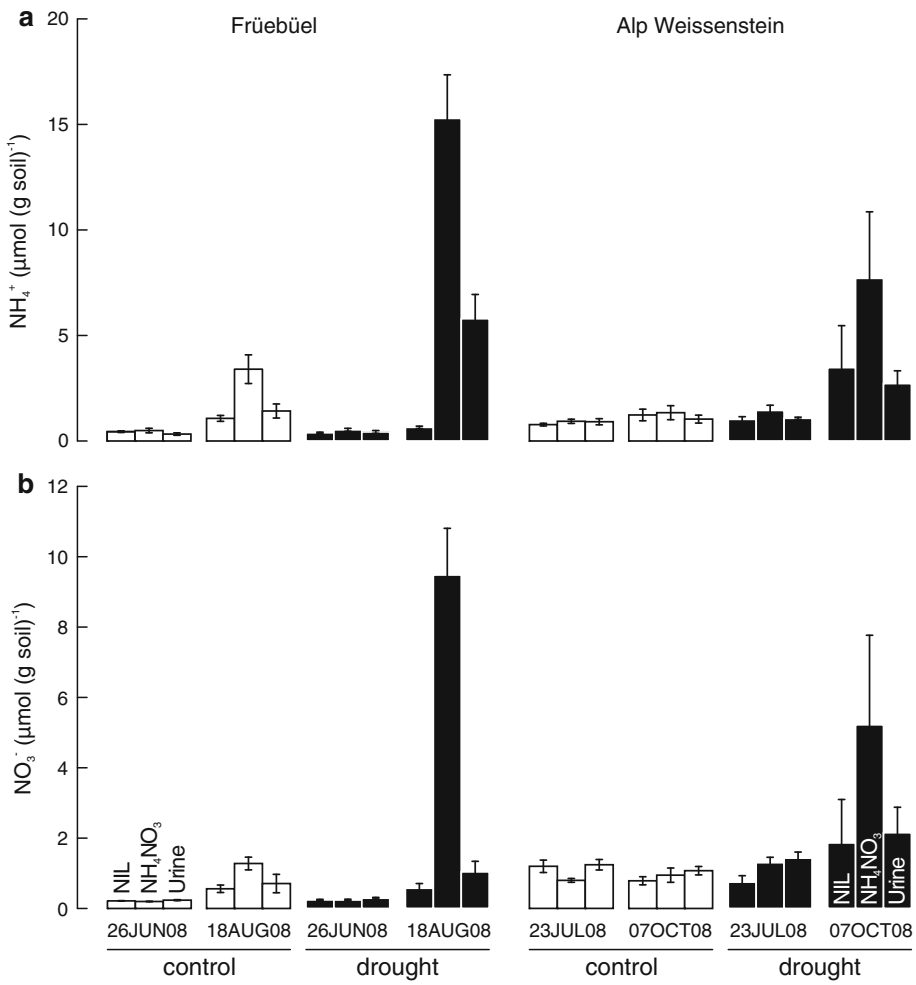

Fig. 1 Extractable **a** NH_4^+ and **b** NO_3^- in dependence of site (Früebüel and Alp Weissenstein), simulated drought and fertilizer treatment

Table 3 Statistical analyses of soil mineral N concentrations (NH_4^+ and NO_3^-), laboratory incubation data (nitrogen mineralisation, potential nitrification, denitrification enzyme activity [DEA]), and microbial functional gene abundances

Hierarchical level	Source of variation	*df*		Statistical signficance[a]								
		Nom.	Denom.	Soil mineral N		Laboratory incubations			Soil microbial gene abundances			
				NH_4^+	NO_3^-	Nitrogen mineralization	Potential nitrification	DEA	AOA	AOB	narG:16S	nirS:16S
Plot	Drought	1	8	**	*	**	NS	NS	NS	NS	NS	NS
	Site × drought	1	8	NS	NS	*	NS	NS	–	–	–	–
Subplot	N	2	32	***	***	*	***	***	NS	NS	*	**
	Drought × N	2	32	*	**	NS	NS	*	*	*	NS	NS
	Site × N	2		NS	NS	**	NS	NS	NS	NS	NS	NS
	Site × drought × N	2	32	NS	NS	NS	NS	NS	–	–	–	–
Time	Time	1/2/3	[b]	***	***	NS	NS	***	***	***	***	***
	Time × drought	1/2/3	[b]	**	*	NS	NS	***	NS	NS	*	NS
	Time × N	2/4/6	[b]	*	***	NS	NS	NS	NS	NS	NS	***
	Time × drought × N	2/4/6	[b]	NS	NS	NS	NS	NS	NS	NS	NS	NS

The statistical tests are grouped by hierarchical level of the experimental design (plot, subplot, or temporal replicates within subplots). Nominator (nom.) and denominator (denom.) degrees of freedom refer to the respective *F* tests. For effects of time, *df* are given for two, three, or four temporal replicates, c.f. respective figures

[a] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant, – test not applicable because factor missing from model

[b] Denominator degrees of freedom for 1, 2, or 3 sampling dates are 54, 108, and 162 (2 sites), and 24, 48, and 72 (1 site only)

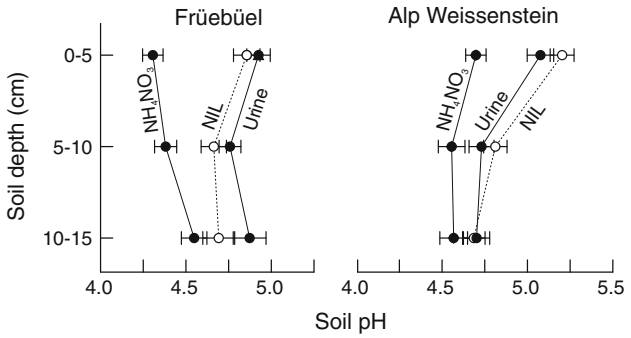

Fig. 2 Soil acidity at Früebüel and Alp Weissenstein in dependence of soil layer, drought, and fertilizer treatment. Data are from summer of the third year (2009), when the experiment was terminated. No fertilizer had been applied in 2009

NO_3^- concentrations (+350 %) while urine had no effect. NO_3^- concentrations increased more when applied under drought than when applied to unsheltered plots (drought × N, $P < 0.01$).

Soil acidity

At both sites, soil acidity (Fig. 2) depended on soil depth ($P < 0.001$), but with contrasting depth profiles at the two sites (site × depth: $P < 0.001$). At Alp Weissenstein, soils showed a pronounced decline of pH with depth, whereas differences between soil layers were smaller at Früebüel.

Soil acidity was not affected by drought. The fertilizer effect on soil acidity ($P < 0.001$) was site-dependent (site × N: $P < 0.05$): while NH_4NO_3 application caused a strong decrease in soil pH at both sites, urine application increased pH at Früebüel but had no effect at Alp Weissenstein. The fertilizers also differed in how they affected the pH at the different soil depths (N × depth: $P < 0.001$). The acidification caused by NH_4NO_3 decreased with soil depth, while the increase in pH due to urine application was independent of soil depth.

Mineralization and nitrification

Drought increased N mineralization rates (Fig. 3; +14 %, $P < 0.01$; Table 3). When analyzing each site separately, this drought-induced increase was only significant at Alp Weissenstein (+20 %, $P < 0.01$). Fertilization did not affect mineralization rates at Früebüel while at Alp Weissenstein both fertilizers increased mineralization rates by about +15 % (site × N: $P < 0.05$).

Potential nitrification (Fig. 4) was not affected by drought. On average, fertilization increased potential nitrification rates ($P < 0.01$). This effect was essentially driven by the urine treatment ($P < 0.001$ for linear contrast comparing NIL and urine treatments), whereas NH_4NO_3 had no statistically significant effect (averages even were lower on many dates).

Denitrification activity

DEA was affected by drought (Fig. 5; Table 3), and this effect depended on time ($P < 0.001$ for time × drought).

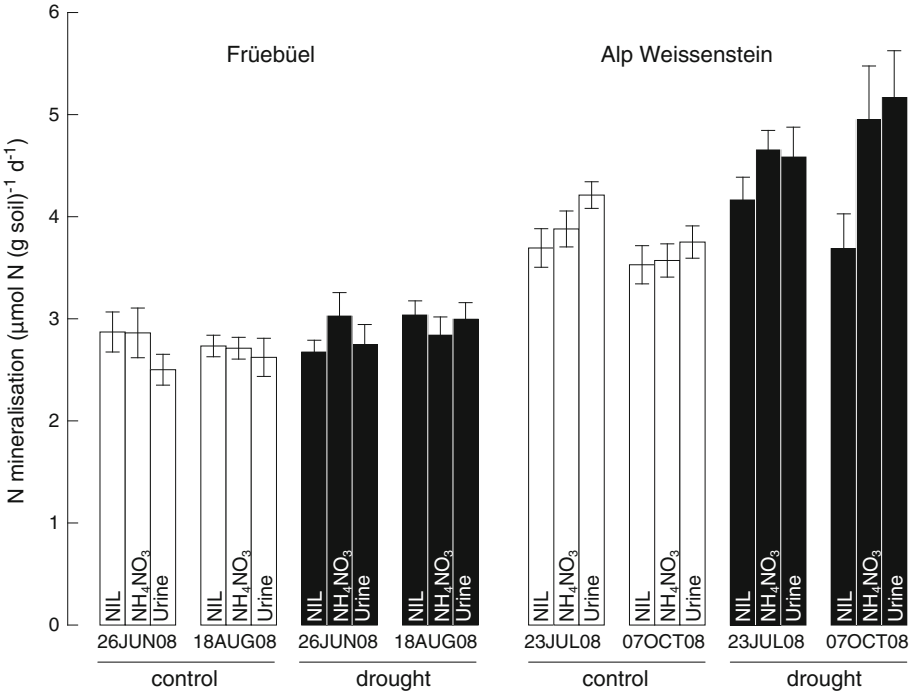

Fig. 3 Nitrogen mineralization as measured in laboratory incubations in dependence of site (Früebüel and Alp Weissenstein), simulated drought and fertilizer treatment

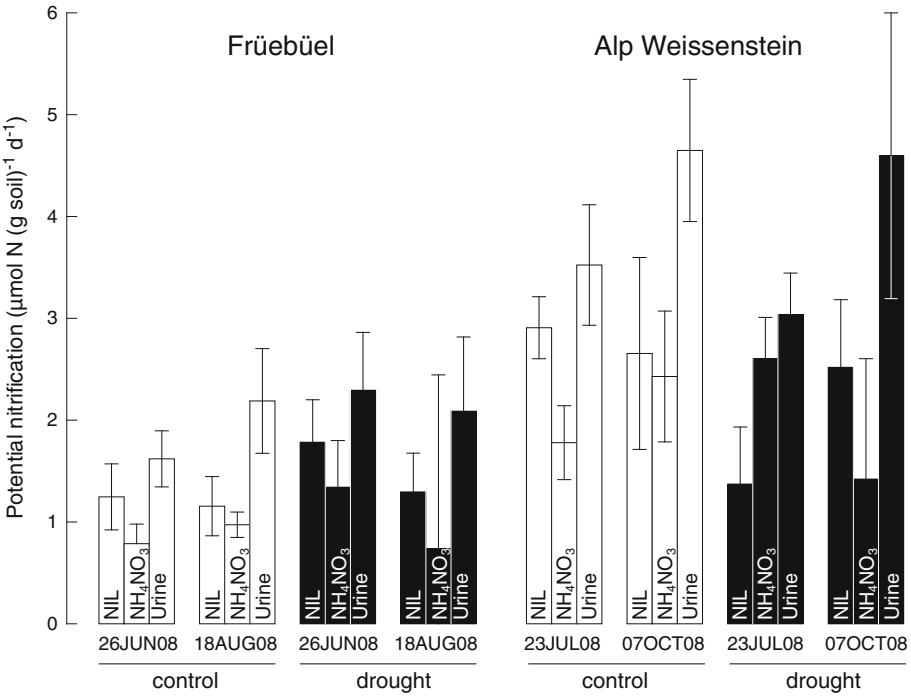

Fig. 4 Potential nitrification in dependence of site (Früebüel and Alp Weissenstein), simulated drought and fertilizer treatment, before and several weeks after fertilizer application

Effects of N were significant ($P < 0.01$), and these were drought-dependent ($P < 0.05$ for drought × N). Partitioning the N-effects using linear contrasts revealed that these effects were essentially driven by the NH_4NO_3 treatment ($P < 0.001$ for the 23 % effect of NH_4NO_3 relative to NIL; $P < 0.05$ for the dependency of this effect on drought), while urine had only a weak positive effect on DEA (+14 %, no significant interaction with time or drought).

Nitrifier and denitrifier functional gene abundance

Drought had no effect on the abundance of ammonia oxidizing bacteria and archaea (expressed as *amoA* copy numbers). Drought also had no effect on the relative abundance of *narG*, *nirK* and *nosZ* (standardized by the abundance of 16S copy numbers; Fig. 6; Online Resource 1) but slightly reduced the relative abundance of *nirS* (−7 %; $P < 0.05$).

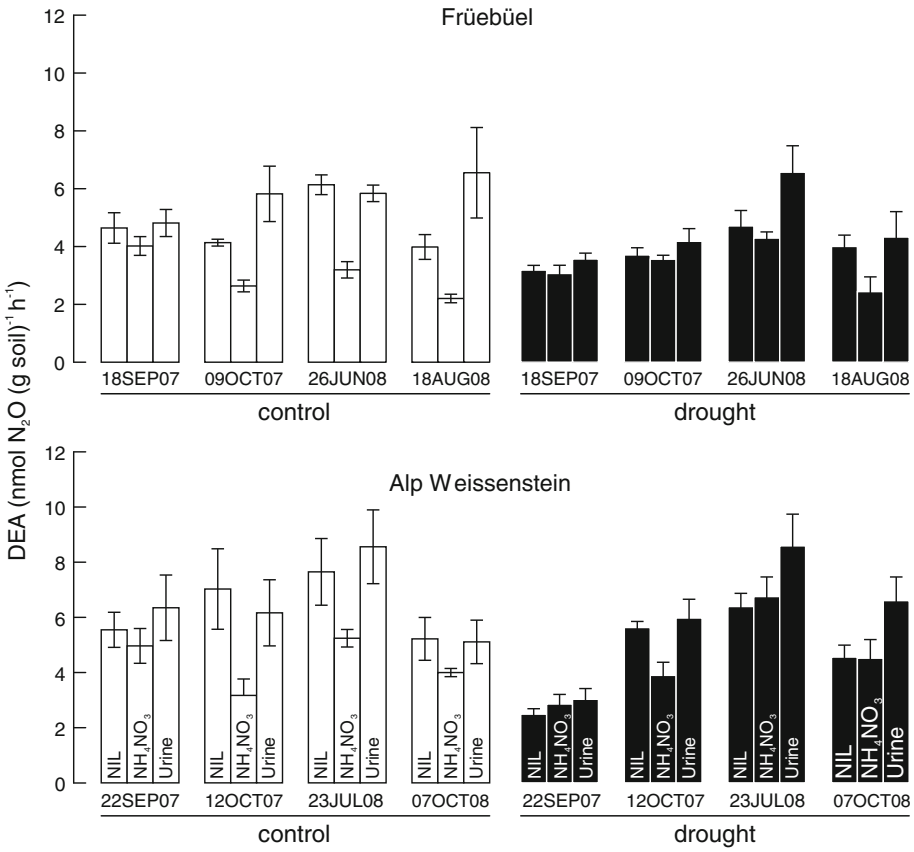

Fig. 5 Denitrifying enzyme activity at the two sites Früebüel and Alp Weissenstein, in dependence of simulated drought and fertilizer application

The N fertilizers increased *amoA* copy numbers for both bacteria and archaea, but only in the absence of drought ($P < 0.05$ for N × drought). This effect originated essentially from the application of NH_4NO_3, which increased AOA and AOB in the control treatment but not under drought (AOA: $P < 0.05$ for NH_4NO_3; $P < 0.01$ for NH_4NO_3 × drought; AOB: $P < 0.001$ for effect of NH_4NO_3, $P < 0.01$ for NH_4NO_3 × drought; no significant effects of urine or urine × drought; linear contrasts within N).

NH_4NO_3 application reduced *narG*:16S and *nirS*:16S, an effect which progressively built up over time, reaching approximately −40 and −20 % after fertilizer application in the second year of treatment ($P < 0.001$). On the other hand, urine application did not affect the relative abundance of any gene investigated in this study.

Discussion

The present study was designed to test effects of drought and N application on soil N cycling processes, focusing in particular on nitrification and denitrification. Further goals were to test which mechanisms were driving the changes in soil N_2O emissions we had previously observed (Hartmann and Niklaus 2012; cf. Table 1). Overall, our results indicate than soil N cycling and N_2O emissions were affected by complex interactions between drought, mineral N availability, soil acidification, and plant growth. Response patterns were by and large equal at Früebüel and Alp Weissenstein, despite many site differences including vegetation, soil, management intensity, and climate, indicating that our findings are robust and possibly generalizable for temperate pasture.

Effects of drought

Massively increased soil NH_4^+ and NO_3^- concentrations after fertilizer application to drought-treated plots indicate that the applied fertilizer N was not efficiently removed from soil solution. We argue that this accumulation of mineral N was driven by (1) a cessation of plant growth and associated root N uptake when soils became very dry under the rain exclusion roofs, and (2) a reduced activity of soil nitrifying micro-organisms. Slowed plant growth was evident in the field, with plants showing signs of loss of turgor pressure and wilting during the most extreme periods of drought. The elevated soil NH_4^+ concentrations we measured also suggest that nitrifiers did not oxidize large amounts of NH_4^+. This reasoning is compatible with published evidence that nitrifier activity is sensitive to desiccation. Linn and Doran (1984) reported optimum soil

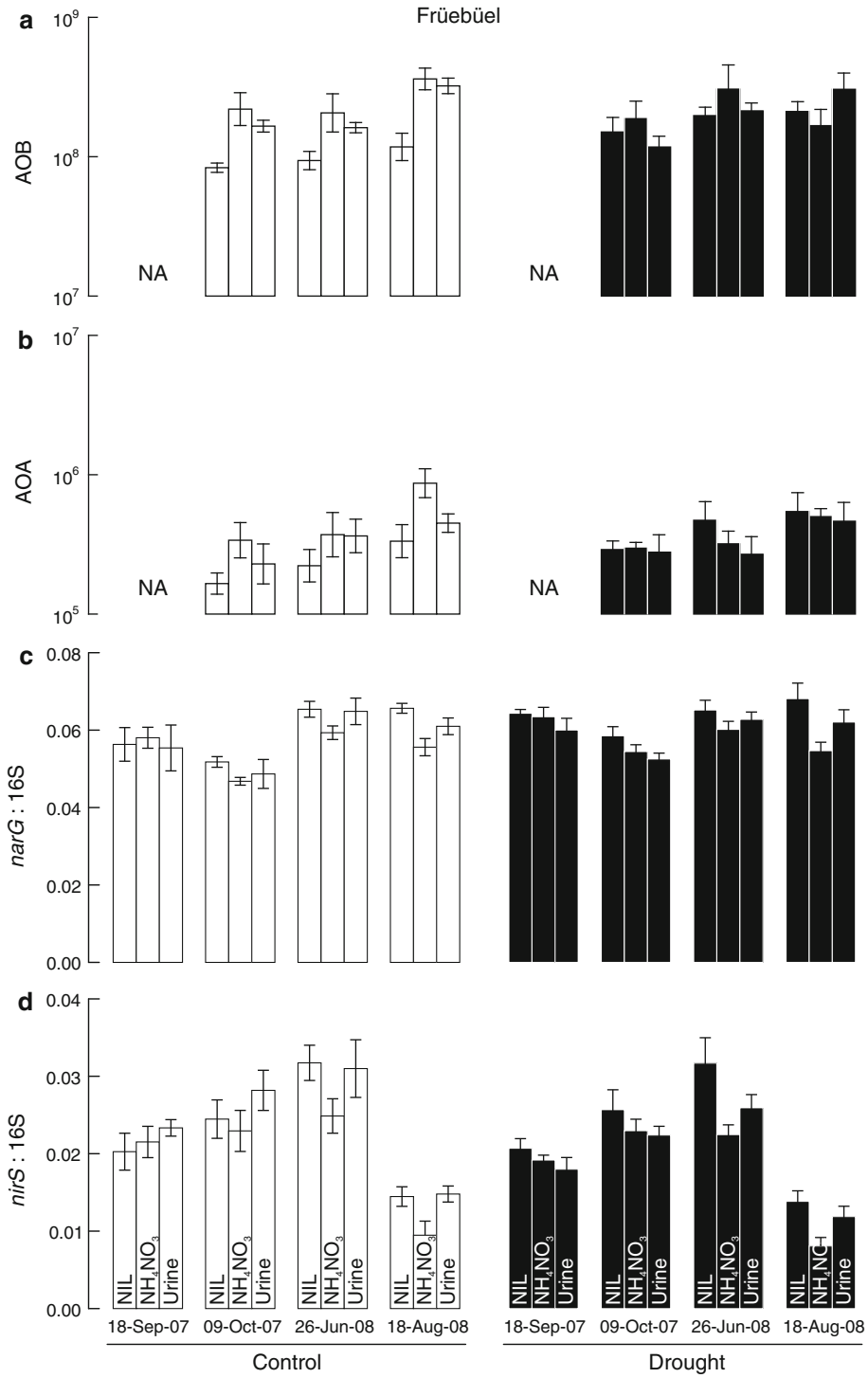

Fig. 6 **a**, **b** Abundance of *amoA* genes for ammonia oxidizing bacteria (*AOB*) and archaea (*AOA*). **c**, **d** Abundance of denitrifier genes coding for nitrate (*narG*) and nitrite (*nirS*) reductase; these data are expressed relative to 16S rRNA. Data are for the low-altitude site Früebüel, no measurements were conducted for soils from Alp Weissenstein

moisture of 60 % water-filled pore space for nitrification. Stark and Firestone (1995) conducted controlled experiments in which NH_3 availability and soil moisture were manipulated independently and showed decreasing nitrification rates with decreasing water potential; below −0.5 MPa, nitrification was substantially inhibited. In our study, drought effects on nitrification were, however, not evident in laboratory measurements of potential nitrification; we argue that this is because these were conducted in a slurry assay in which, unlike in the field, moisture was not limiting. 16S rRNA gene abundances also did not respond to drought, suggesting that only the activity of nitrifying soil bacteria and archaea was reduced but not their community size. Persistent nitrifier populations under drought have also been reported by Davidson (1992), who found that nitrifying bacteria were well adapted to survive

severe drought stress in a grassland with long summer dry seasons, and that their activity increased rapidly after soil rewetting.

Denitrifying enzyme activity (DEA) did respond only little to drought (on some dates), or not at all (average over all dates). This contrasts with many studies that reported a positive correlation of DEA and soil moisture (e.g., Frank and Groffman 1998; Groffman et al. 1991). The absence of effects in our study suggests that the denitrifying enzyme potential had endured the extended drought period, or that de novo synthesis of enzymes occurred so quickly that the activity of newly produced enzymes was captured in the DEA assay. Peterjohn (1991) reported that after rewetting of dry soils, a significant portion of denitrifying enzyme content originates from an enzyme pool capable of tolerating extended drought periods, enabling a rapid increase in denitrification when conditions become favorable. On the other hand, the expression of reductase enzyme genes can recover within hours once soil O_2 is depleted (Smith and Tiedje 1979), and denitrification be activated within minutes to hours after soil rewetting (Davidson, 1992). In our study, unaltered denitrifier functional gene abundances suggest that denitrifier populations persisted through drought; this persistence may have resulted from the fact that denitrification is just one oxidative pathway of denitrifying bacteria, i.e., most denitrifiers can use oxygen instead of nitrogen oxides as electron acceptors, which is energetically even more favorable. Therefore, the availability of oxidizable carbon may be the more important determinant of the size of the microbial population capable of denitrification (Murray et al. 1990).

In our study, drought appeared to increase N mineralization rates, but we believe that this in fact may be a reversal of effect direction compared to field conditions. The weak positive response we observed in the incubation assay may have occurred because decomposition was reduced in the field and more substrate, therefore, was available afterwards in the laboratory assay. N mineralization is generally correlated with soil moisture (e.g., Booth et al. 2005; Fisk et al. 1998; Pilbeam et al. 1993), but mineralization can peak upon rewetting after drought (recently reviewed by Borken and Matzner 2009).

Effects of N application

Application of cattle urine increased potential nitrification, whereas application of NH_4NO_3 did not. The dominant nitrogenous compound in urine is urea, which quickly hydrolyzes to NH_4^+ in soils. Therefore, both urine and NH_4NO_3 will have resulted in approximately the same quantity of NH_4^+ added to soils, although with a slightly different temporal dynamic because NH_4^+ release may have been delayed in the case of urine. However, NH_4NO_3 application also resulted in soil acidification, and we argue that nitrification was inhibited at the lower pH in the NH_4NO_3-treated plots, i.e., that nitrification rates would have been higher if pH had not dropped. Soil acidification is mainly the result of ion exchange processes during plant nutrient uptake; roots generally exchange NH_4^+ for a proton (H^+), thereby acidifying the rhizosphere. NH_4^+ oxidation by nitrifiers also results in net H^+ formation if not all NO_3^- produced is assimilated by plants (Bolan et al. 1991). However, in the case of urine, urea hydrolysis results in NH_3 which combines with H^+ to NH_4^+, so that the overall process is neutral. Autotrophic nitrifying bacteria often show highest activities in the neutral to alkaline range (reviewed by Prosser 1989), although nitrifying archaea might tolerate more acidic conditions (Nicol et al. 2008). In general, however, nitrification strongly drops when soil pH approaches ca. 4 (Persson and Wiren 1995; Ste-Marie and Pare 1999).

Denitrification rates are generally highest in neutral to alkaline soils (Dalal et al. 2003; Page et al. 2009; Simek and Cooper 2002). In our study, denitrification enzyme activity, as measured in the laboratory assay, progressively dropped with time in the NH_4NO_3 application treatment, and so did the relative abundance of associated functional genes. We argue that soil acidification drove this decrease, either by a direct effect or indirectly due to reduced substrate availability as a consequence of lower nitrification rates. We believe that a reduction in denitrification under NH_4NO_3 application also occurred in the field, and we argue that the effect on N_2O fluxes was smaller than could have been expected based on the double amount of N that had been applied in the NH_4NO_3-treated compared to the urine-treated plots. Further evidence for this reasoning emerges from the observation that the ratio of N_2O emitted from soils fertilized with NH_4NO_3 to N_2O emitted from cattle urine-treated plots decreased in the course of the 2-year treatment (from $\sim$0.8 in 2007 to $\sim$0.4 in 2008; data from Früebüel in the absence of drought; cf. Table 1).

Mechanisms driving N_2O fluxes

How did the response patterns we found for potential nitrification, denitrification (DEA), and functional gene abundances relate to the field-measurements of N_2O fluxes? Interestingly, effects of drought were not reflected to a large extent in the data reported here, although reduced soil moisture was one of the most important factors controlling N_2O fluxes in the field, in particular in the presence of N fertilizers (cf. Table 1).

N fertilizers massively increased N_2O fluxes, but these effects did not manifested in DEA and denitrifier functional genes. This may appear surprising, but again may be attributed to denitrification being just an alternative oxidative pathway under anaerobic conditions, so that a lack

of nitrogen oxides under oxic conditions may not have any negative consequences for the respective organisms. N fertilizers did, however, manifest in increased nitrifier populations, as is evidenced in increased 16S rRNA gene abundances. Finally, indirect effect of NH_4NO_3 application via topsoil acidification were very prominently reflected in both DEA and denitrifier functional gene abundances, and in potential nitrification rates (but not in *amoA* gene abundances which even tended to increase).

Overall, our data suggest that DEA is only poorly correlated to field N_2O emissions, at least under the conditions we investigated. Over time and across treatments, denitrification was more strongly controlled by environmental conditions than by microbial community sizes and activity potentials. Interestingly, N_2O fluxes were reflected to a larger extent in nitrification than in denitrification potentials and microbial population sizes, despite nitrification being a relatively minor source of N_2O. There are two likely reasons for this phenomenon. First, the nitrification process is essential in providing nitrifiers with energy for growth. Second, nitrification is a strong controller of denitrification and associated N_2O emissions because it is upstream of denitrification in the general sequence of soil N transformations.

Conclusions

Effects of drought significantly interacted with effects of N application. This suggests that responses of pasture N cycling to drought will differ between excreta patches and relatively unaffected areas. These interactive effects also differed between processes of the soil N cycle, suggesting that the spatial heterogeneity in pastures needs to be taken into account when predicting changes in N cycling and associated N_2O emissions in a changing climate.

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