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Evidence that acidification-induced declines in plant diversity and productivity are mediated by changes in below-ground communities and soil properties in a semi-arid steppe

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Summary

1. Anthropogenic acid deposition–induced soil acidification is one of the major threats to biodiversity, ecosystem functioning and services. Few studies, however, have explored in detail how above-ground changes in plant species richness and productivity resulting from soil acidification are mediated by effects on below-ground biota and soil properties.

2. To increase our understanding of this linkage, we collected data on below- and above-ground communities and soil properties in a 3-year field experiment with seven levels of acid addition rate to build-up broad intensities of soil acidification in the semi-arid Inner Mongolian grassland.

3. Acid addition directly elevated concentrations of soil Al^{3+} ions, decreased the base cations Ca^{2+}, Mg^{2+} and Na^{+}, and increased soil moisture and available phosphorus. Acid addition also appears to have altered the soil microbial community via changes in H^{+} and Al^{3+} ions and altered the nematode community via changes in H^{+} ions and soil moisture.

4. The observed changes in soil N availability (i.e. net N mineralization, NO_3^--N and NH_4^+-N) could be explained by mediating changes in the H^{+} and Al^{3+} ions, microbial community (i.e. community structure, bacteria and fungi/bacteria as indicated by phospholipid fatty acids analysis) and the nematode community (i.e. total abundance, taxa richness and maturity index).

5. Declines in plant species richness and productivity were greater at high intensities of soil acidification in the second sampling year than in the first sampling year**.** The changes in plant community observed were mostly explained by soil nutrient pathways (e.g. N availability or base mineral cations), which were in turn regulated by the soil microbial or nematode communities as well as by the direct effects of the increase in H^{+} or Al^{3+} ions.

6. *Synthesis*. Our results suggest that the below-ground microbial and nematode communities are more sensitive to soil acidification than the plant communities are, and further that soil acidification–induced changes in plants are mediated by changes in below-ground communities and soil nutrients. These findings improve our understanding of the links between below- and above-ground communities in the Inner Mongolia grassland, especially in the context of anthropogenic acid enrichment.

Key-words: base mineral cations, nitrogen cycling, plant species richness, plant–soil (below-ground) interactions, productivity, semi-arid steppe, soil aluminium ions, soil microbial organisms, soil nematodes, structural equation modelling

Introduction

Soil acidification (decrease in soil pH) is one of the most important consequences of dramatic increases in anthropogenic acid deposition originating largely from atmospheric sulphur dioxide (SO_2) and nitrogen oxides (NO_x) in fossil fuel combustion and agricultural fertilization (Blake, Johnston & Goulding 2007; Zhao *et al.* 2009; Yang *et al.* 2012). It has been suggested that soil acidification is a major problem in Chinese grassland and agricultural systems (Zhao *et al.* 2009; Yang *et al.* 2012), although the rate of acid deposition has

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reduced across Europe and North America since the 1990s (Oulehle *et al.* 2011). For instance, anthropogenic acid deposition decreased soil pH by 0.63 units, on average, in the surface soil layer across northern China's grasslands during the last two decades (Yang *et al.* 2012). This reduction in soil pH may lead to declines in biodiversity and ecosystem functioning as predicted by previous studies (Blake, Johnston & Goulding 2007; Stevens *et al.* 2010). Generally, it is well documented that soil acidification has reduced the diversity and productivity of above-ground plant communities in part because of direct increases in ions of H^+ and Al^{3+} (Stevens *et al.* 2010; van den Berg *et al.* 2011).

There remains a major gap in our understanding about how soil acidification affects above-ground communities, however. It is unknown that the degree to which acidification itself (e. g. the direct toxicity of H^+ and Al^{3+}) is the primary driver of changes in plant communities or whether changes in other factors like nutrient availability and the microbial and other below-ground communities mediate the effects (Bardgett & Wardle 2010; Putten *et al.* 2013). Only a few field experiments have measured both the direct and indirect impacts of soil acidification on below- and above-ground communities in grassland ecosystems, and a formal evaluation of mediating pathways has not been conducted.

Several hypotheses regarding soil acidification–induced reduction in plant diversity and productivity are focused on the direct effects of toxic ions on plants and indirect effects of changes in below-ground communities and soil properties on above-ground plant community. First and as noted in the previous paragraph, soil acidification increases soil H^+, Al^{3+} and NH_4^+ ions, which can be directly toxic to plant roots (Kochian 1995; Van Den Berg *et al.* 2005; Poschenrieder *et al.* 2008) and soil organisms (Kuperman & Edwards 1997). Secondly, soil acidification reduces base mineral cations (e.g. Ca^{2+}, Mg^{2+}, and Na^+) at the soil's cation exchange complex, which represent a reservoir of plant nutrients and are indicators of soil fertility (Kochian 1995; Kuperman & Edwards 1997). Thirdly, soil acidification also directly changes soil microbial (Kuperman & Edwards 1997; Rousk *et al.* 2010a) and nematode communities (Williams & Dusenbery 1990; Raty & Huhta 2003), which can in turn alter plant diversity, community structure and productivity (Van Der Heijden, Bardgett & Van Straalen 2008; Bardgett & Wardle 2010). Finally, soil acidification is known to indirectly modify key ecosystem processes such as N mineralization and N cycling by altering the soil microbes and nematodes (Freckman 1988; Ferris *et al.* 1998; Djigal *et al.* 2004). However, the relative contributions of these mechanisms or pathways to soil acidification–induced changes in plant diversity and productivity have not been studied in detail with field experiments (Bardgett & Wardle 2010). In addition, previous studies concerning these mechanisms have largely focused on soils of acidic origin; the relative importance of these mechanisms has yet to be explored in soils of alkaline origin.

Our objective in this study was to evaluate the plausibility of different mechanisms whereby soil acidification can affect plant communities via effects on below-ground communities, soil properties and their linkages to the plant communities. We established a 3-year field experiment with seven levels of acid addition rate at a semi-arid Inner Mongolia grassland, which is part of a widely distributed grassland across the Eurasian Steppe region (Bai *et al.* 2004; Chen *et al.* 2013). The semi-arid Inner Mongolia grassland, together with soils of alkaline origin, enables us to test the four interrelated hypotheses that have been proposed to be the major mechanisms underpinning soil acidification–induced plant diversity loss and changes in ecosystem functioning. Specifically, we attempt to address two questions: first, how do above-ground plant community (i.e. species richness and productivity), below-ground microbial and nematode communities, and soil properties (Ca^{2+}, Mg^{2+}, Na^+, and available P), and N cycling (net N mineralization, soil NH_4^+-N and NO_3^--N) respond to different rates of acid addition in the Inner Mongolia grassland? secondly, what appear to be the major mechanisms mediating soil acidification–induced reductions in plant species diversity and alterations in productivity and N cycling?

To answer the first of the above questions, we used analysis of variance and regression to assess the net effects of different levels of acid addition on above- and below-ground communities, soil properties and N cycling. To address the second question, we hypothesize that soil acidification may affect plant diversity and ecosystem functioning via multiple direct and indirect pathways. We established an a priori model of how biotic and abiotic variables may relate to each other and to ecosystem functioning responses to acid addition and used structural equation modelling (SEM) to evaluate support in the data for different hypothesized mediating pathways (Fig. S1 in Supporting Information).

Materials and methods

STUDY SITE

This study was conducted at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS, 43°38′N, 116°42′E) of the Chinese Academy of Sciences, which is located in the Xilin River Basin of Inner Mongolia, China, at an altitude of *c.* 1200 m a.s.l. (Bai *et al.* 2004). The semi-arid continental climate is characterized by a mean annual (1982–2009) precipitation of 334 mm and a mean annual temperature of 0.9 °C. Precipitation mainly falls in the growing season (June–August), which is coincident with high temperatures. The site has a dark chestnut soil (Calcic Chernozem according to ISSS Working Group RB, 1998), with a loamy-sand texture (Bai *et al.* 2010). Before the experiment began, the plant community was dominated by *Leymus chinensis* (Trin.) Tzvel., a C_3 perennial rhizomatous grass that is widely distributed in the Eurasia steppe region. Other common plant species at the experimental site included *Stipa grandis* P. Smirn., *Agropyron cristatum* (L.) Gaertn., *Achnatherum sibiricum* (L.) Keng, *Cleistogenes squarrosa* (Trin.) Keng, *Carex korshinskyi* Kom., *Chenopodium aristatum* L., *Salsola collina* Pall., and *Chenopodium glaucum* L. (Bai *et al.* 2004).

SOIL ACIDIFICATION EXPERIMENT AND PLANT SAMPLING

In 2009, a 15-m × 20-m location with fairly uniform vegetation was designated within the permanent research plots of IMGERS. There

were seven treatments and five replicates for each treatment. The treatments included seven levels of acid addition rate (0, 2.76, 5.52, 8.28, 11.04, 13.80 and 16.56 mol H^+ m^{-2}) in the form of sulphuric acid solution. A total of 35 plots were laid out in a randomized block design; each plot was 2-m × 2-m and was surrounded by an iron sheet fence that extended 20 cm into the soil and 5 cm above the soil. Plots were also separated by 1-m walkways. Acid was added in September 2009, June 2010 and September 2010. At each time, each dose of 98% sulphuric acid was diluted in 80 L of well water. Before and after the acid solution was sprayed, each plot received 30 and 50 L of well water, respectively, to diminish the direct damage of acid to plants and soil organisms. In late August 2010 and 2011, plant species number and plant community cover (as a proxy for productivity) were determined in a 0.5-m × 0.5-m quadrat located in the northeast part of each 2-m × 2-m plot.

SOIL SAMPLING AND ANALYSIS

In late August 2010 and 2011, four soil cores (2 cm diameter, 0–15 cm deep) were randomly collected from each plot and combined to form one composite soil sample per plot. After gentle mixing and removal of roots, the moist soil was passed through a 2-mm-mesh sieve and separated into two parts. One part was maintained fresh for determination of soil moisture and net N mineralization rates and for extraction of nematodes and microorganisms. The second part was air-dried for determination of the soil pH, available phosphorus and extractable cations (Al^{3+}, Ca^{2+}, Mg^{2+}, and Na^+). A 20-g subsample of moist soil was oven-dried at 105 °C for 24 h to determine soil moisture. Soil pH was measured in a 1 : 2.5 (soil : water) suspension. Available phosphorus was determined by the method of Robertson (1999). Briefly, 2 g of air-dried soil per sample was placed in a 250-mL Erlenmeyer flask containing 40 mL of 0.5 M $NaHCO_3$. The soil suspension was shaken for 30 min at 150 rpm and then filtered before 5 mL of the filtrate and 4 mL of a solution (1000 per mL: ascorbic acid, 5.28 g; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 6 g; $(KSbO{\cdot}C_4H_4O_6)$, 0.1454 g; concentrated sulphuric acid (H_2SO_4), 148 mL) were mixed in a flask, and the final volume was adjusted to 25 mL with deionized water. Twenty minutes after mixing, the absorbance of each standard and sample was recorded at a wavelength of 882 nm with a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

SOIL EXTRACTABLE CATIONS AND NET N MINERALIZATION

The extractable cations (Al^{3+}, Ca^{2+}, Mg^{2+}, and Na^+) were measured using a modified BCR sequential extraction (Rauret *et al.* 2000). A 1-g subsample of air-dried soil per sample was placed in a 50-mL polypropylene centrifuge tube. A 20-mL volume of 0.11 mol L^{-1} of acetic acid was added to the tube, which was then shaken at 30 rpm for 16 h at 22 °C. The extract was separated from the solid phase by centrifugation at 3000 rpm for 20 min. The supernatant liquid was decanted into a 50-mL polypropylene centrifuge tube and stored at 4 °C before analysis. The contents of the extractable cations were determined with an inductive coupled plasma emission spectrophotometer (Thermo 6300, Thermo Electron, Milford, MA, USA).

Net N mineralization rates were determined using the aerobic incubation procedure described by Evans *et al.* (2001). A 20-g subsample of field-moist soil per sample was placed in a 125-mL specimen cup, which was then closed with a perforated plastic cap to allow gas exchange while minimizing evaporation. These subsamples were incubated for 30 days at room temperature (*c.* 24 °C). NH_4^+-N and NO_3^--N concentrations were determined by extracting inorganic N at 100 rpm for 2 hours from subsamples with 100 mL of 2 mol L^{-1} KCl before and after incubation. The extract was subjected to colorimetric determination on a 2300 Kjeltec Analyzer Unit (FOSS, Höganäs, Sweden). Net N mineralization was calculated as the change in total inorganic N content from the start until the end of incubation. All results are expressed on the basis of dry soil mass.

SOIL MICROBIAL AND NEMATODE COMMUNITIES

The microbial community in soil samples was assessed using phospholipid fatty acids (PLFAs). Phospholipid fatty acids were extracted from the soil as described by Bossio *et al.* (1998). The resultant fatty acid methyl esters were separated, quantified and identified using capillary gas chromatography (GC). Qualitative and quantitative fatty acid analyses were performed with an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) and the MIDI Sherlock Microbial Identification System (MIDI Inc., Newark, DE, USA). Fatty acids (FAs) were quantified by calibration against standard solutions of FAME 19:0 (Matreya Inc., State College, PA, USA), which was added as an internal standard at 50 ng mL^{-1}. The abundance of each individual FAs was expressed as fatty acid nmol g^{-1} dry soil in a given sample against an internal standard (methyl ester C19:0; Sigma-Aldrich, Taufkirchen, Germany). The structure of microbial community was analysed for each sampling year with a principal component analysis (PCA) based on the relative molar abundances of the entire fatty acids (mol-% of the 23 most abundant FAs) after standardizing to unit variance. The PC1 scores of the entire FAs for 2010 and 2011 were conducted as the indicators for the microbial community structure. Moreover, we conducted the FAs which were specific to bacteria (i14:0, a15:0, i15:0, i16:0, 10Me16:0, a17:0, i17:0, 16:1ω7c, 17:1ω8, 18:1ω9, 18:1ω7c, cy17:0 and cy19:0), fungi (18:2ω6,9) and fungi/bacteria as microbial community components (Olsson *et al.* 1995; Frostegård, Tunlid & Bååth 2011).

Nematodes were extracted from 50 g of moist soil per sample by the Baermann funnel method for 48 h (Barker 1985). After fixation in 4% formalin solution, nematodes were counted with the aid of an inverted microscope, and the first 100 individuals encountered were identified to genera. All nematodes were identified when there were fewer than 100 individuals in a sample. Based on feeding habits and life-history characteristics, soil nematodes were assigned to five trophic groups and functional guilds (Yeates *et al.* 1993; Ferris, Bongers & De Goede 2001). A soil nematode functional guild consisted of taxa with the same feeding habits and with the same inferred function in soil food webs (Ferris, Bongers & De Goede 2001). The five trophic groups were conducted as the indicator of the nematode community component, including plant-feeding, bacterial-feeding, fungal-feeding, omnivorous and carnivorous nematodes. Because carnivorous nematodes were found infrequently in our experiment, we included carnivorous nematodes in an omnivorous + carnivorous trophic group.

To characterize nematode community structure in each sample, we calculated nematode ecological indices as follows (Bongers 1990; Yeates, Wardle & Watson 1999): (i) taxa richness $R = (S-1)/\ln(N)$, where S is the number of genera, and N is the total number of nematodes; (ii) maturity index $MI = \Sigma v(i){\cdot}f(i)$ for non-plant-parasitic nematode families, where $v(i)$ is the colonizer–persister (c–p) of taxon i according to their r and K characteristics (Bongers 1990), and $f(i)$ is the frequency of taxon i in a sample; (iii) plant–parasite index $PPI = \Sigma v(i){\cdot}f(i)$ for plant-parasitic nematode families. The PPI is identical in concept to the MI but is based only on the life-history charac-

teristics of the plant-parasitic nematodes. MI and PPI are used to evaluate the functional responses of soil nematodes to resource and environmental change (Bongers 1990).

STATISTICAL ANALYSES

Statistical analyses were performed using the SPSS 17.0 software package (SPSS, Chicago, IL, USA). Mixed linear models across all response variables were performed using acid addition treatment, year and their interactions as fixed effects and block as random effects. One-way ANOVAs with Duncan's multiple-range tests were performed across all response variables to compare the acid addition effects for each rate in 2010 and 2011. The data of response variables were transformed with the natural logarithm before the analysis to improve normality. The response of variables to soil acidification was further examined using linear regression with acid addition rate treated as a continuous variable.

Structural equation modelling (SEM) was performed to analyse different hypothetical pathways that may explain soil acidification linkages to ecosystem functioning responses (Fig. S1). To facilitate our analysis and interpretations, we classified all variables into seven effect and response functional groups before SE models were evaluated. These groups include (i) soil acidification (Δ pH reduction); (ii) soil extractable Al^{3+} ions; (iii) soil properties (i.e. soil moisture, available phosphorus and extractable Ca^{2+}, Mg^{2+} and Na^{+}); (iv) N cycling (net N mineralization rate, soil NH_4^+-N and NO_3^--N); (v) soil microorganisms (PC1 of entire FAs, bacterial FAs, fungal FAs and the fungi/bacteria); (vi) soil nematodes (total nematode abundance, taxa richness, maturity index and PPI); and (vii) plant community (species richness and total community cover). Because variables in each functional group are often correlated, we used PCA to create multivariate functional indexes (principal components) for each functional group with multiple variables and each acidification intensity (Chen *et al.* 2013). For each functional group with multiple variables, the first principal component (PC1), which explained 42–86% of the total variance, was used in the subsequent SEM analysis (Table S1). Within each functional group, only variables that showed significant correlations with PC1 were used to explain the relationships between the functional groups.

Results

RESPONSES OF SOIL ABIOTIC PROPERTIES AND PLANT COMMUNITY

ANOVA and regression analyses showed that, for both sampling years, acid addition reduced soil pH but elevated Al^{3+} content (Table 1). Soil pH decreased across the acid addition gradient by 0.8–2.3 units in 2010 and by 1.1–3.5 units in 2011 (Table 1). Similarly, the content of Al^{3+} increased from 39 to 68 mg kg^{-1} in 2010 and from 37 to 83 mg kg^{-1} in 2011. With respect to soil base cations and available P, acid addition did not change the contents of Ca^{2+}, Mg^{2+} and Na^{+} but increased soil moisture and available P in 2010; in 2011, acid addition decreased the contents of Ca^{2+}, Mg^{2+} and Na^{+} and increased soil moisture and available P. For both years, acid addition decreased the rate of net N mineralization from 0.1 to −6.8 μg kg^{-1} day^{-1} and soil NO_3^--N from 3.4 to 0.7 mg kg^{-1} dry soil but increased soil NH_4^+-N from 1.3 to 25.9 mg

Table 1. Responses of soil pH, Al^{3+}, extractable P, soil moisture, extractable Ca^{2+}, extractable Mg^{2+} and extractable Na^{+} to acid addition in years 2010 and 2011

		Level of acid addition (mol m^{-2})										
Response variable	Years	0.00	2.76	5.52	8.28	11.04	13.80	16.56	Slope	r^2	*P* value	*F* value	*P* value
Soil pH	2010	7.3 (0.0)^{a}	6.5 (0.2)^{b}	6.2 (0.2)^{b}	5.8 (0.1)^{bc}	5.4 (0.2)^{cd}	5.0 (0.4)^{d}	5.0 (0.3)^{d}	−0.13	0.69	**< 0.01**	7.4	**< 0.01**
	2011	7.7 (0.1)^{A}	6.6 (0.2)^{B}	6.3 (0.2)^{B}	5.4 (0.3)^{C}	4.8 (0.1)^{CD}	4.2 (0.2)^{D}	4.2 (0.3)^{D}	−0.24	0.86	**< 0.01**		
Al^{3+} (mg kg^{-1})	2010	39 (1)^{c}	44 (1)^{c}	45 (2)^{c}	44 (2)^{c}	51 (3)^{bc}	59 (8)^{a}	68 (9)^{ab}	1.61	0.41	**< 0.01**	24.6	**< 0.01**
	2011	37 (2)^{D}	42 (2)^{D}	47 (6)^{CD}	60 (4)^{C}	72 (3)^{B}	93 (4)^{A}	83 (6)^{AB}	3.43	0.78	**< 0.01**		
Soil moisture (%)	2010	6.9 (0.3)^{c}	6.4 (0.3)^{c}	6.6 (0.2)^{c}	7.2 (0.3)^{c}	7.0 (0.5)^{bc}	8.3 (0.9)^{a}	8.2 (0.4)^{ab}	0.11	0.26	**< 0.01**	32.9	**< 0.01**
	2011	6.3 (0.1)^{E}	6.0 (0.2)^{E}	7.0 (0.6)^{DE}	7.7 (0.5)^{CD}	8.7 (0.5)^{C}	13.5 (0.7)^{A}	11.1 (0.5)^{B}	0.40	0.66	**< 0.01**		
P (mg kg^{-1})	2010	2.4 (0.1)^{c}	3.5 (0.2)^{bc}	3.8 (0.4)^{bc}	4.7 (0.2)^{bc}	5.5 (0.6)^{b}	8.1 (1.7)^{a}	8.0 (0.9)^{a}	0.36	0.59	**< 0.01**	201.2	**< 0.01**
	2011	3.3 (0.3)^{D}	4.7 (0.5)^{D}	8.1 (1.3)^{C}	13.0 (1.2)^{B}	15.7 (1.0)^{A}	18.5 (0.3)^{A}	18.1 (1.1)^{A}	1.03	0.87	**< 0.01**		
Ca^{2+} (mg kg^{-1})	2010	2500 (31)^{a}	2396 (85)^{a}	2602 (333)^{a}	2352 (94)^{a}	2352 (163)^{a}	2079 (121)^{a}	2322 (175)^{a}	−14.69	0.11	**0.07**	91.0	**< 0.01**
	2011	2484 (49)^{A}	1963 (61)^{B}	1788 (145)^{B}	1579 (313)^{BC}	1551 (154)^{BC}	1065 (93)^{D}	1182 (101)^{CD}	−76.84	0.61	**< 0.01**		
Mg^{2+} (mg kg^{-1})	2010	233 (25)^{a}	241 (34)^{a}	245 (35)^{a}	228 (25)^{a}	222 (19)^{a}	242 (29)^{a}	235 (22)^{a}	−0.16	0.01	0.93	2.1	0.17
	2011	313 (7)^{A}	263 (5)^{B}	234 (8)^{BC}	207 (10)^{CD}	183 (9)^{DE}	151 (5)^{F}	180 (18)^{EF}	−8.70	0.76	**< 0.01**		
Na^{+} (mg kg^{-1})	2010	34 (4)^{a}	37 (4)^{a}	35 (7)^{a}	34 (4)^{a}	32 (3)^{a}	32 (5)^{a}	31 (2)^{a}	−0.28	0.04	0.28	31.6	**< 0.01**
	2011	27 (6)^{AB}	32 (8)^{A}	18 (4)^{AB}	13 (3)^{AB}	18 (6)^{AB}	15 (4)^{B}	13 (3)^{B}	−1.02	0.19	**<0.01**		

Values are means of five replicate plots with SE in parentheses for each level of acid addition. Regression was performed using a linear model with acid addition level as a continuous predictor. A mixed linear model was conducted to determine the difference in variables between the two sampling years. Different superscript alphabets indicate significant differences among the levels of acid addition for each year (one-way ANOVA, $P < 0.05$). Mean values in the same row quoted with different superscript alphabets are different at the level $\alpha < 0.05$. Bold values indicate the statistical significance determined at $P < 0.05$.

kg^{-1} dry soil (Fig. 1). The responses of all variables except Na^+ to acid addition were greater in 2011 than in 2010 based on mixed linear models.

Plant species richness and total plant cover (used as a proxy for productivity) remained largely unchanged across the acid addition gradient in 2010 (Fig. 2), except for species richness at the highest acid addition rate (16.56 mol H^+ m^{-2}). In 2011, however, both species richness and plant cover declined with increasing acid addition rate (Fig. 2). Relative to the untreated control, species richness decreased by 2–14%, while plant cover did not change at low and moderate levels of acid addition (2.76, 5.52 and 8.28 mol H^+ m^{-2}). Species richness decreased by 22–53% and plant cover decreased by 29–66% at high levels of acid addition (11.04, 13.8 and 16.56 mol H^+ m^{-2}) compared with the control plots (Fig. 2). Plant richness was lower in 2011 than in 2010 based on a mixed linear model, while plant cover did not significantly differ between the 2 years (Fig. 2).

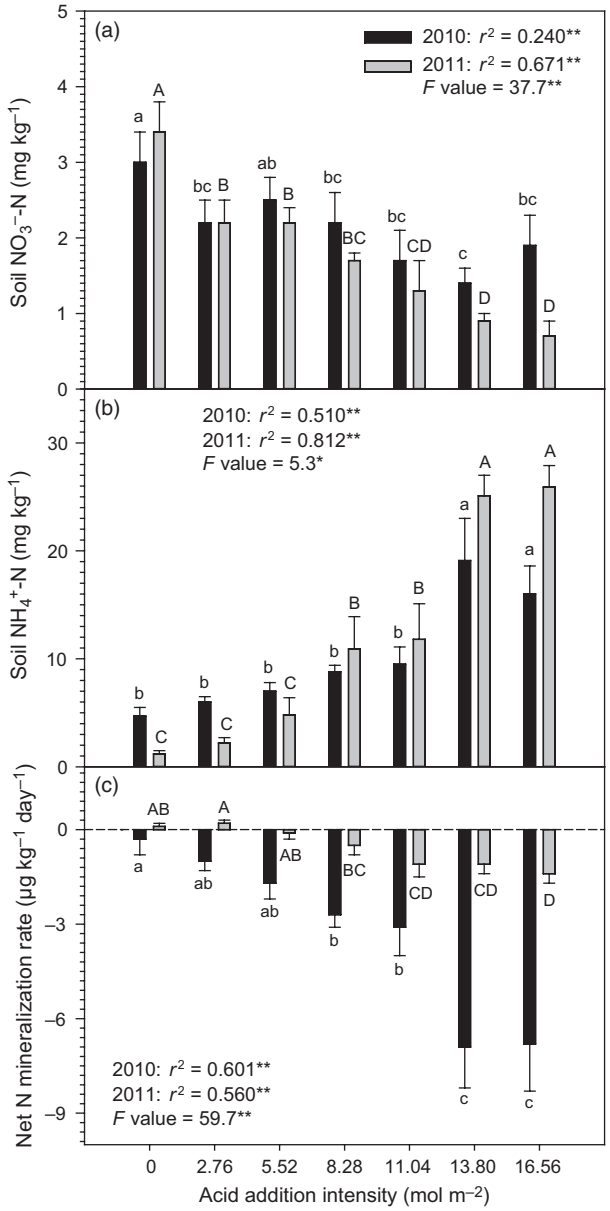

Fig. 1. Responses of soil nitrogen cycling (a–c) to acid addition level in years 2010 and 2011. (a) soil NO_3^--N; (b) soil NH_4^+-N; and (c) net N mineralization rate. Bars represent the means of five replicate plots (error bars denote SE). Regression was estimated each year using a linear model with acid addition level as a continuous predictor. Different letters indicate significant differences among the levels of acid addition (one-way ANOVA, $P < 0.05$). A mixed linear model was used to determine the difference in variables between the two sampling years (F value). Significant differences are reported as NS, $P > 0.05$; *, $P < 0.05$; and **, $P < 0.01$.

RESPONSES OF SOIL MICROBIAL AND NEMATODE COMMUNITIES

PC1 alone explained 47% of the total variance in the entire FAs in 2010 and 50% in 2011, indicating that the PC1 appeared to be sufficient to represent the soil microbial community structure (Fig. S2). In 2010, the relative abundances of 16:0, 16:1ω5, 16:1ω7c, a17:0, cy17:0 and 10Me18:0 dominated PC1 at low levels of acid addition,

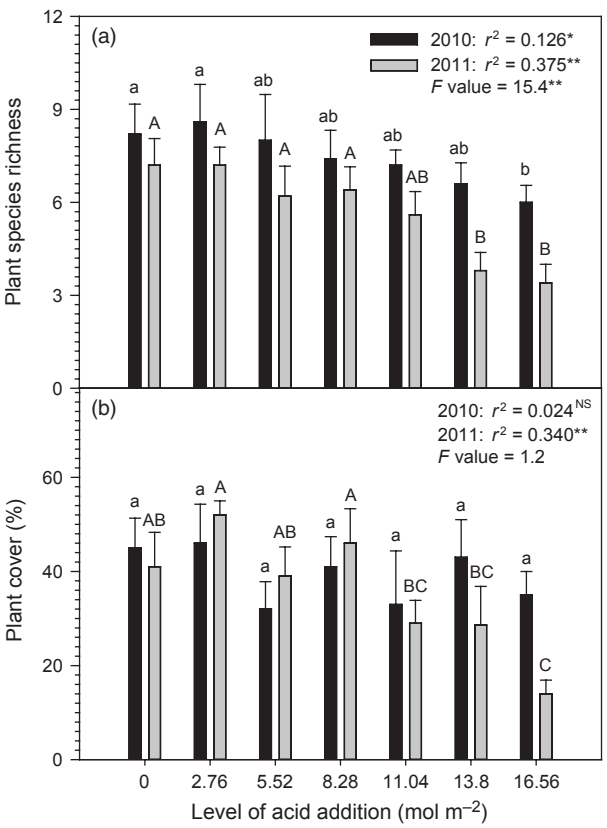

Fig. 2. Plant species richness (a) and community cover (b) responses to acid addition in years 2010 and 2011. Bars represent means of five replicate plots (error bars denote SE). Regression was estimated each year using a linear model with acid addition level as a continuous predictor. Different letters indicate significant differences among the levels of soil acid addition (one-way ANOVA, $P < 0.05$). A mixed linear model was used to determine the difference in variables between the two sampling years (F value). Significant differences are reported as NS, $P > 0.05$; *, $P < 0.05$; and **, $P < 0.01$.

and 14:0, a15:0, i15:0 and br17:0 dominated PC1 at high levels of acid addition (Fig. S2 and Fig. S3). In 2011, PC1 was dominated by i14:0, 16:1ω5 16:1ω7c, br17:0, 18:1ω9 and 20:00 at low levels of acid addition and by 14:0, a15:0, i16:0, i17:0, a17:0 and cy19:0 at high levels of acid addition (Fig. S2 and Fig. S3). The PC1 revealed that the PLFA patterns for both years were significantly correlated with different levels of acid addition rate, although the PLFA community structure in 2010 was different from that in 2011 (Fig. 3a).

ANOVA and regression analyses further confirmed that acid addition substantially altered the soil microbial community components in 2011 but not in 2010 (Figs 3b–e and S3). In 2010, acid addition did not affect total fatty acids, fungal fatty acids, bacterial fatty acids or fungi/bacteria ratio. While in 2011, acid addition significantly increased fungal fatty acids by up to 49% and fungi/bacteria ratio by up to 120% relative to control plots, and it decreased total fatty acids by up to 47% and bacterial fatty acids by up to 40%. Total fatty acids, bacterial fatty acids and fungal fatty acids were less abundant in

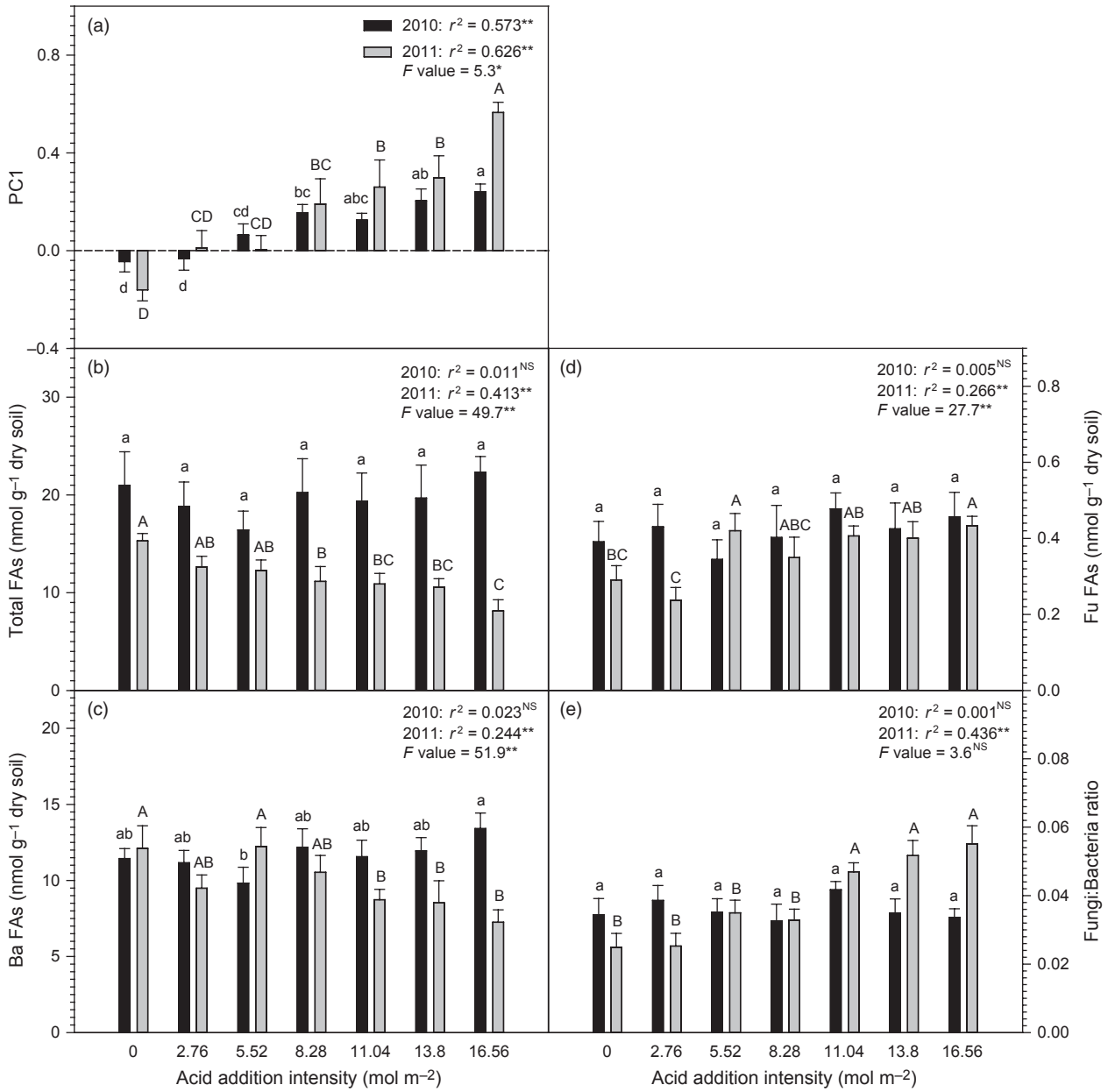

Fig. 3. Responses of soil microbial community (a–e) to acid addition in years 2010 and 2011. (a) PC1 of entire FAs; (b) total FAs; (c) bacterial FAs; (d) fungal FAs; and (e) fungi/bacteria. Bars represent the means of five replicate plots (error bars denote SE). Regression was estimated each year using a linear model with acid addition level as a continuous predictor. Different letters indicate significant differences among the levels of soil acid addition (one-way ANOVA, $P < 0.05$). A mixed linear model was used to determine the difference in variables between the two sampling years (F value). Significant differences are reported as NS, $P > 0.05$; *, $P < 0.05$; and **, $P < 0.01$.

2011 than in 2010, but the fungi/bacteria did not differ between the 2 years. The responses of soil microbial community to acid addition were greater at high levels of acid addition (11.04, 13.8, and 16.56 mol m^{-2}) than those at low and moderate levels of acid addition (2.76, 5.52, and 8.28 mol H^+ m^{-2}).

For both sampling years, soil acidification clearly altered the soil nematode community (Fig. 4 and Table S2). Acid addition increased the total number of soil nematodes due to an increase in all nematode trophic groups in 2010 and to an increase in bacterivores and fungivores but not in herbivores, omnivores or carnivores in 2011 (Fig. 4a–e). Nematode taxa richness in 2011 was decreased by acid addition but did not show a clear trend in 2010 (Fig. 4f). Acid addition increased the nematode maturity index in 2010 but decreased it in 2011 (Fig. 4g). For both years, acid addition decreased the PPI (Fig. 4h). Total nematode numbers was 49% lower and taxa richness was 50% lower in 2011 than in 2010 (Fig. 4). In general, greater responses of soil nematode community to acid addition were found at the high levels of acid addition in both years.

PATHWAYS DETERMINING SOIL MICROBIAL AND NEMATODE COMMUNITIES

Most variables or categories examined in this study were correlated with one another, making this data set appropriate for SEM analysis (Table S3). SEM analyses performed separately for each year supported the conclusion that soil acidification directly altered soil Al^{3+} ions and soil resources and base cations (soil moisture, available P, Ca^{2+}, Mg^{2+} and Na^+) in both years. In 2010, direct paths in our model from soil acidification directly to soil microbial community (increase in PC1-score of community structure) and nematode community (increases in total nematode abundance and maturity index and decrease in PPI) was observed (Table S3). Because of increases in soil moisture and available phosphorus and decreases in Ca^{2+} contents, model results suggest the soil resources pathway also affected the nematode community. In 2011, the changes in soil microbial community (increases in PC1-sore of community structure, fungal FAs and fungi/bacteria ratio and decrease in bacterial FAs) were apparently affected by both soil acidification and soil Al^{3+} ions pathways (Fig. 5). Model results suggest soil acidification also altered the nematode community, as indicated by increases in total nematode abundance and PPI and decrease in taxa richness and maturity index. Variations in nematode taxa richness, maturity index and PPI were related to pathways of soil acidification and soil resources (Fig. 5). In addition, there was a significant residual correlation between the soil microbial and soil nematode communities in both years (Fig. 5). Such residual correlations suggest some additional factors constraining responses (such as promotion of soil nematodes or a coevolution in responses).

PATHWAYS DETERMINING SOIL N CYCLING AND PLANT COMMUNITY

In 2010, the soil acidification, Al^{3+} ion content and soil microbial community pathway together explained 82% of the total variance in soil N cycling (Fig. 5). The increases in contents of H^+ and Al^{3+} ion and PC1-score of community structure were associated with decreases in the net N mineralization rate and soil NO_3^--N and increases in soil NH_4^+-N. In 2011, the increases in contents of H^+ and Al^{3+} ion along with changes in microbial community (via increases in PC1-sore of community structure, fungal FAs and fungi/bacteria ratio and decrease in bacterial FAs) and nematode community (increases in total abundance and parasite index and decreases in taxon richness and maturity index) explained 80% of the total variance in soil N cycling (Fig. 5).

Surprisingly, both soil acidification and soil Al^{3+} ions appear to have had no direct effect on the plant community in both years, independent of indirect pathways (Fig. 5). In 2010, observed changes in N cycling could explain about 26% of the total variance in plant community. The decrease in plant species richness could be mainly attributed to changes in soil N cycling (decrease in net N mineralization rate and increase in NH_4^+-N) (Fig. 5). In 2011, changes in N cycling and soil resources could explain about 68% of the total variance in plant community. The changes in the plant community (i.e. decreases in plant species richness and community cover) were mainly related to changes in soil N cycling (decrease in net N mineralization rate and increase in NH_4^+-N) and base mineral cations (decreases in Ca^{2+}, Mg^{2+} and Na^+).

Discussion

EFFECTS OF SOIL ACIDIFICATION ON MICROBIAL COMMUNITY

Our study provides evidence that soil acidification–induced changes in pH and Al^{3+} are two key factors shaping the soil microbial community. First, soil acidification–induced changes in pH appear to exert a profound effect on the microbial community composition in this study. At individual microbial fatty acids level, our results showed that monounsaturated fatty acids 16:1ω5, 16:1ω7c, 17:1ω8 and 18:1ω9 decreased with decreasing soil pH, and i16:0, i17:0 and cy19:0 increased with decreasing soil pH. These findings are corroborated by previous studies in arable soil (Aciego Pietri & Brookes 2009; Rousk, Brookes & Bååth 2010b) and forest soil (Nilsson *et al.* 2007; Aliasgharzad, Mårtensson & Olsson 2010). At microbial functional group level, we found that a high level of acid addition favoured fungal fatty acid and determined the total and bacterial fatty acids, which are consistent with previous studies in temperate grassland (Grayston *et al.* 2001) and boreal forest (Högberg, Högberg & Myrold 2007). However, the response of total fatty acids, fungal fatty acids and fungi/bacteria in our experiment was inconsistent with those in the Hoosfield acid strip (arable soil) in a long-term liming experiment (pH 4.0–8.3), which documented only small effects of soil pH on total fatty acids, fungal fatty acids and fungi/bacteria (Rousk, Brookes & Bååth 2009). The different results in our experiment vs. those in the Hoosfield acid strip experiment could be due to the following: (i) in the Hoosfield acid

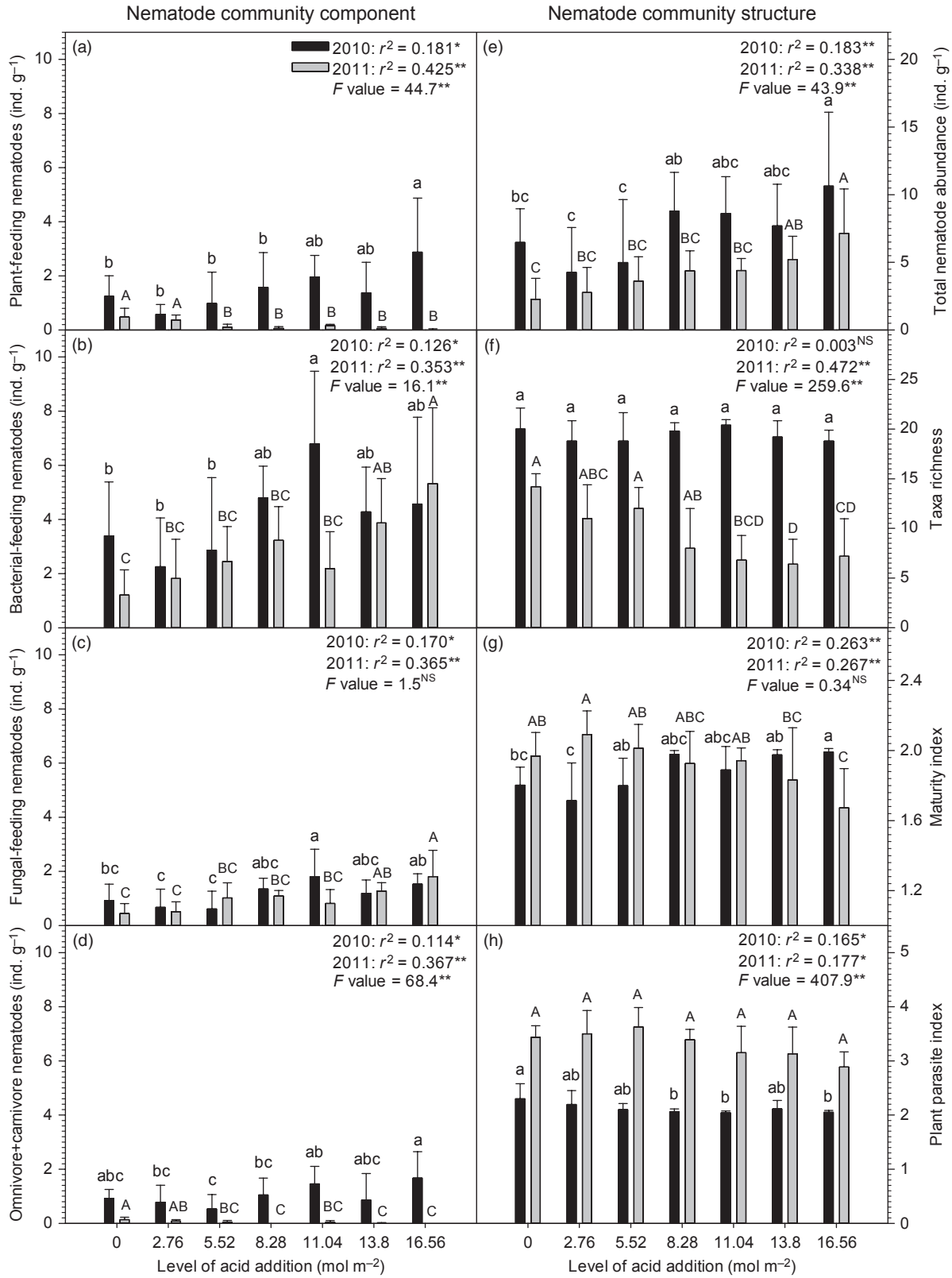

Fig. 4. Responses of soil nematode community components (a–d) and structure (e–h) to acid addition in years 2010 and 2011. (a) plant-feeding nematodes; (b) bacterial-feeding nematodes; (c) fungal-feeding nematodes; (d) omnivorous + carnivorous nematodes; (e) total nematode abundance; (f) taxa richness; (g) maturity index; and (h) plant–parasite index. Bars represent means of five replicate plots (error bars denote SE). Regression was estimated each year using a linear model with acid addition level as a continuous predictor. Different letters indicate significant differences among the levels of soil acid addition (one-way ANOVA, $P < 0.05$). A mixed linear model was used to determine the difference in variables between the two sampling years (F value). Significant differences are reported as NS, $P > 0.05$; *, $P < 0.05$; and **, $P < 0.01$.

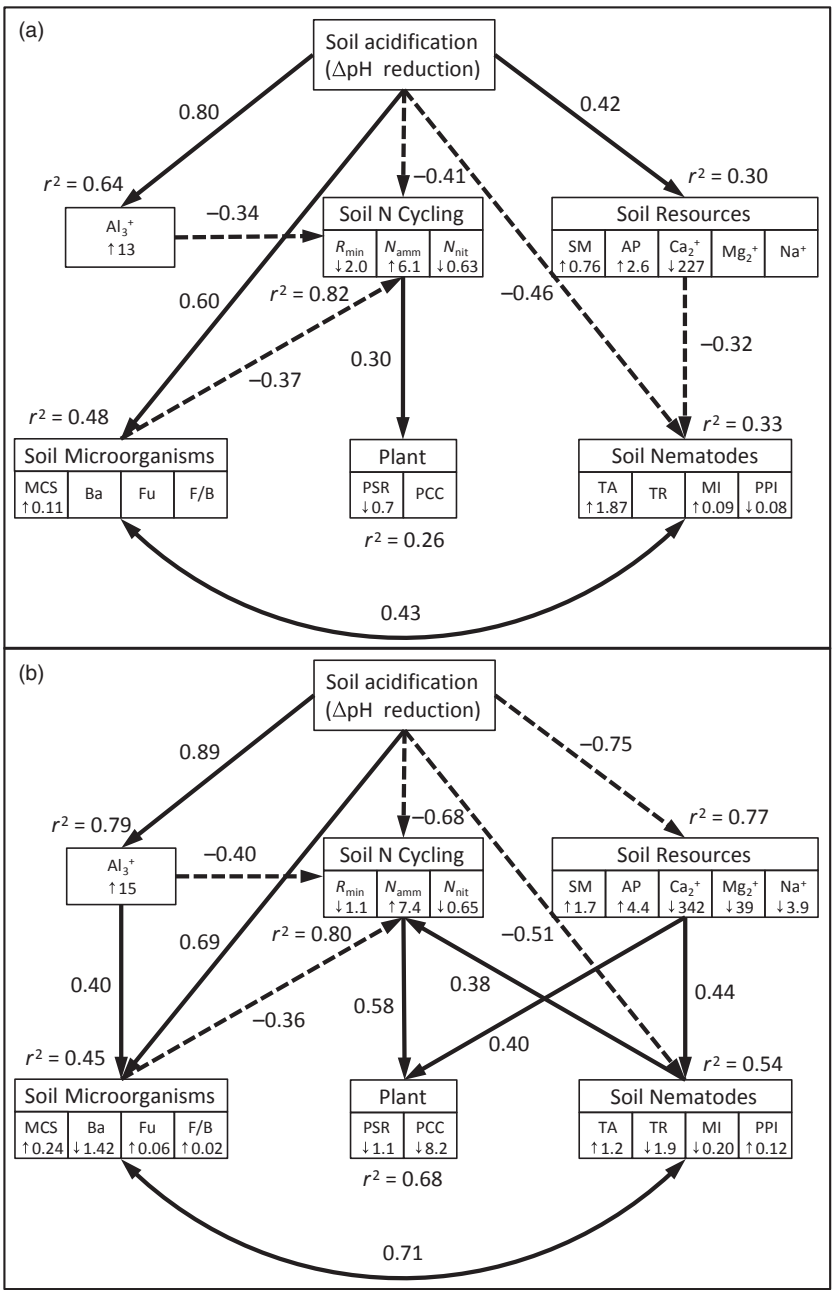

Fig. 5. Structural equation model analysis (SEM) examining the effects of soil acidification on the plant community via pathways of soil resources and soil communities in year (a) 2010 and (b) 2011. Square boxes indicate variables included in the model: Δ pH reduction, $pH_{control} - pH_{treatment}$; the ion of Al^{3+}; soil resources and base cations includes soil moisture (SM), available phosphorus (AP), extractable Ca^{2+}, extractable Mg^{2+} and extractable Na^{+}; soil N cycling includes the net N mineralization rate (R_{min}), soil NH_4^+-N (N_{amn}) and soil NO_3^--N (N_{nit}); the soil microorganisms includes microbial community structure (MCS), bacteria (Ba), fungi (Fu) and the fungi/bacteria (F/B) as indicated by phospholipid fatty acids analysis; the soil nematodes includes total nematode abundance (TA), taxa richness (TR), maturity index (MI) and plant–parasite index (PPI); the plant community includes plant species richness (PSR) and plant community cover (PCC). The symbols '↑' and '↓' indicate a significant increase or decrease, respectively, in the response of the variables to Δ pH reduction. The number in each square box indicates the response to Δ pH reduction (slope of linear model with Δ pH reduction as a continuous predictor, e.g., for Al^{3+}, mg kg^{-1} per unit pH). Results of model fitting: (1) 2010: χ^2=0.222, P = 0.637, d.f. = 1, n = 35; (2) 2011: χ^2 = 1.869, P = 0.172, d.f. = 1, n = 35 (Note high P-values associated with χ^2 tests indicate *good* model fit to data; i.e., no significant discrepancies). Solid and dashed arrows indicate significant positive and negative effects ($P < 0.05$), respectively; the pathways without significant effects did not show ($P > 0.05$). r^2 values associated with response variables indicate the proportion of variation explained by relationships with other variables. Values associated with solid arrows represent standardized path coefficients.

strip, the acidic original soil is limed by (application of base cations) and dominated with fungi biomass, whereas in our experiment, the alkaline original soil is acidified (application of protons) and dominated with bacteria biomass. Once the pH was changed, these two pathways could be quite different in terms of responses by soil microorganisms due to the fact

that bacteria could exhibit narrow optimal-pH range while wide optimal-pH range for fungi (Rosso *et al.* 1995); (ii) barley had been grown on the Hoosfield acid strip, resulting in stable productivity and a stable plant community. In contrast, the chance for soil acidification to affect the plant community and thereby the microbial community might be greater at our semi-arid grassland site than that at the Hoosfield acid strip due to the underlying plant–soil feedback interactions (Putten *et al.* 2013).

Secondly, soil acidification leads to an increase in Al^{3+} concentration, which has been considered to be the main factor inhibiting soil microbial biomass and structure (Piña & Cervantes 1996; Illmer, Obertegger & Schinner 2003; Joner *et al.* 2005). In this study, we found that low Al^{3+} concentration of 37–60 mg kg^{-1} (0.9–1.5 p.p.m.) at low levels of acid addition (2.76, 5.52 and 8.28 mol H^+ m^{-2}) did not change soil microbial community structure and composition (e.g. total fatty acids, bacterial fatty acids, fungal fatty acids and fungi/bacteria ratio) in the semi-arid Inner Mongolia grassland with soils of alkaline origin. However, high Al^{3+} concentration of 51–83 mg kg^{-1} (1.3–2.1 p.p.m.) at high levels of acid addition (11.04, 13.8 and 16.56 mol m^{-2}) decreased total fatty acids and bacterial fatty acids but increased fungal fatty acids and fungi/bacteria ratio, particularly in the third year (2011). Our findings are consistent with previous research suggesting that, below pH 5, total microbial biomass and bacterial biomass could be inhibited by Al^{3+} ions (>1.4 p.p.m.) in forest soil (Piña & Cervantes 1996; Joner *et al.* 2005). Similarly, Rousk *et al.* (2010a) found that, below pH 4.5, the universal inhibition of all microbial variables derived from increased release of free aluminium. Additionally, the decrease in bacterial fatty acids but increase in fungal fatty acids and fungi/bacteria ratio in the third year could be ascribed to that the fungi was more tolerant to Al^{3+} and H^+ ions over bacteria (Piña & Cervantes 1996; Kuperman & Edwards 1997).

EFFECTS OF SOIL ACIDIFICATION ON NEMATODE COMMUNITY

Our results demonstrate that the effects of soil acidification on nematode community differed substantially between the 2 years. In 2010, acidification had beneficial effects on all nematode trophic groups. In 2011, acidification had beneficial effects on bacterivorous and fungivorous nematodes but detrimental effects on plant-feeding and omnivorous + carnivorous nematodes. We also found that the responses of all trophic groups to acid addition were correlated with their responses to soil moisture in 2010. Such results and the fact that nematodes require free water to maintain activity and reproduce suggest that soil moisture could be a key factor influencing the abundance of soil nematodes in our semi-arid steppe. Previous studies have documented a positive relationship between soil moisture and total nematode abundance or the abundance of specific trophic groups, particularly the abundance of plant-feeding and bacterivorous nematodes (Todd, Blair & Milliken 1999). With the decrease in plant production in response to acid addition in 2011, plant-feeding nematodes were subjected to nutritional limitation but not moisture limitation. The decrease in omnivorous + carnivorous nematodes in 2011 was correlated with the decrease in soil pH, but this cannot be explained by a reduction in food supply because acidification increased bacterivorous and fungivorous nematodes in that year. Hence, the decrease might be explained by a direct detrimental effect of H^+ ions on omnivorous + carnivorous nematodes (Hyvönen & Persson 1990; Kuperman & Edwards 1997). The abundance of soil nematodes is less affected by Al^{3+} concentration than by H^+ concentration (Nagy 1999; Shao *et al.* 2012), although the abundance of omnivorous + carnivorous nematodes was also associated with Al^{3+} concentrations in present study.

Our experiment also demonstrated that soil acidification altered the soil nematode maturity index, nematode species richness and PPI. The negative influence of acid addition on taxa richness was due to a reduction in the number of genera of plant-feeding and omnivorous + carnivorous nematodes (data not show). Previous studies have confirmed that environmental stress can reduce soil community diversity (Nagy 1999; Suominen 1999; Bardgett *et al.* 2005) and fertilization can decrease the nematode maturity index (Suominen 1999). In the semi-arid grassland, our results with soil nematodes support the idea that soil acidification reduces below-ground diversity. In response to acid addition, the maturity index, which indicates the responses of soil nematodes to resource and environmental changes, increased in 2010 but decreased in 2011. This is easily explained by the increase in all nematode trophic groups, especially those in the higher c–p classes (e.g. omnivorous + carnivorous nematodes) with increasing acid addition in 2010, but they decreased in 2011. We found that soil acidification reduced the PPI, which is consistent with the view that a gradual decrease in plant primary production will decrease the abundance and diversity of plant-parasitic nematodes (Kuperman & Edwards 1997; Bardgett *et al.* 2005).

EFFECTS OF SOIL ACIDIFICATION ON N CYCLING

ANOVA and SEM analyses of the two sampling years indicate that the effects of H^+ and Al^{3+} ions were two major pathways whereby acidification can affect soil N cycling in the semi-arid steppe. The magnitude of the toxic effects of H^+ and Al^{3+} ions on N cycling was greater at the high levels of acid addition than that at the low to moderate levels of acid addition. The toxic effects were expressed in three ways, including inhibition of N mineralization, accumulation of exchangeable NH_4^+-N and shutdown of exchangeable NO_3^--N nitrification in the topsoil. This can be easily explained because nitrification was inhibited at low pH or high Al^{3+} concentrations and, hence, the transfer rate of soil NH_4^+-N to soil NO_3^--N was inhibited (Van Den Berg *et al.* 2005; Aciego Pietri & Brookes 2008). As a consequence, soil NH_4^+-N produced from the mineralization of soil organic matter can be expected to accumulate.

Our results indicate N cycling was also affected by interactions between the soil microbial community and the nematode community, although the effect is estimated to be less

than the effects of H^+ and Al^{3+} ions. This is reasonable, given the recognized importance of soil microorganisms for N cycling and other ecosystem processes (Van Der Heijden, Bardgett & Van Straalen 2008; Bardgett & Wardle 2010). Soil acidification decreased total and bacterial fatty acids, which are the most abundant and important groups of net mineralization associated with grasses (Van Der Heijden, Bardgett & Van Straalen 2008). In addition, the soil nematode community structure, but not total nematode abundance, also affected soil N cycling via the changes in taxa richness, the maturity index and the PPI. Soil nematodes generally stimulate N mineralization directly by the excretion of N and indirectly by grazing on soil microorganisms, thereby stimulating microbial turnover and production (Van Der Heijden, Bardgett & Van Straalen 2008). Approximately 30% of total net N mineralization is due to the direct effects of soil fauna (Freckman 1988; Ferris *et al.* 1998; Djigal *et al.* 2004).

MECHANISMS UNDERLYING SOIL ACIDIFICATION–INDUCED CHANGES IN PLANT COMMUNITY

Our results support the conclusion that the soil acidification–induced decreases in plant species richness and plant cover (a proxy for productivity) result from indirect effects on soil N cycling and soil base mineral cations rather than direct toxicity of soil H^+ and Al^{3+} ions to plants. Many studies have documented that soil acidification increases Al^{3+} concentrations and that high Al^{3+} concentrations can be directly toxic to plants (Kochian 1995; Van Den Berg *et al.* 2005). In our experimental site, however, the toxic effects of the H^+ and Al^{3+} ions pathways did not directly contribute to the observed decrease in biomass production and plant species richness. This is perhaps because the Al^{3+} concentrations in the present study (1–2 p.p.m.) were too low to damage living root development (0–20 cm deep) based on our measurement using soil core in the third sampling year (Lan unpubl. data). As reported in previous research, soil acidification reduced the availability of base mineral cations (Ca^{2+}, Mg^{2+}, and Na^+) and inhibited plant uptake of mineral cations, leading to a loss of acid-sensitive species (Kochian 1995; Van Den Berg *et al.* 2005; Bowman *et al.* 2008). Loss of base mineral cations, in particular Ca^{2+}, has been implicated in the declining health of plants subjected to acid deposition and has been linked to increased susceptibility to other stresses (e.g. Al^{3+} toxicity and soil eutrophication) (Kuperman & Edwards 1997; Poschenrieder *et al.* 2008).

A change in soil N cycling was one of the primary factors associated with a reduction in plant species and plant productivity. We believe this reduction probably occurred because of the reduced availability of N caused by the reduced rate of N mineralization and the shutdown of nitrification. The availability of soil N and other nutrients greatly affects plant species diversity and biomass production, and this is especially true for the nutrient-poor semi-arid steppe in the current study. Our results are consistent with previous findings that plant communities are greatly affected by soil nutrients (Bai *et al.* 2010; van den Berg *et al.* 2011). In addition to reducing the supply of N to plants, the altered N cycle caused NH_4^+-N to accumulate in soil, and NH_4^+ can be toxic to plants (De Deyn, Raaijmakers & Van der Putten 2004a; Van Den Berg *et al.* 2005).

Looking at the whole system, in both years, the responses of the soil microbial and nematode communities to acid addition were much stronger than that of the plant community. This was consistent with our inference that changes in the plant community induced by soil acidification resulted from changes in the below-ground communities (Van Den Berg *et al.* 2005; Bowman *et al.* 2008). That supports our decision to not include feedback by plants on the soil communities in our models (although such a feedback was documented in previous studies) (De Deyn *et al.* 2004b; Putten *et al.* 2013). That the soil microbial and nematode communities were more sensitive or responded more quickly than the plant community to soil acidification is, however, consistent with other reports concerning short-term responses to N deposition and other soil disturbances (De Deyn, Raaijmakers & Van der Putten 2004a).

IMPLICATIONS FOR ECOSYSTEM FUNCTIONING AND SERVICES UNDER GLOBAL CHANGE

Our findings show that soil acidification can substantially alter the soil microbial and soil nematode communities, which may in turn contribute to reduced plant species diversity and productivity. The declines of plant species diversity and productivity observed can be explained by changes in soil nutrients (N availability and base mineral cations), which appear to be regulated by the soil microbial and nematode communities, though not by the direct effects of soil H^+ and Al^{3+} ions. Researchers have predicted that future climatic change will increase the rate of N deposition (Vitousek *et al.* 1997). If anthropogenic acid deposition persists and without mitigation, our findings indicate that the semi-arid grassland soils on the Mongolia plateau would be acidified, leading to an altered below-ground community, a loss in biodiversity and a probable reduction in the ability of the grassland ecosystems to provide goods and services (Cardinale *et al.* 2012). In the context of global climate change and the potential for increased acid deposition, our findings have important implications for understanding the effects of soil acidification on ecosystem services and species diversity in the Inner Mongolia grassland and beyond.

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Supporting Information

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

Table S1. Results of a principal components analysis (PCA) of five groups (soil resources, soil N cycling, soil microorganisms, soil nematodes, and plant) in years 2010 and 2011.

Table S2. Responses of soil nematode taxa to acid addition in years 2010 and 2011.

Table S3. Bivariate correlations among Δ pH reduction, Al^{3+}, and PC1 of five groups included in the SEM model in years 2010 and 2011.

Figure S1. Conceptual model of the effects of soil acidification on soil microbial and nematode communities and linkages to changes in the plant community.

Figure S2. The soil microbial community structure from principal component analysis (PCA) based on the relative mole abundances of the entire fatty acids in years (a) 2010 and (b) 2011.

Figure S3. Influence of soil acid addition on the individual fatty acids in years 2010 and 2011.