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Intra- and Inter-annual Variation of Soil Microbial and Enzymatic Response to Water and Nitrogen Addition in a Chinese Semi-arid Steppe

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The knowledge about the effects of precipitation change and N deposition on soil microbe and enzymes is still limited for now. A field experiment was conducted with water- and N-addition treatments in a Chinese semi-arid grassland, and the dynamic changes of invertase, β-glucosidase, urease, dehydrogenase and substrate-induced respiration were monitored in 2011and 2012 to investigate the response of soil enzymes and microorganisms to increasing precipitation and N deposition. The results showed that the tested enzymes and substrate-induced respiration had significant intra- and inter-annual variations. The tested enzyme activities peaked at the beginning of the growing season in 2012, when the soil inorganic N was lower than that in 2011, but the substrate-induced respiration was not synchronous with the enzymes. Water addition had negative effect on the enzymes and substrate-induced respiration. N addition improved the activities of C-acquiring enzymes but decreased the activity of N-acquiring enzyme in the lower-precipitation year, while stimulated all four enzymes in the higher-precipitation year. There was a significant interaction between water addition and N addition on soil enzymes and microorganisms. This study highlighted that the inter-annual fluctuation of climates could be a non-ignorable interference to the effects of water and N treatments on soil extracellular enzymes and microorganisms.

Key words: Water addition, N addition, enzyme, substrate-induced respiration, intra/inter-annual variation.

Nitrogen (N) deposition has increased dramatically due to anthropogenic activities since the industrial revolution[1,2]. The rate of N deposition has far exceeded its natural rate, even up to an order of magnitude in large regions of the world[2]. Meanwhile, variation in global and regional precipitation regime has also been predicted by many climate models[3, 4]. As primary factors for the living of microorganisms, the changes in the N and water inputs in ecosystem would inevitably influence the microorganism-driving carbon (C) and N cycles in soil, and bring feedbacks to global changes. Therefore, researches on changes of the ecosystem processes, especially those of soil processes, under predicted scenario of N and water inputs are crucial for us to better foresee the future terrestrial C and N balance and provide data support for global change forecasting models.

As the key participants and regulators of nutrient cycle, soil microorganisms and extracellular

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enzymes are important driving forces of terrestrial C and N cycles, and are very sensitive to the change of environmental factors such as precipitation and N deposition[5]. The effects of environmental factors on C and N cycles are achieved generally through influencing amounts and activity of soil microorganisms and extracellular enzymes. The responses of soil extracellular enzyme activity (EEA) have been widely studied under different water[6,7] or N conditions[8,9]. It has been reported that water addition could promote EEA in water-limited soil, generally due to the direct effects though enhancing diffusion of soil enzymes and substrates[10] and the indirect effects through improving microbial metabolism and substrate input from plant[11]. However, water addition could also negatively influence soil microbes and extracellular enzymes[12]. It has been documented that β-glucosidase activity is lower in soil with higher moisture[13]. Experiments conducted by Sinsabaugh *et al.*,[14] and Henry *et al.*,[15] have shown that increasing N input depresses soil polyphenol oxidase activity while stimulates the activity of soil polysaccharide-degrading enzymes and phosphatase, which are attributed to the increasing C demand by microbes. It has also been reported that N addition could affect soil N-acquiring EEA positively[16], negatively[17] and neutrally[18], depending on the background nutrient level of soil[14, 19], as well as the plant-microbe nutrient competition and quality and quantity of litter input[20].

Generally, the influences of water and N addition on soil EEA are inconsistent in various studies, because they were subject to different soil properties, plants growths, local climates, and other environmental factors. Moreover, environmental changes do not exist independently, but interact with each other in reality, which should be taken fully into consideration by researches of global changes[21, 22]. The changes in N deposition and precipitation changes also interact. But till now, attentions have been mostly paid to the interactive effects between N deposition and precipitation/water changes on the aboveground plant[23, 24], while seldom on the soil microbial ecological processes. Studies about the influence of N- and water-input changes on soil EEA and microorganisms are critical for us to reveal the mechanism of terrestrial ecosystem responses and feedback to global changes.

Temperate semi-arid steppe is the most extensive terrestrial ecosystem in China, and the fragile eco-environment has made it very sensitive to climate change[25, 26]. In this study, a manipulative field experiment was carried out in the temperate semi-arid steppe of Northern China. Soil was sampled during growing seasons in two continuous years to determine the activity of four soil extracellular enzymes, including invertase (INV), β-glucosidase (GLU), urease (URE) and dehydrogenase (DEH), which were chosen because INV and GLU are considered as C-acquiring enzymes, while URE is N-acquiring enzyme, and activity of DEH provide the physiological state of the microbial community[27]. Soil substrate-induced respiration (SIR) was also determined to indicate soil microbial biomass[28]. It was hypothesized that water and N additions would stimulate EEAs and SIR, considering the deficiency of water and N in this area; furthermore, effects of water and N additions on EEAs and SIR would interact with each other. It was also supposed that soil EEAs and SIR under treatments would vary inter- and intra-annually. This study aimed to find out (1) the effects of water and N additions on soil microbial activity and EEAs, (2) whether there exists interactive effect between water and N on soil EEAs and microbial activity, (3) the intra- and inter-annual variations of the four extracellular enzymes and SIR under water and N addition, and (4) whether the intra- and inter-annual variations in climate influence the response of soil microbes and enzymes to experimental water and N additions.

MATERIALS AND METHODS

Site description

The experiment was conducted in the semi-arid steppe in Xilin River Basin, Inner Mongolia, China (43°26' - 44°39' N, 115°32' - 117°12' E, 1,265 m asl), and in the vicinity of the Inner Mongolian Grassland Ecosystem Research Station, Chinese Academy of Sciences. The soil was classified as chestnut soil (Chinese classification) or calcic-orthic aridisol (U.S. Soil Taxonomy Classification), with a particle composition of 60% sand, 21% clay, and 19% silt.

The climate condition in the area is moderate temperate monsoon climate with mean annual temperature of -0.4 °C. The monthly mean temperature ranges from -21.4 °C (January) to 18.5 °C (July). The mean annual precipitation is 350-450 mm, 70% of which falls between June and September. The dominant populations of plants are *Leymus chinensis*, *Stipa grandis*, *Agropyron michnoi* and *Cleistogenes squarrosa*. The site was used for grazing prior to enclosure, with a grazing intensity of approximately 2.25 sheep per hectare.

Experimental design

The experiment plots were established in the selected *L. chinensis* steppe with plain topography and relatively homogeneous soil and plant. Forty plots (5 rows × 8 columns, 8 m × 8 m for each plot) were laid out in a 51 m × 78 m fenced area and separated by 1 m buffers. The fenced area also included marginal buffers (3.5 m). There were totally eight treatments composed by the combination of two water- and four N-addition levels, and five replications were prepared for each treatments. The replicated treatments were arranged using a randomized block design.

The two levels of water addition were zero (W0, the water control) and 15% surplus of average annual precipitation (W15, 51.7 mm·y^{-1}), according to the predictions of future increasing rate of precipitation at the end of the 21st century in North China[4,29,30]. Water addition was carried out during the local rainy season of each experimental year (from June to September), according to the rainfall proportion of each month versus that of the four months. In order to set each addition to the medium amount of the daily rainfalls in this area, the monthly amount was split into halves for two applications, except for only one addition in September. Water was evenly added into the plots using backpack sprayers.

The amount of N addition was decided on the basis of the current N deposition level and the future change of N deposition in the next 50 years[31]. The N additions were conducted using NH_4NO_3 with four levels, i.e. 0 kg N·ha^{-1}·y^{-1} (N0, the N control), 50 kg N·ha^{-1}·y^{-1} (N50), 100 kg N·ha^{-1}·y^{-1} (N100), and 200 kg N·ha^{-1}·y^{-1} (N200). The N applications were carried out in the rainy season of late June and early August (late July for 2012) every year, considering that the main pulsing N-inputs in this area are often along with rainfall[32]. The amount of each N addition was a half of the N treatment level.

Soil and plant biomass sampling

Soils were sampled on May 12, June 21, August 14 and September 20 in 2011 and May 17, June 19, August 6 and September 23 in 2012 from three plots of the five replications for each treatment. The second (in June) and the third (in August) samplings for each year were arranged before the first and after the second fertilizer application, respectively. Sampling would not be implemented until two days after the water additions (if there was any) and seven days after N additions to avoid the pulse effects of treatments. Six soil cores (3.5 cm diameter × 10 cm depth) were taken from each plot and mixed. Soil samples were transferred immediately to the lab in an icebox, sieved through 2 mm mesh, and then stored at 4 °C before analyses. The determination of soil enzyme and substrate-induced respiration were finished within two weeks. The plant biomass was calculated by adding the aboveground and underground plant biomass together. All the standing plants in a 1 m × 1 m quadrat, which was randomly selected in the plots, were clipped to determine the aboveground plant biomass. The underground plant biomass was measured through evacuating soil measured by 0.4 m × 0.4 m × 0.4 m in the quadrat and collecting the roots from the evacuated soil.

Precipitation data collecting

Precipitation data during the experimental years were collected from the National Meteorological Information Centre, China Meteorological Administration (daily record). The precipitation of each day between two contiguous sampling times was summed as monthly rainfalls. Considering that the durations between the successive two samplings were about a month, precipations of previous 30 days were summed up as the seasonal rainfall of the first sampling.

Soil physiochemical properties

Soil moisture (Mois) was determined by oven-drying at 105 °C for 24 h. Soil inorganic N (SIN, including NH_4^+ and NO_3^-) was extracted from the 10 g of fresh soil with 50 ml of 2 M KCl and determined using a continuous-flow ion auto-analyzer (Bran and Luebbe, Norderstedt, Germany). Total soil organic carbon (TOC) was determined by dry combustion (Vario TOC cube, Elementar, Hanau, Germany).

Soil extracellular enzyme activity and substrate-induced respiration

Invertase activity (INV) was assayed using 15 ml of 8% sucrose as substrate. The sucrose solution, along with 5 ml of phosphate buffer (pH 5.5) and toluene, was added with 5 g of soil, then they were incubated at 37 °C for 24 h. After the filtrate of the soil suspension reacted with 3,5-dinitrosalicylic acid, the filtrate was colorimetrically determined at 508 nm. The result was shown as μg glucose·g^{-1}·h^{-1}.

Activity of β-glucosidase (GLU) was determined by adding 1 g of soil with 0.25 ml of toluene, 4 ml of modified universal buffer (MUB) (pH 6.0), and 1 ml of 0.5 mol·L^{-1} p-nitrophenyl-β-D-glucoside (PNG) solution. The mixture was incubated at 37 °C for 1 h, and the enzymatic reaction was stopped by adding 1 ml of 0.5 mol·L^{-1} $CaCl_2$ and 4 ml of 0.1 mol·L^{-1} tris (hydroxymethyl) aminomethane buffer (pH 12). The absorbance at 410 nm was read using spectrophotometer. The activity of GLU was expressed as μg PNG·g^{-1}·h^{-1}.

Urease (URE) activity was also determined using colorimetric method. 5 g of soil was added with 10 mL of 10% urea solution, 20 ml of phosphate solution (pH 6.7) and 1 ml of toluene, and incubated at 37 °C for 24 h, and the released ammonium was determined through the indophenol blue method. The color density was read at 580 nm, and the result of urease activity was shown as mg NH_3-N·g^{-1}·h^{-1}.

Activity of dehydrogenase (DEH) was measured based on the spectrophotometric determination of triphenyl formazan at 485 nm, and the measurement was proceeded by adding 2 ml of 1% triphenyltetrazolium chloride to 5 g of soil, and incubating at 37 °C for 24 h. The activity of DEH was shown as μl H^+·g^{-1}·h^{-1}. All enzyme activities determination were carried in duplicate for each sample and expressed by the dry weight equivalent soil.

For soil substrate-induced respiration (SIR) measurement, a jar was injected with 10 ml of 0.1 mol L^{-1} NaOH solution, and 10 g of fresh soil mixed thoroughly with 60 mg of glucose was hanged in the jar using a doubly wrapped cloth bag. Then, the jar was sealed and incubated in darkness at 30 °C for 24 h. CO_2 released from soil was absorbed by NaOH solution and measured by titration.

Statistical analysis

Repeated-measures ANOVA (RMANOVA) was used to analyze the effects of treatments (water and N additions) and sampling time on soil and plant properties, soil EEAs, and SIR for each year using monthly data. Monthly data were averaged for each year to analyze the effects of water, N and year, using three-way ANOVA instead of RMANOVA because there was only two years' observation in this study. To get rid of the interaction between time and the treatments, two-way ANOVA was carried out to test the effects of water and N treatments for each month, and one-way ANOVA was used to determine the effect of N treatments under different water treatments to get rid of the interaction between water and N additions. All statistical analyses of ANOVAs were performed using SPSS 20.0 (IBM Corp., New York, USA). Redundancy analysis (RDA) was also carried out for each year to analyze the relationship between environmental factors (soil, plant and climate properties) and soil EEAs as well as SIR, using CANOCO software (version 4.5, Microcomputer Power, USA). Difference was considered significant when $p<0.05$ unless there was specific instruction.

RESULTS

Intra- and inter-annual variations

The rainfall across the whole growing-season of 2012 (424.9 mm) increased by 236.45% than that of 2011 (179.7 mm). Moreover, the monthly precipitations in 2012 were also larger than the corresponding monthly precipitations in 2011 (Fig. 1a). The seasonal mean aboveground and underground plant biomasses were also higher in 2012 (Fig. 1b, c), while the seasonal mean soil inorganic N content decreased by approximate 46% in 2012 comparing to that in 2011 (Fig. 1d). During the growing seasons, soil inorganic N increased from June to September in 2011, while it showed a decreasing trend in the growing season of 2012 (Fig. 1d). Aboveground biomass basically increased monthly for both years (Fig. 1b), and underground biomass also had a temporal increasing trend in 2011, while it was relatively higher in June than in other months of 2012 (Fig. 1c).

Soil EEAs and SIR also varied

Table 1. Significance (*p* values) of treatments and time (month and year respectively) and their interactions affecting on activity of soil extracellular enzymes and soil substrate-induced respiration

	DEH	INV	URE	GLU	SIR
2011 (based on RMANOVA)					
W	0.000	0.000	0.000	0.000	0.000
N	0.000	0.000	0.000	0.003	0.000
M	0.000	0.000	0.000	0.000	0.000
W × N	0.000	0.005	0.000	0.000	0.000
M × W	0.000	0.000	0.000	0.000	0.000
M × N	0.000	0.000	0.000	0.000	0.000
M × W × N	0.000	0.000	0.000	0.000	0.000
2012 (based on RMANOVA)					
W	0.000	0.000	0.000	0.000	0.000
N	0.000	0.022	0.041	0.069	0.000
M	0.000	0.000	0.000	0.000	0.000
W × N	0.000	0.000	0.000	0.023	0.024
M × W	0.000	0.000	0.000	0.000	0.000
M × N	0.000	0.000	0.000	0.000	0.000
M × W × N	0.000	0.000	0.000	0.000	0.000
2011-2012 (based on Three-way ANOVA)					
W	0.000	0.480	0.000	0.000	0.000
N	0.000	0.000	0.000	0.005	0.000
Y	0.000	0.000	0.000	0.000	0.000
W × N	0.000	0.001	0.000	0.000	0.000
Y × W	0.000	0.000	0.000	0.034	0.782
Y × N	0.000	0.000	0.000	0.028	0.028
Y × W × N	0.000	0.000	0.000	0.000	0.001

W: water addition, N: N addition, M: month, Y: year;
DEH: dehydrogenase, INV: invertase, URE: urease, GLU: β-glucosidase, SIR: substrate-induced respiration

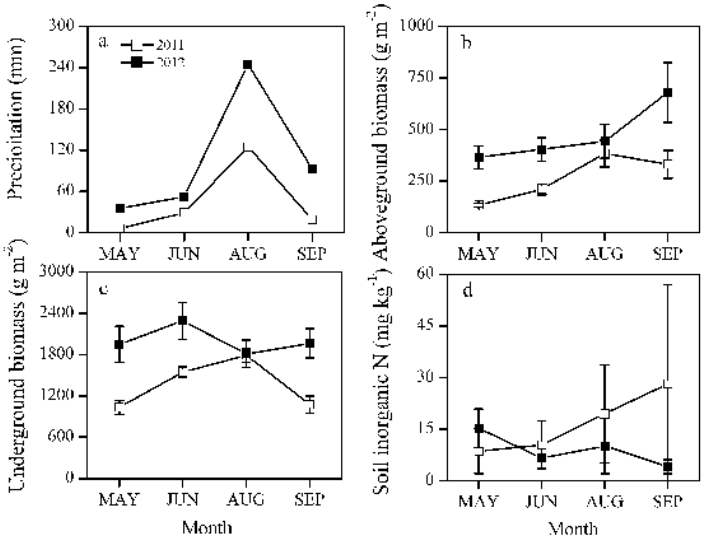

Fig. 1. Intra- and inter-annual variation of precipitation(a), plant aboveground (b) and underground (c) biomass, soil inorganic N (d)

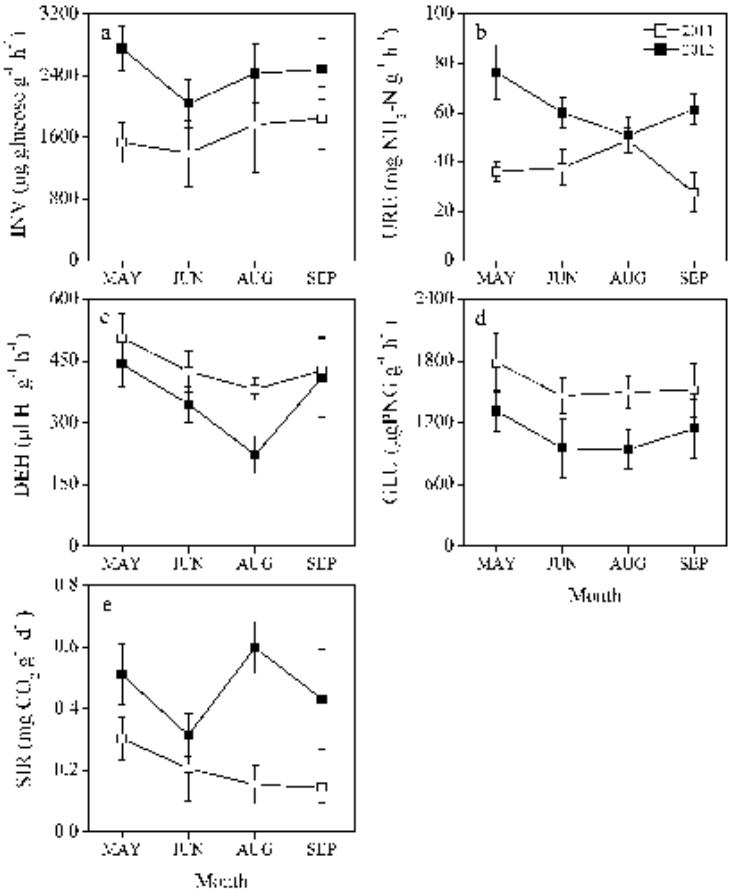

Fig. 2. Intra- and inter-annual variation of activities of invertase (a), urease (b), dehydrogenase (c), β-glucosidase (d) and substrate-induced respiration (e). INV: invertase, URE: urease, DEH: dehydrogenase, GLU: β-glucosidase, SIR: substrate-induced respiration

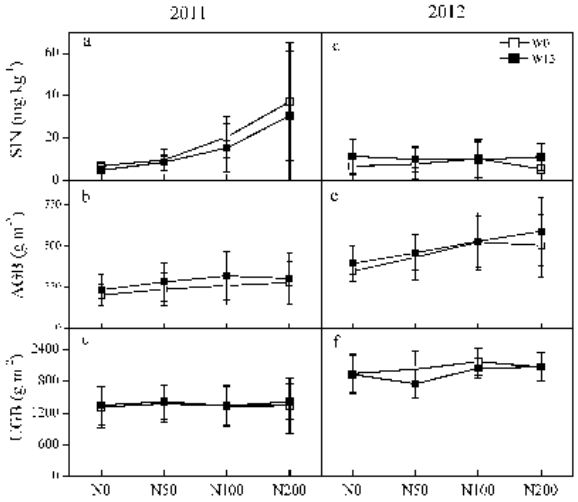

Fig. 3. Effects of water and N addition on soil inorganic N and plant biomass in 2011 (a-c) and 2012 (d-f). SIN: soil inorganic N, AGB: aboveground biomass, UGB: underground biomass, W: water addition, N: N addition

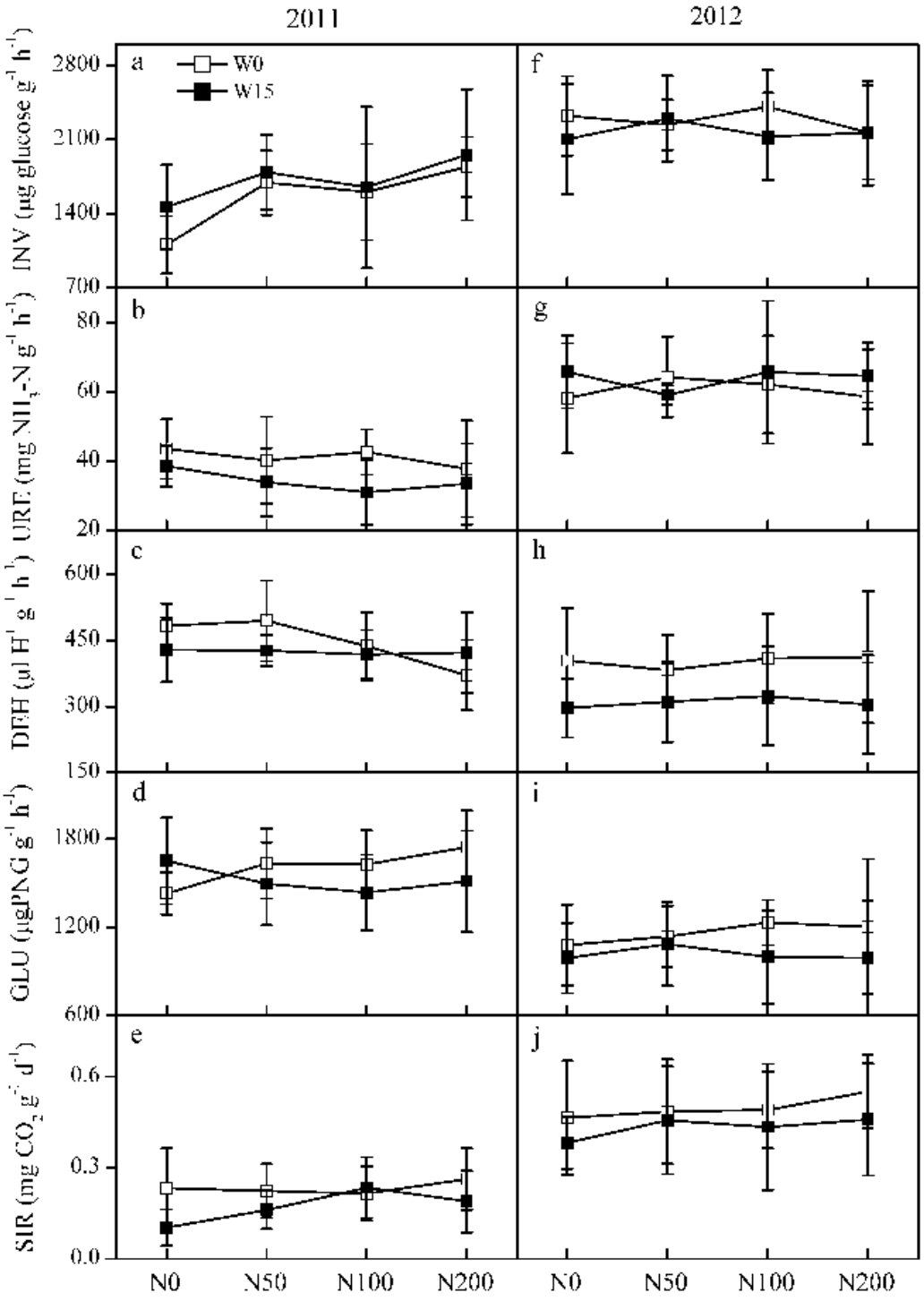

Fig. 4. Effects of water and N addition on soil enzymes and substrate-induced respiration in 2011 (a-e) and 2012 (f-j). INV: invertase, URE: urease, DEH: dehydrogenase, GLU: β-glucosidase, SIR: substrate-induced respiration, W: water addition, N: N addition

significantly between 2011 and 2012 (Table 1, $p<0.001$). The activities of invertase and urease, as well as soil SIR were significantly higher in 2012 (Fig. 2a, b, e), while the activities of dehydrogenase and β-glucosidase were significantly higher in 2011 (Fig. 2c, d). There also existed significant intra-annual variations of the soil EEAs and SIR in both years (Table 1, $p<0.001$). In 2011, activities of invertase and urease reached peaks in August (Fig. 2a, b), while dehydrogenase and β-glucosidase got their highest level in May (Fig. 2c, d). Nevertheless, in 2012, the four EEAs were all highest in May and lowest in August (Fig. 3a-d). Soil SIR in 2011 was also highest in May, while the highest level in 2012 occurred in August (Fig. 2e).

Treatments effects

Water addition decreased soil inorganic N in 2011 (Fig. 3a), while increased plant aboveground biomass in both years (Fig. 3b, e). N addition increased soil inorganic N in 2011 (Fig. 3a) and aboveground biomass in both years (Fig. 3c, d).

Generally, water and N additions significantly influenced soil EEAs and SIR (Table 1). Water addition decreased soil SIR and EEAs from the overall-two-years point of view, except for invertase which had insignificant response to water addition (Fig. 4). The RMANOVA results also showed negative response of soil EEAs and SIR to water addition in 2011 and 2012, with exceptions for invertase in 2011 and urease in 2012, which were insignificantly influenced and increased by water addition, respectively (Fig. 4).

For the treatment of N additions, activities of invertase and β-glucosidase and soil SIR showed similar responses to different levels of N additions from the overall view of the two years. They were all increased by the N addition, and the high N addition (N200) led to the highest activities of invertase and β-glucosidase and SIR (Fig. 4). However, activities of the other two enzymes, urease and dehydrogenase, were decreased by N addition and their activities were lowest at the high N addition (N200) (Fig. 4). For each year, responding patterns of soil SIR to N additions (Fig. 4 f, j) were all in accordance with the overall trend of the two years. The effects of N additions on EEAs in 2011 (Fig. 4a-f) also generally synchronized with those of the whole experimental period from 2011 to 2012, while the effects in 2012 showed a little difference. The results in 2012 showed that the four EEAs were all improved by N additions and the EEAs were the highest at the moderate level of N addition (N100) (Fig. 4f-j). Results of multivariate analyses indicated that there were significant interactive effects on soil EEAs between N addition and time (month and year) as well as between water and N additions (Table 1).

Related environmental factors

According to the results of redundancy analysis (RDA), the first two principal components accounted for 43.l% and 6.0% of the total variance

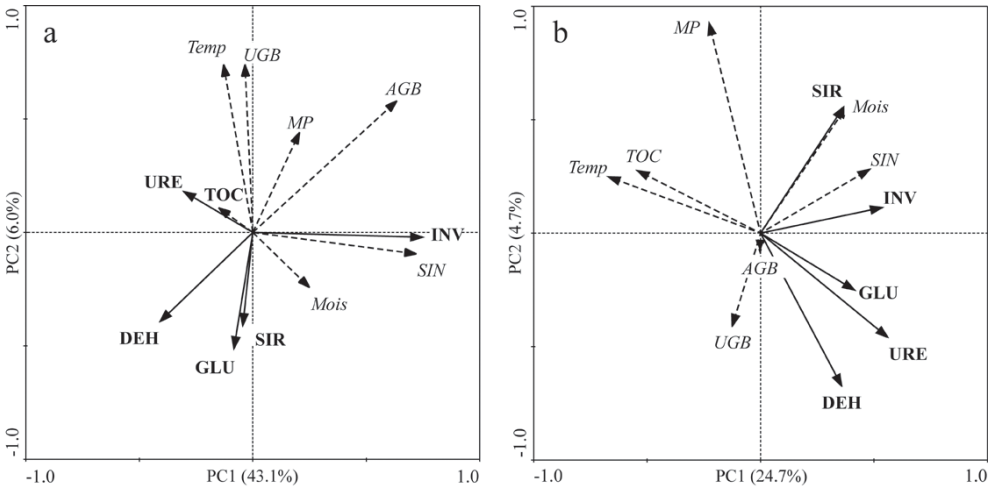

Fig. 5. Redundancy analysis of soil enzymes and substrate-induce respiration in 2011 (a) and 2012 (b). The solid arrows denotes enzymes (INV: invertase, URE: urease, DEH: dehydrogenase, GLU: β-glucosidase), and substrate-induced respiration (SIR). The dashed arrow denotes environmental factors (SIN: soil inorganic N, AGB: plant aboveground biomass, UGB: plant underground biomass, Temp: temperature, Mois: moisture, MP: monthly precipitation)

in 2011, respectively (Fig. 5a), and accounted for 24.7% and 4.7% of the total variance in 2012, respectively (Fig. 5b). Monte Carlo permutation test results showed that the change of soil inorganic N explained the most variation of soil EEAs and SIR in 2011 (23% of variability, p=0.002), and other variability predictors at significant levels in 2011 were soil moisture (12% of variability, p=0.002), plant aboveground biomass (9% of variability, p=0.002) and plant underground biomass (4% of variability, p=0.004). In 2012, factors that significantly affected the changes of EEAs and SIR were soil temperature (11% of variability, p=0.004), moisture (11% of variability, p=0.004) and monthly precipitation (4% of variability, p=0.016).

DISCUSSION

Significant inter- and intra-annual variation of soil EEAs and SIR

There were significant inter- and intra-annual variations in soil EEAs and SIR as hypothesized. The inter-annual variation pattern was different among the four soil EEAs. Activities of invertase and urease were higher in 2012, while those of dehydrogenase and β-glucosidase were higher in 2011. A similar situation among soil EEAs happened in the intra-annual variation pattern in 2011. Activities of invertase and urease peaked in August, while dehydrogenase and β-glucosidase were highest in May. From the results of correlation analysis based on the overall two years (date not shown), activities of invertase and urease were positively related to soil moisture, plant aboveground and underground biomass, and annual precipitation, while activities of dehydrogenase and β-glucosidase were negatively related to these environmental factors. Soil substrate-induced respiration was also found higher in 2012. The results indicated that the increased activities of invertase and urease in 2012 were due to the higher plant litter input and soil microbial biomass, which was caused by the higher precipitation this year. Previous studies have reported that soil EEAs could be stimulated by enhanced soil mineral N[17,33]. In this study, soil inorganic N got increased in August of 2011, which might make an important contribution to the increase of invertase and urease activities. However, the reason for the different temporal-variation patterns of the activities of dehydrogenase and β-glucosidase, which were higher in 2011 inter-annually and highest in May of 2011 intra-annually, was not very clear for now. The higher activities of dehydrogenase and β-glucosidase might indicate that the substrates of these enzymes were higher at the time[34].

Seasonal-variation patterns of soil EEAs in 2012 was different from those in 2011. In 2012, soil EEAs were all at their highest levels in May, while declined in August. This result was in agreement with some other studies[35, 36]. The reason for the lowest soil EEAs in August of 2012 was probably the soil N-limitation[34]. In this study, soil inorganic N remained low in August even after the N additions, and it probably could be attributed to the much higher precipitation in the growing season of 2012. On one hand, the greater precipitation in 2012 led to leaching of inorganic N, and on the other hand, higher precipitation simulated plant growth, resulting in more plant absorption of inorganic N. Therefore, the competition for N nutrient from plants in the aggravatingly N-limited soil would likely depress growth or/and activity of microorganisms and result in a suppressing effect on soil EEAs. On the other side of the above discussion, the results of stimulating effects in the relatively N-rich year and depressing effects in the relatively N-limited year also indicated that the studied soil was in N deficiency.

Soil SIR in 2011 also peaked in May, similar to the seasonal variation of dehydrogenase. However, in 2012, soil SIR peaked in August, different from the variation patterns of the EEAs. Soil SIR is an indicator of potential activity of microbe, and is closely related to soil microbial biomass[28]. The results indicated that soil microbial biomass, which was indicated by SIR, was not always synchronous with EEAs. The result agreed with former studies[9, 12] which indicated that changes in microbial enzymes production were independent to changes in microbial biomass. Soil extracellular enzymes were produced by soil microbes to be assistant in their growth[37]. However, extracellular enzyme in soil could be trapped by soil clay and aggregate or limited by the restriction of specific substrate availability[38,39], causing the asynchronization in the variation between soil

enzymes and microbial biomass/SIR.

Water and N addition effects on soil EEAs and SIR

Water addition generally decreased soil EEAs except for the insignificant invertase, which, however, was also decreased by water addition in 2012. The result was in contrary to the hypothesis. However, there were some evidences suggesting that water addition could attenuate soil EEAs by improving enzyme efficiency in water-stressed environment[15], which was similar to the water limited ecosystem in the experiment. Therefore, further researches are required to examine how the soil extracellular enzyme efficiency reacts to water addition in this area. The responding patterns of EEAs to water addition in each year was basically the same as the overall response of the two years, except for urease in 2012, which was increased by water addition. Urease is associated with N acquisition and reflects the microbial demands for N nutrients. Therefore, the increased activity of urease under water addition in 2012 probably indicated a shortage of N for soil microbes. The point was further proved by the decrease of soil inorganic N content in 2012 comparing to that in 2011, as well as the decrease of soil inorganic N by water addition. Water addition basically decreased soil SIR, which might result from the competition for nutrients between microorganisms and plants. Water addition improved plant growth, and enhanced nutrient competition with soil microbes. The RDA results also showed negative relationship between SIR and plant biomass.

The four soil extracellular enzymes showed different responses to N addition based on the overall analysis of the two years, Activities of β-glucosidase and invertase, including C acquisition, were increased by high level of N addition (N200), while activities of urease, related to N acquisition, and dehydrogenase were depressed by N addition. Same variation patterns also existed in 2011 based on yearly analyses. This result was in concert with many other reports[8, 15, 40]. N addition could increase the microbial demand for liable organic C, leading to the improvement of the polysaccharide-degrading enzyme activity[12, 15, 41], and hinder N cycling by reducing the activity of enzymes involved in organic N mineralization[17]. The result further provided an evidence for this popular viewpoint, which is mainly referred as the allocation theory[42,43]. In addition, N addition was found to decrease the activity of dehydrogenase, indicating a depression of soil microbial metabolic rates by N addition[17].

Contrasting to the different responses of the four EEAs to N addition in 2011, the responses were consistent in 2012, and the activities of the four EEAs were all improved by N addition. The positive effects of N addition on all the four enzymes might be attributed to the decrease of soil inorganic N in 2012. Hence, N addition ameliorated metabolic N stress for soil microorganisms and improved microbial production of enzymes[14, 44]. Soil SIR was improved by N addition not only in each year but also across the two years, suggesting a positive effect of N input for soil microbial biomass in this study, and further proving the N deficiency of the tested soil.

There was significant interactive effect between water and N additions on soil EEAs and SIR (Table 1). However, the results showed unstable and complex interactive effects between water and N additions on soil enzymes and microbial biomass (SIR). Taking invertase as an example (data not shown), N addition increased the activity of invertase by 159-206% without water addition and by 126-139% with water addition in August of 2011, suggesting that water addition decreased the affecting extent of N addition by averaged 30% approximately. The averaged decreasing effect by water addition had sustained but attenuated to 24% in September of 2011, while turned into a basically increasing effect at the beginning of the growing season in the second year, 2012. Moreover, the interactive effect on the invertase activity became unremarkable in August of 2012, after the N application for the second year. The reason for the complicated and changing interactive effects could be attributed to the interference by other factors, such as the effective duration of treatments, direct impact from seasonal and inter-annual climate conditions and indirect impact from the alteration of plant and soil properties. More researches are needed to explore the exact mechanisms of interaction between water and N additions.

Main environmental factors affecting soil EEAs and SIR

According to the results of RDA in 2011, soil inorganic N content and soil moisture, which were the factors directly affected by experimental

treatments, played the most important roles in affecting soil EEAs and SIR, and plant aboveground and underground biomass were also significant influencing factors. However, in 2012, factors significantly affecting the responses of EEAs and SIR were soil temperature, moisture and monthly precipitation, which were climate factors or the factors that were directly related to climate. The shift in the main regulating factors implied that responses of soil microbial activities and extracellular enzymes to experimental treatments were significantly influenced by inter-annual climate fluctuation. Other studies also reported that despite the significant treatment effects, the degrees of responses of soil EEAs and microbial activity to treatments were smaller comparing to the inter-annual variation of EEAs[9, 12]. Latest study conducted by Bell *et al*.[45] found that 25% shifts of precipitation in a desert grassland could induce significant changes in soil microorganisms and nutrient properties. In this study, shift of precipitation in the growing season of 2012 was up to 136% comparing to that of 2011, and the shift could lead to dramatic changes in soil microbial and enzymatic properties.

CONCLUSIONS

The results of this study suggested that biomass of soil microorganisms and activity of extracellular enzymes had significant intra- and inter-annual variations in the semi-arid steppe. However, changes of microbial biomass could be seasonally asynchronized with the activities of soil enzymes. The strong interference by inter- and intra-annual precipitation variations resulted in unstable and complex responses of soil microorganism and enzymes to water, N and their interactions. Nevertheless, there still existed significant water and N addition effects on soil microorganism and extracellular enzymes. Water addition generally decreased microbial biomass and enzyme activities, while N addition could stimulate microbial demands for carbon acquisition while suppress microbial N acquisition. This result provided an evidence for the theory that when the availability of a nutrient increases, soil microbes would reduce the amounts and activities of the enzymes that could catalyze substrates and produce the incremental nutrient.

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