Agriculture, Ecosystems and Environment 326 (2022) 107785

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Heavy rainfall in peak growing season had larger effects on soil nitrogen flux and pool than in the late season in a semiarid grassland

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ARTICLE INFO

Keywords:
Climate extremes
Grassland
N cycling
Seasonal timing
Soil functional gene

ABSTRACT

Increasing heavy rainfalls can strongly affect ecosystem nitrogen (N) cycling processes and thereby alter soil N fluxes and pools. However, the effects of heavy rainfalls on soil N fluxes and pools are poorly understood, particularly with regards to high rainfall timing under field conditions. We conducted a 3-year (2014–2016) manipulative experiment in which heavy rainfall was imposed in middle (plant peak growing stage) or late (plant senescent growth stage) growing season in a semiarid grassland of Inner Mongolia, China to explore the responses of N_2O fluxes and soil total N contents and the underlying microbial mechanisms. Mid-season heavy rainfall promoted soil N_2O emissions by 65% on average across the three years, attributable mainly to increases in denitrifying *nirK* and *nirS* abundances induced large denitrification at higher soil water contents. However, archaeal and bacterial *amoA* and *narG* genes did not change significantly due probably to counteracting effects of increased soil water content (positive) and soil pH (negative). Mid-season heavy rainfall led to 17% reduction in soil total N by the end of the last year of the study (2016), partly due to the enhanced accumulated N_2O emissions over the three years. In contrast, late-season heavy rainfall did not change N_2O emissions and soil total N contents even though soil water content, soil pH and *nirK* and *nirS* abundance were significantly increased, perhaps due to limitation by low temperature. Timing of the heavy rainfall events during the plant growing season strongly influenced their impacts on soil N fluxes and pools and heavy rainfalls in the peak stage of plant growth may potentially cause a positive feedback to global warming and exacerbate N limitation in terrestrial ecosystems.

1. Introduction

One of the characteristics of global climate change is the increase in frequency, intensity, and extent of heavy rainfalls (Donat et al., 2016; Fischer and Knutti, 2016; Jian et al., 2020), which can have profound impacts on ecosystem nitrogen (N) cycling processes, such as nitrification, denitrification, and resultant nitrous oxide (N_2O) emission (Greaver et al., 2016). Soil N_2O flux variation would further influence the size of soil N pool (Fowler et al., 2013). N_2O is the third most important anthropogenic greenhouse gas with a global warming potential 265 times that of carbon dioxide over a 100-year lifespan (Stocker, 2014). Soil N content was highly related to multiple ecosystem structure and functions, such as carbon uptake and microbial community (Yang et al., 2021; Widdig et al., 2020). As such, understanding the effects of heavy rainfalls on ecosystem N_2O flux and soil N pool is particularly important for assessing global warming potential and ecosystem functions.

Ecosystem N cycling processes are mainly driven by microorganisms. For example, each process in nitrification and denitrification is

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https://doi.org/10.1016/j.agee.2021.107785
Received 20 May 2021; Received in revised form 30 October 2021; Accepted 18 November 2021
Available online 25 November 2021

performed by a specialized group of nitrifiers and denitrifiers (e.g., ammonium-oxidizing and nitrite-reducing microorganisms) which contain specific functional genes (e.g., *amoA* and *nirK/nirS*) encoding corresponding oxido-reductase (e.g., ammonia monooxygenase and Cu-containing/haem-containing cd1 nitrite reductase) (Kuypers et al., 2018). Changes in precipitation are expected to fundamentally affect N_2O flux and/or soil N pool through directly altering the abundance and structure of nitrifiers and denitrifiers (J. Chen et al., 2017a; X. Chen et al., 2017b; Sun et al., 2018; Zhang et al., 2017, 2013), in addition to indirectly changing soil conditions, such as soil moisture, aeration, and redox potential (J. Chen et al., 2017a; X. Chen et al., 2017b; Patil et al., 2010; Sun et al., 2018). However, the microbial mechanisms of N_2O fluxes responses to precipitation variation are not well understood due to limited data of soil functional genes (gene markers for nitrifiers and denitrifiers) currently available (Z. Li et al., 2020b; L. Li et al., 2020b).

Recently, several meta-analysis studies suggested that increased precipitation promoted terrestrial N_2O emissions, but unchanged soil total N content based on current field manipulative studies at a global scale (Z. Li et al., 2020b; L. Li et al., 2020b; Yan et al., 2018). Note that most of these manipulative studies focused on the scenario that every precipitation event proportionately increased in magnitude over the growing season or whole year, rather than the scenario that abrupt heavy rainfall events within few days. Nevertheless, the two precipitation scenarios may have different ecological impacts (Reyer et al., 2013). Chronic increases in precipitation at a seasonal or yearly scale would produce better soil moisture conditions for nitrifiers and denitrifiers activity (Yang et al., 2018; Zhang et al., 2018). However, heavy rainfall-induced inundation may lead to soil water saturation and inhibit microbial activity, especially for aerobic nitrifiers. Additionally, chronic increases in precipitation may promote N mineralization and N availability, providing substrate for nitrification and denitrification processes, thus ultimately accelerating N_2O emissions (Liu et al., 2017). In contrast, dramatic heavy rainfalls are likely to cause flood and ecosystem N loss from topsoil via leaching and runoff (Greaver et al., 2016; Kasper et al., 2019), thereby potentially limiting production of N_2O. On the other hand, large precipitation events possibly induce abrupt N_2O emission pulse (Petrakis et al., 2017), in particular after a dry period (Groffman et al., 2009). In some cases, consistent high rainfalls can suppress N_2O emission (Rowlings et al., 2015). Hence, speculating heavy rainfall effects on soil N_2O emissions, based on past relatively long-term evenly increased precipitation manipulative experiments, may not be straightforward.

Previous limited studies have suggested that high intensity precipitation occurred in different periods of a season had discrepant impacts on multiple ecological processes (Craine et al., 2012). For example, experimental heavy rainfalls in late-growing season consistently reduced below-ground and total biomass while heavy rainfalls in mid-growing season had little effects in a semiarid grassland (Li et al., 2019). In another semiarid grassland, deluge at mid-season caused the greatest increase in soil respiration, aboveground net primary production and canopy greenness than those in early- and late-season (Post and Knapp, 2020). Jian et al. (2020) found that yield variability of early rice in China was negatively affected by the frequency of extreme precipitation from the end of flowering to doughty stages but not in other stages. Likewise, seasonal timing may also largely regulate heavy rainfall effects on soil N_2O emissions and soil N content. However, we still lack an understanding of whether responses of soil N_2O flux and N pool to heavy rainfalls depend on the seasonal timing.

Here, we conducted a 3-year field manipulative experiment in which heavy rainfall was respectively imposed in middle and late plant-growing seasons in a semiarid grassland of Inner Mongolia, China. The seasonal dynamic of N_2O fluxes, soil inorganic and total N content, soil functional genes were measured. The specific questions addressed in this study were: (1) what are the effects of heavy rainfall on N_2O fluxes and soil N pool? (2) what are the biological mechanisms for N_2O fluxes following heavy rainfalls? (3) how does the seasonal timing of an event heavy rainfall regulate its effects on soil N_2O fluxes and soil N pools?

2. Materials and methods

2.1. Study site

This study was conducted in a long-term fenced (since 1979) semi-arid grassland at the Inner Mongolia Grassland Ecosystem Research Station in the Xilin River Basin (43°20′ N, 116°40′ E, 1200 m a.s.l), Inner Mongolia, China. Mean annual air temperature (1953–2017) was 2.5 °C and mean annual precipitation was 281 mm, of which 86% (~ 242 mm) falls during the growing season from May to September. The soil is classified as dark chestnut in Chinese soil classification or Calcis-orthic Aridisol in US Soil Taxonomy, with 60% sand, 21% clay and 19% silt. The soil water content is 0.29 m^3 m^{-3} at field capacity and 0.12 m^3 m^{-3} at wilting point. The plant community was mainly dominated by perennial rhizome and bunch grasses, including *Leymus chinensis*, *Stipa grandis*, *Achnatherum sibiricum* and *Agropyron cristatum*.

2.2. Experimental design

In this study, different from the ETCCDI extreme precipitation indices calculated on a daily basis (Zhang et al., 2011), we considered heavy rainfall as uninterrupted rainfall over a multi-day period that induce multi-day flooding or waterlogging. Based on the long-term climate records (1953–2012) at this site, the longest continuous period with daily precipitation during the growing season was 20 d. The 99th percentile of total effective precipitation over any continuous 20 days within the growing season was 282 mm. Thus, we defined the heavy rainfall treatment at this site as 282 mm rainfall in total applied uniformly over 20 d (14.1 mm d^{-1}). The experimental design of our study was similar to that in the EVENT experiment conducted in central Europe, where heavy rainfall was defined as 170 mm rainfall over 14 days (Jentsch et al., 2009; Kreyling et al., 2008), and another study conducted in Russia (120 mm over 6 days, Koide et al., 2010). The heavy rainfall treatment was imposed in the middle or late growing season. Therefore, three treatments were involved in the manipulative experiment: ambient control (Control), heavy rainfall in middle growing season (HR-mid, middle heavy rainfall), and heavy rainfall in late growing season (HR-late, late heavy rainfall). The periods of HR-mid were from 17 June to 6 July in 2014, and from 27 June to 16 July respectively in 2015 and 2016. The periods of HR-late were from 20 August to 8 September in these three years.

There were three replicates for each treatment and twelve plots (2 m × 2 m each) randomly located in four blocks. To prevent potential water exchange across plot boundaries, metal sheets were installed around the plots to a depth of 40 cm, with 10 cm extended above the ground. Rain-exclusion shelters (3 m length × 3 m width, height 1.8 m) covered the treatment plots to prevent natural rainfall into plots during the heavy rainfall treatment periods. Heavy rainfall treatments at 14.1 mm per day were applied by hand using a sprinkling can and local groundwater, as described in detail by Li et al. (2019).

2.3. N_2O measurements

A chamber-gas chromatograph method was used to assess N_2O fluxes. Briefly, one square stainless-steel frame (50 cm length × 50 cm width, 10 cm height with 3 cm extending aboveground) with water groove on the upper surface of the edge was installed in each plot in May 2012. Gas samplings were conducted between 9:30 and 11:30 a.m., when the N_2O fluxes would represent the daily mean values in this grassland (Dong et al., 2000). During the measurements, a stainless-steel static chamber (50 cm length × 50 cm width × 50 cm height), covered with thick foam for heat insulation and fitted with two fans inside to mix the chamber air, was placed on top of the frame with the bottom edges inserted into the water groove. Water was added into the groove to prevent gas exchange across internal and external of chamber. Then, gas samples were taken from the headspace of the chamber after 0, 10, 20, 30, 40 min, successively. These samples were subsequently analyzed for N_2O concentration using a gas chromatograph equipped with an electron capture detector (Agilent 7890A GC System, Palo Alto, CA, USA). The N_2O flux was calculated as the slope of linear regressions from the measured gas concentrations with time. Positive and negative N_2O flux values represent net ecosystem emission and uptake, respectively (Li et al., 2016).

2.4. Measurement of soil physicochemical properties

Soil water content (SWC) in the top 20 cm depth and soil temperature (ST) at 10 cm below the surface were measured in situ by time domain reflectrometry probes (TDR 300, Spectrum Technologies, Inc. CST, USA) and soil thermometers (TL-883, Tonglixing technology Co., Ltd., China), respectively. SWC was measured approximately every 5 days in 2015 and 2016 and approximately every 10 days in 2014. ST was measured at the same time as N_2O measurement (Li et al., 2019).

Soil was sampled at the end of the growing season after the treatments were finished (around 24 September). Three soil cores (0–10 cm) were collected, using steel augers with a 3 cm diameter, from each treatment plot and then composited for each plot. All soil samples were sieved to ≤ 2 mm immediately. Subsequently, each soil sample was separated into two parts: one of them was air-dried for measuring pH and total N (TN), and the other was kept at − 20 °C in a freezer for functional gene abundance analysis and physicochemical properties analysis, including inorganic N content (SIN; sum of NH_4^+ and NO_3^-), dissolved organic carbon (DOC), and microbial biomass C (MBC) as detailed below.

Briefly, soil pH value was measured using a pH meter (STARTER 3100, Ohaus Instruments Co. Ltd. Shanghai, China) in a 1:5 dry soil-water suspension. TN was analyzed using the micro-Kjeldahl method. SIN was determined using a continuous flow spectrophotometer (AutoAnalyzer 3 System; SEAL Analytical GmbH, Norderstedt, Germany) after the soil samples were extracted with 0.5 M K_2SO_4. DOC and MBC was measured using the chloroform fumigation–extraction method. Briefly, a pair of fresh soils (10 g dry weight equivalent for each) were fumigated for 24 h with ethanol-free $CHCl_3$ and unfumigated as control, respectively. After that, both the fumigated and unfumigated soil samples were extracted by shaking for 30 min in 60 mL of 0.5 M K_2SO4. Then, the extracts were filtered and analyzed for DOC by a Total Organic Carbon Analyzer (Elementar vario TOC, Elementar Co., Germany). MBC was calculated as the differences between DOC contents in the fumigated and unfumigated control samples using a conversion factor of 0.45 (Li et al., 2020a).

2.5. Soil functional genes measurements

We used PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) to extract DNA from 0.30 g of soil, as described by suppliers. Then, the DNA solutions were diluted 1:3 with water for the subsequent polymerase chain reaction (PCR) and real-time PCR amplifications, to attenuate the potential effects of PCR inhibitors.

Copy numbers of archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS*, *narG*, and nosZ genes were quantified using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with the degenerated primers (Table S1). The reaction mixtures (20 μL) were comprised of template DNA (1 μL, DNA, cDNA, or serially diluted standards), Maxima™ SYBR Green (10 μL) or ROX (2 ×, Thermo Fisher Scientific, Waltham, MA, USA), forward primer (0.5 μL, 20 μmol L^{-1}), reverse primer (0.5 μL, 20 μmol L^{-1}), and nuclease-free water (8.0 μL). We used plasmids harboring the corresponding DNA fragments to construct standard curves. All DNAs were analyzed in triplicate using three no template controls to check for reagent contamination. PCR runs started with an initial denaturation and enzyme activation step for 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C, followed by 30 s at the annealing temperatures (Table S1), 40 s at 72 °C, and finally 30 s at 80 °C. Fluorescence signals at 80 °C were recorded to attenuate influences of primer dimers. Then, melting curve analysis was used to test the specificities of the PCR products.

2.6. Statistical analyses

We used a mixed-effect model to test heavy rainfall treatments effects on growing season-mean N_2O, SWC, and ST, soil physicochemical properties (DOC, MBC, and SIN) and abundance of soil functional genes (archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS, narG*, and nosZ), and their temporal trends while accounting for repeated measurements. Heavy rainfall imposed in the two periods (HR-mid and HR-late) and year were treated as fixed effects and plot replication was included as a random effect term. A first-order autoregressive temporal covariance structure was assumed in all models. Another mixed-effect model (HR-mid and HR-late as fixed effects and plot replication as a random effect term) was also used to test HR-mid and HR-late effects on TN in 2016. The mixed-effect models were performed using the NLME package. Treatment effects were considered to be statistically significant at $P \leq 0.05$ and marginally significant at $0.05 < P \leq 0.10$ given the small number of treatment replicates (n = 3). A *post-hoc* Duncan test was used to compare mean differences of the above variables across the three treatments each year. Additionally, linear regression was used to correlate accumulated N_2O emission across the three years with TN in 2016. All statistical analyses were conducted in R version 3.4.4 (R Core Team, 2018).

Structural equation modeling (SEM) was used to quantify the direct and indirect impacts of heavy rainfall on N_2O fluxes. Given N_2O flux responses to heavy rainfalls varied with heavy rainfall timing (Tables 1 and 2), we performed the SEM separately for mid- and late-season heavy rainfall treatments. Heavy rainfall-induced changes in soil physiochemical properties (SWC and pH) and soil functional gene abundances were included in the SEM to explore the effects of heavy rainfall on N_2O fluxes. ST, SIN, MBC, and DOC were excluded from the final SEM because these variables were not significantly affected by heavy rainfalls and the SEM excluding these variables was more efficient (i.e. smaller Akaike information criterion). Adequate model fit was indicated by a nonsignificant chi-squared test ($P > 0.05$). The SEM analysis was performed using the AMOS 25 software (IBM, SPSS, Armonk, NY, USA).

3. Results

3.1. Soil water content and soil temperature

Heavy rainfall plots received nearly double the amount of total precipitation over the growing season (256, 481, and 495 mm in 2014; 242; 487, and 501 mm in 2015; 182, 425, 439 mm in 2016 for three

Table 1
P-values from mixed-effect model analyses of HR-mid (heavy rainfall imposed in the mid-growing season) and HR-late (heavy rainfall imposed in the late-growing season) effects on soil physicochemical properties and growing season-mean N_2O fluxes across all the three years.

	SWC	ST	N_2O	DOC	MBC	TIN	Soil pH
HR-mid	**< 0.01**	0.19	**< 0.01**	0.85	0.16	0.13	**0.04**
HR-late	**< 0.01**	0.39	0.44	0.72	0.85	0.64	**< 0.01**
Year	**< 0.01**	**0.03**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	0.80
Hid-mid × year	**< 0.01**	0.36	**0.03**	0.30	0.47	0.20	0.56
HR-late × year	**< 0.01**	0.95	**0.01**	**0.04**	0.95	0.69	**0.05**

P-values in bold are statistically significant to an alpha value of 0.05.
SWC: growing season mean soil water content; ST: growing season mean soil temperature; DOC: soil dissolved organic carbon; MBC: microbial biomass carbon; and TIN: inorganic N contents.

Table 2
P-values from mixed-effect model analyses of HR-mid (heavy rainfall imposed in the mid-growing season) and HR-late (heavy rainfall imposed in the late-growing season) effects on soil functional genes abundances across all the three years.

	Archaeal amoA	*Bacterial amoA*	*Archaeal and Bacteria amoA*	*nirK*	*nirS*	*nirK and nirS*	*narG*	*nosZ*
HR-mid	0.79	0.67	0.76	0.63	0.23	**0.04**	0.21	0.30
HR-late	0.15	0.96	0.22	**0.03**	**0.03**	**< 0.01**	0.43	0.07
Year	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
HR-mid × year	0.61	0.36	0.43	**< 0.01**	0.31	**< 0.01**	**0.03**	**< 0.01**
HR-late × year	0.14	0.52	0.22	**< 0.01**	**0.01**	**< 0.01**	0.30	**< 0.01**

P-values in bold are statistically significant to an alpha value of 0.05.

treatments, respectively; Fig. 1i–l). As a result, heavy rainfall clearly increased soil water content during periods of treatment and for c. 2–3 weeks thereafter, increasing the seasonal mean SWC by 14–27% (Fig. 1d) regardless of seasonal timing (Fig. 1a–d). However, seasonal dynamic and seasonal-mean soil temperature were not affected by either heavy rainfall treatment (Fig. 2e–h).

3.2. N_2O fluxes

At the seasonal scale, the mid-season heavy rainfall significantly stimulated N_2O emissions compared to the ambient Control ($P < 0.01$, Table 1). The positive effects were more obvious in 2014 and 2016 than in 2015 (Fig. 2a–c). In 2014 during the mid-season heavy rainfall period, we observed a large N_2O emission pulse which continued for more than three weeks after the treatment (Fig. 2a). In contrast, the late-season heavy rainfall did not affect N_2O fluxes ($P = 0.44$, Table 1).

3.3. Soil physicochemical properties

Heavy rainfalls significantly increased soil pH ($P = 0.04$, Table 1 and Fig. 3d) while having no effects on dissolved organic carbon and microbial biomass carbon, regardless of seasonal timing ($P = 0.85$ and 0.16 respectively, Table 1 and Fig. 3a–b). Although soil inorganic N content was, on average, always lower in the mid-season heavy rainfall treatment than the control, there was sufficient variation among plots that no significant differences were identified ($P = 0.13$ and 0.64 for HR-mid and HR-late, respectively; Table 1 and Fig. 3c).

3.4. Soil functional genes

Heavy rainfalls did not change abundances of archaeal and bacterial *amoA* regardless of the heavy rainfall timing ($P > 0.10$ for all; Table 2 and Fig. 3a–c). Overall, abundances of *nirK* and *nirS* did not change following the mid-season heavy rainfall events ($P = 0.63$ and 0.23 respectively), except for significant increases in 2015, while they did increase significantly following the late-season heavy rainfall, especially in 2016 ($P = 0.03$ for both genes; Table 2 and Fig. 4d and e). Both the mid- and late-season heavy rainfalls significantly increased the total abundance of *nirK* and *nirS* ($P = 0.04$ and $P < 0.01$ for HR-mid and HR-late, respectively; Table 2 and Fig. 4f). The effects of the heavy rainfall treatments on *narG* and *nosZ* abundances also differed among years. The *narG* abundance significantly decreased following mid-season heavy rainfall in 2015 but increased following late-season heavy rainfall in 2016; the *nosZ* abundance significantly increased in 2015 but decreased in 2016 following mid-season heavy rainfall and significantly decreased in 2014 but increased in 2015 following late heavy rainfall, compared to the ambient control (Table 2 and Fig. 4g and h).

3.5. Relationships among soil physiochemical properties, soil functional genes and N_2O fluxes

Structural equation modeling adequately fit the data for the mid-

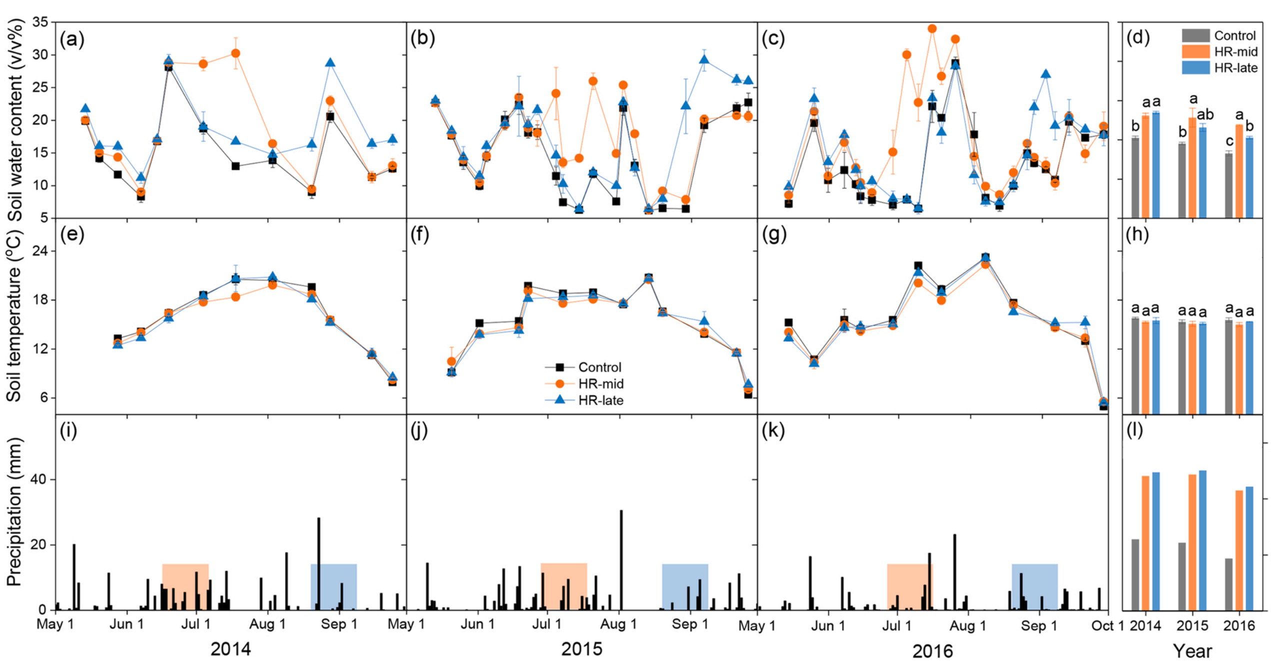

Fig. 1. Seasonal dynamics and annual averages of soil water content and soil temperature (e–h), and daily and total precipitation (i–l) over the growing season for the three treatments during 2014–2016. Control: ambient control; HR-mid: heavy rain imposed in the mid-growing season; and HR-late: heavy rainfall imposed in the late-growing season. The orange and blue shaded bars in i–k indicate periods of HR-mid and HR-late treatments, respectively. Different letters indicate significant differences ($P \leq 0.05$) among the treatments each year. The error bars indicate 1 SE.

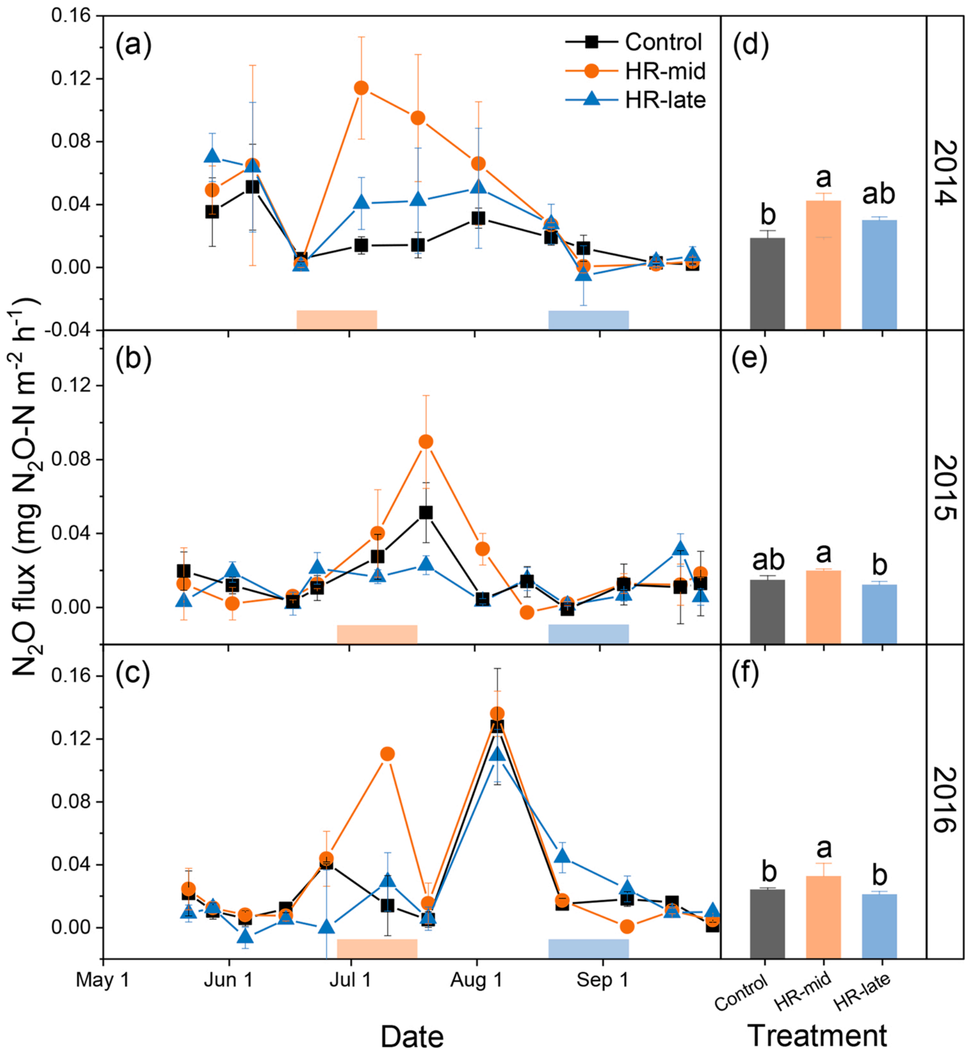

Fig. 2. Seasonal dynamics (a–c) and average (d–f) of N_2O fluxes over the growing season for the three treatments during 2014–2016. Control: ambient control; HR-mid: heavy rain imposed in the mid-growing season; and HR-late: heavy rainfall imposed in the late-growing season. The orange and blue shaded bars in a–c indicate periods of HR-mid and HR-late treatments, respectively. Different letters indicate significant differences ($P \leq 0.05$) among the treatments each year. The error bars indicate 1 SE.

season heavy rainfall treatment, explaining 74% of the N_2O flux variation ($P = 0.34$; Fig. 5a). The mid-season heavy rainfall had positive effects on soil water content, which further had positive effects on the sum of *nirK* and *nirS* abundances and ultimately contributed to N_2O flux increases. Similarly, soil water content had positive effects on the sum of archaeal and bacterial *amoA* copies as well as *narG* copies, while the mid-season heavy rainfall induced-higher soil pH had negative effects on these two variables. Neither soil water content nor soil pH had significant effects on *nosZ* abundance. We found multiple positive relationships among soil functional genes. Except for the sum of *nirK* and *nirS*, other soil functional genes had weak relationships with N_2O flux.

In contrast, the above SEM model did not fit the data for the late heavy rainfall treatment ($P = 0.01$; Fig. 5b), although some structural pathways were significant.

3.6. Relationships between soil total N and accumulated N_2O fluxes

Mid-season heavy rainfall significantly reduced soil total N in 2016, while late heavy rainfall had little effects (Fig. 6a). There was a strongly negative relationship between soil total N and accumulated N_2O flux over the three growing seasons (Fig. 6b).

4. Discussion

This study examined the seasonal patterns and biotic mechanisms of N_2O flux and N pool variation in response to heavy rainfalls applied near the middle and end of the growing season. Our results provided, to the best of our knowledge, the first experimental evidence that seasonal timing of heavy rainfalls strongly regulates total N_2O fluxes over the plant growing season. This advance agrees generally with previous observational and manipulative studies suggesting that the effects of extreme precipitation regimes (e.g. drought and heavy rainfall) on terrestrial ecosystems (e.g., CO_2 flux, biomass, and phenology) are influenced by the seasonal timing of the extremes (Li et al., 2019; Post and Knapp, 2020; Zeppel et al., 2014).

4.1. Mid-season heavy rainfall promotes N_2O fluxes

In this study, N_2O fluxes were largely promoted by short-term mid-season heavy rainfall (Fig. 1), which is consistent with the past findings that increased precipitation at seasonal or annual scale had positive effects on N_2O fluxes (Brown et al., 2011; Z. Li et al., 2020b; L. Li et al., 2020b; Yue et al., 2019). These demonstrated the strong role of precipitation amount and soil water availability in regulating soil N_2O emission (Rowlings et al., 2015). In this grassland, mid-season heavy rainfall favored N_2O emission and was probably the primary cause of increases in the abundance of denitrifying *nirS* and *nirK* at high soil water content (Fig. 4c). The *nirS* and *nirK* genes exist in many microorganisms such as Proteobacteria and Bacteroidetes and can respectively encode Cu-containing and haem-containing cd1 nitrite reductases, which carry out the denitrification process by reducing nitrite to nitric oxide (Graf et al., 2014; Kuypers et al., 2018). Heavy rainfalls should

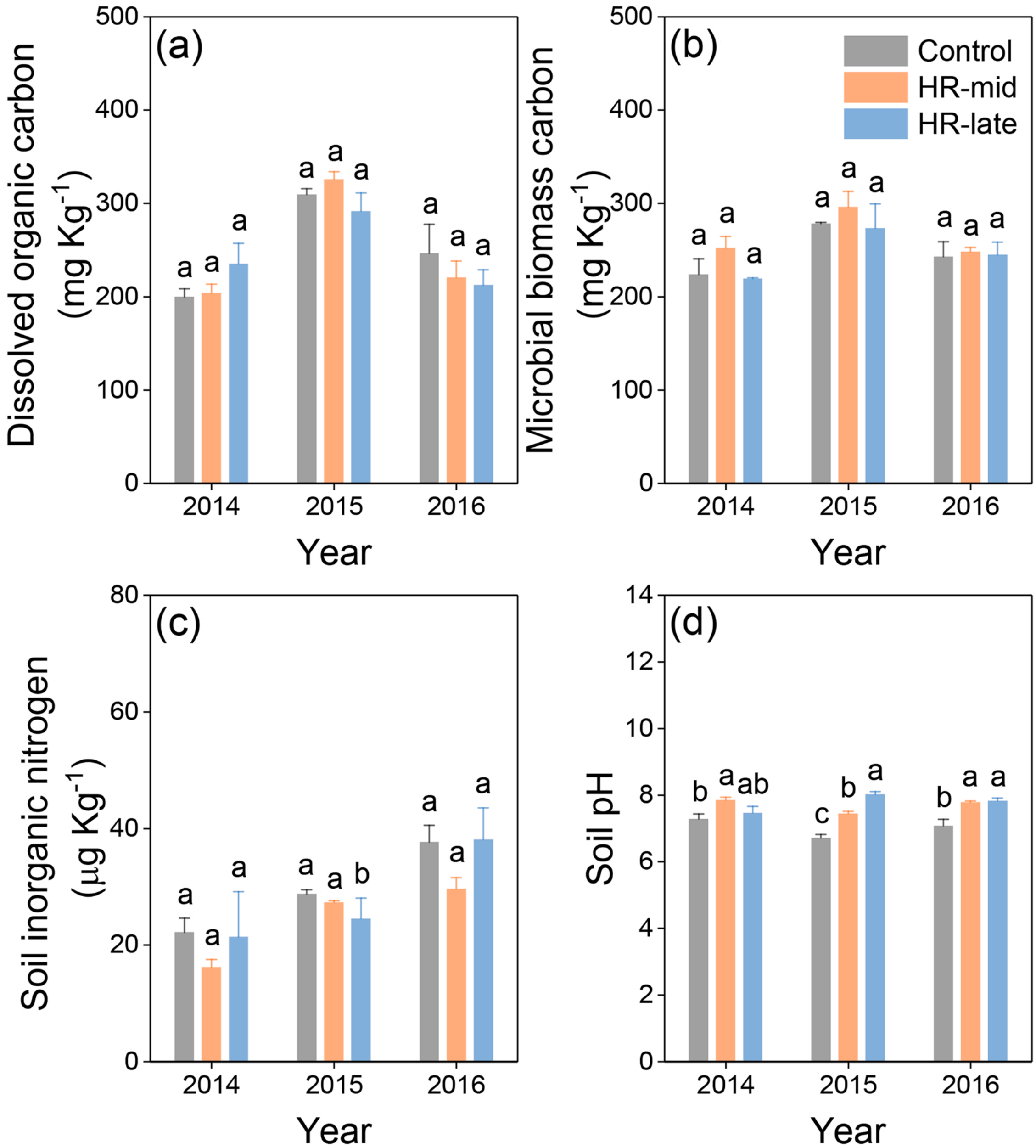

Fig. 3. Soil physicochemical properties for the three treatments during 2014–2016. Control: ambient control; HR-mid: heavy rain imposed in the mid-growing season; HR-late: heavy rainfall imposed in the late-growing season. Different letters indicate significant differences ($P \leq 0.05$) among treatments each year. The error bars indicate 1 SE.

have eliminated water limitation for the growth and proliferation of denitrifying microorganisms in this semiarid grassland. Additionally, anaerobic soil conditions during and shortly after the mid-season heavy rainfall treatment period could also benefit activities of denitrifying microorganisms, through partly or completely saturating soil porosity and thus limiting O_2 diffusion and enhancing O_2-consuming soil respiration (Fig. S1).

High soil water content also had positive effects on ammonia oxidizers (represented by archaeal and bacterial *amoA* gene abundance) and denitrifying *narG* (Fig. 4a). However, the abundances of these functional genes were not significantly affected by mid-season heavy rainfall because high soil pH, also induced by mid-season heavy rainfall (Fig. 3d), had negative effects on these functional genes (Fig. 5a). In other words, the positive effects of increased soil water content and the negative effects of increased soil pH counteracted each other, resulting in unchanged abundances of archaeal and bacterial *amoA* and *narG* genes following the mid-season heavy rainfall. In many cases, soil pH has been identified as a chief explanatory environmental variable for nitrifiers and denitrifiers as well as N_2O emissions at regional scales (Hu et al., 2013; Wang et al., 2018), although the underlying mechanisms are still not fully understood. The negative relationships between soil pH and archaeal and bacterial *amoA* as well as *nraG* in the weak acidic to alkaline soils (pH 6.7–8.2) in this study were supported by findings from a pH gradient study (4.5–7.5) (Nicol et al., 2008) and a transect study across Inner Mongolia grasslands (Zhou et al., 2021), in which multiple N transformation genes (archaeal and bacterial *amoA* gene, *narG and nosZ*) had significantly negative relationships with soil pH in the range of 6.5–8.5. Furthermore, higher soil pH (7.5 vs. 5.5) was found to suppress transcription of archaeal *amoA* (Jung et al., 2019). Similar to the results herein, an intensified precipitation seasonality study demonstrated that increases in N_2O emissions associated with wet-season storms were mainly due to increased *nirS* abundance, rather than other

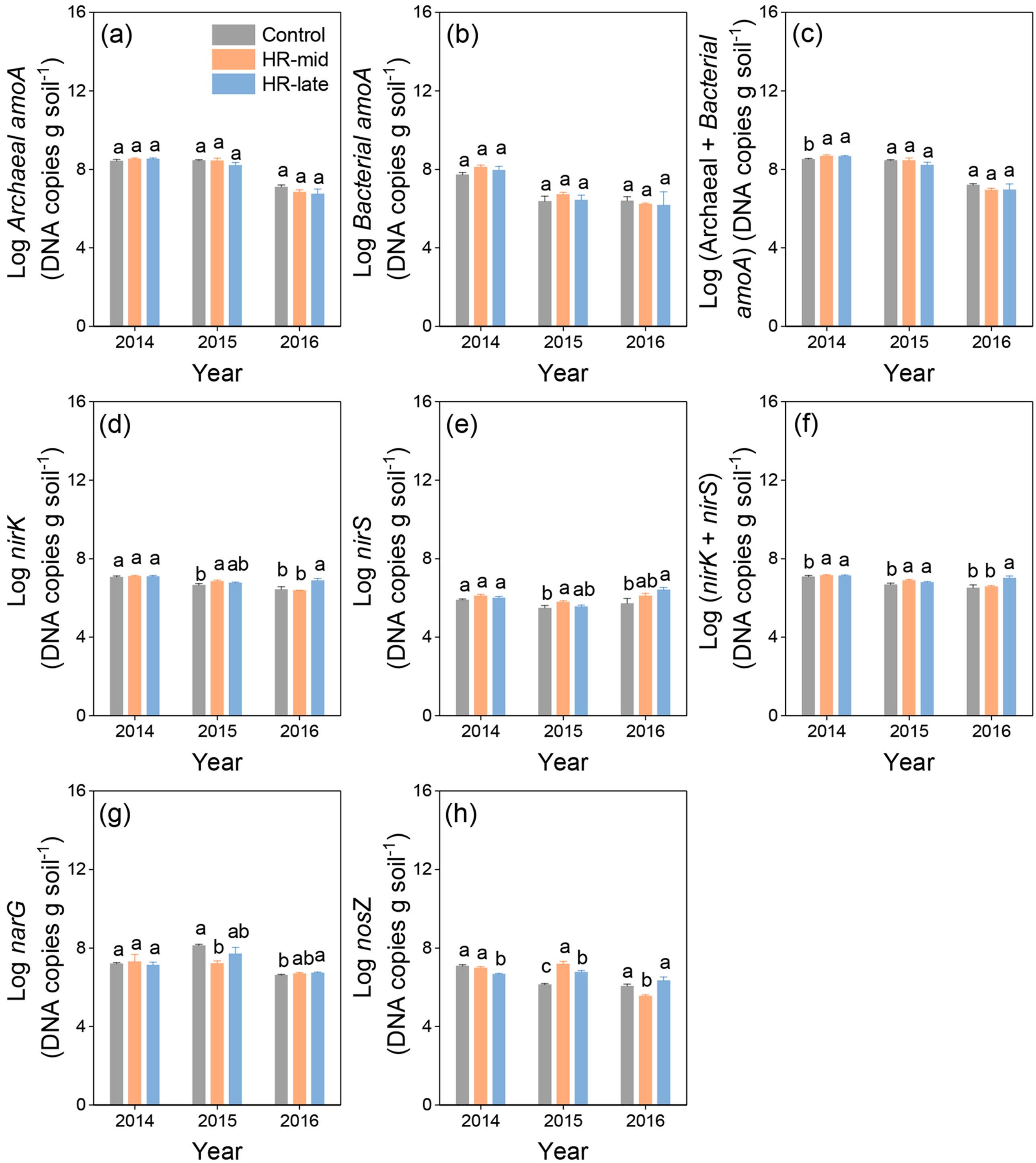

Fig. 4. Soil functional genes abundances for the three treatments during 2014–2016. Control: ambient control; HR-mid: heavy rain imposed in the mid-growing season; HR-late: heavy rainfall imposed in the late-growing season. All data have been transformed. Different letters indicate significant differences ($P \leq 0.05$) among the treatments each year. The error bars indicate 1 SE.

N transformation functional genes, in a subtropical forest (Chen et al., 2019).

We also found close correlations among different functional genes (Fig. 5a). This might be because multiples functional genes co-existed in a single microorganism. For example, Bartossek et al. (2010) reported that *nirS* and *nirK* were present in ammonia-oxidizing bacteria and archaea in soils and other environments. The *nirS* gene was frequently observed to co-exist with *nosZ* (Graf et al., 2014).

4.2. Mid-season heavy rainfall reduces soil N pool

Soil N substrates are normally expected to be important factors regulating nitrification, denitrification, and N_2O emissions. Regionally or globally, soil total nitrogen content alone or combined with inorganic nitrogen was considered as the most important limiting control of nitrification, denitrification and soil N_2O emission (Gutlein et al., 2018; Z. Li et al., 2020a, 2020b). However, in this study, soil inorganic N and total N not only non-significantly and significantly decreased in

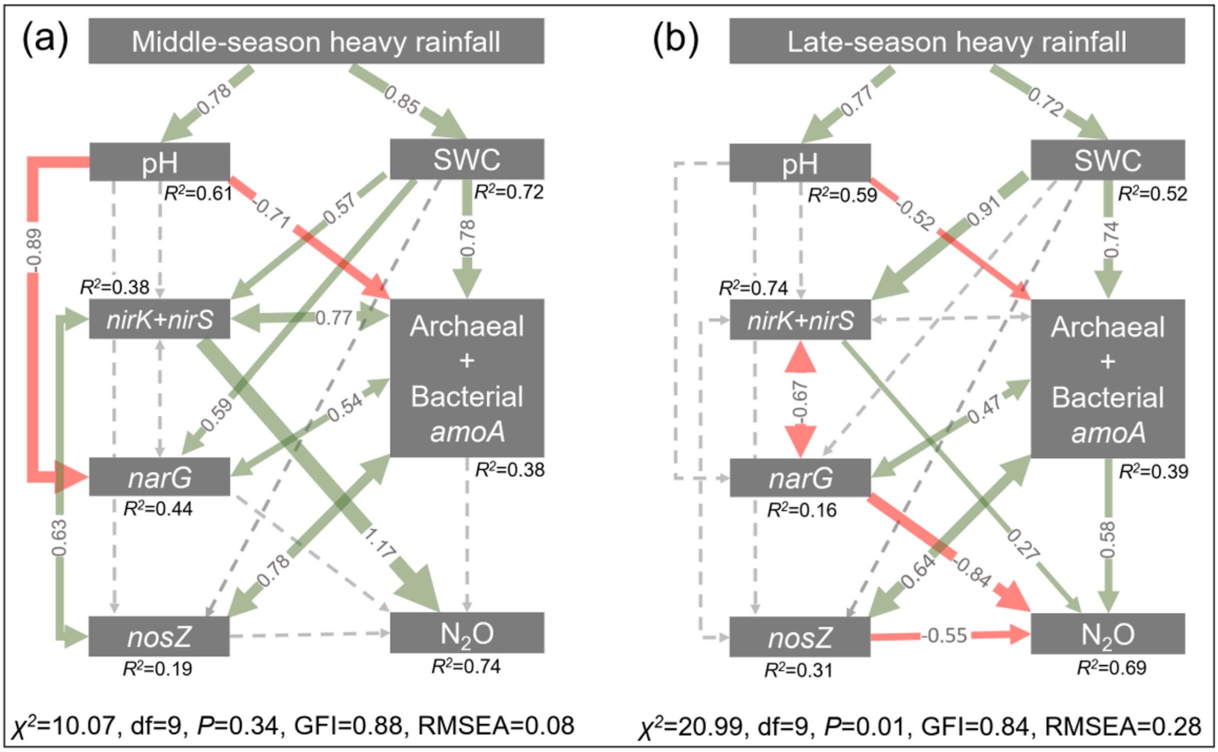

Fig. 5. Structural equation model showing the effects of abiotic factors and biotic factors on N_2O flux under mid-season (a) and late-season (b) heavy rainfall. The red and green arrows are the significantly positive and negative relationships ($P \leq 0.05$), respectively, while grey dashed lines are the non-significant relationships ($P > 0.05$). Numbers on the arrows are standardized coefficients. R^2 values represent the proportion of variance explained for each dependent variable. SWC: soil water content.

response to mid-season heavy rainfall (Figs. 3c and 6a), but also showed negative relationships with seasonal mean N_2O flux and accumulated N_2O flux over three growing seasons, respectively (Figs. 6b and S2). There may be several reasons for these unexpected phenomena at the ecosystem scale. First, high soil water status under the mid-season heavy rainfall treatment might increase N mineralization, increasing soil inorganic N availability (Guntiñas et al., 2012; Knoepp and Swank, 2002). On the other hand, as discussed above, the increased N_2O emissions in the mid-season heavy rainfall indicated soil N loss from soil to air as gaseous N. Second, heavy rainfalls could have caused N runoff and leaching (Greaver et al., 2016; Kasper et al., 2019). Collectively, it is likely that the positive effects of mid-season heavy rainfall on soil inorganic N availability from mineralization were overwhelmed by the negative effects of leaching and denitrification, leading to net soil inorganic N loss. Recurrence of these processes would then result in reduction of soil total N, which could restrain ecosystem development from a long-term perspective. Consistent with our results, a recent forest study indicated that increased frequency of large precipitation events in the wet season reduced annual inorganic N primarily through leaching and secondly via gaseous N emissions (Chen et al., 2019).

As the experimental plots were enclosed by metal flashings to prevent lateral surface flow, heavy rainfall-induced N loss through runoff from terrestrial ecosystems to aquatic ecosystems or from the 'upstream' ecosystems to the 'downstream' ecosystems could be larger at regional scales in reality. Besides, an earlier study suggested that winter cover crops can efficiently mitigate extreme climate effects on soil inorganic N leaching (Iqbal et al., 2018). Thus, mid-season heavy rainfalls might lead to more serious N loss in grazed grasslands due to low plant cover and reduced protective litter (Hu et al., 2016; Sun et al., 2020) relative to our fenced experimental grassland.

4.3. Minor effects of late-season heavy rainfall on N_2O flux and soil N pool

Similar to the mid-season heavy rainfall, the late heavy rainfall also largely increased soil water content, soil pH and denitrifying *nirk* and *nirS* (Figs. 1a–d, 3d and 4a–c). Nevertheless, N_2O fluxes remained stable in responses to the late heavy rainfall (Fig. 2). The SEM model fitted the data well for the mid-season heavy rainfall treatment but not for the late-season heavy rainfall treatment (Fig. 5). This highlighted that heavy rainfalls in different stages of the growing season had different effects of on the ecosystem N cycling. Actually, we failed to find a model that fit the data for the late-season heavy rainfall treatment based on the parameters used in this study, indicating that there were no clear direct and indirect effects of late-season heavy rainfall on N_2O flux. We also did not find increased N_2O emissions during the treatment period. This suggests that although the abundance of microorganisms that contained *nirK* and *nirS* genes increased, either they could not encode the corresponding oxido-reductase (Cu-containing and haem-containing cd1 nitrite reductases); or the encoded enzymes could not catalyze the corresponding denitrification processes (nitrite reduction to nitric oxide), due to cooler temperatures in the late growing season (Fig. 1e–g). Our previous study suggested that either ecosystem CO_2 emission or uptake was accelerated by heavy rainfall in middle growing season but not in late growing season in this semiarid grassland (Li et al., 2019). Likewise, positive effects of a mid-season deluge event on soil respiration, dominant species flowering, and aboveground net primary productivity were remarkably larger than those of a late deluge event in another grassland (Post and Knapp, 2020). Together, these results indicate that heavy rainfall in late growing season had limited influences on ecological functions and biogeochemical cycling processes when metabolic processes of plants and microbes were constrained by temperature instead of soil water availability.

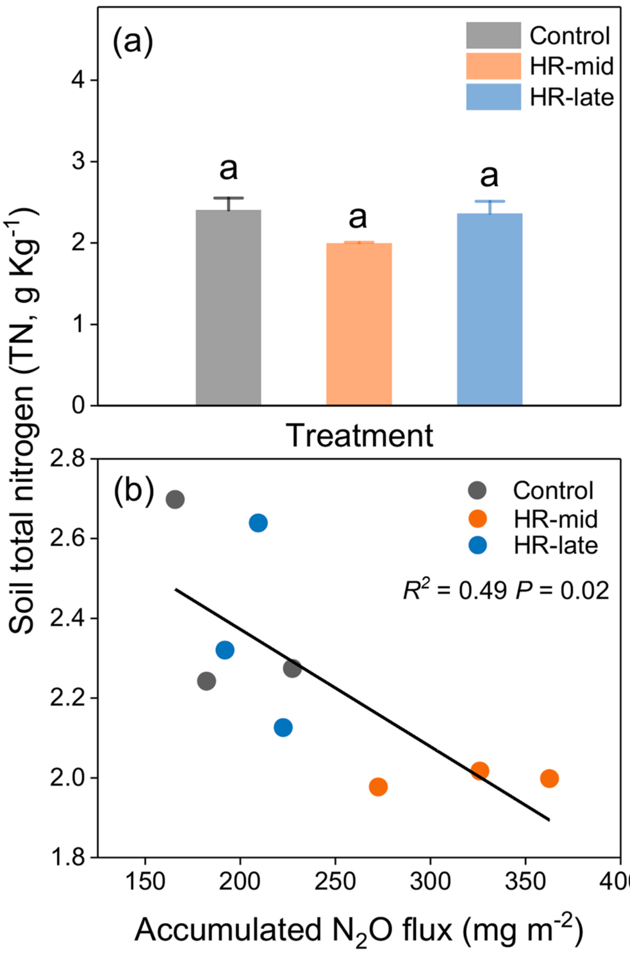

Fig. 6. Soil total nitrogen for three treatments in 2016 (TN, a) and the relationship between accumulated N_2O flux across the three years (b). HR-mid: heavy rainfall imposed in the middle-growing season; HR-late: heavy rainfall imposed in the late-growing season. The error bars indicate 1 SE. *P*-values from mixed-effect model analyses of HR-mid and HR-late effects on TN were 0.04 and 0.81, respectively.

4.4. Limitations

There were certain limitations within our experiment. First, the experimental heavy rainfall design was mainly based on the longest recorded period of continuous rainfall and the extreme total effective precipitation amount over the periods studied. However, the rainfall size and duration each day was not taken into consideration, which could have played important roles in regulating soil N cycling processes. For example, recent studies suggested that the net raindrop-induced ejection of N in rice paddies of China were positively correlated with intensities from 20, 40, 60, to 100 mm h^{-1}, regardless of site locations or rice-growing stages (Wu et al., 2020). The rainfall size and duration may vary substantially on different days under natural conditions, while the simulated rainfall events might have introduced some artificial treatment effects.

Second, the measurements of N_2O emissions in this study had to be conducted with an interval of 10–15 days during the five months of the grass growing season due to limitation by weather conditions, workload and funding. In this case, potentially large N_2O pulses induced by natural heavy rainfall events that occurred during the measurement intervals might have been missed out (Groffman et al., 2009; Petrakis et al., 2017), although the overall treatment effects should be successfully detected with the current measurement frequencies. As a result, N_2O emissions in the ambient control treatment might have been underestimated, which could lead to overestimation of the positive effects of mid-season heavy rainfall on annual N_2O emission.

Third, similar to many studies, the abundances of soil functional genes were used to indicate specific functional microbes in this study. However, the genes present in soil may not necessarily be expressed and different community compositions of a specific microbial functional group may have different effects on N_2O production in soil. Gene abundances do not always correspond to the amount of N-cycling enzymes produced (Nannipieri et al., 2019). Therefore, to improve understanding of the underlying microbial mechanisms, there remains a need to quantify community compositions and activities of these N cycling microorganisms as well as N-cycling enzymes.

5. Conclusions

Projected increases in heavy rainfalls may alter soil N biogeochemical processes and N pools, including nitrification, denitrification and N_2O fluxes. Based on the heavy rainfall × seasonal timing manipulative experiment in a semiarid grassland, our results revealed that N_2O flux responses to heavy rainfall depended on the seasonal timing, with mid-season heavy rainfall promoting N_2O emission while late heavy rainfall having no significant effects. Positive effects of mid-season heavy rainfall on N_2O fluxes could be attributed mainly to increases in denitrification under high soil water content. Consequently, mid-season heavy rainfall led to soil total N loss through denitrification and possible leaching. Therefore, mid-season heavy rainfall can potentially cause a positive feedback to global warming and exacerbate N limitation in terrestrial ecosystems in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This project was funded by the National Natural Science Foundation of China (Grant nos. 42041005 and 32101313), the China Postdoctoral Science Foundation (Grant no. 2021M693138), the CAS Strategic Priority Research Programmer (A) (Grant no. XDA19030202), and the Fundamental Research Funds for the Central Universities (Grant no. E1E40511). J. Biederman's contributions were supported by the US Department of Agriculture, Agricultural Research Service. USDA is an equal-opportunity employer.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2021.107785.

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