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Soil extractable organic C and N contents, methanotrophic activity under warming and degradation in a Tibetan alpine meadow

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ABSTRACT

The Tibetan alpine meadow ecosystem is an important part of the Eurasian grasslands and is experiencing intense warming at approximately three times the global warming rate and rapid degradation. However, little is known about the effect of warming and degradation and their interactions on ecosystem functions like soil carbon (C) and nitrogen (N) pools and methane (CH_4) uptake in this region. Here, we selected a long-term simulated warming site in a Tibetan alpine meadow with different degradation levels. After 4 years of warming, we analyzed soil total C (TC) and total N (TN) contents, extractable organic C (EOC) and extractable organic N (EON) contents as well as methanotrophic activity, abundance and community structure. Soil EOC and EON contents were measured through hot water extraction, whereas methanotrophic activity was measured along a gradient of CH_4 concentrations in laboratory incubations. Michaelis–Menten kinetics analysis [maximal rate of velocity (V_{max}) and half-saturation constant (K_m)] was used to quantify changes in methanotrophic activity among the treatments. Active methanotrophic communities in the natural soils were measured via DNA-based stable isotope probing (SIP). The results showed that warming significantly increased soil EON contents, whereas degradation significantly decreased soil TC and TN contents, and EOC and EON contents. Methanotrophic activity was significantly lower at different levels of degradation but no significant effects were observed under warming. Changes in soil methanotrophic abundance among the treatments followed the same trend, but warming and degradation had no interactive effects on methanotrophic activity and abundance. Active methanotrophic communities in the natural meadow soils were dominated by *Methylosinus* (a Type II methanotroph). In conclusion, our results indicate that soil C and N pools and CH_4 oxidation capability were influenced more strongly by degradation than warming. However, warming may have an additional effect on the stability of these important ecosystem processes, regardless of degradation in this region.

1. Introduction

The alpine meadows of the Tibetan Plateau cover an area of 0.49×10^5 km^2 (Zhao et al., 2005), comprising a substantial part of the Eurasian grassland ecosystems that provide important services such as livestock grazing and soil carbon (C) storage (Dong et al., 2010; Babel et al., 2014). However, over the past few decades, the alpine meadow ecosystem has experienced rapid degradation, and the annual degradation rates can reach up to 34.5% in certain areas near the sources of the Yangtze and Yellow rivers, caused by overgrazing as a result of

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human activities (Zhou et al., 2005; Wang et al., 2010). This degradation has resulted in a variety of ecological consequences, including the loss of soil C and nutrients, and a decline in aboveground plant productivity (Wu et al., 2014; Su et al., 2015; Liu et al., 2018). As well as rapid degradation, this alpine meadow ecosystem is predicted to suffer from intense warming at approximately three times the global warming rate (Qiu, 2008; Chen et al., 2013; IPCC, 2013). Therefore, much attention has been paid to whether warming accelerates the degradation of the alpine meadow ecosystem. Previous studies have shown that warming increases soil C and nitrogen (N) pools by increasing the rates of plant-derived litter decomposition in the Tibetan alpine meadows (Rui et al., 2011), whereas degradation can cause substantial losses of soil C and N (Shi et al., 2013; Liu et al., 2018). However, it is still unclear how warming and degradation in combination affect soil C and N cycling and important ecosystem functions such as methane (CH_4) uptake in these alpine meadows.

It has previously been found that soil C and N contents can remain stable following the application of various management events, both immediately and in the short term, potentially due to buffering effects driven by resilient soil processes (Haynes, 2005; Zhou et al., 2013). However, the active fractions of soil C and N contents, the extractable organic C (EOC) and extractable organic N (EON) pools, act as a short-term reservoir of nutrients for plants and microorganisms (Ghani et al., 2003; Zhou et al., 2013) and have been reported to respond rapidly to changes in management practices (Haynes, 2005). Soil EOC and EON contents extracted by hot water can include dissolved organic matter derived from microbial biomass (Chen and Xu, 2008), and from root exudation and breakdown of soil aggregates (Ros et al., 2010). In previous studies, soil EOC and EON contents obtained via hot water extraction were have already been widely used to indicate the active fractions of soil total C (TC) and total N (TN) contents in different terrestrial ecosystems (Ghani et al., 2003; Bu et al., 2010; Zhou et al., 2013). To date, only a few studies have studied the changes in soil EOC and EON contents under warming and degradation in these alpine meadows (Fu et al., 2012).

Besides soil EOC and EON, CH_4 is sensitive to warming and degradation, and is often used as an indicator to explore the effect of environmental changes on grasslands. Atmospheric CH_4 is the second most important greenhouse gas after CO_2, contributing about 25% to global warming with about 25–30 times the warming potential of CO_2 on a molecular basis (IPCC, 2013). Aerated soils of terrestrial ecosystems act as important CH_4 sinks, which consumes about 30 Tg CH_4 from the atmosphere per annum (IPCC, 2013; Tate, 2015). The soil CH_4 sink is mediated by a group of aerobic microorganisms, namely CH_4-oxidizing bacteria (methanotrophs) (Kolb, 2009). The *pmoA* gene encodes a subunit for particulate CH_4 monooxygenase, a key enzyme in the methane oxidation pathway, and has been widely used as a molecular marker to detect methanotrophic abundance and diversity in soils (Kolb, 2009; Tate, 2015). Management practices can greatly influence soil methanotrophic activity through changes in soil properties (Kolb, 2009; Tate, 2015). Among these soil properties, methanotrophic activity is mainly influenced by soil moisture contents and soil temperature (Einola et al., 2007; Li et al., 2015).

In general, warming can increase soil methanotrophic activity by enhancing microbial metabolism (Einola et al., 2007; Urmann et al., 2009). By comparison, degradation tends to decrease soil moisture contents in alpine meadow systems (Börjesson et al., 2004; Su et al., 2015) and may have negative effects on soil methanotrophic activity (Zhou et al., 2014). Given these inconsistent effects of warming and degradation on soil methanotrophic activity, it is important to clarify the combined effects of warming and degradation for understanding the factors controlling CH_4 flux in the alpine meadow ecosystem more clearly. Until now, most previous studies have investigated soil methanotrophic activity by using laboratory incubation at atmospheric CH_4 concentrations (Zhou et al., 2008; Zheng et al., 2012), but only a few other studies have used Michaelis–Menten kinetic analysis [maximal rate of velocity (V_{max}) and half-saturation constant (K_m)] for observing soil methanotrophic activity along a gradient of CH_4 concentrations (Gulledge et al., 2004; Xu and Inubushi, 2009; Krause et al., 2015 Judd et al., 2016). In this study, we measured CH_4 oxidation kinetics to quantify the effects of warming and degradation separately in these alpine meadows, because kinetic analysis can help to get a more reliable representation constant for soil methanotrophic activity compared with measuring CH_4 oxidation potential (Gulledge et al., 2004; Judd et al., 2016).

To investigate the effects of warming and degradation on soil EOC and EON contents and methanotrophic activity, we selected a long-term warming site along a gradient of Tibetan alpine meadow with different degradation levels established in 2012. In this study, we tested the hypothesis that the potential positive effect of warming on methanotrophic activity would offset the negative effects of degradation to some extent. We examined the responses of soil EOC and EON contents as well as soil TC and TN contents to warming and degradation, and quantified the response of methanotrophic activity and abundance under warming and degradation in the alpine meadows. Soil methanotrophic abundance was measured by quantitative polymerase chain reaction (qPCR) analysis of *pmoA* genes. We also combined high-throughput sequencing and DNA-based stable isotope probing (DNA-SIP) after $^{13}CH_4$ incubation to detect active methanotrophs associated with soil CH_4 oxidation (Dumont et al., 2011).

2. Materials and methods

2.1. Experimental sites

We selected three sites with different degradation levels: the natural alpine meadow (the control, CK), a moderately degraded alpine meadow (MD) and a heavily degraded alpine meadow (HD) near the Naqu Ecological and Environmental Observation and Research Station, China (Fig. 1), which is located in the center of the Tibetan Plateau (31°17′N, 92°06′E, 4501 m above sea level). At each site, eight plots were randomly chosen. Of these, four plots were covered by open-top chambers (OTCs) to simulate warming and the other four plots were set as controls. Cylindrical OTCs made of solar transmitting plastic were 1.5 m in diameter at the base, 1.0 m diameter at the top and 0.5 m in height. The OTC platform was established in 2012 and the associated warming effect has been described previously by Cui et al. (2017).

The station lies in the center of the Tibetan Plateau and experiences a typical continental plateau climate. This region (plateau subfrigid zone) belongs to a semiarid and subhumid monsoon climate (Jin et al., 2018). The mean annual precipitation is ~406 mm and mean annual temperature is about -2.1 °C at the Naqu station. The local climate is characterized by strong solar radiation with long, cold winters and short, cool summers. These alpine meadows provide important grazing grounds for livestock, where the dominant plant species is *Koenigia islandica*, with more than 90% canopy coverage (Li et al., 2016). The abundance of the dominant plant species and other descriptors of the plant community present in the three alpine meadow sites are given in Table S1. The mainly soil texture in this region is sandy loam. Clay and silt accounted for 17.1% and 46.9% at the control sites, respectively, but accounted for 6.4% and 33.3%, respectively, at the heavily degraded sites (Li et al., 2016).

2.2. Soil sampling and measurements of soil physiochemical properties

Soil samples were collected in August 2016 by using a diagonal sampling pattern (i.e., one point at each corner and one in the center of each plot) with a soil auger (5 cm in diameter) at a depth of 0–10 cm within each plot. This depth range was used as the majority of root biomass and reactive soil C and N pools are present in this soil layer in these alpine meadows, and past research in similar settings indicated that soil properties in this depth range were most sensitive to climate

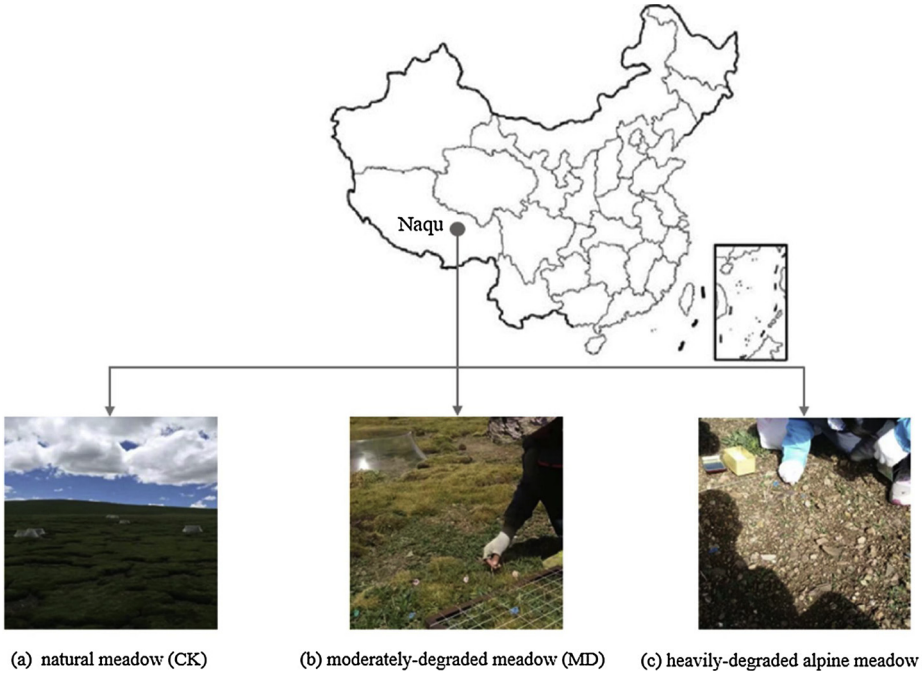

Fig. 1. Photos showing the warming and degradation experiment plots: natural meadow (CK) (a), a moderately degraded meadow (MD) (b) and a heavily degraded alpine meadow (HD) (c) in an alpine meadow of the Tibetan Plateau.

change (Zhou et al., 2013). The soil cores were immediately mixed thoroughly and kept in a cooler (4 °C). After passing the samples through a 2-mm sieve to remove roots and stones, the soil samples were stored at 4 °C prior to analysis. Soil moisture content was determined after the samples were oven-dried at 105 °C overnight. Soil pH was measured at a 1:2.5 dry soil/water ratio. After air-dried soil samples were finely ground, soil TC and TN contents were determined on a Vario MICRO cube elemental analyzer (Elementar, Germany).

2.3. Measurements of soil EOC and EON contents

Soil NH_4^+-N, NO_3^--N, EOC and EON contents were determined in hot water extracts (Zhou et al., 2013). Briefly, field soil samples (5 g) were extracted with 50 mL of hot water and incubated at 70 °C for 16 h in Falcon tubes. After that, the Falcon tubes were rotated in an end-to-end shaker at 120 rpm for 1 h and then the supernatant was filtered through Whatman No. 42 paper. The inorganic N contents (sum of NH_4^+-N and NO_3^--N) was measured on a Smartchem Discrete Auto Analyzer (Smartchem200, AMS, Italy). Soil EOC and total soluble N contents in the soil extracts were determined with a Multi N/C 3100 total organic C analyzer fitted with a total N unit (Analytik Jena, Germany). Soil EON contents were calculated by subtracting extractable inorganic N contents (NH_4^+-N and NO_3^--N) from total soluble N for each soil sample.

2.4. Measurements of soil methanotrophic activity under different CH_4 concentrations under warming and degradation

Approximately 10 g of fresh soil from each sample was weighed into 50-mL containers and then placed into 1-L glass jars. A hole in the center of lid was sealed with a rubber septum for gas sampling. All jars were flushed with fresh air and pre-incubated for two weeks to minimize the effect of soil disturbance on CH_4 oxidation rate measurements. The soil samples were incubated with one of a gradient of CH_4 concentrations [i.e., 2, 10, 20, 500, and 1000 ppm] in the dark at 22 °C for one week. At the beginning and end of the incubation, gas samples were collected from the headspace of jars with a 30-mL syringe. The concentrations of CH_4 during the incubation were determined on a gas chromatograph equipped with a flame ionization detector (7890B GC, Agilent, USA). The CH_4 oxidation rates were calculated according to the differences in CH_4 concentrations in the headspace over the incubation time (Zhou et al., 2008). Check standards were measured once every 10 samples and the coefficient of variation of all standards for one run was less than 5%. In addition, five jars containing ambient air were processed via the same protocol as the control for checking gas leakage. The soil CH_4 oxidation rate was expressed as μg kg^{-1} dry soil h^{-1}.

Methanotrophic activity data were log transformed to produce distributions better suited for plotting and subsequent analysis. Michaelis–Menten kinetics were used to calculate the constant (K_m) and velocity (V_{max}) for soil methanotrophic activity (Judd et al., 2016). Values of apparent K_m and V_{max} were determined from Lineweaver–Burk plots of methanotrophic activity versus CH_4 concentration.

2.5. Soil genomic DNA extraction

Soil genomic DNA was extracted from 0.5 g of each sample using the procedures described previously elsewhere (Zhou et al., 2008; Bu et al., 2018). In brief, soil was placed in a 2-mL screwcap tube containing a mixture of ceramic and silica particles (Bio101, USA) and the mixture was homogenized for 30 s in a FastPrep bead beater cell disrupter (MP Biomedicals, USA). After the nucleic acids had been precipitated and washed twice in 75% (vol/vol) ethanol, the DNA was re-suspended in 100 μL of double-distilled water. Furthermore, the crude extract was purified with the Qiagen Gel Extraction Kit (Qiagen Inc., Germany). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.6. Measurements of methanotrophic abundance by qPCR

The abundance of the *pmoA* gene of methanotrophs was quantified by real-time qPCR with the primers of *A189* (forward) and *A650* (reverse) (Bourne et al., 2001). The reactions were performed on the ViiA 7 Real-Time PCR System (Applied Biosystems, USA). The qPCR mixture (10 μL) consisted of 5 μL 2 × TB Green Premix Ex Taq II (Tli RNaseH Plus), 0.2 μL 50 × ROX reference dye, 0.4 μL of each primer (10 μM),

Table 1
F-values showing the effects of warming, degradation level and their interactions on soil moisture content, total carbon (TC) and total nitrogen (TN) contents, carbon to nitrogen ratios (C:N), extractable organic carbon (EOC) and extractable organic nitrogen (EON) contents, EOC:EON ratios, and NH_4^+-N and NO_3^--N contents in a Tibetan alpine meadow.

Parameter	Moisture	TC	TN	C:N	EOC	EON	EOC:EON	NH_4^+-N	NO_3^--N
Warming (W)	2.68	0.44	0.99	1.48	2.81	4.71*	0.07	4.83*	0.03
Degradation (D)	75.47***	45.09***	39.95***	1.58	53.84***	47.32***	0.33	28.59***	3.46*
W × D	0.31	0.13	0.32	1.11	0.35	1.62	0.51	0.91	0.44

Significance level.
$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$.

3 μL ddH_2O and 1 μL of the DNA template. The cycling conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and a final elongation at 72 °C for 5 min. As a copy number standard, a synthetic DNA fragment containing binding sites for the primers *A189F* and *A650R* placed at the appropriate distance according to their amplicon size of 461 bp was created (Wcgene Biotechnology, China). The standard was obtained by a 10-fold dilution series of plasmids containing *pmoA* gene fragments. Soil methanotrophic abundance data for each soil sample were transformed by a common logarithm.

2.7. High-throughput sequencing of the pmoA gene

Illumina MiSeq sequencing of *pmoA* genes was used to characterize the methanotrophic community structure. A DNA sample from an ambient control site was selected for PCR amplification with the primers *A189F* and *A650R*. The PCR mixture (25 μL) consisted of 5 μL 5× reaction buffer, 5 μL 5 × GC buffer, 2 μL dNTP (2.5 mM), 0.25 μL Q5 DNA Polymerase (New England Biolabs, UK), 1 μL of each primer (10 μM), 8.75 μL ddH_2O and 2 μL of the DNA template. The PCR reaction procedure was the same as described above and the PCR cycles were run 30 times. The products were determined by 2% agarose gel electrophoresis and quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific Inc., USA). Finally, the products were submitted to the Illumina MiSeq platform (Majorbio, China) for sequencing.

We assembled paired-end reads with FLASH (Magoč and Salzberg, 2011). The taxonomic classification was implemented with the mothur command classify.seqs by a naïve Bayesian classifier (Wang et al., 2007). We used a cutoff value of 80% to control data quality and compared the sequences against the *pmoA* database complied by Dumont et al. (2014). We obtained ˜55,459 reads of *pmoA* genes in the natural soils. These sequences have been deposited in the DNA DataBank of Japan (DDBJ) biosample database with the accession number SAMD00154612.

2.8. Measurements of active methanotrophic communities using DNA-SIP

Approximately 10-g (dry weight equivalent) soil samples from the ambient control site were incubated with and without 2000 ppm $^{13}C-CH_4$ in the headspace at 22 °C in the dark for 3 weeks. The soil samples without $^{13}C-CH_4$ were set as controls. The incubation procedures were as described above. To make sure that the $^{13}CH_4$ had been used by the methanotrophs as a substrate, concentrations of $^{13}CH_4$ in the headspace were periodically monitored weekly during the incubation. After a 3 week incubation period, the soil total genome DNA was extracted, and DNA-SIP fractionation performed as described previously (Zheng et al., 2014). According to previous work (Cai et al., 2016), DNA fractions were separated into a total of 15 gradient fractions. An AR200 digital hand-held refractometer (Reichert, Inc., USA) was used to determine the refractive index of each fraction. The fractionated DNA was recovered by PEG-6000 precipitation and re-suspended in 30 μL of a TE buffer, allowing selection of only the heavy fractions (the fourth, fifth and sixth) for further 16S rRNA high-throughput sequencing on the Illumina MiSeq sequencing platform (Majorbio, China). The high-throughput sequencing data were analyzed as described by Bu et al. (2018). Every soil sample obtained ˜32,000 reads. These sequences have been deposited in the DDBJ biosample database with the accession number SAMD00154611.

2.9. Statistical analysis

Two-way analysis of variance (ANOVA) was used to determine the main and interactive effects of warming and degradation on soil moisture contents, pH, TC and TN contents, C:N ratios, EOC and EON contents, EOC:EON ratios, NH_4^+-N and NO_3^--N contents, methanotrophic activity (K_m and V_{max}) and abundance. All analyses of variance were performed in R (R Core Team, 2014). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Soil EOC and EON contents and other properties under warming and degradation

Degradation significantly decreased soil moisture contents but warming, with or without degradation, had no effect (Table 1 and Fig. 2a). Neither warming nor degradation changed soil pH (all $P > 0.05$, Fig. S1). There were no interactive effects of warming and degradation on soil moisture contents or soil pH (Table 1 and Fig. S1).

Warming induced numerical increases in soil TC and TN contents, EOC contents, EOC:EON ratios and NO_3^--N contents across the degradation levels, but these changes were not statistically significant. Warming significantly increased EON and NH_4^+-N contents across the degradation levels, whereas degradation significantly decreased all these properties (Table 1, Figs. 2 and 3). There were no interactive effects of warming and degradation on soil TC, TN, EOC, EON, NH_4^+-N or NO_3^--N contents in these alpine meadows (Table 1).

3.2. Soil methanotrophic activity and abundance under warming and degradation

The kinetic analysis showed that warming had no significant effects on K_m and V_{max} for soil methanotrophic activity, although the mean values were higher under the warming treatment than in the ambient control, with the exception of the moderately degraded sites (Tables 2 and S2, Figs. 4 and S3). However, degradation significantly decreased K_m and V_{max} regardless of the warming treatment (Table 2 and Fig. 4). Warming and degradation had no interactive effects on K_m and V_{max} in these alpine meadows (Table 2).

Measurements of *pmoA* gene copy numbers showed that warming significantly increased methanotrophic abundance but degradation had a significant negative effect (Table 2 and Fig. 5). Additionally, warming

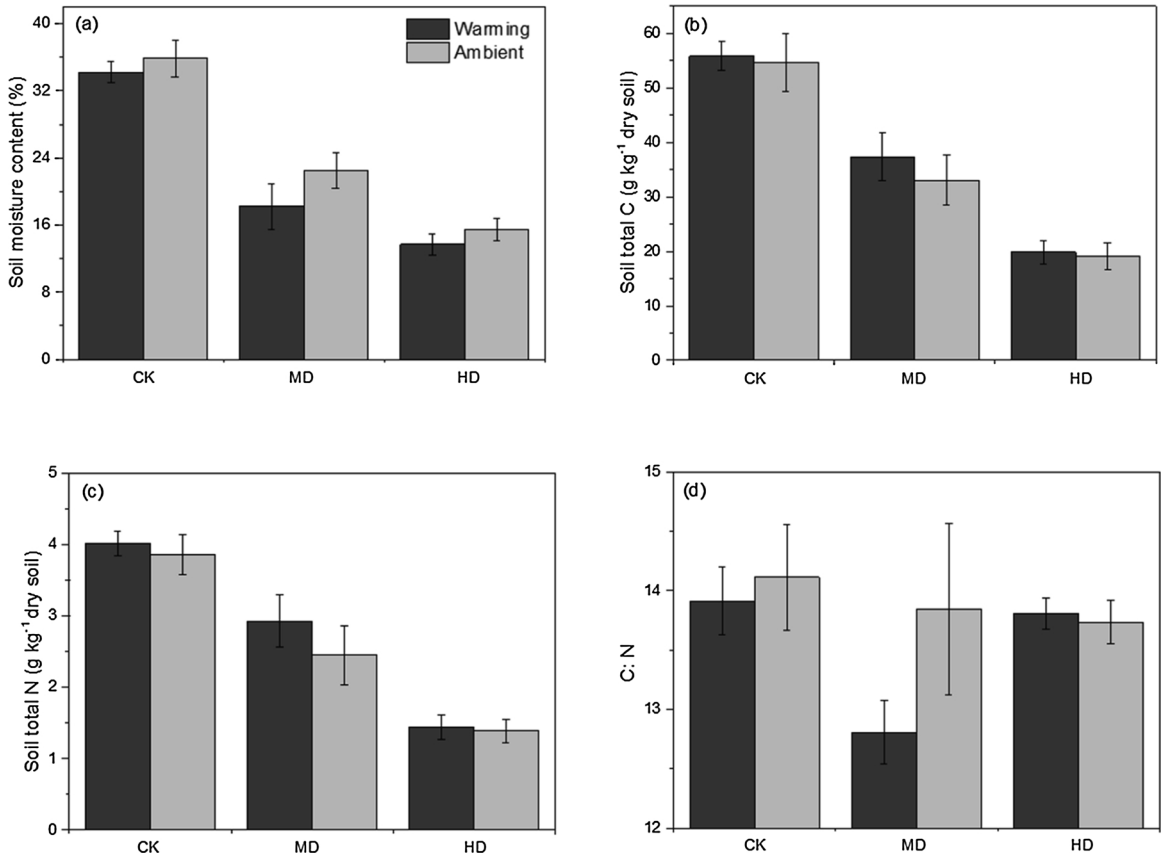

Fig. 2. Differences in soil moisture content (a), total carbon (C) (b) and nitrogen (N) (c) contents as well as ratios of total C to N (d) in a Tibetan alpine meadow subjected to warming along a degradation gradient. CK, natural meadow; MD, moderately degraded meadow; HD, heavily degraded meadow.

and degradation in combination did not have any interactive effects on methanotrophic abundance (Table 2).

3.3. Active methanotrophic communities in ambient control soils

Based on the $^{13}C-CH_4$ labeling in combination with DNA-SIP, only the methanotrophic genus *Methylosinus* (a Type II methanotroph) was identified as active in the fourth fraction of ^{13}C-DNA in the soils (Fig. 6). No active methanotrophs were detected in the fifth and sixth heaviest fractions. Other facultative methanotrophic genera (i.e., the methylotrophic genus *Methylobacterium*) were detected in all the fractions of ^{13}C-DNA (Fig. S4). We also detected another facultative methylotrophic genus (*Methylotenera*) in the fifth heaviest fraction of ^{13}C-DNA.

High-throughput sequencing of *pmoA* showed that methanotrophic communities were dominated by Type II methanotrophs in the natural soil (Table S3).

4. Discussion

4.1. Effects of warming and degradation on soil TC, TN, EOC and EON contents

As expected, significant differences in soil TC and TN contents were not detected after 4 years of warming (Table 1 and Fig. 2b, c). Compared with soil TC and TN contents, soil EOC and EON are much more sensitive to warming, which was supported by the significant differences in soil EON contents among the treatments (Table 1 and Fig. 3). As an active component of soil TC and TN contents, soil EOC and EON pools are closely related to aboveground plant biomass (Fu et al., 2012; Zhou et al., 2012), and can be used as one of the most sensitive indicators for determining subtle changes within an ecosystem (Ghani et al., 2003). Soil EOC and EON pools can be measured by different extraction methods, such as KCl extraction, cool water extraction and hot water extraction as used in this study. In another comparison of these methods, we found that hot water extraction can obtain more reliable soil EOC and EON pools than the other methods (Zhou et al., 2013).

In this study, we found that 4 years of warming tended to increase soil TC, TN, EOC and EON contents, and they showed consistent trends along a degradation gradient (Figs. 2 and 3). Warming increased soil EOC and EON contents, which was supported by higher amounts of soil DNA under warming, as the amount of soil DNA can act as an indicator of soil microbial biomass (Fig. S2). This result is also consistent with previous studies that showed higher soil C and N pools under warming (Rui et al., 2011). The reason for this might be attributed to higher aboveground biomass, which was supported by higher plant cover under warming (Wang et al., 2012). On the other hand, the wide alpine meadows on the Tibetan Plateau has been experiencing different levels of degradation and has suffered large losses of soil C and N stocks (Liu et al., 2018). Here, we also found that degradation significantly decreased soil C and N pools (Table 1 and Figs. 2 and 3), which was consistent with previous results (Su et al., 2015; Liu et al., 2018). The reason for this might be the lower aboveground plant biomass and cover in the alpine meadows of the Tibetan Plateau and the higher soil compaction under degradation (Wu et al., 2014).

It is worth note that there was no interactive effect of warming and degradation on soil C and N pools (Table 1, Figs. 2 and 3). Given that warming increased EOC and EON contents to some extent irrespective of degradation, warming may support plant growth and ecosystem recovery in these alpine meadows, as the active fractions of soil C and N pools can provide short-term nutrients for plants and microorganisms.

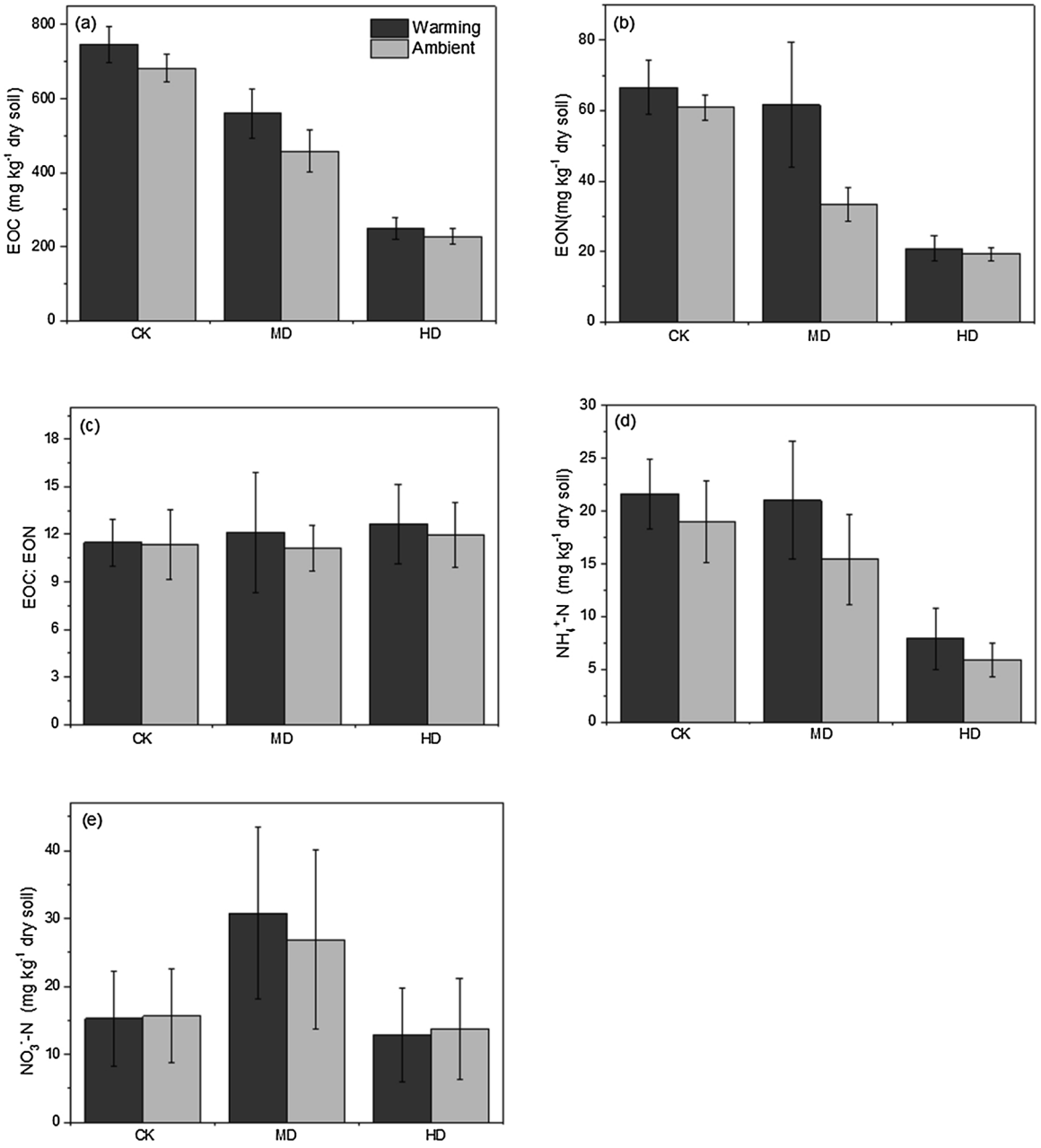

Fig. 3. Differences in soil extractable organic carbon (EOC; a) and extractable organic nitrogen (EON; b) contents, EOC:EON (c), NH_4^+-N (d) and NO_3^--N (e) contents in a Tibetan alpine meadow subjected to warming along a degradation gradient. CK, natural meadow; MD, moderately degraded meadow; HD, heavily degraded meadow.

Table 2
F-values showing the effects of warming, degradation level and their interactions on methanotrophic *pmoA* gene copy numbers and the growth rate of methanotrophic activity in a Tibetan alpine meadow.

Parameter	Log_{10} (methanotrophic *pmoA* gene copies)	K_m (CH_4 concentration (ppm))	V_{max} (μg CH_4 h^{-1} kg^{-1} dry soil)
Warming (W)	6.15*	0.017	0.208
Degradation (D)	6.87**	13.871***	14.149***
W × D	2.41	1.438	2.032

Significance level.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$. K_m, half-saturation constant; V_{max}, maximal rate of velocity; ppm, parts per million.

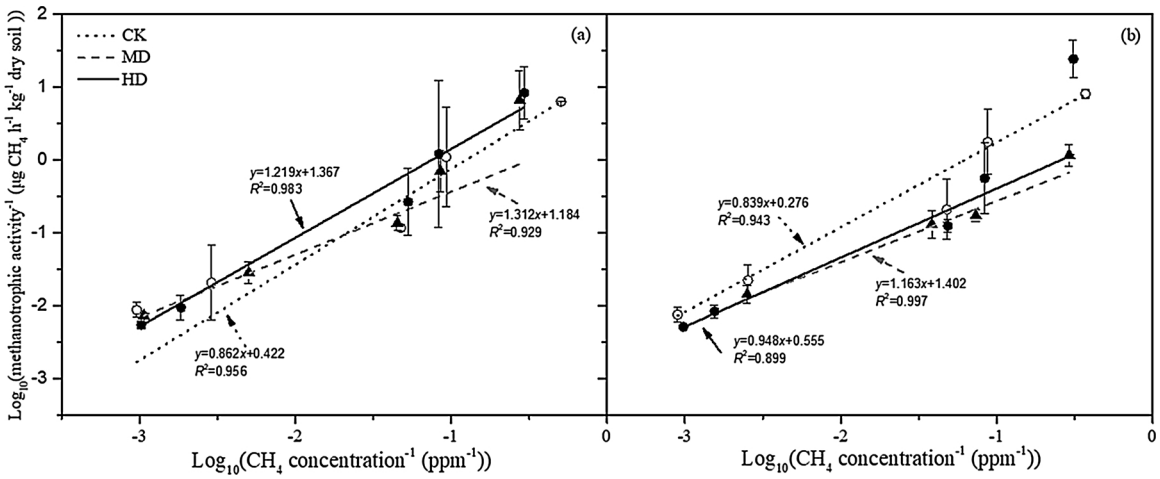

Fig. 4. Kinetics of methanotrophic activity in a Tibetan alpine meadow under ambient temperatures (a) and warming (b). Methanotrophic activity was measured at 2, 10, 20, 500 and 1000 ppm CH_4 concentrations. CK, natural meadow; MD, moderately degraded meadow; HD, heavily degraded meadow.

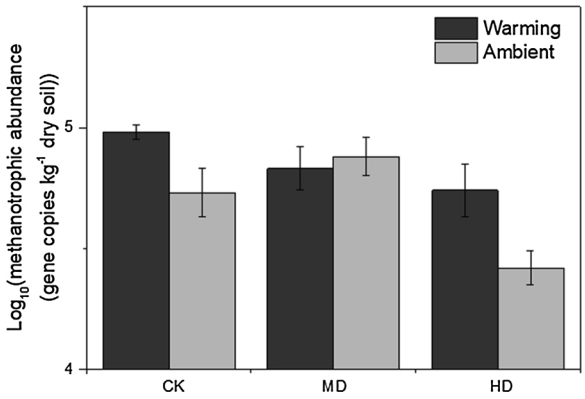

Fig. 5. Differences in soil methanotrophic abundance based on the logarithm of *pmoA* gene copy numbers in a Tibetan alpine meadow subjected to warming along a degradation gradient. CK, natural meadow; MD, moderately degraded meadow; HD, heavily degraded meadow.

4.2. Effects of warming and degradation on soil methanotrophic activity and abundance

The Tibetan alpine meadows act as a significant soil CH_4 sink, and the mean rate of oxidation strength (an average of -71.5 ± 2.5 μg CH_4 m^{-2} h^{-1}) (Wei et al., 2015) was greater than that reported in temperate grasslands (-52.6 ± 6.5 μg CH_4 m^{-2} h^{-1}) (Wang et al., 2009) and in subtropical forest ecosystems (-60.2 ± 1.8 μg CH_4 m^{-2} h^{-1}) (Butterbach-Bahl et al., 2002). Warming has been reported to increase soil CH_4 uptake in the Tibetan alpine meadow environment (Chen et al., 2013). Soil methanotrophic activity is responsible for soil CH_4 uptake, as evidence shows that methanotrophic activity has a good relationship with CH_4 uptake in grassland ecosystems (Zhou et al., 2008; Zheng et al., 2012; Nazaries et al., 2013; Lin et al., 2015).

Consistent with previous results (Zheng et al., 2012), we found that warming tended to increase soil methanotrophic activity (Table 2 and Fig. 4). The reason for this could be attributed to the increased microbial metabolism and enzyme activity associated with CH_4 oxidation under warming, confirmed by higher methanotrophic abundance under warming (Fig. 5). On the other hand, we found that degradation significantly decreased soil methanotrophic activity (Table 2 and Fig. 4), which was supported by lower soil methanotrophic abundance (Fig. 5).

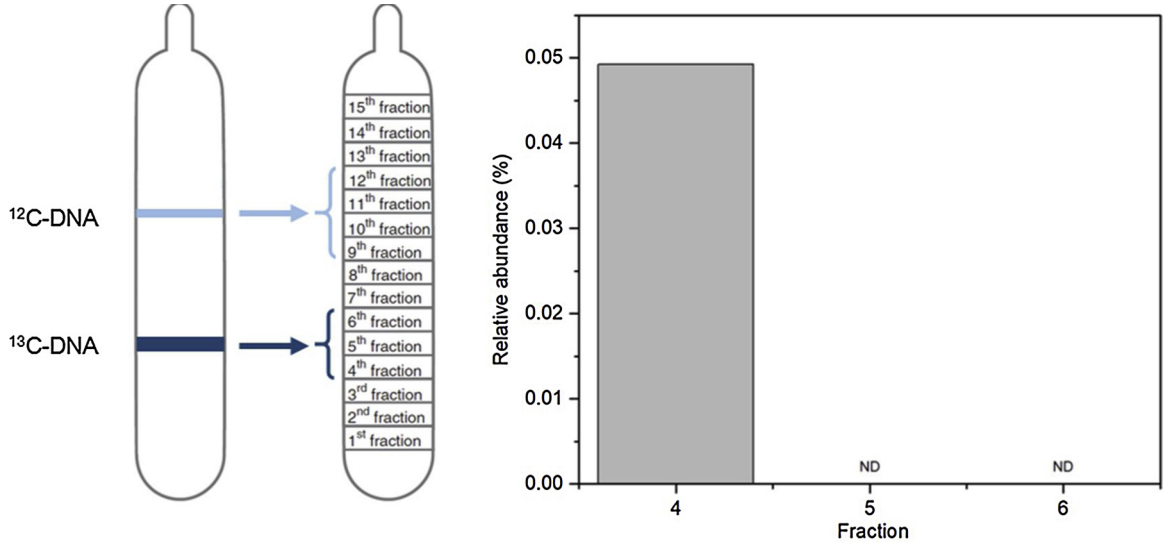

Fig. 6. *Methylosinus* found in the fourth, fifth and sixth fractions of DNA in soils incubated with 2000 ppm $^{13}CH_4$ for three weeks collected from a Tibetan alpine meadow, as detected by 16S rRNA high-throughput sequencing. ND, not detected.

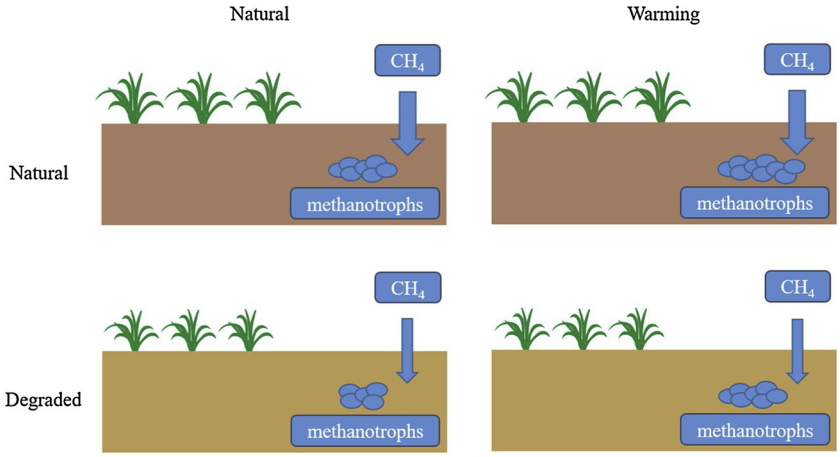

Fig. 7. Schematic diagram showing changes in soil C and N pools as well as methanotrophic activity and abundance in alpine meadows of the Tibetan Plateau under warming and degradation. The changes in the soil C and N pools are indicated by the soil color: the lighter color indicates lower soil C and N contents, the darker color indicates higher soil C and N contents. The width of the arrows indicates the strength of soil methanotrophic activity and number of blue methanotrophs indicates methanotrophic abundance.

The reason for this might be the lower soil moisture content under degradation (see Table 1). Our previous study has shown that soil methanotrophic activity followed a hump pattern in response to soil moisture content (Zhou et al., 2014), with the highest methanotrophic activity seen at optimal soil moisture contents, with lower activity seen when soil moisture contents increased or decreased. In addition, many studies have stated that among all soil properties, soil moisture contents, to some extent, outweigh all the other properties in driving community structure and methanotroph activity (Fest et al., 2015).

4.3. The active methanotrophic communities in ambient control soils

Previous studies showed that methanotrophic communities in soils of the Tibetan alpine meadows were dominated by USCγ (a Type I methanotroph) according to the clone library of *pmoA* genes (Zheng et al., 2012) and by using high-throughput sequencing of *pmoA* genes (Kou et al., 2017). In contrast with these studies, we found that soil methanotrophic communities were dominated by *Methylocystis* (a Type II methanotroph) according to high-throughput sequencing of *pmoA* genes in these alpine meadows (Table S3). We acknowledge that these *pmoA* primer sets used in this study could cause some bias, although these *pmoA* primers have been widely used before (Bourne et al., 2001; Kolb, 2009). In this study, based on 16S rRNA high-throughput sequencing in combination with DNA-SIP, the active soil methanotrophic communities were dominated by *Methylosinus* (a Type II methanotroph) (see Fig. 6), which is different from the result with *pmoA* genes. The reason for this could be twofold. First, only a fraction of methanotrophs were active, though a large number of methanotrophs were detected (Dumont et al., 2011). Second, the DNA-SIP used in this study was based on laboratory incubation at 2000 ppm of $^{13}CH_4$ in the headspace and the CH_4 concentration was much higher than the ambient CH_4 concentration in this region. The higher CH_4 concentration might stimulate the growth of other facultative communities of methanotrophs such as methylotrophs, which was partly supported by the higher abundance of the methylotrophic genus *Methylobacterium* (see Fig. S4). The DNA-SIP method has been widely used to detect active methanotrophic communities in landfills (Dumont et al., 2011) and wetland soils (Cai et al., 2016) containing high CH_4 concentrations. Further study is needed to test if the method is feasible for detecting active methanotrophic communities in grasslands or forest ecosystems under ambient atmospheric CH_4 concentrations.

5. Conclusion

After 4 years of warming, we found a significantly increase in soil EON contents, and non-significant increases in soil TC and TN contents as well as EOC contents in the Tibetan alpine meadows. The effect of degradation was considerably stronger, significantly decreasing soil TC and TN contents as well as EOC and EON contents. By using Michaelis–Menten kinetic analysis of soil methanotrophic activity, we found that warming tended to increase soil methanotrophic activity, whereas degradation significantly decreased it, which was supported by changes in soil methanotrophic abundance among the treatments (Fig. 7). However, warming and degradation had no interactive effects on methanotrophic activity and abundance. The methanotrophic community structure was dominated by Type II methanotrophs and the active methanotrophs were dominated by *Methylosinus* (a Type II methanotroph) in natural soils. Overall, the important ecosystem functions of soil C and N pools and CH_4 oxidation capacity are more strongly influenced by degradation than by warming. Indeed, under warming these processes display a certain resilience to degradation in this region.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2019.03.020.

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