Plant Soil (2016) 406:101–116
DOI 10.1007/s11104-016-2868-3

Microbial acclimation triggered loss of soil carbon fractions in subtropical wetlands subjected to experimental warming in a laboratory study

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Received: 12 November 2015 / Accepted: 21 March 2016 / Published online: 29 March 2016
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Abstract
Aims Wetlands store a substantial amount of soil organic carbon (SOC), and their response to climate warming is critical for predicating global carbon (C) cycling in future climate change.
Methods To understand whether warming causes substantial C loss in wetland soils, a 6-year microcosm experiment was carried out to examine the impact of rising temperature (3–5 °C) on SOC and its two fractions (labile versus recalcitrant) in six types of wetland soils with varied nutrient status.
Results Warming decreased SOC contents in nutrient-enriched soils by invoking a large loss in recalcitrant organic C fractions, while in nutrient-poor soils SOC loss was limited by substrate limitation. With low temperature ranges in the winter (1–10 °C), warming increased the microbial capacity for recalcitrant organic C acquisition greater than that for labile organic C fractions. A relatively higher cross-site contribution of fungi in warmed soils as one strategy of microbial acclimation to rising temperature implies an adjustment of microbial C utilization patterns, leading to substantial C loss in wetland soils.
Conclusions In order to maintain the functional roles of wetlands for C sequestration, our results further suggested that more attention should be paid to nutrient-enriched wetlands in future climate warming scenarios.

Keywords Soil organic carbon · Experimental warming · Wetland · Microbe · Extracellular enzymes

Responsible Editor: Per Ambus.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-016-2868-3) contains supplementary material, which is available to authorized users.

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Introduction

Cool and anaerobic conditions favor reduced rates of decomposition, resulting in high accumulation of soil organic carbon (SOC) in wetland soils (Fissore et al. 2009). The projected rise in global surface temperature (2.6–4.8 °C increase) (IPCC 2013) is altering wetland habitats and promoting soil carbon (C) loss through accelerated decomposition rates (Verhoeven et al. 2006). Although potential consequences of such warming on SOC in the forest, tallgrass prairies, and tundra have been extensively studied (von Luetzow and Koegel-Knabner 2009), inland wetlands with great potential for C sequestration have received limited attention.

SOC is comprised of a continuum of organic fractions with varying turnover rates (Davidson and Janssens 2006; Marin-Spiotta et al. 2009), which chemically divides into labile and recalcitrant C fractions (Belay-Tedla et al. 2009). Labile organic carbon (LOC) only constitutes a very small amount of SOC, while its fast-cycling and readily degradable properties largely determine its rapid and strong response to environmental changes (Melillo et al. 2002; Bradford et al. 2008). However, the majority of SOC is comprised of recalcitrant fractions through a biologically-controlled humification process (Branco de Freitas Maia et al. 2013), and the ultimate fate of soil C storage is determined by recalcitrant organic fractions with relatively slow turnover rates and their large amounts (Belay-Tedla et al. 2009). Large SOC loss always invokes decreased recalcitrant organic C (REC). Temperature sensitivity of REC has been well recognized for its critical role in determining the fate of SOC over decades to centuries in global warming scenarios (Fierer et al. 2005a; Davidson and Janssens 2006; Conant et al. 2008). Long-term observations regarding how different organic C fractions (LOC versus REC) with distinct ecological functions respond to warming may better improve our knowledge about the fate of SOC stored in wetlands.

Extensive studies have revealed a rapid decline in readily degradable substrates in soils when subjected to warmed stimulation (Melillo et al. 2002; Eliasson et al. 2005; Bradford et al. 2008; Conant et al. 2008). The "apparent" acclimation of soil respiration to warming is mainly due to the substrate limitation when soil microorganisms consume much more readily degradable C in the first stage of warming (Eliasson et al. 2005). Such rapidly declined fractions of readily degradable substrates allocated to microbial utilization may alleviate C loss through decreased microbial biomass and associated reduction in enzyme production before any detectable changes in SOC occur. As a result, SOC remains unchanged or the effect of warming on SOC is negligible. However, beyond the "substrate-limitation" theory, Allison et al. (2010) suggest that the SOC response to warming is dependent on changed microbial physiology and communities adapted to new environments, leading to an upward adjustment of C utilization and accelerated SOC loss.

In addition to temperature, soil nutrient content also plays an important role in soil C decomposition from wetlands. Soil respiration and C decomposition have been found much higher in nutrient-enriched wetland soils compared to others (Wang et al. 2012). Along an organic matter gradient at the Charles City Wetland, soils heavily loaded with organics released significantly more CO_2 than those that have received little or no organic matter (Winton and Richardson 2015). As a result, microbes were found more sensitive with increasing nutrient status in wetlands (Wright et al. 2009). The high sensitivity of microbial communities in response to changes in environmental conditions, such as rising temperature, may serve as sensitive indicators of changes in soil C decomposition in wetlands. In permafrost soils of high nutrient contents, large C loss was observed (Schuur et al. 2009). The permafrost melting due to rising temperature may alleviate diffusion constraints on enzyme activity, and thus generate substantial SOC losses, indicating the importance of nutrient availability in determining the fate of wetland soil C destiny (Mack et al. 2004). Therefore, it is assumed that nutrient-enriched soils may be less constrained by loss in nutrient availability under warming. Despite this, warming effects on wetland soils differing in soil nutrient contents are rarely studied.

In our previous studies, increased C fluxes from nutrient-enriched wetland soils have been observed during 1–2.5 years of warming (Wang et al. 2012). However, we further found a decline in fractions of available substrates utilized by microbes, which exerted a potentially negative feedback on initially accelerated C loss after 4.5 years of warming (Wang et al. 2014). Up to now, we still lack the synthesized results to give an overall framework of how microbial acclimation to warming influences patterns of C loss under sustained warming scenarios (1–6 years), and whether there is divergent responses to warming among our different wetland soils. In the present study, a real-time temperature-controlled system was developed in May 2008 which simulates warming scenarios to investigate SOC dynamics during a 6-year incubation of soils from six subtropical wetlands (Zhang et al. 2012). All of these wetlands belong to inland shallow and enclosed areas of freshwater with an annual mean water depth of 1.2 m. To avoid litter being incorporated into soils that can counteract SOC loss, the aboveground biomass of the dominant macrophytes were periodically cut manually during observation. A humid habitat in the soils was continuously maintained to avoid the possibility of warming-induced reductions in soil moisture. After this,

we were able to focus our research solely on warming effects on SOC in the wetlands. The objectives of this study are to investigate (*i*) whether there is substantial SOC loss in studied wetland soils when subjected to experimental warming, (*ii*) whether such SOC loss is dependent on different wetland soils (nutrient-poor versus nutrient-enriched), and the underlying mechanisms mediated by microbial acclimation, and (*iii*) which environmental factors act as key regulators influencing the stocks of soil C in wetlands.

Materials and methods

Microcosm configuration and sample description

A custom-built novel microcosm (Online Resource 1) simulating climate warming under a minute-scale for both daily and seasonal temperature variations was developed using separately monitored water-bath jackets under both current ambient water temperature conditions (control) and simulated warming conditions of 3–5 °C above ambient water temperature (warmed). The temperature difference was automatically set at 5 °C, while the in situ measured temperature difference ($p < 0.001$) was 3–5 °C and never exceeded 5 °C. Seasonal temperature variations had a broad range from 1.2 °C in winter to 35.7 °C in summer (Online Resource 1). Specifics regarding the configuration and corresponding operation of this microcosm system since May 2008 have been reported previously (Zhang et al. 2012). This device offered a high resolution temperature comparison, good reproducibility, and the ability to simulate realistic warming conditions.

The study sites were located within the delta of the Yangtze River (China). The climate in this area is subtropical monsoon, with an annual average rainfall of 1380 mm and an annual average air temperature of 27.8 °C in the summer and 6.37 °C in the winter (2005–2010 inclusive) from the China Meteorological Data Sharing Service System (http://cdc.cma.gov.cn/). Six wetlands with shallow waterbodies (0.8–1.5 m deep) differing in land uses and nutrient status were selected (Online Resource 2). In brief, YaTang riverine (YT) wetland is polluted by duck farms, while XiaZhuHu (XZ) wetland is impacted by aquaculture. Both of them are heavily loaded with nutrients, typical of contaminated wetlands with the highest trophic status. BaoYang (BY), XiXi (XX), JinHu (JH), and ShiJiu (SJ) wetlands are generally preserved for tourism and used as water reservoirs, typical of recovered wetlands. The organic C contents of YT and XZ soils are much higher ($p < 0.01$) that in JH and SJ soils (Online Resource 2). Particularly, SJ originated from a waterlogged paddy field with the lowest nutrient level among the six selected wetlands. The native vegetation found in these wetlands included some floating-leaved (e.g., *Trapa spp*.) and emergent (e.g., *Phragmites communis*, and *Acorus calamus*) aquatic plants. The observed dominant macrophytes in each specific wetland are shown in Online Resource 2.

Intact soil cores (0–20 cm in deep) sampled from in situ wetland soils using a stainless steel column sampler (10 cm in inner diameter) were filled into transparent PVC wetland columns (12 soil cores for each wetland site). After sediment filling, each column was refilled with 20 cm of ambient overlying water. These wetland columns were put in the microcosm system (Online Resource 1; for each wetland site: controls, $n = 6$; warmed, $n = 6$) in May 2008 and have been in continuous operation. Details regarding preparation of wetland columns were described previously (Zhang et al. 2012).

Laboratory analysis of soil samples

We began our samplings only after a 1.5-year preincubation of these wetland columns (started in May 2008) in order to ensure the stability of this temperature-controlled microcosm system. Each 0–5 cm undisturbed top soil core in a vertical profile was then collected from these columns.

Total SOC and different fractions In order to reveal annual variations in the responses of soil C contents to warming, soil samples were collected in November or December of each year (2009–2013 inclusive, at the end of each year) during the 6-year incubation. These samples were freeze-dried and then ground in a mortar and pestle before being passed through a 1-mm mesh sieve. A standard potassium dichromate oxidation method (Bao 2000) was used to determine SOC. LOC was determined using a modified $KMnO_4$ oxidation procedure (Mirsky et al. 2008; Mandal et al. 2011). In brief, 20 ml alkaline $KMnO_4$ solution (0.02 mol·L^{-1} $KMnO_4$ and 0.1 mol·L^{-1} $CaCl_2$) was added to each tube filled with 5 g freeze-dried soil samples. After shaking and settling, the absorbance at 550 nm of the supernatant aliquot was measured using a UV-2550 spectrophotometer (Shimadzu Corporation, Japan). The amount of

oxidized C was determined by the change in concentration of alkaline $KMnO_4$ solution. Such oxidized C could represent the fractions of readily degradable substrates involved in enzymatic reactions of microbial-mediated decomposition of LOC (Mirsky et al. 2008). The REC was calculated as the difference between SOC and LOC.

Mass-specific potential extracellular enzyme activity Microbial metabolisms are greatly influenced by surrounding temperature. In order to understand how microbes drive soil C loss in wetlands, soil samples were collected in two seasons with contrasting temperature ranges, i.e., July 16 (summer samples) and December 15 (winter samples) of 2013 at the last year of the 6-year warming period. Fresh samples were immediately transported to laboratory for analysis. Soil microbial biomass carbon (SMBC) was determined using the fumigation-extraction method described by Wu et al. (1990). Potential extracellular enzymatic activities, indicative of the maximum enzymatic activities, were measured under saturated substrate concentrations to represent in situ enzymatic reactions without substrate constraint (Wallenstein and Weintraub 2008) and were divided as hydrolytic or oxidative. For hydrolytic enzymatic activity, four enzymes (α-1, 4-glucosidase [AG]; β-1, 4-glucosidase [BG]; cellobiohydrolase [CBH]; β-1, 4-xylosidase [BXYL]) involved in labile C cycles were measured using fluorescent dye-conjugated substrates. For these assays, standard methods were followed according to previous reports (German et al. 2011). In brief, sample suspensions were prepared by homogenizing 1.0 g fresh soil samples with 125 mL of 50 mM sodium acetate buffer. The pH of the sodium acetate buffer was adjusted to 7.0 in order to match the in situ pH of soil samples (German et al. 2011). For each soil sample, 12 replicate wells were simultaneously used in a 96-well solid black fluorescent plate (Corning Inc., Costar 3603, USA). In each well, 200 μL of soil suspension and 50 μL of 200 μM substrate were first added. After that, sodium acetate butter (50 mM), fluorescent dye reference standard (10 μM), and fluorescent dye-conjugated substrate (200 μM) were selectively added separately or pairwise in each well following the procedures for calculation of enzymatic activity (German et al. 2011). Fluorescent plates were incubated in the dark at a constant temperature of 20 °C for 4 h, and then 10 μL of 1.0 M NaOH was added to each well to terminate the reaction and enhance the fluorescent intensity of the dye. A time frame of 60 s between the addition of NaOH and reading of plates was accurately controlled to minimize any potential variations in enzyme activities (German et al. 2011). Fluorescent intensities were measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Inc., Winooski, VT, USA) with 365 nm excitation and 460 nm emission filters.

For oxidative enzymatic activity, two kinds of oxidative enzymes involved in recalcitrant C degradation (phenol oxidase [PHENOX] and peroxidase [PEROX]) were analyzed by colorimetric methods according to Li et al. (2008). Briefly, both enzymes were assayed spectrophotometrically at 430 nm using 1,2,3-benzenetriol (PAPG) as the substrate, followed by quantification of the red PAPG oxidation product. For PHENOX assays, 10 mL of 1 % PAPG was added to 1 g of soil and homogenized before incubation in the dark at 30 °C for 3 h. After incubation, 4 mL of citric and phosphoric acid buffer (pH = 4.5) was added. For PEROX assays, 10 mL of 1 % PAPG and 2 mL of 0.5 % H_2O_2 were added to 1 g of soil and homogenized before incubation in the dark at 30 °C for 1 h. After incubation, 2.5 mL of 0.5 mol·L^{-1} HCl was added. For both enzymes, 35 mL of diethyl ether was used to absorb the oxidation product; subsequently, absorbance of the solution was measured. Labile C acquisition capacity (L-acq) and recalcitrant C acquisition capacity (R-acq) (Huang et al. 2011) were estimated using the following formulas:

$$\text{L-acq}\left(\text{nmol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}\right) = \text{Hydrolytic enzymatic activity of AG} + \text{BG} + \text{CBH} + \text{BXYL} \quad (1)$$

$$\text{R-acq}\left(\text{nmol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}\right) = \text{Oxidative enzymatic activity of PHENOX} + \text{PEROX} \quad (2)$$

To make variables comparable, we normalized L-acq and R-acq to SMBC because both decreased, followed by a decline in SMBC after the 6-year warming period, and we assumed that warming would increase microbial metabolic rates and reduce the allocation of C to growth per unit microbial biomass. Therefore, mass-specific L-acq and R-acq were calculated further using the following formulas:

$$\text{Mass-specific L-acq} = \text{L-acq}\left(\text{nmol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}\right)/\text{SMBC} \quad (3)$$

$$\text{Mass-specific R-acq} = \text{R-acq}\left(\text{nmol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}\right)/\text{SMBC} \quad (4)$$

Quantitative polymerase chain reaction (PCR) of microbial communities Soil samples collected on December 15, 2013 were used for this analysis. DNA from 0.2 g of fresh samples was immediately extracted using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, USA). Each 20 μL quantitative PCR (qPCR) reaction contained 10 μL of iQ™ SYBR Green Supermix (Bio-Rad, Munich, Germany), 0.4 μL each of 10 μM forward and reverse primers, 8.2 μL of sterile DNA-free water, and 1.0 μL of diluted DNA as the template. Primer sets for the bacteria (Eub338, Eub518), fungi (ITS1f, 5.8 s), and archaea (ARCH21f, ARCH958r) were reported previously (Cytryn et al. 2000; Fierer et al. 2005b). The reaction was carried out in a Biorad CFX96 system (Bio-Rad, Munich, Germany) with the following protocol: 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 60 s. Three plasmid standards containing a specific target region of the ribosomal gene for each of bacteria, archaea, and fungi, respectively were generated. Three standard curves were obtained from a 10-fold serial dilution of these plasmid standards, the concentration of which ranged from 5×10^{-8} to 5×10^{-3} ng DNA per reaction. From the C_t values linked to the standard curve, the absolute gene copies of bacteria, fungi, and archaea were calculated. All qPCR reactions were run in quadruplicate for each DNA sample. Due to the differential amplification efficiency of certain taxa, we confined our results with the relative ratios of gene copies (i.e., fungal-to-bacterial and archaeal-to-bacterial).

In situ measurements of environmental factors

Through the whole year (2013), water table level relative to the soil surface (horizontal soil line centered on zero) in each wetland soil column (Online Resource 1) was manually measured bimonthly. At the same time, the dissolved oxygen (DO) concentration and pH in overlying water were measured at the bottom of the each water column to a depth of 2 cm above the surface soil (sediment-water interface) using a portable DO meter (HQ30d, HACH Corporation, USA) and a pH reader (SensoDirect pH 200, Lovibond, German), respectively. The moisture of soil samples was determined after drying at 105 °C for 24 h. The annual average value of water table level was 4.3 ± 2.5SE cm lower under warming. Accompanied by this, the annual average value of DO in overlying water was 7.65 mg L^{-1} in controls, while it was 8.88 mg L^{-1} in warmed samples. The pH and soil moisture remained unchanged ($p > 0.05$) between the treatments (control versus warmed; Online Resource 3).

Statistical analyses

The degree of similarity for the responses of three variables, including LOC, REC and SOC to experimental warming (2009–2013, inclusive) for the six studied wetlands was analyzed both by linear model distance-based principal component analysis (PCA) between samples ($n = 6$) and hierarchical clustering analysis based on squared Euclidian distance to show whether the responses of these variables were consistent or different among these soils. The data, calculated as percent changes (%) between treatments (control versus warmed), were used in these analyses since the absolute values varied with different soils. These soil C-related indices (LOC, REC, and SOC) were further examined using repeated measures three-way analysis of variance (repeated ANOVA) to test (*i*) whether these variables differed with geographic location (site), treatment (control versus warmed), and sampling time in order to determine whether changes in SOC subjected to warming varied with observation years and whether SOC loss is sustainable, and (*ii*) the interaction effects among the three factors to determine whether warming effects on these variables are dependent on studied wetland soils or sampling time. Similar to the C-related indices, microbial variables (SMBC, L-acq, R-acq, mass-specific L-acq, mass-specific R-acq, fungus-to-bacteria ratio, and archaea-to-bacteria ratio) were also examined by repeated measures ANOVA. Student's *t*-tests were further used to test whether there were significant differences in these variables for each specific wetland between treatments (control versus warmed). To understand how microbial changes were derived by environmental changes, a redundancy analysis (RDA) plus variation partitioning was performed using CANOCO for Windows version 4.5 to link all microbial variables to the environmental variables.

Q_{10} is defined as the proportional change of a biological reaction when temperature increases by 10 °C (Sierra 2012). Here, the Q_{10} value of mass-specific L-acq and R-acq is given to calculate the effect of rising temperature on C acquisition capacity per biomass in

summer versus winter samples according to the following equation (Leifeld and Fuhrer 2005):

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)} \quad (5)$$

where R_2 and R_1 are mass-specific L-acq or mass-specific R-acq detected in soil samples under control and warming, respectively, T_1 is ambient water temperature, and T_2 is simulated warming conditions of 3–5 °C above ambient water temperature in the summer or winter. To explain why there were significant changes in fungal communities in response to warming, linear regression analysis of fungus-to-bacteria ratio against environmental factors was performed for soil samples from the six wetlands. Before analysis, the data were tested for the assumption of normality, and thus data for the fungus-to-bacteria ratio, temperature, and DO concentration were $\log_e$-transformed to meet requirements. A stepwise method was used in linear regressions to evaluate whether other environmental variables beyond temperature explained the relative changes in fungal communities significantly. The regression model with R^2 value was reported, where insignificant ($p > 0.05$) variables were removed. All statistical analyses described above were conducted using SPSS for windows (version 17.0).

Results

SOC and different fractions

Both PCA and hierarchical clustering analysis clearly showed distinct responses of LOC, REC, and SOC in different wetland soils to warming (Online Resource 4), implying that warming impacts on different soil C pools were dependent on the wetland studied. In details, dynamics in C responses to warming in XZ and YT wetland soils clustered together and showed higher clustering degrees compared to other wetlands. In hierarchical clustering analysis, consistently SJ and JH wetlands also clustered together, and separated from other wetlands with the furthest similarity distances (Online Resource 4). Three-way ANOVA further showed that there was a significant ($p < 0.001$) interaction effect in soil C pools between site and warming. We classified these six wetlands with respect to LOC, REC, and SOC contents into three groups (Fig. 1), i.e., nutrient-low (SJ & JH), nutrient-moderate (XX & BY), and nutrient-enriched soils (XZ & YT). Results showed that significant ($p < 0.05$) decreases in LOC occurred for all studied wetlands throughout the observation period (except for 2010), ranging from 11.3 % to 24.7 %. REC concentrations under warming regimes consistently showed significant ($p < 0.05$ or 0.01) decreases in nutrient-enriched and –moderate soils compared to controls during 2010–2013, a lag time response compared to LOC. Approximately 10 % of the SOC was lost from these soils after a 6-year warming period, and nutrient-enriched wetland soils showed the strongest responses to warming with respect to SOC loss (Fig. 1, by 10.6 % on average). For nutrient-poor wetland soils (containing 1.2–2.7 % of SOC per dry soil), loss in REC (by 1.93 % on average) and SOC (by 4.10 % on average) between control and warmed samples were minor and insignificant ($p > 0.05$, except for SJ collected in 2011) throughout the 6-year period. Three-way ANOVA also revealed that the loss in SOC subjected to warming when compared to controls did not vary ($p = 0.388$) with sampling year since 2010. Therefore, no further warming-induced decomposition of SOC occurred since 2010. Within the same levels of soil nutrient status, the interaction effect between site and warming was also insignificant ($p > 0.05$), further indicating soil C temporal changes under warming may have responded in a similar way for the same soil groups.

Mass-specific extracellular enzymatic activity

Experimental warming generally decreased ($p < 0.001$) SMBC in samples collected from both the summer and winter of 2013, after the 6-year warming period (Table 1). For example, for soil samples collected in the winter, SMBC ranged from 0.51 $mg \cdot g^{-1}$ (SJ) to 1.71 $mg \cdot g^{-1}$ (YT) in controls and from 0.37 $mg \cdot g^{-1}$ (SJ) to 1.12 $mg \cdot g^{-1}$ (YT) in warmed samples. SMBC did not vary with sampling season as no significant differences were found in SMBC between summer and winter samples by three-way ANOVA. Accompanying the decreased SMBC, potential enzymatic activities decreased. Of the four hydrolytic enzymes, AG, BG, and BXYL decreased by 22.1 %, 17.4 %, and 14.3 %, respectively, for all summer samples as a whole, while these values were 25.0 %, 18.2 %, and 20.1 % for winter samples. CBH remained unchanged between treatments for all samples collected in the summer and winter (Table 1). The percent change in AG was highest among

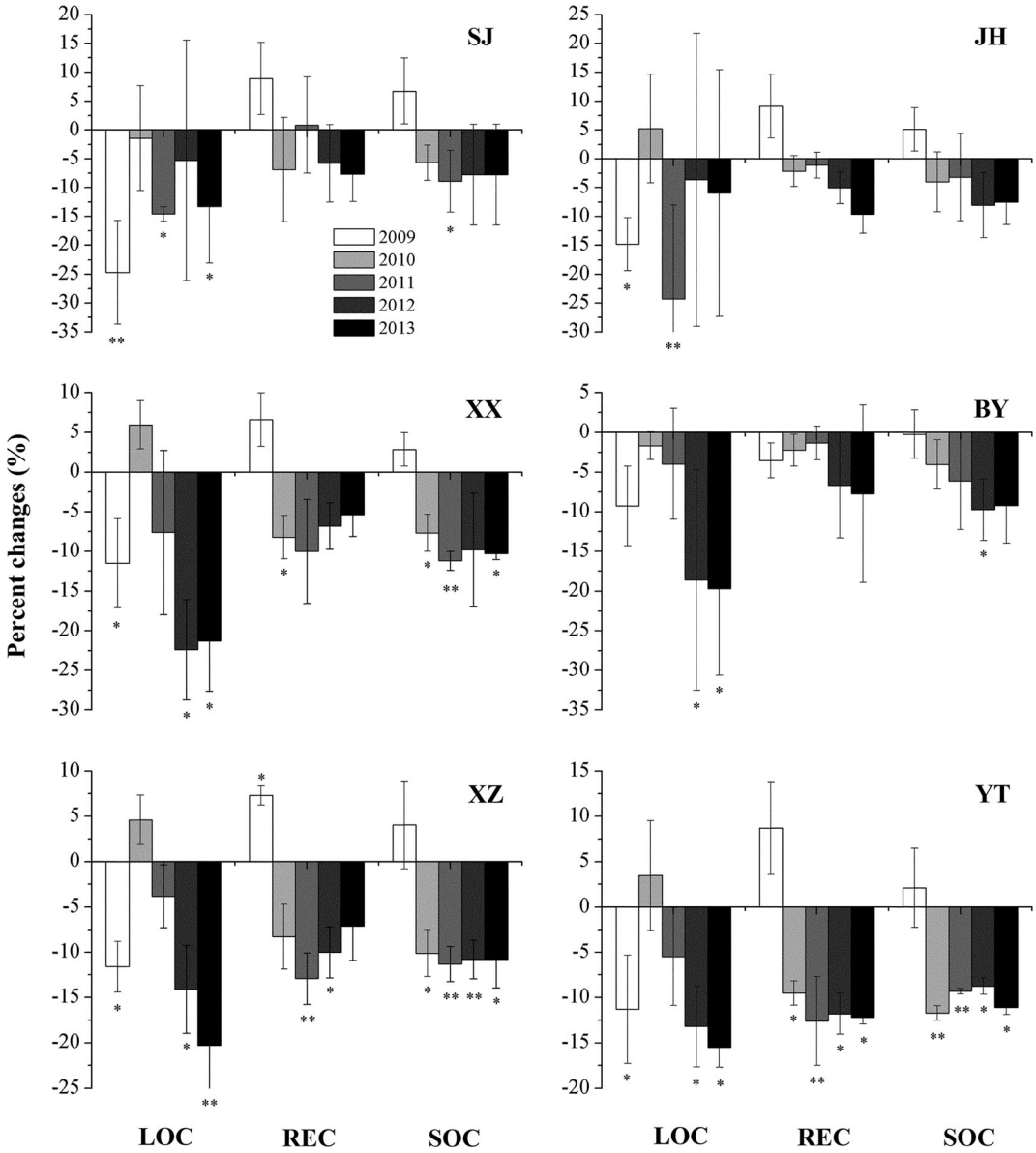

Fig. 1 Percent changes (%) in soil carbon fractions in warmed wetland soils compared to controls. Labile organic carbon (LOC; $g \cdot kg^{-1}$), recalcitrant organic carbon (REC; $g \cdot kg^{-1}$), and soil organic carbon (SOC; $g \cdot kg^{-1}$) are shown in each plot. Soil samples were collected in December of each year (2009–2013 inclusive). SJ and JH belong to nutrient-poor sites, XX and BY belong to nutrient-moderate sites, and XZ and YT belong to nutrient-enriched sites based on pre-clustering analysis (see Online Resource 4). $*p < 0.05$, $**p < 0.01$ between warmed and control samples by Student's *t*-test. *Error bars* in each plot represent standard errors

all hydrolytic enzymes accompanied by decreased SMBC. Similar to SMBC, the response of hydrolytic enzymes to warming is cross-site and independent of the soil studied. For example, BXYL decreased by 27.7 % and 23.1 % for SJ (nutrient-poor soil) and YT (nutrient-enriched soil), respectively (Table 1). Of the two oxidative enzymes (PHENOX and PEROX), the percent change in PEROX activity (decreased by 8.51 %) in response to warming was much lower than that of PHENOX (decreased by 16.2 %; Table 2). When normalized to SMBC, mass specific L-acq (Fig. 2 a and c) and mass specific R-acq (Fig. 2 b and d) showed obvious seasonal patterns between the treatments (control versus warmed). Winter samples generally showed greater responses to warming than summer samples, especially for JH, XX, and YT. For example, the Q_{10}

Table 1 Soil microbial biomass carbon (SMBC; $mg \cdot g^{-1}$), and potential enzymatic activities ($nmol \cdot h^{-1} \cdot g^{-1}$) of four hydrolytic enzymes, α-1, 4-glucosidase (AG); β-1, 4-glucosidase (BG); cellobiohydrolase (CBH); and β-1, 4-xylosidase (BXYL) involved in labile carbon cycling

Wetlands[a]	SMBC		AG		BG		CBH		BXYL	
	Control	Warmed	Control	Warmed	Control	Warmed	Control	Warmed	Control	Warmed
Summer samples (collected in June)										
SJ	0.56 ± 0.06	**0.33** ± 0.04**[b]	143 ± 8.54	**116** ± 2.75**	233 ± 21.1	**169** ± 16.3**	64.2 ± 5.62	**63.5* ± 2.58**	644 ± 239	465 ± 29.2
JH	0.68 ± 0.11	0.57 ± 0.07	118 ± 11.1	107 ± 13.4	262 ± 49.1	**191* ± 12.5**	70.4 ± 5.47	65.9 ± 3.87	477 ± 43.9	417 ± 27.3
XX	1.09 ± 0.12	1.00 ± 0.11	218 ± 15.0	**182* ± 14.0**	425 ± 47.4	389 ± 37.5	79.5 ± 4.49	**73.9* ± 2.90**	470 ± 39.5	453 ± 32.4
BY	1.12 ± 0.14	**0.92* ± 0.05**	171 ± 5.79	**150** ± 7.52**	314 ± 34.1	**271* ± 9.93**	73.3 ± 3.41	**74.7* ± 3.56**	540 ± 40.5	514 ± 22.8
XZ	1.37 ± 0.08	**1.13* ± 0.04**	145 ± 9.49	**122* ± 11.8**	298 ± 71.0	236 ± 28.7	61.7 ± 3.50	61.1 ± 3.14	560 ± 19.8	**483* ± 24.6**
YT	1.55 ± 0.12	**1.18* ± 0.06**	258 ± 19.5	**177** ± 17.4**	786 ± 54.4	**511** ± 59.3**	97.2 ± 4.77	**86.3* ± 7.29**	677 ± 43.7	**521** ± 22.0**
p-value	**< 0.001**		**< 0.001**		**< 0.001**		0.054		**0.006**	
Winter samples (collected in December)										
SJ	0.51 ± 0.17	0.38 ± 0.14	188 ± 14.4	**158* ± 17.7**	381 ± 43.2	**200** ± 19.0**	164 ± 15.0	151 ± 9.78	158 ± 6.88	**126** ± 7.82**
JH	0.69 ± 0.08	**0.49* ± 0.08**	404 ± 67.2	337 ± 9.15	437 ± 75.0	422 ± 80.7	166 ± 24.7	174 ± 10.27	420 ± 49.1	**342* ± 33.7**
XX	1.02 ± 0.10	**0.62** ± 0.07**	222 ± 22.5	**168* ± 21.6**	163 ± 10.4	154 ± 10.0	150 ± 13.1	151 ± 10.2	221 ± 72.1	156 ± 17.5
BY	0.84 ± 0.08	0.73 ± 0.05	227 ± 40.2	**173* ± 8.40**	182 ± 17.4	167 ± 9.62	162 ± 12.3	155 ± 10.0	152 ± 8.15	**118** ± 13.5**
XZ	1.24 ± 0.03	**0.97** ± 0.08**	209 ± 22.8	**167* ± 13.1**	571 ± 74.9	**194** ± 19.5**	159 ± 9.22	164 ± 6.52	160 ± 13.1	**134* ± 8.62**
YT	1.70 ± 0.08	**1.16* ± 0.23**	292 ± 29.7	266 ± 45.2	1016 ± 86.0	**821* ± 83.9**	178 ± 12.2	162 ± 11.3	162 ± 6.50	**140* ± 13.0**
p-value[c]	**< 0.001**		**<0.001**		**<0.001**		0.411		**<0.001**	

[a] Soil samples were collected in summer and winter of 2013 from six studied wetlands between treatments (control versus warmed). The data are expressed as mean ± standard error. SJ, ShiJiu; JH, JinHu; XX, XiXi; BY, BaoYang; XZ, XiaZhuHu; YT, YaTang

[b] Bold indicates a significant difference with warming compared to controls with $*p < 0.05$ or $**p < 0.01$ by Student's *t*-test

[c] The bottom row indicates *p*-values from ANOVA conducted to test for differences between treatments across all wetlands as a whole

Table 2 Potential enzymatic activity (nmol·h^{-1}·g^{-1}) of two oxidative enzymes, phenol oxidase (PHENOX) and peroxidase (PEROX), involved in recalcitrant carbon degradation

Wetlands[a]	Summer samples (June)				Winter samples (December)			
	PHENOX		PEROX		PHENOX		PEROX	
	Control	Warmed	Control	Warmed	Control	Warmed	Control	Warmed
SJ	694 ± 23.6	**579** ± 43.4**[b]	1269 ± 35.0	**1136* ± 58.2**	566 ± 18.4	**494** ± 9.82**	1252 ± 89.6	1187 ± 66.7
JH	395 ± 15.4	348 ± 40.0	2038 ± 16.2	**1800** ± 44.2**	557 ± 35.1	**487* ± 22.5**	2027 ± 90.7	**1659* ± 193**
XX	573 ± 37.7	**438** ± 30.0**	4219 ± 332	3974 ± 212	998 ± 210	957 ± 220	4408 ± 297	4424 ± 185
BY	1276 ± 27.7	**912** ± 51.1**	5022 ± 453	5063 ± 216	1603 ± 177	**1066** ± 95.6**	4592 ± 152	4472 ± 167
XZ	564 ± 37.2	521 ± 64.6	3297 ± 96.3	**2996* ± 147**	956 ± 145	**480** ± 103**	3647 ± 47.4	**2964** ± 108**
YT	514 ± 18.6	**468* ± 20.8**	3817 ± 135	**3252** ± 57.3**	981 ± 29.7	**805** ± 24.1**	4689 ± 158	**4301* ± 110**
p-value[c]	**< 0.001**		**< 0.001**		**< 0.001**		**0.001**	

[a] Soil samples were collected in summer and winter of 2013 from six studied wetlands between treatments (control versus warmed). The data are expressed as mean ± standard error. SJ, ShiJiu; JH, JinHu; XX, XiXi; BY, BaoYang; XZ, XiaZhuHu; YT, YaTang

[b] Bold indicates a significant difference with warming compared to controls with *$p < 0.05$, **$p < 0.01$ by Student's *t*-test

[c] The bottom row indicates *p*-values from ANOVA conducted to test for differences between treatments across all wetlands as a whole

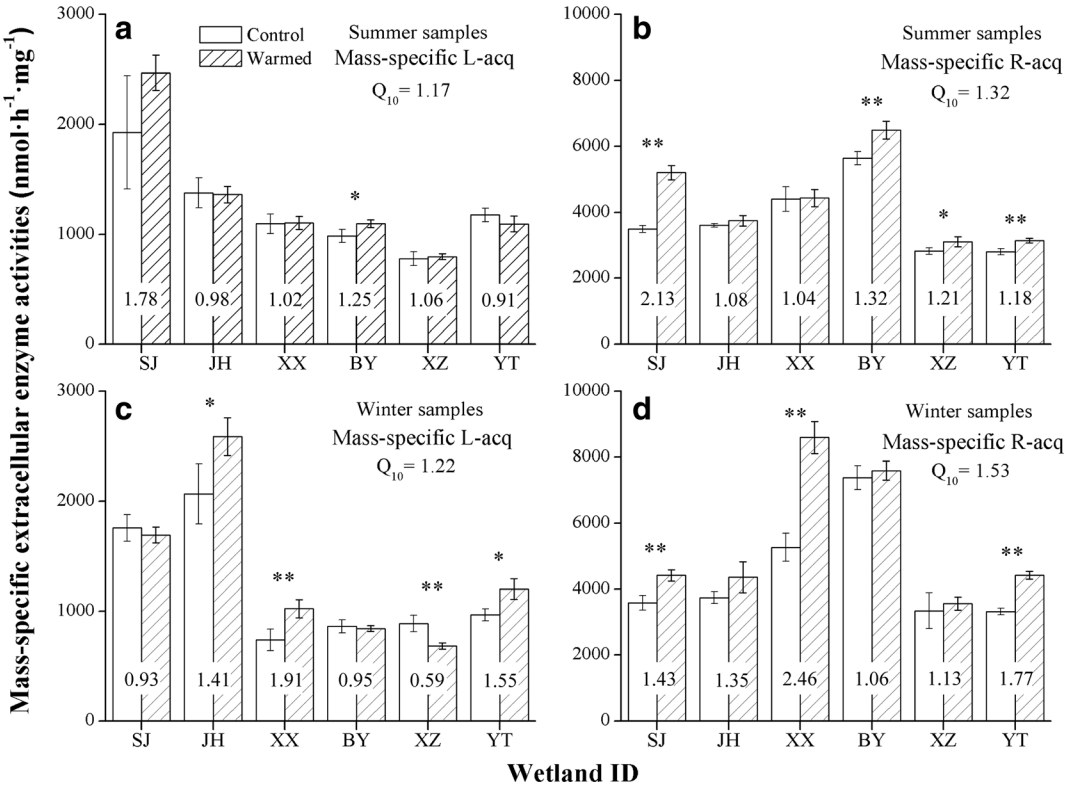

Fig. 2 Shifts in mass-specific carbon acquisition activities subjected to warming. Mass-specific labile organic carbon acquisition activities (mass-specific L-acq: **a** & **c**), mass-specific recalcitrant organic carbon acquisition activities (mass-specific R-acq: **b** & **d**) are calculated by normalizing L-acq and R-acq to microbial biomass carbon, respectively. L-acq are the sum activities of four hydrolytic enzymes, and R-acq are the sum activities of two oxidative enzymes. Q_{10} value for each wetland soil (SJ, ShiJiu; JH, JinHu; XX, XiXi; BY, BaoYang; XZ, XiaZhuHu; YT, YaTang) between treatments (control versus warmed) is listed in the middle of each bar chart. The mean for these Q_{10} values is shown in each plot. *$p < 0.05$, **$p < 0.01$ between warmed and control samples by Student's *t*-test. *Error bars* in each plot represent standard errors

value of YT was 1.55 (warmed) versus 0.91 (controls) for mass-specific L-acq, and 1.77 versus 1.18 for mass-specific R-acq. When comparing mass-specific enzyme utilization patterns between REC and LOC, we also found the Q_{10} value of mass-specific R-acq for each of all sites was generally greater than that for LOC. Experimental warming did not significantly affect mass-specific L-acq for summer samples (except for BY; Fig. 2).

Microbial community composition shifts

The relative abundance of the fungus-to-bacteria ratios were enhanced consistently across all study sites with warming (expect for SJ; Fig. 3). For example, the fungus-to-bacteria ratio for JH was averagely 4.19 % in warmed samples, while it was 2.97 % in controls. No significant changes were observed for the archaea-to-bacteria ratio, and shifts in relative abundance of archaea under warming were also inconsistent. For example, the archaea-to-bacteria ratio for JH increased from 0.61 % to 0.82 % under warming, while it decreased from 0.26 % to 0.15 % for wetland site XX. We regressed the fungi changes against *in-situ* observed environmental factors from all studied wetlands. The results showed that the fungi changes were significantly ($p < 0.01$) explained by temperature, DO and water table level as indicated by the multiple regression model [0.480·ln (*temperature*) - 0.624·ln (*DO*) - 1.207·*water table level*, $R^2 = 0.176$]. The soil pH and soil moisture explained less ($p > 0.05$) in fungi changes.

Linking microbial changes to environmental factors

In the winter, experimental warming exerted a stronger effect on the changing patterns of the microbial variables (54.7 % and 18.9 % explained for axis 1 and axis 2, respectively) than those in the summer (48.2 % and 13.4 % explained) (RDA, Fig. 4). Winter sample points were also clearly separate between treatments (control

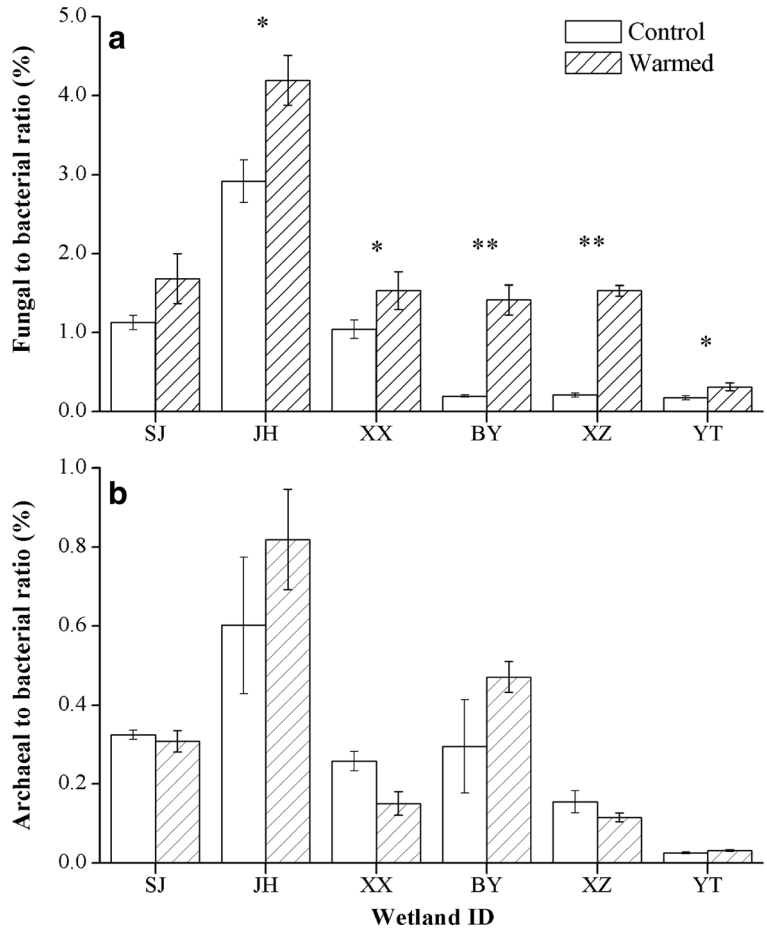

Fig. 3 Shifts in soil microbial communities subjected to warming. The relative abundance of bacterial, fungal, and archaeal ribosomal gene copies expressed as fungus-to-bacteria (**a**) and archaea-to-bacteria (**b**) ratios detected in soil samples (SJ, ShiJiu; JH, JinHu; XX, XiXi; BY, BaoYang; XZ, XiaZhuHu; YT, YaTang) between treatments (control versus warmed) in 2013 using quantitative PCR assays. *$p < 0.05$, **$p < 0.01$ between warmed and control samples by Student's *t*-test. *Error bars* in each plot represent standard errors

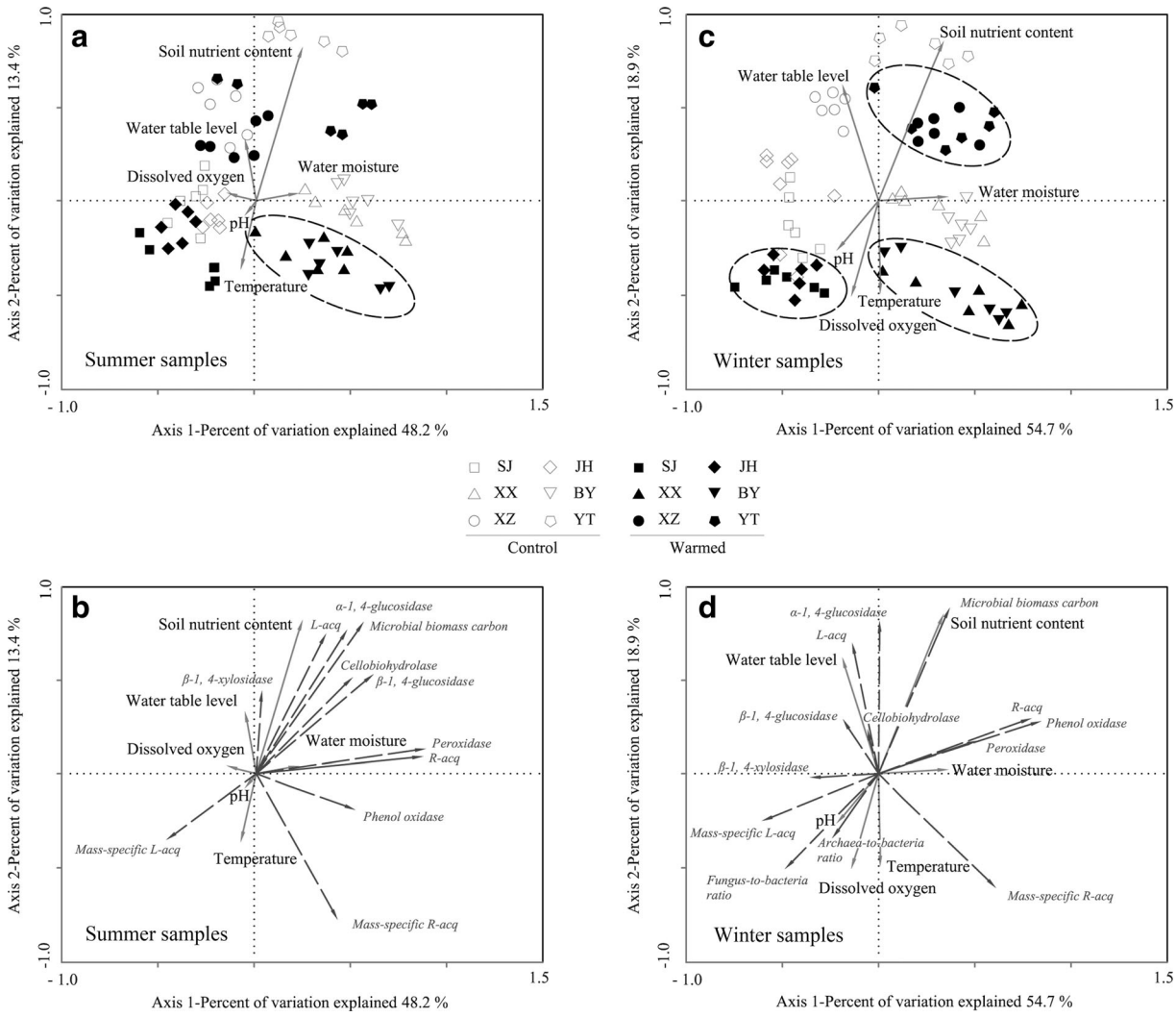

Fig. 4 Redundancy analysis (RDA) for linking microbial variables to environmental variables. Four biplots are illustrated, two for summer samples (**a** and **b**) and the other two for winter samples (**c** and **d**). The biplots **a** and **c** show samples and environmental (explanatory) variables in the ordination space, and the biplots **b** and **d** show microbial (response) and environmental variables in the ordination space. The microbial variables include α-1, 4-glucosidase, β-1, 4-glucosidase, cellobiohydrolase, β-1, 4-xylosidase, soil microbial biomass, L-acq, R-acq, mass-specific L-acq, mass-specific R-acq, fungus-to-bacteria ratio (only measured in winter samples), and archaea-to-bacteria ratio (only measured in winter samples). The environmental variables include temperature, soil nutrient content, water table level, dissolved oxygen, pH, and soil moisture. The Monte Carlo permutation test with 499 random permutations was performed to show the relative importance of these environmental variables (Further see Online Resource 5)

versus warmed). Soil nutrient content and water table level were the most significant variables driving microbial changes (Online Resource 5). Water table level was positively correlated with nutrient-enriched soil samples, indicating the warming-induced lower water table level disfavors soil organic accumulation. The simulated temperature was positively correlated with mass-specific R-acq, mass-specific L-acq, and fungus-to-bacteria ratio, whereas it was negatively correlated with enzymatic activities and microbial biomass (Fig. 4). RDA-based variation partitioning was further performed to separate the effects of environmental variables on the shifts of microbial properties (Online Resource 5). Soil nutrient content (11.5 % and 13.2 %) and warming-induced environmental variables (46.5 % and 51.4 %) explained variations, which were much higher than those explained by temperature itself (1.8 % and 0.8 %). The interaction between these

environmental factors was mainly found in winter samples with a total variation of 9.4 %. Based on our RDA model, about 37.9 % and 25.2 % of the microbial variation remained unexplained by the above environmental variables in summer and winter samples, respectively.

Discussion

In the present work, we found that 6-year experimental warming led to approximately 10 % of SOC loss in wetlands characterized by nutrient-enriched soils compared to controls, which was equivalent to a net loss of almost 2.6 kg of C per square meter within 0–5 cm top soils. Our work assists the development of new insights into how climate warming may influence microbial-mediated C dynamics in wetland soils.

Does substantial C loss depend on the nutrient status of wetland soils?

Submerged soils take on the characteristics of a distinct ecosystem differing from other terrestrial ecosystems. Water contained in wetland soils may act as an important dispersion medium that can alleviate diffusion constraints on enzyme activity since the combined warming and drying have shown to suppress microbial activities (Allison and Treseder 2008). Such alleviation may generate a large SOC loss, especially when soils are nutrient-enriched. It was reported that soils with externally added nutrients, which relieved nutrient constraints on enzyme activity, have shown greater biomass and SOC loss in global warming scenarios (Mack et al. 2004), explaining the substantial C loss that occurred in wetland soils with high nutrient status. Such increased decomposer biomass and enzyme production were observed in nutrient-enriched wetland soils after our 2.5 years of warming in a previous study (Wang et al. 2012). Contrastingly, in nutrient-poor soils, initial warming led to a rapid loss in LOC, and subsequent diffusion processes may be readily constrained by limited available substrates when their nutrient contents were initially low, exerting a negative feedback to warming. As a consequence, SOC loss in SJ and JH after the 6-year warming period compared to controls was insignificant ($p > 0.05$).

However, with sustained 6-year warming, a loss in LOC was accompanied by a decrease in SMBC and enzyme production for all wetland soils studied (Table 1). Such a trend was opposite to the nutrient-enriched or –moderate soils observed under short-term (2.5 years) warming (Wang et al. 2012). Such a decrease in SMBC is mainly due to the decline in available C allocated to growth when LOC was reduced, indicating that LOC is important in determining the SOC contents by influencing SMBC when microbes mineralize organic matters (Wang et al. 2012). The decreased biomass could also be a result of higher maintenance costs (higher energy needs) when catabolism processes (such as heterotrophic respiration) respond relatively stronger to temperature than biomass production (Allison and Treseder 2008). For nutrient-poor versus nutrient-enriched, the difference is that there was a lag time for substrate limitation in nutrient-enriched soils that was only observed after the occurrence of substantial SOC loss, but not before.

How do microbes acclimate to warming in wetland soils?

Beyond "substrate-limitation" aforementioned, altered microbial physiology and community are responsible for C loss. The higher Q_{10} value in winter (Fig. 2) suggests that enzyme acquisition capacity per microbial biomass was preferentially impacted by warming during cold seasons. Obviously, low temperature may upwardly adjust the response to warming (Al-Raei et al. 2009). Q_{10} of mass-specific R-acq was generally greater than that for LOC, indicating that enzymes involved in the decomposition of high molecular weight compounds exhibit a higher sensitivity to warming (Trasar-Cepeda et al. 2007). Consistently, increased oxidative enzyme activity with coupled elevated temperature and addition of nitrogen have been reported to be an important mechanism for changed recalcitrant C in forest soils (Waldrop and Firestone 2004). Such shifts may have great implications for an alteration in the magnitude and direction of C-climate feedback in global models through adjusted microbial physiology.

Microbial species in a given ecosystem have unique habitats and temperature optima. Our 1-year warming increased fungal biomass as evidenced by the unique fatty acids of living cell membranes as biomarkers (Zhang et al. 2012). Our 2.5 years warming was further shown to increase fungal diversity and relative abundance of *Ascomycota* and *Basidiomycota* (Wang et al. 2012). A shift to the relative dominance of fungi appears

to be consistent when comparing those previous results with these obtained in the present study (Fig. 3). Fungi are better at mobilizing and decomposing stable C through relocating and recycling limited substrates through cytoplasm translocation (Ma et al. 2012). In particular, fungi can be linked with rapid decreases in REC fractions through enhanced enzyme activities, mainly including polyphenol oxidase and PEROX (Jassey et al. 2011). Freeman et al. (2004) pointed out that the activity of PHENOX may be a key regulator of wetland C cycling since many recalcitrant C-degrading enzymes are oxidative. The shifted composition of the microbial community (Fig. 3) may be key to explaining the altered strategy to use different substrates, and thus suggests an adaptation of soil decomposers to the new environment. However, it appears that the responses of the relative abundance of fungi to warming may vary with studied ecosystems, treatment times, and could be even accounted by the changes in other warming-induced factors. For example, Frey et al. (2008) and Rinnan et al. (2007) showed a significant reduction in the ratio of fungi to bacteria in forest soils and arctic heaths soils, respectively, the opposite effect as observed in this study. However, such shifts were mainly recorded after 12–15 years of chronic warming treatments. In tundra and boreal ecosystems (Allison and Treseder 2008), warming and drying decreased both bacterial and fungal absolute abundance, and further caused a shift in relative abundance among different fungal taxa.

Which environmental factors are key in driving microbial changes?

Decreased water table level accelerates C loss, especially for shallow water bodies (Reddy et al. 1998). Chimner and Cooper (Chimner and Cooper 2003) reported that up to two-fold more CO_2 was released from sites where the water table had been artificially lowered. In laboratory incubations, addition of molecular oxygen enhanced the activity of PHENOX nearly seven-fold, showing rapid decomposition of phenolic compounds (Freeman et al. 2004). Aerobic respiration and slight limitations in soil water moisture seems to be favorable for fungal growth, since fungi rely more on aerobic conditions (Zhang et al. 2005). However, when water table level decreased severely, excessively high moisture stress may alleviate decomposition processes by depressing microbial activity (Allison and Treseder 2008).

Is carbon loss sustainable under long-term experimental warming?

Although microbial communities and their physiology also shifted in nutrient-poor wetlands (i.e., SJ and JH), substantial C loss was not observed in these soils. It indicates that substrate limitation is a foremost mechanism in determining C loss with the microbial shift as only a complimentary one. It is well known that substrate limitation is caused by absolute decrease in microbial biomass and enzymes, while altered microbial community and physiology only represent the shifts in relative values. It has been suggested that physical protection from microbial attack and interactions with soil minerals are likely to be the main reason for the persistent nature of soil organic matter (Schmidt et al. 2011), since pedological processes operating in a given soil type influence the distribution of decomposers and substrates. The relatively more mineral contents in nutrient-poor soils may lead to substrate inaccessible to the decomposers. Substrate availability is usually constrained in nutrient-low wetland soils (i.e., JH and SJ, low in organic contents in soil matrix) due to steric hindrance and physical disconnection between organics and minerals. More unevenly distributed organics in soil particles and water-saturated pores will also enhance variability within samples (Schmidt et al. 2011), and may mask the observed differences in SOC content between treatments, as was seen by the great variability in SOC of SJ (error bars, Fig. 1). The "cancelling effect" reported by von Luetzow and Koegel-Knabner (2009) especially in C-limited soils, adds constraints on the affinity of enzymes for substrates, increasing soil heterogeneity and hence the uncertainty of warming effects. Even for nutrient-enriched soils, experimental warming-induced increments in decomposition rates will slow down over time through a decreased substrate accessibility at the expense of partial C loss in these soils under warming. Such an increased physical isolation between SOC-minerals may eventually protect SOC from further decomposition (Schmidt et al. 2011).

Implications for C dynamics when subjected to warming

Our 3–5 °C increase in simulated temperature are comparable to several soil warming studies with a modest increase (1 to 3 °C) in soil temperature (Sowerby et al. 2005; Zhang et al. 2005; Rinnan et al. 2007; Darrouzet-

Nardi et al. 2015). For example, Zhang et al. (2005) showed the increased fungi dominance in the tallgrass prairie when subjected to 2 °C increase in soil temperature. Even a small increase (less than 1 °C) in soil temperature was sufficient to invoke increased enzymatic activity across heathland soils (Sowerby et al. 2005), and the soil warming by 2 °C had a substantially negative effect on C balance from biocrust soils (Darrouzet-Nardi et al. 2015). Additionally, about 1 to 3 °C increase in temperature may cause microbial changes to a lesser extent or with a longer time lag in responses (Rinnan et al. 2007), indicating that the lower simulated temperature increase may reflect similar results with our 3–5 °C increase, but to a lesser extent. Air warming by 2 °C using infrared heaters also led to the decreased soil organic matter and microbial biomass (Yoshitake et al. 2015), which was consistent with our study. However, air warming (1 to 2 °C) of an alpine meadow using open-top chambers and a tallgrass prairie using infrared heaters have both shown the significantly increased soil microbial biomass (Belay-Tedla et al. 2009; Zhang et al. 2015). Such contrasting results mainly resulted from warming-induced gradual increases in plant biomass input, which masked the direct effects of warming on soils. The disproportional loss in different organic C fractions in this study may lead to a low-quality soil remaining. Since low quality soils have been demonstrated to be more sensitive to temperature than high-quality soils (Davidson and Janssens 2006), over much longer time periods (e.g., decades to centuries), a greater amount of soil C will be lost than expected for a warmer world (Belay-Tedla et al. 2009). From this study, the possible underlying mechanisms for C dynamics in wetland soils under warming are summarized in Table 3.

Conclusions

The overall effect of different environmental factors should be systematically analyzed to explore how rising temperature determine SOC in wetlands. Predicting soil C storage in wetlands under a global warming scenario has some implications for the study on future climate. First, nutrient-enriched soils are more susceptible to rising temperatures than the nutrient-poor with respect to SOC loss. Second, a lag time for substrate limitation occurred in nutrient-enriched soils, and a certain amount of SOC would be initially lost from these soils until

Table 3 The possible underlying mechanisms for carbon (C) dynamics in studied wetland soils with warming

	Short-term observation (2.5 years)	Long-term observation (6 years)	Implications for decades warming
Nutrient-poor wetland soils[a]			
Whether C was loss[e]	No	No	Uncertain
Underlying mechanisms	Substrate limitation[b]	Substrate limitation[b]	Reduced soil quality increase its sensitivity to warming
	Low substrate accessibility[c]	Low substrate accessibility[c]	Microbial acclimation to warming[d]
Nutrient-enriched wetland soils[a]			
Whether C was loss[e]	Yes	Yes, but not sustainable	Yes, but uncertain whether sustainable
Underlying mechanisms	Substrate not limited[ref.]	Substrate limitation[b]	Reduced soil quality increase its sensitivity to warming
	Microbial acclimation to warming[d]	Low substrate accessibility[c]	Microbial acclimation to warming[d]

[a] Wetlands investigated in this study belong to inland subtropical wetlands with shallow waterbodies 0.8–1.5 m in depth; organic matter ranges from 14.6–25.5 $g \cdot kg^{-1}$ for nutrient-poor soils, and ranges from 114 to 65.7 $g \cdot kg^{-1}$ for nutrient-enriched soils; there is no C incorporated into soils from aboveground plant biomass, and no warming-induced reduction in soil moisture

[b] Substrate limitation is a direct result of a decrease in soil microbial biomass and enzymes

[c] Low substrate accessibility is a direct result of spatial inaccessibility and physical disconnection between organics and minerals

[d] Microbial acclimation includes community shifts, altered physiology, and thermal adaptation

[e] If C loss is sustainable, it means C loss kept increasing year by year

[ref.] Referring to our previous work titled "linkage of soil carbon pools and microbial functions in subtropical freshwater wetlands in response to experimental warming." published in *Applied and Environmental Microbiology* 2012, *78*, (21), 7652–7661

substrate limitations and inaccessibility become a bottleneck for further decomposition. Moreover, altered C utilization patterns imply that the microbial-mediated decomposition of recalcitrant pools will be preferentially accelerated with warming compared to labile C. Finally, the decrease in water table level may be one warming-induced factor to understanding microbial community shifts towards fungal dominance, which may further imply a major mechanism of microbial acclimatization to new environments in wetland soils.

Acknowledgments This work was supported by the National Natural Science Foundation of China (41373074, 31500409), National Departments of Science and Technology, and Water Resource (2013GB23600658, 201301092), and Zhejiang Science and Technology Innovation Program (2015F50002).

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