Hydrobiologia (2015) 743:221–235
DOI 10.1007/s10750-014-2039-6

PRIMARY RESEARCH PAPER

Redox potential and microbial functional gene diversity in wetland sediments under simulated warming conditions: implications for phosphorus mobilization

Zhijian Zhang · Hang Wang · Jizhong Zhou · Hongyi Li · Zhili He · Joy D. Van Nostrand · Zhaode Wang · Xinhua Xu

Received: 17 February 2014 / Revised: 31 August 2014 / Accepted: 8 September 2014 / Published online: 14 October 2014

Abstract Microbial-driven biogeochemical cycles in wetlands impacted by global warming pose a potential downstream eutrophication risk. However, the consequences of ongoing warming on the functional and metabolic potential of sediment microbial communities are largely unknown. We incubated sediment samples under both ambient temperature conditions (control) and simulated warming conditions of 5°C above ambient temperature (warmed) using a novel field microcosm system. In warmed samples, we observed in situ a decreased thickness of the oxidized sediment layer and associated lower sediment redox potential. GeoChip 4.0, a comprehensive functional gene microarray, demonstrated that many functional genes that are involved in oxidation–reduction reactions and in phosphorus (P) degradation were preferentially enriched under warming conditions. The enriched genes included those genes encoding carbon monoxide dehydrogenase, acetyl-CoA carboxylase biotin carboxylase (*ppc*), and ribulose-1,5-bisphosphate carboxylase (Rubisco) for carbon fixation; nitrate reductases (*narG*) and nitrous oxide reductases (*nosZ*) for denitrification; cytochrome c for metal reduction; and exopolyphosphatase (*ppx*) for polyphosphate degradation. The redox potential was one of the most significant parameters linked to microbial functional gene structure. These results demonstrate that the enhanced hypoxia and

Handling editor: Stefano Amalfitano

Electronic supplementary material The online version of this article (doi:10.1007/s10750-014-2039-6) contains supplementary material, which is available to authorized users.

Z. Zhang (✉) · H. Wang · H. Li · X. Xu (✉)
College of Environmental and Resource Science, Zhejiang University, 886th Yuhangtang Ave, Hangzhou 310058, China
e-mail: zhangzhijian@zju.edu.cn

X. Xu
e-mail: xuxinhua@zju.edu.cn

Z. Zhang
China Academy of West Region Development, Zhejiang University, Hangzhou 310058, China

J. Zhou · Z. He · J. D. Van Nostrand
Department of Microbiology and Plant Science, Institute for Environmental Genomics, University of Oklahoma, Norman, OK 73019, USA

J. Zhou · Z. He · J. D. Van Nostrand
State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China

Z. Wang
State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Science, Nanjing 210008, China

anaerobic metabolic pathways accelerated sediment P mobilization in freshwater wetland subject to warming, raising the potential of water eutrophication.

Keywords Biogeochemical cycling · Functional gene · Warming · Freshwater wetland

Introduction

Climate projections for the twenty first century suggest that the average global surface temperature has increased by 0.74°C since 1850 and is likely to increase a further 1.1–6.4°C by the end of this century (Solomon et al., 2007). The elevated temperature would most likely exacerbate some symptoms of eutrophication in hypertrophic shallow and enclosed areas of freshwater ecosystems, such as small lakes, ponds, or riparian zones, where anthropogenic nutrient inputs intermittently accumulate on the surface of the bottom sediment (Verhoeven et al., 2006; Feuchtmayr et al., 2009; Wang et al., 2010). These heavily nutrient-loaded sediments are sensitive to elevated temperatures, and the subsequent mobilization of phosphorus (P) from mineral-bound or polyphosphate forms (Sondergaard et al., 2003) leads to positive feedback that causes eutrophication. Temperature increases accelerate the microbially mediated biogeochemical cycling of P at the sediment–water interface. Although our previous study demonstrated that P release was greatly influenced by elevated temperature (Liu et al., 2014), the interaction between the ongoing warming of water bodies and the underlying responses of key microbial processes involved in P cycling is more complex and may also be controlled by other warming-induced environmental factors. However, until now, a gap between climate warming and P release had not been fully explored in detail.

Rising temperature is a priming factor that decreases the solubility of oxygen in water columns (Vaughan et al., 2009) and enhances metabolic rates as the latter accelerates microbial-driven oxygen consumption in surface sediments. Oxygen depletion that is accompanied by expanded hypoxic zones (Diaz & Rosenberg, 2008) has serious consequences for freshwater ecosystems, which are coping with worldwide eutrophication because many biogeochemical processes are regulated by oxidation–reduction reactions (Rabenhorst & Castenson, 2005; Vaughan et al., 2009). The sediment–water interface provides a habitat for diverse microbes that produce enzymes that catalyze oxidation–reduction reactions (Brune et al., 2000; Vuillemin et al., 2013). The physicochemical characteristics of the sediment–water interface result in a distinct stratification of various microbial communities that have adapted to the diverse sediment layers. For instance, most phosphate-accumulating bacteria and ammonia-oxidizing bacteria may exist in the uppermost oxidized sediment layer, whereas the lower layer, with a prevalence of anoxic conditions, benefits the growth of denitrifying and iron-reducing bacteria (Himmelheber et al., 2009). The microbial response to shifts in the redox potential at the sediment–water interface may greatly influence the microbial distribution and metabolic potential, which further modulates microbial metabolism through the intrinsic balance between aerobic and anaerobic pathways. Internal eutrophication caused by hypoxia and the subsequent P availability for many hypertrophic freshwater areas has been well-documented (Sondergaard et al., 2003). Two primary mechanisms of microbial processes that contribute to P mobilization and release are the redox-sensitive dissimilatory reduction of metal oxide-bound P under anoxic conditions, and the decomposition of organic matter, which liberates P from sediment aggregates (Reitzel et al., 2007; Henderson et al., 2012). It is widely accepted that organic matter decomposition in sediments is highly temperature-dependent (Gudasz et al., 2010; Weedon et al., 2013). Therefore, to comprehensively understand the effects of global warming on freshwater, we must know whether and how the sediment redox potential influences microbial processes at elevated temperatures. Because of the complexity and variety of microbial functional attributes across biomes (Fierer et al., 2012), currently the process-level functional metabolism stimulated by warming-induced redox potential shifts has remained largely unknown. Although previous studies have focused on some element cycles (e.g., carbon, sulfur, and iron) mediated by warming-induced redox potential shifts (Sanz-Lazaro et al., 2011), a comprehensive profiling of various processes involved in a wide range of elements cycles at the molecular level may better improve our understanding regarding the impact of warming on sediment P mobilization.

Therefore, our objective was to link climate warming and sediment redox potential in a freshwater ecosystem to evaluate the potential risk of P mobilization and release during 2-year field incubations using

an in situ, computerized microcosm that simulates warming scenarios with 5°C differences (Zhang et al., 2012). The sediment redox potential in three nutrient-enriched wetland sediments was measured using both field observation and lab-scale microbial activity assays, and the associated microbial response was examined by a novel functional gene microarray, GeoChip 4.0 (Hazen et al., 2010). Through rigorous statistical tests, we identified the essential environmental factors to be linked to microbial functional gene structure, which had long implications for freshwater sediment responding to climate stress. We hypothesized that ongoing warming would preferentially alter microbial functional and metabolic potentials through the enhancement of anaerobic pathway.

Materials and methods

Microcosm configuration

A custom-built, novel microcosm (Fig. S1) that stimulates climate warming was developed under both present-day ambient temperature conditions (control) and simulated warming conditions of 5°C above the ambient temperature (warmed) (Fig. S2). The details regarding the configuration of this microcosm system and its corresponding operation were described previously (Zhang et al., 2012) or can be found in the Supporting Information (A-1).

Study sites and sampling

The study sites were located within the delta of the Yangtze River in southeast China. The basic descriptions of the three wetlands in situ are shown in Table S1. Briefly, the YaTang riverine wetland (YT) is in an advanced state of eutrophication, with the highest sediment organic matter (114 g kg^{-1}), nutrient (total P: 2,530 mg kg^{-1}), and water content (68.7%) of all the study sites. The XiaZhuhu aquaculture wetland (XZ) and the wetland in the XiXi National Wetland Park (XX) are in a mesoeutrophic state, where the organic matter and the P content in the XZ sediment are nearly twofold compared with those values in the XX sediment. Transparent polyvinyl chloride wetland columns filled with selected sediments and overlying water were placed in the microcosm system (Fig. S1) in May 2008 and have been in continuous operation since then. Some floating-leaf aquatic plants grew in the wetland columns, such as *Trapa incisa, Lemna minor,* and *Azolla imbricata*. All external factors, including plant growth, were not statistically different between treatments when samples were taken after 1–2 years incubation in this study. The details for preparing the wetland columns (with six replicates for each wetland site, $n = 6$) were described previously (Zhang et al., 2012) or can be found in the Supporting Information (A-2).

Investigation of in situ dissolved oxygen, oxidized layer, and redox potential

From May 2010 to February 2011, a period that covers all four seasons, three selected parameters, including the dissolved oxygen (DO) concentration, the thickness of the oxidized sediment layer and the redox potential at the uppermost sediment, were simultaneously investigated in wetland columns per 3 months. The DO concentration was measured at the bottom of the water column within a depth of 2 cm above the uppermost sediment using a portable DO meter (HQ30d, HACH Corporation, US). A distinct interface between a light surface layer (oxidized layer) and a dark deep layer formed along the sediment profile due to the diffusion of dissolved oxygen into the sediment surface. The thickness of this oxidized layer was measured with a ruler (read to the nearest millimeter) around the sediment core in a vertical profile (Jensen & Andersen, 1992). We measured the redox potential in the uppermost surface of the sediment using a redox electrode (SensoDirect pH200, LOVIBOND, Germany).

Genetic analyses of sediment samples

In Dec 2010, immediately after water sampling, approximately 100 g of fresh sediment from a homogenized 0–5 cm top sediment core was collected from each wetland column using a thin-walled plastic core tube. Six separate DNA samples were extracted from the sediment for each site and were pooled to form a composite extract, which was then amplified, labeled, and hybridized using a functional gene microarray (GeoChip 4.0) as described previously (Hazen et al., 2010; He et al., 2010; Lu et al., 2012). After hybridization, spots were scored as positive when the signal to noise (SNR) was ≥ 2.0 and when the coefficient of variation (CV) of the background was <0.8.

Biochemical analysis of P release from sediment to water

From February 2009 to November 2010, the overlying water in the water column and the sediment pore water were sampled from each wetland column at approximately 60-day intervals to examine P concentrations using the spectrophotometric method of Murphy & Riley (1962) with a continuous flow analyzer (Auto-Analyzer III, BRAN + LUEBBE, Germany).

Laboratory incubation for sediment oxygen demand and reducing capability

In the summer (Jul 2010) within the period of the in situ measurements (May 2010 to February 2011), the sediment oxygen demand (SOD) and the reducing capability were determined in samples from YT wetland columns. For the measurement of SOD, 0–5 cm of the top sediment core was placed in a glass container with a 10-cm water column above the core under anoxic conditions. At each sampling time point, the DO concentration in the water column was measured. The SOD was further calculated in units of mmol O_2 m^{-2} day^{-1}. Potential nitrate, sulfate, and ferric iron reduction rates were measured by the addition of electron acceptors (KNO_3: 140 mg N l^{-1}; K_2SO_4: 20 mg S l^{-1}; and $FeC_6H_5O_7{\cdot}H_2O$: 200 mg Fe l^{-1}) and substrate acetate (20 mmol l^{-1}) to stimulate the activity of specific microbial communities (Wei, 2002; Martins et al., 2011). Details regarding these measurements are found in the Supporting Information (A-3). The incubations were terminated when the measured concentrations in the liquid or trace gas forms reached (near-) constant values.

Statistical analysis

The in situ investigation of the DO, the thickness of the oxidized layer, and the redox potential, as well as P concentration dynamics, were examined using an ANOVA, which was followed by post hoc multiple comparisons using Duncan's multiple range test. The SOD and the sediment reducing capability were examined using Student's *t* test for each single sampling time point.

Because only a composite extract for each wetland was analyzed using the microarray GeoChip 4.0, we did not statistically perform between-site differences subjected to warming. The pre-processed GeoChip data, which had low quality spots for the three integrated wetlands removed, were analyzed using different statistical methods: (i) the microbial diversity index and a paired Student's *t* test; (ii) the hierarchical clustering of genes to determine the microbial functional community structure; (iii) a non-parametric multivariate analysis of variance (MANOVA) of differences in specific gene functional communities; (iv) a detrended correspondence analysis (DCA) of key gene functional categories; (v) a redundancy analysis (RDA) for linking microbial communities to environmental variables (Lepš & Šmilauer, 2003); and (vi) a partial RDA for co-variation analysis (variation partitioning analysis, VPA) to examine the proportion of variation that is solely explained by each single environmental variable (Borcard et al., 1992; Lepš & Šmilauer, 2003). Details for the statistical tests are provided in the Supporting Information (A-4).

Results

Shifts in functional genes in response to warming

More functional genes (22.2% on average) were detected in warmed samples compared with the control. The gene diversities that were involved in key biogeochemical categories, i.e., carbon, nitrogen, phosphorus, sulfur, and iron cycling, were higher in warmed samples based on Shannon–Weiner and Simpson's indices (Table S2). Warming consistently shifted these genes in a similar pattern for all tested wetlands, as indicated by DCA (Fig. S3). Clustering of the detected genes produced six major groups of different functional gene signals, with a significant shift in the abundance of genes in Group 3 and Group 6 ($P < 0.05$ or 0.01) under warming (Fig. 1). Most genes were actually aggregated in one or two groups, which brought new insight on significantly changed gene categories. Group 6 primarily contained genes that were involved in carbon fixation and denitrification, which accounted for 16.3 and 12.9% of the total genes in this group, respectively. These values were much higher compared with those values in other groups. In contrast, approximately 56.2% of the genes that are related to aerobic methane oxidation and 70.8% of the genes that are related to nitrification were clustered in Group 3, including those genes with a high

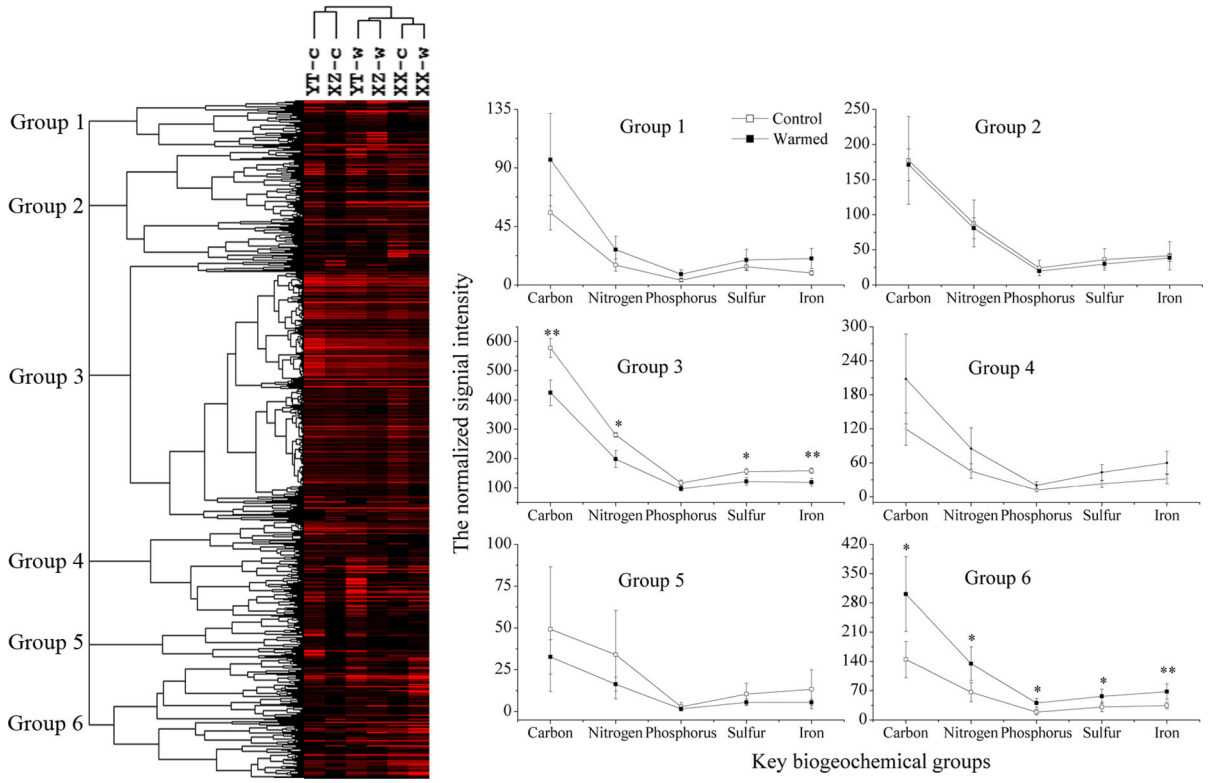


Fig. 1 Hierarchical clustering of all functional genes involved in key biogeochemical categories (i.e., carbon, nitrogen, phosphorus, sulfur, and iron cycling) detected in sediment samples from tested wetlands. *Brighter red* coloring indicates higher signal intensities. The relative abundance of detected genes indicated by mean values of normalized signal intensities was plotted in six separate groups (Group 1, Group 2, Group 3, Group 4, Group 5, and Group 6). *Error bars* are ±standard deviation. *Asterisks* represent significant paired Student's *t* test differences between control and warmed samples (*$P < 0.05$, **$P < 0.01$)

Table 1 Statistical analysis of the effects of experimental warming on selected functional gene categories using a non-parametric multivariate analysis of variance (MANOVA). The *P*-values smaller than 0.05 are bold significance

Functional gene categories	*F* value	*P* value
Carbon fixation	0.286	**0.046**
Methanogenesis	0.037	0.509
Denitrification	0.254	0.087
Assimilatory/dissimilatory nitrogen reduction	0.284	**0.001**
Iron reduction	0.280	**0.011**
Sulfur reduction	0.205	0.384
Selected genes related to microbial anaerobic metabolism	0.271	**0.022**
Total detected genes	0.296	0.113

abundance in control samples. To confirm these patterns, a MANOVA (Table 1) showed that not all genes significantly ($P = 0.113$) changed under warming, whereas warming preferentially led to significant ($P < 0.05$) or marginally significant ($P < 0.1$) changes in the abundance of specific genes that are related to microbial anaerobic metabolism (including denitrification, assimilatory/dissimilatory nitrogen reduction, and iron reduction) and those genes that are involved in carbon fixation. Shifts in individual gene categories due to warming are discussed below.

Carbon fixation genes

Carbon fixation genes showed significantly ($P < 0.05$) higher (by 23.6%) abundances in warmed samples compared with the control (Fig. 2A). A clustering of all detected carbon fixation genes (Fig. S4) showed that all warmed samples were clustered together and well separated from control samples, particularly for genes in Group 2. In warmed samples, genes that encode carbon

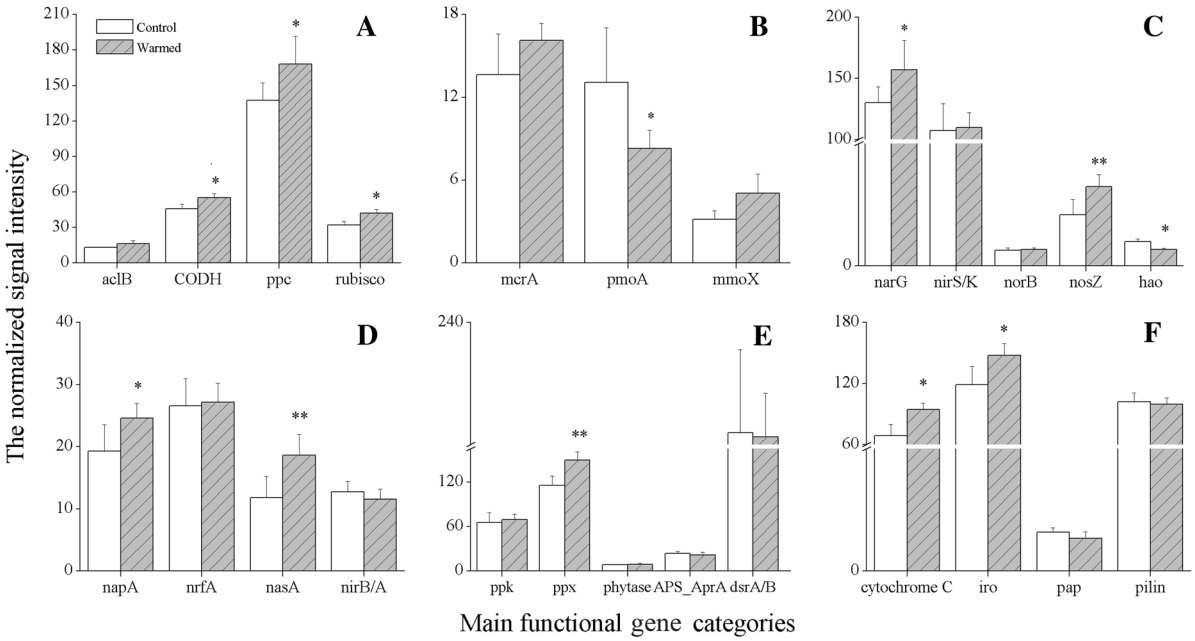


Fig. 2 The relative abundance of functional genes indicated by mean values of normalized signal intensities detected in sediment samples from tested wetlands involved in **A** carbon fixation (i.e., *aclB*, CODH, *ppc,* and *rubisco*); **B** methanogenesis (i.e., *mcrA*) and aerobic methane oxidation (i.e., *pmoA* and *mmoX*); **C** denitrification (i.e., *narG*, *nirS/K*, *norB*, and *nosZ*) and nitrification (i.e., *hao*); **D** dissimilatory nitrogen reduction (i.e., *napA* and *nrfA*) and assimilatory nitrogen reduction (i.e., *nasA* and *nirB/A*); **E** phosphorus biosynthesis and degradation (i.e., *ppk*, *ppx*, *and phytase*) and sulfur reduction (i.e., *APS_AprA* and *dsrA/B*); **F** iron reduction (i.e., cytochrome c) and utilization (i.e., *iro*, *pap*, and *pilin*). *Error bars* are +standard deviation. The differences between control and warmed treatments were tested by paired Student's *t* test (*$P < 0.05$, **$P < 0.01$)

monoxide dehydrogenase (CODH), acetyl-CoA carboxylase biotin carboxylase (*ppc*), and ribulose-1,5-bisphosphate carboxylase (*rubisco*) were primarily derived from *Pseudomonas carboxydohydrogena*, *Rhodobacter sphaeroides ATCC 17025*, *Dyadobacter fermentans DSM 18053*, and some uncultured *Proteobacteria.*

Methanogenesis and aerobic methane oxidation

The abundance of genes that encode the alpha subunit of methyl coenzyme M reductase (*mcrA*) and particulate methane monooxygenase (*mmoX*) remained unchanged. The significantly ($P = 0.034$) lower abundance of *pmoA* (decreased by 34.5%), which encodes methane monooxygenase, was found in warmed samples compared with the control (Fig. 2B).

Nitrogen cycling

Genes that are involved in denitrification (Fig. 2C) and in dissimilatory/assimilatory nitrogen reduction (Fig. 2D) accounted for 61.1% of nitrogen-cycling genes, which represent the primary nitrogen metabolic pathway in the studied sediments. The abundance of all detected genes that are involved in denitrification was significantly higher ($P = 0.042$, by 13.0%) in warmed samples, particularly for *narG*, which encodes nitrate reductases ($P = 0.047$, by 16.3%), and *nosZ*, which encodes nitrous oxide reductases ($P = 0.006$, by 54.7%). No significant differences were found in *nirS/K*, which encodes nitrite reductase, or *norB*, which encodes nitric oxide reductase (Fig. 2C). In contrast, the abundance of *hao*, which encodes hydroxylamine oxidoreductase in nitrifiers (an indication of nitrification activity), was 16.5% lower under warming (Fig. 2C). For assimilatory nitrogen reduction, the abundance of *nasA*, which encodes nitrate reductase, increased by 45.1% in warmed samples. For dissimilatory nitrogen reduction, the gene that encodes periplasmic nitrate reductase (*napA*) was also significantly higher under warming, whereas

Table 2 Maximum, half-incubation and average values of sediment oxygen demand (SOD, mmol m^{-2} day^{-1}) and substrate-induced nitrate reduction rates (NRR, μg g^{-1} h^{-1}), ammonium production rates (APR, μg g^{-1} h^{-1}), sulfite production rates (SPR, μg g^{-1} day^{-1}), hydrogen sulfide production rates (HPR, μg g^{-1} day^{-1}), ferrous iron production rates (FPR, μg $g^{-1}h^{-1}$), and ferric iron reduction rates (FRR, μg g^{-1} h^{-1}) measured in sediment samples (July 2010) from YaTang riverine wetland (YT) as an example under control and warmed treatments. Data with significant *P*-values (<0.05 or 0.01) are in bold

Reaction rates	Max		Half-incubation		Average	
	Control	Warmed	Control	Warmed	Control	Warmed
SOD	84.1 (16.7)	**114* (29.0)**	74.2 (10.1)	80.4 (8.16)	58.4 (7.02)	**72.6* (11.7)**
N-related						
NRR	18.5 (8.32)	**43.6** (4.21)**	38.5 (6.28)	41.1 (5.07)	29.5 (4.09)	**35.6* (3.74)**
APR	0.74 (0.10)	**1.36** (0.20)**	0.37 (0.02)	**0.58** (0.04)**	0.45 (0.08)	**0.74** (0.17)**
S-related						
SPR	3.05 (0.52)	3.85 (1.65)	**2.01* (0.41)**	1.39 (0.47)	1.98 (0.23)	2.53 (0.33)
HPR	1.60 (0.17)	2.66 (1.10)	1.60 (0.17)	2.66 (1.10)	1.29 (0.10)	**2.18* (0.23)**
Fe-related						
FPR	40.6 (2.29)	45.6 (6.07)	1.70 (0.83)	**7.63** (0.59)**	16.3 (6.23)	20.5 (5.90)
FRR	96.7 (28.7)	**177** (20.0)**	14.7 (18.1)	**29.7* (5.82)**	27.3 (10.8)	**47.3* (19.8)**

c-type cytochrome nitrite reductase (*nrfA*) between two treatments remained unchanged (Fig. 2D).

Phosphorus utilization and sulfur reduction

The abundance of *ppx*, which encodes exopolyphosphatase for inorganic polyphosphate degradation, significantly ($P = 0.007$) increased by 29.6% in warmed samples. Phytase, which is involved in phytate degradation, and *ppk,* which is required for polyP biosynthesis, remained unchanged (Fig. 2E). Most of the detected genes for sulfur reduction were derived from uncultured sulfate-reducing bacteria. However, none of sulfur reduction genes were significantly impacted by experimental warming (Fig. 2E).

Iron reduction and utilization

The cytochrome c genes showed higher (by 32.4%) abundance in warmed samples and were well-separated from the control by clustering (Fig. 2F, Fig. S5). Most of these genes were derived from *Rhodobacter* and *Geobacter* populations. For iron utilization, the abundance of genes that are involved in iron uptake (*iro*) was also higher (by 24.2%) under warming (Fig. 2F), which suggests the possibility of an increased microbial acquisition of iron.

Functional activity assays

Sediment oxygen demand

The SOD values were higher ($P < 0.05$) in warmed samples (Table 2), which ranged from 32.3 to 114 mmol m^{-2} day^{-1}, compared with the control (31.3–84.1 mmol m^{-2} day^{-1}). These results were consistent with DO concentration dynamics that were measured in incubated sediment cores, where rapid oxygen uptake occurred when the DO concentration was above 3.5 mg l^{-1} (Fig. S6).

Reducing capability of electron acceptors

Anoxic assays assessed substrate-induced sediment reduction rates after the addition of terminal electron acceptors (i.e., nitrate, sulfate, and ferric iron). Throughout incubations, most reduction processes occurred during the first half of the incubation period (Table 2). Then, reaction rates declined over the second half of the incubation period and reached (near-) constant values on the 3rd, 5th, and 9th day for nitrate reduction (Fig. S7), sulfite production (Fig. S8), and ferric iron reduction (Fig. S9), respectively. The warmed samples showed lower average concentrations of NO_3^- within the 5-day incubation and higher ($P < 0.01$) maximum NO_3^- reduction rates (1.25 mg l^{-1}, 43.6 μg g^{-1} h^{-1},

respectively) compared with the control (1.11 mg l^{-1}, 18.5 μg g^{-1} h^{-1}, respectively). Sulfate reduction rates were also slightly higher in warmed samples, as indicated by maximum sulfite production rates of 3.85 μg g^{-1} day^{-1}, compared with the control (3.05 μg g^{-1} day^{-1}, Table 2). Subsequently, higher ($P < 0.05$) Fe(III) reduction rates were observed, with approximately 3.26 mg g^{-1} of Fe(II) produced within the 14-day incubation in warmed samples and 2.91 mg g^{-1} for the control. Similarly, in the gas phase, a greater release of NH_4 ($P < 0.01$) and H_2S ($P < 0.05$) was observed under warming, with cumulative releases of as much as 96.8 and 12.4 mg kg^{-1}, respectively, compared with 68.8 and 7.64 mg kg^{-1} in the control (Figs. S7, S8).

Redox as a predominant factor that influences the microbial functional structure

For in situ measurements, DO concentrations at the bottom of the water column were lower in warmed samples compared with the control (Table 3), with the lowest values in the summer (August 2010) compared with other seasons (Fig. S10). Experimental warming led to decreased thickness of the oxidized layer, which ranged from 0.53 (YT) to 1.75 cm (XX), compared with 0.73 (YT) to 1.92 cm (XX) in the control. This decrease was confirmed by 5.2 (XZ)–11.8% (XX) lower redox potential in the uppermost centimeter of the sediment in warmed samples (Table 3). When comparing among the three wetlands, highly eutrophic sediments in the YT tended to be more hypoxic with the lowest DO concentration, redox potential, and thickness of the oxidized layer among sediments in three wetlands (Table 3). Greater amounts of dissolved P were also detected in the pore water and overlying water of YT (Table S3), as well as more enriched functional genes compared with the other wetlands. In response to experimental warming, DO concentrations calculated from the mean value of different months decreased by 9.73% for YT, compared with 4.50% and 7.90% for XZ and XX, respectively (Table 3). Similarly, the thickness of the oxidized layer was reduced by 27.4% for YT, as well as by 8.72 and 8.33% for XZ and XX, respectively. Therefore, in YT wetland columns, for example, warming induced an additional 4.0 mg l^{-1} P in pore water and 0.52 mg l^{-1} P in overlying water (Table 3). For other parameters, pH and water contents in sediments were not significantly changed in our study.

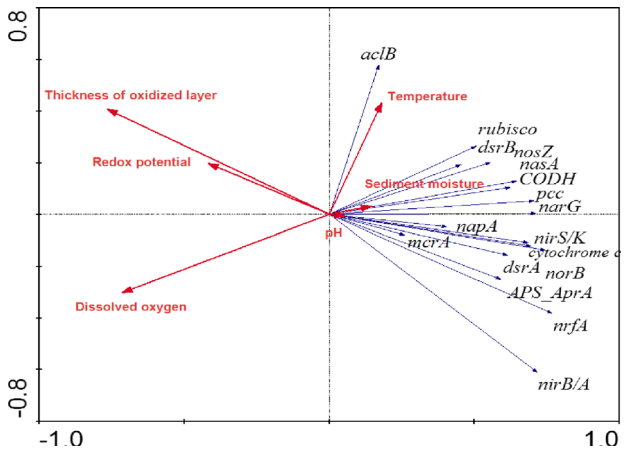
Of the five selected environmental variables (Fig. 3), RDA demonstrated that the parameters of the DO, the thickness of the oxidized layer, and the redox potential had higher F values and longer arrows compared with the sediment moisture and pH, which indicated a strong link between the redox variables and microbial functional gene differences. The increased temperature was positively ($P = 0.002$) correlated with the selected functional genes, whereas the redox potential was negatively ($P = 0.004$) correlated, which suggested that rising temperatures and lower redox potentials are beneficial for these selected genes. Based on the VPA results, redox variables (i.e., the DO, the thickness of the oxidized layer, and the redox potential) explained 41.4% ($P = 0.002$) of the total variations, whereas the temperature and other factors (sediment moisture and pH) independently explained 5.6 and 2.3% of variations, respectively. Approximately 51.3% of the community functional variations, which were based on the GeoChip data, remained unexplained by the above selected variables. Since there are more redox variables (i.e., DO, thickness of oxidized layer, redox potential) than others (i.e., temperature) in the VPA, and these parameters may strongly depend on each other, 41.4% of variations may be an overestimate. Due to this, a further VPA excluding redox variables was conducted, and the results showed that without redox potential, other parameters failed to significantly explain ($P > 0.05$) the variations of selected functional genes among studied samples. Our statistical analysis finally validated our hypothesis that the warming-induced decrease in redox conditions at the sediment–water interface greatly impacted microbial-mediated anaerobic metabolism.

Field phosphorus variations in water under low redox potential

Integrated data from different sampling months using a two-way ANOVA showed that experimental warming significantly increased P concentrations by 30.6 (XZ)–67.2% (XX) in pore water and marginally increased ($P < 0.1$) P concentrations in overlying water (Table 3, Table S3). In each specific month, higher ($P < 0.05$, Students' t test) P concentrations in overlying water under warming were also observed for

Table 3 Insitu observations for dissolved oxygen (DO) concentration (mg l^{-1}) at the bottom of water, thickness of top oxidized sediment layer (cm), redox potential of uppermost sediment centimeter (mV) (May 2010–February 2011) and phosphorus (P) concentrations (mg l^{-1}) in the pore water and overlying water (2009–2010) in wetland columns incubated under treatments (control vs. warmed) with sediments collected from three tested wetlands. Data with significant *P*-values (<0.05 or 0.01) are in bold. Indices with the same superscript in each row are not significantly different at the 0.05 levels

Parameters	YT			XZ			XX		
	Control	Warmed	*P* value	Control	Warmed	*P* value	Control	Warmed	*P* value
DO concentration	8.42	7.61[a]	0.075	10.0	9.55[c]	0.306	9.23	8.50[b]	**0.001**
thickness of oxidized layer	0.73	0.53[a]	**<0.001**	1.72	1.57[b]	**0.017**	1.92	1.76[c]	**0.008**
Redox potential	231	212[a]	**0.006**	297	262[b]	**<0.001**	307	291[c]	**0.035**
Pore water P concentration	12.2	16.2[a]	**0.033**	1.22	2.04[b]	**<0.001**	1.60	2.09[b]	**0.032**
Overlying water P concentration	2.49	3.01[a]	0.067	0.16	0.23[b]	0.136	0.21	0.24[b]	0.094



Environmental variables	F	*p*-value
Thickness of oxidized layer	37.1	**0.002**
Temperature	6.19	**0.002**
Redox potential	4.17	**0.004**
Dissolved oxygen	3.67	**0.016**
Sediment moisture	2.26	0.082
pH	0.59	0.646

Fig. 3 GeoChip 4.0 data redundancy analysis (RDA) for signal intensities of selected genes related to microbial oxidation–reduction metabolism detected in sediment samples from tested wetlands and environmental variables. Forward selections with Mante Carlo significance test were performed to show the relative importance of environmental variables with *F* values and *P* values in a decreasing order. Data with significant *P* values (<0.05 or 0.01) are in *bold*. See Fig. 2 for functional category of each specific gene

samples that were collected in May and Jul, whereas the release of P from sediment to pore water seemed occur more strongly in response to experimental warming compared with the subsequent upward transfer into overlying water (Table S4). For seasonal dynamics, P concentrations exhibited large seasonal variations with greater ($P < 0.05$) P release observed in Jul and Sep compared with other months (Table S3).

Discussion

Hypoxia and subsequent eutrophication events are a global problem. Many coastal and marine ecosystems have forecasted expanding hypoxic zones, which will be followed by elevated temperature and associated environmental changes (Diaz & Rosenberg, 2008; Conley et al., 2009). Metabolic rates increase exponentially with rising temperatures (Brown et al., 2004; Gudasz et al., 2010), leading to increased responses of benthic microbes to hypoxia. With the growth of excess anthropogenic nutrient inputs in recent years, elucidating the warming-induced major environmental changes and the associated microbial-mediated key functional processes is a crucial scientific step forward in understanding biogeochemical cycling in freshwater ecosystems in the context of global warming. Our results showed that experimental warming decreased the oxygen concentration and the redox potential in nutrient-enriched freshwater sediments (Table 3; Fig. S10) and enhanced the potential of anaerobic metabolism in benthic microbial processes (Tables 1, 2),

which caused sediment P mobilization and release (Table 3, Table S3). Seasonally, the lowest DO concentration and redox potential all occurred in the summer, and continuously increased and then maintained at the highest values in the spring or winter (Fig. S10). Such dynamics of DO and redox potential were consistent with observations for P release, which showed that the higher P concentrations were found in Jul and Sep compared to those in other seasons. At seasonal scales, the important roles of temperature can be well reflected by the impacts of our real-time manipulated warming experiment.

Functional gene diversity pattern

Elevated temperatures may affect sediment metabolic potential in both direct and indirect ways. Compared with historical climate data, the temporal trend of elevated air temperature has led to an acceleration of global soil respiration (Bond-Lamberty & Thomson, 2010; Weedon et al., 2013). Additionally, our previous study showed that the microbial metabolic activity and the abundance of functional genes that are involved in carbon degradation positively increased with experimental warming (Wang et al., 2012). These results suggest that elevated temperatures would directly enhance the microbial metabolic potential for the decomposition of sediment organic matter. However, such a stimulatory effect may diminish over time due to the thermal acclimation of microbial physiology (Bradford et al., 2008; Zhou et al., 2012), which partially explains some unchanged gene abundances in warmed samples, as shown in Fig. 2. Potential indirect effects, such as warming-induced environmental changes, may greatly alter microbial metabolic pathways. In forest soils, the reduced soil moisture under warming suppressed microbial metabolism is an example (Allison & Treseder, 2008). In nutrient-enriched freshwater sediments, the lower redox potential under warming increased the contribution of anaerobic metabolism to microbial activities (Table 2). The data represented here reflect this idea; temperature alone explained 5.6% of the total variations of selected functional genes compared with redox variables at 44.1% (Fig. 3). For specific functional process, carbon-degrading genes were studied in details, which showed a close linkage with total carbon contents, and different carbon fractions in sediments (Wang et al., 2012). The enriched carbon fixation genes (Fig. 2A, Fig. S4) suggest that warming may benefit the growth of some benthic anoxygenic photosynthetic bacteria. These bacteria usually colonize in photic hypoxic zones and can use small organic compounds as electron donors to synthesize their primary photosynthetic pigment (Goericke, 2002). A range of metal-reducing microbes, including *Desulfovibrio* and *Geobacter*, could utilize Fe(III) as the electron acceptor in their physiological oxidation–reduction reactions. The transformations of bio-available metals, especially iron performed by metal-reducing microbes, were supported by our decreased iron-binding P content in sediments (Wang et al., 2013). Accompanied by P release, iron oxides could be reduced by iron-reducing microbes. In this process, cytochrome c genes are oxidized through anaerobic metabolic pathways (Lovley et al., 1993). Similarly, enriched genes included those genes that are involved in denitrification processes, such as *nosZ*, which encodes nitrous oxide reductases (Fig. 2). When oxygen is limited, facultative or obligate anaerobes can use other electron acceptors, such as nitrate, ferric iron, sulfate, and even carbon dioxide for the anaerobic oxidation of organic compounds (Achtnich et al., 1995; Sanz-Lazaro et al., 2011). The warming-induced lower redox potential (Table 3; Fig. S10) reflects shifts in microbial functional genes with the potential preferential enhancement of some anaerobic metabolism (Fig. 2; Table 1).

The functional gene diversity was compared with microbial structure analysis performed in the same wetland columns using phospholipid fatty acids methods in our previous study by Zhang et al., (2012). The relative abundance of sulfate-reducing bacteria, methane-oxidizing bacteria, anaerobic bacteria, aerobic bacteria, actinomycetes, and protozoa increased in XZ and XX samples, while YT had decreased abundance in these microbial groups. This indicates that shifts in gene abundance may not always be consistent with shifts in microbial taxon, particularly since some defined aerobic bacteria can survive under anaerobic conditions.

Additionally, not all anaerobic-related genes were enriched under experimental warming. Our study primarily led to a higher potential of microbially mediated denitrification and metal reduction processes, except for methanogenesis and sulfur reduction (Table 1). The microbial utilization of multiple electron acceptors in a competitive environment usually

follows a strictly decreasing order of energy yield (Reddy et al., 1998). Methanogenesis and sulfate reduction only occur under a strongly reduced redox status (<−200 and <−100 mV, respectively) after the sequential reduction of nitrate and ferric iron (Achtnich et al., 1995; Jerman et al., 2009). In shallow freshwater ecosystems, molecular oxygen dissolved in the overlying water is capable of reoxidizing reduced chemical species, which ensures a continuous supply of nitrate, ferric iron, and other electron acceptors that cycle back into sediments, preventing a sharp decrease in the redox potential (Reddy et al., 1998; Sanz-Lazaro et al., 2011). Although the redox potential (ranging from 150 to 400 mV, Fig. S10) was mainly observed at the topmost sediment layer, no significant shifts in the abundance of genes for methanogenesis and sulfur reduction under experimental warming implied that the average level of redox potential at water–sediment interface may generally be higher than −100 mV (Fig. 2B, E). In contrast, for typical coastal or marine sediments with a strongly reduced redox status, there is a great potential for warming impacts on sulfate reduction rates (Sanz-Lazaro et al., 2011). No sulfate reduction potential was enhanced under warming in our study for freshwater sediments, which was one of the most predominant differences compared to the results in coastal or marine sediments reported by Sanz-Lazaro et al., (2011). Similar findings by Liang et al., (2012) using the comprehensive functional gene array GeoChip 2.0 showed that *dsrA/B* (responsible for sulfite reduction) and *mcrA* (responsible for methanogenesis) were enriched only when the redox status decreased from iron-reducing to sulfate reduction conditions. In general, iron reduction may out-compete methanogenesis for substrate utilization (Reddy et al., 1998). However, methane emission through methanogenesis has been verified to be a ubiquitous process in freshwater sediments under sustained water-logging and anoxic conditions when ferric iron is depleted (Jerman et al., 2009), which indicates that launching a long-term and dynamic observation to track the in situ sediment redox potential of freshwater ecosystems in response to warming is required in future studies. Overall, our lab-scale assays confirmed the changes in functional gene abundances: nitrate and ferric iron reduction rates were significantly higher in warmed samples compared with the control, whereas the differences in the average values of sulfite production rates between treatments were insignificant (Table 2). Lab-scale measurements of specific functional activities (Table 2) could provide evidence for the microbially mediated transformation potential of natural systems under ideal conditions. However, inconsistencies between the results from the lab-scale assay and the DNA signal intensities detected by microarrays may still exist because microbes could only utilize one single electron acceptor for each assay in our laboratory incubation, differing from a more "native" environment where multiple electron acceptors could be selectively used.

Implication for phosphorus mobilization

We investigated three wetlands along a gradient of sediment organic matter and nutrient contents (Table S1) to determine which wetland was more susceptible to experimental warming in terms of redox potential shifts. The in situ observation results (Table 3) showed that the YT wetland had a higher potential of oxygen deficiency occurring at the sediment–water interface compared with the other wetlands, which indicated that rising temperatures may have greater implications for hypereutrophic than for mesoeutrophic or oligotrophic freshwater. This observation is reasonable because when the available substrates as electron donors are affluent, then rising temperatures could substantially increase the threshold of sediment metabolic capacity (Sanz-Lazaro et al., 2011). In recent years, anthropogenic discharge greatly enhanced the nutrient loads in freshwater sediments, which accelerated the expansion of hypoxic zones (Diaz & Rosenberg, 2008; Conley et al., 2009; Korosi et al., 2013). High responses of hypereutrophic freshwater to experimental warming imply that the impacts of human activities on hypoxia would be preferentially triggered or even amplified by future climate warming. As a result, bottom water hypoxia may cause eutrophication through the enhanced regeneration of P from sediments (Conley et al., 2009; Zhang et al., 2012; Nurnberg et al., 2013), considering the high requirement for P by phytoplankton in water bodies. Previous study showed that carbon can adjust its cycling to enhance the dynamics of P (Reddy et al., 1998), indicating the close interrelationships between elements cycles in freshwater ecosystems. For the three tested wetlands, sediments with greater mineral content responded to a

lesser extent to experimental warming in term of P mobilization compared to those where the sediment had high organic content (Table 3). Not only does carbon affect P cycling, but also P may play more important roles in carbon dynamics. For example, our previous study also demonstrated that exogenous phosphorus inputs into farmland lands decreased the complexity of organic carbon (Liu et al., 2014), and thus increased its reactivity in dissolved forms. Recently, ecological stoichiometry as a promising field provides new insights into the sustainable C–P balance in soil ecosystems; how carbon responds to disturbance may be one of the determining incentives in predicting P release from a view of integrated wetland ecosystems.

Two steps of P release include the dissolution of particulate P to the pool of dissolved P in pore water and a diffusion process that is responsible for the release of dissolved P into the overlying water. This dissolved P can be absorbed with minerals or taken up by microbes. Increased temperature promotes P release through the accelerated desorption and decomposition of organic matter. In addition to the enriched *ppx* gene (Fig. 2E), many genes involved in carbon degradation were also enriched in our warmed samples (Wang et al., 2012), which contributed to P liberation from the breakdown of sediment aggregates. There was evidence in our previous study (Zhang et al., 2012) that warming led to higher phosphatase secretion, which may help explain the relatively higher P concentrations in the pore water under a warmer condition (Table 3). However, compared with these results, the major effect of increased temperature on P release invokes chemically and microbially mediated redox reactions (Sondergaard et al., 2003; Hui et al., 2013). P itself does not participate in redox reactions; however, the classic P cycling model is redox-sensitive based on the coupling of Fe and P cycles. Warming also promoted the redistribution of different mobile P forms in sediments with a significant decrease in iron-binding P contents (Wang et al., 2013). Lower redox weakens the ability of P that is bound to Fe(III) oxides (Sondergaard et al., 2003). In chemical or microbial dissimilatory Fe(III) reduction processes (as seen with enriched cytochrome c genes, Fig. 2F), Fe(III), which is an electron acceptor, is reduced to soluble Fe(II), which liberates metal oxide-bound P from sediments into pore water (Penn et al., 2000). Denitrification processes benefit P release because accumulated NO_3^-, similar to oxygen, can inhibit Fe(III) reduction (Smayda, 2005). When sulfate is reduced to sulfide, this molecule can bind to Fe(II). As a result, the released P will no longer be captured by iron, which is bound to sulfide, leading to more P availability (Hupfer & Lewandowski, 2008). Therefore, both denitrification and sulfate reduction can indirectly stimulate P release. Some microbial groups, such as denitrifying P-accumulating organisms, which use nitrate as an electron acceptor, could accumulate polyphosphate in extremely large amounts under aerobic conditions and release these accumulations when anoxia is prevalent (Hupfer et al., 2007), contributing to P release (Martins et al., 2011). However, Hupfer & Lewandowski, (2008) pointed out that redox-insensitive P release or retention and other P dynamic mechanisms should also be considered in a case of anoxic conditions beyond the above-discussed classic P cycling model. Through diffusion processes, the mobilized P in pore water could further transfer into overlying water. As a result, P concentration dynamics were consistent with the increased P fluxes under warming reported in our previous study (Wang et al., 2013). However, relative to pore water, P concentrations in overlying water were less responsive to experimental warming (Table S4). One reason for this observation is that a proportion of released P may chemically bind to the re-precipitated Fe(III) compounds (Sondergaard et al., 2003; Kleeberg et al., 2013) in water columns or through the activity of lithographic Fe(II)-oxidizing bacteria that live on the sediment surface. P dynamics in water bodies are also dependent on other variables, such as water chemical properties or aquatic plants (Cheng et al., 2010; Zhang et al., 2012), which could cause large seasonal fluctuations (Table S3) and, thus, high variability in concentration. In light of this observation, the ecology of benthic microbes could most likely explain P release in overlying water in less detail than those microbes in pore water. However, the accumulated dissolved P in pore water indicates a steeper P gradient along a sediment profile and, thus, poses a high potential risk for P transfer into water bodies under subsequent warming conditions in nutrient-enriched wetlands.

In this work, a microarray analysis for many functional genes was performed to show shifts in the

microbial metabolic potential in response to warming. The application of microarray analyses to farmlands (Reeve et al., 2010) and grasslands (Zhou et al., 2012) demonstrated a significant correlation between specific gene group diversity and related soil processes. These results suggest that the functional gene microarray is a promising tool to predict changes in microbially mediated processes in response to environmental disturbances. However, whether the microbial taxonomic structure was shifted from aerobe to anaerobe dominance under experimental warming remains unclear. Using phospholipid fatty acid technology, our previous study showed a greater total microbial biomass in warmed sediments relative to the control, which contributed to the high excretion of phosphatase and the subsequent P release (Zhang et al., 2012). However, the specificity of this technology to distinguish these two types of microbes (aerobes vs. anaerobes) is limited because many facultative anaerobes have similar characteristics of specific fatty acids to those characteristics for aerobes (Frostegard et al., 2011). In addition, functional characteristics are not strictly related to taxonomic units because most generalist species may harbor essentially identical genes (Yergeau et al., 2012). Another possibility arose from the fact that even some obligate anaerobes could survive in the oxidized layer and maintain a functional metabolism under oxygen stress or could be quickly reactivated by anoxic conditions (Angel et al., 2012). For instance, some species, such as *Desulfovibrio desulfuricans*, are capable of changing metabolic pathways from sulfate reduction to nitrate or oxygen reduction under changing environmental conditions (Cypionka, 1994), which enhances the variability in P release (Table S3). Microbial adaptation to life at the sediment–water interface is an important mechanism in modulating biological processes (Brune et al., 2000; Vuillemin et al., 2013). Determining how the profiles of microbes respond to varying environmental conditions at both taxonomic and functional levels remain challenging due to their functional versatility. In recent studies, metagenomic sequencing has proven to be a reliable and informative tool for understanding microbial taxonomic diversity and the functional attributes that cross various terrestrial biomes (Gilbert et al., 2008; Fierer et al., 2012, 2013). Therefore, further exploration of the linkage between microbial ecology from different perspectives and P dynamics in sediments using GeoChip integrated high-throughput sequencing for future studies is a worthy endeavor.

Conclusion

The elevated temperature and associated environmental shifts due to climate change may accelerate hypoxia in many coastal and marine ecosystems. In this study, the simulated experimental warming enriched the anaerobic-related functional genes, and enhanced the potential of anaerobic metabolic pathways, mainly denitrification and metal reduction. Such preferentially altered microbial functional and metabolic potentials were closely ($P < 0.01$) linked with warming-induced lower redox potential in sediments. As a consequence, the release of redox-sensitive P from sediments may be triggered under ongoing warming scenarios, which increases the potential of water eutrophication, especially in organic-enriched wetlands.

Acknowledgment This work was supported by the National Natural Science Foundation of China (41373074), National Ministry of Science and Technology (2013GB23600658), and National Ministry of Water Resources (201301092). This work was also partially supported by the United States Department of Energy, Biological Systems Research on the Role of Microbial Communities in Carbon Cycling Program (DE-SC0004601), and the Oklahoma Bioenergy Center (OBC).

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