Microbial Ecology

Salinity affects the composition of the aerobic methanotroph community in alkaline lake sediments from the Tibetan Plateau

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Abstract:	Lakes are widely distributed on the Tibetan Plateau, which plays an important role in natural methane emission. Aerobic methanotrophs in lake sediments reduce the amount of methane released into the atmosphere. However, no study to date has analyzed the methanotroph community composition and their driving factors in sediments of these high-altitude lakes (>4000 m). To provide new insights on this aspect, the abundance and composition in the sediments of six high-altitude alkaline lakes (including both freshwater and saline lakes) on the Tibetan Plateau were studied. The quantitative PCR, terminal restriction fragment length polymorphism and 454-pyrosequencing methods were used to target the pmoA genes. The pmoA gene copies ranged 10^4 -10^6 per gram fresh sediment. Type I methanotrophs predominated in Tibetan lake sediments, with Methylobacter and uncultivated type Ib methanotrophs being dominant in freshwater lakes and Methylomicrobium in saline lakes. Combining the pmoA-pyrosequencing data from Tibetan lakes with other published pmoA-sequencing data from lake sediments of other regions, a significant salinity and alkalinity effect (P=0.001) was detected, especially salinity, explained ~25% of methanotroph community variability. The main effect was Methylomicrobium being dominant (up to 100%) in saline lakes only. In freshwater lakes, however, methanotroph composition was relatively diverse, including Methylobacter, Methylocystis, and uncultured type Ib clusters. This study provides the first methanotroph data for high-altitude lake sediments (>4000 m) and shows that salinity is a driving factor for the community composition of aerobic methanotrophs.
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Reviewers' comments:

Reviewer #1:

1. Abstract needs tremendous improvement. Remove codes.
   - We have deleted the abbreviation (Line 33, 41) and improved the abstract (mainly Line 23-27).

2. Information regarding sequencing technologies/primers, is needed.
   - We have added more information in Line 132-136 about the pyrosequencing technologies.
     We used primer set A189f-mb661r for all methodologies which target *pmoA* gene, including qPCR, T-RFLP and pyrosequencing. We have added the primers' sequence and reference at the first place we mentioned it. Line 113-114: "forward primer A189f (5'-GGNGACTGGGACTTCTGG-3') and reverse primer mb661r (5'-CCGGMGCAACGTCYTTACC-3') [27]".

3. It is not clear why using both RFLP and 454 is better to determine diversity. Both methodologies have limitations. Discuss.
   - We have added one paragraph in discussion section to discuss this topic (Line 331-338).

4. Line 72. "haloalkalitorerant" would be haloalkalitolerant?
   - Thank you for your very careful reading. We have changed it in Line 74.

Line 210: "methantrophic" would be methanotrophic?
   - Thank you very much for finding such small mistakes. We have changed it in Line 214.

Line 248: Authors should make clear what method was used to "... first checked for a significant explanation of each environmental factor..."
   - We have clarified the method and reorganized this sentence (Line 252-254).

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Journal: Microbial Ecology

Title: Salinity affects the composition of the aerobic methanotroph community in alkaline lake sediments from the Tibetan Plateau

Running title: Salinity drives methanotroph community in lakes

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Abstract

Lakes are widely distributed on the Tibetan Plateau, which plays an important role in natural methane emission. Aerobic methanotrophs in lake sediments reduce the amount of methane released into the atmosphere. However, no study to date has analyzed the methanotroph community composition and their driving factors in sediments of these high-altitude lakes (>4000 m). To provide new insights on this aspect, the abundance and composition in the sediments of six high-altitude alkaline lakes (including both freshwater and saline lakes) on the Tibetan Plateau were studied. The quantitative PCR, terminal restriction fragment length polymorphism and 454-pyrosequencing methods were used to target the *pmoA* genes. The *pmoA* gene copies ranged 10^4 -10^6 per gram fresh sediment. Type I methanotrophs predominated in Tibetan lake sediments, with *Methylobacter* and uncultivated type Ib methanotrophs being dominant in freshwater lakes and *Methylomicrobium* in saline lakes. Combining the *pmoA*-pyrosequencing data from Tibetan lakes with other published *pmoA*-sequencing data from lake sediments of other regions, a significant salinity and alkalinity effect (P=0.001) was detected, especially salinity, explained ~25% of methanotroph community variability. The main effect was *Methylomicrobium* being dominant (up to 100%) in saline lakes only. In freshwater lakes, however, methanotroph composition was relatively diverse, including *Methylobacter*, *Methylocystis*, and uncultured type Ib clusters. This study provides the first methanotroph data for high-altitude lake sediments (>4000 m) and shows that salinity is a driving factor for the community composition of aerobic methanotrophs.

Keywords: aerobic methanotrophs, high-altitude lake, *Methylomicrobium,* salinity, Tibetan plateau

Introduction

Methane is an important greenhouse gas and its concentration has increased 150% since 1750 [1]. Lakes are estimated to be responsible for 6-16% of total natural methane emission, which is greater than oceanic emission [2]. Methane emission in lake sediments is ultimately regulated by microbial production and oxidation, including the actions of methanogens and methanotrophs. Aerobic methanotrophs within the oxic lake sediment surface layer can consume ~93% of the methane produced in deeper sediments [3], fulfilling an important ecosystem service.

Methanotrophs belonging to the phylum Proteobacteria [4] are widespread in diverse habitats. They are commonly divided into two groups: type I methanotrophs in the family *Methylococcaceae* (Gammaproteobacteria) and type II methanotrophs in the family *Methylocystaceae* and *Beijerinckiaceae* (Alphaproteobacteria) [5]. Methanotrophs in Gammaproteobacteria are further divided into type Ia and type Ib. The genera *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosarcina* belong to type Ia, while *Methylocaldum*, *Methylococcus* and uncultivated cluster FWs, LWs and RPCs are grouped as type Ib methanotrophs [6, 7]. Methanotrophs can be identified in environmental samples not only by 16S rRNA gene analysis but also by the detection of the *pmoA* gene, which encodes the β-subunit of particulate methane monooxygenase (pMMO) [8].

A large diversity of different methanotrophs has been reported in different lake ecosystems [9, 10, 11]. In sediments of freshwater lakes, such as Lake Constance, arctic Lake Qalluuraq and Yunnan lakes, type I methanotrophs dominated over type II methanotrophs [10, 11, 12, 13]. Especially *Methylobacter* (type Ia) is always one of the dominant genera. However, because of unclear classification of uncultivated clusters, methanotrophs in the type Ib group have not been well described in the former lake studies. Methane oxidation activity has also been found in the water column and the sediment of saline and alkaline lakes [14, 15]. Methanotroph communities of saline and alkaline lakes are dominated by type I methanotrophs [9, 16], and many of these haloalkaliphilic and haloalkalitolerant type I methanotrophs have also been isolated in pure culture [17, 18]. In the meantime, type II methanotrophs were also isolated from saline and alkaline lakes indicating that they are an important component of the methanotroph community [19, 20].

There are several thousand lakes on the Tibetan Plateau with a total area of about 36,900 km^2 accounting for about 49.5% of the total lake area in China [21]. These lakes are characterized by high altitude and low annual mean temperature [22]. These environmental conditions could favor an aerobic methanotroph community distinct from warmer climates, as it is generally thought that low temperature might be of advantage for type I methanotrophs [11, 23]. Most of the Tibetan lakes are alkaline and some of them are more or less saline [24, 25, 26], so that the gradual effects of salinity can be studied. However, the methanotroph composition in these lakes has not been reported previously.

Our main objectives were to obtain information on the presence of aerobic methanotrophs in lake sediments of the Tibetan region and to investigate the effect of salinity on their community composition. Therefore, sediments from six lakes were sampled on the Tibetan Plateau. We applied *pmoA* gene pyrosequencing coupled with terminal restriction fragment length polymorphism (T-RFLP) and quantitative PCR (qPCR) to detect the relative abundance of the main methanotroph groups in these lake sediments. We also summarized other published studies on methanotroph community composition in lake sediments from various other regions and compared them with our results from the lake sediments of the Tibetan Plateau.

Materials and Methods

Characteristics of the lakes

The six lakes sampled were Bangong Co (BGC), Zongxiong Co (ZXC), Lagor Co (LGC), Qige Co (QGC), Long Co (LC) and Zhangnai Co (ZNC), which were all located on the Tibetan Plateau (Figure 1). The altitude of these lakes was all above 4,000 m. Triplicate samples from the surface sediment (0-5 cm) were collected from each lake in August 2012 at or near the site of maximum water depth using a sediment grab sampler. The salinity, pH and conductivity of lake water overlying the sediment were measured using the Hydrolab DS5 Quality Multiprobe (Hach, Loveland, CO, USA). The sediment samples were kept in a cool box during transportation and stored in the laboratory at -20°C. The total organic carbon (TOC) and total nitrogen (TN) of sediments were determined by dichromate oxidation and Kjeldahl digestion,

respectively.

DNA extraction and quantitative PCR

Total DNA was extracted from 0.5 g sediment using the FastDNA® SPIN Kit (MP Biomedical). The copy numbers of *pmoA* gene were determined by quantitative PCR using forward primer A189f (5'-GGNGACTGGGACTTCTGG-3') and reverse primer mb661r (5'-CCGGMGCAACGTCYTTACC-3') [27] as described previously [28]. Quantitative PCR was performed using an iCycler instrument (Bio-Rad) and the SYBR Green System (Sigma-Aldrich).

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP fingerprinting of *pmoA* genes from the lake sediment DNA was performed using the A189f-mb661r primers with the forward primer A189f FAM-labeled. PCRs were performed on a Bio-Rad instrument with the following cycling conditions: initial denaturation (94°C, 4 min), followed by 38 cycles of denaturation (94°C, 1 min), annealing (54°C, 1 min) and elongation (72°C, 1 min), and final extension (72°C, 10 min). Each 50 μl PCR reaction contained 25 μl Premix Taq DNA Polymerase (TaKaRa Co), 0.5 μM each primer and 1 μl template. GenElute PCR Clean-up kit (Sigma) was used to purify the PCR product. Approximately 100 ng of purified product was then digested with the restriction endonuclease *Msp*I (TaKaRa Co) for 1 h at 37°C. Reactions were stopped by incubating at 65°C for 15 min. The digested products were sent to Sangon Biotech Company (Shanghai, China) for T-RF

separation.

Pyrosequencing and data analysis

Amplicon pyrosequencing was performed using the same primer set (A189f-mb661r without FAM-label) as for qPCR and T-RFLP fingerprinting. Each sample had an A189f primer with a 6-bp individual barcode for further identification. PCR was performed in 50 ml volumes using the same conditions as described for T-RFLP analyses. Finally, the PCR products from each sample were quantified by Qubit dsDNA HS Assay Kit (Life Technologies) and pooled together at equal concentration. Roche GS-FLX 454 pyrosequencing was carried out at Meiji Biotechnology Company (Shanghai, China) using standard procedures. The pyrosequencing data were processed as described previously [29]. In short, reads were first sorted to samples based on the unique barcode and then sequences with low quality or shorter than 400 bp were deleted in Mothur (v. 1.27) [30]. Taxonomy analyses based on a *pmoA* taxonomy database (Supplement of [29]) were done using the Bayesian/Wang methods (cutoff=80%). The pyrosequencing reads (raw data) were deposited under the study number SRP068717 in the NCBI Sequence Read Archive.

Data collection and analysis

Besides data of the 6 lakes we measured on Tibetan Plateau, the *pmoA* sequence data in sediments of other 22 lakes were collected from literatures. All the *pmoA* sequences had been obtained using the primer sets A189f-mb661r or A189f-A682r.

Only those studies were included that were based on DNA extracted from lake sediments, while those based on DNA from lake water or from enrichments of lake sediments were not included. Eighteen of the studied lakes are freshwater lakes, including four lakes in Germany (Lakes Dagow, Stechlin [31]; Lakes Plußsee, Schöhsee [32]) and fourteen on the Yunnan Plateau in China [12, 13]). Only sequences in lake Plußsee and Schöhsee were amplified using A189f-A682r primer set. All the *amoA* sequences obtained in these two lakes were deleted for further OTU analysis. The other four analyzed lakes were saline lakes in Russia (Lakes Suduntuiskii, Gorbunka, Khuzhirta [9]) and India (Lonar Lake [33]). The *pmoA* sequences from all these lake sediments were downloaded from the website of the National Center for Biotechnology Information (NCBI).

Distance matrices were calculated in ARB [34] based on the 133 amino acid residues of the downloaded *pmoA* sequences and the high quality *pmoA* sequences obtained in the present study. OTUs were assigned based on a cut-off of 7% protein dissimilarity using the average linkage algorithm implemented in Mothur. Two *pmoA* sequences from Lonar Lake were shorter than 400 bp length and excluded from the calculations. Instead, they were subsequently assigned to OTUs using a maximum-parsimony method. A neighbor-joining phylogenetic tree based on representative OTU sequences and related sequences was built using ARB. A heatmap was generated based on methanotroph composition data using the R functions heatmap.2 (package gplots) and hclust with Euclidean distances and Ward distance functions (ward. D2). The phylogenetic tree and heatmap were combined in Adobe Illustrator CS6 according to the position of the OTUs in the tree.

Statistical analysis

DUNCAN test was performed to test significant differences of *pmoA* gene copy numbers, TOC and TN between different lakes using agricolae package in R [35]. The canonical correspondence analysis (CCA) method [36] was used to investigate the effects of environmental factors on the methanotroph community composition. First, CCA analysis was conducted for each candidate environmental factor (including salinity and pH of lake water, TOC and TN of lake sediment) to find out which factor has significant effect. Only salinity and pH have significant impacts and were used as environmental factors. The 22 lakes having pH values were used in the analysis. The pH factor was used as scale variable, while the salinity factor was used as nominal variable because 8 out of 22 lakes only had salinity categories (i.e., fresh lake or saline lake) instead of numerical salinity values. The 14 lakes with numerical salinity values were classified into categories using the method proposed by Hammer (1986) [37]. In this method, lakes were classified into 5 categories: fresh (less than 0.5 g L^{-1} salinity), subsaline (0.5-3 g L^{-1}), hyposaline (3-20 g L^{-1}), mesosaline (20-50 g L^{-1}), and hypersaline (more than 50 g L^{-1}) according to their salinity. Because there are only 2 salinity categories in the other 8 lakes, the subsaline, hyposaline, mesosaline and hypersaline lakes were further grouped as saline lakes to generate a classification result of 2 categories. In addition, we also conducted a CCA analysis taking both the salinity and pH factors as scale variables using data of the 14 lakes with numerical salinity values. The variables for methanotroph community composition included the relative abundance of 28 non-singleton OTUs. An ANOVA like permutation test

function (anova.cca) was run to detect the significant environmental factors contributing to the methanotroph community composition. All CCA statistical analyses were performed using the vegan package in R [38].

Results

The six lakes studied are located in different geographic zones along a west-east transect on the Tibetan Plateau (Figure 1). The lakes have different environmental and limnological characteristics (Table 1). All of the lakes were alkaline (pH 8.7-10.4), but had different water salinities (and conductivities). Three of the lakes (ZXC, QGC and BGC) were considered as freshwater lakes, three (LC, ZNC and LGC) as saline lakes with salinities ranging from 1.55 g/L (3000 μS cm^{-1}) to 43.8 g/L (63649 μS cm^{-1}). TOC of lake sediments was between 21.8 g/kg and 97.4 g/kg; and TN was between 1.1 g/kg and 5.1 g/kg (Table 1). TOC and TN of lake sediments in ZXC and BGC were significantly higher than those in the other four lakes ($P<0.05$).

Methanotroph abundances were determined by qPCR analyses of the *pmoA* gene copies. Lake LC, which has a salinity of 1.55 g/L, had *pmoA* gene copies of 10^6 g^{-1} fresh sediment. Higher *pmoA* gene copies were detected in the sediments of freshwater lakes (ZXC, QGC and BGC, 10^6 g^{-1} fresh sediment) than that in the saline lakes (ZNC, 10^4 g^{-1} fresh sediment and LGC, 10^5 g^{-1} fresh sediment) ($p<0.05$) (Table 1 and Figure S1).

The composition of the methanotroph community in Tibetan lake sediments was determined by T-RFLP and pyrosequencing analysis of the *pmoA* genes. Six main T-RF fragments were detected (Figure 2A). The affiliation of these T-RFs to phylogenetic groups of methanotrophs is shown in Table S1. We compared the abundances obtained by T-RFLP and pyrosequencing and found very good agreement (Figure 2A, B). Type I methanotrophs were found to be dominant in all Tibetan lake sediments. In the freshwater lakes (ZXC and QGC), sequences of the uncultivated type Ib groups RPCs and FWs (both main T-RF 79 bp) accounted for more than 90% and type Ia sequences belonging to *Methylobacter* (main T-RF 505 bp) made up the rest. In the sediment of freshwater BGC, *Methylobacter* accounted for 80% of the detected sequences. However, in the sediments of saline lakes (LC, ZNC and LGC) the type Ia genus *Methylomicrobium* (main T-RF 437 bp) was the by far most abundant methanotroph. Its relative abundance is almost 100% in lakes ZNC and LGC and is 57% in LC. The lake LC contained the type II methanotroph genus *Methylocystis* as the second most abundant (42%) group. In the other lakes *Methylocystis* was only detected at very low (<1%) relative abundance.

In addition to the data from the Tibetan Plateau lakes, we also summarized the published methanotroph community compositions in sediments of lakes from other geographical regions. These included both freshwater and saline lakes, and also lakes having neutral or alkaline pH (Figure 3). All these lakes have *pmoA* OTU numbers between 2 to 13. The coverage was generally higher 76% and most higher than 90% meaning that these *pmoA* sequences represent the methanotrophs in these lakes quite

well. The average methanotroph diversity was higher in freshwater than saline lakes (Table S2). Using Euclidean distances and Ward distance functions, saline lakes and freshwater lakes were separated into two different groups (Figure 3). The relative abundance of *Methylomicrobium* was clearly higher in sediments of saline compared with freshwater lakes. *Methylobacter* (OTU-4), *Methylocystis* (OTU-5), and the uncultured clusters Lake Cluster-2 (OTU-6), FWs (OTU-3) and LWs (OTU-7, 8, 13 and 14) were common methanotrophs in freshwater lakes. Besides these abundant clusters, other methanotroph groups were also present in these lakes, especially among type I methanotrophs (Figure 3). The type Ia-affiliated sequences detected include *Methylomonas*, *Methylosarcina* and RPC-2 clusters. Besides Lake Cluster-2, FWs and LWs, the clusters RPCs, RPC-1, *Methylocaldum*_rel and *Methylothermus* within type Ib group were detected. *Methylothermus* species were only found in two saline lakes in Russia. Among the type Ic group, upland cluster USCγ was detected in BGC, ZNC and LGC, and upland cluster JR-3 was detected in BGC and QGC. Among type II methanotrophs, *Methylocystis* species were common and *Methylosinus* and *Methylocapsa* were also detected in saline lakes in Russia.

We first performed CCA and the ANOVA like permutation test (anova.cca) for each environmental factor to check which factor has a significant explanation for the methanotroph community matrix. TOC and TN data of Tibetan and Yunnan Plateau lakes were collected and they accounted for only 6.7% (P=0.22) and 5.9% (P=0.34) of the variability of the methanotroph community composition, respectively, which are not significant. Salinity and pH both significantly accounted for the variability of

methanotroph community (P=0.001) and were selected to perform CCA analysis. The contribution of salinity and pH to the total variability of the methanotroph community matrix was 33.1%, of which CCA1 accounted for 25.7% and CCA2 for 7.4% of total variability. CCA1 described salinity difference of lakes, reflected by a distribution of OTUs and lakes along CCA1 (Figure 4). CCA2 showed the effects of pH on the methanotroph community composition. We also performed CCA analysis with 14 lakes whose salinity and pH values were known. The results confirmed our conclusion that salinity is the most important factor in shaping methanotroph community (Figure S1). The methanotroph cluster *Methylomicrobium* was only abundant in saline and alkaline lakes. The freshwater lakes TP-QGC and TP-ZXC with alkaline pH, different from other freshwater lakes with lower alkalinity, showed higher relative abundances of FWs and RPCs.

Discussion

Salinity has been reported as the driving factor in shaping bacterial and archaeal community composition in Tibetan lake sediments [25, 26, 39, 40]. We found a significant effect of lake water salinity on methanotroph community composition in Tibetan lake sediments and other studied sediments (P=0.001). The main difference was *Methylomicrobium* species being abundant in sediments of lakes with water salinity higher than 1.5 g/L (0.15% w/v, 0.026 M). This salinity seems to be the threshold for the presence of *Methylomicrobium* species. This observation is

consistent with the description of *Methylomicrobium* strains as being NaCl-requiring methanotrophs (0.05 M Na^+), isolated from hypersaline and alkaline lake sediments [41, 42, 43].

Sediment pH was found to be the best predictor for the community structure of bacteria in lake sediments on the Tibetan Plateau [24]. We found that besides salinity, pH was also an important factor shaping the methanotroph community composition of lake sediments. However, our results showed that *Methylomicrobium* species were absent in the non-saline lakes TP-QGC and TP-ZXC (pH > 9), and the pH optima of *Methylomicrobium* species isolated from hypersaline and alkaline lakes was 6.5-10.5 [43]. This indicates that it is salinity rather than pH that will select for *Methylomicrobium* in lake sediments. The fact that the saline lakes analyzed here were all high pH with a span of 1 pH unit means that we could not test the effect of pH on the methanotroph community in these lakes. However, the CCA analysis indicated pH was the main factor in separating the freshwater lakes TP-QGC and TP-ZXC (pH > 9) with other freshwater lakes with lower alkalinity. Therefore, salinity is the primary factor with pH having a secondary effect on methanotroph community structure in lake sediments.

Methanotroph community composition has been previously studied in other saline lakes, albeit sometimes using different primer sets than those used here or targeting lake water rather than sediment. PCR products were obtained from DNA purified from Mongolian soda lake sediments using primers specific for *Methylomicrobium* [44] and *Methylomicrobium* species were found to be abundant in

the water of Mono Lake, California [15, 20]. Besides *Methylomicrobium*, which seems to be abundant in saline lakes, other genera like *Methylobacter*, *Methylothermus* and *Methylocystis* are commonly found in saline lakes [9, 15, 20].

DNA stable-isotope probing experiments in Lonar and Transbaikal soda lake sediments showed that *Methylomicrobium* species were not only abundant but were also responsible for the consumption of methane [15, 45]. Therefore, we may infer that *Methylomicrobium* is also active in consuming methane in the Tibetan lake sediments. *Methylomicrobium* appear to have adapted to the high salinity by the synthesis of osmoprotectants, such as ectoine, sucrose and glutamate [16, 43]. The formation of macro-molecular glycoprotein structures (S-layers) on the cell surface might be another adaptation to elevated salt concentration [16, 43].

Our results showed that type I methanotrophs were the dominant methanotrophs in the lake sediments on the Tibetan Plateau. They were relatively diverse in the non-saline freshwater lake sediments, including *Methylobacter* and uncultured clusters (Lake Cluster-2, FWs and LWs). In our results, all these clusters were abundant in sediments of the three freshwater lakes (BGC, ZXC and QGC). The Lake Cluster-2 and FWs clusters were similar to sequences found in freshwater lakes in Germany and on the Yunnan Plateau in China [10, 12, 13, 31, 32]. *PmoA* sequences of uncultivated type Ib methanotrophs have also been previously detected in Lake Constance [46], wetlands of Lake Kevaton [47] and the Zoige wetlands [48, 49].

Type II methanotrophs (e.g. *Methylocystis*) are ubiquitous methanotroph

inhabitants of many ecosystems [50, 51, 52] and often dominate among methanotrophs in acidic peat wetlands [51, 53]. We found that *Methylocystis* was a common methanotroph in most of the freshwater lake sediments. In addition, molecular studies of methanotrophs in soda lakes from Mongolia, Southeastern Transbaikalia and the United States, plus the isolation of *Methylocystis* from Southeastern Transbaikalia further indicates the presence of type II methanotrophs in saline and alkaline lakes [9, 15, 19, 44]. The detection of *Methylocystis* in alkaline Tibetan lakes suggests that *Methylocystis* are widely distributed in alkaline lake sediments.

In methanotroph community profiling studies, t-RFLP analysis targeting the *pmoA* gene has often been used [31, 54, 55]. However, T-RFLP has a limited phylogenetic resolution, and particular t-RFs can co-occur in more than one phylotype [55]. The different T-RFs need to be assigned to particular *pmoA* clusters by using *pmoA* sequencing data. Pyrosequencing allows linking particular *pmoA* clusters to T-RFLP fingerprints, and also allows deeply analyzing *pmoA* sequences for phylogenetic affiliation. We used these two techniques for analysis of *pmoA*. Both T-RFLP and pyrosequencing showed consistent methanotroph community composition.

In summary, our data show that salinity is the most important factor shaping methanotroph community composition in surface sediments of the Qinghai-Tibetan Lakes and other lakes around the world.

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Conflict of interest: None declared.

References

1. IPCC (2014) Climate Change 2014: Synthesis Report. Contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, pp 151.
2. Bastviken D, Cole J, Pace M et al (2004) Methane emissions from lakes: Dependence of lake characteristics, two regional assessments, and a global estimate. Global Biogeochem Cycles18:GB4009. doi:10.1029/2004gb002238
3. Frenzel P, Thebrath B and Conrad R (1990) Oxidation of methane in the oxic surface layer of a deep lake sediment (Lake Constance). FEMS Microbiol Ecol 73:149-158. doi:10.1128/aem.01350-08
4. Bowman J (2000) The Methanotrophs. The families Methylococcaceae and Methylocystaceae. In: Dworkin M (ed.). The Prokaryotes. Springer, New York, pp 266-289.
5. Semrau JD, DiSpirito AA and Yoon S (2010) Methanotrophs and copper. FEMS Microbiol Rev 34:496-531. doi:10.1111/j.1574-6976.2010.00212.x
6. Knief C (2015) Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on *pmoA* as molecular marker. Front Microbiol 6:1346. doi:10.3389/fmicb.2015.01346
7. Lüke C and Frenzel P (2011) Potential of *pmoA* amplicon pyrosequencing for methanotroph diversity studies. Appl Environ Microb 77:6305-6309. doi:10.1128/aem.05355-11
8. Dumont MG and Murrell JC (2005) Community-level analysis: Key genes of aerobic methane oxidation. Methods Enzymol 397:413-427. doi:10.1016/s0076-6879(05)97025-0
9. Lin JL, Radajewski S, Eshinimaev BT et al (2004) Molecular diversity of methanotrophs in Transbaikal soda lake sediments and identification of potentially active populations by stable isotope probing. Environ Microbiol 6:1049-1060. doi:10.1111/j.1462-2920.2004.00635.x
10. Rahalkar M, Deutzmann J, Schink B et al (2009) Abundance and activity of methanotrophic bacteria in littoral and profundal sediments of lake Constance (Germany). Appl Environ Microb 75:119-126. doi:10.1128/aem.01350-08
11. He R, Wooller MJ, Pohlman JW et al (2015) Methane-derived carbon flow through microbial communities in arctic lake sediments. Environ Microbiol 17:3233-3250. doi:10.1111/1462-2920.12773
12. Yang YY, Zhao Q, Cui YH et al (2015) Spatio-temporal variation of sediment methanotrophic microorganisms in a large eutrophic lake. Microb Ecol 71:9-17. doi:10.1007/s00248-015-0667-7
13. Liu Y, Zhang JX, Zhao L et al (2015) Aerobic and nitrite-dependent methane-oxidizing microorganisms in sediments of freshwater lakes on the Yunnan Plateau. Appl Microbiol Biotechnol 99:2371-2381. doi:10.1007/s00253-014-6141-5
14. Khmelenina VN, Eshinimaev BT, Kalyuzhnaya MG et al (2000) Potential activity of methane and ammonium oxidation by methanotrophic communities from the soda

lakes of Southern Transbaikal. Microbiology 69:460-465. doi:10.1007/BF02756771
15. Lin JL, Joye SB, Scholten JCM et al (2005) Analysis of methane monooxygenase genes in Mono Lake suggests that increased methane oxidation activity may correlate with a change in methanotroph community structure. Appl Environ Microb 71:6458-6462. doi:10.1128/aem.71.10.6458-6462.2005
16. Trotsenko YA and Khmelenina VN (2002) The biology and osmoadaptation of haloalkaliphilie methanotrophs. Microbiology 71:123-132. doi:10.1023/a:1015183832622
17. Khmelenina VN, Kalyuzhnaya MG, Starostina NG et al (1997) Isolation and characterization of halotolerant alkaliphilic methanotrophic bacteria from Tuva soda lakes. Curr Microbiol 35:257-261. doi:10.1007/s002849900249
18. Sorokin DY and Kuenen JG (2005) Chemolithotrophic haloalkaliphiles from soda lakes. FEMS Microbiol Ecol 52:287-295. doi:10.1016/j.femsec.2005.02.012
19. Eshinimaev BT, Khmelenina VN and Trotsenko YA (2008) First isolation of a type II methanotroph from a soda lake. Microbiology 77:628-631. doi:10.1134/s0026261708050196
20. Carini S, Bano N, LeCleir G et al (2005) Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). Environ Microbiol 7:1127-1138. doi:10.1111/j.1462-2920.2005.00786.x
21. Bian D, Yang Z, Li L et al (2006) The response of lake area change to climate variations in north Tibetan Plateau during last 30 years. Acta Geol Sin 5:510-518
22. Zheng M (1997) An introduction to saline Lakes on the Qinghai-Tibet plateau. Springer Netherlands
23. He R, Wooller MJ, Pohlman JW et al (2012) Identification of functionally active aerobic methanotrophs in sediments from an arctic lake using stable isotope probing. Environ Microbiol 14:1403-1419. doi:10.1111/j.1462-2920.2012.02725.x
24. Xiong JB, Liu YQ, Lin XG et al (2012) Geographic distance and pH drive bacterial distribution in alkaline lake sediments across Tibetan Plateau. Environ Microbiol 14:2457-2466. doi:10.1111/j.1462-2920.2012.02799.x
25. Liu YQ, Yao TD, Jiao NZ et al (2013) Salinity impact on bacterial community composition in five high-altitude lakes from the Tibetan Plateau, Western China. Geomicrobiol J 30:462-469. doi:10.1080/01490451.2012.710709
26. Liu YQ, Priscu JC, Xiong JB et al (2016) Salinity drives archaeal distribution patterns in high altitude lake sediments on the Tibetan Plateau. FEMS Microbiol Ecol doi: 10.1093/femsec/fiw033.
27. Costello AM and Lidstrom ME (1999) Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl Environ Microb 65:5066-5074.
28. Kolb S, Knief C, Stubner S et al (2003) Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl Environ Microb 69:2423-2429. doi:10.1128/aem.69.5.2423-2429.2003
29. Deng YC, Cui XY, Lüke C et al (2013) Aerobic methanotroph diversity in Riganqiao peatlands on the Qinghai-Tibetan Plateau. Env Microbiol Rep

5:566-574. doi:10.1111/1758-2229.12046
30. Schloss PD, Westcott SL, Ryabin T et al (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microb 75:7537-7541. doi:10.1128/aem.01541-09
31. Dumont MG, Pommerenke B, Casper P et al (2011) DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. Environ Microbiol 13:1153-1167. doi: 10.1111/j.1462-2920.2010.02415.x
32. Kim OS (2007) Diversity of functional genes in the aquatic nitrogen cycle. PhD Thesis, Christian-Albrechts-Universität, Kiel.
33. Antony CP, Kumaresan D, Hunger S et al (2013) Microbiology of Lonar Lake and other soda lakes. ISME J 7:468-476. doi:10.1038/ismej.2012.137
34. Ludwig W, Strunk O, Westram R et al (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363-1371. doi:10.1093/nar/gkh293
35. De Mendiburu F, Simon R. (2015) Agricolae-Ten years of an open source statistical tool for experiments in breeding, agriculture and biology. PeerJ PrePrints 3:e1748 https://doi.org/10.7287/peerj.preprints.1404v1
36. Anderson MJ and Willis TJ (2003) Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. Ecology 84:511-525. doi:10.1890/0012-9658(2003)084[0511:CAOPCA]2.0.CO;2
37. Hammer UT (1986) Saline lake ecosystems of the world. Dr W Junk, Dordrecht
38. Oksanen J, Blanchet FG, Kindt R et al (2016) vegan: Community Ecology Package.
39. Liu XL, Hou WG, Dong HL et al (2015) Distribution and diversity of Cyanobacteria and eukaryotic algae in Qinghai-Tibetan lakes. Geomicrobiol J doi: 10.1080/01490451.2015.1120368.
40. Yang J, Ma LA, Jiang HC et al (2016) Salinity shapes microbial diversity and community structure in surface sediments of the Qinghai-Tibetan Lakes. Sci Rep 6:25078. doi:10.1038/srep25078
41. Kaluzhnaya M, Khmelenina V, Eshinimaev B et al (2001) Taxonomic characterization of new alkaliphilic and alkalitolerant methanotrophs from soda lakes of the Southeastern Transbaikal region and description of *Methylomicrobium buryatense* sp.nov. Syst Appl Microbiol 24:166-176. doi:10.1078/0723-2020-00028
42. Kalyuzhnaya MG, Khmelenina V, Eshinimaev B et al (2008) Classification of halo(alkali)philic and halo(alkali)tolerant methanotrophs provisionally assigned to the genera *Methylomicrobium* and *Methylobacter* and emended description of the genus *Methylomicrobium*. Int J Syst Evol Micr 58:591-596. doi:10.1099/ijs.0.65317-0
43. Khmelenina VN, Shchukin VN, Reshetnikov AS et al (2010) Structural and functional features of methanotrophs from hypersaline and alkaline lakes. Microbiology 79:472-482. doi:10.1134/s0026261710040090
44. Sorokin DY, Gorlenko VM, Namsaraev BB et al (2004) Prokaryotic communities of the north-eastern Mongolian soda lakes. Hydrobiologia 522:235-248. doi:10.1023/B:HYDR.0000029989.73279.e4
45. Antony CP, Kumaresan D, Ferrando L et al (2010) Active methylotrophs in the sediments of Lonar Lake, a saline and alkaline ecosystem formed by meteor impact.

ISME J 4:1470-1480. doi:10.1038/ismej.2010.70
46. Deutzmann JS, Woerner S and Schink B (2011) Activity and diversity of methanotrophic bacteria at methane seeps in eastern lake Constance sediments. Appl Environ Microb 77:2573-2581. doi:10.1128/aem.02776-10
47. Siljanen HMP, Saari A, Krause S et al (2011) Hydrology is reflected in the functioning and community composition of methanotrophs in the littoral wetland of a boreal lake. FEMS Microbiol Ecol 75:430-445. doi:10.1111/j.1574-6941.2010.01015.x
48. Yun JL, Ma AZ, Li YM et al (2010) Diversity of methanotrophs in Zoige wetland soils under both anaerobic and aerobic conditions. J Environ Sci 22:1232-1238. doi: 10.1016/s1001-0742(09)60243-6
49. Yun JL, Zhuang GQ, Ma AZ et al (2012) Community structure, abundance, and activity of methanotrophs in the Zoige wetland of the Tibetan Plateau. Microbial Ecol 63:835-843. doi: 10.1007/s00248-011-9981-x
50. Chen Y, Dumont MG, Cebron A et al (2007) Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. Environ Microbiol 9:2855-2869. doi:10.1111/j.1462-2920.2007.01401.x
51. Chen Y, Dumont MG, McNamara NP et al (2008) Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. Environ Microbiol 10:446-459. doi:10.1111/j.1462-2920.2007.01466.x
52. Kip N, van Winden JF, Pan Y et al (2010) Global prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems. Nature Geosci 3:617-621. doi:10.1038/ngeo939
53. Dedysh SN (2009) Exploring methanotroph diversity in acidic northern wetlands: molecular and cultivation-based studies. Microbiology 78:655-669. doi:10.1134/S0026261709060010
54. Horz HP, Yimga MT, Liesack W (2001) Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67: 4177-4185. doi: 10.1128/AEM.67.9.4177-4185.2001
55. Reim A, Lüke C, Krause S, et al (2012) One millimetre makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific activity at the oxic-anoxic interface in a flooded paddy soil. ISME J 6:2128-2139. doi: 10.1038/ismej.2012.57
56. Wang SM, Dou HS (1998) Chinese Lake Records. Science Press, Beijing
57. Schlegel I and Scheffler W (1999) Seasonal development and morphological variability of Cyclotella ocellata (Bacillariophyceae) in the eutrophic Lake Dagow (Germany). Int Rev Hydrobiol 84:469-478. doi: 10.1002/iroh.199900041
58. Salka I, Cuperova Z, Masin M et al (2011) Rhodoferax-related *pufM* gene cluster dominates the aerobic anoxygenic phototrophic communities in German freshwater lakes. Environ Microbiol 13:2865-2875. doi:10.1111/j.1462-2920.2011.02562.x
59. Furtado ALS, Casper P and Esteves FA (2001) Bacterioplankton abundance, biomass and production in a Brazilian coastal lagoon and in two German lakes. Anais Da

Academia Brasileira De Ciencias 73:39-49. doi:10.1590/s0001-37652001000100005
60. Krambeck HJ, Albrecht D, Hickel B, Hofmann W, Arzbach HH (1993) Limnology of the Plußsee. In: Overbeck J, Chrost RJ (eds) Microbial ecology of Lake Plußsee. Springer-Verlag, New York, pp 1-23.

Figure Legends

Figure 1 Location of the investigated six lakes, Long Co (LC), Bangong Co (BGC), Zongxiong Co (ZXC), Qige Co (QGC), Zhangnai Co (ZNC) and Lagor Co (LGC), on the Tibetan Plateau.

Figure 2 Relative abundances of different *pmoA* clusters in the six lake sediments based on (A) T-RFLP and (B) pyrosequencing analyses. Means ± SD, n=3.

Figure 3 Neighbor-joining phylogenetic tree based on representative OTUs and related *pmoA* sequences. The heatmap next to phylogenetic clusters shows the relative abundance of the sequencing reads assigned to each OTU. OTUs that are relatively abundant (≥10%) in lake sediments are marked in red; horizontal cluster codes indicate the different lake sediments from which the *pmoA* sequences originated. The first two capital letter indicate the location; i.e. DE = Germany; IN = India; RUS = Russia; TP = Tibetan Plateau; YN = Yunnan. The following letters indicate the particular lake. Values of pH and salinity were collect from the following literatures: pH and salinity of Yunnan lakes [56]; pH of DE-Dag [57]; pH of DE-Ste [58]; salinity of DE-Dag and DE-Ste [59]; pH of DE-Plu [60]; pH of RUS lakes and salinity of RUS-Gor [9]; pH and salinity of IN-Lon [33].

Figure 4 The effect of lake salinity and pH on methanotroph community composition was determined by canonical correspondence analysis (CCA). Relative abundances of

non-singleton OTUs were used for the methanotroph community matrix. Only 22 lakes with known pH values (Figure 3) were included in the analysis. Salinity separated them into two groups as saline (pink circle) versus freshwater (light blue circle) lakes. Characters next to symbols identify different lakes, the first two capital letter of the names indicating the location (i.e., DE = Germany; IN = India; RUS = Russia; TP = Tibetan Plateau; YN = Yunnan) and the following letters indicating the particular lake. OTUs are shown as red plus signs (+). Abundant OTUs (≥10%) are shown in bold and were labeled with their cluster affiliations, such as *Methylomicrobium*, *Methylocystis*, *Methylobacter*, Lake Cluster-2 and FWs.

Figure 1

Click here to download Figure Figure 1_Lake location.tif

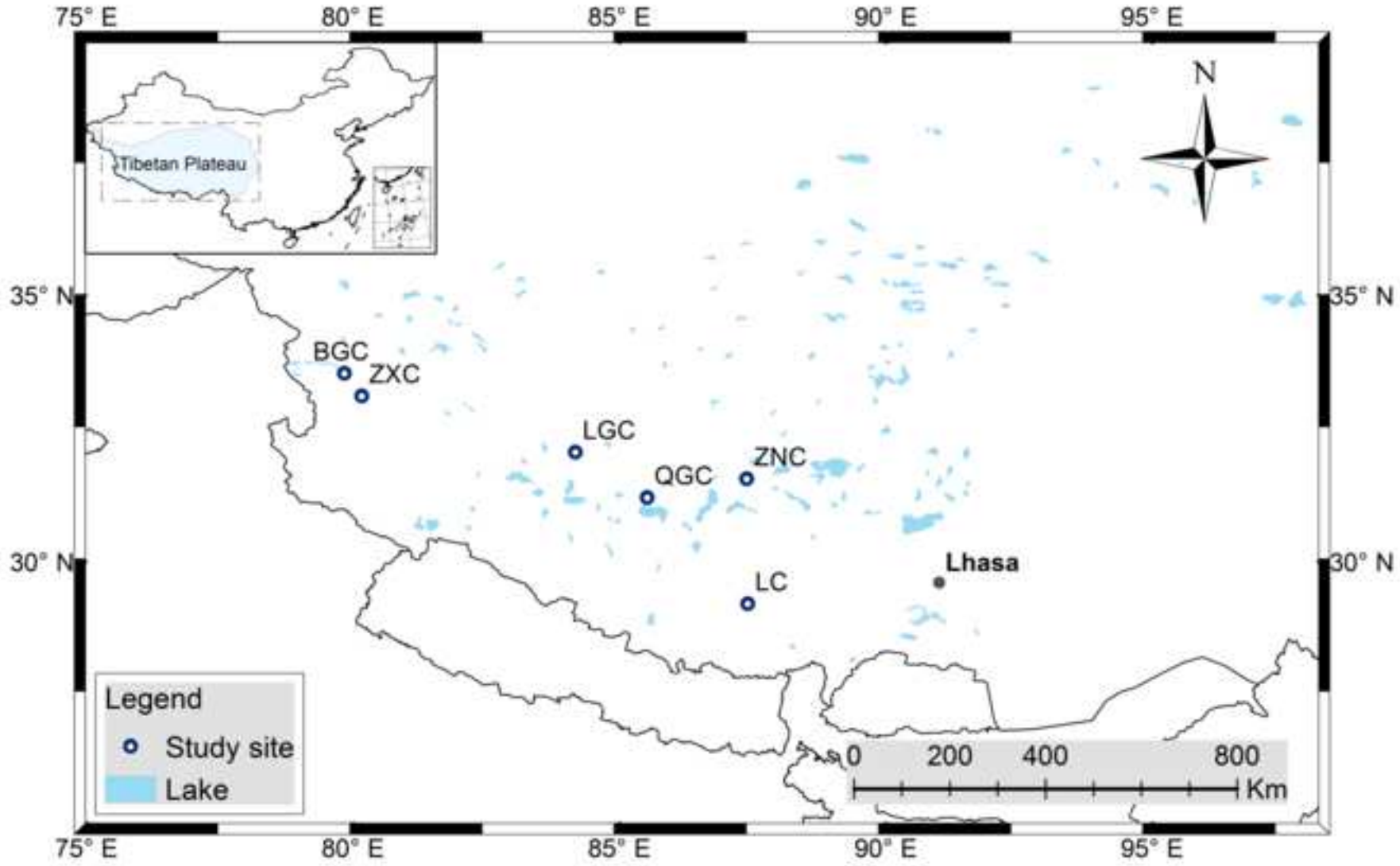

Figure 2

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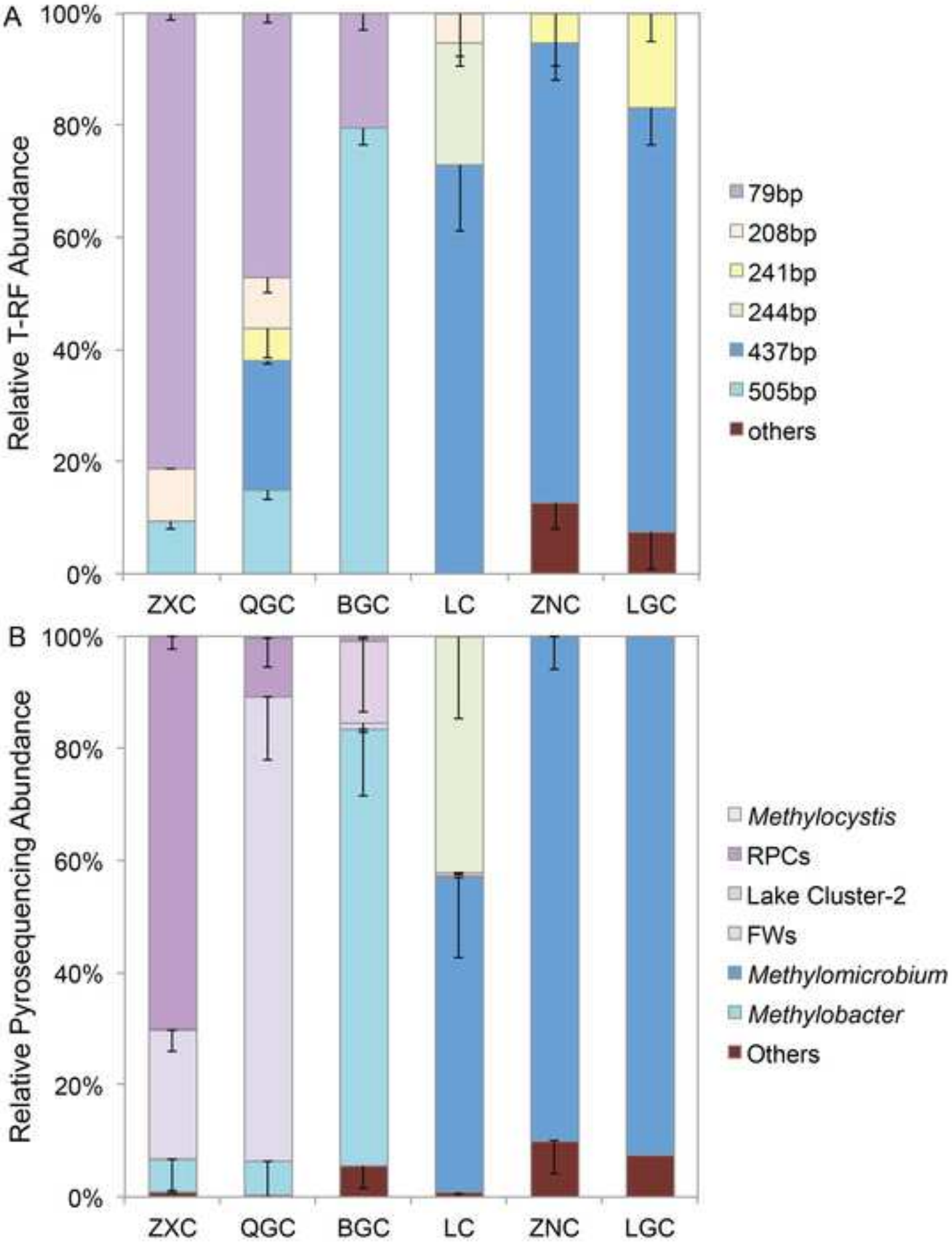

Figure 3

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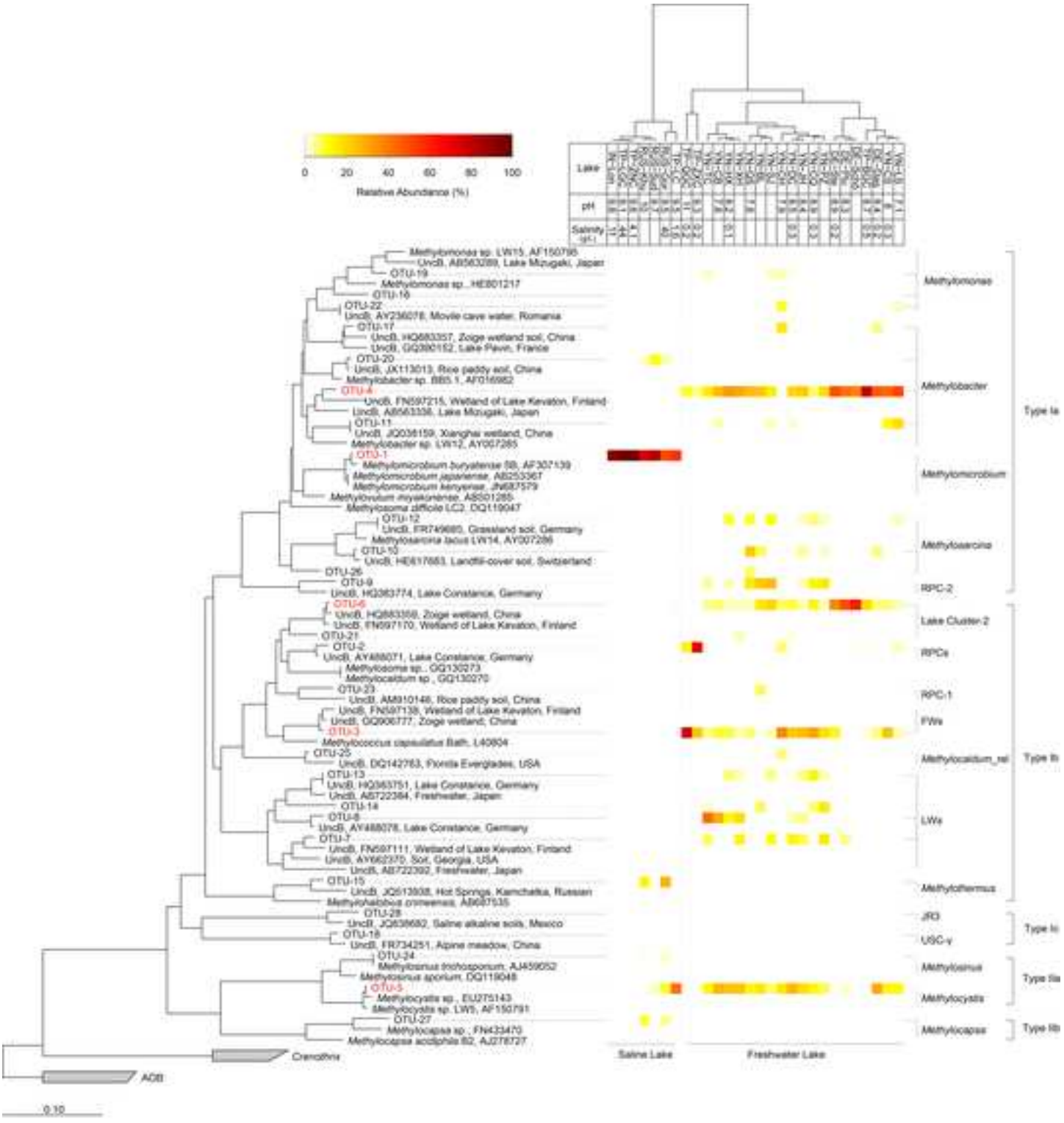

Figure 4

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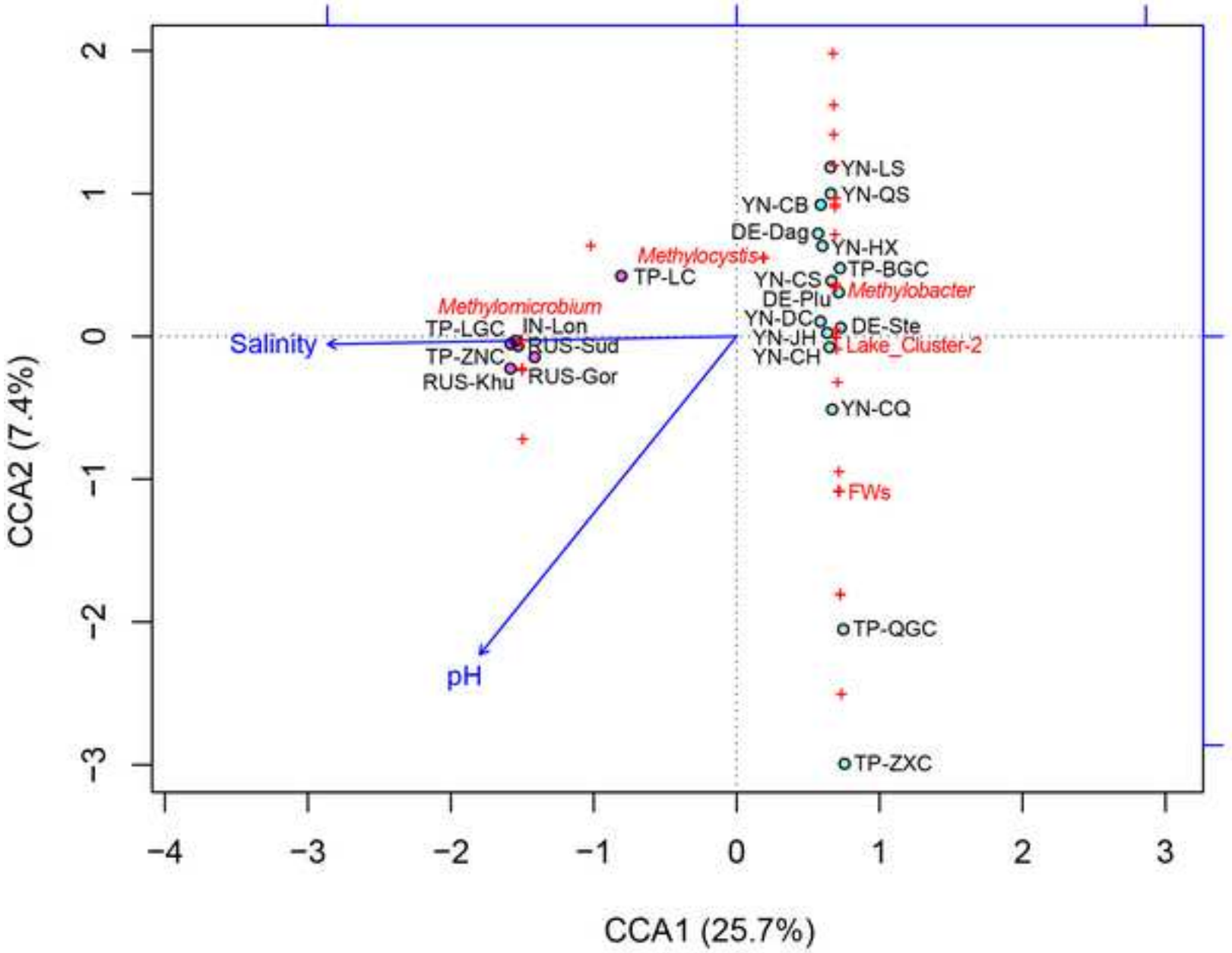

Table

Table 1 Overview of physical and chemical properties of lake water and sediment in six lakes of the Tibetan Plateau.

Lake	Short Name	Location	Altitude (m)	Sediment			Water			
				pmoA copies ($\times 10^5$ g^{-1} fresh sediment)	TOC (g/kg)	TN (g/kg)	Depth (m)	Salinity (g/L)	Conductivity ($\mu S\ cm^{-1}$)	pH
Zongxiong Co	ZXC	N33°06′, E80°09′	4324	30.5±10.4	97.4±13.7	3.62±0.33	1.2	0.15	314	9.34
Qige Co	QGC	N31°11′, E85°31′	4663	32.6±1.86	34.8±0.9	1.37±0.11	0.6	0.18	374	10.48
Bangong Co	BGC	N33°31′, E79°50′	4212	16.2±5.74	81.2±5.0	5.12±0.5	37	0.47	948	8.74
Long Co	LC	N29°12′, E87°24′	4264	26.6±16.5	25.3±3.1	1.37±0.04	25.4	1.55	3000	9.45
Zhangnai Co	ZNC	N31°32′, E87°23′	4581	0.2±0.11	38.2±3.4	1.90±0.32	13.7	4.06	7335	9.59
Lagor Co	LGC	N32°03′, E84°10′	4442	1.16±0.17	21.8±0.9	1.14±0.03	19.6	43.77	63649	9.11

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