Ammonia impacts methane oxidation and methanotrophic community in freshwater sediment

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Abstract

Lacustrine ecosystems are an important natural source of greenhouse gas methane. Aerobic methanotrophs are regarded as a major regulator controlling methane emission. Excess nutrient input can greatly influence carbon cycle in lacustrine ecosystems. Ammonium is believed to be a major influential factor, due to its competition with methane as the substrate for aerobic methanotrophs. To date, the impact of ammonia on aerobic methanotrophs remains unclear. In the present study, microcosms with freshwater lake sediment were constructed to investigate the influence of ammonia concentration on aerobic methanotrophs. Ammonia influence on the abundance of pmoA gene was only observed at a very high ammonia concentration, while the number of pmoA transcripts was increased by the addition of ammonium. pmoA gene and transcripts differed greatly in their abundance, diversity and community compositions. pmoA transcripts were more sensitive to ammonium amendment than pmoA gene. Methane oxidation potential and methanotrophic community could be impacted by ammonium amendment. This work could add some new sights towards the links between ammonia and methane oxidation in freshwater sediment.

Keywords: Ammonium; Freshwater lake; Methane oxidation; Methanotroph; pmoA gene; pmoA transcripts
1. Introduction

Methane is a major product of carbon metabolism in freshwater lakes, and also a critical greenhouse gas in the atmosphere (Bastviken et al., 2004). Aerobic methane oxidation performed by bacterial methanotrophs is a major pathway controlling methane emission. Up to 30–99% of the total methane produced in anoxic sediment can be oxidized by methanotrophs (Bastviken et al., 2008). Methane oxidation in freshwater lakes can be greatly influenced by the environmental changes (e.g. eutrophication) induced by anthropogenic activities (Borrel et al., 2011).

The increasing input of nutrients into freshwater lakes has greatly raised the availability of dissolved organic carbon (DOC), nitrogen and phosphorus, and also exerted a considerable influence on aerobic methane oxidation (Liikanen and Martikainen, 2003; Veraart et al., 2015). Among various types of nutrients, ammonia has attracted great attention. Ammonium and methane have similar chemical structure, and ammonium is known to compete with methane for the binding site of methane monooxygenase, a key enzyme in methane oxidation (Bédard and Knowles, 1989). On the other hand, a high concentration of oxygen in lake water might also inhibit methane oxidation (Rudd and Hamilton, 1975), and excess ammonia can lead to the competition between methane oxidizers and ammonium oxidizers for oxygen. With high oxygen availability or low in-situ nitrogen content, methane oxidation can
also be stimulated by the addition of ammonium (Rudd et al., 1976). Hence, the effect of ammonium on methane oxidation is complex (Bodelier and Laanbroek, 2004), and previous studies have documented contradictory results, such as inhibition (Bosse et al., 1993; Murase and Sugimoto, 2005; Nold et al., 1999), no effect (Liikanen and Martikainen, 2003), or stimulation (Bodelier et al., 2000; Rudd et al., 1976). The effect of ammonium on methane oxidation might largely depend on the characteristics of the studied ecosystem and in-situ environment (Bodelier and Laanbroek, 2004; Borrel et al., 2011).

To date, previous studies about the ammonium effect on methane oxidation in freshwater lakes mainly focused on either oxidation rate or net methane flux (Bosse et al., 1993; Liikanen and Martikainen, 2003; Murase and Sugimoto, 2005). However, methanotrophs play a fundamental role in regulating methane emission from freshwater sediment (Bastviken et al., 2008). The abundance, transcription, and community structure of aerobic methanotrophs may also be affected by the extra input of ammonium (Shrestha et al., 2010). The difference in methanotrophic community structure can further lead to various responses of methane oxidation to nitrogen content (Jang et al., 2011; Mohanty et al., 2006; Nyerges and Stein, 2009). Therefore, identification of the variation of methanotrophic community can be helpful to understand how ammonium input influences methane oxidation process. The community change of methanotrophs under ammonium stress has been observed in
various soils, such as agriculture soil (Seghers et al., 2003; Shrestha et al., 2010) and landfill soil (Zhang et al., 2014). The results of these previous studies suggested that the effect of ammonium on methanotroph community might be habitat-related. A recent filed work suggested that in-situ ammonia concentration might be a key regulating factor of methanotrophic community structure in freshwater lake sediment (Yang et al., 2016). However, the direct evidence for the influence of ammonium (or ammonia) on methanotroph community in freshwater lake sediment is still lacking. Hence, in the present study, microcosms with freshwater lake sediment were constructed to investigate the ammonium influence on methane oxidation potential and the abundance, transcription and community structure of aerobic methanotrophs.

2. Materials and methods

2.1. Sediment characteristics

Dianchi Lake is a large shallow lake (total surface area: 309 km²; average water depth: 4.4 m) located in southeast China (Yang et al., 2016). This freshwater lake is suffering from anthropogenically-accelerated eutrophication (Huang et al., 2017). Surface sediment (0–5 cm) (24.9286N, 102.6582E) were collected using a core sampler from the north part of Dianchi Lake in October, 2017. In-situ dissolved oxygen (DO) and ammonium nitrogen (NH₄⁺-N) in overlying water were 8.37 mg/L and 344 µM, respectively. Sediment total organic carbon (TOC), total nitrogen (TN), the ratio of TOC to TN (C/N), nitrate nitrogen (NO₃⁻-N), ammonium nitrogen (NH₄⁺-
N), total phosphorus (TP), and pH were 41.3 g/kg, 3.95 g/kg, 10.5, 12.3 mg/kg, 364 mg/kg, 0.60 g/kg, and 7.2, respectively. Sediment (2 L) was transported to laboratory at 4°C for incubation experiment.

2.2. Experimental setup

Sediments were placed at room temperature for 24 h and then homogenized. The homogenized sediments were centrifuged at 5000 rpm for 10 min to determine the initial ammonia concentration of pore water. A series of 50-mL serum bottles (as microcosms) were added with 10 mL of sediment aliquot (containing about 0.1 g dry sediment). A total of 111 microcosms were constructed, including three autoclaved ones used as the control for the measurement of methane oxidation potential. Six treatments (A–F) were set up. The microcosms with treatments B–F were added with 1 mL of NH₄Cl at the levels of 5, 20, 50, 100, and 200 mM, respectively, while the microcosm with treatment A was amended with 1 mL diluted water as the blank control. For each treatment, 18 microcosms were constructed, including half used for molecular analyses and another half for methanotrophic potential measurement. These microcosms were closed with butyl rubber stoppers and incubated for 14 days at 25°C at 100 rpm in dark.

At each sampling time point (day 1 (12 h after incubation), day 7 or day 14), triplicate sediment samples of each treatment were transferred into Falcon tubes, and then
centrifuged at 5000 rpm for 10 min. The supernatant was filtered with a 0.2-μm syringe filter, and its ammonia level was measured using Nessler reagent-colorimetry. The sediment was mixed up and immediately used for nucleic acid extraction. In addition, at each sampling time, for methanotrophic potential measurement, another three bottles of each treatment were opened and shaken to provide ambient air, then closed again with butyl rubber stoppers. Headspace air (1 mL) was replaced by CH₄ (99.99%) with an air-tight syringe. Samples were shaken vigorously to mix. After incubation at 25°C, 100 rpm for 24 h, 0.1 mL of headspace gas was taken and measured using a GC126 gas chromatograph equipped with a flame ionization detector. Autoclaved control was also processed to exclude methane loss due to dissolution or airtightness.

2.3. Nucleic acid extraction, reverse transcription and quantification

Sediment DNA and RNA were extracted with PowerSoil DNA Isolation Kit (MoBio) and PowerSoil Total RNA Isolation Kit (MoBio, USA), respectively. The quality and concentration of extracted nucleic acids were examined with Nanodrop 2000 (Thermo Fisher Scientific, USA). RNA was diluted to a similar concentration before further analysis. Real-time PCR of pmoA gene was performed on a CFX Connect cycler (Bio-Rad, USA), using the primer set A189f/mb661r following the conditions reported in our previous study (Liu et al., 2015). Reactions were carried out using a TransStart Top Green qPCR Kit (Transgen, China) following the manufacturer’s
instructions. Gene transcripts were quantified in a one-step RT-qPCR using a TransScript Green One-step qRT-PCR Kit. Melting curve analyses were carried out at the end of PCR run to check the amplification specificity. Each measurement was carried out with three technical replicates. Standard curve was constructed with \textit{pmoA} gene clones, and the efficiency and r-square were 91.5\% and 0.998, respectively.

2.4. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting

DNA \textit{pmoA} gene fragment was amplified with primer sets A189f/mb661r, with the forward primer A189f modified with FAM at 5'-end. PCR reactions were performed as previously described (Liu et al., 2015). Two-step RT-PCR was carried out on RNA. In the first step, RNA was reversely transcribed into cDNA with \textit{pmoA} gene specific primer using One-step gDNA removal and cDNA synthesis kit (Transgen Biotech Co., LTD, China). The 20-μL reaction solution contained 1 μL EasyScript RT/RI Enzyme Mix, 1 μL gDNA remover, 10 μL 2×ES Reaction Mix, 2 pmol of gene specific primers and 1 μL RNA template. The reaction mixture was incubated at 42°C for 30 min, and the enzymes were deactivated at 85°C for 5 s. In the second step, 1 μL cDNA was used as template in \textit{pmoA} gene PCR amplification, proceeded following the same protocol with DNA.

The fluorescently labeled PCR products were purified using a TIANquick Mini Purification Kit (TIANGEN Bitotech Co., Ltd, China). Approximately 20 ng of
purified PCR products were digested with restriction endonuclease BciT130 I (Takara Bio Inc., Japan) following the conditions recommended by the manufacturer’s instruction. Electrophoresis of digested amplicons was carried out by Sangon Biotech (China) using an ABI 3730 DNA analyzer (Thermo Fisher Scientific, USA). The length of T-RFs was determined by comparing with internal standard using the GeneScan software. Terminal restriction fragments (T-RFs) with similar length (less than 2 bp difference) were merged, and T-RFs shorter than 50 base pairs (bp) or longer than 508 bp were removed from the dataset. Relative abundance of each fragment equaled to the ratio of its peak area to the total area. Minor T-RFs with relative abundance less than 0.5 % were excluded for further analysis. The Shannon diversity indices of pmoA gene and transcripts were calculated based on DNA and RNA T-RFs, respectively.

2.5. Cloning, sequencing and phylogenetic analysis

pmoA gene clone library was generated with mixed DNA PCR products using a TA cloning kit (TransGen Biotech Co., LTD, China). Randomly picked clones were subjected to sequencing. The in silico cut sites of these pmoA sequences were predicted using the online software Restriction Mapper (http://www.restrictionmapper.org). The sequences of each T-RF, together with their reference sequences from the GenBank database, were used for phylogenetic analysis. A neighbor-joining tree was conducted with MEGA 7 (Kumar et al., 2016), and
bootstrap with 1000 replicates was carried out to check the consistency. The phylogenetic tree was visualized using iTOL v4.2 (Letunic and Bork, 2016). The sequences used in phylogenetic analysis were deposited in GenBank database, and the accessions were shown in Fig. 3.

2.6. Statistical analysis

Two-way ANOVA (analysis of variance) was carried out to determine the effect of ammonia concentration and incubation time on CH₄ oxidation potential, gene abundance and transcription. One-way ANOVA followed by Student-Newman-Keuls test was adopted to detect the difference among treatments. The analysis was carried out in R, using R packages stats (version 3.4.4) and agricolae (version 1.2-8).

Moreover, the comparison of methanotrophic communities in different microcosms, using Redundancy Analysis (RDA) and clustering analysis, was carried out with R package Vegan (version 2.4-6) (Oksanen et al., 2018). Permutation test was carried out to detect the margin effect of variables (treatment and time). Clustering analysis was carried out based on Bray-Curtis dissimilarity, to demonstrate the variation of microbial community structure during incubation.

3. Results

3.1. Methane oxidation potential
Ammonium was found to quickly deplete in each ammonium added microcosm (Fig. S1). Methane oxidation potential (MOP) varied from 0.77 (in the microcosm with treatment F on day 1) to 1.94 (in the microcosm with treatment F on day 14) mmol/g dry sediment day (Fig. 1), while autoclaved control did not show notable methane oxidation (data not shown). Based on two-way ANOVA, both ammonium concentration (treatment) and incubation time had significant effects on MOP ($P < 0.01$), and their interaction was also significant ($P < 0.05$). The MOP in the microcosm with treatment A (with no external ammonium addition) did not show a significant difference among incubation times ($P > 0.05$). Based on post-hoc test (Fig. 1, Table S1), at each time, the microcosm with treatment B had slightly higher MOP than the microcosm with treatment A. At days 1 and 7, the microcosms with treatments C, D and E had slightly lower MOP than the un-amended microcosm. However, at each time, no statistical difference in MOP was observed among the microcosms with treatments A–E. Moreover, the microcosm with treatment F (with the highest ammonium addition) tended to have significantly lower MOP than other microcosms on day 1 ($P < 0.05$), but significantly higher MOP on day 14 ($P < 0.05$). On day 7, no statistical difference in MOP was found between the microcosm with treatment F and any other microcosms.

3.2. *pmoA* gene and transcript abundance
Two-way ANOVA indicated that the number of both *pmoA* gene and transcripts was significantly influenced by ammonium concentration and incubation time (*P* < 0.01) (Fig. 2a and 2b). The abundance of *pmoA* gene in the microcosm with treatment A showed no significant difference among times (0.05 < *P* < 0.1). On day 1, the microcosms with treatments C and D had higher (but not significantly) *pmoA* gene abundance than other microcosms. However, at days 7 and 14, the microcosm with treatment F (with the highest ammonium addition) had the highest *pmoA* gene abundance.

At each time, *pmoA* transcripts in the un-amended microcosm was less abundant than those in amended microcosms. On day 1, the highest number of transcripts was observed in the microcosm with treatment C, followed by the microcosms with treatments D, E and F. The microcosm with treatment B had much lower *pmoA* transcript abundance than other ammonium added microcosms (*P* < 0.05) (Table S1). On day 7, *pmoA* transcript abundance tended to increase with the level of added ammonium, although statistical difference in *pmoA* transcript abundance was only observed between treatment F and other treatments. On day 14, no significant difference in *pmoA* transcript abundance was detected among treatments (*P* > 0.05).

The ratio of transcripts to *pmoA* gene varied with ammonium concentration and incubation time (Fig. S2). The ratio tended to decrease with time in ammonium
amended microcosms. Moreover, at days 1 and 7, the ratio tended to increase with the increasing ammonium concentration.

3.3. T-RFLP fingerprinting

In silico analysis of the cloned pmoA sequences showed that restriction enzyme BciI could well capture pmoA gene diversity and present a good resolution among different subgroups of aerobic methanotrophs. Most of the T-RFs retrieved in the current study could be assigned to certain methanotrophic groups, while some of the T-RFs from pmoA transcripts could not match the cut site predicted from the sequences in clone library. The obtained pmoA sequences could be grouped into four clusters (Fig. 3), which could be convincingly affiliated with known methanotrophic organisms. Three clusters were affiliated with Type I methanotrophs (Gammaproteobacteria), which could be further divided into several subgroups.

Cluster 1 contained 157 bp, 242 bp and 338 bp T-RFs that could be related to Type Ia methanotrophs, the most frequently detected methanotrophs in freshwater lakes (Borrel et al., 2011). The 157 bp and 338 bp T-RFs might be affiliated with Methylobacter and Methylophillum, respectively. However, the 242 bp T-RF could not be convincingly assigned to a certain genus because of the highly similar pmoA sequences of Type Ia organisms. Cluster 2 was composed of three different T-RFs, and could be affiliated with Methylococcus and Methyloparacoccus. Cluster 3 included the T-RFs of 91 bp and 508 bp, which might be closely related to...
Candidatus Methylospira. Both cluster 2 and cluster 3 could be affiliated with Type Ib methanotrophs, but they distinctly differed in phylogeny and morphology (Danilova et al., 2016). Cluster 4 comprised of the T-RFs of 217 bp, 370 bp and 403 bp, and it was phylogenetically related to Type IIa methanotrophs (Methylocystaceae in Alphaproteobacteria). The 403 bp T-RF was likely affiliated with Methylosinus, while 217 bp and 370 bp T-RFs could not be convincingly assigned to a single genus.

The 508 bp fragment could be affiliated with either Methylospira or unknown Type Ia methanotroph. Considering the low abundance of 508 bp T-RF (<0.5% in DNA TRFLP profile and approximately 2% in RNA TRFLP profile), and in order to avoid incorrect annotation, this T-RF was excluded from further analysis.

3.4. T-RFLP diversity and profiles of pmoA gene and transcripts

Diversity of each community was calculated based on T-RFLP results. In the current study, the T-RFs with relative abundance more than 5% in at least one sample or with average relative abundance more than 2% in all samples were defined as major T-RFs. For a given sample, the total number of T-RFs and the number of major T-RFs were greater in RNA T-RFLP profile than in DNA T-RFLP profile. On day 1, ammonium amended microcosms tended to have lower pmoA gene diversity than un-amended microcosm, while an opposite trend was found at days 7 and 14 (Table 1). For a given sample, pmoA transcript showed higher Shannon diversity than pmoA
Ammonium amended microcosms tended to have lower pmoA transcript diversity than un-amended microcosm. In the microcosms with treatments A–D, pmoA transcript diversity tended to increase with time. However, the Shannon diversity of transcriptional T-RFs experienced an increase followed by a decrease in the microcosms with treatments E and F.

A total of 11–14 T-RFs were retrieved from T-RFLP analysis of DNA samples. Most of them (including all major T-RFs) could be well assigned to certain methanotrophic groups (Figs. 3 and 4a). In all DNA samples, Type Ia and Type IIa methanotrophs dominated methanotrophic communities. On day 1, the 242 bp T-RF (Methylobacter-related Type Ia methanotrophs) comprised about 50% of methanotrophic communities. The 370 bp T-RF (Type IIa methanotrophs) also showed a considerable proportion (20–25%). The addition of ammonium tended to induce no considerable change of methanotrophic community structure after 12-hour incubation. After 7 and 14 days of incubation, the proportions of major T-RFs illustrated an evident variation. The proportion of Type Ia methanotrophs (157 bp, 242 bp and 338 bp; marked in green) decreased with time, while Type IIa methanotrophs (217 bp and 370 bp, marked in pink) increased. The proportion of Methylococcus-related Type Ib methanotrophs (marked in blue) also increased, especially the 145 bp T-RF, whereas the proportion of Methylospira-related Type Ib methanotrophs (91 bp, marked in yellow) did not show a notable variation.
A total of 14–38 T-RFs were retrieved from T-RFLP analysis of RNA samples, but most of them were only detected in a few samples with low relative abundance (Fig. 4b). Among the major transcript T-RFs, only 4 transcript T-RFs could be assigned to a known methanotrophic group, and on day 1 they comprised of a considerable part of methanotrophic community in un-amended microcosm (43–72%) and of in amended microcosms (22–72%), while the other 7 T-RFs were not found in pmoA gene library as well as DNA T-RFLP profiles. Compared with pmoA gene, the community structure of pmoA transcripts was more sensitive to external ammonium addition. The addition of ammonium induced a marked shift in pmoA transcriptional community structure after 12-h incubation. The proportion of 242 bp increased, but the proportion of 91 bp decreased. After 1 or 2 weeks’ incubation, the microcosms with treatments B, C, D and E had similar transcriptional community structure as the un-amended microcosm. However, the microcosm with treatment F (with the highest ammonium addition) encountered a remarkable increase in 91 bp (Ca. Methylospira-related Type Ib methanotrophs). Moreover, the 370 bp T-RF, accounting for up to one fourth (average) of DNA T-RFs, was only detected on day 14, with relative abundance of 0.8–2.9%.

3.5. Clustering and statistical analysis of TRFLP profiles
DNA- and RNA-based methanotrophic community structures were characterized with hierarchal clustering based on Bray-Curtis dissimilarity (Fig. 5). *pmoA* community structure was quite stable during the whole incubation period. Most of the samples on day 1 were grouped together. Samples B7, D7, E7, B14, C14, D14, E14 and F14 were clustered into another group. Sample D1 was distantly separated from other samples, higher dissimilarity of transcriptional community structures could be observed among samples. The samples on day 1 were still close to each other, and they were clearly separated from the samples at day 7 and 14. Samples A7, A14, B7, B14, D7, E7 and F7 could form a clade, while samples C7, D14 and E14 formed another clade.

Moreover, sample F14 was distantly separated from other samples.

RDA with permutation test was carried out to test the potential relationship between each major T-RF and factors (treatment and incubation time). The result indicated that incubation time had a significant impact on DNA-based methanotrophic community composition ($P<0.01$), while ammonium concentration did not exert a significant influence ($P>0.05$). The constrained variables could explain up to 74.4% of total variance. However, most of the explained variance (73.7% out of 74.4%) was related to constrained axis 1, and only the first axis was significant ($P=0.029$). In addition, for RNA-based methanotrophic community, treatment and time were able to explain 76.0% of total variance. Only incubation time had a significant effect on RNA-based
methanotrophic community composition ($P < 0.01$), and only the first constrained axis was significant ($P < 0.01$). These results indicated that after the addition and with the depletion of ammonium, the community compositions of both $pmoA$ gene and transcripts could undergo a considerable shift.

4. Discussion

4.1. Effect of ammonium on MOP

The current study showed that a high dosage of ammonium could present a temporary inhibition effect on methane oxidation. The result was consistent with several previous studies (Bosse et al., 1993; Murase and Sugimoto, 2005; Nold et al., 1999). These studies indicated that the addition of ammonium might inhibit methane oxidation in water and sediment of freshwater lake. However, to date, the minimal inhibit concentration for methane oxidation in lake sediment is still unclear. Bosse et al. (1993) pointed out that methane oxidation in littoral sediment of Lake Constance could be partially inhibited when ammonium concentration in pore water was higher than 4 mM. In contrast, methane oxidation in sediment of hyper-eutrophic Lake Kevätön was not obviously affected by a continuous water flow containing up to 15 mM of ammonium (Liikanen and Martikainen, 2003). Lake Kevätön and Dianchi Lake had similar average water depth, and the overlying water of sediment in both lakes had very high levels of ammonium (Liikanen and Martikainen, 2003). In the present study, inhibition was only observed in the microcosm with a very high
ammonium dosage (with 17.3 mM ammonium in overlying water on day 1), while no
evident inhibition was found in the other ammonium amended microcosms, even at
high dosages. This suggested that methane oxidation might depend on ammonium
dosage, which was sustained by the result of two-way ANOVA. The minimal inhibit
concentration for methane oxidation in Dianchi Lake was much higher than that in
Lake Constance (Bosse et al. 1993). Hence, the minimal inhibition concentration for
methane oxidation could be lake-specific.

Despite a very high dosage of ammonium, sediment MOP was only partially
inhibited. This might be explained by two facts. The studied sediment sample
originated from a eutrophic lake, which suffered from high ammonium input.
Methanotrophs in this kind of ecosystem could effectively oxidize methane under the
condition of high ammonia concentration (Liikanen and Martikainen, 2003). This was
consistent with the above-mentioned lake-related minimal inhibition concentration for
methane oxidation. On the other hand, the affinity of pMMO (pmoA encoding
protein) to methane is much higher than that to ammonium (Bédard and Knowles,
1989). As a result, when the methane concentration is high enough, as a common case
for the measurement of MOP, methanotrophs should be able to consume a
considerable amount of methane.
A recovery of MOP after a single-shot fertilization has been reported in forest soil (Borjesson and Nohrstedt, 2000). In this study, it was noted that after the depletion of ammonium, sediment MOP could also get a quick recovery. The highest ammonium dosage eventually stimulated sediment MOP in the long run (about two weeks). Considering the increase of pmoA gene abundance and the change of RNA-based methanotrophic community structure, this might be attributed to an adaption to the environment. The initial decrease of MOP could be explained by the competition between methane and ammonium for pMMO (Bédard and Knowles, 1989), while the subsequent increase of MOP might be the consequence of the shift in methanotrophic community structure (Seghers et al., 2003; Shrestha et al., 2010) and the increase of pmoA gene abundance and transcription.

4.2 Effect of ammonium on pmoA gene and transcript abundance

So far, little is known about the changes of methanotrophic abundance and transcripts induced by external ammonium amendment. Alam and Jia (2012) reported that the addition of 200 μg of nitrogen/g dry weight soil (in ammonium sulfate) showed no significant influence on pmoA gene abundance in paddy soil. However, in ammonium-amended rhizospheric soil microcosms, pmoA gene abundance slightly increased after 29 days’ incubation (Shrestha et al., 2010). In this study, after 7 days’ incubation, the sediment microcosm with the highest ammonium dosage had much higher pmoA gene abundance than un-amended microcosm and other amended
microcosms with lower dosage, whereas no significant difference of pmoA gene abundance was detected between un-amended microcosm and amended microcosms (except for the treatment with the highest ammonium dosage). After 14 days’ incubation, the microcosm with the highest ammonium dosage also had much higher DNA-based methanotrophic abundance than other amended microcosms. Hence, the present study further provided the evidence that the addition of ammonium, depending on dosage, could influence freshwater sediment DNA-based methanotrophic abundance, which was in agreement with the result of two-way ANOVA. Dianchi Lake had been suffering from eutrophication for over 30 years (Huang et al., 2017). It could be assumed that methanotroph community in this lake had been adapted to high in-situ ammonia concentration. As a result, only extremely high dosage of ammonium could pose a significant impact on DNA-based methanotrophic abundance.

At each time, the microcosm with no external ammonium addition had lower abundance of pmoA transcripts than each amended microcosm. This suggested the addition of ammonium could influence the transcription of pmoA gene. The stimulation of pmoA transcription by the addition of ammonium could be attributed to the competition between methane and ammonium for the binding site of pMMO (Bédard and Knowles, 1989). This was also verified by the similar number of transcripts in these amended microcosms after the considerable reduction of
ammonium. At days 1 and 14, the abundance of *pmoA* transcripts differed greatly in different amended microcosms. This suggested that ammonium dosage could influence the number of *pmoA* transcripts, which was consistent with the result of two-way ANOVA.

### 4.3. Effect of ammonium on DNA- and RNA-based methanotrophic community compositions

Several previous studies have investigated the influence of ammonium amendment on soil methanotrophic community structure (Alam and Jia, 2012; Mohanty et al., 2006; Shrestha et al., 2010), yet information about the influence of ammonium amendment on freshwater methanotrophic community structure is still lacking. In this study, immediately after ammonium addition (after 12-h incubation), the relative abundance of Type I (especially Type Ia) methanotrophs transcripts increased, instead of Type II. This coincided with the result reported in rice and forest soils (Mohanty et al., 2006). This also suggested that a high level of ammonia favored the growth of Type I methanotrophs and they might play an important role in methane oxidation in ammonia-rich lake. However, both DNA- and RNA-based T-RFLP profiles indicated that the addition of ammonium lead to an increase in the ratio of Type II to Type I methanotrophs in two weeks, which was contrary to the results observed in some previous studies in soil ecosystems (Alam and Jia, 2012; Bodelier et al., 2000; Mohanty et al., 2006). These previous studies found that Type I methanotrophs had a
numerical advantage over Type II at a high ammonia concentration. Our recent field study suggested that the abundance of Type II methanotrophs in sediment of Dianchi Lake was closely correlated to the concentration of ammonia (Yang et al., 2016). Therefore, the response of methanotrophs to ammonia might depend on the type of ecosystem. In addition, the community compositions of pmoA genes and transcripts could be divergent, and DNA-based and RNA-based methanotrophs could show different responses to ammonium addition (Shrestha et al. 2010). In this study, compared with pmoA gene, the community structure of pmoA transcripts was more sensitive to external ammonium addition. This was in a consensus with the result of a previous study on the effect of ammonium addition on methanotrophs in root and rhizospheric soils (Shrestha et al. 2010).

5. Conclusions

This was the first microcosm study on the influence of ammonium on freshwater lake sediment methanotroph community. In freshwater lake sediment microcosm, methane oxidation potential and methanotrophic community could be influenced by ammonium amendment. Ammonia concentration had a significant impact on methanotrophic abundance and diversity, but exerted no evident influence on community structure. Compared with pmoA gene, transcripts were more sensitive to external ammonium addition. Further works are necessary in order to elucidate the influence of ammonium on methane oxidation in freshwater sediment.
Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

This work was financially supported by National Natural Science Foundation of China (No. 41571444), and National Basic Research Program of China (No. 2015CB458900).

References


Table 1 Numbers of T-RFs and T-RF-based Shannon diversity. For sample name, upper case letters refer to treatment while digits indicate sampling time.

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</tr>
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</tr>
<tr>
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<tr>
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<tr>
<td>F14</td>
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</table>
Fig. 1. Change of methane oxidation potential in the microcosms with different treatments. Error bar indicates standard deviation ($n=3$). Asterisk indicates the significance between experiment group and control group ($P<0.05$). ‘ns’ indicates no significant difference among treatments at a given time.
Fig. 2. Changes of pmoA gene (a) and transcript (b) abundance in the microcosms with different treatments. Error bar indicates standard deviation (n=3). Asterisk indicates the significance between experiment group and control group (P<0.05). ‘ns’ indicates no significant difference among treatments at a given time.

(a)

![Graph showing changes of pmoA gene abundance](image1)

(b)

![Graph showing changes of pmoA transcript abundance](image2)
Fig. 3. Phylogenetic tree of obtained pmoA sequences and reference sequences from the GenBank database. The predicted cut sites were shown after the accession numbers of sequences. The dots at branches represent the support values from bootstrap test. Branch support values of no less than 50 were dotted. The bar represents 1% sequence divergence based on neighbor-joining algorithm.

Tree scale: 0.01
Fig. 4. T-RFLP profiles based on pmoA gene (a) and transcripts (b). For sample name, upper case letters refer to treatment while digits indicate sampling time.
Fig. 5. *pmoA* gene (a) and transcripts (b)-based cluster diagrams of similarity values for samples with different treatments. Dissimilarity levels are indicated above the diagram. For sample name, upper case letters refer to treatment while digits indicate sampling time.