Ammonia impacts methane oxidation and methanotrophic community in freshwater sediment

Yuyin Yang[1], Jianfei Chen[1], Shuguang Xie[1,*], Yong Liu[2]

[1]State Key Joint Laboratory of Environmental Simulation and Pollution Control, College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China

[2]Key Laboratory of Water and Sediment Sciences (Ministry of Education), College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China

* Corresponding author. Tel: 86-10-62751923. Email: xiesg@pku.edu.cn (S Xie)

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). Therefore, aerobic methane oxidation is an important biochemical process in freshwater lakes. And this process 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, ammonium, an essential compound in nitrogen cycling, 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 ammonium can lead to the competition between methane

64 oxidizers and ammonium oxidizers for oxygen. With high oxygen availability or low
65 in-situ nitrogen content, methane oxidation can also be stimulated by the addition of
66 ammonium (Rudd et al., 1976). Besides, ammonium might also induce differential
67 expression of pMMO encoding genes (Dam et al., 2014). Hence, the effect of
68 ammonium on methane oxidation in natural ecosystems is complex (Bodelier and
69 Laanbroek, 2004), and previous studies have documented contradictory results, such
70 as inhibition (Bosse et al., 1993; Murase and Sugimoto, 2005; Nold et al., 1999), no
71 effect (Liikanen and Martikainen, 2003), or stimulation (Bodelier et al., 2000; Rudd et
72 al., 1976). The effect of ammonium on methane oxidation might largely depend on
73 the characteristics of the studied ecosystem and in-situ environment (Bodelier and
74 Laanbroek, 2004; Borrel et al., 2011).
75
76 To date, previous studies about the ammonium effect on methane oxidation in
77 freshwater lakes mainly focused on either oxidation rate or net methane flux (Bosse et
78 al., 1993; Liikanen and Martikainen, 2003; Murase and Sugimoto, 2005). However,
79 methanotrophs play a fundamental role in regulating methane emission from
80 freshwater sediment (Bastviken et al., 2008). The abundance, transcription, and
81 community structure of aerobic methanotrophs may also be affected by the extra input
82 of ammonium (Shrestha et al., 2010). The difference in methanotrophic community
83 structure can further lead to various responses of methane oxidation to nitrogen
84 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. Especially, many freshwater lakes in China have been suffering from eutrophication. The methanotrophic communities in these ecosystems have been under high ammonium pressure. The response pattern of methanotrophic community to ammonium pressure in eutrophic lakes might be different from that in oligotrophic lakes. Hence, in the present study, microcosms with eutrophic freshwater lake sediment were constructed to investigate the ammonium influence on methane oxidation potential and the abundance, transcription and community structure of aerobic methanotrophs. The object of this current study was to demonstrate how different concentrations of ammonium nitrogen impacted the structure and function of aerobic methanotrophic communities in freshwater lakes with long-term eutrophication.

2. Materials and methods

2.1. Sediment characteristics

Dianchi Lake is a large shallow lake (total surface area: 309 km^2; 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. The pH and water dissolved oxygen (DO) were immediately measured with electrode sensors. Ammonium nitrogen (NH_4^+-N) was measured using Nessler's reagent spectrophotometric method. Physicochemical properties of sediment were determined according to the literature (Wang, 2012). In-situ dissolved oxygen (DO) and ammonium nitrogen (NH_4^+-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 (NO_3^--N), ammonium nitrogen (NH_4^+-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 NH_4Cl 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^{o}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

147 closed again with butyl rubber stoppers. Headspace air (1 mL) was replaced by CH_4
148 (99.99%) with an air-tight syringe. Samples were shaken vigorously to mix. After
149 incubation at 25^{o}C, 100 rpm for 24 h, 0.1 mL of headspace gas was taken and
150 measured using a GC126 gas chromatograph equipped with a flame ionization
151 detector. Autoclaved control was also processed to exclude methane loss due to
152 dissolution or airtightness.
153
154 *2.3. Nucleic acid extraction, reverse transcription and quantification*
155 Sediment DNA and RNA were extracted with PowerSoil DNA Isolation Kit (MoBio)
156 and PowerSoil Total RNA Isolation Kit (MoBio, USA), respectively. The quality and
157 concentration of extracted nucleic acids were examined with Nanodrop 2000 (Thermo
158 Fisher Scientific, USA). RNA was diluted to a similar concentration before further
159 analysis. Real-time PCR of *pmoA* gene was performed on a CFX Connect cycler
160 (Bio-Rad, USA), using the primer set A189f/mb661r following the conditions
161 reported in our previous study (Liu et al., 2015). Reactions were carried out using a
162 TransStart Top Green qPCR Kit (Transgen, China) following the manufacturer's
163 instructions. Gene transcripts were quantified in a one-step RT-qPCR using a
164 TransScript Green One-step qRT-PCR Kit. Melting curve analyses were carried out at
165 the end of PCR run to check the amplification specificity. Each measurement was
166 carried out with three technical replicates. Standard curve was constructed with *pmoA*
167 gene clones, and the efficiency and r-square were 91.5% and 0.998, respectively.

168

169 *2.4. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting*

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

181

182 The fluorescently labeled PCR products were purified using a TIANquick Mini
183 Purification Kit (TIANGEN Bitotech Co., Ltd, China). Approximately 20 ng of
184 purified PCR products were digested with restriction endonuclease *BciT130* I (Takara
185 Bio Inc., Japan) following the conditions recommended by the manufacturer's
186 instruction. Electrophoresis of digested amplicons was carried out by Sangon Biotech
187 (China) using an ABI 3730 DNA analyzer (Thermo Fisher Scientific, USA). The
188 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. A total of 93 *pmoA* sequences were retrieved and in silico cut sites of these *pmoA* sequences were predicted using the online software Restriction Mapper (http://www.restrictionmapper.org). Several 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 CH_4 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 F with 200 mM ammonium on day 1) to 1.94 (in the microcosm F with 200 mM ammonium 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 (5 mM ammonium) had slightly higher MOP than the microcosm with control group (A). At days 1 and 7, the microcosms with 20-100 mM ammonium addition had slightly lower MOP than the un-amended microcosm. However, at each time, no statistical difference in MOP was observed among the microcosms with treatment A-E (0-100 mM ammonium addition). Moreover, the microcosm with treatment F (200 mM ammonium) 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 control group (A) showed no significant difference among times ($0.05 < P <0.1$). On day 1, the microcosms with treatment C and D (20-100 mM ammonium) 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 of 200 mM) 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 (20 mM), followed by the microcosms with treatments D, E and F (50-200 mM). The microcosm with treatment B (5 mM) 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 *BciT130* I 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 *Methylomicrobium*, 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*

292 in *Alphaproteobacteria*). The 403 bp T-RF was likely affiliated with *Methylosinus*,
293 while 217 bp and 370 bp T-RFs could not be convincingly assigned to a single genus.
294
295 The 508 bp fragment could be affiliated with either *Methylospira* or unknown Type Ia
296 methanotroph. Considering the low abundance of 508 bp T-RF (<0.5% in DNA
297 TRFLP profile and approximately 2% in RNA TRFLP profile), and in order to avoid
298 incorrect annotation, this T-RF was excluded from further analysis.
299
300 *3.4. T-RFLP diversity and profiles of pmoA gene and transcripts*
301 Diversity of each community was calculated based on T-RFLP results. In the current
302 study, the T-RFs with relative abundance more than 5% in at least one sample or with
303 average relative abundance more than 2% in all samples were defined as major T-
304 RFs. For a given sample, the total number of T-RFs and the number of major T-RFs
305 were greater in RNA T-RFLP profile than in DNA T-RFLP profile. On day 1,
306 ammonium amended microcosms tended to have lower *pmoA* gene diversity than un-
307 amended microcosm, while an opposite trend was found at days 7 and 14 (Table 1).
308 For a given sample, *pmoA* transcript showed higher Shannon diversity than *pmoA*
309 gene. Ammonium amended microcosms tended to have lower *pmoA* transcript
310 diversity than un-amended microcosm. In the microcosms with treatments A–D (0-50
311 mM ammonium), *pmoA* transcript diversity tended to increase with time. However,

312 the Shannon diversity of transcriptional T-RFs experienced an increase followed by a
313 decrease in the microcosms with treatments E and F (100-200 mM ammonium).
314
315 A total of 11–14 T-RFs were retrieved from T-RFLP analysis of DNA samples. Most
316 of them (including all major T-RFs) could be well assigned to certain methanotrophic
317 groups (Figs. 3 and 4a). In all DNA samples, Type Ia and Type IIa methanotrophs
318 dominated methanotrophic communities. On day 1, the 242 bp T-RF (*Methylobacter*-
319 related Type Ia methanotrophs) comprised about 50% of methanotrophic
320 communities. The 370 bp T-RF (Type IIa methanotrophs) also showed a considerable
321 proportion (20–25%). The addition of ammonium tended to induce no considerable
322 change of methanotrophic community structure after 12-hour incubation. After 7 and
323 14 days of incubation, the proportions of major T-RFs illustrated an evident variation.
324 The proportion of Type Ia methanotrophs (157 bp, 242 bp and 338 bp; marked in
325 green) decreased with time, while Type IIa methanotrophs (217 bp and 370 bp,
326 marked in pink) increased. The proportion of *Methylococcus*-related Type Ib
327 methanotrophs (marked in blue) also increased, especially the 145 bp T-RF, whereas
328 the proportion of *Methylospira*-related Type Ib methanotrophs (91 bp, marked in
329 yellow) did not show a notable variation.
330
331 A total of 14–38 T-RFs were retrieved from T-RFLP analysis of RNA samples, but
332 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 (5-100 mM ammonium) 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 methantrophic 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 methantrophic community, treatment and time were able to explain 76.0% of total variance. Only incubation time had a significant effect on RNA-based methantrophic 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

394 high dosages. This suggested that methane oxidation might depend on ammonium
395 dosage, which was sustained by the result of two-way ANOVA. The minimal inhibit
396 concentration for methane oxidation in Dianchi Lake was much higher than that in
397 Lake Constance (Bosse et al. 1993). Hence, the minimal inhibition concentration for
398 methane oxidation could be lake-specific.
399
400 Despite of a very high dosage of ammonium, sediment MOP was only partially
401 inhibited. This might be explained by two facts. The studied sediment sample
402 originated from a eutrophic lake, which suffered from high ammonium input.
403 Methanotrophs in this kind of ecosystem could effectively oxidize methane under the
404 condition of high ammonia concentration (Liikanen and Martikainen, 2003). This was
405 consistent with the above-mentioned lake-related minimal inhibition concentration for
406 methane oxidation. On the other hand, the affinity of pMMO (*pmoA* encoding
407 protein) to methane is much higher than that to ammonium (Bédard and Knowles,
408 1989). As a result, when the methane concentration is high enough, as a common case
409 for the measurement of MOP, methanotrophs should be able to consume a
410 considerable amount of methane.
411
412 A recovery of MOP after a single-shot fertilization has been reported in forest soil
413 (Borjesson and Nohrstedt, 2000). In this study, it was noted that after the depletion of
414 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 methantrophic 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 methantrophic 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 methantrophs 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 methanortrophs had a numerical advantage over Type II at a high ammonia concentration. Our recent field study suggested that 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).

4.4 TRFLP fingerprinting

T-RFLP has been a popular approach to capture microbial community diversity. It has been also widely used in community studies of methylotrophs (Mohanty et al., 2006; Pester et al., 2004; Shrestha et al., 2010). However, the most widely used digestive enzyme, *MspI*, may not be suitable for all kinds of samples. For example, samples from littoral Lake Constance only resulted in a few T-RFs. And most of the clones from Type I were at the same T-RF length of 248 bp (Pester et al., 2004). *MspI* enzyme also generated only very few T-RFs for the sediment samples from Dianchi Lake. The number of T-RFs based on a certain digestive enzyme might partly depend on the in-situ microbial community features. Since T-RF was only related to the first cleavage site, if a specific point mutation got widespread (especially when the

population was limited), it might notably impact the T-RF pattern. Efforts were made to avoid some of the weakness of T-RFLP and improve the taxonomic resolution, including the application of multiplex T-RFLP (Elliott et al., 2012) and the usage of primers labelled with different fluorochromes (Deutzmann et al., 2011). Here, we tried to choose a suitable enzyme based on in-silico analysis. A previous NGS result of methylotrophic community in the same region (under accession number SRP131884) was used as a reference. The *pmoA* sequences were grouped into OTUs at 0.03 cutoff, and the representative sequences of each OTU were used in mapping. We tested all the enzymes listed in http://www.restrictionmapper.org, and calculated the proportion of each T-RF. A simplified T-RF map (including the enzymes which could cut over 80% of total sequences) was shown in Fig S3. We expected that a good digestive enzyme should: (1) generate no or few T-RFs smaller than 50 bp or larger than 500 bp; (2) generate T-RFs having at least 2 bp differences among each other; (3) generate more T-RFs to retrieve the diversity; (4) be consistent with taxon and phylogenetic tree (i.e., the same T-RF should not be affiliated to very distantly related taxa). Based on these rules, most of the enzymes were easy to exclude. *MspI* and *BciT130I*, the same as *HpaII* and *EcoRII*, respectively, were further tested using a neighbor-joining tree. *BciT130I* was able to generate more different T-RFs, and thus could more ideally reflect methylotrophic diversity. It also had a better taxonomic resolution than *MspI*.

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

Alam, M., Jia, Z., 2012. Inhibition of methane oxidation by nitrogenous fertilizers in a

paddy soil. Front. Microbiol. 3, 246.

Bastviken, D., Cole, J., Pace, M., Tranvik, L., 2004. Methane emissions from lakes: Dependence of lake characteristics, two regional assessments, and a global estimate. Glob. Biogeochem. Cycle 18, GB4009.

Bastviken, D., Cole, J., Pace, M., de Bogert, M., 2008. Fates of methane from different lake habitats: Connecting whole-lake budgets and CH_4 emissions. J. Geophys. Res.-Biogeosci.113, G02024.

Bédard, C., Knowles, R., 1989. Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. 53, 68–84.

Bodelier, P.L.E., Roslev, P., Henckel, T., Frenzel, P., 2000. Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. Nature 403, 421–424.

Bodelier, P.L.E., Laanbroek, H.J., 2004. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. FEMS Microbiol. Ecol. 47, 265–277

Borjesson, G., Nohrstedt, H.O., 2000. Fast recovery of atmospheric methane consumption in a Swedish forest soil after single-shot N-fertilization. For. Ecol. Manage. 134, 83–88.

Borrel, G., Jezequel, D., Biderre-Petit, C., Morel-Desrosiers, N., Morel, J.P., Peyret, P., Fonty, G., Lehours, A.C., 2011. Production and consumption of methane in freshwater lake ecosystems. Res. Microbiol. 162, 832–847.

561 Bosse, U., Frenzel, P., Conrad, R., 1993. Inhibition of methane oxidation by
562 ammonium in the surface layer of a littoral sediment. FEMS Microbiol. Ecol. 13,
563 123–134.
564 Dam, B., Dam, S., Kim, Y., Liesack, W., 2014. Ammonium induces differential
565 expression of methane and nitrogen metabolism-related genes in *Methylocystis* sp.
566 strain SC2, Environ. Microbiol. 16, 3115–3127.
567 Danilova, O.V., Suzina, N.E., Van De Kamp, J., Svenning, M.M., Bodrossy, L.,
568 Dedysh, S.N., 2016. A new cell morphotype among methane oxidizers, a spiral-
569 shaped obligately microaerophilic methanotroph from northern low-oxygen
570 environments. ISME J. 10, 2734–2743.
571 Deutzmann, J. S. S., Wörner, S. and Schink, B., 2011. Activity and diversity of
572 methanotrophic bacteria at methane seeps in eastern Lake Constance sediments,
573 Appl. Environ. Microbiol. 77, 2573-2581.
574 Elliott, G., Thomas, N., MacRae, M., Campbell, C., Ogden, I., Singh, B., 2012.
575 Multiplex T-RFLP Allows for increased target number and specificity: Detection of
576 *Salmonella enterica* and six species of *Listeria* in a single test. Plos One, 7, e43672.
577 Jang, I., Lee, S., Zoh, K.D., Kang, H., 2011. Methane concentrations and
578 methanotrophic community structure influence the response of soil methane
579 oxidation to nitrogen content in a temperate forest. Soil Biol. Biochem. 43, 620–
580 627.
581 Huang, C.C., Yao, L., Zhang, Y.L., Huang, T., Zhang, M.L., Zhu, A.X., Yang, H.,

582 2017. Spatial and temporal variation in autochthonous and allochthonous
583 contributors to increased organic carbon and nitrogen burial in a plateau lake. Sci.
584 Total Environ. 603, 390–400.
585 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics
586 analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
587 Letunic, I., Bork, P., 2016. Interactive tree of life (iTOL) v3, an online tool for the
588 display and annotation of phylogenetic and other trees. Nucleic Acids Res. 44,
589 W242–W245.
590 Liikanen, A., Martikainen, P.J., 2003. Effect of ammonium and oxygen on methane
591 and nitrous oxide fluxes across sediment–water interface in a eutrophic lake.
592 Chemosphere 52, 1287–1293.
593 Liu, Y., Zhang, J.X., Zhao, L., Li, Y.Z., Yang, Y.Y., Xie, S.G., 2015. Aerobic and
594 nitrite-dependent methane-oxidizing microorganisms in sediments of freshwater
595 lakes on the Yunnan Plateau. Appl. Microbiol. Biotechnol. 99, 2371–2381.
596 Mohanty, S.R., Bodelier, P.L., Floris, V., Conrad, R., 2006. Differential effects of
597 nitrogenous fertilizers on methane-consuming microbes in rice field and forest
598 soils. Appl. Environ. Microbiol. 72, 1346–1354.
599 Murase, J., Sugimoto, A., 2005. Inhibitory effect of light on methane oxidation in the
600 pelagic water column of a mesotrophic lake (Lake Biwa, Japan). Limnol.
601 Oceanogr. 50, 1339–1343.

602 Nold, S.C., Boschker, H.T.S., Pel, R., Laanbroek, H.J., 1999. Ammonium addition
603 inhibits 13C-methane incorporation into methanotroph membrane lipids in a
604 freshwater sediment. FEMS Microbiol. Ecol. 29, 81–89.
605 Nyerges, G., Stein, L.Y., 2009. Ammonia cometabolism and product inhibition vary
606 considerably among species of methanotrophic bacteria. FEMS Microbiol. Lett.
607 297, 131–136.
608 Oksanen. J, Blanchet, F.G., Friendly, M., Kindt, R., Legendre P., McGlinn, D.,
609 Minchin, P.R., O'Hara, R. B., Simpson G.L, Solymos, P., Stevens M.H.H.,
610 Szoecs, E., Wagner, H., 2018. Vegan, Community ecology package, version 2.4-
611 6, Available at, https,//CRAN.R-project.org/package=vegan.
612 Pester, M., Friedrich, M., Schink, B., Brune, A., 2004. *pmoA*-based analysis of
613 methanotrophs in a littoral lake sediment reveals a diverse and stable community
614 in a dynamic environment, Appl. Environ. Microbiol. 70, 3138-3142.
615 Rudd, J.W.M., Hamiltion, R.D., 1975. Factors controlling rates of methane oxidation
616 and the distribution of the methane oxidizers in a small stratified lake. Arch.
617 Hydrobiol. 75, 522–538.
618 Rudd, J.W.M., Furutani, A., Flett, R.J., Hamilton, R.D., 1976. Factors controlling
619 methane oxidation in Shield Lakes: The role of nitrogen fixation and oxygen
620 concentration. Limnol Oceanogr, 21, 357–364.
621 Seghers, D., Top, E.M., Reheul, D., Bulcke, R., Boeckx, P., Verstraete, W., Siciliano,
622 S.D., 2003. Long-term effects of mineral versus organic fertilizers on activity

and structure of the methanotrophic community in agricultural soils. Environ. Microbiol. 5, 867–877.

Shrestha, M., Shrestha, P.M., Frenzel, P., Conrad, R., 2010. Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. ISME J 4, 1545–1556.

Veraart, A.J., Steenbergh, A.K., Ho, A., Kim, S.Y., Bodelier, P.L.E., 2015. Beyond nitrogen: The importance of phosphorus for CH_4 oxidation in soils and sediments. Geoderma 259, 337–346.

Wang, S. H., 2012. Manual for sediment mass investigation and assessment. Science Press, Beijing.

Yang, Y.Y., Zhao, Q., Cui, Y.H., Wang, Y.L., Xie, S.G., Liu, Y., 2016. Spatio-temporal variation of sediment methanotrophic microorganisms in a large eutrophic lake. Microb. Ecol. 71, 9–17.

Zhang, X., Kong, J.Y., Xia, F.F., Su, Y., He, R., 2014. Effects of ammonium on the activity and community of methanotrophs in landfill biocover soils. Syst. Appl. Microbiol. 37, 296–304.

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.

Sample	DNA		RNA	
	T-RFs	Shannon	T-RFs	Shannon
A1	11	1.55	23	2.55
B1	11	1.55	23	2.23
C1	11	1.49	22	2.17
D1	11	1.27	14	1.70
E1	11	1.48	20	2.12
F1	12	1.66	25	2.55
A7	12	1.74	28	2.92
B7	12	1.80	32	3.09
C7	13	1.77	27	2.86
D7	12	1.79	18	2.46
E7	14	1.89	30	2.98
F7	13	1.70	24	2.70
A14	13	1.70	36	3.17
B14	12	1.79	38	3.09
C14	12	1.83	33	2.96
D14	12	1.87	34	3.08
E14	12	1.83	32	2.89
F14	12	1.79	20	2.18

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.

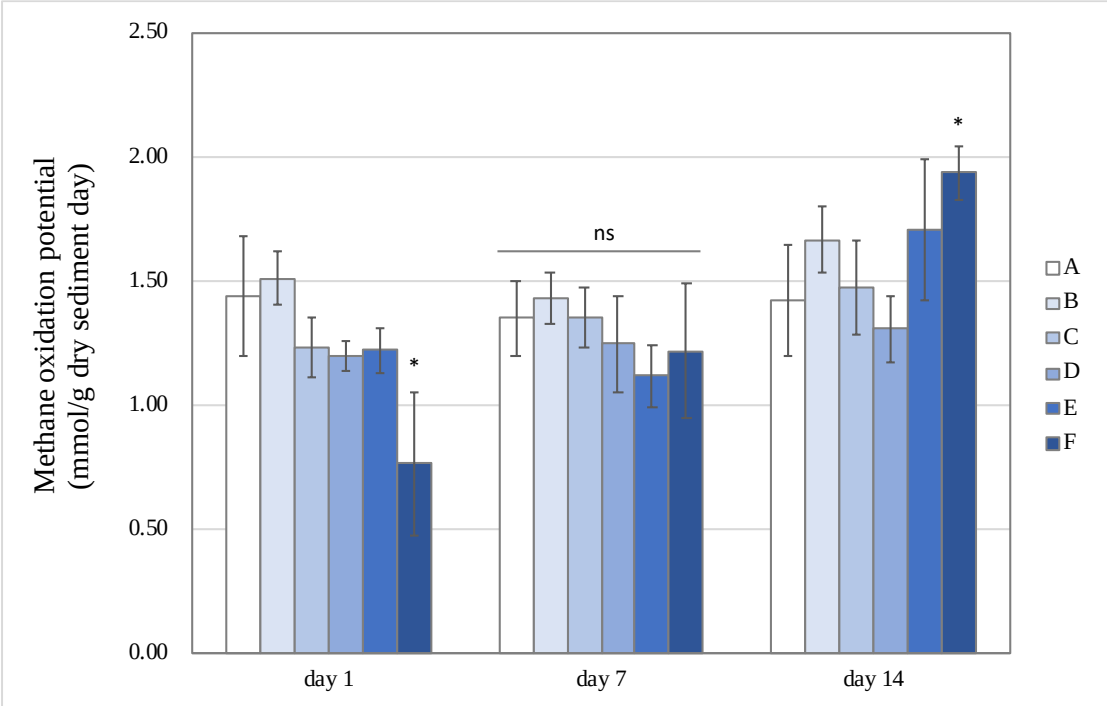
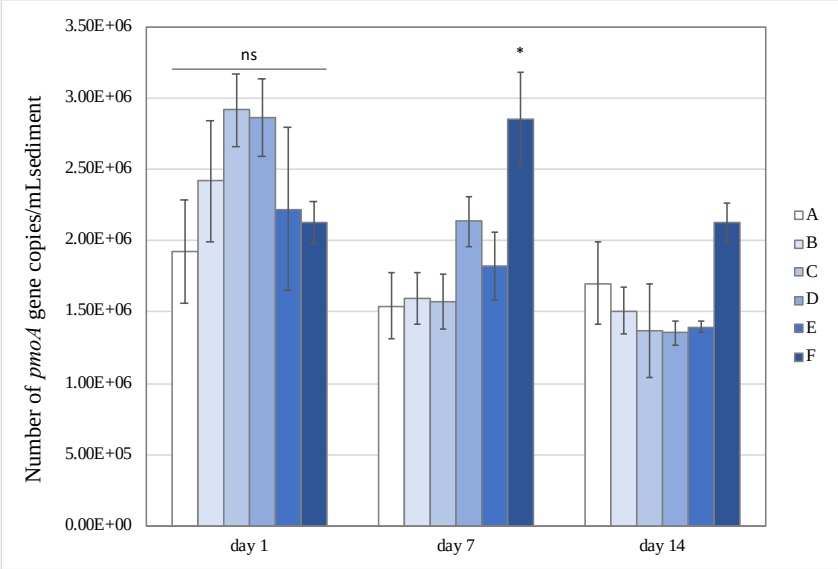


Fig. 2. Changes of *pmoA* gene (a) and transcript (b) abundance in the microcosms with different treatments. Error bar indicates standard deviation (*n*=3). Asterisk indicates the significance between experiment group and control group ($P < 0.05$). 'ns' indicates no significant difference among treatments at a given time.

(a)



(b)

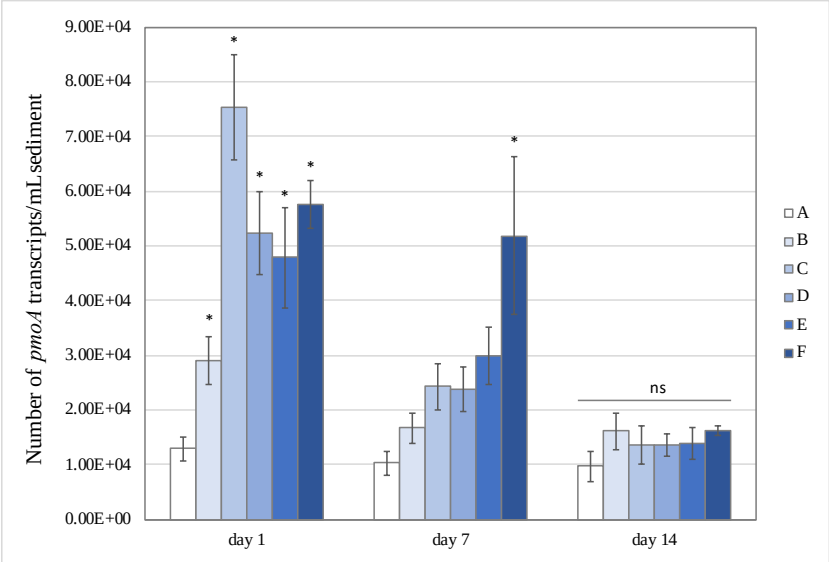


Fig. 3. Phylogenetic tree of obtained *pmoA* sequences and reference sequences from the GenBank database. The predicted cut sites were shown after the accession numbers of sequences. The dots at branches represent the support values from bootstrap test. Branch support values of no less than 50 were dotted.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

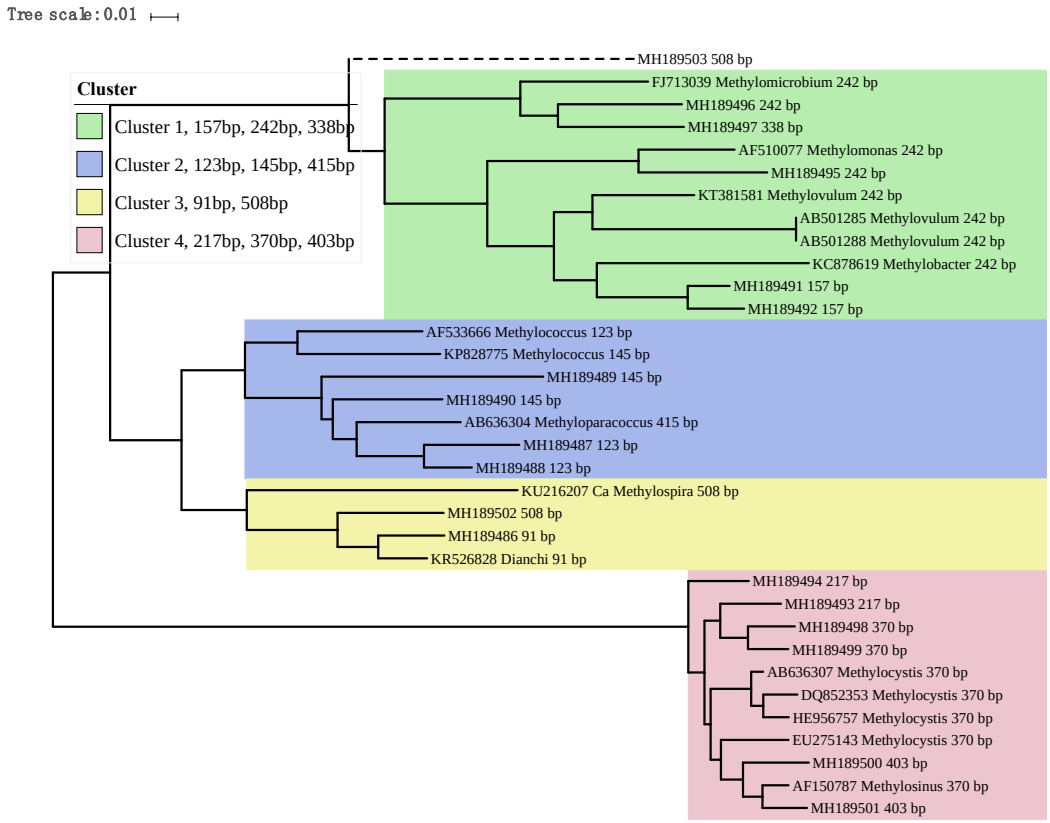
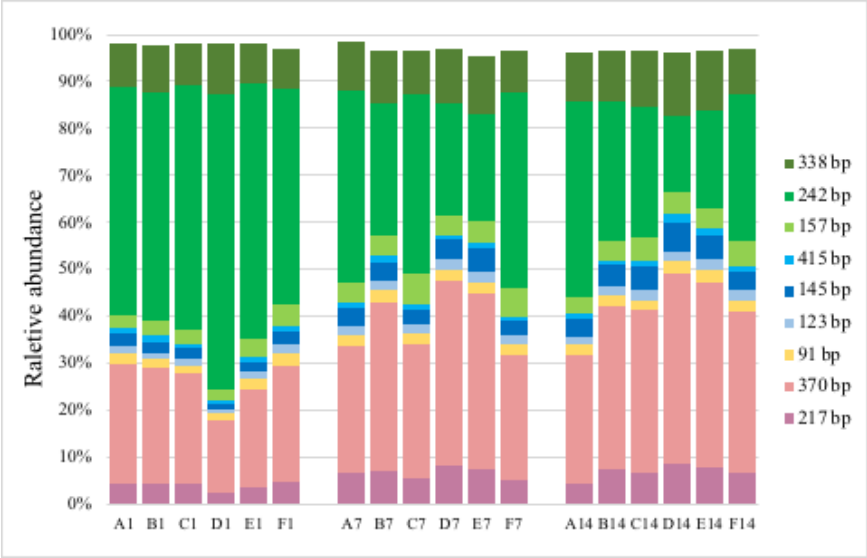
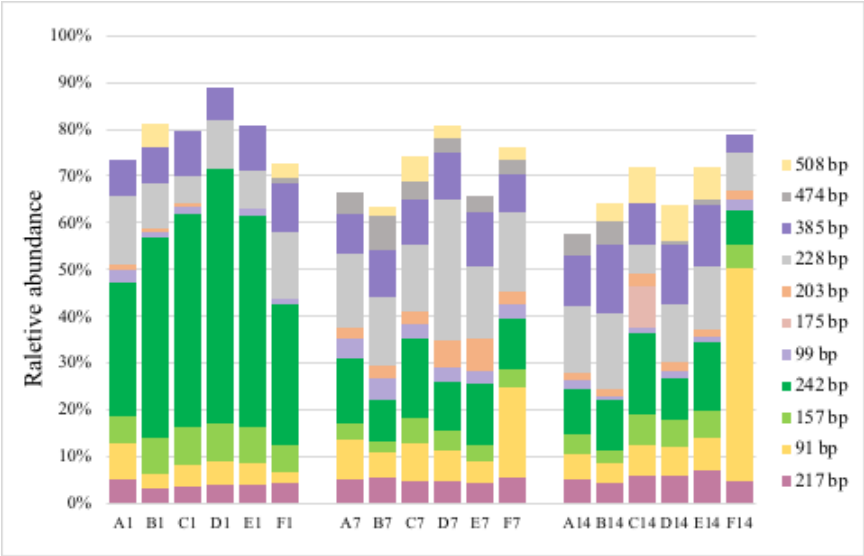


Fig. 4. T-RFLP profiles based on *pmoA* gene (a) and transcripts (b). For sample name, upper case letters refer to treatment while digits indicate sampling time.

(a)



(b)



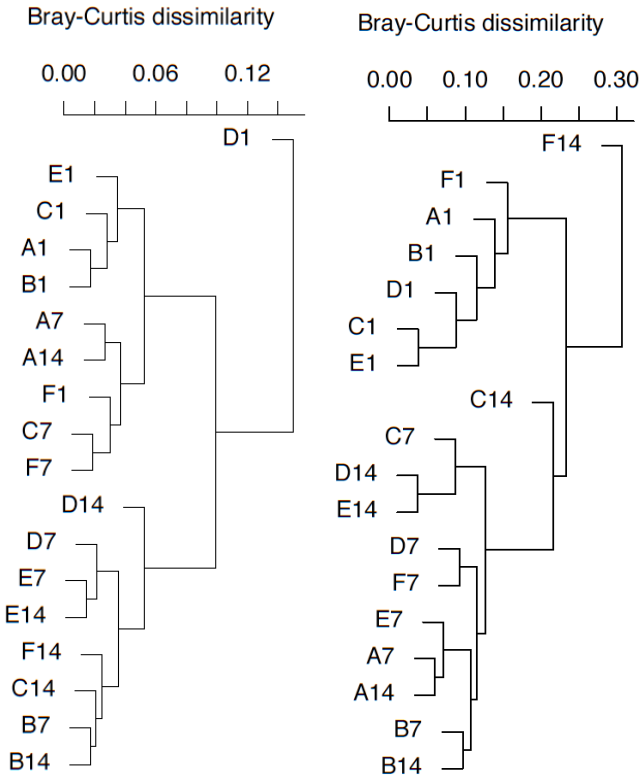
713 **Fig. 5.** *pmoA* gene (a) and transcripts (b)-based cluster diagrams of similarity values

714 for samples with different treatments. Dissimilarity levels are indicated above the

715 diagram. For sample name, upper case letters refer to treatment while digits indicate

716 sampling time.

717



718

719 **(a)** **(b)**

720

721

722

723

724

725

Supplementary Material

Table S1 Multiple comparison of treatments by means of Student-Newman-Keuls post-hoc testing. In each column, different characters indicate significant difference at 0.05 level.

treatment	MOP		*pmoA* abundance		transcript abundance	
	Day 1	Day 14	Day 7	Day 14	Day 1	Day 7
A	a	b	b	ab	d	b
B	a	ab	b	b	c	b
C	a	b	b	b	a	b
D	a	b	b	b	b	b
E	a	ab	b	b	b	b
F	b	a	a	a	b	a

Fig. S1. Change of ammonium concentration of pore water in the microcosms with different treatments

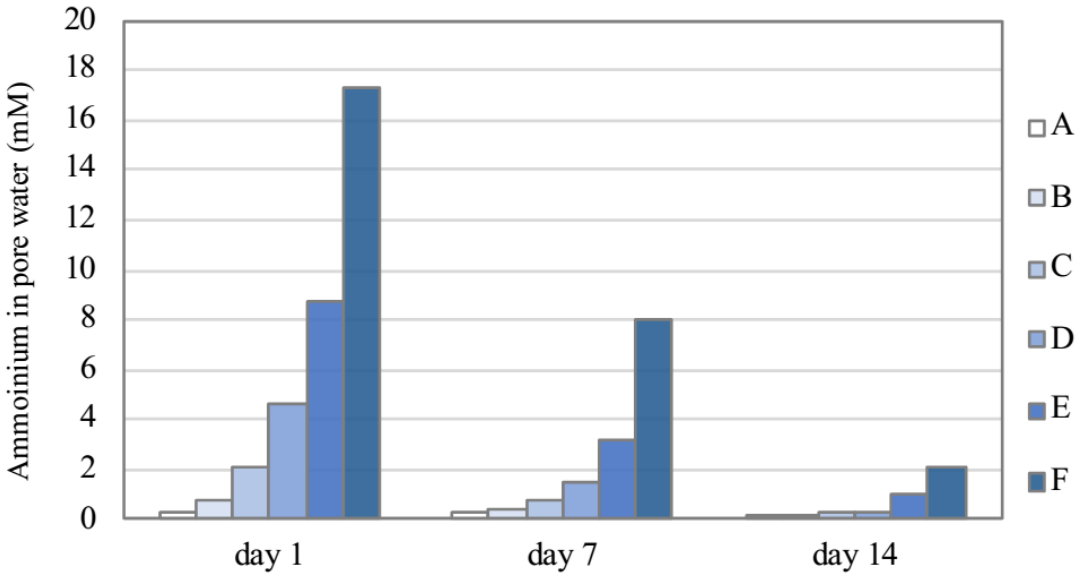


Fig. S2. The ratio of transcripts to *pmoA* gene in the microcosms with different treatments

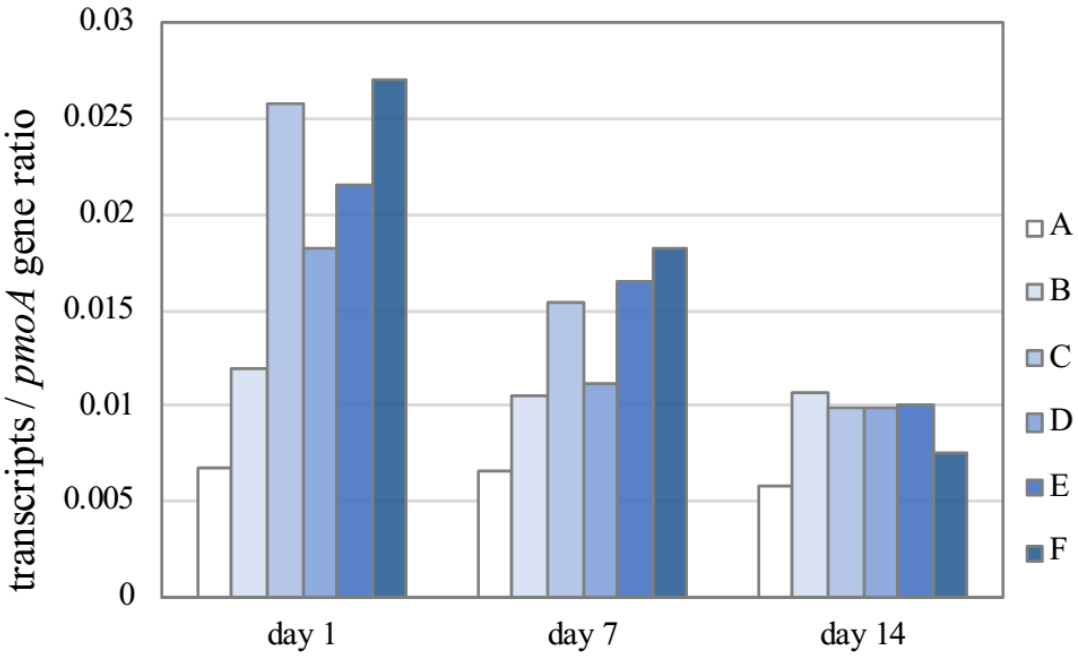


Fig. S3. Prediction of NGS representative sequences based on T-RFs