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To cite this article: Dayong Zhao, Xinyi Cao, Jin Zeng, Rui Huang & Zhongbo Yu (2017) Effects of macrobenthic bioturbation on the abundance and community composition of ammonia-oxidizing prokaryotes under different temperatures, *Journal of Freshwater Ecology*, 32:1, 405-414, DOI: [10.1080/02705060.2017.1320307](https://doi.org/10.1080/02705060.2017.1320307)

To link to this article: <https://doi.org/10.1080/02705060.2017.1320307>



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Published online: 08 May 2017.



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Effects of macrobenthic bioturbation on the abundance and community composition of ammonia-oxidizing prokaryotes under different temperatures

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ABSTRACT

In order to investigate the effects of macrobenthos on the abundance and community composition of ammonia-oxidizing prokaryotes under different temperature conditions, laboratory microcosms containing two kinds of macrobenthos (*Corbicula fluminea* and *Tubificid* worms) were constructed. Real-time polymerase chain reaction (PCR) and clone libraries were applied to analyze the ammonia-oxidizing archaea (AOA) and bacteria (AOB) communities in the surface sediments. The lowest abundances of the archaeal and bacterial *amoA* gene were found in the samples cultured under 28 °C (archaeal *amoA* gene abundance, 2.71×10^6 copies/g dry sediment; bacterial *amoA* gene abundance, 1.17×10^7 copies/g dry sediment) of the *C. fluminea* group. However, there was no significant difference in terms of the abundance of archaeal *amoA* gene in all *Tubificid* worms treatment groups. Compared to the community composition of AOB, greater variations in the community composition of AOA were observed among the three different temperature groups.

ARTICLE HISTORY


Received 19 December 2016
Accepted 3 April 2017



KEYWORDS

Bioturbation; temperature; abundance and community composition; ammonia-oxidizing prokaryotes; macrobenthos

Introduction

Nitrification plays a critical biogeochemical role in both individual ecosystems and the global nitrogen cycle (Beman & Francis 2006), in which the oxidation of ammonia is the rate-limiting and important step of the whole nitrogen cycling processes. For a long time, this process is considered to be performed by ammonia-oxidizing bacteria (AOB) (Kowalchuk & Stephen 2001). However, recent environmental genomic studies demonstrated that the ammonia-oxidizing archaea (AOA) have the similar function of AOB (Venter et al. 2004; Könneke et al. 2005; Francis et al. 2007). Previous studies have proved the important functions of AOA in various ecosystems including soils (Leininger et al. 2006), lake sediments (Zeng et al. 2012; Zhao et al. 2013), rhizosphere sediments (Zhao et al. 2014), marine water and sediments (Francis et al. 2005; Mincer et al. 2007) and hot springs (Zhang et al. 2008). Through feeding, growth and metabolism activity, the bioturbation of macrobenthos could affect the transportation of dissolved oxygen exchange at the sediment–water interface and these effects would also promote the release of nitrogen from the sediment (Aller 2001). Previous studies have investigated the effects of bioturbation of macrobenthos on the cycling of nutrient elements in the aquatic ecosystem (Mermillod-Blondin et al. 2004). Zhang et al (2010) found that the

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 Supplemental data for this article can be accessed at  <https://doi.org/10.1080/02705060.2017.1320307>.

bioturbation of *Tubificid* worms resulted in the destruction of the surface sediment, which influenced the properties and biochemical processes of lake sediments. The bacterial community composition in lake sediment would also be influenced by the bioturbation of macrobenthos (Zeng, Zhao, Liu et al. 2014). Moreover, bioturbation processes could increase the amount of electron acceptors for ammonia oxidation, and provide suitable microenvironments for the growth and metabolism of ammonia-oxidizing micro-organisms (Satoh et al. 2007). Additionally, previous studies have demonstrated that temperature could influence the community composition and activity of macrobenthos (Nie et al. 2011). Variations in the temperature could affect the activity of the macrobenthos (Nie et al. 2011), and then influence the sediment properties and the abundance and composition of the ammonia-oxidizing prokaryotes.

Corbicula fluminea and *Tubificid* worms are commonly found macrobenthos in eutrophic lake sediments (Cai et al. 2010). Previous studies have demonstrated that temperature would influence the activities of macrobenthos (Nie et al. 2011). However, it is still unknown whether the bioturbation of macrobenthos would influence the abundance and community composition of the ammonia-oxidizing micro-organisms under different temperatures.

In the present study, microcosms were constructed in the laboratory under different temperatures to investigate the response of ammonia-oxidizing micro-organisms under the bioturbation of two commonly found macrobenthos (*Tubificid* worms and *C. fluminea*). Real-time polymerase chain reaction (PCR) and clone libraries were applied to analyze the abundance and community composition of ammonia-oxidizing micro-organisms. The aim of this study is to seek the effects of temperature on the abundance and community composition of ammonia-oxidation micro-organisms under the bioturbation of macrobenthos.

Materials and methods

Sediment collection and construction of the microcosms

C. fluminea and *Tubificid* worms were collected by Peterson Grab in Pukou, which is located in the western part of Lake Taihu (N 31.295556°, E 119.937499°) and in severe eutrophic status (Lu et al. 2011; Cai et al. 2012). Sediment samples were collected using a core sampler (DM60, Mingyu, China). Both the lake water and sediments were sampled and rapidly transferred to the laboratory.

Microcosms were constructed in polyethylene glass columns to simulate the natural lake environment. The top 12 cm of the collected sediment cores were sectioned into 2 cm intervals, and the samples at the same depth were pooled together. The sediments were then sieved with mesh to remove the macrofauna and large particles. The prepared sediment samples were then fully homogenized and placed in polyethylene glass columns with 2 cm intervals corresponding to their original depths. Each of the columns was wrapped with aluminum foil in the sediments part and added 10 cm of lake water with an intravenous needle. The constructed microcosms were stabilized at room temperature for a week. The constructed microcosms were divided into *C. fluminea* and *Tubificid* worms groups. The biomasses added in each column were 500 ind./m² for *C. fluminea* and 9000 ind./m² for *Tubificid* worms (Cai et al. 2010). The microcosms were cultivated in incubators at three different temperatures, 15, 22 and 28 °C, respectively. Each temperature had three replicates for each kind of macrobenthos. The microcosms were cultivated under a 12:12 h light/dark cycles for 60 days. The surface sediment samples (0–1 cm) were collected using the self-made PVC pipes, which could accurately collect the sediment samples at a certain depth without disturbing the surrounding sediment and had been authorized a patent from the State Intellectual Property office of China (ZL 201220054471) (<http://www.pss-system.gov.cn/>).

Chemical analysis

A vacuum freeze dryer was employed to dry the sediment samples (ALPHA1-2, CHRIST, Germany). Sediment properties of samples of both before and after the incubation were investigated.

Total nitrogen (TN) and total phosphorus (TP) in the sediment samples were digested using $K_2S_2O_8$ and analyzed with spectrophotometer (Jin & Tu 1990). Ammonia nitrogen (NH_4^+-N) and nitrate ($NO_3^- -N$) were extracted by 2 M KCl and measured with a continuous flow analyzer (San++, SKALAR, Netherlands).

DNA extraction and real-time quantitative PCR

Extraction of the total DNA from sediment samples was carried out with the DNA extraction kit (Mobio PowerSoil DNA Isolation kit, USA). The concentration of the extracted DNA was determined using a BioPhotometer (Eppendorf, Hamburg, Germany).

Real-time quantitative PCR was performed to analyze the copy numbers of the archaeal and bacterial *amoA* gene in different sediment samples on the basis of SYBR Green I using an IQ5 Thermocycler (RG65HD, Corbett, Australia). The primers Arch-*amoA*F/Arch-*amoA*R (Francis et al. 2005) and *amoA*-1F/*amoA*-2R (Rotthauwe et al. 1997) were chosen for the amplification of the archaeal and bacterial *amoA* genes, respectively. Plasmid DNA was prepared and the concentrations of DNA were measured. Plasmid DNA was tenfold serially diluted for the construction of the standard curves ($3.40 \times 10^2 - 3.40 \times 10^8$ archaeal *amoA* gene copies per μ l and $1.59 \times 10^2 - 1.59 \times 10^8$ bacterial *amoA* gene copies per μ l). The 20 μ l PCR mixtures included 5 ng of the DNA template, 1 \times SYBR Premix Ex TaqTM buffer (Takara, Japan), and 0.2 μ M of each archaeal or bacterial *amoA* primer. The thermal cycling program for archaeal *amoA* PCR amplification was 3 min at 95 °C, 45 cycles of 30 s at 95 °C, 1 min at 53 °C, 20 s at 72 °C, and finally, 7 min at 72 °C. The thermal cycling program for bacterial *amoA* PCR amplification was: 3 min at 95 °C, 45 cycles of 30 s at 95 °C, 1 min at 50 °C, 20 s at 72 °C, and finally, 7 min at 72 °C. The specificity of the PCR products was examined by both the 2% agarose gel electrophoresis and melting curve analysis. Data analysis was carried out with the Rotor-Gene 6000 software package. The amplification efficiencies were 0.96–1.04 for the archaeal *amoA* gene and 0.95–1.04 for the bacterial *amoA* gene.

Construction of the clone libraries

Clone libraries were constructed for the three different temperature treatment groups. Archaeal and bacterial *amoA* gene fragments were amplified with the primers described earlier, whereas the cycle number of PCR amplification was reduced to 30 for both of the archaeal and bacterial *amoA* genes. Triplicate PCR products were pooled, gel purified (Axygen PCR cleanup purification kit) and cloned by the pGEM-T vector (Promega, Madison, WI, USA). The ligation products were transformed to *Escherichia coli* competent cells (DH5a, Takara, Japan). Picked transformants were grown overnight on Luria-Bertani (LB) agar plates containing 100 μ g/ml ampicillin, 40 μ g/ml X-Gal and 24 μ g/ml Isopropyl β -D-Thiogalactoside (IPTG). The clones were checked by PCR amplification using vector primers (T7 and SP6) and sent to Shanghai Majorbio Biotechnology Co., Ltd. for DNA sequencing.

The vector sequences of the obtained results were removed by the DNASTar software package (Lasergene 14.1) (<http://www.dnastar.com/>). All of the sequences were compared with the sequences deposited in the GenBank database using BLAST. Operational taxonomic units (OTUs) were defined at 5% nucleotide differences. Multiple sequence alignments of the most related environmental sequences and clone sequences were performed with ClustalX (Thompson et al. 1997). Indices of diversity including the number of OTUs, Chao 1 (S_{Chao1}) and Shannon (H) indexes were calculated with the software program DOTUR (Schloss & Handelsman 2005). Neighbor-joining phylogenetic trees (based on Jukes–Cantor distances) were constructed based on alignments of the nucleic acid sequences using MEGA 4.0 (Tamura et al. 2007).

Nucleotide sequence accession numbers

The archaeal and bacterial *amoA* gene sequences obtained in this study have been deposited in the GenBank database under the accession numbers KJ688859-KJ689248.

Results

Sediment properties

Sediment properties of samples of both before and after the incubation were shown in Figure 1. The concentrations of TN, TP, ammonia nitrogen and nitrate nitrogen differed among the treatment groups under different temperatures (Figure 1). For the *C. fluminea* group, the sediment samples cultured under 15 °C maintained the highest concentrations of nitrate nitrogen (Figure 1). For the *Tubificid* worms group, remarkable reduction in the concentrations of ammonia and nitrate nitrogen was found in 22 and 28 °C (Figure 1).

Abundance of the archaeal and bacterial *amoA* gene

The abundances of both archaeal and bacterial *amoA* genes were analyzed by the real-time PCR analysis (Figure 2). The abundances of the bacterial *amoA* gene were remarkably higher than those of the archaeal *amoA* gene in all groups. For the *C. fluminea* group, the abundances of both archaeal and bacterial *amoA* genes were initially increased and then decreased with the elevated temperatures and the lowest abundances of the archaeal and bacterial *amoA* genes were found in the samples cultured under 28 °C (archaeal *amoA* gene abundance, 2.71×10^6 copies/g dry sediment; bacterial *amoA* gene abundance, 1.17×10^7 copies/g dry sediment). However, there was no significant difference in terms of the abundance of archaeal *amoA* gene in all *Tubificid* worms treatment groups. The highest abundance of bacterial *amoA* gene was found in the sediment samples cultured under 22 °C (1.30×10^7 copies/g dry sediment) (Figure 2).

Diversity and phylogenetic analysis of the archaeal and bacterial *amoA* gene

In order to understand the diversity of the archaeal and bacterial *amoA* gene in different sediment samples, clone libraries were constructed and the diversity indexes including Shannon (*H*) and S_{Chao1} of the each clone library were calculated (Table 1). For the archaeal *amoA* gene, the highest diversity was found in the *C. fluminea* group cultured under 28 °C. Samples cultured under 22 °C maintained the lowest diversity of archaeal *amoA* gene for both kinds of macrobenthos (Table 1). For the bacterial *amoA* gene, the highest diversity was found in the samples cultured under 15 °C for both kinds of macrobenthos (Table 1).

Phylogenetic analysis was performed to investigate the community composition of both AOA and bacteria derived from different sediment samples (Figures S1 and S2). Compared to the community composition of AOB, greater variations in the community composition of AOA were observed among the three different temperatures. *Nitrosopumilus* was the dominant AOA group in all sediment samples except the samples cultured under 22 °C of the *C. fluminea* group (Figure 3). *Nitrososphaera* became the most abundant AOA group in the samples cultured under 22 °C of the *C. fluminea* group. *Nitrosotalea* occupied small percentages of the AOA group in the samples cultured under 28 °C for both kinds of macrobenthos (Figure 3).

Nitrosomonas oligortopa was the dominant AOB group in all sediment samples (Figure 3). *Nitrosomonas communis* covered 13.6%–48.6% of the AOB in the *C. fluminea* treatment groups; however, they just occupied a small proportion (2.9% under the 15 °C treatment group) of the AOB group in the *Tubificid* worms treatment groups. *Nitrosospira* was found in all of the *C. fluminea* treatment groups; however, it was absent in all of the *Tubificid* worms treatment groups (Figure 3).

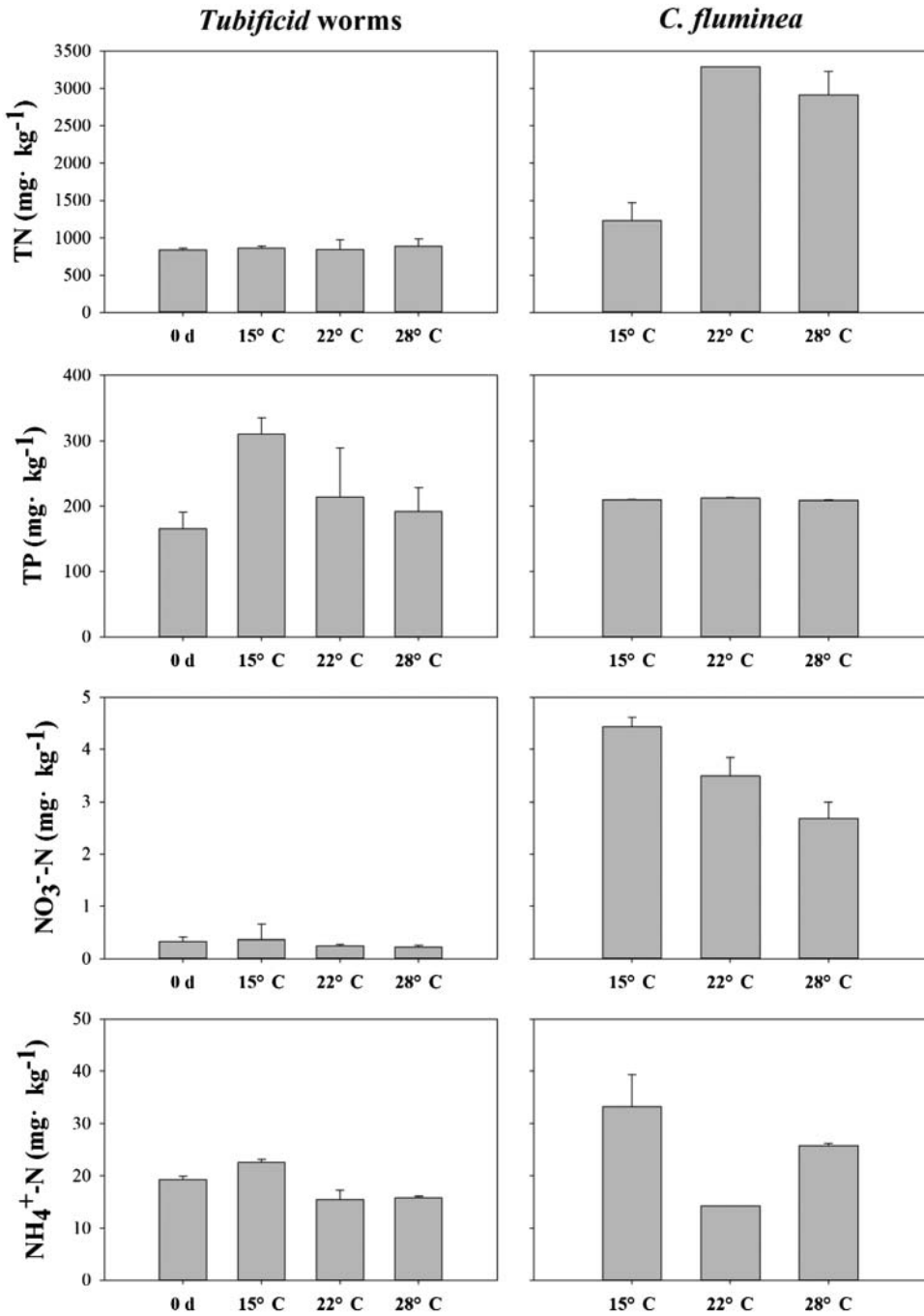


Figure 1. Properties of the surface sediment samples incubated under different temperatures. Left column for *Tubificid* worms and right column for *C. fluminea*.

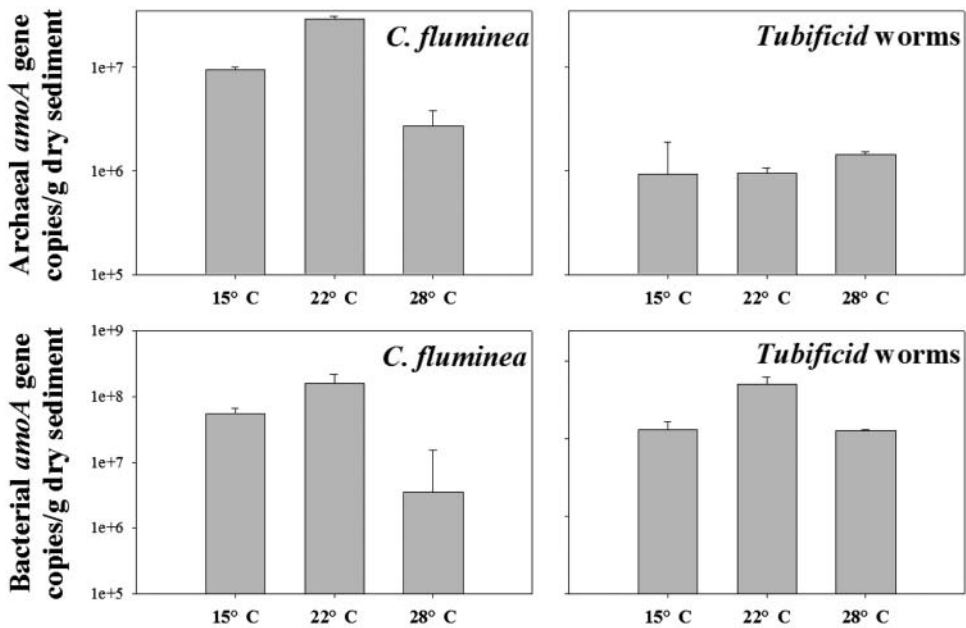


Figure 2. Abundance of the archaeal and bacterial *amoA* gene detected in surface sediment samples incubated under different temperatures.

Relationships between environmental factors and abundance and diversity of archaeal and bacterial *amoA* gene

The bioturbation of macrobenthos changed the sediment properties of different treatment groups. In order to investigate the relationships between sediment properties and the abundance or diversity of the archaeal and bacterial *amoA* gene, the Spearman correlation coefficients were calculated and the results were shown in Table S1. The abundance of the archaeal *amoA* gene was positively correlated with the concentrations of TN ($P < 0.05$), whereas there was no significant correlation found between bacterial *amoA* gene abundance and TN (Table S1). Nitrate exhibited significantly positive relations with the number of detected OTUs of bacterial *amoA* gene (Table S1).

Table 1. Diversity of the archaeal and bacterial *amoA* gene in sediment samples with the bioturbation of *C. fluminea* or *Tubificid* Worms under different temperatures.

Clone library	Clone numbers		OTUs		S_{Chao1}		H	
	Archaeal <i>amoA</i> gene	Bacterial <i>amoA</i> gene	Archaeal <i>amoA</i> gene	Bacterial <i>amoA</i> gene	Archaeal <i>amoA</i> gene	Bacterial <i>amoA</i> gene	Archaeal <i>amoA</i> gene	Bacterial <i>amoA</i> gene
<i>C. fluminea</i>								
15 °C	32	36	2	13	2	18.25	0.31	2.12
22 °C	39	35	2	8	2	9.5	0.27	1.66
28 °C	17	22	2	6	2	16	0.60	0.90
<i>Tubificid</i> worms								
15 °C	23	35	4	3	7	4	0.53	0.26
22 °C	24	33	1	2	1	2	0	0.14
28 °C	22	22	2	1	2	1	0.18	0

Note: OTUs were defined as 5% difference in nucleic acid sequences. H (Shannon–Wiener) and S_{Chao1} were calculated by the DOTUR software package.

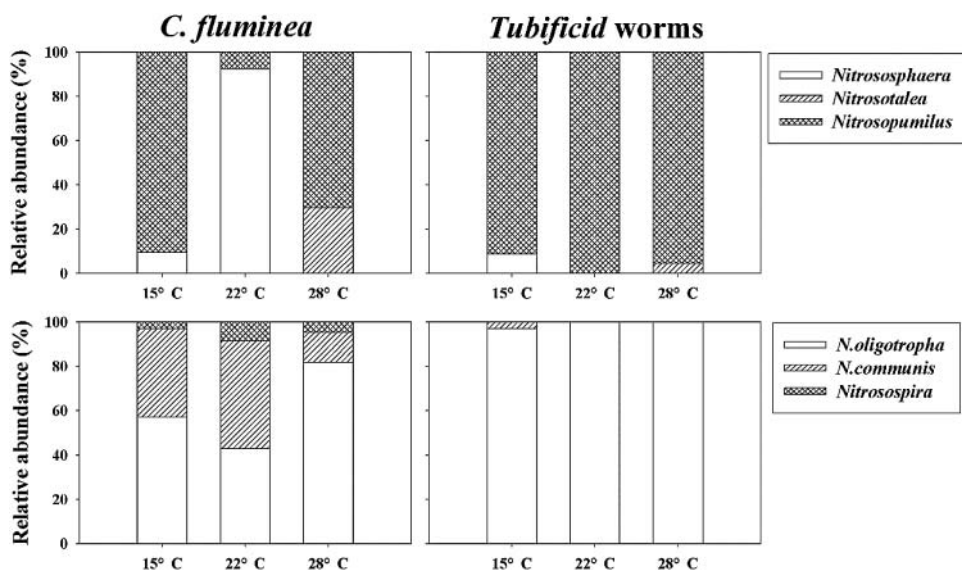


Figure 3. Relative percentages of the different ammonia-oxidizing archaea and bacteria groups detected in surface sediment samples incubated under different temperatures.

Discussion

In the present study, the bioturbation effects of *C. fluminea* and *Tubificid* worms on the abundance and community composition of AOA and bacteria under different temperatures were investigated. Remarkable differences in the abundance and community composition of ammonia-oxidizing micro-organisms in different treatment groups have been found.

Bioturbation results in the sediment reconstruction and biological flushing. For the *Tubificid* worms group, positive correlation was found between the temperature and the release amount of nitrogen from the surface sediment (Figure 1). As the temperature increased from 15 to 28 °C, *Tubificid* worms would increase the frequency of fibrillation to get more dissolved oxygen, which would promote the release of nitrogen and phosphorus from the surface sediment (Nie et al. 2011). Huang et al. (2016) constructed the microcosms to investigate the bioturbation effects of *Tubificid* worms in the lake ecosystem and found that the bioturbation of *Tubificid* worms promoted the release of nitrogen and phosphorus from the surface sediment to the overlying water. The highest abundance of the archaeal and bacterial *amoA* gene was found in the samples cultured under 22 °C of the *C. fluminea* group and no significant correlation was found for temperature and the abundance of the archaeal and bacterial *amoA* gene (Figure 2). This was consistent with the results of Cao et al. (2011), who investigated the abundances of archaeal and bacterial *amoA* genes in surface sediments from the Pearl River estuary to the South China Sea (from 2.9 to 21.3 °C). Wang et al. (2014) found that the abundance of archaeal and bacterial *amoA* gene decreased with the increasing densities of *C. fluminea* in the microcosms cultured under 25 °C, suggesting the abundance of the *amoA* gene negatively correlated with the intensity of bioturbation. The results were not consistent with the present study if the intensity of bioturbation was positively correlated with the temperature. It could be inferred that temperature was the dominant factor controlling the abundance of ammonia-oxidizing micro-organisms from 15 to 22 °C; however, bioturbation turned to be the most important factor under 28 °C.

For the *Tubificid* worms group, the results obtained in the present study indicated that the abundance of the archaeal *amoA* gene was comparable among the three temperature groups. Similar result was also found in the microcosms cultured under the 28 °C conditions (Wu et al. 2013). Zeng,

Zhao, Yu et al. (2014) reported the positive correlation between the abundance of archaeal *amoA* gene and temperature in the microcosms. However, the elevated temperature would increase the intensity of bioturbation (Nie et al. 2011), which would decrease the abundance of the archaeal *amoA* gene (Huang et al. 2016). The comparable abundance of the archaeal *amoA* gene among different groups could be attributed to the combined effects of temperature and bioturbation.

In the present study, the lowest diversity of the archaeal *amoA* gene was found in the samples cultured under 22 °C for both the *C. fluminea* and *Tubificid* worms groups. Similar result has been found by Zeng, Zhao, Yu et al. (2014) that the lowest diversity of the archaeal *amoA* gene was found in the medium temperature group (25 °C). Other study also reported the insignificant correlation between temperature and the diversity of the archaeal *amoA* gene (Cao et al. 2011).

Compared to the community composition of AOB, greater variations in the community composition of AOA were observed among the three different temperatures in the present study. A previous study conducted in the soil microcosm also found clearly changed community structure of the archaeal ammonia oxidizers other than bacterial ammonia oxidizers under different temperatures (10–30 °C) (Tourna et al. 2008). AOA and AOB maintained different responses to the variation of temperatures. Some species of AOA isolated from terrestrial hot springs (*Nitrosocaldus yellowstonii*) can grow at temperatures as high as 74 °C (de la Torre et al. 2008). However, AOB have not been found in environments consistently over 40 °C (Hatzenpichler 2012).

The bacterial *amoA* gene sequences obtained in the present study were mostly belonged to *Nitrosomonas oligotropha* lineage, which is often found in freshwater lakes; however, its ecophysiological traits remain unclear (Zeng, Zhao, Yu et al. 2014). Genus of *Nitrosomonas* has been considered to have a competitive advantage in the eutrophic environment (Koops et al. 2003), which is consistent with the higher concentrations of nitrogen and phosphorus in sediments of the present study. Moreover, only small percentages of *Nitrospira* lineage were found in the *C. fluminea* treatment groups; however, this cluster was absent from all the *Tubificid* worms treatment groups, suggesting the different effects induced by different macrobenthos.

Conclusions

In conclusion, the present study focused on the effect of bioturbation on the community composition and abundance of AOA and AOB under different temperatures. Remarkable differences in the abundance and community composition of ammonia-oxidizing micro-organisms were observed among different temperatures and macrobenthos groups. The lowest abundances of the archaeal and bacterial *amoA* genes were found in the samples cultured under 28 °C of the *C. fluminea* group, whereas there was no significant difference of the abundance of archaeal *amoA* gene in all *Tubificid* worms treatment groups. Compared to the community composition of AOB, greater variations in that of AOA were observed among the three different temperatures. The data obtained in the present study would be helpful for further understanding the nitrogen cycling process in the freshwater ecosystem.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 41371098], [grant number 41571108], [grant number 41671078]; Natural Science Foundation of Jiangsu Province, China [grant number BK20151614]; Special Fund of State Key Laboratory of Hydrology-Water Resources and Hydraulic Engineering [grant number 20145027312], [grant number 20155019012]; Fundamental Research Funds for the Central Universities [grant number 2015B14214].

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