Response to associate editor

Comments to Authors:

(1) Your manuscripts have been critically reviewed by three independent referees, and these comments have been addressed. However, after a careful check, there are still some concerns that might have been overlooked, and part of results was over-interpreted. For example, it is not appropriate to say "The cell-specific nitrification rates were estimated to be in the range of 0.41 to 0.79 fmol N per AOA cell per hour, which is consistent with earlier estimates in estuary environments. This study demonstrated that AOA were widely involved in nitrification in this hot spring." The reason is that AOA in estuary environment might not be the same as those detected in this study from hotsprings. The comments are listed below for your reference.

Reply:

The authors greatly appreciate the associate editor's constructive comments. We have revised it carefully according to your comments.

(2) P2L4. Replace "unclear" with "poorly understood", and it may be rephrased as follows "However, it remains poorly understood about the link of in situ nitrification activity to taxonomic identities of ammonia oxidizers in high-temperature environments".

Reply:

We have changed it and please see this change in Line 13 to 14, Page 1.

(3) P2L9. Three significant figures might be preferred. For example, 4.81 instead of 4.8.

Reply:

We have changed it throughout the revised manuscript.

(4) P2L13. "Candidatus Nitrosocaldus yellowstonii". Candidatus should be in italic,

while Nitrosocaldus yellowstonii is NOT ITALIC.

Reply:

We have changed it throughout the revised manuscript.

(5) P2L7. The abstract can be rephrased as follows: “”

*The in situ ammonia oxidation rates measured by the $^{15}N–NO_3$ pool dilution technique in the surface sinter and bottom sediments were 4.8 and 5.3 nmolNg^{-1} h^{-1}, respectively. Real-time quantitative PCR (qPCR) indicated that the archaeal 16S rRNA genes and amoA genes were present in the range of 0.128 to 1.96*10^{8} and 2.75 to 9.8*10^{5} and gene copies g-1 respectively, while bacterial amoA genes were not detected. Phylogenetic analysis of archaeal 16S rRNA genes showed high sequence similarity to thermophilic “Candidatus Nitrosocaldus yellowstonii”, which represented the most abundant operation taxonomic units (OTU) in both surface and bottom sediments. The archaeal predominance was further supported by fluorescence in situ hybridization (FISH) visualization. The cell-specific rate of ammonia oxidation was estimated to range from 0.41 to 0.79 fmol N archaeal cell^{-1} h^{-1}, being almost ***-fold higher than that of thermophilic “Candidatus Nitrosocaldus yellowstonii”. It implies that phylogenetic relatedness may not necessitate similarity in physiology of ammonia oxidation. These results suggest the importance of archaea rather than bacteria in driving the nitrogen cycle in terrestrial geothermal environments, and highlight the important of effort to cultivate thermophilic AOA in a wide variety of hotsprings.*

Reply:

We have changed it according to the associate editor’s valuable comments.

(6) P3L9. Please add reference for the first evidence of thermophilic AOA (2008 José R. de la Torre et al, 2008 EM; and Wagner group 2008 PNAS)

Reply:

We have added it and please see this change in Line 54, Page 2.

(7) P3L13-14. Rephrased as: previous studies suggested that AOA play important roles in nitrification in oligotrophic environments such as oceanic water (reference) and acid soils (reference).

Reply:

We have changed it and please see this change in Line 44, Page 2.

(8) P4L6. delete "extremely"

Reply:

We have deleted it.

(9) P4L11. In situ nitrification remains largely unknown and very few studies have explicitly…

Reply:

We have changed it.

(10) P5L3. Please state that this is ^{15}N pool dilution technique for nitrification measurement in situ.

Reply:

We have stated it in the revised manuscript.

(11) P5L5. Rephrased as follows. Meanwhile, potential nitrification activity was determined in the presence of high ammonium concentration: B1 (SS slurry+$^{15}NO_3$+$^{15}NH_4^+$)

Reply:

We have changed it and please see this change in Line 162, Page 6.

(12) P5L20. Pls add the brand of the GC-IRMS here.

Reply:

The brand of the GC-IRMS is Thermo Scientific from USA. We have added it in revised manuscript.

(13)P7L19. Replace "16S rDNA" with "16S rRNA gene", replace "and" with "using universal primers"

Reply:

We have changed it and please see this change in Line 272, Page 9.

(14)P7L20-25. Please specify how many different clone libraries constructed. For example, the authors obtained 16S rRNA genes amplicons of total bacteria (27f-1492r), archaea (21f-958r).

Reply:

We have added it in the revised manuscript and please see the details in Line 293-294, Page 11.

*(15)P9L12. Rephrased as follow: Archaeal and bacterial populations were determined by quantify their 16S rRNA genes with *** (reference) and *** primer pairs (reference), respectively. In addition, the abundance of AOA and AOB were quantified using *** and *** primers, respectively.*

Reply:

We have changed it and please see this change in Line 250-254, Page 9.

(16)P9L23. Pls specify the probes used for AOA visualization.

Reply:

We only use Cren679 probe to detect Crenarchaea cells in the samples (The information of Cren679 probe is list in table 1). We have changed it and please see this change in Line 250-254, Page 9.

(17)P10L23-24. As far as I understand, the authors made clone libraries for archaeal communities (21f-958r), bacterial communities (27f-1492r), and archaeal amoA genes. pls specify it clearly here.

Reply:

We have added the relevant information in the revised manuscript and please see the details in Line 293-294, Page 11.

*(18)P11L10, rephrased as follows. "…, the near-in situ rates of ammonia oxidation were estimated to be *** and *** using ^{15}N pool dilution technique, respectively."*

Reply:

We have changed it and please see this change in Line 307-308, Page 11.

(19)P11L13. Rephrased as follows: "In the meantime, the nitrate concentration increased from 2.84±2 to 3.25±2 μM in the surface sediments and from 2.33±3 to 2.62±3 μM in the bottom sediments, lending further support for strong nitrification activity under in situ conditions in the hotsprings.

Reply:

We have changed it and please see this change in Line 310-313, Page 11.

(20)P11L14. Furthermore, the potential activity of ammonia oxidation was determined with ammonium amendments. The nitrate concentration increased significantly upon the addition of NH_4^+, and the ammonia rates recorded in the surface sediments and bottom sediments (with NH_4^+) were 5.7±0.6 and 7.1±0.8 nmolNg^{-1} h^{-1}, respectively.

Reply: We have changed it and please see this change in Line 314-318, Page 12.

(21)P11L19. Please specify the number of cloned 16S rRNA genes here.

Reply:

We have added it and please see this change in Line 320, Page 12.

(22)P12L16-27. As referee suggested, pls refer to the taxonomic classification proposed by Pester 2012 EM paper. For example, Nitrososphaera lineage, Nitrotalea lineage; N. yellowstonii lineage, and N.maritimus lineage. And the old term usually

refers to group 1.1b, group 1.1a and group 1.1a-associated. The readers will be confused by cluster A, B and C. The authors can put the representative AOA sequences (e.g from group 1.1a or 1.1b) together with sequences in cluster A and B. Thus it would be easy for readers to understand.

Reply:

We have updated the taxonomic classification of AOA-amoA according to the associate editor's suggestion. Please see this change in section 3.4.

(23) P14L25. It seems plausible that archaeal amoA genes were detected ony in the Gongxiaoshe hot spring

Reply:

We have changed it.

(24) P16L2. Please replace "amoA transcripts" with "amoA genes"

Reply:

We have changed it and please see this change in Line 465, Page 17.

(25) P17L1. Rephrased as follow. "Therefore, it implies that phylogenetic relatedness does not necessarily indicate similarity in the physiology of thermophilic AOA, and ammonia oxidation activity of thermophilic AOA in this study might be stronger than those detected in other hotsprings from US (reference) and Iceland (reference). These results also suggest the importance of cultivation studies for comparative analysis of environmentally representative AOA in a wide variety of hotsprings.

Reply:

We have changed it and please see this change in Line 507-512, Page 18.

Response to Anonymous Referee #1

Received and published: 13 November 2015

We greatly appreciate the reviewer's constructive comments. Below, we address all the comments and questions point-by-point. The original reviewer's comments are italicized and our responses to the reviewer's comments follow.

General comment

(1). *This manuscript aimed at addressing contribution to nitrification in a high-temperature hot spring. The high abundances of ammonia oxidizing archaea (AOA) were determined by qPCR. The number of the studies on nitrification process and corresponding source microorganisms is still rare. Therefore, this paper is timely and the idea behind is interesting. However, it is just a single site in Tengchong hot spring system and could not represent the whole region. The results just simply show the data and lack logical writing. Some paper close related are not cited or fully discussed in this manuscript (Hou et al. 2013; Xie et al. 2014). The authors could investigate more sites to get a statistic results for the nitrification process in the region, compare with other geothermal systems and get some overall rationales for the similarities or differences among them.*

Reply:

Thanks for the reviewer's constructive comments. We agree with that more data of AOA activity from different sites will enhance our understanding of the nitrification process in the whole Tengchong Geothermal Field. However, in this study, our research goals primarily focus on verifying the hypothesis that Archaea rather than Bacteria drive the ammonia oxidation in high-temperature territorial hot spring environments (we have pointed it out more clearly in the revised paper). Therefore, we selected a representative site in which the ammonia oxidation driven by Archaea might be active in Tengchong Geothermal Field to test this hypothesis.

There are two reasons for us to choose Gongxiaoshe hot spring as the research site to test the hypothesis in this study: 1) Ammonia concentration in Gongxiaoshe hot spring water is 102.61 μg L^{-1}, thermodynamically favorable to ammonia oxidation; 2) The ammonia-oxidizing archaea "*Candidatus Nitrosocaldus yellowstonii*" were

dominant in hot spring water and no AOB amoA genes were detected in the hot spring.

In the revised paper, we have rewritten the Introduction section to make the research goals more clearly. In addition, we have added more information on the selection of the site and added more discussions on the difference in ammonia oxidation rates among Gongxiaoshe hot spring and other geothermal systems. The papers close related to this work have also been cited and discussed in the revised paper.

Specific comments of the reviewer #1

(1). *Page 16258, line 6-14, Hou et al. (2013, Plos one) and Xie et al. (2014, EM) have shown the dominated of AOA in the Gongxiaoshe hot spring. It should be mentioned here.*

Reply:

We have added it in revised manuscript. Please see this change in Line 86, Page 3.

(2). *Page 16260, line 20. What is the brand for the GC-IRMS?*

Reply:

The brand of the GC-IRMS is Thermo Scientific from USA. We have added it in revised manuscript. Please see this change in Line 181, Page 7.

(3). *Page 16262, line 10-20. How many sequences have you got? What is the representation of those sequences? Hou et al. (2013, Plos one) have already published thousands of 16S rRNA sequences from this site, why do not you cite some to build the phylogenetic tree?*

Reply:

A total of 152 archaeal clone sequences were obtained in this study. The coverage ranged from 90% to 93%. In addition, we got 141 bacterial clone sequences in this study. The coverage ranged from 76% to 83%. We have randomly selected

forty 16S rRNA sequences from the Short Read Archive database at NCBI (Hou et al., 2013) to build new phylogenetic trees. The new phylogenetic trees have also been added in revised manuscript (Figure 4 and Figure S2).

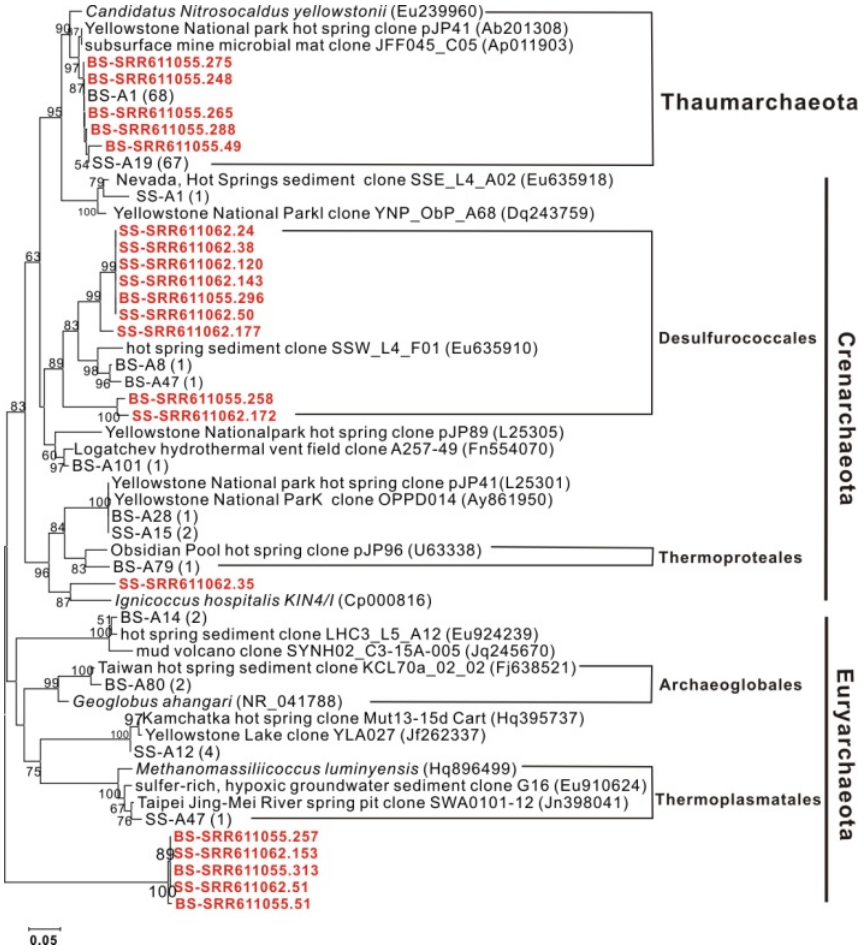

Figure 4. Archaeal phylogenetic tree based on 16S rRNA gene sequences, including various 16S rRNA gene clones obtained from the Gongxiaoshe hot spring sediments (SS and BS) and cited some sequences from Hou et al. (2013) (marked in red). Bootstrap confidence values are obtained using 1000 replicates, and values greater than 50% are indicated at the nodes. The scale bar represents the expected number of changes per nucleotide position.

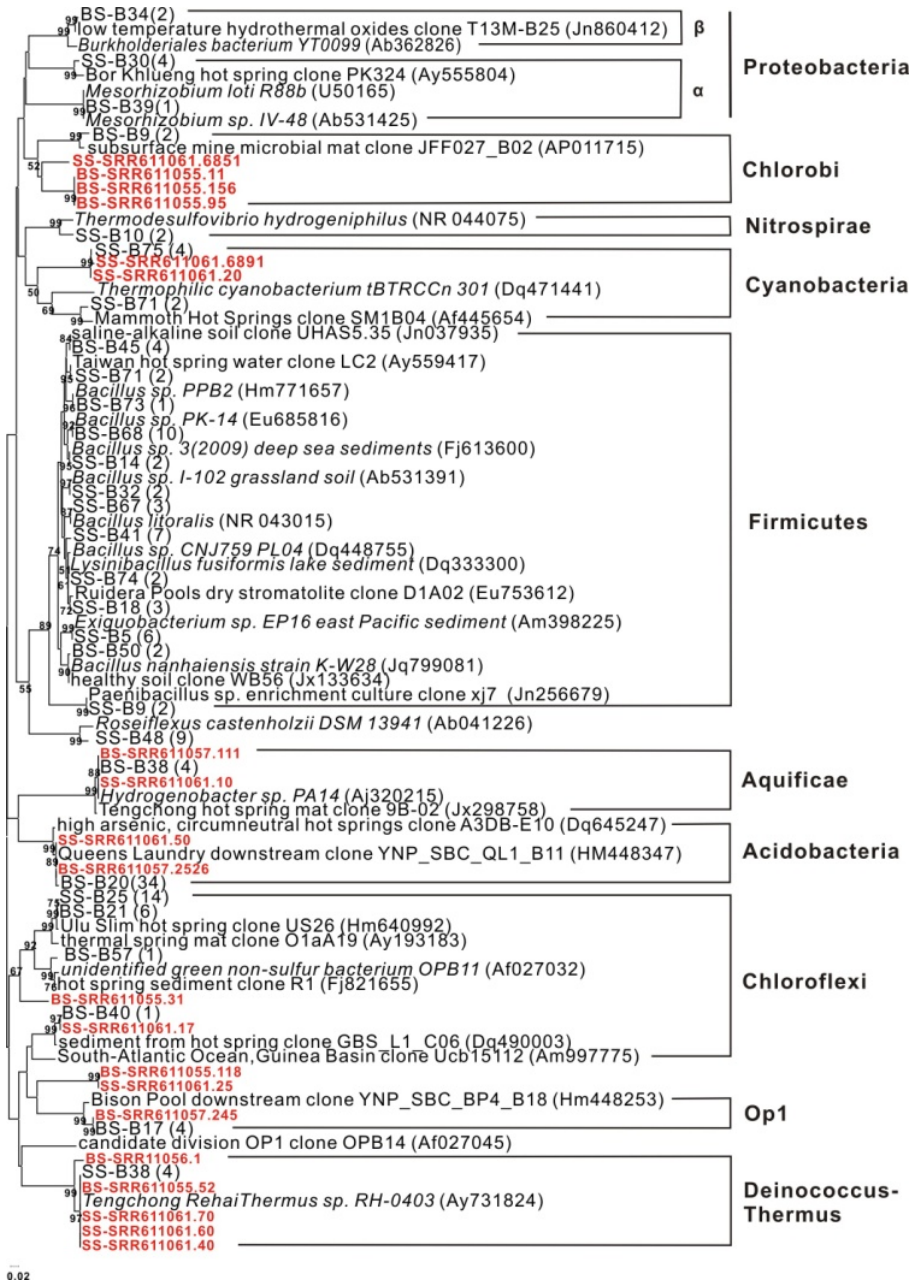

Figure S2. Bacterial phylogenetic tree based on 16S rRNA gene sequences, including various 16S rRNA gene clones obtained from the Gongxiaoshe hot spring sediments (SS and BS) and cited some sequences from Hou et al. (2013) (marked in red). Bootstrap confidence values are obtained using 1000 replicates, and values greater than 50% are indicated at the nodes. The scale bar represents the expected number of changes per nucleotide position.

(4). *Page 16262, line 25. All the "amoA" should be italics.*

Reply:

We have changed it in revised manuscript.

(5). *Page 16263, line 11-20. How specific these qPCR reactions are?*

Reply:

The PCR conditions are as follows: 10 min at 50°C, 2 min at 95°C; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C; 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C to make the melting curve. We have added it in revised manuscript.

(6). *Page 16263, line 11-20. What are the efficiencies and R2 values of the qPCR assays?*

Reply:

The efficiencies of the qPCR runs are 87.8-105.6% (R^2=0.992-0.999) for 16S rDNA and 102% (R^2=0.998) for AOA. We have added it in the revised manuscript.

(7). *Page 16267, line 1-4. What is the meaning of the phylotype close to soil AOA? Does it mean that the soil might be one source of the hot spring AOA? Should do some discussion here.*

Reply:

It is interesting that some phylotypes from Gongxiaoshe hot spring are closely related to soil AOA. One possibility is that some of the *amoA* genes obtained in this study may derive from soil AOA, particularly those sequences in cluster *Nitrosotalea* and cluster *Nitrososphaera*, which have been widely found in sediments and soils. Those AOA from soil might have evolved multiple times and have adapted to high-temperature environments. We have added some discussion in the revised manuscript. Please see this change in Line 437-442, Page 16.

(8). *Page 16267 line 16. Xie et al.(2014, EM) has shown similar results by lipid marker. Should be mentioned here.*

Reply:

Thanks for the reviewer's suggestion. We have added it in the revised manuscript.

(9). *Page 16268 line 5-25. This paragraph just generally described the AOA research in geothermal hot springs. No discussion about your own data. Should be specific about how the environmental factors controlling the AOA in Gongxiaoshe hot spring.*

Reply:

We are very grateful for the reviewer's comments. A specific paragraph has been added in the revised manuscript to discuss the environmental factors (e.g. temperature and ammonia concentration) controlling the AOA in Gongxiaoshe hot spring as follows.

Temperature is likely a very important factor controlling microbial community structure in Gongxiaoshe hot spring. This interpretation is supported by the results of qPCR (Fig. 2b and Fig. S2). The sediment samples from the bottom of pool (T=77 °C) are dominated by Archaea, whereas the sediment samples from the margin of pool (T=55 °C) are dominated by Bacteria. In addition, no AOB were detected in both bottom and margin samples, indicating that it might be difficult for AOB to inhabit in high-temperature hot spring environments (Lebedeva et al., 2005; Hatzenpichler et al., 2008). Additionally, the abundance of AOA amoA gene in bottom sediments is slightly higher than that in margin sediments, reflecting that although AOA can adapt to a wide range of temperature, higher temperature could be more favorable to the growth of AOA (de la Torre, et al., 2008; Hatzenpichler et al., 2008; Jiang et al., 2010). Ammonia concentration may be another factor that influences the potential activity of AOA and AOB in hot springs. Because AMO in AOA has a much higher affinity for the substrate compared to a similar process in AOB, the ability of AOA to compete for ammonia in oligotrophic hot spring environments is also quite higher than that of AOB (Hatzenpichler et al., 2008). In Gongxiaoshe hot spring, the ammonia concentration is 102.61 $\mu g\ L^{-1}$ that is lower compared to other hot springs with high ammonia concentrations. This relatively low ammonia concentration may also be one of the reasons for the absence of AOB in Gongxiaoshe hot spring.

Response to Anonymous Referee #2

Received and published: 26 November 2015

We greatly appreciate the reviewer's constructive comments. Below, we address all the comments and questions point-by-point. The original reviewer's comments are italicized and our responses to the reviewer's comments follow.

General comment

(1). *In this study the authors investigated nitrification activity along with the community composition and abundance of ammonia oxidizing prokaryotes in the sediment of a hot spring in China. The authors detected ammonia oxidizing archaea related to Nitrosocaldus yellowstonii, and abundances of archaeal amoA genes were sufficient to explain the observed nitrification rates while bacterial ammonia oxidizers were not detected. The authors concluded that nitrification in these terrestrial geothermal environments is driven by archaea. The manuscript addresses an interesting topic, however, my major concern is that the amount of data presented here is rather limited. Only two samples were taken and analyzed.*

Reply:

Thanks for the reviewer's constructive comments. In this study, our research goal is to verify the hypothesis that Archaea rather than Bacteria drive ammonia oxidation in high-temperature hot spring environments (We have rewritten the Introduction section and stated this point more clearly). To test this hypothesis, we selected Gongxiaoshe hot spring in Tengchong Geothermal Field as a representative hot spring in which ammonia oxidation driven by Archaea might be active, based on relatively high concentration of ammonia and widely presence of AOA genes in Gongxiaoshe hot spring. Although just two sediment samples were recovered from the margin and the bottom of the pool for incubation experiments and molecular analyses, we think those samples are representative for the current study for following two reasons:

1) Gongxiaoshe hot spring is a small pool with a diameter of ~300 cm and a depth of ~130 cm. Hot spring water in the pool is well mixed and water chemistry shows no difference in different areas of the pool.
2) Sediments of Gongxiaoshe hot spring are found to be only present at the margin of

the pool and at the bottom of the pool, representing two typically sedimentary environments in this pool. Samples recovered from the same sedimentary environment (e.g. bottom of the pool) show no difference in mineralogy and geochemistry.

We have stated this point more clearly in the revised version.

(2). *it is not clear from the manuscript if these samples were at least taken in triplicates. In order to confirm the message that ammonia oxidizing archaea dominate nitrification in this hot spring environment, results of replicate samples showing the same trend would make the outcome more convincing, including the molecular analyses.*

Reply:

In this study, 16S rDNA and archaeal amoA genes were determined in triplicate. We set up reactors in duplicate for four ^{15}N stable isotope tracing experiments. The results of replicate samples show the same trend. We have added more information in the materials and methods, in Line 167, Page 7.

(3). *Moreover, it is not clear in what way this study is different from the previous studies targeting ammonia oxidation in hot springs that the authors refer to. Here, the authors should point out more clearly in the introduction what new insight into ammonia oxidation in hot springs they expected to gain from their study, and/or why their experimental approach was going beyond what previous studies already did, especially in light of the fact that the amount of data presented in this manuscript is rather limited. Here, more clear research questions or hypotheses would help to better define the research goals of this study.*

Reply:

Thanks for the reviewer's valuable suggestion. To our knowledge, previous studies targeting ammonia oxidation in hot springs mainly focused on archaeal amoA gene (AOA) via a variety of molecular approaches (e.g. qPCR, 16S rRNA gene library and CARD-FISH). The results from these studies suggested that

ammonia-oxidizing archaea (AOA) may be ubiquitous in high-temperature environments and even more abundant than their bacterial counterparts, which has led to a hypothesis that Archaea rather than Bacteria drive ammonia oxidation in high-temperature terrestrial hot spring environments. This hypothesis, however, still needs to be verified. Currently, our knowledge about the activity of AOA in such high-temperature environments is largely constrained, especially due to the data deficiency of ammonia oxidation rates. *In-situ* incubation experiments are urgently required to verify the potential activity of AOA and their contribution to ammonia oxidation at such high temperature environments. In this study, we determine not only the community structure of AOA but also their potential contribution to nitrification, in combination of culture-based ^{15}N pool dilution techniques and uncultured-based molecular approaches (FISH, qPCR and a 16S rRNA gene library). We have rewritten the Introduction section to state more clearly the research goals of this study.

(4). *The discussion also needs to be restructured in order to focus more on the key findings of this work. A substantial part of the discussion deals with the estimated per cell activities, however, I have some concerns regarding the assumptions on which this estimation was based (see specific comment below).*

Reply:

Thanks for the reviewer's constructive comments. We have restructured the Discussion section. More discussions have been made to focus on the key findings of this work, for examples, the variation of *AOA amoA* gene and nitrification rates.

Specific comments of the reviewer #2

(1). *title: Please add "Community composition" at the beginning.*

Reply:

We have added it. Please see this change in Line 1, Page 1.

(2). *p. 16256, l. 12: operational taxonomic units*

Reply:

We have corrected it.

(3). *p. 16256, l. 14: rather write AOA-amoA than just AOA because this only refers to gene abundances*

Reply:

We have corrected it.

(4). *p. 16257, l. 13: ...in the function of their ecosystem. Which ecosystem?*

Reply:

The terrestrial and marine ecosystem. We have changed it in the revised manuscript.

(5). *p. 16257, l. 22: Which temperature was the optimum temperature? please give the number here.*

Reply:

The optimum temperature is 65-72 °C. We have added it in the revised manuscript (Line 22, Page 16257).

(6). *p. 16258, l. 24: The last sentence is the conclusion of the whole work and should rather not appear in the introduction.*

Reply:

We have deleted it.

(7). *p. 16259, l. 16-17: Why were the water samples diluted prior to storage?*

Reply:

Once hot spring water cools, chemical components (e.g. silica) in water would become supersaturated and spontaneously precipitate in the bottles. The way to avoid this is to dilute the water samples prior to storage.

(8). *p. 16261, l. 20-21: Please give references for the primers A21F and A958R.*

Reply:

We have added references for the primers A21F and A958R and primers Eubac27f and Eubac1492r (Line 207-211, Page 8).

Reference:

Delong, E. F. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA, 89: 5685-5689, 1992.

Lane, D. J. 16S/23S rRNA sequencing, p. 115-175. In E. Stackebrandt and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. Wiley, Chichester, UK, 1991.

(9). *p. 16265, l. 13: The differences in nitrate concentrations described here are very small. What was the detection limit of the method?*

Reply:

The detection limit of this method is 0.2 ppm.

(10). *p. 16265, l. 16. What is meant by ammonia rates, ammonia oxidation rates? Please specify.*

Reply:

Sorry for this mistake. “Ammonia rates” should be “Ammonia oxidation rates”. We have corrected it in revised manuscript.

(11). *p. 16266, l. 13: What does "extremely similar" mean, can you give percent sequence identity here?*

Reply:

We have deleted “extremely”. The percent sequence identity information has been added in the revised paper (Page 342, Line 13).

(12). *p. 16266,l. 17-24: The phylogeny of AOA-amoA is not update. Please follow the phylogeny suggested by Pester et al. 2012, Environmental Microbiology.*

Reply:

We have updated the phylogeny of AOA-*amoA* according to the reviewer's suggestion (Fig. 5). Please see the details in section 3.4, Page 13.

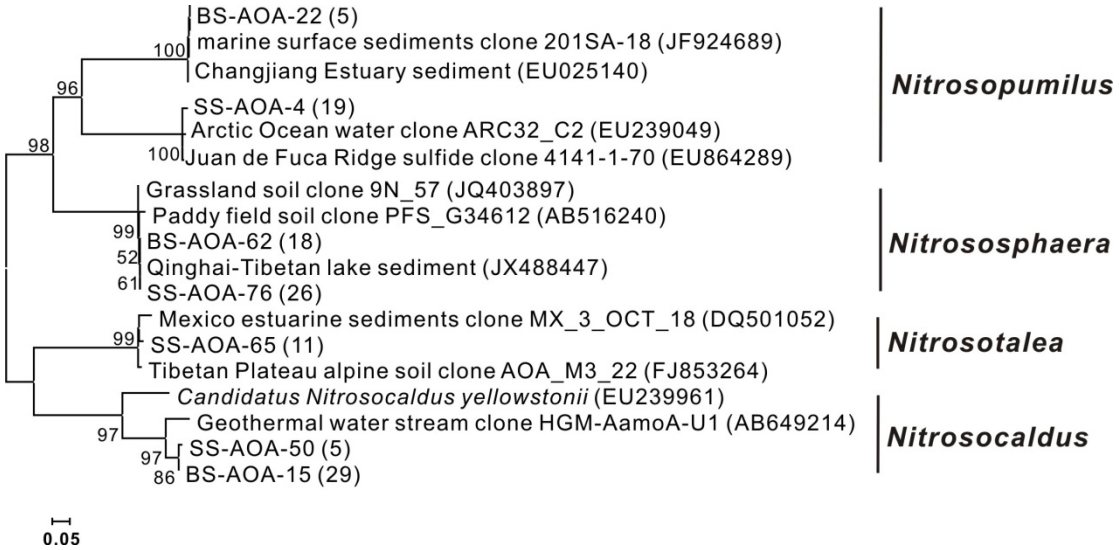

Figure 5. The phylogenetic tree of archaeal amoA genes is cloned from the Gongxiaoshe hot spring sediments (SS and BS). The tree is constructed using the neighbor-joining method, and bootstrap confidence values over 50% (1000 replicates) are shown. The scale bar represents the expected number of changes per nucleotide position.

(13). *p. 16267, l. 17: The differences in gene abundances are not convincing, a factor 3 differences could still be within the error range of the qPCR method. Here, the authors should be careful not to over-interpret the differences.*

Reply:

Thanks for valuable suggestion. We have rephrased this sentence as follows:

The copy numbers of archaeal amoA genes in the surface and bottom sediments are 2.75×10^5 *and* 9.80×10^5 *gene copies* g^{-1} *sediment, respectively.*

(14). *p. 16269, l. 1: ...for archaeal 16S rRNA genes, please add*

Reply:

We have added it.

(15). *p. 16269, l. 20-21: The message here is unclear, how does this sentence go*

together with the information about AOA-amoA gene abundances in the sentence before?

Reply:

We have changed the sentence as fellow:

The bacterial amoA genes were not detected, indicating that AOB is absent or is a minority in this hot spring ecosystem.

(16). *p. 16270, l. 1-2: The method section only describes DNA-based work. By which approach did the authors measure archaeal amoA transcripts?*

Reply:

We didn't measure archaeal *amoA* transcripts in this study. We are sorry for this mistake.

(17). *p. 16270, l. 22: This study giving the average amoA gene copy number per cell was published in 1997, long before ammonia oxidizing archaea were first described. I wonder if the authors can really use this number for their estimations of per cell activity.*

Reply:

Bernander and Poplawski (1997) demonstrated that single cell of thermophilic archaea contained two genomes in stationary phase. Although it is long before ammonia oxidizing archaea (AOA) were first described, we assume that this number can be also used to estimate the per cell activity in this study, due to thermophilic and archaeal nature of AOA harbored in high-temperature Gongxiaoshe hot spring. This method was also adopted by Dodsworth et al. (2011) to estimate the per cell nitrification activity of AOA in two US Great Basin hot springs. However, as suggested by reviewer, some uncertainties of this method may still exist, with respect to the stage of cell cycle and the diversity of archaea. We have added a sentence to state these uncertainties in the revised paper.

Reference:

Bernander, R. and Poplawski, A.: Cell cycle characteristics of thermophilic archaea, J. Bacteriol.,

179, 4963-4969, 1997.

Dodsworth, J. A., Hungate, B. A., and Hedlund, B. P.: Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea, Environ. Microbiol., 13, 2371–2386, 2011.

Response to H. Jiang, (Received and published: 9 December 2015).

We greatly appreciate Dr. Jiang's constructive comments. Below, we address all the comments and questions point-by-point. The original reviewer's comments are italicized and our responses to the reviewer's comments follow.

General comment

Terrestrial geothermal environments are very important settings for research on biogeochemical cycle of elements. Ammonia oxidation is the first and rate-limiting step of nitrification in nature environments. The manuscript by Chen et al describes a study on composition of ammonia-oxidizing archaea and their contribution to nitrification in a high-temperature hot spring. Their results showed that AOA were widely involved in nitrification whereas bacterial amoA was not detected in studied hot spring, indicating dominance of archaea in driving the nitrogen cycle in terrestrial geothermal environments. The results are very important for our understanding on N biogeochemical cycle in hot springs. However, I have some concerns as listed below:

(1). *P16L5: "A weak but significant correlation was found between the abundances of the archaeal amoA and gross nitrification rates, which were consistent with the results reported by Isobe et al. (2012)". P16l20: "By conducting correlation analysis between the gross nitrification rates and abundances of amoA in the two samples".*
It is not a scientific way to described statistical correlation on only two samples.

Reply:

We agree with the reviewer's valuable comments and have rephrased the sentences as follows.

"High abundance of ammonia-oxidizing archaea corresponds to high ammonia oxidation rate, which is consistent with the results reported by Isobe et al. (2012)".

"To understand the relationship between the ammonia oxidation rates and abundances of amoA in the two samples, we specifically estimated the contribution of archaeal cells to nitrification"

(2). *As the manuscript showed that the cell-specific nitrification rates were estimated to be in the range of 0.41 to 0.79 fmol N cell-1 h-1, which is consistent with earlier estimates in estuary environments. These results are two magnitude higher than those for AOA in reported US hot springs (0.008-0.01 fmolN cell-1 h-1; Dodsworth et al., 2011). In P14L7, the author said "The ammonia or ammonium concentration and temperature are controlling factors of the distribution of AOA", and P14L17 "The ammonia concentration and potential activity of AOA and AOB showed an obvious positive correlation". The pH and Temperature showed no significant difference between the GXS hot spring (Temp:77 degree C, pH7.7, NH_4^+ concentration:102.61 μg/L, amoA copies: 2.75-9.8*10^5 gene copies g^{-1} of dry weight) and the GBS hot spring (Temp:81 degree C, pH7.2, NH_4^+ concentration: 663 μg/L, amoA copies: 3.5-3.9*10^8 gene copies g-1 of dry weight). However, the GBS hot spring possesses high amoA gene copies and NH_4^+ concentration. Such ammonia oxidation difference between the authors' and Dodsworth et al. (2011) is of interest. The author should include this point into the discussion on controlling factors of cell-specific nitrification rates.*

Reply:

We are very grateful for the reviewer's constructive comments. More discussions have been made to focus on controlling factors of cells specific nitrification rates in revised manuscript as follows.

To understand the relationship between the ammonia oxidation rates and abundances of amoA in the two samples, we specifically estimated the contribution of archaeal cells to nitrification. By assuming two amoA copies per cell (Bernander and Poplawski, 1997) and by comparing the ammonia oxidation rates with the qPCR results of AOA amoA per gram, the cell-specific nitrification rates were estimated to be 0.41 fmol N cell^{-1}h^{-1} and 0.79 fmol N cell^{-1}h^{-1} in the surface and bottom sediments of the hot spring, respectively. These results are much higher than those for AOA in the GBS hot spring [0.008-0.01 fmol N cell^{-1}h^{-1}(Dodsworth et al., 2011b)]. It is

interesting that although the GBS hot spring possesses higher amoA gene copies (3.5-3.9 × 10^8 gene copies g^{-1} of dry weight) and higher NH_4^+ concentration (663 μg L^{-1}), it exhibits a lower cell-specific nitrification rate than Gongxiaoshe hot spring. This may imply that both the abundance of AOA and the NH_4^+ concentration are not important factors that control the cell-specific nitrification rates in high-temperature hot spring environments. The difference in cell-specific nitrification rates between the Gongxiaoshe hot spring and the GBS hot spring may reflect the difference of AOA population structure in those two hot springs (Gubry-Rangin et al., 2011; Pester et al., 2012). In line with this AOA heterogeneity, cell-specific nitrification rates do not reflect the overall AOA abundance or NH_4^+ concentration in these AOA-dominated hot springs. Alves et al. (2013) reported a similar case that soil dominated by AOA (clade A) exhibited the lowest nitrification rates, in spite of harboring the largest AOA populations.

(3). *P3L21: "A thermophilic autotrophic AOA Ca. N. yellowstonii)": the bracket should be removed.*

Reply:

We have removed it in the revised manuscript.

(4). *Page 6 line 4-7, how many bottles for each experiment treatment?*

Reply:

We set up reactors in duplicate for four experiments, eight bottles for each experiment. We have added this information in the revised manuscript.

(5). *Page 9, section 2.7, the qPCR conditions should be at least briefly given here. In addition, the qPCR efficiency should also be presented.*

Reply:

Thanks for the reviewer's valuable suggestion. We have added following information on the qPCR conditions in the revised manuscripts.

The PCR conditions were as follows: 10 min at 50°C, 2 min at 95°C; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C; 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C to make the melting curve.

The efficiencies of the qPCR runs were 87.8-105.6% (R^2=0.992-0.999) for 16S rDNA and 102% (R^2=0.998) for AOA.

(6). *Page 10, line 12, did the authors forget archaeal probe here? There is Arch915 probe targeting total archaea in table1.*

Reply:

We did not use archaeal probe to perform FISH experiment. We apologize for this confusion. We have deleted relevant information in table 1.

(7). *Page 13, line 24-26, based on Fig.3, I cannot get the information on cell relative abundance of Crenarchaea. The cells shown in Fig. 3 are all Crenarchaea (I assume the green ones are). Are the two dyes for archaea and Crenarchaea probes same or different? If different, two pictures should be taken at the same place for total archaea and Crenarchaea, which will reveal whether the observed cells are Crenarchaea or other group of archaea. If same, how did the authors distinguish crenarchaea cells from others?*

Reply:

We apologize for this confusion. The abundance of archaeal *amoA* genes in this study is determined by qPCR, instead of FISH. We only use Cren679 probe to detect Crenarchaea cells in the samples, so the green cells observed in Fig. 3 are all Crenarchaea. We have added a sentence to make this point more clearly in the revised paper.

Community c~~C~~omposition of ammonia-oxidizing archaea and their contribution to nitrification in a high temperature hot spring

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Abstract

The oxidation of ammonia by microbes ~~and associated organisms~~ has been shown to occur in diverse natural environments. However, it remains poorly understood about the link of *in situ* nitrification activity to taxonomic identities of ammonia oxidizers in high-temperature environments.~~the contribution of ammonia oxidizing archaea to nitrification in high temperature environments remains unclear.~~ Here, we studied *in situ* ammonia oxidation rates and the abundance of ammonia-oxidizing archaea (AOA) in surface and bottom sediments at 77 °C in the Gongxiaoshe hot spring, Tengchong, Yunnan, China. The *in situ* ammonia oxidation rates measured by the ^{15}N-NO_3^- pool dilution technique in the surface ~~sinter~~ and bottom sediments were 4.80 and 5.30 nmol N $g^{-1}h^{-1}$, respectively. Real-time quantitative PCR (qPCR) indicated that the archaeal 16S rRNA genes and *amoA* genes were present in the range of 0.128 to 1.96 × 10^8 and 2.75 to 9.80 × 10^5 gene copies g^{-1} sediment, respectively, while bacterial *amoA* was not detected. ~~Relative abundances of Crenarchaea in both samples were determined by fluorescence in situ hybridization (FISH).~~ Phylogenetic analysis of 16S rRNA genes showed high sequence similarity to thermophilic '*Candidatus* Nitrosocaldus yellowstonii', which represented the most abundant operational taxonomic units (OTU) in both surface and bottom sediments. The archaeal predominance was further supported by fluorescence in situ hybridization (FISH) visualization. ~~Furthermore, bacterial *amoA* was not~~

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~~detected in this study. Quantitative PCR (qPCR) indicated that AOA and 16S rRNA genes were present in the range of 2.75 to 9.80 × 10^5 and 0.128 to 1.96 × 10^8 gene copies g^{-1} sediment.~~ The cell-specific ~~nitrification~~ rate of ammonia oxidation~~s~~ was~~ere~~ estimated to ~~to be in the~~ range from ~~of~~ 0.410 to 0.790 fmol N archaeal $cell^{-1}$ h^{-1}, higher than those in the two US Great Basin hot springs. These results suggest ~~which is consistent with earlier estimates in estuary environments. This study demonstrated that AOA were widely involved in nitrification in this hot spring. It further indicated~~ the importance of archaea rather than bacteria in driving the nitrogen cycle in terrestrial geothermal environments.~~.~~

1 Introduction

Nitrogen is a key element controlling the species composition, diversity, dynamics, and functioning of many ecosystems (Vitousek et al., 1997). Despite of recent processes in our understanding of nitrogen cycling activities in soils, fresh and marine waters, and sediments (Francis et al., 2005; He et al., 2007; Beman et al., 2008; Jia and Conrad., 2009; Konneke et al., 2005; Nicol and Schleper, 2006), gaps in knowledge associated with high temperature ecosystems have prevailed (Zhang et al., 2008a).~~Geothermal systems harbor phylogenetically and functionally distinct microbial communities under their wide range of physical and chemical conditions. Previous studies have demonstrated that thermophilic, chemotrophic and phototrophic microorganisms are ubiquitous in global geothermal environments, as they play an important role in the biogeochemical cycle of elements (*Pierson et al., 1999; Huber et al., 2000; Shock et al., 2010*).~~ Recently, more~~any~~ studies have elucidated nitrogen metabolism and cycling in high-temperature hot spring ~~geothermal~~ ecosystems (Dodsworth et al., 2011b; Nishizawa et al., 2013; Gerbl et al., 2014). In such systems, there has been evidence of microbial communities oxidizing ammonia, the first and rate-limiting step of nitrification (Reigstad et al., 2008; Hatzenpichler et al., 2008~~Dodsworth et al., 2011a~~). ~~The ammonia monooxygenase subunit A (*amoA*) gene can be used to quantify and characterize ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) in natural environments. When metagenomic and laboratory isolation methods were performed on samples, the results indicated that mesophilic crenarchaea significantly oxidized ammonia. However, the function of mesophilic crenarchea in its ecosystem was previously unknown.~~

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~~Moreover, it has been found that AOA exceed AOB in abundance of by several orders of magnitude in many terrestrial and marine systems (*Wuchter et al., 2006; Leininger et al., 2006; Mincer et al., 2007*). Previous studies suggested that AOA play a significant role in the function of their ecosystem.~~

~~Following the discovery that the archaeal amoA gene is ubiquitous in moderate environments, more attention has been paid to the AOA in higher temperature environments.~~ Since the occurrence of a putative archaeal *amoA* gene in hot spring~~geothermal~~ environments was first reported by Weidler et al. (2007) and Spear et al. (2007), thaumarchaeota possessing ammonia monooxygenase (AMO) have been obtained from some terrestrial hot springs in the USA, China and Russia~~; these species harbor AOA *amoA* gene markers~~ (Pearson et al., 2008; Zhang et al., 2008a). ~~A thermophilic autotrophic AOA (*N. yellowstonii*) was enriched from a hot spring at an optimum temperature; thereafter, ammonia oxidation was carried out in the temperature range of 65 to 72℃, representing a separate ammonia oxidizing lineage (De la Torre et al., 2008).~~

~~Reigstad et al. (2008) measured the nitrification rates in an acidic hot spring *in situ*, suggesting that considerable oxidation of ammonia occurs in many terrestrial hot springs. Dodsworth and colleagues (2011b) measured the oxidation rate of ammonia in two US Great Basin hot springs, indicating that this process was driven by ammonia oxidizing archaea. Data from these studies serve as strong evidence supporting the notion that archaeal ammonia oxidizers play an important role in the biogeochemical nitrogen cycle of terrestrial, geothermal environments. However, further investigations are needed to ascertain the abundance and diversity of ammonia oxidizing archaea in these extremely high temperature biotopes, as well as the rate of nitrification catalyzed by these species in the aforementioned biotopes.~~

~~In the Ruidian geothermal area, Gongxiaoshe is a large, circumneutral site that is dominated by carbonate depositing springs.~~ Previous studies targeting ammonia oxidation in hot springs mainly focused on archaeal *amoA* gene (AOA) via a variety of culture-independent approaches (e.g. 16S rRNA clone library, biomarkers) (Weidler et al., 2007; Francis et al., 2007; Zhang et al., 2008a; Jiang et al., 2010; Xie et al., 2014). The results from these studies

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suggested that ammonia-oxidizing archaea (AOA) may be ubiquitous in high-temperature environments and even more abundant than their bacterial counterparts, which has led to a hypothesis that Archaea rather than Bacteria drive ammonia oxidation in high-temperature hot spring environments. This hypothesis, however, still needs to be verified. Currently, our knowledge about the activity of AOA in such high-temperature environments is largely constrained, especially due to the data deficiency of ammonia oxidation rates (Reigstad et al., 2008; Dodsworth et al., 2011b; Li et al., 2015). *In situ* incubation experiments are urgently required to verify the potential activity of AOA and their contribution to ammonia oxidation in such high temperature environments. In this study, we selected the Gongxiaoshe hot spring at Tengchong Geothermal Field as a representative site to test the hypothesis that Archaea rather than Bacteria drive ammonia oxidation in high-temperature hot spring environments. The reasons for choosing the Gongxiaoshe hot spring as the research site are: 1) Ammonia concentration in the Gongxiaoshe hot spring water is 102.61 μg L^{-1}, thermodynamically favorable to ammonia oxidation (Shock et al., 2005); 2) Ammonia-oxidizing archaea "*Candidatus* Nitrosocaldus yellowstonii" were dominant in hot spring water and no AOB *amoA* genes were detected in the hot spring (Hou et al., 2013), indicating that the ammonia oxidation driven by Archaea might be active. Here, in combination of culture-independent (fluorescence *in situ* hybridization, quantitative PCR and clone library) and culture-dependent (^{15}N pool dilution technique) approaches, we provide direct evidences that AOA are indeed responsible for the major portion of ammonia oxidation in high-temperature hot spring environments.

~~A variety of studies have focused on the microbial communities thriving in Gongxiaoshe hot spring, as the water of this hot spring is enriched with ammonia (*Zhang et al., 2008; Hou et al., 2013; Edwards et al., 2013*), indicating that ammonia oxidation and possibly other N transformations may be widespread at this site. However, very few studies have explicitly characterized the abundance and community composition of AOA or determined the extent to which the thermophilic ammonia oxidizing microorganisms of this hot spring are involved in nitrification.~~

~~In this study, our aim was to conduct quantitative analyses of the ammonia oxidizing~~

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~~microorganism populations and their contribution to nitrification in the Gongxiaoshe hot spring. To determine the nitrification activity in hot springs under *in situ* high temperature conditions, the ^{15}N pool dilution technique was employed to trace the nitrate produced from ammonia oxidation in the sediments (surface and bottom) of the hot spring. The rate measurements were correlated with the censuses of microbial populations and the *amoA* functional gene in the surface and bottom samples. Fluorescence in situ hybridization (FISH) and quantitative PCR (qPCR) specific for ammonia oxidizing microorganisms were performed in this process.~~

~~Our results imply that nitrification is driven by archaea rather than by bacteria in the geothermal environment.~~

2 Materials and methods

2.1 Site description and chemical measurements

Gongxiaoshe hot spring is a small pool with a diameter of ~300 cm and a depth of ~130 cm (Fig. 1). Hot spring water in the pool is well mixed and water chemistry shows no difference in different areas of the pool (Zhang et al., 2008b). Sediments of Gongxiaoshe hot spring are found to be only present at the margin of the pool and at the bottom of the pool, representing two typically sedimentary environments in this pool. The samples from the pool margins and sediments from the bottom of the spring, designated SS (Surface Sediments) and BS (Bottom Sediments), respectively, were collected using sterile equipment in April 2013. ~~Gongxiaoshe is an octagonal pool with a concrete wall and iron railing. Around the pool margins, the area is filled with sinter characterized as a $CaCO_3$ precipitate. The surfaces of the sinter are either partially or completely covered with dark green microbial mats (Fig 1). At the bottom of the hot spring, there is loose cream colored sediment containing carbonates. The sinter samples from the pool margins and sediments from the bottom of the spring, designated SS (Surface Sinter) and BS (Bottom Sediment), respectively, were collected using sterile equipment in April 2013.~~ During transportation, all of the samples were packed with dry ice. ~~After transporting the samples to the laboratory,~~ T~~t~~hey were then stored in a freezer at –80 °C in lab for further analysis.

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Temperature and pH were measured *in situ* in the hot water spring. Temperature was determined with an iButton thermometer (DS1922T, Dallas Semiconductor, USA). The pH was measured using a pH Meter (SevenGo™ pH meter SG2, Mettler Toledo, USA). Water samples for cation and anion analysis were filtered through a syringe filter with a 0.22 ~~-~~μm filtration membrane; these samples were diluted 10 times with deionized water and stored in 100 ~~ml~~ mL polypropylene bottles in the field because an analysis was carried out after two days. The cation concentrations were determined using an IRIS Advantage ICP-AES, whereas the anion (F^-, SO_4^{2-}, Cl^-) concentrations were determined using the Ion Chromatography System (DIONEX ICS-1500, Thermo Scientific, USA). The HCO_3^- concentration was measured using the Gran titration method (Appelo and Postma, 1996). The NH_4^+-N and NO_3^--N concentrations were determined using a Nutrient Analyzer (Micromac 1000, Partech, UK).

2.2 ^{15}N stable isotope tracing of nitrification activity

Gross N nitrification rates were determined *in situ* by the ^{15}N pool dilution technique. All of the nitrification measurements were conducted in 500 ~~ml~~ mL polycarbonate culture flasks (Nalgene) with a silicone plug that contained 400 ~~ml~~ mL of mud (~1/3 sediment by volume). Two subsamples were collected from the bottom and surface sediments with 350 μ~~l~~ L of $K^{15}NO_3$ (485 μmol L^{-1} ~~μmol/L~~, at 10% ^{15}N). For each sample, two experiments were conducted to measure the in situ nitrification activity: A1 (SS slurry + $^{15}NO_3^-$) and A2 (BS slurry + $^{15}NO_3^-$). Meanwhile, potential nitrification activity was determined in the presence of high ammonium concentration~~Two experiments involving ionic ammonium were conducted to determine the potential nitrification activity~~: B1 (SS slurry + $^{15}NO_3^-$ + $^{14}NH_4^+$) and B2 (BS slurry + $^{15}NO_3^-$ + $^{14}NH_4^+$). Two pairs of duplicate reactors were set up in four experiments. The reactors were incubated near the *in situ* conditions of the hot spring water at 77 °C for 30 and 120 min. At certain time intervals (e.g., 30 min, 120 min), 80 ~~-~~mL~~l~~ aliquots were collected from the experimental reactors ~~A1, A2, B1, and B2~~ with sterile serological pipettes and transferred to acid-cleaned 250 ~~-~~mL~~l~~ polypropylene bottles. Prior to filtration, 40 ~~ml~~ mL of

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KCl (3 M) was added to each sample bottle, and the samples were shaken at 120 rpm for 1 h and then centrifuged at 1600 ×*g* for 10 min (Reigstad et al., 2008). The supernatant was filtered through a syringe filter containing a 0.22 -µm filtration membrane; the supernatant was subsequently stored in acid-cleaned 60 -mL~~l~~ polypropylene bottles at 4 °C, and analysis was performed after 2 days.

In the laboratory, the concentrations of NH_4^+ and NO_3^- in the filtrate were determined by a Nutrient analyzer (Micromac-1000, UK). The NO_3^- ($^{15}NO_3^-$ and $^{14}NO_3^-$) ions of the filtrates were converted to N_2O by denitrifying bacteria (*Pseudomonas aureofaciens*) lacking N_2O reductase activity, and N_2O was quantified by coupled gas chromatography isotope ratio mass spectrometry (GC-IRMS, Thermo Scientific, USA) (Dodsworth et al., 2011a). The ammonia oxidation~~gross nitrification~~ rates were calculated using the equations of Barraclough. D. (1991) as were the concentrations and N isotope ratios of NO_3^- in the samples incubated for 30 and 120 min, respectively.

2.3 DNA extraction and purification

DNA was extracted by the SDS-based extraction method described by Zhou et al. (1996), with some modifications. Briefly, approximately 5 -g samples were frozen with liquid nitrogen and milled three times. Then the powdered samples were mixed with 13.5 ~~ml~~ mL of DNA extraction buffer and 100 -µL~~l~~ of proteinase K (10 mg -ml^{-1}) in tubes; these tubes were horizontally shaken at 225 rpm for 30 min~~utes~~ at 37 °C. After shaking, 1.5 ~~ml~~ mL of 20% SDS was added, and the samples were incubated in a water bath; the temperature of the water bath was maintained at 65 °C for 2 h~~ours~~. During this period, the tubes were subjected to gentle end-over-end inversions every 15 to 20 min~~utes~~. The supernatant fluids were collected after subjecting the tubes to centrifugation at 6000 ×*g* for 10 min at room temperature; the collected supernatant tubes were subsequently transferred into 50 ~~ml~~ mL centrifuge tubes. The supernatant fluids were mixed with an equal volume of chloroform: isoamyl alcohol solution (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with a 0.6 volume of isopropanol at room temperature; this process was carried out for at least 1 h~~our~~.

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Crude nucleic acids were obtained by centrifugation at 16,000 × *g* for 20 min at room temperature; these crude nucleic acids were washed with cold 70% ethanol and resuspended in sterile deionized water; the final volume of this solution was 100 μl. The crude nucleic acids were purified with a Cycle-Pure Kit (Omega, USA). These crude nucleic acids were then resuspended in the elution buffer, and the final volume of the solution mixture was 50 μl; this solution was stored at –80 °C.

2.4 PCR and clone library construction

16S rRDNA gene was amplified with purified genomic DNA as templates using and universal bacterial primers. The primer pairs A21f A21F (5′-TTC CGG TTG ATC CCT G CCG GA-3′) and A958R (5′-CCC GGC GTT GAA TC AAT T-3′) were chosen for Archaea (Delong, 1992) and Eubac27Ff (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Eubac1492Rr (5′-GGT TAC CTT GTT ACG ACT T-3′) were chosen for bacteria (Lane, 1991). In a total volume of 50 μl, the reactions were performed using 1.25 U of Taq DNA polymerase (Takara, Japan). The amplification conditions were as follows: an initial denaturation was carried out at 94 °C for 4 minutes, and then, the same denaturation was continued at 94 °C for 1 minute. Thereafter, annealing was carried out at 55 °C for 45 seconds, while extension was conducted at 72 °C for 60 seconds; the process was repeated for 30 cycles, followed by a final extension step at 72 °C for 10 minutes. The PCR products were excised after being separated by gel electrophoresis; a gel-extraction kit (Omega, USA) was used to purify the products in accordance with the manufacturer's instructions. The purified PCR products were cloned into pMD20-T vectors (Takara, Japan) and transformed into competent *Escherichia coli* DH5α cells. To select the positive clones, colony PCR was used to determine the presence of correctly sized inserts containing vector-specific primers M13f (5′-GTA AAA CGA CGG CCA G-3′) and M13r (5′-CAG GAA ACA GCT ATG AC-3′).

2.5 Sequencing and phylogenetic analysis

All of the clones were sequenced by the dideoxynucleotide chain-termination method. In this procedure, an ABI 3730 capillary electrophoresis sequencer (Applied Biosystem, Inc., USA)

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was coupled with the T-vector universal primers M13f and M13r. The whole sequence of each clone was spliced using DNAMAN software (version 6.0), and the vector sequences were deleted; the presence of chimeras was checked using the Greengenes chimera check tool (Bellerophon server) (Huber et al., 2004). The program DOTUR was used to determine the operation taxonomic units (OTU~~s~~) for each sequence; 97% similarity was considered as the cut-off for the chimeric sequences. To find closely related sequences in the GenBank and EMBL databases for phylogenetic analysis, none of the chimeric sequences were submitted to the Advanced BLAST search program. Phylogenetic trees were constructed using the neighbor-joining method and the software MEGA (version 5.05). A bootstrap analysis was used to provide confidence estimates of the tree topologies.

2.6 Amplification of *amoA* (ammonia monooxygenase subunit A)-related sequences.

Archaeal *amoA* gene fragments were amplified using the primer pair Arch-amoAF (5´-STA ATG GTC TGG CTT AGA CG-3´) and Arch-amoAR (5´-GCG GCC ATC CAT CTG TAT GT-3´) (Francis et al., 2005). Bacterial *amoA* genes were also tested using the bacterial primer sets amoA 1F (5'-GGG GTT TCT ACT GGT GGT-3´) and amoA 2R (5'CCC CTC KGS AAA GTT CCT TC-3´) (Rotthauwe et al. 1997). PCR cycling was performed by the method of Francis et al. (2005). In this method, PCR products from SS and BS were recovered from the gel slices using a gel-extraction kit (Omega, USA) in accordance with the manufacturer's instructions. The purified PCR products from each type of sample were cloned into the pMD20-T vectors (Takara, Japan) and transformed into competent *Escherichia coli* DH5α cells. Cloning and sequencing were performed according to the above-mentioned process. Forty to fifty randomly selected colonies per sample were analyzed for the presence of insert archaeal *amoA* gene sequences.

2.7 Quantification of 16S rDNA and *amoA* Genes

Archaeal and bacterial populations were determined by quantify their 16S rRNA genes with 344F-518R (Øvreas et al., 1998) and 518F-786R primer pairs (Muyzer et al., 1993),

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respectively. In addition, the abundance of AOA and AOB were quantified using amo196F-amo277R (Treusch et al., 2005) and amoA-1F and amoA-2R (Rotthauwe et al., 1997) primers, respectively. All sample and standard reactions were performed in triplicate. ~~The numbers of 16S rDNA and archaeal *amoA* genes were determined by an ABI 7500 real-time PCR system (Applied Biosystems Inc., USA); T~~the SYBR Green I method was used for this analysis. The 20 -μL~~l~~ reaction mixture contained 1 μ~~l~~ L of template DNA (10 ng), a 0.15 μM concentration of each primer, and 10 μ~~l~~ L of Power SYBR Green PCR master mix (Applied Biosystems Inc., USA); this reaction mixture was analyzed with ROX and SYBR Green I. The PCR conditions were as follows: 10 min at 50 °C, 2 min at 95 °C; 40 cycles consisting of 15 s at 95 °C and 1 min at 60 °C; 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C to make the melting curve (Wang et al., 2009). Melting curve analysis was performed after amplification, and the cycle threshold was set automatically using system 7500 software ~~(1.3)~~v2.0 Patch 6. The efficiencies of the qPCR runs were 87.8-105.6% (R^2=0.992-0.999) for 16S rDNA and 102% (R^2=0.998) for AOA. Primers targeting different genes ~~were selected according to previous studies (Muyzer et al. 1993; Øvreas et al., 1998; Treusch et al., 2005) and~~ are listed in Table 1.

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2.8 Sample processing for FISH

To visualize Crenarchaea cells~~AOA~~ *in situ*, FISH was performed according to the procedure described by Orphan et al. (2002, 2009). Small aliquots of sediment were fixed overnight at 4 °C using 2 % formaldehyde in 1×PBS [145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4 (pH =7.4)]; these aliquots of sediments were washed twice with 1×PBS and stored at –20 °C~~C~~ in ethanol: PBS (1:1, vol/vol) medium. The total supernatant was filtered through a polycarbonate filter (Millipore) under low vacuum (<5 psi; 1psi=6.89 kPa). Filters were cut into suitably sized pieces and transferred onto untreated, round, 1 -in~~ch~~ glass slides. The transfer of filters onto glass slides was performed according to the procedure described by Murray et al. (1998). In this process, 5 μ~~l~~ L of a 1×PBS solution was spotted onto a glass slide that was scored with a diamond pen prior to mapping, and half of the freshly prepared filter was used to invert the sample onto the slide; this inverted sample was then air-dried.

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Prior to FISH, the samples on the glass slides were treated with an EtOH dehydration series (50%, 75%, and 100% EtOH), dried, and stored at –20 °C. Hybridization and wash buffers were prepared according to the procedure described by Pernthaler et al., 2001. Here, 20 µl L of hybridization buffer containing 35% or 20% formamide was added to the samples on the glass slides. FITC or CY3-labeled oligonucleotide Cren679 probes described by *Stahl and Amann,(1991) and* Labrenz et al. (2010), respectively, waswere added to the hybridization buffer so that the final solution had a concentration of 5 ng /µlL^{-1}.

The hybridization mixtures on the slides were incubated for 1.5 h at 46 –°C in a pre-moistened chamber. After hybridization, the slides were transferred into a preheated wash buffer and incubated for an additional 15 min at 48 –°C. The samples were rinsed in distilled water and air-dried in the dark. The microscopic images of the hybridized samples were recorded on a Leica Imager (Leica, DMI 4000B, Germany).

2.9 Nucleotide sequence accession numbers

The clone libraries for archaeal communities (21F-958R), bacterial communities (27F-1492R), and archaeal amoA genes(amoAF-amoAR) were constructed. All of the small-subunit rRNA gene sequences and the *amoA* sequences were deposited in the GenBank/EMBL nucleotide sequence database under the following accession numbers: KP784719 to KP784760 for partial 16S rRNA gene sequences; and KP994442 to KP994448 for the *amoA* sequences.

3 Results

3.1 Water chemistry

The hot water (of the spring had a temperature of 77.06 °C and pH= of 7.7) ; it contained Ca (20.25 mg/ L^{-1}), K (41.97 mg L^{-1}mg/L), Mg (3.986 mg L^{-1}mg/L), Na (313.3 mg L^{-1}mg/L), SiO_2 (130.3 mg L^{-1}mg/L), HCO_3^- (963 mg L^{-1}mg/L), NH_4^+-N (102.61 µg /L^{-1}), NO_3^--N (7.68 µg L^{-1} µg/L), F^- (9.158 mg L^{-1}mg/L), Cl^- (418.9 mg L^{-1}mg/L) and SO_4^{2-} (24.96 mg L^{-1}mg/L). The bottom water had a temperature of 77 °C, higher than the surface water that had a temperature of 55 °C. Thise hot spring was previously categorized as a Na-HCO_3 spring (Zhang et al., 2008) because ofdue to the high concentration of alkaline metal ions (K, Na, and

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Ca) (Zhang et al., 2008b).

3.2 Ammonia oxidation rates

In the surface and bottom sediments (without NH_4^+ stimulation), the near *in situ* rates of ammonia oxidation ~~rates calculated from the ^{15}N-NO_3^- pool dilution data~~ were estimated to be 4.80 ± 0.2 and 5.30 ± 0.5 nmol N $g^{-1}h^{-1}$ using ^{15}N-NO_3^- pool dilution technique, respectively. In the meantime ~~samples with no added NH_4^+~~, the nitrate concentration increased from 2.84 ± 2 μM to 3.25 ± 2 μM in the surface sediments and from 2.33 ± 3 μM to 2.62 ± 3 μM in the bottom sediments, further providing evidences for strong nitrification activity under *in situ* conditions in the hotsprings. Furthermore, the potential activity of ammonia oxidation was determined with ammonium amendments. ~~These results agreed with the incubation experiments amended with NH_4^+.~~ The nitrate concentration increased significantly upon ~~continuously with~~ the addition of NH_4^+, and the ammonia oxidation rates recorded in the surface sediments and bottom sediments (with NH_4^+) were 5.70 ± 0.6 and 7.10 ± 0.8 nmol N $g^{-1}h^{-1}$, respectively.

3.3 Archaeal community composition and phylogenetic analysis.

A total of 152 archaeal clone sequences were obtained in this study. Phylogenetic analysis showed the distribution of the clone sequences into three monophyletic groups: Thaumarchaeota, Crenarchaeota, and Euryarchaeota (Fig 4). In this study, the most abundant archaeal phylum was Thaumarchaeota. Among them, two phylotypes~~,~~ (SS-A19 and BS-A1), were the most dominant archaeal lineage, representing 89% and 86% of the cloned archaeal sequences in surface and bottom sediments, respectively. These sequences were closely related to the thermophilic, autotrophic, ammonia-oxidizing archaeal "~~'~~*Ca.* N. yellowstonii~~'~~" (de la Tarre et al., 2008). The seven archaeal OTUs found here belonged to Crenarchaeota, which contains sequences recovered from hydrothermal vents and hot spring environments. In addition, two phylotypes (BS-A47 and BS-A8) that were branched with uncultured sequences belonged to *Desulfurococcales*, which was also recovered from sediments of the hot spring. Euryarchaeota also occurred in both the sediments, but with relatively low abundances.

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Phylotype BS-A80 is associated with *Geoglobus ahangari*, which belongs to *Archaeoglobales* and is capable of oxidizing organic acids (Kashefi, ~~K~~et al., 2002). SS-A12, which represents four clones recovered from the surface sediments, showed 93% similarity to an uncultured archaeal clone that was recovered from the Spring River. SS-A47 belonged to the *Thermoplasmatales* that were 96% similar to their nearest neighbor sequence, which were collected from the Spring River. The other euryarchaeotal sequences BS-14 and BS-A80 were ~~extremely~~ similar to their uncultured counterparts (from 96 to 99% identity), which were mostly recovered from high-temperature geothermal environments.

3.4 Community analysis of AOA

~~In this study, a~~A total of 113 archaeal *amoA* gene fragments were obtained from the two samples. They were all branched within the four~~three~~ distinct clusters of archaeal *amoA* sequences: Cluster *Nitrosopumilus*, *Nitrososphaera*, *Nitrosotalea*, *Nitrosocaldus* (Fig 5).~~cluster A with subclusters A1 to A2, cluster B, and cluster C (Fig 5). Cluster A contained 4 phylotypes (57% of total OTUs) representing 68 sequences (60% of the total sequences). This cluster had two major clades whose bootstrap confidence value was 99%.~~ *Nitrosopumilus* Cluster~~Clade A1~~ contained phylotypes SS-AOA-4 and BS-AOA-22, which branched with large numbers of sequences recovered from the sediments and water samples in the marine or fresh environments. The other clade, Cluster *Nitrososphaera*~~A2~~, has two~~2~~ phylotypes representing 44 sequences. OTU BS-AOA-62 contained 18 sequences, which was closely related to sequences from soil. The clone SS-AOA-76 clustered within clade *Nitrososphaera*~~A2~~ and showed up to 99% sequence identity to an uncultured archaeon clone GHL2_S_AOA_19 (JX488447) obtained from lake sediment.

Cluster *Nitrosotalea*~~Cluster B~~ had 1 phylotype (SS-AOA-65) with 11 sequences (12% of the total sequences). The closely related sequences in this cluster included characteristic crenarchaeotal group sequences that were obtained from alpine soil (with 98% identity). Another clone, MX_3_OCT_18 (DQ501052), from estuary sediment was 96% similar.

Cluster *Nitrosocaldus*~~Cluster C~~ contained ~~2~~ two phylotypes (BS-AOA-15 and SS-AOA-50) with 34 sequences (30% of the total sequences). They were closely related to the geothermal

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water sequences, with 95-99% similarity. Furthermore, Cluster *Nitrosocaldus*~~cluster C~~ mainly represented previously described ThAOA/HWCG III (Prosser and Nicol, 2008). Notably, the recently reported *amoA* gene sequence of "*Ca.*~~*ndidatus* *Nitrosocaldus*~~ N. yellowstonii" (EU239961) (De la Torre et al., 2008) showed 85% sequence identity to clones BS-AOA-15 and SS-AOA-50.

3.5 Quantitative PCR

The qPCR results (Fig. 2b) indicated that the abundance of the archaeal 16S rRNA gene in the two samples was similar, ranging from 1.28 to 1.96×10^7 gene copies ~~per gram~~g^{-1} of dry weight of sediments. However, the abundance of the bacterial 16S rRNA gene varied greatly, ranging from 6.86×10^6 to 4.25×10^8 gene copies g^{-1}~~per gram~~ of dry weight of sediments (~~supplementary materials, Fig~~ Fig. S2 in the Supplement). ~~The copy numbers of archaeal amoA genes in the bottom sediments (9.80×10~~5 gene copies per gram of dry weight) ~~is three times higher than those of the surface sediments (2.75×10~~5 gene copies per gram of dry ~~weight).~~ The copy numbers of archaeal *amoA* genes in the surface and bottom sediments are 2.75×10^5 and 9.80 ×10^5 gene copies g^{-1} sediment, respectively. The copy numbers of the archaeal 16S rRNA genes in the bottom sediments were significantly higher than those of the bacterial 16S rRNA genes, with a ratio of 28.57. However, in surface sediments, the ratio of bacterial 16S rRNA genes to archaeal 16S rRNA genes is 3.32.

3.6 FISH

FISH was used to analyze the relative abundance of Crenarchaea in two samples. As expected, most metabolically active Crenarchaea cells and aggregated cells were detected by FISH probes (Cren679) (Fig 3). Based on the qPCR results~~fluorescence signals~~, a high abundance of crenarchaea in the hot spring sediments harbored *amoA* genes, providing strong evidence supporting the important role of Crenarchaea in the oxidation of ammonia.

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4 Discussion

4.1 Environmental factors affecting the occurrence of ammonia-oxidizing microorganisms

Temperature is likely a very important factor influencing microbial community structure. This interpretation is supported by the results of qPCR (Fig. 2b and Fig. S2). The sediment samples from the bottom of pool (T=77 °C) are dominated by Archaea, whereas the sediment samples from the margin of pool (T=55 °C) are dominated by Bacteria. In addition, no AOB were detected in both bottom and margin samples, indicating that it might be difficult for AOB to inhabit in high-temperature hot spring environments (Lebedeva et al., 2005; Hatzenpichler et al., 2008). Additionally, the abundance of AOA *amoA* gene in bottom sediments is slightly higher than that in margin sediments, reflecting that although AOA can adapt to a wide range of temperature, higher temperature could be more favorable to the growth of AOA (de la Torre, et al., 2008; Hatzenpichler et al., 2008; Jiang et al., 2010).~~The ammonia or ammonium concentration and temperature are controlling factors of the distribution of AOA. The thermophilic AOA *amoA* genes that were retrieved from hot springs mediate ammonia oxidation at temperatures of 45 to 97 °C (de la Torre, et al., 2008; Hatzenpichler et al., 2008; Jiang et al., 2010). So far, only two enrichments of thermophilic ammonia oxidizing archaeota (i.e., *C. Nitrosocaldus yellowstonii* and *C. Nitrososphaera gargensis*) have been shown to perform nitrification at high temperatures. The Gongxiaoshe hot spring, with a high temperature of 77 °C, does not favor AOB, which is in accord with the observation that it is difficult for AOB to exist in a high temperature geothermal environment (40–55 °C) (Lebedeva et al., 2005; Hatzenpichler et al., 2008).~~

Ammonia concentration may be another factor that influences the potential activity of AOA and AOB in hot springs.~~The ammonia concentration and potential activity of AOA and AOB showed an obvious positive correlation. Martens-Habbena et al. (2009) found that the affinity of '*Candidatus Nitrosopumilus maritimus*' strain SCM1 for ammonia/ammonium was among the highest affinities reported for microbial substrates, as it exceeds that of AOB by more than 200-fold. Their study also proved the existence of oligotrophic ammonia oxidizers among thaumarchaeota. Moreover, they provided evidence for the ability of thaumarchaeota to~~

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~~compete for ammonia in oligotrophic hot spring environments (*Hatzenpichler et al., 2008).*~~ Because AMO in AOA has a much higher affinity for the substrate compared to a similar process in AOB, the ability of AOA to compete for ammonia in oligotrophic hot spring environments is also substantially higher than that of AOB (Hatzenpichler et al., 2008). In Gongxiaoshe hot spring, the ammonia concentration of 102.61 μg L^{-1} is lower compared to other hot springs with high ammonia concentrations. This relatively low ammonia concentration may possibly be responsible for the absence of AOB in Gongxiaoshe hot spring. ~~, it has been suggested that archaeal amoA genes were detected only in the Gongxiaoshe hot spring.~~

4.2 Composition and abundance of AOA

The rarefaction curves (~~supplementary materials,~~ Fig. S3) for archaeal 16S rRNA genes and *amoA* genes in the surface and bottom sediment samples reached a plateau, and their coverage values were relatively high (89-99%). This result indicated that a large part of the archaeal/*amoA* diversity at this spring was probably included in the archaeal/*amoA* clone libraries. The majority of archaeal sequences were closely related to '*Ca.* N. yellowstonii', a known AOA, which may be responsible for the oxidation of ammonia in this spring.

In this study, phylogenetic analyses of archaea *amoA* genes showed that *Candidatus* Nitrosocaldus yellowstonii dominated in both of the samples. This result also agreed with previous hot spring observations reported by Dodsworth et al., (2011b) ;and Hou et al. (2013). According to the sequences retrieved from NCBI, *Nitrosotalea and Nitrososphaera* clusters~~Cluster A2 and Cluster B~~ were closely related to the cluster soil~~,~~. One possibility is that some of the *amoA* genes obtained in this study may derive from soil AOA, particularly those sequences in cluster *Nitrosotalea* and cluster *Nitrososphaera*, which have been widely found in sediments and soils. Those AOA from soil might have evolved multiple times and have adapted to high-temperature environments.~~whereas cluster A1 was related to the cluster marine and cluster sediments published in previous studies (*Francis et al.,2005; Park et al., 2006).~~ Based on the analysis of the real-time PCR and FISH methods, our data indicate that the abundance of AOA is relatively high in both samples. The archaeal *amoA* gene copy numbers ranged from 2.75 to 9.80$\times 10^5$ per gram dry weight of sediments in this study. This is

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comparable to the abundance in other hot water springs [10^4-10^5 copies g^{-1}~~copies/g~~ (Dodsworth et al., 2011b)], but is lower than the abundance of the archaeal *amoA* gene in non-thermal environments, such as paddy rhizosphere soil [10^6-10^7 copies ~~/~~g^{-1} (Chen et al., 2008)] and marine sediments [10^7-10^8 copies g^{-1}~~copies/g~~ (Park et al., 2008)]. The bacterial *amoA* genes were not detected, indicating that~~This result indicates the absence or minority of~~ AOB is absent or is a minority in this hot spring ecosystem. A predominance of archaeal *amoA* genes versus bacterial *amoA* genes indicated that ammonia oxidation may be due to the activity of archaea in the Gongxiaoshe hot spring.

4.3 The role of AOA in the nitrification of terrestrial geothermal environments

In the surface and bottom sediments (without NH_4^+), the ammonia oxidation rates calculated from the ^{15}N-NO_3^- pool dilution data were 4.80 ± 0.2 and 5.30 ± 0.5 nmol N $g^{-1}h^{-1}$, respectively. The ammonia oxidation rates recorded in the surface sediments and bottom sediments (with NH_4^+) were 5.70 ± 0.6 and 7.10 ± 0.8 nmol N $g^{-1}h^{-1}$, respectively. Moreover, the rates reported here were comparable with those observed in the two US Great Basin (GB) hot springs [5.50–8.60 nmol N $g^{-1}h^{-1}$(Dodsworth et al., 2011b)] and in two acidic (pH = 3, T = 85 °C) Iceland hot springs [2.80-7.00 nmol NO_3^- $g^{-1}h^{-1}$ (Reigstad et al., 2008)]. However, the rates reported in this study were lower than those observed in some wetland sediments and agricultural soils [85-180 nmol N $g^{-1}h^{-1}$ (White and Reddy, 2003; Booth et al., 2005)].

~~In the experiments carried out in this study, the gross nitrification rates of the hot spring varied slightly between 4.8 to 7.1 nmol N $g^{-1}h^{-1}$.~~ The ammonia oxidation rates in bottom sediments (without NH_4^+) ~~(5.3 ± 0.5 nmol N $g^{-1}h^{-1}$)~~ were slightly higher than those observed in surface sediments (without NH_4^+) ~~(4.8 ± 0.2 nmol N $g^{-1}h^{-1}$)~~. This result agrees with the distribution of archaeal *amoA* genes~~transcripts~~, which were found to be in higher abundance in the bottom sediment than in the surface sediment. High abundance of ammonia-oxidizing archaea corresponds to high ammonia oxidation rates, ~~A weak but significant correlation was found between the abundances of the archaeal amoA and gross nitrification rates,~~ which were consistent with the results reported by Isobe et al. (2012). Compared with~~In contrast to~~ the incubation experiments~~the surface sediments'~~ unamended with NH_4^+ ~~(4.8 ± 0.2 nmol N $g^{-1}h^{-1}$)~~, the ammonia oxidation rate appeared to be stimulated after amendment with NH_4^+ (1

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M) ~~(5.7 ± 0.6 nmol N $g^{-1}h^{-1}$)~~. There are indications that the ammonia concentration is an important factor affecting the rates of nitrification (Hatzenpichler et al., 2008). ~~Similarly, the rates of ammonia oxidation in the bottom sediments were increased by the addition of NH_4^+. Moreover, the rates reported here were comparable with those observed in the two US Great Basin hot springs [5.5–8.6 nmol N $g^{-1}h^{-1}$ (Dodsworth et al., 2011b)] and in two acidic (pH = 3, T = 85 °C) Iceland hot springs [2.8–7.0 nmol NO_3^- $g^{-1}h^{-1}$ (Reigstad et al., 2008)]. However, the rates reported in this study were lower than those observed in some wetland sediments and agricultural soils [85–180 nmol N $g^{-1}h^{-1}$ (White and Reddy, 2003; Booth et al., 2005)]. Furthermore, most studies specifically mention the ammonia oxidation rates, rather than complete nitrification (ammonia oxidation plus nitrite oxidation). Those studies neither determined the occurrence of NO_2^- oxidation nor accounted for the possibility of nitrate loss by denitrification (Dodsworth et al., 2011a).~~

To understand the relationship ~~By conducting correlation analysis~~ between the ammonia oxidation~~gross nitrification~~ rates and abundances of *amoA* in the two samples, we specifically estimated the contribution of archaeal cells to nitrification. By assuming two *amoA* copies per cell (Bernander and Poplawski, 1997) and by comparing the ammonia oxidation rates with the qPCR results of AOA *amoA* per gram (however, some uncertainties of this method may still exist, with respect to the stage of cell cycle and the diversity of archaea), the cell-specific nitrification rates were estimated to be 0.410 fmol N $cell^{-1}h^{-1}$ and 0.790 fmol N $cell^{-1}h^{-1}$ in the surface and bottom sediments of the hot spring, respectively. These results are much higher than those for AOA in US hot springs [0.008-0.01 fmol N $cell^{-1}h^{-1}$ (Dodsworth et al., 2011b)]. It is interesting that although the GBS hot spring possesses higher *amoA* gene copies (3.50-3.90×10^8 gene copies g^{-1} of dry weight) and higher NH_4^+ concentration (663 μg L^{-1}), it exhibits a lower cell-specific nitrification rate than Gongxiaoshe hot spring. This may imply that both the abundance of AOA and the NH_4^+ concentration are not important factors that control the cell-specific nitrification rates in high-temperature hot spring environments. The difference in cell-specific nitrification rates between the Gongxiaoshe hot spring and the GBS hot spring may reflect the difference of AOA population structure in those two hot springs (Gubry-Rangin et al., 2011; Pester et al., 2012). In line with this AOA heterogeneity,

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cell-specific nitrification rates do not reflect the overall AOA abundance or NH_4^+ concentration in these AOA-dominated hot springs. Alves et al. (2013) reported a similar case where~~that~~ soil dominated by AOA (clade A) exhibited the lowest nitrification rates, in spite of harboring the largest AOA populations.

~~When the nitrification rates in the North Sea and Colne estuary were measured *in situ*, it was found that they had maximum values of 0.3 fmol N cell^{-1}h^{-1} (Wuchter et al., 2006) and 3.1 fmol N cell^{-1}h^{-1} (Li et al., 2014), respectively. Therefore,~~ These results also suggest the importance of cultivation studies for comparative analysis of environmentally representative AOA in a wide variety of hotsprings.~~it can be deduced that the estimated activity of AOA in the Gongxiaoshe hot spring is similar to the activity of AOA in an estuary environment. The changes in the *amoA* gene abundance were specifically associated with the high nitrification rates in the two sediment samples, thereby implying that AOA plays an important role in the nitrogen cycle of Gongxiaoshe hot water spring.~~

5 Conclusions

Combination of ^{15}N-NO_3^- pool dilution and molecular analyses demonstrate that the oxidation of ammonia by AOA occurs actively in the high-temperature Gongxiaoshe geothermal system. ~~The observation of t~~The presence of considerable *in situ* nitrification rates in the hot spring is likely due to two dominant groups ~~in the hot springs~~ that include phylotypes that are closely related to the autotrophic AOA '*Ca.* N. yellowstonii'~~.~~. The detection of archaeal *amoA* genes and the absence of AOB indicate that archaeal ammonia oxidizers, rather than AOB, ~~may~~ significantly contribute to the nitrification in the Gongxiaoshe geothermal systems. Due to the AOA heterogeneity, cell-specific nitrification rates may not reflect the overall AOA abundance or NH_4^+ concentration in the AOA-dominated hot springs. Our results shed light on the importance of AOA in driving the oxidation of ammonia in high-temperature environments, which may be ubiquitous in other terrestrial hot springs on Earth~~in the world~~.

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Table 1. FISH probes and quantitative PCR primer sets used in this study

Application	Probe/ Primer set	Specificity	sequence(5'-3')	FA(%)/ AT(℃)[a]	Reference
FISH	Cren679~~Ar ch915~~	crenarchaeotal ~~archaea~~	TTTTACCCCTTCCTTCCG~~CTGCT CCCCCGCCAATTCCT~~	35~~20~~	Labrenz M, et al. 2010~~Stahl, 1991~~
	~~Cren679~~	~~crenarchaeota~~	~~TTTTACCCCTTCCTTCCG~~	~~35~~	~~Labrenz M, et al. 201~~
qPCR	518F	Bacteria	CCAGCAGCCGCGGTAAT	57	Muyzer et al. 1993
	786R		GATTAGATACCCTGGTAG		
	344F	Archaea	ACGGGGCGCAGCAGGCGCGA	60	Øvreas et al., 1998
	518R		ATTACCGCGGCTGCTGG		
	amo196F	Archaeal	GGWGTKCCRGGRACWGCMAC	60	Treusch et al., 2005
	amo277R	amoA	CRATGAAGTCRTAHGGRTADCC		

[a]FA, Formamide; AT, Annealing Temperature

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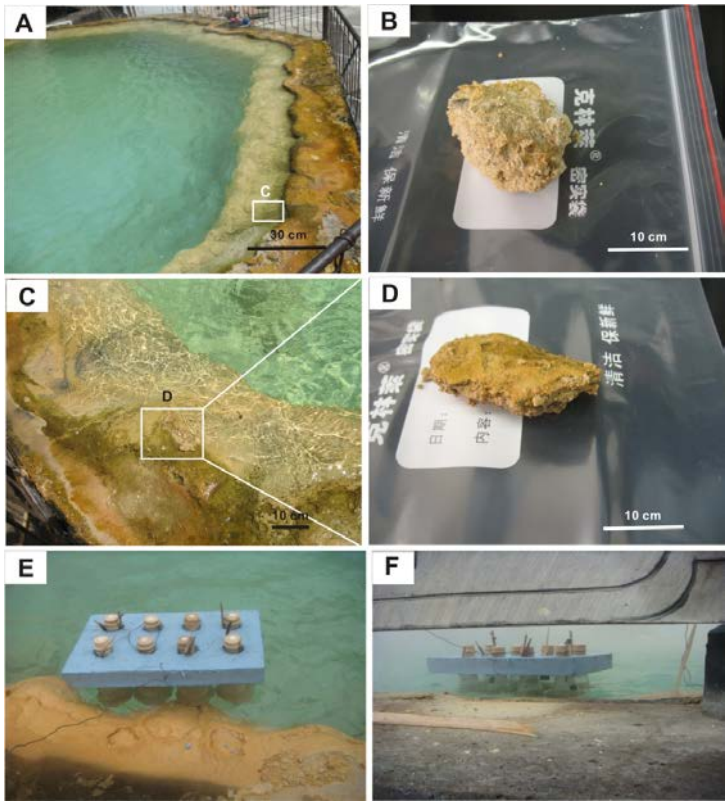

Figure 1. The Gongxiaoshe hot spring, located in the Ruidian geothermal area. ~~A~~(**a**). A full view of the spring~~,~~ ; ~~B~~(**b**). ~~Bottom~~ Bottom sediments of the hot spring, designated as BS~~,~~ ; ~~C~~(**c**). ~~An~~ An enlarged view of the white box from Fig~~ure~~ 1a~~A~~, surface sediments~~inter~~ of the hot spring~~,~~ ; ~~D~~(**d**). Surface sediments~~sinter~~ of the hot spring~~,~~ designated as-SS~~).~~); ~~E~~ and (**e, f**)~~F~~, *In situ* nitrification activity and potential nitrification activity experiments in the field.

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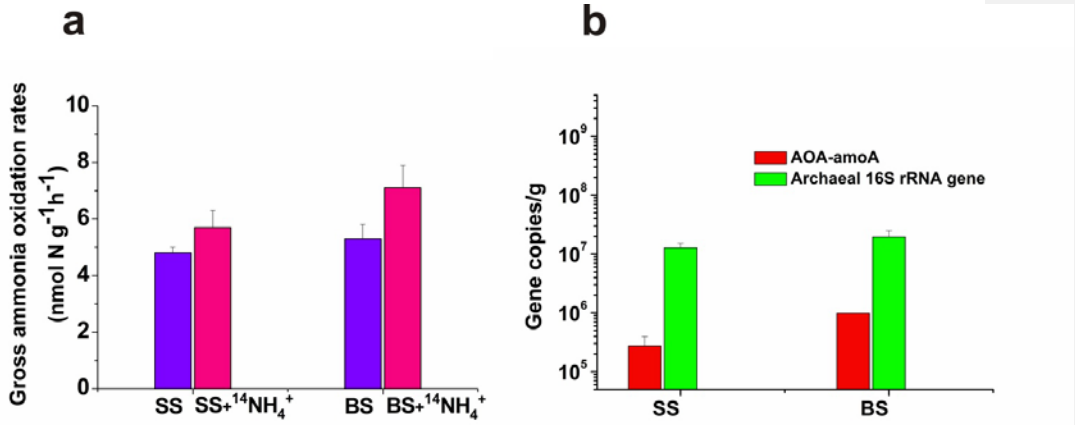

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823 **Figure 2.** ~~(~~**a**~~).~~ Gross ammonia oxidation rates calculated from $^{15}N\text{-}NO_3^-$ pool dilution

824 experiments on amended (add $^{14}NH_4^+$) or unamended SS and BS sediment slurries. It defines

825 that the amendment with "$^{15}NO_3$" represents *in situ* nitrification activity, while $^{15}NO_3$ plus

826 $^{14}NH_4$ is considered as potential nitrification activity. Bars represent the mean and standard

827 error of the mean (n = 3) for 30 and 120 min incubation.

828 ~~b~~(**b**)~~.~~ Abundance of archaeal 16S rRNA genes and archaeal amoA genes for SS and BS

829 samples collected from Gongxiaoshe hot spring. Data are expressed as gene copies per gram

830 of sediment (dry weight). Error bars represent the standard deviation of the mean (n=3).

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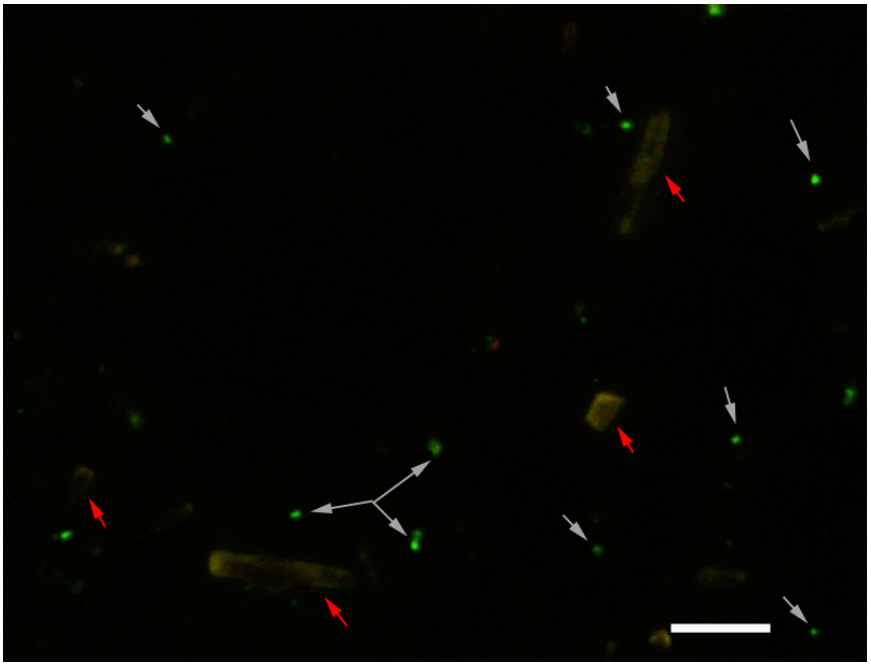

Figure 3. Epifluorescence photomicrograph of Crenarchaeota cells and cell aggregates. (White and red arrows show the cells and carbonate crystals, respectively. Scale bar corresponds to 20 μm)

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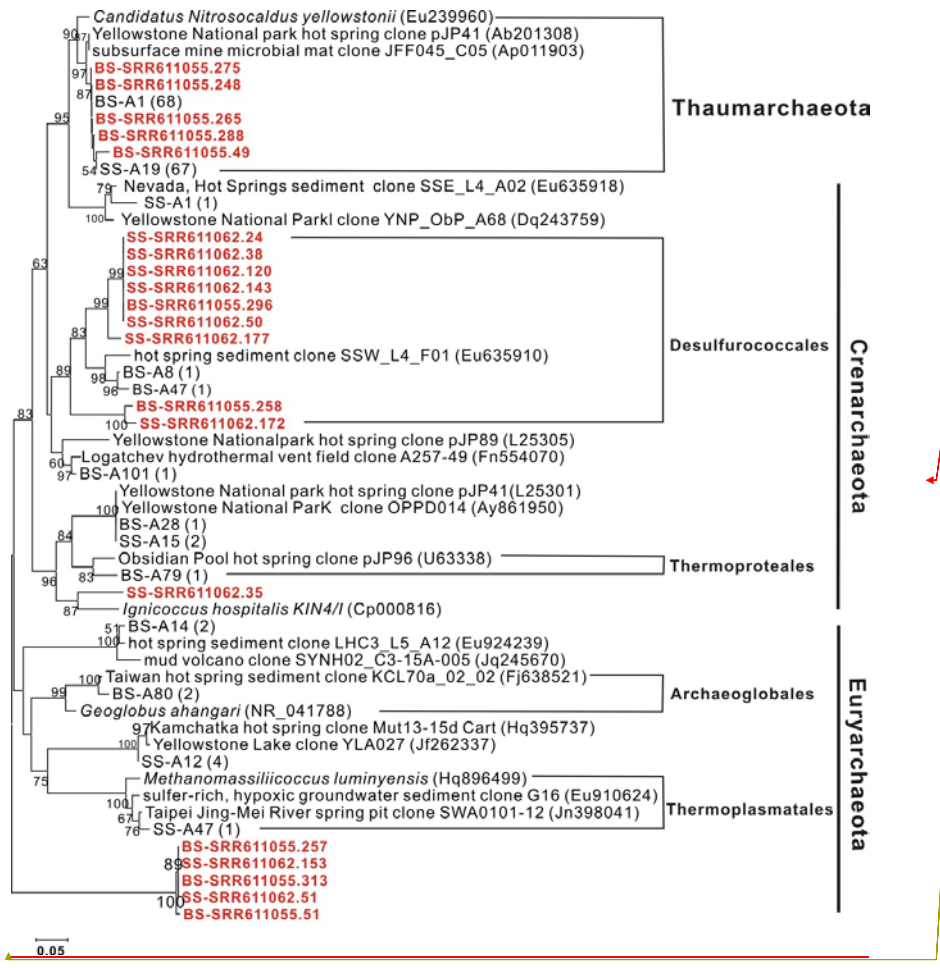

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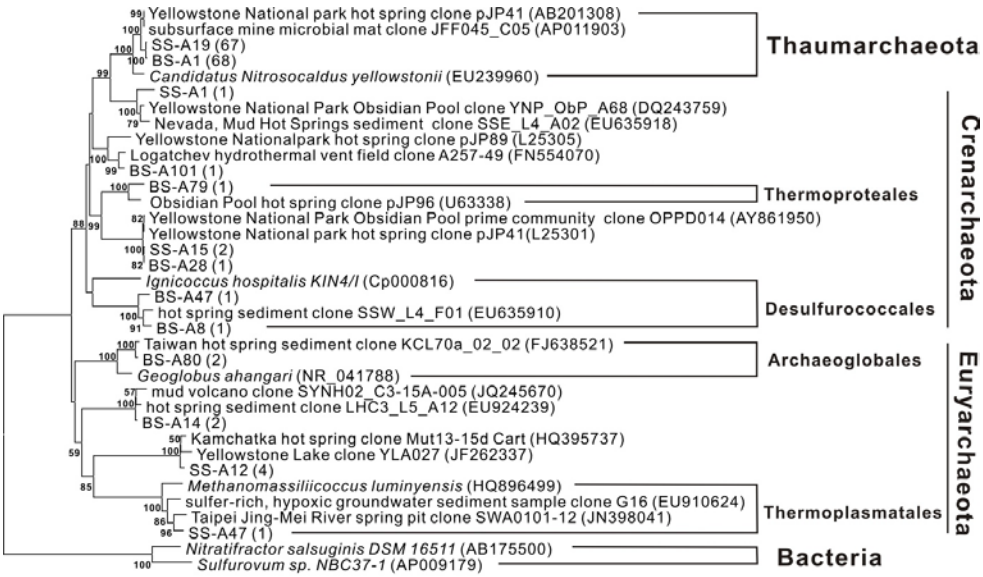

Figure 4. Archaeal phylogenetic tree based on 16S rRNA gene sequences, including various 16S rRNA gene clones obtained from the Gongxiaoshe hot spring sediments (SS and BS) and cited some sequences from Hou et al. (2013) (stained by red). The tree is constructed using the neighbor-joining method, and bootstrap confidence values over 50% (1000 replicates) are shown. The scale bar represents the expected number of changes per nucleotide position.

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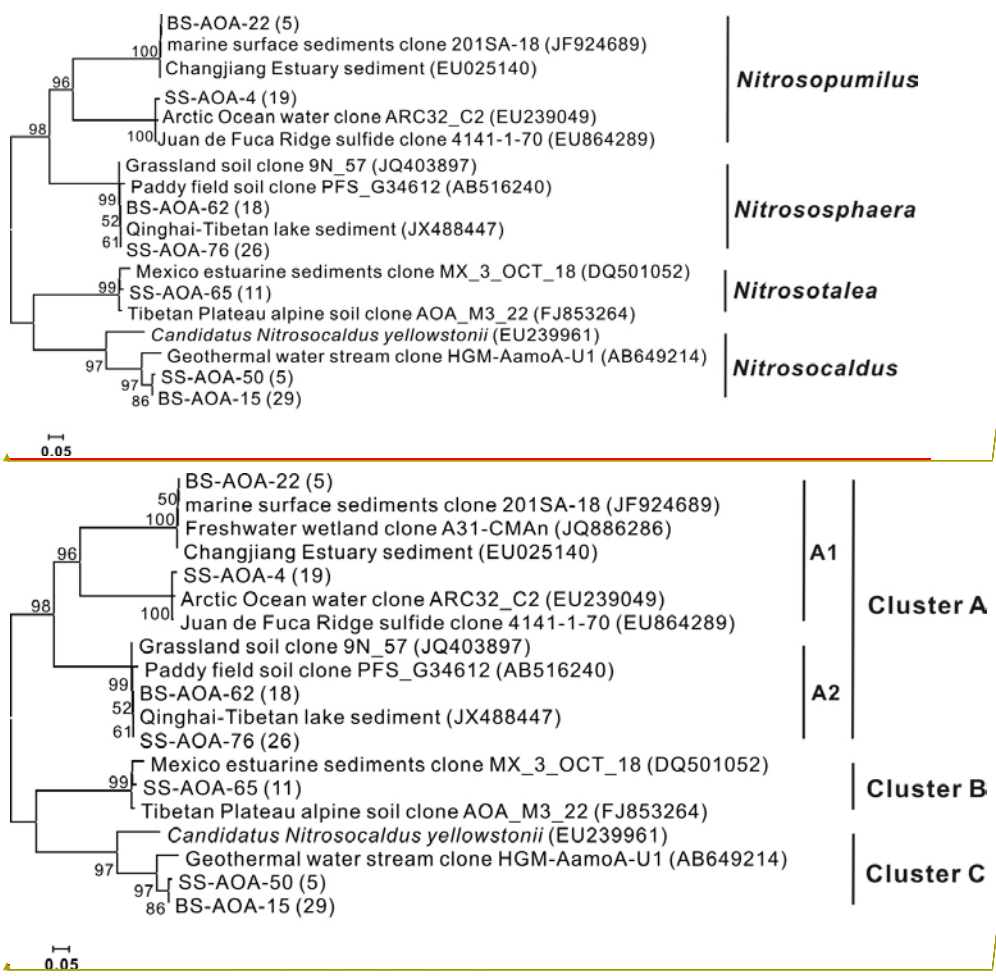

Figure 5. The phylogenetic tree of archaeal amoA genes is cloned from the Gongxiaoshe hot spring sediments (SS and BS). The tree is constructed using the neighbor-joining method, and bootstrap confidence values over 50% (1000 replicates) are shown. The scale bar represents the expected number of changes per nucleotide position.

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