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Enrichment and characteristics of ammonia-oxidizing archaea in wastewater treatment process

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Abstract: High purity ammonia-oxidizing archaea (AOA) culture containing a single AOA strain was enriched from the filtering materials of biological aerated filter. The concentration of AOA reached 3.27×10^7 copies/mL, while its proportion was 91.40%. The AOA *amoA* gene sequence belonged to *Nitrososphaera* cluster. Ammonia concentration significantly influenced the growth of AOA in culture, while total organic carbon (TOC) concentration had no obvious effect. The optimum ammonia concentration, temperature, pH and DO concentration for growth of AOA were 1 mM, 30 ˚C, 7.5 and 2.65 mg/L, respectively. Under the optimum growth conditions, the AOA abundance and ammonia oxidation rate were 3.53×10^7 copies/mL and 2.54×10^{-10} mg/(copies • d).

Keywords: ammonia-oxidizing archaea (AOA); biological aerated filter (BAF); enrichment; growth conditions; ammonia oxidation rate

1 Introduction

Nitrification played an important role in nitrogen removal from wastewater[1]. Nitrification consists of nitritation ($NH_4^+\rightarrow NO_2^-$) and nitrataion ($NO_2^-\rightarrow NO_3^-$). It had been long believed that ammonia oxidizing bacteria (AOB) was the main microorganisms involved in the nitritation process. However, the discovery of ammonia-oxidizing archaea (AOA) demonstrated that archaea could oxidize ammonia as well.

Similar to AOB, AOA owned *amoA* gene as well, which could code for ammonia monooxygenase, i.e. the key enzyme responsible for ammonia oxidation. Compared to AOB, AOA could be easier to find in various natural environments[2-3], which implied its importance in nitrogen cycle.

Although AOA distributed widely in nature, its cultivation and isolation was extremely difficult. Up till now, some AOA isolates had been obtained, such as, *Nitrosopumilus maritimus* SMC1 from aquarium[4], strains PS0 and HCA1 from coastal marine[5], *N. devanaterra* Nd1 and *Nitrosotalea* sp. Nd2 from acidophilic agricultural soil[6-7], *Nitrososphaera viennensis* EN76 from garden soil [8], *Candidatus* Nitrosocosmicus franklandus from arable soil[9]. Besides that, cultures containing a single strain of AOA as the only ammonia-oxidizer were also enriched, including "*Candidatus Nitrosocaldus yellowstonii*"[10], "*Candidatus Nitrosotalea devanaterra*"[11], "*Candidatus Nitrosoarchaeum koreensis*"[12] and so on, and most of them were enriched from natural environments. However, few studies had been conducted on the enrichment of AOA from wastewater treatment plants (WWTPs)[13].

Previous studies had confirmed the existence of AOA in wastewater treatment processes[14-18]. Nevertheless, the reported abundance, distribution and diversity of AOA varied a lot under different conditions. Most studies indicated that AOB were the dominant strains in the wastewater treatment process. Meanwhile, only a few studies showed that AOA strains outnumbered AOB strains in wastewater treatment process. Furthermore, the role of AOA in nitrogen removal in wastewater treatment process was still unclear.

Based on the known AOA *amoA* gene sequences from literatures, AOA could be divided into five major clusters: *Nitosopumilis* cluster, *Nitrososphaera* cluster, *Nitrosocaldus* cluster, *Nitrosotalea* cluster and *Nitrosophaera* sister cluster[19]. According to these studies, only a few AOA *amoA*

sequences were collected from wastewater treatment process, and most of them belonged to *Nitrososphaera* cluster.

Compared with AOB, AOA could thrive at low dissolved oxygen (DO) level and have a higher ammonia affinity[20]. Many biological reactions, such as, autotrophic nitrogen removal and denitrifying sulfide removal (DSR) process, were more adapted to the environments with low DO level[21-22]. Therefore, compared with AOB, AOA could be easier to accompany with nitrogen and sulfide removal[23-24]. Furthermore, it has been found that the biotransformation of micropollutants were involved with the cometabolism of ammonia-oxidizing microorganisms[25]. For example, *Nitrososphaera gargensis*, a kind of AOA culture, could degrade two kinds of pharmaceuticals, mianserin and ranitidine[26]. Thus AOA could play an important role in pollutants removal, and the investigation of AOA in WWTPs is necessary.

In this study, AOA contained culture was enriched, which was from the filtering materials of biological aerated filter in a WWTP. Culture growth and ammonia oxidation was investigated. Besides that, the environmental factors including ammonia concentration, pH, temperature and DO concentration on AOA in culture were also investigated. Orthogonal experiments were designed to obtain the optimum condition for the growth of AOA strain.

2 Material and methods

2.1 Sources of the samples

Filters materials was sampled from a biological aerated filter (BAF) in a full-scale municipal WWTP. (ShenZhen, China). The treatment capacity of the BAF was 50,000 m^3/d. The height of the

BAF was 7 m, while the height of the filter layer was 3.5 m. The samples were taken from seven sampling points simultaneously. The sampling points were equally distributed along the filter layer vertically. The main characteristics of influent and effluent of BAF were listed in the Table S1. The BOD, ammonia, nitrite, nitrate, total nitrogen (TN) and suspended solids (SS) concentrations were analyzed using the standard methods[27]. DO concentration and pH value were measured by DO meter (YSI-550A, YSI, USA) and pH meter (FE20, METTLER TOLEDO, Switzerland), respectively.

2.2 The preparation of initial inoculums

10 mL of the filter materials sample was mixed and transferred into a centrifuge tube and 10 mL of sterile water was added. After that, the centrifuge tube was placed on vortex mixer for 20 min to separate biofilms from filters and form the cell suspension. The cell suspension would be used as the initial inoculums for further enrichment.

2.3 Optimization of the AOA enrichment

During the experiments, different kinds of mediums and antibiotics were used to optimize the enrichment of AOA. Two types of medium (A and B) were used in experiments. Medium A was a synthetic medium as described by De la Torre et al.[10], which was the combination of the filtered mixtures of 10 mL KH_2PO_4 (0.03 mol/L), 1 mL NH_4Cl (1 mol/L), 1 mL $NaHCO_3$ (1 mol/L), 1 mL selenium tungstic acid (Table S2), 1mL vitamin mixtures (Table S3) and 1 mL trace elements (Table S4) as well as the mixtures of 1 g/L NaCl, 0.4 g/L $MgCl_2 \cdot 6H_2O$, 0.5 g/L KCl, 0.1 g/L $CaCl_2$ and 0.1 g/L KBr.

Medium B was another kind of synthetic medium, which was the combination of the filtered mixtures of 1 mL NH_4Cl (1 mol/L), 2 mL $NaHCO_3$ (1 mol/L), 1 mL sodium pyruvate (0.5 mol/L), 1 mL Fe-EDTA solution (Table S5) and 1 mL trace elements (Table S4) and the mixtures of 1 g/L NaCl, 0.4 g/L $MgCl_2 \cdot 6H_2O$, 0.5 g/L KCl, 0.1 g/L $CaCl_2$ and 0.2 g/L KH_2PO_4.

Antibiotics were used to select against the co-cultured bacteria in initial phase of enrichment process. As shown in Table S6, 7 kinds of antibiotics were prepared, and each kind of antibiotics were combined with medium A and medium B, respectively. Therefore, 14 batch tests were designed to obtain the optimum medium and antibiotics for AOA enrichment.

The pH value of mediums was adjusted to 7.0 and the concentration of ammonia was kept at 0.5-1.0 mM during the enrichment process. Enrichment cultures were incubated at 37 ℃ in dark without shaking. At the end of each cycle (12 days), the amount of AOA, AOB, archaea and eubacteria in enrichment cultures were measured by qPCR and 10% of the culture was transferred to 90 mL fresh medium for next cycle. After 3 cycles, culture was filtrated to exclude AOB and contaminated fungi before further enrichment.

2.4 DNA extraction, PCR amplification and construction of clone libraries

DNA was extracted from the frozen AOA cells harvested from the enrichment culture using Fast DNA SPIN Kit for Soil (MP, USA). The concentration of DNA was measured by a NanoDrop Spectrophotometer (ND-2000, Thermo Fisher Scientific, Germany). The *amoA* genes, Eubac gene

and Arch gene were amplified by PCR using the primers listed in Table S7.

The PCR amplification was performed using Mighty AmpTM (Takara,China) by the following procedure: 2 min initial denaturation at 98℃, followed by 35 cycles of 98℃ for 30 s, annealing at 55℃for 30 s, and 72℃ for 1 min; and final extension at 72℃ for 5 min. The PCR products were pooled by 1.2% agarose gel electrophoresis to check the presence of the target gene. After purifying with MiniBest Agarose Gel DNA Extraction Kit Ver.3.0 (Takara, Japan) and inserted into pMD®19-T Vector (Takara, Japan), ligated vectors were transformed into *Escherichia coli* DH5α competent cells. The positive clones were sent to The Beijing Genomics Institute (BGI) for sequencing. The obtained sequences sharing at least 97% *amoA* gene nucleotide identity were assigned to one operational taxonomic units (OTUs) using Mothur v.1.26.0[28]. The representative sequences were aligned to the database of the National Center for Biotechnology Information (NCBI) using BLAST. Neighbor-joining phylogenetic trees were constructed with the obtained and similar sequences using MEGA 5.1 (bootstrap value was set at 1000 replicates to estimate the reliability of phylogenetic reconstruction).

2.5 Quantification of PCR

All quantification reactions were performed with a qPCR system (LightCycler1.5, Roche Applied Science, Germany). After purification, the PCR products were ligated into pMD®19-T Vector (Takara, Japan) and transformed into *Escherichia coli* DH5α competent cells. Then the plasmid DNA, which used for making the standard curve, was extracted from the positive clones. The concentration of the plasmid DNA was determined with Nanodrop2000 and the process was

repeated above five times to ensure the accuracy of measurement. The gene copy numbers were calculated based on the detected concentration of plasmid. The standard samples were diluted for eight-gradient serial dilutions of plasmid DNA and the threshold cycle (C_t) value was determinate. Then, the standard curve between C_t value and the logarithm of the copy numbers was established. The qPCR was amplified in a 20-μL reaction mixture consisting of 10 μL SYBR Premix Ex Taq™ Ⅱ, 0.8 μL of each primer, 2 μL of template DNA, and complemented with RNase-free water. The reaction conditions were: initial denaturation at 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s, annealing for 30 s (AOA and AOB at 55℃, Archaea at 53℃and Eubacteria at 60℃), and final extension at 72℃ for 1 min. The dissolution curve was measured immediately after the PCR reaction at 65- 95°C. The results were accurate when the melting temperature (T_m) value was above 80°C and the dissolution curve was single peak. The correlation coefficients (R^2) were 0.9988 for AOA *amoA*, 0.9996 for AOB *amoA*, 0.9976 for eubacteria and 0.9982 for archaea.

2.6 Growth experiments of culture

The growth experiments were conducted in 500 mL Erlenmeyer flasks. 10 mL culture S1 (the culture with the AOA proportion of 91.40%, which will be described below) and 190 mL medium D (medium B + bran hydrolysate, which will be described below) were transferred into Erlenmeyer flasks and cultivated at 37℃. The ammonia oxidizer activity and growth were assessed by analyzing of ammonia concentrations and the abundance of AOA *amoA* genes.

2.7 Orthogonal experiments

The orthogonal experiments were carried out in 150 mL Erlenmeyer flasks. 100 mL medium D was

added into the triangle bottle. Four independent effective variables including initial ammonia concentration, temperature, pH and DO concentration, were selected. Each operating variables was sat at three levels. The ranges and levels of the variables in actual units were presented in Table 1. The abundances and ammonia oxidation rates of AOA were measured or calculated as responses. To achieve the designated DO concentration, different amount of medium were added into Erlenmeyer flasks. The concentration of ammonia was adjusted by adding NH_4Cl (1 g/L). The pH value was adjusted to the designated value by HCl (1 mol/L) or NaOH (1 mol/L).

Table 1 Experimental range and levels of the independent variables

	Ammonia concentration (mM)	Temperature (℃)	pH	DO concentration (mg/L)
Range and level	1	25	7	2.03
	2	30	7.5	2.65
	3	37	8	2.88

3 Results

3.1 Description of the BAF

The main characteristics of influent and effluent in BAF were shown in Table S1. The concentrations of BOD decreased substantially from 38.0 mg/L to 6.9 mg/L and the concentration of SS decreased from 44 mg/L to 10 mg/L, which suggested that most of the BOD and SS were removed after treatment of BAF. The concentration of ammonia was decreased from 10.6 mg/L to 3.3 mg/L and nitrate was increased from 2.7 to 9.0 mg/L. It was mainly due to that the nitrification reactions occurred in the BAF and the ammonia was mainly oxidized to nitrate. The temperature of the water was kept at 24℃ and pH decreased from 7.5 to 6.6, which might cause by nitrification in BAF. Due to the aeration from the bottom of BAF, the DO concentration increased from 0.94 mg/L

to 4.57 mg/L.

Filter samples collected from different heights of BAF filter layer were analyzed by qPCR. The abundance of AOA was ranged between 6.32×10^3 copies/ng DNA and 3.8×10^4 copies/ng DNA, the ratio of AOA/AOB varied from 845 to 2784 (Fig.S1). The results suggested that AOA were dominant strains in the filter layer of BAF. The distribution and relative abundance of AOA *amoA* genes in BAF were showed in Fig.S2. Based on AOA *amoA* genes sequences, 6 clone libraries were constructed and 8 OTUs were observed at 3% nucleotide cut-off. OTU-5 and OTU-8 were the dominant OTU types in the filter layer of BAF. OTU-8 was the dominant strain between the height of 2 m and 3.5 m and OUT-5 was dominant strain between the height of 4 m and 5 m. The phylogenetic tree of AOA *amoA* gene in BAF was shown in Fig.S3.

3.2 Establishment of highly purified AOA cultures

The cell suspension was used as the initial inoculums for AOA enrichment. Based on qPCR results, the concentrations of AOA, AOB, archaea and eubacteria in initial inoculums 4.37×10^4 copies/mL, 9.72×10^2 copies/mL, 2.21×10^7 copies/mL and 1.15×10^9 copies/mL， respectively. At the initial stage of the enrichment, different kinds of medium and antibiotics were used and compared as described in section 2.4.

The concentrations of AOA, AOB, archaea and eubacteria were measured after 3 cycles (12 days per cycles) and the results were shown in Table 2. According the results, medium B was found more favorable for AOA growth and the highest AOA proportion was 41.23% while the combined

antibiotics comprised of streptomycin, kanamycin, ampicillin, carbenicillin and tetracycline were added. The concentrations of AOA in the culture (hereafter referred to as culture F1) were 1.13×10^7copies/mL, about 258 times of the initial inoculums. Besides, AOB *amoA* genes were not detected in the culture F1.

Table 2 The effects of mediums and antibiotics on AOA enrichment

No.	Antibiotics addition	Medium	AOA (copies/mL)	AOB (copies/mL)	Archaea (copies/mL)	Eubacteria (copies/mL)	AOA proportion
1	a	A	8.35×10^4	6.38×10^4	8.31×10^5	9.25×10^6	0.83%
2	b	A	1.04×10^5	3.01×10^2	9.16×10^5	3.12×10^6	2.58%
3	c	A	1.31×10^5	1.86×10^2	6.28×10^5	1.09×10^6	7.63%
4	d	A	1.15×10^5	NA	5.91×10^5	8.98×10^5	7.72%
5	e	A	9.27×10^4	NA	5.36×10^5	8.73×10^5	6.58%
6	f	A	NA	NA	2.39×10^5	5.31×10^5	NA
7	g	A	NA	NA	2.24×10^5	4.96×10^5	NA
8	a	B	7.85×10^6	6.37×10^4	9.21×10^6	9.23×10^7	7.73%
9	b	B	9.16×10^6	4.28×10^2	1.03×10^7	4.39×10^7	16.96%
10	c	B	9.71×10^6	1.02×10^2	1.16×10^7	2.69×10^7	25.22%
11	d	B	1.13×10^7	NA	1.33×10^7	1.45×10^7	41.23%
12	e	B	8.97×10^6	NA	1.06×10^7	1.23×10^7	38.01%
13	f	B	4.16×10^4	NA	3.11×10^6	1.04×10^7	0.34%
14	g	B	3.17×10^4	NA	3.23×10^6	9.17×10^6	0.26%

NA=Not determined

Although the proportion of AOA in the culture F1 achieved by 41.23%, a lot of co-cultured microorganisms still existed. To obtain highly purified AOA culture, filters were used for further enrichment. 10 mL of the culture F1 was filtrated through filters and mixed with 90 mL medium B (antibiotics were added as well). After 3 cycles, AOA *amoA* genes could not be detected in the cultures, which implied that medium B was no longer suitable for AOA's growth. It is possible due to the fact that the co-exist microorganisms might provide some substances, which were necessary for AOA growth. To avoid the situation, vitamin solution, bran hydrolysate and sterilized domestic

sewage were selected as complement and added in medium B, respectively (named as medium C, medium D and medium E, severally). 10 mL of the cultures were filtrated and separately transferred to 90 mL medium C, D and E. After 3 cycles, the abundances of AOA in the cultures were measured and the results were shown in Table 3. No AOA *amoA* genes could be detected in the cultures with medium C, showing that vitamin was not the necessary substances for growth of AOA. The abundance of AOA in cultures with medium D achieved by 3.27×10^7 copies/mL and the proportion of AOA was reached to 91.40%. The abundance of AOA in cultures with medium E was 7.61×10^6 and the proportion of AOA was 50.3%. The results showed that medium D was more suitable for the growth of AOA.

Table 3 Effects of different mediums and pore sizes of filter for AOA enrichment

Medium	Pore size of filer (μm)	AOA (copies/mL)	Archaea (copies/mL)	Eubacteria (copies/mL)	AOA proportion
B	0.45	NA	2.05×10^6	1.92×10^6	NA
	0.8	NA	4.13×10^7	1.71×10^7	NA
C	0.45	NA	3.11×10^7	6.28×10^6	NA
	0.8	NA	8.51×10^6	7.55×10^7	NA
D	0.45	3.27×10^7	3.31×10^7	2.69×10^6	91.4%
	0.8	2.85×10^7	3.46×10^7	8.91×10^6	65.5%
E	0.45	7.61×10^6	9.03×10^6	6.11×10^6	50.3%
	0.8	5.95×10^6	1.24×10^7	1.49×10^7	21.8%

NA=Not determined

Furthermore, compared to filters with pore size of 0.8 μm, more eubacteria could be screened out by filters with pore size of 0.45 μm. The culture with the AOA proportion of 91.40% was used for further analysis, and was referred to as culture S1.

The phylogenetic tree of AOA *amoA* gene sequences of culture S1 was shown in Fig.1. According to the results, only OTU-8 was found, suggesting that culture S1 contained a single AOA. The obtained AOA *amoA* gene sequences belong to *Nitrososphaera* cluster. The OTU-8 was substantially correlated with the HZNAOA7 from Dongjiang sediment and the similarity was above 99%. Meanwhile, the AOA *amoA* genes sequences of culture S1 was found less than 95% of identity with the two isolated pure AOA strains.

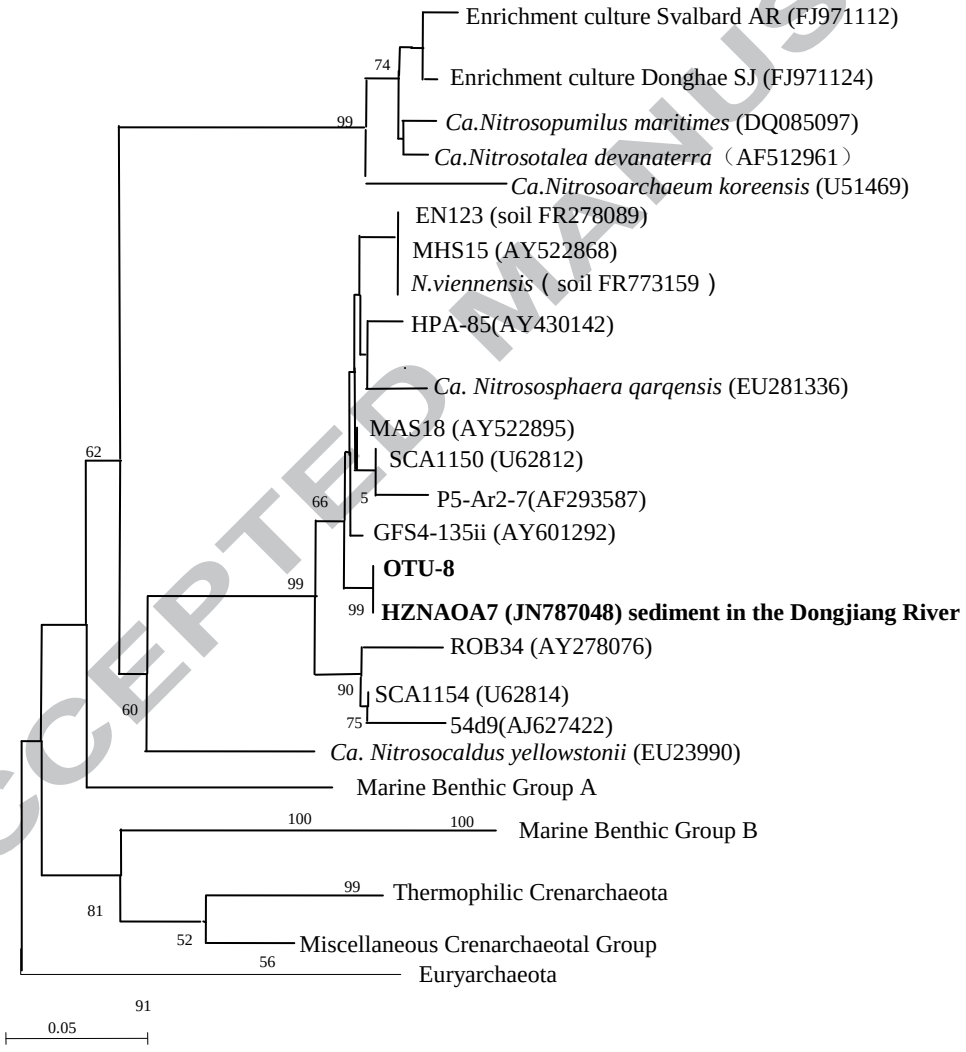


Fig.1 Phylogenetic tree of AOA *amoA* gene sequences in culture S1

The results of ammonia oxidation by culture S1 were shown in Fig.2. The growth of culture S1 was

very slow and the generation time was more than 20 hours during the tests. Ammonia was completely converted to nitrite after 12 days; the AOA abundance increased accordingly and achieved the highest amount (3.31×10^{7} copies/mL) on the 10th day. The highest ammonia oxidation rate (ammonia oxidation by per AOA in one day) of culture S1 was 2.27×10^{-10} mg/（copies·d）on the 7th day and decreased gradually with the decrease of ammonia concentration.

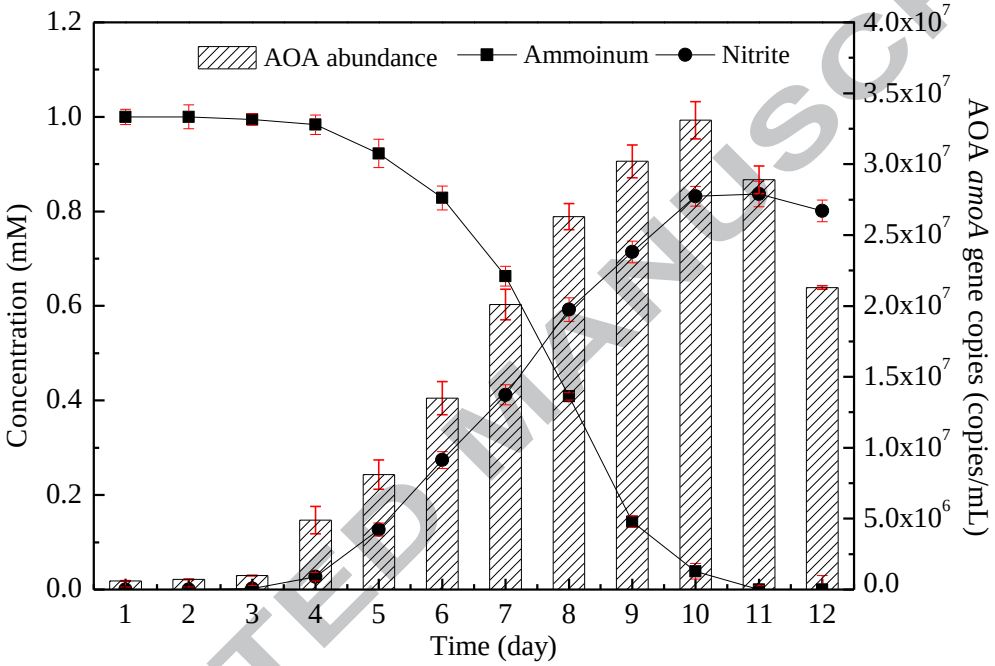
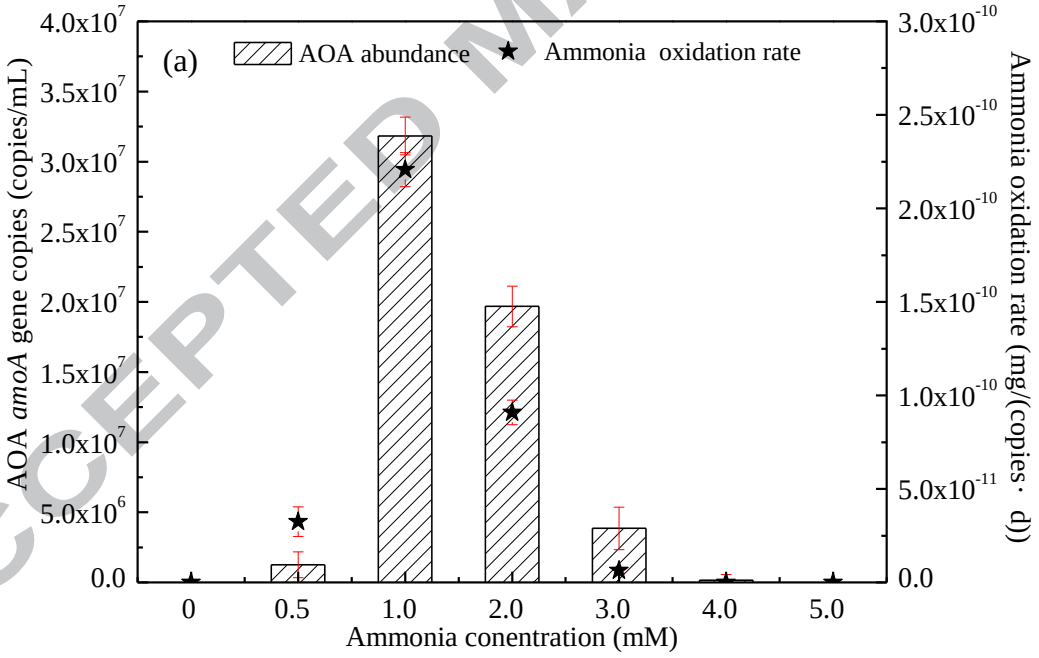


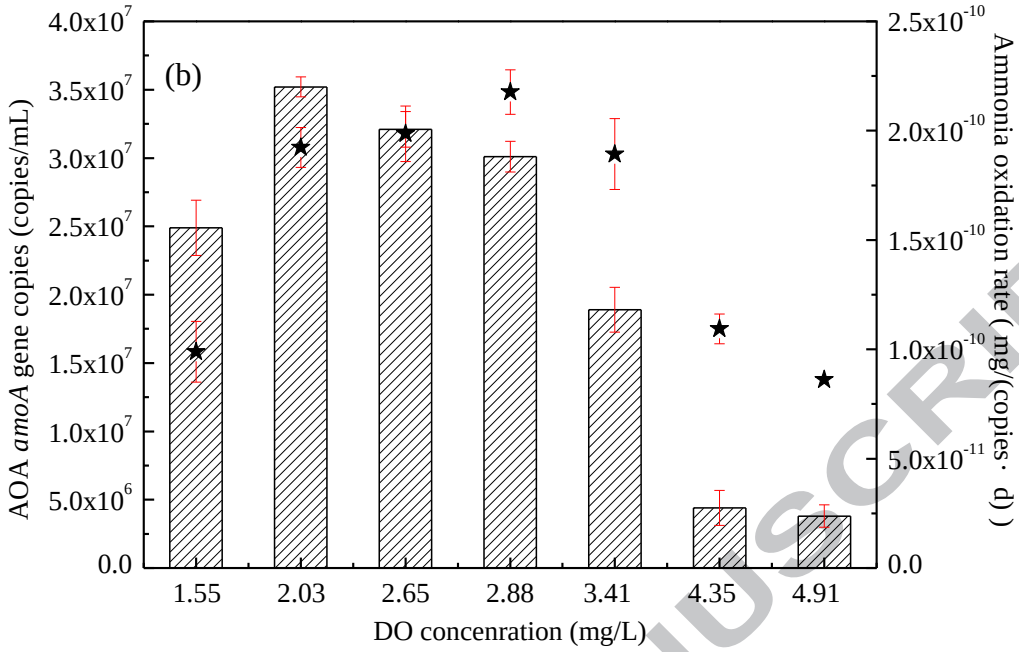
Fig.2 Ammonia oxidation by the enrichment culture S1

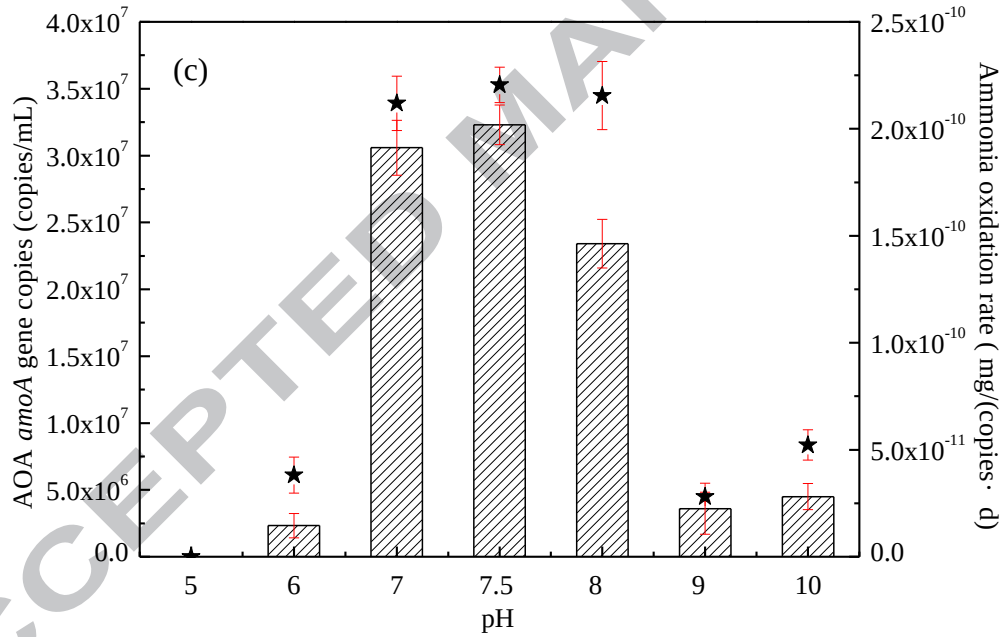
3.3 Influence of environmental factors on ammonia oxidation by culture S1

Five factors may influence ammonia oxidation by archaea including ammonia concentrations, temperature, pH, DO and total organic carbon (TOC) concentrations were assessed during the tests. For each influence factor, the abundance of AOA and ammonia oxidation rate were measured and compared, the results were shown in Fig. 3. When the initial ammonia concentration was 1 mM, the highest abundance of AOA was achieved at 3.18×10^{7} copies/mL. The growth of AOA in culture S1 was inhibited when ammonia concentration was 3 mM (see Fig. 3 (a)). For the DO

concentrations, the optimum level was 2.03 mg/L and the growth of AOA was inhibited while DO concentration was higher than 4 mg/L (see Fig. 3(b)). As shown in Fig. 3(c), the optimum pH was 7.5 for the growth of AOA, meaning that the AOA in culture S1 was mesophilic and neutrophilic. The temperature ranged from 20 to 45℃ was found suitable for the growth of AOA and the optimum temperature was 37℃ (see Fig. 3(d)). In addition, AOA in culture S1 could not survival under extreme temperature conditions. Besides, during the experiments, the AOA abundances and ammonia oxidation rates were maintained at 3.0×10^{7} copies/mL and 2.0×10^{-10} mg/（copies·d） while the TOC concentration was adjusted at 0, 19, 37, 56 and 75 mg/L by adding $C_6H_{12}O_6$ (2.5 g/L), meaning that TOC concentration might have no influence on growth of AOA.







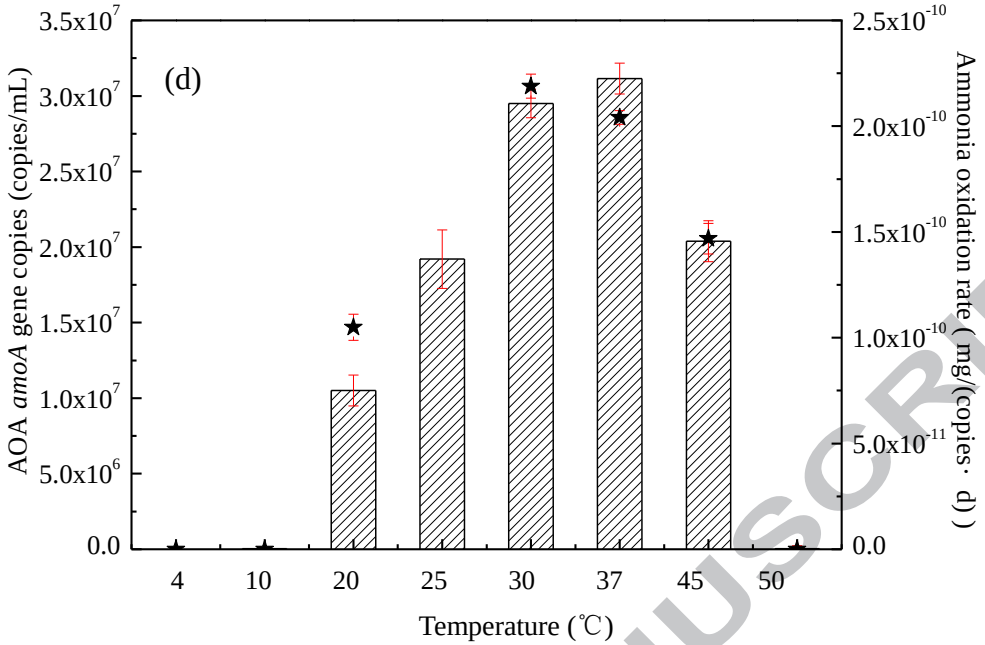


Fig.3 Effects of environmental factors on AOA's growth (a) Ammonia concentrations (b) DO concentrations (c) pH (d) Temperature

Based on the results, the orthogonal experiments were designed to obtain the optimum conditions for the growth of AOA and the results were shown in Table 4. The optimum conditions were obtained while the ammonia concentration was 1mM, temperature was 30℃, pH was 7.5 and DO concentration was 2.65 mg/L, respectively. Under the optimum condition, the AOA abundance and ammonia oxidation rate were 3.51×10^{7} copies/mL and 2.41×10^{-10} mg/（copies·d）. The order of environmental factors on target index was evaluated by range analysis (*R*). The *R* values of environmental factors were shown in Table 5. According to the results, the *R* values of ammonia concentration for AOA abundance and ammonia oxidation rate were highest (2.73 and 1.92), suggesting that ammonia concentration was the primary factor for AOA's growth.

Table 4 The results of orthogonal experiments

No.	Ammonia concentration (mM)	Temperature (℃)	pH	DO concentration (mg/L)	AOA abundance ($\times10^7$ copies/mL)	Ammonia oxidation rate ($\times10^{-10}$mg/(copies·d))
1	1	25	7	2.88	2.96	2.13
2	1	30	7.5	2.65	3.51	2.41
3	1	37	8	2.03	3.09	2.54
4	2	25	7.5	2.03	1.17	0.93
5	2	30	8	2.88	1.25	0.89
6	2	37	7	2.65	1.97	0.74
7	3	25	8	2.65	0.32	0.59
8	3	30	7	2.03	0.59	0.49
9	3	37	7.5	2.88	0.46	0.45

Table 5 Range analysis of orthogonal experiments

	Ammonia concentration	Temperature	pH	DO concentration
R of AOA abundance	2.73	0.30	0.29	0.37
R of ammonia oxidation rate	1.92	0.20	0.04	0.16

3.4 Verification experiments

According to the results of previous experiments, the optimum ammonia concentration, temperature, pH and DO concentration for AOA growth were 1 mM, 37℃, 7.5 and 2.03 mg/L, respectively. The results were different from the results obtained from orthogonal experiments. Hence, the two conditions were compared and the results were shown in Table 6. Based on the results, the optimum condition sat by orthogonal experiments (test No.2), was more suitable for culture S1 while AOA abundance and ammonia oxidation rate were 3.53×10^7copies/mL and 2.54×10^{-10} mg/d. Therefore, the optimum ammonia concentration, temperature, pH and DO concentration for culture S1 were 1 mM, 30℃, 7.5 and 2.65 mg/L, respectively.

Table 6 The results of verification experiments

No.	Ammonia concentration (mM)	Temperature (℃)	pH	DO concentration (mg/L)	AOA *amoA* gene abundance (10^7 copies/mL)	Ammonia oxidation rate (10^{-10} mg/（copies·d）)
1	1	37	7.5	2.03	3.38±0.09	2.18±0.07
2	1	30	7.5	2.65	3.53±0.11	2.54±0.16

4 Discussion

During the experiments, AOA culture S1 originated from a BAF process containing a single AOA strain affiliated with *Nitrososphaera* cluster was enriched. The AOA proportion of initial inoculums was only 0.04%. During the first enrichment stage, medium B, was found more suitable for the growth of AOA. The medium had also been used for the isolation and enrichment of AOA from soil environments (*Nitrososphaera viennensis* EN 76) and hot springs (*Nitrosocaldus yellowstonii* HL72)[8, 10]. After the first enrichment, the AOA proportion increased to 41.23%. During the second enrichment stage, it was found that the filter with the pore size of 0.45 μm was more suitable to eliminate the co-existence bacteria, which implied that the size of AOA was smaller than 0.45 μm. Most kinds of the AOA were reported as rod or coccoid archaea, with the size ranged between 0.15 to 0.95 μm. After filtration, medium B was found no longer suitable for the growth of AOA in culture. Therefore, bran hydrolysate was added in the medium, and the AOA proportion was finally achieved by 91.40% (Fig.4). This phenomenon indicated that AOA might depend on certain kind of co-existed bacteria in culture which could provide some kinds of substances with the similar function as bran hydrolysate. According to Tourna et al[8], the generation time of *Nitrososphaera viennensis* EN 76 could increase from 23 days to 2 days by the addition of pyruvate.

During the experiments, we tried to eliminate the co-cultured microorganisms and obtained the pure AOA strain. Plate screening and serial dilution method was used but eubacteria could not be thoroughly removed. We also tried to isolate it under the inversed fluorescent microscope with the manipulate arm, but the methods did not work either. After the enrichment, OTU-8 was the only OTU remained in culture S1, which was the dominate strain at the height from 2 to 3.5m in the filter layer of BAF.

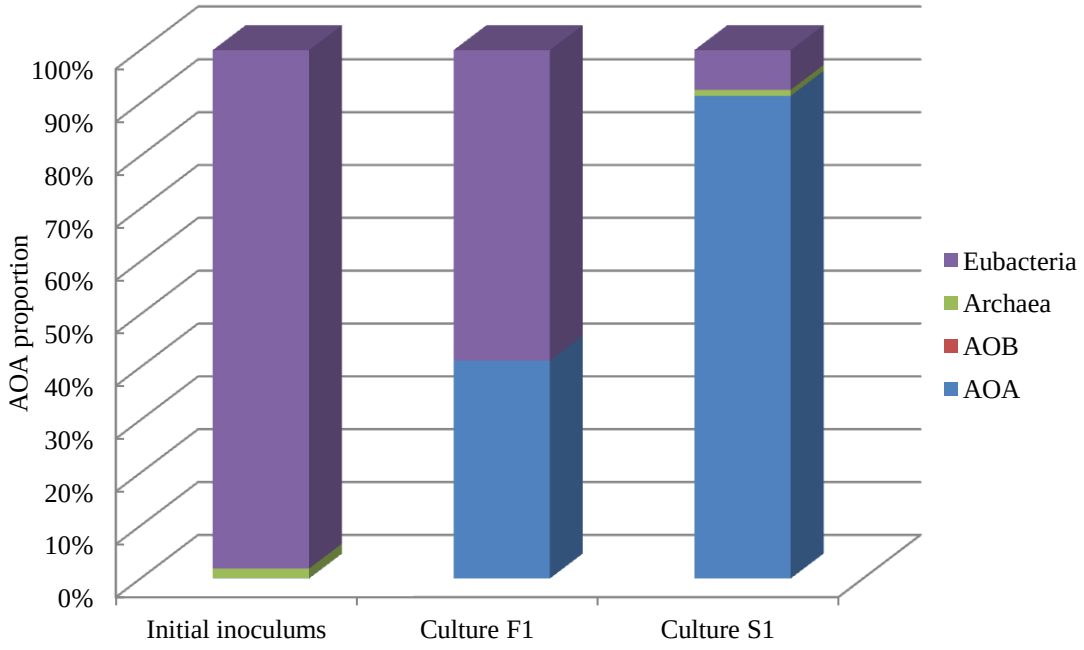


Fig.4 Comparison of the AOA proportion in different cultures

The influent was introduced from the bottom of BAF and the ammonia concentration of influent was 11.98 mg/L. The orthogonal experiments showed that optimum ammonia concentration for culture S1 was 1 mM (14 mg/L), which was agreed with the actual situation in BAF. Tolerance of ammonia concentration by culture S1 was about 4 mM. The optimum concentrations were similar to some previous works on AOA enrichment cultures, such as, culture AR (tolerance up to 4 mM) and "*Candidatus Nitrososphaera gargensis* Ga9.2" (optimum was 1 mM and tolerance was 3.1

mM)[29-30], but lower than "*nitrosoarchaeum koreensis* MY1" (tolerance up to 10 mM)[12, 31] and "*Nitrososphaera viennensis* EN76" (up to 15 mM)[8, 32]. The optimum temperature for culture S1 was 30℃ and the culture could still survive at 50℃ while it was seriously repressed. The optimum temperature for most AOA strains were ranged between 22℃ and 28℃, including "*Nitrosopumilus maritimus* SCM1" (28℃)[33], "*Nitrosoarchaeum limnia* BG20" (22℃)[34-35] and "*Nitrosotalea devanaterra* Nd1" (25℃)[11]. There were also some AOA strains, which could tolerance higher temperature. For example, the optimum temperature for "*Nitrosocaldus yellowstonli* HL72" enriched from hot spring sediment ranged from 65℃ to 72℃[10]; for "*Nitrososphaera viennensis* EN76" which was isolated from garden soil, the optimum temperature was 42℃[8]. In a word, the majority of AOA isolates and enrichment cultures are mesophilic or moderately thermophilic[36]. Previous studies have demonstrated that AOA could thrive in the environments with very low DO level and achieve higher ammonia oxidation rate than AOB[37]. "*Ca.Nitrosopumilus maritimus*" achieved maximum oxygen uptake rate at the DO concentration of 0.96 mg/L[38]. Similar results also showed that AOA performed nitrification at the maximum growth rate with the DO level of 0.64 mg/L[39]. While, controversial result was also exhibited. High abundance of AOA was found in WWTP with the DO concentration of 3.25 mg/L[40]. In this study, the optimum DO concentration for AOA in culture S1 was 2.65 mg/L. Although most AOA could grow with bicarbonate or carbon dioxide as carbon source, the addition of simple organic compounds, such as pyruvate and carboxylic acids, could also strongly accelerate growth of AOA. Thus, some AOA might have a mixotrophic metabolism[41]. However, in this study, the TOC concentration ranged from 0 to 75 mg/L by adding sodium acetate had no influence of TOC on AOA growth and ammonia oxidation.

5 Conclusions

The AOA culture S1 containing a single AOA strain was enriched from a biological aerated filter. It was found that the AOA *amoA* genes of culture S1 was achieved by 3.27×10^7 copies/mL and the AOA proportion was 91.40%. The AOA *amoA* gene sequence in culture S1 was belong to *Nitrososphaera* cluster and substantially correlated with the HZNAOA7 from Dongjiang sediment. The optimum ammonia concentration, temperature, pH value and DO concentration for AOA growth in culture S1 were 1 mM, 30℃, 7.5 and 2.65 mg/L, respectively. Under the optimum condition, the AOA abundance and ammonia oxidation rate were 3.53×10^7 copies/mL and 2.54×10^{-10} mg/(copies·d).Besides that, TOC concentration showed no influence on AOA growth.

Acknowledgments

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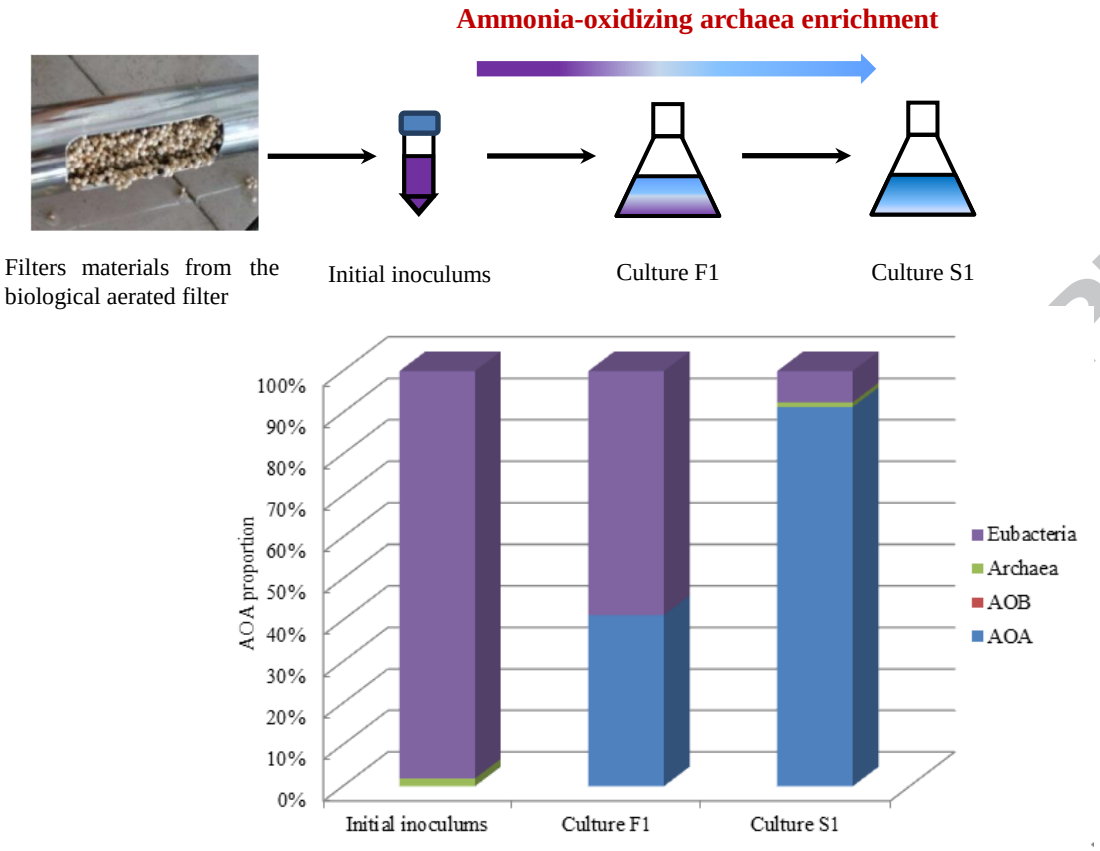
Graphical Abstract



Highlights:

- High purity AOA culture was enriched from the filtering materials of biological aerated filter.
- The AOA amoA gene sequence belonged to *Nitrososphaera* cluster.
- Ammonia concentration significantly influenced the growth of AOA in culture.
- The concentration of AOA attained 3.27×10^{7} copies/mL, while its proportion was 91.40%.