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Abundance and Diversity of Nitrifying Microorganisms in Marine Recirculating Aquaculture Systems

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Abstract: Recirculating aquaculture systems (RAS) are important for water quality management in aquaculture facilities, and can help resume water consumption. However, information about the community structure of the micro-ecosystem existing in biofilters, especially the participation of the known nitrifying groups (i.e., AOA, AOB, NOB, and comammox *Nitrospira*), remains to be fully clarified. In this research, we compared the community structures in three RAS systems operated at different temperatures in a marine aquarium, through both *amoA*-targeted qPCR assay and 16S rRNA-targeted next-generation sequencing. As result, AOA was the primary nitrifier in the biofilters and was typically abundant and diverse in high-temperature samples (ca. 25 °C). NOB's relative abundance patterns were numerically similar to that of AOA, suggesting a cooperation relationship between AOA and NOB in the marine RAS system. AOB was at a comparable level with AOA in medium-temperature samples (ca. 19 °C), while their abundance sharply decreased in high-temperature samples. The number of observed OTUs of AOA in high-temperature samples was 1.9 and 1.5 times as much as that detected in low (ca. 10 °C) and medium temperature samples respectively, suggesting a much more diverse and predominant occurrence of AOA at high temperatures. Comammox *Nitrospira* was only detected at a low level in the biofilter samples, suggesting a negligible contribution to the nitrification process in such ammonia-limited, saline biofilms. Although comammox *Nitrospira* cannot be detected by 16S rRNA-based analysis, the high diversity and abundance of NOB that were detected in high-temperature samples indicated the prospective possibility of the occurrence of complete ammonia oxidation at high temperatures.

Keywords: marine RAS; nitrification; ammonia-oxidizing archaea (AOA); ammonia-oxidizing bacteria (AOB); nitrite-oxidizing bacteria (NOB); comammox *Nitrospira*

1. Introduction

Recirculating aquaculture systems (RAS) have been widely applied in aquariums and fish farming global wide [1–3]. In RAS, water is recirculated through biofilters to remove solid waste and also purify water quality; purified water is then sent back to aquaculture tanks to reduce water consumption. Compared to conventional flow-through aquaculture systems, RAS remarkably reduce 90–99% water consumption [4,5], lower fish disease risk, and contribute to effective management [6]. On the filter surface of the RAS system, microorganisms functioning in water purification aggregate and form a biofilm with high diversity [7,8]. Among these, nitrifying microorganisms existing in the biofilm are critical to the removal of nitrogenous compounds generated from fish metabolites and feed waste. The key process is nitrification, which converts ammonium into nitrate through nitrite to reduce toxic chemical risks for aquarium organisms. Conventional nitrification
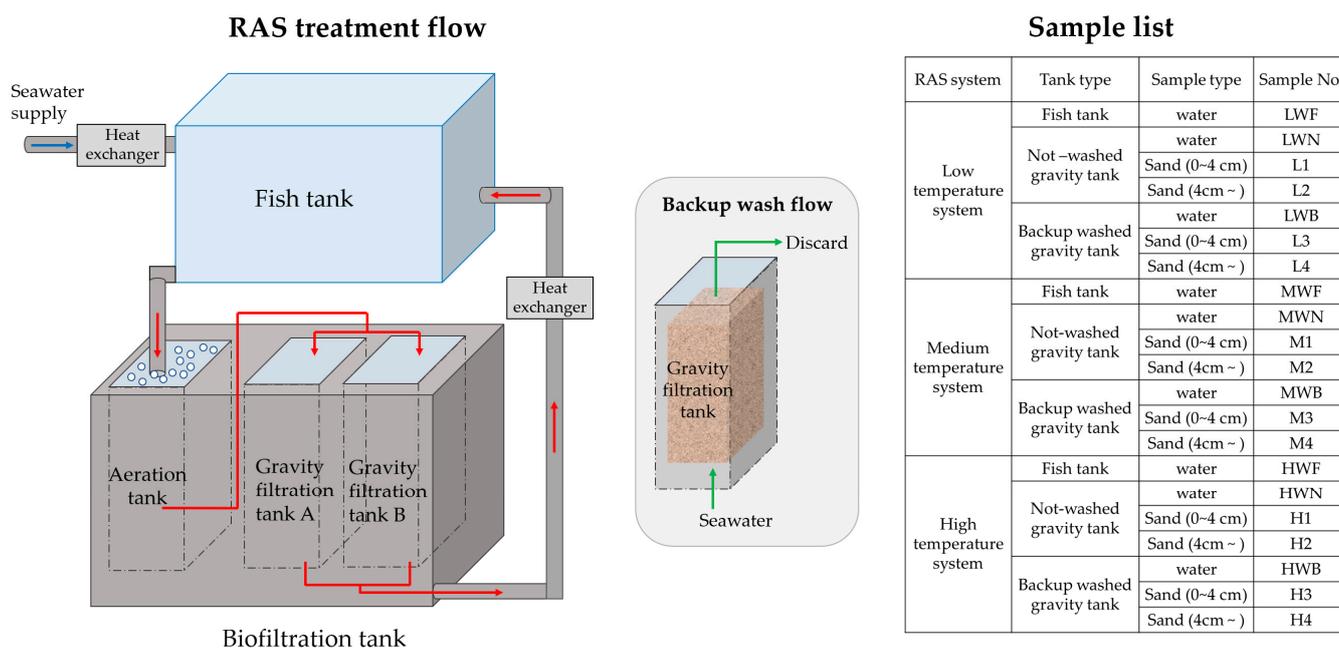
had been considered as a two-step reaction performed separately by two groups of microorganisms: ammonia oxidizers including ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) conduct the first oxidation from ammonia to nitrite [9,10], while nitrite-oxidizing bacteria (NOB) conduct the next oxidation to nitrate [11]. In addition to that, recent studies found that some *Nitrospira* (comammox *Nitrospira*) were able to independently catalyze the complete ammonia oxidation to nitrate, which largely changed the conventional understanding of nitrification [12,13]. Later, the occurrence of comammox *Nitrospira* has been reported in some municipal and natural systems, though their participation in different environments remains to be fully understood, and the key factors that drive the composition of comammox *Nitrospira* and canonical ammonia oxidizers stay unknown.

Although general microbial communities and abundances of the nitrifiers (especially AOA and AOB) have been analyzed in some RAS [14–17], we still lack information on 1. general community structure difference under different operation temperatures; 2. relative compositions and diversities of nitrifying microorganisms including the perspective of comamox *Nitrospira*; 3. the niche difference of nitrifying microorganisms and the possible factors that drive the difference. To solve the above questions, we present a comparative study of microbial communities in three RAS systems operated under different temperatures in a marine aquarium, mainly focusing on the distribution and diversity of nitrifying microorganisms (AOA, AOB, and comammox *Nitrospira*).

2. Materials and Methods

2.1. Sampling

Water samples and biofiltration sand samples were collected from a public aquarium (Aqua World Ibaraki Prefectural Oarai Aquarium, Ibaraki, Japan) on March 1st, 2021. Three RAS systems that operate under continuous thermal control at different temperatures were chosen as the research targets (water temperatures: ca. 10 °C, 19 °C, and 25 °C, respectively). According to the water temperatures, the three RAS systems were named "Low-temperature system" (ca. 10 °C), "Medium temperature system" (ca. 19 °C), and "High-temperature system" (ca. 25 °C). Each RAS system shares identical construction and flow strategy shown in Figure 1. In each RAS system, about 5 to 10% of fresh seawater is supplied to the fish tank after temperature adjustment. Used water from the fish tank is then flowed through an aeration tank and filtered through a gravity filtration tank (each filled with 1 m-depth grain of sand as filtration materials). Filtered water is sent back to the fish tank after temperature adjustment. To remove the organic suspension in the surficial sands in gravity filtration tanks, a backup wash is carried out routinely every 1 or 2 months (depending on the situation). When either one of the two gravity filtration tanks is under backup-wash treatment, the other gravity filtration tank is kept used for water filtration. For water samples, ca. 2 L of water was collected from each fish tank and gravity filtration tank in the three RAS systems, stored in 2 L PE bottles at 4 °C during transportation. Then, ca. 1 L of water samples were filtered through 0.2 μm pore-size polycarbonate filters, and the filters were saved at −30 °C for the use of DNA extraction. For the use of inorganic nutrient measurements, ca. 500 mL of water samples were filtered through GF/C glass fiber filters, and the filtrate was saved at 4 °C. For Biofilm samples, 6–10 cm from the surface to the depth of the sand sample were collected by a probe-type sediment sampler (AMP401, ISIS, Osaka, Japan) from one newly backup-washed gravity filtration tank, and one not-washed gravity filtration tank in each RAS system. The sand samples were kept at 4 °C during transportation, and then divided into two parts: the upper 4 cm depth layer (0 to 4 cm depth), and the lower layer (4 cm and deeper) and stored at −30 °C for further use. The abbreviation names of water samples and biofilm samples were listed in Figure 1.

RAS treatment flow

Sample list

RAS system	Tank type	Sample type	Sample No.
Low temperature system	Fish tank	water	LWF
	Not –washed gravity tank	water	LWN
		Sand (0~4 cm)	L1
		Sand (4cm ~)	L2
	Backup washed gravity tank	water	LWB
		Sand (0~4 cm)	L3
		Sand (4cm ~)	L4
Medium temperature system	Fish tank	water	MWF
	Not-washed gravity tank	water	MWN
		Sand (0~4 cm)	M1
		Sand (4cm ~)	M2
	Backup washed gravity tank	water	MWB
		Sand (0~4 cm)	M3
		Sand (4cm ~)	M4
High temperature system	Fish tank	water	HWF
	Not-washed gravity tank	water	HWN
		Sand (0~4 cm)	H1
		Sand (4cm ~)	H2
	Backup washed gravity tank	water	HWB
		Sand (0~4 cm)	H3
		Sand (4cm ~)	H4

Figure 1. Schematic diagram of the aquarium RAS system. Sample type and its abbreviation name (samples No.) are shown in the sample list on the right.

2.2. Water Chemistry Analysis

Physiochemical data (pH, DO, and water temperature) in each tank of the three RAS systems were measured in situ using a waterproof portable multi-water quality logger (AS810, AS ONE Corporation) during sampling. NH_4^+-N were determined by the indophenol-blue method (JIS K 0102 42.2). NO_2^--N were determined by the diazotization method (JIS K 0102 43.1.1). NO_3^--N were determined by the brucine method (JIS K 0102 43.2.4). PO_4^{3-}-P were determined by the molybdenum blue method (JIS K 0102 46.1.1). The measurements of inorganic nutrients were carried out within 36 h after sampling.

2.3. DNA Extraction and Quantitative PCR Assays

DNA was extracted from ca. 0.5 g of wet sand or ca. 1 L of seawater (filtered through 0.2μm pore-size polycarbonate filters), using DNeasy PowerBiofilm Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quantitative PCR (qPCR) assays were performed on StepOnePlusTM Real-time PCR System (Applied Biosystems, Foster City, CA, USA) equipped with StepOneTM Plus v2.3 software. The copy numbers of archaeal *amoA*, bacterial *amoA*, and comammox *amoA* in all samples were determined in triplicate on non-diluted samples using the SYBR green method, and the copy number of bacterial and archaeal 16S rRNA genes were determined by TaqMan probe method. For real-time PCR analysis, either THUNDERBIRD® SYBR® qPCR Mix or THUNDERBIRD® Probe qPCR Mix (TOYOBO corp., Osaka, Japan) was used. Primers used in this research are shown in Table 1. Thermal conditions and primer concentrations were optimized for the specificity and efficiency of the amplifications of each target gene. Efficiencies of all real-time PCR assays ranged from 90.3% to 99.8%, with R^2 values > 0.99 for standard curves. Standards for qPCR were prepared from environmental clones as described previously [18].

Table 1. Primer/probe information used in qPCR assays.

Target Gene	Primer/Probe	Sequence	Ref.
Thaumarchaeal *amoA*	amoA-for	STAATGGTCTGGCTTAGACG	[19]
	amoA-rev	GCGGCCATCCATCTGTATGT	
Beta-proteobacterial *amoA*	amoA-1F	GGGGTTTCTACTGGTGGT	[20]
	amoA-rNew	CCCCTCBGSAAAVCCTTCTTC	[21]

Table 1. *Cont.*

Target Gene	Primer/Probe	Sequence	Ref.
Comammox *Nitrospira amoA*	Ntsp-amoA162F	GGATTTCTGGNTSGATTGGA	[18]
	Ntsp-amoA359R	WAGTTNGACCACCASTACCA	
Archaeal 16S rRNA	ARCH1-1369F	CGGTGAATACGTCCCTGC	[22]
	ARCH2-1369F	CGGTGAATATGCCCCTGC	
	PROK 1541R	AAGGAGGTGATCCRGCCGCA	
	Tm1389F	CTTGTACACACCGCCCGTC	
Bacterial 16S rRNA	BACT1369F	CGGTGAATACGTTCYCGG	[22]
	PROK1492R	GGWTACCTTGTTACGACTT	
	Tm1389F	CTTGTACACACCGCCCGTC	

2.4. 16 S rRNA Amplicon Sequencing

DNA collected from each sample was amplified using the 515f and 806r primer set [23] for prokaryotes' 16S rRNA V4 region. Two 20 µL independent PCR reactions were performed using TaKaRa Ex Taq® Hot Start Version (TaKaRa Bio Inc., Shiga, Japan) at 94 °C for 2 min followed by 20 cycles of 94 °C (30 s), 50 °C (30 s), and 72 °C (30 s), and a final extension step at 72 °C for 5 min (1st PCR); 94 °C for 2 min followed by 8 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s), and a final extension step at 72 °C for 5 min (2nd PCR). All amplicons were sequenced using a Miseq system (Illumina, San Diego, CA, USA) by Fasmac (Genetic Analysis Division, Fasmac, Kanagawa, Japan). Raw reads were checked by USEARCH (ver. 8.0.1623_i186linux64) to examine the quality and remove chimera. The microbial composition was determined by Qiime (ver. 1.9.0), and sequences were clustered into operational taxonomic units (OTUs) defined by 97% similarity. The representative OTU sequences were determined by BLAST+ (ver. 2.2.29).

2.5. Nucleotide Sequence Accession Numbers

The raw reads of the 16S rRNA V4 region of all 12 samples in this research were deposited in the DDBJ Sequence Read Archive (DRA) under accession number DRA013133 (DRX320677-DRX320688, DRR331680-DRR331691).

2.6. Statistical Analysis

The student's *t*-test was performed using SPSS software ver. 26.0 (IBM corp., New York, NY, USA). Differences were considered significant at *p*-values ≤ 0.05. Bioinformatic analysis was performed using R statistical language ver. 4.1.3 (https://www.r-project.org/) (accessed on 1 March 2022) and the OmicStudio tools (https://www.omicstudio.cn/tool) (accessed on 1 June 2022).

3. Results

3.1. Water Quality in Each Tank

The results of water quality in each tank are shown in Table 2. The pH in all tanks was in the range of 7.4 to 7.7. The average dissolved oxygen (DO) in mg/L in each system was 8.93 in the low-temperature system, 7.88 in the medium-temperature system, and 6.00 in the high-temperature system. In all tanks, ammonium was detected at low levels (<10 µg/L), and nitrite was not detectable. For nitrate concentration, the highest nitrate concentration was detected in the medium-temperature system (125.88 µg/L on average), and the lowest nitrate concentration was detected in the low-temperature system (34.81 µg/L on average). In all three systems, the nitrate concentrations in fish tanks were slightly higher than in gravity filtration tanks. Low concentrations of the three forms of nitrogen compounds indicated that the biofiltration systems were operating efficiently. In all three systems, the average phosphate concentrations were 1.19 µg/L in the low-temperature system, 3.72 µg/L in the medium-temperature system, and 1.26 µg/L in the high-temperature system.

Table 2. General information on the three filtration systems in the present study. Values are shown by average $\pm$ standard deviation (n = 3).

Sample No.	Temp (°C)	pH	DO (mg/L)	NH_4^+-N (µg/L)	NO_3^- -N (µg/L)	PO_4^{3-}-P (µg/L)
LWF	10.0	7.6	8.90	2.54 ($\pm$0.68)	41.71 ($\pm$3.37)	1.07 ($\pm$0.03)
LWN	10.1	7.6	9.03	2.35 ($\pm$1.11)	32.43 ($\pm$3.79)	1.28 ($\pm$0.13)
LWB	10.1	7.6	8.87	5.07 ($\pm$1.33)	30.29 ($\pm$2.67)	1.21 ($\pm$0.19)
MWF	19.0	7.5	7.90	1.86 ($\pm$4.07)	138.50 ($\pm$15.35)	3.55 ($\pm$0.06)
MWN	18.4	7.7	8.04	6.43 ($\pm$0.09)	113.86 ($\pm$6.93)	3.84 ($\pm$0.03)
MWB	18.4	7.7	7.70	1.96 ($\pm$0.32)	125.29 ($\pm$2.77)	3.77 ($\pm$0.12)
HWB	26.8	7.4	5.28	3.32 ($\pm$0.05)	98.86 ($\pm$5.15)	1.35 ($\pm$0.17)
HWN	24.7	7.5	6.58	2.64 ($\pm$0.17)	81.71 ($\pm$8.37)	1.14 ($\pm$0.09)
HWB	24.7	7.5	6.15	3.51 ($\pm$0.80)	88.86 ($\pm$7.16)	1.28 ($\pm$0.03)

3.2. Quantification Results of Archaeal, Beta-Proteobacterial, and Comammox Nitrospira Amoa Genes

The populations of three types of nitrifiers were determined by quantification of *amoA* genes of thaumarchaeota, beta-proteobacteria, and comammox *Nitrospira* using qPCR. As the result (Figure 2), AOA was dominant to AOB and comammox *Nitrospira* in most samples with one exception in medium temperature system (i.e., M4). The abundance of AOA slightly fluctuated from 3.8×10^7 to 2.3×10^8 copies/g sand, except for one sample in the high-temperature system (i.e., 2.6×10^9 copies/g sand in H3). AOA was 5.0-fold higher than AOB in the low-temperature system, 1.8-fold higher in the medium-temperature system, and 101.0-fold higher in the high-temperature system. While the average ratio of AOA to comammox *Nitrospira* in the three systems was 543.5, 180.8, and 20,976.0 respectively. This indicated that the predominance of AOA over the other two nitrifier counterparts was greater in the high-temperature system than in the low or medium temperatures. In contrast to the preference for the high temperature of AOA, the abundance of AOB sharply decreased by one magnitude in the high-temperature system. Among the three systems, AOB was most numerically abundant in medium temperature samples (7.0×10^7 copies/g sand on average, maximum of 1.1×10^8 copies/g sand (M3)). For the comammox *Nitrospira*, its *amoA* gene was detected in all samples but with relatively low values (3.1×10^4 to 8.0×10^5 copies/g sand). Based on quantitative data by qPCR, comammox *Nitrospira* only comprised less than 0.5% of all nitrifiers in most samples except for two samples in the lower layer in the high-temperature system (i.e., H2 and H4). The numerical inferiority of comammox *Nitrospira* indicated that complete nitrification may not be a primary strategy for biofilm microorganisms in our samples.
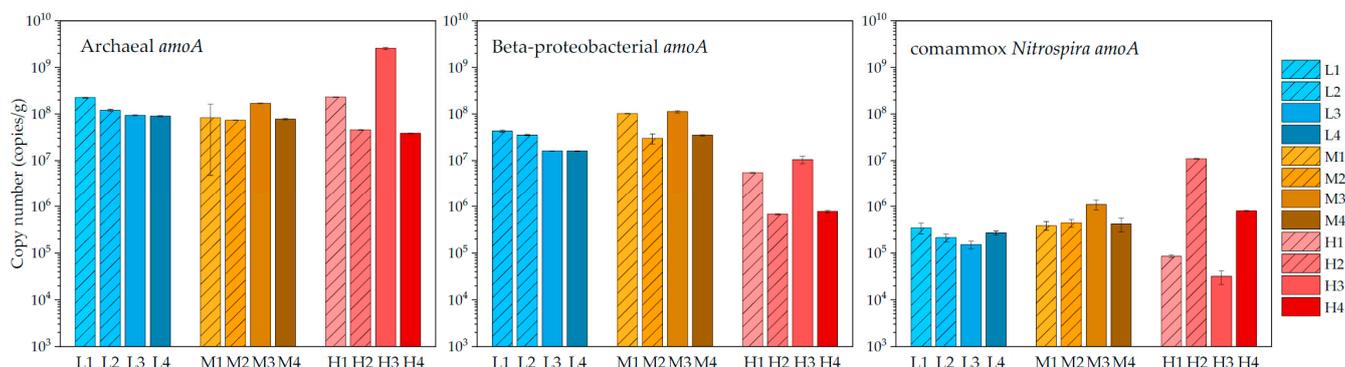
Figure 2. Quantitative result of *amoA* genes of nitrifying microorganisms biofilm samples. ((**left**): archaeal *amoA*; (**middle**): beta-proteobacterial *amoA*; (**right**): comammox *Nitrospira amoA*).

To test the significance of the difference in microbial abundances between backup-washed samples and not-washed samples (and between upper-layer samples and lower-layer samples), Student's t-tests were conducted for these two pairs of samples. As result, al-though there were numerical differences between backup-washed samples and not-washed

samples (and between upper-layer samples and lower-layer samples), the differences were not statistically significant in these two pairs of samples ($p > 0.05$).

In addition to microorganisms in the biofilm of bio-filtration systems, planktonic nitrifiers present in water may also contribute to the removal of ammonia. As result (Table 3), the average abundances of AOA, AOB, and comammox *Nitrospira* were 2.6×10^4 copies/mL water, 2.9×10^2 copies/mL water, and 1.3×10^1 copies/mL water, respectively. In accordance with the results of biofilm, AOA was also the dominant nitrifying microorganism in the planktonic phase.

Table 3. Quantitative result of *amoA* genes of nitrifying microorganisms (planktonic phase) in water samples (copies/mL). Values are shown by average $\pm$ standard deviation (n = 3).

Sample No.	AOA	AOB	Comammox *Nitrospira*
LWF	1.76×10^4 (± 659.14)	1.50×10^2 (± 23.54)	1.6×10^1 (± 2.40)
LWN	5.04×10^3 (± 49.83)	1.17×10^2 (± 19.01)	3.81×10^1 (± 7.91)
LWB	2.40×10^4 (± 678.11)	1.37×10^2 (± 3.33)	2.23×10^1 (± 0.26)
MWF	2.13×10^4 (± 747.40)	5.94×10^2 (± 86.85)	3.87×10^0 (± 1.01)
MWN	1.84×10^4 (± 260.69)	5.52×10^2 (± 23.31)	8.7×10^0 (± 0.44)
MWB	2.03×10^4 (± 253.31)	6.57×10^2 (± 12.38)	7.54×10^0 (± 0.32)
HWF	5.2×10^4 (± 1177.35)	1.82×10^2 (± 19.52)	1.18×10^1 (± 3.7)
HWN	4.17×10^4 (± 1122.59)	1.55×10^2 (± 10.35)	9.91×10^0 (± 1.08)
HWB	3.36×10^4 (± 686.65)	8.13×10^1 (± 11.69)	5.33×10^0 (± 1.56)

3.3. Microbial Communities Based on 16S rRNA V4 Region Amplicon Sequencing

A total of 991,060 reads were retrieved from 12 biofilm samples, forming 20,662 unique OTUs at 97% identity. An average of 70,978 clean reads were retrieved, and an average of 4011 OTUs were identified per sample (Table 4).

Table 4. A general summary of the results of next-generation sequencing.

	Raw Reads	Clean Reads	AOA Reads	AOB Reads	NOB Reads	OTU No.	AOA OTU No.	AOB OTU No.	NOB OTU No.
L1	69,800	59,133	1443	1220	1177	3543	36	32	17
L2	77,781	65,949	2328	2275	2645	3732	41	49	28
L3	68,202	57,731	1401	1569	1193	3513	34	36	24
L4	69,915	59,308	2089	2631	3098	3565	50	40	28
M1	87,868	74,951	4433	5026	3135	3450	60	64	25
M2	82,719	70,304	2813	3114	2756	3281	38	47	24
M3	85,189	72,402	2283	5402	1733	4011	34	60	23
M4	101,204	85,612	4569	6095	4666	4961	69	72	33
H1	96,814	77,850	8808	2368	2689	4265	87	37	49
H2	90,945	76,243	6676	2503	4300	4937	74	63	47
H3	77,410	64,298	6222	3390	4492	4412	79	70	59
H4	83,213	69,846	5273	2521	4313	4470	63	60	43

At the phylum level (Figure 3), *Proteobacteria* dominated in all 12 biofilm samples, accounting for approximately 50% of all reads. Following *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* also shared considerable proportions and predominated especially in medium-temperature samples and low-temperature samples. While in the high-temperature samples, *Thaumarchaeota* was the second predominant phylum (avg. 9.3%), followed by *Actinobacteria*, *Planctomycetes*, *Bacteroidetes*, and *Nitrospira,* with almost even ratios (avg. for those communities were between 5.5 to 6.9%). At the phylum level, although the difference between backup washed and not-washed samples can be observed in some samples (ie. the relative abundances of *Acidobacteria* and *Bacteroidetes* in medium-temperature samples), the difference in phylum communities between washed and not washed samples was not statistically significant (student's *t*-test, $p > 0.05$). The difference in phylum communities

between the upper layer and lower layer samples was not statistically significant either (student's t-test, $p > 0.05$).
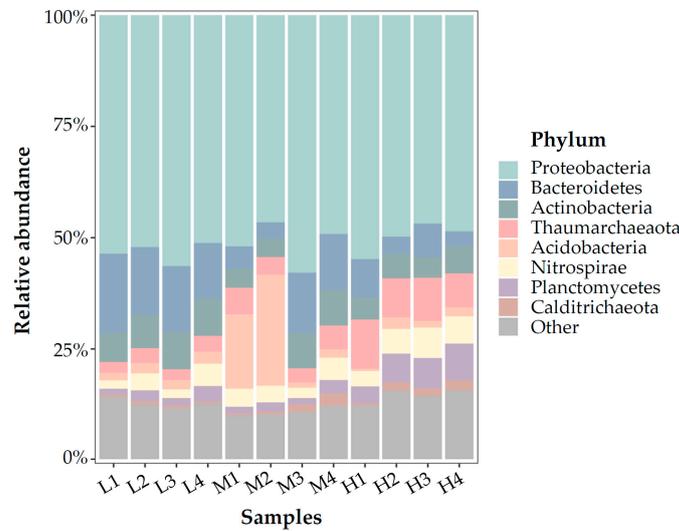

Figure 3. Taxonomic classification at the phylum level.

At the family level (Figures 4 and 5), *Woeseiaceae* (*proteobacteria*) were the most abundant phylum, accounting for 9.9% to 15.5% of all reads. Besides that, *Rhodobacteraceae*, *Nitrosopumilaceae*, *Saprospiraceae*, and *Thermoanaerobaculaceae* also shared considerable proportions. The relative abundance of *Nitrosopumilaceae* was much higher in high-temperature samples than in other samples. Similarly, we can find some other phyla which were particularly abundant in the high-temperature samples while absent or negligible in other temperatures (ie. *Pirellulaceae, Kiloniellaceae, Vibrionaceae, Nitrosococcaceae*). In contrast to that, some phyla occupied higher percentages in low or medium-temperature samples than in the high-temperature samples (i.e., PS1_clade, *Thioalkalispiraceae*). When comparing backup washed with not washed samples (or compared upper layer with lower layer), some notable differences in community structure at the family level could be found in the relative abundances of some phyla, especially in medium temperature samples. However, the student's t-test used for two pairs of samples showed no significant difference between washed and not-washed samples (nor between upper and lower layer samples).
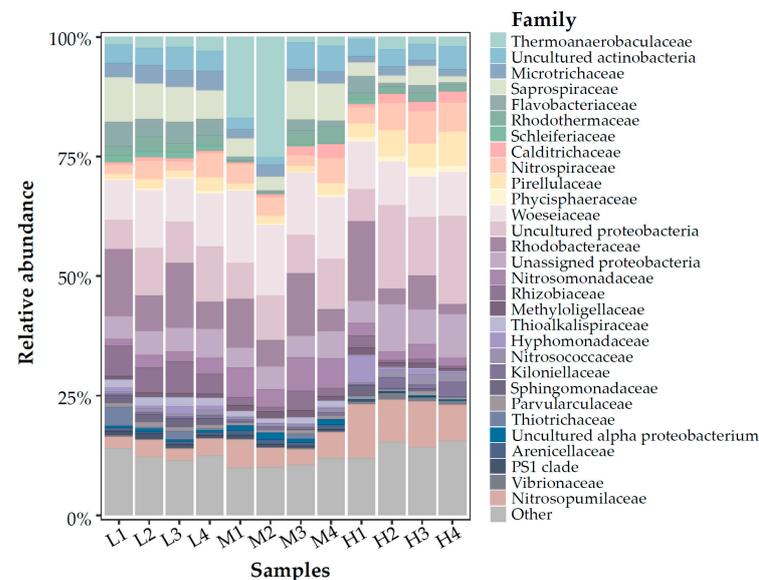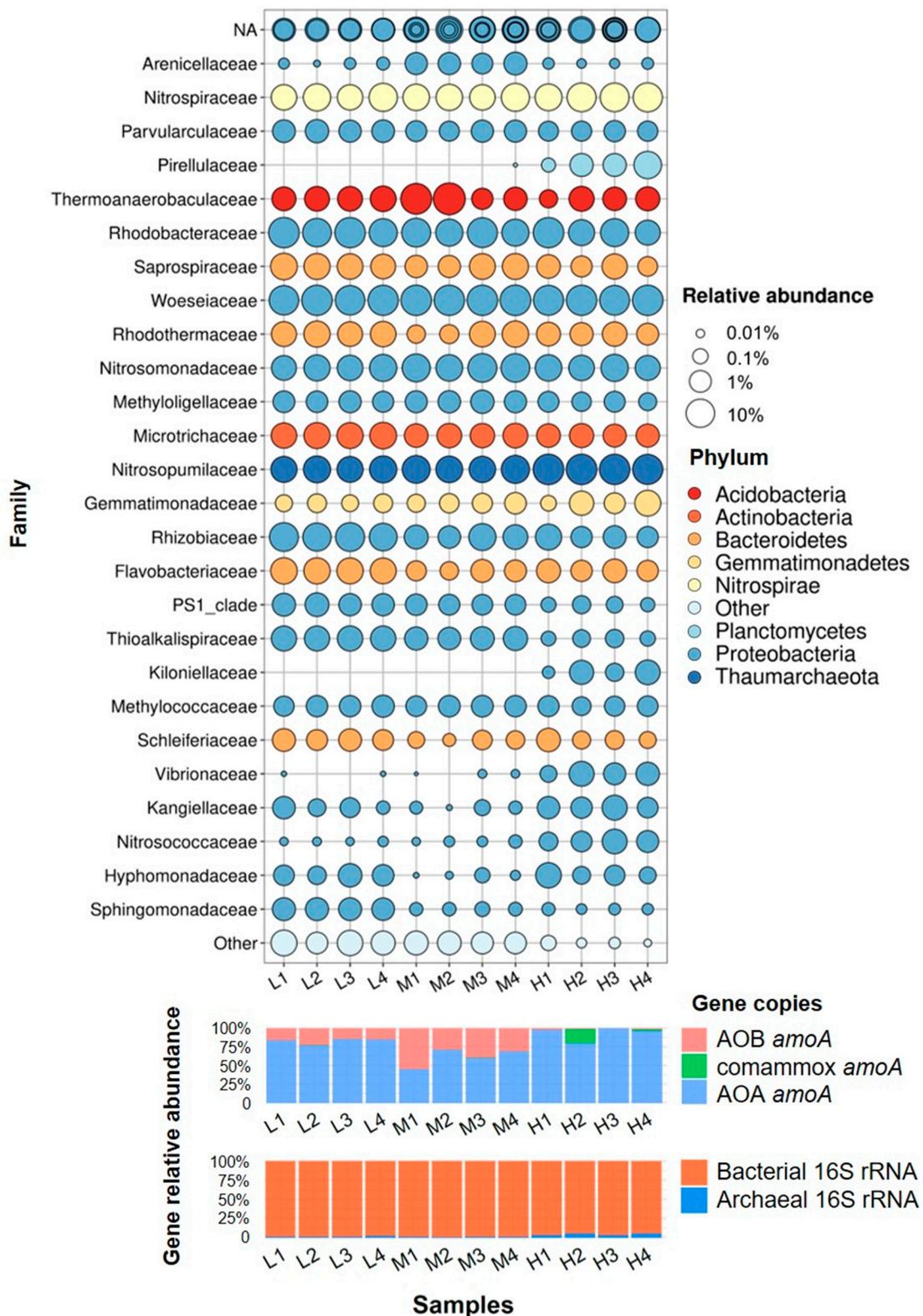

Figure 4. Taxonomic classification at the family level.

Figure 5. Relative abundance bubble plot of 16S-v4 gene profile at the family level (**up**), quantification results of *amoA* gene via qPCR (**middle**), quantification result of 16S rRNA gene via qPCR (**down**).

The reads of the known family that are involved in nitrification (ammonia oxidation and nitrite oxidation) were extracted and plotted in Figure 6 (Here, the abundance was described as the read number of each family). As known as AOA, *Nitrosopumalaceae* were the important nitrifiers in all samples and predominant in the high-temperature samples, which

is in accordance with qPCR results and relative-abundance results. Another known AOA, *Nitrososphaeraceae* was only detected in some high-temperature samples with extremely few reads. For AOB, *Nitrosomonadaceae* were the dominant AOB in all samples, and their abundances were comparable with AOA in medium-temperature samples. Another known AOB, *Nitrosococcaceae* showed slightly higher abundance in high-temperature samples than others, but they were much less abundant than *Nitrosomonadaceae* in all samples. The sum of AOB (*Nitrosomonadaceae* and *Nitrosococcaceae*) was highest in medium temperature samples (avg. 6.4%), which was in accordance with that observed by the qPCR method. For nitrite oxidizers, *Nitrospiraceae* were the dominant NOB, and their abundance in all samples was relatively even. In general, the total abundance of nitrifiers (based on amplicon sequencing reads) was higher in high-temperature samples than in low-temperature samples.
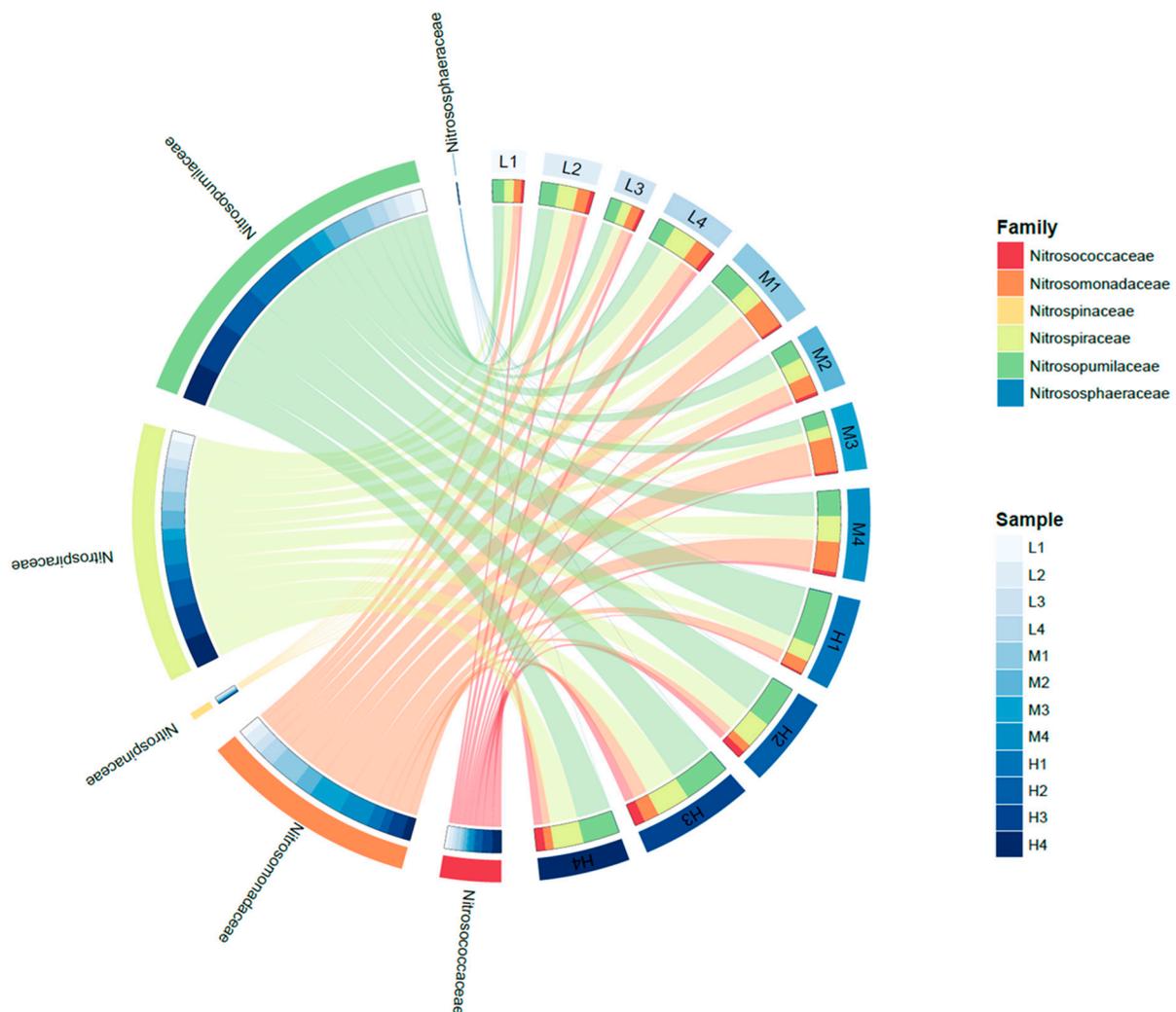

Figure 6. Circus plot of abundances of nitrifying microorganisms in all 12 samples. Abundances were represented by reading numbers of each family via 16S v4 amplicon sequencing. (AOB: *Nitrosococcaceae, Nitrosomonadaceae*; NOB: *Nitrospinaceae, Nitrospiraceae*; AOA: *Nitrosopumilaceae, Nitrososphaeraceae*).

4. Discussion

4.1. Microbial Community in Marine Aquarium Gravel Biofilm

Similar to our results, *Proteobacteria* were found to be the most predominant phylum in both marine [14,17,24,25] and freshwater RAS biofilters [8,16,26]. Proteobacteria in the RAS system is usually diverse [17,27,28], playing important roles in reactions like nitrification, anammox, and denitrification [7,8,29]. We also identified *Bacteroidetes* (AVG 9.9%), *Actinobacteria* (AVG 6.4%), *Thaumarchaeota* (AVG 5.6%), *Acidobacteria* (AVG 5.1%),

Nitrospira (AVG 4.2%), *Planctomycetes* (AVG 3.4%) and *Calditrichaeota* (AVG 1.2%) as minor-predominant phyla in our samples. Most of these phyla were also common in other RAS biofilters, while some of those results analyzed only the bacteria domain so the existence of *Thaumarchaeota* was not mentioned [14,15,17,25]. The composition of the minor-predominant phyla in the present study varied in each sample. In the low and medium-temperature systems, specific phyla showed remarkable predominance over other phyla (i.e., *Bacteroidetes* and *Actinobacteria* in the low-temperature system, *Acidobacteria*, and *Bacteroidetes* in the medium-temperature system). In contrast to this, the composition of these minor predominant phyla was more even in the high-temperature system than in others. At the OTU level, PCA analysis (Figure 7) also showed community compositions in high-temperature samples were distinct from that in low or medium-temperature samples, and the composition in medium-temperature samples may vary markedly to the sampling position in biofilters. Moreover, rarefaction curves for Chao 1 and Shannon index values (Figure 8) revealed that the high-temperature system held the highest diversity in all samples. Similar to the present results, Lee et al. (2016) [15] applied 16S rRNA amplicon sequencing to compare bacterial diversity for RAS biofilters at 15 °C, 20 °C, and 25 °C and observed lower diversity (by Shannon index) in low-temperature samples than in others. And prior to the next-generation sequencing, Urakawa et al. [30], reported sharply decreased diversities of both AOA and AOB in cold-water tanks (5.5 °C) compared to moderate-temperature tanks (ca. 19 °C) using sanger sequencing. In the present research, using next-generation sequencing, the diversity of the general microbial community and nitrifiers at three temperatures generally follows the conclusion of Urakawa et al. [30], but the diversity in low-temperature samples was not as low as predicted. In fact, some medium-temperature samples (i.e., M1 and M2) shared the lowest diversity among all samples. These result differences may be due to the different sequencing technologies, as next-generation sequencing tends to target more non-predominant species.
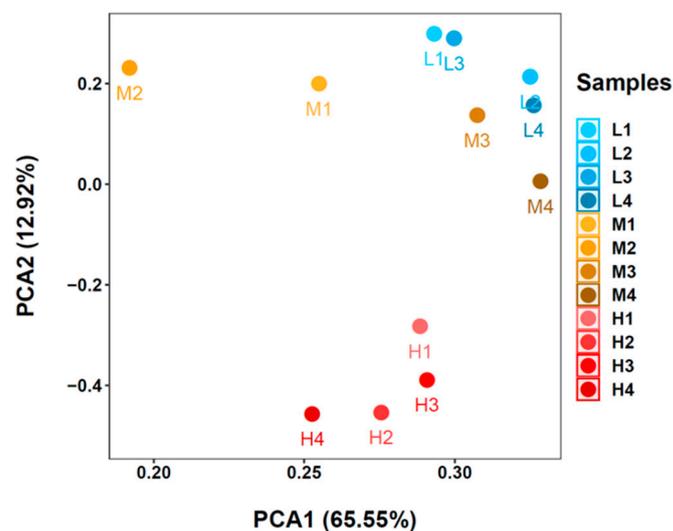
Figure 7. Principal coordinate analysis (PCA) on the composition of microbial communities at operational taxonomic units (OTU) level.

Although *Chloroflexi* was also reported as one of the most frequent phyla by its high relative abundance in many RAS systems [14,15,17,25], the relative abundance of *Chloroflexi* was low in all our samples (average value less than 1%). The absence of *Chloroflexi* in our study may be related to the filtration strategy (which is, sand gravel bed in the present study), as the above works of literature which reported considerable existence of *Chloroflexi* were generally based on strategies like MBBR (Moving bed biofilm reactor) filtration. Similar to this, Huang et al. [14] also reported *Chloroflexi*'s absence in their sand filter samples, while using the same methods they recorded abundant *Chloroflexi* in all other

biofilter samples, suggesting *Chloroflexi* may only be common in MBBR and submerged type RAS systems, but not in sand-filter RAS systems.
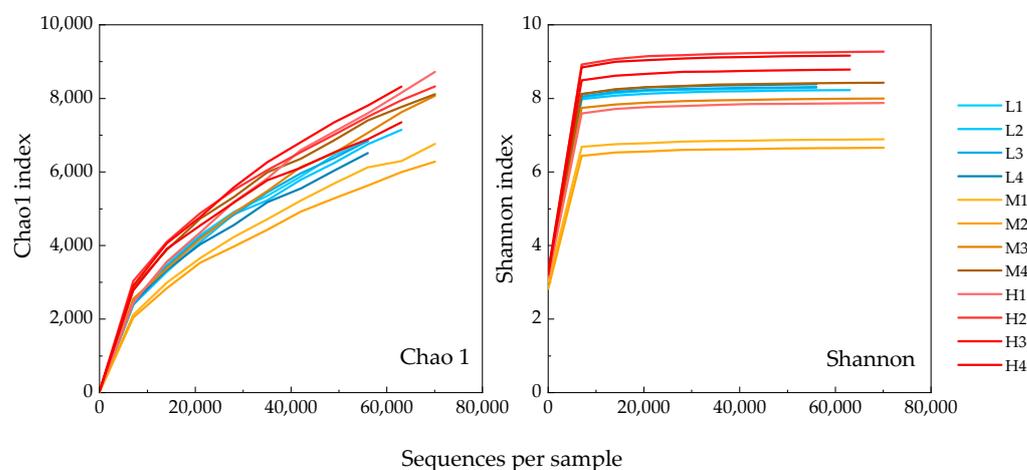
Figure 8. Rarefaction results of Chao1 and Shannon index of observed OTUs in all samples.

In addition to the filter type, community structures in RAS systems may also vary with their operational controls and sampling positions [16,28,31], suggesting each RAS system may hold a unique "microbial fingerprint". In support of this idea, differences in taxa proportions and existence were observed in samples under different operation periods (before or after backup wash), and samples at different depths (the surface 4 cm or below 4 cm depth). Although these differences were not statistically significant, our data suggested that ignoring this spatial difference in RAS systems may cause numerical deviation in microbial composition, which may lead to different conclusions when other factors were taken into consideration.

4.2. Nitrifying Microorganisms

Thaumarchaeota play an important role in the marine nitrogen cycle and contribute to ammonia removal in marine RAS systems. In the present study, we found the dominance of AOA over AOB in almost all the samples (except for M1), which is supported by both *amoA*-based qPCR assay and 16S rRNA-based NGS results. Similar to this result, the predominance of thaumarchaeal ammonia oxidizers over bacterial compartments has been broadly reported in RAS biofilters and marine environments [16,17,30,32–36]. In such ammonia-depleted environments, AOA seemingly out-compete AOB by having higher ammonia affinity than AOB [36–39]. Besides ammonia concentration, the temperature is also considered a niche segregation factor for the relative abundance of AOA and AOB. Experiments based on isolated strains showed that most AOB strains were mesophilic [40], while AOA strains seemed to prefer higher temperatures (25 °C for *Nitrosopumilus maritimus* HCA1, 26 °C for *Nitrosopumilus maritimus* PS0, 35 to 40 °C for *Candidatus Nitrososphaera* sp.JG1, 60 to 74 °C for *Candidatus Nitrosocaldus yellowstonii*) [41–43]. By quantifying *amoA*-gene in an engineered water treatment system, Lin et al. [44] found that AOA's numerical predominance over AOB was the greatest at 35 °C (Ratio AOA/AOB was 2.19×10^3), lowest at 20 °C (6.78×10^2), but still high at 10 °C (which is, 1.25×10^3). Similarly, the ratio of AOA to AOB in the present study was 101.0, 1.8, and 5.0 in the average of the high–, medium- and low-temperature systems respectively. In another research modeling soil nitrification at different temperatures (4–42 °C), Taylor et al. [45] found that the optimum temperature for AOA was 12 °C higher than for AOB. These results suggested that AOA may be more adaptive to non-moderate temperatures than AOB, and can numerically thrive at high temperatures. Prior to next-generation sequencing, using sanger sequencing on archaeal and bacterial *amoA* gene, Urakawa et al., [30] reported sharply-decreased phylogenetic diversity of both archaeal and bacterial *amoA* in cold-water biofilter samples (5 °C) compared to moderate water tanks (19.0 and 19.9 °C). Using

next-generation sequencing, the present study recorded the highest AOA diversity in high-temperature samples (OTU observed: 75.7 ± 8.7), while the AOA diversity at medium and low temperatures was at similar levels (medium: 50.3 ± 14.7, low: 40.3 ± 6.2). For AOB, diversity at high and medium temperatures was similar (medium: 60.8 ± 9.0, high: 57.5 ± 12.4), and decreased to 39.3 (±6.3) at low temperatures. Nevertheless, these results suggested that temperature might have an impact on the abundance, diversity, and relative ratio of AOA and AOB.

Following the oxidation of ammonia to nitrite, the oxidation of nitrite to nitrate is the second step in nitrification, performed by seven genera belonging to four phyla of bacteria [46]. Among these NOBs, *Nitrospinae* is particularly predominant in meso- and bathy-pelagic seawaters and sediments [47–50]; while *Nitrospirae* is the most diverse and abundant NOB in engineered systems [51,52] and is also ubiquitous in nature [46]. In the present study, *Nitrospirae* was the dominant NOB in all samples, comprising 1.9% to 6.9% of all clean reads. To be mentioned, the relative abundance of *Nitrospirae* was higher in the high-temperature system (AVG 5.5%) than in medium (AVG 3.8%) or low-temperature systems (AVG 3.2%). Moreover, there were 42.8 (±5.6) *Nitrospirae* OTUs in high-temperature samples, which is about twice as much as that in medium (21 ± 3.6) and low-temperature samples (17.3 ± 4.2). This indicated that high temperatures may be beneficial for the growth and diversity of *Nitrospirae*. In contrast to Nitrospirae, only tens to hundreds of *Nitrospinae* reads were detected, accounting for 0.1% of all clean reads in general. Genus of *Nitrobacter*, *Nitrococcus*, *Nitrotoga*, *Nitrolancea*, and *Candidatus Nitromaritima* were not detected. The abundance patterns of NOB generally followed the patterns of AOA, suggesting a cooperation relationship between AOA and NOB in the marine RAS system.

As newly recognized nitrifying bacteria, the existence of comammox *Nitrospira* has been widely reported in freshwater systems [53,54], estuaries [55,56], paddy and forest soils [57,58], drinking and wastewater treatment systems [18,59,60] and some RAS biofilters [13,16]. However, the involvement of comammox *Nitrospira* in marine environments remains unclear. Current knowledge on comammox *Nitrospira* showed it had higher ammonium affinity than most canonical ammonia oxidizers [61,62], and also tend to aggregate in densely-packed cells [63–65], and therefore comammox *Nitrospira* may have an advantage in ammonia-limited and biofilm environments [62]. Based on these characteristics, the target marine aquarium RAS system in the present study was supposed to be an appropriate habitat for comammox *Nitrospira*, as this habitat was low in ammonia concentrations, and was functioning by biofilms on the surface of gravel sands. However, our qPCR results indicated a low contribution of comammox *Nitrospira* in nitrification in the marine RAS systems. As comammox *Nitrospira* cannot be distinguished from canonical NOB by 16S rRNA gene-based methods [62], we were not able to extract comammox-like reads from next-generation sequencing results. The reason that drives the failure of comammox *Nitrospira* in the competition with AOA and AOB in the marine RAS in the present study may be salinity. Most of the above-mentioned existence of comammox *Nitrospira* was in freshwater systems. In the present study, salinity was not measured, but based on a previous report in the same area [30], salinity in Oarai seawater should be around 33 to 34 PSU. In a large-scale investigation of the distribution of comammox *Nitrospira* across several estuary tidal wetlands in China, Sun et al. [56] found the abundance of comammox *Nitrospira* was 2.21 to 5.44 fold lower than canonical ammonia oxidizers, suggesting salinity as a negative impacting factor on comammox *Nitrospira*. Similarly, another study targeting 10 locations along Yangtze Estuary revealed that both comammox *Nitrospira* and potential nitrification rates were negatively correlated to salinity ($p = −0.463$ ** and $−0.573$ **, respectively. ** $p < 0.01$) [55]. These results suggested that salinity may be an important selective factor shaping the relative composition of comammox *Nitrospira* and canonical ammonia oxidizers.

Nevertheless, the observed OTU numbers of NOB in the present study were considerable in high-temperature samples (49.5 on average), and the comammox *amoA* copy number was relatively high in some high-temperature samples (i.e., H2 and H4). As NOB

is versatile in its metabolic capability [46], and considering the rare species which may be ignored by the current clustering methods (97% similarity), there may be the possibility that some NOB at high temperatures may participate in the comammox process but are not detectable using current primers. In Liu et al.'s research [66], although comammox *Nitrospira* was found to be less abundant than AOA, its abundance exceeded AOB in 4 out of 6 sampling sites in mangrove ecosystems, which includes sites with salinity as high as 55 PSU. These results suggested that the influence of salinity may be complex as comammox *Nitrospira* may be capable to adapt to some extreme high salinity environments. Thus, although generally less significant, we suggest that the potential comammox process may minorly contribute to ammonia removal in high-temperature saline waters.

5. Conclusions

In the present study, we analyzed microbial communities in biofilters in three RAS systems in a marine aquarium using both quantitative PCR and next-generation amplicon sequencing. Both qPCR and next-generation sequencing showed AOA was the primary nitrifier, which predominance and diversity were most remarkable in high-temperature samples. AOB was relatively important in medium-temperature samples, as it shared a comparable level of abundances and diversities with AOA in these samples. The abundance pattern of NOB was numerically similar to that of AOA, suggesting a cooperation relationship between AOA and NOB in the marine RAS system. The abundance of comammox *Nitrospira* was low in all samples, suggesting a negligible contribution to nitrification in the marine RAS system. Here we suggest, that in such an ammonia-limited saline biofilm environment, AOA and NOB together performed most of the nitrification generally, while AOB shared the second importance, especially at moderate temperatures (near 19 °C). Salinity may be the main factor that drives the relative composition of canonical ammonia oxidizers and comammox *Nitrispira*, while the temperature may play a significant role in determining the diversity and abundance of AOA and AOB.

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