Enriched Microbial Communities for Ammonium and Nitrite Removal from Recirculating Aquaculture Systems

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Research

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Abstract

Background: The aim of this study was the enrichment of high-performance microbial communities in biofilters for removal of ammonium and nitrite from aquaculture water.

Methods: Ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) were enriched from different environmental water samples. The microbial communities with higher ammonium and nitrite removal activity were selected and adapted to different temperatures [9 ºC, 15 ºC, room temperature (25 ºC), and 30 ºC]. The expression of genes involved in nitrification including ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR) were measured in temperature-adapted AOB and NOB microbiomes. The microbial species present in the selected microbiomes were identified via 16s rRNA sequencing.

Results: The microbial communities containing *Nitrosomonas oligotropha* and *Nitrobacter winogradskyi* showed the highest ammonium and nitrite removal activity at all temperatures used for adaptation. Furthermore, the microbial communities do not contain any pathogenic bacteria. They also exhibited the highest expression of AMO and NXR genes. Using the enriched microbial communities, we achieved a 288% and 181% improvement in ammonium and nitrite removal over the commonly used communities in biofilters at 9 °C, respectively.

Conclusions: These results suggest that the selected microbiomes allowed for a significant improvement of water quality in a recirculating aquaculture system (RAS).

Background

Water consumption is very high in aquaculture, thus strategies for reusing aquaculture water have a high priority. The basic prerequisite for reusing aquaculture waters is a nitrification treatment [1, 2]. This process involves a two-step reaction, often catalyzed by aerobic autotrophic microorganisms using carbon dioxide as an energy source [3, 4]. At first, ammonia-oxidizing microorganisms (AOM) oxidize ammonia to hydroxylamine (NH_2OH), using the enzyme called ammonia monooxygenase (AMO). This process will be completed by the enzyme called hydroxylamine oxidoreductase, which produces nitrite. In the next step, nitrite-oxidizing bacteria (NOB) will oxidize nitrite to nitrate (NO^{3-}), using the enzyme called nitrite oxidoreductase (NOR or NXR) [5, 6].

Continuous removal of ammonium and nitrite is also required in recirculating aquaculture systems (RAS), and this is achieved by biofilters. Biofilters for water quality improvement are separated from the fish breeding part of RAS [7] and their activity critically depends on the microbial community they contain [8]. The activity of AOB and NOB in biofilter communities decreases with temperature reduction [9–11]. The removal of harmful compounds is optimal at 25°C and can be compromised at lower temperatures (below 15°C) [9, 12–14]. In order for ammonium and nitrite removal to function at lower temperatures, the AOB and NOB must adapt their metabolic activity [15, 16]. Rainbow trout is a species living optimally in cold water (9–15°C) rearing systems [2, 10]. Therefore, microbial adaptation to lower temperatures is necessary in biofilters used in aquatic rearing systems for rainbow trout [17].

Biofilters systems commonly do not operate at maximum efficiency, because the microbial communities present in biofilter systems are natural, and they are rarely supplemented with specific species or engineered [18]. While the natural microbial communities in biofilters can reduce pollutants such as ammonium or nitrite, they can also be destroyed by chemicals and drugs, such as antibiotics present in RAS [19]. Therefore, maintaining the communities of autotrophic AOB and NOB is essential in a RAS system. Bio-augmentation or microbial enrichment of such microbial communities could be used as an effective strategy for ensuring productive water treatment [20].

Microbial enrichment is a procedure for increasing the percentage of a specific microorganism group in the community [21]. This process is usually performed by providing the nutrients and optimal conditions for the desired microorganisms, thus allowing them to outcompete other species [22]. The aim of this study was the enrichment of autotrophic AOB and NOB species adapted to low temperatures in biofilter communities used in rainbow trout RAS. Considering the key role of these microorganisms in the nitrification process in biofilter systems, we argue that the adapted and enriched nitrifying microbial community presented in this study could play an important role in the treatment of aquaculture water at low temperatures.

Material And Methods

Sampling

For AOB and NOB microbiome enrichment, water samples were collected from different water sources in Gothenburg, Sweden artificial lake [57°41'02.6"N 11°56'50.0"E] (SDL), river [57°41'49.4"N 11°55'04.5"E] (GR), and natural lake [57°40'42.4"N 12°03'25.7"E 57°40'42.4"N 12°03'25.7"E] (DL). Additionally, two wastewater samples with high concentration of ammonium (WW1) and nitrite (WW2) were also utilized.

AOB and NOB enrichment

3 ml of water samples (SDL, GR, DL, WW1 and WW2) were inoculated in 47 ml of AOB liquid medium with high concentration of ammonium (containing 279 mg/ml ammonium, pH 7.2 [23, 24]) and 47 ml of NOB liquid medium with high concentration of nitrite (containing 427 mg/ml nitrite and pH 8.4–8.6 [25, 26]). Samples were grown in 100 ml sterile flasks at room temperature (RT) at 170 rpm (all flasks were covered by aluminum foil). To continuously supply the alkalinity for the optimal growth of AOB and NOB bacteria, bromothymol blue was added to the medium [27] to monitor the color change corresponding to pH. Thus, when the medium color turned to yellow, the pH was adjusted.

Samples were transferred to a fresh culture medium after 80% consumption of ammonium or nitrite (approximately after 2 weeks). We have repeated this procedure three times in a consecutive manner. Next, the samples with the highest ammonium (WW1 and GR) and nitrite removal (SDL and WW2) activity were selected after 2 weeks.

Microbiome identification by 16S rRNA **sequencing**

The genomic DNA of selected microbiomes was extracted using the Ultra clean Qiagen kit according to the manufacturer's protocol. The 16S rRNA fragments were amplified using a thermocycler (c1000 touch

thermal cycler, BioRad, USA) after preparation with primstar PCR kit, using the following primers: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-GGT TAC CTT GTT ACG ACT T-3' (Table 1). The PCR amplified products were purified using GeneJET PCR Purification Kit (Thermo Scientific). The size and quality of 16S rRNA fragments (expected size 1.5 kb) were checked by agarose gel electrophoresis (Supplemental Fig. 1). The amplified 16S rRNA fragments of selected microbiomes were sequenced at the Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark. 2 × 300 bp pooling library samples endured paired-end sequencing production until ≈ 40000 paired-end reads. The data were analyzed using the ezbiocloud bioinformatics platform (bacterial and archaeal community analysis, https://www.ezbiocloud.net/) [28].

Table 1
Primers and PCR conditions for amplification of 16S rRNA in enriched microbiomes

Primer	Sequence	PCR conditions						
		Denaturation		Annealing		Extension		Number of cycles
		Temperature (°C)	Time (s)	Temperature (°C)	Time (s)	Temperature (°C)	Time (s)	
s27F	5'-AGA GTT TGA TCC TGG CTC AG-3'	95	30	50	30	72	60	27
1492R	5'-GGT TAC CTT GTT ACG ACT T-3'							

Temperature adaptation

3 ml of each selected sample was transferred to 250 ml flasks containing 97 ml of either AOB or NOB culture medium. Samples were grown at different temperatures: 9°C, 15°C, RT, and 30°C. The ammonium and nitrite concentrations in the culture samples were determined weekly. When nearby 80% of the ammonium and nitrite were consumed, 3 ml of the culture was transferred to a fresh either AOB or NOB culture medium. It was repeated three times for a period of one and half months [23, 24].

Quantitative PCR (q-PCR)

Specific q-PCR primers for AMO and NXR genes were designed for AOB (*Nitrosmonas oligotropha*) and NOB (*Nitrobacter winogradskyi*) group strains, respectively. Furthermore, a constitutively expressed gene (16S rRNA gene) was used for selected strains as an internal reference (Supplementary Table S1).

RNA was extracted from microbial community samples adapted to different temperatures and stored at -80°C. cDNA was synthesized from the isolated RNA. The expression of AMO and NXR genes was analyzed by q-PCR according to Rahimi et al 2020 [29]. The relative values of gene expression were assessed using Agilent Technologies Stratagene Mx30005P and were calculated according to the manufacturer's instructions [21].

SEM Microscopy analysis

For SEM analysis, 15 ml of microbial cultures were centrifuged at 6000 rpm for 3 min. The microbial cells were then fixed overnight using 3% of glutaraldehyde. The fixed cells were dehydrated using graded series of ethanol (40%, 50%, 60%, 70%, 80%, 90%, and 100%) for 10 min each. Thin films were prepared by using dehydrated samples on cover glass and dried for 24 h at RT. The dried samples were then sputter coated with gold (5 nm) before imaging. SEM imaging was performed with JEOL JSM 6301F (Carl Zeiss AG, Jena, Germany).

Ammonium and nitrite measurments in biofilter water in the presence of AOB-GR and NOB-WW2 microbiomes

Water from a RAS trout biofilter system was obtained from the Department of Environmental Sciences, University of Gothenburg, Sweden. Ammonium and nitrite concentrations were increased by addition of ammonium sulphate and sodium nitrite stock solutions to the biofilter water. 3 ml of the AOB-GR microbiome (9°C- and 15°C-adapted) were mixed with 47 ml low ammonium concentration water (biofilter water) (0.5 mg/ml), 47 ml medium ammonium concentration water (5 mg/ml), and 47 ml high ammonium concentration water (35 mg/ml). 3 ml of the NOB-WW2 microbiome (9°C- and 15°C-adapted) were mixed with 47 ml low nitrite concentration water (0.4 mg/ml) (biofilter water), 47 ml medium nitrite concentration water (4 mg/ml), and 47 ml high nitrite concentration water (8 mg/ml). In a refrigerated incubator, 3 replicates of each mixture were grown at 9°C and 15°C at 170 rpm. To evaluate the effect of selected microbiomes and comparing their performance in a natural RAS biofilter water, negative control groups was used that include high, medium, and low ammonium/nitrite concentration waters without adding the selected microbiomes.

Statistical Analysis

The normality of data was evaluated by the Kolmogorov-Smirnov analysis test. One way ANOVA was used for comparing data means. The significance level among different treatments was determined by the Tukey test, at 5% level. Statistical analysis was performed by SPSS 17 software and the graphs were drawn by Microsoft excel 2019.

Results

The environmental water samples enriched with AOB and NOB microbial communities significantly remove ammonium and nitrite

Different water sources have different limnological conditions (from eutrophic to oligotrophic) that support growth of different bacterial species [30, 31]. Therefore, to achieve microbial communities with the highest ammonium and nitrite removal activity, different water sources in Gothenburg, Sweden, were selected. These included an artificial lake (SDL), a river (GR), a natural lake (DL), and two different types of wastewater (WW1 and WW2). The ammonium, nitrite, and nitrate concentrations, pH and temperature indices were measured in the samples collected from these water sources (Fig. 1a). When anaerobically treated sewage sludge is dewatered, reject water is generated that contains high concentrations of ammonium [32]. The

concentration of ammonium and nitrate was highest in raw reject water sample which we refer to as WW1. We also used the reject water treated with nitritation (ammonia oxidation to nitrite) and anammox [33], which we refer to as WW2 that contains high concentration of nitrite (Fig. 1a). By contrast, the lowest concentrations of ammonium, nitrite and nitrate were related to DL, SDL, and DL samples, respectively.

Our results indicated that the ammonium removal and nitrite production activities were high in GR, WW1, and SDL samples (Fig. 1b), whereas the WW2 and DL exhibited higher nitrite removal and nitrate production (Fig. 1c). Considering that the selected microbial communities were related to the municipal sewage, river, and lakes, the possibility of enriching autotrophic AOB and NOB bacteria from the selected sources seemed logical [34–36]. Therefore, GR, WW1, and SDL were selected for further experiments as AOB-enriched microbial communities, and WW2 and DL samples were selected as NOB-enriched microbial communities. To increase the efficiency of these communities at lower temperatures (9 and 15°C), temperature adaptation was performed at the next stage.

Temperature-adapted AOB and NOB microbiomes are able to remove ammonium and nitrite at different temperatures

We have evaluated the performance of the selected microbiomes in terms of ammonium (Fig. 2a-g) and nitrite (Fig. 3a-g) removal at different temperatures: 9 ºC, 15 ºC, RT, and 30 ºC. RT was overall the optimal temperature for ammonium removal. At this temperature, the GR sample was able to completely remove ammonium after 216 h (Fig. 2b). SDL and WW1 microbiomes where somewhat slower, reaching complete ammonium removal after 288 h and 480 h, respectively. The ammonium removal activity was reduced in all microbiomes at 30°C compared to RT, but the highest ammonium removal rate was still found in the GR sample, completely removing ammonium after 288 h (Fig. 2a). At 15°C, the ammonium removal was further reduced. The GR sample was still the best performer, requiring 384 h for complete removal (Fig. 2c). Decreasing the temperature to 9 ° C dramatically decreased ammonium removal of all sample, but the GR was still the best performer (Fig. 2d). Due to the poor performance of the WW1 microbiome in ammonium removal at all tested temperatures, it was excluded from further experiments (Fig. 2a-d).

The NOB-enriched microbiomes (WW2 and DL) exhibited the best nitrite removal rate at 30°C (Fig. 3a-d). At this temperature, nitrite was completely removed by the WW2 sample after 144 h (Fig. 3a). Nitrite removal decreased in both samples with decreasing temperatures (Fig. 3b-d). The WW2 sample consistently showed a higher rate of nitrite removal at all temperatures (Fig. 3a-d).

In order to correlate metabolic activity of ammonium and nitrite removal with expression of key genes at population level, we investigated the expression of key genes AMO and NXR, involved these respective processes. Highest expression levels of AMO and NXR were observed in GR and WW2 samples, respectively (Fig. 2e, Fig. 3e). The expression of both AMO and NXR was highest in the temperature range of 25–30°C. This result was consistent with the highest ammonium and nitrite removal activity of GR and WW2 at RT and 30°C, respectively.

Key nitrifying bacteria are present in selected AOB and NOB microbiomes

Although a biofilter system is separate module of the RAS during fish farming, it is nevertheless important to ensure that there are no pathogenic bacteria (especially primary pathogens) in the microbial community before its addition to the biofilter section [37]. In our study, the GR and WW2 microbiomes have had the best performance in ammonium and nitrite removal, but we also need to consider if these microbiomes contained any pathogenic species. Therefore, the microbiomes underwent identification of bacterial strains. The results showed that *Nitrosmonas oligotropha* (21.6% of all DNA reads in the metagenome) and *Nitrobacter winogradskyi* (6.1% of all DNA reads in the metagenome) were the dominant species in the GR and WW2 microbiomes, respectively (Fig. 2f, Fig. 3f). These species are known to be involved in the nitrification process [38, 39]. With such high prevalence of *N. oligotropha* and *N. winogradskyi*, it should be possible to visualize these species using the SEM microscopic observation of the bacterial communities. In the GR sample, we were able to identify many rod-shaped bacteria resembling the general description of the genus *Nitrosomonas* in terms of shape and size [40] (Fig. 2f). Similarly, in the WW2 sample, we were able to identify cells corresponding to the general description of the genus *Nitrobacter* (Fig. 3f).

The bacteria pathogens of aquatic animals and humans that are commonly encountered in RAS was previously listed [41–47]. Presence of these bacteria was investigated in the microbial communities of GR and WW2 samples. According to our results, there were no pathogenic bacteria in the GR and WW2 communities.

GR and WW2 microbiomes efficiently remove ammonium and nitrite from biofilter water at 9 and 15°C

To test the activity of the selected microbiomes (GR and WW2) for use on RAS biofilters, they were further tested in biofilter water samples with different concentrations of ammonium (0.5, 5, and 35 mg/ml) and nitrite (0.4, 4, and 8 mg/ml). The ammonium and nitrite removal activities of GR and WW2 were investigated in these water samples at 9 and 15°C (Fig. 4a-f, Fig. 5a-f). The results showed that the respective ammonium and nitrite removal activity of the GR and WW2 samples was higher than the negative control groups (natural biofilter water) at all tested doses of ammonium and nitrite at the selected temperatures (9 and 15°C) (Fig. 4g, Fig. 5g). The ammonium and nerite removal activity of our microbial communities was expectedly slower at 9°C than at 15°C. The slow removal activities at 9 °

C was not specific to our microbial communities, as it was also observed in the negative control group (Fig. 4b, Fig. 4d, Fig. 4f, Fig. 5b, Fig. 5d, Fig. 5f). In spite of slow removal activities at 9°C, the removal rate of GR and WW2 was much higher than that of the negative control sample, that is commonly found in RAS systems. The ammonium removal by GR was improved by 288% over the control (Fig. 4g), and nitrite removal by WW2 was improved by 181% over the control microbial community (Fig. 5g).

Discussion

Intensive rearing of aquatic animals includes fish, crustaceans and bivalves that grow densely in the systems with recirculating water. Increase of animal density in water results in the higher concentration of pollutants (ammonium and nitrite). The biofilters of these systems, whose microorganisms are colonized, have a certain capacity for water treatment, pollutants removal and water reuse. Meanwhile, the hygienic conditions are not suitable for breeding [48, 49]. If a way is found to eliminate the pollutants by up to 10%,

the capacity of the system can be increased, which is quite economical [50–52]. Nitrification in RAS biofilters is driven mostly by the nitrifying bacteria [53, 54]. These bacteria mostly comprise slow-growing species of the genera *Nitrospira, Nitrobacter, Nitrosomonas, Nitrococcus*, and *Nitrosococcus* [55, 56]. When a RAS system is getting started, it is possible to add a slow growing microbial community to the system without fish [54, 57]. Thus, a community enriched with suitable nitrifying bacteria could be added to a starting RAS to ensure optimal operation of the biofilter [10].

Metagenomic and metatranscriptomic techniques are helpful for investigating microbial communities and enable taxonomic analysis and classification of bacterial varieties [58]. In this study, we showed that the GR microbial community, selected for its AOB activity and enriched with *N. oligotropha*, could be used for effective ammonium removal at both high and low temperatures in a trout aquaculture biofilter water system. *N. oligotropha* is a Gram-negative, rod-shaped species with aerobic metabolism [59]. In our selected NOB-enriched community, the WW2 sample, *N. winogradskyi* was found as the dominant denitrifier. *N. winogradskyi* is a Gram-negative bacterium that plays a key role in the nitrogen cycle by converting nitrite to nitrate as the end product of ammonium oxidation in nitrification process [60].

During fish farming, RAS system moves towards the nitrificant bacteria that have adapted to the system. Biofilters play an essential role in these systems. Proteobacteria sp., Bacteriodetes sp., Nitrospirae sp., Planctomycetes sp., Rhizobiaceae., and Chloroflexi sp that Nitrosomonas sp., Nitrospira sp. and Nitrobacter sp. are the most common microbial communities in biofilter systems [61–63]. In order to compare the performance of our selected microbiomes with the natural colonized AOB and NOB bacteria, the temperature-adapted GR and WW2 microbiomes were added to the RAS biofilter water. The results demonstrated the positive effect of selected microbiomes containing *N. oligotropha* and *N. winogradskyi* on nitrite and nitrate production in biofilter water at low temperatures (9 and 15°C), respectively.

It has been shown that *N. oligotropha* isolated from Austin Lake used mainly the AMO enzyme for ammonium removal [59]. Hence, we confirmed that the AMO gene was highly expressed in the GR microbiome, corresponding to peak ammonium removal activity (Fig. 2e). The negative effect of lower temperatures on ammonium removal was accompanied by lower expression of the AMO gene (Fig. 2e). Nitrite gets reduced to nitrate by NXR enzyme, whose differential expression was detected in the WW2 microbiome (Fig. 3e). NXR expression correlated very well with the nitrite removal activity of the WW2 community (Fig. 3).

Conclusions

In fact, we report a strategy for bio-augmentation of RAS biofilters enriched with autotrophic AOB and NOB bacteria. Using the enriched microbial community GR, we achieved a 288% improvement in ammonium removal over the commonly used communities in biofilters at 9°C (Fig. 6). Similarly, the enriched community WW2 improved nitrite removal by 181% compared to the commonly used communities in biofilters at 9°C (Fig. 6). It is important to emphasize that no pathogenic bacteria were identified in the GR and WW2 microbiomes, making them eminently suitable for application in low temperature RAS for trout breeding. We would therefore propose bio-augmentation with GR and WW2 as a safe and effective improvement to the

standard procedures in RAS for trout breeding. The surface colonization of these microbiomes needs to be further studied using different biofilters, in order to optimize colonization and maximize their effectiveness.

Abbreviations

AOB
Ammonia-oxidizing bacteria; NOB:Nitrite oxidizing bacteria; AMO:ammonia monooxygenase; (NXR):nitrite oxidoreductase; SDL:Sweden artificial lake; GR:Gothenburg River; DL:natural lake; WW1:high concentration of ammonium wastewater; WW2:high concentration of nitrite wastewater; RT:room temperature; RAS:Recirculating aquaculture system; 16S rRNA:16S ribosomal RNA; PCR:Polymerase chain reaction; q-PCR; Quantitative PCR; ANOVA:Analysis of variance

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Please contact authors for 16S rRNA data requests.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AN, SR, GR, HF and IM designed the project. AN and SR performed the laboratory works. AN, SR and IM performed library preparation, sequencing, and metagenomics analysis. AN and SP prepared microbiome samples and photographed with SEM. All authors contributed to the preparation of the manuscript.

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Figures

a)

	WW1	WW2	DL	SDL	GR
pH	7.5	8.23	7.4	7.6	7.3
NH_4-N (mg/l)	1006	22.4	0.006	0.051	0.135
NO_2-N (mg/l)	1.9	76.6	0.025	0.024	0.026
NO_3-N (mg/l)	82	2.2	0.053	0.352	0.079

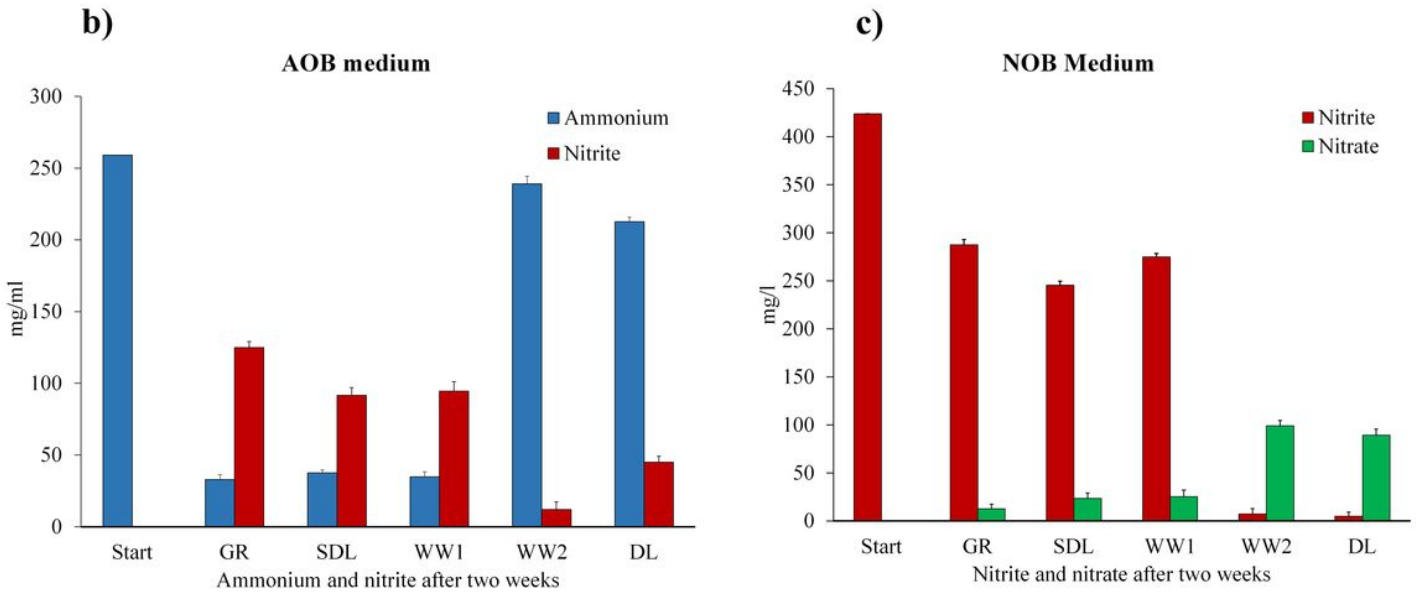

Figure 1

The characteristics of microbiomes collected from different water sources. a Water quality indicators in collected samples from different sources. b Ammonium removal and nitrite production in the AOB enriched microbiomes after two weeks. c Nitrite removal and nitrate production in the NOB enriched microbiomes after two weeks.

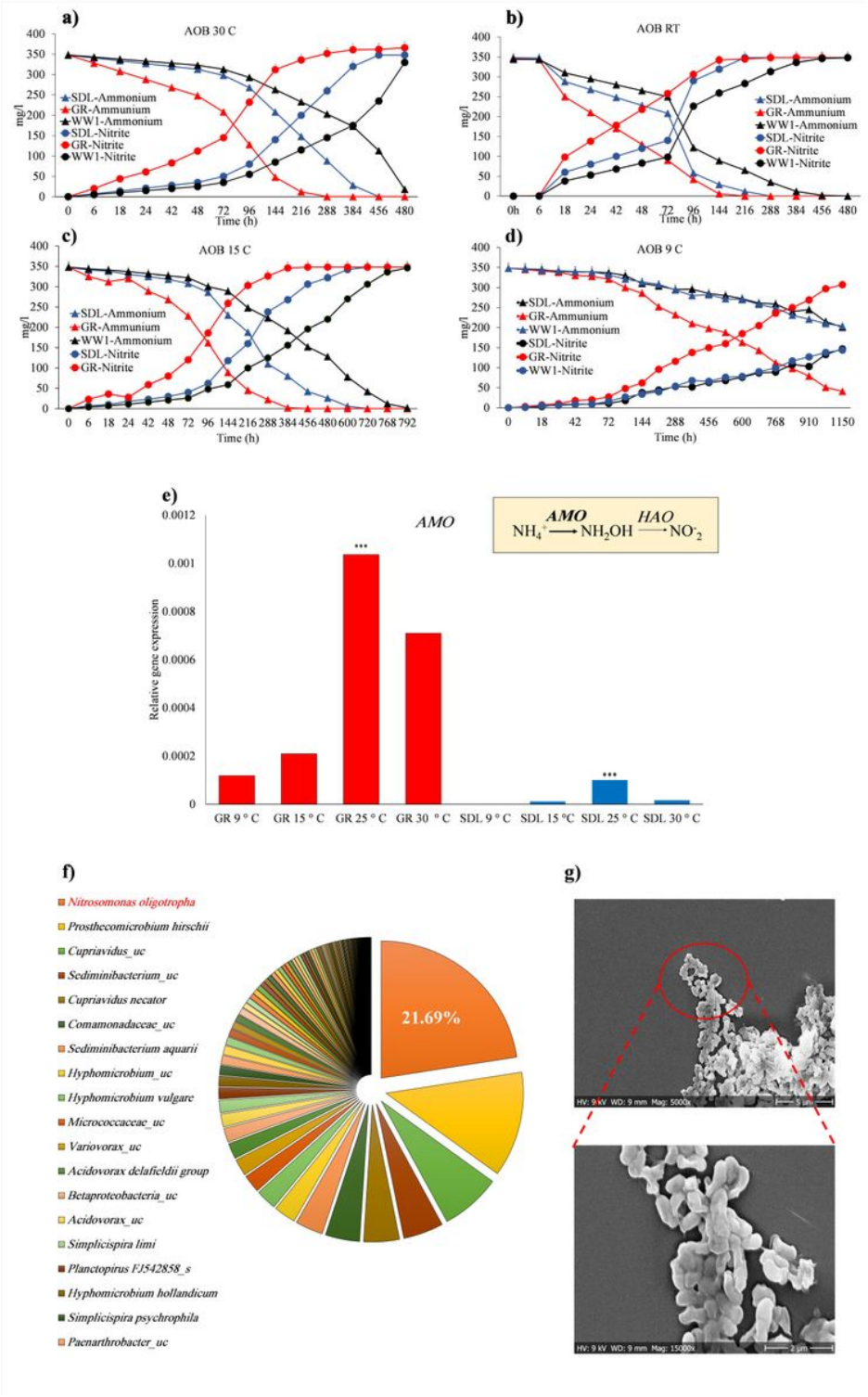

Figure 2

Ammonium removal and nitrite production using: a 30 °C-adapted AOB microbiomes, b temperature-adapted AOB microbiomes, c 15 °C-adapted AOB microbiomes, and d 9 °C-adapted AOB microbiomes. e Ammonia monooxygenase (AMO) gene expression in the different temperature-adapted AOB microbiomes. f Bacterial population (species) in GR microbiome g Microscopic observation of Nitrosomonas oligotropha in GR microbiome.

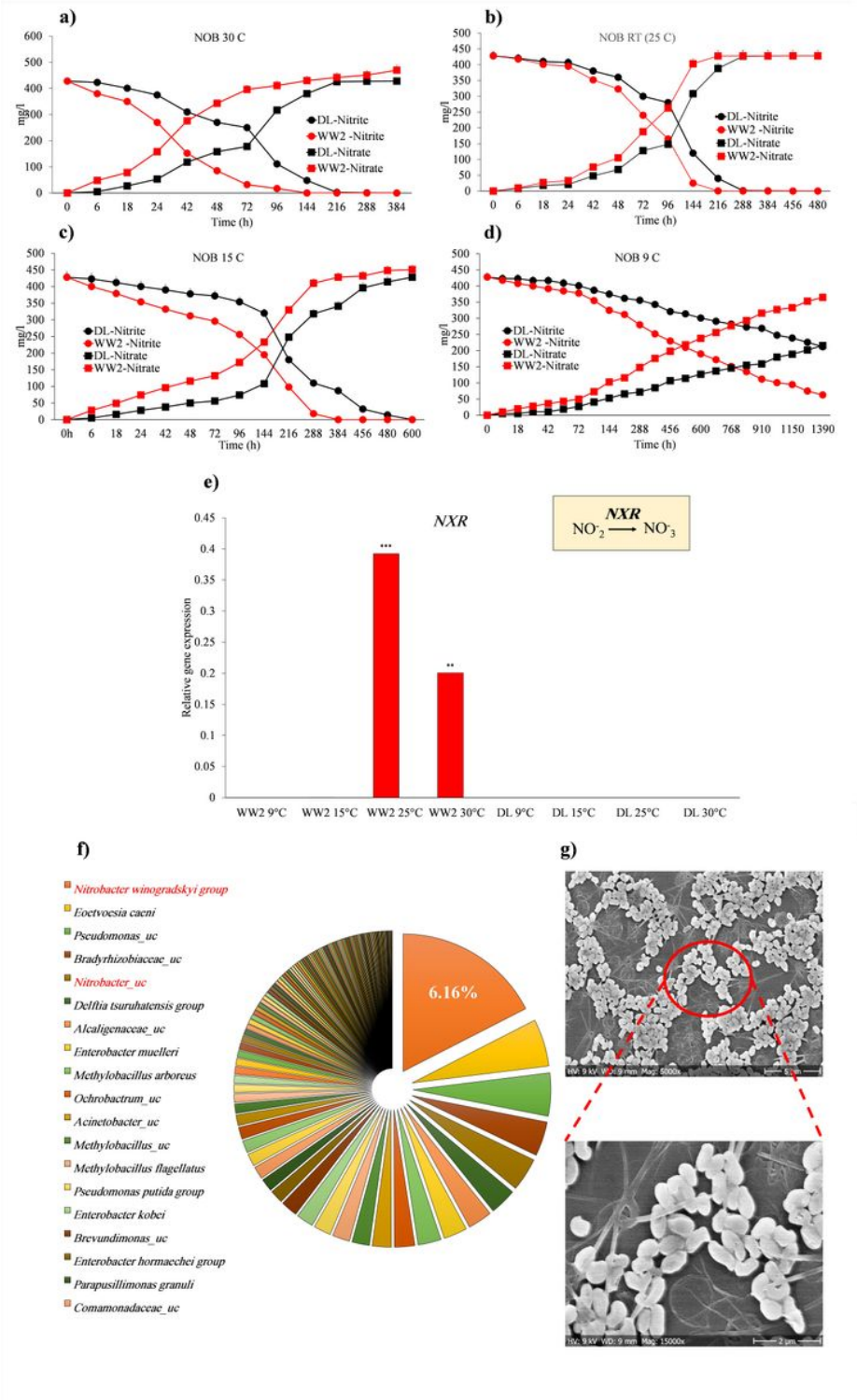

Figure 3

Nitrite removal and nitrate production using a 30 °C-adapted NOB microbiomes, b temperature-adapted NOB microbiomes, c 15 °C-adapted NOB microbiomes, and d 9 °C-adapted NOB microbiomes. e Nitrite oxidoreductase (NXR) gene expression in the different temperature-adapted AOB microbiomes. f Bacterial population (species) in WW2 microbiome g Microscopic observation of Nitrobacter winogradskyi in WW2 microbiome.

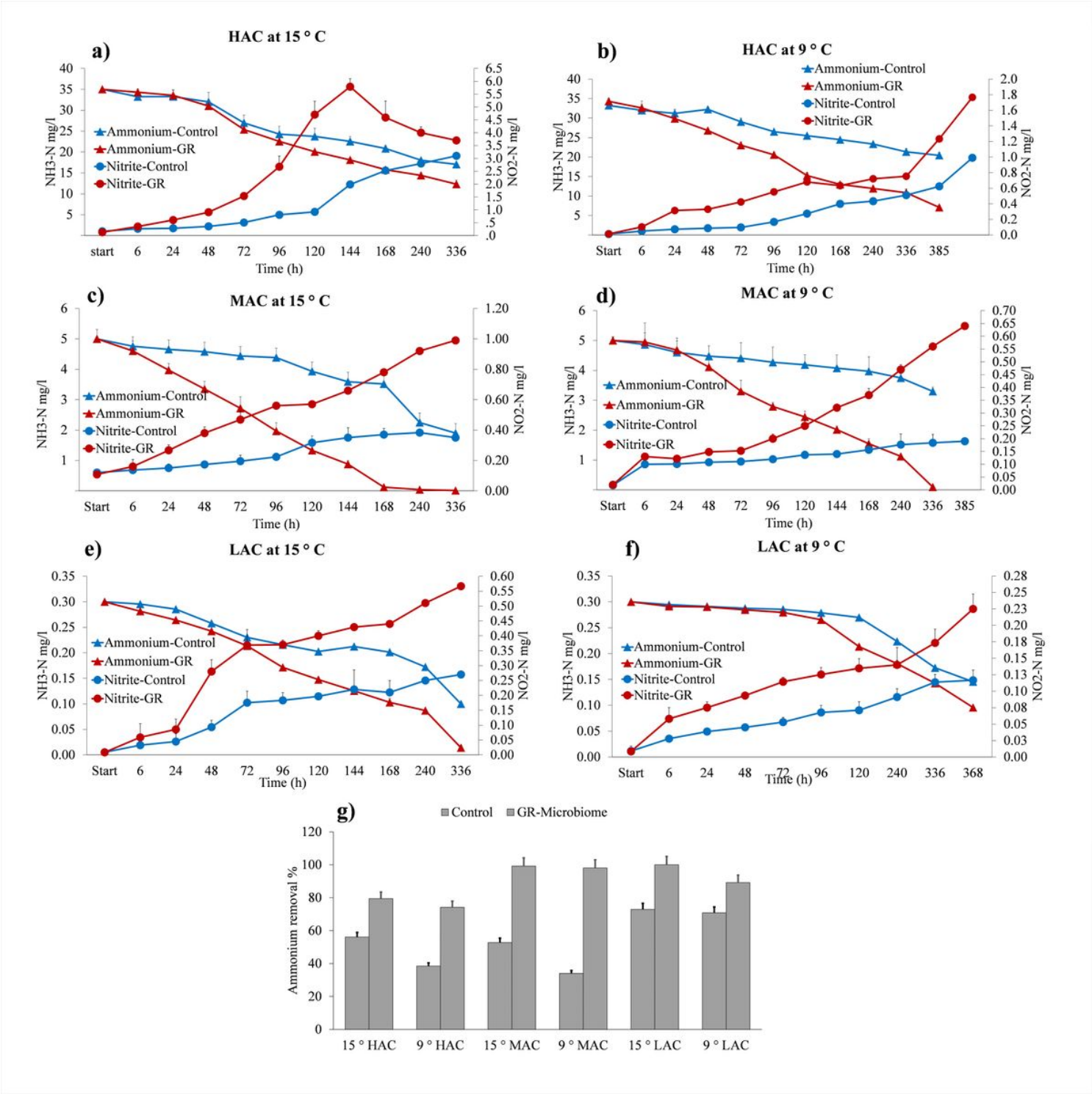

Figure 4

Ammonium removal activity using GR microbiome at different concentrations of ammonium and cold temperatures. a At high ammonium concentration (HAC, 35 mg/ml) and 15 °C, b at HAC and 9 °C, c at medium ammonium concentration (MAC, 5 mg/ml) and 15 °C, d at MAC and 9 °C, e at low ammonium concentration (LAC, 0.5 mg/ml) and 15 °C, f at LAC and 9 °C. g Ammonium removal percentage at different ammonium concentrations (HAC, MAC, and LAC) and temperatures (9 and 15 °C) using GR microbiome.

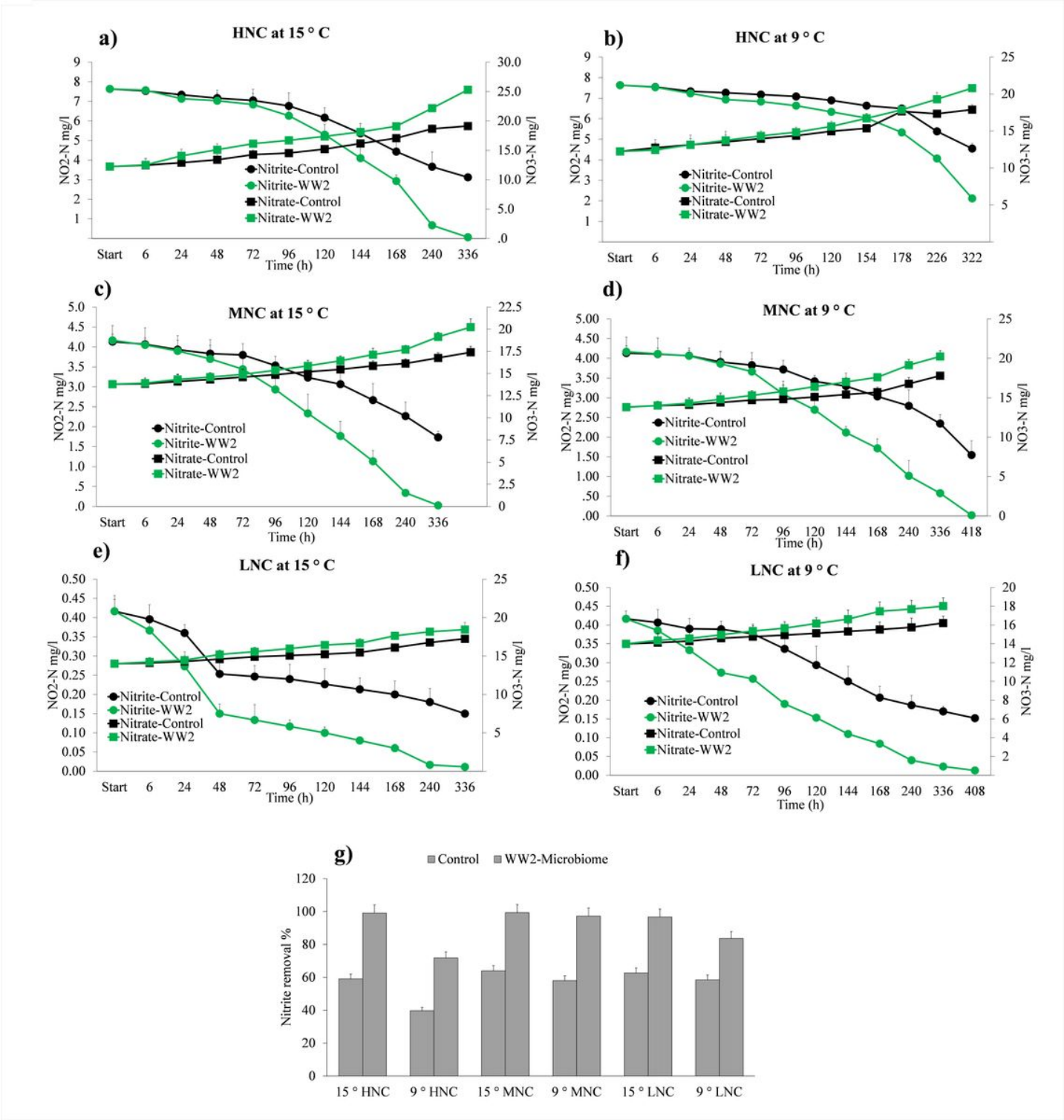

Figure 5

Nitrite removal activity using WW2 microbiome at different concentrations of nitrite and cold temperatures. a At high nitrite concentration (HNC, 8 mg/ml) and 15 °C, b at HNC and 9 °C, c at medium nitrite concentration (MNC, 4 mg/ml) and 15 °C, d at MNC and 9 °C, e at low nitrite concentration (LNC 0.4 mg/ml) and 15 °C, f at LNC and 9 °C. g Nitrite removal percentage at different concentrations (HNC, MNC, and LNC) and temperatures (9 and 15 °C) using WW2 microbiome.

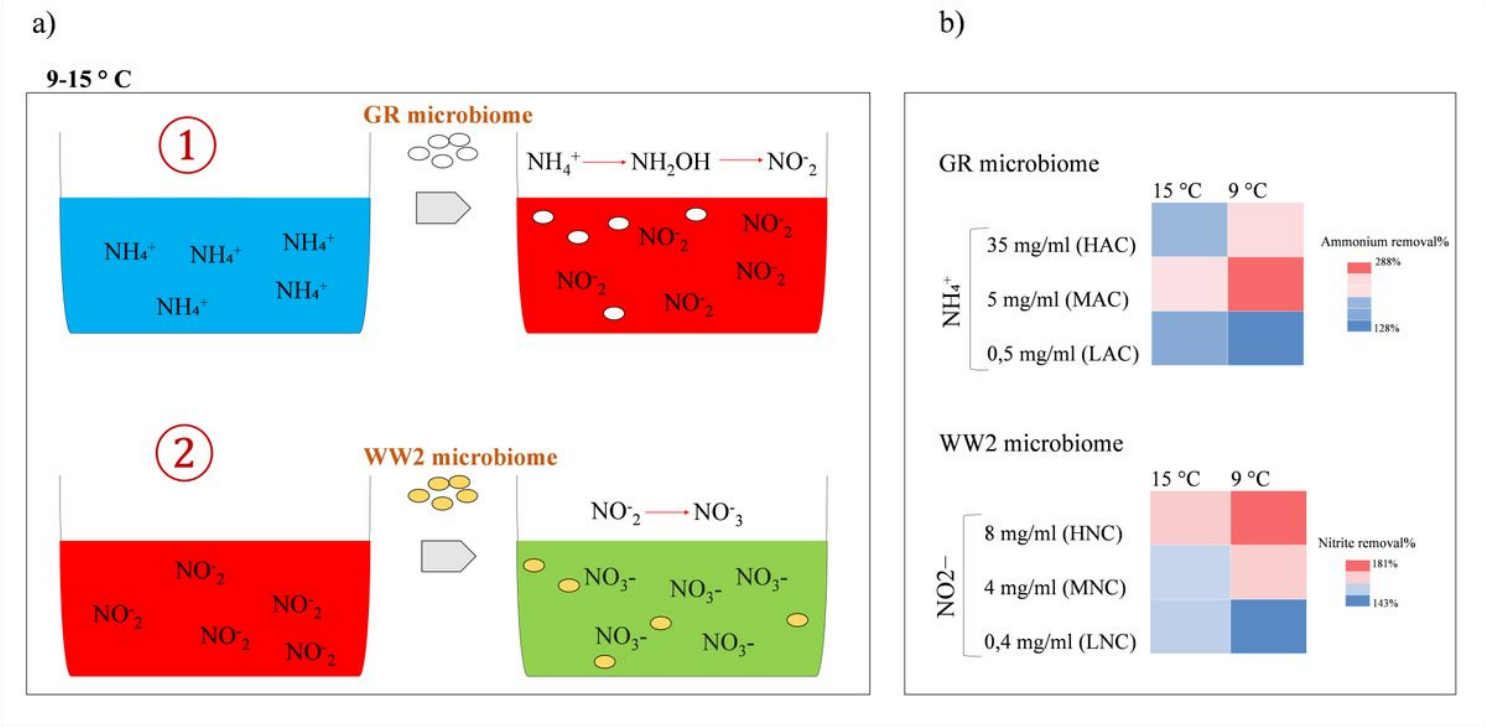

Figure 6

Schematic representation of GR and WW2 microbiomes performance for ammonium and nitrite removal at cold temperatures. a Bioaugmentation of GR and WW2 microbiomes degrade ammonium and nitrite at 9-15 °C. b Heatmap of improvement of ammonium and nitrite removal percentage by GR and WW2 microbiomes compared with the negative control group at different concentrations of ammonium (HAC, MAC, and LAC) and nitrite (HNC, MNC, and LNC) in cold conditions (9 and 15 °C).