Characters of a nitrobacter enrichment culture from a freshwater aquaculture pond

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ABSTRACT
To isolate nitrite-oxidizing bacteria to control the nitrite concentration in freshwater aquaculture systems, a Nitrobacter enrichment culture was obtained from a freshwater shrimp pond using a continuous enrichment and gradient dilution method. There was only one species of Nitrobacter in the enrichment culture, with an abundance of more than 99%. The Nitrobacter strain was identified as Nitrobacter vulgaris XY-12 on the basis of its 16S rRNA gene and NxrA gene sequences. The effects of pH, temperature, salinity and nitrate on the nitrification activity of the enrichment culture were examined, and its ability to remove nitrite in aquaculture water was also tested. The results showed that the optimum pH for the nitrite-oxidation activity of the enrichment culture was 7.5, the optimum temperature range was 27 °C–30 °C, the optimum salinity was 0.38%, the half maximal inhibitory concentration (IC50) of NO3−–N was 400 mg L−1, and the optimum concentration of NO2−–N was 200 mg L−1. In optimum conditions, the maximum NO2−–N removal rate afforded by the enrichment was 1.62 mg L−1 h−1, and the minimum doubling time was 12 ± 2 h. Moreover, XY-12 could multiply in field aquaculture water, and the maximum NO2−–N removal rate was 0.15 mg L−1 h−1. These results indicated that the enrichment had good application prospects in aquaculture waters.

Introduction
Nitrite is one of the main pollutants in aquaculture water, especially when the temperature is high because ammonia-oxidizing bacteria have a greater growth rate and can out-compete nitrite-oxidizing bacteria (NOB) at temperatures higher than 25 °C [1,2]. A concentration of NO2−–N in water >1 mg L−1 is harmful to aquatic organisms [3].

NOB mainly include five groups: Nitrobacter, Nitrospira, Nitrospina, Nitrococcus and Candidatus Nitrotoga [4]. Generally, Nitrospira, Nitrospina and Nitrobacter were considered to be the main NOB in aquaculture water. Brown et al. [5] found that Nitrospira was the main dominant genus in shrimp recirculating aquaculture water. Hüpeden et al. [6] found that Nitrotoga was the main dominant genus in a low-temperature recirculating aquaculture water system. Navada et al. [7] reported that Nitrobacter was the main dominant genus in some brackish water biofilms. Nitrobacter and Nitrospira were the main NOB in a freshwater recirculating aquaculture system (RAS) [8] and in a moving-bed biofilm reactor (MBBR) [9].

Nitrobacter was the earliest NOB to be studied and was mainly divided into four species: N. winogradskyi, N. hamburgensis, N. vulgaris and N. alkalicus [10]. In natural water environment and aquaculture waters, N. winogradskyi was found to be the dominant NOB in brackish water [11], marine environment and coastal shrimp RAS [12]. N. vulgaris was widely distributed in freshwater, brackish water, seawater [13] and freshwater aquarium biofilter [14], while N. alkalicus [15] and N. hamburgensis [16,17] were the dominant species in sediments of soda lakes and freshwaters, respectively.

To date, most of the known purified Nitrobacter strains have been isolated from soil, sand filters of waterworks, sewage treatment plants and freshwater rivers [15, 18–20]. However, only one strain of N. winogradskyi ZS-1 was reportedly isolated from brackish aquaculture water [11], and there was no report of Nitrobacter isolates from freshwater aquaculture waters.

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The application of NOB in aquaculture water is considered to have great potential in the control of nitrite concentration. However, the sensitivity to environmental factors, accompanied by the slow growth of NOB [1, 2], has greatly prevented the application of NOB in aquaculture systems, especially when the water temperature is high [1, 2]. Thus, the isolation of a fast-growing and high-temperature tolerant NOB would be of benefit for the application of NOB in aquaculture water. Therefore, in this study, a high-temperature tolerant *Nitrobacter* enrichment culture was obtained from a freshwater aquaculture pond, and the effects of various environmental factors on its ability to remove nitrite were examined.

**Materials and methods**

**Sampling**

The water sample was collected from a shrimp pond (N30°41′49.6″E113°46′51.0″) located in Hanchuan, Hubei Province, China.

The collected water sample was subjected to 16S rRNA gene high-throughput sequencing. The sample DNA was extracted by a HiPure soil DNA kit B kit (Magen, Guangzhou, China), and the DNA concentration was determined by a qubit@ds DNA HS assay kit (Magen). The primers used for the template of polymerase chain reaction (PCR) amplification of the V3–V4 region of the 16S rRNA gene were F- CCTACGGGRBGCASCAGKVRVGAAT and R-GGACTACNVGGGTWTCTAATCC [21]. The reaction system was contained in 25 μL: buffer, 2.5 μL; deoxyribonucleoside (dNTP) mixture, 2 μL; upstream and downstream primers, 1 μL; Taq DNA, 0.5 μL; template, 2 μL; and ultrapure water, 16 μL. The reaction conditions were 24 cycles of predenaturation at 94 °C for 3 min, denaturation at 94 °C for 5 s, annealing at 57 °C for 90 s, extension at 72 °C for 10 s, and final extension at 72 °C for 5 min. The PCR products were sequenced by Genewiz Co. Ltd. (Suzhou, China).

For the enrichment of NOB, 1 mL of sample was inoculated into 100 mL of enrichment medium (NaHCO₃ 0.4 g, KH₂PO₄ 1 g, K₂HPO₄ 1.31 g, EDTA 2 mg, ZnSO₄·7H₂O 1.1 mg, CoCl₂·6H₂O 0.8 mg, MnCl₂·4H₂O 2.55 mg, CuSO₄·4H₂O 0.8 mg, Na₂MoO₄·2H₂O 0.1 mg, CaCl₂·2H₂O 2.75 mg, FeCl₃·6H₂O 2.5 mg, MgSO₄·7H₂O 88.8 mg and ddH₂O 1000 mL) [22]. In the mixture, the NO₂⁻–N concentration in the form of NaNO₂ was adjusted to 20 mg L⁻¹ and the pH was set to 7.4–7.6. The mixture was cultured at 27 °C, 150 rpm for 35 days. During this period, the concentration of nitrite was measured every 7 days. After 35 days, 1 mL of the enriched culture was counted on a BD FACS Calibur flow cytometer to determine its total bacterial count.

To purify the enrichment culture, when the total bacterial count (determined by flow cytometry, see below) reached ~3.0 x 10⁶ cells mL⁻¹, take 1 mL enriched sample and dilute it 10⁷ times by step dilution method, so that there were only approximately three bacteria in 100 mL. Subsequently, 100 mL of the diluted sample was equally divided into 20 tubes (5 mL per tube), which were then incubated at 27 °C, 150 rpm for 30 days. The positive samples were identified by measuring the concentration of NO₂⁻–N on the 30th day.

**Nxr gene sequencing**

The amplification of the *NxrA* gene was conducted as described by Franck et al. [23] using the primers F1370 F1 nxrA CAG ACC GAC GTG TGC GAA AG and F2843 R2 nxrA TCC ACA AGG AAC GGA AGG TC. The amplification of the *NxrB* gene was conducted as described by Pester et al. [24] using the primers nxrB 169f TAC ATG TGG AAC A and nxrB 638r CGG TTC TGG TCR ATC A. All primers were synthesized by Invitrogen (Shanghai, China), and the PCR product was sequenced by Shanghai Sangon Biotech Co., Ltd.

**Physiological characterization**

To prepare the inoculum, 5 mL of the positive sample with the highest nitrification activity was inoculated into 100 mL of medium containing 20 mg L⁻¹ NO₂⁻–N and the mixture was cultured at 27 °C, 150 rpm until the NO₂⁻–N removal rate reached ~0.2 mg L⁻¹ h⁻¹.

To determine the optimum temperature of the nitrification activity, 9 mL of inoculum was inoculated into 900 mL of medium containing 20 mg L⁻¹ NO₂⁻–N. Subsequently, 50-mL samples of the mixture were collected into 100-mL flasks and incubated at 18 °C, 21 °C, 24 °C, 27 °C, 30 °C and 33 °C each for 4 days (pH 7.5, 150 rpm).

To determine the optimum pH of the nitrification activity, 9 mL of inoculum was inoculated into 900 mL of medium containing 20 mg L⁻¹ NO₂⁻–N. Subsequently, 50-mL samples of the mixture were collected into 100-mL flasks and the pH was set to 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The samples were then incubated at 27 °C, 150 rpm for 4 days.

To determine the salinity tolerance of the enrichment culture, a 10.5 mL inoculum was inoculated into 1050 mL of medium containing 20 mg L⁻¹ NO₂⁻–N. Subsequently, 50-mL samples of the mixture were collected into 100-mL flasks, and the salinity was set to 0.13%, 0.38%, 0.63%, 0.88%, 1.13%, 1.38% and 1.63%.
The samples were then incubated at pH 7.5, 27 °C, 150 rpm for 4 days.

To determine the nitrate tolerance of the enrichment culture, a 10.5-mL inoculum was inoculated into 1050 mL of enrichment medium containing 20 mg L\(^{-1}\) NO\(_2\)\(^-\)-N. Subsequently, 50-mL samples of the mixture were collected into 100-mL flasks, and the NO\(_2\)\(^-\)-N concentration (in the form of NaNO\(_3\)) was set to 0, 25, 50, 100, 200, 400 and 800 mg L\(^{-1}\), respectively. Moreover, NaCl was added to those mixtures so that the initial salinity was the same for all the treatments. The samples were then incubated at pH 7.5, 27 °C, 150 rpm for 4 days.

For all of the tests of temperature, pH, salinity and nitrate concentration described above, subsamples were collected every 24 h to measure the concentration of NO\(_2\)\(^-\)-N; moreover, the concentration of NO\(_3\)\(^-\)-N was measured at the end of the experiment.

In the test to determine the optimum nitrite concentration (the maximum initial NO\(_2\)\(^-\)-N concentration was up to 600 mg L\(^{-1}\)), because the high initial concentration of nitrite significantly influenced the sensitivity of the measurement of NO\(_2\)\(^-\)-N, dissolved oxygen (DO) uptake, rather than the elimination of NO\(_3\)\(^-\)-N used in the former tests, was applied to indicate the nitrification activity. To achieve a considerable decrease in DO within an hour, 2700 mL of inoculant was concentrated by centrifugation (27 °C, 5432 g, 15 min). Subsequently, 27 mL of the concentrated sample was added to 1350 mL of basic medium and divided equally into 27 flasks. The initial NO\(_2\)\(^-\)-N concentration was then set to 10, 25, 50, 100, 200, 300, 400, 500 and 600 mg L\(^{-1}\). Next, the flasks were completely sealed and incubated for 1 h at 27 °C with 10 rpm of magnetic stirring, during which the DO uptake was measured with a Hamilton Bonaduz AG dissolved oxygen probe every 15 min.

According to the results of the tests, a 1.5-mL inoculum was inoculated into 150 mL of modified basic medium (pH7.5; salinity, 0.38%; NO\(_3\)\(^-\)-N, 200 mg L\(^{-1}\)). Subsequently, 50-mL samples of the mixture were collected into 100-mL flasks and incubated at 27 °C, 150 rpm for 7 days. The concentration of NO\(_2\)\(^-\)-N, OD\(_{600}\) and total bacterial count were determined every 12 h; moreover, the concentration of NO\(_3\)\(^-\)-N was measured at the end of the experiment.

### Nitrification activity in aquaculture water

The test water samples were collected from a freshwater shrimp pond, NanHu Lake (a freshwater lake) and brackish water (salinity 1%). NaNO\(_2\) was added to 250 mL of freshwater from shrimp pond, NanHu Lake and Brackish water, respectively, to yield an initial concentration of NO\(_3\)\(^-\)-N of ~13 mg L\(^{-1}\). Subsequently, a 2.5-mL inoculum of the enrichment culture was added to the tested treatments, whereas the control treatments were inoculated with bacteria-free medium. The samples were then incubated at pH 7.5, 27 °C, 150 rpm for 8 days. Subsamples were collected every 24 h to measure the concentration of NO\(_3\)\(^-\)-N, and the concentration of NO\(_3\)\(^-\)-N was measured at the end of the experiment.

### Measurement and data analysis

Triplicate independent tests were conducted for all experiments. NO\(_2\)\(^-\)-N and NO\(_3\)\(^-\)-N were detected via the N-(1-naphthalene)-diaminoethane spectrophotometric method and the phenol disulfonic acid method, respectively.

The total bacterial count was determined via flow cytometry, as follows: A 1 mL water sample was filtered through a 2000 mesh sieve to remove big particles in the water. Subsequently, SYBR Green I was added to obtain a final concentration of 1× in the sample, followed by its incubation at 25 °C for 10 min [25] and measurement by a BD FACS Calibrated flow cytometer at 488 nm excitation.

The oxidation ratio of NO\(_2\)\(^-\)-N was calculated as follows:

\[
A(\%) = \frac{C_i - C_0}{C_i} \times 100\%
\]

where \(C_0\) is the concentration of NO\(_3\)\(^-\)-N (mg L\(^{-1}\)) at the end of the experiment and \(C_i\) is the initial concentration of NO\(_3\)\(^-\)-N (mg L\(^{-1}\)).

The generation time was calculated as follows:

\[
T(h) = \frac{\ln 2 \times \Delta t}{\ln(B_i) - \ln(VB_{t-1})},
\]

where \(B_i\) is the total bacterial count of the enrichment culture and \(\Delta t\) is the time interval.

### Data analysis

Origin 2017 was used to plot the data. Data are mean values with standard deviation (±SD). Analysis of variance (ANOVA) tests (with multiple comparisons of LSD) were performed using SPSS 20.0.

### Results and discussion

**The NOB in the enrichment was identified as *N. vulgaris***

Only one NOB strain was identified by high-throughput sequencing in the field sample, although its relative abundance was only 0.0687‰. Moreover, the partial
16S rRNA gene sequence of the NOB was 100% identical to that of *N. vulgaris* (NR 042449.1).

In the NOB enrichment culture, as shown in Figure 1, only one NOB strain was identified via high-throughput sequencing, with a relative abundance of 99.1%. Its partial 16S rRNA gene was 100% identical to that of *N. vulgaris* (NR042449.1).

According to the phylogenetic tree of the NxrA gene sequence (Figure 2), the NOB in the enrichment had the closest genetic distance to *N. vulgaris*, and its identity was 100% compared with *Nitrobacter vulgaris* (DQ421375.1). Nitrite oxidoreductase (NXR) is a key enzyme in NOB [26], and NxrA and NxrB were used in the PCR amplification of *Nitrobacter* [13] and *Nitrospira* [24], respectively. In the present study, the amplification of the NxrB gene yielded negative results, indicating that there was no *Nitrospira* in this enrichment. Hence, the only NOB strain detected in the enrichment was termed *N. vulgaris* XY-12.

Till now, *N. vulgaris* has been found in different kinds of waters, but particularly in low nitrite environment [19]. Cai et al. [13] found that *N. vulgaris* was the main *Nitrobacter* in the biofilter of a freshwater fish tank through enrichment culture. Navada et al. [7] found that *N. vulgaris* was the main NOB in the nitrifying filter of brackish water with salinity of 12 %. However, the abundance of *N. vulgaris* in a biofilm reactor of RAS was significantly higher when the salinity was 6 %–12 % than the salinity of 32 % [27].

**Physiological characteristics of *N. vulgaris* XY-12**

As shown in Figure 3(a), an obvious nitrification activity of XY-12 was observed when the temperature was between 21 °C and 30 °C, and the highest nitrification activity occurred at 27 °C–30 °C (with a maximum nitrification rate of 0.46 mg L\(^{-1}\) h\(^{-1}\) and an oxidation ratio of NO\(_2^-\)–N of 97%–100%). Temperature can directly affect the growth and metabolism of microorganisms by affecting the activity of key enzymes and the optimum cell membrane fluidity for microbial growth [28]; moreover, it can indirectly affect the activity of nitrifying bacteria by affecting pH and FNA [29]. Huang et al. [30] found that *Nitrobacter* exhibited a negative correlation with temperature and a higher abundance under lower temperature conditions in aerobic activated sludge bioreactors. However, Sorokin et al. [15] reported that the optimum temperature of *Nitrobacter* AN1 was 25 °C–28 °C. In this paper, the optimum temperature of XY-12 was between 27 °C and 30 °C (Figure 3(a)), which was higher than that reported for AN1. From the aspect of application, the relatively higher optimum temperature of XY-12 might contribute to its application in high-temperature conditions, at which the accumulation of nitrite is more likely to occur [1].

As shown in Figure 3(b), XY-12 had obvious nitrification activity between pH 6.0 and 7.5. The nitrification activity reached 0.457 mg L\(^{-1}\) h\(^{-1}\) at pH 7.5 (with an oxidation ratio of NO\(_2^-\)–N of 92%) but was only 52% of the maximum nitrification activity at pH 6.0. Thus, the optimum pH for XY-12 was between 6.5 and 7.5. pH is an essential factor in the oxidation of nitrite, which can influence NOB directly by affecting their metabolism or indirectly by affecting the ionization equilibrium of NO\(_2^-\)/HNO\(_2\) [31]. As shown in Table 1, the isolated *Nitrobacter* grew well under neutral or alkaline conditions, i.e. Zhang et al. [33] found that the optimum pH of nitrifying bacteria was 7–9 in a sequencing batch reactor [33], which is comparable with that observed for XY-12. From the perspective of application, XY-12 may be able to adapt to the pH range in freshwater aquaculture systems, which is usually between 6.5 and 8.5, and particularly favours relative low pH conditions when the toxic effect of HNO\(_2\) is more pronounced.

As shown in Figure 3(c), the nitrification activity of XY-12 was obvious at a salinity of 0.38%–0.63% (with an oxidation ratio of NO\(_2^-\)–N of 87%–93%). In this salinity range, the nitrification activity reached up to 0.32 mg L\(^{-1}\) h\(^{-1}\) when the salinity was 0.38%, whereas it was only 25% of the maximum nitrification activity when the salinity was 0.63%, indicating that the

![Figure 1. Proportion of various bacteria in the enrichment culture.](image)
optimum salinity for the nitrification of XY-12 was \(~0.38\%\) and that the half maximal inhibitory concentration was between 0.38% and 0.63%. Salinity could affect cell osmotic pressure, which in turn modulates the transfer of substrates and oxygen, thereby affecting the rate of nitrification [34–36]. The tolerance of salinity varies between different Nitrobacter strains. For instance, the nitrite oxidation rate of \(N.\) \textit{winogradskyi} decreased to 42% when the salinity reached 1.17% [37], whereas the nitrite oxidation rate of \(N\textit{itrobacter}\) sp. was not affected when the salinity was up to 1.5% [38]. In this paper, the nitrification activity of XY-12 was highest at a salinity of 0.38% but was completely inhibited when the salinity reached 1.13%. As the salinity of freshwater aquaculture is usually lower than 0.5%, XY-12 could at least adapt to the salinity of freshwater aquaculture waters.

High nitrate concentrations inhibit the nitrification activity [39]. As shown in Figure 3(d), XY-12 had significant nitrification activity when the initial \(NO_3^-\) concentration was between 0 and 400 mg L\(^{-1}\), and the highest nitrification activity of 0.462 mg L\(^{-1}\) h\(^{-1}\) was reached at a \(NO_3^-\) concentration of 20 mg L\(^{-1}\) (with an oxidation ratio of \(NO_2^-\) of 86%). When the \(NO_3^-\) concentration was 400 mg L\(^{-1}\), the nitrification activity decreased to only 46% of the maximum nitrification activity, indicating that the optimum \(NO_3^-\) concentration range for XY-12 was 0–200 mg L\(^{-1}\) and the \(IC_{50}\) was 400 mg L\(^{-1}\). There are few studies of the nitrate inhibition of \(Nitrobacter\); moreover, the \(IC_{50}\) of \(N.\) \textit{winogradskyi} ZS-1 (isolated from brackish aquaculture water) was only 100 mg L\(^{-1}\) [11], which was much lower than that of XY-12.

As shown in Figure 4, XY-12 exhibited a strong DO consumption during the first 15 min, when the initial concentration of \(NO_2^-\) was 50–200 mg L\(^{-1}\). Moreover, the DO consumption rate was highest when the initial concentration of \(NO_2^-\) was 200 mg L\(^{-1}\) \((p < 0.05)\), indicating that the optimum initial concentration of \(NO_2^-\) was approximately 200 mg L\(^{-1}\). \(Nitrobacter\) is considered to be an \(r\)-strategist with low substrate affinity [40]. Generally, the optimum \(NO_2^-\) concentration of NOB ranges from 100 to 1500 mg L\(^{-1}\) [41]. Thus, the affinity of XY-12 was higher than that of most other NOB. For instance, the optimum \(NO_2^-\) concentration of \(N.\) \textit{winogradskyi} ZS-1 was as high as 500 mg L\(^{-1}\) [11], which was much higher than that of XY-12 (200 mg L\(^{-1}\)). From the perspective of application, its relatively high affinity to \(NO_2^-\) might benefit the application of XY-12 in freshwater aquaculture systems because the \(NO_2^-\) concentration is usually no more than 20 mg L\(^{-1}\) in these systems [3].

In optimum culture conditions, XY-12 removed \(~50\) mL\(^{-1}\) \(NO_2^-\) in 3 days (Figure 5), with a maximum \(NO_2^-\) removal rate of approximately 1.62 mg L\(^{-1}\) h\(^{-1}\) and an oxidation ratio of \(NO_2^-\) of 97%. Moreover, according to the total bacterial count, the shortest generation time of XY-12 was 12 ± 2 h. Thus, the growth of XY-12 was comparable to \(N.\) \textit{vulgaris} \textit{Ab1} isolated by Bock [19], but was much faster than that.
Figure 3. Effects of temperature (a), pH (b), salinity (c) and nitrate (d) on the removal efficiency of nitrite (mean ± SD).

Table 1. Adaptability of different *Nitrobacter* strains to various environmental factors.

| Strain                        | Optimum pH | Optimum salinity (%) | Optimum temperature (°C) | Optimum NO$_2$−N growth concentration (mg L$^{-1}$) | Half inhibitory concentration of NO$_3$−N (mg L$^{-1}$) | Generation time (autotrophic conditions) (h) | Isolation source                                |
|-------------------------------|------------|----------------------|--------------------------|---------------------------------------------------|----------------------------------------------------|-----------------------------------------------|------------------------------------------------|
| This study                    | 6.5–7.5    | 0.38                 | 27–30                    | 200                                               | 400                                                | 12 ± 2                                        | Freshwater aquaculture pond                     |
| *Nitrobacter agilis* [20]     | 6.5–8.5    | n.d.                 | 30                       | n.d.                                              | n.d.                                               | 16                                            | Freshwater aquaculture pond                     |
| *Nitrobacter vulgaris’ Z’* [19]| 7.5–8.0    | n.d.                 | 23–28                    | n.d.                                              | n.d.                                               | 140                                           | Sand filter                                    |
| *Nitrobacter winogradskyi ZS-1* [11] | 7–9        | n.d.                 | 25–32                    | 500                                               | 100                                                | n.d.                                          | Coastal *Pseudosciaena crocea* rearing pond     |
| *Nitrobacter strain Nevada* [19] | 7.6–7.8    | n.d.                 | 20–30                    | n.d.                                              | n.d.                                               | 12                                            | Desert soil                                    |
| *Nitrobacter alkalicus AN1* [15] | 9.5        | 2                    | 25–28                    | n.d.                                              | n.d.                                               | 18                                            | Sediments                                     |
| *Nitrobacter hamburgensis X14* [32] | 7.5        | n.d.                 | 25                       | n.d.                                              | n.d.                                               | 40                                            | Soil                                           |
| *Nitrobacter Y* [18]          | 7.5–8.0    | n.d.                 | 25–30                    | n.d.                                              | n.d.                                               | 18*                                           | Soil                                           |

*Note*: n.d., not mentioned; * mixed-trophic condition.
of *N. vulgaris* 'Z' [19]. The previously reported generation time of *Nitrobacter* was 12–43 h (Table 1), suggesting that XY-12 is among the fastest-growing *Nitrobacter* strains. Because the growth of NOB is usually very slow [16], a NOB strain with rapid proliferation might contribute to its population establishment and a subsequent efficient removal of nitrite.

As shown in Figure 6, the removal of nitrite by XY-12 in various aquaculture waters was obvious: the concentrations of nitrite in all tested treatments were significantly lower than those observed for the control treatments (*p* < 0.05). In the freshwater shrimp pond water (Figure 6(a)), the maximum rate of removal of NO$_2^-$–N in the tested treatment was 0.15 mg L$^{-1}$ h$^{-1}$, and NO$_2^-$–N in the tested treatment was below the detection limit within 7 days, whereas the rate of removal of NO$_2^-$–N in the control treatment was only 0.02 mg L$^{-1}$. To verify the growth of XY-12 in the field sample, the samples of the tested treatment were subjected to 16S rRNA gene high-throughput sequencing, with the results indicating that the abundance of XY-12 increased from 0.034‰ to 0.58% within 7 days. Hence, XY-12 multiplied and contributed greatly to the removal of nitrite in the freshwater shrimp water. Similarly, Lin and Zhu [42] reported that inoculated *Nitrobacter* became one of the dominant species in a shrimp pond within 48 h after inoculation.

In NanHu Lake water (Figure 6(b)), the average removal rate of NO$_2^-$–N in the tested treatment was
0.03 mg L$^{-1}$ h$^{-1}$, which was also significantly higher than the control treatment.

In brackish water (Figure 6(c)), XY-12 was affected by salinity: there was no significant difference between the tested treatment and the control treatment in the first 4 days. However, from the fifth day, the removal rate of nitrite in the tested treatment increased significantly, and the average NO$_2^-\text{N}$ removal rate reached 0.07 mg L$^{-1}$ h$^{-1}$, which was comparable with the NO$_2^-\text{N}$ removal rate observed in freshwater shrimp pond water. In other words, after 4 days of inhibition by the high salinity in the brackish water, the nitrification activity of XY-12 finally recovered. Comparable to our result, Navada et al. [27] also found that, after 14 days of gradual increasing of 10 ‰ salinity, the nitrification rate was 35% higher comparing to a one-step increasing of salinity.

Moreover, the oxidation ratios of NO$_2^-\text{N}$ were all higher than 85% for the three tested treatments (Table 2), suggesting that nitrification was the main route used for the removal of nitrite in those tested aquaculture waters. Kuhn et al. [43] added a commercial bacterial agent containing $N$. winogradskyi to a circulating aquaculture system, and the concentration of NO$_2^-\text{N}$ was maintained at 1 mg L$^{-1}$ within 28 days. Ren et al. [44] added NOB to a shrimp-breeding pond; the concentration of NO$_2^-\text{N}$ in the experimental treatment was less than 0.02 mg L$^{-1}$ within 7 days. Comparably, nitrite in various aquaculture waters was obviously removed within 7–8 days in the present study.

By comparison of the nitrification activity of XY-12 in different media and in aquaculture water, we noted that the NO$_2^-\text{N}$ removal rate ranged greatly from 0.03 mg L$^{-1}$ h$^{-1}$ (in NanHu Lake water, with the NO$_2^-\text{N}$ concentration of 13 mg L$^{-1}$) to 1.62 mg L$^{-1}$ h$^{-1}$ (in optimum culture condition, with the NO$_2^-\text{N}$ concentration of 200 mg L$^{-1}$). The result was in accordance with the understanding that Nitrobacter is a typical r-strategist and grows faster at relatively high NO$_2^-\text{N}$ concentrations [40].

According to its characteristics, the potential bioaugmentation of XY-12 in wastewater treatment of RAS at high temperature will include two approaches. One is that, if the hydraulic retention time (HRT) is significantly longer than the generation time of XY-12 (about 12 h), which is quite common for the wastewater treatment of RAS [45,46], the enrichment can be seeded to the system directly. Otherwise, in the conditions of shorter HRT, since nitrifying bacteria are suitable for adhesive growth [47], XY-12 should be fixed in some carriers, such as filters [48] or MBBR [49], to avoid being washed out by the high flow rate. Thus, future research should pay special attention to the practical technology of how to use such a NOB enrichment in different aquaculture scenes, especially when the commercial cost is concerned.

### Conclusions

In this study, XY-12, a $N$. vulgaris enrichment culture with high nitrification efficiency, was isolated and identified from freshwater aquaculture water. Efficient nitrification was achieved in a wide range of temperatures and pH values, and XY-12 had a good removal effect of nitrite in both freshwater and brackish aquaculture water. These characteristics will be of great value for application in aquaculture waters.

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### Disclosure statement

Authors have no conflict of interest to declare.

### Data availability

The data that support the findings of this study are available in the public domain: https://blog.csdn.net/weixin_57734968/article/details/116226160.

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