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Photobacterium sp. NNA4, an efficient hydroxylamine-transforming heterotrophic nitrifier/aerobic denitrifier

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An efficient heterotrophic nitrifying/aerobic denitrifying strain, *Photobacterium* sp. NNA4 was isolated from a recirculating aquaculture system (RAS). NNA4 was capable of utilizing ammonia, nitrate or nitrite as sole N-source with maximal removal rates of 12.5 mg/L/h for NH_4^+–N, 16.4 mg/L/h for NO_3^--N, and 4.5 mg/L/h for NO_2^--N, respectively. Optimal nitrification conditions were: sodium succinate as C-source, 30–37°C, NaCl 1–4%, pH 7.0–8.0, dissolved oxygen 5.89 mg/L, C/N > 10. Gas chromatography/mass spectrometry and gas chromatography/isotope ratio mass spectrometry analyses showed that N_2 and N_2O were aerobic denitrification products of nitrite and nitrate. NNA4 could tolerate high concentration of hydroxylamine and displayed efficient hydroxylamine-transforming capability. Hydroxylamine oxidoreductase activity using potassium ferricyanide as electron acceptor was 0.042 U. Results revealed that strain NNA4 could oxidize NH_2OH directly to N_2O at aerobic conditions. In view of its high removal ability of inorganic nitrogen pollutants and broad salinity tolerance range, NNA4 has great potential in denitrification treatment of types of wastewater with either low salinity (e.g., municipal facilities) or high salinity (e.g., aquaculture, seafood processing).

[**Key words:** *Photobacterium* sp. NNA4; Heterotrophic nitrification; Aerobic denitrification; Hydroxylamine-transforming; Nitrogen removal; Wastewater treatment]

Increased availability of reactive nitrogen in many ecosystems resulting from human activities has led to steadily increasing eutrophication and consequent adverse effects on ecosystem health and human health (1). Biological nitrogen removal is the major method because of considerations of cost, removal efficiency, and ease of implementation. Biological nitrogen removal most commonly involves two steps: (i) nitrification by autotrophic nitrifying bacteria aerobically; (ii) denitrification by heterotrophic denitrifying bacteria anaerobically (2). Many heterotrophic nitrifiers able to perform nitrification and denitrification processes simultaneously have been reported during the past few decades, such as *Alcaligenes faecalis* strain No. 4 (3), *Pseudomonas stutzeri* (4), *Bacillus* strain N31 (5), *Achromobacter xylosoxidans* CF-S36 (6), *Marinobacter* sp. NNA5 (7), *Agrobacterium* sp. LAD9 (8) and *Enterobacter cloacae* CF-S27 (9). These heterotrophic nitrifiers/aerobic denitrifiers are highly effective for biological nitrogen removal because they can function in a single reaction tank under constant running conditions. Thus, the potential applicability of heterotrophic nitrifiers is greater than that of traditional nitrifiers. The nitrogen removal pathway in heterotrophic nitrifiers/aerobic denitrifiers remains poorly understood. Differing characteristics of nitrogen removal pathways have been reported in different heterotrophic nitrifiers, and there may be unknown nitrogenous gas producing pathway existed in heterotrophic nitrification coupled with aerobic denitrification. Certain heterotrophic nitrifiers, e.g., *A. faecalis* strain No. 4 and NR, *Acinetobacter calcoaceticus* HNR, apparently perform heterotrophic nitrification and producing N_2 and nitrous oxide (N_2O) simultaneously via hydroxylamine aerobically, but do not perform aerobic nitrite/nitrate denitrification (3,10,11). Hydroxylamine (NH_2OH) is a key intermediate compound in nitrification processes in heterotrophic/autotrophic ammonia-oxidizing bacteria and ammonia-oxidizing archaea (12,13). Traditional concept considered that ammonium is oxidized to NH_2OH by ammonia monooxygenase, and then NH_2OH is transformed to nitrite by hydroxylamine oxidoreductase (HAO), and NO_2^- is further oxidized to nitrate (14). Recent research found that hydroxylamine metabolism is closely related to the emissions of N_2O, a significant greenhouse gas (15,16). Therefore, investigation of hydroxylamine metabolism pathways in additional heterotrophic nitrifying bacteria is needed.

A novel heterotrophic nitrifying/aerobic denitrifying bacterium, termed NNA4, was isolated from a marine recirculating aquaculture system (RAS). Its performance was characterized, and emission nitrogenous gas were analyzed precisely. Hydroxylamine utilization capability and HAO activity were determined in *in vitro* experiments. Our findings revealed that heterotrophic nitrification bacterium NNA4 may perform the pathway of hydroxylamine oxidation directly to N_2O, and help to further understanding the metabolism of hydroxylamine and also the characterizations of heterotrophic nitrifying/aerobic denitrifying bacteria. In view of its heterotrophic nitrification/aerobic denitrification capability and broad salinity tolerance range, NNA4 and similar strains are potentially useful microbial resources in biological denitrification treatment of wastewater.

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MATERIALS AND METHODS

Media Compositions of media used (per liter artificial seawater) were as follows. Enrichment medium (EM): 1.0 g of $(NH_4)_2SO_4$, 2.5 g of yeast extract, 5.0 g of tryptone. Artificial seawater (ASW) was prepared according to Bruns et al. (17). Basal medium (BM) was prepared as described previously (7). Media NM-1, NM-2, DM-1, and DM-2 (Table 1) were prepared by adding specific amounts of four nitrogen sources to BM. $NH_2OH{\cdot}HCl$ was dissolved in water, pH adjusted to 7.0, sterilized by filtration through 0.2 μm-pore-size membrane, and added to autoclaved media as needed.

Isolation, identification of heterotrophic nitrifying/aerobic denitrifying strains A Water sample was collected from an RAS located in Tianjin, China. One milliliter sample was inoculated into 100 mL EM and incubated on a rotary shaker (140 rpm) at 30°C for 1 week. Resultant culture (0.1 mL) was spread and incubated at 30°C for 2 days. Single colonies were picked and individually tested for nitrifying and denitrifying activity.

PCR amplification and sequencing of 16S rRNA gene, and phylogenetic analysis were performed as described previously (7). 16S rRNA gene was PCR amplified using bacterial universal primers 27F and 1492R, and sequenced by Sino Genoma Corp (Beijing, China). The PCR steps was carried out as follows: 94°C for 5 min; 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min; and final extension at 72°C for 10 min. An almost-complete 16S rRNA gene sequence of strain NNA4 (1417 nt) obtained sequence was compared with available 16S rRNA gene sequences in GenBank databases using the BLAST program on NCBI (http://www.ncbi.nlm.nih.gov) and also on the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) by using identity analysis. Phylogenetic tree was constructed by using neighbor-joining methods with the MEGA 5 program package. The robustness of the tree topologies was assessed using bootstrap analyses based on 1000 replications.

Capabilities of heterotrophic nitrification/aerobic denitrification The heterotrophic nitrifying/aerobic denitrifying abilities on four inorganic nitrogen substrates were determined using four media (Table 1). Cells were cultured in NM-1 medium for 1 d, harvested, and washed with ASW. One percent of medium volume of cell suspension was inoculated into these media and incubated aerobically (160 rpm, 30°C). Media without inoculation were used as controls.

Effects of six factors on heterotrophic nitrification Characteristics of heterotrophic nitrification under various culture conditions were evaluated by single-factor experiments (carbon source, temperature, salinity, initial pH, dissolved oxygen (DO), C/N ratio). The carbon sources used were sodium acetate, sodium succinate, sodium citrate, sodium pyruvate, potassium sodium tartrate, and glucose. Baseline experimental conditions were: initial nitrogen concentration 140 mg/L, initial pH 7.5, NaCl 30 g/L, C/N ratio 10, and incubated aerobically (160 rpm, 30°C) for 48 h. Effects of changes in temperature, salinity, initial pH, DO, and C/N ratio on ammonia removal were evaluated using NM-1 medium. Temperature was adjusted to 16°C, 23°C, 30°C, 37°C, and 45°C. Initial pH was adjusted to 5.0–10.0 (interval of 1.0) using diluted HCl or NaOH solution. In salinity experiments, NaCl concentration was set at 0–6% (interval of 1%). Shaking speed in aeration experiments was adjusted to 50, 100, 160, and 200 rpm. C/N ratio was adjusted to 2, 4, 6, 8, 10, 15, and 20, with fixed NH_4^+–N concentration 140 mg/L. Experiments were performed in triplicate with 1% inoculum size of cell suspension. Values shown are mean ± SD from triplicate experiments.

Hydroxylamine-transforming capability assay Hydroxylamine stability has been linked to pH and to absence of nitrite and metal ions (18,19). To eliminate interference from spontaneous decomposition, PBSS buffer (PBS buffer which contained 12.5 mmol/L sodium succinate) was used as solution buffer. Cells were cultured in NM-1 medium for 24 h and thallus were harvested by centrifugation at 3000 rpm. The pellet was washed 3 times with PBS (pH 7.4) and resuspended in PBSS containing 2.5 mmoL/L hydroxylamine. Cell suspension was adjusted to OD_{600} = 1.0 ± 0.05 and incubated at 30°C. Samples were taken at various times, and hydroxylamine, nitrite, and N_2O levels were measured. Non-inoculated sample, and sample inoculated with heat-inactivated cells (OD_{600} = 1.0 ± 0.05), were used as negative controls.

HAO enzyme activity assay NNA4 cells were cultured in NM-1 for 24 h as above, harvested by centrifugation, and washed 3 times with 20 mmoL/L PBS (pH 7.5). For preparation of cell-free extracts, NNA4 cell suspensions were lysed by ultrasonication and then centrifuged (11,190 ×*g*, 4°C, 15 min) to remove intact cells and cell debris. HAO enzyme activity assay was conducted according to Otte et al. (20). One unit (U) was defined as the enzyme amount required for transforming 1 μmol potassium ferricyanide per minute.

Detection of nitrogenous gases Gaseous nitrogen products of denitrification were analyzed by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) and gas chromatography/mass spectrometry (GC/MS) (7,21). NNA4 was incubated in basal medium containing 50% $K^{15}NO_3$ or 50% $Na^{15}NO_2$ (by atomic fraction, Spectra Corp., Dallas, TX, USA). Cultures were aerobic incubated in sealing bottles with one tenth liquid loading amount and cultured at 30°C on a rotary shaker (150 rpm). Gaseous products from headspace were detected after inoculation for one week. Both gas detection devices were equipped with GS–Carbon Plot (30 m × 0.32 mm × 3.0 μm, Agilent Technologies Inc., Santa Clara, CA, USA). N_2O were analyzed by GC/MS (model 7890A/5975C, Agilent). N_2 were analyzed by GC/IRMS (Thermo Fisher Scientific Trace GC ultra/DELTA V Advantage Isotope Ratio MS, Thermo Fisher Scientific, Waltham, MA, USA). For detection of nitrogenous gases using hydroxylamine as substrate, $^{15}NH_2OH{\cdot}HCl$ was added to PBSS buffer as substrate, and final concentration was adjusted to 2.5 mmoL/L. Nitrogenous gases (N_2O and N_2) of denitrification were analyzed by GC/MS and GC/IRMS as described above.

Detection of *napA* gene Aerobic denitrification was confirmed by amplification of *napA* gene, which encodes periplasmic nitrate reductase. PCR amplification was performed according to standard methods (22). PCR product was sequenced by Sino Genomax Company (Beijing, China). Phylogenetic analysis was performed as described previously (7).

Analytical methods Samples were taken periodically for determination of cell optical density (OD_{600}) using a spectrophotometer (model UV-7200; UNICO, Shanghai, China), and then centrifuged (10,000 ×*g*, 2 min) to obtain supernatant for determination of ammonium, nitrite, nitrate and hydroxylamine levels. The concentrations of the first three substrates were measured using an Aquakem analyzer (model Aquakem 600, Thermo Fisher Scientific) as described previously (7). The concentration of hydroxylamine was measured by the method of Frear and Burrell (23). Protein concentration was determined using Bradford method. Each treatment was performed in triplicate, and results are presented as mean ± standard deviation.

TABLE 1. Amounts of nitrogen sources added to basal medium for preparation of media NM-1, NM-2, DM-1, and DM-2.

Medium	Nitrogen source (g/L)			
	$(NH_4)_2SO_4$	$NH_2OH{\cdot}HCl$	KNO_3	$NaNO_2$
NH_4^+–N nitrification medium (NM-1)	0.66			
NH_2^+–N nitrification medium (NM-2)		0.348		
NO_3^-–N denitrification medium (DM-1)			1.0	
NO_2^-–N denitrification medium (DM-2)				0.28

RESULTS

Isolation and identification of bacterial strains Many bacterial strains were isolated from the RAS water sample, and strain NNA4 showed quite efficiency of heterotrophic nitrification and aerobic denitrification. Therefore, it was selected for further experiments, and deposited in China General Microbiological Culture Collection Center (CGMCC) under accession number CGMCC 1.13496. Strain NNA4 was a gram-staining-negative, heterotrophic, facultatively anaerobic bacterium. Its 16S rRNA gene sequence obtained was deposited in NCBI GenBank (MG270575). Phylogenetic analysis based on 16S rRNA gene sequence indicated that NNA4 is a member of the genus *Photobacterium*, showing highest sequence similarity (99.8%) to *P. ganghwense* FR1311^T (Fig. 1).

Heterotrophic nitrification/aerobic denitrification capability of NNA4 Heterotrophic nitrification capability of NNA4 was evaluated using ammonia (medium NM-1, Table 1) as sole nitrogen source. From 0 to 6 h, cell growth was slow, NH_4^+–N removal rate was low, and nitrite and nitrate accumulated (Fig. 2A). From 6 to 15 h, cells were in logarithmic phase, nitrite and nitrate levels dropped rapidly, the majority of NH_4^+–N consumption occurred in this phase, and maximal removal rate of NH_4^+–N was achieved at 12.5 mg/L/h. After 15 h, cells were in stable phase, OD_{600} remained fairly constant (1.4–1.6), and NH_4^+–N removal percentage was >70%.

Aerobic denitrification capability of NNA4 using nitrate or nitrite (medium DM-1 or DM-2, Table 1) as sole nitrogen sources is shown in Fig. 2B and C. NNA4 grew well in both these media. Using nitrate as sole nitrogen source (Fig. 2B), from 0 to 6 h, cell growth rate was slow and NO_3^-–N removal was low. After 6 h, cells were in logarithmic phase, nitrate level decreased significantly, and nitrite accumulation was maintained and reflected cell growth rate. The majority of nitrate consumption was occurred during this period, and maximal nitrate removal rate was achieved at 16.4 mg/L/h. After 18 h, cells were in stable growth phase, OD_{600} reached its maximal value (1.373), nitrate was consumed completely, and nitrite reached concentration ~59 mg/L and then decreased

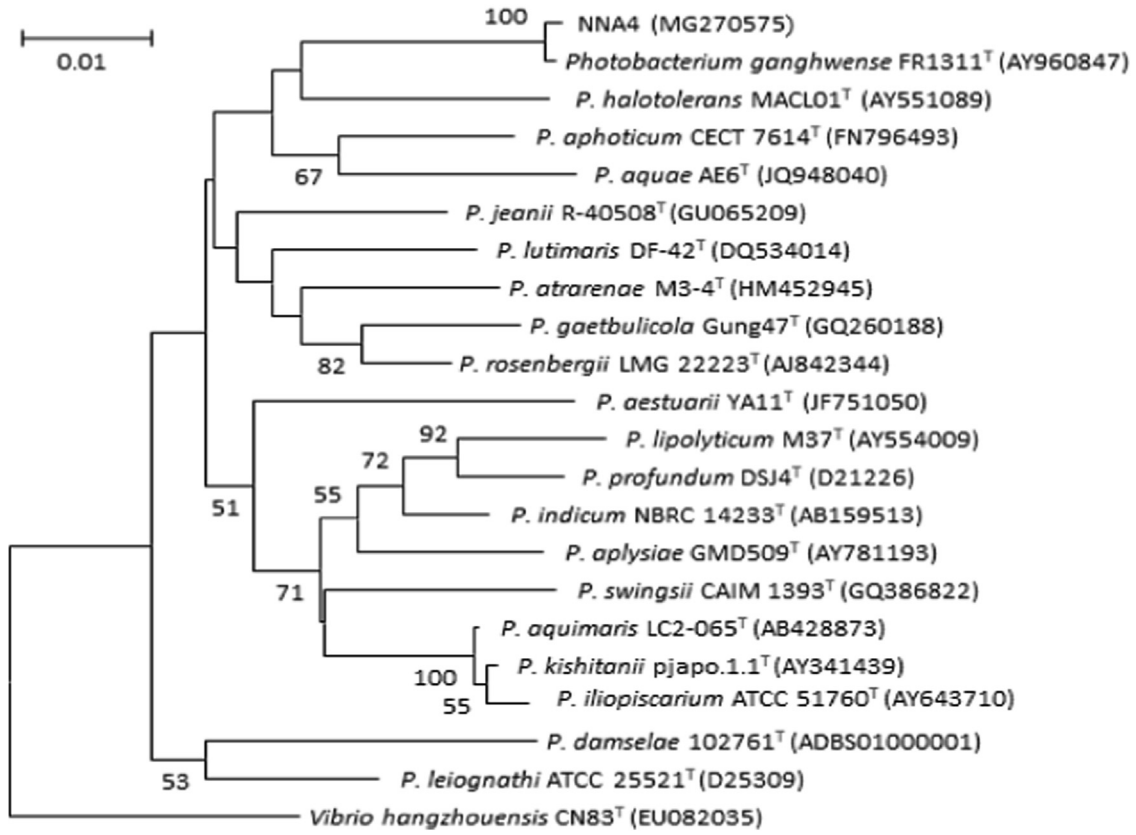

FIG. 1. Neighbor-joining phylogenetic tree for NNA4 based on 16S rRNA gene sequences. Bootstrap values >50 % (based on 1000 replications) are shown at branch points.

rapidly. After 24 h, OD_{600} declined slightly, perhaps because of cellular lysis in decay phase. Using nitrite as sole nitrogen source (Fig. 2C), cells grew slowly from 0 to 12 h, in logarithmic phase from 12 to 24 h, and in stable growth phase after 24 h. During logarithmic growth phase, nitrite level decreased and maximal NO_2^-–N removal rate (4.5 mg/L/h) was attained.

Effects of various factors on heterotrophic ammonium removal Effects of various factors on nitrification efficiency were also investigated. Nitrification capability of NNA4 using six carbon sources is shown in Table 2. Cell growth and ammonia removal capability of NNA4 were good when sodium succinate, sodium citrate, sodium acetate, or sodium pyruvate was used as sole carbon source, but minimal when glucose or potassium sodium tartrate was used. No accumulation of nitrite and nitrate was observed for any of these carbon sources. Our findings indicate that sodium succinate was the optimal carbon source for NNA4 among those tested, with the highest observed values of cell growth (OD_{600} 0.974) and ammonia removal (total NH_4^+–N removal 51.9%).

The heterotrophic nitrification performance of NNA4 at different temperatures is shown in Fig. 4A. As temperature increased from 16°C to 30°C, ammonium removal increased from 15% to 66%. When temperature further increased from 37°C to 45°C, ammonium removal dropped sharply. The optimal temperature range was 30–37°C for growth and heterotrophic nitrifying activity of

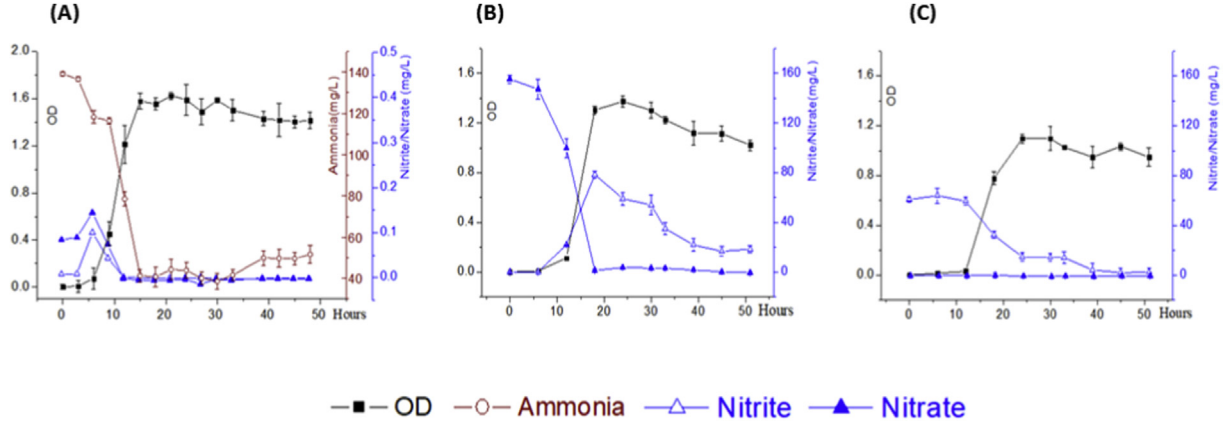

FIG. 2. Growth parameters (OD_{600}, ammonia level, nitrite/nitrate ratio) as a function of time under aerobic conditions using ammonia (A), nitrate (B), and nitrite (C) as sole nitrogen source. Values shown are mean ± SD from triplicate experiments.

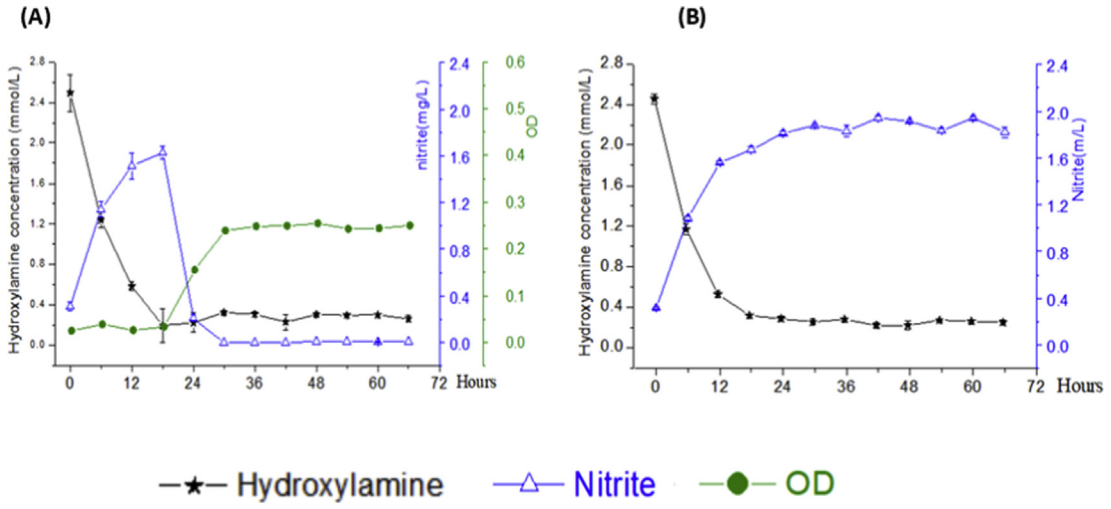

FIG. 3. Growth parameters (hydroxylamine level, nitrite level, and OD_{600}) as a function of time under aerobic conditions using hydroxylamine as sole nitrogen source. (A) Sample inoculated with strain NNA4. (B) Non-inoculated blank control. Values shown are mean ± SD from triplicate experiments.

NNA4. The heterotrophic nitrification performance of NNA4 at different initial pH values was shown in Fig. 4B. At initial pH 7.0, NNA4 reached maximal biomass (OD_{600} 1.23) and NH_4^+–N removal 61%. Maximal NH_4^+–N removal (63%) was attained for initial pH 8.0. For pH < 6.0 (acidic condition), almost no OD_{600} increase or ammonium removal occurred. For pH 10.0, cell growth and ammonium removal rate declined. The heterotrophic nitrification performance of NNA4 in the presence of different NaCl concentrations is shown in Fig. 4C. Growth and ammonium removal rate of NNA4 were stable for NaCl concentrations 1–4%, but declined rapidly for NaCl concentration 5%. Rotation speed had notable effect on ammonium removal and cell growth (Fig. 4D). An increase of rotation speed from 50 rpm (DO 4.62 mg/L) to 160 rpm (DO 5.89 mg/L) was associated with increases of bacterial biomass and ammonia removal efficiency. However, these parameters did not show further improvement as rotation speed rose to 200 rpm (DO 6.29 mg/L). Effects of C/N ratio on ammonium removal and cell growth are shown in Fig. 4E. Ammonia removal efficiency was low at C/N ratio < 6, increased as ratio climbed from 6 to 10, and did not differ significantly among ratios from 10 to 20 (>95% in each case).

Detection of nitrogenous gases Nitrogenous gases of aerobic denitrification were detected by GC/MS and GC/IRMS, as described above. GC/MS analysis revealed production of N_2O at 361 ppm and 45.2 ppm when nitrate or nitrite was used as substrate, respectively (Fig. 5A). When labeled $Na^{15}NO_2$ or $K^{15}NO_3$ was used as substrate, GC/IRMS analysis revealed production of N_2, the end product of denitrification (Fig. 5B). $\delta^{15}N/^{14}N$ ratios for labeled $Na^{15}NO_2$ (4.730) and $K^{15}NO_3$ (36.131) were much higher than that for blank control (0.228).

Hydroxylamine-transforming capability Strain NNA4 could grow well on hydroxylamine as sole nitrogen source and tolerate hydroxylamine higher than 10 mmoL/L (Fig. 6). As shown in Fig. 3A, hydroxylamine concentration declined and cells grew with a notable increase of OD_{600} using hydroxylamine (medium NM-2, Table 1) as sole nitrogen source. However, hydroxylamine concentration also declined in the blank control, presumably because of spontaneous decomposition of hydroxylamine in the system (Fig. 3B). Nitrite curves clearly differed between inoculated sample and blank control, i.e., nitrite concentration in the inoculated sample increased in the early stage and then declined to near zero in time-dependent manner, whereas it increased continuously in the blank control. Results of these experiments were inaccurate because of spontaneous decomposition of hydroxylamine. Thus, actual hydroxylamine utilization capability may be higher than the measured value.

Fig. 7 shows the results when the interference of metal ions was excluded by using PBSS buffer. Concentration curves for hydroxylamine, nitrite, and N_2O using hydroxylamine as substrate are detected. For non-inoculated sample and inoculated sample with heat-inactivated cells, hydroxylamine concentration decreased very slowly and production of nitrite and N_2O was very low, indicating that spontaneous decomposition of hydroxylamine occurred but at a very slow rate. Therefore, interference from this phenomenon could be essentially excluded. For sample inoculated with NNA4 cell suspension with a high OD value (OD_{600} = 1.0 ± 0.05), hydroxylamine concentration decreased quickly, >80% of hydroxylamine was removed by 3 h, and average hydroxylamine removal rate was 0.70 mmoL/L/h. Nitrite concentration increased quickly up to 1 h, and subsequently fluctuated between 0.11 and 0.13 mg/L. Release of nitrogenous gases of N_2O was associated with decline of hydroxylamine concentration, and the N_2O release curve was consistent with the hydroxylamine change curve.

For HAO enzyme activity detection, HAO activity of strain NNA4 was measured as 0.042 U and specific activity 0.009 U/mg protein when potassium ferricyanide was used as electron acceptor.

TABLE 2. Nitrification capability of NNA4 using six carbon sources.

Carbon source	OD_{600}	NO_2^-–N (mg/L)	NO_3^-–N (mg/L)	NH_4^+–N		
				Initial (mg/L)	Final (mg/L)	Removal percentage (%)
Sodium succinate	0.974 ± 0.034	0	0	144	69.4 ± 0.22	51.9
Sodium acetate	0.846 ± 0.053	0	0	152	80.4 ± 0.10	47.0
Sodium citrate	0.934 ± 0.038	0.06	0.03	136	85.8 ± 0.04	36.9
Sodium pyruvate	0.717 ± 0.017	0	0.01	136	82.2 ± 0.21	39.6
Potassium sodium tartrate	0.002 ± 0.003	0.08	0.11	152	149 ± 0.31	2.0
Glucose	0.165 ± 0.062	0.06	0.04	144	102.6 ± 0.32	28.7

Values shown are mean ± SD from triplicate experiments.

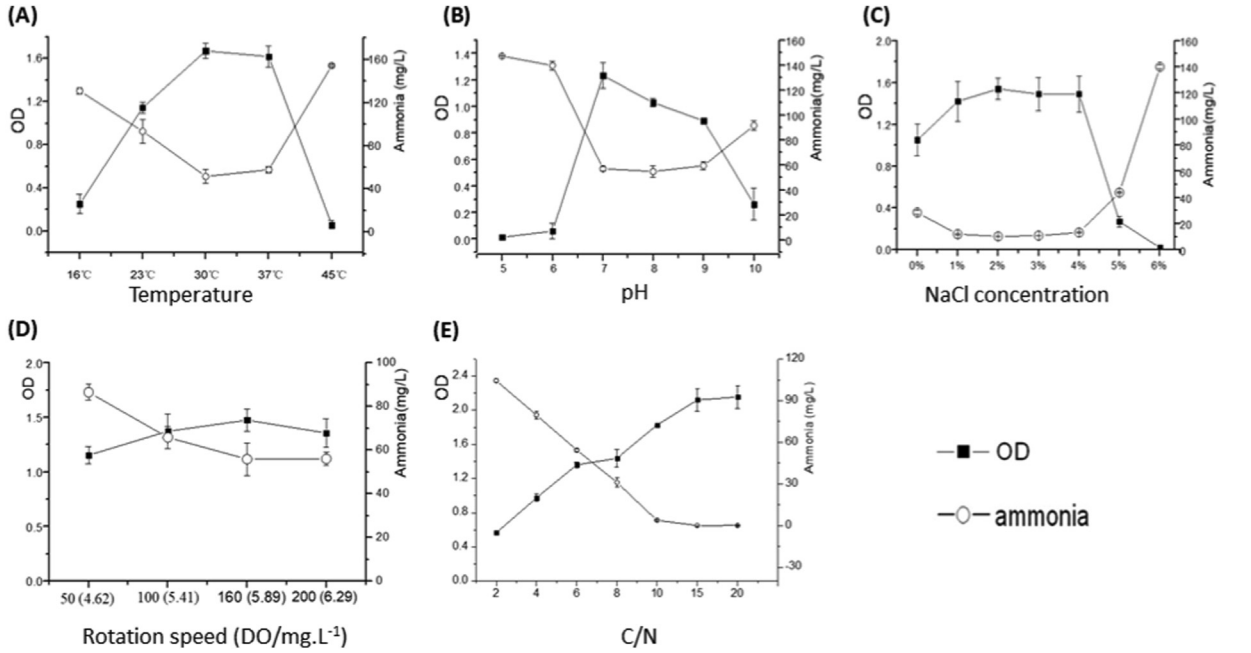

FIG. 4. Effects of temperature (A), initial pH (B), NaCl concentration (C), DO (D), and C/N ratio (E) on cell growth and heterotrophic nitrification capability of NNA4. Values shown are mean ± SD from triplicate experiments.

Detection of *napA* gene PCR of the *napA* gene resulted in amplification of a 1526 nt fragment. The GenBank accession number of *napA* gene is MG344624. The NapA amino acid sequence of NNA4 was deduced using PFAM (http://xfam.org) and ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder), and compared to available sequences in the GenBank database with online BLAST program. The NapA of strain NNA4 showed 67.6% sequence identity with that of a denitrifying bacterium, *Shewanella denitrificans* (24).

DISCUSSION

An efficient heterotrophic nitrifying/aerobic denitrifying strain, NNA4, was isolated and identified as *Photobacterium* sp. This species is primarily marine, and widely distributed in seawater and in marine animals (25). To date, no study has addressed heterotrophic nitrification/aerobic denitrification in *Photobacterium*.

NNA4 is capable of growth on ammonium, nitrate, or nitrite as sole nitrogen source, and is an effective heterotrophic nitrifier/aerobic denitrifier. Trace nitrite and nitrate were detected when ammonium was used as sole nitrogen source for strain NNA4, which probably resulted from the conversion of nitrite or nitrate to nitrogen gas by denitrifying progress. Similar results were also reported in previous studies such as *Vibrio* strain SF16 (26). The above findings demonstrate that NNA4 performed simultaneous nitrification and denitrification (SND) pathway as proposed by Richardson et al. (31). As compared to conventional process, SND has advantages as simplifying the system design and highly accelerating rate of nitrogen removal in a single aeration phase (27). NNA4 showed high efficiency of both aerobic denitrification and heterotrophic nitrification. The maximal ammonia–N and nitrate–N removal rate reached to 12.5 mg/L/h and 16.4 mg/L/h, respectively, which were much higher than rates reported previously for other heterotrophic nitrifier/aerobic denitrifier, e.g., *Vibrio diabolicus* SF16 (2.29 mg NH_4^+–N/L/h; 2.83 mg NO_3^-–N/L/h) (26), *E. cloacae* CF-S27 (11.6 mg NH_4^+–N/L/h; 15.1 mg NO_3^-–N/L/h) (9), *P. stutzeri* YG-24 (8.75 mg NH_4^+–N/L/h; 7.73 mg NO_3^-–N/L/h) (27). The high efficiency of heterotrophic nitrification/aerobic denitrification capability showed a future application of strain NNA4 for industrial water treatment.

The influence of different factors on nitrification efficiency was investigated. Carbon sources typically serve as sources of both energy and electron for heterotrophic bacteria, and are important factors determining heterotrophic nitrification capability (28). In this study, sodium succinate was the optimal carbon source for NNA4. Krebs cycle intermediates such as succinate.

NNA4 showed the maximum growth and ammonia removal rate at the temperatures ranging 30–37°C, which was similar to *A. faecalis* No. 4 (3). In addition, pH is another factor affecting microbial activity, the optimum pH for growth and ammonia removal performance of strain NNA4 was 7–8, in consistence with many nitrifying bacteria whose optimal pH for heterotrophic nitrification was reported to be slightly alkaline or neutral (3,28,29). More free ammonia existing in culture medium is presumably conductive to ammonia monooxygenase, so that the slightly alkaline environment can be beneficial to heterotrophic nitrification (29). The optimum NaCl concentration for growth and nitrification performance of strain NNA4 was 1–4%. Excessive salinity would cause cell lysis and enzyme activity loss (30). Thus, NNA4 should be effective in treatment of types of wastewater with either low salinity (e.g., municipal facilities) or high salinity (e.g., aquaculture, seafood processing).

N_2O and N_2 were the aerobic denitrification products on nitrite and nitrate. GC/MS analysis revealed production of N_2O when nitrate or nitrite was used as substrate. For GC/IRMS analysis of N_2, $\delta^{15}N/^{14}N$ ratio values >1 indicate that $^{15}N_2$ was generated according to previous report (21). The ratio was increased due to the generation of ^{15}N-labeled N_2. The GC/IRMS analysis results revealed production of N_2 when ^{15}N-labeled nitrate or nitrite was used as substrate. The above findings demonstrate that NNA4 is an aerobic denitrifier and follows the complete denitrification pathway as proposed by Richardson et al. (31). The *napA* gene which encode periplasmic nitrate reductase is related to aerobic nitrate respiration and denitrification (26). Aerobic denitrification capability of NNA4 is further confirmed by the presence of *napA* gene.

Hydroxylamine, a key intermediate compound in nitrification processes as mentioned above, is toxic to microorganisms even at millimolar concentrations, and has strong mutagenic effects on

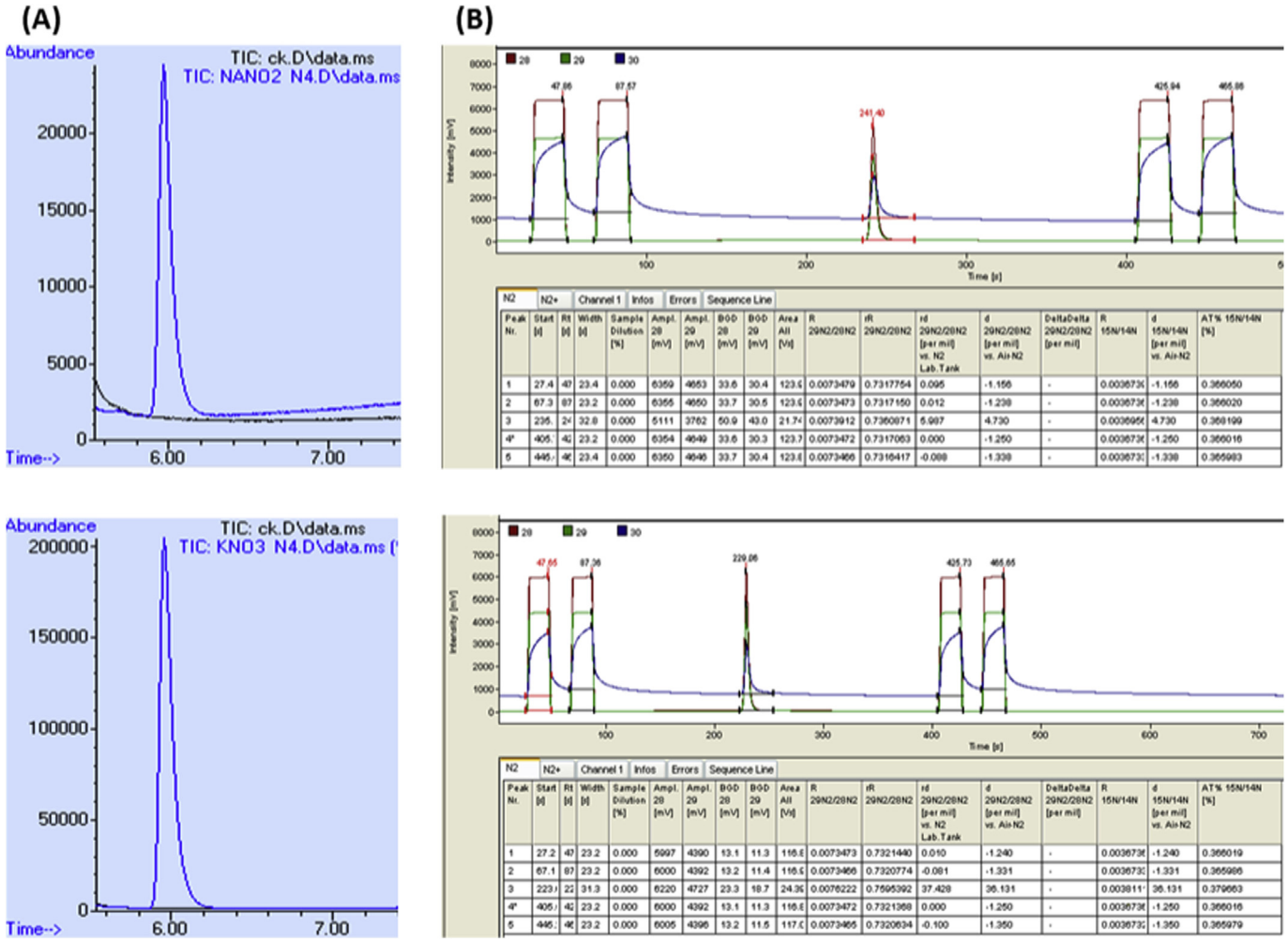

FIG. 5. Detection of nitrogenous gases by GC/MS (A) and GC/IRMS analysis (B). Sodium nitrite (upper) or potassium nitrate (lower) was used as substrate.

numerous phages, viruses, bacteria, fungi, protozoa, *Drosophila*, and plants (32). Up to date, only a few nitrifying bacteria had been found to be capable of growing mixotrophically on hydroxylamine in the presence of other nitrogen sources; these include the autotrophic nitrifier *Nitrosomonas europaea* (33) and heterotrophic nitrifiers such as *Pseudomonas* PB16 (34), *A. faecalis* No. 4 (3), and *E. cloacae* CF-S27 (9). However, strain NNA4 grew well in inorganic salt medium with hydroxylamine up to >10 mmoL/L as sole N-source, suggesting that NNA4 was quite efficient in hydroxylamine utilization and a unique heterotrophic nitrifier with strong hydroxylamine tolerance ability whose mechanism remained to be further investigated. In addition, hydroxylamine is unstable and can spontaneously be decomposed (35), not only making study of this intermediate compound difficult, but also needing non-inoculating blank controls. However, the above previous studies did not include non-inoculating blank controls and thus did not take into account the spontaneous decomposition of hydroxylamine. The hydroxylamine utilization rates reported may therefore have been inaccurate.

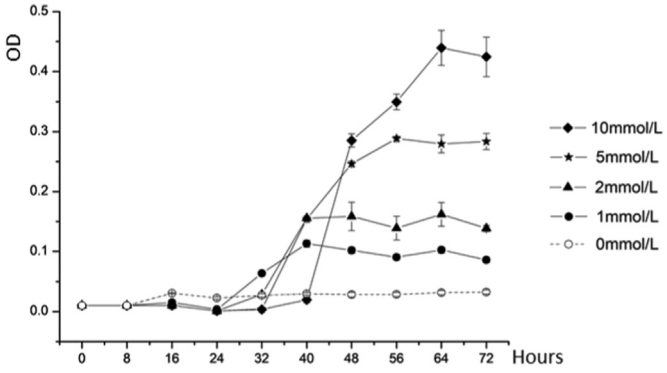

FIG. 6. Growth of NNA4 on different concentrations of hydroxylamine as sole source nitrogen source.

When hydroxylamine spontaneously decomposition was reduced using PBSS buffer to exclude the interference of metal ions, NNA4 also displayed quite efficient hydroxylamine-transforming capability. The hydroxylamine concentration decreased quickly with NNA4, in comparison with non-inoculated controls and heat-treated controls (Fig. 7). Corresponding, large amount of N_2O was detected with the decrease of hydroxylamine (Fig. 7). Though nitrite was also detected at the same time, the nitrite concentration is not stoichiometric with the hydroxylamine decrease concentration. All these results suggested that NNA4 could transform hydroxylamine directly to N_2O. This ability of NNA4 was quite different from previously described heterotrophic/autotrophic nitrifiers which commonly transformed hydroxylamine to nitrite by HAO (14); but consistent with *N. europaea*, an autotrophic ammonia-oxidizing bacterium could oxidize NH_2OH directly to N_2O via cytochrome P460 under aerobic conditions as reported recently (15,16).

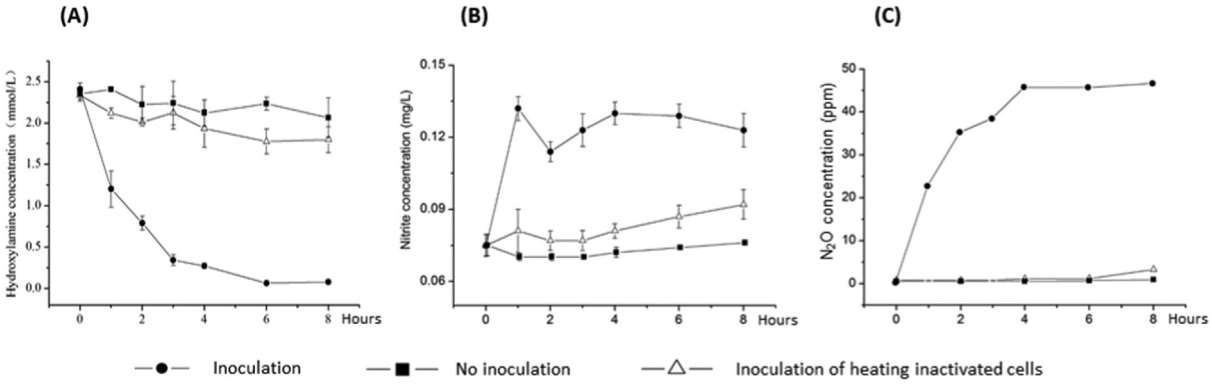

FIG. 7. Concentration curves for hydroxylamine (A), nitrite (B), and N_2O (C), using hydroxylamine as substrate without interference from metal ions.

The above findings indicate that NNA4 is a unique heterotrophic nitrifier/aerobic denitrifier. It could tolerate high concentration of hydroxylamine and displayed efficient hydroxylamine-transforming capability. Majority of hydroxylamine was transformed to N_2O with only small amount of nitrite produced, revealed that strain NNA4 may oxidize NH_2OH directly to N_2O. Our research helps to further understand the metabolism of hydroxylamine and also the characterizations of heterotrophic nitrification/aerobic denitrification bacteria. In view of its heterotrophic nitrification/aerobic denitrification capability and broad salinity tolerance range, NNA4 has great potential in denitrification treatment of wastewater with either low or high salinity.

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References

1. **Finlay, J. C., Small, G. E., and Sterner, R. W.:** Human influences on nitrogen removal in lakes, Science, **342**, 247–250 (2013).
2. **Zhu, L., Ding, W., Feng, L. J., Kong, Y., Xu, J., and Xu, X. Y.:** Isolation of aerobic denitrifiers and characterization for their potential application in the bioremediation of oligotrophic ecosystem, Bioresour. Technol., **108**, 1–7 (2012).
3. **Joo, H. S., Hirai, M., and Shoda, M.:** Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4, J. Biosci. Bioeng., **100**, 184–191 (2005).
4. **Takaya, N., Catalansakairi, M. A. B., Sakaguchi, Y., Kato, I., Zhou, Z., and Shoun, H.:** Aerobic denitrifying bacteria that produce low levels of nitrous oxide, Appl. Environ. Microbiol., **69**, 3152–3157 (2003).
5. **Huang, F., Pan, L., Lv, N., and Tang, X.:** Characterization of novel *Bacillus* strain N31 from mariculture water capable of halophilic heterotrophic nitrification–aerobic denitrification, J. Biosci. Bioeng., **124**, 564–571 (2017).
6. **Padhi, S. K. and Maiti, N. K.:** Molecular insight into the dynamic central metabolic pathways of *Achromobacter xylosoxidans* CF-S36 during heterotrophic nitrogen removal processes, J. Biosci. Bioeng., **123**, 46–55 (2017).
7. **Liu, Y., Ai, G. M., Miao, L. L., and Liu, Z. P.:** *Marinobacter* strain NNA5, a newly isolated and highly efficient aerobic denitrifier with zero N_2O emission, Bioresour. Technol., **206**, 9–15 (2016).
8. **Chen, Q. and Ni, J.:** Ammonium removal by *Agrobacterium* sp. LAD9 capable of heterotrophic nitrification-aerobic denitrification, J. Biosci. Bioeng., **113**, 619–623 (2012).
9. **Padhi, S. K., Tripathy, S., Mohanty, S., and Maiti, N. K.:** Aerobic and heterotrophic nitrogen removal by *Enterobacter cloacae* CF-S27 with efficient utilization of hydroxylamine, Bioresour. Technol., **232**, 285–296 (2017).
10. **Zhao, B., Yi, L. H., Hughes, J., and Xiao, F. Z.:** Heterotrophic nitrogen removal by a newly isolated *Acinetobacter calcoaceticus* HNR, Bioresour. Technol., **101**, 5194–5200 (2010).
11. **Zhao, B., An, Q., He, Y. L., and Guo, J. S.:** N_2O and N_2 production during heterotrophic nitrification by *Alcaligenes faecalis* strain NR, Bioresour. Technol., **116**, 379–385 (2012).
12. **Arp, D. J., Sayavedrasoto, L. A., and Hommes, N. G.:** Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea, Arch. Microbiol., **178**, 250–255 (2002).
13. **Vajrala, N., Martenshabbena, W., Sayavedrasoto, L. A., Schauer, A., Bottomley, P. J., Stahl, D. A., and Arp, D. J.:** Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea, Proc. Natl. Acad. Sci. USA, **110**, 1006–1011 (2013).
14. **Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L., and Siegrist, H.:** Mechanisms of N_2O production in biological wastewater treatment under nitrifying and denitrifying conditions, Water Res., **46**, 1027–1037 (2012).
15. **Caranto, J. D., Vilbert, A. C., and Lancaster, K. M.:** *Nitrosomonas europaea* cytochrome P_{460} is a direct link between nitrification and nitrous oxide emission, Proc. Natl. Acad. Sci. USA, **113**, 14704–14709 (2016).
16. **White, C. J. and Lehnert, N.:** Is there a pathway for N_2O production from hydroxylamine oxidoreductase in ammonia-oxidizing bacteria? Proc. Natl. Acad. Sci. USA, **113**, 14474–14476 (2016).
17. **Bruns, A., Rohde, M., and Berthe-Corti, L.:** *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment, Int. J. Syst. Evol. Microbiol., **51**, 1997–2006 (2001).
18. **Harper, W. F., Takeuchi, Y., Riya, S., Hosomi, M., and Terada, A.:** Novel abiotic reactions increase nitrous oxide production during partial nitrification: modeling and experiments, Chem. Eng. J., **281**, 1017–1023 (2015).
19. **Kock, A. and Bange, H. W.:** Nitrite removal improves hydroxylamine analysis in aqueous solution by conversion with iron(III), Environ. Chem., **10**, 64–71 (2013).
20. **Otte, S., Schalk, J., Kuenen, J. G., and Jetten, M. S.:** Hydroxylamine oxidation and subsequent nitrous oxide production by the heterotrophic ammonia oxidizer *Alcaligenes faecalis*, Appl. Microbiol. Biotechnol., **51**, 255–261 (1999).
21. **Ai, G. M., Zheng, H. Y., Zhang, M., and Liu, Z. P.:** Isotopic confirmation of occurrence of microbial denitrification based on N_2 and N_2O production monitored by gas chromatography/isotope ratio mass spectrometry and gas chromatography/mass spectrometry, Chin. J. Anal. Chem., **39**, 1141–1146 (2011).
22. **Klatte, T., Evans, L., Whitehead, R. N., and Cole, J. A.:** Enzymology and ecology of the nitrogen cycle: four PCR primers necessary for the detection of periplasmic nitrate reductase genes in all groups of proteobacteria and in environmental DNA, Biochem. Soc. Trans., **39**, 321–326 (2011).
23. **Frear, D. S. and Burrell, R. C.:** Spectrophotometric method for determining hydroxylamine reductase activity in higher plants, Anal. Chem., **27**, 1664–1665 (1955).
24. **Simpson, P. J. L., Richardson, D. J., and Codd, R.:** The periplasmic nitrate reductase in *Shewanella*: the resolution, distribution and functional implications of two NAP isoforms, NapEDABC and NapDAGHB, Microbiology, **156**, 302–312 (2010).
25. **Farmer, J. J. and Hickman-Brenner, F. W.:** The genera *Vibrio* and *Photobacterium*, pp. 508–563, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H. and Stackebrandt, E. (Eds.), The prokaryotes, 3rd ed. Springer, New York (2006).
26. **Duan, J., Fang, H., Su, B., Chen, J., and Lin, J.:** Characterization of a halophilic heterotrophic nitrification–aerobic denitrification bacterium and its application on treatment of saline wastewater, Bioresour. Technol., **179**, 421–428 (2015).
27. **Li, C., Yang, J., Wang, X., Wang, E., Li, B., He, R., and Yuan, H.:** Removal of nitrogen by heterotrophic nitrification–aerobic denitrification of a phosphate accumulating bacterium *Pseudomonas stutzeri* YG-24, Bioresour. Technol., **182**, 18–25 (2015).
28. **Ren, Y. X., Yang, L., and Liang, X.:** The characteristics of a novel heterotrophic nitrifying and aerobic denitrifying bacterium, *Acinetobacter junii* YB, Bioresour. Technol., **171**, 1–9 (2014).

29. **Zhang, Q. L., Liu, Y., Ai, G. M., Miao, L. L., Zheng, H. Y., and Liu, Z. P.:** The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7, Bioresour. Technol., **108**, 35–44 (2012).
30. **Uygur, A. and Kargi, F.:** Salt inhibition on biological nutrient removal from saline wastewater in a sequencing batch reactor, Enzyme Microb. Technol., **34**, 313–318 (2004).
31. **Richardson, D. J., Wehrfritz, J. M., Keech, A., Crossman, L. C., Roldan, M. D., Sears, H. J., Butler, C. S., Reilly, A., Moir, J. W., and Berks, B. C.:** The diversity of redox proteins involved in bacterial heterotrophic nitrification and aerobic denitrification, Biochem. Soc. Trans., **26**, 401–408 (1998).
32. **Gross, P.:** Biologic activity of hydroxylamine: a review, Crit. Rev. Toxicol., **14**, 87–89 (1985).
33. **de Bruijn, P., van de Graaf, A. A., Jetten, M. S. M., Robertson, L. A., and Kuenen, J. G.:** Growth of *Nitrosomonas europaea* on hydroxylamine, FEMS Microbiol. Lett., **125**, 179–184 (1995).
34. **Jetten, M. S., De, B. P., and Kuenen, J. G.:** Hydroxylamine metabolism in *Pseudomonas* PB16: involvement of a novel hydroxylamine oxidoreductase, Antonie Van Leeuwenhoek, **71**, 69–74 (1997).
35. **Soler-Jofra, A., Stevens, B., Hoekstra, M., Picioreanu, C., Sorokin, D., Loosdrecht, M. C. M. V., and Pérez, J.:** Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitritation of reject water, Chem. Eng. J., **287**, 720–726 (2016).