Characterization of a novel heterotrophic nitrifying and aerobically denitrifying bacterium, *Halomonas* sp. Z8, as a potential bioagent for wastewater treatment

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Abstract

Aim : The aim of the present study was to identify and characterize the functions of key microbes mediating nitrification.

Methodology : After sampling the biofilm from a submerged biofilter in a marine aquaculture system, selective media were used to isolate microbial strains involved in nitrification. Isolates were identified using physiological and biochemical assays and sequencing of 16S rRNA gene. Nitrogen removal under different conditions was characterized. Nitrogen removal pathway was characterized by a ¹⁵N tracer experiment. Representation of key microbes in the biofilm was characterized by metagenomics analysis.

Results : Single-factor tests showed that *Halomonas* sp. strain Z8 exhibited good heterotrophic nitrification and aerobic denitrification abilities, with maximum NH₄⁺-N, NO₂⁻-N and NO₃⁻-N removal rates of 2.37, 1.28 and 1.7 mg N l⁻¹ hr⁻¹, respectively. The ¹⁵N isotope tracer experiment confirmed the aerobic nitrogen removal pathway of strain Z8. Average NO₃⁻ removal efficiencies were all above 80% in an aerated moving bed bioreactor inoculated with strain Z8 and employed to treat synthetic marine aquaculture wastewater. Metagenomic microbial community analysis revealed that *Halomonas* sp. Z8 was one of the dominant taxa at genus level, suggesting a vital role in removing nitrate from bioreactor.

Interpretation : The results of the present study indicate the potential of *Halomonas* sp. Z8 for treating wastewater in marine re-circulating aquaculture systems.

Key words: Aerobic denitrification, Aquaculture, Biofiltration, *Halomonas* sp. Z8, Heterotrophic nitrification

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Introduction

Aquaculture production continues to grow to meet the increasing global demand for seafood, with an average annual growth rate of 5.8% and production amounting to 80 million metric tons (FAO, 2018). Land-based, intensive marine aquaculture production using recirculating aquaculture systems (RAS) offer potential for bio-secure, environmentally-contained production. However, discharge of wastewater from marine aquaculture operations into the adjacent sea without effective treatment may pose threat to marine life, local environmental resources, and human activities such as bathing and fishing (Grigorakis and Rigos, 2011). Indeed, characterization of RAS wastewater (Piedrahita, 2003) showed high concentrations of suspended solids, organic matter, and nutrients such as nitrogen and phosphorous. Hence, aquaculture wastewater must be treated properly before discharge into nearby areas.

Biological treatment processes are widely used to remove nitrogenous compounds and other pollutants from aquaculture waste streams at high efficiency and low cost (Wheaton, 1993). Conventional biological nitrogen removal processes include autotrophic nitrification under aerobic conditions and heterotrophic denitrification under anaerobic conditions. With the ongoing development of biofiltration technology, there have been reports of new microbes utilizing different nitrogen removal pathways, including nitrataion, nitrifier denitrification, anaerobic ammonium oxidation (anammox), and combined pathways (Ahn, 2006). Aerobic denitrification was proposed by Robertson and Kuenen (1984) based on their work using aerobically grown cultures of *Thiosphaera pantotropa* (now known as *Paracoccus denitrificans* (Ludwig et al., 1993), showing the possibility for simultaneous nitrification and denitrification processes under aerobic conditions. Later, numerous bacteria capable of combined heterotrophic nitrification and aerobic denitrification have been isolated (Joo et al., 2005; Sarioglu et al., 2012; Zhang et al., 2012; He and Li, 2015; Chen et al., 2016; Liu et al., 2016).

Some of these bacteria have been evaluated for potential use in biological nitrogen removal systems (Padhi et al., 2013; Shoda and Ishikawa, 2014; He and Li, 2015). However, the majority of heterotrophic nitrification and aerobic denitrification bacteria used to treat freshwater may not perform efficiently in high-saline water, as high salinity can cause cell plasmolysis due to a dramatic increase in osmotic pressure and change in microbial metabolism (Vyrides and Stuckey, 2009). Only few bacteria, such as *Aeromonas* sp. (Chen et al., 2014), *Halomonas campisalis* (Guo et al., 2013), *Marinobacter* sp. (Zheng et al., 2012), *Bacillus methylotrophicus* (Zhang et al., 2012) and *Vibrio diabolus* SF16 (Duan et al., 2015) have been isolated and applied for the treatment of saline wastewaters; of these, only *Marinobacter* sp., isolated from a marine RAS biofilter (Zheng et al., 2012), has been applied for treating aquaculture wastes. In this study, a novel haloduric heterotrophic nitrifying and aerobically denitrifying bacterium, *Halomonas* sp. Z8, was

isolated and identified using phenotypic and phylogenetic analyses. Their abilities for heterotrophic nitrification and aerobic denitrification have been characterized. In addition, the nitrogen removal pathway for the strain was confirmed using ^{15}N isotope tracing method. Finally, the strain was inoculated into a biological aerated filter to treat saline wastewater, and the performance and microbial community of biofilter were characterized.

Materials and Methods

Isolation and identification of bacterial strain: Enrichment denitrifying medium (EDM) used to enrich the bacteria contained (per liter of distilled water) 3 g beef extract, 5 g peptone, 1 g KNO_3 , and 30 g NaCl ; pH 7.2-7.4. A modified bromothymol blue (BTB) medium used for bacterial isolation contained (per liter of distilled water) 1 g KNO_3 , 1 g $\text{C}_6\text{H}_5\text{Na}_3\text{O}_2 \cdot 2\text{H}_2\text{O}$, 1 g KH_2PO_4 , 1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05g CaCl_2 , 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ml BTB medium (1% ethanol), 20 g agar, and 30 g NaCl ; pH 7.2-7.4. Three different heterotrophic nitrification and aerobic denitrification media (HNM, per liter distilled water) contained 1.1 g CH_3COONa , 1 g KH_2PO_4 , 5 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 g NaCl , 0.361 g KNO_3 (for HNM-1 medium), or 0.246 g NaNO_2 (for HNM-2 medium), or 0.191 g NH_4Cl (for HNM-3 medium) and 3 ml of trace elements solution; pH 7.2-7.4. The trace elements solution contained (per liter of distilled water) 50.0 g EDTA, 5.06 g ZnSO_4 , 2.2g CaCl_2 , 5.5g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0g $(\text{NH}_4)_6\text{Mn}_7\text{O}_2 \cdot 4\text{H}_2\text{O}$, 1.57 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1.61 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; pH 7.2-7.4.

A sample of biofilm was obtained from the submerged biofilter used for treatment of re-circulating water of an aquaculture system at Laizhou Mingbo Aquatic Co. Ltd., Yantai, China. The sample was incubated with 100 ml of EDM on a rotary shaker (150 rpm) at 30°C. Nitrate and nitrite concentrations were monitored twice a day using diphenylamine reagent and Griess reagent methods (APHA et al., 2018). A 15% (V/V) bacteria suspension liquid was inoculated into freshly autoclaved EDM when nitrate content decreased, and this procedure was repeated four times. Serial dilutions (from 10^4 - 10^7) of the resulting bacterial suspension were spread onto BTB agar plate medium and incubated at 30°C for 3 days. Blue colonies were selected from BTB agar plates, transferred to solid HNM-1 medium, and held at 30°C until visible colonies were formed. A total of eight bacterial colonies were picked up and streaked onto fresh agar plates with the aim of obtaining pure colonies. The isolates were tested individually for denitrifying activity using HNM-1 medium. The Z8 strain with the highest nitrogen removal rate was selected for further experiments.

Bacterial growth was measured by monitoring optical density (OD_{600}) using a UV-7200 Unico Spectrophotometer (Shanghai Instruments Co., Ltd., Shanghai, China). Concentrations of ammonium ($\text{NH}_4^+\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$), and total nitrogen (TN) were determined by hypochlorite oxidation photometry, N-(1-naphthalene)-diaminoethane photometry, zinc-cadmium reduction and potassium persulfate

oxidation-UV methods, respectively (APHA, 1998). Micrographs of Z8 were observed using a Philips Model TM-1000 scanning electron microscope (SEM) using the following procedure: Strain Z8 was first fixed with 2.5% glutaraldehyde in phosphate buffer for more than 4 hrs; washed three times in phosphate buffer; then post-fixed with 1% OsO_4 in phosphate buffer for an hour and washed three times in phosphate buffer. The specimen was first dehydrated graded series of ethanol (30, 50, 70, 80, 90, 95 and 100%) for about 15 to 20 min at each step, transferred to a mixture of alcohol and isoamyl acetate (v:v=1:1) for about 30 minutes, and then transferred to pure isoamyl acetate for about an hour. At the end, the specimen was dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid CO_2 . The dehydrated specimen was coated with gold-palladium, and observed and photographed using SEM.

Physiological and biochemical analyses of strain Z8, including catalase and oxidase activities, hydrogen sulfide production, Voges-Proskauer test, methyl red test, indole production and glucose fermentation citrate test were performed according to Bergey's Manual (Holt *et al.*, 1994). To identify the microbe, the 16S rRNA gene of isolated bacterium Z8 was amplified by polymerase chain reaction using bacterial universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TTGGYTACCTGTACGACT-3') and sequenced by Majorbio Biopharm Technology Co. Ltd. (Shanghai, China). DNA sequence alignment and search were performed using the Basic Local Alignment and Search Tool (BLAST). A consensus phylogenetic tree was constructed by neighbor-joining method implemented within the MEGA5.0 program (Tamura *et al.*, 2011).

Nitrogen removal performance under different conditions:

Single-factor experiments were conducted in flasks using media (100 mg l⁻¹ of HNM-1, HNM-2, or HNM-3) to assess how C/N ratio, temperature, pH, dissolved oxygen (DO) and salinity affected the nitrogen removal efficiency of strain Z8. Sodium acetate was selected as the sole carbon source for all the assays as it proved the best carbon source in our previous study (unpublished data). As C/N ratio is an important factor affecting heterotrophic nitrification and aerobic denitrification, different C/N ratios (10, 15, 20, 25, or 30) were utilized to determine optimal C/N ratios for the bacterium. To produce such ratios, for medium HNM-1, for example, using CH_3COONa (C% = 24/82) and KNO_3 (N% = 14/101), for a C/N ratio = 10, our use of 1.75g of CH_3COONa and 0.361g of KNO_3 yielded a C/N ratio = $(1.75 \times 24/82) / (0.361 \times 14/101) = 10.2$. Masses of reagents required to obtain other C/N ratios were calculated similarly. Other experimental conditions were: initial nitrogen concentration 100 mg l⁻¹-N (using NaNO_3 , NaNO_2 , or NH_4Cl), initial pH 7, culture temperature 25°C, shaking speed 160 rpm and salinity 30‰. Temperature is one of the important parameters affecting nitrogen removal process, so the culture temperature was adjusted to 20, 25, 30 or 35°C. Other experimental conditions were: C/N ratio 20, pH 7, shaking speed 160 rpm and salinity 30‰. Bacterial growth was affected significantly by pH (Guo *et al.*, 2014), initial pH was adjusted to 6, 7, 8, 9, or 10 to assess nitrogen removal performance, and other experimental conditions were: C/N ratio 20, temperature 25°C,

shaking speed 160 rpm and salinity 30‰. In the aerobic denitrification process, both oxygen and nitrate act as electron acceptors, but oxygen also can inhibit nitrous oxide reductase, which may enhance the production of N_2O (Yang *et al.*, 2012). In order to clarify the effect of DO concentration on the nitrogen removal of strain Z8, different DO concentrations were achieved by changing the shaking speeds (80, 120, 160, or 200 rpm). Other experimental conditions were: C/N ratio 20, pH 7, temperature 25°C and salinity 30‰. As salinity affects the nitrification or denitrification efficiencies of particular microbes, salinity was set to 0-5% (w/v) by adjusting NaCl concentration in the medium. Other experimental conditions were: C/N ratio 20, pH 7, temperature 25°C and shaking speed of 200 rpm.

All the experiments were conducted in 160 ml flasks with 50 ml of sterile medium with an inoculation dosage of 1% (v/v). The concentrations of nitrate nitrogen, ammonium nitrogen and nitrite nitrogen were determined. One ml of pure-culture of strain Z8 was inoculated in 250 ml conical flask containing 100 ml HNM-1, HNM-2 or HNM-3, incubated at 25°C and 160 rpm for 48 hrs. Samples were taken periodically for the determination of OD600, nitrate, nitrite and ammonia nitrogen. All experiments were performed in triplicate. Strain Z8 was inoculated in 100 ml HNM-1, HNM-2 or HNM-3 containing K^{15}NO_3 , $\text{Na}^{15}\text{NO}_2$ or $^{15}\text{NH}_4\text{Cl}$, respectively, in 250 ml serum bottles in a nitrogen gas-free system. The bottles were first evacuated, fully aerated with pure oxygen, and sealed tightly with a rubber stopper. The bottles were then incubated at 25°C at 160 rpm in a rotary shaker. The ^{15}N isotopic ratios of N_2O and N_2 in the headspace of the sample were measured after 48 hrs in a hermetic incubation by isotope ratio mass spectrometry (MAT271, Thermo-Finnigan, San Jose, CA, USA) at the Shanghai Engineering Research Center of Stable Isotopes (Shanghai, China). The relative ^{15}N isotopic abundance ($\delta^{15}\text{N}$) was calculated by the equations given by Mariotti (1983) and Ye *et al.* (2016).

Denitrification performance in aerated moving bed bioreactor:

A MBBR system was set up to treat saline wastewater. The system contained a submerged biofilter, wastewater tank, peristaltic pump, air blower, air pump, and heater. The submerged biofilter was modified with a PVC bucket (13 cm inner diameter, 25 cm high) with a capacity of 2.2 l. The inlet port was located close to the bottom opposite the outlet port at a height of 18 cm. The biofilter utilized Kaldness K3 polyethylene beads (190 g) for biofilm colonization. Air from the blower was forced through a diffuser to maintain dissolved oxygen and to provide mixing. The reactor was placed in a temperature-controlled ($25 \pm 1^\circ\text{C}$) water bath. To investigate the denitrification performance of isolated strain in an engineering application, the halophilic bacterium Z8 was activated and 10 ml mixture was inoculated into the MBBR to set up the biofilter. Synthetic marine waste water was used (modified with the HNM-3 medium) with high nitrate concentration (50, 150 and 250 mg l⁻¹) in the experiment. The pump was not started for one day in order to allow the establishment of bacteria before starting continuous feeding. Flushing the reactors with a large flow rate washed most

of the inoculum from the reactors. The pump was then turned on to provide wastewater flow through biofilter at a flow rate of 9.12 ml min⁻¹. The hydraulic retention time in the biofilter was 4 hrs. Waters samples from the influent and effluent of the biofilters were analyzed on alternate days.

Microbial community analysis in MBBR: Biofilm was collected to investigate the abundance of bacterium Z8 in MBBR. Continuous feeding was maintained until a steady-state culture, as evidenced by a stable removal rate, was attained for each particular inlet nitrate concentration. The microbial community was analyzed by high-throughput Illumina-MiSeq DNA sequencing method used in our previous study (Huang et al., 2016). Raw data were deposited to NCBI short-reads archive database (accession number PRJNA415345).

Results and Discussion

Cells of strain Z8 were white and exhibited a short rod shape with a size of approximately 0.3–0.5 µm diameter and

1.2–1.5 µm length. Strain Z8 was Gram negative when stained and showed positive results in oxidase reaction, catalase reaction, starch hydrolysis and sugar fermentation tests. The results of urea hydrolysis, lipid hydrolysis, gelatin liquefaction, Voges–Proskauers and methyl red tests were negative. The 16S rRNA gene sequence of strain Z8 (1177 bp) was determined. A search of archived sequences in Gen Bank showed high homology to Accession Number MF575810, indicating that strain Z8 was closely related to *Halomonas venusta* (Fig. 1). Strain Z8 was deposited in Marine Culture Collection of China (MCCC NO.1K03474).

Carbon is essential for cell growth and nitrate reduction processes, and aerobic denitrification is related to heterotrophic nitrification. An optimal C/N ratio is usually required for a bacterium exhibiting both heterotrophic nitrification and aerobic denitrification. In the present study, the C/N ratio significantly ($p < 0.05$) affected the ammonium-N and nitrate-N removal efficiencies (Table 1). The removal efficiencies for ammonium-N and nitrate-N increased with the C/N ratio from 10 to 30, and the

Fig. 1: Phylogenetic tree of strain Z8 and related bacteria based on partial 16S rRNA sequence. Tree was constructed using neighbor-joining method with bootstrap values from 1000 replications.

Fig. 2: Cell growth and nitrogen removal characteristics of strain Z8 using (A) ammonium, (B) nitrite or (C) nitrate as nitrogen source. Symbols: OD600, circles; ammonium, squares; nitrite, triangles; and nitrate, diamonds.

highest removal efficiencies of 99.87 and 99.95 for $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, respectively, were observed at a C/N ratio of 30. The lowest removal efficiency attained at low C/N ratio of 10 and might be due to exhaustion of carbon source for heterotrophs. The nitrite-N removal rates sharply decreased at high C/N ratios, and were only 7.23% and 15.73 at ratio of 25 and 30, respectively. Hence, the optimal C/N ratio might be considered as 20, a ratio higher than those reported by Joo *et al.* (2005), Taylor *et al.* (2009) and Chen and Ni (2012). The C/N ratio test showed the potential

of Z8 for wastewater treatment, where carbon content is usually high. Strain Z8 exhibited efficient nitrogen species removal ability over a wide range of temperatures (Table 1). Ammonium-N removal rates were higher than 99% and temperature did not affect removal rates ($p > 0.05$). Nitrate removal efficiencies were similar and higher than 90%. Nitrite removal rate declined ($p < 0.05$) with increasing temperature from 20°C to 35°C, although the removal rates were above 80%. These observations showed that strain Z8 was a mesophilic bacterium maintaining high

Fig. 3: Denitrification performance of mixed bed biological reactor inoculated with strain Z8.

nitrogen removal efficiency across moderate temperatures. Strain Z8 performed efficient nitrification in acid, neutral and alkaline environments. The ammonium-N removal rates were more than 93% at pH 6-10. Strain Z8 exhibited excellent nitrite removal efficiencies at both neutral and alkaline environments with removal rates above 95% at pH between 7 and 10, but poor removal efficiency at pH 6. The nitrate removal rate peaked at 91.91% at pH 7 and diminished at higher pH values.

Denitrification is generally thought to occur under anoxic or at low DO concentration for different kinds of denitrifiers (Ji et al., 2015). The effect of DO concentration on nitrogen removal was investigated by adjusting the shaking speed used in our assays. As shown in Table 1, the nitrate and nitrite removal rates were more than 99% at 160 and 200 rpm, which were significant by higher ($p < 0.05$) than those at low DO conditions (at shaking speeds of 80 or 120 rpm). These results showed that strain Z8 could tolerate high DO concentrations ($> 5 \text{ mg l}^{-1}$), similar to the results of *Pseudomonas stutzeri* strain YZN-001 (Zhang et al., 2011), D6 (24), and X31 (Ji et al., 2014). Similarly, strain Z8 strain exhibited high average removal rates under 1-5‰ but not at 0 ppt salinity. Hence, the strain might be suitable for application in saline wastewater treatment. We used ammonium-N as the sole nitrogen source to investigate the heterotrophic nitrification activity of strain Z8. A significant decrease in ammonium concentration was observed and ammonium was almost undetectable by 24 hr (Fig. 2a), reaching 0.02 mg l^{-1} at 48 hr. Cell growth curves for strain Z8 measured as OD_{600} showed a lag phase over the first 8 hr, followed by exponential growth from 8 to 32 hr, and then reached stationary phase. Ammonium removal efficiency reached 97.2% at 24 hr, with a nitrification rate of 2.37

$\text{mg NH}_4^+\text{-N l}^{-1} \text{ hr}^{-1}$ (Fig. 2a). The intermediate nitrite was accumulated during early part of the experiment and eventually declined to zero around 48 hr.

Aerobic denitrification abilities were evaluated using nitrite and nitrate as the sole nitrogen sources under anaerobic condition, with results respectively shown in Figs. 2b and 2c. Cell growth curves for both compounds showed a long initial lag phase, which might be due to the strain needing time to adjust its metabolism in order to adapt to the environment. The strain then began to grow rapidly with the decrement of nitrate or nitrite, and then reached the stationary phase. The removal efficiency of nitrite reached a peak value of 97.8%, with a removal rate of $1.7 \text{ mg NO}_2\text{-N l}^{-1} \text{ hr}^{-1}$ at 32 hr. The removal efficiency of nitrate reached 92.9%, with a removal rate of $1.28 \text{ mg NO}_3\text{-N l}^{-1} \text{ hr}^{-1}$ at 40 hr. No intermediate products accumulated with decrease in nitrite content during the experiment (Fig. 2b); nitrite accumulated with decrease in nitrate content and then reduced to a final concentration of 0.04 mg l^{-1} after 48 hr showing that nitrate was first converted in to nitrite and then to nitrogen gas.

To investigate the metabolic pathway of strain Z8, an ^{15}N isotope tracing experiment was conducted. The final products of heterotrophic nitrification and aerobic denitrification were detected by isotope ratio mass spectrometry, and the results showed that $^{15}\text{N}_2\text{O}$ and $^{15}\text{N}_2$ were detected after incubation in the respective media. Using HDM-1, which had nitrate as N source, 107‰ $\delta^{15}\text{N}$ of N_2 and 1001‰ of N_2O were detected. Using HDM-2, which had nitrite as N source, 88‰ $\delta^{15}\text{N}$ of N_2 and 798‰ of N_2O were detected. Using HDM-3 media, which used ammonium as

Fig. 4: Relative frequencies of (A) phyla and (B) genera or other taxa detected in biofilter in all periods.

Table 1: TAN, nitrite and nitrate removal efficiencies for strain Z8 in 48 hr under different conditions^a

Factor	Level			
		NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N
C/N ratio	10	52.06±0.53 ^a	85.94±0.82 ^a	81.54±1.77 ^c
	15	71.77±0.20 ^b	99.29±0.03 ^a	99.31±2.16 ^a
	20	99.23±0.60 ^c	92.28±0.12 ^a	91.91±2.27 ^b
	25	99.50±0.36 ^c	7.23±1.42 ^b	99.75±0.51 ^a
	30	99.87±0.22 ^c	15.73±1.92 ^c	99.95±0.06 ^a
Temperature (°C)	20	99.7±0.12 ^a	99.89±2.19 ^a	-
	25	99.23±0.60 ^a	92.19±0.12 ^b	91.91±2.26 ^b
	30	99.14±0.04 ^a	84.53±0.72 ^c	96.27±0.38 ^a
	35	99.69±0.18 ^a	81.22±1.94 ^c	99.40±0.13 ^a
pH	6	98.26±0.17 ^a	10.53±2.29 ^a	26.61±2.67 ^a
	7	99.23±0.60 ^a	92.19±0.12 ^{bc}	91.91±2.26 ^b
	8	94.99±2.46 ^{ab}	95.45±3.64 ^c	84.66±3.69 ^c
	9	93.31±1.19 ^b	98.66±0.03 ^c	31.99±5.19 ^a
	10	93.12±0.31 ^b	98.61±0.03 ^c	32.63±3.86 ^a
Shaking speed (rpm)	80/4.37	99.85±0.03 ^a	56.06±2.56 ^a	29.95±16.42 ^a
DO (mg l ⁻¹)	120/5.72	98.09±0.18 ^b	70.49±3.22 ^b	44.76±13.96 ^a
	160/6.08	99.23±0.60 ^b	92.19±0.12 ^c	91.91±2.26 ^b
	200/7.11	98.82±0.82 ^b	98.89±0.22 ^a	99.53±0.03 ^c
Salinity (%)	0	1.89±0.02 ^b	3.09±0.22 ^b	1.01±0.13 ^b
	2	95.72±2.35 ^a	98.96±0.00 ^a	90.21±5.64 ^a
	2.5	95.42±2.26 ^a	98.77±0.06 ^a	92.80±2.89 ^a
	3.0	98.82 ± 0.82 ^a	98.89 ± 0.22 ^a	99.53 ± 0.03 ^b
	3.5	96.64±0.40 ^a	98.74±0.02 ^a	96.79±4.34 ^{ab}
	4	93.73±0.89 ^a	98.74±0.04 ^a	99.73±0.28 ^b
	5	93.84±1.25 ^a	98.64±0.08 ^a	-

^aValues followed by different letters in the same column were significantly different at $p < 0.05$

the N source, 121‰ $\delta^{15}\text{N}$ of N_2 and 890‰ of N_2O were observed. Because all values of $\delta^{15}\text{N}$ were greater than zero, we concluded that the ^{15}N -labelled gaseous nitrogen in the gas samples came from the ^{15}N -labelled culture. The results also suggest that strain Z8 can aerobically convert K^{15}NO_3 and $\text{Na}^{15}\text{NO}_2$ to $^{15}\text{N}_2\text{O}$ and $^{15}\text{N}_2$. $^{15}\text{NH}_4\text{Cl}$ was aerobically converted to N_2O and N_2 since the labelled ^{15}N in $^{15}\text{NH}_4\text{Cl}$ was detected in ^{15}N -labelled N_2O and N_2 . Hence, the aerobic metabolic pathway of strain Z8 might be $\text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$ for nitrification and $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ for denitrification.

A continuous moving bed biological reactor (MBBR) was set up to assess denitrification ability under aerobic conditions. We used nitrate as the sole N source in the influent. The performance of MBBR inoculated with strain Z8 is shown in Fig. 3. In period I and II, the influent nitrate concentration was 48.3 mg l⁻¹ and 149.3 mg l⁻¹, and the average effluent nitrate concentration was about 1.4 and 14.5 mg l⁻¹. The majority of nitrate was removed in MBBR, and the removal efficiency was more than 90%. With a further increase in influent nitrate concentration in period III, the nitrate removal efficiency decreased, but was maintained above

80%. Apart from fluctuation over the first few days, no obvious nitrite accumulation in the effluent was observed during the whole experiment (data not shown). The microbial community of biofilter was characterized in order to gain insight into the relative importance of our focal strain, *Halomonas* sp. Z8. The microbial communities sampled at different time-periods were analyzed using Illumina high-throughput DNA sequencing. Among all the samples, 11 phyla were detected through the experiment (Fig. 4A). Of all the samples, Proteobacteria (average relative abundance, 62.8%) and Bacteroidetes (average, 14.7%) were the most dominant phyla, followed by Cloacimonetes (average, 8.3%), Spirochaetae (average, 3.1%) and Firmicutes (average, 2.5%).

At genus level, samples from three periods exhibited similar bacterial community richness (Fig. 4B). The dominant genera in three samples were unclassified *Rhodobacteraceae* (7.2-14.2%), *Cohaesibacter* (6.4-13.1%), *Desulfopila* (0.4-4.78%), and *Marinicella* (1.1-4.5%). The abundance of aerobic denitrification bacterium *Halomonas* Z8 was 3.3%, 4.6% and 7.9% for period I, II and III, showing increasing frequency with rising inlet nitrate concentrations. As other common denitrifying bacteria (e.g., *Pseudomonas*, *Alkaligenes* or *Bacillus* species)

were not found in MBBR biofilter, bacterium *Halomonas* sp. Z8 likely played a vital role in removing nitrate MBBR. Our results showed that the bacterium *Halomonas* sp. Z8 could remove ammonium, nitrite and nitrate efficiently under high-salinity, aerobic conditions with little accumulation of intermediates. The average removal rates were more than 90% under 1-5‰ salinity (w/v), but not at 0 ppt salinity.

The latter observation may be due to the fact that NaCl is essential for maintaining the enzymatic activities. When *Halomonas* sp. Z8 was isolated, heterotrophic carbon (CH_3COONa) was used in the media and synthetic wastewater; strain Z8 could perform nitrification and denitrification with carbon as its energy source, indicating that the isolate has a heterotrophic phenotype. Because of its heterotrophic nitrification and aerobic denitrification abilities under saline conditions (1-5‰), strain Z8 can be characterized as a halophilic bacterium (Ventosa et al., 1998). This characteristic expands its potential scope of application, regardless of the treatment of municipal wastewater with low salinity or other wastewaters with high salinity, such as aquaculture and seafood-processing wastewaters. Yoshie et al. (2006) also characterized a *Halomonas* bacterium that clustered closely with *H. venusta* that effectively mediated denitrification at 2‰ or 10‰ salinity with better results at higher salinity. We were able to identify *Halomonas* bacterium more thoroughly because the database for 16S rRNA sequence has improved over the intervening time; our strain also was more effective at higher salinity.

Ammonium removal efficiency in this study reached 97% at 24 hr (Fig. 2a), with a nitrification rate of $2.37 \text{ mg NH}_4^+-\text{N l}^{-1} \text{ hr}^{-1}$. These results were similar to two other halophilic bacteria, $2.29 \text{ mg NH}_4^+-\text{N l}^{-1} \text{ hr}^{-1}$ for *Vibrio diabolicus* SF16 (Duan et al., 2015) and $2.15 \text{ mg NH}_4^+-\text{N l}^{-1} \text{ hr}^{-1}$ for *Bacillus methylotrophicus* (Zhang et al., 2012), and lower than $8.33 \text{ mg NH}_4^+-\text{N l}^{-1} \text{ hr}^{-1}$ for *Halomonas campisalis* ha³ (Guo et al., 2013). The nitrate removal rate for strain Z8 was similar to that of *Marinobacter* sp. ($1.23 \text{ mg NO}_3^--\text{N l}^{-1} \text{ hr}^{-1}$; Zheng et al. 2012), and less than that of *Vibrio diabolicus* Sf16 ($2.83 \text{ mg NO}_3^--\text{N l}^{-1} \text{ hr}^{-1}$; Duan et al., 2015).

Heterotrophic nitrification and aerobic denitrification bacteria showed significantly different N_2O and N_2 production under aerobic denitrification conditions. In this study, strain Z8 produced more N_2O than N_2 , similar to the results reported for *Providencia rettgeri* YL (Ye et al., 2016), *Pseudomonas alcaligenes* (Su et al., 2006), *Pseudomonas stutzeri* (Takaya et al., 2003) and *Alcaligenes faecalis* TUD (Otte et al., 1996). N_2O was the dominant product perhaps due to oxygen tolerance of nitrous oxide reductase (Nor) enzyme of the bacterium. High concentration of oxygen may decrease the activity of Nor, which is related to the conversion of N_2O to N_2 (Berks et al., 1995). Halophilic microbes pose a wide range of biotechnological applications, including degradation of wastes borne in saline waters (Zhuang et al., 2010). In our study, we observed high rate of nitrate removal in MBBR, indicating that the filter seeded with

Halomonas sp. Z8 could treat saline wastewater from RAS effectively. In commercial marine RAS operations, the wastewater contain high levels of nitrate and organic carbon (from uneaten feed and fish excretion).

However, the C/N ratio of wastewater from commercial recirculating aquaculture system generally lower whereas is this it was optimal. Hence, a suitable carbon source is required and carbon should be added to the reactor for effective treatment of many aquaculture wastewaters. Use of different carbon sources affect denitrification performance (Xu et al., 2019), suggesting the advisability of evaluating different sources for use with aquaculture wastewaters. Solid-phase denitrification, in which biodegradable polymers provide both attachment and a source of carbon, can be applied for the treatment of aquaculture wastewater (Wang and Chu, 2018). Denitrifying sludge accumulated over time was found effective as seed sludge for establishing a denitrifying community for wastewater treatment (Yoshie et al., 2006b). Collectively, these findings can contribute to the advancement of aquaculture wastewater treatment processes.

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References

- Ahn, Y. H.: Sustainable nitrogen elimination biotechnologies, A review. *Process Biochem.*, **41**, 1709-1721 (2006).
- APHA (American Public Health Association): American Water Works Association, Water Environment Federation Standard Methods for the Examination of Water and Wastewater. 23rd Edn., American Public Health Association, Washington, D.C. (2018).
- Berks, B.C., S.J. Ferguson, J.W. Moir and D.J., Richardson: Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta*, **1232**, 97-173 (1995).
- Chen, J., S. Gu, H. Hao and J. Chen.: Characteristics and metabolic pathway of *Alcaligenes* sp. TB for simultaneous heterotrophic nitrification-aerobic denitrification. *Appl. Microbiol. Biotechnol.*, **100**, 9787-9794 (2016).
- Chen, M., W.C. Wang, Y. Feng, X.H. Zhu, H.Z. Zhou, Z.L. Tan and X.D. Li: Impact resistance of different factors on ammonia removal by heterotrophic nitrification-aerobic denitrification bacterium *Aeromonas* sp. N-02. *Biores. Technol.*, **167**, 456-461 (2014).
- Chen, Q. and J. Ni: Ammonium removal by *Agrobacterium* sp. LAD9 capable of heterotrophic nitrification – aerobic denitrification. *J. Biosci. Bioeng.*, **113**, 619-623 (2012).
- Duan, J., H. Fang, B. Su, J. Chen, J. and J. Lin: Characterization of a halophilic heterotrophic nitrification-aerobic denitrification bacterium and its application on treatment of saline wastewater. *Biores. Technol.*, **179C**, 421-428 (2015).
- FAO: Fishery and Aquaculture Statistics, Food and Agriculture

- Organization of the United Nations, Rome (2018).
- Grigorakis, K. and G. Rigos: Aquaculture effects on environmental and public welfare-the case of Mediterranean mariculture. *Chemosphere*, **85**, 899-919 (2011).
- Guo, L.Y., Z.X. Hu, F. Fang, T. Liu, X.M. Chuai and L.Y. Yang: Trophic status determines the nirS-denitrifier community in shallow freshwater lakes. *Ann. Microbiol.*, **64**, 999-1006 (2014).
- Guo, Y., X.M. Zhou, Y.G. Li, K. Li, C.X. Wang, J.F. Liu, D. Yan, Y.L. Liu, D.H. Yang and J.M. Xing: Heterotrophic nitrification and aerobic denitrification by a novel *Halomonas campisalis*. *Biotechnol. Lett.*, **35**, 2045-2049 (2013).
- He, T.X. and Z.L. Li: Identification and denitrification characterization of a novel hypothermia and aerobic nitrite-denitrifying bacterium, *Arthrobacter arilaitensis* strain Y-10. *Desalin. Water Treat.*, **57**, 19181-19189 (2015).
- Holt, J., N. Krieg, P. Sneath and J. Staley: Bergey's Manual of Determinative Bacteriology. 9th Edn., Lippincott, Williams & Wilkins, Philadelphia, PA (1994).
- Huang, Z., R. Wan, X. Song, Y. Liu, E. Hallerman and D. Dong: Metagenomic analysis shows diverse, distinct bacterial communities in biofilters among different marine recirculating aquaculture systems. *Aquacult. Inter.*, **24**, 393-1408 (2016).
- Ji, B., H. Wang and K. Yang: Tolerance of an aerobic denitrifier (*Pseudomonas stutzeri*) to high O₂ concentrations. *Biotechnol. Lett.*, **36**, 719-722 (2014).
- Ji, B., K. Yang, L. Zhu, Y. Jiang, H. Wang, J. Zhou and H. Zhang: Aerobic denitrification: A review of important advances of the last 30 years. *Biotechnol. Bioeng.*, **20**, 643-651 (2015).
- Joo, H., M. Hirai and M. Shoda: Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. *J. Biosci. Bioeng.*, **100**, 184-191 (2005).
- Liu, Y., G.M. Ai, L.L. Miao and Z.P. Liu: Marinobacter strain NNA5, a newly isolated and highly efficient aerobic denitrifier with zero N₂O emission. *Biores. Technol.*, **206**, 9-15 (2016).
- Ludwig, W., G. Mittenhuber and C.G. Friedrich: Transfer of *Thiosphaera pantotropa* to *Paracoccus denitrificans*. *Int. J. Syst. Bacteriol.*, **43**, 363-367 (1993).
- Mariotti, A.: Atmospheric nitrogen is a reliable standard for natural ¹⁵N abundance measurements. *Nature*, **303**, 685-687 (1983).
- Otte, S., N.G. Grobbsen, L.A. Robertson, M.S. Jetten and J.G. Kuenen: Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. *Appl. Environ. Microbiol.*, **62**, 2421-2426 (1996).
- Padhi, S.K., S. Tripathy, R. Sen, A.S. Mahapatra, S. Mohanty and N.K. Maiti: Characterization of heterotrophic nitrifying and aerobic denitrifying *Klebsiella pneumoniae* CF-S9 strain for bioremediation of wastewater. *Int. Biodeterior. Biodegrad.*, **78**, 67-73 (2013).
- Piedrahita, R.H.: Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. *Aquaculture*, **226**, 35-44 (2003).
- Robertson, L.A. and J.G. Kuenen: Aerobic denitrification: A controversy revived. *Arch. Microbiol.*, **139**, 351-354 (1984).
- Sarioglu, O.F., R. Suluyayla and T. Tekinay: Heterotrophic ammonium removal by a novel hatchery isolate *Acinetobacter calcoaceticus* STB1. *Int. Biodeterior. Biodegrad.*, **71**, 67-71 (2012).
- Shoda, M. and Y. Ishikawa: Heterotrophic nitrification and aerobic denitrification of high-strength ammonium in anaerobically digested sludge by *Alcaligenes faecalis* strain No. 4. *J. Biosci. Bioeng.*, **117**, 737-741 (2014).
- Su, J.J., K.S. Yeh and P.W. Tseng: A strain of *Pseudomonas* sp. isolated from piggery wastewater treatment systems with heterotrophic nitrification capability in Taiwan. *Curr. Microbiol.*, **53**, 77-81 (2006).
- Takaya, N., M. Catalan-Sakairi, Y. Sakaguchi, I. Kato, Z. Zhou and H. Shoun: Aerobic denitrifying bacteria that produce low levels of nitrous oxide. *Appl. Environ. Microbiol.*, **69**, 3152-3157 (2003).
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molec. Biol. Evol.*, **28**, 2731-2739 (2011).
- Taylor, S.M., Y. He, B. Zhao and J. Huang: Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL. *J. Environ. Sci.*, **21**, 1336-1341 (2009).
- Ventosa, A., J.J. Nieto and A. Oren: Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.*, **62**, 504-544 (1998).
- Vyrides, I. and D.C. Stuckey: Adaptation of anaerobic biomass to saline conditions: Role of compatible solutes and extracellular polysaccharides. *Enz. Microb. Technol.*, **44**, 46-51 (2009).
- Wang, J. and L. Chu: Biological nitrate removal from water and wastewater by solid-phase denitrification process. *Biotech. Adv.*, **34**, 1103-1112 (2016).
- Wheaton, F.W.: Aquacultural Engineering. Krieger Publ. Co., Malabar, FL (1993).
- Xu, Z., X. Dai and X. Chai: Effect of different carbon sources on denitrification performance, microbial community structure and denitrification genes. *Sci. Total. Envir.*, **634**, 195-204 (2018).
- Yang, X., S. Wang and L. Zhou: Effect of carbon source, C/N ratio, nitrate and dissolved oxygen concentration on nitrite and ammonium production from denitrification process by *Pseudomonas stutzeri* D6. *Bioresour. Technol.*, **104**, 65-72 (2012).
- Ye, J., B. Zhao, Q. An and Y. Huang: Nitrogen removal by *Providencia rettgeri* strain YL with heterotrophic nitrification and aerobic denitrification. *Environ. Technol.*, **37**, 2206-2213 (2016).
- Yoshie S., T. Ogawa, H. Makino, H. Hirosawa, S. Tsuneda and A. Hirata: Characteristics of bacteria showing high denitrification activities in saline wastewater. *Lett. Appl. Microbiol.*, **42**, 277-283 (2006a).
- Yoshie, S., H. Makino, H. Hirosawa, K. Shirotani, A. Tsuneda and A. Hirata: Molecular analysis of halophilic bacterial community for high-rate denitrification of saline industrial wastewater. *Environ. Biotech.*, **72**, 182-189 (2006b).
- Zhang, J., P. Wu, B. Hao and Z. Yu: Heterotrophic nitrification and aerobic denitrification by the bacterium *Pseudomonas stutzeri* YZN-001. *Bioresour. Technol.*, **102**, 9866-9869 (2011).
- Zhang, Q.L., Y. Liu, G.M. Ai, L.L. Miao, H.Y. Zheng and Z.P. Liu: The characteristics of a novel heterotrophic nitrification aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. *Bioresour. Technol.*, **108**, 35-44 (2012).
- Zheng, H.Y., Y. Liu, X.Y. Gao, G.M. Ai, L.L. Miao and Z.P. Liu: Characterization of a marine origin aerobic nitrifying-denitrifying bacterium. *J. Biosci. Bioeng.*, **114**, 33-37 (2012).
- Zhuang, X., Z. Han, Z. Bai, G. Zhuang and H. Shim: Progress in decontamination by halophilic microorganisms in saline wastewater and soil. *Environ. Pollut.*, **158**, 1119-1126 (2010).