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Diversity of culturable aerobic denitrifying bacteria in a rotating biological contactor combined with anaerobic-anoxic-oxic-oxic wastewater treatment system

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ABSTRACT

Recent studies have confirmed the existence of aerobic denitrifying bacteria, particularly exploring their diversity and heterotrophic nitrification-aerobic denitrification capability in a wastewater treatment plant (WWTP). In this study, the sewage treatment technology, which combined a rotating biological contactor (RBC) with an anaerobic-anoxic-oxic-oxic (A^2/O^2) process, demonstrated strong removal efficiency for nitrate pollutants. Five types of activated sludge in a WWTP were used to isolate 226 strains of aerobic denitrifying bacteria, which were classified into 12 genera based on the 16S rDNA. *Pseudomonas stutzeri*, *Pseudomonas monteilii*, and *Gordonia cholesteroliborans* were the most abundant aerobic denitrifying bacteria in the five types of activated sludge. *P. stutzeri*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas chengduensis*, *Beijerinckia fluminensis*, and *Arthrobacter protophormiae* demonstrated strong heterotrophic nitrification-aerobic denitrification capacity. *G. cholesteroliborans*, *Pseudomonas pseudoalcaligenes*, *P. chengduensis*, and *B. fluminensis* also showed aerobic denitrifying capacity, which was not reported previously. The Venn diagram and the relative abundance diagram revealed the different structures of aerobic denitrifiers among the five types of activated sludge. This study may provide a reference for screening and application of aerobic denitrifying bacteria in WWTPs.

Keywords: Wastewater treatment system; Nitrogen removal; Heterotrophic nitrification; Aerobic denitrification; Aerobic denitrifying bacteria

1. Introduction

The rapid development of agriculture, industrialization, and urbanization has become the main cause of water nitrogen pollution including nitrate pollution. As nitrate compounds released through industrial and domestic activities have accumulated in lakes, rivers, and wetlands worldwide, nitrate pollution has become a severe environmental problem [1]. Wastewater contains excessive amounts of nitrates and nitrites, which could lead to eutrophication, resulting in the increase of phytoplankton biomass and sometimes algal toxins [2]. Furthermore, excessive levels of nitrate in drinking water could lead to stomach cancer [3,4].

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Therefore, the reduction of nitrate content in water is crucial for controlling water pollution. Traditionally, nitrate removal has been performed through denitrification by anaerobic heterotrophs [5]. However, anaerobic heterotrophs are more sensitive to the dissolved oxygen (DO) content in water, which is a key factor affecting the transformation of nitrate. Therefore, separation of aerobic and anoxic tanks leads to complex problems, such as increased costs due to large area requirement [6,7]. Most denitrifying bacteria are facultative anaerobic bacteria, such as *Proteus*, *Pseudomonas*, *Micrococcus*, *Bacillus*, and *Achromobacter*.

Several studies have reported the existence of aerobic denitrifying bacteria. *Thiosphaera pantotropha* (now known as *Paracoccus pantotrophus*) [8] was first isolated from a wastewater treatment plant (WWTP) in 1983 [9]. Since then, numerous aerobic denitrifying bacteria have been isolated. *Pseudomonas aeruginosa* was isolated from oil-contaminated soil in 1990 [10], *Alcaligenes faecalis* No. 4 was isolated from sewage sludge in 2005 [11], *Zoogloea* sp. N299 was isolated from a drinking water reservoir in 2015 [12], and *Bacillus cereus* PB88 was isolated from shrimp water in 2018 [13]. These aerobic denitrifying bacteria exhibited several advantages in terms of nitrate removal and some of them possessed heterotrophic nitrification ability [14]. Although there are many studies that reported the existence of aerobic denitrifying bacteria in different environmental sites, only a few studies on the characterization and distribution of aerobic denitrifying bacteria in WWTP have been reported.

In this study, we attempted to isolate aerobic denitrifiers from the activated sludge of a rotating biological contactor (RBC) combined with an anaerobic-anoxic-oxic-oxic (A^2/O^2) wastewater treatment system [15,16]. In addition, the performance of aerobic denitrification and heterotrophic nitrification-aerobic denitrification was investigated [17].

2. Materials and methods

2.1. WWTP description and sample collection

The samples were collected from a full-scale wastewater WWTP located in Qinghai Province, China. The wastewater treatment system consisted of the RBC and A^2/O^2 parts (Fig. 1). The DO content of the RBC tank, anaerobic tank 1, anoxic tank 2, oxic tank 3, and oxic tank 4 were 0.88 ± 0.42, 0.08 ± 0.04, 0.34 ± 0.34, 2.99 ± 1.11, and 2.04 ± 1.28 mg/L, respectively. Furthermore, the water quality was tested, and the result showed that the effluent water quality met the national grade A standard.

Activated sludge samples were collected from the RBC tank, anaerobic tank 1, anoxic tank 2, oxic tank 3, and oxic tank 4 on July 8, 2016. Sample collection was conducted in the steady-state of the wastewater treatment system. Collected samples were stored at –4°C.

2.2. Isolation of aerobic denitrifying bacteria

Three types of media were used to isolate the aerobic denitrifiers as introduced by Lv et al. [18] enrichment medium (EM), denitrification medium (DM), and screen medium (GN). The solid medium was prepared by adding two agar powder to the liquid medium.

Five grams of activated sludge was transferred into 250 mL EM, which contained several glass beads incubated at 30°C with shaking at 150 rpm for 10 d. During this phase, 25 mL mixture of EM and microorganisms were aspirated while 25 mL of the fresh EM were inoculated into the flask cultures and incubated under the same conditions for 48 h. After 10 d of incubation, 1 mL mixture was transferred into 250 mL DM, incubated at 30°C with 150 rpm. During the enrichment stage, 25 mL of a mixed solution of DM and microorganisms were aspirated and replaced with fresh DM every 48 h. After 8 d of incubation, the 0.1 mL mixture was spread on a solid DM medium. Single colonies were isolated from the plates and purified by several streaks to obtain pure colonies. Then, the isolated colonies were incubated in a glass tube, which contained 3 mL GN for confirmation of denitrifying bacteria. The isolates were incubated at 30°C with a shaking speed of 150 rpm. After 48 h incubation, blue colonies were sorted out as denitrifying bacteria.

2.3. DNA extraction and PCR amplification

DNA was extracted using the cetyltrimethylammonium bromide method [19,20]. The 16S rDNA from the isolated aerobic denitrifying bacteria was amplified by polymerase chain reaction (PCR). A fragment of approximately 1,500 bp was amplified using the bacterial universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [21]. The PCR conditions were pre-denaturing step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1.5 min, and 1 cycle at 72°C for 10 min [18]. The amplified products were sequenced using the Sanger sequencing method and all the sequences were compared in the GenBank database using the BLAST program.

2.4. Sequence classification and phylogenetic analysis

MEGA 4.0 was used to construct the bootstrap-tested neighbor-joining phylogenetic analysis. All sequences acquired from Sanger sequencing were classified in taxonomic ranks according to the NCBI 16S rDNA database [22]. The MOTHUR software was used to form the operational taxonomic unit (OTU) table [23,24].

2.5. Assessment of aerobic denitrification capacity

To detect the aerobic denitrification ability of the cultural aerobic denitrifiers, 24 isolates were selected as representative strains of the 24 OTUs. These strains were incubated in the Luria–Bertani medium (LB) at 30°C and 160 rpm for 12 h (reached logarithmic phase). The LB medium was supplemented with 10 g/L tryptone, 5 g/L yeast, and 5 g/L NaCl. The bacteria were harvested by centrifugation at 5,000 rpm for 5 min, and the bacterial pellets were washed thrice with 1 × PBS (phosphate-buffered saline) to remove the excess medium. The bacteria were then incubated into 1 mL DM (NO_3^-–N concentration was 50 mg/L) at 150 rpm at 30°C for 96 h. Samples were collected from the bottles to determine total nitrogen (TN). All experiments were performed in triplicates. TN concentration was determined

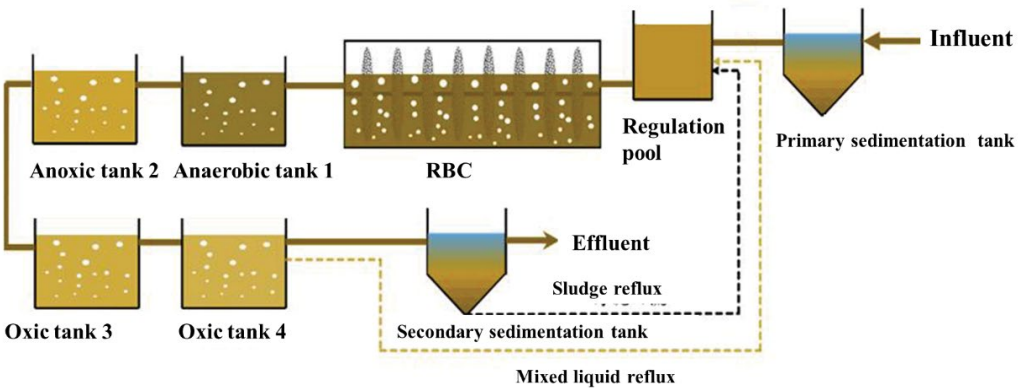

Fig. 1. Process flow diagram of the WWTP system.

using an alkaline potassium persulfate digestion-UV spectrophotometer [25].

2.6. Assessment of heterotrophic nitrification and aerobic denitrification capacity

To evaluate the heterotrophic nitrification and aerobic denitrification ability of the cultural aerobic denitrifiers, 24 representative isolates were tested. The heterotrophic nitrifying medium (HM) consisted of 0.24 g/L $(NH_4)_2SO_4$, 55 g/L $Na_2HPO_4{\cdot}12H_2O$, 1.5 g/L KH_2PO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 4.0 g/L sodium citrate, and 0.2 (volume ratio) of trace element solution which included 50.0 g/L EDTA-Na_2, 2.2 g/L $ZnSO_4$, 5.5 g/L $CaCl_2$, 5.06 g/L $MnCl_2{\cdot}4H_2O$, 5.0 g/L $FeSO_4{\cdot}7H_2O$, 1.57 g/L $CuSO_4{\cdot}5H_2O$, and 1.61 g/L $CoCl_2{\cdot}6H_2O$. The final pH of the HM was adjusted to 7.0. All used media were sterilized by autoclaving at 121°C for 20 min. The NH_4^+–N concentration of HM was 50 mg/L. The methods were as same as in the previous chapter.

3. Results

3.1. Screening of aerobic denitrifying bacteria from five types of activated sludge

Two hundred and twenty-six strains of aerobic denitrifying bacteria were selected, all of them showed a positive reaction with bromothymol blue due to increased pH. A total of 24 OTUs were clustered (Supplementary Table S1) at a cutoff of 0.01, which was generally considered as the label for species [26]. The classification of OTUs relative to the NCBI 16S rDNA reference database is shown in Table 1. Of the 24 OTUs, 13 were isolated from RBC, 11 were isolated from anaerobic tank 1, 14 were isolated from anoxic tank 2, 10 were isolated from oxic tank 3, 10 were isolated from oxic tank 4 (Fig. 2 and Table S1). The community structures were studied specifically in the five types of activated sludge.

The 13 OTUs isolated from the RBC were grouped into six genera, namely, *Pseudomonas* (*Pm.*), *Rhodococcus* (*Rc.*), *Gordonia* (*G.*), *Achromobacter* (*Ac.*), *Bacillus (B.)*, and *Stenotrophomonas* (*St.*) (Fig. 3 and Fig. S1). These 13 OTUs contained 53 strains of aerobic denitrifying bacteria. Concurrently, OTU15 (unclassified *Bacillus* sp.), OTU20 (*Pm. pseudoalcaligenes*), and OTU21 (*St. acidaminiphila*) were found only in the RBC tank.

Thirty-four strains were identified as *Pseudomonas*, which consisted of 13 strains of *Pm. stutzeri* (R16, R18, R19, etc.), 9 strains of *Pm. guguanensis* (R03, R04, R05, etc.), 6 strains of *Pm. mendocina* (R29, R30, R34, etc.), 4 strains of *Pm. chengduensis* (R13, R14, R15, and R56), 2 strains of *Pm. monteilii* (R12 and R35) and *Pm. pseudoalcaligenes* (R28). In addition, the strain R45 could not be identified due to high similarity (99.93) with *B. aerius* 24°K, *B. stratosphericus* 41KF2a, and *B. altitudinis* 41KF2b (Supplementary Table S2). Four strains of unclassified *Achromobacter* sp. (R41, R42, R43, and R51) exhibited the same similarity with *Ac. denitrificans* NBRC 15125 and *Ac. agilis* LMG 3411.

In the samples from anaerobic tank 1, all 11 OTUs consisted of a group of 42 strains (Fig. 4), which were clustered into five genera, namely, *Pseudomonas* (*Pm.*), *Rhodococcus* (*Rc.*), *Gordonia* (*G.*), *Achromobacter* (*Ac.*), and *Arthrobacter* (*Ar.*), based on sequence assignment using the NCBI database (Fig. S2). The most abundant culturable aerobic denitrifiers were *Pseudomonas*, which included 29 strains. Thirteen isolates, including A121, A118, A115, and A123, were assigned to *Pm. Stutzeri*; nine isolates, including A103, A106, A104, and A107, were assigned to *Pm. guguanensis* (Fig. 4). In the genus *Gordonia*, A130, A131, A132, A135, and A133 were assigned to *G. malaquae*, and A101 and A136 to *G. cholesterolivorans* (Fig. 4). In addition, the culturable aerobic denitrifiers also included *Achromobacter* sp. (A134, A141), *Azoarcus protophormiae* (A139), and *Rhodococcus* sp. (A137, A138, A140).

Fourteen OTUs consisted of 58 strains of bacteria in the samples from anoxic tank 2 (Fig. 5). Using the NCBI database, these 58 strains were clustered into eight genera, namely, *Pseudomonas* (*Pm.*), *Rhodococcus* (*Rc.*), *Gordonia* (*G.*), *Achromobacter* (*Ac.*), *Azoarcus* (*Az.*), *Bacillus (B.)*, *Pannonibacter* (*Pa.*), and *Nocardia* (*N.*) (Fig. S3). Thirty-four isolates belonged to the *Pseudomonas*, which were the most abundant culturable aerobic denitrifiers in the anoxic tank 2. The *Pseudomonas* consisted of 13 strains of *Pm. stutzeri* (A225, A228, A224, etc.), 9 strains of *Pm. guguanensis* (A208, A211, A204, etc.), 6 strains of *Pm. mendocina* (A235, A231, A249, etc.), 3 strains of *Pm. alcaliphila* (A213, A215 and A214), 2 strains of *Pm. monteilii* (A236 and A212) and *Pm. chengduensis* (A260) (Fig. 5).

The second-most abundant genus was *Gordonia*, which included seven strains. The *Gordonia* consisted of *G. malaquae*

Table 1
Classification of OTUs of culturable aerobic denitrifiers in the WWTP system relative to the NCBI 16S rDNA reference database

OTUs	Representative isolate	Taxonomic classification		
		Order	Genus	Species
OTU1	A226	Pseudomonas	*Pseudomonas*	*Stutzeri*
OTU2	R10	Pseudomonas	*Pseudomonas*	*Guguanensis*
OTU3	A113	Pseudomonas	*Pseudomonas*	*Mendocina*
OTU4	A132	Actinobacteridae	*Gidonia*	*Malaquae*
OTU5	A248	Burkholderiales	*Achromobacter*	Unclassified
OTU6	A137	Actinobacteridae	*Rhodococcus*	*Rhodochrous*
OTU7	A202	Actinobacteridae	*Rhodococcus*	Unclassified
OTU8	A129	Pseudomonas	*Pseudomonas*	*Monteilii*
OTU9	A241	Rhodocyclales	*Azoarcus*	*Communis*
OTU10	A101	Actinobacteridae	*Gordonia*	*Cholesterolivorans*
OTU11	A112	Pseudomonas	*Pseudomonas*	*Chengduensis*
OTU12	A229	Bacillales	*Bacillus*	*Velezensis*
OTU13	A134	Burkholderiales	*Achromobacter*	*Mucicolens*
OTU14	A424	Burkholderiales	*Achromobacter*	*Aegrifaciens*
OTU15	R45	Bacillales	*Bacillus*	Unclassified
OTU16	A247	Actinobacteridae	*Nocardia*	*Puris*
OTU17	A414	Burkholderiales	*Achromobacter*	*Xylosoxidans*
OTU18	A415	Pseudomonadales	*Acinetobacter*	*Haemolyticus*
OTU19	A413	Rhizobiales	*Beijerinckia*	*Fluminensis*
OTU20	R28	Pseudomonas	*Pseudomonas*	*Pseudoalcaligenes*
OTU21	R50	Xanthomonadales	*Stenotrophomonas*	*Acidaminiphila*
OTU22	A245	Rhodobacterales	*Pannonibacter*	*Phragmitetus*
OTU23	A419	Actinobacteridae	*Rhodococcus*	*Ruber*
OTU24	A139	Actinobacteridae	*Arthrobacter*	*Protophormiae*

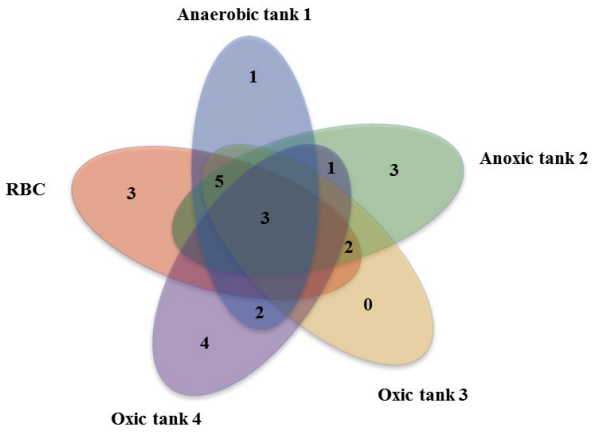

Fig. 2. Venn diagram of OTUs at a cut-off of 0.01 for the culturable aerobic denitrifying bacteria. Numbers in the overlapping parts represent the shared OTUs between different phases. RBC, Rotating biological contactor.

(A237, A238, A239, etc.) and *G. cholesterolivorans* (A201 and A252). The *Rhodococcus* consisted of unclassified *Rhodococcus* sp. (A256, A202, and A244) and *Rc. rhodochrous* (A253, A254, and A258). The strains *Rc. jialingiae* djl–6–2, *Rc. qingshengii* djl–6, and *Rc. degradans* CCM 4446 exhibited sequence similarity, therefore they were identified as the unclassified *Rhodococcus* sp. Therefore, strains A202, A256, and A244 could not be identified at the species level based on the 16S rDNA sequence. In addition, *Az. communis* (A241, A242, and A243), *Pa. phragmitetus* (A245), *B. velezensis* (A229), and *N. puris* (A247) were also isolated.

Ten OTUs consisted of 50 strains of bacteria in the samples from oxic tank 3. These 50 strains were clustered into four genera, namely, *Pseudomonas* (*Pm.*), *Rhodococcus* (*Rc.*), *Gordonia* (*G.*), and *Achromobacter* (*Ac.*) (Fig. 6).

As with the anoxic tank 2, the most abundant culturable aerobic denitrifier was the genus *Pseudomonas*, which consisted of 12 strains of *Pm. stutzeri* (A322, A318, A317, etc.), 9 strains of *Pm. guguanensis* (A303, A311, A305, etc.), 6 strains of *Pm. mendocina* (A330, A331, A328, etc.), 4 strains of *Pm. chengduensis* (A352, A313, A314, and A315), and 2 strains of *Pm. monteilii* (A312 and A334) (Fig. S4). Second only to *Pseudomonas* was *Gordonia*, which consisted of 5 strains of *G. malaquae* (A335, A336, A337, etc.) and *G. cholesterolivorans* (A344 and A301). In addition, 4 strains of unclassified *Achromobacter* sp. (A340, A341, A342, and A347) were also isolated from the oxic tank 3, which exhibited similarity with *Ac. denitrificans* NBRC 15125 and *Ac. agilis* LMG 3411.

Ten OTUs consisted of 23 strains of bacteria in the samples from oxic tank 4, (Fig. 7). These 23 strains were

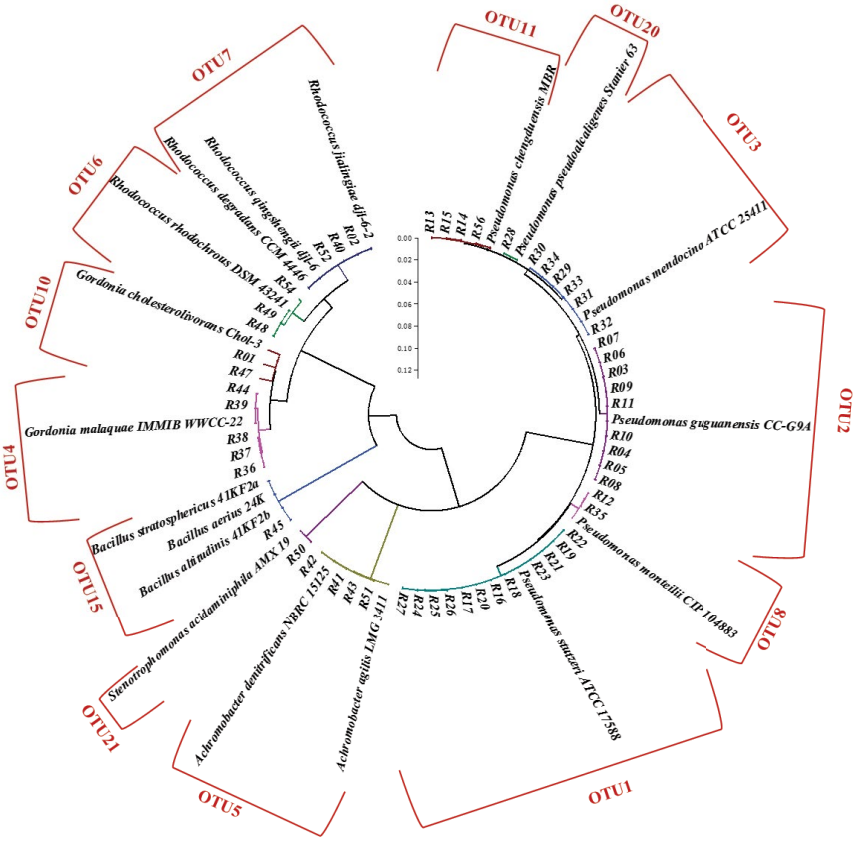

Fig. 3. Phylogenetic tree (circular mode) of the culturable aerobic denitrifying isolates in the RBC tank. Vertical scale bar in the middle represents the distance (dissimilarity) between the isolates and reference strains.

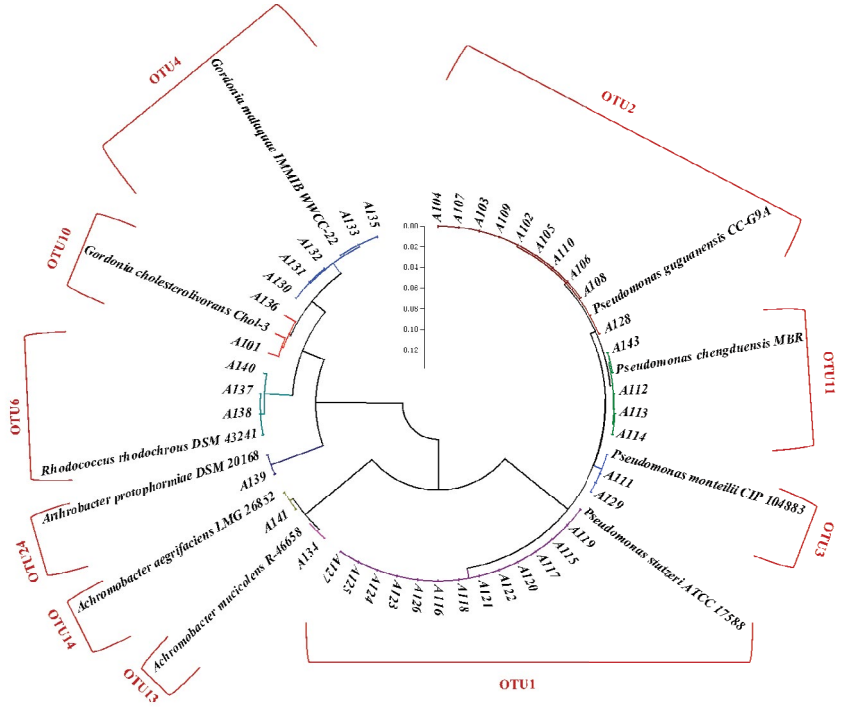

Fig. 4. Phylogenetic tree (circular mode) of the culturable aerobic denitrifying isolates in anaerobic tank 1. Vertical scale bar in the middle represents the distance (dissimilarity) between the isolates and reference strains.

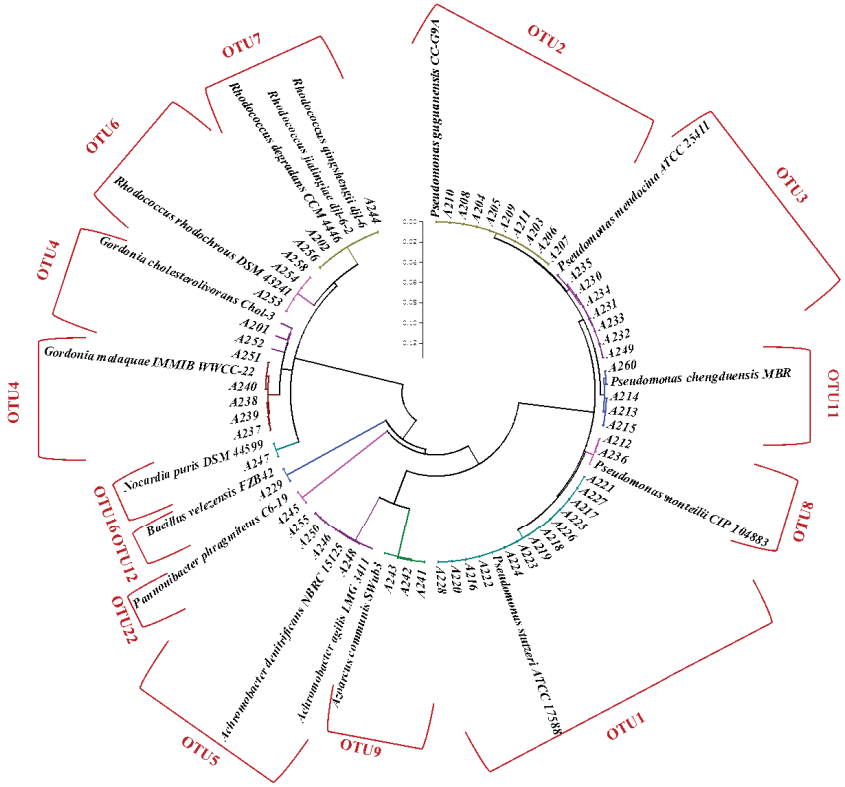

Fig. 5. Phylogenetic tree (circular mode) for the culturable aerobic denitrifying isolates in anoxic tank 2. Vertical scale bar in the middle represents the distance (dissimilarity) between the isolates and reference strains.

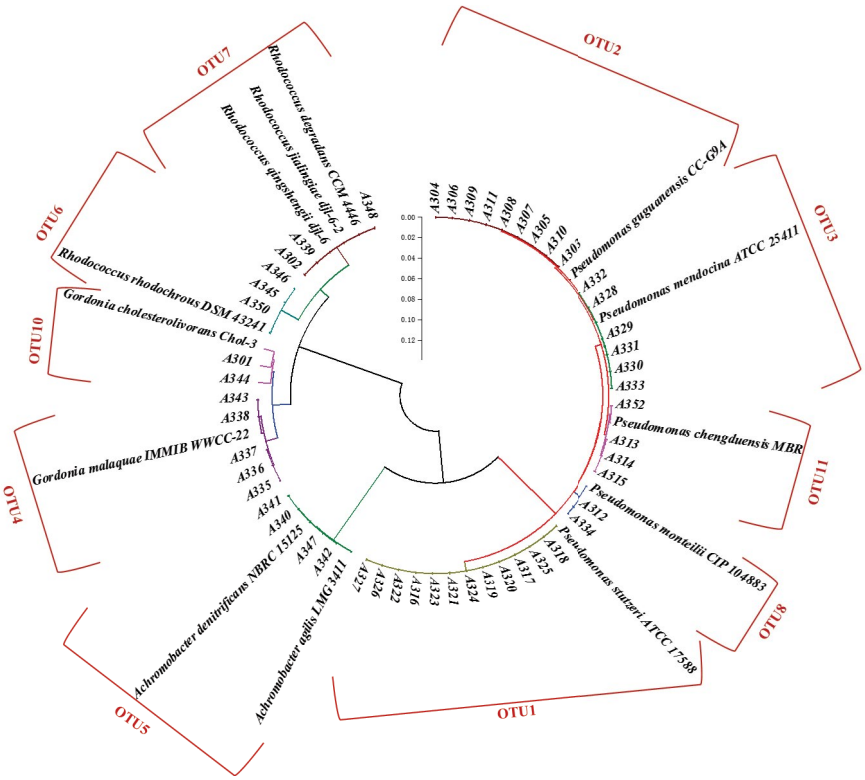

Fig. 6. Phylogenetic tree (circular mode) for the culturable aerobic denitrifying isolates in oxic tank 3. Vertical scale bar in the middle represents the distance (dissimilarity) between the isolates and reference strains.

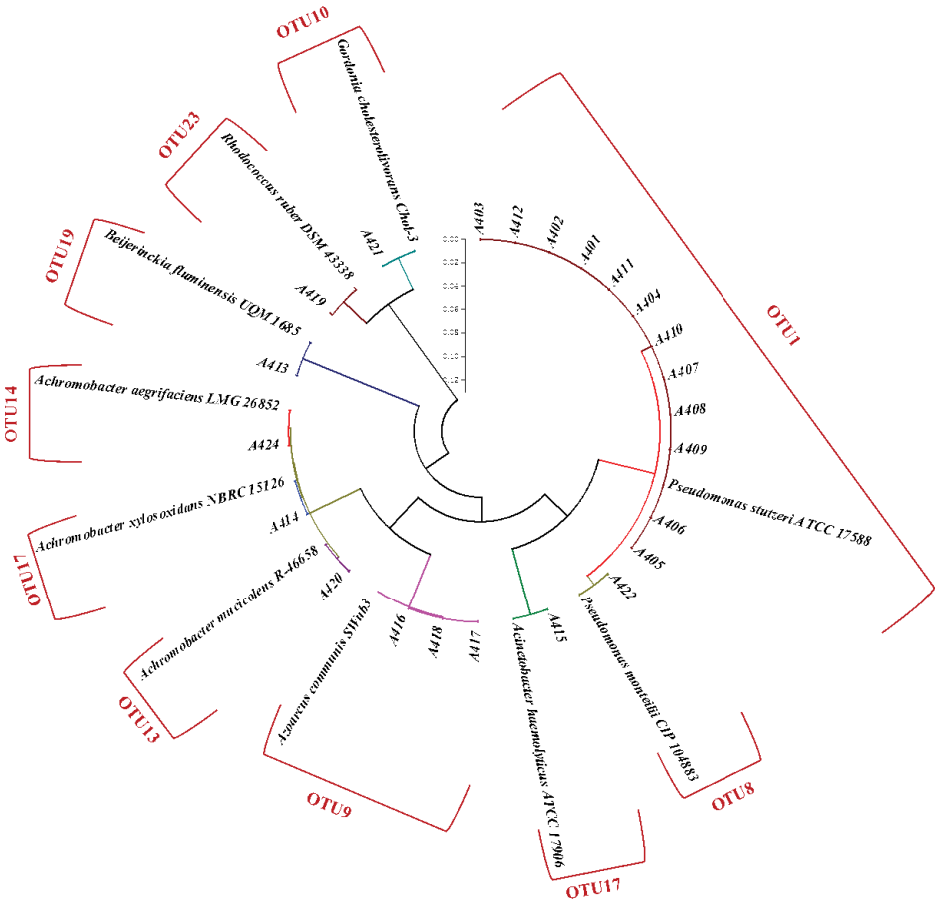

Fig. 7. Phylogenetic tree (circular mode) for the culturable aerobic denitrifying isolates in oxic tank 4. Vertical scale bar in the middle represents the distance (dissimilarity) between the isolates and reference strains.

clustered into seven genera, namely, *Pseudomonas* (*Pm.*), *Rhodococcus* (*Rc.*), *Gordonia* (*G.*), *Achromobacter* (*Ac.*), *Azoarcus* (*Az.*), *Acinetobacer* (*An.*), and *Beijerinckia* (*Bj.*). The dominant genus in oxic tank 4 was *Pseudomonas*, which included 12 strains of *Pm. stutzeri* (A407, A412, A411, etc.) and *Pm. monteilii* (A422) (Fig. S5). The secondary genus was *Achromobacter*, which consisted of *Ac. xylosoxidans* (A414), *Ac. mucicolens* (A420), and *Ac. aegrifaciens* (A424). In addition, *Bj. Fluminensis* (A413), *An. haemolyticus* (A415), *Az. Communis* (A416, A417, and A418), *G. cholesterolivorans* (A421), and *Rc. Ruber* (A419) were also present in oxic tank 4.

3.2. Community analysis of culturable aerobic denitrifiers in activated sludge of RBC, anaerobic tank 1, anoxic tank 2, oxic tank 3, and oxic tank 4

The Venn diagram showed the shared and unique OTUs among RBC, tank 1, tank 2, tank 3, and tank 4. Three OTUs were found in the samples from all five activated sludge, and unique OTUs were also observed in RBC, anaerobic tank 1, oxic tank 3, and oxic tank 4. The community structures of different tanks were analyzed further based on OTU diversity. Fig. 8 summarizes the relative abundance of the isolates based on the OTU level. The three OTUs found in the five activated sludge samples included OTU1 (*Pm. stutzeri*), OTU8 (*Pm. monteilii*), and OTU10 (*G. cholesteroliborans*) (Fig. 2). OTU1 (*Pm. stutzeri*) accounted for 31, 23, 24, 52, and 23 of the culturable aerobic denitrifiers in tanks 1, 2, 3, 4, and RBC tank, respectively. The abundance of OTU8 (*Pm. monteilii*) and OTU10 (*G. cholesteroliborans*) were in the range of 3.5–4.8.

OTU1 (*Pm. stutzeri*) was the most dominant OTU among all the culturable aerobic denitrifiers in the five samples, and accounted for more than 22. OTU2 (*Pm. guguanensis*) accounted for 24, 16, 18, and 17 of the culturable aerobic denitrifiers in tanks 1, 2, 3, and RBC tank, respectively, while no strain of OTU2 was isolated from tank 4. There were a few unique OTUs in the oxic tank 4, such as OTU17 (*Ac. xylosoxidans*), OTU18 (*An. haemolyticus*), OTU19 (*Bj. fluminensis*), and OTU23 (*Rc. ruber*), all of which occupied 4.35. OTU9 (*Az. communis*) accounted for 13.04 and 5.26 of the culturable aerobic denitrifiers in oxic tank 4 and anoxic tank 2, respectively, but did not contain any strain in other samples (Fig. 8).

Among the 24 OTUs, OTU24 (*Ar. protophormiae*) was only included in the anaerobic tank 1; OTU14 (*Ac. aegrifaciens*), OTU16 (*N. puris*), and OTU22 (*Pa. phragmitetus*) were only included in anoxic tank 2; OTU17 (*Ac. xylosoxidans*), OTU18 (*Ac. haemolyticus*), OTU19 (*Bj. fluminensis*), and OTU23 (*Rc. ruber*) were only included in oxic tank 4; and OTU15

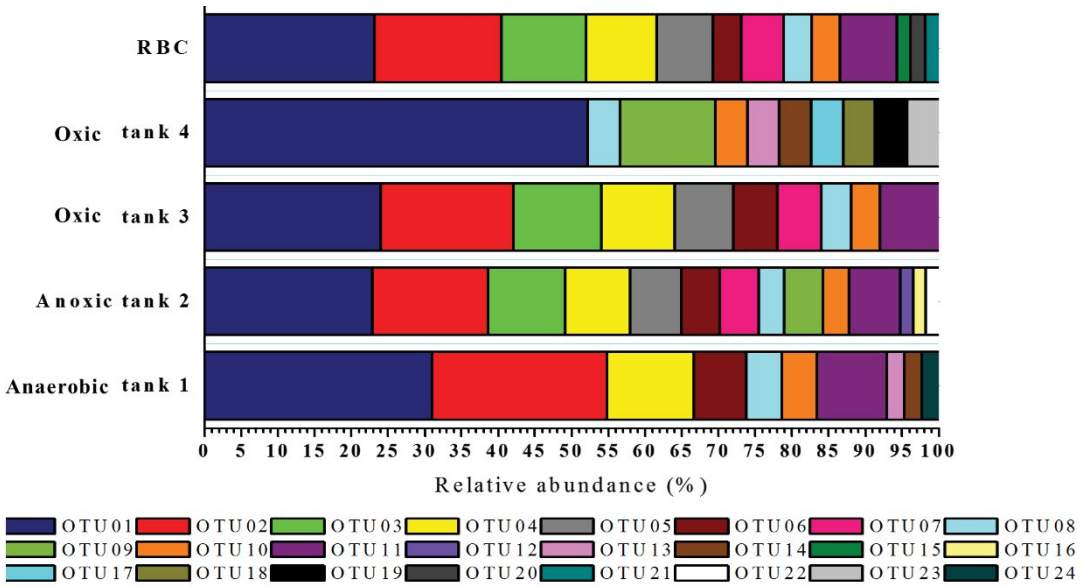

Fig. 8. Relative abundance of aerobic denitrifying bacteria in RBC, anaerobic tank 1, anoxic tank 2, aeration tank 3, and aeration tank 4.

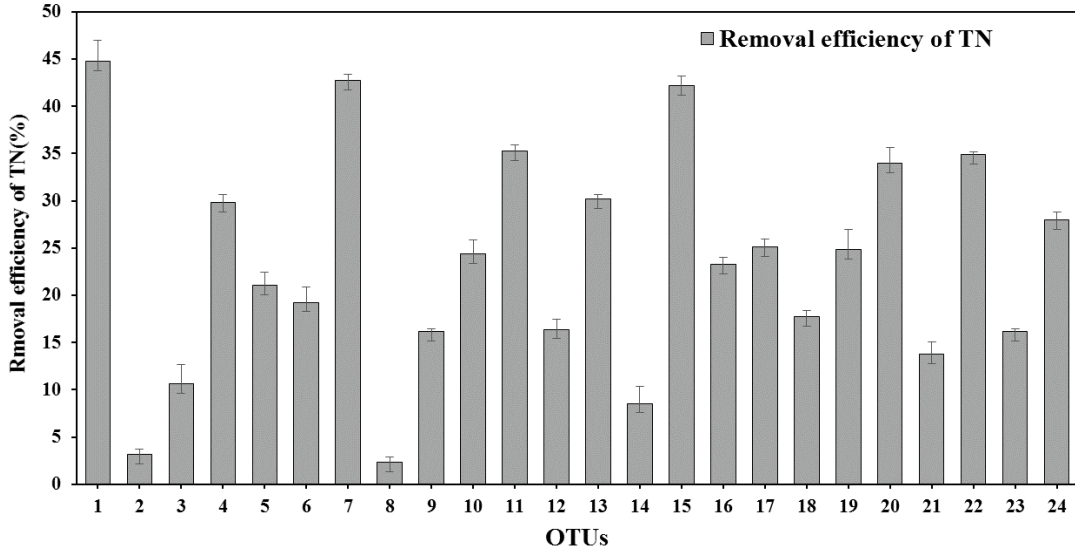

Fig. 9. Aerobic nitrification performance of 24 representative strains. TN removal efficiency reflects the aerobic denitrifying capacity.

(unclassified *Bacillus* sp.), OTU20 (*Pm. pseudoalcaligenes*), and OTU21 (*St. acidaminiphila*) were only included in the RBC tank.

3.3. Aerobic denitrification capability and heterotrophic nitrification-aerobic denitrification capability

The aerobic denitrification capability of representative strains was tested. The result showed that all 24 representative strains possessed aerobic denitrifying capacity, and their TN (total nitrogen) removal efficiencies were in the range of 2.3–45 (Fig. 9). The top three aerobic denitrifying bacteria were OTU1 (*Pm. stutzeri*), OTU7 (*Rc.* sp. unclassified), and OTU15 (unclassified *Bacillus* sp.), which reduced TN by 45, 43, and 42, respectively. The lowest TN removal efficiency was exhibited by the OTU8 (*Pm. monteilii*) at 2.3, and the TN removal efficiency of OTU2 (*Pm. guguanensis*) was 3.2 at the same time.

Simultaneously, the heterotrophic nitrification-aerobic denitrification capacities of the isolates were also evaluated. Fig. 10 shows that the TN removal efficiency of the 24 representative strains was in the range of 1.9–15. Under heterotrophic nitrifying (HM medium in this study) conditions, the top three aerobic denitrifying bacteria with high TN removal efficiency were OTU18 (*Acinetobacter haemolyticus*), OTU19 (*Beijerinckia fluminensis*), and OTU11 (*Pseudomonas*

chengduensis), and the TN removal efficiencies were 15, 14, and 15, respectively.

Combining the aerobic denitrification capabilities with the heterotrophic nitrification-aerobic denitrification capabilities for the 24 strains, the excellent heterotrophic nitrification-aerobic denitrifying bacteria were identified to be OTU1 (*Pm. stutzeri*), OTU20 (*Pm. pseudoalcaligenes*), OTU11 (*Pm. chengduensis*), OTU19 (*Bj. fluminensis*), and OTU24 (*Ar. protophormiae*).

4. Discussion

In this study, three species-OTU1 (*Pm. stutzeri*), OTU8 (*Pm. monteilii*), and OTU10 (*G. cholesteroliborans*) were found to be the most abundant aerobic denitrifying bacteria in five different activated sludge samples of a WWTP. Several studies have reported on the aerobic denitrification ability of *Pseudomonas*, such as *Pseudomonas thivervalensis* [27]. *Pseudomonas nautica* [28], *Pseudomonas mendocina* [29]. *Pm. stutzeri*, and *Pm. monteilii* were also found to possess denitrifying ability under different aerobic conditions. For example, *Pm. stutzeri* YZN-001 exhibited heterotrophic nitrification and aerobic denitrification ability, with the notable ability to remove ammonium at low temperatures [30]. The strain *Pm. stutzeri* KTB (isolated from activated sludge flocci) exhibited heterotrophic nitrification-aerobic denitrification ability [31]. Thus, the dominant existence of the *Pm. stutzeri* in the culturable aerobic denitrifying community indicated that *Pm. stutzeri* might be a remarkable candidate for biological removal of nitrogen compounds from wastewater. In addition, aerobic denitrifying *Pm. stutzeri* have been found in a wide range of environments, such as soil [32], wastewater, and groundwater [33]. Similar to *Pm. stutzeri*, *Pm. monteilii* is also widespread in environments, such as deep sea sediment [32], banana rhizospheric soil [34], and tannery waste soil [35]. Simultaneously, *Pm. monteilii* have shown aerobic denitrification ability in previous studies. For example, *Pm. monteilii* H97 [36] and *Pm. monteilii* LKX-1were isolated from the soil and rural sewage, respectively. Both of them were proven to possess aerobic denitrification capability. *G. cholesteroliborans* Chol-3 was first isolated from a sewage sludge sample, and recognized as a member of a novel species within the genus *Gordonia*. Several *Gordonia* sp. possessed aerobic denitrification capability, including *G. sihwensis*, *G. terrae*, and *G. malaquae* [37], but no studies have reported the aerobic denitrifying ability of *G. cholesteroliborans*.

OTU9 (*Az. communis*) was abundant in oxic tank 4. No previous studies have directly reported the aerobic denitrification capacity of *Az. communis*. Previous studies had found that *Az. Communis*, which was isolated from refinery oily sludge, has a strictly aerobic metabolism and is able to form N_2O from nitrate [38]. This conclusion was also supported by this present study, given that oxic tank 4 was under aeration, and *Az communis* uses aerobic metabolism. Furthermore, *Az. communis* can contribute N_2 to the plant [39] as well as denitrifying toluene degraders [38]. This conclusion may be related to the aerobic denitrification of the *Az. communis*. In this study, *Az. communis* could remove nitrate. However, the transformation pathway needs to be further studied.

OTU1 (*Pm. stutzeri*), OTU7 (unclassified *Rhodococcus* sp.), and OTU15 (unclassified *Bacillus* sp.) exhibited a strong aerobic denitrification ability. Several studies have reported on the aerobic denitrification ability of *Pm. stutzeri*. For example, a *Pm. stutzeri* strain XL-2 had been shown to remove 97.9 of nitrate with an initial concentration of approximately 100 mg/L [40]. Moreover, aerobic denitrifying bacterium *Pm. stutzeri* KTB have been shown to possess high nitrogen removal efficiency [19]. Simultaneously, *Pm. stutzeri* C3 was

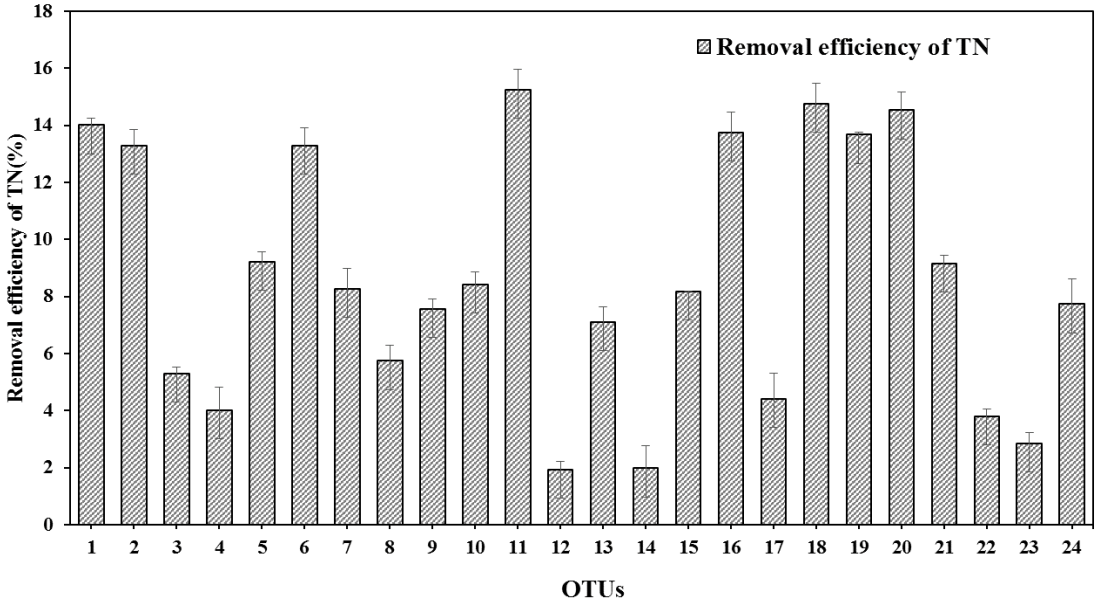

Fig. 10. Denitrification performance of 24 representative strains. TN removal efficiency reflects the heterotrophic nitrifying-aerobic denitrifying capacity.

isolated from activated sludge and the isolate possessed an average nitrate removal efficiency of 95.8 in 24 h [41]. In this study, the TN removal efficiencies of *Pm. stutzeri* were only 14.0 and 44.7 when cultivated in HM (Heterotrophic nitrifying medium) and DM (Denitrifying medium), respectively, which may be related to factors such as temperature, carbon source, nitrogen source, and inoculation quantity. Thus, the heterotrophic aerobic-denitrification ability of *Pm. stutzeri* under different conditions needs to be further studied. The strain *Rc. jialingiae* djl–6–2 has been isolated from diverse environmental samples, such as sludge of a carbendazim wastewater treatment facility [41] and soil, but no studies have reported on the heterotrophic aerobic-denitrification ability of *Rc. jialingiae*. The strain *B.* MS30 was isolated from a deep-sea hydrothermal vent, and had the ability to produced nitrite from ammonia under aerobic conditions [42–44]. The strain *B. aerius* was isolated from cryogenic tubes used for collecting air samples from high altitudes [45,46], but no studies have reported on its aerobic denitrification ability. Simultaneously, *B. stratosphericus* 41KF2b, *B. altitudinis* 41KF2b, and *B. aerius* 24K have been shown to be very similar to one another [47,48]. This result also agrees with our study (Supplementary Table S1).

In this study, OTU20 (*Pm. pseudoalcaligenes*) showed a very strong capacity for heterotrophic nitrification-aerobic denitrification, which was not reported in previous studies. Several studies had reported that *Pm. pseudoalcaligenes* could remove diverse pollutants, such as nitrobenzene [49], phenol, and salicylate [50,51], but no studies have reported on the capacity of *Pm. pseudoalcaligenes* for aerobic denitrification. In addition, *Pm. chengduensis*, *Bj. Fluminensis*, and *Ar. protophormiae* all showed a strong capacity for heterotrophic nitrification-aerobic denitrification in this study, which no previous studies had reported on.

The uneven distribution of microbial diversity shown by the Venn diagram (Fig. 2) indicated that the structure of aerobic denitrifiers might be different among the phases of these five sampling points. The anoxic tank 2 and the RBC tank had a richer community of aerobic denitrifying bacteria than others, which had 14 and 13 OTUs, respectively. Concurrently, the structure of aerobic denitrifiers in oxic tank 4 was significantly different from the others (Fig. 8). Thus, the factors leading to diversity of culturable community structure in these five sampling points need further study.

5. Conclusion

Two hundred and twenty-six strains of aerobic denitrifying bacteria were isolated from a special sewage treatment reactor, which were classified into 12 genera. This study provided a reference for screening aerobic denitrifying bacteria and increased the possibility to promote the application of aerobic denitrifying bacteria in WWTPs. However, the effect of varying conditions, such as pH, carbon to nitrogen ratio (C:N), hydraulic retention times, and sludge retention time, on these denitrifiers has not been revealed. In addition, functional genes need to be studied.

A sewage treatment technology, which combined RBC and A^2/O^2 exhibited strong removal efficiency of nitrate. Simultaneously, the capacity of heterotrophic nitrification-aerobic denitrification was also studied. This study provides a reference for studying aerobic denitrification bacteria in WWTPs. Based on this study, aerobic denitrifying bacteria could be isolated from different WWTP samples for comparative studies. Taken together, the optimal working conditions of aerobic denitrifying bacteria with strong heterotrophic nitrification aerobic denitrification ability should be investigated for application in WWTP.

Author Contributions

Z.B. designed and directed the experiments. Z.H., P.L., X.L., and M.S. performed the experiments, Z.H., P.L., and T.G. analyzed the data and wrote the main text of manuscript. J.L., X.L., and Y.Z. contributed to the interpretation of the results. All authors reviewed and improved the manuscript.

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Supplementary information:

Table S1
Aerobic denitrifying bacteria in each OTU at cutoff of 0.01

OTUs	Strains of aerobic denitrifying bacteria
Otu01	A226, A115, A116, A117, A118, A119, A120, A121, A216, A217, A218, A219, A220, A221, A222, A316, A317, A318, A319, A320, A321, A401, A402, A403, A404, A405, A406, R16, R17, R18, R19, R20, R21, A126, A227, A326, A411, R23, A122, A123, A124, A125, A223, A224, A225, A322, A323, A324, A325, A407, A408, A409, A410, R22, A127, A228, A327, A412, R24, R25, R26, R27
Otu02	R10, A107, A108, A109, A208, A209, A210, A308, A309, A310, R08, R09, A110, A211, A311, R11, A102, A203, A303, R03, A105, A206, A306, R06, A103, A104, A204, A205, A304, A305, R04, R05, A106, A207, A307, R07, A128
Otu03	A113, A214, A314, R14, A114, A215, A315, R15, A143, A260, A352, R56, A235, A332, R33, A230, A231, A232, A233, A234, A249, A328, A329, A330, A331, A333, R29, R30, R31, R32, R34
Otu04	A132, A131, A238, A336, R37, A130, A237, A335, R36, A239, A337, R38, A133, A240, A338, R39, A135, A251, A343, R44
Otu05	A248, A246, A250, A255, A342, A347, A341, A340, R51, R43, R42, R41
Otu06	A137, A138, A253, A254, A345, A346, R48, R49, A140, A258, A350, R54
Otu07	A202, A302, R02, A244, A339, R40, A256, A348, R52
Otu08	A129, A111, A212, A312, R12, A236, A334, R35, A422
Otu09	A241, A416, A242, A243, A417, A418
Otu10	A101, A201, A301, R01, A421
Otu11	A112, A213, A313, R13
Otu12	A424, A141
Otu13	A134, A420
Otu14	A229
Otu15	R45
Otu16	A247
Otu17	A414
Otu18	A415
Otu19	A413
Otu20	R28
Otu21	R50
Otu22	A245
Otu23	A419
Otu24	A139

Table S2
The aerobic denitrifying bacteria in the tank of rotating biological contactor and the closest relative reference strains in NCBI database

Strain Number	Related GenBank sequence	Closest relative (NCBI accession No.)	Identity
R01	MH773363	*Gordonia cholesterolivorans* strain Chol-3 (NR_044445.1)	97.56
		Rhodococcus jialingiae strain djl-6–2 (NR_115708.1)	100
R02	MH773364	*Rhodococcus qingshengii* strain djl-6 (NR_043535.1)	100
		Rhodococcus degradans strain CCM 4446 (NR_145886.1)	100
R03	MH773365	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.1)	99.01
R04	MH773366	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.1)	99.15
R05	MH773367	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.1)	99.15
R06	MH773368	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.1)	99.22
R07	MH773369	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.2)	98.37
R08	MH773370	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.2)	99.08
R09	MH773371	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.3)	99.08
R10	MH773372	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.3)	99.08
R11	MH773373	*Pseudomonas guguanensis* strain CC-G9A (NR_135725.1)	99.05
R12	MH773374	*Pseudomonas monteilii* strain CIP 104883 (NR_024910.1)	99.57
R13	MH773375	*Pseudomonas chengduensis* strain MBR (NR_125523.1)	98.86
R14	MH773376	*Pseudomonas chengduensis* strain MBR (NR_125523.1)	99.64
R15	MH773377	*Pseudomonas chengduensis* strain MBR (NR_125523.1)	99.71
R16	MH773378	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.93
R17	MH773379	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.93
R18	MH773380	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.93
R19	MH773381	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.93
R20	MH773382	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.93
R21	MH773383	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.93
R22	MH773384	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.86
R23	MH773385	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.86
R24	MH773386	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.86
R25	MH773387	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.86
R26	MH773388	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	100
R27	MH773389	*Pseudomonas stutzeri* strain ATCC 17588 (NR_041715.1)	99.29
R28	MH773390	*Pseudomonas pseudoalcaligenes* strain Stanier 63 (NR_037000.1)	99.22
R29	MH773391	*Pseudomonas mendocina* strain ATCC 25411 (NR_114477.1)	99.29
R30	MH773392	*Pseudomonas mendocina* strain ATCC 25411 (NR_114477.1)	99.29
R31	MH773393	*Pseudomonas mendocina* strain ATCC 25411 (NR_114477.1)	99.29
R32	MH773394	*Pseudomonas mendocina* strain ATCC 25411 (NR_114477.1)	99.93
R33	MH773395	*Pseudomonas mendocina* strain ATCC 25411 (NR_114477.1)	99.22
R34	MH773396	*Pseudomonas mendocina* strain ATCC 25411 (NR_114477.1)	99.29
R35	MH773397	*Pseudomonas monteilii* strain CIP 104883 (NR_024910.1)	99.79
R36	MH773398	*Gordonia malaquae* strain IMMIB WWCC-22 (NR_115020.1)	99.57
R37	MH773399	*Gordonia malaquae* strain IMMIB WWCC-22 (NR_115020.1)	99.49
R38	MH773400	*Gordonia malaquae* strain IMMIB WWCC-22 (NR_115020.1)	99.71
R39	MH773401	*Gordonia malaquae* strain IMMIB WWCC-22 (NR_115020.1)	100
		Rhodococcus jialingiae strain djl-6-2 (NR_115708.1)	100
R40	MH773402	*Rhodococcus qingshengii* strain djl-6 (NR_043535.1)	100
		Rhodococcus degradans strain CCM 4446 (NR_145886.1)	100
R41	MH773403	*Achromobacter denitrificans* strain NBRC 15125 (NR_113732.1)	99.57
		Achromobacter agilis strain LMG 3411 (NR_152013.1)	99.57
R42	MH773404	*Achromobacter denitrificans* strain NBRC 15125 (NR_113732.1)	99.64
		Achromobacter agilis strain LMG 3411 (NR_152013.1)	99.64
R43	MH773405	*Achromobacter denitrificans* strain NBRC 15125 (NR_113732.1)	99.50
		Achromobacter agilis strain LMG 3411 (NR_152013.1)	99.50

(Continued)

Table S2 Continued

Strain Number	Related GenBank sequence	Closest relative (NCBI accession No.)	Identity
R44	MH773406	*Gordonia malaquae* strain IMMIB WWCC-22 (NR_115020.1)	99.93
		Bacillus aerius strain 24K (NR_118439.1)	99.93
R45	MH773407	*Bacillus stratosphericus* strain 41KF2a (NR_042336.1)	99.93
		Bacillus altitudinis strain 41KF2b (NR_042337.1)	99.93
R47	MH773408	*Gordonia cholesterolivorans* strain Chol-3 (NR_044445.1)	98.48
R48	MH773409	*Rhodococcus rhodochrous* strain DSM 43241 (NR_116689.1)	99.78
R49	MH773410	*Rhodococcus rhodochrous* strain DSM 43241 (NR_116689.1)	99.78
R50	MH773411	*Stenotrophomonas acidaminiphila* strain AMX 19 (NR_025104.1)	99.58
R51	MH773412	*Achromobacter denitrificans* strain NBRC 15125 (NR_113732.1)	99.43
		Achromobacter agilis strain LMG 3411 (NR_152013.1)	99.43
		Rhodococcus jialingiae strain djl-6-2 (NR_115708.1)	99.93
R52	MH773413	*Rhodococcus qingshengii* strain djl-6 (NR_043535.1)	99.93
		Rhodococcus degradans strain CCM 4446 (NR_145886.1)	99.93
R54	MH773414	*Rhodococcus rhodochrous* strain DSM 43241 (NR_116689.1)	99.79
R56	MH773415	*Pseudomonas chengduensis* strain MBR (NR_125523.1)	99.93

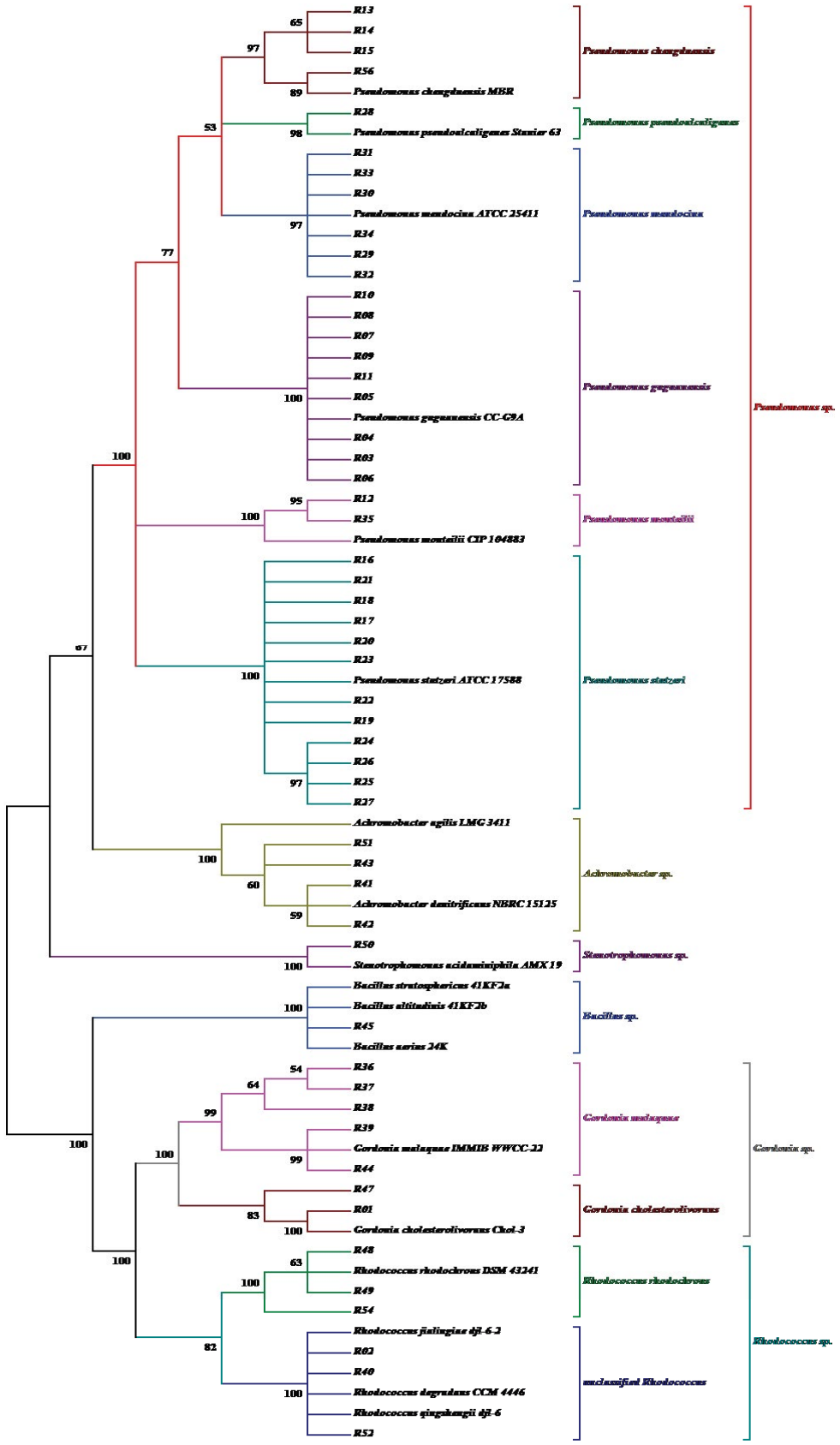

Fig. S1. Phylogenetic tree (rectangular mode) for the culturable aerobic denitrifying isolates from the tank of rotating biological contactor. The numbers next to the branches represent the boostrap values.

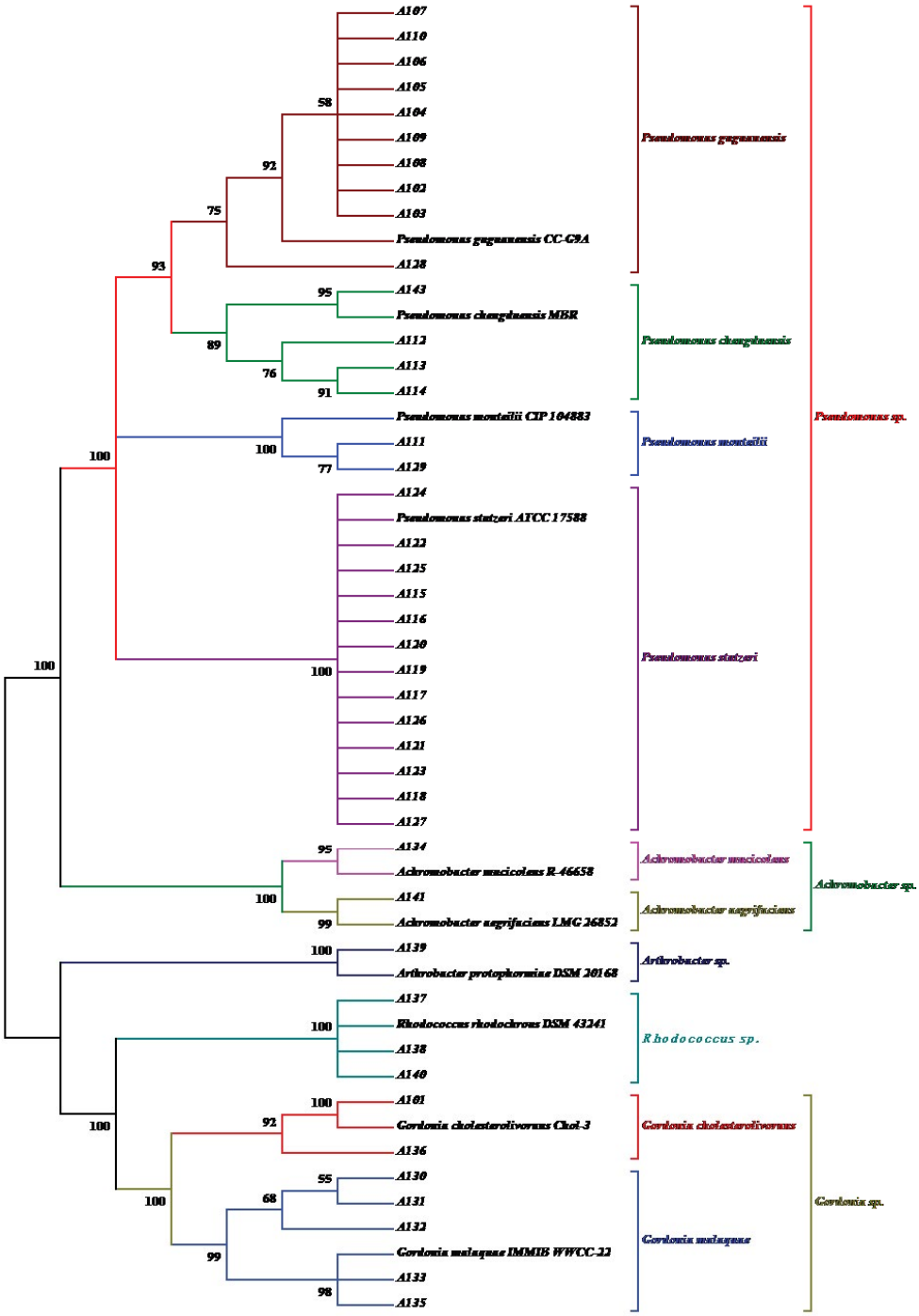

Fig. S2. Phylogenetic tree (rectangular mode) for the culturable aerobic denitrifying isolates from the anaerobic tank 1. The numbers next to the branches represent the boostrap values.

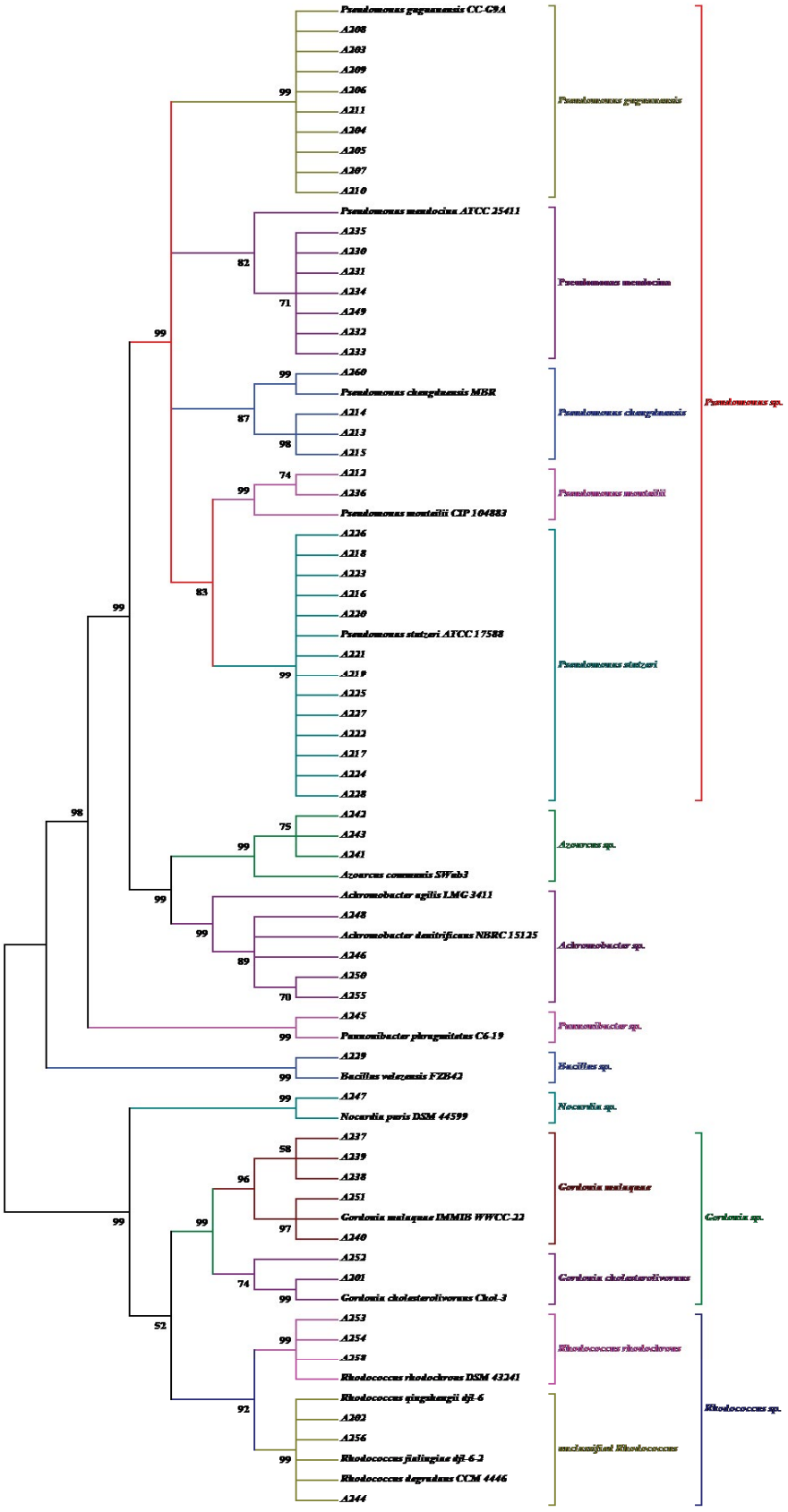

Fig. S3. Phylogenetic tree (rectangular mode) for the culturable aerobic denitrifying isolates from the anoxic tank 2. The numbers next to the branches represent the boostrap values.

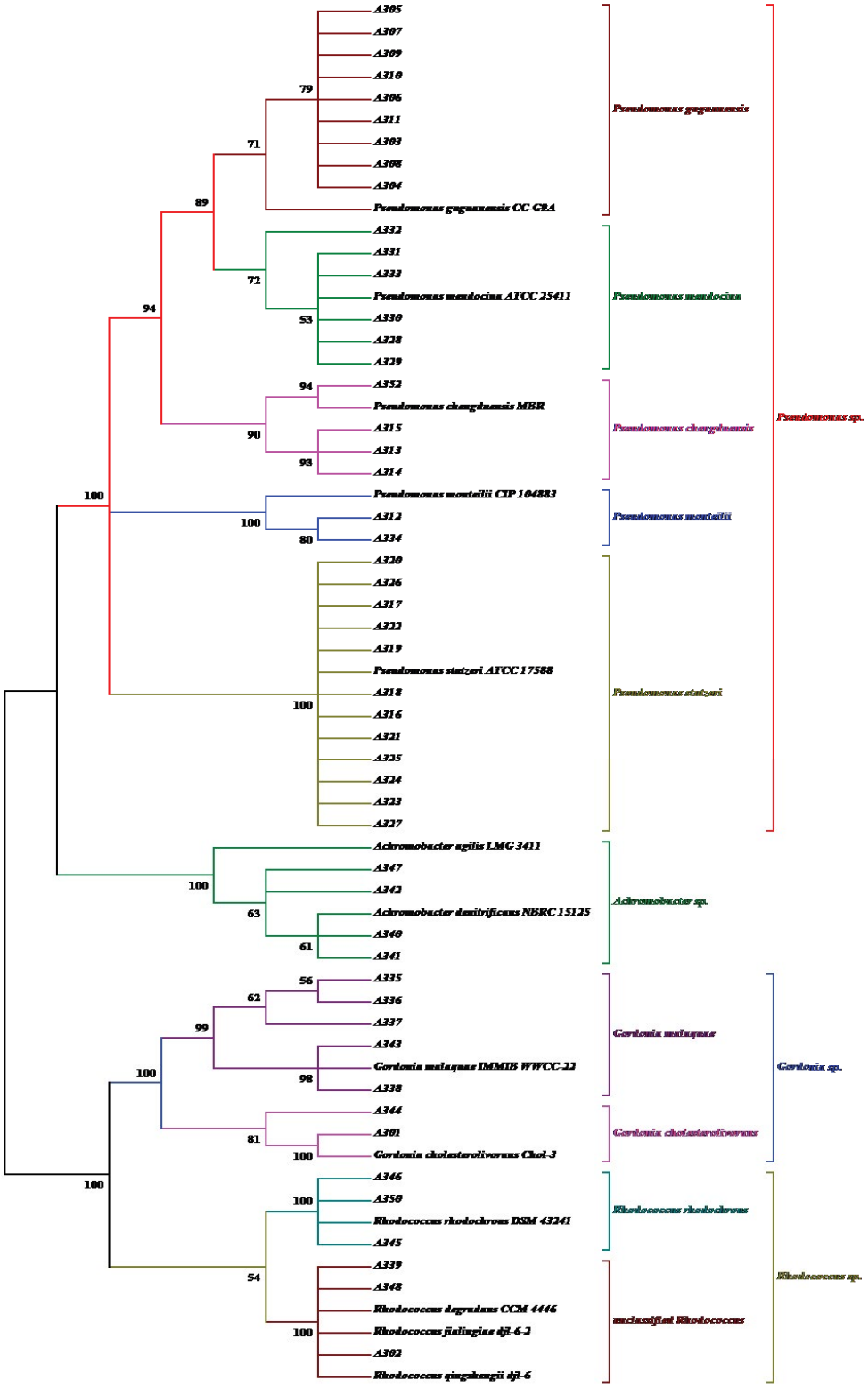

Fig. S4. Phylogenetic tree (rectangular mode) for the culturable aerobic denitrifying isolates from the oxic tank 3. The numbers next to the branches represent the boostrap values.

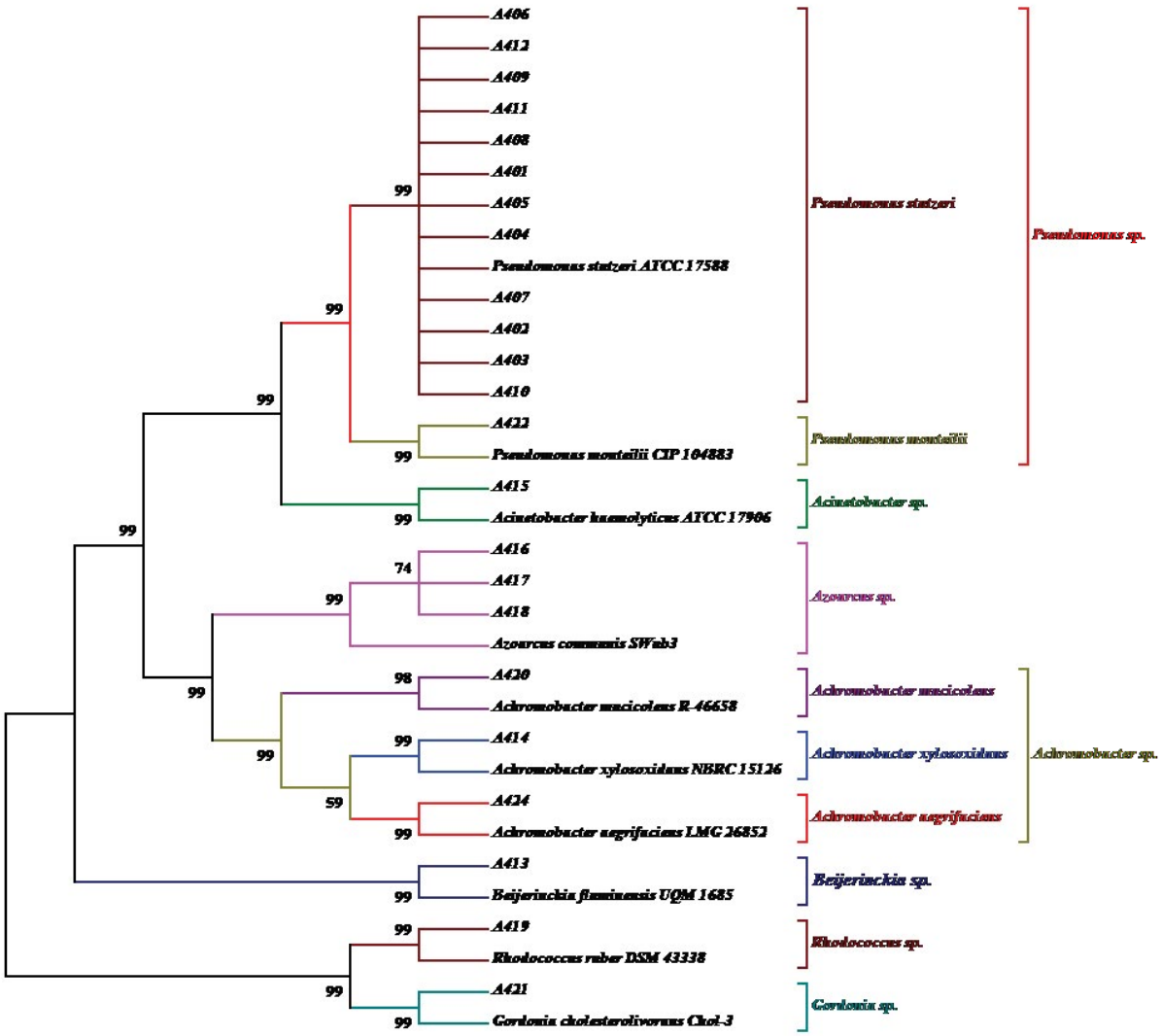

Fig. S5. Phylogenetic tree (rectangular mode) for the culturable aerobic denitrifying isolates from the oxic tank 4. The numbers next to the branches represent the boostrap values.