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Study on Screening and Denitrification Characteristics of Highly Efficient Aerobic Denitrifying Bacteria from Lake Sediments

Zhouying Xu · Kunyuan Xiao · Xinshuo Li · Xiangling Zhang · Yihui Ban · Yinghe Jiang

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Abstract Owing to rapid industrial and agricultural development, nitrate (NO_3^--N) in the aquatic environment has been increasing constantly, which poses a serious threat to human health. The NO_3^--N can be reduced by aerobic denitrifying bacteria to gaseous nitrogen under aerobic conditions. In this study, three mixed aerobic denitrifying bacteria (NH, XF, and WU) were screened from various lake sediments. The NO_3^--N removal efficiencies reached over 97% at 36 h incubation in the medium and 72.89%, 67.33%, and 80.22% at 55 h incubation in eutrophic lake water. Nitrogen balance results showed that aerobic denitrifying bacteria transformed 41.22%, 24.56%, and 47.99% of the total nitrogen into gaseous nitrogen, respectively. High-throughput sequencing results showed the dominant bacteria were *Proteobacteria* and *Pseudomonas*. The relative abundance of functional genes for denitrification was predicted utilizing PICRUSt2, which was consistent with the trend of the quantitative microbial element cycling results. This study will provide a novel approach for applying microbial remediation in aqueous environment.

Keywords Aerobic denitrifying bacteria · Nitrate removal · Eutrophication · QMEC · Denitrification gene

1 Introduction

Recently, due to the discharge of industrial wastewater, domestic sewage, aquaculture wastewater, the concentration of nitrate nitrogen (NO_3^--N) in the aqueous environment has been increasing considerably, which exacerbates the water eutrophication and causes serious consequences for human health and aqueous environment balance (Rajta et al., 2020). When NO_3^- enters the human body, its toxicity multiplies to 11 times (Sebaei & Refai, 2021). Thus, excessive NO_3^- (> 10 mg/L) can cause leukemia in humans and nitrate poisoning in animals (Rajta et al., 2020). Moreover, NO_3^- is an important indicator for evaluating the degree of groundwater pollution. Groundwater utilization and ecological value can be seriously affected by excessive NO_3^- (Elisante & Muzuka, 2016).

Compared with other nitrogen removal processes, biological denitrification is widely utilized because of its lower cost, simpler operation, and less secondary pollution (He et al., 2019). Conventional biological

Z. Xu · K. Xiao · X. Zhang · Y. Jiang (✉)
School of Civil Engineering and Architecture, Wuhan University of Technology, Wuhan 430070, Hubei, China
e-mail: whlgdx218@126.com

Z. Xu · Y. Ban
Weihai Research Institute of Wuhan University of Technology, Weihai 264300, Shandong, China

X. Li · Y. Ban (✉)
School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, Wuhan 430070, Hubei, China
e-mail: banyihui@whut.edu.cn

denitrification processes require the maintenance of a strict anaerobic environment and suitable growth conditions (Kuypers et al., 2018). In actual environment or operation, this will certainly increase the cost and difficulty (Hao et al., 2022). Traditional biological methods are therefore not optimal for remediating water quality in rivers and lakes with excessive NO_3^--N. Fortunately, a bacterial strain capable of converting NH_4^+-N to NO_3^--N and then to N_2 had been isolated (Robertson & Kuenen, 1990), which provided a novel direction for denitrification theory. Subsequently, Some researchers studied aerobic denitrifying bacteria, such as *Pseudomonas mendocina* TJPU04 (He et al., 2019), *Psychrobacter* sp. S11 (Zheng et al., 2011), *Pseudomonas stutzeri* YG-24 (Li et al., 2015), *Paracoccus thiophilus* LSL 251 (Zhang et al., 2018), *Methylobacterium gregans* DC-1 (Hong et al., 2019), *Acinetobacter haemolyticus* ZYL (Wang et al., 2021), and *Bacillus* sp. ST20 (Hoang et al., 2022). These bacteria were found to utilize various carbon sources as nutrient and electron sources for aerobic denitrification. *Pseudomonas stutzeri* YG-24 and *Pseudomonas mendocina* TJPU04 were also reported to perform simultaneous heterotrophic nitrification and aerobic denitrification (He et al., 2019; Li et al., 2015).

Although there have been many studies on aerobic denitrifying bacteria and fungi, most of them have concentrated on the denitrifying performance and optimal working conditions of single strains (Fu et al., 2022; Xia et al., 2020; Zheng et al., 2011). In contrast to single pure bacterium, synergistic effects within the native mixed bacteria and the signaling molecules among microbes can increase the enzymatic activity of them (Gatsios et al., 2021; Guo et al., 2018; Whiteley et al., 2017). As a result, native mixed bacteria are better able to adapt to water quality, utilize carbon sources, and remove pollutants. At present, native mixed aerobic denitrifying bacteria (MADB) are rarely concerned.

The denitrification is a complex process because the corresponding enzymes need to be encoded by a number of functional genes (*narGHI*, *napAB*, *nirKS*, *norBC*, *nosZ*, etc.) (Kuypers et al., 2018). Previous studies have predicted denitrification pathways based on the analysis of one or a few of these functional genes (Xia et al., 2020; Zhu et al., 2022), but all functional genes are rarely investigated. Furthermore, other studies had shown that functional genes predict nitrogen cycling more reliably than microbial analysis (Kuypers et al., 2018). Therefore, to obtain a completer and more reliable denitrification pathway, it is worth analyzing all currently recognized functional denitrification genes (Graham et al., 2016).

This study explored the growth and denitrification performance of MADB, followed by applying the screened MADB to actual lake water. The nitrogen transformation during this process and the characteristics of the microbial community were investigated. Additionally, based on PICRUSt2 and QMEC, the study analyzed the denitrification functional genes and metabolic pathways of the bacterial community. Consequently, this study will provide a reference for further research and lay the foundation for data support in various scale applications. It also will offer choices for bacterial sources and ensure safeguards for the remediation of micro-polluted water environments.

In the present study, several groups of MADB were screened in lake sediments with the expectation of removing NO_3^--N from complex eutrophic waters by aerobic denitrification. The specific objectives were as follows: (1) to screen MADB with good performance in sediments under aerobic conditions; (2) to examine the growth and denitrification performance of MADB at 130 rpm and 30°C; (3) to examine the denitrification capacity of the screened MADB under eutrophic lake water; and (4) to analyze the composition of bacterial communities and predict functional bacterial genes for elucidating denitrification pathways.

2 Materials and Methods

2.1 Denitrification Medium

Denitrifying medium (DM) contained the following ingredients (per liter): 4.7 g $C_4H_4Na_2O_4$, 1 g KNO_3, 1.5 g KH_2PO4, 5.0 g Na_2HPO_4•$7H_2O$, 2 ml trace element solution, 0.05 g $MgSO_4$, pH = 7 ± 0.5. Trace element solution (per liter): 100 mg EDTA, 4.4 mg $ZnSO_4$, 11 mg $CaCl_2$, 10.2 mg $MnCl_2$·$4H_2O$, 10 mg $FeSO_4$•$7H_2O$, 2.2 mg $(NH_4)_6Mo_7O_{24}$•$4H_2O$, 3.2 mg $CuSO_4$·$5H_2O$, 3.2 mg $CoCl_2$•$6H_2O$. The DM was autoclave sterilized at 121 ℃ for 30 min prior to be uses, and the inoculation was carried out in the ultraclean workbench.

2.2 Screening of MADB

150 ml of surface layer sediment was collected from Nan Hu Lake (30.51° N, 114.36° E), Xing Fu Park (30.51° N, 114.34° E), and the pond in Wuhan University of Technology (30.52° N, 114.34° E) in Wuhan, China. 100 ml of the surface layer sediment was placed in a 1-L conical flask containing 500 ml of DM. Conical flasks were sealed with tissue culture breathable films and cultivated at 130 rpm and 30℃ in a shaking incubator. Fresh sterilized DM culture medium was added into the flasks when the total nitrogen (TN) concentrations were lower than 10 mg/L, and continuous culture was conducted until the TN removal efficiencies was stable. The stable mud-water mixtures were diluted to 10^{-3} by 10-fold serial dilution method. 1 ml diluted sample was coated on solid DM medium and cultured in a biochemical incubator at 30℃ until the colonies formed. The colonies on the petri dishes were rinsed with phosphate buffer solution (PBS) to obtain the mixed bacteria named as NH, XF, and WU. The MABD were suspended in 25% glycerin and stored at − 80 °C until further study. Microbes need to be cultured twice before use to eliminate the influence of glycerol.

2.3 Growth Process and Denitrification Characteristics of MADB

Bacteria in logarithmic growth phase (5 ml, $OD_{600} = 0.3 \pm 0.05$) were inoculated into a conical flask containing sterilized fresh DM (100 ml). The bacteria were cultured for 36 h at 130 rpm (DO = 5.90 ± 0.81 mg/L) and 30℃ in a shaking incubator. During the period, samples were filtered through a 0.22-μm membrane filter at regular intervals. The concentrations of NO_3^--N, NH_4^+-N, NO_2^--N, and chemical oxygen demand (COD) were analyzed according to standard methods (APHA, 2017). The OD_{600} was measured to study the concentration of microbial cells. Each experiment was performed in triplicate ($n = 3$).

2.4 Nitrogen Conversion of MADB in Eutrophic Lake Water

The eutrophic lake water was collected from Ye Zhi Lake (Wuhan, China), where the pH was 7.0 ± 0.6 as well as the concentration of TN, NO_3^--N, NO_2^--N, NH_4^+-N, COD, and dissolved oxygen (DO) were 15.72 ± 0.03, 14.54 ± 0.27, 0, 0.40 ± 0.13, 26 ± 3.27, and 5.67 mg/L, respectively. Sodium succinate and inorganic salts were added to the eutrophic lake water to provide nutrients needed for growth. One hundred milliliters of eutrophic lake water in a 250-ml conical flask was inoculated with 5 ml MADB ($OD_{600} = 0.5 \pm 0.05$), and the uninoculated lake water was used as a blank control to eliminate the influence of indigenous microbes. For the calculation of intracellular TN (ITN), samples were treated with a cell crusher (Shanghai Lichen Technology Co., Ltd., Shanghai, China) to obtain bacterial suspensions before measurement of ITN. All samples were treated to 150 cycles using a 150 W cell crusher (Luffing rod, 6 mm diameter). One cycle consisted of 5 s of sonication and 5 s of rest. The bacterial suspensions were processed by centrifugation (8000 rpm) and measured according to standard methods (APHA, 2017).

2.5 Microbial Diversity and Functional Gene Analysis

To study the community composition, the three MADB were filtered through 0.22-μm sterilized membrane after 36 h of incubation, and the filtered membranes were stored at − 80°C for further analysis. DNA was extracted using ALFA-SEQ Advanced Soil DNA Kit (Guangzhou Mchip Bio-Tech Co., Ltd, Guangzhou, China). The concentration and purity were measured using the NanoDrop One (Thermo Fisher Scientific, MA, USA). The V3–V4 region of the 16S rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGG AGGCAGCA-3′) and 806R (5′-GGACTACHVGGG TWTCTAAT-3′). Primers were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, USA). PCR reactions, containing 25 μL 2 × Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 μL each primer(10 μL), and 3 μL DNA (20 ng/μL) template in a volume of 50 μL were amplified by thermocycling: 5 min at 94°C for initialization; 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 30 s extension at 72°C, followed by 10 min final elongation at 72°C. The PCR instrument was BioRad S1000 (Bio-Rad Laboratory, CA, USA).

The length and concentration of the PCR product were detected by 1% agarose gel electrophoresis. PCR products were mixed in equidensity ratios

according to the GeneTools Analysis Software (Version4.03.05.0, SynGene). Mixture of PCR products was purified with E.Z.N.A. Gel Extraction Kit (Omega, USA). Sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA). At last, the library was sequenced on an Illumina Nova6000 platform and 250 bp paired-end reads were generated. The sequencing was carried out by the Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China). In addition, PICRUSt2 (version 2.3.0) was used to predict the function of 16S rRNA gene data in the Magigene cloud platform (cloud.magigene.com). The abundance prediction of KO (KEGG Orthology) function and KEGG pathway were obtained by comparison with KEGG (kyoto encyclopedia of genes and genomes) database. The functional genes (*napA*, *nirS*, *nosZ*) of XF were detected by Quantitative microbial element cycling (QMEC). Amplification was operated on the Wafergen SmartChip Real-time PCR system (Wafergen, Fremont, CA). The procedure was the same with a previous study (Chen et al., 2020).

2.6 Statistical Analysis Methods and Calculations

In this study, the calculation formula of Nitrogen conversion is listed as follows:

$$\text{organic nitrogen}\left(N_{org}\right) = TSN - \left(NO_3^- - N\right) - \left(NH_4^+ - N\right) - \left(NO_2^- - N\right) \quad (1)$$

$$\text{Intracellular TN (ITN)} = TN - TSN \quad (2)$$

$$\text{Gaseous N} = [(\text{Initial TN} - TSN)/\text{Initial TN}] \times 100\% \quad (3)$$

TSN and ITN indicate total soluble nitrogen and intracellular total nitrogen, respectively. TN, NO_3^--N, NH_4^+-N, and NO_2^--N were the data at the end of the experiment.

All figures were drawn using Origin, 2010 software (OriginLab, USA) and R (version 3.6.3). Mean, standard deviation (SD) were calculated using Excel 2019 (Microsoft, USA).

3 Results and Discussion

3.1 Cell Growth

Figure 1 shows the growth and COD removal performance of the three MADB (NH, XF, WU). In an aerobic environment, the three MADB were cultured in DM for 36 h. In the beginning, they grew slowly because the bacteria were in the stage of adapting to the environment (Hong et al., 2019). After an adaptation period of about 6 h, NH and XF entered the logarithmic growth stage, and then the cell concentration increased sharply, reaching 1.73 and 1.18, respectively, at 36 h. In contrast, WU immediately entered the logarithmic growth phase after a shorter adaptation period (about 3 h) and reached the stable phase at 18 h. The microbial cell concentration reached the maximum (OD_{600}=0.98) at 24 h (Fig. 1c). The difference of the adaptation period between the three MADB reflected that WU could adapt to the medium more quickly and grow faster than NH and XF. Nevertheless, the adaptation periods of the three MADB were shorter than LJH1, HH3, and XK1 (Zhang et al., 2022). With the growth of MADB, the concentrations of COD in the culture medium decreased gradually. After 36 h of cultivation, NH, XF, and WU exhibited COD removal efficiencies of 82.35%, 81.25%, and 85.71%, respectively. Furthermore, their respective COD utilization efficiencies were 77.23, 71.72, and 77.23 mg/L•h, which surpassed that of *Pseudomonas mendocina* TJPU04 (54.23 mg/L•h) (He et al., 2019). Accordingly, the above results demonstrated the excellent environmental adaptability and COD removal ability of the three MADB.

3.2 Nitrogen Removal Characteristics

The denitrification characteristics of the three MADB were shown in Fig. 2. After 36 h culture in the medium with the initial NO_3^--N concentration of 136 mg/L, the NO_3^--N concentration in the medium of NH, XF, and WU decreased to 2.76, 0.96, and 0.13 mg/L, respectively. The removal efficiencies of the three MADB were more than 97%, among which the removal

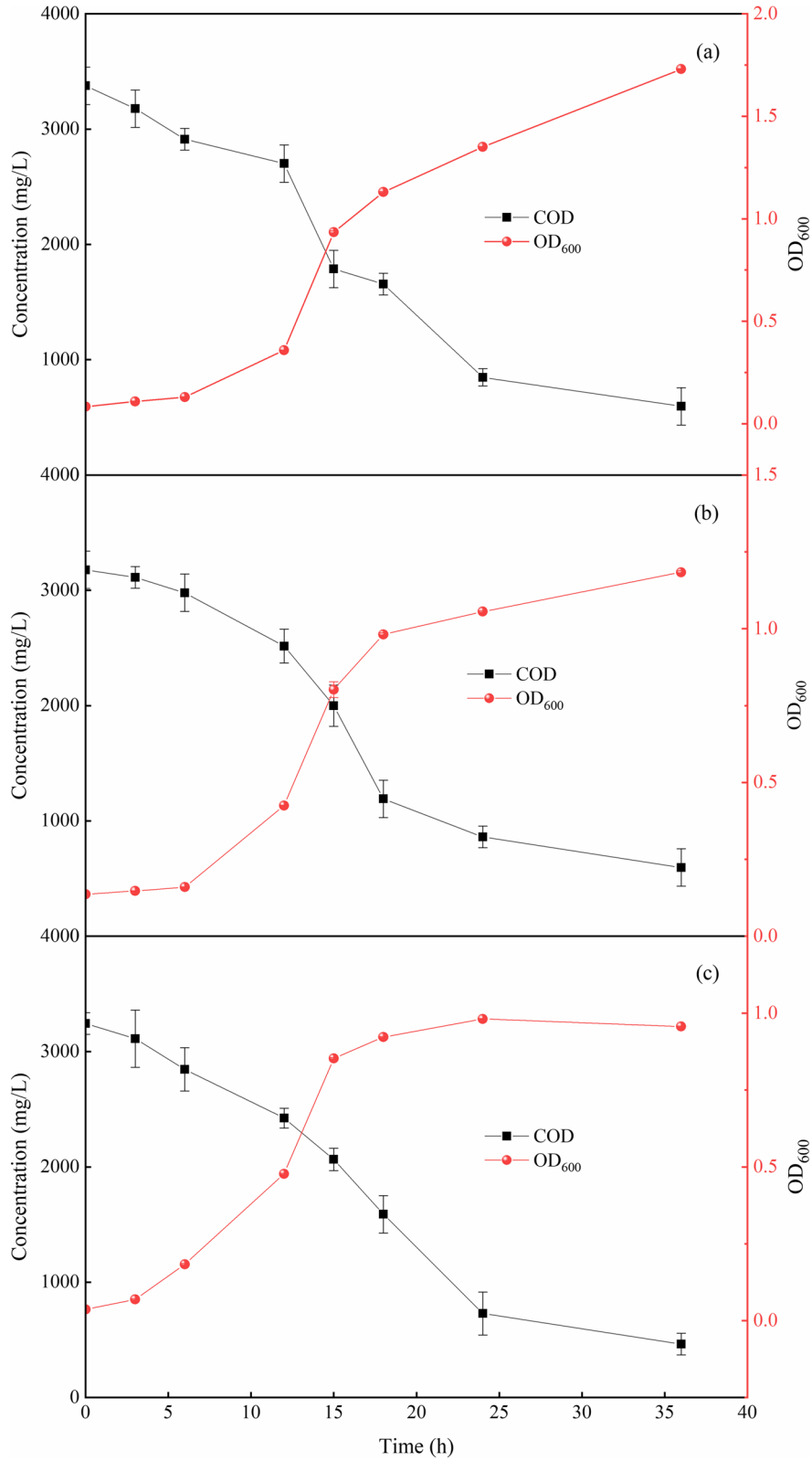

Fig. 1 Cell growth curves and COD concentrations of the three MADB of NH (**a**), XF (**b**), and WU (**c**) inoculated in DM

efficiencies of WU reached 99.91%. The NO_3^--N removal efficiencies of three MADB were higher than the single strain XL-2 (97.9%) (Zhao et al., 2018) and Mix-CADB LJH1 (93.84%) (Zhang et al., 2022). Following 18-h incubation period, a considerable reduction in NO_3^--N concentration was observed, with NH, XF, and WU exhibiting average removal efficiencies of 7.24, 7.40, and 7.41 mg/L•h, respectively. These efficiencies were comparable to that of strain *P. stutzeri* YG-24 (7.73 mg/L•h) (Li et al., 2015) and superior to *P. mendocina* TJPU04 (5.60 mg/L•h) (He et al., 2019).

Additionally, three MADB exhibited varying degrees of NO_2^--N accumulation. The NO_2^--N concentrations of NH and XF increased during 9–15 h, and with the highest cumulative NO_2^--N levels reaching a maximum of 44.95 mg/L for NH. XF reached the highest of 19.66 mg/L and then decreased to less than 0.2 mg/L during 15–24 h. In contrast, WU accumulated a small amount of NO_2^--N during the 6–9-h period, but then the amount decreased. A certain amount accumulated again during the 12–18-h period, reaching the highest level of 8.77 mg/L, and then decreased to 0.14 mg/L within 6 h (Fig. 2c). Zhao et al. (2018) also reported the accumulation of NO_2^--N during the experiment, reaching a maximum of 44.7 mg/L. This phenomenon in MADB might stem from the considerably higher activity of nitrate reductase compared to nitrite reductase (Lycus et al., 2018).

As illustrated in Fig. 2, WU exhibited an earlier NO_2^--N accumulation time compared to NH and XF, possibly due to its shorter adaptation period. However, the NO_2^--N accumulation in WU was considerably lower than that in NH and XF, which could be attributed to WU's robust reducing ability towards NO_2^--N. WU was capable of timely reducing NO_2^--N generated during denitrification to N_2, thereby mitigating the inhibitory effects of NO_2^--N on bacterial growth (Zhang et al., 2022). There was also a slight accumulation of NH_4^+-N, and the accumulation of NH_4^+-N at 0 h might be brought in by bacteria in the logarithmic growth stage. Furthermore, NH_4^+-N accumulation was observed after 18 h, potentially resulting from the demise of numerous fast-growing bacteria due to inadequate nutrient supply. The bacteria underwent autolysis and released NH_4^+-N. This phenomenon was also reported in previous studies (He et al., 2019). Apparently, three MADB presented considerable performance for denitrification under aerobic condition and the performance of WU was particularly excellent.

3.3 Treatment of Eutrophic Lake Water and Nitrogen Conversion Analysis

The three MADB were inoculated in the eutrophic lake water collected from Ye Zhi Lake and cultured under aerobic condition for 55 h. The pollutant concentrations in the eutrophic lake water and the results were shown in Table 1. The results showed that the pollutants in the control group (lake water without inoculation) were almost not removed after the culture, which indicated that the local microbes of Ye Zhi Lake had little influence on the nitrogen removal. The inoculation of MADB resulted in varying degrees of nitrogen reduction in the eutrophic lake water, with WU exhibiting the most superior denitrification performance. The concentrations of TN and NO_3^--N in WU decreased to 4.79 mg/L and 2.86 mg/L, respectively, with the corresponding removal efficiencies reaching 69.09% for TN and 80.22% for NO_3^--N. After 55 h of culture, there was a slight increase in NH_4^+-N, probably due to the dissolution of dead bacterial cells, and varying degrees of NO_2^--N accumulation were also observed. Among the MADB, WU exhibited the lowest level of NO_2^--N accumulation.

Due to the complex composition of the aqueous environment and the variety of carbon compounds, the indigenous microbial will compete with the inoculated bacteria for nutrition. Hence, the treatment effect may be affected by the change of water quality when single strain is used to treat the actual sewage. However, compared with the limitations of single strain, mixed bacteria have more prospects because of their rich microbial species and diverse metabolic pathways. As a complex microbial community, mixed bacteria exhibit a high degree of ecological stability and resilience to environmental fluctuations (Weiland-Brauer, 2021).

Besides, the nitrogen balance of three mixed bacteria was analyzed. As shown in Table 2, 0.25, 0.24, and 0.34 mg N were assimilated in NH, XF, and WU for microbial growth, accounting for 15.61%, 14.59%, and 21.40% of the initial total nitrogen, respectively. The intracellular N obtained in the present study were lower than the previous reports. Single strain *P. stutzeri* T13 assimilated about 44.89% of NO_3^--N into biomass (Sun et al., 2017). Zhang et al. (2022) found mixed cultures LJH1, HH3, and XK1 assimilated about 36.16%, 40.98%, and 22.99% of NO_3^--N

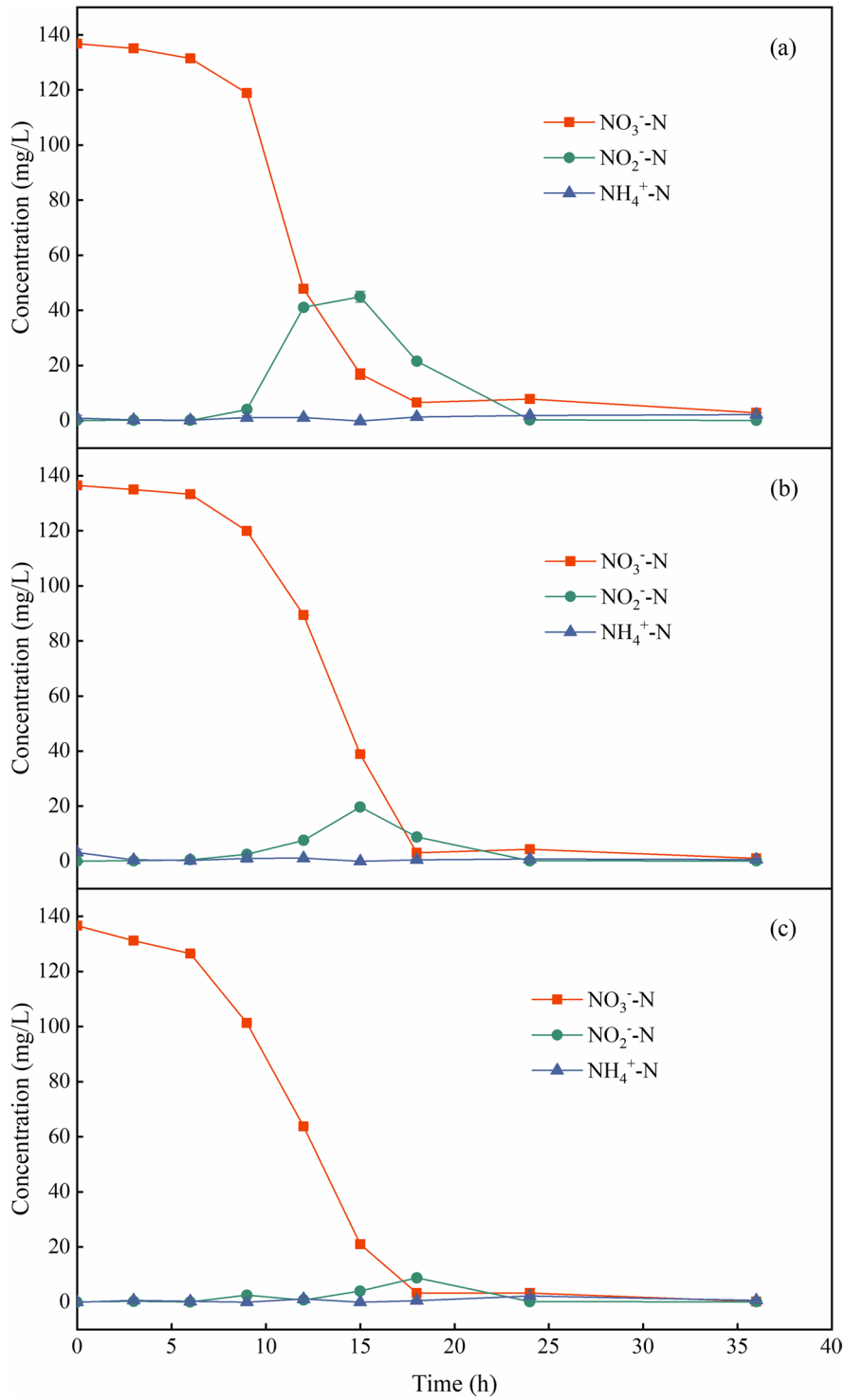

Fig. 2 Nitrogen concentrations of the three MADB of NH (**a**), XF (**b**), and WU (**c**)

into biomass, respectively. It is noteworthy that NH, XF, and WU achieved nitrogen removal efficiencies of 41.21%, 24.56%, and 47.99%, respectively, in the form of gas from eutrophic lake water. These efficiencies are lower than those achieved by mixed cultures of LJH1 (52.56%), HH3 (46.93%), and XK1 (62.82%)

Table 1 Nitrogen concentration of aerobic denitrifying mixed bacteria of NH, XF, and WU treating eutrophic lake water bodies under aerobic conditions

	TN (mg/L)	NO_3^--N (mg/L)	NH_4^+-N (mg/L)	NO_2^--N(mg/L)
Lake water	15.72 ± 0.30	14.54 ± 0.27	0.40 ± 0.13	0.09 ± 0.01
Lake water without inoculation	15.51 ± 0.13	14.45 ± 0.24	0.47 ± 0.16	0.26 ± 0.01
NH inoculation	6.89 ± 0.25	3.92 ± 0.37	0.34 ± 0.12	2.59 ± 0.04
NH removal efficiency (%)	55.61	72.89	27.66	-
XF inoculation	9.82 ± 0.19	4.72 ± 0.28	4.27 ± 0.37	1.47 ± 0.04
XF removal efficiency (%)	36.68	67.33	-	-
WU inoculation	4.79 ± 0.17	2.86 ± 0.33	0.34 ± 0.12	1.29 ± 0.03
WU removal efficiency (%)	69.09	80.22	27.66	-

Values are means ± standard deviation (SD) ($n = 3$)

Table 2 Nitrogen balance of MADB of NH, XF, and WU treating eutrophic lake water under aerobic conditions (unit: mg)

	Initial TN	Final nitrogen				Intracellular N	Gaseous N (%)
		NO^-_3-N	NH_4^+-N	NO_2^--N	N_{Org}		
NH inoculation	1.59 ± 0.026	0.39 ± 0.037	0.03 ± 0.012	0.26 ± 0.004	0.003	0.25	41.21
XF inoculation	1.61 ± 0.033	0.47 ± 0.028	0.35 ± 0.037	0.15 ± 0.004	0.014	0.24	24.56
WU inoculation	1.57 ± 0.034	0.29 ± 0.033	0.03 ± 0.012	0.13 ± 0.003	0.030	0.34	47.99

Values are means ± standard deviation (SD) ($n = 3$)

(Zhang et al., 2022), but higher than single strains of *Stenotrophomonas maltophilia* DQ01 (19.63%) (Jia et al., 2019), *P. stutzeri* AD-1 (17.74%) (Qing et al., 2018), and *Acinetobacter* sp. YT03(28.33%) (Li et al., 2019). However, it needed to be noted that this study was different from some previous studies (Jia et al., 2019; Li et al., 2019; Qing et al., 2018). The object of this experiment was the eutrophic lake water containing NO_3^--N, while the former study treated synthetic wastewater. The complexity of the water quality may be one of the factors that influence the efficiency of nitrogen removal. Taken together, both NH and WU showed excellent denitrification performance compared to XF in the treatment of synthetic wastewater and eutrophic lake water. The results of this study will have a wide application potential.

3.4 Composition and Analysis of MADB

In order to further analyze the difference of nitrogen removal among the three MADB and find the efficient bacteria in aerobic denitrification process, the community structure of the three MADB was determined. Figure 3 shows the composition of the MADB at the phylum and genus levels, respectively. RDA (redundancy analysis) was also used to reveal the relationship between environmental factors and dominant genus-level microbes (Fig. 4). The purpose was to further elucidate the experimental nitrogen content related bacterial.

At the phylum level, Proteobacteria accounted for 71%, 80%, and 99% in NH, XF, and WU, respectively, and the abundance of Proteobacteria was considerably higher in WU than the other two groups of bacteria. The majority of aerobic denitrifying bacteria was Proteobacteria, as previous studies have shown (Kou et al., 2021; Zhang et al., 2022). Bacteroidetes accounted for a total composition of 28%, 11%, and 0.04%. In addition, Patescibacteria made up 9% of the XF and less than 0.1% of the NH and WU. In addition to Proteobacteria, Bacteroidetes were found in large numbers in biological wastewater treatment reactors and constructed wetlands due to their good ability to remove pollutants (Guan et al., 2015; Xiao et al., 2020).

At the genus level, Proteobacteria accounted for 35%, 40%, and 50% in NH, XF, and WU, respectively, and *Pseudomonas* accounted for 27%, 16%, and 47%, respectively. According to Fig. 4, the

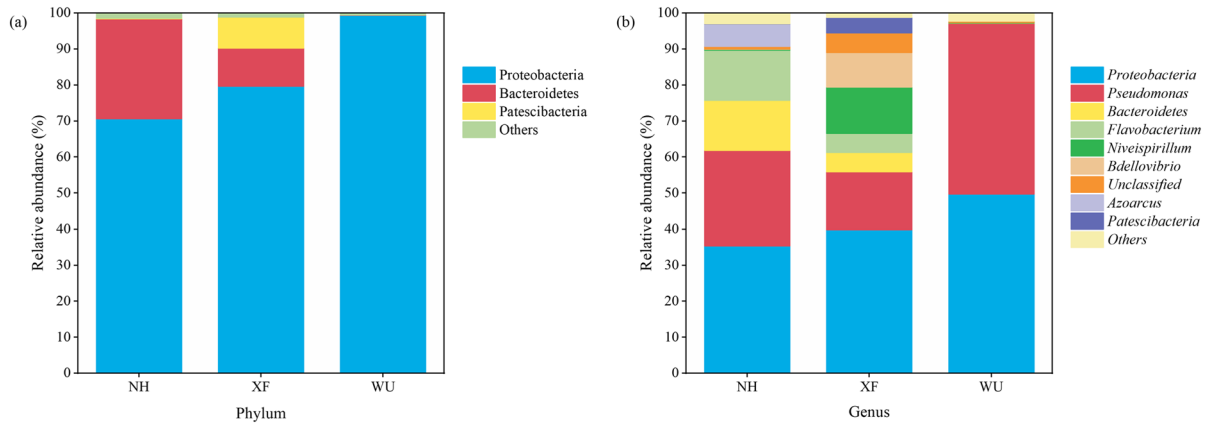

Fig. 3 Relative abundance of major taxa of microbial communities of three MADB. **a** At the phylum level (top 3); **b** at the genus level (top 9). Phyla and genus that comprise < 0.1% of the bacteria in all treatments are grouped into "Others"

abundance of Proteobacteria and *Pseudomonas* was positively correlated with R(NO_3^--N). Similar to the results of previous studies, Proteobacteria and *Pseudomonas* were the dominant homogeneous genera in the aerobic denitrification bacteria (Zhang et al., 2022). Therefore, it could be hypothesized that Proteobacteria and *Pseudomonas* were the primary contributing bacterium in MADB for NO_3^--N removal. *Bacteroidetes* (14%), *Flavobacterium* (14%), and *Azoarcus* (6%) were more abundant in NH than the other two groups. *Bacteroidetes* had been also found in nitrification–denitrification tanks in wastewater treatment plants (Juretschko et al., 2002). *Flavobacterium* had been widespread in wastewater, sediment, and activated sludge (Cai et al., 2018; Liu et al., 2017). *Azoarcus* had been divided into two main groups, nitrogen-fixing bacteria that had grown in the root system but had difficulty surviving outside the root system (Wartiainen et al., 2008) and aromatic hydrocarbons-degraded bacteria under denitrification conditions (Lee et al., 2014). *Azoarcus* found in this study may belong to the second class of denitrifying bacteria. The abundance of *Bacteroidetes*, *Flavobacterium*, and *Azoarcus* were positively correlated with NO_2^--N (Fig. 4), indicating that these genera might be the contributing factor to the accumulation of NO_2^--N, and have a competitive relationship with nitrite-reducing bacteria in NH. *Niveispirillum* (13%) and *Bdellovibrio* (10%) were more abundant in XF than the other two groups. Bacteria of the genus *Niveispirillum* usually have the ability to fix nitrogen. Cai et al. (2015) had isolated a bacterial strain of *Niveispirillum* with the ability to fix nitrogen in eutrophic Lake Taihu. *Bdellovibrio* were predatory bacteria that attacked and consumed other bacteria (Dwidar et al., 2012). The relative abundance of the nitrate removal-contributing bacterium Proteobacteria in XF was higher than NH but lower than WU. However, the NO_3^--N removal efficiency in XF was the lowest among the three MADB. It might be due to the nitrogen-fixing ability of *Niveispirillum* and the attack of *Bdellovibrio* on Proteobacteria and other bacteria with denitrifying functions (Cai et al., 2015; Dwidar et al., 2012). In addition, the relative abundance of *Pseudomonas* in XF was the lowest. It had been demonstrated in many wastewater treatment systems that *Pseudomonas* was a highly important nitrogen-removing bacterium due to its strong nitrification ability (Zhang et al., 2019). The reasons of poor nitrogen removal performance of XF could be attributed to the presence of *Niveispirillum* and *Bdellovibrio*. As a whole, the data obtained in the present study (Fig. 2, Fig. 4 and Table 1) showed that WU exhibited better performance in both synthetic wastewater and eutrophic lake water, probably due to the higher abundance of efficient bacteria (Proteobacteria and *Pseudomonas*) in the aerobic denitrification process. The unique microbial composition in XF might be the reason for the lower pollutant removal efficiency than NH and WU.

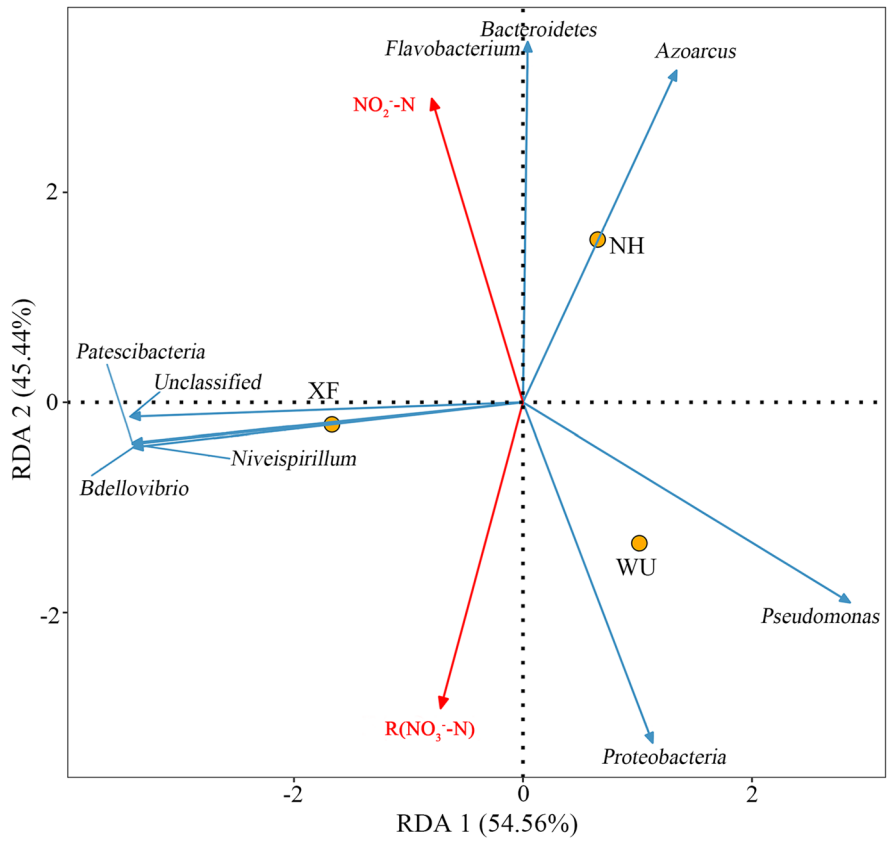

Fig. 4 Redundancy analysis (RDA) of the relationships between the top 9 relative abundances at the genus level and environmental factors. NO_2^--N represented the accumulation of NO_2^--N; R(NO_3^--N) represented the removal rate of NO_3^--N. Blue arrows represented the genera of the microorganisms. Red arrows represented environmental factors. Orange dots represented the samples

3.5 Prediction and Analysis of Denitrification Pathways of MADB

To further investigate the denitrification pathways within the microbial community, PICRUSt2 was used for predictive profiling of relevant functional genes. The denitrification pathway of MADB was shown in Fig. 5a. Ten functional genes of denitrification (*narG*, *narH*, *narI*, *napA*, *napB*, *nirS*, *nirK*, *norB*, *norC*, *nosZ*) were obtained, and the relative abundances of each are shown in the bubble diagram in Fig. 5b. In addition, three genes (*napA*, *nirS*, and *nosZ*) were detected by QMEC in the XF at 0 h and 36 h, and their trends were consistent with those predicted by PICRUSt2 (Fig. 5c).

The gene *narGHI* and *napAB* are responsible for encoding nitrate reductase Nap and Nar. Among these, the expression of *narGHI* is favored under anaerobic conditions, whereas that of *napAB* is favored under aerobic conditions (Wang et al., 2019). The genes *napAB* encodes periplasmic nitrate reductase, which is preferentially expressed under aerobic conditions. Aerobic denitrifying bacteria typically use Nap enzyme for nitrate reduction, representing the primary difference between aerobic and anaerobic denitrifying bacteria. Therefore, *napAB* has been widely used as a marker gene for the identification of aerobic denitrifying bacteria (Wang et al., 2019). The relative abundance of *napAB* was much higher than that of the *narGHI* in the three MADB. This result was in line with what had been reported previously.

The genes *nirS* and *nirK* encode Cd-type and Cu-type nitrite reductase, respectively. Nitrite reductase is responsible for the conversion of NO_2^--N to NO, which is a crucial step in denitrification (Xia et al., 2020). In addition, the relative abundance of *nirK* was found to be considerably higher than that of *nirS* in the three MADB. Previous studies had also found that *nirK* was more abundant in aerobic than anaerobic environments, while *nirS* was more abundant in anaerobic environments (Li et al., 2021; Zhang et al., 2019). In the three MADB, the relative abundance of the gene *nirK* was the lowest in NH, while it was the highest in WU. The difference might be attributed to the accumulation of NO_2^--N in the XF during the denitrification process, with WU exhibiting the least accumulation.

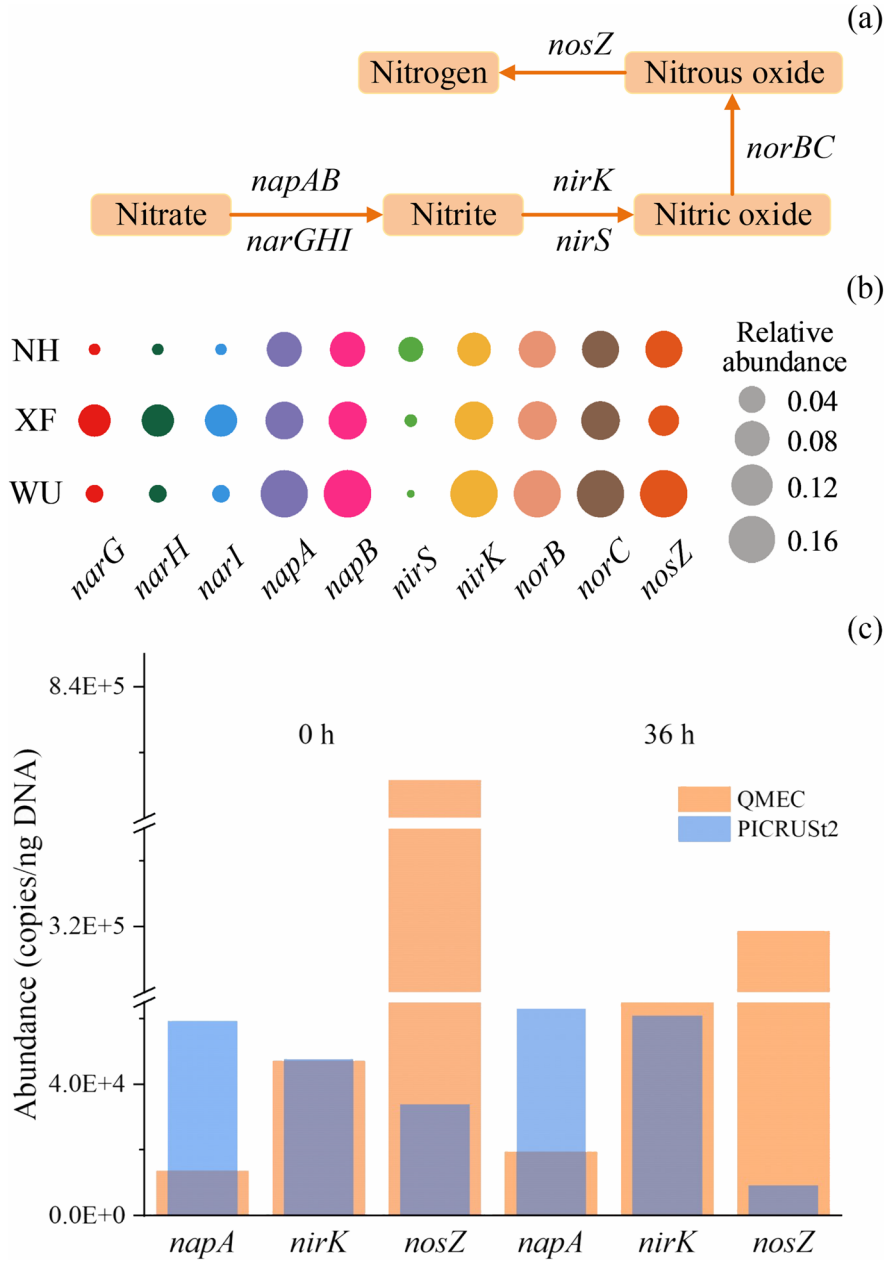

Fig. 5 The abundance of nitrogen cycling functional genes and metabolic pathway diagram. **a** Denitrification metabolic pathways. **b** The relative abundance bubble plot of MADB functional genes. **c** Comparison of functional gene results between QMEC and PICRUSt2 in XF in 0 h and 36 h

Nitric oxide reductase (Nor), encoded by *norBC*, is responsible for converting nitric oxide to nitrous oxide, thereby maintaining low levels of NO in the cell. Nor prevents adverse effects on cellular metabolism that could be caused by high levels of NO (Pang & Wang, 2021).

The gene *nosZ* express oxynitrite reductases responsible for catalyzing the electron transfer of oxynitrite reductase and the reduction of N_2O to N_2, respectively (Kuypers et al., 2018). Previous studies had shown that the gene *nosZ* contributed to the removal of TN (Liang et al., 2021). In three MADB, the abundance of *nosZ* was the highest in WU, which might explain the higher TN removal efficiencies observed in WU. In addition, a higher abundance of the gene *nosZ* could potentially be more favorable in reducing the emission of the greenhouse gas N_2O during denitrification processes.

4 Conclusion

Three efficient MADB NH, XF, and WU, with nitrate removal efficiencies exceeded 97% after 36-h incubation in DM, were screened from different lake sediments. After the application of the MADB in the treatment of eutrophic lake water, the NO_3^--N removal efficiencies reached 72.89%, 67.33%, and 80.22%, and removal efficiencies of TN in the form of gas reached 41.22%, 24.56%, and 47.99%, respectively. The results of high-throughput sequencing showed the MADB were mainly composed of *Proteobacteria* and *Pseudomonas*. MADB has the potential to remove NO_3^--N from both synthetic wastewater and actual lake water. The denitrification pathway predicted by PICRUSt2 and QMEC results followed the same trend as the PICRUSt2 results. Therefore, MADB might be a reference for the remediation of water quality in rivers and lakes.

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Data Availability Data will be made available on request.

Declarations

Competing Interests The authors declare no competing interests.

References

APHA. (2017). *Standard methods for the examination of water and wastewater* (23rd ed.). American Public Health Association.

Cai, H., Wang, Y., Xu, H., Yan, Z., Jia, B., Maszenan, A. M., & Jiang, H. (2015). *Niveispirillum cyanobacteriorum* sp nov., a nitrogen-fixing bacterium isolated from cyanobacterial aggregates in a eutrophic lake. *International Journal of Systematic and Evolutionary Microbiology, 65*, 2537–2541.

Cai, H., He, W., Wang, Y., Yan, Z., Wang, C., Xu, H., & Shao, K. (2018). *Flavobacterium aurantiibacter* sp nov., an orange-pigmented bacterium isolated from cyanobacterial aggregates in a eutrophic lake. *International Journal of Systematic and Evolutionary Microbiology, 68*(6), 1839–1844.

Chen, Q.-L., Ding, J., Li, C.-Y., Yan, Z.-Z., He, J.-Z., & Hu, H.-W. (2020). Microbial functional attributes, rather than taxonomic attributes, drive top soil respiration, nitrification and denitrification processes. *Science of the Total Environment, 734*, 139479.

Dwidar, M., Monnappa, A. K., & Mitchell, R. J. (2012). The dual probiotic and antibiotic nature of Bdellovibrio bacteriovorus. *Bmb Reports, 45*(2), 71–78.

Elisante, E., & Muzuka, A. N. N. (2016). Assessment of sources and transformation of nitrate in groundwater on the slopes of Mount Meru, Tanzania. *Environmental Earth Sciences, 75*(3), 15.

Fu, W., Song, G., Wang, Y., Wang, Q., Duan, P., Liu, C., Zhang, X. and Rao, Z. (2022). Advances in research into and applications of heterotrophic nitrifying and aerobic denitrifying microorganisms. *Frontiers in Environmental Science, 10*, 887093.

Gatsios, A., Kim, C. S., & Crawford, J. M. (2021). Escherichia coli small molecule metabolism at the host-microorganism interface. *Nature Chemical Biology, 17*(10), 1016–1026.

Graham, E. B., Knelman, J. E., Schindlbacher, A., Siciliano, S., Breulmann, M., Yannarell, A., Bemans, J. M., Abell, G., Philippot, L., Prosser, J., Foulquier, A., Yuste, J. C., Glanville, H. C., Jones, D. L., Angel, F., Salminen, J., Newton, R. J., Burgmann, H., Ingram, L. J., … Nemergut, D. R. (2016). Microbes as engines of ecosystem function: When does community structure enhance predictions of ecosystem processes? *Frontiers in Microbiology, 7*, 214.

Guan, W., Yin, M., He, T., & Xie, S. (2015). Influence of substrate type on microbial community structure in vertical-flow constructed wetland treating polluted river water. *Environmental Science and Pollution Research, 22*(20), 16202–16209.

Guo, Y., Yang, R., Zhang, Z., Wang, X., Ye, X., & Chen, S. (2018). Synergy of carbon and nitrogen removal of a co-culture of two aerobic denitrifying bacterial strains, *Acinetobacter* sp GA and *Pseudomonas* sp GP. *Rsc Advances, 8*(38), 21558–21565.

Hao, Z.-L., Ali, A., Ren, Y., Su, J.-F. and Wang, Z. (2022). A mechanistic review on aerobic denitrification for nitrogen removal in water treatment. *Science of the Total Environment, 847*, 157452.

He, X., Sun, Q., Xu, T., Dai, M., & Wei, D. (2019). Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a novel halotolerant bacterium *Pseudomonas mendocina* TJPU04. *Bioprocess and Biosystems Engineering, 42*(5), 853–866.

Hoang, P. H., Nguyen, T. M., Le, N. C. T., Phan, K. S., Mai, T. T. T., & Ha, P. T. (2022). Characterization of isolated aerobic denitrifying bacteria and their potential use in the treatment of nitrogen-polluted aquaculture water. *Current Microbiology, 79*(7), 209.

Hong, P., Shu, Y., Wu, X., Wang, C., Tian, C., Wu, H., Donde, O. O., & Xiao, B. (2019). Efficacy of zero nitrous oxide emitting aerobic denitrifying bacterium, *Methylobacterium gregans* DC-1 in nitrate removal with strong auto-aggregation property. *Bioresource Technology, 293*, 122083.

Jia, Y., Zhou, M., Chen, Y., Luo, J., & Hu, Y. (2019). Carbon selection for nitrogen degradation pathway by *Stenotrophomonas maltophilia*: Based on the balances of nitrogen, carbon and electron. *Bioresource Technology, 294*, 122114.

Juretschko, S., Loy, A., Lehner, A., & Wagner, M. (2002). The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment

plant analyzed by the full-cycle rRNA approach. *Systematic and Applied Microbiology, 25*(1), 84–99.

Kou, L., Huang, T., Zhang, H., Wen, G., Li, N., Wang, C., & Lu, L. (2021). Mix-cultured aerobic denitrifying bacterial communities reduce nitrate: Novel insights in micro-polluted water treatment at lower temperature. *Science of the Total Environment, 796*, 148910.

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology, 16*(5), 263–276.

Lee, D.-J., Wong, B.-T., & Adav, S. S. (2014). *Azoarcus taiwanensis* sp nov., a denitrifying species isolated from a hot spring. *Applied Microbiology and Biotechnology, 98*(3), 1301–1307.

Li, C., Yang, J., Wang, X., Wang, E., Li, B., He, R., & Yuan, H. (2015). Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a phosphate accumulating bacterium *Pseudomonas stutzeri* YG-24. *Bioresource Technology, 182*, 18–25.

Li, B., Lv, R., Xiao, Y., Hu, W., Mai, Y., Zhang, J., Lin, L., & Hu, X. (2019). A novel nitrite-base aerobic denitrifying bacterium *Acinetobacter* sp. YT03 and its transcriptome analysis. *Frontiers in Microbiology, 10*, 2580.

Li, Y., Ling, J., Chen, P., Chen, J., Dai, R., Liao, J., Yu, J., & Xu, Y. (2021). *Pseudomonas mendocina* LYX: A novel aerobic bacterium with advantage of removing nitrate high effectively by assimilation and dissimilation simultaneously. *Frontiers of Environmental Science & Engineering, 15*(4), 1–10.

Liang, D., He, W., Li, C., Wang, F., Crittenden, J. C., & Feng, Y. (2021). Remediation of nitrate contamination by membrane hydrogenotrophic denitrifying biofilm integrated in microbial electrolysis cell. *Water Research, 188*, 116498.

Liu, H., Lu, P., Jin, L., & Zhu, G. (2017). *Flavobacterium luticocti* sp nov., isolated from wastewater. *International Journal of Systematic and Evolutionary Microbiology, 67*(2), 369–373.

Lycus, P., Soriano-Laguna, M. J., Kjos, M., Richardson, D. J., Gates, A. J., Milligan, D. A., Frostegard, A., Bergaust, L., & Bakken, L. R. (2018). A bet-hedging strategy for denitrifying bacteria curtails their release of N2O. *Proceedings of the National Academy of Sciences of the United States of America, 115*(46), 11820–11825.

Pang, Y., & Wang, J. (2021). Various electron donors for biological nitrate removal: A review. *Science of the Total Environment, 794*, 148699.

Qing, H., Donde, O. O., Tian, C., Wang, C., Wu, X., Feng, S., Liu, Y., & Xiao, B. (2018). Novel heterotrophic nitrogen removal and assimilation characteristic of the newly isolated bacterium *Pseudomonas stutzeri* AD-1. *Journal of Bioscience and Bioengineering, 126*(3), 339–345.

Rajta, A., Bhatia, R., Setia, H., & Pathania, P. (2020). Role of heterotrophic aerobic denitrifying bacteria in nitrate removal from wastewater. *Journal of Applied Microbiology, 128*(5), 1261–1278.

Robertson, L. A., & Kuenen, J. G. (1990). Combined heterotrophic nitrification and aerobic denitrification in thiosphaera-pantotropha and other bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 57*(3), 139–152.

Sebaei, A. S., & Refai, H. M. (2021). Hazard index: Probabilistic risk exposure of nitrate and nitrite in Egyptian fruits and vegetables. *International Journal of Environmental Analytical Chemistry, 101*(10), 1477–1484.

Sun, Y., Feng, L., Li, A., Zhang, X., Yang, J., & Ma, F. (2017). Ammonium assimilation: An important accessory during aerobic denitrification of *Pseudomonas stutzeri* T13. *Bioresource Technology, 234*, 264–272.

Wang, X. J., Wang, W. Q., Zhang, Y., Sun, Z. T., Zhang, J., Chen, G. H., & Li, J. (2019). Simultaneous nitrification and denitrification by a novel isolated *Pseudomonas* sp. JQ-H3 using polycaprolactone as carbon source. *Bioresource Technology, 288*, 121506.

Wang, Y., Zou, Y.-L., Chen, H., & Lv, Y.-K. (2021). Nitrate removal performances of a new aerobic denitrifier, *Acinetobacter haemolyticus* ZYL, isolated from domestic wastewater. *Bioprocess and Biosystems Engineering, 44*(2), 391–401.

Wartiainen, I., Eriksson, T., Zheng, W., & Rasmussen, U. (2008). Variation in the active diazotrophic community in rice paddy - nifH PCR-DGGE analysis of rhizosphere and bulk soil. *Applied Soil Ecology, 39*(1), 65–75.

Weiland-Brauer, N. (2021). Friends or foes-microbial interactions in nature. *Biology-Basel, 10*(6), 496.

Whiteley, M., Diggle, S. P., & Greenberg, E. P. (2017). Progress in and promise of bacterial quorum sensing research. *Nature, 551*(7680), 313–320.

Xia, L., Li, X., Fan, W., & Wang, J. (2020). Heterotrophic nitrification and aerobic denitrification by a novel *Acinetobacter* sp. ND7 isolated from municipal activated sludge. *Bioresource Technology, 301*, 122749.

Xiao, J., Huang, J., Huang, M., Chen, M., & Wang, M. (2020). Application of basalt fiber in vertical flow constructed wetland for different pollution loads wastewater: Performance, substrate enzyme activity and microorganism community. *Bioresource Technology, 318*, 124229.

Zhang, H., Zhao, Z., Chen, S., Kang, P., Wang, Y., Feng, J., Jia, J., Yan, M., Wang, Y., & Xu, L. (2018). *Paracoccus versutus* KS293 adaptation to aerobic and anaerobic denitrification: Insights from nitrogen removal, functional gene abundance, and proteomic profiling analysis. *Bioresource Technology, 260*, 321–328.

Zhang, H., Zhao, Z., Li, S., Chen, S., Huang, T., Li, N., Yang, S., Wang, Y., Kou, L., & Zhang, X. (2019). Nitrogen removal by mix-cultured aerobic denitrifying bacteria isolated by ultrasound: Performance, co-occurrence pattern and wastewater treatment. *Chemical Engineering Journal, 372*, 26–36.

Zhang, H. H., Shi, Y. J., Ma, B., Huang, T. L., Zhang, H., Niu, L. M., Liu, X., & Liu, H. Y. (2022). Mix-cultured aerobic denitrifying bacteria augmented carbon and nitrogen removal for micro-polluted water: Metabolic activity, coexistence and interactions, and immobilized bacteria for reservoir raw water treatment. *Science of the Total Environment, 838*, 156475.

Zhao, B., Cheng, D. Y., Tan, P., An, Q., & Guo, J. S. (2018). Characterization of an aerobic denitrifier *Pseudomonas stutzeri* strain XL-2 to achieve efficient nitrate removal. *Bioresource Technology, 250*, 564–573.

Zheng, H., Liu, Y., Sun, G., Gao, X., Zhang, Q., & Liu, Z. (2011). Denitrification characteristics of a marine origin psychrophilic aerobic denitrifying bacterium. *Journal of Environmental Sciences, 23*(11), 1888–1893.

Zhu, W., Zhang, X., Yuan, S., Sang, W., Tang, X., & Zhang, S. (2022). Effects of different aeration strategies on removal of organics, nitrogen and phosphorus in sequencing batch biofilm reactor (SBBR): Performance, microbial

community and nitrogen cycling pathways. *Journal of Water Process Engineering, 49*, 103118.