Environmental Science and Pollution Research (2020) 27:9307–9317
https://doi.org/10.1007/s11356-019-07551-3

RESEARCH ARTICLE

A lab-scale study on heterotrophic nitrification-aerobic denitrification for nitrogen control in aquatic ecosystem

Zixia Qiao[1] · Yaoguo Wu[1] · Jin Qian[1,2] · Sihai Hu[1] · Jiangwei Chan[1] · Xiaoyan Liu[1] · Ran Sun[1] · Wendong Wang[3] · Bo Zhou[1]

Received: 2 February 2019 / Accepted: 29 December 2019 / Published online: 8 January 2020

Abstract

Nitrogen (N) loss is generally caused by denitrification under anaerobic conditions and the N loss in the heterotrophic nitrification–aerobic denitrification (HN–AD) system is of recent research interest. However, previous studies are generally focused on pure cultures-based system and the information on HN–AD in the complex aquatic ecosystem is limited. In this study, HN-AD system was established in the mixed cultures of the sediments and the performances of HN-AD were evaluated under different conditions. Further, the N loss mechanism in HN–AD system was explored. The study found that the N was lost in the sediment cultures with ammonium-N (NH_4^+–N) (or) and nitrate-N (NO_3^-–N) as N source under aerobic conditions. The highest N loss rate was achieved under the TOC/TN mass ratio of 10 with citrate as the carbon source. Under this condition, the N loss percentages of NH_4^+–N (201.91 mg/L) and NO_3^-–N (130.00 mg/L) reached 99.61% and 100.00%, respectively, which were higher than those in the pure HN–AD strains reported in the literature. High NH_4^+–N removal efficiencies were also achieved at low C/N mass ratio and high NH_4^+–N concentration (493.12 mg L^{-1}). The N loss pathway in the system was investigated by adding Na_2WO_4 as the nitrate reductase inhibitor. The study found that the N was not lost via partial nitrification/denitrification pathway, i.e., $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O$ (N_2), instead via full nitrification/denitrification pathway, i.e., $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2O$ (N_2), since nitrate was a key intermediate. The variation in NH_4^+–N, NO_3^-–N, and NO_2^-–N concentrations in the HN–AD processes further confirmed the N transformation pathway. Therefore, HN–AD may occur and cause N loss in natural aquatic ecosystems. The results of this study demonstrate that N was lost through HN-AD and that the well-cultured HN-AD sediments could be useful biological tool to remediate eutrophic water bodies.

Keywords Nitrogen loss · Heterotrophic nitrification-aerobic denitrification · Aquatic ecosystems · Mixed culture

Introduction

Over the last few years, the usage of artificial N fertilizer has doubled to achieve high crop yields to cater for the rapidly growing human population (Galloway et al. 2008; Lunau et al. 2013; Tilman et al. 2002), leading to increased nitrogen-saturation of the terrestrial ecosystem. Accordingly, significant amounts of ammonium–N (NH_4^+–N) and nitrate–N (NO_3^-–N) have entered into water bodies such as lakes, reservoirs, and seas (Zhou et al. 2007) via tributary rivers, resulting in deterioration of aquatic ecosystems, adverse public health outcomes, and local economy worldwide (Hu et al. 2018; Qian et al. 2018a; Wang et al. 2017; Liang et al. 2005). Furthermore, the excess N can cause eutrophication and hypoxia in aquatic ecosystems, destruction of habitats for resident organisms, and reduction of species diversity (Lanoux et al.

Responsible editor: Boqiang Qin

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-019-07551-3) contains supplementary material, which is available to authorized users.

✉ Yaoguo Wu
wuygal@nwpu.edu.cn

[1] Department of Applied Chemistry, Northwestern Polytechnical University, Xi'an 710129, China

[2] Research & Development Institute, Northwestern Polytechnical University in Shenzhen, Shenzhen 518057, China

[3] School of Human Settlements and Civil Engineering, Xi'an Jiaotong University, Xi'an 710049, China

2013; Lenihan and Peterson 1998; Roberts et al. 2014). Thus, the removal and reduction of N in aquatic ecosystems are critical.

A biological approach involving autotrophic nitrification and denitrification is extensively applied for N removal from wastewater and nitrogen-polluted water bodies (Kuypers et al. 2018; Qian et al. 2018b). Nitrification is the aerobic oxidation of ammonium to nitrate ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) by autotrophs including ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), whereas denitrification is the anaerobic stepwise reduction of nitrate to N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) by heterotrophs. Because of the different requirements for dissolved oxygen (DO) and organic carbon, the nitrification and denitrification are operated in separate aerobic and anoxic reactors, respectively, making the conventional biological N removal (BNR) system complex and expensive. Thus, the alternative heterotrophic nitrification-aerobic nitrification (HN-AD) system is extensively studied to overcome the drawbacks of BNR.

Thiosphaera pantotropha capable of performing both heterotrophic nitrification and aerobic denitrification was firstly found in a wastewater treatment plant in 1984 (Robertson and Kuenen 1984). Thereafter, several HN–AD strains including *Alcaligenes faecalis* No.4 (Joo et al. 2005), *Providencia rettgeri* YL (Taylor et al. 2009), *Rhodococcus* sp. CPZ24 (Chen et al. 2012), *Chryseobacterium* sp. R31 (Kundu et al. 2014), and *Pseudomonas stutzeri* strain T1 (Guo et al. 2013) were isolated and characterized in wastewater treatment plants and natural water ecosystems, suggesting that there is a great potential to apply the HD-AD for the remediation of nitrogen-laden water body. HN–AD have several advantages compared with the autotrophic nitrification or heterotrophic denitrification, including the occurrence of nitrification and denitrification within the same systems, faster growth, and ability to perform denitrification under high DO levels and at low temperatures (Choi et al. 2016).

Consequently, more researchers have focused on N removal using HN-AD strains in nitrogen-laden water body. For example, Duan et al. (2015) used HN-AD strain SF-16 to treat saline wastewater and found that the average NH_4^+–N and TN removal efficiencies reached 97.14% and 73.92%, respectively. Tang et al. (2018a, b) applied aerobic denitrifiers coupled with a denitrification agent in the sediment of an urban river for the bioremediation of N pollution and explored the mechanism of aerobic denitrifiers on urban river sediment remediation. Guo et al. (2013) demonstrated that HN-AD strain *Pseudomonas stutzeri* strain T1, which was isolated from a shallow lake, showed both rapid removal rate and efficiency for NH_4^+–N and NO_3^-–N at 60% and 75%, respectively. They also confirmed that the water quality of the sample improved from Grade V to Grade II due to the addition of this strain. Consequently, the HN-AD bacteria are a promising alternative for N removal in nitrogen-polluted water body.

However, the HN-AD has never been investigated with mixed cultures such as in the sediments, and the feasibility of HN-AD in the aquatic ecosystem requires a comprehensive investigation. In this study, a mixed cultures-driven HN-AD system was established in a simulated aquatic ecosystem with the objectives to investigate (1) the effects of organic carbon source, C/N ratio, and NH_4^+–N concentration on HN–AD performance and (2) the mechanism of N loss in HN-AD system.

Materials and methods

Study area

Qixiang Lake (34° 1′ 30″–34° 1′ 40″ N, 108° 45′ 30″–108° 45′ 40″ E) is located in the campus of Northwest Polytechnical University, Shaanxi, China (Fig. 1), covers a surface area of 0.03 km^2, and has 1.4-m mean water depth and 3-m maximum water depth. The lake area has a typical continental monsoon climate characterized by hot and rainy summer, and cold and dry winter, with a mean annual temperature of 15.6 °C (Quan et al. 2013). The mean annual precipitation is approximately 554 mm and the rainfall is concentrated in the summer and autumn (Song et al. 2010).

Eutrophication is a significant problem in the lake for many years, especially in summer (Fig. S1), while the N content was relatively lower in spring without any significant eutrophication. It was hypothesized that microorganisms could have removed N in the lake. Thus, water and sediment samples were collected from the lake in spring to investigate the N removal performance of the microorganisms.

Water and sediment sampling

Water and sediments samples were collected from 20 fixed sites in Qixiang Lake on March 15, 2018, using a TC–Y sampler (TECH Instrument in Shenyang, China) and a homemade grab sampler, respectively, and then mixed and homogenized to form a composite sample. The sediment samples were immediately air-dried and sieved through a 2-mm sieve before further use. The physicochemical properties of water and sediments samples are shown in Table 1.

Mixed cultures in HN–AD

A total of 300 g sediments were inoculated into 1000–mL brown glass bottles, then 800–mLof lake water was added into each bottle with NH_4^+–N (or) and NO_3^-–N as N source. According to Choi et al. (2016), optimum DO in aeration tank for the HN–AD process should be above 2 mg/L. Thus, approximately 0.2 vvm (air volume/liquid volume/min) of mild aeration was supplied using a lab-scale air pump in this study to maintain DO between 2 and 3 mg/L (Fig. 2).

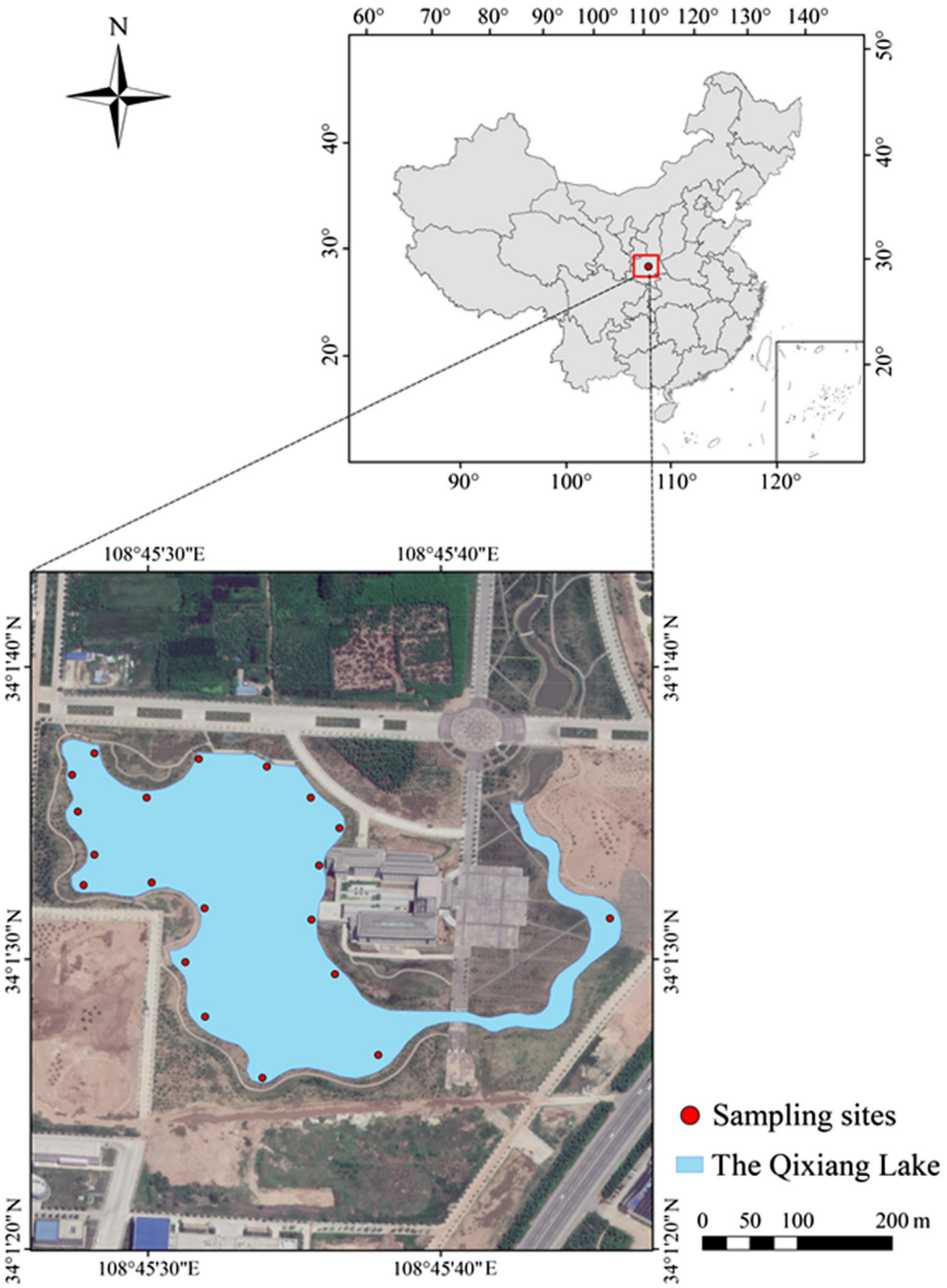

Fig. 1 Sampling sites in the Qixiang Lake of China

Establishment of HN–AD in mixed cultures

As indicated in Table 2, two sets of microbial mixed cultures (each set contained three mixed cultures) were applied to confirm the establishment of HN-AD: (1) the establishment of HN–AD by adding NH_4^+–N or NO_3^-–N as the single N source and (2) the establishment of HN–AD by adding NH_4^+–N and NO_3^-–N as the mixed N source. For the single N source of mixed cultures, four different media were inoculated into one set of mixed cultures at different periods. The performance of HN-AD was further examined by adding both NH_4^+–N (205 mg/L) and NO_3^-–N (230 mg/L) with C/N ratio of 10 in the other set of mixed cultures.

Effect of operational factor on the performance of HN–AD

The HN–AD system was operated under different conditions, including different carbon source, TOC/TN mass ratios, and NH_4^+–N concentrations. Three organic compounds, namely glucose, ethanol and citrate, were employed as the sole carbon source to maintain a TOC/TN mass ratio of 10. The effect of

Table 1 Physicochemical properties of the water and sediment samples [$N = 3$, mean (std. err.)]

	Temperature (°C)	pH	DO (mg/L)	Cond (μs)	TOC	Ammonium-N	Nitrate-N	Nitrite-N	A_{surf} (m^2/g)	Pore size (nm)	Ca	Na	K	Mg	Cl^-
Water	9.8 (0.1)	9.01 (0.02)	9.99 (0.05)	347 (5)	4.01 (0.01) mg/L	0.05 (0.01) mg/L	4.05 (0.01) mg/L	0.15 (0.01) mg/L	–	–	7.89 (0.05) mg/L	9.23 (0.09) mg/L	2.27 (0.07) mg/L	1.37 (0.05) mg/L	0.76 (0.02) mg/L
Sediment	9.5 (0.1)	8.67 (0.03)	–	–	72.33 (1.13) mg/kg	45.91 (0.52) mg/kg	40.05 (0.03) mg/kg	0.58 (0.01) mg/kg	7.27 (1.02)	92.75 (5.05)	94.23 (0.15) (mg/Kg)	38.43 (0.11) (mg/Kg)	13.75 (0.09) (mg/Kg)	17.41 (0.02) (mg/Kg)	–

– not detected

TOC/TN mass ratio (citrate as a carbon source) on HN–AD was examined by varying the ratio between 0 and 20 at a fixed NH_4^+–N amount of 200 mg/L. Similarly, the NH_4^+–N concentration in lake water was adjusted to 75.99, 201.91, 267.02, and 482.52 mg/L by adding the citrate as sole carbon source (TOC/TN mass ratio of 10) to study the effect of NH_4^+–N concentration on HN–AD.

N transformation pathway

Ammonium and nitrate were separately used as the sole N source at TOC/TN mass ratio of 10 to study the possible N loss pathway of the HN-AD in the mixed cultures. Furthermore, Na_2WO_4 (1.7 mmol L^{-1}) was added to inhibit the nitrate reductase (Nar) to further investigate the aerobic metabolic pathway during the nitrification and denitrification (Chen et al. 2016).

Analytical methods

The pH and oxidation reduction potential (ORP) were determined using a water quality meter (SX700, Shanghai, China) (Wu et al. 2019), while TN and TOC were analyzed using a TOC/TN analyzer (N/C3000ChD, Jena, Germany). NH_4^+–N, NO_2^-–N, and NO_3^-–N concentrations were analyzed after filtering the samples through the disposal 0.45-μm Millipore filters (pore size 0.45 μm, H9059, Heng Odd, Beijing, China). NH_4^+–N was determined using Nessler's reagent spectrophotometry (iodine mercury method), while NO_2^-–N was analyzed by N-(1-naphthalene)-diaminoethane photometry method. NO_3^-–N was calculated using the absorbance at 220 nm after subtracting the two times background absorbance value at 275 nm. Metal contents (Ca, Na, K, and Mg) were determined by the ion emission spectrometer (ICP-2070, Baird, USA), while Cl^- was analyzed by ion chromatography (IC) (792Basic IC, Metrohm, Switzerland).

pH in the sediment was determined using a soil:water (1:1) extract (Pei et al. 2010), whereas total organic matter (TOC) in the sediment phase was analyzed by using a TOC analyzer (N/C3000ChD, Jena, Germany). Total N (TN) was measured using the Kjeldahl method after digesting samples in a digestor using sulfuric acid/mercuric oxide catalyst. To extract mineral N in the sediments, 5 g aliquots of moist sediments were taken and shaken in 25 mL of 1 M KCl for 1 h before filtration through a membrane filter (Pei et al. 2010). NH_4^+–N, NO_2^-–N, and NO_3^-–N concentrations in the filtered extracts were then determined using the relevant methods described above. Specific surface area and microporosity of the sediments were analyzed using the N_2-BET method (Tristar II 3020, Micromeritics, USA). Metal contents (Ca, Na, K, and Mg) of sediment were determined by an ion emission spectrometer (ICP-2070, Baird, USA).

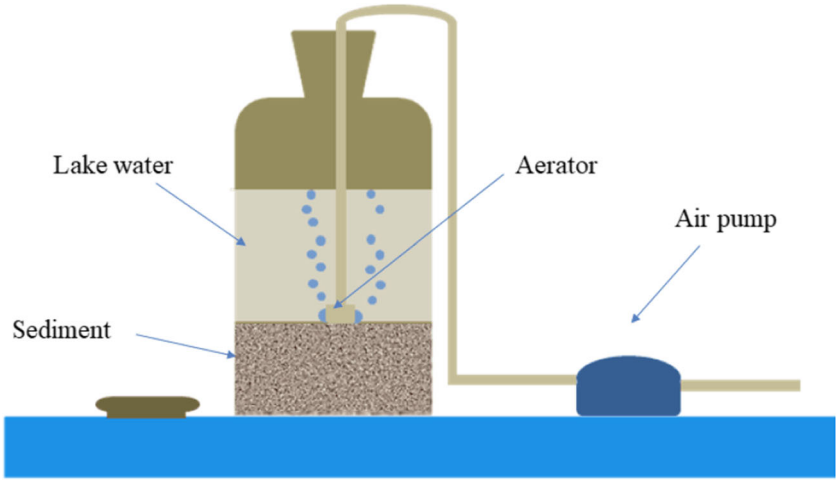

Fig. 2 Simulated aquatic ecosystem for mixed cultures

Results and discussion

Establishment of HN-AD in mixed cultures

Utilization of NH_4^+–N or NO_3^-–N

Four different media were added into one set of mixed cultures to examine whether HN-AD could occur in the stimulated aquatic ecosystem (Fig. 3). The ORP value fluctuated, but was above 100 mV in the mixed cultures during the test (Fig. S2), suggesting an aerobic condition in the simulated ecosystem.

In the mixed cultures with NH_4^+–N as the sole N source in Period I, the NH_4^+–N concentration decreased gradually at a removal rate of approximately 4.32 mg/L/day in the 42-day period by the heterotrophic nitrification due to the simultaneous consumption of total organic carbon (TOC) (Fig. S3). Although NO_3^-–N was present due to nitrification with NH_4^+–N as the initial N source (Eq. 1), the increased concentration of generated NO_3^-–N was significantly lower compared with the decreased NH_4^+–N concentration. In other words, N was unexpectedly lost via pathways such as aerobic denitrification. A temporary low level accumulation of NO_2^-–N was observed, confirming that nitrification took place during Period I.

$$NH_4^+ + O_2 + H_2O \rightarrow NO_3^- + 4e^- + 6H^+ \quad (1)$$

Heterotrophic nitrification was confirmed by adding both NH_4^+–N and external organic carbon from the 43rd to 85th days in Period II. The maximum NH_4^+–N removal rate was 12.01 mg/L/day when citrate was used as the external carbon source, which was 2.78 times higher than that in the absence of an external carbon source. During this process (Period II), NO_3^-–N, and NO_2^-–N accumulations were low, indicating that citrate could significantly promote aerobic denitrification. Guo et al. (2013) suggested that citrate could contribute to either NH_4^+–N or NO_3^-–N removal by HN–AD strain with a TOC/TN mass ratio of 10.

A total of 130.00 mg/L NO_3^-–N source was added as the sole N in the mixed cultures during Period III since the 86th day to further confirm the existence of aerobic denitrification. The NO_3^-–N concentration decreased gradually at a rate of 1.15 mg/L/day from the 86th to 123rd days, suggesting that aerobic denitrification occurred in the mixed cultures. Furthermore, approximately 85% of TN was removed from the mixed cultures in Period III. Unlike the autotrophic nitrification, NO_2^-–N concentration was lower than the detection limit of 0.08 mg/L in the aerobic denitrification process.

When citrate was added to the mixed cultures after the 123rd day in Period IV, the NO_3^-–N concentration decreased dramatically with a removal rate of 14.10 mg/L/day. A significant

Table 2 Experimental conditions for the establishment of HN-AD

	Periods	Organic carbon	Nitrogen source
Establishment of HN–AD (single nitrogen source)	Period I	–	NH_4^+–N 200 mg/L
	Period II	Potassium citrate 2 g-C/L	NH_4^+–N 200 mg/L
	Period III	–	NO_3^-–N 130 mg/L
	Period IV	Potassium citrate 0.88 g-C/L	NO_3^-–N 88 mg/L
Establishment of HN–AD (mixed nitrogen source)	The whole period	Potassium citrate 4.35 g-C/L	NH_4^+–N 205 mg/L and NO_3^-–N 230 mg/L

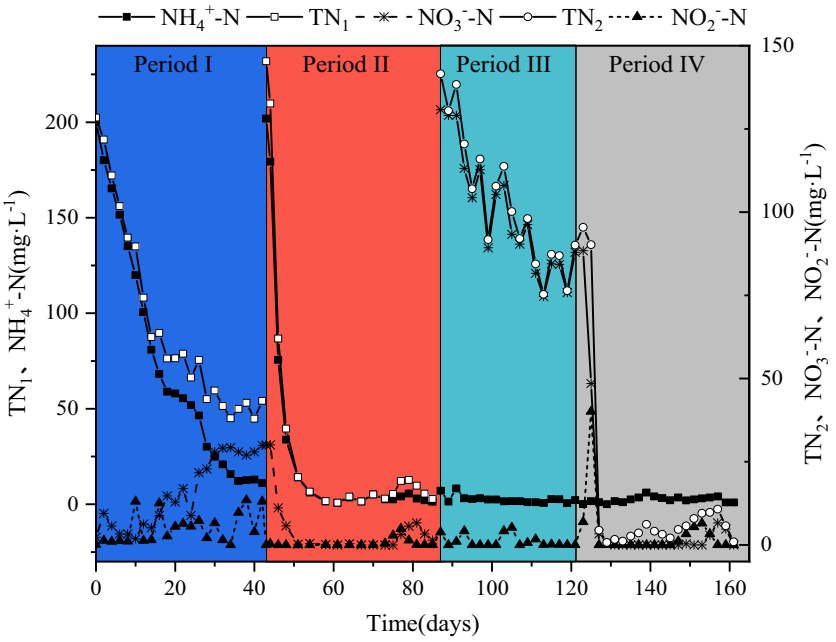

Fig. 3 Changes in the concentration of N species at different periods when the N source was either ammonium (Period I and II) or nitrate (Period III and IV) in the mixed cultures

accumulation of NO_2^-–N was accompanied by the decrease of NO_3^-–N. The maximum NO_2^- concentration was 40.08 mg/L within 4 days from the 123rd to the 127th day and was completely removed since the 129th day, confirming the nitrite generation and consumption during this process. The trend of NO_2^-–N variation is similar to the NO_3^-–N concentration variation pattern, further confirming the aerobic denitrification in the mixed cultures.

The findings confirmed that HN–AD occurred in the simulated natural aquatic ecosystem and that the system could remove N using either NH_4^+–N or NO_3^-–N as N source under aerobic conditions. A total of 201.91 mg/L NH_4^+–N and 130.00 mg/L NO_3^-–N were completely transformed to other N species and finally to N_2O or N_2 with the TN removal efficiencies reaching approximately 99.61% and 100%, respectively, which are higher than that by the pure HN–AD strain (Table 3). This may be attributed to the abundance of functional microorganism in our mixed cultures and is in agreement with the study by Choi et al. (2016), which reported that the mixed strains were more effective than the pure strain to remove N with TOC/TN of 12.5.

Simultaneous utilization of NH_4^+–N and NO_3^-–N

Both NH_4^+–N and NO_3^-–N were used as mixed N sources at TOC/TN ratio of 10 to further confirm HN–AD in the other set of mixed cultures. As per Fig. 4, the NH_4^+–N and NO_3^-–N concentrations declined immediately with the removal rate of 24.48 and 28.56 mg/L/day, respectively, while the TN removal efficiency increased with the decrease of NH_4^+–N and NO_3^-–N concentrations. It is worthy to note that a lag phase of 2-day period existed for the NO_3^- removal and decreased rapidly in the following days similar to *Alcaligenes* sp. TB (Chen et al. 2016). Yao et al. (2013) and Zhao et al. (2010) also demonstrated that the pure HN-AD strains growth had a lag phase when

Table 3 N loss percentage through HN-AD

Microorganisms	Nitrogen source	Removal percentage (%)	References
Acinetobacter sp. Y16	NH_4^+–N	66.00	(Huang et al. 2013)
	NO_3^-–N	56.73	
Pseudomonas stutzeri strain T1	NH_4^+–N	60.00	(Guo et al. 2013)
	NO_3^-–N	75.00	
Rhodococcus sp. CPZ24	NH_4^+–N	85.00	(Chen et al. 2012)
	NO_3^-–N	67.00	
Vibrio diabolicus SF16	NH_4^+–N	91.80	(Duan et al. 2015)
	NO_3^-–N	99.70	
The mixed culture	NH_4^+–N	99.60	This study
	NO_3^-–N	100.00	

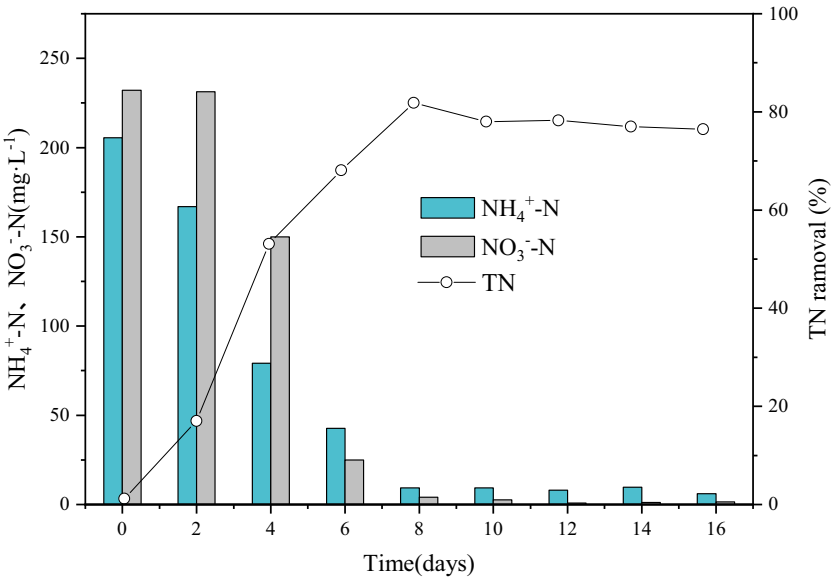

Fig. 4 The NH_4^+-N, NO_3^-N, and TN concentration changes when both NH_4^+-N and NO_3^--N were N sources in the mixed cultures

nitrate was supplied as the N source. The simultaneous NH_4^+–N and NO_3^-–N degradation demonstrated that the mixed cultures possessed HN–AD capability.

Effect of experimental parameter on HN–AD

Types of organic carbon

Organic carbon serves as the energy and electron source for HN–AD bacteria (Joo et al. 2005). As shown in Fig. 5, three organic carbon types such as glucose, citrate, and ethanol could enhance the activities of HN-AD. Similar NH_4^+–N and TN removal efficiencies suggested that HN–AD bacteria were insensitive to these organic carbons in the mixed cultures. Nevertheless, glucose was not beneficial for N removal by *P. stutzeri* strain T1 (Guo et al. 2013) at the TOC/TN mass ratio of 10 and inhibit the HN–AD bacterial activity. The growth of the strain L7 could be enhanced by glucose when citrate is with the TOC/TN mass ratio of 6 (Zhang et al. 2012), indicating that the mixed cultures had good tolerance to different organic carbon types. This might be explained by the interaction of many kinds of microorganisms in the studied aquatic ecosystem (Zhang et al. 2012).

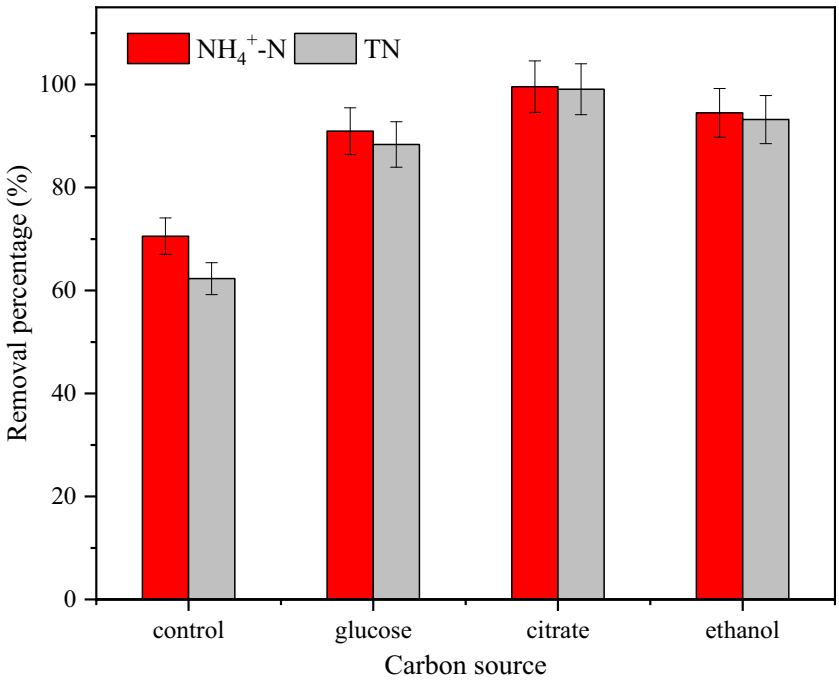

Fig. 5 Effect of organic carbon on the HN-AD efficiency at the 18th day with NH_4^+-N as the N source

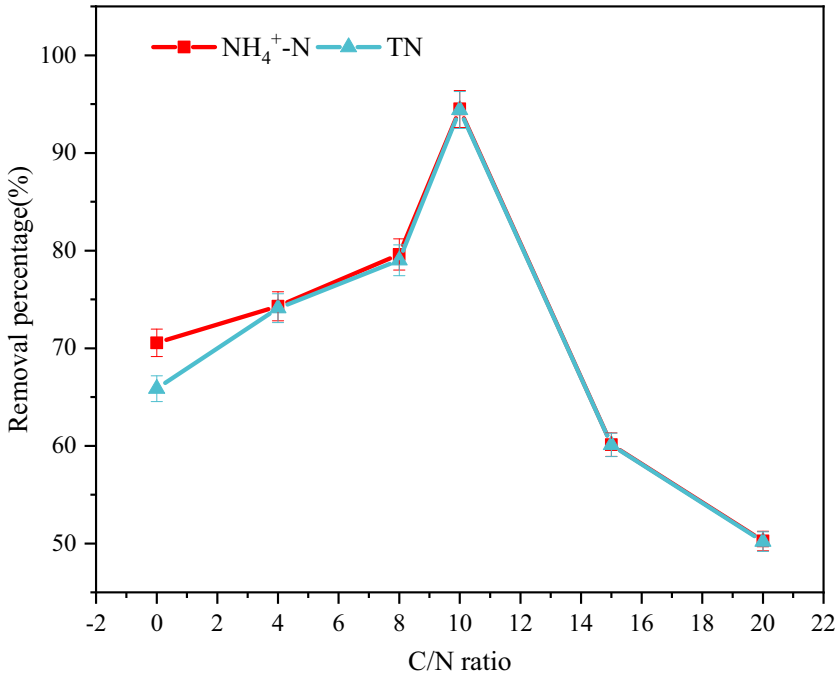

Fig. 6 Effect of TOC/TN mass ratio on the HN-AD efficiency at the 18th day with NH_4^+-N as the N source

TOC/TN mass ratio

Figure 6 shows the effect of TOC/TN mass ratio on HN-AD performance within 18 days. Significant differences were observed with TOC/TN ratios ranging from 0 to 20. Within the suitable ratio of TOC/TN (0–10), more NH_4^+–N and TN were lost at higher TOC/TN. When external organic carbon was absent (TOC/TN mass ratio of 0) in the mixed cultures, N loss was observed with NH_4^+–N and TN removal percentages of 74.10% and 65.86%, respectively. The maximum NH_4^+–N and TN consumption occurred at TOC/TN mass ratio of 10, although further increase in TOC/TN mass ratio yielded a decrease in N removal efficiency indicating that the N loss occurs at an appropriate TOC/TN mass ratio, whereas the mixed cultures enter a nutritional state at high organic carbon (Becquevort et al. 2007). Most investigations on N loss by heterotrophic nitrifying bacteria were also conducted at TOC/TN mass ratio of 10 (Guo et al. 2013; Joo et al. 2005; Kundu et al. 2014; Taylor et al. 2009). The optimized TOC/TN mass ratio could lead to efficient denitrification without any nitrate and nitrite accumulation (Chen et al. 2016). Therefore, TOC/TN mass ratio of 10 was used in the subsequent tests.

NH_4^+-N concentration

Rapid N loss occurred when the initial NH_4^+–N concentration was higher in the mixed cultures with the TN removal rates of 4.69, 11.01, and 21.03 mg/L/day, at the initial NH_4^+–N concentrations of 85.53, 201.91, and 493.12 mg/L, respectively (Table 4). The NH_4^+–N and TN removal rates increased with the increase in initial NH_4^+–N concentration, suggesting that high NH_4^+–N concentration did not inhibit the performance of HN–AD in the mixed cultures. The tolerance of HN-AD mixed cultures to NH_4^+–N was higher than that of heterotrophic nitrifier *Providencia rettger* YL, which had the maximum tolerance threshold of 300 mg/L NH_4^+–N (Taylor et al. 2009). This finding confirmed that the mixed cultures were superior to the pure strains in NH_4^+–N removal at high concentration, indicating the practical applications in actual aquatic ecosystems where ammonium concentration tends to be high.

Table 4 HN-AD ability with different NH_4^+–N concentrations

Initial NH_4^+–N (mg/L)	Average NO_2^-–N (mg/L)	Removal rate of NH_4^+–N (mg/L/day)	Removal rate of TN (mg/L/day)
85.53 ± 0.005	0.81 ± 0.006	4.75 ± 0.025	4.69 ± 0.021
201.91 ± 0.012	0.05 ± 0.005	11.18 ± 0.005	11.01 ± 0.025
493.12 ± 0.008	0.03 ± 0.009	21.71 ± 0.023	21.03 ± 0.009

Values are means ± SD for triplicate at the 18th day

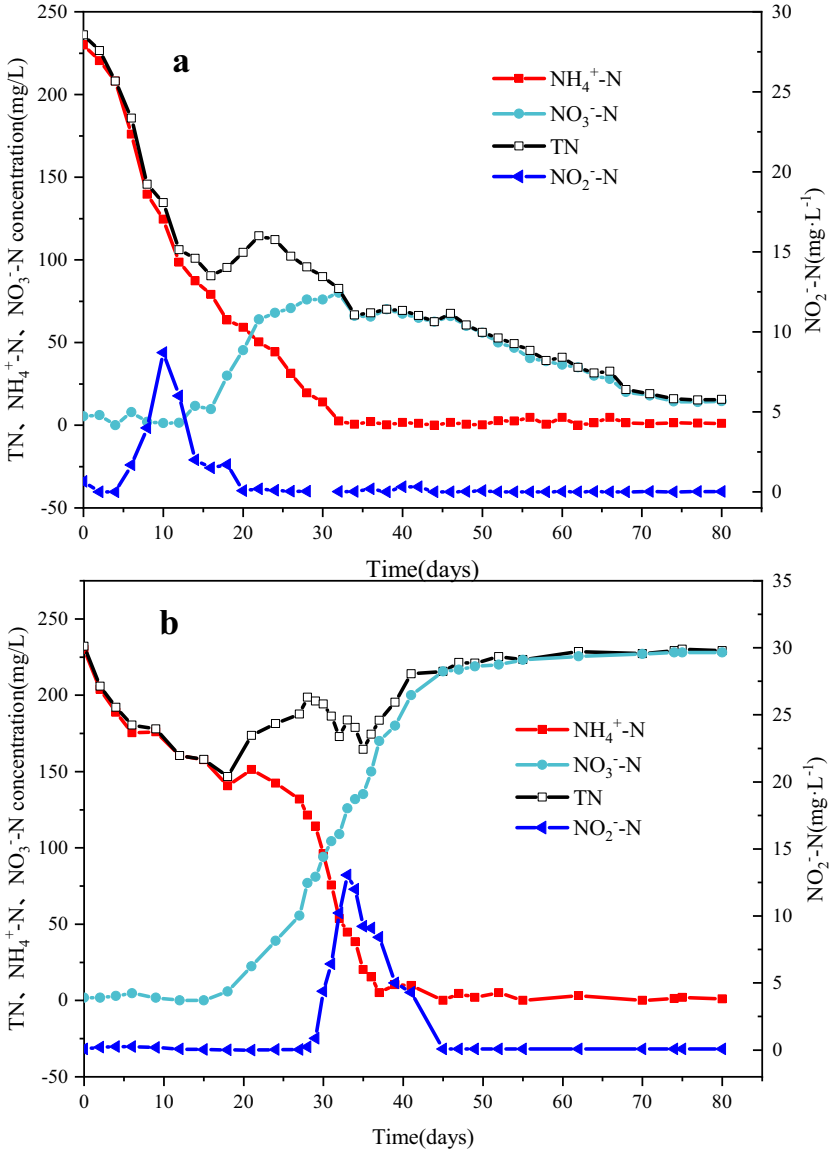

Fig. 7 Utilization of NH_4^+–N **a** without inhibition and **b** with Na_2WO_4 as the Nar reductase inhibitor. ([NH_4^+–N = 230 mg/L, 8.4 g K_2HPO4, 3.6 g KH_2PO4, [Na_2WO_4] = 500 mg/L)

N transformation pathway via HN–AD

Ammonium and nitrate were separately used as the N sources in the mixed cultures to determine the possible N loss pathways (Fig. 3). When NH_4^+–N was the N source, N loss in the mixed cultures showed simultaneous HN-AD. The accumulation of N species suggested that the rate of heterotrophic nitrification was higher than that of aerobic denitrification; thus, the N transformation pathways can be described as $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2O$ (N_2). When NO_3^-–N was the N source, N was lost with the accumulation of NO_2^-–N, further confirming the aerobic denitrification pathway of $NO_3^- \rightarrow NO_2^- \rightarrow N_2O$ (N_2).

To further investigate the aerobic metabolic pathway of HN–AD, tests without inhibitor (Fig. 7a) and with Na_2WO_4 as the Nar reductase inhibitor (Fig. 7b) were conducted with NH_4^+–N as the sole N source under aerobic conditions. As shown in Fig. 7a, NH_4^+–N was completely removed within 30 days in the mixed cultures without Na_2WO_4, indicating that aerobic nitrification had occurred. In contrast, NO_3^-–N was a final product with its concentration increasing to the maximum of 80 mg/L on 32nd day and then gradually decreasing below its detection limit, indicating subsequent aerobic denitrification. In the mixed cultures with Na_2WO_4 as nitrate reductase inhibitor, the denitrification was completely inhibited with an accumulation of NO_3^-–N concentration of 235.13 mg/L (Fig. 7b). As per the N mass balance, 100% NH_4^+–N were converted to NO_3^-–N, confirming that N was not lost during the nitrification process and that NO_3^-–N was an intermediate during the N transformation process.

Production of N_2 has been widely accepted as a result of HN–AD in wastewater and nitrogen-polluted water bodies (Huang et al. 2013; Joo et al. 2005; Zhang et al. 2012). However, N_2O is generally the main end product instead of N_2

due to the inhibition of nitrous oxide reductase by O_2 (Aboobakar et al. 2013). The two main pathways for aerobic N removal and gaseous N production by heterotrophic bacteria (Richardson et al. 1998) are the heterotrophic nitrification and coupled aerobic denitrification in wastewater (Chen et al. 2016; Chen and Ni 2012; Taylor et al. 2009) or nitrogen-polluted water bodies (Huang et al. 2013). In addition, conversion of ammonium to N_2O and/or N_2 via NH_2OH (Frear and Burrell 1955) by pure HN-AD strains isolated from wastewater treatments is also considered as another route (Joo et al. 2005; Zhao et al. 2010). In such case, the conversion of NH_4^+–N to NO_3^-–N should not be detected by 100% with Na_2WO_4 as nitrate reductase inhibitor (Fig. 7b). Nevertheless, the N balance showed that almost all of NH_4^+–N was converted to NO_3^-–N in this simulated aquatic ecosystem when Na_2WO_4 was added, suggesting that N was not lost via partial nitrification/denitrification pathway (Chen et al. 2016), i.e., $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O$ (N_2), instead via the full nitrification/denitrification pathway, i.e. $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2O$ (N_2). Concentrations of NH_4^+–N, NO_3^-–N, and NO_2^-–N varied in the HN–AD processes (Fig. 3) further confirming the N transformation pathway. This pathway is generally consistent with that of *Acinetobacter* sp. Y16 isolated from source water (Huang et al. 2013).

Conclusions

This study demonstrates that HN–AD could occur in the mixed culture of a stimulated aquatic ecosystem. With citrate as organic carbon source at 10 C/N mass ratio, the N loss percentages of NH_4^+–N (201.91 mg/L) and NO_3^-–N (130.00 mg/L) were 99.61% and 100.00%, respectively, especially, the high efficiencies were kept at low C/N mass ratio and high NH_4^+–N concentration (493.12 mg/L). Using Na_2WO_4 as the nitrate reductase inhibitor, the study found that N loss was not via $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O$ (N_2), instead via $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2O$ (N_2). NH_4^+–N, NO_3^-–N, and NO_2^-–N concentration variations in the HN–AD processes further confirmed the N transformation pathway, confirming that HN–AD may spontaneously occur and cause N loss in natural aquatic ecosystems. Further, future works will focus on the gene analysis of microorganisms through high-throughput sequencing, which will permit us to get more information on the heterotrophic nitrification-aerobic denitrification in aquatic ecosystems.

Funding information This work was financially supported by the National Natural Science Foundation of China (Program no. 41601338 and no. 51608444), the Natural Science Basic Research Plan in Shaanxi Province of China (Program no. 2018JQ4019), Science, Technology and Innovation Commission of Shenzhen Municipality (no. JCYJ20170306153655840), National Training Program of Innovation and Entrepreneurship for Undergraduates (S201910699176), and Fundamental Research Funds for the Central Universities (Program No. 3102018zy042).

References

Aboobakar A, Cartmell E, Stephenson T, Jones M, Vale P, Dotro G (2013) Nitrous oxide emissions and dissolved oxygen profiling in a full-scale nitrifying activated sludge treatment plant. Water Res 47: 524–534

Becquevort S, Lancelot C, Schoemann V (2007) The role of iron in the bacterial degradation of organic matter derived from Phaeocystis antarctica. Biogeochemistry 83:119–135

Chen J, Gu S, Hao H, Chen J (2016) Characteristics and metabolic pathway of *Alcaligenes* sp. TB for simultaneous heterotrophic nitrification-aerobic denitrification. Appl Microbiol Biotechnol 100:9787–9794

Chen P, Li J, Li QX, Wang Y, Li S, Ren T, Wang L (2012) Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium *Rhodococcus* sp. CPZ24. Bioresour Technol 116:266–270

Chen Q, Ni J (2012) Ammonium removal by *Agrobacterium* sp. LAD9 capable of heterotrophic nitrification-aerobic denitrification. J Biosci Bioeng 113:619–623

Choi K-J, Zhang S, Song JH, Hwang S-J (2016) Aerobic denitrification by a heterotrophic nitrifying-aerobic denitrifying (HN-AD) culture enriched activated sludge. KSCE J Civ Eng 21:2113–2118

Duan J, Fang H, Su B, Chen J, Lin J (2015) Characterization of a halophilic heterotrophic nitrification-aerobic denitrification bacterium and its application on treatment of saline wastewater. Bioresour Technol 179:421–428

Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA (2008) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320:889–892

Guo L, Chen Q, Fang F, Hu Z, Wu J, Miao A, Xiao L, Chen X, Yang L (2013) Application potential of a newly isolated indigenous aerobic denitrifier for nitrate and ammonium removal of eutrophic lake water. Bioresour Technol 142:45–51

Hu SH, Wu YG, Zhang YJ, Zhou B, Xu X (2018) Nitrate removal from groundwater by heterotrophic/autotrophic denitrification using easily degradableorganics and nano-zero valent iron as co-electron donors. Water Air Soil Pollut 229:9

Huang X, Li W, Zhang D, Qin W (2013) Ammonium removal by a novel oligotrophic Acinetobacter sp. Y16 capable of heterotrophic nitrification-aerobic denitrification at low temperature. Bioresour Technol 146:44–50

Joo HS, Hirai M, Shoda M (2005) Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. J Biosci Bioeng 100:184–191

Kundu P, Pramanik A, Dasgupta A, Mukherjee S, Mukherjee J (2014) Simultaneous heterotrophic nitrification and aerobic denitrification by *Chryseobacterium* sp. R31 isolated from abattoir wastewater. Biomed Res Int 2014:436056

Kuypers MMM, Marchant HK, Kartal B (2018) The microbial nitrogen-cycling network. Nat Rev Microbiol 16:263–276

Lanoux A, Etcheber H, Schmidt S, Sottolichio A, Chabaud G, Richard M, Abril G (2013) Factors contributing to hypoxia in a highly turbid, macrotidal estuary (the Gironde, France). Environ Sci-Proc Imp 15: 585

Lenihan HS, Peterson CH (1998) How habitat degradation through fishery disturbance enhances impacts of hypoxia on oyster reefs. Ecol Appl 8:128–140

Liang ZP, Feng YQ, Liang ZY, Meng SX (2005) Kinetic of adsorption of urea nitrogen onto chitosan coated dialdehyde cellulose under catalysis of immobilized urease. Chin Chem Lett 16:697–700

Song LY, Song JX, Zhang WJ (2010) Temporal and spatial distribution and long- term variation trend of precipitation in Xi'an. J Arid Land Resour Environ 24:85–89

Lunau M, Voss M, Erickson M, Dziallas C, Casciotti K, Ducklow H (2013) Excess nitrate loads to coastal waters reduces nitrate removal efficiency: mechanism and implications for coastal eutrophication. Environ Microbiol 15:1492–1504

Pei YS, Wang J, Wang ZY, Yang ZF (2010) Characteristics of ammonia-oxidizing and denitrifying bacteria at the river-sediment interface. Procedia Environ Sci 2:1988–1996

Qian J, Zhang MK, Pei XJ, Zhang Z, Niu JT, Liu Y (2018a) A novel integrated thiosulfate-driven denitritation (TDD) and anaerobic ammonia oxidation (anammox) process for biological nitrogen removal. Biochem Eng J 139:68–73

Qian J, Zhang MK, Wu YG, Niu JT, Chang X, Yao HR, Hu SH, Pei XJ (2018b) A feasibility study on biological nitrogen removal (BNR) via integrated thiosulfate-driven denitratation with anammox. Chemosphere 208:793–799

Quan Q, Luo W, Sheng B, Jia ZH (2013) Effects of climate variability and land-use change on stormwater runoff in Xi'an, China for the past 57 years. Disaster Adv 6:198–203

Richardson DJ, Wehrfritz JM, Keech A, Crossman LC, Roldan MD, Sears HJ, Butler CS, Reilly A, Moir JWB, Berks BC, Ferguson SJ, Thomson AJ, Spiro S (1998) The diversity of redox proteins involved in bacterial heterotrophic nitrification and aerobic denitrification. Biochem Soc T 26:401–408

Roberts K, Kessler A, Grace M, Cook P (2014) Increased rates of dissimilatory nitrate reduction to ammonium (DNRA) under oxic conditions in a periodically hypoxic estuary. Geochim Cosmochim Ac 133:313–324

Robertson LA, Kuenen JG (1984) Aerobic denitrification - old wine in new bottles? Anton van Leeuw 50:525–544

Frear DS, Burrell RC (1955) Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal Chem 27: 1664–1665

Tang Y, Li M, Xu D, Huang J, Sun J (2018a) Application potential of aerobic denitrifiers coupled with a biostimulant for nitrogen removal from urban river sediment. Environ Sci Pollut Res 25:5980–5993

Tang Y, Li M, Zou Y, Lv M, Sun J (2018b) Mechanism of aerobic denitrifiers and calcium nitrate on urban river sediment remediation. Int Biodeterior Biodegrad 126:119–130

Taylor SM, He Y, Zhao B, Huang J (2009) Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL. J Environ Sci 21: 1336–1341

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671

Wang WD, Han Y, Liu H, Zhang K, Yue Q, Bo LL, Wang XC (2017) Pollutant removal performance of an integrated upflow-constructed wetland filled with haydites made of Al-based drinking water treatment residuals. Environ Technol 38:1111–1119

Wu YG, Fan L, Hu SH, Wang SC, Yao HR, Wang KF (2019) Role of dissolved iron ions in nanoparticulate zero-valent iron/H_2O_2 Fenton-like system. Int J Environ Sci Technol 16:4551–4562

Yao S, Ni J, Ma T, Li C (2013) Heterotrophic nitrification and aerobic denitrification at low temperature by a newly isolated bacterium, *Acinetobacter* sp. HA2. Bioresour Technol 139:80–86

Zhang QL, Liu Y, Ai GM, Miao LL, Zheng HY, Liu ZP (2012) The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. Bioresour Technol 108:35–44

Zhao B, He YL, Hughes J, Zhang XF (2010) Heterotrophic nitrogen removal by a newly isolated *Acinetobacter calcoaceticus* HNR. Bioresour Technol 101:5194–5200

Zhou Q, Takenaka S, Murakami S, Seesuriyachan P, Kuntiya A, Aoki K (2007) Screening and characterization of bacteria that can utilize ammonium and nitrate ions simultaneously under controlled cultural conditions. J Biosci Bioeng 103:185–191