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High-rate partial-denitrification via effluent residual nitrate controlling and microbial mechanism of nitrite accumulation by carbon dosage optimization

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ABSTRACT

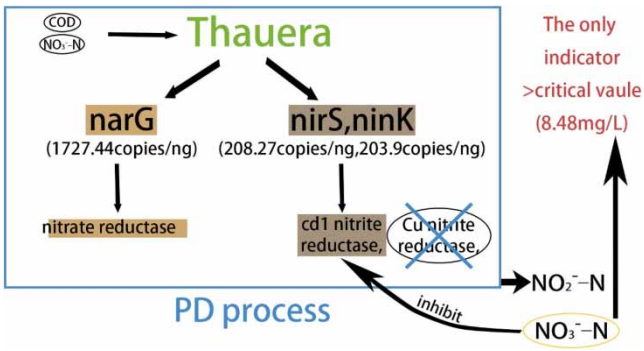
The high-rate partial-denitrification via effluent residual nitrate controlling by carbon dosage optimization was investigated based on the analysis of microbial mechanism of nitrite accumulation in this study. With the COD/N was changed from 4.0 to 1.8 and the effluent nitrate was above 8.48 mg/L, the nitrate accumulation ratio (NAR) and nitrate removal ratio (NRR) were achieved to 60 and 90%, respectively. With the electron donor starvation (EDS) strategy, the nitrite accumulation was increased, which is related to the reduced utilization of carbon sources. In addition, the rapidly increased of *Thauera* (0.21% to 53.29%) and inhibited of *Others* and *Unclassified* (96.93% to 16.99%), and the significantly different expression between reductase genes contributed to nitrite production (narG, 1,727.44 copies/mg) and nitrite reduction (*nirS*, 208.27 copies/mg; *nirK*, 203.94 copies/mg) commonly involved to PD start-up and stable operation. While another reactor can be quickly started by controlling effluent residual nitrate within 19 days.

Key words: COD dosage, effluent nitrate residual, nitrate reductase gene, nitrite accumulation, partial-denitrification, *Thauera*

HIGHLIGHTS

- Partial denitrification process was quickly start up within 19 days via EDS strategy.
- Critical concentration of NO_3^--N inhibiting NO_2^--N reduction was proved to be exist.
- The average NAR and NRR could reached 60 and 89%, respectively.
- Thauera increased from 0.21% to 53.29% when effluent NO_3^--N was above the critical value.
- Nir gene expression was 4.2 times than the Nir gene so that nitrite was accumulated.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Anaerobic ammonium oxidation (Anammox) is an autotrophic nitrogen removal process that transforms ammonia and nitrite which respectively acts as an electron donor and acceptor with a ratio of 1: 1.32 to nitrogen from wastewater (Equation (1)) (Arora *et al.* 2021). Researchers are increasingly pay attention to Anammox, mostly owing to its superiorities related to

operational costs (mainly aeration costs), excess sludge treatment and lack of carbon sources are over the traditional biological nitrogen removal (Trinh *et al.* 2021). However, the sustainable supply of nitrite is an essential step for Anammox process. To address this challenge, partial nitrification (PN) that converts ammonia to nitrite and partial denitrification (PD) that transforms nitrate to nitrite are favored by researchers (Ji *et al.* 2019; Lotti *et al.* 2019). To date, most of the Anammox plants have been established in worldwide are used to treating wastewater with high ammonia and low carbon to nitrogen (C/N) ratio (Mao *et al.* 2017). When it was used to treat the municipal wastewater, the concentration of free ammonia (FA) and free nitrous acid (FNA) which can inhibit the nitrite oxidizing bacteria (NOB) is low, causing the decreasing of nitrite accumulation in PN process (Qiu *et al.* 2021). On the contrary, PD can not only remove organic matter and nitrogen at the same time, but also the operational environment (mainly pH and dissolved oxygen (DO)) is more conducive to Anammox process. Furthermore, PD coupling with Anammox can achieve 100% removal of nitrogen in theoretical. Therefore, because of the difficulty of PN to stably generate nitrite, researchers have a preference on PD owing to its easily control and stability of producing nitrite (Chen *et al.* 2021).

PD is to control the conventional denitrification process to nitrite as the final product without further reduction (Equation (2)) (Chen *et al.* 2021). As the reports, denitrification process mainly involves two types reductase, that is nitrate reductase (Nar) and nitrite reductase (Nir), respectively (Zhang *et al.* 2019a, 2019b, 2019c). Over the years, PD was mainly achieved through: (1) Using carbon sources that preferentially transfer electrons to Nar, eg. Acetic acid, glycerin (Van Rijin *et al.* 1996; Le *et al.* 2021); (2) Controlling C/N ratio and reaction time to stop the denitrification reaction in time; (3) Inhibiting the expression of *nirK* gene belonging to Cu type Nir by high pH or enriching the *Taueria* with only *nirS* gene (Etchebehre *et al.* 2005). Fundamentally, PD is mostly achieved through enriching bacteria capable of nitrite accumulation (Du *et al.* 2019). For instance, *Halomonas* and Saccharibacteria were enriched from a methylotrophic denitrifying culture (Li *et al.* 2016). Li and coworkers achieved PD (nitrite accumulation ratio (NAR) >90%) with *Halomonas* as the dominant bacterial among functional population on the 120th day (Li *et al.* 2016). *Thauera* was enriched by limiting carbon source and controlling pH (Shi *et al.* 2019). Additionally, nitrite accumulation of PD process is also influenced by functional genes that encoding enzyme. However, there are almost no research has been reported that employs effluent residual nitrate as the only indicator to startup PD process.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

$$1.32NO_3^- + 0.55CH_3COO^- + 0.088NH_4^+ \rightarrow 1.32NO_2^- + 0.088C_5H_7NO_2 + 0.66HCO_3^- + 0.198H^+ + 0.264H_2O \quad (2)$$

In this study, high-rate PD process was achieved via effluent residue nitrate controlling by EDS strategy. And the effluent residual nitrate was used as an indicator of electron donor starvation (EDS), not the effluent COD. The main aims of this study were: (1) To evaluate the critical effluent residue nitrate for inhibiting nitrite reduction via in-situ tests; (2) To observe the evolution of microbial community to EDS strategy through 16S rRNA gene sequencing; (3) To reveal the microbial mechanism of nitrite accumulation by metagenomic sequencing. The results of this study will help to rapidly startup practical PD process and long-term stable operation of PD process.

2. METHODS

2.1. Reactor configurations

The experiment was carried out in SBR with effective working volume of 4 L and volume exchange ratio of 50%. The mixture liquid suspend solid (MLSS) of the seed sludge which was collected from a domestic sewage treatment plant (Ganzhou, China) is 2–3 gVSS/L. The composition of the synthetic wastewater was 5 mg/L $CaCl_2$; 5 mg/L $MgCl_2$; 2 mg/L KH_2PO_4; 80 mg/L NO_3^--N and 1 mL/L trace elements solution. The trace elements solution contained (g/L):15 EDTA, $ZnSO_4{\cdot}7H_2O$ 0.43, $CuSO_4{\cdot}5H_2O$ 0.25, $NiCl_2{\cdot}6H_2O$ 0.19, $MnCl_2{\cdot}4H_2O$ 0.99, $CoCl_2{\cdot}6H_2O$ 0.24, $NaMoO_4{\cdot}2H_2O$ 0.22, H_3BO_4 0.014. And sodium acetate was using as organic carbon source, its dosage was depending on the demand at different stages.

2.2. Operation strategy

The SBR was operated in a cycle of 5 steps: 9 min for feeding, 1 min for adding chemicals, 120 min for stirring and reaction, 40 min for settling and 10 min for discharging. The temperature and mechanical mixing were set at a room temperature

ranging 15–35 °C and 120 rpm, respectively. In-situ tests were performed at different operating periods to observe the change of nitrogen in the reactor.

2.3. Analytical methods

Concentrations of NO_3^--N and NO_2^--N were daily measured via standard methods (Federation and Association 2005). COD was determined by potassium dichromate method (Federation and Association 2005). The MLVSS were determined using the gravimetric method (Federation and Association 2005). In this study, the nitrite accumulation ratio (NAR) and nitrate reduction ratio (NRR) were calculated as Equations (4) and (5), respectively.

$$NAR = ([NO_2^- - N]_{eff}/[NO_3^- - N]_{inf} \times 100\% \quad (3)$$

$$NRR = ([NO_3^- - N]_{inf} - [NO_3^- - N]_{eff})/[NO_3^- - N]_{inf} \times 100\% \quad (4)$$

2.4. High-throughput sequencing and microbial community analysis

Sludge sample were obtained from the seed sludge and after the end of stage I (0–11 d), II (12–42 d), III (43–52 d), IV (53–63 d),which were named B0, B1, B2, B3, B4, respectively. Briefly, total genomic deoxyribonucleic acid (DNA) was extracted from sludge sample use bacterial DNA isolation kit (MOBIO Laboratories, Inc, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of 16S rRNA gene was performed with primer 341F/806R (341F: CCTACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC) in V3-V4 region. DNA isolation was performed in accordance with established protocols described by PCR amplification was performed in Eppendorf Mastercycler under the conditions described by Antwi *et al.* (2017a, 2017b, 2017c). Illumina Miseq platform (Vazyme Biotech Co., Ltd) was used to conduct 16 rRNA gene sequences.

2.5. Real-time fluorescence quantitative PCR

Select SYBR Green I dye method for real-time polymerase chain reaction (qPCR) to detect the amount of gene expression during the reaction (VanGuilder *et al.* 2008). First find the coding sequence (CDS) of the target gene in NCBI, then use the Beacon Designer (version 7.9) software for primer design. Entrusted Shenggong Bioengineering (Shanghai) Co., Ltd to synthesize primers. After obtaining the sludge sample, refer to the instruction manual of the OMEGA kit (EZNATM Mag-Bind Soil DNA Kit) to centrifuge the sample, collect the precipitate, weigh, and perform DNA extraction, and then use the DNA ladder as a reference to perform agarose gel (1.5%) Electrophoresis to check DNA integrity. Details of operation procedures of DNA isolation and PCR amplification were referred to the previous study (Antwi *et al.* 2019; Zhang *et al.* 2019a, 2019b, 2019c).

3. RESULTS AND DISCUSSION

3.1. Reactor performance

The process performance was depicted as Figure 1. Sufficient carbon source was added for improving denitrification activity on phase-I. The sludge has a strong denitrification performance when the effluent nitrate concentration was stable below 3 mg/L and was no accumulation of nitrite in the effluent. Therefore, in-situ test was performed on the 11th day to determine the peak nitrite accumulation (Figure 2). The nitrite increased first and then decreased, indicating that nitrite accumulation was really existed during the denitrification process. The accumulated nitrite began to decrease gradually at 15th min, and the corresponding nitrate concentration was 8.48 mg/L at this time. Hence, the nitrate concentration inhibiting the reduction of nitrite could be determined as 8.48 mg/L.

There were two methods to control the effluent nitrate concentration above 8.48 mg/L according to Figure 2: (1) Shortening the reaction time within 15 min; (2) Inhibiting the bacteria activities by reducing the dosage of carbon source. The method of electron donor starvation was selected to control the effluent nitrate concentration since the dosage of carbon source only needed to be adjusted according to the influent concentration.

The dosage of carbon source was reduced once the effluent residual nitrate was below the critical value (8.48 mg/L) on phase-II–IV. It was observed that the effluent residual nitrate first rose above the critical value (8.48 mg/L) and then decreased gradually, and it was accompanied with a gradual increase of the effluent nitrite simultaneously. While the effluent nitrite was stopped rising when the effluent residual nitrate was dropped below 8.48 mg/L, indicating that the controlling of

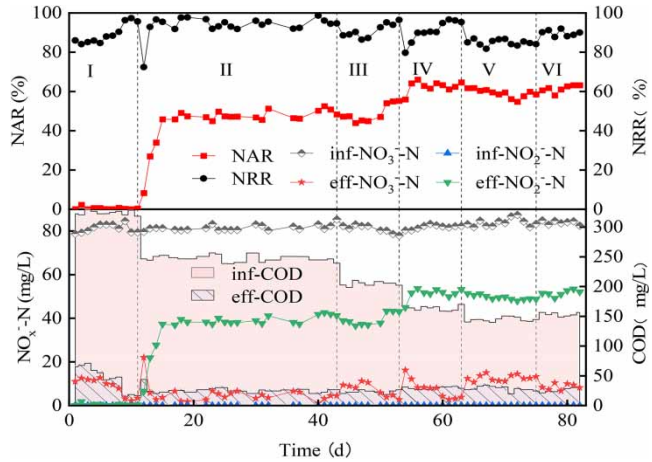


Figure 1 | Long-term performance of the reactor 1.

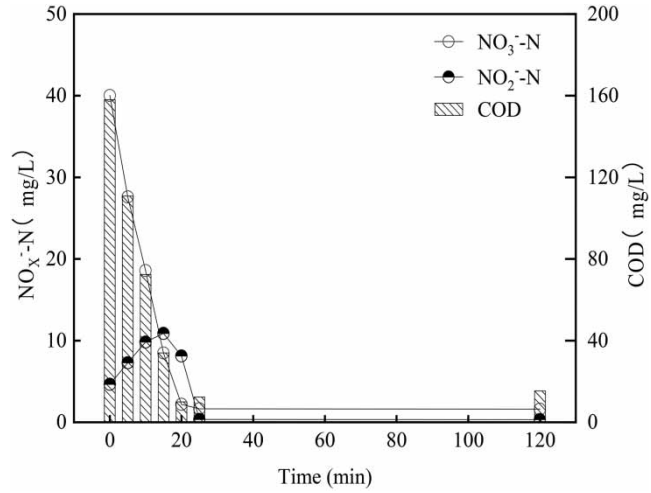


Figure 2 | Nitrogen conversion on 11th day via in-situ tests.

residual nitrate in effluent above 8.48 mg/L can indeed achieve PD and realize the accumulation of nitrite. At the end of phase-IV, the average effluent nitrite, average nitrate and COD concentration was 51.79 mg/L, 4.57 mg/L and 20–30 mg/L, respectively. The average NAR and NRR was 62.87% and 93.36%, respectively.

On the phase-V (64–75 d), the dosage of carbon source was reduced from 160 mg/L to 144 mg/L, then the effluent nitrite was 51.33 mg/L, NAR and NRR were 61.72% and 85.04%, respectively. And the effluent nitrate increased to 12.43 mg/L. However, the effluent nitrate concentration was always above 8.48 mg/L, and the effluent nitrite did not rise any more. The average effluent nitrite and nitrate concentration was 49.38 mg/L and 12.71 mg/L, respectively, and the average NAR and NRR were 58.89% and 84.84%, respectively. While the residual COD concentration was still 20–30 mg/L. In conclusion,

Table 1 | NO_3^--N and chemical oxygen demand (COD)/ NO_3^--N in the influent in different stages

	Phase I Day 0–11	Phase II Day 12–42	Phase III Day 43–52	Phase IV Day 53–63	Phase V Day 64–75	Phase VI Day 76–82
NO_3^--N (mg/L)	80	80	80	80	80	80
COD/NO_3^--N	4	3	2.5	2	1.8	1.9

PD has been successfully start-up on the phase-IV when the effluent nitrate was raised above 8.48 mg/L and did not decrease for a long time.

On phase-VI (76–82 d), the effluent residual nitrogen was very close to phase- IV when the dosage of carbon source was adjusted back to 152 mg/L. But it can be seen that the fine tuning of influent COD has significant impact on the effluent residual nitrate. Hence, it is essential to control the influent COD concentration to reach the standard of effluent total nitrogen.

In-situ test was performed again on the 90th day to reveal the characteristics of nitrogen conversion. The nitrogen changes were depicted as Figure 3. The nitrogen conversion process can be clearly divided into two stages (Table 2). The denitrification rate of stage-I (93 mg N/(L·h)) was 15 times higher than stage-II (6.04 mg N/(L·h)). The COD was dropped from 44.96 mg/L to 14.26 mg/L on stage-I, and almost stopped declining on stage-II, indicating that the available COD may have been consumed on stage-I. It has been reported that under aerobic conditions, acetate can be converted into poly 3-hydroxybutyrate which can be stored in cells as an effective electron donor for denitrification. Denitrification was continued after COD consumption and the reaction rate was slow, indicating that the electron donor was stored (Carucci *et al.* 2001) and there was an endogenous denitrification process (Zhang *et al.* 2019a, 2019b, 2019c). Therefore, the effluent nitrite concentration will not only depend on the COD in the influent, but also the reaction time. The reaction time would affect the accumulation of nitrite because of the existence of endogenous denitrification, so it should be paid attention in the subsequent pilot or production-scale studies.

3.2. Microbial community analysis

3.2.1. Diversity of bacterial community in reactor 1

The number of operational taxonomic units (OTU) was the highest in the seed sludge (Table 3). The number of OTU was decreased significantly with the operation of the reactor 1. The amount of OTU was only 1/3 of seed sludge after successful acclimation of R1 sludge (B4), which was suggested that certain bacteria were suppressed or eliminated under the limitation of electron donors and species diversity was declined. Moreover, the same result was obtained by richness indices (ACE and Chao1) (Table 3). Therefore, controlling the effluent nitrate concentration was an effective selective pressure.

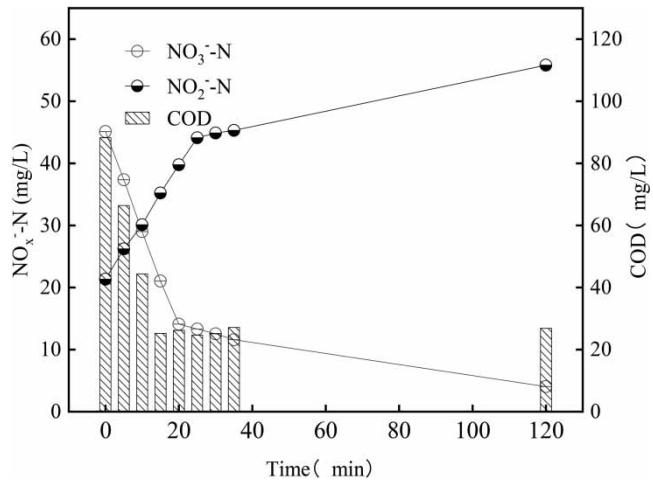


Figure 3 | Nitrogen conversion on 90th day via in-situ tests.

Table 2 | Characteristics of nitrogen conversion on 90th day

Stage	Time (min)	Nitrate change (mg/L)	Nitrite change (mg/L)	COD change (mg/L)	Denitrification rate (mg N/(L·h))
I	0–15	45.11–14.11	21.34–39.77	44.96–14.26	93
II	15–120	14.11–4.03	39.77–55.82	14.26 ± 2.15	6.04

Table 3 | Alpha diversity statistics

	Sequence reads			Richness indices		Diversity indices		
Sample	Raw	Filtered	Number of OTU	ACE	Chao1	Simpson	Shannon	Good's coverage
B0	108,259	82,770	2,726	3,076.39	2,948.77	0.014	5.775	0.99
B1	87,970	76,508	1,960	2,495.97	2,359.01	0.033	4.777	0.99
B2	89,198	84,726	956	1,427.00	1,263.02	0.099	3.337	1.00
B3	81,102	77,971	794	1,516.87	1,155.11	0.095	3.099	1.00
B4	73,360	69,875	832	1,584.23	1,195.12	0.297	2.291	1.00

3.2.2. Succession of microbial community composition of reactor 1

The microbial community structure at the phylum, class, order, family and genus level was analyzed to reveal the effect of EDS strategy on microbial community. After being treated with EDS strategy, the *Proteobacteria* and *Bacteroidetes* with an abundance of 65.19% and 22.19% had become the dominant phyla finally on phase-V compared with the seed sludge (30.32% and 19.80%) (Figure 4(a)). *Proteobacteria* and *Bacteroidetes* were one of the dominant bacteria in many denitrification environments and were the main promoters of denitrification process (Zhou *et al.* 2019). It suggested that the EDS strategy may effectively improve the denitrification performance of the system.

The *Betaproteobacteria* (*Proteobacteria* phyla) (59.80%) had become the dominant bacteria finally at class level compared with seed sludge (12.26%) (Figure 4(b)). However, it was worth noting that the abundance of *Cytophagia* bacteria under the EDS strategy had increased 21.02% on phase-V. This bacterium belongs to the class of *Bacteroidetes*, but there was no detailed report on the function of this bacterium in relevant literature. Therefore, the relationship between *Cytophagia* bacteria and nitrite accumulation remains to be determined.

Species identified as *Cytophagia* bacteria at the class level were further identified as *Cytophagales* at the order level, and *Cytophagia* and *Cytophagales* had a same relative abundance in each phase, indicating that *Cytophagales* is endemic to *Cytophagia*. The *Rhodocyclales* which is the main species of *β-proteobacteria* phyla had a relative abundance of 56.42%

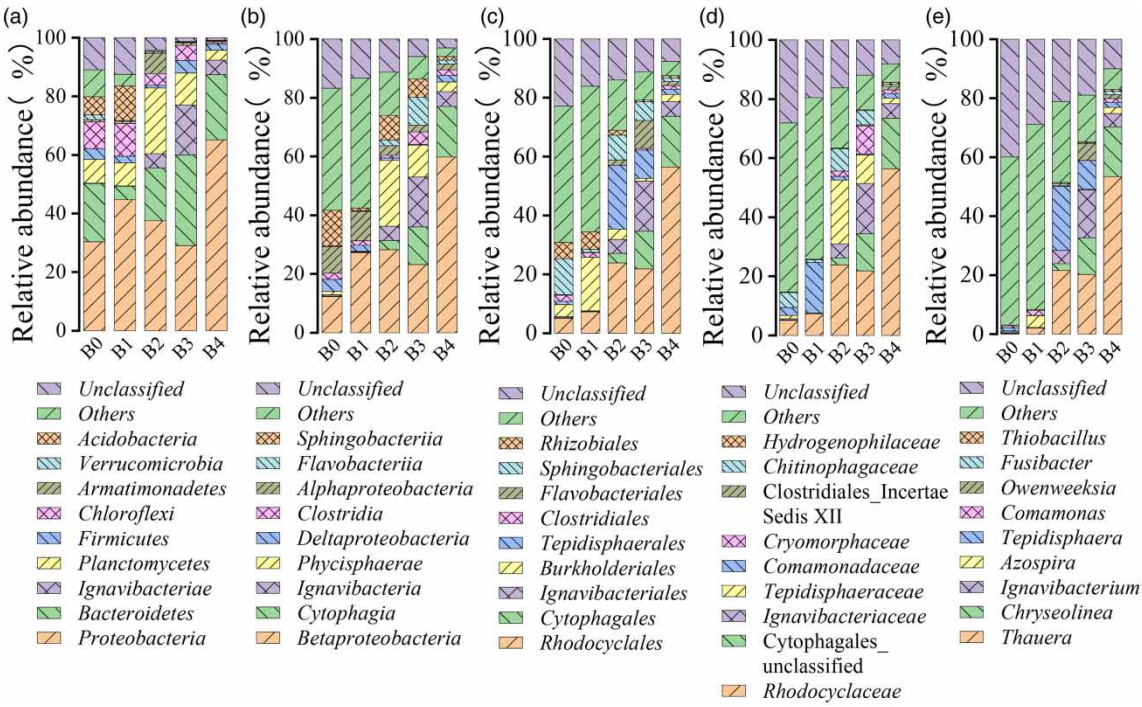


Figure 4 | Relative abundance of microbial communities as revealed by High-throughput gene sequences: (a) Phylum; (b) Class; (c) Order; (d) Family; (e) Genus.

finally on phase-V (Figure 4(c)). *Rhodocyclales* bacteria is consist of three families, namely *Rhodocyclaceae*, *Azonexaceae*, and *Zoogloeaceae*, all of which are heterotrophic bacteria that can use oxygen, nitrate or nitrite as electron receptors (Boden *et al.* 2017).

Rhodocyclaceae (56.42%) belonging to *Rhodocyclales* order were the dominant bacteria finally on phase-V at family level. There was no further classification of *Cytophagales*i (Figure 4(d)). The *Rhodocyclaceae* family contained 18 genera, including *Thauera*, *Azoarcus*, *Azospira* and other fungi. They have diverse functions that can be found in both polluted and unpolluted waters of soil, sewage treatment plants, ponds, rivers and aquifers.

The microbial community structure of each sample at genus level was further analyzed (Figure 4(e)). *Thauera* (46.97%) and *Chryseolinea* (17.06%) were the dominant bacteria finally on phase-V. *Thauera* was one of the dominant bacteria in many studies related to partial denitrification (Shi *et al.* 2019; Si *et al.* 2019). Most *Thauera* genera have been reported to capable to reduce of nitrate or nitrite via heterotrophic denitrifying process (Morgan-Sagastume *et al.* 2008; Bakken *et al.* 2012; Liu *et al.* 2013). The relative abundance of *Thauera* was increased from 0.02% to 46.97%, indicating that the PD process was successfully achieved. For example, transient accumulation of nitrite was observed during nitrate reduction by *Thauera.aminoaromatica* and *Thauera.phenylacetica.* Some *Thauera* species can not only carry out partial denitrification to obtain the accumulation of nitrite, but also carry out whole-process denitrification to continue to reduce the accumulated nitrite to nitrogen (Liu *et al.* 2013). In general, the gradual increase of *Thauera* explained the gradual increase of the accumulation rate of nitrite. What's more, the relative abundance of *Chryseolinea* increased from 0.24% to 17.03%. The increased abundance of *Chryseolinea* may be due to the uncomplete removal of dissolved oxygen (DO) from the water. *Chryseolinea* was reported to be an aerobic chemotrophic bacterium (Kim *et al.* 2013) that could remove nutrients from water (Kim *et al.* 2013; Xu *et al.* 2018). There was no effect of *Chryseolinea* bacteria on the partial denitrification performance has been observed during the 82-day operation of the reactor. However, attention should be paid to the enrichment of *Chryseolinea* bacteria on the consumption of COD in the practical application.

3.3. Mechanism of species selection and nitrite accumulation

The process of denitrification involves a variety of specific enzymes including nitrate reductases (Nar), nitrite reductase (Nir), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) (Tavares *et al.* 2006). Nar is encoded by the *narG* gene, while Nir is encoded by *nirK* or *nirS* gene (Simon & Klotz 2013). The coding sequences (CDS) of the *narG*, *nirK* and *nirS* genes were obtained in NCBI and primers were designed by Beacon Designer (version 7.9) software (Table 4).

The expression of target genes was analyzed via real-time fluorescence quantitative PCR. The expression level of *narG*, *nirK* and *nirS* genes were 1,727.44 copies/mg of wet sludge, 208.27 copies/mg of wet sludge and 203.94 copies/mg of wet sludge, respectively (Table 5). The expression level of Nar gene was 4.2 times of Nir gene, indicating that nitrate reduction rate was

Table 4 | Primer sequence of target gene

Target gene	Direction of primer	Primer Sequence (5′–3′)	Starting position	Length of the product
narG	Forward	ACCGAGAAACTGTTGAAC	3,229	98
	Reverse	CTGTAGGTGGAGTGGATG	3,326	
nirK	Forward	CAAGGAGCTTGTTTATGAC	621	82
	Reverse	CTTGAAATTGCCGTTCTC	702	
nirS	Forward	GAAGAACCATCCGCAGTA	1,281	82
	Reverse	ATGGGTCTTGATGAACAG	1,362	

Table 5 | Expression of target gene

Gene	Reductase	Expression (copies/mg wet sludge)
narG	nitrate reductase	1,727.44
nirK	Cu-type nitrite reductase	208.27
nirS	cd1-type nitrite reductase	203.94

much higher than nitrite reduction rate, so the nitrite accumulation was achieved. This result was consistent with the research of Qian's *et al.* (2019) study. Each type of bacteria contains only one kind of nitrite reductase according to reports, and *Thauera* contained only cd1 nitrite reductase encoded by the *nirS* gene (Etchebehere & Tiejie 2005). It suggested that there was a low level of Cu-type reductase, and explained the low expression of *nirK* gene. The expression of *nirS* gene was inhibited by nitrate, resulting in low levels of cd1-type nitrite reductase (Li *et al.* 2016).

The species selection mechanism achieving a large enrichment of *Thauera* via EDS strategy may be related to the transfer of electron donors. Studies have shown that electron donors that provided by acetate have a priority on reaching nitrate reductase upstream of the denitrification respiratory chain (Van rijin *et al.* 1996). Therefore, the limitation of electron donors would give priority to partial denitrification when effluent nitrate was controlled above the critical value (8.48 mg/L). *Thauera* species can not only obtain the accumulation of nitrite by partial denitrification, but also continue to transfer the accumulated nitrite to nitrogen by whole-process denitrification (Liu *et al.* 2013), enabling *Thauera* bacteria to carry out normal metabolism and maintain their own growth and reproduction even if only partial denitrification was carried out. Meanwhile, some bacteria were difficult to complete normal metabolism due to the lack of electron donors, then *Thauera* bacteria gradually became the dominant bacteria.

3.4. Rapid start-up experiment of PD reactor 2

The rapid start-up experiment of the PD reactor was carried out by controlling the effluent residual nitrate in reactor 2. And the operating conditions of reactor 2 were consistent with the reactor 1 except that the initial nitrate concentration of influent was 100 mg/L. The performance of reactor 2 was shown as Figure 5. According to the first experiment, the dosage of carbon source was reduced when every time the nitrate of effluent was below the critical value. The decreasing of carbon source dosage was not fixed, so the increasing value of effluent residual nitrate was also different. After five times of carbon source dosage reducing, the reactor 2 was successfully started on the 19th day. Its performance removing the adaption period (0–19 d) was shown in Table 6. Therefore, it can be seen that the rapid start-up of PD by controlling the effluent residual nitrate via EDS strategy was effective and repeatable.

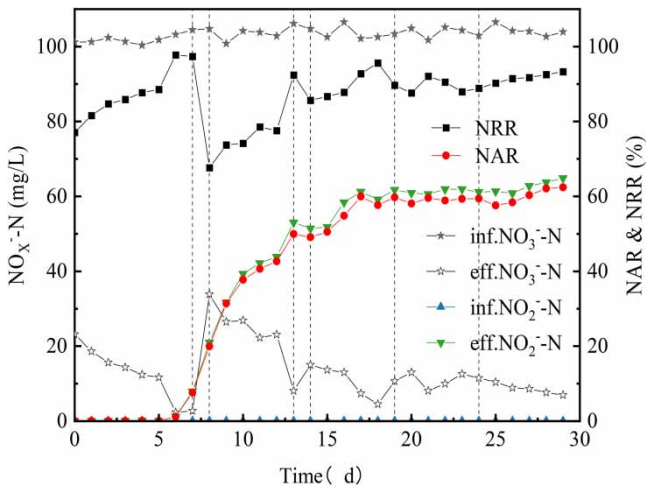


Figure 5 | Operation performance of reactor 2.

Table 6 | Comparison of the performance of reactor 1 and 2

Reactor	NO_3^--inf (mg/L)	NO_3^--eff (mg/L)	NO_2^--eff (mg/L)	NAR (%)	NRR (%)
1	83.2 ± 3.6	12.71	49.38	58.89	84.84
2	102.1 ± 2.1	10.66	61.75	59.74	89.68

3.5. Significance of the study

PD process with the characteristics of efficiency, less carbon source, easy control and long-term stable operation, can offer sustainable nitrite source for Anammox and open up a novel way for engineering application of Anammox (Celia *et al.* 2017; Cui *et al.* 2020). At present, PD process coupled Anammox has gained good nitrogen removal efficiency in small scale (Singh *et al.* 2021). However, the investigation of microbial process mechanism has not been systematically carried out.

This study revealed the evolution in PD system under EDS strategy, and performed an analysis of nitrite accumulation performance, microbial community and expression of related reductase. These results indicated that the critical value (8.48 mg/L) of residual nitrate in effluent to inhibit nitrite reduction can be determined by EDS strategy, and remained the residual nitrate higher than that critical value so as to maximize nitrite accumulation. Additionally, the significant enrichment of *Thauera* (0.21%–53.29%) and the significantly different expression between reductase genes contributed to nitrite production (narG, 1,727.44 copies/mg) and nitrite reduction (*nirS*, 208.27 copies/mg; *nirK*, 203.94 copies/mg) commonly involved to PD start-up and stable operation. These results provided some support for further understanding of the microbial mechanism of nitrite accumulation in PD process.

4. CONCLUSION

The PD process was achieved within 11 days and stably operated via EDS strategy by employing the effluent residual nitrate as the only indicator. The critical value (8.48 mg/L) of effluent residual nitrate inhibiting nitrite reduction was determined by in-situ test. There was an inhibition of nitrite reduction when the effluent residual nitrate was higher than the critical value. It was confirmed that the stable operation of PD process can be achieved quickly by controlling the effluent residual nitrate above the critical value. In addition, a significant enrichment of *Thauera* (0.21% to 53.29%) was obtained through high-throughput sequencing. What's more, the expression of nitrate reductase (narG, 1,727.44 copies/mg) was 4.2 times than nitrite reductase (nirK and nirS, 208.27 copies/mg and 203.94 copies/mg) via real-time fluorescence. *Thauera* only contains cd1 type nitrite reductase, while nitrate inhibits cd1 type nitrite reductase. Thus, nitrite accumulation can realize by controlling effluent nitrate above critical value.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Antwi, P., Li, J., Boadi, P. O., Meng, J., Koblah Quashie, F., Wang, X. & … Buelna, G. 2017a Efficiency of an upflow anaerobic sludge blanket reactor treating potato starch processing wastewater and related process kinetics, functional microbial community and sludge morphology. *Bioresource Technology* **239**, 105–116.

Antwi, P., Li, J., Boadi, P. O., Meng, J., Shi, E., Chi, X. & … Ayivi, F. 2017b Dosing effect of zero valent iron (ZVI) on biomethanation and microbial community distribution as revealed by 16S rRNA high-throughput sequencing. *International Biodeterioration & Biodegradation* **123**, 191–199.

Antwi, P., Li, J., Opoku Boadi, P., Meng, J., Shi, E., Xue, C. & … Ayivi, F. 2017c Functional bacterial and archaeal diversity revealed by 16S rRNA gene pyrosequencing during potato starch processing wastewater treatment in an UASB. *Bioresource Technology* **235**, 348–357.

Antwi, P., Zhang, D. C., Luo, W. H., Xiao, L. W., Meng, J., Kabutey, F. T., Ayivi, F. & Li, J. Z. 2019 Performance, microbial community evolution and neural network modeling of single-stage nitrogen removal by partial-nitritation/anammox process. *Bioresource Technology* **284**, 359–372.

Arora, S. A., Nawaz, A., Qyyum, A. M., Ismail, S., Aslam, M., Tawfik, A., Yun, M. C. & Lee, M. 2021 Energy saving anammox technology-based nitrogen removal and bioenergy recovery from wastewater: inhibition mechanisms, state-of-the-art control strategies, and prospects. *Renewable and Sustainable Energy Reviews* **135**, 110126.

Bakken, L., Bergaust, L., Liu, B. & Frostegard, A. 2012 Regulation of denitrification at the cellular level: a clue to the understanding of N2O emissions from soils. *Philosophical Transactions of Royal Society B-Biological Sciences* **367** (1593), 1226–1234.

Boden, R., Hutt, L. & Rae, A. 2017 Reclassification of Thiobacillus aquaesulis (Wood & Kelly, 1995) as Annwoodia aquaesulis gen. nov., comb. nov., transfer of Thiobacillus (Beijerinck, 1904) from the Hydrogenophilales to the Nitrosomonadales, proposal of Hydrogenophilalia class. nov within the 'Proteobacteria', and four new families within the orders Nitrosomonadales and Rhodocyclales. *International Journal of Systematic and Evolutionary Microbiology* **67**, 1191–1205.

Carucci, A., Dionisi, D., Majone, M., Rolle, E. & Smurra, P. 2001 Aerobic storage by activated sludge on real wastewater. *Water Research* **35** (16), 3833–3844.

Celia, M. C. B., Jia, M. S., Mark, C. M. V. L., Eveline, I. P. V. & Mari, K. H. W. 2017 Evaluating the potential for dissimilatory nitrate reduction by anammox bacteria for municipal wastewater treatment. *Bioresource Technology* **233**, 363–372.

Chen, H., Tu, Z., Wu, S., Yu, G. L., Du, C. Y., Wang, H., Yang, E. Z., Zhou, L., Deng, B., Wang, D. B. & Li, H. L. 2021 Recent advances in partial denitrification-anaerobic ammonium oxidation process for mainstream municipal wastewater treatment. *Chemosphere* **278**, 130436.

Cui, B., Yang, Q., Liu, X. H., Wu, W. J., Liu, Z. B. & Gu, P. C. 2020 Achieving partial denitrification-anammox in biofilter for advanced wastewater treatment. *Environment International* **138**, 105612.

Du, R., Cao, S., Peng, Y., Zhang, H. & Wang, S. 2019 Combined PD (PD)-Anammox: a method for high nitrate wastewater treatment. *Environment International* **126**, 707–716.

Etchebehere, C. & Tiejie, J. 2005 Presence of two different active nirS nitrite reductase genes in a denitrifying Thauera sp. from a high-nitrate-removal-rate reactor. *Applied and Environmental Microbiology* **71** (9), 5642–5645.

Federation, W.E., Association, A.P.H. 2005 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association (APHA), Washington, DC, USA.

Ji, J., Peng, Y., Li, X. & Zhang, Q. 2019 Stable long-term operation and high nitrite accumulation of an endogenous partial-denitrification (EPD) granular sludge system under mainstream conditions at low temperature. *Bioresource Technology* 121634.

Kim, J., Alkawally, M., Brady, A., Rijpstra, W. & Dunfield, P. 2013 Chryseolinea serpens gen. nov., sp nov., a member of the phylum Bacteroidetes isolated from soil. *International Journal of Systematic and Evolutionary* **63**, 654–660.

Le, T., Peng, B., Su, C. Y., Massoudieh, A., Torrents, A., Omari, A. A., Murthy, S., Wett, B., Chandran, K., Barbadillo, C., Bott, C. & Clippeleir, D. H. 2021 Impact of carbon source and COD/N on the concurrent operation of partial denitrification and anammox. *Water Environment Research* **91** (3), 185–197.

Li, W., Lin, X. & Chen, J. 2016 Enrichment of denitratating bacteria from a methylotrophic denitrifying culture. *Applied Microbiology and Biotechnology* **100** (23), 10203–10213.

Liu, B., Mao, Y., Bergaust, L., Bakken, L. & Frostegard, A. 2013 Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. *Environmental Microbiology* **15** (10), 2816–2828.

Lotti, T., Burzi, O., Scaglione, D., Ramos, C. A., Ficara, E., Pérez, J. & Carrera, J. 2019 Two-stage granular sludge partial nitritation/anammox process for the treatment of digestate from the anaerobic digestion of the organic fraction of municipal solid waste. *Waste Management* **100**, 36–44.

Mao, N., Ren, H., Geng, J. & Ding, K. X. 2017 Engineering application of anaerobic ammonium oxidation process in wastewater treatment. *Microbiology and Biotechnology* **33** (8), 153.

Morgan-Sagastume, F., Nielsen, J. & Nielsen, P. 2008 Substrate-dependent denitrification of abundant probe-defined denitrifying bacteria in activated sludge. *Fems Microbiology Ecology* **66** (2), 447–461.

Qian, W., Ma, B., Li, X., Zhang, Q. & Peng, Y. 2019 Long-term effect of pH on denitrification: high pH benefits achieving partial-denitrification. *Bioresource Technology* **278**, 444–449.

Qiu, S. K., Li, Z. B., Hu, Y. S., Shi, R., Liu, L., Liu, R., Shi, L., Chen, L. J. & Zhan, X. M. 2021 What's the best way to achieve successful mainstream partial nitritation-anammox application? *Critical Reviews in Environmental Science and Technology* **51** (10), 1045–1077.

Shi, L., Du, R. & Peng, Y. 2019 Achieving partial denitrification using carbon sources in domestic wastewater with waste-activated sludge as inoculum. *Bioresource Technology* **283**, 18–27.

Si, Z., Peng, Y., Yang, A., Zhang, S., Li, B. & Wang, S. 2019 Rapid nitrite production via partial denitrification: pilot-scale operation and microbial community analysis. *Environmental Science-Water Research & Technology* **6** (3), 864–864.

Simon, J. & Klotz, M. G. 2013 Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **1827** (2), 114–135.

Singh, V., Ormeci, B., Mishra, S. & Hussain, A. 2021 Simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) – a review of critical operating parameters and reactor configurations. *Chemical Engineering Journal* **19**, 133677.

Tavares, P., Pereira, A. S., Moura, J. J. G. & Moura, I. 2006 Metalloenzymes of the denitrification pathway. *Journal of Inorganic Biochemistry* **100** (12), 2087–2100.

Trinh, P. H., Lee, H. S., Jeong, G., Yoon, H. & Park, D. H. 2021 Recent developments of the mainstream anammox processes: challenges and opportunities. *Journal of Environmental Chemical Engineering* **9** (4), 105583.

VanGuilder, H. D., Vrana, K. E. & Freeman, W. M. 2008 Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* **44** (5), 619–626.

Van Rijin, J., Tal, Y. & Barak, Y. 1996 Influence of volatile fatty acids on nitrite accumulation by a pseudomonas stutzeri strain isolated from a denitrifying fluidized bed reactor. *Applied and Environmental Microbiology* **62** (7), 2615–2620.

Xu, J., He, J., Wang, M. & Li, L. 2018 Cultivation and stable operation of aerobic granular sludge at low emperature by sieving out the batt-like sludge. *Chemosphere* **211**, 1219–1227.

Zhang, M., Wang, S. Y., Ji, B. & Liu, Y. 2019a Towards mainstream deammonification of municipal wastewater: partial nitrification-anammox versus partial denitrification-anammox. *Science of the Total Environment* **692** (20), 393–401.

Zhang, X. J., Chen, Z., Zhou, Y., Ma, Y. P., Ma, C., Li, Y., Liang, Y. H. & Jia, J. P. 2019b Impacts of the heavy metals Cu (II), Zn (II) and Fe (II) on an Anammox system treating synthetic wastewater in low ammonia nitrogen and low temperature: Fe (II) makes a difference. *Science of the Total Environment* **648** (15), 798–804.

Zhang, D., Su, H., Antwi, P., Xiao, L., Liu, Z. & Li, J. 2019c High-rate partial-nitritation and efficient nitrifying bacteria enrichment/out-selection via pH-DO controls: efficiency, kinetics, and microbial community dynamics. *Science of the Total Environment* **692**, 741–755.

Zhou, B., Duan, J., Xue, L., Zhang, J. & Yang, L. 2019 Effect of plant-based carbon source supplements on denitrification of synthetic wastewater: focus on the microbiology. *Environmental Science and Research* **26** (24), 24682–24694.

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